Cent. Eur. For. J. 69 (2023) 214–223 sciendo DOI: 10.2478/forj-2023-0010
CENTRAL EUROPEAN Forestry Journal

ORIGINAL PAPER

Inconsistent phenotypic differentiation at physiological traits in Norway spruce (*Picea abies* Karst.) provenances under contrasting water regimes

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Abstract
Norway spruce is expected to suffer from drought stress and other manifestations of climate change. This study relies on a manipulative experiment with drought-stressed and well-watered (control) seedlings, comprising five provenances of Norway spruce distributed along a steep elevational transect from 550 to 1,280 m a.s.l. within the natural range. Seedlings were subjected to measurement of physiological traits (content of phytohormones and monoterpenes, slow and fast chlorophyll *a* fluorescence kinetics, gas exchange, hyperspectral indices), and genotyping at 8 nuclear microsatellite loci. Comparison of the coefficient of differentiation at neutral loci (F_{ST}) vs. differentiation at phenotypic traits (P_{ST}) was used to identify traits underlying divergent selection. In total, 18 traits exhibited a significant $P_{ST} - F_{ST}$ difference. However, the consistency in differentiation patterns between drought-stressed and control plants was limited, only three traits exhibited signals of selection under both treatments. This outcome indicates that the identified differentiation patterns can only be interpreted in the context of environmental setup of the experiment, and highlights the importance of common gardens in adaptation research, as they allow both elimination of environment-induced phenotypic variation and studying genotype-by-environment interaction in physiological responses to environmental stresses.

Key words: adaptation; phenotypic differentiation; drought stress; plasticity; divergent selection

Editor: Matteo Campioli

1. Introduction

The rules for the transfer of forest reproductive material (FRM) in Europe as defined by the Directive 105/1999/EC (European Communities 1999) rely on the concept of regions of provenance, which are defined as 'the area or group of areas subject to sufficiently uniform ecological conditions in which stands or seed sources showing similar phenotypic or genetic characters are found', while in mountainous countries, they may be subdivided into smaller units respecting elevational zonation. The basic idea in the background is that of local adaptation: seed sources and reforestation sites within a provenance region share similar climates. Local populations are expected to be adapted to the local environments, while non-local seed sources, located in distant regions and thus having evolved under different environmental conditions, are expected to be genetically differentiated. Consequently, fitness of non-local sources may be lower, which may be reflected in lower yield and higher mortality rates. Although the reasoning for regions of provenance is purely genetic, any type of genetic information is rarely used for their delineation; in most countries, they are designed based on climatic or phytogeographic regions, while administrative subdivision of a country also plays a role (Konnert et al. 2015). Currently, especially under consideration of the ongoing climate change, the limitations of this concept have been acknowledged and development of alternatives is recommended (Gömöry et al. 2021). Nevertheless, true alternatives require profound understanding of the physiological basis of fitness-related traits, knowledge of

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their genetic control and their underlying evolutionary mechanisms.

Two categories of evolutionary forces determine the patterns of genetic differentiation (and, consequently, differentiation in genetically controlled phenotypic traits), namely random processes such as mutation, genetic drift and gene flow, and evolutionary response to environmental pressures by means of natural selection. Distinguishing between them is a major challenge for both marker-based and phenotype-related studies. Moreover, genetic as well as phenotypic structures within any set of populations may either be homogenized by stabilizing selection or differentiated by divergent selection associated with local adaptation. A standard approach to quantify the effects of neutral and adaptive processes on variation of a quantitative phenotypic trait is the comparison of neutral differentiation (typically assessed using some type of neutral markers such as nuclear microsatellites) measured by the coefficient of differentiation F_{ST} (Wright 1951) vs. differentiation at the trait, measured by the coefficient of quantitative differentiation (Q_{ST}; Spitze 1993). The latter is estimated from additive between- and within-population variance components of the respective phenotypic trait, and is expected to measure differentiation at the trait-controlling gene(s) (McKay & Latta 2002). If populations are at drift-migration equilibrium, a difference between Q_{ST} and F_{ST} is a sign of natural selection: $Q_{ST} > F_{ST}$ is an indication of diversifying selection (local adaptation), while $Q_{ST} < F_{ST}$ is a sign of stabilizing (homogenizing) selection (Merilä & Crnokrak 2001).

Neutral differentiation (F_{ST}) can easily be assessed using markers; this assessment is routinely done in natural populations in situ and does not require any specific arrangement. On the other hand, a reliable estimation of Q_{ST} requires manipulative experiments relying on family-based experimental designs to distinguish additive and non-additive genetic effects and to avoid environmental and maternal effects on quantitative traits (Merilä & Crnokrak 2001). As a proxy of Q_{ST} in a situation, when the available data do not allow direct estimation of Q_{ST}, coefficient of phenotypic differentiation P_{ST} was proposed by Leinonen et al. (2006). In wild populations studied in situ, the use of P_{ST} is problematic, as additive genetic effects can hardly be separated from environmental effects, and phenotypic plasticity is completely ignored (Pujol et al. 2008). However, provenance experiments as a type of common gardens, where environmental variation is controlled at least at the macro-site scale, allow overcoming some of the mentioned problems (Leinonen et al. 2008; Merilä & Hendry 2013). An additional problem with the $P_{ST} - F_{ST}$ comparison results from the fact that the expression of any quantitative trait is affected by the environment, and a phenotypic response to an environmental stimulus may differ between genotypes (genotype-by-environment interaction). Therefore, the outcome of the analysis may depend from the experimental setup.

Norway spruce (*Picea abies* Karst.) is a good candidate for studies of differentiation at the physiological level. In contrast to its traditional status of the commercially most successful species in Europe (Jandl 2020), it is currently considered one of the species most endangered by climate change (Vitali et al. 2018), and both natural spruce ecosystems and plantations outside its natural range decline in many areas across the whole distribution range (Schurman et al. 2018). The knowledge of evolutionary mechanisms underlying variation in traits associated with fundamental physiological processes such as photosynthesis or water exchange is essential for defining reasonable rules or recommendations for the use of forest reproductive materials. A trait employed to guide the choice of seed sources must be under genetic control (at least partially). Both observational and manipulative experiment demonstrated responsiveness of physiological parameters to environmental stresses (Pollastrini et al. 2017; Marozas et al. 2019; Hájíčková et al. 2021), but the evidence for their genetic basis is often lacking.

As drought stress is currently considered the main threat for the persistence of Norway spruce in Central Europe (Lévesque et al. 2013), we studied phenotypic differentiation among Norway spruce provenances distributed along a steep altitudinal and climatic gradient in a manipulative experiment, where phenotypic responses under drought stress were compared to those under normal water supply. The objective of the study was identification of physiological traits responsive to divergent selection, and identification of differences in differentiation patterns resulting from different stress exposure.

2. Materials and methods

2.1. Experimental material

A manipulative experiment comprising 5 provenances originating within the natural range of Norway spruce in Slovakia was established in 2015; their basic characteristics are presented in Table 1. Seeds were received from the gene bank of forest trees of Slovakia (OZ Semenoles Liptovský Hrádok) and sown in a nursery. After 2 years, seedlings were transplanted into peat substrate in 3 L pots, which were then placed in a nursery bed in the Arboretum Mlyňany. After further 2 years, seedlings in pots were transported to the AgroBioTech centre of the Slovak University of Agriculture in Nitra, Slovakia, and placed there for 6 weeks in a climatized room for acclimation. During subsequent 24 days, from May 25 to June 19, 2019, each provenance underwent two treatments – control and drought, while nine seedlings per treatment and provenance were used. The control seedlings were irrigated to a constant weight of 2.7 kg every second day. For drought-stressed seedlings, watering was completely excluded for the whole duration of the

treatment. During the experiment, day-time (14 hours) was simulated with a light intensity of 300 µmol m^{-2} s^{-1}, 45% relative humidity and temperature of 23 °C, while during the night-time (10 hours) light was switched off, and relative humidity of 55% and temperature of 17 °C were maintained. An automated phenotyping line Plant Screen™ Conveyor System equipped with a FieldSpec2 spectroradiometer available at AgroBioTech was used to measure hyperspectral indices at 3-day intervals throughout the experiment. Moreover, gas exchange as well as rapid and slow kinetics of chlorophyll a fluorescence were measured manually, also at 3-day intervals. As we were interested in the outcome of the drought stress, only the measurements done on June 19 (the last day of the experiment) were taken into account. At the end of the experiment, plant material was collected for the analyses of the contents of phytohormones content (performed at the Institute of Experimental Botany of Czech Academy of Sciences, Prague, Czech Republic), monoterpenes and photosynthetic pigments. Seedlings were randomly rearranged after each measurement on conveyor belts (each 3 days). The details about physiological traits as well as recording and/or analytical procedures can be found in Table 1.

Table 1. List of the studied Norway spruce provenances.

Provenance	Basic Material code	Longitude	Latitude	Elevation [m a.s.l.]	MAT [°C]	MAP [mm]
Čadca	pab225CA-003	49°24′	18°42′	550	7.1	908
Beňuš	pab235BR-062	48°50′	19°45′	750	6.2	880
Habovka	pab216TS-840	49°15′	19°39′	920	5.1	1,073
Liptovský Mikuláš	pab216LM-028	49°04′	19°41′	1,100	4.2	1,138
Brezno	pab217BR-167	48°50′	19°25′	1,280	3.1	1,113

2.2. DNA Extraction, Microsatellite Analysis and Sequencing

After finishing physiological measurements, plant material was also taken from the measured seedlings for DNA analyses. Total genomic DNA was isolated from 10–20 mg of silica-dried needles per seedling using a modified CTAB protocol following Doyle & Doyle (1987). DNA concentration and quality was assessed with NanoDrop (Thermo Fisher, Waltham MA, USA).

Eight nuclear microsatellites (nSSR) were analyzed as strictly neutral nuclear loci: WS0022.B15, WS00111.K13, WS0016.O09, WS0092.A19 (Rungis 2004), PAAC23 (Scotti et al. 2000), pgGB5, paGB3 (Besnard 2003), and Pa28 (Fluch 2011) in two multiplex reactions. 5 µl reaction mixtures contained 2.5 µl of Qiagen Multiplex PCR kit, 1 µl of Q-solution (Qiagen), 1 µl of DNA, primers and water to final volume. The first multiplex reaction used the following concentrations of primers: 0.1 µM WS0022.B15, 0.08 µM pgGB5, 0.1 µM paGB3, in the second multiplex reaction, concentrations were 0.05 µM Pa28, 0.15 µM WS00111.K13, 0.1 µM WS0016.O09, 0.07 µM PAAC23 and 0.08 µM WS0092.A19. Thermal profile used for amplification was as follows: polymerase activation and denaturation at [94 °C, 15 minutes] – [94 °C, 30 seconds – 58 °C, 90 seconds at – 90 seconds at 72 °C] × 32, – [60 °C, 20 minutes]. Amplified fragments were separated on an ABI 3130 sequencer, the resulting raw data were analysed in GeneMapper 4.0 (Applied Biosystems).

2.3. Data analysis

To reveal potential substructure in the nSSR data, Bayesian analysis of population structure was conducted using STRUCTURE 2.3.4. (Pritchard et al. 2000), which was run 10 times for each $K = 1$ to 9, with a burn-in period of 500,000 and subsequent 2,000,000 iterations to determine the number of clusters. The admixture model was used, along with sampling locations as prior information to assist the clustering. The choice of the most probable number of clusters K was based on posterior probabilities of cluster membership and the ΔK measure (Evanno et al. 2005), using the program STRUCTURE HARVESTER (Earl 2012). Clusters were aligned using CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) using full search.

Variation between treatments and among provenances was assessed using a two-way analysis of variance (ANOVA); both treatment and provenance were considered fixed-effect factors. Procedure GLM of SAS 9.1.3 (SAS/STAT® Software, SAS Institute) was used.

To identify traits, which are potentially under divergent selection, quantitative and neutral differentiation were compared (Spitze 1993). Differentiation at neutral loci (quantified by F_{ST}) relied on nSSR data. As null alleles contribute to overestimation of genetic differentiation among populations, multilocus estimate of F_{ST} was calculated using the correction procedure (ENA; excluding null alleles) to judge the effect of null alleles on differentiation estimates, employing the FREENA program (Chapuis & Estoup 2007). Confidence intervals for F_{ST} were estimated from 10,000 bootstrap replicates over loci. As the family structure of the tested provenances was unknown, and thus the additive component of phenotypic variation could not be estimated, the coefficient of quantitative differentiation Q_{ST} was approximated by the coefficient of phenotypic differentiation P_{ST} following Brommer (2011):

$$P_{ST} = \frac{c\sigma_B^2}{c\sigma_B^2 + 2h^2\sigma_W^2} = \frac{\frac{c}{h^2}\sigma_B^2}{\frac{c}{h^2}\sigma_B^2 + 2\sigma_W^2} \quad [1]$$

where σ_B^2 and σ_W^2 are the between-population and within-population components of variance of the quantitative trait, respectively, h^2 is the narrow-sense heritability and c is the proportion of the total phenotypic variance which is due to additive genetic effects across populations. Variance components (σ_B^2, σ_W^2) were estimated using the VARCOMP procedure (SAS). Estimates were done

under the null assumption $c = h^2$. Confidence intervals of P_{ST} were estimated from 600 bootstraps, each involving resampling of individuals within provenances. P_{ST} was considered to differ from F_{ST} when their confidence intervals did not overlap. Moreover, to assess the robustness of the inference on the $P_{ST} - F_{ST}$ difference, the critical c/h^2 ratio was estimated, at which the 95% confidence intervals of P_{ST} and F_{ST} touch (Brommer 2011). The more the critical c/h^2 ratio differs from one, the stronger is the evidence of selection being the mechanism behind differentiation in the studied phenotypic trait.

3. Results

Analysis of variance of the scored physiological traits revealed that gasometry parameters and parameters of slow kinetics of chlorophyll *a* fluorescence generally reacted on the drought treatment across all provenances (Table 2). Among the other traits, significant between-treatment differences were rather exceptional (e.g., ABA and α-terpinolene content, photochemical efficiency). On the other hand, isoprenoid contents and several hyperspectral parameters showed significant inter-provenance variation or treatment-by-provenance interaction, which indicates differences in provenance responses to drought stress.

In the analysis of the nSSR dataset under the STRUCTURE procedure, the Δ*K* measure (Evanno et al. 2005) indicated $K = 2$ as the most probable number of groups (Fig. 1a). However, it must be reminded that by principle, Δ*K* does not allow inference on $K = 1$, i.e., absence of any structure. The mean log posterior probability ln(*P*(*X*|*K*)) did not exhibit an abrupt change at $K = 2$, the transition was smooth; therefore, the maximum of the Δ*K* curve cannot be considered a sufficient evidence for the presence of two groups. The proportion of groups was around 50% in all individuals and all provenances, no geographical trend could be identified, which is another indication for the absence of any structure (Fig. 1b).

The overall differentiation at neutral nSSR loci was negligible ($F_{ST+ENA} = 0.0054$; 95% CI = –0.0002 – 0.0089), as expected. Phenotypic traits, for which the confidence interval of P_{ST} did not overlap with that of F_{ST} either in control or drought-stressed plants, are listed in Table 3. Only the cases when $P_{ST} > F_{ST}$ are listed; first because we were primarily interested in divergent selection, but also because F_{ST} is very low (including the upper limit of the confidence interval), so expectedly for no trait P_{ST} was significantly lower than F_{ST}. Isoprenoid contents were most represented among traits showing excessive phenotypic differentiation, especially in control plants. Hyperspectral indices, which are indicators of the content of photosynthetic pigments, exhibited significant inter-provenance differentiation primarily in drought-stressed seedlings. On the other hand, none of P_{ST} estimates for directly measured contents of chlorophylls and carotenoids or rapid kinetics of chlorophyll a fluorescence was significant. With a few exceptions, the critical c/h^2 ratios were less than 0.3, i.e., much lower than 1, giving a fairly good support for an adaptive basis of variation of the respective trait. However, only for three traits (sabinene hydrate, α-terpinolene and normalized difference vegetation index) signals of selection were identified both in control (well-watered) and drought-stressed seedlings, meaning that adaptive significance depends from the environmental context.

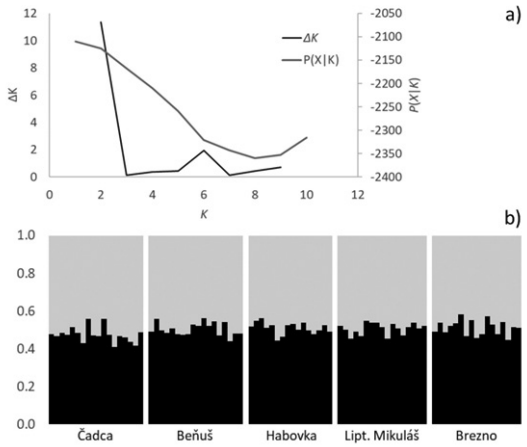

Fig. 1. Results of the Structure analysis: a) assessment of the number of groups following Evanno et al. (2005) b) ancestry of individuals inferred by the Structure analysis for the number of clusters $K = 2$.

Table 2. Two-way analysis of variance of physiological traits (probabilities associated with F-tests).

Trait	Associated probability		
	Treatment	Provenance	T × P
Gasometry			
CO_2 assimilation rate (*A*)	< 0.0001	0.0157	0.1649
stomatal conductance (*Gs*)	< 0.0001	0.5688	0.5230
intercellular CO_2 concentration (*Ci*)	0.0270	0.5468	0.6314
transpiration rate (*E*)	< 0.0001	0.5070	0.4368
intrinsic water use efficiency (*WUEi*)	0.0125	0.6074	0.6672
water use efficiency (*WUE*)	0.0510	0.5076	0.6099
Fast kinetics of chlorophyll *a* fluorescence			
F_v/F_m	0.0042	0.0750	0.3000
Area	0.0143	0.1456	0.1549
δETo	0.7079	0.9087	0.4736
δREo	0.2902	0.8954	0.8220
ϕRE1o	0.2026	0.9076	0.8546
ABS/RC	0.2956	0.5071	0.3066
Piabs	0.6472	0.8447	0.4484
Slow kinetics of chlorophyll *a* fluorescence			
YII	0.0003	0.3524	0.1929
YNPQ	0.0620	0.4499	0.1738
YNO	0.0042	0.3664	0.1482
NPQ	0.0047	0.3288	0.0203
qN	0.0091	0.3592	0.1106
qP	0.0004	0.3829	0.0866
qL	0.0003	0.3505	0.0288
ETR	0.0004	0.3767	0.2190

Trait	Associated probability		
	Treatment	Provenance	T × P
Content of photosynthetic pigments			
chlorophyll *a*	0.3138	0.4241	0.5624
chlorophyll *b*	0.2322	0.1311	0.4898
chlorophyll *a* + *b*	0.2184	0.2448	0.5718
Carotenoids	0.1184	0.0702	0.3181
Content of proline and phytohormones			
proline	0.1378	0.1245	0.0904
abscisic acid (ABA)	< 0.0001	0.4647	0.6338
abscisic acid glucose ester (ABA-GE)	0.1618	0.8246	0.3905
indole-3-acetic acid (IAA)	0.5599	0.9535	0.1605
indole-3-acetyl-aspartate (IAA-Asp)	0.1302	0.0566	0.0078
jasmonic acid (JA)	0.2210	0.5978	0.9090
Content of isoprenoids			
α-pinene	0.0874	0.0034	0.0091
β-pinene	0.1131	0.0269	0.0674
camphene	0.0444	0.0047	0.0048
Δ-3-carene	0.4574	0.5924	0.1229
myrcene	0.1748	0.0395	0.1300
limonene	0.4774	0.0842	0.6336
γ-terpinene	0.2809	0.5024	0.0356
α-terpinolene	0.0006	0.0129	0.0013
sabinene hydrate	0.0737	<.0001	0.0107
camphor	0.2086	0.5211	0.0580
bornyl acetate	0.0013	0.0654	0.0044
Hyperspectral indices			
BGI1	0.2237	0.4200	0.6913
GI	0.5306	0.9453	0.4061
NGRR	0.6607	0.2789	0.6504
RGRI	0.8866	0.3919	0.5500
PRI1	0.0739	0.3677	0.2835
REIP	0.3383	0.8685	0.0598
$NDVI_{750}$	0.8603	0.0939	0.9116
NDVI	0.3886	0.0297	0.6320
mNDVI	0.5029	0.0039	0.4599
MCARI1	0.3703	0.0499	0.2439
CHLgreen	0.7258	0.6695	0.9439
ACI	0.1720	0.6573	0.8082
ARI2	0.4863	0.3346	0.5802
ANTH	0.7387	0.0006	0.0592
CRI2	0.6747	0.0145	0.2418
CAR	0.4785	0.0009	0.4194

Abbreviations: *Fv/Fm* – maximal quantum yield of PSII photochemistry, *Area* – area above the OJIP curve, *δETo* – probability with which a PSII trapped electron is transferred from PSII beyond reduced QA, *δREo* – probability with which a PSII trapped electron is transferred from reduced QA beyond PSI, *ϕRE1o* – probability with which a PSII trapped electron is transferred from PSII electron acceptor side to PSI acceptor side, *ABS/RC* – size of antenna complex per one active reaction centre, *PIabs* – photosynthetic performance index, *YII* – effective quantum yield of PSII, *YNPQ* – quantum yield of regulated energy dissipation in PSII, *YNO* – quantum yield of non-regulated energy dissipation in PSII, *NPQ* – non-photochemical quenching, *qN* – coefficient of non-photochemical quenching, *qP* – coefficient of photochemical quenching, *qL* – fraction of PSII centres that are open, *ETR* – electron transport rate, BGI1 – blue/green pigment index, – GI greenness index, NGRR – normalized green/red ratio, RGRI – red/green pigment index, PRI1 – photochemical reflectance index, REIP – red-edge inflexion point, $NDVI_{750}$ – red-edge normalized difference vegetation index, NDVI – normalized difference vegetation index, mNDVI – modified normalized vegetation index, modified chlorophyll absorption reflectance index 1 – MCARI1, CHLgreen – chlorophyll index at green range, anthocyanin content index – ACI , anthocyanin reflectance index – ARI2, ANTH – anthocyanin, CRI2 – carotenoid content index, CAR – carotenoids.

4. Discussion

4.1. Choice of physiological variables

As heat and drought mostly act together in nature and their physiological effects are hardly separable, our study focused on physiological parameters, which are influenced by these two climatic factors. Gas exchange, water use and photosynthesis are processes known to be affected by climatic extremes, and genes participating in their genetic control are supposed to be targeted by natural selection (Teskey et al. 2014). This was the motivation for the choice of parameters of fast and slow kinetics of chlorophyll a fluorescence, which are related to functioning of photosystem II (Banks 2018), as well as the contents of photosynthetic pigments. Another class of the analyzed bioactive substances included free proline and phytohormones (abscisic acid, indole-3-acetic acid, jasmonic acid and their derivatives), as they are also associated with drought and heat response (Wilkinson & Davies 2010; Ullah et al. 2018). Finally, volatile organic compounds (primarily monoterpenes) were targeted as substances emitted at high rates from plants and counteracting photooxidative stress (Tattini et al. 2015; Feng et al. 2019). Hyperspectral indices constituted the last group of the assessed variables, as they are considered to be a simple and cheap proxy of biochemical and physiological parameters, useful also for genomic and adaptation studies (Li et al. 2014; Roberts et al. 2018). Modern devices allow measurement of hyperspectral indices at a single-plant level, which allows both their use in manipulative experiments and upscaling to the stand level. We focused mainly on indices related to photosynthetic pigments and photochemical efficiency.

4.2. Neutral vs. adaptive variation

Climate, especially temperature regime and water availability, is generally considered a major factor affecting the distribution of genetic diversity among natural tree populations (Mosca et al. 2018; Jordan et al. 2020). This results in moderate to high levels of among-population genetic variation for adaptive traits along climatic gradients, documented by numerous common-garden experiments (König 2005; Alberto et al. 2013). However, in addition to local adaptation, parallel clines of nuclear gene frequencies and phenotypic traits may also result from neutral processes affecting the whole genome, such as genetic drift, gene flow, migration/colonization etc., combined with purely environmental basis of phenotypic expression. As already mentioned, disentangling these two groups of factors underlying the observed population structures is not easy, especially when geography correlates with environment. For instance, isolation by distance, i.e. decrease of dispersal rates with increasing

Table 3. Coefficients of phenotypic differentiation P_{ST} for the scored traits in control and drought-stressed seedlings.

Trait	Control			Drought		
	P_{ST}	CI–95%	c/h^2*	P_{ST}	CI–95%	c/h^2*
proline	0.1463	0.0192–0.6276	0.457			
IAA-aspartate				0.2932	0.0114–0.6171	0.779
α-pinene	0.2158	0.1123–0.5224	0.071			
β-pinene	0.1103	0.0385–0.4075	0.224			
camphene	0.2003	0.0981–0.5202	0.083			
myrcene	0.1150	0.0509–0.3910	0.168			
α-terpinolene	0.1274	0.0386–0.4469	0.224	0.2689	0.1655–0.5848	0.040
sabinene hydrate	0.4120	0.2447–0.6970	0.302	0.2104	0.0707–0.5738	0.050
camphor	0.0699	0.0158–0.3284	0.561			
bornyl acetate				0.1889	0.1018–0.4451	0.052
CO_2 assimilation rate	0.1358	0.0301–0.5550	0.289			
NPQ	0.1618	0.0436–0.5463	0.197			
qL	0.1106	0.0144–0.4431	0.615			
NDVI	0.0883	0.0104–0.3428	0.017	0.1886	0.0863–0.4634	0.095
mNDVI				0.1708	0.0774–0.4559	0.107
CRI2	0.1262	0.0158–0.4114	0.560			
CAR				0.2026	0.0932–0.4896	0.087
ANTH				0.1589	0.0572–0.4658	0.148

Abbreviations: see Table 2*the critical c/h^2 ratio, at which the confidence intervals of P_{ST} and F_{ST} touch.

geographic distance resulting in restricted gene flow, may produce geographical clines in gene frequencies parallel with climatic gradients, i.e. gradient of continentality in the longitudinal direction or gradient of photoperiod and temperatures with increasing latitude (Savolainen et al. 2007; Meirmans 2012). Migration routes (in Europe, mainly in the context of Holocene colonization), which often follow latitudinal gradient, may also produce clines, as colonization is frequently associated with founder events at the front edge of migration, recurrently depauperating gene pools of newly established populations (Comps et al. 2001). The effects of these processes need not necessarily affect all loci identically, but they do not avoid genes with adaptive significance and may produce falsely positive correlations between environmental variables and allele frequencies. This was the main motivation for limiting the geographical extent of our study. Of course, spatial scale is a matter of the steepness of the environmental gradient and the reach of gene dispersal rather than physical distances among populations (Vasemägi & Primmer 2005). In our case, the number of provenances was too small for a reliable testing of isolation by distance. Nevertheless, spatial scale of pollen dispersal in various spruce species is generally much bigger than the size of the territory covered by the current study (Burczyk et al. 2004; O'Connell et al. 2007; Haselhorst et al. 2019); except differences in flowering phenology associated with altitude, we do not suppose any barriers in gene exchange among the studied provenances. Moreover, there is no evidence for mixing of different gene pools in Slovakia, as all provenances very probably have identical glacial origin. Norway spruce was widely distributed in Central Europe during the Vistulian period and refugial populations were present in the Western Carpathians also during the full glacial (Ravazzi 2002; Latalowa & van der Knaap 2006), while genetic data also suggest common origin (Gömöry 1992; Tollefsrud et al. 2009). There is also no indication of former bottlenecks, founder events or similar phenomena. The signals of selection observed in our study are thus very unlikely to have been produced by neutral processes.

Phenotypic differentiation coefficients P_{ST} are not always reliable indicators of selection. P_{ST} differs from Q_{ST}, as the phenotypic variation confuses the genetic and environmental component (Pujol et al. 2008). P_{ST} reflects also those components of phenotypic differentiation, which are due to non-additive genetic effects, such as dominance and epistasis, or epigenetic carryover effects. This problem may be partially overcome by using a provenance experiment, where all seedlings have been raised and treated in identical way in a nursery, which means that environmental variation is expected to be the same across all tested provenances. Consequently, the c/h^2 ratio is expected to approach 1, which is the null assumption as formulated by Brommer (2011). Nevertheless, quantification of the critical c/h^2 ratio, at which the confidence intervals of P_{ST} and F_{ST} begin to overlap, allows testing the robustness of this expectation: the more the critical ratio differs from one, the stronger is the evidence that selection (divergent selection in our case) is the mechanism underlying phenotypic differentiation.

Among the measured physiological traits, monoterpene contents appeared to be most differentiated. Generally, the contents of isoprenoids show high heritability (Tognetti et al. 2000), and even monogenic control was suggested for several monoterpenes (Yazdani et al. 1982). Several isoprene derivatives are known to participate in adaptation to environmental stresses (Loreto & Schnitzler 2010). Even though mechanisms of response to abiotic stress are not exactly known, terpenes are supposed to counteract reactive oxygen species, which are induced by heat or drought stress (Kopaczyk et al. 2020). However, terpene emissions in conifers vary among populations (Tognetti et al. 2000; Kleiber et al. 2017; van Meeningen et al. 2017), which may reflect adaptive response to past selective pressures. Another group showing signals of

divergent selection is represented by hyperspectral indices, which represent a heterogeneous set of parameters reflecting vegetation structure, biochemistry and physiological processes. In this context, it was surprising that phenotypic differentiation was absent for directly measured contents of photosynthetic pigments, while hyperspectral indices quantifying carotenoids or anthocyanin showed significant P_{ST}. However, it must be noted that reflectance spectra are always related to a wider complex of factors, which means that they are generally polygenic. Higher number of underlying quantitative trait loci (QTL) combined with loci potentially affecting expression levels of QTL offer broader possibilities for adaptive differentiation. In contrast to the mentioned two groups of physiological variables, gasometric and chlorophyll fluorescence traits generally do not show signal of divergent selection, maybe because of a low heritability (Čepl et al. 2016). In our case, we found only three exceptions, namely the CO_2 assimilation rate and two parameters of slow chlorophyll *a* fluorescence kinetics.

The results suggest that the phenotypic response to selection in physiological traits depends from the environmental context. Except normalized difference vegetation index, α-terpinolene and sabinene hydrate contents, the evidence for inter-provenance phenotypic differentiation is inconsistent between control and drought-stressed seedlings. The outcomes of ANOVA showed at least partly the same: in many traits the treatment-by-provenance interaction is significant, implying that the response to drought stress is provenance-specific. This may indicate that the basis of inter-provenance variation is heritable but not necessarily genetic. Genetic control of most studied physiological traits is unknown and probably polygenic. Plastic treatment-dependent trait expression is an indication of a strong epigenetic component in trait variation. Conifers with their huge genomes are prone to epigenetic regulation of phenotypic variation, which includes not only trans-generation memory but also transient or short-term effects (Bräutigam et al. 2013). A wide range of epigenetic mechanisms could provide a means for altering gene expression after stress events (Bruce et al. 2007). Epigenetics as such is not in contradiction with the hypothesis of divergent selection as the basis of the observed variation: along with allelic variation at genes controlling physiological traits, selection may target also sequences or sites regulating their activity, such as miRNAs sequences or differentially methylated sites (Browne et al. 2021).

5. Conclusions

The observed inconsistency between treatments is an indication that drought affects the expression of QTL underlying the respective traits (Verta et al. 2013). For example, terpene profiles of conifers (including Norway spruce) are drought-responsive, while the response was shown to be population-specific both in manipulative experiments and *in situ* observational studies (Turtola et al. 2003; Winner et al. 2004; Lüpke et al. 2016). The role of allelic variation at trait-controlling structural genes vs. regulatory sequencies is thus an issue requiring further clarification. Nevertheless, a caveat demonstrated by this study is that phenotypic differentiation levels depend from the environmental setup of the experiment. This is important when physiological traits are intended to guide the choice of seed sources and transfer of forest reproductive materials. Practical application of such traits for this purpose must be preceded by a detailed assessment of their variation on a large set of populations representative for the territory of interest, allowing identification of geographical trends (which naturally is not feasible with five provenances), while the measurement needs to be performed under stressful conditions.

On the other hand, the study also demonstrated the value of common gardens in adaptation research (Lepais & Bacles 2014). Provenance experiments (conducted either under field conditions, in a nursery, a laboratory, a climatic chamber, a phytotron etc.) allow both an at least partial elimination of environment-induced phenotypic variation and studying genotype-by-environment interaction in phenotypic responses to environmental stresses.

Acknowledgements

This work was supported by the grants of the Slovak Grant Agency for Science VEGA 1/0029/20 and the Slovak Research and Development Agency APVV-21-0270. The help of the AgroBioTech Research Centre of the Slovak University of Agriculture in Nitra, namely Marián Brestič, Marek Kovár and Marek Živčák, with recording of hyperspectral indices is highly appreciated. We are also grateful to Miroslav Blaženec, Peter Fleischer, Hana Húdoková, Anna Kracinová, Eva Pšidová and Lenka Sarvašová for their technical assistance during the experiment.

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