Tracing the acclimation of European beech (*Fagus sylvatica* L.) populations to climatic stress by analyzing the antioxidant system

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Through a common garden (provenance) experiment, we investigated the metabolic responses to climatic stress with regard to the acclimation potential of different European beech (*Fagus sylvatica* L.) populations. Selected enzymatic and non-enzymatic antioxidants were analyzed in leaves. Peroxidase (POD) and polyphenol oxidase (PPO) enzyme activity, total protein content as well as ABTS [2,2'-azino-bis-(3-etylbenzothiazoline)-6-sulphonic acid] antioxidant capacity were measured in the leaves of selected populations. Major leaf polyphenols were identified and their relative amounts were compared. Significant correlations were found between phenotypic (diameter growth) response to simulated climatic stress and the activity (and amount) of selected chemical components. The concentrations of certain polyphenols, POD enzyme activity, and total protein content may be chemical indicators of the acclimation potential of populations and may contribute to the forecasting of climate change effects, which can aid in the selection of suitable propagation material for adaptive silviculture.

Keywords: Beech, Drought Stress, Antioxidants, Phenotypic Plasticity, Provenance Trial, Climate Change

Introduction

The global decline of forests is an apparent consequence of climate change (Allen et al. 2015). Various analyses including those of common garden tests, large scale inventory data, or modeling growth along ecological/climatic transects confirmed the negative effects that increasing warming and decreasing precipitation have on growth and vitality at the species level. These analyses also emphasize the role of genetics in climatic resilience (Linnakoski et al. 2019).

European beech (*Fagus sylvatica* L.), a common stand-forming forest tree species in Europe, is currently facing decline and even local extinction in areas exposed to hotter and more extreme droughts, especially at the xeric, low-elevation distribution limits (Mátyás et al. 2010, Stojanovic et al. 2013). Compared to conifers (Sáenz-Romero et al. 2019), beech has demonstrated higher adaptability and acclimation potential. In a European network of common gardens, beech has displayed modest between-population phenotypic differentiation, indicating a good adaptability of the species (Von Wuehlisch & Alia 2011).

To maintain functional flexibility under fluctuating conditions, sessile organisms with extremely long life cycles, such as forest trees, are highly dependent on mechanisms to sustain physiological and developmental processes. During acclimation to stress, photosynthesis products are allocated to secondary metabolites that influence growth. Types and concentrations of metabolites may vary by species and by genotypes within species (Isah 2019). Although such research has already been conducted for some plant species (Grace & Logan 1996, Pennycooke et al. 2005), results for forest trees are limited to cuttings (Popovic et al. 2016, Zhang et al. 2012) or involve mature trees growing in forest ecosystems (Luwe 1996, Haberer et al. 2008, Zolfaghari et al. 2010).

Within-species genetic differences may influence the vulnerability of beech populations, especially at the continental (Berki et al. 2009, Lakatos & Molnár 2009, Czúcz et al. 2011) and Mediterranean habitats (Penuelas et al. 2007). Research on the role of metabolic processes and their links to inherited resilience in acclimation may support the development of adaptive measures in forestry in view of projected changes.

Through biochemical analysis focused on the antioxidant system – a major defense pathway of plants – the present work assessed stress amounts and the effects these had on European beech. Most stress factors trigger oxidative stress, which is interpreted as a shift in the balance of oxidants and antioxidants toward the dominance of the oxidants (Sies 1991). Oxidative stress is mostly realized by reactive oxygen species (ROS) that are normally present in the biochemical processes of living organisms. ROS levels rise during periods of oxidative stress, which subsequently affects various parts of living cells (Foyer & Noctor 2005) by triggering reaction chains that cause irreversible damage to living organisms. Plants activate their complex antioxidant pathways to block cell and tissue damage, which has been the subject of numerous studies and reviews (Del Río 2015, Gupta et al. 2018).

Regarding molecular structure, antioxidants can be enzymatic and non-enzymatic. Polyphenols are important types of non-enzymatic antioxidants that participate in defense reactions against biotic and abiotic stress in plants (Dalmagro et al. 2018, Achmadi 2019). Furthermore, polyphenols are also responsible for color effects (plant pigments), biochemical signaling in ripening, growth processes, and hormone regulating effects (Tanase et al. 2019). The most recent and detailed investigation on beech leaf polyphenols was conducted by Cadahía et al. (2015), Aranda et

al. (2017) and Hofmann et al. (2017a). These studies used the HPLC-MS/MS technique to identify the main polyphenolic compounds in beech leaves.

The most important enzymes involved in oxidative stress defense are catalase, superoxide dismutase, and the enzymes responsible for glutathione transformation (oxidase, peroxidase, and reductase). The investigation of the enzymatic antioxidant system in the stress response of beech has proven that peroxidase (POD) and polyphenol oxidase (PPO) enzymes participate in defense reactions and can, thereby, be possible indicators of acclimation triggered by climatic stress (Haberer et al. 2008, Zolfaghari et al. 2010, Visiné Rajczi et al. 2018). Earlier allozyme study results support the climatic sensitivity of peroxidases. Significant geographic trends in both the latitudinal (Comps et al. 1998) and altitudinal (Comps et al. 1990) distribution of peroxidase isomers have been observed, which may – parallel to random impacts – also indicate the effect of climatic gradients on allozyme frequency across the European range of beech. As both enzymatic and non-enzymatic antioxidants are essential to the proper life functions of living organisms, we have supposed that the concentration of certain polyphenols and antioxidant enzyme activity could be chemical indicators of the acclimation (phenotypic plasticity) potential of populations. These indicators could forecast future responses to climate change within the populations.

The present work investigated the level changes of selected compounds in the leaf antioxidant systems of different beech provenances as they reacted to translocation stress. Acclimation stress intensity was approximated by the environmental change of populations being transferred from their places of origin to a common garden test, thereby providing a "space-for-time" projection of expectable future responses. Our study is the first to investigate the effect of acclimation stress on antioxidant types and concentrations in different European beech provenances. The research aimed to prove that the differentiated responses to stress exposure of beech provenances can be tracked by monitoring selected antioxidants. Some of these compounds may characterize the acclimation processes at the molecular level, and their changes may provide insights into drought-triggered processes in forest trees, thus helping to develop strategies for future afforestation.

Materials and methods

Basic experimental data and the investigated provenances

The European Cooperation in Science and Technology "Evaluation of Beech Genetic Resources for Sustainable Forestry" COST BeechE52 – a European research consortium – established a network of close to 20 beech provenance tests across Europe in 1998 (Von Wuehlisch & Alia 2011). The test contains 36 different provenances, predominantly from Western and Central Europe (Fig. 1). In these regions, nearly all the provenances in the network experience warmer and drier climates than they would at their origin sites (Horváth & Mátyás 2014). Seedlings were centrally raised in a nursery garden near Hamburg (Germany). After two years, the seedlings were transferred to the European trial sites. The Hungarian experimental site within the network was established in the south-western part of the country near Bucsuta (Zala County). Experimental plots were arranged randomly, with three repetitions (Horváth & Mátyás 2014, 2016). The Hungarian location is particularly interesting for studying acclimation because it is situated at the low-elevation edge of the climate-zonal distribution of European beech, *i.e.*, at the xeric limit.

Six provenances with largely varying original climatic backgrounds were selected for the current study. The populations from Farchau (Germany), Torup (Sweden), and Gråsten (Denmark) have adapted to a cooler and wetter Atlantic climate. Pidkamin originates from the eastern-continental limit of the species in the Ukraine. Magyaregregy and Bánokszentgyörgy, represent the two Hungarian provenances, with the latter containing trees of local origin. Tab. 1 lists the main climatic data of provenances. The mean diameter of the selected representative trees within the populations, which were used for correlating biochemical parameters with phenotype, were measured during the annual leaf sampling of trees that were 16-19 years old (see Tab. S1 in Supplementary material).

Climate data

Fifty years of temperature and precipitation data (1951-2000) for the original provenance locations were extracted from the WorldClim database (Hijmans et al. 2005). Weather data from the nearest (18 km) meteorological station in Nagykanizsa were used for the Bucsuta test site (Horváth & Mátyás 2014). Employed here as a drought index, Ellenberg's climate quotient (EQ – Ellenberg 1988) was calculated from the temperature quotient of the hottest month (July in Central Europe, T_{07}) and annual precipitation (P_{ann} – eqn. 1):

$$EQ = 1000 \cdot T_{07} \cdot P_{ann}^{-1} \tag{1}$$

To characterize the climate at the original locations to which the populations were adapted, the EQ quotients were calculated from the 50-year mean July temperatures (T_{07}, °C) and the 50-year mean annual precipitations (P_{ann}, mm). Fifteen-year averages (1998-2013), from planting to measurement, were used for the EQ value cal-
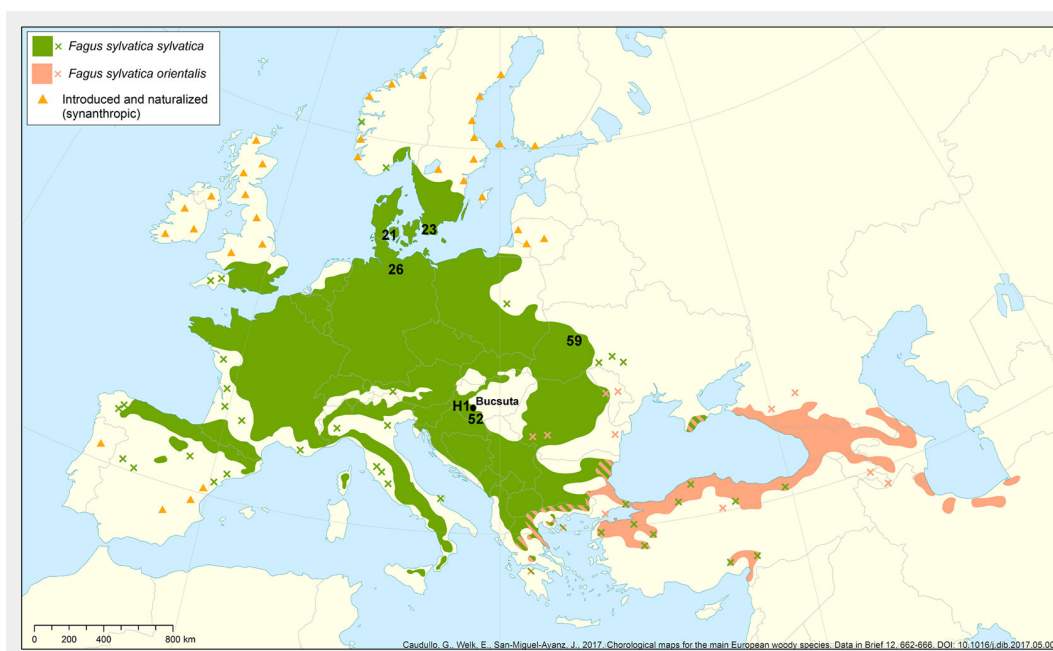

Fig. 1 - The provenances represented in the beech provenance test in Bucsuta (Hungary) with the investigated populations: 21: Gråsten (DK); 23: Torup (S); 52: Magyaregregy (H); H1: Bánokszentgyörgy (H); 59: Pidkamin (UA); 26: Farchau (D). Adapted from Caudullo et al. (2017).

culation of the Bucsuta test site, representing the reference period to which the trees were responding.

Ecodistance (ΔEQ) was calculated as the climate difference between the test location and the original location (Mátyás 1994). For the assessment of climatic change due to population transfer, we used the difference between the earliest available past climate data for the origin of a provenance and the data for the 15-year test period in Bucsuta (Horváth & Mátyás 2016). Depending on the EQ value of the original location, negative ecodistance values represent cooler/wetter conditions, while positive differences reflect warmer/drier conditions for the transferred populations at the test site.

Sample collection for chemical analysis

For the polyphenol and ABTS antioxidant capacity investigations, a sample collection was conducted at the end of June 2014. The sample collection was as follows: eight trees were assigned from each of the investigated six provenances and altogether 40 leaves were sampled randomly from two different positions (sunned leaves and shaded leaves) of each tree canopy. Leaf samples were put in dry ice immediately after collection and stored there until extraction.

Sample collection for the investigation of the enzymatic antioxidant system was completed at the end of June, in three subsequent years (2015-2017). Four trees were selected from the previously assigned eight trees in each of the six provenances. Leaf collection (20 randomly picked sunned leaves and 20 shaded leaves from each tree) and sample storage were executed as previously described.

Enzyme activity analysis

Frozen leaf samples were transferred to a grinder and ground into a fine powder in a frozen state. About 0.5 g leaf powder was homogenized vigorously with 10 ml phosphate puffer (pH: 5.6, with 80 g L^{-1} PVP40) for four minutes then centrifuged at 6000 min^{-1} for 10 minutes. The supernatant was collected and taken to analysis. Total protein content was assayed according to Bradford (1976). Bovine serum albumin (92%) was used as a standard to quantify total protein content of the samples. The POD enzyme activity was determined using the method of Shannon et al. (1966) measuring absorbance change at 480 nm and regarding 0.01 ΔA min^{-1} as 1 Unit. The PPO enzyme activity was assayed according to the method of Flurkey & Jen (1978) at 420 nm, taking 0.001 ΔA min^{-1} as 1 Unit. All measurements were conducted in triplicates.

Investigation of non-enzymatic antioxidants

Extraction

Leaves were treated for two minutes by applying 750 W microwave energy to inac-

tivate their polyphenol-oxidizing enzymes and avoid the oxidation of polyphenols during the extraction process (Hofmann et al. 2015). Leaves were ground and 0.15 g of the ground powder was extracted with 15 ml methanol:water 80:20 v/v by stirring for 24 h in the dark. Extracts were filtered through a 0.45 μm cellulose acetate membrane filter.

ABTS assay of antioxidant capacity

The ABTS assay was run as described by Stratil et al. (2007) at 734 nm, using the ABTS•+ radical ion and trolox standard. Reaction time was 10 min. ABTS antioxidant capacity was evaluated as mg trolox g^{-1} dry leaf units. Measurements and evaluations were run in triplicates.

The HPLC-PDA-ESI-MS/MS separation and relative quantitative determination of leaf polyphenols

The separation and quantitative assessment of leaf polyphenols was completed using high-performance liquid chromatography separation and photodiode array as well as tandem electrospray mass spectrometry detection (HPLC-PDA-ESI-MS/MS). In an earlier study, we identified 44 compounds, 38 of which were identified by name (Hofmann et al. 2017a).

For chromatographic separation, a Shimadzu LC-20® type high-performance liquid chromatograph was used. This was coupled to a Shimadzu SPD-M20A® type diode array detector (PDA – Shimadzu Corporation, Kyoto, Japan) and an AB Sciex 3200 QTrap® triple quadrupole/linear ion trap LC/MS/MS detector (AB Sciex, Framingham, MA, USA). A Phenomenex Kinetex C18®, 150 × 4.6 mm, 2.6 μm core-shell column was applied for the separation at 40 °C. The mobile phase (1.2 mL min^{-1}) gradient of A (H_2O + 0.1% HCOOH) and B (CH_3CN + 0.1% HCOOH) was run as follows: 10% B (0-1 min), 12% B (8 min), 18% B (10 min), 22% B (13 min), 28% B (19 min), 98% B (23 min), 98% B (23-32 min), 10% B (33 min), 10% B (33-40 min). Prior to chromatographic separation, extracts were diluted two-fold with pure methanol:water 80:20 v/v solution and 4 μl of the diluted extracts were injected. The

PDA detection was executed in the wavelength range of 250-380 nm. Flow-splitting was applied in front of the mass spectrometer using a split valve, which allowed 0.6 mL min^{-1} flow to enter the ion source. Negative electrospray ionization mode with the following settings was employed: ion spray voltage: -4500V; curtain gas (N_2) pressure: 30 psi; spray gas (N_2) pressure: 40 psi; drying gas (N_2) pressure: 30 psi; ion source temperature: 500 °C.

The MRM (multiple reaction monitoring) transitions, characteristic to the mass spectrometric fragmentation of each of the compounds as well as compound-optimized settings of the mass spectrometer, used for subsequent quantitative analysis, were determined by the direct infusion of the extracts into the mass spectrometer as described in Hofmann et al. (2017a). Quantitative assessment of the compounds was achieved using relative quantification, which involved the determination of peak areas by monitoring the respective MRM channel for each compound. Chromatographic data were acquired and processed using the software Analyst® v. 1.6.1.

Chemicals

Conventional distillation equipment was used to produce double distilled water for extraction and chromatography. Acetonitrile (LC-MS grade) was obtained from VWR-International (Budapest, Hungary). Potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), sodium hydrogen phosphate, potassium dihydrogen phosphate, formic acid (98%), bovine serum albumin (92%), 3,3'-diaminobenzidine, polyvinylpyrrolidone (PVP-40), and Whatman GF/A glass fiber filter paper were procured from Sigma-Aldrich (Budapest, Hungary). Pyrocatechin, Coomassie Brilliant Blue G-250, ethanol, methanol, phosphoric acid, hydrogen peroxide were purchased from Reanal (Budapest, Hungary). Quercetin was obtained from Carl Roth GmbH (Karlsruhe, Germany).

Tab. 1 - Main geographic and climatic data (including EQ) of sampled provenances at the original location for the period 1951-2000 and their ecodistance (ΔEQ), calculated for the test site Bucsuta (from Horváth & Mátyás 2014). (T_{07}): July mean temperature; (P_{ann}): annual precipitation; (ΔEQ): eco-distance (see text).

Provenance (reg. number)	Country	Elev. (m a.s.l.)	T_{07} (°C)	P_{ann} (mm)	EQ index	ΔEQ
Farchau (26)	Germany	55	17.3	676	25.6	3.86
Pidkamin (59)	Ukraine	-	18.1	612	29.6	-0.13
Torup (23)	Sweden	40	16.6	634	26.2	3.27
Gråsten (21)	Denmark	45	15.8	780	20.3	9.19
Magyaregregy (52)	Hungary	400	19.0	707	26.9	2.57
Bánokszentgyörgy (H1)	Hungary	200	20.0	747	26.8	2.67
Bucsuta test site	*Hungary*	*220*	*20.8*	*707*	*29.4*	*-*

Tab. 2 - Correlation matrices using the data from 2015, 2016, and 2017 (see also Tab. S1 in Supplementary material). Significant correlations (for p < 0.15 for n=6; |R| ≥ 0.664) are marked with an asterisk.

Year	Variables	Total protein content (µg g⁻¹)	POD (U ug⁻¹)	PPO (U ug⁻¹)	Ecodistance ΔEQ	Average stem diameter (cm)
2015	Total protein content (µg g⁻¹)	1.000	-0.674*	-0.755*	0.854*	-0.251
	POD (U ug⁻¹)	-	1.000	0.161	-0.808*	0.575
	PPO (U ug⁻¹)	-	-	1.000	-0.439	-0.393
	ΔEQ	-	-	-	1.000	-0.341
	Average stem diameter (cm)	-	-	-	-	1.000
2016	Total protein content (µg g⁻¹)	1.000	-0.286	-0.075	0.708*	-0.032
	POD (U ug⁻¹)	-	1.000	0.430	-0.474	0.833*
	PPO (U ug⁻¹)	-	-	1.000	-0.737*	0.583
	ΔEQ	-	-	-	1.000	-0.339
	Average stem diameter (cm)	-	-	-	-	1.000
2017	Total protein content (µg g⁻¹)	1.000	-0.024	0.016	0.679*	0.191
	POD (U ug⁻¹)	-	1.000	-0.310	-0.445	0.941*
	PPO (U ug⁻¹)	-	-	1.000	-0.306	-0.068
	ΔEQ	-	-	-	1.000	-0.354
	Average stem diameter (cm)	-	-	-	-	1.000

Statistical evaluation

Biochemical results were correlated with the climatic parameters (Ellenberg index: EQ, and ecodistance: ΔEQ) as well as with provenance growth (average stem diameter). Correlation analysis was implemented by the evaluation of Pearson correlation coefficients. For the comparison of samples, ANOVA analysis was run by applying the Tukey HSD calculation method for *post-hoc* tests. In order to fulfil the requirements of the ANOVA analysis, measurement values were first checked for normal distribution, and then the variables were inspected for the homogeneity of variances using Bartlett's Chi-square test. All statistical tests were performed using Statistica® v. 12 (StatSoft Inc., Tulsa, OK, USA).

Results

Enzymatic antioxidants

Data of this primary evaluation of POD, PPO, and leaf protein content are presented in Tab. S1 (Supplementary material). A significant (p<0.05) increase of the total protein content was found in some of the provenances from 2015 to 2017 (Farchau, Pidkamin, Magyaregregy); however, this tendency was not observed in the other provenances. No significant change was discovered in the POD and PPO activities by comparing different years of sampling, and there were only a few notable changes of activity among provenances within each year.

As the simple ANOVA evaluation of primary data did not show any apparent and unequivocal results, linear correlation analysis was applied to find possible relationships between chemical parameters as well as growth characteristics and ecodistance. Linear correlation analysis results are summarized in Tab. 2. According to these, there was a significant positive correlation (p<0.15) between average stem diameter and POD enzyme activity in 2016 and 2017, while in 2015 the relationship displayed the same tendency; however, this tendency proved insignificant.

Fig. 2 depicts the correlation equation of 2017, which clearly shows that the Atlantic provenances with poorer growth and survival rates (Gråsten, Torup) display the lowest POD enzyme activity. Interestingly, Pidkamin is located closest to the weak performing provenances in Fig. 2 and was characterized by low POD activity and stem diameter. Vigorous height growth was observed in this provenance (not shown), contrary to low diameter, which is not reflected in Fig. 2.

PPO enzyme has shown a negative correlation with ecodistance, yet the relationship was significant only in 2016. Ecodistance was also correlated negatively with POD, but significance was only evidenced for the 2015 data.

There was a notable positive correlation between total protein content and ecodistance in all three investigation years. Provenances that had originally adapted to a warmer and drier climate (*e.g.*, Pidkamin) had lower total protein levels and showed better growth performance compared to the provenances with higher ecodistance (*e.g.*, Gråsten).

Fig. 3 displays the correlation for 2017. Fig. 3 also demonstrates the shifting of the climate index of Bánokszentgyörgy, next to Bucsuta, which is regarded as a local control population. This population should have been theoretically positioned at the ΔEQ=0 position in Fig. 3, but the EQ value of the local climate has already shifted due to the warmer and drier weather of the past 15 years. The Hungarian provenance Magyaregregy appears as an outlier. Investigations have proven that this provenance is of unknown origin, and that it was planted as part of afforestation in an area of decayed forests. Furthermore, it has grown under the influence of local climate.

The non-enzymatic antioxidant system

Primary results

The results on the ABTS antioxidant capacity as well as average stem diameter from 2014 and Ellenberg's climate quotient are summarized in Tab. 3.
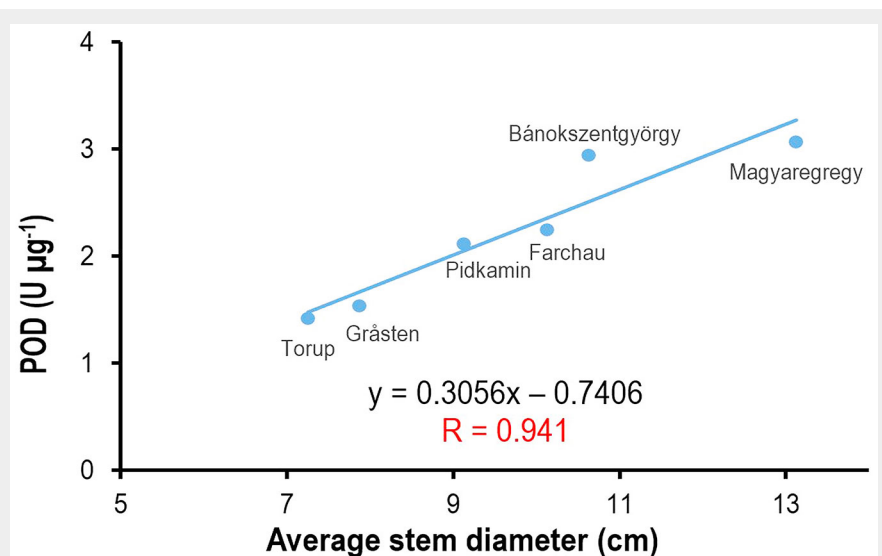

Fig. 2 - Correlation between average stem diameter and POD enzyme activity using the data from 2017.

$$y = 0.3056x - 0.7406$$
$$R = 0.941$$

According to Tab. 3, the provenances with the poorest growth parameters (Gråsten, Torup) had the highest ABTS levels in their leaves, yet differences were not always significant. Many types of compounds contribute to ABTS antioxidant capacity. Fig. S1 in Supplementary material shows an example of a PDA chromatogram of beech leaf extract.

The amounts of individual compounds were assessed by their respective MRM peak areas instead of determining absolute concentrations as detailed above. The MRM peak areas corresponding to one given compound in the eight individual trees of a given provenance were averaged. The averaged peak areas for each compound and provenance are included in Tab. S2 (Supplementary material). According to Tab. 3 and Tab. S2, apparent differences existed not only in ABTS antioxidant capacity, but also in polyphenolic composition. In the poorest growing provenances (previously characterized with the highest ABTS values), the levels of some polyphenols were also the highest (*e.g.*, Caffeic acid-*O*-hexoside, Unknown 2; Quercetin-*O*-hexoside 1 and 2; Quercetin-*O*-pentoside; Kaempferol-*O*-pentoside) or, surprisingly, the lowest (Unknown 1, 3 and 6; Procyanidin B dimer 5 and 6; Procyanidin C trimer 6).

Correlation analysis of primary data

From the Tab. 3 data it was assumed that translocation induced more intense stress in the individuals of these provenances. It was also assumed that the elevated ABTS
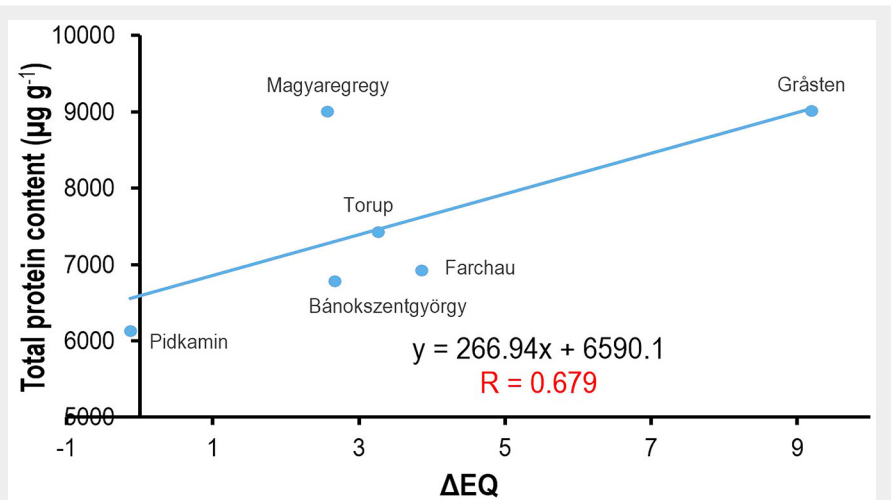

Fig. 3 - Correlation between ecodistance (ΔEQ) and total protein content using the data from 2017.

$$y = 266.94x + 6590.1$$
$$R = 0.679$$

Tab. 3 - Average stem diameters, ABTS antioxidant capacity, and Ellenberg's climate quotient (EQ) of the investigated provenances for the samples from 2014. Results are given as mean ± std. deviation when applicable. Significant differences at p<0.01 level (n=8) are denoted with different upper case letters in a given column.

Provenance	ABTS (mg trolox g⁻¹ dw)	Average stem diameter (cm)	EQ
Farchau (26)	120.7 ± 49.7 [a]	6.4 ± 2.5 [a]	25.59
Pidkamin (59)	155.8 ± 27.9 [a]	7.4 ± 1.7 [ab]	29.58
Torup(23)	202.1 ± 33.2 [ab]	5.1 ± 2.2 [a]	26.18
B.szentgyörgy (H1)	163.5 ± 78.7 [a]	8.6 ± 2.8 [ab]	26.77
M.egregy(52)	178.2 ± 54.9 [a]	11.4 ± 4.6 [b]	26.87
Gråsten (21)	296.2 ± 84.4 [b]	5.6 ± 1.7 [a]	20.26

Tab. 4 - Correlation of polyphenol levels with ABTS levels, Ellenberg's climate quotients (EQ), as well as with average stem diameter (ASD). Statistically significant (p < 0.05, n=6; |R| ≥ 0.812) correlation coefficients are marked with an asterisk.

Compound	ABTS	EQ	ASD	Compound	ABTS	EQ	ASD
Quercetin-*O*-hexoside 1	0.937*	-0.756	-0.202	Coniferin isomer	-0.359	-0.265	-0.197
Coniferin derivative 2	0.919*	-0.706	-0.001	Coniferin derivative 3	-0.392	0.791	0.311
(−)-Epicatechin	0.903*	-0.781	-0.429	Procyanidin B dimer 5	-0.558	0.493	0.808
Quercetin-*O*-hexoside 2	0.889*	-0.678	-0.107	Procyanidin C trimer 8	-0.561	0.872*	0.216
Quercetin-*O*-pentoside	0.876*	-0.693	-0.256	Unknown 1	-0.572	0.677	0.790
(+)-Catechin	0.873*	-0.834*	-0.084	Unknown 3	-0.594	0.707	0.677
Caffeic acid-O-hexoside	0.872*	-0.632	-0.393	Procyanidin B dimer 6	-0.639	0.097	0.137
Procyanidin C trimer 3	0.870*	-0.851*	-0.222	Procyanidin C trimer 7	-0.665	0.784	0.057
Procyanidin B dimer 4	0.825*	-0.838*	-0.143	Feruloylthreonic acid	-0.683	0.314	0.679
Procyanidin C trimer 4	0.817*	-0.757	-0.312	Procyanidin C trimer 5	-0.694	0.304	0.379
Kaempferol-O-hexoside 2	0.815*	-0.627	-0.059	Naringenin-*C*-hexoside 2	-0.733	0.539	0.344
Quercetin-*O*-glucuronide	0.811	-0.555	-0.445	Unknown 4	-0.759	0.656	0.582
Procyanidin B dimer 2	0.761	-0.513	-0.330	Unknown 5	-0.785	0.793	0.642
Kaempferol-*O*-hexoside 1	0.740	-0.505	-0.014	Naringenin-*C*-hexoside 1	-0.807	0.638	0.304
Kaempferol-*O*-pentoside	0.732	-0.531	-0.156	Naringenin-*C*-hexoside 3	-0.824*	0.672	0.237
Unknown 2	0.650	-0.435	-0.078	Procyanidin C trimer 2	-0.829*	0.869*	0.075
Procyanidin C trimer 1	0.605	-0.525	0.192	Procyanidin B dimer 1	-0.849*	0.441	0.033
Coniferin derivative 1	0.534	0.007	-0.098	Procyanidin B dimer 8	-0.849*	0.778	0.013
Chlorogenic acid isomer 2	0.519	-0.200	-0.174	Unknown 6	-0.900*	0.848*	0.658
Chlorogenic acid isomer 1	0.382	0.163	0.514	Procyanidin B dimer 3	-0.908*	0.574	0.498
Chlorogenic acid isomer 3	0.251	0.135	0.765	Procyanidin C trimer 6	-0.944*	0.688	0.497
Kaempferol-*O*-deoxyhexoside	-0.343	0.660	0.469	Procyanidin B dimer 7	-0.954*	0.782	0.172

antioxidant capacities may be a response to this stress.

We surmised that a significant positive correlation between ABTS antioxidant capacity levels and the concentration of a given compound indicates a strong influence on the antioxidant properties of the leaf extracts; hence, it is an "efficient" antioxidant compound, which is likely to contribute significantly to the stress response of the plant (Hofmann et al. 2017b).

Tab. 4 summarizes the correlation analysis results. According to these, Quercetin-O-hexoside 1 (R=0.937), Quercetin-O-hexoside 2 (R=0.889), Coniferin derivative 2 (R=0.919), (+)-Catechin (R=0.873), (−)-Epicatechin (R=0.903), Quercetin-O-pentoside (R=0.876), Caffeic acid-O-hexoside (R=0.872), Kämpferol-O-hexoside 2 (R=0.815), Procyanidin B dimer 3 (R=0.825), Procyanidin C trimer 3 (R=0.870) and Procyanidin C trimer 4 (R=0.817) were the most efficient antioxidants. Interestingly, some compounds also indicated noteworthy negative correlations (R<-0.812). A possible explanation is that these compounds may have pro-oxidant effects in beech leaf extracts assessed by the ABTS method. The antioxidant behavior of isomers (especially those of Procyanidin B and C isomers) seems to be markedly different. Generally, the antioxidant and radical scavenging abilities of condensed procyanidins increases with the growing degree of polymerization (DP – Pedan et al. 2016, Zhang et al. 2017), yet evidence for significant differences between the antioxidant efficiency of procyanidin isomers with similar DP also exists (Hofmann et al. 2017b).

Obviously, many types of compounds contribute to the antioxidant capacity of leaf extracts. This raises the question of whether there are any special indicator compounds that influence ABTS antioxidant capacity significantly and also act as indicators of translocation stress response. To examine these effects, a linear correlation analysis between polyphenol concentration and EQ and average stem diameter was completed (Tab. 4).

The correlations have shown that the provenances with higher EQ (originating from warmer and more arid regions of Europe) had lower levels of some of the most efficient antioxidant compounds, indicated by a significant negative correlation ((+)-Catechin, Procyanidin C trimer 3 and Procyanidin B dimer 4). It may be concluded that the provenances originally adapted to a drier and warmer climate do not tend to produce efficient antioxidant polyphenols in excess, as they are better acclimated to the warmer/drier test conditions in Bucsuta. These provenances have also shown better diameter growth compared to low EQ provenances with weaker acclimation potential. Weaker acclimation potential of provenances to local conditions was not only evidenced by smaller diameters, but also by a higher tree mortality rate (data not shown).

Findings are also in accordance with earlier results on growth responses (Horváth & Mátyás 2016). Average stem diameter, as a direct measure of provenance growth and performance, showed no significant correlation with either of the identified compounds. Interestingly, some compounds (Procyanidin C trimer 2 and 8, Unknown 6) have shown elevated levels (significant positive correlations with the EQ value) in these provenances. This finding requires further explanation.

Discussion

In general, increased antioxidant concentrations are associated with increased stress, caused mostly by elevated ozone levels, UV radiation, drought, and extreme temperature conditions in forest trees (Luwe 1996, Haberer et al. 2008).

The POD and PPO enzymes in plants have several known functions (e.g., in lignin biosynthesis, ethylene production, indole-3-acetic acid metabolism, enzymatic browning, heartwood formation, etc.). The increase of their activity is considered a non-specific and general response to oxidative stress in living cells (Laukkanen et al. 1999, Tang & Newton 2004, Gupta et al. 2018).

For Pinus (Zheng et al. 2012) and Picea (Bae et al. 2010, Pukacki & Kaminska-Rozek 2013) spp. the increased amount of antioxidant enzymes in the needles was already reported during the acclimation process.

According to present results, the provenances of poorer growth performance were characterized by the lower POD enzyme activities of the leaves. The tendency was the same with PPO enzyme, yet correlation relationships were not significant. Normally, oxidative stress triggers an increase of POD levels in tree leaves (Boughalleb & Mhamdi 2011, Chakhchar et al. 2015a, 2015b, Song et al. 2015) while the changes of PPO activity are variable, depending on species, stress tolerance, and circumstances (Vahdati & Lotfi 2013, Song et al. 2015, Li et al. 2020). According to the correlation between POD vs. stem diameter, a decrease of activity in the provenances with lower stem diameters was experienced, which together with the generally lower tree vigor and higher tree mortality rate indicates that their long-term acclimation has not been successful, leading to decreased enzyme activities in the exhaustion phase of the stress response (Selye 1950). The positive correlation of POD with growth (Tab. 2.) shows that the general increase experienced in the leaves of other tree species during stress also applies here.

The variation of the correlations between PPO vs. diameter of the three investigated years also supports the previous statements on the PPO enzyme activity variability, depending on different factors.

The general negative correlations between enzyme activities and ecodistance also support the finding that provenances originally adapted to warmer and drier climates tend to have higher enzyme activities, yet this may be influenced by many factors, which is reflected by the weak correlation coefficients.

According to Baniulis et al. (2020) the comparative analysis of the protein content in the needles of pine seedlings provide insights into adaptation processes at the cellular level and the adaptive capacity within plant species.

According to Zhang et al. (2012), the total (Bradford) protein content in the leaves of Populus cathayana cuttings increased under drought stress. Conversely, Kala & Godara (2011) found decreased total protein content in the leaves of Ziziphus mauritiana L. under moisture stress (water deprivation), which was attributed to increased protease activity or decreased protein synthesis or a combination of both of these factors.

According to Korotaeva et al. (2015) the accumulation of dehydrins localized mostly in chloroplast and in the mitochondria membrane system was observed in pine needles during acclimation experiments. Generally, the types of proteins in leaves vary greatly; therefore, the changes in the total amount of proteins is extremely complex and can be influenced by many factors. In our findings, the provenances originally adapted to a cooler and wetter climate (e.g., Gråsten) also had higher levels of total proteins and also exhibited poorer growth under higher drought stress. In this respect, the total protein content of leaves may not only be a marker of drought, but of other environmental stress responses in tree leaves as well.

Under stress, plant polyphenol composition can change due to increased phenylpropanoid metabolism (Dixon & Paiva 1995, Isah 2019). Polyphenols may account for the major portion of the antioxidant activity of tree leaves, as Lee et al. (2009) demonstrated with olive leaves.

However, the levels of different polyphenolic compounds can also behave differently under stress conditions, for example in water-stressed poplar plants, the water deficit increased some polyphenol levels, including flavonoids (chrysin, myricetine, kaempferol) and isoferulic acid in roots as well as total phenols and antioxidant capacity in the leaves (Popovic et al. 2016). One explanation for the differing behavior of polyphenolic compounds is their varied role in defense and signaling processes in plants. Moreover, under certain circumstances, polyphenols can act as pro-oxidant compounds (Smirnoff 2005, Hofmann et al. 2017b), demonstrating that their concentration may decrease in the living tissue during defense reactions. The different behaviors of beech leaf polyphenols was also indicated by the significant correlations between ABTS antioxidant capacity and by the EQ vs. polyphenol concentration, as evidenced both by positive and negative values (Tab. 4). According to many recent reports detailed in Isah (2019), the produc-

tion of secondary metabolites, including polyphenols, increase in drought as well as in cold stress; however, in most cases this is accompanied by a decrease of biomass production. This is in accordance with the findings as the highest ABTS antioxidant capacities were found for the provenances with the poorest growth parameters (average stem diameter).

The role of individual polyphenolic compounds depends on the antioxidant efficiency of these compounds and on the potential of participating in plant defense reactions. According to Tab. 4, the most efficient compounds contributing to ABTS antioxidant power (showing the best positive correlations with the ABTS levels) in beech leaves were Quercetin-*O*-hexosides, Quercetin-*O*-pentoside, Coniferin derivative 2, (+)-Catechin and (−)-Epicatechin. According to Tab. S2 (Supplementary material), these compounds have generally higher concentrations in the provenances with poorer growth, which also confirms the role of these compounds in stress response processes in beech. According to Di Ferdinando et al. (2012) only a few glycosylated flavonoids are effective antioxidants compared to their respective aglycone part; however, the present study found the glycoside conjugates of quercetin to be the most efficient polyphenolic antioxidants. The flavan-3-ols (+)-Catechin and (−)-Epicatechin have been known to be efficient antioxidants in other plant tissues (Hofmann et al. 2017b) and contribute significantly to the defense reaction in beech leaf tissues (Feucht et al. 1994, 1997) and the defense reaction in other plant tissues (Feucht et al. 1996, Feucht & Treutter 1999, Hofmann et al. 2008). Some compounds had pro-oxidant effects. The roles of these compounds need to be clarified in the future.

The complex roles that different types of secondary metabolites play in the antioxidant system of beech was shown by Stajner et al. (2013), who pointed out that the adaptability of different beech provenances to environmental factors was best in the case of high FRAP value and free proline and soluble proteins contents, which proves the combined effects of different types of metabolites.

The results of the current study are in accordance with the results of Berini et al. (2018), outlining that the presence and concentration of secondary metabolites may be adaptive. Results demonstrated the concentration change of antioxidants related to the sensed climatic change in beech provenances. The correlations between metabolite concentrations and growth rate indicate that the application of antioxidants as stress indicators is only partly possible due to the complex effects of various abiotic factor combinations (Berini et al. 2018). Regarding the genetic background of the relatively high acclimatization potential of beech, the distinction between *a priori* coded genetic responses, acclimation and/or epigenetic effects is a

secondary issue, as the latter must be linked to genetics as well (Münzbergová et al. 2018).

Conclusions

The present study investigated general relationships between acclimation and leaf antioxidant properties in beech by provenance trials. Our study is among the first to show that genetic differences in observed acclimation processes between populations do affect antioxidant types and concentrations. We have concluded that the response to translocation-simulated climatic stress depends on the original genetic adaptation of the provenances, and that differences may be tracked using the illustrated biochemical analyses. Statistical analyses of variance did not always indicate significant differences between provenances because of highly unexplained variation; but correlation analyses revealed significant trends between biochemical parameters and growth as well as for simulated climate stress. Certain polyphenol concentrations, POD enzyme activity, and total protein content may serve as chemical indicators of the acclimation potential of populations and may contribute to the forecasting of climate change effects. In the course of planning adaptive forest management, differences in acclimation potential have to be considered when deciding about priorities for tree species and provenances. Knowledge of phytochemical processes and indicators of acclimation may support such decisions. Future research studies should focus on the roles of other compounds in enzymatic and non-enzymatic antioxidant systems and on the structural elucidation of the compounds labelled as "unknown".

Acknowledgements

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Supplementary Material

Tab. S1 - POD and PPO enzyme activity, total protein content and average stem diameter at breast height of the investigated provenances.

Tab. S2 - Average (n=8) peak areas for each compound according to provenance.

Fig. S1 - Separation of a beech leaf extract - PDA (250-380 nm) chromatogram.

Link: Visi-Rajczi_3542@suppl001.pdf