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Applicability of Beech (*Fagus Sylvatica* L.) Leaf Polyphenols as Indicators for Growth and Acclimation and Their Potential Contribution to Sustainable Forestry

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Sustainable forestry is one of the most important challenges of the 21st century, which requires the cultivation of species adapted to the changing climate and, in the case of existing species, the selection of climate-resistant propagation materials. European beech (*Fagus sylvatica* L.) is the most important hardwood tree species in Europe. Its ecological and economic importance is outstanding. The species, however, is significantly affected by the change in climate, so it is essential to select the appropriate propagating material for future cultivation and afforestation. The aim of this work was to measure and evaluate the polyphenolic composition of the leaves of different beech provenances to find relationships between average stem diameter, climate index (EQ), ABTS (2,2'-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid) antioxidant capacity and polyphenolic composition to assess the adaptability of the tested beech provenances using factor analysis. According to the results from the altogether 44 identified polyphenols, flavonoid (quercetin, kaempferol) glycosides and flavan-3-ol compounds were the strongest antioxidants, and the two types of compounds probably participate differently in the antioxidant defence system, but both have a significant role. A significant relationship between the concentration of individual polyphenols (Procyanidin B dimer 5, Feruloylthreonic acid, Unidentified 1) and average stem diameter was found: these compounds and factors can be chemical indicators of growth and climatic adaptation and can be used in predicting the future effects of climate change and in the future selection of beech propagation material.

1. Introduction

European beech (*Fagus sylvatica* L.) is one of Europe's most significant hardwood species from an ecological and economic point of view. It is particularly sensitive to the drying of the climate and stress, so its future sustainability raises many questions. Climate projections predict significant warming and drought throughout Hungary (Bartholy et al., 2014). While the tree species accounted for 5.5 % of Hungary's forest area (103 thousand hectares) in 2019 (URL1), according to forecasts, by 2050, only 1 % of the country's territory can possess optimal climatic conditions for the species (Gálos and Führer, 2018). In 1998 a provenance trial was initiated by the International Union of Forest Research Organizations (IUFRO) throughout Europe to study the climatic adaptability of the species (von Wuehlisch and Alia, 2011). Offspring of beech populations, adapted to the climate of purposefully chosen sites of origin were planted at assigned test sites. According to the hypothesis, the climatic difference between the sites of origin and the test site imitates rapid climate change and reveals the possibilities and limitations of inherited adaptability (Horváth and Mátyás, 2014). The Hungarian test location was Bucsuta (Zala county), located in an ecologically privileged location, closest to the xeric limit of the tree species (Horváth and Mátyás, 2016).
The trees at the test sites are exposed to several stressors at the same time, which can affect the functioning of the cell and the entire organism, from genes to plant metabolites. The vast majority of stress factors trigger

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oxidative stress, which is a shift in the balance between oxidants and antioxidants in favour of oxidants (Sies, 1991). In response, the plant activates its enzymatic and non-enzymatic antioxidant systems to eliminate reactive oxygen species and damaging free radicals. The glutathione system and other specific stress proteins, oxidase enzymes and their isoenzymes, and some polyphenols indirectly characterize plant stress through changes in their qualitative and quantitative spectra and can be considered biomarkers (Vieito et al., 2018) of the stress response (Tausz et al., 2004). In beech leaves, (Formato et al., 2022) and bark (Dübeler et al., 1997) the role of polyphenols against environmental stress has been already investigated in detail.

During the year 2013, the general state of the non-enzymatic antioxidant system of six selected beech provenances (Farchau, Pidkamin, Torup, Gråsten, Bánokszentgyörgy, Magyaregregy) was assessed in the Bucsuta provenance trial through the quantitative evaluation of polyphenolic compounds and the determination of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) antioxidant capacity of the leaves (Visi-Rajczi et al., 2021). By the assessment of the polyphenolic composition of the leaves of the different beech provenances relationship with the average trunk diameter, climate index, sun exposure and the ABTS antioxidant capacity was established using multivariate statistical analysis (factor analysis).

This study is the first to investigate the effect of acclimation stress in different European beech provenances by the assessment of the leaf polyphenolic antioxidant system using multivariate statistical methods. To the best of our knowledge, such an evaluation has not been yet conducted. The present research investigated if stress responses in different beech provenances could be characterized by chemical analysis of selected leaf metabolites, and that among these metabolites, which may be stress indicators that characterise acclimation and adaptation at the molecular level. The results of the work aimed at answering the following questions in particular:

1. Which compounds are most responsible for the measured ABTS antioxidant capacity, i.e. which leaf polyphenols could participate most effectively in defence reactions in case of biotic and abiotic stress in the case of beech?
2. How does sunlight (exposure to light) affect the polyphenol content and ABTS antioxidant capacity of the leaves, thus the defence capacity (resilience)?
3. Are there compounds that can be used as tree growth indicators or related to climate adaptation?

2. Materials and methods

2.1 Sample collection and processing

Figure 1. depicts the origin of the investigated provenances and the location of the test site (Bucsuta) compared with the occurrence of beech. Six provenances were selected for the investigations and 8 trees of each of the provenances were sampled in June 2013 (Visi-Rajczi et al., 2021). The average diameter at the breast height of the trees (ASD) was determined for each provenance. From each tree, 30 leaves were collected from different parts of the canopy. Leaves were stored in dry ice until the extraction process. Before extraction leaves were treated to inactivate their polyphenol-oxidizing enzymes (2 min at 750 W in a household microwave oven), then the leaves were ground and the polyphenols were extracted as follows: 0.15 g ground leaves were mixed with 15 ml MeOH:H_2O 4:1 (v/v) mixture and stirred for 24 h in the dark, then the extract was filtered through a 0.45 µm cellulose acetate filter, diluted twice fold and taken to analysis

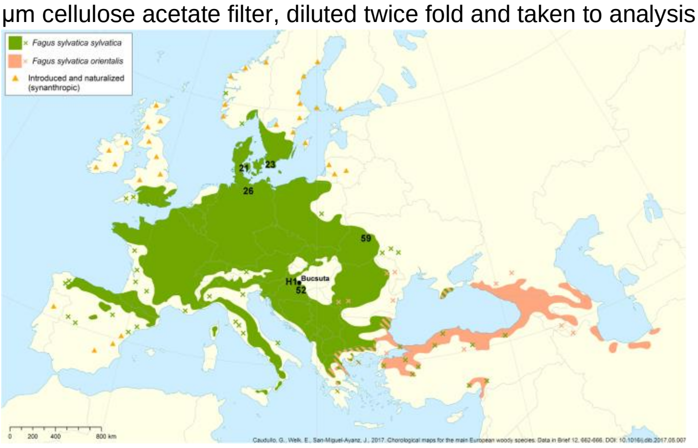

Figure 1: Origin of the investigated provenances and the location of the test site (Bucsuta) compared with the occurrence of beech. Investigated provenances: 21: Gråsten (DK), 23: Torup (S), 52: Magyaregregy (H), H1: Bánokszentgyörgy (H), 59: Pidkamin (UA), 26: Farchau (D)

The aim of this work was to measure and evaluate the polyphenolic composition of the leaves of different beech provenances to find relationships between average stem diameter, climate index (EQ), sun exposure factor, ABTS (2,2'-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid) antioxidant capacity and polyphenolic composition to assess the adaptability of the tested beech provenances using factor analysis.

2.2 Ellenberg climate quotient (EQ)

For the characterization of the climate of the origin of the provenances the Ellenberg climate quotient (EQ) was used, which is calculated from the ratio of the mean temperature of the warmest month (July, T_{07} [°C]) and annual precipitation (P_{ann}, [mm]) as shown in Eq(1) (Ellenberg, 1988):

$$EQ = 1000 \cdot T_{07} \cdot {P_{ann}}^{-1} \quad (1)$$

2.3 Sun exposure factor (Sun)

For all samples, the average of the light intensity was measured which the leaves were exposed to using a Sunche HS1010A type handheld light meter. From the measured light intensity, a sun factor was calculated by normalizing light intensity values to the 0-100 scale, where 0 corresponded to the leaves exposed to the lowest light intensity and 100 to the samples exposed to the highest intensity.

2.4 ABTS antioxidant capacity

The ABTS assay was run as described by Stratil et al. (2007) at 734 nm, using the $ABTS^{\bullet+}$ 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.

2.5 Chromatographic analysis

For the identification and quantitative assessment of polyphenolic compounds high-performance liquid chromatography/photodiode array detection/tandem electrospray mass spectrometry (HPLC-PDA-ESI-MS^n) has been applied. Detailed methods for analysis and evaluation are included in the work of Hofmann et al. (2017). In this study, the authors have identified 44 polyphenolic compounds from beech leaves. The results and list of compounds are consistent with later non-targeted studies on the polyphenol profiling of beech leaves (Formato et al., 2021).

2.6 Statistical evaluation

Factor analysis was run using the Statistica 12 software (StatSoft Inc, Tulsa, USA). For data analysis and interpretation, the most commonly-used varimax normalized method was applied.
The scheme of the sample collection, chemical analysis and evaluation is summarized in Figure 2.

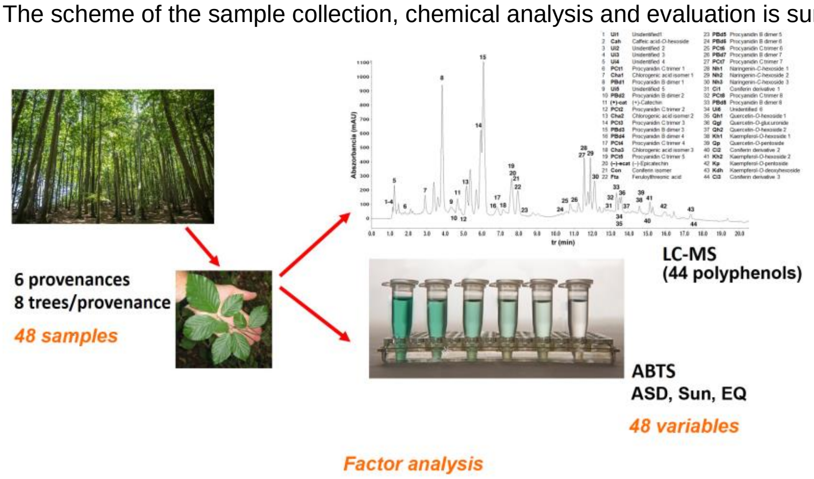

Figure 2: Graphical scheme of the study approach

3. Results and discussion

Factor analysis was applied for the evaluation of the data. Factor analysis helps to find connections between independent variables (vectors). Factors are calculated from the transformation of individual vectors, grouped by variance. In this manner, compounds are grouped in a way that also respects their relationship with antioxidant capacity (ABTS), environmental parameters (Sun, EQ) and growth (ASD).

In the factor analysis, first, the scree-plot was determined (Figure 3), in which the sum of variance of the eigenvalues for the analysis is plotted in descending order. In the scree-plot, it can be seen that the sum of variance for the first four eigenvalues is higher than 95 % of the sum of all the eigenvalues, which indicates that the 48 variables (44 polyphenol concentrations, ABTS, Sun, ASD, EQ) can be investigated and well displayed in a four-dimensional factor space, where the four factors correspond to > 95 % of the total variance of the eigenvalues.

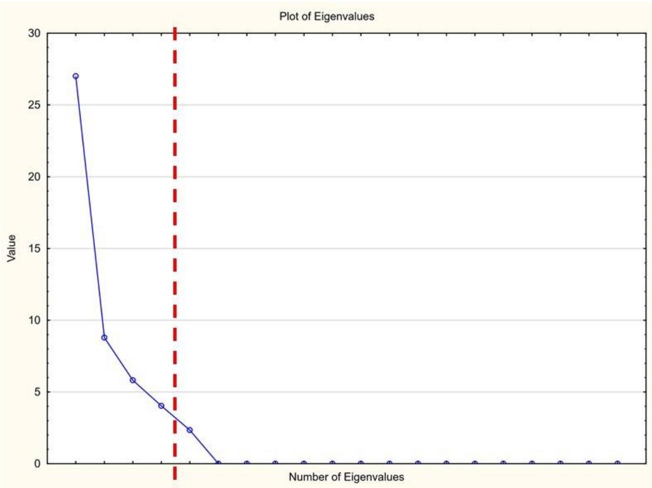

Figure 3: The scree plot

By plotting the 4 factors against one another the factor coordinates of the individual vectors can be displayed, and the vectors closely related to each other can be revealed. The results are presented in Figure 4 (Factor 1 vs. Factor 2) and Figure 5 (Factor 1 vs. Factor 4). The vectors that best define each factor (loading > |0.7|) were marked in both figures. The + or – signs indicate whether the factor weights (loadings) are positive or negative. It was supposed that the polyphenol vectors closely located to each other are also behaving similarly and the vectors “clustered” closely together have a significant relationship with either of the ABTS, Sun, ASD or EQ vectors that they are located closely to.

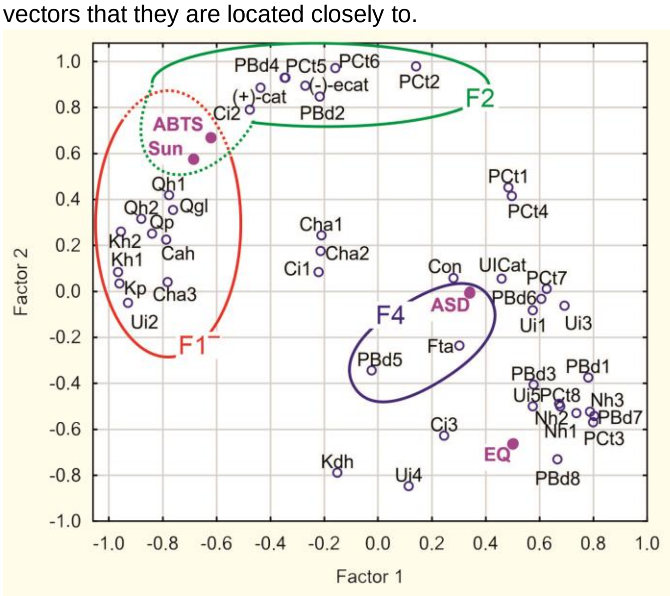

Figure 4: Results of the factor analysis; factor loadings Factor 1 vs. Factor 2. F1 denotes the group of vectors corresponding to Factor 1, and F2 and F4 are used similarly. For interpretation of tags of compounds, please refer to Figure 2

Based on Figure 4, it can be stated that ABTS antioxidant capacity and Sun are most closely related to factors F1 and F2 and they are also close to each other in the F1 vs F2 factor space, indicating their close relation, which can be explained that strong sunlight (UV radiation) increases the accumulation of polyphenols in leaves (Holub et al., 2019). Nevertheless, the factor coordinates of both vectors are at the limit of the significance for both of the factors, which can be interpreted as both influencing F1 and F2 equally, but none of F1 and F2 are the exclusive antioxidant factors.

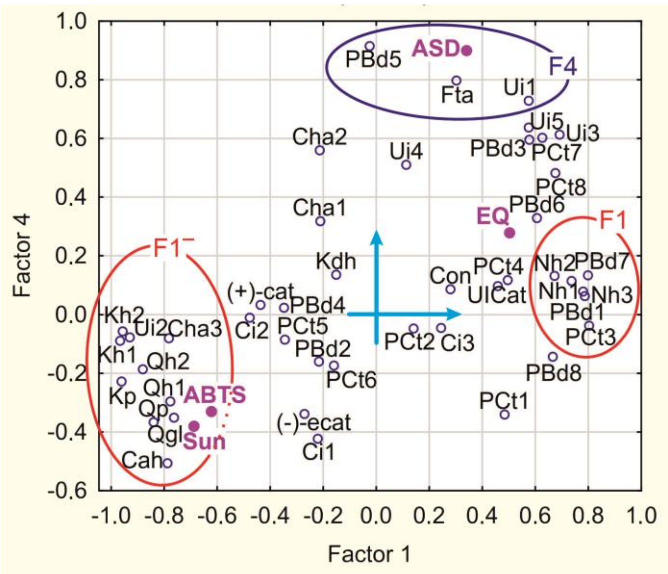

Figure 5: Results of the factor analysis; factor loadings Factor 1 vs. Factor 4. F1 denotes the group of vectors corresponding to Factor 1, and F4 is used similarly. For interpretation of tags of compounds, please refer to Figure 2

Factor 1 has a strong significant relationship with flavonoid (quercetin, kaempferol) glycosides, while Factor 2 has a strong and significant relationship with catechins and procyandins. According to this, both Factors 1 and 2 are "antioxidant factors", since ABTS has a relationship with both of these factors. Based on the results, it can be assumed that there are two types of antioxidant mechanisms in beech leaves with regard to polyphenols, i.e., the compounds connected with Factors 1 and 2 are all strong antioxidants, but they exert their effects in different ways. As polyphenols play key roles in the growth, regulation and structure of plants (Watson, 2014), it seems feasible that there must be compounds of those concentrations that are directly correlated with the growth performance of plants. According to Figure 5, the average trunk diameter (ASD) showed the strongest correlation with Factor 4 (loading > 0.9) and the vectors close to ASD (PBd5 (Procyanidin B dimer 5): 0.915, Fta (Feruloylthreonic acid): 0.798, Ui1 (Unidentified 1): 0.729) can be indicators of tree growth, thereby also indicators of the future adaptability of the investigated beech provenances. Future tests need, however, be carried out to study the effects and precise functions of these compounds.

4. Conclusion

Sustainable management of beech in Europe requires the proper choice of propagation material for growing healthy beech forests that can withstand changing climatic conditions. The assessment of plant stress via antioxidant compounds as markers can help to elaborate methods for this purpose. In the present work leaf polyphenols and ABTS antioxidant capacity, as well as Ellenberg's climate index, average stem diameter, and sun exposure factor have been assessed and evaluated using factor analysis based on an international provenance trial at the Bucsuta test site. According to the results in the polyphenolic antioxidant system of beech leaves, flavonoid (quercetin, kaempferol) glycosides and flavan-3-ol compounds were shown to be the strongest antioxidants. The two types of compounds probably participate differently in the antioxidant defence system, but both have a significant and determining role. Strong relationships were found between the concentration of Procyanidin B dimer 5, Feruloylthreonic acid, Unidentified 1 and the average stem diameter, which suggest that these compounds are indicators of growth in the beech provenances. Certain polyphenol concentrations may serve as chemical indicators of the acclimation potential of populations and may contribute to the forecasting of the effects of climate change. The applicability of the results for forestry practice requires further research and evaluation. Future measurements will focus on the analysis of other provenances and a larger number of trees to investigate the applicability of the results.

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