Secondary metabolites profile and physiological leaf traits in wild and cultivated *Corylus avellana* under different nutritional status

*Granata Mirko U1*, Francesco Bracco1, Rosangela Catoni1, Valeria Cavalloro1, Emanuela Martino1

**Abstract**

Leaf secondary metabolites production and physiological leaf traits were analyzed in *Corylus avellana* wild type (WT) and *cultivar* (cv. ‘Tonda Gentile Trilobata’, TGT) under different nutrient supplies. Three treatments were applied: control treatment with no fertilizer supply (WT$_C$ and TGT$_C$), low nutrient treatment (WT$_{LN}$ and TGT$_{LN}$) and high nutrient treatment (WT$_{HN}$ and TGT$_{HN}$). The analysis of leaf extracts showed a higher concentration of Quercitrin and Myricitrin, with the highest concentrations of both the compounds in WT than TGT. This result can be related to the ecological role of flavonoids, including also antimicrobial properties, which resulted more useful in the understory forest form which hazelnut wild type originates. Therefore, their lower concentration in TGT can be relate to the genetic background of TGT *cultivar* with a lesser intrinsic need to produce such compounds and justified by a usual growth under more controlled environmental conditions, including also pest and disease control.

**Experimental Section**

*Plant material and experimental design*

Experiment was carried out from April to July 2018 at the Botanical Garden of the University of Pavia (WGS84 UTM 32N 512814 m E 5003584 m N). Saplings of *C. avellana* cv. ‘Tonda Gentile Trilobata’ (TGT) (n= 30; mean height = 70 ± 5cm) from Alba (CN, Piedmont) and saplings of *C. avellana* wild type (WT) (n = 30; mean height = 65 ± 4 cm) from the understory of a deciduous forest, were grown in pots (diameter = 20cm, volume = 6L). Pots contained an organic commercial substrate (COMPO BIO, COMPO GmbH, Germany) with the following composition: organic carbon (C) 35%, humic carbon 11%, organic nitrogen (N) 1.4%, carbon on total nitrogen ratio of 25, peat (65%). The pH was 6.0–7.0.

At the beginning of April 2018 saplings were randomly assigned to three different nutrient treatments defined on the basis of the nutritional needs of the species. A complete randomized design (CRD) with ten replicates per treatment was established and an organic mineral NPK fertilizer (Nutri ONE Universal liquid fertilizer) was applied twice a month. The fertilizer composition was: total N 5.5%, total P 5%, total K 7.5%, total C 4.5%, soluble Fe 0.1%, soluble
Mg 0.03% and soluble Zn 0.04%. In particular, thirty saplings of TGT and WT were split in ten with no nutrient treatment and used as control (TGT\textsubscript{C} and WT\textsubscript{C}, respectively), ten were subjected to low nutrient treatment (TGT\textsubscript{LN} and WT\textsubscript{LN}, respectively, 0.5 ml equal to 0.62 g of fertilizer applied per pot twice a month) and ten were subjected to high nutrient treatment (TGT\textsubscript{HN} and WT\textsubscript{HN}, respectively, 2 ml equal to 2.48 g of fertilizer applied per pot twice a month).

Microclimate measurements were carried over the study period. In particular, air temperature (\(T_a, ^\circ C\)) and relative humidity (RH, %) were recorded at 5 min intervals by HOBO data loggers (H08-003-02, Onset HOBO Data Loggers, Cape Cod, MA); the photosynthetic photon flux density [PPFD, \(\mu\text{mol(photons)} \text{ m}^{-2} \text{s}^{-1}\)] was recorded at 5 min interval by Sunshine SensorBF3 (Delta-T Device, UK). Over the entire period, \(T_a\) ranged between 10.8°C to 28.4°C, RH from 48% to 97%, PPFD from 1226 to 1600 \(\mu\text{mol(photon)} \text{ m}^{-2} \text{s}^{-1}\).

**HPLC-UV/PAD analysis**

*C. avellana* leave extracts were analyzed using a ThermoFinnigan(Japan) high performance liquid chromatography-photodiode array system (HPLC-UV/PAD) equipped with Surveyor autosampler, a Surveyor pump and a Surveyor PDA Plus Detector. Experimental data were acquired and interpreted exploiting XCalibur software.

Each sample was dissolved in 50% water and 50% MeOH (5 mg mL\(^{-1}\)) and filtered with a 0.45 \(\mu\text{m}\) GH Polypro (GHP) membrane before injection into the HPLC-system. The samples were analyzed on a Chromolith Performance RP-18endcapped (4.4 × 50 mm) column. The mobile phase is composed by Water (A) and MeOH (B), both containing 0.1\% (\(\text{v/v}\)) formic acid and the composition gradient was: from 15 to 30% B in 8 min, to 45% B in 7 min, to 52% B in 5 min, to 100% B in 2 min,15% B until 4 min followed by a re-equilibration step of 4 min. The flow rate was 2 mL min\(^{-1}\) and the elution was performed at room temperature. The UV detection was fixed at 264 nm.

The analytical conditions reported above ensure a good resolution of Quercitrin, Myricitrin and Afzelin with respect to the other metabolites present in the extract. A representative chromatographic profile of the extract is reported in Figure 4. Their peaks were unequivocally identified comparing their UV spectrum with those of the standard analyzed in the same conditions and by analyzing the chromatographic fingerprints of the extracts spiked with pure standards. Quantitative determinations of Quercitrin and Myricitrin were performed using the external standard procedure by means of a six point calibration curve with three replicate measurements for each calibration point. By contrary, Afzelin quantification was not possible because its content in the extracts was below the limit of quantification. Data related to HPLC method validation are reported in the SI.
**Extraction Procedure**

Dried *C. avellana* leaves were ground with a blade-mill (A10 IKA-Werke GmbH & Co. Staufen, Germany) to obtain a homogenous powder, just before performing the extractions. The so obtained raw material was then washed with both Hexane (100mL/2g of raw material, overnight) and Dichloromethane (100mL/2g of raw material, 7h) at room temperature under magnetic stirring. Matrices were then filtered and dried. Then, 500mg of the so obtained powder were dispersed in 15mL of solvent and subjected to microwave heating (2 min ramping, 5 min hold time, maximum pressure 120 psi, maximum potency 200W, temperature 80°C, 1 cycle) using Discover® SP instrument, CEM Corporation, Matthews, NC, USA. The considered solvents were Ethyl Acetate, Ethanol 80% and Ethanol 100%. The mixture was left to cool at room temperature filtered and concentrated under reduced pressure. Results are summarized in Table 1. The best procedure in term of extraction yield and chromatographic profile was achieved using Ethanol 100% as solvent. This solvent coupled with the microwave conditions previously described was exploited to extract all the different matrices previously washed with Hexane and Dichloromethane.

**S.1 HPLC-UV/PAD analysis**

Quantitative determinations of Quercitrin and Myricitrin (Figure S1) were performed using the external standard procedure by means of a six point calibration curve with three replicate measurements for each calibration point.

Results are summarized in Table 2. The statistical control of the method was assessed by performing three injections of reference standard at the beginning of every measurement session on different days.

The method accuracy was evaluated by assessing the analytes recovery by single-point standard addition method, spiking 3.00 g of samples with 0.1 mg of quercitrin and, then with 0.3mg of myricitrin. The recovery found by comparison of the spiked amount of analytes to the amount found in the non-spiked samples was as high as 97% and 98% respectively.

**Gas Exchange**

Gas exchange measurements were carried out in the morning (8.00 – 10.00 h) on ten fully expanded leaves of TGT and WT per each treatment three times in June and July with comparable weather condition. Measurements were carried out by an infrared gas analyzer (LCPro+, ADC, UK), equipped with a leaf chamber (PLC, Parkinson Leaf Chamber). Net assimilation rate \( A_N, \mu\text{mol(CO}_2\text{)} \text{m}^{-2} \text{s}^{-1} \), stomatal conductance \( g_s, \text{mol(H}_2\text{O) m}^{-2} \text{s}^{-1} \), transpiration rate \( E, \text{mmol(H}_2\text{O) m}^{-2} \text{s}^{-1} \), photosynthetic photon flux density [PPFD,
\( \mu \text{mol(photons)} \text{ m}^{-2} \text{s}^{-1} \) and sub-stomatal CO\(_2\) concentration (C\(_i\), ppm) were measured. Relative carboxylation efficiency \([C_E, \text{mol (CO}_2\text{)} \text{ fixed mol}^{-1}\text{(CO}_2\text{)} \text{ present in the sub-stomatal spaces}]\) was calculated by the ratio of net photosynthesis and sub-stomatal CO\(_2\) concentration (Flexas et al. 2001).

**Chlorophyll Fluorescence**

Chlorophyll fluorescence measurements were carried out in the morning at the same time of gas exchange on ten fully expanded leaves of TGT and WT per each treatment. Measurements including maximum PSII photochemical efficiency (F\(_{V}/F_{M}\)), actual quantum yield of photosynthesis of light-adapted leaves (\(\Phi_{PSII}\)) and electron transportation rate (ETR) were carried out by a portable modulated fluorometer (OS5p, Opti-Sciences, USA) on fully expanded leaves. For measurements of F\(_{V}/F_{M}\), leaves were first dark-adapted for 30 minutes by leaf clips then a saturating pulse was applied to measure initial (F\(_0\)) and maximum (F\(_M\)) fluorescence.

F\(_{V}/F_{M}\) was estimated as:

\[
F_{V}/F_{M} = (F_{M}-F_{0})/F_{M}
\]

\(\Phi_{PSII}\) was calculated on light-adapted leaves, according to Genty et al. (1989) as:

\[
\Phi_{PSII} = (F_{M}-F_{S})/F_{M}'
\]

where F\(_{M}'\) was the maximum fluorescence obtained with a light-saturating pulse (~8000 \(\mu \text{mol m}^{-2} \text{s}^{-1}\)) and F\(_S\) was the steady-state fluorescence of illuminated leaves (1600 \(\mu \text{mol m}^{-2} \text{s}^{-1}\)).

ETR [\(\mu \text{mol(e}^{-}) \text{ m}^{-2} \text{s}^{-1}\)] was calculated according to Krall and Edwards (1992) as:

\[
ETR = (\Phi_{PSII}) \times \text{PPFD} \times 0.5 \times 0.84
\]

**Data analysis**

To test the nutrient availability effects on the considered parameters a one-way ANOVA was carried out. Differences between means were compared using the Tukey test. Differences were considered significant if \(P\) was at least \(\leq 0.05\). All the statistic tests were performed by a statistical software package (Statistica 8.0, Statsoft, USA). The data obtained represented the means with standard error (SE).

**Acknowledgments**

This work was supported by the ’Natural Reserve Bosco Siro Negri’ funded by The Ministry of the Environment and Protection of Land and Sea of Italy.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**References**


**Table 1.** Comparison of the extraction solvents in term of yields and chromatographic profile.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Yield</th>
<th>Chromatographic profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>3.42%</td>
<td>![Ethyl acetate chromatogram]</td>
</tr>
<tr>
<td>Ethanol 80%</td>
<td>31.96%</td>
<td>![Ethanol 80% chromatogram]</td>
</tr>
<tr>
<td>Ethanol 100%</td>
<td>24.88%</td>
<td>![Ethanol 100% chromatogram]</td>
</tr>
</tbody>
</table>

**Table 2:** results obtained from the calibration curves of the considered analytes

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration range</th>
<th>Equation</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercitrin</td>
<td>0.03-0.20mg/mL</td>
<td>$y=27490x+112.88$</td>
<td>$R^2=0.9984$</td>
</tr>
<tr>
<td>Myricitrin</td>
<td>0.25-1.00mg/mL</td>
<td>$Y=22473x-3351.2$</td>
<td>$R^2=0.9789$</td>
</tr>
</tbody>
</table>
**Figure S1:** HPLC-UV/PAD profile ($\lambda = 264$ nm) and calibration curve of Quercitrin standard (A) and Myricitrin standard (B) obtained under the chromatographic conditions described in the article.