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Protocol Article

Trehalose determination in Norway spruce (*Picea abies*) roots. Analytics matters

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A B S T R A C T

We present concise results of method validation for trehalose quantitation by LC-MS/MS in spruce ectomycorrhizal roots in order to describe spruce health status, mainly in connection to contamination by a pathogenic fungus, *Gemmamyces piceae*. The procedure is based on Rogatsky et al. (2005) developed for human plasma. We found out that the best extraction yield was achieved with 80% methanol/water (v/v) solution and optimal extraction temperature was set between 50-60°C. In contrast to previous papers, we minimized the activity of trehalase enzyme by putting root samples into liquid N_2 immediately after root excavation, followed by freeze-drying in order to stop trehalase activity. Higher content of trehalose was recorded in healthy trees, confirming the idea that ectomycorrhiza plays a significant role in plant-pathogen interactions.

A R T I C L E I N F O
Method name:
Keywords: Trehalose, Spruce roots, Liquid chromatography - Mass spectrometry, Mycorrhiza
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Specifications table

Subject Area	Environmental Science
More specific subject area	Plant physiology
Method name:	
Protocol name	NA
Reagents/tools	**Reagents** liquid nitrogen methanol (HPLC grade) caesium acetate (LC-MS grade) acetonitrile (HPLC grade) trehalose dihydrate (analytical standard) ethanol (denatured with benzene, GR) **Equipment** Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA) with a triple quadrupole mass spectrometer (Q Trap 3200; ABSciex, USA). HILIC (amino) column
Experimental design	Analytical method validation was performed using spiked pooled blank samples of healthy Norway spruce (*Picea abies*) roots. Root samples from Norway spruce were taken in the Ore Mountains (Krušné hory) near Červený Hrádek (GPS 50°31′07.9"N 13°27′03.0"E) in the Czech Republic. Samples were taken from both healthy (n = 10) trees and trees affected by the pathogenic fungi *Gemmamyces piceae* causing bud blight disease ($n = 10$). Tree age was between 50 and 80 years.
Trial registration	NA
Ethics	
Value of the Protocol	- Optimization of the field sampling procedure. - Trehalose quantitation in ectomycorrhizal spruce roots.

Description of protocol: Method details

Background

The non-reducing disaccharide trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) has been considered to help plants survive in inconvenient environmental conditions; nevertheless, its biotic interactions are still little understood [1]. We focused on Norway spruce (*Picea abies*) affected by the deadly pathogen *Gemmamyces piceae* and trehalose content in the roots as the biomarker of mycorrhizal quality, resulting in higher tree vitality and resistance [3].

In the past, carbohydrates in spruce ectomycorrhizal roots were analyzed as trimethylsilyl derivatives by GC-FID or by HPAEC-PAD [2]. The disadvantages of these techniques are high polarity of trehalose, insufficient sensitivity, or co-eluting substances (e.g., sucrose). Many discrepancies between previously published values were probably caused by the use of different analytical techniques. Moreover, the different approach in ectomycorrhizal root sampling and trehalose analysis by LC-MS/MS revealed the source of different published results.

Our research goals were i) to establish a proper methodology for field root handling followed by extraction and quantitation of trehalose, and ii) to compare trehalose content in the roots damaged by a fungal pathogen (*Gemmamyces piceae*) and healthy ones.

Final procedure:

Root sampling

1. Fine roots < 2 mm were collected from a soil sample taken with a soil auger (6 cm i.d., stuck 15 cm deep).
2. Excavated roots were extracted from soil samples using tweezers, quickly washed with water and submerged in a plastic bag into liquid nitrogen.

3. In the laboratory, samples were removed from liquid nitrogen and stored in a freezer at −80 °C until further analysis.
4. Within three weeks, the samples underwent freeze-drying (for 196 h; Lyovac GT 2, Steris, Germany).

Sample extraction

1. 5-7 g of freeze-dried roots were ground in a laboratory mill (IKA, A11 basic, Germany).
2. 25 mg of the root powder was weighed into a 2-mL plastic microcentrifuge tube (Eppendorf, Germany) and 1.5 mL methanol/water mix (80/20 v/v) added.
3. The tube was shaken vigorously for a couple of seconds to wet the sample and placed in a thermoshaker (TS-100, Biosan, Latvia) for 30 min at 50 °C and 1,000 rpm.
4. The samples were then centrifuged (Eppendorf, Minispin plus) at 12,500 rpm for 10 min and the liquid phase filtered through a nylon syringe filter (0.25 µm) into a 2-mL HPLC vial.

LC-MS/MS analysis

We developed an optimized extraction procedure for determination of trehalose followed by LC-MS/MS detection using Cs^+ ion attachment, based on Rogatsky's assay developed for human plasma [4]. Trehalose analysis was carried out using an Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA) equipped with a binary pump, coupled with a triple quadrupole mass detector (Q Trap 3200; ABSciex, USA). The analytes were separated by isocratic elution (with a column flush step) on a Luna NH_2 column (150 × 2 mm, 3 μm, 100 A; Phenomenex, USA). The operating conditions were as follows: flow rate 350 µL/min; column temperature 25 °C; injection volume 1 μL. The binary mobile phase consisted of (A) 0.1 mM caesium acetate in water and (B) acetonitrile. Isocratic conditions of 20% A and 80% B were kept for 10 min, increased to 50% A and 50% B at 11 min and remained constant for 1.5 min, then returned to original conditions of 20% A and 80% B and remained there until minute 15. The autosampler was kept at 10 °C. Trehalose (as m/z [M+Cs], e.i. precursor m/z 565, product m/z 133) was detected and quantified using ESI source operated in positive mode with the following parameters: Q1 mass 475.1 Da; Q3 mass 133.0 Da; curtain gas 30 psi; collision gas: medium; ion spray voltage: 5500 V; source gas temperature 600°C; GS1 60 psi and GS2 60 psi. Data were gathered using Chromeleon 6.8 and Analyst 1.4 software.

Method validation parameters

Method optimization and validation was performed on spiked blank spruce roots (spike level 15 µg/g). Before spiking, complete removal of trehalose from tested samples was achieved using naturally present trehalase. Due to co-elution with saccharose in real samples, quantification of trehalose was based on peak height. A five-point matrix matched linear calibration curve was made using trehalose dihydrate of 5, 10, 20, 30, and 40 µg/mL. Linearity was R^2 (square of the correlation coefficient) = 0.9989, residuals <4%. Limit of detection (as 3 × S/N) = 1.3 µg/mL which corresponds to 78 ng/mg of sample matrix; limit of quantitation (as 10 × S/N) = 4.35 µg/mL, which corresponds to 261 ng/mg of sample matrix. Average recovery on spike level 15 µg/g was 96%. Within-day precision (4%) and day-to-day precision (8%) are expressed as relative standard deviation from average measured value. During method validation the following parameters were optimized: i) extraction solvent mixture, ii) extraction temperature, and iii) extraction time. The results of these test are presented in Supporting Information. All validation analyses were performed in triplicate.

Statistical analysis

The data are presented in µg trehalose/g DW with standard deviation; see Supporting Information (Table S1). Datasets were tested for homogeneity (Levene test) and normality (Shapiro-Wilk test). After fulfilling the statistical presumptions, one-way ANOVA was used. Differences were tested at the level of $p = 0.05$. Tukey HSD test was used for detailed analysis. Statistical analysis was performed using Statistica 13.3 (TIBCO Software, USA).

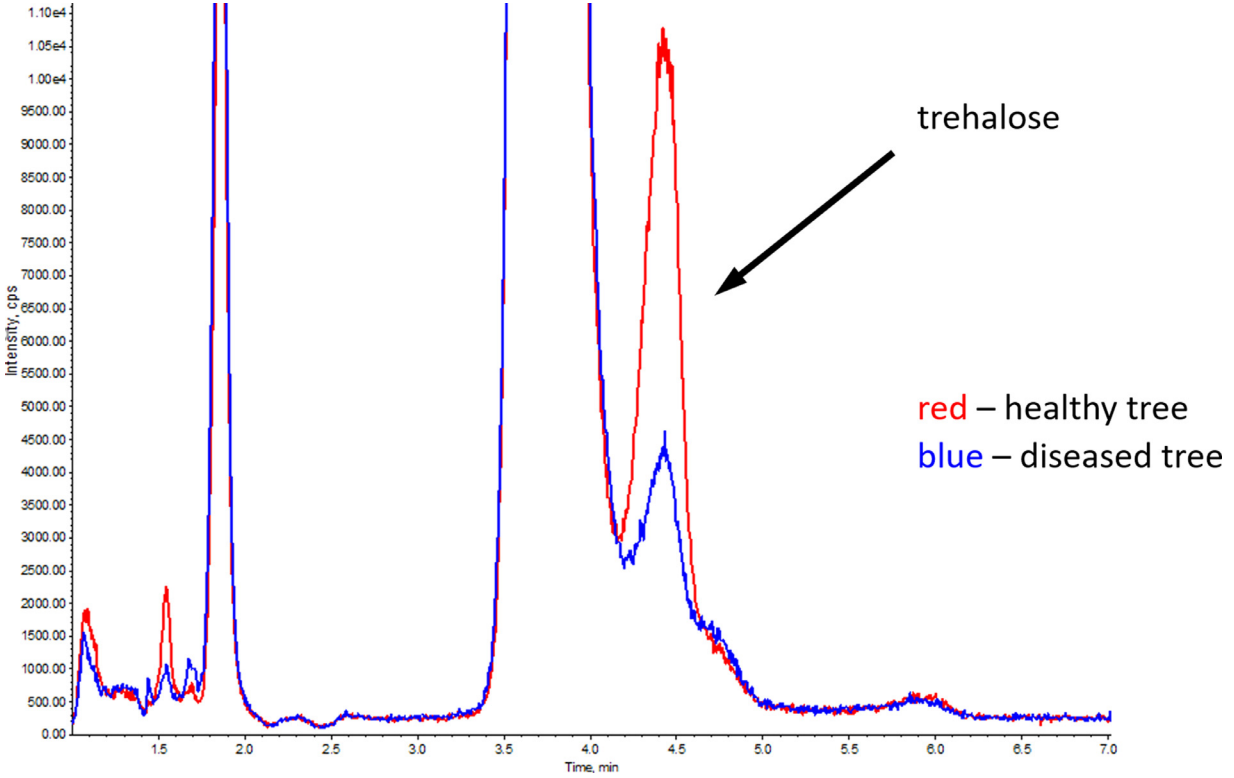

Fig. 1. Chromatogram comparison from a freeze-dried material of a healthy (red) and diseased (blue) tree. Retention time of trehalose is about 4.5 min.

Results from the testing dataset

From our results, it is evident that diseased trees do differ significantly from healthy ones ($p = 0.01$). The mean value of trehalose in healthy spruce roots was 739 ± 96 µg trehalose/g DW (mean ± SD) as compared to 624 ± 88 µg trehalose/g DW in the damaged group (Fig. 1).

Conclusion

The key factors for accurate trehalose quantitation in ectomycorrhizal roots samples are swift material collection, immediate sample cooling in liquid N_2, storage at −80 °C, and freeze-drying. From the combinations of extracting solvents with varying proportions of either ethanol or methanol in water, the best extraction yield was achieved with 80% methanol/water (v/v) solution (Supporting Information Table S2). In contrast to this, the methodology based on ethanolic mixtures predominantly used in other papers recovered about 10% less trehalose in our experiments. Depending on the nature of the sample, the extraction efficacy was 90–99%. The optimum extraction temperature lay between 50 and 60 °C, depending on the matrix (Supporting Information Table S3). Previous studies always used hot ethanol mixes to stop trehalase activity. We minimized the activity of trehalase by putting root samples into liquid N_2 immediately after ectomycorrhizal root excavation and by subsequent freeze-drying. The optimum extraction time was found to be 30 min, even though a small proportion of samples was already fully extracted after 15 min (Supporting Information Table S4). Using lower amounts of sample than 25 mg did not provide reproducible results for trehalose in some samples (SD < 32%, $n = 5$) and higher amounts are inconvenient due to the large saccharose content, which then interferes with the trehalose peak.

In trehalose LC-MS analysis the main difficulty is finding out the appropriate conditions for separation of trehalose from other carbohydrates. In our work, the mobile phase was modified to achieve good separation. But the novelty of our approach was to develop and optimize extraction procedure for quantitaion of trehalose in root samples. To the best of our knowledge, there is no publication where an optimal extraction procedure is described in detail for analysis of spruce ectomycorrhyzal roots. Because of the complex matrix of plant materials, the optimization of extraction is quite important.

The validated method was used on set of samples where half of the sampled trees were affected by *Gemmamyces piceae*. The results showed that these trees had a lower content of trehalose in root samples than trees without this pathogen.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found at doi:10.1016/j.mex.2020.101207.

References

[1] O. Fernandez, L. Béthencourt, A. Quero, R.S. Sangwan, C. Clément, Trehalose and plant stress responses: friend or foe? Trends in Plant Sci. 15 (2010) 409–417.

[2] R. Hampp, C. Schaeffer, T. Wallenda, C. Stülten, P. Johann, W. Einig, Changes in carbon partitioning or allocation due to ectomycorrhiza formation: biochemical evidence, Can. J. Bot. 73 (1995) 548–556.
[3] M. Niederer, W. Pankow, A. Wiemken, Trehalose synthesis in mycorrhiza of Norway spruce: an indicator of vitality, Eur. J. For. Pathol. 19 (1989) 14–20.
[4] E. Rogatsky, H. Jayatillake, G. Goswami, V. Tomuta, D. Stein, Sensitive LC MS quantitative analysis of carbohydrates by Cs^+ attachment, J. Am. Soc. Mass Spectrom. 6 (2005) 1805–1811.