FSL 2: ^{1}H NMR- based metabolite profiling of tropane alkaloids in *Duboisia* spec.

Sophie Friederike Ullrich[1,2], Andreas Rothauer[1], Oliver Kayser[2]

[1]Boehringer Ingelheim Pharma GmbH und Co. KG, Binger Str. 173, D-55216 Ingelheim am Rhein, Germany, e-mail: sophie_friederike.ullrich@boehringer-ingelheim.com (corresponding author)
[2]Department of Biochemical and Chemical Engineering, TU Dortmund, Emil-Figge-Str. 66, D-44227 Dortmund, Germany

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Abstract

Duboisia R.Br. (*Solanaceae*) is the main source of the tropane alkaloid scopolamine, which is an important precursor of various active pharmaceutical ingredients due to its anticholinergic properties. As only little is known about the metabolite composition among the different species, NMR-based metabolic profiling was done in order to elucidate primary and secondary metabolism in *Duboisia* especially focusing on the tropane alkaloid pathway. For this purpose, plants of five different genotypes (*Duboisia myoporoides*, *D. leichardtii* and hybrids of *D. myoporoides* and *D. leichhardtii*) were cultivated under strictly controlled conditions in climate chambers, leaf and root extracts were prepared and measured via ^{1}H NMR. 14 different metabolites could be identified using 1D- and 2D-NMR techniques. Principal component analysis of the NMR data allowed a clear distinction between *Duboisia* hybrids and the wild types, which could be again subgrouped in *D. myoporoides* and *D. leichhardtii,* based on the metabolites identified.

Keywords: ^{1}H NMR, Metabolomics, Scopolamine, Alkaloids, *Duboisia*, *Solanaceae*

Introduction

Duboisia R.Br., which belongs to the family of *Solanaceae*, is indigenous to Australia and used as principal source of the active substance scopolamine (Barnard, 1952; Palazón et al., 2008). Until today, the synthetic production of scopolamine is expensive and no alkaloid levels competitive to field grown plants could be achieved by overexpressing biosynthetic genes in regenerated plants or by using cell culture systems (Foley, 2006; Hashimoto, 2003). Thus, the industrial production providing scopolamine is largely based on agricultural field plant cultivation (Foley, 2006).

For optimizing the breeding process and the plant cultivation, ^{1}H NMR-based metabolic profiling was applied as it allows a deeper insight into the primary and secondary metabolism of *Duboisia* with special focus on the tropane alkaloid biosynthesis. Therefore, samples of different species (three different wild types and two hybrids of *Duboisia myoporoides* and *D. leichhardtii*) were analysed choosing roots and leaves for extraction, as scopolamine is biosynthesized in the roots from where it is transported to the leaves, its main storage location (Wink, 1987).

Materials and Methods

Plant material

All plants under investigation belong to the genus *Duboisia* R.Br., family *Solanaceae,* and were supplied by Boehringer Ingelheim (Germany). Wild types of *Duboisia myoporoides* R.Br. (A) and *Duboisia leichhardtii* F.Muell. (B, C) and hybrids of *Duboisia myoporoides* R.Br. and *Duboisia leichhardtii* F.Muell. (D,E) were cultivated in climate chambers under strictly controlled conditions at 25 °C exposed to 12 h of light per day with an intensity of 110 µmol/m^2·s. After sampling, all plant material was frozen immediately using liquid nitrogen and stored at –80 °C. After grinding in liquid nitrogen and freeze-drying, all samples were extracted and subsequently measured via ^{1}H NMR.

Extraction

Samples were prepared according to a protocol published by Kim et al. with slight changes (Kim, 2010). 20 mg of the freeze-dried material was weighed into a 2 ml - centrifuge tube. 0.5 ml of CD_3OD and 0.5 ml of D_2O, containing 0.29 mM TSP-*d4*, buffered with KH_2PO_4 (90 mM) and adjusted to pH 6.0 using 1.0 M NaOD, were added. After vortexing 1 min at room temperature and 5 min of ultrasonication, the samples were centrifuged for another 5 min at 13,000 g at room temperature and 300 µl of supernatant were filtered into a 3 mm - NMR tube.

Measurements

The NMR experimental parameters were chosen based on the protocol of Kim et al. (Kim et al., 2010). ^{1}H NMR spectra were measured at 25 °C on a 600 MHz DMX-600 spectrometer operating at 600.13 MHz and equipped with a TCI cryoprobe and Z-gradient system. The resulting spectra were manually phased and baseline corrected with the help of TopSpin (ver. 3.1 Bruker).

Results

^{1}H NMR- based metabolite profiling

By using CD_3OD-KH_2PO_4 buffer in D_2O (1:1, v/v) for extraction, a wide range of polar metabolites was covered, including sugars, amino acids and secondary metabolites like flavonoids or tropane alkaloids. As the NMR-signals were often overlapping, it was first necessary to select the characteristic signals of possible metabolite-candidates. Subsequently, all metabolites were further verified by using 2D-NMR techniques like ^{1}H-^{1}H-correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC). All in all, 14 different primary and secondary metabolites could be identified in the leaves of *Duboisia* using NMR data. Regarding the root extracts, only sugars like glucose and sucrose and traces of scopolamine could be detected due to the very low concentration of single metabolites.

Comparison of different genotypes

Comparing the wild types of *Duboisia myoporoides* (A) and *Duboisia leichhardtii* (B, C) with the hybrids of *Duboisia myoporoides* and *D. leichhardtii* (D, E) by using principal component analysis (PCA), a separation into three different groups is possible based on the metabolites identified. Principal component 1 (PC1) divides the samples into wild types and hybrids, whereas principal component 2 (PC2) further subgroups the wild types into *Duboisia myoporoides* and *D. leichhardtii*.

The loading plot displays the identified metabolites, which are responsible for the group differentiation into wild types and hybrids according to PC1. Scopolamine and signals, which could be generally assigned to tropane alkaloids, were found in higher concentrations in *Duboisia* hybrids. Sugars like glucose or sucrose and amino acids like proline or threonine were more present in the wild types *Duboisia myoporoides* and *D. leichhardtii*. Hence, the tropane alkaloid biosynthesis seems to be enhanced in case of the hybrids, especially the last step of the biosynthesis, namely the conversion of hyoscyamine via 6β-hydroxy-hyoscyamine to scopolamine. Hyoscyamine was significantly increased in the wild types, whereas the hybrids of *Duboisia* contained higher amounts in scopolamine. This could be due to a higher expression level or activity of the responsible enzyme, the hyoscyamine 6β-hydroxylase (H6H). But additional proteomic and transcriptomic data will be needed for a final assessment.

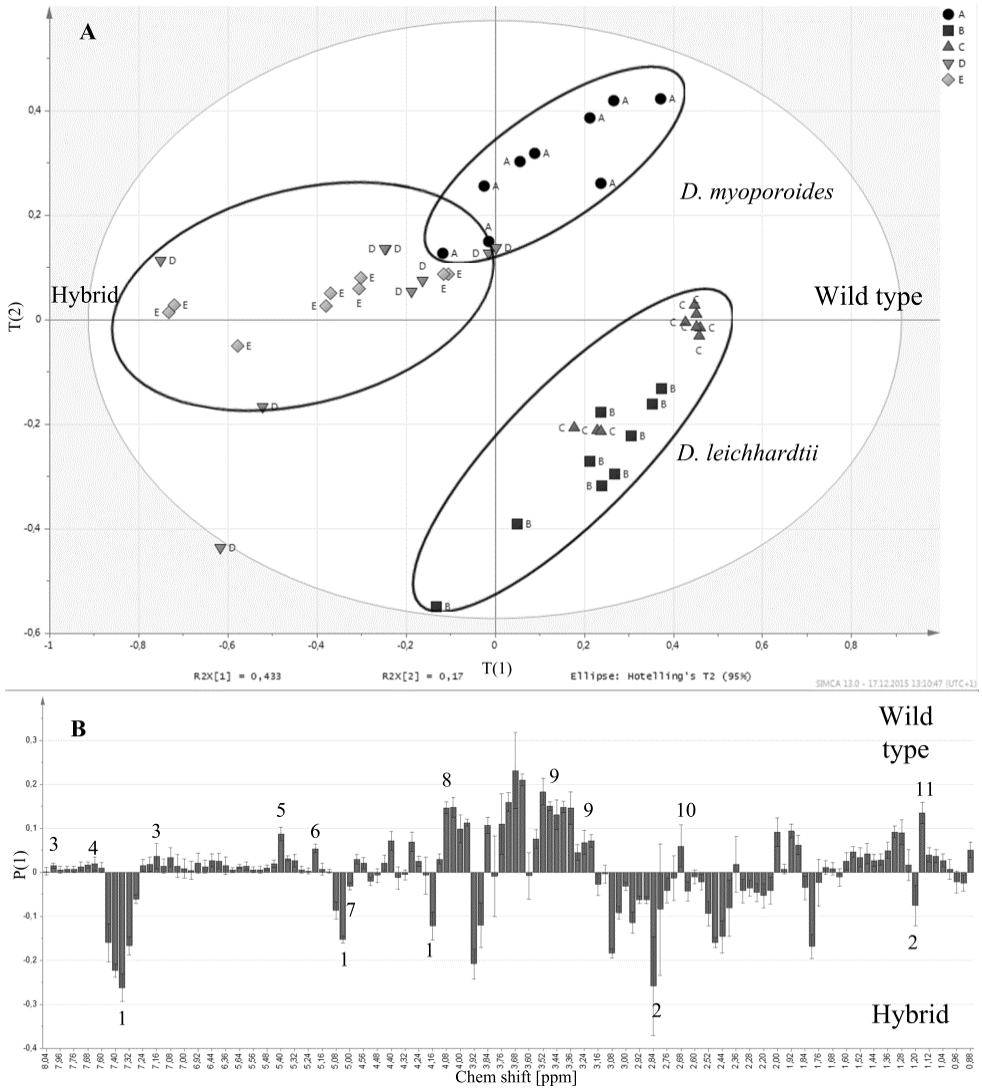

Figure 1 Score plot of PC1 and PC2 (A) and loading column plot of PC1 (B) as results of principal component analysis (PCA); data obtained by ^{1}H NMR comparing the leaf extracts of different genotypes (A-E) of *Duboisia*. Signals of: tropane alkaloids (1), scopolamine (2), scopoletin (3), quercetin (4), sucrose (5), glucose (6), 6β-hydroxy-hyoscyamine (7), proline (8), myo-inositol (9), hyoscyamine (10) and threonine (11).

This NMR analysis allows a fast and easy comparison of different samples of *Duboisia* by grouping them based on their metabolite composition. In addition, it can be applied in order to select promising genotypes and optimised cultivation conditions for the production of scopolamine.

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