

Influence of abiotic factors on growth and biosynthesis of secondary plant components in *Duboisia* species

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"Look deep into nature, and then you will understand everything better."

Albert Einstein

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Abstract

The Australian plant genus *Duboisia* has a long history of use dating back to the Aborigines and still today, it is main source of the medicinally used tropane alkaloids. Thereof scopolamine and its derivatives are often applied as anticholinergic agents in the treatment of postoperative nausea and vomiting, motion sickness as well as gastrointestinal, renal and biliary spasms. Until today, the global supply in scopolamine is mainly covered by large scale field cultivation of hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii*.

The objective of this work was to evaluate the impact of abiotic elicitors on growth and biosynthesis of secondary plant components in *Duboisia* species with special focus on scopolamine. Thereby, ¹H NMR-based metabolite profiling was applied for a global analysis of primary and secondary metabolism comparing different genotypes, plant organs, growth stages and cultivation conditions. An appropriate HPLC-MS method was developed and validated for a detailed analysis of tropane alkaloids. The results demonstrate that the abundance of tropane alkaloids in *Duboisia* species is largely influenced by genetic characteristics as well as by environmental conditions, whereas the plant age plays an inferior role. In order to systematically analyse the influence of temperature, light and macronutrients on scopolamine biosynthesis and plant growth, plants of three different genotypes were grown in climate chambers under controlled conditions. The data analysis hereby reveals that especially the light intensity as well as the nitrogen supply have a major impact on the scopolamine and biomass production.

All in all, this research contributes to a better understanding of the interaction of abiotic factors with alkaloid metabolism and plant growth. Prospectively, the employment of the methods established within this work will help to select promising genotypes in breeding as well as suitable cultivation conditions for an optimised production of scopolamine.

Zusammenfassung

Die in Australien beheimatete Pflanzengattung *Duboisia*, die historisch schon von den Aborigines verwendet wurde, ist bis heute die Hauptquelle der medizinisch genutzten Tropanalkaloide. Davon werden Scopolamin und dessen Derivate häufig als anticholinerge Wirkstoffe in der Behandlung von postoperativer Übelkeit und Erbrechen, Reiseübelkeit sowie gastrointestinalen, renalen und biliären Krämpfen angewandt. Bis heute wird die globale Marktversorgung mit Scopolamin hauptsächlich über den großflächigen Freilandanbau von Hybriden aus *Duboisia myoporoides* und *Duboisia leichhardtii* gewährleistet.

Das Ziel dieser Arbeit war die Untersuchung des Einflusses von abiotischen Faktoren auf Wuchs und Biosynthese sekundärer Pflanzeninhaltsstoffe, insbesondere des Scopolamins, in *Duboisia*. Dabei wurde ¹H NMR Metabolite Profiling für einen allgemeinen Vergleich des Primär- und Sekundärstoffwechsels verschiedener Genotypen, Pflanzenorgane, Altersgruppen und Anbaubedingungen eingesetzt. Darüber hinaus wurde eine passende HPLC-MS Methode für die Gehaltsbestimmung der enthaltenen Tropanalkaloide entwickelt und validiert. Die Ergebnisse zeigen, dass die Menge an Tropanalkaloiden in *Duboisia* stark von genetischen Merkmalen sowie von Umwelteinflüssen abhängt, während der Entwicklungsstand eine untergeordnete Rolle spielt. Um den Einfluss der Umweltfaktoren Temperatur, Licht und Makronährstoffe auf die Scopolaminbiosynthese und den Wuchs systematisch zu untersuchen, wurden Pflanzen dreier Genotypen unter kontrollierten Bedingungen in Klimakammern angebaut. Die Datenanalyse zeigt einen hohen Einfluss von Lichtintensität und Stickstoffversorgung auf die Scopolaminproduktion und das Pflanzenwachstum; diese Parameter können gezielt zur Steigerung von Scopolamin- und Biomasseproduktion genutzt werden.

Zusammenfassend trägt diese Forschungsarbeit zu einem besseren Verständnis der Interaktion von abiotischen Faktoren mit dem Alkaloidstoffwechsel und dem Pflanzenwachstum bei. Zukünftig werden die in dieser Arbeit entwickelten Methoden dazu beitragen, vielversprechende Genotypen in der Züchtung zu selektieren und geeignete Anbaumethoden für eine optimierte Scopolaminproduktion auszuwählen.

Chapter 1: Scope of the thesis

1.1. Aims and Objectives

The aim of this work is to analyse the impact of abiotic elicitors on growth and biosynthesis of secondary plant components in *Duboisia* species, especially focusing on its pharmaceutically active ingredient scopolamine. For this purpose, a suitable controlled cultivation system for *Duboisia* as well as appropriate analytical methods for the qualitative and quantitative analysis of the plant extracts need to be developed and validated. The objectives of this cumulative thesis are worked out as individual chapters including the following contents:

Chapter 2 provides an introduction to this thesis and gives an overview of the current state of knowledge with special regard to botanical origin, pharmacology, biosynthesis as well as agricultural and biotechnological production of scopolamine.

Chapter 3 analyses the impact of environmental factors and genetic constitution on biomass production and the biosynthesis of scopolamine and its derivatives in *Duboisia* species. In this study, ¹H NMR- and HPLC-MS-based metabolic profiling of leaf and root extracts are performed using three wild types and two hybrids of *Duboisia myoporoides* R.Br. and *Duboisia leichhardtii* F.Muell. at different developmental stages grown under controlled conditions in climate chambers as well as under agricultural field cultivation.

Chapter 4 systematically evaluates the influence of temperature (20, 24, 28 °C), light (50 – 300 μmol/m²·s; 12, 18, 24 hours per day) and macronutrients (N, Ca, K) on growth and scopolamine biosynthesis using plants of three different genotypes (wild type of *Duboisia myoporoides* R.Br., hybrids of *Duboisia myoporoides* R.Br. and *Duboisia leichhardtii* F.Muell.) grown in climate chambers under strictly controlled conditions.

Chapter 5 highlights and discusses the key findings and gives an outlook on future challenges in this field of research.

Chapter 2: Introduction

Sophie Friederike Ullrich, Hansjörg Hagels, Oliver Kayser

Sophie Friederike Ullrich (SFU), Hansjörg Hagels (HH) and Oliver Kayser (OK) jointly planned and designed the chapter; SFU performed the literature review; SFU wrote the manuscript with inputs from all coauthors; OK oversaw the entire project as senior supervisor of SFU.

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Abstract

Tropane alkaloids are present in many different plants of the Solanaceae family and widely known for their anticholinergic properties. Among them, most valued and increasingly demanded is scopolamine, also known under the name of hyoscine, which is used as pharmaceutical active substance in the treatment of postoperative nausea and vomiting, motion sickness and gastrointestinal, renal and biliary spasms for instance. It naturally occurs in various plant genera, e.g. *Anisodus*, *Anthocercis*, *Atropa*, *Brugmansia*, *Datura*, *Duboisia*, *Hyoscyamus*, *Mandragora* and *Scopolia* and the purified substance has a long history of use dating back to the 19th century. Until today, the supply in scopolamine is mainly covered by large scale field plant cultivation of hybrids between *Duboisia myoporoides* and *Duboisia leichhardtii*. Biotechnological approaches optimising the alkaloid biosynthesis, for example the use of callus cultures or genetically transformed hairy root cultures, are not competitive by now. The aim of this review is to give a comprehensive overview regarding the current knowledge on botanical origin, pharmacology, biosynthesis as well as agricultural and biotechnological production of scopolamine.

Keywords

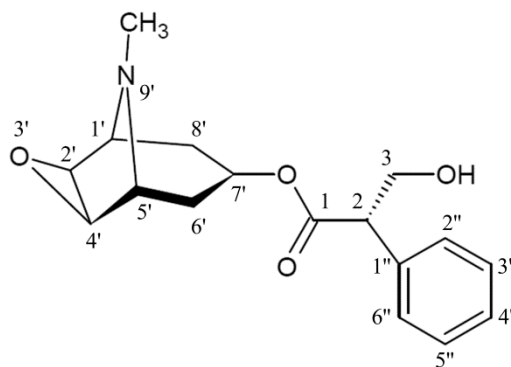
Anticholinergics, Hyoscine, *Solanaceae*, Tropane alkaloids

2.1. Introduction

Tropane alkaloids have been medicinally used for centuries due to their anticholinergic properties and are of great significance until today. They are chemically classified by their bicyclic tropane ring (*N*-methyl-8-azabicyclo [3.2.1] octane), which is characteristic for a group of approximately 200 compounds naturally occurring in mostly solanaceous plants, including scopolamine, hyoscyamine or cocaine (Grynkiewicz and Gadzikowska 2008). One widely used substances among them is scopolamine, also known under the name of hyoscine, which acts as competitive antagonist at muscarinic acetylcholine receptors and thereby exhibits a parasympatholytic effect (Palazón et al. 2008). Its commercial demand is assessed about 10-fold higher than for hyoscyamine due to its fewer adverse effects and higher physiological activity (Zhang et al. 2004; Yun 1992; Palazón et al. 2003). It is a more powerful mydriatic and suppressant of salivation than hyoscyamine, shows weaker spasmolytic effects and possesses central depressing effects already at low therapeutic doses, which can be used for example in the treatment of motion sickness (EFSA 2013; Finkel et al. 2009). Moreover, it is used as substrate for semisynthetic drugs like tiotropium bromide or scopolamine-*N*-butyl bromide. That is why there is a longstanding interest to increase the scopolamine production using large scale plant cultivation or different biotechnological approaches. Until today, its demand is continuing, as scopolamine and its derivatives like *N*-butyl scopolamine are pharmaceutically applied in many different therapeutic areas, e.g. as antiemetics or spasmolytics, and are still expanded to further applications, e.g. Clozapine-induced hypersalivation (Takeuchi et al. 2015). Moreover, other classes of compounds that can be substituted for these plant derived drugs are lacking (Oksman-Caldentey 2007). In order to further optimize the scopolamine production, an extensive knowledge on its botanical sources, biosynthetic pathway as well as agricultural and biotechnological production is required. This review gives a comprehensive overview on botanical origin, biosynthesis and medicinal applications focusing especially on the production of scopolamine.

2.2. Botanical origin

Scopolamine is to be found in many different plant genera of the *Solanaceae* family and predominantly occurs in form of (-)-scopolamine (Armstrong et al. 1987) (Figure 2-1).



(-)-Scopolamine ((-)-Hyoscyine)
 IUPAC name: (1R,2R,4S,5S,7S)-9-Methyl-3-oxa-9-azatricyclo[3.3.1.0^{2,4}]non-7-yl (2S)-3-hydroxy-2-phenylpropanoate
 Molecular formula: C₁₇H₂₁NO₄

Figure 2-1: Structure, IUPAC name and molecular formula of scopolamine

Until the 19th century, scopolamine itself was not identified as a separate substance, but was applied in form of plant extracts containing mixtures of different anticholinergic agents (Thearle and Pearn 1982; Gryniewicz and Gadzikowska 2008). Scopolamine naturally occurs within the “x = 12” clade (Figure 2-2) in the subfamily Solanoideae, tribes Hyoscyameae and Datureae, as well as in the subfamily Nicotianoideae, tribe Anthocercideae (taxonomy according to Olmstead (Olmstead et al. 2008)).

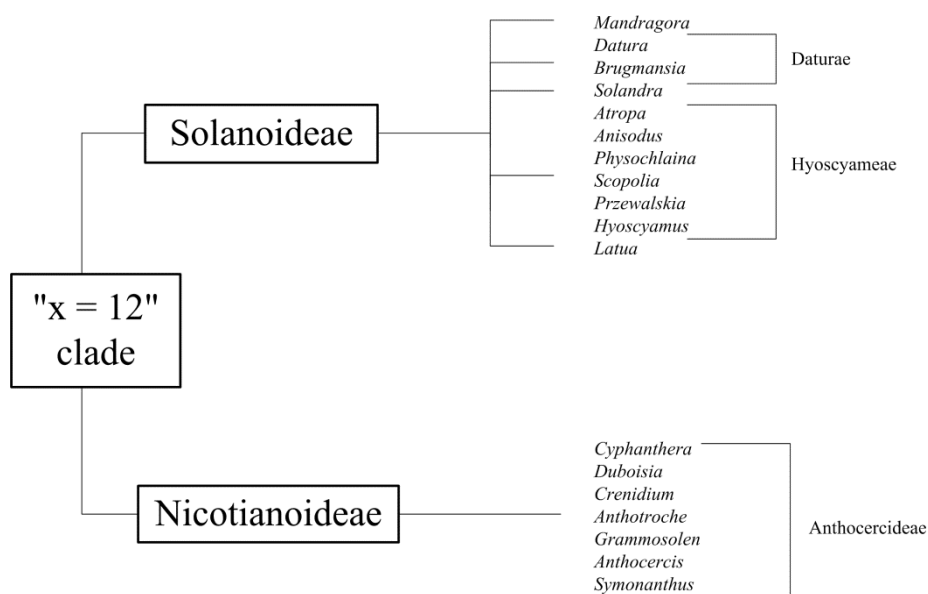


Figure 2-2: Taxonomy of the botanical sources of scopolamine in the “x = 12” clade within the *Solanaceae* family

Within the subfamily Solanoideae, it has been identified in *Solandra* (Evans et al. 1972), in *Latua*, (Munoz and Casale 2003) and in *Mandragora* (Jackson and Berry 1979). Referring to the tribe Datureae, it has been reported to be present in *Datura* (Evans and Wellendorf 1959) and *Brugmansia* (Griffin and Lin 2000). It is also occurring in *Iochroma*, tribe Iochrominae (Berger 2011) as well as in *Lycium*, tribe Lycieae (Rätsch 2012). Furthermore, plant genera belonging to the tribe Hyoscyameae, namely *Scopolia* (Samoryadov and Minina 1971), *Hyoscyamus*, *Physochlaina*, *Przewalskia* (Griffin and Lin 2000), *Atropa* (Phillipson and Handa 1976), *Atrophante* (Ripperger 1995) and *Anisodus* (Kai et al. 2007) are all known to contain scopolamine. Besides, *Duboisia* (Hills et al. 1953), *Anthocercis*, *Cyphanthera*, *Crenidium* (El Imam and Evan 1984), *Anthotroche* (Evans and Ramsey 1981), *Symonanthus* and *Grammosolen* (Evans and Ramsey 1983) of the tribe Anthocercideae all belong to the scopolamine-containing solanaceous plants. Apart from the family of Solanaceae, scopolamine has been identified in *Benthamia alyxifolia* within the Loranthaceae living as a parasite on *Duboisia myoporoides* and thereby accumulating scopolamine in its leaves (Rätsch 2012).

2.3. Pharmacology

2.3.1. Ethnopharmacological use

Until the nineteenth century, scopolamine was not separated as pure substance, but applied in extracts and tinctures containing a mixture of the plant-derived anticholinergics. The first record on the medicinal use of scopolamine and its derivatives can be traced back to the ancient Egyptians in 1500 BC. The use of one of its parent plants, namely *Hyoscyamus*, is described in the Ebers Papyrus, one of the oldest preserved medical documents. Therein, it is recommended in the treatment of “magic in the belly”, probably signifying colic and abdominal pain (Thearle and Pearn 1982; Gyermek 1997). Hereinafter, herbal preparations, which are nowadays known to contain scopolamine, were listed in several herbal books, e.g. *Hyoscyamus* in “De Materia Medica” of Dioscorides or Gerarde’s “The Herball or Generall Historie of Plantes” (Gerarde 1597; Osbaldeston 2000). *Hyoscyamus*, also known as Henbane and widespread in different regions all over the world, was also used as a potent ingredient in the various midnight brews and ointments prepared by witches in the Middle Ages (Carter 2003).

Chapter 2

Datura species are largely distributed in warm climates all over the world (Griffin and Lin 2000) and have been used for their hallucinogenic properties for a long time. Their geographical origin is not fully elucidated yet (Rätsch 2012). It is assumed that *Datura* plants, also known under the name of thornapple, devil's trumpet or Jimson weed, were brought to Europe around 1500 AD, where they were utilized as potions by medieval witches at that time (Perger 1864; Thearle and Pearn 1982). They have also been applied by the Jivaroan people of Brazil (Thearle and Pearn 1982) and can be assigned to the Hindu god Shiva to whom the thornapples are provided as small offerings until today (Soni et al. 2012).

Different plant parts of *Atropa*, indigenous to Europe, North Africa and Western Asia, have been traditionally applied for ages for diverse purposes, e.g. the treatment of arthritis and muscle spasms as well as acute infections, asthma, colitis and many more (Nisar et al. 2013; Godara et al. 2014). Moreover, extracts of *Atropa belladonna*, commonly called belladonna or deadly nightshade, have also been used as poison already during the Roman empire (Thearle and Pearn 1982).

Scopolia, distributed in Europe as well as in East Asia, was used as mydriatic in the 19th century by applying an alkaloid-mixture called "Scopolein" extracted from its roots (Schmidt and Henschke 1888). *Brugmansia* has been cultivated by indigenous people in Central and South America until today due to its hallucinogenic, narcotic and medicinal properties. It is applied externally for broken bones, swellings and also in case of stomach or arthritic pain. Moreover, it is taken orally in case of pain of childbirth, untreatable illness or severe accidents, probably due to its narcotic properties (Shepard 2009). *Solandra*, endemic to South America, is associated with the Huichole god of wind and sorcery Kieri Tewiari and has been used by the Huichol of Mexico as a hallucinogenic agent to date (Knab 1977). *Latua*, native to Chile, is still relatively unknown compared to other plant genera and is mainly used by shamans and sorcerers on-site because of its ability to produce delirium, hallucinations and trance-like states (Plowman et al. 1971). *Mandragora* plants have been traditionally applied for medicinal purposes for a long time and are related to various myths due to their hallucinogenic effects. For instance Joan of Arc, burned as a heretic in 1431, was accused of the witches' crime to own Mandrake among other things (Ungricht et al. 1998). *Duboisia*, endemic to Australia and New Caledonia, has been historically used by the Aborigines as a fish poison added to a billabong and, similar to *Scopolia*, its extract was used by ophthalmologists due to its mydriatic properties already in the 19th century (Foley 2006). All

exemplary listed types of application can be traced back to the pharmacological properties of scopolamine and related tropane alkaloids.

2.3.2. Contemporary therapeutical use

Scopolamine itself is used in different therapeutic areas due to its anticholinergic effects. Fields of application are postoperative nausea and vomiting, motion sickness and hypersalivation by using a transdermal patch (Scopoderm[®]) as well as induced mydriasis and cycloplegia in diagnostic procedures by using eye-drops (Isopto[®] Hyoscine) (Apfel et al. 2010; Choi et al. 1964; Mato et al. 2010; Nachum et al. 2006). It can also be used against death rattle in terminal care for symptom relief at the end of life in patients with Parkinson's disease by continuous intravenous or subcutaneous infusion and can be applied intravenously as antidepressant (Jaffe et al. 2013; Wildiers et al. 2009; Perez et al. 2010). Tiotropium bromide (Spiriva[®], Tiova[®]), a semi synthetic scopolamine derived active substance (Figure 2-3), is applied as an inhalative drug against chronic obstructive pulmonary disease (Barr 2006). Furthermore, its derivatives scopolamine-*N*-methyl bromide (Pamine[®]) (Figure 2-3), administered orally, as well as scopolamine-*N*-butyl bromide (Buscopan[®]) (Figure 2-3), given as tablet, suppository or injection, are known for their antispasmodic effects.

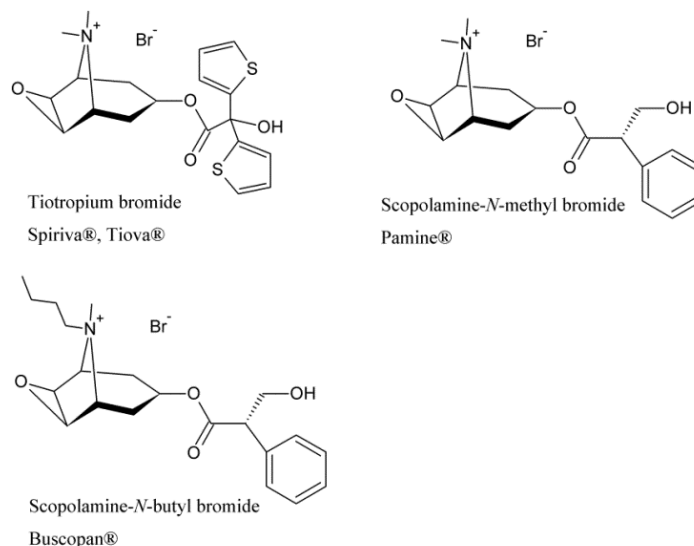


Figure 2-3: Structures of semisynthetic derivatives of scopolamine on the market

Chapter 2

Scopolamine-*N*-butyl bromide is available on the market since the early fifties (Figure 2-4) and is for example used in the treatment of abdominal pain, bladder spasms, and can also be applied against hypersalivation (Tytgat 2007; Takeuchi et al. 2015).

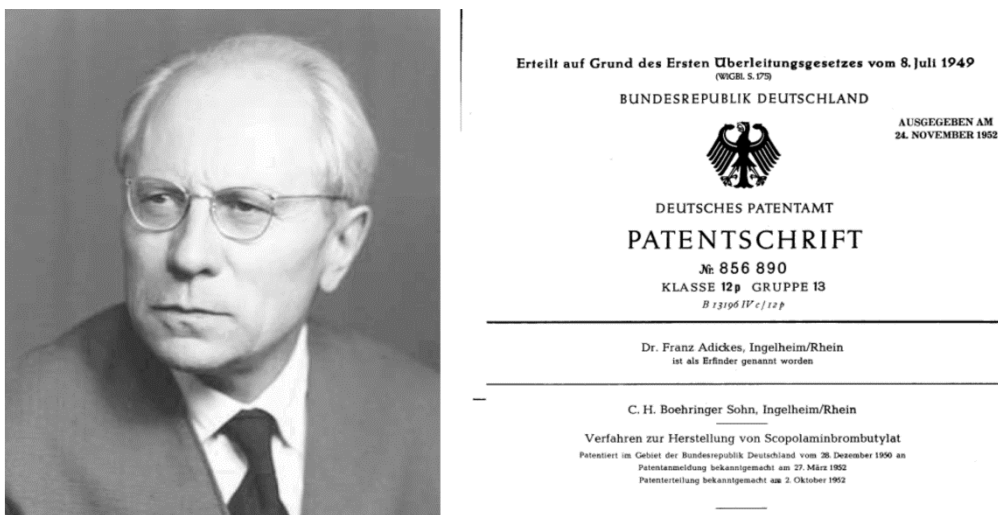


Figure 2-4: Prof. Franz Adickes, chemist, who is named as inventor in the patent specification DE 856 890 of scopolamine-*N*-butyl bromide approved in 1952

In contrast to scopolamine, scopolamine-*N*-butyl bromide is not passing the blood-brain barrier due to its quaternary ammonium salt structure and therefore showing no side effects concerning the central nervous system (CNS) (Tytgat 2008). It is commercially produced in large scale by derivatizing the plant extracted (-)-scopolamine with 1-butylbromide via *N*-alkylation (Figure 2-5).



Figure 2-5: Derivatization of plant-derived (-)-scopolamine to scopolamine-N-butyl bromide (Buscopan®), manufactured and distributed by Boehringer Ingelheim Pharma GmbH und Co.KG since 1951

2.3.3. Mechanism of action

Scopolamine is inhibiting the activity of the muscarinic acetylcholine receptors (mAChR). The mAChRs have widespread and diverse functions in the peripheral and in the CNS, e.g. the regulation of heart rate, smooth muscle contraction and glandular secretion (Bymaster et al. 2003). Five subtypes of the mAChRs exist, namely M1-M5 (Staskin and Zoltan 2007). M1 receptors are located in the CNS, in autonomic ganglia and glands. M2 receptors are widely expressed in the CNS as well as in heart, smooth muscle and autonomic nerve terminals. M3 receptors are also to be found in the CNS and present in smooth muscle and heart. M4 receptors are predominantly located in the forebrain, M5 receptors at low levels in the CNS and the periphery (EFSA 2013). Scopolamine acts at all five muscarinic receptor subtypes with relatively equal potency (Witkin et al. 2014). M1-M5 are coupled to heterotrimeric G-proteins and grouped based on the intracellular signalling pathway activated by ligand binding (Strang et al. 2010). The signalling pathways of M1, M3 and M5 are associated with the $G_{q/11}$ protein family and consist of an activation of the phospholipase C. M2 and M4 are linked with the $G_{i/o}$ protein family and inhibit the adenylyl cyclase activity (Volpicelli and Levey 2004; Caulfield and Birdsall 1998). By blocking the parasympathetic system, many different

physiological effects are observed, which can be pharmaceutically used. The main anti-muscarinergic effects consist of a decreased production of salivary, bronchial and glandular secretions, mydriasis, cycloplegia, increased heart rate, inhibited micturition, reduced gastrointestinal tone and gastric acid secretion.

2.3.4. Pharmacokinetics

Doses in (-)-scopolamine vary according to the indication with a maximum oral daily dose of 1.2 mg (Corallo et al. 2009). The pharmacokinetic parameters are largely dependent on the dosage form. In case of oral administration, the bioavailability is limited ranging between 3% and 27%. Maximum plasma levels are to be found 23.5 ± 8.2 minutes after oral intake. Regarding ocular administration, scopolamine is rapidly, efficiently and systemically absorbed. In case of transdermal application, the systemic availability is comparable to an intravenous infusion suggesting a high transdermal bioavailability. Data concerning metabolism and renal excretion are limited, glucuronide or sulfate conjugation are supposed to be involved in biotransformation (Renner et al. 2005). An urinary excretion of ca. 30 % parent compound in addition to Phase II conjugates is observed within 24 hours after oral administration (EFSA 2013).

2.3.5. Toxicity

Toxic doses of (-)-scopolamine cause restlessness, disorientation, hallucinations and delirium due to CNS stimulation. In the event of an overdose, CNS stimulation is followed by central depression and death through respiratory paralysis. The lethal dose is estimated to be about 100 mg (-)-scopolamine (EFSA 2013). Intoxications with scopolamine mainly occur in the following categories: unintended ingestions, intended ingestions of plant material and poisoning due to its abuse (Beyer et al. 2009). Unintended ingestions are mostly due to contamination of food, e.g buckwheat or corn, and via intake of plant parts of *Brugmansia*, *Datura* or of *Atropa*, as for example the fruits of *Atropa belladonna* are sometimes confused with blackberries and the seeds of *Datura stramonium* are mixed up with those of poppy (Adamse and van Egmond 2010; Koleva et al. 2012). In Germany, *Brugmansia* belongs to the four plant genera leading to the most severe poisonings by botanicals in children (Pietsch et al. 2009).

Chapter 2

In different regions all over the world, the seeds or flowers of *Datura* are intentionally taken by adolescents and young adults as a hallucinogenic agent leading to severe intoxications (Hall et al. 1977; Kintz et al. 2006). The same applies to *Brugmansia*, native to South America and spread over different regions worldwide, which is also frequently abused by adolescent recreational drug users (Kim et al. 2014).

2.3.6. Abuse as “Truth serum”

Early in the 20th century, medical doctors began to employ scopolamine, along with morphine and chloroform, as an agent for easing the pain of childbirth, inducing the so-called “twilight sleep” (Foley 2006). This sleep was characterized not only by a state of anaesthesia, but also by complete amnesia, in which the expectant mother lost all memories of the birth process. Moreover, doctors remarked that women in twilight sleep became talkative and were able to answer their questions accurately without remembering it later (Bimmerle 1993). In 1922, Robert House, an American obstetrician experienced with the usage of scopolamine in child birth, suggested a similar technique to be employed in the interrogation of suspected criminals. He also started to use scopolamine in the questioning of prisoners to show its potential in criminal investigations, published his results and thereby became famous as the “father of truth serum” (Geis 1959). However, only a few cases in which scopolamine was used for police interrogation came to public notice (Bimmerle 1993). These days, scopolamine derived from *Brugmansia*, commonly labelled Devil’s breath in this context, is widely abused by criminals in South America in order to render their victims unconscious and rob them. In Columbia for instance, unofficial data estimate scopolamine incidents to be at approximately 50,000 per year (OSAC 2014).

2.4. Biosynthesis

2.4.1. Plant biosynthetic pathway

Scopolamine is known to be mainly biosynthesized in the roots, from where it is transported to the leaves, its main storage location, which has been proved by using classical grafting experiments (Wink 1987; De Luca and St Pierre 2000). The complete biosynthetic pathway is not yet fully understood, but most of the enzymes involved are identified and characterized

(Figure 2-6). The tropane alkaloid biosynthesis starts with the amino acids ornithine and/or arginine, which are transformed to putrescine by the ornithine decarboxylase (OrnDC, EC 4.1.1.17) and/or arginine decarboxylase (ArgDC, EC 4.1.1.19) (Hashimoto and Yamada 1994; Hashimoto et al. 1989). The putrescine *N*-methyltransferase (PMT, EC 2.1.1.53) then catalyses the *S*-adenosylmethionine (SAM)-dependent *N*-methylation of putrescine, the first specific reaction guiding the flux of nitrogen away from polyamine biosynthesis to alkaloid biosynthesis (Hibi et al. 1992). Subsequently, the *N*-methylputrescine oxidase (MPO, EC 1.4.3.6) catalyses the oxidative deamination of *N*-methylputrescine to 4-methylaminobutanal, which spontaneously cyclizes to form the *N*-methylpyrrolinium cation (Heim et al. 2007). The next biosynthetic step is not yet fully elucidated and still topic of controversial discussions. It is presumed that the *N*-methylpyrrolinium cation condenses with acetoacetic acid yielding hygrine, which is further converted to tropinone (Ziegler and Facchini 2008). Tropinone is subsequently reduced by the tropinone-reductase I or II yielding either tropine or pseudotropine, the ratio of products being affected by the respective activity of both enzymes (Dräger 2006). Tropine, being a precursor of scopolamine, is formed via the tropinone-reductase I (TR-1, EC 1.1.1.206) and condenses with the phenylalanine-derived phenyllactate to littorine (Nakajima and Hashimoto 1999; Ziegler and Facchini 2008; Hashimoto et al. 1992), whereas the tropinone-reductase II (TR-2, EC 1.1.1.236) converts tropinone to pseudotropine, a precursor of the calystegines (Zhang 2005). After tropine being condensed with the phenylalanine-derived phenyllactate to littorine, hysocyamine is formed via an intramolecular rearrangement of littorine (Robins 1995, 1994). The mechanism for the rearrangement of littorine, containing a phenyllactic acid ester at C-3 of the tropine unit, to hysocyamine with its tropic acid ester moiety has been under debate for a long time and still, it is not completely understood. Hysocyamine is most likely formed via hysocyamine-aldehyde in a two-step reaction catalysed by Cyp80F1, probably with an alcohol dehydrogenase involved as second enzyme (Li et al., 2006). As last step of the biosynthetic pathway, the hysocyamine 6 β -hydroxylase (H6H, EC 1.14.11.11) converts hysocyamine via 6 β -hydroxy-hysocyamine to scopolamine (Hashimoto et al. 1993a).

In order to further elucidate the tropane alkaloid pathway, new approaches are currently under way, e.g. the use of isotope ratio monitoring by ^{13}C NMR spectrometry. Romek et al. successfully showed that *N*-methylpyrrolinium, a precursor of scopolamine as well as of nicotine, introduces similar isotope distribution patterns in the two target compounds, whereas the other atoms of both alkaloids, being of different origins, reflect their specific metabolic origin (Romek et al. 2016). Prospectively, those measured ^{13}C distribution patterns can be

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targeted used in order to clarify aspects of enzymatic reactions still to be identified, as position-specific observations allow deductions as to the putative reaction mechanism involved.

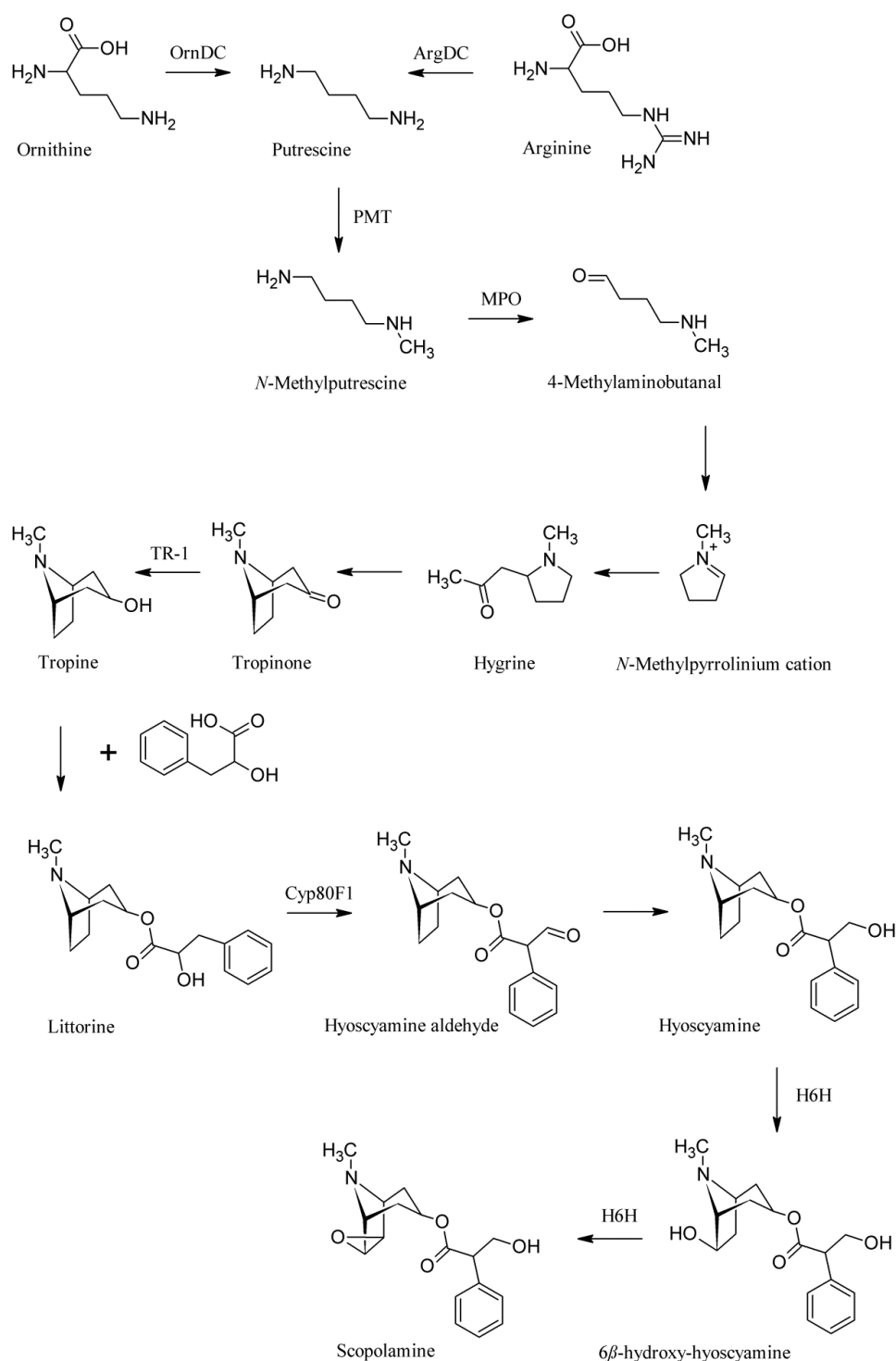


Figure 2-6: Biosynthesis of scopolamine. Abbreviations: ornithine decarboxylase (OrnDC), arginine decarboxylase (ArgDC), putrescine-*N*-methyltransferase (PMT), *N*-methylputrescine oxidase (MPO), tropinone-reductase I (TR-1), littorine mutase/ monooxygenase (Cyp80F1), hyoscyamine 6β-hydroxylase (H6H)

2.4.2. Structure elucidation and full chemical synthesis

Scopolamine was first isolated from and named after *Scopolia*, native in Europe and Asia, by Ernst Schmidt and Hermann Henschke in 1888 (Schmidt and Henschke 1888; Schmidt 1892). A few years later, Willstätter was the first scientist who completely elucidated the structure of the tropane alkaloid ring and developed a chemical synthesis of tropine providing the possibility to produce hyoscyamine (Willstätter 1901). In 1917, Robinson published a short method for the synthesis of tropinone by using succinaldehyde, methylamine and an acetonedicarboxylic acid calcium salt, a proposal, which is of great significance to date (Robinson 1917; Humphrey and O'Hagan 2001). Based on Robinson's synthesis of tropinone, it was supposed that the *in vivo* reactions could be similar to the chemical synthesis, which led to further progress in the elucidation of the tropane alkaloid pathway, exemplified in the following with ornithine and arginine. Leete showed the non-proteinogenic amino acid ornithine, as an equivalent to succinaldehyde, to be involved in the biosynthesis of the tropane skeleton by feeding *Datura stramonium* plants with radioactive labelled [2-¹⁴C] ornithine (Leete et al. 1954). As the metabolism of arginine is closely related to ornithine, it has also been proved to take part in the tropane alkaloid metabolism as a precursor (Walton et al. 1990). Robins demonstrated that arginine mainly contributes to the production of tropane alkaloids and that the responsible enzymes for the conversion of arginine and ornithine, namely the OrnDC and ArgDC, interact with each other (Robins et al. 1991).

The correct structure of scopolamine was first postulated by Gadamer and Hammer in 1921, but could not be reliably verified at that time (Gadamer and Hammer 1921). In 1923, Willstätter and Berner suggested four potential projection formulas of scopolamine (Willstätter and Berner 1923). Thirty years later, in 1953, Fodor and Kovács suggested the piperidine ring in the chair form, in analogy to Meinwald (Fodor and Kovács 1953; Meinwald 1953). In 1959, the first total synthesis of scopolamine was published by Dobó et al. starting from tropane-3 α ,6 β -diol via 3 α -Acetoxytrop-6-ene to 3 α -acetoxy-6 β ,7 β -epoxytropane, the latter acylated with O-acetyltropoyl chloride and hydrolysed to scopolamine (Dobo et al. 1959). Recently, a second approach to synthetically produce scopolamine was published by Nocquet and Opatz in a nine step reaction with 6,7-dehydrotropine as key intermediate including two different approaches to form the tropane skeleton with similar yields. The last reaction step, the chemoselective epoxidation of the 6,7-double bond, proved to be the limiting step of the full synthesis with only 16 % yield. Nevertheless, since higher yield was reported for related substrates, various possibilities for the optimisation of the last reaction

step exist and need to be further investigated (Nocquet and Opatz 2016). Until today, the chemical synthesis does not seem to be competitive to direct extraction of plant material, as the synthetic routes are still expensive, low yielding and include too many reaction steps (Kai et al. 2007).

2.5. Production

2.5.1. Industrial production

Down to the present day, industrial production of scopolamine is based extensively on field cultivation of hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii* mainly in Australia (Figure 2-7), associated with drying, extraction, isolation and purification at different sites (Foley 2006).



Figure 2-7: Photograph of the Boehringer Ingelheim Pty Limited *Duboisia* Farms located next to Kingaroy, Australia

Overseas plantations of *Duboisia* species have also been started in other regions like India or South America (Williams 2013; Mangathayaru 2013). Related plant genera like *Atropa* spp. or *Datura* spp. are not used for commercial production due to their lower content in

scopolamine (0.2-0.8 % compared to 2-4 % of total alkaloids in *Duboisia* spp.) (Gryniewicz and Gadzikowska 2008). Main global producers providing plant material are Boehringer Ingelheim Pty Limited as well as Alkaloids of Australia Pty Limited, both located in Kingaroy, Australia, and Alkaloids Corporation in Calcutta, India. Also involved in the processing to produce pure scopolamine are the Fine Chemicals Corporation in Cape Town, South Africa, and Phytex Australia Pty Ltd in Sydney, Australia.

2.5.2. Agricultural cultivation

Rosenblum already stated in 1954 that various environmental factors, e.g. the daily exposure to sunlight and the soil composition, are able to mask genetic characteristics in the field (Rosenblum 1954). This makes it difficult to get satisfactory results in field trials, especially by comparing plants grown in different geographical regions. However, in the past decades some field trials were carried out on *Duboisia* and related plants. In Queensland, Australia, Luanratana and Griffin observed the alkaloid content to decrease in autumn and winter in field grown *Duboisia* plants and assumed the low temperatures to be responsible for the decreased amount in tropane alkaloids (Luanratana and Griffin 1980b). As seasonal variations have also been observed in *Duboisia* hybrids grown in Japan (Ikenaga et al. 1985), plants were cultivated under controlled temperature in greenhouses in Thailand and showed stable scopolamine contents (Luanratana et al. 1990). Until now, a systematic assessment of the impact of individual climate factors such as temperature, humidity, light intensity and duration on the alkaloid biosynthesis and biomass production is lacking.

Not only the climate, but also the soil composition and fertilisation have an influence on the alkaloid biosynthesis and plant development in *Duboisia* and related plant genera. In outdoor grown *Duboisia* plants in Queensland, Australia, Luanratana and Griffin observed no correlation between alkaloid yield and fertilisation with nitrogen, potassium and sulfur (Luanratana and Griffin 1980b). Field trials in Saudi Arabia with *Datura innoxia* using Sangral[®] compound fertiliser showed increased scopolamine and hyoscyamine levels up to a concentration of 600 kg/ha, decreasing again at higher amounts (Al-Humaid 2004). By harvesting five *Hyoscyamus* species originating from different geographical origins in Iran including soil analysis, Nejadhabibvash et al. demonstrated positive correlations between nitrogen, phosphorus, potassium, calcium and yield in scopolamine and hyoscyamine (Nejadhabibvash et al. 2012).

In contrast to field trials, experimental set-ups using hydroponic culture under glasshouse conditions allow a better differentiation regarding the specific effect of individual nutrients on alkaloid and biomass production, which will be specified in the following section. Referring to nitrogen, contradictory results have been published (Alaghemand et al. 2013; Luanratana and Griffin 1980a). Luanratana and Griffin observed a decrease in total alkaloids at higher nitrogen levels in case of *Duboisia* plants in contrast to Alaghemand measuring the highest content in scopolamine and hysocyamine with increased nitrogen concentrations using *Hyoscyamus* plants. Smolenski described a reduced biomass production and an elevated yield of total nitrogen and alkaloids, as the ratio of calcium to potassium was increased in *Atropa* plants (Smolenski et al. 1967). Moreover, Luanratana observed higher potassium levels leading to a significant increase in the percentage of scopolamine in hydroponically cultivated *Duboisia* plants (Luanratana and Griffin 1980a).

The previously described experiments show that there is a lot of potential in order to improve the large scale cultivation of scopolamine producing plants. In a first step, systematic trials under controlled, reproducible conditions should be conducted in order to eliminate environmental influences that cannot be clearly allocated. Secondly, the knowledge gained by those trials could be transferred to field-grown plants and tested within specific field trials.

2.5.3. Biotechnological Production

Callus and Cell cultures

In 1957, West and Mika were the first scientists using callus cultures of *Atropa belladonna* instead of intact plants in order to verify the site of tropane alkaloid biosynthesis and thereby detecting atropine in root callus (West Jr. and Mika 1957). From now on, many different approaches were applied in order to use callus or suspension cultures for the targeted production of tropane alkaloids. The first publications showed the alkaloids in undifferentiated cultures to be low concentrated or hardly present compared to intact plants (Tabata et al. 1972; Staba and Jindra 1968; Stohs 1969). Endo and Yamada successfully demonstrated the production of tropane alkaloids in non-transformed roots derived from callus cultures of *Duboisia*. Interestingly, no alkaloids could be detected in the callus cultures used for differentiation indicating that the alkaloid production in *Duboisia* species is associated with organogenesis of roots (Endo 1985). This is most likely due to the specific location of key enzymes, e.g. the pericycle-specific location of the PMT and H6H (Suzuki et

al. 1999; Hashimoto et al. 1991). Since the roots are supposed to be the main site of tropane alkaloid biosynthesis, from then on the focus was placed on *in vitro* root cultures (Parr 1989).

Hairy root cultures

During the eighties, *Agrobacterium rhizogenes*-transformed root cultures of many different plant genera (*Atropa*, *Datura*, *Duboisia*, *Hyoscyamus*, *Scopolia*) were used in order to improve the production of hyoscyamine and/or scopolamine (Kamada et al. 1986; Jaziri et al. 1988; Mano et al. 1986; Mano et al. 1989). *A. rhizogenes* induces hairy root disease marked by extensive proliferation of roots originating from an infected plant wound (White and Nester 1980). The hairy root phenotype is generally characterized by fast growth and genetic stability in contrast to conventional root cultures. Furthermore, the secondary metabolites synthesized by hairy roots are comparable to those of intact parent roots and found in similar or even higher amounts (Sevón and Oksman-Caldentey 2002). By using hyoscyamine-producing hairy root cultures of *Datura stramonium*, Payne et al. were also able to show that those cultures preserve the biosynthetic capacity of their mother plant and that formation of the desired products is less susceptible to manipulation than in callus or cell suspension cultures (Payne et al. 1987).

Many different cultivation parameters were optimised including culture media, pH-value, carbon source, nutrient concentrations and the use of biotic and abiotic elicitors, such as methyl jasmonate, chitosan, salicylic acid or silver nitrate improving the scopolamine production (Dupraz et al. 1994; Pitta-Alvarez and Giulietti 1995; Pitta-Alvarez and Giulietti 1999; Pitta-Alvarez et al. 2000; Palazón et al. 2008). The use of the wound stress hormone methyl jasmonate is still of current interest, as its stimulation of the tropane alkaloid biosynthesis is not fully understood and seems to be species dependent (Ryan et al. 2015). Previous studies reported a lack of jasmonate stimulation of tropane alkaloid biosynthesis in *Atropa belladonna* or *Hyoscyamus muticus* (Biondi et al. 2000; Suzuki et al. 1999), whereas methyl jasmonate-induction was successful in cultured roots of *Datura stramonium* (Zabetakis et al. 1999). Furthermore, systematic and repeated selection of promising lines for further culturing was applied and the influence of somaclonal variation was evaluated systematically (Maldonado-Mendoza et al. 1993; Sevón et al. 1998). Subroto et al. also tested the co-culture of *Atropa belladonna* shooty teratomas and hairy roots using hormone-free medium, thereby mimicking the whole plant by providing the possibility for localized metabolite synthesis as well as for transportation of compounds between organs. This led to a

3-11 times increased accumulation in scopolamine compared to the average levels found in leaves of intact plants (Subroto et al. 1996). Nevertheless, no breakthrough in the biotechnological production of tropane alkaloids was achieved by that, which is shown in the following examples. In *Duboisia myoporoides* hairy root cultures the average content in scopolamine was significantly increased compared to the parent lines after several selections (3.2 % per dry weight compared to 0.15 % / dw), but at the same time the growth rate was decreased (Yukimune et al. 1994). Decreased growth combined with high alkaloid production and vice versa was also observed by Sauerwein and Shimomura using hairy roots of *Hyoscyamus albus* for testing different media and sucrose concentrations (Sauerwein and Shimomura 1991). And even though the alkaloid content in *Datura candida* hairy root cultures was 1.6- and 2.6-fold higher (up to 0.68 % / dw) compared to the aerial parts and roots of the parent plants, this is still not competitive with the commercially grown *Duboisia* hybrids containing up to 4 % of tropane alkaloids in their dried leaves (Gryniewicz and Gadzikowska 2008; Christen et al. 1989). Moreover, one major restriction for the commercial production of scopolamine by hairy root cultures remains the scale up to an industrial relevant level. Different bioreactor systems have been tested so far, including modified airlift and stirred tanks for *Datura metel* and *Brugmansia candida*, connective flow reactors for *Hyoscyamus muticus* as well as bubble-column and spray bioreactors for *Hyoscyamus niger* and *Scopolia parviflora*, and efforts to synthesize scopolamine in bioreactor systems appropriate for large scale production are still ongoing (Cardillo et al. 2010; Cusido et al. 1999; Carvalho 1998; Min et al. 2007; Jaremicz et al. 2014).

Genetic engineering

The gene isolation, purification and sequencing as well as the characterisation of the related key enzymes within the tropane alkaloid pathway (PMT, EC 2.1.1.53; TR-1, EC 1.1.1.206; H6H, EC 1.14.11.11) resulted in new approaches in the biotechnological production of scopolamine (Hashimoto et al. 1989; Matsuda et al. 1991; Hashimoto et al. 1992). Plant cell cultures, bacterial cultures as well as transgenic plants were used as hosts for the overexpression of one or multiple genes in order to increase the biosynthesis of scopolamine (Figure 2-8). The results of those researches will be discussed in detail within the following sections.

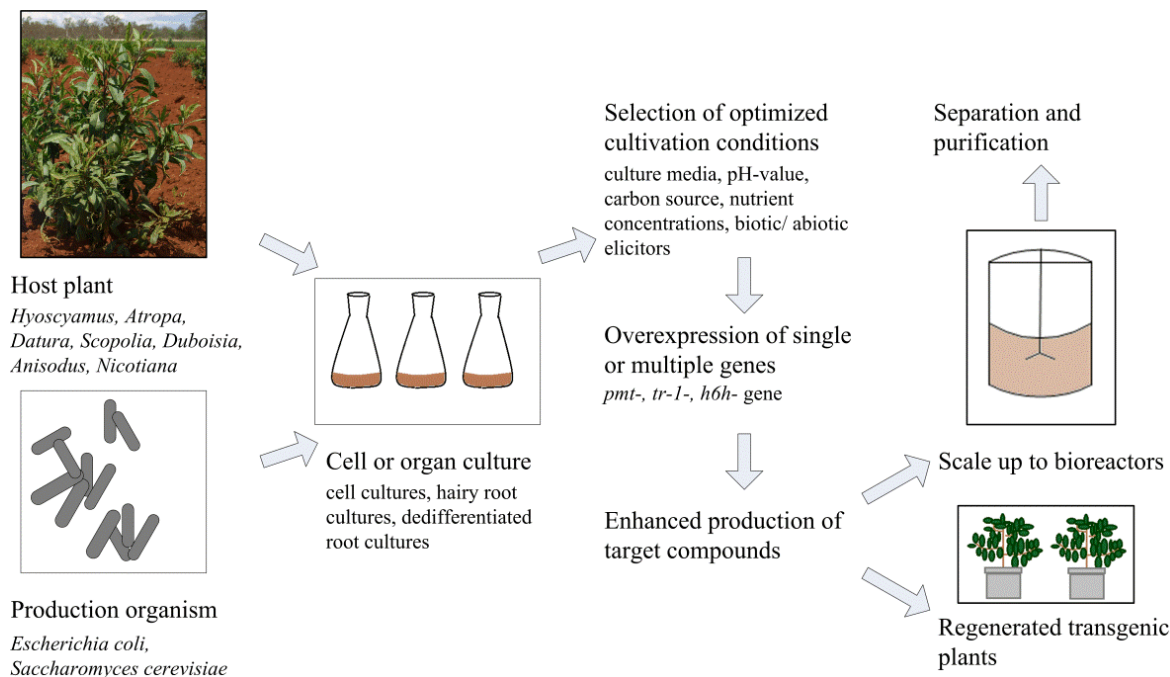


Figure 2-8: Genetic engineering approaches in order to enhance the scopolamine production

a) *Overexpression of the pmt gene*

In 2001, Sato et al. showed transgenic hairy root clones of *Atropa belladonna* with a 5-fold increased *pmt* transcript level to have quantitatively as well as qualitatively similar alkaloid profiles compared to the wild type (Sato et al. 2001). This indicates that the overexpression of the PMT enzyme might be not sufficient to boost the tropane alkaloid synthesis later in the biosynthetic pathway. In 2002, Moyano et al. introduced the *pmt* gene in *Duboisia* hybrids and found the *N*-methylputrescine levels of the resulting hairy roots to be 2–4-fold higher compared to wild type roots, but again no significant increase in tropane alkaloids (Moyano et al. 2002). Only shortly thereafter, Moyano et al. genetically engineered hairy root cultures of *Datura metel* and *Hyoscyamus muticus* by overexpressing the *pmt* gene and observed a more rapid ageing, growth reduction as well as an accumulation of higher amounts of tropane alkaloids than in control hairy roots. Thereby, hyoscyamine and scopolamine production were both increased in hairy root cultures of *Datura*, whereas in *Hyoscyamus* only hyoscyamine was found in higher amounts compared to controls (Moyano et al. 2003). In 2005, the overexpression of the *pmt* gene in *Scopolia parviflora* improved its production of hyoscyamine and scopolamine. However, the yields were only comparable to or even lower than those synthesized by other species in the absence of such transformation (Lee et al. 2005). Furthermore, the *pmt* gene has been introduced in *Hyoscyamus niger*, thereby showing a

significant increase in PMT activity and more than 5-fold higher contents of *N*-methylputrescine compared to wild type hairy roots, but no increase in tropane alkaloids. Zhang et al. also demonstrated that the exposure of the roots to the elicitor methyl jasmonate positively affects both polyamine and tropane biosynthetic pathways in *Hyoscyamus* (Zhang et al. 2007). Those data already show that the same biosynthetic pathway in related plant species might be differently regulated. Moreover, the transgene allows bypassing of the endogenous control of the metabolic flux to the alkaloids including metabolic changes not directly related to the transgene presence. Furthermore, most pathways do not have a single rate-limiting step, but the flux is controlled by multiple enzymes and feedback inhibition by the end-product (Kholodenko et al. 1998). This makes it difficult to predict the effect of the overexpression of single genes.

b) Overexpression of the tr-1 gene

The tropinone reductases TR-1 and TR-2 are another branch point within the biosynthesis of scopolamine. The TR-1 is thereby responsible for the biosynthesis of hyoscyamine and scopolamine via tropine, whereas the TR-2 catalyses the formation of calystegines via pseudotropine. Richter et al. showed that overexpression of the *tr-1* or *tr-2* gene in *Atropa belladonna* led to a higher enzyme activity and an increase in the respective enzyme products pseudotropine or tropine. Moreover, high pseudotropine levels resulted in an accumulation of calystegines in the roots and a high expression of the *tr-1* increased hyoscyamine by a factor of three and scopolamine by a factor of five compared to controls (Richter et al. 2005).

c) Overexpression of the h6h gene

The hyoscyamine 6 β -hydroxylase (H6H) is the last enzyme of the tropane alkaloid pathway which is needed in order to convert hyoscyamine into scopolamine and has been the main target of transgenic approaches in order to improve the scopolamine biosynthesis in hairy root cultures and regenerated plants. In 1992, Yun et al. used hyoscyamine-rich *Atropa belladonna* plants for the overexpression of the *h6h* gene from *Hyoscyamus niger*. The single primary transformed plant and its subsequent generations almost exclusively stored scopolamine in the aerial plant parts indicating an nearby complete conversion of hyoscyamine to scopolamine (Yun 1992). Shortly afterwards, Hashimoto et al. successfully engineered *Atropa belladonna* hairy roots using the *h6h* gene of *Hyoscyamus niger*. Thereby, increased amounts and high

enzyme activities of the H6H were detected in the engineered hairy roots, which contained up to 5-fold higher levels in scopolamine and elevated amounts of its direct precursor 6 β -hydroxy-hyoscyamine compared to the wild type (Hashimoto et al. 1993b). *Hyoscyamus muticus* hairy roots overexpressing the same gene contained up to a 100-fold increased amounts in scopolamine compared to controls, but hyoscyamine still remained the main alkaloid indicating an incomplete conversion to the target substance scopolamine. Moreover, a large variation in the tropane alkaloid pattern was observed among the 43 positive clones with only 22 of these clones showing elevated levels of scopolamine, probably depending on the *h6h* expression level (Jouhikainen et al. 1999). The *h6h* gene was also introduced into the genome of a *Duboisia* hybrid rich in scopolamine. The resulting engineered hairy root lines contained increased amounts in scopolamine up to a factor of three with regard to the wild type hairy roots, but there was no significant enhancement in scopolamine production in the engineered regenerated plants compared to controls (Palazón et al. 2003). Rahman et al. used the *Duboisia leichhardtii* for the overexpression of the *h6h*, which led to high variations among the *h6h* gene positive clones. In case of clone 117 the conversion of hyoscyamine to scopolamine was almost complete with a rate of more than 95 %, whereas only half as much scopolamine than hyoscyamine was produced by clone 16 (Rahman et al. 2006). Transgenic hairy roots of *Atropa baetica* overexpressing the *h6h* showed hyoscyamine to be entirely converted into scopolamine. In the best clone, scopolamine accumulated in 9-fold higher amounts compared to wild type plants (Zárate et al. 2006). Moreover, Moyano et al. demonstrated that plant cells, which do not naturally produce secondary compounds of interest, are able to do so after overexpression of the responsible gene and feeding with a suitable precursor. They used dedifferentiated root cultures of *Nicotiana tabacum* carrying the 35S-*h6h* gene from *Hyoscyamus niger* and successfully showed these cell cultures to be able to bioconvert 18 % of exogenous hyoscyamine into scopolamine using a 5l turbine stirred tank bioreactor (Moyano et al. 2007).

However, apart from the high variations in alkaloid production and conversion rate of hyoscyamine to scopolamine which is to be found within the previous examples, also morphological anomalies were detected, both making it difficult to achieve a stable production of scopolamine. Even though hyoscyamine-rich plants, such as *Hyoscyamus* or *Atropa*, were actually converted into a potential source of scopolamine by significantly increasing its production, the amounts in scopolamine obtained so far are still too low for a commercial application.

d) Engineering two steps

Besides the expression of single genes, the approach of multiple gene expression was pursued in order to further enhance the scopolamine production. This is illustrated within the examples given in the following. In 2004, the *pmt* and *h6h* gene were introduced and overexpressed in transgenic *Hyoscyamus niger* hairy root cultures. Transgenic hairy root lines overexpressing both genes accumulated significantly higher amounts of scopolamine (up to 411 mg/l) compared to the wild type and single gene transgenic lines overexpressing the *pmt* or the *h6h* gene (Zhang et al. 2004). This indicates that transgenic hairy roots overexpressing both *pmt* and *h6h* gene may have an enhanced flux in the tropane alkaloid biosynthetic pathway that increases the yield in scopolamine, thereby being more effective than plants hosting only one of the two genes. This was also shown by overexpressing *pmt* and *tr-1* gene in *Anisodus acutangulus* producing significantly higher levels of tropane alkaloids compared to the wild type and single gene transformed lines. But as the H6H responsible for the conversion of hyoscyamine to scopolamine was not overexpressed, hyoscyamine remained the major alkaloid being only transformed to scopolamine on a small scale (Kai et al. 2011b). A simultaneous expression of the *tr-1* and *h6h* gene in *Anisodus acutangulus* again led to a significant increase in tropane alkaloids compared to controls and single gene transformed lines. The levels in hyoscyamine, 6 β -hydroxy-hyoscyamine (anisodamine) and α -hydroxyscopolamine (anisodine) could be significantly increased as well as those of scopolamine; however, the latter was still low concentrated compared to its precursors (Kai et al. 2012). As the examples show, the effect of overexpressing two enzymes at once is difficult to predict and also undesired reactions might be favoured by that, for example the conversion from scopolamine to α -hydroxyscopolamine, a reaction probably catalysed by an unknown hydroxylase. Still, a co-overexpression of multiple biosynthetic enzymes might be a promising strategy, including the PMT responsible for the first committed step in the biosynthesis, the TR-1 as branch-controlling enzyme and the metabolically downstream enzyme H6H to the target substance scopolamine.

Protein engineering

Another approach that was applied was the use of bacteria cultures, such as *Escherichia coli*, for the production of scopolamine. Hashimoto et al. already used *E. coli* in 1993 in order to characterize the H6H of *Hyoscyamus niger* by using a fusion protein with maltose-binding protein. Even though the expression level of the fusion protein was low, they were able to

show that in case of a sufficient expression of the *h6h* gene, scopolamine was synthesized without substantial accumulation of its precursor 6 β -hydroxy-hyoscyamine. In contrast to that, a limiting amount of *h6h* in relation to the supply of hyoscyamine led to an increase in 6 β -hydroxy-hyoscyamine (Hashimoto et al. 1993a). The gene encoding H6H in *Anisodus acutangulus* was cloned by Kai et al. and expressed in *E. coli* by using a His-tag or GST-tag fusion protein. Hereby, the bifunctional assay with His-AaH6H and GST-AaH6H led to a conversion of hyoscyamine (at a concentration level of 40 mg/l) into scopolamine at 32 and 31 mg/l and 6 β -hydroxy-hyoscyamine at 3.4 and 3.1 mg/l, respectively (Kai et al. 2011a). Only recently, random mutagenesis and site-directed saturation mutagenesis were applied for the enhancement of the hydroxylation activity of H6H from *Anisodus acutangulus* using *E. coli*. One double mutant, namely AaH6HM1(S14P/K97A), possessed a 3.4 times improved hydroxylation activity and an 2.3-fold enhanced *in vivo* epoxidation activity in comparison to the wild type enzyme. The total yield in scopolamine was 97 % (1.068 g) by using this mutant in a 5 l bioreactor (working volume: 3 l) with a space time yield of 251 mg/l/d (Cao et al. 2015). This is a considerable improvement compared to Hashimoto et al. and Kai et al., where scopolamine was produced from hyoscyamine with space time yields of 4.1 mg/l/d and 16 mg/l/d by cultivating recombinant *E. coli* cells in shake flasks, and shows that protein engineering in *E. coli* might be promising for further scale up. Not only *Escherichia coli*, but also *Saccharomyces cerevisiae* was applied as production organism for scopolamine. In this case, the *h6h* isolated from *Brugmansia candida* was used for functional expression, untagged and with a His-tag. The tagged enzyme converted hyoscyamine into 6 β -hydroxy-hyoscyamine (35.7 % after 15 h of incubation), whereas the untagged protein was able to produce scopolamine as well as 6 β -hydroxy-hyoscyamine (7.6 and 83.3 % respectively) (Cardillo et al. 2008). Even though *S. cerevisiae* has a long history of application in industry and combines the advantages of unicellular organisms with the ability of protein processing together with the lack of endotoxins, the yields in scopolamine are too low for commercial exploitation due to the incomplete conversion of hyoscyamine. Furthermore, the histidine tag fused to the protein and the epitope seems to reduce the capability of the enzyme to synthesize 6 β -hydroxy-hyoscyamine and particularly scopolamine. If a large scale production of high amounts in scopolamine by microorganisms, competitive to extraction from plant material, will be possible in the future, remains questionable.

Doubled Haploidy and Polyploidisation

The use of double haploid plants might also be one strategy towards homozygous lines, thereby improving breeding by the selection of lines showing enhanced biomass production and yielding high levels of the desired medicinal compounds. Microspore-derived embryos and haploid plants have already been successfully generated in case of *Atropa belladonna* and *Hyoscyamus muticus* (Chand and Basu 1998; Zenkteler 1971). However, compared to crops like wheat or barley, only little effort has been undertaken with regard to medicinal plants so far (Ferrie 2009). In order to successfully use this technique in breeding and cultivation, more work in this field of research has to be done.

Another promising strategy towards an enhanced scopolamine production will be the use of polyploid plants, which may differ in their cytological, biochemical, genetic and physiological character providing unique tolerances and developmental patterns. Within previous research, autopolyploids of many medicinal plants showed increased production of tropane alkaloids (Levin 1983). Lavania and Srivastava observed up to 22.5 % enhanced production of tropane alkaloids as well as higher fertility in artificial autotetraploids of *Hyoscyamus niger* (Lavania and Srivastava 1991). Only recently, Belabbassi et al. studied the effects of polyploidisation in hairy roots of *Datura stramonium* revealing the content of hyoscyamine to be increased up to 276 % in tetraploid lines using salicylic acid as elicitor (Belabbassi et al. 2016). Dehghan et al. reported tetraploid plants of *Hyoscyamus muticus* to increase scopolamine production up to 200 % compared to their diploid counterparts, however, this did not apply to the corresponding induced hairy root cultures. Moreover, manipulation of the ploidy level and adaption of the culture conditions successfully shifted the scopolamine/hyoscyamine ratio towards scopolamine, even though hyoscyamine is known to be the main alkaloid *Hyoscyamus* (Dehghan et al. 2012).

These examples illustrate that it is worth to continue working with polyploid plants and to apply this knowledge also in order to optimise breeding in *Duboisia* hybrids towards improved production plants.

2.6. Conclusions

Solanaceous plants containing scopolamine have been medically used for centuries and scientific research on the active compounds including scopolamine started more than 100 years ago. Still, the global demand in scopolamine and its semisynthetic derivatives is

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increasing due to their various therapeutic applications, e.g. motion sickness or gastrointestinal spasms. Worldwide, many different botanical sources of scopolamine have been identified and characterized. From those plant extracts of the aerial parts of field-grown hybrids of *Duboisia* are mainly used for its commercial production by today.

Alternatives to the agricultural production of scopolamine continue unabated. But the full chemical synthesis, being very complex and therefore quite expensive, is economically not feasible by now. Moreover, efforts using biotechnological approaches (genetically modified bacteria, plant cell cultures and transgenic plants) in order to optimise the output in scopolamine are ongoing. They are complicated by the complex interactions and various factors influencing the tropane alkaloid biosynthesis including biosynthetic steps which are not fully elucidated yet.

The metabolic engineering results achieved so far reveal that a thorough knowledge of all steps of the biosynthetic pathway is necessary including the sequences of the responsible enzymes and their regulation in order to optimise the biosynthesis in transgenic plants as well as in cell cultures towards the desired medicinal product scopolamine. Due to the high occurrence of multiple rate-limiting steps and the interaction of metabolic pathways and their metabolites involved, it is difficult to predict the results of overexpressing a single or multiple genes involved in the targeted pathway. Even though more and more knowledge is gained regarding the enzymes involved, their localisation and catalytic activities, there are still bottlenecks to be found within the tropane alkaloid pathway. In order to fully elucidate the complete pathway, systematic research combining proteomics, transcriptomics and metabolomics and the use of metabolite correlation networks will be helpful. Besides, the selection of the most suitable host is crucial, as not only in cell cultures of the botanical sources of scopolamine like *Duboisia* or *Hyoscyamus*, but also in *Echerichia coli* or *Saccharomyces cerevisiae* cultures as well as plants like *Nicotiana tabacum*, a biotechnological production has been shown to be realizable. In addition, a stable and reproducible production system is required, which also needs to bring along the necessary characteristics for a scale-up to industrial levels for commercialisation. Until now, these requirements are not fully met and further studies are ongoing.

A promising strategy in order to improve the industrial production in scopolamine and to cover its rising demand will be the further optimisation of the field cultivation of *Duboisia*. Previous research revealed that the genotype, the climate as well as the fertilisation have an impact on the alkaloid biosynthesis and plant development in *Duboisia* indicating a high

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potential for improving the large scale cultivation of scopolamine producing plants. However, as various biotic and abiotic interactions make it difficult to clearly allocate individual influencing factors towards higher scopolamine and biomass production, further experiments are needed. In a first step, the impact of genotype, plant age and type of cultivation should be assessed in a targeted manner. Secondly, systematic trials under controlled, reproducible conditions should be conducted in order to characterise the key drivers towards a higher scopolamine and biomass production and to be able to systematically apply those findings in future.

2.8. References

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Chapter 3: Discrimination of wild types and hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii* at different growth stages using ^1H NMR-based metabolite profiling and tropane alkaloids-targeted HPLC-MS analysis

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SFU, AR and OK jointly planned and designed the chapter; SFU performed the plant cultivation, sampling and processing within the climate chambers and took care of the HPLC-MS method validation and analysis; NA sampled and processed the field grown *Duboisia* plants, SFU, YHC and LC jointly planned, performed and analysed the NMR measurements; SFU did the data analysis and wrote the manuscript with inputs from all coauthors; OK oversaw the entire project as senior supervisor of SFU.

3.1. Abstract

Duboisia species, which belong to the family of Solanaceae, are commercially cultivated in large scale, as they are main source of the pharmaceutically-used active compound scopolamine. In this study, ^1H NMR-based metabolite profiling linking primary with secondary metabolism and additional quantification via HPCL-MS with special focus on the tropane alkaloids were applied to compare leaf and root extracts of three wild types and two hybrids of *Duboisia myoporoides* and *D. leichhardtii* at different developmental stages grown under controlled conditions in climate chambers and under agricultural field plantation. Based on the leaf extracts, a clear distinction between the *Duboisia* hybrids and the wild types *Duboisia myoporoides* and *D. leichhardtii* using principal component analysis of ^1H NMR data was observed. The average content in scopolamine in the hybrids of *Duboisia* cultivated in climate chambers increased significantly from month 3 to 6 after potting of the rooted cuttings, however, less pronounced for the examined wild types. The *Duboisia* hybrids grown in climate chambers showed higher growth and contained more sugars and amino acids than *Duboisia* hybrids grown in the field, which in contrast showed an enhanced flux towards tropane alkaloids as well as flavonoids. For a more detailed analysis of tropane alkaloids, an appropriate HPLC-MS method was developed and validated. The measurements revealed large differences in the alkaloid pattern within the different genotypes under investigation, especially regarding the last enzymatic step, the conversion from hyoscyamine to scopolamine by the hyoscyamine 6 β -hydroxylase. Scopolamine was found in highest concentrations in *Duboisia* hybrids (20.04 ± 4.05 (genotype D) and 17.82 ± 3.52 mg/g dry wt (genotype E)) followed by *Duboisia myoporoides* (12.71 ± 2.55 mg/g dry wt (genotype A)), both showing a high selectivity for scopolamine in contrast to *Duboisia leichhardtii* (3.38 ± 0.59 (genotype B) and 5.09 ± 1.24 mg/g dry wt (genotype C)) with hyoscyamine being the predominant alkaloid. The results of this study clearly demonstrate that the abundance of tropane alkaloids in *Duboisia* species is largely influenced by genetic characteristics as well as environmental conditions.

Key words

Duboisia myoporoides, *Duboisia leichhardtii*, *Duboisia* hybrids, Solanaceae, Metabolomics, ^1H NMR, HPLC-MS, Alkaloids, Scopolamine

3.2. Introduction

Duboisia is a native Australian plant (Barnard 1952), which belongs to the family of Solanaceae within the subfamily Nicotianoideae, tribe Anthocercideae (Olmstead et al. 2008). Like related plant genera such as *Atropa*, *Brugmansia*, *Datura*, *Hyoscyamus* and *Scopolia* (Griffin and Lin 2000), it is known to contain tropane alkaloids as specialised plant components (Rätsch 2012). Thereof pharmaceutically most important are scopolamine and hyoscyamine (Hashimoto et al. 1991), which are medically used due to their strong anticholinergic properties (Witkin et al. 2014). The worldwide demand for scopolamine is considered to be ca. 10-fold higher in respect to hyoscyamine and its racemate atropine, which is presumably due to its higher physiological activity and lower adverse effects (Oksman-Caldentey 2007; Palazón et al. 2008).

Efforts to optimise alkaloid biosynthesis by using modern plant biotechnology in *Duboisia* are pursued undiminished. Callus cultures and regenerated shoot cultures only gave low yields in tropane alkaloids (Foley 2006). Also, by overexpressing biosynthetic genes in regenerated plants and by using hairy root cultures, no significant increase in alkaloid levels were achieved compared to the amount extracted from plant material (Palazón et al. 2003; Hashimoto and Yamada 2003). Currently, industrial manufacturing operations providing scopolamine and derivatives are largely based on field plant cultivation of hybrids of *Duboisia myoporoides* and *D. leichhardtii* (Foley 2006).

The biosynthesis of tropane alkaloids is known to mainly take place in the roots, from where they are transported to the aerial parts of the plants (Hashimoto et al. 1991; Wink 1987). The complete biosynthetic pathway is not yet fully elucidated (Humphrey and O'Hagan 2001), but important enzymes were identified and characterised, for example the putrescine *N*-methyltransferase (PMT, EC 2.1.1.53) and the hyoscyamine 6 β -hydroxylase (H6H, EC 1.14.11.11). Tropane alkaloids originate from putrescine, which is synthesised from the amino acids ornithine or arginine (Hashimoto et al. 1989). The PMT then catalyses the *S*-adenosylmethionine-dependent *N*-methylation of putrescine and represents the pathway defining entry step in the tropane alkaloid biosynthesis (Hibi et al. 1992). Tropine is subsequently formed via the tropinone-reductase I (TR-1, EC 1.1.1.206) (Nakajima and Hashimoto 1999), whereas the tropinone-reductase II (TR-2, EC 1.1.1.236) converts tropinone to pseudotropine, a precursor of the calystegines (Hashimoto et al. 1992; Ripperger 1995). After tropine being condensed with the phenylalanine-derived phenyllactate to littorine, hyoscyamine is formed via an intramolecular rearrangement of littorine (Armstrong et al.

1987; Armstrong et al. 1991). This reaction via hyoscyamine-aldehyde is catalysed by Cyp80F1, probably with an alcohol dehydrogenase involved (Li et al. 2006). The H6H finally converts hyoscyamine via 6 β -hydroxy-hyoscyamine to scopolamine (Figure 3-1) (Hashimoto et al. 1993; Perger 1864).

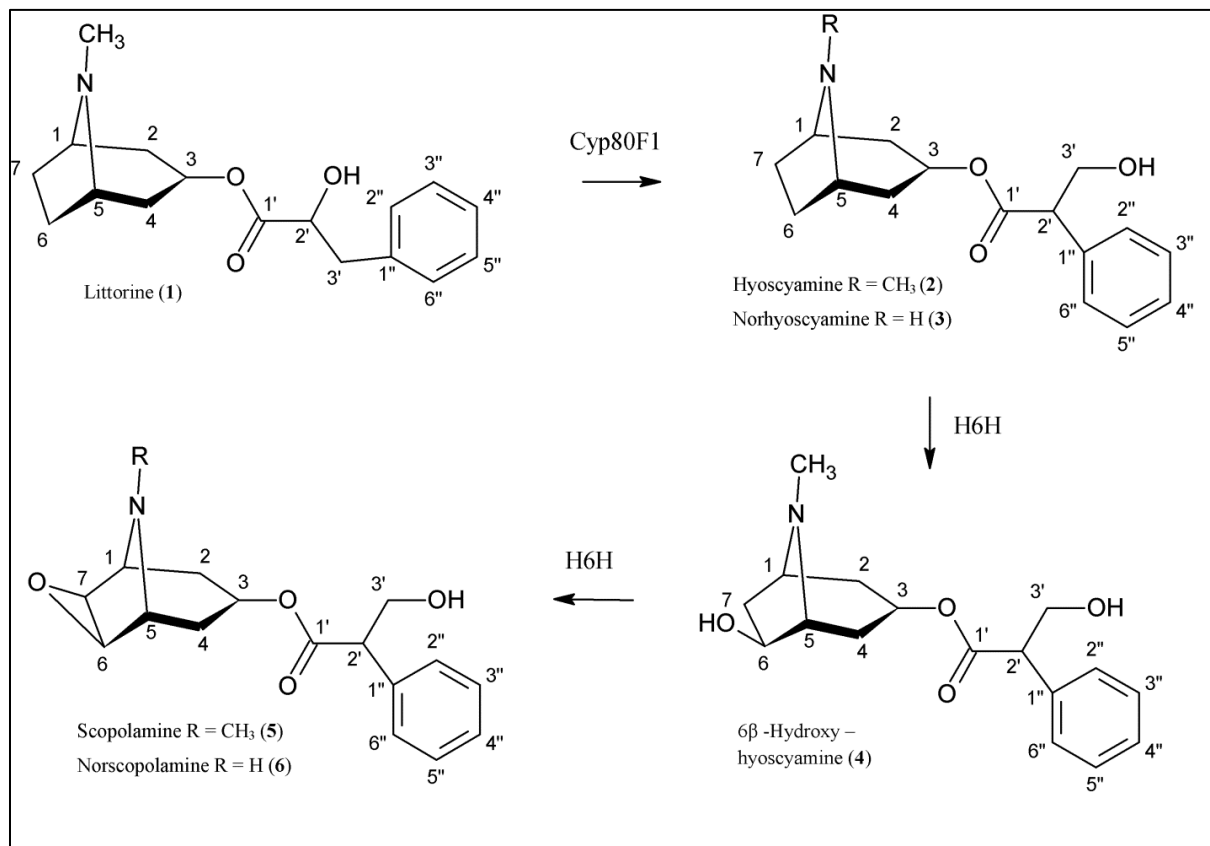


Figure 3-1: Biosynthesis of scopolamine starting from littorine. Selected compounds are discussed in the manuscript. The carbon numbering system for each compound is used in the following for NMR assignment.

To optimise the breeding process as well as the plant cultivation, metabolic profiling is helpful in order to get an overview of the plant metabolism in *Duboisia* and to assess the qualitative and quantitative occurrence of specific metabolites important for growth and alkaloid production. For this purpose, root and leaf extracts using 3- and 6-months old plants of various genotypes (three different wild types and two hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii*) of field-grown plants and plants grown under controlled cultivation in climate chambers are analysed. For the selection of the appropriate analytical method, nuclear magnetic resonance spectroscopy has a great advantage over other techniques because of its simple and fast sample preparation and high-speed throughput (Colquhoun 2007). It is often used for metabolic profiling, as it is a non-destructive method allowing the detection of

compounds of any polarity depending on the extraction method. The weakness of NMR lies in its low sensitivity compared to mass spectroscopy (Kim et al. 2011), so that only compounds at the upper end of the concentration range with levels above 50 μM are detected, which makes it difficult to identify and quantify minor compounds. As it mostly allows an untargeted analysis of a large group of metabolites which might be biosynthetically related to the tropane alkaloid biosynthesis, ^1H NMR is considered for a first macro-level analysis, whereas HPLC-MS is used in addition to provide more detail allowing a quantification of all major tropane alkaloids occurring in *Duboisia*. As plant extracts are very complex in composition, it is difficult to perform a visual comparison of large numbers of spectra or chromatograms. Multivariate statistical methods, like principal component analysis (PCA), are able to compress data into a more easily managed form (Colquhoun 2007). This makes it possible to relate one sample to others and identify important similarities and differences by comparing the relative proportion of metabolites to improve breeding and adapt cultivation.

Duboisia hybrids are the main source of scopolamine and commercially cultivated for decades due to their high content in tropane alkaloids, around half thereof being scopolamine (Foley 2006). Despite this, only little systematic research on the metabolite composition and on factors influencing breeding and cultivation has been done so far. Previous work on *Duboisia* has revealed that, in the field, various environmental influences, e.g. the daily exposure to sunlight and the soil composition, may mask genetic characteristics (Rosenblum 1954) and that the alkaloid content is decreased in autumn and winter presumably due to cold weather (Luanratana and Griffin 1980). This makes it difficult to obtain significant and reproducible results from field trials. To date, there has never been a detailed study looking at both primary and secondary metabolites in *Duboisia* wild types and hybrids at different developmental stages under fully comparable growth conditions with regard to field-grown plants. The strictly controlled cultivation in climate chambers allows minimising seasonal influences and interactions with other organisms. The knowledge whether the alkaloid production is age-dependent helps to decide about the optimal time point for harvesting or taking samples within the breeding process. The use of wild types in addition to hybrids offers a holistic view including naturally occurring plants and those obtained by repeated systematic selection. This is crucial in order to pick out promising genotypes being highly selective for scopolamine and to optimise the plant cultivation towards more biomass and scopolamine production.

3.3. Methods and Materials

3.3.1. Plant material

All plants under investigation in this study belong to the genus *Duboisia* R.Br. (family Solanaceae, subfamily Nicotianoideae, tribe Anthocercideae) 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 grown in climate chambers (CLF PlantMaster, CLF Plant Climatics GmbH, Wertingen, Germany) under strictly controlled conditions at 25 °C exposed to 12 h of light per day with an intensity of 110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ (lamp: Eye Cera Arc PAR36, 3500 K). The plants were fertilised by using drip irrigation containing Wuxal Super (Manna, Düsseldorf, Germany) in a concentration of 0.1 vol% (Plant number: TU-FUL-1-50). Roots and young / mature leaves were harvested after 3 and 6 months of growth, respectively. Additionally, samples of field-grown plants (3- and 6-months old, grown from March/June to September 2014) belonging to genotype E were taken on a commercial plantation of *Duboisia* hybrids close to Kingaroy in Queensland, Australia (Plant number: TU-FUL-50-60). Voucher samples were kept in the department of biochemical and chemical engineering at the Technical University of Dortmund.

After determination of the fr. wt, all samples were frozen immediately using liquid nitrogen and stored afterwards at $-80\text{ }^{\circ}\text{C}$. After grinding in liquid nitrogen and freeze-drying over 48 h, all samples were extracted and subsequently measured by ^1H NMR.

3.3.2. Chemicals and reagents

$\text{CH}_3\text{OH}-d_4$, D_2O formic acid and TSP- d_4 were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Ortho-phosphoric acid (85 wt%) was obtained from Carl Roth GmbH (Karlsruhe, Germany). Methanol (HPLC gradient grade) was obtained from Fisher Scientific GmbH (Schwerte, Germany) and acetonitrile (HPLC gradient grade) was purchased from VWR International GmbH (Darmstadt, Germany). Reference compounds of the alkaloids (scopolamine (purity = 99.3 %), hyoscyamine sulfate (purity = 99.7 %), littorine (purity = 94.2 %), aposcopolamine (purity = 99.5 %), norscopolamine (purity = 99.8 %), norhyoscyamine sulfate (purity = 100 %), 6-hydroxyhyoscyamine hydrobromide (purity = 97.4 %), 7-hydroxyhyoscyamine hydrobromide (purity = 85.8 %)) were received from

Boehringer Ingelheim Pharma GmbH und Co. KG (Ingelheim, Germany). In addition, scopolamine-D3 hydrobromide (purity = 99 %) was ordered by EQ Laboratories GmbH (Augsburg, Germany). The identity of the reference compounds was verified via ^1H NMR and HPLC-MS.

3.3.3. NMR analysis

Extraction - Samples were prepared according to a protocol established by Kim et al., in particular the NMR-based metabolomic analysis of plants (Kim 2010), with slight modifications. Ca. 20 mg of the freeze-dried *Duboisia* plant material was transferred into a 2 ml - centrifuge tube. 0.5 ml of $\text{CH}_3\text{OH}-d_4$ and 0.5 ml of D_2O were added. The D_2O -soln. was previously buffered with KH_2PO_4 (90 mM) and adjusted to pH 6.0 by adding 1.0 M NaOD. In addition, TSP-*d*4 was added to D_2O in a concentration of 0.29 mM. After vortexing 1 min at room temp., ultrasonication was performed using a Branson 5510E-MT (Branson Ultrasonics, Danbury, CT, USA) for 15 min. The samples were centrifuged for 5 min at 13,000 g at room temp., finally 300 μl of supernatant were filtered directly into a 3 mm - NMR tube.

Measurements - The NMR experimental parameters of ^1H NMR spectroscopy, ^1H - ^1H -correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) were chosen according to the protocol of Kim et al. (Kim et al. 2010). ^1H NMR spectra were measured at 25 °C on a 600 MHz DMX-600 spectrometer (Bruker AXS Advanced X-ray Solutions GmbH, Karlsruhe, Germany) operating at a ^1H NMR frequency of 600.13 MHz and equipped with a TCI cryoprobe and Z-gradient system. CD_3OD was used as internal lock. For 1D- ^1H NMR spectra a total of 32,768 data points were recorded covering a spectral window of 12,019 Hz with 64 scans including water suppression using presaturation. All data was zero-filled to 65,536 points. An exponential window function with a line broadening factor of 0.3 Hz was applied prior to Fourier transformation. The resulting spectra were manually phased and baseline corrected using TopSpin (ver. 3.1 Bruker).

^1H - ^1H correlation spectroscopy (COSY) spectra were acquired with pre-saturation ($\gamma B_1 = 50$ Hz) using a relaxation delay of 1.5 s. A data matrix of 256 x 2,048 points covering 7,209.8 x 7,213.3 Hz was recorded with 4 scans per increment. Data were zero-filled to 4,096 x 4,096 points prior to Fourier transformation.

For recording heteronuclear multiple bond correlation (HMBC) spectra a data matrix of 512 x 2,048 points covering 34,713.8 x 6,008.2 Hz was used including 32 scans per increment at frequencies of 600.13 and 150.92 Hz. The relaxation delay was set to 1.5 s and a coherence transfer delay optimised for a long range coupling of 8 Hz was applied.

Data analysis - The ^1H NMR spectra were automatically reduced to ASCII files. The ^1H NMR spectra of all extracts were scaled to TSP (δ 0.0) and reduced to integrated regions of equal width (0.04 ppm) from δ 0.0 – 10.0 ppm. The regions of δ 4.7 – 4.9 and δ 3.28 – 3.34 were removed from the analysis due to the residual signals of water and methanol. Bucketing was performed by AMIX software (ver. 3.0 Bruker) with scaling on total intensity (bucket width 0.04 ppm).

Principal component analysis (PCA) and the orthogonal partial least square discriminant analysis (OPLS-DA) were performed with SIMCA-P software (v. 13.3, MKS Umetrics AB, Umeå, Sweden). Scaling of the data was based on the Pareto method, which reduces the relative importance of large values while emphasising weaker peaks that may have more biological relevance.

3.3.4. HPLC-MS

Extraction - After grinding the dried leaves (Retsch Ultra Centrifugal Mill of type ZM 100 with a 1 mm strainer insert), 50 mg +/- 1 mg were weighed into a 15 ml plastic tube. 10 ml of ortho-phosphoric acid 0.5 vol% were added and ultrasonication was performed using a Sonorex Super 10P Digital (Bandelin electronic GmbH & CoKG, Berlin, Germany) sonicator at 30 °C for 15 min. After shaking the extracts at 200 rpm for 18 h on an Orbitron-shaker purchased from Infors AG (Bottmingen, Switzerland), ca. 1 ml of soln. was filtered into an eppendorf tube. The sample was then transferred to a vial using 250 μl of extract, 200 μl ortho-phosphoric acid 0.5 vol% and 50 μl Scopolamine-D3 in a concentration of 7 mg/l (leading to a final concentration of 0.7 mg/l).

Standard solutions - Standard solns. of scopolamine, hyoscyamine sulfate, littorine, norscopolamine, norhyoscyamine sulfate and 6-hydroxyhyoscyamine hydrobromide were prepared using concentrations from 0.25 $\mu\text{g/l}$ to 50 mg/l in ortho-phosphoric acid 0.5 vol%. In addition, scopolamine-D3 (dissolved in methanol) was added as internal standard in a final concentration of 0.7 mg/l.

Measurements - The used HPLC is a 1260 series UHPLC consisting of a 1200 series Degasser, a 1260 Infinity Bin Pump, a 1200 series HiP-ALS SL+ Autosampler, a 1260 Infinity TCC column oven and a 1260 Infinity 1260 DAD Detector manufactured by Agilent Technologies (Böblingen, Germany). A Kinetex Core Shell C18 column (100 x 2.1 mm, 2.7 μm) by Phenomenex (Aschaffenburg, Germany) was used. In addition, an ESI-qTOF-MS-system (Bruker Daltonik GmbH, Bremen, Germany) was coupled to the LC system.

A gradient grade method based on two different mobile phases was applied (mobile phase A/mobile phase B: 0-1 min (9/1), 1-9.1 min (9/1 – 6/4), 9.1-17 min (9/1)). Mobile phase A consisted of ultrapure H_2O with 0.1 vol% of FA, while mobile phase B consisted of MeOH-ACN (4:1 v/v). The injection volume was set to 1 μl , column temp. was held at 30 $^\circ\text{C}$, the flow rate was 0.35 ml/min and scopolamine, hyoscyamine and other minor alkaloids were detected by their mass-to-charge ratio and quantified by the mass intensity of their molecular ion peak $[\text{M}+\text{H}]^+$. Internal mass calibration of each analysis was performed by using 10 mM sodium formate in methanol/water, 1:1 v/v.

Eluted compounds were detected from m/z 90 to 700 using a Micro-TOF-Q compact quadrupole time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ion source in positive ion mode using the following instrument settings: nebuliser gas, nitrogen, 2.0 bar; dry gas, nitrogen, 8 l/min, 220 $^\circ\text{C}$; capillary, 3800 V; end plate offset, 500 V; funnel 1 RF, 220 Vpp; funnel 2 RF, 250 Vpp; in-source CID energy, 0 V; hexapole RF, 250 Vpp; quadrupole ion energy, 2 eV; collision gas, nitrogen; collision energy, 8 eV; collision RF 300 Vpp; transfer time, 70 μs ; prepulse storage, 5 μs ; spectra rate, 2 Hz.

Data analysis and quantification - HPLC-MS measurement of tropane alkaloids was performed in biological triplicates with a threefold determination of content. The statistical evaluation by means of the Kruskal–Wallis test was done using Microsoft Office Excel with Engine Room (MoreSteam, Powell, USA) as add-in.

3.4. Results and discussion

3.4.1. ^1H NMR- based metabolite profiling

By using $\text{CH}_3\text{OH}-d_4\text{-KH}_2\text{PO}_4$ buffer in D_2O (1:1, v/v), a wide range of metabolites is detected, including sugars, amino acids and secondary metabolites like flavonoids or tropane alkaloids (Figure 3-2).

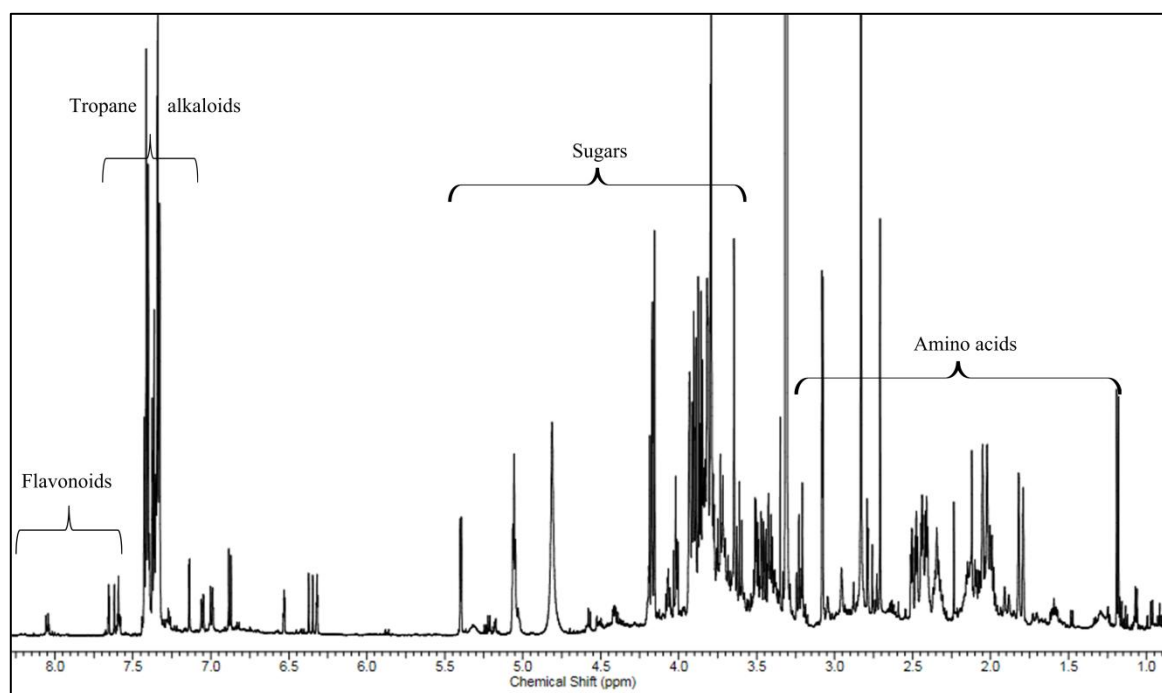


Figure 3-2: ^1H NMR spectrum of a *Duboisia* leaf extract (Genotype E, cultivated in the field for 3 months) demonstrating the signal richness and the wide range in metabolites.

The individual metabolites in the plant extract were identified by comparing NMR data with the in-house library of the Natural Products Laboratory of the Institute of Biology in Leiden, literature data (Verpoorte et al. 2007) and with the Human Metabolome Database as well as the Spectral Database for Organic Compounds. The identified primary and secondary metabolites in the leaves of *Duboisia* are listed in Table 3.1.

Table 3.1: Characteristic ^1H chemical shifts of all metabolites identified in extracts of *Duboisia* plants

Identified metabolites	Chemical shifts (ppm) and coupling constants (Hz)
Alanine [15]	δ 1.47 (H-3, d, $J = 7.2$ Hz)
Choline [16]	δ 3.21 (N-CH ₃ , s)
Proline [17]	δ 4.05 (H-2, dd, $J = 8.6, 6.4$ Hz), δ 2.32 (H-3, m)
Threonine [14]	δ 1.31 (H-4, d, $J = 6.6$ Hz)
α -Glucose [12]	δ 5.16 (H-1, d, $J = 3.8$ Hz)
β -Glucose [12]	δ 4.56 (H-1, d, $J = 7.9$ Hz)
Sucrose [13]	δ 5.39 (H-1, d, $J = 3.8$ Hz), δ 4.15 (H-3', d, $J = 8.5$ Hz)
Myo-Inositol [11]	δ 4.00 (H-2, t, $J = 2.8$ Hz), δ 3.60 (H-4, H-6, t, $J = 9.9$ Hz), δ 3.45 (H-1, H-3, dd, $J = 9.9, 2.9$ Hz), δ 3.22 (H-5, t, $J = 9.3$ Hz)
Chlorogenic acid [7]	δ 7.60 (H-7', d, $J = 15.9$ Hz), δ 7.13 (H-2', d, $J = 2.1$ Hz), δ 7.04 (H-6', dd, $J = 8.4, 2.0$ Hz), δ 6.87 (H-5', d, $J = 8.4$ Hz), δ 6.35 (H-8', d, $J = 15.9$ Hz), δ 5.31 (H-3, td, $J = 10.3$ Hz, 4.8 Hz)
6 β -hydroxy-hyoscyamine [4]	δ 5.01 (H-3, t, $J = 4.8$ Hz), δ 4.56 (H-6, m)
Hyoscyamine [2]	δ 2.71 (N-CH ₃ , s), δ 1.88 (H-2, d, $J = 16.5$ Hz), δ 1.58 (H-7, m)
Scopolamine [5]	δ 3.78 (H-6, d, $J = 3.7$ Hz), δ 3.07 (H-7, d, $J = 2.9$ Hz), δ 2.83 (N-CH ₃ , s), δ 2.01 (H-4, d, $J = 16.5$ Hz), δ 1.78 (H-2, d, $J = 16.5$ Hz)
Scopoletin [8]	δ 7.99 (H-4, d, $J = 9.4$ Hz), δ 7.20 (H-5, s), δ 6.89 (H-8, s)
Kaempferol [10]	δ 8.04 (H-2', H-6', d, $J = 9.0$ Hz), δ 6.98 (H-3', H-5', d, $J = 9.0$ Hz), δ 6.51 (H-8, d, $J = 2.4$ Hz), δ 6.3 (H-6, d, $J = 2.4$ Hz)
Quercetin [9]	δ 7.64 (H-2', d, $J = 2.5$ Hz), δ 7.57 (H-6', dd, $J = 8.5, 2.5$ Hz), δ 6.98 (H-5', d, $J = 8.5$ Hz), δ 6.51 (H-8, d, $J = 2.0$ Hz), δ 6.30 (H-6, d, $J = 2.0$ Hz)

In case of the root samples, only sugars including glucose and sucrose and traces of scopolamine were detected, possibly due to the immediate transport of the biosynthesised tropane alkaloids to the leaves, which form the main storage location. Therefore, all further data analysis and metabolite identification were followed up by using leaf samples of *Duboisia*. As the signals in the obtained NMR spectra were often overlapping, it was necessary to first select the characteristic signals of possible metabolite-candidates. In a

second step, those metabolites, which were not sufficiently confirmed by ^1H NMR data analysis, were further verified by using ^1H - ^1H -correlated spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) (Figure 3-3).

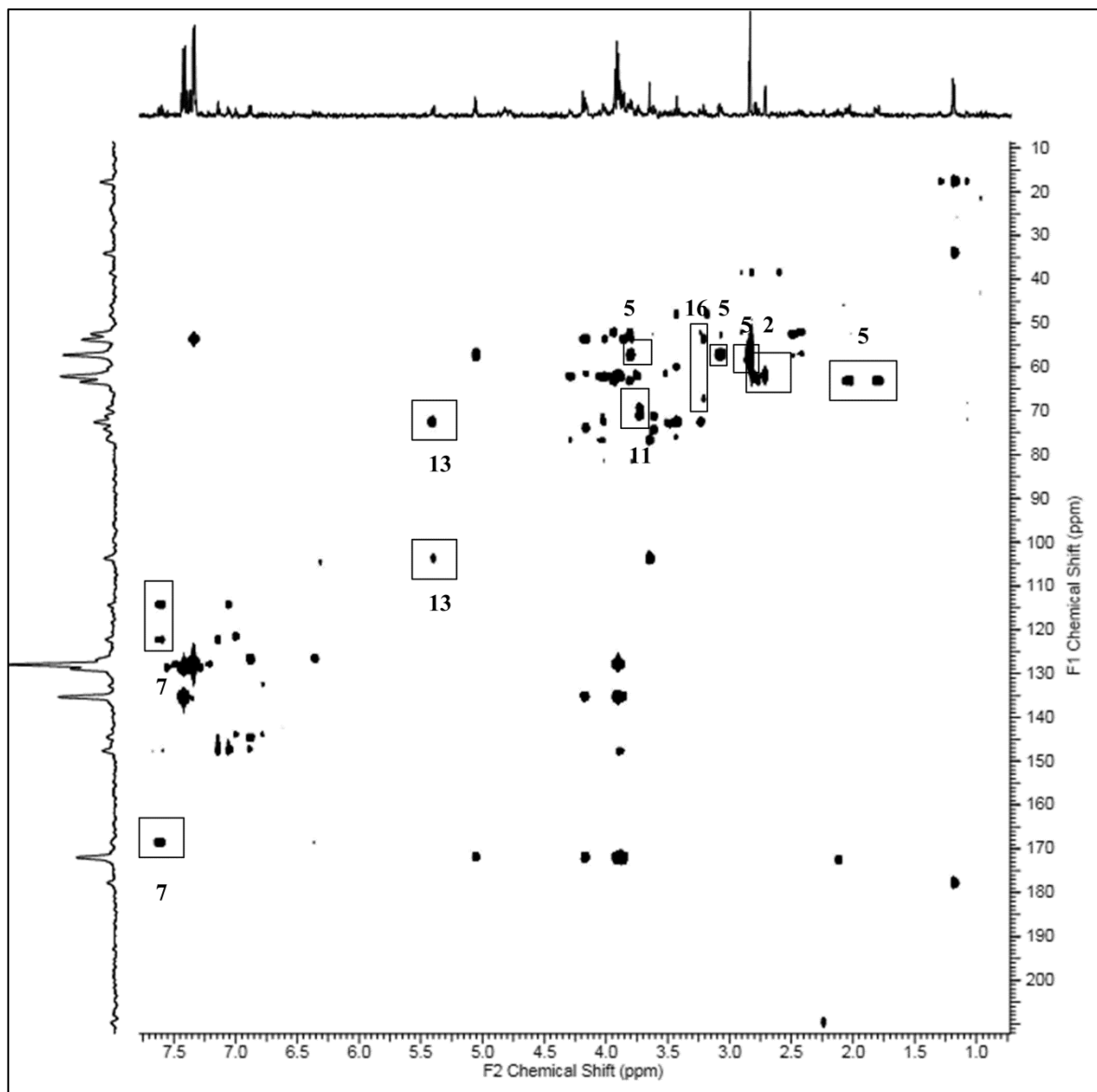


Figure 3-3: HMBC-spectrum of a *Duboisia* leaf extract (Genotype E, cultivated in the field for 3 months). Cross signals of the following metabolites are shown: scopolamine (5) with cross signals [ppm]: 3.78/57.3, 3.07/57.3, 2.83/57.3, 2.01/63.4, 1.78/63.4, sucrose (13) with cross signals [ppm]: 5.39/103.62, 5.39/72.56, *myo*-inositol (11) with cross signals [ppm]: 3.60/74.5, 3.60/71.0, hyoscyamine (2) with cross signals [ppm]: 2.71/61.99, chlorogenic acid (7) with cross signals [ppm]: 7.60/168.5, 7.60/122.2, 7.60/114.3 and choline (16) with cross signals [ppm]: 3.21/67.5, 3.21/53.7.

Regarding the group of tropane alkaloids (Figure 3-1), several characteristic signals for each compound were assigned in all genotypes, although many signals overlapped. Especially in position H-6 and H-7 of the tropane ring a clear differentiation was possible, as hyoscyamine is not substituted, while 6 β -hydroxy-hyoscyamine is substituted in position H-6 and scopolamine has an epoxide ring at H-6 and H-7 (El-Shazly et al. 1997; Ishimaru and Shimomura 1989). In addition to the tropane alkaloids, other important secondary metabolites were identified. The presence of scopoletin as well as kaempferol- and quercetin-glycosides in *Duboisia* hybrids was confirmed, which is noteworthy as flavonoids like quercetin and kaempferol are sharing phenylalanine as a precursor derived from the shikimate pathway with the tropane alkaloids.

Not only signals of the tropane alkaloids, but also those of several primary metabolites were assigned. This is of interest, as sugars and amino acids are essential for plant growth and therefore their concentration likely correlates with biomass production. We detected *myo*-inositol, which plays a role in intracellular and intercellular communication for coordination of cell growth (Stevenson et al. 2000), as well as sucrose and glucose, which are known to be important in the regulation of plant growth due to their function as signalling molecules, for example by controlling photosynthetic gene expression (Rolland and Sheen 2005). The main amino acids identified in *Duboisia* leaf extracts were alanine, choline, proline and threonine. They are not only needed in order to build up proteins and other *N*-containing metabolites, but are also involved in osmoregulation, ion transport and stomatal opening (Rai 2002).

3.4.2. Comparison of different genotypes

Comparing the wild types of *Duboisia myoporoides* (A) and *Duboisia leichhardtii* (B, C) with the hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii* (D, E) by using principal component analysis (PCA), a separation into three groups was obtained by the score plot of PC1 and PC2 scores. Principal component 1 divided the samples into wild types and hybrids, thereby explaining 43.4 % of the variance (Figure 3-4), whereas principal component 2 (16.9 %) separated the wild types again into plants that were categorised as *Duboisia myoporoides* and those that were assigned to *Duboisia leichhardtii*. The loading plot (Figure 3-4) shows the identified metabolites, which were responsible for the group differentiation into wild types and hybrids.

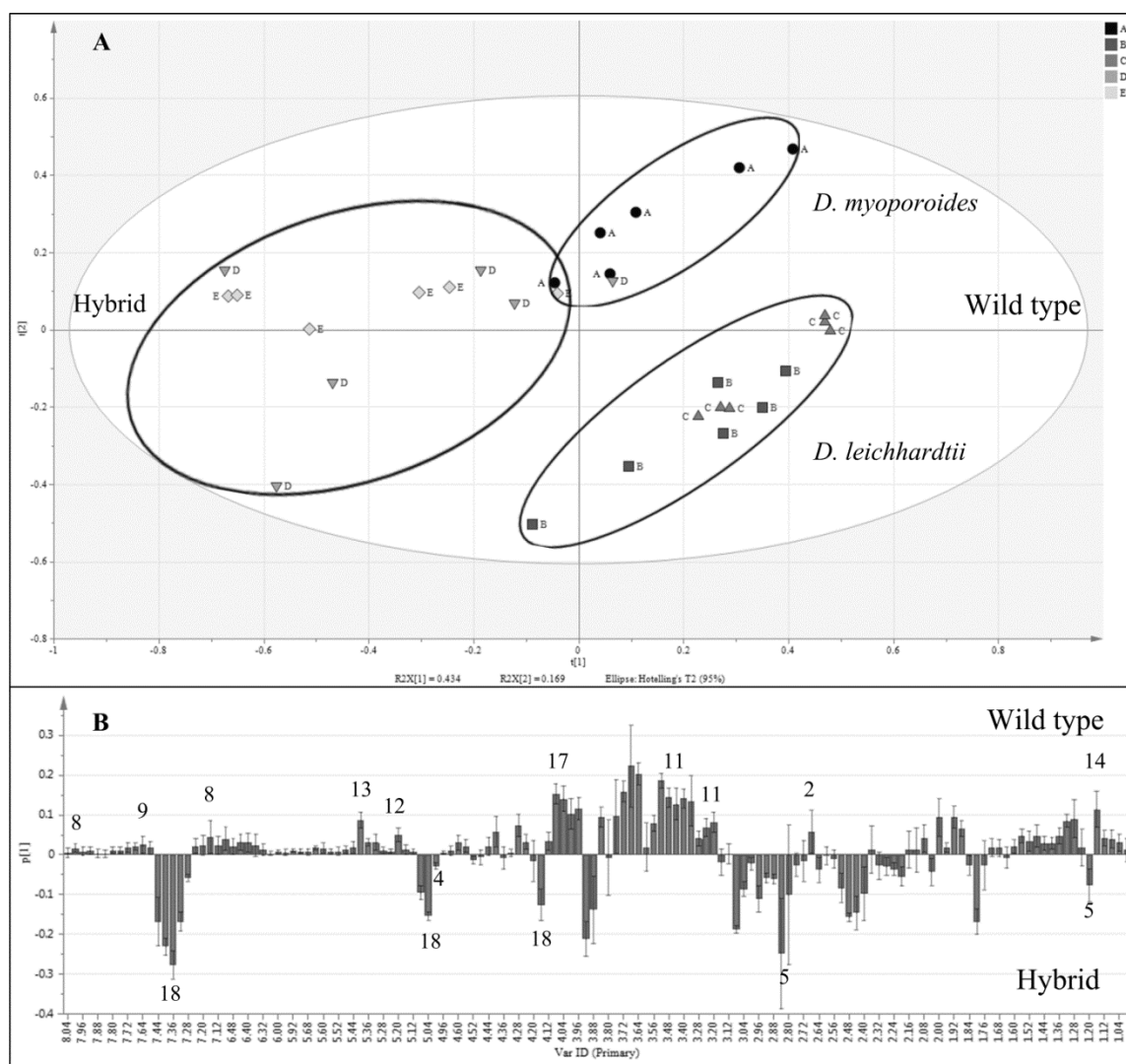


Figure 3-4: A) Score plot of principal component analysis (PCA), results obtained by ^1H NMR using PC1 and PC2 comparing the leaf extracts of different genotypes (A-E) of *Duboisia*. B) Loading column plot of principal component analysis (PCA), results obtained by ^1H NMR using PC1 comparing the leaf extracts of different genotypes (A-E) of *Duboisia*. Signals of tropane alkaloids (18), scopolamine (5), scopoletin (8), quercetin (9), sucrose (13), glucose (12), 6 β -hydroxy-hyoscyamine (4), proline (17), *myo*-inositol (11), hyoscyamine (2) and threonine (14) are assigned.

The signals at δ 4.16, δ 5.04 and δ 7.32, δ 7.35 as well as δ 7.40 were assigned to the tropane ring and the tropic acid group of tropane alkaloids as scopolamine and its direct precursor 6 β -hydroxy-hyoscyamine and were found at higher quantities in the hybrids of *Duboisia*. In contrast to that, glucose and sucrose, amino acids like proline and threonine as well as hyoscyamine were more abundant in the wild types *Duboisia myoporoides* and *Duboisia leichhardtii*. Hence, in the *Duboisia* hybrids the tropane alkaloid biosynthesis seems to be strongly increased, especially the conversion from hyoscyamine to scopolamine via 6 β -hydroxy-hyoscyamine. The quantification via HPLC-MS showed that hyoscyamine was the

predominant alkaloid in *Duboisia leichhardtii* with concentrations up to 8.86 ± 1.17 mg/g dry wt, whereas it was less abundant in *Duboisia myoporoides* as well as *Duboisia* hybrids with scopolamine as major compound. This might be due to a higher expression level or activity of H6H, the enzyme responsible for catalysis of this reaction. Additional proteomic and transcriptomic data are however needed in order to allow a final assessment. In order to choose promising genotypes of *Duboisia* for large scale cultivation, their selectivity for scopolamine plays a key role. *Duboisia myoporoides* and the two examined *Duboisia* hybrids show a high conversion rate from hyoscyamine to scopolamine compared to related plant genera like *Atropa* or *Hyoscyamus*, which are known to be rich in hyoscyamine, but poor in scopolamine.

3.4.3. Comparison of different growth stages

The comparison of different growth stages (3 and 6 months after potting of the rooted cuttings) of the two hybrids (genotypes D and E) via PCA showed a clear separation according to the plant age revealing interesting differences in the metabolite profile with PC1 and PC2 explaining 75.1 % of variation (Figure 3-5). In contrast to that, the wild types (genotypes A, B and C) did not differ significantly in the composition based on their age (data not shown). The loading plot (Figure 3-5) presents some of the metabolites responsible for this age-dependent group differentiation in *Duboisia* hybrids.

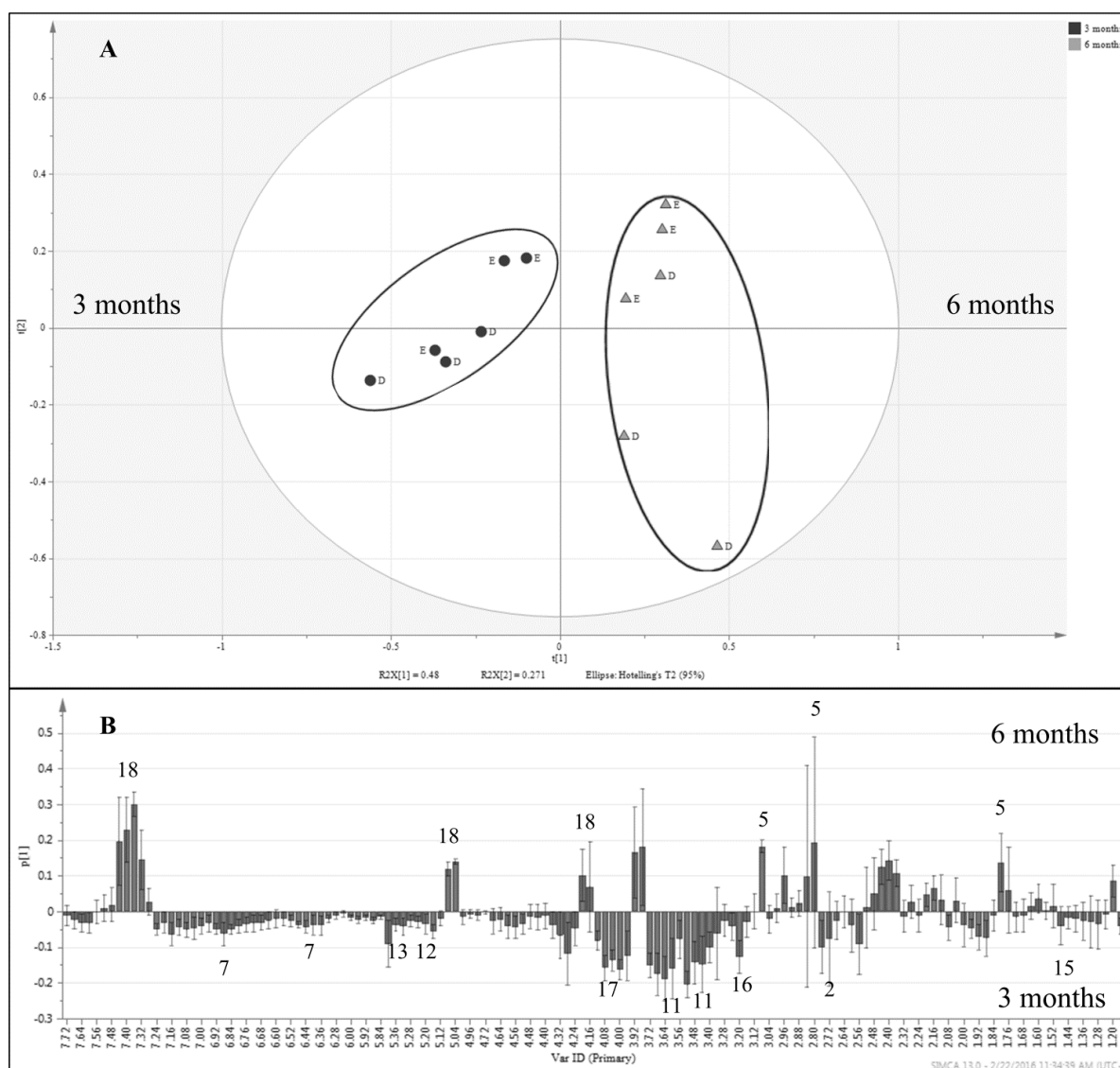


Figure 3-5: A) Score plot of principal component analysis (PCA), results obtained by ^1H NMR using PC1 and PC2 comparing the leaf extracts of *Duboisia* hybrids (D, E) according to the plant age (3 and 6 months grown plants). B) Loading column plot of principal component analysis (PCA), results obtained by ^1H NMR using PC1 comparing the leaf extracts of *Duboisia* hybrids (D, E) according to the plant age (3 and 6 months grown plants). Signals of tropane alkaloids (18), scopolamine (5), sucrose (13), glucose (12), proline (17), *myo*-inositol (11), hyoscyamine (2), chlorogenic acid (7), choline (16) and alanine (15) are assigned.

After three months of cultivation, sugars (glucose, sucrose) and amino acids (proline, choline, and alanine) involved in plant growth were higher conc. compared to plants cultivated for 6 months. The levels of scopolamine and its direct precursors were increased after 6 months of growth. In case of the hybrids, there may be a boost in secondary metabolism with increasing age of the plants. According to Yamamura and Tsuji, plants investing more resources in the biosynthesis of defence substances are able to protect themselves better from herbivory attacks, however this goes along with decreased biomass production (Yamamura and Tsuji

1995). Van Dam et al. state that plants growing slowly allocate more resources to defence (Van Dam et al. 1996). These theories might explain the metabolite distribution in 3 and 6 months grown *Duboisia* plants. Interestingly, this did not apply to the wild types, where the scopolamine and hyoscyamine levels remained comparable independent of the growth stage.

3.4.4. Impact of growth stage and genotype on the metabolite distribution

In order to simultaneously analyse the impact of the two investigated factors age and genotype on the metabolite distribution, the orthogonal partial least square discriminant analysis (OPLS-DA) models of growth stage (3 months vs. 6 months) and genotype (wild type vs. hybrids) were represented using a SUS (shared and unique structures)-plot (Figure 3-6).

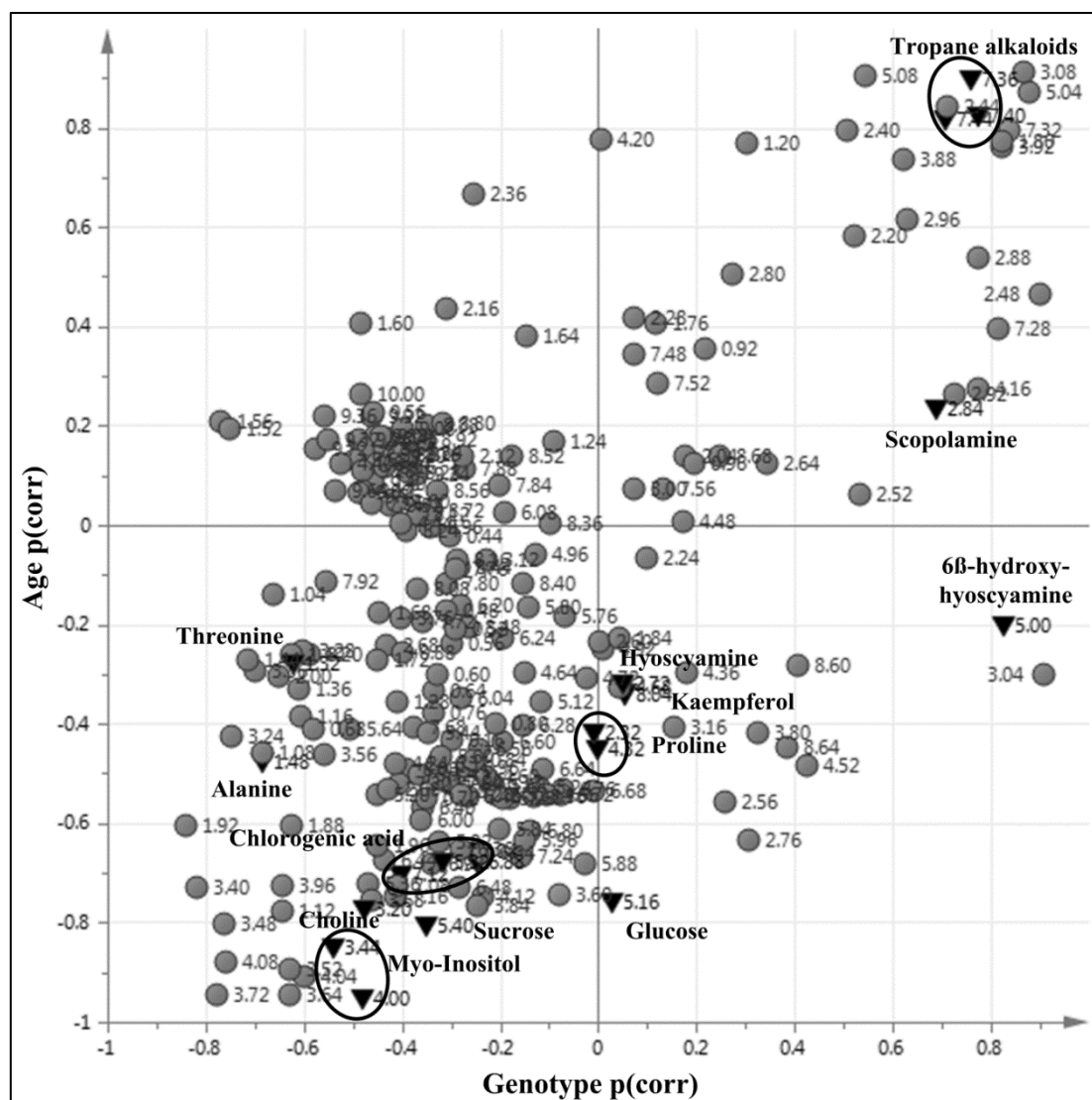


Figure 3-6: SUS-Plot showing the shared and unique correlation structures between age (3 months and 6 months old hybrids) and genotype (wild types and hybrids) using OPLS-DA models.

The plot indicates scopolamine and 6β-hydroxy-hyoscyamine to be of high importance in the discrimination of *Duboisia* hybrids against wild types, being predominant in hybrids, whereas especially proline, choline as well as glucose, sucrose and *myo*-inositol are influential in the separation of 3 months and 6 months grown plants. About the target compound scopolamine it can be stated that the genotype is more influential to its quantitative occurrence than the age of the investigated plant. This indicates that already in the early stage of growth a clear distinction of different genotypes based on their scopolamine level is possible, e.g. in regard to breeding and selection.

3.4.5. Comparison of different cultivation conditions

Genotype E, hybrid of *Duboisia myoporoides* and *D. leichhardtii*, is commercially used for the production of scopolamine and therefore cultivated in large scale in the field in Queensland, Australia. Samples of genotype E grown in the field and in climate chambers under controlled conditions were taken after 3 months and 6 months and compared by using PCA (Figure 3-7).

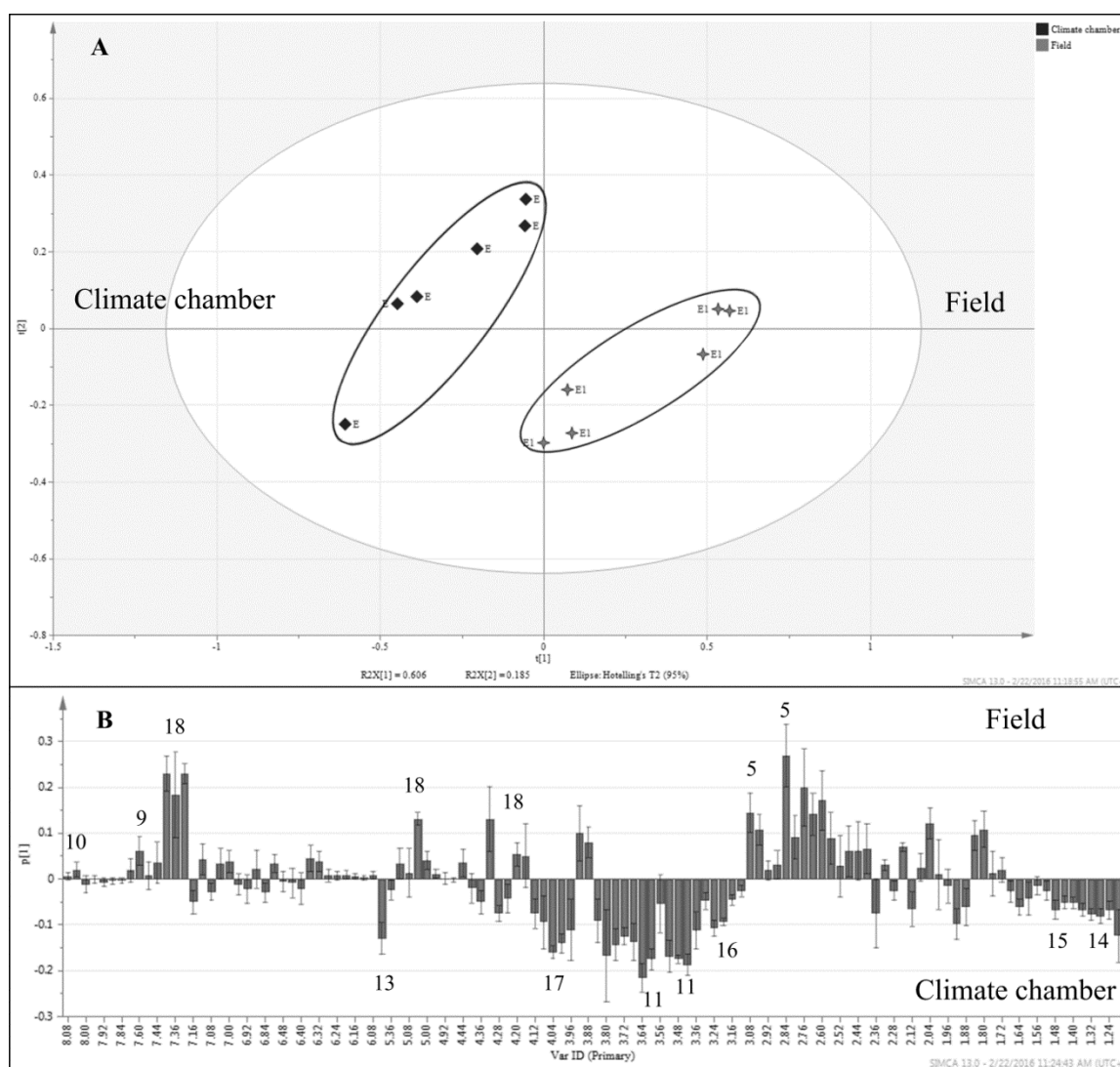


Figure 3-7: A) Score plot of principal component analysis (PCA), results obtained by ^1H NMR using PC1 and PC2 comparing the leaf extracts of *Duboisia* hybrids (genotype E) comparing different cultivation conditions (climate chamber vs. field). B) Loading column plot of principal component analysis (PCA), results obtained by ^1H NMR using PC1 comparing the leaf extracts of *Duboisia* hybrids (genotype E) comparing different cultivation conditions (climate chamber vs. field). Signals of tropane alkaloids (18), scopolamine (5), quercetin (9), sucrose (13), proline (17), *myo*-inositol (11), threonine (14), choline (16), alanine (15), and kaempferol (10) are assigned.

As the score plot showed, PC1 and PC2 were able to explain 79.1 % of total variation by clearly separating plants grown outdoors from those grown in climate chambers. The loading plot shows (Figure 3-7) flavonoids like quercetin and kaempferol, as well as other major secondary compounds like scopolamine, to be more abundant in field-grown plants, whereas primary metabolites like sugars and amino acids were mainly found in the plants grown indoors under strictly controlled conditions. This might be due to greater interaction of field-grown plants with other organisms and exposure to climatic changes, in contrast to plants cultivated solely indoors in isolation from their natural environment. Flavonoid production can be enhanced by environmental stresses, e.g. exposure to solar ultraviolet B radiation (Xu et al. 2008), for example kaempferol and quercetin are able to protect plants against photooxidation. This might explain higher flavonoid concentrations in field-grown plants when exposed to natural sun light compared to plants grown in climate chambers using artificial illumination containing low UV A and no UV B radiation. The significantly higher content in scopolamine may be explained by the interaction of field-grown plants with animals and other plant species, as tropane alkaloids are reportedly produced as chemical defence against insects and herbivores (El-Shazly et al. 1997). Moreover, the flux towards secondary metabolism (flavonoids, alkaloids) in field-grown plants is further demonstrated by looking at the plant height. On average, the plants grown in climate chambers showed more than double the size compared to field grown plants after 6 months of growth (112 ± 1.4 cm compared to 52.7 ± 7 cm). In contrast, field-grown plants developed a higher number of lateral shoots (see also supplementary material), possibly due to the different light conditions (intensity, wavelength, period) compared to indoor grown plants, as light is supposed to have a major impact on branching in plants (Leduc et al. 2014).

3.4.6. HPLC-MS method validation for quantification of tropane alkaloids

In order to investigate the tropane alkaloid composition in more detail, an appropriate HPLC-MS method was developed and validated. An extraction with ortho-phosphoric acid 0.5 vol% was chosen in order to extract high amounts of tropane alkaloids by simultaneously decreasing the content of other secondary metabolites, like e.g. phenolic compounds, in the leaf extract. Scopolamine, norscopolamine, 6 β -hydroxy-hyoscyamine, hyoscyamine, norhyoscyamine, littorine and aposcopolamine were identified and quantified via HPLC-MS

using scopolamine-D₃ as internal standard. All compounds were available as pure substances and were utilised in order to determine selectivity, accuracy, precision and range, including LoD and LLoQ.

Selectivity was tested by measuring the extraction solvent o-phosphoric acid 0.5 vol%, standard solns. and plant extracts. The MS-chromatogram (Figure 3-8) shows the most abundant alkaloids in *Duboisia* plants corresponding to their pseudo molecular ions [M+H]⁺. Alkaloids with a similar molecular weight, namely norscopolamine, hyoscyamine, littorine, were all baseline-separated.

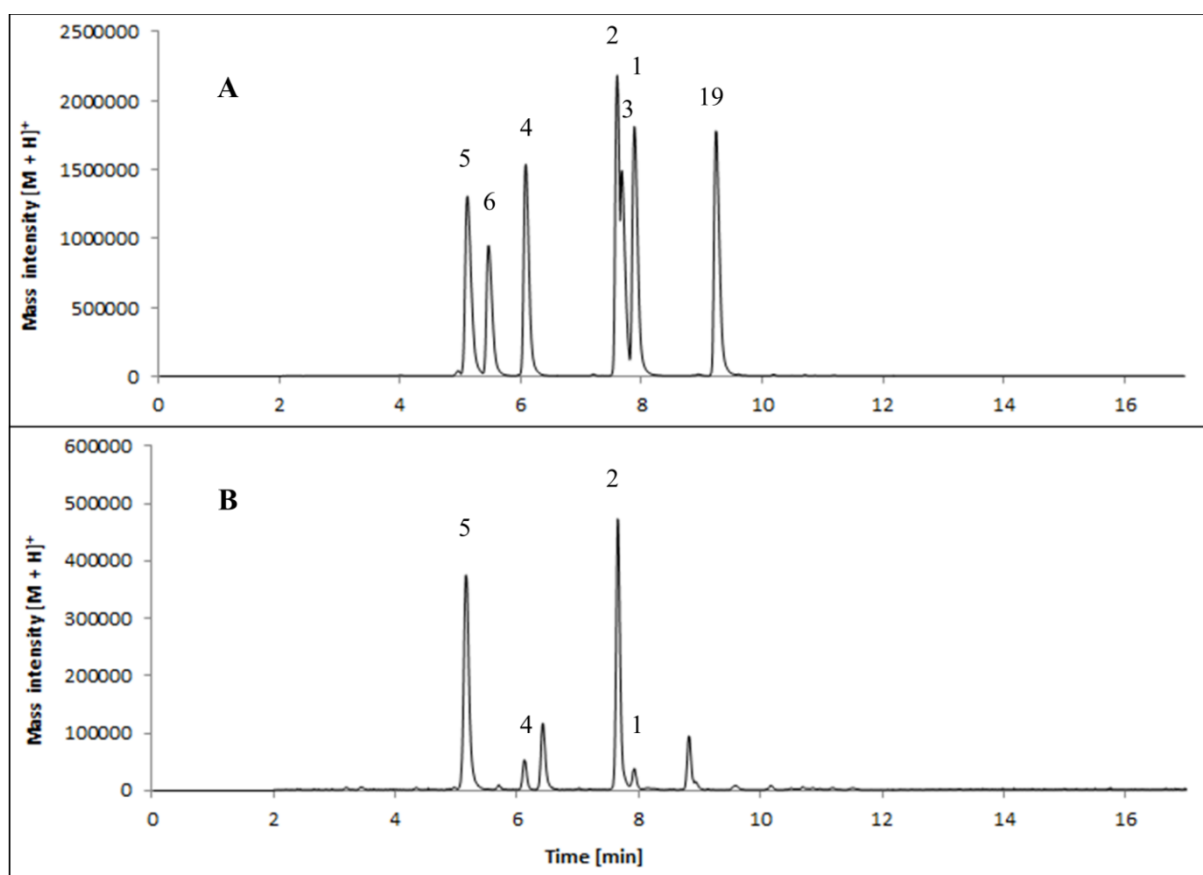


Figure 3-8: MS-chromatogram of the most abundant alkaloids in *Duboisia* spec.: scopolamine (5), norscopolamine (6), 6 β -hydroxy-hyoscyamine (4), hyoscyamine (2), norhyoscyamine (3), littorine (1), aposcopolamine (19). A) Standard soln. at a concentration level of 20 mg/l. B) Leaf extract of genotype C (*Duboisia leichhardtii*).

In order to investigate accuracy and precision, a fivefold determination of 10 different concentrations of standard solns. in a concentration range of 0.16 – 50 mg/l was done while intra- and interday variations were also considered. The accuracy was determined by dividing

the measured concentration of the individual standard solns. multiplied with 100 by their theoretical calculated concentration. The results meet the requirements with an accuracy of +/- 0.1 to 6.4 % compared to the theoretical value at concentrations of 0.31 – 50 mg/l and a deviation lower than 15 % at a concentration level of 0.16 mg/l, which was set as the lower limit of quantification (LLoQ). Analysing the precision, the observed values for the entire concentration range (0.31 – 50 mg/l), including LLoQ, showed a coefficient of variation (CV) between 1.11 – 4.02 % (LLoQ = 7.43 %) for scopolamine, 0.7 – 4.16 % (LLoQ = 8.05 %) for norscopolamine, 0.65 – 2.86 % (LLoQ = 4.05 %) for 6 β -hydroxy-hyoscyamine, 0.8 – 4.98 % (LLoQ = 6.87 %) for hyoscyamine, 0.93 – 5.98 % (LLoQ = 10 %) for norhyoscyamine, 1.12 – 3.86 % (LLoQ = 6.54 %) for littorine and 0.46 – 4.51 % (LLoQ = 11.14 %) for aposcopolamine. The range was evaluated by using 10 concentration levels measuring standards starting from 0.16 mg/l up to 50 mg/l. In order to describe an adequate calibration function, different models were tested. Taking all 10 concentration levels into consideration, a quadratic model was more appropriate than a linear regression model. This became evident from visual inspection as well as the residual variance, which was much larger for the linear fit than the quadratic model. The Mandel's test, which was additionally used for comparing the linear model with the quadratic one, again confirmed the better suitability of the quadratic model for calibration. In addition, the limit of detection (LoD) and the lower limit of quantification (LLoQ) were verified. Therefore, standard solns. containing low concentrations (0.25 – 25 μ g/l) of the substance of interest and blank samples containing the extraction solvent o-phosphoric acid 0.5 vol% were repeatedly measured. The lower limit of quantification (LLoQ) was defined as 0.16 mg/l, as the signal to noise ratio was > 10 and the analyte peak was clearly identifiable and reproduced with an accuracy of 80 – 120 % and a precision of 20 %. The LoD was specified on the basis of a signal to noise ratio > 3, which results in a concentration of 12.5 μ g/l for all tropane alkaloids, apart from hyoscyamine (LoD = 1.25 μ g/l).

This developed method provides the opportunity of a rapid, selective and accurate quantification of the main tropane alkaloids occurring in *Duboisia* and related plant genera.

3.4.7. Tropane alkaloid profile

Altogether, samples of 15 plants belonging to five different genotypes (A - E) were measured in a threefold determination of content (Figure 3-9).

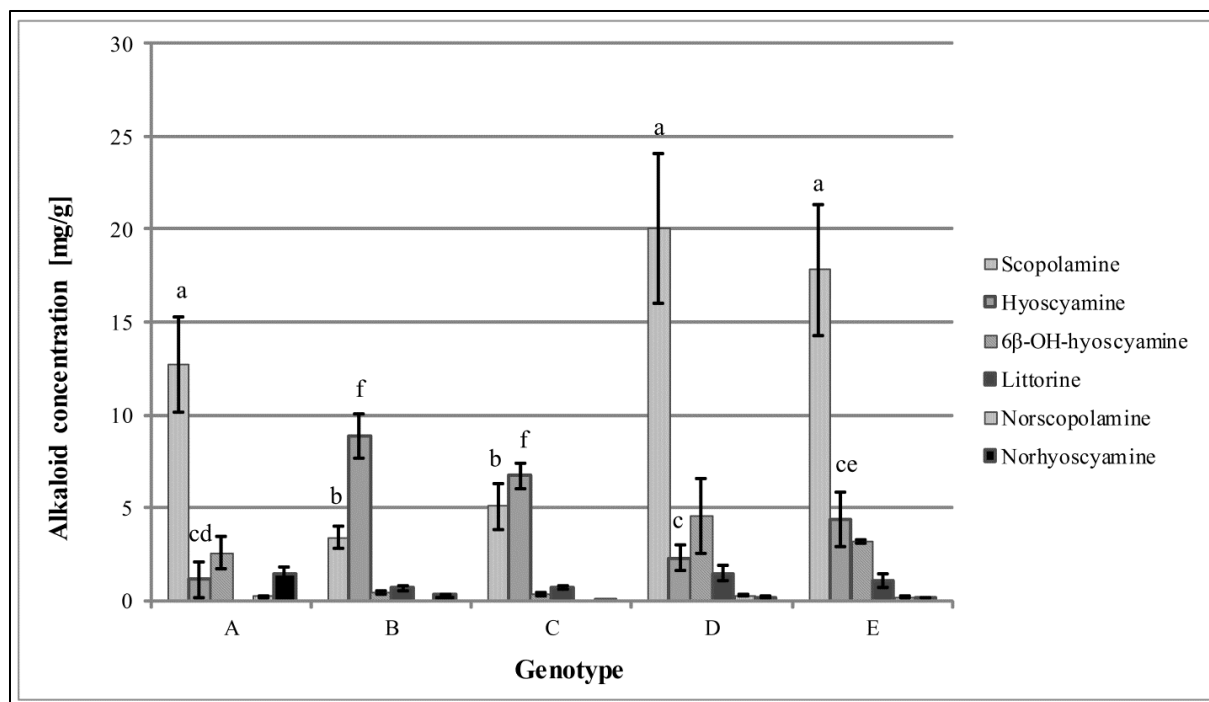


Figure 3-9: Alkaloid profile of leaf extracts from *Duboisia myoporoides* (A), *Duboisia leichhardtii* (B, C) and the hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii* (D, E) grown in climate chambers for 3 months, quantification via HPLC-MS. Means and standard deviations were calculated for all tropane alkaloids quantified and the amount in scopolamine and hyoscyamine was further analysed by means of the Kruskal-Wallis test (different letters are symbolising statistical differences in the alkaloid levels between the genotypes).

The results showed clear differences in the quantity of the alkaloids depending on the genotype. The highest average content in scopolamine was found in the two hybrids D and E with concentrations of ca. 20.04 ± 4.05 and 17.82 ± 3.52 mg/g dry wt respectively, followed by 12.71 ± 2.55 mg/g dry wt in *Duboisia myoporoides* (A). In contrast to that, plants belonging to *Duboisia leichhardtii* (B, C) showed a very low selectivity for scopolamine (3.38 ± 0.59 and 5.09 ± 1.24 mg/g dry wt), but high concentrations of hyoscyamine ranging up to 8.86 ± 1.17 and 6.71 ± 0.68 mg/g dry wt respectively. This might be due to a lower expression or activity of the H6H, responsible for the conversion of hyoscyamine via 6β-hydroxy-hyoscyamine to scopolamine, in *Duboisia leichhardtii*, which results in the high quantities of hyoscyamine, but additional proteomic and transcriptomic data are needed to verify this hypothesis. The other tropane alkaloids were found at very low levels except for 6β-hydroxy-hyoscyamine, which was even higher conc. than hyoscyamine in case of *Duboisia myoporoides* and the hybrids.

The results of the HPLC-MS-measurements match very well with the NMR-data. The group separation according to genotype corresponds to the score plot distinguishing between hybrids

and wild types (Figure 3-4), whereof the latter were further sub-grouped into *Duboisia myoporoides* and *Duboisia leichhardtii*. Comparing the different alkaloid profiles, genotype D and E are closely related as well as genotype B and C show a similar alkaloid distribution. The alkaloid profile of *Duboisia myoporoides* resembles those of the hybrids of *Duboisia*. However, all alkaloids in *Duboisia myoporoides* were found in significantly lower quantities compared to the hybrids, except of norhyoscyamine.

It is noteworthy that without genetic engineering the selectivity for scopolamine compared to its precursors and degradation products is high in *Duboisia myoporoides* and *Duboisia* hybrids making these valuable plant sources, in contrast to *Duboisia leichhardtii*, which accumulates significantly more hyoscyamine and shows less conversion to scopolamine.

Prospectively, it will be of interest to have a closer look at pathways closely related to the scopolamine biosynthesis, e.g. the biosynthesis of nicotine and its derivatives or of the calystegines. The latter also belong to the group of tropane alkaloids and have been already identified in *Duboisia leichhardtii* (Kato 1997). Together with scopolamine and its derivatives, they share tropinone as a common precursor, which is then converted to pseudotropine via the TR-2. If the biosynthesis of calystegines was favoured over that of the medicinally used tropane alkaloids, this would channel away key intermediates needed for the biosynthesis of scopolamine implicating new strategies towards its higher production, e.g. a knock-down or knock-out of the TR-2.

3.5. Conclusion

Duboisia is the main source for scopolamine and has been commercially cultivated for decades (Foley 2006). However to the best of our knowledge, there has never been a detailed study looking at both primary and secondary metabolites, correlating primary with secondary metabolism using wild types and hybrids under fully comparable growth conditions, and comparing the results with those of field-grown plants. This is of great importance in order to better understand the plant metabolism and allow optimisation of the breeding process and the plant cultivation towards higher biomass and scopolamine production.

Analysis of the metabolite profile using PCA revealed a clear separation between hybrids and wild types; the latter again sub-grouped in leaf extracts of *Duboisia myoporoides* and *Duboisia leichhardtii*. In case of the wild types, primary metabolites like sugars and amino acids involved in plant growth were found in higher concentrations, whereas the hybrids

showed significantly higher levels of scopolamine and related tropane alkaloids, which was further confirmed by HPLC-MS analysis. Nevertheless, some direct precursors of scopolamine, especially hyoscyamine, were more abundant in the wild types. This indicates that the enzyme responsible for the last conversion step in the biosynthesis of scopolamine (H6H) might be less expressed and/or less active in the wild types. This was especially the case for the hyoscyamine-rich *Duboisia leichhardtii* in contrast to *Duboisia myoporoides* and *Duboisia* hybrids, which showed a high selectivity for scopolamine.

The simultaneous analysis of the two investigated factors age and genotype by OPLS-DA using an SUS-plot shows that the genotype is more influential to the quantitative occurrence of scopolamine than the age of the investigated plant suggesting that young plants of different genotypes can already be compared and selected based on their scopolamine level, e.g. in breeding research.

The investigation of indoor and outdoor cultivation of hybrid E, used for commercial production, revealed large differences in the respective metabolite composition. The plants grown in climate chambers contained more sugars and amino acids as well as higher growth than the field-grown plants, which in contrast were significantly increased in tropane alkaloids as well as flavonoids showing the high impact of environmental factors enhancing the flux towards secondary metabolism in *Duboisia*. This HPLC-MS- and ¹H NMR-based analysis easily allows the comparison of different samples of *Duboisia* and grouping according to their genetic origin, type of cultivation and developmental stage. In summary, it can be stated that genetic characteristics as well as environmental conditions have a high impact on the amount of tropane alkaloids present in *Duboisia* species. The levels in scopolamine and hyoscyamine are largely genotype-dependent and a further analysis of proteomic and transcriptomic data is highly recommended for further elucidation of the biosynthetic regulation in the plant, especially with regard to key enzymes as the PMT, TR-1 and H6H. Prospectively, the employment of these analytical methods can help to select promising genotypes and suitable cultivation conditions for the production of scopolamine, e.g. by simulating different growing conditions monitoring biomass and alkaloid production or rapid scanning of recently bred plants.

3.6. References

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4. Chapter 4: Influence of Light, Temperature and Macronutrients on Growth and Scopolamine Biosynthesis in *Duboisia* species

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SFU, HH, AR and OK jointly planned and designed the cultivation trials; SFU conducted the experiments and did the data evaluation; SFU wrote the manuscript and prepared the figures with input from all co-authors; all authors furthermore provided critical review and revision of the manuscript.

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4.1. Abstract

Scopolamine is used in the pharmaceutical industry as a precursor in organic synthesis of different classes of important active substances and is extracted in large scale from field grown *Duboisia* plants. Previous research revealed that plant growth as well as production of scopolamine and derivatives strongly varies depending on abiotic factors. However, only little systematic research has been done on the influence of environmental conditions on scopolamine and biomass production so far. In order to extend knowledge in this field, plants of three different genotypes (wild type of *Duboisia myoporoides*, hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii*) were grown in climate chambers under controlled conditions in order to systematically analyse the influence of temperature (20, 24, 28 °C), light (50 – 300 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 12, 18, 24 hours per day) and macronutrients (N, Ca, K) on growth and scopolamine biosynthesis. The data indicate that the light intensity and daily exposure to light have a major impact on the scopolamine production and the plant development, whereas temperature only shows a minor influence. Nitrogen (N) positively affects biomass production, but is negatively correlated with scopolamine content. Calcium (Ca) shows a negative influence on the scopolamine biosynthesis at increased levels as well. Potassium (K) neither affects biomass nor scopolamine production within the tested concentration range. All in all, it can be concluded that especially the light intensity and the nitrogen supply are important regulating variables which can be applied in a targeted manner for influencing the scopolamine and biomass production.

Key Words

Solanaceae, *Duboisia*, Light, Temperature, Macronutrients, Scopolamine, Biosynthesis

4.2. Introduction

Duboisia myoporoides R.Br. and *D. Leichardtii* F.Muell., indigenous to Australia (Rahman et al. 2006) and belonging to the family of Solanaceae, contain tropane alkaloids, which are utilised as anticholinergic drugs in human medicine along with their derivatives (Gryniewicz and Gadzikowska 2008). Thereof pharmaceutically most valued and increasingly demanded is scopolamine, which is for example applied in form of scopolamine-*N*-butylbromide for the treatment of abdominal pain and bladder spasms (Hashimoto et al. 1993). Up to now, industrial manufacturing operations providing scopolamine are widely based on agricultural field plant cultivation of hybrids of *Duboisia myoporoides* R.Br. and *D. leichardtii* F.Muell. (Foley 2006).

It is generally estimated that an increase in crop yield up to 70 % would be feasible, if the environmental conditions were near the optimum for a given plant (Król et al. 2015). This is why in case of *Duboisia* it is of great interest to further optimise the cultivation by increasing the knowledge concerning the impact of environmental factors on scopolamine and biomass production.

In the past decades some field trials regarding the influence of fertilisation (Luanratana and Griffin 1980a) and season dependent harvesting on the alkaloid profile of *Duboisia* have been undertaken (Luanratana et al. 1990). Therefrom it is supposed that the alkaloid content decreases in autumn and winter due to cold weather (Luanratana and Griffin 1980b). Additionally, nitrogen, potassium and calcium are known to influence the alkaloid biosynthesis and plant growth in *Duboisia* and related plant genera. In case of nitrogen, contradictory statements have been published (Alaghemand et al. 2013; Luanratana and Griffin 1980a); Luanratana observed a decrease in total alkaloids at higher nitrogen levels in case of *Duboisia*, whereas Alaghemand measured the highest content in scopolamine and hyoscyamine under elevated nitrogen concentrations using *Hyoscyamus* plants. The growth of *Atropa* plants is supposed to be affected by the ratio of calcium to potassium in the nutrient medium (Smolenski et al. 1967). Smolenski described a reduced biomass production and an elevated yield of total nitrogen and alkaloids, as the ratio of calcium/potassium was increased. Moreover, Luanratana observed an increase in potassium leading to a significant increase in the percentage of scopolamine in *Duboisia* plants (Luanratana and Griffin 1980a).

Previous work has also shown that outdoor various environmental influences, e.g. the daily exposure to sunlight and the soil composition, may mask genetic characteristics (Rosenblum 1954; Ikenaga et al. 1985). In contrast to that, plants being grown under controlled

temperatures in greenhouses in Thailand showed stable scopolamine contents (Luanratana et al. 1990). This underlines the difficulty to get significant and reproducible results in field-trials, especially by comparing plants cultivated in different geographical regions.

By now, no systematic assessment of the impact of individual climate factors like temperature or light on the alkaloid biosynthesis and biomass production in *Duboisia* and related plant genera has been documented. This is why within this study different genotypes of the plant (*Duboisia myoporoides*, hybrids of *Duboisia myoporoides* and *Duboisia leichardtii*) are examined in climate chambers under strictly controlled conditions by varying climate conditions and nutrient supply. Thereby, temperature is chosen to be investigated, as it is crucial for plant growth and development (Yan 2013). High as well as low temperatures are able to negatively affect plant performance and productivity (Zinn et al. 2010) including many changes in structure and function of the photosynthesis which can be affected by high or low temperatures (Wahid et al. 2007). Moreover, the light intensity and illumination time are investigated, as light is of course an important abiotic factor influencing photosynthesis and thus plant growth. The photosynthesis rate is reduced under low light intensities (Zheng et al. 2011), whereas high light intensities may produce photoinhibition due to a disequilibrium between energy supply and consumption (Demmig-Adams and Adams III 1992). Regarding nutrients, the focus was placed on nitrogen, potassium and calcium. In case of nitrogen, a strong causal correlation between leaf nitrogen content and photosynthesis is known to be present across many species (Evans 1989). Moreover, nitrogen is also involved in the biosynthesis of amino acids needed for the synthesis of tropane alkaloids as scopolamine, which are also nitrogen containing compounds. Calcium and potassium are also required for plant development in large amounts and have been reported to affect growth and alkaloid yield (Smolenski et al. 1967; Luanratana and Griffin 1980a).

All in all, this work will contribute to a better understanding of the interaction between environmental conditions and plant metabolism in *Duboisia*. Furthermore, the knowledge gained will be useful to improve plant cultivation in the greenhouse as well as in the field.

4.2.1. Experimental Design

Figure 4-1 shows the design of the cultivation parameters light and temperature varying light exposure, light intensity and temperature.

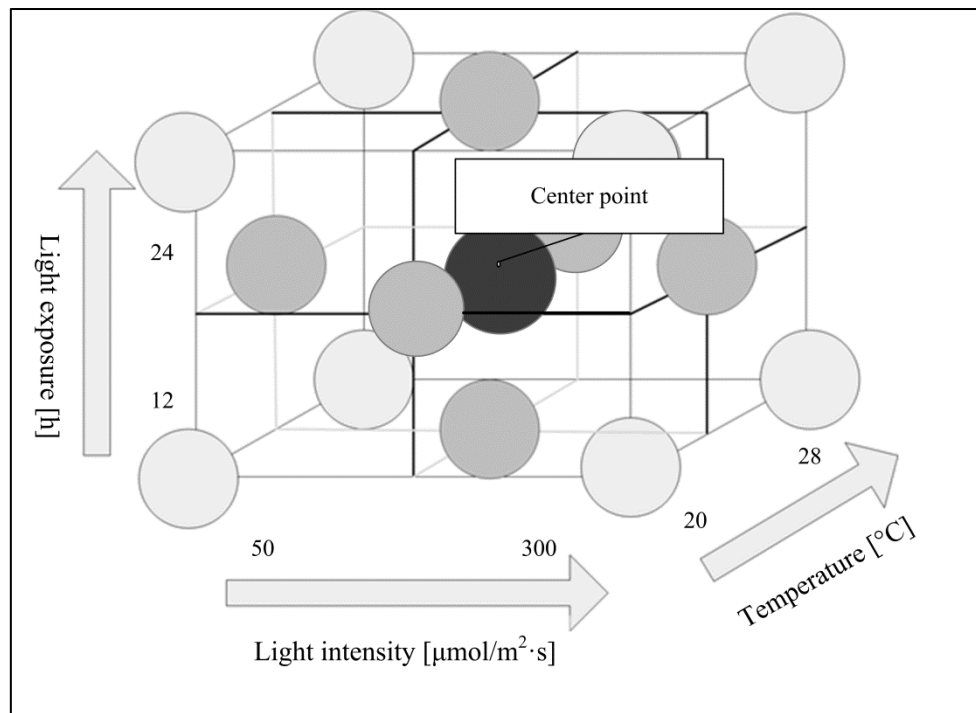


Figure 4-1: Experimental design of the cultivation parameters light and temperature

Design of experiment has been used as a tool in order to decrease the number of trials to be conducted from 27 to 15 by keeping a high degree of validity (Cavazzuti 2013). A response surface model was created in order to identify the correlation between light exposure, light intensity and temperature and to select the optimal cultivation conditions. As not all experiments could be performed simultaneously, a center point was added to each experimental run (including 4-5 different cultivation conditions) providing a measure of process stability and variability. In order to not only detect linear, but also quadratic effects, all factors were investigated at three levels. A central composite face centered (CCF) - design has been chosen because of its ability to build up a model on linear effects, quadratic effects and interactions between the three factors investigated. And in contrast to other related designs it creates an experimental design which allows to work within the narrow technical limits of the climate chambers, especially regarding temperature. The resulting regression model is the following:

$$Y = a + b_1t + b_2l + b_3i + b_{12}tl + b_{13}ti + b_{23}li + b_{11}t^2 + b_{22}l^2 + b_{33}i^2$$

$Y = \text{effect variable (scopolamine [mg/g], biomass [g])}$

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$a = \text{intercept}$

$b = \text{regression coefficient}$

$t, l, i = \text{cause variables (} t = \text{temperature [}^\circ\text{C}], l = \text{daily exposure to light [h], } i = \text{light intensity [}\mu\text{mol}\cdot\text{m}^2/\text{s])}$

In order to systematically analyse the influence of different nutrients on the biomass and alkaloid profile, an appropriate experimental design has been developed using the same model as in the examination of light and temperature (Figure 4-2).

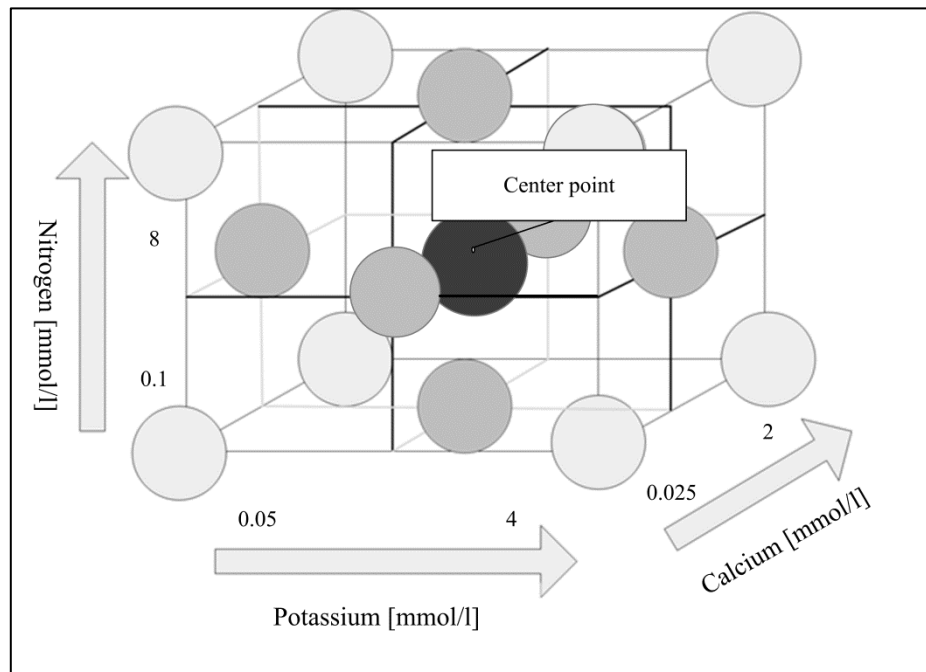


Figure 4-2: Experimental design for nitrogen, potassium and calcium

The nutrient trial was focused on nitrogen, potassium and calcium; all other nutrients left were kept at constant concentration levels. The nutrient salts and related concentrations were chosen based on the Hoagland solution, which has been developed especially for hydroponic cultivation of plants (Hoagland and Arnon 1950) and has already been used for *Duboisia* and other related alkaloid containing plant species like *Nicotiana* (Ju et al. 1983; Luanratana and Griffin 1980a). The chemical composition of the individual compounds was selected by taking possible chemical incompatibilities and their bioavailability into account. NH_4NO_3 was used instead of KNO_3 as it has been shown to increase the nitrogen availability for

secondary metabolism and thereby the alkaloid biosynthesis in *Datura* plants (Demeyer and Dejaegere 1992) and does not influence the potassium level. The appropriate concentration range for nitrogen, potassium and calcium within the main experiments was determined by using pretests at various nutrient levels.

4.2.2. Chemicals and Reagents

Acetone (100 %), O-phosphoric acid (85 %), boric acid, 2-(N-morpholino) ethanesulfonic acid as well as calcium carbonate, cobaltous chloride hexahydrate, disodium hydrogen phosphate dehydrate, potassium sulfate and zinc sulfate heptahydrate were purchased from Carl Roth GmbH (Karlsruhe, Germany). Formic acid, manganese (II) chloride tetrahydrate and calcium chloride dihydrate were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Methanol HPLC gradient grade was bought by Fisher Scientific GmbH (Schwerte, Germany) and acetonitrile HPLC gradient grade was purchased from VWR International GmbH (Darmstadt, Germany). Ammonium nitrate, sodium molybdate tetrahydrate and magnesium sulfate heptahydrate were purchased from AppliChem GmbH (Darmstadt, Germany) and ethylenediaminetetraacetic acid iron (III) monosodium salt was bought from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Copper (II) sulfate pentahydrate was obtained from Merck GmbH (Schwalbach am Taunus, Germany). Reference compounds of the alkaloids (scopolamine, hyoscyamine sulfate, littorine, aposcopolamine, norscopolamine, norhyoscyamine sulfate, 6-hydroxyhyoscyamine hydrobromide, 7-hydroxyhyoscyamine hydrobromide) were received from Boehringer Ingelheim Pharma GmbH and Co. KG (Ingelheim, Germany).

4.2.3. Plant Material

All plants under investigation in this study belong to the genus *Duboisia* R.Br., family Solanaceae, and were supplied by Boehringer Ingelheim Pharma GmbH and Co. KG (Ingelheim, Germany). Wild types of *Duboisia myoporoides* R.Br. (A) (Plant voucher number: TU-FUL-1A-114A) and hybrids of *Duboisia myoporoides* R.Br. and *Duboisia leichhardtii* F.Muell. (B,C) (Plant voucher number: TU-FUL-1B-95B, TU-FUL-1C-95C) were grown in climate chambers (CLF PlantMaster, CLF Plant Climatics GmbH, Wertingen, Germany) under strictly controlled conditions. As Table 4.1 shows, the selected lines differ in their

average scopolamine content as well as in their morphology. Deposit samples were kept in the department of biochemical and chemical engineering at the Technical University of Dortmund.

Table 4.1: Measurement of the different clone characteristics in the climate chambers after 6 weeks of cultivation at 24 °C with 18 h of light exposure at 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$

Line	Scopolamine content (mg/g of dry weight)	Foliation (1 = min., 9 = max.)	Leaf shape (1 = narrow, 9 = broad)
A	4.5	7	8
B	9.8	7	4
C	8.7	6	8

4.2.4. Plant Cultivation

Within the climate chambers (PlantMaster GroBanks, CLF PlantClimatics GmbH, Wertingen, Germany), rooted cuttings of *Duboisia* were potted in expanded clay (Euro pebbles by Plagron, Ospel, Netherlands) and placed in nutrient solution. Philips Master TL-D 58 W/840 (4000 K)-lamps were used for lighting combined with LEDs additionally increasing the dark red light region to imitate sunrise and sunset.

In order to examine the influence of light and temperature, 304 *Duboisia* plants, thereof 16 per climate condition (6 plants belonging to line A, 5 plants belonging to line B and C, respectively) were grown indoors in climate chambers under standardised conditions fertilised with Wuxal 0.1 % in water (Wuxal Super®, Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany). They were initially generated from cuttings, taken in Ingelheim and transferred to Dortmund 7 weeks after having formed roots.

In order to examine the influence of different macronutrients, 64 rooted cuttings of *Duboisia myoporoides* (4 plants per condition) were cultivated at three different concentration levels of nitrogen, potassium and calcium. Nitrogen was applied in the form of NH_4NO_3 , calcium as $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ and potassium as K_2SO_4 . In Table 4.2 the other nutrients all held at constant concentrations are shown. Ultrapure water was used including 2-(N-morpholino) ethane

sulfonic acid as a buffering agent. The nutrient solution was exchanged every two weeks. Cultivation was done at 24 °C with 18 h of light per day at an intensity of 110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$.

Table 4.2: Nutrients held at constant concentrations for hydroponic culture, buffered with 2-(N-morpholino) ethanesulfonic acid, exchange every two weeks

Element	P	Mg	Fe	B	Mn	Co	Zn	Mo	Cu
Concentration [mg/l]	15.48	12.15	1.12	0.270	0.11	0.118	0.099	0.0072	0.032
Concentration [mmol/l]	0.5	0.5	0.12	0.025	$2\cdot 10^{-3}$	$2\cdot 10^{-3}$	$0.15\cdot 10^{-3}$	$0.075\cdot 10^{-3}$	$0.5\cdot 10^{-3}$
Compound applied	Na_2HPO_4 ·2 H_2O	MgSO_4 ·7 H_2O	NaFe-EDTA	H_3BO_3	MnCl_2 ·4 H_2O	CoCl_2 ·6 H_2O	ZnSO_4 ·7 H_2O	MoNa_2O_4 ·4 H_2O	CuSO_4 ·5 H_2O

Data loggers were used in each experimental run recording values for temperature and humidity every 10 minutes (Onset HOBO U12-012, Metrics GmbH, Hagen). Additionally, the respective light intensity was reviewed and adjusted, if seen as necessary, on a regular basis. The plant high was measured as well as the number of lateral shoots was counted periodically. In addition, pictures of all plants were taken from a constant camera position with defined camera settings before harvesting. As scopolamine-storage is mainly located in the leaves (Luanratana and Griffin 1982), the total quantity of leaves was collected plant by plant after 7 weeks of cultivation. Hereinafter the fresh weight was measured and the plant material was dried over 24 hours at a temperature of 60 °C. After drying, the dry weight was noted and the dried leaves were stored in air-tight packaging for the subsequent determination of alkaloid content via HPLC-MS.

In greenhouse, three replicates each (line A, B and C) were planted in moist soil, fertilised with Ferty 3 and Ferty 7 (Ferty® Basis 3 and Ferty® Basis 7, Planta Düngemittel GmbH, Regenstauf, Germany) and watered every day. During autumn and winter time, additional lighting and heating in the greenhouse was necessary, but only under restricted control.

4.2.5. Tropane Alkaloid Assay

All samples have been prepared according to the HPLC-MS-method of Ullrich et al. (Ullrich et al. 2016).

4.2.6. Chlorophyll and Carotenoid Assay

The extraction and quantification of chlorophyll *a* and *b* and carotenoids was done according to the method published by Lichtenthaler and Wellburn in 1983 using 100 vol% acetone (Lichtenthaler and Wellburn 1983). Fresh *Duboisia* leaves were cut into small pieces of approximately 1 cm in diameter of which 100 mg were weighed in. A small amount of acetone, sea sand and a spade point of calcium carbonate were added to the plant material which was then thoroughly grinded by using pestle and mortar. The acetone phase was then transferred to a 20 ml volumetric flask including that part of the solvent, which was repeatedly used for rinsing of the pestle and mortar. The resulting extract was made up to 20 ml with acetone. 2 ml of the sample were subsequently filtered into a quartz cuvette for further photometric measurements.

The optical density of the solution was measured with a UV-1800 Shimadzu UV spectrometer (Shimadzu Deutschland GmbH, Duisburg, Germany) at 662, 645 and 470 nm. Pure acetone was used for zero adjustment. Within the measured concentration range, no dilution steps were necessary.

The respective pigment concentration was calculated using the following formulas:

$$c_{\text{Chlorophyll } a} = 11.75 E_{662} - 2.35 E_{645} \text{ [}\mu\text{g/ml]}$$

$$c_{\text{Chlorophyll } b} = 18.61 E_{645} - 3.96 E_{662} \text{ [}\mu\text{g/ml]}$$

$$c_{\text{Carotinoids}} = \frac{1000 E_{470} - 2.27 c_{\text{Chlorophyll } a} - 81.4 c_{\text{Chlorophyll } b}}{227} \text{ [}\mu\text{g/ml]}$$

On this basis, the pigment content was calculated as follows:

$$C_{\text{Pigment}} = \frac{\text{Extractant [ml]} \times \text{Pigment concentration [ml]}}{\text{Weight [mg]}} \text{ [mg/g]}$$

4.2.7. Data Analysis and Quantification

The HPLC-MS measurement of tropane alkaloids was done in a threefold determination of content. The statistical evaluation by means of the *Kruskal–Wallis test* was done using Microsoft Office Excel with Engine Room (MoreSteam, Powell, USA) as add-in. All data were furthermore analysed by multiple linear regression using MODDE 9.1 (Umetrics, Malmö, Sweden).

4.3. Results

4.3.1. Influence of Light and Temperature on Biomass and Scopolamine Production

After completion of all experiments, MODDE 9.1 was applied in order to build up a first model for the influencing factors light intensity (Int), light exposure (Lig) and temperature (Temp) on scopolamine [mg/g] and biomass [g] in *Duboisia myoporoides* (A).

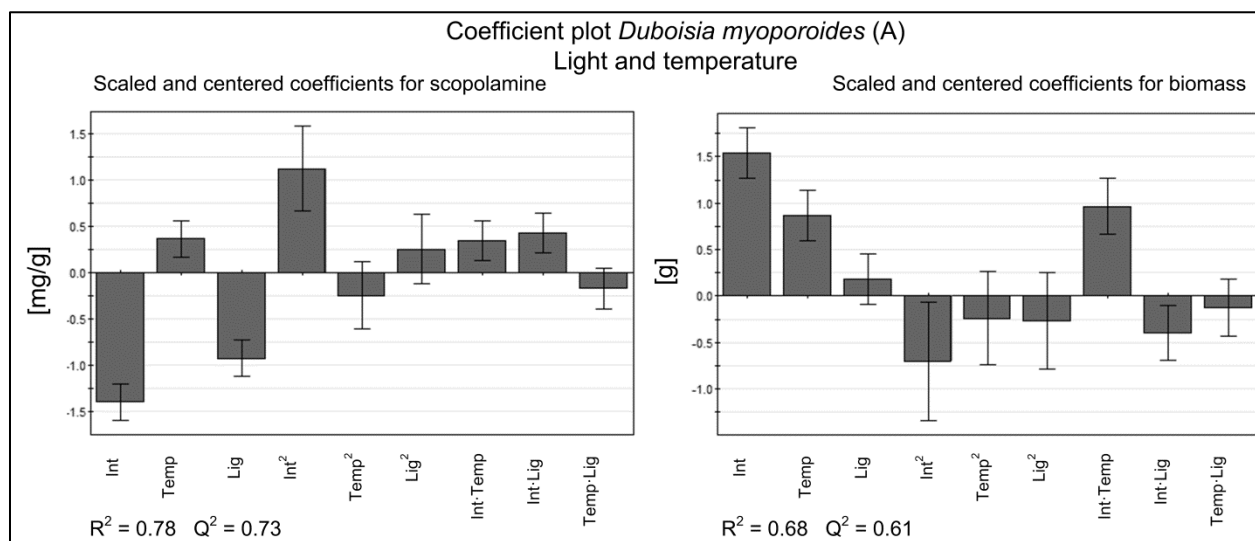


Figure 4-3: Coefficient plot for the influencing factors light intensity (Int), light exposure (Lig) and temperature (Temp) on the average scopolamine content [mg/g] and the biomass [g] in *Duboisia myoporoides* (A) with MODDE 9.1

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The coefficient plot (Figure 4-3) displays the multiple linear regression (MLR) coefficients with their respective confidence intervals. The data are scaled (encoded units: -1, 0, 1) and centred in order to make the coefficients comparable. The size of each coefficient represents the change in the response (scopolamine/biomass), when changing from 0 to high. The coefficient is considered as significant different from the noise, when its confidence interval does not cross zero.

Not only the coefficients for light intensity, light exposure and temperature, but also their interactions and quadratic effects are considered. Scopolamine [mg/g] is negatively influenced by high intensities of light and long daily illumination time. Temperature has only a minor influence on the scopolamine content. In addition, the influence of light intensity on the scopolamine content is described by combining a linear with a quadratic regression model. This means that the scopolamine content is considerably decreased by increasing the light intensity from 50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ to 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$. Any further increase in light intensity does not lead to an additional reduction in scopolamine [mg/g].

Looking at the biomass [g], a positive influence of light intensity and temperature is to be found, whereas the illumination time doesn't have any significant effect on the biomass production. The relation of light intensity to biomass is characterised by a linear regression model in combination with a quadratic function. All three influencing factors show interactions.

The Contour plot visualizes the predicted response values of scopolamine [mg/g] and biomass [g] spanned by two influencing factors (light intensity and light exposure) at the three different temperature levels examined.

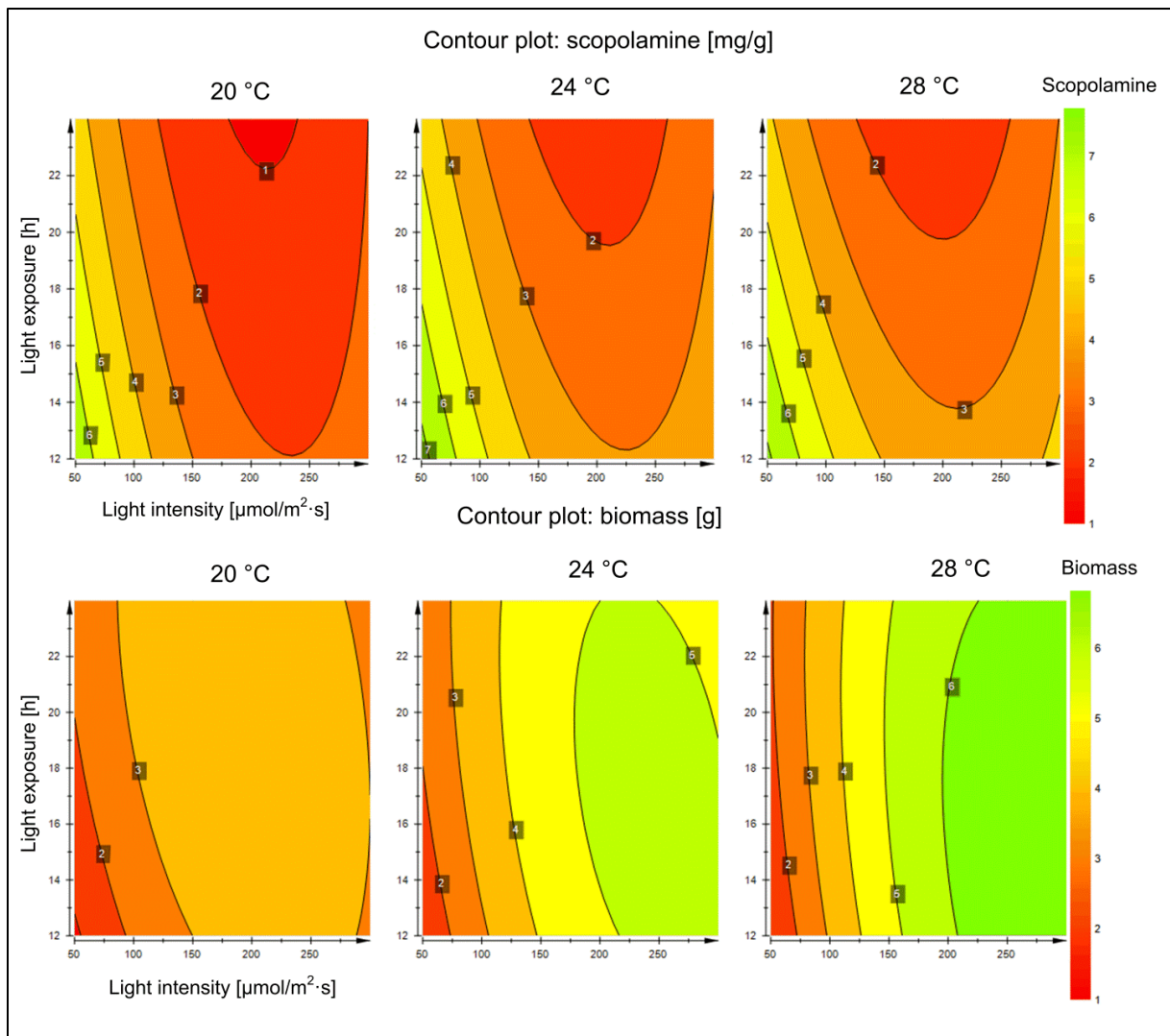


Figure 4-4: Contour plot on scopolamine [mg/g] and biomass [g] in *Duboisia myoporoides* (A) varying light exposure [h], light intensity [$\mu\text{mol}/\text{m}^2\cdot\text{s}$] and temperature [$^{\circ}\text{C}$] using MODDE 9.1

This makes it easier to understand the regression model created by MODDE 9.1. Looking at Figure 4-4, the three different plots at 20, 24 and 28 °C are looking almost similar. This is consistent with the coefficient plot (Figure 4-3) showing only little influence of temperature on the average scopolamine. Regarding biomass, low temperatures lead to a major decrease in leaf production and especially high intensities of light seem to be important for optimal biomass production, which is further illustrated by images of individual plants (Figure 4-5).

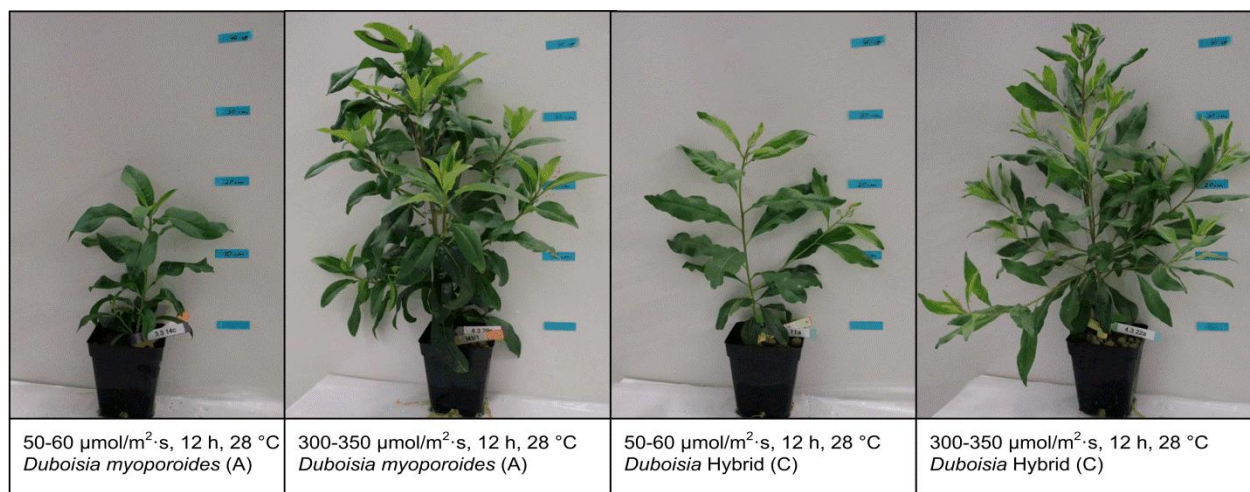


Figure 4-5: Pictures showing the biomass production of 7-week-old plants grown at 28 °C with 12 h of light exposure per day using a light intensity of 50-60 and 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ respectively

The model created by MODDE 9.1 is in line with the observations regarding biomass and scopolamine production, which were made by comparing single test runs with each other (Figure 4-6). Moreover, Figure 4-6 shows scopolamine to be the predominant alkaloid independent of the respective cultivation condition, always followed by its direct precursor 6 β -hydroxy-hyoscyamine. Hyoscyamine, which is known to be the predominant alkaloid in related plant genera as *Atropa* or *Hyoscyamus*, is only present in traces.

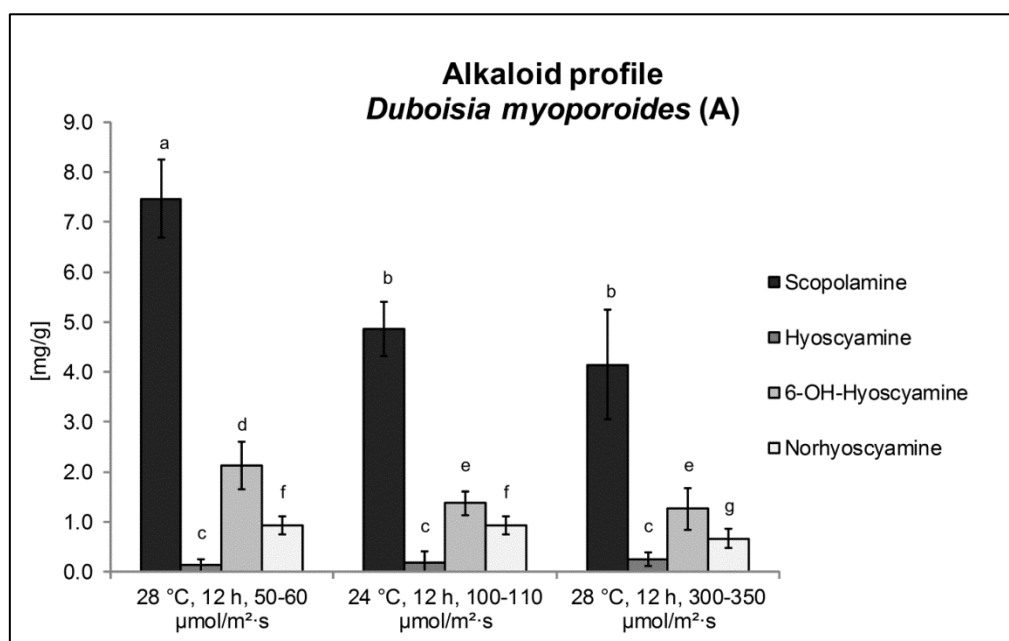


Figure 4-6: Alkaloid profile of 7-week-grown *Duboisia* plants of *Duboisia myoporoides* A (5-6 replicates/cultivation condition) under different experimental settings (temperature [°C],

daily exposure to light [h], light intensity [$\mu\text{mol}/\text{m}^2\cdot\text{s}$]), *Kruskal-Wallis test* with $P = 0.05$ (different letters = statistical significant differences)

In order to validate the model established with MODDE, different parameters were calculated. R^2 is the percentage of variation of the response covered by the model describing how well the model fits the data. A large R^2 (> 0.5) is a necessary condition for a good model. Q^2 describes how well the model is able to predict new data. This is tested by using cross validation. The Model validity is a measure of the explanatory power of the model. If the model validity is larger than 0.25, the model shows no lack of fit (LOF) meaning that the model error is in the same range as the pure error. If the model validity is less than 0.25, there is a significant LOF to be found. This means that the regression model is not able to adequately describe the functional relationship between all influencing factors investigated and the respective response variable. There are many causes of lack of fit, which result in poor model validity. If the model has a true lack of fit, usually not only the model validity, but also R^2 , Q^2 and the reproducibility will be small. The reproducibility is the variation of the response under the same experimental conditions (pure error), which is tested with the help of the repeated centre points compared to the total variance of the response (should be above 0.5 for a good model).

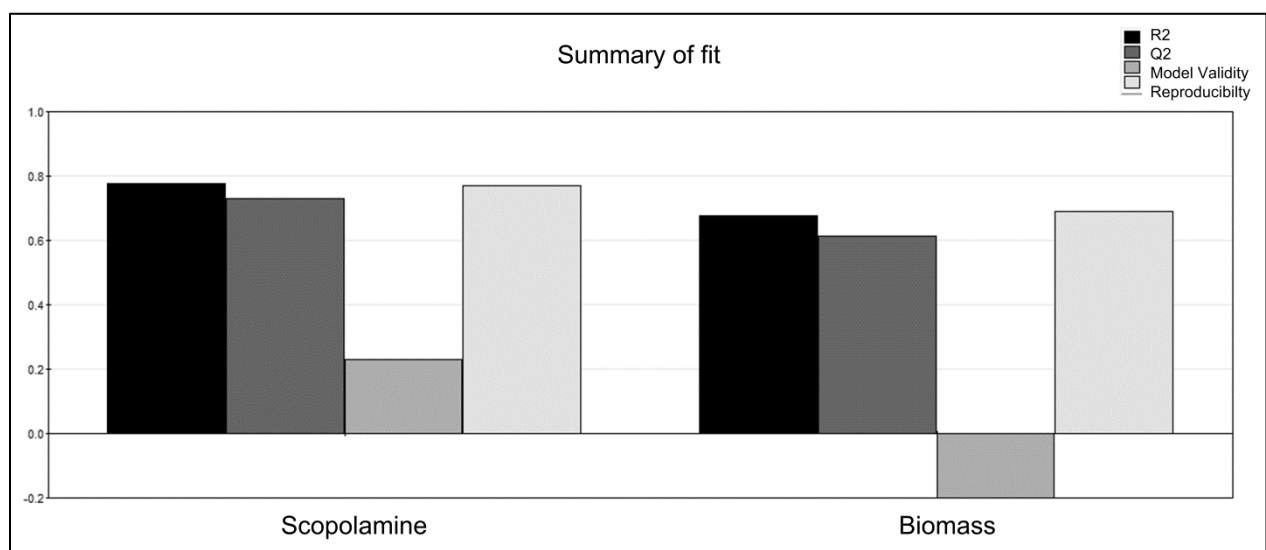


Figure 4-7: Summary of fit for both models (biomass [g] and scopolamine [mg/g]) varying light and temperature in *Duboisia myoporoides* (A) with MODDE 9.1

The requirements for a good model are fulfilled for all 4 parameters considered in the summary of fit in case of the response scopolamine (Figure 4-7). Looking at the biomass, Q2, R2 and reproducibility meet the requirements, but the model validity is extremely low. This might be due to outliers within the single test runs.

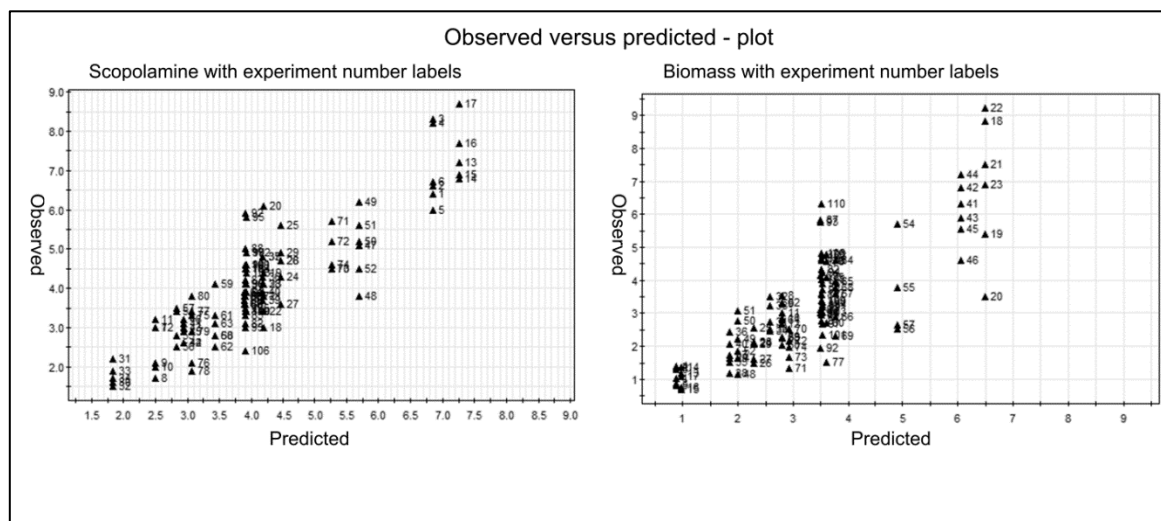


Figure 4-8: Observed versus predicted - plot for scopolamine [mg/g] and biomass [g] varying light and temperature in *Duboisia myoporoides* (A) with MODDE 9.1

Figure 4-8 shows the observed values (Y) compared to the predicted values (X) for biomass [g]. On average 5 plants per experimental condition were analysed, each replicate was assessed separately (1 biological replicate = 1 number in the observed versus predicted - plot). It can be seen that there is a clear correlation, but some values are differing a lot comparing the measured value with the predicted value. This is probably due to high variations within the biological replicates. This is furthermore confirmed by the replicate plot (Figure 4-9) showing repeated experiments on the same stick. The main part of the replicates within one cultivation condition is located quite close to each other (e.g. 1-6), but some plants are showing high variations (e.g. 18-23).

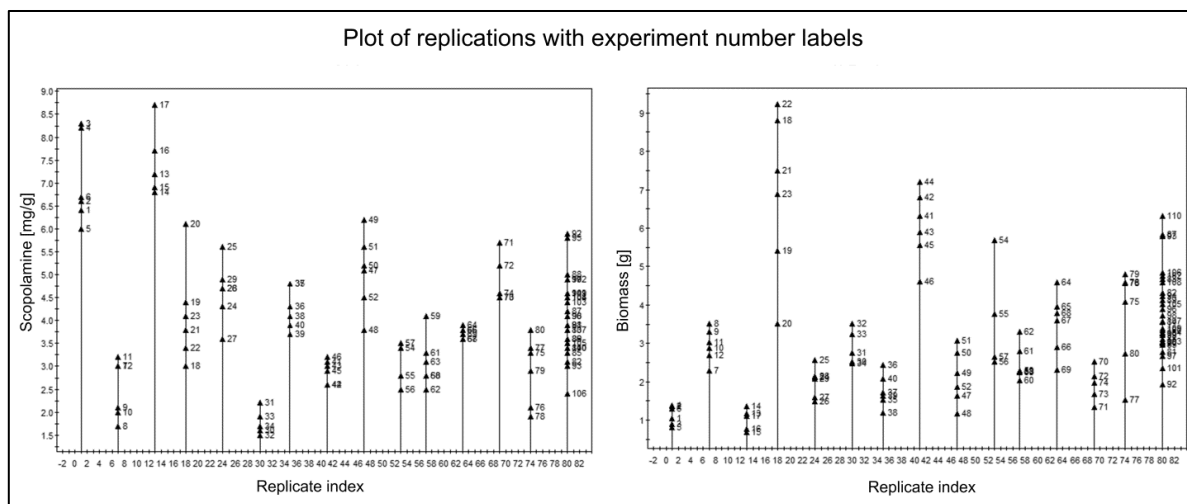


Figure 4-9: Replicate plot for scopolamine [mg/g] and biomass [g] varying light and temperature in *Duboisia myoporoides* (A) with MODDE 9.1

The data analysis of the *Duboisia* hybrids (data not shown) reveals that the observed effects are similar to those observed in *Duboisia myoporoides* (A). Scopolamine production is always negatively influenced by increased light intensity up to $350 \mu\text{mol}/\text{m}^2 \cdot \text{s}$ and light exposure up to 24 h/d, whereas biomass is positively affected by higher light intensity ($350 \mu\text{mol}/\text{m}^2 \cdot \text{s}$) and temperature ($28 \text{ }^\circ\text{C}$). In case of the hybrids, longer illumination time has an additional positive effect on biomass production in contrast to *Duboisia myoporoides*.

4.3.2. Influence of Macronutrients on Biomass and Scopolamine Production

MODDE 9.1 has also been applied in order to build up a model for the influencing factors nitrogen (N), potassium (K) and calcium (Ca) on scopolamine [mg/g] and biomass [g] production in *Duboisia myoporoides* (A). As the coefficient plot illustrates (Figure 4-10), the scopolamine level is negatively influenced by nitrogen and calcium, whereas the biomass production is significantly affected only by nitrogen. No interactions can be detected.

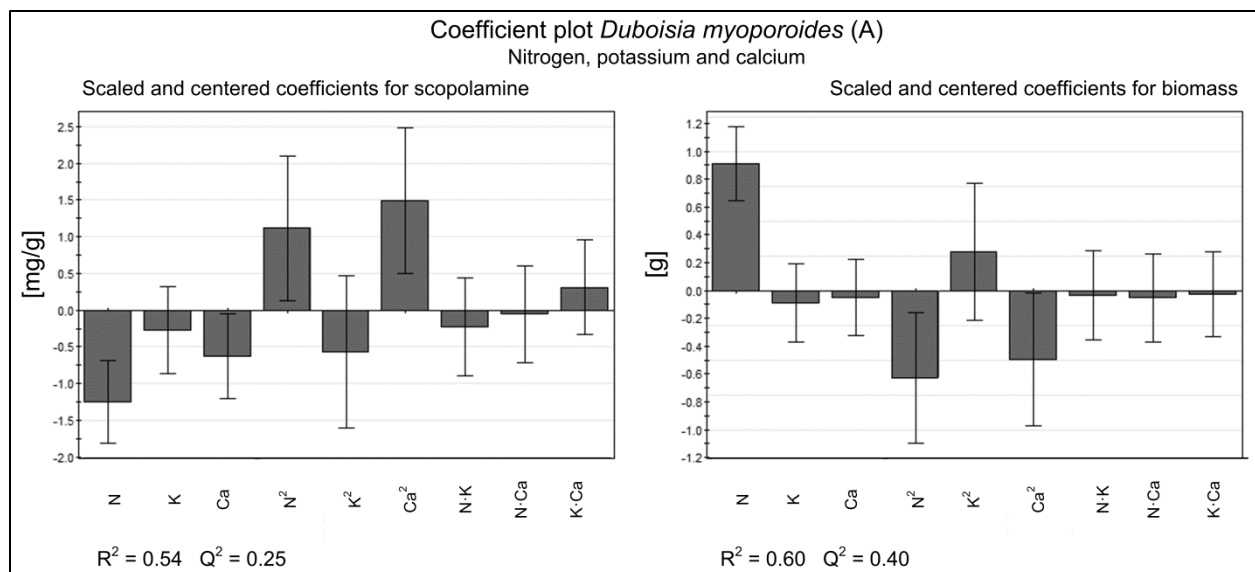


Figure 4-10: Coefficient plot for the nutrients nitrogen (N), potassium (K) and calcium (Ca) on the average scopolamine content [mg/g] and the biomass [g] in *Duboisia myoporoides* (A) with MODDE 9.1

The influence of nitrogen and calcium is described by combining a linear with a quadratic regression model. This also becomes apparent by looking at single test runs comparing lowest (N: 0.1 mmol, K: 0.05 mmol, Ca: 0.025 mmol), medium (N: 4 mmol, K: 2 mmol, Ca: 1 mmol) and highest levels (N: 8 mmol, K: 4 mmol, Ca: 2 mmol) (Figure 4-11). A significant increase in biomass from averagely 0.5 to 2.3 g is to be found comparing lowest to medium nutrient concentrations, whereas the biomass is not further increased at highest nutrient levels, where saturation is likely to be reached. In contrast, the average content in scopolamine is decreased from 8.9 to 5.5 mg/g comparing lowest to medium nutrient levels.

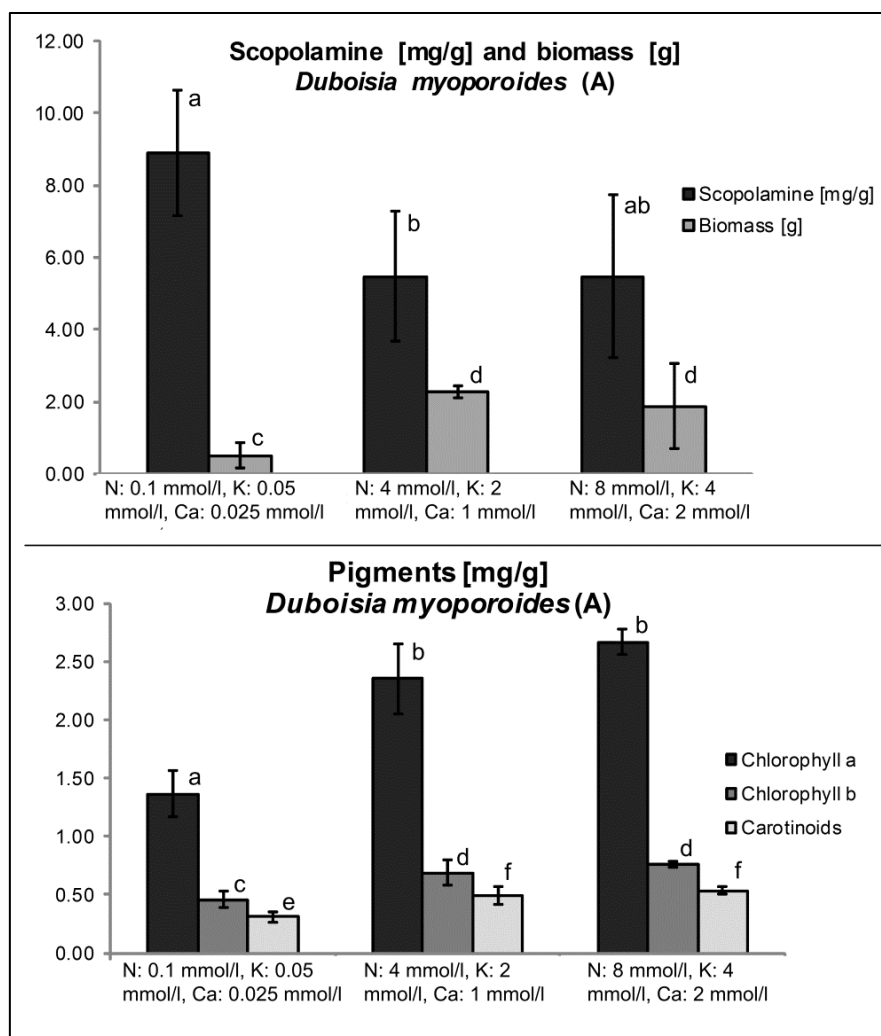


Figure 4-11: Scopolamine concentration [mg/g], biomass production [g] and pigment levels [mg/g] of 7-week-grown *Duboisia* plants of *Duboisia myoporoides* A (3-4 replicates/cultivation condition) varying nitrogen, potassium and calcium levels, *Kruskal-Wallis test* with $P = 0.05$ (different letters = statistical significant differences)

In addition to the measurement of biomass and scopolamine, the concentration of photosynthetic pigments was analysed (Figure 4-11). At lowest nutrient levels (N: 0.1 mmol, K: 0.05 mmol, Ca: 0.025 mmol), chlorophyll *a* and *b* as well as carotenoids are reduced by about 30%, probably due to insufficient nutrient supply. This correlates with a decreased photosynthesis rate also explaining the biomass production being reduced to one quarter comparing lowest to medium nutrient levels (N: 4 mmol, K: 2 mmol, Ca: 1 mmol). This is also visible looking at individual examples of plants at low, intermediate and high nutrient supply (Figure 4-12).

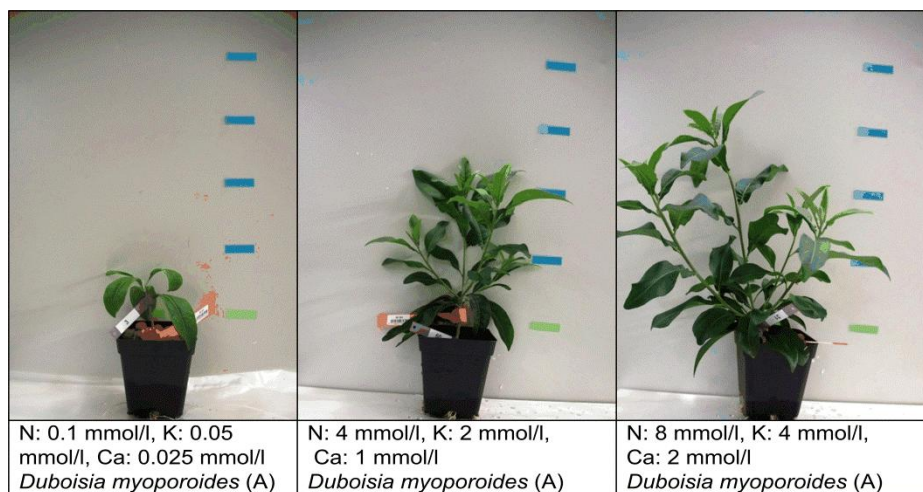


Figure 4-12: Pictures showing the biomass production of 7-week-old plants grown at different levels in nitrogen, potassium and calcium

In summary, the examination of nitrogen, potassium and calcium on growth and alkaloid biosynthesis revealed nitrogen to have a major impact on biomass as well as on scopolamine production.

4.3.3. Comparison to Greenhouse Cultivation

Concurrently to the experiments in climate chambers, 3 rooted cuttings of lines A, B and C were planted on soil and grown under greenhouse conditions during all 4 successive experimental runs. This allows a comparison of the development and the biological variation of the plants grown on soil and exposed to natural daylight to those in hydroponic culture using artificial lighting under strictly controlled growing conditions. A high variability is found between the different runs, probably due to the seasonal changes which can only be controlled in a very limited way compared to the climate chambers. Especially in case of run 4, which took place during the winter months, a really low biomass (line A: 0.6 g, line B: 0.5 g, line C: 0.2 g) is to be found for all of the three lines under investigation. Thereby, the highest average biomass after 7 weeks of greenhouse cultivation accounts only for around 0.6 g, whereas in the climate chambers up to 4.5 g on average are reached. In contrast to biomass, the content in scopolamine is quite stable and shows slightly higher average values under greenhouse cultivation than in climate chambers.

4.4. Discussion

The influence of light, temperature and nutrients on scopolamine and biomass production has been systematically evaluated under controlled growth in climate chambers and discloses light intensity and nitrogen supply to play a key role.

Regarding light intensity, light saturation of the photosynthesis seems to be nearly reached at 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, as only a slight increase in growth is observed compared to 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$. The negative correlation of scopolamine with regard to increasing light intensity and illumination time might occur due to a shift from primary to secondary metabolism at reduced light exposure (12-14 h/d). In total, the highest average scopolamine level with approximately 7.5 mg/g is to be found at the lowest intensity of light combined with a decreased biomass production of a total of 1 ± 0.3 g dried leaves after 7 weeks of cultivation. This matches very well with the hypothesis of Van Dam stating that the biosynthesis of secondary metabolites is reinforced under favourable growing conditions, especially under slow grow (Van Dam et al. 1996), which is the case at 50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$. At a first glance it may appear that a decrease in biomass leads to less number of leaves meaning less storage space for alkaloids and therefore a higher content in scopolamine. By growing plants at 28 °C with 12 h exposure to a light intensity of 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ per day for instance, on which level the highest biomass (6.9 ± 2.2 g) is produced, one would expect a decrease in scopolamine. Indeed, the scopolamine level remains comparable to cultivation conditions where significantly less biomass has been produced. This shows that a higher production of biomass providing more storage space in leaves does not necessarily cause a decrease in the average leaf content in scopolamine by a dilution of the biosynthesised alkaloids. Moreover, probably not only the aerial plant parts, but also the biomass of roots, where tropane alkaloids are biosynthesised, have an impact at this point. The root mass likely correlates with the leaf mass, as *Duboisia* plants producing higher leaf mass also show enhanced root production (data not shown) and are therefore able to synthesise more tropane alkaloids, which are subsequently transported and distributed within the leaves.

The positive correlation between temperature and growth within the tested range can be explained by an increased photosynthesis rate leading to higher biomass production (Yan 2013). The optimum temperature for the biochemical reactions seems not yet to be exceeded at 28 °C.

By increasing nitrogen supply up to 4 mmol/l, biomass production is significantly enhanced from averagely 0.5 to 2.3 g combined with a reduction in scopolamine levels from 8.9 to

5.5 mg/g. This supports the observation of Luanratana and Griffin, who also noticed a decrease in alkaloid production in *Duboisia* through increased nitrogen supply within their field trials (Luanratana and Griffin 1980a). As growth is strongly reduced at lowest nutrient supply and only slightly increased at highest levels, the average nutrient supply consisting of 4 mmol/l N, 2 mmol/l K and 1 mmol/l Ca can be considered as optimal and applied as basic value in hydroponics of *Duboisia* and related plant genera. The optimum concentrations for field grown plants will need to be evaluated separately, as they strongly depend on the soil composition and require the consideration of environmental concerns, e.g. the ground water salination.

Interestingly, it seems that both target values scopolamine and biomass are influenced in an opposite manner. The secondary plant constituent scopolamine is increasingly produced under reduced light intensity (50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$) and illumination time (12-14 h/d), whereas the primary metabolism necessary for growth and biomass production seems to be enhanced through increased nutrient supply and high light intensity (300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$), probably due to a higher photosynthesis rate, which is also in accordance with the measured chlorophyll contents (see Figure 4-11) and literature data (Demmig-Adams and Adams III 1992; Evans 1989; Ku 1977). Especially the light intensity appears to be an important regulating variable, which can be applied in a targeted manner in order to improve the large scale production of scopolamine. In greenhouse for example, high intensities of light (300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$) might be used in the beginning of cultivation in order to increase biomass production, whereas reduced light intensities (50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$) prior to harvesting will possibly increase the average content in scopolamine. As temperature has only a minor influence, additional heating in the greenhouse would probably cost more than it benefits plant growth.

4.5. Conclusion

The controlled cultivation of *Duboisia myoporoides* and hybrids of *Duboisia myoporoides* and *D. leichhardtii* in climate chambers allows a statistically significant analysis of the influence of abiotic factors, namely light, temperature and nutrients, on scopolamine and biomass production.

In conclusion, this study contributes to a better understanding of the interaction of abiotic factors with alkaloid metabolism and plant growth, revealing light intensity and nitrogen supply to have a major impact. In contrast to field cultivation, the controlled cultivation of

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Duboisia in climate chambers opens up the possibility of producing well predictable amounts of plant material and scopolamine, independently from seasonal fluctuations. Moreover, it can be used in order to improve the already existing farming of *Duboisia* and related plant genera.

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5. Chapter 5: Discussion and Outlook

5.1. Discussion

Duboisia is the main source of the pharmaceutically valuable tropane alkaloids, in particular of scopolamine (Foley 2006). As neither its full chemical synthesis nor its biotechnological production are competitive by now (Palazón et al. 2003; Hashimoto and Yamada 2003), this work was focused on the optimisation of plant-based production.

Despite the commercial cultivation of *Duboisia* since decades, little is known on the metabolic regulation of tropane alkaloid production depending on environmental conditions as well as on plant age, genotype and method of cultivation. This work largely contributes to filling this gap by not only systematically analysing the influence of those factors, but also by providing new approaches for an optimised cultivation and breeding of those plants.

The climate change makes a stable scopolamine production in *Duboisia* more and more challenging. Moreover, the season of harvest is also supposed to influence the yield in scopolamine. Field trials within the past decades showed the alkaloid content to be decreasing in autumn and winter in field grown *Duboisia* plants in Queensland, Australia (Luanratana and Griffin 1980). Seasonal variations have also been observed in *Duboisia* hybrids grown in Japan (Ikenaga et al. 1985). But these data are difficult to interpret since they do not allow drawing a conclusion on individual influencing factors affecting the scopolamine biosynthesis, as a clear allocation is not possible. Furthermore, various environmental factors in the field are known to be able to mask genetic characteristics (Rosenblum 1954) and make it difficult to get meaningful results in field trials, e.g. by comparing plants grown in different geographical regions or at different seasons. Therefore, we developed a controlled hydroponic cultivation system in climate chambers allowing us to systematically analyse the impact of selected environmental factors, including their interactions, on the scopolamine biosynthesis and growth of *Duboisia*.

In order to obtain an overview with regard to the influence of diverse *Duboisia* genotypes at different growth stages, cultivated indoors and outdoors, on the primary and secondary metabolism, leaf and root extracts were prepared and analysed via ¹H NMR and HPLC-MS. The 1D- and 2D-NMR analysis allowed to get a global overview of the metabolite composition, whereas the HPLC-MS analysis was more focused on a qualitative as well as quantitative analysis of tropane alkaloids.

The data analysis revealed that the specific genotypes can be divided according to their chemical composition into hybrids and wild types, the latter subclassified into *Duboisia myoporoides* and *D. leichhardtii*. Thereby, the wild types generally showed higher levels in

sugars and amino acids compared to the hybrids which showed a higher flux towards scopolamine. Some direct precursors of scopolamine were more abundant in the wild types, especially hyoscyamine occurring as main alkaloid in *Duboisia leichhardtii*, which was further validated via quantification with HPLC-MS. This points out that the H6H, responsible for the conversion of hyoscyamine to scopolamine, might be less expressed and/or less active in *Duboisia leichhardtii*. A strong dependence of plant secondary metabolite production on genotypic variations has already been reported for many medicinal plants, e.g. *Panax quinquefolius*, *Eucalyptus globulus* or *Arabidopsis* (Schlag and McIntosh 2013; Külheim et al. 2011; Kliebenstein et al. 2001).

Apart from genetic differences at species, variety and genotype levels, environmental conditions are also known to influence the primary and secondary plant metabolism in medicinal plants (Chen et al. 2015). In the leguminous plant Menggu Huangqi (*Astragalus membranaceus*), applied in traditional Chinese medicine, the metabolic fingerprinting showed growth locations (cultivated *versus* wild) to have even greater impacts on the metabolite composition and quantity than the genotypes (Duan et al. 2012). In Astragali Radix, for example used in the treatment of diabetes or cardiovascular diseases, significant differences between wild and cultivated *Astragalus* plants were present. This did not only apply to the primary, but also to the secondary metabolite production (Li et al. 2015). In Asian and American ginseng, the main differences between wild and cultivated plants were identified as ginsenosides, which were higher concentrated in wild grown American ginseng (Zhao et al. 2015). Significant differences in primary and secondary metabolite composition are also found comparing field grown plants of *Duboisia* to those cultivated in climate chambers. The plants grown under controlled conditions contained higher amounts in primary metabolites as sugars and amino acids combined with increased vertical growth than the field-grown plants, which showed significantly higher levels of tropane alkaloids as well as flavonoids. This might be due to various abiotic and biotic interactions in the field, such as temperature, light, drought, wind and insects. Moreover, the continuous nutrient supply in climate chambers via irrigation with nutrient solution and the constant light regime are possibly responsible for the increased growth compared to outdoor cultivation. Besides, the light spectrum in climate chambers is not fully comparable to natural daylight and might therefore also induce a different growth habit. It is furthermore noteworthy that the samples of the field-grown plants only reflect one single snapshot and might show a different behaviour with regard to growth and metabolite composition depending on the time of sampling.

In addition, it was of great interest to examine the influence of the plant age (3 months, 6 months after planting) on the metabolite distribution. After 3 months, sugars like glucose or sucrose and amino acids as proline, choline and alanine involved in plant growth were more abundant compared to 6 months of cultivation. In the hybrids, scopolamine and its direct precursors were increased after 6 months of growth. This indicates that there might be an enhanced flux towards secondary metabolism with increasing plant age. However, this did not apply to the wild types, where the scopolamine and hyoscyamine levels remained comparable independent of the growth stage. In order to verify the hypothesis of a stronger flux towards tropane alkaloids with increasing plant age, more times of harvest should be tested, for example once a month within a period of 1-2 years.

The synchronous evaluation of age and genotype by OPLS-DA revealed the genotype to be more influential to the quantitative occurrence of scopolamine than the age of the investigated plant. Prospectively, this might allow the comparison and selection of young plants of different genotypes based on their scopolamine level in breeding research already at early stage of growth.

All in all, the impact of environmental factors, developmental stage as well as genotype on biomass production and the biosynthesis of scopolamine and its derivatives in *Duboisia* species was comprehensively analysed by applying NMR-based metabolic profiling and targeted HPLC-MS analysis of tropane alkaloids.

Additionally, the influence of light intensity, illumination time, temperature as well as supply in nitrogen, potassium and calcium on scopolamine and biomass production was systematically evaluated using our controlled cultivation system in climate chambers. The data analysis hereby revealed especially the light intensity as well as the nitrogen supply to have a large effect on scopolamine as well as on biomass production in *Duboisia myoporoides* as well as in hybrids of *Duboisia myoporoides* and *D. leichardtii*. Unfortunately, light intensity and nitrogen load do influence those effect variables in an opposite manner, meaning that no setting was detected which would be able to increase both, biomass as well as scopolamine yield. Whereas biomass is positively affected by high irradiation and increased supply in NH_4NO_3 , the opposite holds true for the secondary metabolite scopolamine. These observations regarding secondary metabolism are in consistence with those of other species and metabolic pathways. Ibrahim and Jaafar for example observed an enhanced production of flavonoids and phenolics in *Orthosiphon stimanus* under low irradiance (Ibrahim and Jaafar 2012). The terpenoid indole alkaloid vinblastine did also accumulate under low light (Liu et al.

2011). Regarding the inverse relationship of biomass and production of secondary metabolites, Van Dam et al. found that plants growing slowly allocate more resources to defence (Van Dam et al. 1996). Yamamura and Tsuji also noted that plants investing more resources in the biosynthesis of defence substances show a decreased biomass production (Yamamura and Tsuji 1995). The correlation between nitrogen supply and biomass production is in line with our expectations, as nitrogen is already known to positively influence the growth rate of plants up to a certain level (Hirose 1988; Evans 1989).

The systematic evaluation of the impact of temperature, light and macronutrients on biomass and tropane alkaloid production in *Duboisia* confirms previous research, but also detects new findings. In this context, the positive influence of low light intensity on the biosynthesis of scopolamine is particularly noteworthy.

5.2. Outlook

As previously described, the growth and scopolamine production in *Duboisia* is largely influenced by illumination levels as well as by supply with nitrogen. But as yield in biomass and scopolamine biosynthesis respond contrarily, it would make sense to adapt cultivation parameters during growth. Especially under controlled greenhouse conditions the total yield might be improved by starting cultivation with the optimised parameters for an enhanced biomass production (high light intensity, high levels in nitrogen) and modify them to those that improve scopolamine synthesis (low light intensity, low levels in nitrogen) a few weeks prior to harvest.

Other abiotic factors deserve to be looked at more closely, as they might also impact the yield in plant material as well as secondary metabolite production, namely humidity and UV light. A systematic evaluation of those specified parameters was technically not realizable in our climate chambers. However, Binder et al. reported UV-B light to increase the terpenoid indole alkaloid production in hairy roots of *Catharanthus roseus* (Binder et al. 2009). The exposure of *Psychotria brachyceras* cuttings to UV-B radiation significantly enhanced leaf brachycerine concentrations, which is a monoterpene indole alkaloid (Nascimento et al. 2012). This effect has also been observed with regard to tropane alkaloids; Qin et al. reported UV-B stressed hairy root cultures to accumulate more scopolamine due to a very strong increase of gene expression (Qin et al. 2014), which renders UV-B radiation interesting for further investigation. Regarding humidity, accumulation of alkaloids has been reported to be

greater at high humidity in *Nicotiana tabacum* (Linskens and Jackson 1994). Not only the alkaloid accumulation, but also the biomass production can be affected by humidity. Daily totals of net photosynthesis were observed as higher for leaves at increased humidity (Bunce 1983), which is also in accordance with increased biomass production (Beadle and Long 1985).

Our improved knowledge on cultivation and metabolism and established analytical methods can be applied in future research in the fields of breeding and production as well as expanded to other sectors, e.g. in quality control of *Duboisia* plant material. As NMR provides a fingerprint of the plant extracts, many anomalies in metabolite composition can be detected at first sight allowing for example the comparison of different batches. The additional HPLC-MS analysis allows to not only analysing the final products, but also the accumulation of precursors and degradation products within the alkaloid pathway. Moreover, a chemotaxonomic classification of the breeding stock as well as of *Duboisia* plants of unknown origin collected from the wild is facilitated by applying those NMR- and HPLC-MS methods. In addition, this metabolome analysis is a first step towards the elucidation of the tropane alkaloid pathway, which is crucial not only in order to optimise scopolamine production in *Duboisia* plants, but also for further progress in the biotechnological production of scopolamine. Prospectively, in order to fully explore the tropane alkaloid pathway, including all responsible enzymes, bottlenecks and interactions with other pathways, systematic research combining proteomics, transcriptomics and metabolomics and the use of metabolite correlation networks will be required.

5.3. Conclusion

In summary, this work contributes to an increased knowledge of the interaction of abiotic factors with alkaloid metabolism and plant growth in *Duboisia*. It shows especially genotype and environment to have a strong impact on growth and metabolite composition, in particular towards the tropane alkaloid biosynthesis. In contrast to that, the plant age seems to be less influential with regard to the metabolite production.

In addition, a comprehensive and systematic analysis of the impact of abiotic factors on growth and alkaloid biosynthesis in *Duboisia* was performed for the first time and reveals light intensity and nitrogen supply to largely affect biomass and scopolamine production, whereas temperature as well as calcium and potassium levels play only a secondary role.

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All in all, this work provides an appropriate controlled cultivation system as well as suitable analytical methods for the systematic assessment of metabolic processes and plant development in *Duboisia* and related plant genera. The future application of these methods will help to improve breeding and selection as well as indoor and outdoor cultivation of *Duboisia* for an enhanced production of scopolamine.

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Appendix

- I. List of Abbreviations
- II. List of Tables
- III. List of Figures
- IV. List of Original Contributions
- V. *Curriculum Vitae*

I. List of Abbreviations

<i>ACN</i>	Acetonitrile
<i>ArgDC</i>	Arginine decarboxylase
<i>Ca</i>	Calcium
<i>CCF</i>	Central composite face centered
<i>CNS</i>	Central nervous system
<i>COSY</i>	Correlation spectroscopy
<i>CV</i>	Coefficient of variation
<i>Cyp80F1</i>	Cytochrome P450 80F1
<i>D</i>	Deuterated
<i>DAD</i>	Diode array detector
<i>D₂O</i>	Deuterium oxide
<i>ESI-qTOF-MS</i>	Electrospray ionization quadrupole time-of-flight mass spectrometry
<i>eV</i>	Electron volt
<i>FA</i>	Formic acid
<i>fr</i>	Fresh
<i>GST-tag</i>	Gluthation-S-transferase-tag
<i>H6H / h6h</i>	Hyoscyamine 6 β -hydroxylase (enzyme / gene)
<i>HIS-tag</i>	Polyhistidine-tag

List of Abbreviations

<i>HMBC</i>	Heteronuclear multiple-bond correlation spectroscopy
<i>H NMR</i>	Proton nuclear magnetic resonance spectroscopy
<i>HPLC-MS</i>	High-performance liquid chromatography-mass spectrometry
<i>Hz</i>	Hertz
<i>J</i>	Coupling constant
<i>K</i>	Potassium
<i>LED</i>	Light-emitting diode
<i>LoD</i>	Limit of detection
<i>LLoQ</i>	Lower limit of Quantification
<i>LOF</i>	Lack of fit
<i>mAChR</i>	Muscarinic acetylcholine receptors
<i>MHz</i>	Mega hertz
<i>MPO</i>	<i>N</i> -methylputrescine oxidase
<i>m/z</i>	Mass to charge ratio
<i>N</i>	Nitrogen
<i>NaOD</i>	Sodium deuterioxide
<i>OPLS-DA</i>	Orthogonal partial least square discriminant analysis
<i>OrnDC</i>	Ornithine decarboxylase
<i>PC</i>	Principal component
<i>PCA</i>	Principal component analysis
<i>PMT / pmt</i>	Putrescine <i>N</i> -methyltransferase (enzyme / gene)

List of Abbreviations

<i>Q²</i>	Fraction of the variation of the response predicted by the model
<i>R²</i>	Fraction of the variation of the response explained by the model
<i>RF</i>	Radio frequency
<i>rpm</i>	Revolutions per minute
<i>ROS</i>	Reactive oxygen species
<i>SAM</i>	<i>S</i> -adenosylmethionine
<i>soln.</i>	Solution
<i>SUS</i>	Shared and unique structures
<i>TCC</i>	Thermostatted column compartment
<i>TR-1 / tr-1</i>	Tropinone-reductase I (enzyme / gene)
<i>TR-2 / tr-2</i>	Tropinone-reductase II (enzyme / gene)
<i>TSP</i>	Trimethylsilyl propanoic acid
<i>UHPLC</i>	Ultra-high-performance liquid chromatography
<i>UVA / B</i>	Ultraviolet A / B
<i>V_{pp}</i>	Peak to peak voltage
δ	Chemical shift
γB_1	Pre-saturation, Larmor frequency

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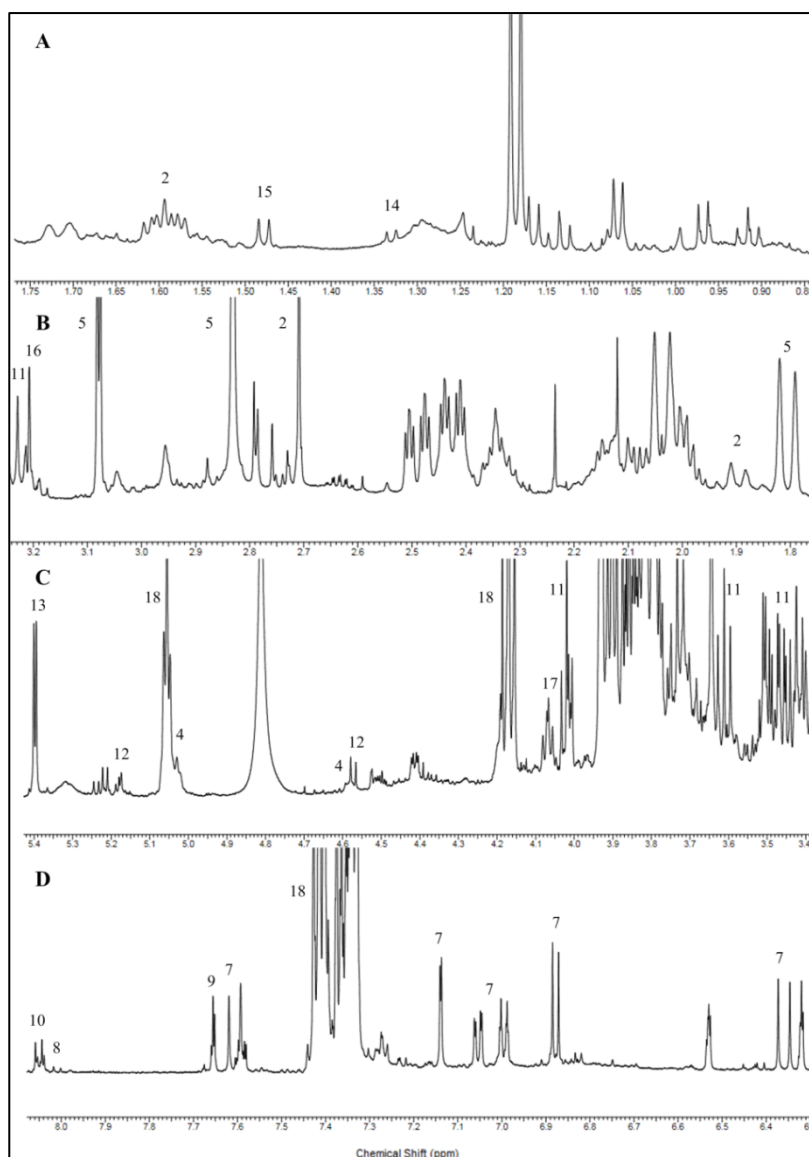
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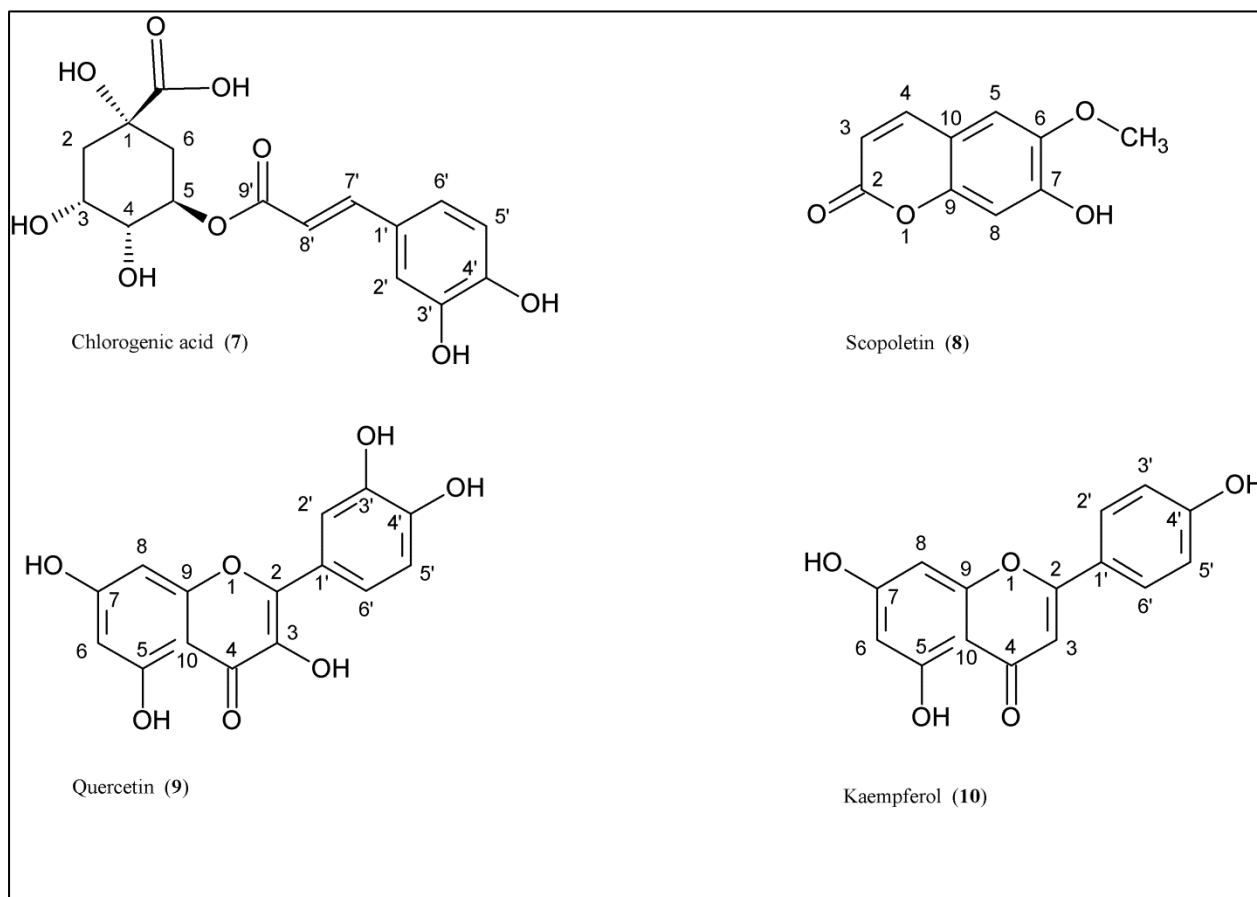
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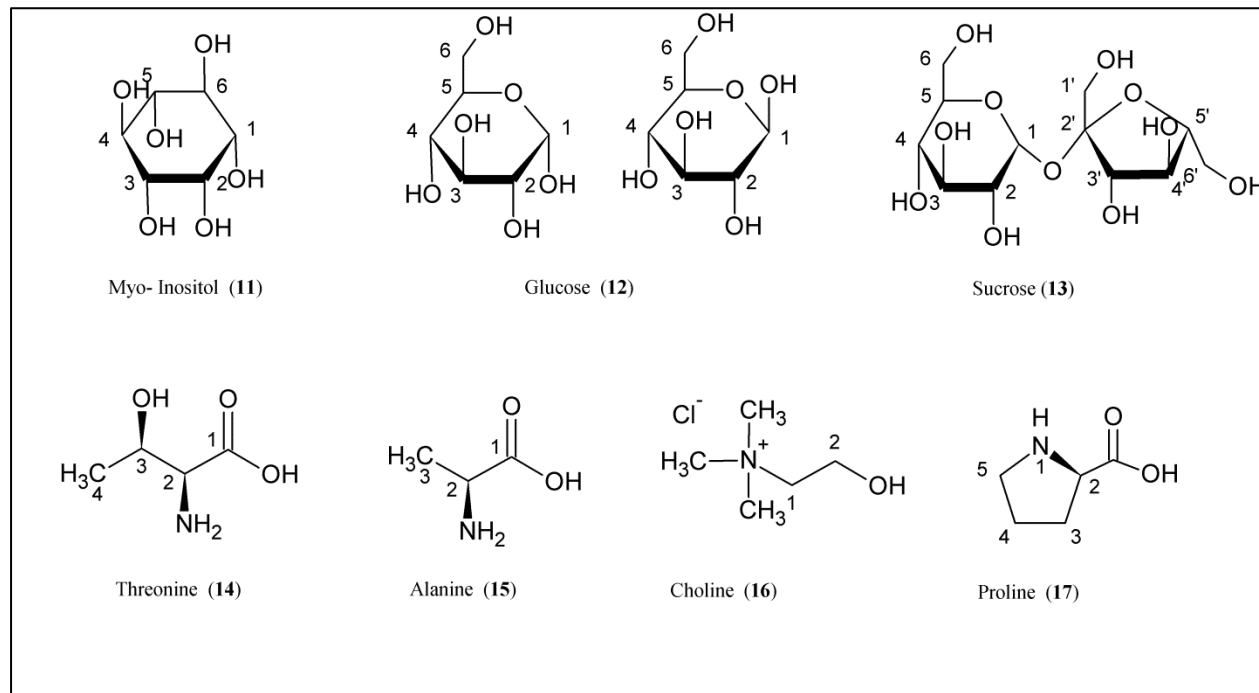
Supplementary Material

II. Selected secondary compounds detected in *Duboisia*. The carbon numbering system for each compound is used in the following for NMR assignment.



Supplementary Material

III. Selected primary compounds detected in *Duboisia*. The carbon numbering system for each compound is used in the following for NMR assignment.



Supplementary Material

IV. Data on plant height and side branches

<i>Duboisia myoporoides</i>					
Plant	Line	Number of branches	Height [cm]	Average	SD
3M B KK M1	A	8	48.1	51.70	4.26
3M B KK M2	A	7	50.6		
3M B KK M3	A	5	56.4		
6M B KK M1	A	27	121.3	112.53	8.80
6M B KK M2	A	17	103.7		
6M B KK M3	A	21	112.6		
<i>Duboisia leichhardtii</i>					
Plant	Line	Number of branches	Height [cm]	Average	SD
3M B KK M14	B	21	54.2	48.67	6.44
3M B KK M16	B	27	50.2		
3M B KK M17	B	16	41.6		
6M B KK M11	B	63	180	112.93	58.30
6M B KK M19	B	37	74.3		
6M B KK M20	B	44	84.5		
3M B KK M21	C	28	80.1	83.63	4.22
3M B KK M22	C	28	82.5		
3M B KK M23	C	26	88.3		
6M B KK M26	C	44	154.1	156.93	3.13
6M B KK M28	C	48	156.4		
6M B KK M29	C	48	160.3		

Supplementary Material

<i>Duboisia</i> hybrids					
Plant	Line	Number of branches	Height [cm]	Average	SD
3M B KK M3	D	8	31.1	28.80	3.16
3M B KK M4	D	7	30.1		
3M B KK M5	D	9	25.2		
6M B KK M2	D	21	58.4	60.77	6.67
6M B KK M7	D	22	68.3		
6M B KK M10	D	17	55.6		
3M B KK M15	E	6	59.8	60.40	3.64
3M B KK M17	E	7	64.3		
3M B KK M19	E	8	57.1		
6M B KK M12	E	12	110.4	111.77	1.35
6M B KK M14	E	15	111.8		
6M B KK M16	E	10	113.1		
Field grown <i>Duboisia</i> hybrids					
Plant	Line	Number of branches	Height [cm]	Average	SD
3M B FL M1	E	2	26	26.00	4.00
3M B FL M2	E	0	30		
3M B FL M3	E	4	22		
6M B FL M4	E	18	60	52.67	7.02
6M B FL M5	E	20	52		
6M B FL M6	E	25	46		
10M B FL M7	E	38	103	107.67	5.69
10M B FL M8	E	42	106		
10M BJ FL M9	E	23	114		

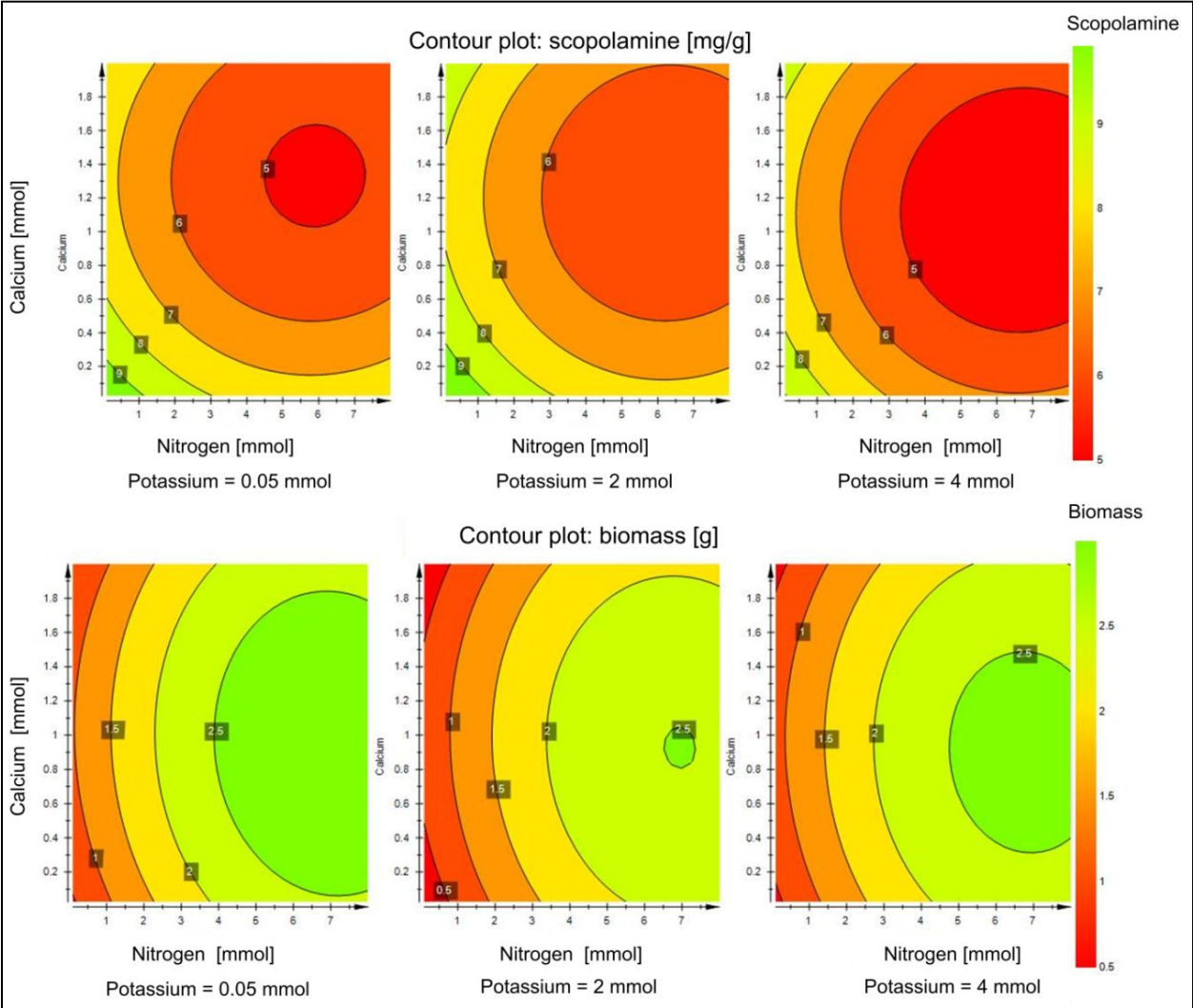
Supplementary Material

V. Overview of method validation parameters of tropane alkaloids occurring in leaf extracts of *Duboisia* spec. analysed by HPLC–MS

Compound	rt [min]	[M + H] ⁺	El. Comp.	Calibration curve	Correlation coefficient	LoD [µg/l]	LLo Q [mg/l]	Average accuracy [%]	Precision CV [%]	Range [mg/l]
Scopolamine	5.19	304.16	C ₁₇ H ₂₁ NO ₄	$y = -0.000506 x^2 + 0.777501 x + 0.043357$	0.9994	12.5	0.16	97.14 – 104.76	1.11 – 4.02	0.16 - 50
Norscopolamine	5.56	290.14	C ₁₆ H ₁₉ NO ₄	$y = 0.000559 x^2 + 0.443673 x + 0.017087$	0.9996	12.5	0.16	97.59 – 104.24	0.7 – 4.16	0.16 - 50
6β-hydroxy-hyoscyamine	6.17	306.17	C ₁₇ H ₂₃ NO ₄	$y = -0.000047 x^2 + 0.721242 x + 0.018286$	0.9997	12.5	0.16	97.62 – 104.25	0.65 – 2.86	0.16 - 50
Hyoscyamine	7.71	290.18	C ₁₇ H ₂₃ NO ₃	$y = -0.001670 x^2 + 1.019226 x + 0.034757$	0.9995	1.25	0.16	96.55 – 105.25	0.8 – 4.98	0.16 - 50
Norhyoscyamine	7.80	276.16	C ₁₆ H ₂₁ NO ₃	$y = -0.000258 x^2 + 0.697377 x + 0.037818$	0.9996	12.5	0.16	97.26 – 104.73	0.93 – 5.98	0.16 - 50
Littorine	7.98	290.18	C ₁₇ H ₂₃ NO ₃	$y = -0.000096 x^2 + 0.937253 x + 0.024966$	0.9997	12.5	0.16	97.42 – 104.5	1.12 – 3.86	0.16 - 50
Aposcopolamine	9.34	286.14	C ₁₇ H ₁₉ NO ₃	$y = -0.000733 x^2 + 0.981796 x + 0.064763$	0.9997	12.5	0.16	97.6 – 106.39	0.46 – 4.51	1.16 - 50

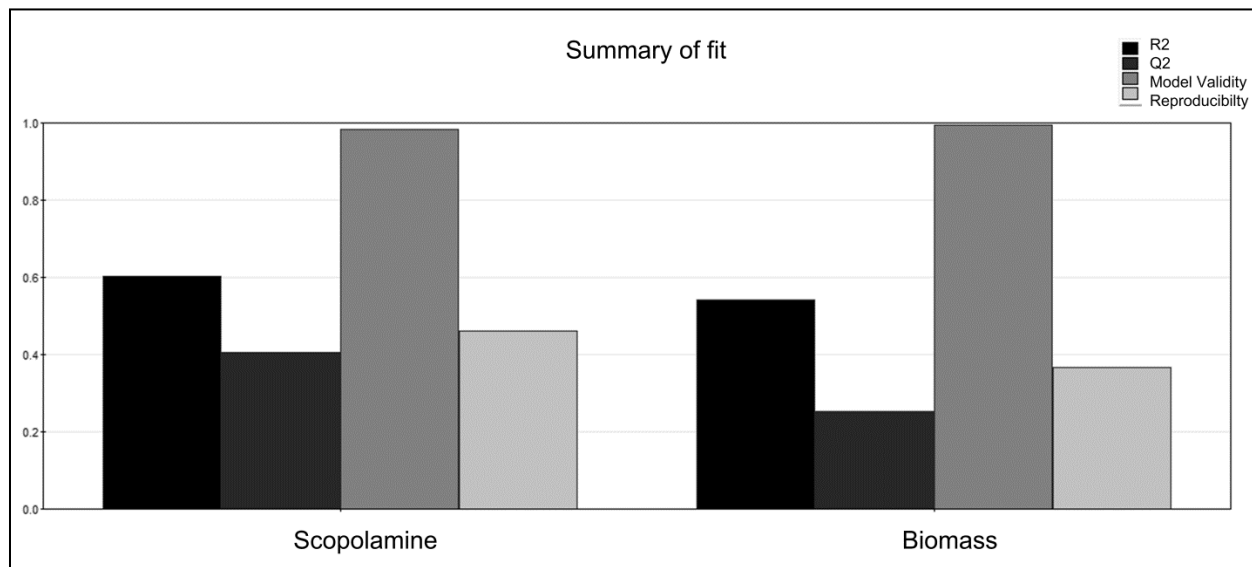
Chapter 4

VI. Contour plot on scopolamine [mg/g] and biomass [g] in *Duboisia myoporoides* (A) varying nitrogen, potassium and calcium using MODDE 9.1

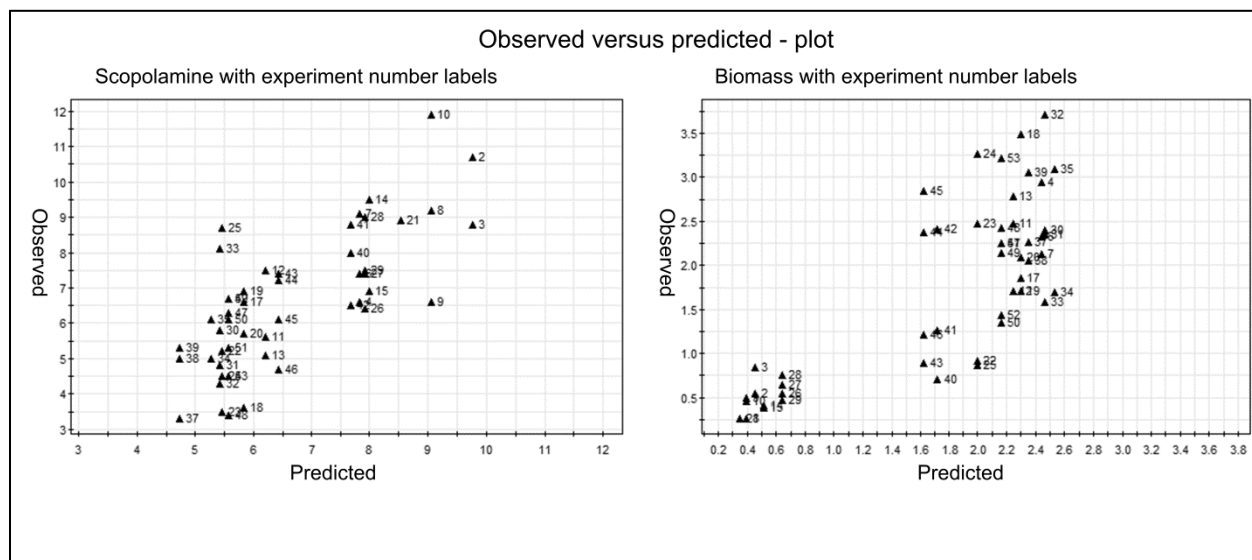


Supplementary Material

VII. Summary of fit for both models (scopolamine [mg/g] and biomass [g]) varying nitrogen, potassium and calcium in *Duboisia myoporoides* (A) with MODDE 9.1

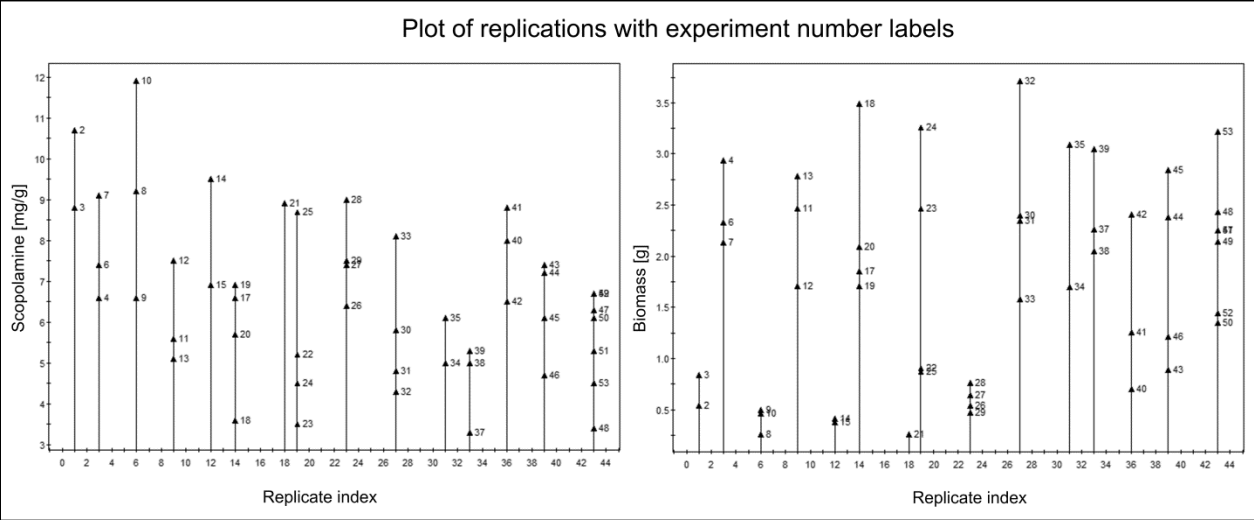


VIII. Observed versus predicted - plot for scopolamine [mg/g] and biomass [g] varying nitrogen, potassium and calcium in *Duboisia myoporoides* (A) with MODDE 9.1



Supplementary Material

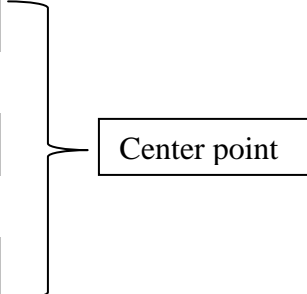
IX. Replicate plot for scopolamine [mg/g] and biomass [g] varying nitrogen, potassium and calcium in *Duboisia myoporoides* (A) with MODDE 9.1



Supplementary Material

X. Different climate conditions that were tested in the climate chambers

Order of runs	Light exposure/ day [h]	Light Intensity [$\mu\text{mol}/\text{m}^2\cdot\text{s}$]	Temperature [$^{\circ}\text{C}$]
1	24	50-60	28
1	24	300-350	28
1	24	100-110	24
2	12	50-60	28
2	12	300-350	28
2	12	100-110	24
2	18	100-110	28
3	12	300-350	20
3	24	300-350	20
3	18	50-60	24
3	18	100-110	20
4	12	50-60	20
4	24	50-60	20
4	18	300-350	24
1	18	100-110	24
1	18	100-110	24
2	18	100-110	24
3	18	100-110	24
4	18	100-110	24



Center point

Supplementary Material

XI. Different nutrient settings that were tested in the climate chamber

Exp. number	Nitrogen [mmol]	Potassium [mmol]	Calcium [mmol]
1	0.1	0.05	0.025
2	8	0.05	0.025
3	0.1	4	0.025
4	8	4	0.025
5	0.1	0.05	2
6	8	0.05	2
7	0.1	4	2
8	8	4	2
9	0.1	2	1
10	8	2	1
11	4	0.05	1
12	4	4	1
13	4	2	0.025
14	4	2	2
15	4	2	1
16	4	2	1

Center point

Supplementary Material

XII. Average values and standard deviation for temperature and humidity recorded by data loggers every 10 minutes during the cultivation trial

Run	Cultivation condition	Temperature [°C]	Humidity [%]
1	24 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	24 ± 0.1	61.6 ± 0.8
1	24 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	23.8 ± 0.2	61.2 ± 1.0
1	28° C, 50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 24 h	28.0 ± 0.2	48.7 ± 0.6
1	24 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 24 h	24.1 ± 0.2	60.4 ± 0.7
1	28 °C, 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 24 h	28.0 ± 0.3	48.6 ± 0.7
2	24 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	24.1 ± 0.2	61.2 ± 1.0
2	28 °C, 50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 12 h	27.8 ± 0.5	48.7 ± 1.3
2	24 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 12 h	24.1 ± 0.1	60.5 ± 0.9
2	28 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	28.1 ± 0.2	48.8 ± 1.4
2	28 °C, 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 12 h	27.3 ± 0.6	51.5 ± 1.8
3	20 °C, 50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 24 h	19.8 ± 0.1	66.5 ± 0.8
3	24 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	24.1 ± 0.2	51.1 ± 0.8
3	20 °C, 50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 12 h	20.0 ± 0.2	64.7 ± 0.9
3	24 °C, 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	23.6 ± 0.3	52.2 ± 0.9
4	20 °C, 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 24 h	19.9 ± 0.6	65.4 ± 2.2
4	24 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	23.9 ± 0.3	50.8 ± 1.1
4	20 °C, 300-350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 12 h	19.9 ± 0.3	64.3 ± 1.1
4	20 °C, 100-110 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	20.0 ± 0.2	64.4 ± 0.9
4	24 °C, 50-60 $\mu\text{mol}/\text{m}^2\cdot\text{s}$, 18 h	24.1 ± 0.3	51.7 ± 1.0

V. List of Original Contributions

Peer reviewed articles

Ullrich, S. F., Averesch, N.J.H., Castellanos, L., Choi, Y.H., Rothauer, A., Kayser, O., 2016, Discrimination of wild types and hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii* at different growth stages using ¹H NMR-based metabolite profiling and tropane alkaloids-targeted HPLC-MS analysis, *Phytochemistry* 131, 44-56

Ullrich, S.F., Hagels, H., Kayser, O., 2016, Scopolamine: a journey from the field to clinics. *Phytochem Reviews* (doi:10.1007/s11101-016-9477-x)

Sasse, J., Schlegel, M., Borghi, L., **Ullrich, S.F.**, Lee, M., Liu, G.-W., Giner, J., Kayser, O., Bigler, L., Martinoia, E., Kretzschmar, T., 2016, *Petunia hybrida* PDR2 is Involved in Herbivore Defense by Controlling Steroidal Contents in Trichomes, *Plant Cell & Environment* 39(12), 2725-2739 (doi: 10.1111/pce.12828)

Ullrich, S.F., Rothauer, A., Hagels, H., Kayser, O., 2016, Influence of Light, Temperature and Macronutrients on Growth and Scopolamine Biosynthesis in *Duboisia* species, *Planta Medica* (under Review)

Conference proceedings

Ullrich, S.F., Rothauer, A., Kayser, O., 2016, ¹H NMR-based metabolite profiling of tropane alkaloids in *Duboisia* spec., *Julius-Kühn-Archiv* 453, 79-82

Ullrich, S.F., Hagels, H., Kayser, O., 2014, Einfluss von abiotischen Faktoren auf Wuchs und Scopolamin-Biosynthese in *Duboisia myoporoides*, *Julius-Kühn-Archiv* 446, 31-34

Poster presentations

Kohnen, L., **Ullrich, S.F.**, Averesch, N.J.H., Kayser, O., Principal Studies on Scopolamine Biosynthesis in *Duboisia* spec. for Heterologous Reconstruction of Tropane Alkaloid Biosynthesis, *Metabolic Engineering* 11, 26.-30.06.2016, Kobe, Japan

List of Original Contributions

Oral presentations

Ullrich, S.F., Rothauer, A., Kayser, O., ¹H NMR-based metabolite profiling with special focus on tropane alkaloids in *Duboisia* species, 9th Joint Natural Products Conference (JNPC2016), 24.-27.07.2016, Copenhagen, Denmark

Ullrich, S.F., Rothauer, A., Kayser, O., ¹H NMR-based metabolite profiling of tropane alkaloids in *Duboisia* spec., 6th International Symposium Breeding Research on Medicinal and Aromatic Plants (BREEDMAP 6), 19.-23. 06.2016, Quedlinburg, Germany

Ullrich, S.F., Hagels, H., Kayser, O., Influence of abiotic factors on growth and biosynthesis of secondary plant components in *Duboisia* sp., 2nd DISCO Progress & Review Meeting, 03.-05.11.2015, Brussels, Belgium

Ullrich, S.F., Hagels, H., Kayser, O., Einfluss von abiotischen Faktoren auf Wuchs und Scopolamin-Biosynthese in *Duboisia* sp., 7. Tagung Arznei- und Gewürzpflanzenforschung, 14.- 17.09.2014, Vienna, Austria

VI. Curriculum Vitae

Personal Information

Name: Sophie Friederike Ullrich

Nationality: German

Date of birth: 21st of July 1986

Place of birth: Mainz

Work Experience

Since 10/2016 Quality Assurance Manager, Hexal AG, Holzkirchen, Germany

01/2013 – 07/2016 PhD student, Technische Universität Dortmund, Germany in cooperation with Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany

02/2015 – 07/2015 Manager of Breeding and Cultivation (Maternity-leave replacement), Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany

05/2012 – 10/2012 Pharmacist (practical year), Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany

11/2011 – 04/2012 Pharmacist (practical year), Adler-Apotheke, Mainz, Germany

10/2008 – 02/2009 Graduate assistant, section of pharmaceutical technology, Johannes-Gutenberg-Universität, Mainz, Germany

International Experience

08/2015 University of Leiden, The Netherlands

Research stay

09/2009 – 04/2010 Université de Bourgogne, Dijon, France

Exchange student (Erasmus)

Education

04/2007 – 11/2012 Johannes Gutenberg-Universität, Mainz, Germany

Degree: state examination 1.5 (A) in pharmaceutics

09/1997 – 03/2006 Rabanus-Maurus-Gymnasiums, Mainz, Germany

Degree: Abitur (High school diploma) 1.5 (A)