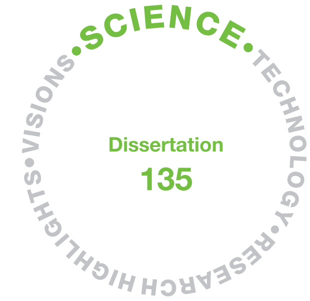


The killer of Socrates exposed – Coniine in the plant kingdom

Hannu Hotti

VTT SCIENCE 135

The killer of Socrates exposed – Coniine in the plant kingdom

Hannu Hotti

VTT Technical Research Centre of Finland Ltd

Doctoral Programme in Plant Sciences

Department of Agricultural Sciences
Faculty of Agriculture and Forestry
University of Helsinki

Thesis for the degree of Doctor of Science (Agriculture and Forestry) to be presented with due permission for public examination and criticism in auditorium 1041 in Biocentre 2 (Viikinkaari 5), at University of Helsinki, on the 14th October 2016 at 12 o'clock.

ISBN 978-951-38-8459-8 (Soft back ed.)
ISBN 978-951-38-8458-1 (URL: http://www.vttresearch.com/impact/publications)

VTT Science 135

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8458-1

Copyright © VTT 2016

JULKAISIJA – UTGIVARE – PUBLISHER

Teknologian tutkimuskeskus VTT Oy
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
Puh. 020 722 111, faksi 020 722 7001

Teknologiska forskningscentralen VTT Ab
PB 1000 (Teknikvägen 4 A, Esbo)
FI-02044 VTT
Tfn +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland Ltd
P.O. Box 1000 (Tekniikantie 4 A, Espoo)
FI-02044 VTT, Finland
Tel. +358 20 722 111, fax +358 20 722 7001

Cover image: Hannu Hotti

Juvenes Print, Tampere 2016

Preface

This work was carried out as a joint Ph.D. project at VTT Technical Research Centre of Finland Ltd and at the Department of Agricultural Sciences, Faculty of Agriculture and Forestry, University of Helsinki during the years 2009-2016. The Plant Biotechnology and Metabolomics research team at VTT provided facilities and expertise in plant and cell culture together with possibilities for the chemical analysis of small molecules. I received support from the Gerbera Laboratory of the Department of Agricultural Sciences in cloning and analysing polyketide synthase genes and their products. The management of VTT is acknowledged for providing excellent working facilities. This study was funded by a Doctoral Program in Plant Sciences, VTT, the Academy of Finland (project number 138808, PKRed), Oskar Öflunds Stiftelse, Eteläsuomalaisten ylioppilaiden säätiö, Societas pro Fauna et Flora Fennica, Otto A. Malmin lahjoitusrahasto and University of Helsinki.

I am grateful to my supervisors, docent Heiko Rischer, docent Kirsi-Marja Oksman-Caldentey and professor Teemu Teeri. Your commitment, advice, and support through these long years enabled me to complete this project. Especially I thank my main supervisor Heiko Rischer, who always had time and advice for my work.

I thank my follow-up committee, whose members alongside my supervisors were Anneli Ritala and Anna Kärkönen. Also, I thank fellow members in the PKRed-project, Suvi Häkkinen, Juha Kontturi and Milla Pietiäinen along with the project leaders, Heiko Rischer, and Teemu Teeri, for monthly meetings to discuss various research topics related to the project.

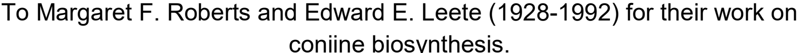
I thank my co-authors Heiko Rischer, Tuulikki Seppänen-Laakso, Gopal Peddinti, Suvi Häkkinen, Philipp Meier, Mikko Arvas and Teemu Teeri. With you, I have learned a lot about academic writing and all that goes with it.

I am grateful for all the advice and expertise I received from Jaana Rikkinen, Tuuli Teikari, and Airi Hyrkäs. My gratitude also goes to Kaj-Roger Hurme in the Isotope Laboratory of the Faculty of Agriculture and Forestry for all things related to radioactive work. I thank Lieven Sterck from VIB for *Conium* transcriptome assembly and Kean-Jin Lim from the Gerbera Laboratory for Trinity-assembly. I thank all the current and past members of the Plant Biotechnology and Metabolomics research group for assistance and friendship.

Words cannot express all the gratitude I feel towards my family for what I have in my heart and my soul. Special thanks go to my parents, Anne and Risto Hotti, who

supported me throughout all these years and had the patience to listen to my reflections on my research. I also thank my dear grandmother, Pirkko Hotti, my sister Heidi, brother-in-law Antti and niece Emilia Manninen. Finally, I thank my aunt Marjaana and uncle Paavo Pelkonen for inspiring me to reach my academic dream.

In Helsinki September, 2016
Hannu Hotti



To Margaret F. Roberts and Edward E. Leete (1928-1992) for their work on coniine biosynthesis.

Vinum poturus rex, memento te bibere sanguinem terra;
cicuta hominum venenum est, cicuta vinum.

When you are about to drink wine, o King, remember that you are about to drink the blood of earth. Hemlock is a poison to man, wine a poison to hemlock.

-Pliny the Elder, 14.5

Quippe videre licet pinguescere saepe cicuta barbigeras pecudes,
homini quae est acre venenum.

Sooth, as one oft many see the bearded goats batten upon hemlock
which to man is violent poison.

-Lucretius, De rerum natura, 5.899-900.

Academic dissertation

Supervisors	Docent Heiko Rischer VTT Technical Research Centre of Finland Ltd Espoo, Finland
	Docent Kirsi-Marja Oksman-Caldentey VTT Technical Research Centre of Finland Ltd Espoo, Finland
	Professor Teemu Teeri Department of Agricultural Sciences University of Helsinki, Helsinki, Finland
Reviewers	Professor Riitta Julkunen-Tiitto Department of Biology University of Eastern Finland, Joensuu, Finland
	Professor Dr. Robert Verpoorte Institute of Biology Leiden University, Leiden, the Netherlands
Opponent	Professor Dr. Heribert Warzecha Department of Biology Technische Universität Darmstadt, Darmstadt, Germany
Custos	Professor Teemu Teeri Department of Agricultural Sciences University of Helsinki, Helsinki, Finland

List of publications

This thesis is based on the following original publications, which are referred to in the text as Articles I–IV. The publications are reproduced with kind permission from the publishers.

I Hotti, H., Seppänen-Laakso, T., Arvas, M., Teeri, T.H. & Rischer, H. 2015. Polyketide synthases from poison hemlock (*Conium maculatum* L.). FEBS Journal 282, 4141-4156. doi: 10.1111/febs.13410.

II Meier, P., Hotti, H. & Rischer, H. 2015. Elicitation of furanocoumarins in poison hemlock (*Conium maculatum* L.) cell culture. Plant Cell, Tissue and Organ Culture 123(3), 443-453. doi: 10.1007/s11240-015-0847-7.

III Hotti, H., Häkkinen, S.T., Seppänen-Laakso, T. & Rischer, H. 2016. Polyketides in *Aloe* plant and cell cultures. Manuscript.

IV Hotti, H., Gopalacharyulu, P., Seppänen-Laakso, T. & Rischer, H. 2016. Metabolite profiling of the carnivorous pitcher plants *Darlingtonia* and *Sarracenia*. Submitted to PLOSONE.

Nota bene: The following results have not been published in the aforementioned articles:

- Transcriptome sequencing of *Conium maculatum*
- Testing of optimal pH for CPKS1 and CPKS5

Author's contributions

I. The author planned the work together with Heiko Rischer and Teemu Teeri. The author carried out the experimental work, except for the phylogenetic tree analysis and analysis of enzyme product structures. The author interpreted the data and had the main responsibility for writing the publication under the supervision of Heiko Rischer and Teemu Teeri.

II. The author planned the work together with Philipp Meier and Heiko Rischer. The author carried out the development of the tested cell line. The author co-authored the publication writing with Philipp Meier under the supervision of Heiko Rischer.

III. The author planned the work together with Heiko Rischer and Suvi Häkkinen. The author carried out the experimental work, except for analysis of the structure of *N*-methylconiine. The author interpreted the data with Suvi Häkkinen and had the main responsibility for writing the publication under the supervision of Heiko Rischer.

IV. The author planned the work together with Gopal Peddinti, Tuulikki Seppänen-Laakso, and Heiko Rischer. The author carried out the experimental work. The author co-authored the publication, writing with Gopal Peddinti under the supervision of Heiko Rischer.

Contents

Preface ... **3**

Academic dissertation ... **6**

List of publications ... **7**

Author's contributions ... **8**

Contents ... **9**

List of abbreviations ... **11**

1. Introduction ... **13**

1.1 Secondary metabolites ... 13
1.1.1 Biosynthetic classification - Polyketides ... 14
1.1.2 Structural classification - Alkaloids ... 16
1.2 Piperidine alkaloids ... 21
1.2.1 Hemlock alkaloids and their chemistry ... 21
1.2.2 Plants containing hemlock alkaloids ... 24
1.2.2.1 Poison hemlock (*Conium maculatum* L.) ... 24
1.2.2.2 *Aloe* sp. ... 27
1.2.2.3 Yellow pitcher plant (*Sarracenia flava* L.) ... 30
1.2.2.4 Other plants possibly containing hemlock alkaloids ... 32
1.2.3 Biosynthesis of hemlock alkaloids ... 33
1.2.4 Biological activity of coniine alkaloids ... 39
1.2.4.1 Medicinal use of poison hemlock and coniine alkaloids ... 41
1.2.4.2 Toxicity to animals ... 42
1.2.4.3 Teratogenicity to animals ... 43
1.2.4.4 Toxicity to humans ... 43
1.2.4.5 Socrates ... 44
1.3 Elicitation ... 46
1.4 Plant cell culture ... 48

2. Aims ... **50**

3. Materials and methods ... **51**

3.1 Transcriptome sequencing ... 52

4. Results **53**
4.1 Polyketide synthases of poison hemlock and *Sarracenia* sp. 53
4.1.1 *Conium* polyketide synthase 1 56
4.1.2 *Conium* polyketide synthase 2 56
4.1.3 *Conium* polyketide synthase 5 57
4.1.4 PKSs of *Sarracenia* sp. 61
4.1.5 Enzymatic testing 61
4.2 Callus induction 67
4.2.1 Poison hemlock 67
4.2.2 *Aloe* sp. 68
4.3 Elicitation of poison hemlock callus 72
4.3.1 Biomass accumulation 72
4.3.2 Elicitation 73
4.4 Micropropagation of *Aloe viguieri* 76
4.5 Alkaloid detection in plant material 78
4.5.1 Alkaloids in *Aloe* sp. 78
4.5.2 Coniine in *Sarracenia* sp. 80

5. Discussion **82**
5.1 Hemlock alkaloid biosynthesis and its relation to polyketide synthases 82
5.2 Callus induction 87
5.2.1 Poison hemlock 87
5.2.2 *Aloe* sp. 88
5.3 Elicitation of poison hemlock cell cultures 88
5.4 Micropropagation of *A. viguieri* 90
5.5 Alkaloid analysis 91

6. Conclusions **94**

References **97**

Appendices

Appendix A: Sequences of short CPKSs
Appendix B: Alignment of PKSs of *Sarracenia* sp.
Appendix C: *In vitro* testing of CPKS1
Appendix D: *In vitro* testing of CPKS2
Appendix E: *In vitro* testing of CPKS5
Appendix F: Mass spectra of elicitated furanocoumarins
Appendix G: Results of *A. viguieri* micropropagation
Appendix H: Selected GC-MS chromatograms of examined plant material
Appendix I: Publications I-IV

Abstract
Tiivistelmä

List of abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2PS	2-Pyrone synthase
AAT	L-Alanine:5-keto-octanal aminotransferase
ACP	Acyl-carrier protein
ACS	Acridone synthase
BA	6-Benzylaminopurine
CHS	Chalcone synthase
CoA	Coenzyme A
CPKS	*Conium* polyketide synthase
CR	γ-Coniceine reductase
CSAM	*S*-Adenosyl-L-methionine:coniine methyltransferase
CTAL	*p*-Coumaryl triacetolactone
DCW	Dry cell weight
EC_{50}	Half maximal effective concentration
FCW	Fresh cell weight
GC-MS	Gas chromatography-mass spectrometry
IC_{50}	Half maximal inhibitory concentration
KIN	Kinetin
LC-MS	Liquid chromatography-mass spectrometry
MS	Murashige and Skoog medium
MW	Molecular weight
NAA	Naphthalene acetic acid
nAChR	Nicotic acetylcholine receptor

OLS	Olivetol synthase
ORF	Open reading frame
PKS	Polyketide synthase
PVP	Polyvinylpyrrolidone
R_t	Retention time
SIM	Selected ion monitoring
STS	Stilbene synthase
TAL	Triacetolactone
TLC	Thin layer chromatography
UPLC	Ultra performance liquid chromatography
VPS	Valerophenone synthase
WP	Woody plant medium

1. Introduction

1.1 Secondary metabolites

Secondary metabolites are compounds which are not directly responsible for the normal growth, development or reproduction of an organism (Fraenkel, 1959; Croteau et al., 2000). These compounds have wide and varied functions, including resistance against herbivores and microbes, and a role as signalling molecules in plant-plant, plant-animal and plant-microbe interactions (Wink, 2010). Humans utilize a number of plant secondary metabolites for various purposes such as spices, perfumes, dyes, poisons, stimulant hallucinogens, medicines and biopesticides (Wink, 2009).

Secondary metabolites can be classified, for example, on the basis of their biosynthetic origin in plants: terpenoids, polyketides, alkaloids, and phenylpropanoids. Alkaloids are a group of secondary metabolites which are nitrogenous organic molecules mainly originating from amino acids including lysine, tyrosine and arginine (Croteau et al., 2000). It has been estimated that a single plant species may produce between 5 000 and 20 000 individual primary and secondary compounds, although many of them are present in the plant only in trace amounts and are often overlooked (Wink, 2010). Estimates of known numbers of secondary metabolites in 2010 are presented in Table 1; some of these estimates have since increased.

Table 1 Numbers of known secondary metabolites from plants (Wink, 2010).

Type of compound	Number[a]
Nitrogen-containing	
Alkaloids	21 000
Non-protein amino acids	700
Amines	100
Cyanogenic glycosides	60
Glucosinolates	100
Alkamides	150
Lectins, peptides, polypeptides	2 000
Without nitrogen	

Monoterpenes (C_{10})[b]	2 500
Sesquiterpenes (C_{15})[b]	5 000
Diterpenes (C_{20})[b]	2 500
Triterpenes, steroids, saponins (C_{30}, C_{27})[b]	5 000
Tetraterpenes (C_{40})[b]	500
Flavonoids, tannins	5 000
Phenylpropanoids, lignin, coumarins, lignans	2 000
Polyacetylenes	1 500
Polyketides	750

[a] Approximate number of known structures.
[b] The total number of terpenoids was over 22 000 in 2010.

1.1.1 Biosynthetic classification - Polyketides

Polyketides as a group are very versatile and include for example chalcones, stilbenes, pyrones and acridones (Figure 1) (Shi et al., 2008). They all have different chemical structures and properties. Common to all polyketides is the use of malonate-derived acetate units in elongation of the polyketide chain, but the starter unit and number of elongations vary. They also share a common intermediate state in which they have multiple keto groups. Polyketides have different functions in plants, *e.g.* flower colour, UV protection, defence against pathogens, interaction with microorganisms and fertility in some plants (Schröder, 1997). Polyketides are used in different medical applications such as anti-inflammatory, anticarcinogenic, antitumour and antibiotic drugs (Shi et al., 2008).

Triacetolactone
Resveratrol
1,3-Dihydroxy-*N*-methylacridone
Naringenin chalcone
Humulone
Δ^1-THC acid A
Xanthone

Figure 1 Some examples of polyketide compounds in plants.

Polyketides are formed by a polyketide synthase (PKS). The enzymes are theorized to be evolutionarily derived from fatty acid synthase (Austin & Noel, 2003). PKSs can be classified into three different groups according to their structure (Flores-Sanchez & Verpoorte, 2009; Shen, 2003). Type I enzymes are modular and have multiple active sites. Each active site makes one assembly or modification of the carbon chain. As a carrier molecule they use acyl carrier protein (ACP), which

also anchors the growing polyketide chain. They are known from fungi and from bacteria. Type II enzymes are multienzyme complexes. They are found from soil-borne and marine Gram-positive actinomycetes. They also use ACP as the carrier molecule.

Type III PKSs have two active sites per dimer and can form homodimers. Their size is 40-45 kDa and they use coenzyme-A (CoA) as carrier molecule instead of ACP. They can use a wide variety of starter molecules and elongate them using acetate from malonate (Figure 2). One of the most common type III PKSs is chalcone synthase, which forms naringenin chalcone in a flavonoid pathway from one *p*-coumaryl-CoA and three malonyl-CoAs (Figure 2A). Other starter units in addition to *p*-coumaryl-CoA include a number of substituted cinnamoyl, benzoyl, and short- and medium-length fatty acyl substrates. Usually, the enzymes make 1-7 condensation reactions with malonyl-CoA. After the elongation reaction, the polymer usually forms a ring structure by heterocyclic lactone formation, or carbon-carbon bonding via intramolecular Claisen-condensation or aldol addition (Austin & Noel, 2003). Hitherto the only known alkaloids formed in the polyketide pathway are acridones (Maier et al., 1993) (Figure 2C) and quinolones (Resmi et al., 2013). Type III PKSs are found in plants, fungi, and bacteria.

A 4-coumaryl-CoA + 3 x malonyl-CoA → (CHS) naringenin chalcone

B acetyl-CoA + 2 x malonyl-CoA → (2PS) TAL

C *N*-methylanthraniloyl-CoA + 3 x malonyl-CoA → (ACS) 1,3-dihydroxy-*N*-methylacridone

D isovaleroyl-CoA + 3 x malonyl-CoA → (VPS) phloroisovalerophenone

Figure 2 Different type III PKS reactions and their products: A chalcone synthase (CHS) and naringenin chalcone (a phloroglucinol), B 2-pyrone synthase (2PS) and triacetolactone (TAL, a pyrone), C acridone synthase (ACS) and 1,3-dihydroxy-*N*-

methylacridone (an alkaloid), and D valerophenone synthase (VPS) and phlorovalerophenone (a phloroglucinol).

1.1.2 Structural classification - Alkaloids

Alkaloids are a structurally diverse group of nitrogen-containing secondary metabolites. They have been identified in plants, especially in angiosperms, in which more than one in five species produces alkaloids.Bacteria, fungi, marine invertebrates, arthropods, frogs and a few birds and mammals also contain alkaloids (Wink, 2007). The role of alkaloids is quite important in plants as they have antibacterial, antifungal, antiviral, anti-herbivory and herbicidal activities (Wink, 2007). These properties and pharmaceutical activities (*e.g.* towards different neuroreceptors) make them important for applications in medicine and biotechnology. Most of them are derived from amino acids as a precursor, such as ornithine, lysine or tryptophan. It has been estimated that there are approximately 21 000 different alkaloids (Wink, 2010). Alkaloids can be classified either by their biosynthetic origin (amino acids, purines, terpenes and polyketides (Roberts, 1998)) or by their structure (Table 2).

Table 2 The classification of alkaloids by their structure according to Hesse (2002).

True alkaloids			
Class	Common structure	Example	Structure
Pyrrolidine derivatives		Hygrine	
Tropane derivatives		Atropine	
Pyrrolizidine derivatives		Retronecine	
Piperidine derivatives		Coniine	
Quinolizidine derivatives		Lupinine	
Indolizidine derivatives		Swainsonine	

Pyridine derivatives		Nicotine	
Isoquinoline derivatives		Papaverine	
Oxazole derivatives		Texamine	
Isoxazole derivatives		Ibotenic acid	
Thiazole derivatives		Nostocyclamide	
Quinazoline derivatives		Febrifugine	
Acridine derivatives		Rutacridone	
Quinoline derivatives		Quinine	

Indole derivatives		Non-isoprene indole alkaloids
		Physostigmine (eserine)
		Semiterpenoid indole alkaloids
		Ergotamine
		Monoterpenoid indole alkaloids
		Vincristine
Imidazole derivatives		Stevensine
Purine derivatives		Caffeine
Protoalkaloids (nitrogen in a side chain)		
β-Phenylethylamine derivatives		Tyramine

Colchicine alkaloids	Colchicine
Muscarine	Muscarine
Benzylamine	Capsaicin
Polyamine alkaloids	
Putrescine derivatives	Paucine
Spermidine derivatives	Lunarine
Spermine derivatives	Verbascine
Peptide (cyclopeptide) alkaloids	
13-Membered cycle	Nummularine P

14-Membered cycle		Scutianine F	
15-Membered cycle		Mucronine A	
Pseudoalkaloids			
Diterpenes		Aconitine	
Steroids		Solanidine	

1.2 Piperidine alkaloids

Piperidine alkaloids (Figure 3) contain a six-membered saturated heterocyclic ring, *i.e.* piperidine nucleus, in their structure. Robinson (1917) suggested that piperidine alkaloid biosynthesis in plants starts from the amino acid lysine. This has later been expanded by recognising that besides lysine, the carbon backbone of piperidine can also originate from acetate, mevalonate or monoterpenes (*e.g.* alkaloid skytanthine) (Panter & Keeler, 1989). Piperidine alkaloids are found in many genera, for example, *Nicotiana*, *Conium*, *Lobelia*, *Pinus*, *Punica*, *Duboisia*, *Sedum*, *Withania*, *Carica*, *Hydrangea*, *Dichroa*, *Cassia*, *Prosopis*, *Genista*, *Ammodendron*, *Lupinus*, *Liparia* and *Collidium* (Panter et al., 1988a). Fire ants (*Solenopsis* sp.), as an example outside the plant kingdom, also contain simple piperidine alkaloids (Jones et al., 1982). Over 700 members of this alkaloid class are currently known (Verpoorte et al., 1991).

Coniine — *Conium maculatum*
Pipecolic acid
Pinidine — *Pinus* spp.
Swainsonine — *Swainsona* spp.
Euphococcine — *Euphorbia atoto*
Sedamine — *Sedum* spp.
Lobeline — *Lobelia* spp.
Solenopsin — Fire ant (*Solenopsis* spp.)

Figure 3 Some piperidine alkaloids found in plants and arthropods. They are structurally similar but are formed in different biosynthetic pathways.

Chemical synthesis of piperidines, *e.g.* of coniine, is a much-researched area and methods include Mannich-type reaction, Michael addition, ring-closing metathesis, iminium ion cyclization, Diels-Alder reaction and Pd^{II}-catalyzed reaction of nitrogen nucleophile (Hande et al., 2008). Other ways to synthesize these compounds include intramolecular N-C bond formation, C-C bond formation, intermolecular reactions and modification of already available six-membered nitrogen heterocycles (Denić et al., 2013).

1.2.1 Hemlock alkaloids and their chemistry

Currently, there are thirteen known hemlock alkaloids (Figure 4), which can be classified according to how many carbon atoms they have in their backbone. The most simple class, C_6, has only one member, 6-methylpiperidine. The C_8-class has nine confirmed and two theoretical members. The C_{10}-class is the most recent in hemlock alkaloids, as its first members were discovered in 1997. In the

literature, there are hints that more alkaloids could be found. In the following is a detailed description of each known and two theoretical hemlock alkaloids.

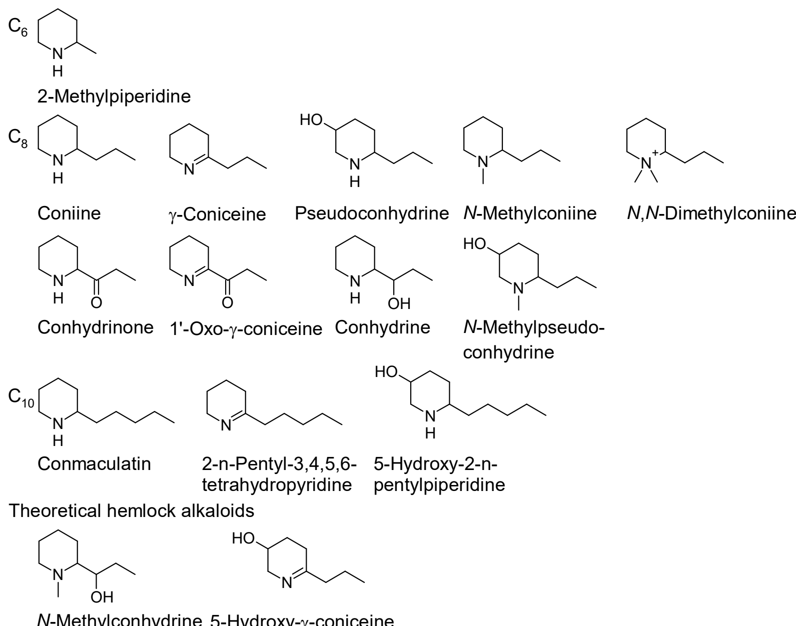


Figure 4 Classification of naturally occurring poison hemlock (*Conium maculatum* L.), *Sarracenia flava* L. and *Aloe* spp. alkaloids according to their carbon number. Theoretical hemlock alkaloids are *N*-methylconhydrine (Roberts, 1985) and 5-hydroxy-γ-coniceine (Leete & Adityachaudhury, 1967).

2-Methylpiperidine. $C_6H_{13}N$. MW 99.17 g/mol. CAS number 109-05-7. Also known as 2-pipecoline, α-pipecoline, α-methylpiperidine. The boiling point is 120°C (Büchel & Korte, 1962) and the melting point is -4°C (Bradley et al., 2014). 2-Methylpiperidine is an alkaloid found from poison hemlock (*Conium maculatum* L.) (Cromwell, 1956; Holstege et al., 1996).

Coniine. $C_8H_{17}N$. MW 127.23 g/mol. CAS number 458-88-8. Also known as 2-propylpiperidine, cicutine, conicine. The boiling point is 166°C and the melting point -2°C (Reynolds, 2005). Coniine was first isolated from poison hemlock by Giseke (1826). Its structure was established by Hofmann (1881) and it was synthesized by Ladenburg (1886), thus making it the first alkaloid to be formulated and synthesized. Coniine has a stereocenter at position C-2, leading to two stereoisomers, (*S*) and (*R*), of which the former is the naturally occurring (Reynolds, 2005).

γ-Coniceine. $C_8H_{15}N$. MW 125.21 g/mol. CAS number 1604-01-9. Also known as 2-propyl-1,4,5,6-tetrahydropyridine. The boiling point is 171°C (Reynolds, 2005)

and the predicted melting point is -4.56°C (US EPA, 2016). γ-Coniceine was isolated from poison hemlock by Wolffenstein (1895). He also determined the chemical formula, but the structure was determined as late as 1961 by Beyerman et al. by hydrogen nuclear magnetic resonance and infrared. The alkaloid was first synthesized by Gabriel (1909). γ-Coniceine is the principal alkaloid in leaves and young tissues of poison hemlock (Cromwell, 1956).

Pseudoconhydrine. $C_8H_{17}NO$. MW 143.23 g/mol. CAS number 140-55-6. Also known as 6-propyl-3-piperidinol, Ψ-conhydrine. The predicted boiling point is 246°C (US EPA, 2016) and the melting point is 105°C (Reynolds, 2005). Pseudoconhydrine was isolated from poison hemlock by Merck (1891). Its structure was finally determined by Yanai & Lipscomb (1959). It is an isomer of conhydrine with a hydroxyl group at C-5. This alkaloid is usually present in poison hemlock as a minor alkaloid but in one American strain it is reported to be a major component and it originates from γ-coniceine (Leete & Adityachaudhury, 1967).

***N*-Methylconiine**. $C_9H_{19}N$. MW 141.25 g/mol. CAS number 35305-13-6. Also known as 1-methyl-2-propylpiperidine, methylconiine. The predicted melting and boiling points are -6.2°C and 177.6°C, respectively (US EPA, 2016). It was first synthesized from coniine and its formula was determined by von Planta & Kekulé (1854). The alkaloid's structure was determined by Wolffenstein (1894) from poison hemlock extract.

***N,N*-Dimethylconiine**. $C_{10}H_{22}N$. MW 156.24 g/mol. CAS number 329270-32-8. Also known as 1,1-dimethyl-2-propylpiperidinium. The predicted melting and boiling points are 144.4°C and 383.2°C, respectively (US EPA, 2016). It was isolated from *Aloe sabaea* (Blitzke et al., 2000).

Conhydrinone. $C_8H_{15}NO$. MW 141.21 g/mol. CAS number 97073-23-9. Also known as 1-(2-piperidynyl)-1-propanone. The predicted melting point is 34.9°C (US EPA, 2106) and the boiling point is 94°C (Hess & Eichel, 1918). It was isolated from poison hemlock by Leete & Olson (1972). They also showed that biosynthetically conhydrinone originates from γ-coniceine.

1'-Oxo-γ-coniceine. $C_8H_{13}NO$. MW 139.19 g/mol. CAS number 80933-75-1. Also known as 1-(3,4,5,6-Tetrahydro-2-pyridinyl)-1-propanone. The predicted melting and boiling points are 24.9°C and 213°C, respectively (US EPA, 2016). It was found from poison hemlock by Holstege et al. (1996).

Conhydrine.$C_8H_{17}NO$. MW 143.23 g/mol. CAS number 495-20-5. Also known as 2-(1-hydroxypropyl)piperidine, α-ethyl-2-piperidinemethanol, 2-(α-hydroxypropyl)-piperidine. The melting point is 121°C (Reynolds, 2005) and the predicted boiling point is 241°C (US EPA, 2016). It was found from poison hemlock in (+) form by Wertheim (1856).

***N*-Methylpseudoconhydrine**. $C_9H_{19}NO$. MW 157.25 g/mol. CAS number 78962-69-3. Also known as 1-methyl-6-propyl-3-piperidinol. The melting point is 157°C (Roberts & Brown, 1981) and the predicted boiling point is 238.6°C (US EPA, 2016). The alkaloid was found in a South-African yellow-flowered *Conium* sp. (Roberts & Brown, 1981).

Conmaculatin. $C_{10}H_{21}N$. MW 155.28 g/mol. CAS number 33354-97-1. Also known as 2-pentylpiperidine. The predicted melting point is 30°C (US EPA, 2016) and the boiling point is 207-208°C (Wegler & Pieper, 1950). The alkaloid was first found from Serbian poison hemlock (Radulović et al., 2012).

2-n-Pentyl-3,4,5,6-tetrahydropyridine. $C_{10}H_{19}N$. MW 153.26 g/mol. CAS 5832-23-5. The predicted melting and boiling points are 8.4°C and 212°C, respectively (US EPA, 2016). The alkaloid was discovered from poison hemlock (Lang & Smith, 1997). The compound has also been found from fire ant (*Solenopsis*) species (Jones et al., 1982).

5-Hydroxy-2-n-pentylpiperidine. $C_{10}H_{21}NO$. MW 171.28 g/mol. CAS number 220088-35-7. Also known as 6-pentyl-3-piperidinol. The predicted melting and boiling points are 66°C and 280°C, respectively (US EPA, 2016). It was found in poison hemlock (Lang & Smith, 1997).

Other possible hemlock alkaloids. Research performed by Cromwell (1956) and Fairbairn & Suwal (1961) indicated that poison hemlock has alkaloids which have larger molecular size and are more polar. Castells et al. (2005) found an unknown alkaloid from poison hemlock. Its MS fragmentation pattern (*m/z*: 125 (M^+, 1), 124 (6), 110 (18), 97 (100), 96 (31), 82 (9), 69 (7), 55 (15)) is similar to that of γ-coniceine, thus making it a possible γ-coniceine isomer, but the location of the double bond is unknown. Roberts (1985) postulated the possible existence of *N*-methylconhydrine (Figure 4) in poison hemlock after a series of enzymatic studies. Leete & Adityachaudhury (1967) theorized that 5-hydroxy-γ-coniceine could be an intermediate product in the biosynthesis route of pseudoconhydrine from γ-coniceine.

1.2.2 Plants containing hemlock alkaloids

1.2.2.1 Poison hemlock (*Conium maculatum* L.)

Poison hemlock (*C. maculatum*) belongs to the family Apiaceae, which comprises 434 genera and 3780 species depending on classification (Stevens, 2013). The genus *Conium* has one to four members, of which three are South African (The Plant List, 2015). Poison hemlock is native to Europe, northern Africa, and western Asia. It has also been introduced to North America, South America, Australia and New Zealand (Holm et al., 1997). In southern Finland it is rare and in northern

Finland up to the Oulu region only occasional. Probably it has been continually re-introduced along with cereals, ley seed and ballast until the middle of 20th century (Jonsell & Karlsson, 2010).

The old Roman name for poison hemlock was *cicuta*, which is used nowadays for water hemlock (*Cicuta virosa* L.). The modern name comes from the Greek word 'Konas', to whirl about due to consumption caused by ataxia, tremor, and convulsions. The Latin word *maculatum* means spotted, and indeed the plant has reddish spots on the stem and the leaf stalk. Hemlock has been written hemlic/hymelic (Anglo-Saxon), hymlice, hymlic, hemeluc, hemlake, hemlocke and hemlock (first used in William Shakespeare's play 'Life of Henry the Fifth') (Vetter, 2004). Common names for the plant include poison parsley, carrot fern, conium, spotted hemlock, spotted cowbane, spotted corobane, carrot weed, California or Nebraska fern, musquash root and poison root (de Landoni, 1990; Drummer et al., 1995; Mitich, 1998; USDA Forest Service, 2015).

Poison hemlock (2n = 22) is a herbaceous biennial plant (Figure 5), but it can also be a winter annual or a short-lived perennial. The stem (Figure 5A) is stout and can achieve a height of 1-2 m, in the Nordic countries 1.2-1.5 m. It is straight, branched mainly in the upper part, its texture is smooth and coloured pale green with purple spots. The basal part is 4-8 mm thick and terete or angled. The cross section of the stem is hollow or rarely solid, except at the nodes where it has fine, shallow, longitudinal ridges. The inflorescences (Figure 5B) are large, compound umbels which are open and 4-6 cm across. The terminal umbel, which is situated at the top, blooms first. The flowers are small in large, loose clusters with a circle of narrow bracts at the base. The poison hemlock has five petals, which are white, incurved and devoid of sepals. The bracts are involucre lanceolate, acuminate and inconspicuous. The fruit is a schizocarp, oval to circular in outline and forms a compound of two grey or brown mericarps (seeds). The seeds are narrowly ovate, 1.2-2 mm wide, 2-3 mm long, with a slightly extended apex, the dorsal side strongly convex with 5 prominent wave ridges from top to bottom and they weight about 0.5 mg. Its taproot (Figure 5C) is long, 5-11 mm thick, whitish and usually unbranched. The leaves (Figure 5D) are present as a rosette from the crown and alternately on the stem (Figure 5E). They are 20-40 cm long with 3 or 4 times pinnate leaf compounds and their segments are toothed or deeply cut. The base of the petiole tends to sheath the stem and is shorter on upper leaves (Holm et al., 1997; Jonsell & Karlsson, 2010).

Figure 5 A Flowering poison hemlock, **B** flowers and developing seeds, **C** taproot, **D** leaf and **E** rosette (photos by H. Hotti).

All tissues of poison hemlock contain alkaloids in abundance (Corsi & Biasci, 1998). The main alkaloid of flower buds and flowers is γ-coniceine, which is transformed during the fruit development into coniine and further into *N*-methylconiine, which are the main alkaloids in mature fruit (Cromwell, 1956). The volatile alkaloids in flowers may attract pollinators (Roberts, 1998), especially frequently Diptera (Nitao, 1987).

There is a "coniine layer" in fruit which starts to develop during week 3 (after fertilization); by week 7 the walls are completely thickened. During week 3 the fruits also contain the maximum amount of alkaloids, which can be as much as 3% of dry weight (Fairbairn & Challen, 1959; Corsi & Biasci, 1998). The role of alkaloids in fruits may be related to protection of seeds before germination, as the coniine layer is lost during germination (Roberts, 1998). There are secretory structures, called vittae, in seedlings, vegetative organs, flowers and fruits, which are possible sites for alkaloid biosynthesis (Corsi & Biasci, 1998).

Poison hemlock is a cross-breeding species (Gulezian et al., 2012). The plant is a prolific seed producer with 1700-39000 seeds per plant and can dominate small areas if left unchecked, at the same time creating a seed bank (Panter et al., 1988b; Baskin & Baskin, 1990). The seed dispersal occurs from mid-September to late February, as the stalks persist through winter, and by late December 95% of the seeds have been dispersed (Baskin & Baskin, 1990; Pokorny & Sheley, 2012).

Its seeds do not have a dormancy restriction, which allows them to germinate as soon as the prevailing conditions permit. Depending on the year, 40-85% of seeds will germinate when there is a suitable temperature difference. Generally, poison hemlock seeds remain viable for 3-6 years (Baskin & Baskin, 1990; USDA Forest Service, 2015).

Poison hemlock belongs to a group of widely spread weeds (Vetter, 2004). It is a pioneer plant and thus an invasive species (USDA Forest Service, 2015). The plant's invasiveness is due to its ability to grow in very different locations, such as in cultivated fields, waste areas, along ditch banks or fences, around barnyards, waterways and along roadsides (Smith et al., 1984; Panter et al., 1988b). The general habitat is moderately dry, mull-rich and nutrient-rich bare soil with good moisture in the light-open area (Panter et al., 1988b; Jonsell & Karlsson, 2010). The plant tolerates a high level of heavy metals (arsenic, cadmium, lead) in soil (Gulezian et al., 2012).

Poison hemlock can contaminate harvested hay, as it grows in alfalfa fields. It also grows in grass pastures and meadows, causing a risk of poisoning to grazing livestock, especially in the spring when there is little else to eat and poison hemlock is one of the first plants to emerge (in the USA) (Panter et al., 1988b). Thus, poison hemlock is part of the overall problem caused by introduced weeds, which result in about $1-2 billion/year losses in forage crops in the USA (DiTomaso, 2000; Pimental et al., 2005).

Parts of poison hemlock have been confused with many edible Apiaceae-plants; its leaves with parsley (*Petroselinum crispum* (Mill.) Fuss), roots with parsnip (*Pastinaca sativa* L.) and seeds with anise (*Pimpinella anisum* L.) (Smith et al., 1984; Krezelok et al., 1996; Vetter, 2004). Other mix-ups are with *e.g. Chaerophyllum* sp., *Anthriscus sylvestris* (L.) Hoffm and *Aethusa cynapium* L. (Jonsell & Karlsson, 2010).

Poison hemlock should be eradicated as soon as it is noticed. A 3-6 year approach is needed, as the plant stand cannot be effectively controlled in one year (USDA Forest Service, 2015). For chemical control of poison hemlock, suitable compounds include hexazinone (active over 90%); metribuzin (over 90%); terbacil (over 90%); glyphosate at 1.1 kg/ha (97-98%); and 2,4-dichlorophenoxyacetic acid (2,4-D) at 1.1 kg/ha (97-98%) (Smith et al., 1984; Jeffery & Robinson, 1990; Woodard, 2008; USDA Forest Service, 2015). The spraying should be performed when seedlings emerge or before the flowering stalk begins to develop in order to stop the invasion (Smith et al., 1984). Physical control should be applied before the plants produce seeds. Manual methods include hoeing, digging, cutting and grubbing. Recommended mechanical methods are mowing and tillage. For biological control, the hemlock moth (*Agonopterix alstroemeriana* Clerk) may be used (USDA Forest Service, 2015).

1.2.2.2 *Aloe* sp.

The genus *Aloe* is a member of the family Xanthorrhoeaceae (the Angiosperm Phylogeny Group, 2003). About 400 species comprise the genus, which is native

to most of Africa south of the Sahara Desert, the Arabian Peninsula, Madagascar and several smaller islands in the Indian Ocean (Figure 6) (Viljoen et al., 1998). The general feature of the habitat of *Aloe* is an arid or semi-arid region, more specifically dry forests, open woodland, grassland, bare rock surfaces and cliff faces (Carter et al., 2011). Humans have used aloes for several millennia at least, as *A. vera* (L.) Burm.f. was used as a medicine in China and India about 2 400 years ago (Rowley, 1997). Nowadays they are mainly ornamental plants in warm climates. In traditional medicine, the aloes have a role in treating burns, skin disorders and as a purgative. In cosmetics, extract of aloe is used *e.g.* in soaps. In many African cultures cut aloes are dried and used as such in dyes. Poisonous *A. ruspoliana* Baker is used to kill hyenas in eastern Africa.

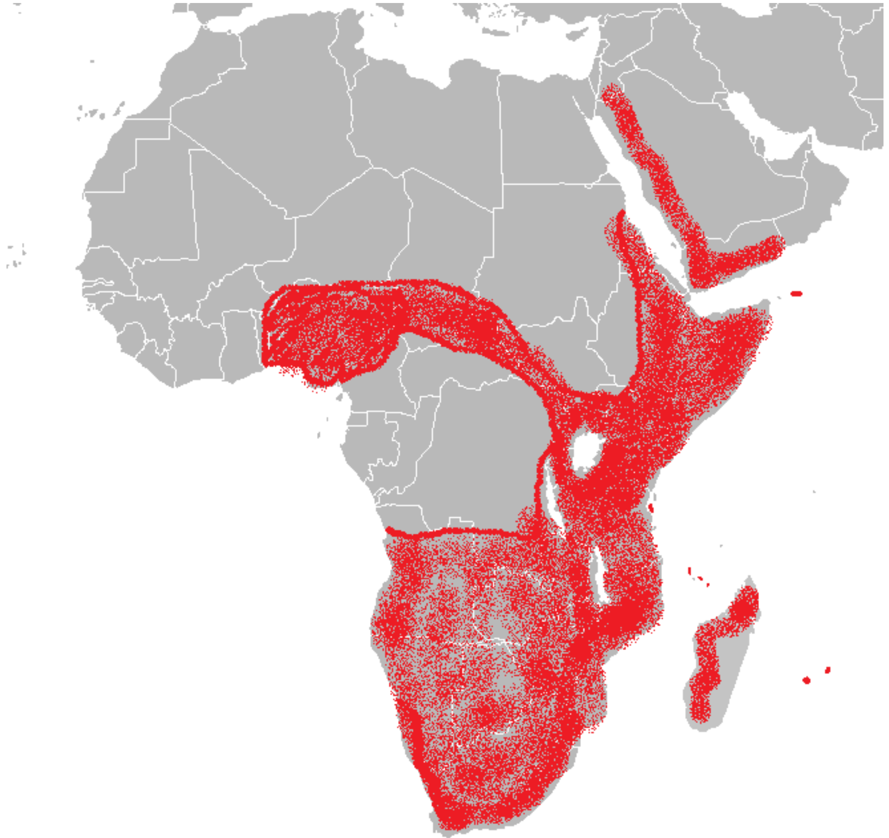


Figure 6 Distribution of the genus *Aloe* (red) according to Carter et al. (2011).

Aloe species are rich in secondary compounds. These include various chromones, anthraquinones, anthrones, coumarins, pyrones, flavonoids and sterols (Dagne et

al., 2000). Very few alkaloids have been described from the genus, namely the purines hypoxanthine and xanthine from *A. ferox* Mill. (Loots et al., 2007), and the tyramine-derivated phenylethylamines in 18 *Aloe* species (Nash et al., 1992). Piperidine alkaloids are present in twelve species (Table 3) (Dring et al., 1984; Nash et al., 1992; Blitzke et al., 2000).

Table 3 Hemlock alkaloids reported from *Aloe* (Dring et al., 1984; Nash et al., 1992; Blitzke et al., 2000).

Aloe species	Alkaloids
A. ballyii Reynolds	γ-coniceine, conhydrinone
A. deltoideodonta Baker	γ-coniceine, a trace of pseudo-conhydrine
A. descoingsii Reynolds	coniine, conhydrine
A. gariepensis Pillans	γ-coniceine, conhydrine
A. globuligemma Pole Evans	coniine, conhydrine
A. gracilicaulis Reynolds & P.R.O. Bally	γ-coniceine
A. ibitiensis Perrier	γ-coniceine
A. krapholiana Marloth.	coniine, conhydrine
A. ortholopha Christian & Milne-Redh.	coniine, conhydrine
A. ruspoliana Baker	γ-coniceine
A. sabaea Schweinf. (syn. *A. gillilandii* Reynolds)	γ-coniceine, coniine, *N,N*-dimethylconiine
A. viguieri Perrier	coniine, traces of γ-coniceine

Aloe gariepensis Pillans is stemless or has a short stem which is about 50 cm high and is covered with dried leaves. The stems grow alone or sometimes branched, forming small clusters. Leaves form a dense rosette, and their shape is from erect to slightly curved, lance-shaped and they are 30-40 × 5-8 cm. The leaves are coloured from dull green to reddish brown, and they have depressed lines and copiously white spots when they are young (Figure 7A). When the leaves become mature they have a few white spots on the top of the leaf. The leaf margins are horny, with sharp reddish-brown teeth 2-3 mm long and about 1 cm apart. The aloe grows the valley of the Orange River, formerly known as the Gripe River, which is the border between Namibia and the Northern Cape Province of South Africa. There it grows on steep rocky slopes and crevices at altitudes of 150-800 m (Carter et al., 2011).

Aloe globuligemma Pole Evans has creeping, rooting stems, which are up to 50 cm long with a tendency to form dense groups. From one stem grows about 20 leaves, which are lance-like, erectly spreading and curved towards the centre. Leaf size is 45-60 × 8-11 cm and the leaves are coloured bluish green (Figure 7B). The leaf margins have hard, pinkish, brown-tipped teeth, which are 2 mm long and 1 cm apart from each other. This aloe is widespread from near Steelpoort, in the Lydenburg District of South Africa, extending northward to Zimbabwe, where it is plentiful in the Sabi Valley near the border with Mozambique, and to the west

around Plumtree and just into Botswana. The plant favours undulating stretches of bushveld with deep sandy soils that may be rich in heavy metals (Carter et al., 2011). *A. globuligemma*, used as a herbal medicine, has caused deaths in rural Africa (Drummond et al., 1975). Parry and Matambo (1992) studied the toxicity of *A. globuligemma* as it is used as a traditional herbal medicine in Zimbabwe. Its crude extract has LD_{50} < 250 mg/kg on mice and the toxic symptoms were reported to be similar to those of poison hemlock poisoning.

Aloe viguieri Perrier grows solitary or forms small groups. The stem is slender and can grow up to 1 m long, often being pendulous. The stem has 12-16 leaves, which are shaped lanceolate-attenuate. The leaf is 30-40 × 8-9 cm and 1 cm thick. The leaf colour is light green or greyish, with linear bands on the surface (Figure 7C). The leaf margins have a distinct, white, cartilaginous border and minute, white, horny close-set teeth, which are about 0.5 mm long and 1-2 mm apart from each other. This aloe grows in the Toliara Province of Madagascar. The plant is restricted to the coastal belt between the Onilahy and Fiherenana Rivers and occurs along the river valleys for some distance inland, growing on steep banks or cliffs of Eocene limestone (Carter et al., 2011).

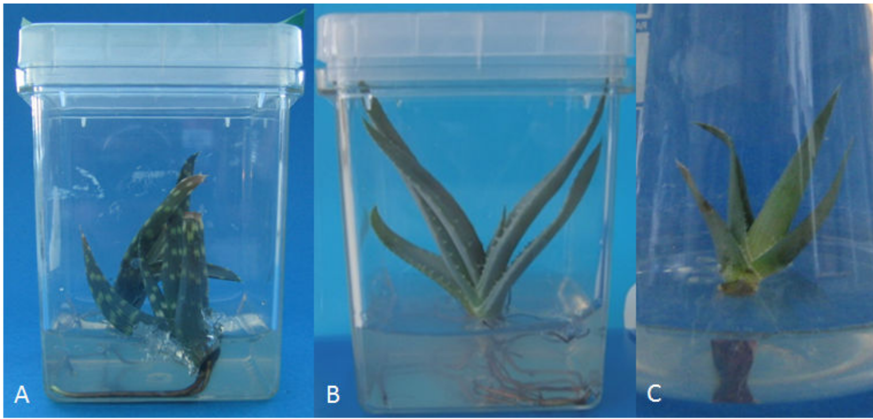


Figure 7 **A** *A. gariepensis*, **B** *A. globuligemma* and **C** *A. viguieri* growing as *in vitro* plants (Article III).

1.2.2.3 Yellow pitcher plant (*Sarracenia flava* L.)

Sarraceniaceae is a New World carnivorous plant family. It includes three genera, *Darlingtonia*, *Heliamphora,* and *Sarracenia.* In *Sarracenia,* there are 8-11 species depending on the classification (McPherson, 2006; Ellison et al., 2012) (Figure 8). The native range of *Sarracenia* is the eastern seaboard of North America. The main feature of their native habitat is that it is nutrient-poor, in an acidic and wet environment comprising swamps, fens and grassy plains. All *Sarracenia* species are insectivorous *i.e.* they attract, capture, and digest insects to supplement their nutrient uptake. A common feature of all *Sarracenia* is that they lure insects to

their elongated tubular leaves. *S. psittacina* Michx. hides the entry/exit hole using multiple translucent false entries so that trapped insects finally tire and fall to their death. Other *Sarracenia* species utilize downward pointing hairs and waxy substances in their pitchers to trap insects.

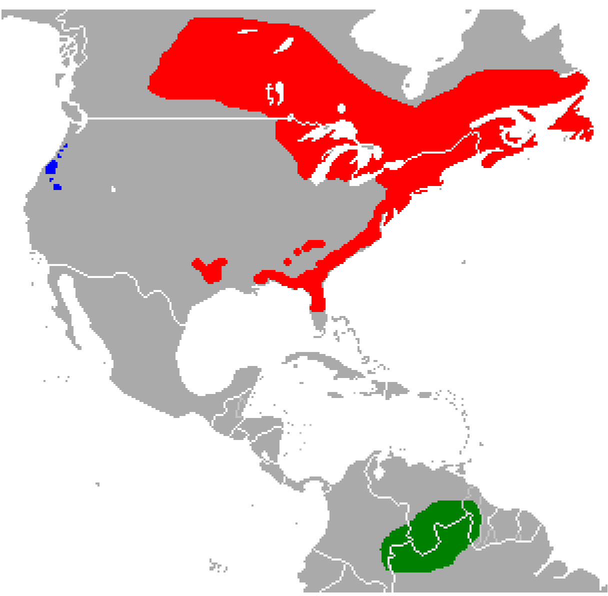


Figure 8 Distribution map of *Sarracenia* (red), *Heliamphora* (green), and *Darlingtonia* (blue).

The yellow pitcher plant *(Sarracenia flava L.)* (Figure 9) is a carnivorous plant which feeds on insects. *S. flava*'s native range is an arc from south-eastern Virginia across the Atlantic and Gulf coastal plains of the Carolinas, Georgia, western Florida and southeastern Alabama. The habitat is marshy savannah, wetter seeps, shallow standing water, sphagnous seep bogs, open-canopy boggy pine forest and marl fens. The recognised varieties of *S. flava* are *S. flava* var. *flava, S. flava* var. *atropurpurea, S. flava* var. *cuprea, S. flava* var. *maxima, S. flava* var. *ornata, S. flava* var. *rubricorpora* and *S. flava* var. *rugelii.* The summer leaves are pitchers up to 120 cm tall with mouths up to 12 cm wide, and have a narrow, flattened border. The lid is mounted on an erect column with markedly recurved margins to more than halfway to midline, near circular when viewed from the top and up to 20 cm across. The tip of the lid is filamentous, sharply pointed with no keel. The colouring of the pitcher ranges from yellowish green to yellow and to deep red depending on the variety. The winter leaves are called phyllodia; they are sword-shaped, straight and 20-40 cm long. The robust rhizome grows parallel to the surface of the ground. The flower scapes appear before the pitchers in the spring, and are slightly shorter than mature pitchers. The flower is 4-7 cm in diameter. The petals are strap-shaped with round ends, 3-5 cm long and coloured

yellow. The sepals are yellowish green. The pistil is umbrella-shaped and yellow-yellowish green. The flowers have a strong, musty odour resembling feline urine. The mature seed capsule is up to 2 cm in length. The flowering time is in the native range from mid-March to early May (Schnell, 2002; McPherson, 2006). *S. flava* is reported to contain coniine (Mody et al., 1976). Mody et al. (1976) tested isolated coniine with fire ants and found that the compound paralysed them. Harborne (1982) suggested that coniine not only paralyses insects but also entices them into the pitcher.

Figure 9 The yellow pitcher plant (*S. flava*) (photo by H. Hotti).

1.2.2.4 Other plants possibly containing hemlock alkaloids

There are other plants which on the basis of their mousy smell are speculated to contain hemlock alkaloids, but no detailed investigations have been made. Such plants include upright spurge (*Euphorbia stricta* L.) and crown imperial (*Fritillaria imperialis* L.), both of which have a reportedly mousy smell when crushed (Reynolds, 2005). *Fritillaria* spp. also contain alkaloids, but they are steroid-based (Li et al., 2001). Hébert & Haim (1898) speculated that different aroids (*Amorphophallus* sp., *Arisarum* sp., *Arum* sp., *Caladium* sp.) could contain coniine

alkaloids. Raffauf (1970) continued this list with the genera *Sarcolobus, Punica,* and *Parietaria*. Power & Tutin (1905) examined fool's parsley (*Aethusa cynapium*) and reported it to contain coniine or a related alkaloid. Later research has shown that the then identified compound is aethusin (cynapine), a polyacetate (Teuscher et al., 1990).

Semnostachya menglaensis Tsui (Apocynaceae), a rare plant from the Yunnan province of China, has 1'-oxo-γ-coniceine as a major component and conhydrinone in its volatile oil. The plant contains four other simple alkaloids (Figure 10) (Naef et al., 2005). Bermuda grass (*Cynodon dactylon* (L.) Pers.), Poaceae, is known to contain conhydrine (Jananie et al., 2012). There is another report of the presence of the same alkaloid in lemon balm (*Melissa officinalis* L.), Lamiaceae (Pereira et al., 2014). *Pimpinella acuminate* (Edgeworth) C. B. Clarke has been reported to contain coniine, *N*-methylconiine, 1-methyl-2-butylpiperidine and 1-methyl-2-pentylpiperidine (Ashraf et al., 1979).

1-(3,4,5,6-Tetrahydropyridin-2-yl)-ethanone
1-(1,4,5,6-Tetrahydropyridin-2-yl)-ethanone
1-(3,4,5,6-Tetrahydropyridin-2-yl)-propan-1-one
1-(1,4,5,6-Tetrahydropyridin-2-yl)-propan-1-one
Conhydrinone
1-(Pyridin-2-yl)-propan-1-one

Figure 10 Alkaloids of *Semnostachya menglaensis* (Naef et al., 2005).

1.2.3 Biosynthesis of hemlock alkaloids

Poison hemlock is the only plant species in which hemlock alkaloid biosynthesis has been studied. Leete (1963) fed uniformly labelled $[^{14}C]$-L-lysine, $[2-^{14}C]$-DL-lysine and $[1,5-^{14}C]$-cadaverine to poison hemlock. These feedings resulted in negligible activity in the alkaloids. Cromwell & Roberts (1964) fed to poison hemlock uniformly labelled $[^{14}C]$-L-lysine, $[^{14}C]$-Δ^1-piperideine and $[^{14}C]$-Δ^1-piperideine-2-carboxylic acid together with $[6-^{14}C]$-DL-α-aminoadipic acid, $[1,5-^{14}C]$-cadaverine and $[2-^{14}C]$-propionate, which were incorporated into γ-coniceine. The hypothesis of coniine biosynthesis based on these results is summarised in Figure 11. Leete (1963 & 1964) theorized on the basis of his results obtained with uniformly labelled $[^{14}C]$-lysine that lysine underwent metabolism in the plant, finally ending up as acetate, which was incorporated into γ-coniceine and then into coniine. However, because lysine was not incorporated directly into coniine, Leete (1963 & 1964) proposed an alternative hypothesis that acetate, rather than lysine, could function as the carbon source for coniine.

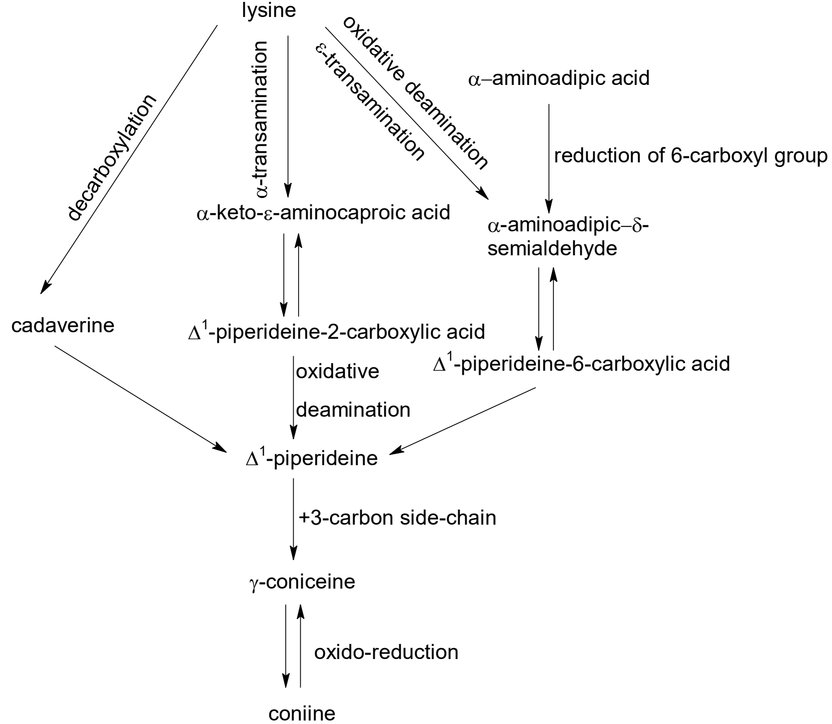


Figure 11 Biosynthesis of coniine from lysine according to Cromwell & Roberts (1964).

Leete (1963 & 1964) (Figure 12) fed poison hemlock plants sodium [1-^{14}C]-acetate and the activity from labelled acetate was detected in the carbons of coniine and conhydrine. Via systematic degradation it was established that almost all the activity was in the even-numbered carbons and equally distributed between these positions (C-2 26%, C-3 1.6%, C-4 22%, C-5 1%, C-6 24%, C-1' 1.3%, C-2' 22%, C-3' 1.6%). He postulated that the possible formation route could be from one acetyl-CoA and three malonyl-CoAs. Thus, the carbon backbone of coniine would originate from acetates via the intermediary of a poly-β-keto acid (polyketide) (Leete, 1963 & 1964; Roberts, 1998; Reynolds, 2005).

4 x acetate → [poly-β-keto acid intermediate] → coniine

Figure 12 Poison hemlock plants were fed sodium [1-^{14}C]-acetate and the radioactive carbon (blue dots) was found in the even numbered carbons of coniine (Leete, 1963 & 1964).

Leete (1970) fed poison hemlock plants labelled sodium [1-^{14}C]- and [2-^{14}C]-octanoate. The plants were harvested after seven days and the labelled carbon was found to be present in γ-coniceine. He postulated that octanoic acid is activated to 5-keto-octanoic acid and/or cleaved to acetyl-CoA via the Krebs cycle and processed further into γ-coniceine. In other words, octanoic acid is not used directly as such but via oxidation. Leete & Olson (1970) fed plants sodium [1-^{14}C]-acetate, [1-^{14}C]-octanoic acid, [6-^{14}C]-5-keto-octanoic acid and [6-^{14}C]-5-keto-octanal, and the plants were harvested after 24 h. The incorporation rates were for [1-^{14}C]-acetate 0.009%, for [1-^{14}C]-octanoic acid 0.07%, for [6-^{14}C]-5-keto-octanoic acid 0.61% and for [6-^{14}C]-5-keto-octanal 1.1%. They noticed that octanoic acid is not the preferred precursor for the alkaloids as it has a poor incorporation rate. The compound could lead to the formation of 5-keto-octanoid acid, which is probably a step before alkaloid formation, as its activity was in the carbon C-1' of formed alkaloids. Leete & Olson (1972) theorized that 5-keto-octanal and 5-keto-octanoic acid are the most probable precursors for alkaloid biosynthesis. They hypothesized that 5-keto-octanal goes to transamination to form γ-coniceine. Roberts (1998) proposed that the key to hemlock alkaloid production could be the availability of 5-keto-octanal. Pineapple (*Ananas comosus* (L.) Merr.) uses 5-keto-octanoic acid in the biosynthesis of δ-octalactone (Figure 13A) (Engel et al., 1989). Leete (1971) fed [1-^{14}C]-hexanoic acid to poison hemlock, by which it was incorporated to coniine at only a poor rate. He noted that chain elongation of hexanoic acid is possible in the form of hexanoyl-CoA with malonyl-CoA. Furthermore, 2-methylpiperidine is plausibly produced in an analogous manner from 5-ketohexanoic acid.

Leete & Adityachaudhury (1967) fed plants [^{14}C-1']-γ-coniceine, and later labelled coniine and pseudoconhydrine were isolated. The compounds had the labelled carbon in the correct position. Dietrich & Martin (1968) fed poison hemlock plants with [^{14}C]-CO_2. γ-Coniceine, coniine, and *N*-methylconiine were isolated after 4 h exposure in molar ratios of 20:1:1, respectively. Dietrich & Martin (1969) noticed that a compound "D" could be linked to alkaloid biosynthesis as it had a rapid turnover during a monitored experiment. Koleoso et al. (1969) identified the compound "D" as 3-formyl-4-hydroxy-2*H*-pyran (Figure 13B).

A δ-Octalactone

B Compound "D"

Figure 13 A δ-Octalactone (Engel et al., 1989) **B** Compound "D" (Koleoso et al., 1969).

Nitrogen of hemlock alkaloids is introduced in a transaminase-catalysed reaction between 5-keto-octanal and L-alanine by L-alanine:5-keto-octanal aminotransferase (AAT) (Figure 14) (Roberts, 1971). The enzyme has no reverse

activity, *i.e.* the reaction is unidirectional, L-alanine:5-ketooctanal to pyruvate:γ-coniceine with an activity of 10 U/mg protein. Serine, glutamic acid, 3-aminobutyric acid and 6-amino hexanoic acid can also function as nitrogen donors for AAT (Roberts, 1977). The transaminase has two isozymes, A and B, with the same molecular size, 56.23 kDa (Roberts, 1978). Isozyme A is mitochondrial (Roberts, 1981) and its K_m for 5-keto-octanal is 1.6 mM and for L-alanine 27 mM, with a pH optimum of 7.5-8.5 (Roberts, 1978). Isozyme B is chloroplastic (Roberts, 1981), and its K_m for 5-keto-octanal is 0.14 mM and for L-alanine 55 mM, with a pH optimum of 8.5 (Roberts, 1978). Roberts (1981) suggested that especially the isozyme B would be the transaminase responsible for alkaloid formation. An AAT isolated from spinach (*Spinacia oleracea* L.) leaves is also capable of forming γ-coniceine from 5-keto-octanal with L-alanine (Unger, 1977). The cyclization after transamination is a non-enzymatic reaction (Roberts, 1977; Unger, 1977).

γ-Coniceine is a precursor of coniine (Fairbairn & Challen, 1959; Leete & Adityachaudhury, 1967; Dietrich & Martin 1968 & 1969; Fairbairn & Ali, 1968b), which is formed by γ-coniceine reductase (CR). The enzyme was isolated from metabolically active leaves and unripe fruits of hemlock, in which coniine is a major alkaloid. The CR is NADPH-dependent (Roberts, 1975). Conhydrinone (Leete & Olson, 1972) and pseudoconhydrine (Leete & Adityachaudhury, 1967) are derived from γ-coniceine.

N-Methylconiine is made from coniine via methylation (Dietrich & Martin, 1968 & 1969).The hemlock plants were fed [methyl-^{14}C]-L-methionine and the methyl group was incorporated into *N*-methylconiine (Roberts, 1974a). The enzyme behind the reaction is *S*-adenosyl-L-methionine:coniine methyltransferase (CSAM) (Roberts, 1974b), which was isolated from unripe fruits. The actual donor of the methyl group is *S*-adenosyl-L-methionine. The enzyme's optimal reaction rate is 140 nmol coniine/h/mg protein with K_m 1.55 mM and its optimal pH is 8.2. The enzyme can use coniine, conhydrine and pseudoconhydrine as substrates to produce *N*-methylated alkaloids. CSAM works best with pseudoconhydrine, then coniine and most poorly with conhydrine (Roberts, 1985).

Cromwell (1956) deduced that the biosynthesis of alkaloids is more likely to occur in shoots than in roots. This conclusion was based on the fact that the sap of a decapitated plant's roots was devoid of alkaloids, and there was no accumulation over a period of one week. Fairbairn & Suwal (1961) noted that seedling roots did not contain alkaloids and there were alkaloids in the roots of the second-year plants before spring growth. During the growth season, no alkaloids were detected in the roots. [^{14}C]-Labelled alkaloids in seeds were not found in germinating cotyledons, as γ-coniceine of seedlings is synthesized *de novo* (Roberts, 1985). The key enzymes of alkaloid production (AAT, CR, and CSAM) are active during leaf expansion. This activity ceases when the leaf has matured and a similar situation prevails in the fruits (Roberts, 1998).

In poison hemlock, the change of accumulation from γ-coniceine to saturated alkaloids may be associated with active growth, and this is also reversible (Fairbairn & Challen, 1959; Fairbairn & Ali, 1968b). Coniine content can vary by over 100% and γ-coniceine by over 400% during the daytime. The amount of γ-

coniceine peaks around midday, when coniine is absent. There are also hourly and daily changes in alkaloid concentration (Fairbairn & Suwal, 1961). Roberts (1981) suggested that there is a close link between illumination and alkaloid production. Biosynthesis of pseudoconhydrine is apparently dependent on environmental factors, as the same variety produced conhydrine outdoors and pseudoconhydrine in a greenhouse (Leete & Adityachaudhury, 1967). γ-Coniceine is the dominant alkaloid during the rainy season and coniine in the dry season (Fairbairn & Challen, 1959). Lang & Smith (1997) concluded that alkaloid production varies according to temperature and moisture conditions.

Fairbairn & Challen (1959) hypothesized that hemlock alkaloids could be connected to an oxidation-reduction mechanism. γ-Coniceine and coniine are theorized to have a similar function to NAD^+ and NADH in poison hemlock (Fairbairn & Ali, 1968b; Reynolds, 2005). However, increased alkaloid production is associated with general improvement in growth and vigour. In poison hemlock, volatile alkaloids may attract pollinators (Roberts, 1998), and they are part of the host plant recognition of the hemlock moth (Castells & Berenbaum, 2006). Alkaloids may also be a defensive measure. If plants are eaten, more alkaloids are produced (López et al., 1999; Castells et al., 2005), as the alkaloid biosynthesis is to some extent inducible.

In the poison hemlock plant, especially the fruit, the alkaloids are stored as compounds which become more complex as time passes. When these compounds are broken down they yield γ-coniceine, coniine, and some other alkaloids (Fairbairn, & Ali, 1968a & 1968b). Coniine alkaloids are quite stable once they are formed, at least for nine days (Leete & Olson, 1972), and are present in non-volatile form (Castells & Berenbaum, 2008a).

Figure 14 Proposed biosynthesis of coniine in poison hemlock according to Fairbairn & Challen (1959), Leete (1963 & 1964), Leete & Adityachaudhury (1967), Dietrich & Martin (1968 & 1969), Fairbairn & Ali (1968), Leete & Olson

(1970 & 1972) and Roberts (1971, 1974b, 1975, 1977 & 1985). Abbreviations: AAT L-alanine:5-keto-octanal aminotransferase, CSAM *S*-Adenosyl-L-methionine:coniine methyltransferase, CR γ-coniceine reductase.

1.2.4 Biological activity of coniine alkaloids

The hemlock alkaloids affect the mammalian system so that respiration is first stimulated, then depressed, becoming cyanosed, and death apparently occurs because of respiration failure (Bowman & Sangvi, 1963). This is due to the fact that these alkaloids are neurotoxins and teratogens (Reynolds, 2005; Green et al., 2010). Coniine is a nicotic acetylcholine receptor (nAChR) antagonist. Its teratogenic action may be related to its ability to activate (stimulate) and subsequently, desensitize (depress) nAChRs, as this leads to inhibition of foetal movement (Green et al., 2010). However, the larvae of the hemlock moth need coniine in order to develop properly (Castells & Berenbaum, 2006).

One of the first studies of the biological effects of coniine in the body was performed by Moore & Row (1898). They injected subcutaneously 10-20 mg coniine into a frog weighing about 25 g. This led to complete muscular paralysis of the animal. When the nerves of the frog were stimulated with electricity, there was no response due to paralysis of the intramuscular part of the muscular nerves. The coniine poisoning first affected the peripheral nervous system. In a rabbit, 50-60 mg coniine applied to the superior cervical ganglion induced a partial paralysis, which rapidly passed off. The authors also observed that coniine slowed down amphibian and mammalian hearts. Coniine caused dilation of arterioles even to threefold the normal diameter. In mammals, coniine caused first a slight quickening and marked deepening of the respiration. Later, the breathing became slower and the individual breaths became shallower. When coniine (20-70 mg) was injected into a cat or a dog, their respiration became feebler and finally halted due to peripheral paralysis of the respiratory muscles. There was no clear result to indicate the fatal dose. Moore & Row (1898) concluded that coniine has similar physiological roles to those of nicotine and piperidine, only with varying intensity.

Bowman & Sangvi (1963) studied the effects of coniine, *N*-methylconiine, and γ-coniceine on the body. Coniine (15-50 μg/ml) and γ-coniceine (5-15 μg/ml) caused contraction of isolated guinea pig ileum and rabbit duodenum. The action arose from stimulation of parasympathetic ganglia. *N*-Methylconiine did not cause any effect on the tested material. Blood pressure dropped with a coniine dosage of 0.5-2 mg/kg, *N*-methylconiine 1-4 mg/kg and γ-coniceine 0.2-0.5 mg/kg. In isolated rabbit heart, a decrease in the strength of beating was recorded with coniine at a dosage of 2 mg, *N*-methylconiine at 4 mg, and γ-coniceine at 0.2 mg. Generally, hemlock alkaloids intravenously or intra-arterially caused a small increase in venous outflow in skeletal muscle blood flow. The authors observed that respiration was first stimulated and then depressed during their animal testing. Coniine caused reportorial stimulation and depression at dosages of 1-4 mg/kg, whereas for γ-coniceine a lower dosage of only 0.3-1 mg/kg was sufficient. *N*-Methylconiine had no stimulatory effect; only depression was recorded in high

doses due to neuromuscular block. Small dosages of coniine (20-30 μg) or γ-coniceine (10-20 μg) slowly increased the respiratory rate and depth, larger dosages caused depression of respiration and later respiratory failure.

Coniine blocks transmission through the superior ganglion and the neuromuscular junction (Sampson et al., 1966). It blocks acetylcholine in nictitating membrane. In frog neuromuscular endplate, coniine first reduces the amplitude and then causes depolarization of the membrane. In cat's spine, the alkaloid causes depression and excitement. The depression is manifested by a decrease of monosynaptic response and the depression of post-tetanic potentiation. The excitement is the production of spontaneous waves and discharges recorded from the ventral root and blockade of direct and recurrent postsynaptic inhibition.

Coniine's half maximal inhibitory concentration (IC_{50}) is in rat diaphragm 314 μM, chick leg muscle 70 μM, maternal rat brain 1100 μM, foetal rat brain 820 μM and chick brain 270 μM (Forsyth et al., 1996). Its binding to neuroreceptors and acetylcholine-related enzymes is in the adrenergic receptor $Alpha_2$ 260 μM, in the serotonin receptor 5-HT2 492.7 μM, and in the acetylcholine receptors muscarinic AChR 2071 μM and nAChR 19 μM, and butylcholine esterase 327.5 μM (Wink et al., 1998).

Coniine, γ-coniceine and *N*-methylconiine block or reduce the response of muscle to acetylcholine (Bowman & Sangvi, 1963) due to alkaloid binding to the nicotinic receptor of neuromuscular cells (Forsyth et al., 1996; Lee et al., 2008). When compared to different alkaloids in nAChR of foetal rat muscle, the efficiency is from the highest to the lowest: nicotine > coniine > tubocurarine > lobeline (Cooper et al., 1996). The toxicities of coniine enantiomers on human tumour cell line TE-671 expressing human foetal nAChR are in order from the highest to the lowest: γ-coniceine > (–)-coniine > (–)-*N*-methylconiine > (±)-coniine > (±)-*N*-methylconiine > (+)-coniine > (+)-*N*-methylconiine (Lee et al., 2008; Lee et al., 2013a). The exact values for different coniine enantiomers are presented in Table 4. (—)-Coniine elicits more effectively electrical changes in TE-671 cells. It also inhibits foetal movement in goats (Green et al., 2013).

Table 4 The half maximal effective concentration (EC_{50}) of cell lines expressing nAChR *in vitro* for different alkaloids and their enantiomers (Green et al., 2010; Lee et al., 2013a).

	EC_{50} of the cell line expressing nAChR	
Alkaloid	TE-671	SH-SY5Y
(–)-coniine	115 μM	9.6 μM
(±)-coniine	208 μM	51.4 μM
(+)-coniine	900 μM	10.2 μM
γ-coniceine	1.3 μM	-
(–)-*N*-methylconiine	105 μM	-
(±)-*N*-methylconiine	405 μM	-
(+)-*N*-methylconiine	3000 μM	-

The hemlock alkaloids behave partly similarly to curare but still differently (de Boer, 1950; Bowman & Sangvi, 1963). The effects of hemlock alkaloids resemble those of nicotine in both the central and peripheral nervous system. Coniine potentiates morphine's analgesic activity (Arihan et al., 2009). Hemlock alkaloids have analgesic (pain relieving) and anti-inflammatory activity in rats at 200 mg/kg (total alkaloids) (Madaan & Kumar, 2012). Coniine has antinociceptive activity (preventing transmission of harmful signals in the nervous system) via nicotinic receptors at a dosage of 20 mg/kg in mice (Arihan et al., 2009). Conmaculatin has a strong peripheral and central antinociceptive activity in mouse over a dosage range of 10-20 mg/kg (Radulović et al., 2012).

How hemlock alkaloids are metabolised in the mammalian system(s) is still unknown (Panter & Keeler, 1989). The microsomes of rat and chick liver did not biotransform coniine during the testing (15 min). A more likely route to remove coniine is excretion (via urine or faeces) rather than biotransformation (metabolism or catabolism) (Forsyth et al., 1996). In insects, cytochrome P450 is involved when piperidine alkaloids are detoxified (Castells & Berenbaum, 2008b). In rats piperidine alkaloid piperine is excreted via faeces (3%) and the rest of it via catabolism in the liver in the form of conjugated uronic acids, sulphates and phenols which are excreted via urine (Bhat & Chandrasekhara, 1986).

1.2.4.1 Medicinal use of poison hemlock and coniine alkaloids

Poison hemlock has been used as a medicine externally to treat herpes, erysipelas (also known as Ignis sacer, holy fire, and St. Anthony's fire; a bacterial skin infection caused by *Streptococcus pyogenes* Rosenbach) and breast tumours. The Greek and Arabian physicians used the plant to cure indolent tumours, swellings, and pains in the joints. Poison hemlock was used in antiquity to wither testicles and to shrink breasts (Bloch, 2001). The juice of poison hemlock together with seeds of betony (*Stachys officinalis* (L.) Trevis. ex Briq.) and fennel (*Foeniculum vulgare* Mill.) mixed into wine were used for the treatment of the bite of a mad dog (rabies). The plant has been the last resort antidote for strychnine and other virulent poisons. Religious sects in the 1400s and 1500s used roasted roots to relieve pains of gout. From the 1760s onwards the plant was used as a cure for cancerous ulcers. Tinctures and extracts made from hemlock have been used as a sedative and an anodyne (analgesic). Its antispasmodic effects were used to treat tetanus, asthma, epilepsy, whooping cough, angina, chore and stomach pains (de Landoni, 1990; Daugherty, 1995; Mitich, 1998; Vetter, 2004; Arihan et al., 2009; Madaan & Kumar, 2012). In Finnish folk medicine, poison hemlock has been used as a powder, plaster and poultice to treat hardened glands, cramp and malignant wounds (Linnilä et al., 2003).

Dried leaf and juice of poison hemlock were part of the official London and Edinburgh pharmacopoeias of 1864-1898. The last official recognition of the medical use of poison hemlock was in 1938 in the British Pharmaceutical Codex (Bowman & Sangvi, 1963). The reason for discontinuation appears to be the

difficulty of manufacturing a medicine with even quality: different preparations varied in potency (Panter & Keeler, 1989). If poison hemlock is used internally it must be carefully administrated, as narcotic poisoning with paralysis and loss of speech may result from overdosage (Mitich, 1998; Vetter, 2004).

The coniine alkaloids could serve as a starting point for the synthesis of specific and less toxic spinal relaxants (de Boer, 1950; Bowman & Sangvi, 1963). Bowman & Sangvi (1963) noted that the hemlock alkaloids do not have a local anaesthetic effect, or it is very weak. Later research has found that coniine has a local anaesthetic effect in mice and rats (Arihan et al., 2009; Madaan & Kumar, 2012).

1.2.4.2 Toxicity to animals

Various mammals have been reported to have eaten poison hemlock and been poisoned as a result: cattle (*Bos taurus* L.) (Penny, 1953; Galey et al., 1992; Binev et al., 2007; Swerczek & Swerczek, 2012), pigs (*Sus scrofa domesticus* Erxleben) (Buckingham, 1937; Edmonds et al., 1972; Dyson & Wrathall, 1977; Panter et al., 1983; Widmer, 1984; Hannam, 1985; Markham, 1985; Panter et al., 1988a), horses (*Equus ferus caballus* L.) (MacDonald, 1937; Keeler et al., 1980; Nice et al., 2015), deer (Swerczek & Swerczek, 2012), tule elks (*Cervus canadensis nannodes*) (Jessup et al., 1986), goats (*Capra aegagrus hircus* L.) (Copithorne, 1937; Holstege et al., 1996), sheep (*Ovis aries* L.) (Panter et al., 1988a & 1988c) and rabbits (*Oryctolagus cuniculus* L.) (Short & Edwards, 1989). Coniine is deadly to several birds: quails (*Coturnix corturnix* L.), chickens (*Gallus gallus* L.) and turkeys (*Meleagris gallopavo* L.) (Frank & Reed, 1986). General signs of poison hemlock toxicosis on mammals are muscular weakness, incoordination, trembling, nervousness, ataxic gait, knuckling at the fetlock joints, excessive salivation, bloating, intestinal irritation, rapid and weak pulse, loss of appetite, cyanotic membranes, dilating pupils, initial central nervous system stimulation, then depression and finally death from respiratory paralysis (all symptoms not always present) (Keeler et al., 1980; Panter et al., 1988b; Nice et al., 2015). Some of these signs appear within 1 hour of consumption, followed by respiratory paralysis in 2-3 hours, and some of the symptoms come later (in 3-4 days) (Keeler et al., 1980; Nice et al., 2015). The treatment for animals includes nerve and heart stimulants with large doses of mineral oil and purgatives to empty the digestive tract (Nice et al., 2015).

Different species have different reactions to coniine. Its toxicity varies from the lowest to the highest tolerance as follows: cows < mares < ewes (Keeler et al., 1980). For example, to cows coniine is toxic in a daily dosage of 3.3-6.6 mg/kg (Keeler et al., 1980) and about 1 kg of poison hemlock is also deadly (Nice et al., 2015). The lethal level for pigs is 1 g/kg of seeds and 8 g/kg of the plant (Panter et al., 1983). Coniine, piperidine, and 2-ethylpiperidine are toxic to cattle, with classic symptoms of hemlock poisoning. 2-Methylpiperidine, 2-piperidine-ethanol, conyrine, 3-methylpiperidine and *N*-methylpiperidine are not toxic. Inhalation of coniine or crushed green plant material does not cause toxication (Keeler & Balls,

1978). The primary toxicants to livestock are coniine and γ-coniceine (Panter et al., 1988b).

1.2.4.3 Teratogenicity to animals

Coniine and hence poison hemlock are teratogenic to animals (from the most intense to the least): cows > sows > ewes (Panter et al., 1988c). Piperidine, 2-methylpiperidine, 2-ethylpiperidine, 2-piperidineethanol, conyrine, 3-methylpiperidine and *N*-methylpiperidine are not teratogenic (Keeler & Balls, 1978). For a piperidine alkaloid to be a teratogen it must have at least three carbons in the 'tail' and only one or no double bonds in the ring structure (Keeler & Balls, 1978; Keeler et al., 1983). The reason behind the teratogenic malformations is alkaloid-induced foetal immobilization (Bunch et al., 1992). The malformations sometimes resolve spontaneously after birth in 8-10 weeks. Poison hemlock plant material causes multiple congenital contractures and cleft palate. Multiple congenital contractures include torticollis, scoliosis, lordosis, arthrogryposis, rib cage anomalies, overextension, flexure and rigidity of joints (Panter et al., 1990).

1.2.4.4 Toxicity to humans

A historic poem of *koneion* (hemlock) intoxication by Nicander of Colophon (204-135 B.C.) in Alexipharmaca (Bloch, 2001) describes how the effects progress in the human body:

"*Take note of the noxious draught which is hemlock, for this drink assuredly loses disaster upon the head bringing the darkness of night: the eyes roll, and men roam the streets with tottering steps and crawling upon their hands; a terrible choking blocks the lower throat and the narrow passage of the windpipe; the extremities grow cold; and in the limbs the stout arteries are contracted; for a short while the victim draws breath like one swooning, and his spirit beholds Hades.*"

Reese's (1884) description is as follows: a headache, "imperfect vision", pharyngeal pain, vomiting, drowsiness, gradual paralysis of extremities and death at last "from apnea". Other symptoms may be convulsions, coma, violent delirium, salivation and involuntary discharges from the bladder and bowels. Modern major clinical effects on humans are irritation of oral mucosa, salivation, nausea, emesis, slight abdominal pain, diarrhoea (uncommon), bradycardia, miosis, hypertension to tachycardia, hypotension, mydriasis, seizures following ascendant muscle paralysis and respiratory failure. Diagnosis uses blood gases, electrolytes, and a plant sample. First-aid and management procedures are immediate gastric lavage or emesis following activated charcoal with a purgative drug. Treatment is mainly to ensure adequate respiratory function (de Landoni, 1990). The important factor is to get the patient quickly into hospital care, as the poisoning symptoms appear quite rapidly. With the help of artificial breathing such as intubation, it is possible to save the patient's life.

Coniine is toxic to humans and 3 mg produces symptoms. Up to 150-300 mg coniine can be tolerated, which translates to 6-8 leaves (6 g) (de Landoni, 1990; Rizzi et al., 1991; Biberci et al., 2002). Accidental ingestion of poisonous plants (*e.g.* poison hemlock) can also be sourced to herbal medication that has been incorrectly prepared or an incorrect plant has been used due to misidentification (Beyer et al., 2009).

1.2.4.5 Socrates

Socrates died in 399 B.C. He was sentenced to death because of corrupting the youth of Athens and failing to recognize the city's traditional gods. The sentencing was passed with only a small majority. The execution was carried out with a dose of poison potion called *pharmakon* according to the tradition. The death of Socrates (Figure 15) is described in Plato's Socratic dialogue, Phaedo (Daugherty, 1995; Scutchfield & Genovese, 1997) (English translation by Jowett, 1892):

Crito made a sign to the servant, who was standing by; and he went out, and having been absent for some time, returned with the jailer carrying the cup of poison. Socrates said: "You, my good friend, who are experienced in these matters, shall give me directions how I am to proceed." The man answered: "You have only to walk about until your legs are heavy, and then to lie down, and the poison will act." At the same time he handed the cup to Socrates, who in the easiest and gentlest manner, without the least fear or change of colour or feature, looking at the man with all his eyes, Echecrates, as his manner was, took the cup and said: "What do you say about making a libation out of this cup to any god? May I, or not?" The man answered: "We only prepare, Socrates, just so much as we deem enough." "I understand," he said: "but I may and must ask the gods to prosper my journey from this to the other world — even so — and so be it according to my prayer." Then raising the cup to his lips, quite readily and cheerfully he drank off the poison. And hitherto most of us had been able to control our sorrow; but now when we saw him drinking, and saw too that he had finished the draught, we could no longer forbear, and in spite of myself my own tears were flowing fast; so that I covered my face and wept, not for him, but at the thought of my own calamity in having to part from such a friend. Nor was I the first; for Crito, when he found himself unable to restrain his tears, had got up, and I followed; and at that moment, Apollodorus, who had been weeping all the time, broke out in a loud and passionate cry which made cowards of us all. Socrates alone retained his calmness: "What is this strange outcry?" he said. I sent away the women mainly in order that they might not misbehave in this way, for I have been told that a man should die in peace. "Be quiet then, and have patience." When we heard his words we were ashamed, and refrained our tears; and he walked about until, as he said, his legs began to fail, and then he lay on his back, according to the directions, and the man who gave him the poison now and then looked at his feet and legs; and after a while he pressed his foot hard, and asked him if he could feel; and he said, "No;" and then his leg, and so upwards and upwards, and showed us that he was cold and stiff. And he felt them himself, and

said: "When the poison reaches the heart, that will be the end." He was beginning to grow cold about the groin, when he uncovered his face, for he had covered himself up, and said — they were his last words — he said: "Crito, I owe a cock to Asclepius; will you remember to pay the debt?" "The debt shall be paid", said Crito; "is there anything else?" There was no answer to this question; but in a minute or two a movement was heard, and the attendants uncovered him; his eyes were set, and Crito closed his eyes and mouth.

Figure 15 Jacques-Louis David (1748-1825): The death of Socrates (Source: Wikimedia).

Often the poison that killed Socrates has been suggested to have been poison hemlock (Dayan, 2009). Plato's description of the painless death of Socrates might be true based on the results of research by Arihan et al. (2009), according to which coniine has antinociceptive activity. It may have been a mixture of *koneion* (poison hemlock) and for example opium, myrrh, and wine (de Boer, 1950; Daugherty, 1995; Scutchfield & Genovese, 1997; Bloch, 2001; Arihan et al., 2009; Dayan, 2009). Correctly performed the poison, as in Plato's description, would speed the death without unnecessary suffering (Daugherty, 1995). Theophrastus' Enquiry into Plants describes how to prepare hemlock for quick and painless death; poppy and other similar herbs are mixed with it. However, there is no information concerning which hemlock plant the recipe uses, poison hemlock (*C. maculatum*), water hemlock (*Cicuta* sp.) or water dropwort (*Oenanthe crocata* L.) (Sullivan, 2001).

There are several factors supporting the theory that poison hemlock was one of the components of the poison administered to Socrates. The paralysis started

from the feet, death was due to respiratory failure, the feeling of “cold” and “stiff-“stiffness” was in the calves and spread upwards to the chest, the loss of feeling in the legs and the fact that death appears to have been quite quick on the basis of the dialogue. The negatives for poison hemlock are that there was no abdominal pain, nausea, vomiting or diarrhoea (Dayan, 2009). A Scottish physician, John Harley, tested poison hemlock on himself in the 19th century and his description agrees rather well with Plato’s (Bloch, 2001). When coniine and opium are used together and tested on rats, the effects of the mixture are quicker than either alone. The symptoms are depression of respiration, very strong cyanosis, paralyzed skin sensitivity, almost absent convulsions, strong muscle paralysis, pronounced paralyzing action of coniine and lowered anaesthetic power of opium. Opium and poison hemlock were used together to speed up the death of Socrates according to de Boer (1950). His results could explain the skin effects in the legs and the last-minute speaking of Socrates. Poison hemlock alone would have needed administration of quite a large amount of the plant to reach the necessary dose (de Boer, 1950).

One possibility is that the description of Socrates’ death has some artistic licence and is not entirely accurate (Daugherty, 1995), or there might be some confusion in the description of symptoms, as Plato’s writing is in fact a quotation of Crito (Scutchfield & Genovese, 1997; Dayan, 2009). It is possible that Plato wanted to present a beautified picture of Socrates’ passing without all the gruesome details (Scutchfield & Genovese, 1997). Bloch (2001) concluded that Plato described correctly the poisoning of Socrates once all the confusing layers are peeled away. Sullivan (2001) supported the notion that poison hemlock was behind the poisoning of Socrates without poetic licence, as the description in Phaedo is quite clear.

1.3 Elicitation

An elicitor is a factor, either physical or chemical, which initiates or enhances the biosynthesis of target compounds when introduced in small amounts to a living cell system (Namdeo, 2007). The elicited compounds usually ensure the survival of plant cells when they are stressed by environmental factors or phytopathogens (Wang, 2007; Patel & Krishnamurthy, 2013). Elicitors can be classified into endogenous and exogenous as well as abiotic and biotic. Exogenous elicitors include chemical compounds originating from outside the target cell, such as fatty acids, polysaccharides, peptides and enzymes. Endogenous elicitors are made inside the target cell by induction of intracellular biotic or abiotic signals (Namdeo, 2007). Biotic elicitors may be directly released by microorganisms and recognized by the plant cell (*e.g.* cellulase), formed by the action of microorganisms on plant cell wall (e.g. pieces of pectin), formed by plant enzymes on microbial cell walls (*e.g.* chitosan), or they may be compounds which are endogenous and constitutive in nature, formed or released by the plant cell in response to various stimuli (Namdeo, 2007). Abiotic elicitors range from physical factors (*e.g.* UV-light,

temperature) to chemical compounds (*e.g.* heavy metal salts of *e.g.* lead and copper) (Namdeo, 2007; Patel & Krishnamurthy, 2013).

Elicitation is used to enhance secondary metabolite production when an established plant cell culture produces the compound of interest in very low amounts or none at all (Namdeo, 2007). For example, cell cultures of *Taxus* spp. have been induced by arachidonic acid to produce taxol (Veersham et al., 1995), and *Papaver somniferum* L. has been elicitated with fungal spores to produce morphine and codeine (Heinstein, 1985). Elicitation of plant cell cultures may be promising, as it has shown favourable results in the production of antibiotics and other similar compounds in microbial systems. However, elicitation enhances secondary metabolism in plants or plant cells *in vitro* but the exact mechanism of action is not well understood (Namdeo, 2007). Several factors have an influence on elicitation, such as elicitor concentration and selectivity, duration of elicitor exposure, age of the culture, cell line, growth regulation, nutrient composition, and quality of cell wall materials, among others (Ganapathi & Kargi, 1990). Thus, a clearly defined elicitor is a better option when dealing with a poorly known metabolite biosynthesis route, rather than an undefined elicitor for example yeast extract, in which the exact elicitor(s) may be unknown.

Poison hemlock cell cultures have been elicited with fresh medium, alkaloids (coniine, papaverine) and polyamines (spermidine, cyclic AMP) to produce quinolizidine alkaloids (Wink & Witte, 1983). Whole hemlock plants have been treated with copper(II) chloride, causing accumulation of furanocoumarins (xanthotoxin, isopimpinellin, bergapten, psoralen, scopoletin, isoscopoletin, umbelliferone and coumarins) (Al-Barwani & Eltayeb, 2004). When hemlock moth feeds on poison hemlock, this action increases alkaloid production (Castells et al., 2005). This provides an indication of the inducibility of coniine biosynthesis in poison hemlock. *A. vera* cell cultures have been elicitated with ammonium nitrate, sucrose, titanium oxide and silver to enhance aloin production (Raei et al., 2014). Furthermore, the same aloe's root cultures have been elicitated with methyl jasmonate, salicylic acid and ethephon to produce more aloe emodin and chrysophanol (Lee et al., 2013b).

Alginic acid is a polysaccharide which is composed of a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate and its C-5 epimer α-L-guluronate residues. It is a component of brown algae cell walls and pathogenic bacteria (Wang, 2007).

Cellulase is an enzyme which breaks down a major cell wall component, cellulose, into its basic building blocks, glucose. It is present in fungi, bacteria, and protozoa.

Chitosan is a polysaccharide ((1-4)-β-linked *N*-acetyl-D-glucosamine) which is produced by deacetylation of chitin found in cell walls of fungi or in the exoskeleton of crustaceans such as crabs or shrimps (Holopainen *et al*., 2009; Ruiz-García & Gómez-Plaza, 2013). The breakdown products of chitosan may induce a defense response in plants (Wang, 2007).

Ethephon (2-chloroethylphosphonic acid) is a salt which when metabolized by a plant releases the natural gaseous plant hormone ethylene (Holopainen et al.,

2009). Ethephon activates stilbene production and increases the number of path-pathogenesis-related proteins in grapevine (*Vitis vinifera* L.), leading to protection against fungal pathogens (Belhadj et al., 2008).

Methyl jasmonate (methyl-3-oxo-2-(2*Z*)-2-pentenyl-cyclopentaneacetate) is a derivate from jasmonic acid. Both compounds are plant hormones formed under various biotic stresses via the octadecanoid pathway (Holopainen *et al*., 2009; Ruiz-García & Gómez-Plaza, 2013). The pathway starts with an oxygenation of free linolenic acid, which is followed by a further reaction catalysed by a lipoxygenase. Methyl jasmonate and jasmonic acid induce genes controlling the secondary metabolic pathways (Holopainen et al., 2009).

Salicylic acid is synthesized from chorismate via isochorismate. Infection of the plants by a broad range of pathogens results in an increase in salicylic acid levels both at the site of infection and in distant tissues (Santner et al., 2009). Salicylic acid induces activation of the enzyme phenylalanine ammonia lyase and causes accumulation of secondary metabolites in the flavonoid pathway (Ruiz-García & Gómez-Plaza, 2013).

Silver and copper salts are among many heavy metal salts which can be used as an elicitor (Bota & Deliu, 2011). These salts cause an abiotic stress which elicits secondary metabolite accumulation.

1.4 Plant cell culture

Plant cell culture is the growth of cells in isolation from the intact plant in different culture systems. These systems are usually aseptic and provide the culture all needed inorganic salts, sucrose, vitamins, plant hormones and water (Moscatiello et al., 2013). The grown cells have characteristics of callus cells, and on the solid medium are called callus cultures. When these cultures are transferred to liquid medium then they are called suspension cultures.

Plant cell cultures can be initiated almost from every part of the plant. The callus tissue develops into wounds of cut-up plant when plated *in vitro* and provided with suitable plant hormones, such as cytokinins and auxins. The correct conditions to initiate and sustain plant cell cultures depend on the plant species and starting tissue, and must be identified through experimentation. There are two main differences between animal and plant cell cultures: cultured plant cells can grow indefinitely without a limitation to the number of cell divisions as in the animal system, and plant cell cultures are totipotent, *i.e.* with suitable hormonal stimulation they can regenerate into whole plants (Moscatiello et al., 2013).

One of the uses for cell cultures is to produce secondary metabolites, especially when isolation of target compounds from whole plants is not realistic (Oksman-Caldentay & Inzé, 2004). Such cases are for example paclitaxel production in *Taxus* spp. suspension culture (Choi et al., 2001; Patel & Krishnamurthy, 2013), or to enable someday the production of vincristine and vinblastine in *Catharanthus roseus* (L.) G.Don suspension cultures (Oksman-Caldentay & Inzé, 2004).

Poison hemlock cell cultures have been initiated in a wide range of different conditions (Nétien & Combet, 1970; Carew & Bainbridge, 1976; Wink et al., 1980a & 1980b; Wink et al., 1981; Schoofs et al., 1983). The cell cultures have been used in biotransformation studies (Carew & Bainbridge, 1976; Wink et al., 1980a), elicitation of non-typical alkaloids for the species (Wink & Witte, 1983) and to study piperidine alkaloid content (Mahrenholz & Carew, 1966; Nétien & Combet, 1970).

Aloe callus cultures have also been specifically initiated for genetic transformation (Velcheva et al., 2010) and the production of certain compounds (Yagi et al., 1998). Callus formation has been reported for *A. arborescens* (Kawai et al., 1993; Bedini et al., 2009), *A. bellatura* (Tatsuo & Amano, 1985), *A. ferox* (Racchi, 1988), *A. polyphylla* (Ramsay & Gratton, 2000), *A. pretoriensis* (Groenewald et al., 1975), *A. saponaria* (Yagi et al., 1983; Baek et al., 2009) and *A. vera* (Roy & Sarkar, 1991; Yagi et al., 1998; Velcheva et al., 2010; Rathore et al., 2011; Raei et al., 2014).

2. Aims

The biosynthesis of coniine in poison hemlock has previously been studied: how the compound is formed and what enzyme types are involved. However, the formation of the carbon backbone of coniine has been something of a black box. On the basis of available information about fatty acid biosynthesis in the early 1960s, Leete (1963 & 1964) theorized that acetyl-CoA and three malonyl-CoAs could be a starting point for coniine biosynthesis. Before the current study, the only plants known to contain hemlock alkaloids were twelve aloes, poison hemlock, and yellow pitcher plant. A few studies have postulated that poison hemlock callus could contain alkaloids, but no systematic studies were performed on whether the piperidine alkaloid biosynthesis is inducible or not.

The overall aim of the work was to examine hemlock alkaloid biosynthesis at different levels and to study its distribution in the plant kingdom:

1. To identify and characterize the gene and the encoded enzyme for PKS that participates in coniine formation in poison hemlock.
2. To produce callus of coniine-containing plants for biosynthesis studies.
3. To induce or enhance coniine biosynthesis in callus of target plants in order to study coniine biosynthesis.
4. To develop *in vitro* techniques for coniine-containing plants for easy propagation to produce high-quality plants for experimental needs.
5. To develop different chromatographic methods to analyse hemlock alkaloids.

3. Materials and methods

The materials and methods used in this study are summarized in Table 5, except for the transcriptome sequencing (Section 3.1). All details have been described in the original publications (I-IV).

Table 5 Materials and methods used in this study. The Roman numerals refer to the four original publications.

Materials or methods	Publication
Analysis of enzyme products (including structural analysis with liquid chromatography-mass spectrometry (LC-MS)*)	I
Callus induction	II, III
Cloning of PKS genes	I
Coniine detection in low quantities with gas chromatography-mass spectrometry (GC-MS) selected ion monitoring (SIM)	IV
Determination of the enzyme kinetic parameters	I
Elicitation of poison hemlock callus*	II
Enzyme assay of PKSs	I
Enzyme production, purification of PKSs	I
GC-MS	II, III, IV
Genomic DNA isolation	I
Growth curve of poison hemlock callus*	II
Homology modelling	I
In vitro germination of *Aloe* seeds	III
Micropropagation of *A. viguieri*	III
Phylogenetic analysis of *PKS*-genes*	I
Plant material: Poison hemlock	I, II
Plant material: *Aloe* sp.	III
Plant material: *Sarracenia* sp.	IV
Secondary metabolite extraction	II, III, IV
Surface sterilisation of plant material	II, III
Statistical analysis*	III
Thin layer chromatography (TLC)	III
Viability testing*	II

Note: Methods marked with a star were conducted by the co-authors.

3.1 Transcriptome sequencing

Total RNA was isolated from root, stem, leaf, flower and fruit of poison hemlock in duplicate with the pine tree method (Monte & Somerville, 2003). Root, stem, and leaf samples were collected from a greenhouse-grown second-year plant during the winter of 2011. The plant was grown from seeds collected from Viikki, Helsinki (60.238482°N, 25.033406°E). Flower and developing fruit were collected in July 2011 from Pasila (60.214250°N, 24.917459°E), Helsinki. Fresh plant material was ground in liquid nitrogen. Plant material (1 g) was added to 6 ml isolation buffer (2% w/v CTAB, 2% w/v PVP K 30, 100 mM Tris-HCl pH 8, 25 mM EDTA, 2 000 mM NaCl, 0.5 g/l spermidine, 2% v/v β-mercaptoethanol), which was heated to 65°C and β-mercaptoethanol was added just before heating. Plant material and isolation buffer were mixed by inverting several times. 6 ml chloroform-isoamyl alcohol (24:1) was added, shaken, and centrifuged for 5 minutes at 7 000 g in ambient conditions. The aqueous phase was taken to a new tube, carefully avoiding breaking the middle phase. The aqueous phase was then washed with chloroform-isoamyl alcohol once more. 0.3 x V 8 000 mM LiCl was added to the aqueous phase and the vial was kept at 4°C overnight. The solution was centrifuged at 4°C for 20 min at 7 000 g and the supernatant was removed after centrifugation. The RNA pellet was dissolved in 500 µl SSTE (1 000 mM NaCl, 0.5% w/v SDS, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). After dissolving, 500 µl chloroform-isoamyl alcohol (24:1) was added and centrifuged at 4°C at maximum speed. The aqueous phase was transferred to a new tube, 1 ml 96% ethanol was added and the mixture was kept at -20°C at least for 2 h. Then the material was centrifuged at 22 000 g for 20 min. The supernatant was removed carefully and the pellet was dried in a fume hood for a few minutes. Finally, the pellet was dissolved in 50 µl RNase-free water. The possible genomic DNA was removed with DNase (RNase free, Fermentas, Leon-Rot, Germany). The quality of the RNA was measured with an Agilent 2100 Bioanalyzer and the mRNA fraction was paired-end sequenced by the Illumina platform at Biomedicum Functional Genomics Unit (Helsinki, Finland).

The obtained reads were assembled and annotated at the VIB Department of Plant Systems Biology, University of Gent (Belgium). They were also later re-assembled with the Trinity-program (Grabherr et al., 2011) and re-annotated with Pannzer (Koskinen, 2011).

The figures for individual gene expression were generated using the Excel program (Microsoft, Redmond, WA, USA). The expression of individual genes was analysed by searching matching reads from all libraries with the blast program (Altschul et al., 1997). The number of blast hits (at a specified stringency) was normalized by dividing it by the multiplication product of the target gene length and the number of reads in the target transcriptome, and finally scaled by multiplying by 10^9 to whole numbers for better visualization (Equation 1).

Equation 1 Calculation of individual gene expression in the target transcriptome.

$$Expression = \frac{number\ of\ blast\ hits}{number\ of\ reads\ in\ transcriptome \times target\ gene\ length} \times 10^9$$

4. Results

4.1 Polyketide synthases of poison hemlock and *Sarracenia* sp*.*

Poison hemlock was examined for a PKS-enzyme participating in coniine biosynthesis. For this purpose, *PKS*-genes of the plant were cloned using a homology-based approach (Article I). Five fragments were obtained with PCR when genomic DNA was used as a template and the primers were deg-CHS-R and -F (Helariutta et al., 1995). They were 118 bp long and named C*pks1-5*. The closest Blast hits for the fragments were type III plant PKSs. The 5' and 3' ends of *Cpks1* and *Cpks2* were cloned with an RACE-based technique. An attempt was made to clone *Cpks3*-*5* using cDNA from leaf, stem, root, flowers and developing seeds via RACE, but without success.

In the transcriptome sequencing, the libraries each contained 0.5-17.4 million pairs of 140 bp reads. Together the root, leaf and stem libraries contained 63 383 contigs, of which 29 773 contigs were less than 450 bp long. The contigs from all libraries were analysed with the Pannzer-program and eventually 14 928 contigs were annotated. The sequence of *Cpks5* and seven more fragments numbered *Cpks6*-*12* were obtained via transcriptome analysis.

The fragments of *Cpks3, 4, 10, 11* and *12* did not have overlapping regions with other fragments. The fragments *Cpks6* and *9* did have an overlapping region, although they were not a continuation of the same gene. *Cpks1, 2, 5, 6, 7* and *9* were deposited in GenBank under the numbers KP726914, KP726915, KP726916, KP726917, KP726918, and KP726919, respectively. The sequences of *Cpks3, 4, 8* and *10-12* are presented in Table A1 (Appendix A).

Cpks1 and *Cpks2* sequences both have over 90% amino acid sequence identity with type III plant PKSs in the databank and exhibit the highest sequence similarity with carrot *CHS2* (97%) and *CHS1* (93%), respectively. *Cpks5* has the highest similarity with carrot *CHS1* (81%) (Table 6) and otherwise it shares over 70% identity with type III PKSs.

Table 6 Sequence similarities between different PKSs at the amino acid level (%).

	CPKS1	CPKS2	CPKS5	G2PS1	DcCHS1	DcCHS2	DcCHS9	GCHS1	MsCHS2	PcCHS
CPKS1	100	81	72	72	82	97	82	87	81	97
CPKS2		100	83	70	93	81	92	82	80	81
CPKS5			100	63	81	71	80	72	71	71
G2PS1				100	69	72	69	74	70	71
DcCHS1					100	81	99	82	81	81
DcCHS2						100	81	88	81	98
DcCHS9							100	81	80	81
GCHS1								100	83	87
MsCHS2									100	81
PcCHS										100

The identified genes were analysed in the context of the whole type III PKS protein family. An exhaustive phylogenetic analysis of the family was carried out. All protein sequences belonging to the family were retrieved from TrEMBL and supplemented with closest matches of the identified genes from NCBI's environmental and patent databases. A subset of the whole phylogeny of relevant plant proteins is shown in Figure 16. Fragments CPKS7 and 8 were not included due to inability to align them reliably. The expected result was that the full-length genes of poison hemlock clustered with other known Apiaceae genes. CPKS2 and CPKS5 appear to be poison hemlock specific paralogues and orthologues of carrot CHS1 and CHS9, and of *Glehnia littoralis* CHSs. The fragment of CPKS6 clusters together with them. CPKS1 is an orthologue of carrot CHS2 together with parsley CHS and *Bupleurum chinense* Bc-753f. CPKS3 and 4 fragments are almost identical, as they cover the same part of the gene and thus are either recent paralogues, errors or alleles. CPKS12 is considerably separate from the other CPKSs. CPKS10 and 11 cluster together with *Arabidopsis thaliana* PKSA and B, which participate in the biosynthesis of sporopollenin, a component of the outer pollen wall (Kim et al., 2010). The branch encompassing the identified genes includes known non-CHS/stilbene synthase (STS), CHS and STS genes, whereas other non-CHS/STS and ketoacyl synthase genes are found elsewhere in the

phylogeny. Although the grouping of the *CHS*-genes is evident, the phylogenetic analysis does not clearly separate the functions of the genes (Figure 16).

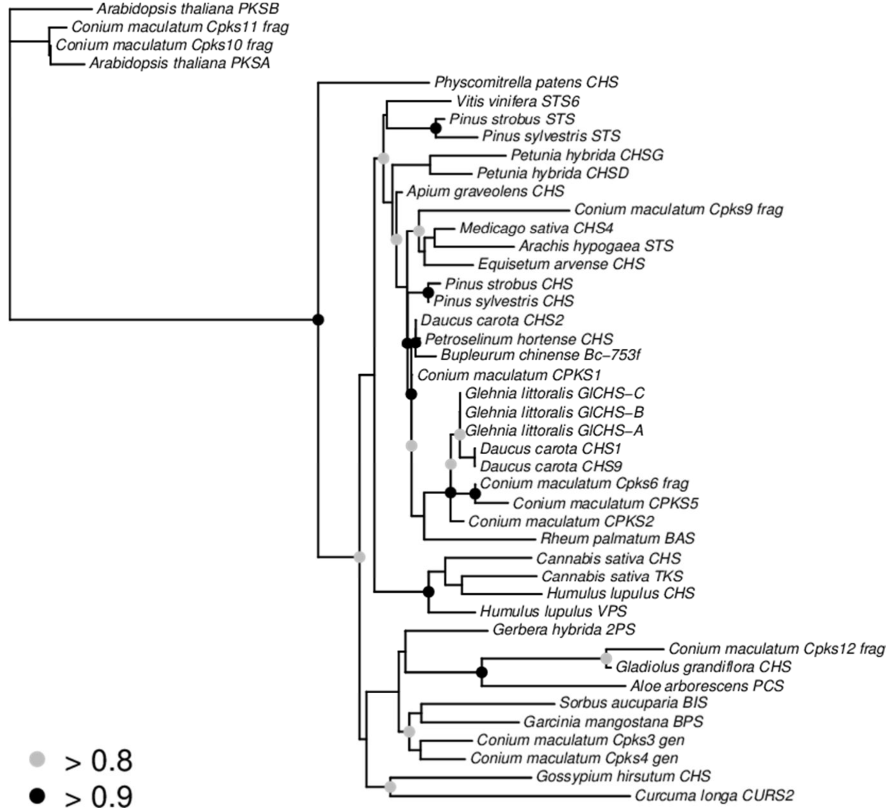


Figure 16 A phylogenetic tree of PKS III family proteins. Nodes are coloured according to their support values, with nodes less than 0.8 support being left unlabelled. frag: Fragments obtained in transcriptome sequence analysis, gen: genomic DNA fragment via PCR with degenerated primers. Abbreviations: 2PS, 2-pyrone synthase; BAS, benzalacetone synthase; BIS, biphenyl synthase; BPS, benzophenone synthase; CHS, chalcone synthase; CURS, curcumin synthase; OLS, olivetol synthase; PCS, pentaketide chromone synthase; PKS, polyketide synthase; STS, stilbene synthase; TKS, 3,5,7-trioxododecanoyl-CoA synthase. GenBank accession numbers: *Aloe arborescens* PCS (AAX35541.1), *Apium graveolens* CHS (AH007394.1), *Arabidopsis thaliana* PKSA (NP171707.1), *Arabidopsis thaliana* PKSB (NP567971.1), *Arachis hypogaea* STS (BAA78617.1), *Bupleurum chinense* Bc-753f (FG341967.1), *Cannabis sativa* CHS (AY082343.1), *Cannabis sativa* TKS (OLS) (BAG14339.1), *Conium maculatum* CPKS1 (KP726914), *Conium maculatum* CPKS2 (KP726915), *Conium maculatum* CPKS5 (KP726916), *Conium maculatum* CPKS6 (KP726917), *Conium maculatum* CPKS7 (KP726918), *Conium maculatum* CPKS9 (KP726919), *Curcuma longa* CURS2 (BAH85780.1), *Daucus carota* CHS1 (Q9ZS41.1), *Daucus carota* CHS2

(Q9ZS40.1), *Daucus carota* CHS9 (Q9SB26.1), *Equisetum arvense* CHS (AB030004.1), *Garcinia mangostana* BPS (AEI27291.1), *Gerbera hybrida* 2PS (CAA86219.2), *Gladiolus grandiflora* CHS (ADM18303.1), *Glehnia littoralis* GlCHS-A (AB374260.1), *Glehnia littoralis* GlCHS-B (AB374261.1), *Glehnia littoralis* GlCHS-C (AB374262.1), *Gossypium hirsutum* CHS (AEO96987.1), *Humulus lupulus* CHS (BAB47196.1), *Medicago sativa* CHS4 (AAB41559.1), *Petroselinum crispum* CHS (1001151A), *Petunia hybrida* CHSD (CAA32733.1), *Petunia hybrida* CHSG (CAA32735.1), *Physcomitrella patens* CHS (DQ275627.2), *Pinus sylvestris* CHS (CAA43166.1), *Pinus sylvestris* STS (AAB24341.2), *Pinus strobus* CHS (CAA06077.1), *Pinus strobus* STS (CAA87012.1), *Rheum palmatum* BAS (AAK82824.1), *Sorbus aucuparia* BIS (D2DRC4.1), *Vitis vinifera* STS6 (JQ868692.1) (Article I).

4.1.1 *Conium* polyketide synthase 1

Cpks1 contains an 1191 bp open reading frame (ORF) that encodes a 43.4 kDa protein with p*I* 6.20 (Article I). The protein consists of 397 amino acids including the conserved cysteine-histidine-asparagine catalytic triad (Austin & Noel, 2003). The active site size of the enzyme is 945 $Å^3$ and the homodimer's active sites are identical. The gene is mainly expressed in leaf stem, leaf, and flower (Figure 17).

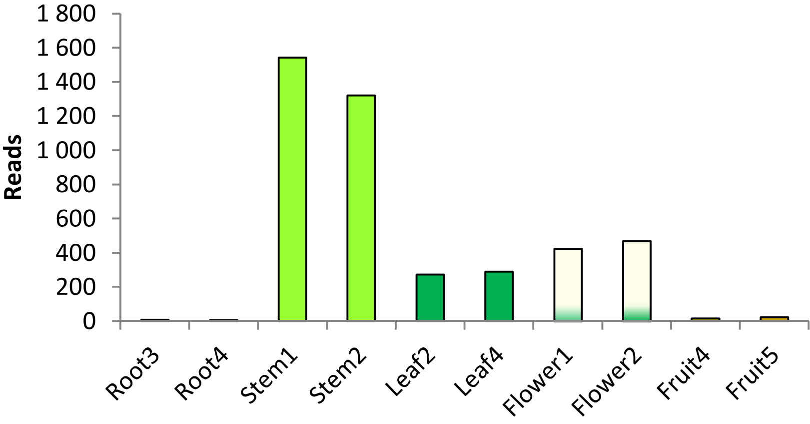


Figure 17 Expression of *Cpks1* in different parts of poison hemlock. The expression is analysed via transcriptome analysis, normalized based on the number of hits, length of gene and size of the transcriptome, and confidence level is 1e-50. Each organ has two transcriptomes sequenced.

4.1.2 *Conium* polyketide synthase 2

Cpks2 contains an 1170 bp ORF that encodes a 42.6 kDa protein with p*I* 6.59 (Article I). The protein consists of 389 amino acids. *Cpks2* also exhibits the conserved cysteine-histidine-asparagine catalytic triad (Austin & Noel, 2003). The

enzyme's active site size is 940 Å^3 and the homodimer's two active sites are identical. *Cpks2* is expressed in leaf stem and flowers (Figure 18).

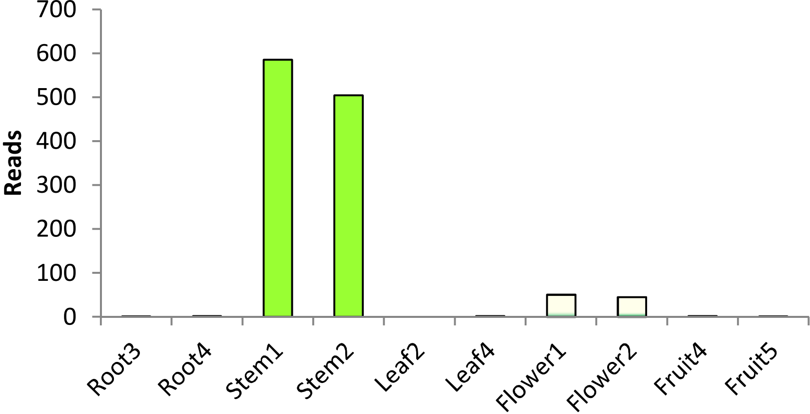


Figure 18 Expression of *Cpks2* in different parts of poison hemlock. The expression is analysed via transcriptome analysis, normalized based on the number of hits, length of gene and size of the transcriptome, and confidence level is 1e-50. Each organ has two transcriptomes sequenced.

4.1.3 *Conium* polyketide synthase 5

Cpks5 contains an 1167 bp ORF that encodes a 42.6 kDa protein with p*I* 6.62 (Article I). The protein consists of 388 amino acids. The enzyme has a conserved cysteine-histidine-asparagine catalytic triad (Austin & Noel, 2003). The active site size for chain A is 934 Å^3 and for chain B is 858 Å^3 according to the model obtained with the CASTp-program. Cpks5 is expressed mainly in leaf stem, flowers and developing fruit (Figure 19). Seven amino acids of the CPKS5 active site are changed (Figure 20; Figure 21; Table 7) and there is one deletion at position 86 (using alfalfa (*Medicago sativa* L.). CHS2 numbering (Junghans et al., 1993)).

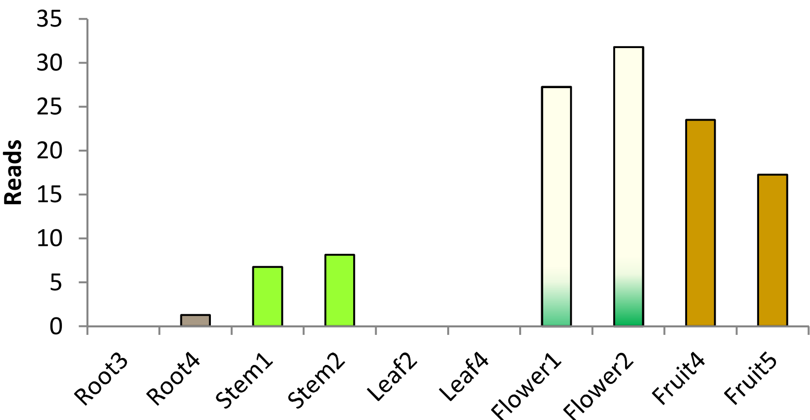


Figure 19 Expression of *Cpks5* in different parts of poison hemlock. The expression is analysed via transcriptome analysis, normalized based on the number of hits, length of gene and size of the transcriptome, and confidence level is 1e-50. Each organ has two transcriptomes sequenced.

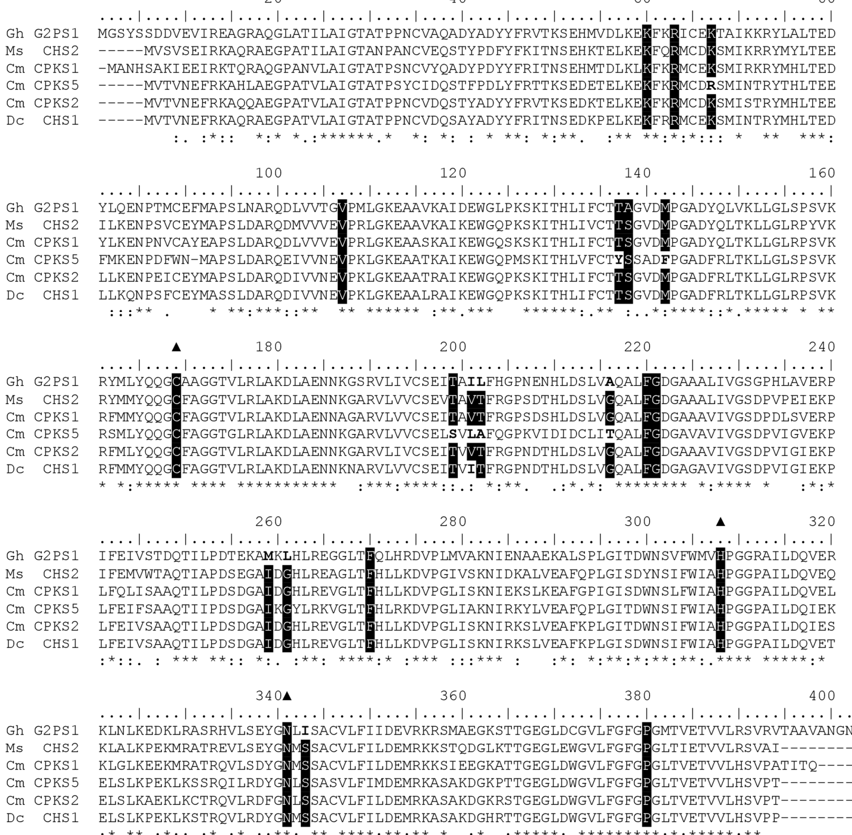


Figure 20 Analysis of deduced amino acid sequences encoded by poison hemlock (*C. maculatum*) *CHS*-like genes. A comparison between *M. sativa* (Ms) CHS2, *D. carota* (Dc) CHS1, *Gerbera hybrida* (Gh) G2SP1 and *C. maculatum* (Cm) CPKS1, CPKS2, and CPKS5. Functionally important conserved residues are highlighted with a black background and changed amino acids in those positions are in bold. ▲ indicates the catalytic triad (Cysteine-Histidine-Asparagine) (Article I).

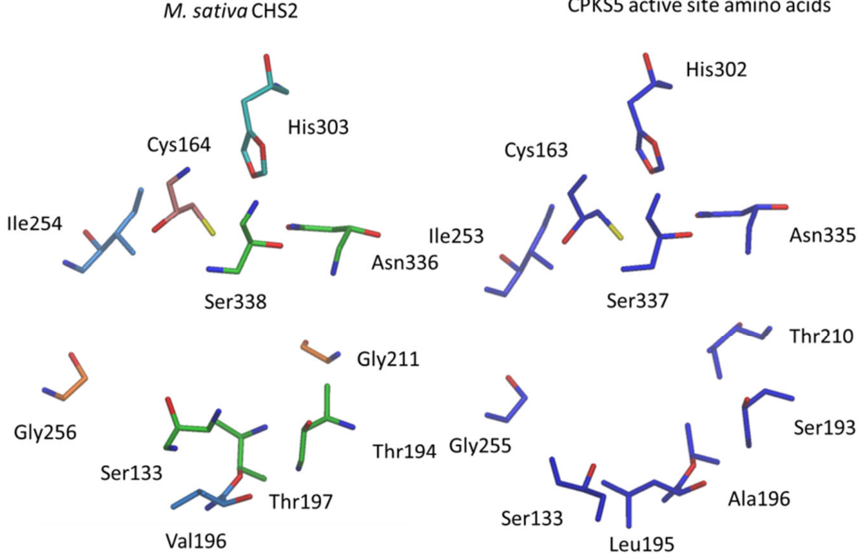


Figure 21 Active site amino acids of *M. sativa* CHS2 and CPKS5.

Table 7 Comparison of alfalfa *(M. sativa)* CHS2 and poison hemlock (*C. maculatum)* CPKS5 active site amino acids. Underlined amino acids are changed and numbering is according to the enzyme's own (Article I).

Alfalfa CHS2	Poison hemlock CPKS5
Catalytic triad	
Cysteine 164	Cysteine 163
Histidine 303	Histidine 302
Asparagine 336	Asparagine 335
"Gatekeepers"	
Phenylalanine 215	Phenylalanine 214
Phenylalanine 265	Phenylalanine 264
CoA-binding tunnel	
Lysine 55	Lysine 55
Arginine 58	Arginine 58
Lysine 62	Arginine 62
Initiation pocket	
Isoleucine 254	Isoleucine 253
Glycine 256	Glycine 255
Phenylalanine 265	Phenylalanine 264
Elongation pocket	
Threonine 132	Tyrosine 131
Serine 133	Serine 132

Threonine 194	Serine 193
Valine 196	Leucine 195
Threonine 197	Alanine 196
Glycine 216	Glycine 215
Serine 338	Serine 337
Other functions	
Valine 98	Valine 97
Methionine 137	Phenylalanine 136
Glycine 211	Threonine 210
Phenylalanine 375	Phenylalanine 374

4.1.4 PKSs of *Sarracenia* sp.

Publicly available transcriptomes of *S. psittacina* and *S. purpurea* were examined for candidate PKS-genes for coniine biosynthesis in plant species. When transcriptomes were analysed using the tblastn-program with *M. sativa* CHS2 as a template, the results were 8 and 12 sequences, respectively, for PKSs when the stringency was set to 1e-10 (Article IV). Correct reading frames were selected and aligned with each other after the nucleotide sequences were translated to amino acid sequences. This resulted in three unique contigs per species. Of these, one represents the N-terminus and two the C-terminus when compared to full-length PKS-enzyme. None of the contigs cover the middle part of the PKS-enzyme sequence but they contain all conserved amino acids in the active site in the observed area (Austin & Noel, 2000) when compared to other full-length PKSs (Figure B1, Appendix B).

4.1.5 Enzymatic testing

Various starters, for example, *p*-coumaryl- and benzoyl-CoA, were used to test the substrate specificity of CPKS1, CPKS2, and CPKS5 and the results were analysed with ultra-performance liquid chromatography (UPLC) (Article I). CPKS1 and CPKS2 have similar starter-CoA utilization (Table 8). With malonyl-, acetyl- or acetoacetic-CoA as a starter substrate, CPKS1 and CPK2 produce triacetolactone (A3) as the main compound, with small amounts of tetraketide lactone (A4) as a side product. Lactones Be3 and Be4 are produced from benzoyl- and malonyl-CoA. For CPKS1, the main compound is Be4, with a small amount of Be3 8 (Figure C1D, Appendix C), whereas CPKS2 produced Be3 and Be4 in equal relative amounts in the UPLC chromatogram (Figure D1D, Appendix D). They both produce naringenin chalcone (C4) as the only product, without any side products, from one *p*-coumaryl-CoA and three malonyl-CoAs. When hexanoyl- or octanoyl-CoA are supplied as reaction starters for CPKS1 and CPKS2, the formed main lactone is triketide with little in the side tetraketide lactone. With butyryl-CoA as a starter, the main product is the triketide lactone Bu3 for both enzymes. Lactone Bu4a and phloroglucinol Bu4b are produced in equal relative amounts as they are

products of alternative foldings at the tetraketide stage. CPKS1 and CPKS2 form only the diketide quinolone N2 in relatively low amounts when *N*-methylanthraniloyl-CoA is the starter.

CPKS5 forms the corresponding triketide lactones as main products from acetyl-, acetoacetyl-, benzoyl-, butyryl-, hexanoyl- and octanoyl-CoA with malonyl-CoA. It forms the corresponding tetraketide lactones in low amounts as side products (Figure E1, Appendix E). When malonyl-CoA is available alone, CPKS5 hardly utilizes it as a starter to form A3 and A4. The main difference between CPKS5 and CPKS1 or CPKS2 is in the butyryl-CoA products. There are no phloroglucinol derivatives among the products obtained with CPKS5. The enzyme does not catalyse any product formation from *p*-coumaryl-CoA or *N*-methylanthraniloyl-CoA.

The identities of reaction products were determined by their relative retention times (R_t), UV-spectra and masses (Table 9). Triacetolactone (A3) and naringenin chalcone (C4) were used as pure compound standards in the analyses. Lactones A3, A4, H3, H4, Be3, and Be4 were identified on the basis of their elution order, UV-spectra and masses (Karppinen et al., 2008). Lactones O3 and O4 were identified based on their elution order when compared to the similar lactones H3 and H4. Their mass of $[M\text{-}H]^-$ was 28 amu higher than the mass of H3 and H4, respectively, which corresponds to one C_2H_4 unit as the used starter-CoA was octanoyl-CoA.

Lactones Bu3 and Bu4 were identified on the basis of their elution order and mass (Jindaprasert et al., 2008). Phlorobutyrophenone (Bu4b) was identified based on its fragmentation pattern (*m/z* 195, 151 (100), 125), which is similar to that of phlorocaprophenone (Ghosh et al., 2008), the only difference being in its mass (28 amu lower). Quinolinone N2 was identified based on mass (Resmi et al., 2013).

Table 8 Products formed in *in vitro* experiments (Article I). More detailed information is available in Appendix C for CPKS1, Appendix D for CPKS2 and Appendix E for CPKS5.

	Enzyme		
Starter-CoA	CPKS1	CPKS2	CPKS5
Malonyl-CoA	A3*	A3*	A3*
	A4	A4	A4
Acetyl-CoA	A3*	A3*	A3*
	A4	A4	A4
Acetoacetyl-CoA	A3*	A3*	A3*
	A4	A4	A4
Benzoyl-CoA	Be3	Be3	Be3*
	Be4*	Be4	Be4
Butyryl-CoA	Bu3*	Bu3*	Bu3*
	Bu4a	Bu4a	Bu4a
	Bu4b	Bu4b	N.D.
p-Coumaryl-CoA	C4	C4	N.D.
Hexanoyl-CoA	H3*	H3*	H3*
	H4	H4	H4
N-Methylanthraniloyl-CoA	N2	N2	N.D.
Octanoyl-CoA	O3*	O3*	O3*
	O4	O4	O4

*Main product; N.D. not detected

Table 9 *In vitro* reaction products using different starters. Structures, retention times and UV-absorbance were determined with UPLC-MS (Article I).

Starter-CoA	Polyketide level	Product	IUPAC name	Rt (min)	λ_{max} (nm)	*m/z* $[M-H]^-$	Structure
Acetyl-/Acetoacetyl-CoA	Triketide	A3	4-Hydroxy-6-2*H*-pyran-2-one	2.16	283	125	
	Tetraketide	A4	4-Hydroxy-6-(2-oxopropyl)-2*H*-pyran-2-one	1.78	282	167	
Benzoyl-CoA	Triketide	Be3	4-Hydroxy-6-phenyl-2*H*-pyran-2-one	4.31	283	187	
	Tetraketide	Be4	4-Hydroxy-6-(2-oxo-2-phenylethyl)-2*H*-pyran-2-one	4.13	319	229	
Butyryl-CoA	Triketide	Bu3	4-Hydroxy-6-propyl-2*H*-pyran-2-one	3.77	284	153	
	Tetraketide	Bu4a	4-Hydroxy-6-(2-oxopentyl)-2*H*-pyran-2-one	3.59	284	195	

	Tetraketide	Bu4b	1-(2,4,6-Trihydroxy-phenyl)butan-1-one	4.89	225.7, 284.7	195	
p-Coumaryl-CoA	Tetraketide	C4	5,7-Dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4*H*-chromen-4-one	4.66	289	271	
Hexanoyl-CoA	Triketide	H3	4-Hydroxy-6-pentyl-2*H*-pyran-2-one	5.02	284	181	
	Tetraketide	H4	4-Hydroxy-6-(2-oxoheptyl)-2*H*-pyran-2-one	4.87	284	223	
N-Methylanthraniloyl-CoA	Diketide	N2	4-Hydroxy-1-methylquinolin-2(1H)-one	3.84	228, 274, 315	174	
Octanoyl-CoA	Triketide	O3	6-Heptyl-4-hydroxy-2*H*-pyran-2-one	6.14	225.7, 284.7	209	
	Tetraketide	O4	4-Hydroxy-6-(2-oxononyl)-2*H*-pyran-2-one	5.92	225.7, 283.2	251	

The enzyme kinetic parameters of CPKS5 were tested with the starters acetyl-, butyryl-, hexanoyl- and octanoyl-CoA with [2-^{14}C]malonyl-CoA. Total activity per reaction was formed primarily by triketide compounds, with minuscule amounts of tetraketides. Since PKSs can decarboxylate malonyl-CoA to acetyl-CoA, which in turn acts immediately as a starter (Eckermann et al., 2003), the base level for the kinetic measurements of the substrate feeding is always elevated. To measure enzymatic activity, optimum pH was investigated using 57 µM butyryl-CoA over a pH range of 5.2-7.2 (Figure 22). Based on the results the optimal pH for CPKS1 is 6.6 and for CPKS5 6.2.

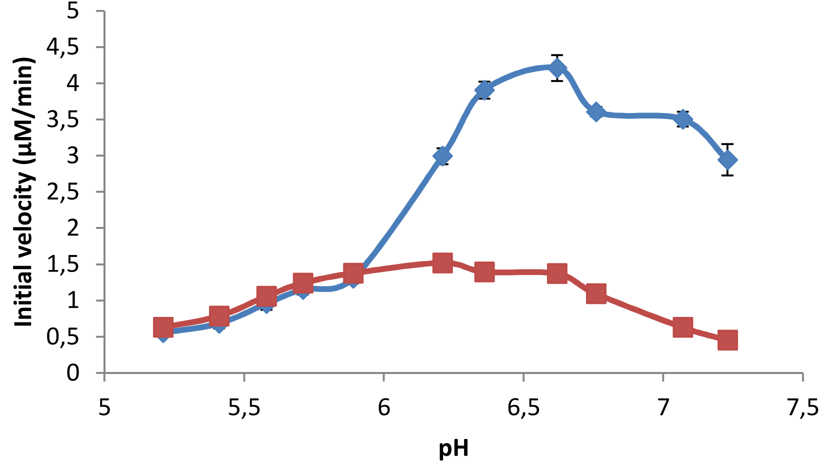


Figure 22 pH-optima of (♦) CPKS1 and (■) CPKS5. The reactions were performed with 5 µg of purified enzyme, 57 µM butyryl-CoA, 198 µM malonyl-CoA and 2 µM [2-^{14}C]malonyl-CoA in the pH-range 5.2-7.2.

The enzymatic parameters for butyryl-CoA were calculated and the results were fitted into the Michaelis-Menten kinetics model. The calculated parameters for butyryl-CoA consumption by CPKS5 using the Lineweaver-Burk plot (R^2 = 0.9946) were: K_m = 6.63±0.26 µM, k_{cat} = 0.63±0.01 min^{-1}, k_{cat}/K_m = 1595±49 s^{-1} M^{-1}. To compare butyryl-CoA usage by CPKS1 and CPKS2, their parameters were also calculated. The kinetic parameters for CPKS1 were: K_m = 4.14±0.96 µM, k_{cat} = 1.73±0.15 min^{-1}, k_{cat}/K_m = 7178±1306 s^{-1} M^{-1} at pH 7.0; and for CPKS2 K_m = 5.9±3.11 µM, k_{cat} = 1.70±0.05 min^{-1}, k_{cat}/K_m = 5522±2114 s^{-1} M^{-1} at pH 7.0. The detected activity increased little or not at all from the base level when acetyl-, hexanoyl- or octanoyl-CoA were used as a starter substrate for CPKS5 (Figure 23). These results demonstrate that CPKS5 favours butyryl-CoA as a starter among the tested compounds.

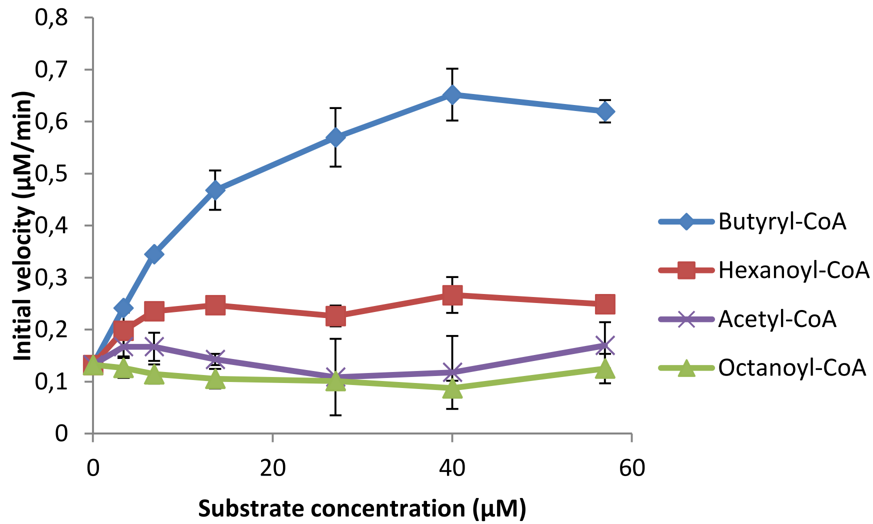


Figure 23 Kinetic testing of CPKS5 at pH 6.2. The reactions were performed with 5 µg of purified enzyme, 0-100 µM starter-CoA, 198 µM malonyl-CoA and 2 µM [2-^{14}C]malonyl-CoA (Article I).

4.2 Callus induction

4.2.1 Poison hemlock

Callus was induced from poison hemlock in order to study alkaloid biosynthesis and its inducibility. To initiate callus, leaf stalks were chosen as the target plant material (Article II). The initiation frequency was assessed after 36 days. Out of the 50 callus initiation plates, 15 plates were contaminated either with mould or bacteria and therefore discarded. Poison hemlock leaf stalks formed calli on Woody plant (WP) medium containing 0.063-0.25 mg/l 6-benzylaminopurine (BA) and 1-4 mg/l naphthalene acetic acid (NAA) (Table 10). The best hormone combination to induce callus was 0.188 mg/l BA and 1, 2 or 4 mg/l NAA. The production of stable and multiplying lines took over six months. The formed lines were mostly green, except one white, and the texture varied from hard to soft but none was hyperhydrated. The established callus lines were later transferred to WP medium with 3 mg/l NAA and 0.125 mg/l BA for easier maintenance.

Table 10 Callus formation on poison hemlock (*C. maculatum*) leaf stalks grown on WP medium (Article II).

Hormone concentration		**Callus formation and survival**	
BA (mg/l)	NAA (mg/l)	Survival	Percentage of explants (n = 20) forming callus (%)
0	0	+	40
0.063	0	-	0
0.125	0	+	25
0.188	0	+	20[a]
0.250	0	-	0[a]
0	1	-	0[a]
0.063	1	+	30
0.125	1	++	30
0.188	1	++	100
0.250	1	++	50[a]
0	2	-	0
0.063	2	+	90[a]
0.125	2	-	0[b]
0.188	2	++	100[a]
0.250	2	++	60[a]
0	3	-	0[a]
0.063	3	++	25
0.125	3	++	65
0.188	3	+	25
0.250	3	+	30[a]
0	4	-	0
0.063	4	+	30[a]
0.125	4	+	80[a]
0.188	4	++	100
0.250	4	+	80

- No callus formation; + Callus formation; ++ Callus formation and callus surviving the establishment phase; [a](n = 10); [b](n = 0)

4.2.2 *Aloe* sp.

Callus was induced from *A. gariepensis* and *A. viguieri* in order to study alkaloid biosynthesis and its inducibility. The callus was induced on leaves or stalk of *A. gariepensis* and *A. viguieri* using two different hormone regimes on Murashige and

Skoog (MS) medium: 10.0 mg/l NAA and 0.2 mg/l BA; and 6.0 mg/l 2,4-D (Article III). The callus induction was evaluated after 50 days. On medium containing NAA and BA, 64% of the *A. gariepensis* explants produced callus, whereas on medium containing 2,4-D only 5% responded (Table 11). By contrast, *A. viguieri* explants produced callus (40%) only on 2,4-D and did not respond to the NAA and BA hormone combination.

Table 11 Callus induction in *A. gariepensis* and *A. viguieri* after 50 days of culture with different combinations of plant growth regulators (Article III).

Species	Hormone concentration			Number of explants	Number of explants producing callus	Explants producing callus (%)
	2,4-D (mg/l)	NAA (mg/l)	BA (mg/l)			
A. gariepensis	6	0	0	20	1	5
	0	10	0.2	25	16	64
A. viguieri	6	0	0	25	10	40
	0	10	0,2	20	0	0

The callus induction medium contained the supplements polyvinylpyrrolidone (PVP), adenine and ascorbic acid to prevent browning of medium and explants. With the additives, the explants survived the callus initiation phase. There was a noticeable reduction in medium browning and growth of calli when the medium supplements were left out. Root formation was observed on some of the explants of *A. gariepensis* on MS with 10.0 mg/l NAA and 0.2 mg/l BA. Calli of both species grew more vigorously on MS with 2.0 mg/l 2,4-D and subculturing was made every second week without browning of the medium. Finally, the 2,4-D content was decreased to 1.0 mg/l and the subculturing was reduced to a three-week interval without any detrimental effect (Figure 24).

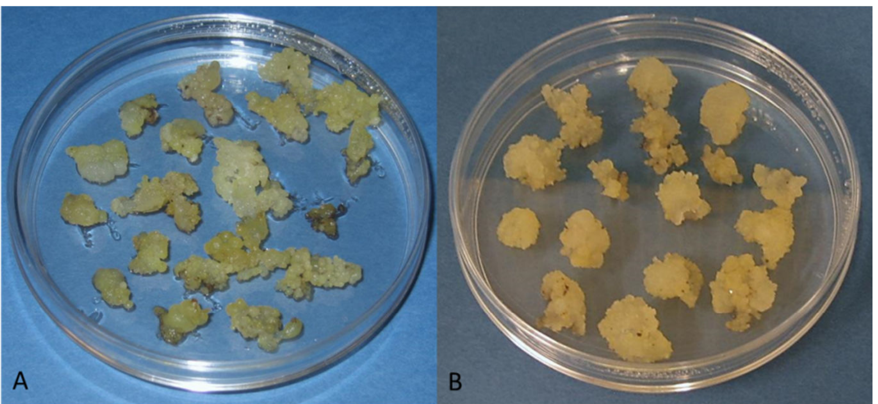


Figure 24 Callus of **A** *A. gariepensis* and **B** *A. viguieri* (Article III).

Callus fresh weight development was followed for three weeks. The calli were grown in the dark at 25°C. Three lines from both species were selected and labelled as A-C (*A. gariepensis*) and D-E (*A. viguieri*) based on vigorous growth and healthy appearance (without browning or leaching of phenolics into the medium). Statistical analysis did not reveal significant differences in the original starting weights (Table 12). All six lines grew similarly during weeks I and II, without statistically significant changes in growth except for line D. This callus line showed the highest and statistically significantly different growth rate in comparison to the other lines throughout the duration of the experiment. Generally, *A. viguieri* calli grew faster than *A. gariepensis* (28-86% weight increase versus 12-48%). The greatest weight increase for all lines occurred in week II. Among the *A. gariepensis* lines, C exhibited the greatest weight increase, on average 37% per week, whereas line B showed the slowest growth altogether, with 21% average increase. The fresh weight of the *A. viguieri* line D increased most, on average 72% per week, and line F was slowest, on average 39%, which was still higher than any of the *A. gariepensis* lines.

The establishment of the suspension cultures for *A. gariepensis* and *A. viguieri* calli took over six months. During this time period, the subculturing interval was two weeks in order to counter leaching of polyphenols into the medium. The leaching slowed down or ceased altogether and no hyperhydricity was observed in calli. In each subculturing cycle, the callus was cut into smaller pieces with a spoon, which led to the size of callus becoming smaller as the goal was a fine suspension of cell culture for easier maintenance.

Table 12 Fresh weight of selected callus lines of *A. gariepensis* and *A. viguieri* measured over 3 weeks (Article III).

		Callus weight (fresh weight)								
		Week 0	Week I		Week II		Week III[1]		Average increase/week	
Species	Callus line	Weight (g)	Weight (g) (Increase)	Increase (%)	Weight (g) (Increase)	Increase (%)	Weight (g) (Increase)	Increase (%)	Weight (g)	Increase (%)
A. gariepensis	A	0.146±0.049	0.170±0.058 ($0.024 \pm 0.014_{ab}$)	17	0.236±0.078 ($0.066 \pm 0.026_{a}$)	40	0.313±0.099 ($0.077 \pm 0.034_{a}$)	33	0.056±0.024	30
	B	0.096±0.048	0.107±0.052 ($0.012 \pm 0.011_{a}$)	13	0.136±0.066 ($0.028 \pm 0.019_{a}$)	28	0.168±0.091 ($0.032 \pm 0.031_{a}$)	22	0.024±0.020	21
	C	0.128±0.052	0.162±0.074 ($0.034 \pm 0.029_{ab}$)	25	0.240±0.120 ($0.078 \pm 0.054_{a}$)	45	0.331±0.196 ($0.105 \pm 0.090_{a}$)	40	0.072±0.058	37
A. viguieri	D	0.120±0.049	0.195±0.081 ($0.076 \pm 0.038_{b}$)	65	0.363±0.146 ($0.168 \pm 0.084_{b}$)	90	0.607±0.243 ($0.243 \pm 0.114_{b}$)	67	0.162±0.079	74
	E	0.133±0.034	0.177±0.044 ($0.044 \pm 0.014_{ab}$)	34	0.254±0.082 ($0.082 \pm 0.034_{a}$)	47	0.345±0.117 ($0.083 \pm 0.085_{a}$)	45	0.070±0.045	42
	F	0.096±0.033	0.123±0.056 ($0.027 \pm 0.035_{ab}$)	27	0.187±0.106 ($0.064 \pm 0.058_{a}$)	51	0.285±0.252 ($0.098 \pm 0.156_{a}$)	38	0.063±0.083	39

Statistically significant differences in weight. Means not sharing the same letter are significantly different (Tukey HSD, $p < 0.05$). Comparisons were made between the lines at a particular time-point. [1]Results analysed with One-way ANOVA, except for cases when the homogeneity of group variances did not fulfil the criteria of ANOVA (Levene $p<0.05$) and in those cases Tamhane's T2 was used.

4.3 Elicitation of poison hemlock callus

4.3.1 Biomass accumulation

A growth curve was generated to choose an optimal time point for elicitation studies to induce coniine biosynthesis in poison hemlock cell culture. Cell line 2-3 (Figure 25) was selected from the generated cell lines for testing due to its bright green colour, visibly fast growth and soft texture (Article II). An inoculum of 20 g/l fresh cell weight (FCW) exhibited 90% viability after one week (versus 80% for 10g/l and 75% for 40.0 g/l), and hence the concentration with the highest viability was chosen for the elicitation experiments. Before actual elicitations were performed, a growth curve experiment was carried out with different measured indicators: biomass accumulation (FCW and dry cell weight (DCW)), use of nutrients (pH and conductivity) and sugars. FCW and DCW increased rapidly when the exponential phase started on day four until the growth curve entered the stationary phase on day 12 (Figure 26A). The maximal biomass accumulation was reached on day 12 of the experiment (312.2±13.1 g/l FCW and 12.08±0.34 g/l DCW). Conductivity decreased rapidly from 2.736±0.032 mS/cm at day 0 to 1.261±0.025 mS/cm at day 6, due to entering the exponential phase, followed by a gradual reduction to 1.163±0.006 mS/cm by the end on day 13 (Figure 26B). Before day 8, pH-values ranged from 5.10±0.01 to 4.84±0.07. On day 13 the pH-values had decreased to 4.25±0.10. The glucose level in the medium increased from 0.25% on day 0 to 3.0% on day 3 due to hydrolysation of sucrose in the medium. The sugar content then decreased linearly to 0.5% on day 11, where it remained until the end of the experiment. Despite high viability, a colour change was observed from greenish cells on day 9 to a bleached green appearance on day 13. Generally, cell aggregates broke down into smaller ones, or into single cells, as the culture aged.

Figure 25 Callus line 2-3 of poison hemlock (Article II).

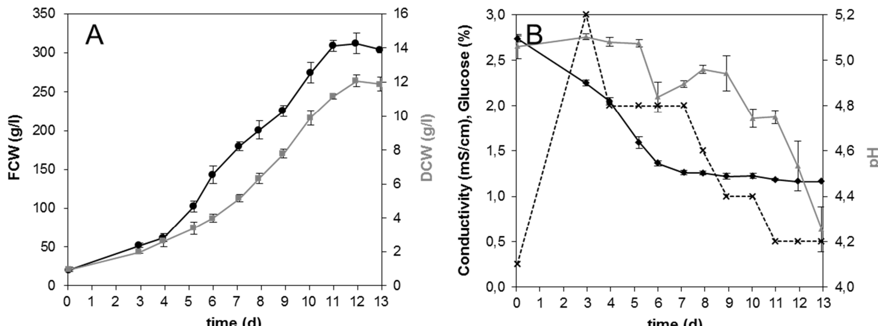


Figure 26 Growth of poison hemlock cells (line 2-3) in WP medium. Biomass accumulation of (**A**) fresh cell weight (FCW) (●) and dry cell weight (DCW) (■) is shown as well as (**B**) conductivity (♦), pH (▲) and glucose content (×) of the medium until stationary phase. SD was calculated from n = 5 samples until day 10 and thereafter from n = 3 (Article II).

4.3.2 Elicitation

Suspension culture of poison hemlock cell line 2-3 was elicited with alginic acid (550 mg/l), cellulase (27.5 mg/l), chitosan (110 mg/l), copper(II) sulphate (275 mg/l), ethephon (110 mg/l), methyl jasmonate (12.3 mg/l), salicylic acid (15.2 mg/l) and silver nitrate (275 mg/l) to induce synthesis of hemlock alkaloids. Samples were collected on days 1, 3 and 5 after elicitation (Article II). Cells and medium were separated and extracts were prepared from them which were analysed by thin layer chromatography (TLC). Extracts of medium harvested on day three after elicitation were used for further secondary metabolite analysis due to pre-test TLC results. Samples, which were collected three days after elicitation, indicated the presence of multiple compounds and were therefore further analysed by gas chromatography-mass spectrometry (GC-MS).

The elicited or non-elicited callus cultures were devoid of hemlock alkaloids. GC-MS chromatograms of analysed samples were examined for peaks of known alkaloids at the characteristic R_t (coniine, R_t = 4.55 min; γ-coniceine, R_t = 5.25 min; *N*-methylconiine, R_t = 5.15 min; conhydrine, R_t = 7.40 min; conmaculatin, R_t = 7.69 min). Peaks present at these retention times did not have the characteristic fragments (Table 13), and no hemlock alkaloids were identified via database match.

Table 13 Characteristic GC-MS fragmentation for selected piperidine alkaloids (Article III).

Compound	R_t (min)	Match (%)	Note	Characteristic fragments
Coniine	4.55	86%	*a*	127 (M^+), 126, 98, 84 (base peak), 70, 56
γ-Coniceine	5.25	72%	*b*	125 (M^+), 124, 110, 97 (base peak), 82, 70
N-Methylconiine	5.05		*b*	141 (M^+), 112, 99, 98 (base peak), 70
Conhydrine	7.40	78%	*a*	143 (M^+), 114, 96, 84 (base peak), 67, 56, 41
Conmaculatin	7.69		*b*	155 (M^+), 154, 140, 126, 112, 96, 84 (base peak), 77, 70, 56, 41

[a] Identified by comparison of retention data and mass spectral data with reference material. [b] Identified by comparison to known spectra (Holstege et al., 1996; Radulović et al., 2012; Palisade Complete 600K Mass Spectral Library).

Among the tested elicitors, only cell wall components (alginic acid, chitosan), an enzyme (cellulase) and heavy metals (copper(II) sulphate, silver nitrate) resulted in secondary metabolite formation. Non-elicited cultures and elicitation with plant hormones (ethylene released from ethephon, methyl jasmonate, and salicylic acid) did not trigger a similar response. The comparison of mass spectra of sample extracts with the Palisade Complete 600K Mass Spectral Library, the NIST Mass Spectral Search Program, and literature data revealed the presence of several furanocoumarins, which were identified as psoralen, marmesin, xanthotoxin, bergapten, columbianetin, isopimpinellin and oroselone (Figure 27; Appendix F). Only those with strict match databases (84% or higher) and/or the spectra matched with the literature are listed in Table 14. Furanocoumarins were mainly present in the medium and to a lesser extent in the cell fraction. Bergapten and isopimpinellin were produced by all effective elicitors. Isopimpinellin was the most abundant both in the medium (max. 43.23% peak area of selected peaks) and in the cells (max. 10.97%). Chitosan caused the most diverse furanocoumarin production, including linear and angular furanocoumarins, both in the media and in cells. Alginic acid had a similar effect, but not all compounds were found in the cell fraction at comparable concentrations. Elicitation with cellulase caused only bergapten and isopimpinellin biosynthesis, both accruing in the cells and in the medium, whereas columbianetin was only found in the medium. Unlike the cell wall elicitors, furanocoumarins elicited with copper(II) sulphate were only present in trace amounts in cell extracts and comparably low relative amounts in the medium. Silver nitrate elicitation only triggered columbianetin accumulation in the medium.

Table 14 Furanocoumarins detected by GC-MS in SCAN mode in medium and cell extracts of poison hemlock at three days after elicitation. Furanocoumarins of each extract are listed individually in the order of their R_t. Library matches (Qual.) of peaks greater than 90% and their associated CAS numbers are listed. Those peaks with lower than 90% match and identified on the basis of literature data are presented in parentheses (Article II).

Elicitor	R_t (min.)	Qual. (%)	CAS No.	Name	Area (%) of medium extract	Area (%) of cell extract
Non elicited (Neg)	-	-	-	no furanocoumarins	-	-
Alginic acid (550 mg/l)	14.88	96	66-97-7	Psoralen	5.63	trace
	15.90	98	298-81-7	Xanthotoxin	14.66	1.68
	16.02	98	484-20-8	Bergapten	12.17	2.95
	16.42	84	1760-27-6	(Oroselone)	0.99	-
	16.75	86	13849-08-6	(Marmesin)	1.94	-
	16.82	96	482-27-9	Isopimpinellin	43.23	5.58
	17.18	93	3804-70-4	Columbianetin	5.66	trace
Cellulase (27.5 mg/l)	16.03	98	484-20-8	Bergapten	30.52	1.34
	16.82	98	482-27-9	Isopimpinellin	38.42	4.79
	17.20	84	3804-70-6	(Columbianetin)	1.68	-
Chitosan (110 mg/l)	14.87	94	66-97-7	Psoralen	11.07	0.68
	15.78	99	298-81-7	Xanthotoxin	15.78	3.15
	16.02	98	484-20-8	Bergapten	7.46	3.83
	16.42	95	1760-27-6	Oroselone	1.33	3.54
	16.75	[a]	13849-08-6	(Marmesin)	0.57	0.98
	16.82	98	482-27-9	Isopimpinellin	25.49	10.97
	17.17	93	3804-70-4	Columbianetin	7.77	3.40
Copper(II) sulphate (275 mg/l)	15.93	96	298-81-7	Xanthotoxin	2.80	trace
	16.04	90	484-20-8	Bergapten	0.86	-

	16.83	90	482-27-9	Isopimpinellin	3.62	-
	17.19	[a]	3804-70-4	(Columbianetin)	4.26	-
Silver nitrate (275 mg/l)	17.19	[a]	3804-70-4	(Columbianetin)	3.37	-
Ethephon (110 mg/l)	-	-	-	no furanocoumarins	-	-
Methyl jasmonate (12.3 mg/l)	-	-	-	no furanocoumarins	-	-
Salicylic acid (15.2 mg/l)	-	-	-	no furanocoumarins	-	-

[a] Identification based on literature comparison.

	R_1	R_2
Psoralen	H	H
Xanthotoxin	OCH_3	H
Bergapten	H	OCH_3
Isopimpinellin	OCH_3	OCH_3

Columbianetin

Oroselone

Marmesin

Figure 27 Furanocoumarins elicited in poison hemlock callus line 2-3 (Article II).

4.4 Micropropagation of *Aloe viguieri*

Micropropagation of *A. viguieri* was planned using a computer-assisted experimental design to propagate high-quality plants (Article III). Statistics of the experiment demonstrated the significance and good predictive power when three parameters were at levels 0.06-0.25 mg/l (BA), 0.1-0.4 mg/l (NAA), and four, five and six weeks (time) when one outlier was omitted (Table G1, Appendix G). The quality of the fit of the polynomial model equation is expressed as coefficient of determination (R^2) and it should be over 0.5. Other factors are: The predictive

power of the model (Q^2), which should be over 0.1 to make a statistically significant model; the validity of the model, which should be 0.25 to conclude the trueness of the model; and the reproducibility of the model, which should be 0.5 for repeatability of the experiment (Umetrics, 2011). The R^2 was 0.952, which indicated that 95.2% of the variability of the response was explained by the model. The Q^2 was 0.821, the validity of the model was 0.982 and the reproducibility was 0.808. Plantlets emerged already within four weeks from the axillary buds at the base of the mother plant. The highest plantlet number (5) was achieved with BA 0.25 mg/l and NAA 0.4 mg/l (Figure 28). Hormone concentrations lower than 0.25 mg/l BA and 0.4 mg/l NAA produced only up to two plantlets (Table G1, Appendix G).

Further investigation of the same model design with higher BA (0.25-4.0 mg/l) and NAA (0.4-2.0 mg/l) levels at the same time points as previously (four, five and six weeks) did not allow fitting of a good quality model due to low significance (R^2 = 0.267) with a poor predictive power (Q^2 = -0.804). Thus, a more optimal hormone combination for *A. viguieri* micropropagation could not be ascertained. Although a hormone combination of 4.0 mg/l BA and 1.2 mg/l NAA produced seven *A. viguieri* plantlets within four weeks and nine plantlets in six weeks (Table G2, Appendix G), the formed plantlets were hyperhydrated and consequently exhibited poor viability.

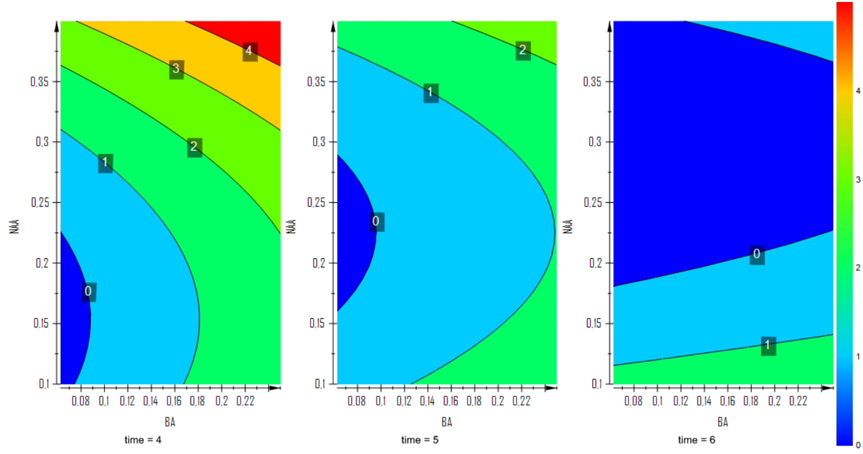


Figure 28 Contour plot of *A. viguieri* micropropagation (factors: BA, NAA, and time; response: plantlet number). Altogether 17 experiments were performed. Model parameters were R^2 = 0.952 and Q^2 = 0.821 with one outlier (Article III).

4.5 Alkaloid detection in plant material

4.5.1 Alkaloids in *Aloe* sp.

In vitro leaves and roots of *A. gariepensis*, *A. globuligemma* and *A. viguieri* together with generated callus lines of *A. gariepensis* and *A. viguieri* (Section 4.2.2) were analysed with GC-MS for their piperidine alkaloid content. The identification of coniine in *Aloe* samples was based on the R_t (4.55±0.05 min) and mass spectrum, especially the base peak (*m/z* 84), of the commercial reference (Article III). γ-Coniceine was recognized using the mass spectral database (number 28670, Palisade Complete 600K Mass Spectral Library) and comparing to literature data (Holstege et al., 1996; Table 13). The retention time (5.05±0.05 min) and base peak (*m/z* 98) of *N*-methylconiine were derived from poison hemlock (Holstege et al., 1996) (Figure H1E, Appendix H). In addition, *N*-methylconiine did not form trimethylsilyl derivatives with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, thus marking the presence of a tertiary amino group. Conhydrine was also present in reference material, poison hemlock. Its spectrum matched with the spectral database (number 20048, Palisade Complete 600K Mass Spectral Library). However, conhydrine was absent in all studied *Aloe* material. In GC-MS analyses of a coniine, a dilution series showed that in SCAN mode the detection limit was 10 µg/ml, which corresponded to 0.01 mg/g dry weight.

Neither the leaves of *in vitro* grown *A. gariepensis* nor any of the derived callus lines contained alkaloids, but roots contained traces of coniine and *N*-methylconiine (Table 15). *In vitro* grown leaves of *A. globuligemma* contained coniine, *N*-methylconiine and γ-coniceine, whereas roots did not contain any alkaloids. Leaves and roots of *in vitro* grown *A. viguieri* contained coniine, *N*-methylconiine, and γ-coniceine. None of the analysed *A. viguieri* callus lines contained piperidine alkaloids.

Table 15 Piperidine alkaloids in *A. gariepensis*, *A. globuligemma* and *A. viguieri* plants and cell cultures (Article III). Piperidine alkaloids of each extract are listed individually in the order of their R_t. Library matches (Qual.) of peaks and their associated CAS number are listed. Those peaks with only the base peak fragment present are presented in parentheses (Article III).

Species	Sample	R_t (min)	Qual. (%)	CAS No.	Name	Are (%) of extract
A. gariepensis	leaf, *in vitro*	-	-	-	no piperidine alkaloids	-
	root, *in vitro*	4.55	*a*	458-88-8	(Coniine)	trace
		5.05	*a*	35305-13-6	(*N*-Methylconiine)	trace
	callus line A	-	-	-	no piperidine alkaloids	-
	callus line B	-	-	-	no piperidine alkaloids	-
	callus line C	-	-	-	no piperidine alkaloids	-
A. globuligemma	leaf, *in vitro*	5.85[c]	*b*	458-88-8	Coniine	0.53
		6.40[c]	*b*	35305-13-6	*N*-Methylconiine	10.93
		6.50[c]	*b*	1604-01-9	γ-Coniceine	4.24
	root, *in vitro*	-	-	-	no piperidine alkaloids	-
A. viguieri	leaf, *in vitro*	4.55	*b*	458-88-8	Coniine	4.98
		5.05	*b*	35305-13-6	*N*-Methylconiine	1.48
		5.25	90	1604-01-9	γ-Coniceine	1.89
	root, *in vitro*	4.55	86	458-88-8	Coniine	0.26
		5.05	*a*	35305-13-6	(*N*-Methylconiine)	trace
		5.25	*a*	1604-01-9	(γ-Coniceine)	trace
	callus line D	-	-	-	no piperidine alkaloids	-
	callus line E	-	-	-	no piperidine alkaloids	-
	callus line F	-	-	-	no piperidine alkaloids	-

[a] Identified on the basis of the base peak fragment present (coniine *m/z* 84, γ-coniceine *m/z* 97, *N*-methylconiine *m/z* 98);
[b] Identified by comparison to known spectra (Holstege et al., 1996; Palisade Complete 600K Mass Spectral Library);
[c] Different GC-MS analysis conditions.

4.5.2 Coniine in *Sarracenia* sp.

S. flava and seven other *Sarracenia* species were analysed to determine whether they contain coniine, of which which *S. flava* is known to have very little (Mody et al., 1976). A selected ion monitoring (SIM) method was developed for GC-MS to detect coniine in low concentrations. Based on the fragmentation pattern of coniine (Table 13), characteristic ions *m/z* 56, 70, 80, 84 (base peak), and 126 (mass peak) were selected (Article IV). Ions *m/z* 80, 84, and 126 are specific for coniine; by contrast ions *m/z* 56 and 70 are shared with many other molecules.

With the GC-MS method coniine eluted at a constant retention time (6.33±0.01 min) in spiked barley material and poison hemlock leaf extract. The analyses of the samples were based on SCAN mass spectra and were compared to a database. Pure coniine matched the database with 86%, or in plant matrix 78-86% identity. Due to the stable retention time, a match lower than 90% was acceptable especially since the ions 80, 84, and 126 exhibited the same relative abundances when detected in the matrix as in pure coniine.

The limit of detection for coniine in SIM was 1 µg/ml, which corresponded to 1 µg/g dry weight. Using SIM as a detection method, coniine was identified from *S. alata*, *S. flava*, *S. leucophylla*, *S. minor*, *S. oreophila*, *S. psittacina*, *S. purpurea* and *S. rubra* (Table 16). Out of these, *S. flava* and *S. alata* samples contained coniine at the detection limit, whereas other samples had coniine at somewhat higher levels. Depending on the used classification system, the plants *S. alabamensis* and *S. rosea* also contain coniine. *S. jonesii* was not part of the studied set. In *S. minor* var. *okefenokeensis* pitcher and *S. oreophila* lid, no coniine was detected. During the handling of samples, *S. leucophylla* pitcher and *S. psittacina* (Gulf Giant) lid were lost.

Table 16 Coniine detection in *Sarracenia* spp. (Article IV).

Species	Lid	Pitcher
Sarracenia alata 'Black Tube'	x	x*
Sarracenia alata	x	x
Sarracenia flava	x*	-
Sarracenia flava var. *atropurpurea*	x*	x*
Sarracenia flava var. *maxima*	x*	x*
Sarracenia flava var. *ornata*	x	x*
Sarracenia leucophylla	x	†
Sarracenia minor var. *okefenokeensis*	x	-
Sarracenia oreophila	-	x
Sarracenia psittacina (Gulf Giant)	†	x
Sarracenia psittacina	x	x
Sarracenia purpurea subsp. *burkii*	x	x
Sarracenia purpurea subsp. *venosa*	x	x
Sarracenia purpurea subsp. *venosa* var. *burkii* f. *luteola*	x	x
Sarracenia purpurea subsp. *venosa* var. *montana*	x	x
Sarracenia rubra subsp. *alabamensis*	x	x
Sarracenia rubra subsp. *gulfensis*	x	x

Explanation: x present, - not present, *within the limit of detection (1 µg/ml), † sample was lost during analysis

5. Discussion

5.1 Hemlock alkaloid biosynthesis and its relation to polyketide synthases

Previously, coniine alkaloid biosynthesis has been studied in poison hemlock and several key reactions and enzymes have been identified. However, alkaloid biosynthesis in *Aloe* sp. or *Sarracenia* sp. has not been studied. How similar or different they are when compared to poison hemlock is unknown (Reynolds, 2005).

The problem of identifying the enzyme responsible for carbon backbone formation of coniine alkaloids was approached by isolating and characterizing full-length genes of type III PKSs expressed in tissues of poison hemlock (Article I). CPKS1, 2 and 5 were isolated together with fragments of CPKS3, 4 and 6-12 from poison hemlock. Based on the phylogenetic analysis alone the functions of CPKS1, 2 and 5 in poison hemlock could not be assigned (Weng & Noel, 2012). The number of obtained reads was in a similar range to that obtained from *Gerbera hybrida* libraries (2-18 million reads) (Bashandy et al., 2015). *Sarracenia psittacina* and *S. purpurea* transcriptome libraries contained fewer contigs (46275 and 36681, respectively) than poison hemlock (Srivastava et al., 2011). The differentiated expression pattern between CPKS1 and CPKS2 is expected, as other plant species, *e.g. Gerbera* (Deng et al., 2014), exhibit similar patterns when there are multiple members of the same gene family present.

CPKS1 and CPKS2 appear as typical CHSs based on their substrate acceptance (Article I). They catalyse naringenin chalcone production from *p*-coumaryl- and malonyl-CoA *in vitro,* like the reference enzymes parsley CHS and gerbera GCHS1. They do not produce resveratrol, and neither are any side products detected such as bisnoryangonin or *p*-coumaryl triacetolactone (CTAL). CPKS1 and CPKS2 are neither acridone nor quinolone synthases, since they barely accept *N*-methylanthraniloyl-CoA and form only quinolone N2 in tiny amounts. No unusual compounds were detected from benzoyl-CoA or any other starter in addition to the expected tri- and tetraketide lactones formed from corresponding starters. By contrast, CPKS5 differs from CHSs at the sequence level and in terms of substrate acceptance. CPKS5 displays a conserved catalytic triad, cysteine-164, histidine-303 and asparagine-336 (amino acids are numbered according to alfalfa CHS2 [Junghans et al., 1993]), like all active type III PKSs

(Austin & Noel, 2003). Phenylalanine-215 and phenylalanine-265, that function as gatekeepers controlling traffic to and from the active site, are also conserved (Austin & Noel, 2003). Only the glycine-211 to threonine substitution affects the gatekeeper phenylalanines. It also changes the pocket shape of the starter binding site (Austin & Noel, 2003). Bibenzyl synthase shows the same substitution (Austin & Noel, 2003).

Furthermore, there are six more amino acids changed in the active site of CPKS5 (Article I). Methionine-137 to phenylalanine substitution causes a significant change in the active site due to the bulkier side group. The amino acid at this position protrudes into the other monomer's active site, affecting its cyclization pocket (Jez et al., 2000) and leading to unsymmetrical active sites in the homodimers of the enzyme according to the computer model of CPKS5. Residues 132-137 are among the positions differentiating STS and CHS, and these specific residues are responsible for the alternative cyclization of STS versus CHS (Austin & Noel, 2003).

In the CoA-binding tunnel (Austin & Noel, 2003), lysine-62 is replaced by arginine, representing a conservative change (Article I). In CHS, lysine-62 contributes hydrogen bonds to CoA binding (Jez et al., 2000), and in the case of CPKS5 arginine brings more hydrogens into the binding.

There are four amino acids, threonine-132, threonine-194, valine-196 and threonine-197, in the elongation pocket, which are changed to tyrosine, serine, leucine and valine, respectively (Article I). The side group tyrosine is more hydrophobic and bulkier than threonine-132. In *Garcinia mangostana* benzophenone synthase, a mutation of threonine-133 to leucine, which corresponds to position 132 in alfalfa CHS2, causes a decrease in the active site volume and as a consequence, the majority of the enzymatic products are triketide lactones (Nualkaew et al., 2012). Jez et al. (2000) reported that threonine-132 may dictate starter molecule specificity and in *Sorbus aucuparia* biphenyl synthase a change to alanine at the same position influences the cyclization mechanism (Liu et al., 2007). This change in position 132 could explain CPKS5's preference to produce triketide lactones (Article I).

Threonine-194 to serine in CPKS5 constitutes a conservative change, since the amino acids threonine and serine are similar in size and chemical properties (Article I). Austin & Noel (2003) noted that position 194 is important for modulating substrate specificity and proper cyclization, together with positions 132 and 197. Valine-196 to leucine causes a change towards polar uncharged and bulkier substitution. This position also influences folding, as evidenced in the *Antirrhinum majus* CHS1 mutation valine-196 to methionine. Here a loss of naringenin chalcone formation is caused and CTAL is produced due to a change from Claisen condensation to lactonization and lowered pH optimum (Hatayama et al., 2010). The same change is in CPKS5's position 196 and may be the reason for lactonization and lowered pH-optimum when compared to CPKS1. Threonine-197 to alanine makes the side chain properties more hydrophobic. In fact, *A. arborescens* aloesome synthase displays the same change as in CPKS5 (Abe et al., 2004). Mutation studies revealed that this position controls substrate acceptance (Austin

& Noel, 2003; Abe et al., 2005), chain length (Abe et al., 2005; Mizuuchi et al., 2009) and the number of malonyl-CoA condensations (Mizuuchi et al., 2009) by steric modulation of the active site.

Many PKSs, such as CHS, are able to use aliphatic CoAs as starters *in vitro* (Jez et al., 2002). A wide variety of plant PKSs use acetyl-CoA as a starter, for example gerbera 2PS (Eckermann et al., 1998), *Hypericum perforatum* octaketide synthase HpPKS2 (Karppinen et al., 2008) and *Drosophyllum lusitanicum* hexaketide synthase DluHKS (Jindaprasert et al., 2008). Utilization of longer starters, butyryl-CoA or hexanoyl-CoA, is less often reported. There are two described cases for butyrate acceptance: In the fern *Dryopteris marginalis* labelled butyrate was used in the biosynthesis of margaspidin, a butyrylphloroglucinol derivative (Gordon et al., 1968), and incorporation into csypyrone B1 by *Aspergillus oryzae* CsyB has also been reported (Seshime et al., 2010; Hashimoto et al., 2013). The only report concerning hexanoyl-CoA utilisation is for *Cannabis sativa* olivetol synthase (OLS) (Taura et al., 2009).

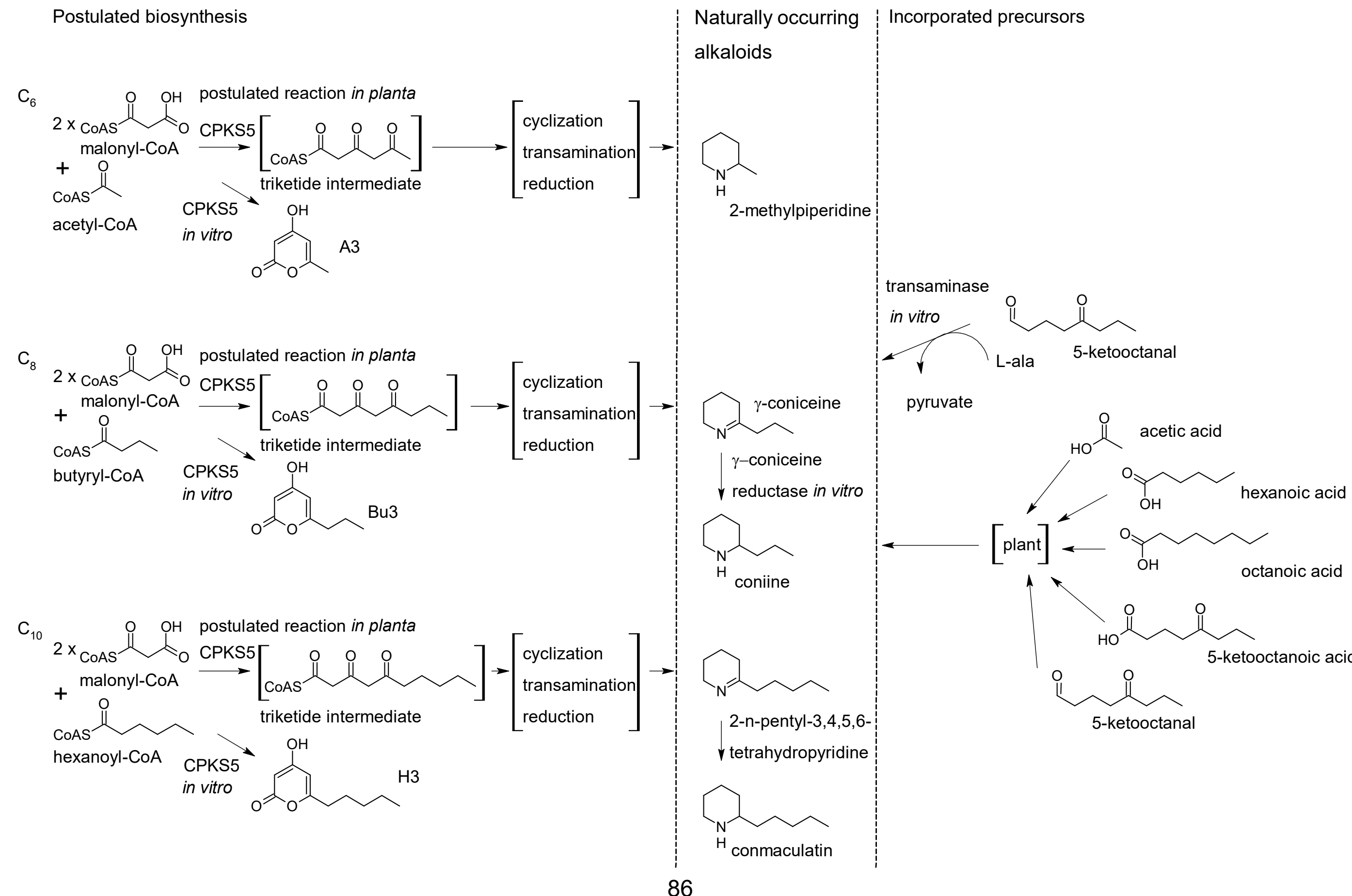
CHSs also have the capacity to use butyryl-CoA (Jez et al., 2002). CPKS1 and CPKS2 are able to use butyryl-CoA *in vitro* (CPKS1 at pH 7: k_{cat}/K_m 7178 s^{-1} M^{-1}). The efficiency of CPKS5 is lower (at pH 6.2 with k_{cat}/K_m 1595 s^{-1} M^{-1}) (Article I). However, whereas CPKS1 and CPKS2 have the ability to use *p*-coumaryl-CoA, CPKS5 has apparently lost this feature while retaining the capacity to use butyryl-CoA.

CPKS5 has a low affinity for acetyl- and octanoyl-CoA (Article I). These two precursors are also relatively poor starters for other Apiaceae PKSs, such as for example parsley CHS (Schüz et al., 1983). Increasing the starter amount did not increase activity either with acetyl-CoA, as reported for *A. arborescens* pentaketide chromone synthase (Abe et al., 2005), or with octanoyl-CoA. Among the tested aliphatic starter-CoAs, CPKS5 kinetically favours butyryl-CoA, which is elongated twice with malonyl-CoA forming the triketide lactone Bu3.

Srivastava et al. (2011) analysed the transcriptomes of *S. psittacina* and *S. purpurea*. In the transcriptomes of both species three contigs are present which belong to PKS (Article IV). They may be two to three PKS-genes, as there is one contig in the beginning and two at the end. Due to the middle part being missing from the transcriptomes of both species, the N-terminal and the C-terminal contigs cannot be assigned to each other as each contig could potentially belong to an active gene alone (Article IV). However, due to contigs not being full-length sequences a direct candidate PKS-gene for coniine biosynthesis in *Sarracenia* sp. cannot be assigned. There might be possible mutations in the active site of enzymes which could be outside the observed sequence area, as the important mutations of CPKS5 are situated there (Article I).

Leete's hypothesis (Leete, 1963 & 1964), proposing a tetraketide backbone of coniine formed from one acetyl-CoA and three malonyl-CoAs, remains unsupported because a PKS favouring acetyl-CoA as a starter could not be identified. On the other hand, the substrate preference of CPKS5 correlates with the alkaloid presence in poison hemlock. It is tempting to speculate that the same enzyme could be responsible for the polyketide formation of all C_6, C_8 and C_{10} alkaloids in

the plant. Such a scenario would involve condensation of either acetyl-, butyryl-, or hexanoyl-CoA with two malonyl-CoAs to form a triketide (Figure 29). C_8-alkaloids are the most common (*e.g.* Cromwell, 1956), consistently with butyryl-CoA being the favoured starter among those tested. C_{10}-alkaloids are minor alkaloids. Conmaculatin is a relatively new finding (Radulović et al., 2012) that probably escaped earlier analyses due to its low amounts. Hexanoyl-CoA is a suitable starter but may not be preferred naturally. The only C_6-alkaloid, 2-methylpiperidine, has occasionally been reported (Cromwell, 1956; Holstege et al., 1996), although CPKS5 does not utilize acetyl-CoA very well under the tested conditions (Article I). According to the *in vitro* substrate tests there could theoretically be C_{12}-alkaloids in poison hemlock, but hitherto these have not been observed.



Postulated biosynthesis
Naturally occurring alkaloids
Incorporated precursors
C6
2 x CoAS
O OH
malonyl-CoA
+
CoAS
acetyl-CoA
postulated reaction in planta
CPKS5
triketide intermediate
CPKS5
in vitro
A3
cyclization
transamination
reduction
2-methylpiperidine
C8
butyryl-CoA
Bu3
γ-coniceine
γ–coniceine
reductase in vitro
coniine
C10
hexanoyl-CoA
H3
2-n-pentyl-3,4,5,6-
tetrahydropyridine
conmaculatin
transaminase
in vitro
L-ala
pyruvate
5-ketooctanal
acetic acid
hexanoic acid
octanoic acid
5-ketooctanoic acid
plant

Figure 29 Proposed biosynthetic pathways leading to coniine. The C_6-, C_8- and C_{10}-alkaloids in the middle were all isolated from poison hemlock. Putative condensation products catalysed by CPKS5 are shown together with their corresponding *in vitro* derailment products on the left. Incorporated substrates and known reactions previously reported in the literature are shown on the right (Article I).

In the light of the current investigation Leete's labelling results *in planta* (Leete, 1963 & 1964) could be interpreted so that fed acetate is first activated into acetyl-CoA, part of which is further processed into malonyl-CoA. Acetyl-CoA is then elongated with malonyl-CoA by fatty acid synthase to form butyryl-CoA (Seshime et al., 2010), resulting in the labelling of even-numbered carbons when using ^{14}C-labelled acetate substrate. Then butyryl-CoA is elongated twice with malonyl-CoA containing ^{14}C originating from the fed acetate (Figure 30).

Figure 30 An alternative explanation of how the labelling pattern of ^{14}C described by Leete (1963 & 1964) could be explained. Blue dots present the labelled carbon and FAS is fatty acid synthase.

5.2 Callus induction

5.2.1 Poison hemlock

Previously, poison hemlock callus has been induced on MS medium containing either complex supplements, such as 15% coconut milk (Nétien & Combet, 1970), or plant hormones in specific amounts, for example 0.5 mg/l kinetin (KIN) and 1 mg/l 2,4-D (Wink et al., 1980b; Wink et al., 1981; Schoofs et al., 1983). Due to the observed better growth of whole hemlock plants on WP than on MS-medium, WP was chosen as the basal medium (H. Hotti, personal observation). Callus induction is easily achieved with a wide range of different hormone combinations of NAA and BA on fully defined WP medium (Article II). Combinations of 0.188 mg/l BA with either 1, 2 or 4 mg/l NAA are the most efficient to induce callus formation and at the same maintain the formed cell lines viable over the establishment phase. From the available auxins, NAA is generally preferred because of the mutagenic effects of 2,4-D (Enan, 2009). In order to minimize hormone concentrations in the long-term maintenance, calli were maintained on WP with 3 mg/l NAA and 0.125

mg/l BA (Article II). The same medium without a gelling agent was suitable for cell suspension cultures, which showed a typical growth curve for batch mode operation. The formed callus lines in liquid culture can be used for elicitation (Section 5.3) or biotransformation studies (Carew & Bainbridge, 1976; Wink et al., 1980a).

5.2.2 *Aloe* sp.

The induction of callus has been achieved in several *Aloe* species with the aim for example to produce specific compounds (Yagi et al., 1983 & 1998). In most studies MS medium has been used together with a variety of different hormone combinations (Article III). Most commonly the auxin of choice is either 2,4-D or NAA together with the cytokinins KIN or BA. 2,4-D is chosen due to its capacity in different *Aloe* species to promote callus induction (Groenewald et al., 1976; Racchi, 1988; Roy & Sarkar, 1991) and cell division in culture (Velcheva et al., 2010). Only 40% of *A. viguieri* explants formed callus on MS containing 6 mg/l 2,4-D (Article III), which is the medium which Rathore et al. (2011) used for *A. vera* with over 75% induction rate. *A. gariepensis* responded better to a hormone combination of 10.0 mg/l NAA and 0.2 mg/l BA, with 64% of explants producing callus (Article III). Yagi et al. (1998) used the same hormone combination for callus induction of *A. vera* in darkness (100% induction rate). Overall the achieved callus induction rates for *A. viguieri* and *A. gariepensis*, while generally low, are in line with previously reported rates for *A. arborescens* (27.7% and 40% in light) (Bedini et al., 2009). However, for long-term maintenance Rathore et al. (2011) recommended using lower hormone concentrations because otherwise callus cultures may become hyperhydrated. Roy & Sarkar (1991) employed 1 mg/l 2,4-D and 0.2 mg/l KIN, whereas it was determined that *A. gariepensis* and *A. viguieri* callus lines are kept viable with 1 mg/l 2,4-D without KIN (Article III). In Article III PVP, ascorbic acid and adenine were initially used as antioxidants to reduce the leaching of phenolic compounds and to prevent the browning of tissues and the surrounding culture medium. The use of PVP is based on its ability to absorb polyphenols and prevent further oxidation of phenolic compounds (Sathyanarayana & Varghese, 2007). Roy & Sarkar (1991) noted in their research that PVP was the most efficient antioxidant in preventing browning of the medium when compared to ascorbic acid and activated charcoal.

Previously only suspension cultures from *A. saponaria* (Yagi et al., 1983), *A. arborescens* (Liu et al., 2003) and *A. vera* (Raei et al., 2014) have been described. The transfer of *A. viguieri* and *A. gariepensis* callus cultures from solid to the liquid medium was unproblematic; however it took a considerable time to achieve a useable liquid culture for research purposes (Article III).

5.3 Elicitation of poison hemlock cell cultures

Before the effects of elicitation on poison hemlock suspension culture could be studied, a time point for this purpose was determined by making a growth curve of

the chosen cell line (Article II). During the first seven days of the experiment, the conductivity decreased rapidly as the nutrient salts were consumed by the plant cells. The enzymatic hydrolysis of sucrose to glucose and fructose caused the monitored glucose levels initially to increase in the medium and then to decrease as the sugars were used by the culture. The late exponential or early stationary phase is usually considered optimal for elicitor addition (Baldi et al., 2009). Based on the growth-related data collected, day nine was selected for initiation of elicitation.

In accordance with an earlier report (Nétien & Combet, 1971), non-elicited hemlock suspension cultures did not contain coniine, γ-coniceine or *N*-methylconiine, but treatment with a wide variety of elicitors including biotic cell wall compounds, plant hormones as well as abiotic heavy metals also failed to trigger alkaloid biosynthesis (Article II). By contrast, earlier attempts to elicit poison hemlock cell culture with coniine, spermidine, and cyclic AMP resulted in the production of quinolizidine alkaloids, which are compounds that do not naturally occur in poison hemlock (Wink & Witte, 1983). Wink et al. (1980a) also initiated an accumulation of quinolizidine alkaloids when they fed sparteine and 17-oxosparteine into hemlock cell culture. Roberts (1981) and Fairbairn & Suwal (1961) observed a connection between lighting conditions and alkaloid production in hemlock plants. Although this was considered by growing cultures in illuminated conditions and using green cultures in the experiments, there might be some other critical factors lacking for piperidine alkaloid biosynthesis to occur (Article II). It is known that poison hemlock plants do possess specialized ducts, called 'vittae', which function as the accumulation site for coniine alkaloids (Fairbairn & Challen, 1959; Corsi & Biasci, 1998). Therefore, a certain degree of cell differentiation might be a prerequisite for the biosynthesis or storage of coniine alkaloids.

The presence of furanocoumarins in elicited culture of poison hemlock, as a member of the Apiaceae, is expected in some conditions (Article II). Many species of this family (*e.g. Ammi majus*, carrot, parsnip) produce furanocoumarins such as psoralen, xanthotoxin, bergapten and isopimpinellin in suspension cell cultures after elicitation with a fungal elicitor (Ekiert, 2000). Furthermore, elicitation of whole poison hemlock plants with copper(II) chloride clearly increased the contents of constitutive antifungal furanocoumarins, xanthotoxin, isopimpinellin and umbelliferone (Al-Barwani & Eltayeb, 2004). The highest concentrations of these furanocoumarins were detected in poison hemlock leaves three days after elicitation, which is a similar timeframe to that which was observed in the tested cell cultures (Article II). Notably, the last missing steps in the biosynthesis of furanocoumarins, which is the prenylation of umbelliferone at two positions subsequently leading to the formation of either linear or angular versions, have been elucidated only very recently (Karamat et al., 2014).

The differential accumulation of furanocoumarins in the medium or the cells after elicitation with various elicitors was unexpected, but not completely unprecedented. Furanocoumarins are also released into the medium after elicitation of *Ruta graveolens* (Diwan & Malpathak, 2011) and *Glehnia littoralis* (Ishikawa et al., 2009) cell cultures. Diwan & Malpathak (2011) reported that in their elicitation

system a number of furanocoumarins are dependent on the elicitor, of which the most efficient is yeast extract. In the tested system biotic compounds, alginic acid, cellulase and chitosan, were also the most efficient (Article II). Zobel & Brown (1993) reported that furanocoumarin composition and concentration varies in unelicited suspension cultures of carrot and parsnip. Zobel & Brown (1993) and Ishikawa et al. (2009) speculated that there is a selective transporter system behind the furanocoumarin extrusion into the medium.

5.4 Micropropagation of *A. viguieri*

Only seven out of 400 *Aloe* species have a micropropagation protocol so far, and none of the species contain hemlock alkaloids. The published studies use MS as the basal medium, sometimes in liquid form (Barringer et al., 1996). However, there is no clear consensus on optimal hormone combinations across species (Article III). The most commonly used cytokinin is BA, which is used in the range 1.0-2.5 mg/l in combination with the auxin NAA at 0.1-1.0 mg/l. *A. viguieri* was chosen as an example from among the poison aloes, aiming at good quality plants from micropropagation employing the experimental design software, MODDE 9.0. The central composite face design model indicated significance and good predictive power when the BA and NAA levels were within the range 0.06-0.4 mg/l and 0.1-0.4 mg/l, respectively, and sampling times were after four, five and six weeks. In the best case when BA was 0.25 mg/l and NAA 0.4 mg/l, *A. viguieri* produced up to five new shoots from axillary buds of the mother plant within four weeks. The study design, therefore, adds another suitable tool to the earlier described statistically supported micropropagation strategies for *Aloe* (Barringer et al., 1996; Chukwujekwu et al., 2003; Liao et al., 2004; Hashemabadi et al., 2008; Abadi & Kaviani, 2010).

Although high hormone concentrations have been recommended for the micropropagation of other species, for example 2.0 mg/l BA and 1.0 mg/l NAA for *A. arborescens* (Bedini et al., 2009), *A. viguieri* plantlets produced at 2.13 or 4.0 mg/l BA and 1.2 mg/l NAA were of poor quality, exhibiting hyperhydration despite high abundance (up to seven shoots from a single mother rosette) (Article III). In this setup with BA and NAA levels in the range of 0.25-4.0 mg/l and 0.4-2.0 mg/l, respectively, a robust model could not be derived.

Hyperhydration (in older literature known as vitrification) is a physiological disorder in *in vitro* cultures that adversely affects growth and regeneration ability. Thus, the plant material cannot be maintained or propagated properly. It has been suggested that hyperhydration is due to a higher concentration of plant growth regulators and consequent increased water potential of the medium. For example, BA is known for its hyperhydricity-inducing tendencies in shoot culture (Leshem et al., 1988; Tsay, 1998; Thomas et al., 2000). *A. viguieri* plants become hyperhydrated when the BA level exceeds 2.0 mg/l (Article III), which is higher than in the case of *A. polyphylla*, for which a concentration over 1.0 mg/l BA induces hyperhydricity (Chukwujekwu et al., 2003). Abrie & van Staden (2001) noted that seed-

lings of *A. polyphylla* become hyperhydrated quite easily, and that lowering of plant hormone levels decreased the level of hyperhydricity. BA and gelrite as gelling agent lead to a change of cytokinin-type from isoprenoid to aromatic, thus increasing hyperhydricity. Ivanova et al. (2006) theorized that a partial reason for this is upregulation of endogenous cytokinin levels. Gelrite also lowers the shoot multiplication rate and causes hyperhydricity in *A. polyphylla,* as the structure of gelrite allows increased absorbance of various compounds (Ivanova & van Staden, 2011). In the micropropagation of *A. viguieri* a partial reason for hyperhydration might be the use of gelrite as a gelling agent (Article III). Ivanova & van Staden (2008) noted that a high concentration of ammonium ions together with high levels of cytokinins, especially BA, leads to hyperhydricity and recommended changing cytokinin if possible for example to zeatin.

An alternative route for micropropagation is via a callus phase, which has been developed for *A. arborescens* (Bedini et al., 2009), *A. ferox* (Racchi, 1988) and *A. vera* (Roy & Sarkar, 1991; Velcheva et al., 2010; Rathore et al., 2011). The selected micropropagation method (directly from differentiated plant material or via callus) depends on the species and the purpose (Velcheva et al., 2010). As there is a method developed for initiation of callus from *A. viguieri* (Article III), it opens an alternative route for micropropagation in cases when there are not enough mother plants available for conventional micropropagation.

5.5 Alkaloid analysis

Earlier, coniine alkaloids have been reported from poison hemlock (*e.g.* Cromwell, 1956), *S. flava* (Mody et al., 1976), and twelve *Aloe* species (Dring et al., 1984; Nash et al., 1992; Blitzke et al., 2000). These studies have been conducted using a variety of chromatographic methods, *e.g.* Dring et al. (1984) and Nash et al. (1992) used TLC and paper chromatography. They identified hemlock alkaloids from *Aloe* sp. using different colouring reagents, such as Dragendorff's and compared the findings with authentic alkaloids from poison hemlock or synthetic references using R_f-values. In Articles II, III & IV the method of choice was GC-MS, as this method can detect and identify alkaloids with a sensitivity about ten times higher than that of TLC. The detection sensitivity of GC-MS can be increased further using different methods such as SIM when the compound under investigation is known to be in low concentration in the studied matrix (*e.g.* pitchers of *S. flava*). Mass spectrometry also allows the identification of unknown compounds without an array of authentic compounds.

In contrast, to the aforementioned earlier studies (Dring et al., 1984; Nash et al., 1992), it was found that γ-coniceine is the major alkaloid both in *A. globuligemma* and in *A. viguieri* (Article III). The presence of γ-coniceine was also detected in *A. viguieri* roots using the base peak ion as a marker. Nash et al. (1992) reported that *A. gariepensis* contains γ-coniceine but in the studied material the alkaloid was not present in leaves. Interestingly, Blitzke et al. (2000) speculated that γ-coniceine

could be a starting point for the biosynthesis of a chlorinated derivative in *A. sabaea*.

The presence of coniine in *A. globuligemma* (Nash et al., 1992) and *A. viguieri* leaves (Dring et al., 1984) was confirmed, but an additional alkaloid was also found in *A. viguieri* roots (Article III). This is the first report of the occurrence of *N*-methylconiine in the genus *Aloe*. *N*-Methylconiine is present in the unrelated plant, poison hemlock (Cromwell, 1956) and a similar alkaloid, *N,N*-dimethylconiine, has been reported from *A. sabaea* (Blitzke et al., 2000). *N*-Methylconiine was detected in *A. globuligemma* leaves (*in vitro*) and roots (*in vitro*), and in *A. viguieri* leaves (*in vitro*). Based on the detected ion *m/z* 98, it is possible that *N*-methylconiine is also present in low amounts in *A. gariepensis* and *A. viguieri* roots (Article III).

Different growth conditions or genotypes could explain why conhydrine was absent in *A. gariepensis* and *A. globuligemma* (Article III), in contrast to the results of Nash et al. (1992), who found it in the leaves of both species. Detection problems can be ruled out since conhydrine was clearly detected in poison hemlock reference samples (Article III). It has been observed that alkaloid content in poison hemlock depends on the population, weather, location, season and age of the plant (Fairbairn & Challen, 1959). Thus, the absence of alkaloids in *A. gariepensis* can be explained for example by the size of the sampled plant *in vitro* versus that of Nash et al. (1992). One strain of poison hemlock, for example, produces conhydrine as a major alkaloid in outdoor conditions but pseudoconhydrine in the greenhouse (Leete & Adityachaudhury, 1967).

None of the callus lines of aloe (A-F) contained piperidine alkaloids (Article III). Similar observations were made by Nétien & Combet (1971) and in Article II with poison hemlock callus lines. In the latter case, not even elicitation triggered alkaloid accumulation, although the selected callus was green and grown in light, in contrast to the *Aloe* calli. Several factors might affect alkaloid production in *Aloe*. Roberts (1981) suggested that coniine alkaloid biosynthesis occurs in the chloroplasts localised in the green aerial parts of poison hemlock. The biosynthesis might furthermore be dependent on specialized tissues (Fairbairn & Challen, 1959; Corsi & Biasci, 1998). In completely undifferentiated callus the biosynthetic capacity might therefore be blocked. The synthetic plant hormone, 2,4-D, could be another reason for the absence of hemlock alkaloids in *Aloe* callus (Article III). There are reports that 2,4-D generally exhibits an inhibitory role in secondary metabolism (Tabata & Hiraoka, 1976; Knobloch & Berlin, 1980; Morris, 1986; Stalman et al., 2003). It can either alter the alkaloid profile, *e.g.* in *Leucojum aestivum* callus (Ptak et al., 2013), or inhibit the production altogether as for *Nicotiana* alkaloids (Furuya et al., 1971; Tabata & Hiraoka, 1976; Ishikawa et al., 1994) and terpenoid indole alkaloids (Rischer et al., 2006).

In contrast to the report made by Mody et al. (1976), Romeo et al. (1977) did not detect any alkaloids or volatile amines in *Sarracenia* plant material which they examined with paper chromatography. The findings of Mody et al. (1976) were confirmed and it was also found that coniine occurs at least in seven other species, *e.g. S. purpurea* (Article IV).

Mody et al. (1976) reported 5 mg of coniine from 45 kg fresh plant material via steam distillation. These results also similarly indicate that coniine is present in low amounts in the studied plant material. Knowledge of whether coniine is biosynthesized in *Sarracenia* sp. in the lid, hairs, extrafloral nectar, mouth, middle or lower part of the pitcher is lacking. The exact location of biosynthesis of coniine in *Sarracenia* sp. needs further study. There are indications of possible locations of its biosynthesis in poison hemlock and methods for identifying the location do exist (Corsi & Biasci, 1998). In Article IV, the division to lid and pitcher might have influenced the result, because the whole pitcher was ground up and only a small portion of it was used for chemical analysis.

An important question is the function of coniine in *Sarracenia* sp. Why should plants living in nutrient-poor environments produce a nitrogen-consuming compound if there are no visible benefits? Butler & Ellison (2007) studied nitrogen acquisition of *S. purpurea* and observed that the plant's pitchers are efficient in prey capture and thus could greatly enhance the available nitrogen for the following growth season. Mody et al. (1976) postulated that coniine could be an insect-paralyzing agent because *S. flava*, in which they found coniine, paralyzed fire ants. Another function for coniine could be attracting insects, as indicated by Roberts (1998), who found coniine to be one of the floral scent compounds in poison hemlock. When these factors are taken into account, investment in coniine biosynthesis by a *Sarracenia* plant could indicate that coniine is an enhancing mechanism for prey capture due to its dual role in insect attraction and paralysis.

6. Conclusions

In this thesis coniine alkaloid biosynthesis was studied on multiple levels: compound, gene, enzyme and organism. Previously, only several key enzymes for hemlock alkaloid biosynthesis have been known. The interesting part of the puzzle is how the carbon backbone of coniine is formed. Based on radioactive feeding studies there have been several theories which employ a PKS or a similar enzyme. During this study, a PKS, CPKS5, was discovered and was then concluded to be the enzyme behind the carbon body biosynthesis from one butyryl-CoA and two malonyl-CoAs based on *in vitro* results. Further testing is needed to confirm whether CPKS5 is the key enzyme for the alkaloid biosynthesis *in planta*.

Calli from two poison aloes and poison hemlock were initiated and studied for their possible alkaloid contents. Poison hemlock callus was easy to induce and maintain under a wide range of hormone combinations. *Aloe* callus initiation took a long time, and depending on the species they responded well only to one treatment. Calli provide a tool for biosynthetic studies if they can be induced to produce hemlock alkaloids. However, in this thesis, the tested methods did not cause alkaloid production in poison hemlock but instead furanocoumarins were synthesized in the elicited cultures. The reason could be a transcriptional block (need for specialized tissues) or the availability of building blocks for alkaloids, such as 5-keto-octanal.

In order to investigate on the compound level, new tools were developed for sensitive detection, especially in the case of coniine in *Sarracenia* sp. The new GC-MS SIM-method led to the discovery of coniine in seven new *Sarracenia* species and at the same time confimed the presence of this alkaloid in *S. flava*.

Three poison aloes, *A. gariepensis*, *A. globuligemma* and *A. viguieri*, were studied further on the plant level. A new aloe alkaloid, *N*-methylconiine, was also discovered. Micropropagation of *A. viguieri* was studied using computer-assisted experimental design and good quality plants were obtained with low levels of phytohormones. It was observed that *A. viguieri* suffers from hyperhydricity quite easily if the hormone levels are too high. This provides a tool to produce plants easily for further experimental needs.

This study opens the door for further investigation of coniine biosynthesis in poison hemlock. The next steps would be to identify other enzymes, *e.g.* transaminase, in the pathway with the help of transcriptome analysis, looking for genes

which have similar expression patterns to that of CPKS5. This could also help to elucidate what kind of block exists in poison hemlock callus that prevents coniine biosynthesis. As the price of sequencing has come down in recent years, a comparative transcriptome analysis between poison hemlock, *Aloe* sp. and *Sarracenia* sp. could reveal similarities and differences between these plant species concerning the hemlock alkaloid route. With the help of this knowledge, a modified coniine could be re-introduced as a medicine, after its disappearance from the pharmacopeia at the turn of the 19th and 20th centuries. There is a need for a painkiller which affects the nervous system without an addictive influence.

References

Abadi, H.D. & Kaviani, B. 2010. *In vitro* proliferation of an important medicinal plant *Aloe* - A method for rapid production. Australian Journal of Crop Science 4, 216-222.

Abe, I., Utsumi, Y., Satoshi, O. & Noguchi, H. 2004. The first plant type III polyketide synthase that catalyzes formation of aromatic heptaketide. FEBS Letters 562, 171-176.

Abe, I., Utsumi, Y., Oguro, S., Morita, H. & Noguchi, H. 2005. A plant type III polyketide synthase that produces pentaketide chromone. Journal of the American Chemical Society 127, 1362-1363.

Abrie, A.L. & van Staden, J. 2001. Micropropagation of the endangered *Aloe polyphylla*. Plant Growth Regulation 33, 19-23.

Al-Barwani, F.M. & Eltayeb, E.A. 2004. Antifungal compounds from induced *Conium maculatum* L. plants. Biochemical Systematics and Ecology 32, 1097-1108.

Altschul, S., Madden, T., Schäffer, A, Zhang J., Zhang, Z., Miller, W. & Lipman, D. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acid Research 25, 3389-3402.

Angiosperm Phylogeny Group, the. 2003. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG II. Botanical Journal of the Linnaean Society 141, 399-436.

Arihan, O., Boz, M., Iskit, A.B. & Ilhan, M. 2009. Antinociceptive activity of coniine in mice. Journal of Ethnopharmacology 125, 274-278.

Ashraf, M., Ahmad, R., Asghar, B. & Bhatty, M.K. 1979. Studies on the essential oils of the Pakistani species of the family Umbelliferae. Part XX. *Pimpinella acuminate* (Edgew) Clarke (jungle anise) seed oil. Pakistan Journal of Scientific and Industrial Research 23(1-2), 79-81.

Austin, M.B. & Noel, J.P. 2003. The chalcone synthase superfamily of type III polyketide synthases. Natural Products Review 20, 79-110.

Baek, J.-H., Kim, M.-U., Kang, T.-S., Hur, W. & Lee, S.-Y. 2009. Polysaccharide characteristics from hot water extracts of *Aloe saponaria* callus. Korean Society for Biotechnology and Bioengineering Journal 24, 59-64.

Baldi, A., Srivastava, A.K. & Bisaria, V.S. 2009. Fungal elicitors for enhanced production of secondary metabolites in plant cell suspension cultures. In book: Varma, A. & Kharkwal, A.C. (eds.) Soil Biology 18 - Symbiotic Fungi. Springer-Verlag, Berlin. pp 373-380.

Barringer, S.A., Mohamed-Yasseen, Y. & Splittstoesser, W.E. 1996. Micropropagation of endangered *Aloe juvenna* and *A. volkensii* (Aloaceae). Haseltonia 4, 43-45.

Bashandy, H., Pietiäinen, M., Carvalho, E., Lim, K.-J., Elomaa, P., Martens, S. & Teeri, T.H. 2015. Anthocyanin biosynthesis in gerbera cultivar 'Estelle' and its acyanic sport 'Ivory'. Planta 242(3), 601-11.

Baskin, J.M & Baskin, C.C. 1990. Seed germination ecology of poison hemlock, *Conium maculatum*. Canadian Journal of Botany 68, 2018-2024.

Bedini, C., Caccia, R., Triggiani, D., Mazzucato, A., Soressi, G.P. & Tiezzi, A. 2009. Micropropagation of *Aloe arborescens* Mill: A step towards efficient production of its valuable leaf extracts showing antiproliferative activity on murine myeloma cells. Plant Biosystems 143, 233-240.

Belhadj, A., Telef, N., Cluzet, S., Bouscaut, J., Corio-Costet, M.F. & Merillon, J.M. 2008. Ethephon elicits protection against *Erysiphe necator* in grapevine. Journal of Agricultural and Food Chemistry 56, 5781-7.

Beyer, J., Drummer, O.H. & Maurer, H.H. 2009. Analysis of toxic alkaloids in body samples. Forensic Science International 185, 1-9.

Beyerman, H.C., van Leeuwen, M., Smidt, J. & van Veen, A. 1961. γ-Coniceine of *Conium maculatum* L., a revision of the generally accepted structure. Recueil 80, 513-525.

Bhat, B.G. & Chandrasekhara, N. 1986. Studies on the metabolism of piperine: Absorption, tissue distribution and excretion urinary conjugates in rats. Toxicology 40, 83-92.

Biberci, E., Altuntas, Y., Çobanoglu, A & Alpinar, A. 2002. Acute respiratory arrest following hemlock (*Conium maculatum*). Clinical Toxicology 40(4), 517-518.

Binev, R., Mitev, J. & Miteva, T. 2007. Intoxication with poison hemlock (*Conium maculatum* L.) in calves. Trakia Journal of Sciences 5(3-4), 40-50.

Blitzke, T., Porzel, A., Masoud M. & Schmidt, J. 2000 A chlorinated amide and piperidine alkaloids from *Aloe sabaea*. Phytochemistry 55, 979-982.

Bloch, E. 2001. Hemlock poisoning and the death of Socrates: Did Plato tell the truth? Journal of the International Plato Society, March 2001. https://www3.nd.edu/~plato/bloch.htm. Accessed 19.4.2015.

de Boer, J. 1950. The death of Socrates. A historical and experimental study on the actions of coniine and *Conium maculatum*. Archives internationales de pharmacodynamie et de thérapie 83(4), 473-490.

Bota, C. & Deliu., C. 2011. The effect of copper sulphate on the production of flavonoids in *Digitalis lanata* cell cultures. Farmacia 59(1), 113- 118.

Bowman, W.C. & Sangvi, I.S. 1963. Pharmacological actions of hemlock (*Conium maculatum* L.) alkaloids. Journal of Pharmacy and Pharmacology 15(1), 1-25.

Bradley, J.-C., Lang, A. & Williams, A. 2014. Jean-Claude Bradley double plus good (highly curated and validated) melting point dataset. https://dx.doi.org/10.6084/m9.figshare.1031638.v1. Accessed 16.2.2016.

Buckingham, J.L. 1937. Poisoning in a pig by hemlock (*Conium maculatum*). The Veterinary Journal 92, 301-302.

Bunch, T.D., Panter, K.E. & James, L.F. 1992. Ultrasound studies of the effects of certain poisonous plants on uterine function and fetal development in livestock. Journal of Animal Science 70, 1639-1643.

Butler, J.L. & Ellision, A.M. 2007. Nitrogen cycling dynamics in the carnivorous northern pitcher plant, *Sarracenia purpurea.* Functional ecology 21, 835-843.

Büchel, K.H. & Korte, F. 1962. Acyl-lactone rearrangement. XXIV. Synthesis of hemlock alkaloids by the reaction principle of the acyl-lactone rearrangement. Chemische Berichte 95, 2460-2464.

Carew, D.P. & Bainbridge, T. 1976. Biotransformation with plant tissue cultures. Lloydia 39(2-3), 147-149.

Carter, S., Lavranos, J.J., Newton, L.E. & Walker, C.C. 2011. *Aloe* – the definitive guide. Kew Publishing. Royal Botanical Gardens, Kew, Richmond, Surrey, UK. 720 p.

Castells, E., Berhow, M.A., Vaughn, S.F. & Berenbaum, M.R. 2005. Geographic variation in alkaloid production in *Conium maculatum* populations experiencing differential herbivory by *Agonopterix alstroemeriana.* Journal of Chemical Ecology 31(8), 1693-1709.

Castells, E. & Berenbaum, M.R. 2006. Laboratory rearing of *Agonopterix alstroemeriana*, the defoliating poison hemlock (*Conium maculatum* L.) moth, and effects of piperidine alkaloids on preference and performance. Physiological Ecology 35(3), 607-615.

Castells, E. & Berenbaum, M.R. 2008a. Host plant selection by monophagous herbivore is not mediated by quantitative changes in unique plant chemistry: *Agonopterix alstroemeriana* and *Conium maculatum*. Arthropod-Plant Interactions 2, 43-51.

Castells, E. & Berenbaum, M.R. 2008b. Resistance of the generalist moth *Trichoplusia ni* (Noctuidae) to a novel chemical defence in the invasive plant *Conium maculatum.* Chemoecology 18, 11-18.

Choi, H.K., Yun, J.H., Kim, S.L., Son, J.S., Kim, H.R., Kim, J.H., Choi, H.J. & Hong, S.S. 2001. Enhanced production of paclitaxel by semi-continuous batch process (SCBP) in suspension culture of *Taxus chinensis*. Enzyme and Microbial Technology 29**,** 583-586.

Chukwujekwu, J.C., Fennell, C.W. & van Staden, J. 2003. Optimisation of the tissue culture protocol for the endangered *Aloe polyphylla*. South African Journal of Botany 68, 424-429.

Cooper, J.C., Gutbrod, O., Witzermann, V. & Methfessel, C. 1996. Pharmacology of the nicotinic acetylcholine receptor from fetal rat muscle expressed in *Xenopus* oocytes. European Journal of Pharmacology 309, 287-298.

Copithorne, B. 1937. Suspected poisoning of goats by hemlock (*Conium maculatum*). The Veterinary Record 49(33), 1018-1019.

Corsi, G. & Biasci, D. 1998. Secretory structures and localization of alkaloids in *Conium maculatum* L. (Apiaceae). Annals of Botany 81, 157-162.

Cromwell, B.T. 1956. The separation, micro-estimation and distribution of the alkaloids of hemlock (*Conium maculatum* L.). Biochemical Journal 64, 259-266.

Cromwell, B.T. & Roberts M.F. 1964. The biogenesis of γ-coniceine in hemlock (*Conium maculatum* L.). Phytochemistry 3, 369-375.

Croteau, R., Kutchan, T.M. & Lewis, N.G. 2000. Chapter 24: Natural products (secondary metabolites). In book: Buchanan, B.B., Gruissem, W. & Jones R.L. (eds.) Biochemistry & molecular biology of plants. American Society of Plant Physiologists. Rockville, Maryland, USA. pp. 1250-1318.

Dagne, E., Bisrat, D., Viljoen, A. & van Wyk, B.-E. 2000. Chemistry of *Aloe* species. Current Organic Chemistry 4, 1055-1078.

Daugherty, D.G. 1995. The death of Socrates and the toxicology of hemlock. Journal of Medical Biography 3, 178-182.

Dayan, D.A. 2009. What killed Socrates? Toxicological considerations and questions. Postgraduate Medical Journal 85, 34-37.

Deng, X., Bashandy, H., Ainasoja, M., Kontturi, J., Pietiäinen, M., Laitinen, R. A. E., Albert, V. A., Valkonen, J. P. T., Elomaa, P. & Teeri, T. H. 2014. Functional diversification of duplicated chalcone synthase genes in anthocyanin biosynthesis of *Gerbera hybrida*. New Phytologist 201, 1469–1483.

Denić, M., Blagovejić, P. & Radulović, N. 2013. Synthetic approaches to coniine and other 2-alkyl piperidines. FACTA UNIVERSITATIS. Series: Physics, Chemistry and Technology 11(1), 1-26.

Dietrich, S.M.C. & Martin, R.O. 1968. Biosynthesis of *Conium* alkaloids using carbon-14 dioxide. Interrelation of γ-coniceine, coniine, and *N*-methylconiine. Journal of the American Chemical Society 90(7), 1921-1923.

Dietrich, S.M.C. & Martin, R.O. 1969. The biosynthesis of *Conium* alkaloids using carbon-14 dioxide. The kinetics of ^{14}C incorporation into the known alkaloids and some new alkaloids. Biochemistry 8(10), 4163-4172.

DiTomaso, J.M. 2000. Invasive weeds in rangelands: Species, impacts, and management. Weed Science 48, 255-265.

Diwan, R. & Malpathak, N. 2011. Bioprocess optimization of furanocoumarin elicitation by medium renewal and re-elicitation: A perfusion-based approach. Applied Biochemistry and Biotechnology 163, 756-764.

Dring, J.V., Nash, R.J., Roberts, M.F. & Reynolds, T. 1984 Hemlock alkaloids in *Aloes*. Occurrence and distribution of γ-coniceine. Planta Medica 50, 442-443.

Drummer, O.H., Roberts, A.N., Bedford, P.J., Crump, K.L. & Phelan, M.H. 1995. Case report – Three deaths from hemlock poisoning. The weed that killed Socrates is common in many parts of Australia. The Medical Journal of Australia 162, 592-593.

Drummond, R.B., Gelfand, M. & Mavi, S. 1975. Medicinal and other uses of succulents by the Rhodesian African. Excelsa 5, 51-56.

Dyson, D.A. & Wrathall, A.E. 1977. Congenital deformities in pigs possibly associated with exposure to hemlock (*Conium maculatum*). The Veterinary Record 100, 241-242.

Eckermann, S., Schröder, G., Schmidt, J., Strack, D., Edrada, R.A., Helariutta, Y., Elomaa, P., Kotilainen, M., Kilpeläinen, I., Proksch, P., Teeri, T.H. & Schröder, J. 1998. New pathway to polyketides in plants. Nature 396, 387-390.

Eckermann, C., Schröder, G., Eckermann, S., Strack, D., Schmidt, J., Schneider, B. & Schröder, J. 2003. Stilbenecarboxylate biosynthesis: A new function in the family of chalcone synthase-related proteins. Phytochemistry 62**,** 270-286.

Edmonds, L.D., Selby, L.A. & Case, A.A. 1972. Poisoning and congenital malformations associated with consumption of poison hemlock by sows. Journal of the American Veterinary Medical Association 160(9), 1319-1324.

Ekiert, H. 2000. Medicinal plant biotechnology: the Apiaceae family as the example of rapid development. Pharmazie 55, 561-567.

Ellison, A.M., Butler, E.D., Hicks, E.J., Naczi, R.F.C., Calie, P.J., Bell, C.D. & Davis, C.C. 2012. Phylogeny and biogeography of the carnivorous plant family Sarraceniaceae. PLoS ONE 7(6), e39291. doi:10.1371/journal.pone.0039291

Enan, M.R. 2009. Genotoxicity of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D): Higher plants as monitoring systems. Journal of Forest and Environmental Science 25, 147-155.

Engel, K.H., Heidlas, J., Albrect, W. & Tressel, R. 1989. Biosynthesis of chiral flavour and aroma compounds in plants and microorganisms. ACS Symposium series 388, 8-22.

Fairbairn, J.W. & Ali, A.A.E.R. 1968a. The alkaloids of hemlock (*Conium maculatum* L.) – III. The presence of bound forms in the plant. Phytochemistry 7, 1593-1597.

Fairbairn, J.W. & Ali, A.A.E.R. 1968b. The alkaloids of hemlock (*Conium maculatum* L.) – IV. Isotopic studies of the bound forms of alkaloids in the plant. Phytochemistry 7, 1599-1603.

Fairbairn, J.W. & Challen, S.B. 1959. The alkaloids of hemlock (*Conium maculatum* L.). Distribution in relation to the development of the fruit. Biochemical Journal 72, 556-561.

Fairbairn, J.W. & Suwal, P.N. 1961. The alkaloids of hemlock (*Conium maculatum* L.). – II Evidence for a rapid turnover of the major alkaloids. Phytochemistry 1, 38-46.

Flores-Sanchez, I.J. & Verpoorte R. 2009. Plant polyketide synthases: A fascinating group of enzymes. Plant Physiology and Biochemistry 47, 167-174.

Forsyth, C.S., Speth, R.C., Wecker, L., Galey, F.D. & Frank, A.A. 1996. Comparison of nicotinic receptor binding and biotransformation of coniine in the rat and chick. Toxicology Letters 89, 175-183.

Fraenkel, G.S. 1959. The raison d'êntre of secondary plant substances. These odd chemicals arose as a means of protecting plant from insects and now guide insects to food. Science 129(3361), 1466-1470.

Frank, A.A. & Reed, W.M. 1986. *Conium maculatum* (poison hemlock) toxicosis in a flock of range turkeys. Avian Diseases 31(2), 386-388.

Furuya, T., Kojima, H. & Syono, K. 1971. Regulation of nicotine biosynthesis by auxins in tobacco callus tissues. Phytochemistry 10:1529-1532

Gabriel, S. 1909. Synthese des γ-Coniceins. Berichte der Deutschen Chemischen Gesellschaft 42, 4059–4062.

Galey, F.D., Holstege, D.M. & Fisher, E.G. 1992. Toxicosis of dairy cattle exposed to poison hemlock (*Conium maculatum*) in hay; isolation of *Conium* alkaloids in plants, hay, and urine. Journal of Veterinary Diagnostic Investigation 4, 60-64.

Ganapathi G & Kargi F. 1990. Recent advances in indole alkaloid production by *Catharanthus roseus* (Periwinkle). Journal of Experimental Botany 41, 259-267.

Ghosh, R., Chhabra, A., Phatale, P.A., Samrat, S.K., Sharma, J., Gosain, A., Mohanty, S., Saran, D. & Gokhale, R.S. 2008. Dissecting the functional role of polyketide synthases in *Dictyostelium discoideum*: Biosynthesis of the differentiation regulating factor 4-methyl-5-pentylbenzene-1,3-diol. The Journal of Biological Chemistry 283**,** 11348-11354.

Giseke, A.L. 1826. Über das wirksame Princip des Schierlings *Conium maculatum*. Archiv des Apotheker-Vereins im nördlich Deutschland 20, 97–111.

Gordon, P.G., Penttila, A. & Fales, H.M. 1968. The incorporation of sodium butyrate into methylenebis(butyrylphloroglucinols) by a novel biosynthetic pathway. Journal of the American Chemical Society 90, 1376-1377.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N. & Regev, A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29, 644–652.

Green, B.T., Lee, S.T., Panter, K.E., Welch, K.D., Cook, D., Pfister, J.A. & Kem, W.R. 2010. Actions of piperidine alkaloid teratogens at fetal nicotinic acetylcholine receptors. Neurotoxicology and Teratology 32, 383-390.

Green, B.T., Lee, S.T., Welch, K.D., Pfister, J.A. & Panter, K.E. 2013. Fetal muscle-type nicotinic acetylcholine receptor activation in TE-671 cells and inhibition of fetal movement in a day 40 pregnant goat model by optical isomers of the piperidine alkaloid coniine. The Journal of Pharmacology and Experimental Therapeutics 344, 295-307.

Groenewald, E.G., Koeleman, A. & Wessels, D.C.J. 1975. Callus formation and plant regeneration from seed tissue of *Aloe pretoriensis* Pole Evans. Zeitschrift für Pflanzenphysiologie 75, 270-272

Gulezian, P.Z., Ison, J.L. & Granberg, K.J. 2012. Establishment of an invasive species (*Conium maculatum*) in contaminated roadside soil in Cook County, Illinois. The American Midland Naturalist 168(2), 375-395.

Hande, S.M., Kawai, N. & Uenishi, J. 2008. An efficient synthesis of 2- and 2,6-substituted piperidines using Pd^{II}-catalyzed 1,3-chirality transfer reaction. Journal of Organic Chemistry 74, 244-253.

Hannam, D.A.R. 1985. Hemlock (*Conium maculatum*) poisoning in the pig. The Veterinary Record 116, 322.

Harborne, J.B. 1982. Introduction to ecological biochemistry. 2nd edition. Academic Press. London, UK. 278 p.

Hashimoto, M., Ishida, S., Seshimi, Y., Kitamoto, K. & Fujii, I. 2013. *Aspergillus oryzae* type III polyketide synthase CsyB uses a fatty acyl starter for the biosynthesis of csypyrone B compounds. Bioorganic & Medicinal Chemistry 23, 5637-5640.

Hatayama, M., Unno, H., Kusunoki, M., Takahashi, S., Nishino, T. & Nakayama, T. 2010. Production of tetraketide lactones by mutated *Antirrhinum majus* chalcone synthases (AmCHS1). Journal of Bioscience and Bioengineering 110, 158-164.

Hébert, A. & Heim, F. 1898. Recherches sur les principes actifs de quelques aroïdées. Bulletin de la Société Chimique de France 17, 664-669.

Heinstein, P.F. 1985. Future approaches to the formation of secondary natural products in plant cell suspension cultures. Journal of Natural Products 48, 1-9.

Helariutta, Y., Elomaa, P., Kotilainen, M., Griesbach, R.J., Schröder, J. & Teeri, T.H. 1995. Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in *Gerbera hybrida* (Asteraceae). Plant Molecular Biology 28**,** 47-60.

Hess, K. & Eichel, A. 1918. Alkaloids of the pomegranate tree. IV. A scheme for the isolation of the pure pelletierine alkaloids. Determination of the constitution of methylisopelletierine ("methylpelletierine," "isomethylpelletierine"). Transformation of conhydrine into methylisopelletierine. Constitution of conhydrine. Journal of the Chemical Society, Abstracts 114(1), 34-35.

Hesse, M. 2002. The alkaloids: Nature's curse or blessing? Verlag Helvetica Chimica Acta. Zürich, Switzerland. 414 p.

Hofmann, A.W. 1881. Einwirkung der Wärmeauf die Ammoniumbasen. Berichte der Deutschen Chemischen Gesellschaft 14, 705–713.

Holm, L., Doll, J., Holm, E., Pancho, J. & Herberger, J. 1997. Chapter 27. *Conium maculatum* L. In: *World Weeds – Natural histories and distribution*. John Wiley & Sons, Inc. New York, Chichester, Weinheim, Brisbane, Singapore, Toronto. Pp. 221-225.

Holstege, D.M., Galey, F.D., Johnson, B. & Seiber, J.N. 1996. Determination of alkaloid exposure in a model ruminant (goat) using a multiresidue screening method. Journal of Agricultural and Food Chemistry 44, 2310-2315.

Holopainen, J.K., Heijari, J., Nerg, A. M., Vuorinen, M. & Kainulainen, P. 2009. Potential for the use of exogenous chemical elicitors in disease and insect pest management of conifer seedling production. The Open Forest Science Journal 2, 17-24.

Ishikawa, A., Kuma, T., Sasaki, H., Sasaki, N., Ozeki, Y., Kobayashi, N. & Kitamura, Y. 2009. Constitutive expression of bergaptol *O*-methyltransferase in *Glehnia littoralis* cell cultures. Plant Cell Report 28, 257-265.

Ishikawa, A., Yoshihara, T. & Nakamura, K. 1994. Jasmonate-inducible expression of a potato cathepsin D inhibitor-GUS gene fusion in tobacco cells. Plant molecular biology 26, 403-414.

Ivanova, M., Novák, O., Strnad, M. & van Standen, J. 2006. Endogenous cytokinins in shoots of *Aloe polyphylla* cultured *in vitro* in relation to hyperhydricity, exogenous cytokinins and gelling agents. Plant Growth Regulation 50, 219-230.

Ivanova, M. & van Staden, J. 2008. Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of in vitro regenerated shoots of *Aloe polyphylla*. Plant, Cell, Tissue and Organ Culture 92, 227-231.

Ivanova, M. & van Staden, J. 2011. Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*. Plant, Cell, Tissue and Organ Culture 104, 13-21.

Jananie, R.K., Priya, V. & Vijayalakshmi, K. 2012. Secondary metabolites of *Cynodon dactylon* as an antagonist to angiotensin II type1 receptor: Novel in silico drug targeting approach for diabetic retinopathy. Journal of Pharmacology and Pharmacotherapeutics 3(1), 20-25.

Jeffery, L.S. & Robinson, L.R. 1990. Poison-hemlock (*Conium maculatum*) control in alfalfa (*Medicago sativa*). Weed Technology 4, 585-587.

Jessup, D.A., Boermans, H.J. & Kock, N.D. 1986. Toxicosis in tule elk caused by ingestion of poison hemlock. Journal of the American Veterinary Medical Association 189(9), 1173-1175.

Jez, J.M., Austin, M.B., Ferrer, J.-L., Bowman, M.E., Schröder, J. & Noel, J.P. 2000. Structural control of polyketide formation in plant-specific polyketide synthases. Chemistry and Biology 7, 919-930.

Jez, J.M., Bowman, M.E. & Noel, J.P. 2002. Expanding the biosynthetic repertoire of plant type III polyketide synthases by altering starter molecule specificity. Proceedings of the National Academy of Sciences of the United States of America 99, 5319-5324.

Jindaprasert, A., Springob, K., Schmidt, J., De-Eknamkul, W. & Kutchan, T.N. 2008. Pyrone polyketides synthesized by a type III polyketide synthase from *Drosophyllum lusitanicum*. Phytochemistry 69, 3043-3053.

Jones, T.H., Blum, M.S. & Fales, H.M. 1982. Ant venom alkaloids from *Solenopsis* and *Monorium* species: Recent developments. Tetrahedron 38(13), 1949-1958.

Jonsell, B. & Karlsson, T. 2010. Flora Nordica 6. The Swedish Museum of Natural History. Stockholm, Sweden. 298 p.

Jowett, B. 1892. The Dialogues of Plato translated into English with Analyses and Introductions. 3rd edition. Oxford University Press. London, UK. pp. 157-266.

Junghans, H., Dalkin, K. & Dixon, R.A. 1993. Stress responses in alfalfa (*Medicago sativa* L.). 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. Plant Molecular Biology 22**,** 239-253.

Karamat, F., Olry, A., Munakata, R., Koeduka, T., Sugiyama, A., Paris, C., Hehn, A., Bourgaud, F. & Yazaki, K. 2014. A coumarin-specific prenyltransferase catalyzes the crucial biosynthetic reaction for furanocoumarin formation in parsley. The Plant Journal 77, 627-638.

Karppinen, K., Hokkanen, J., Mattila, S., Neubauer, P. & Hohtola, A. 2008. Octaketide-producing type III polyketide synthase from *Hypericum perforatum* is expressed in dark glands accumulating hypericins. FEBS Journal 275**,** 4329-4342.

Kawai, K., Beppu, H., Koike, T., Fujita, K. & Marunouchi, T. 1993 Tissue culture of *Aloe arborescens* Miller var. *natalensis* Berger. Phytotherapy Research 7, S5-S10.

Keeler, R.F. & Balls, L.D. 1978. Teratogenic effects in cattle of *Conium maculatum* and conium alkaloids and analogs. Clinical Toxicology 12(1), 49-64.

Keeler, R.F., Balls, L.D., Shupe, J.L. & Crowe, M.W. 1980. Teratogenicity and toxicity of coniine in cows, ewes and mares. The Cornell Veterinarian 70, 19-26.

Kim, S.S., Grienenberger, E., Lallemand, B., Colpitts, C.C., Kim, S.Y., de Azevedo Souza, C., Geoffroy, P., Heintz, D., Krahn, D., Kaiser, M., Kombrink, E., Heitz, T., Suh, D.Y., Legrand, M. & Douglas, C.J. 2010. LAP6/POLYKETIDE SYNTHASE A and LAP5/POLYKETIDE SYNTHASE B encode hydroxyalkyl α-pyrone synthases required for pollen development and sporopollenin biosynthesis in *Arabidopsis thaliana*. The Plant Cell 22(12), 4045-66.

Knobloch, K.-H. & Berlin, J. 1980. Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *Catharanthus roseus* (L.) G. Don. Zeitschrift für Naturforschung 35c, 551-556.

Koleoso, O.A., Dietrich, S.M.C. & Martin, R.O. 1969. The biosynthesis of *Conium* alkaloids. Identification of a novel nonnitrogenous base from *Conium maculatum* as 3-formyl-4-hydroxy-2*H*-pyran. Biochemistry 8(10), 4172-4177.

Koskinen, P. 2011. Pannzer. http://ekhidna.biocenter.helsinki.fi/pannzer/Home.html. Accessed 6.11.2015.

Krezelok, E.P., Jaconsen, T.D. & Aronis, J.M. 1996. Hemlock ingestions: The most deadly plant Exposures. Journal of Toxicology 34(5), 601-602.

Ladenburg, A. 1886. Versuche zur Synthese des Coniin. Berichte Deutschen der Chemischen Gesellschaft 19(1), 439–441.

de Landoni, J.H. 1990. *Conium maculatum* L. (PIM 144). http://www.inchem.org/documents/pims/plant/conium.htm. Accessed 9.5.2015.

Lang, D.G. & Smith, R.A. 1997. Two new alkaloids of *Conium maculatum*, and evidence for a tautomeric form for "γ"-coniceine. In book: Garland T. & Barr, A.C. (eds.) Toxic plants and other natural toxicants. CAB International. Oxford, UK. Pp. 419-422.

Lee, S.T., Green, B.T., Welch, K.D., Pfister, J.A. & Panter, K.E. 2008. Stereoselective potencies and relative toxicities of coniine enantiomers. Chemical Research in Toxicology 21, 2061-2064.

Lee, S.T., Green, B.T., Welch, K.D., Jordan, G.T., Zhang, Q., Panter, K.E., Hughes, D., Chang, C.-W.T., Pfister, J.A. & Gardner, D.R. 2013a. Stereoselective potencies and relative toxicities of γ-coniceine and *N*-methylconiine enantiomers. Chemical Research in Toxicology 26, 616-621.

Lee, Y.S., Ju, H.K., Kim, Y.J., Lim, T.-G., Uddin, M.R., Kim, Y.B., Baek, H., Kwon, S.W., Lee, K.W., Seo, H.S., Park, S.U. & Yang, T.J. 2013b. Enhancement of anti-Inflammatory activity of *Aloe vera* adventitious root extracts through the alteration of primary and secondary metabolites via salicylic acid elicitation. PLoS ONE 8(12): e82479. doi:10.1371/journal.pone.0082479

Leete, E. 1963. The Biosynthesis of coniine four acetate units. Journal of the American Chemical Society 85(21), 3523-3524.

Leete, E. 1964. Biosynthesis of the hemlock alkaloids. The incorporation of the acetate-1-C^{14} into coniine and conhydrine. Journal of the American Chemical Society 86, 2509-2513.

Leete, E. 1970. Biosynthesis of coniine from octanoic acid in hemlock plants (*Conium maculatum*). Journal of the American Society 92(12), 3835.

Leete, E. 1971. Biosynthesis of the hemlock and related piperidine alkaloids. Accounts of Chemical Research 4, 100-107.

Leete, E. & Adityachaudhury, N. 1967. Biosynthesis of the hemlock alkaloids – II. The conversion of γ-coniceine to coniine and Ψ-conhydrine. Phytochemistry 6, 219-223.

Leete, E. & Olson, J.O. 1970. 5-Oxo-octanoic acid and 5-oxo-octanal, precursors of coniine. Journal of the Chemical Society D: Chemical Communications 23, 1651-1652.

Leete, E. & Olson, J.O. 1972. Biosynthesis and metabolism of the hemlock alkaloids. Journal of the American Chemical Society 94(15), 5472-5477.

Leshem, B., Werker, E. & Shalevi, D.P. 1988. The effect of cytokinins on vitrification in melon and carnation. Annals of Botany 62, 271-276.

Li, S.-L., Lin, G., Chan, S.-W. & Li, P. 2001. Determination of the major isosteroidal alkaloids in bulbs of *Fritillaria* by high-performance liquid chromatography coupled with evaporative light scattering detection. Journal of Chromatography A 909, 207-214.

Liao, Z., Chen, M., Tan, F., Sun, X. & Tang, K. 2004. Micropropagation of endangered Chinese aloe. Plant, Cell, Tissues and Organ Culture 76, 83–86.

Linnilä, K., Savikko, S. & Terttu Lempiäinen (eds.). 2003. Elias Lönnrotin Flora Fennica I-III. Kustannusosakeyhtiö Tammi. Hämeenlinna, Finland. 404+400+415 p.

Liu, Y., Takatsuki, H., Yoshikoshi, A., Wang, B. & Sakanishi, A. 2003. Effects of ultrasound on the growth and vacuolar H^+-ATPase activity of *Aloe arborescens* callus cells. Colloid Surface B 32,105-116.

Liu, B., Raeth, T., Beuerle, T. & Beehues, L. 2007. Biphenyl synthase, a novel type III polyketide synthase. Planta 225, 1495-1503.

López, T.A., Cid, M.S. & Bianchini, M.L. 1999. Biochemistry of hemlock (*Conium maculatum* L.) alkaloids and their acute and chronic toxicity in livestock. A review. Toxicon 37, 841-865.

Loots, D.T., van der Westhuizen, F.H. & Botes, L. 2007. *Aloe ferox* leaf gel phytochemical content, antioxidant capacity, and possible health benefits. Journal of Agricultural and Food Chemistry 55, 6891-6896.

MacDonald, H. 1937. Hemlock poisoning in horses. The veterinary Record 49(38), 1211-1212.

Madaan, R. & Kumar, S. 2012. Screening of alkaloidal fraction of *Conium maculatum* L. aerial parts for analgesic and anti-inflammatory activity. Indian Journal of Pharmaceutical Sciences 74(5), 457-460.

Mahrenholz, R.M. & Carew D.P. 1966. *Conium maculatum* tissue culture: nutrition and alkaloid formation. Lloydia 29 (4), 376.

Maier, W., Baumert, A., Schumann, B., Furukawa, H. & Gröger, D. 1993. Synthesis of 1,3-dihydroxy-*N*-methylacridone and its conversion to rutacridone by cell-free extracts of *Ruta graveolens* cell cultures. Phytochemistry 32, 691–698.

Markham, K. 1985. Hemlock poisoning in piglets. The Veterinary Records 116(1), 27.

McPherson, S. 2006. Pitcher plants of the North America. The McDonald & Woodward Publishing Company. Blacksburg, Virginia, USA. 320 p.

Merck, E. 1891. Reported as “pseudoconhydrine”. The Pharmaceutical Journal 21, 662.

Mitich, L.W. 1998. Poison-hemlock (*Conium maculatum* L.). Weed Technology 12, 194-197.

Mizuuchi, Y., Shi, S.-P., Wanibuchi, K., Kojima, A., Morita, H., Noguchi, H. & Abe, I. 2009. Novel type III polyketide synthases from *Aloe arborescens*. FEBS Journal 276, 2391-2401.

Mody, N.V., Henson, R., Hedin, P.A., Kokpol, U. & Miles, D.H. 1976. Isolation of insect paralysing agent coniine from *Sarracenia flava*. Experientia 32, 829-830.

Monte, D. & Somerville, S. 2003. Pine tree method for isolation of plant RNA. In DNA Microarrays. In book: Bowtell, D. & Sambrook, J. (eds.) A molecular cloning manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York, USA. pp. 124–126.

Moore, B., & Row, R. 1898. A comparison of the physiological actions and chemical constitution of piperidine, coniine and nicotine. The Journal of Physiology 22(4), 273-295.

Morris, P. 1986. Regulation of produce synthesis in cell cultures of *Catharanthus roseus*. III. Alkaloid metabolism in cultured leaf tissue and primary callus. Planta Medica 121, 127-132.

Moscatiello, R., Baldan, B. & Navazio, L. 2013. Plant cell suspension cultures. Methods in Molecular Biology 953, 77-93.

Naef, R., Velluz, A., Mayenzet, F., Starkenmann, C. & Sun, H.-D. 2005. Volatile constituents of *Semnostachya menglaensis* Tsui. Journal of Agricultural and Food Chemistry 53(23), 9161-9164.

Namdeo, A. G. 2007. Plant cell elicitation for production of secondary metabolites: A review. Pharmacognosy Reviews 1 (1), 69-79.

Nash, R.J., Beaumont, J., Veitch, N.C., Reynolds, T., Benner, J., Hughes, C.N.G., Dring, J.V., Bennett, R.N. & Dellar, J.E. 1992. Phenylethylamine and piperidine alkaloids in *Aloe* species. Planta Medica 58, 84-87.

Nétien, G. & Combet, J. 1970. Etude comparative dans la composition chimique des cultures de tissus de *Conium maculatum* cultivées *in vitro*. I. Variation des substances azotées. Paris Soc Biol Compt Rend 165(1), 103-107.

Nice, G., Johnson, B., Bauman, T. & Jordan, T. 2015. Poison hemlock – the toxic parsnip. Purdue Extension – Weed Science, Purdue University. West Lafayette, IN, USA. http://www.carrotmuseum.co.uk/PHemlock03.pdf. Accessed 9.5.2015.

Nitao, J.K. 1987. Test for toxicity of coniine to a polyphagous herbivore, *Heliothis zea* (Lepidoptera: Noctuidae). Environmental Entymology 16(3), 656-659.

Nualkaew, N., Morita, H., Shimokawa, Y., Kinjo, K., Kushiro, T., De-Eknamkul, W., Ebizuka, Y. & Abe, I. 2012. Benzophenone synthase from *Garcinia mangostana* L. pericarps. Phytochemistry 77, 60-69.

Oksman-Caldentey, K.-M. & Inzé, D. 2004. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends in Plant Science 9(9), 433-440.

Panter, K.E., Keeler, R.F., Buck, W.B. & Shupe, J.L. 1983. Toxicity and teratogenicity of *Conium maculatum* in swine. Toxicon, supplement 3, 333-336.

Panter, K.E., Bunch, T.D., Keeler, R.F. & Sisson, D.V. 1988a. Radio ultrasound observation of the fetotoxic effects in sheep from ingestion of *Conium maculatum* (poison-hemlock). Clinical Toxicology 26(3-4), 175-187.

Panter, K.E., Keeler, R.F. & Baker, D.C. 1988b. Toxicosis in livestock from the hemlocks (*Conium* and *Cicuta* spp.). Journal of Animal Science 66, 2407-2413.

Panter, K.E., Bunch, T.D. & Keeler, R.F. 1988c. Maternal and fetal toxicity of poison hemlock (*Conium maculatum*) in sheep. American Journal of Veterinary Research 49(2), 281-283.

Panter, K.E. & Keeler, R.F. 1989. Chapter 5 - Piperidine alkaloids of poison hemlock (*Conium maculatum*). In book: Cheeke, P.R. (ed.) Toxicants of plant origin, volume I. CRC Press. Boca Raton, Florida, USA. pp. 109-132.

Panter, K.E., Bunch, T.D., Keeler, R.F., Sisson, D.V. & Callan, R.J. 1990. Multiple congenital contractures (MCC) and cleft palate induced in goats by ingestion of piperidine alkaloid-containing plants: Reduction in fetal movement as the probable cause. Clinical Toxicology 28(1), 69-83.

Parry, O. & Matambo, C. 1992. Some pharmacological actions of aloe extracts and *Cassia abbreviata* on rats and mice. Central African Journal of Medicine 38(10), 409-414.

Patel, H. & Krishnamurthy, R. 2013. Elicitors in plant tissue culture. Journal of Pharmacognosy and Phytochemistry 2, 60-65.

Penny, R.H.C. 1953. Hemlock poisoning in cattle. The Veterinary Record 65(42), 669-670.

Pereira, R.P., Boligon, A.A., Appel, A.S., Fachinetto, R., Ceron, C.S., Tanus-Santos, J.E., Athayde, M.L. & Rocha, J.B.T. 2014. Chemical composition, antioxidant and anticholinesterase activity of *Melissa officinalis*. Industrial Crops and Products 53, 34-45.

Pimental, D., Zuniga, R. & Morrison, D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52, 273-288.

von Planta, A. & Kekulé, A. 1854. Beiträge zur Kenntnis einiger flüchtiger Basen. Annalen der Chemie und Pharmacie 89, 129–155.

Plant List, the. 2015. A working list of all plant species. http://www.theplantlist.org/tpl1.1/search?q=Conium. Accessed 4.11.2015.

Pokorny, M. & Sheley, R. 2012. Poison hemlock *Conium maculatum.* MT200013AG, MontGuide, Montana State University Extension. Bozeman, MT, USA. http://store.msuextension.org/publications/AgandNaturalResources/MT200013AG.pdf. Accessed 9.5.2015.

Power, F.D. & Tutin, F. 1905. Chemical examination of *Aethusa cynapium*. Journal of the American Chemical Society 27(12), 1461-1476.

Ptak, A., El Tahchy, A., Skrzypek, E., Wójtowicz, T. & Laurain-Mattar, D. 2013. Influence of auxins on somatic embryogenesis and alkaloid accumulation in *Leucojum aestivum* callus. Central European Journal of Biology 8, 591-599.

Racchi, M.L. 1988. Using *in vitro* culture to study the biosynthesis of secondary products in *Aloe ferox*. Rivista di Agricoltura Subtropicale e Tropicale 82, 707-714.

Radulović, N., Đorđević, N., Denić, M., Pinheiro., M.M.G., Fernandes, P.D. & Boylan, F. 2012. A novel alkaloid from poison hemlock (*Conium maculatum* L., Apiaceae): Identification, synthesis and antinociceptive activity. Food and Chemical Toxicology 50, 274-279.

Raei, M., Angaji, S.A., Omidi, M. & Khodayari, M. 2014. Effect of abiotic elicitors on tissue culture of *Aloe vera*. International Journal of Biosciences 5(1), 74-81.

Raffauf, R.F. 1970. A handbook of alkaloids and alkaloid-containing plants. Wiley. New York, London, Sydney, Toronto. 1275 p.

Ramsay, M.M. & Gratton, J. 2000. Propagation of *Aloe polyphylla* Schönl. ex Pillans from callus derived from leaf tissue. Botanical Gardens Micropropagation News 2, 61-64.

Rathore, M.S., Chikara, J. & Shekhawat, N.S. 2011. Plantlet regeneration from callus cultures of selected genotype of *Aloe vera* L. An ancient plant for modern herbal industries. Applied Biochemistry and Biotechnology 163, 860-868.

Reese, J.J. 1884. Text-book of medical jurisprudence and toxicology. Reprint 2013, Forgotten Books, London, UK. Pp. 386-387.

Resmi, M.S., Verma, P., Gokhale, R.S. & Soniya, E.V. 2013. Identification and characterization of a type III polyketide synthase involved in quinolone alkaloid biosynthesis from *Aegle marmelos* Correa. The Journal of Biological Chemistry 288**,** 7271-7281.

Reynolds, T. 2005. Hemlock alkaloids from Socrates to poison aloes. Phytochemistry 66, 1399-1406.

Rischer, H., Orešič, M., Seppänen-Laakso, T., Katajamaa, M., Lammertyn, F., Ardiles-Diaz, W., Van Montagu, M.C.E., Inzé, D., Oksman-Caldentey, K.-M. & Goossens, A. 2006. Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proceeding of National Academy of Sciences 103, 5614-5619.

Rizzi, D., Basile, C., di Maggio, A., Sebastio, A., Introna Jr, F., Rizzi, R., Scatizzi, A., de Marco, S. & Smialek, J.E. 1991. Clinical spectrum of accidental hemlock poisoning: Neurotoxic manifestations, rhabdomyolosis and acute tubular necrosis. Nephrology Dialysis Transplantatation 6, 393-943.

Roberts, M.F. 1971. The formation of γ-coniceine from 5-ketooctanal by a transaminase of *Conium maculatum*. Phytochemistry 10, 3057-3060.

Roberts, M.F. 1974a. Origin of the methyl carbon of methyl coniine in *Conium maculatum*. Phytochemistry 13, 1841-1845.

Roberts, M.F. 1974b. An S-adenosyl-L-methionine; coniine methyltransferase from *Conium maculatum*. Phytochemistry 13, 1847-1851.

Roberts, M.F. 1975. γ-Coniceine reductase in *Conium maculatum*. Phytochemistry 14, 2393-2397.

Roberts, M.F. 1977. Purification and properties of L-alanine:5-ketooctanal aminotransferase from *Conium maculatum*. Phytochemistry 16, 1381-1386.

Roberts, M.F. 1978. Separation of the formation of γ-coniceine and aliphatic amines from GOT activity in *Conium maculatum*. Phytochemistry 17, 107-112.

Roberts, M.F. 1981. Enzymatic synthesis of γ-coniceine in *Conium maculatum* chloroplasts and mitochondria. Plant Cell Reports 1, 10-13.

Roberts, M.F. 1985. Alkaloid production in *Conium* fruit. Journal of Pharmacy and Pharmacology 37, 141P.

Roberts, M.F. 1998. Enzymology of alkaloid biosynthesis. In book: Roberts, M.F. & Wink, M. (eds.) Alkaloids: biochemistry, ecology, and medicinal applications. Springer. New York, New York, USA. Pp. 109-146.

Roberts, M.F. & Brown, R.T. 1981. A new alkaloid from South African *Conium* species. Phytochemistry 20, 447-449.

Robinson, R. 1917. LXXV. - A theory of the mechanism of the phytochemical synthesis of certain alkaloids. Journal of the Chemical Society, Transactions 111, 876-899.

Romeo, J.T., Bacon, J.D. & Marby, T.J. 1977. Ecological considerations of amino acids and flavonoids in *Sarracenia* species. Biochemical Systematics and Ecology 5, 117-120.

Rowley, G.D. 1997. A history of succulent plants. Strawberry Press. Mill Valley, CA, USA. 409 p.

Roy, S.C. & Sarkar, A. 1991. *In vitro* regeneration and micropropagation of *Aloe vera* L. Scientia Horticulturae 47, 107-113.

Ruiz-García, Y. & Gómez-Plaza, E. 2013. Elicitors: a tool for improving fruit phenolic content. Agriculture 3, 33-52.

Sampson, S.R., Esplin, D.W. & Zablocka, B. 1966. Effects of coniine on peripheral and central synaptic transmission. The Journal of Pharmacology and Experimental Therapeutics 152(2), 313-324.

Santner, A., Calderon-Villalobos, L.I.A. & Estelle, M. 2009. Plant hormones are versatile chemical regulators of plant growth. Nature Chemical Biology 5(5), 301-7.

Sathyanarayana, B.N. & Varghese, D.B. 2007. Plant tissue culture: Practices and new experimental protocols. I.K. International Publishing House Pvt. Ltd., New Delhi, India. 316 p.

Schnell D.E. 2002. Carnivorous plants of the United States and Canada. 2nd edition. Timber Press, Inc. Portland, Oregon, USA. 468 p.

Schoofs, G., Teichmann, S., Hartmann, T. & Wink, M. 1983. Lysine decarboxylase in plants and its integration in quinolizidine alkaloid biosynthesis. Phytochemistry 22, 65-69.

Schröder, J. 1997. A family of plant-polyketide synthases: facts and predictions. Trends in Plant Science 2(10), 373-378.

Schüz, R., Heller, W. & Hahlbrock, K. 1983. Substrate specificity of chalcone synthase from *Petroselinum hortense*. Formation of phloroglucinol derivatives from aliphatic substrates. Journal of Biological Chemistry 258, 6730-6734.

Scutchfield, F.D. & Genovese, E.N. 1997. Terrible death of Socrates: Some medical and classical reflections. The Pharos of Alpha Omega Alpha Honor Medical Society 60(3), 30-33.

Seshime, Y., Juvvadi, P.R., Kitamoto, K., Ebizuka, Y. & Fujii, I. 2010. Identification of csypyrone B1 as the novel product of *Aspergillus oryzae* type III polyketide synthase CsyB. Bioorganic & Medicinal Chemistry 18, 4542-4546.

Shen, B. 2003. Polyketide biosynthesis beyond the type I, II, and III polyketide synthase paradigms. Current Opinion in Chemical Biology 7, 285-295.

Shi, S., Morita, H., Wanibuchi, K., Mizuuchi, Y., Noguchi, H. & Abe, I. 2008. Enzymatic synthesis of plant polyketides. Current Organic Synthesis 5, 250-266.

Short, S.B. & Edwards, W.C. 1989. Accidental *Conium maculate* poisoning in the rabbit. Veterinary and Human Toxicology 31(3), 54-57.

Smith, L.J., Thill, D.C., Callihan, R.H. & Lish, J.M. 1984. Poison hemlock. A threat to man and livestock. Current Information Series No. 632. Cooperative Extension Service Agricultural Experiment Station, College of Agriculture, University of Idaho.

Srivastava, A., Rogers, W.L., Breton, C.M., Cai, L. & Malmberg, R.L. 2011. Transcriptome analysis of *Sarracenia*, an insectivorous plant. DNA Research 18, 253-261.

Stalman, M., Koskamp, A.-M., Luderer, R., Vernooy, J.H.J., Wind, J.C., Wullems, G.J. & Croes, A.F. 2003. Regulation of anthraquinone biosynthesis in cell cultures of *Morinda citrifolia*. Journal of Plant Physiology 160, 607-614.

Stevens, P.F. 2013. Angiosperm Phylogeny Website. Version 13, September 2013. http://www.mobot.org/MOBOT/research/APweb/. Accessed 18.4.2016.

Sullivan, J. 2001. A note on the death of Socrates. Classical Quarterly 51(2), 608-610.

Swerczek, T.W. & Swerczek, S.J. 2012. Spotted hemlock poisoning in a herd of Angus cattle. Journal of the American Veterinary Medical Association 240(11), 1280-1281.

Tabata, M. & Hiraoka, N. 1976. Variation of alkaloid production in *Nicotiana rustica* callus cultures. Physiologia Plantarum 38, 19-23.

Tatsuo, K. & Amano, M. 1985. Callus formation in stamens of *Aloe bellatura*. Annals of the Tsukuba Botanical Garden 3, 19-26.

Taura, F., Tanaka, S., Tagushi, C., Fukamizu, T., Tanaka, H., Shoyama, Y. & Morimoto, S. 2009. Characterization of olivetol synthase, a polyketide putatively involved in cannabinoid biosynthetic pathway. FEBS Letters 583, 2061-2066.

Teuscher, E., Greger, H. & Adrian, V. 1990. 1. Investigation of the toxicity of *Aethusa cynapium* L. (Hundspetersilie). Pharmazie 45(7), 537-8.

Thomas, P., Mythili, J.B. & Shivashankara, K.S. 2000. Explant, medium and vessel aeration affect the incidence of hyperhydricity and recovery of normal

plantlets in triploid watermelon. The Journal of Horticultural Science and Biotechnology 75, 19-25.

Tsay, H.-S. 1998. Effects of medium composition at different recultures of on vitrification of carnation (*Dianthus caryophyllus*) *in vitro* shoot proliferation. Acta Horticulturae 461, 243-249.

Umetrics. 2011. Design for experiments. Umetrics academy: Training in multivariate technology, IID1066. Umetrics AB. Malmö, Sweden.

Unger, W. 1977. Enzymatische *In-vitro*-synthase von γ-Conicein durch eine Aldehyd-aminosäure-transaminase aus Spinatblättern. Planta medica 31, 262-265.

USDA Forest Service. 2015. Field Guide for managing poison hemlock in the Southwest. TP-R3-16-18. Albuquerque, NM, USA. http://www.fs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb5410121.pdf

US EPA. 2016. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.11. United States Environmental Protection Agency, Washington, DC, USA.

Veersham, C., Srinivasan, V. & Shuler, M.L. 1995. Elicitation of *Taxus* Sp. cell cultures for production of taxol. Biotechnology Letters 17, 1343-1346.

Veerporte, R., van der Heijden, R., van Gulik, W.M. & ten Hoopen H.J.G. 1991. Plant biotechnology for the production of alkaloids: Present status and prospects. In book: Brossi, A. (ed.) The alkaloids, Vol. 40. New York: Academic Press. New York, New York, USA. Pp. 1-188.

Velcheva, M., Faltin, Z., Vardi, A., Hanania, U., Eshdat, Y., Dgani, O., Sahar, N. & Perl, A. 2010. *Aloe vera* transformation: the role of Amberlite XAD-4 resin and antioxidants during selection and regeneration. In Vitro Cellular & Developmental Biology 46, 477-484.

Vetter, J. 2004. Poison hemlock (*Conium maculatum* L.). Food and Chemical Toxicology 42, 1373-1382.

Viljoen, A.M., van Wyk, B.-E. & van Heerden, F.R. 1998. Distribution and chemotaxonomic significance of flavonoids in *Aloe* (Asphodelaceae). Plant Systematics and Evolution 211, 31-42.

Wang, B.Y. 2007. Environmental biodegradation research focus. Nova Science Publishers. Hauppauge, NY, USA. 320 p.

Wegler, R. & Pieper, G. 1950. The addition of alkylpyridines to butadiene and styrene in the presence of alkali metal. Chemische Berichte 83, 6-10.

Weng, J.-K. & Noel, J.P. 2012. Structure-function analyses of plant type III polyketide synthases. In book: Hopwood, D. (ed.) Methods in enzymology, volume 515. Natural product biosynthesis by microorganisms and plants, part A. Academic Press, San Diego, CA, USA. Pp. 317-335.

Wertheim, T. 1856. Über ein neues Alkaloïd in *Conium maculatum*. Justus Liebig's Annalen der Chemie und Pharmacie 100, 328–339.

Widmer, W.R. 1984. Poison hemlock toxicosis in swine. Veterinary Medicine 79(3), 405-408.

Wink, M., Hartmann, T. & Witte, L. 1980a. Biotransformation of cadaverine and potential biogenetic intermediates of lupanine biosynthesis by plant cell suspension culture. Planta Medica 40, 31-39.

Wink, M., Witte, L., Schiebel, H.-M. & Hartmann, T. 1980b. Alkaloid pattern of cell suspension cultures and differentiated plants of *Lupinus polyphyllus.* Planta Medica 38, 238-245.

Wink, M., Witte, L. & Hartmann, T. 1981. Quinolizidine alkaloid composition of plants and of photomixotrophic cell suspension cultures of *Sarothamnus scoparius* and *Orobanche rapum-genistae*. Planta Medica 43, 342-352.

Wink, M. & Witte, L. 1983. Evidence for a wide-spread occurrence of the genes of quinolizidine alkaloid biosynthesis. Induction of alkaloid accumulation in cell suspension cultures of alkaloid-'free' species. FEBS Letters 159(1-2), 196-200.

Wink, M., Schmeller, T. & Latz-Brüning, B. 1998. Modes of action of allelochemical alkaloids: Interaction with neuroreceptors, DNA, and other molecular targets. Journal of Chemical Ecology 24(11), 1881-1937.

Wink, M. 2007. Ecological roles of alkaloids. In book: Fattorusso, E. & Taglialatela-Scafati, O. (eds.) Modern alkaloids – structure, isolation, synthesis and biology. WILEY-VCH Verlag GmbH & Co. KGaA. Weinheim, Baden-Württenberg, Germany. pp. 3-24.

Wink, M. 2009. Chapter 1: Introduction. Annual Plant Reviews 39, 1-20.

Wink, M. 2010. Chapter 1: Introduction: Biochemistry, physiology and ecological functions of secondary metabolites. Annual Plant Reviews 40, 1-19.

Wolffenstein, R. 1894. Über Conium-Alkaloïde. Berichte der Deutschen Chemischen Gesellschaft 27, 2611–2615.

Wolffenstein, R. 1895. Über Coniumalkaloïde. Berichte der Deutschen Chemischen Gesellschaft 28, 302–305.

Woodard, C.A. 2008. Poison hemlock (*Conium maculatum* L.): biology, implications for pastures and response to herbicides. A thesis for Master of Science, University of Missouri. Columbia, MO, USA. 81 p.

Yagi, A., Shoyama, Y. & Nishioka, I. 1983. Formation of tetrahydroanthracene glucosides by callus tissue of *Aloe saponaria*. Phytochemistry 22, 1483-1484.

Yagi, A., Hine, N., Asai, M., Nakazawa, M., Tateyama, Y., Okamura, N., Fujioka, T., Mihashi, K. & Shimomura, K. 1998. Tetrahydroanthracene glucosides in callus tissue from *Aloe barbadensis* leaves. Phytochemistry 47, 1267-1270.

Yanai, H.S. & Lipscomb, W.N. 1959. The structure of Ψ-conhydrine. Tetrahedron 6, 103–108.

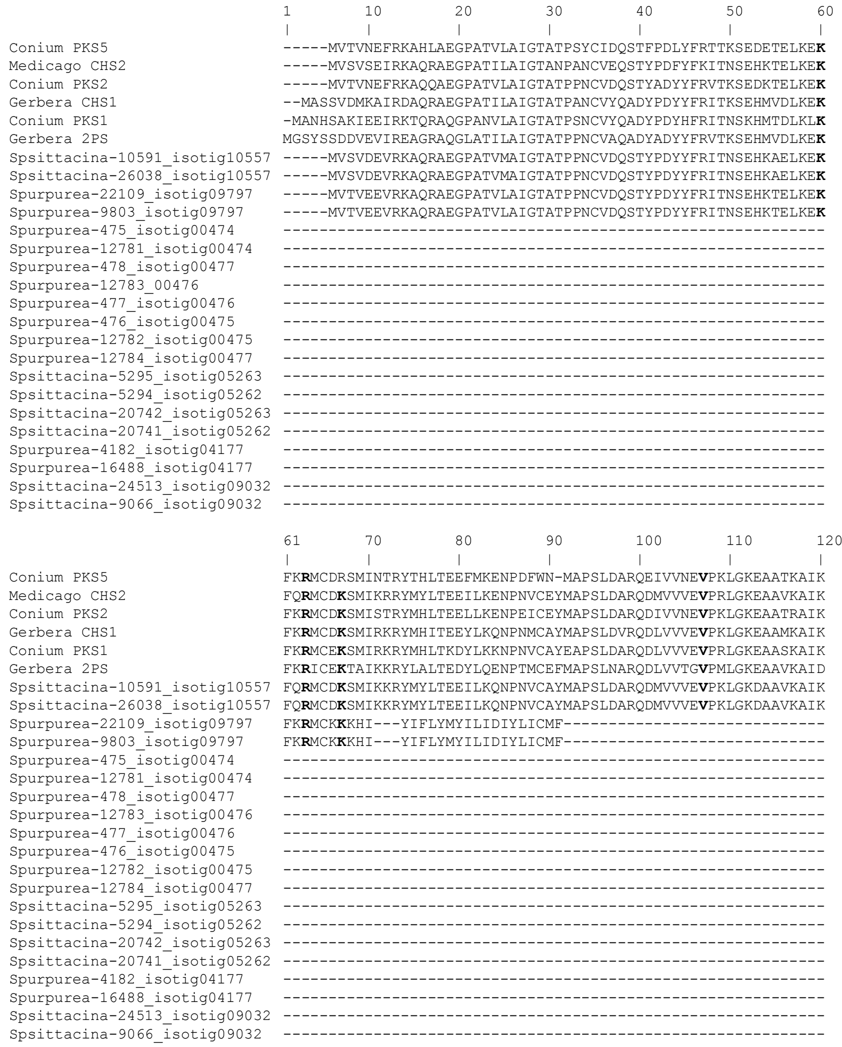
Zobel, A.M. & Brown, S.A. 1993. Furanocoumarins on the surface of callus cultures from species of Rutaceae and Umbelliferae. Canadian Journal of Botany 71, 966-969.

Appendix A: Sequences of short CPKSs

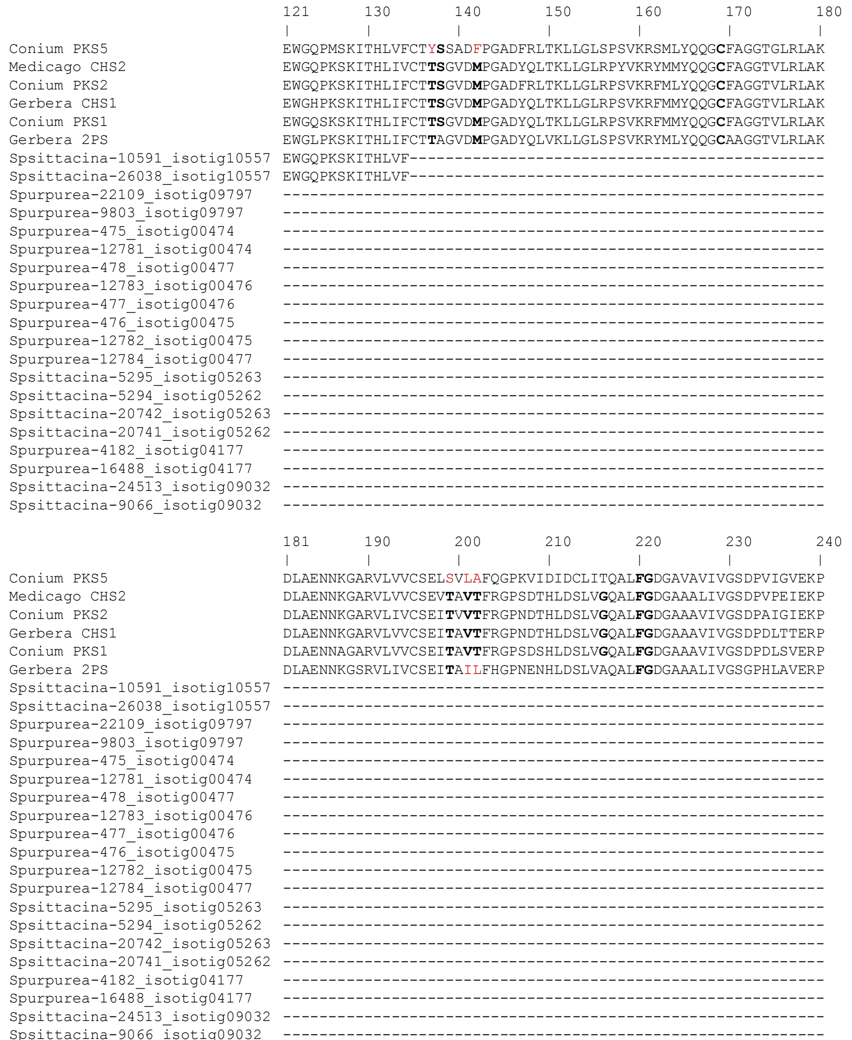
Table A1 Short sequences of poison hemlock (*Conium maculatum*) polyketide synthases. *Cpks3* and *Cpks4* are obtained via PCR with degenerated primers from genomic data. *Cpks8-12* are from transcriptome sequence analysis (Article I).

Gene name	Sequence
Cpks3	5′-CCAACCAATGTCGAAAATCACCCACCTCATCTTTTGCACCACTAGCAGT GTTGATATGCCTGGTGCAGACTACCGTCTAACCAAGCTTCTCGGGCTCAATC CATCCATCAGGCGCTTC
Cpks4	5′-ACAGCCAAAGTCCGAAATCACTCACCTAATCTTTTGCACCACTAGCATT GCTGATTTACCTGGTGCGGACTTTAGGCTTACCAAGCTTCTCGGGCCCAGTC CGTCAGTCAAGCACTCC
Cpks8	5′-AACGATAGCACCCTCACTATCCGGAAGGATGGTTTGGGCAGCAGAAACA ATTTCAAACAATGGTTTTTCTAGTCCAAGAATCGGGTCTGAGCCTACTATAA CCGAACTAGCCCCGTCTCCAAACAAGGCCTGGCCTACAAGACTATCGACATG ATTATCATTTGGT
Cpks9	5′-CTGGTGATGGGCTAGAATGGGGTGTTGCTTTTGGTTTCGGGCCGGGTCT CACTGTCGAAACTGTTGTGCTGCATAGTATGCCGATTGCTACTGTTCCTTGA GATGCAAGGTGATGAATCGGAATGTTTAATAACGGGATCCAGTTTTTCATTG TGTGCTGTAATAATTGCTCAAAACTTTATGGCGGTAGTATCATGTTATGTTC CCATGCACAGGTGTACCTTACGAGGTTACACCTGTTTTTTGGACCGGGTAGT ATTTGGATTCCCTCCGTTTCATCCGTTTCTTTATACTTTTTATTTTACCG
Cpks10	5′-TCGACGGTAGGGTTTCAGAAGAGGGGATAAATTTCAAGTTAGGAAGAGA CCTTCCACAAAAAATAGAGGACAACATTGAGGAGTTTTGCAAGAAGGTGATG GCGAAAGAAGGCATAAAGAAGTTCAATGATCTGTTTTGGGCAGTTCATCCAG GTGGACCGG
Cpks11	5′-CTTGCTTTAGCATTCGGACCGGGGATTACATTTGAAGGCATTCTCATGC GTAGCCTCTAAGTTTGTTTAACTACTCAAATTACTCAATTCACACTAAACTT ATGTTCACATAAAGTTTCTTAAGTTATCATCTGCTGCAATAGATATGATATG CTAATGAATATGAATGAGATTTCAGCTGTT
Cpks12	5′-GGCCTTGTTTGGGGATGGAGCTGTTGCGGTGATAGTGGGCTCAGACCCT GTTACTGGAGTTGAGAAACCGTTGTTCGAGATTGCTTCTGCAGCTCAAACCA TCCTTCCGGATAGTGATGGTGCTATCCAGGGTCATCTTCGGAAGATGGGACT TACTTTTCACCTTCTTAA

Appendix B: Alignment of PKSs of *Sarracenia* spp.



```
                                1        10        20        30        40        50        60
                                |        |         |         |         |         |         |
Conium PKS5                     -----MVTVNEFRKAHLAEGPATVLAIGTATPSYCIDQSTFPDLYFRTTKSEDETELKEK
Medicago CHS2                   -----MVSVSEIRKAQRAEGPATILAIGTANPANCVEQSTYPDFYFKITNSEHKTELKEK
Conium PKS2                     -----MVTVNEFRKAQQAEGPATVLAIGTATPPNCVDQSTYADYYFRVTKSEDKTELKEK
Gerbera CHS1                    --MASSVDMKAIRDAQRAEGPATILAIGTATPANCVYQADYPDYYFRITKSEHMVDLKEK
Conium PKS1                     -MANHSAKIEEIRKTQRAQGPANVLAIGTATPSNCVYQADYPDYHFRITNSKHMTDLKLK
Gerbera 2PS                     MGSYSSDDVEVIREAGRAQGLATILAIGTATPPNCVAQADYADYYFRVTKSEHMVDLKEK
Spsittacina-10591_isotig10557   -----MVSVDEVRKAQRAEGPATVMAIGTATPPNCVDQSTYPDYYFRITNSEHKAELKEK
Spsittacina-26038_isotig10557   -----MVSVDEVRKAQRAEGPATVMAIGTATPPNCVDQSTYPDYYFRITNSEHKAELKEK
Spurpurea-22109_isotig09797     -----MVTVEEVRKAQRAEGPATVLAIGTATPPNCVDQSTYPDYYFRITNSEHKTELKEK
Spurpurea-9803_isotig09797      -----MVTVEEVRKAQRAEGPATVLAIGTATPPNCVDQSTYPDYYFRITNSEHKTELKEK
Spurpurea-475_isotig00474       ------------------------------------------------------------
Spurpurea-12781_isotig00474     ------------------------------------------------------------
Spurpurea-478_isotig00477       ------------------------------------------------------------
Spurpurea-12783_00476           ------------------------------------------------------------
Spurpurea-477_isotig00476       ------------------------------------------------------------
Spurpurea-476_isotig00475       ------------------------------------------------------------
Spurpurea-12782_isotig00475     ------------------------------------------------------------
Spurpurea-12784_isotig00477     ------------------------------------------------------------
Spsittacina-5295_isotig05263    ------------------------------------------------------------
Spsittacina-5294_isotig05262    ------------------------------------------------------------
Spsittacina-20742_isotig05263   ------------------------------------------------------------
Spsittacina-20741_isotig05262   ------------------------------------------------------------
Spurpurea-4182_isotig04177      ------------------------------------------------------------
Spurpurea-16488_isotig04177     ------------------------------------------------------------
Spsittacina-24513_isotig09032   ------------------------------------------------------------
Spsittacina-9066_isotig09032    ------------------------------------------------------------

                                61       70        80        90        100       110       120
                                |        |         |         |         |         |         |
Conium PKS5                     FKRMCDRSMINTRYTHLTEEFMKENPDFWN-MAPSLDARQEIVVNEVPKLGKEAATKAIK
Medicago CHS2                   FQRMCDKSMIKRRYMYLTEEILKENPNVCEYMAPSLDARQDMVVVEVPRLGKEAAVKAIK
Conium PKS2                     FKRMCDKSMISTRYMHLTEELLKENPEICEYMAPSLDARQDIVVNEVPKLGKEAATRAIK
Gerbera CHS1                    FKRMCDKSMIRKRYMHITEEYLKQNPNMCAYMAPSLDVRQDLVVVEVPKLGKEAAMKAIK
Conium PKS1                     FKRMCEKSMIRKRYMHLTKDYLKKNPNVCAYEAPSLDARQDLVVVEVPRLGKEAASKAIK
Gerbera 2PS                     FKRICEKTAIKKRYLALTEDYLQENPTMCEFMAPSLNARQDLVVTGVPMLGKEAAVKAID
Spsittacina-10591_isotig10557   FQRMCDKSMIKKRYMYLTEEILKQNPNVCAYMAPSLDARQDMVVVEVPKLGKDAAVKAIK
Spsittacina-26038_isotig10557   FQRMCDKSMIKKRYMYLTEEILKQNPNVCAYMAPSLDARQDMVVVEVPKLGKDAAVKAIK
Spurpurea-22109_isotig09797     FKRMCKKKHI---YIFLYMYILIDIYLICMF-----------------------------
Spurpurea-9803_isotig09797      FKRMCKKKHI---YIFLYMYILIDIYLICMF-----------------------------
Spurpurea-475_isotig00474       ------------------------------------------------------------
Spurpurea-12781_isotig00474     ------------------------------------------------------------
Spurpurea-478_isotig00477       ------------------------------------------------------------
Spurpurea-12783_isotig00476     ------------------------------------------------------------
Spurpurea-477_isotig00476       ------------------------------------------------------------
Spurpurea-476_isotig00475       ------------------------------------------------------------
Spurpurea-12782_isotig00475     ------------------------------------------------------------
Spurpurea-12784_isotig00477     ------------------------------------------------------------
Spsittacina-5295_isotig05263    ------------------------------------------------------------
Spsittacina-5294_isotig05262    ------------------------------------------------------------
Spsittacina-20742_isotig05263   ------------------------------------------------------------
Spsittacina-20741_isotig05262   ------------------------------------------------------------
Spurpurea-4182_isotig04177      ------------------------------------------------------------
Spurpurea-16488_isotig04177     ------------------------------------------------------------
Spsittacina-24513_isotig09032   ------------------------------------------------------------
Spsittacina-9066_isotig09032    ------------------------------------------------------------
```



121 130 140 150 160 170 180
Conium PKS5 EWGQPMSKITHLVFCTYSSADFPGADFRLTKLLGLSPSVKRSMLYQQGCFAGGTGLRLAK
Medicago CHS2 EWGQPKSKITHLIVCTTSGVDMPGADYQLTKLLGLRPYVKRYMMYQQGCFAGGTVLRLAK
Conium PKS2 EWGQPKSKITHLIFCTTSGVDMPGADFRLTKLLGLRPSVKRFMLYQQGCFAGGTVLRLAK
Gerbera CHS1 EWGHPKSKITHLIFCTTSGVDMPGADYQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRLAK
Conium PKS1 EWGQSKSKITHLIFCTTSGVDMPGADYQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRLAK
Gerbera 2PS EWGLPKSKITHLIFCTTAGVDMPGADYQLVKLLGLSPSVKRYMLYQQGCAAGGTVLRLAK
Spsittacina-10591_isotig10557 EWGQPKSKITHLVF--
Spsittacina-26038_isotig10557 EWGQPKSKITHLVF--
Spurpurea-22109_isotig09797 --
Spurpurea-9803_isotig09797 --
Spurpurea-475_isotig00474 --
Spurpurea-12781_isotig00474 --
Spurpurea-478_isotig00477 --
Spurpurea-12783_isotig00476 --
Spurpurea-477_isotig00476 --
Spurpurea-476_isotig00475 --
Spurpurea-12782_isotig00475 --
Spurpurea-12784_isotig00477 --
Spsittacina-5295_isotig05263 --
Spsittacina-5294_isotig05262 --
Spsittacina-20742_isotig05263 --
Spsittacina-20741_isotig05262 --
Spurpurea-4182_isotig04177 --
Spurpurea-16488_isotig04177 --
Spsittacina-24513_isotig09032 --
Spsittacina-9066_isotig09032 --
181 190 200 210 220 230 240
Conium PKS5 DLAENNKGARVLVVCSELSVLAFQGPKVIDIDCLITQALFGDGAVAVIVGSDPVIGVEKP
Medicago CHS2 DLAENNKGARVLVVCSEVTAVTFRGPSDTHLDSLVGQALFGDGAAALIVGSDPVPEIEKP
Conium PKS2 DLAENNKGARVLIVCSEITVVTFRGPNDTHLDSLVGQALFGDGAAAVIVGSDPAIGIEKP
Gerbera CHS1 DLAENNKGARVLVVCSEITAVTFRGPNDTHLDSLVGQALFGDGAAAVIVGSDPDLTTERP
Conium PKS1 DLAENNAGARVLVVCSEITAVTFRGPSDSHLDSLVGQALFGDGAAAVIVGSDPDLSVERP
Gerbera 2PS DLAENNKGSRVLIVCSEITAILFHGPNENHLDSLVAQALFGDGAAALIVGSGPHLAVERP
Spsittacina-10591_isotig10557 --
Spsittacina-26038_isotig10557 --
Spurpurea-22109_isotig09797 --
Spurpurea-9803_isotig09797 --
Spurpurea-475_isotig00474 --
Spurpurea-12781_isotig00474 --
Spurpurea-478_isotig00477 --
Spurpurea-12783_isotig00476 --
Spurpurea-477_isotig00476 --
Spurpurea-476_isotig00475 --
Spurpurea-12782_isotig00475 --
Spurpurea-12784_isotig00477 --
Spsittacina-5295_isotig05263 --
Spsittacina-5294_isotig05262 --
Spsittacina-20742_isotig05263 --
Spsittacina-20741_isotig05262 --
Spurpurea-4182_isotig04177 --
Spurpurea-16488_isotig04177 --
Spsittacina-24513_isotig09032 --
Spsittacina-9066_isotig09032 --

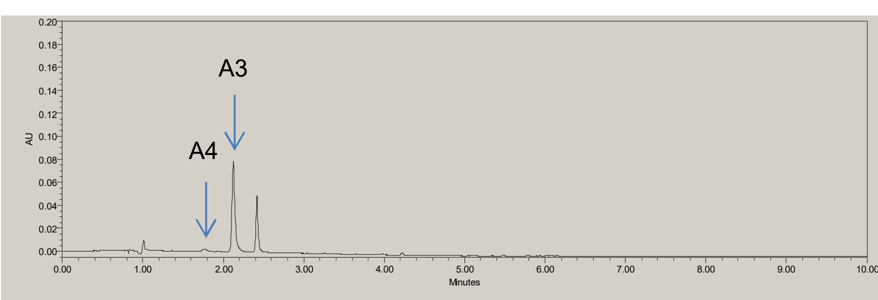
```
                              241       250       260       270       280       290       300
                              |         |         |         |         |         |         |
Conium PKS5                   LFEIFSAAQTIIPDSDGAIKGYLRKVGLTFHLRKDVPGLIAKNIRKYLVEAFQPLGITDW
Medicago CHS2                 IFEMVWTAQTIAPDSEGAIDGHLREAGLTFHLLKDVPGIVSKNITKALVEAFEPLGISDY
Conium PKS2                   LFEIVSAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIRKSLVEAFKPLGITDW
Gerbera CHS1                  LFEMVSAAQTILPDSEGAIDGHLREVGLTFHLLKDVPGLISKNIEKALTTAFSPLGINDW
Conium PKS1                   LFQLISAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLKEAFGPIGISDW
Gerbera 2PS                   IFEIVSTDQTILPDTEKAMKLHLREGGLTFQLHRDVPLMVAKNIENAAEKALSPLGITDW
Spsittacina-10591_isotig10557 ------------------------------------------------------------
Spsittacina-26038_isotig10557 ------------------------------------------------------------
Spurpurea-22109_isotig09797   ------------------------------------------------------------
Spurpurea-9803_isotig09797    ------------------------------------------------------------
Spurpurea-475_isotig00474     ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-12781_isotig00474   ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-478_isotig00477     ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-12783_isotig00476   ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-477_isotig00476     ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-476_isotig00475     ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-12782_isotig00475   ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-12784_isotig00477   ----------------------------TFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spsittacina-5295_isotig05263  -----------RAHSEGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spsittacina-5294_isotig05262  -----------RAHSEGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spsittacina-20742_isotig05263 -----------RAHSEGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spsittacina-20741_isotig05262 -----------RAHSEGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLVEAFQPLGISDW
Spurpurea-4182_isotig04177    ---------------------------------------------------------SDW
Spurpurea-16488_isotig04177   ---------------------------------------------------------SDW
Spsittacina-24513_isotig09032 --------------------------------------------------RSSHVGISDW
Spsittacina-9066_isotig09032  --------------------------------------------------RSSHVGISDW

                              301       310       320       330       340       350       360
                              |         |         |         |         |         |         |
Conium PKS5                   NSIFWIAHPGGPAILDQIEKELSLKPEKLKSSRQILRDYGNLSSASVLFIMDEMRKASAK
Medicago CHS2                 NSIFWIAHPGGPAILDQVEQKLALKPEKMNATREVLSEYGNMSSACVLFILDEMRKKSTQ
Conium PKS2                   NSIFWIAHPGGPTILDQIESELSLKAEKLKCTRQVLRDFGNLSSACVLFILDEMRKASAK
Gerbera CHS1                  NSIFWIAHPGGPAILDQVELKLGLKEEKLRATRHVLSEYGNMSSACVLFIIDEMRKKSSE
Conium PKS1                   NSLFWIAHPGGPAILDQVESKLGLKEEKMRATRQVLSDYGNMSSACVLFILDEMRKKSIE
Gerbera 2PS                   NSVFWMVHPGGRAILDQVERKLNLKEDKLRASRHVLSEYGNLISACVLFIIDEVRKRSMA
Spsittacina-10591_isotig10557 ------------------------------------------------------------
Spsittacina-26038_isotig10557 ------------------------------------------------------------
Spurpurea-22109_isotig09797   ------------------------------------------------------------
Spurpurea-9803_isotig09797    ------------------------------------------------------------
Spurpurea-475_isotig00474     NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-12781_isotig00474   NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-478_isotig00477     NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-12783_isotig00476   NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-477_isotig00476     NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-476_isotig00475     NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-12782_isotig00475   NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-12784_isotig00477   NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spsittacina-5295_isotig05263  NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spsittacina-5294_isotig05262  NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spsittacina-20742_isotig05263 NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spsittacina-20741_isotig05262 NSLFWIAHPGGPAILDKVEEKLALKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSAE
Spurpurea-4182_isotig04177    NSLFWIAHPGGPAILDQVELKLGLKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSVD
Spurpurea-16488_isotig04177   NSLFWIAHPGGPAILDQVELKLGLKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSVD
Spsittacina-24513_isotig09032 NSLFWIAHPGGPAILDQVELKLGLKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSVE
Spsittacina-9066_isotig09032  NSLFWIAHPGGPAILDQVELKLGLKPEKLRATRHVLSEYGNMSSACVLFILDEMRKKSVE
```

```
                             361      370       380       390       400       410       420
                             |        |         |         |         |         |         |
Conium PKS5                  DGKPTTGEGLDWGVLFGFGPGLTVETVVLHSVPT--------------------------
Medicago CHS2                NGLKTTGEGLEWGVLFGFGPGLTIETVVLRSVAI--------------------------
Conium PKS2                  DGKRSTGEGLDWGVLFGFGPGLTVETVVLHSVPT--------------------------
Gerbera CHS1                 NGAGTTGEGLEWGVLFGFGPGLTVETVVLHSVPTTVTVAV--------------------
Conium PKS1                  EGKATTGEGLDWGVLFGFGPGLTVETVVLHSVPATITQ----------------------
Gerbera 2PS                  EGKSTTGEGLDCGVLFGFGPGMTVETVVLRSVRVTAAVANGN------------------
Spsittacina-10591_isotig10557 ------------------------------------------------------------
Spsittacina-26038_isotig10557 ------------------------------------------------------------
Spurpurea-22109_isotig09797  ------------------------------------------------------------
Spurpurea-9803_isotig09797   ------------------------------------------------------------
Spurpurea-475_isotig00474    NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spurpurea-12781_isotig00474  NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spurpurea-478_isotig00477    NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spurpurea-12783_isotig00476  NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spurpurea-477_isotig00476    NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spurpurea-476_isotig00475    NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spurpurea-12782_isotig00475  NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spurpurea-12784_isotig00477  NGLKTTGEGLEWGVLFGFGPGSPSRLLCSTA-----------------------------
Spsittacina-5295_isotig05263 NGLKTTGEGLEWGVLFGFGPGLTVETVVLHSLST--------------------------
Spsittacina-5294_isotig05262 NGLKTTGEGLEWGVLFGFGPGLTVETVVLHSLST--------------------------
Spsittacina-20742_isotig05263 NGLKTTGEGLEWGVLFGFGPGLTVETVVLHSLST--------------------------
Spsittacina-20741_isotig05262 NGLKTTGEGLEWGVLFGFGPGLTVETVVLHSLST--------------------------
Spurpurea-4182_isotig04177   DGLKTTGEGLEWGVLFGFGPGLTVETVVLHSVTA--------------------------
Spurpurea-16488_isotig04177  DGLKTTGEGLEWGVLFGFGPGLTVETVVLHSVTA--------------------------
Spsittacina-24513_isotig09032 DGLKTTGEGLEWGVLFGFGPGLTVETVVLHSVTA--------------------------
Spsittacina-9066_isotig09032 DGLKTTGEGLEWGVLFGFGPGLTVETVVLHSVTA--------------------------
```

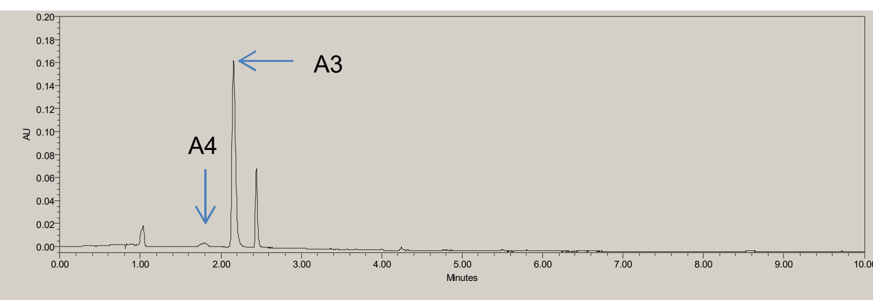
Figure B1 The alignment of PKSs in *Sarracenia* spp. together with selected PKSs translated into an amino acid sequence (Article IV). Conserved amino acids of the active site are in bold and coloured amino acids are mutated amino acids of the active site. GenBank accession numbers: *Conium maculatum* CPKS1 (KP726914), *Conium maculatum* CPKS2 (KP726915), *Conium maculatum* CPKS5 (KP726916), *Gerbera hybrida* 2PS (CAA86219.2), *Gerbera hybrida* CHS1 (Z38096.1), *Medicago sativa* CHS2 (L02902.1).

Appendix C: *In vitro* testing of CPKS1

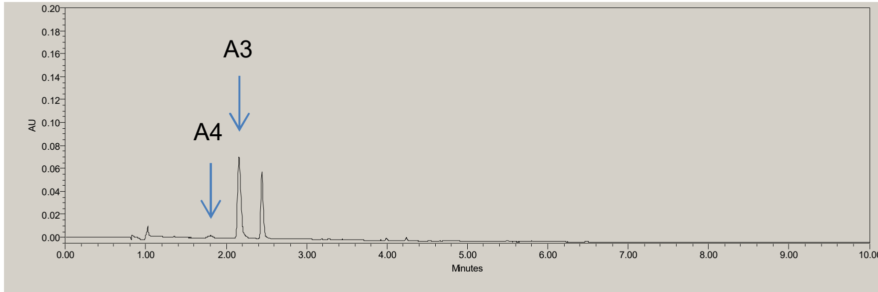
A



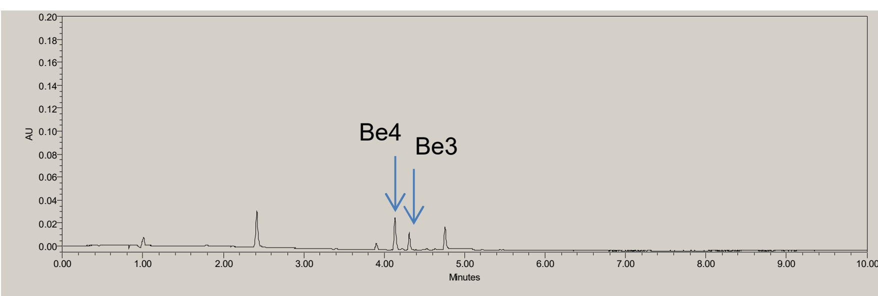
B



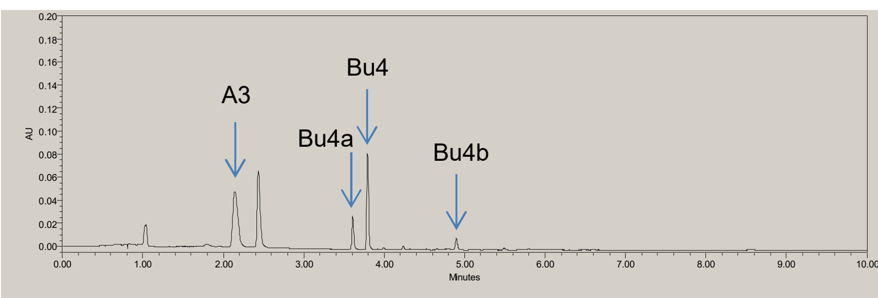
C



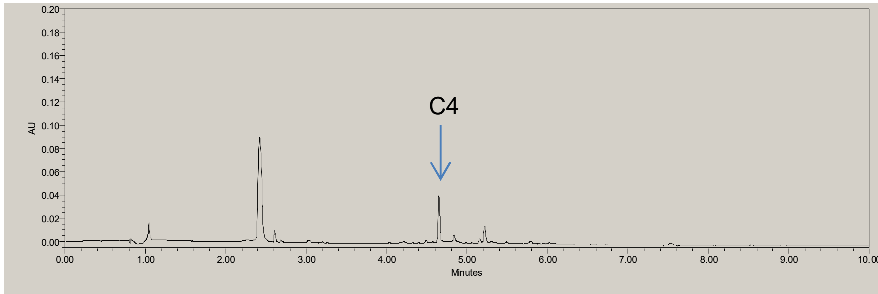
D



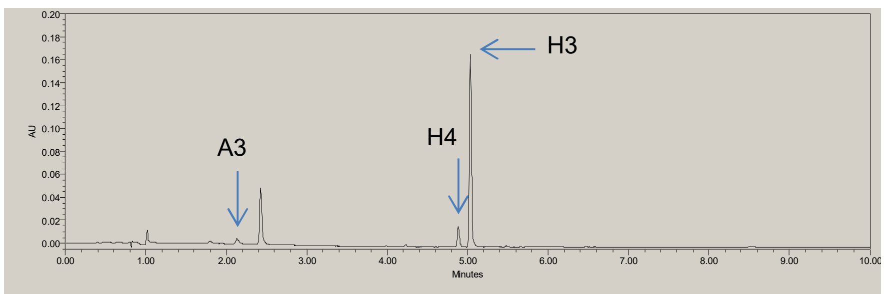
E



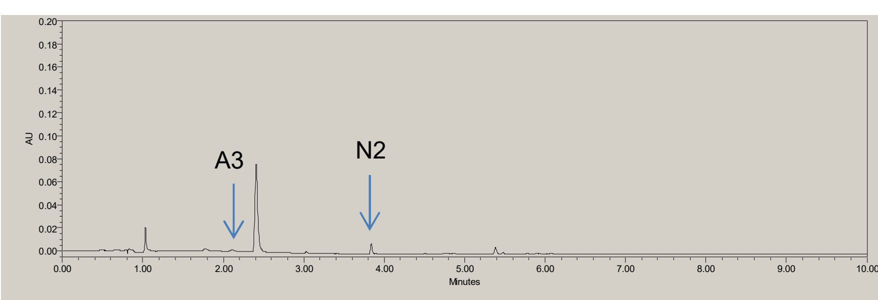
F



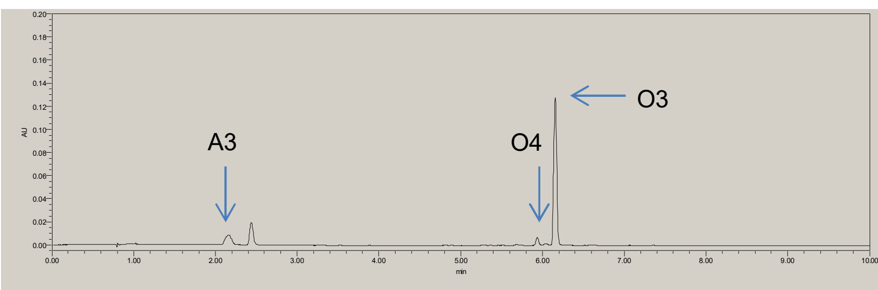
G



H



I



J

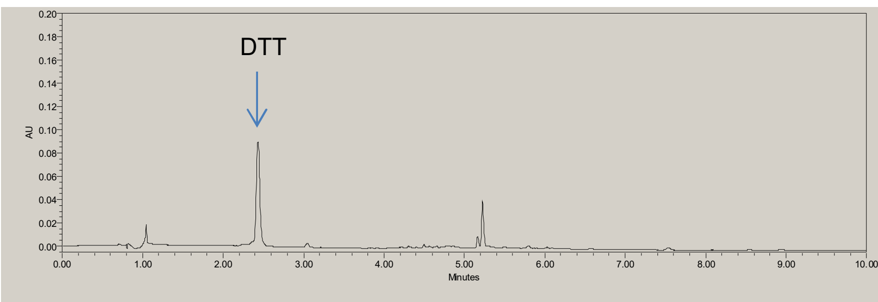
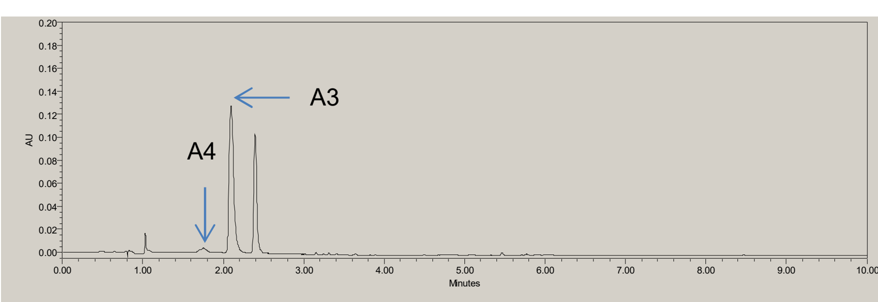


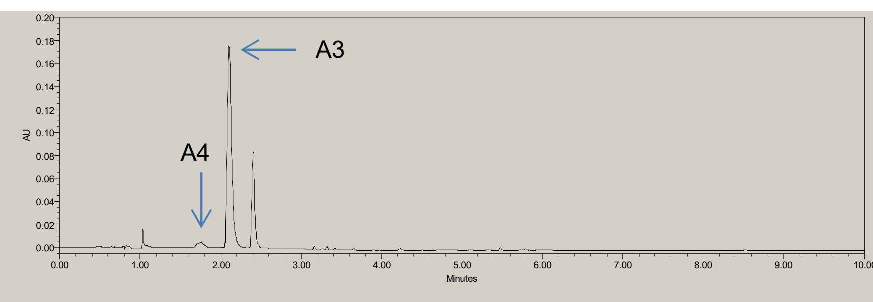
Figure C1 *In vitro* testing of CPKS1. **A** acetoacetyl-CoA, **B** acetyl-CoA, **C** malonyl-CoA, **D** benzoyl-CoA, **E** butyryl-CoA, **F** p-coumaryl-CoA, **G** hexanoyl-CoA, **H** *N*-methylanthraniloyl-CoA, **I** octanoyl-CoA and **J** boiled enzyme.

Appendix D: *In vitro* testing of CPKS2

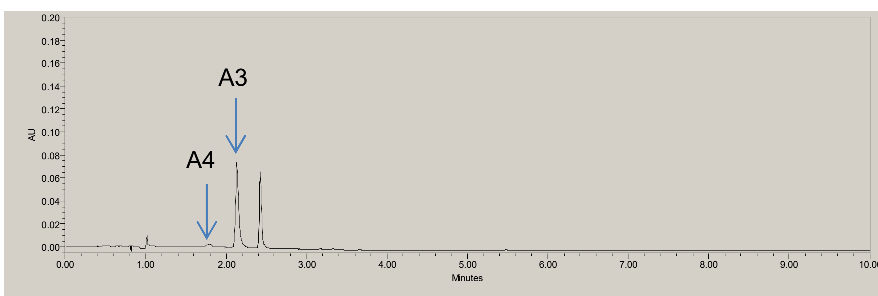
A



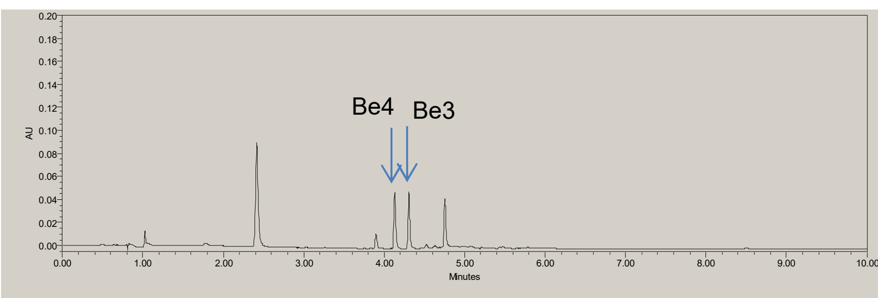
B



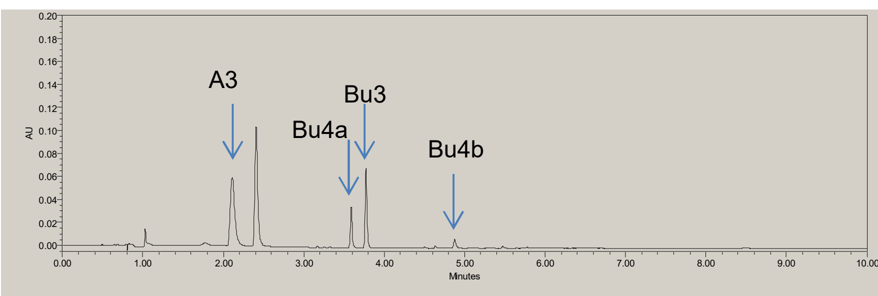
C



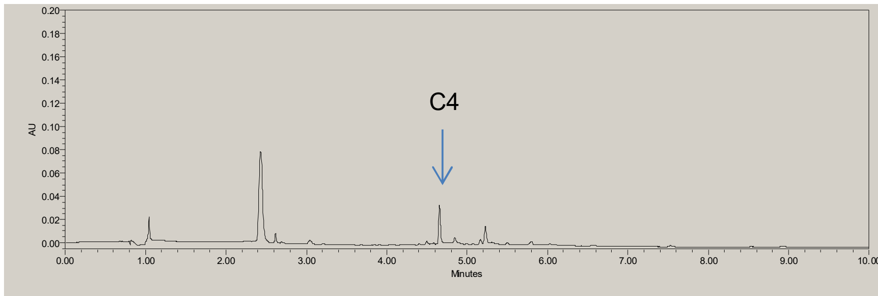
D



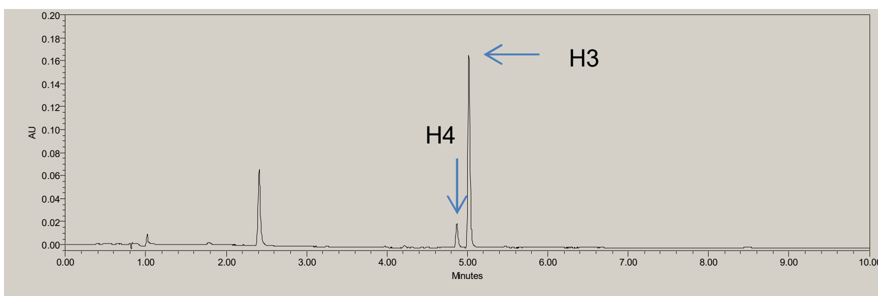
E



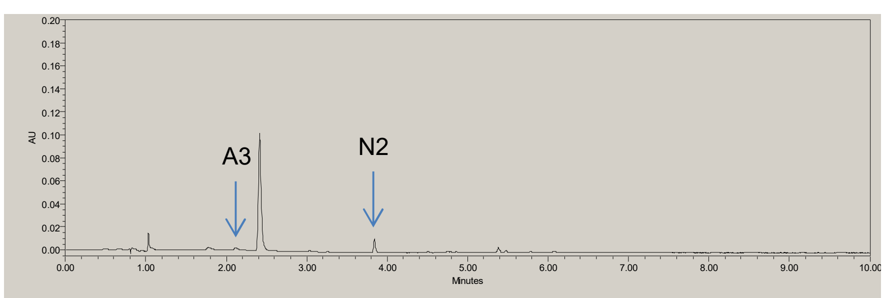
F



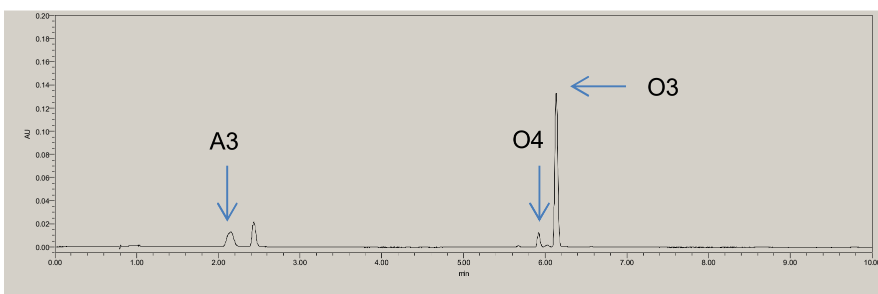
G



H



I



J

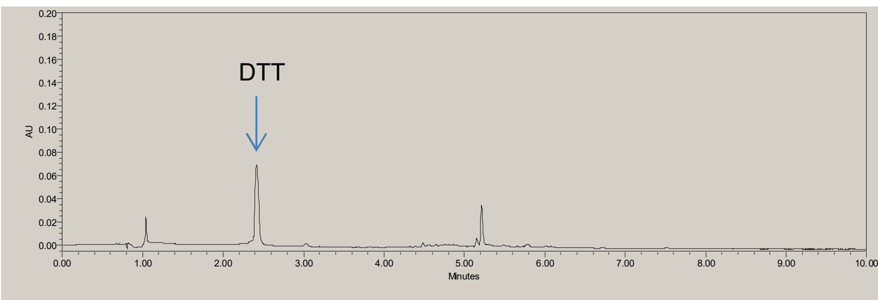
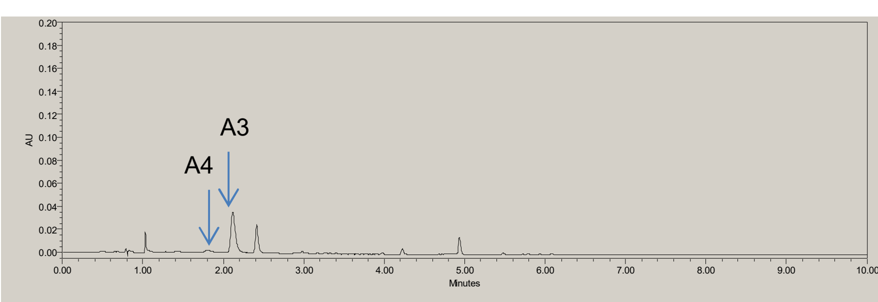


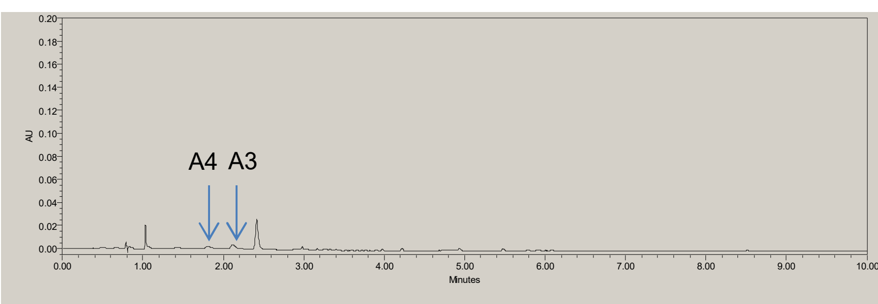
Figure D1 *In vitro* testing of CPKS2. **A** acetoacetyl-CoA, **B** acetyl-CoA, **C** malonyl-CoA, **D** benzoyl-CoA, **E** butyryl-CoA, **F** p-coumaryl-CoA, **G** hexanoyl-CoA, **H** *N*-methylanthraniloyl-CoA, **I** octanoyl-CoA and **J** boiled enzyme.

Appendix E: *In vitro* testing of CPKS5

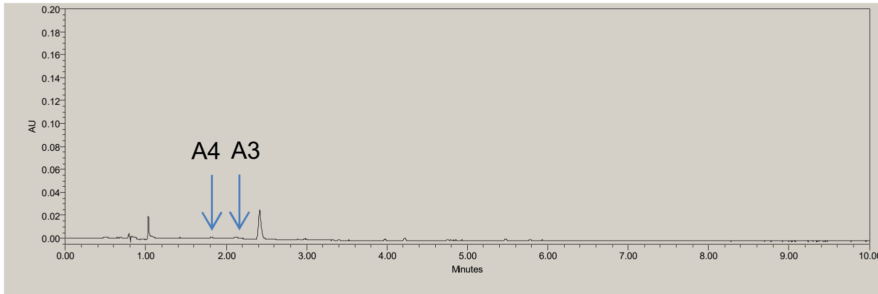
A



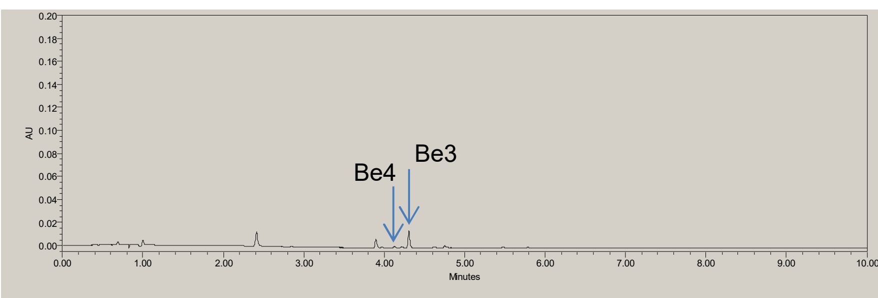
B



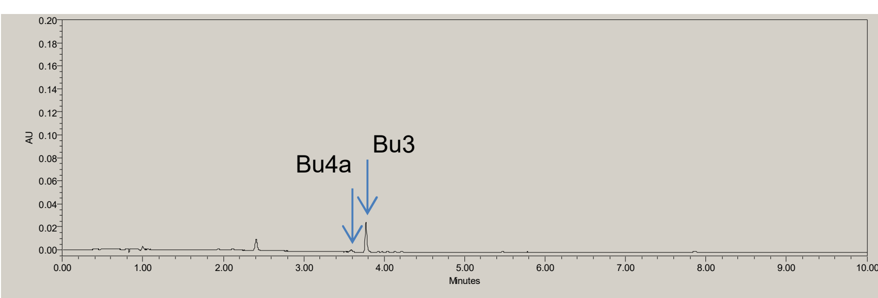
C



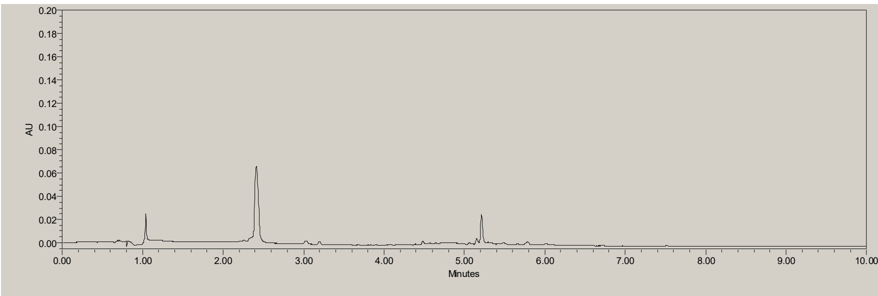
D



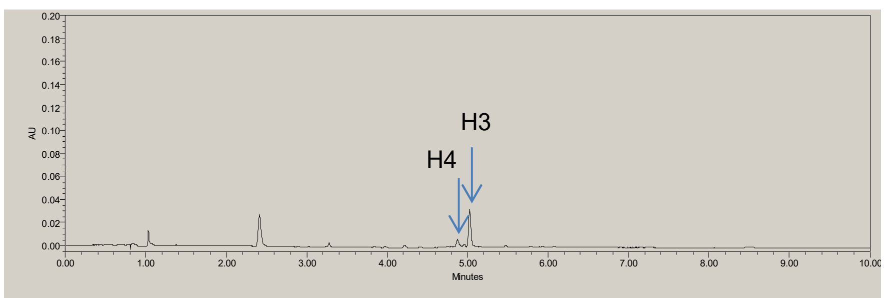
E



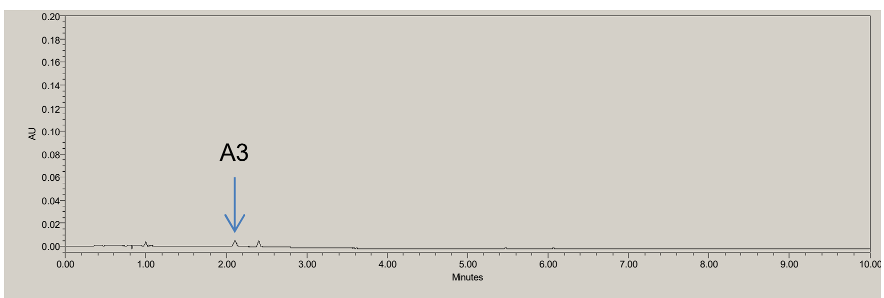
F



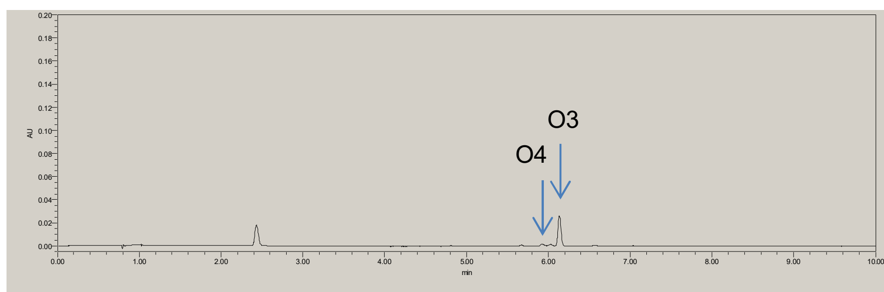
G



H



I



J

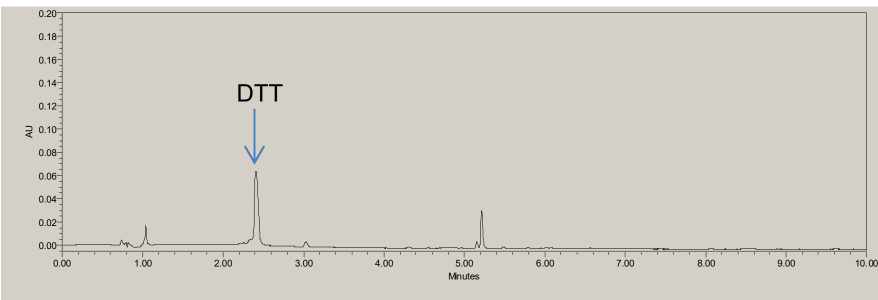
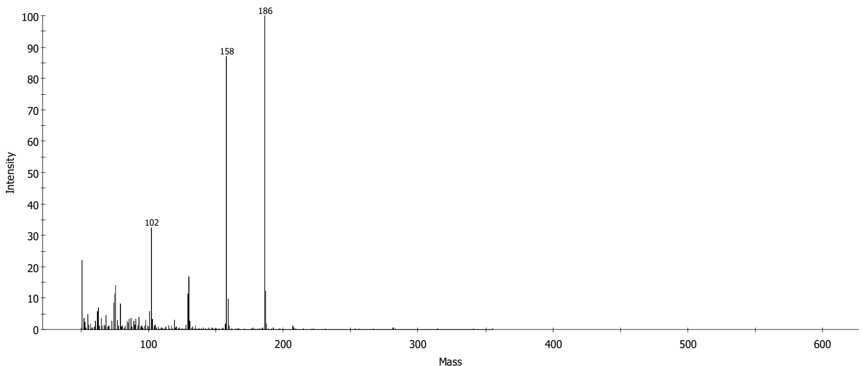


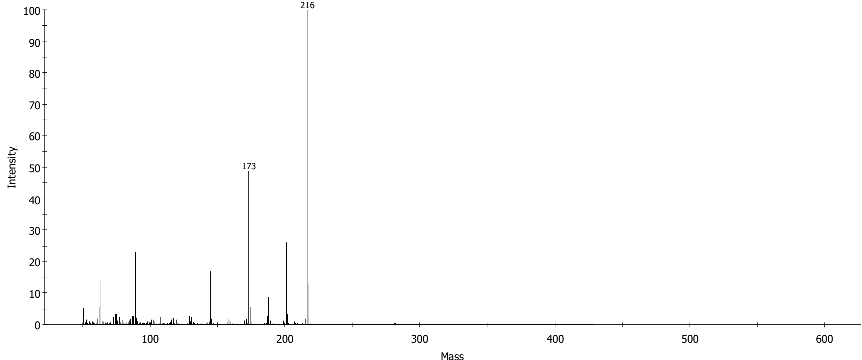
Figure E1 *In vitro* testing of CPKS5. **A** acetoacetyl-CoA, **B** acetyl-CoA, **C** malonyl-CoA, **D** benzoyl-CoA, **E** butyryl-CoA, **F** *p*-coumaryl-CoA, **G** hexanoyl-CoA, **H** *N*-methylanthraniloyl-CoA, **I** octanoyl-CoA and **J** boiled enzyme.

Appendix F: Mass spectra of elicitated furanocoumarins

A

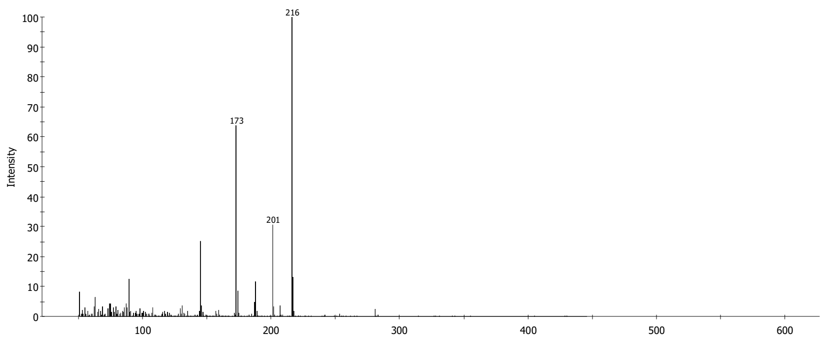


B



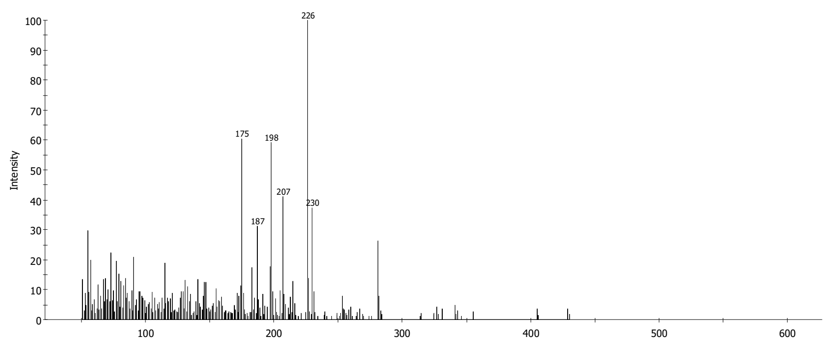
C

Time 16.0274 mins, Scan# 1749, m/z 326.083, Rel Int 56.44%



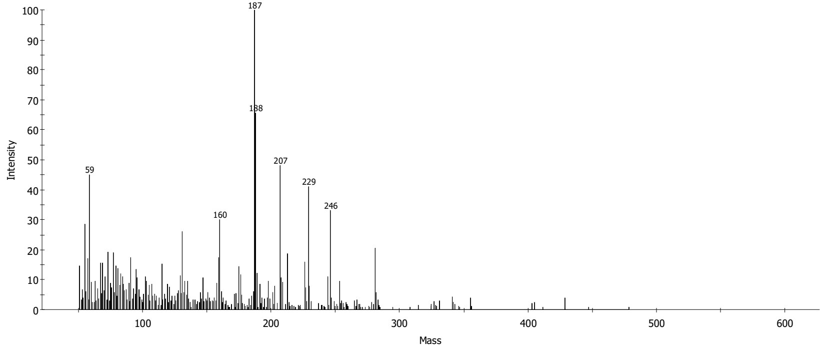
D

Time 16.4216 mins, Scan# 1812, m/z 330.362, Rel Int 71.17%



E

Time 16.7467 mins, Scan# 1864, m/z 285.435, Rel Int 73.01%



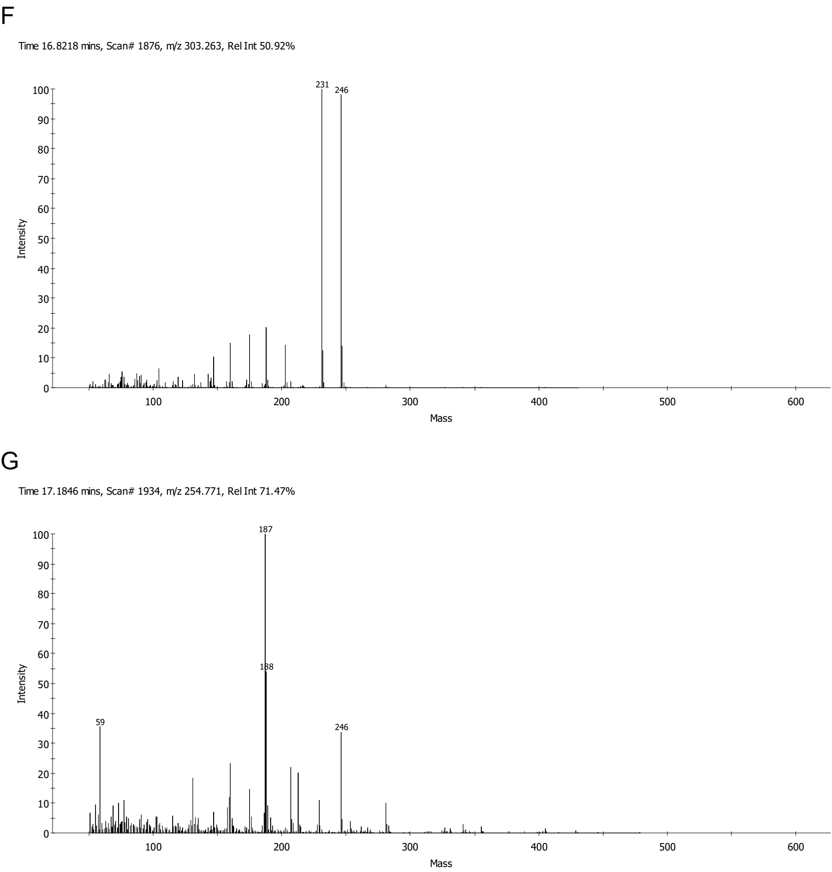


Figure F1 Mass spectra of furanocoumarins. **A** Psoralen, **B** Xanthotoxin, **C** Bergapten, **D** Oroselone, **E** Marmesin, **F** Isopimpinellin, **G** Columbianetin (Article II).

Appendix G: Results of *A. viguieri* micropropagation

Table G1 *A. viguieri* plantlet formation with different BA and NAA combinations and treatment durations (Article III).

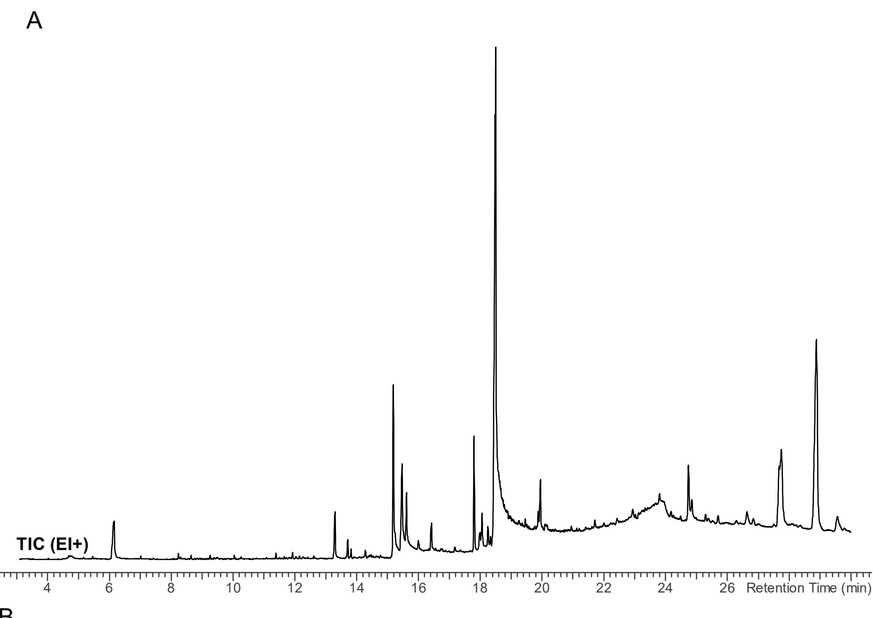
BA (mg/l)	NAA (mg/l)	Time (weeks)	Number of plantlets	Data included/excluded
0.06	0.1	4	0	included
0.25	0.1	4	2	included
0.06	0.4	4	0	excluded
0.25	0.4	4	5	included
0.06	0.1	6	1	included
0.25	0.1	6	2	included
0.06	0.4	6	0	included
0.25	0.4	6	0	included
0.06	0.25	5	0	included
0.25	0.25	5	1	included
0.16	0.1	5	1	included
0.16	0.4	5	2	included
0.16	0.25	4	1	included
0.16	0.25	6	0	included
0.16	0.25	5	1	included
0.16	0.25	5	0	included
0.16	0.25	5	0	included

Table G2 *A. viguieri* plantlet formation with different BA and NAA combinations. Results were recorded after 4, 5 and 6 weeks (Article III).

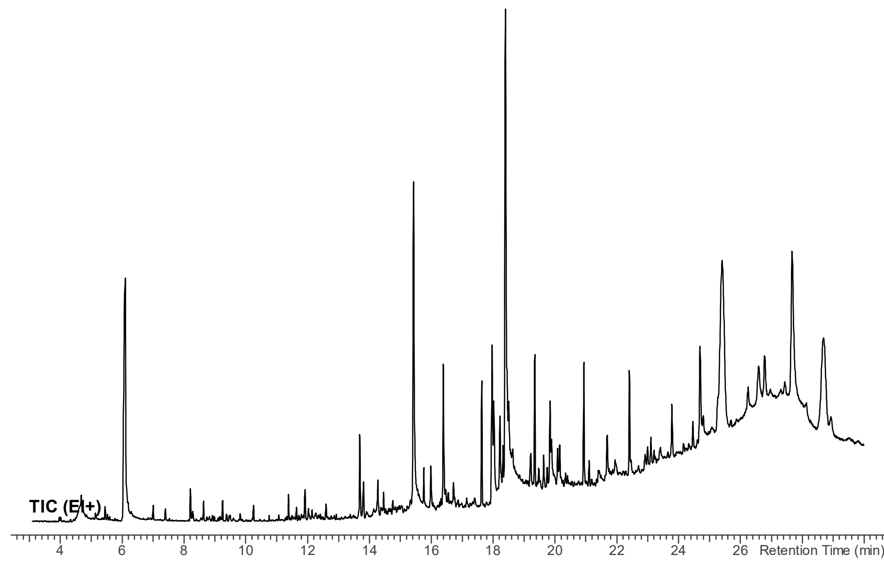
		Number of plantlets		
BA (mg/l)	NAA (mg/l)	4 weeks	5 weeks	6 weeks
0.25	0.4	0	0	0
0.25	2	0	0	0
4	0.4	1	1	1
4	2	0	1	1
2.13	0.4	2	2	2
2.13	2	3	4	5
0.25	1.2	2	2	2
4	1.2	7	7	9
2.13	1.2	1	8	8
2.13	1.2	2	2	2
2.13	1.2	0	3	3

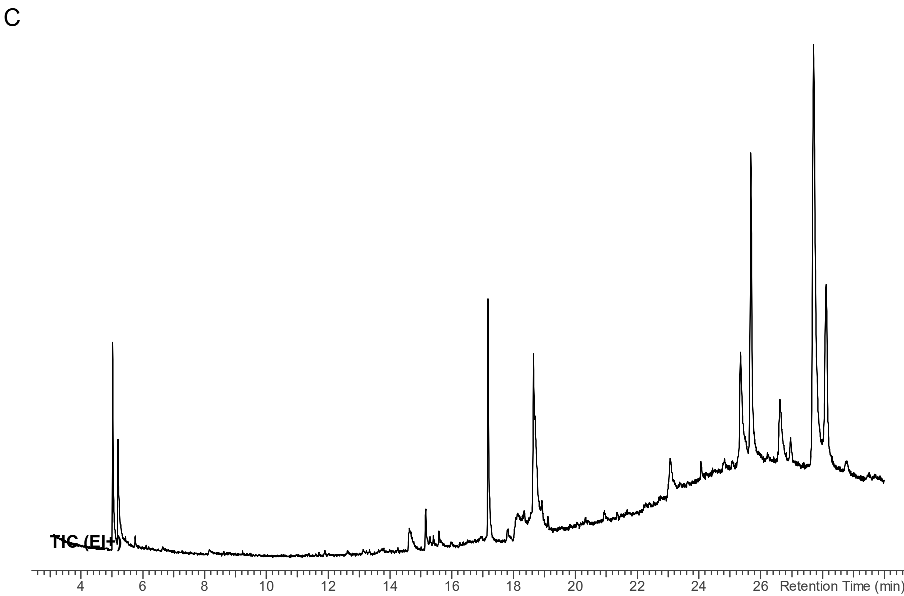
Appendix H: Selected GC-MS chromatograms of the examined plant material

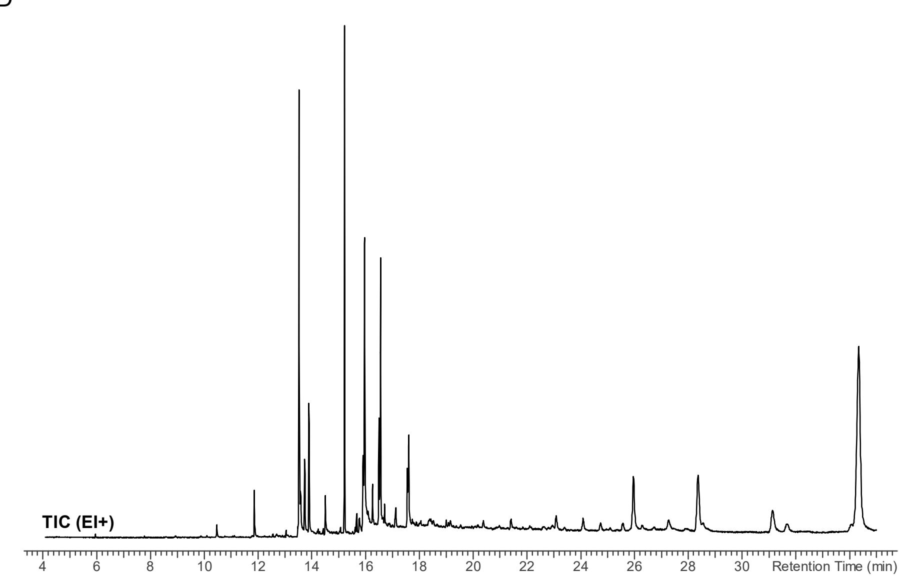
A



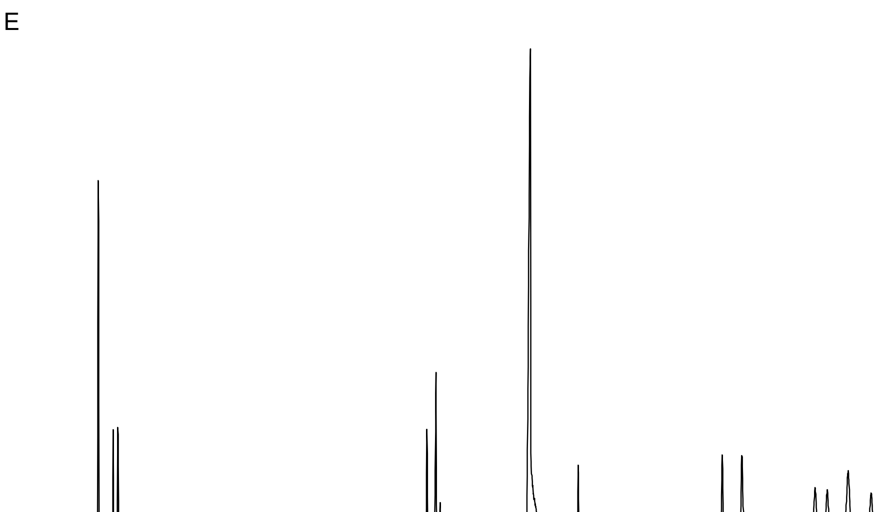
B



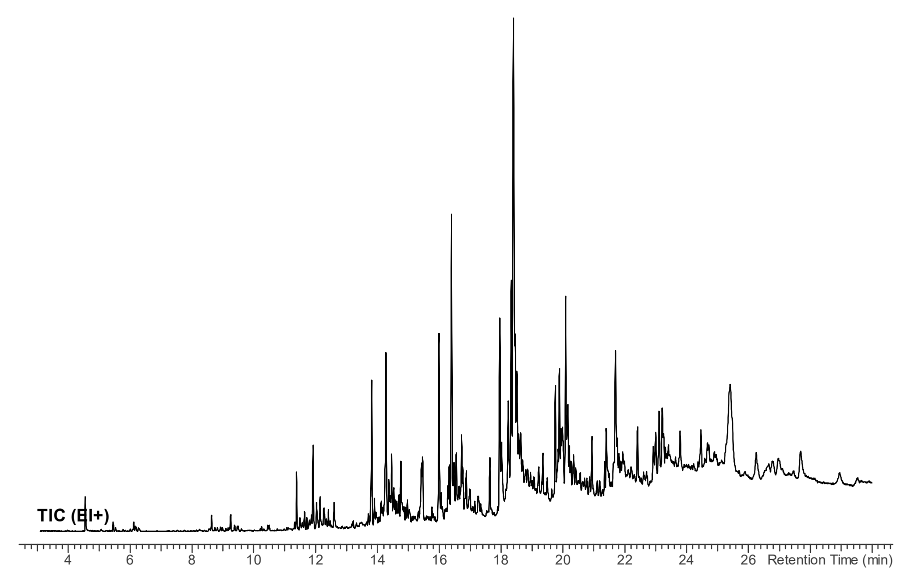

C
TIC (EI+)
4
6
8
10
12
14
16
18
20
22
24
26
Retention Time (min)

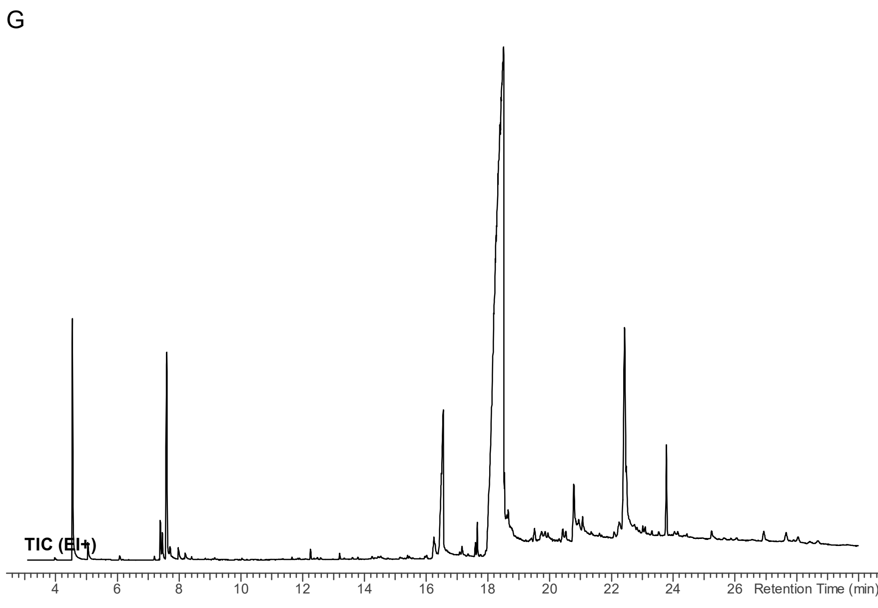

D
TIC (EI+)
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Retention Time (min)

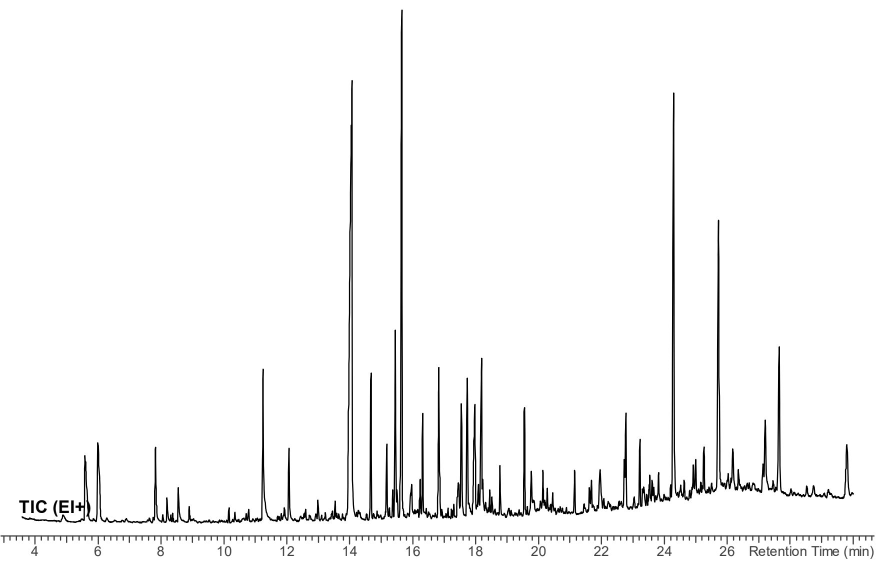
E



F




G
TIC (EI+)
4 6 8 10 12 14 16 18 20 22 24 26
Retention Time (min)


H
TIC (EI+)
4 6 8 10 12 14 16 18 20 22 24 26
Retention Time (min)

I

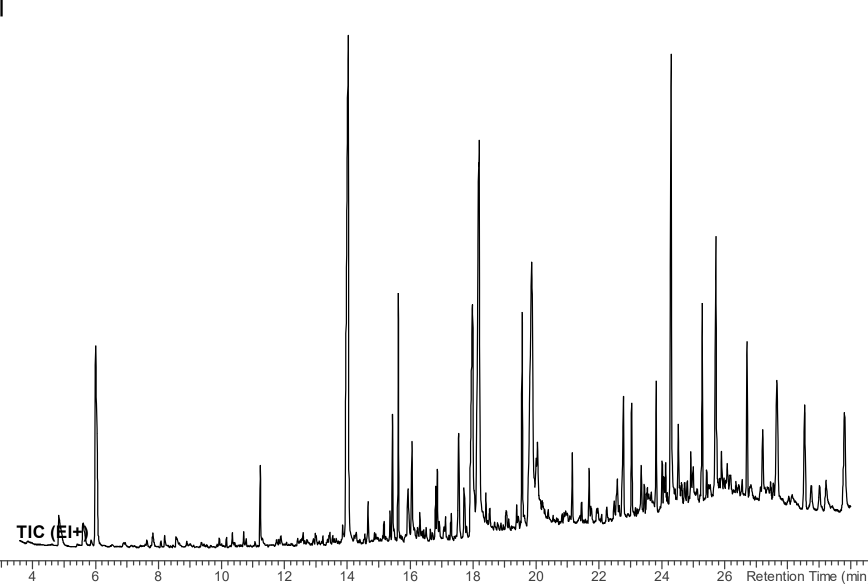


Figure H1 The selected GC-MS chromatograms of examined plant material. **A** *Aloe gariepensis* leaf, **B** *A. gariepensis* root, **C** *A. globuligemma* leaf, **D** *A. globuligemma* root, **E** *A. viguieri* leaf, **F** *A. viguieri* root, **G** *Conium maculatum* developing seed, **H** *Sarracenia flava* lid, **I** *S. flava* pitcher.

ARTICLE I

Polyketide synthases from poison hemlock (*Conium maculatum* L.)

FEBS Journal 282, 4141–4156.
Copyright 2015 FEBS.
Reprinted with permission from the publisher.

Polyketide synthases from poison hemlock (*Conium maculatum* L.)

Hannu Hotti[1], Tuulikki Seppänen-Laakso[1], Mikko Arvas[1], Teemu H. Teeri[2] and Heiko Rischer[1]

1 VTT Technical Research Centre of Finland Ltd., Espoo, Finland
2 Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland

Keywords
alkaloids; coniine; *Conium maculatum* (poison hemlock); polyketide synthase; secondary metabolites

Correspondence
H. Rischer, VTT Technical Research Centre of Finland Ltd., PO Box 1000, Espoo FI-02044 VTT, Finland
Fax: +358 20 722 7071
Tel: +358 20 722 4461
E-mail: heiko.rischer@vtt.fi

(Received 10 February 2015, revised 4 August 2015, accepted 7 August 2015)

doi:10.1111/febs.13410

Coniine is a toxic alkaloid, the biosynthesis of which is not well understood. A possible route, supported by evidence from labelling experiments, involves a polyketide formed by the condensation of one acetyl-CoA and three malonyl-CoAs catalysed by a polyketide synthase (PKS). We isolated PKS genes or their fragments from poison hemlock (*Conium maculatum* L.) by using random amplification of cDNA ends (RACE) and transcriptome analysis, and characterized three full-length enzymes by feeding different starter-CoAs *in vitro*. On the basis of our *in vitro* experiments, two of the three characterized PKS genes in poison hemlock encode chalcone synthases (CPKS1 and CPKS2), and one encodes a novel type of PKS (CPKS5). We show that CPKS5 kinetically favours butyryl-CoA as a starter-CoA *in vitro*. Our results suggest that CPKS5 is responsible for the initiation of coniine biosynthesis by catalysing the synthesis of the carbon backbone from one butyryl-CoA and two malonyl-CoAs.

Database
Nucleotide sequence data are available in GenBank/EMBL/DDBJ under the accession numbers KP726914, KP726915, KP726916, KP726917, KP726918, and KP726919

Introduction

Alkaloids are secondary metabolites that are classified as basic natural compounds containing nitrogen [1]. Typically, they exert pharmacological effects on humans and animals. Poison hemlock (*Conium maculatum* L.) (Apiaceae) is known to accumulate toxic alkaloids such as the notorious coniine [2,3], predominantly in vegetative organs and fruits [4]. The philosopher Socrates was sentenced to death by drinking poison hemlock juice in 399 BC in ancient Greece. Plato, a pupil of Socrates, documented this case of hemlock poisoning for the first time in his treatise *Phaedo* [5]. Later, coniine was the first alkaloid to be chemically synthesized [6].

Hemlock alkaloids strongly affect the nervous system. Coniine binds to the nicotinic neuromuscular receptors [7]. The most poisonous form is (–)-coniine; (+)-coniine is less poisonous. In mice, the LD_{50} for (–)-coniine is 7.0 $mg{\cdot}kg^{-1}$ intravenously, and that for (+)-coniine is 12.1 $mg{\cdot}kg^{-1}$ [7]. The oral LD_{50} for coniine is 100 $mg{\cdot}kg^{-1}$. Poison hemlock is dangerous to humans when consumed accidently. There have been cases of confusion with edible Apiaceae species, such

Abbreviations
CHS, chalcone synthase; PK, polyketide; PKS, polyketide synthase; RACE, random amplification of cDNA ends; STS, stilbene synthase; UPLC, ultra-HPLC.

as parsley leaves (*Petroselinum crispum*), parsnip root (*Pastinaca sativa*), or anise seeds (*Pimpinella anisum*) [2,8].

Despite its toxicity, poison hemlock has been used as a medicine. Externally, it was used for treating herpes, erysipelas (a bacterial infection), and breast tumours [2]. Dried leaves and juice of poison hemlock were included in the official pharmacopoeias of London and Edinburgh from 1864 to 1898. The plant had its last official recognition as a medicine in the British Pharmaceutical Codex of 1934 [9]. Obviously, the plant extract is difficult to administer internally, because of the thin line between therapeutic and poisonous levels [2,10].

Twelve different piperidine alkaloids, including the infamous coniine, have been identified in poison hemlock (Fig. 1). Hemlock alkaloids can be categorized according to the number of carbon atoms in their molecules: 2-Methylpiperidine contains six carbons [11,12]; coniine, γ-coniceine, *N*-methylconiine [11], conhydrine, conhydrinone [13], pseudoconhydrine, *N*-methylpseudoconhydrine [14] and 1′-oxo-γ-coniceine [12] contain eight carbons; and 2-n-pentyl-3,4,5,6-tetrahydropyridine, 5-hydroxy-2-n-pentylpiperidine [15] and conmaculatin [16] contain 10 carbons.

Leete [17] suggested that the carbon backbone of coniine is formed via a poly-β-keto acid. He showed that feeding poison hemlock with [1-^{14}C]acetate resulted in coniine with evenly labelled carbons. By contrast, [2-^{14}C]DL-lysine was not incorporated into coniine at all [18]. Therefore, coniine is not lysine-derived like many other piperidine alkaloids. When labelled 5-oxooctanal, 5-oxooctanoic acid, octanoic acid and hexanoic acid were fed to the plant, they were incorporated in turn into the carbon backbone of coniine [13,19–21]. Leete and Olson concluded that 5-oxooctanal and 5-oxooctanoic acid are plausible precursors for γ-coniceine, and hypothesized that 2-methylpiperidine could be formed in a similar manner from 5-oxohexanoic acid [21].

The carbon backbone of coniine presumably originates from the polyketide (PK) pathway [2,3,22]. The common process of PK biosynthesis, in which a starter-CoA is elongated by malonyl-CoA 1–7 times before the formed product is released by the enzyme [23], is responsible for the generation of many natural products, including flavonoids and stilbenes. For coniine, Leete [17,18] suggested that acetyl-CoA initially functions as a starter unit, which is elongated by three malonyl-CoAs (Fig. 2). The formed tetraketide intermediate would go through several reduction steps. The nitrogen of L-alanine is subsequently introduced in a transaminase-catalysed reaction [24]. Two isozymes, mitochondrial transaminase A and chloroplastic transaminase B, were found to perform this reaction in hemlock [25,26], but even transaminase originating from spinach (*Spinacia oleracea*) leaf extract is functional [27]. Cyclization to γ-coniceine occurs spontaneously [28]. Coniine is finally derived from γ-coniceine by γ-coniceine reductase catalysis

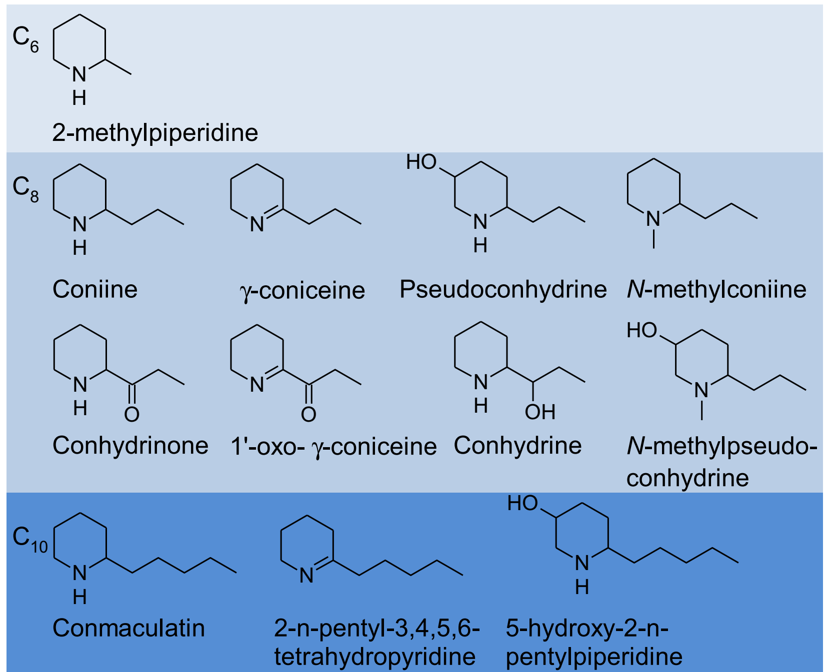


Fig. 1. Classification of naturally occurring poison hemlock (*C. maculatum*) alkaloids according to their carbon numbers.

Acetyl-CoA + 3 x malonyl-CoA → [Tetraketide intermediate] → Reductions → 5-oxooctanal → Transamination, Cyclization → γ-coniceine

Fig. 2. Biosynthesis of coniine in poison hemlock (*C. maculatum*) according to Leete [17,18] and Roberts [24,28].

[29], which is NADPH-dependent. It is believed that all other poison hemlock alkaloids are derived from coniine. Although several enzymes involved in coniine biosynthesis have been functionally characterized, their amino acid and coding gene sequences remain unknown.

Among the alkaloids, only the biosyntheses of acridones [30] and quinolones [31] have been shown to involve plant specific (type III) PK synthase (PKS). In both cases, the assumed starter unit already contains nitrogen. There is evidence suggesting that naphthylisoquinolines are also PKs, receiving their nitrogen via transamination [32]. Reynolds [3], Roberts [22] and Vetter [2] proposed that coniine biosynthesis involves a PK. Therefore, we searched for a non-chalcone synthase (CHS)/stilbene synthase (STS)-type enzyme that could start the condensation reaction with acetyl-CoA according to Leete's hypothesis [17,18]. Several acetyl-CoA-accepting PKSs are known, e.g. 2-pyrone synthase from *Gerbera hybrida* [33]. In this study, we describe three PKSs from poison hemlock and their possible roles in coniine formation. Interestingly, a non-CHS/STS enzyme found in this study kinetically favours C_4 esters, leading to a new suggestion concerning how the carbon backbone of coniine is formed. In contrast to Leete's hypothesis, our theory includes only two condensations and one reduction reaction.

Results

Cloning of CPKSs

A homology-based approach was used to clone PKS genes from poison hemlock. PCR with genomic DNA as the template and the primers deg-CHS-R and deg-CHS-F [34] (Table S1) provided 118-bp fragments for which the closest BLAST hits were plant type III PKSs. We obtained five different fragments with this method, and named them *Cpks1–Cpks5*. We cloned the 5′ and 3′ ends of *Cpks1* and *Cpks2* by random amplification of cDNA ends (RACE). The cloning of *Cpks3*, *Cpks4* and *Cpks5* with cDNA from leaf, stem, root, flowers and developing seeds via RACE was unsuccessful. However, the sequences of *Cpks5* and of seven more fragments numbered *Cpks6–Cpks12* were obtained through transcriptome analysis (H. Rischer & T. H. Teeri, unpublished). The fragments of *Cpks3*, *Cpks4*, *Cpks10*, *Cpks11* and *Cpks12* do not have overlapping regions with other fragments. The fragments *Cpks6* and *Cpks9* do have overlapping regions. *Cpks1*, *Cpks2*, *Cpks5*, *Cpks6*, *Cpks7* and *Cpks9* have been deposited in GenBank under the accession numbers KP726914, KP726915, KP726916, KP726917, KP726918, and KP726919, respectively. The sequences of *Cpks3*, *Cpks4*, *Cpks8* and *Cpks10–Cpks12* are shown in Table S2.

Cpks1 and *Cpks2* sequences both share >90% amino acid sequence identity with plant type III PKSs in the databank, and show the highest sequence similarity with carrot (*Daucus carrota*) *CHS2* (97%) and *CHS1* (93%), respectively.

Cpks1 contains a 1191-bp ORF that encodes a 43.4-kDa protein with a pI of 6.20. The protein consists of 397 amino acids, including the conserved Cys-His-Asn catalytic triad (Fig. 3) [23]. The active site size of the enzyme is 945 Å^3, and the homodimer's active sites are identical.

Cpks2 contains a 1170-bp ORF that encodes a 42.6-kDa protein with a pI of 6.59. The protein consists of 389 amino acids, and shares 81% identity with *Cpks1* and 83% identity with *Cpks5*. *Cpks2* also contains the conserved Cys-His-Asn catalytic triad [23]. The enzyme's active site size is 940 Å^3, and the homodimer's two active sites are identical.

Cpks5 contains a 1167-bp ORF that encodes a 42.6-kDa protein with a pI of 6.62. The protein consists of 388 amino acids, and shares 72% identity with *Cpks1*. It shares over 70% amino acid sequence identity with type III PKSs in the databank, and shows the highest sequence similarity (81%) with carrot *CHS1*. The enzyme contains the conserved Cys-His-Asn catalytic triad [23]. The active site size for chain A is 934 Å^3, and that for chain B is 858 Å^3, according to the model obtained with the CASTP program. Seven amino acids of the CPKS5 active site are changed (Table 1), and there is one deletion at position 86 [according to alfalfa (*Medicago sativa*) CHS2 numbering] [35].

All three full-length poison hemlock enzymes were overexpressed in *Escherichia coli* as His_6-tagged proteins, and were purified by affinity chromatography

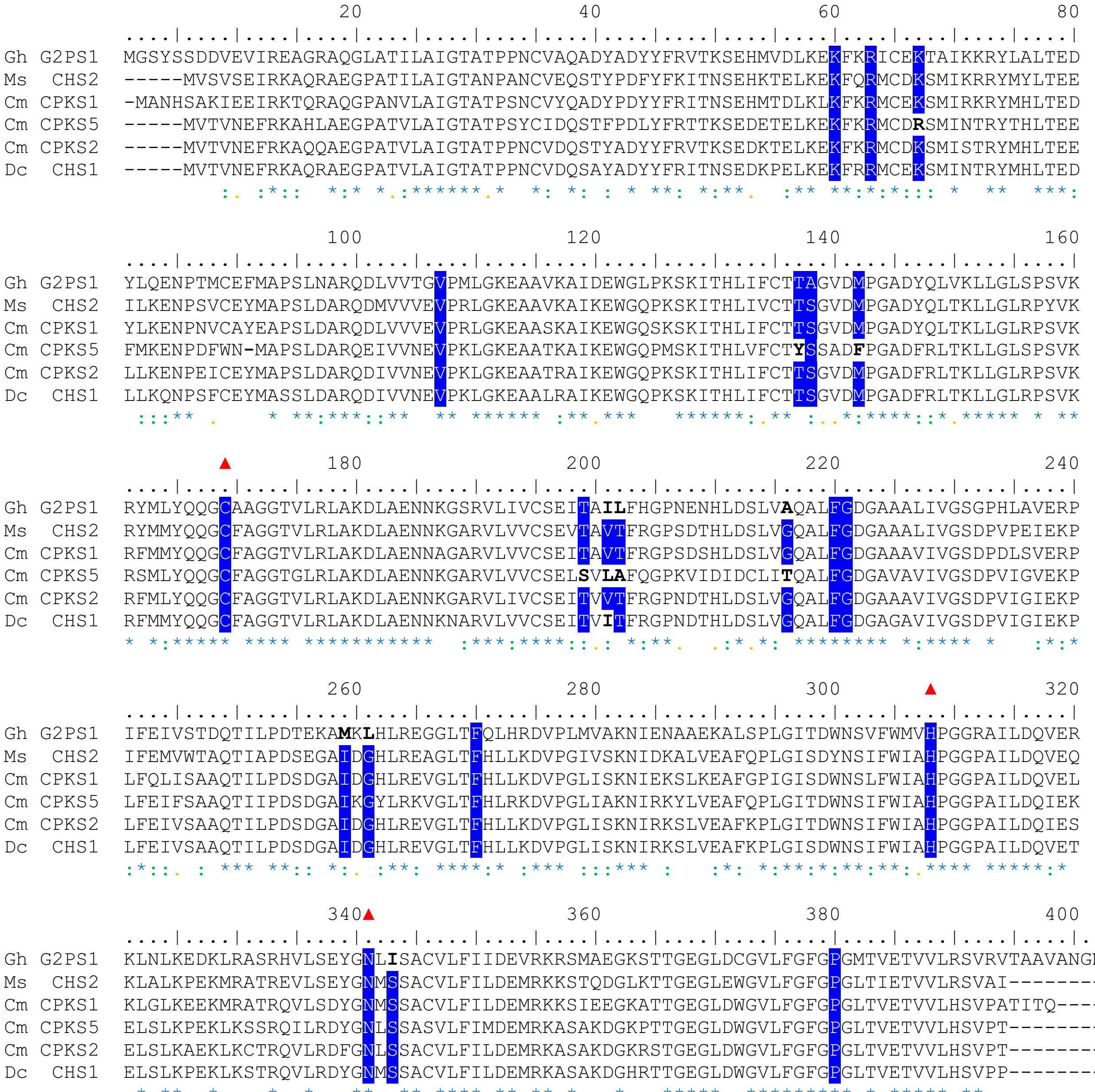


Fig. 3. Analysis of deduced amino acid sequences encoded by poison hemlock (*C. maculatum*) *CHS*-like genes: comparison between *M. sativa* (Ms) CHS2, *D. carota* (Dc) CHS1, *G. hybrida* (Gh) G2SP1, and *C. maculatum* (Cm) CPKS1, CPKS2, and CPKS5. Functionally important conserved amino acids are highlighted with a blue background, and changed amino acids in these positions are in bold. ▲ indicates the catalytic triad (Cys-His-Asn).

on an Ni^{2+}–nitrilotriacetic acid agarose matrix for enzymatic characterization.

Phylogenetic tree

In order to analyse the identified genes in the context of the whole type III PKS protein family, we carried out an exhaustive phylogenetic analysis of the family. All protein sequences belonging to the family were retrieved from TrEMBL and supplemented with closest matches of the identified genes from NCBI's environmental and patent databases. A subset of the whole phylogeny of relevant plant proteins is shown in Fig. 4. Fragments CPKS7 and CPKS8 were not included, because they

Table 1. Comparison of alfalfa (*M. sativa*) CHS2 and poison hemlock (*C. maculatum*) CPKS5 active site amino acids. The underlined amino acids are changed, and the numbering is according to either alfalfa CHS2 or poison hemlock CPKS5.

Alfalfa CHS2	Poison hemlock CPKS5
Catalytic triad	
Cys164	Cys163
His303	His302
Asn336	Asn335
‘Gatekeepers’	
Phe215	Phe214
Phe265	Phe264
CoA-binding tunnel	
Lys55	Lys55
Arg58	Arg58
Lys62	Arg62
Initiation pocket	
Ile254	Ile253
Gly256	Gly255
Phe265	Phe264
Elongation pocket	
Thr132	Tyr131
Ser133	Ser132
Thr194	Ser193
Val196	Leu195
Thr197	Ala196
Gly216	Gly215
Ser338	Ser337
Other functions	
Val98	Val97
Met137	Phe136
Gly211	Thr210
Phe375	Phe374

could not be aligned reliably. As expected, the full-length poison hemlock genes cluster with Apiaceae genes. CPKS2 and CPKS5 appear to be poison hemlock-specific paralogues and orthologues of carrot CHS1 and CHS9, and *Glehnia littoralis* CHSs. The fragment CPKS6 is clustered together with them. CPKS1 is an orthologue of carrot CHS2 together with parsley CHS and *Bupleurum chinense* Bc-753f. CPKS3 and CPKS4 are almost identical and cover the same part of the gene, and hence are either recent paralogues or errors. CPKS12 is somewhat distinct from the others. CPKS10 and CPKS11 cluster together with *Arabidopsis thaliana* PKSA and PKSB, giving a hint of similarity in function. The branch containing the identified genes includes known non-CHS/STS, CHS and STS genes, whereas further non-CHS/STS and ketoacyl synthase genes are found elsewhere in the phylogeny. Although grouping of CHS genes is evident, phylogenetic analysis does not clearly separate the functions of the genes (Fig. 4).

Substrate specifity

Various starter substrates were used to assay the substrate specifity of CPKS1, CPKS2, and CPKS5, and the results were analysed with ultra-HPLC (UPLC). CPKS1 and CPKS2 had similar starter-CoA acceptance (Table 2). They formed naringenin chalcone (C4) as the only product from one *p*-coumaryl-CoA and three malonyl-CoAs. Lactones Be3 and Be4 were produced from benzoyl-CoA and malonyl-CoA. The main product for CPKS1 was Be4, with a small amount of Be3, whereas CPKS2 produced Be3 and Be4 in equal amounts. With malonyl-CoA, acetyl-CoA or acetoacetyl-CoA as a starter substrate, the enzymes produced triacetolactone (A3), with small amounts of tetraketide lactone (A4). The same occurred when hexanoyl-CoA or octanoyl-CoA were supplied as reaction starters for CPKS1 and CPKS2. With butyryl-CoA as a starter, the main product was the triketide lactone Bu3. Lactone Bu4a and phloroglucinol Bu4b were produced in equal amounts, as they are products of alternative foldings at the tetraketide stage. CPKS1 and CPKS2 formed only diketide quinolone N2 in relatively low amounts with *N*-methylanthraniloyl-CoA as a starter.

CPKS5 did not catalyse any product formation from *p*-coumaryl-CoA or N-methylanthraniloyl-CoA. The enzyme formed the corresponding triketide lactones as main products from acetyl-CoA, acetoacetyl-CoA, benzoyl-CoA, butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA with malonyl-CoA. It formed the corresponding tetraketide lactones in low amounts. The main difference between CPKS5 and CPKS1 or CPKS2 was in the butyryl-CoA products. There were no phloroglucinol derivatives among the products obtained with CPKS5.

Enzyme product identification

The identities of reaction products were determined from their relative retention times, UV spectra, and masses (Table 3). Triacetolactone (A3) and naringenin chalcone (C4) were used as pure compound standards in the analyses. Lactones A3, A4, H3, H4, Be3 and Be4 were identified on the basis of their elution order, UV spectra, and masses [36]. Lactones O3 and O4 were identified in a similar manner as H3 and H4, based on their elution order. Their masses of $[M–H]^-$ were 28 Da higher than the masses of H3 and H4, respectively, which corresponds to one C_2H_4 unit, as the starter-CoA used was octanoyl-CoA.

Lactones Bu3 and Bu4 were identified on the basis of their elution order and mass [37]. Phlorobutyrophe-

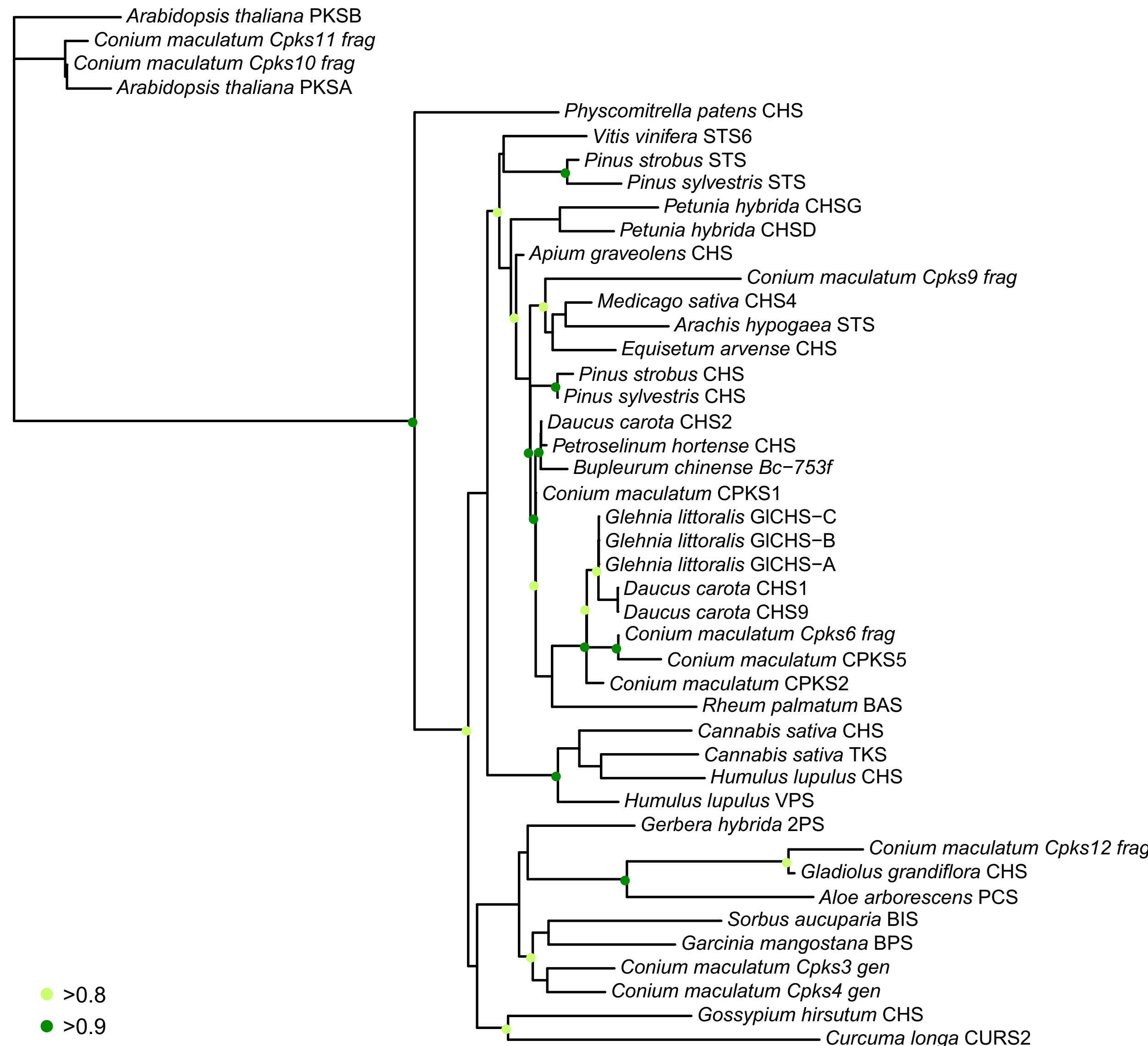


Fig. 4. A phylogenetic tree of PKS III family proteins. Nodes are coloured according to their support values, with nodes having < 0.8 support being left unlabelled. *frag*, fragments obtained in transcriptome sequence analysis; *gen*, genomic DNA fragment obtained via PCR with degenerated primers. BAS, benzalacetone synthase; BIS, biphenyl synthase; BPS, benzophenone synthase; CURS, curcumin synthase; OLS, olivetol synthase; PCS, pentaketide chromone synthase; 2PS, 2-pyrone synthase; TKS, 3,5,7-trioxododecanoyl-CoA synthase. GenBank accession numbers: *A. arborescens* PCS, AAX35541.1; *Apium graveolens* CHS, AH007394.1; *Ar. thaliana* PKSA, NP171707.1; *Ar. thaliana* PKSB, NP567971.1; *Arachis hypogaea* STS, BAA78617.1; *B. chinense* Bc-753f, FG341967.1; *Ca. sativa* CHS, AY082343.1; *Ca. sativa* TKS (OLS), BAG14339.1; *C. maculatum* CPKS1, KP726914; *C. maculatum* CPKS2, KP726915; *C. maculatum* CPKS5, KP726916; *C. maculatum* CPKS6, KP726917; *C. maculatum* CPKS7, KP726918; *C. maculatum* CPKS9, KP726919; *Curcuma longa* CURS2, BAH85780.1; *D. carota* CHS1, Q9ZS41.1; *D. carota* CHS2, Q9ZS40.1; *D. carota* CHS9, Q9SB26.1; *Equisetum arvense* CHS, AB030004.1; *Ga. mangostana* BPS, AEI27291.1; *G. hybrida* 2PS, CAA86219.2; *Gladiolus grandiflora* CHS, ADM18303.1; *Gl. littoralis* GlCHS-A, AB374260.1; *Gl. littoralis* GlCHS-B, AB374261.1; *Gl. littoralis* GlCHS-C, AB374262.1; *Gossypium hirsutum* CHS, AEO96987.1; *Humulus lupulus* CHS, BAB47196.1; *M. sativa* CHS4, AAB41559.1; *P. crispum* CHS, 1001151A; *Petunia hybrida* CHSD, CAA32733.1; *Pe. hybrida* CHSG, CAA32735.1; *Physcomitrella patens* CHS, DQ275627.2; *Pinus sylvestris* CHS, CAA43166.1; *Pi. sylvestris* STS, AAB24341.2; *Pinus strobus* CHS, CAA06077.1; *Pi. strobus* STS, CAA87012.1; *Rheum palmatum* BAS, AAK82824.1; *S. aucuparia* BIS, D2DRC4.1; *Vitis vinifera* STS6, JQ868692.1.

none (Bu4b) was identified on the basis of on its fragmentation pattern [*m*/*z* 195, 151 (100), 125], which is similar to that of phlorocaprophenone [38]. This mass was 28 Da lower, which corresponds to C_2H_4, as the starter-CoA used was butanoyl-CoA. Quinolinone N2 was identified on the basis of mass [31].

Table 2. Products formed in the *in vitro* experiments. ND, not detected.

	Enzyme		
Starter-CoA	CPKS1	CPKS2	CPKS5
Malonyl-CoA	A3[a]	A3[a]	A3[a]
	A4	A4	A4
Acetyl-CoA	A3[a]	A3[a]	A3[a]
	A4	A4	A4
Acetoacetyl-CoA	A3[a]	A3[a]	A3[a]
	A4	A4	A4
Benzoyl-CoA	Be3	Be3	Be3[a]
	Be4[a]	Be4	Be4
Butyryl-CoA	Bu3[a]	Bu3[a]	Bu3[a]
	Bu4a	Bu4a	Bu4a
	Bu4b	Bu4b	ND
p-Coumaryl-CoA	C4	C4	ND
Hexanoyl-CoA	H3[a]	H3[a]	H3[a]
	H4	H4	H4
N-Methylanthraniloyl-CoA	N2	N2	ND
Octanoyl-CoA	O3[a]	O3[a]	O3[a]
	O4	O4	O4

[a] Main product.

Enzyme kinetics of CPKS5

We tested the enzyme kinetic parameters of CPKS5 by using acetyl-CoA, butyryl-CoA, hexanoyl-CoA and octanoyl-CoA starters with [2-^{14}C]malonyl-CoA. Total activity per reaction was accounted for primarily by triketide production, with minuscule amounts of tetraketides. As PKSs can decarboxylate malonyl-CoA to acetyl-CoA, which in turn acts immediately as a starter [39], the base level for the kinetic measurements of the substrate feeding is always elevated (Fig. 5). We calculated enzymatic parameters for butyryl-CoA, and fitted the results into the Michaelis–Menten kinetics model. We calculated the parameters for butyryl-CoA consumption by CPKS5 using the Lineweaver–Burk plot ($R^2 = 0.9946$). The values for CPKS5 were $K_m = 6.63 \pm 0.26$ μM, $k_{cat} = 0.63 \pm 0.01$ min^{-1} and $k_{cat}/K_m = 1595 \pm 49$ s^{-1}·M^{-1} at pH 6.2. To compare butyryl-CoA usage by CPKS1 and CPKS2, we also calculated their parameters. The kinetic parameters for CPKS1 were $K_m = 4.14 \pm 0.96$ μM, $k_{cat} = 1.73 \pm 0.15$ min^{-1} and $k_{cat}/K_m = 7178 \pm 1306$ s^{-1}·M^{-1} at pH 7, and CPKS2 had similar values. The detected activity increased little or not at all from the base level when acetyl-CoA, hexanoyl-CoA or octanoyl-CoA was used as a starter substrate for CPKS5. These results demonstrate that CPKS5 favours butyryl-CoA as a starter among the tested compounds.

Discussion

We approached the problem of identifying the enzyme responsible for carbon backbone formation of coniine alkaloids by isolating and characterizing type III PKSs expressed in tissues of poison hemlock. We identified three full-length genes, *Cpks1*, *Cpks2*, and *Cpks5*, together with fragments of *Cpks3*, *Cpks4*, and *Cpks6–Cpks12*, from poison hemlock. On the basis of the phylogenetic analysis alone, the functions of CPKS1, CPKS2 and CPKS5 in poison hemlock could not be assigned [40].

CPKS1 and CPKS2 appear to be typical CHSs, according to their substrate acceptance. They catalyse naringenin chalcone production from *p*-coumaryl-CoA and malonyl-CoA *in vitro*, like the reference enzymes CHS from parsley and GCHS1 from gerbera. They did not produce resveratrol, and neither did we detect any side products such as bisnoryangonin or *p*-coumaryl triacetolactone. CPKS1 and CPKS2 are neither acridone nor quinolone synthases, as they barely accept *N*-methylanthraniloyl-CoA and form only quinolone N2. We did not detect any unusual compounds formed from benzoyl-CoA or any other starter. In contrast, CPKS5 differs from CHSs at the sequence level and in terms of substrate acceptance. CPKS5 contains a conserved catalytic triad, i.e. Cys164, His303 and Asn336 (amino acids are numbered according to alfalfa CHS2 [35]), like all active type III PKSs [23]. Phe215 and Phe265, which function as gatekeepers controlling traffic to and from the active site, are also conserved [23]. Only the G211T substitution affects the gatekeeper phenylalanines. It also changes the pocket shape of the starter binding site [23]. Bibenzyl synthase shows the same substitution [23].

Furthermore, there are six more amino acids changed in the active site of CPKS5. The M137F substitution causes a significant change in the active site, owing to the bulkier side group. The amino acid at this position protrudes into the other monomer's active site, affecting its cyclization pocket [41]. Positions 132–137 are among the positions differentiating STS and CHS, and the specific amino acids at these positions are responsible for the alternative cyclization of STS versus CHS [23].

In the CoA-binding tunnel [23], Lys62 is replaced by Arg, which is a conservative change. In CHS, Lys62 contributes hydrogen bonds to CoA binding [41].

There are four amino acids, Thr132, Thr194, Val196, and Thr197, in the elongation pocket, which were changed to Tyr, Ser, Leu, and Val, respectively. The side group of Tyr is more hydrophobic and

Table 3. *In vitro* reaction products obtained with different starters. Retention times and UV absorbances were determined with UPLC-UV. Masses were determined with UPLC-Orbitrap.

Starter-CoA	Polyketide level	Product	IUPAC name	Retention time (min)	λ_{max} (nm)	*m/z* $[M+H]^+$	Proposed structure
Acetyl-/Acetoacetyl-CoA	Triketide	A3	4-Hydroxy-6-2*H*-pyran-2-one	2.16	283	127.03859 (calculated for $C_6H_7O_3^+$ 127.03897)	
	Tetraketide	A4	4-Hydroxy-6-(2-oxopropyl)-2*H*-pyran-2-one	1.70	282	169.04921 (calculated for $C_8H_9O_4^+$ 169.04954)	
Benzoyl-CoA	Triketide	Be3	4-Hydroxy-6-phenyl-2*H*-pyran-2-one	4.31	283	189.03085 (calculated for $C_{11}H_9O_3^+$ 189.05462)	
	Tetraketide	Be4	4-Hydroxy-6-(2-oxo-2-phenylethyl)-2*H*-pyran-2-one	4.13	319	231.06790 (calculated for $C_{13}H_{11}O_4^+$ 231.06519)	
Butyryl-CoA	Triketide	Bu3	4-Hydroxy-6-propyl-2*H*-pyran-2-one	3.77	284	155.06964 (calculated for $C_8H_{11}O_3^+$ 155.07027)	
	Tetraketide	Bu4a	4-Hydroxy-6-(2-oxopentyl)-2*H*-pyran-2-one	3.59	284	197.08011 (calculated for $C_{10}H_{13}O_4^+$ 197.08084)	
	Tetraketide	Bu4b	1-(2,4,6-Trihydroxyphenyl)butan-1-one	4.89	225.7, 284.7	197.06825 (calculated for $C_{10}H_{13}O_4^+$ 197.08084)	
p-Coumaryl-CoA	Tetraketide	C4	5,7-Dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4*H*-chromen-4-one	4.66	289	273.07526 (calculated for $C_{15}H_{13}O_5^+$ 273.07575)	
Hexanoyl-CoA	Triketide	H3	4-Hydroxy-6-pentyl-2*H*-pyran-2-one	5.02	284	183.10161 (calculated for $C_{10}H_{15}O_3^+$ 183.10157)	
	Tetraketide	H4	4-Hydroxy-6-(2-oxoheptyl)-2*H*-pyran-2-one	4.87	284	225.11177 (calculated for $C_{12}H_{17}O_4^+$ 225.11214)	

Table 3. (Continued).

Starter-CoA	Polyketide level	Product	IUPAC name	Retention time (min)	λ_{max} (nm)	*m/z* $[M+H]^+$	Proposed structure
N-Methylanthraniloyl-CoA	Diketide	N2	4-Hydroxy-1-methylquinolin-2(1*H*)-one	3.84	228, 274, 315	176.06999 (calculated for $C_{10}H_{10}O_2N^+$ 176.07061)	
Octanoyl-CoA	Triketide	O3	6-Heptyl-4-hydroxy-2*H*-pyran-2-one	6.14	225.7, 284.7	211.13269 (calculated for $C_{12}H_{19}O_3^+$ 211.13287)	
	Tetraketide	O4	4-Hydroxy-6-(2-oxononyl)-2*H*-pyran-2-one	5.92	225.7, 283.2	253.14313 (calculated for $C_{14}H_{21}O_4^+$ 253.14344)	

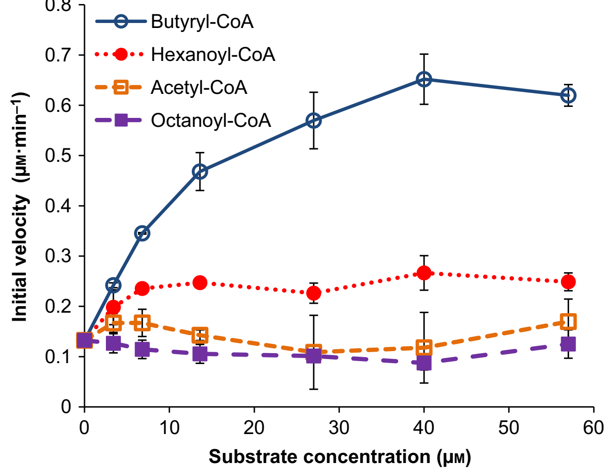


Fig. 5. Kinetic testing of CPKS5. The reactions were performed with 5 µg of purified enzyme, 0–100 µM starter-CoA (acetyl-CoA, butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA), 198 µM malonyl-CoA, and 2 µM [2-^{14}C]malonyl-CoA. The error bars represent the standard deviation of triplicate reactions.

bulkier than that of Thr132. In *Garcinia mangostana* benzophenone synthase, mutation of Thr133 to Leu, which corresponds to position 132 in alfalfa CHS2, causes a decrease in the active site volume, and, as a consequence, the majority of the enzymatic products are triketide lactones [42]. Jez *et al.* [41] reported that Thr132 may dictate starter molecule specificity, and, in *Sorbus aucuparia* biphenyl synthase, a change to Ala at the same position influences the cyclization mechanism [43]. The T194S mutation is a conservative change, as Thr and Ser are similar in size and chemical properties. Austin and Noel [23] noted that position 194 is important for modulating substrate specificity and proper cyclization, together with positions 132 and 197. The V196L mutation causes a change towards polar uncharged and bulkier substitution. This position also influences folding, as shown by the AmCHS1 mutation V196M. Here, loss of naringenin chalcone formation is caused and *p*-coumaryl triacetolactone is produced, owing to a change from Claisen condensation to lactonization and a lowered pH optimum [44]. The T197A mutation changes the side chain properties to hydrophobic. In fact, *Aloe arborescens* aloesone synthase shows the same change as in CPKS5 [45]. Mutation studies revealed that this position controls substrate acceptance [23,46], chain length [46,47] and the number of malonyl-CoA condensations [47] by steric modulation of the active site.

Many PKSs, such as CHS, are able to use aliphatic CoAs as starters *in vitro* [48]. A wide variety of plant PKSs use acetyl-CoA as a starter, e.g. *G. hybrida* 2PS

[33], *Hypericum perforatum* octaketide synthase HpPKS2 [36], and *Drosophyllum lusitanicum* hexaketide synthase DluHKS [37]. Utilization of longer starters, i.e. butyryl-CoA or hexanoyl-CoA, has been reported less often. There are two described cases of butyrate acceptance: labelled butyrate being used in the biosynthesis of margaspidin, a butyrylphloroglucinol derivative, in the fern *Dryopteris marginalis* [49]; and incorporation into csypyrone B1 by *Aspergillus oryzae* CsyB [50,51]. The only report concerning hexanoyl-CoA utilization is for *Cannabis sativa* olivetol synthase [52].

CHSs also have the capacity to use butyryl-CoA [48]. CPKS1 and CPKS2 are able to use butyryl-CoA *in vitro* (CPKS1 at pH 7: k_{cat}/K_m = 7178 $s^{-1}{\cdot}M^{-1}$). The efficiency of CPKS5 is lower (at pH 6.2: k_{cat}/K_m = 1595 $s^{-1}{\cdot}M^{-1}$). However, whereas CPKS1 and CPKS2 have the ability to use *p*-coumaryl-CoA, CPKS5 has apparently lost this feature while retaining the capacity to use butyryl-CoA.

CPKS5 has a low affinity for acetyl-CoA and octanoyl-CoA. These two precursors are also relatively poor starters for other Apiaceae PKSs, e.g. parsley CHS [53]. Increasing the starter amount did not increase activity either with acetyl-CoA, as reported for *A. arborescens* pentaketide chromone synthase [46], or with octanoyl-CoA. Among the tested starter-CoAs, CPKS5 kinetically favours butyryl-CoA, which is elongated twice with malonyl-CoA, forming the triketide lactone Bu3.

As we could not identify a PKS favouring acetyl-CoA as a starter, Leete's hypothesis [17,18] proposing a tetraketide backbone of coniine formed from one acetyl-CoA and three malonyl-CoAs remains unsupported. On the other hand, the substrate preference of CPKS5 correlates with the presence of alkaloids in poison hemlock. It is tempting to speculate that the same enzyme could be responsible for the polyketide formation of all C_6, C_8 and C_{10} alkaloids in the plant. Such a scenario would involve condensation of either acetyl-CoA, butyryl-CoA or hexanoyl-CoA with two malonyl-CoAs to form a triketide (Fig. 6). C_8 alkaloids are the most common [e.g. 11], with butyryl-CoA consistently being the favoured starter among those

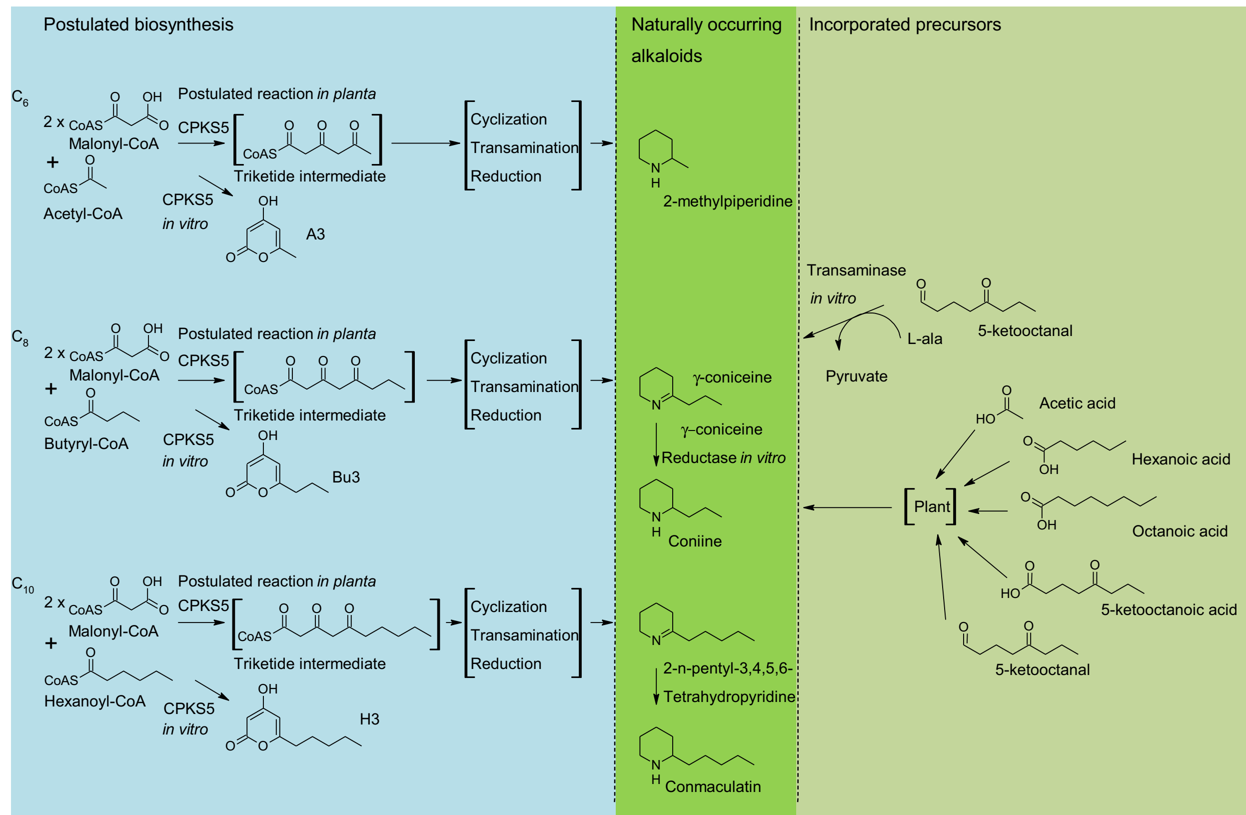


Fig. 6. Proposed biosynthetic pathways leading to piperidine alkaloids. The C_6, C_8 and C_{10} alkaloids in the middle are all known from *C. maculatum*. Putative condensation products whose production is catalysed by CPKS5 are shown together with their corresponding *in vitro* derailment products on the left. Incorporated substrates and known reactions previously reported in the literature are shown on the right.

tested. C_{10} alkaloids are minor alkaloids. Conmaculatin is a relatively new finding [16] that probably escaped detection in earlier analyses because of its low amounts. Hexanoyl-CoA is a suitable starter, but may not be preferred naturally. The only C_6 alkaloid, 2-methylpiperidine, has been occasionally reported [11,12], although CPKS5 does not utilize acetyl-CoA very well under the tested conditions. According to the *in vitro* substrate tests, there could theoretically be C_{12} alkaloids in poison hemlock, but hitherto these have not been observed.

In the light of the current investigation, Leete's labelling results *in planta* [17,18] could be interpreted as follows. Fed acetate is first activated into acetyl-CoA, part of which is then further processed into malonyl-CoA. Acetyl-CoA is then elongated with malonyl-CoA by fatty acid synthase to form butyryl-CoA [50], resulting in the labelling of even-numbered carbons when a ^{14}C-labelled acetate substrate is used. Then, butyryl-CoA is elongated twice with malonyl-CoA containing ^{14}C originating from the fed acetate.

In conclusion, we provide evidence for an alternative biosynthetic route to the alkaloid coniine involving a newly discovered PKS type III. The recombinant CPKS5 preferably catalyses the condensation of butyryl-CoA with two malonyl-CoA units. Owing to the absence of accessory proteins facilitating reduction and cyclization, only pyrone-type derailment products are formed *in vitro*, as is commonly observed for type III PKSs. Therefore, further research *in planta* is needed in order to fully establish the mechanism of alkaloid biosynthesis in poison hemlock.

Experimental procedures

Plant material

Poison hemlock (*C. maculatum* L.) was grown from seeds collected in Helsinki, Finland. The controlled growth chamber conditions were: temperature, 20 °C; humidity, 60%; and a photoperiod of 16 h of light and 8 h of dark. The potting soil was half vermiculite and half peat (Kekkilä Oy, Vantaa, Finland). Flowers and young developing seeds were collected from two locations in Helsinki, Finland, during summer for RNA extraction.

Genomic DNA isolation

Genomic DNA was isolated with an adapted cetyltrimethylammonium bromide method [54]. Isolation buffer (55 mM cetyltrimethylammonium bromide, 1.4 mM NaCl, 20 mM EDTA, 10 mM Tris/HCl, pH 8, 0.3% v/v 2-mercaptoethanol) was heated to 63 °C. Plant material was ground in a Retsch MM301 mill (Germany); three steel balls were added per 2-mL tube for 2 min at 29 Hz. Then 400 μL of prewarmed isolation buffer was added to the tube, which was kept in a heating block (Thermomixer 5436; Eppendorf, Hamburg, Germany) at 63 °C for 30–60 min. Then, 400 μL of phenol and 200 μL of chloroform/isoamyl alcohol (24 : 1) was added. The mixture was vortexed, and centrifuged at 4 °C for 6 min at full speed (21 000 ***g***). The aqueous phase was transferred to a new tube, and, after addition of 0.66 times the volume of ice-cold isopropanol, it was kept at – 20 °C for 5 min. The mixture was then centrifuged (21 000 ***g***) at 4 °C for 10 min. The supernatant was removed, and the pellet was washed with 600 μL of washing buffer (3 mM ammonium acetate in 70% ethanol). The mixture was centrifuged (21 000 ***g***) at 4 °C for 5 min. The supernatant was removed, and the residue was dried in a concentrator (SpeedVac Plus SC110A; Savant, Hobrook, NY, USA) for 1–2 min. The dry pellet was eluted into 40 μL of elution buffer (QIAgen, Valencia, CA, USA), and the DNA concentration was measured spectrophotometrically (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA).

RNA isolation

Total RNA was isolated with the pine tree method [55].

Cloning of polyketide synthase genes

The conserved regions of *Cpks1*–*Cpks5* were amplified with the degenerated primers deg-CHS-F and deg-CHS-R from genomic DNA (Table S1) [34]. PCR was performed with DreamTaq DNA polymerase (Fermentas, Waltham, MA, USA) on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). The PCR conditions were as follows: denaturing at 95 °C for 0.5 min, followed by 10 cycles of amplification at 95 °C for 0.5 min, 53 °C (–1 °C per cycle) for 0.5 min, and 72 °C for 1.5 min, and then 30 cycles of amplification at 95 °C for 0.5 min, 53 °C for 0.5 min, and 72 °C for 2 min, and final extension at 72 °C for 5 min.

Then, 5′ and 3′ ends of *Cpks1* and *Cpks2* were cloned from anchored cDNA, which was synthesized with an ExactSTART Eukaryotic mRNA 5′-RACE and 3′-RACE Kit with Failsafe PCR Enzyme Mix (Epicentre, Chicago, IL, USA), according to the manufacturer's instructions. On the basis of the obtained conserved region sequences, the new primers Cpks1A and Cpks1B were designed to clone *Cpks1*. Similarly, the primers Cpks2A, Cpks2B, Cpks3A, Cpks3B, Cpks4A, Cpks4B, Cpks5A and Cpks5B (Table S1) were designed to clone *Cpks2*–*Cpks5*. 5′ and 3′ ends were cloned according to the manufacturer's instructions.

Full-length *Cpks1* and *Cpks2* genes were cloned from cDNA with the primers Cpks1F, Cpks1R, Cpks2F, and Cpks2R (Table S1). PCR was performed with an

Expand High Fidelity PCR Kit (Roche, Basel, Switzerland), with conditions according to the manufacturer's instructions. The annealing temperatures were 58.7 °C for *Cpks1* and 54.3 °C for *Cpks2*.

The full-length sequence of *Cpks5* and fragments of *Cpks6–Cpks12* were obtained by transcriptome sequencing (H. Rischer and T. H. Teeri, unpublished). On the basis of the observed sequence, *Cpks5* was ordered from Genscript (Piscataway Township, NJ, USA).

Cloning of PKS expression vectors

With *Cpks1* in pGEM-T Easy-plasmid (Promega, Fitchburg, WI, USA) as a template, a 1.2-kb DNA fragment containing the coding region was amplified by PCR with primers Cpks1F-pHIS8 and Cpks1R-pHIS8 (Table S1). Similarly, *Cpks2* was amplified by PCR with primers Cpks2F-pHIS8 and Cpks2R-pHIS8, and *Cpks5* was amplified with primers Cpks5F-pHIS8 and Cpks5R-pHIS8 (Table S1). PCR was performed with an Expand High Fidelity PCR Kit. The PCR conditions were according to the manufacturer's instructions, with the following annealing temperatures: *Cpks1*, 59.2 °C; *Cpks2*, 61.6 °C; and *Cpks5*, 59.7 °C.

The resulting PCR products (1.2 kb) were purified from 1% TBE/agarose gel with an EZNA Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). The PCR products were digested with the restriction enzymes *Eco*RI, *Sal*I, and *Bam*HI (FastDigest; Fermentas), as indicated in Table S1. The receiving plasmid, pHIS8 [56], was similarly treated. *Cpks1*, *Cpks2* and *Cpks5* were then ligated to pHIS8 plasmids with T4 ligase.

Enzyme production

The recombinant plasmids pHIS8-*Cpks1*, pHIS8-*Cpks2* and pHIS8-*Cpks5* were transferred to *E. coli* BL21 (DE3) for protein expression [56]. *E. coli* cells containing the recombinant plasmid were grown in LB liquid medium in the presence of kanamycin (50 mg·L^{-1}) at 37 °C until the $D_{600\ nm}$ of the culture was 0.9–1.2. After cooling of the cultures on ice, protein production was induced with 0.5 mM IPTG. The cultures were grown at 20 °C for 4 h.

Enzyme purification

E. coli cells were harvested by centrifugation at 4000 ***g*** for 20 min, and the supernatant was decanted. Cells were resuspended in a lysis buffer (50 mM Tris/HCl, pH 8, 500 mM NaCl, 20 mM imidazole, pH 8, 20 mM 2-mercaptoethanol, 10% v/v glycerol, 1% v/v Tween-20) [56]. The lysis buffer contained 2 mg·mL^{-1} lysozyme and 20 U·mL^{-1} DNaseI (New England Biolabs, Ipswich, MA, USA). The mixture was incubated at room temperature for 30 min. Then, the tubes were frozen three times in liquid nitrogen, and thawed at room temperature. The lysate was centrifuged at 10 000 ***g*** and 4 °C for 30 min. The cleared lysate was passed through a His SpinTrap column (GE Healthcare, Little Chalfont, Buckinghamshire, UK), which was prepared according to the manufacturer's instructions. The supernatant (600 μL) was applied to the column for centrifugation (100 ***g***, 5 min). The column was washed once with 600 μL of washing buffer (50 mM Tris/HCl, pH 8, 500 mM NaCl, 20 mM imidazole, pH 8, 20 mM 2-mercaptoethanol, 10% v/v glycerol), and this was followed by centrifugation at 100 ***g*** for 2 min. His-tagged proteins were eluted twice with 200 μL of elution buffer (50 mM Tris/HCl, pH 8, 500 mM NaCl, 500 mM imidazole, pH 8, 20 mM 2-mercaptoethanol, 10% v/v glycerol), with centrifugation at 100 ***g*** for 30 s. The elution fractions were pooled, washed and salt exchanged by centrifugation at 4000 ***g*** for 20 min at 4 °C with a centrifugal filter device (Amicon Ultra-15 30K; Millipore, Billerica, MA, USA) so that the final solution contained 5 mM HEPES (pH 7.5), 25 mM NaCl, and 5 mM DTT.

Enzyme assay

The tested enzymes were recombinant CPKS1, CPKS2, and CPKS5, with parsley CHS [57], G2PS1 [33] and GCHS1 [34] as reference enzymes. For enzymatic testing, the reaction mixture contained 20 μg of protein, 100 μM starter-CoA [acetyl-CoA, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, or benzoyl-CoA (all Sigma Aldrich, St Louis, MO, USA), *N*-methylanthraniloyl-CoA (MicroCombiChem, Wiesbaden, Germany), or *p*-coumaryl-CoA (MicroCombiChem)] and 200 μM malonyl-CoA (Sigma Aldrich) in 100 μL of 0.1 M potassium phosphate buffer (pH 7). The reaction mixture was incubated at 30 °C for 90 min. Reactions were stopped with 20 μL of acetic acid or 100 μL of 1 M Tris/HCl (pH 8.5) for *p*-coumaryl-CoA. For ring formation, the samples were incubated for a further 15 min at 37 °C. Final products were extracted twice with 250 μL of ethyl acetate. Collected ethyl acetate fractions were combined and evaporated to dryness under N_2 flow. The products were dissolved in 100 μL of 50% methanol.

Product analysis

The enzymatic end product analysis method was adapted from Karppinen *et al.* [36]. A Waters ACQUITY UPLC (Waters, Milford, MA, USA) system and a Waters ACQUITY UPLC BEH C18 2.1 × 100-mm column with a particle size of 1.7 μm (Waters) were used to separate the biosynthetic products. The UPLC eluents were 0.1% acetic acid in UPLC-grade water (A) and acetonitrile (B) (HPLC grade; Rathburn Chemicals Ltd, Walkerburn, Scotland, UK). The initial gradient condition was 90% A

and 10% B; this was changed linearly to 60% B in 4 min, and this was followed by 6 min of isocratic elution and 2 min of equilibration in the initial conditions, resulting in a total analysis time of 12 min. The eluent flow rate was 0.3 mL·min^{-1} and the column temperature was 35 °C; the injection volume was 7 µL. A Waters ACQUITY photodiode array detector was used for the measurement of online UV spectra of the biosynthetic products. A range of 200–400 nm was monitored.

UPLC-MS and UPLC-MS/MS runs were performed on a Waters Micromass Quatro micro MS combined with a Waters Acquity UPLC equipped with an Acquity BEH C_{18} column (100 × 2.1 mm, 1.7 µm) at 35 °C. The solvent flow was 0.3 mL·min^{-1}, and the program started with 90% A and 10% B; the proportion of B was increased to 60% in 4 min, and maintained there up to 10 min. The column was equilibrated to the initial conditions in 5 min. The analytes were detected in ESI-MS negative ion mode from 100 to 600 m/z with a capillary energy of 2.4 kV and a cone energy of 30 V. The source temperature was 125 °C, and the desolvation gas flow and temperature were 800 L·h^{-1} and 350 °C, respectively. In MS/MS mode, the collision energy was 15 V. The analytes were also detected before MS with a photodiode array detector scanning from 210 to 420 nm. The MS data were collected with Waters MASSLYNX software (version 4.1). Accurate masses were determined with a UPLC instrument combined with a TriVersa Nanomate (Advion Biosciences, Ithaca, NY, USA) and an LTQ-Orbitrap LX (Thermo Fisher Scientific, San Jose, CA, USA) in positive ion mode, by the use of chip-based nanoelectrospray with 1.7-kV spray voltage. Identifications were based on the accurate mass (target mass resolution R = 60 000 at 400 m/z) of the protonated molecular ion. Data were processed with THERMOXALIBUR software (Thermo Fisher Scientific).

Determination of the kinetic parameters of CPKS5

Determination of the kinetic parameters of CPKS5 was carried out in triplicate with a reaction mixture containing 0.1 M citric acid/0.2 M Na_2HPO_4 buffer, 5 µg of protein, 0–100 µM starter-CoA (acetyl-CoA, butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA), 198 µM malonyl-CoA and 2 µM [2-^{14}C]malonyl-CoA (52.4 mCi·mmol^{-1}; PerkinElmer, Zaventem, Belgium) at a final pH of 6.2. Kinetic parameters were also determined for CPKS1 and CPKS2 with 0–100 µM butyryl-CoA. Reactions were incubated at 30 °C for 20 min after addition of [2-^{14}C]malonyl-CoA, and they were ended by addition of 20 µL of acetic acid. Reaction products were extracted twice with 250 µL of ethyl acetate. The ethyl acetate extract was transferred to a scintillation bottle with 3 mL of scintillation liquid (Optiphase Hisafe 3; PerkinElmer, Groningen, the Netherlands). The reaction products were analysed with a scintillation counter (1414 Liquid scintillation counter; Winspectral, Wallac, Finland). The kinetic parameters were calculated for all products of the enzyme reactions together.

Homology modelling

Structural modelling was performed on the basis of homology with the web-based SWISS MODEL workspace [58–60]. The template for CPKS5 was the alfalfa CHS2 (1BI5, chain A) homodimer, which shows 70.88% sequence identity with CPKS5. To calculate active site volumes the CASTP online program was used [61].

Phylogenetic analysis

In order to carry out a phylogenetic analysis of the identified candidate genes, comprehensive collection of the genes for the protein family type III PKS (IPR011141) was performed. Initially, all proteins (2422 from TrEMBL and 175 from SwissProt) belonging to IPR011141 were collected from Interpro [62]. In order to retrieve a suitable subset for phylogenetic analysis, these sequences were clustered with BLASTCLUST [63] (length coverage threshold –L = 0.7 and score coverage threshold –S = 80; that is, sequences in the same cluster are 80% identical along at least 70% of their length). From each cluster, a representative was then picked primarily by prioritizing sequences that were included in SwissProt, and secondarily by use of an annotated EC number, yielding a total of 547 sequences. In order to include coverage of the latest database submissions, a BLAST search was then carried out for the candidate genes in NCBI sequence databases (with a cut-off E-value of 1 × 10^{-30} and a maximum number of hits of 5) including patent and environmental sequence databases, providing 35 additional sequences having a significant hit to IPR011141. Sequences that were not full length as compared with other PKS sequences (e.g. clearly missing the beginning or ending of the ORF) were removed, and some previously published sequences were manually added, to arrive at the final dataset of 589 sequences. Alignment of the sequences was then carried out with MAFFT [64], the alignment was trimmed with TRIMAL [65], and the final phylogeny was constructed with FASTTREE [66], all with default settings. An R-script [67] with library ape [68] was used to plot and process the results.

Acknowledgements

We thank Kari Kammiovirta for providing poison hemlock seeds and their collection location, Tuuli Teikari, Jaana Rikkinen, Dagmar Schwenk and Kirsi Bromann for their advice and help with cloning of the genes, Airi Hyrkäs and Heli Nygren for assistance

with UPLC analysis, Kaj-Roger Hurme for technical advice on radioactivity experiments, Kaisa Marjamaa for help with interpreting enzyme kinetic results, Philipp Meier for discussions, and Joseph M. Jez for providing the pHIS8 vector. This work was supported by the Finnish Doctoral Programme in Plant Science, Societas pro Fauna et Flora Fennica, Eteläsuomalaisten ylioppilaiden säätiö, Oskar Öflunds Stiftelse, the Otto A. Malm Foundation (all to H. Hotti), VTT, and the Academy of Finland (grant 138808 to T. H. Teeri and H. Rischer).

Author contributions

H. Hotti, T. H. Teeri and H. Rischer planned experiments. H. Hotti, T. Seppänen-Laakso and M. Arvas performed experiments. H. Hotti, T. Seppänen-Laakso and M. Arvas analysed data. H. Hotti and H. Rischer wrote the paper.

References

1 IUPAC (2014) Compendium of Chemical Terminology – Gold Book. Institute of Chemical Technology, Prague, 1670 p.

2 Vetter J (2004) Poison hemlock (*Conium maculatum* L.). *Food Chem Toxicol* **42**, 1373–1382.

3 Reynolds T (2005) Molecules of interest – Hemlock alkaloids from Socrates to poison aloes. *Phytochemistry* **66**, 1399–1406.

4 Corsi G & Biasci D (1998) Secretory structures and localisation of alkaloids in *Conium maculatum* L. (Apiaceae). *Ann Bot London* **81**, 157–162.

5 Plato (1892) Phaedo. In *The Dialogues of Plato translated into English with Analyses and Introductions* (Jowett B, ed), 3rd edn, pp. 157–266. Oxford University Press, London.

6 Ladenburg A (1886) Versuche zur Synthese des Coniin. *Ber Dtsch Chem Ges* **19**, 439–441.

7 Lee ST, Green BT, Welch KD, Pfister JA & Panter KE (2008) Stereoselective potencies and relative toxicities of coniine enantiomers. *Chem Res Toxicol* **21**, 2061–2064.

8 Colombo ML, Marangon K, Locatelli C, Giacche M, Zulli R & Restani P (2009) Hemlock poisoning due to plant misidentification. *J Pharm Sci Res* **1**, 43–47.

9 Bowman WC & Sanghvi IS (1963) Pharmacological actions of hemlock (*Conium maculatum*) alkaloids. *J Pharm Pharmacol* **15**, 1–25.

10 Holm L, Doll J, Holm E, Pancho J & Herberger J (1997) World Weeds: Natural Histories and Distribution. Wiley, New York, USA.

11 Cromwell BT (1956) The separation, micro-estimation and distribution of the alkaloids of hemlock (*Conium maculatum* L.). *Biochem J* **64**, 259–266.

12 Holstege DM, Galey FD, Johnson B & Seiber JN (1996) Determination of alkaloid exposure in a model ruminant (goat) using a multiresidue screening method. *J Agric Food Chem* **44**, 2310–2315.

13 Leete E & Olson JO (1972) Biosynthesis and metabolism of the hemlock alkaloids. *J Am Chem Soc* **94**, 5472–5477.

14 Roberts MF & Brown RT (1981) A new alkaloid from South African *Conium* species. *Phytochemistry* **20**, 447–449.

15 Lang DG & Smith RA (1997) Two new alkaloids of *Conium maculatum*, and evidence for a tautomeric form for "γ"-coniceine. In Toxic Plants and Other Natural Toxicants (Garland T & Barr AC, eds), pp. 419–422. CAB International, Oxford, UK.

16 Radulović N, Đorđević N, Denić M, Pinheiro MMG, Fernandes FD & Boylan F (2012) A novel toxic alkaloid from poison hemlock (*Conium maculatum* L., Apiaceae): identification, synthesis and antinociceptive activity. *Food Chem Toxicol* **50**, 274–279.

17 Leete E (1963) The biosynthesis of coniine from four acetate units. *J Am Chem Soc* **85**, 3523–3524.

18 Leete E (1964) Biosynthesis of the hemlock alkaloids. The incorporation of acetate-1-C^{14} into coniine and conhydrine. *J Am Chem Soc* **86**, 2509–2513.

19 Leete E (1970) Biosynthesis of coniine from octanoic acid in hemlock plants (*Conium maculatum*). *J Am Chem Soc* **92**, 3835.

20 Leete E & Olson JO (1970) 5-Oxo-octanoic acid and 5-oxo-octanal, precursors of coniine. *J Chem Soc D* **23**, 1651–1652.

21 Leete E (1971) Biosynthesis of the hemlock and related piperidine alkaloids. *Acc Chem Res* **4**, 100–107.

22 Roberts MF (1998) Enzymology of alkaloid biosynthesis. In Alkaloids: Biochemistry, Ecology, and Medicinal Applications (Roberts MF & Wink M, eds), pp. 109–146. Springer, New York, USA.

23 Austin MB & Noel JP (2003) The chalcone synthase superfamily of type III polyketide synthases. *Nat Prod Rep* **20**, 79–110.

24 Roberts MF (1971) The formation of γ-coniceine from 5-ketooctanal by transaminase of *Conium maculatum*. *Phytochemistry* **10**, 3057–3060.

25 Roberts MF (1978) Separation of the formation of γ-coniceine and aliphatic amines from GOT activity in *Conium maculatum*. *Phytochemistry* **17**, 107–112.

26 Roberts MF (1981) Enzymatic synthesis of γ-coniceine in *Conium maculatum* chloroplasts and mitochondria. *Plant Cell Rep* **1**, 10–13.

27 Unger W (1977) Enzymatische In-vitro-synthase von γ-Conicein durch eine Aldehyd-aminosäure-transaminase aus Spinatblättern. *Planta Med* **31**, 262–265.

28 Roberts MF (1977) Purification and properties of L-alanine: 5-ketooctanal aminotransferase from *Conium maculatum*. *Phytochemistry* **16**, 1381–1386.

29 Roberts MF (1975) γ-Coniceine reductase in *Conium maculatum*. *Phytochemistry* **14**, 2393–2397.
30 Maier W, Baumert A, Schumann B, Furukawa H & Gröger D (1993) Synthesis of 1,3-dihydroxy-*N*-methylacridone and its conversion to rutacridone by cell-free extracts of *Ruta graveolens* cell cultures. *Phytochemistry* **32**, 691–698.
31 Resmi MS, Verma P, Gokhale RS & Soniya EV (2013) Identification and characterization of a type III polyketide synthase involved in quinolone alkaloid biosynthesis from *Aegle marmelos* Correa. *J Biol Chem* **288**, 7271–7281.
32 Bringmann G, Wolhfarth M, Rischer H, Grüne M & Schlauer J (2000) A new biosynthetic pathway to alkaloids in plants: acetogenic isoquinolines. *Angew Chem Int Ed* **39**, 1464–1466.
33 Eckermann S, Schröder G, Schmidt J, Strack D, Edrada RA, Helariutta Y, Elomaa P, Kotilainen M, Kilpeläinen I, Proksch P *et al.* (1998) New pathway to polyketides in plants. *Nature* **396**, 387–390.
34 Helariutta Y, Elomaa P, Kotilainen M, Griesbach RJ, Schröder J & Teeri TH (1995) Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in *Gerbera hybrida* (Asteraceae). *Plant Mol Biol* **28**, 47–60.
35 Junghans H, Dalkin K & Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.). 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family. *Plant Mol Biol* **22**, 239–253.
36 Karppinen K, Hokkanen J, Mattila S, Neubauer P & Hohtola A (2008) Octaketide-producing type III polyketide synthase from *Hypericum perforatum* is expressed in dark glands accumulating hypericins. *FEBS J* **275**, 4329–4342.
37 Jindaprasert A, Springob K, Schmidt J, De-Eknamkul W & Kutchan TN (2008) Pyrone polyketides synthesized by a type III polyketide synthase from *Drosophyllum lusitanicum*. *Phytochemistry* **69**, 3043–3053.
38 Ghosh R, Chhabra A, Phatale PA, Samrat SK, Sharma J, Gosain A, Mohanty S, Saran D & Gokhale RS (2008) Dissecting the functional role of polyketide synthases in *Dictyostelium discoideum*: biosynthesis of the differentiation regulating factor 4-methyl-5-pentylbenzene-1,3-diol. *J Biol Chem* **283**, 11348–11354.
39 Eckermann C, Schröder G, Eckermann S, Strack D, Schmidt J, Schneider B & Schröder J (2003) Stilbenecarboxylate biosynthesis: a new function in the family of chalcone synthase-related proteins. *Phytochemistry* **62**, 270–286.
40 Wemg J-K & Noel JP (2012) Structure-function analyses of plant type III poleketide synthases. In Methods in Enzymology, Volume 515. Natural Product Biosynthesis by Microorganisms and Plants, Part A (Hopwood D, ed.), pp. 317–335. Academic Press, San Diego, CA, USA.
41 Jez JM, Austin MB, Ferrer J-L, Bowman ME, Schröder J & Noel JP (2000) Structural control of polyketide formation in plant-specific polyketide synthases. *Chem Biol* **7**, 919–930.
42 Nualkaew N, Morita H, Shimokawa Y, Kinjo K, Kushiro T, De-Eknamkul W, Ebizuka Y & Abe I (2012) Benzophenone synthase from *Garcinia mangostana* L. pericarps. *Phytochemistry* **77**, 60–69.
43 Liu B, Raeth T, Beuerle T & Beehues L (2007) Biphenyl synthase, a novel type III polyketide synthase. *Planta* **225**, 1495–1503.
44 Hatayama M, Unno H, Kusunoki M, Takahashi S, Nishino T & Nakayama T (2010) Production of tetraketide lactones by mutated *Antirrhinum majus* chalcone synthases (AmCHS1). *J Biosci Bioeng* **110**, 158–164.
45 Abe I, Utsumi Y, Satoshi O & Noguchi H (2004) The first plant type III polyketide synthase that catalyzes formation of aromatic heptaketide. *FEBS Lett* **562**, 171–176.
46 Abe I, Utsumi Y, Oguro S, Morita H & Noguchi H (2005) A plant type III polyketide synthase that produces pentaketide chromone. *J Am Chem Soc* **127**, 1362–1363.
47 Mizuuchi Y, Shi S-P, Wanibuchi K, Kojima A, Morita H, Noguchi H & Abe I (2009) Novel type III polyketide synthases from *Aloe arborescens*. *FEBS J* **276**, 2391–2401.
48 Jez JM, Bowman ME & Noel JP (2002) Expanding the biosynthetic repertoire of plant type III polyketide synthases by altering starter molecule specificity. *Proc Natl Acad Sci USA* **99**, 5319–5324.
49 Gordon PG, Penttila A & Fales HM (1968) The incorporation of sodium butyrate into methylenebis (butyrylphloroglucinols) by a novel biosynthetic pathway. *J Am Chem Soc* **90**, 1376–1377.
50 Seshime Y, Juvvadi PR, Kitamoto K, Ebizuka Y & Fujii I (2010) Identification of csypyrone B1 as the novel product of *Aspergillus oryzae* type III polyketide synthase CsyB. *Bioorg Med Chem* **18**, 4542–4546.
51 Hashimoto M, Ishida S, Seshimi Y, Kitamoto K & Fujii I (2013) *Aspergillus oryzae* type III polyketide synthase CsyB uses a fatty acyl starter for the biosynthesis of csypyrone B compounds. *Bioorg Med Chem* **23**, 5637–5640.
52 Taura F, Tanaka S, Tagushi C, Fukamizu T, Tanaka H, Shoyama Y & Morimoto S (2009) Characterization of olivetol synthase, a polyketide putatively involved in cannabinoid biosynthetic pathway. *FEBS Lett* **583**, 2061–2066.
53 Schüz R, Heller W & Hahlbrock K (1983) Substrate specificity of chalcone synthase from *Petroselinum*

hortense. Formation of phloroglucinol derivatives from aliphatic substrates. *J Biol Chem* **258**, 6730–6734.
54 Murray MG & Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* **8**, 4321–4325.
55 Monte D & Somerville S (2003) Pine tree method for isolation of plant RNA. In DNA Microarrays. A Molecular Cloning Manual (Bowtell D & Sambrook J, eds), pp. 124–126. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
56 Jez JM, Ferrer J-L, Bowman ME, Dixon RA & Noel JP (2000) Dissection of malonyl-coenzyme A decarboxylation from polyketide formation in the reaction mechanism of a plant polyketide synthase. *Biochemistry* **39**, 890–902.
57 Reimold U, Kroger M, Kreuzaler F & Hahlbrock K (1983) Coding and 3′ non-coding nucleotide sequence of chalcone synthase mRNA and assignment of amino acid sequence of the enzyme. *EMBO J* **2**, 1801–1805.
58 Arnold K, Bordoli L, Kopp J & Schwede T (2006) The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195–201.
59 Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J & Schwede T (2009) Protein structure homology modelling using SWISS-MODEL Workspace. *Nat Protoc* **4**, 1–13.
60 Biasini M, Bienert S, Waterhouse A, Arnold K, Studer S, Schmidt T, Kiefer F, Cassarino TG, Bertoni M, Bordoli L *et al.* (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* **12**, W252–258.
61 Dundas J, Ouyang Z, Tseng J, Binkowski A, Turpaz Y & Liang J (2006) CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. *Nucleic Acid Res* **34**, W116–W118.
62 Hunter S, Jones P, Mitchell A, Apweiler R, Attwood R, Bateman A, Bernand T, Binns D, Bork P, Burge S *et al.* (2012) InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acid Res* **40 (D1)**, D306–312.
63 Altschul SF, Gish W, Miller W, Meyers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
64 Katoh S (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**, 772–780.
65 Capella-Gutiérrez S, Silla-Martinez JM & Gabaldón T (2009) trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* **25**, 1972–1973.
66 Price MN, Dehal PS & Arkin AP (2010) FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLoS One* **5**, e9490.
67 R Core Team (2014) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
68 Paradis E, Claude J & Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289–290.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. Primer sequences used for cloning.

Table S2. Short sequences of poison hemlock (*Conium maculatum*) polyketide synthases.

ARTICLE II

Elicitation of furanocoumarins in poison hemlock (*Conium maculatum* L.) cell culture

Plant Cell, Tissue and Organ Culture 123(3), 443–453.
Copyright 2015 Springer Science+Business Media.
Reprinted with permission from the publisher.

ARTICLE III

Polyketides in *Aloe* plants and cell cultures

Manuscript.

Copyright 2016 Authors.

Polyketides in *Aloe* plants and cell cultures

Hannu Hotti, Suvi T. Häkkinen, Tuulikki Seppänen-Laakso and Heiko Rischer

VTT Technical Research Centre of Finland Ltd., Tietotie 2, Espoo, P.O. Box 1000, FI-02044 VTT, Finland

Author for correspondence: Heiko Rischer

Tel: +358 20 722 4461

Fax: +358 20 722 7071

Email: heiko.rischer@vtt.fi

Abstract

(150-250 words)

The genus *Aloe* L. is native to Sub-Saharan Africa, the Arabian Peninsula and Madagascar. Generally, the plants are rich in secondary metabolites such as flavonoids and anthraquinones. Only twelve species out of about 400 contain piperidine alkaloids. *In vitro* cultivated *Aloe gariepensis* Pillans*, A. globuligemma* Pole Evans and *A. viguieri* H. Perrier were investigated for their polyketide content using gas chromatography-mass spectrometry. Micropropagation of *A. viguieri* was investigated using statistical experimental design. Up to five plantlets of good quality were produced from a mother plant using Murashige and Skoog (MS) medium with 3.0 g L^{-1} gelrite, 0.25 mg L^{-1} 6-benzylaminopurine (BA) and 0.4 mg L^{-1} naphthalene acetic acid (NAA). Callus induction was investigated using MS medium containing a combination of 10.0 mg L^{-1} NAA and 0.2 mg L^{-1} BA, or only 6.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). *A. gariepensis* formed callus with NAA and BA and *A. viguieri* responded to 2,4-D. For long-term callus maintenance MS with 1.0 mg L^{-1} 2,4-D was used for both species. The callus of neither species contained piperidine alkaloids after elicitation with chitosan or salicylic acid. Suspension cultures of both *Aloe* species contained chrysophanol and chrysarobin, the latter representing a new record for the genus. *A. globuligemma* and *A. viguieri* were found to contain coniine, γ-coniceine and also *N*-methylconiine, an alkaloid not previously reported from *Aloe* sp. These *in vitro* techniques for piperidine-containing *Aloe* pave the way for future investigations of alkaloid biosynthesis.

Key message

(max 30 words)

In vitro techniques (micropropagation, callus, suspension) were developed for *Aloe gariepensis*, *A. globuligemma* and *A. viguieri.* Polyketide-derived metabolites were monitored and two new compounds, *N*-methylconiine and chrysarobin, recorded for *Aloe* sp.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzylaminopurine; CCF, central composite face; DW, dry weight; FW, fresh weight; GC-MS, gas chromatography-mass spectrometry; KIN, kinetin; MS, Murashige and Skoog medium (Murashige and Skoog 1962); MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NAA, naphthalene acetic acid; OKS, octaketide synthase; PKS, polyketide synthase; PVP, polyvinylpolypyrrolidone; TLC, thin-layer chromatography

Keywords

(4-6 words)

alkaloids, *Aloe*, coniine, callus, micropropagation, polyketides

Introduction

The genus *Aloe* L., Xanthorrhoeaceae, comprises about 400 species native to Sub-Saharan Africa, the Arabian Peninsula, and Madagascar (Viljoen et al. 1998). In many cultures *Aloe*, especially *A. vera*, has long been used as a traditional medicine. The aloe products from different parts of the plant, which include the latex, gel, and whole leaf, have been used as laxatives, in creams for skin ointments, and as a treatment for several diseases. Members of the genus exhibit a wide range of secondary metabolites of medicinal value, for example aloe emodin from *A. ferox* (Loots et al. 2007) and barbaloin (aloin A) from *A. vera* (Smith and Smith 1851). Remarkably many compounds in *Aloe* are derived from polyketides (Fig. 1A). Type III polyketide synthases (PKS) (Mizuuchi et al. 2009) are the key condensing enzymes in these pathways.

Anthraquinones (Fig. 1B) are a class of natural compounds which has a planar three aromatic ring structure with two keto groups at the 9- and 10-positions (Malik and Müller 2016). *Aloe* anthraquinones are made by octaketide synthase (OKS) belonging to type III PKS superfamily from one acetyl-CoA and seven malonyl-CoAs (Abe et al. 2005). Anthraquinones are found in different genera including *Aloe* (Reynolds 1997), *Rheum, Rumex*, *Rhamnus* and *Senna* (Steglich et al. 2000). The compounds have multiple uses in textile dying, paints, imaging devices, foods, cosmetics, and pharmaceuticals with diverse targets such as anticancer, laxative and anti-inflammatory (Malik and Müller 2016).

60 Only a limited number of *Aloe* species contain alkaloids including the purines
61 hypoxanthine and xanthine from *A. ferox* (Loots et al. 2007), and the tyramine-derived
62 phenylethylamines are found in 18 *Aloe* species (Nash et al. 1992). Hitherto twelve *Aloe* species
63 have been reported to contain piperidine alkaloids (Table 1) (Dring et al. 1984; Nash et al. 1992;
64 Blitzke et al. 2000) including coniine, γ-coniceine and conhydrine (Fig. 1C). Interestingly, these
65 alkaloids are otherwise only known from phylogenetically distant dicotyledonous plants, *i.e.* poison
66 hemlock (*Conium maculatum* L.) (Giseke 1827) and the yellow pitcher plant (*Sarracenia flava* L.)
67 (Mody et al. 1976). So far the biosynthesis of coniine alkaloids has only been investigated in poison
68 hemlock. Theoretically, the carbon backbone of the alkaloids in *Aloe* could be either a tetraketide
69 polyketide derivative (Leete 1963, 1964) or a triketide (Hotti et al. 2015). The nitrogen of L-alanine
70 is introduced in a transaminase-catalyzed reaction (Roberts 1971) followed by a non-enzymatic
71 cyclization to form γ-coniceine (Roberts 1977). Coniine formation from γ-coniceine is finally
72 catalysed by NADPH-dependent γ-coniceine reductase (Roberts 1975). There are reports that
73 poisonous aloes, among them *A. globuligemma*, have been administered as medicines, causing
74 deaths in rural Africa (Drummond et al. 1975). Parry and Matambo (1992) studied the toxicity of *A.*
75 *globuligemma* due to its use as a traditional herbal medicine in Zimbabwe. The crude extract is
76 toxic, with an LD_{50} of less than 250 mg kg^{-1} in mice, which exhibited symptoms similar to those of
77 poison hemlock intoxication. In some cases toxic and beneficial aloes have been confused due to
78 misidentification (Reynolds 2005).

79 The biotechnology of *Aloe* is underdeveloped and underutilized (Rathore et al. 2011),
80 although many species are already endangered in their natural habitats. Commercial and research
81 interest has driven the development of micropropagation methods for a certain number of species
82 (Grace 2011). *A. vera* (syn. *A. barbadensis*) is certainly the most prominent example due to its
83 economic importance (Gantait et al. 2011), and *A. polyphylla* is a representative example of an
84 endangered species (Ivanova and van Staden 2008). It must be emphasized that generally species-
85 specific micropropagation protocols are required. Some of these methods proceed via callus
86 formation (Roy and Sarkar 1991; Bedini et al. 2009; Rathore et al. 2011), but callus cultures have
87 also been specifically initiated for genetic transformation (Velcheva et al. 2010) and the production
88 of certain compounds (Yagi et al 1998). Callus formation has been reported for *A. arborescens*
89 (Kawai et al. 1993; Bedini et al. 2009), *A. bellatura* (Tatsuo and Amano 1985), *A. ferox* (Racchi
90 1988), *A. polyphylla* (Ramsay and Gratton 2000), *A. pretoriensis* (Groenewald et al. 1975), *A.*
91 *saponaria* (Yagi et al. 1983; Baek et al. 2009) and *A. vera* (Roy and Sarkar 1991; Yagi et al. 1998;

92 Velcheva et al. 2010; Rathore et al. 2011). Piperidine alkaloid-producing *Aloe* species have hitherto
93 not attracted any attention in this respect.

94 Here we report the *in vitro* cultivation of *A. gariepensis*, *A. globuligemma* and *A.*
95 *viguieri*. Greenhouse-grown specimens of all three species have been reported to contain hemlock
96 alkaloids (Dring et al. 1984; Nash et al. 1992). We focused on establishing micropropagation for *A.*
97 *viguieri* and cell culture development together with elicitation for *A. gariepensis* and *A. viguieri*,
98 while monitoring alkaloid and other polyketide derived compounds in order to provide a basis for
99 future biochemical investigations. Interestingly, one of the piperidine alkaloids and of the
100 anthraquinones were detected in this work and identified in *Aloe* for the first time, illustrating the
101 potential for further compound discoveries.

102 Materials and Methods

103 Plant material

104 *Aloe gariepensis* Pillans, *A. globuligemma* Pole Evans, and *A. viguieri* H. Perrier seeds were
105 obtained from B & T World Seeds (Paguignan, France). Poison hemlock (*Conium maculatum* L.)
106 seeds were collected in Helsinki, Finland. Barley (*Hordeum vulgare* L. 'Golden Promise') was
107 grown from seeds and used as alkaloid-free reference material.

108 Poison hemlock and barley were grown under greenhouse conditions at 20°C, 60%
109 humidity, and a 16/8 h photoperiod. The potting soil consisted of half vermiculite and half peat
110 (Kekkilä Oy, Vantaa, Finland).

111 Surface sterilization and *in vitro* germination

112 *A. gariepensis* (n = 10), *A. globuligemma* (n = 10) and *A. viguieri* (n = 5) seeds were surface
113 sterilized by dipping them into 70% ethanol for three minutes, followed by immersion in 2%
114 sodium hypochlorite containing a drop of Tween-20 (Sigma-Aldrich, Lyon, France) for 20 minutes
115 and washing three times with sterile ultrapure water. Seeds were placed on Murashige and Skoog
116 (MS) medium (Murashige and Skoog 1965) (Duchefa Biochemie B.V., Haarlem, the Netherlands)
117 with 3.0 g L^{-1} gelrite at pH 5.8 for germination. The *in vitro* plants were kept on MS medium
118 without hormones and transferred to fresh medium every second month.

119 Micropropagation

120 *A. viguieri* plants were grown on 50 mL MS medium with 30.0 g L^{-1} sucrose and 3.0 g L^{-1} gelrite in
121 250 mL wide neck Erlenmeyer flasks. The medium was supplemented with 0.10-2.00 mg L^{-1}

122 naphthalene acetic acid (NAA) and 0.06-4.00 mg L^{-1} 6-benzylaminopurine (BA) (Sigma-Aldrich,
123 St. Louis, MO, USA). Effects were recorded after 4, 5, and 6 weeks.

124 Experiments and data analyses were performed with the experimental design software
125 MODDE 9.0 (Umetrics, Malmö, Sweden). To minimize the necessary number of plants, the central
126 composite face (CCF) design was applied to investigate effects of different variables on the number
127 of initiated plantlets. In CCF the studied parameters are within a certain distance of the central
128 point, and to determine repeatability the central point was measured in triplicate. Studied factors
129 were initially: 0.06, 0.16 and 0.25 mg L^{-1} BA and 0.1, 0.25 and 0.4 mg L^{-1} NAA after four, five and
130 six weeks. The equation for the polynomial model is:

$$\mathbf{y} = -6.59524 + 27.5048 \times x_1 + 13.7619 \times x_2 + 1.71429 \times x_3 + 51.4286 \times (x_3)^2 - 4.19048 \times (x_1 \times x_3) - 7.38095 \times (x_2 \times x_3)$$

131 where x_1 = [BA], x_2 = [NAA] and x_3 = [time]. In the follow-up experiment the parameters were:
132 0.25, 2.13, 4.0 mg L^{-1} BA and 0.4, 1.2, 2.0 mg L^{-1} NAA, with monitoring after four, five and six
133 weeks.

134 Callus initiation and maintenance

135 Explants were derived from *A. gariepensis* and *A. viguieri in vitro* plants. Leaves or stems were cut
136 into pieces and were placed in Petri dishes (⌀ 9 cm; 5 pieces/plate) containing MS medium with 1.0
137 g L^{-1} polyvinylpolypyrrolidone (PVP) (average mol wt 40 000, Sigma-Aldrich, St. Louis, MO,
138 USA) (Roy and Sarkar 1991), 18.34 mg L^{-1} adenine and 100.0 mg L^{-1} L-ascorbic acid (Sigma-
139 Aldrich, Shanghai, China) (Rathore et al. 2011). The hormone combinations were 10.0 mg L^{-1} NAA
140 and 0.2 mg L^{-1} BA (Yagi et al. 1998) or only 6.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D)
141 (Sigma-Aldrich, St. Louis, MO, USA) (Rathore et al. 2011). The callus initiation plates were
142 incubated in a growth chamber (Sanyo versatile environmental test chamber, Sanyo Electric Co.,
143 Ltd., Takamatsu, Japan) with a 16/8h photoperiod at 25°C/23°C (day/night) for 50 days. In the
144 beginning the formed calli were kept on the initiation media with the same hormone combinations,
145 in the dark and were transferred every week. Six weeks after initiation, callus was transferred to MS
146 supplemented with 2.0 mg L^{-1} 2,4-D without PVP, adenine and ascorbic acid. Nine weeks later
147 callus cultures were transferred to MS medium with 1.0 mg L^{-1} 2,4-D and kept in darkness at 25°C
148 for maintenance.

149 ## Growth curve

150 The fresh weight (FW) of calli was recorded for three weeks to choose cell lines for further
151 experimentation. Three lines of both species were selected and labelled as A-C (*A. gariepensis*) and
152 D-E (*A. viguieri*) based on vigorous growth and healthy appearance (without browning or excessive
153 leaching of phenolics into the medium). For each selected line 12 pieces were weighed at the
154 beginning of cultivation and then once every week. The average FW increase of the calli was
155 calculated for each line. Statistical analyses of callus growth were performed with One-way
156 ANOVA and Tamhane's T2 using IBM SPSS Statistics software (Version 22).

157 The growth curves for suspension cultivations were performed in sterilized 125 mL
158 glass Erlenmeyer flasks containing 25 mL MS medium with 1.0 mg L^{-1} 2,4-D. Inoculum with
159 biweekly sub-cultured cells of lines C and D was added to each flask at a concentration of 50.0 g L^{-1}
160 ± 1.0 g L^{-1} FW. At each time point a series of three replicates was investigated (in total 33 flasks for
161 each cell line). Flasks were placed on an orbital shaker (130 rpm) at 24°C and cultured in darkness.
162 Conductivity and pH of the medium as well as the FW and dry weights (DW) of the cell mass were
163 determined for all replicates at each time point.

164 ## Elicitation

165 Individual elicitors were added to cell lines C and D to test secondary metabolite production.
166 Erlenmeyer flasks (125 mL) containing 25 mL MS medium with 1.0 mg L^{-1} 2,4-D were inoculated
167 with 50.0 g L^{-1} ± 1.0 g L^{-1} FW of biweekly subcultured cells. At day 8 and day 14 for cell lines C
168 and D, respectively, elicitors were added at the concentrations 110 mg L^{-1} for chitosan (Sigma-
169 Aldrich, St. Louis, MO, USA) (Meier et al. 2015) and 138 mg L^{-1} for salicylic acid (Sigma-Aldrich,
170 St. Louis, MO, US) (Lee et al. 2013). Cultures were harvested on the 1st, 3rd and 5th day after
171 elicitation (Carew and Bainbridge 1976). Chitosan stock solution of 0.1 M acetic acid in water at
172 pH 5.0 was prepared according to Sévon et al. (1992). Adverse effects of acetic acid to cell lines C
173 and D was tested in an additional flask at the highest volume tested in the elicitor experiment.
174 Samples treated with stock solution solvent were collected after one, three and five days. Cells and
175 growth medium were separated by vacuum filtration and cells were lyophilized.

176 ## Metabolite extraction

177 Freeze-dried plant material (100 ± 10 mg) was used for secondary metabolite extraction according
178 to Häkkinen et al. (2005). Lipids were removed from the plant material with 3.0 mL petroleum
179 ether (Sigma-Aldrich, St. Louis, MO, USA). The plant material was diluted with 2.0 mL ultrapure
180 water and a pH above 9 was obtained by addition of 10% ammonium hydroxide (Merck KGaA,
181 Darmstadt, Germany) solution. Metabolites were extracted twice with 2.0 mL dichloromethane

182 (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK). From elicited cells secondary metabolites
183 were extracted as previously described except that lipids were not removed, 5 mL 10% ammonium
184 solution and 6 mL dichloromethane was used. The medium was made alkaline with 1 M NaOH and
185 extracted twice with 10 mL dichloromethane. Finally, the dichloromethane extracts were
186 concentrated to 100 µL for further analysis. For compound identification, selected samples were
187 further trimethylsilylated with 25 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)
188 (Pierce, Rockford, IL, USA) at 70°C for 20 minutes.

189 Thin layer chromatography

190 The thin layer chromatography (TLC) method was adapted from Lopez et al. (2004) by employing
191 dichloromethane instead of chloroform. The eluent was dichloromethane-ethanol (65:35 v/v), and
192 TLC-plates (silica gel 60 F_{254}, 20 × 20 cm aluminium sheets, Merck KGaA, Darmstadt, Germany)
193 were sprayed with Dragendorff's reagent (Fluka Analytical, Buchs, Switzerland) to stain alkaloids
194 red. The TLC-method was established using commercial coniine (Sigma-Aldrich, Munich,
195 Germany), coniine-spiked barley material and poison hemlock extract. To monitor the alkaloid
196 extraction steps petroleum ether, alkaline water, and dichloromethane fractions were individually
197 subjected to TLC.

198 Gas chromatography-mass spectrometry

199 Commercial coniine was used to develop the gas chromatography-mass spectrometry (GC-MS)
200 method and to determine the detection limit. A GC-MS system consisting of an Agilent 6890A GC
201 and an Agilent 5973N MS detector (Agilent Technologies Inc., Santa Clara, CA, USA) and a
202 Combipal automatic sampler (Varian Inc., Palo Alto, CA, USA) was used to study the elicitation
203 extracts. Analyses were performed on a diphenyl dimethyl polysiloxane RTX-5MS (Restek,
204 Bellefonte, PA, USA) silica capillary column (15 m × 250 µm with a phase thickness of 0.25 µm)
205 The oven temperature programme started from 40°C (1 min) and increased at a rate of 10°C min^{-1}
206 up to 270°C (6 min) resulting in a total running time of 40 minutes. Helium with a flow rate of 1.2
207 mL min^{-1} was used as the carrier gas (split ratio 15:1 or 20:1). Injection port temperature was 250°C
208 and the injection volume 2.0 µL. MSD was operated in electron impact mode at 70 eV and the data
209 were collected in SCAN mode at a mass range of *m/z* 40-650. The chromatograms were integrated
210 by using MSD ChemStation software (E.02.01.1177; Agilent Technologies, Inc., Santa Clara, CA,
211 USA). Compounds were identified on the basis of relevant literature, reference substance and
212 spectral libraries such as Palisade Complete 600K Mass Spectral Library (Palisade Mass
213 Spectrometry, Ithaca, NY, USA), and the NIST Mass Spectral Search 2.0 Program (The Standard

214 Reference Data Program of the National Institute of Standards and Technology, Gaithersburg, MD,
215 USA).

216 *In vitro* samples of *A. globuligemma* were analysed and identified as above except by
217 using an HP-5MS silica capillary column (25 m × 0.2 mm) with 0.33 µm phase thickness (Agilent
218 Technologies, Inc., Santa Clara, CA, USA). The temperature program started at 50°C (1 min), and
219 increased at 10°C min^{-1} up to 300°C.

220 Derivatized samples (1 µL aliquots) were analysed in splitless mode by an Agilent
221 GC-MS instrument consisting of a 5975MSD and a 7890A GC fitted with a 30 m × 0.25 mm (0.25
222 µm) VB-5 silica capillary column (Valco Instruments Co. Inch., Houston, TX, USA). The
223 temperature program started at 50°C (1 min) and increased at 10°C min^{-1} up to 240°C. The data
224 were collected at a mass range of *m/z* 50-600. Identification was performed as above.

225 Results

226 Surface sterilization and seed germination

227 Half of the ten *A. gariepensis* and *A. globuligemma* seeds, respectively, and two of the five *A.*
228 *viguieri* seeds germinated on MS medium following surface sterilization. The first seedlings
229 emerged already within 30 days, but some seeds only germinated after four months. Mould or
230 bacterial contaminations were not observed.

231 Micropropagation

232 Statistics indicated the good predictive power of the experimental design with the parameters BA
233 and NAA at levels of 0.06-0.25 mg L^{-1} and 0.1-0.4 mg L^{-1}, respectively, and the time points four,
234 five and six weeks when one outlier was omitted (Supplemental Table 1). The quality of the fit of
235 the polynomial model equation, which is expressed as coefficient of determination $R^2 = 0.952$ (>
236 0.5) (Umetrics 2011), indicates that 95.2% of the variability of the response is explained by the
237 model. The predictive power was $Q^2 = 0.821$ (> 0.1), confirming the statistical significance of the
238 model. Model validity was 0.982 (> 0.25) and reproducibility was 0.808 (> 0.5) (Umetrics 2011).
239 Plantlets were initiated already within four weeks from the axillary buds at the base of the mother
240 plant (Fig. 2). The highest plantlet number (5) was achieved with BA 0.25 mg L^{-1} and NAA 0.4 mg
241 L^{-1} (Fig. 3). At hormone concentrations lower than 0.25 mg L^{-1} BA and 0.4 mg L^{-1} NAA, only up to
242 two plants were obtained (Supplemental Table 1).

243 Further investigation with the same model design involving higher BA and NAA levels, *i.e.* 0.25-4.0 mg L^{-1} and 0.4-2.0 mg L^{-1}, respectively, but with the same time points as previously (four, five and six weeks) did not result in a good quality model due to low significance (R^2 = 0.267) and a poor predictive power (Q^2 = -0.804). Therefore *A. viguieri* micropropagation could not be improved with higher hormone concentrations. Although a hormone combination of 4.0 mg L^{-1} BA and 1.2 mg L^{-1} NAA resulted in seven *A. viguieri* plantlets within four weeks and nine plantlets in six weeks (Supplemental Table 2), the plants were hyperhydrated and had poor viability.

251 Callus initiation

252 Callus induction frequency on MS medium with two different hormone regimes was evaluated 50 days after initiation. On medium containing 10.0 mg L^{-1} NAA and 0.2 mg L^{-1} BA, 16 out of the 25 *A. gariepensis* explants (64%) produced callus, whereas on medium containing 6.0 mg L^{-1} 2,4-D only 1 in 20 (5%) responded. By contrast *A. viguieri* explants did not produce any callus on the former medium but instead 10 out of 25 (40%) initiated callus on the medium containing 2,4-D.

257 Initially the medium supplements PVP, adenine and ascorbic acid were used to prevent browning. However, established calli could be maintained on medium devoid of these agents. There was also some root formation from the explants of *A. gariepensis* on MS with 10.0 mg L^{-1} NAA and 0.2 mg L^{-1} BA. On MS supplemented with 2.0 mg L^{-1} 2,4-D calli from both species grew more vigorously when sub-cultured every second week. Finally the 2,4-D content in the medium was decreased to 1.0 mg L^{-1} and the sub-culturing to a three-week interval, without any detrimental effect (Figs. 4D and E).

264 Growth curve

265 Callus FW development was monitored for three weeks in darkness at 25°C (Table 2). Three lines of both species were selected and labelled as A-C (*A. gariepensis*) and D-E (*A. viguieri*) based on vigorous growth and healthy appearance (without browning or leaching of phenolics into the medium). Statistical analysis did not reveal significant differences in the original starting weights of the calli (Table 2). All six callus lines grew similarly for two weeks, without statistically significant changes in callus growth except for line D, which displayed the highest and significantly different growth compared to the other lines throughout the whole experimental period. Generally *A. viguieri* grew faster than *A. gariepensis* (weight increase 28-86% vs. 12-48%). The greatest weight increase for all lines occurred during week two. Among *A. gariepensis*, line C exhibited the greatest weight increase, on average 38% per week, whereas line B showed the slowest overall growth with an

275 average increase of 21%. The fresh weight of the *A. viguieri* line D increased most, on average 72%
276 per week.

277 Based on the initial testing of FW increase on a plate, cell lines C and D were chosen
278 for further experimentation. For both cell lines, an inoculum of 50 g L^{-1} FW was used in growth
279 curves. For cell line C there was a two day lag period after which FW increased to its highest point,
280 268.23 ± 25.28 g L^{-1}, on day 23 (Fig. 5A). DW of cell line C increased until day 9 when it reached
281 stationary phase. The maximal DW was reached on day 19 (12.31 ± 1.16 g L^{-1}) and thereafter it
282 started to decrease. Conductivity decreased rapidly from 5.46 mS cm^{-1} (day 0) to 3.33 ± 0.17 mS
283 cm^{-1} (day 14) (Fig. 5B) followed by a gentle reduction to 3.05 ± 0.16 mS cm^{-1} until the end point on
284 day 23. The starting pH (5.0) decreased rapidly to 4.3 ± 0.1 on day 2 and then increased steadily to
285 4.6 ± 0.1 on day 23.

286 For cell line D there was a two day lag period after which FW increased to its highest
287 point, 203.84 ± 5.17 g L^{-1}, on day 23 (Fig. 5C). DW of cell line D increased until day 14 when it
288 reached stationary phase. The maximal DW was reached on day 19 (11.29 ± 0.23 g L^{-1}) and
289 thereafter it started to decrease due to the cultures entering the death phase. Conductivity decreased
290 rapidly from 5.46 mS cm^{-1} (day 0) to 3.30 ± 0.18 mS cm^{-1} (day 14) (Fig. 5D) followed by a gentle
291 reduction to 2.99 ± 0.04 mS cm^{-1} until the end point on day 23. The starting pH (5.0) decreased
292 rapidly to 4.3 ± 0.0 on day 2 and then increased steadily to 4.7 ± 0.1 on day 23.

293 Elicitation

294 Based on growth curves of cell line C and D, days 8 and 14, respectively, were chosen for the
295 elicitations. Samples were collected on days 1, 3 and 5 after elicitation with chitosan (110 mg L^{-1})
296 or salicylic acid (138 mg L^{-1}) and extracts were subjected to GC-MS analysis. Cells and culture
297 medium were separated and analysed individually for secondary metabolites.

298 None of the elicited or non-elicited cell cultures contained hemlock alkaloids (Table
299 3). Although, when *A. gariepensis* cell line C or *A. viguieri* cell line D were elicited with salicylic
300 acid, cells often died in culture and produced a distinctive smell reminiscent of coniine. In several
301 samples characteristic base peaks (*m/z*) for coniine, γ-coniceine and/or *N*-methylconiine were
302 present, however, the concentrations were at the detection limit and the fragments therefore
303 originate from the background.

304 In suspension cultures, secondary metabolites chrysarobin, chrysophanol, and terpenes
305 xanthoperol and *p*-meth-8(10)-en-9-ol were found (Table 4). The relative amount of chrysarobin

306 and chrysophanol were from 0.2 to 1.0% and 0.13-0.31% of peak area, respectively, in *A.*
307 *gariepensis* cell line C extracts. Chrysarobin was absent in the media of non-elicited as well as in
308 acetic acid and chitosan (110 mg L^{-1}) elicited samples in contrast to the salicylic acid elicited
309 samples where its proportion was 1.18-1.32% of peak area. Chrysophanol was not present in the
310 media.

311 In *A. viguieri* cell line D, chrysarobin and chrysophanol were not present in media
312 extracts without elicitor and acetic acid treatment. When present, the proportions of chrysarobin and
313 chrysophanol were 0.57-1.92% and 0.09-0.65% of peak area, respectively. Chrysarobin was present
314 in media when elicited with chitosan or salicylic acid. In addition, salicylic acid elicitation increased
315 peak areas of both compounds and induced production of four unidentified compounds. The
316 unknown compound 1 was tentatively identified as a pyrrolidine with MW = 305 and a long carbon
317 side chain (Table 5). The unknown compounds 2 (MW = 239), 2 (MW = 258) and 3 (MW = 257)
318 tentatively contained a three carbon ring structure, and thus they might be anthraquinone type
319 compounds, based on fragmentation patterns of suggested compounds as a match in NIST-
320 program's database. The unknown compound 3 was found only in the media and the unknown
321 compounds 2 and 4 were present only in the cell extract.

322 Alkaloid analysis

323 The extraction method for alkaloids was established using commercial coniine, coniine-spiked
324 barley material (without natural alkaloid content), and poison hemlock extract (naturally containing
325 coniine). Examination of the different extraction steps (petroleum ether, alkaline water,
326 dichloromethane) by TLC revealed that only the dichloromethane fraction contained alkaloids. In
327 all cases coniine (R_f = 0.04) was detected on the TLC-plate. A dilution series of reference coniine
328 analysed on a TLC-plate showed that a spot containing minimally about 0.1 µg coniine could be
329 reliably detected.

330 GC-MS analyses of a coniine dilution series proved to have a higher sensitivity
331 (detection limit 10 µg mL^{-1}; corresponding to 0.01 mg g^{-1} dry weight) than TLC. Coniine was
332 identified based on the retention time (4.55 ± 0.05 min) and the base peak (*m/z* 84) of the
333 commercial reference. γ-Coniceine identification was based on the mass spectral database (number
334 28670, Palisade Complete 600K Mass Spectral Library) and comparison to literature (Holstege et
335 al. 1996; Table 6). The retention time (5.05 ± 0.05 min) and the base peak (*m/z* 98) of *N*-
336 methylconiine were derived from reference samples (*C. maculatum*) naturally containing this
337 alkaloid. *N*-methylconiine has the base peak *m/z* 98, molecular mass *m/z* 141 and typical fragments

338 (*m/z* 112, 99, 70) as reported in the literature (Holstege et al. 1996). In addition, *N*-methylconiine
339 did not react with MSTFA to produce trimethylsilyl derivatives, indicating a tertiary amino group.
340 Conhydrine was present in the poison hemlock which was used as reference material. Its spectrum
341 matched the database (number 20048, Palisade Complete 600K Mass Spectral Library). However,
342 conhydrine was not detected in any studied *Aloe* material.

343 Neither the leaves of *in vitro* grown *A. gariepensis* nor any of the derived callus lines
344 contained alkaloids, but roots contained traces of coniine and *N*-methylconiine (Table 7). *In vitro*
345 grown leaves of *A. globuligemma* contained coniine, *N*-methylconiine and γ-coniceine, whereas
346 roots contained only *N*-methylconiine. Leaves and roots of *in vitro* grown *A. viguieri* contained
347 coniine, *N*-methylconiine and γ-coniceine. None of the *A. viguieri* callus lines contained piperidine
348 alkaloids.

349 Discussion

350 Micropropagation

351 Micropropagation protocols have hitherto only been developed for seven out of 400 *Aloe* species,
352 none of which produce hemlock alkaloids. The published studies have used MS basal medium,
353 however, there is no clear consensus on optimal hormone combinations across species. Most
354 commonly the cytokinin BA is used in a range between 1.0 and 2.5 mg L^{-1} in combination with the
355 auxin NAA at 0.1-1.0 mg L^{-1}. *A. viguieri* was chosen as an example of poison aloes, with the aim of
356 producing good quality plants by micropropagation employing the experimental design software
357 MODDE 9.0. The CCF design model indicated significance and good predictive power when the
358 BA and NAA levels were in the range of 0.06-0.4 mg L^{-1} and 0.1-0.4 mg L^{-1}, respectively, and
359 sampling times were after four, five and six weeks. In the best scenario, *i.e.* at 0.25 mg L^{-1} BA and
360 0.4 mg L^{-1} NAA, *A. viguieri* produced up to five new shoots from axillary buds of the mother
361 rosette within four weeks. The study design therefore adds another suitable tool to the earlier
362 described statistically supported micropropagation strategies for *Aloe* (Barringer et al. 1996;
363 Chukwujekwu et al. 2002; Liao et al. 2004; Hashemabadi et al. 2008; Abadi and Kaviani 2010).
364 Although high hormone concentrations have been recommended for the micropropagation of other
365 species, *e.g.* 2.0 mg L^{-1} BA and 1.0 mg L^{-1} NAA for *A. arborescens* (Bedini et al. 2009), *A. viguieri*
366 plantlets produced at 2.13 or 4.0 mg L^{-1} BA and 1.2 mg L^{-1} NAA were of poor quality, exhibiting
367 hyperhydration despite high abundance (up to seven shoots from the mother rosette). In this setup
368 with BA and NAA levels between 0.25-4.0 mg L^{-1} and 0.4-2.0 mg L^{-1} respectively, a robust model
369 could not be derived due to the high concentration of BA (Leshem et al. 1988; Tsay 1998; Thomas

370 et al. 2000). Hyperhydration (in older literature called vitrification) is a physiological disorder in *in*
371 *vitro* cultures that adversely affects growth and regeneration ability. *A. viguieri* plants became
372 hyperhydrated when the BA level exceeded 2.0 mg L^{-1}. Similarly, it has been shown that more than
373 1.0 mg L^{-1} BA triggers hyperhydricity in *A. polyphylla*, although inducing the highest number of
374 shoots (Chukwujekwu et al. 2002). Abrie and van Staden (2001) reported that decreasing plant
375 hormone concentrations decreased the level of hyperhydricity in seedlings of *A. polyphylla*. Another
376 crucial parameter potentially responsible for hyperhydricity is the gelling agent in the medium such
377 as gelrite which decreases the shoot multiplication rate and causes hyperhydricity in *A. polyphylla*
378 (Ivanova and van Staden 2011).

379 Micropropagation via a callus phase has been developed for *A. arborescens* (Bedini et
380 al. 2009), *A. ferox* (Racchi 1988) and *A. vera* (Roy and Sarkar 1991; Velcheva et al. 2010; Rathore
381 et al. 2011). The selected micropropagation method (directly from differentiated plant material or
382 via callus) depends on the species and the purpose (Velcheva et al. 2010).

383 Callus induction

384 Callus has been induced in several *Aloe* spp. for different purposes, *e.g.* to produce
385 specific compounds (Yagi et al. 1983, 1998). In most studies MS medium has been used together
386 with a variety of different hormone combinations. Most commonly the auxins 2,4-D and NAA are
387 used together with the cytokinins kinetin (KIN) and BA. 2,4-D can promote callus induction
388 (Groenewald et al. 1976; Racchi 1988; Roy and Sarkar 1991) and cell division (Velcheva et al.
389 2010) in different *Aloe* species. Only 40% of *A. viguieri* explants formed callus on MS containing 6
390 mg L^{-1} 2,4-D, the medium which Rathore et al. (2011) used for *A. vera* with over 75% induction
391 rate. *A. gariepensis* responded better to a hormone combination of 10.0 mg L^{-1} NAA and 0.2 mg L^{-1}
392 BA, with 64% of explants producing callus. Yagi et al. (1998) used the same hormone combination
393 for callus induction of *A. vera* in darkness (100% induction rate). Overall the achieved callus
394 induction rates for *A. viguieri* and *A. gariepensis* are in line with previously reported rates (Bedini et
395 al. 2009) for *A. arborescens* (27.7% and 40% in light). However, for long-term maintenance
396 Rathore et al. (2011) recommend lower hormone concentrations, otherwise callus cultures may
397 become hyperhydrated. Roy and Sarkar (1991) used 1 mg L^{-1} 2,4-D and 0.2 mg L^{-1} KIN, whereas
398 we found that 1 mg L^{-1} 2,4-D without KIN suffices for *A. gariepensis* and *A. viguieri* callus lines. In
399 our study we initially used PVP, ascorbic acid and adenine as antioxidants to reduce the leaching of
400 phenolic compounds and to prevent browning of tissues and surrounding culture medium. The use
401 of PVP is based on its ability to absorb polyphenols and prevent further oxidation of phenolic
402 compounds (Sathyanarayana and Varghese 2007). Roy and Sarkar (1991) noted that PVP was a

more efficient antioxidant than ascorbic acid and activated charcoal in preventing browning of the medium.

The transfer of *A. viguieri* and *A. gariepensis* callus cultures from solid to liquid medium was unproblematic. Previously only suspension cultures from *A. saponaria* (Yagi et al. 1983) and *A. arborescens* (Liu et al. 2003) have been described. The same medium without gelling agent enables cell suspension cultures exhibiting a typical growth curve in a batch mode. Conductivity decreased in both suspension cultures due to nutrient salt consumption of the plant cells during first the 14 days. The late exponential or early stationary phase is usually considered optimal for elicitation (Baldi et al. 2009). On the basis of all the growth-related data collected, day 8 and day 14 were chosen for cell lines C and D, respectively.

Polyketides in *Aloe*

Coniine alkaloids are interesting due to their fragmentary distribution in non-related plant taxa (dicots vs. monocots), and especially among *Aloe*. Their biosynthesis in *Aloe* is unknown, but could be similar to that in poison hemlock due to the occurrence of γ-coniceine, which is reported to be the common precursor alkaloid in the pathway towards coniine (Leete 1970; Leete and Olson 1970; Roberts 1971; Leete and Olson 1972; Hotti et al. 2015).

In previous studies Dring et al. (1984) and Nash et al. (1992) used TLC and paper chromatography to study alkaloid contents of *Aloe*. They conducted ground-breaking research when they identified hemlock alkaloids from *Aloe* for the first time using different coloring reagents, such as Dragendorff's, and compared the findings with authentic alkaloids from *C. maculatum* or synthetic references using R_f-values. In our study we employed GC-MS to detect and identify alkaloids, as the method is about ten times more sensitive than TLC. MS allows the identification of unknown compounds without an array of authentic compounds.

In contrast to the cited earlier studies (Dring et al. 1984; Nash et al. 1992), we found that γ-coniceine is the major alkaloid both in *A. globuligemma* and in *A. viguieri*. We also detected the presence of γ-coniceine in *A. viguieri* roots, using the base peak ion as a marker. Nash et al. (1992) reported that *A. gariepensis* contains γ-coniceine but in our material the alkaloid was not present in leaves. Interestingly, Blitzke et al. (2000) speculated that γ-coniceine could be a starting point for the biosynthesis of a chlorinated derivative in *A. sabaea*.

The presence of coniine in *A. globuligemma* (Nash et al. 1992) and *A. viguieri* leaves (Dring et al. 1984) was confirmed, but additionally coniine was also found in *A. viguieri* roots. To

434 our knowledge, we here report the occurrence of *N*-methylconiine in the genus *Aloe* for the first
435 time. *N*-methylconiine is present in the unrelated plant, poison hemlock (Cromwell 1956) and a
436 similar alkaloid, *N*,*N*-dimethylconiine, has been reported from *A. sabaea* (Blitzke et al. 2000). We
437 detected *N*-methylconiine in *A. globuligemma* leaves and roots, and in *A. viguieri* leaves. Based on
438 the detected ion *m/z* 98 it is possible that *N*-methylconiine is also present in low amounts in *A.*
439 *gariepensis* and *A. viguieri* roots.

440 Different growth conditions or genotypes could explain why conhydrine was absent in
441 *A. gariepensis* and *A. globuligemma*, in contrast to the results of Nash et al. (1992) who found it in
442 the leaves of both species. Detection problems can be ruled out since we clearly detected
443 conhydrine in *C. maculatum* reference samples. It has been observed that alkaloid content in poison
444 hemlock depends on the population, weather, location, season, and age of the plant (Fairbairn and
445 Challen 1959). A certain strain of poison hemlock for example produces conhydrine as a major
446 alkaloid in outdoor conditions but pseudoconhydrine in the greenhouse (Leete and Adityachaudhury
447 1967).

448 None of the callus lines (A-F) contained piperidine alkaloids, nor when suspension
449 cultures of lines C and D were elicited with chitosan or salicylic acid. Similar observations were
450 made by Nétien and Combet (1971) and Meier et al. (2015) with poison hemlock callus lines. In the
451 latter case not even elicitation triggered alkaloid accumulation, although the selected callus was
452 green and grown in light, in contrast to the *Aloe* calli, which were white/light yellow and grown in
453 darkness.

454 Roberts (1981) suggested that coniine alkaloid biosynthesis occurs in the chloroplasts
455 localized in the green aerial parts of poison hemlock. In addition to the demand of specialized
456 tissue, several other factors might affect alkaloid production in *Aloe.* In *Conium* specialized ducts,
457 called 'vittae', specifically accumulate coniine and might be involved in alkaloid biosynthesis
458 (Fairbairn and Challen 1959; Corsi and Biasci 1998). In completely undifferentiated calli the
459 biosynthetic capacity might therefore be blocked. The synthetic plant hormone, 2,4-D, could be
460 another reason for the absence of hemlock alkaloids in *Aloe* callus. There are reports that 2,4-D
461 generally exhibits an inhibitory role in secondary metabolism (Tabata and Hiraoka 1976; Knobloch
462 and Berlin 1980; Morris 1986; Stalman et al. 2003). It can either alter the alkaloid profile *e.g.* in
463 *Leucojum aestivum* callus (Ptak et al. 2013), or inhibit the production altogether as in the case of
464 tobacco alkaloids (Furuya et al. 1971; Tabata and Hiraoka 1976; Ishikawa et al. 1994) and terpenoid
465 indole alkaloids (Rischer et al. 2006).

466 Chrysophanol and chrysarobin were observed from cell and media extracts of cell
467 lines C and D. Elicitation with chitosan increased relative peak areas of the compounds. The most
468 noticeable effect was triggered with salicylic acid for both cell lines as it increased several times the
469 peak areas of both compounds and induced production of three unknown compounds. Lee et al.
470 (2013) elicited root culture of *A. vera* with salicylic acid and noticed 5-13 fold increase in
471 chrysophanol production. They also noticed that salicylic acid treatment increased expression of
472 OKS transcripts leading to anthraquinone productions. In that study, elicitation with 138 mg L^{-1}
473 salicylic acid the accumulation of chrysophanol was shown to be maximal in media after 3 days and
474 in roots after 2 days. In our experiments the maximal accumulation of chrysophanol in cell line D
475 occurred after 3 days.

476 Chrysophanol, an anthraquinone, is known to occur in *Rheum*, *Rumex*, *Rhamnus*, and
477 *Senna* species (Steglich et al. 2000). It is also known from *A.vera* and exhibit anti-inflammatory
478 activity (Lee et al. 2013). Chrysarobin, an anthrone, is found from the stem of *Cassia singueana*
479 (Ibrahim et al. 2013) and Chinese rhubarb (Han et al. 2013). There is no previous report of this
480 compound in *Aloe*.

481 *cis-p*-Menth-8(10)-en-9-ol is a terpenoid which has been found previously from
482 *Valeriana jatamansii* (Yang et al. 2006), *Syzygium densiflorum* (Deepika et al. 2013), *Calligonum*
483 *comosum* (Dawidar et al. 2012) and fruit of *Mangifera indica* 'Keitt' (Zhou et al. 2014).
484 Xanthoperol is a known terpenoid from several conifers which include *Taxodium distichum*
485 (Kusumoto et al. 2014), *Juniperus formosa* (Kuo & Yu 1997), and *Calocedrus* (syn. *Libocedrus*)
486 *formosana* (Lin et al. 1975). Both compounds were accumulated in *Aloe viguieri* cell line D five
487 days after elicitation with or without acetic acid.

488 Our target was to induce biosynthesis of piperidine alkaloids. We did not find them in
489 our elicited suspension cultures but instead we had anthraquinones and also increased their
490 production. To answer why anthraquinones were observed but not piperidine alkaloids we can form
491 a hypothesis for this purpose. When *A. vera* roots were elicited with salicylic acid Lee et al. (2013)
492 observed that malonyl-CoA levels were lower than during the non-elicited situation. Acetyl-CoA
493 was not measurable at all. At the same time they reported elevated OKS transcript levels. In *in vitro*
494 environment OKS of *A. arborescens* has catalytic activity k_{cat} = 0.094 min^{-1} and K_m = 95 μM for
495 malonyl-CoA (Abe et al. 2005) and CPKS5 of *C. maculatum* has k_{cat} = 0.63 and K_m = 6.63 for
496 butyryl-CoA. When Leete's original hypothesis of coniine biosynthesis from one acetyl-CoA and
497 three malonyl-CoAs (1963, 1964) is taken into an account as a possible route to coniine formation,

498 this could offer an explanation as to why there are no piperidine alkaloids. Of these two
499 biosynthesis routes anthraquinones are more "powerful" in suspension culture environment due to
500 malonyl-CoA from available pool is channelled to that route. Apparently piperidine alkaloids also
501 need more specialized tissues as they do in case of poison hemlock (Fairbairn and Challen 1959;
502 Corsi and Biasci 1998).

503
Conclusion

504 We studied three *Aloe* species, *A. gariepensis*, *A. globuligemma* and *A. viguieri*, which were chosen
505 on the basis of previous reports on their alkaloid content. Micropropagation of *A. viguieri* on MS
506 containing 0.25 mg L^{-1} BA and 0.4 mg L^{-1} NAA led to five high-quality plantlets per mother rosette
507 within four weeks. Lower hormone levels produced fewer plantlets, whereas higher hormone
508 concentrations produced more but hyperhydrated plantlets. Here we report for the first time *N*-
509 methylconiine from *Aloe* and γ-coniceine from *A. globuligemma*. Explants of *A. viguieri* and *A.*
510 *gariepensis* generated callus on MS medium supplemented with 6.0 mg L^{-1} 2,4-D and with a
511 combination of 10.0 mg L^{-1} NAA and 0.2 mg L^{-1} BA, respectively. Additives (PVP, ascorbic acid
512 and adenine) were used in callus induction medium to reduce stress. Calli of both species have been
513 maintained for over two years on 1.0 mg L^{-1} 2,4-D on MS without the additives. *Aloe* callus can be
514 grown in suspension culture without any visible deterioration such as hyperhydration. Neither callus
515 cultures of *A. gariepensis* nor of *A. viguieri* contained hemlock alkaloids with or without elicitation
516 with salicylic acid or chitosan. Anthraquinones chrysophanol and chrysarobin were present in
517 examined cell and media extracts. Elicitation with salicylic acid increased the concentration of
518 chrysophanol.

519
Author contributions

520 H.H., S.T.H., T.S.-L., H.R. planned the experiments; H.H., T.S.-L. performed the experiments;
521 H.H., S.T.H., T.S.-L., H.R. analysed the data; H.H., S.T.H., H.R. wrote the paper.

522
Acknowledgements

523 We thank Professor Teemu Teeri for discussions, Kaarina Viljanen for GC-MS analysis, and
524 Docent Riitta Puupponen-Pimiä and Professor Riitta Julkunen-Tiitto for commenting on the
525 manuscript. This research was supported by funding from VTT, Academy of Finland (grant 138808
526 to HR), Finnish Doctoral Program in Plant Science, Oskar Öflunds Stiftelse, Eteläsuomalaisten
527 ylioppilaiden säätiö and the Otto A. Malm Foundation (all to HH).

528 ## References

529 Abadi HD, Kaviani B (2010) *In vitro* proliferation of an important medicinal plant *Aloe* - A method
530 for rapid production. Aust J Crop Sci 4:216-222

531 Abe I, Oguro S, Utsumi Y, Sano Y, Noguchi H (2005) Engineered biosynthesis of plant
532 polyketides: chain length control in an octaketide-producing plant type III polyketide synthase. J
533 Am Chem Soc 127:12709-12716

534 Abrie AL, van Staden J (2001) Micropropagation of the endangered *Aloe polyphylla*. Plant Growth
535 Regul 33:19-23

536 Baek J-H, Kim M-U, Kang T-S, Hur W, Lee S-Y (2009) Polysaccharide characteristics from hot
537 water extracts of *Aloe saponaria* callus. KSBB Journal 24:59-64

538 Baldi A, Srivastava AK, Bisaria VS (2009) Fungal elicitors for enhanced production of secondary
539 metabolites in plant cell suspension cultures. In: Varma A, Kharkwal AC (ed) Soil Biology 18 -
540 Symbiotic Fungi. Springer-Verlag, Berlin, pp 373-380.

541 Barringer SA, Mohamed-Yasseen Y, Splittstoesser WE (1996) Micropropagation of endangered
542 *Aloe juvenna* and *A. volkensii* (Aloaceae). Haseltonia 43-45

543 Bedini C, Caccia R, Triggiani D, Mazzucato A, Soressi GP, Tiezzi A (2009) Micropropagation of
544 *Aloe arborescens* Mill: A step towards efficient production of its valuable leaf extracts showing
545 antiproliferative activity on murine myeloma cells. Plant Biosystems 143:233-240

546 Blitzke T, Porzel A, Masoud M, Schmidt J (2000) A chlorinated amide and piperidine alkaloids
547 from *Aloe sabaea*. Phytochemistry 55:979-982

548 Carew DP, Bainbridge T (1976) Biotransformations with plant tissue cultures. Lloydia 39:147-149.

549 Chukwujekwu JC, Fennell CW, van Staden J (2003) Optimisation of the tissue culture protocol for
550 the endangered *Aloe polyphylla*. S Afr J Bot 68:424-429

551 Corsi G, Biasci D (1998) Secretory Structures and localization of alkaloids in *Conium maculatum*
552 L. (Apiaceae). Annals of Botany 81:157-162

553 Cromwell BT (1956) The separation, micro-estimation and distribution of alkaloids of hemlock
554 (*Conium maculatum* L.). Biochem J 64:259-266

555 Dawidar A-AM, Abdel-Mogib M, El-Sherbiny IM, Saeed AA (2012) Terpenes, steroids and
556 shikiimates in *Calligonum comosum* L. Mansoura Journal of Chemistry 39:91-105

557 Deepika N, Eganathan P, Sujanapal P, Parida A (2013) Chemical composition of *Syzygium*
558 *benthamianum* (Wt. ex Duthie) Gamble essential oil - an endemic and vulnerable tree species.
559 Journal of Essential Oil-Bearing Plants 16:289-293

560 Dring JV, Nash RJ, Roberts MF, Reynolds T (1984) Hemlock alkaloids in *Aloes*. Occurrence and
561 distribution of γ-coniceine. Planta Med 50:442-443

562 Drummond RB, Gelfand M, Mavi S (1975) Medicinal and other uses of succulents by the
563 Rhodesian African. Excelsa 5, 51-56

564 Fairbairn JW, Challen SB (1959) The alkaloids of hemlock (*Conium maculatum* L.). Distribution in
565 relation to the development of the fruit. Biochem J 72:556-561

566 Furuya T, Kojima H, Syono K (1971) Regulation of nicotine biosynthesis by auxins in tobacco
567 callus tissues. Phytochemistry 10:1529-1532

568 Gantait S, Mandal N, Das PK (2011) *In vitro* accelerated mass propagation and *ex vitro* evaluation
569 of *Aloe vera* L. with aloin content and superoxide dismutase activity. Nat Prod Res 25:1370-1378.

570 Giseke AL (1827) Extract from several notebooks of the archives of the society of pharmacy of
571 northern Germany. J Pharm Sci Accessoires 13: 366-367

572 Grace OM (2011) Current perspectives on the economic botany of the genus *Aloe* L.
573 (Xanthorrhoeaceae). S Afr J Bot 4:980-987

574 Groenewald EG, Koeleman A, Wessels DCJ (1975) Callus formation and plant regeneration from
575 seed tissue of *Aloe pretoriensis* Pole Evans. Z Pflanzenphysiol 75:270-272

576 Han C, Wang J, Li Y, Cui Y (2013) *In vitro* antimicrobial activity and effect on *E. coli* integrity of
577 cinnamon essential oil and rhubarb ethanol extract. Food Sci Technol Res 19:1155-1163

578 Hashembadi D, Kaviani B (2008) Rapid micro-propagation of *Aloe vera* L. via shoot multiplication.
579 Afr J Biotechnol 7: 1899-1902

580 Holstege DM, Galey FD, Johnson B, Seiber JN (1996) Determination of alkaloid exposure in a
581 model ruminant (goat) using a multiresidue screening method. J Agric Food Chem 44:2310-2315

582 Hotti H, Seppänen-Laakso T, Arvas M, Teeri TH, Rischer H (2015) Polyketide synthases from
583 poison hemlock (*Conium maculatum* L.). FEBS J. doi: 10.1111/febs.13410

584 Häkkinen ST, Moyano E, Cusidó RM, Palazón J, Piñol MT, Oksman-Caldentey K-M (2005)
585 Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing heterologous
586 hyoscyamine-6β-hydroxylase. J Exp Bot 56:2611-2618

587 Ibrahim MA, Koorbanally NA, Islam S (2013) *In vitro* anti-oxidative activities and GC-MS analysis
588 of various solvent extracts of *Cassia sangueana* parts. Acta Poloniae Pharmaceutica – Drug
589 Research 70:709-719.

590 Ishikawa A, Yoshihara T, Nakamura K (1994) Jasmonate-inducible expression of a potato cathepsin
591 D inhibitor-GUS gene fusion in tobacco cells. Plant molecular biology 26:403-414

592 Ivanova M, van Staden J (2008) Effect of ammonium ions and cytokinins on hyperhydricity and
593 multiplication rate of in vitro regenerated shoots of *Aloe polyphylla*. Plant Cell Tiss Organ Cult
594 92:227-231

595 Ivanova M, van Staden J (2011) Influence of gelling agent and cytokinins on the control of
596 hyperhydricity in *Aloe polyphylla*. Plant Cell Tiss Organ Cult 104:13-21

597 Kawai K, Beppu H, Koike T, Fujita K, Marunouchi T (1993) Tissue culture of *Aloe arborescens*
598 Miller var. *natalensis* Berger. Phytother Res 7:S5-S10

599 Knobloch K-H, Berlin J (1980) Influence of medium composition on the formation of secondary
600 compounds in cell suspension cultures of *Catharanthus roseus* (L.) G. Don. Z Naturforsch 35:551-
601 556

602 Kuo Y-H, Yu M-T (1997) 6-Oxoferruginol and 6α-acetoxyferruginol, new abietane type diterpenes
603 from the heartwood of *Juniperus formosa*. Journal of Natural Products 60:648-650

604 Kusumoto N, Aburai N, Ashitani T, Takahashi K, Kimura K-I (2014) Pharmacological prospects of
605 oxygenated abietane-type diterpenoids from *Taxodium distichum* cones. Advances in Biological
606 Chemistry 4:2162-2191

607 Lee YS, Ju HK, Kim YJ, Lim T-G, Uddin MR, Kim YB, Baek H, Kwon SW, Lee KW, Seo HS,
608 Park SU, Yang T-J (2013) Enhancement of anti-inflammatory activity of *Aloe vera* adventitious
609 root extracts through the alteration of primary and secondary metabolites via salicylic acid
610 elicitation. PLoS ONE 8:e82479. doi:10.1371/journal.pone.0082479

611 Leete E (1963) The biosynthesis of coniine from four acetate units. J Am Chem Soc 85:3523-3524

612 Leete E (1964) Biosynthesis of the hemlock alkaloids. The incorporation of acetate-1-C^{14} into
613 coniine and conhydrine. J Am Chem Soc 86:2509-2513

614 Leete E (1970) The biosynthesis of coniine from octanoic acid in hemlock plants (*Conium*
615 *maculatum*). J Am Chem Soc 92:3835

616 Leete E, Adityachaudhury N (1967) Biosynthesis of the hemlock alkaloids – II. The conversion of
617 γ-coniceine to coniine and Ψ-conhydrine. Phytochemistry 6:219-223

618 Leete E, Olson JO (1970) 5-Oxo-octanoic acid and 5-oxo-octanal, precursors of coniine. J Chem
619 Soc D:1651

620 Leete E, Olson JO (1972) Biosynthesis and metabolism of the hemlock alkaloids. J Am Chem Soc
621 94:5472-5477

622 Leshem B, Werker E, Shalevi DP (1988) The effect of cytokinins on vitrification in melon and
623 carnation. Ann Bot 62: 271-276

624 Liao Z, Chen M, Tan F, Sun X, Tang K (2004). Micropropagation of endangered Chinese aloe.
625 Plant Cell Tiss Organ Cult 76: 83–86

626 Lin YT, KUO YH, Chang BH (1975) Studies on the extractive constituents of the bark of
627 *Libocedrus formosana* Florin. II. Journal of the Chinese Chemical Society (Taipei, Taiwan) 22:331-
628 334.

629 Liu Y, Takatsuki H, Yoshikoshi A, Wang B, Sakanishi A (2003) Effects of ultrasound on the
630 growth and vacuolar H^+-ATPase activity of *Aloe arborescens* callus cells. Colloid Surface B
631 32:105-116

632 Loots DT, van der Westhuizen FH, Botes L. 2007. *Aloe ferox* leaf gel phytochemical content,
633 antioxidant capacity, and possible health benefits. J Agric Food Chem 55:6891-6896

634 Lopez T, de la Torre M Cid M (2004) An efficient TLC method for analysis of gamma-coniceine
635 and coniine in *Conium maculatum* L. foliage. J Planar Chromatogr - Mod TLC 17:218-223

636 Malik EM, Müller CE (2016) Anthraquinones as pharmaceutical tools and drugs. Medicinal
637 Research Reviews 36:705-748

638 Meier P, Hotti H, Rischer H (2015) Elicitation of furanocoumarins in poison hemlock (*Conium*
639 *maculatum* L.) cell culture. Plant Cell Tiss Organ Cult. doi: 10.1007/s11240-015-0847-7

640 Mizuuchi Y, Shi, S-P, Wanibuchi K, Kojima A, Morita H, Noguchi H, Abe I (2009) Novel type III
641 polyketide synthases from *Aloe arborescens*. FEBS J 276:2391-2401

642 Mody NV, Henson R, Hedin PA, Kokpol DH (1976) Isolation of the insect paralysing agent coniine
643 from *Sarracenia flava*. Experimentia 32:829-830

644 Morris P (1986) Regulation of produce synthesis in cell cultures of *Catharanthus roseus*. III.
645 Alkaloid metabolism in cultured leaf tissue and primary callus. Planta Med 121:127-132.

646 Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue
647 cultures. Physiol Plant 15(3):473-497

648 Nash RJ, Beaumont J, Veitch NC, Reynolds T, Benner J, Hughes CNG, Dring JV, Bennett RN,
649 Dellar JE (1992) Phenylethylamine and piperidine alkaloids in *Aloe* species. Planta Med 58:84-87

650 Nétien G, Combet J (1971) Etude comparative dans la composition chimique des cultures de tissus
651 de *Conium maculatum* cultivées *in vitro*. I. Variation des substances azotees. Paris Soc Biol Compt
652 Rend 165:103-107

653 Parry O, Matambo C (1992) Some pharmacological actions of aloe extracts and *Cassia abbreviata*
654 on rats and mice. Cent Afr J Med 38:409-414

655 Ptak A, El Tahchy A, Skrzypek E, Wójtowicz T, Laurain-Mattar D (2013) Influence of auxins on
656 somatic embryogenesis and alkaloid accumulation in *Leucojum aestivum* callus. Cent Eur J Biol
657 8:591-599

658 Racchi ML (1988) Using *in vitro* culture to study the biosynthesis of secondary products in *Aloe*
659 *ferox*. Riv Agr Subtrop Trop 82:707-714

660 Ramsay MM, Gratton J (2000) Propagation of *Aloe polyphylla* Schönl. ex Pillans from callus
661 derived from leaf tissue. Bot Gardens Micropropag News 2:61-64

662 Rathore MS, Chikara J, Shekhawat NS (2011) Plantlet regeneration from callus cultures of selected
663 genotype of *Aloe vera* L. An ancient plant for modern herbal industries. Appl Biochem Biotechnol
664 163:860-868

665 Rischer H, Orešič M, Seppänen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van
666 Montagu MCE, Inzé D, Oksman-Caldentey K-M, Goossens A (2006) Gene-to-metabolite networks
667 for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc Natl Acad Sci USA
668 103:5614-5619

669 Reynolds T (1997) Comparative chromatographic patterns of leaf exudate components from *Aloe*
670 section *Pachydendron* Haw. Botanical Journal of the Linnean Society 125:45-70

671 Reynolds T (2005) Molecules of interest – Hemlock alkaloids from Socrates to poison aloes.
672 Phytochemistry 66:1399-1406

673 Roberts MF (1971) The formation of γ-coniceine from 5-ketooctanal by a transaminase of *Conium*
674 *maculatum*. Phytochemistry 10: 3057-3060

675 Roberts MF (1975) γ-Coniceine reductase in *Conium maculatum*. Phytochemistry 14: 2393-2397

676 Roberts MF (1977) Purification and properties of L-alanine:5-ketooctanal aminotransferase from
677 *Conium maculatum*. Phytochemistry 16:1381-1386

678 Roberts MF (1981) Enzymatic synthesis of γ-coniceine in *Conium maculatum* chloroplasts and
679 mitochondria. Plant Cell Rep 1:10-13

680 Roy SC, Sarkar A (1991) *In vitro* regeneration and micropropagation of *Aloe vera* L. Sci Hortic
681 47:107-113

682 Sathyanarayana BN, Varghese DB (2007) Plant tissue culture: Practices and new experimental
683 protocols. I.K. International Publishing House Pvt. Ltd., New Delhi, India

684 Sévon N, Varjonen T, Hiltunen R, Oksman-Caldentey K-M (1992) Effect of sucrose, nitrogen and
685 copper on the growth and alkaloid production of transformed root cultures of *Hyoscyamus muticus*.
686 Planta Medica 58:609-610.

687 Smith T, Smith H (1851) On aloin: the cathartic principles of aloes. Mon J Med Sci 12:127-131

688 Stalman M, Koskamp A-M, Luderer R, Vernooy JHJ, Wind JC, Wullems GJ, Croes AF (2003)
689 Regulation of anthraquinone biosynthesis in cell cultures of *Morinda citrifolia*. J Plant Physiol
690 160:607-614

691 Steglich W, Fugmann B, Lang-Fugmann (eds.) (2000) RÖMPP Encyclopedia natural products. 1st
692 edition. Georg Thieme Verlag, Stuttgart Germany. 748 p.

693 Tabata M, Hiraoka N (1976) Variation of alkaloid production in *Nicotiana rustica* callus cultures.
694 Physiol Plant 38:19-23

695 Tatsuo K, Amano M (1985) Callus formation in stamens of *Aloe bellatura*. Ann Tsukuba Bot Gard
696 3:19-26

697 Thomas P, Mythili JB, Shivashankara KS (2000) Explant, medium and vessel aeration affect the
698 incidence of hyperhydricity and recovery of normal plantlets in triploid watermelon. J Hort Sci
699 Biotech 75: 19-25

700 Tsay H-S (1998) Effects of medium composition at different recultures of on vitrification of
701 carnation (*Dianthus caryophyllus*) *in vitro* shoot proliferation. Acta Hort 461: 243-249

702 Umetrics (2011) Design for experiments. Umetrics academy: Training in multivariate technology,
703 IID1066. Umetrics AB, Malmö, Sweden

704 Velcheva M, Faltin Z, Vardi A, Hanania U, Eshdat Y, Dgani O, Sahar N, Perl A (2010) *Aloe vera*
705 transformation: the role of Amberlite XAD-4 resin and antioxidants during selection and
706 regeneration. In Vitro Cell Dev-Pl 46:477-484

707 Viljoen AM, van Wyk B-E, van Heerden FR (1998). Distribution and chemotaxonomic significance
708 of flavonoids in *Aloe* (Asphodelaceae). Plant Syst Evol 211:31-42

709 Yagi A, Shoyama Y, Nishioka I (1983) Formation of tetrahydroanthracene glucosides by callus
710 tissue of *Aloe saponaria*. Phytochemistry 22:1483-1484

711 Yagi A, Hine N, Asai M, Nakazawa M, Tateyama Y, Okamura N, Fujioka T, Mihashi K,
712 Shimomura K (1998) Tetrahydroanthracene glucosides in callus tissue from *Aloe barbadensis*
713 leaves. Phytochemistry 47:1267-1270

714 Yang Z, Peng Q, Yang M, Wang D (2006) Analysis of *Valeriana jatamansii* volatile components
715 by simultaneous distillation and extraction/GC-MS methods. Zhongguo Yaoxue Zazhi 41:74-75

716 Zhou Y, Wu H, Yao Q, Wang S, Wei C, Ma X (2014) Changes of aroma components in 'Keitt'
717 mango during the postharvest period. Agricultural Biotechnology 3:14-17

718 Online resources

719 Online Resource Table 1: *A. viguieri* plantlet formation with different BA, NAA combinations and
720 treatment durations.

721 Online Resource Table 2: *A. viguieri* plantlet formation with different BA and NAA combinations.
722 Results were recorded after 4, 5 and 6 weeks.

723 Tables

724 **Table 1.** Hemlock alkaloids reported from *Aloe* (Dring et al. 1984; Nash et al. 1992; Blitzke et al.
725 2000).

Species	Alkaloids
A. ballyii Reynolds	γ-coniceine, conhydrinone
A. deltoideodonta Baker	γ-coniceine, a trace of pseudoconhydrine
A. descoingsii Reynolds	coniine, conhydrine
A. gariepensis Pillans	γ-coniceine, conhydrine
A. globuligemma Pole Evans	coniine, conhydrine
A. gracilicaulis Reynolds & P.R.O. Bally	γ-coniceine
A. ibitiensis Perrier	γ-coniceine
A. krapholiana Marloth.	coniine, conhydrine
A. ortholopha Christian & Milne-Redh.	coniine, conhydrine
A. ruspoliana Baker	γ-coniceine
A. sabaea Schweinf. (syn. *A. gillilandii* Reynolds)	γ-coniceine, coniine, *N,N*-dimethylconiine
A. viguieri Perrier	coniine, a trace of γ-coniceine

726

727 **Table 2.** Fresh weight of selected callus lines (each n = 12) monitored for a period of three weeks.

		Callus weight (fresh weight)								
		Week 0	Week I		Week II		Week III[1]		Average increase/Week	
Species	Callus line	Weight (g)	Weight (g) (Increase)	Increase (%)	Weight (g) (Increase)	Increase (%)	Weight (g) (Increase)	Increase (%)	Weight (g)	Increase (%)
A. gariepensis	A	0.15±0.05	0.17±0.06 ($0.02±0.01_{ab}$)	17	0.24±0.08 ($0.07±0.03_{a}$)	40	0.31±0.1 ($0.08±0.03_{a}$)	33	0.06±0.02	30
	B	0.10±0.05	0.11±0.05 ($0.01±0.01_{a}$)	13	0.14±0.07 ($0.03±0.02_{a}$)	28	0.17±0.09 ($0.03±0.03_{a}$)	22	0.02±0.02	21
	C	0.13±0.05	0.16±0.07 ($0.03±0.03_{ab}$)	25	0.24±0.12 ($0.08±0.05_{a}$)	45	0.33±0.20 ($0.11±0.09_{a}$)	40	0.07±0.06	37
A. viguieri	D	0.12±0.05	0.200±0.08 ($0.08±0.04_{b}$)	65	0.36±0.15 ($0.17±0.08_{b}$)	90	0.61±0.24 ($0.24±0.11_{b}$)	67	0.16±0.08	74
	E	0.13±0.03	0.18±0.04 ($0.04±0.01_{ab}$)	34	0.25±0.08 ($0.08±0.03_{a}$)	47	0.35±0.12 ($0.08±0.09_{a}$)	45	0.07±0.05	42
	F	0.10±0.03	0.12±0.06 ($0.03±0.04_{ab}$)	27	0.019±0.11 ($0.06±0.06_{a}$)	51	0.29±0.25 ($0.10±0.16_{a}$)	38	0.06±0.08	39

728 Statistically significant differences in weight. Means not sharing the same letter are significantly different (Tukey HSD, $p < 0.05$). Comparisons were made
729 between the lines at a particular time-point. [1]Results analysed with One-way ANOVA, except for cases in which the homogeneity of group variances did not
730 fulfil the criteria of ANOVA (Levene $p<0.05$); in these cases Tamhane's T2 was used.

731

732 **Table 3.** The presence of hemlock alkaloid base peak fragments in elicited samples.

Cell line	Elicitor	Days after elicitation	Time point 4.55 min Cells m/z	4.55 min Liquid m/z	5.05 min Cells m/z	5.05 min Liquid m/z	5.25 min Cells m/z	5.25 min Liquid m/z
Aloe gariepensis line C	No elicitor	1	84[a]	84[a]	98[a]	98[a]	-	97[a]
		3	84[a]	84[a]	-	98[a]	-	97[a]
		5	84[a]	84[a]	-	98[a]	-	97[a]
	Acetic acid	1	84[a]	84[a]	-	-	-	97[a]
		3	84[a]	-	-	98[a]	-	97[a]
		5	84[a]	84[a]	-	-	-	97[a]
	Chitosan (110 mg L^{-1})	1	-	84[a]	-	-	97[a]	97[a]
		3	84[a]	84[a]	-	-	-	97[a]
		5	-	84[a]	98[a]	-	97[a]	97[a]
	Salicylic acid (138 mg L^{-1})	1	84[a]	84[a]	98[a]	98[a]	97[a]	97[a]
		3	-	84[a]	-	98[a]	-	97[a]
		5	-	84[a]	-	98[a]	-	97[a]
Aloe viguieri line D	No elicitor	1	-	84[a]	-	-	-	97[a]
		3	84[a]	84[a]	-	98[a]	-	97[a]
		5	-	84[a]	-	98[a]	-	97[a]
	Acetic acid	1	-	84[a]	-	98[a]	-	97[a]
		3	-	84[a]	-	98[a]	97[a]	97[a]
		5	-	84[a]	98[a]	98[a]	97[a]	97[a]
	Chitosan (110 mg L^{-1})	1	84[a]	84[a]	-	98[a]	-	97[a]
		3	84[a]	84[a]	-	98[a]	-	97[a]
		5	-	84[a]	-	98[a]	97[a]	97[a]
	Salicylic acid (138 mg L^{-1})	1	-	84[a]	-	98[a]	97[a]	97[a]
		3	84[a]	84[a]	-	98[a]	-	97[a]
		5	84[a]	84[a]	-	98[a]	97[a]	97[a]

733 Explanation: - not present. [a] Ion present in trace amounts.

734 **Table 4.** Secondary metabolites found in non-elicited and elicited suspension cultures of cell lines

735 C and D.

Cell line	Elicitor	Day of collection	R_t (min)	CAS No	Name	Cell extract Match (%)	Cell extract Area (%)	Medium extract Match (%)	Medium extract Area (%)
Aloe gariepensis cell line C	No elicitor	1	19.443	491-59-8	Chrysarobin	97	0.20	-	-
			19.649	481-74-3	Chrysophanol	97	0.13	-	-
		3	19.442	491-59-8	Chrysarobin	97	0.83	-	-
		5	19.442	491-59-8	Chrysarobin	95	0.53	-	-
			19.649	481-74-	Chrysophanol	97	0.24	-	-

				3					
	Acetic acid	1	19.442	491-59-8	Chrysarobin	97	0.47	-	-
			19.649	481-74-3	Chrysophanol	98	0.21	-	-
		3	19.442	491-59-8	Chrysarobin	97	0.49	-	-
			19.649	481-74-3	Chrysophanol	97	0.16	-	-
		5	19.443	491-59-8	Chrysarobin	97	0.59	-	-
			19.649	481-74-3	Chrysophanol	98	0.25	-	-
	Chitosan (110 mg L^{-1})	1	19.442	491-59-8	Chrysarobin	97	0.67	-	-
			19.649	481-74-3	Chrysophanol	98	0.25	-	-
		3	19.443	491-59-8	Chrysarobin	97	0.64	-	-
			19.649	481-74-3	Chrysophanol	98	0.31	-	-
		5	19.443	491-59-8	Chrysarobin	96	0.33	-	-
			19.649	481-74-3	Chrysophanol	98	0.22	-	-
	Salicylic acid (138 mg L^{-1})	1	19.470	491-59-8	Chrysarobin	97	0.24	94	1.18
			19.691	481-74-3	Chrysophanol	98	0.4	-	-
			21.049	-	Unknwon compound 2	-	0.87	-	-
			21.318	-	Unknown compound 3	-	-	-	1.86
			25.014	-	Unknown compound 4	-	0.67	-	-
		3	19.443	491-59-8	Chrysarobin	97	0.47	92	1.27
			19.649	481-74-3	Chrysophanol	98	0.14	-	-
			21.318	-	Unknown compound 3	-	-	-	2.22
			24.952	-	Unknown compound 4	-	1.06	-	-
		5	19.449	491-59-8	Chrysarobin	97	1.01	95	1.32
			19.649	481-74-3	Chrysophanol	98	0.29	-	-
			21.325	-	Unknown compound 3	-	-	-	2.39
			24.959	-	Unknown compound 4	-	1.57	-	-
Aloe viguieri cell line D	No elicitor	1	-	-	No compounds	-	-	-	-

	3	19.442	491-59-8	Chrysarobin	96	0.57	-	-
		19.649	481-74-3	Chrysophanol	97	0.30	-	-
	5	19.656	481-74-3	Chrysophanol	96	0.09	-	-
		23.793	15714-13-3	*p*-Menth-8(10)-en-9-ol, *cis*-	93	0.19	-	-
Acetic acid	1	19.649	481-74-3	Chrysophanol	96	0.22	-	-
	3	19.656	481-74-3	Chrysophanol	98	0.52	-	-
	5	19.518	-	Unknown compound 1	-	0.96	-	-
		27.965	57377-89-6	Xanthoperol	92	1.64	-	-
Chitosan (110 mg L^{-1})	1	19.512	-	Unknown compound 1	-	1.27	-	-
	3	19.449	491-59-8	Chrysarobin	97	0.72	-	-
		19.656	481-74-3	Chrysophanol	96	0.30	-	-
	5	19.456	491-59-8	Chrysarobin	97	0.77	95	1.26
		19.663	481-74-3	Chrysophanol	98	0.40	-	-
Salicylic acid (138 mg L^{-1})	1	19.456	491-59-8	Chrysarobin	97	1.92	-	-
	3	19.463	491-59-8	Chrysarobin	97	1.17	95	1.28
		19.670	481-74-3	Chrysophanol	98	0.65	-	-
		21.325	-	Unknown compound 3	-	-	-	2.15
	5	19.463	491-59-8	Chrysarobin	97	1.00	95	1.19
		19.670	481-74-3	Chrysophanol	98	0.55	-	-
		21.325	-	Unknown compound 3	-	-	-	2.07
		24.986	-	Unknown compound 4	-	1.93	-	-

Table 4. Mass spectra of unknown compounds from elicited suspension cultures of cell lines C (*Aloe gariepensis*) and D (*Aloe viguieri*).

Name	R_t (min)	Mass spectra
Unknown compound 1	19.518	305 (M^+), 290, 276, 262, 248, 234, 220, 208, 199, 194, 180, 168, 154, 149, 140, 124, 112, 98 (base peak), 85, 79, 69, 67,

		57, 55, 44, 41
Unknown compound 2	21.049	239 (M^+, base peak), 224, 210, 196, 180, 165, 152, 139, 126, 115, 104, 96, 90, 83, 77, 69, 63, 57, 51, 41
Unknown compound 3	21.325	258 (M^+, base peak), 240, 229, 225, 212, 201, 197, 183, 173, 165, 155, 141, 127, 115, 99, 91, 85, 77, 71, 65, 57, 43
Unknown compound 4	24.952	257 (M^+, base peak), 239, 228, 224, 210 196, 181, 173, 165, 152, 139, 128, 115, 105, 98, 90, 83, 77, 69, 63, 57, 43

739 **Table 6.** Characteristic GC-MS fragments for relevant piperidine alkaloids (Holstege et al. 1996;
740 Palisade Complete 600K Mass Spectral Library) and anthraquinones (NIST Mass Spectral Search
741 2.0 Program).

Compound	R_t (min)	Match (%)	Note	Characteristic ions
Coniine	4.55	86%	*a*	127 (M+), 126, 98, 84 (base peak), 70, 56
γ-Coniceine	5.25	90%	*b*	125 (M+), 124, 110, 97 (base peak), 82, 70
N-Methylconiine	5.05		*b*	141 (M+), 112, 99, 98 (base peak), 70
Conhydrine	7.40	78%	*a*	143 (M+), 114, 96, 84 (base peak), 67, 56, 41
Chrysarobin	19.44	97%	*c*	240 (M+, base peak), 225, 211, 197, 194, 181, 165, 152, 139, 127, 120, 115, 111, 105, 97, 89, 82, 76, 74, 69, 96, 53, 51
Chrysophanol	19.67	98%	*c*	254 (M+, base peak), 237, 226, 209, 197, 180, 169, 155, 152, 141, 127, 115, 105, 99, 90, 76, 65, 63, 57, 51, 43

742 [a]Identified by comparison of retention time and mass spectra with reference material. [b]Identified by
743 comparison of spectra (Holstege et al. 1996; Palisade Complete 600K Mass Spectral Library).
744 [c]Identified by comparison of spectra (NIST Mass Spectral Search 2.0 Program).

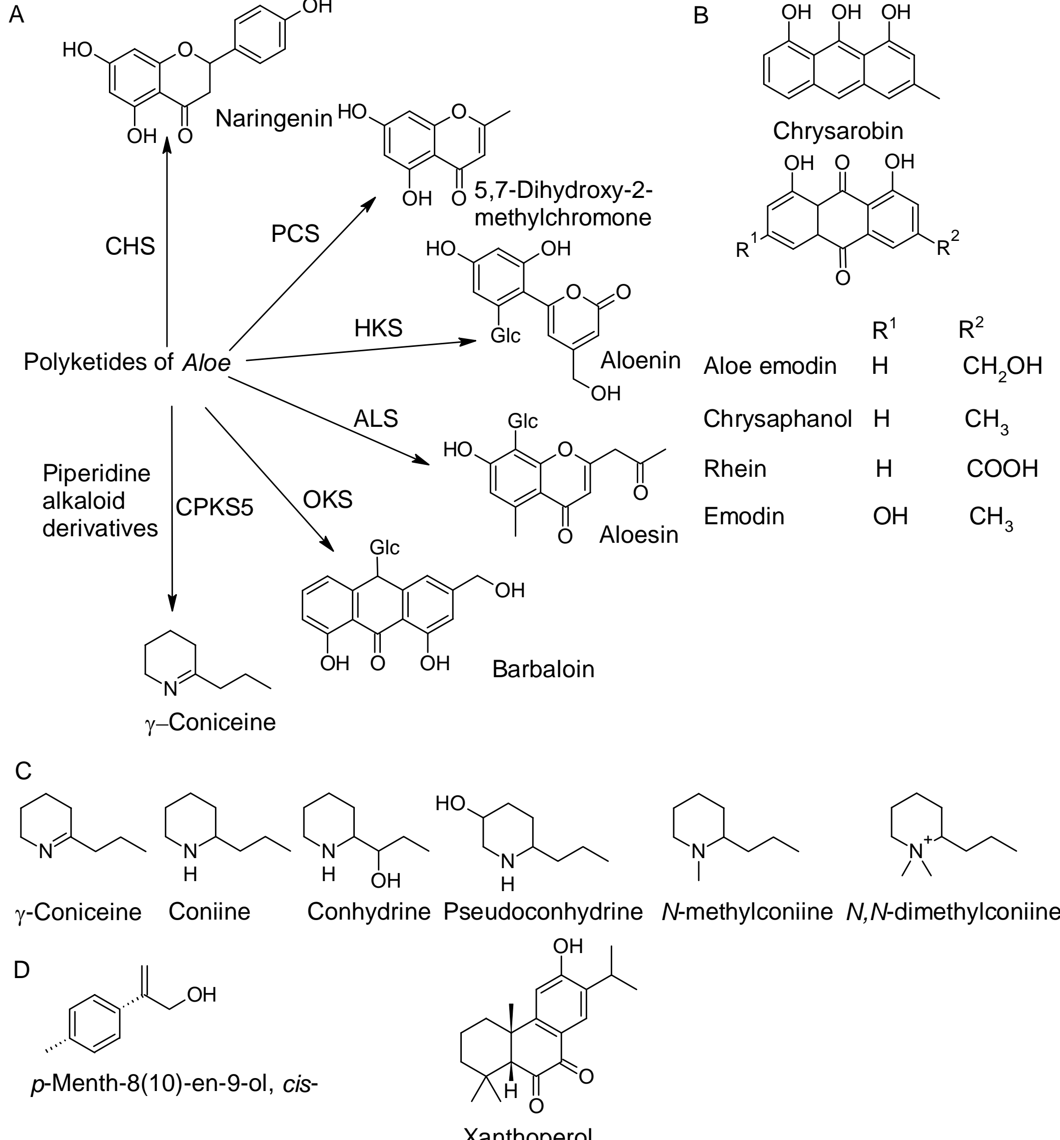
745 **Table 7.** Piperidine alkaloids in *A. gariepensis*, *A. globuligemma* and *A. viguieri* plants and cell
746 cultures. Alkaloid is brackets if there is trace of it.

Species	Sample	R_t (min)	Match (%)	CAS No.	Name	Are (%) of extract
A. gariepensis[d]	leaf, *in vitro*	-	-	-	no piperidine alkaloids	-
	root, *in vitro*	4.55	*a*	458-88-8	(Coniine)	trace
		5.05	*a*	35305-13-6	(*N*-Methylconiine)	trace
A. globuligemma	leaf, *in vitro*	5.85[c]	*b*	458-88-8	Coniine	0.53
		6.40[c]	*b*	35305-13-6	*N*-Methylconiine	10.93
		6.50[c]	*b*	1604-01-9	γ-Coniceine	4.24
	root, *in vitro*	-	-	-	no piperidine alkaloids	-
A. viguieri[e]	leaf, *in vitro*	4.55	*b*	458-88-8	Coniine	4.98
		5.05	*b*	35305-13-6	*N*-Methylconiine	1.48
		5.25	90	1604-01-9	γ-Coniceine	1.89

root, *in vitro*	4.55	86	458-88-8	Coniine	0.26
	5.05	*a*	35305-13-6	(*N*-Methylconiine)	trace
	5.25	*a*	1604-01-9	(γ-Coniceine)	trace

747 [a] Identified on the basis of the base peak fragment present (coniine *m/z* 84, γ-coniceine *m/z* 97, *N*-
748 methylconiine *m/z* 98); [b] Identified by comparison to known spectra (Holstege et al. 1996; Palisade
749 Complete 600K Mass Spectral Library); [c] Different GC-MS analysis conditions. [d] Cell lines A-C of
750 *A. gariepensis* did not contain piperidine alkaloids. [e] Cell lines D-F of *A. viguieri* did not contain
751 piperidine alkaloids.

752 ## Figures

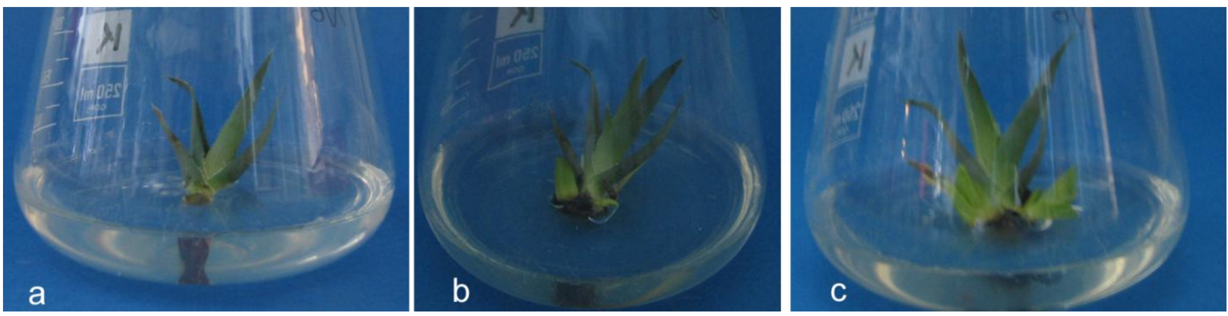


753

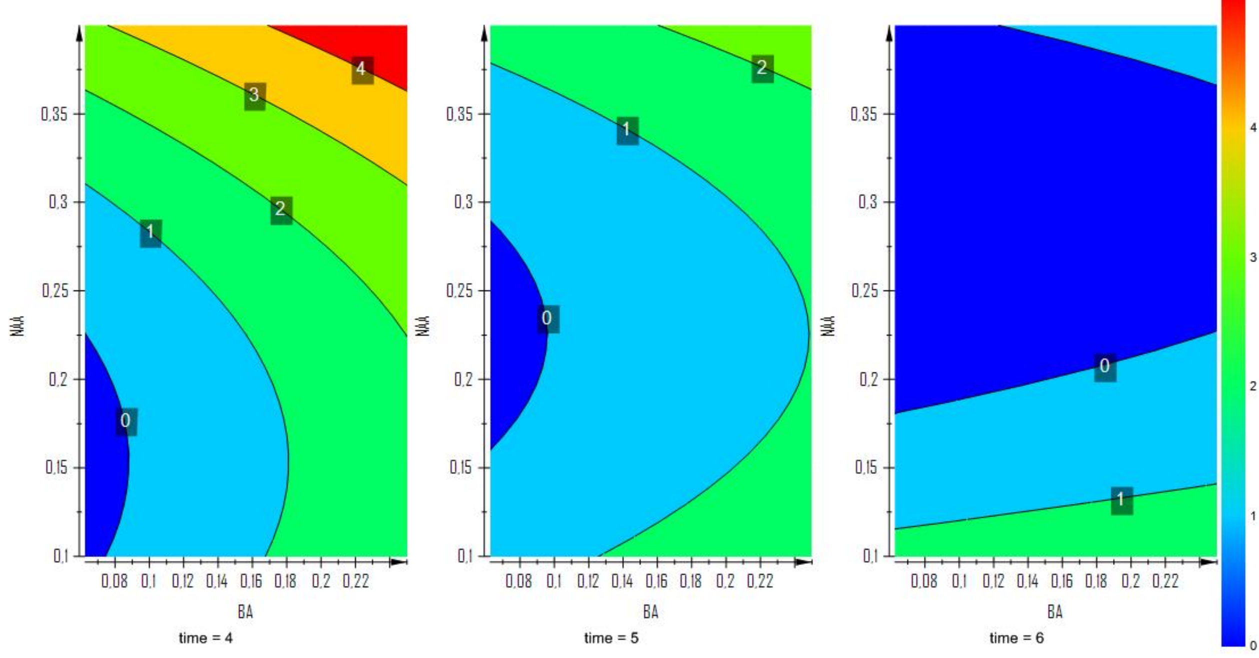
754 **Fig. 1 A** Known polyketide derivatives in *Aloe*. CHS chalcone synthase, PCS pentaketide chromone
755 synthase, HKS hexaketide synthase, ALS aloesin synthase, OKS octaketide synthase, CPKS5

756 *Conium* polyketide synthase 5. **B** A few anthraquinones from *Aloe*. **C** The structures of known
757 piperidine alkaloids from *Aloe* (Dring et al. 1984; Nash et al. 1992; Blitzke et al. 2000). **D** Minor
758 compounds found from *Aloe* suspension cultures.

759

760 

761 **Fig. 2** Typical development of a *A. viguieri* plant after **A** 2 weeks, **B** 4 weeks and **C** 6 weeks.



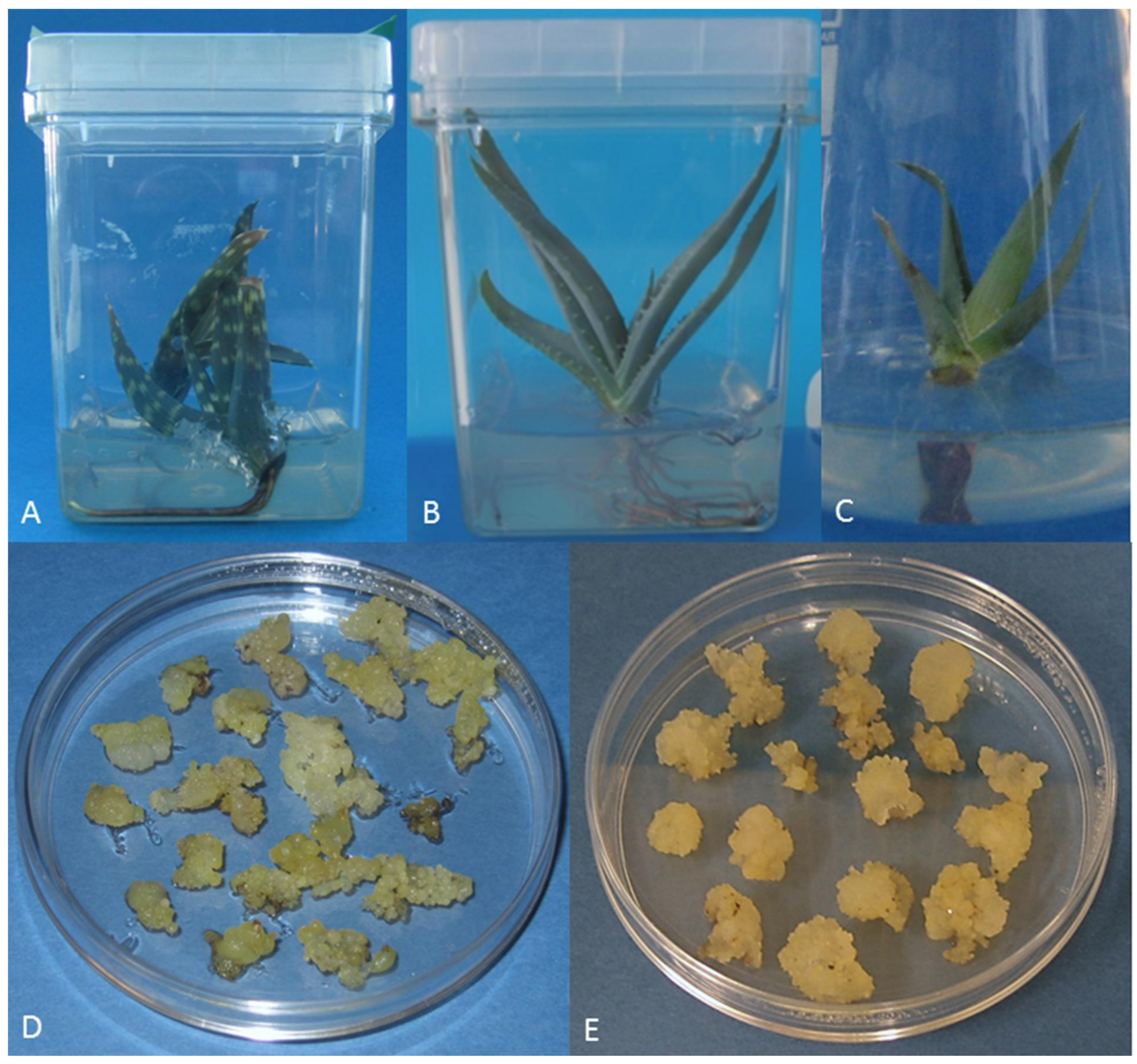
762

763 **Fig. 3** Contour plot of *A. viguieri* micropropagation (factors: BA, NAA and time; response: plantlet

764 number). Altogether 17 experiments were performed. Model parameters: R^2 = 0.952, Q^2 = 0.821

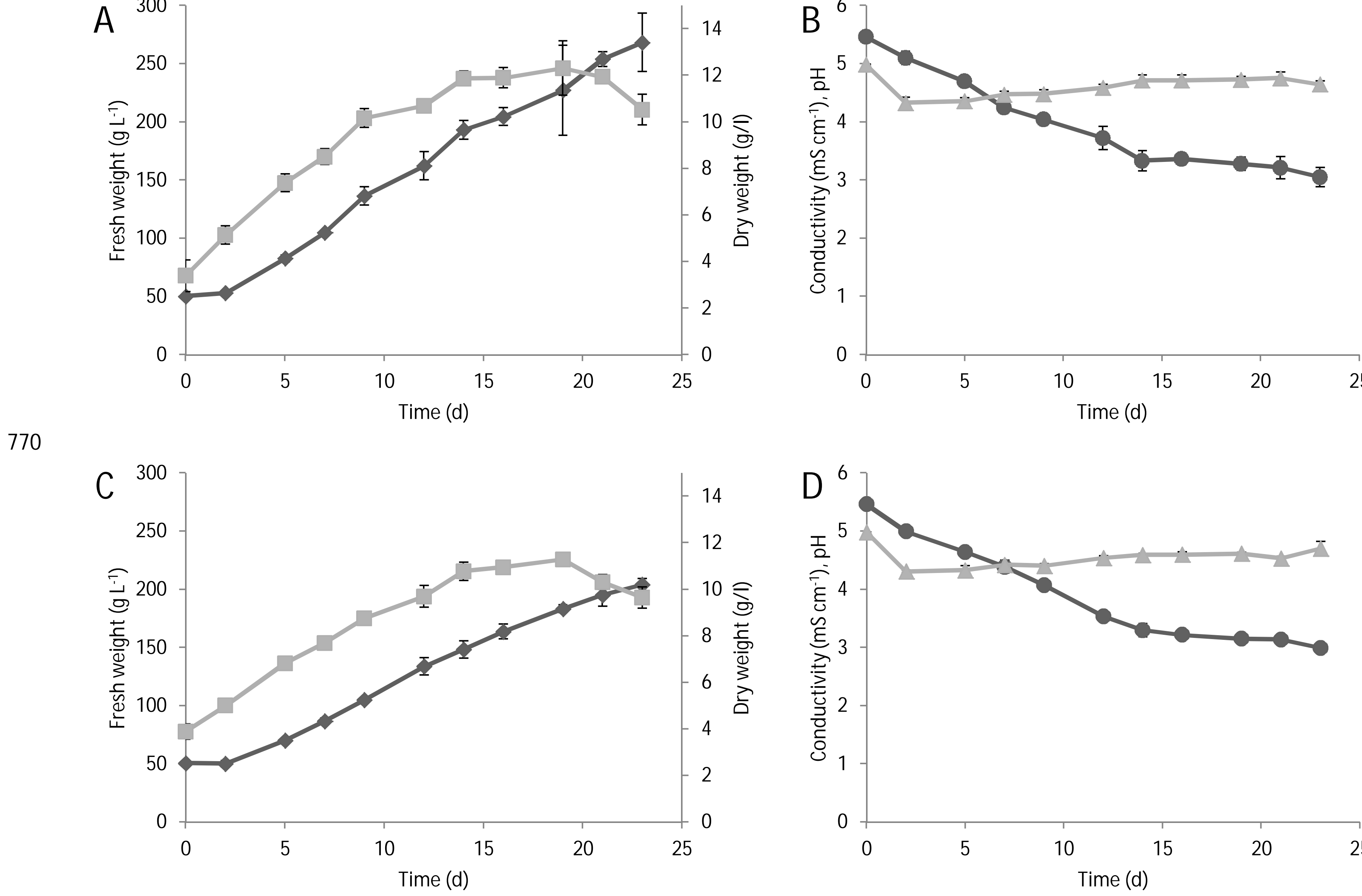
765 with one outlier.

766



767 **Fig. 4** Plantlets of **A** *A. gariepensis* **B** *A. globuligemma* **C** *A. viguieri* and callus from **D** *A.*
768 *gariepensis* and **E** *A. viguieri*

769



770

771

772 **Fig. 5 AB** Growth curve of *A. gariepensis* cell line C and **CD** Growth curve of *A. viguieri* cell line D in MS medium with 1 mg L^{-1} 2,4-D.
773 Biomass accumulation of **AC** fresh weight (diamond) and dry weight (square) is shown as well as **BD** conductivity (circle) and pH (triangle). SD
774 was calculated from n = 3 samples.

775

776

ARTICLE IV

Metabolite profiling of the carnivorous pitcher plants *Darlingtonia* and *Sarracenia*

Submitted to PLOS ONE.
Copyright 2016 Authors.

1 Metabolite profiling of the carnivorous pitcher plants *Darlington* *ia* and *Sarracenia*

2

3

4 Hannu Hotti[1¶], Peddinti Gopalacharyulu[2¶], Tuulikki Seppänen-Laakso[1], Heiko Rischer[1*]

5

6

7 [1]VTT Technical Research Centre of Finland Ltd, Espoo, Finland

8 [2]Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki, Finland

9

10 *Corresponding author

11 E-mail: heiko.rischer@vtt.fi (HR)

12

13 [¶]These authors contributed equally to this work.

14

15 *Short title:* Phytochemical analysis of *Darlingtonia* and *Sarracenia*

16

17

18

19

20

21

22

Abstract

Sarraceniaceae is a New World carnivorous plant family comprising three genera: *Darlingtonia*, *Heliamphora*, and *Sarracenia*. The plants occur in nutrient-poor environments and have developed insectivorous capability in order to supplement their nutrient uptake. *Sarracenia flava* contains the alkaloid coniine, otherwise only found in *Conium maculatum*, in which its biosynthesis has been studied, and several *Aloe* species. Its ecological role and biosynthetic origin in *S. flava* is speculative. The aim of the current research is to investigate the occurrence of coniine in *Sarracenia* and *Darlingtonia* and to identify common constituents of both genera, unique compounds for individual variants and floral scent chemicals. In this comprehensive metabolic profiling study, we looked for compound patterns that are associated with the taxonomy of *Sarracenia* species. In total, 57 different *Sarracenia* and *D. californica* accessions were used for metabolite content screening by gas chromatography-mass spectrometry. The resulting high-dimensional data were studied using a data mining approach. The two genera are characterized by a large number of metabolites and huge chemical diversity across distinct species. By applying feature selection for clustering and by integrating new biochemical data with existing phylogenetic data, we demonstrate that the chemical composition of the species can be explained by their known classification. Although transcriptome analysis did not reveal a candidate gene for coniine biosynthesis, the use of a sensitive selected ion monitoring method enabled the detection of coniine in eight *Sarracenia* species, showing that it is more widespread in the genus than previously believed.

Introduction

Sarraceniaceae is a New World carnivorous plant family comprising three genera: *Darlingtonia* Torr. (monotypic), *Heliamphora* Benth. (ca. 23 species [1]) and *Sarracenia* L. (ca. 11 species [2]). The distribution of *Darlingtonia* is limited to a few locations at the western coast of North America, *Heliamphora* occurs mainly on tepuis of the Guiana Highlands in South America and *Sarracenia* is the most widespread genus in the family, found in the eastern coastal plains of North America.

48 *Darlingtonia californica*, *Sarracenia,* and *Heliamphora* are able to compete in nutrient-poor
49 habitats due to their insectivorous nature, *i.e.* the ability to attract, capture, and digest insects to
50 supplement their nutrient uptake. A common feature for all three genera is that they lure insects to
51 their elongated tubular leaves. In order to attract insects, they produce extrafloral nectar [3], emit
52 insect attractants [4], and most species are brightly coloured. They utilize various methods to
53 capture their prey. *Darlingtonia californica* and *S. psittacina*, for example, hide their entry/exit hole
54 from the inside, displaying multiple translucent false exits so that insects finally get exhausted and
55 fall into the pitcher. Other *Sarracenia* and *Heliamphora* species utilize downward pointing hairs
56 and waxy surfaces in their pitchers to trap insects.

57

58 The family is relatively poorly described in terms of chemical constituents [5], which is
59 surprising given the fact that *Sarracenia* species have long been used as traditional medicine by
60 many aboriginal communities in North America, and have attracted renewed pharmaceutical
61 interest due to recent investigations revealing their cytoprotective activities in cell models [6].
62 *Darlingtonia californica* has not been chemically investigated at all so far but several insect-
63 attracting constituents have been described from the spoon-shaped lid structures of pitchers of two
64 *Heliamphora* species [4]. Also, various compounds found in *Sarracenia* have been reported,
65 including volatiles [7,8], flavonoids [9-11], phytochemicals [12-14] and pitcher fluid composition
66 [3,11,15,16]. Sarracenin, an enol diacetal monoterpene, was first identified in *S. flava* [17] and later
67 found in a number of *Sarracenia* [18] and *Heliamphora* [4] species. *Sarracenia flava* is the most
68 studied species with respect to its chemical composition [7,8,19,20]. Interestingly, *S. flava* contains
69 coniine [21], a toxic alkaloid, which is otherwise only known from the unrelated *Conium*
70 *maculatum* (Apiaceae) and several *Aloe* species (Xanthorrhoeaceae) [22,23]. In *C. maculatum,* a
71 polyketide synthase (PKS) initiates the biosynthesis of coniine [24]. The original study [21] referred
72 to earlier research on *S. purpurea,* indicating that it could also contain coniine or related alkaloids.

73 Mody et al. [21] speculated that coniine functions in *S. flava* to paralyze insects, whereas Harborne
74 [25] postulated insect attraction. Systematic investigations of the compound's wider occurrence in
75 the genus have hitherto not been performed. In order to follow up on the earlier findings in *S. flava*
76 and to expand our knowledge on coniine distribution in *Sarracenia,* we aimed at investigating a
77 number of accessions using a sensitive gas chromatography-mass spectrometry (GC-MS) method
78 applying selected ion monitoring (SIM) to detect coniine reliably in plant material even at low
79 concentrations. Additionally, the transcriptomes of *S. psittacina* and *S. purpurea* were analysed for
80 encoded candidate PKSs putatively involved in coniine biosynthesis.

81 Three previous studies derived the phylogeny of *Sarraceniaceae* using gene sequence data with
82 incongruent results [2628]. Stephens et al. [2] recently addressed this inconsistency by applying a
83 target enrichment approach to assess the phylogenetic relationships among 75 *Sarracenia*
84 accessions. Unlike the mutations from highly conserved genomic loci, the chemical composition
85 usually differs between closely related species and hence is not suitable for deriving reliable
86 taxonomies [29]. In a biochemical profiling study of volatiles, Jürgens et al. [8] applied an approach
87 based on multidimensional scaling to study the similarities among different species. They then used
88 similarity percentage analysis (SIMPER) to obtain compounds that explained the highest amount of
89 dissimilarity among the samples. Thus, Jürgens et al. [8] focused on the variability in the data
90 without focusing directly on the phylogenetic structure. The phylogeny, on the other hand, may
91 explain the chemical diversity of the species. The aim of our current study is to provide a
92 comprehensive catalogue of chemical constituents of *Sarraceniaceae* and to examine the extent to
93 which the known phylogenetic information explains chemical composition of the plants. Therefore,
94 we employed a comprehensive metabolic profiling approach using GC-MS to detect all ions in
95 SCAN mode in a large sample collection. The genus *Sarracenia* comprises 44 recognised
96 intraspecific taxa [30] within 11 *Sarracenia* species [2]. In contrast only one *Darlingtonia* species is
97 known occurring in a geographically restricted area. Our collection was selected to adequately

98 cover the diversity of pitcher plants that had been examined in phylogenetic studies [26]. We found
99 common chemical constituents among the plants, unique compounds for individual variants and
100 possible floral scent chemicals as classified according to Knudsen et al. [29] and studied whether
101 the biochemical profiles can be explained by the taxonomy presented in Stephens et al. [2].

102 Materials and Methods

103 Plant material

104 Pitchers of cultivated plants were investigated in order to level off environmental effects.
105 *Sarracenia* L. (56 accessions) and *Darlingtonia californica* Torr. were provided by C. Klein,
106 Germany (http://www.carnivorsandmore.de). Metabolite and coniine content screening was
107 performed using global metabolomics in a set of 48 accessions (Table 1) that contained one *D.*
108 *californica* and 47 *Sarracenia* accessions. The *Sarracenia* accessions included *S. alata* Alph.Wood
109 (5 accessions), *S. flava* L. (11 accessions), *S. leucophylla* Raf. (5 accessions), *S. minor* Walt. (3
110 accessions), *S. oreophila* (Kearney) Wherry (2 accessions), *S. psittacina* Michx. (4 accessions), *S.*
111 *purpurea* L. (13 accessions) and *S. rubra* Walt. (4 accessions).

112 Targeted metabolomics for sensitive detection of coniine was performed in 17 accessions,
113 including eight accessions that were also analyzed using global metabolomics (Table 2). These
114 accessions included *S. alata* (2 accessions), *S. flava* (4 accessions), *S. leucophylla* (1 accession), *S.*
115 *minor* (1 accession), *S. oreophila* (1 accession), *S. psittacina* (2 accessions), *S. purpurea* (4
116 accessions) and *S. rubra* (2 accessions).

117 Cultivated poison hemlock (*Conium maculatum* L.) and barley (*Hordeum vulgare* L. 'Golden
118 Promise'), were used as alkaloid-containing or alkaloid-free reference material, respectively.

119 **Table 1. List of *Darlingtonia* and *Sarracenia* accessions for metabolite profiling by GC-MS (SCAN).**

Species	Newer classification (according to [2])	Sample number	Sample numbering in [2][a]	Growth form	Origin	Coniine in Lid			Coniine in Pitcher		
						m/z 80	*m/z* 84	*m/z* 126	*m/z* 80	*m/z* 84	*m/z* 126
Darlingtonia californica		18	SAMN03354579			-	x[f]	-	-	x	-
Sarracenia alata		14	SAMN03354583[b]	blood form	DeSoto, Mississippi	x[g]	x[g,h]	x[h]	-	x	-
Sarracenia alata		46	SAMN03354583[b]	blood form	Stone, Mississippi	x[i]	x[f,i]	-	x[i]	x[f,i]	-
Sarracenia alata		28	SAMN03354584[c]		Citronelle, Alabama	x	x[h]	-	x	x[h]	-
Sarracenia alata		40	SAMN03354586[b]		Robertson, Texas	-	x	-	x[i]	x[i]	-

Sarracenia alata		42	SAMN03354583[b]		Perry Co. Mississippi	-	x	-	x[i]	x[i]	-
Sarracenia flava		20	SAMN03354588[d]			x	-	-	x	x[h]	-
Sarracenia flava var. *atropurpurea*		31	SAMN03354589[b]		Bloodwater, Florida	x[i]	x[i]	x	x[i]	x[i]	x
Sarracenia flava var. *atropurpurea*		35	SAMN03354589[b]		Bay County, Florida	x	x[h]	-	x	x[h]	-
Sarracenia flava var. *atropurpurea*		1	SAMN03354589[b]		Bloodwater, Florida	x	x[h]	-	-	x[f]	-
Sarracenia flava var. *cuprea*		10	SAMN03354591[d]			x	x[f]	-	x	x[h]	-
Sarracenia flava var. *flava*		11	SAMN03354593[b]		Dinwiddie, Virginia	x[g]	x[g,h]	x[g]	x[g]	x[g,h]	x[g]
Sarracenia flava		21	SAMN03354590[b]		near Shallotte,	x[i]	x[i]	-	x[g]	x[g,h]	x[g]

var. *heterophylla*					North Carolina						
Sarracenia flava var. *maxima*		44	SAMN03354593[d]			x	x[h]	-	x[g]	x[g,h]	x[g]
Sarracenia flava var. *ornata*		29	SAMN03354592[b]		Sandy Creek, North Carolina	x	x[h]	x[j]	x[g]	x[g,h]	x[g]
Sarracenia flava var. *rubricorpora*		8	SAMN03354594[b]		Apalachicola, Florida	x[g]	x[g,h]	x[g]	x[i]	x[i]	-
Sarracenia flava var. *rugelii*		32	SAMN03354596[d]			x[i]	x[i]	-	x[i]	x[f,i]	-
Sarracenia leucophylla		33	SAMN03354604[b]		Splinter Hills Bog, Alabama	-	x	-	x	x[h]	-
Sarracenia leucophylla		17	SAMN03354603[d]	Big pink lip	Apalachicola, Florida	x[g]	x[g,h]	x[g]	x[i]	x[i]	-
Sarracenia leucophylla		12	SAMN03354605[d]	Pubescent, covered with white hairs		-	x	x	x	x[h]	-

Sarracenia leucophylla 'Schnell's Ghost'		45	SAMN03354606[d]			-	x	x	x[i]	x[f,i]	-
Sarracenia leucophylla var. *alba*		26	SAMN03354608[d]			x[g]	x[g,h]	x[g]	x[g]	x[g,h]	x[g]
Sarracenia minor		15	SAMN03354609[d]	large form		x	x[f]	-	x[i]	x[i]	-
Sarracenia minor		4	SAMN03354610[d]	small form		-	x	-	x[i]	x[i]	-
Sarracenia minor var. *okefenokeensis*		5	SAMN03354614[e]			x	x[h]	-	x	x[h]	-
Sarracenia oreophila		22	SAMN03354616[d]			x[g]	x[g,h]	x[g]	x[g]	x[g,h]	x[g]
Sarracenia oreophila		27	SAMN03354615[d]		Sand Hill, Alabama	x[g]	x[g,h]	x[g]	x	x[h]	-

Sarracenia psittacina f. *heterophylla*		6	SAMN03354621[d]	Yellow flower		x[g]	x[g,h]	x[g]	x	x[h]	-
Sarracenia psittacina f. *heterophylla*		24	SAMN03354623[b,d]		Baldwin County, Alabama	x	x[h]	-	x[i]	x[i]	-
Sarracenia psittacina		13	SAMN03354626[b]	Gulf giant	Wewahitchka, Florida	-	x	-	x[g]	x[g,h]	x[g]
Sarracenia psittacina		43	SAMN03354628[e]	Yellow flower		x	x[h]	-	x	x[h]	-
Sarracenia purpurea subsp. *purpurea*		16	SAMN03354629[e]		Switzerland	x	x[f]	-	x	x[h]	-
Sarracenia purpurea subsp. *purpurea*		19	SAMN03354630[e]			x[g]	x[g,h]	x[g]	x[g]	x[g,h]	x[g]
Sarracenia		38	SAMN03354631[e]	extreme		-	x	-	x	x[h]	-

purpurea subsp. *purpurea* f. *heterophylla*				dense growth form							
Sarracenia purpurea subsp. *venosa*		36	SAMN03354633[d,e]			-	x	-	x	x[i]	-
Sarracenia purpurea subsp. *venosa*		47	SAMN03354634[d,e]		Tom's Swamp	-	x	-	-	x	-
Sarracenia purpurea subsp. *venosa*		30	SAMN03354663[d,e]	All green		x	x[i]	-	-	x	-
Sarracenia purpurea subsp. *venosa*		37	SAMN03354632[b]		Tyrrel County, North Carolina	-	x	-	-	x[f]	-
Sarracenia purpurea subsp.	*S. rosea*	34	SAMN03354637[d,e]	small strongly		x[i]	x[f,i]	-	x	x[h]	-

venosa var. *burkii*				waving form							
Sarracenia purpurea subsp. *venosa* var. *burkii*	*S. rosea*	7	SAMN03354640[d,e]		Carteret, North Carolina	x	x[h]	-	x	x[h]	-
Sarracenia purpurea subsp. *venosa* var. *burkii*	*S. rosea*	39	SAMN03354639[d,e]	Giant		x[i]	x[f,i]	-	x	x[h]	-
Sarracenia purpurea subsp. *venosa* var. *burkii* f. *luteola*	*S. rosea* f. *luteola*	48	SAMN03354638[d,e]	veinless form		x	x[h]	-	x[g]	x[g,h]	x[g]
Sarracenia purpurea subsp. *venosa* var.		41	SAMN03354635[e]			x[g]	x[g,h]	x[g]	†	†	†

montana											
Sarracenia purpurea subsp. *venosa* var. *montana*		9	SAMN03354636[e]		Chipola, Florida	x[g]	x[g,g]	x[g]	x[i]	x[i]	-
Sarracenia rubra subsp. *alabamensis*	*S. alabamensis*	2	SAMN03354582[e]			x	x[h]	-	x	x[h]	-
Sarracenia rubra subsp. *gulfensis*		25	SAMN03354647[d]			x	x[h]	-	-	x[f]	-
Sarracenia rubra subsp. *jonesii*	*S. jonesii*	3	SAMN03354599[b]		Cesars Head, South Carolina	-	-	-	-	-	-
Sarracenia rubra subsp. *wherryi*	*S. alabamensis* subsp. *wherryi*	23	SAMN03354650[e]			x	x[h]	-	x	x[h]	-

120 x mass *(m/z)* present, - not present, † not analysed

121 [a] Given a corresponding sample when applicable, otherwise c.

122 [b] Based on collection location.

123 [c] Mississippi accessions were used as they are the closest geographical location for this sample.

124 [d] Drawn lots, if there are more than two options from which to choose.

125 [e] Based on the same variety if collection location is not available.

126 [f] Low intensity fragment.

127 [g] Masses *m/z* 80, 84 and 126 are present in correct proportions.

128 [h] Mass *m/z* 80 has greater intensity than *m/z* 84.

129 [i] Masses (*m/z*) have the same relative intensity.

130 [j] Mass *m/z* 126 has the greatest intensity of the three selected ions.

131 **Table 2.** ***Sarracenia*** **accessions for targeted coniine analysis by GC-MS (SIM).**

Species	Newer classification (according to [2])	Sample number[1]	Growth form	Origin	Coniine in Lid	Coniine in Pitcher
Sarracenia alata 'Black Tube'					x	x*
Sarracenia alata			Wide hood	Stane County, Mississippi	x	x
Sarracenia flava		20			x*	-
Sarracenia flava var. *atropurpurea*					x*	x*
Sarracenia flava var. *maxima*		44			x*	x*
Sarracenia flava var. *ornata*					x	x*
Sarracenia leucophylla				Citronelle, Alabama	x	†
Sarracenia minor var. *okefenokeensis*		5			x	-
Sarracenia oreophila			typical form		-	x
Sarracenia psittacina		13	Gulf giant		†	x

Sarracenia psittacina		43	Yellow flower		x	x
Sarracenia purpurea subsp. *burkii*			Veinless		x	x
Sarracenia purpurea subsp. *venosa*		36			x	x
Sarracenia purpurea subsp. *venosa* var. *burkii* f. *luteola*	*S. rosea* f. *luteola*	48	veinless form		x	x
Sarracenia purpurea subsp. *venosa* var. *montana*		41			x	x
Sarracenia rubra subsp. *alabamensis*	*S. alabamensis*			Chilton County, Alabama	x	x
Sarracenia rubra subsp. *gulfensis*					x	x

132 x present; - not present; x* trace, close to limit of detection (1 μg/ml); † not analyzed

133 [1] Included in metabolite profiling (Table 1)

134

135 Metabolite extraction

136 Lids and pitchers were separated, washed with tap water and ground up. Fresh (2 g; metabolite
137 profiling) or freeze dried (200 mg; coniine analysis) plant material was used for extraction as
138 described in [31]. Lipids were removed from the plant material with 3.0 ml petroleum ether (puriss

139 p.a., Sigma-Aldrich Munich, Germany). The plant material was diluted with 2.0 ml ultrapure water
140 and a pH above 9 was obtained by addition of 10% ammonium hydroxide solution (25% stock
141 solution, pro analysi, Merck KGaA, Darmstadt, Germany). Metabolites were extracted twice with
142 2.0 ml dichloromethane (HPLC grade, Rathburn Chemicals Ltd, Walkerburn, Scotland, UK). The
143 combined dichloromethane extracts were evaporated to dryness and dissolved in 100 µl
144 dichloromethane for further analysis.

145 Gas chromatography-mass spectrometry

146 Samples (1 µl) were analysed by GC-MS consisting of a 6890A Series GC (Agilent Technologies,
147 Inc., Santa Clara, CA, USA) combined with an Agilent 5973 Network MSD and a Combipal
148 automatic sampler (Varian Inc., Palo Alto, CA, USA). Analytes were separated by an Agilent HP-
149 5MS capillary column (25 m × 0.2 mm i.d, 0.33 µm). The temperature program started at 50°C with
150 1 min holding time and then increased at 10°C/min up to 300°C. MSD was operated in electron
151 impact mode at 70 eV.

152 Pure coniine (Sigma-Aldrich, Munich, Germany) was used as the reference compound in
153 developing the GC-MS method. To determine the detection limit of coniine in the SIM-method, 1,
154 5, 10 and 20 µg was spiked into alkaline water and extracted as described in [31]. Cotinine (20
155 µg/sample) (Sigma-Aldrich, Munich, Germany) was used as an internal standard.

156 PKS-encoding genes in transcriptomes of *S. psittacina* and *S. purpurea*

157 Available transcriptomes of *S. psittacina* (accession number SRX060168 in the NCBI database) and
158 *S. purpurea* (accession number SRX060177 in the NCBI database) [32] were analyzed for PKSs
159 using Geneious (version 9.0.4) [33]. The tblastn algorithm in Geneious was used to search the
160 sequence database with the *Medicago sativa* CHS2 amino acid sequence [34] as the template and a
161 stringency setting of 1e-10. The obtained nucleotide sequence hits were translated to amino acid

162 sequences, and the correct reading frames were chosen and aligned using the Geneious alignment
163 option.

164 Data handling

165 Peaks in GC-MS chromatograms were integrated automatically using MSD ChemStation software
166 (version E.02.01.1177, Agilent Technologies, Inc., Santa Clara, CA, USA). Peaks were identified
167 with the Palisade Complete 600K Mass Spectral Library (Palisade Mass Spectrometry, Ithaca, NY,
168 USA) and the NIST Mass Spectral Search Program (The Standard Reference Data Program of the
169 National Institute of Standards and Technology, Gaithersburg, MD, USA). The computer-generated
170 identifications were sorted manually with a cut-off at 70% identification [35] into an Excel
171 spreadsheet (Microsoft, Redmond, WA, USA) according to their chemical structure, elution time
172 and origin. When peaks with same retention time were identified as different hydrocarbons in
173 multiple samples, they were treated as n-alkanes at the specific retention time. The relative peak
174 abundances were used in the data input.

175 Data mining

176 The metabolite data were treated in two formats: (1) a qualitative format representing presence (*i.e.*
177 concentration level above the detection limit) or absence (concentration level below the detection
178 limit) of a compound in a sample, by coding the presence and absence as 1 and 0, respectively, and
179 (2) a quantitative or continuous format in which the concentration level is given as the percentage of
180 the total peak area. The main aim of our data mining was to visualize any patterns present in the
181 data. Towards this goal, it was first noted that the current data are very high dimensional (*i.e.*
182 contain a large number of compounds), very sparse (91.35% zeros in the lids dataset and 91.86% in
183 the pitchers dataset), and that the distinct species show huge chemical diversity (*i.e.* the metabolite
184 composition of different plants is largely distinct). Therefore, it is reasonable to expect that only a
185 small proportion of compounds are likely to be useful for clustering the samples. A feature selection

186 approach for clustering [36] was applied to identify the most important features required for
187 deriving hierarchical clusters. This approach computes and reweights the overall dissimilarity
188 matrix while applying a lasso-type penalty, which results in a dissimilarity matrix that is sparse in
189 features [36]. This sparse clustering was applied using the R package sparcl. In order to compute the
190 hierarchical clustering with the qualitative format of the data, the hamming distance was used as the
191 dissimilarity measure. For the quantitative format of the data, the Euclidean distance was used. The
192 complete linkage method was used for the clustering.

193 In order to compare the phylogenetic structure with the chemical profiles, the MP-EST
194 accession tree from [2] was downloaded. Then the accessions in the two studies were mapped based
195 on the location of sample collection, which resulted in a many-to-many mapping (Table 1) with one
196 or more of 42 nodes in the phylogenetic tree matching one or more of 48 species in our study. From
197 this, 36 possible bijective maps were enumerated, and compound-based distances corresponding to
198 each bijective map were calculated as follows. The distance between every pair of accessions was
199 calculated using hamming distance for the binary and Euclidean distance for the continuous data of
200 the selected metabolite features. These distances are referred to as species-level distances (SLD)
201 below. Using the clades resolved in the MP-EST accession tree (*i.e. D. californica, S. flava, S.*
202 *psittacina, S. minor, S. purpurea* complex, *S. rubra* complex, *S. alata*, *S. leucophylla*, and *S.*
203 *oreophila*), distances within and between the clades were calculated. A within-clade distance
204 (WCD) was calculated as the average of all pairwise SLDs of accessions within the clade. A
205 between-clade distance (BCD) was calculated as the average of all SLDs of accession-pairs across
206 the pair of clades. Average species-level and clade-level distance matrices were calculated over all
207 36 bijective maps to derive the average within-clade (aWCD) and between-clade distances (aBCD),
208 as well as the average species-level distances (aSLD). These averaged distances were used to assess
209 how well the metabolite data supports the phylogenetic structure. If the phylogenetic structure
210 explains the compound data, the aWCDs are expected to be lower than the aBCDs. This was

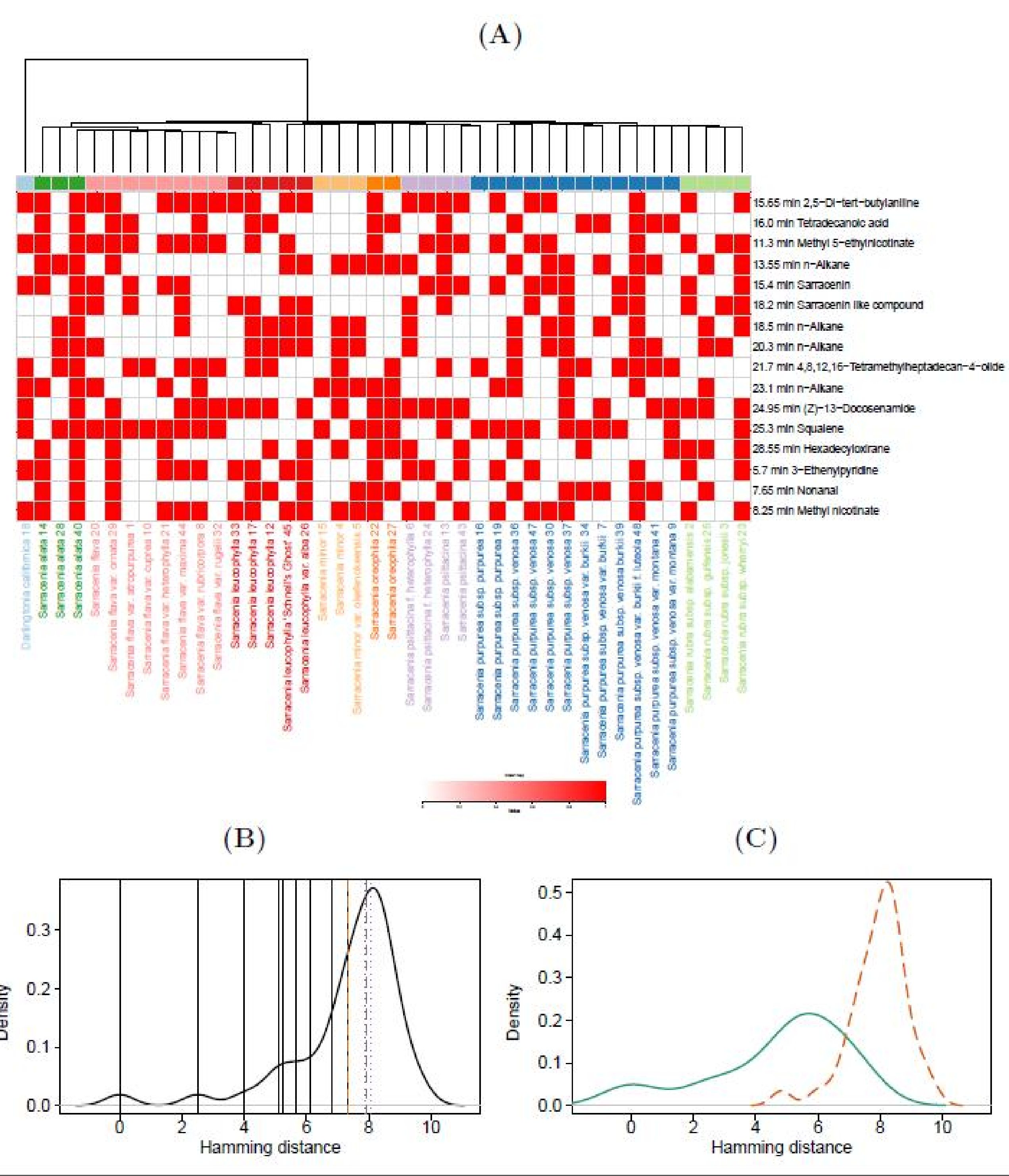
211 assessed by comparing aWCDs against not only aBCDs but also aSLDs as an additional test. More

212 precisely, we (I) visualized aWCDs against the background distance distribution formed by aSLDs

213 (Figs 1B, 2B, S1B and S2B), (II) visualized the difference between the distribution of aWCDs and

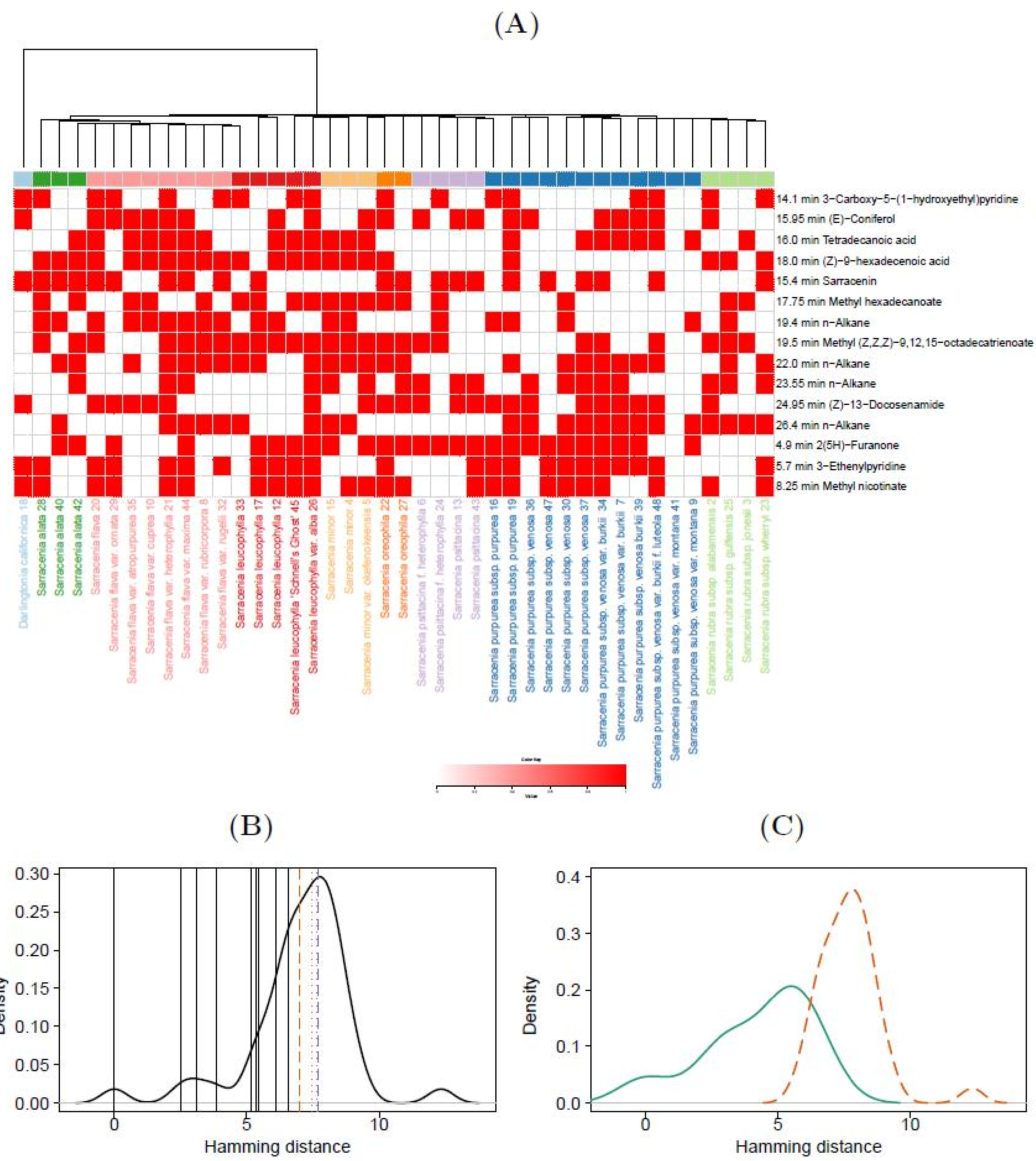
214 aBCDs (Figs 1C, 2C, S1C and S2C) and (III) performed one-sided Wilcoxon's rank sum tests to

215 assess whether aWCDs are less prelevant than aBCDs.



216

217 **Fig 1. Visualization of selected metabolite features from the qualitative data of lids.** (A) Heat
218 map visualization of selected metabolite features from the qualitative data of lids. The phylogenetic
219 tree from [2] is displayed as the column dendrogram. Six samples of our dataset (11, 31, 35, 38, 42,
220 and 46) are omitted from this heat map based on the sample selection procedure described in the
221 Methods section. (B) Comparison of average within-clade distances (aWCDs) against the
222 background distribution of average species-level distances (aSLDs) and average between-clade
223 distances (aBCDs). Distribution of aSLDs was calculated using qualitative data of the selected
224 metabolite features and displayed in a density plot. The black vertical lines mark the individual
225 aWCDs. The orange dashed and dotted lines show the mean and median of aSLDs. The purple
226 dashed and dotted lines show the mean and median of aBCDs. (C) Comparison of aWCDs (green
227 continuous density line) with aBCDs (orange dashed density line).



228

229 **Fig 2. Visualization of selected metabolite features from the qualitative data of pitchers.** (A) Heat map visualization of selected metabolite features from the qualitative data of pitchers. The phylogenetic tree from [2] is displayed as the column dendrogram. Six samples of our dataset (1, 11, 14, 31, 38, and 46) are omitted from this heat map, based on the sample selection procedure described in the Methods section. (B) Comparison of average within-clade distances (aWCDs)

234 against the background distribution of average species-level distances (aSLDs) and average
235 between-clade distances (aBCDs). Distribution of aSLDs was calculated using qualitative data of
236 the selected metabolite features and displayed in a density plot. The black vertical lines mark the
237 individual aWCDs. The orange dashed and dotted lines show the mean and median of aSLDs. The
238 purple dashed and dotted lines show the mean and median of aBCDs. (C) Comparison of aWCDs
239 (green continuous density line) with aBCDs (orange dashed density line).

240 In order to visualize the metabolite features selected for clustering alongside the phylogenetic
241 structure presented in [2], the best mapping of samples between the MP-EST accession tree and our
242 compound data was obtained. The best bijective map is expected to result in the maximum BCD
243 and minimum WCD among all possible bijective maps. To achieve this objective, we chose the map
244 that yields the maximum difference between the mean values of BCD and WCD *i.e. mean*(BCD) –
245 *mean*(WCD) for these visualizations (Figs 1A, 2A, S1A and S2A). Thus, the heat maps shown in
246 Figs 1A, 2A, S1A and S2A contain only one sample from our compound dataset for each node in
247 the MP-EST accession tree chosen to maximize the *mean*(BCD) – *mean*(WCD). Since only 42
248 nodes in the accession tree map to our dataset, each heat map omits 6 samples from our study. In
249 particular, the samples numbered 31 and 46 (Table 1) were omitted in all four heat maps (Figs 1A,
250 2A, S1A and S2A). Apart from these two samples, 11, 35, 38, and 42 were omitted from Fig 2A;
251 14, 35, 37, and 44 were omitted from S1A Fig; 1, 11, 14, and 38 were omitted from Fig 2A; and 1,
252 11, 38, and 42 were omitted from S2A Fig.

253 All the statistical analyses and visualizations were performed using the R statistical software
254 [37] and its packages such as gplots, sparcl, metadar (http://code.google.com/p/metadar), ihm
255 (http://code.google.com/p/ihm), and RColorBrewer.

256 # Results

257 ## Coniine identification and occurrence in *Sarracenia*

258 With the GC-MS method used, coniine elutes at a constant retention time (6.33±0.01min) even in
259 spiked barley material and *C. maculatum* leaf extract. The samples were analysed on the basis of
260 their SCAN mass spectra and were compared to a database. Pure coniine matched the database with
261 86%, or in plant matrix with 78%-86% identity. The retention time of coniine was very stable, and
262 the ions 80, 84, and 126 exhibited the same relative abundances in the sample matrix and in the
263 coniine reference substance (Fig 3). Therefore, a match lower than 90% can be considered
264 acceptable. Using the SCAN mode, coniine was detected in *S. alata*, *S. flava*, *S. leucophylla*, *S.*
265 *oreophila*, *S. psittacina* and *S. purpurea* (incl. *S. rosea*) (Table 1). In *D. californica*, only the
266 fragment *m/z* 84 was detected, whereas in *S. jonesii* (3) none of the ions were detected at 6.33 min.

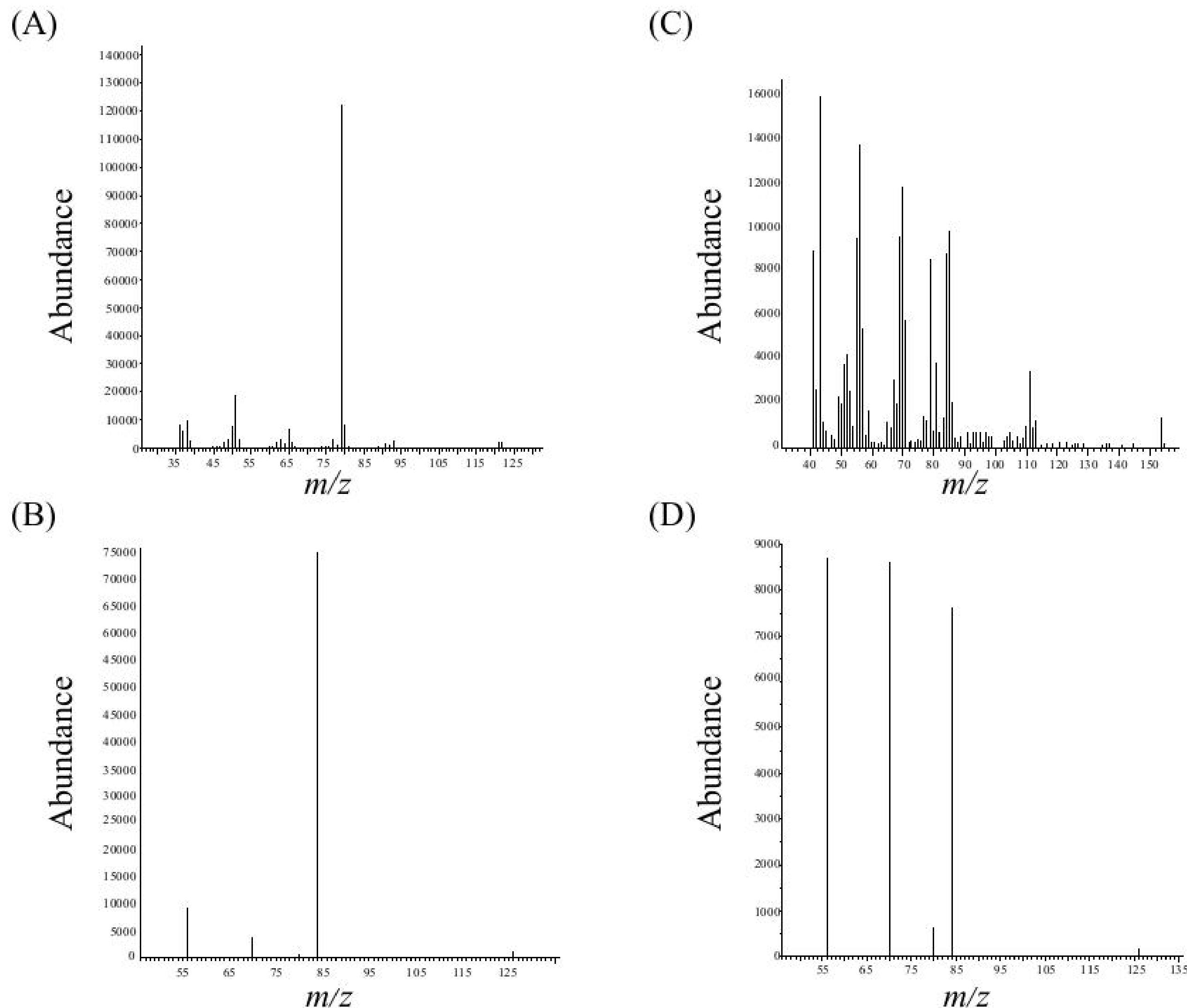


Fig 3. Mass spectrum of coniine reference substance and detection of coniine in the sample matrix. Mass spectrum of pure coniine in SCAN mode (A) and selected fragments in SIM mode (B). Coniine detection in sample matrix (*S. flava)* in SCAN (C) and SIM modes (D).

In order to detect coniine at low concentrations, we operated the GC-MS in SIM mode. Based on the fragmentation pattern of coniine (*m/z* 43, 56, 70, 80, 84, 97, 110, and 126), the characteristic ions *m/z* 56, 70, 80, 84 (base peak) and 126 (mass peak) were selected. The fragments *m/z* 80, 84, and 126 are specific for coniine, in contrast to the ions *m/z* 56 and 70, which are shared with many other molecules.

276 The limit of detection for coniine in SIM was 1 µg/ml, which corresponds to 1 µg/g dry weight. 277 Using SIM detection, coniine was identified from *S. alata*, *S. flava*, *S. leucophylla*, *S. minor*, *S.* 278 *oreophila*, *S. psittacina*, *S. purpurea* (incl. *S. rosea*) and *S. rubra* (incl. *S. alabamensis*) (Table 2). 279 Of these, *S. flava* and *S. alata* samples only contained coniine traces close to the detection limit, 280 whereas other samples accumulated clearly higher levels of coniine. No coniine was detected in the 281 pitchers of *S. minor* var. *okefenokeensis* or the lids of *S. oreophila*.

282 PKSs in *Sarracenia* transcriptomes

283 *Sarracenia psittacina* and *S. purpurea* transcriptomes were analysed using the tblastn algorithm 284 with the stringency set to 1e-10 and *M. sativa* CHS2 as a template, resulting in 8 and 12 sequences, 285 respectively. Correct reading frames were selected and aligned with each other after the nucleotide 286 sequences were translated to amino acid sequences. This resulted in three unique contigs per 287 species. Of these, one represents the N-terminus and two the C-terminus when compared to full-288 length PKS-enzyme. None of the contigs cover the middle part of the PKS-enzyme sequence, but 289 they do contain all the conserved amino acids in the active site in the observed area [38] when 290 compared to other full-length PKSs (S3 Fig).

291 Metabolite profiles

292 The metabolite profiles of lids and pitchers were analysed separately. In addition to analysing the 293 metabolite profiles using the quantitative (concentration) data, we also investigated the qualitative 294 (presence or absence) data in which compounds with non-zero concentration levels (*i.e.* with levels 295 above the detection limits) were treated as present and compounds with levels below the detection 296 limits as absent.

297 The manually aligned lid dataset consisted of a total of 560 compounds detected in at least one 298 sample. Among these, there were library matches (≥70%) for 69 alcohols, 70 aldehydes and

299 ketones, 53 esters, 58 ethers, 30 carboxylic acids and sterols, 45 hydrocarbons (including some
300 identified as alkanes), 148 n-alkanes, 75 nitrogen compounds, and 12 sulphur compounds.
301 However, each individual plant's lid contained an average of only 48 compounds. The lid sample of
302 *S. purpurea* subsp. *purpurea* (16) contained the lowest number of compounds (n = 20) and *S. rubra*
303 subsp. *wherryi* (23) had the highest number of compounds (n = 85). The barplot in S4 Fig shows the
304 distribution of compounds across all the lid samples. Furthermore, every lid sample had on average
305 approximately six compounds uniquely found in that sample but in no other sample, one of which
306 could be classified as a floral scent component which had previously been detected from intact
307 flowers [29]. *Sarracenia leucophylla* (17) displayed the highest number (n = 4) of floral scent
308 compounds (Table 3). The sample *S. purpurea* subsp. *venosa* var. *burkii* (39) is an exception in that
309 it did not accumulate unique compounds, whereas *S. flava* var. *atropurpurea* (35) had the largest
310 number (n = 18) of unique compounds. S1A Table shows the compounds unique to each sample
311 along with their concentration levels. Finally, when we compared the lid samples in pairs, we
312 observed that, on average, every lid sample contained 32 unique compounds (S2A Table).

313 **Table 3. Unique compounds for each *Darlingtonia* and *Sarracenia* accession in lids and**
314 **pitchers.**

	Lids		Pitchers	
Species/strain	Unique compounds	Floral scent compounds [29]	Unique compounds	Floral scent compounds [29]
Darlingtonia californica 18	5	0	16	0
Sarracenia alata 14	4	1	3	1
Sarracenia alata	14	2	1	1

46				
Sarracenia alata 28	3	1	2	0
Sarracenia alata 40	5	1	3	0
Sarracenia alata 42	2	0	11	0
Sarracenia flava 20	4	0	13	1
Sarracenia flava var. *ornata* 29	12	3	4	1
Sarracenia flava var. *atropurpurea* 31	4	0	25	6
Sarracenia flava var. *atropurpurea* 35	18	1	11	1
Sarracenia flava var. *atropurpurea* 1	10	2	11	2
Sarracenia flava var. *cuprea* 10	2	1	5	0
Sarracenia flava var. *flava* 11	9	1	12	1
Sarracenia flava	5	2	4	1

var. *heterophylla* 21				
Sarracenia flava var. *maxima* 44	1	0	8	1
Sarracenia flava var. *rubricorpora* 8	2	1	3	0
Sarracenia flava var. *rugelii* 32	3	0	4	0
Sarracenia leucophylla 33	5	0	11	0
Sarracenia leucophylla 17	15	4	5	1
Sarracenia leucophylla 12	14	3	0	0
Sarracenia leucophylla 'Schnell's Ghost' 45	16	3	1	0
Sarracenia leucophylla var. *alba* 26	10	0	19	3
Sarracenia minor 15	7	0	1	0
Sarracenia minor	5	2	9	0

4				
Sarracenia minor var. *okefenokeensis* 5	15	3	16	6
Sarracenia oreophila 22	7	2	7	0
Sarracenia oreophila 27	6	0	3	0
Sarracenia psittacina f. *heterophylla* 6	1	1	0	0
Sarracenia psittacina f. *heterophylla* 24	3	1	1	0
Sarracenia psittacina 13	10	3	9	5
Sarracenia psittacina 43	3	0	5	0
Sarracenia purpurea subsp. *purpurea* 16	1	0	0	0
Sarracenia purpurea subsp. *purpurea* 19	1	0	8	4
Sarracenia	4	1	5	2

purpurea subsp. *purpurea* f. *heterophylla* 38				
Sarracenia purpurea subsp. *venosa* 36	15	2	17	1
Sarracenia purpurea subsp. *venosa* 47	3	0	2	0
Sarracenia purpurea subsp. *venosa* 30	2	0	1	0
Sarracenia purpurea subsp. *venosa* 37	4	2	9	0
Sarracenia purpurea subsp. *venosa* var. *burkei* 34	3	1	3	0
Sarracenia purpurea subsp. *venosa* var. *burkei* 7	5	0	5	0
Sarracenia purpurea subsp.	0	0	10	5

venosa var. *burkei* 39				
Sarracenia purpurea subsp. *venosa* var. *burkei* f. *luteola* 48	4	0	2	0
Sarracenia purpurea subsp. *venosa* var. *montana* 41	11	1	0	0
Sarracenia purpurea subsp. *venosa* var. *montana* 9	13	0	2	1
Sarracenia rubra subsp. *alabamensis* 2	2	0	7	1
Sarracenia rubra subsp. *gulfensis* 25	6	0	7	0
Sarracenia rubra subsp. *jonesii* 3	3	0	7	2
Sarracenia rubra subsp. *wherryi* 23	12	3	11	4

Average	6,4	1,0	6,6	1,1

The pitcher dataset contained 589 compounds detected in at least one sample. Among these, there were library matches (≥ 70%) for 67 alcohols, 60 aldehydes and ketones, 72 esters, 60 ethers, 52 carboxylic acids and sterols, 50 hydrocarbons (including those identified as alkanes), 139 n-alkanes, 74 nitrogen-containing compounds and 15 sulphur-containing compounds. Each individual plant's pitcher sample had an average of 48 compounds. The pitcher sample *S. purpurea* subsp. *venosa* var. *montana* (41) did not contain a single compound at a detectable concentration level and *S. leucophylla* var. *alba* (26) had the highest number of compounds (n = 78). The barplot in S5 Fig shows the distribution of compounds across all the pitcher samples. Furthermore, every pitcher sample had approximately seven unique compounds and, on average, one of them can be considered as a floral scent component [29]. *Sarracenia flava* var. *atropurpurea* (31) and *S. minor* var. *okefenokeensis* (5) had the highest number (n = 6) of floral scent compounds (Table 3). Four samples, *S. leucophylla* (12), *S. psittacina* f. *heterophylla* (6), *Sarracenia purpurea* subsp. *purpurea* (16) and *S. purpurea* subsp. *venosa* var. *montana* (41) did not contain unique compounds, whereas *S. flava* var. *atropurpurea* (31) had the highest number of unique compounds (n = 25). S1B Table shows the compounds unique to each sample along with their concentration levels. Similar to the lids, pitcher pairs had an average of 32 unique compounds (S2B Table).

A sarracenin-like compound was found at an elution time of 18.2 min. Its mass peak was *m/z* 225, major fragments *m/z* 180 and 138, and further fragments were *m/z* 162, 120, 93, 67 and 43.

Selection of metabolites

Overall, both the lid and pitcher datasets are very sparse, with 91.35% zeros in the lid dataset and 91.86% in the pitcher dataset. These datasets are also high dimensional, as described above, with 560 and 589 compounds, respectively, in the lid and pitcher datasets. We performed sparse

337 hierarchical clustering of the data in order to reduce the dimensionality of the datasets and identify
338 the compounds important for clustering. The metabolite features selected using the qualitative and
339 quantitative formats of the data are visualized as heat maps (S6-S9 Figs).

340 Integration of phylogenetic clustering

341 The MP-EST accession tree presented in [2] was integrated with metabolite profiling data. Firstly,
342 the selected metabolite features were visualized as heat maps with the MP-EST accession tree (Figs
343 1A, 2A, S1A and S2A). Since the best bijective map between the samples of the two studies was
344 selected for these visualizations, six samples from our compound dataset are omitted from each of
345 the heat maps (Figs 1A, 2A, S1A and S2A). Secondly, the MP-EST accession tree was used to
346 assess whether the metabolite profiles support the clade-level classification of the plant family. This
347 was done by comparing the aWCDs against aBCDs as well as the background distance distribution
348 formed by the aSLDs. The aWCDs were lower than aBCDs (Figs 1, 2, S1 and S2), indicating that
349 the compound data was consistent with the clade-level classification. From the qualitative data of
350 lids, all aWCDs were less than the mean and median values of the aBCDs. In comparison to the
351 background distribution, eight out of nine aWCDs were less than the mean of the aSLDs and all the
352 aWCDs were less than the median of the aSLDs (Fig 2B). Finally, the aWCDs were significantly
353 lower than the aBCDs (Wilcoxon test *P*-value = 1.42e-05; Fig 2C). From the qualitative data of
354 pitchers, all aWCDs were less than the mean and median values of the aBCDs as well as the aSLDs
355 (Fig 2B), and the aWCDs were significantly lower than aBCDs (*P*-value = 5.109e-06; Fig 2C). The
356 quantitative data weakly supported the clade-level classification (S1 and S2 Figs). From the
357 quantitative data of lids, seven out of nine aWCDs were lower than the mean and median values of
358 the aBCDs and aSLDs (S1B Fig), and the difference between aWCDs and aBCDs was marginally
359 significant (*P*-value = 0.02; S1C Fig). From the quantitative data of pitchers, all aWCDs were lower
360 than the mean of aBCDs, eight out of nine aWCDs were lower than the mean of aSLDs, seven
361 aWCDs were less than the median of aBCDs, and six aWCDs were less than the median of aSLDs

362 (S2B Fig). The difference between aWCDs and aBCDs was marginally significant (*P*-value = 363 0.004; S2C Fig).

364 Discussion

365 Coniine in *Sarracenia* sp.

366 The presence of coniine has been reported from poison hemlock and twelve *Aloe* species [22,23]. 367 The only report of coniine in Sarraceniaceae is by Mody et al. [21], who isolated 5 mg of coniine 368 from 45 kg fresh pitchers of *S. flava* via steam distillation. This is in contrast to the results of 369 Romeo et al. [11], who did not detect any alkaloids or volatile amines in *Sarracenia*. We have now 370 confirmed the findings of Mody et al. [21] and also found that coniine occurs, often in low amounts, 371 in at least seven other species, *e.g. S. purpurea* (Table 2). It remains unknown where exactly 372 coniine is biosynthesized in *Sarracenia* spp., since the compound was detected both in lids and in 373 the actual pitchers. Biosynthesis of coniine has been studied in poison hemlock. In this case the 374 carbon backbone is derived from the iterative coupling of butyryl-CoA and two malonyl-CoAs by a 375 PKS, CPKS5 [24]. According to our analysis, genes encoding such enzymes are present in the 376 transcriptomes [32] of *S. psittacina* and *S. purpurea*. Both species harbour three contigs which 377 present two to three PKSs. The exact number could not be determined because the N-terminal 378 contig cannot be assigned to either of the C-terminal contigs. The contigs do not represent full-379 length sequences and therefore it is impossible to clearly assign them as PKSs for coniine 380 biosynthesis in *Sarracenia* spp. Important mutations might be located outside the observed area, 381 preventing distinction from chalcone synthases involved in anthocyanin synthesis [9,10].

382 An important question is the function of coniine in *Sarracenia*. Why should plants living in 383 nutrient-poor environments produce a nitrogenous compound if there are no benefits? Butler and 384 Ellison [39] studied nitrogen acquisition of *S. purpurea* and reported that the pitchers are in fact

385 very efficient in prey capture and could thus greatly enhance the available nitrogen for the
386 following growth season. Mody et al. [21] postulated that coniine could be an insect-stunning agent.
387 Coniine did indeed paralyze fire ants, but probably the tested concentrations were not physiological
388 [21]. Another function for coniine could be insect attraction, as suggested by Harborne [25] and
389 Roberts [40], who identified coniine as a floral scent compound in poison hemlock. In conclusion, it
390 appears that an investment in coniine biosynthesis could have a double benefit by enhancing both
391 insect attraction and retention.

392 Metabolite profiles of *Sarracenia* and *Darlingtonia*

393 There are several previous reports on *Sarracenia* volatiles [7,8]. For example, Miles et al. [7]
394 reported benzothiazole, benzyl alcohol, heptadecane and tridecane from *S. flava,* which we also
395 found from *Sarracenia* spp. Nonanal, a floral scent compound widespread in the plant kingdom
396 [28], was found from *Sarracenia* spp. lids in our study. The compound is known to attract
397 mosquitos [41], and Miles et al. [7] described it as one of *S. flava*'s volatile organic compounds.
398 The Venus flytrap (*Dionaea muscipula*), another carnivorous plant, emits this volatile organic
399 compound when it is feeding on fruit flies (*Drosophila melanogaster*) [35].

400 Sarracenin (Fig 4A) has previously been reported from *S. flava* [17], *S. alata*, *S. leucophylla*, *S.*
401 *minor* and *S. rubra* [18]. Our study confirms the presence of this compound in all the
402 aforementioned species, except *S. minor*. Our study revealed several new species containing
403 sarracenin, namely, *S. psittacina*, *S. purpurea* and *D. californica*. The compound is volatile and
404 attracts insects to *Heliamphora* sp. [4]. A possible explanation of why *S. minor* did not accumulate
405 sarracenin in our study could be that our samples were not feeding on insects at the time of
406 collection, and as a result, they did not synthesize the compound [4].

(A)

Sarracenin Methyl 5-ethylnicotinate Methyl nicotinate (*E*)-Coniferol 2,5-Di-tert-butylaniline

4,8,12,16-Tetramethylheptadecan-4-olide (*Z*)-13-Docosenamide Coniine

(B)

Actinidine *p*-Cymene -Eudesmol Isochiapin B

Pulegone Vanillin Lagumicine 6-Nitro-2,3-dimethylindole

Fig 4. Compounds identified in *Sarracenia* and *D. californica*. (A) Common and (B) specific constituents of *Sarracenia* and *D. californica.*

We also found (*Z*)-13-docosenamide (erucamide) to be a common compound in *Sarracenia* spp. and *D. californica*. It has previously been reported from *H. tatei* and *H. heterodoxa* [4], where it is a possible lubricating component of the nectar.

Other common compounds from *Sarracenia* sp. and *D. californica* are carboxylic acids (fatty acids) such as tetradecanoic, hexadecanoic and (*Z*)-9-hexadecenoic acids. All three are floral scent compounds and the latter is known from *Hydnora africana* [42]. Hexadecanoic acid is emitted by the Venus fly trap as a volatile organic compound after feeding [35].

417 *Sarracenia* spp. display a huge variety of unique compounds which are found only in their lid
418 and/or pitcher. Actinidine is a floral scent compound known from *Sauromatum guttatum* [43] and
419 an insect pheromone in Hymenoptera [44]. *Trans*-Jasmone acts either as an insect attractant or
420 repellent depending on the insect species. Pulegone (Fig 4B) is a floral scent compound on *Tilia* sp.
421 [45] and *Agastache* sp. [46], and functions as an insecticide [47]. 14-β-Pregna is a sex pheromone
422 of the insect *Eurygaster maura* [48]. Lagumicine was found from *S. oreophila* lid. Previously it had
423 been found from *Alstonia angustifolia* var. *latifolia* [49]. Miles et al. [17] suggested that terpene
424 indole alkaloids could be synthesized in *Sarracenia* spp. based on the possible cleavage of
425 sarracenin.

426 The studied accessions of Sarraceniaceae are characterized by a large number of diverse
427 metabolites, with nearly 600 metabolites identified in lids as well as in pitchers. They are also
428 characterized by a huge chemical diversity, as the metabolite compositions of different plants were
429 largely distinct. Unlike mutation data from highly conserved genomic loci, the data that mainly
430 displays wide heterogeneity of samples is not suitable for constructing taxonomies. Knudsen et al.
431 [29] concluded that the usability of floral scent compounds in chemotaxonomy is limited because
432 chemical composition usually differs between closely related species. The composition may also
433 vary among genera of a specific family as it may vary among species of a given genus. Thus, the
434 chemical composition alone is of little use for phylogenetic estimates above the genus level. As
435 expected, clustering derived from our data alone does not agree with the phylogenetic structure of
436 the accessions (see the column dendrograms in S6-S9 Figs).

437 The available phylogenetic information, on the other hand, may help us to understand the
438 current data. We sought to explain the metabolite composition of plants with the known
439 phylogenetic information from [2]. We successfully demonstrated that the metabolite data conform
440 with the clade-level classification of the plant family and hence that the phylogeny can explain the
441 metabolite composition of the plants to some extent. Notably, whereas the qualitative data could be

442 largely explained by phylogeny (Figs 1 and 2), the concordance of quantitative data with the clade-
443 level classification was relatively weaker (S1 and S2 Figs). Thus, we speculate that evolution may
444 more directly affect the presence or absence of specific chemicals than the exact amount in which
445 the chemicals are present.

446 We have limited the focus of the current data mining to cataloguing and visualizing the data.
447 Given the dominance of zeroes, the current datasets may benefit from computational methods
448 specially designed for zero-inflated or left-censored data. But such a detailed computational
449 analysis is out of the scope of this biochemical profiling study.

450 Conclusion

451 Studied accessions of Sarraceniaceae possessed a diverse variety of compounds. Lids and pitchers
452 were studied separately and approximately 600 compounds were detected in both collections. The
453 accessions also showed huge diversity, with every accession containing unique compounds. Coniine
454 was newly detected in seven *Sarracenia* species in addition to the known source, *S. flava*. However,
455 we could not identify a specific candidate gene involved in coniine biosynthesis in *Sarracenia* spp.
456 Among the common constituents of Sarraceniaceae are sarracenin, erucamide, and nonanal. By
457 integrating existing phylogenetic information of Sarraceniaceae, we successfully demonstrated that
458 the phylogeny can explain the metabolite composition of the plants. Phylogeny explained the
459 presence or absence of compounds more strongly than their concentrations.

460 Acknowledgments

461 We thank Kaarina Viljanen, Anna-Liisa Ruskeepää and Airi Hyrkäs for GC-MS analyses, Jan
462 Schlauer and Teemu Teeri for commenting on a draft of the manuscript, Michael Bailey for
463 proofreading and language editing and Russell L. Malmberg for providing contigs of *S. psittacina*
464 and *S. purpurea* transcriptomes.

465 # Author contributions

466 Conceived and designed the experiments: HH PG HR. Performed the experiments: HH PG TS-L.
467 Analyzed the data: HH PG TS-L. Wrote the paper: HH PG HR.

468 # References

469 1. McPherson S, Wistuba A, Fleischmann A, Nerz J. Sarraceniaceae of South America. Poole,
470 Dorset, England: Redfern Natural History Productions; 2011.

471 2. Stephens JD, Rogers WL, Heyduk K, Cruse-Sanders JM, Determann RO, Glenn TC,
472 Malmberg RL. Resolving phylogenetic relationships of the recently radiated carnivorous
473 plant genus *Sarracenia* using target enrichment. Mol Phylogenet Evol. 2015; 85: 76-87.

474 3. Dress WJ, Newell SJ, Nastase AJ, Ford JC. Analysis of amino acids in nectar from pitchers
475 of *Sarracenia purpurea* (Sarraceniaceae). Am J Bot. 1997; 84: 1701-1706.

476 4. Jaffé K, Blum MS, Fales HM, Mason RT, Cabrera A. On insect attractants from pitcher
477 plants of the genus *Heliamphora* (Sarraceniaceae). J Chem Ecol. 1995;21: 379-384

478 5. Schlauer J, Nerz J, Rischer H. Carnivorous plant chemistry. Acta Bot Gall. 2005;15(2): 187-
479 195.

480 6. Harris CS, Asim M, Saleem A, Haddad PS, Arnason JT, Bennett SAL. Characterizing the
481 cytoprotective activity of *Sarracenia purpurea* L., a medical plant that inhibits glucotoxicity
482 in PC12 cells. BMC Complement Altern Med. 2012;12: 245.

483 7. Miles DH, Kokpol U, Mody NV. Volatiles of *Sarracenia flava*. Phytochemistry. 1975;14:
484 845-846.

485 8. Jürgens A, El-Sayed AM, Suckling DM. Do carnivorous plants use volatiles for attracting
486 prey insects? Funct Ecol. 2009;23: 875-887.

487 9. Sheridan PM, Mills RR. 1998. Presence of proanthocyanidins in mutant green *Sarracenia*
488 indicate blockage in late anthocyanin biosynthesis between leucocyanidin and pseudobase.
489 Plant Sci. 1998;135: 11-16.

490 10. Sheridan PM, Griesbach RJ. Anthocyanidins of *Sarracenia* L. flowers and leaves.
491 Hortscience. 2001;36: 384.

492 11. Romeo JT, Bacon JD, Marby TJ. 1977. Ecological considerations of amino acids and
493 flavonoids in *Sarracenia* species. Biochem Syst Ecol. 1977;5: 117-120.

494 12. Hu J-F, Starks CM, Williams RB, Rice SM, Norman VL, Olson KM, Hough GW, Goering
495 MG, O'Neill-Johnson M, Eldridge GR. Secoiridoid glycosides from the pitcher plant
496 *Sarracenia alata*. Helv Chim Acta. 2009; 92: 273-280.

497 13. Muhammad A, Haddad PS, Durst T, Arnason JT. 2013. Phytochemical constituents of
498 *Sarracenia purpurea* L. (pitcher plant). Phytochemistry. 2013;94: 238-242
499 14. Cieniak C, Walshe-Roussel B, Liu R, Muhammad A, Saleem A, Haddad PS, Cuerrier A,
500 Foster BC, Arnason JT. Phytochemical comparison of the water and ethanol leaf extracts of
501 the Cree medicinal plant, *Sarracenia purpurea* L. (Sarraceniaceae). J Pharm Pharm Sci.
502 2015;18(4): 484-93.

503 15. Cipollini Jr DF, Newell SA, Nastase AJ. Total carbohydrates in nectar of *Sarracenia*
504 *purpurea* L. (Northern Pitcher Plant). Am Midl Nat. 1994;131: 374–377.

505 16. Deppe JL, Dress WJ, Nastase AJ, Newell SJ, Luciano CS. Diel variation of sugar amount in
506 nectar from pitchers of *Sarracenia purpurea* L. with and without insect visitors. Am Midl
507 Nat. 2000;144: 123-132.

508 17. Miles DH, Kokpol U, Bhattacharayya J, Atwood JL, Stone KE, Bryson TA, Wilson C.
509 Structure of sarracenin. An unusual enol diaceal monoterpene from the insectivorous plant
510 *Sarracenia flava*. J Am Chem Soc. 1976;98: 1569-1573.

511 18. Newman T, Ibrahim S, Wheeler JW, McLaughlin WB, Petersen RL, Duffield RM.
512 Identification of sarracenin in four species of *Sarracenia* (Sarraceniaceae). Biochem Syst
513 Ecol. 2000;28: 193-195.

514 19. Miles DH, Kokpol U, Zalkow LH, Steindel SJ, Nabors JB. Tumor inhibitors I: Preliminary
515 investigation of antitumor activity of *Sarracenia flava*. J Pharm Sci. 1974;63: 613-615.

516 20. Miles DH, Kokpol U. Tumor inhibitors II: Constituents and antitumor activity of *Sarracenia*
517 *flava*. J Pharm Sci. 1976; 65: 284-285.

518 21. Mody NV, Henson R, Hedin PA, Kokpol U, Miles DH. Isolation of insect paralysing agent
519 coniine from *Sarracenia flava*. Experientia. 1976;32: 829-830.

520 22. Dring JV, Nash RJ, Roberts MF, Reynolds T. Hemlock alkaloids in Aloes. Occurrence and
521 distribution of γ-coniceine. Planta Med. 1984;50: 442-443.

522 23. Nash RJ, Beaumont J, Veitch NC, Reynolds T, Benner J, Hughes CNG, Dring JV, Bennett
523 RN, Dellar JE. Phenylethylamine and piperidine alkaloids in *Aloe* species. Planta Med.
524 1992;58: 84-87.

525 24. Hotti H, Seppänen-Laakso T, Arvas M, Teeri TH, Rischer H. Polyketide synthases from
526 poison hemlock (*Conium maculatum* L.). FEBS J. 2015;282(21): 4141-56.

527 25. Harborne JB. Introduction to ecological biochemistry. 2nd ed. London, UK: Academic
528 Press; 1982.

529 26. Ellison AM, Butler ED, Hicks EJ, Naczi RF, Calie PJ, Bell CD, et al. Phylogeny and
530 biogeography of the carnivorous plant family Sarraceniaceae. PLoS One. 2012;7(6):e39291.

531 27. Bayer RJ, Hufford L, Soltis DE. Phylogenetic relationships in Sarraceniaceae based on rbcL
532 and ITS sequences. Syst Bot. 1996;21: 121-134.

533 28. Neyland R, Merchant M. Systematic relationships of Sarraceniaceae inferred from nuclear
534 ribosomal DNA sequences. Madroño. 2006;53: 223-232.

535 29. Knudsen JT, Eriksson R, Gershenzon J, Ståhl B. Diversity and distribution of floral scent.
536 Bot Rev. 2006;72: 1-120

537 30. McPherson S, Schnell D. Sarraceniaceae of North America. Poole, UK: Redfern Natural
538 History Productions Ltd; 2011.

539 31. Häkkinen ST, Moyano E, Cusidó RM, Palazón J, Piñol MT, Oksman-Caldentey K-M.
540 Enhanced secretion of tropane alkaloids in *Nicotiana tabacum* hairy roots expressing
541 heterologous hyoscyamine-6β-hydroxylase. J Exp Bot. 2005;56: 2611-2618.

542 32. Srivastava A, Rogers WL, Breton CM, Cai L, Malmberg RL. Transcriptome analysis of
543 *Sarracenia*, an insectivorous plant. DNA Res. 2011;18: 253-261.

544 33. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious
545 Basic: an integrated and extendable desktop software platform for the organization and
546 analysis of sequence data. Bioinformatics. 2012;28(12): 1647-9.

547 34. Junghans H, Dalkin K, Dixon RA. Stress responses in alfalfa (*Medicago sativa* L.). 15.
548 Characterization and expression patterns of members of a subset of the chalcone synthase
549 multigene family. Plant Mol Biol. 1993; 22: 239-253.

550 35. Kreuzwieser J, Scheerer U, Kruse J, Burzlaff T, Honsel A, Alfarraj S, et al. The Venus
551 flytrap attracts insects by the release of volatile organic compounds. J Exp Bot. 2014;65(2):
552 755-66.

553 36. Witten DM, Tibshirani R. A framework for feature selection in clustering. JASA.
554 2010;105(490): 713-726.

555 37. R Core Team. R: A language and environment for statistical computing. R Foundation for
556 Statistical Computing, Vienna, Austria. 2014. Available: http://www.R-project.org/.

557 38. Austin MB, Noel JP. The chalcone synthase superfamily of type III polyketide synthases.
558 Nat Prod Rep. 2003;20(1): 79-110.

559 39. Butler JL, Ellision AM. Nitrogen cycling dynamics in the carnivorous northern pitcher
560 plant, *Sarracenia purpurea*. Funct Ecol. 2007;21: 835-843.

561 40. Roberts MF. Enzymology of alkaloid biosynthesis. In: Roberts MF, Wink M, editors.
562 Alkaloids: Biochemistry, ecology, and medicinal applications. New York, USA: Springer;
563 1998. pp. 109-146.

564 41. Syed Z, Leal WS. Acute olfactory response of *Culex* mosquitoes to a human- and bird-
565 derived attractant. Proc Natl Acad Sci U S A. 2009;106(44): 18803-8.

566 42. Burger BV, Munro ZM, Visser JH. Determination of plant volatiles 1: analysis of the insect-
567 attracting allomone of the parasitic plant *Hydnora africana* using Grob-Habich activated
568 charcoal traps. J High Resol Chromatogr. 1988;11: 496-499.

569 43. Borg-Karlson A-K, Valterová I, Nilsson LA. Volatile compounds from flowers of six
570 species in the family Apiaceae: Bouquets for different pollinators? Phytochemistry.
571 1993;35(1):111-9.

572 44. Janssen E, Bestmann HJ, Hölldobler B, Kern F. *N,N*-dimethyluracil and actinidine, two
573 pheromones of the ponerine ant *Megaponera foetens* (Fab.) (Hymenoptera: Formicidae). J
574 Chem Ecol. 1995;21(12): 1947-55.
575 45. Buchbauer G, Remberg B, Jirovetz L. Comparative headspace analysis of living and fresh
576 cut lime tree flowers (*Tiliae flores*). Flav Fragr J. 1995;10: 221-224.
577 46. Wilson LA, Senechal NP, Widrlechner MP. Headspace analysis of the volatile oils of
578 *Agastache*. J Agric Food Chem. 1992;40: 1362-1366.
579 47. Franzios G, Mirotsou M, Hatziapostolou E, Kral J, Scouras ZG, Mavragani-Tsipidou P.
580 Insecticidal and genotoxic activities of mint essential oils. J Agric Food Chem. 1997;45:
581 2690-2694.
582 48. Durak D, Kalender Y. Fine structure and chemical analysis of the metathoracic scent gland
583 of *Eurygaster maura* (Linnaeus, 1758) (Heteroptera: Scutelleridae). Folia Biol (Krakow).
584 2007;55(3-4): 133-41.
585 49. Kam TS, Choo YM. Alkaloids from *Alstonia angustifolia*. Phytochemistry. 2004;65(5):
586 603-8.

587 # Supplementary material

588 **S1 Table. Unique compounds with their concentration percentages in metabolite samples**.
589 Unique compounds found in *Sarracenia* and *D. californica* (lids and pitchers separately).

590 **S2 Table**. **Sample comparison in pairs.** Samples are compared to each other in lids and pitchers
591 separately.

592 **S1 Fig. Visualization of selected metabolite features from the quantitative data of lids.** (A)
593 Heat map visualization of selected metabolite features from the quantitative data of lids. The
594 phylogenetic tree from [2] is displayed as the column dendrogram. Six samples of our dataset (14,
595 31, 35, 37, 44, and 46) are omitted from this heat map, based on the sample selection procedure

described in the Methods section. (B) Comparison of average within-clade distances (aWCDs) against the background distribution of average species-level distances (aSLDs) and average between-clade distances (aBCDs). Distribution of aSLDs was calculated using qualitative data of the selected metabolite features and displayed in a density plot. The black vertical lines mark the individual aWCDs. The orange dashed and dotted lines show the mean and median of aSLDs. The purple dashed and dotted lines show the mean and median of aBCDs. (C) Comparison of aWCDs (green continuous density line) with aBCDs (orange dashed density line).

S2 Fig. Visualization of selected metabolite features from the quantitative data of pitchers. (A) Heat map visualization of selected metabolite features from the quantitative data of pitchers. The phylogenetic tree from [2] is displayed as the column dendrogram. Six samples of our dataset (1, 11, 31, 38, 42, and 46) are omitted from this heat map, based on the sample selection procedure described in the Methods section. (B) Comparison of average within-clade distances (aWCDs) against the background distribution of average species-level distances (aSLDs) and average between-clade distances (aBCDs). Distribution of aSLDs was calculated using qualitative data of the selected metabolite features and displayed in a density plot. The black vertical lines mark the individual aWCDs. The orange dashed and dotted lines show the mean and median of aSLDs. The purple dashed and dotted lines show the mean and median of aBCDs. (C) Comparison of aWCDs (green continuous density line) with aBCDs (orange dashed density line).

S3 Fig. Alignment of *Sarracenia* PKSs with selected plant-PKSs translated into an amino acid sequence. Conserved amino acids of the active site are bolded and coloured amino acids indicate mutated amino acids of the active site. GenBank accession numbers: *Conium maculatum* CPKS1 (KP726914), *Conium maculatum* CPKS2 (KP726915), *Conium maculatum* CPKS5 (KP726916), *Gerbera hybrida* 2PS (CAA86219.2), *Gerbera hybrida* CHS1 (Z38096.1), *Medicago sativa* CHS2 (L02902.1).

620 **S4 Fig. Barplot of distribution of compounds across lid samples.**

621 **S5 Fig. Barplot of distribution of compounds across pitcher samples.**

622 **S6 Fig. Heat map of selected features obtained from qualitative data of lids.**

623 **S7 Fig. Heat map of selected features obtained from quantitative data of lids.**

624 **S8 Fig. Heat map of selected features obtained from qualitative data of pitchers.**

625 **S9 Fig. Heat map of selected features obtained from quantitative data of pitchers.**

Series title and number

VTT Science 135

Title

The killer of Socrates exposed – Coniine in the plant kingdom

Author(s)

Hannu Hotti

Abstract

Poison hemlock *(Conium maculatum* L.) is a poisonous plant which is speculated to have been the main ingredient of the toxic potion given to the philosopher Socrates. The plant's main compound of the thirteen known piperidine alkaloids is coniine. The compound is also present in twelve *Aloe* species and an unrelated carnivorous plant, *Sarracenia flava* L. Coniine is a toxic alkaloid, the biosynthesis of which is not well understood. A possible route, supported by evidence from labelling experiments, involves a polyketide formed by a condensation reaction of one acetyl-CoA and three malonyl-CoAs catalysed by a polyketide synthase (PKS). This study focused on identification and characterization of PKS-genes involved in coniine formation, induction of callus from plants containing hemlock alkaloids and investigation of the possibility to elicitate the alkaloid pathway in cell culture in order to understand coniine biosynthesis. Plant materials involved in different stages of this study were investigated for their alkaloid content using gas chromatography-mass spectrometry (GC-MS).

PKS-genes or their fragments were isolated from poison hemlock using the rapid amplification of cDNA ends -method and transcriptome analysis. Three expressed enzymes were characterized by feeding different starter-CoAs in vitro. Based on the results of *in vitro* experiments, two of the three characterised *PKS-genes* in poison hemlock are chalcone synthases (CPKS1, CPKS2) and one is a novel type PKS (CPKS5). CPKS5 kinetically favours butyryl-CoA as a starter-CoA *in vitro*. These results suggest that CPKS5 is responsible for the initiation of coniine biosynthesis by catalysing the synthesis of the carbon backbone from one butyryl-CoA and two malonyl-CoAs.

In order to induce coniine biosynthesis in poison hemlock cell culture, different elicitors were tested. Alginic acid, cellulase, chitosan, silver nitrate and copper(II) sulphate all induced the production of furanocoumarins. Extracts contained bergapten, columbianetin, isopimpinellin, marmesin, oroselone, psoralen and xanthotoxin, but no piperidine alkaloids. Plant hormones (ethylene, methyl jasmonate, and salicylic acid) were not able to induce furanocoumarin biosynthesis. The relative amount of furanocoumarins was generally higher in the medium than in the cells.

Aloe gariepensis and *A. viguieri* formed callus which did not contain piperidine alkaloids. Micropropagation of *A. viguieri* was investigated using computer-generated statistical experimental design. Up to five plantlets of good quality were produced from a mother plant. *In vitro* cultivated *A. gariepensis*, *A. globuligemma* and *A. viguieri* were found to contain coniine, -coniceine and also *N*-methylconiine, an alkaloid not previously reported from *Aloe* spp. In order to analyse low concentrations of coniine alkaloids in *Sarracenia* species, a sensitive GC-MS method was developed. *S. flava* was confirmed to contain coniine, as were seven other *Sarracenia* species.

These results together represent a single step in the long road to understanding the distribution of coniine in the plant kingdom. The hemlock alkaloids are more widely distributed than was previously believed. Different *in vitro* techniques, such as cell cultures, will be valuable in the investigation of the coniine biosynthesis route. The identification of the first enzyme (CPKS5) of this route at the gene level will open the door for further discovery with the help of the sequenced transcriptome of poison hemlock's different organs.

ISBN, ISSN, URN	ISBN 978-951-38-8459-8 (Soft back ed.) ISBN 978-951-38-8458-1 (URL: http://www.vttresearch.com/impact/publications) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online) http://urn.fi/URN:ISBN:978-951-38-8458-1
Date	October 2016
Language	English, Finnish abstract
Pages	146 p. + app. 119 p.
Name of the project	
Commissioned by	
Keywords	alkaloids, Aloe, coniine, Conium maculatum, polyketide, polyketide synthase, Sarracenia, secondary metabolites
Publisher	VTT Technical Research Centre of Finland Ltd P.O. Box 1000, FI-02044 VTT, Finland, Tel. 020 722 111

Julkaisun sarja ja numero

VTT Science 135

Nimeke

Sokrateen tappaja esille – koniini kasvikunnassa

Tekijä(t)

Hannu Hotti

Tiivistelmä

Myrkkykatko (*Conium maculatum* L.) on myrkyllinen kasvi, jonka arvellaan olleen pääainesosa Sokrateelle annetussa myrkyssä. Eniten kasvissa on sen tunnetuista kolmestatoista piperidiinialkaloidista koniinia. Tuo yhdiste tunnetaan myös kahdeltatoista *Aloe-lajilta* sekä lihansyökasvi *Sarracenia flavalta* L. Koniini on myrkyllinen yhdiste, jonka biosynteesiä ei kunnolla tunneta. Erään ehdotuksen, jota radioaktiivisuuskokeiden tulokset tukevat, mukaan polyketidisyntaasi (PKS) aloittaisi reitin muodostamalla polyketidin yhdestä asetyyli-CoA:sta ja kolmesta malonyyli-CoA:sta. Tässä tutkimuksessa keskityttiin tunnistamaan ja karakterisoimaan *PKS*-geenit, jotka ovat mukana koniinin biosynteesissä. Lisäksi tutkittiin koniinialkaloideja sisältävillä kasveilla kalluksen muodostusta sekä mahdollisuutta elisitoida alkaloidituotanto kasvisoluviljelmässä, jotta koniininbiosynteesiä ymmärrettäisiin paremmin. Tutkimuksen eri vaiheissa käytetty kasvimateriaalin alkaloidisisältö tutkittiin kaasukromatografiamassaspektrometrillä (GC-MS).

PKS-geenejä tai niiden paloja eristettiin myrkkykatkolta käyttämällä RACE-menetelmää ja traskriptomianalyysiä. Kolme ilmenevää entsyymiä karakterisoitiin antamalla niille erilaisia aloittaja-CoA:ta koeputkessa. Näiden kokeiden perusteella kaksi kolmesta myrkkykatkon *PKS*-geeneistä oli kalkonisyntaaseja (CPKS1, CPKS2) ja yksi uudenlainen PKS (CPKS5). Kineettisesti CPKS5 käyttää parhaiten butyryyli-CoA:ta aloittaja-CoA:na koeolosuhteissa. Näiden tulosten perusteella CPKS5 aloittaa koniinin biosynteesin muodostamalla yhdisteen hiilirungon yhdestä butyryyli-CoA:sta ja kahdesta malonyyli-CoA:sta.

Erilaisia elisiittoreita testattiin myrkkykatkon soluviljelmässä, jotta voitaisiin selvittää mahdollisuutta koniinin kertymiseen. Algiinihappo, sellulaasi, kitosaani, hopeanitraatti ja kupari(II)sulfaatti saivat aikaiseksi furanokumariinien kertymisen viljelmiin. Uutteet sisälsivät bergapteenia, kolumbianetiinia, isopimpinelliiniä, marmesiinia, oroselonea, psoraleenia ja ksantotoksiinia, mutta eivät yhtään piperidiinilalkaloidia. Kasvihormonit (etyleeni, metyylijasmonaatti ja salisyylihappo) eivät saaneet aikaiseksi furanokumariinituotantoa. Furanokumariinien suhteelliset määrät olivat yleisesti suuremmat kasvatusalustassa kuin soluissa.

Aloe gariepensis ja *A. viguieri* muodostivat kallusta, joka ei sisältänyt piperidiinialkaloideja. *A. viguierin* mikrolisäystä tutkittiin tietokoneavusteisella koeasettelulla ja jopa viisi hyvänlaatuista pikkukasvia saatiin muodostumaan yhdestä emokasvista. *In vitro*-kasvatetuista *A. gariepensikestä*, *A. globuligemmasta* ja *A. viguierista* löydettiin koniinia, -konikeiinia sekä uutena *Aloe*-suvulle *N*-metyylikoniinia. Jotta *Sarracenia*-lajeille tyypillinen matala koniinipitoisuus saataisiin tutkittua, kehitettiin herkkä GC-MS-menetelmä. Tällä menetelmällä saatiin varmistettua koniini *S. flavassa* sekä seitsemässä muussa *Sarracenia*-lajissa.

Näiden tulosten yhteisvaikutuksena ymmärryksemme koniinin biosynteesin selvittämisessä on mennyt eteenpäin. Nämä alkaloidit ovat levinneet laajemmalle kuin aikaisemmin on tiedetty. Erilaiset *in vitro* -menetelmät, kuten kalluksen tutkiminen, ovat arvokkaita, kun tutkitaan koniinin biosynteesireittiä. Tuon reitin ensimmäisen entsyymin, CPKS5:n, löytäminen auttaa tunnistamaan reitin muut entsyymit myrkkykatkon eri osien sekvensoidusta transkriptomeista.

ISBN, ISSN, URN

ISBN 978-951-38-8459-8 (nid.)
ISBN 978-951-38-8458-1 (URL: http://www.vtt.fi/julkaisut)
ISSN-L 2242-119X
ISSN 2242-119X (Painettu)
ISSN 2242-1203 (Verkkojulkaisu)
http://urn.fi/URN:ISBN:978-951-38-8458-1

Julkaisuaika

Lokakuu 2016

Kieli

Englanti, suomenkielinen tiivistelmä

Sivumäärä

146 s. + liitt. 119 s.

Projektin nimi

Rahoittajat

Avainsanat

alkaloidit, Aloe, Conium maculatum, koniini, polyketidi, polyketidisyntaasi, Sarracenia, sekundäärimetabolia

Julkaisija

Teknologian tutkimuskeskus VTT Oy
PL 1000, 02044 VTT, puh. 020 722 111

The killer of Socrates exposed – Coniine in the plant kingdom

Coniine, a piperidine alkaloid, is known from poison hemlock (*Conium maculatum* L.), twelve *Aloe* species and *Sarracenia flava* L. Its biosynthesis is not well understood, although a possible route starts with a polyketide formed by a polyketide synthase (PKS). This study focused on identification and characterization of PKS-genes involved in coniine formation, induction of callus from plants containing hemlock alkaloids and investigation of the possibility to elicitate the alkaloid pathway in cell culture in order to understand coniine biosynthesis. Plant materials involved in different stages of this study were investigated for their alkaloid content using gas chromatography-mass spectrometry. A novel type of PKS, CPKS5, was identified as the starter candidate for the initiation of coniine biosynthesis by catalysing the synthesis of the carbon backbone from one butyryl-CoA and two malonyl-CoA moieties. When elicitated, poison hemlock cell cultures produced furanocoumarins but no piperidine alkaloids. The hemlock alkaloids are wider distributed than previously has been thought among *Sarracenia,* and *Aloe* spp. contain a new alkaloid for the genus. These results together pave the way towards possible utilization of hemlock alkaloids.

ISBN 978-951-38-8459-8 (Soft back ed.)
ISBN 978-951-38-8458-1 (URL: http://www.vttresearch.com/impact/publications)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8458-1