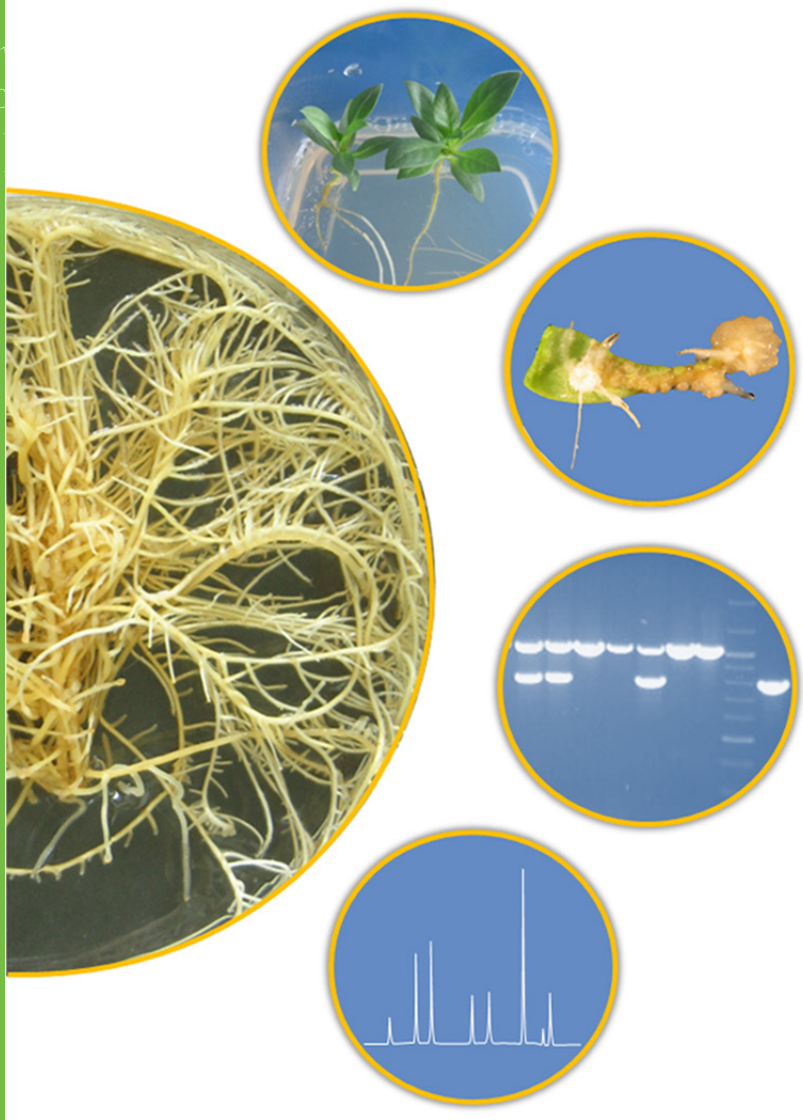

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Dissertation
93

Alkaloids of *in vitro* cultures of *Rhazya stricta*

Amir Akhgari

VTT

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Alkaloids of *in vitro* cultures of *Rhazya stricta*

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Abstract

Rhazya stricta Decne. (Apocynaceae) is a traditional medicinal plant in the Middle East and South Asia. It produces a large number of terpenoid indole alkaloids (TIAs), some of which possess important pharmacological properties. This study focused on the establishment of biotechnological production tools of *R. stricta*, namely undifferentiated cell cultures, and an *Agrobacterium rhizogenes*-mediated transformation method to obtain hairy roots expressing heterologous genes from the early TIA pathway. As *Rhazya* alkaloids comprise a wide range of structures and polarities it was necessary first to develop different analytical methods to determine the alkaloid contents and changes in their profiles in transgenic cultures and after various treatments. Targeted and non-targeted analyses from cell and organ cultures were carried out using gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and nuclear magnetic resonance (NMR) spectroscopy.

Callus cultures were successfully initiated from five different explants on modified B5 medium containing phytohormones. The phenotypes of the calli varied, but as was expected the callus cultures accumulated lower levels of alkaloids than wild type hairy roots and adventitious roots. Surprisingly, calli derived from stems had elevated levels of strictosidine lactam compared to other cultures.

Transformation experiments revealed that only leaves but not cotyledons, hypocotyls or stem segments were susceptible to *Agrobacterium* infection and subsequent root induction. The transformation efficiency varied from 22% to 83% depending on the gene. Wild type and *gus* hairy root clones contained twofold higher amounts of alkaloids than adventitious roots. A total of 17 TIAs, including glycosylated alkaloids, were identified from hairy root extracts by UPLC-MS. GC-MS analysis allowed the separation of the most volatile and non-polar alkaloids in a single run. The composition of typical non-polar alkaloids indicated the occurrence of 20 TIAs belonging to nine different groups. The quantities of these alkaloids varied between clones in the order eburenine, vincanine, vallesiachotamine and yohimbine isomer II. The occurrence of pleiocarpamine, fluorocarpamine, vincamine, ajmalicine, and yohimbine isomers, analysed by GC-MS, and serpentine and its isomer, tetrahydrosecodinol as well as tabersonine, analysed by UPLC-MS, is reported here for the first time from *R. stricta*.

Methyl jasmonate, a well-known elicitor, caused a significant increase in the total alkaloid content of wild type hairy roots as determined by NMR analyses. Detailed targeted analyses by GC-MS showed that the contents of eight out of ten studied alkaloids increased compared to non-elicited cultures. Another studied elicitor, chitosan, did not have any effect on individual alkaloid contents.

Transgenic hairy root clones did not exhibit phenotype differences. Multivariate analysis from NMR data showed a clear discrimination between transformed and wild type/*gus* cultures. This was most probably due to differences in primary metabolites, as the total alkaloid content did not vary between different hairy roots and controls. In general, the production of individual TIAs, analysed by HPLC, was repressed in hairy roots transformed with geraniol synthase (*ges*) and geraniol 8-oxidase (*g8o*) genes compared to the wild types. Overexpression of the strictosidine synthase (*str*) gene resulted in a higher accumulation of serpentine, whereas the production of strictosidine lactam was decreased. There were no significant differences in the contents of other alkaloids compared to the wild type hairy roots.

In conclusion, a simple and efficient gene transfer method is reported for *R. stricta* for the first time. New analytical methods were established which enabled comprehensive investigation of the alkaloids. These data might serve as a basis for further utilization of biotechnological methods for *R. stricta* and its further metabolic engineering.

Keywords hairy roots, transgenic roots, medicinal plant, organ and cell culture, plant secondary metabolites, elicitation, chemical analysis

Tiivistelmä

Alkaloidit *Rhazya stricta* Decne. *in vitro* -viljelmissä

Rhazya stricta Decne. (Apocynaceae) on Lähi-idästä ja Kaakkois-Aasiasta kotoisin oleva perinteinen lääkekasvi. Se tuottaa lukuisia terpeeni-indolialkaloideja (TIA), joista useilla on tärkeitä farmakologisia ominaisuuksia. Tämä tutkimus keskittyi bioteknologisten menetelmien kehittämiseen *R. stricta* -kasville. Näihin menetelmiin lukeutuivat erilaistumattomien solukkoviljelmien perustaminen sekä *Agrobacterium rhizogenes* -bakteerin avulla aikaansaadut siirtogeeniset karvajuuret, jotka ilmensivät varhaisen TIA-synteesireitin geenejä. *Rhazya*-alkaloidit muodostavat laajan kirjon kemiallisia, erilaisen polaarisuuden omaavia rakenteita. Tästä syystä oli tärkeää kehittää uusia analyysimenetelmiä alkaloidi-pitoisuuksien määrittämiseen ja alkaloidiprofiilien muutosten seuraamiseen eri siirtogeenisten linjojen ja käsittelyiden välillä. Kohdennetut ja kohdentamattomat analyysit solu- ja solukkoviljelmistä suoritettiin kaasukromatografia-massaspektrometrialla (GM-MS), korkean erotuskyvyn neste-kromatografialla (HPLC), erittäin korkean suorituskyvyn nestekroma-tografialla (UPLC-MS) ja ydinmagneettisella resonanssispektroskopialla (NMR).

Kallusviljelmät perustettiin viidestä eri kasvinosasta modifioidulle, kasvutekijöitä sisältävälle B5-kasvatusalustalle. Kallusten fenotyypit vaihtelivat, mutta kuten oletettua, ne tuottivat pienempiä määriä alkaloideja kuin villityypin juuret tai karvajuuret. Yllättäen varren soluista perustetut kallusviljelmät tuottivat suurempia määriä striktosidiinilaktaamia kuin muut soluviljelmät.

Vain lehdet, toisin kuin sirkkalehdet, alkeisvarret tai varren osat, olivat alttiita agrobakteeri-infektiolle ja siten karvajuurten muodostumiselle. Transformaatio-tehokkuus vaihteli 22 ja 83 %:n välillä riippuen kohdegeenistä. Villityypin sekä *gus*-geeniä ilmentävät karvajuurilinjat tuottivat kaksinkertaisia määriä alkaloideja verrattuna tavallisiin juuriin. UPLC-MS -menetelmällä karvajuurista tunnistettiin kaikkiaan 17 terpeeni-indolialkaloidia, mukaan lukien glykosyloidut alkaloidit. GC-MS -analyysi mahdollisti myös kaikkein helpoimmin haihtuvien, poolittomien alkaloidien erottelun samalla kertaa. Tyypillisten poolittomien alkaloidien koostumus viittasi 20 TIA:n esiintymiseen, jotka voitiin edelleen jakaa yhdeksään ryhmään. Suurimmat vaihtelut alkaloidien pitoisuuksissa eri solulinjojen välillä havaittiin ebureniinin, vinkaniinin, vallesiakotamiinin ja johimbiinin isomeeri II:n kohdalla, tässä järjestyksessä. GC-MS:lla analysoitua pleiokarpamiinia, fluorokarpamiinia, vinkamiinia, ajmalisiinia ja johimbiini-isomeereja sekä UPLC-MS:lla analysoitua serpentiiniä ja sen isomeereja, tetrahydrosekodinolia ja tabersoniinia ei ole tätä ennen ole raportoitu *R. stricta* -kasvista.

NMR-analyysi osoitti metyylijasmonaatin, joka on yleinen elisiittori, lisäävän merkittävästi alkaloidien kokonaismäärää villityypin karvajuurissa. Kohdennettu GC-MS -analyysi osoitti, että kahdeksan alkaloidin pitoisuus kymmenestä lisääntyi

verrattuna käsittelemättömiin karvajuuriin. Toisaalta kitosaanilla, toisella elisiittorilla, ei havaittu olevan vaikutusta yhdenkään yksittäisen alkaloidin pitoisuuteen.

Siirtogeeniset karvajuuret eivät poikenneet fenotyypiltään villityypin karvajuurista. NMR-datan monivarianssianalyysi osoitti kuitenkin selkeän eron siirtogeenisten ja villityypin tai *gus*-karvajuurien välillä. Tämä johtui todennäköisimmin eroista primäärimetaboliittien pitoisuuksissa, koska alkaloidien kokonaispitoisuudessa ei ollut eroa eri karvajuurilinjojen ja kontrollien välillä. Yksittäisten HPLC:lla analysoitujen terpeeni-indolialkaloidien tuotto oli yleisesti vähentynyt geraniolisyntaasi- (*ges*) sekä geranioli-8-oksidaasi-geenejä (*g8o*) ilmentävissä karvajuurilinjoissa verrattuna villityypin karvajuuriin. Striktosidiinisyntaasi-geenin (*str*) yli-ilmeneminen johti kohonneeseen serpentiini pitoisuuteen ja pienentyneeseen striktosidiinilaktaamin tuottoon. Muiden alkaloidien kohdalla ei havaittu merkittävää eroa villityypin karvajuuriin.

Tässä työssä esitetään ensimmäistä kertaa yksinkertainen ja tehokas geeninsiirtomenetelmä *R. stricta* -kasville. Lisäksi kehitettiin joukko uusia analyysimenetelmiä alkaloidien kokonaisvaltaiseen analysoimiseen. Nämä tulokset luovat pohjan bioteknologisten menetelmien laajemmalle käytölle *R. stricta* -kasvin kohdalla ja sen metabolian muokkaamiselle.

Avainsanat karvajuuri, siirtogeeniset juuret, lääkekasvi, solukko- ja soluviljelmä, kasvien sekundaari metaboliitit, elisitaatio, kemiallinen analyysi

Preface

This work was carried out as a joint PhD thesis at VTT Technical Research Centre of Finland Ltd. and at the Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki during the years 2008-2015 and benefitted considerably from the well-established cooperation between the two institutes. The Plant Biotechnology and Metabolomics research team at VTT provided facilities and excellent expertise for plant cell and organ culture, genetic engineering as well as chemical analysis of metabolites. I received invaluable support from the Division of Pharmaceutical Biosciences for isolation of natural products and their chemical analysis. It has been truly a privilege to work with the two institutions. The management, including vice president Anu Kaukovirta-Norja, is acknowledged for providing excellent working facilities at VTT. The Graduate School of Pharmaceutical Research (GSPR), The Finnish Society of Sciences and Letters, COST Action (FA1006 PlantEngine) and VTT grants are gratefully acknowledged for their financial support.

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Amir Akhgari

Academic dissertation

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List of abbreviations

7-DLGT, *7-dlgt*	7-deoxyloganetic acid glucosyltransferase
7-DLH, *7-dlh*	7-deoxyloganic acid hydroxylase
8-HGO, *8-hgo*	8-hydroxygeraniol oxidoreductase
BAP	6-benzylaminopurine
CaMV	cauliflower mosaic virus
CR	cathenamine reductase
CTAB	cetyl trimethyl ammonium bromide
D4H, *d4h*	deacetylvindoline-4-hydroxylase
DAT, dat	deacetylvindoline-4-*O*-acetyltransferase
DCM	dichloromethane
DCI-MS	desorption chemical ionization-mass spectrometry
DMAPP	dimethylallyl diphosphate
EI	electron impact ionization
ESI	electrospray ionization
FAB-MS	fast atom bombardment-mass spectrometry
G8O, *g8o*	geraniol 8-oxidase
G8H, *g8h*	geraniol 8-hydroxylase
G10H, *g10h*	geraniol 10-hydroxylase
GC-MS	gas chromatography-mass spectrometry
GES	geraniol synthase
GLUC	5-bromo-4-chloro-3-indolyl glucuronide
GPP	geranyl diphosphate

GPPS, *gpps*	geranyl diphosphate synthase
GUS, *gus*	β-glucuronidase
HPLC	high performance liquid chromatography
HR-EI-MS	high resolution-electron impact mass spectrometry
IDI, *idi*	isopentenyl diphosphate isomerase
IO, *io*	iridoid oxidase
IPP	isopentenyl diphosphate
IS, *is*	iridoid synthase
IS	internal standard
LAMT, *lamt*	loganic acid *O*-methyltransferase
LOD	limit of detection
LOQ	limit of quantification
MeJA	methyl jasmonate
MEP	methyl erythritol phosphate pathway
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
MVA	mevalonate
NAA	α-naphthaleneacetic acid
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NMR	nuclear magnetic resonance
NMT	*N*-methyltransferase
OPLS	orthogonal partial least squares
OPLS-DA	orthogonal projection to latent structures-discriminant analysis
ORCA3, *orca3*	octadecanoid-derivative responsive *Catharanthus* AP2-domain
PCA	principal component analysis
PCR	polymerase chain reaction
PDA	photodiode array detector
POD	peroxidase
R	reductase

rol B	rooting-locus gene B
RP-HPLC	reversed phase high performance liquid chromatography
SG, *sg*	strictosidine β-D-glucosidase
SLS, *sls*	secologanin synthase
STR, *str*	strictosidine synthase
T16H	tabersonine-16-hydroxylase
THAS	tetrahydroalstonine synthase
TDC, *tdc*	tryptophan decarboxylase
TIA	terpenoid indole alkaloid
TIC	total ion current
TLC	thin-layer chromatography
TMS	trimethylsilyl derivative
UPLC	ultra performance liquid chromatography
UV	ultraviolet
virD	virulence gene D

1. Introduction

The genus *Rhazya* is a member of the Apocynaceae family and comprises two species, *R. stricta* Decne. and *R. orientalis* Decne. *R. stricta* is an evergreen small, glabrous, erect, toxic shrub, which is widely distributed in the Middle East and Indian sub-continent and has been used as a folk medicine to treat several diseases, *e.g.* fever and chronic rheumatism (Atta-ur-Rahman *et al.* 1989b). Alkaloids in *R. stricta* were first detected by Hooper (1906), and since the early 1960s they have been widely reported from this plant. These alkaloids, including a number of isomers, have been classified into 17 sub-groups (Atta-ur-Rahman *et al.* 1989b). The phytochemical, pharmacological and ethnobotanical studies of *R. stricta* have been extensively reviewed (Atta-ur-Rahman *et al.* 1989b, Ali *et al.* 2000, Gilani *et al.* 2007). At least 15 TIAs from *R. stricta* have been subjected to pharmacological and toxicological assays, which have indicated the predominantly anticancer and antibacterial potential of this plant (Gilani *et al.* 2007). Furthermore, a crude ethanolic extract of the fruit has shown antibacterial and lipoxygenase activities, as well as acetylcholinesterase inhibitory effect (Sultana and Khalid 2010). Recently, Baeshen *et al.* (2012) reported that an ethanol extract inhibited cellular growth and colony formation of human breast cancer cell clones through apoptosis induction.

TIAs, like many other secondary metabolites, are usually present in small quantities in plants and their chemical synthesis *in vitro* is often difficult and uneconomical because of their large size and very complex stereochemical structures, *e.g.* the presence of multiple chiral centres (Ziegler and Facchini 2008). For example, the dimeic indole alkaloids vincristine and vinblastine have nine stereocentres. Biotechnological methods for sustainable production of phytochemicals are attractive alternatives for the production of high-value compounds (Rischer *et al.* 2013). Cell and somatic hybrid suspension cultures of *R. stricta* have previously been established and the major TIAs in the cell suspension cultures have been investigated (Pawelka and Stöckigt 1986, Kostenyuk *et al.* 1991, Gerasimenko *et al.* 2001b). However, undifferentiated *in vitro* cell cultures (*e.g.* callus cultures) are often genetically unstable and synthesize valuable secondary metabolites only at very low levels (Sevón and Oksman-Caldentey 2002, Verpoorte *et al.* 2002). By contrast, organ cultures, *e.g.* hairy roots, may exhibit significantly improved, stable production of secondary

metabolites at high levels in comparison to callus and cell suspension cultures and even to intact plants (Oksman-Caldentey and Inzé 2004). Hairy roots can also produce a range of secondary metabolites that are not present in the parent plant (Georgiev *et al.* 2007, Chandra and Chandra 2011). Furthermore, a transgenic root system offers great potential for biotechnological approaches such as elicitation, biotransformation and metabolic engineering through *Agrobacterium rhizogenes*.

The genetic transformation of *R. stricta* either by *Agrobacterium* or with any other method has not hitherto been reported. In this study an *A. rhizogenes*-mediated transformation method was developed and accumulation/composition of TIAs in *R. stricta* transgenic hairy roots was investigated. The plant produces complex alkaloids with a variety of structures and polarities. There are no reports on comprehensive chemical analyses of alkaloids from *R. stricta* crude extracts. Therefore, multiple technologies were developed for targeted (GC-MS, LC and LC-MS) and non-targeted (NMR) analysis of TIAs.

Part of the results of the present study has been published in Akhgari *et al.* 2015a, and submitted for publication Akhgari *et al.* 2015b. Also some results are presented in a manuscript Akhgari *et al.* 2015c.

2. Review of the literature

2.1 The importance of secondary metabolites for human well-being

Plants produce a wide variety of chemical constituents, including both primary and secondary metabolites. Primary metabolites are essential for plant growth and development. Secondary metabolites are low molecular weight compounds, typically less than 3 000 Daltons, fulfil important functions in the interaction between plants and their biotic and abiotic environment, *e.g.* in protection against ultraviolet light as well as in various defence-related reactions (Ziegler and Facchini 2008). Biosynthetic pathways leading to secondary metabolites are often long and complex and the biosynthetic enzymes are usually localized in different cell types and different subcellular compartments. In order to minimize the risk of auto-intoxication, secondary metabolites are usually targeted into compartments with low metabolic activity, usually the vacuole and the extracellular space (Oksman-Caldentey and Inzé 2004). Secondary metabolites are structurally classified into five groups according to their biosynthetic origin: alkaloids, flavonoids, isoprenoids (*e.g.* terpenoids), phenylpropanoids and polyketides. Some compounds (*e.g.* alkaloids) are found only in a few plant families, whereas flavonoids for example are widely distributed in plant kingdom.

Natural products are often produced in minor quantities (less than 1% of the total dry weight) and the production depends greatly on the physiological and developmental stage of the plant. The number of identified secondary metabolites exceeds 100 000, but the actual number in nature is much higher since only 10-30% of plants have been chemically investigated (Wink 2010, Miralpeix *et al.* 2013). Only 10% of estimated 50 000 medicinal plants currently utilised by humans can be cultivated, due to their dependence on specific environmental conditions. For instance, the natural habitat of many such plant species are in tropical rain forests (Rischer *et al.* 2013).

In addition to ecological roles of secondary metabolites, they have long been exploited as sources of pharmaceuticals. For example, opioids from opium poppy *Papaver somniferum* L. have been used since Neolithic times (McCurdy and Scully 2005). The link between specific constituents and their medicinal properties was recognized more than 200 years ago when 1805 the first alkaloid, morphine

was isolated from *P. somniferum* and its narcotic activity was confirmed. The role of natural products in drug discovery and drugs which are derived directly or indirectly from plants and are commercially available or in preclinical development stage have been extensively reviewed (Cragg *et al.* 2009, Newman and Cragg 2009, Cragg and Newman 2012, Cragg and Newman 2012, Rischer *et al.* 2013).

Medicinal plants are still widely used in folk medicine and a major source of novel drugs. Secondary metabolites also have potential as lead compounds in drug discovery (Newman and Cragg 2012). Some examples of plant-derived compounds with pharmacological activities are listed in **Table 1**.

Table 1. Examples of plant-derived compounds with pharmacological activities*.

Product	Type**	Function	Plant species
Ajmaline	TIA	Antiarrhythmic	*Rauwolfia serpentina* (L.) Benth. ex Kurz
Ajmalicine	TIA	Antihypertensive	*Catharanthus roseus* (L.) G. Don
Artemisinin	SL	Antimalarial	*Artemisia annua* L.
Berberine	BIA	Intestinal ailment	*Coptis japonica* (Thunb.)Makino
Camptothecine	QA	Anticancer	*Camptotheca acuminate* Decne.
Capsaicin	A	Counterirritant, analgesic	*Capsicum frutescens* L.
Codeine	BIA	Analgesic, antitussive	*Papaver somniferum* L.
Colchicine	PIA	Anticancer	*Colchium autumnale* L.
Digoxin	CG	Antiarrhythmic	*Digitalis lanata* Ehrh.
Diosgenin	SSA	Steroidal precursor	*Dioscorea deltoidea* Wall. ex Kunth
Ellipticine	TIA	Anticancer	*Ochrosia elliptica* Labill.
Emetine	TTIA	Antiamoebic	*Carapichea ipecacuanha* (Brot.) L.Andersson
Morphine	BIA	Analgesic	*Papaver somniferum* L.
Podophyllotoxin	L	Anticancer	*Podophyllum peltatum* L.
Quinidine	QA	Antiarrhythmic	*Cinchona spp.*
Quinine	QA	Antimalarial	*Cinchona ledgeriana* Moens

Rescinamine	TIA	Antihypertensive	*Rauwolfia spp.*
Reserpine	TIA	Antipsychotic, antihypertensive	*Rauwolfia serpentina* (L.) Benth. ex Kurz
Scopolamine	TA	Antiemetic	*Duboisia spp.*
Serpentine	TIA	Sedative	*Catharanthus roseus* (L.) G. Don
Shikonin	NQ	Antibacterial	*Lithospermum erythrorhizon* Siebold & Zucc.
Strychnine	TIA	Neurotoxin	*Strychnos nux-vomica* L.
Paclitaxel	DT	Anticancer	*Taxus brevifolia* Nutt.
Toxiferine	TIA	Curare toxin, muscle relaxant	*Strychnos toxifera* Schomb. ex Benth
Tubocurarine	BIA	Muscle relaxant	*Chondrodendron tomentosum* Ruiz & Pavón
Vinblastine Vincristine	TIA	Anticancer	*Catharanthus roseus* (L.) G. Don
Vincamine	TIA	Peripheral vasodilator	*Vinca minor* L.
Yohimbine	TIA	Aphrodisiac	*Pausinystalia johimbe* (K.Schum.) Pierre ex Beille

*Dewick 2002, Osbourn and Lanzotti 2009, Miralpeix *et al.* 2013.
**A: amide; BIA: benzylisoquinoline alkaloid; CG: cardiac glycoside; DT: diterpene; L: lignin; NQ: naphthoquinone; PIA: phenethylisoquinoline alkaloid; QA: quinoline alkaloid; SL: sesquiterpene lactone: SSA: steroidal saponin aglycone; TIA: terpenoid indole alkaloid; TTIA: terpenoid tetrahydroisoquinoline alkaloid.

The use of semi-synthetic derivatives of natural products as pharmaceuticals began at the end of the nineteenth century, *e.g.* acetylated salicylic acid from willow bark (*Salix alba* L.) yielded analgesic aspirin (Rainsford 2004). From then on, several semi-synthetic derivatives have been designed to improve the potency, selectivity, or pharmacokinetic parameters of the naturally-occurring compounds. For example, derivatives of podophyllotoxin, *i.e.* etoposide, etopophos and teniposide (Canel *et al.* 2000) and of camptothecin, *i.e.* irinotecan and topotecan (Sirikantaramas *et al.* 2007) have been developed for cancer therapy. The prominent role of natural products in pharmaceutical discovery is remarkable. Between 1981 and 2010, about 50% of new anticancer, 70% of antibacterial and 60% of antiparasitic drugs originated directly or indirectly from natural resources (Newman and Cragg 2012).

It is worth mentioning that during the past 30 years 27% of all the new drug entities released to the market were either natural products or were derived from natural products. In addition, 20% of drugs were synthesized after the molecule

was first identified from natural resources (Rischer *et al.* 2013). According to WHO, 11% of the 252 essential drugs are exclusively generated from plants (Raskin *et al.* 2002). In addition, approximately 25% of the drugs prescribed in industrialized countries contained at least one compound directly or indirectly derived from natural origin (Newman and Cragg 2012).

In addition to their pharmaceutical importance, secondary metabolites are also used as flavours, fragrances, colorants, pheromones and insecticides (**Table 2**), and consequently have economic value (El-Sayed and Verpoorte 2007). For example, the global market for flavour and fragrance compounds was 20 billion dollars in 2007, with an annual increase of 11 to 12% (Hansen *et al.* 2009).

Table 2. Examples of natural products with non-medicinal use*.

Products	Plants
Color/dye	
Anthocyanin	*Aralia cordata* Thunb.
Shikonin	*Lithospermum erythrorhizon* Siebold & Zucc.
Berberine	*Coptis japonica* (Thunb.)Makino
Flavour/taste	
Vanillin	*Vanilla planifolia* Jacks. ex Andrews
Thioglucosinolates	*Wasabia japonica* Matsum.
Hop bitter acids	*Humulus lupulus* L.
Menthol	*Mentha arvensis* L.
Picrocrosin/safranal	*Crocus sativus* L.
Fragrances/aroma	
Rose oil	*Rosa damascena* Mill.
Lavender oil	*Lavandula angustifolia* Mill.
Cinnamaldehyde	*Cinnamomum verum* J.Presl
Insecticide	
Azadirachtin	*Azadirachta indica* A. Juss.
Pyrethrin	*Chrysanthemum cinerariaefolium* (Trevir.) Sch. Bip.

*Dewick 2002, Rao and Ravishankar 2002, Osbourn and Lanzotti 2009.

In addition to the pharmaceutical applications of terpenes and iridoids, these classes of compounds can also be used for non-medicinal purposes. For example, geraniol, an acyclic monoterpene alcohol, is a component of essential oils in many fragrant plant species (Yang *et al.* 2005). It has a rose-like odour and is used in perfumes and as an aromatic fragrance in wine (Herrero *et al.* 2008). Iridoids such as nepetalactone isomers can be used as repellents against tropical mosquitoes (Birkett *et al.* 2011).

Plant-derived alkaloids: More than 200 000 structures of secondary metabolites from plants have been isolated (Springob and Kutchan 2009). It has been estimated that among them, the alkaloid group contains more than 21 000 compounds that are distributed in monocots, dicots and gymnosperms (Wink 2010). Alkaloids occur in about 20% of plant species. Over 12 000 alkaloids have been structurally well characterized. Alkaloids are not evenly distributed within a particular plant. Some alkaloids accumulate in the roots (reserpine), whereas others may be concentrated predominantly in the leaves (nicotine), the fruits (strychnine), the bark (quinine) or the latex (morphine) (Cordell *et al.* 2001). The majority of alkaloids have been found to be derived from amino acid precursors: tyrosine, phenylalanine, anthranilic acid, tryptophan/tryptamine, ornithine/arginine, lysine, histidine and nicotinic acid (Yang and Stöckigt 2010).

Due to their versatile nature, alkaloids can be defined only rather loosely as low molecular weight, heterocyclic nitrogen-containing organic substances of natural origin. However, the simple amino acids and proteins, and nitrogen-containing glycosidic substances such as the aminoglycoside antibiotics are excluded from the defined classification (Buckingham *et al.* 2010). The presence of one or more nitrogen atoms results in primary, secondary or tertiary amine and donates basicity of the alkaloid and, in turn, facilitates their isolation and purification since water-soluble salts can be formed in the presence of mineral acids (Buckingham *et al.* 2010, Samuelsson 2004). The degree of basicity varies significantly depending on the structure of the alkaloid and the presence and location of other functional groups. However, some alkaloids are naturally neutral (Buckingham *et al.* 2010). Alkaloids containing quaternary amines are also found in nature. Alkaloids are generally considered to be plant products; nevertheless, they also occur in humans, animals, insects and microorganisms (Dewick 2002).

Alkaloids are often classified according to the nature of the carbon skeleton, *e.g.* indole pyrrolidine, piperidine, quinolone and isoquinoline alkaloids. Due to their structural complexity, some alkaloids fit into more than one category. However, the carbon skeletons of amino acids, the precursors for alkaloid biosynthesis, principally remain intact in the alkaloid structures. Classification of alkaloids based on their amino acid precursors therefore represents an informative approach. Alkaloids are derived from ornithine (pyrrolidine and tropane alkaloids), lysine (piperidine, quinolizidine and indolizidine alkaloids), nicotinic acid (pyridine alkaloids), tyrosine (phenylethylamines, tetrahydroisoquinoline and amaryllidaceae alkaloids), tryptophan (terpenoid indole, quinoline, pyrroloindole and ergot alkaloids), anthranilic acid (quinazoline, quinoline and acridine alkaloids), histidine (imidazole alkaloid) and purine (saxitoxin and tetrodotoxin alkaloids). However, acetate-derived, phenylalanine-derived and steroidal alkaloids are synthesized by amination reactions and are not derived from amino acids (Dewick 2002).

2.2 Botanical description and ethnopharmacology of *Rhazya stricta*

The genus *Rhazya* belongs to the order Gentianales, family Apocynaceae and subfamily Rauwolfioideae. The genus comprises two species, *Rhazya stricta* Decne. and *Rhazya orientalis* Decne (Ali *et al.* 2000). *Rhazya* species have been named after the Iranian scientist Zakariya Razi (854-925 C.E), better known as Rhazes or Rasis in the west.

R. stricta (**Figure 1**) is indigenous to the Middle East (Iran, Iraq, Saudi Arabia, Oman, Qatar, United Arab Emirates) and South Asia (Afghanistan, India and Pakistan) (Miller and Morris 1988, Gilani *et al.* 2007). In Iran *R. stricta* grows in scattered populations in the Persian Gulf area in the southern part of the country, mainly in the Hormozgan, Kerman and Sistan-Baluchestan provinces. It is known among the local people as 'Eshväräk' (Persian: اشورک).

Figure 1. *Rhazya stricta* plant (Photos by courtesy of Prof. A.M. Assaeed, King Saud University, Saudi Arabia).

R. stricta is a diploid 2n=22 (Noori-Daloii *et al.* 1996), evergreen erect shrub approximately 40-100 cm high, with a smooth central stem and dense semi-erect branches. The plant leaves are alternate, entire, coriaceous, fleshy, yellowish green, 7-10 × 1-2 cm in size, oblong-lanceolate. They are acute and attenuate at the base, shortly petiolate or sessile and their midrib is conspicuous. Flowers are about 3.5 mm across, fragrant, white, bract foliaceous, bracteoles subulate-lanceolate, acute, about 2.5 mm long. Calyx is deeply divided and the lobes ovate, acute-acuminate, and long. Corolla are 1-1.6 cm long, dilated above the middle; lobes about 2.5 mm long, ovate, acute spreading. Disc is cup-shaped. The style is about 3 mm long, filiform, stigma shortly bibbed and rounded. The fruits consist of two follicles, 5-7 × 0.5 cm, straight or slightly curved with pale yellow or brown colour, glabrous and striated. Seeds are about 6 mm long, brown, rugose, shortly winged. The flowering period is from December to March (Jafri 1966, Nazimuddin and Qaiser 1983, Western 1989).

R. stricta has long been used as a folk medicine to cure various ailments in the Middle East. **Table 3** summarizes the use of *R. stricta*. The plant is used

traditionally by the Selimani tribes in Kerman, Iran, to accelerate wound healing (Khaksari *et al.* 2000). The infusion of leaves is used for the treatment of syphilis, sore throat, general debility, chronic rheumatism, joint affections, pain and is used in bitter tonics for fever (Adam 1998). The leaves and fruits are also used for the treatment of burns and rashes (Bashir *et al.* 1994a, Qureshi *et al.* 2007).

Table 3. Ethnopharmacological use of *R. stricta*.

Use	Plant part	Area	Reference
Anti-inflammatory	Whole plant	Persian Gulf area	Tanira *et al.* 1996
Antipyretic	Leaves	Oman	Miller and Morris 1988
Constipation, intestinal diseases	Roots, leave, branches	Pakistan	Ahmad *et al.* 2005
Diabetes	Whole plant	Persian Gulf area	Al-Gonemi 1992, Bashir *et al.* 1994a, Tanira *et al.* 1996
	Roots, leaves	Pakistan	Ahmad *et al.* 2005
Foot burning	Fresh leaves	Pakistan	Sultana *et al.* 2006
Heat effects	Seeds	Pakistan	Qureshi *et al.* 2007
Helminthiasis	Whole plant	United Arab Emirates	Tanira *et al.* 1996, Al-Gonemi 1992
Pimples, acne	Fresh leave, seeds	Pakistan	Sultana *et al.* 2006, Qureshi *et al.* 2007
Rheumatism	Whole plant	India	Dymock *et al.* 1893
	Fruits, leaves	Pakistan	Chopra *et al.* 1956
Skin diseases	Fruits, leaves	Pakistan	Ahmad *et al.* 2004
	Whole plant	United Arab Emirates	Bashir *et al.* 1994a
Sore throat, general debility	Whole plant	India	Dymock *et al.* 1893
		Pakistan	Chopra *et al.* 1956
Stomach pain, colic	Whole plant	United Arab Emirates	Bashir *et al.* 1994a
Syphilis	Leaves	Saudi Arabia	Adam 1998
Tooth ache	Branches	Pakistan	Ahmad *et al.* 2005, Sultana *et al.* 2006
Urinary tract disorders	Whole plant	India, Pakistan	Gilani *et al.* 2007
Vermifuge, purgative	Leaves	Saudi Arabia	Al-Yahia *et al.* 1990
Wounds	Whole plant	Iran	Khaksari *et al.* 2000

As mentioned earlier, *R. stricta* has been widely used in folk medicine to treat various diseases. The pharmacological properties of *R. stricta* extracts and the isolated compounds have been studied in a number of *in vitro* and animal models. These studies are briefly discussed in the following section.

Anticancer and cytotoxic activity: Rhazinilam is a unique molecule due to its activity on tubulin. This compound induces spiralization of tubulin, as vinblastine, and inhibits the cold-induced disassembly of microtubules in a manner similar to paclitaxel (David *et al.* 1994). Many semi-synthetic analogues of rhazinilam have also been investigated in attempts to increase their biological activity (David *et al.* 1997, Dupont *et al.* 1999, Baudoin *et al.* 2002, Edler *et al.* 2009).

Sewarine has been reported to possess marked oncolytic activity (Siddiqui *et al.* 1966). Sewarine, vallesiachotamine and tetrahydrosecamine have also exhibited cytotoxic activity against Eagle's KB carcinoma of the nasopharynx in a cell culture model (Mukhopadhyay *et al.* 1981). In the same model tetrahydrosecamine**,** a dimeric indole alkaloid and tetrahydrosecaminediol, a derivative of tetrahydrosecamine, were also shown to be cytotoxic. The monomeric indole alkaloid 16-epi-*Z*-isositsirikine from the leaves of *R. stricta* displayed antineoplastic activity in both the eagle's KB carcinoma of the nasopharynx *in vitro* and in P-388 lymphocytic leukaemia *in vivo* test systems (Mukhopadhyay *et al.* 1983).

A synthetic compound, *dl*-1-(1-Oxo-3,4-*threo*-3,4,5-trihydroxy-l-pentyl)-β-carboline, derived from 1-formyl-β-carboline present in *R. stricta*, has displayed cytotoxic activity against human promyelocytic leukemia cells HL 60 and human diploid embryonic lung fibroblast cells (Abdel-Moty *et al.* 1997). Strongly basic alkaloid fraction of *R. stricta* leaves has been shown to induce a key enzyme, NAD(P)H:quinone oxidoreductase 1 (Nqo1), involved in cancer chemoprevention in murine and human hepatoma cells (El Gendy *et al.* 2012).

Antibacterial and antifungal activities: Stemmadenine has been shown to exhibit antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* (Mariee *et al.* 1988). An alcoholic extract of *R. stricta* leaves and fruits exhibited antibacterial activity *in vitro* against *Streptococcus pyogenes*, *S. aureus*, *P. aeruginosa* and *Salmonella typhi* (Sultana and Khalid 2010). Alcoholic extract of *R. stricta* fruit also exhibited lipoxygenase and cholinesterase activity.

Studies have shown that fractionated methanol and chloroform extracts possess significant antifungal activity against *Trichophyton longifusis*, *Aspergillus flavus*, *C. albicans* and *Fusarium solani* (Khan and Khan 2007). Chloroform and methanol extracts of the roots of *R. stricta* have shown antimicrobial and anti-fungal activities against *S. aurous*, *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, *Aspergillus terreus*, *A. flavus* and *C. albicans*. Tetrahydrosecamine, isolated from the roots of the plant, exhibited a broad spectrum of antimicrobial activity. Strictanol has also been reported to be active against *E. coli* and *P. aeruginosa* (Bashir *et al.* 1994a,b).

Antioxidant activity: *R. stricta* extract possesses an antioxidant activity in rats, as demonstrated by Ali (2002). The methanolic extract of the leaves exhibited the

highest total phenolic content and an antioxidant potential comparable with antioxidants such as α-tocopherol and the synthetic antioxidant butylated hydroxyanisole (Iqbal *et al.* 2006).

Effect on arachidonic acid (AA) metabolism: Rhazimine has been shown to be a dual and selective inhibitor of platelet activating factor (PAF)-induced platelet aggregation and AA metabolism *in vitro* (Saeed *et al.* 1993). These effects might provide beneficial anti-inflammatory and anti-platelet-activating factor (PAF) effects in addition to those of conventional non-steroidal anti-inflammatory drugs. Furthermore, butanol extracts of *R. stricta* have been found to considerably diminish carrageenan-induced inflammation (Wasfi *et al.* 1995, Tanira *et al.* 1996).

Effects on serum lipid profile concentrations: Aqueous extract of *R. stricta* leaves significantly decreases the concentration of triglycerides (TGs), low density lipoprotein- cholesterol (LDL-c), cholesterol, uric acid and creatinin, but increases the concentration of high density lipoprotein-cholesterol (HDL-c) in male Wistar rats (Baeshin *et al.* 2009). It triggers all these activities without affecting liver enzymes or kidney functions. These discoveries may have a positive impact on cardiovascular patients and may provide a new therapeutic strategy to reduce hypertriglyceridemia.

Other activities: The effect of topical administration of *R. stricta* aqueous extract on cutaneous wound healing has been evaluated in male rats and been shown to be comparable to phenytoin cream (Khaksari *et al.* 2000). Extracts of *R. stricta* have also been shown to possess antidiabetic, herbicidal, nematicidal, pesticidal, larvicidal, hypotensive and leucopenic activities as reviewed by Ali *et al.* (2000) and Gilani *et al.* (2007).

2.3 Phytochemistry of *Rhazya stricta*

R. stricta is rich in terpenoid indole alkaloids (TIAs). Other alkaloids belonging to the piperidines, *e.g.* strictimine (Atta-ur-Rahman and Zaman 1984a), and quinolines, *e.g.* strictigine (Atta-ur-Rahman *et al.* 1996), have also been found in the plant. Strictigine was the first 4-vinylquinoline alkaloid isolated from nature. A total of 75 alkaloids of *R. stricta* have previously been classified into 17 sub-groups (Atta-ur-Rahman *et al.* 1989b). A few non-alkaloidal constituents such as flavonoids (Sultana *et al.* 2005, Zaman 1990), glycosides (Andersen *et al.* 1986), terpenoids (Zaman 1990, Abdl-Moghib *et al.* 1998, Sultana and Khalid 2010), shikimate aromatics (Abdl-Moghib *et al.* 1998) and fatty acid esters (Zaman 1990, Atta-ur-Rahman *et al.* 2008 , Sultana and Khalid 2010) have also been isolated from *R. stricta*.

Hitherto, more than 100 alkaloids have been isolated, structurally characterized and identified from the intact plants (Gilani *et al.* 2007), largely from leaves (Ali *et al.* 2000) but also from stem, roots and fruits (with or without seeds). The majority of the alkaloids which have been identified from *R. stricta* since 1989 fit into the classification by Atta-ur-Rahman *et al.* (1989b). These alkaloids are presented in **Table 4** in the order of the subgroup they belong to.

Chatterjee *et al.* (1961) reported that the total alkaloid content in *R. stricta* reached 8-10%. They reported that an alcoholic extract of air-dried roots and leaves yielded quebrachamine, rhazine (akuammidine) and rhazinine. Structural features of rhazine (Chatterjee *et al.* 1962) and rhazinine (Ganguli *et al.* 1962) were also characterized by the group. In addition, rhazidine, a minor alkaloid of *R. stricta*, was isolated (Chaudhury *et al.* 1963a,b). The occurrence of minor alkaloids, geissoschizine and rhazinilam along with two known alkaloids in the leaves was discovered by the same group (Banerji *et al.* 1970). This was the first report of the isolation of rhazinilam. Detailed structural elucidation of rhazinaline and geissoschizine was also reported (Chatterjee *et al.* 1976). The isolation of geissoschizine from *R. stricta* was of biogenetic importance, since it was postulated as an immediate precursor for the biosynthesis of rhazine, strictamine, secamines and the aspidosperma and eburnamine -type alkaloids (Battersby and Hall 1969, Scott 1970, Zenk and Juenger, 2007, Facchini and De Luca, 2008).

Chromatography of the petroleum-soluble leaf alkaloids yielded the most abundant alkaloid (+)-eburenine along with (-)-quebrachamine, and a mixture of (±)- and (+)-vincadifformine. Chemical reduction of (+)-eburenine produced (+)-aspidospermidine or a mixture of (+)-aspidospermidine and (-)-quebrachamine, (Smith and Wahid 1963).

Aspidospermidine, eburnamine, eburnamenine, eburnamonine, quebrachamine and eburenine (1,2-didehydroaspidospermidine) have been isolated from *R. stricta* leaves (Schnoes *et al.* 1962), and later the group also isolated strictamine (Schnoes *et al.* 1966).

Sewarine was first isolated from the leaves of *R. stricta* (Siddiqui *et al.* 1966). Its structure was further confirmed on the basis of chemical reactions and spectral data (Ahmad *et al.* 1970, Ahmad *et al.* 1971), indicating that sewarine is a phenolic alkaloid with the structure 10-hydroxyakuammicine. Sewarine was only the second phenolic akuammicine derivative to be isolated from natural sources. Previously, 11-hydroxyakuammicine had been isolated from *Vinca erecta* Regel & Schmalh and named vinervine (Ahmad *et al.* 1970). Hitherto, sewarine has only been reported from *R. stricta* and *Rauwolfia volkensii* (K.Schum.) Stapf (Akinloye and Court 1981). Ahmad *et al.* (1977) isolated strictamine, strictalamine and vincanine (nor-C-fluorocurarine) from air-dried leaves of *R. stricta*, and their structures and absolute configurations were determined by X-ray spectroscopy and chemical correlations. Reduction of strictamine followed by oxidation yielded compounds identical with strictalamine and vincanine.

Dimeric indole alkaloids, secamine, isomers of dihydrosecamines and tetrahydrosecamines (Evans *et al.* 1968a), presecamine, dihydropresecamine and tetrahydropresecamine (Cordell *et al.* 1970a) have been isolated from the leaves of *R. stricta*. Monomeric secodine-type alkaloids, tetrahydrosecodine and dihydrosecodine have been isolated from the leaves of *R. stricta* (Cordell *et al.* 1970b).

In 1981, the isolation of polyneuridine, along with the known compounds vallesiachotamine and sewarine from leaves and tetrahydrosecamin from roots, was reported by the Cordell group (Mukhopadhyay *et al.* 1981).

Table 4. Alkaloid composition in *R. stricta.*

Compound	Origin	Formula	MW	Reference
Ajmaline-type				
Leepacine**	L	$C_{21}H_{22}N_2O_3$	350	Habib-ur-Rehman 1987, Atta-ur-Rahman *et al.* 1991
Strictisidine	L	$C_{21}H_{22}N_2O_3$	350	Khanum 1986
Akuammicine-type				
Stemmadenine	L	$C_{21}H_{26}N_3O_2$		Mariee *et al.* 1988
Aspidosperma-type				
1,2-Dehydroaspido-spermidine-*N*-Oxide**	R	$C_{19}H_{24}N_2O$	296	Atta-ur-Rahman and Zaman 1986f, Atta-ur-Rahman and Zaman 1988a, Zaman 1990
15β-Hydroxyvinca-difformine**	L	$C_{21}H_{26}N_2O_3$	354	Atta-ur-Rahman *et al.* 1988b, Fatima 1988
(+)-Aspidospermidine	L[1] A.P[2]	$C_{19}H_{26}N_2$	282	[1]Schnoes *et al.* 1962, [1]Habib-ur-Rehman 1987, [2]Abdbl-Mogib *et al.* 1998
Aspidospermidose**	L	$C_{25}H_{34}N_2O_5$	422	Atta-ur-Rahman *et al.* 1987b, Habib-ur-Rehman 1987
Aspidospermiose**	L	$C_{24}H_{32}N_2O_5$	428	Habib-ur-Rehman 1987, Habib-ur-Rehman and Atta-ur-Rahman 1996
Eburenine (synonym 1,2-didehydroaspido-spermidine)	L[1] F[2] A.P[3]	$C_{20}H_{24}N_2$	280	[1]Schnoes *et al.* 1962, [1]Smith and Wahid 1963, [2]Abdel Sattar *et al.* 1994, [3]Abdel-Mogib *et al.* 1998
Isosaifinine***	R	$C_{19}H_{26}N_2O_4$	330	Abbas1995
N-acetylaspido-spermidine	L[1], R[2]	$C_{21}H_{28}N_2$	324	[2]Atta-ur-Rahman *et al.* 1991, [1]Sultana 1992

Continues→

(-)-Quebrachamine	L[1] S[2] F[3]	$C_{19}H_{26}N_2$	282	[1]Schnoes *et al.* 1962, [1]Chatterjee *et al.* 1976, [1]Jewers *et al.* 1980, [1]Fatima 1980, [2]Miana *et al.* 1982, [1]Ahmad *et al.* 1983, [1]Khanum 1986, [1]Habib-ur-Rehman 1987, [3]Abdel Sattar *et al.* 1994
Rhazidigenine-*N*-oxide	S	$C_{19}H_{26}N_2O_2$	314	Miana *et al.* 1982
Saifine**	R	$C_{19}H_{26}N_2O_3$	330	Abbas 1995, Atta-ur-Rahman *et al.* 1995
Saifinine***	R	$C_{19}H_{26}N_2O_3$	330	Abbas 1995
Strictanine**	F	$C_{20}H_{26}N_2O_2$	326	Atta-ur-Rahman and Malik1987
Strictanol (synonyms rhazidine, rhazidigenine)	L[1] R[2] F[3]	$C_{19}H_{26}N_2O$	298	[2]Chaudhury *et al.* 1963a,b, [1]Chatterjee *et al.* 1976, [1]Fatima 1980, [1]Habib-ur-Rehman 1987, [1]Atta-ur-Rahman *et al.* 1987a,g, [3]Atta-ur-Rahman and Malik 1987, [2]Qureshi 1991, [2]Bashir *et al.* 1994a, [2]Abbas 1995
Strictibine**	L	$C_{13}H_{11}NO_2$	213	Habib-ur-Rehman 1987, Habib-ur-Rehman and Atta-ur-Rahman 1996
Strictimidine***	L	$C_{19}H_{24}N_2O$	296	Fatima 1988
(-)-Vincadifformine	R	$C_{21}H_{26}N_2O_2$	338	Zaman 1990
(+)-Vincadifformine	L	$C_{21}H_{26}N_2O_2$	338	Smith and Wahid 1963, Fatima 1980, Habib-ur-Rehman 1987, Atta-ur-Rahman *et al.* 1989b
(±)-Vincadifformine	L	$C_{21}H_{26}N_2O_2$	338	Smith and Wahid 1963, Habib-ur-Rehman 1987, Atta-ur-Rahman *et al.* 1989b

Continues→

Vincadine	F	$C_{21}H_{28}N_2O_2$	340	Malik 1985a, Atta-ur-Rahman and Malik 1985a
Aspidospermatin-type				
Condylocarpine	L[1] F[2]	$C_{20}H_{22}N_2O_2$	322	[2]Malik 1985, [1]Atta-ur-Rahman *et al.* 1986d, [1]Habib-ur-Rehman 1987
Tubotaiwine (synonym dihydrocondylocarpine)	R	$C_{20}H_{24}N_2O_2$	324	Zaman1990, Atta-ur-Rahman, *et al.* 1991
Corynantheine-type				
16-Epi-*Z*-isositsirikine	L	$C_{20}H_{22}N_2O_2$	354	Mukhopadhayay *et al.* 1983, Habib-ur-Rehman 1987
16*R*-19,20-*E*-isositsirikine acetate**	R	$C_{23}H_{28}N_2O_4$	396	Atta-ur-Rahman, *et al.* 1991
Bhimberine** (synonym rhazimanine)	L[1] F[2]	$C_{21}H_{26}N_2O_3$	354	[2]Malik 1985, [2]Atta-ur-Rahman *et al.* 1986a, [1]Habib-ur-Rehman 1987
Bhimberine *N*-oxide	L	$C_{21}H_{26}N_2O_4$	370	Habib-ur-Rehman 1987
Dihydrocorynantheol	R	$C_{19}H_{26}N_2O$	298	Atta-ur-Rahman and Zaman 1985, Zaman 1986c
Geissoschizine	L	$C_{21}H_{24}N_2O_3$	352	Banerji *et al.* 1970, Chatterjee *et al.* 1976, Atta-ur-Rahman *et al.* 1986
Rhazimanine	L[1] F[2] R[3]	$C_{21}H_{26}N_2O_3$	354	[2]Atta-ur-Rahman*et al.* 1986d, [2]Atta-ur-Rahman *et al.* 1986, [2]Malik 1985, [1]Habib-ur-Rehman 1987, [1]Sultana 1992, [3]Bashir *et al.* 1994a
Dimeric alkaloids				
16'-Hydroxy-rhazisidine***	L	$C_{40}H_{48}N_4O_3$	632	Khanum 1986
3,14-Dehydrorhazigine***	L	$C_{40}H_{48}N_4O_2$	616	Khanum 1986
Bisstrictidine***	L	$C_{38}H_{44}N_4$	556	Khanum 1986

Continues→

Decarbomethoxytetra-hydrosecamine isomers	R	$C_{40}H_{54}N_4O_2$	622	Atta-ur-Rahman and Zaman 1988a, Zaman 1990, Abbas 1995
Didemethoxycarbonyl-tetrahydrosecamine	R	$C_{38}H52N_4$	564	Atta-ur-Rahman *et al.* 1986d, Zaman 1990
Dihydrosecamine**	L	$C_{42}H_{54}N_4O_4$	678	Evans *et al.* 1968a
Dihydropresecamine**	L	$C_{42}H_{54}N_4O_4$	678	Cordell *et al.* 1970a
Presecamine**	L	$C_{42}H_{52}N_4O_4$	676	Cordell *et al.* 1970a, Atta-ur-Rahman *et al.* 1989b
Rhazigine***	L	$C_{40}H_{50}N_4O_2$	618	Khanum 1986
Rhazisidine***	L	$C_{40}H_{46}N_4O_2$	614	Khanum 1986
Secamine***	L	$C_{42}H_{52}N_4O_4$	676	Evans *et al.* 1968a
Strictimine	R	$C_{15}H_{28}N_2O$	252	Atta-ur-Rahman and Zaman 1984a, Zaman 1990
Tetrahydropresecamine	L	$C_{42}H_{56}N_4O_4$	680	Cordell *et al.* 1970a, Atta-ur-Rahman *et al.* 1989b
Tetrahydrosecamine	L^1 R^2	$C_{42}H_{56}N_4O_5$	680	[1]Evans *et al.* 1968a, [1]Mukhopadhyay *et al.* 1981, [2]Abbas 1995, [2]Bashir *et al.* 1994a
Eburnamine-type				
16*R*, 21*R*, *O*-Methyleburnamine**	R	$C_{20}H_{26}N_2O$	310	Atta-ur-Rahman, *et al.* 1991, Zaman 1990
(+)-21*S*-Eburnamenine	L^1 R^2	$C_{19}H_{22}N_2$	278	[1]Schnoes *et al.* 1962, [1]Habib-ur-Rehman 1987, [2]Zaman 1990, [2]Atta-ur-Rahman *et al.* 1991, [1]Sultana 1992
Dihydroeburnamenine**	R	$C_{19}H_{24}N_2$	280	Atta-ur-Rahman *et al.* 1991, Zaman 1990
Eburnamine	L	$C_{19}H_{24}N_2O$	296	Schnoes *et al.* 1962, Habib-ur-Rehman 1987
(+)-Eburnamonine	L	$C_{19}H_{22}N_2O$	294	Schnoes *et al.* 1962, Khanum 1986
Heteroyohimbin-type				
Hexahydroalstonine***	L	$C_{21}H_{26}N_2O_4$	354	Sultana 1992

Continues→

Strictosamide	L	$C_{26}H_{30}N_2O_8$	498	Habib-ur-Rehman 1987, Atta-ur-Rahman *et al.* 1991
Tetrahydroalstonine	L[1] F[2]	$C_{21}H_{24}N_2O_3$	352	[2]Atta-ur-Rahman and Malik 1984f, [2]Malik 1985, [1]Habib-ur-Rehman 1987
Hunterburine-type				
Isovallesiachotamine	F	$C_{21}H_{22}N_2O_3$	350	Atta-ur-Rahman and Malik 1984e, Malik 1985
Rhazinine (synonym antirhine)	L[1] R[2]	$C_{19}H_{24}N_2O$	296	[1]Banerji *et al.* 1970, [1]Chatterjee *et al.* 1976, [1]Fatima 1980, [1]Habib-ur-Rehman 1987, [2]Zaman 1990
Vallesiachotamine	L[1] F[2]	$C_{21}H_{22}N_2O_3$	350	[1]Mukhopadhyay *et al.* 1981, [2]Malik 1985
Marvine-type				
Strictine**	L	$C_{20}H_{20}N_2O_3$	336	Atta-ur-Rahman and Khanum 1987f, Khanum 1986
Picraline-type				
N_b-methyl-strictamine	L	$C_{21}H_{25}N_2O_2$	337	Atta-ur-Rahman *et al.* 1987c
Rhazimal	L	$C_{21}H_{22}N_2O_3$	350	Ahmad *et al.* 1983, Atta-ur-Rahman and Habib-ur-Rehman 1986b, Fatima 1980, Habib-ur Rehman 1987
Rhazimol	L[1] R[2]	$C_{21}H_{24}N_2O_3$	352	[1]Ahmad *et al.* 1983, [1]Atta-ur-Rahman and Zaman 1986, [1]Fatima 1980, [2]Zaman 1990
Rhazinaline	L	$C_{21}H_{22}N_2O_3$	350	Banerji *et al.* 1970, Chatterjee *et al.* 1976
Rhazinol**	L	$C_{19}H_{22}N_2O$	294	Ahmad *et al.* 1983, Fatima 1980, Habib-ur-Rehman 1987

Continues→

Strictalamine**	L	$C_{19}H_{20}N_2O$	292	Ahmad *et al.* 1977, Fatima 1980, Habib-ur-Rehman 1987
Strictamine	L	$C_{20}H_{22}N_2O_2$	322	Schnoes *et al.* 1966, Banerji *et al.* 1970, Chatterjee *et al.* 1976, Ahmad *et al.* 1977, Ahmad *et al.* 1983, Atta-ur-Rahman and Habib-ur-Rehman. 1986, Fatima 1980, Sultana 1992
Quinoline-type				
17- Methoxy-1,17-dihydrorhazimine**	L	$C_{22}H_{26}N_2O_4$	382	Atta-ur-Rahman *et al.* 1989b
19-Z-Isorhazicine***	R	$C_{21}H_{24}N_2O_4$	368	Zaman 1990
2-Methoxy-1,2-dihydro-rhazimine**	L	$C_{22}H_{26}N_2O_4$	382	Atta-ur-Rahman and Khanum 1985b
Isorhazicine**	L	$C_{21}H_{24}N_2O_4$	368	Khanum 1986, Atta-ur-Rahman and Khanum 1987e, Habib-ur Rehman 1987, Fatima 1988
Rhazicine	L	$C_{21}H_{24}N_2O_4$	368	Atta-ur-Rahman and Khanum 1984b, Khanum 1986, Atta-ur-Rahman and Khanum 1987e, Habib-ur-Rehman 1987, Fatima 1988
Rhazimine	L	$C_{21}H_{22}N_2O_3$	350	Atta-ur-Rahman and Khanum 1984c, Khanum 1986, Habib-ur-Rehman 1987, Sultana 1992
Strictigine	R S L	$C_{19}H_{22}N_2$	278	Atta-ur-Rahman *et al.* 1996
Rhazinilam-type				
Leuconolam***	L	$C_{19}H_{22}N_2O_3$	326	Khanum1986

Continues→

N-Methylleuconolam**	R	$C_{20}H_{24}N_2O_3$	340	Atta-ur-Rahman *et al.* 1995, Zaman 1990, Qureshi 1991
Rhazinilam	L	$C_{19}H_{22}N_2O$	294	Banerji *et al.* 1970, De Silva *et al.* 1972, Chatterjee *et al.* 1976, Jewers *et al.* 1980, Habib-ur-Rehman 1987
Sarpagine-type				
(-)-19-*E*-Akuammidine***	R	$C_{21}H_{24}N_2O_3$	352	Zaman 1990
Polyneuridine	L	$C_{21}H_{24}N_2O_3$	352	Mukhopadhyay *et al.* 1981
Rhazine (synonym akuammidine)	L[1] S[2] F[3] R[4]	$C_{21}H_{24}N_2O_3$	352	[1]Chatterjee *et al.* 1976, [2]Miana *et al.* 1982, [3]Abdel Sattar *et al.* 1994, [1]Ahmad *et al.* 1983, [1]Jewers *et al.* 1980, [2]Malik 1985, [1]Khanum 1986, [1]Fatima 1980, [4]Bashir *et al.* 1994a
Secodine-type				
Dihydrosecodine**	L	$C_{21}H_{28}N_2O_2$	340	Cordell *et al.* 1970b
Tetrahydrosecodine	L	$C_{21}H_{30}N_2O_2$	342	Cordell *et al.* 1970b
Strictosidine-type				
Strictosidine	L	$C_{27}H_{34}N_2O_9$	530	Smith 1968
Strychnos-type				
Bharhingine**	L	$C_{19}H_{20}N_2O$	368	Habib-ur-Rehman 1987, Atta-ur-Rahman *et al.* 1987a
Rhazizine**	L	$C_{19}H_{20}N_2O$	368	Fatima 1988, Atta-ur-Rahman *et al.* 1989a
Sewarine	L	$C_{20}H_{22}N_2O_3$	338	Siddiqui *et al.* 1966, Ahmad *et al.* 1970, Ahmad *et al.* 1971, Mukhopadhyay *et al.* 1981
Stricticine	L	$C_{20}H_{22}N_2O_3$	338	Fatima 1980, Atta-ur-Rahman *et al.* 1987d, Fatima 1988

Continues→

Vincanicine	L	$C_{20}H_{22}N_2O_3$	322	Habib-ur-Rehman 1987, Atta-ur-Rahman *et al.* 1987a
Vincanine	L	$C_{19}H_{20}N_2O$	292	Ahmad *et al.* 1977, Fatima 1980, Ahmad *et al.* 1983
Yohimban-type				
Pseudoyohimbine***	R	$C_{21}H_{26}N_2O_3$	354	Abbas 1995
Miscellaneus-alkaloids				
Betaine	L	$C_5H_{11}NO_2$	117	Hassan *et al.* 1977
1-Carbomethoxy-β-carboline	L	$C_{13}H_{10}N_2O_2$	226	Habib-ur-Rehman 1987, Habib-ur Rehman and Atta-ur-Rahman 1996
2-Ethyl-3[2-(3-ethylpiperdine)ethyl]-indole	R	$C_{19}H_{28}N_2$	284	Zaman 1990, Atta-ur-Rahman, *et al.* 1991
Strictazine**	L	$C_{21}H_{24}N_2O_4$	368	Fatima 1988
Rhaziattarine**	L	$C_{19}H_{20}N_2O$	292	Sultana 1992

L: leaf; R: root; F: fruit; S: seed; AP: aerial part. Superscript number indicates the original study. *Fruit refers to legumes or seed pods without seeds. **These alkaloids have hitherto been reported only from *R. stricta* and not from other plant species. *** These compounds were reported in various PhD theses but not in peer-reviewed scientific journals.

Later, they described the isolation of a monomeric indole alkaloid, 16-epi-*Z*-isositsirikine, from the leaves of *R. stricta* and *C. roseus* (Mukhopadhyay *et al.* 1983). It was shown that rhazinilam is a neutral alkaloidal artefact possibly formed from vincadifformine during the extraction procedure (De Silva *et al.* 1972). It was postulated that vincadifformine is first transformed via eburenine to 5,21-dihydrorhazinilam and finally to rhazinilam (Ratcliffe *et al.* 1973, David *et al.* 1997).

In 1988 Mariee and co-workers isolated stemmadenine from *R. stricta*. This was the first time the compound had been reported from the genus (Mariee *et al.* 1988). In 1994 Bashir *et al.* isolated strictanol, tetrahydrosecamine, rhazine and rhazimanine from roots of the plant (Bashir *et al.* 1994a). This was the first report of strictanol, akuammidine and rhazimanine from the roots of *R. stricta*. Quebrachamine, eburenine and rhazine had already been reported from the leaves of *R. stricta* (Chatterjee *et al.* 1962, Smith and Wahid 1963). They were later isolated from the fruits (without seeds) of the plant (Abdel Sattar *et al.* 1994). Abdbl-Mogib *et al.* (1998) presented the GC-MS identification of aspidospermidine and eburenine along with six non-alkaloidal components from fractionated extracts of *R. stricta* aerial parts.

Atta-ur Rahman and co-workers have isolated more than 40 alkaloids from the leaves, stems, roots, fruits and seeds of *R. stricta*. Some of them had already been reported from the plant (*e.g.* rhazine, quebrachamine, tubotaiwine), but several were reported for the first time in *R. stricta*. Among these, the first glycosidic alkaloids with an oxidized sugar unit, *i.e.* aspidospermidose (Atta-ur-Rahman *et al.* 1987b) and aspidospermiose (Habib-ur-Rehman and Atta-ur-Rahman 1996), were isolated from a natural source. Some new indole alkaloids, 16*R*-19,20-*E*-isositsirikine acetate, leepacine and dihydroeburnamenine (syn. eburan), were also isolated (Atta-ur-Rahman *et al.* 1991) and these compounds have not hitherto been reported from any other plant species (Buckingham *et al.* 2010).

In addition to previously reported dimeric alkaloids (Evans *et al.* 1968a, Cordell *et al.* 1970a), new dimeric alkaloids, di-demethoxycarbonyltetrahydrosecamine and isomers of decarbomethoxytetrahydrosecamine, were isolated from the roots of the plant (Atta-ur-Rahman *et al.* 1986d, Atta-ur-Rahman and Zaman 1988a). Moreover, a known glycoalkaloid, strictosidine lactam (syn. strictosamide), which had previously been isolated from *Nauclea latifolia* Smith (Rubiaceae) (Brown *et al.* 1977), was isolated from *R. stricta* (Atta-ur-Rahman *et al.* 1991). A review comprising 75 alkaloids from *R. stricta* and *R. orientalis* along with their spectral data was published in 1989 by Atta-ur-Rahman *et al.* (1989b).

Analytics of terpenoid indole alkaloids (TIAs)

Two general procedures have mainly been used for the isolation of TIAs from *R. stricta*: (1) Separation of alkaloids based on their different pK_a values and (2) separation of alkaloids based on their different polarities.

Alkaloid fractions have been further separated and purified by thin-layer chromatography (TLC), column chromatography (CC) and counter-current distribution (CCD) methods. For example, the ethanolic crude extract of *R. stricta* fruits or roots has been subjected to liquid-liquid extraction with chloroform and water at different pH values. The fractions, subjected to repeated preparative TLC (PTLC), resulted in the isolation of strictanol, strictanine (Atta-ur-Rahman and Malik 1987g), vincadine (Atta-ur-Rahman and Malik 1985a) and tubotaiwine (Atta-ur-Rahman *et al.* 1991).

In most cases, a combination of separation methods has been required to obtain pure compounds. For example, in the isolation of rhazizine (Atta-ur-Rahman *et al.* 1989a), 1-carbomethoxy-β-carboline, strictibine, aspidospermiose (Atta-ur-rahman *et al.* 1996), vallesiachotamine (Mukhopadhayay *et al.* 1981) and eburnamenine (Atta-ur-Rahman *et al.* 1991), CC of *R. stricta* crude extracts was first performed. The fractions were then purified by TLC to obtain pure compounds.

The identification and structural elucidation of compounds in *R. stricta* has been performed by high resolution-electron impact-mass spectrometry (HR-EI-MS) and nuclear magnetic resonance spectroscopy (NMR). Gas chromatography-MS (GC-MS), fast atom bombardment-MS (FAB-MS), desorption chemical ionization-MS

(DCI-MS) and UV spectroscopy have also been applied for analysis of the pure alkaloids (Atta-ur-Rahman *et al.* 1989b, Kostenyuk *et al.* 1995, Aimi *et al.* 1996, Sheludko *et al.* 1999). *R. stricta* extracts can also be fractionated by TLC, medium performance liquid chromatography (MPLC) and high performance liquid chromatography (HPLC) and consequently subjected to GC-MS for identification of compounds (Abdbl-Mogib *et al.* 1998).

GC-MS is a suitable and reproducible technique for the identification and quantification of thermostable, low molecular weight and non-polar compounds in complex mixtures. With this method the identification of unknown alkaloids can be carried out by searching against library spectra of pure compounds (Fiehn *et al.* 2000, Villas-Bôas *et al.* 2005). Chromatographic separation and spectral characteristics can also be improved by derivatization, *e.g.* through trimethylsilylation for separation of alkaloid isomers (Molyneux *et al.* 2002) as well as through acylation as shown in the analysis of steroidal glycoalkaloid aglycons (Laurila *et al.* 1999). Selected ion monitoring (SIM) offers the possibility to increase the sensitivity and selectivity of analyses by avoiding the interference of co-eluting components and enables the detection of minor compounds. The SIM-mode can be used for quantification based on the intensities of selected ions (Li *et al.* 2012).

One of the most effective methods for the qualitative and quantitative analysis of alkaloids is reversed phase high performance liquid chromatography (RP-HPLC) because of its high resolution power and possibilities for automatisation. Liquid chromatography on reversed-phase C18 columns is the most frequently used method in the analysis of alkaloids (Stöckigt *et al.* 2002, Tikhomiroff and Jolicoeur 2002, Hisiger and Jolicoeur 2007). The HPLC-UV method has been utilized for the separation of *R. stricta* alkaloids. HPLC separation has revealed the presence of 11 known compounds and more than 15 additional unidentified alkaloids as minor components (Pawelka and Stöckigt 1986).

Water-soluble basic alkaloids possess high polarity, causing decreased affinity to the lipophilic stationary phase applied. Ionic interaction with deprotonated silanol groups, and hydrophobic and hydrogen bonding may lead to additional retention mechanisms and eventually to peak tailing. The main problem is the strong interaction of basic compounds with anionic silanol functions, resulting in strong tailing and sometimes total loss of signal (Petruczynik and Waksmundzka-Hajnos 2013). A feasible method of avoiding the ionic interactions is the addition of an ion pair reagent *e.g.* sodium alkylsulphonate (hexane-, heptane-, octane-, and decanesulphonate) to the mobile phase to promote the formation of ion pairs leading to electrically neutral analytes (Kostenyuk *et al.* 1991, Stöckigt *et al.* 2002).

An RP-HPLC method has also been developed for the separation of alkaloids using phosphate buffer and ion pair reagent from somatic hybrid cell cultures of *Rauwolfia serpentina* × *R. stricta* (Gerasimenko *et al.* 2001b). It is helpful to use standard compounds in the identification of alkaloids in HPLC-UV-MS, since chromatographic and spectroscopic properties of standard compounds can be compared with those present in the extract. Nevertheless, the availability of

authentic standard compounds is limited since only a few pure compounds of *R. stricta* are commercially available and therefore components must be isolated and purified from crude extracts, which makes the identification more complex and time consuming (Stöckigt *et al.* 2002).

Recently, an improvement in the chromatographic separation of alkaloids was achieved by the introduction of ultra-performance liquid chromatography (UPLC) (Han *et al.* 2008, He *et al.* 2011, Sun *et al.* 2011). It has been shown that if the stationary phase particle size decreases to less than 2.5 µm it results in a significant improvement in separation efficiency (Wren and Tchelitcheff 2006). Transferring the method from HPLC to UPLC has potential advantages in terms of improved speed and resolution.

NMR offers a potentially powerful tool for unpurified extracts. It is an unbiased, rapid, reproducible, non-destructive method and stable over time, while requiring only simple sample preparation. NMR can simultaneously detect primary metabolites, *e.g.* carbohydrates, amino acids, organic acids, fatty acids, amines, esters, ethers and secondary metabolites such as alkaloids, terpenes and flavonoids. NMR-based metabolomics has been widely used in metabolomics studies in diverse fields of plant physiology and classification. For example, it has been applied to distinguish transgenic plants from non-transgenic plants and to inspect the potential effect of transgenic events on plants (Choi *et al.* 2004, Kim *et al.* 2010a). NMR profiling techniques followed by multivariate data analysis can be applied to investigate the metabolic differences of *R. stricta* hairy roots transformed with early key genes from the TIA biosynthesis pathway to find characteristic metabolites responsible for the differentiation of control cultures and transformed clones.

2.4 Terpenoid indole alkaloids (TIAs) and their biosynthesis

Terpenoid indole alkaloids (TIAs) comprise a family of over 3000 compounds with diverse structures, and among them are several pharmacologically valuable compounds. Structurally diverse TIAs have mainly been isolated from the tropical plant families, Apocynaceae, Loganiaceae, Nyssaceae and Rubiaceae as well as from all of the Gentianales (Springob and Kutchan 2009). Several products belonging to the TIA group that have been isolated commercially from plants are presented in **Table 4**, including the antihypertensive ajmalicine and the antitumor agents vinblastine and vincristine from *C. roseus* L. (periwinkle), the antipsychotic and antihypertensive reserpine from *Rauwolfia* species, and the neurotoxin strychnine from *Strychnos nux-vomica* L.

Significant progress in TIA biosynthesis pathway characterization has been made in the medicinal plant *C. roseus* (**Figure 2**), a member of the Apocynaceae family that produces approximately 130 TIAs, some of which have high pharmacological value (Zhao *et al.* 2013). The plant was originally investigated for potential hypoglycaemic activity; further analysis of its extract revealed the anticancer activity of several bisindole alkaloids, in particular vinblastine and

vincristine (Noble, 1990), as well as antihypertensive agents such as ajmalicine and serpentine. Commercial vinblastine (Velbe®) and vincristine (Oncovin®) are currently used as therapy against Hodgkin's disease and non-Hodgkin's lymphomas, rhabdomyosarcoma, and Wilm's tumor (van der Heiden *et al.* 2004). Several derivatives of vincristine or vinblastine have also been developed to decrease toxicity and increase effectiveness, *e.g.* vindesine (Eldisine®), vinorelbine (Navelbine®) and a fluorinated derivative, vinflunine (Javlor®). The production of these drugs still relies on the *C. roseus* plant for production of the monomeric precursors, vindoline and catharanthine (Zhao and Verpoorte 2007).

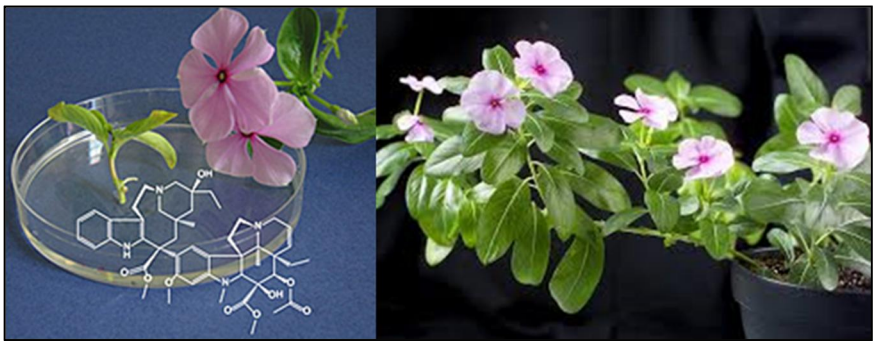


Figure 2. *Catharanthus roseus,* a model plant for the study of TIA biosynthesis (source: VTT webpage).

Terpene indole alkaloids originate from the coupling of the tryptamine (indole moiety) and a C_{10} part from the iridoid glucoside secologanin (terpene moiety) by the enzyme strictosidine synthase to provide the central intermediate, 3α(*S*)-strictosidine. Tryptamine is derived from conversion of the amino acid L-tryptophan, an aromatic amino acid from the shikimate pathway, catalyzed by tryptophan decarboxylase (Verpoorte *et al.* 2000). Tryptophan decarboxylase (TDC) was first isolated from *C. roseus* (De Luca *et al.* 1989) and then several *tdc* genes were cloned and characterized from other plant species, such as *Camptotheca acuminata* Decne. (Lopez-Meyer and Nessler 1997), *Ophiorrhiza pumila* Champ. ex Benth. (Yamazaki *et al.* 2003), *Rauwolfia verticillata* (Lour.) Baill. (Liu *et al.* 2012), *Oryza sativa* L. (Kang *et al.* 2007) and *Capsicum annuum* L. (Park *et al.* 2009). TDC is encoded by a single gene (*tdc*) in *C. roseus* (De Luca *et al.* 1989) and by two autonomously regulated genes in *C. acuminata* (Lopez-Meyer and Nessler 1997). The enzyme TDC channels tryptophan from primary metabolism into the terpenoid indole alkaloid pathway. This reaction represents a branching point from primary metabolism into a secondary pathway (Canel *et al.* 1998).

Secologanin biosynthesis in *C. roseus* can be divided into two phases (Oudin *et al.* 2007). The first includes biosynthesis of isopentenyl diphosphate (IPP) through the methyl erythritol phosphate pathway (MEP, the mevalonate-

independent pathway) in seven enzymatic steps, starting from the condensation of pyruvate with D-glyceraldehyde 3-phosphate (El-Sayed and Verpoorte, 2007). The second phase of secologanin biosynthesis, which is known as the iridoid pathway (**Figure 3**), begins with the activation of IPP through isomerisation to dimethylallyldiphosphate (DMAPP). Two different pathways to produce isopentenyl diphosphate (IPP), the central precursor of terpenoids, exist in plants. One pathway is the cytosolic mevalonate (MVA) pathway leading to the formation of triterpenes (sterols) and certain sesquiterpenes (Lange and Croteau 1999). The other is the mevalonate-independent (MEP) pathway leading to the formation of monoterpenes, diterpenes and tetraterpenes (carotenoids) in the plastids (Arigoni *et al.* 1997).

^{13}C labeling experiments followed by NMR and treatment of cells (Imbault *et al.* 1996, Contin *et al.* 1998,) and hairy root (Hong *et al.* 2003) cultures with specific enzyme inhibitors of the MVA and MEP pathways, respectively, demonstrated that the MEP pathway is the major route for the biosynthesis of secologanin in *C. roseus*.

However, cross-talk between the MVA and the MEP pathways has been reported in several studies, reviewed by Hemmerlin *et al.* (2012). For instance, incorporation of labelled mevalonate (Contin *et al.* 1998), mevalonolactone (Schuhr *et al.* 2003) from the MVA pathway and 1-deoxy-D-xylulose from the MEP pathway (Arigoni *et al.* 1997) into loganin and leutin, products of the MEP pathway, and phytosterols, products of the MVA pathway, respectively, has been reported. It was postulated that the cross-talk is highly complex and depends on the presence or absence of a certain precursor supplied to the cells, the nature of the precursor supplied and the experimental conditions (Schuhr *et al.* 2003). Moreover, interaction between plastidial MEP pathway and the cytosolic MVA pathway has been confirmed by feeding experiments in conjunction with blocking specific steps using chemical inhibitors (Hemmerlin *et al.* 2003).

Feeding labelled 1-deoxy-D-xylulose (DX) from the MEP pathway to tobacco BY-2 cells in the presence of the MVA pathway inhibitor, mevinolin, resulted in DX incorporation into sterols, a product of the mevalonate pathway. In addition, feeding mevalonate to tobacco BY-2 cells in the presence of an MEP pathway inhibitor, fosmidomycin, could overcome the growth inhibition by fosmidomycin and result in mevalonate incorporation into plastoquinone, which normally derives from the MEP pathway (Hemmerlin *et al.* 2003).

Although the iridoid pathway has been studied for decades (Loyola-Vargas *et al.* 2007), several steps in the pathway remained unknown. Until 2011, only the genes and enzymes of three steps in the pathway were well characterized. One of these is geraniol 8-oxidase (G8O), earlier known as geraniol 10-hydroxylase, G10H (Collu *et al.* 2001), which is located in the third step in the pathway. The other two are last steps in the iridoid pathway, loganic acid O-methyltransferase, LAMT (Murata *et al.* 2008) and secologanin synthase, SLS (IrmLer *et al.* 2000) (**Figure 3**). The enzymes for the remaining steps were recently discovered and the corresponding genes were cloned and their functionality characterized (Geu-

Flores *et al.* 2012, Simkin *et al.* 2013, Asada *et al.* 2013, Rai *et al.* 2013, Salim *et al.* 2013, Miettinen *et al.* 2014)

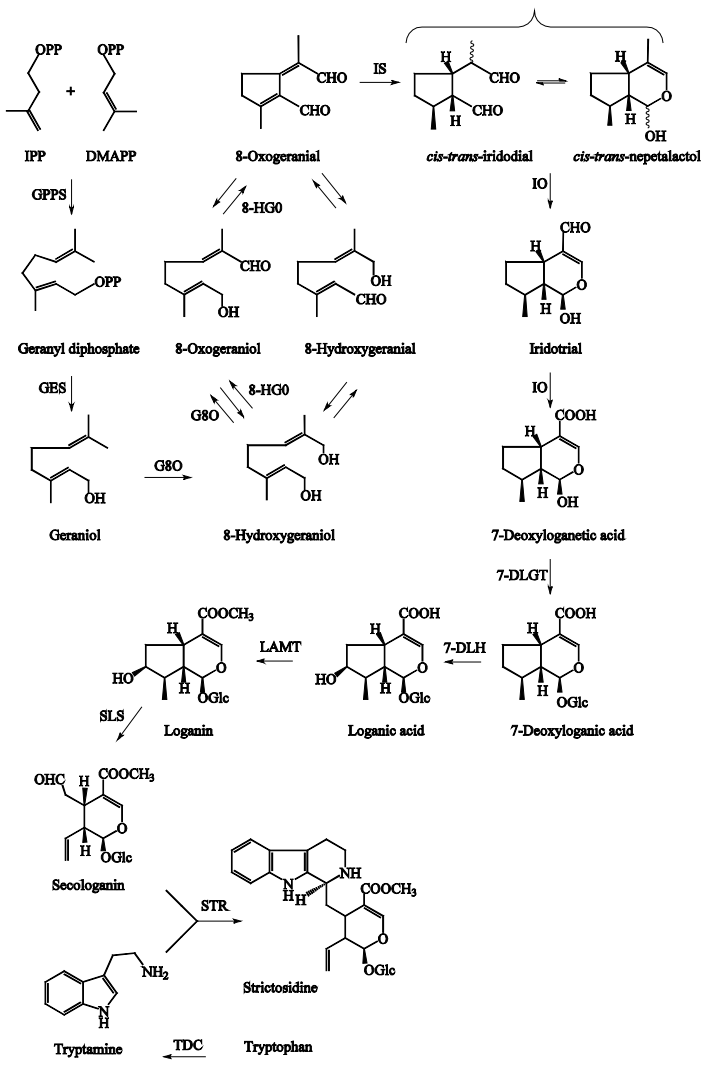


Figure 3. The iridoid pathway in *C. roseus* leading to strictosidine (adopted from Miettinen *et al.* 2014). IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; Glc: glucose; GPPS: geranyl diphosphate synthase, GES: geraniol synthase; G8O: geraniol 8-oxidase; 8-HGO: 8-hydroxygeraniol oxidoreductase; IS: iridoid synthase; IO: iridoid oxidase; 7-DLGT: 7-deoxyloganetic acid glucosyltransferase; 7-DLH: 7-deoxyloganic acid hydroxylase; LAMT: loganic acid O-methyltransferase; SLS: secologanin synthase; STR: strictosidine synthase; TDC: tryptophan decarboxylase.

In the first step of the iridoid pathway towards secologanin biosynthesis, isopentenyl diphosphate (IPP) is activated to dimethylallyldiphosphate (DMAPP) by IPP isomerase (IDI). DMAPP condenses head to tail with one IPP to form geranyl diphosphate (GPP) in the presence of geranyl diphosphate synthase (GPPS) (Verpoorte *et al.* 1997, El-Sayed and Verpoorte 2007). Genes encoding IPP isomerase have been isolated from *Arabidopsis thaliana* (L.) Heynh. (Okada *et al.* 2008) and *Clarkia breweri* (A.Gray) Greene (Blanc and Pichersky 1995). Furthermore, two isoforms of IPP isomerase in *Rubia tinctorum* L., *Cinchona robusta* Trimen and *C. roseus* have previously been detected, of which one form was present only after induction by a fungal elicitor (Ramos-Valdivia *et al.* 1997,1998). In *C. roseus* a single gene (*CrIDI*) encodes two IDI isoforms (*Cr*IDI1 and *Cr*IDI2). Transcriptomic data reveal that *Cr*IDI1 is the mainly expressed isoform in *C. roseus*, whereas *Cr*IDI2 is expressed at a very low level. Interestingly, *Cr*IDI1 protein has triple subcellular targeting (Guirimand *et al.* 2012).

GPP, the entry point to the formation of the terpene moiety, is subsequently dephosphorylated to geraniol (Bouvier *et al.* 2005, Hedhili *et al.* 2007). GPPSs have been characterized in a few plant species and exist in two distinct forms (Nagegowda 2010). The homodimeric GPPS exists in angiosperms, *e.g. A. thaliana* (Bouvier *et al.* 2000) and in gymnosperms, *e.g. Abies grandis* (Douglas ex D. Don) Lindley (Burke and Croteau 2002), *Picea abies* (L.) H.Karst. (Schmidt and Gershenzon 2008), whereas the heterodimeric GPPS has only been found in some angiosperm plant species *e.g. Antirrhinum majus* L., *C. breweri* (Tholl *et al.* 2004) and *Mentha piperita* L. (Chang *et al.* 2010). Rai *et al.* (2013) discovered three genes encoding GPPSs in *C. roseus*; mitochondrial homomeric GPPS (*Cr*GPPS), and its plastidial heteromeric large subunit (*Cr*GPPS.LSU) and small subunit (*Cr*GPPS.SSU), in which the heteromeric GPPSs provide GPP flux for TIA biosynthesis.

The next step in the iridoid biosynthesis is the conversion of GPP to monoterpenoid geraniol through geraniol synthase (GES), which is encoded by the *ges* gene. The GES enzyme removes the diphosphate group of GPP and generates a carbocation site (**Figure 3**). Geraniol is a key intermediate channelling the carbon skeletons to the biosynthesis of the TIAs (Degenhardt *et al.* 2009). The geraniol synthase (*ges*) gene has previously been characterized from *Cinnamomum tenuipilum* Kosterm (Yang *et al.* 2005), *Ocimum basilicum* L. (Iijima *et al.* 2004), *Perilla frutescens* (L.) Britton and *Perilla citriodora* (Makino) Nakai (Ito and Honda 2007). Recently geraniol synthase has been characterised from *Vitis vinifera* L. (Martin *et al.* 2010)*, Valeriana officinalis* L. (Dong *et al.* 2013), *Lippia dulcis* (Trevir.) Moldenke (Yang *et al.* 2011, Dong *et al.* 2013) and *C. roseus* (Simkin *et al.* 2013). The *Cr*GES open reading frame of 1770 base pairs (bp) encodes a protein of 589 amino acids with a mass of 67.7 kDa. The *ges* gene from *C. roseus* (*Cr*GES) encodes a protein which shares 37-67% identity with GES proteins from other plant species. The highest protein similarity was found between *Cr*GES and *Vo*GES (67%), *Ld*GES (66%) and *Ob*GES (63%), respectively. Furthermore, *Cr*GES shares 57% identity with *Vv*GES, 42% with

*Ct*GES and 36% with *Pf*GES. In addition, *Ld*GES possesses 63% identity with *Vo*GES (Dong *et al.* 2013).

The secologanin pathway continues from geraniol and consists of eight enzymes catalysing successive oxidation, reduction, glycosylation and methylation reactions. Geraniol is subsequently hydroxylated at its C-8 position by the NADPH-depended mono-oxygenase cytochrome P450 (CYP) enzyme, geraniol-8-oxidase (G8O), to generate 8-hydroxygeraniol (Collu *et al.* 2001). It was shown that G8O requires a protein partner, cytochrome P450 reductase (CPR) to function (Meijer *et al.* 1993a). Recently, Höfer *et al.* (2013) found that *C. roseus* G8O is a multi-functional oxidase, catalysing the two successive oxidation steps (**Figure 3**); it further oxidizes the C-8 position of 8-hydroxygeraniol to 8-oxogeraniol. Additionally, it was revealed that G8O has an important role in the biosynthesis of flavonoids (Sung *et al.* 2011).

G8O, which is known also as geraniol-8-hydroxylase (G8H) and commonly misnamed geraniol 10-hydroxylase (G10H), belongs to the CYP76B subfamily and was nominated to CYP76B6 (Collu *et al.* 2001). The G8O enzyme was characterized in *C. roseus* (Meehan and Coscia 1973, Meijer *et al.* 1993b, Collu *et al.* 2001) and the cDNA was cloned by Collu *et al.* (2001) from a *C. roseus* cell culture. The isolated cDNA sequence contained a 1482 bp open reading frame (ORF) encoding a protein of 493 amino acids with a molecular mass of 55.7 kDa (Collu *et al.* 2001). CYP76B6 protein possesses 67% similarity with CYP76B2 and 66% with CYP76B5 from *Petunia hybrida* D. Don ex W.H. Baxter, 61% with CYP76B1 from *Helianthus tuberosus* L. and 53% with CYP76B3 and CYP76B4 from *Medicago sativa* L. (Collu *et al.* 2001).

McFarlane *et al.* (1975) demonstrated that G8O is feedback inhibited by catharanthine, vinblastine and vindoline. It was also found that there is a close association between G8O activity and alkaloid accumulation. Schiel *et al.* (1987) showed that G8O activity and TIA production are enhanced in induction medium but supressed when *C. roseus* cell cultures are exposed to excess phosphate in the medium. Furthermore, it was demonstrated that hormones, elicitors and feeding of 8-hydroxygeraniol to hairy root or cell cultures of *C. roseus* caused up-regulation of the *g8o* gene and an increase in the enzyme activity and consequently TIA production (Collu *et al.* 2002, Suttipanta *et al.* 2007). Therefore, it has been proposed that G8O is rate limiting for the biosynthesis of secologanin and TIAs.

In the next two successive and reversible oxidation steps which are catalyzed by 8-hydroxygeraniol oxidoreductase (8-HGO), 8-hydroxygeraniol is converted into 8-oxogeraniol or 8-hydroxygeranial and then into 8-oxogeranial. In the presence of NAD^+, 8-HGO oxidizes the hydroxy group to an aldehyde in the substrates to produce the intermediate 8-oxogeranial. Recently, Miettinen *et al.* (2014) discovered these missing steps, including the relevant genes and enzymes. From their findings and that of from Höfer *et al.* (2013) it was concluded that two enzymes, G8O and 8-HGO, can contribute to the formation of the 8-oxogeraniol (**Figure 3**).

Iridoid synthase (IS) catalyzes an NAD(P)H-dependent reduction step with a subsequent cyclization of 8-oxogeranial into the first bicyclic iridoid, *cis-trans*-nepetalactol (Geu-Flores *et al.* 2012). Nepetalactol, which is the general precursor of the other iridoids in plants, is considered to occur in equilibrium with its open dialdehyde form, iridodial. Iridoid synthase (IS), formerly NADPH-dependent 8-oxogeranial cyclase or monoterpene cyclase, was partially purified from cell-free extracts of *R. serpentina* (Uesato *et al.* 1986,1987) and *C. roseus* hairy roots (Sánchez-Iturbe *et al.* 2005), although the properties of the enzyme remained unknown. Recently, Geu-Flores *et al.* (2012) characterized the biochemical mechanism for the biosynthesis of iridoids and its corresponding enzyme and gene from *C. roseus*.

The enzyme CYP76A26, named iridoid oxidase (IO), catalyzes the conversion of iridodial into 7-deoxyloganetic acid (Miettinen *et al.* 2014). Although iridotrial was previously proposed as an intermediate of the secologanin pathway (Loyola-Vargas *et al.* 2007), it has been shown that IO can convert *cis-trans*-iridodial and *cis-trans*-nepetalactol into 7-deoxyloganetic acid without the release of an iridotrial intermediate.

In the next step, 7-deoxyloganetic acid is glucosylated by the enzyme 7-deoxyloganetic acid glucosyltransferase (7-DLGT), forming 7-deoxyloganic acid using uridine diphosphate glucose (UDP)-glucose as the sugar donor (Miettinen *et al.* 2014).

Then the formation of the loganic acid is catalyzed by hydroxylation of 7-deoxyloganic acid at the C-7 position by 7-deoxyloganic acid hydroxylase (7-DLH, CYP72A224) (Miettinen *et al.* 2014). 7-DLH activity was detected in microsomal preparations from *Lonicera japonica* Thunb. cell cultures (Katano *et al.* 2001) and *C. roseus* cell suspensions (IrmLer *et al.* 2000). CYP72A224 belongs to the same P450 subfamily as secologanin synthase (SLS; CYP72A1), which catalyzes the conversion of loganin to secologanin.

A bioinformatical approach was also used in the studies by the De Luca group to screen large databases of annotated transcripts from various TIA-producing plant species in order to select candidate genes for 7-DLGT (Asada *et al.* 2013) and 7-DLH (Salim *et al.* 2013). Virus-induced gene silencing (VIGS) of these genes in *C. roseus* plants confirmed their roles in secologanin biosynthesis.

The iridoid pathway continues by methylation of the carboxyl group of loganic acid to loganin by loganic acid *O*-methyltransferase (LAMT). This enzyme has previously been isolated from *C. roseus* seedlings (Madyastha *et al.* 1973, Madyastha and Coscia 1979) and the gene was cloned from the same plant by Murata *et al.* (2008). It has been demonstrated that LAMT from crude *C. roseus* leaf extracts is able to methylate loganic acid but not 7-deoxyloganic acid, suggesting that hydroxylation preceeds *O*-methylation (Madyastha *et al.* 1973, Murata *et al.* 2008). However, studies with *L. japonica* cell cultures suggested that *O*-methylation could precede hydroxylation, since 7-deoxyloganin could be converted into loganin (Yamamoto *et al.* 1999).

In the last step of the iridoid pathway, cleavage of the cyclopentane ring of loganin forms the secologanin. The enzyme catalysing this reaction is secologanin

synthase (SLS). Its corresponding gene and enzyme were previously characterized in *C. roseus* (IrmLer *et al.* 2000) and *L. japonica* (Yamamoto *et al.* 2000).

Strictosidine, a monoterpene indole alkaloid glycoside, is a universal precursor of the terpenoid indole and related alkaloids and was first isolated from *R. stricta* (Smith 1968, Patthy-Lukáts *et al.* 1997). STR enzyme, which is encoded by a single *str* gene, was initially isolated from plant cell suspensions of *C. roseus* (DeWaal *et al.* 1995). At least seven STR isoforms have been identified in *C. roseus* and they are probably a result of post-translational modification of the enzyme (McKnight *et al.* 1991). The *str* gene was cloned from *R. serpentina* (Hampp and Zenk 1988, Kutchan *et al.* 1988, McKnight *et al.* 1990, Bracher and Kutchan 1992) and later from Rubiaceae species such as *Cinchona* (Stevens *et al.* 1993) and *Ophiorrhiza* (Yamazaki *et al.* 2003). STR from *R. serpentina* is a monomeric precursor protein with 344 amino acids that exhibits 100, 79, and 58% identity to STR from *Rauwolfia mannii* Stapf, *C. roseus* and *O. pumila*, respectively (Ma *et al.* 2006). The intermediate 3α(*S*)-strictosidine is a building block for TIAs in Apocynaceae and also in the quinoline alkaloids found in *Cinchona* and *Ipecacuanha* (Oudin *et al.* 2007).

The biosynthetic pathways leading to different classes of indole alkaloids branch off after strictosidine. In the first switching point deglucosylation is catalyzed by the highly substrate-specific strictosidine β-D-glucosidase, SG (Geerlings *et al.* 2000) to yield the highly reactive open-ring dialdehyde intermediate. SG enzyme and its gene have been described from *Catharanthus* (Geerlings *et al.* 2000) and *Rauwolfia* (Hemscheidt and Zenk, 1980, Gerasimenko *et al.* 2002, Barleben *et al.* 2007). The unstable dialdehyde is converted to 4,21-dehydrocorynantheine aldehyde (O'Connor and Maresh 2006), followed by spontaneous conversions to yield either 4,21-dehydrogeissoshizine or equally equilibrated to cathenamine (the enol form of 4,21-dehydrogeissoshizine) (Facchini and St-Pierre 2005, Barleben *et al.*, 2007). Consequently, cathenamine or 4,21-dehydrogeissoshizine enters multiple pathways and is further converted to different types of indole alkaloids with structurally diverse carbon skeletons (Gerasimenko *et al.* 2002, Barleben *et al.* 2007) (**Figure 4**,**5**).

Sarpagan and ajmalan- (*e.g.* ajmaline, rhazine, and vallesiachotamine), corynanthe- (ajmalicine, tetrahydroalsonine, serpentine and yohimbine (**Figure 4**), strychnos- (preakuammicine), aspidosperma- (tabersonine), iboga- (catharanthine) (**Figure 5)**, quinoline- (camptothecin and quinine) and bisindole-type (vinblastine and vincristine; **Figure 6**) alkaloids are major classes of terpene indole alkaloids derived from (deglycosylated) strictosidine (O'Connor and Maresh 2006).

Vallesiachotamine and its isomer isovallesiachotamine are biosynthesized directly from a deglycosylated strictosidine, a dialdehyde, which is not a common intermediate of TIA biosynthesis (Djerassi *et al.* 1966, O'Connor and Maresh 2006). In addition, it was shown that heterologously expressed strictosidine synthase in *E. coli* produced strictosidine, which was converted to vallesiaschotamines (Shen *et al.* 1998). Strictosidine is a reactive compound and

after mild hydrolysis forms vallesiachotamine and isovallesiachotamine (Smith *et al.* 1971).

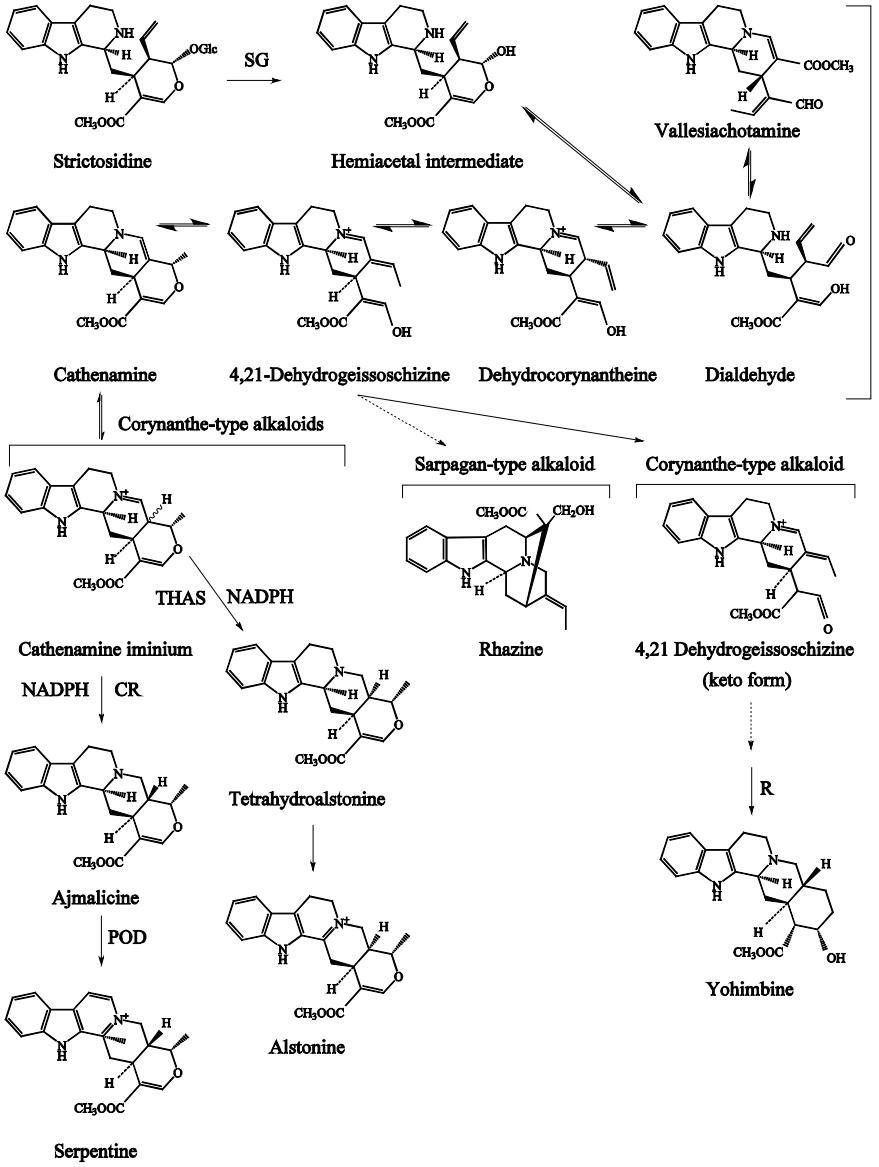


Figure 4. Biosynthesis of the terpenoid indole alkaloids in *C. roseus* from strictosidine (Modified from O'Connor and Maresh 2006, El-Sayed and Verpoorte 2007, Dewick 2009). SG: strictosidine β-D-glucosidase; THAS: tetrahydroalstonine synthase; CR: cathenamine reductase; R: reductase; POD: peroxidase; NADPH: nicotinamide adenine dinucleotide.

Ajmalicine, serpentine and tetrahydroalsonine belong to the corynanthe-type alkaloids. In the biosynthesis of these alkaloids, cathenamine is reduced to form ajmalicine, via an intermediate cathenamine iminiume, by the enzyme cathenamine reductase (CR) which uses NADPH as a cofactor (Stöckgit 1978, Hemscheidt and Zenk 1985) (**Figure 4**). Two different cathenamine reductases are known, one converting cathenamine to ajmalicine (Stöckgit 1978) and the other cathenamine to tetrahydroalstonine. Conversion of the iminium form of cathenamine to tetrahydroalstonine is catalyzed by tetrahydroalstonine synthase (THAS, cofactor NADPH), tetrahydroalstonine is finally oxidized to alstonine (**Figure 4**).

A partially purified NADPH dependent reductase, described as tetrahydroalstonine synthase and isolated from a *C. roseus* cell line, catalyzed the conversion of cathenamine to tetrahydroalstonine *in vitro* (Hemscheidt and Zenk 1980). Ajmalicine is further oxidized to form serpentine by peroxidase (POD) in the vacuoles (Blom *et al.* 1991). However, the oxidation enzyme has not been cloned from plants, but the conversion of ajmalicine to serpentine from peroxidases present in vacuoles of *C. roseus* has been observed (Pfitzner and Stöckigt 1982, Sottomayor *et al.* 2004). In addition, light stimulates oxidation of ajmalicine to serpentine in cells and tissue culture (Loyola-Vargas *et al.* 1992, Zhao *et al.* 2001).

Rhazine (syn. akuammidine) belongs to the sarpagan- alkaloids; it has been hypothesized that it is formed from 4,21-dehydrogeissoschizine through cathenamine (Verpoorte *et al.* 2002). 4,21-Dehydrogeissoschizine represents a key branch point intermediate leading to the biosynthesis of diverse TIAs (Ziegler and Facchini 2008). Geissoschizine, a precursor of dehydrogeissoschizine, which was isolated from *R. stricta* and other related plants, was regarded as one of the key immediate precursors in the biosynthesis of alkaloids such as rhazine and the strictamine group (Banerji *et al.* 1970, Chatterjee *et al.* 1976).

However, further studies have shown that dehydrogeissoschizine is indeed the central intermediate for biosynthesis of a large number of polycyclic indole alkaloids (Stöckigt 1978, Stöckigt *et al.* 1980, Qureshi and Scott 1968, O'Connor and Maresh 2006). The enzymes that convert deglycosylated strictosidine to yohimbine have not been identified. However, a direct biosynthetic route may involve homoallylic isomerization of the keto dehydrogeissoschizine followed by 1,4 conjugate addition and reduction (Kan-Fan and Husson 1980, Dewick 2002).

It is believed that preakuammicine (strychnos-type intermediate) is the common precursor diverting strictosidine to aspidosperma (*e.g.* tabersonine/vindoline, vincadifformine)-, and iboga (*e.g.* catharanthine)-type alkaloids. The formation of preakuammicine from 4,21-dehydrogeissoschizine has also been proposed by Scott and Qureshi (1969) and Dewick (2002) (see **Figure 5**). Reduction of preakuammicine yields stemmadenine, which rearranges to form the acrylic ester dehydrosecodine. The latter compound functions as an intermediate for the aspidosperma and the iboga skeletons (Battersby *et al.* 1969) (**Figure 5**).

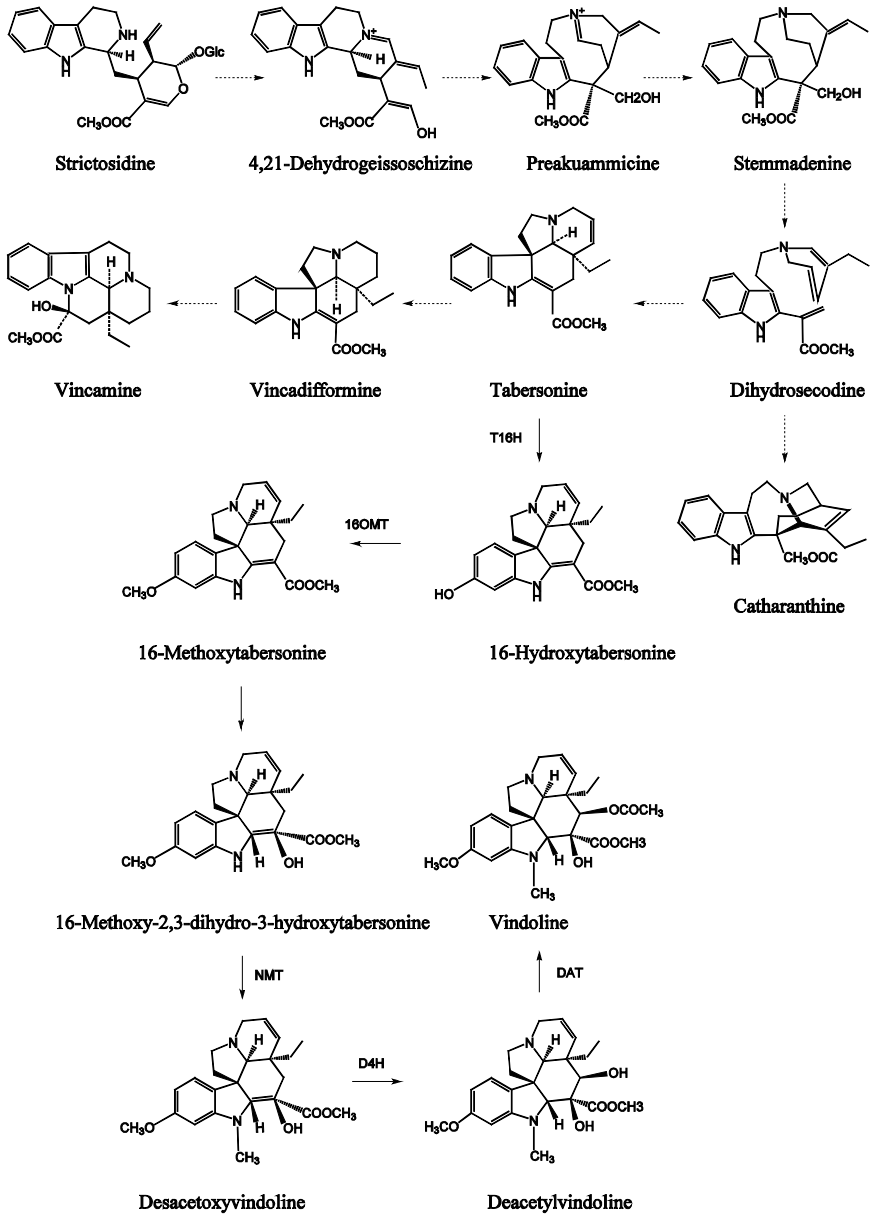


Figure 5. Proposed biosynthetic pathway of aspidosperma-type (tabersonine, vindoline) and iboga-type (catharantine) alkaloids (no enzymatic information is available for some of the proposed steps; Adapted from O'Connor and Maresh 2006 and Dewick 2002). T16H: tabersonine-16-hydroxylase; HTOM: 16-hydroxytabersonine 16-*O*-methyltransferase; NMT: *N*-methyltransferase; D4H: desacetoxyvindoline 4-hydroxylase; DAT: deacetylvindoline-4-*O*-acetyltransferase.

El-Sayed *et al.* (2004) reported that feeding of stemmadenine to a *C. roseus* suspension resulted in higher accumulation of catharanthine and tabersonine. In addition, it was also shown that tabersonine is converted to vincadifformine via 6,7-reductase. This step is uncharacterized at the genetic/enzymatic level (Giddings *et al.* 2011).

Vincamine, an eburnamine-type alkaloid, also originates from tabersonine in a series of reactions (Dewick 2002). The enzymes and genes involved in the catharanthine biosynthetic pathway have not been identified (El-Sayed and Verpoorte 2007), although the vindoline biosynthesis pathway branching from tabersonine has been intensively studied (Loyola-Vargas *et al.* 2007).

Channelling of the flux from intermediate 4,21-dehydrogeissoschizine to vindoline is catalyzed by six enzymatic steps (**Figure 5**). The route includes the hydroxylation of C-16 of tabersonine to form 16-hydroxyltabersonine by cytochrome P-450 dependent tabersonine-16-hydroxylase (T16H) (St. Pierre and De Luca 1995, Schröder *et al.* 1999, Besseau *et al.* 2013), which is further methylated to 16-methoxytabersonine by 16-O-methyltransferase (16OMT). This enzyme has been purified (Cacace *et al.* 2003) and the gene was cloned from *C. roseus* (Levac *et al.* 2008). In the next step hydration of the 2,3-double bond of 16-methoxytabersonine forms 16 methoxy-2,3-dihydro-3-hydroxytabersonine via an uncharacterized hydroxylase. The subsequent step catalyzed by *N*-methyltransferase (NMT) converts the previous intermediate to desacetoxyvindoline. NMT activity has been detected only in differentiated plants, not in plant cell cultures (Dethier and De Luca 1993, Liscombe *et al.* 2010). The two terminal steps of vindoline biosynthesis are catalyzed by a light-regulated deacetylvindoline-4-hydroxylase (D4H) (Vázquez-Flota *et al.* 1997) and by deacetylvindoline-4-*O*-acetyltransferase (DAT) (St-Pierre *et al.* 1998), respectively (**Figure 5**). The two genes encoding these enzymes have been cloned from the plant but have not been reported from the plant cell cultures.

An important step of the bisindole alkaloid biosynthesis is the dimerization of vindoline and catharanthine catalyzed by α-3',4'-anhydrovinblastine synthase to produce anhydrovinblastine (Sottomayor *et al.* 2004). This enzyme, known as *Cr*Prx1*,* belongs to the class III basic peroxidases (Costa *et al.* 2008). Anhydrovinblastine is converted into vinblastine via hydroxylation of the double bond and finally vincristine is formed through oxidation of the *N*-methyl group (**Figure 6**).

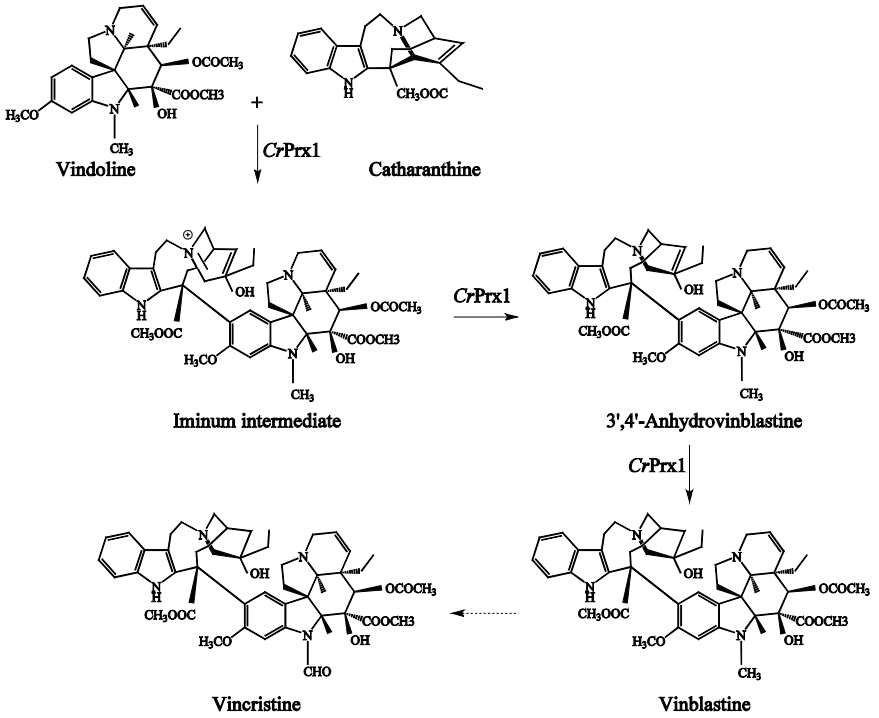


Figure 6. Vinblastine and vincristine biosynthesis from dimerization of vindoline and catharanthine (Adopted from O'Connor and Maresh 2006).

Subcellular localization of TIAs

TIA biosynthesis pathways are under strict developmental regulation and are coordinately regulated by 30 enzymatic steps involving at least 35 known intermediates (van der Heijden *et al.* 2004, Facchini and De Luca 2008). The pathways are located in at least four different cell types, and five different subcellular compartments are involved. In the aerial tissues, *tdc* and *str* are highly expressed in the epidermis of leaf (**Figure 7**), and they are also localized in protoderm and cortical cells around the root apical meristem (Vázquez-Flota et al. 1997, St. Pierre *et al.* 1999, IrmLer *et al.* 2000, Guirimand *et al.* 2011a).

In situ mRNA hybridization and immune-cytochemistry studies revealed that MEP pathway genes are predominantly expressed in the internal phloem associated parenchyma (IPAP) cells. IPAPs are located in the periphery of stem pith or intraxylary on the upper part of the vascular bundles in leaves (Burlat *et al.* 2004, Oudin *et al.* 2007, Mahroug *et al.* 2007). Similarly, hybridization also demonstrated that transcripts of *ges, is, 8-hgo, io, 7-dlgt* and *7-dlh* along with *g8o* are localized in the IPAPs (Geu-Flores *et al.* 2012, Simkin *et al.* 2012, Asada *et al.* 2013, Miettinen *et al.* 2014) (**Figure 7**). Loganic acid is the intermediate

transferred from IPAPs to epidermis, then methylated in the cytosol, and subsequently further oxidized in the endoplasmic reticulum (ER) to yield secologanin. It is thought that the transportation of loganic acid controls fluxes in secologanin production (IrmLer *et al.* 2000, St-Pierre *et al.* 1999, Yamamoto *et al.* 2000).

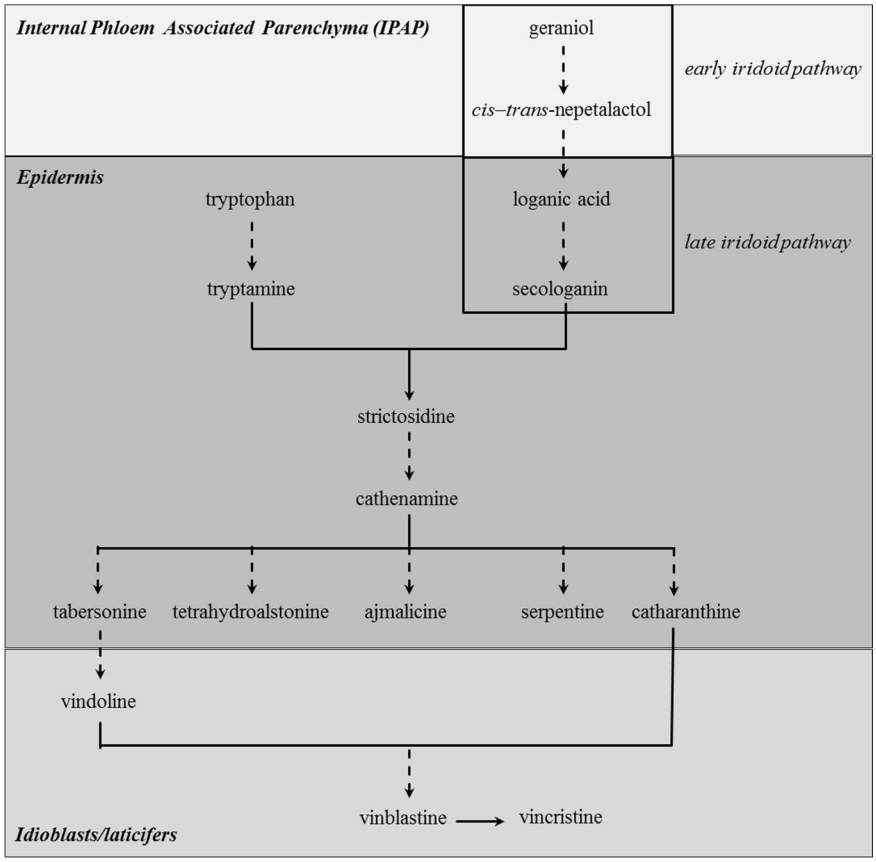


Figure 7. Tissue localization of TIA biosynthesis (Adopted from Miettinen 2013).

The carborundum abrasion (CA) approach showed that the MEP pathway and early steps of the seco-iridoid pathways take place in IPAP cells, whereas the last two steps of the seco-iridoid pathway are localized in the epidermis (Miettinen *et al.* 2014, **Figure 7**).

The MEP pathway occurs in plastids (Roytrakul and Verpoorte, 2007). Green fluorescent protein (GFP) localization indicated that *C. roseus* geranyl diphosphate synthase (*Cr*GPPS) is present in mitochondria (Rai *et al.* 2013). Transient transformation of *C. roseus* cells with a yellow fluorescent protein-fusion construct revealed that geraniol synthase (GES) is localized in plastid stroma and

stromules (Simkin *et al.* 2012). The use of GFP fusions in *C. roseus* revealed the occurrence of geraniol-8- oxidase (G8O), iridoid oxidase (IO), 7-deoxyloganic acid hydroxylase (7-DLH) and secologanin synthase (SLS) in the ER (Guirimand *et al.* 2011a, Miettinen *et al.* 2014). Iridoid synthase (IS) and loganic acid O-methyltransferase (LAMT) are localized in the cytosol (Geu-Flores *et al.* 2012), whereas 8-hydroxygeraniol oxidoreductase (8-HGO) and 7-deoxyloganetic acid glucosyl transferase (7-DLGT) exist in both cytosol and nucleus (Miettinen *et al.* 2014).

Cathenamine, catharanthine, serpentine, ajmalicine and tetrahydroalstonine are localized in the cytosol of epidermal cells (De Luca *et al.* 2014) (**Figure 7**). The subcellular localization of pathway enzymes involved in vindoline biosynthesis and dimerization with catharanthine to form vinblastine and vincristine are poorly understood. The subcellular localization studies indicated that *CrPrx1* is localized in vacuoles (Costa *et al.* 2008). The last two enzymes of vindoline biosynthesis, deacetylvindoline-4-hydroxylase (D4H) and deacetylvindoline-4-*O*-acetyltransferase (DAT) (Vázquez-Flota *et al.* 1997, St-Pierre *et al.* 1998), are expressed only in idioblast and laticifer cells (St-Pierre *et al.* 1999, Guirimand *et al.* 2011b) (**Figure 7**).

Transport of TIAs

Compartmentation and localization of the TIA pathways are considered as a regulatory mechanism, since it necessitates the transport of different metabolites from one location to another, where the metabolites are further transformed. Different types of transporters have been suggested to be involved in the trafficking of intermediates (Yazaki *et al.* 2006, De Luca *et al.* 2014). The H^+ antiporter and ABC-transporters (ATP-binding-cassette, ABC) have been proposed to be involved in the transportation of strictosidine, ajmalicine, catharanthine and vindoline across the tonoplast (Roytrakul and Verpoorte, 2007). An ABC multi drug-resistant transporter of *C. japonic*a (*Cj*MDR1) has been heterologously transformed to *C. roseus* cell cultures, resulting in significantly enhanced accumulation of ajmalicine and tetrahydroalstonine (Pomahačová *et al.* 2009).

A catharanthine ABC-transporter (*Cr*TPT2) which is mainly expressed in the epidermis of young leaves was recently cloned and functionally characterized (Yu and De Luca 2013). It was shown that catharanthine induced *CrTPT2* expression and its silencing redistributes catharanthine into the leaves, leading to an increase of dimeric alkaloid levels. It is thought that *Cr*TPT2 may be specific to TIA-producing plant species, where it facilitates the secretion of alkaloids to the leaf surface. Recently the vacuolar transport mechanism of the main TIAs, vindoline, catharanthine, and α-3',4'-anhydrovinblastine, which are accumulated in *C. roseus* leaves, was characterized using a tonoplast vesicle system (Carqueijeiro *et al.* 2013). It was claimed that TIAs are actively taken up by *C. roseus* mesophyll vacuoles through a vacuolar transport mediated by a proton-driven antiport and not by ABC transporters.

2.5 Biotechnological approaches for production of TIAs

The production of TIAs is typically very low in plants and strongly depends on the vegetative stage, stress and nutrient accessibility conditions (Verpoorte *et al.* 2002). Therefore, isolation of alkaloids from intact plants is laborious and costly. For example, 15 tons of dried *C. roseus* leaves are required for the production of 28 g of vinblastine (Noble 1990). The chemical complexity of TIAs in general and the complicated structure of dimeric alkaloids in particular (*e.g.* vinblastine and vincristine) have hitherto hampered their chemical synthesis *in vitro*, mainly due to difficulties in achieving the correct stereochemical structure, which is critical for their activity (Miyazaki 2007). For example, in vincristine the stereochemistry at C-16 is *S* whereas at C-14' it is *R* and at C-20' it is *S*. Inversion of the C-16 configuration from *S* to *R* results in a complete loss of activity, as does the C-14' conversion from *R* to *S*.

Biotechnological approaches, such as cell and organ cultures, could provide alternative and efficient methods for the production of valuable TIAs. However, the yield of TIAs is often too low or even absent in the cell cultures. Therefore, cell cultures have not found commercial applications despite extensive studies on the optimization of growth and production media and cell line selection (Miralpeix *et al.* 2013). This is usually due to the fact that production is controlled in a tissue-specific manner and de-differentiation results in loss of production capacity (Verpoorte *et al.* 2002). In *C. roseus* the valuable constituents, vinblastine and vincristine, fail to accumulate in cell cultures due to the absence of the precursor of biosynthesis, vindoline. On the other hand, in *C. roseus* plants, vindoline is abundant but catharanthine is limited. By contrast, *C. roseus* cell cultures are able to synthesize higher levels of catharanthine than in intact plants (Zhao and Verpoorte 2007). Therefore, a chemical semi-synthesis of vinblastine by coupling two monomeric building blocks, *i.e.* catharanthine isolated from plant cell cultures and vindoline from cultivated plants has been proposed (Misawa and Goodbody 1996).

Metabolic engineering is an approach to manipulate flux through both primary and secondary metabolic pathways using gene transfer technology, allowing for the redirection of carbon flux towards products of interest (Lau *et al.* 2014). Therefore, engineering of the metabolic flux in the TIA pathway by the introduction of rate-limiting genes appears an interesting platform to boost the production of TIAs (Glenn *et al.* 2013). However, lack of knowledge of the enzymes responsible for TIA biosynthesis, particularly in the secologanin pathway, has hindered the engineering of the pathway (Miettinen *et al.* 2014). On the other hand, the recent finding of missing genes in the secoiridoid pathway opens new opportunities for TIA metabolic engineering.

Hairy root cultures offer a convenient strategy for the production of plant secondary metabolites, particularly alkaloids and their derivatives (Sevón and Oksman-Caldentey 2002). Hairy roots possess several attractive features,

including high genetic stability (compared to un-differentiated cultures) and rather fast growth rates (compared to normal roots) in hormone-free media (Giri and Narasu 2000, Ono and Tian 2011). Moreover, a transgenic root system offers great potential for elicitation, biotransformation and metabolic engineering through the Ri plasmid of *Agrobacterium rhizogenes*. Since metabolic pathways are controlled at multiple levels, the focus of metabolic engineering has shifted from the introduction of a single gene to the simultaneous overexpression of several biosynthetic genes. *C. roseus* hairy roots accumulate indole alkaloids more than suspension cultures (Ciau-Uitz *et al.* 1994). After the initiation of suspension cultures from hairy roots, the alkaloid content diminished in the cell suspensions, but after regeneration of hairy roots the alkaloid content reached the original level (Moreno-Valenzuela *et al.* 1998).

In TIA biosynthesis, the early efforts at metabolic engineering focused on the expression of *tdc* and *str* genes in *C. roseus* cell cultures (Facchini 2001). However, in most cases the overexpression of genes from the indole biosynthesis pathway, such as *tdc* in *C. roseus* cell or hairy root cultures, only led to higher levels of tryptamine, while the contents of the majority of the studied alkaloids did not change (Mérillon *et al.* 1986, Goddijn *et al.* 1995, Canel *et al.* 1998, Whitmer *et al.* 2002, Hughes *et al.* 2004a,b, Hong *et al.* 2006). Similar results were obtained after feeding the cultures with indole precursors, tryptophan or tryptamine (Facchini and DiCosmo 1991, Whitmer *et al.* 1998).

Geraniol feeding of *C. roseus* cell (Lee-Parsons and Royce 2006) and hairy root cultures (Morgan and Shanks, 2000) resulted in an increase in the production of ajmalicine and tabersonine. This may suggest that the biosynthetic step leading to the production of geraniol is critical in TIA biosynthesis. This was later confirmed by Simkin *et al.* (2013), who observed that the treatment of *Catharanthus* cells with methyl jasmonate increased *Cr*GES transcript levels.

It has been reported that the amount of catharanthine increases through the overexpression of *g8o* or both *g8o* and o*rca3* genes in *C. roseus* hairy roots (Wang *et al.* 2010). On the other hand, the overexpression of *orca3* alone or the co-overexpression of *orca3* and *g8o* in *C. roseus* have been shown to stimulate the accumulation of monomeric alkaloids, strictosidine, vindoline and catharanthine (Pan *et al.* 2012). However, only a slight increase in the accumulation of the bisindole alkaloids anhydrovinblastine and vinblastine was observed, indicating that more regulatory factors need to be induced. The overexpression of *orca3* resulted in an increase in ajmalicine and serpentine concentrations but a decrease in tabersonine, lochnericine, and hörhammericine concentrations in *C. roseus* hairy roots (Peebles *et al.* 2009). Overexpression of *orca2* in *C. roseus* hairy roots also led to an increase in catharanthine and vindoline content (Liu *et al.* 2011).

In several cases, overexpression of the *str* gene in *C. roseus* has been shown to be directly correlated with the TIA content (Canel *et al.* 1998; Whitmer *et al.* 2002, Dutta *et al.* 2005). In *C. roseus* cell lines overexpressing *tdc* or *str* the level of alkaloid biosynthesis can be increased by feeding loganin, and even greater increase in alkaloid production can be achieved by feeding both loganin and

tryptamine (Moreno *et al.* 1993, Whitmer *et al.* 2002). There are also other reports that feeding loganic acid, loganin or secologanin to *C. roseus* cells often stimulates the accumulation of TIAs (El-Sayed and Verpoorte 2007, Oudin *et al.* 2007). Of the iridoid precursors, loganin is most efficiently incorporated into indole alkaloids. Thus, TIA accumulation appears to be much more dependent on the enzymes of the terpene pathway than of the indole pathway.

Transcription factors

The biosynthetic steps of TIA production in *C. roseus* are also under transcriptional control. Transcription factors (TFs) regulate the expression of specific genes through sequence-specific DNA-binding and protein-protein interactions (Gantet and Memelink 2002). They can both activate or repress gene expression, resulting in either increased or decreased messenger RNA production (van der Fits and Memelink 2000, Memelink and Gantet 2007). Hitherto, seven putative activators (ORCA2, ORCA3, *Cr*BPF1, *Cr*MYC1, *Cr*MYC2, *Cr*WRKY1 and *Cr*WRKY2) and five putative promotor repressors (ZCT1, ZCT2, ZCT3, GBF1and GBF2) have been found to be involved in terpenoid indole alkaloid biosynthesis in *C. roseus* (Patra *et al.* 2013).

The well-known TFs regulating TIA biosynthesis are the jasmonates-responsive ORCA2 and ORCA3 (octadecanoid-responsive *Catharanthus* AP2-domain proteins) from the plant-specific AP2/ERF (APETALA2/ethylene-responsive factor). The expression of *orca* genes is induced by jasmonates, a major and essential signalling pathway inducing TIA biosynthesis (van der Fits and Memelink, 2001).

For *C. roseus* cell suspension cultures overexpressing *orca3*, an increase in TIAs was not observed. This was due to the fact that *g8o* was not up-regulated under ORCA3 over-expression (van der Fits and Memelink 2000). Only when loganin was fed to cells overexpressing *orca3* was an increase in TIAs observed. In hairy roots, neither *tdc* nor *g8o* were up-regulated when ORCA3 was overexpressed (Peebles *et al.* 2009). When loganin, tryptophan or their mixture were fed to *C. roseus* hairy roots overexpressing ORCA3, no significant increases in TIA metabolites were observed. This finding confirms the potential of different regulatory mechanisms of TIA biosynthesis in cell suspension cultures and differentiated tissues such as hairy roots. Overexpression of transcription factors simultaneously with other rate-limiting genes appears to be a more effective and promising approach for the control of metabolic pathways. It has been shown that the expression of *orca3* in cell and hairy root cultures of *C. roseus* elevates the expression of *tdc*, *str*, *ges*, secologanin synthase (*sls*) and desacetoxyvindoline 4-hydroxylase (*d4h*) (van der Fits and Memelink 2000, Rischer *et al.* 2006). This indicates that *orca3* is a central regulator of TIA biosynthesis as well as of the biosynthesis of their precursors. However, *orca3* does not regulate the expression of *g8o*, strictosidine β-D-glucosidase (*sd*) or deacetylvindoline-4-*O*-acetyltransferase (*dat*) (Li *et al.* 2013). These observations suggest that the *g8o* is the bottle-neck of the TIA biosynthesis pathway and is regulated by other JA- or

elicitor-responsive transcription factors (Verpoorte and Memelink 2002). In addition, it has been shown that *orca2* increases the expression of *str, tdc* and *g8o* genes in *C. roseus* (Li *et al.* 2013; Menke *et al.* 1999).

Elicitation

Plants often biosynthesize defensive secondary metabolites following attacks by microorganisms and/or herbivores. As a result of the attack the plant produces compounds known as elicitors. Elicitors activate signal transduction pathways that generate secondary signals within plants. The three major plant secondary signalling molecules are jasmonate or its derivatives (JAs) (Balbi and Devoto 2008), ethylene (Wang *et al.* 2002) and salicylic acid (Shah 2003). Production of these hormones leads to cascades of events responsible for the physiological and morphological responses to the external stress. Among these three key regulatory signals, JAs are considered to be the most important molecules for induction of the biosynthesis of a variety of secondary metabolites, including alkaloids, terpenoids, glucosinolates and phenylpropanoids in different plant species (Zhao et *al.* 2005).

Addition of elicitors in plant cell and organ cultures is a common method of enhancing secondary metabolites for metabolic, enzymatic or regulatory studies. This strategy mimics the plant's natural response to pathogen attack or wounding by activating JA biosynthesis, and JA then induces TIA biosynthetic gene expression. In *C. roseus*, the mRNA levels of several key TIA pathway genes, including *tdc*, *g8o*, *str*, *sd* and cytochrome P450 reductase (*cpr*), increase significantly after exposure to jasmonic acid (van der Fits and Memelink 2000, Collu *et al.* 2001).

A comprehensive profiling analysis of *C. roseus* by combining genome-wide transcript profiling of cDNA-amplified fragment-length polymorphism with metabolic profiling of elicited *C. roseus* cell cultures has resulted in the identification of genes most probably involved in TIA metabolism. Some of these genes may be the missing links in the biosynthesis of monomeric TIAs (Rischer *et al.* 2006). 417 differentially expressed transcript tags have been discovered and the majority of these represented new sequence information, 37% of which was not similar to any known plant genes. In addition, a metabolic network was established linking metabolites and gene expression profiles (Rischer *et al.* 2006, Oksman-Caldentey *et al.* 2007)

Biotechnology of *Rhazya stricta*

Since 1986, only a few classical cell culture studies on *R. stricta* have been performed to investigate their capacity to produce alkaloids. When the present study was started, there were no genomic data for *R. stricta*. Furthermore, there were no report on transformation of *R. stricta*. However, the complete sequences of the plastid and mitochondrial genomes of *R. stricta* have been realized only recently (Park *et al.* 2014). Additionally, phytochrome-like genes in the plant were characterized using a *de novo* genome assembly of next generation sequence data (Sabir *et al.* 2013). The temporal foliar transcriptome of the plant in its natural environment has also been reported (Yates *et al.* 2014).

Pawelka and Stöckigt (1986) developed a cell suspension culture from the plant. The 4X- medium, modified B5 medium (Ulbrich and Zenk 1979) containing indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic (2,4-D), α-naphthaleneacetic acid (NAA) and kinetin (KIN) was used for calli initiation. Major alkaloids were identified from the culture.

Calli from various explants of *R. stricta* including roots, cotyledons, stems and leaves have been established (Omar 1988). It was found that calli induction was optimal on Murashige and Skoog (MS) (Murashige and Skoog 1962) medium supplemented with IAA and NAA for roots, stems and leaves. Calli could be initiated from cotyledons and leaves on MS media supplemented with 2,4-D. Regeneration took place from the stem-derived calli, supplemented on the media containing NAA and grown in the dark. Suspension cultures were established from leaf-derived calli on MS media supplemented with enriched sucrose, inositol and 2,4-D. Suspension cultures from root calli on White's media (White 1943) supplemented with 2,4-D, enriched with vitamins and casein hydrolysate, were also established. Somatic embryogensis could be achieved with NAA only or NAA in combination with KIN. Alkaloid contents of all calli were analysed by TLC. The root calli contained more alkaloids, whereas calli from leaves and stems had the same amounts and cotyledon-derived calli had the lowest concentration of alkaloids.

Somatic hybrid cell suspension cultures of *R. serpentina* × *R. stricta* have also been developed by Stöckgit's group, and alkaloid diversity was investigated in the undifferentiated cells and elicited cultures (Kostenyuk *et al.* 1991,1995, Aimi *et al.* 1996, Kitajima *et al.* 1996, Sheludko *et al.* 1999, Sheludko *et al.* 2000). A new monoterpenoid indole alkaloid, 3-oxo-rhazinilam, was isolated from intergeneric somatic hybrid cells (Gerasimenko *et al.* 2001a). The alkaloids which were identified from *R. stricta* and hybrid cell suspension cultures are presented in **Table 5**.

Recently the micropropagation of *R. stricta* has been established from nodal segments containing axillary buds (El-Tarras *et al.* 2012). Shoot proliferation was achieved in MS medium with 6-benzylaminopurine (BAP) and KIN. Subsequently roots were formed on the same medium supplemented with indole-3-butyric acid (IBA).

Table 5. Major alkaloids identified from *R. stricta* and hybrid *Rauwolfia serpentina* × *R. stricta* cell cultures.

Compound	Origin	Formula	MW	Reference
β-Carboline*	Hybrid cell culture	$C_{11}H_8N_2$	168	Aimi *et al.* 1996
1-(β-Carbolin-1-yl)-3,4,5-tri-hydroxy-1-pentanone*	Hybrid cell culture	$C_{16}H_{16}N_2O_4$	300	Aimi *et al.* 1996, Kitajima *et al.* 1996
16(*R*)-19, 20(*E*)-Isositsirikine*	Hybrid cell culture	$C_{21}H_{26}N_2O_3$	354	Aimi *et al.* 1996
17-O-Acetylajmaline	Elicited hybrid culture	$C_{22}H_{28}N_2O_3$	368	Sheludko *et al.* 1999
17-O-Acetyl-norajmaline	Elicited hybrid culture	$C_{21}H_{26}N_2O_3$	354	Sheludko *et al.* 1999
17-O-Acetyl-nortetraphyllicine	Elicited hybrid culture	$C_{22}H_{26}N_2O_2$	336	Sheludko *et al.* 1999
17-O-Acetylrauglucine	Elicited hybrid culture	$C_{28}H_{38}N_2O_8$	530	Sheludko *et al.* 1999
17-O-Acetyl-tetraphyllicine	Elicited hybrid culture	$C_{21}H_{24}N_2O_2$	350	Sheludko *et al.* 1999
1-Acetyl-β-carboline*	Hybrid cell culture	$C_{13}H_{10}N_2O$	210	Aimi *et al.* 1996
1-Methoxycarbonyl-β-carboline*	Hybrid cell culture	$C_{13}H_{10}N_2O_2$	226	Aimi *et al.* 1996
21-Hydroxysarpagan-glucoside	Elicited hybrid culture	$C_{25}H_{32}N_2O_7$	470	Sheludko *et al.* 1999
5(*S*)-5-Carbomethoxy-strictosidine*	Hybrid cell culture	$C_{29}H_{36}N_2O_{11}$	588	Aimi *et al.* 1996
5(*S*)-5-Carboxy-strictosidine*	Hybrid cell culture	$C_{28}H_{34}N_2O_{11}$	574	Aimi *et al.* 1996
Ajmalicine	Elicited hybrid culture	$C_{21}H_{24}N_2O_3$	352	Sheludko *et al.* 1999
Ajmaline	Elicited hybrid culture	$C_{20}H_{26}N_2O_2$	326	Sheludko *et al.* 1999
Akuammicine*	*R. stricta* cell culture	$C_{20}H_{22}N_2O_2$	322	Pawelka and Stöckigt 1986
Deserpidine	Elicited hybrid culture	$C_{32}H_{38}N_2O_8$	578	Sheludko *et al.* 1999

Continues→

Eburenine*	[1]*R. stricta* cell culture, [2]Elicited hybrid culture	$C_{19}H_{24}N_2$	280	[1]Pawelka and Stöckigt 1986, [2]Sheludko *et al.* 2000
Eburnamine*	*R. stricta* cell culture	$C_{19}H_{24}N_2O$	296	Pawelka and Stöckigt 1986
Eburnamonine*	*R. stricta* cell culture	$C_{19}H_{22}N_2O$	294	Pawelka and Stöckigt 1986
Isomer of isositsirikine*	Elicited hybrid culture	$C_{21}H_{26}N_2O_3$	354	Sheludko *et al.* 1999
Isomer of yohimbine	Elicited hybrid culture	$C_{21}H_{26}N_2O_3$	354	Sheludko *et al.* 1999
Isomer of yohimbine oxindole	Elicited hybrid culture	$C_{21}H_{26}N_2O_4$	370	Sheludko *et al.* 1999
Isomer of yohimbine pseudoindoxyl	Elicited hybrid culture	$C_{21}H_{26}N_2O_4$	370	Sheludko *et al.* 1999
Macrophylline	Elicited hybrid culture	$C_{20}H_{21}N_3O$	319	Sheludko *et al.* 1999
Norajmaline	Elicited hybrid culture	$C_{19}H_{24}N_2O_2$	312	Sheludko *et al.* 1999
Perakine	Elicited hybrid culture	$C_{21}H_{22}N_2O_3$	350	Sheludko *et al.* 1999
Raucaffricine	Elicited hybrid culture	$C_{27}H_{32}N_2O_8$	512	Sheludko *et al.* 1999
Rescinnamine	Elicited hybrid culture	$C_{35}H_{42}N_2O_9$	634	Sheludko *et al.* 1999
Reserpine	Elicited hybrid culture	$C_{33}H_{40}N_2O_9$	608	Sheludko *et al.* 1999
Rhazine*	*R. stricta* cell culture	$C_{21}H_{24}N_2O_3$	352	Pawelka and Stöckigt 1986,
Rhazinilam*	Elicited hybrid culture	$C_{19}H_{22}N_2O$	294	Sheludko *et al.* 2000
Sarpagine	Elicited hybrid culture	$C_{19}H_{22}N_2O_2$	310	Sheludko *et al.* 1999, Sheludko *et al.* 2000
Stemmadenine*	[1]*R. stricta* cell culture, [2]Hybrid cell culture, [3]Elicited hybrid culture	$C_{21}H_{26}N_2O_3$	354	[1]Pawelka and Stöckigt 1986, [2]Kostenyuk *et al.* 1995, [3]Sheludko *et al.* 2000

Continues→

Strictosidine*	Elicited hybrid culture	$C_{27}H_{34}N_2O_9$	530	Sheludko *et al.* 1999, Sheludko *et al.* 2000
Strictosidine lactam*	[1]*R. stricta* cell culture, [2]Hybrid cell culture, [3]Elicited hybrid culture	$C_{26}H_{30}N_2O_8$	498	[1]Pawelka and Stöckigt 1986, [2]Sheludko *et al.* 1999, [3]Sheludko *et al.* 2000
Suaveoline	Elicited hybrid culture	$C_{20}H_{21}N_3$	303	Sheludko *et al.* 1999
Tabersonine*	[1]*R. stricta* cell culture, [3]Elicited hybrid culture	$C_{21}H_{24}N_2O_2$	336	[1]Pawelka and Stöckigt 1986, [3]Sheludko *et al.* 2000
Tetrahydrosecodine*	*R. stricta* cell culture	$C_{21}H_{30}N_2O_2$	342	Pawelka and Stöckigt 1986
Tubotaiwine*	[1]*R. stricta* cell culture, [2]Hybrid cell culture, [3]Elicited hybrid culture	$C_{20}H_{24}N_2O_2$	324	[1]Pawelka and Stöckigt 1986, [2]Kostenyuk *et al.* 1995, [3]Sheludko *et al.* 2000
Vallesiachotamine isomers*	[1]*R. stricta* cell culture, [2]Hybrid cell culture, [3]Elicited hybrid culture	$C_{21}H_{22}N_2O_3$	350	[1]Pawelka and Stöckigt 1986, [2]Kostenyuk *et al.* 1995, [3]Sheludko *et al.* 1999, [3]Sheludko *et al.* 2000
Vincadifformine*	*R. stricta* cell culture	$C_{21}H_{26}N_2O_2$	338	Pawelka and Stöckigt 1986
Vincanine*	*R. stricta* cell culture	$C_{19}H_{20}N_2O$	292	Pawelka and Stöckigt 1986
Vinorine	Elicited hybrid culture	$C_{21}H_{22}N_2O_2$	334	Sheludko *et al.* 1999
Vomilenine	[1]Hybrid cell culture, [2]Elicited hybrid culture	$C_{21}H_{22}N_2O_3$	350	[1]Kostenyuk *et al.* 1995, [2]Sheludko *et al.* 1999, [2]Sheludko *et al.* 2000

*Alkaloids occur in *Rhazya* species. Superscript numbers refer to the reported references.

3. Aims of the study

The main aim of the work described in this thesis was to establish various *in vitro* culture systems for *R. stricta* and to investigate their alkaloid compositions. This study represents the first biotechnological attempt to develop transgenic hairy root cultures of *R. stricta*. For comparison, callus cultures from different explants were initiated and their alkaloid contents were studied.

The specific aims of the study were

- Establishment of cell and organ cultures and the development of transformation systems for *R. stricta*.
- Establishment of an elicitation procedure to stimulate secondary metabolite accumulation in *R. stricta*.
- Heterologous introduction of key genes involved in the early biosynthesis of terpenoid indole alkaloids (TIAs), *i.e.* geraniol synthase (*ges*), geraniol 8-oxydase (*g8o*) and strictosidine synthase (*str*).
- Development of qualitative and quantitative targeted (GC-MS, HPLC-DAD and UPLC-MS) and non-targeted (NMR) analytical methods for metabolites of the transgenic cultures.

4. Experimental

4.1 Plant material

Seeds of *R. stricta* were collected from Iran (Hormozgan province, Minab zone in the Persian Gulf area 27°08′48″ N, 57°04′48″ E) with a necessary permit (no. 14179/244/25) from the National Plant Gene-Bank of Iran (NPGBI). Prof. Gh.R. Amin (Faculty of Pharmacy, Tehran University of Medical Sciences, Iran) and BSc H. Rigi (Natural resource organization, Hormozgan province, Iran) confirmed the identification of plant material.

The seeds were rinsed with sterile water twice for 15 min using a magnetic stirrer, surface-sterilized with ethanol for five min, and stirred with sterilized water overnight to induce swelling of the pericarps. Seeds were then further sterilized with ethanol for 30 sec, followed by treatment with 5% (v/v) sodium hypochlorite solution (NaClO) supplemented with a few drops of Tween 20 in an ultrasonic bath (Fritsch, Laborette 17) for 15 min and finally rinsed five times with sterile water. Seeds were allowed to germinate for two days at 24±1°C in the dark on sterile moist filter paper for two to three days.

Seedlings were then placed on modified Gamborg B5 medium as described by Oksman-Caldentey *et al.* (1991), solidified with 0.3% (w/v) gelrite (Carl Roth GmbH; Karlsruhe, Germany) in 9 cm petri dishes at 24±1°C under a standard cool white fluorescent light with a flux rate of 30-40 µmol/mm^2 s and a 16 h photoperiod. Prior to the addition of gelrite, the medium was adjusted to pH 5.8 and then sterilized by autoclaving at 121°C for 20 min. After two weeks, the seedlings were transferred aseptically to sterile plastic boxes containing solid modified Gamborg B5 medium.

4.2 Chemicals

All chemicals and solvents were of analytical grade and are presented in **Tables 6** and **7**. Polymerase chain reaction (PCR) reagents were from Fermentas (GmbH Germany). Primers for the *virD*, *rolB*, *gusA*, *ges* and *g8o* genes were obtained from Sigma and for *str* from Oligomer Oy (Finland).

Table 6. List of chemicals for *in vitro* cultures of *R. stricta* and *A. rhizogenes*.

Chemical	Supplier
Agar	Difco (NJ, USA)
Agarose (SeaKem® LE Agarose)	Lonza Rockland, Inc. (Rockland, ME, USA)
$(NH_4)_2SO_4$	Merck (Darmstadt, Germany)
6-Benzylaminopurine (BAP)	Sigma-Aldrich (St. Louis, MO, USA)
Basta®	Duchefa (The Netherlands)
$CaCl_2 \cdot 2H_2O$	Sigma-Aldrich (St. Louis, MO, USA)
Cefotaxime sodium	Duchefa (The Netherlands)
Cetyl trimethyl ammonium bromide	Sigma-Aldrich (St. Louis, MO, USA)
Chitosan	Sigma-Aldrich (St. Louis, MO, USA)
$CoCl_2 \cdot 6H_2O$	Merck (Darmstadt, Germany)
$CuSO_4 \cdot 5H_2O$	Merck (Darmstadt, Germany)
D-mannitol	Sigma-Aldrich (St. Louis, MO, USA)
Ethanol (Etax)	Altia (Rajamäki, Finland)
Ethidium bromide	Sigma-Aldrich (St. Louis, MO, USA)
$FeSO_4 \cdot 7H_2O$	Sigma-Aldrich (St. Louis, MO, USA)
Gelrite	Carl Roth GmbH (Karlsruhe, Germany)
H_3BO_3	Sigma-Aldrich (St. Louis, MO, USA)
Hygromycin B	Sigma-Aldrich (St. Louis, MO, USA)
Isoamyl alcohol	Sigma-Aldrich (St. Louis, MO, USA)
Isopropanol	Rathburn Chemicals (Walkerburn, Scotland)
$K_2HPO_4 \cdot 3H_2O$	Merck (Darmstadt, Germany)
Kanamycin	Sigma-Aldrich (St. Louis, MO, USA)
KI	Merck (Darmstadt, Germany)
KNO_3	Merck (Darmstadt, Germany)
Mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
Meronem	AstraZeneca (UK)
Methyl jasmonate (MeJA)	Duchefa (The Netherlands)
$MgSO_4 \cdot 7H_2O$	Merck (Darmstadt, Germany)
$MnSO_4 \cdot H_2O$	Sigma-Aldrich (St. Louis, MO, USA)

myo-Inositol	Sigma-Aldrich (St. Louis, MO, USA)
$Na_2EDTA \cdot 2H_2O$ (Titriplex III)	Sigma-Aldrich (St. Louis, MO, USA)
NaCl	Sigma-Aldrich (St. Louis, MO, USA)
NaClO	AppliChem GmbH (Darmstadt, Germany)
$Na_2MoO_4 \cdot 2H_2O$	Merck (Darmstadt, Germany)
α-Naphthaleneacetic acid (NAA)	Sigma-Aldrich (St. Louis, MO, USA)
Nicotinic acid	Merck (Darmstadt, Germany)
Phenol	VWR International (PA, USA)
Purified water	PURELAB® Ultra Analytic Water Purification System (ELGA LabWater, High Wycombe, UK)
Pyridoxine hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Rifampicin	Sigma-Aldrich (St. Louis, MO, USA)
Sucrose	Dansukker (Finland)
Spectinomycin dihydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Thiamine hydrochloride	Merck (Darmstadt, Germany)
Tris-hydrochloride (Trizma® base)	Sigma-Aldrich (St. Louis, MO, USA)
Woody Plant Medium	Duchefa (The Netherlands)
X-GLUC (5-bromo-4-chloro-3-indolyl glucuronide)	Duchefa (The Netherlands)
Yeast extract	Difco (NJ, USA)
$ZnSO_4 \cdot 7H_2O$	Merck (Darmstadt, Germany)

Table 7. List of chemicals used in chemical analyses.

Chemical	Supplier
2,4'-Dipyridyl	Tokyo Kasei (Tokyo, Japan)
Acetonitrile	Rathburn Chemicals (Walkerburn, Scotland)
Ajmalicine hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Ammonia solution (25%)	Merck (Darmstadt, Germany)
Ammonium acetate	Sigma-Aldrich (St. Louis, MO, USA)
Deuterium oxide (99.90%)	Euriso-top (Sain Aubin, France)
Dichloromethane	Rathburn Chemicals (Walkerburn, Scotland)
Ibogaine hydrochloride	Carl Roth GmbH (Karlsruhe, Germany)
Formic acid	Sigma-Aldrich (St. Louis, MO, USA)
Methanol D4 (99.80%)	Euriso-top (Sain Aubin, France)
N-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)	Pierce (IL, USA)
Petroleum ether	Sigma-Aldrich (St. Louis, MO, USA)
Potassium dihydrogen phosphate (KH_2PO_4)	Riedel-de haën (C.O.O., Germany)
Purified water	PURELAB® Prima (ELGA LabWater, High Wycombe, UK)
Sodium 1-heptanesulphonate	Sigma-Aldrich (St. Louis, MO, USA)
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck (Darmstadt, Germany)
Sulphuric acid (95-97%)	Sigma-Aldrich (St. Louis, MO, USA)
Tabersonine	Sigma-Aldrich (St. Louis, MO, USA)
Tetrahydroalstonine	Sigma-Aldrich (St. Louis, MO, USA)
Vincamine	Sigma-Aldrich (St. Louis, MO, USA)
Vincanine	Accurate Chemical & Scientific Corporation (NY, USA)
Yohimbine hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)

4.3 Establishment of calli

Root, hypocotyl, leaf and stem segments with or without nodes were excised from four-week old seedlings, divided into segments of 0.5-1.0 cm and cultured on modified Gamborg B5 solidified medium with gelrite (0.3 w/v) and supplemented with 1 mg/L BAP and 0.75 mg/L NAA in 16 h/day illumination at 25±1 °C. In the

case of leaf explants, entire leaves were also placed on the hormone-containing medium. The initiated calli were harvested and evaluated based on their colour, texture and size after four weeks. Calli obtained were subcultured in the same fresh media at four-week intervals.

4.4 *Agrobacterium* strain and vectors

The wild type agropine strain of *A. rhizogenes* LBA 9402 was used. Transformed *A. rhizogenes* harbouring the Gateway™ (Invitrogen) overexpression vector pH7WGD2-GUS (Karimi *et al.* 2002) containing a cauliflower mosaic virus (CaMV) 35S promoter-GUS fusion sequence and the hygromycin phosphotransferase (*hph*) selectable marker was obtained from VIB, Ghent, Belgium, (Karimi *et al.* 2002) (http://gateway.psb.ugent.be/vector/show/pH2GW7/search/index/overexpression/any/).

The heterologous genes *str, g8o* (previously named as *g10h*) from *C. roseus* and *ges* from *V. officinalis* were received from University of Leiden (The Netherlands) and University of Wageningen (The Netherlands), respectively.

A. rhizogenes LBA 9402 carrying the pBIN2.4VoGES1 vector based on Gateway® technology (Ritala *et al.* 2014), containing a neomycin (kanamycin) phosphotransferase II gene (*NptII*) as a selectable marker, was used for the expression of geraniol synthase (*ges*) in *R. stricta* hairy roots. This vector is augmented with an artificial plastid-targeting signal, driven by the double-enhanced CaMV 35S promoter.

A. rhizogenes LBA 9402 carrying the pCambia 3300-G10H vector containing a CaMV 35S promoter and a phosphinothricin acetyl transferase gene (*bar*), with a herbicide (*e.g.* Basta®) resistance gene as a selectable marker, was used for transformation of hairy roots with the geraniol 8-oxidase (*g8o*) gene.

Gene specific primers were designed to amplify a 1059 base pair coding DNA sequence of *str* gene (GenBank: X61932.1) from the pLC-1gR-*str* plasmid construct carrying the *str* gene. For Gateway cloning, *attB* recombination sites were added before the gene specific sequence. Forward primer, 5´- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT Tat ggc aaa ctt ttc tga atc taa at-3´; reverse primer, 5´- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA cta gct aga aac ata aga att tcc c- 3´ (capital letters represent the adapters). The resulting PCR product was then inserted into pDONR™221 vector (Invitrogen) via BP clonase™ II mediated recombination reaction, creating an entry clone according to the manufacturer's protocol.

The entry clone was sequenced and the gene fragment from the intermediate entry clone was subsequently inserted in destination vector pH2GW7 (Karimi *et al.* 2002) (http://gateway.psb.ugent.be/vector/show/pH2GW7/search/index/overexpression/any), using the LR™ clonase II recombination reaction according to the manufacturer's protocol, resulting in an expression clone. The construct contained the 35S promoter sequence and the *hph* selectable marker. The

expression construct was sequenced and subsequently transformed into *A. rhizogenes* LBA 9402 by electroporation (1 cm cuvette, 2.5 kV, 25 µF, 200 Ω).

Prior to infection, bacteria from a -80°C stock were grown for 48 h at 28±1°C on solidified YMB medium (Hooykaas *et al.* 1977) supplemented with 100 mg/L rifampicin for wild type and transformed bacteria), 100 mg/L spectinomycin for bacteria transformed by the *gusA* and *str* genes and 50 mg/L kanamycin for bacteria transformed by *ges* and *g8o*.

4.5 Development of transgenic hairy root cultures

Eight two month-old seedlings were used for the preparation of explants for wild type hairy root induction as follows: cotyledons (n=16), hypocotyl segments (n=16), leaves with petiole (n=42) and excised stem segments with nodes and internodes (n=40). Three two month-old seedlings were used for the preparation of explants from cotyledons (n=6), hypocotyl segments (n=6), leaves with petiole (n=15) and excised stem segments with nodes and internodes (n=12) for inducing GUS hairy root clones. The apices were removed from both cotyledons and leaves and the leaf midrib (abaxial side) was wounded by a sterile syringe needle loaded with bacterial culture. The explants were placed on the adaxial side on modified Gamborg B5 medium (Ø9 cm petridishes containg 20 mL of media). Excised stem segments were pricked either at the node or the internode. Explants wounded in the same manner using a sterile needle but without bacteria served as controls. The explants were kept for two days in the dark at 24±1°C for co-cultivation. After two days, the explants were placed on medium supplemented with 500 mg/L cefotaxime and 10 mg/L meronem to eliminate residual bacteria and kept in the dark at 24±1°C.

Emerging putative transgenic hairy roots (1-1.5 cm in length) of infected explants were designated as independent clones, excised and placed on medium containing antibiotic (cefotaxime and meronem) for four weeks. In the case of developing GUS hairy roots the medium contained a selection reagent (2 mg/L hygromycin). Then root tips (1 cm in length) were sub-cultured on solid antibiotic/hormone-free medium and maintained at 24±1°C in the dark.

The established gene transfer method was used for transformation of hairy roots with the heterologous genes *ges*, *g8o* and *str* using leaf explants (for each n=50). The transformation procedures were the same as for initiation of wild type hairy roots. Data for all transformed roots was recorded eight weeks after the initial inoculation.

4.6 Selection conditions for transformed hairy roots

The natural resistance of *R. stricta* to hygromycin, kanamycin and a herbicide Basta® was tested in order to identify the best selection conditions by growing five randomly chosen wild type hairy root clones (presence of *rolB* gene confirmed by PCR) with three replicates each on medium supplemented with different

concentrations (0, 1, 1.5, 2, 5, 12.5, 25, 50, 75, 100, 150, 200, 300 and 400 mg/L) of antibiotics and the herbicide. The growth and colour of hairy roots were recorded after four weeks.

4.7 GUS staining analysis

Histochemical GUS assay in the emerged transformed and non-transformed roots (wild type hairy roots as negative controls) was carried out according to Jefferson *et al.* (1987). The roots were placed in 1 mL of X-GLUC solution (5-bromo-4-chloro-3-indolyl glucuronide) and incubated at 37±1°C until the characteristic blue colour appeared (2-16 h).

4.8 Genomic DNA extraction and PCR

From all the different putative hairy root clones and non-transformed roots, 50-100 mg was weighed and immediately frozen. Then frozen roots were powdered by a mixer mill (Retsch MM301, Haan, Germany) with stainless steel beads (4 mm diameter) for 2 min at 29 Hz. Total genomic DNA was extracted with the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980).

PCR amplifications were performed separately with primers specific to the Ri plasmid virulence gene *virD* (Hamill *et al.* 1991) and to the Ri plasmid gene *rolB* (Sevón *et al.* 1995) for the transformed hairy root clones. The PCR initial denaturation (95°C/1.5 min), final complete denaturation (95°C/30 sec), extension (72°C/1.5 min) and final extension (72°C/7 min) were similar for the genes. Denaturation, extension and annealing were performed in 35 cycles, except for *g8o,* which included 30 cycles. Annealing time for all the genes was 1 min. Primers, annealing temperatures and expected PCR-amplified fragments of each gene used in the study are presented in **Table 8**.

The amplification reaction was performed on a G-Storm GS482 thermal cycler (G-Storm & Kapa Biosystems, UK). The PCR reaction mixtures were loaded directly onto 1% agarose/TE (Tris/EDTA) gel for electrophoretic analysis. A 100 base or 1 KB ladder (Fermentas, GmbH Germany) was used as a molecular marker for the PCR-amplified DNA fragments.

Table 8. List of oligonucleotide primers, annealing temperatures and expected PCR product sizes.

Gene	Primers	Annealing temperature (°C)	Expected PCR product (bp)
virD	F: 5'-ATG TCG CAA GGA CGT AAG CCC A-3' R: 5' -GGA GTC TTT CAG CAT GGA GCA A-3'	59	450
rolB	F: 5'-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3' R: 5' -TTA GGC TTC TTT CTT CAGGTT TAC TGC AGC-3'	47	780
gusA	F: 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG C-3'* R: 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG G-3'*	64	~1900
ges	F: 5'-CGA CAC TTA TGG CTC GTA TG-3' R: 5' -ACC GAC TCG TTA CAA GAA GG-3'	64	850
g8o	F: 5'-GAC GTC GGC CGA GCT GCT TT-3' R: 5'- CGA CCA GCA CCG AAC GGA AT-3	65	797
str	F: 5'-ATG GCA AAC TTT TCT GAA TCTAAA T-3' R: 5'-CTA GCT AGA AAC ATA AGA ATT TCC C-3'	61	1059

*Primers contain ATTB1 and ATTB2 regions of the Gateway vector. F: forward primer, R: reverse primer.

4.9 Light and media experimental setup

Once the root cultures were established, the axenic cultures (root tips, 100 mg) were transferred to hormone-free liquid medium and kept in the darkness at 24±1°C, using a rotary shaker at 110 rpm for four weeks for growth and alkaloid production. The axenic maintenance cultures were sub-cultured at four-week intervals on the solid medium. The effects of two different nutrient media, Woody Plant Medium (Lloyd and McCown 1980) and modified Gamborg B5 medium (Oksman-Caldentey *et al.* 1991), and two light conditions (total darkness or 16 h light: 8 h dark photoperiod, light intensity about 30-40 µmol/mm^2 s Osram cool white/ Osram fluora, 1:1 Watt basis) on the growth and alkaloid content of ten

randomly selected wild type hairy root clones (in triplicate) were investigated. Hairy roots (100 mg fresh weight) were transferred to 100 mL flasks containing 20 mL of liquid modified Gamborg B5 medium or Woody Plant Medium and grown in the dark at 24±1°C on a rotary shaker (110 rpm) for four weeks. In the other experiment 100 mg of wild type hairy roots were transferred to flasks containing 20 mL of liquid modified Gamborg B5 medium and grown on a rotary shaker (110 rpm) either in darkness or under a 16 h light:8 h dark photoperiod for four weeks at 24±1°C.

The roots were harvested after four weeks, rinsed with distilled water, vacuum filtered and the fresh weights were recorded. Samples were immediately frozen in liquid nitrogen and kept at -80°C. Then the samples were lyophilised (Steris Lyovac GT2, Hürth, Germany), dry weights were determined and alkaloids extracted.

4.10 Elicitation of wild type hairy roots

Elicitation was carried out with methyl jasmonate (MeJA) and chitosan. Methyl jasmonate stock solution (25 mM) was prepared in 40% (v/v) ethanol and then filter-sterilized. Chitosan was prepared according Sevón *et al.* (1992).

From a randomly selected wild type hairy root (clone 9) grown on solid medium, root tips (500 mg) were transferred into a 500 mL flask containing 200 mL hormone-free liquid modified Gamborg B5 medium and subcultured in the dark at 24±1°C on a rotary shaker (110 rpm) for four weeks to obtain a fresh hairy root culture. From this culture, 100 mg of root tips were transferred to 50 mL flasks containing 20 mL of the same medium and cultured under the same conditions.

Methyl jasmonate or chitosan were added aseptically to 21-day-old hairy root cultures in order to reach final concentrations of 50, 100 and 200 µM or 100 and 250 mg/L, respectively. For control cultures equal volumes of 40% ethanol were added. The roots were harvested after 1, 3, 5 and 7 days of elicitation. After harvesting, the roots were washed with sterile water, vacuum-filtered, excess water was removed with a paper towel and fresh weight was determined. The roots were rapidly frozen in liquid nitrogen, lyophilised and dry weight was determined. All treatments were performed in triplicate.

4.11 Sample preparation for chemical analyses

4.11.1 GC-MS

Alkaloids were extracted from the samples as follows: lyophilised powdered samples (50 mg dry weight) were spiked with 2,4'-dipyridyl (50 µL, 1 mg/mL) and extracted with 1 mL of 10% sulphuric acid (v/v) in an ultrasonic bath for 30 min. The extracts were centrifuged at 11 000 rpm for 5 min and 900 µL aliquots of the acidic supernatant were transferred to other tubes. The extraction was repeated

and combined aqueous phases were basified to pH 10 with 25% ammonia solution, vortexed and left for 30 min at room temperature. Alkaloids were then extracted with 1 mL of dichloromethane. The mixture was vortexed and centrifuged at 4 000 rpm for 15 min and an 800 µL aliquot was separated. The extraction was repeated and the dichloromethane fractions were combined and gently evaporated to dryness under nitrogen flow and immediately dissolved into 75 µL of dichloromethane to be injected to GC-MS.

In few cases, derivatizations were made for silylable pure substances and plant extracts as follows: DCM samples were evaporated to dryness, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) reagent (25 µL) was added and samples were kept at 100°C for 30 min.

4.11.2 HPLC-PDA and UPLC-PDA-MS

Alkaloids were selectively extracted from roots for HPLC analysis as follows: lyophilised powdered samples (50 mg dry weight) were de-fatted three times by adding 1 mL petroleum ether followed by vortexing, sonication for 30 min and centrifugation at 4 000 rpm (3 220×g) at room temperature for 15 min. Petroleum ether was discarded and de-fatted samples were dried under nitrogen flow. They were extracted with 2 mL of 10% sulphuric acid (v/v) three times in an ultrasonic bath for 30 min. The mixture was then centrifuged at 4 000 rpm for 30 min and the acidic aqueous phases were combined and basified to pH 10 with 25% ammonia solution, vortexed and left for 2 h at room temperature. Aqueous phases were then extracted three times with dichloromethane, vortexed and centrifuged at 4 000 rpm for 30 min. The dichloromethane fractions were combined, evaporated to dryness, dissolved in 500 µL methanol and filtered (GHP Acrodisc® 0.2 µm, Pall Life Science, USA) prior to the HPLC analysis. For extracting nutrition media, the medium was lyophilised and the same extraction procedures as for alkaloid extraction from hairy roots, including pH adjustment in different steps, were also carried out. All organic solvents were of analytical grade.

4.11.3 NMR

Aliquots of 50 mg of lyophilised powdered hairy roots were transferred to eppendorf tubes and 1.5 mL of extraction solution (methanol D_4:KH_2PO_4 buffer in D_2O, pH 6.0, 1:1, v/v) was added. After vortexing for 1 min, the samples were sonicated for 10 min. Supernatant was collected by centrifugation (10 000 rpm, 10 min) and transferred to an eppendorf tube. After another centrifugation step (10 000 rpm, 1 min), the supernatant was collected for NMR analyses. A volume of 600 µL of the supernatant was transferred to a 5 mm NMR tube.

4.12 Chromatographic and spectroscopic analyses

4.12.1 GC-MS

GC-MS method was applied for the identification and quantification of alkaloids in 20 wild type hairy root clones. The method was also used for the quantification of alkaloids in elicited samples. The alkaloids were analysed with an Agilent 7890A GC combined with a 5975C mass selective detector (MSD) using an Rtx®-5MS silica capillary column (15 m, 0.25 mm i.d., 0.25 µm phase thickness). The oven temperature increased from 70 °C to 270°C at a rate of 10°C/min. Total run time was 50 min. Helium was used as the carrier gas in constant flow mode at 1.2 mL/min. The injector, ion source and interface temperatures were 250°C, 230°C and 240°C, respectively, with a split ratio of 25:1. Samples (1 µL) were injected by a Gerstel Maestro MPS 2 sampling system (Gerstel GmbH & Co. KG, Müllheim an der Ruhr, Germany). MSD was operated in electron impact (EI) mode at 70 eV and the full scan data (m/z 40-600) was collected at a scan rate of 2.6 scans/s and 1200 EMV. In this study, instead of GC-MS-SIM, quantitative analyses of alkaloids from 20 hairy root clones were based on extracted base peak abundances from a total ion chromatogram (TIC), divided by those of 2,4'-dipyridyl (internal standard, IS).

Identification of the compounds was based on retention times, GC-MS library comparisons (The Wiley® Registry of Mass Spectral Data, John Wiley and Sons, Inc., Electronic data Division, New York, USA) and literature data. Chromatographic data were collected and evaluated using Chemstation Software. For the identification, probability based matching (PBM) was utilized. This is a reverse search procedure weighting mass and abundance values of the peaks according to their uniqueness. The probability is indicated by a confidence index (*K*): $K_j=U_j+A_j+W_j-D$. The match confidence (*K*) value is the sum of *Ki* values for each matching peak, where *Uj* (uniqueness) and *Aj* (abundance) are the $-\log_2$ probabilities that a peak of such mass and abundance will occur in the reference file, *W* is the window tolerance for an abundance match, and *D* (dilution factor) represents the fraction of reference abundance used (McLafferty *et al.* 1974).

Method validation

All validation tests, *i.e.* linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, solvent extraction recovery and hairy root extraction recovery, were performed using internal standard (2,4'-dipyridyl, IS) and reference compounds (vincanine, ibogaine and tetrahydroalstonine). Extracted basepeak ions from total ion current (TIC) analyses were used in experiments.

Linearity: Pure substances were prepared in dichloromethane at a concentration of 1 mg/mL. The vincanine stock solution was then diluted to a concentration range from 5 to 100 µg/mL (six points) and in the case of tetrahydroalstonine and ibogaine from 1 to 50 µg/mL (six points). The samples (in 75 µL dichloromethane) including 50 µg of IS were analysed in triplicate to obtain the calibration curve.

Peak areas of alkaloids were divided by that of IS and the ratios were plotted against the corresponding concentrations of pure compounds.

The calibration curves were linear over a wide concentration range and the slope values indicated that ibogaine and tetrahydroalstonine had the highest responses. The linear regression equations and coefficients of correlation from TIC analyses were: vincanine (y= 0.0103x–0.0556; r^2 = 0.999), ibogaine (y = 0.0256x–0.0829; r^2= 0.998) and tetrahydroalstonine (y= 0.0291x–0.1216; r^2= 0.996), where y and x represent the relationship between the peak area ratio (compound/IS) and the corresponding calibration concentrations, respectively. The regression equations and coefficients of vincanine (y= 0.0065x; r^2= 0.997) and tetrahydroalstonine (y= 0.0117x; r^2= 0.998) from extracted ions from TIC analyses were also obtained.

Limits of detection (LOD) and limits of quantification (LOQ): The instrumental LOD was defined as the lowest concentration of vincanine, tetrahydroalstonine and ibogaine which produced a signal three times higher than that of the base (n=6). The LOQ was determined as the lowest concentration of samples which showed tenfold higher signal than the base (n=6). These parameteres were determined to evaluate the sensitivity of the method. The LOD were found to be 2.5 µg/mL for vincanine and 0.5 µg/mL for ibogaine and tetrahydroalstonine. The LOQ for vincanine was 5.0 (7.5% RSD) and for ibogaine and tetrahydroalstonine 1.0 µg/mL (7% RSD).

Repeatability: Repeatability experiments, conducted for intra-assay and inter-assay analyses, were determined by six replicate injections of a mixture of IS, vincanine, tetrahydroalstonine and ibogaine solutions on the same day and on three consecutive days, respectively. In addition, the repeatability was determined from repeated injections of a single *Rhazya* sample spiked with 10 µg of ibogaine. The values are expressed as relative standard deviation (RSD%).

The RSDs of intra-day repeatabilities ranged 3.2-4.7%, 1.2-3.9% and 1.3-3.4% for vincanine, Ibogaine and for tetrahydroalstonine, respectively. The RSDs of inter-day repeatabilities were 7.9%, 3.7% and 3.4%, respectively. The method was shown to be reproducible and reliable with regard to both intra-day and inter-day repeatability. In the case of the analysis of a *Rhazya* sample, repeated injections exhibited less than 3% RSD for vincanine, ibogaine and tetrahydroalstonine. In addition, the extracted ion monitoring from TIC analysis of 20 hairy root clones revealed the RSD% of t_R between 0.01%-0.5% for 12 major alkaloids.

Extraction recovery from standard solution: The recovery (accuracy of the method) was determined by extracting mixtures by the extraction protocol described in section **4.11.1.** The test mixtue consisted of IS (50 µg), vincanine (5 and 50 µg/75µL DCM), tetrahydroalstonine and ibogaine (5 and 25 µg/75µL DCM for both) solutions at high (n=3) and low (n=3) concentration levels, respectively. The DCM was evaporated and the mixture was dissolved in 75 µL of DCM. The concentrations were obtained by using calibration curves from linearity tests and were compared to the added amounts. Recoveries were expressed as percentages of the added amounts. Mean recoveries were in the range of 107.4%

(8.3% RSD), 104.6% (7.9% RSD) and 94.8 (8.5% RSD) for standards at low concentration levels of vincanine, tetrahydroalstonine and ibogaine, respectively. Mean recoveries were in the range of 96.5% (4.1% RSD), 96.6% (7.8% RSD) and 101.1 (8.7% RSD) for standards at high concentration levels of vincanine, tetrahydroalstonine and ibogaine, respectively.

Extraction recovery from spiked hairy root samples: The extraction recovery was determined by spiking hairy root samples (50 mg, n=6) with 50 µg/50 µL DCM of IS, vincanine and tetrahydroalstonine (both 25 µg/25 µL DCM) and Ibogaine (10 µg/10 µL DCM) prior to the extraction. DCM was evaporated from root samples. The extraction protocol was carried out as described in section **4.11.1**. The recoveries of vincanine, ibogaine and tetrahydroalstonine were calculated by comparing the concentrations obtained from the standard compounds and spiked samples. Extraction recovery was expressed as a percentage of the recovered amount compared to the added amount according to the following formula:

$$\text{Recovery (\%)} = (\text{amount found} - \text{original amount})/\text{amount spiked} \times 100\%$$

Mean recoveries from spiked *Rhazya* crude extract were 105.6% (6.5% RSD) for vincanine and 94.5 (7.2% RSD) for tetrahydroalstonine. Ibogaine recovery from spiked *Rhazya* crude extract was 94.3% (6% RSD). The compounds did not show any major matrix effect influencing extraction efficiency. The results indicate that the current GC-MS method is reproducible.

4.12.2 HPLC-PDA

HPLC analysis was performed with a Waters 2996 system coupled to a Waters 996 photodiode array detector monitoring a wavelength of 200-450 nm. An Xterra C18 column (5 µm, 250 mm × 4.6 mm; Waters) connected to an Xterra MS C18 pre-column (5 µm, 20 mm × 3.9 mm, Waters) was used. The mobile phase was according to Gerasimenko *et al.* (2001b) with slight modifications, and consisted of sodium dihydrogen phosphate (39 mM) and heptane-sulphonic acid buffer (2.5 mM, pH 2.5) (A): acetonitrile (B), running as a gradient from 90:10 to 80:20 within 10 min, 10 min hold in 80:20, to 65:35 within 20 min, and to 20:80 within 10 min (total run time 50 min), followed by a 5 min hold and eventually to 90:10 within 5 min with a 5 min hold in the end. The flow rate was 1 mL/min, injection volume 20 µL and detection wavelength 255 nm.

Repeatability of the HPLC-PDA method: The repeatability of the analytical procedure was evaluated by assessing the intra-assay and inter-assay repeatability of the HPLC method. The evaluation was based on the peak areas of the seven major compounds present in the extracts. Intra-assay repeatability was deduced from ten replicate injections (20 µL) of a *R. stricta* wild type hairy root extract within one day. Inter-assay repeatability was calculated based on six injections carried out on three consecutive days (n= 3×6). The values are expressed as relative standard deviation (RSD%).

The intra-day repeatability of the developed HPLC method for seven alkaloids of crude *Rhazya* extracts, expressed as the RSD% of the peak areas, ranged from 2.1 to 2.8%. The inter-day repeatability ranged from 3.4% to 4.7%.

Comparisons were made with a sample analysed by HPLC with and without the ion pair reagent. Use of the ion pair reagent avoided the peak broadening and overlapping that was otherwise rather pronounced at 13-35 min in the run, with simultaneous decreases in retention times.

4.12.3 UPLC-PDA-MS

The UPLC-MS runs were performed on a Waters Micromass Quattro micro™ triple quadrupole mass spectrometer with an electrospray source, combined with Waters Acquity Ultra Performance LC (UPLC) with a photodiode array detector (PDA). The column was an Acquity UPLC™ BEH C18 (100 mm × 2.1 mm, 1.7 µm) with a precolumn and the analyses were run at 25°C. The solvent system was made alkaline as described earlier (Rischer *et al.* 2006). It consisted of a mixture of 10 mM ammonium acetate (pH 10; A) and acetonitrile (B). The flow rate was 0.4 mL/min. The gradient started with 35% B, increased to 50% B in 15 min and remained constant up to 20 min.

The runs were performed both in positive (ESI^+) and negative (ESI^-) electrospray ionization mode and the data was collected in a mass range of m/z 100-850. The capillary and cone energies were 2.5 kV and 40 V in ESI^+ and 2.5 kV and 20 V in ESI^- mode, respectively. Source temperature was 125°C and desolvation temperature 350°C; desolvation gas flow was 800L/h. The PDA detector scanned wavelengths from 200 to 420 nm.

For the UPLC-PDA-MS analyses, pure samples of vincanine, yohimbine I, vincamine and tabersonine were available. These compounds were used for identification of *Rhazya* alkaloids by comparing retention times and spectroscopic data from UPLC-UV- MS. For other alkaloids than those for which standards were available, the comparisons were made using literature data. In addition, extracted molecular ions from TIC were utilised to confirm the identification. All the runs lasted 20 min.

The solvent system for the UPLC method, without ion pair reagent, was studied. Two isocratic runs were tested using either 55% or 65% solvent A (10 mM ammonium acetate, pH 10 in water) and 45% or 35%, respectively, solvent B (acetonitrile).

Three gradient runs were also tested. The solvent proportions were changed in 15 min from 35% to 50% (solvent B). In the first gradient run, the solvent system contained 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). In the second gradient run, the concentration of formic acid in water was tenfold (1%). In the last gradient run, the solvents contained 10 mM ammonium acetate, pH 10 in water (solvent A) and acetonitrile (solvent B).

The first isocratic run showed better separation compared to the second run, but major peaks still overlapped during the first three min. Therefore, smaller

compounds were separated better. In the second isocratic run, the majority of the compounds were eluting very rapidly, even before three minutes.

In the first gradient run all the major peaks eluted in the first two minutes. In the second gradient run, with 1% formic acid, several minor components co-eluted with three major peaks in the middle of the run. The best separation of these experiments was obtained in the third gradient run (solvent A, pH 10/acetonitrile solvent B), and these conditions were used in the UPLC-PDA-MS analyses of three representative wild type hairy root clones 2, 3 and 10.

4.12.4 NMR

The NMR spectra were recorded at 22°C on a 600 MHz Bruker Avance III NMR spectrometer equipped with a QCI cryoprobe and SampleJet sample changer. The residual water signal was suppressed by 4 s volume selective presaturation using Bruker's pulse program noesygppr1d. The number of scans was 128 and the number of dummy scans was 16. 64k points were recorded with a spectral width of 20 ppm and a 0.5 Hz line-broadening window function was used prior to the Fourier transformation.

The transgenic clones were compared both to wild type control clones and to GUS clones carrying the *gus* gene by using ^{1}H-NMR and multivariate analysis. Data analysis was carried out at the host laboratory (Institute of Biology, Natural Products Laboratory, Leiden University, The Netherlands). The ^{1}H-NMR spectra were automatically reduced to ASCII files. Spectral intensities were scaled to internal standard (TSP) at 0.0 ppm and reduced to integrated regions of equal width (0.04) using AMIX software (v.3.7 Bruker Biospin). Principle component analysis (PCA) with scaling based on Pareto, partial least-square-discriminant analysis (PLS-DA) and orthogonal partial squares analysis (OPLS) with a scaling method based on unit variance (UV) were performed with SIMCA-P+13.0 (Umetrics, Umeå, Sweden).

4.13 Statistical analysis

Statistical analyses were performed using SPSS (version 21.0, Chicago, IL, USA). The data from experiments measuring alkaloid contents in different *in vitro* cultures was subjected to analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences test to determine the significance between means. Probability level $p \leq 0.05$ was considered significant. Student's *t*-test was used to compare the difference between means of two groups. Data was expressed as mean ± RSD%. SIMCA-P+13.0 software was used for NMR data analyses.

5. Results

5.1 *In vitro* cultures of *R. stricta*

5.1.1 Undifferentiated cell cultures

To obtain plant material for initiation of calli and hairy roots, seeds were sterilized and *in vitro* germinated (**Figure 8A, B**). Because of high contamination in the germinated seedlings, a two-step sterilization procedure of the seeds (see materials and methods) was used to improve significantly the rate of obtaining sterile seedlings. With this method the germination rate was 73.3% (n=15) and contaminated seedlings were not observed. Pericarps of seeds were thick, which makes difficult germination. Stirring the seeds in sterile water overnight induced swelling of the pericarp.

Explants were prepared from roots, hypocotyls, cotyledons, leaves and stems of ten *in vitro* seedlings. Explants were placed on modified Gamborg B5 solidified medium supplemented with 1 mg/L BAP and 0.75 mg/L NAA. After three to nine days, calli were initiated from explants. Hypocotyls (**Figure 8C**) and stems (**Figure 8D**) produced high biomass, which grew faster after subculturing and persisted for a long time without marked changes in their colour. Calli were induced from cut sections and edges of cotyledons (**Figure 8E**) and leaves (**Figure 8F**). Roots (**Figure 8G**) and hypocotyls (**Figure 8C**) initiated calli earlier than other explants.

The colour and texture of calli varied; root-derived calli were yellow with a watery and friable texture, whereas stems produced green and hard calli. Stem explants produced calli with a rigid texture. The calli derived from different explants retained their growth and colour after successive subculturing, at four week intervals, in medium containing the hormones for more than six years. **Table 9** presents some characteristics of the different callus lines.

Table 9. Characteristics of calli initiated originated from different explants.

Calli type	Days until callus initiation	Texture	Colour
Roots	2-4	friable	yellow
Hypocotyls	2- 5	soft	greenish yellow
Cotyledons	4- 8	soft	greenish yellow
Stems	4- 7	rigid	green
Leaves	5- 9	soft	greenish yellow

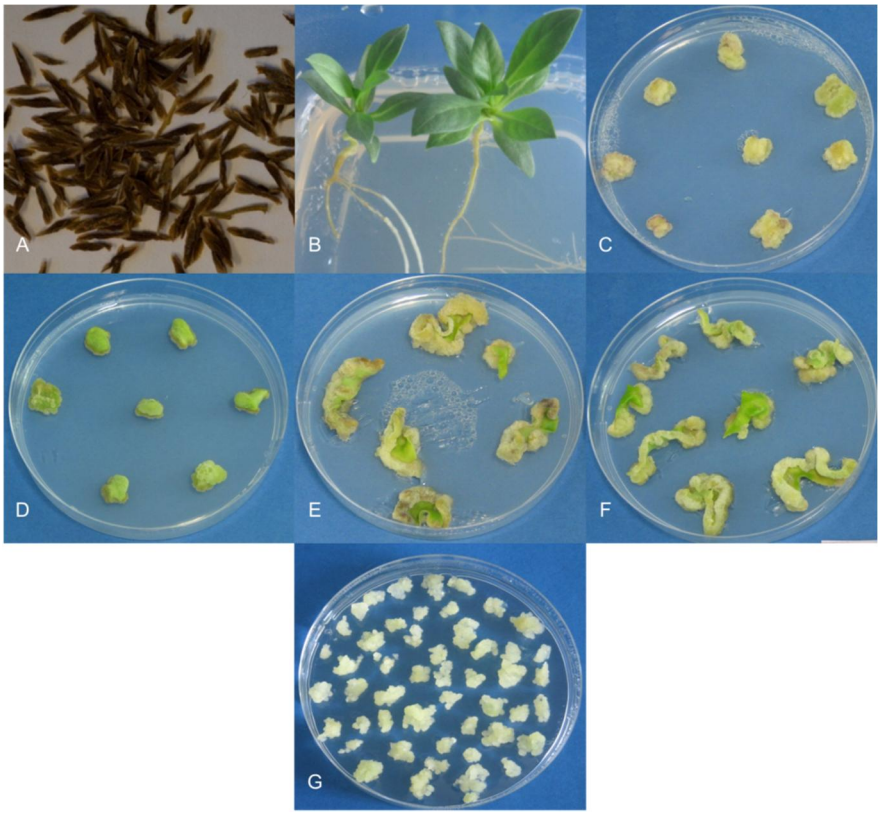


Figure 8. Callus induction in *R. stricta*. Seeds were surface sterilized (**A**) and aseptic seedlings cultured on modified Gamborg's B5 containing 3% sucrose and 3% gelrite (**B**); calli initiated from four weeks-old hypocotyls (**C**), stems (**D**), cotyledon (**E**), leaves (**F**) and roots (G) of *R. stricta*, respectively, on the media supplemented with BAP 1 mg/L and NAA 0.75 mg/L after three to ten days.

5.1.2 Wild type and GUS hairy roots

R. stricta wild type and GUS hairy roots were induced to be used as controls. The needle-wounded infection sites turned dark within 1-2 weeks in all explants. Within 3-4 weeks after injury white to light yellow callus appeared (**Figure 9A**), with or without *A. rhizogenes* infection. Most hairy roots emerged within two to six weeks after inoculation, mainly from callus on the leaf midrib including leaf petioles (**Figure 9A**). Only leaves were susceptible to *Agrobacterium* infection and subsequent root induction. Cotyledons, hypocotyls or stem segments did not produce hairy roots. Bigger leaves located at the base of seedlings were more susceptible to infection than smaller ones. Adventitious roots developed only from hypocotyl, leaf and stem segments. However, these roots did not elongate after sub-culturing.

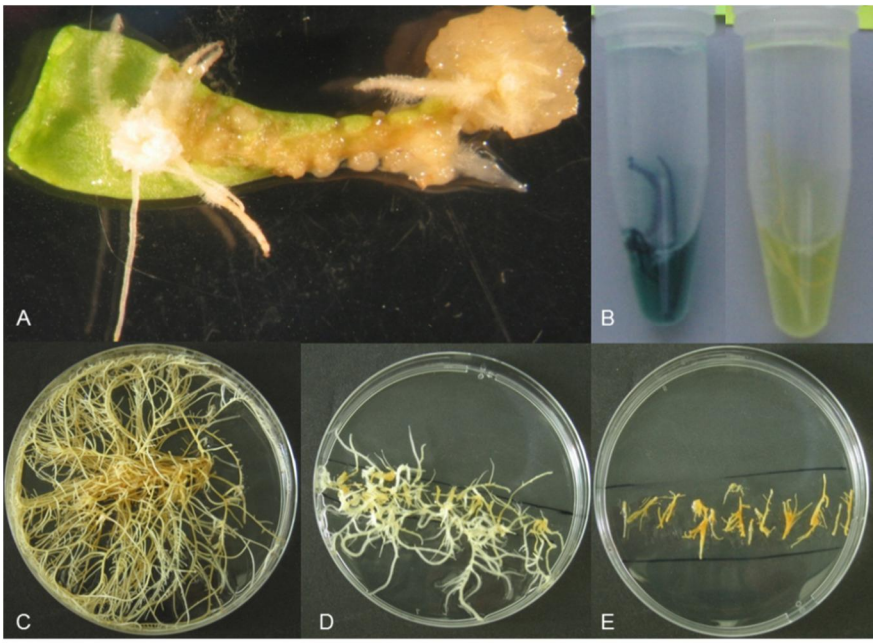


Figure 9. (**A**) Development of *R. stricta* wild type hairy roots six weeks after inoculation with *A. rhizogenes*. (**B**) Histochemical GUS assay of a *R. stricta* transgenic GUS clone (left) and a wild type control (right). Wild type hairy roots of *R. stricta* grown on hygromycin-free media (**C**) on media supplemented with 1 mg/L (**D**) and 2 mg/L hygromycin (**E**), respectively, four weeks after transfer to selection conditions.

Selection for antibiotic resistance

Hygromycin: The characteristic hygromycin tolerance of *R. stricta* wild type hairy roots was tested in order to find the optimal hygromycin concentration for selection conditions of transformed GUS clones. In the presence of 1 mg/L

hygromycin (**Figure 9D**), hairy roots exhibited normal growth with white root tips but grew somewhat slower compared to control cultures (**Figure 9C**). At a concentration of 1.5 mg/L hygromycin hairy roots had white root tips, which indicated they were alive, but did not elongate. Concentrations of 2 mg/L hygromycin and higher proved lethal for wild type hairy root clones, which turned yellowish within 2-3 weeks and died (**Figure 9E**). Therefore, a concentration of 2 mg/L hygromycin was chosen for selection of putative GUS hairy root clones.

Kanamycin: Wild type hairy root clones grown at concentrations of 1 to 12.5 mg/L kanamycin exhibited the same growth properties as observed for control kanamycin-free cultures. At a concentration of 25-75 mg/L, 25% of roots had growing white root tips. However, a concentration above 75 mg/L (100-400 mg/L) of kanamycin completely inhibited the development of hairy roots and the roots turned brownish. Therefore, a kanamycin concentration of 100 mg/L was selected for screening transformed *R. stricta* hairy roots with constructs carrying kanamycin resistance.

Basta®: A low concentration of Basta® (1 mg/L) completely inhibited the growth of wild type hairy roots and turned them brownish. Basta® concentrations of 0.1-0.75 mg/L were tested to determine an appropriate concentration for selection of roots. At concentrations of 0.1-0.5 mg/L the roots had white tips, whereas their growth was stopped at a concentration of 0.75 mg/L. Therefore, 0.75 mg/L was selected to screen transformed hairy roots when constructs with the Basta resistance gene (*bar*) were used for transformation of *R. stricta*.

Transformed roots were characterized by rapid branching and highly plagiotropic growth, and showed vigorous growth in hormone-free liquid and solid medium in the dark. After 4-5 weeks of continuous culture in either liquid or solid medium, the hairy roots started to develop a dark-coloured central zone indicating necrosis. However, the root tips retained their proliferative capacity even after ten weeks of liquid/solid culture initiation. Intact *in vitro* roots grew slowly in hormone-free medium in the dark. For over five years until the present time, *R. stricta* hairy roots have been maintained by sub-culturing at four week intervals on solid Gamborg B5 medium.

A. rhizogenes strain LBA9402 harbouring the recombinant pH7WGD2::CaMV 35S::*gusA* binary vector was used for initiating transgenic hairy root clones from cotyledons, hypocotyls, leaves and stem segments of axenic *R. stricta* seedlings. However, similar to the wild type root induction, putative GUS hairy roots were produced only from leaf explants. Taking into account the results of the hygromycin sensitivity test, selection and proliferation of putative transgenic (GUS) hairy root clones was carried out on hormone-free solid medium supplemented with 2 mg/L hygromycin, 500 mg/L cefotaxime and 10 mg/L meronem. Eventually ten clones out of twelve putative clones survived and were subjected to the GUS assay and PCR. All ten GUS clones were subjected to the histochemical assay and turned blue with different intensities. Among the transgenic hairy root clones, six clones exhibited high levels of GUS activity. A wild type hairy root was used as a negative control and no blue colour was observed even after incubation for 72 h at 37±1°C (**Figure 9B**).

PCR, first with *virD* and then with *rolB* specific primers, was performed for wild type and GUS hairy root clones. Absence of the *virD* gene (450 bp) indicated all the hairy roots to be *Agrobacterium*-free (**Figure 10A,B**). The PCR products showed the expected size of 780 bp fragment for *rolB* (**Figure 10C,D**) and ~2 kb for *gusA* (**Figure 10E**). Neither *rolB* nor *gusA* fragments were amplified from adventitious *i.e.* non-transformed roots.

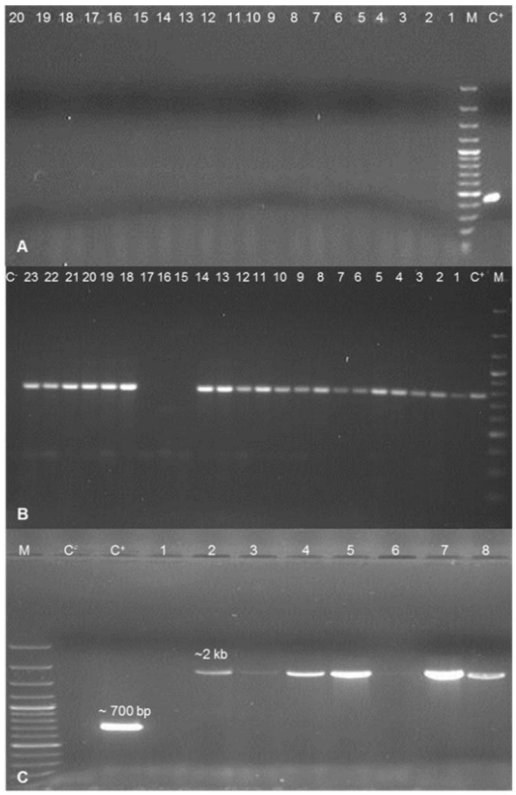


Figure 10. PCR analyses for confirmation of absence of *vir D* and transformation *rolB* and *uidA* (*gus*) genes into *R. stricta* hairy roots. (**A**) Absence of the *virD* gene in wild type. C^+, the positive control (wild type plasmid carrying the *virD* gene). M, 100 bp DNA ladder. (**B**) Amplification of 780 bp fragments from *Agrobacterium*-free wild type clones confirms transformation of hairy root clones with *rolB*. C^+, positive control (wild type plasmid carrying the *rolB* gene). M, 100 bp DNA ladder. (**C**) Screening of GUS clones (partially presented). C^+, as a positive control (Gateway® vector construct carrying a fragment (Nt PYL4) derived from the cDNA-AFLP set of methyl jasmonate-modulated genes (Goossens et al. 2003)). C^-, negative control (PCR product with GUS specific genes from a wild type hairy root). PCR amplified ATTB sites (ATTB 1 and ATTB2) of the Gateway and *uidA* gene and resulted in a fragment with ~ 2 KB. M, 100 bp DNA ladder. Absence of the *virD* and presence of *rolB* genes in GUS clones are presented in **Appendix A1, B1**.

5.1.3 Transformed hairy roots with early key TIA biosynthesis genes

The *A. rhizogenes* strain LBA9402 harbouring the pCambia 3300-G10H construct and Gateway-based recombinant vectors including pBIN2.4VoGES1 and pH2GW7-*STR* was inoculated into leaves. Hairy roots were transformed with the same procedure as was used for initiation of wild type hairy roots.

All putative hairy roots transformed with genes from the TIA pathway were *virD*-free. These *Agrobacterium*-free hairy roots were first screened for the presence of *rolB* by PCR. DNA of all the individual *virD*-free hairy roots which integrated *rolB* into their genome was isolated and PCR was carried out using specific primers designed using heterologous gene sequences. As a positive control (C^+), fragments were also amplified from the plasmids containing corresponding genes, and DNA of a wild type root was used as a negative control. Clones for which PCR amplified the expected 850, 797 and 1059 bp fragments for *ges*, *g8o* and *str*, respectively, were designated as separate clones (**Figure 11**).

PCR with *g8o* gene specific primers and wild type clone genomic DNA as a template resulted in two amplified fragments. One of the fragments had 797 bp length, the same size of the amplified gene from the plasmid containing the *g8o* gene, whereas another fragment had a size of ~1 KB indicating that the *g8o* gene in *Rhazya* had partial homology with the *g8o* from *C. roseus*. However, PCR did not amplify the *ges* and *str* genes from wild type hairy roots.

Table 10 summarizes the total number of leaf explants used for induction of transformed roots, the number of explants generating transformed hairy roots and putative hairy roots obtained within 8 weeks after the initial inoculation, the transformation efficiency for all transformed hairy root clones and the ratio of transformed clones to putative hairy roots.

Table 10. Hairy root induction by *A. rhizogenes* on leaf explants of *R. stricta*.

Root type	Infected	Leaves generated transformed roots	Putative hairy roots	Transformed hairy roots	Efficiency (%)*
Wild**	42	18	27	20	74
gus	15	7	12	10	83
ges	50	22	37	10	27
g8o	50	16	31	10	32
str	50	26	44	10	22

*Frequency of inoculated leaf explants giving rise to at least one transformed root was assessed six weeks after co-culture with *A. rhizogenes*. **Wild type hairy roots possessed only the *rolB* gene.

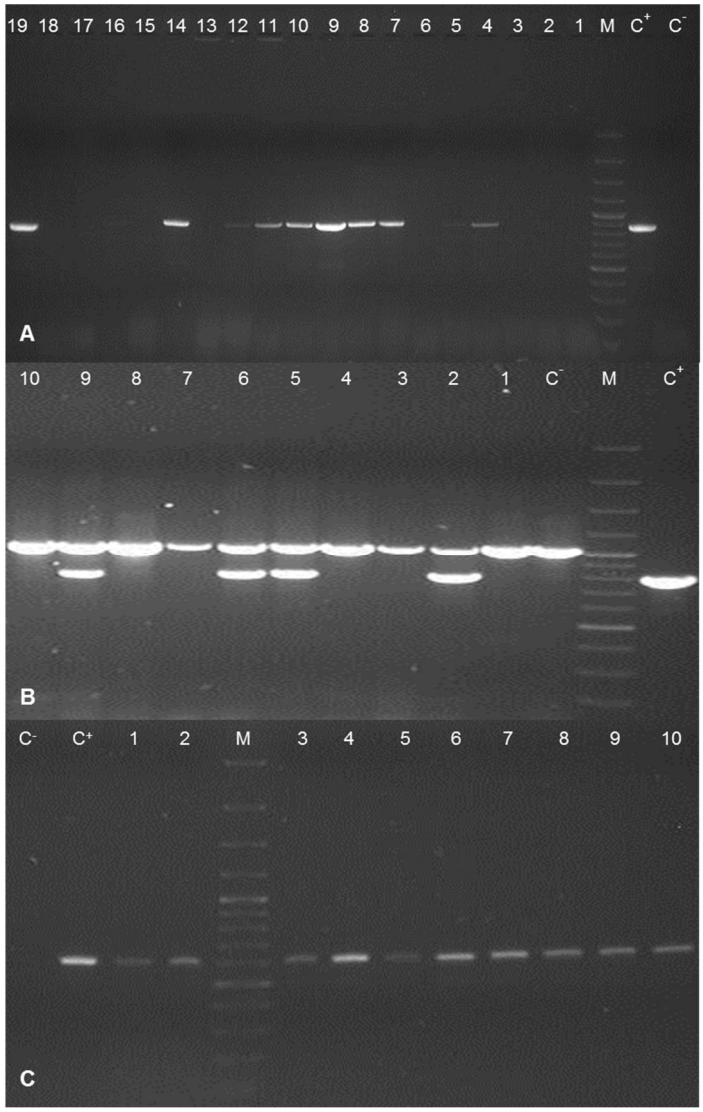


Figure 11. PCR analyses for conformation of transformation of early key TIA genes into *R. stricta* hairy roots. (**A**) PCR amplification of the 850 bp segment of *ges*, C^+, positive control (plasmid carrying the *ges* gene). C^-, negative control (DNA isolated from a wild type hairy root). M, a 100 bp DNA ladder. (**B**) PCR amplified a 797 bp fragment of *g8o* (partially presented). C^+, positive control (plasmid carrying the *g8o* gene). C^-, negative control (DNA isolated from a wild type hairy root). M, a 100 bp DNA ladder. (**C**) PCR amplification of the 1059 bp fragment, full length coding DNA sequence of *str*. C^+, positive control (plasmid carrying the *str* gene). C^-, negative control (DNA isolated from a wild type). M, a 100 bp DNA ladder. Absence of the *virD* and presence of *rolB* genes in *ges*, *g8o* and *str* hairy roots clones are presented in **Appendix A2-4, B2-4**.

5.2 Alkaloid accumulation in *R. stricta in vitro* cultures

5.2.1 Alkaloid composition

GC-MS method was developed to enable identification of alkaloids in a single run. Chromatographic separation of *R. stricta* hairy root alkaloid extracts was performed using an Rtx®-5MS column. Analysis of extracts from *R. stricta* hairy roots in TIC mode indicated the presence of 20 monomeric TIAs with molecular weights ranging from 278 to 354. Typical TIC and extracted iongrams are presented in **Figure 12A,B** and the chemical structures of some identified compounds in **Figure 13**.

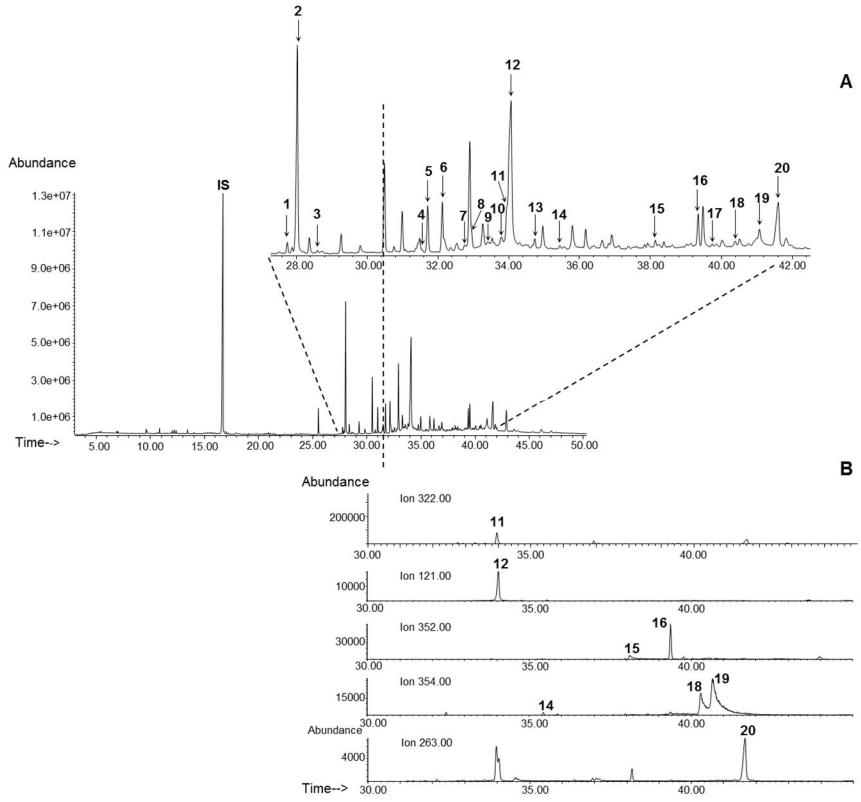


Figure 12. Total ion GC-MS (**A**) and extracted ion analyses (**B**) of *R. stricta* wild type hairy root alkaloids. Numbers refer to compounds in **Table 11**.

For quantification of compounds, extracted ion analyses were performed to avoid interfering fragments from overlapping compounds. GC separation and disturbing fragments at the penetrated baseline (**Figure 12A**) can also be caused by *e.g.* thedecomposition of dimeric alkaloids present in *Rhazya* or by free

phytosterols. Therefore, after each analysis of a crude extract a cleaning run was carried out. When studying spectra profiles, baseline subtraction was often necessary in order to improve the reliability of comparisons with the spectral library. The 20 alkaloids identified from *R. stricta* hairy root extracts are presented in **Table 11**.

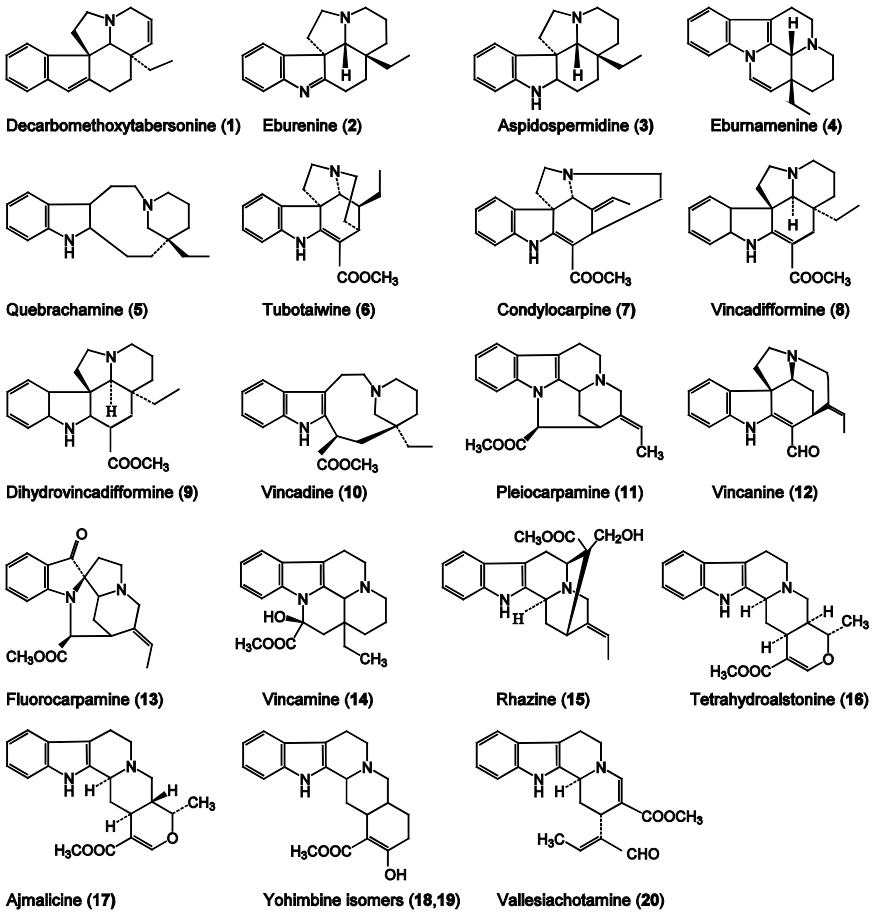


Figure 13. Structures of monoterpenoid indole alkaloids identified in this study. Numbers refer to compounds in **Table 11**.

The chromatographic conditions allowed the separation of single alkaloid peaks in the GC-MS system between 28 and 42 min (**Figure 12A**) and provided a large amount of mass spectral data with good matching values (>90%). Their fragmentation patterns and relative abundances were in line with those presented in the literature. In addition, the retention times and fragmentation patterns of vincanine (**12**), vincamine (**14**), tetrahydroalstonine (**16**), ajmalicine (**17**) and

yohimbine I (**18)** were in accordance with the alkaloid standards used in the study. The reported mass spectra were chosen on the basis of their similarity to measured mass spectra (**Table 11**). Minor differences in the data compared to the reported mass spectral data are due to the fact that most of the fragmentation data in early literature was obtained by high resolution mass spectrometry from pure isolated compounds. The mass spectra of the alkaloids are presented in **Appendix C**.

GC-MS analysis indicated that eburenine (**2**) is the most abundant alkaloid in *R. stricta* hairy roots. The present study reports six new compounds (**11**, **13**, **14**, **17**, **18** and **19**) for *Rhazya* species (**Figure 12**). Using the GC-MS method, two yohimbine isomers (**18** and **19**) with similar fragmentation were separated with retention times very close to each other. Compound **18** was identified as yohimbine (isomer I, **Table 11**), having the same retention time and fragmentation as the pure reference compound.

The minor alkaloids decarbomethoxytabersonine (**1**) and dihydro-vincadifformine (**9**) were identified in the extracts (**Table 11**). The spectrum of the latter alkaloid was distorted by other closely eluting compounds with much higher concentrations. In addition rhazinilam, with a molecular mass of 294 and eluting at 32.38 min, was detected in a very low concentration in hairy root extracts. Rhazinilam had a low match due to only a few fragments in the library spectrum and therefore the identification was considered to be only tentative and it was not included in **Table 11**.

Trimethylsilylation

Vincanine (**12)**, vincamine (**14)**, rhazine (**15)**, tetrahydroalstonine (**16)**, yohimbine isomer I (**18)**, yohimbine isomer II (**19)** and vallesiachotamine (**20**) (**Figure 13**) were the seven compounds in hairy root extracts which had been derivatized by trimethylsilylation ([−$Si(CH_3)_3$]). Fragment m/z 73 is a typical indicator for TMS derivatization. Due to the free functional groups of the alkaloids, *e.g.* hydroxyl and/or secondary amino groups, the silylation process resulted in either mono- or di-TMS derivatives. Consequently, the molecular size increased by 72 and 144 atomic mass units, respectively. In addition, *Rhazya* extracts contain silylable non-alkaloid constituents such as amines, free organic acids and phytosterols.

The separation and spectral characteristics of TMS-derived alkaloids were studied. The same fragmentation patterns that were observed for derivatized pure compounds (**12**, **14**, **16** and **18**) were also observed in the extract (**Table 12**). As the pure alkaloids **15**, **19** and **20** were not available; the identification was considered tentative (**Table 12**). Rhazine-di-TMS (**15**) showed a sharp peak and without any interfering fragments. On the contrary, the two non-derivatized yohimbine isomers (**18**-**19**) showed peak broadening and partial overlapping. In this case, trimethylsilyl derivatization is a method of choice to obtain sharper peaks and baseline separation. Extracted ion monitoring using basepeak ion m/z 425 resulted in clearly improved resolution as shown in **Appendix D**. Di-TMS-derivatives of yohimbine isomer I (**18**) and II (**19**) exhibited fragmentation patterns

which were similar to those of the pure yohimbine isomer I. Improved separation between partially co-eluted pleiocarpamine (**11**) and vincanine (**12**) was achieved after silylation of the latter.

In non-silylated samples, vallesiachotamine isomer (41.62 min) showed characteristic ions with m/z 279 (100), 263 (97) and 350 [M^+] (**Table 11**). However, silylation resulted in the separation of two mono-TMS isomers with equal peak areas and with identical spectra (**Table 12**). In addition, these isomers had the same characteristic ion m/z 279 (100) as observed in non-silylated vallesiachotamine.

Table 11. Alkaloids detected in *R. stricta* hairy root extracts by GC-MS. Peak numbers refer to constituents in **Figure 12**.

Peak	Compound	t_R (min)	Molecular formula	MW	Measured EI mass spectrum	Reported EI mass spectrum	References	Library match (%)
	2,4'-Dipyridyl (IS)	16.71	$C_{10}H_8N_2$	156	156 (100), 155 (42), 129 (41), 128 (19),51(16), 78 (13), 157 (11), 130 (11)	156, 155 , 129, 51, 78, 128, 79, 15	http://webbook.nist.gov	99
	Ibogaine (IS)	36.65	$C_{20}H_{26}N_2O$	310	136 (100), 310 (80), 135 (73), 225 (56), 149 (37), 122 (37), 155 (25), 148 (19)	136, 135, 310, 225, 82, 122,, 186	Hearn *et al.* 199	99
1	Decarbomethoxy-tabersonine	27.75	$C_{19}H_{22}N_2$	278	278 (100), 169 (41), 249 (39), 250 (38), 208 (38), 156 (36), 170 (36), 222 (33)	n.a	n.a	99
2	Eburenine (1,2-dide hydroaspidospermidine)	28.04	$C_{19}H_{24}N_2$	280	280 (100), 210 (90), 251 (47), 194 (39), 208 (30), 211 (24), 281 (22), 125 (22)	280 (100), 194, 210, 251	Rakhimov *et al.* 1970	98
3	Aspidospermidine	28.60	$C_{19}H_{26}N_2$	282	124 (100), 254 (17), 282 (16), 125 (11), 130 (11), 281 (8), 144 (7), 152 (7)	282, 254, 225, 210, 152, 144, 130, 124	Biemann *et al.* 1963	97
4	Eburnamenine	31.56	$C_{19}H_{22}N_2$	278	208 (100), 249 (90), 278 (33), 206 (28), 247 (26), 193 (23), 209 (22), 248 (18)	208 (100), 56 (93), 249 (89), 55 (61), 70 (54), 69 (44), 278 (34), 193 (31), 206 (26), 220 (14)	*Atta-ur-Rahman *et al.* 1991, Budzikiewicz *et al.* 1964, Kováčik *et al.* 1969, Zheng *et al.* 1989	98
5	Quebrachamine	31.72	$C_{19}H_{26}N_2$	282	282 (100), 110 (72), 125 (56), 124 (55), 157 (44), 143 (38), 138 (37), 156 (29)	282, 110, 125, 124, 157, 143, 138, 144, 156, 96	*Biemann *et al.* 1962, Budzikiewicz *et al.* 1964, Biemann *et al.* 1963	99
6	Tubotaiwine (Dihydrocondylocarpine)	32.13	$C_{20}H_{24}N_2O_2$	324	180 (100), 229 (95), 167 (90), 181 (51), 194 (48), 182 (44), 168 (40), 324 (37), 267 (34)	324, 293, 279, 267, 253, 229, 197, 193, 180, 167	Atta-ur-Rahman *et al.* 1989b	95

Continues→

7	Condylocarpine	32.76	$C_{20}H_{22}N_2O_2$	322	322 (100), 252 (59), 235 (57), 279 (38), 130 (27), 323 (25), 220 (25), 249 (25)	322 (100), 235 (91), 121 (77), 252 (73), 279 (39), 307 (34), 267 (15)	Atta-ur-Rahman *et al*. 1989b	95
8	Vincadifformine	32.96	$C_{21}H_{26}N_2O_2$	338	124 (100), 338 (20), 125 (10), 339 (5), 168 (4), 167 (4), 154 (4), 253 (3)	124 (100), 338 (28), 167 (5), 154 (5), 353 (3), 180 (3), 110 (3), 109 (3), 309 (1), 214 (1)	Djerassi *et al.* 1962, Budzikiewicz *et al.* 1964, *Zheng *et al*. 1989	95
9	Dihydrovincadifformine	33.41	$C_{21}H_{28}N_2O_2$	340	124 (100), 125 (12), 156 (11), 340 (10), 154 (6), 129 (5), 127 (5), 122 (5)	124, 254, 125, 340, 130, 144, 143	Djerassi *et al.* 1962, *Budzikiewicz *et al*. 1964	93
10	Vincadine	33.82	$C_{21}H_{28}N_2O_2$	340	210 (100), 340 (81), 110 (78), 215 (62), 124 (48), 138 (38), 169 (31), 154 (29)	340, 325, 311, 309, 281, 215, 210, 138, 124, 110	Atta-ur-Rahman *et al*. 1985a	94
11	Pleiocarpamine	33.95	$C_{20}H_{22}N_2O_2$	322	263 (100), 180 (99), 322 (85), 234 (39), 232 (35), 181 (25), 167 (25), 194 (23)	263 (100), 180 (100), 322, 234 (51), 154 (25), 108 (25)	Atta-ur-Rahman *et al*. 1983	99
12	Vincanine (Nor-*C*-fluorocurarine)	34.07	$C_{19}H_{20}N_2O$	292	121 (100), 292 (53), 167 (47), 249 (36), 194 (33), 168 (32), 180 (31), 277 (30)	121(100), 292 (83), 57 (26), 263 (20), 167 (20), 249 (17), 180(16), 277 (9)	Shakirov *et al*. 1996	99
13	Fluorocarpamine	34.75	$C_{20}H_{22}N_2O_3$	338	121 (100), 338 (47), 265 (27), 130 (17), 172 (15), 122 (14), 160 (13), 106 (12)	121 (100), 338, 265 (14), 279 (11), 160 (10), 231 (6), 193 (6)	Atta-ur-Rahman *et al*. 1983	96
14	Vincamine	35.44	$C_{21}H_{26}N_2O_3$	354	354 (100), 252 (82), 295 (64), 224 (51), 267 (47), 353 (39), 251 (38), 266 (34)	354 (100), 252 (70), 267 (52), 224 (47), 295 (38), 237 (27), 294 (24), 266 (21)	Budzikiewicz *et al.* 1964, *Kováčik *et al.* 1969, Shakirov *et al*. 1996	96
15	Rhazine (Akuammidine)	38.12	$C_{21}H_{24}N_2O_3$	352	249 (100), 352 (95), 169 (88), 168 (70), 321 (52), 351 (51), 279 (29), 182 (25)	352 (100), 351 (73), 249 (65), 169 (59), 321 (45), 168 (42), 293 (19), 335 (11)	Budzikiewicz *et al.* 1964, *Shakirov *et al*. 1996	97
16	Tetrahydroalstonine	39.36	$C_{21}H_{24}N_2O_3$	352	352 (100), 351 (91), 156(49), 337 (35), 169 (28), 353 (23), 223 (22), 168 (17)	352 (100), 351 (87), 156 (74), 223 (38), 337 (31), 169 (27), 353 (25), 321 (6)	Budzikiewicz *et al.* 1964, *Atta-ur-Rahman *et al.* 1984f	99
17	Ajmalicine (δ-Yohimbine)	39.74	$C_{21}H_{24}N_2O_3$	352	352 (100), 351 (98), 156 (59), 184 (43), 169 (40), 353 (25), 170 (24), 168 (23)	352 (100), 156 (63), 351 (61), 169 (16), 170 (10), 225 (8), 321 (5), 337 (5)	Budzikiewicz *et al.* 1964, Shakirov *et al*. 1996	93

Continues→

18	Yohimbine isomer I	40.22	$C_{21}H_{26}N_2O_3$	354	353 (100), 354 (79), 355 (23), 171 (14), 169 (14), 156 (9), 133 (9), 170 (8)	353 (100), 354 (82), 169 (38), 156 (17), 170 (19), 143 (14), 184 (15)	Budzikiewicz *et al.* 1964, *Betz *et al.* 1995	97
19	Yohimbine isomer II	40.68	$C_{21}H_{26}N_2O_3$	354	353 (100), 354 (71), 169 (29), 184 (13), 355 (12), 170 (12), 156 (11), 183 (10)	353 (100), 354 (82), 169 (38), 156 (17), 170 (19), 143 (14), 184 (15)	Budzikiewicz *et al.* 1964, *Betz *et al.* 1995	94
20	Vallesiachotamine	41.61	$C_{21}H_{22}N_2O_3$	350	279 (100), 263 (97), 221 (73), 350 (53), 291 (48), 265 (42), 307 (40), 322 (33)	263 (100), 279 (97), 221 (77), 264 (60), 350 (50), 291 (50), 322 (46), 265 (42), 209 (38), 307 (30)	Mukhopadhyay *et al.* 1981, *Kostenyuk *et al.* 1995	99

*The presented mass spectral data taken from this reference; n.a: data not available.

Table 12. GC-MS data in retention order of TMS-derivatives of pure reference alkaloids and their identification in *R. stricta* hairy roots. Peak numbers refer to compounds in **Table 11.**

Peak	Compound	t_R (min)	Molecular formula	MW of TMS-derivative	Fragmentation in derivatized hairy root extract	Fragmentation of derivatized pure compound	Fragmentation of underivatized pure compound
12	Vincanine (mono-TMS)	34.20	$C_{22}H_{28}N_2OSi$	364	364 (100), 73 (89), 365 (31), 246 (25), 363 (25), 247 (24), 259 (22), 230 (21)	364 (100), 73 (61), 365 (29), 363 (24), 246 (20), 259 (19), 247 (18), 349 (17)	121 (100), 292 (54), 167 (42), 249 (36), 194 (31), 168 (29), 180 (28), 222 (25)
14	Vincamine (mono-TMS)	35.05	$C_{24}H_{34}N_2O_3Si$	426	367 (100), 368 (27), 252 (19), 426 (17), 73 (14), 323 (13), 383 (9), 427 (9)	367 (100), 368 (31), 426 (19), 252 (18), 73 (13), 383 (10), 369 (8), 251 (7)	354 (100), 252 (84), 267 (64), 353 (49), 295 (42), 224 (39), 251 (28), 237 (27)
15	Rhazine* (di-TMS)	38.08	$C_{27}H_{40}N_2O_3Si_2$	496	393 (100), 73 (84), 321 (75), 496 (64), 240 (55), 241 (51), 495 (43), 322 (31)	n.a	249 (100), 352 (95), 169 (88), 168 (70), 321 (52), 351 (51), 279 (29), 182 (25)**
16	Tetrahydroalstonine (mono-TMS)	39.25	$C_{24}H_{32}N_2O_3Si$	424	424 (100), 423 (95), 73 (69), 241 (40), 256 (37), 351 (33), 425 (33), 242 (30)	424 (100), 423 (94), 73 (70), 241 (43), 256 (40), 351 (34), 228 (33), 242 (33)	352 (100), 351 (88), 156 (42), 337 (33), 169 (22), 353 (21), 223 (20), 251 (13)
20a	Vallesiachotamine I* (mono-TMS)	40.49	$C_{24}H_{28}N_2O_3Si$	422	279 (100), 280 (23), 422 (23), 73 (19), 363 (18), 317 (12), 281 (8), 421 (7)	n.a	279 (100), 263 (97), 221 (73), 350 (53), 291 (48), 265 (42), 307 (40), 322 (33)**
18	Yohimbine isomer I (di-TMS)	40.77	$C_{27}H_{42}N_2O_3Si_2$	498	425 (100), 426 (83), 427 (31), 169 (20), 73 (17), 411 (13), 184 (11), 170 (11)	425 (100), 426 (91), 427 (28), 169 (19), 411 (17), 184 (12), 170 (12), 156 (11), 73 (10)	353 (100), 354 (68), 169 (22), 355 (14), 170 (12), 156 (11), 184 (9), 168 (7)
20c	Vallesiachotamine * (di-TMS)	40.87	$C_{24}H_{30}N_2O_3Si_2$	494	351 (100), 73 (95), 279 (74), 494 (29), 352 (28), 241 (19), 435 (19), 280 (18)	n.a	279 (100), 263 (97), 221 (73), 350 (53), 291 (48), 265 (42),307 (40), 322 (33)**
20b	Vallesiachotamine II* (mono-TMS)	40.94	$C_{24}H_{30}N_2O_3Si$	422	279 (100), 73 (24), 280 (23), 422 (21), 363 (16), 317 (11), 351 (9), 265 (8)	n.a	279 (100), 263 (97), 221 (73), 350 (53), 291 (48), 265 (42), 307 (40), 322 (33)**
19	Yohimbine isomer II* (di-TMS)	41.08	$C_{27}H_{42}N_2O_3Si_2$	498	425 (100), 426 (92), 427 (29), 129 (24), 396 (19), 73 (16), 357 (14), 75 (14)	n.a	353 (100), 354 (74), 169 (19), 355 (14), 170 (13), 156 (9), 184 (7)**

*Tentatively identified; **Data from the analysis of *R. stricta* hairt root clones; n.a: pure compound was not available.

In order to obtain a comprehensive view of compounds in *Rhazya* hairy root samples they were also subjected to HPLC analysis for monitoring the composition of alkaloids. The alkaloid profile of the crude extract, illustrated in **Figure 14**, showed seven abundant peaks with baseline separation. Non-separated peaks at 46 min were not included in the comparisons. Vincanine (peak **12**) was identified by comparing the t_R and UV (λ_{max} 246, 300 and 365 nm) of the reference and by spiking (**Figure 14B,C**). The maximum at 365 nm was highly intense. Due to its low absorbance at 255 nm, the peak is small when compared to other alkaloids **21**-**26**.

The HPLC-UV data of alkaloids **(Table 13)** indicated that most of the spectra had three intense maxima, and that two of them were identical (**21-22**). In addition, a flat UV maximum at 364-369 nm was typical for compounds **24**-**26**. Compound **23,** which had a low UV maximum at 290 nm, showed absorbance (sh) at 217 nm, close to the maximum, but it was distorted by an impurity from an extra maximum at 227 nm. This alkaloid was assumed to be strictosidine lactam. Peak **5** exhibited three UV maxima at 210, 249 and 307 nm, but a low absorbance at 368 nm. The UV spectra of compounds **25** and **26** bore a close resemblance to each other (**Table 13**). Further identifications obtained by UPLC-PDA-MS analyses are combined and shown below.

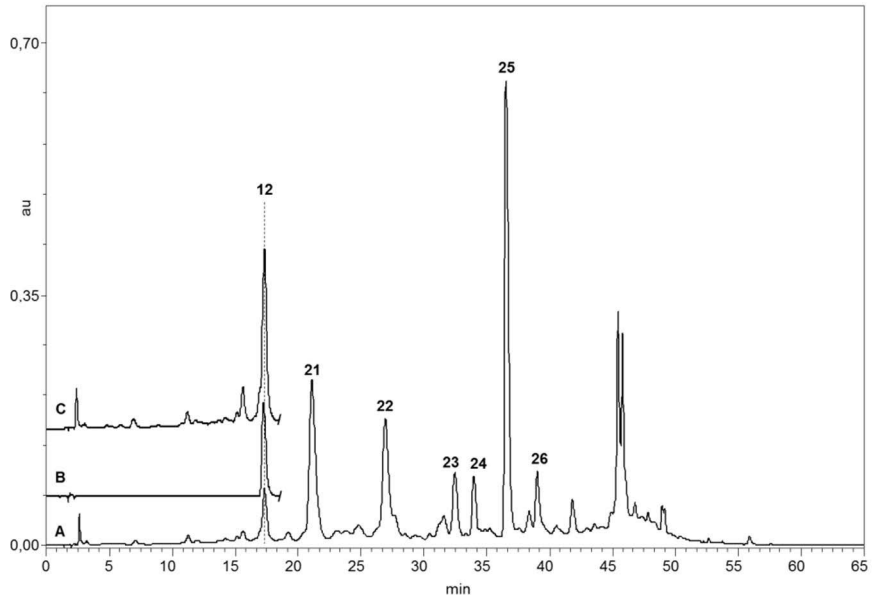


Figure 14. HPLC analysis of a *R. stricta* wild type hairy root extract monitored at 255 nm (**A**). Vincanine standard (**B**) and *Rhazya* extract spiked with vincanine (**C**). Numbers refer to compounds from **Table 13**. au: Absorbance unit.

Table 13. Comparison of HPLC-PDA and UPLC-PDA-MS data of hairy root alkaloids.

Peak	Compound	HPLC-PDA		UPLC-PDA-MS		MW	ES^+ Fragments	ES^-
		t_R* (min)	λ_{max} (nm)	t_R** (min)	λ_{max} (nm)		m/z (rel. int.%)	m/z
12	Vincanine	17.35	243, 300, 364	2.46	246, 300, 365	292	293 (100)	291 (100)
21	Leepacine isomer I	21.13	208, 252, 305	1.95	210, 250, 298sh, 304	350	351 (100)	349 (tr)
22	Leepacine isomer II	26.95	206, 250, 305	2.14	210, 250, 298sh, 305	350	351 (100)	349 (tr)
23	Strictosidine lactam	32.48	217sh, 227, 283, 290	1.31	217sh, 227, 283, 290	498	499 (100), 337 (98)	497 (100), 577 (71)***
24	Serpentine	34.00	206, 249, 308, 364	4.28	210, 249, 307, 368	348	349 (100)	347 (100)
25	Unidentified	36.56	220, 255, 292, 307, 365	12.73	226, 254, 292, 307, 366	628	629 (100), 126 (96)	n.d
26	Unidentified	39.05	220, 255, 292, 307, 366	13.72	225, 250, 291, 308, 365	628	629 (100), 126 (92)	n.d

*from Figure 14; **from Figure 16; ***acetate adduct; tr: traces; n.d: not deteted.

Comparison of UPLC-PDA-MS and HPLC-PDA

For HPLC analyses, phosphate buffer solution, a low pH (2.5) and ion pair reagent were chosen. UPLC conditions and column properties were very different compared with those used in HPLC analysis. In UPLC, the column length and diameter were smaller and particle size, especially, was less than 2 µm. In the solvent systems, the pH was adjusted to 10; however, no ion pair reagent was used in the MS. In addition, injection volume was 2 µL and solvent flow rate 0.4 mL/min. In both methods, the UV spectra appeared very similar, indicating that no changes in UV maxima had occurred either in acidic or alkaline conditions. The greatest difference was seen in the total run time, which was about three times shorter in UPLC analysis.

Compounds **21** and **22** were very similar with regard to their UV and MS data. In both cases, positive and negative ion MS analyses confirmed $[M+H]^+$ ions at m/z 351 and $[M-H]^-$ at m/z 349, respectively (**Table 13)**. The occurrence of leepacine (**Figure 15)** and a related isomer was suggested by the MS data at t_R 2 min, but not exactly by their UV maxima at 306 nm, instead of 298 nm. In addition, GC-MS analyses of *Rhazya* samples indicated $[M+H]^+$ at m/z 350 with the

basepeak of m/z 122 (t_R 36.49 min), which is characteristic of leepacine (Atta-ur-Rahman *et al.* 1991) (not given in **Table 11**).

On the basis of the UV and MS spectra, compound **23** was identified as strictosidine lactam (**Table 13, Figure 15**). Compound **5** displayed four UV maxima at 210, 249, 307, 368 nm and a protonated $[M+H]^+$ ion at m/z 349, and it was identified as serpentine (**Figure 15**). Compounds **25** and **26** had identical UV and MS spectra, but they could not be identified. Since they share a high molecular ion of m/z 629 and possess the characteristic ion m/z 126 for indole alkaloids, these two compounds can be assumed to be dimeric alkaloids.

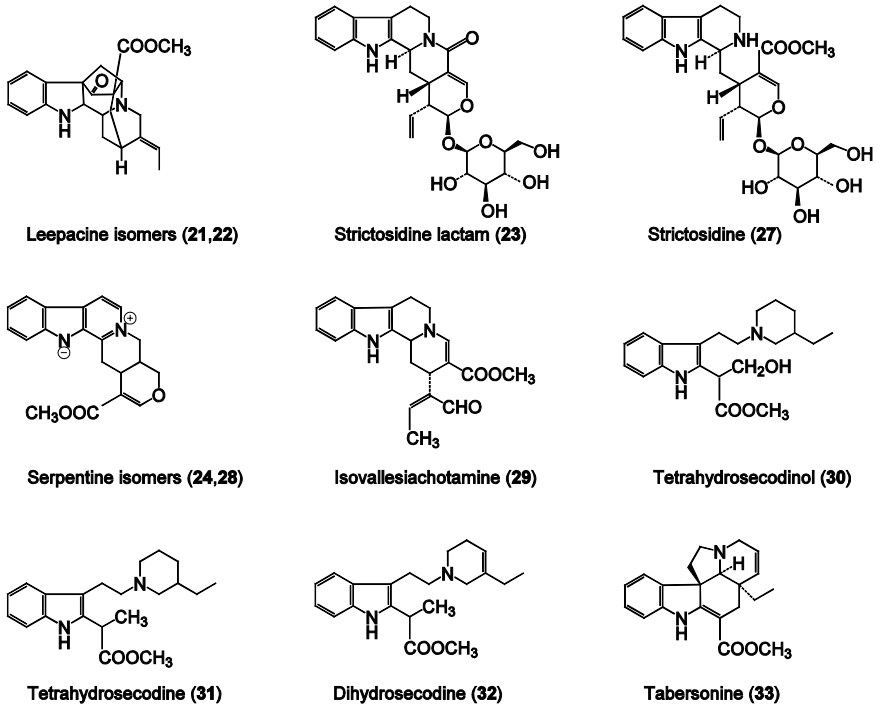


Figure 15. Structures of selected alkaloids identified by UPLC-PDA-MS. Numbers refer to compounds in **Table 13** and **15.**

Reference substances in UPLC-PDA-MS analyses

For identification of alkaloids, four reference substances were analyzed in positive ion mode (**Table 14**). Vincanine exhibited an UV maximum at 365 nm, which was twice as high as that of the second highest maximum (246 nm). A total ion scan of this compound showed a protonated molecular ion ($[M+H]^+$) at m/z 293, and no other major fragments were detected.

Table 14. UPLC-PDA-MS data for reference compounds in positive ion mode.

Pure substance	t_R (min)	UV λ_{max} (nm)	MW	$[M+H]^+$ m/z	Fragments m/z (rel. int.%)
Vincanine	2.45	246, 300, 365	292	293 (100)	
Yohimbine I	3.34	227, 279	354	355 (100)	212 (18), 144 (20), 224 (5)
Vincamine	5.02	228, 280	354	355 (100)	337 (90)
Tabersonine	19.19	229, 298, 330	336	337 (100)	305 (50)

Yohimbine I isomer had a high UV absorbance at 227 nm and low (15%) at 279 nm, and $[M+H]^+$ ion at m/z 355 accompanied by three minor fragments in the order of decreasing abundance at 212, 144 and m/z 224 (**Table 14**). Vincamine, having a similar UV spectrum and MW to yohimbine, could be distinguished by $[M+H]^+$ ion at m/z 355 and by a high fragment m/z 337, due to the loss of water $[M+H-18]^+$.

Tabersonine displayed the most intense UV maximum at 330 nm and also 80% response at 229 and 298 nm (**Table 14**). The molecular ion of this compound was detected at m/z 337, together with a smaller fragment at m/z 305 due to the loss of $[(M+H-CH_3OH)]^+$.

Identification of alkaloids using UPLC-PDA-MS

The UV profile of alkaloids from *Rhazya* extracts which was generated at 255 nm is illustrated in **Figure 16A**. Comparisons between electrospray ionization (ESI) techniques demonstrate that the majority of abundant alkaloids could be identified in positive ion mode (ES^+) (**Figure 16B**). Negative ion analyses (ES^-) exhibited clearly lower intensity and fewer compounds, but cleaner spectra were obtained. Practically no peaks were observed after 10 min of elution (**Figure 16C**). The formation of acetate adducts $[M-H+60]^-$ was also found using negative ion mode (**Table 13**,**15**).

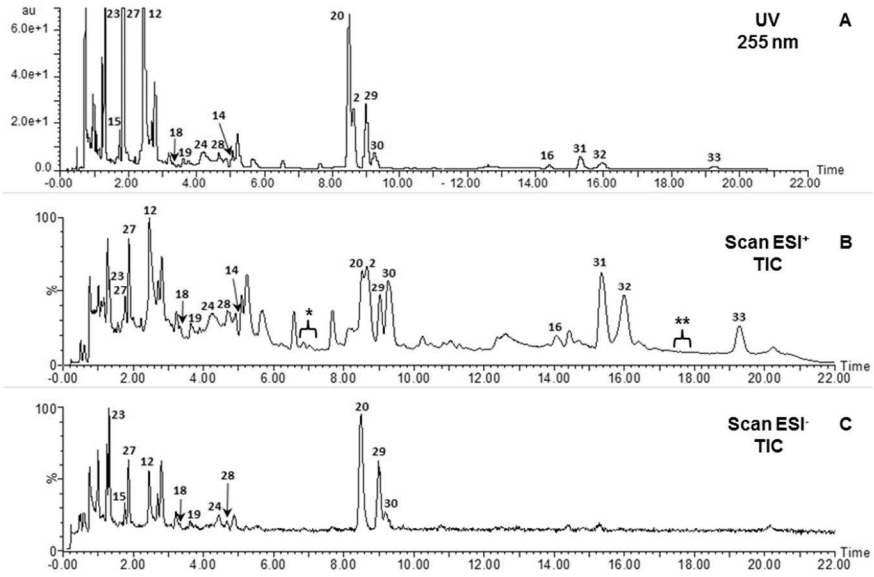


Figure 16. UPLC-PDA-MS chromatograms using UV, MS-ES$^+$ and MS-ES$^-$ iongrams of *R. stricta* alkaloids. *t_R of vincadifformine from clone 3; **t_R of tetrahydrosecamines from clone 10.

It also became evident from the total ion scan (**Figure 16B**) that often nodiscernible baseline separations were found. Therefore, extracted ion recording was used to detect the compounds with the same molecular ion (**Figure 17**), which also allows monitoring the LC-MS spectra at a particular retention time. Selected ion channels at m/z 499 (alkaloid **23**), 531 (**27**), 359 (**30**) and 343 (**31**) displayed symmetric peaks practically without extra signals. The alkaloids were identified on the basis of UV spectra, $[M+H]^+$ and $[M-H]^-$ ions and fragment ions, the data from reference compounds (**Table 15**), GC-MS data (**Table 18**) and literature data. Compound **27**, not found by HPLC, had an UV profile similar to that of alkaloid **23,** exhibited protonated molecular ion [M+1]+ at m/z 531 and an additional fragment m/z 514 ($[M+1-17]^+$) due to the cleavage of a hydroxyl group. Corresponding deprotonated ion $[M-1]^-$ was dectected at m/z 529. This alkaloid was identified as strictosidine (**Table 15**, **Figure 15**).

When comparing the ES$^+$ signals of vincanine (**12**; channel m/z 293, **Figure 17**) and eburenine (peak **2**; channel m/z 281), their intensities appeared equally high. However, the UV chromatogram and total scan iongrams showed considerable peak tailing **(Figure 16A-C**). Thus, neighbouring sharp peaks at the right side of vincanine are strongly distorted by the UV maximum (365 nm) and $[M+H]^+$ m/z 293 of vincanine (**Figure 16B)**.

The $[M+H]^+$ ion m/z 281 of eburenine similarly disturbes the separation of vallesiachotamine isomers (**Figure 16B, Table 15).** Vincanine and eburenine were also the principal alkaloids also in GC-MS analysis.

Table 15. Identification of indole alkaloids of *R. stricta* hairy roots by UPLC-PDA-MS.

No	Compound	t_R (min)	UV λ_{max} (nm)	MW	ES^+ m/z (rel. int.%)	ES^- m/z
23	Strictosidine lactam	1.31	217 (sh), 227, 283, 290	498	337 (100) 499 (98) 267 (12)	497 (100) 335 (tr) 557(71)*
15	Rhazine	1.77	227, 279, 291 (sh)	352	353 (100) 323 (14) 307 (10) 230 (5)	351 (100) 411 (15)* 319 (12)
27	Strictosidine	1.87	228, 270, 280, 290	530	531 (100) 514 (37) 369 (4)	529 (20) 589 (100)*
12	Vincanine	2.46	246, 300, 365	292	293 (100)	291 (100)
18	Yohimbine isomer I	3.33	227, 279	354	355 (100) 212 (6) 144 (11) 224 (4)	353 (100) 413 (88)*
19	Yohimbine isomer II	3.63	226, 279	354	355 (100) 212 (11) 144 (21) 224 (2)	353 (100) 413 (34)*
24	Serpentine isomer I	4.28	210, 248, 307, 368	348	349 (100)	347 (tr)
28	Serpentine isomer II	4.60	210, 248, 307, 368	348	349 (100)	347 (tr)
14	Vincamine	5.03	228, 280	354	355 (100) 337 (82)	n.d
20	Vallesiachotamine isomer I	8.47	222, 292	350	319 (100) 281 (83)** 351 (50) 170 (35)	349 (100)
2	Eburenine	8.69	222, 262	280	281 (100)	n.d

Continues→

29	Vallesiachotamine isomer II	8.99	223, 291	350	319 (100) 351 (56) 170 (49) 281 (30)**	349 (100)
30	Tetrahydrosecodinol	9.27	222, 283, 290	358	126 (100) 359 (62) 341 (49) 246 (30)	357 (tr)
16	Tetrahydroalstonine	14.45	227, 270 (sh), 282, 290	352	353 (100) 144 (60)	n.d
31	Tetrahydrosecodine	15.34	224, 283, 290 (sh)	342	126 (100) 343 (90) 230 (53)	n.d
32	Dihydrosecodine	15.99	224, 280 (sh), 290, 305	340	126 (100) 341 (51) 228 (26)	n.d
33	Tabersonine	19.25	229, 298, 331	336	337 (100) 305 (68) 228 (2)	n.d

*acetate adduct, **distorted by eburenine; n.d: not detected; tr: trace.

Yohimbin isomer I, had the same retention time in the LC-MS analysis of *Rhazya* extract (**Table 15**) as the commercial yohimbin reference substance (**Table 14**). In the extract, two yohimbine isomers were detected having similar UV spectra, and the $[M+H]^+$ and $[M-H]^-$ ions at m/z 355 and m/z 353, respectively. The intensity of yohimbine isomers shown at channel m/z 355 was among the lowest of all alkaloids (**Figure 17)**. In addition, a further yohimbine-like compound was found at 3.26 min with the same fragments as shown for yohimbine isomers. However, the MS spectrum was considerably distorted by ions at m/z 297 (100) and 293 (38).

In the UV and total ion chromatogram (**Figure 16A,B**), the vincamine peak (**14, Table 15**) in *Rhazya* extracts is hardly visible due to co-elution with the following compound. However, the extracted ions channel at m/z 355 (**Figure 17**), showing the same t_R at 5.03 min as the reference compound (**Table 14**). Despite its very low intensity, vincamine displayed a rather symmetric peak with baseline separation.

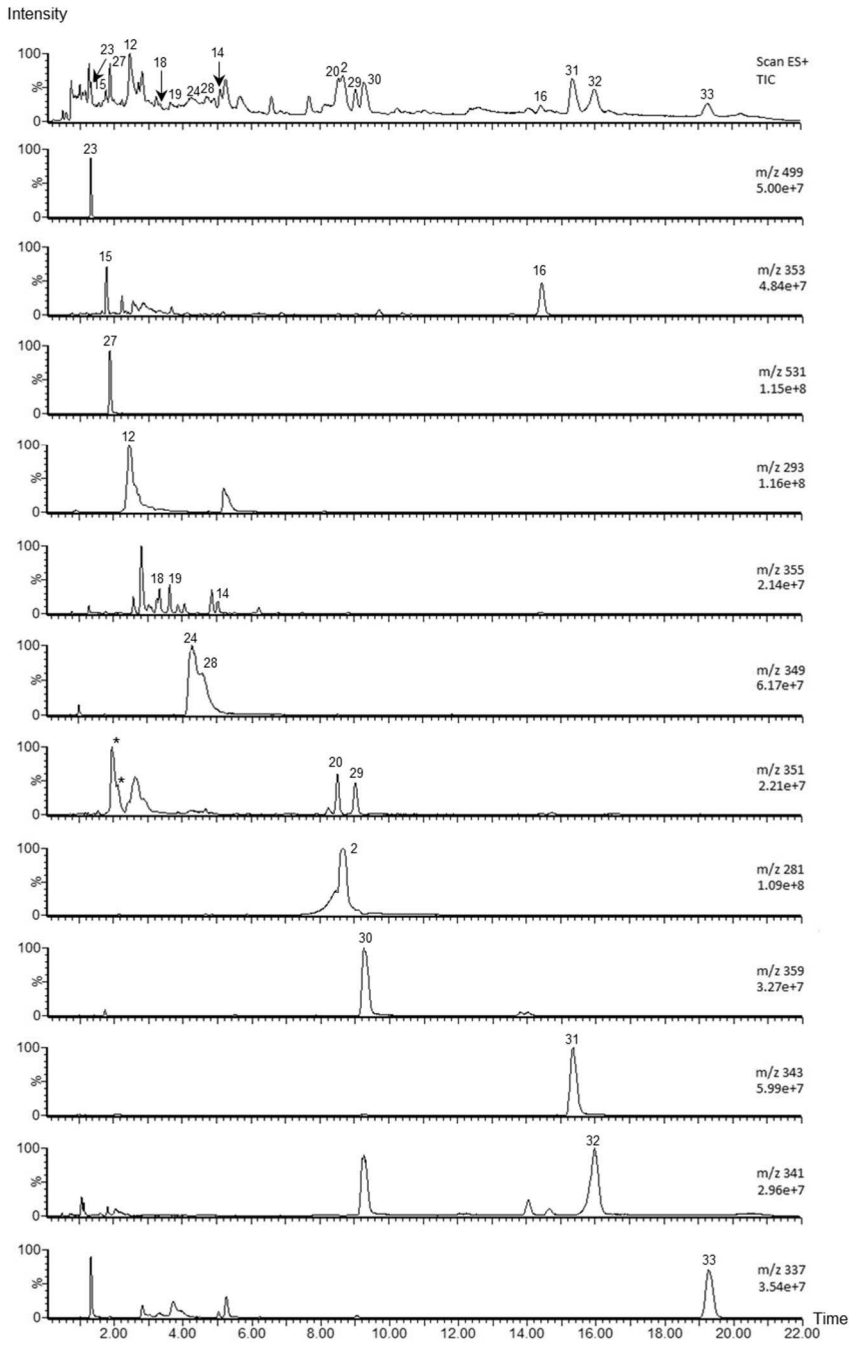


Figure 17. Extracted iongrams (ES^+) from UPLC-PDA-MS-TIC analysis of *R. stricta* alkaloids. *Leepacine isomers (channel m/z 351).

Compound **24** represented a minor constituent in HPLC analysis (**Figure 14**) and its UV spectrum with a flat maximum at 370 nm also showed close similarity with that obtained by UPLC-PDA-MS (compound **24**, **Table 15**). The exact t_R in UPLC was confirmed by extracted ion recording with subsequent MS analysis. Compound **24** displayed $[M+H]^+$ and $[M-H]^-$ ions at m/z 349 and 347 at t_R 4.28 min. However, the same molecular ions were also observed at 4.60 min, indicating an isomer of **24**. No further fragments at higher mass ranges were detected. The alkaloids **24** and 2**8** were identified as serpentine monomers (**Table 15**, **Figure 16**). The occurrence of these isomers in UPLC-MS analysis suggests that peak **24** in HPLC (**Figure 14**) is actually a mixture of two alkaloids.

Vallesiachotamine isomers exhibited equal UV maxima in UPLC and had $[M+1]^+$ ions at m/z 351 and basepeak ions at m/z 319 due to the cleavage of ($[M+1-CH_3OH]^+$). In addition, extracted ion recording (ES^+ channel; m/z 351; **Figure 17**) showed an abundant, broadened peak zone at 2 min composed of two alkaloids which were assumed to be leepacine isomers. At this retention time, clean spectra at m/z 351 were detected, without any interfering signals.

Compounds **30**, **31** and **32** formed a specific group of alkaloids, since they all had the basepeak fragment at m/z 126 (**Table 15**). No fragments at higher mass range were found after the molecular ions. Compound **30** displayed low-abundance UV maxima at 283 and 290 nm. The molecular ion at m/z 359 was followed by m/z 341, due to the loss of water $[M+H-18]^+$, and by m/z 246. The alkaloid was identified as tetrahydrosecodinol. Compound **31**, which had an identical UV with alkaloid **30** and a molecular ion at m/z 343 followed by fragment m/z 230, was identified as tetrahydrosecodine. Compound **32**, having a UV maximum at higher wavelength (λ_{max} 305 nm) and $[M+H]^+$ at m/z 341, was identified as dihydrosecodine for the first time for *R. stricta*. The structures of these compounds are illustrated in **Figure 15**.

Compound **33** showed three UV maxima and protonated ion m/z 337 of the reference substance of tabersonine, followed by the fragment at m/z 305. The structure of tabersonine is shown in **Figure 15.**

A number of compounds also displayed UV maxima resembling the profile of tabersonine. Clones 3 and 10, but not clone 2, showed a broad but symmetric peak at 6-8 min. Using extracted ion recording (m/z 339) from TIC, the peak top was located at 7.08 min (t_R shown in **Figure 16B**). It exhibited UV with three maxima (λ_{max} 228, 299 and 329 nm) and a molecular ion at m/z 339, and a fragment at m/z 307 due to the loss of CH_3OH. Based on this data, the alkaloid was assumed to be vincadifformine. Another compound from clone 2 detected at t_R 5.75 min had an identical UV to vincadifformine (λ_{max} 228, 298 and 329 nm) but showed a molecular ion at m/z 323 and a basepeak fragment at m/z 291 derived from the loss of $[M+1-CH_3OH]^+$, which would rather support the occurrence of akuammicine. Despite the insufficient alkaloid separations, resulting in several broadened peak zones spread along the baseline, the identification of 17 alkaloids was successfully carried out using PDA and MS.

However, MS evidence of a dimeric secamine-type alkaloid was obtained from clone 10. Using extracted ion recording, the MS spectrum was recorded and the

molecular ions were located on TIC. On the basis of the UPLC-MS data, a broad but symmetric peak zone at 16-22 min having a peak at t_R 18.46 min (marked in **Figure 16B**) displayed a UV profile with λ_{max} at 229, 286, 292 and a dimeric molecular ion $[2M+H]^+$ at m/z 681(20). Characteristic basepeaks for secodin-type alkaloids at m/z 126 (100) and the fragment from the parent monomer at m/z 341 (17) were observed, as well as one major interfering fragment at m/z 143. This dimeric alkaloid was identified as tetrahydrosecamine.

5.2.2 Alkaloid accumulation in undifferentiated cell cultures

Alkaloid contents of four-week old undifferentiated calli derived from various explants (each n=10) were analysed by HPLC-PDA (**Appendix E**) and compared with differentiated wild type hairy roots (n=20) and adventitious roots (n=10). The amount of strictosidine lactam (**23**) was statistically higher in hypocotyl, stem and leaf calli than in wild type hairy roots, whereas its concentration did not vary in calli derived from roots and cotyledons compared to the hairy roots. Accumulation of other alkaloids showed a statistically significantly increase in the hairy roots (**Figure 18**). In comparison, the concentrations of vincanine (**12**), leepacine isomer II (**22**), strictosidine lactam (**23**), serpentine (**24**) and an unidentified alkaloid **25** were significantly higher in hairy roots than in adventitious roots; however, the amounts of leepacine isomer I (**21**) and an unidentified alkaloid **26** were not significantly different.

In addition, it was revealed that the content of strictosidine lactam (**23**) was statistically higher in all calli lines compared to adventitious roots. Production of leepacine isomer II (**22**) in leaf calli was not significantly different from that of the adventitious roots. The accumulation of other prominent alkaloids in adventitious roots was statistically significantly higher than in different calli lines (**Figure 19**).

Pairwise comparisons of alkaloid contents in different calli lines were also carried out (**Appendix F**).

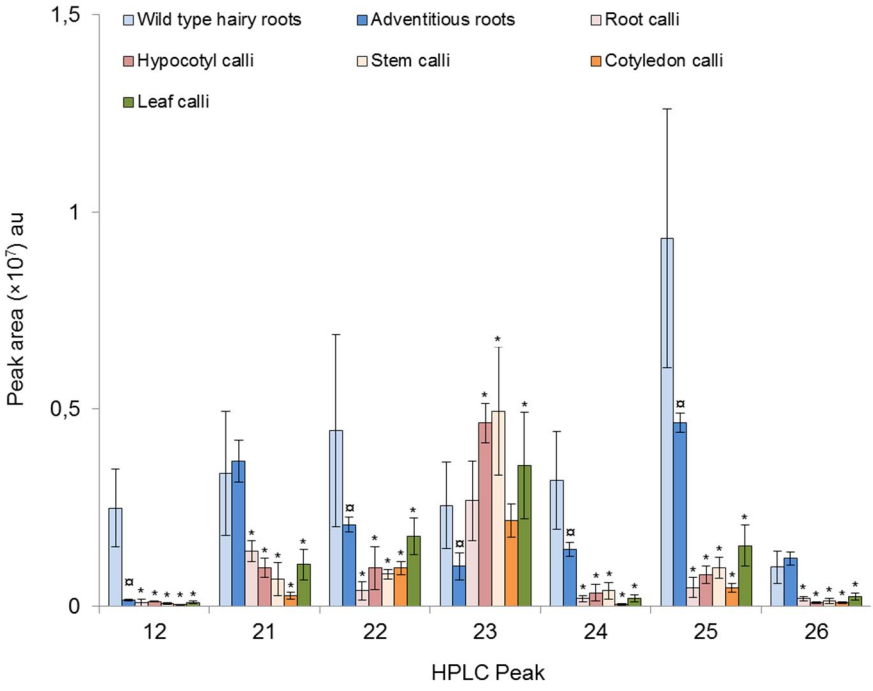


Figure 18. Comparisons of seven major alkaloids in wild type transformed roots (n=20) *vs*. non-transformed adventitious roots (n=10) and five different calli lines (n=10) of *R. stricta*. Bars represent the mean values ± SD. * indicates significant difference in the mean between wild type hairy roots and different calli lines (p<0.05 by ANOVA). ¤ indicates significant difference in the mean between wild type hairy roots and adventitious roots (p<0.05 by ANOVA). Numbers refer to compounds from **Table 13**. au: HPLC peak area.

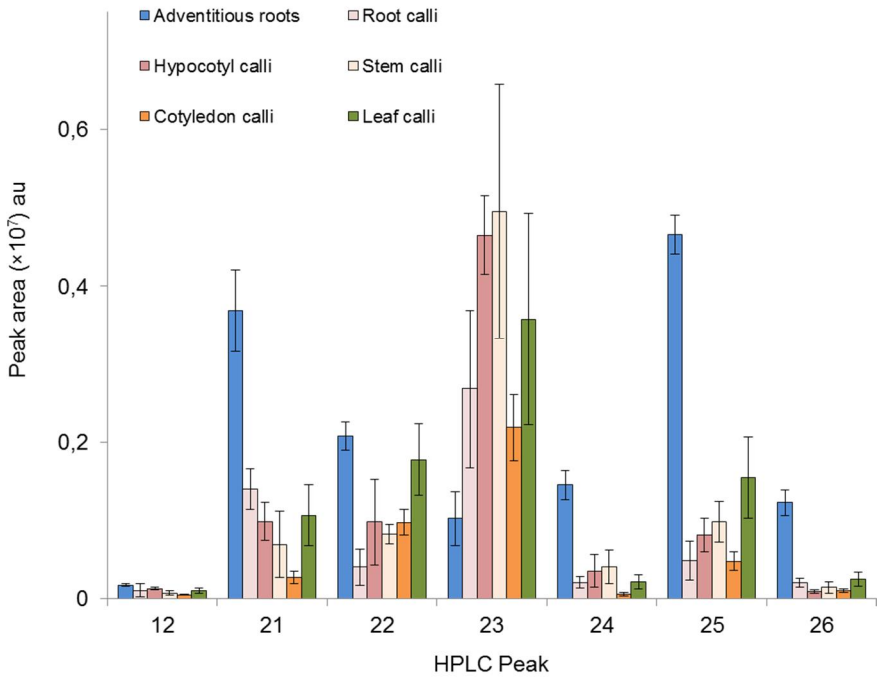


Figure 19. Comparisons of seven major alkaloids in non-transformed adventitious roots (n=10) *vs*. five different calli lines (each n=10) of *R. stricta*. Bars represent the mean values ± SD. * indicates significant difference in the mean between adventitious roots and different calli lines (p<0.05 by ANOVA). Numbers refer to compounds from **Table 13**. au: HPLC peak area.

5.2.3 Alkaloid accumulation in wild type and GUS hairy roots

Four-week old wild type (n=20) and GUS (n=10) hairy roots grown in liquid medium and adventitious roots (n=10) obtained from *in vitro* grown *R. stricta* were examined by HPLC-PDA for their alkaloid production. The results indicated that the accumulation of alkaloids **21**-**24** (leepacine isomers, serpentine and strictosidine lactam) and unidentified alkaloids **25** and **26** between wild type hairy roots and GUS clones did not differ statistically significantly (**Table 16**). Concentrations of vincanine (**12**), on the other hand, were significantly higher in wild type hairy roots. When comparing wild type clones to non-transformed roots they contained significantly higher amounts of vincanine, leepacine isomer II (**22**), strictosidine lactam (**23**), serpentine (**24**) and alkaloid **25** than non-transformed roots. GUS hairy root clones contained significantly higher amounts of vincanine (**12**), leepacine isomer II (**22**), strictosidine lactam (**23**) and serpentine (**24**) compared to non-transformed roots (**Table 16**).

Table 16. Comparisons of seven major alkaloids in wild type hairy roots (n=20, WT), GUS hairy roots (n=10, GUS) and non-transformed adventitious roots (n=10, NT) of *R. stricta*.

Peak	WT M±SD (au)	GUS M±SD (au)	NT M±SD (au)	*p* value WT/GUS	*p* value WT/NT	*p* value GUS/NT
12	2.50×10^{6}	9.57×10^{5}	1.73×10^{5}	$p<0.05$	$p<0.05$	$p<0.05$
	9.86×10^{5}	5.64×10^{5}	2.12×10^{4}			
21	3.37×10^{6}	3.51×10^{6}	3.69×10^{6}	$p=0.96$	$p=0.96$	$p=0.95$
	1.58×10^{6}	1.53×10^{6}	5.25×10^{5}			
22	4.47×10^{6}	3.17×10^{6}	2.07×10^{6}	$p=0.18$	$p<0.05$	$p<0.05$
	2.44×10^{6}	1.33×10^{6}	1.82×10^{5}			
23	2.56×10^{6}	3.77×10^{6}	1.02×10^{6}	$p=0.43$	$p<0.05$	$p<0.05$
	1.10×10^{6}	1.91×10^{6}	3.39×10^{5}			
24	3.19×10^{6}	3.90×10^{6}	1.45×10^{6}	$p=0.45$	$p<0.01$	$p<0.05$
	1.25×10^{6}	2.43×10^{6}	1.83×10^{5}			
25	9.34×10^{6}	5.73×10^{6}	4.66×10^{6}	$p<0.05$	$p<0.05$	$p=0.64$
	3.29×10^{6}	2.59×10^{6}	2.46×10^{5}			
26	9.97×10^{5}	1.08×10^{6}	1.22×10^{6}	$p=0.84$	$p=0.31$	$p=0.70$
	4.06×10^{5}	5.18×10^{5}	1.66×10^{5}			

Significant difference in the peak area mean between different groups is presented as $p<0.05$ (by ANOVA). au: absorbance unit.

Effect of different media and light: The biomass production of wild type hairy roots was similar independent of the media used. In modified Gamborg B5 medium the fresh weight of hairy roots was 3.1 ± 0.5 g, dry weight 1.0 ± 0.3 g and extraction yield 2.4 ± 0.4 mg/50 mg of plant material dry weight. In Woody Plant Medium, the fresh weight of hairy roots grown for four weeks was 3.1 ± 0.4 g, dry

weight 1.0 ± 0.3 g and extraction yield 2.2 ± 0.7 mg/50 mg of plant material dry weight. However, hairy roots grown in liquid modified Gamborg B5 medium produced significantly higher concentration of strictosidine lactam (**23)** and alkaloid **25** compared with hairy roots grown in liquid Woody Plant Medium (**Figure 20**).

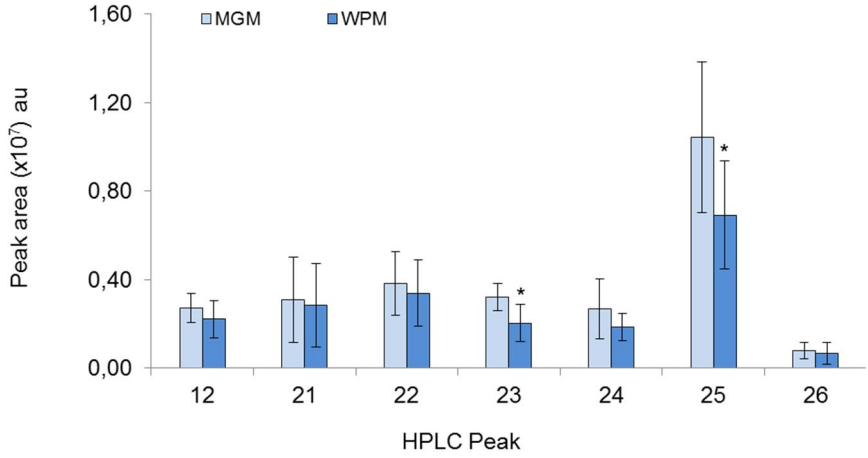


Figure 20. Accumulation of seven major alkaloids in wild type hairy root clones of *R. stricta* grown in dark either in liquid modified Gamborg B5 medium (n=10 in triplicate, white bar, MGM) or in liquid Woody Plant Medium (n=10 in triplicate, dark grey bar, WPM). Bars represent the mean values ± SD. * indicates significant difference in the mean (student's *t* test, $p<0.05$). Numbers refer to compounds from **Table 13**. au: unit absorbance.

Hairy root clones grown in the dark contained statistically significantly higher amounts of vincanine (**12**), strictosidine lactam (**23)**, serpentine (**24**) and alkaloid **25** compared to hairy root clones grown under the 16 h photoperiod. The difference in vincanine concentration was especially pronounced, *i.e.* a sixfold increase compared to the 16 h photoperiod. On the other hand, the amount of leepacine isomer I (**21)** in hairy root clones grown in the dark was significantly lower compared to the hairy root clones grown under the 16 h photoperiod (**Figure 21**). However, the accumulation of leepacine isomer II (**22**) and alkaloid **26** was not statistically different among cultures grown in two different conditions. The fresh weight of hairy roots grown in the dark was statistically significantly higher than that of clones grown under a 16 h photoperiod, but the differences in dry weight and extraction yield were not statistically significant.

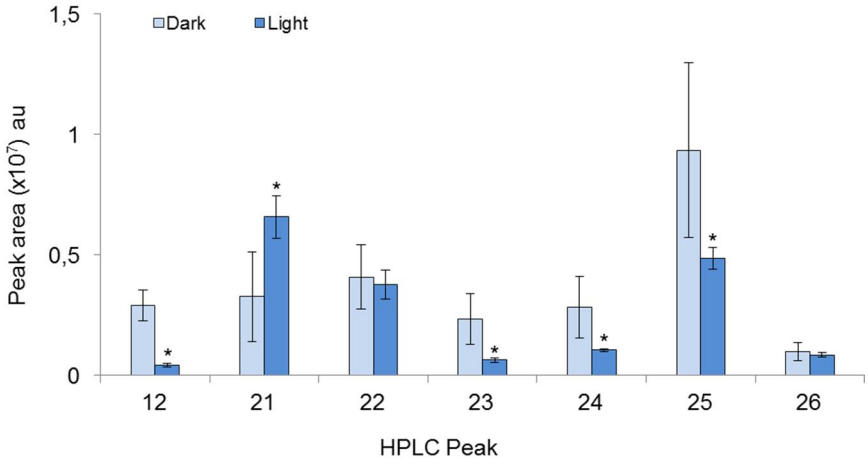


Figure 21. Accumulation of seven major alkaloids in wild type hairy root clones of *R. stricta* grown in modified Gamborg B5 medium either in the dark (dark grey bar) or under a 16 h photoperiod condition (light grey bar). Bars represent the mean values ± SD. * indicate significant differences in the mean (student's *t* test, $p<0.05$). Numbers refer to compounds from **Table 13**. au: unit absorbance.

Variation of alkaloid contents

In order to obtain a broader view of the concentrations of major alkaloids in 20 wild type clones, GC-MS was performed. The results are shown in **Table 17**. The extracted ion monitoring method used for quantification of alkaloids from all hairy root clones and showed excellent RSD of t_R (≤0.5%) for the 12 alkaloids (**Table 17**). The alkaloid profiles were qualitatively rather similar in the majority of the lines but considerable quantitative variation was observed. Eburenine (**2**), vincanine (**12**), yohimbine isomer II (**19**) and vallesiachotamine (**20**) were the major components. Yohimbine isomer II concentrations varied between 0.002 and 0.04% of the DW of hairy roots.

Eburenine was the major alkaloid in all the lines (0.09± 0.06%) followed by vincanine (0.05±0.02%), vallesiachotamine (0.02±0.01%) and yohimbine isomer II (0.02±0.01%). Quebrachamine, tubotaiwine, fluorocarpamine, rhazine, ajmalicine and yohimbine isomer I represented less than 0.01% of *Rhazya* dry powder, on average.

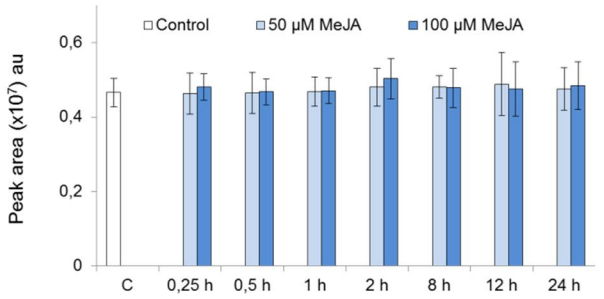
Table 17. Quantitative variation of major alkaloids in hairy root clones based on ratio of base peak and dipyridyl (IS) abundances obtained from extracted ions.

Peak*	Compound	Range (% of DW)	Mean ± SD (% of DW)	RSD (%)	t_R (RSD%)
2	Eburenine	0.009 – 0.040	0.088 ± 0.056	63.1	0.06
5	Quebrachamine	0.001 – 0.009	0.003 ± 0.002	70.2	0.01
6	Tubotaiwine	0.002 – 0.006	0.004 ± 0.001	22.7	0.01
11	Pleiocarpamine	0.005 – 0.017	0.010 ± 0.003	31.0	0.03
12	Vincanine	0.023 – 0.091	0.051 ± 0.017	33.2	0.06
13	Fluorocarpamine	0.001 – 0.003	0.001 ± 0.001	49.7	0.02
15	Rhazine	0.000 – 0.006	0.002 ± 0.001	66.4	0.02
16	Tetrahydroalstonine	0.003 – 0.018	0.010 ± 0.004	43.6	0.04
17	Ajmalicine	0.0004 – 0.002	0.001 ± 0.0004	37.0	0.03
18	Yohimbine isomer I	0.002 – 0.017	0.009 ± 0.004	48.2	0.02
19	Yohimbine isomer II	0.002 – 0.040	0.024 ± 0.012	48.2	0.46
20	Vallesiachotamine	0.009 – 0.036	0.024 ± 0.008	31.5	0.54

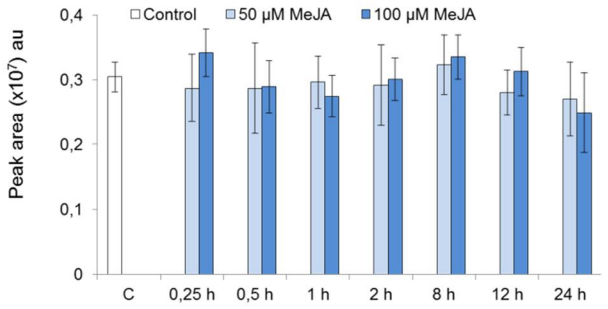
*Numbers refer to compounds in **Table 11**.

5.2.4 Effect of elicitation on wild type hairy roots

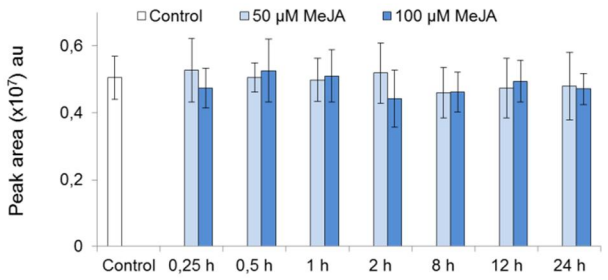
Selection of an adequate elicitor: Preliminary experiments were carried out using methyl jasmonate, MeJA (50 and 100 µM) and chitosan (100 and 250 mg/L). These elicitors were separately added to 21-day hairy root cultures grown in liquid media and they were harvested after 0.25, 0.5, 1, 2, 8, 12 and 24 hours, freeze-dried and their alkaloid alteration was qualitatively analysed by HPLC. All treatments were performed in triplicate and controls were included. HPLC analyses revealed that MeJA at concentration of 50 or 100 µM statistically significantly increased the accumulation of strictosidine lactam (**23)**, serpentine (**24**) and an unidentified alkaloid (**25**) after 24 h (**Figure 22**). However, FW and DW of elicited samples did not significantly change in comparison with control cultures (**Appendix G**). HPLC analyses showed that there were no significant differences in the content of the seven prominent alkaloids in hairy roots elicited with chitosan (**Appendix H**) and in their FW and DW compared to control cultures.



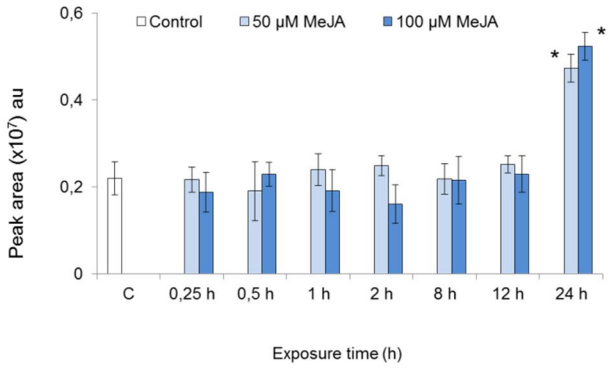
Vincanine (**12**)



Leepacine I (**21**)



Leepacine I (**22**)



Strictosidine lactam (**23**)

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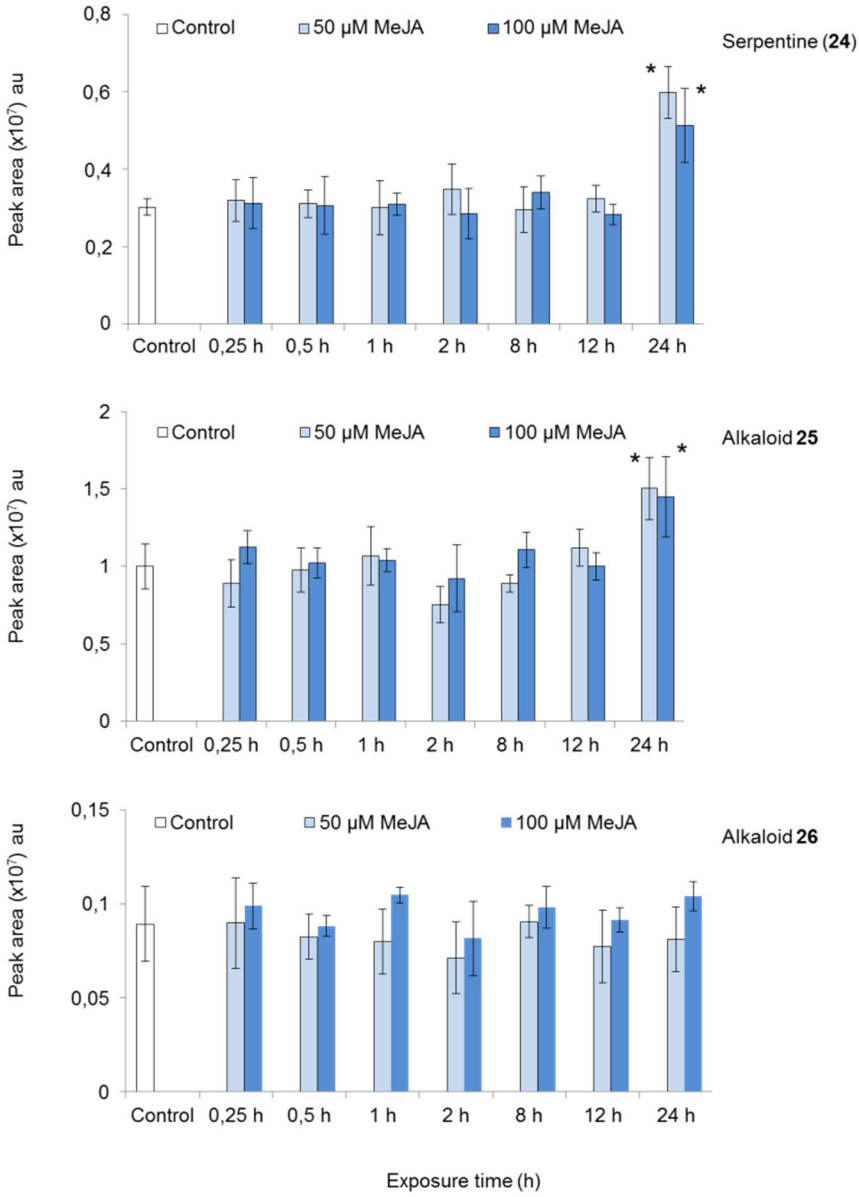
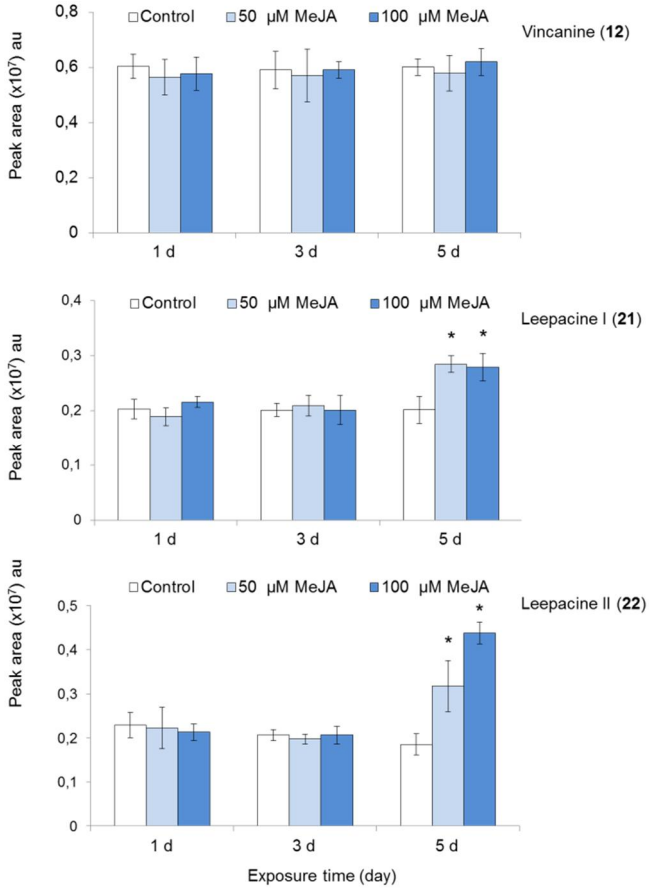


Figure 22. Accumulation of seven major alkaloids analysed by HPLC in *R. stricta* wild type hairy roots elicited with 50 and 100 µM methyl jasmonate (MeJA) after 0.25, 0.5, 1, 2, 8, 12 and 24 hours exposure time. Control (non-elicited cultures) contained 80 µL of 40% ethanol (the same volume of 100 µM MeJA) and were harvested after 24 h. Values presented as mean±SD of three samples for each treatment. * indicates significant difference in the mean between controls and elicited samples (student's *t* test, $p<0.05$).

Another experiment was performed and wild type hairy roots harvested 1, 3 and 5 days after elicitation with 50 and 100 μM MeJA. Results of HPLC analyses showed a clear increase in alkaloids **21** and **22** (leepacine isomers) after five days of MeJA exposure at both concentrations (**Figure 23**). The amounts of strictosidine lactam (**23**) and alkaloid **25** were elevated after elicitation in all experimental conditions. In addition, the accumulation of serpentine (**24**) statistically significantly increased after MeJA exposure of hairy roots at both concentrations after 1 and 3 days. The accumulation of vincanine (**12**) and alkaloid **26** did not vary under the experimental conditions.



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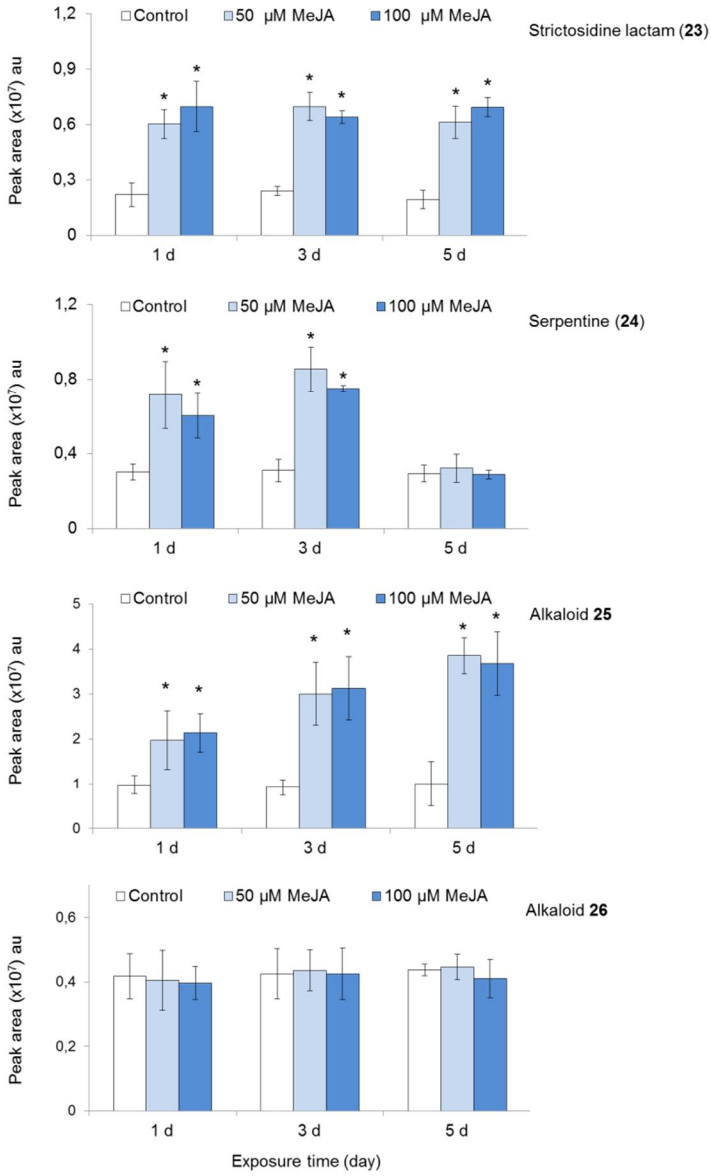


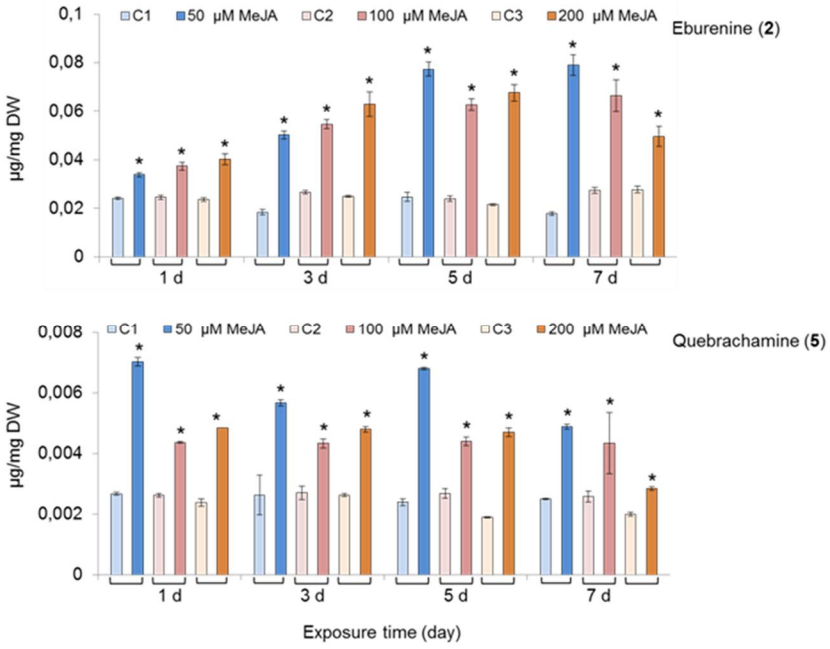
Figure 23. Accumulation of seven major alkaloids analysed by HPLC in *R. stricta* wild type hairy roots elicited with 50 and 100 µM methyl jasmonate (MeJA) after 1, 3 and 5 days (d) exposure time. Controls (non-elicited cultures) contained 80 µL of 40% ethanol (the same volume of 100 µM MeJA) and were harvested at the same time as elicited samples. Values presented as mean±SD of three samples for each treatment. * indicates significant difference in the mean between controls and elicited samples (student's *t* test, $p<0.05$).

Taking these results into account an experiment was designed with 21-day wild type hairy roots from the same clone, elicited with 50, 100 and 200 μM MeJA and harvested after 1, 3, 5 and 7 days. To get a broader view, alkaloid accumulation was analysed by GC-MS.

Alkaloid contents

Alkaloids from elicited samples were quantified using GC-MS extracted ion monitoring data of alkaloids based on base peak abundances divided by those of 2,4'-dipyridyl as IS.

The GC-MS results showed that all three concentration levels of MeJA at all four time points statistically significantly (student's *t* test, $p<0.05$) enhanced the production of eburenine (**2**), quebrachamine (**5**) and fluorocarpamine (**13**) compared with non-elicited hairy roots (**Figure 24**). The same trend was observed for pleiocrpamine (**11**); however, its accumulation did not change after one day of exposure of hairy roots to 100 or 200 μM MeJA. Exposure of hairy roots to MeJA at 50 μM after five days caused the highest accumulation of eburenine, 0.07 μg/mg ±0.002 DW, which was 3.1-fold higher than its control. Hairy roots treated with 50 μM MeJA for seven days also exhibited eburenine production of 0.07 μg/mg ±0.004 DW, which represented a 3-fold increase compared with controls.



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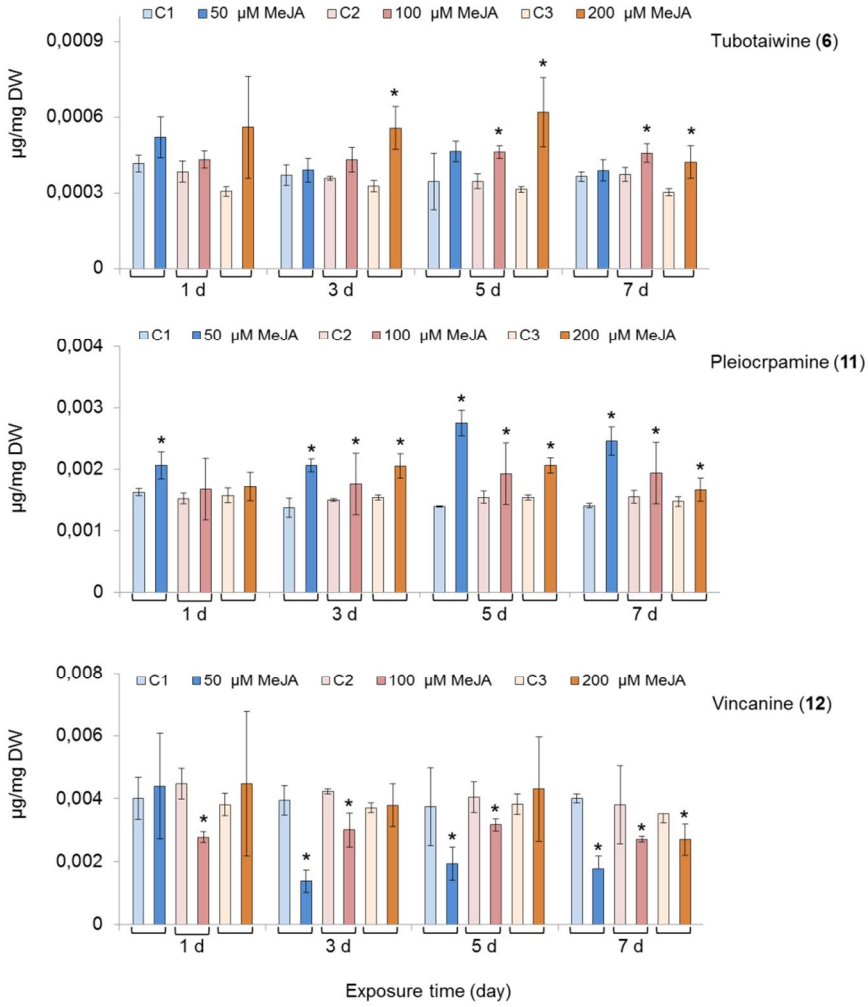
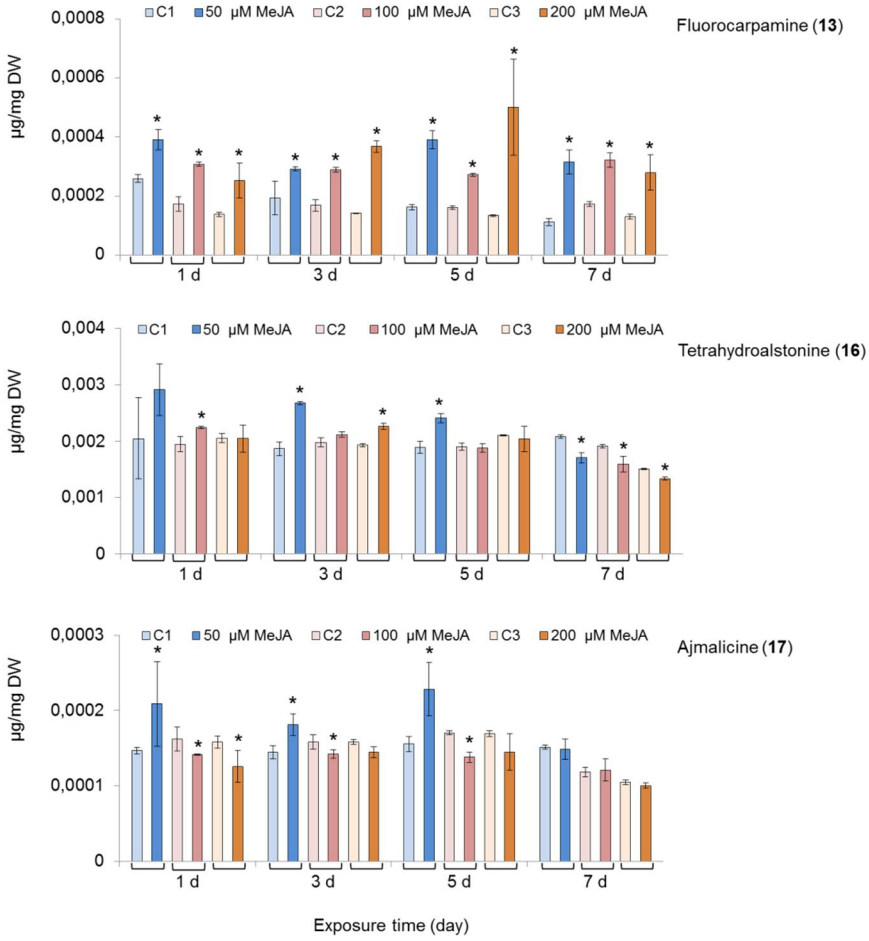

C1
50 μM MeJA
C2
100 μM MeJA
C3
200 μM MeJA
Tubotaiwine (6)
μg/mg DW
0,0009
0,0006
0,0003
0
1 d
3 d
5 d
7 d
Pleiocrpamine (11)
0,004
0,003
0,002
0,001
Vincanine (12)
0,008
0,006
0,004
0,002
Exposure time (day)

Continues→


C1
50 µM MeJA
C2
100 µM MeJA
C3
200 µM MeJA
Fluorocarpamine (13)
µg/mg DW
0,0008
0,0006
0,0004
0,0002
0
1 d
3 d
5 d
7 d
Tetrahydroalstonine (16)
0,004
0,003
0,002
0,001
Ajmalicine (17)
0,0003
0,0002
0,0001
Exposure time (day)

Continues→

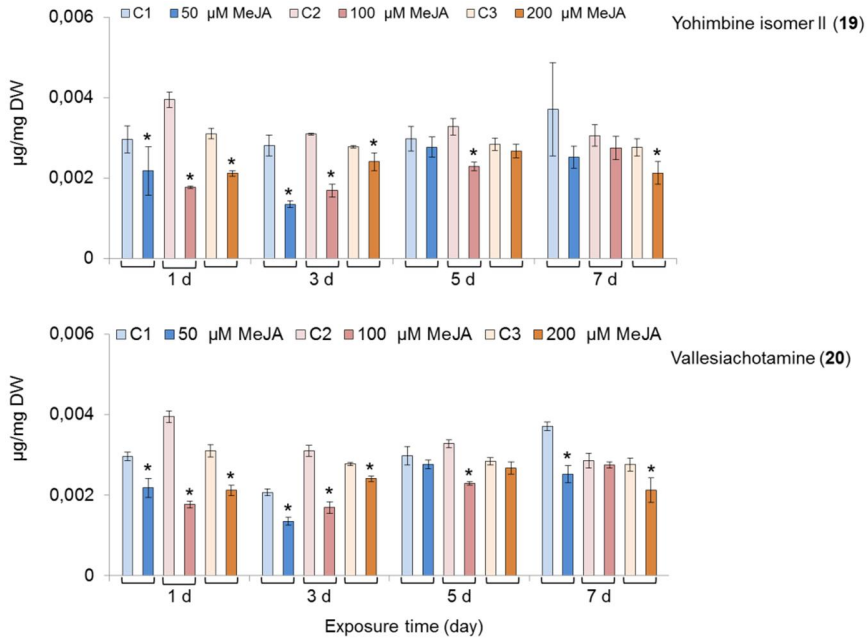


Figure 24. Accumulation of ten major alkaloids analysed by GC-MS in *R. stricta* wild type hairy roots elicited with 50, 100 and 200 µM methyl jasmonate (MeJA) after 1, 3, 5 and 7 days (d) exposure time. C1, C2, C3, controls (non-elicited cultures) contained corresponding amount of 40% ethanol for 50, 100 and 200 µM MeJA, respectively, and were harvested at the same time as elicited samples. Values presented as mean±SD of three samples for each treatment. * indicates significant difference in the mean between controls and elicited samples (student's *t* test, $p<0.05$). Quantitative analysis was based on GC-MS base peak abundances from extracted ions from extracted ions.

It is evident from **Figure 24** that the highest accumulation of quebrachamine (**5**) was achieved in hairy roots exposed to 50 µM MeJA for one and five days, *i.e.* 2.6- and 2.5-fold higher compared to the corresponding control cultures, respectively. Its production reached to 0.007 µg/mg ± 0.0006 DW and 0.006 µg/mg ±0.0002 DW. **Figure 24** shows that among MeJA concentration ranges and incubation times fluorocarpamine (**13**) content was significantly enhanced after hairy roots were treated with 200 µM MeJA for five days. This elicitation condition resulted in a 3.7-fold (0.0004 µg/mg ±0.0001 DW) increased level of fluorocarpamine than in non-elicited roots.

The highest production of pleiocarpamine (**11**) was 1.7- or 1.5-fold compared with the corresponding controls and was achieved when hairy roots were exposed to 50 µM MeJA for five or seven days, respectively; in both conditions its yield was found to be 0.002 µg/mg ±0.0002 DW.

In comparison to the control cultures, hairy roots which were treated with 200 µM MeJA for three, five and seven days and with 100 µM MeJA for five and seven days contained a statistically significantly (student's *t* test, $p<0.05$) higher level of

tubotaiwine (**6**). The maximum level of tubotaiwine was obtained after hairy roots were treated with 200 µM MeJA for five (0.0006 µg/mg ±0.0001 DW) or three (0.0004 µg/mg ±0.00008 DW) days, with 1.9- and 1.7-fold increase compared to their non-elicited controls, respectively (**Figure 24**). Other concentrations and exposure times did not affect the accumulation of tubotaiwine.

Significant increase of tetrahydroalstonine (**16**) was detected in hairy roots exposed to 50 µM MeJA after three and five days and showed 1.4- and 1.2-fold increases compared with non-elicted roots over the same time (**Figure 24**). In addition, MeJA application at 100 µM and 200 µM after one and three days, respectively, statistically promoted the production of tetrahydroalstonine. Exposure of hairy roots to different concentration levels of MeJA for seven days supressed the production of tetrahydroalstonine (**16**); in other experimental conditions the accumulation of the compound did not significantly alter compared to controls.

It was found that the accumulation of ajmalicine (**17**) was significantly increased only at a concentration of 50 µM MeJA one, three and five days after elicitation and led to 1.42-, 1.25- and 1.46-fold increases, respectively, in comparison with control cultures. It was also observed that control cultures contained higher concentrations of ajmalicine than corresponding elicited samples when the MeJA concentration was higher than 50 µM (**Figure 24**).

Data analyses obtained from GC-MS showed that the accumulation of vincanine (**12**) was inhibited when hairy roots were exposed to different concentrations of elicitor for seven days. Similarly, when roots were treated with 100 µM MeJA for one, three and five days as well as with 50 µM for three and five days the production of vincanine was supressed (**Figure 24**). The contents of yohimbine isomer II (**19**) and vallesiachotamine (**20**) were also decreased in elicited hairy roots with different doses of MeJA and exposure times of one and three days in comparison with control cultures. In addition, elicitation with 100 and 200 µM MeJA for five and seven days, respectively, inhibited the production of both alkaloids (**19**,**20**). None of ten studied alkaloids were detected in the media.

5.2.5 Alkaloid accumulation in transformed hairy roots with key TIA genes

Three key genes from the upstream TIA biosynthesis pathway including geraniol synthase (*ges*) and geraniol-8-oxidase (*g8o*) from the terpenoid biosynthesis pathway and strictosidine synthase (*str*), as an intermediate gene between the upstream and downstream TIA biosynthesis pathways, were introduced to *R. stricta* hairy root clones. Four weeks old wild type (n=20), *ges*, *g8o* and *str* (each n=10) *R. stricta* hairy root clones grown in liquid medium were examined for their alkaloid production. The hairy roots were analysed by HPLC-PDA and their alkaloid contents were compared to those of wild type hairy root clones.

It was revealed that the concentrations of vincanine (**12**) and serpentine (**24**) were statistically significantly increased in *str* hairy root clones compared to wild types (**Figure 25**). However, in comparison to control cultures, the accumulation of leepacine isomers (**22**-**23**) and unidentified alkaloids (**25**-**26**) remained the same and stricitosidine lactam (**23**) was decreased in *str* clones.

In transformed *ges* hairy roots the contents of vincanine (**12**) and strictosidine lactam (**23**) were not statistically changed compared to wild type clones, although the amounts of other alkaloids were decreased. Introduction of the *g8o* gene into hairy root clones decreased the production of all studied alkaloids.

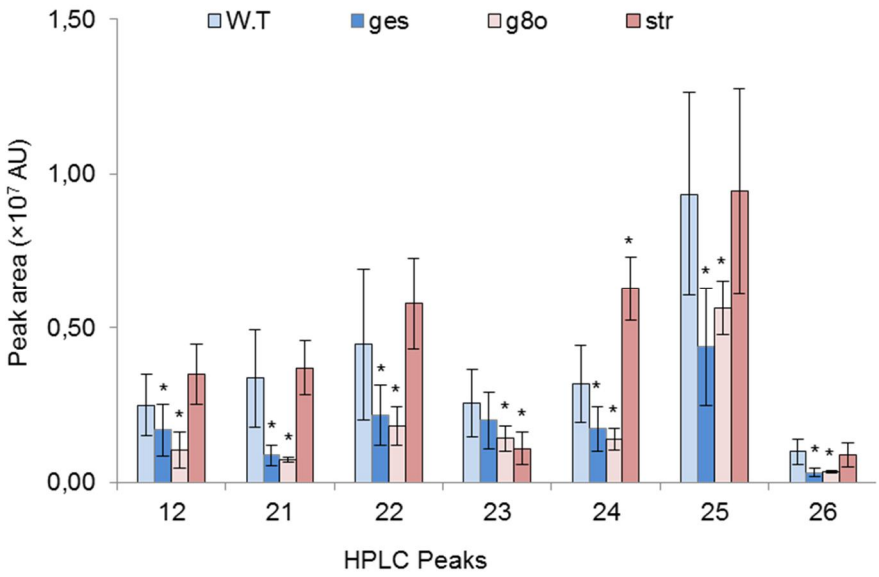


Figure 25. Comparisons of seven major alkaloids in wild type (n=20; white bar, WT), *ges* hairy roots (n=10; light grey bar), *g8o* (n=10; dark grey bar) and *str* hairy root clones (n=10; black bar) of *R. stricta*. Bars represent the mean values ± SD. * indicates significant difference in the mean between wild type hairy roots and different transformed hairy root clones (p<0.05 by ANOVA). Numbers refer to compounds from **Table 13**. au: HPLC peak area.

5.2.6 Monitoring of metabolite changes in hairy roots by NMR

Monitoring of metabolite changes in hairy roots by NMR: D_2O/CD_3OD extracts of transformed and elicited hairy roots were subjected to ^{1}H-NMR analysis. The ^{1}H-NMR spectra were divided into three different regions according to their chemical shift (δ): the aromatic region (δ 6.00- δ 10.00 ppm), the sugar region (δ 3.00- δ 6.00 ppm) and the organic and amino acid region (δ 0.00- δ 3.00 ppm).
Identification of primary metabolites: The high signal intensities in the amino acid region (δ 3.00- δ 0.00) were useful to identify a number of amino and organic acid signals. The ^{1}H-NMR signals of all the common metabolites such as amino/organic acids and carbohydrates were assigned with the use of a ^{1}H-NMR library (in house-library at Leiden University, Natural product group) containing over 500 metabolites.

In the organic and amino acid region, the signals observed included aspartic acid (δ 2.82), citric acid (δ 2.71), malic acid (δ 2.69), succinic acid (δ 2.45), glutamic acid (δ 2.35), proline (δ 2.01), acetic acid (δ 1.93), γ-amino butyric acid (GABA), alanine (δ 1.48), threonine (δ 1.33), valine (δ1.01) and leucine or isoleucine (δ 0.99). Other typical signals were also present in this region, such as sucrose (δ 5.42), α -glucose (δ 5.20), β-glucose (δ 4.59) and fructose (δ 4.08), which were identified in the sugar region.
Differences between *R. stricta* hairy roots: In order to underline the changes in the metabolic profiles between *R. stricta* transformed hairy root clones, first option principal component analysis (PCA) was applied to the bucket data of ^{1}H-NMR. The chemical shifts were used as variables and the samples served as observation data in the data matrix. The data were scaled to the Pareto scaling method. However, this method failed to make a separation. Thus, partial least squares-discriminant analysis (PLS-DA) was applied. In PLS-DA, in addition to the X-matrix of NMR bucket data, five classes were mean-centred and scaled to the unit variance (UV) method. Nevertheless, PLS-DA also did not give clear separation among the transformed clones.

In order to obtain distinguishable and better separation, orthogonal projection to latent structures-discriminant (OPLS-DA) model was applied to the same data (**Figure 26 A**). This model was validated by CV-ANOVA with *p* values of 8.82×10^{-16}. The best separation was achieved with components 1 and 3. Component 1 was found to be responsible for the separation of control hairy roots, wild type and GUS clones from hairy roots which were transformed with early key genes from the TIA pathway. Wild type roots are clustered on the positive side of PC 1, whereas GUS and *ges* clones are on the negative side of component 1 and also have negative component 3 scores. On the other hand, *str* and *g8o* clones were clustered very closely and were located on the negative side of component 3, with positive component 1 scores. As is showed in **Figure 26A**, the transformed roots were located between the wild type and GUS clones and could be separated by component 3. Variable importance in the projection (VIP) plot (**Figure 26B**) was

used to find signals (metabolites) which contributed to the separation of the different groups. Among them the first 20 signals with higher impact on discrimination of groups are shown.

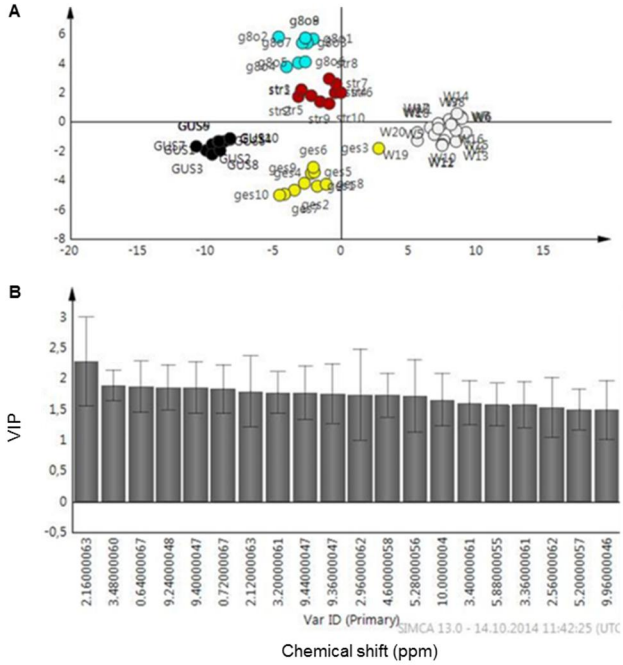


Figure 26. Score plot OPLS-DA (**A**) based on ^{1}H-NMR spectral data from different *R. stricta* transformed hairy root clones and its VIP plot (**B**) sorted based on the largest values responsible for separations of five groups. W: wild type clones (n=20, white circules); GUS: GUS clones (n=10, black circule); *ges* (n=10, yellow circles); *g80* (n=9) and *str* (n=10, red circules) clones. VIP was calculated as the weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate.

Subsequently, multivariate data analysis to discriminate metabolite differences between controls (wild type/GUS clones) and each individual group of transformed hairy roots (*ges*, *g8o* and *str* clones) were performed. The GUS clones were chosen as controls to investigate whether transformation of hairy roots as such has an influence on the metabolite changes of roots.

Control *vs. str* clones

Applying PCA and PLS-DA did not lead to distinguishable separation between s*tr* and wild type and GUS hairy root clones, and therefore OPLS-DA validated by CV-ANOVA with *p* values of 1.91×10^{-16} and 1.49×10^{-10} for *str*/wild type and

str/GUS pairs, respectively, was used. A score plot of OPLS-DA showed clear distinction between different clone groups. Wild type and *str* clones were well separated by component 1. *str* clones were grouped on the positive side of component 1, having zero component 2 scores, whereas wild type samples clustered in the negative side of component 1 and had positive or negative component 2 scores (**Figure 27A**).

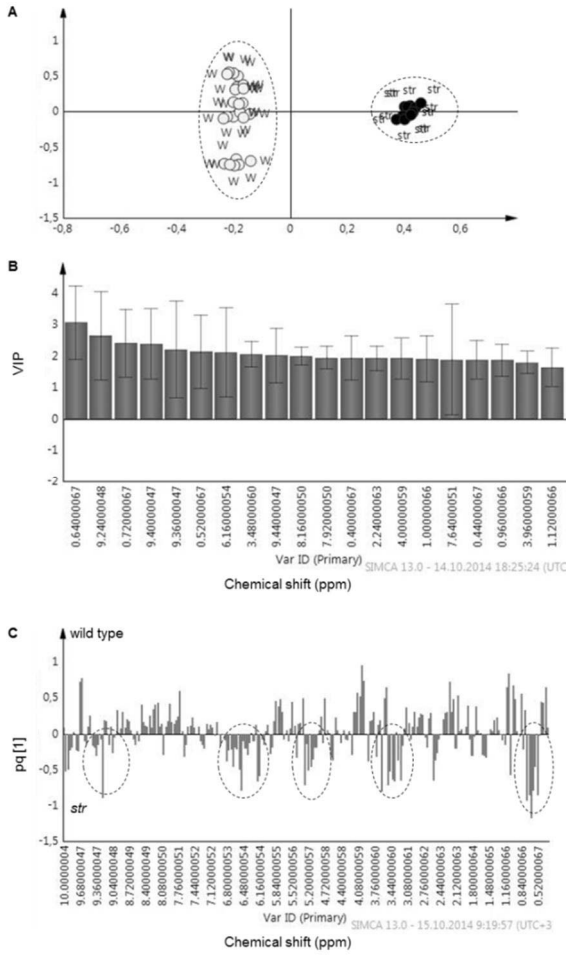


Figure 27. Pairwise multivariate data analysis of ^{1}H-NMR data from *R. stricta* wild type (W, n=20) and from hairy roots transformed with the *str* gene (n=10). The Score plots of OPLS-DA (**A**); VIP plots that show characteristic signals which are more influential for separation of two pairs (**B**) and column plots (**C**) are presented. In the column plot, the area circled with a dashed line indicates that the signals have higher intensities in *str* clones. VIP was calculated as the weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate. pq: correlation of original variables with the first OPLS-DA component.

Twenty signals which participated strongly in the discrimination of wild type and *str* hairy root clones are presented in **Figure 27B** by a VIP plot. By examining the loading column plot several regions of NMR spectra corresponding to different classes of compounds were distinguished which contribute to separation of the groups. *str* samples contained higher levels of compounds having signals at 6.16-6.80 ppm and 9.04-9.36 ppm in the aromatic area of ^{1}H-NMR spectra (**Figure 27C**) compared with wild type clones. In addition, *str* clones possessed higher contents of compounds (sugars) with signals in the 5.20-5.52 ppm and 3.08-3.76 ppm areas. *str* clones can also be recognized from the elevated amount of organic/amino acid having chemical shifts in the area of 0.52 ppm-0.84 ppm.

GUS and *str* clones were separated by component 1. *str* samples were located on the positive side of component 1 and were spread along component 2 with positive or negative scores. GUS clones had mainly negative component 1 and 2 scores (**Figure 28A**). In **Figure 28B**, twenty principal signals for discrimination of GUS and *str* clones are shown using VIP plots. In the comparison of GUS and *str* clones it can be seen from the column plot (**Figure 28C**) that *str* hairy roots differ in the aromatic compound area of the spectrum. GUS and *str* groups also have different contents of organic acids/amino acids (0.52-3.08 ppm zone) and sugars (*e.g.* 4.40-5.52 ppm zone).

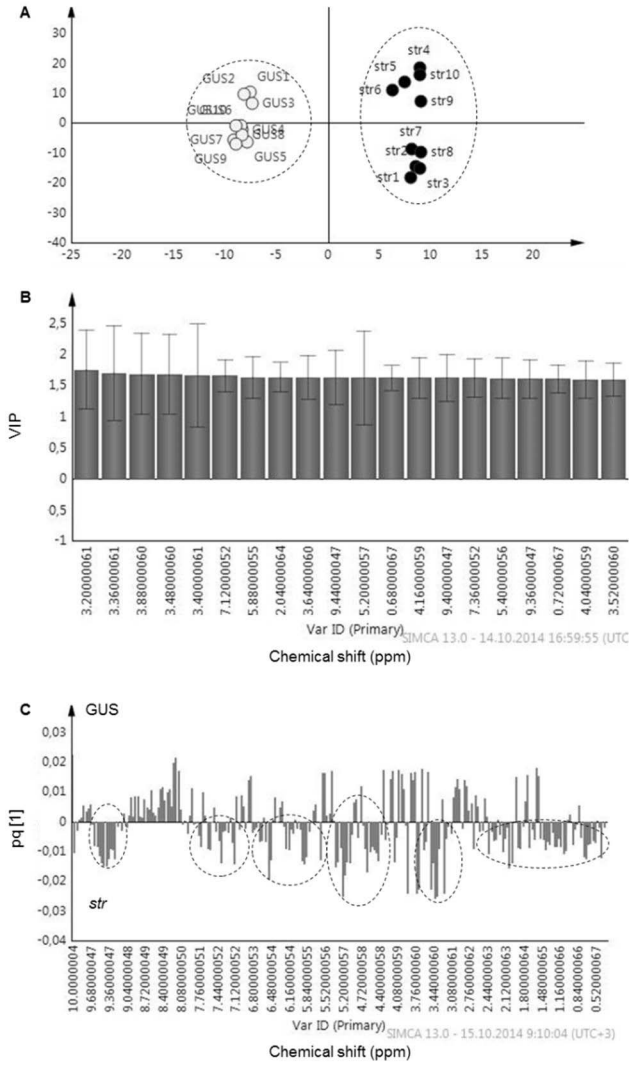


Figure 28. Pairwise multivariate data analysis measured by ^{1}H-NMR data from *R. stricta* GUS clones (n=10) and from hairy roots transformed with the *str* gene (n=10). The Score plots of OPLS-DA (**A**); VIP plots that show characteristic signals which are more influential for separation of two pairs (**B**) and column plots (**C**) are presented. In the column plot, the area circled with a dashed line indicates that the signals are more prevalent in *str* clones. VIP was calculated as the weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate. pq: correlation of original variables with the first OPLS-DA component.

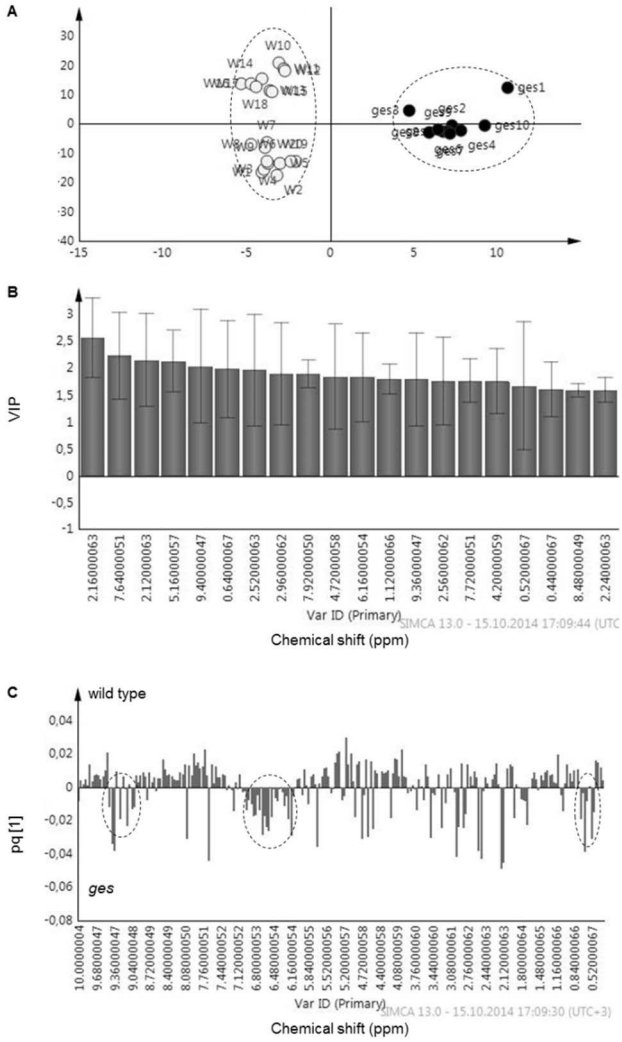
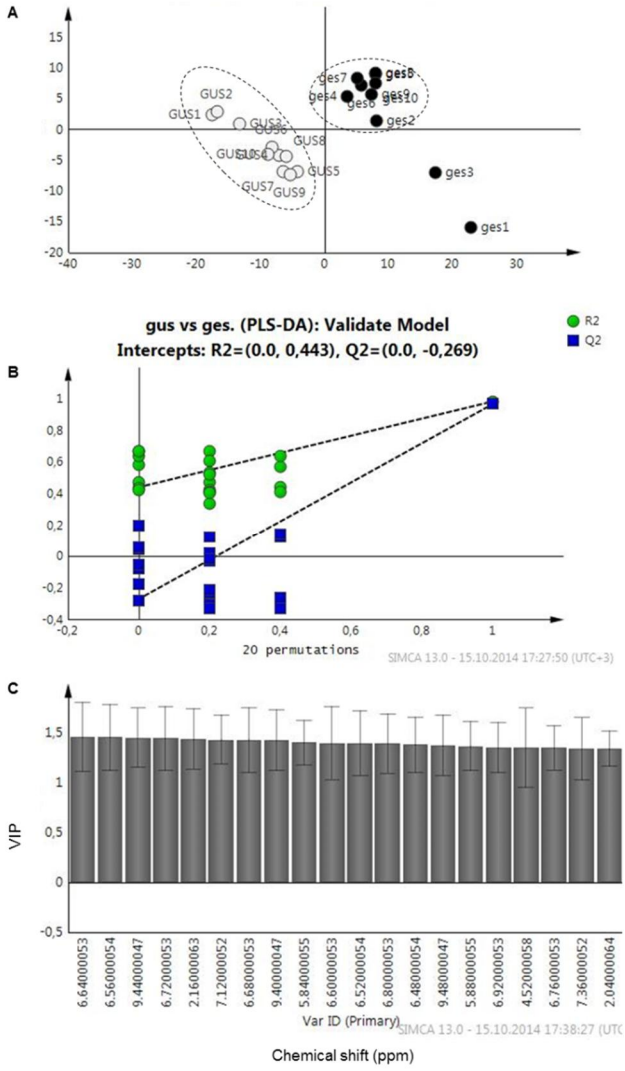


Figure 29. Score plots of OPLS-DA, VIP plots and column plots based on the ^{1}H-NMR spectra from *R. stricta* wild type hairy roots and roots transformed with the *ges* gene. OPLS-DA shows components which were responsible for separation of *R. stricta* wild type hairy roots (W, n=20) and *ges* clones (n=10) (**A**). VIP plots show 20 signals that have higher impact on differentiation of wild type and *ges* clones (**B**); using the chemical shifts, the loading column plot shows signal differences between wild type and *ges* clones (**C**), the area circled with a dashed line designates the signals which are in higher *ges* clones. VIP was calculated as the weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate. pq: correlation of original variables with the first OPLS-DA component.


A
GUS2
GUS1
GUS3
GUS6
GUS8
GUS4
GUS5
GUS7
GUS9
ges7
ges8
ges4
ges9
ges6
ges10
ges2
ges3
ges1
gus vs ges. (PLS-DA): Validate Model
Intercepts: R2=(0.0, 0,443), Q2=(0.0, -0,269)
R2
Q2
B
20 permutations
SIMCA 13.0 - 15.10.2014 17:27:50 (UTC+3)
C
VIP
6.64000053
6.56000054
9.44000047
6.72000053
2.16000063
7.12000052
6.68000053
9.40000047
5.84000055
6.60000053
6.52000054
6.80000053
6.48000054
9.48000047
5.88000055
6.92000053
4.52000058
6.76000053
7.36000052
2.04000064
Var ID (Primary)
SIMCA 13.0 - 15.10.2014 17:38:27 (UTC
Chemical shift (ppm)

Continues→

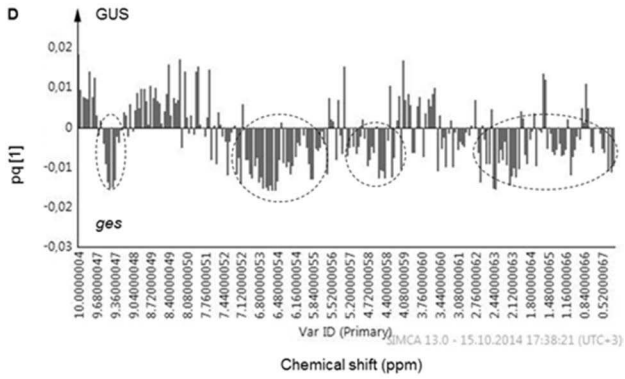


Figure 30. Multivariate data analysis based on the ^{1}H-NMR spectra of *R. stricta* GUS hairy roots as controls (n=10) and transformed roots with the *ges* gene (n=10). Score plots of PLS-DA (**A**); the permutation test for PLS-DA (**B**); VIP plots (**C**) and column plots (**D**). VIP is a weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate.

Control *vs. g8o* clones

The ^{1}H-NMR data from controls and *g8o* clones were also subjected to pairwise analyses; first PCA and then PLS-DA were applied. The two models produced partial separation with overlapping. However, OPLS-DA was applied and validated by CV-ANOVA with a p value of 5.31×10^{-15} for type/*ges* hairy roots and 2.19×10^{-9} for GUS/*ges* pairs. OPLS-DA gave better separation for the wild type. As is shown in **Figure 31A**, wild type and *g8o* samples were separated by component 1. Wild type hairy root clones clustered on the negative side of component 1, with positive and negative component 2 scores. Transformed *g8o* clones, which were tightly clustered, had positive component 1 scores whereas their component 2 scores were close to zero (**Figure 31A**). The other control set, GUS clones, was also separated from the *g8o* group by component 1, although the two groups were spread along component 2. GUS and *g8o* clones were located on the positive and negative side of component 1, respectively, and both had positive and negative component 2 scores (**Figure 32A**). **Figure 31B**, **32B** present VIP plots with twenty characteristic and the most dominant signals for discrimination of type/*ges* hairy roots and GUS/*ges*, respectively. It is evident from **Figure 31C** that compared with wild types, *g8o* hairy root clones accumulate higher amounts of aromatic compounds which have ^{1}H-NMR spectral signals in 7.72 -9.36 ppm and 6.48 ppm-6.80 ppm, sugars with 3.26 -3.60 ppm and organic acids /amino acids having 2.32 ppm-2.96 ppm and 0.40 ppm-0.72 ppm. It was also revealed that *g8o* lines have significant levels of organic acids /amino acids (0.4-2.64 ppm) in comparison with GUS samples (**Figure 32C**). Furthermore, *g8o* clones have higher contents of aromatic compounds, with signals in the 6.16-6.80 and 9.04-9.68 ppm areas. In the areas 4.24-4.56 ppm and 3.28-3.60 ppm, they also differ from GUS samples.

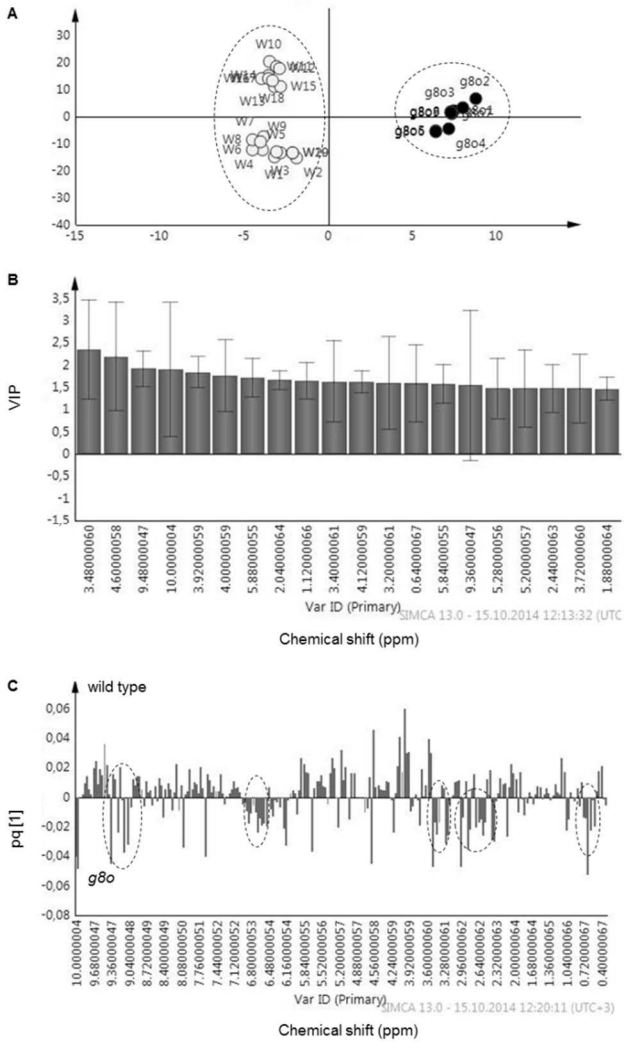


Figure 31. Pairwise multivariate data analysis measured by ^{1}H-NMR data from *R. stricta* wild type (W, n=20) and from hairy roots transformed with the *g8o* gene (n=9). The Score plots of OPLS-DA (**A**); their VIP plots that show characteristic signals which are more influential for separation of two pairs (**B**) and column plots (**C**) are presented. In the column plot, the area circled with a dashed line indicates that the signals are more prevalent in *g8o* clones. VIP is a weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate.

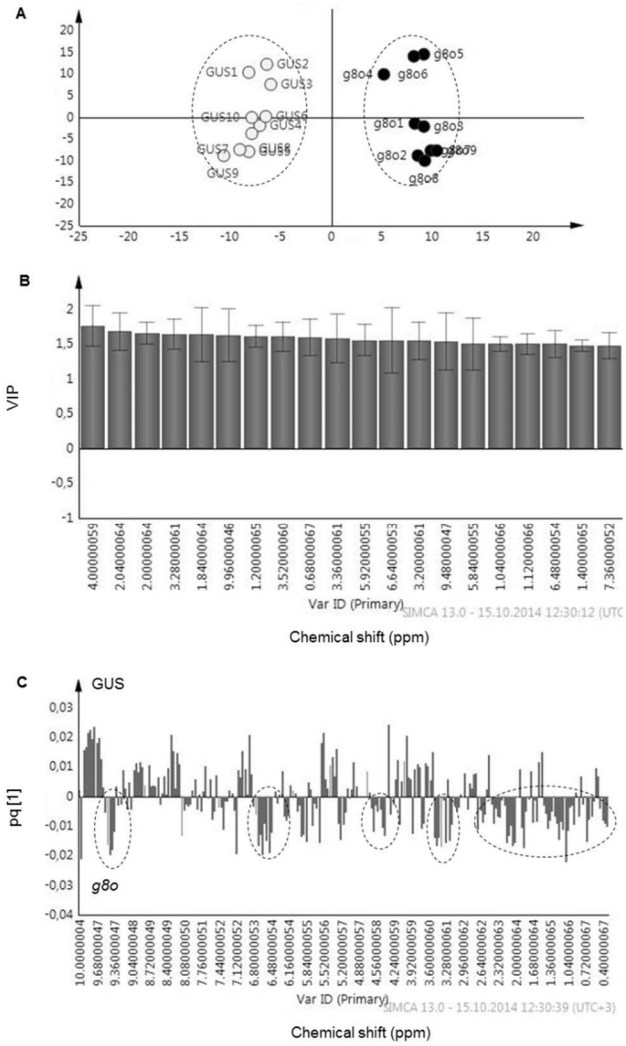


Figure 32. Multivariate data analyses based on the ^{1}H-NMR data from *R. stricta* GUS (n=10) and from hairy roots transformed with the *g8o* gene (n=9). The score plots of OPLS-DA (**A**); their VIP plots that show characteristic signals which are more influential for separation of two pairs (**B**) and column plots (**C**) are presented. In the column plot, the area circled with a dashed line indicates that the signals are more prevalent in *g8o* clones. VIP is a weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate.

Wild type *vs.* GUS hairy roots

Multivariate data analysis based on the ^{1}H-NMR data of wild type and GUS hairy roots was also performed to examine the effect of introduction of a heterologous gene, which does not have any role in TIA biosynthesis, on metabolite alteration. PCA gave a weak separation whereas PLS-DA well separated the GUS lines in a cluster but wild type samples were not clearly grouped and were overlapped by the GUS cluster. Therefore, the OPLS-DA was applied to two separate sets. The OPLS-DA score plot was validated ($p=4.63\times10^{-15}$) and showed two clearly separated groups (**Figure 33A**). It is evident that GUS samples were clustered on the positive side of component 1 whereas their component 2 was close to zero. In addition, wild type hairy roots were assembled on the negative side of component 1 but were almost equally distributed on both the positive and negative sides of component 2 (**Figure 33A**). In the VIP plot twenty signals (chemical shifts) with significant influence for sample separation are shown (**Figure 33B**). It was found that wild type hairy roots have higher amounts of aromatic compounds with a chemical shift between 9.36-9.68 ppm and 7.12-8.08 ppm, whereas GUS clones had higher levels of compounds with chemical shifts between 6.48 and 6.80 ppm (**Figure 33C**). Wild type clones contain higher amounts of sugars (3.92-5.84 ppm zone) and organic/amino acids (1.04-2.32 ppm zone) than GUS hairy roots. However, GUS clones had elevated contents of amino acids with chemical shifts around 0.40-0.72.

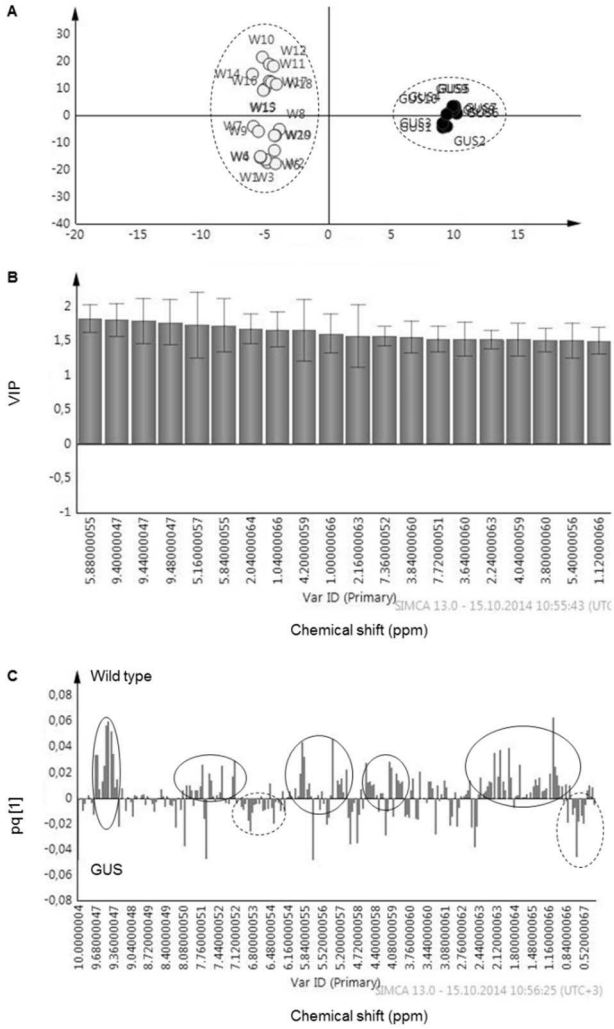


Figure 33. Score plots of OPLS-DA (A), VIP (B) and a loading column plot (**C**) from ^{1}H-NMR spectra data of wild type and GUS hairy roots are presented. Two groups were segregated clearly by component 1 of OPLS-DA (**A**); and twenty highly influential factors discriminating the two groups are shown (**B**); the column plot (**C**) shows a higher level of a different class of compounds in two groups. In the column plot, the areas circled with dashed and continuous lines designate the signals which were higher in GUS and wild type clones, respectively. VIP was calculated as a weighted sum of squares of the OPLS weights. The error bar shows the standard error in the VIP estimate. pq: correlation of original variables with the first OPLS-DA component.

Differences between *R. stricta* elicited and control hairy roots

In order to analyse the changes in metabolic profiles between MeJA-elicited and control wild type hairy roots of *R. stricta*, PCA was applied to the bucket data of ^{1}H-NMR spectra. As shown in **Figure 34A**, PCA clearly distinguished between hairy roots elicited with 50 and 100 µM MeJA for five days and control samples. Elicited samples were well separated from controls by PC 1 (85%). Controls had positive PC 1 as well as positive PC 2 (11%) scores (with one exception, C3). Elicited samples with 50 and 100 MeJA both had the negative PC 1 scores but grouped on the negative and positive PC 23 scores, respectively (**Figure 34A**). It is clear from the column plot that elicited samples had higher amounts of aromatic compounds (in the 6.16-8.40 ppm zone) as well as organic/amino acids (in the 0.40-2.32 ppm zone). The contents of sugars also varied between elicited and non-elicited samples (**Figure 34B**).

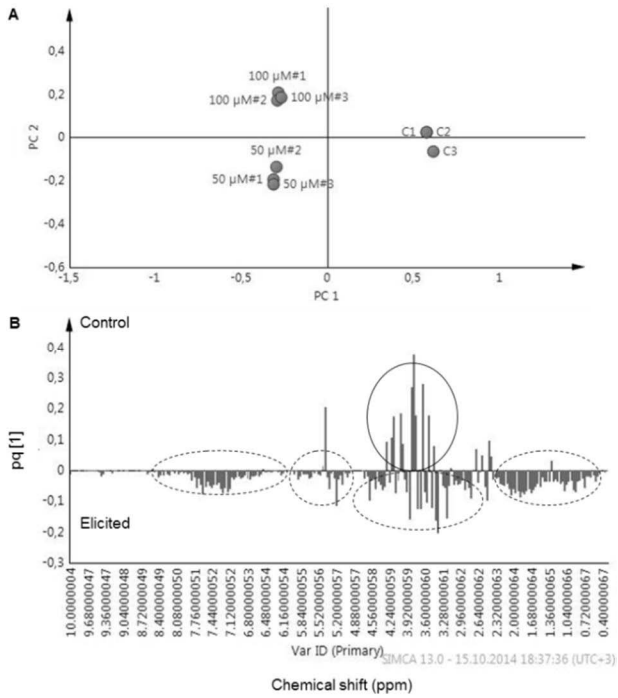


Figure 34. PCA score plots based on ^{1}H-NMR data to compare *R. stricta* hairy roots exposed to 50 (n=3) and 100 (n=3) µM MeJA with the control (n=3) samples (**A**) and loading column plot (**B**); elicited samples were effectively separated by PC 1 (**A**); the column plot shows higher levels of aromatics, sugars and organic/amino acids (B). In the column plot, the area circled with dashed and continuous lines specifies the predominant signals in elicited and control hairy root cultures, respectively. pq: correlation of original variables with the first OPLS-DA component.

Total alkaloid contents in *R. stricta* hairy roots

The sum of values of spectral intensity from ^{1}H-NMR bucket data in the region δ 0.49- δ 0.54 (ppm) was used for quantification of total alkaloids (personal communications with Dr Y.H. Choi, Institute of Biology, Natural Products Laboratory, Leiden University, The Netherlands) in elicited wild type hairy roots and in different transformed hairy root clones. It is clear that elicited samples have approximately sixfold higher total alkaloid contents than non-elicited cultures, whereas there was no significant variance between hairy roots elicited with two different concentrations of MeJA (**Figure 35**). However, it was demonstrated that there were no significant differences between hairy roots transformed with *str*, *ges* and *g8o* genes compared with wild type clones. In addition, it was shown that the *str* and *ges* hairy root clones had higher contents of alkaloids than the GUS control lines (**Figure 36**).

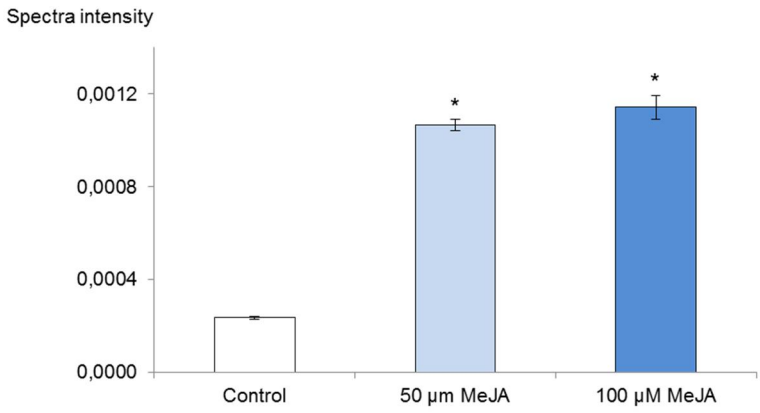


Figure 35. Comparison of total alkaloid based on ^{1}H-NMR data in hairy roots elicited with 50 µM and 100 µM MeJA after five days and non-elicited control cultures (80 µL 40% ethanol, equal volume of 100 µM MeJA) of *R. stricta*. Values presented as mean±SD of three samples for each treatment. * indicates significant difference in the mean between control and elicited samples (student's t test, $p<0.05$).

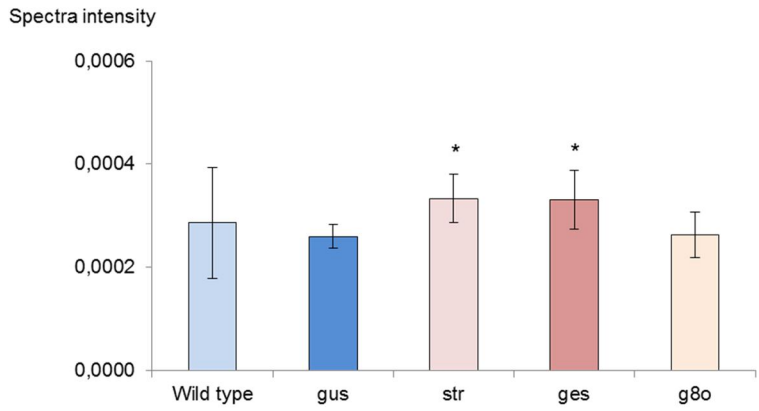


Figure 36. Comparison of total alkaloids based on ^{1}H-NMR data in *R. stricta* transformed clones; *str* (n=10), *ges* (n=10) and *g8o* (n=9) with controls clones wild type (n=20) and GUS (n=10). Values presented as mean±SD. * indicates significant difference in the mean between GUS and transformed clones (p<0.05 by ANOVA). There was not statistically significant difference between wild type control and transformed hairy root clones.

6. Disussion

6.1 Development of *in vitro* cultures

Plant cell and tissue cultures are an alternative to intact plants for the production of valuable secondary metabolites, and the production of novel compounds normally not found in the parent plants has also been reported. As an example, akuammicine is an alkaloid of the *R. stricta* cell culture which has not hitherto been found in the intact plants (Pawelka and Stöckigt 1986). It has also been shown that hairy roots of *R. serpentina* do not produce the major indole alkaloids of the plant, *e.g.* ajmaline, ajmalicine and serpentine (Falkenhagen *et al.* 1993). There are a number of plant cell cultures which have been commercialized for production of secondary metabolites, *e.g.* paclitaxel (Tabata 2006). However, there are still problems in the production of metabolites by cell cultures because of instability of cell lines, low yields, slow growth and scale-up problems (Whitmer *et al.* 2003). Strategies such as screening and selection of highly productive cell lines, manipulation of nutrients, optimizing the culture environment, use of hormones, precursor feeding and elicitation have been shown to increase secondary metabolite production in plant cell cultures. In the current study, two *in vitro* cultures of *R. stricta*, namely undifferentiated calli and differentiated hairy root cultures, were established and their alkaloid profile and the production of major terpenoid indole alkaloids were investigated and compared in the two systems.

Calli were successfully initiated from five explants of *R. stricta* on solidified media with the same hormone treatment (BAP 1 mg/L and 0.75 mg/L NAA). Earlier, Pawelka and Stöckigt (1986) used four different hormones to initiate undifferentiated calli and Omar (1988) reported the initiation of calli from different explants using only auxins. He showed that 3 mg/L IAA was optimal for root-derived calli and higher concentrations of NAA, 10 and 30 mg/L, were needed for callus initiation from leaves and stems, respectively. On the other hand leaves and cotyledons were able to produce calli only in the presence of 2,4-D at a concentration of 0.1-10 mg/L. In the present study, the same concentrations of applied BAP and NAA resulted in different textures and colours in the calli derived from different explants. By contrast, Omar (1988) indicated that low and high concentrations of auxins resulted in friable and firm calli texture, respectively. He also reported that root calli had higher amounts of alkaloids than leaf and stem

calli. Furthermore, a hybrid cell line of *R. serpentina* and *R. stricta* has been shown to have an enhanced alkaloid metabolism resulting in a greater diversity of alkaloids than in the parental species (Kostenyuk *et al.* 1995, Aimi *et al.* 1996, Sheludko *et al.* 1999, 2000).

Induction of transgenic hairy roots by *A. rhizogenes*, a natural plant genetic engineer, is an effective, low cost and simple method to achieve stable transgene expression in plants. Transgenic hairy roots have become a core research tool for the production of secondary metabolites and engineering of metabolic pathways, and are used as a model for functional genomics analysis. Furthermore, hairy roots have the capacity of regenerating transformed plants (Chandra 2012). Expression of reporter genes such as the gusA gene has been used widely in order to follow the development of transformation in plants (Boyko *et al.* 2006). Hairy root cultures have been established from important medicinal plants in the Apocynaceae family from *e.g. C. roseus* (Toivonen *et al.* 1989, Bhadra *et al.* 1993), *R. serpentina* (Falkenhagen *et al.* 1993, Benjamin *et al.* 1993) and *Rauvolfia micrantha* Hook.f. (Sudha *et al.* 2003). In the present study an easy and convenient method for establishing transgenic hairy roots of *R. stricta* through infection with wild type and transformed *A. rhizogenes* LBA 9402 was developed for the first time.

It is known that the virulence of *Agrobacterium* strains varies for different plant hosts and explants (Chaudhuri *et al.* 2005). In this study *A. rhizogenes* LBA 9402 was shown to be suitable for induction of hairy roots in *R. stricta*. This strain has previously been used for inducing hairy roots in many other species, *e.g. Hyoscyamus* spp. (Vanhala *et al.* 1995), *P. somniferum* Album. (Le Flem-Bonhomme *et al.* 2004) and *Picrorhiza kurroa* Royle ex Benth.(Verma *et al.* 2007). However, there are also reports, *e.g.* from *Gentiana macrophylla* Pall. (Tiwari *et al.* 2007) and *Tylophora indica* (Burm. f.) Merr. (Chaudhuri *et al.* 2005), according to which *A. rhizogenes* LBA 9402 failed to induce roots from any explants.

Four explant types including cotyledons, hypocotyls, leaves (including petiole) and stem segments from two-month-old axenic *in vitro* germinated seedlings were used for inoculation with bacteria, but only mature leaves initiated putative hairy roots. In the early literature, it was already reported that appropriate explant selection affects the transformation efficiency and subsequently the induction of hairy roots (Tepfer 1984). In this study, hairy roots emerged from initiated callus on deeply wounded sites of the midrib or on the leaf petioles. Scratching instead of deep wounding did not initiate callus for root formation, although all wounded sites turned dark within two weeks. Even though callus formation was initiated on the cut edges of leaf apices, hairy roots were never generated from this part. The use of deep wounding and leaf explants has been shown to affect hairy root formation *e.g.* in *P. kurroa* (Verma *et al.* 2007). Phloem cells, rich in sucrose and indole-3-acetic acid, are located inside the midrib. According to Nilsson and Olsson (1997), *rolB* and *rolC* genes are regulated by high levels of auxin and sucrose, respectively, and only cells with elevated levels of auxin and sucrose are able to act as root meristem initials and are also ideal targets for *A. rhizogenes* infection. This hypothesis explains why deep wounding is often necessary for successful

hairy root initiation. The results of the present study in hairy root establishment agreed well with the hypothesis. Similar results have also been obtained for hairy root induction in *Pinus halepensis* Mill. (Tzfira *et al.* 1996), *Aesculus hippocastanum* L. (Zdravković-Korać *et al.* 2004) and *G. macrophylla* (Tiwari *et al.* 2007), for example. However, Chaudhuri *et al.* (2005) reported that nodes with intercalary meristem and internodes with a lateral meristem showed high transformation rates in *T. indica,* which is in contrast to the hairy root induction in *R. stricta*.

Excised non-transformed roots of *R. stricta* seedlings grew very slowly, and therefore intact plant roots could not be used for the evaluation of hygromycin resistance as a transformed root selection agent. Hence, in this study wild type hairy roots were established first, and the final concentration of hygromycin for transformed root selection was determined to be 2 mg/L. Transformed GUS hairy roots grew on media supplemented with 2 to 25 mg/L hygromycin, although their growth was slower than that of non-treated control cultures. Hygromycin selection was very effective, since all surviving *R. stricta* GUS hairy root clones on media supplemented with hygromycin exhibited blue colour with various intensities in the GUS staining assay. It has been shown that *C. roseus* (Wang *et al.* 2010) and soybean (Li *et al.* 2010) hairy roots, which integrated the hygromycin gene as a plant selective marker into their genome from T-DNA of the *Agrobacterium* Ri plasmid, were efficiently selected with hygromycin at concentrations of 10 and 20 mg/L, respectively.

The transformation efficiency of *R. stricta* was 74% and 83% for wild type and GUS clones, respectively. This can be considered to be excellent, as transformation efficiencies of different *A. rhizogenes* strains inducing hairy roots in *R. serpentina* leaf explants were reported to range from 11 to 33%, with 26% for the LBA 9402 strain (Mehrotra *et al.* 2014). Only leaves were able to generate hairy roots after *A. rhizogenes* LBA 9402 infection in three to four weeks in *Scutellaria baicalensis* Georgi when leaf, cotyledon, and cotyledonary petioles were compared. Moreover, the transformation efficiency with LBA 9402 was very low (7.4%) compared to the other tested strains (Tiwari *et al.* 2008). Similarly, the transformation efficiency with LBA 9402 in *Saussurea involucrata* (Karel. & Kir.) was low when leaves were used as explants (Fu *et al.* 2005).

After development of a transformation method for *R. stricta*, selected key heterologous genes from the early terpenoid biosynthesis pathway, *ges*, *g8o* and *str* were introduced into plants and hairy roots generated by inoculation of the plant leaves. The procedures of root induction were the same as in wild type hairy roots but the transformation efficiencies ranged from 22 to 32%.

Broad phenotypic variation was observed among *R. stricta* hairy root clones, wild type and GUS clones (as controls) and transformed clones with heterologous genes. In particular, highly branched clones also exhibited rapid growth. The present results agree with the literature, in which root biomass and growth in the transgenic roots has been reported to be much higher than in non-transformed roots. This could be attributed among other factors to the endogenous auxin synthesis in the hairy roots (Benjamin *et al.* 1993, Nilsson and Olsson 1997).

6.2 Identification of the alkaloids

Generally, all the alkaloids occur in multicomponent mixtures and the separation of alkaloids from other groups of natural products is the first requirement for comprehensive and detailed qualitative and quantitative analyses of single alkaloids. Acidic inorganic extractive solvents are commonly used, since they are known to improve the stability and solubility of the alkaloids (Hisiger and Jolicoeur 2007). Therefore, this type of alkaloid-specific extraction utilizing diluted sulphuric acid was also applied in the study.

Due to the complexity and diversity of plant metabolites, it is unlikely that one single analytical method could provide information about all the metabolites present in a plant. NMR spectroscopy combined with multivariate analysis techniques, such as principal component analysis (PCA) or orthogonal partial least square discriminant analysis (OPLS-DA), was applied for distinguishing and classifying of sample sets, *e.g.* control and transformed/elicited samples in terms of the different metabolite level variation. It is known that metabolic pathway fluxes (primary and secondary metabolites) change as a result of genetic transformation (Manetti *et al.* 2004, Choi *et al.* 2004b) and elicitation (Liang *et al.* 2006, Flores-Sanchez *et al.* 2009, Mustafa *et al.* 2009).

Chromatographic methods were also applied for qualitative and quantitative analyses of the plant cell and hairy root materials. For the sample preparation, alkalinization with diluted ammonia and subsequent extraction with organic solvent was used. The selection of appropriate solvent systems in HPLC must be taken into account (Stöckigt *et al.* 2002, Tikhomiroff and Jolicoeur 2002). Although it has been shown that the use of phosphate buffer and hexane sulphonic acid as an ion exchange reagent results in clearly improved separation of alkaloids (Gerasimenko *et al.* 2001b), the use of phosphate buffer and reagent is not compatible with LC-MS analysis.

The HPLC analyses of alkaloids was performed according to the method of Gerasimenko *et al.* (2001b). The HPLC method included slight differences in gradient steps and showed repeatable and reliable intra-day repeatability below 3% and inter-day repeatability below 5% for seven *Rhazya* alkaloids extracted from the hairy roots. In ultra-performance liquid chromatography (UPLC), it is typical that total running times are shorter. In addition, the use of columns with 1.7 μm particle size results in sharp peaks (Han *et al.* 2008). Recent UPLC analysis of quaternary ammonium alkaloids, in which a non-volatile ion pair reagent was not used, showed that detailed optimization of pH is necessary. The results have shown considerable variation in retention times of alkaloids when using mobile phase with a wide pH range of 3-10, and complete separation was obtained at pH 10 (Qiu *et al.* 2012). In order to achieve optimal UPLC conditions for the separation of alkaloids from crude extracts of *Rhazya* hairy roots, the solvent

systems and run conditions were examined. The method was originally adapted from Rischer *et al.* (2006), who analysed *C. roseus* cell suspensions. Two isocratic conditions, consisting of ammonium acetate (A: either 45 or 35) at pH 10 and acetonitrile (B), did not result in good separation due to rapid elution and consequent peak overlapping. Similarly in the gradient run, when solvent A contained 0.1 formic acid with a gradient change from 65% to 50%, alkaloids were retained rapidly and co-eluted. When this same gradient was tested using an isocratic solvent system, the separation appeared to be satisfactory and the major alkaloids were separated within 22 min. By using these conditions the results of repeated injections indicated that good peak shape and sensitivity of various alkaloids could be obtained. These conditions showing improved separation combined with alkaline pH and the use of a BEH C18 column agree well with the analysis of yohimbe bark alkaloids by Sun *et al.* (2011), who reported that this column provided better separation compared to other tested columns. In addition, similar column performance has been reported in the UPLC analyses of alkaloids from *Lindera aggregate* Kosterm. (Han *et al.* 2008), *C. roseus* (Runguphan *et al.* 2009) and *Coptidis* spp. (Qiu *et al.* 2012).

In the present study, GC-MS was applied for thermostable, low molecular weight and non-polar compounds, thus enabling identification and quantification of alkaloids. The alkaloids in **Table 11** comprised nine groups including aspidosperma (also referred to as aspidospermine)-, eburnamine-, aspidospermatin-, pleiocarpaman-, strychnos-, sarpagine-, heteroyohimbine-, yohimbinoid- and hunterburine-type alkaloids. Despite abundant literature on *R. stricta* alkaloids, there are no reports of comprehensive GC-MS data concerning the identification of alkaloids from crude extracts. Therefore, a GC-MS method was developed and validated for the separation, identification and quantitation of the alkaloids in *R. stricta* hairy roots. A GC-MS method has previously been applied for the analysis of major alkaloids in the Apocynaceae family, for example for identification of vindoline and ajmalicine in *C. roseus* cell cultures (Ylinen *et al.* 1990) and hairy root cultures (Bhadra *et al.* 1993). In GC-MS analyses of *R. verticillata* (Lour.) Baill. roots, 12 indole alkaloids were identified among the 39 volatile compounds (Hong *et al.* 2013).

Hitherto, more than 100 alkaloids with wide ranges of molecular weight and polarity have been found in *R. stricta* plants. In this study, hairy root cultures of *R. stricta* were established for the first time and their alkaloid content was analysed in detail both qualitatively and quantitatively with spectroscopic and chromatographic methods. In the following sections these results are discussed more comprehensively.

6.2.1 Novel alkaloids in *R. stricta* hairy roots

Hairy roots are known to produce a spectrum of secondary metabolites that are not present in the parent plant (Oksman-Caldentey and Inzé 2004). In this study we were able to identify for the first time ten new compounds in *R. stricta*, including pleiocarpamine (**11**), fluorocarpamine (**13**), vincamine (**14**), ajmalicine

(**17**), yohimbine isomers (**18**,**19**), serpentine isomers (**24**,**28**), tetrahydrosecodinol (30) and tabersonine (**33**). They have previously been reported in various other species within the Apocynaceae family (Buckingham *et al.* 2010).

Pleiocarpamine (**11**) and fluorocarpamine (**13**) belong to the pleiocarpaman-type alkaloids. Spectral data of (**11**) and (**13**) from GC-MS analyses of *Rhazya* crude extract were in accordance with those reported from isolated pure alkaloids from *C. roseus* (Atta-ur-Rahman and Bashir 1983).

Vincamine (**14**), a member of the eburnamine-type alkaloids, was identified by GC-MS and UPLC-MS, having the same retention time, fragmentation pattern, UV and ES^+-MS as the pure reference compound. Furthermore, these data were also in line with those reported in the literature (Budzikiewicz *et al.* 1964, Kováčik and Kompiš 1969, Shakirov *et al.* 1996). However, vincamine has not been reported in *Rhazya* intact plants or cell cultures.

Ajmalicine and yohimbine isomers have previously been isolated from *R. serpentina* plants (Stöckigt *et al.* 1981) and *R. serpentina* × *R. stricta* hybrid cell suspension cultures (Sheludko *et al.* 1999), whereas they have not been identified in *Rhazya stricta*. Heteroyohimbine-type alkaloid ajmalicine (**17**) and yohimbinoid alkaloids **18** and **19** (yohimbine isomers) possess a tetrahydro-β-carboline moiety and have a pronounced diagnostic peak m/z [M-1]. In the GC-MS study, the corresponding spectral data for ajmalicine (**17**) and yohimbine isomers (**18**,**19**) were supported by the fragmentation patterns from pure compounds and from the literature (Budzikiewicz *et al.* 1964, Shakirov *et al.* 1996, Betz *et al.* 1995). Yohimbine isomers showed molecular ions $[M+H]^+$ and basepeaks at m/z 355 for isomers in UPLC-MS analyses of *Rhazya* hairy root crude extract. The three fragments at m/z 212,144 and 224 are the same as have been reported for direct cleavage of this $[M+H]^+$ of yohimbines (Sun *et al.* 2011). In addition, the UV profile and the ES^+ patterns were also fully consistent with those of the reference compound and reported from yohimbe bark (Zanolari *et al.* 2003). Using the GC-MS and UPLC-MS methods, two yohimbine isomers (**18**,**19**) with similar fragmentation patterns were separated with retention times very close to each other.

Serpentine is a quaternary alkaloid, which is typical for *Rauwolfia* (Stöckigt *et al.* 1997) and *Catharanthus* (van der Heijden *et al.* 2004) species, but it has not been reported in *Rhazya*. According to the literature, the characteristic molecular ion for this compound is $[M+H]^+$ m/z 349 (Stöckigt *et al.* 1997). In the current study, extracted ion recording in ES^+ and ES^- first located two peaks. These two serpentine monomeric isomers (**24**,**28**) exhibited characteristic UV (λ_{max} 248, 307 and 368 nm) and molecular ions $[M+H]^+$ at m/z 349 and $[M-H]^-$ at m/z 347, with corresponding basepeaks. isomer I (**24**) had a higher intensity than isomer II (28). The UV and ES^+-MS data were consistent with serpentine from *C. roseus* extracts with ESI-IT-MS reported by Chen *et al.* (2013) and with HPLC-ESI-MS/MS (Ferreres *et al.* (2010); Suttipanta *et al.* (2011)).

The occurrence of a secodine-type tetrahydrosecodinol (30) in hairy root extracts was confirmed by the UV and ES^+-MS. The peak **30** exhibited UV maxima (λ_{max} 283 and 290 nm) and $[M+H]^+$ m/z 359 with a basepeak at m/z 126, and the

fragment at m/z 341 which has been previously reported for isolated tetrahydrosecodinol from the roots of intact *R. orientalis* plants (Cordell *et al.* 1970a).

Tabersonine (**33**), an aspidosperma-type alkaloid, showed the same UV maxima, a protonated molecular ion at m/z 337 and the characteristic fragment at m/z 305, due to the loss of methanol, as in the pure reference compound. A comparison was also made with literature data (Suttipanta *et al.* 2011). However, some of the compounds have shown a very similar UV profile to that of tabersonine, for example vincadifformine (λ_{max} 228, 299 and 329 nm), akuammicine (λ_{max} 228, 298 and 329 nm and one unkown (λ_{max} 228, 308 and 331 nm). Although these alkaloids are characteristic in *R. stricta* (Pawelka and Stöckigt 1986), their exact retention times could not be located without reference compounds and therefore their identification is not definite. Tabersonine has previously been reported from *R. orientalis* (Zsadon *et al.* 1971) and *R. stricta* cell suspension cultures (Pawelka and Stöckigt 1986, Sheludko *et al.* 2000), but not from *R. stricta* intact plants.

6.2.2 Other alkaloids found in *R. stricta* hairy roots

GC-MS and UPLC-MS analysis indicated that eburenine (**2**) is the most abundant alkaloid in *R. stricta* hairy roots. It is also the most abundant among leaf alkaloids (Smith and Wahid 1963). Eburenine (**2**), aspidospermidine (**3**) quebrachamine (**5**) vincadifformine (**8**) and vincadine (**10**), all identified by GC-MS, belong to the aspidosperma-alkaloids. Among the aspidosperma-type alkaloids vincadifformine (**8**) is a biogenetically important intermediate for the (bio)synthesis of aspidospermidine- and akuammicine-type alkaloids (Biemann *et al.* 1961, Djerassi *et al.* 1962). In the present GC-MS analyses of *Rhazya* hairy root extracts, the fragmentation patterns of eburenine (**2**), aspidospermidine (**3**) and vincadine (**10**) were in line with data from the isolated alkaloids from *V. erecta* (Rakhimov *et al.* 1970), *Aspidosperma quebracho blanco* (Biemann *et al.* 1963) and *R. stricta* (Atta-ur-Rahman *et al.* 1985a), respectively. The MS data of quebrachamine (**5**) and vincadifformine (**8**) were also in line with those reported by Budzikiewicz *et al.* (1964). Eburenine (**2**) and vincadifformine (**8**), along with vincanine (**12**) and rhazine (**15**), have previously been reported from *R. stricta* and/or *R. serpentina* × *R. stricta* cell suspension cultures (Pawelka and Stöckigt 1986, Sheludko *et al.* 2000).

The spectral data for aspidospermatin-type alkaloids, tubotaiwine (**6**) and condylocarpine (**7**), were here in agreement with those reported by Atta-ur-Rahman *et al.* (1989b). Tubotaiwine is formed as a result of the catalytic hydrogenation of condylocarpine (Schumann and Schmid 1963) and it has been shown to be the same compound also reported as dihydrocondylocarpine (Schripsema *et al.* 1987). In both tubotaiwine and dihydrocondylocarpine, the carbon in position 20 has S configuration (Schripsema *et al.* 1987). Tubotaiwine has previously been detected in *R. serpentina* × *R. stricta* cell suspension cultures (Kostenyuk *et al.* 1995, Sheludko *et al.* 2000).

Vincanine (**12**) was presented as a main component together with eburenine (**2**). Vincanine displayed a highly intense UV at 365 nm and a basepeak and $[M+H]^+$ at 293 nm without any other fragments. The data well agreed with the same UV maxima and with MS obtained from HR-MS analyses of isolated vincanine from *Rhazya* (Ahmad *et al.* 1977) and from *Vinca* (Shakirov *et al.* 1996). Vincanine, also called (-)-nor-*C*-fluorocurarine, belongs to the strychnos-type alkaloids, and was already isolated in the 1960s from *V. erecta* and *Diplorhynchus condylocarpon* (Müll.Arg.) Pichon (Stauffacher 1961, Shakirov *et al.* 1996). Since then, it was discovered in *R. stricta* (Ahmad *et al.* 1977) and later on as a major alkaloid in its cell culture (Pawelka and Stöckigt 1986).

In current study, the UV and MS data of rhazine (**15**) were in full accordance with those obtained by trap time-of-flight mass spectrometric (LCMS-IT-TOF) analyses from *Alstonia scholaris* (L.) R.Br. studied by Hou *et al.* (2012) and by Feng *et al.* (2013). Furthermore, its mass fragments and their abundances were in line with those reported in the literature (Budzikiewicz *et al.* 1964, Shakirov *et al.* 1996). Rhazine (syn. akuammidine) was among the first alkaloids to be isolated from *R. stricta* (Chatterjee *et al.* 1961).

Spectral data of tetrahydroalstonine (**16**) was the same as in the pure reference compound and was in line with reported data in the literature (Budzikiewicz *et al.* 1964, Atta-ur-Rahman *et al.* 1984f). This compound has previously been isolated from *R. stricta* but has not been reported from *Rhazya* cell cultures.

In the present analysis, two isomers of vallesiachotamine (**20**,**29**) were identified from *R. stricta* hairy root extracts. The mass spectra of the isomers exhibited typical fragments, $[M+H]^+$ at m/z 351 and a fragment of tetrahydro-β-carboline moiety at m/z 170. These isomers possess high UV absorption in the 290 nm region, which is characteristic for hunterburine-type alkaloids (Djerassi *et al.* 1966). The UV and MS profiles of isomers were well in line with EI-MS or HRMS data reported from *Rhazya* (Mukhopadhyay *et al.* 1981, Atta-ur-Rahman and Malik 1986, Kostenyuk *et al.* 1995) and additionally with those of *Strychnos tricalysioides* Hutch. & M.B. Moss (Waterman and Zhong 1982) and *V. dichotoma* (Djerassi *et al.* 1966). Detailed isomeric analyses by NMR have shown that the hydrogen linked to C19 is in the *trans* position in vallesiachotamine but *cis* in isovallesiachotamine (Waterman and Zhong 1982).

Although in GC-MS analysis compound **20** was identified as vallesiachotamine on the basis of its fragmentation, further derivatization analysis suggested that the corresponding peak (**20**) was a mixture of two co-eluting isomers, vallesia- and isovallesiachotamine. Major mass fragments and their abundances for vallesia- (Mukhopadhyay *et al.* 1981, Kostenyuk *et al.* 1995) and isovallesiachotamine (Atta-ur-Rahman and Malik 1984e) are the same. The fragments m/z 279, 263, 221 and 350 $[M^+]$ are indicative for the two isomers but fragment ion m/z 307, which is notable in the mass spectrum of vallesiachotamine, does not exist in isovallesiachotamine. These isomers were also identified in *R. stricta* cell suspension cultures (Pawelka and Stöckigt 1986, Kostenyuk *et al.* 1995, Sheludko *et al.* 1999, Sheludko *et al.* 2000). Vallesiachotamine was originally isolated from *Vallesia dichotoma* Ruiz et Pav shrub (Apocynaceae) and it was considered to

have an unusual structure (Djerassi *et al.* 1966). Isomeric mixtures of vallesiachotamines have frequently been isolated from plants and reported in the literature, for example from *Rhazya* species (Evans *et al.* 1968b, Mukhopadhyay *et al.* 1981) and suspension cultures of *R. stricta* (Pawelka and Stöckigt 1986, Kostenyuk *et al.* 1995, Sheludko *et al.* 1999, Sheludko *et al.* 2000). Its occurence in *C. roseus* (Kurz *et al.* 1981) and *R. verticillata* (Zhang *et al.* 2013) has also been reported.

Compounds **1**, decarbomethoxytabersonine and **9**, dihydrovincadifformine that were detected in the hairy root extracts are known to be artefacts. Chemical hydrolysis and decarboxylation of tabersonine produces decarbomethoxy-tabersonine (Plat *et al.* 1962, Zsadon and Otta 1973). On the other hand, dihydrovincadifformine can be formed by chemical reduction of vincadifformine (Djerassi *et al.* 1962, Zsadon and Otta 1973). These two artefacts have not been reported earlier from *Rhazya*.

Rhazinilam had a low library match and thus was only tentatively identified. However, its molecular ion m/z 350 and major fragment ions, m/z 265 and 237 (Banerji *et al.* 1970), were detected. Rhazinilam has earlier been reported as a minor constituent of *R. stricta* leaves occurring as an artefact (Banerji *et al.* 1970, De Silva *et al.* 1972). On the other hand, rhazinilam has been identified as one of the major alkaloids in *Rhazya* hybrid suspension cultures (Sheludko *et al.* 2000). It has also been proposed that vincadifformine is a precursor for biosynthesis of rhazinilam, since it is semi-synthesized from vincadifformine, (**8**) via the intermediate eburenine (**2**) (Ratcliffe *et al.* 1973). This artefact might result from the extraction conditions used.

In the present study of *R. stricta*, strictosidine lactam (**23**), strictosidine (**27**), tetrahydrosecodinol (**30**), tetrahydrosecodine (**31**) and dihydrosecodine (**32**) were major alkaloids in UPLC-PDA-MS analysis of hairy roots, whereas they were not identified by GC-MS analyses.

Strictosidine lactam (syn. strictosamide) is a known component in *R. stricta* (Atta-ur-Rahman *et al.* 1991) and also among the major alkaloids in its cell cultures (*e.g.* Pawelka and Stöckigt 1986). In the current study, the UV profile of strictosidine lactam (**23**) and its specific molecular ions in ES^+ and ES^-, basepeak fragments and a low-abundant fragment in ES^+ were in line with UV and MS ($[M^+]$ at m/z 498) from the pure alkaloid of *Rhazya* and analysed with EI-HRMS (Atta-ur-Rahman *et al.* 1991). They were also fully consistent with ESI/MS data from *O. pumila* (Yamazaki *et al.* 2003), *Nauclea pobeguinii* (Pob. ex Pell.) Petit (Dhooghe *et al.* 2008) and *Nauclea latifolia* (Donalisio *et al.* 2013). Moreover, in an animal study, ion trap-TOF mass spectrometer and mass defect filter (LC-IT-TOF-MS) analysis of strictosidine lactam in ES^+ (Liang *et al.* 2011) well agreed with the data in **Table 15**. Strictosidine lactam (**23**) is also the principal alkaloid in *C. roseus* (Kurz *et al.* 1981), and it has also been identified in its hairy root cultures along with ajmalicine, tabersonine, tetrahydroalstonine and yohimbine (Toivonen *et al.* 1989). In addition, strictosidine lactam has been reported as the main alkaloid in *Vinca major* L. (Eilert *et al.* 1987) and in the genus *Nauclea* (Sarcocephalus) (Mesia *et al.* 2010, Xu *et al.* 2012). Therefore, it has also been postulated that it is

most likely a natural constituent of plants exhibiting a number of bioactivities *in vitro* and *in vivo* (Mesia *et al.* 2010, Donalisio *et al.* 2013).

Strictosidine (**27**), which has been long known as an intermediate in the biosynthesis of indole alkaloids (Smith 1968), was among the principal alkaloids in *Rhazya* extract. Its UV and spectral data were well in line with those reported by Yamazaki *et al.* 2003.

Secodine alkaloids tetrahydrosecodine (**31**) and dihydrosecodine (**32**) together with tetrahydrosecodinol (**30**), a new alkaloid for *R. stricta*, are specific TIAs, since the basepeak fragment m/z 126 is typical for alkaloids containing a secodine skeleton with a saturated (**30**,**31**) or unsaturated (**32**) piperidine ring (Evans *et al.* 1968a, Cordell *et al.* 1970a,b). Dihydrosecodine (**32**) displayed a characteristic UV maximum at 305 nm and $[M+H]^+$ at m/z 341, with a basepeak at m/z 126. Already early studies have reported the close relationships between these two alkaloids in *R. stricta* (Cordell *et al.* 1970a). The molecular ion m/z 358 of tetrahydrosecodinol, obtained by EI-MS, readily loses water and produces a radical ion m/z 340 corresponding to $[M^+]$ of 15,20-dihydrosecodine. This structure also allowed the double bond to be located in the piperidine unit. In addition, pure dihydrosecodine had a typical UV maximum of 303 nm (Cordell *et al.* 1970b). Tetrahydrosecodine (**31**), with a UV spectrum similar to that of tetrahydrosecodinol (**30**), showed a molecular ion m/z 343 and basepeak m/z 126 with an additional fragment m/z 230, which are in accordance with literature data. By using EI-MS, this compound exhibited a molecular ion at m/z 342 and basepeak at m/z 126 (Cordell *et al.* 1970a). The high intensity of the basepeak m/z 126 has been considered especially important in the discovery and isolation of secodine-type alkaloids (Cordell *et al.* 1970b). Furthermore, the co-occurrence of tetrahydrosecodine with dimeric secamines and presecamines also indicates the saturated piperidine ring (Cordell *et al.* 1970b). Secodine, as a reactive monomeric alkaloid ($C_{21}H_{26}N_2O_2$) with a MW of 338, is a presumed precursor of the dimeric presecamine group, which in turn produces the secamine group alkaloids. Due to the complexity of the isomeric mixtures, the stereochemistry of tetrahydropresecamine (MW 860) is still not exactly defined. Furthermore, it rearranges quantitatively to secamine in weakly acidic conditions (Buckingham *et al.* 2010). These compounds are common constituents in *Rhazya*, and early isolations showed that both double bonds were saturated in the structure of tetrahydrosecamine. It was also characteristic for this compound that the basepeak at m/z 126 ($C_8H_{16}N$) in the piperidine fragment appeared with unusually high intensity, which additionally allowed easy monitoring of the isolated alkaloid fractions (Evans *et al.* 1968a).

In this study, tetrahydrosecamine was identified from a broad zone with a UV profile λmax at 229, 286 and 292 nm and molecular ion $[2M+H]^+$ m/z 681, with a high-intensity basepeak at m/z 126 (100%). It also showed the $[M+H]^+$ ion of the parent monomer at m/z 341 (15%) and a fragment m/z 309 (51%) due to the loss of methanol from the monomer. The UV (λmax at 224, 284 and 292 nm) is consistent with the results from *Amsonia* roots obtained by Zsadon *et al.* (1975), who used methanol in the isolation of tetrahydrosecamine. The $[M+1]^+$ ion m/z 680

and basepeak at m/z 126 was obtained by HR-EI-MS. The UV and EI-MS data from *Rhazya* were as reviewed by Atta-ur-Rahman *et al.* (1989b).

Presecamines can well appear mainly by non-enzymatic dimerisation of secodine units, either in the cell or during extraction. The importance of pH was shown in a study in which a high yield of tetrahydropresecamines from *R. orientalis* roots was obtained by a procedure which avoided the use of acidic conditions stronger than pH 4.2, whereas the *R. stricta* alkaloids were exposed to a strongly acidic solvent in which most of the presecamine content could have rearranged to secamine (Cordell *et al.* 1970b). Thus, the alkaloid extraction from *R. stricta* in this study, which started with 10% sulphuric acid, would mean that similar re-arrangement to tetrahydrosecamine could have occurred.

In UPLC-MS analysis, two alkaloid peaks having $[M+H]^+$ ions at m/z 351 and UV maxima at 210, 250 and 298 nm (with an extra λ_{max} at 304 nm), were putatively identified as leepacine isomers. It should also be noted that leepacine isolated at pH 6 gives λ_{max} at 207, 250 and 298 nm (Atta-ur-Rahman *et al.* 1991), and that UV maxima can be slightly higher in alkaline than in neutral conditions (Ahmad and Quesne 1970). In addition, the fragmentation obtained from separate GC-MS analysis was consistent with the EI-HR-MS data reported earlier (Atta-ur-Rahman *et al.* 1991).

6.2.3 Variation in alkaloid contents in *in vitro* cultures

In order to investigate the capacity of *R. stricta* callus cultures to produce terpenoid indole alkaloids, quantitative analyses were performed. HPLC analysis was used to compare the contents of major alkaloids in undifferentiated calli with those of differentiated hairy root cultures. The chromatograms revealed that the profile of major alkaloids remained the same between calli and hairy root cultures. The same profiles were also observed regardless of which explant the calli were derived from. Hypocotyl-, stem- and leaf-derived calli accumulated higher amounts of stricitosidine lactam (**23**), whereas the concentrations of the other six major alkaloids were significantly higher in hairy root cultures. The amount of an unidentified alkaloid **26** in leaf-derived explants was significantly (p=5%) higher than in stem-derived calli. Nevertheless, the amounts of other major alkaloids did not vary between the two calli types. This might be due to the fact that in the callus cultures strictosidine is not immediately deglycosylated to form the precursor of TIAs. Instead, a lactam is formed immediately between the amine of strictosidine and the methyl ester derived from the secologanin.

The stability of alkaloid profiles in these undifferentiated cultures was confirmed over a period of six years. However, the quantitation of alkaloid levels has not been determined. The callus cultures, which have higher amounts of stricitosidine lactam (**23**) compared to hairy roots, might lose their capacity to accumulate stricitosidine lactam over a longer time, since undifferentiated cultures typically show a tendency to lose productivity due to somaclonal variation (Whitmer *et al.* 2003). This tendency may be due to the fact that callus formation is a de-differentiation process that gives rise to rapidly proliferating cells, which results in

a negative correlation between cell division and production of secondary metabolites. Prolonged subculturing selects for the most rapidly growing cell types, which can be expected to be metabolically less active.

The variation of alkaloid contents in 20 wild type hairy roots was studied by GC-MS. The highest concentrations of alkaloids in hairy root clones ranged from 0.04 to 0.29% for eburenine. The contents of eburenine (0.040-0.290%), quebrachamine (0.001-0.009) and rhazine (0.001-0.006) from various *Rhazya* hairy root clones were in line with reported data for eburenine (0.12%), quebrachamine (0.002-0.06%) and rhazine (0.015-0.05%) isolated from the different parts of the plant (Smith and Wahid 1963, Chatterjee *et al.* 1976). Wide quantitative variation in this population would be a good basis for further selection of high producers of the desired alkaloids from *Rhazya* wild type hairy root clones.

It is known that the rate of biomass accumulation as well as the production of terpenoid indole alkaloids in *C. roseus* is influenced by nutritional and environmental factors (Toivonen *et al.* 1989, Moreno *et al.* 1995). In this study, it was shown that there were no statistically significant differences in fresh weight, dry weight and alkaloid extraction yields of wild type *R. stricta* hairy roots grown in Woody Plant Medium and modified Gamborg B5 medium. However, the contents of strictosidine lactam (**23**) and serpentine (**25**) in wild type hairy roots grown in modified Gamborg B5 medium were significantly higher than in Woody Plant Medium. Furthermore, the absence of light had a significant effect on the accumulation of alkaloids even though there were no significant differences in dry weight or extraction yield. Accumulation of four out of seven alkaloids was increased in cultures grown in the dark, showing that these compounds were more effectively biosynthesized. On the other hand, the amount of leepacine (**2**) was significantly higher in hairy roots grown under a 16 h photoperiod, indicating that the production of this compound is highly up-regulated by light irradiation. Light is known to regulate not only plant growth and development but also the biosynthesis of both primary and secondary metabolites (Vázquez-Flota and De Luca 1998). The findings agreed with the results of Sander (2009) and Sidwa-Gorycka *et al.* (2009), who stated that light adaptation decreases some metabolite levels by increasing the flux out of the metabolite pools in hairy roots of *C. roseus* and *Ruta graveolens* L., respectively.

The accumulation of two alkaloids out of all seven monitored ones in wild type hairy roots was higher than in GUS hairy root clones. This might be due to the fact that the transformation has a stress effect and can affect the alkaloid accumulation. Kim *et al.* (2010b) reported that GUS hairy root clones of buckwheat (*Fagopyrum esculentum* M.) exhibited higher rutin contents than wild type clones. There are also reports indicating that the expressed GUS gene has no effect on the production of secondary metabolites, *e.g.* decursinol angelate and decursin in *Angelica gigas* Nakai. (Park *et al.* 2010).

6.3 Effect of elicitation on alkaloid accumulation

MeJA has been widely applied as an effective elicitor in plant cell cultures to enhance secondary metabolite production in many species, *e.g. C. roseus* (Rischer *et al.* 2006, Goklany *et al.* 2013). In the current study, principal component analysis (PCA) based on ^{1}H-NMR data could separate hairy roots elicited with 50 and 100 µM MeJA for five days from non-elicited samples. Loading column plots showed that elicited samples contained higher alkaloid contents than non-elicited clones. This was also confirmed by assessing the total alkaloid level. It was revealed that elicited hairy roots had 5-fold higher total alkaloid concentrations than the controls. In addition, the elicited samples had higher concentrations of aromatic and aliphatic compounds than control cultures. The results were in line with the finding from salicylic acid (SA)-elicited *C. roseus* cell suspension cultures monitored by NMR. It was found that the levels of some aliphatic amino acids and organic acids in the SA-treated cells was significantly higher than in control cultures (Mustafa *et al.* 2009). Similarly, ^{1}H-NMR spectroscopy data analysis of JA-treated *Cannabis sativa* L. showed that the contents of phenolic compounds such as phenylalanine, tyrosine, tyrosol, tyramine and tyramine glucoside showed an increase compared with non-elicited samples (Flores-Sanchez *et al.* 2009, Peč *et al.* 2010).

In addition, the effect of an abiotic elicitor, MeJA (50-200 µM), at ten time points (15 min-7 days) was studied in the accumulation of 16 major alkaloids in *R. stricta* wild type hairy roots using HPLC and GC-MS data. Monitoring of seven major alkaloids from HPLC analysis showed that five alkaloids showed increased amounts at different MeJA concentrations (50 and 100 µM) and exposure times of 1-5 days (dose and time-dependent) in comparison with non-treated samples. The GC-MS results showed dose- (50, 100 and 200 µM) and/or time (1-7 days)-dependent increases of eight alkaloids, eburenine, quebrachamine, tubotaiwine, pleiocarpamine, fluorocarpamine, tetrahydroalstonine, ajmalicine and vallesiachotamine, although the elicitation did not result in higher accumulation of vincanine and yohimbine isomer II.

By contrast, exposure of hairy roots to a biotic elicitor chitosan (250 and 500 mg/L) did not change the alkaloid content of selected alkaloids in *R. stricta* hairy roots. The results of this work agreed with those of Sánchez-Sampedro *et al.* (2005); it was shown that different concentrations of chitosan did not promote any increase in silymarin accumulation in *Sylibum marianum* (L.) Gaertn. cell cultures. However, there are reports indicating that chitosan enhanced the production of several secondary metabolites in hairy root cultures, for example, artemisinin (Putalun *et al.* 2007), ginsenosides (Palazón *et al.* 2003) and tropane alkaloids (Sevón *et al.* 1992).

It was demonstrated that exposure of *R. serpentine* × *R. stricta* hybrid cell culture to 100 µM methyl jasmonate elevated indole alkaloids levels after one and five days (Sheludko *et al.* 1999). Ajmalicine, vallesiachotamione, and yohimbine have previously been reported to have higher concentrations in elicited *Rhazya* hybrid culture (Sheludko *et al.* 1999), although in this study the production levels

of yohimbine isomer II or vincanine were not elevated in the elicited hairy roots. A smaller increase of ajmalicine was observed in elicited samples compared to *Rhazya* control culture. The same trend was observed in *C. roseus* MeJA-elicited hairy roots, although the dosage was optimal for other compounds (Goklany *et al.* 2013). In the current elicitation experiments no alkaloids were found in GC-MS analyses of media samples. However, Sheludko *et al.* (1999) reported the presence of the six indole alkaloids in cultivation media. Interestingly, it was observed that even small amounts of ethanol might have an effect.

It was shown that the application of elicitors, *e.g.* MeJA, induces the accumulation of endogenous jasmonic acid that consequently results in increased production of TIAs (Menke *et al.* 1999, Zhao *et al.* 2005). In addition, MeJA induces genes in primary metabolism leading to the formation of TIA precursors (van der Fits and Memelink, 2000). In *C. roseus*, the effects of MeJA dosage on terpenoid indole alkaloid (TIA) biosynthesis are correlated with the relative levels of specific MeJA-responsive transcription factors (*e.g.* ORCAs) (Lee-Parsons *et al.* 2004, Memelink and Gantet 2007). Rischer *et al.* (2006) reported that JA induces expression of a number of TIA biosynthetic genes in *C. roseus* suspension cultures, including geraniol 8-hydroxylase (*g8o*), strictosidine β-D-glucosidase (*sd*) and tryptophan decarboxylase (*tdc*). However, the expression levels of acetyl-CoA:minovincine-*O*-acetyltransferase (MAT) and deacetylvindoline-4-*O*-acetyltransferase (DAT) were not affected. Goklany *et al.* 2013 investigated the expression of transcription factors, TIA pathway genes (*g8o, tdc, str*, and *sd*) and TIA metabolites (secologanin, strictosidine and tabersonine) in *C. roseus* hairy root cultures elicited with a range of MeJA dosages (up to 1 mM).

6.4 Effect of transgenes on alkaloid production

Different *R. stricta* transformed hairy root clones were subjected to ^{1}H-NMR analysis in order to investigate overall metabolite differences between transgenic and control hairy root clones. Various multivariate data analysis methods, in combination with ^{1}H-NMR spectroscopy, were used in order to identify overall metabolic differences between groups. Mainly OPLS-DA of ^{1}H-NMR bucket data exhibited complete separation among the transformed roots and controls, wild type and *gus* clones. VIP plots showing more influential factors on separation of different clones were also presented.

Although there was no significant difference in total alkaloid content between control wild type/GUS hairy roots and transgenic clones, multivariate data analysis (OPLS-DA) resulted in a clear separation of the two groups. VIP and loading column plots indicated that the separation between two sets was mainly due to differences in primary metabolite levels. The ^{1}H NMR spectra from deuterated extracts were found to be dominated by the signals of sugars and amino acids. Therefore, fractionations of extracts, extraction with different solvents (Peč *et al.* 2010) using an SPE column (Ali *et al.* 2011a) or an SPE column-based cation exchange column (*e.g.* MCX), which retains cationic compounds such as alkaloids

resulting in sample clean-up, are an alternative approach to eliminate sugars and amino acids and subsequently avoid spectra overlapping. Limitations in resolution and signal overlapping in ^{1}H-NMR spectra can be overcome by the combination of different NMR technologies, such as J-resolved, COSY, HSQC or HMBC (Ali *et al.* 2011b). In order to obtain a broad view of the biochemical status of different *R. stricta* hairy root cultures, application of these techniques is required.

Although there was no significant difference in total alkaloid content between control wild types and transformed hairy root cultures, the accumulation of individual alkaloids in transformed hairy roots with early key genes from the iridoid pathway, geraniol synthase (*ges*) and geraniol-8-oxidase (*g8o*), and strictosidine synthase (*str*), a key intermediate gene between upstream and downstream TIA biosynthesis pathway, was investigated by HPLC analysis. In transgenic *str* hairy root clones, analysed by HPLC, the accumulation of vincanine (**12**) and serpentine (**24**) was statistically significantly higher (p<0.05) than in wild type hairy root clones, indicating that introduction of the *str* gene into a plant genome affected their biosynthesis. Vincanine is a strychnos-type alkaloid, whereas serpentine is a member of the corynanthe group alkaloids. However, it was suggested that that the strychnos alkaloids are derivatives of the corynanthe alkaloids. This might explain channelling of the strictosidine pool in *str* clones towards simultaneous higher production of (**12**) and (**24**).

On the other hand, introduction of *ges* or *g8o* into roots did not cause elevated concentrations of major compounds compared to control cultures. It has been shown that geraniol is easily converted into other products. For example, when *V. vinifera, Nicotiana benthamiana* Domin and *A. thaliana* were transformed with the *ges* gene from *O. basilicum*, oxidation products of geraniol were detected (Höfer *et al.* 2013, Fischer *et al.* 2013). Similarly, in the leaves of transformed maize with the *ges* gene from *L. dulcis*, geranyl acetate was identified instead of geraniol (Yang *et al.* 2011). This might also be the case in transformed *R. stricta ges* hairy root clones in this work, but further analytical studies are required. There are reports that hairy root clones expressing the *g8o* gene alone, or both the *g8o* and *orca3* genes, accumulate more catharanthine (Wang *et al.* 2010).

The effect of overexpression of the *g8o* alone and with 1-deoxy-D-xylulosesynthase (*dxs*) in *C. roseus* root cultures was also investigated by Peebles *et al.* (2011). The overexpression of single genes resulted in mixed results with regard to the accumulation of TIA metabolite pools, whereas overexpression of the two genes resulted in an increase in several TIAs. The authors concluded that the overexpression of a single gene might be insufficient to overcome the regulation around this branch point, whereas the overexpression of two genes simultaneously may act in concert to push and pull the flux towards the TIA metabolites. Although the overexpression of single genes may increase flux through the pathway, the overexpression of multiple genes may be necessary in order to achieve significant gains in product accumulation. This may especially be true in highly branched TIA biosynthesis pathways, in which precursors can be channelled into a variety of pathways.

As was discussed above, accumulation of the prominent alkaloids varied among the different transformed clones. This can be explained by the fact that following transformation, transgenes are integrated into different sites of the plant genome with diverse levels of expression (Ritala *et al.* 2014). Somaclonal variation can also cause various accumulation levels of alkaloids. However, it must be taken into account that a real-time quantitative reverse transcription PCR (q-RT-PCR) should be performed to investigate the expression levels of selected genes, as it is possible that a gene is integrated to the genome but not expressed, or has a feedback effect on other genes (Hughes *et al.* 2004a,b).

7. Conclusions

Plants are important sources of therapeutics and also provide a large resource of natural compounds for the discovery of novel pharmaceuticals. Drug development and semi-synthesis of promising compounds largely rely on building blocks from plant sources. The classical plant-based pharmaceutical drug production approach has been hindered by low yields and lack of reproducibility. Moreover, due to the complex structures of plant compounds, their chemical synthesis is difficult because multiple steps are required for synthesis and purification, and reaction products are commonly mixtures of isomers and epimers that compromise the biological activity of the products. Biotechnological approaches such as undifferentiated cell cultures and differentiated organ cultures combined with metabolic engineering in medicinal plants are alternative platforms for sustainable and contained production of active compounds. Furthermore, new compounds can be formed in *in vitro* cultures, thus offering new possible opportunities to the pharma industry.

Rhazya stricta has long been used in folk medicine in the Middle East and on the Indian subcontinent to cure various diseases. Because of its biological activities, mainly due to terpenoid indole alkaloids (TIA), the plant has been subjected to phytochemical and pharmacological research focusing on isolation of compounds from the plant and their bioactivity screenings. Phytochemical analysis has identified more than 100 TIAs from the plant. Research has revealed anti-cancer properties of the crude extracts or isolated compounds from *R. stricta*. For example, recent studies indicated that vallesiachotamine inhibits the proliferation of human melanoma cell lines *in vitro* and exhibits significant accumulation of melanoma cells in the G0/G1 growth phase. Another example is rhazinilam, which possesses unique antimitotic properties similar to those of vinblastine and paclitaxel. Therapeutically improved analogues of rhazinilam were also achieved by its chemical modification. In contrast to the diverse descriptions of compounds in *R. stricta* and *in vitro* and *in vivo* bioactivity data deriving from animal and human studies, there are no reports of biotechnological method applications for the stable and extensive production of pharmacologically active compounds in *R. stricta*.

In this study, an *Agrobacterium rhizogenes*-mediated transformation system was developed for *R. stricta* for the first time. The transformed roots were only

obtained from leaf explants and they exhibited stability in growth and production of terpenoid indole alkaloids. Excellent transformation efficiency for control (74%) and marker gene (83%) clones was achieved, thus building firm bases to introduce heterologous genes. The method was further used for introduction of early genes from the TIA biosynthesis pathway in order to investigate their effect on production of known and novel alkaloids. Calli derived from different explants of *R. stricta* as undifferentiated cultures were also initiated to compare their biosynthetic potential with differentiated organ (hairy root) cultures.

Rhazya alkaloids consist of a wide range of structures and polarities, and therefore different analytical methods including HPLC, UPLC-MS and GC-MS were developed for the analyses of target alkaloids. This study provides the first comprehensive analysis of the alkaloid composition of *R. stricta* hairy root crude extracts. Furthermore, in order to obtain a general view of metabolite changes in different transgenic hairy root clones or in elicited samples, NMR spectroscopy coupled with multivariate analysis was also employed and demonstrated that the multivariate models effectively distinguish metabolite differences between controls and transformed/elicited clones. In order to obtain better insights into detailed metabolic changes, further experiments are needed.

As concerns production of alkaloids in two different *in vitro* culture systems, it was revealed that the alkaloid profiles remain the same in calli and hairy root cultures but hairy roots possess a greater capacity for accumulation of the alkaloids than calli. However, the accumulation of strictosidine lactam, exhibiting a number of bioactivities *in vitro* and *in vivo*, was found to be significantly higher in hypocotyl-, stem- and leaf-derived calli than in hairy roots. Suspension cultures might offer the possibility for further investigation of the capacity of calli lines for alkaloid production via elicitation and biotransformation.

In the present study, a total of 33 terpenoid indole alkaloids from *R. stricta* wild type hairy roots were identified. The alkaloids belonged to twelve groups. The quantities of these alkaloids varied between clones, although eburenine, vincanine, vallesiachotamine, yohimbine isomer II, strictosidine lactam, strictosidine, serpentine, tetrahydrosecodinol, tetrahydrosecodine, dihydro-secodine and tabersonine were the major compounds identified. Wide variation in the amount of alkaloids in different hairy root clones offers a basis for screening and selection for high producing clones. In particular, the detailed optimization of the growth media and precursor feeding might further improve the accumulation of alkaloids in such cultures. Moreover, in order to maintain high-producing hairy root cultures, cryopreservation should be considered and developed.

In the current study, the potential of *R. stricta* hairy roots for biosynthesis of novel alkaloids was investigated. The ten terpenoid indole alkaloids which are reported here for the first time from *R. stricta* hairy roots were pleiocarpamine, fluorocarpamine, vincamine, ajmalicine, yohimbine isomers, serpentine and its isomer, tetrahydrosecodinol as well as tabersonine.

NMR analyses showed that the transformation of *R. stricta* hairy roots with single key genes from the TIA biosynthesis pathway did not result in elevated levels of total alkaloid content in comparison with control wild type hairy roots.

However, the accumulation of intact alkaloids might vary, as HPLC analysis showed an increase of two alkaloids, vincanine and serpentinet. More chemical analyses, *e.g.* GC-MS are required to monitor the individual alkaloid contents in transgenic hairy root clones. It was clear that by overexpression of a single gene from the pathway, the flux was only moderately increased. Since metabolic pathways are controlled at multiple levels, targeting many steps in the same pathway can help to control metabolic flux in a more predictable manner. Expression of double-gene or multi-gene cassettes in *R. stricta* hairy roots, including transcription factor genes, in order to understand better the TIA biosynthesis has to yet be performed.

It is well known that the biosynthesis of secondary metabolites can be induced by stress conditions (*e.g.* elicitor). In this study, methyl jasmonate resulted in a five-fold increase in the total alkaloid content of wild type hairy roots determined by NMR analyses. Detailed targeted analyses by GC-MS showed that the contents of eight out of ten studied alkaloids increased compared to non-elicited cultures. Expression of several genes, for example *str* and *g8o,* is co-ordinately regulated by elicitation, jasmonate being the intermediate signal required. Therefore, the elicitation of transgenic *R. stricta* hairy roots might even have a further effect on alkaloid accumulation.

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Appendix A: PCR analyses of *vir D* gene

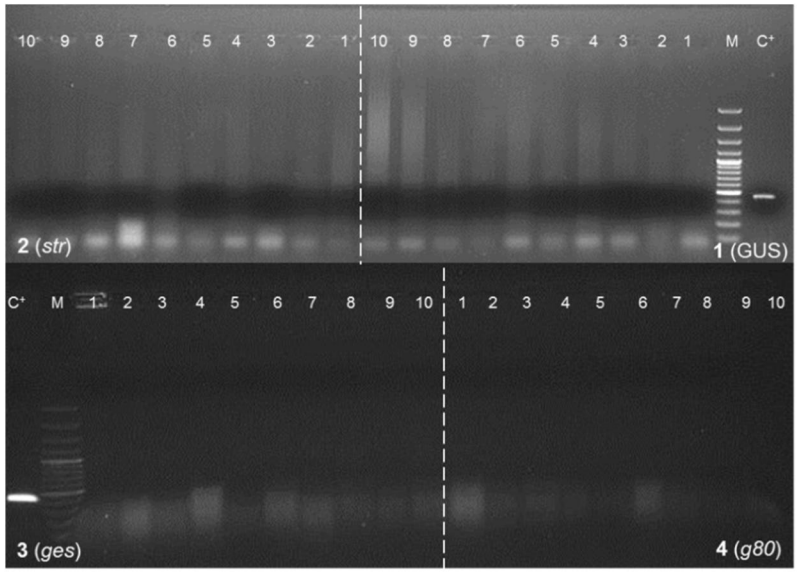


Figure A. PCR analyses for confirmation of absence of *vir D* gene in *R. stricta* (**1**) GUS, (**2**) *str*, (**3**) *g8o* and (**4**) *ges* hairy root clones. C^+, the positive control (wild type plasmid carrying the *virD* gene). M, 100 bp DNA ladder.

Appendix B: PCR analyses of *rolB* gene

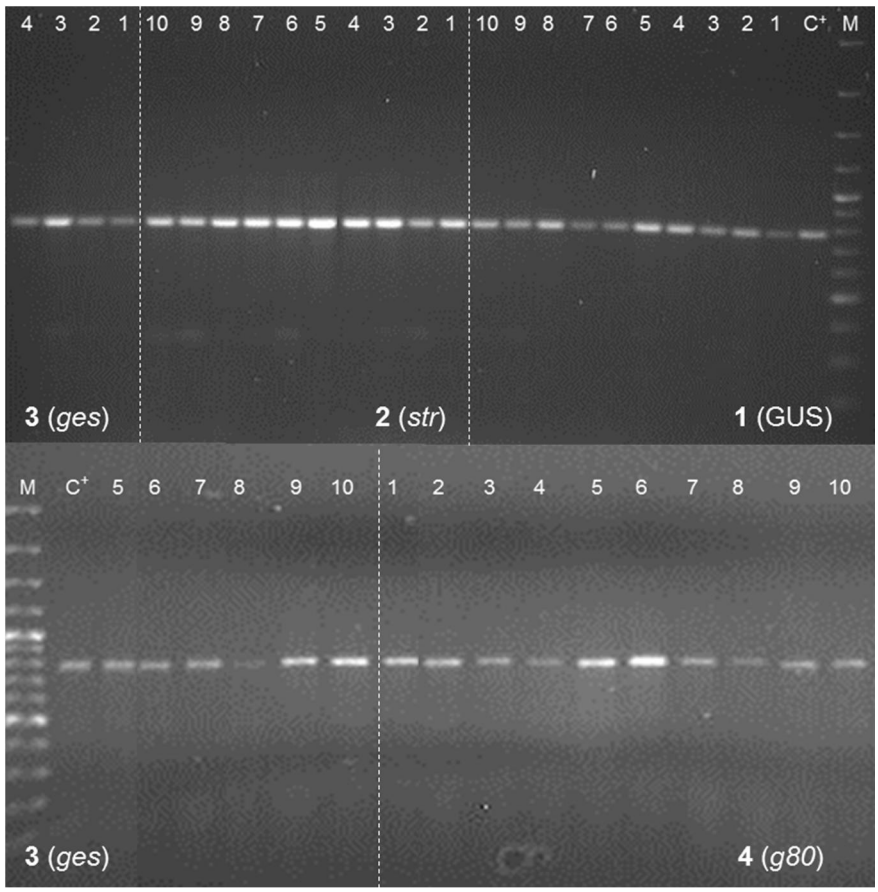
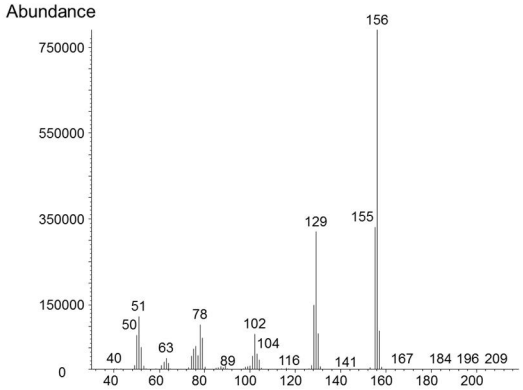
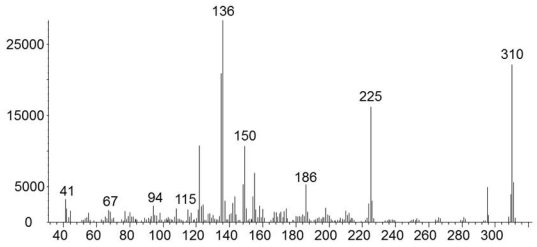


Figure B. PCR analyses for confirmation of transformation of *rolB* gene into *R. stricta* (**1**) GUS, (**2**) *str*, (**3**) *g8o* and (**4**) *ges* hairy root clones. C^+, positive control (wild type plasmid carrying the *rolB* gene). M, 100 bp DNA ladder.

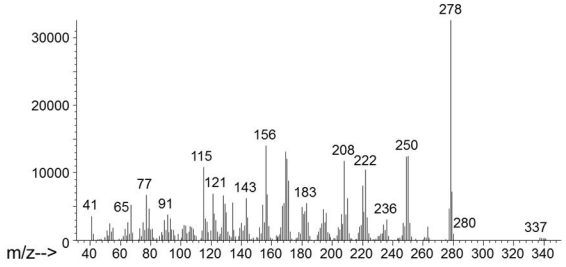
Appendix C: GC-MS spectra of the target alkaloids



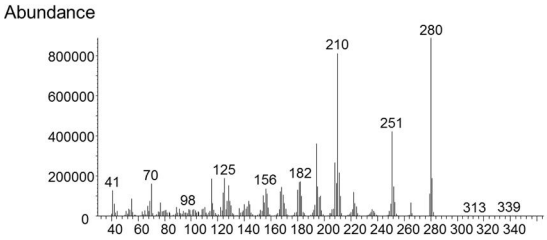
2,4'-Dipyridyl (IS)



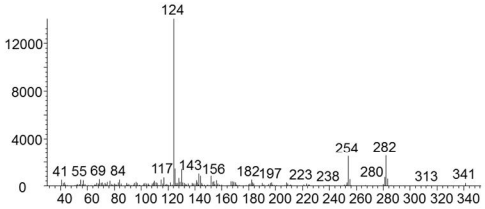
Ibogaine



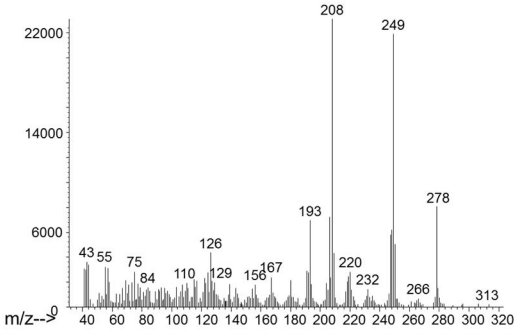
Decarbomethoxytabersonine (**1**)



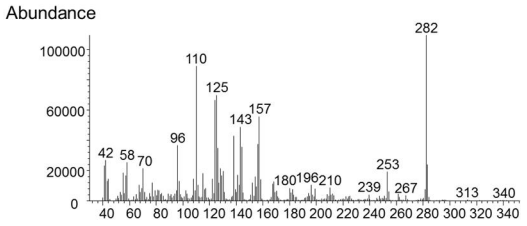
Eburenine (**2**)



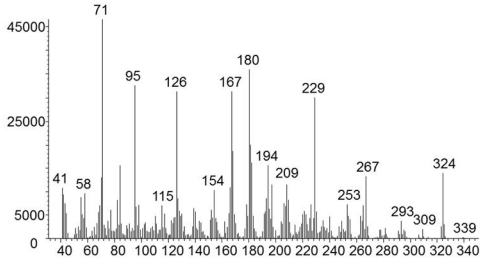
Aspidospermidine (**3**)



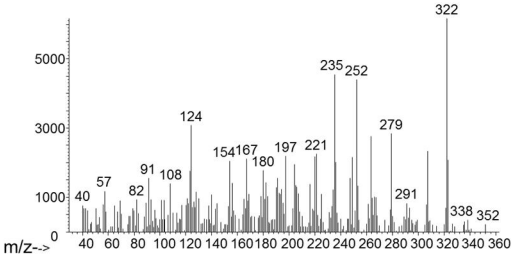
Eburnamenine (**4**)



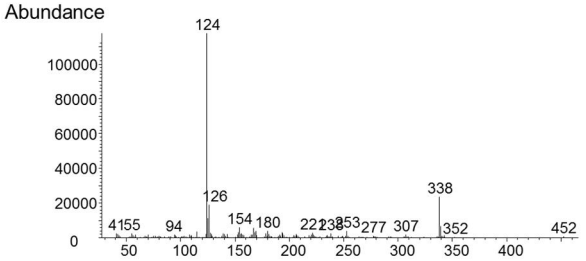
Quebrachamine (**5**)



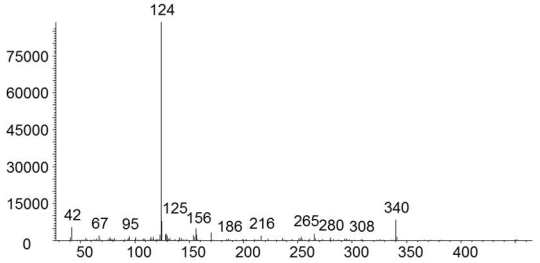
Tubotaiwine (**6**)



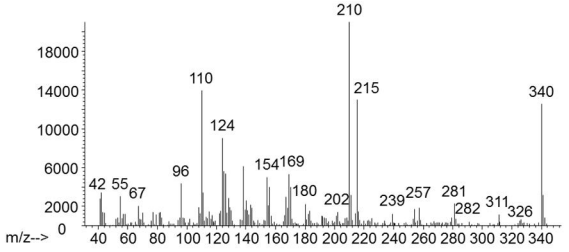
Condylocarpine (**7**)



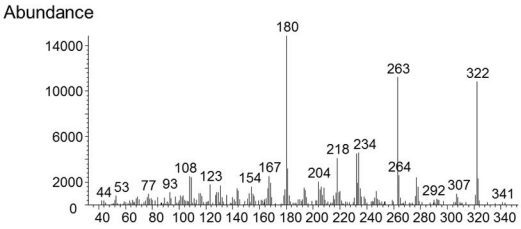
Vincadifformine (**8**)



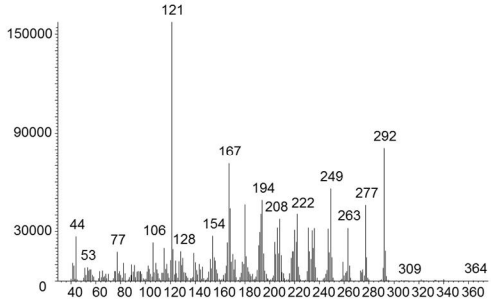
Dihydrovincadifformine (**9**)



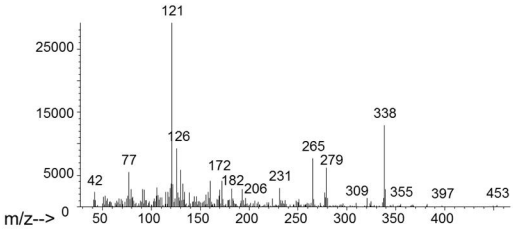
Vincadine (**10**)



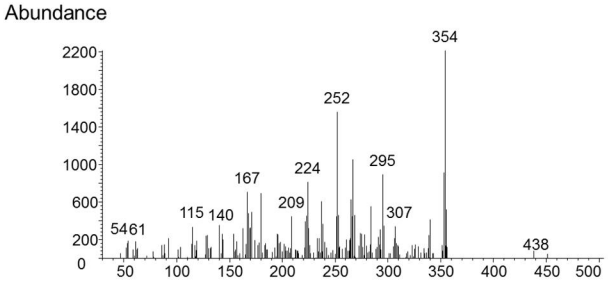
Pleiocarpamine (**11**)



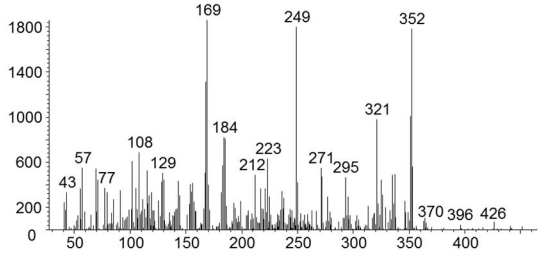
Vincanine (**12**)



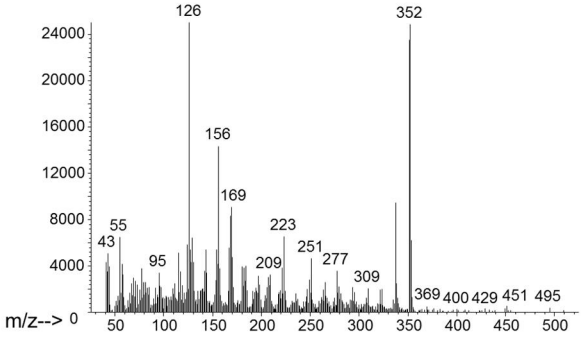
Fluorocarpamine (**13**)



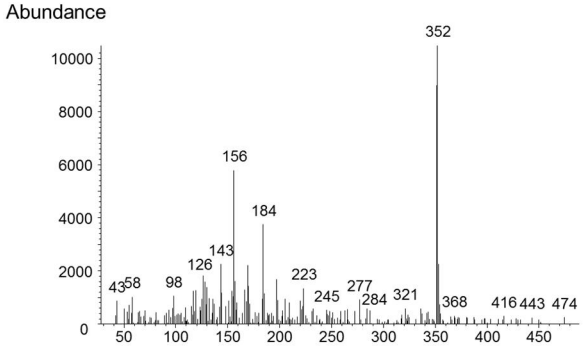
Vincamine (**14**)



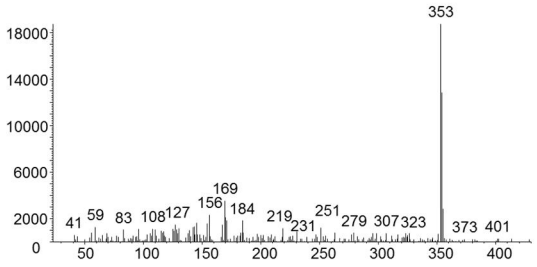
Rhazine (**15**)



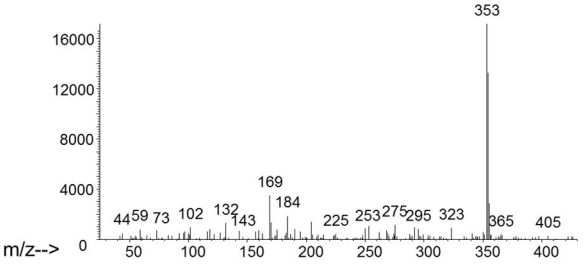
Tetrahydroalstonine (**16**)



Ajmalicine (**17**)



Yohimbine isomer I (**18**)



Yohimbine isomer II (**19**)

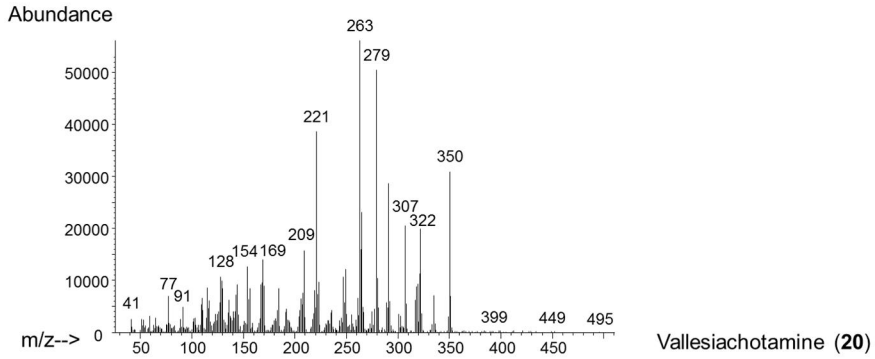


Figure C. GC-MS spectra of the target alkaloids. Compounds refer to constituents in **Table 11**.

Appendix D: GC-MS separation of yohimbin isomers

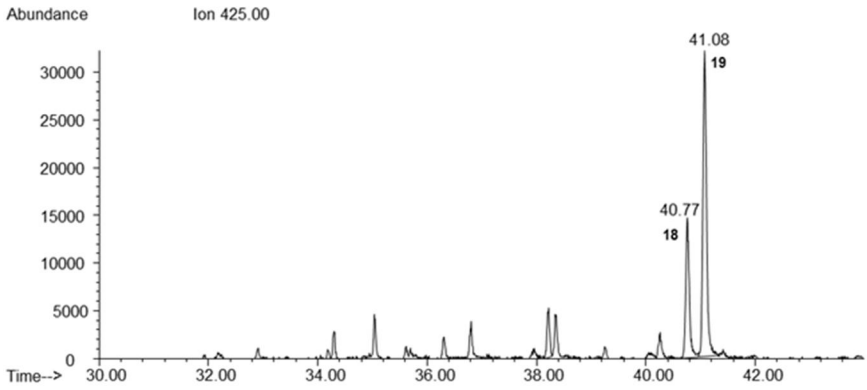
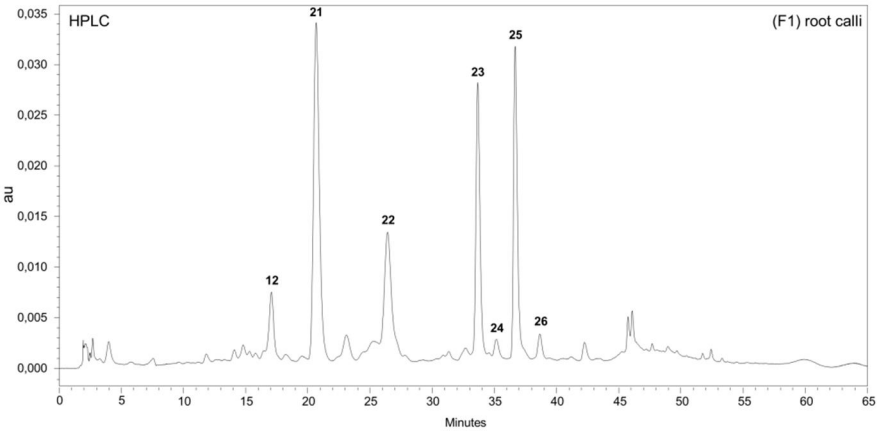
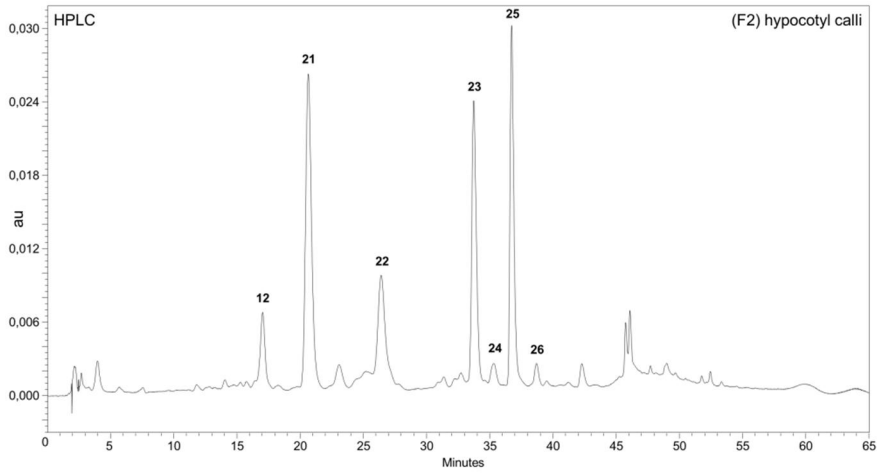


Figure D. GC-MS separation of yohimbin isomers I and II in silylated *Rhazya* sample using extracted ion m/z 425. Peak numbers refer to constituents in **Table 11**.

Appendix E: HPLC analyses of *R. stricta* calli line extracts





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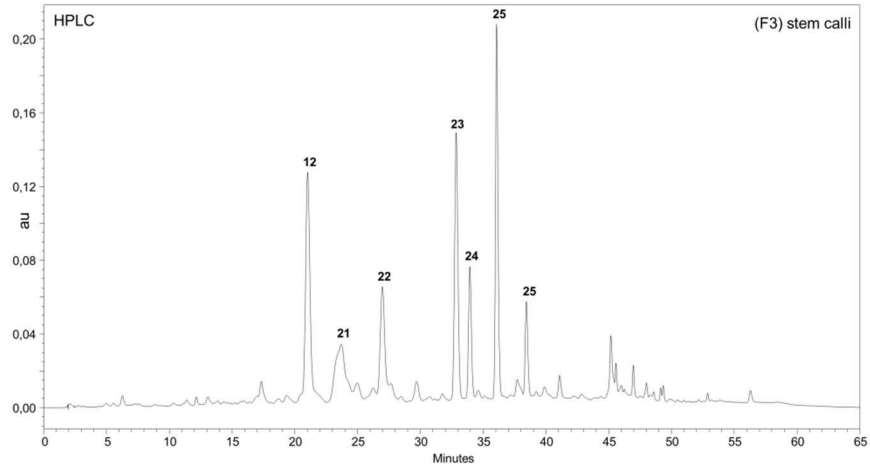
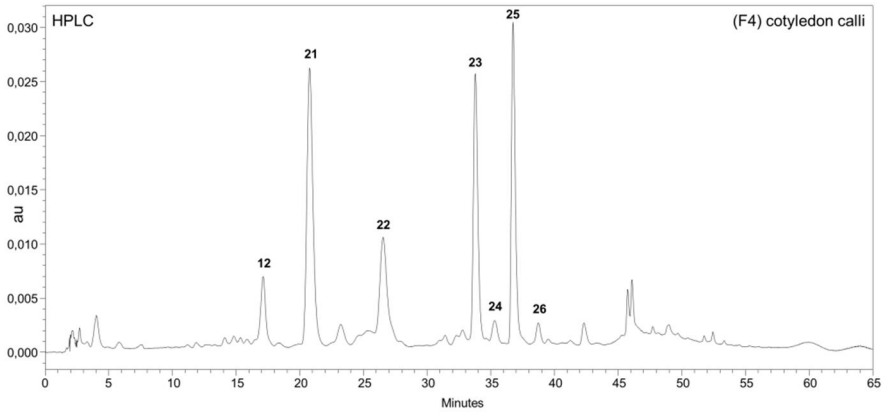

HPLC
(F3) stem calli
au
Minutes
12
21
22
23
24
25
25


HPLC
(F4) cotyledon calli
au
Minutes
12
21
22
23
24
25
26

continues→

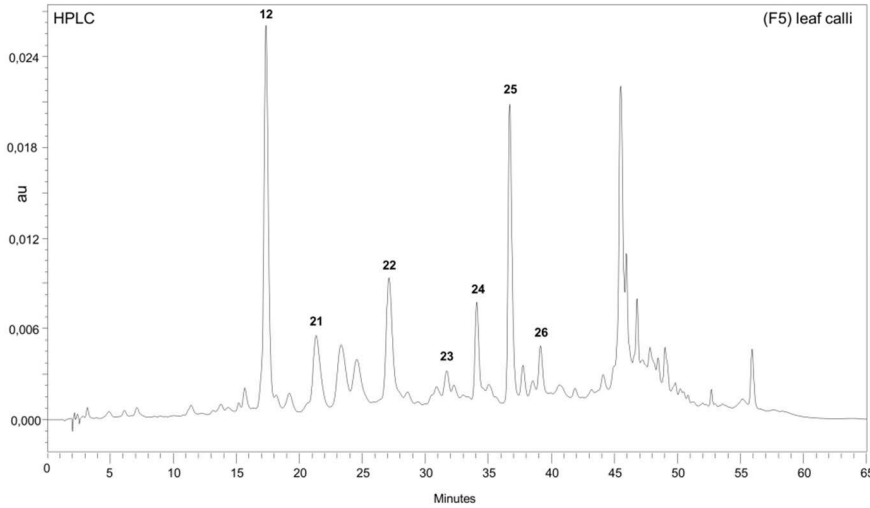


Figure E. HPLC analyses of *R. stricta* calli line extracts monitored at 255 nm. (**F1**) root calli; (**F2**) hypocotyl calli; (**F3**) stem calli; (**F4**) cotyledon calli and (**F5)** leaf calli. Numbers refer to compounds from **Table 13**. au: Absorbance unit. For the HPLC conditions see page 73, section **4.12.2**.

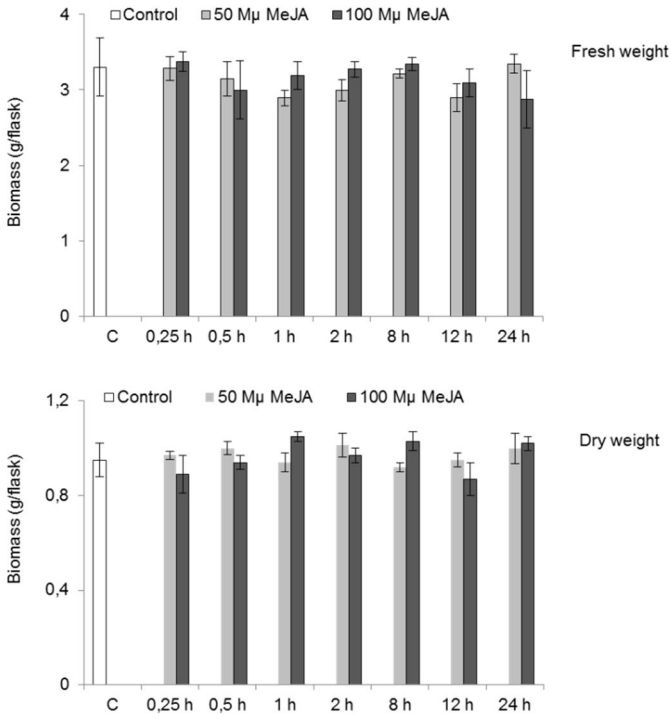
Appendix F: Pairwise comparisons of seven major alkaloids in five different calli lines

Peak	Root calli M±SD (au)	Hypocotyl calli M±SD (au)	Stem calli M±SD (au)	Cotyledon calli M±SD (au)	Leaf calli M±SD (au)
1	$1.1\times10^{5}\pm8.7\times10^{4}$	$1.3\times10^{5}\pm1.4\times10^{4}$	$6.2\times10^{4}\pm1.1\times10^{4}$	$4.8\times10^{4}\pm5.7\times10^{3}$	$1.0\times10^{5}\pm3.5\times10^{4}$
2	$1.4\times10^{6}\pm2.6\times10^{5}$	$1.0\times10^{6}\pm2.4\times10^{5}$	$6.9\times10^{5}\pm4.2\times10^{5}$	$2.7\times10^{5}\pm8.3\times10^{4}$	$1.1\times10^{6}\pm3.9\times10^{5}$
3	$4.0\times10^{5}\pm2.3\times10^{5}$	$9.8\times10^{5}\pm5.5\times10^{5}$	$8.2\times10^{5}\pm1.2\times10^{5}$	$4.8\times10^{5}\pm1.7\times10^{5}$	$2.9\times10^{6}\pm3.5\times10^{6}$
4	$2.7\times10^{6}\pm1.0\times10^{6}$	$4.7\times10^{6}\pm5.0\times10^{5}$	$4.6\times10^{6}\pm1.1\times10^{6}$	$2.5\times10^{6}\pm3.0\times10^{5}$	$4.0\times10^{6}\pm7.0\times10^{5}$
5	$2.0\times10^{5}\pm7.5\times10^{4}$	$2.5\times10^{5}\pm2.1\times10^{5}$	$4.1\times10^{5}\pm2.1\times10^{5}$	$5.7\times10^{4}\pm2.5\times10^{4}$	$2.1\times10^{5}\pm8.5\times10^{4}$
6	$4.8\times10^{5}\pm2.5\times10^{5}$	$8.1\times10^{5}\pm2.2\times10^{5}$	$9.8\times10^{5}\pm2.6\times10^{5}$	$4.8\times10^{5}\pm1.2\times10^{5}$	$1.5\times10^{6}\pm5.2\times10^{5}$
7	$2.0\times10^{5}\pm5.8\times10^{4}$	$9.8\times10^{4}\pm2.0\times10^{4}$	$9.8\times10^{4}\pm3.6\times10^{4}$	$1.0\times10^{5}\pm1.1\times10^{5}$	$4.7\times10^{5}\pm8.2\times10^{4}$

Peak	R/H	R/S	R/C	R/L	H/S	H/C	H/L	S/C	S/L	C/L
1	1	1	1	1	p<0.01	p<0.01	1	p<0.01	0.2	p<0.01
2	0.12	p<0.01	*p*<0.01	1	1	p<0.01	1	0.33	1	p<0.01
3	0.4	*p*<0.01	1	1	1	0.77	1	p<0.01	1	1
4	*p*<0.01	*p*<0.01	1	0.11	1	p<0.01	0.72	p<0.01	1	p<0.01
5	1	0.44	*p*<0.01	1	1	0.7	1	p<0.01	0.6	p<0.01
6	0.19	*p*<0.01	1	*p*<0.01	1	p<0.01	p<0.01	p<0.01	0.25	p<0.01
7	*p*<0.01	*p*<0.01	0.78	*p*<0.01	1	1	p<0.01	1	p<0.01	p<0.01

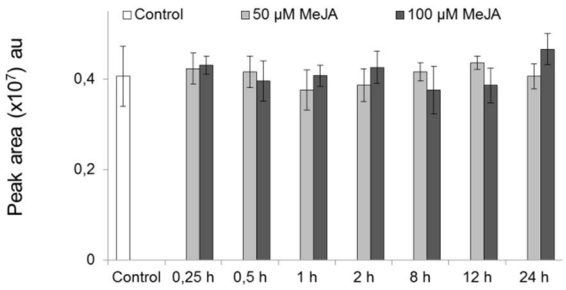
Pairwise comparisons of seven major alkaloids in five different calli lines (n=10) by ANOVA. R: root calli; H: hypocotyl calli; S: stem calli; C: cotyledon calli; L: leaf calli. Data derived from HPLC analysis. au: absorbance unit.

Appendix G: Biomass production of elicited wild type hairy root clones

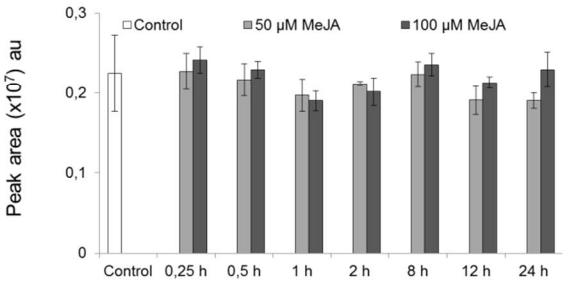


Appendix G. Biomass production of wild type hairy root clones elicited with 50 µM (grey bar) or 100 µM (black) methyl jasmonate after 0.25, 0.5, 1, 2, 8, 12 and 24 hours exposure time. Control cultures (white bar) contained 80 µL of 40% ethanol and were harvested after 24 h.

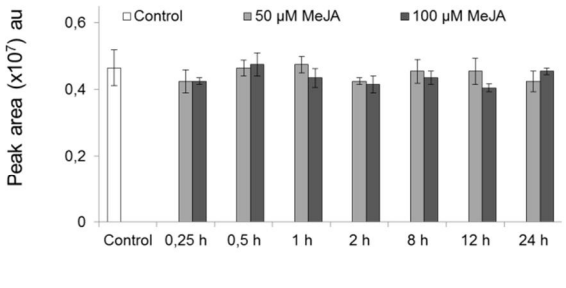
Appendix H: Elicitation experiments with chitosan



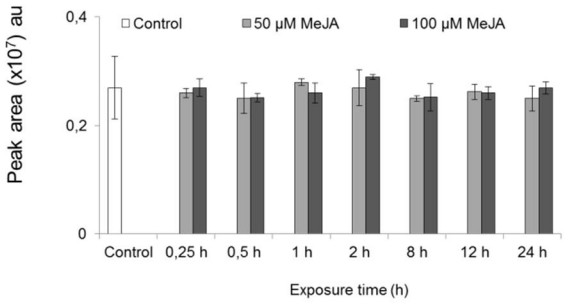
Vincanine (**12**)



Leepacine I (**21**)



Leepacine II (**22**)



Strictosidine lactam (**23**)

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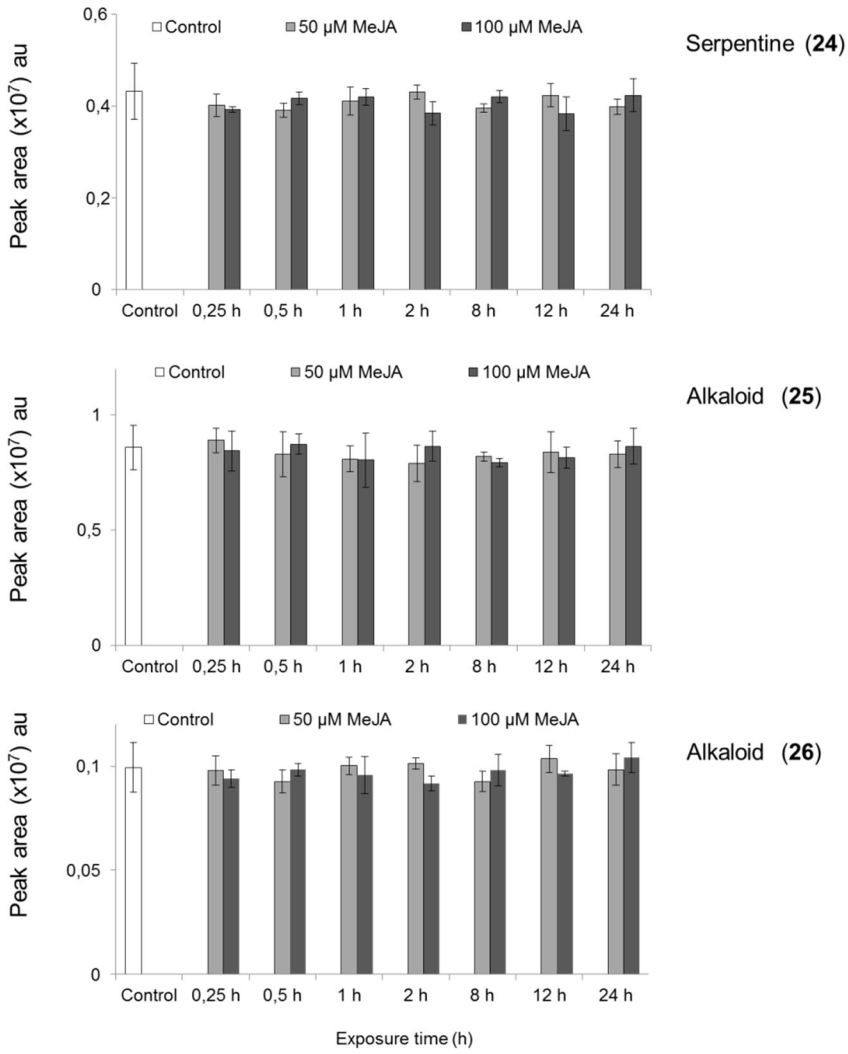

Appendix H. Accumulation of seven major alkaloids in *R. stricta* wild type hairy roots elicited with 250 and 500 mg/L chitosan after 0.25, 0.5, 1, 2, 8, 12 and 24 hours exposure time analysed by HPLC. Control contained 800 µL of 0.1 M acetic acid and were harvested after 24 h. Values are presented as mean±SD of three samples for each treatment.

Series title and number

VTT Science 93

Title	**Alkaloids of *in vitro* cultures of *Rhazya stricta***
Author(s)	Amir Behzad Akhgari Nazarlou
Abstract	*Rhazya stricta* Decne. (Apocynaceae) is a traditional medicinal plant in Middle East and South Asia. It contains a large number of terpenoid indole alkaloids (TIAs), some of which possess interesting pharmacological properties. This study was focused on biotechnological production tools of *R. stricta*, namely undifferentiated cell cultures, and an *Agrobacterium rhizogenes*-mediated transformation method to obtain hairy roots expressing heterologous genes from the early TIA pathway. *Rhazya* alkaloids comprise a wide range of structures and polarities, therefore, many analytical methods were developed to investigate the alkaloid contents in *in vitro* cultures. Targeted and non-targeted analyses were carried out using gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), ultra performance liquid chromatography-mass spectrometry (UPLC-MS) and nuclear magnetic resonance (NMR) spectroscopy. The callus cultures accumulated lower levels of alkaloids than wild type hairy roots and adventitious roots. Surprisingly, calli derived from stems contained elevated levels of strictosidine lactam compared to other cultures. The results of transformation experiments revealed that only leaves were susceptible to *Agrobacterium* infection and subsequent root induction. The transformation efficiency varied from 22% to 83% depending on the gene. A total of 17 TIAs, including glycosylated alkaloids, were identified from hairy root extracts by UPLC-MS. GC-MS indicated the occurrence of 20 non-polar TIAs belonging to nine different groups. The occurrence of pleiocarpamine, fluorocarpamine, vincamine, ajmalicine, and yohimbine isomers, analysed by GC-MS, and serpentine and its isomer, tetrahydrosecodinol as well as tabersonine, analysed by UPLC-MS, is reported here for the first time from *R. stricta*. Detailed targeted analyses by GC-MS showed that the contents of eight out of ten studied alkaloids increased as a result of elicitation by methyl jasmonate (MeJA). Multivariate analysis of NMR data showed a clear discrimination between transformed and control cultures. In conclusion, a simple and efficient gene transfer method is reported for *R. stricta* for the first time. New analytical methods were established which enabling comprehensive investigation of the alkaloids. These data serve as a basis for further utilization of biotechnological methods for *R. stricta* and its further metabolic engineering.
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Alkaloids of *in vitro* cultures of *Rhazya stricta*

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Calli derived from stems contained elevated levels of strictosidine lactam compared to other *in vitro* cultures. It was revealed that only leaves were susceptible to *Agrobacterium* infection and subsequent root induction. The transformation efficiency varied from 22% to 83% depending on the gene. A total of 17 TIAs were identified from hairy root extracts by UPLC-MS. GC-MS indicated the occurrence of 20 non-polar TIAs. The occurrence of pleiocarpamine, fluorocarpamine, vincamine, ajmalicine, and yohimbine isomers, analysed by GC-MS, and serpentine and its isomer, tetrahydrosecodinol as well as tabersonine, analysed by UPLC-MS, is reported here for the first time from *R. stricta*. GC-MS analyses showed that the contents of eight out of ten studied alkaloids increased as a result of elicitation by methyl jasmonate. Multivariate analysis of NMR data showed a clear discrimination between transformed and control cultures.

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