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ORIGINAL ARTICLE

Establishment of transgenic *Rhazya stricta* hairy roots to modulate terpenoid indole alkaloid production

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Abstract
Key message **Transgenic hairy roots of *R. stricta* were developed for investigation of alkaloid accumulations. The contents of five identified alkaloids, including serpentine as a new compound, increased compared to non-transformed roots.**
Abstract *Rhazya stricta* Decne. is a rich source of pharmacologically active terpenoid indole alkaloids (TIAs). In order to study TIA production and enable metabolic engineering, we established hairy root cultures of *R. stricta* by co-cultivating cotyledon, hypocotyl, leaf, and shoot explants with wild-type *Agrobacterium rhizogenes* strain LBA 9402 and *A. rhizogenes* carrying the pK2WG7-*gusA* binary vector. Hairy roots initiated from the leaf explants 2 to 8 weeks. Transformation was confirmed by polymerase chain reaction and in case of GUS clones with GUS staining assay. Transformation efficiency was 74 and 83 % for wild-type and GUS hairy root clones, respectively. Alkaloid accumulation was monitored by HPLC, and identification was achieved by UPLC-MS analysis. The influence of light (16 h photoperiod versus total darkness) and media composition (modified Gamborg B5 medium versus Woody Plant Medium) on the production of TIAs were investigated. Compared to non-transformed roots, wild-type hairy roots accumulated significantly higher amounts of five alkaloids. GUS hairy roots contained higher amounts two of alkaloids compared to non-transformed roots. Light conditions had a marked effect on the accumulation of five alkaloids whereas the composition of media only affected the accumulation of two alkaloids. By successfully establishing *R. stricta* hairy root clones, the potential of transgenic hairy root systems in modulating TIA production was confirmed.

Keywords *Rhazya stricta* (Apocynaceae) · Medicinal plant · *Agrobacterium rhizogenes* · Transgenic hairy roots · GUS reporter gene · Terpenoid indole alkaloid (TIA)

Introduction

The genus *Rhazya* (Apocynaceae) 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. This species has been used as a folk medicine to treat several diseases, e.g. fever and chronic rheumatism (e.g. Gilani et al. 2007). Phytochemistry of *R. stricta* has been studied extensively and led to the isolation and structural elucidation of more than 100 constituents from different parts of the plant, most of which are terpenoid indole alkaloids (TIAs) (Gilani et al. 2007) categorized in 17 different groups (Atta-ur-Rahman et al. 1989). TIAs are a structurally diverse class of alkaloids, many of which are pharmacologically valuable compounds, e.g. vinblastine, vincristine and ajmaline. TIAs originate from the coupling of tryptamine (indole moiety) with the iridoid glucoside

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secologanin (terpene moiety) facilitated by strictosidine synthase to provide the central intermediate, strictosidine. The biosynthetic pathway towards secologanin, tryptamine and some alkaloids, e.g. serpentine, has been characterized (O'Connor and Maresh 2006; Miettinen et al. 2014). However, the biosynthetic steps for the production of several alkaloids, e.g. strictosidine lactam, from strictosidine are not yet resolved.

Many of the *Rhazya* alkaloids have been isolated from other Apocynaceae plants, too and are thus Apocynaceae-specific. However, aspidospermiose, aspidospermidose, leepacine, strictanine, strictibine and strictanol (Atta-ur-Rahman et al. 1989) are species-specific TIAs i.e. have been reported so far only from *R. stricta*.

At least 15 of the identified TIAs from *R. stricta* have been subjected to pharmacology and toxicology assays indicating predominantly their anti-cancer and anti-bacterial potential (Gilani et al. 2007). Furthermore, the crude ethanolic extract of *R. stricta* fruit has shown anti-bacterial and lipoxygenase activities, as well as acetylcholinesterase inhibitory effect (Sultana and Khalid 2010). It was reported that the crude extract of *R. stricta* potently inhibited cellular growth and colony formation of human breast and lung cancer cell clones through apoptosis induction (Baeshen et al. 2012; Elkady 2013). At least in the case of rhazinilam, the promising anticancer activity of the natural product from *R. stricta* has resulted in the structural improvement of the lead compound through medicinal chemistry (Edler et al. 2009).

TIAs, as secondary metabolites, are usually present in small quantities in plants, and their total chemical synthesis is often difficult and uneconomical because of their very complex stereochemical structures (Ziegler and Facchini 2008). Biotechnological methods for sustainable production of phytochemicals are attractive alternatives for the production of high-value compounds (Rischer and Oksman-Caldentey 2005). Generally undifferentiated in vitro cell cultures (e.g. callus cultures) are genetically unstable and synthesize valuable secondary metabolites at very low levels (Sevón and Oksman-Caldentey 2002). On the contrary, organ cultures, e.g. hairy roots which do not require the addition of growth regulators for their cultivation can make a significant contribution to the stable and contained production of secondary metabolites in comparison to callus and cell suspension cultures and even to intact plants (Oksman-Caldentey and Inzé 2004). Hairy roots are known to produce a spectrum of secondary metabolites that are not present in the parent plant. Furthermore, transgenic roots offer potential for genetic engineering (Georgiev et al. 2007). Several reviews have been devoted to medicinal plant hairy root cultures and their applications during the last few decades (e.g. Sevón and Oksman-Caldentey 2002; Ono and Tian 2011).

Callus and cell suspension cultures of *R. stricta* have been established, and major indole alkaloids in cell suspension cultures have been investigated (Pawelka and Stöckigt 1986). Furthermore, hybrid cell cultures of *Rauwolfia serpentina* and *Rhazya stricta* have been developed (Kostenyuk et al. 1995; Sheludko et al. 1999, 2000), and their alkaloid patterns have been studied (Gerasimenko et al. 2001). In contrast to the diversity of compounds described in *R. stricta* and in vitro and in vivo bioactivity data derived from human and animal studies, there are not reports on metabolic engineering of *R. stricta* aiming at a sustainable production of the phytochemicals. Genetic transformation of *R. stricta* has so far neither been reported.

Our interest in *Rhazya* alkaloids covers: the development of hairy root cultures and the analytical investigation by HPLC/UPLC-MS and GC–MS. This report describes (1) the initiation of transgenic hairy roots by inoculating different explants from in vitro germinated *R. stricta*; (2) PCR analysis of putative hairy roots in order to determine the transformation efficiency; (3) identification of several alkaloids by correlating high performance liquid chromatography (HPLC) and ultra performance liquid chromatography- mass spectrometry (UPLC-MS) data; (4) monitoring of selected TIAs in the established hairy roots by a HPLC method; and (5) effects of major physical and chemical factors on hairy root growth and accumulation of alkaloids.

Materials and methods

Plant material

Seeds of *R. stricta* were collected in Iran (Hormozgan province, Minab zone in Persian Gulf area 27°08′48″N 57°04′48″E) with a necessary permit (no. 14179/244/25) from National Plant Gene-Bank of Iran (NPGBI).

Sterilization and germination of seeds

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 s, 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 2 days at 24 ± 1 °C in the dark on sterile moist filter paper for 2–3 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^{-1} 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 2 weeks, the seedlings were transferred aseptically to sterile plastic boxes containing solid-modified Gamborg B5 medium.

Preparation of *A. rhizogenes*

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 (https://gateway.psb.ugent.be/vector/show/pH7WG2D/search/index/overexpression/any). Prior to infection, the 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) and 100 mg/l spectinomycin (for bacteria transformed by *gusA* gene).

Establishment of hairy root transformation

Eight 2 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 2 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. Both from cotyledons and leaves the apices were removed and the leaf midrib (abaxial side) wounded by a sterile syringe needle loaded with bacterial culture. The explants were placed on the adaxial side on modified Gamborg B5 medium. Excised stem segments were pricked either at the node or the internode. Explants wounded in the same manner using a sterile needle without bacteria served as controls. The explants were kept for 2 days in the dark at 24 ± 1 °C for co-cultivation. After 2 days, leaves and stems were placed on medium supplemented with 500 mg/l cefotaxime (Duchefa, The Netherlands) and 10 mg/l meronem (AstraZeneca, UK) to eliminate residual bacteria and kept in the dark at 24 ± 1 °C.

Emerging putative transgenic hairy roots (1–1.5 cm in length) at the wounded sites of infected explants were designated as independent clones, excised and placed on antibiotic (cefotaxime and meronem) containing medium for 4 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 antibiotic/hormone-free medium and maintained at 24 ± 1 °C in the dark. Data for transformed root formation were recorded 8 weeks after the initial inoculation.

Establishment of selection conditions

The natural hygromycin resistance of *R. stricta* was tested in order to find the right selection condition by growing five randomly chosen wild-type hairy root clones (presence of *rolB* gene confirmed by PCR) with each three replicates 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 hygromycin (Duchefa, The Netherlands). The growth and colour of hairy roots were recorded after 4 weeks.

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 (1987). The roots were placed in 1 ml of X-GLUC solution (5-bromo-4-chloro-3-indolyl glucuronide; Duchefa, The Netherlands) and incubated at 37 ± 1 °C until the characteristic blue colour appeared (2–16 h).

Genomic DNA extraction and PCR amplification

Total genomic DNA from 50 to 100 mg fresh weight of all different putative hairy root clones and of non-transformed root was extracted with the acetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). A mixer mill (Retsch MM301, Haan, Germany) was used to crush the frozen roots with stainless steel beads (4-mm diameter) for 2 min at 29 Hz.

PCR amplifications were performed separately with primers (Sigma) specific for the Ri plasmid gene *rolB* (Sevón et al. 1995), for the Ri plasmid virulence gene *virD* (Hamill et al. 1991) and for the *gusA* reporter gene, which contains ATTB1 and ATTB2 regions of the Gateway vector 5′-GGGGACAAGTTTGTACAAAAAAGCAG GCT-3′ and 5′-GGGGACCACTTTGTACAAGAAA GCTGGGT-3′. The amplification reaction was performed with a G-Storm GS482 thermal cycler (G-Storm and Kapa Biosystems, UK). The PCR reaction mixtures were loaded directly onto 1 % agarose/TE (Tris/EDTA) gel for electrophoretic analysis. A 100 base pair ladder (Fermentas, GmbH Germany) was used as a molecular marker for the PCR-amplified DNA fragments.

Effect of light and media

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 4 weeks for growth and alkaloid production. The axenic maintenance cultures were subcultured at 4-weeks 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^{-1} 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 4 weeks.

In the other experiment, 100 mg of wild-type hairy roots was 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 4 weeks at 24 ± 1 °C.

Extraction of alkaloids

Alkaloids were selectively extracted from roots according to Samuelsson and Bohlin (2010), 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 4000 rpm (3220×*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 4000 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 4000 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 and UPLC-MS analysis. Culture medium was lyophilised, and the described extraction procedure, including pH adjustment at different steps was followed. All organic solvents were of analytical grade. Purified water was obtained by PURELAB® Ultra Analytic Water Purification System (ELGA LabWater, High Wycombe, UK).

HPLC analysis of alkaloids

HPLC analysis was performed with a Waters 2996 system coupled to a Waters 996 photodiode array detector monitoring a wavelength range of 200–450 nm. Xterra C18 column (5 µm, 250 mm × 4.6 mm; waters) connected to Xterra MS C18 precolumn (5 µm, 20 mm × 3.9 mm, waters) was employed. The mobile phase was according to Gerasimenko et al. (2001), 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. Reference vincanine was purchased from Accurate Chemical & Scientific Corporation, USA.

UPLC-MS analysis of alkaloids

The UPLC-MS runs were performed on a Waters Micromass Quattro micro™ triple quadrupole mass spectrometer with electrospray source combined with Waters Acquity Ultra Performance LC (UPLC) with 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 stayed constant up to 20 min.

The runs were performed both in positive (ESI^+) and negative (ESI^-) electrospray ionization mode, and the data were 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 was scanning wavelengths from 200 to 420 nm.

Reference vincanine was used for identification of *Rhazya* vincanine by their comparing retention times, HPLC–UV, UPLC-UV, and MS data. For other alkaloids, comparisons were made with the literature. In addition, extracted molecular ions from TIC were utilized to confirm the identification.

Precision of the HPLC method

The precision of the analytical procedure was evaluated by assessing the repeatability and intermediate precision of the

HPLC method. The evaluation was based on the peak areas of the seven major compounds present in the extracts. Repeatability (intra-assay precision) was deduced from ten replicate injections (20 µl) of a *R. stricta* wild-type hairy root extract within a day, and intermediate precision (inter-day precision) was calculated based on six injections carried out in three consecutive days ($n = 3 \times 6$). The precision is expressed as the coefficient of variation of intra-day and inter-day measurements.

Statistical analysis

Statistical analyses were performed using Systat 13 statistical software (Systat Software Inc, USA). The data were subjected to analysis of variance (ANOVA) with Bonferroni test to determine the significance between means. Probability level $p \leq 0.05$ was considered significant. Data are expressed as mean ± standard deviation.

Results

Seed sterilization and germination

Because of high contamination in the germinated seedlings, a two-step sterilization procedure of the seeds 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 at all. Pericarps of seeds were thick, which makes difficult the germination of seedlings. Stirring the seeds in sterile water overnight induced swelling of the pericarp.

Initiation of hairy roots

R. stricta wild-type hairy root clones were induced to be used as controls. The needle-wounded infection sites turned dark within 1–2 weeks in all explants. Within

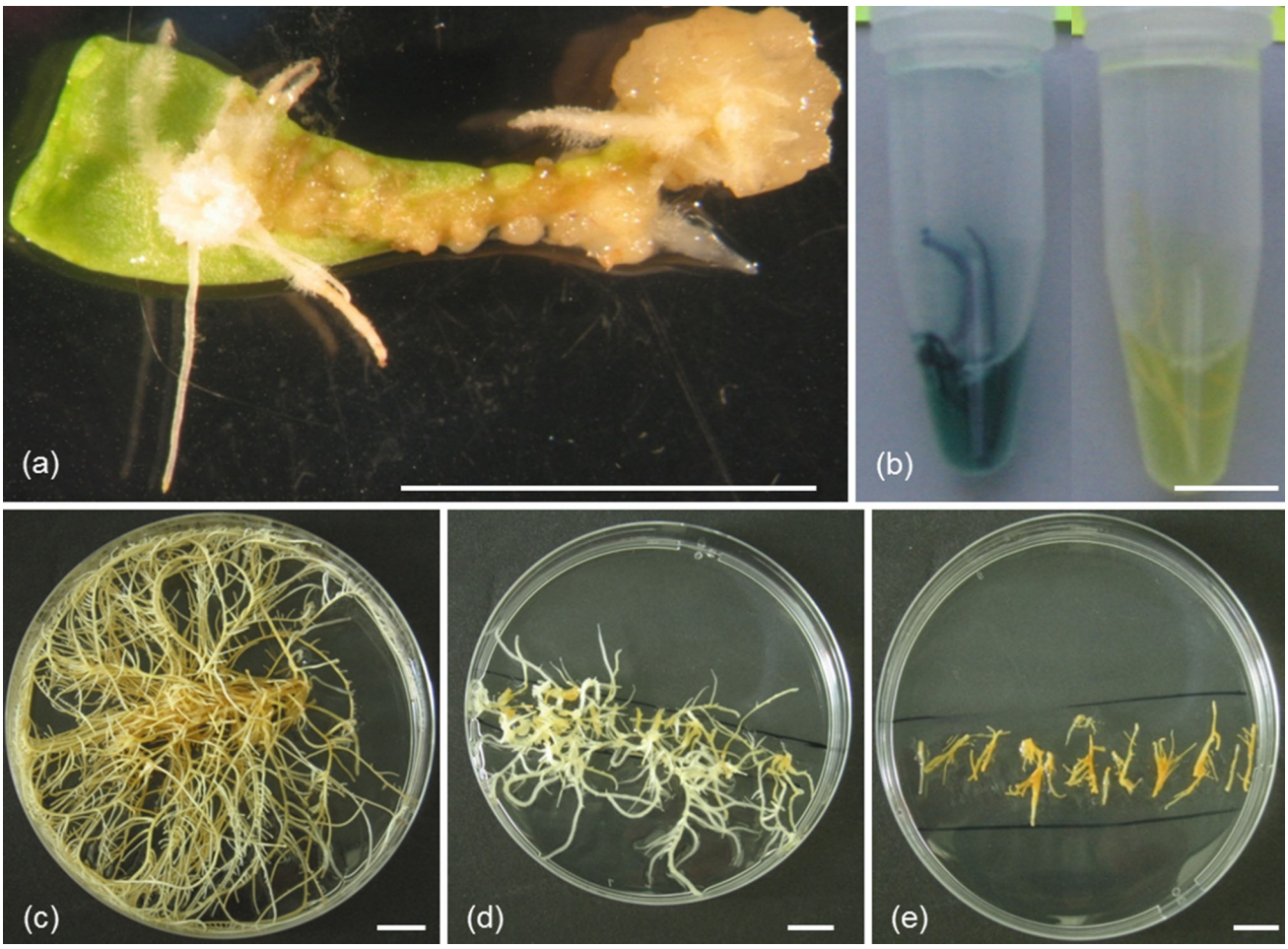

Fig. 1 **a** Development of *R. stricta* wild-type hairy roots 6 weeks after inoculation with *A. rhizogenes*. **b** Histochemical GUS assay of a *R. stricta* transgenic GUS clone (*left*) and a wild-type control (*right*). **c** wild-type hairy roots of *R. stricta* grown on hygromycin-free media, **d**, **e** on media supplemented with 1 and 2 mg/l hygromycin, respectively, 4 weeks after transfer to selection conditions

Table 1 Hairy root induction by *A. rhizogenes* on leaf explants of *R. stricta*

Root type	Total infected leaves	Leaves generated transformed hairy roots	Putative hairy roots	Transformed hairy roots	Transformation efficiency (%)[a]
Wild	42	18	27	20	74
GUS	15	7	12	10	83

[a] Frequency of inoculated leaf explants giving rise to at least one transformed root was assessed 8 weeks after co-culture with *A. rhizogenes*

3–4 weeks after injury, white to light yellow callus appeared (Fig. 1a), independently of *A. rhizogenes* infection. Most hairy roots emerged within 2–6 weeks after inoculation mainly from callus on the leaf midrib including leaf stalks (Fig. 1a). Only leaves were susceptible to *Agrobacterium* infection and subsequent root induction but neither cotyledons, hypocotyls nor stem segments. 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. These roots, however, did not elongate after sub-culturing. Transformed roots, containing the *rolB* gene, showed rapid growth and extensive branching in both liquid and solid medium (Fig. 1c). Altogether 27 putative wild-type hairy roots were obtained from leaf explants within 8 weeks after the initial inoculation (Table 1).

Hygromycin sensitivity of *R. stricta* wild-type hairy roots

The inherent 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, hairy roots exhibited normal growth with white root tips but grew somewhat slower compared to control cultures (Fig. 1d). 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 (Fig. 1e). Therefore, a concentration of 2 mg/l hygromycin was chosen for selection of putative GUS hairy root clones.

Generation of transgenic GUS hairy roots

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, all putative GUS hairy roots were only produced from leaf explants. Taking into account, the results of the hygromycin sensitivity test, selection and proliferation of putative transgenic (GUS) hairy root clones were carried out on hormone-free solid medium supplemented with 2 mg/l hygromycin, 500 mg/l cefotaxime and 10 mg/l meronem. Eventually ten GUS hairy root clones out of 12 putative clones survived and were subjected to the GUS assay and PCR.

Confirmation of genetically transformed hairy roots

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 blue colour was not observed even after incubation for 72 h at 37 ± 1 °C (Fig. 1b).

The absence of *virD* gene indicated all the hairy roots to be *Agrobacterium*-free (Online Resource 1). The PCR products showed the expected size of 780 bp fragment for *rolB* and ~2 kb for *gusA*. From 20 out of 27 *virD*-free putative wild-type hairy roots, the *rolB* fragment was amplified (74 %). From ten *virD*-free GUS clones out of twelve putative GUS hairy roots, *rolB* and *gusA* were amplified (83 %). Table 1 summarizes all results including transformation efficiency calculated as the percentage of inoculated explants that initiated at least one transformed root (Alpizar et al. 2006).

Growth and morphology of generated roots

Transformed roots characterized by rapid branching and highly plagiotropic growth 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 10 weeks of liquid/solid culture initiation. Intact in vitro roots grew slowly in hormone-free medium in the dark. Meanwhile, *R. stricta* hairy roots have been maintained by sub-culturing in 4 weeks intervals on solid Gamborg B5 medium for over 6 years.

HPLC–PDA analysis

A typical HPLC chromatogram of a hairy root crude extracts (Fig. 2a) showed seven abundant peaks with

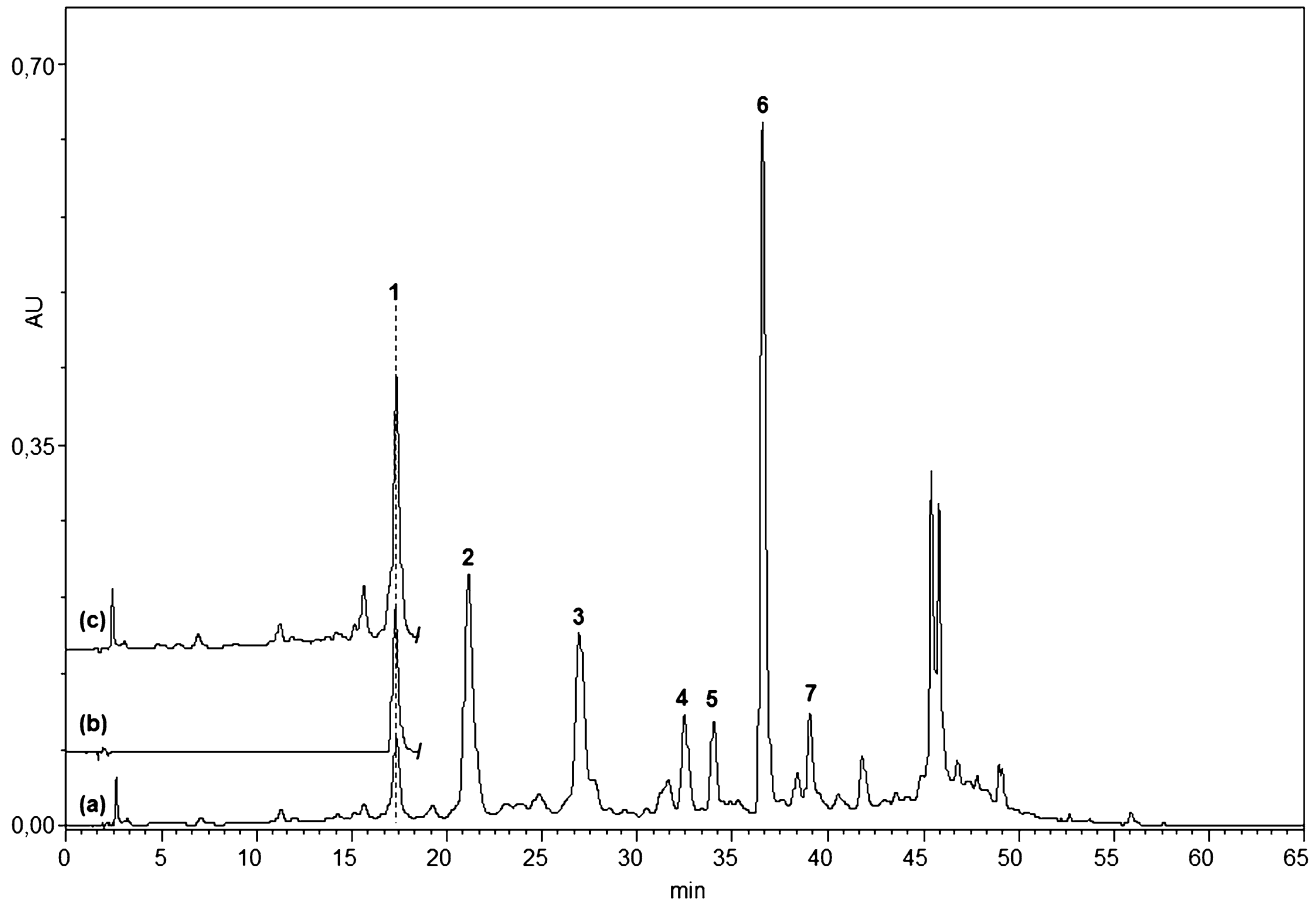

Fig. 2 A chromatogram of a *R. stricta* wild-type hairy root extract detected at 255 nm. **a** The peaks are numbered according to their retention time from *1* to *7*. *Peak **1*** was identified as vincanine based on the comparison of retention time and UV spectrum and by spiking a sample with vincanine standard. Overlay **b** the partial chromatogram of vincanine standard. Overlay **c** represents the partial chromatogram of a sample which has been spiked by vincanine. *AU* UV absorbance unit

baseline separation which were labelled in the order of increasing retention time as compounds **1**–**7**. Non-separated peaks at 46 min were not included in comparisons. In extracts of the culture media alkaloids were not detected.

Vincanine (peak **1**, Online Resource 2) was identified (Table 2) by comparing the t_R and UV of reference (Fig. 2b) and by spiking (Fig. 2c). The HPLC–UV data of alkaloids indicated that most of the spectra had three intense maxima, and two of them were identical (**2**–**3**). Also, a flat maximum at 365–367 nm was typical for compounds **5**–**7**. Compound **4** had a high absorbance at 227 nm followed by low intense maxima at 283 and 290 nm. Peak **5** exhibited intense maxima at 210, 249 and 307 nm, but a low absorbance at 367 nm. In HPLC analysis, compound **6** exhibited an abundant sharp peak accompanied by a minor alkaloid **7**. The UV spectra of both bore a close similarity to each other (Table 2). Further identifications were obtained by UPLC-PDA-MS analyses.

Alkaloid composition analysed by UPLC-PDA-MS

Identifications of seven alkaloids of *Rhazya* extract were based on resemblance between UV data from HPLC analyses (Table 2) and UV (Online Resource 3) and MS data from UPLC-MS (Table 2). The UPLC chromatogram generated at 255 nm is illustrated in Online Resource 4. All alkaloids were identified in positive (ES^+) (Online Resource 4) and negative ion (ES^-) mode, except alkaloids **6** and **7** by ES^- only (Table 2). The identification was further confirmed by extracted ion monitoring (Online Resource 4).

In the extract, vincanine had identical UV absorption (Online Resource 3) and molecular ion at *m/z* 293 (Table 2, Online Resource 4) as the pure reference. Compounds **2** and **3** had very similar UV spectra, and the MS data confirmed molecular ions at *m/z* 351 ES^+ and at *m/z* 349 ES^-, respectively (Table 2). The occurrence of leepacine **2** and its isomer **3** (Online Resource 2) was suggested by the extracted ion monitoring (Online Resource 4).

Table 2 Comparison of HPLC–UV and UPLC-PDA-MS data of hairy root alkaloids

Peak	Compounds	HPLC–UV		UPLC-MS					References
		t_R^a (min)	λ_{max} (nm)	t_R^b (min)	λ_{max} (nm)	MW	$[M+H]^+$ fragments *m/z* (rel. int. %)	$[M-H]^-$ fragments *m/z* (rel. int. %)	
1	Vincanine	17.35	243, 300, 364	2.46	246, 300, 365	292	293 (100)	291 (100)	Ahmad et al. (1977); Shakirov et al. (1996)
2	Leepacine isomer I	21.13	208, 252, 305	1.95	210, 250, 298sh, 304	350	351 (100)	349 (tr)	Atta-ur-Rahman et al. (1991)
3	Leepacine isomer II	26.95	206, 250, 305	2.14	210, 250, 298sh, 305	350	351 (100)	349 (tr)	Atta-ur-Rahman et al. (1991)
4	Strictosidine lactam	32.48	227, 283, 290	1.31	217 sh, 227, 283, 290	498	499 (100), 337 (98)	497 (100), 577 (71)[c]	Atta-ur-Rahman et al. (1991); Yamazaki et al. (2003); Dhooghe et al. (2008); Donalisio et al. (2013)
5	Serpentine	34.00	206, 249, 308, 367	4.28[d]	210, 248, 307, 367	348	349 (100)	347 (100)	Suttipanta et al. (2011); Chen et al. (2013); Ferreres et al. (2010)
				4.60[e]	210, 249, 307, 367	348	349 (100)	347 (100)	
6	Unidentified	36.56	220, 255, 292, 307, 365	12.73	226, 254, 292, 307, 366	628	629 (100), 126 (96)	n.d	
7	Unidentified	39.05	220, 255, 292, 307, 366	13.72	225, 250, 291, 308, 365	628	629 (100), 126 (92)	n.d	

tr traces, *n.d* not detected

[a] From Fig. 2

[b] From Online Resource 3

[c] Acetate adduct

[d] Isomer I

[e] Isomer II

According to the UV spectra and MS fragmentation, compound **4** was identified as strictosidine lactam (Table 2, Online Resource 2). UPLC-UV analysis showed weak shoulders at 217, 222, 283, and 290 nm (Table 2, Online Resource 3), while the two first ones were strongly overlapped by 227 nm in HPLC–UV.

Compound **5a**, which displayed four intense UV maxima and molecular ions (Table 2) at *m/z* 349 (ES^+) and at *m/z* 347 (ES^-), respectively, was identified as serpentine isomer I (Online Resource 2). It was a minor alkaloid in HPLC analysis (Fig. 2), but its UV spectrum appeared identical with that obtained by UPLC (Table 2, Online Resource 3). A compound having the same molecular ions and UV profile, which eluted close to **5a** indicating an isomer of serpentine (Table 2), was designated serpentine isomer II (**5b**, Online Resource 4). The occurrence of these isomers (Online Resource 1) in UPLC-MS analysis suggests that peak **5** in HPLC (Fig. 2) is actually a mixture of two alkaloids. Isomer I (**5a**) had higher intensity than isomer II (Online Resource 4, channel *m/z* 349).

In the case of the compounds **6** and **7**, UPLC-UV showed a broadened peak zone with very low UV intensity (Online Resource 4). These alkaloids had fairly similar UV spectra (Table 2, Online Resource 2). Although they shared base peak and molecular ion at *m/z* 629 and with fragment at *m/z* 126 (Table 2), they could not be identified.

Alkaloid accumulation in hairy roots

The repeatability of the developed HPLC method expressed as the coefficient of variation of the peak areas of the seven major peaks in the extract was 2.1–2.8 %. The intermediate precision ranged between 3.4 and 4.7 %. Four-week-old wild-type ($n = 20$), GUS hairy root clones ($n = 10$) grown in liquid medium and the roots ($n = 10$) obtained from in vitro grown *R. stricta* were subjected to HPLC analysis for monitoring the accumulation of alkaloids.

A comparison of wild-type hairy roots with GUS hairy root clones indicated that the accumulation of leepacine

isomer I (**2**), leepacine isomer II (**3**), strictosidine lactam (**4**), serpentine (**5**) and unidentified alkaloid **7** did not differ significantly (Fig. 3a). Concentrations of vincanine (**1**) and unidentified alkaloid **6**, on the other hand, were significantly higher in wild-type hairy roots. GUS hairy root clones had significantly higher concentrations of vincanine and compound **4** compared to non-transformed roots (Fig. 3a). Finally, wild-type hairy roots contained significantly higher amounts of vincanine (**1**), leepacine isomer II (**3**), strictosidine lactam (**4**), serpentine (**5**) and alkaloid **6** than non-transformed roots.

Effect of light and different media on hairy root growth and alkaloid production

The biomass production of wild-type hairy roots was independent of the media used. In Woody Plant Medium the fresh weight of hairy roots grown for 4 weeks was 3.1 ± 0.4 g, dry weight 1.0 ± 0.3 g and extraction yield 2.4 ± 0.7 mg/50 mg dry weight of plant material. 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 2.2 ± 0.4 mg/50 mg dry weight of plant material. However, hairy roots grown in liquid modified Gamborg B5 medium contained a significantly higher amount of compounds **4** and **6** compared with hairy roots grown in liquid Woody Plant Medium (Fig. 3b).

Hairy root clones grown in the dark contained significantly higher amounts of vincanine and compounds **4**, **5** and **6** compared to hairy root clones grown under the 16 h photoperiod. The difference in vincanine concentration was especially pronounced, i.e. a six-fold increase compared to the 16 h photoperiod. On the other hand, the amount of compound **2** in hairy root clones grown in the dark was significantly lower compared to the hairy root clones grown under the 16 h photoperiod (Fig. 3c). There was a statistically significant difference in the fresh weight between hairy roots grown in the dark or under a 16 h photoperiod ($n = 10$) but the differences in dry weight and extraction yield were not statistically significant (Fig. 3d).

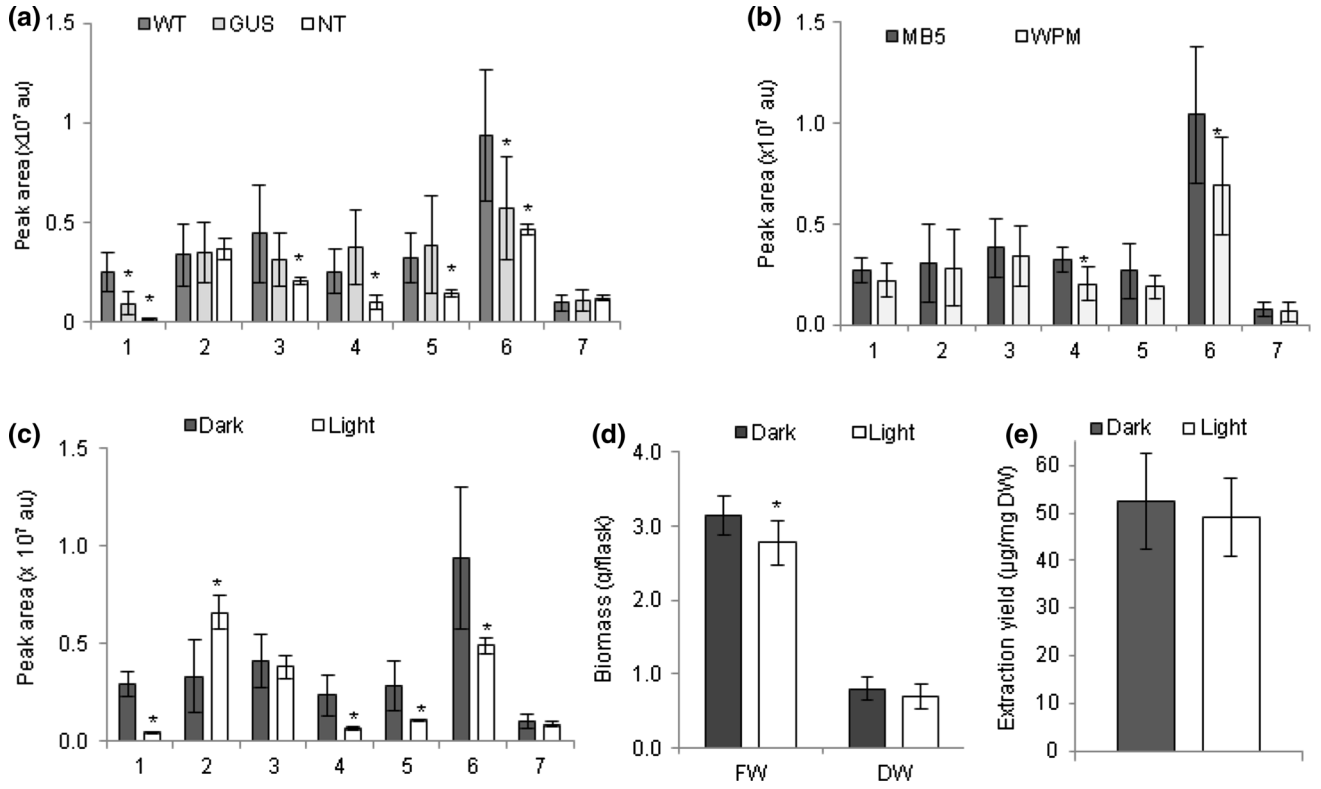

Fig. 3 Accumulation of compounds **1**–**7** in (**a**) wild-type hairy roots ($n = 20$; *black bar*, WT), GUS hairy roots ($n = 10$; *grey bar*, GUS) and non-transformed roots ($n = 10$; *white bar*, NT) of *R. stricta* grown in modified Gamborg B5 media. Wild-type hairy roots were used as indicators in the pairwise significance tests (**b**) wild-type hairy root clones of *R. stricta* grown either in liquid modified Gamborg B5 media ($n = 10$ in triplicate, *grey bar*, MB5) or in liquid Woody Plant Media ($n = 10$ in triplicate, *white bar*, WPM) (**c**) wild-type hairy root clones of *R. stricta* grown either in the dark (*black bar*) or under a 16 h photoperiod condition (*white bar*) grown in modified Gamborg B5 media. **d** Biomass production of wild-type hairy root clones grown either in the dark (*black bar*) or under a 16 h photoperiod condition (*white bar*). **e** Extraction yields of wild-type hairy root clones grown either in the dark (*black bar*) or under a 16 h photoperiod condition (*white bar*). *Bars* represent the mean values ± SD. *Significant difference in the mean (student's *t* test, $p < 0.05$). *Numbers* refer to compounds from Table 2. *AU* absorbance unit

Discussion

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 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* (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. *Catharanthus roseus* (Parr et al. 1988), *Hyoscyamus* spp. (Vanhala et al. 1995), *P. somniferum* (Le Flem-Bonhomme et al. 2004) and *Picrorhiza kurroa* (Verma et al. 2007). However, there are also reports, e.g. from *Gentiana macrophylla* (Tiwari et al. 2007) and *Tylophora indica* (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 2-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 2 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* (Tzfira et al. 1996), *Aesculus hippocastanum* (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 generated, and the final concentration of hygromycin for transformed root selection was 2 mg/l. Transformed GUS hairy roots grew on media supplemented with 2–25 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 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 4 weeks in *Scutellaria baicalensis* 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* was low when leaves were used as explants (Fu et al. 2005).

Broad phenotypic variation was observed among *R. stricta* hairy root clones. 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 have 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).

In the present study, two analytical methods including HPLC–PDA and UPLC-PDA-MS were used for the separation and identification of major alkaloids from *R. stricta* hairy roots. The use of phosphate buffer and hexane sulphonic acid as an ion exchange reagent resulted in clearly improved separation of alkaloids. However, the use of phosphate buffer and reagent is not compatible with LC–MS analysis. In the case, when isolated or commercially pure alkaloids have been available, e.g. vincanine, 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, we developed a LC–MS method with a compatible solvent system to facilitate compound identification.

The optimised HPLC method was shown to be repeatable and reliable with intra-assay precision below 3 % and inter-day precision below 5 %. The method enabled the monitoring of seven major components in the extracts, of which two compounds eluting after 40 min were not satisfactorily resolved (Fig. 2). In general, *R. stricta* wild-type and GUS hairy root clones contained higher concentrations of the detected compounds than seedling roots (Fig. 3a).

It is known that secondary metabolite biosynthesis in hairy root cultures is influenced by chemical and physical factors (Toivonen et al. 1989). We showed that there were not 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, concentrations of strictosidine lactam (**4**), serpentine (**5**) and compound **6** in transformed roots grown in modified Gamborg B5 medium were significantly higher than in roots grown in Woody Plant Medium (Fig. 3b). Furthermore, the absence of light had a significant effect on the accumulation of alkaloids even though there were not significant differences in dry weight (Fig. 3d) and extraction yield (Fig. 3e) in different lighting conditions. Production of four out of seven compounds monitored by HPLC increased in cultures grown in the dark (Fig. 3c). On the other hand, the concentration of leepacine isomer II (**3**) 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 (Fig. 3c). 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). Our findings are in line 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*, respectively.

Among the terpenoid indole alkaloids, vincanine (**1**) is a typical strychnos-type alkaloid which 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 our UPLC-PDA-MS analyses, the spectroscopic data (Table 2, Online Resource 3 and 4) agreed well with high-resolution mass spectrometry (HRMS) analyses of isolated vincanine from *Rhazya* (Ahmad et al. 1977) and from *Vinca* (Shakirov et al. 1996).

In the current analysis, extracted ion recording showed two alkaloid peaks (Online Resource 4) with identical MS data and UV profiles which were identified as leepacine isomers (Table 2, Online Resource 1). These data were in accordance with those reported by Atta-ur-Rahman et al. (1991) except the slight maximum at 298 nm and at 305.

Strictosidine lactam (**4**) is a highly polar alkaloid containing a sugar moiety (Atta-ur-Rahman et al. 1991) in *R. stricta* and its cell cultures (e.g. Pawelka and Stöckigt 1986). The present results were in line with three UV maxima and MS data (Table 2, Online 3) obtained for isolated alkaloid from *Rhazya* analysed by EI-HRMS (Atta-ur-Rahman et al. 1991). The MS data for alkaloid **4** from other plant species (Yamazaki et al. 2003; Dhooghe et al. 2008; Donalisio et al. 2013) have been also consistent with the present results.

Serpentine (**5**) is a quaternary alkaloid which is typical for *Rauwolfia* (Stöckigt et al. 1997) and *Catharanthus* (van der Heijden et al. 2004) species, but it had not been reported in *Rhazya* so far. In this study, extracted ion recording by ES^+ (Online Resource 4, channel *m/z* 349) and ES^- first located two peaks. The present UV and ES^+-MS data (Online Resource 3, Table 2) agree well with that of serpentine from *C. roseus* extracts (Suttipanta et al. 2011) and with its isomer reported by Chen et al. (2013) and Ferreres et al. (2010).

The high intensity of the fragment *m/z* 126 in **6** and **7** (Table 2) would suggest the occurrence of secodine-type indole alkaloids as previously reported from *R. stricta* (Evans et al. 1968; Cordell et al. 1970a, b).

Conclusion

A. rhizogenes-mediated transformation of *R. stricta* was developed, and hairy roots were generated. The suitability of hygromycin as a selective agent for screening of transgenic cultures showed to be efficient. The alkaloid pattern of hairy root clones was investigated by HPLC and UPLC-MS, and five indole alkaloids were identified. Most of the alkaloids, monitored by HPLC, significantly accumulated in hairy roots compared to non-transformed roots. We also showed that most alkaloids accumulated in higher amounts in cultures grown in the dark as compared to those grown in the light. The developed method enables metabolic engineering of *R. stricta* hairy root clones with the aim of increasing the production of selected terpenoid indole alkaloids.

Author contribution statement Conceived and designed the experiments: AA, IL, HV, HR. Performed the experiments: AA, TY, and IL. Analysed the data: AA, IL. Contributed to the writing of the manuscript: AA, TY, IL HV, KMOC, and HR.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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