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BASIC AREAS
Article

Rhizobium rhizogenes-mediated transformation of *Rhodiola rosea* leaf explants

Marta Iraburu Martínez[1], Gregorio Barba-Espín[1,2], Bruno Trevenzoli Favero[1], Henrik Lütken[1*]

1. University of Copenhagen - Faculty of Science - Department of Plant and Environmental Sciences - Tåstrup, Denmark.
2. Centro de Edafología y Biología Aplicada del Segura - Departmento de Mejora Vegetal - Grupo de Biotecnología de Frutales - Murcia, Spain.

ABSTRACT: *Rhodiola rosea* L. is an endangered medicinal plant distributed in mountains and in high latitude regions. For its conservation, sustainable methods for the obtaining of its bioactive compounds must be developed. This work hypothesized that leaf, stem and rhizome explants of *R. rosea* from different geographical origins respond differently to inoculation with *Rhizobium rhizogenes* agropine strain ATCC43057. The objective was to generate *R. rosea* hairy roots (HRs) containing *rol*-genes. These HRs could be cultivated under axenic conditions for the extraction of the medical compounds rosavinoids and salidroside. Hereby, production of bioactive compounds could be improved per plant biomass. Thirteen *R. rosea* accessions of Alpine, Scandinavian, Nordic Gene Bank (NGB) and Russian origins were compared for their explant survival and HR formation. Significant differences were observed among plants from different geographical origins, where the NGB leaf explants exhibited up to 70% of HR formation and the Russian accessions did not exhibit HRs at all. Moreover, maintaining explants in light conditions after *R. rhizogenes* inoculation resulted in higher explant survival and HR formation rate (35%) when compared with explants kept in darkness (9%). Taken together, an efficient HR formation in roseroot by inoculation of *R. rhizogenes* following culturing in light was reported as a required step. This work represents a stepping-stone to *R. rosea* HR cultivation in bioreactors as well as regenerating whole plants. Hence, it is initiating a novel route towards high-throughput production of bioactive compounds as well preventing depletion of natural roseroot populations.

Key words: *Agrobacterium rhizogenes*, explant survival, golden root, hairy root formation.

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Rafael Vasconcelos Ribeiro

*Corresponding author:
hlm@plen.ku.dk

INTRODUCTION

The medicinal plant *Rhodiola rosea* L. (roseroot, golden root or arctic root) has multiple uses in traditional and modern medicine, such as treating mental and physical fatigue, stress-induced depression, anxiety and high-altitude sickness (Anghelescu et al. 2018). Its wide application range is based on its health-promoting and adaptogenic properties, including mental and physical stimulation (avoiding fatigue and stress) (Zhang et al. 2016; Bangratz et al. 2018). The plant's pharmacological activity is based on the phenylpropenoid compound rosavin and its derivatives, which are only present in *R. rosea*, and salidroside, found in most *Rhodiola* species (Peschel et al. 2018).

Increasing demand of *R. rosea*-derived products has led to over-exploitation of plants from their natural habitats, which has placed *R. rosea* as an endangered species in several countries and posing the imminent risk of low quality and adulteration of roseroot related products (Booker et al. 2016). Since cultivation of this plant is challenging and costly, new

sustainable approaches for obtaining *R. rosea* products are needed to avoid depletion of its natural sources. In roseroot, several clonal propagation approaches targeting both the content of secondary metabolites and conservation have been pursued e.g. induction of organogenic and callus cultures, regeneration, and micropropagation. However, the outcome often varies significantly (as reviewed by Tasheva and Kosturkova 2012a; Grech-Baran et al. 2015). A promising strategy for the enhancement of bioactive compounds in plants is the insertion of *root oncogenic loci* (*rol*) genes from the soil-born bacterium *Rhizobium rhizogenes*, which has been reported to increase the content of ginsenosides in *Panax ginseng* (Kochan et al. 2018), artimisinin in *Artemisia* spp. (reviewed by Kayani et al. 2018), among other medicinal plants (Singh et al. 2018).

The distinctive morphology of hairy roots can allow a marker-free selection of the successful inoculations (Weber et al. 2008; 2010; Dehghan et al. 2012). The objective of the current study was to establish hairy root cultures (HR) of *R. rosea* following transformation with *R. rhizogenes*. The effect of different explants from thirteen accessions of this endangered species from the Alps, Russia, Scandinavia and the Nordic Gene Bank have been studied and presented in this paper. This work hypothesize that leaf, stem and rhizome explants of *R. rosea* from different geographical origins respond differently to inoculation with *Rhizobium rhizogenes* agropine strain ATCC43057.

MATERIALS AND METHODS

Plant material

As *R. rosea* is known to be recalcitrant, different phenotypes/accessions of the species were tested. A total of 13 *R. rosea* accessions were used; *R. rosea* plants from Russian (R1 and R2), Scandinavian (S1 and S2) and Alpine (A1 and A2) origin (Strictly Medicinal, Williams, OR, US) as well as plants from the Nordic gene bank in Øsaker, Østfold, Norway (NGB1 to NGB7) were kindly provided by Gitte Bjørn Kjeldsen, Agrotech, Taastrup, Denmark. *Rhodiola rosea* plants of group A were revived from dormancy period in August 2016 (NGB1-5, 7) and group B plants were revived in December 2016 (A1, A2, R1, R2, S1, S2 and NGB6). Prior to inoculation, harvested leaves were surface-sterilized in 70% (v/v) ethanol for 1 min, followed by 15 min in 1.5% NaOCl (v/v) (AppliChem Panreac, Darmstadt, Germany) and 0.03% Tween20 (v/v) (MERCK, Hohenbrunn, Germany). Once sterilized, leaves were rinsed 3 times in sterile water and dried on sterile filter paper.

Inoculation with *Rhizobium rhizogenes*

The experiment targeted comparison of various *R. rosea* plant accessions as well as plant organs in respect to hairy root formation. Leaves, stems and rhizomes of *R. rosea* were inoculated with *R. rhizogenes* agropine strain ATCC43057 containing plasmid pRiA4 (Slightom et al. 1985; Jouanin et al. 1987). The inoculation process was based on Hegelund et al. (2017) with minor modifications. Bacteria were grown in malt, yeast and agar (MYA) medium (Tepfer and Casse-Delbart 1987).

Following sterilization, the base of the *R. rosea* leaves was cut off, in order to create fresh wounds for inoculation and these pieces were immersed into either inoculation (*R. rhizogenes* in MYA OD_{600} = 0.5) or control (MYA) solution for 30 min. Subsequently, explants were dried on filter paper, transferred to cocultivation media [half strength Murashige and Skoog medium (Murashige and Skoog 1962) with vitamins (Duchefa Biochemie), 15 $mg \cdot L^{-1}$ acetosyringone (Sigma-Aldrich, St. Louis, MI, USA), 4·gL^{-1} gelrite (Duchefa Biochemie), pH 6] and incubated in darkness at room temperature for 48 h. The explants were then washed in sterile water containing 10 $mg \cdot L^{-1}$ timentin (ticarcillin/clavulanate, 15:1 mixture, Duchefa Biochemie), dried on filter paper and transferred to R-medium which consisted of half strength MS with 100 $mg \cdot L^{-1}$ timentin and 0.5 $mmol \cdot L^{-1}$ arginine (Sigma-Aldrich).

Hairy root induction

The effect of four genotypes, i.e. Alpine, Scandinavian, NGB and Russian, separated in two growth locations, i.e. outdoor and greenhouse, in the responsiveness to *R. rhizogenes* A4 was tested. Additionally, the light inducing effect was investigated by keeping explants either in darkness or in light (225 $\mu mol \cdot m^{-2} \cdot s^{-1}$) after cocultivation. Following inoculation, the explants were kept at room temperature and regularly monitored for first HR appearance (days), explants with HR, HR formation

rate (%) and contamination (%), i.e. fungi and/or bacterial growth. As a WT strain of *R. rhizogenes*, i.e. no antibiotic selection marker was used in the inoculation. Explants of *Kalanchoë blossfeldiana* 'Molly', highly responsive to this bacterial strain (Christensen et al. 2008), were used as a positive control. The HR clusters were separated from the explant once 2 cm growth was achieved, followed by maintenance in R-medium and sub-cultured to fresh medium every three weeks.

DNA extraction and polymerase chain reaction (PCR)

Hairy roots were harvested after approximately 9 weeks and minimum 3 series of subculturing to ensure the establishment of axenic putatively transformed lines. The DNA was extracted from the hairy roots with the DNA isolation kit from TaKaRa-Clontech (TaKaRa Bio Inc., Shiba, Japan) according to the manufacturer's instructions. A NanoDrop (ThermoFischer, Waltham, MA, US) was used to measure DNA concentration and purity. Specific primer sets for fragments of *rol*B, *aux*1, *vir*D2 and *Rractin* (control) (Table 1) were used. Polymerase chain reaction products were amplified in a DNA thermal cycler (MyCycler, Biorad, Hercules, CA, USA) with the following program: 95 °C for 10 min, 40 cycles of [95 °C for 30 s, 57 °C for *rol*B and *aux*1 / 52 °C for *vir*D2 and *Rractin* for 30 s, 72 °C for 30 s] and 72 °C for 7 min. For separation of the amplified PCR products, these were mixed with GelRed (Biotium, Hayward, CA, USA) and subjected to TAE 1.5% agarose gel electrophoresis at 100 V for 55 min. Finally, the products were visualized under UV-light.

Table 1. Polymerase chain reaction (PCR) primers.

Gene	Sequence	Product size (bp)
*rol*B*	5´-GATATCCCGAGGGCATTTTT-3´ 5´-GAATGCTTCATCGCCATTTT-3´	182
*Aux*1	5´-CATAGGATCGCCTCACAGGT-3´ 5´-CGTTGCTTGATGTCAGGAGA-3´	199
*Vir*D2	5´-AGTCGTGATCGCAAGGAGAT-3´ 5´-TGTCTCCAATGCAATCCGTA-3´	445
Rractin	5´-TGGAGAAGATCTGGCATCAC-3´ 5´-CAGCCTGAATGGCAACATAC-3´	171

*Lütken et al. 2012. *Vir*D2 and *Rractin* primers were designed using NCBI sequences from *Rhizobium rhizogenes* agropine strain ATCC43057 and JX431891.1, respectively as queries in Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/)

Statistical analysis

All the statistic studies were performed with the GraphPad Prism 7.03 program, where significance was assessed through t-test ($p < 0.05$). The transformation overview is presented in Table 2. Each individual plant provided explants to at least two independent transformation experiments on which the number of explants varied according to availability, ranging from 20 to 80.

RESULTS AND DISCUSSION

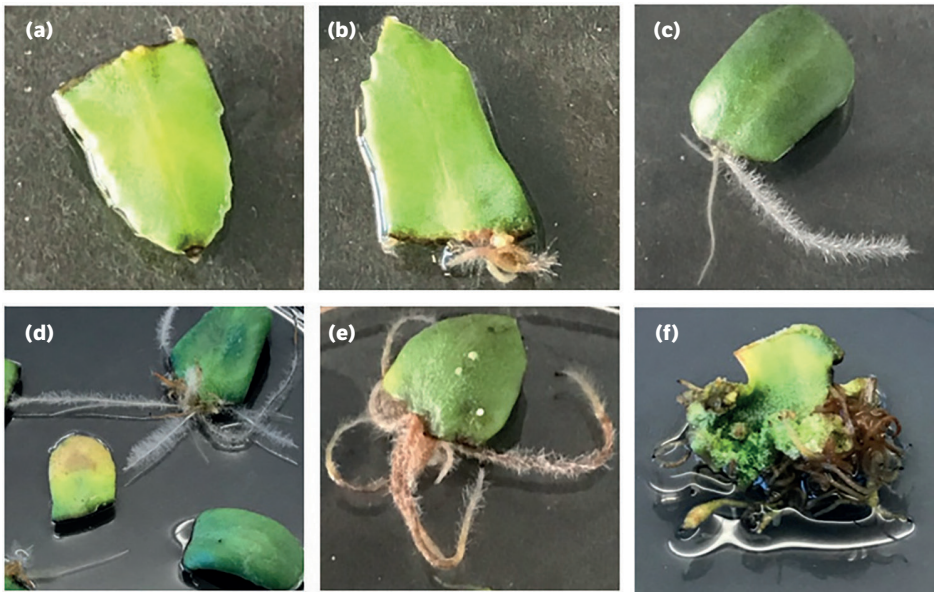
In the current study, hairy roots were successfully induced from leaves of *R. rosea* (Fig. 1) of diverse geographical origin by *R. rhizogenes* strain A4. In addition, different culture conditions were evaluated in terms of viability of surface sterilized explants and HR formation rate. This represents an initial step towards the obtaining of a transformed *R. rosea* plant.

Cultivating *R. rosea* in greenhouse decreased *in vitro* contamination

Leaf explants from experiments TE1-TE3 were collected from plants kept outdoor (Taastrup, Denmark) (TE1 and TE2 from plant NGB1 and TE3 from plant NGB2), which caused a high contamination rate (100% in TE2 and TE3) and the discard of the plant material. Such difficulties related to the surface sterilization of *R. rosea* plants from collected wild material were also reported by Khapilina et al. (2016) and Tasheva and Kosturkova (2010), who tested several sterilization

Table 2. Overview of inoculation experiments. †: HR = hairy roots, ⅃: Rr = *Rhodiola rosea*, ‡: Kb = *Kalanchoë blossfeldiana*

Transformation event (TE)	Plant material	Plant species	Dormancy revival	Transformed (+) / control (-)	Plant location outdoor(O)/ greenhouse (G)	In vitro conditions light / darkness ☼ ☾	Number of explants	First HR† (days)	Explants with HR	HR formation rate (%)	Contamination rate (%)
TE1	NGB1	*Rr* ⅃	8/16	+	O	☾	53	-	-	-	46
	NGB1	*Rr*	8/16	-	O	☾	26	-	-	-	75
TE2	NGB1	*Rr*	8/16	+	O	☾	79	-	-	-	100
	NGB1	*Rr*	8/16	-	O	☾	34	-	-	-	100
TE3	NGB2	*Rr*	8/16	+	O	☾	67	-	-	-	100
	NGB2	*Rr*	8/16	-	O	☾	31	-	-	-	100
TE4	NGB3	*Rr*	8/16	+	G	☾	80	27	15	18.8	0
	NGB3	*Rr*	8/16	-	G	☾	32	-	-	-	0
TE5	NGB3	*Rr*	8/16	+	G	☾	40	-	-	-	25
	NGB3	*Rr*	8/16	-	G	☾	21	-	-	-	0
TE6	NGB4	*Rr*	8/16	+	G	☾	61	29	5	8.2	0
	NGB4	*Rr*	8/16	-	G	☾	23		-	-	0
TE7	NGB5	*Rr*	8/16	+	G	☼	38	36	7	18.4	0
	NGB5	*Rr*	8/16	-	G	☼	21	-	-	-	0
	NGB6	*Rr*	12/16	+	G	☼	53	14	36	67.9	0
	NGB6	*Rr*	12/16	-	G	☼	27	-	-	-	0
TE8	NGB7	*Rr*	8/16	+	G	☼	50	26	10	20	-
	NGB7	*Rr*	8/16	-	G	☼	25	-	-	-	-
	R1	*Rr*	12/16	+	G	☼	50	-	-	-	16.6
	R1	*Rr*	12/16	-	G	☼	25	-	-	-	-
	S1	*Rr*	12/16	+	G	☼	50	20	6	12	-
	S1	*Rr*	12/16	-	G	☼	25	20	3	12	-
	A1	*Rr*	12/16	+	G	☼	50	23	2	4	17
	A1	*Rr*	12/16	-	G	☼	25	18	3	12	-
TE9	S1	*Rr*	12/16	+	G	☼	40	22	5	12.4	16.6
	S1	*Rr*	12/16	-	G	☼	20	26	1	5	33.2
	A1	*Rr*	12/16	+	G	☼	40	-	-	-	67.6
	A1	*Rr*	12/16	-	G	☼	20	-	-	-	33.2
TE10	R2	*Rr*	12/16	+	G	☼	50	-	-	-	16.6
	R2	*Rr*	12/16	-	G	☼	25	-	-	-	-
	S2	*Rr*	12/16	+	G	☼	50	-	-	-	33.2
	S2	*Rr*	12/16	-	G	☼	25	-	-	-	-
	A2	*Rr*	12/16	+	G	☼	50	35	11	22	-
	A2	*Rr*	12/16	-	G	☼	25	-	-	-	-
TE11	R2	*Rr*	12/16	+	G	☼	50	-	-	-	-
	R2	*Rr*	12/16	-	G	☼	25	-	-	-	33.2
	S2	*Rr*	12/16	+	G	☼	50	-	-	-	20
	S2	*Rr*	12/16	-	G	☼	25	-	-	-	-
	A2	*Rr*	12/16	+	G	☼	50	32	5	10	16.6
	A2	*Rr*	12/16	-	G	☼	25	-	-	-	-
Positive control		*Kb*‡	-	+	G	☾	20	14	20	100	0
		Kb	-	-	G	☾	10	-	-	-	0



Note: (a) 2 days, (b) 1 week, (c) 2 weeks, (d) 4 weeks, (e) 6 weeks, (f) 8 weeks. Bars represent 1 cm.

Figure 1. Representative stages of hairy roots on leaves of *Rhodiola rosea* transformed with *Rhizobium rhizogenes*.

methods on different explant tissues, and only 3 out of 14 combinations resulted in successful decontamination. Moreover, it is also likely that some of the contamination encountered could be due to overgrowth of *R. rhizogenes*, and additional washes with 10 mg·L^{-1} timentin and several rounds of subculture were conducted to inhibit *R. rhizogenes* (data not shown).

In order to avoid severe contamination due to an uncontrolled environment, the subsequent transformation experiments were performed using material from plants kept in a greenhouse environment for a week prior to inoculation. This resulted in much lower contamination rates for the greenhouse plant material (TE4-12) with an average contamination of 9% versus outdoor sourced (TE1-3) with an average of 86% (Table 2).

In vitro light exposure positively influenced hairy root formation

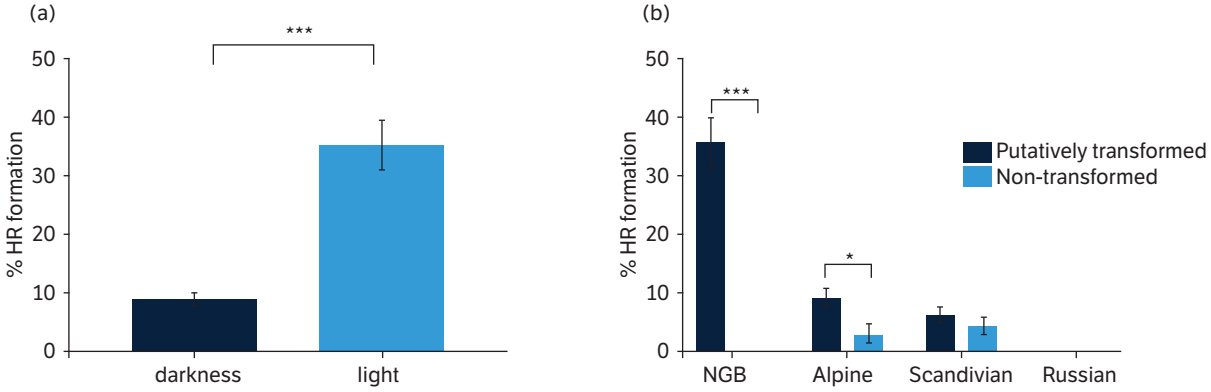
To investigate the optimal growth conditions to induce HR formation, the presence or absence of light on the HR regeneration phase were investigated. This study found that the inoculated explants produced more HR (35 ± 4 %) under constant light, while the explants kept in darkness conditions had lower HR formation (9 ± 1%) (Fig. 2 a). This dynamic change is described in the dark TE1-6 and light-conditioned T7-11 (Table 2). In more details, TE4 using leaves from NGB3 exhibited the first hairy root (HR) 27 days after inoculation and the percentage of explants forming HRs reached 19%. TE5 was also performed on plant material from NGB3 as a repetition. However, the plant utilized in the experiment exhibited yellowish and weak leaves and the explants did not develop hairy roots. Hence, lack of consistency among independent inoculation experiments were generally encountered.

As reflected in the literature (Flem-Bonhomme et al. 2004; Grech-Baran et al. 2014), plant explants transformed with *R. rhizogenes* are typically kept in darkness for root formation, emulating roots naturally growing inside soil where there is no light. However, *R. rosea* is a plant that grows in northern regions, where light intensity is high and days are long during summer (Li et al. 2016). Moreover, periodic light was proven to have a positive effect on the performance of the tissue and the root generation in *in vitro* cultures of *Glycine max* and *Agastache foeniculum* by modulating organogenesis and growth (Nourozi et al. 2016; Chen et al. 2018). Therefore, in the present work a novel strategy was pursued to compare the effect of darkness and light on explant survival.

In a pilot experiment, 24 explants from a *R. rosea* plant of Russian origin were placed in darkness and 24 explants of the same plant were subjected to a light intensity of approximately 225 μmol·m^{-2}·s^{-1} and a light period of 16 h. After 17 days, the explants kept in darkness started exhibiting necrosis, while explants kept in light still maintained their green color (data not shown). This showed that *R. rosea* explants kept in light displayed longer viability than those kept in darkness. Leaf morphology was variable among plants from different geographical origins (Fig. 3) and they also responded differently to inoculation

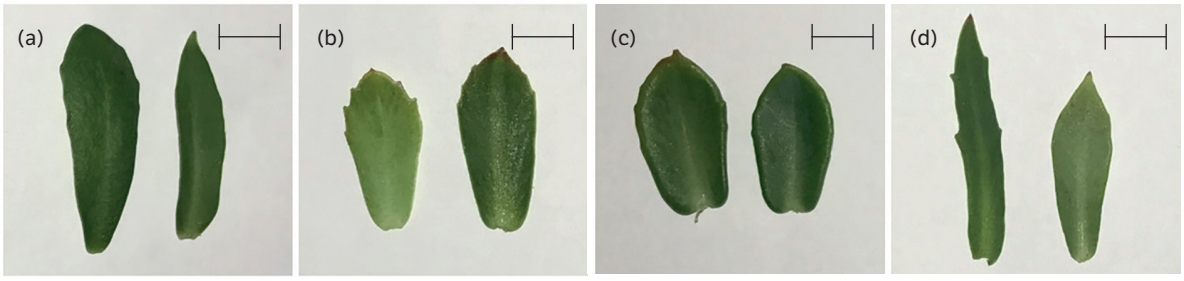
(Fig. 2 b). Hence, the interplay between plant origin, genotype and leaf morphology is an important factor to take into consideration.

Overall, statistical analysis revealed that HR formation rate was significantly ($p < 0.001$) higher when the explants were kept in light prior to inoculation (Fig. 2a). This is supported by Siegień et al. (2013), who tested the effect of light on shoot regeneration and root genesis on explants and sterile plantlets of *Linum usitatissimum*. In that study, explants exhibited 30% higher shoot regeneration when cultured in light than when cultured in darkness, and root formation was similar in both conditions. Light has also proven to have a positive effect on *Artemisia annua* hairy root cultures obtained by inoculation with *R. rhizogenes*. In that study, hairy root cultures were exposed to five different light intensities. The lowest hairy root growth was observed in darkness and hairy root growth was greater as light intensity increased (Liu et al. 2002). However, another study performed on *Stevia rebaudiana* reported that hairy root organogenesis should be induced in darkness, while the subsequent growth of HR-cultures performed well only under continuous light of approximately 40 µmol photons·m^{-2}·s^{-1} (Pandey et al. 2016). From these contradictory results, it can be deducted that the effect of light on organogenesis and performance of *in vitro* plant cultures differs significantly among plant species.



Note: (a) Effect of light on HR formation rate, (b) influence of geographical origin on HR formation rate. * = $p < 0.05$, *** = $p < 0.001$.

Figure 2. Effect of light and geographical origin in response to transformation.



Note: (a) NGB, (b) Alpine, (c) Scandinavian, (d) Russian. Bars represent 1 cm.

Figure 3. Morphological differences between plants.

Leaves were the most responsive tissue to transformation

Moreover, in TE5, stem explants were also used in order to test a different plant organ. However, the explants exhibited necrosis 2 weeks after inoculation (data not shown). Similarly, rhizomes were assessed as source of explants for inoculation, however no response was observed in terms of HR formation, and severe contamination developed and all the plant material died fast (data not shown).

Geographical origin strongly affected the transformation rate

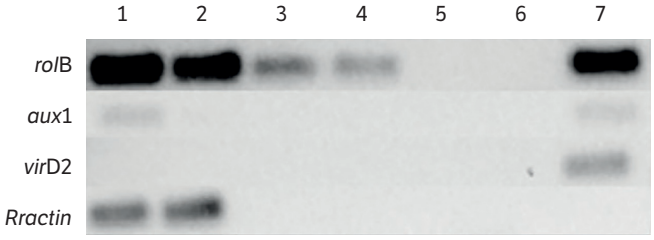
Several attempts succeeded in generating micropropagated vegetative material of *R. rosea* species (Tasheva and Kosturkova, 2010; 2013; 2014). However, when conducting *Rhizobium*-mediated inoculation of *R. rosea* leaves and calli, Tasheva and Kosturkova (2012 b) observed that most of the explants were dead, infected or exhibited necrosis within 2–4 weeks after inoculation. In the current study, HR formation was observed on leaf explants originating from the Nordic Gene Bank, Alpine, and Scandinavian regions, leading to the first successful report of HR formation in this species.

Moreover, the HR formation rate in NGB and Alpine plants in inoculations where explants were kept in light conditions was significantly higher ($p < 0.001$ for NGB and $p < 0.05$ for Alpine) than in explants of Scandinavian and Russian origin (Fig. 2b). *Rhodiola rosea* is a highly variable species, and major morphological differences are observed not only among plants from different countries but also among accessions from the same country (NGB 2005; Serebryanaya and Shipunov 2009). Likewise, morphological differences among the plants from different geographical origin (Nordic Gene Bank, Scandinavian, Russian, Alpine) were observed in the present study (Fig. 2b). This high variability can affect the way *R. rosea* responds to inoculation with *R. rhizogenes*. This is in accordance with results revealed in this work, where plants grown at the same conditions, but with different geographical origins, responded differently to inoculation with *R. rhizogenes*.

Hairy root phenotype was confirmed by PCR

In the current study, an agropine *R. rhizogenes* strain was used, hereby the transferred (T) DNA is part of a root inducing (Ri) plasmid, which harbors two distinct regions. The T_L-DNA contains the *rol*-genes *rol*A, *rol*B, *rol*C and *rol*D, among others (Tepfer 2017; Otten 2018; Desmet et al. 2019), while the T_R-DNA carries two genes involved in auxin synthesis (*aux*1 and *aux*2) (Camilleri and Jouanin 1991) and a *rol*B homolog, called $rolB_{Ir}$ (Bouchez and Camilleri 1990). T_L and T_R regions of the Ri-plasmid can be integrated in the plant DNA independently, but the hairy root development is mainly attributed to the presence of the *rol*-genes from the T_L region (Halder and Jha 2016). This system is often preferred to plant/cell callus and suspension cultures as it has a strong potential of mimicking the multienzyme biosynthetic potential of the parent plants with a relative low-cost production and without significant loss of metabolic activity (Banerjee et al. 2012; Häkkinen et al. 2016).

In order to confirm successful transformation, hairy roots derived from different accessions (NGB, Alpine and Scandinavian) were selected for PCR analysis. The *rol*B fragment represents the T_L-DNA integration into the plant genome, and its presence was detected in all the putatively transformed samples, and it was absent in the nontransformed sample (Fig. 4). Hence, *R. rosea* – although being in the same family (Crassulaceae) as *Kalanchoë* – seems not to form adventitious roots to a similar extend (Christensen et al. 2008). Additionally, nontransformed adventitious "hairy-looking" roots can develop from non-inoculated tissue. This process is highly dependent on the plant species being transformed and up to 50% adventitious root formation has been observed on non-inoculated leaf explant in comparison to inoculated (Christensen et al. 2008). Hence, this issue needs to be taken in to consideration when assessing the hairy-root formation. An *aux*1 fragment was chosen as representative for T_R-DNA and was only present in the NGB transformed sample, indicating the combined T_L+/T_R+ insertion. The independent integration of T_L and T_R-DNA into the plant genome has a ratio favoring T_L insertion alone instead of both T_L and T_R (Roychowdhury et al. 2015; Halder and Jha 2016), which was also the case



Note: 1. NGB transformed, 2. Alpine transformed 1, 3. Alpine transformed 2, 4. Scandinavian transformed, 5. Scandinavian nontransformed, 6. Water, 7. *R. rhizogenes* A4 Ri-plasmid.

Figure 4. PCR products from DNA from hairy roots.

observed in this study. Moreover, a *vir*D fragment, which is a part of the Ri-plasmid not integrated in the plant genome, was only present in the plasmid (positive control). Hence, absence of contamination by *R. rhizogenes* in the samples was demonstrated, verifying that the lines were true transformants. *Rractin* was chosen as a reference gene for *R. rosea*; however, only 2 of the 5 samples showed presence of this gene fragment (Fig. 4), likely due to the un-sequenced status of *R. rosea* and its high genetic variability (György et al. 2012). Although, further growth of the obtained HRs, both in Erlenmeyer flasks and bioreactors, was not achieved (data not shown), this work represents a stepping-stone to *R. rosea* HR cultivation as well as regeneration of whole plants. Hence, a novel route towards high-throughput production of bioactive compounds in HRs as well preventing depletion of natural roseroot populations is being outlined.

CONCLUSION

This study reports, for the first time, effective transformation of *R. rosea* with *R. rhizogenes* and the obtaining of viable hairy root cultures. The results indicated that light has a positive effect on survival of leaf explants of *R. rosea* after bacterial inoculation and resulted in higher HR formation rates. Additionally, differences in response to inoculation of *R. rosea* plants from different geographical origin, genotype and morphology were observed. Overall, the plant material provided by the Nordic Gene Bank was the most responsive to *R. rhizogenes* inoculation. Therefore, further studies on superior HR lines from this source should be conducted towards the obtaining of transformed *R. rosea* plants.

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AUTHOR'S CONTRIBUTION

Conceptualization, Martínez M. I. and Lütken H.; Methodology: Martínez M. I.; Investigations, Martínez M. I. and Barba-Espín G.; Writing – Original Draft, Martínez M. I. and Lütken H.; Writing – Review and Editing, Favero B. T. and Lütken H.; Resources, Favero B. T. and Lütken H.; Supervision: and Barba-Espín G and Lütken H.

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