Introgression of rol *genes from rhizogenic* Agrobacterium *strains into* Escallonia *spp.*

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ORIGINAL ARTICLE

Introgression of *rol* genes from rhizogenic *Agrobacterium* strains into *Escallonia* spp.

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Abstract

The introgression of *rol*-genes of rhizogenic *Agrobacterium* into the plant genome induces changes in plant phenotype and physiology. However, only limited experience with this technique is available for woody ornamentals. To induce new variation within the *Escallonia* genus, several species were co-cultivated with rhizogenic *Agrobacterium* strains. Co-cultivation of three rhizogenic *Agrobacterium* strains (Arqua1, LMG 63 and MAFF210266) with four *Escallonia* species (*E. illinita*, *E. myrtoidea*, *E. rosea*, and *E. rubra*), resulted in hairy roots production with a varying efficiency. Co-cultivation of *E. rubra* with MAFF210266, and *E. myrtoidea* with LMG63 did not yield hairy roots, while co-cultivation of *E. rubra* leaves with LMG63 was most successful for hairy root production (up to 80.6%). In addition, the efficiency of hairy root induction depended on the explant type (leaves or nodal sections). The presence of inserted *rol*-genes and *aux*-genes in hairy roots was molecularly confirmed using qPCR. Few shoots regenerated from hairy roots, but regeneration needs to be optimized for efficient implementation of *rol*-genes introgression in *Escallonia* breeding.

Key Message

This research provides a protocol for the production of hairy roots with *rol*-genes inserted after co-cultivation of several species of *Escallonia* with rhizogenic *Agrobacterium* strains.

Keywords Hairy roots · *rol* genes · Woody ornamental breeding · *Agrobacterium rhizogenes*

Introduction

Rhizogenic *Agrobacterium* strains are naturally-occurring soil-dwelling organisms, containing transfer-DNA (T-DNA) on which, among others, four *rol*-genes (root oncogenic loci) (*rolA*, *rolB*, *rolC*, *rolD*) are located (Huffman et al. 1984; Desmet et al. 2019). When the T-DNA is transferred into the host plant cell and inserted in the host plant DNA (Chilton et al. 1982; White et al. 1985), a neoplastic growth of hairy roots occurs, which produce and exude opines (Vladimirov et al. 2015). Since these opines are the source of energy and food for the *Agrobacterium* strains in the rhizosphere, the gene-transfer of rhizogenic *Agrobacterium* strains to plant hosts can be seen as a survival strategy of the bacteria.

Based on this naturally occurring system, artificial co-cultivation of plants with different rhizogenic *Agrobacterium* strains has been attempted for many genera (Mauro et al. 2017). Regenerants originating from hairy roots, may contain the T-DNA of the bacteria, which can cause typical changes in plant phenotype and physiology (Georgiev et al. 2012). Plants with *rol*-genes inserted can display compactness due to a loss of apical dominance and a decrease in internode length, wrinkled leaves, alterations in flowering time, an increased rooting ability and different secondary metabolite concentrations (Tepfer 1984; Bulgakov 2008;

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Christensen and Müller 2009). Although many woody species are known for their recalcitrance in vitro (Rugini et al. 1991; Rastogi et al. 2008), *rol*-genes were introduced successfully in several woody species. *Lavandula* × *intermedia* plants with introgression of *rol*-genes displayed a compact growth and extensive lateral branching, a delay in flowering time of 1 month and shortened flower stalks (Tsuro and Ikedo 2011). *Betula pendula* with *rol*-genes showed a slower and more bushy growth, smaller leaves and a larger root system, while *B. pendula* with both *rol*- and *aux*-genes inserted, displayed a vigorous growth (Piispanen et al. 2003). The introduction of *rol*-genes has been attempted for *Hibiscus rosa-sinensis* (Christensen et al. 2009), but no successful regeneration was reported yet. Furthermore, transformations of woody species with transgenic rhizogenic *Agrobacterium* strains have also been successfully conducted on *Malus baccata* rootstocks (Wu et al. 2012), *Actinidia deliciosa* (Rugini et al. 1991) and *Aesculus hippocastanum* (Zdravković-Korać et al. 2004).

The present study aims to set-up a protocol for *rol*-gene introduction in *Escallonia* using wild type strains of *A. rhizogenes*. The genus *Escallonia* Mutis ex L.f., contains about 40 flowering woody species and about 20 botanical varieties, originating from South America. Most species are evergreen, have white to red flowers, a honey fragrance, and resin glands on leaves and branches (Bean and Murray 1989). *Escallonia* is used as a hedging plant, especially in coastal regions. Depending on the species, *Escallonia* can thrive in USDA zones from 10 to 7b, corresponding to mean minimum temperatures ranging from − 1.1 to − 14.9 °C (Hoffman and Ravesloot 1998). Numerous hybrids and cultivars have been described (Krüsmann 1960; Bean and Murray 1989; Hillier Nurseries 1991), resulting from harvests in the wild and selections from open pollinated seedling populations. Several developed cultivars were granted the Royal Horticultural Society's Award of Garden Merit, e.g. 'Apple Blossom', 'Donard Radiance', 'Iveyi', 'Peach Blossom', 'Pride of Donard', and 'Crimson Spire', indicating their added value in the garden (Royal Horticultural Society 2018). However, due to the fact that *Escallonia* is not very cold resistant, cultivars of this genus are underused in gardens. If breeding would enable to create cultivars with more cold resistance, compactness, and flower density, taken together with the evergreen characteristic which they already have, the commercial value of *Escallonia* would increase. Possibilities for breeding by polyploidization are evaluated (Denaeghel et al. 2018), but further breeding efforts in *Escallonia* are limited. The introgression of *rol*-genes into *Escallonia*, could result in plants with increased compactness and flower density, which would already meet this highly demanded characteristics in the market segment of woody ornamentals in general, and for *Escallonia* in particular.

In this study *Escallonia* was co-cultivated with three rhizogenic *Agrobacterium* strains to develop a protocol for *rol*-gene introduction. First, the hairy root induction efficiency of these strains was evaluated. Second, several media and approaches were attempted to regenerate shoots from the resulting hairy roots. Finally, the presence of *rol*-genes in the hairy roots and the regenerated shoots was determined.

Materials and methods

Plant material and growth conditions

In this study, we used one genotype within each of four *Escallonia* species: *E. illinita*, *E. myrtoidea*, *E. rosea*, and *E. rubra* (Table 1). Young, non-woody shoots of plants grown in 3 L container (peat based substrate, 1.5 kg/m^3 fertilizer: 12 N:14P:24 K + trace elements, pH 5.0–6.5, EC 450 µS/cm) in a frost free greenhouse, were initiated in vitro according to the procedure described by Denaeghel et al. (2018). Multiplication of in vitro shoots was done on a medium containing MS macro-nutrients (Murashige and Skoog 1962), 0.15 mg/L 6-benzylaminopurine (BAP) and 0.05 mg/L 1-naphthaleneacetic acid (NAA). Cultures were renewed every 12 weeks. Both leaves and nodes of in vitro shoots were used in the co-cultivation experiments.

Table 1 Overview of the *Escallonia* species used

Genotype	Acquisition	Accession number	Herbarium voucher[a]
E. illinita Presl	DN	–	BR0000025666403V
E. myrtoidea Bertero ex DC	RBGE	20,130,304	BR0000025666458V
E. rosea Griseb	HG	–	–
E. rubra (Ruiz & Pav.) Pers	RBGE	19,924,317*B	BR0000025666236V

DN tree nursery De Neve, Oosterzele, Belgium, *RBGE* Royal Botanical Garden Edinburgh, Edinburgh, Scotland, UK, *BMG* Botanical Garden Meise, Meise, Belgium, *HG* Hillier Gardens, Ampfield, Romsey, UK, – information not known

[a]Voucher numbers assigned by the National Botanical Garden, Meise, Belgium for addition to the herbarium

Storage and maintenance of rhizogenic *Agrobacterium* strains

Three strains were used in this study: Arqua1, LMG63 and MAFF210266 (Table 2). These strains were characterized in detail by Desmet et al. (2019). The bacteria were grown in dark conditions at 28 °C for 48 h and then kept at 4 °C in the dark. MAFF210266 was grown on a solid YEG medium (yeast extract glucose broth: 10 g/L yeast, 10 g/L D-glucose, 1 g/L $(NH_4)_2SO_4$, 0.25 g/L KH_2PO_4, 15 g/L Bacto-agar) while Arqua1 and LMG63 were grown on a solid MYA medium (malt yeast agar: 5 g/L yeast extract, 0.5 g/L casein hydrolysate, 8 g/L mannitol, 4.1 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L NaCl, 15 g/L Bacto-agar, pH 6.6) (Leroy et al. 2006). Every 4 weeks, a new culture of the three strains was started on YEG or MYA.

Co-cultivation of *Escallonia* explants with rhizogenic *Agrobacterium* strains

One bacterial colony was transferred to 50 mL liquid MYA (for LMG63 and Arqua1) or 50 mL liquid YEG (for MAFF210266), and incubated in the dark on a rotary shaker (150 rpm) for 24 h at 28 °C. The density of the liquid bacterial culture was analyzed with a spectrophotometer (iMark Microplate Reader, Bio-Rad Laboratories, Hercules, California, USA) and cultures were diluted to an optical density between 0.5 and 0.6 OD (measured at 600 nm).

A schematic overview of the different steps for co-cultivation of *Escallonia* explants with *A. rhizogenes* is shown in Fig. 1. Leaf explants (0.5–0.8 cm^2) were cut from young (maximum 12 weeks) in vitro leaves, always containing a midrib and a cut edge all around. Nodal explants cut from young in vitro shoots (maximum 12 weeks) were 0.3–0.6 cm long. Per species–strain combination (Table 3), 60 explants of each type were co-cultivated with bacteria, and 20 explants of each type were used in a control treatment (application with MYA or YEG without bacteria). Cultures were incubated with the bacterial suspension (15 mL) in Petri dishes (Ø 9 cm) in the dark on a rotary shaker (80 rpm) for 30 min. Subsequently, the explants were air dried on sterile paper for 1–2 min to remove redundant bacterial suspension or liquid medium and placed on solid MS medium (30 g/L sucrose, MS salts and vitamins, 6 g/L agar (plant tissue culture grade agar No. 4, Neogen), pH 5.9 ± 0.1) with 20 mg/L acetosyringone (5 leaf explants and 5 nodal explants per Petri dish). The treated explants were placed in the dark in a growth chamber (ambient temperature 23 ± 1 °C) for 48 h.

Table 3 Number of experiments performed for each *Escallonia* species–bacterial strain combination

Genotype	Arqua1	LMG63	MAFF210266
E. illinita	3	2	1
E. myrtoidea	1	1	–
E. rosea	3	1	–
E. rubra	2	1	1

After co-cultivation, all explants were submerged in liquid MS with 500 mg/L cefotaxime and stirred regularly for 20 min. Then the explants were air dried for 1–2 min on sterile filter paper, placed on solid MS with 500 mg/L cefotaxime and 100 mg/L timentin (5 leaf explants and 5 nodal explants per Petri dish), and incubated in the dark at 23 ± 1 °C. Every 2 weeks, the MS medium containing antibiotics was renewed. Data on the number of roots per explant (>1 cm) and the time needed for rooting were collected until a maximum of 12 weeks post co-cultivation. The cumulative percentage of rooted leaf and nodal explants for the different combinations of rhizogenic *Agrobacterium* strains and the *Escallonia* species was analyzed statistically in R (R Development Core Team 2008, version 3.6.0) using the Welch two sample t-test.

Shoot regeneration

The hairy roots (>1 cm) were excised and put on the regeneration medium (10 root explants per Petri dish, Fig. 1). Different medium compositions for shoot regeneration were tested (Table 4), all based on MS salts and vitamins supplemented with 30 g/L sucrose and solidified with 6 g/L agar (plant tissue culture grade agar No. 4, Neogen) (pH 5.9 ± 0.1). Three different approaches were applied: (1) continuous exposure to a medium without added plant hormones (RM00) (2) continuous exposure to media supplemented with low amounts of either BAP and NAA, thidiazuron (TDZ) or kinetin (KIN) (RM01–06), and (3) a shock treatment during 4–8 weeks on a medium with a relatively high concentration (1 mg/L to

Table 2 Overview of rhizogenic *Agrobacterium* strains used

Strain	Opine-type	Plasmid	Supplier	References
Arqua1	Agropine	pRiA4	D. Vereecke, VIB	White et al. (1985) and Jouanin et al. (1987)
LMG63	Mannopine	pRi8196	BCCM/LMG (Belgian Coordinated Collections of Micro-organisms)	Trypsteen et al. (1991) and Desmet et al. (2019)
MAFF210266	Mikimopine	pRi1724	NARO Genetic Resources Center Japan	Tsuro and Ikedo (2011)

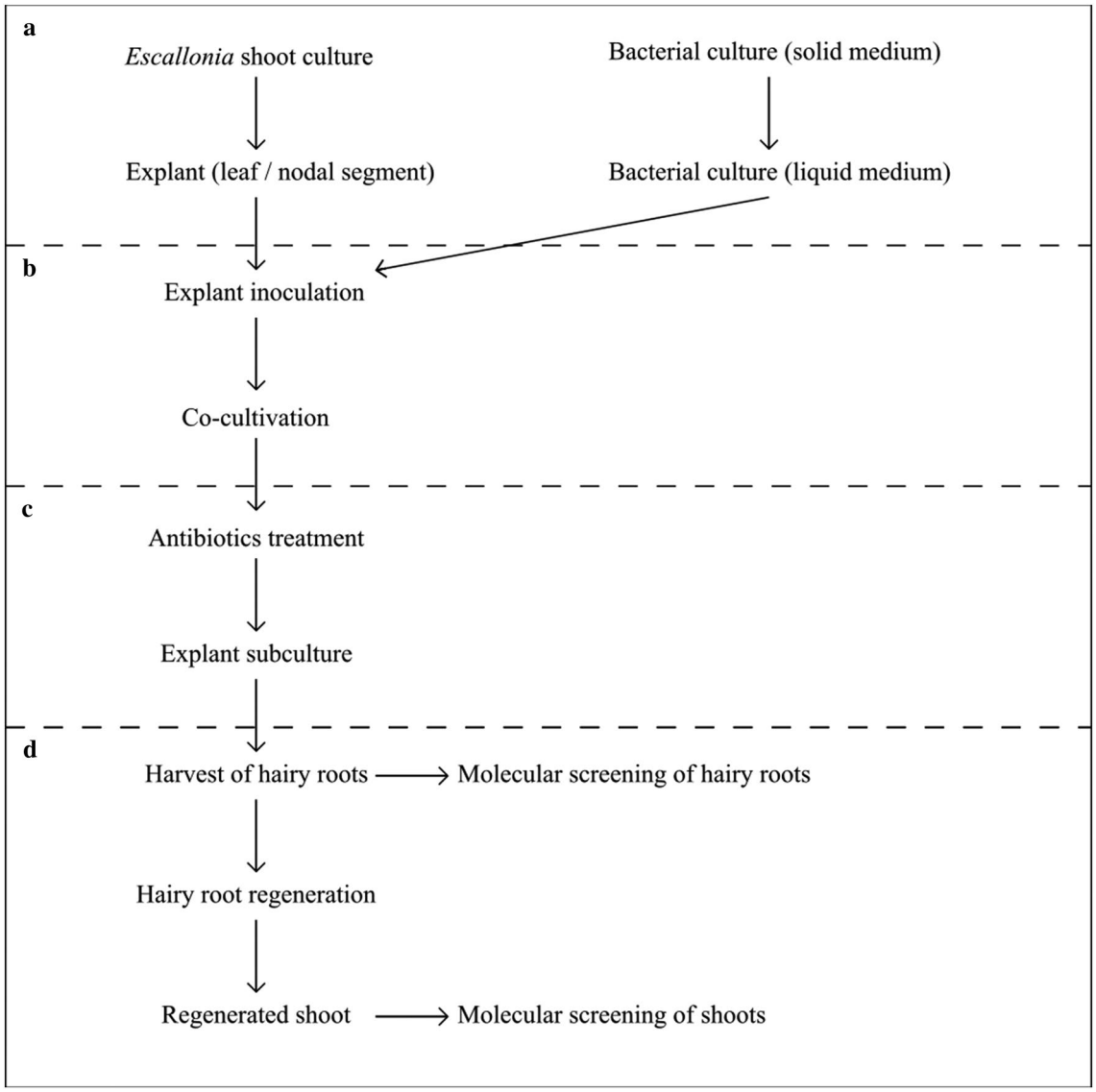

Fig. 1 Schematic overview of the subsequent steps in the co-cultivation and regeneration process of shoots from *Escallonia* hairy roots. **a** Preparation, **b** co-cultivation, **c** subculture and **d** regeneration

7 mg/L) of cytokinins (BAP or TDZ) followed by a medium containing a sevenfold lower cytokinin concentration (RM10 followed by RM12) or tenfold lower cytokinin concentration (RM07 followed by RM01, RM08 by RM09 and RM11 by RM13). The hairy roots were placed in the growth chamber (ambient temperature 23 ± 1 °C, photoperiod 16 h, light intensity 35 µmol/m^2/s, bottom cooling 18 ± 1 °C), and the regeneration medium was renewed every 4 weeks.

Molecular screening of shoots and roots

The process of molecular screening of hairy roots and regenerated shoots is shown in Fig. 1. DNA extraction was performed according to the modified CTAB (cetyltrimethylammonium bromide) DNA isolation protocol (Doyle and Doyle 1990) using 100 mg of young hairy root tips of minimum 8 weeks old or 100 mg of leaf material of regenerated shoots. Leaf tissue of the in vitro stock plants was used as a negative control. A sample of the rhizogenic *Agrobacterium* strains was included as a positive control. Bacterial DNA extraction was conducted according to Desmet et al. (2019). DNA integrity was checked with universal plant its-u3 and its-u4 primers (Cheng et al. 2016). Both on plant and bacterial DNA, *virD2* A and C′ primers of Haas et al. (1995) were used to identify the presence of the bacterial *virD2*-gene and *rol*-gene specific primers were used to confirm introgression of T-DNA in the plant. For Arqua1, primers for *rolA*,

Table 4 Phytohormonal supplementation of the MS-based regeneration media (RM) for shoot formation on *Escallonia* hairy roots

Medium	6-Benzylaminopurine (mg/L)	Thidiazuron (mg/L)	Kinetin (mg/L)	1-Naphthaleneacetic acid (mg/L)
RM00	–	–	–	–
RM01	–	0.1	–	–
RM02	–	0.5	–	–
RM03	–	–	0.1	–
RM04	–	–	0.5	–
RM05	0.1	–	–	0.1
RM06	0.5	–	–	0.1
RM07	–	1.0	–	–
RM08	3.0	–	–	–
RM09	0.3	–	–	–
RM10	7.0	–	–	–
RM11	–	3.0	–	–
RM12	1.0	–	–	–
RM13	–	0.3	–	–

Table 5 Primers for amplification of *rol*-genes present on the Ri plasmid of LMG63

Gene	Sequence (5′–3′)	Amplicon (bp)
rolA	F: atggaactagccggaataaa	278
	R: tcccgtaggtttgaattttt	
rolB	F: ctatctcaggcttcatcacg	233
	R: cttattcgtccacttgcttg	
rolC	F: gatgcaatgcttctatggag	310
	R: accatggcttaccaccttat	
rolD	F: aggctgcctatgtcaacgat	119
	R: gaggtgcttgttctccttgc	

rolB, *rolC* and *rolD* of Lütken et al. (2012a, b) were used for this purpose, for LMG63 specific *rol*-primers (*rolA*, *rolB*, *rolC* and *rolD*) were developed based on WGS data of Desmet et al. (2019) (Table 5). Roots transformed with Arqua1 were also tested for the presence of both *aux*-genes (*aux1* and *aux2*) using the primers of Lütken et al. (2012a). The qPCR mix contained 300 nM of both primers and 2 × SensiFAST SYBR® No-ROX Mix (Bioline Reagents Ltd., London, UK). Cycling conditions were 2 min at 95 °C, followed by 40 cycles of 5 s 95 °C, 10 s 59 °C (*its* and *rol*-genes Arqua1)/56 °C (*rol*-genes LMG63 and *aux*-genes)/55 °C (*virD*) and 20 s 72 °C, with data acquisition at the end of every cycle. Melting curve analysis is performed as follows: 5 s 95 °C, 1 min 61 °C and heating to 97 °C with a ramp rate of 0.06 °C/s. Data acquisition occurs 10 times for every °C.

Results

Hairy root formation on *Escallonia* species

The phenotypic difference between roots produced on explants inoculated with a rhizogenic *Agrobacterium* strain and on control explants was clearly visible for all species (Fig. 2). In control treatments, roots occasionally formed on nodal segments but not on leaves. Wild type roots were smooth, thin and did not show branching. In contrast, roots formed on inoculated explants were thicker, more branched and grew vigorously with numerous root hairs. Only roots with this particular latter phenotype were further considered as hairy roots. The different bacteria–host combinations showed varying efficiencies in terms of hairy root production (Fig. 3). Arqua1 could infect all tested *Escallonia* species, while this was not the case for LMG63 and MAFF210266.

For all experiments, the average percentage of *E. illinita* explants yielding hairy roots was not significantly different between leaves (39.3 ± 12.2%) and nodes (46.1 ± 14.1%), but the virulence of the strains was different. For example, the three experiments on *E. illinita* with Arqua1 yielded average hairy root percentages of 63.2 ± 13.6% for the leaves and 74.3 ± 9.7% for the nodes, while for the two experiments with LMG63 an average of respectively 23.0 ± 0.1% and 33.8 ± 8.8% was obtained. MAFF210266 yielded 31.7% and 38.1% hairy roots on leaves and nodes, respectively. Explants co-cultivated with Arqua1 showed a higher increase in the number of explants with hairy roots in the first 6 weeks after the co-cultivation, compared to both LMG63 and MAFF210266 (Fig. 3a, b). A total number of 549 (Arqua1), 123 (LMG63) and 74 (MAFF210266) hairy roots were harvested on *E. illinita* explants for regeneration.

For *E. rosea*, co-cultivation of leaf explants with Arqua1 resulted in on average 3.3 ± 2.5% hairy roots, while co-cultivation of nodal explants with Arqua1 yields 36.7 ± 13.7% hairy roots (Fig. 3c, d). The inoculation with LMG63 yielded hairy roots on 0% leaves and on 23.0% nodes (Fig. 3c, d). Overall, for *E. rosea* nodal explants were more responsive to hairy root formation. Explants inoculated with Arqua1 showed a higher increase in hairy root production the first 6 weeks after the co-cultivation compared to explants inoculated with LMG63. In total 100 (Arqua1) and 14 (LMG63) hairy roots were harvested for regeneration after co-cultivation of *E. rosea* explants.

For *E. rubra*, Arqua1 inoculated leaves and nodes produced on average 52.9 ± 6.7% and 33.9 ± 3.9% hairy roots, respectively. Leaves and nodes inoculated with LMG63 yielded 83.9% and 73.8% hairy roots, respectively (Fig. 3e, f). Nodal explants inoculated with MAFF210266 did not

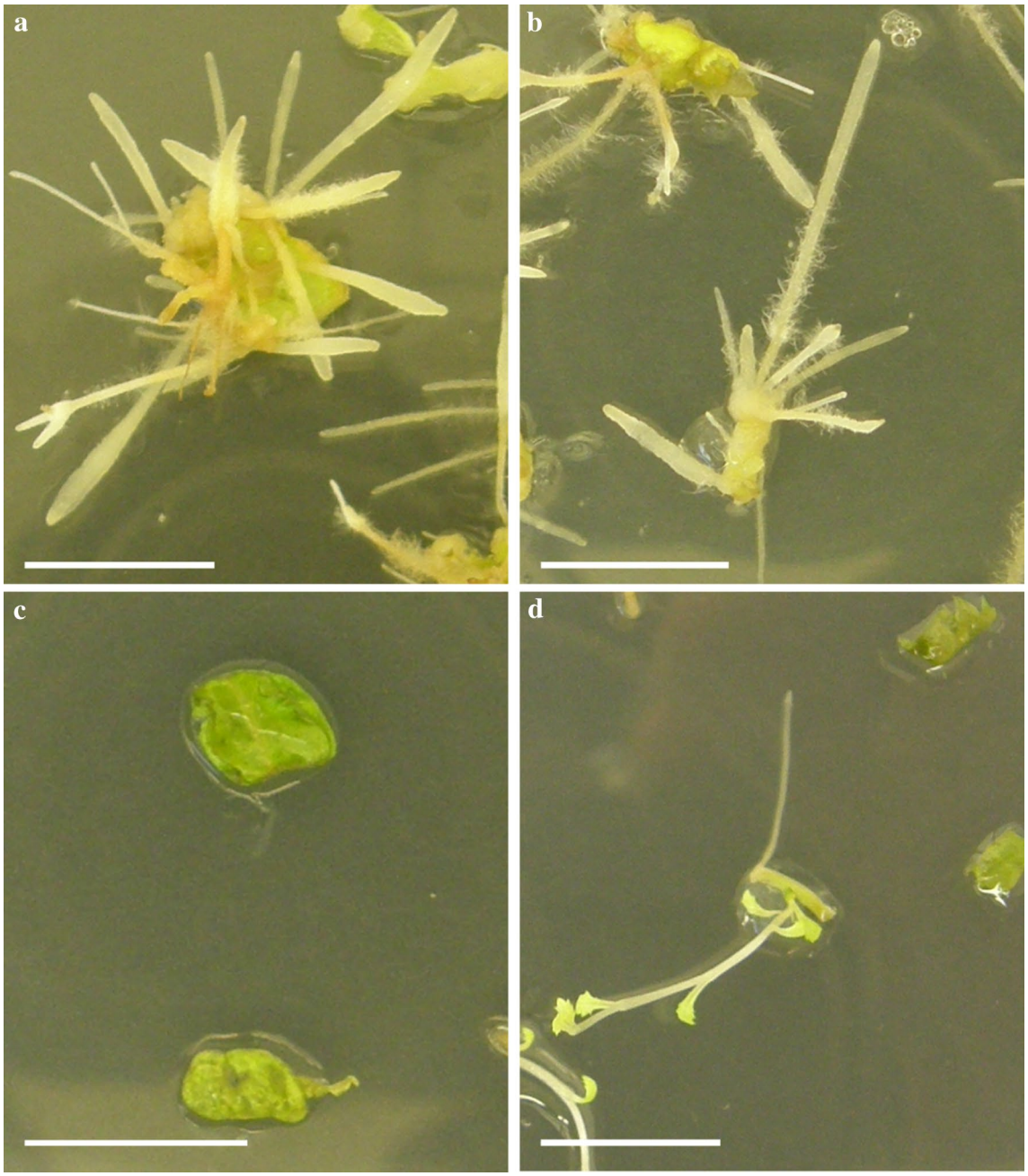

Fig. 2 Hairy roots (**a**, **b**) and control roots (**c**, **d**) produced on *Escallonia illinita* explants after co-cultivation. **a** *Escallonia illinita* leaf explant + Arqua1, **b** *E. illinita* nodal explant + Arqua1, **c** *E. illinita* control leaf explant and **d** *E. illinita* control nodal explant. Scale bar = 1 cm

produce any hairy roots. Explants inoculated with either Arqua1 or LMG63 showed a high increase in hairy root production between 4 and 8 weeks after the co-cultivation (Fig. 3e, f). A total number of 196 (Arqua1) and 166 (LMG63) hairy roots were harvested on *E. rubra* explants and placed on regeneration medium.

Only Arqua1 induced hairy roots on leaf and nodal explants of *E. myrtoidea*, 17.5% and 38.9% respectively. The hairy roots appeared between 4 and 6 weeks after the co-cultivation, with the highest number of roots/explant at 6 weeks (Fig. 3g, h). In total 45 hairy roots were harvested and transferred to regeneration medium.

A subsample of the obtained roots for the different bacteria–plant species combinations were molecularly screened on the presence of *rol*-genes and in the case of Arqua1 also for *aux*-genes (Table 6). All DNA tested positive for the ITS markers, thus confirming sufficient DNA quality. *VirD2* could not be amplified in the 103 hairy

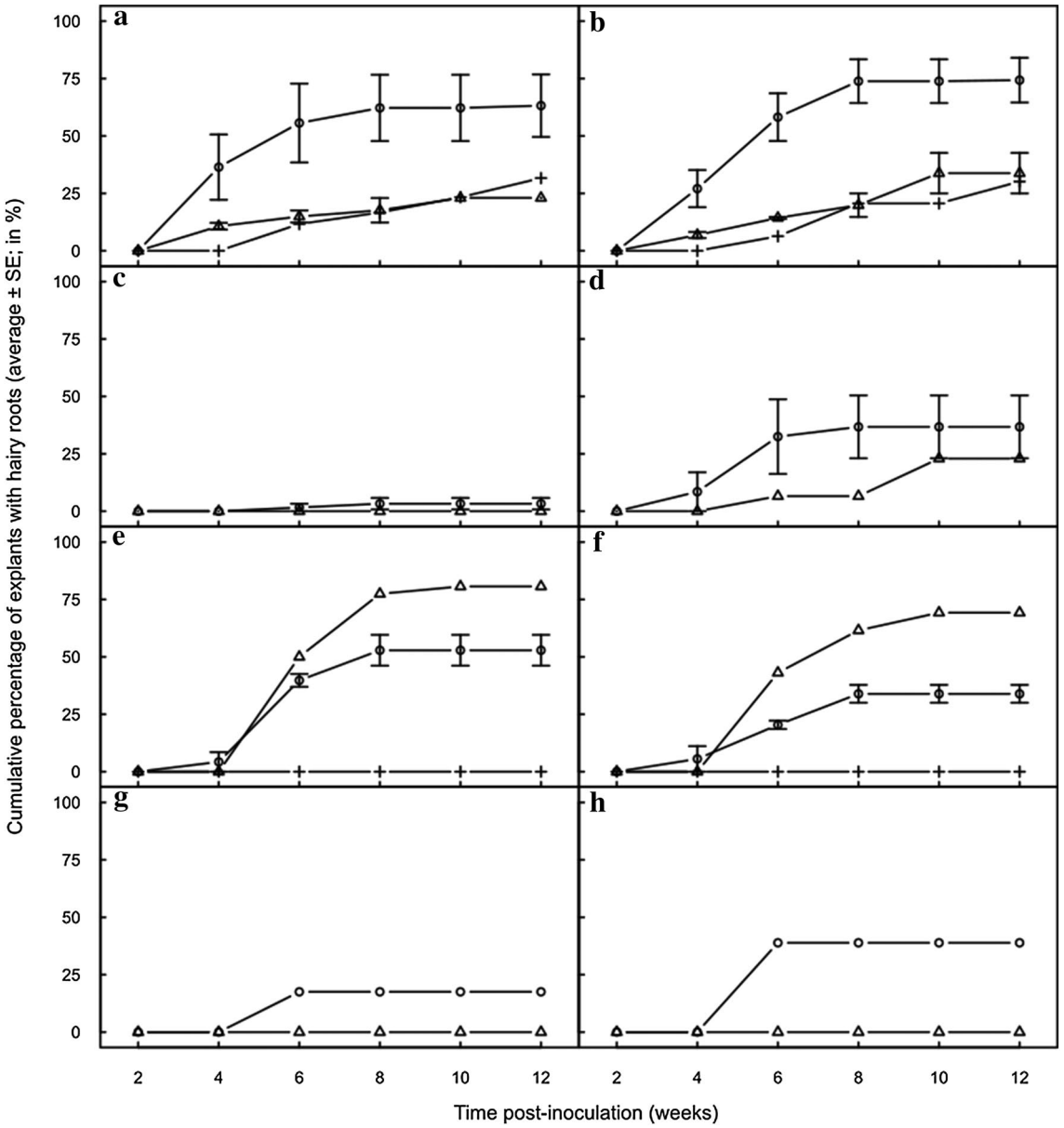

Fig. 3 The average cumulative percentage of leaf (**a**, **c**, **e**, **g**) and nodal (**b**, **d**, **f**, **h**) explants with hairy roots (for repeated experiments ± SE) for the different combinations of rhizogenic *Agrobacterium* strains (Arqua1 open circles, LMG63 open triangles, and MAFF210266 plus) and *Escallonia* species [*E. illinita* (**a**, **b**), *E. rosea* (**c**, **d**), *E. rubra* (**e**, **f**), *E. myrtoidea* (**g**, **h**)] in function of time (in number of weeks post co-cultivation)

root samples from the different *Escallonia* species, indicating bacteria were sufficiently eliminated after the antibiotic treatment. In total, 85.4% of hairy roots contained all four *rol*-genes (Table 6). A higher transformation rate was obtained with Arqua1 (90.0%) compared to LMG63 (75.8%). After inoculation with LMG63, only a single root from *E. rubra* contained *rol*-genes, but for *E. illinita* all 24 tested roots were transformed (Table 6). After inoculation with Arqua1, in four roots of *E. illinita*, two of *E. rosea* and one of *E. rubra* no *rol*-genes were present. In contrast, in these *E. rosea* and *E. rubra* roots presence of *aux*-genes was confirmed, indicating that transfer of the T_R-DNA did occur. In case of *E. illinita* roots, 29.6% did not contain *aux*-genes, but *rol*-genes were present in 50% of these roots. Thus in this case only the T_L-DNA was transferred into the plant. A total of 59 roots derived after inoculation with Arqua1 contained all four *rol*-genes and both *aux*-genes, so both T_R- and T_L-DNA was transferred (Table 6).

Table 6 Presence (+) or absence (−) of the four *rol*- and two *aux*-genes in hairy roots of different *Escallonia* species, induced after explant co-cultivation with Arqua1 or LMG63

Bacterial strain	*Escallonia* genotype	# Hairy roots tested	*rol A*, *B*, *C* and *D*	*aux1* and *2*
LMG63	*E. illinita*	24	+	NT
	E. rubra	8	−	NT
		1	+	NT
Arqua1	*E. illinita*	19	+	+
		4	+	−
		4	−	−
	E. rosea	16	+	+
		2	−	+
	E. rubra	22	+	+
		1	−	+
	E. myrtoidea	2	+	+

NT not tested, genes are not present in the original bacterial strain

Shoot regeneration on hairy roots

A limited amount of shoots regenerated on the harvested hairy roots (Table 7). In total 5 Arqua1 and 4 LMG63 shoots regenerated on a total of 890 Arqua1, 303 LMG63, and 74 MAFF210266 hairy roots (regeneration frequencies: 0.56% for Arqua1; 1.32% for LMG63; 0% for MAFF210266).

Table 7 Shoots regenerated from hairy roots of different *Escallonia* species, with used media and regeneration time

Escallonia genotype	Number of shoots		Medium	Regeneration time (weeks)
	Arqua1	LMG63		
E. illinita	2		RM11	5
	1		RM10	6
	1		RM10 → RM12	4 + 12
	1		RM08 → RM09	4 + 11
		1	RM07	5
E. rosea		1	RM07 → RM01	4 + 4
E. rubra		2	RM07 → RM01	4 + 4

For *E. illinita*, five shoots regenerated after Arqua1 co-cultivation and one after LMG63 co-cultivation (Fig. 4). Two of these Arqua1 shoots were obtained after 5 weeks when using 3.0 mg/L TDZ (RM11). One Arqua1 shoot resulted after 6 weeks using 7.0 mg/L BAP (RM10). The 4th and 5th Arqua1 shoots occurred after the shock treatment with BAP, 4 weeks RM10 followed by 16 weeks RM12, and 4 weeks RM08 followed by 11 weeks RM09, respectively. The LMG63 shoot of *E. illinita* appeared after 5 weeks in 1.0 mg/L TDZ (RM07).

One *E. rosea* LMG63 shoot and two *E. rubra* LMG63 shoots were regenerated after a shock treatment of 4 weeks

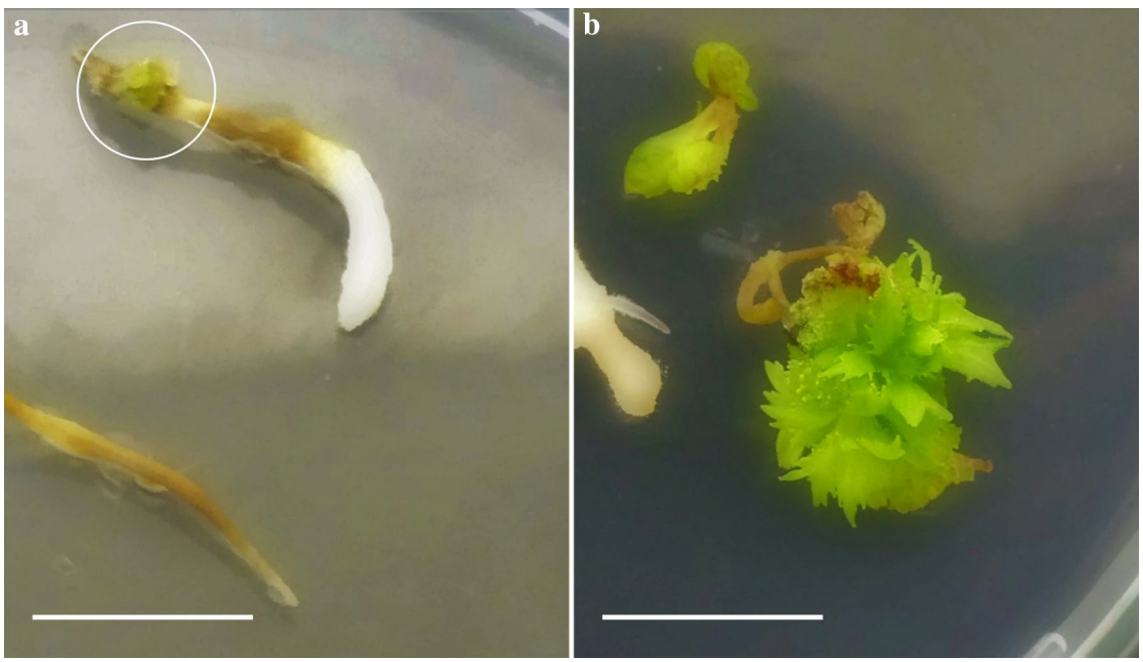

Fig. 4 Shoot regeneration from *Escallonia illinita* hairy root tissue obtained by co-cultivation using LMG63. **a** Induction of shoot primordia on a hairy root and **b** developing shoot 1 month after being separated from the hairy root. Scale bar = 1 cm

in 1.0 mg/L TDZ (RM07) followed by 4 weeks in 0.1 mg/L TDZ (RM01).

In all the regenerated shoots *virD2* was not amplified, consistent to the hairy root screening. However, none of the regenerated shoots contained any of the *rol*- and *aux*-genes.

Discussion

T-DNA transfer into the plant host cell requires a complex interaction of proteins of both the rhizogenic *Agrobacterium* strain and the host plant (Lacroix and Citovsky 2016). As a result strains can display different degrees of virulence depending on the plant genotypes. In our study, hairy roots upon inoculation of plant material with rhizogenic bacteria were successfully obtained. However, the rhizogenic *Agrobacterium* strains showed clear differences with regard to hairy root induction efficiency in the different *Escallonia* species. The most infectious strain for *Escallonia* was Arqua1, capable of inducing hairy roots with confirmed presence of *rol*-genes in all four species. This strain has successfully transformed other woody species e.g. avocado (Prabhu et al. 2017). LMG63 and MAFF210266 are effective in three and one out of the four species, respectively. With regard to infection of woody species, MAFF210266 previously demonstrated its effectiveness, e.g. in *Lavandula* (Tsuro and Ikedo 2011) whereas the successful application of LMG63 is not reported in literature. In addition, we observed plant and strain genotype specific interactive effects: Arqua1 induced more hairy roots in *E. illinita* and *E. rosea* than LMG63, while this was the other way around in *E. rubra*. *E. myrtoidea* displays a high degree of recalcitrance for LMG63. MAFF210266 induces efficient hairy root formation in *E. illinita* only. These effects are reminiscent of the genotype specific patterns of virulence described previously by Porter and Flores (1991) and indicate the importance of the plant–strain interaction (Pitzschke 2013). Similar differences in host-strain reactions are described in poplar (Neb et al. 2017) and in *Coffea* (Alpizar et al. 2006).

Overall, the hairy root induction efficiencies obtained for *Escallonia* are consistent with efficiencies published for other woody species. Both Arqua1 and LMG63 induced hairy roots in up to about 80% of the explants. For MAFF210266 the efficiency was lower, reaching a maximum of 38% explants with hairy roots. Optimum hairy root induction efficiencies reported for other woody species are 61% in *Semecarpus* (Panda et al. 2017), 95% in *Rauwolfia* (Mehrotra et al. 2013), and 60% in *Hibiscus* (Christensen et al. 2009). Other bacterial strains could be investigated to reveal highly compatible plant–strain genotype combinations. Also, the application of a strain specific co-cultivation protocol could lead to higher transformation efficiencies (Desmet et al. 2019).

Shoots can spontaneously regenerate on hairy roots or directly on the inoculated explants (Subotić et al. 2003; Christensen and Müller 2009; Kim et al. 2012; Mehrotra et al. 2013). Regeneration of *Escallonia* hairy roots however remains a bottleneck, even after supplementing regeneration media with various phytohormones. In total, only 9 shoots regenerated on 1278 hairy roots. Woody species in general are known for their recalcitrance in vitro (Rugini et al. 1991; Rastogi et al. 2008). Furthermore, also genotype effects on regeneration potential are reported (Hegelund et al. 2017). We already observed differences in effectiveness of in vitro initiation and multiplication media between *Escallonia* species (Denaeghel et al. 2018), so most likely, also the shoot regeneration procedure will have to be optimized for each species. The shoot induction capacity can be improved by searching for an appropriate cytokinin (type and/or concentration) (Amoo et al. 2011). Additionally, crucial steps in the shoot regeneration process, such as callus induction and shoot induction, could be investigated in order to increase regeneration efficiency (Motte et al. 2014).

None of the *Escallonia* hairy roots induced after co-cultivation with rhizogenic agrobacteria regenerated into *rol*-gene positive shoots. The lack of regeneration of hairy roots after co-cultivation with Arqua1 could be attributed to the transfer of T_R-DNA auxin genes into the plant DNA (White et al. 1985; Jouanin et al. 1987). These *aux1* and *aux2* genes can influence the formation of adventitious roots (Camilleri and Jouanin 1991) which could result in hairy root-like roots without the actual transfer of *rol*-genes, decreasing the relative yield of regenerants containing *rol*-genes. The confirmed presence of auxin genes in Arqua1-hairy roots can also influence potential shoot regeneration capacity by changing the auxin/cytokinin ratio. In *Campanula*, shoots only regenerated on hairy roots that did not contain auxin genes (Hegelund et al. 2017). The *rol*-genes themselves also play a role in shoot regeneration by influencing hormone sensitivity and hormone pathways and thus shoot regeneration capacity. The protein of *rolA* can influence the auxin content (Bettini et al. 2016), *rolB* influences the auxin signal transduction pathway (Maurel et al. 1994), and *rolC* proteins can change the auxin sensitivity (Zuker et al. 2001; Koshita et al. 2002).

The absence of the *virD*2 gene in the hairy roots indicates that the applied treatment with antibiotics was sufficient to remove all bacteria. Moreover, in all four species of *Escallonia*, hairy roots were recovered which contain both *rol* (Arqua1 and LMG63) and *aux* genes (Arqua1). The *rol* positive roots of both Arqua1 and LMG63 always contained all four *rol*-genes. Similarly *aux* positive roots always contained

both *aux1* and *aux2*. This indicates that independent transfer of the T_L-DNA and/or T_R-DNA to the plant occurs as non-fragmented sequences which is reported to be the general mechanism of T-DNA transfer and integration (Choi et al. 2004; Kang et al. 2006; He-Ping et al. 2011; Kim et al. 2012; Petrova et al. 2013). Only a few studies report on the fragmentation of the T-DNA e.g. in *Chrysanthemum cinerariaefolium*, where hairy root lines contained either *rolABC* or *rolBC*, the presence of *rolD* in this study was however not investigated (Khan et al. 2017). In *Bacopa monnieri* 3 regenerants, containing either *rolAB*, *rolABC* or only *rolC*, were obtained (Majumdar et al. 2011).

For *E. rosea* and *E. rubra*, several hairy roots were obtained that carry the *aux* genes but not any of the *rol* genes. Piispanen et al. (2003) found that the presence of *aux* genes in plants resulted in an alleviated version of the Ri phenotype, in which some negative *rol* gene effects such as wrinkled leaves are less pronounced. Therefore, these *aux* containing roots could be very valuable in the creation of unique phenotypes.

In conclusion for this study, we showed the potential of *rol*-gene introduction as a breeding tool for *Escallonia*, and in extension for woody plants in general. We obtained a protocol for hairy root induction in several *Escallonia* species and we developed a screening method enabling to screen for the presence of *rol*-genes and *aux*-genes separately. Hairy roots containing *rol*-genes were induced for all *Escallonia* genotypes in our study. We were able to regenerate few roots, but shoot regeneration from hairy roots remains a bottleneck and the search for an optimal shoot regeneration protocol continues. However, although there were no transformed plants among the regenerants, the regeneration of roots is a step forward in the breeding research of *Escallonia* in particular and woody plants in general.

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Compliance with ethical standards

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

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