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High-Efficiency *Agrobacterium rhizogenes*-Mediated Transgenic Hairy Root Induction of *Lens culinaris*

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Abstract: Previous efforts to transform lentil have been considerably hampered by the crop's recalcitrant nature, giving rise to particularly low transformation and regeneration frequencies. This study aimed at optimizing an *Agrobacterium rhizogenes*-mediated transformation protocol for the generation of composite lentil plantlets, comprised of transgenic hairy roots and wild-type shoots. Transformation was performed by inoculating the cut hypocotyl of young lentil seedlings, while optimization involved the use of different bacterial strains, namely *R1000*, *K599* and *Arqua*, and protocols differing in media composition with respect to the presence of acetosyringone and MES. Composite plantlets had a transgenic hairy root system characterized by an increased number of hairy roots at the hypocotyl proximal region, occasionally showing plagiotropic growth. Overall findings underline that transformation frequencies are subject to the bacterial strain, media composition as well as their combined effect. Among strains tested, *R1000* proved to be the most capable of hairy root formation, while the presence of both acetosyringone and MES in inoculation and culture media yielded considerably higher transformation rates. The transgenic nature of hairy roots was demonstrated by the Ri T-DNA-mediated transfer of the *rolB2* gene and the simultaneous absence of the *virCD* sequence of *A. rhizogenes*. Our findings provide strong evidence that *A. rhizogenes*-mediated transformation may be employed as a suitable approach for generating composite seedlings in lentil, a species whose recalcitrance severely hampers all efforts addressed to transformation and whole plant regeneration procedures. To the best of our knowledge, this is the first report on the development of a non-laborious and time-efficient protocol for the generation of transgenic hairy roots in lentil, thus providing an amenable platform for root biology and gene expression studies in the context of improving traits related to biotic and abiotic stress tolerance.

Keywords: lentil; genetic transformation; *Agrobacterium rhizogenes*; hairy roots; composite plants; root biology studies

1. Introduction

Lentil (*Lens culinaris* Medik) is the most ancient and one of the most important food legume crops worldwide. Its seeds contain essential content of dietary protein, whose contribution to human nutrition is of vital importance in certain areas, mainly in the developing world [1]. In the last few decades, lentil's global demand and consumption shows the highest growth rate in comparison to other major pulses [2]. Although it is a crop ideally suited to addressing issues of growth in marginal environments and/or low input cultivation systems, its yield potential is considerably hampered by various biotic and abiotic stresses as well as its narrow genetic base, which poses serious constraints on breeding efforts for crop improvement. At the same time, the perspectives of obtaining additional gains by molecular breeding approaches seem rather problematic due to the lack of genomic data for genes controlling essential agronomic traits, including those related to biotic and abiotic stress tolerance [3].

The recalcitrant nature of lentil [4,5] further limits the possibilities offered by biotechnology, and genetic engineering in particular, to broaden the available gene pool for crop improvement.

Initial attempts to transform lentil employed various gene transfer approaches, including *Agrobacterium tumefaciens* [6,7], biolistic delivery [8,9] and in planta electroporation [10], while in recent years, the focus has been placed on *A. tumefaciens*-mediated gene transfer [5,11–13]. Despite the progress achieved, the transformation efficiency remains extremely low and highly dependent on a genotype basis, while the transformation procedures are characterized as rather tedious and time-consuming. To circumvent limitations posed by stable transformation, *A. rhizogenes* is increasingly gaining interest as an alternative vehicle for gene transfer in recalcitrant plant species such as lentil.

A. rhizogenes is a Gram-negative, rod-shaped soil bacterium that, due to the possession of a root-inducing (Ri) plasmid, is capable of transferring a T-DNA region into the genome of infected plant cells. The expression of the root locus (rol) genes in the T-DNA region, such as *rolA*, *rolB*, *rolC* and *rolD*, promotes the formation of hairy roots emerging at the wounded surface of explants [14]. *A. rhizogenes*-mediated transformation leads to the generation of composite plants consisting of wild-type shoot and transgenic root tissues [15–17]. Hairy roots are usually non-chimeric and similar in phenotype and structure to wild-type roots, though they are capable of exogenous hormone-independent growth and high lateral branching accompanied by plagiotropic root development [18,19]. *A. rhizogenes*-mediated generation of transgenic roots has been extensively exploited as a tool for promoter analysis as well as phenotype-based functional screens, such as loss-of-function/knock-down assays by RNAi-mediated gene silencing as well as gain-of-function assays by protein overexpression [20]. More importantly, recent findings underline the possibility to practically exploit hairy roots for the large-scale production of bioactive compounds, such as secondary metabolites with medicinal properties [21].

A. rhizogenes-mediated transformation has been realized in a plethora of species belonging to the family of Leguminosae, including both model plants, such as *Lotus japonicus* and *Medicago truncatula* [22,23], and cultivated species, such as *Glycine max* [24,25], *Pisum sativum* [26–28], *Cicer arietinum* [29–32] and *Phaseolus vulgaris* [33]. Relative efforts in lentil, however, are restricted to one study which was characterized as unsuccessful in transforming lentil seedlings through *A. rhizogenes* [34]. In view of lentil's recalcitrance to transformation and regeneration procedures, this study attempts to optimize an *A. rhizogenes*-mediated transformation protocol which allows a fast and non-laborious generation of hairy roots to serve as a tool for gene functional and root biology studies. For this purpose, the optimization of a high-efficiency transformation protocol was pursued, using different bacterial strains and protocols differing in media composition.

2. Materials and Methods

2.1. Plant Material

Transformation experiments were carried out using the lentil cultivar Samos, which is a local accession, conventionally bred and deposited at ELGO Demeter (Institute of Industrial and Fodder Plants, Larissa, Greece).

2.2. Preparation of Inoculum for Transformation

Agrobacterium rhizogenes strains *R1000*, *K599* and *Arqua* were used to transform lentil seedlings. Bacterial strains were grown in 5 mL liquid Luria–Bertani (LB) medium supplemented with appropriate antibiotics (nalidixic acid (25 μg mL^{-1}), rifampicin (50 μg mL^{-1}) and spectinomycin (50 μg mL^{-1}) for *R1000*, *K599* and *Arqua*, respectively) at 28 °C and 160 rpm for 2–3 days or until OD600 = 0.6–1.0 was reached (Figure 1c). Bacterial cells were harvested by centrifugation at 3000 rpm for 5 min and washed in liquid MS. Following centrifugation at 3000 rpm for 5 min, the pellet was re-suspended in liquid MS and used as inoculum for plant transformation (Figure 1d).

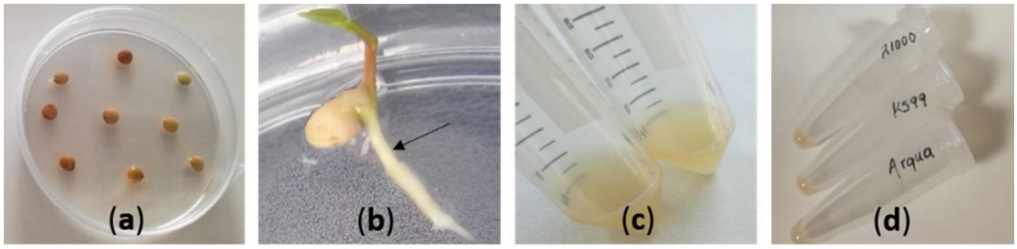

Figure 1. *Agrobacterium rhizogenes*-mediated transformation of lentil. (**a**) Sterilized seeds allowed to germinate; (**b**) three-day-old seedlings used as explants for hairy root induction. The arrow indicates the hypocotyl region, where sectioning was conducted and the existing roots were excised; (**c**) *A. rhizogenes* cultures; (**d**) inoculum used for plant transformation.

2.3. Hairy Root Induction

Three-day-old healthy seedlings with a normal phenotype were employed as explant material for *A. rhizogenes*-mediated transformation, according to the protocol described by Pavli and Skaracis [35] with modifications in relation to age of explants, inoculation method and media composition. In order to determine the optimal media composition for inoculation and hairy root formation, two transformation protocols were followed, differing in the presence of acetosyringone and MES (Table 1).

Table 1. Culture media composition employed for *A. rhizogenes*-mediated hairy root induction in lentil.

Culture Medium	Protocol 1	Protocol 2
Inoculation (MS)	MS: 4.4 g/L Sucrose: 30 g/L -	MS/MES/vitamins: 4.9 g/L Sucrose: 30 g/L Acetosyringone: 150 µM
Co-cultivation	MS: 2.2 g/L Sucrose: 20 g/L Agar: 9.0 g/L -	MS/MES/vitamins: 2.2 g/L Sucrose: 20 g/L Agar: 9.0 g/L Acetosyringone: 100 µM
Emergence	MS: 2.2 g/L Sucrose: 30 g/L Agar: 9.0 g/L	MS/MES/vitamins: 2.46 g/L Sucrose: 30 g/L Agar: 9.0 g/L

Seeds were surface-sterilized in 20% hypochlorite/dH_2O solution containing Tween-20, while gently mixing for 5 min, and washed (4×) with sterile dH_2O. Sterilized seeds were subsequently placed on 1/2 Murashige & Skoog (MS) basal salt medium (Duchefa Biochemie B.V., NL) supplemented with 0.5% PhytoAgar (pH = 5.6) and allowed to germinate under controlled conditions (25 °C, 16 h light/8 h dark cycle) (Figure 1a). After 3 days, seedlings were obtained and used as explants for hairy root induction (Figure 1b). Inoculation was performed by excising the existing roots in the hypocotyl region using a sterile scalpel and dipping the wounded root tip into the bacterial cells. Inoculated lentil seedlings were co-cultivated with *A. rhizogenes* for 3 days (23 °C, 16/8 h light/darkness) in square petri dishes containing co-cultivation medium (Figure 1). Seedlings were subsequently transferred to emergence medium containing cefotaxime (250 mg L^{-1}) so as to eliminate the residual bacteria. After 15 days, seedlings were transferred to fresh emergence medium supplemented with cefotaxime (250 mg L^{-1}). Petri dishes were partially sealed, to enable aeration, and placed vertically in a growth chamber. Non-transformed seedlings cultured under the same conditions were included as controls. The emergence of hairy roots was recorded as a means to provide estimates of transformation efficiency (Figure 2).

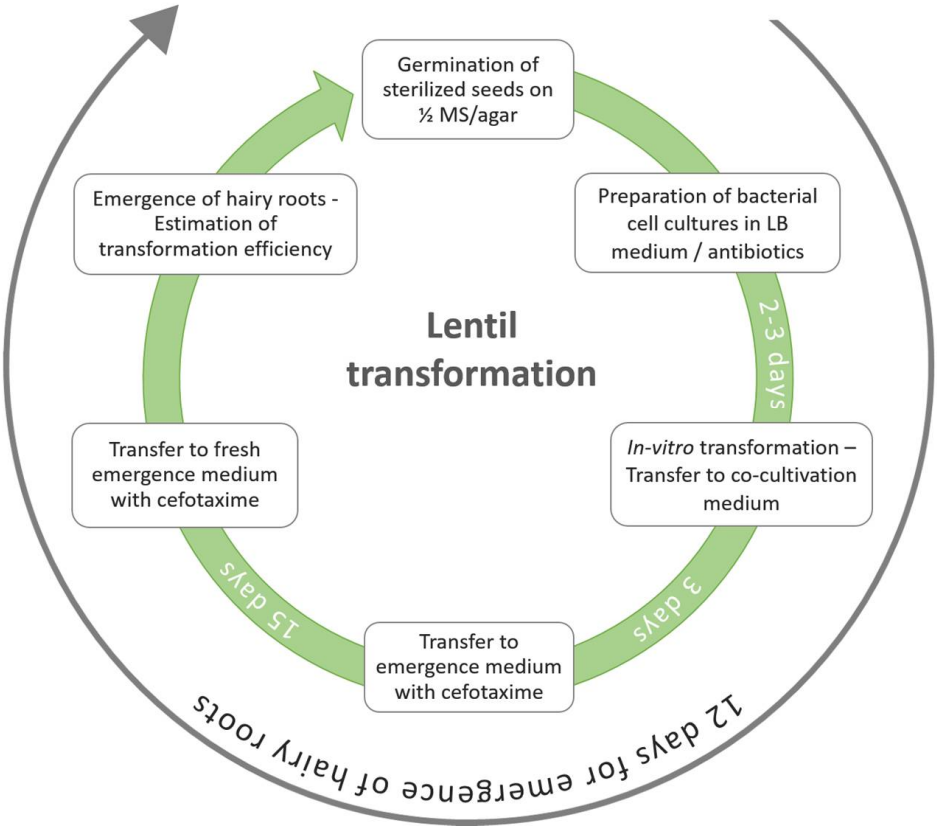

Figure 2. Stepwise protocol for *A. rhizogenes*-mediated hairy root induction in lentil.

2.4. Evaluation of Transformed Roots

Genomic DNA of hairy roots and wild-type roots was extracted using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). DNA was subjected to multiplex PCR, targeting the nucleotide sequences of the *rolB2* and *virCD* genes, to verify T-DNA integration into the plant genome and absence of residual *A. rhizogenes* cells. Targeted sequences were amplified using the following gene-specific primer pairs: *rolB2*-F: 5′-GCTCTTGCAGTGCTAGATTT-3′/*rolB2*-R: 5′-GAAGGTGCAAGCTACCTCTC-3′ [21] and *virCD*-F: 5′-CTCATCAGGCACGCTTG-3′/*virCD*-R: 5′-GCGGATGCTTCAAATGG-3′ [35]. PCR reaction mixture contained 10 ng of genomic DNA, 0.25 µM of each primer, 200 µM dNTPs, 1.25 mM $MgCl_2$, 1× Taq buffer and 1.25 u Taq polymerase (GoTaq Flexi DNA polymerase, Promega) in a final volume of 25 µL. Amplification conditions included initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min and a final elongation at 72 °C for 7 min. Amplification products were analyzed on 1.5% agarose gels stained with ethidium bromide (0.5 mg L^{-1}) and visualized under UV light.

2.5. Statistical Analysis

The 2 × 3 factorial experiment (factors: strain, protocol) followed a completely randomized design, with 3 replications for the 6 treatments. Each experimental unit included 20 explants, and transformation efficiency was expressed as percentage of the explants producing hairy roots. Given that data normality were not rejected (W-test, Prob < 0.115), standard ANOVA was employed, and pertinent comparisons were based on the LSD at the 5% significance level (JMP version 13.0, SAS Institute Inc., Cary, NC, USA, 1989–2019).

3. Results

3.1. Protocol Optimization for the Generation of Composite Plants in Lentil

To optimize a hairy root induction protocol suitable for generating transgenic roots in lentil, *A. rhizogenes*-mediated transformation was pursued using a set of bacterial strains (*R1000*, *K599* and *Arqua*) and protocols differing in composition of inoculation, co-cultivation and emergence media. Transformation was performed by inoculating the hypocotyl of aseptically grown young seedlings. A limited number of lateral roots appeared on the radicle section, shortly after inoculation, in response to the removal of the existing root system both in non-transformed (Figure 3a) and transformed lentil seedlings (Figure 3b–d). However, the emergence of hairy roots was recorded 10–16 days post inoculation, with the exception of seedlings transformed with *Arqua* strain and cultured using the media described in protocol 1 (Table 2). At this timepoint, non-transformed seedlings developed roots of wild-type phenotype and showed an absence of hairy root formation. Three weeks after inoculation, the hairy root phenotype differed significantly from wild-type roots in relation to growth rate, structure and biomass accumulation. In particular, transformed roots exhibited an increased number of hairy root extensions, occasionally exhibiting a plagiotropic development. Figure 4 comparatively depicts the growth patterns of roots in wild-type (Figure 4a) and transformed seedlings (Figure 4b–d) using the different bacterial strains and transformation protocols.

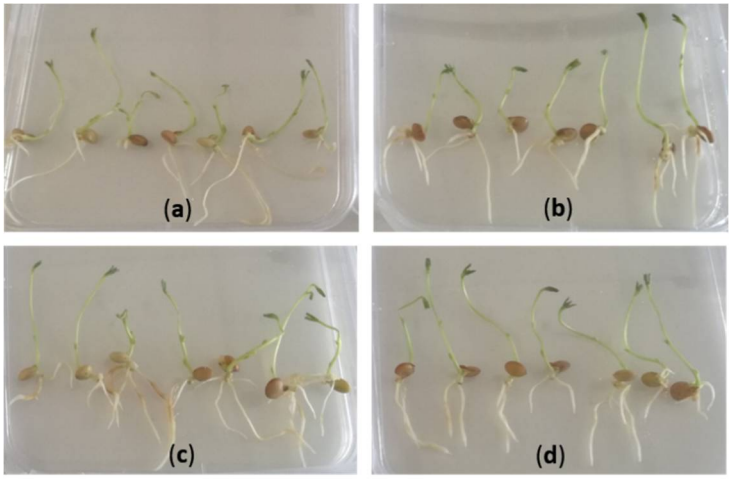

Figure 3. Phenotype of lentil seedlings, 10 days after inoculation. (**a**) Non-transformed seedlings; (**b**) seedlings transformed with *A. rhizogenes* strain *R1000*; (**c**) seedlings transformed with *A. rhizogenes* strain *K599*; (**d**) seedlings transformed with *A. rhizogenes* strain *Arqua*.

Table 2. Effect of *Agrobacterium rhizogenes* strain (*R1000*, *K599*, *Arqua*) and media composition (protocol 1 and 2) on transformation frequency.

***A. rhizogenes* Strain**	**N. Inoculated Explants**		**Time to Hairy Root Emergence (dpt)**		**Seedlings That Formed Hairy Roots (%) [1]**		**MEAN (S)**
	1 [2]	2	1	2	1	2	
R1000	60	60	12 ± 1	10 ± 1	76.7a [3]	95.0a	85.8a
K599	60	60	16 ± 1	15 ± 1	10.0b	38.3c	24.15b
Arqua	60	60	-	15 ± 1	0.0b	56.7b	28.3b
MEAN (P)					28.9a	63.3b	

[1] Transformation efficiency (%) = number of explants producing hairy roots/total number of explants × 100. The seedlings that gave rise to at least one hairy root per plant were considered to be transformed. [2] Protocol applied. [3] Comparisons among strains within each protocol are shown. In addition, comparisons among strains across protocols as well as among protocols across strains are given. Same letters denote no significant differences at the 0.05 level. Interactions (table body) were also found to be significantly different (SED = 5.61).

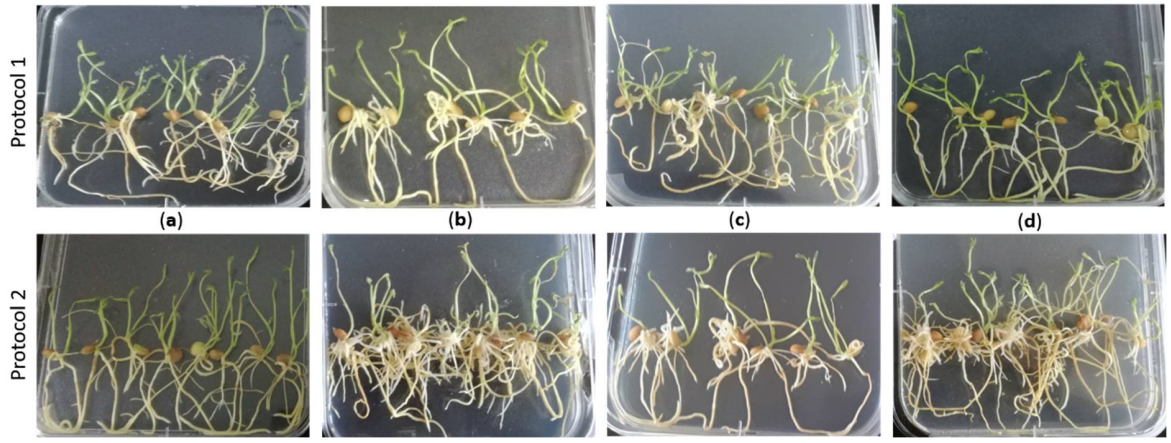

Figure 4. Comparative phenotype of lentil seedlings transformed with different bacterial stains, according to transformation protocols 1 and 2, three weeks after inoculation. (**a**) Non-transformed seedlings; (**b**) seedlings transformed with *A. rhizogenes* strain *R1000*; (**c**) seedlings transformed with *A. rhizogenes* strain *K599*; (**d**) seedlings transformed with *A. rhizogenes* strain *Arqua*.

Although all strains were capable of hairy root induction in lentil, they differed significantly in terms of transformation efficiency, with the proportion of hairy roots generated ranging between 10% and 95% (Table 2). Apart from the bacterial strain, transformation efficiency was considerably affected by media composition, as evidenced by the different transformation frequencies of protocols applied. As such, the presence of acetosyringone and MES both in the bacterial culture prior to inoculation and the co-cultivation and emergence media significantly enhanced the transformation ability of all bacterial strains (Table 2). In the absence of acetosyringone (protocol 1), strain *R1000* proved the most capable of hairy root formation (76.60%), while strains *K599* and *Arqua* gave rise to considerably lower transformation rates (10% and 0%, respectively). The presence of acetosyringone and MES (protocol 2) promoted transformation efficiency, with the respective rates mounting to 95%, 56.6% and 38.3% for strains *R1000*, *Arqua* and *K599*. Such findings are indicative of the fact that bacterial strain and media composition, mainly with respect to acetosyringone, as well as their combined effect, are determinant factors in the efficiency of hairy root induction in lentil. Our findings further underline the superiority of strain *R1000* in generating transgenic hairy roots, independently of the presence of acetosyringone and MES.

3.2. Verification of the Transgenic Nature of Hairy Roots

To confirm the transgenic nature of hairy roots in lentil seedlings, proper integration of the T-DNA was examined by means of a multiplex PCR targeting the *rolB2* gene and the *virCD* sequence of *A. rhizogenes*. The presence of the *rolB2* transgene (423 bp) was detected in all hairy roots, thus confirming successful gene integration (Figure 5). Amplicons corresponding to the *virCD* fragment (1074 bp) of *A. rhizogenes* were only obtained using the bacterial strains (*R1000*, *Arqua*, *K599*) as a template, thus excluding the presence of residual bacteria and verifying the transgenic nature of hairy roots grown. As expected, both the *rolB2* and *virCD* sequences were not detected in roots of non-transformed seedlings (Figure 5).

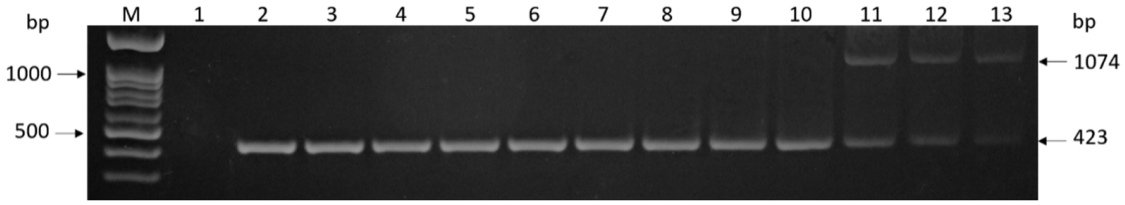

Figure 5. Amplification products obtained by multiplex PCR for the evaluation of transgenic roots. M: DNA Ladder (FastGene 100bp DNA Ladder RTU, NIPPON Genetics Europe GmbH). Lane 1: Roots from non-transformed seedlings, showing absence of both *rol2B* and *virCD* genes. Lanes 2, 3, 4: Roots transformed with *A. rhizogenes* strain *R1000*, carrying the *rolB2* gene. Lanes 5, 6, 7: Roots transformed with *A. rhizogenes* strain *K599*, carrying the *rolB2* gene. Lanes 8, 9, 10: Roots transformed with *A. rhizogenes* strain *Arqua*, carrying the *rolB2* gene. Lanes 11, 12, 13: Bacterial cells from strains *R1000*, *Arqua* and *K599*, carrying both *rol2B* and *virCD* genes. The amplicon of 1074 bp corresponding to *virCD* could only be amplified using bacterial cells as a template, thus verifying the absence of *A. rhizogenes* cells in transgenic roots.

4. Discussion

The in vitro transformation of lentil faces many challenges, thus limiting the possibility of establishing a reliable and readily applicable transformation protocol to be exploited for gene functional studies. Lentil transformation has been in the past pursued through *A. tumefaciens*-mediated gene transfer [5–7,11–13] and biolistic methods [8–10]. However, the efficiency of the abovementioned approaches has been considerably limited by practical implications, as they are rather laborious and time-consuming, along with the recalcitrant nature of the crop giving rise to particularly low transformation frequencies [4]. As an alternative, the *A. rhizogenes*-mediated generation of composite plants, consisting of transgenic roots and wild-type shoot tissues, has proven to be an amenable approach to rapidly generate hairy roots carrying transgenes of interest, as evidenced in a wide range of plant species. In legumes, *A. rhizogenes*-mediated transformation was first researched in *Lotus corniculantus* to facilitate studies on nodule formation [17,36], while later efforts focused on the development of related protocols in model plants [22,23] as well as cultivated species [25,28,32,33]. Given the lack of relative transformation protocols in lentil, this study aimed at optimizing a protocol enabling an efficient generation of transgenic roots to serve as a suitable platform for root biology studies.

A. rhizogenes-mediated transformation of lentil seedlings was pursued using different bacterial strains and protocols differing in composition of inoculation, co-cultivation and emergence media. Our data underline that both strains and media composition as well as their combination are determinant factors in the efficiency of hairy root formation in lentil. As such, significant differences were noted among strains, in terms of both efficiency and time period required to induce hairy root formation in lentil. Strain *R1000* proved to be the most efficient in inducing hairy root formation, giving rise to transformation rates ranging from 76.6% to 95%, while strains *K599* and *Arqua* yielded considerably lower rates. These findings are in agreement with previous reports on the variable ability of *A. rhizogenes* strains to induce the formation of adventitious hairy roots. In legumes, the production of Ri T-DNA-transformed hairy roots has been realized with a variety of bacterial strains. Strain *K599* has proven capable of transforming *Phaseolus vulgaris* [33], *Glycine max* [37] and *Cicer arietinum* [32], while strain *Arqua* has successfully transformed *Medicago truncatula* [38] and *Pisium sativum* [28]. Both *Arqua* and K599 are considered to be *A. rhizogenes* strains of low virulence, thus eliciting a limited number of hairy roots which exhibit a growth pattern comparable to wild-type roots [19,39]. On the other hand, the more virulent strain *R1000* harbors a Ri plasmid with a higher intrinsic capacity [40,41] for T-DNA transfer into the host cells during the infection process [42], thus enabling a more efficient induction of hairy root formation, as evidenced in various plant species [22,35,43,44].

Despite the differential transformation potential per se of strains tested, in all cases, the presence of acetosyringone and MES enhanced the transformation efficiency, as evidenced by the increased number

of composite seedlings with transgenic hairy roots at the hypocotyl proximal region. Acetosyringone, a phenol known to induce the *vir* genes and promote the transfer of Ri T-DNA into the plant cells [45], is routinely applied as a means to enhance the transformation frequency but also decrease the time required for hairy root induction in various plant species [46–48]. Although its contribution to the increase in transformation frequency in lentil was non-essential using the *A. tumefaciens* strain *KYRT1* [12], our findings underline that acetosyringone effectively enhances the transformation rate of infected explants. The observed increase in transformation rate also reflects the positive effect of MES, which has been assigned a role of buffering agent, reducing the excessive acidification of media [49,50]. In this line, our data are in agreement and further reinforce previous reports related to the fact that the stabilization of acidic pH, especially during co-cultivation, is a precondition for a successful infection by *Agrobacterium* and the achievement of increased transformation efficiency [49]. Based on the comparative evaluation of the protocols applied, our findings further support the conclusion that such pH stabilization may counterbalance the adverse effect of sucrose, which has been previously characterized as a non-effective *vir* gene coinducer as it reduces the response of *vir* genes to phenolic inducers like acetosyringone [51]. The phenotypes of transformed roots differed significantly from wild-type roots both in terms of structure and growth rate. Non-transformed seedlings were incapable of hairy root formation, while infected seedlings were characterized by the generation of long hairy root extensions at the hypocotyl proximal region. The transgenic nature of hairy roots was demonstrated by the Ri T-DNA-mediated transfer of the *rolB2* gene, whose function relates to pathogenicity [16]. *A. rhizogenes* transfers two independent T-DNAs, namely TL-DNA and TR-DNA, into the genome of infected plant tissues [52], with the former—carrying ORF 10 (*rolA*), ORF 11 (*rolB*), ORF 12 (*rolC*) and ORF (*rolD*)—being a precondition for hairy root formation [53]. These findings, combined with the simultaneous absence of *virCD* sequence in the hairy roots examined, indicate the efficacy of the developed transformation protocol. Although the generated hairy roots were not selected through the routinely used antibiotic-based resistance, these findings form a basis upon which to explore the possibilities of further improving this protocol to increase the consistency and stability of hairy root production among infected seedlings.

Conclusively, our findings provide strong evidence that *A. rhizogenes*-mediated transformation may be employed as a suitable approach for generating composite seedlings in lentil, a species whose recalcitrance imposes serious constraints on transformation and whole plant regeneration procedures. Based on these preliminary findings, the highest transformation rates may be achieved through the combined positive effects of strain *R1000* and media supplementation with acetosyringone and MES. The generated transgenic hairy roots provide an attractive platform for root biology studies, including those related to nodulation, root developmental processes and interactions of roots with pathogens and abiotic stresses, but also for more practical applications such as the production of valuable secondary metabolites. To the best of our knowledge, this study is the first report on the development of a non-laborious and time-efficient protocol for the generation of transgenic hairy roots in lentil as a shortcut approach to the tedious and low efficiency stable transformation procedures.

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