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Influences of *Agrobacterium rhizogenes* strains, plant genotypes, and tissue types on the induction of transgenic hairy roots in *Vitis* species

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Summary

In this study, we evaluated the influences of 3 *Agrobactrium rhizogenes* strains (15384, A4, and K599), 4 different tissue types (internodes, stem-cut and petiole-cut surfaces on *in vitro* plants, and young shoots excised from *in vitro* plants), and 14 *Vitis* species (*Vitis cinerea*, *V. champinii*, *V. doaniana*, *V. ficifolia*, *V. flexuosa*, *V. girdiana*, *V. jacquemontii*, *V. labrusca*, *V. nesbittiana*, *V. novae-angliae*, *V. palmata*, *V. piasezkii*, *V. treleasii*, and *V. vinifera*) on the induction of transgenic hairy roots. Our results revealed that both 15834 and A4, but not K599, were effective in inducing hairy roots in *Vitis* species and that different *Vitis* species and tissue types responded differently to hairy root induction. Among the 14 species evaluated, *V. champinii*, *V. cinerea*, *V. labrusca*, *V. treleasii*, and *V. vinifera*-1044 produced hairy roots within a 2-week observation period when the induction was carried out on *in vitro* plants. Compared with the tissues of stem-cut and petiole-cut surface, the internode tissue showed a higher efficiency for hairy root induction. We further revealed that when young shoots were excised from *in vitro* plants and used as inoculation material, the overall induction efficiency of hairy roots could be much improved. In addition, we also investigated the feasibility of producing composite plants with transgenic hairy roots and non-transgenic shoots. Although a number of such *in vitro* composite plants were established from inoculated young shoots, none of them were successfully maintained after being transplanted to pot soil.

K e y w o r d s : *Vitis*, *Agrobacterium rhizogenes*, hairy roots, genotype and tissue specificity.

Introduction

Grapevines (*Vitis* spp.) are one of the most important fruit crops and cultivated worldwide. The genome of the most widely cultivated grapevine, *V. vinifera* L., was recently sequenced (Jaillon *et al*. 2007, Velasco *et al*. 2007). The availability of the first grape genome provides an unprecedented opportunity for functional genomics studies of grape genes and genomes through various reversed and forward genetic approaches, such as TILLING-assisted mutation discovery and plant transformation techniques.

Like in many other crops, particle bombardment and *Agrobacterium tumefaciens*-mediated transformation techniques have been developed and routinely used for functional genomics research and transgenic trait development in grapevines (Vidal *et al*. 2010). Although producing stable transgenic grapevines is fairly a routine process, it usually takes more than 18 months for the transgenic plants to be established in pot soil. Apparently, this long process is a hurdle for functional genomics studies, especially when a large number of genes are needed to be evaluated in a relatively short time frame. However, there are cases where functionality of a gene can be adequately or better addressed using certain plant organs/parts instead of a whole plant. In this regard, transgenic hairy roots, induced by *Agrobacterium rhizogenes*, have been extensively used for studying gene functions in root development, nutrient uptake, and resistance to diseases and soil-born pests such as nematodes (Wubben *et al*. 2009). Compared with whole plant transformation, generation of transgenic hairy roots takes only a few weeks (Lupo *et al*. 1994, Torregrosa and Bouquet 1997). The hairy roots, once produced, can be subcultured and multiplied, thus providing abundant plant materials for downstream studies.

Transgenic hairy roots were also generated and used in functional genomics research in grapevines (Cutanda-Perez *et al.* 2009, Gomez *et al*. 2009, Terrier *et al*. 2009). However, these studies involved only a few *Vitis* spp. and single *A. rhizogenes* strains. In the present study, we evaluated 14 *Vitis* spp. and 3 *A. rhizogenes* strains for hairy root induction. The 14 *Vitis* species included the most widely cultivated grapes of *V. vinifera* and *V. labrusca* and also several wild species, such as *V. champinii* and *V. cinerea*, both of which are important sources of germplasm for improving disease resistance and stress tolerance in grapes. Three *A. rhizogenes* strains, 15384, A4 and K599, were evaluated in this study. Although both *A. rhizogenes* strains of 15384 and A4 were previously used in hairy root induction in grapevines (Lupo *et al*. 1994), there is no comprehensive documentation about the differences of these two strains in hairy root induction. *A. rhizogenes* strain K599, a strain widely used in hairy root induction in many other plant species (Collier *et al*. 2005), was also included in this study. In grapevines, stem-cut surface and internodes of *in vitro* plant were commonly used as infection tissues for inducing hairy roots (Franks *et al*. 2006, Lupo *et al*. 1994; Torregrosa and Bouquet 1997). In this study, we investigated the influences of 4 plant tissue types, includ-

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ing internodes, stem-cut and petiole-cut surfaces on *in vitro* plants and young shoots excised from *in vitro* plants, on hairy root induction. To the best of our knowledge, this study was the first comprehensive evaluation of the influences of different *Agrobacterium rhizogenes* strains, plant genotypes, and tissue types on the induction of transgenic hairy roots in grapevines.

Material and Methods

In vitro plant materials: *In vitro* plants of 14 *Vitis* species were screened for hairy root induction (Tabs 1 and 2). The *in vitro V. vinifera* 'Chardonnay' was provided by Dr. B. REISCH at Cornell University and the rest of the *in vitro* plants were obtained from Dr. M. JENDEREK at USDA/ARS, National Center for Genetic Resources Preservation. The *in vitro* plants were cultured in micropropagating medium [MS basal medium (MURASHIGE and SKOOG 1962), 30 g·L^{-1} sucrose, 0.125 mg·L^{-1} IAA (indole-3-acetic acid), 2.5 g·L^{-1} gellan gum, pH 5.8] and subcultured every 8-10 weeks using shoot tips and nodal explants as propagating materials. The *in vitro* plant cultures were maintained at 25 ± 2 ºC under a 16 h photoperiod in a plant growth room.

Agrobacterium rhizogenes strains and binary vector: Three *A. rhizogenes* strains, 15834, A4, and K599, containing a binary vector pBIN61-EGFP-HA (SACCO *et al*. 2009), provided by Dr. P. MOFFETT at University of Sherbrooke, were used for co-cultivation experiments. The binary vector pBIN61-EGFP-HA contains a *gfp* gene for visual observation of GFP expression in transgenic hairy roots and a *npt*II gene for kanamycin resistance. These two marker genes are in separate transcription units with their own promoters and terminators. The pBIN61-EGFP-HA vector was introduced into each *A. rhizogenes* strain through electroporation (SAMBROOK *et al*. 1989).

Hairy root induction: *A. rhizogenes* strain from glycerol stock was cultured overnight at 28 °C in MG/L medium containing 100 mg·L^{-1} kanamycin. Cell density was adjusted to an OD_{600} of 0.5 - 0.7 with MG/L medium and acetosyringone was added at a final concentration of 100 µM prior to inoculation.

Three different types of tissues, including internodes, stem-cut surfaces of the 3rd internode from the top, and petiole-cut surfaces of 8 to 10 week-old *in vitro* plants of 14 *Vitis* species grown in Magenta boxes, were wounded and directly inoculated with *A. rhizogenes* according to the following wounding procedure (Fig. 1). The first 2 visible internodes containing apical shoot and all leaves except those grown close to the bottom of the plants were cut and removed with a scalpel blade to create stem- and petiole-cut surface, respectively, for inoculation. For the convenience of description, we will use stem and petiole below to refer the stem-cut and petiole-cut surface tissues, respectively. The 1st and 2nd internodes below the stem-cut were wounded by multiple 2-mm cuts using a scalpel blade. The wounded surfaces of petioles, stem, and internodes were immediately inoculated with 1, 2, and 5 µl of the prepared agrobacterium inoculum, respectively, using a 10-µl pipette tip. Two to eight *in vitro* plants were used for inoculation of each *A. rhizogenes* strain. The inoculated *in vitro* plants were maintained in Magenta boxes in the plant growth room. Callus formation and hairy root induction were observed daily for 2 weeks. The inoculation experi-

Table 1

Impact of *A. rhizogenes* strains (15834, A4, or K599) on the efficiencies of callus formation and hairy root induction in 14 *Vitis* species at the end of 2-week observation period

Vitis spp. (accession no.)	Strain	No. inoculation tissue[1]	No. calli induced	No. calli generating hairy root	No. hairy roots induced
V. cinerea	15834	25	14	0	0
(DVIT2218)	A4	33	23	1	3
	K599	28	14	0	0
V. champinii	15834	29	11	1	1
(DVIT1281)	A4	21	4	0	0
	K599	22	8	0	0
V. doaniana	15834	18	4	0	0
(DVIT1140)	A4	20	0	0	0
	K599	15	3	0	0
V. ficifolia	15834	24	2	0	0
(DVIT2008)	A4	25	4	0	0
	K599	25	4	0	0
V. flexuosa	15834	18	2	0	0
(DVIT1385)	A4	26	4	0	0
	K599	13	4	0	0
V. girdiana	15834	29	12	0	0
(DVIT1380)	A4	31	4	0	0
	K599	28	9	0	0
V. jacquemontii	15834	40	7	0	0
(DVIT1445)	A4	28	11	0	0
	K599	36	14	0	0
V. labrusca	15834	28	22	13	40
'Concord' (NA)	A4	26	9	0	0
	K599	25	8	0	0
V. nesbittiana	15834	21	0	0	0
(DVIT2235)	A4	25	7	0	0
	K599	20	5	0	0
V. novae-angliae	15834	38	17	0	0
(DVIT1452)	A4	37	3	0	0
	K599	23	2	0	0
V. palmata	15834	65	26	0	0
(DVIT2228)	A4	74	31	0	0
	K599	58	20	0	0
V. piasezkii	15834	28	11	0	0
(DVIT2026)	A4	27	9	0	0
	K599	32	9	0	0
V. treleasii	15834	30	7	3	12
(DVIT1410)	A4	16	8	1	1
	K599	23	8	0	0
V. vinifera-1044	15834	11	8	3	10
(DVIT1044)	A4	33	4	1	1
	K599	23	7	0	0
V. vinifera	15834	13	3	0	0
'Chardonnay' (NA)	A4	27	6	0	0
	K599	10	3	0	0

[1]Inoculation tissues included internodes, stems, and petioles on *in vitro* plants.

Table 2

Impact of tissue types (internodes, stems, and petioles) on the efficiencies of callus formation and hairy root induction in 14 *Vitis* species when the tissues were inoculated with *A rhizogenes* 15834 at the end of 2-week observation period.

Vitis spp. (accession no.)	Type of tissue	No. inoculation tissue	No. callus induced	Callus appearance	No. callus generating hairy root	No. hairy root induced
V. cinerea (DVIT2218)	Internode	9	6	big	0	0
	Stem	9	5	big	0	0
	Petiole	7	4	small	0	0
V. champinii (DVIT1281)	Internode	11	10	big	1	1
	Stem	5	1	big	0	0
	Petiole	13	0	-	0	0
V. doaniana (DVIT1140)	Internode	8	4	small	0	0
	Stem	5	0	-	0	0
	Petiole	5	0	-	0	0
V. ficifolia (DVIT2008)	Internode	9	2	small	0	0
	Stem	7	0	-	0	0
	Petiole	8	0	-	0	0
V. flexuosa (DVIT1385)	Internode	6	2	small	0	0
	Stem	5	0	-	0	0
	Petiole	7	0	-	0	0
V. girdiana (DVIT1380)	Internode	9	7	medium	0	0
	Stem	8	4	small	0	0
	Petiole	12	1	small	0	0
V. jacquemontii (DVIT1445)	Internode	16	7	medium	0	0
	Stem	10	0	-	0	0
	Petiole	14	0	-	0	0
V. labrusca 'Concord' (NA)	Internode	12	11	big	11	37
	Stem	4	3	variable	0	0
	Petiole	12	8	variable	2	3
V. nesbittiana (DVIT2235)	Internode	8	0	-	0	0
	Stem	5	0	-	0	0
	Petiole	8	0	-	0	0
V. novae-angliae (DVIT1452)	Internode	13	7	small	0	0
	Stem	9	2	small	0	0
	Petiole	16	8	variable	0	0
V. palmata (DVIT2228)	Internode	22	14	small	0	0
	Stem	17	10	small	0	0
	Petiole	26	2	small	0	0
V. piasezkii (DVIT2026)	Internode	9	7	big	0	0
	Stem	5	2	big	0	0
	Petiole	14	2	medium	0	0
V. treleasii (DVIT1410)	Internode	7	5	big	3	12
	Stem	8	1	big	0	0
	Petiole	15	1	big	0	0
V. vinifera-1044 (DVIT1044)	Internode	4	4	big	3	10
	Stem	3	2	big	0	0
	Petiole	4	2	small	0	0
V. vinifera 'Chardonnay' (NA)	Internode	4	2	big	0	0
	Stem	3	1	big	0	0
	Petiole	6	0	-	0	0

ments for individual *A. rhizogenes* strains were carried out separately due to the size of the experiments. In a separate experiment, young actively-growing shoots of approximately 1.5 - 2.5 cm were excised from 8 to 14 week-old healthy *in vitro* plants (Tab. 3). Stem ends of the shoots

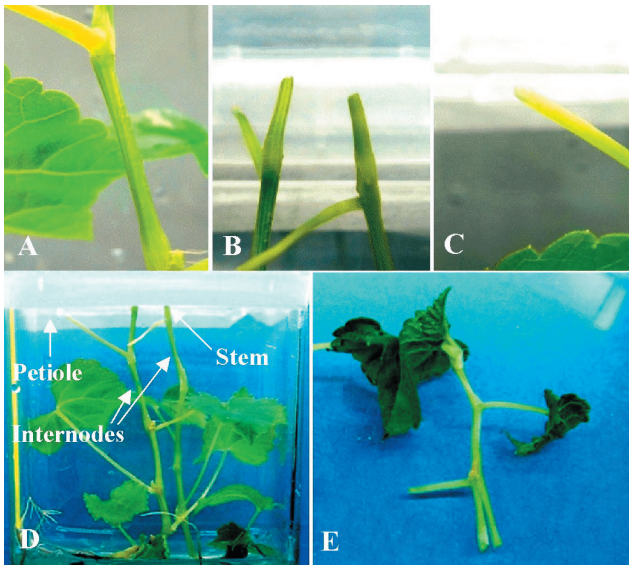

Fig. 1: Illustration of 4 types of wounded tissues used in the study: internodes (**A**), stem-cut surface (**B**), and petiole-cut surface (C) on *in vitro* plants (**D**), and young shoot excised from *in vitro* plants (**E**).

Table 3

Efficiencies of hairy root induction from young shoots excised from *in vitro* plants of 6 *Vitis* species inoculated with *A. rhizogenes* 15834 at the end of 8-week observation period

Vitis spp. (accession no.)	No. shoots used	No. shoots with GFP-positive hairy roots (%)
V. cinerea (DVIT2218)	271	54 (19.9)
V. ficifolia (DVIT2008)	35	0
V. girdiana (DVIT1380)	50	1 (2.0)
V. jacquemontii (DVIT1445)	24	0
V. palmata (DVIT2228)	135	15 (11.1)
V. vinifera 'Chardonnay' (NA)	79	5 (6.3)

were cut open for 0.5 - 1 cm long with a scalpel blade to create wounded area for inoculation. The wounded shoots were submerged in the agrobacterium inoculum of strain 15834 containing 200 µM acetosyringone for 5 to 10 min, blotted on tissue papers, and then transferred onto the co-cultivation medium (HRCO:MS basal medium, 30 g·L^{-1} sucrose, 2.5 g·L^{-1} gellan gum, and 100 µM acetosyringone). The inoculated shoots were co-cultivated at 20 °C under a 16 h photoperiod in a growth chamber. After 14 d of co-cultivation, the shoots were washed twice in sterile water and once in the sterile water containing 400 mg·L^{-1} cefotaxime. The shoots were blotted on tissue papers, then transferred to the bacterial removing medium (HRBR:HRCO medium without acetosyringone, but containing 400 mg·L^{-1} cefotaxime), and maintained in petri dishes in the growth room. After 3 to 4 weeks, hairy roots with GFP expression were isolated and cultured on a hairy root culture medium [HRCU200:LG$_0$ macronutrient (Torregrosa and Bouquet 1997)], MS micronutrient, 25 g·L^{-1} sucrose, 500 mg·L^{-1} casein, 100 mg·L^{-1} myo-inositol, 10 mg·L^{-1} each of nico-

tinic acid, pyridoxine-HCL, and thiamine-HCL, 5 mg·L^{-1} Ca-pantothenic acid, 1 mg·L^{-1} biotin, pH 5.8, 2.5 g·L^{-1} gellan gum, and 200 mg·L^{-1} cefotaxime] supplemented with 20 mg·L^{-1} kanamycin.

Production of *in vitro* composite plants: *In vitro* composite plants consisting of non-transgenic shoots and transgenic hairy roots were generated using the excised shoot approach as described above. After the non-inoculated shoots grew into 5 - 10 cm long and a large number of hairy roots developed from the inoculated stem ends, the composite plants were gently removed from the medium, thoroughly rinsed with tap water to remove adhered agar from the roots, and then transferred to sterile soil. After about 2 months in the greenhouse, the composite plants were removed from soil and their roots were examined for the presence of the GFP marker protein under a fluorescent microscope.

Confirmation of transgenic calli and hairy roots: Two selectable marker genes, *gfp* and *npt*II, were available for selecting transgenic calli and hairy roots in the present study. Transgenic calli and hairy roots were identified for possessing resistance to kanamycin or having GFP expression by using a SZX-ILLB100 Olympus fluorescent stereomicroscope equipped with a GFP-LP filter set 41018 (Chroma Technology Crop., U.S.A). GFP-specific primers (forward: 5'-TCGTGAC-CACCCTGACCTA-3' and reverse: 5'-TAGTTGCCGT-CGTCCTTGA-3') were used in the genomic PCR (95 °C, 4'; 95 °C, 30'', 58 °C, 30'', 72 °C, 1' for 35 cycles; 72 °C, 5') to provide a confirmation test that hairy roots with GFP fluorescence had presence of the *gfp* gene.

Maintenance of hairy root culture: Approximately 1.5 - 2 cm long root tips of *V. cinerea* hairy roots were dissected and transferred onto square plates containing the HRCU200 medium and maintained in the growth room under darkness. Subculture of roots was conducted every 3 to 4 weeks. In addition, 20-30 root tips were cultured in a 250-ml Erlenmeyer flask containing 100 ml of liquid HRCU200 medium. The liquid culture of roots was maintained in darkness at 27 °C in an incubator shaker at the speed of 125 rpm. The liquid medium was refreshed weekly. Subculture was usually conducted in every 4 weeks by transferring 1 - 1.5 cm long actively-growing root tips to fresh medium.

Results and Discussion

Effects of different *A. rhizogenes* strains on callus and hairy root induction: The three *A. rhizogenes* strains, 15834, A4, and K599, exhibited very different capabilities in inducing callus formation on the wounded internodes, stems, and petioles on *in vitro* grapevine plants. Tissues usually began to swell as early as 3 d after being inoculated with 15834 and A4. Calli became visible in the first week for highly responsive plant species. In contrast, calli induced by K599 didn't form until the 2nd week after inoculation. The overall induction frequency across all 3 tissue types of the 14 *Vitis* species was 39 % for the strain 15834, 31 % for K599 and 28 % for A4 (Tab. 1). While the induction frequencies were all in a comparable range, the quality of the induced calli varied quite differently among strains. Calli induced by 15834 and A4 were generally much bigger than those induced by K599. At the end of 2 weeks after agrobacterium infection, hairy roots were formed from sturdy calli on *V. labrusca* 'Concord', *V. champinii, V. treleasii*, and *V. vinifera*-1044 induced by 15834, and *V. cinerea*, *V. treleasii*, and *V. vinifera*-1044 induced by A4. However, no hairy roots were generated from any of the 14 species inoculated with K599. Compared with strain 15384, strain A4 showed a much lower efficiency for hairy root induction. Among the 68 hairy roots produced from infected *in vitro* plants, only 5 were from A4 induction (Tab. 1). All these hairy roots showed detectable GFP florescent signal under a UV light. A genomic PCR test, using primers specific to the *gfp* gene, on the hairy roots from two independent lines confirmed the presence of the *gfp* gene.

Successful induction of transgenic calli and hairy roots has been well documented in *Vitis* species by using strains 15834 (Lupo *et al*. 1994, Torregrosa and Bouquet 1997) and A4 (Lupo *et al*. 1994, Franks *et al.* 2006, Cutanda-Perez *et al*. 2009, Gomez *et al*. 2009, Terrier *et al*. 2009). Our results confirmed the previous observations that both 15834 and A4 can induce hairy roots in *Vitis* species. In addition, we showed that 15834 gave a better induction frequency than A4. K599, also known as NCPPB 2659 (Taylor *et al*. 2006), was found not to be suitable for induction of hairy roots in grapevines. This result was in contrast with that of Collier *et al*. (2005) who successfully used the K599 strain to produce transgenic composite plants from 14 plant species belonging to 9 families in 5 orders. Species specificity could be one of the causes for the observed difference.

Effects of plant genotypes on callus and hairy root induction: Species specificity for callus and hairy root induction was clearly observed among 14 *Vitis* species when *in vitro* plant internodes of these species were inoculated with strain 15834 or A4 (Tab. 2 and Fig. 2). The species specificity pattern from inoculated stem or petiole tissues was difficult to assess because of the limited responses of the two types of tissues to the induction, an interesting observation which will be discussed later. On the basis of the results of callus and hairy root induction from 15834-infected internodes, the 14 *Vitis* species evaluated in this study could be classified into 3 groups. The 1st group consisted of 8 highly responsive species including *V. champinii*, *V. cinerea*, *V. girdiana*, *V. labrusca* 'Concord', *V. palmata*, *V. piasezkii*, *V. treleasii*, *V. vinifera*-1044, and *V. vinifera* 'Chardonnay'. These species all produced medium to big calli. Among these 8 species, *V. labrusca* 'Concord' showed the highest responsiveness to hairy root induction. Eleven of the 12 internodes (91.7 %) inoculated with 15834 generated calli and all the calli (100 %) produced hairy roots within 2 weeks of inoculation. A total of 37 hairy roots were produced from these calli. *V. treleasii* and *V. vinifera*-1044 also showed good responsiveness to 15834 infection. Five of the 7 inoculated internodes of *V. treleasii* generated calli (71.4 %) and 3 of the induced calli (43 %) produced a total

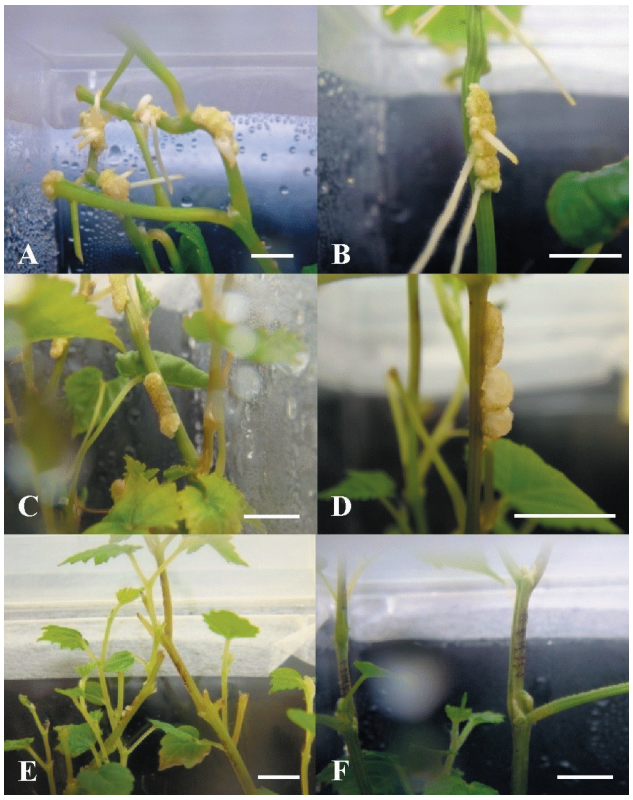

Fig. 2: Calli and hairy root induction from wounded internodes on *in vitro* plants 2 weeks after inoculation with *A. rhizogenes* 15834. Hairy roots were induced on calli from *V. labrusca* 'Concord' (**A**) and *V. vinifera*-1044 (**B**). Big calli, but no hairy roots, were induced from *V. cinerea* (**C**) and *V. vinifera* 'Chardonnay' (**D**). Small-sized calli and no hairy roots were observed from *V. ficifolia* (**E**). No callus was induced from *V. nesbittiana* (**F**). Bars = 1 cm.

of 12 hairy roots. Similarly, all of the 4 inoculated internodes (100 %) of *V. vinifera*-1044 generated calli and 3 of the induced calli (75 %) produced a total of 10 hairy roots. Although big calli were induced on 15834-infected internodes of *V. cinerea*, no hairy roots were generated. It was interesting, however, that a few hairy roots were produced on the A4-induced calli of the species (Tab. 1). Species specificity of hairy root induction was also observed for the A4 inoculation.

The 2nd group, including *V. ficifolia*, *V. flexuosa*, *V. jacquemontii*, and *V. novae-angliae,* were found to be less responsive to *A. rhizogenes* infection compared to those in the 1st group. Species in this group mostly produced small calli and none of them generated hairy roots within the 2-week observation period. Only 1 hairy root was produced on the infected internodes of *V. flexuosa* at the end of 4 weeks after infection. This was similar to the observation from *V. girdiana* in the 1st group that hairy roots were not generated until 4 weeks after agrobacterium inoculation. The 3rd group includes *V. doaniana* and *V. nesbittiana*, which showed resistance to either A4 or 15834 infection. Although some small calli were induced by K599 on these two species, no hairy roots were generated.

Strain 15834 was previously used to induce hairy roots in three grape interspecific rootstocks of 'Gravesac' (*V. berlandieri* x *V. riparia* x *V. rupestris*), '110 Richter' (*V. berlandieri* x *V. rupestris*), and 'Fercal' (*V. berlandieri* x *V. vinifera*)] and six hybrids (*V. vinifera* x *V. rotundifolia*) (Torregrosa and Bouquet 1997). Species or genotype specificity was not reported as they appeared equally susceptible to the *A. rhizogenes*. Three *Vitis* species of *V. rupestris*, *V. riparia*, and *V. vinifera*, and the three hybrids of 'Kober 5BB' (*V. berlandieri* x *V. ripaira*), '110 Richter' (*V. berlandieri* x *V. rupestris*), and LN 33 ('1613 Couderc' x *V. vinifera* cv. Thompson Seedless) were inoculated with strain A4 for hairy root induction in the study of Lupo *et al*. (1994), but species-specific response was not discussed either. These two studies were conducted with relatively a small number of *Vitis* species, which might limit what could be revealed regarding species or genotype specificity. In our study, we observed that the 14 evaluated species showed varying degrees of responsiveness to the infection of three *A. rhizogenes* strains used. 'Concord' is the most important grape cultivar for juice production in the United States (Folwell *et al*. 1994) and also one of the important grape cultivars cultivated in the cold-hardy environments worldwide. Our finding that 'Concord' was highly responsive to *A. rhizogenes*-mediated transformation for callus and hairy root induction provides an additional avenue for carrying out functional genomics research in this important cultivar.

It was previously reported that overall plant vigor and health status could impact hairy root induction in *ex vitro* conditions in common bean and soybean (Estrada-Navarrete *et al*. 2007, Kereszt *et al*. 2007). Torregrosa and Bouquet (1997) reported that 3 month-old *in vitro* plants of grapevines were more suitable for *A. rhizogenes*-mediated hairy root induction than younger plantlets. In this study, we also observed that the overall plant vigor and healthiness was critical for hairy root induction. In general, only plants with good woody stems, robust root systems, and active shoots and leaves should be considered for hairy root induction in grapevines.

Effects of tissue types on callus and hairy root induction: In addition to *A. rhizogenes* strains and plant species/genotypes, types of tissues can also impact the efficiencies of callus and hairy root induction. For example, Lupo *et al*. (1994) reported that *in vitro* plants provided a much better result than greenhouse-grown plants for hairy root induction in grapevines. In our study, wounded internodes, stems, and petioles of *in vitro* plants were inoculated with *A. rhizogenes* to determine whether or not different tissue types might affect the overall efficiencies of callus and hairy root induction. Among the 14 species tested, with the exception of *V. doaniana* and *V. nesbittiana* which were resistant to A4 and 15834 induction, respectively, all showed susceptibility to *A. rhizogenes* infection and generated calli on infected internodes (Tab. 2). Overall, the internode tissue exhibited a much higher rate for callus induction (60 % for 15834 and 59 % for A4) than the stem tissue and petiole tissue combined (52 % for 15834 and 24 % for A4). Within the 2-week observation period after agrobacterium inoculation, *V. labrusca* cv. 'Concord', *V. treleasii*, and *V. vinifera*-1044 generated many visible hairy roots from induced calli on internodes. On the other hand, stem and petiole exhibited

a similar trend for producing small calli and the induction rate of hairy roots from these small calli was much lower (Tab. 2). Clearly, internodes were the most suitable type of tissues for *A. rhizogenes*-mediated hairy root induction on *in vitro* plants of grapevines.

One of the possible explanations for a better efficiency of hairy root induction on internodes is that internodes provided a much larger wounded area for *A. rhizogenes* to infect than stem or petiole tissues. To test this hypothesis, we carried out a follow-up experiment in which young shoots were excised from *in vitro* plants and their stem ends were cut wide open for about 0.5 - 1 cm long to create a large wounded area for inoculation. Because of the limited availability of *in vitro* plants, we were only able to include 6 of the 14 *Vitis* species in this follow-up experiment (Tab. 3). These six species included the four species, *V. cinerea*, *V. girdiana*, *V. palmata*, and *V. vinifera* 'Chardonnay', which were highly responsive to the inoculation of *A. rhizogenes* in forming transgenic calli in our early experiment as described above. Only strain 15384 was used in this experiment. Within 10 d of inoculation, non-transgenic hairy roots showing no GFP expression started emerging around the wounded stem areas. Subsequently transgenic hairy roots, often appearing thicker in diameters, were produced from the wounded stem areas (Fig. 3 A, B). Most of the inoculated shoots generated an average of 1-2 hairy roots, but some of them could have as many as 4 roots. Hairy roots were blight white and thicker than wild-type roots of *in vitro* plants. *V. cinerea* had the highest frequency (19.9 %) of hairy root induction among the 6 *Vitis* species tested, followed by *V. palmata* at 11.1 %, *V. vinifera* 'Chardonnay' at 6.3 %, and *V. girdiana* at 2 %. These results were very interesting, because these four species, although highly responsive to *A. rhizogenes* inoculation with formation of good quality calli, didn't produce transgenic hairy roots when their internodes were used as plant tissue material. No transgenic hairy roots were produced from *V. jacquemontii* and *V. ficifolia* (Tab. 3). Our results revealed that using excised young shoots for hairy root induction was more productive for hairy root induction than using internode, stem, or petiole tissues on *in vitro* plants. In addition to a larger wounded area for agrobacterium infection, the presence of a shoot tip might also provide the needed vigor and other critical factors for promoting hairy root induction on an excised young shoot. It is well known that auxin plays an important role in root initiation and development and it is mainly synthesized in apical meristematic tissues of young shoots. In this regard, the presence of young shoots might provide needed auxin for promoting hairy root induction. A similar observation was recently reported that high frequencies of transformation were obtained from stem segments carrying intercalary meristems (53.6 %) and shoot tips with apical meristems (40.8 %) in 4 types of explants tested for hairy root induction in a herbaceous plant (*Harpagophytum procumbens*) (Grąbkowska *et al*. 2010).

The excised-shoot approach for hairy root induction was easy to perform and handle a large quantity of materials in a relatively short period of time. For example, creating wounding area for inoculation in excised young shoots can be easily performed in a Petri dish. In contrast, wounding and inoculating tissues on *in vitro* plants in Magenta boxes are much laborious and require intensive attention for avoiding accidental contamination of the medium by agrobacterium during the inoculation process. *Agrobacterium* contamination is not an issue for shoot-based approach since it can be easily removed from the exercised young shoots by washing them with sterile water containing 400 $mg \cdot L^{-1}$ cefotaxime and transferring the shoots to HRBR medium.

Torregrosa and Bouquet (1997) generated hairy roots using crushed stems of *in vitro* plants (apices were cut off and 5 mm upper stems were crushed with forceps previously dipped in bacterial inoculum) of 'Gravesac' infected with the strain 15834. This approach was successfully used to generate hairy roots from 'Shiraz' and few other *V. vinifera* cultivars (Cutanda-Perez *et al*. 2009, Gomez *et al*. 2009, Terrier *et al*. 2009). Internodes were used as infected sites to generate hairy roots in several *V. vinifera* cultivars (Lupo *et al*. 1994, Franks *et al*. 2006) and few other *Vitis* species and hybrids (Lupo *et al*. 1994). While these approaches were all successful in inducing hairy roots, their efficiencies were not compared and evaluated. In this study, we demonstrated that on *in vitro* plants, internode was a better tissue for hairy root induction compared to stem and petiole tissues. However, the excised-shoot approach, as described above, was the most effective method for inducing hairy roots in grapevines.

Production of composite plant: The use of young shoot explants for hairy root induction might open a potential avenue for producing composite plants consisting of transgenic roots and non-transgenic scions. Composite plants have many utilities in functional genomics research and transgenic product development (Veena and Taylor 2007) and have been generated through various approaches in several plant species including soybean (Veena and Taylor 2007) and coffee (Alpizar *et al*. 2006). In this study, we tested the feasibility of using excised young shoot explants for generating composite plants in grapevines. The idea was to induce hairy roots from stem ends of the shoots and at the same time to promote shoots growing into scions. We generated about 60 of such *in vitro* composite plants from *V. cinerea* (Fig. 3 C, D), *V. girdiana*, and *V. palmata*. Most of the composite plants did not grow further and died within a month after being transferred to pot soil. Two composite plants survived the transplanting and developed robust roots and scions. However, examination of the roots under fluorescent microscope for the presence of GFP signals revealed that they were non-transgenic. This observation was further confirmed by the failure of PCR amplification of an expected *gfp* fragment in the root DNA samples (data not shown). The reason for the failure in producing stable composite plants is under investigation.

Maintenance of transgenic hairy roots: Fourteen lines of *V. cinerea* hairy roots induced from excised young shoots were cultured on solid medium (Fig. 4 A) and their growth rates during a 3-week culturing period were recorded. In the first week, the average rates of primary root elongation ranged from 1.1-2.3 cm. Some of the root lines became thicker and their diameters grew up to 3 mm. The growth

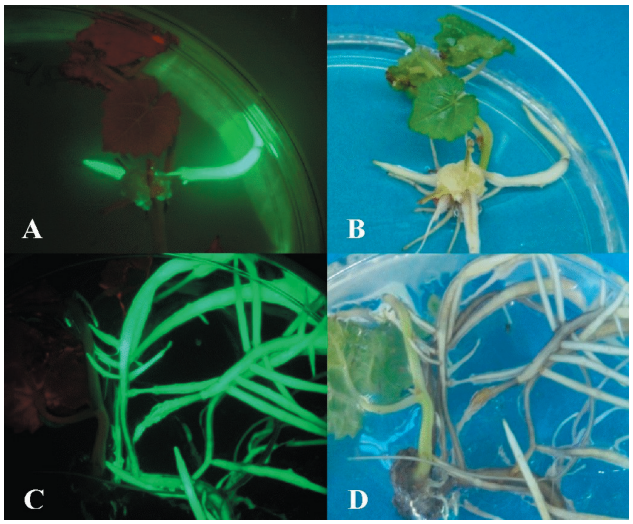

Fig. 3: Transgenic hairy roots were induced from excised young shoot of *V. cinerea* (**A** and **B**). Non-transgenic roots were removed *in vitro* composite plant (**C** and **D**). Transgenic hairy roots were visualized under blue (left) and white light (right). Transgenic hairy roots exhibited the GFP signal under blue light.

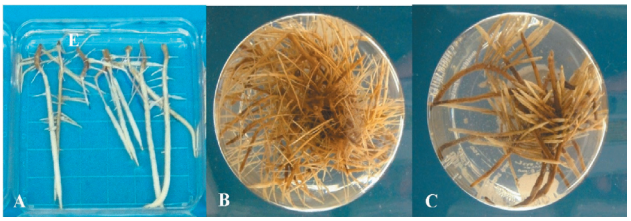

Fig. 4: Growth behavior of *V. cinerea* hairy roots cultured on solid and liquid media. Primary hairy roots cultured on HRCU200 solid medium for 3 weeks (**A**); hairy roots derived from tertiary lateral roots cultured in liquid medium (**B**); and hairy roots derived from primary roots cultured in liquid medium (**C**). The root culture derived from tertiary lateral roots grew faster and healthier than that derived from primary hairy roots.

rates, compared with that of the first week, were generally increased in the 2nd week with average rates of root elongation from 1.1-2.5 cm and then decreased in the 3rd week (0.6-1.7 cm).

Hairy roots appeared growing slower in liquid medium than they did on solid medium. On average, fresh weight of hairy roots in liquid culture increased up to 6 folds in 4 weeks. Young tertiary lateral roots (≤ 1.5 cm long, ≤ 1 m diameter) were found to be the most successful material for establishing hairy root culture (Fig. 4 B) and they grew faster than thicker roots in liquid medium. Although root tips were elongated, the older parts of the roots turned brown in few days. This browning behavior proceeded more rapidly in the culture containing thicker roots (Fig. 4 C). Therefore, refreshing liquid medium weekly was necessary to promote growth of the root culture.

Conclusions

In this study we demonstrated that *Agrobacterium rhizogenes* strains, plant genotypes, and tissue types all had significant impact on the induction of transgenic hairy roots in *Vitis* species. While these observations may not be unexpected, they filled an important knowledge gap in this area and underline the complex nature of the interactions among *Agrobacterium* strains, genotypes and plant physiological status in hairy root induction. Our results should shed important light on the generation of transgenic hairy roots and their applications in functional genomics research of grapevines in the future.

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