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Enhanced Micropropagation and Establishment of Grapevine Rootstock Genotypes

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ABSTRACT

The effect of medium composition, antioxidant and adsorbent of polyphenolic compounds on micropropagation of grapevine rootstocks 3309 Couderc, 110 Richter, 101-14 Millardet et De Grasset, *Vitis riparia* Gloire de Montpellier and Teleki 5C was determined. Shoot length, leaf number and size, as well as root number, weight and length were consistently higher on woody plant medium (WPM) (Lloyd and McCown 1981) relative to full strength or half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) and Martin *et al*. medium (MM) (Martin *et al*. 1987). Supplementing WPM with 37 mg l^{-1} cysteine as antioxidant promoted better rooting and enhanced plant development for most rootstock genotypes. The presence of cysteine in WPM also provided homogeneous growth, regardless of the position of nodal segments on the initial subcultured plants, likely by breaking the apical meristem dominance inhibitory effect on axillary bud proliferation. Our micropropagation protocol facilitated a fast and uniform multiplication of grapevine rootstocks in tissue culture and a successful transfer and growth of micropropagated plants in the greenhouse.

Keywords: Adsorbent of polyphenolics, antioxidant, macronutrients, shoot tip, nodal segments
Abbreviations: **BA**, 6-benzyladenine; **MM**, Martin *et al*. (1987) medium; **MS**, Murashige and Skoog (1962) medium; **PVP**, polyvinylpyrrolidone; ***V. riparia***, *Vitis riparia* Gloire de Montpellier; **WPM**, Lloyd and McCown (1981) woody plant medium; **5C**, Teleki 5C; **101-14**, 101-14 Millardet et De Grasset; **110R**, 110 Richter; **3309C**, 3309 Couderc

INTRODUCTION

Grapevine is one of the most important fruit crops worldwide with over 68 million tons produced essentially as table, wine and raisin grapes on 7.2 million ha in 2010 (Food and Agriculture Organization 2012). Vines are often grafted onto rootstocks to improve survival and production with regard to increased vigor and, more importantly, resistance to abiotic and biotic factors, in particular to phylloxera (*Daktulosphaira vitifoliae*) and nematodes.

Micropropagation is well established for rapidly multiplying *Vitis* sp., primarily *V. vinifera* L., *V. rotundifolia*, *V. labrusca* and hybrids (Chee and Pool 1983; Gray and Benton 1991; Bouquet and Torregrosa 2003; Qiu *et al.* 2004). Micropropagation protocols have been developed for rootstocks but are limited to a few genotypes (Chee and Pool 1983; Bisasi *et al*. 1998; Bouquet and Torregrosa 2003; Shim *et al.* 2003; Santos *et al*. 2005; Machado *et al.* 2007; Alizadeh *et al.* 2010). Micropropagation of *Vitis* sp. is mainly achieved by shoot apex and axillary shoot proliferation on full strength or half strength Murashige and Skoog (1962) medium (MS) with the addition of cytokinins, auxins and, for some genotypes, of antioxidants (Qiu *et al*. 2004) and polyphenolic adsorbents to prevent tissue browning and necrosis (Bouquet and Torregrosa 2003; Maillot *et al*. 2006).

Selecting suboptimal media and using high concentrations of exogenous 6-benzyladenine (BA) can inhibit shoot elongation (Lee and Wetzstein 1990) and continuous exposure to high BA concentration can cause hyperhydricity (Ziv 1991; Singh *et al*. 2004; Banilas and Korkas 2007). Also, activated charcoal (AC) is commonly used for adsorbing polyphenolic compounds produced by *Vitis* sp. explants (Gray and Benton 1991; Bouquet and Torregrosa 2003; Maillot *et al*. 2006) but its use delays explant development due to the binding potential of growth regulators and plant metabolites that are present in the medium (Fridbrog *et al*. 1978; Singh *et al*. 2004).

In this study, an efficient and fast micropropagation protocol was developed for some of the most commonly used grapevine rootstocks worldwide. We assessed the combined effect of tissue culture media [Lloyd and McCown (1981) woody perennial medium (WPM), half and full strength MS and Martin *et al*. (1987) medium (MM)], antioxidants [cysteine and polyvinylpyrrolidone (PVP)] and an adsorbent of polyphenolic compounds (AC) on growth and rooting of micropropagated plants with a special emphasis on achieving a high *ex vitro* establishment rate in the greenhouse.

MATERIALS AND METHODS

Plant material

Potted grapevine rootstocks 3309C (*Vitis riparia* x *V. rupestris*), 110R (*V. berlandieri* x *V. rupestris*), 101-14 (*V. riparia* x *V. rupestris*), 5C (*V. berlandieri* x *V. riparia*) and *V. riparia* that were actively growing at 20 ± 5°C and 150 µE m^{-2} s^{-1} in a greenhouse at the New York State Agricultural Experiment Station, Cornell University, in Geneva, NY were used in this study.

Establishment of shoot tips in tissue culture

Five shoot tips (2 cm in length) were collected per potted plant, including the apical meristem and adjacent axillary buds. Large leaves were removed and shoot tips were surface sterilized in a laminar flow hood, as described previously (Yepes and Aldwinckle, 1994). Briefly, shoot tips were washed with 70% ethanol for 30 sec to remove surface wax. Then, explants were surface sterilized for 15 min with a 1.5% (w/v) sodium hypochlorite solution (10 ml commercial chlorox containing 6% (w/v) sodium hypochlorite

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diluted in 40 ml deionized water) plus 3-4 drops of Tween-20 as a wetting agent. Following sterilization, shoot tips were rinsed with sterile distilled water three times for 5 min and the base of explants (1-5 mm) as well as large leaves were removed.

Sterilized shoot tips (1.5 cm in length) were established on basal MS (Phyto Tech M524) supplemented with 30 g l^{-1} sucrose, B5 vitamins (Gamborg *et al*. 1976), 1 mg l^{-1} BA, and 37 mg l^{-1} cysteine (Sigma C7352). Individual shoots (1-2 cm in length) were subcultured every four weeks on WPM (Sigma M-6774 or Research Products International Corp., M23000) including 37 mg l^{-1} cysteine and 15 g l^{-1} sucrose. After adjusting the pH to 5.8, media were solidified with 5 g l^{-1} agar (Sigma A1296) and dispensed into glass test tubes prior to autoclaving at 121°C and 1.2 kg cm^{-2} for 15 min. One shoot tip was established per test tube and cultures were maintained at 25 ± 2°C and 33-45 µE m^{-2} s^{-1} (16-h photoperiod) for shoot proliferation over four weeks.

Effect of medium composition on micropropagation

A factorial experiment was designed to evaluate the effect of four media (full strength MS, half strength MS, WPM and MM) on growth and rooting of micropropagated grapevine rootstocks. Nodal explants (1.5-2.0 cm in size) containing the apical and one or two axillary buds were used from rooted, elongated micropropagated plants (13 cm in size). For each rootstock, 24-30 explants were used per treatment and each experiment was replicated at least twice. Explants were incubated under cool white fluorescent light at and 25 ± 2°C and 33-45 µE m^{-2} s^{-1} (16-h photoperiod).

Effect of antioxidants and adsorbent of polyphenolic compounds on micropropagation

Another factorial experiment was designed to evaluate the effect of two antioxidants [37 mg l^{-1} cysteine and 10 g l^{-1} or 20 g l^{-1} PVP (Sigma P2307)] and one adsorbent of polyphenolic compounds [3 g l^{-1} AC (Sigma C6289)] in combination with explant position on growth and rooting of micropropagated grapevine rootstocks. Antioxidants were added prior to autoclaving and adjusting the pH level, and AC was added after adjusting the pH. For each rootstock, 24-30 explants were used per treatment and each experiment was replicated at least twice. Nodal explants were collected as described above and numbered starting from the apical meristem to the base of the plantlets with 1 assigned to the most apical explant and 5 to the most basal explant. Culture conditions were as described above.

Transfer of micropropagated plants to soil

Micropropagated plants were gently removed from test tubes using forceps and roots were rinsed in water to discard any remaining tissue culture agar. Prior to establishment on soil, roots were trimmed to about one third in length to stimulate growth. Plants were established in Cornell mix (a mixture of peat, vermiculite, ground limestone, and Uni-mix 10-20-5) in plastic pots (10 cm in diameter) in a greenhouse and covered with plastic bags to avoid dehydration. Plastic bags were gradually opened following active growth (2-3 weeks) at 20 ± 5°C and 75 to 150 µE m^{-2} s^{-1}.

Data collection

Shoot length, root length, number of roots, root weight and leaf area were recorded after four weeks of culture to evaluate optimal conditions for plant development and rooting. Data were compiled and descriptive statistics were obtained for each treatment. Data were subjected to ANOVA to evaluate the combined effect of the different treatments and interactions using SAS® (Statistical Analysis System, version 9.1, SAS Institute Inc., Cary, NC. USA). Significant differences were assessed using Duncan's multiple range test at $P < 0.05$. A SAS mixed model procedure with random (plant) and fixed effects (treatment and genotype) was used to evaluate treatment/genotype interactions and determine the best predictable variable. Leaf area was modeled as a distributional variable. The efficacy of soil transfer of micropropagated plants was calculated as the number of plants that grew in the greenhouse two months post-establishment over the number of plants that were established.

RESULTS AND DISCUSSION

Shoot tips established from greenhouse-grown plants produced 4-5 shoots per explant on medium containing 1 mg l^{-1} BA after four weeks of culture. These initially established plants were used to optimize micropropagation by transferring individual shoots onto WPM with reduced sucrose and no BA. Plants were maintained for up to 6-8 weeks prior to subculturing on varied media.

Of the four media evaluated to optimize elongation and rooting, WPM was the best in terms of overall plant vigor (**Fig. 1**) and rooting (**Fig. 2**). Root number, length and weight as well as leaf number and size were higher on WPM compared to MM, full strength MS and half strength MS (**Table 1**), regardless of the rootstock genotype tested. The genotype × media interaction was significant for all the

Table 1 Effect of medium composition on growth of five grapevine rootstocks.

Genotype	Medium	N	Stem	Leaf	Leaf area[b]					Roots		
			length[c]	number	1	2	3	4	5	Number	Length[c]	Weight[d]
110R	WPM	30	**4.2±0.9****	**5.1±0.9****	1.0±0.7	**2.4±0.7**	**1.6±1.0**	**0.2±0.5**	0	2.4±0.8	**3.7±1.1***	**97.7±34.5****
	MM	30	3.8±0.9	4.7±1.2	**1.6±1.0**	2.2±0.9	0.9±1.0	0.03±0.2	0	**2.5±1.4**	2.4±1.4	49.0±33.0*
	MS	30	2.6±0.8	3.2±1.4	1.0±0.8	1.8±1.0	0.5±0.8	0	0	0.4±1.4	1.3±3.5	2.10±6.30
	1/2MS	30	2.9±0.9	4.1±1.6	0.8±0.5	2.0±1.1	1.2±1.1	0.1±0.4	0	2.3±1.1	2.1±1.2	43.8±24.2
3309C	WPM	30	**5.9±1.5***	**7.3±1.5***	0.3±0.5	**2.4±0.9**	**2.9±0.8**	**1.3±1.3**	**0.3±0.6**	2.8±0.9	**4.1±1.0***	**80.3±19.4***
	MM	30	4.5±1.9**	5.3±1.9**	**1.1±0.9**	1.7±0.9	2.1±1.7	0.4±0.6	0.1±0.3	**2.8±2.1**	2.0±1.3	36.8±27.2
	MS	30	3.7±1.6	4.8±1.9	0.8±0.9	1.8±1.1	0.8±0.9	0.2±0.8	0.03±0.2	2.3±2.1	1.1±1.1	25.6±29.8
	1/2MS	30	4.5±1.6	4.7±1.7	0.8±0.9	1.8±1.2	1.4±1.3	0.6±0.8	0.2±0.5	2.8±1.9	2.0±1.3	46.6±31.3
101-14	WPM	30	3.6±0.9	**4.5±1.2**	0.4±0.6	1.6±1.0	**2.0±1.0**	**0.7±0.9**	**0.1±0.3**	1.8±0.6	**2.9±1.5****	**45.5±23.5****
	MM	30	2.4±1.7	2.2±1.5	0.4±0.6	1.0±0.8	0.8±0.7	0	0	1.2±1.1	1.7±1.6	21.7±23.7
	MS	30	3.4±1.2	3.4±1.2	**0.7±0.9**	1.5±1.0	0.8±1.1	0.4±0.9	0.03±0.2	1.5±1.9	1.0±1.2	14.6±19.5
	1/2MS	30	**4.2±1.2****	4.3±1.5	0.7±0.7	**2.1±1.2**	1.4±1.1	0.03±0.2	0.03±0.2	**3.3±2.1**	2.0±1.4	37.8±26.1
RG	WPM	30	**6.9±2.1****	**5.1±1.4****	0.4±0.6	1.5±1.0	**1.9±1.1**	**0.7±0.7**	**0.6±0.6**	**3.4±1.4**	**4.0±1.6***	**86.8±32.1***
	MM	30	5.5±3.0**	3.7±2.1	0.6±0.6	1.5±1.0	1.0±0.9	0.6±0.8	0	2.3±1.6	2.9±1.9**	39.2±24.9
	MS	30	3.9±1.6	3.3±1.3	**0.7±0.9**	1.5±1.0	0.8±1.1	0.4±0.9	0.03±0.2	1.9±1.6	1.9±1.7	30.4±26.6
	1/2MS	30	5.5±2.4**	3.5±1.2	0.4±0.8	1.4±1.0	1.0±0.9	0.5±0.7	0.2±0.4	3.3±2.1	2.0±1.4	37.8±26.1
5C	WPM	24	**3.2±0.6**	**3.9±0.8**	**0.8±0.6**	**1.0±0.8**	**1.0±0.9**	**0.5±0.6**	**0.6±0.7**	**2.5±0.8**	3.0±0.6**	**63.8±19.4****
	MM	24	2.0±0.0	1.7±0.6	0.2±0.4	0.3±0.6	0.5±0.6	0.5±0.5	0.2±0.5	1.1±0.7	1.8±1.0	15.0±11.6
	MS	24	2.2±0.4	1.8±1.0	0.3±0.5	0.4±0.6	0.7±0.8	0.4±0.5	0.04±0.2	0.1±0.3	0.1±0.4	0.8±2.8
	1/2MS	24	2.0±0.2	1.8±0.5	0.1±0.3	0.5±0.7	0.7±0.8	0.5±0.6	0	1.8±0.8	**3.0±0.9****	4.22±17.0

[a]Data (mean±SE) from two independent experiments were collected after 30 days of culture. N = number of plants per replicate; [b]Number of leaves for each leaf size area category with 1: 0-0.5 mm^2; 2: 0.6-10 mm^2; 3: 11-15 mm^2; 4: 16-20 mm^2; and 5: 21-25 mm^2; [c]Length in cm; [d]Weight in mg. Values significantly different in each column are indicated by * ($P = 0.05$) or ** ($P = 0.01$). Highest parameter values for each rootstock are indicated in bold.

Table 2 Analysis of variance table against the null hypothesis (grapevine rootstock and medium composition have no effect on rooting and growth of micropropagated shoot tips).

Source	Df	Root number		Root length		Root weight		Stem length		Leaf number		Leaf area	
		F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F
Genotype	4	18.73	<0.0001	9.6	<0.0001	20.1	<0.0001	69.61	<0.001	81.64	<0.0001	53.27	<0.0001
Medium	3	27.69	<0.0001	102.42	<0.0001	151.96	<0.0001	27.40	<0.0001	54.76	<0.0001	16.73	<0.0001
Genotype*Medium	12	3.47	0.0003	5.19	<0.0001	6.94	<0.0001	4.68	<0.0001	5.54	<0.0001	3.60	0.0002

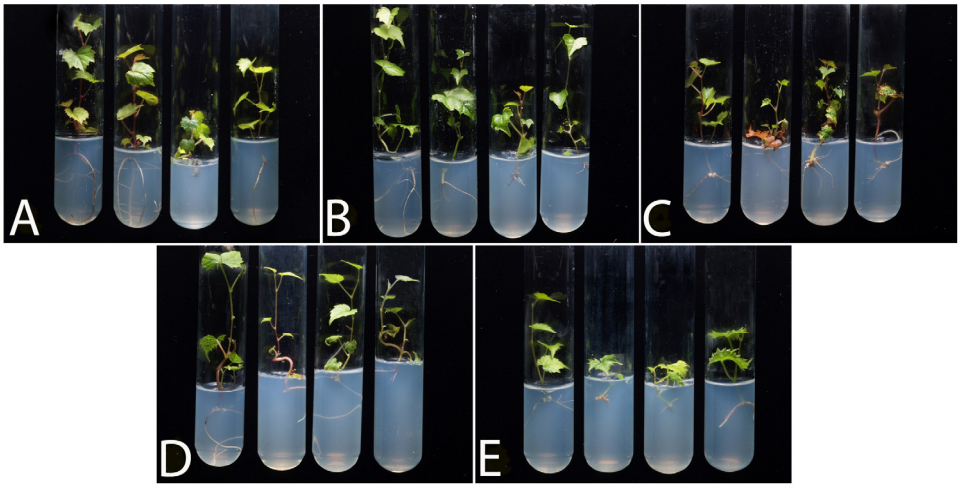

Fig. 1 Effect of media on growth and rooting of (**A**) 110R, (**B**) 3309C, (**C**) 101-14, (**D**) *V. riparia* and (**E**) 5C. Plants were grown on WPM (left), MM (second left), full strength MS (second right) and half strength MS (right) for one month.

Fig. 2 Effect of antioxidant or polyphenolic adsorbent on growth and rooting of (**A**) 110R and (**B**) 3309C micropropagated on WPM supplemented with no antioxidant (left), 37 mg l^{-1} cysteine (second left), 2% PVP (center), 1% PVP (second right) and 3 g l^{-1} activated charcoal (right).

parameters tested ($P < 0.001$) (**Table 2**). ANOVA indicated that root length and root weight were the two parameters most influenced by media treatment while stem length, leaf number and leaf area were influenced by both genotype and media treatment (**Table 2**). Root weight and number were the best parameters to evaluate optimal rooting while root length was the least useful parameter (**Table 2**) since plants with long primary roots did not necessarily develop secondary roots and established poorly in soil.

Plantlets of 110R developed and rooted well on both WPM and MM (**Fig. 1A**) with root initiation occurring slightly earlier on WPM than on MM (10 vs. 15 days). After four weeks of culture, however, root weight doubled for explants on WPM vs. MM (97.7 vs. 49 mg) (**Table 1**) and plantlets developed more and larger leaves in average (4 vs. 2-3 in size categories 2-4) as well as longer stems (4.2 vs. 3.8 mm) (**Table 1**) on WPM compared to MM (**Fig. 1A**). Explants developed very poorly on full strength MS with severe wilting and stunting compared to those on WPM or MM (**Fig. 1A**). Also, plantlets on half strength MS vs. WPM or MM had slightly shorter stems and leaves were overall less developed on full strength MS, half strength MS and MM than on WPM (**Table 1**). For 3309C (**Fig. 1B**), aerial plant development was initially similar on WPM and MM but root development and shoot length was better on WPM (80.3 vs. 36.8 mg in root weight and 4.1 vs. 2.0 mm in shoot length, respectively) (**Table 2**). Also, more and bigger leaves developed on WPM relative to the other media (4 vs. 2 in size categories 3-5) (**Table 1**). For 101-14, plantlets developed well on WPM but grew very poorly on MM due to extensive necrosis of basal leaves and poor aerial growth as well as poor rooting (**Fig. 1C**). On full strength MS, plants grew also poorly with reduced leaf area although negative effects were less pronounced than on MM (**Fig. 1C**). Aerial parts, especially leaf area, developed more on WPM compared to half strength MS or full strength MS (**Table 1**). For RG, plantlets developed well on WPM but exhibited poor rooting and poor secondary root development and elongation on MM and full strength MS (**Table 1**). Fewer plants rooted on full strength MS and MM than on WPM, and several did not root at all (**Fig. 1D**). On half strength MS, RG plants rooted but not as well as on WPM on which roots were longer and thicker with more secondary roots (**Fig. 1D**). Also, in average, more and bigger leaves developed on WPM than on the other media (3 vs. 1-2 in size categories 3-5) (**Table 1**). Marginal leaf chlorosis and even necrosis were observed commonly on full strength and more so on half strength MS. For 5C (**Fig. 1E**), plantlets developed and rooted faster on WPM than on the other media (**Table 1**). Aerial part development was better on half strength MS relative to MM and full strength MS but less roots and big leaves developed than on WPM (**Table 1**).

Overall, among the four media tested, WPM provided the best rooting and plant development for the five grapevine rootstock genotypes. The four media tested differed mainly in the source and concentration of nitrogen with a high (40 mM NO_3^- and 20 mM NH_4^+), intermediate (15 mM NO_3^- and 5 mM NH_4^+) and low (7 mM NO_3^- and 5 mM NH_4^+) nitrate and ammonium salt content for MS, MM and WPM, respectively, suggesting that a high nitrogen level might have an inhibitory effect on elongation and rooting of micropropagated grapevine rootstocks. WPM was further used to test the effect of antioxidants and polyphenolic adsorbent on growth and rooting.

Of the two antioxidants (cysteine and PVP) and one polyphenolic adsorbent (AC) tested, cysteine at 37 mg l^{-1} was the best in terms of rooting and vegetative development (**Table 3**), regardless of the rootstock genotype ($P < 0.001$)

Table 3 Effect of antioxidant or polyphenol adsorbent on growth of five grapevine rootstocks.[a]

Genotype	Antioxidant[b]	N	Stem length[c]	Leaf number	Leaf area[b]					Roots		
					1	2	3	4	5	Number	Length[c]	Weight[d]
110R	None	24	3.3±0.7	4.1±1.2	0.9±0.8	2.0±1.2	0.9±0.9	0.4±0.8	0	1.5±1.3	2.4±1.6	42.2±33.0
	Cysteine	30	**4.3±0.9****	**5.1±0.9****	1.0±0.7	**2.4±0.7**	**1.6±1.0**	0.2±0.5	0	**2.4±0.8**	**3.7±1.1**	**97.7±34.2***
	Act. charcoal	24	3.8±1.3	4.1±1.5	0.3±0.5	1.1±1.1	1.6±1.2	**0.7±0.8**	**0.3±0.5**	1.2±0.9	2.3±1.8	37.0±28.2
	1% PVP	24	2.6±1.1	2.8±1.8	**1.6±1.1**	1.1±1.3	0.2±0.4	0	0	1.2±0.9	2.0±1.4	18.6±13.0
3009C	None	24	6.5±1.5*	5.4±1.3**	0.6±0.8	2.1±1.3	1.9±1.2	0.6±0.7	0.3±0.5	4.3±1.8*	**4.4±1.5***	63.8±27.8**
	Cysteine	30	5.8±1.5	**7.3±1.5***	0.3±0.1	**2.4±0.9**	**2.9±0.8**	**1.3±1.3**	**0.3±0.6**	**4.8±0.9***	4.1±1.0*	**80.3±19.4***
	Act. charcoal	24	**7.1±1.4***	4.9±0.8	0.5±0.7	1.6±0.9	2.0±1.1	0.9±0.8		2.1±0.9	4.0±0.8*	48.5±14.6
	1% PVP	24	2.2±0.4	2.3±0.5	**0.9±0.9**	1.1±0.9	0.3±0.5	0	0	1.0±0.8	1.4±1.0	13.1±10.4
101-14	None	24	3.6±0.9	4.5±1.2	0.4±0.6	1.6±1.0	2.0±1.0	0.7±0.9	0.1±0.3	1.8±0.6	2.9±1.5	45.5±3.1
	Cysteine	30	5.4±1.4	**5.3±1.1****	0.7±0.8	**2.4±1.1**	1.5±1.4	0.6±1.0	0.1±0.3	**3.2±1.7**	3.4±1.3	**54.8±25.1****
	Act. charcoal	24	**6.9±2.5***	5.0±1.4	0.5±0.7	1.6±0.9	**2.0±1.1**	0.9±0.8	0	1.8±0.7	**3.8±0.6***	43.0±12.3
	1% PVP	24	2.2±0.4	2.3±0.5	**0.9±0.9**	1.1±0.9	0.3±0.5	0	0	1.0±0.8	1.4±1.0	13.1±10.4
RG	None	24	4.9±2.4	4.7±2.1	0.4±0.6	1.7±1.3	1.6±1.0	**0.8±0.9**	0.3±0.5	1.6±1.3	2.9±2.2	26.4±21.1
	Cysteine	30	**6.9±2.1***	**5.1±1.4****	0.4±0.6	1.5±1.0	**1.9±1.1**	0.7±0.7	**0.6±0.6**	**4.3±1.4***	**4.0±1.6***	**68.0±32.1****
	Act. charcoal	24	6.3±2.4*	5.2±1.5**	**0.8±0.8**	**1.9±1.1**	1.5±1.2	0.6±0.8	0.5±0.8	2.0±1.0	2.7±1.5	36.4±21.6
	1% PVP	24	2.3±0.7	2.4±0.7	0.6±0.7	1.5±1.0	0.2±0.4	0	0	1.6±1.1	1.2±0.8	18.1±13.9

[a]Data (mean±SE) from two independent experiments were collected after 30 days of culture. N = number of plants per replicate; [b]Number of leaves for each leaf size area category with 1:0-0.5 mm^2; 2:0.6-10 mm^2; 3:11-15 mm^2; 4:16-20 mm^2; and 5:21-25 mm^2; [c]Length in cm; [d]Weight in mg. Values significantly different in each column are indicated by * ($P = 0.05$) or ** ($P = 0.01$). Highest parameter values for each rootstock are indicated in bold.

Table 4 Analysis of variance table against the null hypothesis (antioxidants have no effect on rooting and growth of micropropagated shoot tips).

Source	Df	Root number		Root length		Root weight		Stem length		Leaf number		Leaf area	
		F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F
Genotype	4	18.19	<0.0001	6.78	0.0004	11.08	<0.0001	29.05	<0.0001	9.66	<0.0001	9.48	<0.0001
Antioxidant	3	25.11	<0.0001	53.55	<0.0001	136.48	<0.0001	105.03	<0.0001	123.73	<0.0001	98.07	<0.0001
Genotype*Antioxidant	12	8.87	<0.0001	6.41	<0.0001	11.69	<0.0001	12.90	<0.0001	8.23	<0.0001	5.29	<0.0002

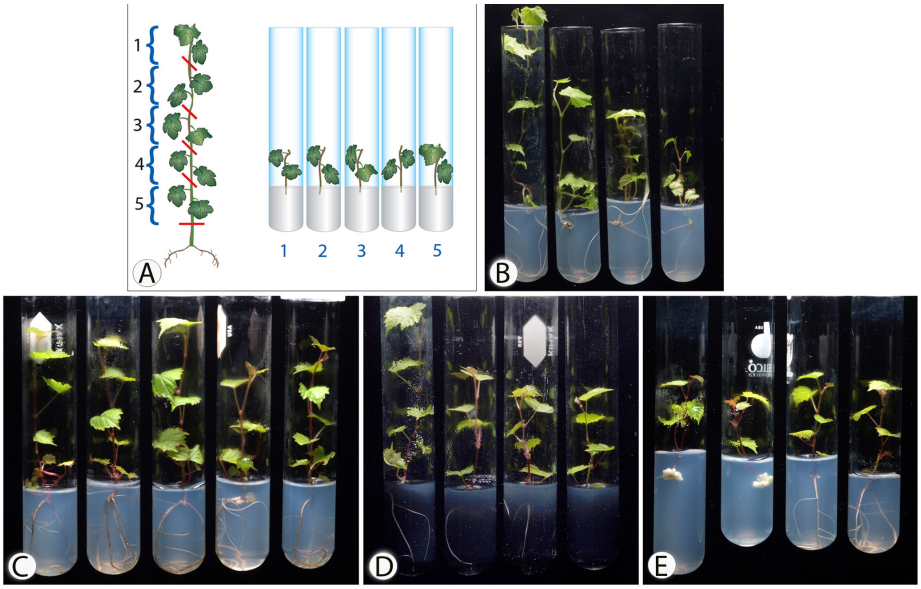

Fig. 3 Effect of nodal segment position from (**A**) subcultured plantlets numbered from 1 (most apical node) to 5 (basal node) and culture on WPM supplemented with (**B**) no antioxidant - *V. riparia* -, (**C**) 37 mg l^{-1} cysteine - 110R -, (**D**) 3 g l^{-1} activated charcoal - 110R -, and (**E**) no antioxidant - 110R -. Note that cysteine breaks the inhibitory effect of apical dominance observed for *V. riparia* and 110R after one month of culture in the absence of antioxidant or presence of activated charcoal. It also prevented callus formation for 110R.

(**Table 4**). Cysteine also promoted uniform proliferation of nodal segments for all rootstocks, except for 101-14, independently of the position of the explant on the initial subcultured plant (**Fig. 3C** vs. **Fig. 3D** and **Fig. 3E**). For antioxidant effect, root weight and leaf area were the best predictor parameters for root and overall plant development, followed by stem length, leaf number, and root number (**Table 3**). ANOVA indicated that root weight, stem length and leaf number were the parameters most influenced by antioxidant treatment followed by leaf area. The genotype x antioxidant interaction was significant for all the parameters tested ($P < 0.001$) (**Table 4**).

110R plants rooted better on WPM with cysteine than on WPM with no antioxidant or supplemented with other antioxidants (**Fig. 2A**). Aerial development was reduced and basal calli developed frequently in the absence of cysteine (**Fig. 2A**). PVP had a negative effect with plants becoming stunted and necrotic (**Fig. 2A**). Also, fewer plants rooted on 1% PVP compared to WPM containing cysteine (65 vs. 100%) and some unrooted plants (25%) formed basal callus. AC delayed rooting by about 2-3 weeks compared to WPM plus cysteine. Also, growth was significantly slower for basal shoot tip explants at position 2, 3 or 4 compared to those from position 1 on AC (**Fig. 3D**). The inhibitory effect of the apical meristem proliferation of axillary buds was overcome by the use of cysteine with a more uniform shoot growth and rooting, regardless of the initial position of nodal segments on mother plants (**Fig. 3C**). Also,

callus formed at the base of explants from positions 1 and 2 in the absence of antioxidants for 110R (**Fig. 3E**). For 3309C, roots developed better on WPM with cysteine than without antioxidant while the addition of 2% PVP caused necrosis (**Fig. 2B**). In the absence of cysteine, the aerial part of the plants developed poorly, basal leaves became necrotic and shoots derived from explants in position 4-5 were stunted (**Table 3**). 1% PVP caused poor rooting and plant development, stunting, and reduced leaf area (> 50%) or no leaf development at all (**Table 3**). Roots developed after 2-3 weeks on medium supplemented with AC versus 10 days on medium supplemented with cysteine, and roots were thinner and with fewer secondary roots (**Fig. 2B**). Interestingly, AC increased stem length but more and bigger leaves developed on WPM with cysteine (5 vs. 3 in size categories 3-5). For 101-14, plants rooted better and had better aerial growth on WPM lacking antioxidant (**Table 3**). PVP caused necrosis of all explants at 2% and very poor development with extensive chlorosis and stunting and very few, if any, roots at 1% (**Table 3**). AC delayed rooting for 2-3 weeks and the progeny of explants from position 4-5 was stunted compared to that from position 1-3 while uniform growth of all explants was observed on WPM plus cysteine. For RG, the addition of cysteine overcame the apical dominance effect observed in the absence of cysteine (**Fig. 3B**) or in the presence of AC. Also, basal leaves were chlorotic and basal explants were stunted when no cysteine was added. PVP at 2% caused necrosis of all explants and chlorosis, while at 1% PVP, stunting and poor rooting occurred with the majority of explants deve-loping no roots (**Table 3**).

Overall, a homogeneous growth and rooting was obtained on WPM supplemented with cysteine while an apical dominance was observed in the presence of AC for 110R, 5C, 3309C and RG (**Fig. 3D** vs. **Fig. 3E**). Rooting occurred 1-3 weeks faster, and stronger and more roots developed in the presence of cysteine compared to PVP and AC. In the absence of antioxidant or AC, calli formed at the base of 110R explants from positions 1 and 2 (**Fig. 3E**).

A total of 450 plants corresponding to the five micropropagated grape rootstocks that grew on WPM supplemented with 37 mg l^{-1} cysteine was transferred from tissue culture to the greenhouse. Successful establishment in soil was achieved with a high survival rate (99%, 447 of 450). In contrast, establishment in the greenhouse was poor (45%, 68 of 152) for plants growing on half strength MS with AC due to poor rooting.

CONCLUSIONS

Homogeneous rooting and growth of micropropagated grapevine rootstocks was achieved on WPM supplemented with 37 mg l^{-1} cysteine. Our protocol facilitated a fast and uniform clonal multiplication of five grapevine rootstocks (3309C, 110R, 101-14, RG and 5C), and a successful transfer and growth of micropropagated plants in the greenhouse. Most reports on micropropagation of grapevine material describe the effect of growth regulators on rooting and development of micropropagated *Vitis* spp. (Bouquet and Torregrosa 2003; Akbas *et al.* 2004; Barreto *et al.* 2006; Banilas and Korkas 2007; Namli *et al.* 2007; Jaskani *et al.* 2008; Aazami 2010; Ikten and Read 2010; Kurmi *et al.* 2011). Our results were consistent with the fact that auxin was not essential for rooting when cysteine was added as antioxidant, confirming earlier reports (Lee and Wetzstein 1990). To our knowledge, this is the first report on the successful use of cysteine as antioxidant in grapevine micropropagation. Our protocol consists of five major steps that can be summarized as follows (**Fig. 4**): 1) Establishment of shoot tips in tissue culture from greenhouse-grown potted plants, 2) Multiplication of shoot tips within one month, 3) Development and rooting of shoot tips within one month, 4) Transfer of plantlets to soil in the greenhouse, and 5) Growth of transferred plantlets in the greenhouse.

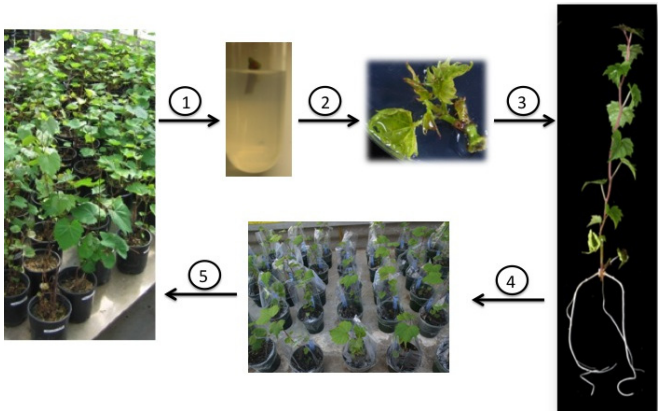

Fig. 4 Five major steps of the micropropagation protocol with (**1**) Establishment of shoot tips in tissue culture from greenhouse-grown potted grapevine rootstocks, (**2**) Multiplication of shoot tips, (**3**) Development and rooting of shoot tips, (**4**) Transfer and (**5**) growth of micropropagated plantlets in the greenhouse.

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