ELIMINATION OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1* AND *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* FROM GRAPEVINE cv. AGIORGITIKO, AND A MICROPROPAGATION PROTOCOL FOR MASS PRODUCTION OF VIRUS-FREE PLANTLETS

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SUMMARY

Grapevine leafroll-associated virus 1 (GLRaV-1) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) were eradicated from *Vitis vinifera* L. cv. Agiorgitiko by combining *in vitro* thermotherapy and tissue culture. GRSPaV is known to be quite recalcitrant to elimination, whereas GLRaV-1 is more easily knocked out. In this study, the effectiveness of two different virus elimination techniques, including meristem- and shoot-tip culture, was evaluated. Results showed that meristem-tip culture combined with thermotherapy was the most effective for eliminating both viruses as confirmed by nested RT-PCR assays. Success rate for GLRaV-1 (91.2%) was higher than for GRSPaV-1 (67.6%). The ratio of virus elimination to survival was higher for meristem-tip culture than for shoot tips (1.108 and 0.469 respectively). The effect of six basal media on *in vitro* shoot proliferation of virus-free explants of cv. Agiorgitiko was also studied and woody plant medium (WPM) proved to be the most effective. The presence of cytokinin 6-benzyladenine (BA) alone resulted in chlorotic plantlets, while supplementation with the auxin naphthaleneacetic acid (NAA) enhanced proliferation rate. Root induction at two temperature regimes (22±2°C and 26±2ºC) revealed that higher temperature was more effective in the presence of IBA (indole-3-butyric acid) rather than NAA.

Key words: thermotherapy, nested RT-PCR, 6-benzyladenine, naphthaleneacetic acid, acclimatization, meristem-tip culture.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is the most widely grown fruit crop worldwide (Péros *et al.*, 1998). One of its greatest health problems is the presence of viruses that are disseminated with nursery stock through vegetative propagation and grafting and reduce productivity in quantity and quality (Walter and Martelli, 1997). Production of virus-free plant material is one strategy to combat these pathogens and contributes to the growth of the viticulture industry, in accordance with the European Union directives for the grapevine. Virus elimination may be achieved through thermotherapy and meristem- or shoot-tip culture, but the combination of both methods is more effective (Grammatikaki *et al.*, 2005).

Viticulture is of great economic importance in Greece. *Vitis vinifera* L. cv. Agiorgitiko is one of the most important Greek red wine varieties, grown in southern Greece for the production of dry, semi-sweet and sweet red V.Q.P.R.D (Vin de Qualité Produit Dans Une Région Déterminée) Nemea wine (Spinthiropulu, 2000). Field monitoring, using ELISA and RT-PCR, has shown that this cultivar is heavily infected by *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Dovas, 2002). GLRaV-1 belongs to the genus *Ampelovirus* and is one of the 9 species of the family *Closteroviridae* involved in grapevine leafroll disease (Gugerli, 2003), responsible for yield losses up to 68% (Walter and Martelli, 1997). GRSPaV is a foveavirus implicated in the aetiology of rugose wood and recently associated with grapevine vein necrosis (Bouyahia *et al.*, 2005).

The production of virus-free 'Agiorgitiko' is important to improve performance of this cultivar. Viruses can be efficiently eliminated *in vitro* when combined with large-scale micropropagation. Grapevine micropropagation was first carried out by Galzy (1961), since then many different procedures have been developed. A crucial problem to be considered in grapevine micropropagation is the genetic variability of *V. vinifera* cultivars (Torregrosa and Bouquet, 1996), which affects the efficiency of *in vitro* techniques and of the micropropagation protocols applied, probably due to the different endogenous plant regulators of each cultivar (Roubelakis-Angelakis and Zivanovitc, 1991; Péros *et al.*, 1998). Therefore, protocols need to be optimized for different cultivars.

Much work has been done on the eradication of GLRaV-1, but for GRSPaV, knowledge is rather limited (Gribaudo *et al.*, 2006). The aim of this study was there-

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fore to develop a simple and efficient protocol for the elimination of GLRaV-1 and GRSPaV from cv. Agiorgitiko by *in vitro* thermotherapy and tissue culture coupled with reliable molecular detection assays. Furthermore, an efficient micropropagation protocol was also developed in order to be able to conserve and mass-produce this very important cultivar.

MATERIALS AND METHODS

Sources of plant material and *in vitro* establishment of explants. Shoots from selected field-grown cv. Agiorgitiko vines infected with GLRaV-1 and GRSPaV were stripped of leaves and washed with tap water. Single node segments were disinfected using 500 ml 2% (w/v) NaOCl plus three drops of Tween 20, shaken on a rotary shaker for 15 min and rinsed three times with sterile distilled water. Explants were cultured in test tubes (100 x 20 mm) containing 10 ml of MS medium (Murashige and Skoog, 1962) supplemented with 4.5 μM BA (6-benzyladenine) and 2.7 μM NAA (α-naphthaleneacetic acid). Explants produced single axillary shoots, which were cut into nodal segments and transferred to 500 ml glass jars containing 125 ml of the same medium. The procedure was repeated every six weeks and material for further experiments was produced after six months.

Virus eradication. *In vitro* thermotherapy was done in a growth chamber (Percival Scientific, I-36LLVL) for 6 weeks, with a 16 h photoperiod using cool white fluorescent light (40 μM m^{-2} s^{-1}). The initial temperature, which was 26°C (day) and 23°C (night), was gradually increased by 3°C/week, so that the final values, maintained for one week, were 40°C (day) and 37°C (night). The procedure was repeated three times and a total of 260 explants were used. Explants were cultured in woody plant medium (WPM) (Lloyd and McCown, 1980).

For post-thermotherapy *in vitro* culture, explants were divided into meristems and shoot tips, both originating from the apical bud. Meristems were 0.1-0.2 mm in length and were cultured in Grammatikaki medium (GRM) (Grammatikaki *et al*., 2005), whereas 0.5 cm long shoot tips were cultured in WPM. After six weeks the plantlets regenerated from meristems and shoot tips (2 cm or more in length) were transferred to fresh WPM.

Nested RT-PCR detection of GLRaV-1 and GRSPaV. Plants were assayed with commercial ELISA kits for the presence of GLRaV-1, -2, -6, -7 (Bioreba, Switzerland), -3 (Agritest, Italy) and -5 (Biorad, USA), *Grapevine fleck virus* (GFKV) (Agritest, Italy), *Grapevine fanleaf virus* (GFLV) (Agritest. Italy), *Grapevine virus A* (GVA) (Biorad, USA), *Grapevine virus B* (GVB) (Agritest, Italy) and *Arabis mosaic virus* (ArMV) (Bioreba, Switzerland). ELISA was followed by RT-PCR using specific primers for the detection of GLRaV-1, -2, -3, -4, -5, -9, GVA, GRSPaV (Routh *et al*., 1998; Meng *et al*., 1999; Alkowni *et al*., 2004; Dovas *et al*., 2006; Gambino *et al*., 2006) and also by generic RT-PCR for the diagnosis of viti-, fovea- and closteroviruses (Dovas and Katis, 2003a).

Total RNA was extracted from 0.2 g of grapevine explants (stems, petioles and leaves) according to Rott and Jelkmann (2001), modified by adding 6% PVP and 0.2 M β–mercaptoethanol to the grinding buffer. GRSPaV diagnosis was based on the generic nested RT-PCR protocol (Dovas and Katis, 2003b) developed for the detection of viti- and foveaviruses, which incorporates degenerate primers targeting a conserved region (198 bp) of the RNA depended RNA polymerase (RdRp). RT-PCR took place in a final volume of 25 μl using 2 μl of RNA and a final concentration of 1 μM of each 'dRWup1' and 'dRWdo2' primers. Nested PCR (20 μl) was performed using 1 μl of the first RT-PCR product, 1 μM of 'dRW nest1' and 1.5 μM of 'dRW nest2'. The cycling profiles were as previously reported (Dovas and Katis, 2003b).

For GLRaV-1 detection, Dovas *et al*. (2006) ramped annealing nested PCR was employed using primers targeting the conserved N terminus (490 bp) of the heat-shock protein 70 homologue (HSP70h) of closteroviruses. A first RT-PCR (25 μl) run for the generic detection of *Closteroviridae* species was done using the primers 'dHSPup1', 'dHSPdo2', 'dHSPdo2C' and 'dHSPup1G' in the concentrations reported (Dovas and Katis, 2003a; Dovas *et al*., 2006), followed by nested PCR, in which 1 μl of the first RT-PCR product was used together with the generic upstream primers 'dHSPnest1', 'dHSPnest2' and the GLRaV-1 specific primer 'LR1-nest4'. The cycling profiles of both assays were as described by Dovas *et al*. (2006).

Micropropagation protocol. *Culture media.* In order to optimize the proliferation rate of explants, six culture media supplemented with 0.13 μM BA and 0.16 μM NAA, were tested: (i) classical MS; (ii) WPM (Lloyd and McCown, 1980), (iii) Galzy (GAL) (Galzy *et al*., 1990), (iv) MS-GAL, i.e. a medium consisting of MS macro- and microsalts and GAL vitamins (Galzy, 1972), without calcium pantothenate and biotin (Chee and Pool, 1982); (v) modified Quoirin and Lepoivre (1977) medium (QL-MS), consisting of QL macro- and microsalts, supplemented with MS vitamins and Fe EDTA (Bottalico *et al*., 1997); (vi) QL-WPM, containing QL macro- and microsalts plus WPM vitamins and MS Fe EDTA.

Effect of plant growth regulators. The effect of 0, 0.5, 1, 1.5, 2, 4, 8 and 16 μM BA in WPM was tested, in comparison with WPM containing BA (0-4 μM) plus 0.1 and 0.3 μM NAA (Heloir *et al*., 1997). These BA concentrations, used after preliminary experiments, had shown that they did not induce severe decline of plantlets, while NAA did not elicit the formation of

large calli at the base of the stem (not shown). WPM without any growth regulators was used as control.

In vitro rooting and acclimatization. Root formation on microcuttings was studied at two different growth temperatures, 22±2°C and 26±2°C. Shoots from 6-week-old plantlets, 4 cm long, coming from virus-free stock were cultured in MS medium (Li and Eaton, 1984), supplemented with different concentrations of either NAA (0.1, 0.3, 0.5, 1 μM) (Chee *et al*., 1984) or IBA (indole-3-butyric acid) (0.1, 0.5, 1, 1.5 μM) (Grammatikaki and Avgelis, 2000).

Rooted and unrooted microshoots, derived from the previous *in vitro* rooting experiment, were rinsed with tap water to remove adhering medium, then planted separately in trays filled with a peat (Klasmann, KTS 1)-perlite 4:1 (v/v) mixture. The plantlets were placed in a greenhouse, under mist at 90% relative humidity (RH) and 50% shading for 10 days. In the following days RH was reduced (5%/day), while light intensity was gradually increased.

Experiments. Nodal explants 1 cm long used in all experiments were randomly selected from virus-free stock material. Fifty explants were used in every treatment and each experiment was repeated twice. All media tested contained 3% (w/v) sucrose, 0.6% (w/v) agar (B & V S. r .L., type S 1000) and were adjusted to pH 5.9 before autoclaving. Cultures were kept at 22±2°C and 16 h photoperiod under cool white fluorescent light (40 μM m^{-2} s^{-1}).

Experiments on the effect of culture media and growth regulators for shoot proliferation lasted 40 days. Shoots number (S), length of microhoots (base to the last visible node) (LM), number of nodes (N), primary root number (R), length of primary roots (LR), proliferation rate (PR) (calculated as SxN), and length of internode segments (IS) were recorded. Data were collected only for shoots longer than 0.5 cm.

Rooting experiments were carried out at 22±2°C over 18 days and at 26±2°C over 10 days. At the end of these periods R and LR were measured for roots longer than 1 mm. Acclimatization lasted 30 days and survival of plantlets was recorded every 10 days. At the end of the acclimatization period S, LM and N and survival expressed as percent were measured.

Measurements of LM, N, PR, IS and LR referred to the average data of S and R, respectively. Experiments followed a randomized complete block design, with ten explants per treatment and five replications. Every experiment was repeated twice. Student's *t*-test was used for comparing data from temperature treatments. Analysis of variance was performed with the general linear model procedure (SPSS 8.0) and mean separation with Duncan's multiple range test ($P<0.05$).

The culture medium found to be optimal was used for growing explants during thermotherapy. The experiments described were performed with virus-free material but when infected plantlets were used, no statistical differences were observed (data not shown). Virus detection, using nested RT-PCR, was repeated every three months for over two years on the virus-free plantlets *in vitro* and *ex vitro* to assess their sanitary status.

RESULTS

Post-thermotherapy survival rates and virus elimination. Only 52.60% of the explants that underwent thermotherapy survived the heat stress and the presence of contaminants (bacteria and fungi). Some surviving plantlets appeared stressed, as indicated by the red colour of their stem, but showed no necrosis at the shoot-tip or elsewhere. Regeneration rate from meristem and shoot-tip culture was 55.7% and 80.3%, respectively. The combination of thermotherapy and tissue culture effectively eliminated both GLRaV-1 and GRSPaV-1 (Fig. 1). However, both viruses were more readily eradicated when thermotherapy was followed by meristem-tip rather than shoot-tip culture. The respective values were 91.2% and 73.8% for GLRaV-1, 67.6% and 50.8% for GRSPaV-1, while 61.8% of meristem-tip and 37.7% of shoot-tip culture were free from both viruses. Interestingly, elimination of GLRaV-1 was easier than that of GRSPaV-1 with both meristem-tip and shoot-tip culture. The ratio of virus elimination to explant survival was higher for meristem-tip than for shoot-tip culture (1.108 and 0.469 respectively).

Micropropagation protocol. *Culture media.* Four (WPM, GAL, QL-MS and QL-WPM) of the six media used showed no significant differences on shoot prolif-

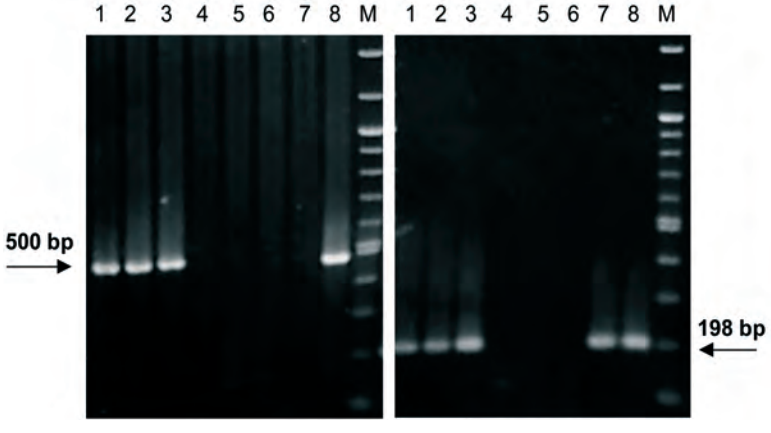

Fig. 1. Agarose gel electrophoretic analysis of nested RT-PCR products, obtained from infected and virus-free plant material of cv. Agiorgitiko. A. GLRaV-1 detection; B. GRSPaV-1 detection. In both gels, lanes 1-3: field-grown and *in vitro* cultured infected plant material; lane 4: healthy control; lanes 5-8: plantlets tested after thermotherapy and *in vitro* culture; M: 100 bp DNA ladder.

Table 1. Effect of basal culture media on *in vitro* shoot proliferation of the grapevine cv. Agiorgitiko.

Media	S	LM (cm)	N	IS (cm)	PR	R	LR (cm)
MS	0.49 [b]	3.73 [bc]	5.40 [b]	0.69 [ab]	2.64 [b]	2.94 [a]	3.73 [c]
WPM	0.98 [a]	4.36 [a]	6.00 [a]	0.73 [a]	5.88 [a]	2.46 [ab]	7.11 [a]
GAL	0.96 [a]	4.46 [a]	6.04 [a]	0.74 [a]	5.79 [a]	2.24 [b]	7.21 [a]
MS-GAL	0.62 [b]	3.58 [bc]	5.61 [ab]	0.64 [b]	3.48 [b]	2.80 [a]	4.40 [c]
QL-MS	0.88 [a]	4.02 [ab]	5.79 [ab]	0.69 [ab]	5.09 [ab]	2.87 [a]	5.78 [b]
QL-WPM	1.01 [a]	3.50 [c]	5.75 [ab]	0.61 [b]	5.81 [a]	2.25 [b]	5.33 [b]

Figures followed by the same letter are not significantly different according to Duncan's range test ($P \leq 0.05$).

Fig. 2. Responses of the grapevine cv. Agiorgitiko to the effects of (a) the six basal culture media; (b) different concentrations (µM) of BA alone; and (c) BA and NAA concentrations (µM). Bars = 1cm.

eration (Table 1, Fig. 2a). Plantlets grown in WPM were vigorous with fully developed green leaves and few long primary roots. In GAL medium, callus developed at the base of the stem. In QL-MS the stems of new plantlets were thinner and the leaves smaller than those from other media tested. QL-WPM yielded the shortest plantlets, although they had a high proliferation rate and the highest number of new shoots/explant. MS and MS-GAL were the least satisfactory, for they produced short chlorotic plantlets.

Effect of growth regulators. The increase of BA concentration from 0.5 to 16 µM produced a higher number of new shoots, whereas the length of plantlets, roots and internodes decreased as compared with the control medium (Table 2, Fig. 2b). When BA concentration above the optimum was used, roots did not grow, small calli developed at the base of the stem and the leaf blades showed downward rolling. No hyperhydricity was observed.

Combination of BA and NAA improved shoot proliferation rate, compared with BA when used alone (Table 3, Fig. 2c). Low BA concentration proved to be the best for growth in the presence of both concentrations of NAA tested. No significant differences were observed between these two media for most parameters studied, but the combination of 0.25 µM BA and 0.3 µM NAA increased proliferation rate. The higher the concentration of BA the lower the stem height, as well as the number and length of roots. BA concentrations higher than 0.25 µM (with both combinations of NAA) increased the number of nodes and the proliferation rate, greatly reducing internode length. Plantlets had chlorotic and downward rolled leaves, developed callus at the stem base and produced no roots.

In vitro rooting and acclimatization. First root formation was observed on the 18th day of culture at 22°C, but only after 10 days at 26°C. Furthermore, at 22°C rooting was less for plantlets treated with IBA than with NAA. At 26°C, root induction followed the same pattern as at 22°C (Table 4). IBA induced longer roots at 26°C, while NAA affected the induction of primary roots and root production at both temperatures. Roots in the control medium were longer but their number and the development ratio were low.

Table 2. Effect of different concentrations of BA on *in vitro* shoot proliferation of the grapevine cv. Agiorgitiko.

Media	S	LM (cm)	N	IS (cm)	PR	R	LR (cm)
Control	0.61 ^{bc}	3.5 ^{a}	5.81 ^{a}	0.61 ^{ab}	3.54 ^{b}	1.18 ^{a}	8.10 ^{a}
0.5 μM BA	0.71 ^{b}	2.4 ^{b}	4.40 ^{cd}	0.55 ^{bc}	3.12 ^{bc}	0.36 ^{b}	6.77 ^{ab}
1 μM BA	0.61 ^{bc}	2.3 ^{bc}	4.00 ^{de}	0.59 ^{ab}	2.44 ^{cd}	0.07 ^{c}	4.49 ^{b}
1.5 μM BA	0.55 ^{c}	1.9 ^{e}	3.67 ^{e}	0.54 ^{bc}	2.02 ^{d}	0.00	0.00
2 μM BA	0.59 ^{bc}	2.0 ^{cde}	4.28 ^{cd}	0.49 ^{bcd}	2.53 ^{cd}	0.00	0.00
4 μM BA	0.99 ^{a}	2.3 ^{bcd}	4.64 ^{bc}	0.50 ^{bc}	4.59 ^{ab}	0.00	0.00
8 μM BA	1.06 ^{a}	2.17 ^{bcde}	4.70 ^{bc}	0.46 ^{cd}	4.98 ^{ab}	0.00	0.00
16 μM BA	1.02 ^{a}	2.03 ^{de}	5.06 ^{b}	0.40 ^{d}	5.16 ^{a}	0.00	0.00

For each parameter the same letter represents values with no significant difference according to Duncan's range test ($P \leq 0.05$).

No callus was formed at 22°C but at 26°C callus formation was stimulated when 0.5 μM NAA and 1 μM IBA were used. At both temperatures, roots were thicker on media supplemented with NAA than in the presence of IBA. At high NAA concentration (1 μM) side effects, such as chlorosis and toxicity symptoms were observed, and were stronger at 26°C. IBA at 0.5 μM and 26°C proved to be the most effective for *in vitro* root formation yielding plantlets with normal appearance and greater number of roots (Table 4).

Acclimatization of both rooted and non-rooted plantlets was successful, with survival above 75% after one month (Table 5). Highest shoot length, node number and survival rate (100%) were recorded for plantlets on rooting medium supplemented with 0.5 μM IBA. Survival rate was higher on media containing IBA than NAA.

DISCUSSION

In this study meristem or shoot tip cultures coupled with thermotherapy were successfully used to eliminate both GLRaV-1 and GRSPaV-1, obtaining a relatively high percentage of virus-free plantlets; our results confirmed that elimination of GLRaV-1 is easier (Mannini, 2003) than GRSPaV (Gribaudo *et al*., 2006). In fact GRSPaV is reported to be particularly difficult to eliminate using meristem tip culture and *in vivo* or *in vitro* thermotherapy (Minafra and Boscia, 2003; Gribaudo *et al*., 2006).

Our results indicated that both viruses could be eliminated even from bigger explants such as shoot tips. Consequently, and in order to avoid the difficulties of meristem-tip culture, shoot tips were used. Although lower percentages of virus-free plantlets were produced

Table 3. Effect of combinations of different concentrations of BA and NAA on *in vitro* shoot proliferation of the grapevine cv. Agiorgitiko.

	Media	S	LM (cm)	N	IS (cm)	PR	R	LR (cm)
0.1 μM NAA	Control	0.68 ^{e}	3.41 ^{d}	5.34 ^{cd}	0.64 ^{bcd}	3.63 ^{de}	2.52 ^{b}	10.07 ^{a}
	0.25 μM BA	1.00 ^{a}	5.27 ^{e}	6.25 ^{ab}	0.84 ^{a}	6.25 ^{bc}	3.18 ^{a}	10.76 ^{a}
	0.5 μM BA	0.88 ^{bcd}	3.29 ^{c}	5.51 ^{cd}	0.60 ^{cd}	4.85 ^{d}	2.34 ^{b}	6.81 ^{a}
	1 μM BA	0.72 ^{de}	2.13 ^{a}	4.83 ^{e}	0.44 ^{e}	3.48 ^{e}	0.76 ^{c}	3.77 ^{b}
	2 μM BA	0.98 ^{ab}	2.88 ^{bc}	5.52 ^{cd}	0.52 ^{d}	5.41 ^{cd}	0.08 ^{d}	2.70 ^{b}
	4 μM BA	1.08 ^{a}	2.63 ^{ab}	6.01 ^{bc}	0.44 ^{e}	6.49 ^{bc}	0.00	0.00
0.3 μM NAA	0.25 μM BA	1.04 ^{a}	5.49 ^{e}	6.70 ^{a}	0.82 ^{a}	6.97 ^{a}	3.10 ^{a}	12.12 ^{a}
	0.5 μM BA	0.95 ^{ab}	4.93 ^{de}	7.09 ^{a}	0.70 ^{b}	6.74 ^{bc}	3.24 ^{a}	10.89 ^{a}
	1 μM BA	0.70 ^{e}	2.80 ^{bc}	5.28 ^{de}	0.53 ^{d}	3.70 ^{de}	2.22 ^{b}	3.83 ^{b}
	2 μM BA	0.86 ^{cde}	2.20 ^{a}	5.88 ^{bcd}	0.37 ^{e}	5.06 ^{cd}	0.30 ^{cd}	2.92 ^{b}
	4 μM BA	0.98 ^{ab}	2.12 ^{a}	5.72 ^{bcd}	0.37 ^{e}	5.61 ^{c}	0.02 ^{d}	0.00

For each parameter the same letter represents values with no significant difference according to Duncan's range test ($P \leq 0.05$).

Table 4. Effect of culture temperature and auxins on rooting of the grapevine cv. Agiorgitiko.

Media	22ºC			26ºC		
	18 days			10 days		
	R	LR (cm)	% Rooting	R	LR (cm)	% Rooting
Control	1.17 ^{e}	0.42 ^{b}	58.00 ^{cd}	1.17 ^{e}	0.59 ^{a}	57.00 ^{d}
0.1 μM NAA	3.23 ^{cd}	0.57 ^{a}	95.00 ^{a}	2.90 ^{d}	0.42 ^{a}	87.00 ^{ab}
0.3 μM NAA	3.77 ^{bc}	0.37 ^{b}	87.00 ^{ab}	4.23 ^{b}	0.26 ^{c}	98.00 ^{a}
0.5 μM NAA	4.20 ^{b}	0.38 ^{b}	82.00 ^{b}	4.20 ^{b}	0.26 ^{c}	88.00 ^{ab}
1 μM NAA	7.67 ^{a}	0.38 ^{b}	98.00 ^{a}	5.28 ^{a}	0.25 ^{c}	93.00 ^{a}
0.1 μM IBA	1.43 ^{e}	0.24 ^{c}	68.00 ^{c}	2.01 ^{e}	0.37 ^{ab}	73.00 ^{c}
0.5 μM IBA	1.80 ^{e}	0.32 ^{bc}	50.00 ^{d}	3.10 ^{cd}	0.42 ^{a}	97.00 ^{a}
1 μM IBA	1.67 ^{e}	0.21 ^{c}	58.00 ^{cd}	3.09 ^{cd}	0.31 ^{bc}	91.00 ^{ab}
1.5 μM IBA	2.80 ^{d}	0.24 ^{c}	80.00 ^{b}	3.87 ^{bc}	0.26 ^{c}	87.00 ^{ab}

For each parameter the same letter represents values with no significant difference according to Duncan's range test ($P \leq 0.05$).

from shoot tips for both GLRaV-1 and GRSPaV-1, the survival rate was significantly higher than in meristem tip culture. The sanitation rate was significantly lower for shoot tips, but more virus-free plantlets were finally produced because of the higher survival rate.

For the development of an efficient micropropagation protocol for mass production of cv. Agiorgitiko, no significant differences (except for LR) were observed among explants cultured on the four basic salt mixtures (WPM, GAL and QL-MS and QL-WPM). Callogenesis noted on GAL rendered this medium inappropriate, whereas the thin stems and short plantlets produced in QL-MS and QL-WPM, respectively, indicated that WPM is the most suitable substrate for micropropagation of Agiorgitiko. Differences in stimulation of shoots may be related to differences in macronutrients, as was previously reported (Galzy, 1969; Roubelakis-Angelakis and Zivanovitc, 1991).

Grönroos *et al*. (1989) suggested that differences in *in vitro* response between genotypes may be related to differences in endogenous content of hormones for several plant species. The same reasoning could explain the great variability among *Vitis vinifera* cultivars (Péros *et al*., 1998; Roubelakis-Angelakis and Zivanovitc, 1991). Some cultivars were reported to proliferate without any auxin addition in the medium (Novak and Juvová, 1983), and Ibañez *et al*. (2005) proposed that explants from cv. Napoleon may function as active centres of auxin synthesis, so its inclusion in the medium was not necessary. Nevertheless, auxin is required for the proliferation of 'Agiorgitiko', indicating that its endogenous auxin is very low, rendering BA alone insufficient for shoot elongation. Rooting of plantlets seems to depend on the nature of the auxin (Heloir *et al*., 1997). In this study NAA produced lower root quality than IBA, while high NAA concentrations caused toxicity, especially at the stem base. These side effects became worse at higher temperatures. However, when IBA was present, even at high concentrations,

Table 5. Effect of auxins on acclimatization of the grapevine cv. Agiorgitiko.

Media	S	LM (cm)	N	% Survival		
				10 days	20 days	30 days
Control	1.03 ^{a}	7.82 ^{d}	6.61 ^{a}	86.10 ^{d}	86.10 ^{d}	85.65 ^{d}
0.1 μM NAA	1.04 ^{a}	11.27 ^{bc}	6.86 ^{a}	96.75 ^{b}	88.45 ^{cd}	85.60 ^{d}
0.3 μM NAA	1.07 ^{a}	11.07 ^{bc}	7.16 ^{a}	89.50 ^{c}	89.25 ^{c}	89.40 ^{c}
0.5 μM NAA	1.16 ^{a}	8.56 ^{cd}	6.60 ^{a}	90.00 ^{c}	81.85 ^{e}	80.00 ^{e}
1 μM NAA	1.18 ^{a}	10.09 ^{bcd}	7.12 ^{a}	74.15 ^{e}	73.30 ^{f}	71.65 ^{f}
0.1 μM IBA	1.32 ^{a}	14.66 ^{a}	7.39 ^{a}	100.00 ^{a}	95.25 ^{b}	94.85 ^{b}
0.5 μM IBA	1.13 ^{a}	11.85 ^{b}	7.27 ^{a}	100.00 ^{a}	100.00 ^{a}	100.00 ^{a}
1 μM IBA	1.09 ^{a}	11.87 ^{b}	7.11 ^{a}	100.00 ^{a}	99.50 ^{a}	99.25 ^{a}
1.5 μM IBA	1.13 ^{a}	11.03 ^{bc}	7.56 ^{a}	96.40 ^{b}	95.80 ^{b}	78.26 ^{e}

For each parameter the same letter represents values with no significant difference according to Duncan's range test ($P \leq 0.05$).
Parameters S, LM and N refer to plants at the end of the acclimatization period.

such problems did not occur. This may be because IBA is slowly oxidized whereas NAA is more stable (Dunlap *et al*., 1986; Nissen and Sutter, 1990). When toxicity was observed the morphological appearance of roots was poor, probably because NAA is more persistent than other auxins (De Klerk *et al*., 1997) and strongly affects the quality of plantlets.

The higher temperature tested resulted in the increased number and length of roots in IBA treatment, although an increased number of shorter roots appeared in the presence of NAA. The auxins differed in their effectiveness and this may reflect differences in uptake, transport and metabolism of explants (De Klerk *et al*., 1997). Higher temperature may promote greater uptake and metabolism of auxins in explants. This may explain the increased toxicity of NAA, which finally inhibits root elongation probably due to the longer culture period at the lower temperature.

Rooting of explants was higher in the presence of IBA than NAA, in agreement with Chee and Pool (1987). The better physiological condition of plantlets obtained in media supplemented with IBA resulted in higher acclimatization rates. Morphology of the plantlets at the end of the *in vitro* root formation phase largely determines their *ex vitro* survival.

Results obtained in this study are considered satisfactory for micropropagation of 'Agiorgitiko'. Only simple nutrient salts and low concentrations of growth regulators were required in order to achieve high proliferation and rooting rates. The plants produced were virus-free and should encourage nurseries to provide healthy materials to growers.

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