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In vitro multiplication and hardening of grapevine plants in aeriated media

Zok, A.[1], Zielinska, A.[2], Oláh, R.[1*] & Szegedi, E.[3]

[1]*Corvinus University of Budapest, Faculty of Horticultural Sciences, Department of Genetics and Plant Breeding, H-1118 Budapest, Villányi út. 29-43, Hungary*
[2]*Hidrokultúra 94 Ltd., H-6600 Szentes, Honvéd út 28., Hungary*
[3]*Research Institute for Viticulture and Enology, H-6001 Kecskemét, POBox 25, Hungary*
e-mail: robert.olah@uni-corvinus.hu

Summary: *In vitro* cultures have widely been used in horticulture for rapid multiplication of new varieties and clones as well as to produce pathogen-free stock material. To improve efficient hardening and transfer *in vitro* grown grapevine plants were multiplied by cutting them into single-node internodes with the whole leaf. Microcuttings including the shoot tips were rooted in granulated perlite moisted with tapwater under sterile conditions. After 2–3 weeks the rooted microcuttings were supplied by nutrients and hardened by gradual opening and finally by complete removal of the lids of jars or plastic boxes used for growth. Using this method microcuttings of *Vitis vinifera* cvs. „Chardonnay", „Cabernet franc", „Riesling" and „Sauvignon blanc" and the rootstock varieties *Vitis riparia x Vitis cinerea* cv. „Börner" and *Vitis berlandieri* x *Vitis rupestris* cv. „Richter 110" formed new roots and shoots and 100% of the tested plants survived the acclimatization procedure. Similar results were obtained when perlite was replaced with rockwool-, or pit-pot blocks. This method may highly increase the efficiency of producing pathogen-free propagating material and new transgenic lines.

Key words: micropropagation, pathogen-free plants, *Vitis vinifera*

Introduction

In vitro cultures started from shoot tips, apical meristems or embryogenic calli have been widely used to produce grapevine propagating material that are free from latent (systemic) virus-, and bacterium infections (*Burr* et al. 1988, *Altmayer* 1989, *Robacker & Chang* 1992, *Thies & Graves* 1992, *Torregrosa* et al. 2001) and to estabish genetically modified plants (*Perl & Eshdat* 1998, *Martinelli & Mandolino* 2001, *Kikkert* et al. 2005). As an alternative method, softwood green internodes and shoot tips have also been used as starting material to propagate grapevines or to produce *Agrobacterium*-free stocks under greenhouse conditions (*Stellmach* 1997, *Thomas & Schiefelbein* 2001, 2004, *Szegedi & Lázár* 2005). Rooting of grapevine cuttings is influenced by several physiological and environmental factors that have mainly been studied on dormant canes (*Fournioux* 1997, *Smart* et al. 2003). For efficient rooting of greenwood grapevine cuttings it was basically important to use aeriated media like volcanic ash (*Stellmach* 1976), perlite (*Szegedi & Lázár* 2005), rockwool (*Pathirana & McKenzie* 2005), or aeriated water (*Stellmach* 1997). The presence of leaves on cuttings also positively influenced the root initiation and subsequent shoot development of grapevine cuttings (*Fournioux* 1997, *Thomas & Schiefelbein* 2004, *Szegedi & Lázár* 2005). The presence of the whole leaf similarly favoured the growth of grapevine microcuttings when they were cultured *in vitro* (*Thomas* 2001). Little is known about the effect of aeriated media on rooting and growth of *in vitro* cultured plants. Newell and his coworkers (*Newell* et al. 2003, 2005) found that aerobic culture conditions significantly improved rooting of endemic Australian plants.

In vitro propagation of grapevines has been well established (*Torregrosa* et al. 2001) and for such cultures media are usually solidified with agar or gelrite. Transfer of *in vitro* plants for growth under greenhouse conditions needs complete removal of agar or gelrite with nutrients prior to planting them into soil to prevent contamination as well as careful hardening to adapt plants for open conditions. To improve the efficiency of hardening intermediate growth in inorganic solution combined with gradual aeriation and the use of ventillated vessels is proposed (*Torregrosa* et al. 2001, *Gribaudo* et al. 2003). Here we show that *in vitro* grapevines, like several other plants (*Hazarika* 2003) can be multiplied and hardened for greenhouse growth in a single step under inorganic conditions. To this end, microcuttings with the whole leaf were grown in aeriated media similarly as described for *in vivo* growth of softwood cuttings.

Material and method

Grapevine (*Vitis vinifera* cvs. „Chardonnay", „Cabernet franc", „Riesling" and „Sauvignon blanc", *Vitis riparia x Vitis cinerea* cv. „Börner" and *Vitis berlandieri* x *Vitis rupestris* cv. „Richter 110") were grown *in vitro* in 270 ml baby food jars filled with 30 ml MS medium (Murashige & Skoog 1962) with half-strength of macroelements, 1% (w/v) saccharose and 0.25% (w/v) gelrite at 14 hrs daily illumination (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Shoots were cut into 1–1.5 cm single-node internodes with a whole leaf attached (*Figure 1*). These microcuttings including the shoot tips were planted into the same type of jars filled with approximately 50 ml of sterile perlite moisted with sterile tapwater slightly acidified with an 1:1 mixture of 5% HNO_3 and 5% H_3PO_4 (pH 6.0–6.2) while 2–3 mm thick water layer appeared at the bottom (*Figure 1*). Jars were closed with their original cover with a spongya-filled hole to provide air exchange. To the remaining part of mother plants (roots and a stem piece with one-two nodes) 5–10 ml of half-strength MS liquid medium was added to regenerate new shoots. For comparative experiments cuttings were planted into 0.6% (w/v) agar made with the same acidified tapwater.

After 18–20 days, when root formation was observable in perlite, planlets were supplied with 10 ml of an inorganic, ammonium-free nutrient solution containing 500 mg/l KNO_3, 150 mg/l $CaCl_2 \cdot 2H_2O$, 250 mg/l $MgSO_4 \cdot 7H_2O$, 150 mg/l $NaH_2PO_4 \cdot H_2O$ (pH adjusted to 6.0 with 3% KOH) supplemented with half-strength FeEDTA and microelements according to the MS medium (Murashige & Skoog 1962). From this time the lids were gradually opened. After four to five weeks completely hardened, fast-growing plants were obtained that were ready to transfer into soil (sterile peat:sand:perlit=1:1:1) for further growth in the greenhouse. Alternatively, cuttings were started in 50 ml cups put in a plastic box (*Figure 1*) under similar conditions. The cover was briefly removed twice a week to assure proper air exchange. In further experiments perlite was replaced with approximately 4x4x4 cm Grodan rockwool (www.grodan.com) or with 3x3x4 cm pit-pot blocks (www.pit-pot.com) moisted also with sterile tapwater slightly acidified with an 1:1 mixture of 5% HNO_3 and 5% H_3PO_4 (pH 6.0–6.2). After rooting the same ammonium-free nutrient solution was used as for perlite. For statistical analysis two-way variance analysis was used at 5% probability.

Figure 1 *In vitro* microcuttings of *Vitis vinifera* cv. „Riesling" (left) planted into perlite separately into a jar (middle) or in larger number into a plastic box (right).

Table 1 Comparison of rooting efficiency of grapevine microcuttings in agar and perlit under different conditions

Variety	No. of experiment	0.6% Agar*	Perlite*
„Börner"/vessels	I.	8/12 (66%)	10/12 (83%)
covered with	II.	12/12 (100%)	8/12 (66%)
spongya filter	III.	10/12 (83%)	12/12 (100%)
average (s. d.)		**83% (17.0)**	**83% (17.0)**
„Börner"/ vessels	I.	2/10 (20%)	5/9 (55%)
covered with	II.	4/11 (36%)	5/12 (33%)
cling film	III.	2/12 (16%)	6/12 (50%)
average (s. d.)		**24% (10.5)**	**46% (11.5)**
„Sauvignon blanc"/	I.	12/12 (100%)	11/12 (91%)
vessels covered	II.	11/12 (91%)	11/12 (91%)
with spongya filter	III.	14/14 /100%)	14/14 (100%)
average (s. d.)		**97% (5.1)**	**94% (5.1)**
„Sauvignon blanc"/	I.	5/11 (45%)	9/11 (81%)
vessels covered	II.	4/11 (36%)	10/12 (83%)
with cling film	III.	3/12 (25%)	6/12 (50%)
average (s. d.)	**35% (10.0)**	**71% (18.5)**	

*no. of rooted/and total no. of cutting (% of rooting)

Results and discussion

In a preliminary experiment grapevine microcuttings (cv. „Chardonnay") were rooted in perlite moisted with sterile tapwater, half-strength MS medium, or with the ammonium-free nutrient solution (see Materials and methods). Microcuttings grown in perlite with MS rapidly become necrotic and died. Plants grown in perlite moisted with ammonium-free nutrient solution remained green, but rooted weakly. When perlite was moisted with tapwater proper rooting and bud germination was observed (data not shown). These observations suggest that MS medium combined with perlite is toxic to grapevine plantlets probably due to its relatively high ammonium concentration (*Murashige & Skoog* 1962) and that low ionic strength (EC: 0.5 mS) promotes root initiation. Thus for further work only sterile tapwater (pH 6.0–6.2) was used to initiate root development.

In a second round of experiments we compared the rooting efficiency of „Sauvignon blanc" and „Börner" microcuttings in water agar (0.6%) and perlite. The frequency of root formation did not differ significantly in these media when air exchange was supported by the use of spongya filters, although the secondary root formation was more intensive in perlite than in agar (data not shown). On the other hand, complete cover of

Table 2 Results of rooting experiments in various media

Variety	Perlite (%)	Grodan rockwool (%)	Pit-pot (%)
„Cabernet franc"	16/16 (100)	16/17 (94)	24/24 (100)
„Chardonnay"	35/35 (100)	13/14 (92)	16/17 (94)
„Riesling"	36/36 (100)	8/8 (100)	10/10 (100)
„Sauvignon blanc"	n. t.*	14/15 (93)	10/10 (100)
„Börner"	12/16 (75)	9/9 (100)	16/16 (100)
„Richter 110"	24/30 (80)	n. t.*	6/10 (60)

*n. t. = not tested

culture vessels with double layers of cling film that did not allow air exchange significantly reduced rooting efficiency. In this case perlite was superior to agar and the differences of rooting efficiency in these two media become significant (*Table 1*). Thus it is basically important to provide regular or permanent air exchange during the rooting process.

Next we have planted single node internodes and tips (*Figure 1*) of *Vitis vinifera* cvs. „Chardonnay", „Cabernet franc", „Riesling" and „Sauvignon blanc", *Vitis riparia x Vitis cinerea* cv. „Börner" and *Vitis berlandieri* x *Vitis rupestris* cv. „Richter 110". As expected, all cuttings of „Chardonnay" (35/35), „Cabernet franc" (16/16) and „Riesling" (36/36) formed well-developed roots within three weeks (*Figure 2*). In the case of cv. „Börner" and „Richter 110" the rooting efficiency was lower (12/16 and 24/30, respectively, *Table 2*). On the four non-rooted „Börner" plants intensive callus growth was observed. This variety forms extremely large leaves *in vitro* that may result in auxin over-production. The same experiments were also carried out with Grodan rockwool (*Figure 3*) and pit-pot blocks moisted with slightly acidified tapwater. Again, the tested cvs. rooted in or nearly 100% within 15–20 days and they initiated shoot growth as well. The results of these experiments are summarized in *Table 2*. For further growth plants were supplied with nutrients as described above. After root formation the lids of jars were gradually opened for acclimatization. Plants rooted in perlite or in Grodan rockwool were transferred into the greenhouse after 4–5 weeks with 100% efficiency since all „Chardonnay" (35/35) and „Riesling" (28/28) plants survived the acclimatization protocol. Four months after the start of experiments they reached approximately 1 m height (*Figure 4*).

Acclimatization of *in vitro* grown plants to the greenhouse or field conditions frequently limits the efficiency of micropropagation techniques. Traditionally whole plants carrying complete root system and 4–5 leaves are used for hardening. To prevent infection the remaining medium should be carefully removed by repeated washing from plants. These plantlets cultured in agar-based media usually have weak, fragile roots that are frequently damaged during this step. To

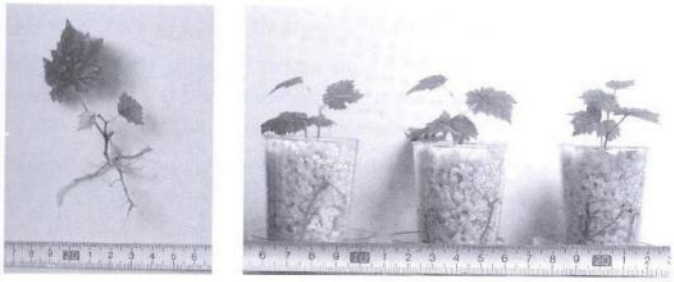

Figure 2 Rooted „Riesling" plantlets. Photos were taken 3 weeks after the start of rooting.

Figure 3 Rooted „Riesling" plantlets. Photos were taken 3 weeks after the start of rooting.

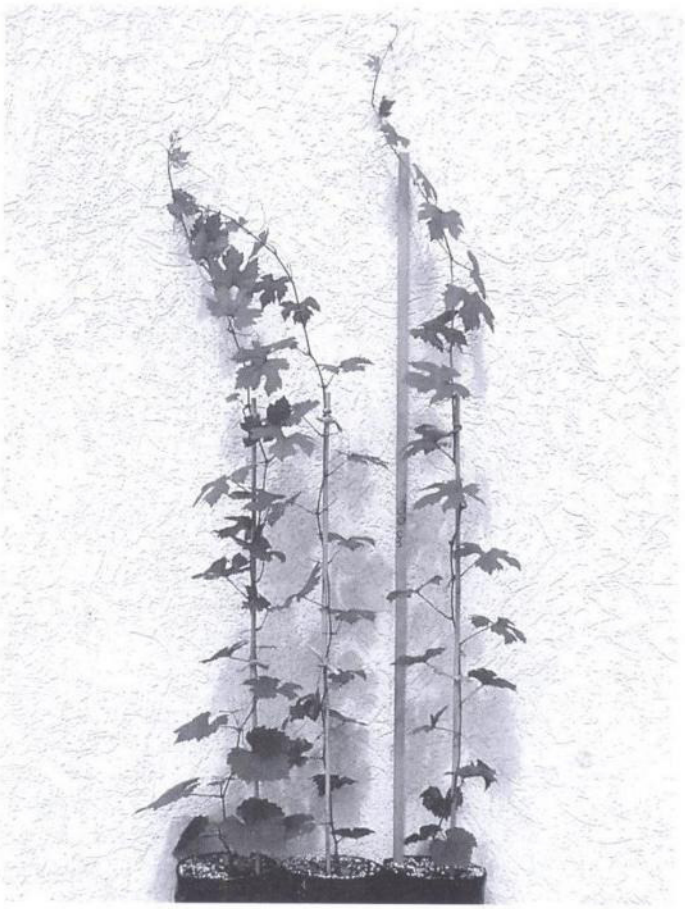

Figure 4 *Vitis vinifera* cv. „Chardonnay" plants have grown over 1 meter after four months.

overcome these difficulties we have used aeriated media combined with inorganic protocols. Photoautotrophic (sugar-free) media have already been widely used for micropropagation of several plants, but few reports have been made for grapevines (*Kozai* et al. 2005). The two-step method described here allows an additional multiplication and acclimatization of *in vitro* plants in a single step resulting 5–6 times more hardened plantlets with strong roots than the traditional technique. The aeriated media used (perlite, rockwool and pit-pot blocks) promoted rapid rooting of microcuttings followed by shoot development without hormones. Additionally, the inorganic conditions used, highly reduce the risk of contamination during the multiplication and acclimatization protocol. Thus this method may greatly increase the efficiency of grapevine micropropagation. A similar technique has been introduced to produce grapevine micrografting but for that case the recovery of plants was only approximately 50% (*Nas & Read* 2003) while for self-rooted plantlets we obtained hardened plants in near to 100% efficiency.

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