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РЕГЕНЕРАЦИЯ ВИНОГРАДНЫХ РАСТЕНИЙ ИЗ КЛЕТОЧНЫХ СУСПЕНЗИЙ И ФОРМИРОВАНИЕ СОЦВЕТИЙ IN VITRO ПОД ВЛИЯНИЕМ РАЗЛИЧНЫХ ВИТАМИНОВ И МИНЕРАЛЬНЫХ ЭЛЕМЕНТОВ ПИТАТЕЛЬНЫХ СМЕСЕЙ

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Эффекты различных концентраций витаминов (тиамин, пиридоксин и никотиновая кислота) и макроэлементов (MS, NN и PG) на развитие глобулярных, сердцевидных и торпедовидных эмбриоидов, проростков с зелеными семядолями и образование побегов из них изучали на виноградной лозе сортов межвидового происхождения Бианка, Подарок Магарача и Интервитис Магарача. Проэмбриогенные каллусы образовывались из эксплантов черешков листьев на твердой полного состава среде NN, с добавлением различных концентраций 2,4-дихлорфеноксиуксусной кислоты (2,4-D) и 6-бензиламинопурина (БА) для каждого культурного сорта и использовались для инициирования клеточных суспензий в жидкой NN-среде, дополненной 1 мг/л 2,4-D и 0.2 мг/л БА. Следующие составы сред были оптимальными для развития различных стадий соматических эмбриоидов и проростков с зелеными семядолями: жидкая NN-среда с добавлением 0.5 мг/л БА - для формирования глобулярных эмбриоидов; жидкая THE-среда с PG макроэлементами, тиамином и пиридоксином в концентрации 5 мг/л каждый и 0.5 мг/л никотиновой кислоты): с добавлением с 0.2 мг/л БА – для развития сердцевидных эмбриодов, с добавкой 0.1 мг/л 1-индол-3-уксусной кислоты (IAA) и 30 мг/л гумата натрия – для развития торпедовидных эмбриоидов и с добавкой 0.5 мг/л гиббереллловой кислоты (GA_3) - для развития проростков с зелеными семядолями. Твердая MS-среда, модифицированная дополнением никотиновой кислоты, пиридоксина и р-аминобензойной кислоты (PAB) в 5 мг/л каждой, 0.5 мг/л - 1 тиамина и 0.5 мг/л БА была наилучшей для развития побегов растений из проростков. Формирование соцветий у растений-регенерантов Подарок Магарача, выращенного на твердой безгормональной среде PG, было вызвано на предварительных этапах добавлением никотиновой кислоты, пиридоксина и PAB в 5 мг/л каждый, 0.5 мг/л 1 тиамина и 0.2 мг/л - 1 БА в жидкой модифицированной HTE-среде для развития проростков с зелеными семядолями, и в твердую модифицированную MS-среду для развития побегов из этих проростков.

Ключевые слова: ВИТИС, КЛЕТОЧНАЯ СУСПЕНЗИЯ, СОМАТИЧЕСКИЕ ЭМБРИОНЫ, РАСТЕНИЯ РЕГЕНЕРАНТЫ, ЦВЕТОК, ВИТАМИНЫ, ИНЕРАЛЬНЫЕ ЭЛЕМЕНТЫ

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GRAPEVINE PLANT REGENERATION FROM CELL SUSPENSIONS AND INFLORESCENCE FORMATION *IN VITRO* UNDER THE INFLUENCE OF DIFFERENT VITAMIN AND MINERAL ELEMENT LEVELS IN NUTRIENT MEDIA

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Effects of different levels of vitamins (thiamine, pyridoxine and nicotinic acid) and macro-elements (MS, NN and PG) on development of globular, heart- and torpedo-stage embryos and plantlets with green cotyledons and on shoot production from them were studied in grapevine interspecific hybrids: cvs. 'Bianca', 'Podarok Magaracha' and 'Intervitis Magaracha'. Pro-embryogenic calli were derived from petiole explants on solid full-strength NN medium supplemented with different levels of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) for each cultivar and used to initiate cell suspensions in liquid NN medium supplemented with 1 mg L^{-1} 2,4-D and 0.2 mg L^{-1} BA. The following formulations of media were optimal for different stages of somatic embryogenesis and for plantlets with green cotyledons development: liquid NN medium supplemented with 0.5 mg L^{-1} BA – for globular embryo formation; liquid HTE medium (with PG macro-elements, thiamine and pyridoxine at 5 mg L^{-1} each and 0.5 mg L^{-1} nicotinic acid): supplemented with 0.2 mg L^{-1} BA – for heart-stage embryo development, supplemented with 0.1 mg L^{-1} indole-3-acetic acid (IAA) and 30 mg L^{-1} sodium humate – for torpedo-stage embryo development and supplemented with 0.5 mg L^{-1} gibberellic acid (GA_3) – for the growth of plantlets with green cotyledons. Solid MS medium modified by the addition of nicotinic acid, pyridoxine and *p*-aminobenzoic acid (PAB) at 5 mg L^{-1} each, 0.5 mg L^{-1} thiamine and 0.5 mg L^{-1} BA was best efficient for shoot production from plantlets. Inflorescence formation in regenerated plants of cv. 'Podarok Magaracha' grown on solid hormone-free PG medium was induced by addition of nicotinic acid, pyridoxine and PAB at 5 mg L^{-1} each, 0.5 mg L^{-1} thiamine and 0.2 mg L^{-1} BA into liquid modified HTE medium for growth of plantlets with green cotyledons and into solid modified MS medium for shoot production from them.

Keywords: VITIS, CELL SUSPENSION, SOMATIC EMBRYO, PLANT REGENERATION, FLOWER, VITAMIN, MINERAL ELEMENT.

Abbreviations

BA 6-benzyladenine; **2,4-D** 2,4-dichlorophenoxyacetic acid; **GA_3** gibberellic acid; **IAA** indole-3-acetic acid; **HTE, M_1MS** and **M_2MS** Zlenko et.al. (2002) media; **MS** Murashige and Skoog (1962) medium; **NN** Nitsch and Nitsch (1969) medium; **PAB** *p*-aminobenzoic acid; **PG** Zlenko et.al. (1995) medium

Introduction

The occurence of chimeric plants can be made lower by marker-assisted selection of genotypes from suspensions of preferably single cells, followed by application of somatic embryogenesis in liquid medium in the presence of a relevant selective factor. Grapevine cell suspensions have successfully been used for obtaining genotypes with fungal resistance (Jayasankar et al. 2000) and for subject to biolistic gene transformation (Kikkert et al. 1996; Striem et al. 2000). Somatic embryos have been used as 'synthetic seeds' by encapsulating in alginate or fluid-drilling gel. Synthetic seed technology requires large numbers of inexpensive, high-quality and synchronously maturing somatic embryos (Gray et al. 1995). Exactly such embryos can be obtained in liquid medium from grapevine cell suspensions (Compton and Gray 1996; Zlenko et al. 2005a).

Effects of 6-benzyladenine (BA) on axillary bud formation and apical dominance of grapevine shoots *in vitro* depend on the levels of vitamins and mineral elements in the media (Zlenko et al. 1995). Solid media with different levels of some mineral elements (e.g., NH_4Cl, KH_2PO_4, $CaCl_2$, $MnSO_4$ and $ZnSO_4$ specify) were optimal for different processes such as: long-term embryogenic callus culture, induction of globular embryo formation, their development into the torpedo-stage embryos and grapevine plant regeneration (Perrin et al. 2001).

Inflorescence formation has been induced in *in vitro* cultures of grapevine explants: one node micro-cuttings with axillary leaves (Martinez et al.1989), tendrils (Srinivasan and Mullins 1978) and shoot tips approximately 1 mm long

(Slenko et al. 2001). Inflorescence and berry formation in one-year-old grapevine regenerated plants after transplant to soil may be induced *in vitro* using nutrient medium of suitable composition. This may be used for checking quality of fruit in transgenic plants.

In this paper we report effects of different concentrations of vitamins (thiamine, pyridoxine and nicotinic acid) and mineral elements (macro-elements) on development of different stages of somatic embryos in liquid media from cell suspensions and subsequent plant regeneration in three grapevine genotypes and on inflorescence formation *in vitro* in regenerated plants of one of them.

Materials and methods

Plant materials

Interspecific hybrids of *Vitis vinifera* L. and a Franco-American hybrids were used: cv. ‘Bianca’ (‘Villard blanc’ × ‘Chasselas Bouvier’), cv. ‘Podarok Magaracha’ [‘Rkatsiteli’ × (‘Mtsvane kakhetinski’ × ‘Sochinski cherny’)] and cv. ‘Intervitis Magaracha’ [(‘Katta Kurgan’ × ‘Shabash krupnoyagodny’) × DRX 100-74-1-5].

Pro-embriogenic cell suspension initiation

Petiole explants excised from *in vitro*-grown plants of the three cultivars were cultured on solid full-strength Nitsch and Nitsch (1969) medium (NN) supplemented with different levels of 2,4-dichlorophenoxyacetic acid (2,4-D) and BA. Specifically (Zlenko and Troshin 1993), 2 mg L^{-1} 2,4-D and 2 mg L^{-1} BA for ‘Bianca’, 2 mg L^{-1} 2,4-D and 1 mg L^{-1} BA for ‘Intervitis Magaracha’ and 0.5 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BA for ‘Podarok Magaracha’. After 91 d in culture, pro-embryogenic calli approximately 200 mg fresh weight were placed

in 100 mL Erlenmeyer flasks containing 20 mL aliquots of liquid full-strength NN medium supplemented with 1 mg L^{-1} 2,4-D and 0.2 mg L^{-1} BA to initiate cell suspensions. The cultures were incubated on a shaker at 60 rpm. After 10 d in culture, suspensions consisting of preferably pro-embryogenic single cells were formed in the three cultivars.

Globular, heart- and torpedo-stage embryo development in liquid media

Pro-embryogenic cell suspensions of the three grapevine cultivars were sub-cultured into full-strength NN liquid medium supplemented with 0.5 mg L^{-1} BA using an inoculum ratio of approximately 1:5 (v:v). Subsequently cell and embryo suspensions were sub-cultured every 21 d into respective fresh media. To achieve large numbers of heart-stage embryos, cell and embryo suspensions of all three cultivars were sub-cultured into liquid HTE medium with 0.2 mg L^{-1} BA (by Zlenko et al. 2005b). HTE medium (heart- and torpedo-stage embryo development, by Zlenko et al. 2002) consists of the following components: PG (plant growth) macro-elements: 308 mg L^{-1} NH_4NO_3, 922 mg L^{-1} KNO_3, 597 mg L^{-1} $MgSO_4$ $7H_2O$, 82 mg L^{-1} KH_2PO_4, 331 mg L^{-1} $CaC1_2$; Fe-EDTA, micro-elements and 0.5 mg L^{-1} nicotinic acid at Murashige and Skoog (1962) medium (MS) concentrations; 20 mg L^{-1} myo-inositol, 5 mg L^{-1} thiamine, 5 mg L^{-1} pyridoxine and 10 g L^{-1} sucrose. Heart-stage embryo (0.3 – 0.8 mm) suspensions of the three cultivars were sub-cultured into liquid HTE medium supplemented with 0.1 mg L^{-1} indole-3-acetic acid (IAA) and 30 mg L^{-1} sodium humate for developing into the torpedo stage. Embryo suspensions of the three cultivars were once more sub-cultured into the above medium to increase the numbers of torpedo-stage embryos.

Experiment I: Germination of torpedo-stage embryos on media containing different vitamin levels (Table I)

Table 1. Effects of vitamin concentrations contained in solid hormone-free modified MS medium on further torpedo-stage embryo development in three grapevine cultivars (Experiment I)

Concentrations (mg L^{-1}) of vitamins in solid hormone-free MS medium modified with 5 mg L^{-1} PAB	Shoot development (%±SE) and other morphological changes in torpedo-stage embryos after 70 d in culture		
	cv. 'Bianca'	cv. 'Intervitis Magaracha'	cv. 'Podarok Magaracha'
MS vitamins: thiamine –0.1, pyridoxine –0.5, nicotinic acid –0.5 (MMS medium)	Overgrowth of brown hypocotyls up to 0.5–1 cm long, roots	Conversion of green hypocotyls into callus, roots	Overgrowth of white embryos up to 0.5–0.8 cm long
TP5 vitamins: thiamine –5.0, pyridoxine –5.0, nicotinic acid –0.5 (M1MS medium)	15 ± 4% shoots, green hypocotyls and green cotyledons up to 0.5 cm long, roots	8 ± 2% shoots, green hypocotyls 1–3 cm long and white cotyledons	Conversion of brown and white hypocotyls 0.3-0.8 cm long and cotyledons into callus, development of secondary torpedo-stage embryos
NP5 vitamins: thiamine –0.5, pyridoxine –5.0, nicotinic acid –5.0 (M2MS medium)	Green hypocotyls 0.4–0.8 cm long and white cotyledons, development of secondary globular embryos	Green hypocotyls 1–2 cm long and white cotyledons, callus	Further development of torpedo-stage embryos is practically arrested, thickening of torpedo-stage embryos

Torpedo-stage embryos of the three grapevine cultivars were removed from liquid HTE medium supplemented with 0.1 mg L^{-1} IAA and 30 mg L^{-1} sodium humate and established on versions of solid hormone-free MS medium modified by addition of 5 mg L^{-1} *p*-aminobenzoic acid (PAB) and different vitamin variants, as reported in Table 1. In experiment (I) 120 torpedo-stage embryos (20 flasks, 6 torpedo-stage embryos per flask) of each cultivar were established on each solid medium. After 70 d in culture, secondary embryo formation and conversion of torpedo-stage embryos into plantlets were assessed and the percentage of plantlets that produced shoots was determined against the total number of torpedo-stage embryos of each cultivar established on each the above indicated solid medium.

Experiment II. Development of somatic embryos, plantlets and plantlet-derived shoots on media with different vitamin and macro-element variants (Table 2)

Table 2. Plant regeneration in the grapevine cultivars ‘Bianca’, ‘Intervitis Magaracha’ and ‘Podarok Magaracha’ as affected by vitamin and mineral element concentrations in liquid media for development of somatic embryos and plantlets with green cotyledons and in solid media for shoot production from plantlets (Experiment II)

Vitamin variants (concentrations, mg L^{-1})	Macro-element variants: MS, NN and PG	Liquid media				Solid media
		Globular embryos (0.5 mg L^{-1} BA; 21 d)	Heart-stage embryos (0.2 mg L^{-1} BA; 21 d)	Torpedo-stage embryos (0.1 mg L^{-1} IAA, 30 mg L^{-1} sodium humate; 21 d)	Development of plantlets (0.5 mg L^{-1} GA_3; 5 d)	Shoot production from plantlets (0.5 mg L^{-1} BA; 50 d)
MS vitamins: thiamine –0.1, pyridoxine –0.5, nicotinic acid –0.5	MS →	+	+	+	+	++
	NN →	++	++	++	++	+
	PG →	++	++	++	++	+
NN vitamins: thiamine –0.5, pyridoxine –0.5, nicotinic acid –5.0	MS →	+	+	+	+	++
	NN →	+++	+	+	+	++
	PG →	+++	+	+	+	++
TP5 vitamins: thiamine –5.0, pyridoxine –5.0, nicotinic acid –0.5	MS →	+	+	+	++	++ (M1MS medium)
	NN →	++	++	++	+++	++
	PG (HTE medium) →	++	+++	+++	+++	++
NP5 vitamins: thiamine –0.5, pyridoxine –5.0, nicotinic acid –5.0	MS →	+	+	+	+	+++ (M2MS medium)
	NN →	+++	+	+	++	++
	PG →	+++	+	+	++	++
P5 vitamins: thiamine –0.5, pyridoxine –5.0, nicotinic acid –0.5	MS →	+	+	+	++	++, roots
	NN →	++	+++	++	++	++, roots
	PG →	++	+++	+++	+++	++, roots

An experiment II was done for every stage of plant regeneration from pro-embryogenic cell suspensions in cvs. ‘Bianca’, ‘Podarok Magaracha’ and ‘Intervitis Magaracha’. Different liquid and solid media with different vitamin variants in different combinations with macro-element variants were used

separately for globular, heart and torpedo-stage embryo development, growth of plantlets with green cotyledons and shoot production from them. The medium versions contained macro-elements, vitamins and growth regulators as reported in Table 2. All the media were completed of Fe-EDTA and micro-elements from MS medium, 20 mg L^{-1} myo-inositol and 10 g L^{-1} sucrose.

Pro-embryogenic cell suspensions of the three grapevine cultivars were initiated in liquid NN medium supplemented with 1 mg L^{-1} 2,4-D and 0.2 mg L^{-1} BA (10 d in culture, see the above sub-heading of pro-embryogenic cell suspension initiation) and sub-cultured into the appropriate media for globular embryo development. This was followed, after 21 d in culture, by successive 21-day sub-culture of embryo suspensions with the medium that proved to be most beneficial for each of the stage of embryo development indicated as symbols (+++), see Table 2. For development of different stages of embryos and plantlets with green cotyledons and hypocotyls each version of liquid medium for each cultivar was in 10 flasks. Finally, after growing plantlets from torpedo-stage embryos in the light for a short time (5 d in culture, by Zlenko et al. 2005b), plantlets approximately 5-8 mm long with green cotyledons and hypocotyls were used for shoot production on the same media, but supplemented with 5 mg L^{-1} PAB and 0.5 mg L^{-1} BA. In the experiment II 100 plantlets (20 flasks, 4 plantlets per flask; and 20 tubes, one plantlet per tube) of each cultivar were sub-cultured from the best for development plantlets liquid media, indicated as symbols (+++), onto each solid medium (Table 2).

For result evaluation of experiment II, formation of globular, heart- and torpedo-stage embryos per flask was assessed using a binocular microscope MBS-6 as from Zlenko et al. (2005a). The number of plantlets approximately 5–8 mm long with green cotyledons and hypocotyls developed from torpedo-stage embryos in liquid medium per flask were counted. The percentage of

plantlets of each cultivar which produced shoots in culture on each version of solid medium was calculated. In order to achieve to a general evaluation of the different media in terms of stimulation of the various morphogenetic stages, the results from the 3 hibrids were pooled and expressed as symbols, i.e., (+++) when the best development was observed in all three cultivars, (++) for intermediate development in all three cultivars or for the case when some of the cultivars did not show reliable differences from the neighboring classes of the evaluation of results, (+) for mediocre development in all three cultivars. Only embryo suspensions and plantlet cultures which showed the best developmental parameters at a given stage in all three cultivars (+++) were sub-cultured to media for a subsequent stage of embryo development or for shoot production.

As for shoot rooting and plant development, shoots produced by plantlets were cut into one-node cuttings bearing one leaf, which were established on a solid medium, formulated as follows: PG macro-elements, half strength MS concentrations for Fe-EDTA and micro-elements, 20 mg L^{-1} myo-inositol, 0.1 mg L^{-1} thiamine, 0.5 mg L^{-1} nicotinic acid, 0.2 mg L^{-1} pyridoxine, 10 g L^{-1} sucrose, 7 g L^{-1} Difco agar and 0.1 mg L^{-1} IAA as growth regulator. If necessary, plants were propagated and transplanted to soil in the greenhouse as described previously (Slenko et al. 2001).

Experiment III: Inflorescence formation in regenerated plants of cv. 'Podarok Magaracha' in vitro (Table 3)

A further experiment was developed in order to investigate both the effects of vitamin variants and *p*-aminobenzoic acid (± PAB) on shoot production from plantlets with green cotyledons, and the subsequent inflorescence formation from regenerated plants developed from single-bud-leaf cuttings were investigated according with the experimental design described in Table 3.

Table 3. Effects of vitamin and p-aminobenzoic acid (PAB) levels added in media on the percentage of shoot production from torpedo-stage embryos of cv. 'Podarok Magaracha' and on subsequent inflorescence formation (%) (Experiment III) (T, thiamine; P, pyridoxine; N, nicotinic acid; SE, standard error)

Conversion of torpedo-stage embryos into plantlets with green cotyledons, 10 d in culture [1]		Shoot production from plantlets, 67 d in culture[2]		Plants with inflorescences (%±SE) [3]
Vitamins (mg L^{-1})	± PAB, (mg L^{-1})	Vitamins (mg L^{-1})	Plantlets with shoots (%±SE)	
TP5 (t–5.0, p–5.0, n–0.5; HTE medium)	0 →	TP5 (t–5.0, p–5.0, n–0.5; M1MS medium)	22 ± 5 →	0
NP5 (t–0.5, p–5.0, n–5.0; modified HTE medium)	0 →	TP5	45 ± 6 →	0
TP5	5 →	TP5	26 ± 5 →	0
NP5	5 →	TP5	53 ± 6 →	0
TP5	0 →	NP5 (t–0.5, p–5.0, n–5.0; M2MS medium)	56 ± 6 →	10 ± 3
NP5	0 →	NP5	18 ± 5 →	25 ± 6
TP5	5 →	NP5	61 ± 7 →	18 ± 5
NP5	5 →	NP5	20 ± 5 →	42 ± 7

[1] Vitamin variants contained in liquid basal and modified HTE media supplemented with 0.2 mg L^{-1} BA and ± PAB.

[2] Vitamin variants + 5 mg L^{-1} PAB contained in solid M1MS and M2MS media supplemented with 0.2 mg L^{-1} BA.

[3] After establishment of single-bud cuttings with one leaf on solid hormone-free PG medium, 91 d in culture.

The initial culture material was a torpedo-stage embryo suspension of cv. 'Podarok Magaracha' developed in liquid HTE medium supplemented with 0.1 mg L^{-1} IAA and 30 mg L^{-1} sodium humate (see sub-heading above). For shoot production in experiment III two hundred plantlets of cv. 'Podarok Magaracha' were sub-cultured from each liquid medium onto each solid medium (50 flasks, 4 plantlets per flask). After that each shoot produced by a plantlet was cut into pieces, and a single-bud cutting with a best developed leaf was taken and established onto solid hormone-free PG medium (one cutting per test tube).

Media and culture conditions

The pH of all media was adjusted to 5.6 with NaOH before autoclaving at 103 kPa for 25 min. Media for development of plantlets with green cotyledons (Experiment II) were supplemented with GA_3 before being autoclaved. Flasks for incubation of cell and embryo suspensions or plantlets contained 20 ml aliquots of liquid media or solid media for culture of torpedo-stage embryos (Experiment I) and plantlets (Experiment II and III) to achieve shoot production. Tubes (200 mm, 22 mm i.d.) contained 6 ml aliquots of solid media (Experiment II and III). Cell and embryo suspensions were incubated on a shaker at 60 rpm in the dark. Incubation of plantlets and plants was in a growth chamber with a 16-h photoperiod under a photon flux density at the culture surface of 55 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Calli, cell and embryo suspensions, plantlets and plants were cultured at 27 ^{0}C.

Statistical analysis

The percentage of shoot production (experiments I, II and III) and inflorescence formation in regenerated plants (experiment III) was calculated by methodic Zlenko et al. (2005b). For that culture flasks with each medium for each cultivar or sub-culture design were numbered and divided into groups each of which contained 12 torpedo-stage embryos (experiment I), or 10 plantlets (experiments II and III), or 10 – 12 single-bud cuttings bearing one leaf (experiment III). The percentage of torpedo stage embryos (experiment I), or plantlets (experiments II and III) which produced shoots, or regenerated plants which formed inflorescences (experiment III) was assessed in each group. This was followed by calculating the average percentage of shoot production or inflorescence formation (± SE, $P < 0.05$).

Results and discussion

Effects of different vitamin levels contained in solid hormone-free modified MS medium on germination of grapevine torpedo-stage embryos (Experiment I)

It has been previously reported that vitamins at increased levels (5 mg L^{-1}) are able to affect morphogenesis in grapevine *in vitro*: nicotinic acid and pyridoxine (NP5 vitamins) inhibite apical domination and induce axillary bud propagation while thiamine and pyridoxine (TP5 vitamins) are beneficial for apical domination (Zlenko et al. 1995). This study showed that, when torpedo-stage embryos (2–3 mm long) were sub-cultured from liquid HTE medium supplemented with 0.1 mg L^{-1} IAA and 30 mg L^{-1} sodium humate onto solid hormone-free modified MS medium with different vitamin variants, different morphogenetic effects are promoted as well (Table 1). Plantlets of 'Bianca' and 'Intervitis Magaracha' had green and white cotyledons, respectively, on the M1MS medium with increased levels of thiamine and pyridoxine (5 mg L^{-1}; TP5 vitamins) and this effect was interrelated with a higher percentage of shoot production in 'Bianca' (15±4%) than in 'Intervitis Magaracha' (8±2%). The ability of somatic embryos to germinate and regenerate into plants on hormone-free medium is genetically determined (Salunkhe et al. 1997; Zlenko et al. 2002, 2005b). In our experiment, green hypocotyls and shoot production were not observed on hormone-free media in cv. 'Podarok Magaracha' in contrast to cvs. 'Bianca' and 'Intervitis Magaracha'. Brown and white hypocotyls and cotyledons of cv. 'Podarok Magaracha' converted to callus on the M1MS medium with the TP5 vitamins. This was accompanied by production of secondary torpedo-stage embryos. On the countrary, secondary globular embryos were formed in 'Bianca' only on the M2MS medium with increased levels of nicotinic acid and pyridoxine (5 mg L^{-1}; NP5 vitamins). Secondary globular or torpedo-stage embryos were not found on any vitamin variant of hormone-free medium in 'Intervitis Magaracha'. When the NP5 vitamins was

used, plantlets with green hypocotyls and white cotyledons developed in ‘Bianca’ and ‘Intervitis Magaracha’. Higher levels of the vitamins tested (TP5 and NP5) had more pronounced morphogenesis effect relative to thouse of the MS medium formulation vitamins.

Grapevine plant regeneration from pro-embryogenic cell suspensions as affected by different medium formulations (Experiment II)

Table 2 presents the results of the experiment planned with the aim to adjust optimal concentrations of vitamins (nicotinic acid, pyridoxine and thiamine) and macro-elements (MS, NN and PG) in media for the development of different stages of somatic embryos, the growth of plantlets with green cotyledons and shoot production from the plantlets, pooling the results obtained from the 3 cultivars. Macroelements and vitamins of liquid NN medium (which contains 5 mg L^{-1} nicotinic acid) were optimal for the formation of globular embryos from cell suspensions. The best results as concerns the heart- and torpedo-stage embryo development (Figure 1) and the growth of plantlets were achieved by

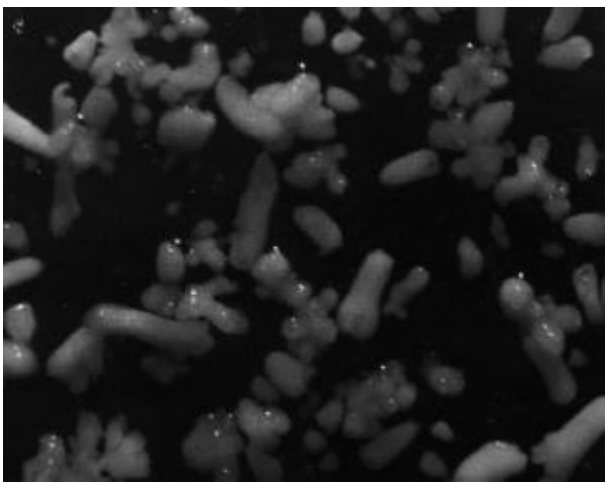

Figure 1.
Globular, heart- and torpedo-stage embryos of cv. ‘Podarok Magaracha’ developed in liquid HTE medium supplemented with 0.1 mg L^{-1} IAA and 30 mg L^{-1} sodium humate after 21 d in culture (Experiment II).

using HTE medium containing the TP5 vitamins with high levels of thiamine and pyridoxine (5 mg L^{-1}) and 0.5 mg L^{-1} nicotinic acid (Table 2). Formation of

abnormal torpedo-stage embryos in grapevine is due to their high levels of endogenous polyamines. This may be overcome by using polyamine synthesis inhibitors (Faure et al. 1991). Putrescine synthesis in grapevine cell suspensions was completely inhibited when NH_4^+ was absent in the medium (Triantaphylidès et al. 1993). Hormone-free modified MS medium lacking the NH_4^+ ions but supplemented with caseine hydrolysate has been found to promote maturation of insufficiently developed zygotic embryos in grapevine (Yamashita et al. 1995). For development of heart- and torpedo-stage embryos HTE medium with PG macro-elements was best efficient due to its lower level of the ions NH_4^+ in comparison with MS and NN media, which may be led to a reduced synthesis of endogenous polyamines in these embryos (Table 2). Modified solid MS medium with a quarter of full levels versus to the full levels of NH_4NO_3 and KNO_3 and increased level of sucrose led to improved numbers of high quality cotyledonary somatic embryos in grapevine (Compton and Gray 1996). The lack of the NH_4^+ ions in solid medium was beneficial for development of grapevine torpedo-stage embryos (Perrin et al. 2001).

MS medium, modified with the addition of high levels of nicotinic acid and pyridoxine (5 mg L^{-1}), 0.5 mg L^{-1} thiamine (NP5 vitamins) and 5 mg L^{-1} PAB (M2MS medium; Table 2), was best efficient for shoot production from the plantlets in all three cultivars (Figure 2a). This may be implicated as a possible cause of the need for high levels of the NH_4^+ ions of MS macro-elements relative to the NN and PG macro-elements for polyamine synthesis and shoot production (Table 2). Ethylene and polyamine synthesis are interrelated: ethylene production decreases when polyamine synthesis increases (Roberts et al. 1984). Grape plants grew better *in vitro* on a medium containing polyamine putrescine (Martin-Tanguy and Carre 1993).

Good shoot production was observed on the medium with an increased level of pyridoxine (5 mg L^{-1}; P5 vitamin version) in ‘Intervitis Magaracha’ while in ‘Bianca’ and ‘Podarok Magaracha’ better shoot production was only on M2MS

medium with increased levels of both pyridoxine and nicotinic acid (NP5). The three cultivars showed a more intense root formation on the solid modified MS medium with an increased level of pyridoxine alone (P5).

Figure 2A.
Regenerated grapevine plants from cell suspensions via somatic embryogenesis in liquid media. (A) Shoot production from plantlets with green cotyledons after 70 d in culture on solid M2MS + 0.5 mg L^{-1} BA medium, from left tube to right tube: cvs. ‘Bianca’, ‘Intervitis Magaracha’ and ‘Podarok Magaracha’ (Experiment II).

Effects of vitamin and PAB levels in culture media on shoot production and subsequent inflorescence formation in regenerated plants of cv. ‘Podarok Magaracha’ (Experiment III)

Cultivar ‘Podarok Magaracha’ differs from cvs. ‘Bianca’ and ‘Intervitis Magaracha’ not only in intense development of secondary embryos and in the lack of shoot production from torpedo-stage embryos cultured on solid hormone-free M1MS medium (Table 1) and reguired medium with BA for that (Zlenko et al. 2002). Another distinguishing feature of this cultivar is its capacity for inflorescence formation in regenerated plants (Table 3).

Regenerated shoots of cv. ‘Podarok Magaracha’ were cut into single-bud cuttings bearing one leaf which were established on hormone-free PG medium for the growth of plants and in some of them inflorescence formation was observed. Inflorescences were formed in 10–42% of plants grown on hormone free PG medium from the single-bud cuttings bearing one leaf taken from shoots developed from plantlets with green cotyledons on solid M2MS medium containing NP5 vitamins + 0.2 mg L^{-1} BA (Table 3). This may be attributed to the fact that, in grapevine, cytokinin is able to induce inflorescence formation *in vitro* by making anlagen arising from terminal or axillary bud apices, undergo intense repeated branching (Srinivasan and Mullins 1978). Higher levels of both nicotinic acid and pyridoxine (5 mg L^{-1}) in NP5 vitamins enhanced the effect of BA with refer to reduction in apical domination and to improve lateral shoot formation (Zlenko et al. 1995).

When liquid HTE medium contained TP5 vitamins + 0.2 mg L^{-1} BA + 5 mg L^{-1} PAB was applied for germination of torpedo-stage embryos and their conversion into plantlets with green cotyledons (Table 3), solid M2MS medium with NP5 vitamins + 0.2 mg L^{-1} BA was best efficient for shoot production (61 ± 7%) from plantlets. Conversely, when the NP5 vitamins was used for the growth of plantlets in liquid modified HTE medium, shoot production was good (53 ± 6%) on solid M1MS + 0.2 mg L^{-1} BA medium containing the TP5 vitamins relative to the other subculture designs. Subculture designs that envisaged the presence of TP5 vitamins in liquid HTE medium (with and without PAB supplementation) for growing of plantlets and NP5 vitamins in solid M2MS medium for shoot production from these plantlets enabled the highest levels of shoot production from plantlets (61 ± 7% and 56 ± 6%, respectively) but were not beneficial for subsequently inflorescence formation in regenerating plants (18 ± 5% and 10 ± 3%, respectively).

The percentage of regenerating plants of cv. ‘Podarok Magaracha’ which formed inflorescences *in vitro* on hormone free PG medium was increased (42 ±

7%; Figure 2b) by the application of liquid modified HTE medium containing NP5 vitamins + 0.2 mg L^{-1} BA + 5 mg L^{-1} PAB for the growth of plantlets, followed by their sub-culturing onto solid M2MS medium with the NP5 vitamin variant + 0.2 mg L^{-1} BA for shoot production from plantlets.

Figure 2B.
Regenerated grapevine plants from cell suspensions via somatic embryogenesis in liquid media. (B) Inflorescence formation in a regenerated plant of cv. 'Podarok Magaracha' developed from one-node cutting with leaf after 91 d in culture on solid PG medium (Experiment III).

Conclusions

The main results of the present study can be summarized as follows:

1. Different levels of vitamins (MS, TP5 and NP5 variants; Table 1) added into hormone-free (MS + 5 mg L^{-1} PAB) medium induced different morphological changes of torpedo-stage embryos in each cultivar under study: formation of secondary embryos, their conversion into plantlets with green hypocotyls and cotyledons and shoot production from these plantlets.

2. An increased level of nicotinic acid (5 mg L^{-1}) in vitamins of liquid NN medium enhanced the effect of BA (0.5 mg L^{-1}) on globular embryo formation from cell suspensions (Table 2).

3. Increased levels of thiamine and pyridoxine (5 mg L^{-1}) were beneficial for development of heart- and torpedo-stage embryos, as well as plantlets with green cotyledons.

4. An increased level of pyridoxine alone (5 mg L^{-1}) led to an intense root development in plants regenerated from plantlets.

5. Nicotinic acid and pyridoxine improved the percentage of shoot production from plantlets that had been grown in liquid medium, containing thiamine and pyridoxine (at 5 mg L^{-1} each) and 0.5 mg L^{-1} GA_3.

6. Macro-elements of liquid NN medium for globular embryo formation from cell suspensions and macro-elements of liquid HTE medium [containing reduced levels of NH_4NO_3 (5.4 fold), KNO_3 (2.1 fold) and KH_2PO_4 (2.1 fold) but 1.6 fold higher level of $MgSO_4$ $7H_2O$ relative to the MS macro-element formulation and the same level of $CaC1_2$] for development of heart- and torpedo-stage embryos and the growth of plantlets with green cotyledons in combination with definite levels of growth regulators and vitamins were best efficient for further shoot production from plantlets established on solid modified MS medium (M2MS) supplemented with 0.5 mg L^{-1} BA.

7. In the cv. ‘Podarok Magaracha’ the highest levels of shoot production from plantlets with green cotyledons and subsequent inflorescence formation were achieved by different vitamin variants (TP5 and NP5) in liquid medium during conversion of torpedo-stage embryos into plantlets and in solid medium onto which they were sub-cultured (Table 3).

8. Increased levels of nicotinic acid and pyridoxine (at 5 mg L^{-1} each) and *p*-aminobenzoic acid at 5 mg L^{-1} with a low level of BA (0.2 mg L^{-1}) et the steps of the growth of plantlets in liquid modified HTE medium and shoot production from them on solid M2MS + 0.2 mg L^{-1} BA medium led to ***in vitro*** inflorescence

formation in several regenerated plants (42 ± 7%) of cv. ‘Podarok Magaracha’ which had been grown from single-bud-leaf cuttings of the above shoots on hormone-free PG medium.

In vitro both inflorescence and berry formation is possible in grapevine (Martinez et al. 1989). Early inflorescence and berry formation in different grapevine cultivars may be induced *in vitro* using medium composition, and quality of fruit of new grape genotypes can be checked in culture flasks or in one-year-old regenerated plants growing in green house.

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