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OPTIMIZATION OF MEDIUM FOR GRAPEVINE PLANT DEVELOPMENT BY APPLYING A MATHEMATICAL DESIGN OF EXPERIMENT

Зленко Валерий Анатольевич
к. с.-х. н.

Котиков Илья Викторович
старший научный сотрудник

Волынкин Владимир Александрович
д. с.-х. н.

Институт винограда и вина «Магарач», Ялта, Автономная Республика Крым

Трошин Леонид Петрович
д. б. н., профессор
Кубанский государственный аграрный университет, Краснодар, Россия

Two methods were developed, one to assess experimental results by reducing a number of developmental parameters to the overall quality, leading to the overall quality criterion, and the other to optimize processes affected by numerous interacting factors, *in-vitro* plant development in this case, by applying a mathematical design of experiment. Single-bud cuttings with one leaf of two *Vitis vinifera* L. genotypes were excised from the central part of two-month-old *in-vitro* grown plants and used as explants. The explants were established on bridges of filtering paper in liquid media and on solid media. Eighteen modifications of these media contained five macro-elements, each at three concentrations, and the distribution of these macro-element concentrations followed the law of random numbers (Experiment I). Parameters characterizing arm, leaf and root development of two-month-old plants of each study genotype established on each liquid or solid medium were reduced to the overall quality of plant development. Since the study genotypes differed in the ability to grow on liquid and solid media with different macro-element concentrations and also in the ability to utilize these macro-elements, the dependence of *in-vitro* plant development on macro-element concentrations was described by different regression equations. That is why the regression equation describing the average outcome of plant development in the two genotypes on liquid and solid media was not as significant and the description of the process was not as adequate (determined) as regression equations calculated for each process. The regression equation which describes the dependence of the average outcome of plant development on macro-element concentrations in Experiment I is as follows: $y_5 = 0.027 + 0.116x_2^2 + 0.109x_2x_4 + 0.106x_2x_3 + 0.114x_4$.

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ОПТИМИЗАЦИЯ СРЕДЫ ДЛЯ РАЗВИТИЯ ВИНОГРАДНЫХ РАСТЕНИЙ *IN VITRO* С ПРИМЕНЕНИЕМ МАТЕМАТИЧЕСКОГО ПЛАНИРОВАНИЯ ЭКСПЕРИМЕНТА

Zlenko Valeriy Anatolyevich
Cand. Agr. Sci.

Kotikov Ilia Viktorovich
senior scientific employee

Volynkin Vladimir Aleksandrovich
Dr. Agr. Sci.

Institut of Vine and Wine "Magarach", 31, Kirov St., 98600 Yalta, Crimea, Ukraine

Troshin Leonid Petrovich
Dr. Sci. Biol., professor
Kuban State Agrarian University, Krasnodar, Russia

С целью оптимизации питательной среды для развития растений винограда в культуре *in vitro* с применением математического планирования эксперимента разработаны два метода: 1) один из них для оценки результатов эксперимента – сведения многих параметров развития растений к одному параметру – критерию качества и 2) другой метод для оптимизации процесса (развития растений), зависящего от многих взаимодействующих факторов – концентраций макроэлементов в среде. Одноглазковые черенки с листом двух генотипов *Vitis vinifera L.*, взятые из центральной части двухмесячных растений, выросших *in vitro*, использовали в качестве эксплантов (для высадки на питательную среду). Экспланты были высажены на мостики из фильтровальной бумаги в жидких средах, а также на твердые среды (с агаром). В 18 вариантах – модификациях этих сред, содержащих пять макроэлементов (каждый из которых в трех концентрациях), концентрации макроэлементов были распределены (в каждом варианте среды) по закону случайных чисел (эксперимент I). Параметры, характеризующие развитие побегов, листьев и корней у двухмесячных растений каждого изучаемого генотипа на каждом варианте жидкой или твердой среды были сведены к одному параметру – критерию качества развития растений. Изучаемые генотипы различались по способности к росту на жидких или твердых средах, содержащих различные концентрации макроэлементов и также по способности к поглощению этих макроэлементов, что позволило описать уравнениями регрессии развитие растений в зависимости от концентраций макроэлементов в среде, ее консистенции (жидкая или твердая) и

Stepwise calculation of macro-element concentrations to optimize *in-vitro* plant development was done based on macro-element concentrations of the initial medium of Experiment I as starting points since that medium was best efficient for this purpose by using regression equation (y_5) and algorithms of multiple curvilinear stepwise regression according to the Box-Wilson method of steepest ascent. Experiment II was undertaken where macro-element concentrations ('steps') were calculated in a stepwise manner to optimize *in-vitro* plant development of the two *V. vinifera* genotypes and the rootstock 'Kober 5BB'. This led to a liquid medium and a solid one which enabled a better plant development in the three genotypes relative to the use of controls: media with ½ MS macro-elements and the initial medium whose macro-element concentrations entered as starting points to calculate 'steps' for optimization of *in-vitro* plant development. The optimized medium contained macro-elements: 318 mg l^{-1} NH_4NO_3 (x_1), 1188 mg l^{-1} KNO_3 (x_2), 370 mg l^{-1} $MgSO_4$ $7H_20$ (x_3) (MS), 370 mg l^{-1} KH_2PO_4 (x_4), 331 mg l^{-1} $CaCl_2$ (x_5) (MS), and other substances at optimum concentrations adjusted earlier: ¼ MS Fe-EDTA, ¼ MS micro-elements, 20 mg l^{-1} myo-inositol, 0.1 mg l^{-1} thiamine (MS), 0.5 mg l^{-1} nicotinic acid (MS), 0.2 mg l^{-1} pyridoxine, 2 mg l^{-1} glycine (MS), 0.1 mg l^{-1} indole-3-acetic acid, 10 g l^{-1} sucrose and, only for solid media, 7 g l^{-1} Difco agar. The optimized medium may be used for propagation of virus-free plants, valuable clones and grapevine genotypes created by gene engineering. The mathematical design of experiment reported in this paper which enables stepwise optimization of *in-vitro* plant development may be used both in agriculture and in the food industry

Keywords: GRAPEVINE, *IN VITRO*, MATHEMATICAL DESIGN OF EXPERIMENT, MINERAL ELEMENT, OPTIMIZATION OF A PROCESS, PLANT DEVELOPMENT, PROPAGATION, *VITIS*.

генотипа. Поэтому уравнение регрессии, описывающее усредненное значение развития растений у двух генотипов на жидкой и твердой средах не было так значимо и описание процесса не было так адекватно (детерминировано), как уравнения регрессии, рассчитанные для каждого из процессов отдельно. Уравнение регрессии, которое описывает зависимость среднего значения развития растений от концентраций макроэлементов в эксперименте I, является следующим: $y_5 = 0.027 + 0.116x_2^2 + 0.109x_2x_4 + 0.106x_2x_3 + 0.114x_4$. Пошаговый расчет концентраций макроэлементов для оптимизации развития растений *in vitro* был дан на базе концентраций макроэлементов в исходной среде эксперимента I, которая была наиболее оптимальной для данного процесса и из которой начинался пошаговый расчет концентраций макроэлементов в сторону оптимизации процесса с применением уравнения регрессии (y_5) и алгоритмов множественной нелинейной пошаговой регрессии по методу Box-Wilson пошагового восхождения. В эксперименте II были взяты концентрации макроэлементов («шаги»), рассчитанные в сторону оптимизации процессов (пошагового восхождения) развития растений *in vitro* двух генотипов *V. vinifera L.* и подвоя Кобер 5ББ. В результате этого эксперимента II были подобраны жидкие и твердые среды, которые способствовали лучшему развитию растений у трех генотипов по сравнению с контрольной средой, содержащей ½ макроэлементов среды MS и исходной средой, из которой начинали пошаговый расчет концентраций макроэлементов в сторону оптимизации процессов развития растений *in vitro*. Оптимизированная среда может быть использована для размножения свободных от вирусов растений ценных клонов и сортов, а также созданных методами генной инженерии генотипов винограда

Ключевые слова: ВИНОГРАД, *IN VITRO*, МАТЕМАТИЧЕСКИЙ ПРОЕКТ ЭКСПЕРИМЕНТА, МИНЕРАЛЬНЫЙ ЭЛЕМЕНТ, ОПТИМИЗАЦИЯ ПРОЦЕССА, РАЗВИТИЕ РАСТЕНИЯ, РАСПРОСТРАНЕНИЕ *ВИТИС*.

Introduction

Grapevine is attacked by viruses, virus-like organisms and mycoplasmas [1], and also by bacteria and fungi which spread through mature canes during propagation of planting material [2]. Plants propagated under sterile conditions *in vitro* are free from fungi, bacteria and other pests. Heat therapy combined with meristem culture enables elimination of nepoviruses and the majority of closteroviruses [3]. In order to produce quality wines, both reputed and newly-bred genotypes have to undergo phytosanitary and clonal selection [4]. *In vitro*

methods allow both rapid propagation of virus-free individual plants and clones and their introduction to any grape-growing region without quarantine restrictions.

Grapevine genotypes differ in the ability to uptake ions from the soil and in the need for these ions: optimum plant development is promoted in different rootstocks and varieties of grapevine by different concentrations of ions in plants, which may be expressed in terms of the efficiency coefficient of each ion [5]. Media with Knop macro-elements were used in the initial research into grapevine propagation [6, 7]. Onward, Galzy [8] tried Knop macro-element formula which was higher in K^+ (10.9 M) and NH_4^+ (2 M). Different levels of Murashige and Skoog [9] (MS) macro-elements were used for grapevine propagation on solid media, such as full-strength MS macro-elements [10, 11], ½ MS macro-elements [12, 13], and ¼ MS macro-elements were applied for growing plants on bridges of filtering paper in liquid medium [14]. Grapevine plants were also propagated *in vitro* on media with macro-elements at concentrations optimized by means of a mathematical design of experiment [15, 16].

The objective of this research was to achieve an optimized medium for plant development *in vitro* for obtaining plants with good vigor and quality and capable to adapt well to *in-vivo* conditions in the greenhouse or in the open ground. Growing viable plants *in vitro* is an important prerequisite for accelerated propagation of virus-free valuable clones of grafted varieties and rootstocks and also transgenic plants with new economical characters.

Materials and methods

Plant materials

The experiments were done using the following grapevine genotypes: the rootstock ***Riparia* × *Rupestris Kober 5BB*** and two cultivars - intraspecific crosses of *Vitis vinifera L.* released by the Institute for Vine and Wine ‘Magarach’: ‘Zhemchug Magaracha’ and the seedless variety ‘Sverkhrannii bessemiannyi’ .

Obtaining initial explants for Experiments I and II and culture conditions

Plants were grown *in vitro* from arm tips 0.5-0.8 mm long following the protocol of Slenko et al. [16]. Single-bud cuttings with one leaf of cvs. ‘Sverkhrannii bessemiannyi’ and ‘Zhemchug Magaracha’ (Experiment I) and also of ‘Kober 5BB’ (Experiment II) were excised from the central part (2nd to

5th nodes) of two-month-old *in-vitro* grown plants and used as explants. The explants were established on bridges of filtering paper in liquid media and on solid media gellified with Difco agar. Before autoclaving at 103 kPa for 25 min, pH of the media was adjusted to 5.6-5.8 with NaOH. The plants were grown in tubes 200 mm long and 22 mm in diameter which contained 7-ml aliquots of relevant liquid or solid medium. The cultures were incubated at 27^0C with 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.

Experiment I with an aim to achieve regression equations describing the dependence of plant development in two cultivars on macro-element concentrations of the liquid and solid media

Modifications of liquid and solid media differed in macro-element concentrations (Tables I and II) but contained the same concentrations of other components that had been used earlier [16]: ¼ Fe-EDTA and ¼ micro-elements of Murashige and Skoog [9] medium (MS), 20 mg l^{-1} myo-inositol, 0.1 mg l^{-1} thiamine (MS), 0.5 mg l^{-1} nicotinic acid (MS), 0.2 mg l^{-1} pyridoxine, 2 mg l^{-1} glycine (MS), 0.1 mg l^{-1} indole-3-acetic acid (IAA), 10 g l^{-1} sucrose and, only for solid media, 7 g l^{-1} Difco agar. In this experiment, different macro-element concentrations were chosen (Table I) based on the previous results [16] and unpublished data (not shown), and the distribution of those concentrations in 18 modifications of liquid and solid media followed the law of random numbers.

Table I. - Meaning of factors (macro-elements) expressed as natural and coded variables in Experiment I

Factors (variables)	Highest level		Intermediate level		Lowest level	
	In natural units, mg l^{-1}	In the coded form	In natural units, mg l^{-1}	In the coded form	In natural units, mg l^{-1}	In the coded form
(x_1) NH_4NO_3	318	1	212	0	106	-1
(x_2) KNO_3	903	2	452	0	226	-1
(x_3) $MgSO_4$ $7H_2O$	370(MS)	0.85	233	0	72	-1
(x_4) KH_2PO_4	230	0.5	170(MS)	0	51	-1
(x_5) $CaCl_2$	436	2	366	0	331 (MS)	-1

Table II presents these media in the ascending order of macro-element concentrations.

Table II. - Design of Experiment I using variables in the coded form (Table I) and results pertaining to plant growth of two grapevine cultivars on bridges of filtering paper in liquid media and on solid media (expressed as units of the overall quality criterion). The variation coefficient (*V*) for characters of growing plants of each cultivar on each modified medium is not more than 31% ($P < 0.05$).

Medium coder	Meaning of factors as variables in the coded form					Plant growth expressed as units of the overall quality criterion after 63 d in culture on liquid and solid modified media					
	NH_4NO_3 (x_1)	KNO_3 (x_2)	$MgSO_4$ $7H_20$ (x_3)	KH_2PO_4 (x_4)	$CaCl_2$ (x_5)	'Sverhrannii bessemiannyi'		'Zhemchug Magaracha'		Average value for the two cultivars	
						Liquid media	Solid media	Liquid media	Solid media	Experimental	Theoretical by equation (y_5)
1.	-1	-1	0	0.5	0	0.017	0.083	0.022	0.105	0.057	0.132
2.	-1	-1	0.85	0	-1	0.022	0.123	0.179	0.222	0.137	0.017
3.	-1	0	-1	0	2	0.012	0.001	0.004	0.005	0.006	0.017
4.	-1	0	-1	0.5	-1	0.103	0.346	0.130	0.057	0.159	0.132
5.	-1	2	-1	0	0	0.165	0.279	0.014	0.081	0.135	0.017
6.	-1	2	0.85	-1	2	0.940	0.550	0.865	0.377	0.683	0.897
7.	0	-1	0	0.5	2	0.009	0.079	0.069	0.014	0.043	0.055
8.	0	-1	0.85	-1	0	0.029	0.172	0.025	0.014	0.060	0.048
9.	0	-1	0.85	0.5	-1	0.017	0.012	0.073	0.105	0.052	0.055
10.	0	0	0	-1	2	0.080	0.008	0.113	0.018	0.055	0.048
11.	0	2	-1	-1	0	0.152	0.120	0.026	0.110	0.102	0.048
12.	0	2	0	-1	0	0.176	0.570	0.017	0.047	0.203	0.048
13.	1	-1	0	-1	2	0.014	0.004	0.026	0.055	0.025	0.060
14.	1	0	-1	0	-1	0.006	0.222	0.236	0.068	0.133	0.210
15.	1	0	0.85	0	2	0.097	0.171	0.209	0.036	0.128	0.210
16.	1	2	0	-1	-1	0.217	0.076	0.160	0.196	0.162	0.060
17.	1	2	0	0	0	0.311	0.098	0.039	0.242	0.173	0.210
18.	1	2	0	0.5	-1	0.990	0.920	0.921	1.000	0.958	0.939

The explants of cvs. ‘Sverkhrannii bessemiannyi’ and ‘Zhemchug Magaracha’ were established in media with different macro-element concentrations (Table II) and cultured for 63 days after which plant development was assessed. Protocols to assess the results obtained and the use of the mathematical design of experiment are described in detail in the relevant subsectors.

As a result, regression equations were raised describing the dependence of plant development in liquid and solid media on macro-element concentrations both for each genotype on an individual basis and for the average outcome of plant development in the two genotypes on these media (Table III).

Table III. - Improvement of statistical characteristics of regression equation (y_5) during sequential selection of equation members describing overall quality criteria of plant development as a function of concentration of substances in modified media (Tables I and II).

Equation member	Coefficient of regression	Level of significance	Coefficient of determination	Standard error
x_2^2	0.116	0.980	0.264	0.197
x_2x_4	0.109	0.988	0.374	0.182
x_2x_3	0.106	0.995	0.493	0.170
x_4	0.114	0.998	0.581	0.163
The free member = 0.027				

Experiment II with an aim to calculate macro-element concentrations in a stepwise manner by using regression equation for *optimization of in vitro plant development*

Macro-element concentrations were calculated in a stepwise manner by using regression equations to optimize plant development in each genotype of Experiment I on relevant liquid or solid media (voluminous data not shown) and also to improve the average outcome of plant development in the two genotypes on these media (Tables IV and V).

Table IV. - Macro-element levels in liquid and solid media optimized based on regression equation (y_5) with a view to improve plant growth in the two grapevine cultivars. In addition to the macro-elements indicated in this Table, each medium versions contained [16]: ¼ Fe-EDTA and ¼ micro-elements of MS medium, 20 mg l^{-1} myo-inositol, 0.1 mg l^{-1} thiamine, 0.2 mg l^{-1} pyridoxine, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} IAA, 10 g l^{-1} sucrose and 7g l^{-1} Difco agar only in solid media. The pH of the media was 5.6–5.8.

Medium code	Levels of macro-elements (mg l^{-1}) in liquid and solid media for plant growth				
	NH_4NO_3 (x_1)	KNO_3 (x_2)	$MgSO_4\,7H_2O$ (x_3)	KH_2PO_4 (x_4)	$CaCl_2$ (x_5)
½MMS	825	950	185	85	166
18 of Table II	318	903	233	230	331
1opt.y_5	318	1067	312	312	331
2opt.y_5	318	1188	370	370	331
3opt.y_5	318	1446	494	482	331

Table V. - Plant development ($P < 0.05$) in three grapevine genotypes after 56 days in culture on bridges of filtering paper in liquid media and on solid media containing macro-elements at levels calculated by using the regression equation (y_5) and other substances indicated in Table IV.

Medium code and consistence: liquid (L) or solid (S)	Plant development in three grapevine genotypes on liquid and solid media of Table IV											
	cv. «Sverkhrannii bessemiannyi»				cv. «Zhemchug Magaracha»				Rootstock «Kober 5BB»			
	Arm length, cm	Arm quality, points	Root length, cm	Root quality, points	Arm length, cm	Arm quality, points	Root length, cm	Root quality, points	Arm length, cm	Arm quality, points	Root length, cm	Root quality, points
MMS, L	6.7±1.0	1.8±0.7	4.6±0.7	1.6±0.5	5.1±0.7	1.6±0.4	4.2±0.6	1.0±0.0	5.3±0.8	2.4±0.5	3.7±0.7	1.5±0.5
-//-, S	5.9±0.8	2.0±0.6	5.8±0.9	3.0±0.6	4.9±0.7	2.1±0.8	3.8±1.0	2.8±0.7	4.2±0.6	1.9±0.6	4.8±0.7	2.8±0.8
18 of Table II, L	7.7±1.0	3.2±0.7	5.9±0.6	2.8±0.9	7.2±0.9	2.8±0.6	5.5±0.7	2.5±0.7	6.5±0.9	3.5±0.7	5.0±0.8	3.5±0.8
-//-, S	7.5±1.0	4.2±0.7	9.0±1.3	3.3±0.7	6.8±0.7	3.1±0.7	6.5±0.5	3.5±0.8	7.4±0.9	3.3±0.5	6.9±0.9	3.5±0.6
1 opt. y_5, L	9.1±1.3	3.2±0.8	5.8±0.7	3.6±0.8	6.0±0.7	3.0±0.7	7.3±0.7	3.2±0.8	6.8±0.9	4.0±0.5	5.3±0.8	3.5±0.7
-//-, S	6.6±0.9	3.5±0.8	8.7±1.2	3.7±0.7	6.3±0.6	3.3±0.5	6.6±0.9	4.0±0.7	5.1±0.7	3.9±0.6	5.4±0.5	3.4±0.5
2 opt. y_5, L	9.4±1.4	4.6±0.4	6.6±0.8	4.3±0.7	8.8±1.2	4.8±0.2	8.1±0.8	4.9±0.1	7.6±1.1	4.9±0.1	6.6±1.0	4.0±0.6
-//-, S	8.1±1.1	5.0±0.0	9.4±1.3	4.3±0.6	7.6±1.0	4.1±0.6	9.0±1.0	5.0±0.0	8.1±1.2	4.2±0.7	7.5±1.0	4.4±0.5
3 opt. y_5, L	8.8±1.1	4.0±0.8	6.0±0.7	3.8±0.8	8.0±1.0	4.4±0.5	7.0±0.8	4.1±0.4	7.8±1.1	4.2±0.5	6.0±0.9	3.6±0.7
-//-, S	7.5±0.9	4.4±0.5	8.7±1.2	3.9±0.7	6.7±0.8	3.6±0.7	7.4±0.8	3.5±0.7	6.9±0.8	3.7±0.5	6.3±0.7	3.8±0.6

To prove, in a significant manner, the efficiency of the protocol with the use of our mathematical design of experiment II (Tables IV and V), explants were also cultured on control media with ½ MS macro-elements and macro-elements of the initial medium of Experiment I which was best efficient for plant development (Tables I and II), enabling the use of its macro-element concentrations as starting points for stepwise calculation of macro-element concentrations to optimize *in-vitro* plant development. Table IV shows modified media with macro-element concentrations: ½ MS, 18 of Table II and calculated in a stepwise manner to optimize plant development in the study genotypes and also concentrations of other components [16] as used in the first experiment. In this experiment II, single-bud cuttings with one leaf of cvs. 'Sverkhrannii bessemiannyi' and 'Zhemchug Magaracha' and also of the rootstock 'Kober 5BB' were excised from the central part (2^{nd} to 5^{th} nodes) of *in-vitro* grown plants and used as explants. The explants were established on media (Tables IV and V) with control macro-element and macro-element concentrations calculated in a stepwise manner to optimize the average outcome of *in-vitro* plant development. The explants of the three genotypes were cultured for 56 days, and plant development was assessed based on the following parameters: arm length (cm), leaf and arm quality (points), main root length (cm), main root quality (points) (Table V). The media were tested, and one was revealed which enabled a better plant development in all three genotypes.

Optimization of component concentrations of the nutrient medium for different grape genotypes is rather a labor-consuming process, and our media (Table VI) may be helping to attempt growing this or that genotype *in vitro*. We suggest that they should be tested to reveal one that will suit best, though we admit that it may fail to be virtually best efficient without raising a mathematical design of experiment to optimize *in-vitro* plant development for the genotype in question.

Table VI. - Composition of nutrient media recommended for plant development of various grapevine genotypes. In addition to the macro-elements indicated in the Table, each modified medium contained [16]: ¼ Fe-EDTA and ¼ micro-elements of MS medium, 20 mg l^{-1} myo-inositol, 0.1 mg l^{-1} thiamine, 0.2 mg l^{-1} pyridoxine, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} IAA, 10 g l^{-1} sucrose and 7 g l^{-1} Difco agar only in solid media. The pH of the media was 5.6-5.8.

Macro-elements	Levels of macro-elements in different liquid and solid modified media, mg l^{-1}					
	Modified medium Slenko et al. [16]	Modified media optimized by using regression equations (y_1-y_5)*				
		'Sverhrannii bessemiannyi'		'Zhemchug Magaracha'		2eq.y_5 medium of Tables IV, V (y_5)
		Liquid medium(y_1)	Solid medium(y_2)	Liquid medium(y_3)	Solid medium(y_4)	
1. NH_4NO_3	308	353	748	424	318	318
2. KNO_3	922	1188	1180	903	922	1188
3. $MgSO_4$ $7H_2O$	597	357	233	216	555	370 (MS)
4. KH_2PO_4	163	324	680	153	170 (MS)	370
5. $CaCl_2$	331 (MS)	331 (MS)	331 (MS)	366	331 (MS)	331 (MS)

* This paper presents the design of experiment to optimize *in-vitro* plant development and the outcomes of the process by using regression equation y_5. Voluminous data referring to optimization by using regression equations y_1-y_4 is not shown.

Assessment of results and calculation of the overall quality criterion of in-vitro plant development

A better *in-vitro* arm or root development requires different concentrations of the ions NH_4^+ and K^+ [17] and macro-elements $MgSO_4$ $7H_2O$ and KH_2PO_4 [16]. That is why to assess *in-vitro* plant development, we used the overall quality criterion which embraces a number of arm and root characteristics: arm length (cm), arm and leaf quality (points, 1-5), main root length (cm), root quality (points, 1-5). The arm and leaf quality was assessed visually as follows: 1-2 for small light-green leaves on thin elongated arms, 3 for medium-size leaves on medium-thick arms, 4 for large dark-green leaves on normally developed arm and 5 for large dark-green leaves with the leaf blade structure typical of a given genotype on relatively thick arms. The root quality was also assessed visually: 1 for black thin or short thick roots, 2 for thin, long root,

partially black or brownish in color, 3 for roots of relatively medium size, 4-5 for long and thick roots, white in color and with lateral ramifications.

The overall quality criterion characterizing leaf, arm and root development was calculated as described in our previous work [15]. Since each character ($Ix_{1,2,...i}$; $IIx_{1,2,...i}$ ...$nx_{1,2,...i}$) varies over a larger or smaller range depending on macro-element concentrations (levels of factors) of the medium, the variation of the character may be re-calculated so that it can remain within the set boundaries which reflect the usefulness of that character. We re-calculated the natural value (x_i) of each character into values ($It_{1,2,...i}$; $IIt_{1,2,...i}$ ...$nt_{1,2,...i}$) which varied within the set boundaries, and then the overall quality criterion (o. q. c.) of *in-vitro* plant development was calculated using the following formulae [15]:

$$Ia = -\frac{Ixmax - M \cdot Ixmin}{M - 1} \qquad (1);$$

$$It_{1,2,...i} = -\frac{Ixi - Ia}{xmax - Ia} \qquad (2);$$

$$o.q.c. = It \cdot IIt \cdot \ldots \cdot nt \qquad (3),$$

were Ixmax; IIxmax … … nxmax and Ixmin; IIxmin … … nxmin are extreme values of characters; xi is the value being estimated of a character; M is a random number determined depending on the usefulness of a character, e.g. if M = 10, ti varies over a range of 0.1-1.0 and if M = 2, ti varies over a range of 0.5-1.0. In this paper M = 10 for the characters of all processes.

Mathematical design of experiment

In order to calculate optimum macro-element concentrations of liquid and solid media for plant development, we applied a mathematical design of experiment using the method of random balance [15, 18]. If the number of factors under study is too large (five factors in our case, each present at three

levels), a complete factorial experiment, leading to a total of 3^5=243 modified media, is difficult. That is why we applied the method of random balance, meaning that the distribution of the concentrations of each macro-element (level of each factor in Table I) in each modified media (a total of 18 modified media shown in Table II) followed the law of random numbers. By using an appropriate software, a design of experiment was adjusted which had no significant correlations among the changes in factor levels in modified media. Based on the design of experiment and on the results obtained, regression equations were calculated which described the dependence of plant development (y_{1-5}) on changes in levels of factors (variables x_1-x_5) in liquid and solid media. The effect of these variables on *in-vitro* plant development was assessed based on the increases in significance and in the determination coefficients of regression equations (Table III). Macro-element concentrations regarded as 'steps' for stepwise optimization of liquid and solid media for plant development in two grapevine genotypes were calculated by using a regression equation describing this process (Table IV). These calculations were done with an aid of algorithms of multiple curvilinear stepwise regressions according to the Box-Wilson method of steepest ascent. Macro-element concentrations for stepwise optimization of *in-vitro* plant development (Tables IV and V) were calculated based on macro-element concentrations as starting points of the medium which was best efficient for plant development in Experiment I (Table II). This was followed by Experiment II. Single-bud cuttings with one leaf of cvs. 'Sverkhrannii bessemiannyi' and 'Zhemchug Magaracha' and also of the rootstock 'Kober 5BB' as explants were established on media whose macro-element concentrations had been calculated for stepwise optimization of *in-vitro* plant development. The results obtained allowed to reveal liquid and solid media which proved to be best efficient for plant development in all three genotypes (Table V).

Statistical analysis

Each modification of liquid or solid medium for each genotype was in 15 replications. The variation boundaries of average values referring to characters concerned were calculated with the level of significant errors at $P < 0.05$.

Results and discussion

Calculation of a regression equation describing the dependence of in-vitro plant development in grapevine genotypes on macro-element concentrations of media (Experiment I)

Table I shows macro-elements (factors) of which each was applied in Experiment I at three concentrations (levels), expressed both as natural units (mg l^{-1}) and in the coded form. Table II shows the design of Experiment I: a total of 18 media, each characterized by random distribution of levels of factors (variables x_1-x_5) presented in the coded form, and the individual and the average outcomes of plant development in cvs. 'Sverkhrannii bessemiannyi' and 'Zhemchug Magaracha' on liquid and solid media expressed as units of the overall quality criterion. For the sake of convenience, the media are presented in an ascending order as concerns their macro-element concentrations. Modifications of media 18 and 6 (onward referred to as medium 18 of Table II and medium 6 of Table II) were best efficient for plant development in the two genotypes both on filtering paper bridges in liquid medium and on solid medium. Medium 18 of Table II was characterized by the following macro-element concentrations: 318 mg l^{-1} NH_4NO_3, 903 mg l^{-1} KNO_3, 233 mg l^{-1} $MgSO_4$ $7H_20$, 230 mg l^{-1} KH_2PO_4 and 331 mg l^{-1} $CaCl_2$. Medium 6 of Table II differed from medium 18 of Table II in the concentrations of NH_4NO_3 (106 mg l^{-1}), $MgSO_4$ $7H_20$ (370 mg l^{-1}), KH_2PO_4 (51 mg l^{-1}) and $CaCl_2$ (436 mg l^{-1}) and contained the same amount of KNO_3.

Regression equations were calculated which described plant development as changes in the overall quality criterion of plant development with changes in

macro-element concentrations (variables x_1-x_5) of media (Tables I and II) in cv. 'Sverkhrannii bessemiannyi' on liquid media (y_1) and on solid media (y_2) and in cv. 'Zhemchug Magaracha' on liquid media (y_3) and on solid media (y_4). A regression equation was calculated which described the average value of the overall quality criterion of plant development in the two genotypes on liquid and solid media (y_5). These regression equations are as follows:

$y_1 = 0.063 + 0.221x_1x_4 + 0.174x_2x_3 + 0.082x_2x_4 + 0.118x_2^2$;

$y_2 = 0.171 + 0.107x_2 + 0.144x_1x_2x_4 + 0.173x_1^2x_4$;

$y_3 = 0.090 + 0.117x_1x_5 + 0.101x_4 + 0.093x_1 + 0.089x_3x_4 + 0.078x_1x_4 + 0.076\ x_3x_5 – 0.075x_1x_3x_4$;

$y_4 = 0.279 + 0.311x_3 – 0.227x_3x_4 + 0.094x_2$;

$y_5 = 0.027 + 0.116x_2^2 + 0.109x_2\ x_4 + 0.106x_2x_3 + 0.114x_4$.

The above-indicated regression equations have the following statistical characteristics: the level of significance (sign.) of regression equation (y_1) is 0.999 and the determination coefficient (det. coef.) is 0.788; y_2 (sign.=0.998, det. coef. = 0.744); y_3 (sign. = 0.999, det. coef. = 0.796); y_4 (sign. = 0.999, det. coef. = 0.695) and y_5 (sign. = 0.998, det. coef. = 0.581). Significance and determination of the equations indicate how well, in terms of reliability and adequacy, the experimental results (Table II) are reflected by the theoretical y_1-y_5 calculated by means of the above-indicated regression equations. Plant development in each study genotype on liquid or solid media (y_1-y_4) and the average outcome of the process (y_5) were differently affected by different macro-elements (variables x_1-x_5) over the concentration ranges applied in Experiment I (Tables I and II). This indicates that the effect of different macro-element concentrations of media on *in-vitro* plant development in each genotype is different. Another finding is that the ability of the genotypes to utilize macro-elements from the medium depends on its consistence, i.e. on whether it is liquid or solid.

The software applied to calculate the regression equations envisages stepwise selection of factors (in the form of equation members) which affect *in-vitro* plant development in the most significant manner. The first members of our regression equations are the most significant and successive addition of new members does further improve statistical characteristics of the equations. For instance, when new members were successively added, this improved significance and determination of equation (y_5) though the largest contribution to the significance of the equation was made by the first members (Table III). In applying our mathematical design of experiment for stepwise optimization of a process (*in-vitro* plant development in this case), there is no need to do preliminary research for selection of factors which exert the most significant influence on the process. This can be avoided since the software applied envisages stepwise selection of factors and their interactions (both in the form of equation members) which affect the process in the most significant manner (Table III).

Selection of medium best efficient for in-vitro plant development in two grapevine genotypes based on media whose macro-element concentrations were calculated in a stepwise manner by using regression equation (y_5) to optimize in vitro plant development (Experiment II)

By using regression equation (y_5) which describes the dependence of the average value of the overall quality criterion of plant development in cvs. ‘Sverkhrannii bessemiannyi’ and ‘Zhemchug Magaracha’ on macro-element concentrations in liquid and solid media, a design of experiment for stepwise optimization of *in-vitro* plant development in these two genotypes was raised. The best-efficient medium 18 of Table II was chosen as the basal one for stepwise optimization of *in-vitro* plant development (Tables IV and V). The concentrations of macro-elements entering regression equation (y_5) as variables x_2, x_3 and x_4 were changed in a stepwise manner while those of macro-elements

presented as variables x_1 and x_5 remained as they were in medium 18 of Table II. Medium with ½ macro-elements of MS medium and medium 18 of Table II (basal one for stepwise optimization of *in-vitro* plant development) were used as controls to check the improvement of plant development on the optimized medium (Tables IV and V). The control media and media ($1opt.y_5$ – $3opt.y_5$) whose macro-element concentrations had been calculated in a stepwise manner, leading to the optimization of plant development, differed only in concentrations of all or several macro-elements while other components were present at the same levels. Plant development in cvs. 'Sverkhrannii bessemiannyi' and 'Zhemchug Magaracha' and in the rootstock 'Kober 5BB' was significantly better ($P < 0.05$) on liquid (L) and solid (S) modifications of the optimized medium ($2opt.y_5$) for all and the majority of study characters than on ½ MMS medium and on medium 18 of Table II, respectively (Tables IV and V). The optimized medium ($2opt.y_5$) of Table IV differed from medium 18 of Table II chosen as basal one for stepwise optimization of *in-vitro* plant development in the concentrations of such macro-elements as KNO_3 (1188 mg l^{-1}), $MgSO_4$ $7H_20$ (370 mg l^{-1}) and KH_2PO_4 (370 mg l^{-1}) and contained NH_4NO_3 (318 mg l^{-1}) and $CaCl_2$ (331 mg l^{-1}) at the same concentrations (Table IV).

Genotypes of the genus *Vitis* differ in the ability to uptake K, Ca and Mg from the soil and in the concentrations of these mineral elements in the plants [5]. Grapevine genotypes also need for their growth different concentrations of mineral substances which enter as sources of soil nitrogen, and some of them are capable of growing on the soils with low nitrogen levels [19]. Besides, nutrient media (substrates) are rich in some mineral elements at the expense of the other, and such insufficiencies result in retarded plant growth. Thus, potassium enrichment of the nutrient solution of four grape genotypes established in water culture led both to higher potassium and lower magnesium levels in all parts of the plants [20]. Plant development on liquid or solid media (as determined by their macro-element concentrations, Tables I and II) in cv. 'Sverkhrannii

bessemiannyi' and in cv. 'Zhemchug Magaracha' considered on an individual basis is described by different regression equations (y_1-y_4), indicating that these two genotypes differ in their needs for these macro-elements and/or in the ability to utilize them from 18 liquid or solid modified media (Table II). Designs to optimize liquid or solid media for each study genotype considered on an individual basis were raised by using regression equations (y_1-y_4). Nevertheless, plant development on these media was not significantly better for the majority of study characters (voluminous data not shown) that on the medium (2opt.y_5) optimized by using regression equation (y_5) common to all study genotypes.

Optimization of macro-element concentrations of media for *in vitro* propagation of some grapevine genotypes by using our mathematical design of experiment is rather a labor-consuming process. That is why Table VI shows best efficient media optimized in this and earlier experiments [16]. Grape genotypes other than those used in this experiment, in the form of one-bud cuttings with one leaf as explants, may be established on media with macro-element concentrations indicated in Table VI. Probably, by doing so, medium best suited for any attempted genotype may be discovered.

Conclusions

The following conclusions arise from applying our mathematical design of experiment to optimize, in a stepwise manner, macro-element concentrations of liquid and solid media for plant development in two grapevine genotypes:

1. Over the applied range of study macro-element concentrations (Table I), the dependence of plant development on these macro-element concentrations of liquid and solid media (Table II) is described by different regression equations, both for each of the two genotypes on an individual basis (y_1-y_4) and for the average outcome of the process in the two genotypes (y_5). This suggests that the two genotypes 'Sverkhrannii bessemiannyi' and 'Zhemchug Magaracha' differ in the pattern of plant development on 18 modified media (Table II) with

different macro-element concentrations and/or in the ability to uptake these macro-elements from media of different composition and consistence (liquid or solid ones). These regression equations (y_1-y_4) are so 'individual' that they may enter as 'visiting cards' for the genotypes and assist in their identification.

2. Our mathematical design of experiment for stepwise optimization of *in vitro* plant development led to significant improvement of plant development in all three genotypes on the optimized (2opt.y_5) medium compared to the controls: ½ MMS medium and 18 of Table II medium chosen as the basal one for stepwise optimization of the process for all and the majority of study characters, respectively (Tables IV and V).

Our protocol to assess experimental results by reducing a number of parameters characterizing a process to an overall quality criterion (equations 1-3) and the mathematical design of experiment reported in this paper may be used in agriculture and in the food industry for optimization of processes affected by numerous interacting factors.

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REFERENCES

1. Martelli, G.P., and Walter, B. 1998. Virus certification of grapevines. In: Hadidi, A. et al. (eds.). Plant virus disease control. St. Paul: American Phytopathological Society (APS) Press. p. 261-276.
2. Krake, L.R., Steele Scott, N., Rezaian, M.A., and Taylor, R.H. 1999. Graft-transmitted diseases of grapevines. Collingwood: CSIRO Publishing. 137 p.
3. Regner, F., Brandt, S., Romann, H., und Stadlhuber, A. 1995. *In vitro*-viruseliminierung bei reben (*Vitis* sp.). Mitteilungen Klosterneuburg **45**: 67-74.
4. Audeguin, L., Boidron, R., Bloy, P., Grenan, S., Leclair, P., et Boursiquot, J.M. 1999. L'Expérimentation des clones de vigne en France. Etats des lieux, méthodologie et perspectives. Progres Agricole et Viticole, Montpellier **116**: 486-491.
5. Scienza, A., Failla, O., und Romano, F. 1986. Untersuchungen zur sortenspezifischen mineralstoffaufnahme bei reben. Vitis **25**: 160-168.
6. Galzy, R. 1961. Confirmation de la nature virale du court-noué de la vigne par des essais de thermothérapie sur des cultures *in vitro*. C. R. Acad. Sci. **253**: 706-708.

7. Gifford, E.M., and Hewitt, W.B. 1961. The use of heat therapy and *in vitro* arm tip culture to eliminate fanleaf virus from grapevine. Amer. J. Enol. Viticult. **12**: 129-130.
8. Galzy, R. 1972. Remarques sur la nutrition minérale des apex de *Vitis rupestris*. C. R. Acad. Sci. **275**: 561-564.
9. Murashige, T., and Scoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**: 473-497.
10. Barlass, M., and Skene, K.G.M. 1978. *In vitro* propagation of grapevine (*Vitis vinifera* L.) from fragmented arm apices. Vitis **17**: 335-340.
11. Silvestroni, O. 1981. Prime esperienze sulla micropropagazione della vite europea. Vignevini **8**: 31-37.
12. Ciccotti, A.M. 1982. Micropropagazione di *Vitis vinifera* L. cvs. 'Moscato d'Amburgo' e 'Pinot Blanc'. Esper. Ric. Nuova Ser. **11**: 73-81.
13. Novak, F.J., and Juvova, Z. 1983. Clonal propagation of grapevine through *in vitro* axillary bud culture. Sci. Hort. **18**: 231-240.
14. Harris, R.E., and Stevenson, J.H. 1982. *In vitro* propagation of *Vitis*. Vitis **21**: 22-32.
15. Zlenko, V.A., Troshin, L.P., and Kotikov, I.V. 1995. An optimized medium for clonal micropropagation of grapevine. Vitis **34**: 125-126.
16. Slenko, W.A., Troschin, L.P., und Kotikow, I.V. 2001. Der einfluss der nährmedienzusammensetzung bei der *in vitro*-vermehrung verschiedener rebgenotypen. Mitteilungen Klosterneuburg **51**: 15-26.
17. Galzy, R. 1969. Remarques sur la croissance de *Vitis rupestris* culivée *in vitro* sur differents milieux nutritifs. Vitis **8**: 191-205.
18. Hartmann, K., Lezki, E., and Shäfer, W. 1977. Trial design in study of tehnological processes. Moscow: 'Mir'. 378 p.
19. Murthy, S.V.K., and Iyengar, B.R.V. 1997. Kinetik parameters of nitrogen absorption in varieties and rootstocks of grape (*Vitis vinifera*). Indian Journal of Plant Phisiology **2**: 232-233.
20. Rühl, E.H. 1993. Effect of K^+ supply on ion uptake and concentration in expressed root sap and xylem sap of several grapevine rootstock varieties. Wein-Wissenschaft (Wiesbaden) **48**: 61-68.
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