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# *In vitro* propagation of grape cultivars and rootstocks for production of pre-basic planting material

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## Abstract

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An optimized and simplified procedure for *in vitro* propagation of pre-basic planting material from different grape cultivars and rootstocks has been developed. A significant advantage of the applied propagation system was that the multiplication and rooting phases occurred simultaneously. The use of the specially modified nutrient medium provided an economically viable production of healthy and hardened plants. It was shown that the genotype is the primary factor determining a high multiplication and economically important efficiency in this process. Applying the optimized micropropagation system, the studied grape genotypes have been successfully propagated *in vitro* and acclimatized to *ex vitro* conditions. Obtained pre-basic plant material of more than 2500 plantlets was cultivated in a nursery for future investigation.

*Keywords:* grape cultivars; rootstocks; *in vitro*; micropropagation; auxins

## Introduction

Grapevine (*Vitis* spp.) is among the most widely grown fruit crops worldwide, which reflects its great economy significance (Reynolds, 2017). Due to its extensive exploitation for wine/table grape production and because of the beneficial health effects of grape metabolites, grapevine got into the spotlight of science and has become a model woody species in plant biotechnology.

Modern plant biotechnology techniques and genetic markers have boosted the development of grape breeding in a dynamic industry with a focus on creating cultivars with enhanced tolerance to pathogens and better adaptation to large ecological factors. As a result, the grapevine genome was fully sequenced, presenting a molecular basis for further studies (Atanassov et al., 2017).

Micropropagation in grapes is used mostly for the production of pre-basic plants, provided to nurseries for establishing increase clean stock blocks to harvest cuttings for the production of material for actual planting of productive vineyards. Genetic homogeneity of grapes that were mass propagated by various *in vitro* methods were assessed using molecular techniques including microsatellite markers, ISSR, and AFLP. In all cases, there was no difference between and among the micropropagated plants and the mother plant (Nookaraju and Agrawal, 2012; Tsvetkov et al.; 2014, Golino et al., 2017). Production of pre-basic pathogen-free plants by clonal micropropagation allows a significant increase in multiplication coefficient and acceleration of the process. The critical factors for the effectiveness of the *in vitro* propagation are the nutrient medium and the genotype specifics, including the existing genetic variability, within and between, the cultivars and rootstocks (Stamp et al., 1990; Botti et al., 1993; Torregrosa et al., 1996; Ibáñez et al., 2005; Skiada et al., 2009). Grape genotypes possess specific requirements and sensitivity to the application of growth regulators (Scienza

et al., 1986, Roubelakis-Angelakis and Zivanovitc, 1991; Péros et al., 1998; Torregrosa et al., 2000; Bouquet and Torregrosa, 2003). The cultivar diversity and polymorphism in the grapes, initially described by Thomas et al. (1993), suggest the specificity of the plant reaction under the wholly changed conditions of in vitro cultivation. The character of this expression represents a particular scientific and practical interest.

The purpose of the study was to determine the effectiveness of an optimized protocol, suitable for micropropagation of both grape cultivars and rootstocks towards commercial propagation, comparing their growth characteristics and obtaining pre-base material for planting in a production nursery and further investigations.

## Materials and Methods

Experimental work was carried out with 12 grape cultivars and 11 rootstocks kindly provided by AMV AGRO, Plovdiv. All preliminary requirements related to the screening of parent donor plants for the presence of viruses have been performed according to Chee et al. (1984) and Skiada et al. (2009). Initial explants were prepared as two-nodal micro cuttings, isolated from green shoots (30-35 cm), developing from winter buds of mature cuttings in a growth chamber.

Sterilization procedure was completed by treatment with 0.1% solution of mercury chloride for 1-2 minutes and following five washes for 3 minutes by sterile water. Single explants were initially cultivated in glass tubes with 10 ml medium in a growth chamber with temperature 25±1ºC, the light intensity of 3000 Lx and 16/8 h photoperiod. Micropropagation procedure was performed by culturing of five two-nodal explants in glass vessels with volume 180 ml, containing 30 ml nutrition medium and duration of the subculture of 45 days (Thomas, 1999).

The optimized composition of the culture medium for micropropagation was based on MS basal medium (Murashige and Scoog, 1962; Murashige, 1974) with the half content of macroelements and supplemented with sucrose (20 g $l^{-1}$) and agar (5.5 g $l^{-1}$). Additionally biotin (0.2 mg $l^{-1}$), Ca-pantothenate (0.5 mg $l^{-1}$), activated charcoal (0.3%) and the auxins indole-acetic acid (IAA 0.2 mg $l^{-1}$) and indole-butyric acid (IBA 0.2 mg $l^{-1}$) were included. pH was adjusted to 6.5 before sterilization by autoclaving for 20 minutes.

Regarding the multiplication efficiency, the following mean values of the growth parameters were counted: number of shoots per explant, maximal shoot length (cm), number of internodes, internode length (cm) and multiplication coefficient (MC), calculated as mean number of new two-nodal cuttings obtained from a single mother explant.

Rooting ability of the studied cultivars and rootstocks was summarized and evaluated in two groups, by the parameters: rooting percentage, average number of roots per plant and average root length.

All data, based on minimum two independent experiments, with three replications of 25 explants per variant, were collected from cultures at the fourth and fifth subculture and analyzed by standard biometrical methods.

Rooted plantlets followed acclimatization and adaptation to ex vitro conditions by planting in the peat-perlite mixture (3:1) in a growth chamber with gradually decreasing atmosphere humidity, temperature 22±1°C, and 16/8 h photoperiod.

## Results and Discussion

Applied multiplication system and growth conditions favored the initial development of the explants and obtaining of plants with well-developed roots and 5-7 leaves (Fig. 1A). Following cultivation on the optimized culture medi-

*A*)

*B*)

**Fig. 1. Plant development of grape cultivars: (*A*) initial explants after three weeks culture on the optimized propagation medium; (*B*) shoots of cv. Syrah in the third subculture**

um, all studied cultivars and rootstocks demonstrated normal growth, forming first roots and subsequently developing shoots on the same culture medium (Fig. 1B).

Comparing the growth characteristics of the studied genotypes we established similar values of the vegetative parameters (Table 1, Table 2).

In both cultivars and rootstocks, the mean number of shoots per explant varies between 1.0 and 1.3. Cultivars Tamianka, Dimiat, Bianka, Syrah, Fetiaska regala and the rootstocks Georgikon, RSB-1, Georgikon 28 and 1616 C developed a single shoot, while Muskat Sandanski, Dornfelder – clone WE700, Gechei zamatosh, Colombard, Lakhedi mezesh, Muskat red and Mavrud proliferated, also forming new shoots from axillary buds. Such growth behavior could be explained by the lack of the cytokinines in the medium, as growth regulators stimulating the proliferation, as well as with the typical for the *Vitis* sp. strong apical dominance.

Variation in the maximal length of the shoots was detected, with ranging from 5.3 to 8.9 cm for the cultivars and from 5.05 to 10.8 cm for the rootstocks. Data comparison showed the highest value for the cultivars Syrah, Colombard, Muskat Sandanski and the rootstocks 101-14 and RSB-1, and the lowest for Gechei zamatosh, Lakhedi mezesh, Bianka and the rootstocks Riparia and 3309.

The mean number of internodes was similar in the studied genotypes. Comparative analysis showed the highest result for cultivars Dornfelder – clone WE700, Muskat red, Syrah, and the rootstocks 101-14, 125 AA, and the lowest for Gechei zamatosh and 1616 C, respectively.

In the micropropagation system, the internode length has importance for the suitable explant preparation and influenc-

**Table 1**
***In vitro* growth parameters of different *Vitis* cultivars (mean values ± standard deviation)**

| Genotype | Number of shoots per explant | Maximal shoot length (cm) | Number of internodes | Internode length (cm) | MC |
|---|---|---|---|---|---|
| Tamianka | 1.0±0.0000 | 5.95±0.6404 | 5.3±0.3262 | 0.95±0.0849 | 2.2±0.2629 |
| Muskat Sandanski | 1.1±0.1054 | 8.20±0.4791 | 6.1±0.3990 | 1.07±0.1176 | 3.4±0.2330 |
| Dornfelder cl.WE700 | 1.3±0.1610 | 7.35±0.5331 | 7.1±0.7927 | 0.90±0.0831 | 3.3±0.4458 |
| Dimiat | 1.0±0.0000 | 8.05±0.8764 | 5.6±0.4766 | 0.68±0.0872 | 2.8±0.4661 |
| Bianka | 1.0±0.0000 | 5.55±0.2658 | 6.0±0.4714 | 0.77±0.0802 | 2.4±0.1721 |
| Gechei zamatosh | 1.1±0.1054 | 5.30±0.2509 | 3.7±0.3531 | 1.12±0.0828 | 1.9±0.1054 |
| Colombard | 1.2±0.1405 | 8.25±0.5457 | 6.1±0.3668 | 1.14±0.1068 | 3.7±0.2744 |
| Lakhedi mezesh | 1.1±0.1178 | 5.40±0.3584 | 4.3±0.3061 | 1.14±0.1312 | 2.3±0.1767 |
| Syrah | 1.0±0.0000 | 8.90±0.4766 | 6.4±0.6324 | 1.16±0.0723 | 3.7±0.2249 |
| Muskat red | 1.3±0.2249 | 7.70±0.2629 | 6.7±0.4172 | 1.00±0.0544 | 3.1±0.1892 |
| Fetiaska regala | 1.0±0.0000 | 6.20±0.2744 | 6.3±0.3262 | 0.79±0.0507 | 2.6±0.2330 |
| Mavrud | 1.3±0.2249 | 6.00±0.3042 | 5.1±0.5079 | 1.11±0.1355 | 2.5±0.2832 |

**Table 2**
***In vitro* growth parameters of different *Vitis* rootstocks (mean values ± standard deviation)**

| Genotype | Number of shoots per explant | Maximal shoot length (cm) | Number of internodes | Internode length (cm) | MC |
|---|---|---|---|---|---|
| Riparia | 1.3±0.1610 | 5.05±0.3552 | 4.90±0.4567 | 0.91±0.0881 | 2.2±0.2629 |
| 68-71 | 1.1±0.1054 | 6.90±0.8270 | 4.80±0.4388 | 1.23±0.1018 | 2.6±0.3912 |
| Gravesak | 1.1±0.1054 | 6.45±0.2283 | 5.50±0.2357 | 1.01±0.0637 | 2.7±0.1610 |
| Georgikon | 1.0±0.0000 | 6.95±0.5744 | 4.00±0.2222 | 1.43±0.1077 | 3.3±0.7036 |
| RSB-1 | 1.0±0.0000 | 8.85±0.5389 | 5.40±0.3912 | 1.38±0.0857 | 3.1±0.2918 |
| 3309 | 1.2±0.1405 | 5.15±0.1932 | 4.70±0.3162 | 0.94±0.0756 | 2.3±0.2744 |
| Fercal | 1.1±0.1054 | 7.05±0.2986 | 5.90±0.5544 | 1.02±0.0899 | 2.9±0.3990 |
| 101-14 | 1.3±0.1610 | 10.8±1.3314 | 7.60±0.5488 | 1.19±0.1346 | 4.7±0.6675 |
| 125 AA | 1.2±0.1405 | 6.80±0.3258 | 6.50±0.5719 | 0.90±0.0544 | 3.2±0.1405 |
| Georgikon 28 | 1.0±0.0000 | 7.40±0.3122 | 5.05±0.5468 | 1.22±0.0733 | 3.4±0.2330 |
| 1616 C | 1.0±0.0000 | 6.35±0.3429 | 3.30±0.1610 | 1.63±0.0861 | 2.2±0.1405 |
| Riparia | 1.3±0.1610 | 5.05±0.3552 | 4.90±0.4567 | 0.91±0.0881 | 2.2±0.2629 |

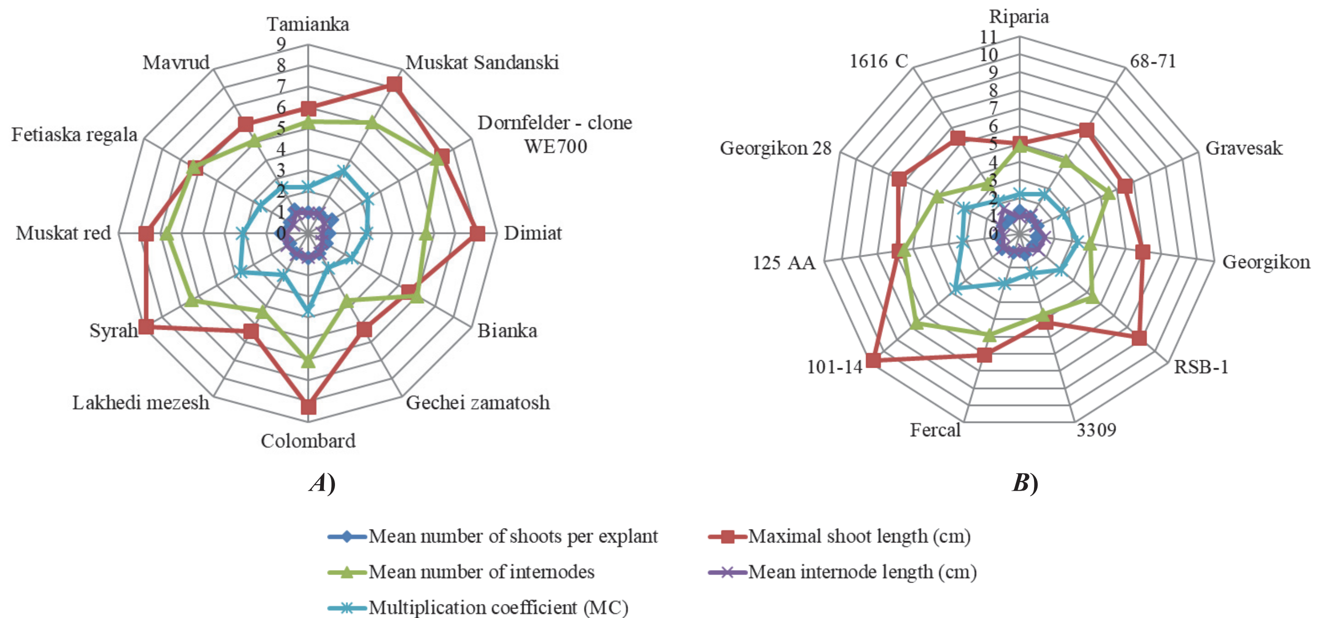


**Fig. 2. Complex evaluation of the growth parameters influencing the efficiency of the micropropagation in the studied grape cultivars (*A*) and rootstocks (*B*)**

ing the in vitro physiology and the subsequent plantlets development. More of the studied cultivars formed internodes with length about 1.0 cm and only in Dimiat it was low. In the rootstocks the parameter was higher, varying between 0.9 for 125 AA and 1.63 cm for 1616 C.

Multiplication coefficient (MC) is a complex indicator of micropropagation efficiency. In our study, it did not reflect significant differences between both groups of plants. The number of cuttings obtained from a single explant varied from 1.9 to 3.7 for the cultivars and from 2.2 to 4.7 for the rootstocks. The specificity of the reaction of individual genotypes revealed some of the genotypes with higher MC like Syrah and Colombard (3.7), Muskat Sandanski (3.4), Dornfelder WE 700 (3.3) and the rootstocks 101-14 (4.7) and Georgikon 28 (3.4). Lower MC demonstrated Gechei zamatosh (1.9), 1616 C (2.2) and Riparia (2.2). These results could be explained on the one hand, by the number of subcultures (4-5), and on the other, with the aim of the study

**Table 3**
**Rooting characteristics of the studied grape genotypes (mean values ± standard deviation)**

| Genotypes | Rooting (%) | Number of roots per plant | Average root length (cm) |
|---|---|---|---|
| Cultivars | 100 | 4,87±0,4172 | 1,90±0,1610 |
| Rootstocks | 100 | 5,73±0,5248 | 2,19±0,2142 |

directed to the unification of the system towards commercial propagation.

The complex evaluation of the growth indicators showed that the multiplication (MC) depends mostly on the length of the plant, which is determined by the number of the formed internodes and their average length (Fig. 2).

The present investigation showed that the application of the optimized multiplication procedure ensures the production of healthy, viable and hardened plants that are successfully adapted. During the cultivation of explants and plant growth, the occurrence of the adverse physiological state of hyperhydricity (vitrification) was not detected.

The calculated rooting percentage was 100%, and the established values of the mean root number and average root length were similar, however slightly higher for the group of the rootstocks (Table 3). After four weeks of cultivation, the formation of secondary roots in all genotypes was observed. The well-developed root system was a prerequisite for successful adaptation and acclimatization of the obtained plants under *ex vitro* conditions.

High adaptation rate (over 95%) was observed for all tested genotypes (Fig. 3A). After the plants reached 25-30 cm of the length, they were transplanted to soil and grown under greenhouse conditions (Fig. 3B, C). Maintaining optimal water and the nutritional regime, some of them grow up to 150 cm by the end of vegetation. To control health status

***A*)**

***B*)**

***C*)**

**Fig. 3. Development of the grape cultivars: (*A*) plants after 21 days acclimatization, (*B*) cultivars, (*C*) rootstocks in an isolated greenhouse**

of adopted plants randomly chosen samples were periodically checked for the presence of viruses.

The optimized culture medium applied does not contain cytokinins. In grape, culture difficulties and shoot vitrification in BAP-containing media have been described (Alizadeh et al., 2010). The significant advantage of the reported propagation system is that the multiplication and rooting phases occurred simultaneously, using a modified medium with auxins only. Moreover, the obtained shoots are long enough for nodal transplantation in next subculture. So in each subculture, one portion of shoots can be put on acclimatization and the rest of them can be divided into two-node cuttings and transplanted to fresh medium for another cycle of *in vitro* growth. Such type of organogenesis is of extreme importance in the mass propagation as the need for preparing different nutrient media for each variety or group of varieties is eliminated. Present efficient micropropagation protocol is an approach for reducing the risk of developing vitrified plants and ensuring the production of quality plant material, leading additionally to cost saving.

## Conclusions

The specificity of the genotype is the crucial factor determining the high multiplication efficiency in the commercial propagation of the grape cultivars and rootstocks. The application of here reported propagation system and culture medium containing a combination of auxins only, provided economically valuable production of healthy, viable and hardened plants. Following the optimized micropropagation protocol, the studied vine genotypes – cultivars and rootstocks were successfully propagated in *in vitro* and adapted to *ex vitro* conditions. The produced pre-basic plant material of more than 2500 plants was established in a production nursery for further investigation.

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