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The Micropropagation Potential and Regenerative Ability of Somatic Embryos of *Vitis Vinifera* ssp. *Sylvestris* (Gmel.) Hegi

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SUMMARY. In this paper are presented the results obtained with *Vitis vinifera* ssp. *sylvestris* accessions subjected to the *in vitro* micropropagation. For clonal propagation were used meristematic tissues (apexes and axillary buds) aiming to establish an efficient protocol for long-term conservation or for virus-free plant recovery (if viruses were detected) applied to accessions belonging to seven different populations. The *in vitro* development showed particular aspects and significant differences among wild populations regarding their competence for differentiation, the moment of differentiation in inoculated explants, the aspect of proliferative structures and the rates of multiplication. The same accessions were tested also for their competence in plant regeneration by either organogenesis or embryogenesis starting from somatic tissues.

Shoot regeneration and normal plants were obtained from petiole explants derived from *in vitro* grown shoots of the seven accessions. Callus induction was obtained with all genotypes on MS media supplemented with BAP and 2,4-D, or IBA, and the best direct adventitious shoot formation was obtained after transfer on medium supplemented with BAP and IAA.

With anther culture, the genetic factors proved to be essential for callus induction and promoting the embryogenic process. Three, out of seven accessions, only male individuals, responded to anther culture by regeneration through embryogenesis on all used media with similar results, forming normal embryos, abnormal embryos and whole plants.

Keywords: embryogenesis, organogenesis, plant regeneration, *Vitis*, wild grapevine accessions.

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Introduction

During the last hundred years, the habitat of *V. vinifera* ssp. *sylvestris,* as wild grapevines, has been reduced due to pathogens or intensive forests and rivers management. Today small populations were identified only in certain ecosystems in Europe, including Romania. The recent papers underlined that *V. vinifera* ssp. *sylvestris* is one of the endangered subspecies and its conservation in germplasm collections could be an important source of plant material useful for the introgression of adaptive traits in cultivated grapevines (Ocete *et al.*, 2011). The ampelographic studies on wild populations have offered valuable information on evolution of cultivated grapevine varieties (Lacombe *et al.,* 2003). The analyses at the molecular level revealed important clues on genetic relationship among cultivated and wild grapevine accessions, and about the degree of genetic variability which could be of great interest for breeders (This *et al*., 2006; Schneider *et al.*, 2009; Arroyo-Garcia and Revilla, 2013).

In our country, many populations with few individuals of wild grapevines were identified in almost all provinces, especially along the main rivers, in areas with high humidity (Pop, 1931; Teodorescu *et al*., 1966; Popa *et al*. 2009). Due to their potential importance as source of valuable genes for improvement the cultivated grapevines, the collecting and establishing *ex situ* collections is essential to protect these wild populations in their native areas, and to prevent their extinction. In a previous paper, were presented the morphological characterization based on OIV descriptors of the individual plants collected from some wild grapevine populations growing along the Danube River, providing valuable information (Popescu *et al*., 2013). The aim was to establish an *ex situ* collection with virus-free plant material and to use it as possible starting plant material for further breeding of grapevine cultivars and rootstocks.

In this paper is presented a synthesis of *in vitro* results obtained with *V. vinifera* ssp. *sylvestris* accessions regarding: a) the regeneration competence of new structures by organogenesis from meristematic tissues, or by embryogenesis from somatic tissues; b) the specific aspects of evolution and multiplication of vegetative structures through the two types of regeneration.

Materials and methods

a. Meristematic tissues culture. Plant material from 7 accessions belonging to different populations of *Vitis vinifera* L. ssp. *sylvestris* was harvested from *ex situ* collection (Table 1). The young shoots of 2-3 cm in length, with apex and 1-2 axillary buds were sterilized by immersion for 3-5 min in 5.2% sodium hypochlorite

(v/v) with a few drops of Tween-20, followed by rinsing with sterile distilled water. From each accession, 50 apexes and 50 axillary buds were inoculated on media with different combinations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). Apexes and axillary buds were initiated on Murashige and Skoog (M&S) media supplemented with 0.5, 1.0 or 1.2 mg/l BAP and 0.1 or 0.5 mg/l IAA. In all types of media were added: 10 mg/l ascorbic acid, 3% sucrose, and 5.5% agar. After 30 days on the initial medium, the new regenerative structures were periodically transferred on fresh medium with reduced sucrose concentration (2%) and modified growth regulators composition (1.0 mg/l BAP and 0.5 mg/l IAA). The culture medium was autoclaved for 20 min at 121°C and 1.2 bars, and the culture vessels were maintained under 16/8 hours' photoperiod, a light intensity of 3,000 lux and 22±2°C temperature. Evaluation of the regenerative processes involved observations on morphological aspects of the new structures and statistical analysis by polynomial regression of the data regarding the number of new explants generated after each transfer, average height of shoots, and the rate of multiplication.

b. The petiole culture was established by using petioles excised from *in vitro* multiplied shoots. The cultures were grown in the dark for the first 5 weeks, on M&S media containing BAP and indole-3 butyric acid (IBA). After that, the petioles were periodically transferred on fresh medium supplemented with 1.0 mg/l BAP and 0.5 mg/l IAA for further shoot regeneration and maintained under 16/8 hours' photoperiod and 22±2°C temperature.

c. For anther culture, the inflorescences in tetrad microsporogenesis phase, were collected before anthesis, kept at 4ºC for 48 h and then sterilized with 0.1% mercury chloride. Under binocular microscope, the anthers were separated and inoculated on solid M&S medium containing 1.0 mg/l BAP, 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 3% sucrose. After six weeks of maintenance in darkness at 20°C, the viable anthers were transferred on fresh medium, a modified M&S medium (double quantity of magnesium sulfate and triple amounts of manganese sulfate and copper sulfate) with the same growth regulators and sucrose. Subsequently, the anthers with calli, or anther-derived calli were sub-cultured every 30 days on fresh modified M&S medium supplemented with 1.0 mg/l BAP, 0.5 mg/l IAA, and 2% sucrose for embryogenesis induction, the maintenance of embryogenic potential and the somatic embryos germination. During these growing phases, the explants were maintained at 16/8 hours photoperiod, and 22±2°C. All the culture media were adjusted to pH 5.8 with NaOH before autoclaving.

Table 1.
Sampling locations, source of plant material, their symbols

Number and symbols	2 individuals: Vs8dm, Vs9dm	1 individual: Vs1	2 individuals: Vs10, Vs3eb	2 individuals: Letea 1, Letea 2
Location	Stârmina Forest	Hinova	Greaca	Letea Forest
Geographic coordinates	22º46'14'' Long.E 44º30'01'' Lat.N	22°46'36" Long.E 44°32'26" Lat.N	26°20'21" Long.E 44°6'33" Lat.N	29° 53'7" Long.E 45°34'24" Lat.N
Altitude (m)	118	100	60	12

Results and discussions

a) The regenerative competence of new structures from meristematic tissues in wild grapevine plants

The results of experiments in which apexes and axillary buds were used as meristematic tissues for the micropropagation of wild grapevine accessions showed that the tested media, similar to those used for cultivated grapevines, were efficient in promoting regenerative processes and ensured the development and growth of vegetative structures. The data collected before each transfer showed different responses induced by different combinations of BAP and IAA. In all tested genotypes, the best results were obtained from explants initiated on medium containing 1.0 mg/l BAP and 0.5 mg/l IAA (Fig. 1). After the fifth passage, considered to be reliable to keep the genetic accuracy of plant material, a similar growth of the shoots from both type of meristematic explants and a very significant higher rate of multiplication from axillary buds were registered (Table 2). While Vs8dm accession showed the lowest rate of multiplication and very short shoots, the Vs9dm accession, harvested from the same location, expressed the best response to *in vitro* culture conditions, giving the highest length of shoots and the best rate of multiplication.

Figure 1. Expression of the rate of multiplication and the shoots raising, starting from axillary buds, with different *Vitis sylvestris* accessions: Letea 2, Vs8dm, Vs10 and Vs1.

Table 2.

Results data registered after five passages and seven months of *in vitro* culture.

Acce-ssion	Average height of shoots (cm) ± SD		Rate of multiplication after the 5th passage		Polynomial regression and coefficient of regresion
	Apex (A)	Buds (B)	Apex (A)	Buds (B)	
Vs1	4.47±1.7	4.38±1.8	11.65	7.14	A: $y = 85,5x^2-408,6x+470,1$ $R^2 = 0,97$
					B: $y = 55,07x^2-272,7x+338,4$ $R^2 = 0,98$
Vs8dm	2.98±1.3	3.58±1.5	4.28	8.58	A: $y = 25,57x^2-114,7x+190,6$ $R^2 = 0,99$
					B: $y = 47x^2-189,9\ 263,6$ $R^2 = 0,97$
Vs9dm	4.35±1.3	4.11±1.9	12.32	31.04	A: $y = 22,45x^3-142,1x^2\ 256,1-48,33$ $R^2 = 0,99$
					B: $y = 56,1x^3-372,6x^2+796,4x-400,6$ $R^2 = 0,99$
Vs10	4.09±1.4	3.53±0.8	7.4	16.31	A: $y = 18,08x^2- 95,8x+172,1$ $R^2 = 0,98$
					B: $y = 17,8x^3-82,3x^2+116,1x+43,3$ $R^2 = 0,99$
Vs3eb	2.99±1.2	3.49±1.4	7.43	19.65	A: $y = 47,5x^2-213,6x+282,4$ $R^2 = 0,98$
					B: $y = 25,6x^3-138,9x^2+243,1x-38$ $R^2 = 0,99$
Letea 1	3.75±1.6	3.55±1.4	19.74	19.53	A: $y = 54,2x^3-421,5x^2+992x-560$ $R^2 = 0,98$
					B: $y = 36,6x^3-242,3x^2+490,8x-199,3$ $R^2 = 0,99$
Letea 2	2.41±1.1	3.70±1.1	16.32	21.12	A: $y = 27,9x^3-176,1x^2+338,1x-97,3$ $R^2 = 0,99$
					B: $y = 36,7x^3-236,1x^2+475,4x-187,3$ $R^2 = 0,99$
Mean	3.58±1.4	3.76±1.4	8.62	17.62	

The explants originated from the two individuals harvested from Greaca, responded to the *in vitro* culture either by differentiating of higher shoots from apexes (Vs10), or by a higher proliferation capacity from axillary buds (Vs3eb).

With the two accessions from Letea Forest were noticed differences regarding the moment of initiation the regenerative processes into the meristematic explants. Letea 2 showed a late response, more evident with apexes. After the third transfer on fresh media for multiplication of Letea 1 and Letea 2, the recorded data proved a similar behavior, these two wild grapevine accessions achieving almost the highest rates of proliferation.

These results confirmed previous observations about the *in vitro* development for wild grapevine accessions (Popescu *et al*., 2013) and proved the differences among investigated genotypes harvested from different populations, or from the same location, regarding the development of proliferative structures and the rate of multiplication. The two types of meristems (apical and lateral) contain cells with embryonic characteristics and expressed their cell totipotentiality by maintaining the cell division and by differentiating into shoots under *in vitro* culture (Cachiță-Cosma, 1987). The evolution of the two types of meristematic explants on the *in vitro* multiplication media, represented by polynomial regression, revealed a normal and adequate development for all tested wild grapevine accessions. The number of new buds and shoots increased progressively with each transfer on fresh medium, but each genotype responded by different rate of multiplication.

b) The competence of regeneration by organogenesis from petiole culture

The processes of regeneration from petiole were expressed in a similar way for all tested genotypes of wild grapevine, either by callus induction in a high proportion without any kind of differentiation on media with BAP and 2,4-D, or by necrosis of petioles on medium containing only BAP as growth regulator (data not shown). Tissue differentiation from the cut ends of petioles and *de novo* formation of meristematic structures from already differentiated tissues has proven to be dependent by genotype and interaction between genotypes and culture medium components. An interesting observation was that the colour and disposition of the calli on the inoculated petioles were the same for accessions harvested from the same location. The calli did not differentiate new structures and later on either necrosed, or maintained their proliferation.

The best percentages of new vegetative structures were obtained when the explants were inoculated on medium containing BAP and IBA. The callus induction was promoted during the first four weeks under darkness. The new regenerated structures (buds, or multiple small shoots) were noticed after transfer on a fresh medium and maintained under light photoperiod. In Table 3 are presented the results obtained after five months of *in vitro* culture of petiole explants.

Relative small amounts of IBA in combination with moderate quantities of BAP were efficient to induce the dedifferentiation at the cut ends and also to trigger the organogenesis processes. During the next four passages, new buds and multiple shoots were regenerated through direct organogenesis from one or both ends of the petioles. After six months from petiole initiation were obtained normal developed plants, able to be transferred to *ex vitro* conditions, from all tested accessions, and the recorded values varied between 5.3% for Vs10 and 17.2% for Letea 2.

c) The competence for regeneration by somatic embryogenesis from anther tissues

The anther culture was tested three years consecutively using different media composition. The best results were obtained by applying the same procedure presented in Table 3. Somatic embryos and whole plants regeneration has been obtained only from individuals with male flowers, and only with three out of the seven tested genotypes (Fig. 2). Our results with wild grapevine confirmed those of Rajasekaran and Mullins (1983), who emphasized the importance of the type of flower for anther culture with grapevine and reported that genotypes with functionally male flowers express a higher embryogenic potential of anthers in comparison with genotypes having hermaphrodite or functionally female flowers.

Table 3.

The differentiation potential from somatic tissues obtained with tested wild grapevine accessions

Type of explant	Accession	Type of regeneration/ Specifications	% from initiated explants	Media composition
Petiole starting from 100 explants	Vs1	Brown calli at the cut ends Direct regeneration of buds	29.1 9.7	Initiation: M&S +1.5 mg/l BAP + 0.1 mg/l IBA + 3% sucrose Passages: M&S + 1.0 mg/l BAP + 0.5 mg/l IAA + 2% sucrose
	Vs8dm	White calli at one cut end Direct regeneration of shoots	87.5 6.3	
	Vs9dm	White calli at the cut ends Direct regeneration of shoots	44.2 10.5	
	Vs10	White calli at one or both cut ends Direct regeneration of shoots	87.2 5.3	
	Vs3eb	White calli at one or both cut ends Direct regeneration of shoots	32.9 7.9	
	Letea 1	White-brownish calli at the cut ends Direct regeneration of shoots	69.1 12.7	
	Letea 2	White-brownish calli at the cut ends Direct regeneration of shoots	79.3 17.2	
Anthers from indivi-duals with male flowers starting from 200 anthers	Vs1	Calli with embryogenic potential % Normal somatic embryos % Abnormal embryos Number of whole plant regenerated by embryogenesis / % of total somatic embryos	18 43.3 56.7 101 / 31.7	Initiation: M&S + 1.0 mg/l BAP + 0.5 mg/l 2,4-D + 3% sucrose Passages: modified M&S +1.0 mg/l BAP + 0.5 mg/l IAA + 2% sucrose
	Vs8dm	-	-	
	Vs9dm	Calli with embryogenic potential % Normal somatic embryos % Abnormal embryos Number of whole plant regenerated by embryogenesis / % of total somatic embryos	0.5 33.3 60.1 1 / 15	
	Vs10	-	-	
	Vs3eb	Calli with embryogenic potential % Normal somatic embryos % Abnormal embryos Number of whole plant regenerated by embryogenesis / % of total somatic embryos	3.5 73.2 26.8 23 / 56.1	
	Letea 1	-	-	
	Letea 2	-	-	

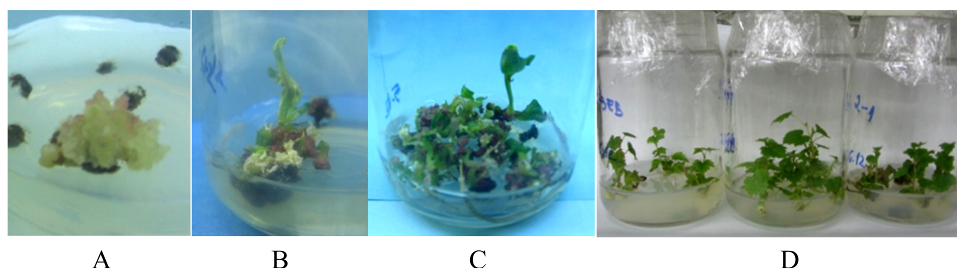

Figure 2. Phases of somatic embryogenesis in anther culture with wild grapevine: A) callus formation; B) regenerated plant with secondary embryos; C) different type of somatic embryos; D) regenerated plant of Vs3eb, Vs9dm and Vs1.

Beside the genetic factors (genotype and type of flower), the induction of somatic embryogenesis from anther tissues could be optimized by media culture composition. Somatic embryogenesis appears to be dependent on the interaction between genotype, explants source and culture medium. Many experiments proved the necessary to develop specific regeneration protocols for each *Vitis* species and *V. vinifera* cultivar (López-Pérez *et al*., 2005). In our case, althought were tested different media composition (data not shown) for anthers initiation, callus induction and promoting the embryogenic process, only three accessions responded by regeneration through embryogenesis on all used media, and with similar results.

The callus derived from anther walls could maintain their proliferative capacity for a long period of time by periodic transfer on fresh media without any other result. But for further evolution is important only the embryogenic callus, which must be selected from the non-embryogenic one and transferred on specific media for embryogenesis induction, the maintenance of embryogenic potential and the somatic embryos germination.

From the total number of somatic embryos, a relative high proportion (between 56.7% for Vs1 and 73.2% for Vs3eb) was formed by abnormal embryos, without ability to develop into normal plants, such as: tube-shaped embryos, cup-shaped embryos, fused embryos, embryos with secondary embryos, or with more cotyledons (data not shown).

The normal somatic embryos became green and developed cotyledons and roots simultaneously. The number of whole plants regenerated from the embryos germinated on media containing moderate quantities of BAP and IAA depend on the number of clusters of normal embryos developed on transferred explants, and also on the proportion of cotyledonary embryos. Thus, a relative high proportion (between 15% for Vs9dm and 56.1% for Vs3eb) from the total somatic embryos regenerated from anther walls, developed into morphologically normal plants, physiologically able to be transferred to *ex vitro* condition (Table 3). The three genotypes, Vs1, Vs9dm and Vs3eb, showing embryogenic competence by somatic cells could be recommended for anther culture, and the other wild grapevine accessions as recalcitrant, or not able to respond to this type of *in vitro* culture.

Conclusions

Similar with cultivated grapevines, the wild grapevine (*Vitis vinifera* subsp. *sylvestris*) responded to *in vitro* culture by organogenesis (in the case of meristematic tissues, or petiole explants), or by embryogenesis (in the case of anther culture).

The genetic factors, culture media composition, added plant growth regulators, type of explants are important for regeneration from meristems and somatic tissues influencing the efficiency of these regenerative processes.

The meristematic structures (apexes and axillary buds) proved to be efficient for large-scale propagation of wild grapevine, aiming to regenerate healthy plants for long-term conservation.

The regenerative potential by organogenesis from petiole explants was comparable among tested genotypes, proving the strong correlation between regenerative competence of this type of explant and media composition supplemented with low quantities of auxin and moderate of citochinine.

The genetic factors (genotype and characteristics of sexual organs of the flowers) are essential in promoting the regenerative process into inoculated anthers. Only three accessions of *Vitis vinifera* subsp. *sylvestris* regenerated whole plants by embryogenesis and only from individuals with functionally male flowers.

The regenerated plants obtained from somatic embryos, either by direct germination on anther walls, or by indirect germination from anther-derived-calli, are valuable for further studies on their genetic uniformity or variability.

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