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Preliminary Characterization of Wild Grapevine Populations (*Vitis vinifera* ssp. *sylvestris*) Grown Along the Danube River

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Abstract

The individuals belonging to three different groups of wild grapevines populations *Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi harvested along, or near the Danube River, were described by means of usual ampelographic methods. The twenty standardized descriptors used for morphological analysis revealed obvious differentiation among analyzed populations. Out of 65 individuals, a half produced flowers with separate sex and a high proportion of them were males (70%). Pollen measurements on light microscope provided information on differences in pollen size among inside wild grapevine populations of *V. sylvestris* with the polar length varying between 15.3 and 23 µm and the equatorial length between 15.5 and 24.4µm. The *in vitro* regenerative potential from meristematic tissue tested with each phenotype showed that the moment of differentiation, the aspect of proliferative structures and the rate of multiplication varied inside these wild grapevine populations, without any correlation with the location of harvesting. Our results provided valuable information about these *Vitis vinifera* ssp. *sylvestris* populations, possible to be used as starting plant material for research in general and further breeding of cultivars and grapevine rootstocks.

Keywords: ampelographic descriptors, diversity, *in vitro* regeneration, pollen morphology

Introduction

Nowadays, wild populations of *Vitis vinifera* ssp. *sylvestris* (Gmelin) Hegi represent an interesting subject for study, in strong correlation with its cultivated forms (ssp. *vinifera*). The main subjects investigated by different groups of researchers, and considered to be of general interest, could be divided as follows: morphological aspects for a complete description of wild populations (Arnold, 1999; Ocete, 2010), studies on the genetic diversity by molecular markers (Grassi *et al*., 2003; Grassi *et al*., 2006; Labra *et al*., 2002; This *et al*., 2004), phytosanitary survey of European wild grapevines (Ocete *et al*., 2011), controversial aspects of *Vitis* genera historical origin (Arroyo-Garcia *et al*., 2006; Sefc *et al*., 2003), evaluation of the genetic distinction between wild and domesticated grapevines (Zecca *et al*., 2010).

In Romania, wild species of *Vitis* were briefly studied, and mainly from the botanical point of view (Pop, 1931; Teodorescu *et al*., 1966). The existence of wild populations of *V. sylvestris* overlapped with *V. vinifera* in almost all Romanian provinces has been mentioned in several papers (Iacob and Scorei, 1997; Popa *et al*., 2007). Detailed description of these plants and also the first attempt to conserve the *Vitis vinifera* ssp. *sylvestris* from the southern part of Romania within germplasm collection was described by Popa *et al*. (2009). In a documented paper, Iacob and Scorei (1997) presented the history of research concerning Euro-Asiatic grapevines, the theories about its phylogeny and the spread areas of these populations in Europe, and in Romania. The authors underlined that *V. sylvestris* as wild grapevines is present as native populations only in restricted areas. Therefore, an inventory of *V. vinifera* ssp. *sylvestris* and monitoring the existing populations should represent a priority for the national research programmes. It represents also the first step required for a complete characterization of the genetic diversity of this subspecies on the Romanian territory.

Taking into consideration that wild grapevines are described as small populations in certain ecosystems in Romania and their habitat is endangered due to reducing the protected areas, or by the enlargement of the surfaces taken under-construction with different purposes, we decided to initiate collecting of wild grapevine individuals and their preservation in *ex situ* collection. The goal of this paper is to present and describe some characteristics of the individual plants collected from wild grapevine populations belonging to the *sylvestris* subspecies.

Materials and methods

The individuals belonging to different populations of *Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi were harvested (as canes) from three geographic locations along, or near the Danube River (Tab. 1). The harvested canes were used to obtain potted plants and after that to establish an *ex situ* collection of wild grapevines. Twenty standardized descriptors were used for morphological analysis of each plant obtained by vegetative multiplication, specific indicators for young shoots, shoots, young and mature leaves: OIV 001, 002, 007, 008, 016, 017, 051, 053, 065, 067, 068, 070, 076, 079, 081.2, 082, 084, 087, 093, 094.

Tab. 1. Sampling locations and number of analyzed wild populations

Plants from	9 different individuals	2 different individuals	5 different individuals
Location	Stârmina Forest	Hinova	Greaca
Geographic coordinates	Long. E 22º 46' 14" Lat. N 44º 30' 01"	Long. E 22° 46' 36" Lat. N 44° 32' 26"	Long. E 26° 20' 21" Lat. N 44° 6' 33"
Mean altitude (m)	118	100	60

Pollen grains from different plants collected from the three locations were examined and measured using an Olympus CX35 light microscope and CellB software for microscopy. Bud flowers were collected just before anthesis and dried at room temperature. For the microscopic observations on pollen size, the samples were stained in 1% acetocarmine and the diameters were measured with a combination of x10; x40 lens for at least 200 pollen grains in each wild grapevine producing flowers.

In order to propagate the collected wild grapevine genotypes, young growth shoots of 2-3 cm, with apex and 1-2 axillary buds, were harvested from potted plants. The surface sterilization of explants was performed 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. The culture media was Murashige and Skoog (M&S) salts supplemented with 0.5-1.0 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l indole-3-acetic acid (IAA). For rooting media was used a half salt concentration M&S media with 1.8 mg/l IAA and 0.022 mg/l kinetin (Vişoiu *et al*., 2000). In all type of media were added: 10 mg/l ascorbic acid, 2-3% sucrose and 5.5% agar. The culture media was autoclaved for 20 min at 121 °C and 1.2 bars and the cultures with meristematic tissues (the apex, or one axillary bud) were maintained under 16-h photoperiod and a light intensity of 3,000 lux and 24±1 °C temperature.

After the initial 30 days on the establishment medium, the new regenerative structures were transferred periodically on fresh medium with reduced sucrose concentration and modified growth regulators composition.

During explants development, the mean number of shoots possible to be multiplied or to be rooted, qualitative aspects related to shoot aspect, colour, vitrification, or callus formation were recorded before each transfer. All the data were statistically analyzed and the obtained regression (linear or polynomial) was considered as an indicator of the *in vitro* regenerative process.

Results and discussion

a) Morphological characterization based on OIV descriptors

The first and summary description of wild grapevine plants was performed at their original habitats, into the forests and wetland areas, with high humidity, often located along the rivers. In one of the habitats, an infestation caused by *Eryophid* mites on the leaves was noticed. No symptoms caused by the subterranean cycle of phylloxera, *Daktulosphaira vitifoliae* (Fitch) (Hemiptera, Phylloxeridae) were found in any areas, presumably due to the forest presence surrounding wild grapevine individuals and to the high level of moisture of the soils, preventing the presence of pathogenic insect. In one case, leaf symptoms of phosphorus deficiency were found, which were attributed to a possible favourable combination of low pH and high levels of iron and aluminium in the soil.

The morphological aspects were registered during the first year of growth to the plants established into the *ex situ* collection. The ampelographical analysis of plants with the OIV morphological descriptors were compared with specific features for *Vitis vinifera* subsp. *sylvestris*. In the Tab. 2 are presented the main features noticed for all analyzed plants as comparison. Two characteristics (number of consecutive tendrils in shoots OIV016, and petiole sinus base limited by vein in mature leaves OIV081-2) were registered to be common for all analyzed populations. Another common character for all studied plants was the occurrence of unisexual flowers, male or functional female flowers on different individuals. Interesting was one individual in the population from Greaca had white skin colour of berries, while all the other individuals producing blue-black berries (not shown in the table).

Variable character differing among individuals within populations from each location were noticed for certain traits related to: the length of tendrils (OIV017), the shape, number of lobes, and color of the upper side of mature leaves (OIV067, 068, 069), and the distribution of anthocyanin coloration on the shoot tips (OIV003) and on the main veins of upper side of blade (OIV070) main veins. Our results revealed also differences between groups of plants from the three locations regarding: the opening of the shoot tip OIV 001, the colour of upper side of young blade (4th leaf) OIV051, density of prostrate hairs between main veins on lower side of the 4th leaf young leaf

OIV053, the density of prostrate hairs between main veins on lower side of mature blade OIV084, density of erect hairs on main veins on lower side of blade OIV087, and depth of upper lateral sinuses in mature leaf OIV094.

Our obtained data based on phenotype evaluation were symilar with those obtained by Ekhvaia and Akhalkatsi (2010) on morphological variability of wild grapevine populations from Georgia. It seems the common characters of all studied populations and the scale of variability within and among populations and individuals are similar with our data.

Although only a limited area of wild grapevine populations was analyzed in this study, our results suggests that substantial diversity has been maintained into the surveyed habitats. These populations need to be protected and efficiently used, either for breeding programmes, or to avoid genetic erosion.

Tab. 2. Ampelographic characters of young shoots, shoots, young and mature leaves of wild grapevine plants from different populations

Code	Stârmina Forest	Hinova Forest	Greaca
OIV 001	5	1	1, 5
OIV 002	1	1, 2	2
OIV 003	3	1, 3	1, 3
OIV 007	3	3	2, 3
OIV 008	2, 3	2, 3	2, 3
OIV 017	7, 9	3, 5	5, 7, 9
OIV 051	2	2	3
OIV 053	3	1	3
OIV 065	5	5	3, 5
OIV 067	3, 4	1, 2	4
OIV 068	3	1, 2	1, 3
OIV 069	5	5	3, 5, 7
OIV 070	1, 2	3	2, 3
OIV 076	2	4	2,3
OIV 079	3	1, 3	3
OIV 082	2	5	2
OIV 084	3	1	3
OIV 087	3	3	1, 3
OIV 093	5, 7	3	5, 7
OIV 094	5	1	5

b) Biometric parameters of pollen grains

Previously palynological studies on grapevine had the main objective to describe the morphological features and to establish intervals of variation of equatorial axis and longitudinal distance of pollen grains in *Vitis vinifera* cultivars in general (Roytchev, 1995). In addition, Gallardo *et al*. (2009) reported the pollen grain dimorphism in 14 different populations of *Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi from Spain. Our results with pollen samples collected from different plants revealed a relative low variation in size of pollen grains (polar axis x equatorial axis). Although the wild populations of grapevines are located at long distances between them, the measurements on pollen grains showed little variation around the specific mean values (Tab. 3). Thus, the polar length varied between 15.3 and 23 μm, with a maximum frequency of 45-50% for pollen grains having a diameter of 19-21 μm ± 1.2. Similarly, the equatorial length varied between 15.5 and 24.4 μm, with a maximum frequency of pollen grains having a diameter of 19-21 μm ± 1.6. For instance, these values are similar to those obtained by Gallardo *et al*. (2009), and are smaller than those specific for *Vitis vinifera* L. subsp. *vinifera*.

Out of 65 obtained and analyzed individuals, in the first year, a half of them produced flowers with separate sex and a high proportion were males (70%). It was noticed that the female flowers produced pollen grains without germination structures (acolporated) and the average dimensions were lower than those from male flowers, ranging between 16.1 and 17 μm.

The studies carried out so far indicate that all analyzed populations produce pollen with size dimorphism similar to wild grapevines, as do *Vitis vinifera* ssp. *sylvestris*.

Also, the measurements have shown the genetic stability of wild grapevine populations in the surveyed areas, and also revealed their great similarity in terms of pollen characteristics.

c) The in vitro regenerative potential of wild grapevine plants

The *in vitro* regenerative potential from meristematic tissues was tested with each analyzed phenotype (Tab. 4). The applied micropropagation protocol was the common one used for *Vitis vinifera* cultivars. With this method, the development and growth of explants proved to be appropriate and the new regenerated plants were obtained from all harvested populations. According to our observations during the *in vitro* development, the moment of differentiation in inoculated explants, the aspect of proliferative structures and the rate of multiplication varied inside each population, without any correlation with the sampling locations.

Thus, the meristematic structures belonging to the six different populations from Stirmina showed the lowest regenerative potential, or rate of multiplication. Normal shoots of 1.5-2.0 cm height and able for rooting were obtained from these populations after 88-150 days of cultures (Fig. 1a).

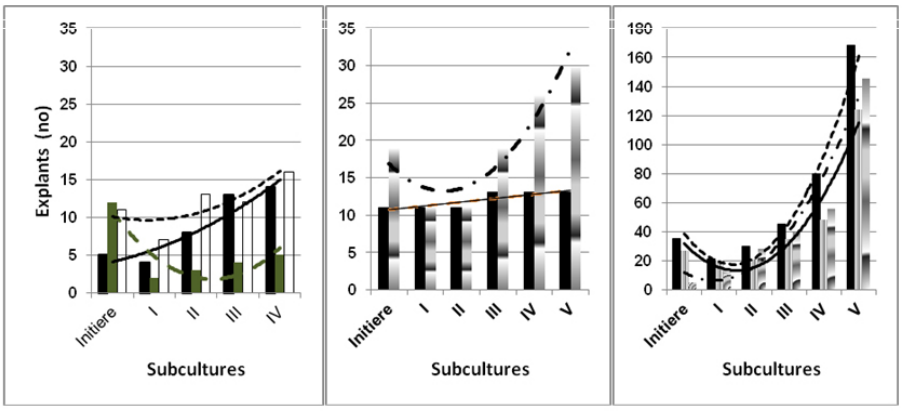
Other two populations from Greaca and one from Hinova revealed relatively higher regenerative potential from meristematic tissues, with an obvious increasing rate of shoot multiplication after the end of the third subculture. Even though they were very good for rooting, the normal shoots of 2.0-2.5 cm height expressed often vitrification symptoms (Fig. 1b).

Tab. 3. Pollen parameters in grapevine wild populations

Location		Interval values (µm)					
		12.5-15.4	15.5-17.4	17.5-19.1	19.2-20.9	21.0-22.6	22.7-24.4
Hinova	Average 20.2		16.4	18.3	20.1	21.6	24.2
	St. Dev. 1.5		0.7	1.2	1.2	0.9	0.5
	Frequency %		2.3	17.7	51.5	26.2	2.3
Starmina	Average 19.5		16.8	18.3	20.0	21.8	
	St. Dev. 4.8		0.4	1.1	1.2	1.1	
	Frequency %		2.7	45.5	33.9	17.8	
Greaca	Average 19.2		16.9	18.4	20.0	21.0	
	St. Dev. 1.3		0.7	0.9	1.2	0.3	
	Frequency %		6.4	42.2	44.9	6.4	
b) Vertical diameters (µm)							
Location		Interval values (µm)					
		12.5-15.4	15.5-17.4	17.5-19.1	19.2-20.9	21.0-22.6	22.7-24.4
Hinova	Average 20.0	15.3	16.3	18.5	19.9	21.6	23.0
	St. Dev. 1.8		0.2	0.8	1.2	1.3	0.2
	Frequency %	0.8	1.5	24.6	47.7	21.5	3.8
Starmina	Average 19.6		17.2	18.3	20.0	21.9	
	St. Dev. 4.7		0.2	1.2	1.2	1.4	
	Frequency %		1.8	40.2	41.9	16.0	
Greaca	Average 19.5		16.9	18.3	20.0	21.3	
	St. Dev. 3.9		0.5	1.2	1.2	0.4	
	Frequency %		1.8	34.9	56.9	6.4	

Tab. 4. Polynomial regression and coefficient of regression calculated for grapevine wild populations

Symbol of populations	Polynomial regression	Coefficient of regression R^2
1 Hrinova	$y=0.3571x^2+0.5571x+3.2$	0.9020
6 Hrinova	$y=1.5714x^2-10.629x+19.8$	0.7796
9 Greaca	$y=0.6429x^2-2.357x+11.8$	0.6609
8 dm Starmina	$y=0.5143x+10.2$	0.7714
10 Greaca	$y=1.5714x^2-7.9143x+23.2$	0.8705
3eb Greaca	$y=7.4107x^2-27.618x+32.1$	0.9302
9dm Starmina	$y=10.964x^2-52.264x+79.8$	0.9807
14 Greaca	$y=8.2679x^2-41.218x+64.7$	0.9411



a. Populations 1, 6 and 9 b. Populations 8dm and 10 c. Populations 3eb, 9dm and 14

Fig. 1. *In vitro* apex induction and the rate of multiplication with explants from different populations of *Vitis vinifera* L. subsp. *sylvestris* (Gmelin)

Plant material of one population from Hinova and two populations from Greaca expressed the highest rate of *in vitro* multiplication. After the fifth subculture were obtained between 10 and 28 of shoot clusters/inoculated explants (Fig. 1c).

Although the culture media assured the establishment of explants and normal development of morphological structures and multiple axillary shoots, significant differences have been found among explants derived from different populations in the regenerative induction processes.

The estimation of apex explants development on tested media by polynomial regression analysis revealed an adequate evolution for each wild grapevine individual subjected to *in vitro* propagation. The best results with the highest rate of multiplication were achieved with apex explants belonging to one population from Starmina and two populations from Greaca.

With axillary buds for culture initiation, similar results were obtained on tissue cultures, but with a slightly higher rate in the multiplication phase (data not shown), which can be suitable for our purposes in further multiplication and stress testing of wild grapevine populations.

Suitable sized shoots of 2.0-2.5 cm were periodically separated and transferred on rooting media. In all cases, with all tested populations, the rooting was promoted after 10-12 days of culture and subsequently the whole grapevine plants regenerated *in vitro* were successfully acclimatized.

Conclusions

The 65 plants belonging to different populations of *V. vinifera* subsp. *sylvestris* (Gmelin) Hegi have been analyzed for morphological characters of young shoot, young leaf and mature leaf. Although the results concern the characteristics expressed by the wild grapevines during the first year of culture, our observations showed some variability within and among studied populations. Therefore, more detailed analyses should be done in the next years in order to fully characterize, morphologically and molecularly, the wild grapevine individuals established in our germplasm collection. We have to take into consideration that the plants were removed from their specific environment and need longer period to express entirely their phenotype traits.

The morphological description, the microscopic measurements on pollen grains and the *in vitro* regenerative potential showed by the wild grapevine plants indicate high polymorphism among populations and also among individuals within each population.

Overall, our results showed that the plants collected from the three locations, although at long distances among them, are different in terms of some morphological features, but seems to be closely related to each other in terms of pollen characteristics.

The new accessions of wild grapevine in our germplasm collection represent a source of valuable plant material for further genetic characterization, and also for biotic and abiotic stress tests. They offer the opportunity to new approaches in associating genotype studies with phenotype evaluation and could ensure appropriate gene resources to specific breeding objectives.

Moreover, the wild populations of grapevine from which samples were collected could be considered as an important source of information about natural mutants possible to be used as resources of plant material for adaptation to environment changing.

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