VINIFERA GENOTYPE BREEDING FOR RESISTANCE TO DOWNY MILDEW BY INTER-SPECIFIC HYBRIDIZATION USING IRRADIATED POLLEN

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ABSTRACT

In order to transfer resistance genes in a *vinifera* variety genome, hybridizations with irradiated pollen were carried out between the following genotypes: Niagara white, Concord and Buffalo, and vinifera variety Donaris recently created. Three rates of gamma irradiation were used: 10 Gy, 20 Gy and 25 Gy. The lethal rate of 50% ranged in between 20 Gy and 25 Gy in case of the hybridization between Donaris and Niagara and between 10 Gy and 20 Gy in case of the other two hybrid combinations. This parameter was determined by taking into account the number of the ovules which differentiated embryos and their ability of development into plants. In all hybrid combinations, the phytohormones added in the culture medium influenced on the ability of differentiating embryos and their evolution into plants. *In vitro* culture of the embryos caused a significant increase in the number of embryos which differentiated, in all combinations and treatments used. Under *in vivo* conditions, the embryo formation rate was poorer, but it increased twice or thrice in case of *in vitro* culture of the embryos. These results prove that the method of *in ovulo* embryo culture is an efficient method for the interspecific hybrid regeneration. Analysis and selection of plants with resistance to *Plasmopara viticola* was made by applying the test of the foliar disks (method Stoudt & Kassenmeyer, 1995). Among 470 plants which were analysed, 73 (15.5%) were selected as having increased resistance to the attack of the pathogen.

Key words: interspecific hybridization, irradiated pollen, resistance to downy-mildew, *Vitis vinifera*.

INTRODUCTION

Conventional breeding for resistance to diseases is based on the combination through hybridization of the resistance genes from one parent, with the quality of the other parent. Varieties belonging to *Vitis vinifera* species are characterised by a good quality of the grapes and by sensitivity to diseases. Therefore, their hybridization with resistant species represents the only conventional method that can be used for obtaining resistant grape varieties (Einset and Pratt, 1975; Galet and Morton, 1990). The resistant species which have been successfully used in interspecific hybridization with *Vitis vinifera* are the following: *Vitis aestivalis* Michx (resistant to downy mildew, powdery mildew, phylloxera and Pierce's disease), *Vitis berlandieri* Planch. (resistant to grey rot, downy mildew, powdery mildew, phylloxera and Pierce's disease), *Vitis candicans* (resistant to grey rot, downy mildew, powdery mildew, Pierce's disease and nematodes), *Vitis labrusca* L. (resistant to downy mildew and powdery mildew), *Vitis riparia* Michx (resistant to grey rot, downy mildew and powdery mildew and phylloxera), *Vitis rupestris* Scheele (resistant to grey rot, downy mildew, powdery mildew, phylloxera and Pierce's disease) and *Vitis rotundifolia* Michx (resistant to downy mildew, some nematodes and Pierce's disease) (Galet and Morton, 1990).

The conventional methods in breeding for resistance by intra/inter-specific hybridization have the following disadvantages:

- they require a long time as a consequence of the very high degree of heterozygosity of the grapevine and of the strong endogammic pressure (Einset and Pratt, 1975; Alleweldt and Possingham, 1988);
- the very small number of hybrid individuals obtained by hybridization;
- the low germination capacity of the seeds.

Stimulation of the genetic recombination process and of the transfer of the resistance genes in *vinifera* variety genom, increase of the hybrid plant number obtained and acceleration of the breeding process for the resistance may be achieved by associating the traditional hybridization method with the *in vitro* rescue technique of the immature embryos.

The rescue technique of the *in vitro* embryos was successfully used for breeding seedless grape varieties (Cain et al., 1983; Spiegel-Roy et al., 1985; Gray et al., 1987; 1990; Goldy and Amborn, 1987; Tsolova, 1990), but also in order to increase the number of interspecific hybrids *Vitis* species x *V. vinifera* obtained (Goldy et al., 1988, 1989; Goldy and Amborn, 1987).

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This experiment aimed at associating the conventional breeding method, the hybridization using irradiated pollen and the technique of rescuing the immature embryos in order to obtain interspecific hybrids that present the quality of *vinifera* varieties and the resistance of the species belonging to *Vitis* genre.

MATERIAL AND METHODS

In order to induce the transfer of resistance genes in the genome of *vinifera* varieties hybridization with irradiated pollen between Niagara white, Concord and Buffalo genotypes and the *vinifera* variety donors, recently created.

Three gamma irradiation doses were used: 10 Gy, 20 Gy and 25 Gy. For each dose, five hybridizations were made. The control was represented by plants obtained by hybridizations with not irradiated pollen.

The genetic origin of the genotypes used in the hybridization is the following:

- Niagara white = Concord/Cassady (*Vitis labrusca*);
- Concord = *Vitis labrusca* (free pollination);
- Buffalo = Herbert/Watkins (*Vitis labrusca/ 4* Vitis vinifera*).

The grapes were harvested at three intervals of time postanthesis: 50 days; 65 days and at full ripening (100 days). The grape berries were sterilised for 1 minute in ethylic alcohol 70%, afterwards they were rinsed for 15 minute in a solution of Na hypochlorite containing several drops of Tween 20. The ovules were aseptically excised after 3 - 4 weeks of preservation in cold conditions (4°C) and cultured in Petri dishes containing 20 ml medium.

The culture medium was represented by the basical Murashige & Skoog (1962) added or not by 1 mg/l indolyl acetic acid (IAA) and 0.5 mg/l GA_3. The medium was supplemented with 20 g/l saccharose, solidified with Difco-Bacto agar 8 g/l and adjusted at 5.6 pH before autoclaving.

Three replications of 25 ovules were used in the experiment.

The embryo cultures were maintained for 2 months in a growth chamber at 25°C ± 2°C then they were transferred on a fresh medium.

The observations concerning embryo germination were made 6-7 weeks after inoculation. The ovules which didn't germinate until 31st of January were excised at their calase according to the method belonging to Valdez and Ulanovski (1997) and mashed on a fresh medium. The cultures were maintained for 30 days in the dark. The vine shoots obtained were rooted on a Murashige & Skoog (1962) medium containing macroelements reduced to a half, supplemented with Kinetine – 0.21 mg/l and IAA – 1.18 mg/l.

The data obtained were analysed by using ANOVA test.

RESULTS AND DISCUSSION

Observations made *in vitro*

In all hybrid combinations, embryo prelevation at 50 days after hybridization determined a very low percentage of germination, no matter what dose was used for irradiating the pollen.

The percentage of germination varied within 0-5.3% in case of Donaris/Niagara hybridization and within 0-4.0% in case of the other hybrid combinations.

The ovules which didn't germinate remained green, but the microscopic analyses evidenced the absence of the embryos in most excised immature seeds, and in some cases, the presence of the globular or heart-shaped embryos. These results suggest that 50 days after pollination, the embryos are in a too early stage of development.

The prelevation of the grape berries 65 days after pollination determined a significant growth of the germinated embryos, the germination rate being correlated with the irradiation dose (Table 1).

The lethal dose 50% ranged within 20 Gy and 25 Gy in case of the hybridization between Donaris and Niagara and between 10 Gy and 20 Gy in case of the other two hybrid combinations. This parameter was determined by considering the number of ovules which differentiated embryos and their capacity of becoming plants.The phytohormones added to the culture medium influenced the embryo differentiation capacity and their further development in all hybrid combinations.

The development rate of the plants was smaller on a medium lacking hormones, and was correlated with the irradiation dose. It registered

values ranging within the limits of 8 - 10% in case of the non-irradiated control, within 9.3 - 14.6% for a dose of 10 Gy, within 12 - 22.6% for a dose of 20 Gy, and 6.6 - 12% for a dose of 25 Gy.

Plant development rate significantly increased on a medium added with growth hormones, being also correlated with the irradiation dose. It registered values ranging in between 16 - 36% in case of the control which wasn't irradiated, within 42.6 - 56% for a dose of 10 Gy, 38.6 - 62.6% for 20 Gy and 14.6 - 24% for 25 Gy (Table 2).

Table 1. In vitro germination embryos according to the irradiation dose and the period of inoculation

Period of inoculation	Donaris/Niagara				Donaris/Concord				Donaris/Buffalo			
	0 Gy	10 Gy	20 Gy	25 Gy	0 Gy	10 Gy	20 Gy	25 Gy	0 Gy	10 Gy	20 Gy	25 Gy
50 day	0	4	2.66	5.33	0	4	0	0	1.3	4	1.3	0
65 day	18.6	42.6	62.6	24	16	48	38.6	14.6	36.0	56	41.3	24

Table 2. Plant differentiation through *in vitro* culture of embryos

Dose	Culture medium	Number of inoculated ovules	Differentiated plants					
			Donaris/Niagara		Donaris/Concord		Donaris/Buffalo	
		No.	No.	%	No.	%	No.	%
0 Gy	a	75	14	18.6	12	16.0	27	36.0
	b	75	6	8.0	8	10.6	8	10.6
10 Gy	a	75	32	42.6	36	48.0	42	56.0
	b	75	11	14.6	12	16.0	7	9.3
20 Gy	a	75	47	62.6	29	38.6	31	41.3
	b	75	17	22.6	9	12.0	9	12.0
25 Gy	a	75	18	24.0	11	14.6	18	24.0
	b	75	9	12.0	5	6.6	5	6.6

a) medium without hormones
b) medium added with hormones

The highest number of hybrid plants (Donaris/Niagara and Donaris/Concord) were obtained after callus formation around the vine explants, but the regenerated plants did not originate from that callus. The irradiation dose significantly influenced upon embryo germination capacity, the highest number of plants being obtained in case of the dose of 20 Gy for the combination Donaris/Niagara (52%) and of 10 Gy for Donaris/Concord (28%) and Donaris/Buffalo (18.6%) (Table 3).

Table 3. Germination of *in ovulo* embryos after callus formation

Dose	Culture medium	Number of inoculated ovules	Donaris/Niagara germination		Donaris/Concord germination		Donaris/Buffalo germination	
		No.	No.	%	No.	%	No.	%
0 Gy	a	75	6	8.0	8	10.6	9	12.0
	b	75	5	6.6	6	8.0	3	4.0
10 Gy	a	75	18	24.0	21	28.0	14	18.6
	b	75	7	9.3	8	10.6	1	1.3
20 Gy	a	75	39	52.0	20	26.6	12	16.0
	b	75	14	18.6	6	8.0	4	5.3
25 Gy	a	75	12	16.0	6	8.0	6	8.0
	b	75	6	8.0	3	4.0	1	1.3

a) medium without hormones
b) medium added with hormones

In the hybrid combination Donaris/Buffalo, the highest number of plants developed through the direct germination of the ovules (Table 4). Direct germination occurred 6-7 weeks after inoculation, when the rootlets appeared and some very large cotyledons differentiated, often presenting morphologic modifications (shape of a cup, absence of hypocotyle, thickness).

Polyembryonic ovules were obtained in a small percentage (0 - 6.6%). The number of plants differentiated from a single ovule ranged between 2-11 in case of Donaris/Niagara, 3-8 in case of Donaris/Concord and 2-7 for Donaris/Buffalo (Table 5).

Table 4. Direct germination of embryos *in ovulo*

Dose	Culture medium	Number of inoculated ovules	Donaris/Niagara germination		Donaris/Concord germination		Donaris/Buffalo germination)	
			No.	%	No.	%	No.	%
0 Gy	a	75	8	10.6	4	5.3	18	24.0
	b	75	1	1.3	2	2.6	5	6.6
10 Gy	a	75	14	18.6	15	20.0	28	37.3
	b	75	4	5.3	4	5.3	6	8.0
20 Gy	a	75	8	10.6	9	12.0	19	25.3
	b	75	3	4.0	3	4.0	5	6.6
25 Gy	a	75	6	8.0	5	6.6	12	16.0
	b	75	3	4.0	2	2.6	4	5.3

a) medium without hormones
b) medium added with hormones

Table 5. Differentiation of the polyembryonic ovules

Dose	Culture medium	Number of inoculated ovules	Donaris/Niagara polyembryonic ovules		Donaris/Concord polyembryonic ovules		Donaris/Buffalo polyembryonic ovules	
			No.	%	No.	%	No.	%
0 Gy	a	75	5	6.6	1	1.3	2	2.6
	b	75	4	5.3	0	0	0	0
10 Gy	a	75	4	5.3	5	6.6	4	5.3
	b	75	1	1.3	4	5.3	0	0
20 Gy	a	75	0	0	2	2.6	0	0
	b	75	2	1.5	0	0	1	1.3
25 Gy	a	75	2	2.6	0	0	0	0
	b	75	0	0	0	0	0	0

a) medium without hormones
b) medium added with hormones

***In vivo* observations**

Seed germination was rather poor in all hybrid combinations, with values between 12.5% (0 Gy) – 20.95% (25 Gy) in case of Donaris/Niagara, 19.28% (0 Gy) – 23.8% (25 Gy) in case of Donaris/Concord and 15.16 % (0 Gy) – 16.31% (25 Gy) in case of Donaris/Buffalo. In all cases, the smallest percentage of germination was registered for the control (0 Gy) and when the dose of 25 Gy was applied. A stimulation of germination was noticed in case of the dose of 10 Gy (Table 6).

Table 6. Results concerning the germination of *in vivo* embryos

	Donaris/Niagara	Donaris/Concord	Donaris/Buffalo

Dose	Number of seeds obtained	Germinated seeds		Number of seeds obtained	Germinated seeds		Number of seeds obtained	Germinated seeds	
		No.	%		No.	%		No.	%
0 Gy	176	22	12.5	140	27	19.28	178	27	15.16
10 Gy	400	102	25.5	300	110	36.6	650	215	33.0
20 Gy	253	79	31.2	182	47	25.8	250	71	28.4
25 Gy	525	110	20.95	378	90	23.8	190	31	16.31

The beginning and the development of the vegetation periods were delayed, being correlated with the irradiation dose.

The germination of the seeds occurred 3-8 days later in comparison with the control which was not irradiated, and it was delayed with 14-47 days in case of Donaris/Niagara, 14-32 days in case of Donaris/Concord and 13-51 days in case of Donaris/Buffalo (Table 7).

Table 7. Dynamics of seed germination

Dose	Germination period (days) and Germination (%)												
	1	4	9	11	18	23	27	31	37	41	49	59	69
	Donaris/Niagara												
0 Gy	18.18	13.6	9.0	27.27	9.0	22.70							
10 Gy		35.2	13.7	19.60	11.7	9.80	3.90	2.94	1.96	0.90			
20 Gy	3.79	11.39	20.25	17.70	30.37	6.32	3.79	2.53	1.26	1.26	1.26		
25 Gy			15.45	24.50	19.0	15.45	8.18	2.70	3.60	2.70	3.60	2.7	1.8
Donaris / Concord													
0 Gy	29.6	11.1	14.80	18.5	7.40	3.7							
10 Gy			16.36	14.5	24.50	19.0	5.45	7.27	5.45	2.70	2.7	1.8	
20 Gy	4.2	10.6	6.38	21.27	29.78	6.38	8.50	6.38	4.25	2.12			
25 Gy		1.1	11.10	18.8	32.20	0	3.33	2.20	2.20	1.10	1.1	1.1	
Donaris / Buffalo													
0 Gy	11.1	18.50	33.30	29.6	7.4								
10 Gy	6.51	8.37	21.86	12.0	27.4	6.51	3.72	2.79	4.65	2.32	2.79	0.46	0.46
20 Gy			12.60	23.9	40.8	25.3	22.5	2.80					
25 Gy		6.45	12.90	6.45	22.5	32.2	3.22	9.67	3.22	3.22			

The dose of 10 Gy had a stimulating effect on plant emergence, except for the hybrid combination Donaris/Concord which showed a large number of plants which emerged when the dose applied was 20 Gy. When compared to the control, the differences were significant both for the dose of 10 Gy, and for that of 20 Gy. The application of a dose of 25 Gy had as a consequence the emergence of a smaller number of plants in all hy brid combinations, the differences in comparison with the control being significantly negative (Table 8).

Table 8. Effect of the irradiation dose upon plants emergence

Genotype	Emerged plants (%) / Dose			
	0 Gy	10 Gy	20 Gy	25 Gy
Donaris/Niagara	59	63.75	62	54.5
Donaris/Concord	70.3	62.7	82.9	68.8
Donaris/Buffalo	66.6	80.9	61.9	54.8

The plant emergence began 4-10 days later in comparison with the control and was delayed with other 9-16 days, depending on the genotype and dose (Table 9).

Table 9. Dynamics of the emergence

Dose	Emergence period (day) and Emerged plants (%)

	5 May	9 May	15 May	17 May	21 May	24 May	31 May	5 June	10 June
Donaris/Niagara									
0 Gy	30.7	23	15.3	38.46	7.7	23			
10 Gy	33.8	15.4	12.3	9.23	15.4	7.69	6.15		
20 Gy			34.6	4.08	22.5	8.16	10.2	10.2	
25 Gy			3.33	20	26.6	13.3	10	15	11.6
Donaris / Concord									
0 Gy		36.8	26.3	15.78	21	5.26			
10 Gy	14.5	20.3	14.5	17.4	8.7	17.4	7.25		
20 Gy	10.25	46.1	10.25	7.7	10.25	7.7	5.12	2.56	
25 Gy		11.3	9.7	14.5	32.2	11.3	12.9	6.45	1.6
Donaris / Buffalo									
0 Gy	77.7	16.3	11.1	22.2	16.6	5.5			
10 Gy	15.8	39	27.9	7.9	6.5	4.65	3.7	3.25	0.5
20 Gy		31.8	20.5	11.36	13.6	11.3	2.27	9.09	
25 Gy			11.8	11.36	9	11.8	0	5.8	

Table 10. Phenotypical modifications induced by the treatment at the level of cotyledons

Dose	Modifications induced at the level of cotyledons (%)					Mutants (%)
	3 - 7 cotyledons	Shape of a cup	Morphologic mutants		modifications of chlorophyllian nature	
			symmetric	asymmetric		
Donaris/Niagara						
0 Gy	7.69		7.69			15.38
10 Gy	12.3	1.5	4.6	3	1.5	21.5
20 Gy	10.2	2	8.16	2	2	22.4
25 Gy	8.3	1.6	1.6	3.3	3.3	18.3
Donaris /Concord						
0 Gy					5.26	5.26
10 Gy	1.4	1.4		1.4		4.34
20 Gy	2.5		10.25	7.7		20.5
25 Gy	3.2		6.4		6.4	16.1
Donaris/Buffalo						
0 Gy						
10 Gy	5.1	0.46	2.3	1.86	0.9	10.69
20 Gy	6.8			2.3	4.5	13.6
25 Gy	1.76	5.8	11.7	1.76		35.29

Table 11. Phenotypical modifications induced by the treatment at the level of the adult plant

Dose	Morphologic mutants (%)				Total number of mutants (%)
	At the level of the leaves:			At the level of the shoot:	
	symmetric	asymmetric	chlorophyllian deficiencies	shortening of the internode length	
Donaris/Niagara					
0 Gy					
10 Gy	3		0.7		3.70
20 Gy		2	6.1		8.16
25 Gy			8.3	5	13.30
Donaris/Concord					
0 Gy	5.26	2.89			8.15
10 Gy		1.60			1.60
20 Gy		11.10	7.7	2.5	21.90
25 Gy	6.4		6.4	1.6	14.40
Donaris/Buffalo					
0 Gy					
10 Gy			2.3	4.5	6.80
20 Gy			1.6	1.76	3.36

25 Gy				

Morphological modifications were noticed in a reduced percentage at the level of the cotyledons (cotyledons having the shape of a cup, the multiplication of the cotyledon number between 3 and 7, chlorophyllian mutants (Table 10) and at the level of the adult plant (symmetric and asymmetric modifications at the level of the leaves, modification of the internode length, chlophyllian mutants) (Table 11).

Comparison concerning the *in vivo* and *in vitro* embryo development

In vitro culture of the embryos had as a consequence a significant increase of the hybrid embryo number which differentiated in all combinations and treatments applied. The development rate of the *in vivo* cultured embryos was lower and increased 2-3 times through *in vitro* culture of the embryos. These results show that the method of *in ovulo* cultured embryos is an efficient method for regenerating the interspecific hybrids.

The analysis and selection of the plants obtained, regarding their level of resistance to *Plasmopara viticola* were made by applying the test of foliar disks (Stoudt and Kassenmeyer method, 1995). Among 470 plants which were analysed, 73 (15.5%) were selected as having an increased resistance to the attack of the pathogen.

Most of the genotypes under breeding were obtained when applying an irradiation dose of 25 Gy, in all hybrid combinations.

CONCLUSIONS

The 50% lethal dose which was determined by considering the number of the ovules which differentiated embryos and their capacity of developing and becoming plants ranged between 20-25 Gy in case of the hybridization between Donaris and Niagara and between 10-25 Gy in case of the other two hybrid combinations.

The highest number of hybrid plants (Donaris/Niagara and Donaris/Concord) were obtained after callus development around the explants, but the regenerated plants did not originate from that callus.

Polyembryonic ovules were obtained in a small percentage (0-6.6%). The number of th plants which differentiated from a single ovule ranged between 2-11 in case of Donaris/Niagara, 3-8 in case of Donaris/Concord and 2-7 in case of Donaris/Buffalo.

Seed germination was rather low in all hybrid combinations. In all cases, the smallest percentage of germination was showed in the control (0 Gy) and when an irradiation dose of 25 Gy was applied. A stimulation of the germination process was noticed when using a dose of 10 Gy.

The beginning and further development of the vegetation periods were delayed, being correlated with the irradiation dose.

In vitro culture of the embryos led to a significant increase in the number of differentiated hybrid embryos in all combinations and treatments. The development rate of the embryos under *in vivo* conditions was lower and increased to 2-3 times through *in vitro* culture of the embryos.

These results show that the method of *in ovulo* culture of embryos represent an efficient method for regenerating the interspecific hybrids.

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