EMBRYOGENESIS IN SEEDLES GRAPES AND HYBRID COMBINATIONS OF (*VITIS VINIFERA L.*)

SOMATIC EMBRYOGENESIS ON SOLID CULTURE IN VITRO - Part II

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ABSTRACT

The possibility for induction of in vitro embryogenesis on solid culture medium from seedless grape cultivars and hybrid combinations, giving an account of the effect of explant age, genotype and growth regulators was investigated. We established that in all studied treatments and genotypes explants isolated 3 days after pollination formed only callus. Comparing the capability for embryo induction, seed buds isolated 10 and 30 days after pollination showed increased regeneration capacity with specifics toward development of somatic or zygotic embryo. The frequency of zygotic embryo formation was higher in the inbreed treatments while the hybrid genotypes produced more somatic embryos. Converting into green plants suitable for micropropagation was observed only from explants isolated 10 and 30 days after pollination. The best embryogenic ability was established for Rusalka3-inbred and the hybrid combination Russalka X Alicante Buchet. Regarding the effect of the culture media the higher concentration of growth regulators in ICI did not promote better frequency of embryo formation than I3 medium, but resulted in obtaining of more green plants with normal development and growth.

Keywords: Seedless grapes, hybrids, seed buds, embryogenesis, in vitro

Introduction

In vitro embryo rescue and somatic embryogenesis in grapes base on the ability for cultivation of isolated immature and mature embryos in optimal conditions and depend on complex of factors. Application of these methods in the grape breeding allows the obtaining of hybrid genotypes from seedless cultivars. Moreover, cultivation of grape seed buds on solid culture medium results in higher number of rescued plants (13, 14). According to Bouquet (6) the polyembryony in *Vitis vinifera* L. could be defined as genotype character and has significant importance for genetic improvement of the culture. Plants obtained from *in ovulo* embryo culture of Thompson seedless have usually zygotic origin (21). In this aspect the genotype is the main factor influencing the reaction of the seed buds in culture. Additionally, the interactions between the genotype and the environmental conditions affect the success of the embryo culture (15, 19). The frequency of germination of stenospermocarpic seed buds obtained from crosses between seedless cultivars cultivated in vitro, as well as the effect of different factors on the process of embryo development have been analyzed (1, 5, 8, 9, 10, 12, 13, 14, 16, 17, 20, 23, 25). Signals controlling gene expression in higher plants and especially for embryogenesis are still under investigation (11). The aim of our study was to establish the frequency of embryogenesis in vitro for the studied seedless grape cultivars and hybrids on solid culture medium and obtaining of plants for future breeding purposes.

Materials and Methods

Plant material. Grape inflorescences from the studied genotypes were collected from the Ampelographic collection of the Dep. Viticulture comparing two different sources of explants: inbreed forms of seedless cultivars Russalka, Russalka 3 and Sultanina and hybrid forms originating from the following crosses: Russalka x Alicante Bouchet, Russalka x [Berlandieri x Riparia SO4], Sultanina x Alicante Bouchet, Sultanina x [Berlandieri x Riparia SO4]. Tinctorial cultivar Alicante Bouchet was especially used as pollinator in the role of phenotypic marker. Scheme of explants isolation was in consideration of the main cytoembryological stages in grape seed bud development, corresponding to the age of explants, respectively 3, 10 and 30 days after pollination (22).

Surface sterilization of the berries was performed by washing for half hour on tap water with liquid commercial detergent, followed by 0.3 % $HgCl_2$ for 10 min and rinsed 3-5 times by sterile distillated water. One hundred seed buds per genotype were isolated aseptically and put in culture media based on MS (18) with some modifications and addition of growth regulators reported as efficient for grape somatic embryogenesis (22, 23, 24) (**Table 1**). Each treatment consisted of 5 replications (5 Petri dishes with 20 explants). All cultures were put in growth

chamber with 24°C±1, 16/8h (day/night) photoperiod and 2500 Lux intensity of white fluorescent lamps (Philips).

Cultivation. The following culture scheme was applied: induction period – 90 days on modified media I3 (2) or ICI (23, 24), maturation and development – up to 90 days on ICR (23, 24) medium with reduced growth regulators concentration and final transfer to solid medium VM (26) (**Table 1**) for converting to green plants and their propagation as individual clones for obtaining of suitable for adaptation plants. Ex vitro acclimatization was done in sterile peat - perlite (3:1) soil mix with gradually decreasing of the atmosphere humidity. All observations and data collection were performed at the end of the respective culture period and reported results based on two independent experiments with totally 4200 explants.

TABLE 1

Composition of the culture media (mg/l)

Content	I3	ICI	ICR	VM
Macro	1/2 MS	1/2 MS	1/2 MS	1/2 MS
Micro	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS
NaFeEDTA	37.6	37.6	37.6	37.6
NH_4NO_3	-	400.0	400.0	-
Boric acid	-	3.8	3.8	3.8
Folic acid	-	1.0	-	-
Biotin	-	-	-	-
Ca pantotenate	-	-	-	0.5
GA_3	0.35	0.7	0.35	-
BAP	0.225	0.45	0.225	-
Kinetine	0.2	0.4	0.2	-
IAA	1.8	4.0	1.8	0.02
IBA	-	-	-	0.5
Sucrose (%)	3.0	-	-	2.0
Glucose	-	1.0	1.0	-
Fructose	-	2.0	2.0	-
Activated charcoal (%)	0.3	0.3	0.3	-
Agar (%)	0.7	0.7	0.7	0.7
pH	5.5	5.7	5.7	6.5

Percentage of explants forming callus, somatic (SE) and zygotic (ZE) embryos were counted. Determination of the type of the formed embryos as somatic (SE) or zygotic (ZE) was done visually.

Results and Discussion

Inbreed forms – influence of the explants age, culture medium and genotype.

Data analysis from the experiments with inbreed forms is presented in **Fig.1**. The youngest explants isolated 3 days after pollination formed only callus. Comparatively more callus was induced on ICI medium (32- 48%) than on I3 (21-40%). The highest percentage of callus was established for Russalka 3 - ICI - 48% and I3 - 40%.

Explants isolated 10 days after pollination demonstrated intensive callus formation in the range of 44-86%. In contrast, ZE and SE development was rather low. The highest percentage of ZE was established for Russalka 3 on I3 medium - 12% and ICI- 10% and subsequently single SE converted to green plants (1-4%).

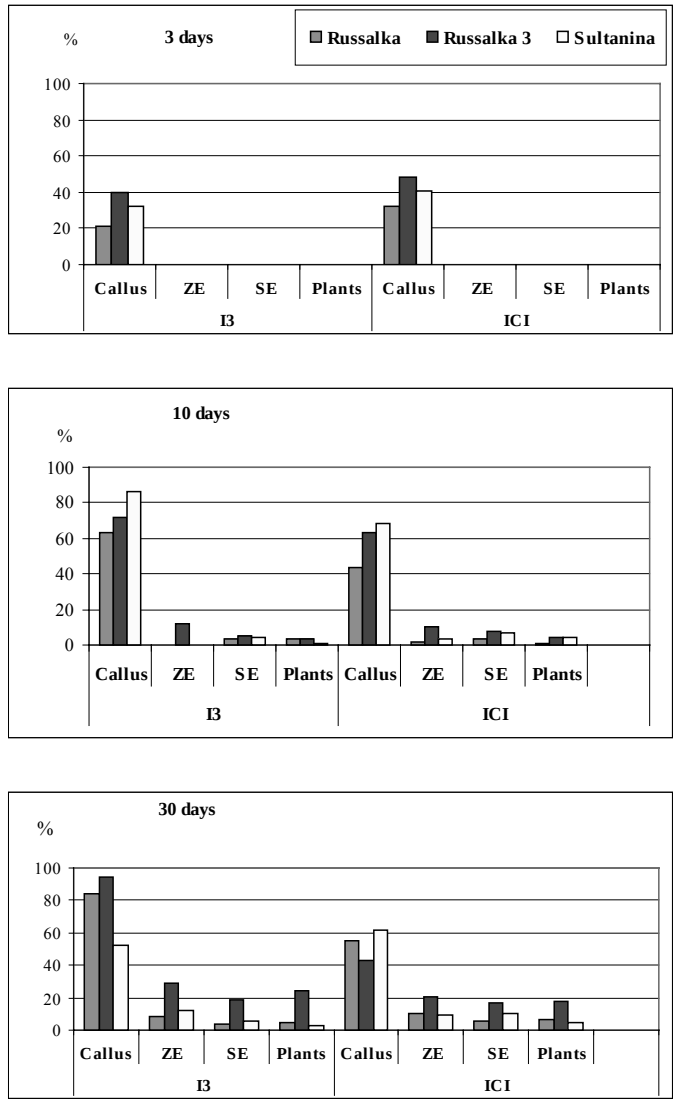


Fig. 1. Inbreed forms – effect of the explants age, culture medium and genotype

The last age - 30 days influenced positively the regeneration ability and the specific of in vitro replay. Behavior of the explants was characterized with increased callus formation (up to 94%) and giving preference to ZE then to SE development. The average frequency of ZE was 16.3% with maximum for Russalka 3 - 29% (I3 medium). The average frequency of SE formation was 10%, with maximum for Russalka 3 - 19% (I3 medium) and 17% (ICI). Green plants development was achieved only from 10 and 30 days explants.

Regarding the effect of the culture medium we established that increased concentration of the growth regulators, addition of NH_4NO_3 and micro elements in ICI did not affect the explants positively. In all the treatments induction medium I3 had similar effect for promotion of higher percentage of callus, comparatively low frequency of ZE and SE formation as well as for obtaining of green plants.

Regarding the effect of the genotype, Russalka 3 showed the highest propensity to embryo structures formation. In the treatment 30 days- I3 this cultivar demonstrated the highest ability for SE formation (up to 20%) and respectively for green plants development (24%).

Hybrid forms – influence of the explants age, culture medium and genotype.

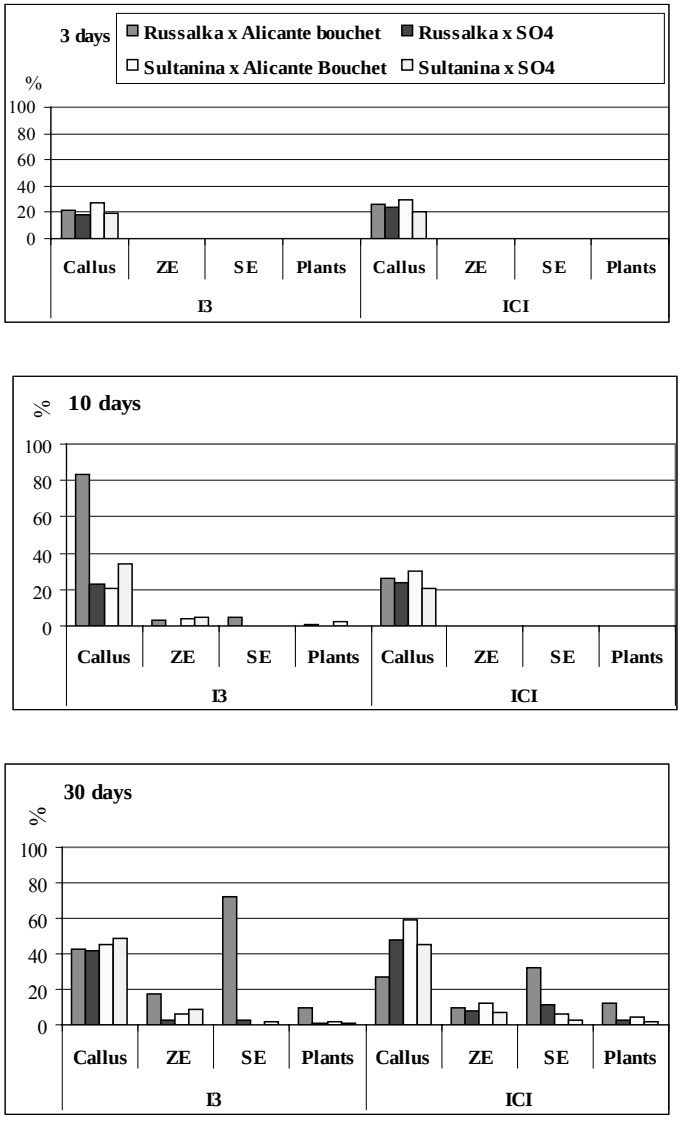


Fig. 2. Hybrid forms – effect of the explants age, culture medium and genotype

Results from the experiments with hybrid forms are presented in **Fig. 2**. The 3-days explants formed only callus - up to 30%. Induction of only callus from the youngest explants and the lack of zygotic (ZE) and somatic embryo (SE) formation could be explained with the citoembryological knowledge that very early isolation of the seed buds from the berries is not favorable for grape embryo development because in this moment (3 days after pollination) the ovule is still in the stage of rest. Time of explants isolation and optimal medium for in vitro cultivation is genotype dependent. As higher is the grade of seedless of the cultivar as earlier has to be started the embryo rescue in vitro (3, 4).

With the increase of explants age (10 days) the frequency of callus formation increased (maximum 83% for Russalka x Alicante Bouchet). Single explants cultivated on I3 medium showed the propensity for ZE and SE. Somatic embryos were obtained only from the cross Russalka x Alicante Bouchet (I3). The frequency of converting to green plants from all the treatments with 10 days explants was low (1- 2%).

In the last date of explants isolation (30 days after pollination) callus induction was observed in average of 40% of explants. The frequency of callus formation varied more between the hybrid genotypes cultivated on ICI medium.

ZE development was higher in the hybrid combination Russalka x Alicante Bouchet (17% for I3 and 10% for ICI) and Sultanina x Alicante Bouchet (12% for I3) comparing to all other treatments (2-6%).

Somatic embryogenesis was characterized with more intensity and frequently with formation of clusters of SE (**Figs. 2, 3**). The average frequency of SE formation varied from 2% (I3) and 3% (ICI) for Sultanina x [Berlandieri x Riparia SO4] to the maximum 72% (I3) and 32% (ICI) for Russalka x Alicante Bouchet. The best convertion of SE to plants was observed for Russalka x Alicante Bouchet – 10-12%. For all other hybrid combinations this percentage was 1 to 4% (**Fig. 2**).

Regarding the effect of the culture medium was established generally, that the increased concentration of the growth regulators in the medium ICI did not promote higher percentage of callus, ZE and SE formation. It was efficient for induction and development of somatic embryos from 30 days explants in all hybrid genotypes and obtaining of more green plants.

Data analysis for the effect of the genotype on the propensity to embryogenesis (ZE and SE) showed interesting results (**Fig. 2**). The hybrid combination Russalka x Alicante Bouchet demonstrated the highest propensity to somatic embryogenesis for both induction media (average 51% for I3 and ICI). Moreover, high frequency of embryogenesis was shown for hybrid forms obtained from crosses where the female parent is Russalka and the pollinator is cultivar Alicante Bouchet. This confirms our

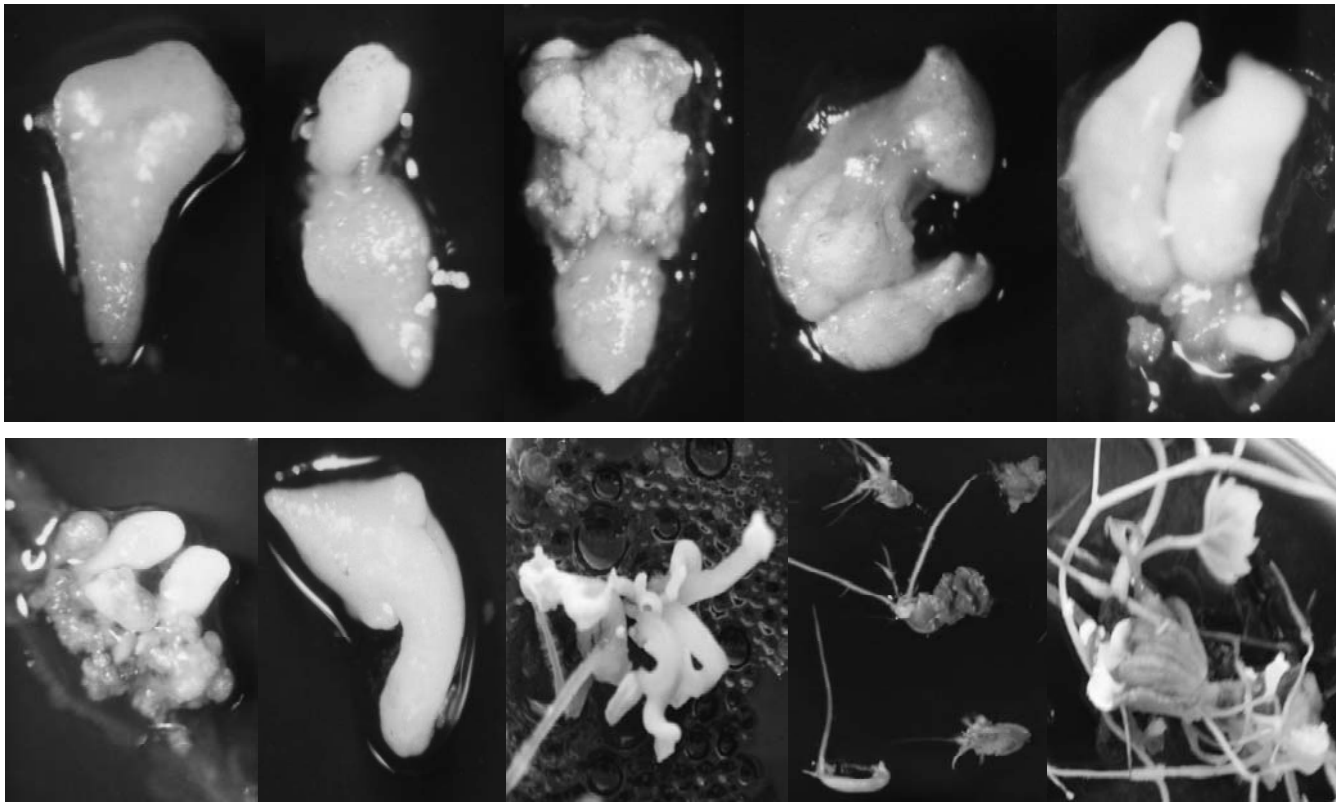
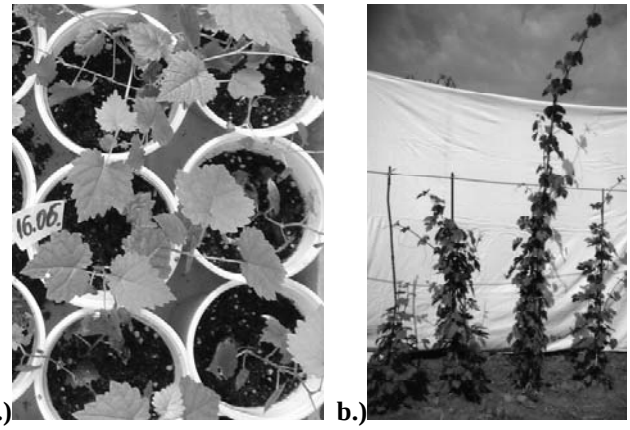


Fig. 3. Induction and subsequent development of grape embryos at different stages with converting to green plants.

previous data (22) for high frequency of somatic embryogenesis in this hybrid combination (Russalka x Alicante Bouchet).



a.) **b.)**

Fig. 4. Grape plants obtained from somatic embryos: a.) following acclimatization and b.) growing in the field conditions for further investigation.

Comparing the origin of explants (from inbreed or hybrid forms) it was established that in the experimental conditions of solid culture medium, both type of explants demonstrated lower propensity for embryogenesis comparing to previous our observations in liquid culture in vitro (22). This could be explained with the better penetration of the growth regulators throughout the explants cultivated in the liquid medium resulting in stronger embryo induction and subsequent development.

Obtained normally developed plants were propagated on VM medium for 3 subcultures. After successful adaptation and acclimatization they were transplanted in the field for further investigations (**Fig. 4**).

Conclusions

The results of our experiments present the possibility for regeneration of plants via embryogenesis from seedless grapes (inbreed and hybrid forms) on solid culture medium. Embryo structures have been formed only from the explants isolated 10 and 30 days after pollination and their frequency correlates positively with the increased explants age. The best embryogenic ability possessed 30 days explants with no significant difference between the studied media. High frequency of embryogenesis was shown from Russalka 3-inbreed and hybrid forms originating from crosses where the female parent is Russalka and the pollinator is Alicante Bouchet.

Present in vitro study demonstrated the obtaining of plants from inbreed seedless grapes and hybrids between seedless and seeded forms and supported the classical breeding program of seedless grapes with additional possibility to overcome the limitations and difficulties like seed abortion and incompatibility.

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