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High embryogenic ability and plant regeneration of table grapevine cultivars (*Vitis vinifera* L.) induced by activated charcoal

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Summary

Somatic embryos and plants from immature anthers or ovaries were obtained from the *Vitis vinifera* cvs Sugraone, Crimson Seedless, Italia and Don Mariano. Explants were cultured on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (4, 7 or 10 µM) and 6-benzyladenine (0.7, 1 or 1.3 µM) for callus induction. Callus frequency depended on genotype, explant type and the culture medium used. Calli were transferred to half strength MS without growth regulators for embryo differentiation. The presence of activated charcoal (AC, 0.25 %) in this medium was essential to obtain somatic embryos in the case of Crimson Seedless, Italia and Don Mariano and to increase the frequency of embryogenic calli in Sugraone (from 5.8 % without AC to 99.5 % with AC). Ovaries and anthers showed different degrees of embryogenic competence. When somatic embryos were placed in a medium with indole-3-acetic acid (10 µM), gibberellic acid (1 µM) and 0.25 % AC, embryo germination was normal, *i.e.* embryos turned green, the hypocotyls and cotyledons started to grow and the apical root axis developed. The percentage of germinated embryos was 100 % for Sugraone and 27.5 %, 38.1 % and 54.5 % for Don Mariano, Italia and Crimson Seedless, respectively. When the germinated embryos were transferred to half strength MS medium in test tubes, 100 % of Crimson, Italia and Don Mariano and 68.3 % of Sugraone embryos developed into normal plants.

Key words: table grapes, activated charcoal, germination, 2,4-dichlorophenoxyacetic acid, indole-3-acetic acid, 6-benziladenine, gibberellic acid, somatic embryo.

Abbreviations: AC = activated charcoal; BA = 6-benzyladenine; 2,4-D = 2,4-dichlorophenoxyacetic acid; GA_3 = gibberellic acid; IAA = indole-3-acetic acid; ABA = abscisic acid.

Introduction

Genetic improvement of grapevine is exacerbated by high levels of heterozygosity and inbreeding depression; it is also a time-consuming process due to the 2-3 years generation cycle (Gray and Meredith 1992). A possible alternative is the application of biotechnology. In grape, adventitious plant regeneration can be achieved from several explant types by both shoot organogenesis and somatic embryogenesis (review: Torregrosa *et al*. 2001, Martinelli and Gribaudo 2001). A successful application of gene technology requires, as a first step, an efficient regeneration method that permits both transformation and regeneration into plantlets. In particular, embryo tissue has been shown to be an optimal cell source for application of genetic transformation techniques in grapevine (Martinelli e*t al*. 2000, Martinelli and Mandolino 2001). Somatic embryogenesis, however, appears to be dependent on the interaction between genotype, explant source and culture medium, and thus, it is necessary to develop specific regeneration protocols for each *Vitis* species and *V. vinifera* cultivar. In this paper, we describe the use of immature anthers and ovaries as valuable explants, the effect of activated charcoal in inducing somatic embryogenesis at high frequency and the effects of IAA, GA_3 and AC on somatic embryo germination of 4 table grape cultivars. This will allow to apply a genetic transformation protocol using *Agrobacterium tumefaciens*.

Material and Methods

Plant material: Inflorescences from *Vitis vinifera* L. cvs Sugraone (Superior Seedless), Crimson Seedless, Italia and Don Mariano (the latter also known as Napoleón, an ancient autochthonous grapevine, seriously affected by virus diseases) were collected in the vineyard 13-15 d before anthesis (Fig. 1 A). They were surface-sterilized for 10 min in sodium hypochlorite (0.4 % active chlorine) and Tween 20 (1 % v/v) and rinsed three times with sterilized distilled water. Ovaries and translucent to green anthers (1-1.5 mm in length) were dissected and used as explants (Fig. 1 B, C). To study the

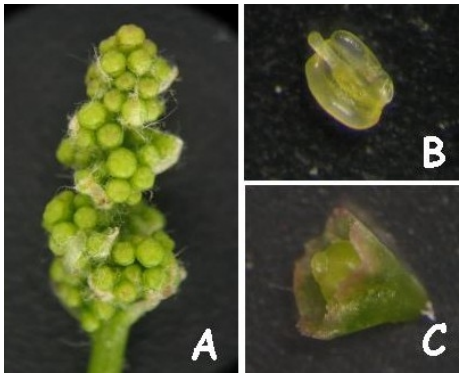

Fig. 1: Flowers and explants from *Vitis vinifera*. A) Inflorescences collected 13-15 d before anthesis. B) Anther explant. C) Ovary. Ovaries were dissected from the calyx before culture.

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effect of chilling on callus induction, inflorescences were kept at 4 ºC for 48 h before being sterilized and cultured.

Callus induction: Anthers and ovaries were placed on 90 mm x 15 mm Petri dishes with 25 ml of callus induction medium consisting of MS medium (Murashige and Skoog 1962) with 100 mg·l^{-1} casein hydrolysate, 100 mg·l^{-1} calcium pantotenate, 10 mg·l^{-1} biotin and 30 g·l^{-1} sucrose. Different combinations and concentrations of 2,4-D (4, 7 and 10 µM) and BA (0.7, 1 and 1.3 µM) were tested. Two independent experiments were carried out and 100 anthers and 30 ovaries per treatment were cultured in each experiment. Cultures were maintained at 25 ºC in the dark for three months and were subcultured on fresh medium every 30 d.

Embryo differentiation: Calli (10-12 mm in length) were divided into halves and subcultured on modified MS (half concentration of macroelements, 100 mg·l^{-1} myo-inositol and 30 g·l^{-1} sucrose, without growth regulators) and supplemented or not with 0.25 % of activated charcoal during 30 d (½ MSAC or ½ MS medium, respectively) for embryo differentiation. The cultures were maintained under the conditions described above.

Embryo germination and plant regeneration: White embryos (1.5-3 mm in length) were cultured for 30 d in 90 mm x 25 mm Petri dishes containing modified MS medium (half concentration of macroelements) with 30 g·l^{-1} sucrose, activated charcoal (0.25 %), IAA (10 µM) and GA_3 (1 µM) for germination. Green germinated embryos with hypocotyls and cotyledons and an apical root axis were transferred to test tubes (25 mm x 120 mm) with 25 ml half strength MS medium, 20 g·l^{-1} sucrose and without growth regulators, where they grew into plantlets (elongation of the root and shoot apex with one or more leaves). All media were adjusted to pH 5.8 with NaOH before adding 8 g·l^{-1} agar Noble (Difco). The cultures in Petri dishes and test tubes, were maintained at a 16 h photoperiod with a photon flux density of 45 µmol m^{-2}s^{-1} provided by fluorescent tubes (Grolux Sylvania).

Acclimatization of plants: Well rooted and elongated plants were transplanted into 10 cm pots with a mixture of 50 % peat and 50 % perlite, covered with a plastic bag and incubated under constant conditions: 16 h photoperiod, 27±1 ºC for two weeks. They were transferred to greenhouse conditions and the plastic bag was gradually raised before being completely removed.

Results and Discussion

Callus induction: Both, immature anthers and ovaries, were able to generate callus. Within 7-9 d, some explants initiated a white unorganised callus, while other explants became dark-brown and died. After about 30 d, two types of callus had developed: white or pale yellow granular calli (Type I) up to 5-8 mm or light-brown and very soft calli (Type II). After three months, the percentage of type I and type II callus obtained depended on genotype, explant type and medium (Tabs 1 and 2). For Sugraone, Crimson Seedless and Italia highest callus frequency was obtained from anthers cultured on 4/1.3 medium (52.5 % type I; 25.0 % type I) and 4/1 (18.1 % type II) respectively. For Don Mariano, highest callus frequency was obtained from ovaries cultured on 4/1 (8.3 % type II). In the case of anthers from Sugraone and Crimson Seedless, callus induction and BA concentration were positively correlated. Investigations are now under way to ascertain whether higher BA concentrations in the culture medium increase callus induction.

Genotype, explant type and culture medium were shown to affect the percentage of callus induction, an effect already described for grapevine by Bouquet *et al*. (1982), Mauro *et al*. (1986) and Stamp and Meredith (1988). The highest percentage of anthers producing callus (52.5 % for Sugraone), is higher than that reported by Perl *et al*. (1995) for Superior (30.4 %) and by Mauro *et al*. (1986) for Cabernet-Sauvignon (43.6 %) but similar to that described by Bouquet *et al*. (1982) for the rootstock *V. riparia* cv. Gloire (51.3 %). The percentages we obtained with ovaries can not be compared since in literature embryogenic frequency is reported rather than the frequency of callus induction (Nakano *et al*. 1997, Martinelli *et al*. 2001). Although Passos *et al*. (1999) described callus formation on the surface of leaf discs for cv. Italia, no somatic embryos were observed.

Table 1

Percentage of callus obtained from anthers of 4 genotypes after three months of culture using various culture media. Type I: White or pale yellow granular calli and type II: light-brown calli. Data from two independent experiments with 4 repetitions per experiment. Data followed by the same letter for each cultivar are not significantly different at P< 0.05 as determined by the Fisher LSD test.

Medium	Sugraone		Crimson Seedless		Italia		Don Mariano	
2,4-D/BA	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II
4/0.7	38.7 ab	0.0 a	1.2 a	0.0 a	0.0 a	10.6 a	0.0 a	1.2 ab
4/1	41.8 ab	0.6 b	2.5 a	0.0 a	0.0 a	18.1 a	0.6 b	3.1 c
4/1.3	52.5 b	0.0 a	25.0 c	0.0 a	0.0 a	17.5 a	0.6 b	1.2 ab
7/0.7	40.6 ab	0.0 a	1.2 a	0.6 b	0.0 a	11.8 a	0.0 a	0.0 a
7/1	48.1 b	0.0 a	4.3 a	0.0 a	0.0 a	13.7 a	0.0 a	0.6 a
7/1.3	48.7 b	0.0 a	6.8 ab	0.0 a	0.0 a	10.6 a	0.6 b	1.2 ab
10/0.7	31.2 a	0.0 a	1.2 a	0.0 a	0.0 a	13.1 a	0.0 a	0.0 a
10/1	45.6 b	0.0 a	5.0 ab	0.0 a	0.0 a	13.1 a	0.0 a	0.6 a
10/1.3	51.8 b	0.0 a	11.8 b	1.2 c	0.0 a	13.1 a	0.0 a	3.1 c

Table 2

Percentage of callus (type I and type II) obtained from ovaries of each genotype after three months of culture using various culture media. Data from two independent experiments with two repetitions per experiment. For details: Tab. 1

Medium	Sugraone		Crimson Seedless		Italia		Don Mariano	
2,4-D/BA	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II
4/0.7	0.0 a	25.0 a	0.0 a	8.3 bc	0.0 a	5.0 ab	0.0 a	1.6 a
4/1	0.0 a	25.0 a	1.6 ab	16.6 d	0.0 a	4.9 ab	0.0 a	8.3 b
4/1.3	0.0 a	30.0 a	6.6 bc	11.6 cd	0.0 a	5.0 ab	1.6 b	5.8 b
7/0.7	0.0 a	30.0 a	0.0 a	3.3 ab	0.0 a	3.3 a	0.0 a	0.0 a
7/1	0.0 a	15.0 a	8.3 c	11.6 cd	0.0 a	8.3 b	0.0 a	0.8 a
7/1.3	0.0 a	25.0 a	1.6 ab	6.7 abc	0.0 a	3.3 a	0.0 a	0.0 a
10/0.7	0.0 a	30.0 a	0.0 a	3.3 ab	0.0 a	5.8 ab	0.0 a	0.0 a
10/1	0.0 a	25.0 a	3.3 abc	0.0 a	0.8 b	3.3 a	0.0 a	0.0 a
10/1.3	0.0 a	15.0 a	0.0 a	1.6 ab	0.0 a	4.1 a	2.4 b	1.6 a

To study the effect of chilling on callus induction, inflorescences were chilled at 4 ºC for 48 h before being cultured. Independently of the callus induction medium used, cold treatment did not improve callus induction and in some cases was even detrimental (Fig. 2). It is a common practice to collect anthers at an early stage and to chill them (4 ºC) for a few days. In our case, a cold treatment of inflorescences had no effect on the callus induction frequency of anthers (Fig. 2 A) or ovaries (Fig. 2 B). These observations contrast with those of Rajasekaran and Mullins (1979), who observed that chilling of flowers (4 ºC for 72 h) greatly improved the ability of anthers to form callus and embryos.

Embryo differentiation: Type I and type II calli (10-12 mm in length) were divided into halves and subcultured on ½ MS or ½ MSAC medium to study the effect of activated charcoal on embryogenesis. The frequencies of embryogenic calli depended on genotype, explant type and the presence of AC in the culture medium (Tab. 3). Independently of the callus induction medium used, the calli cultured on medium with AC showed differentiated embryos after 20-25 d (Fig. 3 B) while in ½ MS without AC embryogenesis was delayed or absent (Fig. 3 A). Embryogenic calli consisted of unorganised callus and somatic embryos of different developmental stages (globular, heart, torpedo and cotyledonary) (Fig. 3 C, D). With regard to the various callus induction media the highest percentage of embryogenic callus was obtained from the anthers of Sugraone and Crimson Seedless cultured on 4/1.3 (2,4-D/BA), from ovaries of Italia cultured on 10/1 (2,4-D/BA), and from ovaries of Don Mariano cultured on 10/1.3 (2,4-D/BA), where, in all cases, calli were embryogenic (data not shown).

We found that addition of AC (0.25 %) to the medium used for callus culture was an essential prerequisite to obtain somatic embryos in the case of Crimson Seedless, Italia and Don Mariano; it greatly increased the frequency of embryogenic calli in Sugraone from 5.8 % in medium without AC to 99.5 % with AC. Moreover, the number of embryos per callus increased as well (data not shown).

Activated charcoal has already been used for culturing different types of grapevine tissues: anther explants to obtain callus (Perl *et al*. 1995), calli from anthers or ovaries to obtain somatic embryo clusters (Martinelli *et al*. 2001; Perl *et al*. 1995), embryogenic callus to maintain cultures (Compton and Gray 1996), proembryogenic masses to obtain mature embryos (Motoike *et al*. 2001), torpedo-stage embryos for conversion into plantlets (Hébert-Soulé *et al*. 1995) or protoplasts to increase plating efficiency and colony formation (Zhu *et al*. 1997). However, media with or without AC have not been compared and, in some cases, authors do not mention the effect of AC. Several reasons have been proposed to explain the positive effect of activated charcoal.

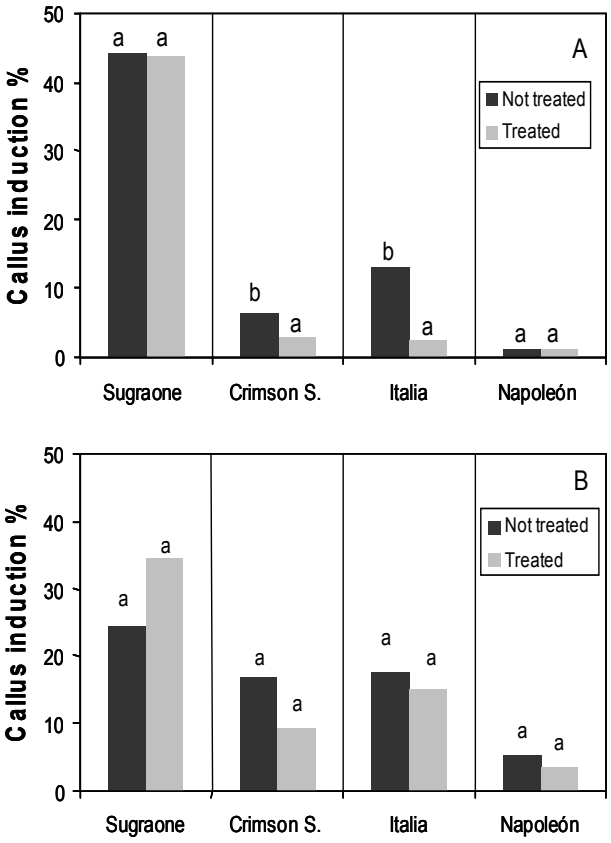

Fig. 2: Effect of chilling (4 ºC, 48 h) on the percentage of callus induction from anthers (A) and ovaries (B). Data from two independent experiments. For each cultivar, the same letter means no significant differences at $P< 0.05$ as determined by the Fisher LSD test.

Table 3

Effects of activated charcoal (0.25 %) on somatic embryo differentiation. Percentage of calli differentiating into somatic embryos after 30 d of culture in ½ MS or ½ MS plus activated charcoal, independently of callus induction medium in which they grew

Cultivar	Explant type	Number of calli cultured	Embryogenic calli in ½ MS, %	Embryogenic calli in ½ MSAC, %
Sugraone	Anthers	223	5.8	99.5
Crimson S.		36	0.0	89.0
Italia		57	0.0	0.0
Don Mariano		19	0.0	15.8
Sugraone	Ovaries	30	0.0	0.0
Crimson S.		40	0.0	22.5
Italia		37	0.0	2.7
Don Mariano		27	0.0	18.5

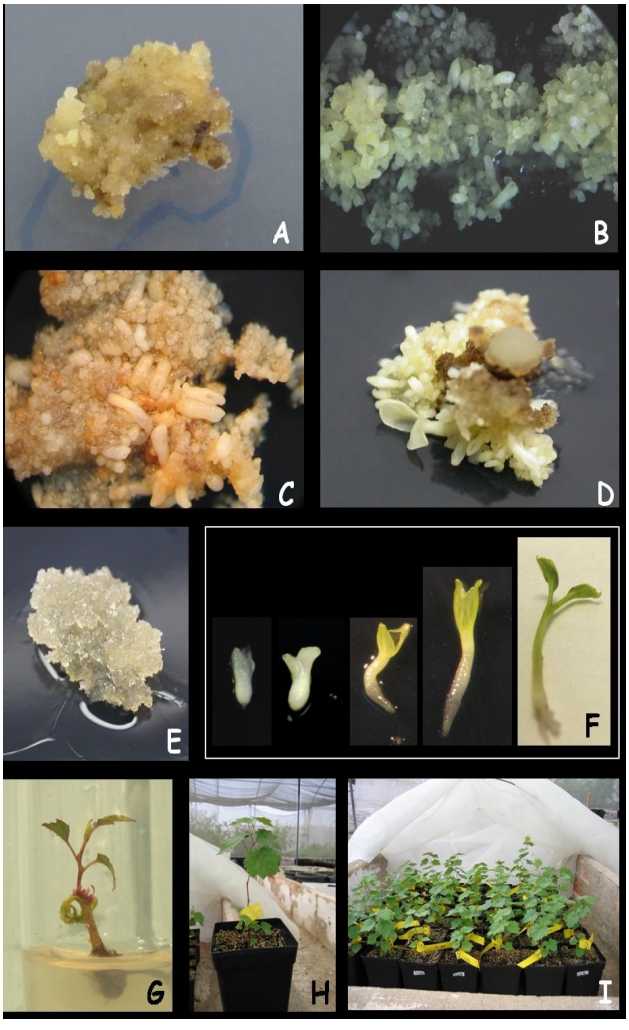

Fig. 3: Embryogenesis and plant regeneration from *V. vinifera*. A,B) Effect of activated charcoal on embryogenesis: callus cultured on ½MS (A) and on ½ MSAC (B). C,D) Calli cultured on ½ MSAC, at different stages of development of the somatic embryos. E) Very soft non-embryogenic calli (type II). F) Different stages of germination of the somatic embryos on medium containing IAA, GA_3 and AC. G) Plant developed on half strength MS in test tubes. H,I) Acclimatisation and culture under greenhouse conditions.

Hébert-Soulé *et al*. (1995) mentioned that the activated charcoal in a ½ MS phosphinothricin-free medium (PPT-free medium) absorbed potential germination inhibitors and remaining hormones and PPT in the medium, permitting the germination of torpedo-embryos and their conversion into plantlets. On the other hand, Zhu *et al*. (1997) observed that AC prevented browning of protoplast-derived cells and consequently cell division continued. Motoike *et al*. (2001) described during the culture of proembryogenic masses the effect of AC in the medium used for embryo development and maturation; AC had a significant effect on embryo development and in long-term maintenance cultures AC may remove ingredients from the culture medium that are essential for the maintenance of proembryogenic masses. In transformation experiments, Torregrosa *et al*. (2002) observed that GS1CA medium (with 2.5 $g \cdot l^{-1}$ AC), when used for embryogenic tissue culture and embryo regeneration, provided the highest transformation efficiency compared with other media. The authors argued that this effect may be due to the absence of 2,4-D or the interaction of auxins and cytokinins, which could cause embryos to mature and make them more susceptible to *Agrobacterium*. Whatever the case, we found that the addition of 0.25 % AC to the medium used for callus culture had a great effect on embryo differentiation, an effect now being tested with others cultivars.

Explant type and genotype were also important for the embryogenic ability of the calli to be expressed (Tab. 3). The highest percentage of embryogenic callus was obtained in anther-derived calli from Sugraone (99.5 %) and Crimson Seedless (89.0 %) and in ovary-derived calli from Italia (2.7 %) and Don Mariano (18.5 %). Although anthers are the most widely used explants for inducing somatic embryogenesis in grapevine, ovaries proved to be more suitable material for establishing somatic embryogenesis in the case of Italia and Don Mariano. Similar results were reported by Martinelli *et al*. (2001), who worked with anthers and ovaries from Chardonnay and Brachetto a grappolo lungo. Both genotypes produced embryos from both explant types, although ovary cultures of Chardonnay gave better results (14 %) than anthers (2 %). The percentage of embryogenic calli obtained from Crimson Seedless and Sugraone are among

the highest. MOZSÁR and VICZIÁN (1996), working with Chasselas, mentioned that, although anthers yielded relatively few calli (5.5 %), almost all (91 %) were embryogenic. On the other hand, PERL *et al.* (1995), working with Superior, found that up to 88 % of proembryogenic lines formed somatic embryos under long-term maintenance conditions.

Callus morphology and embryogenic competence are strongly correlated. The white or pale yellow granular calli (type I) obtained in callus induction medium from both anthers and ovaries were able to differentiate into somatic embryos (Tab. 4 and Fig. 3 B), while light-brown calli (type II) showed no embryogenic competence (Tab. 4 and Fig. 3 E). In addition, granular embryogenic calli showed a lower growth capacity than non-embryogenic calli. Similar differences in callus morphology have been cited in literature. TRAUTMAN and BURGER (1997) reported a yellow granular proembryogenic material emerging from explants (leaves), which was able to differentiate into somatic embryos in a hormone-free culture medium. Moreover NAKANO *et al*. (1997) reported a white to pale yellow embryogenic callus of granular appearance arising from the initial callus when transferred to embryogenesis induction medium (E medium). TORREGROSA *et al*. (1995) reported an embryogenic process initiated in leaf-induced callus and embryogenic structures emerging from a white to yellow typical nodular embryogenic callus bearing globular embryos easy to differentiate. MOTOIKE *et al*. (2001) reported slow growing, friable, white to dark, proembryogenic masses (PEM´s) with a nodular texture, which were able to differentiate into somatic embryos. It is clear that embryogenic callus is easy to identify from its granular or nodular morphology. Accordingly, we are now using this parameter to identify and select calli with embryogenic competence.

Embryo germination and plant regeneration: When somatic embryos were placed in a medium with IAA, GA_3 and AC, normal embryo germination took place. Embryos turned green, hypocotyls and cotyledons grew and an apical root axis developed (Fig. 3 F). Some embryos were abnormal, having one or more than two cotyledons or fused cotyledons. The percentage of germinated embryos (Tab. 5) was 100 % in the case of Sugraone and 27.5 %, 38.1 % and 54.5 % for Don Mariano, Italia and Crimson Seedless, respectively.

Germinated embryos, including abnormal embryos, were transferred on half strength MS medium where 100 % of the embryos from Crimson, Italia and Don Mariano, and 68.3 % from those of Sugraone were able to develop into normal plants (Tab. 5 and Fig. 3 G). The total frequency of plant regeneration with respect to the number of somatic embryos

Table 4

Effects of callus morphology on embryogenic competence. Percentage of calli differentiating into somatic embryos after 30 d of culture in ½ MSAC, independently of callus induction medium in which they grew

Cultivar	Explant type	Number of calli cultured		Embryogenic calli in ½MSAC	
		Type I	Type II	Type I %	Type II %
Sugraone	Anthers	200	0	99	-
Crimson S.		122	11	100	0.0
Italia		0	70	-	0.0
Don Mariano		8	25	100	0.0
Sugraone	Ovaries	0	100	-	0.0
Crimson S.		20	50	82	0.0
Italia		3	40	100	0.0
Don Mariano		7	35	100	0.0

Table 5

Germination and plant regeneration. Frequency of plant regeneration (a) with respect to the number of germinated embryos and (b) with respect to the number of embryos cultured on embryo germination medium

Genotype	Number of embryos cultured on embryo germination medium	Number of germinated embryos (%)	Number of regenerated plants	Frequency of plant regeneration, %	
				a	b
Sugraone	104	104 (100)	71	68	68
Crimson Seedless	99	54 (54.5)	54	100	55
Don Mariano	221	60 (27.5)	60	100	27
Italia	84	32 (38.1)	32	100	38

cultured on germination medium (Tab. 5) was 27 % for Don Mariano, 38 % for Italia, 55 % for Crimson Seedless and 68 % for Sugraone. Within 4-5 weeks, healthy plants were transplanted to soil, where they grew vigorously; morphologically they appeared to be normal. With this very simple protocol, high frequencies of plant regeneration can be obtained within 6 months of initial explant culture.

The results show that 100 % of embryos from Sugraone are able to germinate but have occasional problems in shoot development. Crimson Seedless, Italia and Don Mariano, on the other hand, have some problems in germination, but 100 % of germinated embryos regenerated normal plants.

The efficiency of somatic embryo germination and conversion into plantlets is a key factor for the success of a grapevine regeneration method. The morphological and physiological state of embryo development and the culture conditions are crucial aspects in promoting embryo conversion. Anomalies in the somatic embryos may hinder germination efficiency, and dormancy can be considered as the most critical aspect of embryo conversion (Martinelli and Gribaudo 2001). This could be the case with Crimson Seedless, Italia and Don Mariano, since not all cultured embryos were able to germinate. On the other hand, a blockage of shoot development in germinated embryos, or abnormal or missing shoot apex, or the loss of meristematic characteristics in the shoot apex have been observed in grapevine (Faure *et al*. 1996) and could be the reason why only part of the germinated embryos of Sugraone regenerated normal plants. Various treatments to improve the germination of somatic embryos and their conversion into normal plants have been proposed (Martinelli and Gribaudo 2001). According to Jiménez and Bangerth (2000), the role of endogenous IAA during dormancy breakdown has received little attention. They found no significant differences between chilled and non-chilled proembryogenic callus cultures in endogenous levels of IAA, GAs, Z/ZR and iP/iPA, although, even if not statistically significant, chilled samples contained twice as much endogenous IAA as those cultured under normal conditions. Faure *et al*. (1998) argued that grape somatic embryos do not accumulate ABA and/or IAA in sufficient amounts to permit normal development and subsequent germination. They suggested that the germination of somatic embryos could perhaps be improved by transfer to a growth regulator-enriched medium, while the promoting effect of ABA and IAA on grape somatic embryo germination should be investigated. Spiegel-Roy *et al*. (1985) increased the number of germinated seeds when culturing abortive ovules from Perlette (a seedless cv.) on a medium containing IAA and GA_3. Previous experiments carried out in our laboratory showed that immature zygotic embryos placed on a medium supplemented with IAA, GA_3 and AC germinated into well rooted, elongated and morphologically normal plantlets (García 1999). In our case, the use of IAA (10 µM) and GA_3 (1 µM) along with AC (0.25 %) in the somatic embryo germination medium allowed us to increase plant regeneration frequency up to 68 %, while somatic embryos cultured directly from ½ MSAC to half strength MS medium in test tubes resulted in frequencies under 5 % (data not shown).

Well rooted and elongated plantlets were transplanted to soil and acclimated progressively to greenhouse conditions, where they grew normally (Fig. 3 H, I).

This protocol is being tested successfully with other *Vitis* genotypes, such as the rootstock 1103Paulsen and the *V. vinifera* cvs Red Globe and Dominga (an important autochthonous grapevine from Murcia, Spain).

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