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Somatic embryogenesis from stem nodal sections of grapevine

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Summary

Indirect somatic embryogenesis was obtained for 11 clones of 6 *Vitis vinifera* cultivars: Cabernet-Sauvignon, Chardonnay, Gewürztraminer, Grenache, Merlot and Sauvignon, and for the rootstock Fercal [(*Vitis berlandieri* x *Vitis colombard*) x (*Vitis vinifera* x *Vitis berlandieri*)], starting from vegetative explants of in vitro plantlets. Embryogenic callus was recovered from nodal explants of every tested clone, while leaf explants led to embryogenesis only for the rootstock Fercal. We thus showed that axillary bud microcuttings are valuable explants for inducing somatic embryogenesis in *V. vinifera* and Fercal. Embryogenic cell lines have been maintained through secondary embryogenesis, and some embryos were converted into whole plantlets. A complete protocol for somatic embryogenesis and plant regeneration was therefore designed, using this very simple method.

K e y w o r d s : *Vitis vinifera*, grapevine, axillary bud, nodal explant, somatic embryogenesis.

A b b r e v i a t i o n s : 2,4-D - 2,4-dichlorophenoxyacetic acid, BAP - 6-benzylaminopurine, ENTAV - Etablissement National Technique pour l'Amélioration de la Viticulture, IAA - indole-3-acetic acid, NOA - 2-naphtoxyacetic acid.

Introduction

Genetic variability of grapevine is limited in wine producing cultivars; it is generally improved by clonal selection. Genetic transformation therefore offers unique perspectives for the improvement (Martinelli and Mandolino 2001). For transgenic vine regeneration, somatic embryos have proven to be the best cell source (Martinelli and Gribaudo 2001). In addition, somatic embryogenesis is a useful tool for developmental studies in plants (Dodeman *et al*. 1997, Feher *et al*. 2003, Gaj 2004), and much progress is expected for grapevine in this field. Although reliable protocols for somatic embryogenesis and plant regeneration of grapevine have been described, they are limited to a few genotypes and the efficiency is low due to a low regeneration competence. In most of the studies the starting explants derive from flowers, stamens being particularly used (Martinelli and Gribaudo 2001, Martinelli *et al*. 2001 b, Motoike *et al*. 2001, Nakajima and Matsuta 2003, Morgana *et al*. 2004, Perrin *et al*. 2004). Recently a method was presented to recover embryogenic cell lines from anthers of a large set of *V. vinifera* cultivars (Perrin *et al*. 2004). The free end of the filament was the most frequent site of callus initiation; this is in accordance with a report on somatic embryogenesis from isolated filaments (Nakajima and Matsuta 2003). Other experiments showed that unfertilised ovaries (Nakano *et al*. 1997, Martinelli *et al*. 2001 b, Croce *et al*. 2005) and stigma-style explants (Morgana *et al*. 2004) were more responsive to embryogenic induction than anthers. In order to be independent of the use of flowers being a limiting factor of the protocol, several authors attempted to induce somatic embryogenesis from vegetative explants. Leaf and petiole fragments were successfully used (Martinelli and Gribaudo 2001, Das *et al*. 2002), as well as tendrils (Salunkhe *et al*. 1997), but only from very few cultivars.

In the following article we present a method starting from nodal explants collected from *in vitro*-grown plantlets. A complete protocol was designed for somatic embryogenesis and regeneration of intact plantlets, for 6 *V. vinifera* cultivars and for the rootstock Fercal. The suitability of nodal explants was compared to that of leaf explants for 4 cultivars.

Material and Methods

P l a n t m a t e r i a l : Canes of *V. vinifera* cultivars and of the rootstock Fercal [(*V. berlandieri* x *V. colombard*) x (*V. vinifera* x *V. berlandieri*)] were kindly provided by ENTAV (Etablissement National Technique pour l'Amélioration de la Viticulture) Le Grau du Roi, France: Cabernet-Sauvignon 15 and 169, Chardonnay 76 and 96, Gewürztraminer 48 and 643, Grenache 136 and 435, Merlot 181 and 343, Sauvignon 159 and Fercal 242.

M e d i a a n d c u l t u r e c o n d i t i o n s : All media were Murashige and Skoog (1962) media with half strength major salts. Sucrose and growth regulators were added before autoclaving. The pH level was adjusted to 5.8 by adding 1N NaOH. Activated charcoal was added after adjusting the pH level. Media were solidified by adding 0.7 % Bacto-agar, and sterilized by autoclaving at 115 °C for 30 min. Cultures were kept in the dark or at a 16-h-photoperiod (light intensity: 50 µmol m^{-2} s^{-1}), at 25 °C and a relative humidity of 60 %.

E s t a b l i s h m e n t o f *i n v i t r o* p l a n t s t o c k : Canes were rooted in a mix of Perlite and sand (1/1, v/v), and grown at 25 °C and a 16-h-photoperiod

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(light intensity: 50 µmol m^{-2} s^{-1}). Vegetative shoots (100-150 mm long) were harvested after 4-5 weeks. They were surface-sterilized in a sodium hypochlorite solution (1 %) for 15 min and rinsed three times in sterile water. Microcuttings with a single node were grown in the light in glass tubes (195 mm x 24 mm) on a culture medium containing 20 g l^{-1} sucrose. Clones were propagated every 3 months by axillary bud microcutting.

Induction of embryogenesis: Ten independent experiments (a-j) were carried out. On the whole we tested nodal segments of each clone and leaf fragments of Cabernet-Sauvignon 169, Chardonnay 76, Grenache 136 and Fercal 242, collected from *in vitro* plantlets. The nodal explants (4-6 mm long) consisted of a single axillary bud with short flanking segments of stem and petiole. Eight to twelve of them were placed in Petri dishes (60 mm diameter), and 5-12 dishes were prepared per clone. Segments of whole leaves (about 100 mm^2) were placed, abaxial side to the medium, 10 per Petri dish, and 4-12 dishes were prepared per clone. The first culture was performed on a medium containing 25 g l^{-1} sucrose and a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP), either 2.25 µM:4.5 µM (medium E5), 9 µM:4.5 µM (medium E8) or 4.5 µM:9 µM (medium E9). The plates were incubated in the dark for one month. The explants were transferred onto medium A, containing 60 g l^{-1} sucrose, 2.5 g l^{-1} activated charcoal, 20 µM indole-3-acetic acid (IAA), 10 µM 2-naphtoxyacetic acid (NOA) and 1 µM BAP, and incubated under light. The subculture on medium A was repeated every month until the embryos appeared. Experiments were stopped after 13 months of total culture.

Long-term maintenance of embryogenic callus: A long-term maintenance of the embryogenic ability was obtained by inducing secondary embryogenesis: clusters of embryos (6-8) were placed onto medium E96 (E9 with 60 g l^{-1} sucrose), for one month in the dark. The cultures were then transferred onto medium A.

Regeneration of intact plants: Embryos at the globular- or heart-stage were separated from the embryogenic callus, and grown on a medium containing 25 g l^{-1} sucrose and 0.5 µM BAP. The cultures were incubated in the dark for germination, then transferred to the light to obtain *in vitro* whole plantlets.

Results

Callus induction: The first culture step induced yellow-green callus on any explant of each clone. Cellular proliferation was rather confined to the veins of leaf fragments, while it included the whole nodal explants. When transferred to medium A, callusing explants turned brown. No further growth was observed throughout further cultures on medium A.

Recovery of embryogenic callus: Embryogenic callus was identified when a few embryos appeared on the brownish tissues (Figure, a). Callus formation preceded the differentiation of somatic embryos therefore being an indirect process. The results of the embryogenic callus recovery are shown in Tab. 1. Each clone led to the recovery of embryogenic callus when nodal explants were used. In the whole experiments 50 embryogenic calli were recovered from 2,314 nodal explants. On the contrary, no embryogenic callus was obtained from leaves of *V. vinifera* cvs. Only Fercal 242 produced 4 embryogenic calli from 130 leaf fragments. The recovery efficiency ranged from 0.75 to 4.33 %, depending on the clone. The first step of the protocol involved a medium with varying combinations of 2,4-D and BAP. The use of any combination allowed the emergence of embryogenic calli for any cultivar. On the whole, media with the highest total amount of 2,4-D and BAP (E8 and E9), however, led to the recovery of, respectively, 3.26 and 2.28 times more embryogenic calli than the medium with the lowest total amount of the two growth regulators (E5) (Tab. 2). Embryogenic calli consistently appeared on medium A, during a culture period of 2-12 months after the beginning of the experiments. No correlation was noticed between the clone and the time of appearance. E.g., Chardonnay 96 gave rise to embryogenic calli during a period of 2-9 months of total culture.

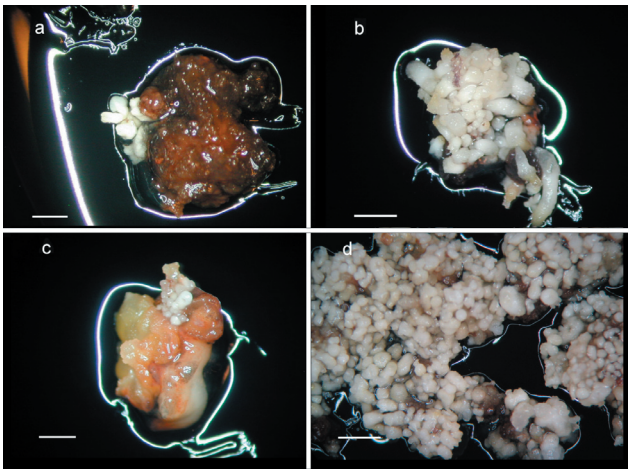

Figure: Somatic embryogenesis from stem nodal sections of grapevine; **(a)** appearance of the first embryos on a brownish callusing nodal explant; **(b)** embryogenic callus exhibiting a continuous and asynchronous production of embryos after two subcultures on medium A; **(c)** small cluster of secondary embryos on a callus derived from a bulk of primary embryos; **(d)** enhanced embryogenic ability of secondary embryogenic tissues; bars = 1 mm.

Long-term maintenance of embryogenic competence: Embryogenic callus was kept on medium A after isolation. It produced embryos in a continuous and asynchronous way. After 2-3 months of culture on medium A, the production of embryos was enhanced (Figure. b). However, the callus progressively lost its embryogenic ability after more subcultures on this medium. The long-term maintenance of embryogenic cell lines was achieved by inducing indirect secondary embryogenesis. Embryos were transferred onto a medium with 2,4-D and BAP (E96). At first dedifferentiation occurred then callus proliferation was induced. New embryogenic clusters appeared after the transfer onto medium A (Figure, c). These secondary embryogenic tissues further exhibited a large and synchronous production of embryos (Figure, d). By us-

Table 1

Recovery of embryogenic calli from nodal explants and leaf fragments of *V. vinifera* and Fercal

Explant type	Clone	Experiment	Total tested explants	Total embryogenic callus[X]	Average efficiency[Y] (%)
Nodal explants	Cabernet Sauvignon 15	a	74	1	1.35
	Cabernet Sauvignon 169	a, f, i	238	5	1.57 ± 1.51
	Chardonnay 76	a, b, g, h	256	7	2.29 ± 4.05
	Chardonnay 96	a, f, g, h	344	7	2.26 ± 1.71
	Gewürztraminer 48	c	80	1	1.25
	Gewürztraminer 643	c, j	175	2	0.75 ± 1.06
	Grenache 136	c, e, i	268	7	3.37 ± 3.07
	Grenache 435	e, g, j	280	9	3.68 ± 2.48
	Merlot 181	c, g	103	2	1.85 ± 2.62
	Merlot 343	c, e, j	252	4	1.20 ± 1.08
	Sauvignon 159	f	133	1	0.75
	Fercal 242	b, h	111	4	3.67 ± 0.70
Leaf fragments	Cabernet Sauvignon 169	i	120	0	0
	Chardonnay 76	d, h	150	0	0
	Grenache 136	i	120	0	0
	Fercal 242	d, h	130	4	4.33 ± 3.30

[X] Embryogenic callus were scored as callus having produced well developed embryos at the cotyledonary stage.
[Y] Average efficiency = mean of efficiencies in the different experiments, calculated as the number of embryogenic callus recovered from 100 tested explants ± standard deviation.

Table 2

Efficiency of embryogenic callus recovery depending on the first culture medium

First medium	Total tested explants	Total embryogenic callus	Recovery efficiency[X] (%)
E5	1041	9	0.86
E8	1180	33	2.80
E9	613	12	1.96

[X] Recovery efficiency = number of embryogenic callus recovered from 100 tested explants.

ing this method, the maintenance of embryogenic cell lines succeeded with each clone. Even after 3 years the very first isolated embryogenic cell lines have still been producing embryos.

Regeneration of intact plants: The regeneration of plantlets was induced by separating globular- or heart-stage embryos from the embryogenic callus and placing them on a culture medium containing 0.5 µM BAP. Embryos first germinated when incubated in the dark for 3-10 d. Intact *in vitro*-plantlets were then recovered after their transfer to the light for 2-4 months. As a result 5-30 % of the separated embryos were converted into whole *in vitro*-plantlets, depending on the clone (data not shown).

Discussion

This article deals with the recovery of embryogenic cell lines from vegetative explants of grapevine, including 11 clones of 6 *V. vinifera* cvs and the rootstock Fercal. Somatic embryogenesis has already been obtained from vegetative explants of very few *V. vinifera* cvs, usually starting from leaves or petioles (Martinelli and Gribaudo 2001, Das *et al*. 2002). Nakano *et al*. (1997) reported somatic embryos derived from leaves of Merlot. However, regeneration through somatic embryogenesis starting from vegetative explants has not yet been described for Cabernet-Sauvignon, Chardonnay, Gewürztraminer, Grenache and Sauvignon neither for Fercal. The protocol we used is close to the one described by Martinelli *et al*. (2001 b) for Chardonnay and Brachetto a grappolo lungo starting from immature anthers and ovaries. A medium containing a combination of an auxin with a cytokinin was used at the first step as usually advised for *Vitis* species (Martinelli and Gribaudo 2001, Martinelli *et al*. 2001 b, Motoike *et al*. 2001, Das *et al*. 2002, Nakajima and Matsuta 2003, Morgana *et al*. 2004, Perrin *et al*. 2004, Croce *et al*. 2005). Significantly more embryogenic calli were recovered when the first medium contained the highest total amount of 2,4-D and BAP (E8 and E9). Moreover, the medium with the highest content of 2,4-D (E8) was the most efficient of the three tested media (E5, E8 and E9). These results suggest that the amounts of growth regulators at the first step of the

protocol, especially that of 2,4-D, are significant for the induction of somatic embryogenesis. This is in accordance with the common idea that 2,4-D is a strong inducer of somatic embryogenesis in plants acting as a growth regulator or a stressor (Feher *et al*. 2003, GAJ 2004).

To our knowledge this is the first time that nodal explants have been tested successfully on grapevine. Usually methods involve reproduction organs which have to be harvested at a precise physiological stage (Martinelli and Gribaudo 2001, Martinelli *et al*. 2001 b, Motoike *et al*. 2001, Nakajima and Matsuta 2003, Morgana *et al*. 2004, Perrin *et al*. 2004). Flowers are collected from grapevines in vineyards or greenhouses, however, the recovery of the flowers remains uncertain and time-consuming. The method we developed with nodal explants is much simpler, vines being easily propagated *in vitro* all the year round. Moreover, nodal explants are very easy to prepare, compared to the reproduction organs obtained by tedious dissection of flowers.

The use of nodal explants has rarely been reported to induce somatic embryogenesis in other plant species. Embryogenic callus has been obtained from apical nodal sections of *in vitro* plantlets of potato (De Garcia and Martinez 1995), and more recently, from the first nodal segment of *in vitro* seedlings of mung bean (Devi *et al.* 2004). Our results show that axillary bud microcuttings are suitable for somatic embryogenesis induction in grape suggesting that they may be of interest for plant species being also recalcitrant to tissue culture.

Under our conditions nodal explants were more responsive to induction of somatic embryogenesis than leaves. In fact, no embryogenic callus was obtained from leaves of *V. vinifera* cvs. Moreover, embryogenic callus appeared on nodal explants of Fercal as soon as 2 months after the beginning of the experiments, whereas at least 5 months were required for leaf segments. In a previous study, Nakano *et al*. (1997) also showed that anthers and ovaries are more suitable than leaves for the induction of somatic embryogenesis of several grapevine cvs. On the whole, these results show that the embryogenic competence strongly depends on the type of explant. Although molecular mechanisms underlying plant embryogenesis are more and more elucidated, very little is known yet about the transition of a cell from the vegetative to the embryogenic state in the context of somatic embryogenesis (Gaj 2004). For this process cells have to change their developmental fate and gain embryogenic potency implying first of all dedifferentiation (Feher *et al*. 2003). Since meristematic cells are not differentiated, they could be more sensitive to embryogenic induction than other somatic cells. Therefore it would be interesting to know if, in our experiments, meristematic cells of axillary buds were implied in the higher embryogenic competence of nodal explants compared to leaf explants.

Embryogenic cell lines have often been maintained through secondary embryogenesis in grapevine (Martinelli and Gribaudo 2001, Martinelli *et al*. 2001 a, Morgana 2004, Croce *et al*. 2005). This part of the protocol is crucial for further genetic engineering experiments or detailed studies of embryogenesis requiring the simultaneous and reproducible production of numerous embryos. Both the necessity and difficulty in maintaining embryogenic cell lines in grapevine were previously underlined (Martinelli *et al*. 2001 a, Perrin *et al*. 2001, 2004). The periodical transfer of embryos onto a medium containing 2,4-D and BAP allowed to keep embryogenic cell lines. The successful use of 2,4-D for maintaining embryogenic capability has already been described for grapevine (Franks *et al*. 1998, Motoike *et al*. 2001). Although primary embryogenic callus was recovered with rather low efficiency, embryogenic cell lines of all clones were maintained. Secondary embryogenic tissues exhibited a strong embryogenic competence in contrast to embryogenic callus first isolated, being a general feature of secondary embryogenesis in plants (Gaj 2004).

Finally, we were able to show that intact plantlets could be regenerated from embryos of each clone, thus designing a complete and new protocol for somatic embryogenesis of grapevine.

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