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Embryogenesis in microspore culture of *Vitis* subspecies

by

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S u m m a r y : Embryoid structures showing epidermal layers have been regenerated from isolated *Vitis* microspores. Experiments were carried out on 8 genotypes of *Vitis* including different donor plant growth conditions, chilling of microspores (4 °C), heat shock (35 °C) and colchicine exposure (10, 25 and 50 mg/l) as induction treatments and incubation of the cultures on 87 different solid and liquid NN and LS media. The reactions of the cultured microspores included enlargement and thickening of the exine, formation of microcalli, calli, globular proembryos and embryoid structures displaying cell differentiation. Callus formation took place mainly on solid LS media with 3 % or 12 % sucrose, whereas most of the embryoids developed on NN media with 3 % sucrose. Both, callus and embryoid formation, were promoted by colchicine treatment. 8 months after transfer of calli and embryoids to subculture media, 4 embryoids still showed cell proliferation.

K e y w o r d s : Vitis, haploid, tissue culture, microspores, colchicine treatment.

A b b r e v i a t i o n s : BA = 6-benzylaminopurine, NOA = β-naphthoxyacetic acid, TDZ = thidiazurone, 2,4-D = 2,4-dichlorophenoxyacetic acid, NN = NITSCH and NITSCH (1969), LS = LINSMAIER and SKOOG (1965).

Introduction

The production of haploid or homozygous *Vitis* plants is a task of great importance for conventional breeding concepts as well as for biotechnological approaches. So far, attempts to regenerate haploid plants by anther culture of *Vitis* failed. Though development of haploid tissue is reported several times (GRESSHOFF *et al.* 1974; RAJASEKARAN *et al.* 1979; ALTAMURA *et al.* 1992), all regenerated plants were diploid (RAJASEKARAN *et al.* 1979; HIRABAYASHI *et al.* 1982; ALTAMURA *et al.* 1992). It may be assumed that the regenerated plants originated from diploid heterozygous anther tissue and not from homozygous microspores. Therefore isolation of microspores from anthers and complete exclusion of anther tissue from the culture are required for regeneration of haploid grapevine plants *in vitro*.

The success of microspore culture depends on the ability of the microspores to switch from gametophytic to sporophytic development (ZAKI *et al.* 1991; TELMER *et al.* 1993) and on the potential of the microspore-derived haploid cells to undergo embryogenesis (TELMER *et al.* 1995). Many factors are known to control the development of cultured microspores: The genotype of microspore donor plants (THURLING *et al.* 1984; MORRISON *et al.* 1986; DUIJS *et al.* 1992; VAN DEN BULK *et al.* 1994), the donor plant growth conditions (THURLING *et al.* 1984; DUNWELL *et al.* 1985), the stage of the microspore development at the time of isolation (MORRISON *et al.* 1986; FAN *et al.* 1988; PECHAN *et al.* 1988; ZAKI *et al.* 1990; TELMER *et al.* 1993; VAN DEN BULK *et al.* 1994), media composition (SATO *et al.* 1989; HAMAOKA *et al.* 1991; VAN DEN BULK *et al.* 1994; DATTA *et al.* 1987) and stress treatments such as high or low temperatures (SATO *et al.* 1989; HAMAOKA *et al.* 1991; DUIJS *et al.* 1992; ALTAMURA *et al.* 1992; TELMER *et al.* 1995) or colchicine treatment (ZAKI *et al.* 1995). Stress treatments are applied to inhibit the normal pollen mitosis and induce the symmetric division of the nucleus, which is considered a prerequisite to sporophytic development (ZAKI *et al.* 1991; TELMER *et al.* 1993). VAN DEN BULK (1994) reported that the stress caused by *in vitro* culturing alone sufficed to trigger sporophytic development of tulip microspores.

The aim of this work was the acquisition of information about the treatment of microspores and culture conditions required for successful microspore culture in *Vitis* sspp. Since no data about experiments with microspore culture of grapevine are available, experiences with other species formed the basis of our work on grapevine.

Material and methods

P l a n t g e n o t y p e s a n d g r o w t h c o n d i t i o n s : Experiments were carried out with 8 *Vitis* genotypes as listed below. 1355/1/116, 1355/3/33 and 1358/1/47 are now registered as Seifert, Rathay and Rösler, respectively. These genotypes and 1358/1/42 are interesting because of their polygene resistance to *Plasmopara viticola* and their tolerance to *Uncinula necator*. They were selected by the Breeding Station of Klosterneuburg. Couderc 3309 and Ganzin 1 are male rootstocks; Grüner

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Veltliner is the most important variety of Austria's commercial viticulture.

Vitis genotypes	Origin
1189-9	Seyve Villard 23804 x Blaufränkisch
1355/1/116 (Seifert)	1189-9 x Blauburger
1355/3/33 (Rathay)	1189-9 x Blauburger
1358/1/47 (Rösler)	1189-9 x Zweigelt
1358/1/142	1189-9 x Zweigelt
Couderc 3309	*V. riparia* x *V. rupestris*
Ganzin 1	Aramon x *V. rupestris*
Grüner Veltliner	*Vitis vinifera* cultivar

To be provided with grapevine inflorescences throughout the period, cuttings were rooted and induced to develop inflorescences at regular intervals following the protocol by MULLINS and RAJASEKARAN (1981). The rooted cuttings were grown either in a greenhouse at 25 °C and 150-200 $\mu mol \cdot m^{-2} s^{-1}$ light intensity or in a growth chamber with a 16 h photoperiod, 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and a day/night temperature of 26/22 °C. Furthermore, inflorescences of field plants were used.

Isolation of microspores: Buds containing mainly microspores at the uninucleate stage were harvested. Microspore stages were first determined by staining the nuclei with Hoechst dye (H 33258) and were later assessed with regard to bud size and anther morphology.

The buds were sterilized for 10 min in a mixture of 30 % Danclor (Commercial bleach; 4.5 % NaOCl) with one drop Tween 20 and rinsed twice in sterile water. Anthers of up to 20 buds were collected in one drop of isolation medium and squeezed to release microspores. The slurry was passed through a net (50 µm mesh size) to exclude anther tissue. Microspores were twice washed in isolation medium and centrifuged at 600 *g* for 3-5 min, then resuspended in isolation medium and plated on petri dishes. The microspores of about 3580 buds were isolated. Seven NN liquid media, differing in sucrose concentration (3, 6 or 9 %) and casein hydrolysate content (1g/l or none), were used for isolating and washing the microspores.

Induction treatments: To induce sporophytic development, microspores or anthers were exposed to several induction treatments including heat shock (35 °C), chilling (4 °C), freezing (-20 °C) and colchicine treatment. Filter sterilized colchicine (Sigma, Vienna/Austria) was added to isolation media supplied with either NOA or 2,4-D to final concentrations of 10, 25 and 50 mg/l. After exposure to colchicine for up to 5 d microspores were washed twice in the isolation medium. Colchicine treatments were performed either at 28 °C or in combination with the temperature treatments at 4 and 35 °C. After completion of the different treatments, all cultures were incubated at 28 °C in the dark.

Culture media and culture conditions: Microspores were incubated in 87 different NN liquid and NN or LS solid media. The NN liquid media contained 3, 6 and 9 % sucrose. Casein hydrolysate was added to some media. NITSCH and NITSCH vitamins were added both to NN and LS media. The sucrose concentration was 3 % in each of the NN media and 3, 6, 12 and 15 % in the LS media. Agar (0.6 %) and gelrite (0.3 %) were used as jelling agents. The plant growth regulators included were NOA (1,2, 3 and 4 mg/l), 2,4-D (0.005, 0.01, 0.05, 0.1, 0.2, 0.5 and 1 mg/l), BA (0.2, 0.5 and 1 mg/l) and TDZ (0.2, 0.5, 1 and 2 mg/l). NOA was either applied alone or in combination with TDZ or BA; 2,4-D was added together with BA. Additionally, media without plant growth regulators were used. For the individual composition of each of the media, see SEFC (1996).

Petri dishes were sealed with parafilm and the microspore cultures were incubated in the dark at 28 °C.

Subculture of microcalli and embryoids: As soon as microcalli or embryoids became macroscopically visible, they were transferred individually to subculture media. Ten different solid NN and LS media were used as subculture media. Their sucrose contents were 2, 3, 6 and 12 %. 1 or 2 mg/l BA were added to 5 of the media, the others were supplied with either 0.2 mg/l NOA, 2 mg/l TDZ or 0.01 mg/l 2,4-D plus 0.5 mg/l BA or did not contain growth regulators at all.

Initially, subcultures were incubated under the same conditions as the microspore cultures. In order to improve development, subcultures were transferred to a growth chamber with a 16-h-photoperiod and a constant temperature of 27 °C.

To facilitate determination of tissue development in subculture, photographs of transferable tissue were taken before and 1-2 weeks after transfer to the subculture media.

Results

Increase of microspore cell volume: Changes in the morphology of the microspores were noticed after 2 d of culture. Two to fifteenfold enlargement of microspores, leading either to cells with granulated cytoplasm or to cells with large vacuoles filling the centers of the cells and with granulated starch arranged around the vacuoles, was observed in up to 50 % of the microspores (Fig. 1). This development occurred in all cultures, but an

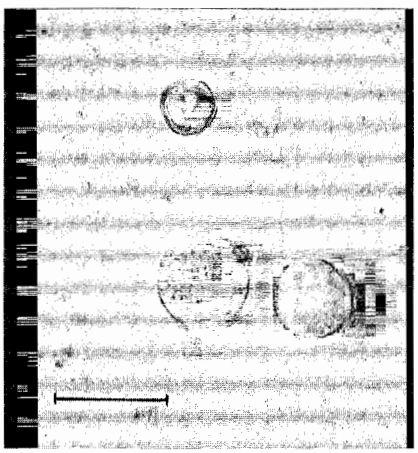

Fig. 1: Enlarged microspores stained with FDA. Bar = 30 µm.

accumulation of these structures was stated in colchicine-treated and chilled cultures. An emergence of membrane fragments was observed to a lesser degree. It is likely that these membranes originate from some of the enlarged, burst microspores.

Pollen tube growth: Some microspores of the genotype 1358/1/47 treated with colchicine and cultured on NN media showed pollen tube growth. Formation of pollen tubes is an indicator of gametophytic development.

Thickening of the microspore exine: Another response to all induction treatments was the enlargement of microspores combined with simultaneous thickening of the exine (Fig. 2). The volume of the microspores increased 10- to 20-fold. The outline of these structures was no longer circular but became irregular. The surface appeared smooth and was streaked with one or two furrows. Most of these structures were found in colchicine treated and chilled cultures. Due to the very thick exine both, Hoechst dye and FDA stain, could not penetrate into the cytoplasm. Therefore, no information about cell or nuclei number and vitality were obtained.

Development of globular proembryos: After 3 months, reddish brown globular proembryos with an approximately 30-fold volume of microspores were observed (Fig. 3). The bodies showed circular or slightly oval forms, a smooth surface and granular contents. Growth decreased and further development of these tissues could not be detected. Altogether, 16 of these tissues were found. Eight of them had developed in colchicine- and heat-treated cultures, 4 in colchicine-treated cultures, two in chilled- and heat-treated cultures and one in a culture of microspores originating from a chilled inflorescence. Except for one proembryo grown in liquid NN medium, the proembryos developed on solid LS media; 9 media had a sucrose content of 3 %, 5 had 12 % and two had 6 and 9 %, respectively. All 4 growth regulators were used in the responsive culture media.

Development of microcalli: With a few exceptions, all cultures showed microcalli with extremely irregular forms and without pigmentation after some weeks of incubation. The development of microcalli was rather abundant with up to 6 microcalli per culture plate and even occurred in the cultures of frozen microspores. The high-

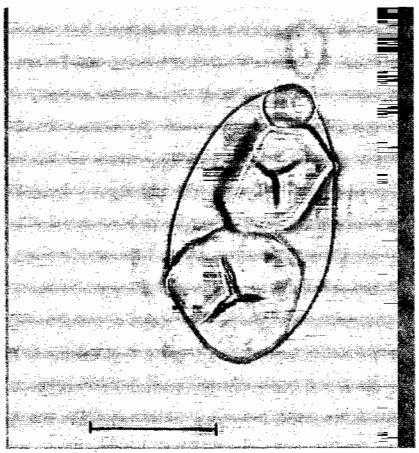

Fig. 2: Changes in surface structure of microspores within three days of cultivation. Bar = 30 µm.

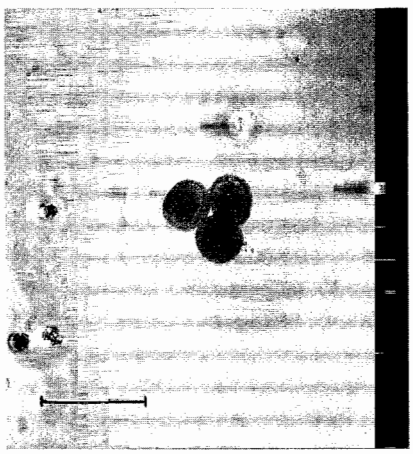

Fig. 3: Globular proembryos. Bar = 100 µm.

Table 1

Callus development in colchicine-treated cultures

Genotype *)	Culture media: Type of medium	Culture media: Sucrose (%)	Culture media: Growth regulators (mg/l)	Colchicine treatment of microspore cultures prior to incubation at 28 °C	Number of calli obtained
1355/3/33 P	LS	12	2 NOA, 0.5 BA	2 days 25 mg/l colchicine	1
1355/3/33 P	LS	12	0.1 2,4-D, 20.5 BA	2 days 25 mg/l colchicine	1
1358/1/47 G	LS	12	0.1 2,4-D, 1 TDZ	2 days 25 mg/l colchicine	2
1358/1/47 P	NN	3	0.05 2,4-D	3 days 10 mg/l colchicine	1
1358/1/47 P	NN	3	3 NOA, 1 TDZ	2 days 10 mg/l colchicine	1
1358/1/47 P	NN	3	4 NOA, 1 TDZ	2 days 10 mg/l colchicine	1
1358/1/42 G	NN	3	1 NOA, 0.2 BA	4 days 10 mg/l colchicine	1
1358/1/42 G	NN	3	3 NOA	4 days 10 mg/l colchicine	1
C 3309 P	NN	3	3 NOA, 1 TDZ	2 days 25 mg/l colchicine	2
1189-9 F	LS	12	0.2 2,4-D, 1 TDZ	1 day 50 mg/l colchicine	1
Veltliner F	LS	3	3 NOA, 0.5 BA	2 days 25 mg/l colchicine	1

*) Plants grown under growth chamber (P), greenhouse (G) or field (F) conditions.

Table 2

Callus development as induced by temperature and colchicine treatment

Genotype *)	Culture media: Type of medium	Culture media: Sucrose (%)	Culture media: Hormones (mg/l)	Treatment of microspore cultures prior to incubation at 28 °C	Number of calli obtained
1355/3/33 G	LS	3	2 NOA, 0.5 BA	2 days 25 mg/l colchicine at 35 ° C, 1 day at 35 °C	1
1355/3/33 G	LS	3	0.05 2,4 D, 0.2 BA	2 days 25 mg/l colchicine at 35 °C, 1 day at 35 °C	1
1355/3/33 P	LS	12	2 NOA, 0.5 BA	2 days 25 mg/l colchicine at 35 °C, 19 days at 35 °C	1
1358/1/47 P	LS	12	0.1 2,4-D, 0.5 BA	4 days 10 mg/l colchicine at 35 °C, 2 days at 35 °C	1
1358/1/47 P	LS	12	0.1 2,4-D, 0.5 BA	3 days 25 mg/l colchicine at 35 °C	2
1358/1/47 P	LS	12	1 NOA, 2 TDZ	3 days 25 mg/l colchicine at 35 °C	1
C 3309 P	LS	3	2 NOA, 0.2 TDZ	4 days 10 mg/l colchicine at 35 °C	1
1355/3/33 P	LS	12	0.1 2,4-D, 0.5 BA	10 days at 35 °C	1
1355/3/33 P	LS	12	0.1 2,4-D, 0.5 BA	4 days at 35 °C	1
1355/3/33 P	LS	3	0.1 2,4-D, 0.5 BA	10 days at 35 °C	1
C 3309 P	LS	3	2 NOA, 0.2 TDZ	4 days at 4 °C, 6 days at 35 °C	1
1358/1/42 G	NN	3	0.1 2,4-D	---	1
1358/1/42 P	NN	3	2 NOA, 0.5 BA	---	2

*) See Tab. 1

est rate of microcalli formation was achieved by using heat shock as an induction treatment. Cultures containing microspores in the tetrad stage produced a comparatively high yield of microcalli. As no change of the morphology of the tetrads was observed, it is assumed that the microcalli originated from young microspores, which had already passed the tetrad stage.

Callus formation: Only a few microcalli developed to calli (Tab. 1 and 2). Callus formation took place exclusively on solid media. 18 of the 28 calli devel-

Table 3

Embryoid formation as induced by temperature and colchicine treatment

Genotype *)	Culture media: Type of medium	Culture media: Sucrose (%)	Culture media: Hormones (mg/l)	Treatment of microspore culture prior to incubation at 28 °C	Number of embryoids obtained
1358/1/47 G	NN	3	2 NOA, 1 TDZ	2 days 10 mg/l colchicine	1
1358/1/47 P	NN**)	6	0.1 2,4 D, 0.5 BA	3 days 10 mg/l colchicine	1
Ganzin 1 G	NN	3	4 NOA, 1 BA	3 days 10 mg/l colchicine	1
C 3309 P	NN	3	0.005 2,4-D	5 days 10 mg/l colchicine	1
C 3309 P	NN	3	3 NOA, 1 TDZ	2 days 25 mg/l colchicine	1
C 3309 P	NN**)	3	4 NOA, 1 BA	3 days 10 mg/l colchicine	1
1358/1/42 G	NN	3	2 NOA, 1 TDZ	2 days 10 mg/l colchicine	1
1358/1/42 G	NN	3	---	4 days chilling of the inflorescences at 4 °C	1
1358/1/42 P	NN	3	2 NOA, 1 TDZ	4 days chilling of the inflorescences at 4 °C	1
1355/1/116 G	NN	3	3 NOA, 1 BA	5 days at 4 °C	1
1355/1/116 F	LS	12	2 NOA, 1 TDZ	1 day at 4 °C, 4 days at 35 °C	1
1189-9 F	LS	15	0.1 2,4-D, 0.5 TDZ	1 day chilling of the inflorescences at 4 °C, 3 days 25 mg/l colchicine	2
1189-9 F	LS	12	0.1 2,4-D, 1 TDZ	1 day chilling of the inflorescences at 4 °C, 3 days 25 mg/l colchicine	1
1355/1/116 G	NN	3	3 NOA, 1 BA	---	2
1358/1/42 P	NN	3	2 NOA, 0.5 BA	---	1

*) see Tab. 1
**) liquid

oped on LS media, the remaining 10 on NN media. The sucrose concentrations of these media were 3 and 12 %. The most responsive genotype was 1358/1/47 with 9 calli followed by 1355/3/33 with 8 calli. Colchicine treatment at 28 °C proved to be the most successful induction method leading to the formation of 13 calli. Combination of heat shock with colchicine treatment induced the formation of 8 calli. Other combinations and incubation without induction treatment resulted in the development of the remaining 7 calli.

Development of embryoids: Embryoid formation was observed mainly on NN media with 3 % sucrose, whereas the 4 LS media, on which embryoids developed, contained 12 and 15 % sucrose (Tab. 3). Three types of embryoids were distinguished. The first showed marked polarity and cellular structure, whereas the second also had a polar form but a less compact structure. The third type formed very compact but rather globular bodies, whose surfaces were non-translucent and did not show a cellular structure (Fig. 4). All embryoids were pigmented.

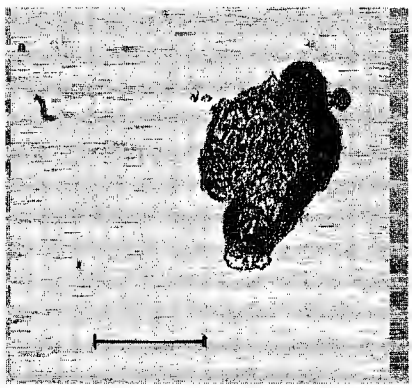

Fig. 4: Microspore-derived embryoid after 6 months of cultivation. Bar = 50 µm.

The efficiency of embryoid formation was <1 % (number of buds: 3580). Treatments with 10 or 25 mg/l colchicine for 2-5 d yielded in total 7 embryoids. Colchicine treatment of microspores isolated from chilled inflorescences and incubation of the microspores without induction treatment led to 3 embryoids each. 2 embryoids were obtained from microspores of chilled inflorescences. Chilling of the microspore cultures and the combination of chilling and heat shock yielded one embryoid (Tab. 3).

Calli and embryoids were transferred to subculture media, but further development was slow. After 2 months of subculture, embryoids did not show reactions to staining with FDA. However, after a period of 8 months, very modest cell proliferation could still be detected on some tissues. Due to the lack of tissue exhibiting high mitotic activity and also of the amount of tissue reqired for flow cytometric analysis, the ploidy level of the tissue could not be examined.

Discussion

Microspore culture has been shown to be successful in many plant species. The most responsive species so far, *Brassica napus*, serves as model plant for studies on sporophytic development *in vitro* (Pechan *et al.* 1991; Custers *et al.* 1994; Telmer *et al.* 1993). In *Vitis*, however, regeneration of haploid plants has been attempted only by means of anther culture (Gresshoff *et al.* 1974; Rajasekaran *et al.* 1979; Altamura *et al.* 1992). The haploid microspore-derived tissue was overgrown by callus from diploid anther tissue (Altamura *et al.* 1992). Therefore, anther culture in *Vitis* has meanwhile been established as a tool for plant regeneration *via* somatic embryogenesis.

As shown with *Capsicum*, anther and microspore culture of the same species do not necessarily require the same culture conditions. While haploid plants can be obtained *via* anther culture, cultured microspores do not develop further than globular embryo stage (Regner 1996). Likewise, it cannot be expected that culture media and treatments employed in anther culture of *Vitis* should be equally favorable to a sporophytic development. As no reports on microspore culture in grapevine have been published, a large number of variations of factors known to influence the development of cultured microspores had to be tested in order to obtain indications for a successful culture of *Vitis* microspores. As only 28 calli and 17 embryoids developed from the microspores of 3580 buds, it is rather difficult to detect a preference for certain conditions. However, most of the cultures which brought forth calli or embryoids were treated with colchicine, lower concentrations (10 and 25 mg/l) being most efficient. This suggests that diploid cells develop more easily to calli or embryoids than haploid material. Chilling of the inflorescences gave rise to several embryoids, but did not induce callus formation. More calli were found in LS media than in NN media, whereas most of the embryoid development took place in NN media. Regarding the sucrose concentration of the culture media it is surprising that at 3 and 12 % sucrose callus development occurred at approximately the same rates, but did not occur in media with intermediate concentrations. Embryoids developed at all sucrose concentrations employed, but clearly preferred the low content (3 %), at which about 75 % of the observed embryoid formation took place. The use of male genotypes did not improve the response to microspore culture.

Neither calli nor embryoids regenerated plants. The ability of embryos to regenerate plants is considered to be genetically determined (Rajasekaran *et al.* 1979) as well as strongly influenced by culture conditions (Fauré *et al.* 1993). It is likely that both factors are responsible for the failure to induce embryoid germination in this work. In grapevine, a lack of vitality of haploid and homozygous material is expected considering the highly heterozygous state of the natural genome. The use of tetraploid microspore donor plants is suggested to avoid at least the haploid phase. Furthermore, genotypes displaying male sterility could be tested on microspore culture (Benito Moreno *et al.* 1988). Chilling of embryos led to germination of somatic embryos in grapevine (Rajasekaran *et al.* 1979) and of haploid embryos in tulip (van den Bulk *et al.* 1994) and might be useful in grapevine microspore subculture as well.

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