INFLUENCE OF THE FLOWER STAGE AND CULTURE MEDIUM ON THE INDUCTION OF SOMATIC EMBRYOGENESIS FROM ANTHER CULTURE IN TUNISIAN GRAPEVINE CULTIVARS

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Abstract

Aims: The aim of this research is to identify the optimal flower developmental stage, in order to establish an efficient regeneration protocol via somatic embryogenesis in several local grapevine (*Vitis vinifera* L.) cultivars, using anther culture.

Methods and results: Immature anthers sampled on fruity cuttings, at three flower developmental stages were cultured on three media [MS (1962), NN (1969) and CP (1987)] to which several phytohormonal combinations were added. Anthers, sampled at the « separated floral buds » stage, corresponding to the tetrad stage of microsporogenesis revealed to be the most appropriate for somatic embryogenesis compared to anthers sampled at the « separated clusters » and « early bloom » stages. Therefore, a unique medium, CP (1987) supplemented with 9 µM of 2,4-D and 11.35 µM of thidiazuron was established for the induction as well as the development of somatic embryos.

Conclusion: The identification of an appropriate flower developmental stage and the optimization of a unique medium allowed establishing a reliable and repetitive regeneration system via somatic embryogenesis, in several local grapevine cultivars.

Significance and impact of study: These embryogenic lines would be particularly appropriate as a target for the genetic transformation, directed towards the induction of tolerance to several biotic and abiotic stresses as well as for bioprospecting.

Keywords: immature anthers, 2,4-D, TDZ, somatic embryogenesis, *Vitis vinifera*

Résumé

Objectif : Ce document vise l'identification du stade inflorescentiel optimal afin d'établir un protocole fiable de régénération par embryogenèse somatique, utilisant la culture d'anthères.

Méthodes et résultats : Des anthères immatures, prélevées à trois stades de développement floral sur des boutures fructifères, ont été cultivées sur trois milieux de base (MS, NN et CP) et plusieurs combinaisons phytohormonales. Les résultats ont montré que les anthères issues du stade « fleurs séparées » correspondant au stade tétrade de la microsporogenèse se prêtent plus favorablement à l'embryogenèse somatique que celles prélevées aux stades boutons floraux séparés et pré-floraison. D'autre part, le milieu unique Chée and Pool (1987) renfermant 9 µM de 2,4-D et 11,35 µM de TDZ a été optimisé pour l'induction et le développement d'embryons somatiques et ce, pour l'ensemble des variétés étudiées.

Conclusion : L'identification du stade inflorescentiel le plus approprié, ainsi que l'optimisation d'un milieu unique, nous ont permis d'établir un protocole de régénération fiable et répétitif par embryogenèse somatique chez plusieurs variétés de vignes tunisiennes.

Signification et impact de l'étude : Ces lignées embryogènes seraient notamment utilisées dans des programmes de transformation génétique visant la régénération de vignes tolérantes aux stress biotique et abiotique ainsi que dans des études de bio-prospection.

Mots clés : anthères immatures, 2,4-D, TDZ, embryogenèse somatique, *Vitis vinifera*

Abbreviations: 2,4,5-T: 2,4,5-trichlorophenoxy-acetic acid, 2,4-D: 2,4-dichlorophenoxyacetic acid, NOA: 2-naphtoxyacetic acid, NAA: 1-naphthaleneacetic acid, IAA: indole-3-acetic acid, BA: 6-benzylaminopurine, Kin: 6-furfurylaminopurine, TDZ: 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea.

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INTRODUCTION

Tunisia possesses a rich phytogenetic patrimony of autochthonous grapevine cultivars well adapted to severe climatic conditions, mainly salt and drought (Ben Salem-Fnayou *et al.*, 2006). In addition, many cultivars present an interesting organoleptic quality (Zemni, 2007). However, several local grapevine cultivars are facing damaging viral diseases, extinction and genetic erosion. Regarding this situation, somatic embryogenesis seems to be an efficient tool for Tunisian genetic resources preservation. Thus, embryogenic material is the target for grapevine genetic transformation (Valat *et al.*, 2006).

Immature anthers are the most commonly used sources for inducing somatic embryogenesis and regenerating grapevine plants (Bouquet *et al.*, 1982; Newton and Goussard, 1990; Bouquet and Torregrosa, 2003). However, this technique is not yet routine; this system even provides poor results with many important cultivars (Martinelli and Gribaudo, 2001; Kikkert *et al.*, 2005).

In literature, authors usually refer to anther culture by « days before bloom » (Carimi *et al.*, 2005), « flower length » (Salunkhe *et al.*, 1999), « anther length » (Stamp and Meredith, 1988a), and « anther external appearance » (Mauro *et al.*, 1986). Few authors were interested to the recognition of the appropriate flower developmental stage that induces somatic embryogenesis (Kikkert *et al.*, 2005), and were much less interested in developing the relationship between the flower developmental stage and anther maturity (Gribaudo *et al.*, 2004).

Moreover, grapevine recalcitrance to tissue culture is still limited by several factors, such as genotype differential responses, arrest at late torpedo-stage (Martinelli and Gribaudo, 2001), culture medium composition (Perrin *et al.*, 2004), and adequate growth regulator combinations (Zlenko *et al.*, 2005).

This study aims to identify the optimal stage for collecting explants, in order to initiate anther culture and establish an efficient regeneration protocol via somatic embryogenesis, a prerequisite for genetic transformation.

MATERIALS AND METHODS

1. Plant materials

Nine Tunisian cultivars (*Vitis vinifera* L.), namely: Ahmar Bou Ahmar, Arich Ahmar, Arich Dressé, Asli , Khamri Tozeur, Muscat d'Alexandrie, Muscat Raf-raf, Sakasly and Turky, grown in the germplasm repository of the Center of Biotechnology of Borj-Cedria, were studied. Anthers were excised from fruity-cuttings (forced canes). Fruity-cuttings were obtained by cultivating three node dormant cuttings in humid pots of sand, during four weeks in a controlled room (28 °C temperature, 90 % relative humidity and 16-h photoperiod). Within two weeks, roots appeared prior to bud development. To induce flowering on these lignified cuttings, primordial bud leaves were regularly removed (Mullins and Rajasekaran, 1981; Ollat *et al.*, 1998 modified later by Bouamama *et al.*, 2004).

2. Observation of anther maturity and disinfection of floral bunches

Inflorescences collected at the « separated floral buds » stage H (Baggiolini, 1952) were gently crushed in a drop of glycerin, directly observed by light microscope and finally selected for starting culture initiation.

Excised inflorescences were disinfected in sodium hypochlorite (6 $g.l^{-1}$) during 15 min, and then rinsed three times in sterile water containing citric acid (0.03 $g.l^{-1}$) in the last bath.

3. Culture media and conditions

To establish an optimal medium for the induction of somatic embryogenesis, three basal media: Murashige and Skoog, 1962 [MS]; Nitsch and Nitsch,1969 [NN] and Chée and Pool,1987 [CP], supplemented with several growth regulator combinations were tested as follows: (9 µM 2,4-D/2.21 µM BA); (9 µM 2,4-D/11.35 µM TDZ); (9 µM 2,4-D/ 2.32 µM Kin); (3.79 µM 2,4,5-T/2.21 µM BA); (3.79 µM 2,4,5-T/11.35 µM TDZ); (3.79 µM 2,4,5-T/2.32 µM Kin); (4.94 µM NOA/2.21 µM BA); (4.94 µM NOA/11.35 µM TDZ); (4.94 µM NOA/2.32 µM Kin); (5.37 µM NAA/ 2.21 µM BA); (5.37 µM NAA/11.35 µM TDZ); (5.37 µM NAA/2.32 µM Kin); (5.7 µM IAA / 2.21 µM BA); (5.7 µM IAA/11.35 µM TDZ) and (5.7 µM IAA /2.32 µM Kin).

Media pH was adjusted to 5.8 by KOH addition. Subsequently, gelrite (3 $g.l^{-1}$) and sucrose (30 $g.l^{-1}$) were added to the media before sterilization (24 min at 116 °C) and distribution in 60 mm Petri dishes (8 ml per plate). Anthers were aseptically plated on their adaxial face on each culture medium. A total number of 25 explants per plate were cultivated through 5 lines (Reustle, pers. comm.) and three replicates were done for each treatment (basal medium and growth regulator combination). The primary calluses were transferred every 4 to 5 week-intervals on the same refreshed solid medium and grown at 24±1 °C, in the dark.

4. Proliferation of embryogenic tissues

Embryogenic calluses were maintained in darkness and sub-cultured in Chée and Pool (1987) basal medium supplemented with 9 µM 2,4-D and 11.35 µM of TDZ. These lines were maintained through embryogenic callus subculture at 4 week-intervals.

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5. Maturation and conversion to somatic embryos and germination to plantlets

The maturation of somatic embryos and their conversion was done on the same medium at 24±1 °C temperature and 16-h photoperiod. Converted somatic embryos were transferred individually into tubes, on Chée and Pool (1987) without phytohormons, to stimulate their germination into plantlets.

6. Morphological observations

Embryogenic calluses and converted somatic embryos were fixed overnight in 3 % glutaraldehyde and then processed according to Rascio *et al.* (1999). Thin sections (1 µm) were cut with an ultra-microtome (Ultracut, Reichert-Jung), stained with « hematoxylin Regaud » and examined using a light microscope.

7. Statistical analysis

Data were analyzed using STATISTICA software. Duncan Multiple Range test was used to determine the significance between the five auxins, the three cytokinins and the three based media cited above (tables 1 and 2) as well as the proliferation and maturation rates (tables 3 and 4).

RESULTS

1. Media effects on the induction of embryogenic calluses

Inflorescences were harvested from fruity-cuttings at three developmental stages: « separated clusters », « separated floral buds » and « early bloom », as shown in (figure 1). These stages correspond respectively to G, H and I as defined by Baggiolini (1952).

At first, the effect of three media (MS, NN, and CP) supplemented with several plant growth regulators on the induction of embryogenesis was studied from immature anthers of Muscat Raf-raf cultivar. The induction of embryogenic calluses, registered after 12 weeks, depended on plant growth regulators as shown in (table 1). Auxins ($p<0.0007$), cytokinins ($p<0.0053$) had a highly significant effect on the induction of somatic embryogenesis, and their interaction was also significant ($p< 0.0235$). Table 2 shows that the presence of TDZ (11.35 µM) and 2,4-D (9 µM) in the CP basal medium significantly enhanced the percentage of embryogenic callus induction from immature anthers in Muscat Raf-raf cultivar (21.33 %). Secondly, NAA/ IAA/ NOA/ 2,4,5-T/ combined to BA/ Kin or TDZ were found to be unsuitable plant growth regulators. Thus, a clear influence of the auxins and cytokinins supplemented to the culture media was noticed on embryogenic induction.

Somatic embryogenesis from anthers was further performed using CP-based medium containing 9 µM of 2,4-D and 11.35 of µM TDZ for all of the nine cultivars cited above.

2. Effects of flower developmental stages on the induction of embryogenic calluses

To identify the appropriate flowering period for embryogenesis induction, anthers from the nine Tunisian cultivars were compared at three phenological stages, « separated clusters », « separated floral buds » and « early bloom ». Anthers were cultivated on Chée and Pool

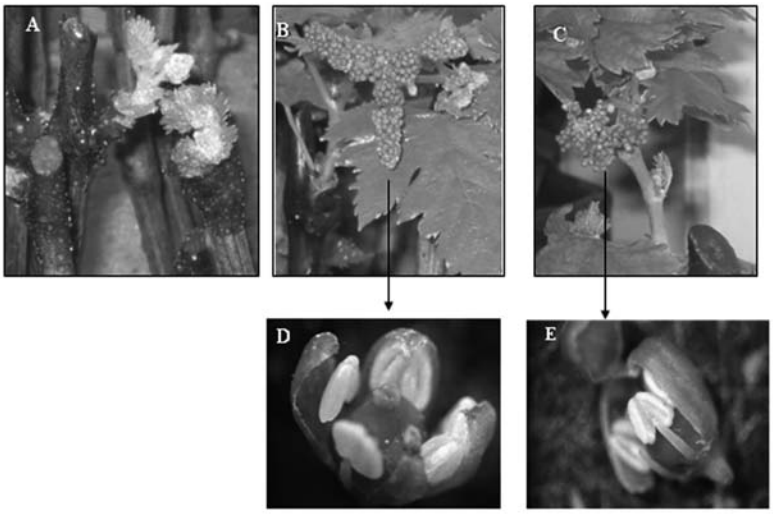


Figure 1 - Different floral developmental stages obtained by fruity-cutting from Tunisian cultivars (*Vitis vinifera*). A, flowers at separated clusters (Baggiolini, 1952). B, flowers at separated floral buds (Baggiolini, 1952). C, Flowers at early bloom, (Baggiolini, 1952). D, Green translucent anthers collected at the separated floral buds. E, Yellowish anthers collected at the early bloom stage.

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Table 1 - Effects of several media, growth regulator combinations and their interactions on somatic embryogenesis induction of Muscat Raf-raf cultivar.

Phytohormons, media and interactions	F	p
Auxins*	4.7888	0.0007*
Cytokinins*	5.2425	0.0053*
Media	0.7057	0.4938
(Auxins x Cytokinins)*	2.2180	0.0235*
(Auxins x Media)	0.7057	0.6868
(Cytokinins x Media)	0.4033	0.8064
(Auxins x Cytokinins x Media)	0.4033	0.9823

*: Asterisks indicate significantly different results at $p<0.05$ significance level, according to ANOVA.

Table 2 - Effect of growth regulator combinations on the percentage of somatic embryogenesis induction of Muscat Raf-raf cultivar (scored after 12 weeks of cultivation).

Auxins	Cytokinins	Basal media	% embryogenic callus
2,4-D	TDZ	CP	21.33a
2,4-D	TDZ	NN	12.00b
2,4-D	BA	CP	10.67b
2,4-D	TDZ	MS	6.67c
2,4-D	BA	NN	6.67c
2,4-D	BA	MS	5.33c
Remaining combinations			< 1.33d

Values with different letters indicate significant differences according to Duncan multiple range test (at $p<0.05$).

(1987) basal medium supplemented with 9 µM of 2,4-D and 11.35 µM of TDZ. As shown in figure 2, the induction of embryogenic calluses depended on the flower developmental stage. The best rates of embryogenic calluses were observed in 8 cultivars (Ahmar Bou Ahmar, Arich Ahmar, Arich Dressé, Asli, Khamri Tozeur, Muscat d'Alexandrie, Muscat Raf-raf and Turky). These rates ranged between 5 and 40 %, depending on the cultivar, when explants were collected at the « separated floral buds », corresponding to tetrad pollen (figure 3). However, anthers with no growth or small embryogenic calluses were observed, when anthers were sampled at both early (mother cell pollen) and late (binucleate pollen) stages, corresponding respectively to « separated clusters » and

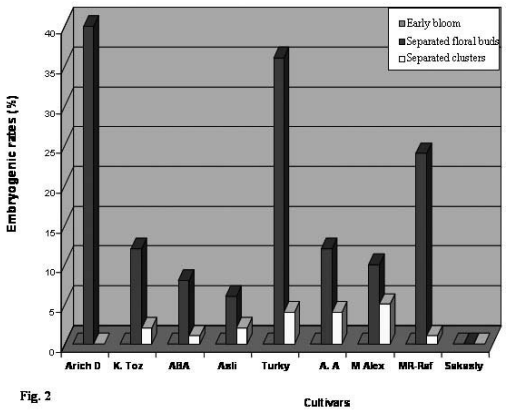


Fig. 2

Figure 2 - Effect of flowering stages on embryogenic induction (%) on Chée and Pool basal medium containing 9 µM of 2,4-D and 11.35 µM of TDZ in nine grapevine cultivars.

Abbreviations: K. Toz.: Khamri Tozeur; ABA: Ahmar Bou Ahmar; M. Alex.: Muscat d'Alexandrie; MR.Raf.: Muscat Raf-raf, Arich D.: Arich Dressé, AA: Arich Ahmar.

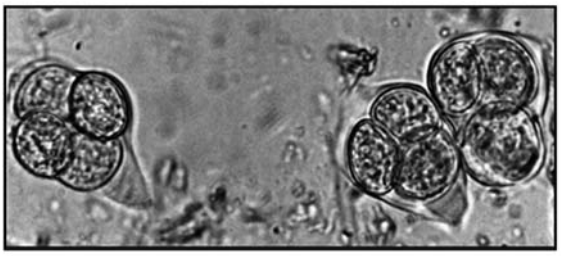


Figure 3 - Microspore at tetrad stage from Muscat Raf-raf cultivar.

« early bloom » (Baggiolini, 1952). Therefore, no response was registered in Sakasly cultivar.

Within 12 weeks of cultivation on CP supplemented with 9 µM 2,4-D and 11.35 µM TDZ, distinctly visible friable and whitish proembryogenic calluses emerged from anthers. Primary calluses produced proembryogenic masses from different anther levels namely, lateral, adaxial and abaxial zones, filaments and entire anthers.

3. Proliferation of embryogenic callus and their development to somatic embryos

The proembryogenic material obtained on CP supplemented with 9 µM 2,4-D and 11.35 µM TDZ, continued proliferating on this same medium, under darkness (figure 4A). An increasing amount of embryogenic calluses was produced in all cultivars, from the third to the fifth sub-culture (table 3). Thus, our strategy, based on a unique medium, seems to be crucial in maintaining the embryogenic competence for more than 5 sub-cultures.

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Table 3 - Effect of the unique medium (Chée and Pool+ 9 µM of 2,4-D and 11.35 µM of TDZ) on embryogenic callus proliferation from the 3rd to the 5th sub-culture.

Cultivars	3rd sub-culture (%)	5th sub-culture (%)
'Arich Ahmar'	37.75 bcd	100.92 a
'Arich Dressé'	60.33 a	75.67 b
'Asli'	30.50 d	77.92 b
Ahmar Bou Ahmar'	44.08 bc	76.17 b
'Muscat Raf-raf"	53.67 a	68.17 bc
'Turky'	35.33 cd	64.42 cd
'Muscat d'Alexandrie'	39.75 bc	69.67 bc
'Khamri Tozeur'	35.50 cd	71.50 bc

Means followed by different letters are significantly different according to Duncan's multiple range test at $p < 0.05$.

The development of somatic embryos was also achieved on the same medium, under 16 h photoperiod conditions in order to induce maturation. At this step, somatic embryos were hard in texture, milky-white and easily distinguishable from embryogenic tissues. Early-developed embryos proceeded towards advanced stages (heart, short and elongated torpedo, precocious cotyledon), developed and differentiated on the surface of embryogenic calluses (figure 4B) and finally resulting in germinated embryos as shown in figure 4C. Chée and Pool medium containing 9 µM of 2,4-D and 11.35 µM of TDZ seems to be efficient for the development of somatic embryos. In fact, table 4 shows significant differences in the mean number of somatic embryos per explant at various stages, with a maximum number of 318.17 in Ahmar Bou Ahmar cultivar. Therefore, a variable effectiveness for somatic embryos formation was noticed between several cultivars (Asli, Ahmar Bou Ahmar, Arich and Turky). While no significant differences were registered between Muscat Raf-raf, Muscat d'Alexandrie and Khamri Tozeur.

Embryogenic callus development and somatic embryos conversion were examined by light microscopy. Embryogenic cells exhibited an active proliferation and progressively initiated clusters, forming numerous huge protruding masses as shown in figure 5A. They grow continuously from the globular to the cotyledonary stage. Somatic embryos formed bipolar axes and a cotyledonary crown and were endowed by a rapid cellular differentiation, which reached precociously and simultaneously a root and a shoot meristem, as shown in figure 5B.

The percentages of germinated somatic embryos that developed into plantlets ranged between 68.9 % in Asli and 95.6 % in Muscat d'Alexandrie cultivars after two weeks of cultivation on CP medium without phytohormons (table 5). Morphologically normal grapevine plants developed from all of the cultivars, excepting Sakasly ; these plants were transferred to *in vivo* conditions within 12 months of cultivation (figure 6A).

The acclimatization of regenerated plants was efficiently done in the greenhouse. In fact, developed *in vitro* plantlets were transferred into small peat pots. About 80 % of *in vitro*-derived plantlets grew larger under ex-vitro, indicating the onset of autotrophy in these controlled conditions (figure 6B).

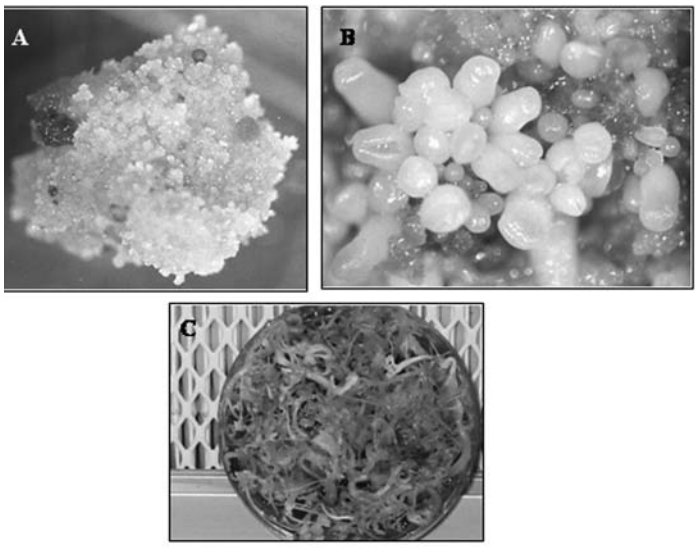


Figure 4 - A: Proembryogenic callus obtained after 3 subcultures on the unique medium from Arich Dressé cultivar (x 20). B: Somatic embryos at different developmental stages (x 20). C: Converted somatic embryos (rooted embryos with 2 cotyledonary leaves) (x 4).

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Table 4 - Effect of the unique medium (Chée and Pool+ 9 µM of 2,4-D µM and 11.35 of TDZ) on the development of somatic embryos under 16 h photoperiod condition.

Cultivars	Total number of somatic embryos	Different stages of somatic embryos		
		Globular	Heart-torpedo	Cotyledonary
'Asli'	234.33 b	151.5 b	51.16 a	31.66 c
'Ahmar B.A'	318.17 a	208.66 a	77.5 b	32 c
'M Alex.' (NS)	220.33	158.5	47.83	14
'Arich Ahmar'	218.5 b	147.66 a	60.5 c	10.33 a
'Turky'	260.5 a	225.16 b	30.33 a	5± c
'M. raf-raf' (NS)	232.83	166.83	56	10.5
'K. Tozeur' (NS)	253.83	190.5	57.16	6.16
'Arich Dressé' (NS)	219.17	158	47.5	13.67

Means of three replicates followed by different letters are significantly different according to Duncan's multiple range test at $p<0.05$.

Abbreviations: Ahmar B.A : Ahmar Bou Ahmar, M. Alex : Muscat d'Alexandrie, M. raf-raf: Muscat Raf-raf, K. Tozeur: Khamri Tozeur.

Table 5 - Germination frequencies of several grapevine cultivars on Chée and Pool basal medium without phytohormons.

Cultivars	Germination frequencies±CI)
'Asli'	68.9±13.67
'Muscat Raf-raf'	88.9±9.28
'Muscat d'Alexandrie'	95.55±6.089
'Arich Ahmar'	70.27±14.93
'Arich Dressé'	90.91±9.96
'Turky'	90±9.41
'Ahmar Bou Ahmar'	92.5±8.26
'Khamri Tozeur'	92.5±8.26

Embryos cultivated on Chée and Pool (1987) without phytohormons at an advanced cotyledonary stage (2 cotyledonary leaves and a root crown).

Each value represents germination frequencies±Confidence Interval.

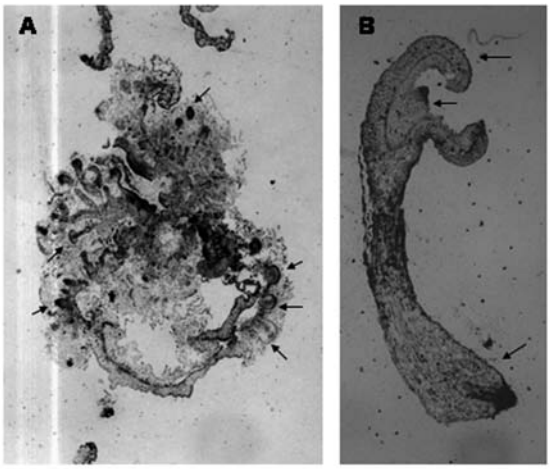


Figure 5 - Development of somatic embryos. A: Longitudinal section of an embryogenic callus showing several precocious and advanced differentiated somatic embryos. B: Cotyledonary stage of the developing embryo (longitudinal section), showing clearly root and shoot meristems.

DISCUSSION

Although the regeneration of grapevine via somatic embryogenesis has been previously reported for several *Vitis* species, including most of the European and American vines (Pinto-Sintra, 2007; Maillot *et al.*, 2006; Kikkert *et al.*, 2005), no reports on a successful somatic embryogenesis exist for Tunisian grapevines. In the present investigation, somatic embryogenesis from anthers was achieved for the first time in eight Tunisian cultivars. It is expected that this system would be employed to establish an efficient genetic transformation scheme for Tunisian grapevines. Hence, we focused on the production of embryogenic cultures, which rapidly grew, proliferate and regenerate. The results of this research show that floral developmental stage and medium composition affect somatic embryogenesis of grapevine. Our

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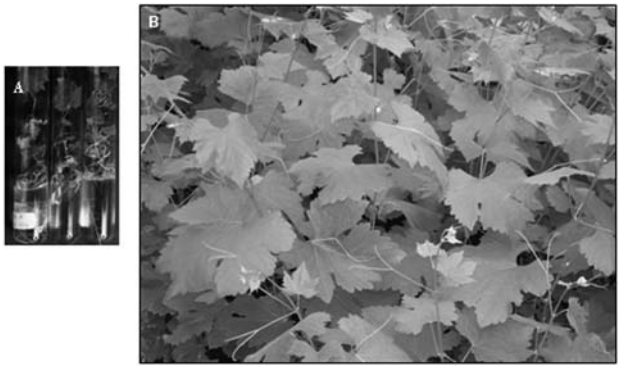


Figure 6 - A: Complete grapevine plantlets on solid Chée and Pool (1987) medium.
B: Acclimatized plantlets regenerated from somatic embryos of several Tunisian cultivars.

experiments revealed that immature anthers excised from inflorescences at « separated floral buds » stage and cultured on CP containing 9 µM of 2,4-D and 11.35 µM of TDZ were competent for the induction of embryogenic calluses for the eight cultivars. Moreover, embryogenic lines, cultured under proliferating conditions (darkness) on the same medium, maintained their embryogenic capacity over 5 subcultures. The levels of 2,4-D and TDZ could be one of the main factors for the induction and morphology gradation from scattered proembryogenic masses on the primary calluses to homogenous embryogenic structures under darkness. The presence of auxin (2,4-D) in the medium is generally essential for somatic embryogenesis induction (Gribaudo *et al.*, 2004), whereas TDZ is better known for its effect in other crops (Jheng *et al.*, 2006). For few years, TDZ has been used to induce somatic embryo formation, similar growth regulators combined to MS basal medium promoted somatic embryogenesis from filaments in grapevines (Nakajima and Matsuta, 2003; Olàh *et al.*, 2003). However, in this study, Chée and Pool (1987) basal medium proved to be more optimal than MS and NN, which were found to be efficient in several studies involving anthers (Gribaudo *et al.*, 2004; Perrin *et al.*, 2004; Martinelli et Gribaudo, 2001). In the literature, the establishment of somatic embryogenesis requires at least two different culture media (induction, differentiation or embryo development) (Kikkert *et al.*, 2005; Pinto-Sintra, 2007). In our case, embryos maturation and their development occurred on the same medium, previously used for embryogenic callus induction and proliferation.

A genotype-specific reaction was observed for all the tested cultivars except Sakasly. Similar results reported that regeneration via somatic embryogenesis is genotype-dependent (Bouquet *et al.*, 1982).

The anthers used for grapevine somatic embryogenesis induction have been described as translucent pale green or white to yellow (Stamp and Meredith, 1988a; Newton and Goussard, 1990; Kikkert *et al.*, 2005). When the microsporogenesis stage is reported, anthers are usually in the tetrad or uninucleate stages (Rajasekaran and Mullins, 1979; Bouquet *et al.*, 1982; Kikkert *et al.*, 2005). Our experiments revealed that the « separated floral bud » stage corresponding to the tetrad pollen, was the most appropriate stage leading to efficient somatic embryogenic rates in eight cultivars, excepting Sakasly which never regenerated somatic embryos from such an explant. Similar results were obtained by Perrin *et al.*, (2004) who approved the efficiency of the « separated floral buds » in inducing somatic embryogenesis on several *Vitis* rootstocks and cultivars. Hollo and Misik (2000) observed better embryogenesis in Riesling cultivar at mid- and late-uninucleate phases, if anthers were originated from forced canes. While Salunkhe *et al.*, (1999) selected anthers from *V. labruscana* at three stages of anther maturity (uninucleate, late uninucleate and tetrads) and obtained embryogenic calluses only from anthers with uninucleate pollen. However, six individual stages (I to VI) were identified by Gribaudo *et al.* (2004) and the best results were obtained when explants were collected at early stages (stages I and II) for Chardonnay and Barbera. For 110R however, these authors reported that somatic embryogenesis occurred more frequently if anther culture started at early and late uninucleate pollen (stages V and VI). The failure of these measures in Sakasly however, could be attributed to the lack of additional treatments such as chilling (Perl *et al.*, 1995). On the other hand, cultural practices and climatic requirements may also influence such a response. Growth conditions, climate and pruning for instance, may affect root and hardwood reserves and consequently flowering ability (Perrin *et al.*, 2004; Lebon *et al.*, 2005).

To conclude, this is the first report on a successful regeneration of Tunisian cultivars *via* somatic embryogenesis using anther culture. This study was based:

i) first, on the optimization of a unique medium for the induction, proliferation and maturation of somatic embryos from eight cultivars.

ii) second, on the identification of an optimal developmental flower stage for inducing embryogenesis from anther culture.

These embryogenic lines would be particularly appropriate as a target for the genetic transformation of Tunisian cultivars, directed towards the induction of tolerance to several biotic and abiotic stresses.

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