ISOLATION AND CULTURE OF LEAF PROTOPLASTS FROM TUNISIAN GRAPES

ISOLATION ET CULTURE DE PROTOPLASTES DE VIGNES TUNISIENNES

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Abstract: Experimental conditions for leaf protoplast isolation and culture were optimised for *in vitro* plants deriving from shoot culture of two Tunisian grape varieties, Sakasly and Muscat d'Alexandrie (*Vitis vinifera* L.). The best yields were obtained from leaves of 4 to 5 weeks old *in vitro* plants, digested for 13 hours under 25 rpm agitation with an enzymatic mixture containing 0.25 % cellulase of *Aspergillus niger*, 0.25 % cellulase of *Penicillium funiculosum*, 0.5 % cellulysin of *Trichoderma viridae*, and 0.2 % macerozyme R-10 of *Rhizopus* sp. More than 50 % of the purified protoplasts had a diameter of 30-40 µm and were rich in chloroplasts. Best aptitude for cell division was found in protoplasts immobilised in sodium alginate layers at a density of $0.5x10^6$ cell/ml, cultivated in CPW-13 medium containing 4 mg/l of NOA and 0.88 mg/l of TDZ. The variety Muscat d'Alexandrie gave better yield whereas Sakasly showed better cell division rates. Formation of micro and macrocallus have been obtained, but the oxidation of the medium has to be solved in order to promote plant regeneration.

Résumé : Les conditions optimales d'isolement et de culture de protoplastes ont été mises au point à partir de vitroplants obtenus par microbouturage de deux variétés de vigne autochtones tunisiennes, Sakasly et Muscat d'Alexandrie (*Vitis vinifera* L.). Les meilleurs rendements ont été obtenus à partir de feuilles prélevées sur des vitroplants âgés de 4 à 5 semaines, digérées pendant 13 h sous une agitation de 25 rpm. La solution de macération était composée de 0,25 % cellulase d'*Aspergillus niger*, 0,25 % cellulase de *Penicillium funiculosum*, 0,5 % cellulysin de *Trichoderma viridae*, et 0,2 % macerozyme R-10 de *Rhizopus* sp. Plus de 50 % des protoplastes purifiés étaient riches en chloroplastes et présentaient un diamètre de 30 à 40 µm. Les protoplastes immobilisés dans des couches d'alginate de sodium, à une densité de 0,5 x 10^6 protoplastes/ml et cultivés sur le milieu CPW-13 additionné de 4 mg/l de NOA et de 0,88 mg/l de TDZ, ont montré une meilleure aptitude à la division. Les meilleurs rendements ont été obtenus avec la variété Muscat d'Alexandrie alors que les meilleurs taux de divisions cellulaires avec la variété Sakasly. Toutefois, les problèmes d'oxydation ont bloqué la prolifération des protoplastes au stade de macrocal et ont par conséquent compromis toute régénération en plantes.

Key words: *Vitis vinifera*, protoplast, macrocallus.

Mots clés : *Vitis vinifera*, protoplaste, macrocal.

INTRODUCTION

Protoplasts represent an interesting material for molecular, biochemical, and physiological studies as well as for the production of genetically transformed plants. Indeed, different and simple techniques could be used, such as electroporation and PEG mediated gene transfer. The selection might be easier and additional techniques like somatic hybridization and selection of somaclonal variants at the protoplast level could be applied (REUSTLE *et al.*, 1995). Nevertheless, the success of these breeding technologies is dependent on the establishment of efficient protoplast-to-plant regeneration procedures.

In grapevine, plant regeneration from protoplasts (REUSTLE *et al.*, 1995; ZHU *et al.*, 1997) and genetic transformation of embryogenic tissue (MARTINELLI and MANDOLINO, 1994; TORREGROSA *et al.*, 1994) have been achieved. However, using leaf protoplasts, several attempts to regenerate plants failed (BREZEANU *et al.*, 1982; LEBRUN, 1985; SHIMIZU, 1985; BARBIER and BESSIS, 1987, 1990; ALLEWELDT and REUSTLE, 1988; REUSTLE and ALLEWELDT, 1990; THEODOROPOULOS and ROUBELAKIS-ANGELAKIS, 1990). In this respect, leaves from *in vitro* plants may represent a convenient source of protoplasts (LEE and WEZSTEIN, 1988; BARBIER AND BESSIS, 1990; KATSIRDAKIS and

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J. Int. Sci. Vigne Vin, 2003, **37**, n°2, 145-153

ROUBELAKIS-ANGELAKIS, 1992a,b), since this kind of material is permanently available and does not require any special equipment or expertise. The difficulties of plant regeneration from leaf protoplasts depend on many factors that affect their isolation and culture (ERIKSON, 1985). Thus, it is necessary to investigate the different parameters involved in order to optimize conditions for isolation, culture and plant regeneration.

The present work represents the first report on protoplasts from Tunisian *Vitis vinifera* genotypes, Sakasly and Muscat d'Alexandrie. The effect of physiological state of donor plant, enzymatic combination, cultivar and maceration conditions have been considered at the isolation step. Composition of culture medium, growth regulator combinations, cell density and culture conditions were analysed during the culture step.

MATERIAL AND METHODS

I - PLANT MATERIAL

Shoots from two Tunisian grape varieties, Sakasly and Muscat d'Alexandrie were cultivated *in vitro* on MURASHIGE and SKOOG medium (1962) supplemented with sucrose 30.0 g/l, agar 8.0 g/l, indole butyric acid (IBA) 0.5 mg/l, and benzylaminopurine (BAP) 0.5 mg/l. The cultivation took place in a room culture where the day temperature is of 25 °C and night temperature of 19 °C, the photoperiod of 12 h and the light intensity of 50-60 µE/m2. s. The resulting plants were micropropagated on growth regulator free M-Cat medium (BEN ABDALLAH *et al.*, 1997) containing the vitamins of MOREL and MARTIN (1955). These vitroplants were transferred to a fresh medium every five weeks.

II - PROTOPLAST ISOLATION AND CULTURE

When the cultivated plants reach a stage of 6 to 8 developed leaves (usually after 4 to 5 weeks), 0.5 g of leaves were harvested and thinly sliced in 15 ml of sucrose solution (200 g/l, pH 5.8). Following an incubation of 10 to 30 min, the sucrose solution was replaced by the maceration mixture (5 g/l BSA (Sigma, type V), 20 mM MES, 1 mM $CaCl_2$, 144 g/l sucrose, 1/10 of MS Macro's and micro's, pH 5.6). Three enzyme combinations were tested (table I). The combination E2 is that used by REUSTLE and ALLEWELDT (1990). The digestion was carried out at 24 °C with 25 rpm or without agitation in the dark for 10, 13 and 16 hours. The digested material was sieved through 100 and 50 µm filters, and then diluted 2 to 3 times with the sucrose solution before to be centrifuged twice at 300 g for 10 min at 4 °C. The resulting floating protoplasts were collected, diluted 10 times with a CPW-13M washing solution (FEARSON *et al.*, 1973; without glucose) containing 18.1 g/l NaCl, and centrifuged at 80 g for 4 min at 4 °C. The purified protoplasts were recovered at the bottom of the tube as a pellet and resuspended in 0.5 to 1 ml of a 0.65 M of mannitol solution, pH 5.8.

To analyse the effect of the physiological state of the donor material, *in vitro* plants aged of 3, 4, 5, and 6 weeks and transferred 2, 3, 4, 5 and 6 times to a fresh medium were used. Different BAP concentrations (0.5, 1.0 and 2.0 mg/l) were added to the M-Cat culture medium to recover protoplast production from senescent material. Two to three samples per treatment were made and repeated several times.

The protoplasts were cultivated either directly as a suspension or after being immobilised in sodium alginate gel layers (KARESH *et al.*, 1991). Suspensions and alginate layers were incubated in four different

Table I - Enzymatic combinations used for protoplast isolation from *in vitro* leaves of the Tunisian grapevine varieties Sakasly and Muscat d'Alexandrie.

Combinaisons enzymatiques employées pour l'isolement des protoplastes à partir des vitrofeuilles des variétés Sakasly et Muscat d'Alexandrie.

Enzyme combination	Enzyme	Concentration (%)
E1	Cellulase (*Aspergillus niger*) (Sigma)	1.5
	Pectolyase Y-23 (Sigma)	0.04
	Macerozyme R-10 (*Rhizopus* sp) (Serva)	0.3
E2	Cellulase of *Aspergillus niger* (Sigma)	0.25
	Cellulase of *Penicillium funiculosum* (Sigma)	0.25
	Cellulysine of *Trichoderma viridae* (Calbiochem)	0.5
	Macerozyme R-10 of *Rhizopus* sp. (Serva)	0.2
E3	Cellulase of *Aspergillus niger* (Sigma)	1
	Pectolyase Y-23 (Sigma)	0.03
	Driselase (Sigma)	0.2

basic media, MS as modified by KARESCH *et al.*, (1991), CPW-13 (FREARSON *et al.*, 1973; with 95 g/l glucose and 13 g/l mannitol), NN-69 (NITSCH and NITSCH, 1969) and W-P (LLOYD and MCCOWN, 1981). Two growth regulator combinations (C1 and C2) were tested with these culture media; the first composed of 4 mg/l naphtoxyacetic acid (NOA) and 0.88 mg/l thidiazuron (TDZ); the second of 1 mg/l 2,4-D and 0.5 mg/l benzylaminopurine (BAP). Densities of 0.25 x 10^6 and 0.5 x 10^6 cell/ml were used. Cultures were incubated in the dark at 24 °C. Cell wall regeneration and divisions were followed regularly by inverted microscope observation (Versus, CETI Belgium).

The same microscope was used to evaluate the quality of the cultivated protoplasts. Protoplasts with an intact plasmic membrane were defined as viable and those having a damaged membrane as non viable. The thickness of the cell contour and the loss of the spherical shape were used as an indicator of cell wall regeneration. The rates of viability, cell wall regeneration and cell division are mean values calculated from 20 microscopic observation fields.

RESULTS AND DISCUSSION

I - PROTOPLAST ISOLATION AND CULTURE

1) Protoplast isolation

Among the three different enzymatic combinations experimented (table I), the E1 combination (1.5 % cellulases, 0.04 % pectolyase Y-23, and 0.3 % macerozyme R-10) gave higher yields of protoplasts from both varieties (table II). However, protoplasts obtained with the combination E2 (0.25 % cellulase of *Aspergillus niger*, 0.25 % cellulase of *Penicillium funiculosum*, 0.5 % cellulysine of *Trichderma viridae* and 0.2 % macerozyme R-10 of *Rhizopus* sp.) were of better quality. The majority of them showed regular spherical shape and a uniform distribution of chloroplasts. In general, less protoplasts with damaged plasmic membrane were obtained with this enzyme combination, probably because the pectolyase was eliminated. Though the yield is lower than with combination E1, the combination E2 was adopted as high percentage of viable protoplasts is necessary for the establishment of cultures. Indeed, this combination had already proved its efficiency in producing competent protoplasts from grapes (REUSTLE and ALLEWELDT, 1990). The use of macerozyme did not seem to affect the protoplast membrane contrarily to pectolyase. This latter is very efficient in the digestion but may damage the protoplast plasmic membrane and affect its viability (NISHIMURA *et al.*, 1984; SHIMIZU, 1985).

Macerations carried out for 13 hours with 25 rpm agitation gave better yields of protoplasts without any effect on their viability (around 70 % for both varieties). Indeed, maceration of the variety Sakasly produced 27.5 x 10^6 protoplasts/ml with agitation against 12.8 x 10^6 protoplasts/ml in the absence of agitation. This may be explained by the fact that the agitation avoids the accumulation of phenolic compounds around the digested cells and their permanent contact with the enzyme solution (BREZEANU and ROSU, 1984). If agitation is not necessary with some plant species, it seems to be benefit with grape leaf tissue and would be recommended due to the rigidity of the cell wall and its high content of phenolic compounds (HASLAM, 1974). Maceration for more than 13 hours showed a decrease of the yield and an increase of the damaged protoplasts (table III). Thus, 13 hours maceration and agitation at 25 rpm revealed to be optimum conditions and were adopted for all of our experiments.

When comparing yields from the two varieties, it was easier to get more protoplasts with better quality from the variety Muscat d'Aleaxandrie than from Sakasly (table II). This is the effect of the genotype which is well known in grapes (REUSTLE *et al.*, 1994), but also in other plant species (WATTS *et al.*, 1974). Indeed, according to BARBIER and BESSIS (1988), the genotype affects the yield as well as the competence of the protoplasts towards the regeneration. Furthermore, REUSTLE *et al.*, (1994) noted that the

Table II - Yield and viability of leaf protoplasts isolated from varieties Sakasly and Muscat d'Alexandrie.

Rendement en protoplastes des feuilles des variétés Sakasly et Muscat d'Alexandrie.

	Genotype			
	Sakasly		Muscat d'Alexandrie	
Enzymatic combination	Yield (10^6 prot/gFW)*	Viability (%)	Yield (10^6 prot/gFW)*	Viability (%)
E1	1.73	54	4.75	58
E2	0.14	70	1.2	80
E3	0.02	45	0.1	50

*FW: fresh weight of leaf material

Table III - Effect of the maceration time on yield and viability of protoplasts from *in vitro* leaves of the variety Sakasly.

Effet de la macération sur le rendement.

Maceration time (h)	Yield (x10^6 prot/ml)	Viability (%)
10	6.3	80
13	95	85
16	28.4	68

Table IV - Effect of age on protoplast isolation from *in vitro* leaves. Case of the variety Sakasly plants from the second subculture.

Effet de l'âge des vitrofeuilles sur l'isolement des protoplastes à partir de la variété Sakasly.

Age (week)	3	4	5	6
Yield (10^6 prot/gFW)*	0.51	5.20	4.98	0.51

*FW: fresh weight of leaf material

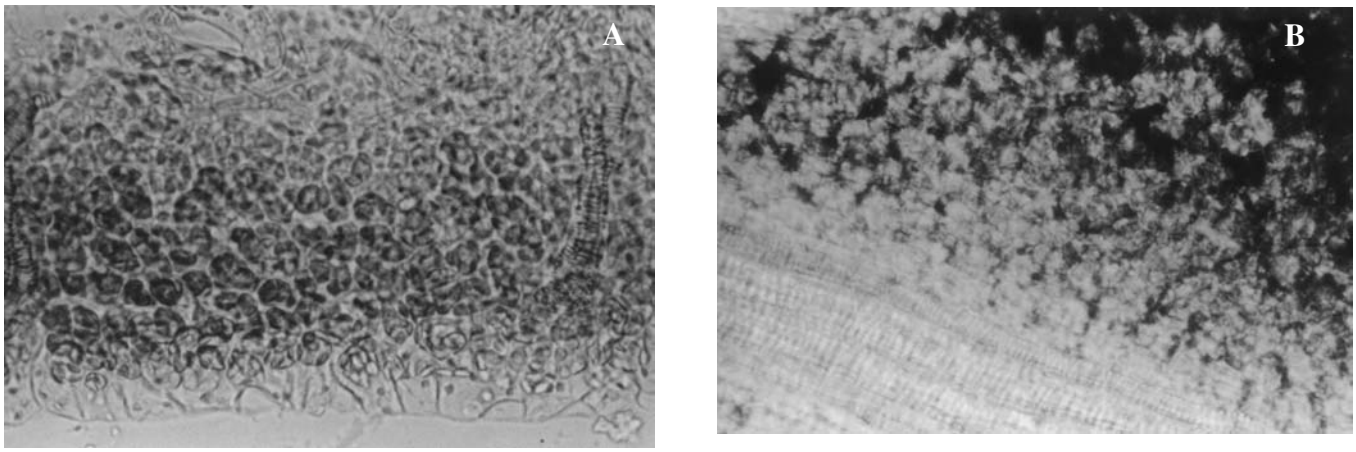

Fig. 1 - Microscopic structure of an *in vitro* leaf section of the variety Sakasly. A- leaf from a young plant (4 weeks old) of the first subculture. B- Leaf from a plant of the 6th subculture (senescent).

Compared to B, the figure shows that in leaves from young plants (A), the parenchyma cells (1) are less rich in chlorophyll, the intercellular spaces (2) more important and the conducting tissues (3) less abundant.

Section de vitrofeuille de la variété Sakasly observée au microscope. A- Feuille de plant âgé de 4 semaines. B- Feuille de plant sénescent.

La comparaison entre A et B montre que le parenchyme (1) de feuilles jeunes (A) est moins riche en chlorophylle, les espaces intercellulaires (2) sont plus importants et les tissus conducteurs (3) moins abondants.

cell division and the microcallus formation in grape protoplast cultures were dependent on the genotype.

II - EFFECT OF THE PHYSIOLOGICAL STATE OF DONOR PLANTS

The best yields of protoplasts were obtained using 4 to 5 week old plants (with 6 to 8 developed leaves) from the second subculture. However, *in vitro* leaves from plants older than 5 weeks gave very poor yields (table IV) and those from plants transferred to a new medium more than six times were unable to produce protoplasts (table V). In these plants, the cell wall seems to become rigid and the mesophyll looses its competence to produce protoplasts. Indeed, the microscopic observation of a leaf section showed that in young leaves (figure 1A) the parenchyma cells are less rich in chloroplasts, the intercellular spaces are more important and the conducting tissues are less abundant than in older leaves (figure 1B). This situation would translate an accumulation of pectin in the middle lamella and an abundance of secondary structures in aged cells. This confirms the report of LEE and WETZSTEIN (1988) on the effect of the leaf age on the yield of protoplasts. On the other hand, WINGER (1998) reported that after several subcultures, the endogenous amount of cytokinins becomes a limiting factor. Thus, when plants were transferred on medium containing

Table V - Effect of the number of transfers on the yield of protoplast from 4 weeks old *in vitro* plants of the variety Sakasly.

Effet du nombre de transferts sur le rendement en protoplastes à partir de la variété Sakasly âgée de 4 semaines.

Number of transfers	2	3	4	5	6
Yield (10^6 prot/gFW)*	4.50	5.70	1.30	0.53	0.00

*FW: fresh weight of leaf material

Table VI - Effect of BAP on yield of protoplast from senescent *in vitro* leaves (6 subcultures) of the variety Sakasly.

Effet du BAP sur le rendement à partir de vitrofeuilles sénescentes (6 subcultures) de la variété Sakasly.

BAP concentration (mg/l)	0.0	0.5	1	2
Yield (10^6 prot/gFW)*	0.00	2.14	0.30	1.10

*FW: fresh weight of leaf material

0.5 mg/l of BAP, we recovered normal yields (table VI). However, no positive correlation can be established between the amount of BAP and the yield. BAP was reported to be efficient in rejuvenilizing plant tissue (CHATIBI, 1999; WINGER, 1998). Furthermore, when working with woody plants like grape, it was recommended to use juvenile material (BARBIER and BESSIS, 1988).

Thus, the experimental conditions giving satisfying yields of protoplasts from *in vitro* leaves of the Tunisian grapevine varieties Sakasly and Muscat d'Alexandrie are:

- 4 weeks old plants from 2nd-3rd subculture as source;

- digestion with enzyme combination E2 for 13 h with agitation.

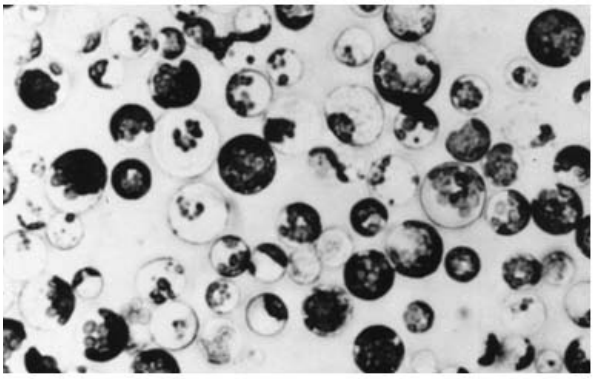

Fig. 2 - Freshly purified protoplasts from the variety Sakasly showing a spherical shape and abundance of chloroplasts.

Protoplastes de la variété Sakasly fraîchement isolés, de forme sphérique et riches en chloroplastes.

1) Protoplast culture

Following their purification, more than 50 % of the protoplasts appear rich in chloroplasts and have a diameter of 30 to 40 µm, which are good criteria of regeneration competence (NISHIMURA *et al.*, 1984) (figure 2). After 7 days of culture, 60 to 80 % of the different protoplast samples, from both varieties, achieved cell wall regeneration. This was independent of the type of the nutrition medium, the nature of growth regulators and the cell density

2) Cell division

The first cell divisions were obtained after a week of culture for both varieties in all of the different situations of culture. However, there were differences in the rates according to the nature of the medium, growth regulator combination, cell density, and genotype (table VII). After 7 days, the rates of first cell division were of 0.2 % to 1.2 % for the genotype Sakasly and 0.2 % to 0.7 % for Muscat d'Alexandrie. For both varieties, the best rates were obtained in sodium alginate immobilised samples, cultivated in CPW-13 medium supplemented with NOA and TDZ and at a density of 0.5 x 10^6 cell/ml (table VII). The second division started after the second week of culture (figure 3). Rates were of 0.2 % to 0.56 % for the variety Sakasly and 0.08 % to 0.38 % for Muscat d'Alexandrie. The best rates were again obtained in the same conditions as those of the first division. Taking into consideration these results, the conditions of culture that promote cell divisions are:

- Immobilisation of protoplasts in sodium alginate gel layers;

- CPW-13 as cultivation medium;

- NOA/TDZ as growth regulator combination;

Table VII - Effect of culture medium, growth regulators and density on the cell wall regeneration and cell division of cultivated protoplasts isolated (using E2) from *in vitro* leaves of varieties Sakasly and Muscat d'Alexandrie (4 weeks old).

Effets du milieu de culture, des régulateurs de croissance et de la densité sur la régénération de la paroi et la division de protoplastes isolés à partir de vitrofeuilles des variétés Sakasly et Muscat d'Alexandrie âgées de 4 semaines.

Variety	Density	Culture Medium	Growth regulator combination	Cell wall regeneration (%)	1st Cell division (%)
Sakasly	0.25x10^6 prot/ml	CPW13	C1	78	0,67
			C2	76.2	0,58
		MW	C1	76.8	0,42
			C2	77.5	0,38
		MS	C1	80.25	0,41
			C2	79.2	0,38
		NN69	C1	77.8	0,31
			C2	69.7	0,20
	0.5x10^6 prot/ml	CPW13	C1	80.2	1.20
			C2	73	0.90
		MW	C1	75.6	0.95
			C2	71	0.85
		MS	C1	69	0.86
			C2	68	0.85
		NN69	C1	65	0.43
			C2	64	0.21
Muscat D'Alexandrie	0.25x10^6 prot/ml	CPW13	C1	74.8	0.45
			C2	73.8	0.38
		MW	C1	70.9	0.39
			C2	71	0.30
		MS	C1	73.8	0.35
			C2	75.8	0.39
		NN69	C1	71.8	0.26
			C2	70.4	0.2
	0.5x10^6 prot/ml	CPW13	C1	75.8	0.70
			C2	75	0.53
		MW	C1	71	0.62
			C2	72	0.51
		MS	C1	76	0.56
			C2	77	0.54
		NN69	C1	73	0.31

C1: 4 mgl^{-1} NOA/ 0.88 mgl^{-1} TDZ. C2: 1 mgl^{-1} 2.4D/ 0.5 mgl^{-1} BAP.

- 0.5x10^6 cell/ml as cell culture density.

Protoplasts of the variety Sakasly revealed to be more competent in cell division than those of Muscat d'Alexandrie. For the protoplasts immobilised in sodium alginate layers, our results confirm the efficiency of this technique in protecting cells from environmental changes and supporting cell division (LEE and WETZSTEIN, 1988) as well as in facilitating the manipulation of cultures (MII *et al.*, 1991; REUSTLE *et al.*, 1994). The CPW-13 medium and the NOA and TDZ growth regulators have already been reported as favourable for cell division and microcallus formation (REUSTLE *et al.*, 1994). The interest of TDZ in particular is known in the protoplast culture (REUSTLE and ALLEWELDT, 1990, 1995; CHUPEAU *et al.*, 1993; HARST, 1995). As reported in previous works (LEE and WETZSTEIN, 1988), the density of 0.5 x 10^6 cell/ml in the cultures gave the best results, with both varieties. We can note, from the above reports, that the conditions for grape protoplasts are quite similar. Though, the limiting factor in the success of plant

regeneration from grape protoplasts seems to be the genotype, the nature and the physiological state of the donor explant rather than the culture conditions. The embryogenic material remains the most convenient explant for grape regeneration from protoplasts (REUSTLE *et al.*, 1995; ZHU *et al.*, 1997).

3) Micro and macrocallus formation

Microcalluses from both varieties have been obtained during the fourth week of cultivation. The best rates did not exceed 0.3 % and appeared only in samples cultivated in CPW-13 medium containing NOA and TDZ and where the density is of 0.5 x 10^6 cell/ml. No microcallus has been observed in samples having a density of 0.25 x 10^6 cell/ml. During the fifth week of culture, only few microcalluses from the variety Sakasly continued their development to macrocallus (figure 4). The majority of the other microcalluses was affected by oxidation and necrosis which led to their death. To try to overcome this oxidation problem, the use of a combination of PVP (1 %), citric acid (200 mg/l), and ascorbic acid (100 mg/l), as well as the renewal of the culture medium solution every 2 to 3 weeks, had delayed the burnishing of the culture medium but did not allow further proliferation of the protoplasts. SADAHARU *et al.* (1990); BARBIER and BESSIS (1988) and NISHIMURA *et al.* (1984) have reported that the use of anti-oxidants in the protoplast culture medium did not ameliorate their cell division. REUSTLE and NATTER (1994) obtained a delay and a decrease of the oxidation, but they were unable to establish a correlation between burnishing of culture medium and protoplast proliferation. Thus, other factors that may affect plant regeneration from leaf protoplasts are still unknown and more investigation is needed to overcome this problem. Several factors have been proposed to contribute to the recalcitrance of protoplasts (ROUBELAKIS-ANGELAKIS, 1993). Among the proposed factors, the oxidative stress induced by active oxygen species generated during normal metabolism or as a response to stress conditions. In this respect, SIMINIS *et al.* (1993, 1994) and FLORENCIANO *et al.* (1992) reported differences in the enzymatic activities involved in the cell defence against oxidation, such as catalase, peroxidase and indole-3-acetic acid oxidase, between recalcitrant protoplasts from grapevine mesophyll and regenerating protoplasts from *Nicotiana tabacum*. PAPADAKIS *et al.*, (2001) reported the detection of high levels of fluorescent lipid peroxides in grapevine protoplasts as a response to the loss of their defence against oxidative stress. Thus, it becomes assumed that a correlation exists between the defence efficiency of grapevine protoplasts against oxidative stress and their recalcitrance. Consequently, polyamines were proposed as protectors against this kind of stress (BOUCHEREAU *et al.*, 1999). Indeed, high endogenous levels of polyamines have been correlated with stress responses in plants (KRAMER *et al.*, 1991; SZIGETI *et al.*, 1996). Low levels of spermine and spermidine detected in grapevine protoplasts, compared to tobacco protoplasts, could refer to induced senescence (TIBURCIO *et al.*, 1993).

Up today, most of the attempts have been unsuccessful to achieve regeneration from grape leaf protoplasts (SHIMIZU, 1985; BARBIER and BESSIS, 1987, 1990; LEE and WEITZSTEIN, 1988; ALLEWELDT and REUSTLE, 1988; REUSTLE and ALLEWELDT, 1990; KATSIRDAKIS and ROUBELAKIS-ANGELAKIS, 1992a,b) and the use of embryogenic material

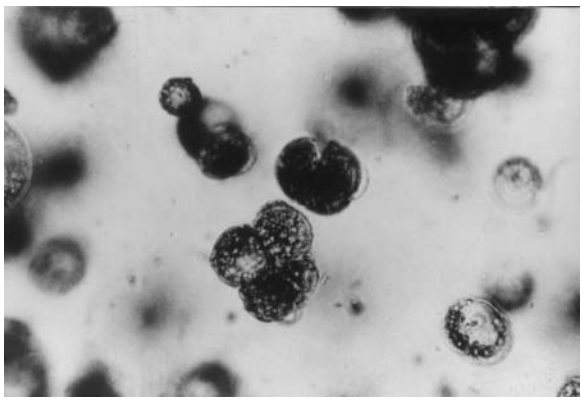

Fig. 3 - Protoplasts from *in vitro* leaves of the variety Sakasly, cultivated at 0.5 x 10^6 prot/ml on CPW-13 medium containing NOA/TDZ, showing the second cell division.

Protoplastes en seconde division de vitrofeuilles de la variété Sakasly cultivés à 0.5 x 10^6 prot/ml sur milieu CPW-13 contenant NOA/TDZ.

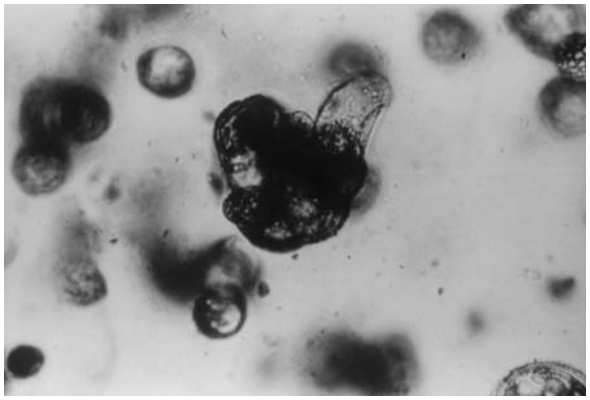

Fig. 4 - Macrocallus obtained from protoplasts of the variety Sakasly immobilised in sodium alginate layer and cultivated in CPW-13 medium containing NOA/TDZ.

Macrocal obtenu à partir de protoplasts de la variété Sakasly immobilisés dans de l'alginate de sodium et cultivés dans le milieu CPW-13 contenant NOA/TDZ.

is recommended when dealing with grape regeneration and genetic transformation.

CONCLUSION

Isolation of leaf protoplasts from *in vitro* cultivated plants of two Tunisian grapevine varieties was possible thanks to the use of a confirmed maceration mixture containing three cellulases and a macerozyme. Four to five week old plants gave the best rates of viable protoplasts. The cultivation of purified protoplasts immobilised in sodium alginate gel layers, in CPW13 medium supplemented with NOA and TDZ allowed cell division and calluses formation. However, these results, obtained for the first time for Tunisian grapes, need more investigation to promote plant regeneration from leaf protoplasts.

REFERENCES

ALLEWELDT G. and REUSTLE G., 1988. Isolation and culture of grapevine protoplasts. In: Puite K.J., Dons J.J.M., Huizing H.J., Kool A.J., Koornneef M., Krens F.A., 1987. Progress in plant protoplast research. *Proceed. of the 7th Intern. Symp., Wageningen*, The Netherlands, December 6-11, Kluwer Academic Publishers, Dordecht, 67-68.

BARBIER M. and BESSIS R., 1987. Isolation and culture of grape leaf protoplasts (*Vitis vinifera* L. var. Chardonnay). *Bull. O.I.V.*, **679-680**, 765-775.

BARBIER M. and BESSIS R., 1988. Effets de différents facteurs contribuant à l'amélioration de l'isolement de protoplastes à partir de feuilles de vigne (*Vitis vinifera* L.). *Bull. Soc. Bot. Fr.*, **135**, Lettres bot., 3, 251-261.

BARBIER M. and BESSIS R., 1990. Isolation and culture of grapevine cv. Chardonnay leaf protoplasts. *Euphytica*, **47**, 39-44.

BEN ABDALLAH F., FNAYOU A. et GHORBEL A., 1997. La sauvegarde des variétés autochtones de vigne tunisiennes par l'utilisation des vitrométhodes. *Progrès Agric. Vitic.*, **114**, 343-347.

BOUCHEREAU A., AZIZ A., LARHER F. and MARTIN-TANGUY J., 1999. Polyamines and environmental changes: recent development. *Plant Sci.*, **140**, 103-125.

BREZEANU A., IORDAN M. and ROSU, A., 1982. Protoplast isolation by enzymatic procedures from *Nicotiana tabacum* L, *Daucus carota* subsp. *Sativus* (hoffm.). Arcangeli, *Vitis vinifera* L. and *Citrillus lanatus* (Thumb). *Mansf. Rev. Roum. Biol.-Biol. Veget.*, **27**, 133-139.

BREZEANU A. and ROSU A., 1984. Isolation and culture of cell protoplasts from the mesophylll callus of *Vitis vinifera* L. *Mansf. Rev. Roum. Biol.-Biol. Veget.*, **29**, 33-37.

CHATIBI A., 1999. Les différentes potentialities de régénération *in vitro* du pistachier (*Pistacia vera* L.) cv. Mateur. *Thèse doctorat sciences biologiques*. Faculté des Sciences de Tunis.

CHUPEAU M.C., LEMOINE M. and CHUPEAU Y., 1993. Requirement of thidiazuron for healthy protoplast development to efficient tree regeneration of hybrid poplar (*Populus tremula* x *P. alba*). *J. Plant Physiol.*, **141**, 601-609.

ERIKSON T.R., 1985. Protoplast isolation and culture. In: Fowke, L.C., Constabel, F., eds. *Plant protoplasts*. CRC Press, Inc. Boca Raton, Florida, p7-15.

FREARSON E., POWER J.B. and COCKING E.C., 1973.The isolation, culture, and regeneration of Petunia leaf protoplasts. *Dev. Biol.*, **3**,130-137.

FLORENCIANO G.E., CALDERON A.A., MUNOZ R. and ROS BARCELO A., 1992. The decarboxylative pathway of indole-3-acetic acid catabolism is not functional in grapevine protoplasts. *J. Exp. Bot.*, **43**, 715-721.

HARST M., 1995. Development of a regeneration protocol for high frequency somatic embryogenesis from explants of grapevines (*Vitis* spp.). *Vitis*, **34**, 27-29.

HASLAM E., 1974. Polyphenols protein interaction. *Biochem J.*, **29**, 285-288.

KARESH H., BILANG R., SCHEID O.M. and POTRUKYS I., 1991. Direct gene transfer to protoplasts of *Arabidopsis thaliana*. *Plant Cell Reports*, **9**, 571-574.

KATSIRDAKIS K.C. and ROUBELAKIS-ANGELAKIS K.A., 1992a. A modified culture medium and culture conditions increase viability and cell wall synthesis in grapevine (*Vitis vinifera* L. cv Sultanina) leaf protoplasts. *Plant Cell Tiss. Org. Cult.*, **28**, 255-260.

KATSIRDAKIS K.C. and ROUBELAKIS-ANGELAKIS K.A., 1992b. Ultrastructral and biochemical aspects of cell wall regeneration in recalcitrant and regenerating leaf protoplasts. *In vitro Cell Dev. Biol.*, **28**, 90-96.

KRAMER G.F., NORMAN H.A., KRIZEK D.T. and MIRECKI R.M., 1991. Influence of UV-B radiation on polyamines, lipid peroxidation and membrane lipids in cucumber. *Phytochemistry*, **30**, 2101-2108.

LEBRUN L., 1985. Isolation and culture of grapevine protoplasts. Moet-Hennesy *Conference on grapevine improvement and in vitro culture*. Paris, pp. 215.

LEE N. and WETZSTEIN Y., 1988. Protoplast isolation and callus production from leaves of cultured *Vitis* ssp. *Plant Cell Reports*, **7**, 531-534.

LLOYD G. and MCCOWN B., 1981. Commercially-feasible micropropagation of Mountain laurel, Kalmia latifolia by use of shoot tip culture. *Intern. Plant Prop. Soc. Proc.*, **30**, 421-427.

MARTINELLI L. and MANDOLINO G., 1994. Genetic transformation and regeneration of transgenic plants in grapevine (*Vitis rupestris* S.). *Ther. Appl. Genet.*, **88**, 621-628.

MII M., ZHOU Y.M., SUGIYAMA T., YANAGIHARA S. and IIZUKA M., 1991. High-frequency callus formation from protoplasts of *Vitis labruscana* Bailey and *Vitis*

thumbergii Sieb. Et Zucc. by embedding in gellan gum. *Sci. Hort*., **46**, 253-260.

MOREL G. et MARTIN C., 1955. Guérison de pommes de terre atteintes de maladies à virus. *C. R. Acad. Agric*., **41**, 472-475.

MURASHIGE T. and SKOOG F, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, **15**, 473-497.

NISHIMURA M., HARA-NISHIMURA I. and ROBINSON S.P., 1984. Isolation of metabolically competent protoplasts from grapevine leaves. *Plant Science Letters*, **37**, 171-175.

NITSCH J.P. and NITSCH C., 1969. Haploid plants from pollen grains. *Science*, **163**, 85-87.

PAPADAKIS A., REUSTLE G. and ROUBELAKIS-ANGELAKIS K.A., 2001. Protoplast technology in grapevine. *In: Molecular biology and biotechnology of the grapevine*. Ed. ROUBELAKIS-ANGELAKIS K.A., Kluwer Academic Publishers, 353-392.

REUSTLE G. and ALLEWELDT G., 1990. Isolation and culture of grapevine protoplasts. *Rev. Roumaine Biol., Biol. Vég*., **29**, 33-37.

REUSTLE G. and NATTER I., 1994. Effect of polyvinylpyrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp.). *Vitis*, **33**, 117-121.

REUSTLE G., HARST M. and ALLEWELDT G., 1994. Regeneration of grapevine (*Vitis* sp.) protoplasts. *Vitis*, **13**, 173-174.

REUSTLE G., HARST M. and ALLEWELDT G., 1995. Plant regeneration of grapevine (*Vitis* sp.) protoplast isolated from embryogenic tissue. *Plant Cell Reports*, **15**, 238-241.

SADAHARU U.I., SUZUKI M., KUBOTA S., MASUDA HMURAKI H. and YAMAKAWA Y., 1990. Cooperative effect of activated charcoal and gellan gum on grape protoplast culture. *Agric. Biol. Chem.*, **54**, 207-209.

SHIMIZU J.I., 1985. Cell regeneration and division of grape mesophylll protoplasts. *J. Plant Physiol*., **119**, 419-424.

SIMINIS C.I., KANELLIS A.K. and ROUBELAKIS-ANGELAKIS K.A., 1993. Differences in protein synthesis and peroxidase isoenzymes between recalcitrant and regenerating protoplasts. *Physiol. Plant.*, **87**, 263-270.

SIMINIS C.I., KANELLIS A.K. and ROUBELAKIS-ANGELAKIS K.A., 1994. Catalase is differentially expressed in dividing and non dividing protoplasts. *Plant Physiol.*, **105**, 1375-1383.

SZIGETI Z., RACZ I., LASZTITY E.D. and LEHOCZKI E., 1996. Are either SOD and catalase or the polyamines involved in the paraquat resistance of *Conyza Canadensis*. *Env. Sci. Health*, **31**, 599-604.

THEODOROPOULOS P.A. and ROUBELAKIS-ANGELAKIS K.A., 1990. Progress in leaf protoplast isolation and culture from virus-free axenic shoot cultures of *Vitis vinefera* L. *Plant Cell Tissue and Organ Culture*, **20**, 15-23.

TIBURCIO A.F., CAMPOS J.L., FIGUERAS X. and BESFORD R.T., 1993. Recent advances in the understanding of polyamine functions during plant development. *Plant Growth Reg*., **12**, 331-340.

TORREGROSA L., LE GALL O., CANDRESSE T. and BOUQUET A., 1994. *In: Vth Intern. Symp. on Grape Breeding*, Yalta, Crimea, Ukraine, 4-10 sept., 91-98.

WATTS J.M., 1974. Problem associated with the production of stable protoplast of tobacco mesophylll. *Ann. Bot.*, **47**, 189-194.

WINGER A., SCHAEWEN A.V., LEEGOOD R.C., LEA P.I. and QUICK W.B., 1998. Regulation of leaf senescence by cytokinin, sugars and light. *Plant Physiol*., **116**, 329-335.

ZHU Y.M., HOSHINO Y., NAKANO M., TAKAYASHI E. and MII M., 1997. High efficient system of plant regeneration from protoplasts of grapevine (*Vitis vinifera* L.) through somatic embryogenesis by using embryogenic callus cultures and activated charcoal. *Plant Sci*., **123**, 151-157.

Manuscrit reçu le 3 septembre 2002 ; accepté pour publication le 12 septembre2003