ELIMINATION OF BROWNING EXUDATE AND IN VITRO DEVELOPMENT OF SHOOTS IN *PISTACIA VERA* L. CV. *MATEUR* AND *PISTACIA ATLANTICA* DESF. CULTURE

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(Received: February 12, 1998. Accepted: February 25, 1999)

ABSTRACT

We report diminution and/or elimination of browning exudate followed by in vitro establishment of in *Pistacia vera* cv. *mateur* and *Pistacia atlantica* explants. Soaking *P. vera* cv. *mateur* explants prior to culture in L-cysteine HCl for 15 min (100 μM) inhibits blackening of the modified Murashige and Skoog medium – MS + 400 mg/l NH_4NO_3 – and of the explants; while shoot formation was increased. The browning in *P. vera* cv. *mateur* and *P. atlantica* explants dissolved when modified MS and Quoirin and Lepoivre – QL.4 – media were supplemented with activated charcoal (from 1 to 3 g l^{-1}) and with 4 and 8 days of darkness. These treatments were enough to eliminate browning from the explants and to improve the shoots elongation, but symptoms of chlorosis were detected. On the other hand, $AgNO_3$ (from 15 to 40 μM) showed a very strong anti-browning effect on the medium and explants of *P. atlantica*. Thus shoot organogenesis was best achieved and the developing sturdy shoots had large and green leaves.

KEY WORDS: *Pistacia vera* cv. *mateur*, *P. atlantica*, anti-browning effect, L-cysteine HCl, activated charcoal, $AgNO_3$, shoot formation.

INTRODUCTION

Tissue culture techniques are used for clonal propagation of *Pistacia* species and some progress has been reported with apical and/or axillary shoots explants taken from juvenile material plant (Barghchi and Alderson 1985; Martinelli 1988; Gonzalez and Frutos 1990; Mederos et al. 1994a, b). However, the in vitro establishment of *Pistacia* species is still problematic, one of the reasons being the rapid browning and/or necrosis of the explants. These problems are at least partly caused by oxidation of polyphenols which are abundant in *Pistacia* species (Barghchi and Alderson 1985; Mederos 1991; George 1996). Also, inhibition of shoot organogenesis and necrosis of the explants are associated with considerable leaking of exudates into the culture medium (Mederos 1991). There are no preliminary reports on reduction of browning in this plant material. The purpose of this study was to eradicate browning compounds and thus, to promote the shoot formation during the establishment of initial cultures from *P. vera* L. cv. *mateur* and *P. atlantica* Desf.

MATERIALS AND METHODS

Plant material, culture medium and culture conditions

One to two month old shoots of *Pistacia vera* cv. *mateur* and *Pistacia atlantica* Desf. were collected between April and July and then, defoliated and disinfected with 1 g l^{-1} benomyl followed by application of 0.5 g l^{-1} $HgCl_2$ plus 0.2 ml Tween 80 (20 min respectively). Then they were rinsed six times in sterile distilled water. After surface sterilization, the explants were cultivated on medium recommended earlier for the successful establishment of shoots (Table 1). The *P. vera* cv. *mateur* shoots were separated into apical buds and nodal segments, 15 mm long and transferred to a modified Murashige and Skoog medium [MS + 400 mg/l NH_4NO_3] (Table 1) (Mederos et al. 1994a, b). The *P. atlantica* shoots were separated into apical buds and nodal segments, 15 mm in size, and transferred to a modified Quoirin and Lepoivre medium (1977) [QL.4] (Quoirin and Lepoivre 1977; Mederos et al. 1997a, b) (Table 1). To investigate the effect of antioxidants on browning exudate, in the first experiment explants were soaked prior to culture in distilled water or in seven antioxidant solutions as, glutathione, ascorbic acid, citric acid, sol-

TABLE 1. Composition of the modified Murashige and Skoog (1962) [1] and Quoirin and Lepoivre (1977) [2] media for achieving organogensis of *Pistacia vera* L. cv. *mateur* [1] and *Pistacia atlantica* Desf. [2] seedlings.

Chemical [1]	Concentration in culture medium	
	modified MS [1]	QL.4 [2]
NH_4NO_3	400.0 mg l^{-1}	800.0 mg l^{-1}
KNO_3	1900.0 mg l^{-1}	1800.0 mg l^{-1}
$Ca(NO)_3.4H_2O$	0.0	200.0 mg l^{-1}
$CaCl_2.2H_2O$	440.0 mg l^{-1}	0.0
$NaNO_3$	0.0	425.0 mg l^{-1}
KH_2PO_4	170.0 mg l^{-1}	2700.0 mg l^{-1}
$MgSO_4.7H_2O$	370.0 mg l^{-1}	3600.0 mg l^{-1}
Myo-inositol	100.0 mg l^{-1}	100.0 mg l^{-1}
Thiamine-HCl	0.4 mg l^{-1}	0.4 mg l^{-1}
Pyridoxine-HCl	0.4 mg l^{-1}	0.4 mg l^{-1}
Sucrose	30.0 g l^{-1}	30.0 g l^{-1}
Difco-Bacto Agar	6.0 g l^{-1}	6.0 g l^{-1}
BAP	1.0 µM	1.0 µM
NAA	0.5 µM	0.5 µM
pH	5.6	5.6

[1] Macronutrients plus micronutrients of MS (1962)

uble polyvinil pyrrolidone (PVP), l-cys HCl, sodium diethyldithiocarbamate [Na-DEDC] or rosmanol at the same concentration (100 µM). The immersion times for every one of these solution were 6, 12, 24, 36, 48 or 72 h for *P. atlantica* and 10, 15, 20 or 25 min for *P. vera* cv. *mateur*. These solutions were shaken periodically. In the second experiment, the compounds used in the previous experiment were added to the culture medium at three concentrations (0, 50 or 100 µM). In the third experiment the culture media were supplemented with activated charcoal at 0, 1, 2 and 3 g l^{-1} and included darkness periods of 4, 8, 10 and 12 days. In the last experiment the effect of $AgNO_3$ (0, 7, 15, 30 and 40 µM) added to the media was tested. The mean values with standard error are given for two repetitions with 12 explants in each treatment. The results were recorded for different periods after the initial culture. Explants were cultured at 24 ± (1°C with 16 h-photoperiod (irradiance of 20 µM m^{-2} s^{-1} provided by cool-white fluorescent lamps) in tubes (diameter 22 mm) on 15 ml. The statistic validity of the results was obtained through the analysis of variance by Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

A fairly rapid blackening of both explants and culture media is very common in *Pistacia* species and our preliminary assays confirmed that the first signs of blackening were

TABLE 2. Effect of immersion time in L-cysteine HCl (100 µM) on explants of *Pistacia vera* cv. *mateur* after 25 of culture. Mean ± S.E. is given.

Immersion time (min)	% of developing shoots	Shoot elongation (mm)	% Explants with brown. & necr.[1]
Control	0.00	0.00^a ± 0.00	100.00
10	83.33	21.60^c ± 0.25	16.67
15	83.33	32.05^e ± 0.28	0.00
20	100.00	25.75^d ± 0.26	0.00
25	83.33	14.35^b ± 0.37	0.00
F	–	637****	–

[1] brown. & necr. = browning and necrosis.
Each value represents the mean of developed shoots. **** = Significance level 0.00001. Means in columns followed by the same letter are not significantly different at 0.05 confidence level (Duncans test).

TABLE 3. Behaviour of the explants of *Pistacia vera* cv. *mateur* [1] and *Pistacia atlantica* Desf. [2] after 25 days of culture on modified MS medium [1] or on QL.4 medium [2] supplemented with chemical compounds at two concentrations. Means ± S.E., is given.

Chemical compounds		% of developing shoots		Shoot length (mm)		% Explants with brown. & necro.[1]	
	(µM)	[1]	[2]	[1]	[2]	[1]*	[2]**
Control	0	0	0	0	0	100	100
Glutathione	50	0	0	0	0	100	100
	100	0	0	0	0	100	100
Ascorbic acid	50	0	0	0	0	100	100
	100	0	0	0	0	100	100
Citric acid	50	0	0	0	0	100	100
	100	0	0	0	0	100	100
PVPs	50	0	0	0	0	100	100
	100	0	0	0	0	75	100
L-cys HCl	50	41.67	0	15.20 ± 1.09	0	16.77	100
	100	25.00	0	10.33 ± 0.57	0	33.33	100
Na-DEDC	50	0	0	0	0	100	100
	100	0	0	0	0	100	100
Rosmanol	50	0	0	0	0	100	100
	100	0	0	0	0	100	100

[1] browning and necrosis. Browning intense (*) and very intense (**). Each value represents the mean of developed shoots.

TABLE 4. Behaviour of the explants of *Pistacia vera* cv. *mateur* [1] and *Pistacia atlantica* Desf. [2] after 25 of culture on modified MS medium [1] or on QL.4 medium [2] supplemented with activated charcoal. Cultures were incubated for 4 and 8 day dark periods. Mean ± S.E. is given.

Activated charcoal ($g\ dm^{-3}$)	% of developing shoots		Shoot length (mm)		Frequency of chlorotic shoots[2]	
	[1]	[2]	[1]	[2]	[1]	[2]
0.0[(1)]	0	0	0	0	0	0
4 days						
1.0	41.67	58.33	1.20 ± 0.37^{f}	12.00 ± 0.44^{b}	+	+
2.0	33.33	66.67	8.25 ± 0.48^{d}	15.50 ± 0.33^{ed}	++	+
3.0	25.00	50.00	6.00 ± 0.41^{cb}	12.00 ± 0.51^{cb}	++	+
8 days						
1.0	41.67	83.33	9.60 ± 0.25^{e}	15.10 ± 0.27^{d}	++	+
2.0	41.67	50.00	5.20 ± 0.20^{b}	12.67 ± 0.29^{dbc}	++	+
3.0	33.33	33.33	3.50 ± 0.29^{a}	9.50 ± 0.30^{a}	++	+
F	–	–	77**	32****	–	–

[(1)]browning and necrosis of 100% of the explants. (2) Clorothic shoots: + = intense, ++ = very intense.
Each value represents the mean of developed shoots. N.S. = No two groups are significantly different and **** = significance level 0.00001. Means in columns followed by the same letter are not significantly different at 0.05 confidence level (Duncans test).

visible after one hour of initial culture. In an early attempt, apical or axillary shoot explants washed with compounds used in the first experiment did not prevent the browning exudate and explants showed no growth. To prevent the leaking of exudates from the cut surface of explants from *P. vera* cv. *mateur* to the medium, presoaking the material with 100 μM L-cysteine HCL for 15 min was found best; shoot formation and elongation were quick (Table 2). However, the incorporation of these antioxidant or reducing agents to culture media (2nd experiment), did not reduce the necrosis and browning effects and shoot formation could not be stimulated or increased (Table 3). Pre-soaking the explants of *P. vera* cv. *mateur* with L-cys HCl solved the problem. However, browning was the most frequently encountered problem with *P. atlantica*. Generally the dark exudate is very difficult to eradicate by pretreatment washes (Barghchi and Alderson 1985; Mederos 1991) and modified culture media (Mederos 1991). Ziv and Halevy (1983), Hildebrandt and Harney (1989) found that antioxidant compounds inhibit shoot formation in *Strelitzia reginae* and *Geranium* species. They also cause browning and necrosis of *Kangarof paws* explants (McComb and Newton 1981). Thus it is necessary to find other alternatives to solve this problem with *P. atlantica* explants. Table 4 shows that 1 $g\ l^{-1}$ of activated charcoal together with dark periods of 4 and 8 days improve the elongation of shoots of *P. atlantica* and *P. vera* cv *mateur*.

In all treatments, symptoms of chlorosis were found at different times after zero time. Scant shoot formation (12%) with these symptoms was also produced at 10 and 12 days of dark incubation. Activated charcoal added to the medium with or whithout dark periods at the beginning of the experiment eliminated browning and was beneficial to shoot formation in several species (Gamborg and Phillips 1995; George 1996). Lee and Skoog (1965), Constantin et al. (1977) and Fridborg et al. (1978) showed that activated charcoal retains phenolic compounds while also stimulating in vitro organogenesis. But these chemicals are not appropriate for *P. vera* cv. *mateur* and *P. atlantica* because the shoots produced in their presence are not vigorous and show symptoms of chlorosis. Even though our preliminary results indicate that, after the sixth transfer to fresh culture medium, the explants from *P. atlantica* ceased to secrete substances into the medium (Mederos et al. 1997a, b) we propose, in order to achieve faster results, an efficient system for controlling exudate release and promoting shoot formation in this species. When effect of $AgNO_3$ in relation to the browning and explants behaviour of *Pistacia atlantica* was evaluated (Table 5), it appeared that 15, 30 and 40 μM $AgNO_3$ showed a very high anti-browning effect on QL.4 medium and explants of *P. atlantica*. Afterwards, organogenesis was best achieved, and the developing shoots were sturdy with large, green leaves. The use of $AgNO_3$ in preliminary assays with *P. vera* cv. *mateur*, showed similar results for eradication of browning exudate but inhibited shoot formation (more than 75%). For this reason, explants from this cultivar were pre-soaked in L-cys HCl (Table 2). $AgNO_3$ has a very slight positive effect on orga-

TABLE 5. Effect of $AgNO_3$ on behaviour of explants of *Pistacia atlantica* Desf. After 15 [1] and 30 [2] of culture on QL.4 medium. Mean ± S.E. is given.

$AgNO_3$ (μM)	% of developing shoots		Shoot elongation (mm)		% Cultures with browning	
	[1]	[2]	[1]	[2]	[1]	[2]
0[(1)]	0	0	0^{a}	0^{a}	0	0
7	100.00	100.00	14.09 ± 0.34^{c}	26.64 ± 0.36^{e}	33	17
15	91.67	91.67	13.40 ± 0.27^{c}	20.50 ± 0.34^{d}	17	0
30	58.33	58.33	11.14 ± 0.34^{b}	16.57 ± 0.43^{c}	0	0
40	33.33	33.33	11.00 ± 0.71^{b}	14.50 ± 0.64^{b}	0	0
F	–	–	17****	163****	–	–

[(1)]browning and necrosis of 100% of the explants.
Each value represents the mean of developed shoots. N.S. = No two groups are significantly different and **, ***, and **** = significance level 0.05, 0.0005 and 0.00001 respectively. Means in columns followed by the same letter are not significantly different at 0.05 confidence level (Duncan's test).

nogenesis of *Hevea brasiliensis* but shows a very slight anti-browning effect on callus from this species (Housti et al. 1992). Our results show that explant behaviour and response to organogenesis is probably genotype dependent. Similar results are reported in other woody species (Coleman and Ernst 1989; Swartz et al. 1990; Mezzetti et al. 1997). These results may be attributed to differences in physiological conditions of the plant material cultured in vitro.

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USUWANIE BRUNATNEGO WYSIĘKU ORAZ TWORZENIE SIĘ PĘDÓW IN VITRO U *PISTACIA VERA* L. CV. *MATEUR* ORAZ *P. ATLANTICA*

STRESZCZENIE

Zaobserwowano spadek lub zanik brunatnego wysięku a następnie rozwój eksplantatów u *Pistacia vera* cv. *mateur* i *P. atlantica*. Moczenie przed kulturą eksplantatów *P. vera* cv. *mateur* w L-cysteinie HCl przez 15 minut zapobiega ciemnieniu zmodyfikowanej pożywki Murashige i Skoog'a – MS + 400 mg/l NH_4NO_3 – i samych eksplantatów w trakcie wzmożonego tworzenia się pędów. Brunatny wysięk u eksplantatów *Pistacia vera* cv. *mateur* i *P. atlantica* zanikał po dodaniu MS i Quoirin oraz Lepoivre – QL.4 – po uzupenieniu pożywek węglem aktywnym (od 1 do 3 gl^{-1}) oraz po 4-8 dniowym zaciemnieniu.

Tego rodzaju postępowanie wystarczało aby usunąć wysięk z eksplantatów i wzmóc wydłużanie się pędów; stwierdzono jednak objawy chlorozy. Natomiast $AgNO_3$ (od 15 do 40 μM) wykazał bardzo silne działanie przeciwwysiękowe na pożywkę i eksplantaty u *P. atlantica*. Uzyskano w ten sposób intensywną organogenezę a rozwijające się po inicjacji mocne pędy miały duże i zielone liście.

SŁOWA KLUCZOWE: *Pistacia vera* cv. *mateur*, *P. atlantica*, usuwanie brunatnego wysięku, L-cysteina HCl, węgiel aktywny, $AgNO_3$, tworzenie się pędów.