ROS MANOL CONTROLS EXPLANTS BROWNING OF HYPERICUM CANARIENSIS L. DURING THE IN VITRO ESTABLISHMENT OF SHOOTS

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ABSTRACT

An efficient method for eradication of browning exudate was developed for Hypericum canariensis. For this purpose the effect of natural products on browning exudates were investigated in four types of culture media: Murashige and Skoog (MS, 1962); Gamborg’s (B5, 1979); Woody Plant Medium (WPM, Lloyd and McCown 1981) and modified Quoirin and Lepoivre (QL4) (Mederos 1991, Mederos et al. 1995) basal macroelements; these basal macroelements were supplemented with the microelement formula described by Murashige and Skoog (MS, 1962). During the establishment of shoots organogenesis potential was achieved in the Murashige and Skoog (MS, 1962) and modified Quoirin and Lepoivre (QL4) (Mederos 1991, Mederos et al. 1995) media after browning exudates was eliminated by rosmanol treatments. Rosmanol is a powerful diterpene antioxidant isolated from Salvia canariensis L., a medicinal species endemic to the Canaries Islands.

KEY WORDS: Hypericum canariensis, browning exudate, rosmanol antioxidant, in vitro organogenesis.

INTRODUCTION

Hypericum canariensis L. (Guttiferae family) is a medicinal plant endemic to the Canary Islands which has been known in traditional medicine with multiple properties, just as antiinflammatory (Pérez de Paz and Medina, 1988); also, antiviral activity of hypercin was investigated by Hudson, López-Bazzocchi et al. (López-Bazzocchi personal communication).

In order to eradicated browning exudate from this species, the effects of different antioxidants supplemented to a solid and liquid media were investigated for to eradicate this symptom that affect in vitro culture of shoots.

MATERIALS AND METHODS

Plant material

Axillary bud explants (12-15 mm) taken from mature 23 year-old plants of Hypericum canariensis L. gathered at Valle Guerra, Tenerife (voucher specimen on file in the Herbarium of the University of La Laguna, TFC 244) were used for this study. Segments of stem were surface-sterilized with 90% ethanol for 2 min and soaked for 8 min in a solution of 1 g l⁻¹ mercuric chloride to which 0.2 ml of tween 80 had been added followed by three times in sterile distilled water, and placed into 150 x 25 test tubes containing 20 ml culture medium.

Culture medium and culture conditions in vitro

In the experiments, the media used were: Murashige and Skoog (MS, 1962); Gamborg’s (B5, 1979); Woody Plant Medium (WPM, Lloyd and McCown 1981) and QL4 (Mederos 1991, Mederos et al. 1995) basal macroelements. All media were supplemented with Fe-EDTA and the microelement formula described by Murashige and Skoog (1962) plus 1 mg l⁻¹ thiamine-HCl, 0.8 mg l⁻¹ pyridoxine-HCl, 100 mg l⁻¹ myoinositol, 20 g l⁻¹ sucrose and 6 g l⁻¹ Difco-Bacto agar. These culture media were supplemented with 10⁻⁷ M BAP combined with 10⁻⁸ M NAA. To investigate the effect of antioxidants on browning exudate, in the first experiment pre soak of explants prior to culture for 6, 12 and 24 hours in ascorbic acid, citric acid, L-cysteine HCl, carnosic acid and/or rosmanol diterpenes at concentrations between 4 x 10⁻⁶ M and 10⁻⁴ M were used. Carnosic acid and rosmanol are powerful antioxidants isolated from Salvia canariensis L. (Luis et al. 1992, Lucia San Andrés et al. 1994). It is a endemic medicinal species to the Canary Islands. In a second experiment each culture media were supplemented with the same antioxidants compounds at the same concentrations used before. The pH was adjusted to 5.6 in all the culture media which were sterilized under 0.5 atmospheres at 115°C for 20 min. The culture were incubated in a growth chamber at 24°C day and night with a 16 hr photoperiod provided by Phillips lights give type of 20 μmol m⁻² s⁻¹ and 75% relative humidity. Forty eight explants
were cultured for each treatment. In each experiment, all explants were transferred twice to the fresh culture medium on the three day intervals. The experiments were repeated two times. The results were analysed using Duncan’s multiple range test. The data presented in tables are average from the independent two experiments.

RESULTS AND DISCUSSION

One of the problems encountered at the moment of establishment of culture of Hypericum canariensis was the presence of a brown exudate arising from the cut tissue; this brown exudate seemed to lead death of the explants. On day two, explants necrosed in culture media used without antioxidants compound (control). Explants browning was relatively more advanced on liquid culture media with respect to solid media and the explants necrosed between two and four days of culture. After day eight (two day after the second subculture), the degree of browning exudate of explants and necrosis was identical in the four solid culture media treated with antioxidants ascorbic acid, citric acid and carnosic acid. Addition of rosmanol to modified Quoirin and Lepoivre – QL. 4-(Mederos 1991, Mederos et al. 1995) and Murashige and Skoog – MS – (1962) media eradicated the browning exudate (Tables 1 and 2); however, explants cultured on B5 and WPM medium with rosmanol showed on day fifteen basal necrosis until the end of the culture. On the other hand, addition of rosmanol to QL.4 medium promoted good shoot development in all treatments; however, addition 6x10^{-6}, 10^{-5}, 2x10^{-5} M of rosmanol to this culture medium produced bigger number of axillary shoot proliferation and were significantly different at P = 0.05 level; better result was obtained in 10^{-5} M rosmanol (Table 1). All these shoots were vigorous, deeply green and had a normally developed leaves. It is possible that antioxidants have a synergistic effect with plant growth regulators (Lis-Baldim 1989). Also, the mean number of multiple shoots by bud obtained in QL. 4 plus 6x10^{-6} M rosmanol was higher and also was significantly different (P = 0.05) with respect the other treatments (Table 1); the percent of explants showing induced multiple shoots were lower in MS medium supplemented with rosmanol (Table 2), whereas shoots development on the other culture media treated with antioxidants treatments was almost nil (data not shown). It is to be emphasized here that, from culture media used in these experiments only QL. 4 and MS induced axillary buds proliferation during the establishment of culture. For axillary shoots induction in MS medium it is necessary to cut the explants whose developed shoots, between thirteen to twenty five days of culture (Table 2). Similar results was reported for Pistacia lentiscus species (Mederos et al. unpublished). Relatively low level of the browning exudates were observed on MS, B5, WPM and QL. 4 media supplemented with rosmanol combined with carnosic acid but after day fifteen, explants necrosed without shoots development. Probably the carnosic acid caused the inhibition of shoots formation of this species as Pistacia species (Mederos 1991). It has also been reported that browning compounds limit the organogenesis in other woody plant species (Bonga and Durzan 1987; El Hadrami et al. 1993; Mamun. Hoodain et al. 1994). There was also a vi-

**TABLE 1. Effect of rosmanol on behaviour of bud explants on modified Quoirin and Lepoivre – QL.4 – macroelement solution after 33 days of culture.**

<table>
<thead>
<tr>
<th>Rosmanol (Mol.)</th>
<th>% of explants developing</th>
<th>Browning exudate</th>
<th>No. of explants with multiple shoots per bud</th>
<th>No. of developing shoots per shoot explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>present</td>
<td>0.0(^a) ± 0.0</td>
<td>0.0(^a) ± 0.0</td>
</tr>
<tr>
<td>4x10^{-6}</td>
<td>91.7</td>
<td>absent</td>
<td>4.0(^b) ± 0.4</td>
<td>2.5(^c) ± 0.3</td>
</tr>
<tr>
<td>6x10^{-6}</td>
<td>100.0</td>
<td>absent</td>
<td>18.0(^d) ± 0.5</td>
<td>5.5(^e) ± 0.5</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>83.0</td>
<td>absent</td>
<td>25.0(^f) ± 0.7</td>
<td>3.6(^g) ± 0.6</td>
</tr>
<tr>
<td>2x10^{-5}</td>
<td>77.0</td>
<td>absent</td>
<td>22.0(^h) ± 0.4</td>
<td>3.0(^i) ± 0.5</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>81.0</td>
<td>absent</td>
<td>6.0(^j) ± 0.5</td>
<td>2.0(^k) ± 0.5</td>
</tr>
</tbody>
</table>

Each value represents the mean of developed shoots. Mean separation in columns by the same letter are not significant different at 0.05 level of confidence (Duncan’s Multiple Range Test).

**TABLE 2. Effect of rosmanol on behaviour of bud explants on modified Murashige and Skoog – MS – macroelement solution after 33 days of culture.**

<table>
<thead>
<tr>
<th>Rosmanol (Mol.)</th>
<th>% of explants developing</th>
<th>Browning exudate</th>
<th>No. of explants with multiple shoots per bud</th>
<th>No. of developing shoots per shoot explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>present</td>
<td>0.0(^a) ± 0.0</td>
<td>0.0(^a) ± 0.0</td>
</tr>
<tr>
<td>4x10^{-6}</td>
<td>83.0</td>
<td>absent</td>
<td>5.0(^e) ± 0.5</td>
<td>2.8(^b) ± 0.3</td>
</tr>
<tr>
<td>6x10^{-6}</td>
<td>71.0</td>
<td>absent</td>
<td>6.0(^e) ± 0.5</td>
<td>3.5(^e) ± 0.2</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>62.5</td>
<td>absent</td>
<td>4.0(^e) ± 0.4</td>
<td>4.0(^d) ± 0.2</td>
</tr>
<tr>
<td>2x10^{-5}</td>
<td>75.0</td>
<td>absent</td>
<td>2.0(^b) ± 0.2</td>
<td>3.5(^e) ± 0.4</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>81.0</td>
<td>absent</td>
<td>2.0(^b) ± 0.2</td>
<td>4.0(^d) ± 0.3</td>
</tr>
</tbody>
</table>

Each value represents the mean of developed shoots. Mean separation in columns by the same letter are not significant different at 0.05 level of confidence (Duncan’s Multiple Range Test).
sible synergistic effect from the use of a mixture of two anti-
oxidants (Lis-Baldim 1989); however, in this case combi-
nining carnosic acid and rosmanol antioxidants into the same 
culture media proved to have not significant benefit (data not 
given). In conclusion, there is now substantial evidence that 
the organogenesis of this interesting medicinal species can 
be improved during the establishment of shoots by treatment 
with rosmanol antioxidant. It is obvious that rosmanol plays a 
central role in the removal of toxins generated by browning 
exudates, even the growth, development and proliferation we 
re improved by rosmanol antioxidants. Also, no reference to 
rosmanol activity in our experiments is to be found in the 
literature.

ACKNOWLEDGEMENTS

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ROSMANOL JAKO KONTROLER BRUNATNIENIJA PRZESZCZEPÓW HYPERICUM CANARIENSIS 
PODczas TWORZENIA PEDOW IN VITRO

STRESZCZENIE

Opracowano wydajną metode usuwania barwiącego na kolor brunatny wysięku dla Hypericum canarien-
sis. W tym celu zbadano wpływ naturalnych produktów na barwiące wysięki w czterech typach pożywek: 
Muraschige i Skoog; Gamborg; pożywkę z roślin drzewistych oraz zmodyfikowaną pożywkę z podstawo-
wych makroelementów Quorin i Lepoivre; podstawowe makroelementy uzupełniono formułą mikroelemen-
tów opisaną przez Murashige i Skoog. W trakcie tworzenia pedów potencjal organogenezy osiągnięty został 
w pożywce Murashige i Skooga zmodyfikowaną za pomocą podstawowych makroelementów Quorin i Le-
opfere po wyeliminowaniu barwiących na brunatno wysięków traktując je rosmanolom. Rosmanol jest sił-
nym diterpenowym antyutleniaczem wyizolowanym z Salvia canariensis L., endemicznej rośliny z Wysp 
Kanaryjskich.

SŁOWA KLUCZOWE: Hypericum canariensis L., barwiąc na brunatno wysięk, antyutleniacz rosmanolowy, 
organogeneza in vitro.