ROSMANOL 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 (QL.4) (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 (QL. 4) (Mederos 1991, Mederos et al. 1995) media after browning exudates was eliminated by rosmanol treatments. Rosmanol is a powerful diterpenic antioxidant isolated from *Salvia canariensis* L., a medicinal species endemic to the Canary 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 anti-inflammatory (Pérez de Paz and Medina, 1988); also, antiviral activity of hypericin was investigated by Hudson, López-Bazzocchi et al. (López-Bazzocchi personal communication).

In order to eradicate 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 QL.4 (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 myo-inositol, 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 4x10⁻⁶ M and 10⁻⁴ M were used. Carnosic acid and rosmanol are powerful antioxidants isolated from *Salvia canariensis* L. (Luis et al. 1992, Lucía 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 atmosphere 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. Fourty eighth 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 browning 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 6×10^{-6} , 10^{-5} ,

2×10^{-5} Mol 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-Baldirim 1989). Also, the mean number of multiple shoots by bud obtained in QL. 4 plus 6×10^{-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 Hossain 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.

Rosmanol (Mol.)	% of explants developing	Browning exudate	No. of explants with multiple shoots per bud	No. of developing shoots per shoot explants
Control	0.0	present	$0.0^a \pm 0.0$	$0.0^a \pm 0.0$
4×10^{-6}	91.7	absent	$4.0^b \pm 0.4$	$2.5^{c,b} \pm 0.3$
6×10^{-6}	100.0	absent	$18.0^d \pm 0.5$	$5.5^f \pm 0.5$
10^{-5}	83.0	absent	$25.0^f \pm 0.7$	$3.6^{e,d} \pm 0.6$
2×10^{-5}	77.0	absent	$22.0^e \pm 0.4$	$3.0^d \pm 0.5$
10^{-4}	81.0	absent	$6.0^{c,b} \pm 0.5$	$2.0^b \pm 0.5$

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 (Duncans 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.

Rosmanol (Mol.)	% of explants developing	Browning exudate	No. of explants with multiple shoots per bud	No. of developing shoots per shoot explants
Control	0.0	present	$0.0^a \pm 0.0$	$0.0^a \pm 0.0$
4×10^{-6}	83.0	absent	$5.0^{d,e} \pm 0.5$	$2.8^b \pm 0.3$
6×10^{-6}	71.0	absent	$6.0^e \pm 0.5$	$3.5^c \pm 0.2$
10^{-5}	62.5	absent	$4.0^c \pm 0.4$	$4.0^d \pm 0.2$
2×10^{-5}	75.0	absent	$2.0^b \pm 0.2$	$3.5^c \pm 0.4$
10^{-4}	81.0	absent	$2.0^b \pm 0.2$	$4.0^d \pm 0.3$

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 (Duncans Multiple Range Test).

sible synergistic effect from the use of a mixture of two antioxidants (Lis-Baldir 1989); however, in this case combining carnosic acid and rosmannol 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 rosmannol antioxidant. It is obvious that rosmannol plays a central role in the removal of toxins generated by browning exudates, even the growth, development and proliferation were improved by rosmannol antioxidants. Also, no reference to rosmannol activity in our experiments is to be found in the literature.

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ROSMANOL JAKO KONTROLER BRUNATNIENIA PRZESZCZEPÓW *HYPERICUM CANARIENSIS* PODCZAS TWORZENIA PĘDÓW *IN VITRO*

STRESZCZENIE

Opracowano wydajną metodę usuwania barwiącego na kolor brunatny wsięku dla *Hypericum canariensis*. W tym celu zbadano wpływ naturalnych produktów na barwiące wsięki w czterech typach pożywek: Murashige i Skoog; Gamborg; pożywkę z roślin drzewiastych oraz zmodyfikowaną pożywkę z podstawowych makroelementów Quoirin i Lepoivre; podstawowe makroelementy uzupełniono formułą mikroelementów opisaną przez Murashige i Skoog. W trakcie tworzenia pędów potencjał organogenezy osiągnięty został w pożywce Murashige i Skooga zmodyfikowaną za pomocą podstawowych makroelementów Quoirin i Lepoivre po wyeliminowaniu barwiących na brunatno wsięków traktując je rosmannolem. Rosmannol jest silnym diterpenowym antyutleniaczem wyizolowanym z *Salvia canariensis* L., endemicznej rośliny z Wysp Kanaryjskich.

SŁOWA KLUCZOWE: *Hypericum canariensis* L., barwiący na brunatno wsięg, antyutleniacz rosmannolowy, organogeneza *in vitro*.