Biotecnología Vegetal Vol. 13, No. 4: 203 - 207, octubre - diciembre, 2013
ISSN 2074-8647, RNPS: 2154 (Versión electrónica)
ISSN 1609-1841, RNPS: 0397 (Versión impresa)

In vitro establishment of *Salvia hispanica* L. plants and callus

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ABSTRACT
We have optimized the culture conditions for the initiation and maintenance of *Salvia hispanica* L. (Chia) plant and callus cultures. Stems section were the best explant source for starting *in vitro* cultures. Both IAA and NAA, at all the concentration tested with or without the addition of citokinines, have induced the development of adventitious roots both in darkness as in light. In darkness, 2,4-D (2.25 μM) has induced the development of *S. hispanica* embryogenic tissues and in light promoted the initiation and establishment of green, and fast-growing calli that lost their friability with time. When subcultured to MS culture media with NAA:kin (0.54 μM:0.46 μM) or Picloram (1.72 μM, 3.43 μM) as plant growth regulators, calli maintained friability for two years in culture. It was possible to detect fatty acid content in the calli obtained but the values remained significant lower than those of seeds.

Key words: callus induction, chia, micropropagation, plant growth regulators.

Establecimiento *in vitro* de plantas y callos de *Salvia hispanica* L.

RESUMEN
El presente trabajo describe la optimización para el establecimiento y mantenimiento de plantas y callos de *Salvia hispanica* L. (Chia). El establecimiento de los cultivos *in vitro* se realizó a partir de secciones de tallos. Tanto el AIA como ANA indujeron el desarrollo de raíces adventicias utilizadas a diferentes concentraciones solas o en combinación con citoquininas en condiciones de luz u oscuridad. En oscuridad, la adición de 2,4-D (2.25 μM) indujo el desarrollo de tejido embriogénico, y en iluminación fue promovida la iniciación y el establecimiento de callos con una alta tasa de crecimiento, los cuales, al principio, fueron verdes pero a lo largo del tiempo fueron perdiendo friabilidad. Por el contrario, cuando se subcultivaron en medio de cultivo MS con las combinaciones de reguladores de crecimiento NAA: kin (0.54 μM:0.46 μM) o Picloram (1.72 μM, 3.43 μM) indujeron la formación de callos y mantuvieron su condición de friables por más de dos años. Fue posible detectar la presencia de aceites esenciales en los callos pero los valores registrados fueron significativamente menores que en las semillas.

Palabras clave: Chia, inducción de callos, micropropagación, reguladores de crecimiento.

INTRODUCTION

Salvia hispanica L. (*Labiateae*), commonly known as Chia, is an annual herbaceous species native to southern Mexico and northern Guatemala (Ayerza and Coates, 1995). It was an important staple food, oil source and medicine for Mesoamericans in pre-Columbian times (Reyes-Caudillo *et al*., 2008). In those days, it was cultured and consumed by the American natives from the area extended from the South western of the United States of America and Mexico to Central America. Nowadays, it is still used in some communities for their nutritional content in the production of beverages and food (Cahill, 2003). Nevertheless, Chia has almost disappeared from the markets for 500 years, at first, presumably for religious persecution and, finally due to the difficulties for establishing Chia culture out from America (Ayerza and Coates, 2011).

In the last times, Chia has been investigated and recommended as industrial crop and functional food due to its content of oil, proteins, antioxidants and fibers (Ixtaina *et al*., 2008). Their seeds have about 25 to 39% oil by weight

and contain the highest proportion of ω-3-linolenic acid (68%), a polyunsaturated fatty acid, of any known plant source (Ayerza and Coates, 2009). Also, they contain higher levels of protein (19-23%) than those of traditional cereals such as wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), rice (*Oryza sativa* L.), oats (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) (Coates and Ayerza, 1996). As for their beneficial effects on health, Reyes-Caudillo *et al*. (2008) have established that the Jalisco and Sinaloa Chia seeds have an antioxidant activity comparable to Trolox® in all of the assay model systems examined. It was also reported that a Chia-based diet inhibits tumor growth and metastasis in some murine tumor models, presumably due to its ω-3- polyunsaturated fatty acid content (Espada *et al*., 2007).

It was recently demonstrated that the evolution of the nutritional quality of Chia is closely related to the ageing of the plant, which is in agreement with the behavior of other oil seed crops. It has a good nutritive value when harvested at a stage before the shooting period (Peiretti and Gai, 2009). Besides, it was established that crop production and the composition in fatty acids, particularly alpha-ω-3 fatty acids, can be ascribed to the different environmental conditions, such as temperature, light, soil type and available nutrients (Ayerza, 1995; Ayerza and Coates, 2004; Peiretti and Gai, 2009).

Thus, in view of its economic potential in chemical industries and foods, the establishment of *in vitro* cultures appeared as an interesting alternative in order to obtain a uniform biomass (regarding genotype, protein and oil content and fatty acid profile) under controlled environmental and nutritional conditions. In that sense, the aim of our work was to establish *in vitro* cultures of Chia.

MATERIALS AND METHODS

Plant material

Salvia hispanica L., *Labiateae* (Chia) seeds were harvested in Salta, Argentina (21º 46' south, 62º 21' 9 west) during January 2011.

Disinfection and establishment

Seeds were surface disinfected by immersion for 20 seconds in ethanol, followed by a treatment during 5-10 minutes with a solution of sodium hypochlorite (4% active chloride) plus 0.1% Triton X-100. Then, it was washed several times with distilled water until the complete elimination of the sterilizing agent. The seeds were germinated on 0.8% agar medium containing MS mineral nutrients (Murashige and Skoog, 1962) with the addition of 5% sucrose and kept in darkness in a culture chamber at 24 ± 2°C.

In vitro plant cultures

Plantlets were maintained in MS mineral nutrients supplemented with 3% sucrose, 0.8% agar and the growth regulators NAA+KIN (1- naphtaleneacetic acid and kinetin, resp) (1.00 μM and 0.10 μM resp.) and subcultured to the fresh medium every 21 days. All cultures were maintained in the room chamber described below under light condition (given by fluorescent lamps at an irradiance of 1.8 $wm^{-2}sec^{-1}$) and 16- hour photoperiod.

Callus induction and culture

Explants from 20-day-old seedlings (hypocotyls and leaves) were transferred to Petri dishes containing 25 ml of semi-solid B5 medium (Gamborg *et al*., 1968), MS medium (Murashige and Skoog, 1962) or MSRT medium (Nigra *et al*., 1989) with the addition of sucrose (3%) as a carbon source. As plant growth regulators we have used different relationships of auxin and kinetin: 2,4-D (2,4-diclorophenoxyacetic acid) (0.45, 2.25, 4.50 μM), IAA (3-indoleacetic acid) (0.57, 2.85, 5.70 μM), NAA (0.54, 2.70, 5.40 μM), 2,4-D : kin (0.45: 0.46, 2.25: 0.46, 4.5: 0.46 μM), 2,4-D: BAP (6- benzylaminopurine) (4.5 : 0.46 μM), IAA : kin (0.57: 0.46 μM, 2.85: 0.46, 4.5M: 0.46 μM), IAA : BAP (0.57: 0.50, 2.85: 0.50, 5.70: 0.50 μM), NAA : kin (0.54: 0.46, 1.00:0.10, 2.68: 0.46, 5.37: 0.46 μM), Picloram (4-amino-3,5,6-trichloropicolinic acid)(0.34, 1.72, 3.43 μM). The cultures were kept in room chamber as described below at 24°C ± 2°C with or without illumination. After 15-20 days in culture we have transferred the developed calli to Petri dishes containing the same fresh medium, the procedure was repeated every 25 days during 6 months.

Fatty acid Analysis

We have also analyzed the fatty acid content in callus obtained and compared it with the values in seeds and micropropagated plants after 2 years in culture. The fatty acid content was determined by acid hydrolysis according to AOAC Official Method 925.32.

Statistical analysis

Data was analyzed by ÷2 and treatments were contrasted by post hoc test (Marascuilo and McSeeney, 1977). All experiments were repeated thrice.

RESULTS AND DISCUSSION

We established the axenic cultures of Chia plants with low rates of microbial contamination (less than 10%). The multiplication rate obtained was 1:3 after two years in culture. Bueno *et al.* (2010) reported the micropropagation of Chia plants form stems with similar results.

The result of assay performed in order to induce friable callus in different salt media, shows the B5 medium (Gamborg *et al*., 1968) was not able to support callus induction. The explants died after three weeks on culture. On the contrary, in the case of MS (Murashige and Skoog, 1962) and MSRT medium (Nigra *et al*., 1989) both were useful for starting callus cultures. The efficiency for callus induction was between 33% and 100% (Table 1).

Considering the influence of the explants source, we could establish that the efficiency for calli initiation was 29% for leaves and 83% for stems when 2,4-D (2.25 µM) was added to the MS media in light conditions. On the other hand, the efficiency was 70% for stems when Picloram was used as plant growth regulator under the same culture conditions. Leaf explants were not useful for starting Chia *in vitro* cultures which is in agreement with the observations made by Bueno *et al.* (2010) when they studied the behavior of Chia in *in vitro* cultures.

Newly initiated callus cultures were a complex mixture of tissue types including white, non-regenerative and green, regenerative tissues. The proportion of the tissue type changed as a function of time in culture, genotype and amount and the type of auxin used. We have analyzed the influence of several auxins with or without the addition of cytokinins (Kinetin or BAP); both in darkness as in light. In all cases IAA and NAA, with or without cytokinins, have had a strong rhyzogenic effect (Table 2). The ability of both types of plant growth regulators of inducing the formation of adventitious roots was already reported in other plant species (Alvarez *et al.,* 1993; Gostin, 2008; Mukherjee *et al*., 2010).

When 2,4-D was the plant growth regulator used, the explants growing in the darkness developed mostly embryogenic tissues. The addition of kinetin improved the initiation of these tissues, a phenomenon that was more evident as the kinetin concentration increased. The fundamental role of the exogenous application of auxins for inducing embryogenesis, and specifically 2,4-D which is considered to be one of the main inductive factors, could be the explanation (Canhoto *et al*., 1992; Ammirato, 1993; Stefanello *et al.,* 2005). On the contrary, when 2,4-D was used alone (2.25 µM) or combined with kin (2,4-D: kin = 0.45 µM : 0.46 µM) in light conditions, green, friable and fast growing calli were obtained. In all cases, after 6 months in culture, those calli turned into a bright, big and tight one.

Table 1. Effect of growth regulators at different concentrations in MS or MSRT culture media in Chia (*S. hispanica*) callus induction rate (%).

Culture medium	2,4-D (µM)			2,4-D : kinetin (µM)			Picloram (µM)		
	0.45	2.25	4.5	0.1:0.46	0.5:0.46	1: 0.46	0.34	1.72	3.43
MS	60a	100b	52a	50a	100b	100b	52a	83b	70b
MSRT	58a	100b	80b	87b	100b	80b	33a	82b	67b

Means values followed by different letters in the same row indicate significant differences (P< 0.05) according to the post hoc test.

Table 2. Rhyzogenic effect of auxins (% roots produced per explants) in *Salvia hispanica* L. (Chia) explants. All the cultures were maintained in MS medium.

Plant growth regulator (μM)		Without citokinine	Kinetin (0.46 μM)	BAP (0.5μM)
	0.45	20a	50c	-
2,4-D	2.25	0	50c	-
	4.50	0	20	-
	0.57	100b	85b	0
IAA	2.75	92b	70b	0
	5.70	90b	75b	0
	0.54	80b	85b	-
NAA	2.70	85b	70b	-
	5.40	70b	50c	-

Means values followed by different letters in the same column indicate significant differences (P< 0.05) according to post hoc test.

Table 3. Fatty acid content (mg g^{-1} fresh weight) of different plant material of Chia (*S. hispanica*).

Plant Material	mg g^{-1}
Seeds	302.2±10
Micropropagated plants	5.6±0.9
Callus in NAA: kin (0.54:0.46 μM)	7.3±1.8
Callus in Picloram (1.72 μM)	6.4±1.5

There is great interest in using all the available breeding technologies to enhance the variation in fatty acid content, to enhance both the nutritional quality as concentration, and domesticate existing Chia varieties. The micropropagated plants and callus cultures could be the starting point to initiate studies in order to obtain agronomic cultures and breeding programs. In the present work we examined the fatty acid content and analyzed that it was detected in plant and callus *in vitro* cultures. The highest content of fatty acid (essential oils) was obtained in the original seeds, as expected. The lowest essential content was obtained in micropropagated plants. Moreover, Table 3 shows no significant differences between treatments in *in vitro* cultures. However, it is important to point out that all cultures produce fatty acid and it can be detected by the methodology described in M&M.

CONCLUSIONS

We have established *in vitro* culture of Chia. The stems are the best explant source for starting *S. hispanica* callus cultures. Also, we have selected as the best culture conditions for starting callus cultures MS culture media with 2,4-D (2.25 μM) as plant growth regulator and 16-h photoperiod. Also, for maintaining friable and fast growing calli, the best combination are MS culture medium with NAA+kin (0.54:0.46 μM) or Picloram (2.25 μM) as plant growth regulators and 16 h photoperiod. In addition, we have determined that the fatty acid content of calli are significantly lower than that of seeds after two years in culture, however, it could be measure. Nevertheless, our results were valuable because of the possibility of establishing Chia callus and plant cultures capable of producing fatty acids.

ACKNOWLEDGMENTS

MAA and PM are research members of CONICET (National Council of Science and Technology) from Argentina. This work was supported by the Agencia Nacional de Ciencia, Tecnología e Innovación Productiva (ANPCyT) from Argentina (PICT2007 N°00552).

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Recibido: 20-08-2013
Aceptado: 27-09-2013