academicJournals

Vol. 12(21), pp. 3204-3207, 22 May, 2013
DOI: 10.5897/AJB2013.12000
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Establishment of callus from *Opuntia robusta* Wendl., a wild and medicinal cactus, for phenolic compounds production

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Accepted 22 April, 2013

In this work, a protocol for the establishment of callus cultures from *Opuntia robusta*, a wild and medicinal cactus, was developed. The effects of plant growth regulators and culture media composition on callus development were evaluated.The best response was observed on Murashige and Skoog medium added with 2,4-dichlorophenoxyacetic acid, benzyladenine, biotin, casein hydrolysate and proline. The exposure of *O. robusta* callus to jasmonic acid increased 1.3-fold and 3-fold total phenolic acids and flavonoids concentration, respectively. The *in vitro* culture from *O. robusta* could be a new approach for the obtainment of metabolites with pharmaceutical and/or nutraceutical value.

Key words: Callus, flavonoids, jasmonic acid, wild *Opuntia*, phenols.

INTRODUCTION

Cactus prickly pear belongs to *Opuntia* genus and has been used as a source of food, building material, natural pigment production and as a medicinal plant. *Opuntia* plants produce tender cladodes, consumed as vegetable and fruits (prickly pear) both highly appreciated due its high nutritional value and nutraceutical properties. Their therapeutic effects have been associated to its antioxidant constituents like vitamin C, vitamin E, carotenoids, glutathione and phenolic compounds such as flavonoids and phenolic acids (Santos-Zea, 2011).

It is known that metabolite and chemical profile in *Opuntia* depends on harvesting season, culture conditions, growth stage and species. In addition, production of young cladodes and prickly pear fruits is affected by plagues and diseases that attack both commercial crops and wild populations of *Opuntia* (Méndez-Gallegos et al., 2008). Plant cell culture (PCC) represents an alternative with biotechnological potential for metabolite production.

In addition, using PCC is possible to increase yield using strategies like optimization of culture conditions, selection of high-producing cell lines, bioreactor design and elicitation.

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Abbreviations: BA, Benzyladenine; **CH,** casein hydrolysate; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **DICAMBA,** 3,6-dichloro-2-methoxybenzoic acid**; IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **KIN,** Kinetin; **NAA,** 1-naphthaleneacetic acid; **PGR,** plant growth regulators; **PICLORAM,** 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; **PPM,** plant preservative mixture; **MS,** Murashige and Skoog medium.

During elicitation, *in vitro* cultures are exposed to compounds that induce metabolite synthesis as a defense mechanism like jasmonic acid (JA) and its methylated form (MeJA). These elicitors increase the expression of phenylpropanoid biosynthetic genes and enzymes and have been used in plant cell cultures to enhance the synthesis of health promoting metabolites (Karuppusamy, 2009). For the establishment of *in vitro* cultures, the participation of PGR is essential. It has been described that auxins affect development, cell growth, embryo formation and callus induction in different species. Kinetin (KIN) and benzyladenine (BA) are synthetic cytokinin commonly used in plant cell culture to generate somatic embryogenesis, organogenesis and callus formation in cacti (Shedbalkar et al., 2010). Addition of amino acids (proline and glutamine) and organic supplements (CH and peptone) also have a promoting effect on *in vitro* culture development stimulating cell proliferation. Vitamins, like biotin improves the physiological response of *in vitro* cultured recalcitrant species (Al-Khayri, 2001).

Previous reports of *in vitro* culture and micropropagation protocols of *Opuntia ficus-indica* and its cultivars have been described (Llamoca-Zárate et al., 1999; García-Saucedo et al., 2005;) but this is the first report of a protocol for *in vitro* culture establishment of *Opuntia robusta*, a wild species, as a potential metabolite production source. *O. robusta* has long and succulent cladodes, highly appreciated for human consumption, in both fresh and pickled forms and effects on glucose and lipid metabolism have been reported (Wolfram et al., 2002).

In this study, a protocol for the establishment of callus culture from *O. robusta* for the phenolic compounds production, was developed. An optimized sterilization protocol for *O. robusta* cladodes was designed and the effect of plant growth regulators (PGR) and different organic nitrogen sources (casein hydrolysate, glutamine, and proline) was evaluated. Since we are particularly interested in the production of flavonoid and phenolic acids (due to their antioxidant properties), the induction of these metabolites by elicitation with JA is also described.

MATERIALS AND METHODS

Collection of plant material

O. robusta donor plants were provided by the National Institute of Agricultural, Livestock and Forest Research at San Luis Potosí. After collection, plants were put in pots and maintained under greenhouse conditions for young cladodes production.

Surface sterilization protocol

Prior to collection of young cladodes, *O. robusta* donor plants were treated with a biocide mixture (containing 3 mg l^{-1} Captan, 3 mg l^{-1} Benlate, 1 ml l^{-1} Previcur, 0.5 g l^{-1} amoxicillin, and 0.4 g l^{-1} ketoconazol). Collected young cladodes were washed with commercial antibacterial detergent and running tap water for 45 min, followed by immersion in the biocide mixture (described above) for 36 h, rinsed in sterile water, and treated with 10% calcium hypochlorite [$Ca(ClO)_2$] during 10 min, followed by 8% $Ca(ClO)_2$ during 5 min. Finally, cladodes were rinsed in sterile water three times.

Culture conditions and callus induction

Sterile explants (1.5 cm^2) were cultured on Murashige and Skoog (MS) basal media (Murashige and Skoog, 1962) supplemented with116 µM myo-inositol, 1.2 µM thiamine-HCl and 30 g l^{-1}sucrose. To evaluate the auxin type effect on callus induction, explants were cultured on MS basal media with 2 mg l^{-1} (IAA), (IBA), (NAA) or (2,4-D). To evaluate the effect of PGR combinations on callus development, explants were cultured on MS basal media with 3 mg l^{-1} IAA or 2,4-D in combination with 1 mg l^{-1} Picloram, Dicamba, BA or KIN. The effect of media supplements (0.1 mg l^{-1} biotin/0.4 g l^{-1} CH, 0.1 mgl^{-1} biotin/500 µM glutamine or proline) on callus development was also tested.

In order to prevent or decrease oxidation of explant, all culture media contained 1% (w/v) activated charcoal (AC), 0.3% polyvinylpyrrolidone (PVP), 0.025 mg l^{-1}ascorbic acid and 0.025 mg l^{-1}citric acid. All media were adjusted to pH 6.7 with 0.1 N KOH solution, solidified with 4.5 g L^{-1} phytagel (SIGMA) and autoclaved at 121°C for 20 min (1.37 x 10^5 Pa). The explants were cultured under dark conditions and callus formation was evaluated three weeks later.

Induction of phenolic compounds production

To promote phenolic compounds production, *O. robusta* calli were cultured on MS basal media with 50 µM MeJA. Elicited and control calli cultures were maintained under same conditions during 15 days and then were lyophilized and stored at -20°C until analysis.

Total phenolic compounds content determination was performed according to Luximon-Ramma et al. (2002). Gallic acid (GA) was used as standard and results were expressed as µmol of GA equivalents (GAE) per g of sample (dry weight). Data correspond to mean value ± standard deviation (SD). Total flavonoids content was performed according to the method reported by Oltica et al. (2007) with brief modifications. Quercetin (Q) was used as standard and the results were expressed as µmol of quercetin equivalents (QE) per g of sample (dry weight). Data were reported as a mean value ± standard deviation (SD). All determinations were made in triplicate.

A completely random experimental design was selected using 20 to 30 explants per treatment. Statistical analysis were performed using the Instat III program the Tukey test (P=0.05) to compare differences between means.

RESULTS AND DISCUSSION

The sterilization of *O. robusta* cladodes surface was a challenging process due to the huge variety of microorganisms including insects, fungus, bacteria and viruses hosted in spines, areoles, hairs and waxes, which density is higher than in *O. ficus-indica* cladodes (Méndez-Gallegos et al., 2008). In order to obtain sterile explants, washing with detergent, immersion in a biocide solution, followed by the application of $Ca(ClO)_2$ solution were required. The sterilization efficiency was 90%.

Auxin type and concentration are determinant in callus development. Therefore, the effect of natural (IAA, IBA) and synthetic auxins (NAA, and 2,4-D) on callus induction from *O. robusta* was primarily evaluated. Callus formation was obtained in 65 to 75% of explants cultured in presence of IAA, IBA or 2,4-D (no significant differences,

Table 1. Effect of auxins on callus induction from cladodes of *Opuntia robusta*

Auxin (2 mg l^{-1})	Callus induction (%)	Callus morphology
IAA	75^a	Moderate friable callus
IBA	75^a	Green compact callus
NAA	33.3^b	Green compact callus
2,4-D	66.6^a	Green compact callus

Data with different letters are statistically different ($p<0.05$).

Table 2. Effect of PGR combinations and supplements on callus formation from *Opuntia robusta.*

PGR (mg l^{-1})	Callus induction (%)	Size of callus (mm)
IAA3	100^a	5-10
IAA3-DICAMBA1	22^c	< 5
IAA3-PICLORAM1	100^a	< 5
IAA3- PICLORAM1-KIN1	100^a	5-10
IAA3- PICLORAM1-KIN1-Bio-gln	100^a	5-10
IAA3- PICLORAM1-KIN1-Bio-CH	100^a	10-13
2,4-D 3	66^b	5-10
2,4-D 3-PICLORAM1-BAP1-Bio-CH	20^c	6-14
2,4-D 3- BAP1-Bio-CH-pro	50^b	6-14

Bio, biotin (0.1 mg l^{-1}); CH, casein hidrolysate (0.4 g l^{-1}); gln, glutamine (500 μM); pro, proline (500 μM).
Values with different letters are statistically different ($p<0.05$).

$p<0.05$). The lowest callus formation (33%) was observed in explants cultured in the presence of NAA (Table 1). However, IAA induced the generation of friable callus, which is a desirable feature in order to obtain suspension cultures. Therefore, sterile explants were cultured on media with 0.5, 1, 2 and 3 mg l^{-1} of IAA to evaluate callus formation. Callus formation occurred in100% of explants cultured with 3 mg l^{-1}IAA, nevertheless, development rate was slow.

It is well known that recalcitrant plants require combinations of PGR, higher concentration of macro-nutrients, additional organic nitrogen sources or vitamins to induce growth or development. To improve growth rate of callus, PGR combinations and addition of media supplements (biotin and CH, glutamine) were tested (Table 2). Callus induction was observed in 100% of explants maintained on media containing IAA alone or in combination with PICLORAM, KIN, biotin/glutamine and biotin/CH but callus formation decreased on medium with IAA/DICAMBA combination.The highest callus amount was obtained in media supplemented with CH/biotin (10 to 12 mm), however, after subcultures, the development of callus was arrested.

It has been shown that 2,4-D promote callus formation and increases growth rates in several cacti species.Thus, 3 mg l^{-1} 2,4-D in combination with PICLORAM and BA, and supplemented with biotin/CH or proline were tested (BA used instead KIN because higher oxidation rates were observed in presence of KIN). Data (Table 2) suggest that 2,4-D, PICLORAM and BA combination induced callus formation, but was lower than callus formation in explants cultured with 2,4-D alone (statistically different, $p<0.05$), however, biomass was similar.On the other hand, callus formation occurred in 50% of explants cultured on media containing 2,4-D and BA, biotin/CH and proline. This calli were friable and its development continued after subcultures. Then, this media was considered as the optimal for the establishment of callus culture of *O. robusta.* The beneficial effect of CH and proline have been attributed to a higher availability of organic nitrogen source, due to the ammonium, derived of transamination reactions of amino acids. CH is also a source of calcium, phosphate, micronutrients, vitamins, and several growth factors that improve shooting and callus development (Khaleda and Al-Forkan, 2006). In addition, proline is an energy source (the oxidation of one molecule of proline yields 30 ATP equivalents), has a reactive oxygen species (ROS) scavenging activity, an osmoprotective role, is considered as a molecular chaperone able to protect protein integrity, and enhance the activities of different enzymes (Szabados and Savoure, 2009). Biotin is a heterocyclic compound that binds covalently to specific carboxylases to facilitate the transfer of CO_2 during carboxylation and decarboxylation reactions, improves the physiological response of *in vitro* cultured recalcitrant species, and embryo development (Al-Khayri, 2001).

The content of phenolic compounds was determined in

Table 3. Production of phenolic acids and flavonoids by callus from *Opuntia robusta*[1].

Metabolite	Control callus (μmol g^{-1})	Elicited callus (μmol g^{-1})	Increment
Phenolic acids[2]	7.50 ± 0.33[b]	9.81± 0.38[a]	1.30
Flavonoids[3]	1.91± 0.08[b]	5.84 ± 0.34[a]	3.06

[1]Values between columns with different letters are statistically different ($p<0.05$).
[2]Phenolic acids content determined as gallic acid equivalents.
[3]Flavonoids content determined as quercetin equivalents.

O. robusta callus. The results show that callus produced 2.3 times more phenolics acids (GAE) than flavonoids (QE). Ellicitation with JA increased 1.3-folds the phenolic acids content and 3-folds the flavonoids concentration in comparison to control callus (Table 3). JA has been widely used in promoting the biosynthesis of both inducible and constitutive secondary metabolites, including several anti-oxidants as α-tocopherol, cyanidineglucosides, resveratrol and flavonoids (Matkowski, 2008).

Comparing our results with other data from literature, the content of phenolic acids in *O. robusta* cultures was 11 times higher than in callus from *Lepidum meyenii* (Wang et al. 2007), while the flavonoids content was two times higher than in cellular suspensions of *Pueraria tuberose* (Goyal and Ramawat, 2008). Further studies must be done to identify and characterize the metabolites from *in vitro* cultures of *O. robusta* species and know if they correspond or not to those present in *O. robusta* plant.In addition, the levels of metabolites could be improved using biotic and abiotic stress.

ACKNOWLEDGMENTS

This work was supported by the FOMIX-GTO Grant No. 2006-CO1-31809, CONACYT- Fondos Bilaterales México Francia (No. 142873) and FAI –UASLP.

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