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Establishment of *in vitro* regeneration system for *Acaciella angustissima* (Timbe) a shrubby plant endemic of México for the production of phenolic compounds

Jannette Alonso-Herrada[a], Félix Rico-Reséndiz[b], Juan Campos-Guillén[b], Ramón G. Guevara-González[a], Irineo Torres-Pacheco[a], Andrés Cruz-Hernández[a,b,*]

[a] Posgradduate and Reseach Division, Engineering Faculty, Centro Universitario Cerro de las campanas s/n, Col. Las campanas, C.P. 76010 Santiago de Querétaro, Qro, Mexico

[b] Chemical Sciences Faculty, Querétaro Autonomous University, Centro Universitario Cerro de las campanas s/n, Col. Las campanas, C.P. 76010 Santiago de Querétaro, Qro, Mexico

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ABSTRACT

Native plants with ecological, medical and industrial potential, as Timbe tree (*Acaciella angustissima*), are categorized as specific targets for biotechnological improvement. The establishment of a tissue culture system as an alternative system of production of secondary metabolites of chemical and pharmaceutical interest was made in this investigation. An *in vitro* method was developed for the production of phenolic compounds from callus cultures of *A. angustissima.* Friable and light yellow colored callus was induced from epicotyl explants on Murashige and Skoog (MS) medium supplemented with 2% sucrose, 0.3% of phytagel, and 2,4-dichlorophenoxyacetic acid (1.5 mg/l) and Benzylaminopurine (5 mg/l). In order to increase the biomass these calli were repeatedly subcultured (3–4 week interval) onto MS medium supplemented with 2,4-dichlorophenoxyacetic acid at 2 mg/l and coconut water at 10%. High frequency shoot regeneration was achieved from epicotyl explants of *A. angustissima*. Adventitious shoots were produced from organogenic callus when it was transferred to MS medium supplemented with Naphthaleneacetic acid at 0.4 mg/l and Benzylaminopurine at 7 mg/l, with shoot induction frequency of 60% and 1.4 shoots by explant. The absence of plant growth regulators allowed a maximum percentage of rooting (85%) and a good number of roots (2.23) per shoot. The total phenolic content varied significantly ($p < 0.05$) in callus of *A. angustissima* in a range between 40.17 and 81.36 mg gallic acid equivalent (GAE/g), 12.95–74.11 mg rutin equivalent (RE/g), and 0.16–0.72 mg catechin equivalents (CE/g) per g dry weight (DW) compared with previous reports in the plant of *A. angustissima*. The shoots with presence of roots were acclimatized to obtain plants, the acclimatized plants were conserved in a growth chamber and nourished with MS medium at 25% for 3–4 weeks, this was essential for optimal survival of the plants (40%). Forty plants were produced per explant over a period of 4–5 months. The result indicates that the callus system could serve like alternative system of production of phenolic compounds for pharmacological, cosmetic, and agronomic industries, based in antioxidant, antimicrobial and antimutagenic properties previously reported.

1. Introduction

Besides production of quality plant material, plant cell and tissue culture methods are currently being used in the secondary metabolites production. Genus *Acacia* is an important source of secondary metabolites, an infinity of these compounds has been reported from a diversity of *Acacia* species, including amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids and seed oils, fluoroacetate, gums, no protein amino acids, terpenes and phenolic compounds such as hydrolysable tannins, flavonoids and

* Corresponding author at: Posgradduate and Reseach Division, Engineering Faculty, Chemical Sciences Faculty Centro Universitario Cerro de las campanas s/n, Col. Las campanas, Santiago de Querétaro, Qro, C.P. 76010, México.
E-mail addresses: jan_847@hotmail.com (J. Alonso-Herrada), fxricoresendiz@gmail.com (F. Rico-Reséndiz), camposj78@hotmail.com (J. Campos-Guillén), ramonggg66@gmail.com (R.G. Guevara-González), torresirineo@gmail.com (I. Torres-Pacheco), andrex1998@hotmail.com (A. Cruz-Hernández).

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condensed tannins (Seigler, 2003). Someone species of genus *Acacia* have a strong antioxidant effect attributed principally to phenolic compounds from the seed in *A. pennatula confusa* and bark of *A. confusa* (Feregrino-Pérez et al., 2011; Wei et al., 2011). In Mexico, genus *Acacia* comprises 64 species, of which *A. cochliancantha*, *A. macracantha*, *A. pennatula* y *A. angustissima* are widely found (Feregrino-Pérez et al., 2011). *Acaciella angustissima* is locally named as timbe, timbre, cantemo or guajillo. *A. angustissima* (Mill.) Kuntze is a leguminous shrub with a distribution from the southern United States to Costa Rica. The specie can be found in arid and semiarid regions of Mexico; *A. angustissima* is used as firewood, the leaves as forage for goats and sheep and its bark has traditionally been extracted for tannins used to tan hide (Rincón-Rosales et al., 2003). Nitrogen fixing shrub, such as *A. angustissima*, form islands of fertility: they increase the content of soil organic matter, prevent erosion and provide refuge for flora and fauna. Over use, overgrazing, forest fires and cultivation have reduced the distribution and density of these trees and has caused a drastic reduction in soil fertility (Reyes-Reyes et al., 2003; Rincón-Rosales and Gutiérrez-Miceli, 2008). Timbe is used in traditional Mexican medicine to alleviate diarrhea and rheumatism. Several studies focused knowledge on the toxicity of *A. angustissima* caused by condensed tannins, phenolic compounds and simple non-protein amino acids. However, has not yet established the relationship of these metabolites and toxicity of the plant (McSweeney et al., 2008, 2005; Smith et al., 2003, 2001). On the other hand, humans have been propagating many species of forest trees such as *Acacia* with traditional methods, in which trees grown from seed and propagated sexually. However, the germination and propagation of this genus are affected by different factors such as type of soil, salt stress and water stress (Morais et al., 2012; Venier et al., 2012). In this sense, tissue culture offers an alternative to vegetative practices and clonal propagation, furthermore it provides high multiplication rates for specific genotypes (Beck et al., 2000). However, there are no reports on the *in vitro* culture of *A. angustissima*. The present investigation has been undertaken to develop an *in vitro* regeneration system for *A. angustissima* via indirect organogenesis with the following objectives: (i) contribute to the development of regeneration protocols that meet production plant biomass and reforestation, (ii) determine the total phenol and phenolic compounds of methanolic extracts of calli in different stages of development, and (iii) generated an alternative production system of phenolic compounds through callus culture of *A. angustissima*.

2. Materials and methods

2.1. Materials

A. angustissima pods were collected in December 2011 from the San Miguelito community 20°31′44″ N, 100° 21′42″ W at 2379 m in Querétaro, México. *A. angustissima* seeds presenting a firmness and dark color were selected, as this is indicative of the seeds maturity. All the macro and micronutrients for tissue culture media, sucrose, agar as well as plant growth regulators were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Seed sterilization

Three methods were tested for the seed sterilization. The first method use a scarification process with sulfuric acid for 15 min and a 2% solution of sodium hypochlorite (*v*/*v*) and polyethylene glycol sorbitan monolaurate (Tween 20) for 30 min. The second method employed a rinse with 2% sodium hypochlorite (*v*/*v*) for 10 min, a treatment with absolute ethanol for 10 min was applied, and final treatment with 70% ethanol (*v*/*v*) for 10 min was included. The third method use scarification process with sulfuric acid for 5 min, afterward, seeds were washed twice with sterile distilled water. Subsequently, the seeds soaked in 2% sodium hypochlorite (*v*/*v*) and Tween 20 for 15 min, a treatment with absolute ethanol for 15 min was applied and final treatment with 70% ethanol (*v*/*v*) for 15 min was applied too; in all methods, the seeds were rinsed with sterile distilled water to remove residues between each treatment. Finally, water excess was removed soaking on sterile filter paper; all processes were carried out under sterile conditions under laminar air-flow hood.

2.3. In vitro *germination*

For germination, the seeds were transferred to Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) for germination and seedling production with sucrose 2% (*w*/*v*), with no plant growth regulators. The medium was autoclaved at 121 °C 15 psi for 15 min, and transferred to baby food flasks. Each flask contained two seeds that were incubated in growth room at 25 ± 2 °C with a photoperiod of 16/8 h (*light*/*dark*). The germination period is intended as the period of time between the germination of the first seed to five days after the germination of the last seed, using as criteria the emergence of radicles.

2.4. In vitro *callus induction*

For callus induction cotyledon, hypocotyls, leaves, epicotyls and cotyledonary nodes of 20-day-old seedlings were used as explants. The media used for callus induction were MS supplemented with 2% sucrose (*w*/*v*), 0.3% of phytagel (*w*/*v*), and plant growth regulators in the following combinations: 2,4-dichlorophenoxyacetic acid [1.5 mg/l] and Benzylaminopurine [5 mg/l] as well as 2,4-dichlorophenoxyacetic acid [1.5 mg/l] and Kinetin [10 mg/l]. The pH of MS medium was adjusted to 5.8 and autoclaved at 121 °C 15 psi for 15 min. After autoclaving, a total of 20 ml of sterile media were poured into 90 × 10 mm Petri dishes under laminar air-flow and were allowed to solidify. The explants were inoculated in the media combinations and incubated in the dark at 25 ± 2 °C with 50–60% relative humidity. The explants from epicotyls and hypocotyls were sectioned about 4–5 mm longitudinally, leaving phloem and xylem in direct contact with the medium. On the other hand, about 3 mm^2 of leaf and cotyledon explants were sectioned and placed in a Petri dish exposing it in direct contact with the medium. Finally, cotyledonary node explants were sectioned in two fragments, which were placed in direct contact with the medium. After induction, calli were regularly subcultured into fresh medium at 20–30 day interval for further proliferation. Once were obtained friable callus from the explants, auxin 2,4-*D* concentration was increased [2 mg/l] and coconut water at 10% (*v*/*v*) was added to the medium for biomass production.

2.5. Growth curve of the callus culture

A. angustissima calli was analyzed by evaluating the dry weight (DW) for 45 days, to obtain growth curve. The initial inoculum was 0.5 ± 0.05 g of biomass in Petri dishes with 20 ml of induction medium. The calli were incubated at 25 ± 2 °C. The dry weight of the callus was evaluated daily and the callus was previously weighed, placed on filter paper, and put in an oven at 55 °C for 2 h. The dry weight (DW) calculation was conducted by subtracting the weight of the filter paper from the dry weight of the callus on the filter paper.