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TISSUE PLANT CULTURE AS A NOVEL INDUSTRIAL STRATEGY TO PRODUCE BIOPHARMACEUTICALS FROM ENDANGERED PLANTS

Abstract:

Global natural antioxidants market is anticipated to grow on account of its increasing demand in food & beverages, cosmetics, pharmaceutical and animal feed. In this regard, we found high content of flavonoids and antioxidant activity (including antioxidant oligoelements) in the seeds of Araucaria araucana (piñones). However, bulk production of these antioxidants is ecologically non-viable since A. araucana is assessed as endangered species with increased extinction risk based in part by an extensive human harvesting of edible piñones. In this context, plant cell culture represents a useful production alternative to direct extraction of valuable secondary metabolites because: (a) a stable and uniform year-round supply of seed tissues or cells is guaranteed since biomass can be continuously produced in vitro, independent of seasonal variations, (b) selected compounds can be produced under controlled conditions, and moreover, (c) industrial production can be achieved while preserving the species. On the other hand, healthy plants can be easy obtained by micropropagation and then, the new plants can be acclimated to replant degraded areas of logged forest. At present, tissue and cell cultures from leaves and seeds, as well as plants, had been obtained in vitro from A. araucana and other native species. Furthermore, data of optimum conditions for in vitro production of antioxidants are being collected. The aim of these experiments is determine the varieties more adequate for the sustainable bio-based production of natural antioxidants by eco-efficient bio-processes and renewable bioresources.

Keywords:

Biotechnology, Sustainability, Conservation, Bioindustry, Bioeconomy

JEL Classification: O31, Q55, Q57

Introduction

Antioxidants-health relation and antioxidant market

Multitudinous results have provided information about the role of oxidative stress, either by increased reactive oxygen (ROS) and nitrogen (NOS) species or diminished antioxidant capacity, in aging and pathophysiology of many diseases. Including cancer, Parkinson's disease, Alzheimer, obesity, type 2 diabetes, neurodegeneration, infertility, pain mechanisms, etc. (Sohrabipour et al, 2013; Li et al., 2015). Interestingly, since maintaining redox equilibrium of the body is important for health maintenance and disease intervention, antioxidant therapy is a promising way of combating the undesirable effects of reactive oxygen species (Deepak et al., 2015; Menon et al., 2016).

Indeed, scientific evidences coupled with a growing trend towards a healthy lifestyle, are driving growth of the global natural antioxidants market on account of its increasing demand in fortified food & beverages, cosmetics, pharmaceutical and animal feed. Thus, natural antioxidants market is expected to reach U$D 4.14 billion by 2022 (Radiant Insights Report). Since then, the antioxidant potential of plants has received a great deal of attention (Kasote et al., 2015). In this regard, the plants have an innate ability to biosynthesize a wide range of fitochemicals. Accordingly, the medical potential of Argentine plants has been recorded on the basis of their traditional use with an ethnobotanical survey over a 26-year period (Goleniowski et al., 2006). Then, antitumor and antioxidant activity were reported (Bongiovanni et al., 2008; Soria et al., 2008). Recently, we found antioxidant capacity in seeds (“piñón”) from *Araucaria araucana* (Pehuen tree) (Sotomayor et al., 2013).

Considerations: Status of the *Araucaria araucana* populations

The Pehuén *(Araucaria araucana* (Molina) K. Koch), reaching 50 m in height, 2.5 m in diameter and up to 1300 years in age, is an evergreen conifer endemic of Argentina and Chile with an actual area of occupancy (AOO) of 392.51 km² which falls within the threshold for Endangered under criterion B2ab(ii,iii,v) (Premoli et al., 2013). In Argentina they are confined to a narrow strip in the Province of Neuquén, between Aluminé Lake and Lolog Lake (latitude from 37º20' to 40º20'S, on the Andes). *A. araucana* is predominantly dioecious and its heavy seed (about 1000 from each female specimen) is gravity-dispersed or assisted by animals (Shepherd et al. 2008). However, because the seeds are very rich in carbohydrates and proteins, and their taste is similar to that of chest nuts, they are subject to intensive human use. Then, Pehuen trees are poor at regenerating, and any regeneration that does occur is principally asexual with trees sprouting from roots (Schilling and Donoso, 1976; data confirm by authors). Moreover, although most stands have some form of protection, especially in “Parque Nacional Lanín” and “Parque Nacional Nahuel-Huapi”, there are severe threats to *A. araucana*, due to the establishment of plantations of exotic tree species within these native stands. Consequently, the population of Pehuen is

continuously declining, and it is therefore considered to be facing a very high risk of extinction in the wild. (Premoli et al., 2013).

Biotechology as a technology that can contribute to sustainable industrial development

Clearly, the development of alternative and complimentary methods to endangered plants extraction, for the production of the biopharmaceuticals (e.g.), is an issue of considerable ecological and socioeconomic importance. In cellular or tissue culture, the isolated cells from the whole plant (or parts derived thereof) are cultivated under appropriate physiological conditions and the desired product is extracted from the cultures. By *in vitro* culture of “piñón”, a number of advantages could be archived: (a) a stable and uniform year-round supply of seminal tissues or cells is guaranteed since biomass can be continuously produced *in vitro*, independent of seasonal variations, (b) selected compounds can be produced under controlled conditions, and moreover, (c) industrial production can be achieved while preserving the species. On the other hand, healthy plants can be easy obtained and then, the new plants can be acclimated to replant degraded areas of logged forest.

The aims of this work were to obtain healthy plants, callus derived of “piñón” and to evaluate their antioxidant capacity in as a new source of antioxidants for the food or cosmetic industry.

Experimental

“Piñón” samples

The “piñón” were collected from adults Pehuenes trees grafted near “Villa Pehuenia“ (38°50'02.3"S 71°12'24.1"W) in April, 2015 and 2016. The thick cover was removed from the seeds and then they were germinated or embryos were separated for callus production.

Germination

Seeds were soaked overnight in running tap water and then disinfected with ethanol 70% (v/v) for 10 min, followed by sodium hypochlorite 1.5% (w/v) for 15 min, and finally washed three times with sterile distilled water. Sterilized seeds were transferred to culture bottles containing 30 ml of agarized (0.8% w/v) MS (Murashige and Skoog) major and minor salt medium with 3% (w/v) sucrose, at pH 5.6. Culture bottles were incubated at 23°C and germination rates were measured 30 days after starting the culture (figure 3, left). The plantlets were kept under a photoperiod of 16/8 light/darkness. The experiment was carried out with 5 randomly selected seeds (replicates) per treatment, and was repeated four times.

Acclimatization

Young plantlets (2 months-old) were washed with sterilized distilled water and potted in sterile culture bags with steam-sterilized peat. Plantlets were acclimated for 3

weeks in a growth chamber and then, acclimated plants were moved to a green-house in 16 cm-wide polypropylene containers adding non-sterile peat.

Callus induction

We established callus cultures from embryos of Pehuen on MS medium fortified with auxins and cytokins (Palacio et al., 2006). Cultures were kept in a growth chamber at 23ºC under a 16/8 h light/dark photoperiod in order to form callus, which were subcultured every 4 weeks.

Preparation of extracts

The embryos were removed from seeds. Embryos and callus extracts were prepared from fresh samples (3 g) finely ground with glass homogenizer in ultrapure water (3:5 w:v) at room temperature. For the flavonoid determination, extraction was carried out in ethanol instead water. The extracts were centrifuged at 3000 rpm for 10 min and supernatants were stored to -20°C in amber glass vials until use.

Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions Fe^{+3}-TPTZ to a blue coloured Fe^{+2}-TPTZ was conducted using method by Benzie and Strain (1996) with minor modifications. The FRAP reagent was prepared by mixing 10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) solution and 1 part of 20 mM FeCl3.6H2O solution and the mixture was incubated at 37°C for 10 minutes. 50 μl of an aliquot of the extract was added to 900 μl of FRAP reagent. Absorbance was measured at 593 nm and the results were expressed as Ascorbic acid equivalents per 100 grams of fresh embryos or callus (mg AAE/100 g FW)

Total phenolic content (TPC)

Total phenolic content was measured by the Folin–Ciocalteu method according to Soria et al. (2014). A solution was created with 100 μl of extract, 100 μl of 2N Folin-Ciocalteau, and then 800 μl of 7.1% (w/v) sodium Bicarbonate solution was added. After 30min of incubation at 37ºC in the dark, absorbance was recorded at 750nm. A standard curve was used to calculate mg equivalents of Gallic acid per 100 grams of fresh embryos or callus (GAE/100 g FW).

Total flavonoid content (TFC)

Total flavonoid content of extracts (aglycone part) was estimated using the aluminum chloride colorimetric method according to Zapata et al. (2013). Each extract in ethanol (100 μl) was separately mixed with 30 μl of 5% (w/v) Sodium nitrite, 60 μl of 10 % AlCl3, 200 µl solution of 1M Sodium hydroxide, and μl 670 of distilled water. The absorbance of this reaction mixture was recorded at 510 nm on spectrophotometer. Quercetin was used as standard and total flavonoid content was expressed as quercetin equivalents per 100 grams of fresh embryos or callus (mg QE/100 g FW).

Statistical analysis

All experiments were conducted at least two times. For each treatment, 14-25 seeds were used. For statistical analysis, all assays were performed in duplicate and quantitative data were expressed as mean of two experiments ± standard deviation.

Results

Germination and acclimatization

In these seeds the embryo keeps the metabolic activity throughout ontogeny, however, the presence of impermeable seminal tegument prevents water entering to embryo (Fig. 1). As shown in Figure 2, left, Pehuen germination was improved by pulling off the cover to “piñón”. Thus, a 100% germination *in vitro* was observed and healthy plants were obtained (Figure 2, right).

In vitro callus Induction from “piñón”

We established callus cultures from embryos of Pehuen on MS medium. Figure 3, left, shows the callus induction results after a two months growth period. The medium with BAP (N6-benzylaminopurine) and 2,4-D (2,4-dichloroacetic acid) produced brown-yellow and friable callus (Fig. 3, left). Between 40 and 60% of callus induction and growth occurred.

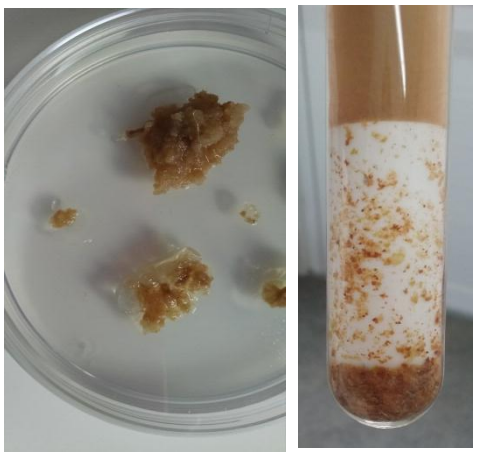
Figure 1: “piñón”

Source: Own photography

Figure 2: *in vitro* germinated plant after 6 weeks on MS medium (left) and acclimated Pehuen after potting into garden soil (right).

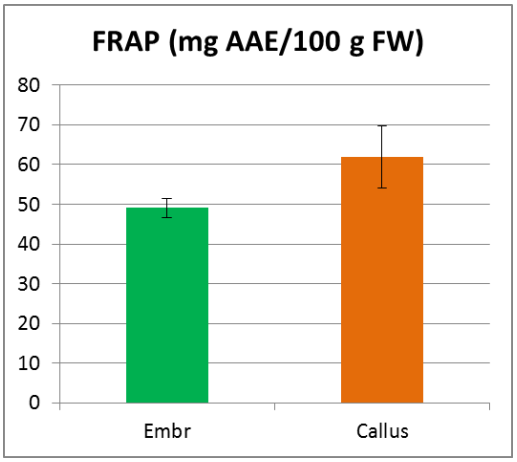
Source: Own photography

Figure 3: Pehuen´s callus and homogenized callus



Source: Own photography

Figure 4: antioxidant capacity in embryos of Pehuen (Embr) or callus (callus): Ferric reducing antioxidant power (FRAP)

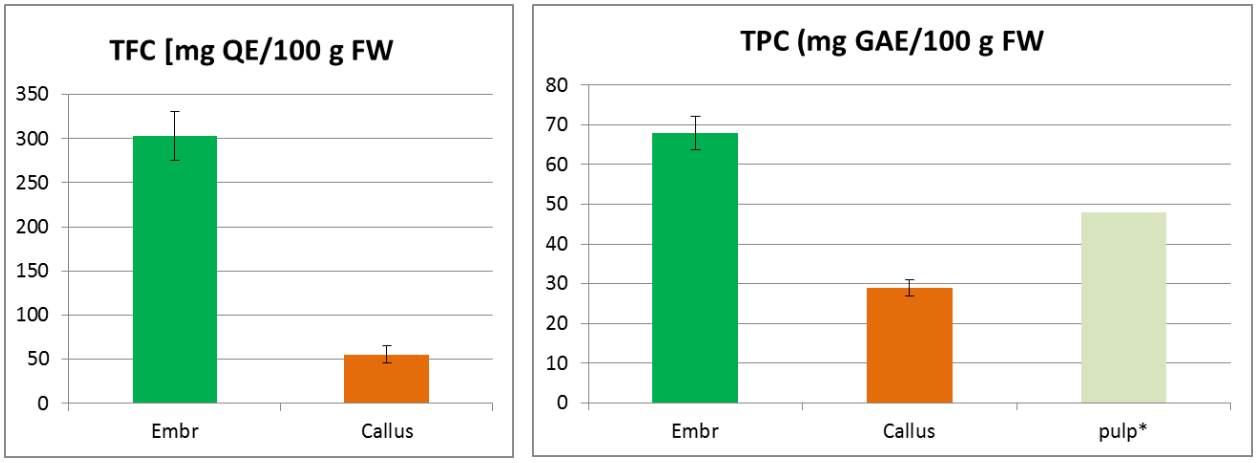


Source: Own results

In vitro production of antioxidants

Other objective of this study was to get information about antioxidant power along original tissue and their *in vitro* derived callus as a potential source of natural antioxidants. The results revealed that callus contain significant amount of antioxidant power at similar level to embryos of Pehuen (*A. araucana*) (FRAP in Fig. 4). However, original tissue (embryos) had more phenols and flavonoids than callus (TFC and TPC in Fig. 5). The results could be indicating the presence of other antioxidant compounds (non-phenols) in callus. New experiments are being performed in order to analyze the biological activity and chemical characterization of extracts. We hope increase the antioxidant power by biotechnological methods, optimizing *in vitro* production of specific metabolites.

Figure 5: antioxidant capacity in embryos of Pehuen (Embr) or callus (callus): Total flavonoid content (TFC); Total phenolic content (TPC).



*Source: Own results. *With comparative propose, TPC reported in pulp of "piñón" is also showed (http://www.portalantioxidantes.com/orac-base-de-datos-actividad-antioxidante-y-contenido-de-polifenoles-totales-en-frutas/).*

Conclusion

The aspects evaluated in this research suggest:

- The *in vitro*-grown plants could be transferred to *ex vitro* conditions, with a view to develop longer-term strategies for the transfer and reintroduction of new Pehuen plants into natural habitat, assisting to conservation and propagation of the specie.

- The callus cultures from Pehuen seeds ("piñón") could are useful for the production of antioxidant metabolites instead of using wild plants. Here we report the induction of callus from Pehuen embryos for continuous production of secondary metabolites.

- Thus, establishment of callus cultures from Pehuen embryos suggesting that "piñón" callus (for cellular suspensions) can be considered an interesting new antioxidant source for the food or cosmetic industry.

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