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Propagation and Conservation of Native Forest Genetic Resources of Medicinal Use by Means of *in vitro* and *ex vitro* Techniques

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In Argentina, there are numerous native species which are an important source of natural products and which are traditionally used in medicinal applications. Some of these species are going through an intense extraction process in their natural habitat which may affect their genetic diversity. The aim of this study was to establish vegetative propagation systems for three native forestal species of medicinal interest. This will allow the rapid obtainment of plants to preserve the germplasm. This study included the following species which are widely used in folk medicine and its applications: *Erythrina crista-galli* or *"seibo"* (astringent, used for its cicatrizant properties and for bronchiolitic problems); *Acacia caven* or *"espinillo"* (antirheumatic, digestive, diuretic and with cicatrizant properties) and *Salix humboldtiana* or *"sauce criollo"* (antipyretic, sedative, antispasmodic, astringent). The methodology included the micropropagation of *seibo*, macro and micropropagation of *Salix humboldtiana* and the somatic embryogenesis of *Acacia caven*. The protocol for *seibo* regeneration was adjusted from nodal sections of seedlings which were obtained from seeds germinated *in vitro*. The macropropagation through rooted cuttings of "*sauce criollo"* was achieved and complete plants of this same species were obtained through both direct and indirect organogenesis using *in vitro* cultures. The somatic embryogenesis for *Acacia caven* was optimized and this led to obtain a high percentage of embryos in different stages of development. We are able to support the conservation of native forest resources of medicinal use by means of vegetative propagation techniques.

Keywords: Micropropagation, native forest species, macropropagation, somatic embryogenesis.

Medicinal plants have been used since ancient times as new therapeutic agents and their uses have been transmitted from generation to generation, either in oral or written forms, up to the present, and this is known as the "traditional therapeutic practice", the use of extracts or active principles of plants, which has been essential to take care of people's health in the first level of attention.

The developed countries as well as the developing ones have increased the use of medicinal plants or their products [1]. Medicinal plants have played a vital role in societies including Argentina for centuries. Most of these plants are wild plants which were available in some forest ecosystems. With the intensity of development and clearing of land many of these wild plants used for medicinal purposes are no longer available in their natural habitat. Now we can address this environmental situation by *ex situ* conservation and propagation of medicinal plants for its sustainable utilization using new and traditional techniques.

In Buenos Aires province (Argentina), there are different native forest species of medicinal interest and some of them are *Salix humboldtiana* or "*sauce criollo*", *Erythrina crista-galli* or "*seibo*" and *Acacia caven* or "*espinillo*".

Salix humboldtiana is distributed in river banks or islands, sometimes in sandy places, too. The different parts of the plant contain salicin. The bark is used in folk medicine as a quinine substitute (it contains glucosides). The decoction of this bark is used "against intermittent fever" (rubber). The bark is bitter and has febrifugal, tonic, sedative and spasmodic properties. It is also astringent [2]. *Erythrina crista-galli* has several important pharmacological uses. It is an astringent and a sedative to heal wounds (3% of bark decoction). It is antihemorrhoidal and used for vaginal lavage in candidiasis cases (bark). It is disinfectant and deodorant, it has cicatrizant properties and it is ahemostatic, emollient for colds, coughs, catarrh, bronchitis and asthmatic pains (the leaves are smoked in a pipe or rolled up like a cigar). It has narcotic, sedative and hypnotic properties: this is attributed to the most inner part

of the bark when it is used in an infusion (it contains several alkaloids). This plant is also used for muscular and rheumatic pains (a balm prepared with its bark and flowers in 70% of alcohol). The leaves are used as antihemorrhoidal for external use and they are antiseptic and astringent [2]. *Acacia caven* or "*espinillo*" is a medicinal plant that lives in South America and it is not tropical. It grows in the arid highlands in the centre and north of the country as well as on the islands in the Paraná River, in very humid areas. The parts of the plant which are used for medicinal purposes are its leaves, stems and seeds. The leaves of this medicinal plant have cicatrizant properties, and the seeds are used as a digestive. The leaves and stems have antiphlogistic and sedative properties (http//www.tusplantasmedicinales.com/).

Vegetative reproduction keeps the parental genotype and its characteristics are preserved in its off springs. Thus, the genotypes of selected trees which are propagated vegetatively reproduce identically and form clones. Grafts, cuttings and layering are the traditional methods of vegetative propagation [3].

In the last few years the use of *in vitro* culture techniques in trees has facilitated the clonation of select phenotypes, the preservation and the manipulation of vegetal material. These techniques allow the multiplication of clones in a short period of time, in any season of the year and in a limited space [4]. By using these techniques, the long time it takes a plant to reach maturity, the low viability of seeds, and the difficulties some species have to propagate by traditional methods can be reduced [5]. Besides, hundreds of clones from the same species can be reproduced *in vitro*. Later, they are taken to a plant nursery and then, they are cultivated in fields where they will develop and finally become a product with a specific economic interest (http://www.biologia.org/).

The aim of this study was to establish vegetative propagation systems for three native trees of medicinal interest: *Salix humboldtiana* (*sauce criollo)*, *Erythrina crista-galli* (*seibo*) and *Acacia caven* (*espinillo*). This will help to obtain plants to conserve germplasm in a rapid way.

Macropropagation of *Salix humboldtiana:* The substrate with the mixture of soil, perlite and vermiculite (6:3, 5:0, 5) was adequate to place the *Salix humboldtiana* cuttings. The cuttings rooted 15 days after they were placed in water. 90% of the cuttings which were treated with IBA rooted, and 75% of them originated complete plants (Figure 1).

In vitro* culture of *Salix humboldtiana: The culture media used for callus, shoot and root induction were adequate. Callus with *de novo* shoots (indirect organogenesis) were obtained approximately 35 days after the leaves were cultured. The shoots formed roots 25 days later. In this way, vitroplants were obtained and they became acclimatized successfully.

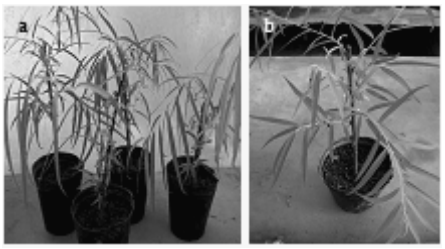

Figure 1 a-b: Macropropagation of *Salix humboldtiana*. Plants obtained by rooting of cuttings.

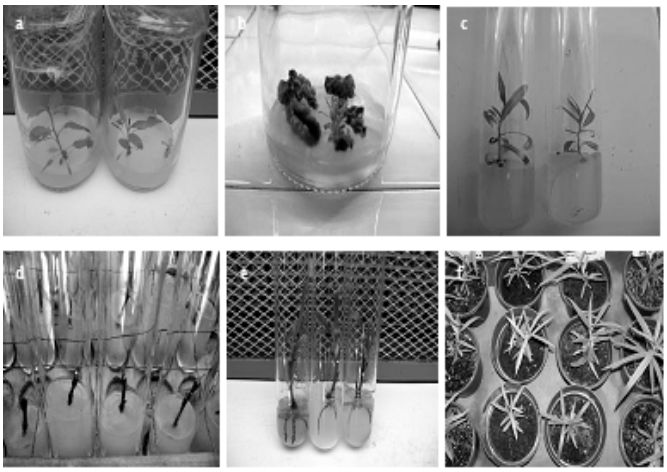

Figure 2a-f: ***In vitro* culture of *Salix humboldtiana*.** a. *In vitro* seedlings of *Salix humboldtiana* obtained from immature embryos. b. Calli obtained from the seedling leaves. c. Shoots formed from calli. d. Shoots from nodal sections. e. Induction of roots from microcuttings. f. Whole plants under greenhouse conditions (acclimatization and hardening).

The percentage of contamination of the nodal sections which grew from fully grown plants was of 80-90% when they were treated with 50% of sodium hypochlorite during 25 minutes and of 60% when they were treated with 50% of sodium hypochlorite during 45 minutes. The WPM culture medium without growth regulators was the best one to obtain preformed shoots and whole plantlets. The first shoots were observed approximately 20 days later, and the roots, 25-30 days after they were cultured *in vitro*.

The culture media with BAP promote the formation of callus on the base of the microcuttings. This callus turned out to be organogenic and the production of shoots was induced by using cytokinins (Figure 2).

Micropropagation of *Erythrina crista-galli :* Growth of the preformed shoots was induced in MS with 1 $mg.L^{-1}$ of BAP and 0.5 $mg.L^{-1}$ of NAA. These shoots were subcultured in a WPM medium with 0.1 $mg.L^{-1}$ of IBA where they elongated. Whole plants were obtained in a WPM rooting medium with 0.1 $mg.L^{-1}$ of NAA. These plants were acclimatized under controlled light and temperature conditions (16 hours of light and 8 hours of darkness, T 21°C +/-2°C) (Figure 3).

Somatic embryogenesis of *Acacia caven:* Direct and indirect somatic embryogenesis were obtained on the

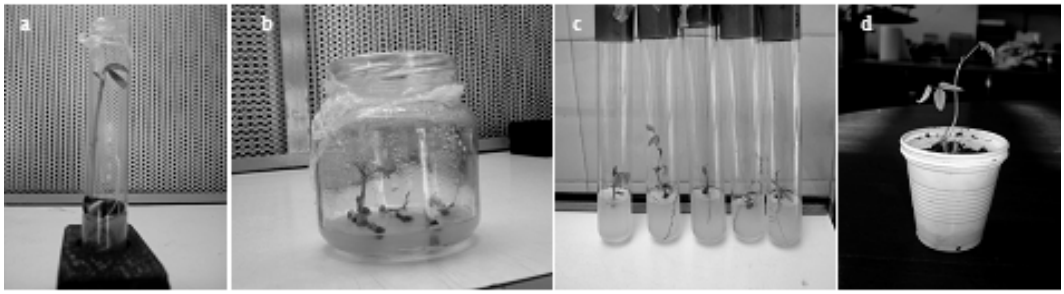

Figure 3a-d: **Micropropagation of *Erythrina crista-galli.*** a. *In vitro* seedlings. b. Shoot elongation from stem section. c. Induction of roots. d. Acclimatization of whole plants.

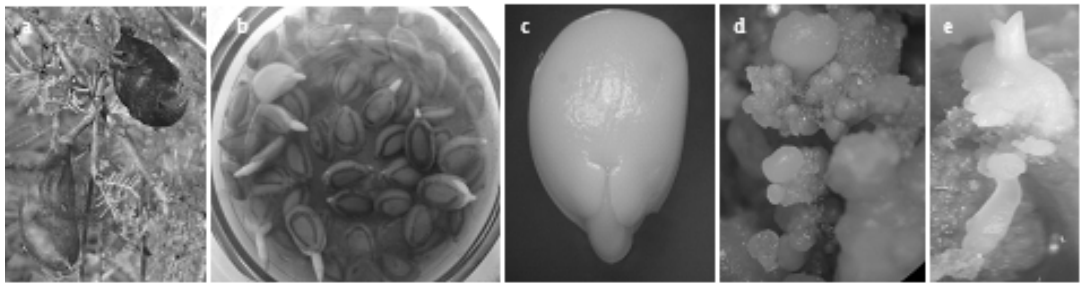

Figure 4a-e: **Somatic embryogenesis of *Acacia caven***.a. Mature fruits of *Acacia caven***.** b y c Seeds in chemical scarification. d. Somatic embryos in a globular and heart stage formed fron cotyledons. e. Embryos in torpedo and cotiledonar stage.

culture medium and the PGR concentration (1 mg.L^{-1} of 2,4 D and 0.1 mg/L^{-1} of BAP) used. The somatic embryos occur directly over the cotyledons, on their adaxial side, or indirectly from the formation of the callus after 6 months of culture. Somatic embryos were observed in different stages of development, and this showed there is no synchronicity in their maturation (Figure 4). The somatic embryos in a globular stage were subcultured on MS medium free from PGR, and they germinated there. 30% of them were converted into complete plants.

Conclusion: When the vegetative propagation techniques of the species mentioned before are adjusted, they contribute to the preservation of the forest genetic resources. Excellent opportunities for scientific research are generated by installing germplasm banks with a wide range of genetic material. This becomes very important if we consider that these species have a great importance in the medicinal use and among others.

These studies pretend to be preliminary stages to achieve the production of native plants of medicinal interest, without the degradation of the genetic base of this resource, which is vitally important for the preservation of the forest resources.

Experimental

This study has been developed at the Centro Experimental de Propagación Vegetativa (C.E.Pro.Ve.) in the Facultad de Ciencias Agrarias y Forestales at Universidad Nacional de La Plata, Buenos Aires, Argentina. In order to reach the objectives, we used vegetative propagation strategies for each species.

For *Salix humboldtiana* or "*sauce criollo*", we used micro and macropropagation systems. Macropropagation was done through the rooting of cuttings with the exogenous application of growth regulators and the optimization of their nutritional requirements. The micropropagation was done through the use of *in vitro* plant tissue culture techniques, following the direct and indirect organogenic pathways.

For *Erythrina crista-galli* or "*seibo*" and *Acacia caven* or "*espinillo*" we used micropropagation following the pathway of organogenesis and somatic embryogenesis. Mother plants which were in good sanitary conditions, optimal growth, and adaptability to the particularities of the local site were chosen as a donating source for the different explants. The methodology included:

Macropropagation of *Salix humboldtiana:* Between 100 and 150 plant stem cuttings with single node each were cut of approximately 30 cm long and from 0.8 to 1.5 cm in diameter. They were treated superficially with 1000 mg. L^{-1} of Bennomyl fungicide for 3 hours in order to avoid the presence of fungi. After that, the bases of the cuttings were dippedin 50 mg. L^{-1} of indole-3-butyric acid (IBA) for 24 hours to induce rooting. The control was not treated with IBA. Then, they were placed in running water for 15 days and they were planted in plastic flowerpots N° 14 (1500 cm^3) with a mixture of soil, perlite and vermiculite (6:3.5:0.5) as substrate [6]. The pots were arranged in randomized block design and replicated three times.

In vitro* culture of *Salix humboldtiana: Explants were nodal sections with internodes (microcuttings) from adult plants and leaves from *in vitro* seedlings obtained from

immature embryos. The embryos were surface sterilized with 10% of commercial sodium hypochlorite (55% active chlorine) for 5 minutes in order to place them *in vitro*, and later obtain the seedlings. The leaves from the seedlings were placed on Murashige & Skoog (MS) [7] basal medium at full strength supplemented with 1 mg.L^{-1} of benzyl amino purine (BAP) and 0.5 mg.L^{-1} of naftalen acetic acid (NAA), 2% sucrose and 7.5 g. L^{-1} agar. Calli were subcultivated on MS medium at full strength with 1mg/L^{-1} of BAP and 1 mg.L^{-1} of NAA. The nodal sections or microcuttings of adult "*sauce"* plant were washed with running water for 5 minutes, and they were surface disinfected with 2000 mg/ L^{-1} of Bennomyl fungicide for 3 hours and 50% of commercial sodium hypochlorite (55% active chlorine) for 25 and 45 minutes. For shoot induction, the following culture media were tested: Woody Plant Medium (WPM) without growth regulators and WPM supplemented with 0.1; 0.5; 1; 1.5; and 2 mg.L^{-1} of the 6- benzilaminopurine (BAP).

The cultures were maintained in the culture room under a regime of 16 h photoperiod (intensity - 40 µEcm-2/min/sec) at 21°C +/-2°C. All experiments were conducted at least three times with 15 replicates each.

Shoots from both explants were placed in a rooting medium with the macro and micronutrients of WPM [8] supplemented with 0.1 mg.L^{-1} of Indolebutyric acid (IBA) (Table 1).

Table 1: Culture media used to the differents explants (leaves and nodal sections) of *Salix humboldtiana*.

Basal media	Growth regulator	mg.L^{-1}	Type of explant
MS complete	BAP/ANA	1:0.5	Leaves
MS complete	BAP/ANA	1:1	Leaves
WPM	IBA	0,1	Shoots
WPM	BAP	0.1; 0.5; 1; 1.5 y 2	Nodal sections
WPM	-	-	Nodal sections

Micropropagation of *Erythrina crista-galli:* In order to induce the shoot proliferation nodal sections from *in vitro* germinated seedlings were used as source of explants. Different culture media with different growth regulators were tested. The explants were cultured in MS medium in a complete, half, and a quarter concentrations, with the addition of different growth regulators: 1 and 2 mg.L^{-1} of BAP, 0.5 mg.L^{-1} of NAA, 1mg.L^{-1} of IBA, 2% sucrose and 7.5 g.L^{-1} of agar, alone or combined. Cultures were incubated at 21°C +/-2°C with a 16 hours photoperiod.

Somatic embryogenesis of *Acacia caven:* In this case, cotyledons from mature seeds were used as explants. These seeds were treated with 98% of sulfuric acid in order to scarification for two hours and they were washed with running water for 10 minutes. Then, they were disinfected with 70% of ethanol during 5 minutes and 20% of sodium hypochlorite (55% active chlorine) for 30 minutes. After that, they were washed 3 times with distilled water under a laminar flow hood and they were put in sterile water during 7 days in order to soften the seed coat and obtain the cotyledons. The latter were sowed in a Murashige & Skoog (MS) basal medium, at half concentration of macro and micronutrients, supplemented with 1 or 2 mg.L^{-1} of 2,4-Dichlorophenoxyacetic acid (2.4-D) and 0.1 mg.L^{-1} of BAP, 3% of sucrose and 7.5 g. L^{-1} agar (Table 2). The cotyledons were placed with their abaxial side in contact with the culture medium and were maintained in the culture room under complete darkness at 21°C +/-2°C.

Table 2: Culture media used in the somatic embryogenesis of *Acacia caven*.

Explants	Media	PGR		Condition
		2.4D (mg.L^{-1})	BAP (mg.L^{-1})	
Cotyledon (Control)	MS/2	0	0	25 +/- 2°C in the darkness.
Cotyledon	MS/2	1	0.1	
Cotyledon	MS/2	2	0.1	

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