European Journal of Medicinal Plants
4(10): 1200-1209, 2014

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Adventitious Bud Formation and Plantlet Regeneration of *Achyrocline satureoides*-A Multipurpose Medicinal Plant

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Authors' contributions

This work was carried out in collaboration between all authors. Author DK performed the experiments. Authors PS and CL designed and instructed the research work. Author CL wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 30th December 2013
Accepted 29th April 2014
Published 30th June 2014

ABSTRACT

Adventitious bud regeneration was achieved from hypocotyls, cotyledons and leaf explants of *Achyrocline satureioides*. Organogenesis was induced from every explant cultured on Murashige and Skoog semisolid medium (plus sucrose 30g·L^{-1}) containing different combinations of 6-benzyladenine (BA) and α-naphtalenacetic acid (NAA) under 116μmol·m^2·s^{-1} photosynthetic photon flux density (PPFD), photoperiod 14 h and at 27±2ºC. The regeneration was similar for every tested explant and varied between 64 and 83%. The number of buds formed per regenerative explants was similar in every treatment (5-8 shoots/explant). In order to stimulate *In vitro* rooting, regenerative leaves were sub cultured from the best induction medium in MS lacking plant growth regulators for the same periods. Every plantlet raised *In vitro* was phenotypically normal and successfully hardened to *ex vitro* conditions. An experimental field plot with 60-day-old *in vitro* regenerated plants was established.

Keywords: Achyrocline satureoides; *regeneration; leaf explants; cotyledon explants; hypocotyls explants.*

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ABBREVIATIONS

AS, adenine sulphate; BA, 6-benzyladenine; 2-iP, 2-iso-pentenyladenine; KIN, Kinetin; PPFD, photosynthetic photon flux density; ZEA, zeatin.

1. INTRODUCTION

Achyrocline satureioides (Lam.) D.C. ("Marcela"), belonging to the Asteraceae family, is a semi-shrub perennial plant which could reach a 30-40cm height, widely distributed in Argentina, Brazil, Paraguay and Uruguay [1]. Its lanceolate leaves with abundant pubescence show an opposite distribution around the stem. Vegetative growth occurs during spring and summer while flowering process takes place in autumn. Flowers are arranged in a terminal inflorescence of yellow color. This specie is disseminated by seeds [2].

The aqueous extracts of aerial parts of *A. satureioides*is widely used in folk medicine in Argentina and other countries of South America for the treatment of several human illnesses, particularly those related to gastrointestinal disorders [3]. Likewise, it is included in some bitter beverages or aperitifs with the purpose of easing digestion process. *A. satureioides* is known to possess a wide spectrum of pharmacological, medicinal and therapeutic properties. In fact, experimental pharmacological studies have shown antioxidant, analgesic, anti-inflammatory, antispasmodic, constipating, sedative, hypocolesterolemic and anticancer effects [4-9]. Also, the ethanol extracts from *A. satureioides* showed insecticidal activity on bloodstream forms of *Trypanosoma cruzi*, a protozoan parasite causing the major health problem in Latin America known as Chagas' disease [10]. Lately, Ruffa et al. [11] reported that the methanol extracts showed cytotoxic activity against a human hepatocellular carcinoma cell line, Hep G2. In addition, Carney et al. [12] informed its antihyperglycemic activity at the same time Gugliucci and Menini [13] concluded that *A. satureioides* would be a natural candidate for studies of herbal complement to diabetes treatment since it combines hypoglycemic, antioxidant and antiglycating tricks.

Phytochemical analysis from various studies have described the presence of flavonoids, caffeoilderivates, coumarins, essential oil and terpenes [14-18] and relationship among these chemical components with pharmacological activities described above. Interestingly, achyrofuran, a new prenylated dibenzofuran, has been isolated from its extract of "Marcela" [12], which significantly lowered blood glucose levels in the db/db mouse model for type 2 diabetes [13]. As a final point, results obtained by Rivera et al. [19] in their studies led them to conclude that the aqueous extracts utilized in the traditional oral administration seems to be devoid of toxic risks.

The unrestricted subtraction of their flowers negatively affects the abundance of *A. satureoides* in the ecosystem because it hinders their natural dissemination by seed. In order to expand cultivation of *A. satureoides*, the first step is the production of high quantities of genetically homogeneous plant material with a desirable chemical composition. Within this context, the use of tissue culture would be of great use for the multiplication and cryopreservation of selected clones. This study describes the procedure developed to induce direct regeneration of shoots and subsequently plantlet production from cotyledons, hypocotyls and leaf culture of *A. satureioides*. As far as we know, neither organogenesis nor somatic embryogenesis of the genus *Achyrocline* has been reported till date.

2. MATERIALS AND METHODS

2.1 Plant Material

The seeds were collected from a wild population grown in the Biological Station Corrientes (BECo), San Cayetano, Corrientes, Argentina (27º33'10.3''S 58º40'46.4''W). To determine the optimal condition for germination during the establishment phase, the seeds were surface sterilized with either 0.8 or 1.2% NaOCl and 0.1% TRITON® for 15 or 20min, and thoroughly washed with sterile distilled water. Afterward, the seeds were cultured in 11 ml glass tubes (1 seed/tube) containing 3ml of semi-solid synthetic Murashige and Skoog [20] medium with $30gr \cdot L^{-1}$ sucrose (MS), pH 5.8, and incubated in a growth room at 27±2ºC with 14h photoperiod subjected to either darkness or 4.5 and $116\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD from white fluorescent lights.

In a second step, from the results obtained, the treatment consisting in the surface sterilization of seeds with 0.8% NaOCl and 0.1% TRITON® for 20 min was chosen to induce adventitious bud regeneration. The seeds were cultured in 110ml glass flask (150 seeds/flask) containing 20 ml of basal medium, and incubated under $116\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD.

2.2 Induction of Shoots and Roots

Hypocotyls, cotyledons, and expanded leaves extracted from15-days-old seedlings obtained by *In vitro* germination of *Achyrocline satureioides* were used for the regeneration experiments. Each explant was transversely divided by two or three cuts and cultured in 11 ml glass tubes containing 3ml MS supplemented with naphtaleneacetic acid (NAA; 0, 0.5, 2.5 or 5µM) and either 6-benzyladenine (BA, 0, 0.5, 2.5, 5 or 15µM), 2-*iso*-pentenyladenine (2-iP), Kinetin (KIN), zeatin (ZEA) or adenine sulphate (AS) at 5µM during 30 days. In order to stimulate *In vitro* rooting, regenerative leaves from the best induction medium were sub cultured in MS medium lacking plant growth regulators for the same periods.

The pH of the media was adjusted at 5.8 with KOH or HCl prior to addition of the gelling agent (Agar Sigma A-1296, 0.65%). The tubes were covered with aluminum foil and autoclaved at $1.46kg \cdot cm^{-2}$ for 20min. The cultures were incubated in the same physical conditions described above.

2.3 Transfer to Soil

The plantlets obtained *In vitro* were carefully washed under running water and set into 200 ml pots filled with a mixture of sterile soil and sand (1:1v/v) plus 0.5g of controlled release micro-fertilize (Osmocote®, N, P, K; 18, 5, 9, 180-day-release) and covered with transparent polyethylene which was subsequently lifted to reduce humidity. They were grown for 30 d under day/night air temperature of 25-27/20-22ºC and substrate temperature of 22-25ºC. 14h photoperiod was kept using 8 cool-white fluorescent lamps (40W) set at 1.8m over the plant which provided $120\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD in a wavelength range of 400-700nm. 60-d-plantlets were set in the field at a distance of 0.7x0.7m.

2.4 Statistical Analysis

Each treatment consisted of 10 explants and the experiment was repeated 3 times. The treatments were arranged randomly on the shelves in the growth room. The results presented are the mean of the replications with the standard error (±SE). Shoot number is presented as the mean of shoots regenerated per morphogenic explants. Regeneration rate is expressed as the average percentage of either hypocotyls cotyledons or leaves that differentiated shoots over the total number of each explants cultured. Analysis of variance (ANOVA) was performed and comparisons of means were conducted using Tukey Multiple Comparison Test with at least significance difference test at 5% level of probability.

3. RESULTS AND DISCUSSION

3.1 Establishment and *In vitro* Germination of Seeds

After 15 days incubation, the establishment of the cultures was successful in all treatments tested Fig. 1A. The germination rate varied between 26.7±3.3 and 56.7±6.7%, depending on the type of disinfectant, concentration and exposure time used Fig. 1B. The best results were obtained when seeds were immersed in an aqueous solution of 0.8% NaOCl plus 0.1% surfactant for 15min. and rinsed several times with sterile distilled water. Although the use of Benzalkonium Chloride, a member of the quaternary ammonium class of biocides recognized for its dual biocidal and detergency properties [21], is widely recommended for surfaces and explants disinfection [22], a pre-treatment with BC 0.05% for 15-20min. negatively affected the *in vitro* germination and growth of *A. satureoides* seedlings. Combination of the best disinfection procedure and lighting conditions produced optimum *In vitro* germination frequency in seeds kept in 14h light photoperiod conditions Fig. 1C.

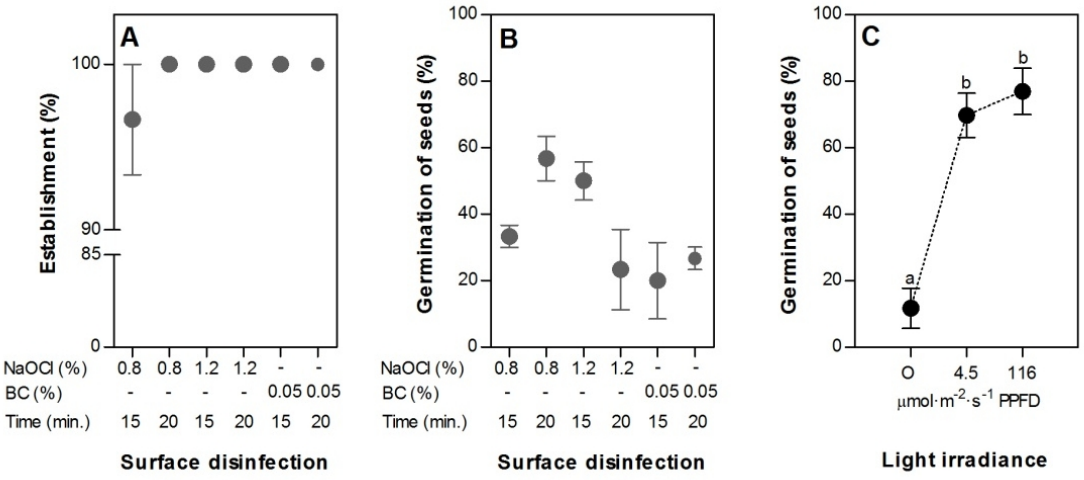

Fig. 1. Effects of different types of surface disinfectants, concentration, time of exposure, and light conditions on the establishment (A) and germination (B, C) of *A. satureioides* seeds

Values are the mean±SEM of three independent experiments. Treatments with different letters are different according to Tukey's Multiple Comparison Test (P=.05)

3.2 Adventitious Bud Regeneration and Production of Multiple Shoots

After 30 days of culture under the environmental conditions described above, the regeneration rate was similar from every tested explant and varied between 64 and 83% Table 1. In general, the regeneration frequencies were greater on MS medium containing NAA at 0.05-0.5μM and BA at 2.5 and 5.0μM than the other treatments. The regeneration rate was similar in all cases, but leaves were chosen as the explant source because of the high quality and growth rate of the regenerated shoots and the facility in obtaining a profuse explant source from them.The system of regeneration was direct without formation of callus and the number of buds formed per regenerative explant was similar in every treatment (5-8 shoots/explant).

Table 1. Effects of NAA and BA on direct shoot organogenesis from *hypocotyls, cotyledons and leaves of Achyroclyne satureoides*

PGRsμM		**Percentage of explants producing shoots**		
		Hypocotyls	**Cotyledons**	**Leaves**
NAA	BA			
0	0.5	0.0±0.0^{c}	11.1±11.1^{b}	6.7±6.7^{e}
	2.5	3.3±3.3^{c}	10.2±6.5^{b}	16.7±3.3^{de}
	5.0	7.4±7.4^{c}	15.7±7.9^{ab}	24.3±9.7^{cde}
0.05	0	3.7±3.7^{c}	0.0±0.0^{b}	0.0±0.0^{e}
	0.5	42.2±8.9^{b}	30.9±12.7^{ab}	27.0±8.5^{bcde}
	2.5	72.4±8.5b^{a}	60.7±3.2^{a}	55.2±2.9^{abc}
	5.0	78.5±0.8^{a}	61.3±12.7^{a}	60.0±5.8^{ab}
0.5	0	0.0±0.0^{c}	2.8±2.8^{b}	3.3±3.3^{e}
	0.5	50.7±10.8^{ab}	47.9±9.0^{ab}	26.7±3.3^{bcde}
	2.5	71.8±8.2^{ab}	46.8±12.3^{ab}	43.3±13.3^{bcd}
	5.0	82.2±9.7^{a}	63.7±10.4^{a}	83.3±8.8^{a}

Values are the mean±SE of three independent experiments; The mean in each column followed by different letters is different according to Tukey's Multiple Comparison Test (P=.05*)*

A high cytokinin to auxin ratio usually results in shoot formation [23]. In *Scaevola aemula*, the addition of BA to the medium has been reported to induce shoots from leaf explants [24]. Later, Wang and Bhalla [25] reported that the inclusion of higher concentrations of NAA in the medium inhibited the production of shoots and roots from the same explants. In the case of *Aloysia polystachya*, another South American medicinal plant, the results of this experiment testified a low concentration of NAA and BA (0.05 and 0.5μM, respectively) is needed to get the highest regeneration rate [26].

Consequently, a MS medium containing NAA 0.05μM was chosen to study the effect of different cytokinins and its precursor, as adenine sulphate, on this morphogenetic process. Fig. 2 shows that the type of cytokinin added to the culture medium affected shoot organogenesis and growth of the regenerated shoots. BA and ZEA promoted adventitious bud regeneration without differences between the explants tested Fig. 2A. Even though, SA plus BA increased the number of phytomers and promoted the micropropagation of strauberry [27], its addition to the culture medium as a unique source of cytokinins did not stimulate the proliferation of adventitious buds from cotyledons or hypocotyls. The inclusion of a low concentration of NAA plus BA in the culture medium promoted the normal growth of shoots Fig. 2B. Although the regeneration rate was not significantly different between ZEA

and BA, the addition of 5μM of ZEA was discarded due to the proliferation of calli. The best result was obtained when the basal medium was supplemented with 0.05μM NAA and 5μM BA, in which 73±3.3% of the hypocotyls formed 3.9±0.3 shoots per responsive explant through a direct system of regeneration without callus proliferation.

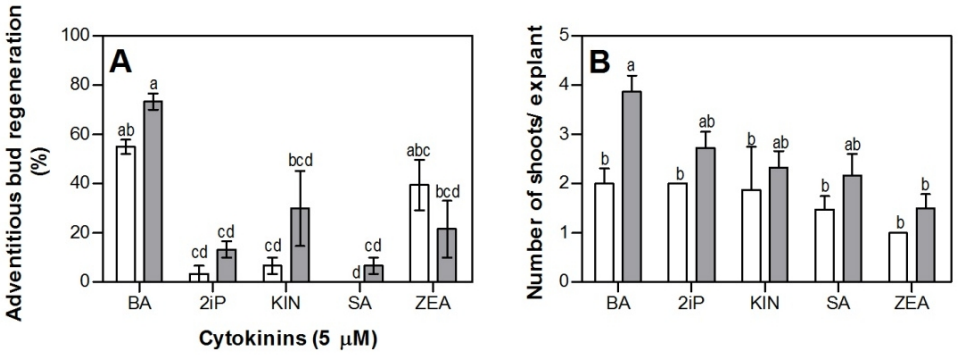

Fig. 2. Effects of different cytokinins on adventitious bud regeneration (A) and proliferation of shoots(B) from either hypocotyls (filled bars) or cotyledons (empty bars) of *A. satureioides* culture in MS plus NAA 0.05μM

Values are the mean±SEM of three independent experiments. Bars with different letters are different according to Tukey's Multiple Comparison Test (P=.05)

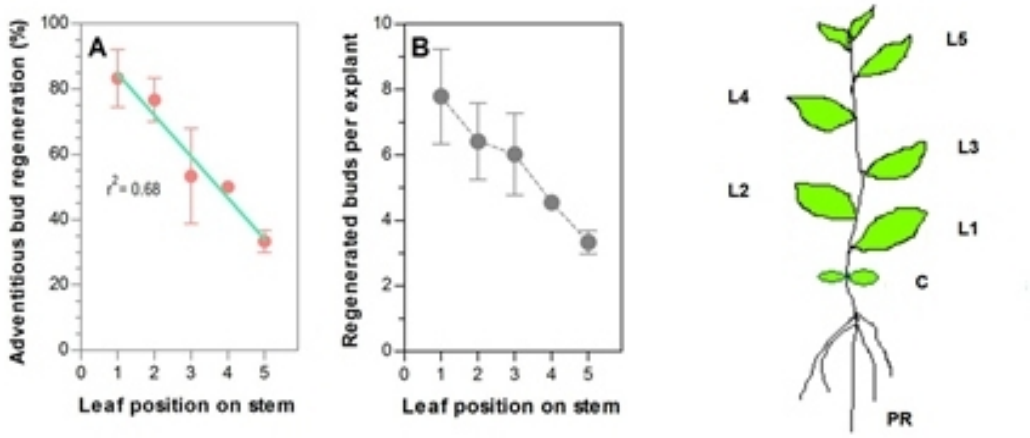

Fig. 3. Effects of leaf position on adventitious bud regeneration (A) and proliferation of buds per regenerative explants (B) from leaves of *A. satureioides* culture in MS plus NAA 0.05μM and BA 5μM

Values are the mean±SEM of three independent experiments. Bars with different letters are different according to Tukey's Multiple Comparison Test (P=.05). Abbreviations: C, cotyledons; L, leaf, PR, primary root

In order to determine how the leaf age affected the rate of regeneration of adventitious buds, the induction medium composed of MS supplemented with NAA 0.05μM and BA 5μM was chosen to study the effect of leaf position on *In vitro* seedling assigning number 1 to the leaf proximate and immediate to the cotyledons.

After 30 days of culture, the adventitious bud regeneration varied between 33±3.3 and 83±8.8%% Fig. 3A, and it was been strongly correlated with the position of the explants in the mother plant. Likewise, the number of buds differentiated per regenerative explants depended on this character and diminished drastically from the explants extracted near to the shoot apical meristem Fig. 3B.

Finally, up to 90% of elongated shoots was rooted in a basal medium lacking plant growth regulators and the rooting process occurred without callus formation Fig. 4 A-B. This morphogenetic process might be successfully stimulated using a basal medium without hormones as reported by Sansberro and Mroginski [28] for *A. polystachya.* After 30 days of culture, the plantlets were carefully washed under running water and set into 200 ml pots filled with a mixture of sterile soil and sand as described in Materials and Methods. Nearly 100% of the 60-d-old acclimatized plantlets were successfully established in soil Fig. 4 C-D.

Fig. 4. Clonal propagation of *Achyrocline satureioides* through *in vitro* regeneration of adventitious buds

A) Shoot-proliferation culture from leaf explants incubated in MS medium supplemented with BA (5μM) and NAA (5μM) under the environmental conditions described in Material and Methods. B) Elongation and rooting phase in MS without plant growth regulators. C) In vitro regenerated plant growing in the field after 90 days of transplantation. Note that the diameter of a single plant reached 80cm. D) Mature plants of A. satureioides (180 days after transference to soil).The bars indicated 1 (A-B), 5 (C), and 10cm (D)

4. CONCLUSION

In conclusion, our study provides a technique for efficient plantlet production for *Achyroclyne satureioides*. The technique described here for direct shoot organogenesis from hypocotyls, cotyledons, and leaf explants and subsequent plantlet regeneration facilitates the rapid propagation. It will also be useful in cryopreservation and genetic studies aimed to improve the essential oil composition of its extracts.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

This work was supported by grants from ANPCyT (PICTO 2011-0213) and SGCyT-UNNE (PI 014/10; PI 005/11). We extend our deep appreciation to anonymous reviewers for their critical comments. P. Sansberro and Claudia Luna are members of the Research Council of Argentina (CONICET).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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