

In vitro plant regeneration of *Spartina argentinensis* Parodi

Regeneración *in vitro* de *Spartina argentinensis* Parodi

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Resumen

Spartina argentinensis Parodi es la especie dominante en comunidades halófitas que ocupan alrededor de 20.000 km² en la Provincia de Santa Fe, Argentina. El objetivo de este trabajo fue desarrollar un método simple para la regeneración de plantas *in vitro* de *S. argentinensis* que podría ser utilizado para la investigación básica y aplicada. Se utilizaron como explantes, segmentos basales de hojas de plantas jóvenes y maduras, puntas de raíces e inflorescencias inmaduras. El medio de cultivo utilizado para callos, brotes e inducción de raíces consistió en la base salina de Murashige y Skoog (MS) suplementado con diferentes reguladores del crecimiento vegetal (2,4-D; BAP ó ANA). La mayoría de los explantes (con excepción de las puntas de raíces) mostró una proliferación celular y formación de callos después de 30 días de cultivo. Sólo las inflorescencias inmaduras regeneraron brotes y raíces cuando los callos se incubaron en sales MS con 2,4-D y BAP (0,1 y 0,01 mg.l⁻¹, respectivamente), posteriormente los callos se transfirieron a medio de inducción de brotes (sales MS, 0,5 mg.l⁻¹ BAP) y luego a medios de inducción de raíz (MS y 0,5 mg.l⁻¹NAA). Las plantas regeneradas se evaluaron para detectar anomalías morfológicas y el contenido de lignina de sus hojas. El análisis histológico de los callos mostró que los brotes y las raíces se originaron vía organogénica. Un bajo porcentaje de las plantas regeneradas mostraron deficiencia de clorofila (plantas albinas) y otras anomalías morfológicas. Entre las plantas regeneradas se detectaron variaciones significativas en el contenido de lignina. El protocolo que se describe en este trabajo podría ser utilizado para la regeneración *in vitro* de plantas de *S. argentinensis* y la selección de variantes somaclonales para futuros planes de mejoramiento.

Palabras clave: *Spartina argentinensis*, *in vitro*, organogénesis, variación somaclonal.

Abstract

Spartina argentinensis Parodi is the dominant species of the temporally-flooded halophyte communities of the Santa Fe Province, Argentina. It occupies around 20 000 km² and it is mainly used as low-cost impute forage for cattle production. The objective of this work was to develop a simple method for *in vitro* plant regeneration of *S. argentinensis* that could be used for fundamental and applied research. Leaf-basal segments from both young and mature plants, roots tips and immature inflorescences were used as explants. Culture media for callus, shoot and root induction consisted of Murashige & Skoog salts (MS) supplemented with different plant growth regulators (2,4-D; BAP or ANA). Most of the explants (with the exception of root tips) showed cell proliferation and callus formation after 30 days of culture. However, only immature inflorescences responded to shoot and root induction when callus were incubated on MS salts plus 2,4-D and BAP (0,1 and 0,01 mg.l⁻¹, respectively), transferred to shoot induction media (MS salts plus BAP, 0.5 mg.l⁻¹) and then to root induction media (MS salts plus NAA 0.5 mg.l⁻¹). Regenerated plants were evaluated for morphological abnormalities and lignin content. Historical analysis of regenerating callus showed that shoots and roots originated via organogenesis. A low proportion of regenerated plants resulted with deficiency in chlorophyll (albino plants) and other morphological

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abnormalities. Between regenerated plants significant variations in the lignin content were detected. The protocol described in this work could be used for *in vitro* plant regeneration of *S. argentinensis* and selecting somaclonal variants for breeding purposes.

Key words: *Spartina argentinensis*, *in vitro*, organogenesis, somaclonal variation.

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Introduction

Spartina argentinensis Parodi (*Poaceae: Chlorideae*) is one of the dominant species found in inland marsh communities of depressed and flooded areas of the Chaco-Pampeana plains at North-Central Argentina (Cabrera and Willink, 1973; Lewis *et al.*, 1990). Traditionally, this area is used for cattle grazing during spring and summer and then abandoned because mature *S. argentinensis* leaves are very tough (due to their high lignin content), and poor in forage quality to be consumed by animals (Baucher *et al.*, 1999). Fire is a frequent management practice in the area (Feldman and Lewis, 2007). It is based on the fact that young leaves arising after fire had low lignin, better digestibility and are more palatable (Feldman and Lewis, 2007). Though some authors sustain that fire should not be considered as a disturbance in the herbaceous plant communities (Ghermandi *et al.*, 2004; Feldman and Lewis, 2005), it certainly affects animals species living within the area and contributes to increases the atmosphere greenhouse gases content (IPPC, 2008). Consequently, this management is a non-environmental friendly practice and should not be encouraged.

One of the main objectives in *S. argentinensis* research is to obtain plants with low lignin content that could be eventually employed for generating new cultivars by traditional breeding strategies. It is expected that improved plants showing a better forage quality, for longer periods, would allow an increment of meat production on marginal areas and contribute to eradicate fire as agronomic practice.

In vitro plant regeneration of *Spartina* species from different explants has been reported. Li *et al.*, (1995) analyzed the *in vitro* behavior of different explants of *S. patens* and regenerated plants from caryopses-derived callus. The same authors reported the *in vitro* plant regeneration of *S. cynosuroides* and *S. alterniflora* from immature inflorescences (Li and Gallagher, 1996) and reported the regeneration of somaclonal variants from mature seeds with important characteristics for marshes restoration. Wang *et al.*, (2003) regenerated plants of *S.*

alterniflora using mature inflorescences and Lu-Zhou *et al.*, (2006) reported the positive effect of the brassinolides in the regeneration ratio of *S. patens*. A high efficient methodology for creating synthetic seeds of *S. alterniflora* has also been described (Utomo *et al.*, 2008).

Larkin and Scowcroft (1981) defined somaclonal variation as any genetic modifications that occurs during the *in vitro* tissue culture and that are transmitted to the progeny. One of the major advantages of this kind of modification in the plant genome for plant breeding is the generation of new variability in species or cultivars with narrow genetic background. The novel variants can be used for creating new varieties within a species after normal crossing. Lu *et al.*, (2006) obtained somaclonal variants in a suspension culture system of triploid bermudagrass (*Cynodon transvaalensis* x *C. dactylon*). Somaclonal mutants with low lignin content were obtained in grass species like *Sorghum sp.* (Cherney *et al.*, 1988), and *Eragrostis curvula* (Jeoung-Mee and Liang, 1998).

The objectives of this research were to develop a simple and efficient protocol for *in vitro* plant regeneration of *S. argentinensis* and to evaluate the presence of variants in lignin content in the regenerated plants.

Materials and methods

Plant material

Plants of *S. argentinensis* were collected from a natural population located nearby Ludueña River, Santa Fe Province, Argentina (32° 45' S; 60° 35' W). Selected individuals were planted in 20 l plastic pots filled with soil of the same area. Plants were kept under natural conditions in the gardens of the Agronomy College of the National University of Rosario, Zavalla, Santa Fe, Argentina. Mature and young leaves, root tips sections and immature inflorescences (approximately 2 days before its emergency from the flag leaf) were used as explants. Moreover, spikelets and caryopses were collected and kept without *lemma* and *palea*, in paper bags at room temperature until culture.

Tissue culture procedure

Culture media consisted of the Murashige and Skoog (1962) salts (MS), supplemented with 30g.l⁻¹ sucrose and different concentrations of ANA, 2,4-D and BAP as plant growth regulators (table 1). All media were adjusted to pH 5.8 by adding HCl or Na(OH) before sterilization at 1.2 PSI for 20 min at 120 0176C.

Explants used were basal segments from leaves from 7-days seedlings and mature leaves; sections of root tips and immature inflorescences. All explants were disinfected by shaking 1 minute in 90 % ethanol, followed by immersion in sodium hypochlorite solutions (15 min, in 1.5 % for leaves and root tips and 20 min in 2.5 % for inflorescences and caryopses prior germination) and rinsed 3 times with sterile distilled water. Disinfected caryopses were placed in Petri dishes for

a week, and after germination young leaves and root apex were cut in 0.5 and 0.2 cm pieces, respectively, and transferred to culture media as described by Bueno *et al.*, (2004). Leaf-basal segments and immature inflorescence were cut in 0.5 cm long sections.

Cultures were initiated in Petri dishes containing the corresponding callus induction media (table 1) including 5 Petri dishes per explant type, and 20 explants per Petri dish. Explants were incubated in the dark at 27 ± 2 °C for callus induction. Callus were transferred to shoot regeneration media (table 1) and were incubated under fluorescent light (16/8 hours of light/dark periods) during 30-40 days. Regenerated shoots were transferred to root regeneration media (table 1) when reached about 3 cm height. Regenerated plants were

Table 1: Composition of media for *S. argentinensis* culture *in vitro*: Murashige and Skoog (1962) salts supplemented with 30 g.l⁻¹ sucrose and plant growth regulators: NAA: naphtaleneacetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid, and BAP: 6-benzylaminopurine. CIL: callus induction media for leaf explants; CIR: callus induction media for roots explants; CIIIn: callus induction media for immature inflorescence explants; SIIIn: shoot induction media for immature inflorescence explants; and RIIIn: root induction media for immature inflorescence explants

Explants	Media	Plant growth regulators (mg.l ⁻¹)		
		2,4-D	BAP	ANA
Leaf bases	CIL1	0.10	0.01	
	CIL2	0.10	0.05	
	CIL3	0.50	0.01	
	CIL4	0.50	0.05	
	CIL5	1.00	0.01	
	CIL6	1.00	0.05	
Root tips	CIR1	0.10	0.01	
	CIR2	0.10	0.05	
	CIR3	0.50	0.01	
	CIR4	0.50	0.05	
	CIR5	1.00	0.01	
	CIR6	1.00	0.05	
Immature inflorescences	CIIIn-1	0.10	0.01	
	CIIIn-2	0.50	0.01	
	CIIIn-3	1.00	0.01	
	CIIIn-4	1.00		
	CIIIn-5	2.00		
	SIIIn-1		0.50	
	SIIIn-2		1.00	
	SIIIn-3		2.00	
	SIIIn-4		3.00	
	SIIIn-5			
	RIIIn-1			
	RIIIn-2			0.50
	RIIIn-3			1.00
RIIIn-4			2.00	

acclimated under greenhouse conditions in 1 liter pots (70:30 soil:sand mixtures) for approximately 30 days, and then transferred to 20 l plastic pots until maturity.

The frequency of callus induction, callus with shoots, and shoots with roots per Petri dish were estimated for each explant type. Differences between media were established by using a χ^2 homogeneity test ($p < 0.05$) (Infostat, 2001).

Histological analysis

Leaves, immature inflorescences, and callus were fixed in a solution of formaldehyde, ethyl alcohol 70%, glacial acetic, and water (30:50:5:15) (FAA) and included in paraffin. Cross and longitudinal sections (18-20 μm) were obtained using a rotator microtome and slices were stained with Safranin-Fast Green (Johannsen, 1940; Dizeo de Strittmater, 1979). Observations were carried out by using a Leica microscope (Model 1349522).

Lignin content determination

The lignin content of leaves from control and experimental plants were determined following the protocol described by Van Soest (1963) modified by Shults *et al.* 1976. Samples were collected and dehydrated until constant weigh in an oven at 80 °C. Each sample was analyzed with at least 3 independent replications. Averages lignin content of controls and experimental plants were compared with ANOVA and Duncan test at $p < 0.05$ (Infostat, 2001). Controls were obtained from caryopses and had the same chronologic age that plants obtained *in vitro* (4 months).

Samples were taken simultaneously from control plants (derived from seeds, physiological age equal to the *in vitro* plants) and *in vitro* regenerated plants growing in greenhouse. For the analysis, was cut the aerial part of the plants from about 10 cm above the remnant left by the cattle. The sheets were dried in oven at 70°C and chopped by a grinder. Then 0.35 g of each sample was placed in a bottle type beaker and added 35 ml FDA solution (sulfuric acid + 20g CTBA cetyltrimethylammonium bromide), was decanted and boiled for 60 minutes in an aluminum block. Subsequently suction filtered into a pyrex crucible of porosity No. 1 or 2 tared, the residue washed several times with hot water for 3 hours was placed in solution with 72% H_2SO_4 , was filtered again with suction and washed repeatedly with hot water to remove excess acid. Was placed in oven at 65° and weighed.

The determination of percent dry weight of lignin was calculated from the following equation:

$$\% \text{ de FDA} = (P2 - P1 * 100) / PM$$

where:

% FDA: Percentage of lignin

P1 = Weighing of the crucible

P2 = weight of dry sample in the crucible

PM = Weight of sample

Results

Explant responses to callus induction media and *in vitro* plant regeneration

Only 7 days-old leaf basal segments and immature inflorescences responded to callus formation. The rest of the explants (basal segments of old leaves and root tips sections), consistently showed no response in any of the callus-induction media tested and after 30 days in culture they appeared completely necrotic without visible growth capacity.

Leaf-basal-segments of 7 days-old leaves showed cell proliferation and callus formation after the first week of culture. Cell proliferation started at the explants borders and after 30 days, compact and light-yellow callus were observed. The percentage of young leaf-basal segments producing callus was relatively low, although significant differences ($p < 0.05$) between culture media were detected (figure 1). The best callus induction medium was CIL4 (MS basal salt plus 0.50 mg.l^{-1} 2,4-D and 0.05 mg.l^{-1} BAP) in which 13 % the explant responded to callus formation. When sections of callus were transferred to regeneration media (table 1) and incubated under fluorescent light, none of them showed shoot or root production though in some cases the presence of green points on callus surface was observed.

Immature inflorescence showed callus formation after 7 days of culture by initiating cell proliferation around the buds of the immature rachis. The best callus induction medium resulted CILn-1 (MS + 0.1 mg.l^{-1} NAA + 0.01 mg.l^{-1} 2,4-D), showing 48 % of the explants with cell proliferation (figure 2). Callus derived from immature inflorescence appeared light-yellow and compact, as callus from basal segments of 7 days-old leaves. After transferring to shoot induction media, all of them developed shoots. The best media was SIIIn-1 where the 30 % of the callus showed shoots (figure 3). Although most of the *in vitro* regenerated plants showed no evident morphological difference with natural *S. argentinensis* plants, chlorophyll-deficient (albino) shoots were observed.

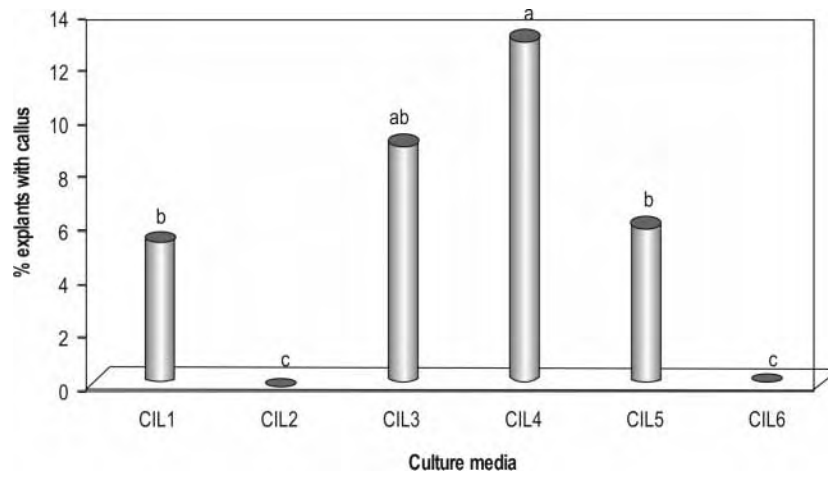


Figure 1: Percentage of callus differentiation from 7 days-old leaf-basal segments of *S. argentinensis* after 30 days in media culture. Bars with the same letter show no significant differences ($p < 0.05$).

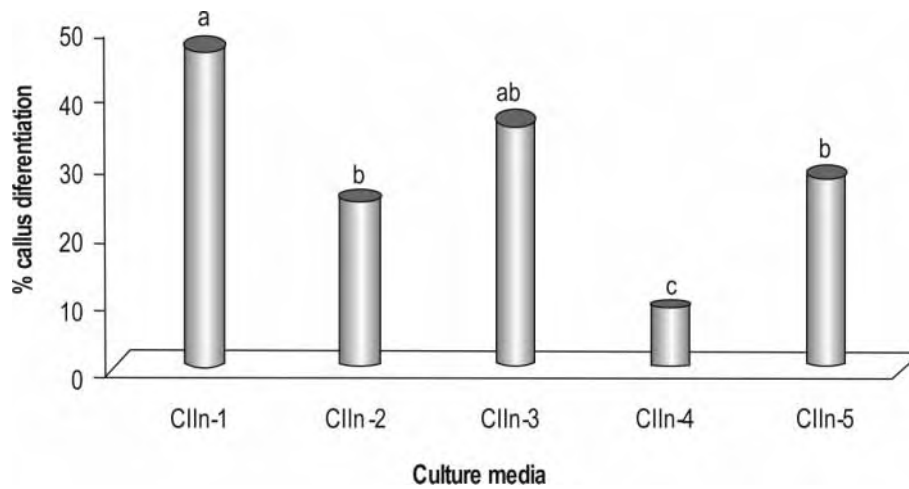


Figure 2: Percentage of callus induction from immature inflorescence explants. Bars with the same letter show no significant differences ($p < 0.05$).

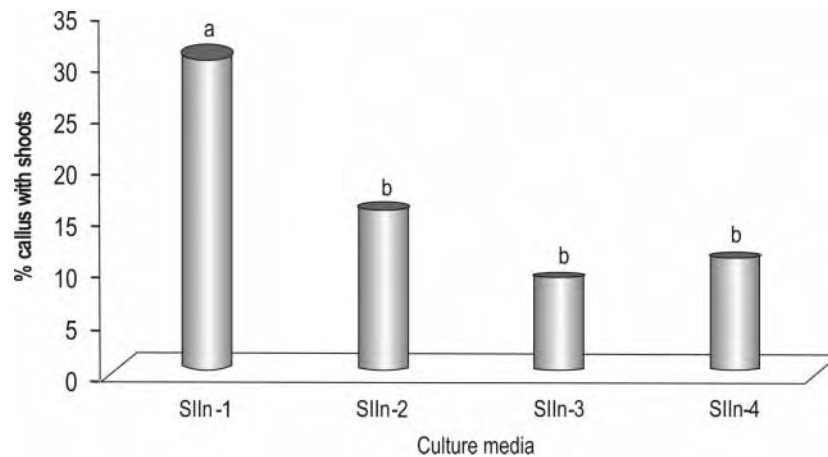


Figure 3: Percentage of immature inflorescence callus with shoots after 30 days in shoot induction media. Bars with the same letter show no significant differences ($p < 0.05$).

After transferring the shoots to root-induction media, roots primordial were observed after one week of culture. The best root-induction media was RRIIn-2 where 42 % of shoots produced roots. Moreover, about the 18.8 % of the shoots develop roots in the absence of NAA (figure 4). *In vitro* regenerated plants transferred to soil and kept under greenhouse conditions developed panicles and set seeds. The whole regeneration process lasted 4 months.

Histological analysis

Histological observations of explants after 7 days of culture showed that leaf basal segments displayed proliferation of meristematic large-nucleated cells, surrounded by less rounded parenquimatous cells and initials of vascular bundles. Leaf and shoot primordia were detectable in callus after 21 days in culture and at day 31 vascular tissues and organized shoot were distinguished. No evidences of somatic embryos were detected in the samples analyzed. Histological observation of callus derived from immature inflorescences performed at 7, 15 and 30 days of cultivation showed some organized sectors with proliferation of isodiametric cells, without formation of embryos or embryogenic zones. After 15 days shoot primordia were detected.

Lignin content determination of control and *in vitro* regenerated plants

S. argentinensis control plants showed on average 9.5 % w/w lignin content with a range form 8 to 11 %

w/w. Analysis of regenerated plants showed that this range was notably increased, with values ranging from 4.5 to 33.1 % (figure 5). This outcome indicates that variants in lignin content were recovered under the assayed tissue culture condition.

Discussion

S. argentinensis is a well adapted species for low and flooded areas of Santa Fe Province, Argentina that was historically used as low-impute forage resource. However, due to its poor forages quality (mainly because to high lignin content in leaves) it limits the cattle production of marginal areas. On the other hand, conversion of lignocellulosic biomass into bioethanol is becoming more than a theoretical possibility due to economical and environmental concerns as well (Tilman *et al.*, 2009). Grasses including species of the *Spartina* genus, are considered suitable for bioethanol production (Sticklen, 2006; Boe and Lee, 2007; Feldman *et al.*, 2011). Most of these C4 grasses are not domesticated species (Sang, 2011) and have several concerns related with high lignin content and the wide range of biomass production (Boe and Lee, 2007; Boe *et al.*, 2009; Gonzalez-Hernandez *et al.*, 2009).

In this work we tested several explants and culture media for developing an *in vitro* culture procedure for efficient and reproducible plant regeneration. Regenerated plants could then be used for both basic and applied researches leading to improve quality for cattle raising or bioethanol production by either traditional or biotechnological procedures.

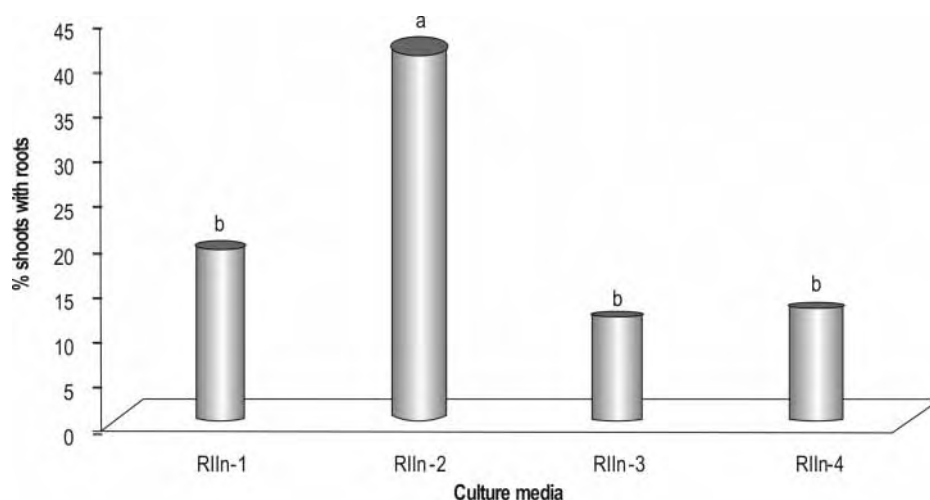


Figure 4: Percentage of shoots from immature inflorescences which differentiated roots when placed in root induction media RIIIn-1; RIIIn-2; RIIIn-3; and RIIIn-4. Bars with the same letter show no significant differences ($p < 0.05$).

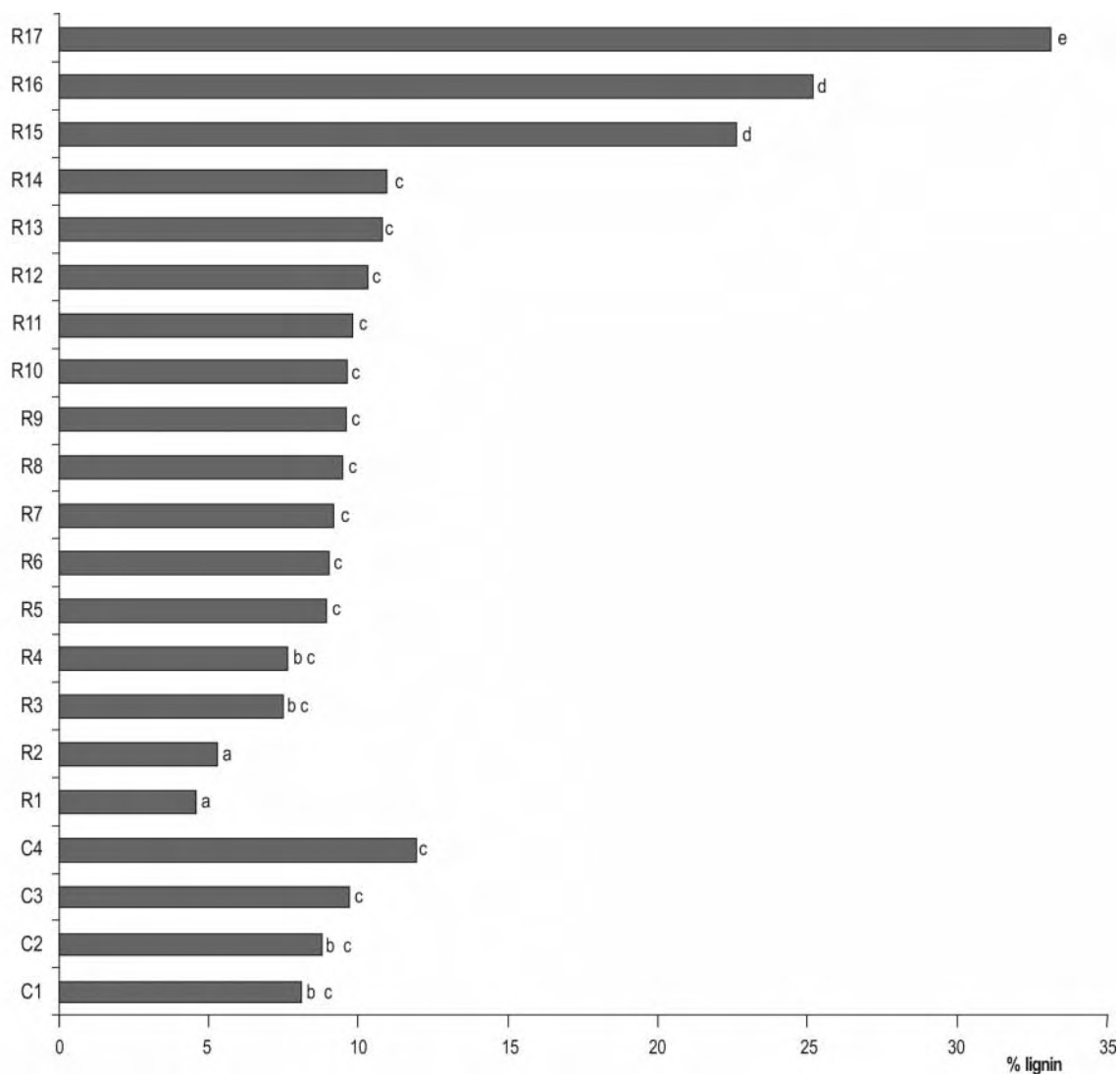


Figure 5: Leaf lignin concentration (%) of control (C) and *in vitro* regenerated plants (R). Bars with the same letter show no significant differences ($p < 0.05$).

Several *Poaceae* species have been considered recalcitrant to tissue culture procedures due to the impossibility of obtain *in vitro* plant regeneration. However, the combined use of 2,4-D and BA reverted this problem in many species. Most protocols recommend the using 1.0-5 mg.l⁻¹ of 2,4-D and 0.01-0.1 mg.l⁻¹ of BA. Moreover, several authors considered that BA should not be included for callus induction media, as well as 2,4-D may be reduced or completely excluded for shoot regeneration and NAA used for root differentiation (Vasil and Vasil, 1981). Thus, the concentration of growth regulators used in this work agree with these antecedents and indicate that a combination of 2,4-D and BAP for callus induction media followed by a transference to a medium supplemented only with BAP allowed shoot regeneration form callus of immature inflorescences. More than 40 % of the callus

transferred to regeneration media developed shoots in these conditions.

Numerous explants were reported useful for *in vitro* plant regeneration of *Poaceae*. Roots were often mentioned as easy explants available during the whole year for initiating the cultures and have been used in different species such as *Calamus manan* (Goh *et al.*, 1999), *Eragrostis curvula* (Echenique *et al.*, 2001), and *Glycine max* (Bueno *et al.*, 2004). Nevertheless, in this experimental system root tips were unable to respond to callus induction. On the other hand, several authors succeeded in regenerating plants using basal leaf segments, *i.e.* Conger *et al.*, 1998, (*Dactylis glomerata*), Jeoung-Mee *et al.*, 1998 (*Tripsacum dactyloides*), Poli *et al.*, 1989 (*Ranunculus serbicus*), and Ramgareeb *et al.*, 2001 (*Cynodon dactylon*). However, under the

conditions used in this experiments, basal segments of leaves were unable to regenerate shoots, coincident with previous reports of Li *et al.*, 1995, and Li and Gallagher, 1996 (*S. patens* and *S. cynosuroides*, respectively) and Wang *et al.*, 2003 (*S. alterniflora*)

It found that a combination of 2,4-D in callus induction media followed by the transference of callus to a medium supplemented only with BAP allowed the shoot regeneration from immature inflorescences. For *S. argentinensis*, immature inflorescences were the best explant tested. This could probably due to the high proportion of meristematic tissue it bears (Roca and Mroginski, 1991; Straub *et al.*, 1992). According with the results of the present work, this explant is recognized as a very useful one for *Poaceae* (Wernicke and Brettell, 1980; Pareddy and Petolino, 1990; Seliskar, 1998; Echenique *et al.*, 2001). The inclusion of ANA in the root-induction media improved the root regeneration from shoots. Interestingly very low concentrations were used, even lower than the concentration reported by Li *et al.*, 1995, for *S. patens*.

Histological analysis showed that plant regeneration was via organogenesis, similarly to the described in *S. patens*, *S. cynosuroides* (Li *et al.*, 1995; Li and Gallagher, 1996, respectively), and *S. alterniflora* (Wang *et al.*, 2003). Using the same explant, plant regeneration via somatic embryogenesis was reported in other species of *Poaceae*, such as *Zea mays* (Pareddy and Petolino, 1990), *Panicum virgatum* (Dutta Gupta and Conger, 1999; Odjacova and Conger, 1999), and *Eragrostis curvula* (Echenique *et al.*, 2001); whereas in *Bouteloua gracilis* both, embriogenic and non-embriogenic regeneration was observed (Agudo-Santacruz *et al.*, 2001).

A high variability in the lignin content was detected between regenerated plants. Particularly, the group of plants in which the lignin content was significantly lower than controls demonstrate the possibility to obtain new material for plant breeding. The protocol described here can be also use for further projects in order to obtain somaclonal variants with low lignin content or other genetic variations as well as it can be used as a regeneration protocol for other wetland grasses.

All procedures, starting from explant extraction until transferring *in vitro* regenerated plants to pots with soil, took about 4 months. Thus, *S. argentinensis* can be successfully regenerated *in vitro* using immature inflorescences as an explant and, as far as we know, this is the first protocol informed. This protocol can be used for screening large numbers of *in vitro* regenerated plants in order to detect somaclonal variation useful for basic and applied research in the species.

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