ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Obtaining new germplasm in *Cenchrus ciliaris* L. through induced-mutation and *in vitro* selection

Obtención de nuevo germoplasma en *Cenchrus ciliaris* L. a través de mutaciones inducidas y selección *in vitro*

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Abstract. *Cenchrus ciliaris* L., a forage grass of wide distribution in the north-west of Argentina, is a tetraploid (4x = 36) and obligate apomictic species. One way of obtaining novel germplasm is by induced mutations. In this work, physical and chemical mutations are combined with *in vitro* selection procedures seeking for new germplasm, with emphasis on salinity and drought tolerance. Mature seeds of *Cenchrus ciliaris* L. cv Biloela were subjected to treatments with X rays (400 Gy) and ethyl methanesulphonate (EMS) water solution (5.5 mM for 24 h). To perform *in vitro* selection, after 7 days of EMS or X rays treatments, germinated seeds were transferred to tubes containing Murashige and Skoog medium supplemented with NaCl or mannitol to simulate salinity and drought conditions, respectively. Fifty-four selected plants were isolated which tolerated 200 mM NaCl and 100 mM mannitol. Both mutagenic agents exhibited similar percentages of induced genetic variation measured through RAPD polymorphisms. This work demonstrated that genetic variability can be generated in *Cenchrus ciliaris* L. using mutagenic agents and *in vitro* selection.

Keywords: *Cenchrus ciliaris*; Genetic improvement; Induced mutations; *In vitro* selection; RAPDs analysis.

Resumen. *Cenchrus ciliaris* L., una forrajera subtropical de amplia distribución en la zona noroeste de Argentina, es un especie tetraploide (4x = 36) y apomíctica obligada. Una forma de obtener nuevo germoplasma es mediante mutaciones inducidas. En este trabajo, mutaciones físicas y químicas se combinaron con técnicas de selección *in vitro* a fin de obtener nuevo germoplama, con énfasis en tolerancia a salinidad y sequía. Semillas maduras de *Cenchrus ciliaris* L. cv Biloela fueron tratadas con rayos X (400 Gray) y con una solución de etil metano sulfonato (5,5 mM EMS durante 24 h). Para realizar la selección *in vitro*, las plántulas que germinaron 7 días después de los tratamientos mutagénicos fueron transferidas a tubos que contenían medio basal Murashige y Skoog, suplementado con NaCl o manitol para simular condiciones de salinidad y sequía, respectivamente. Se obtuvieron cincuenta y cuatro plantas selectas que toleraron 200 mM NaCl y 100 mM de manitol. Ambos agentes mutagénicos exhibieron similares porcentajes de variación genética medida a través de RAPDs. Este trabajo demostró que es posible generar variabilidad genética en *Cenchrus ciliaris* L. mediante el uso de agentes mutagénicos y selección *in vitro*.

Palabras clave: *Cenchrus ciliaris*; Mejoramiento genético; Mutaciones inducidas; Selección *in vitro*; RAPDs.

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Recibido / Received 3.IX.2010. Aceptado / Accepted 16.IV.2011.

INTRODUCTION

Cenchrus ciliaris L. is a subtropical grass widely used for cattle production in semiarid and arid regions in Argentina (Ozias-Akins, 2006; Miles, 2007). Most forage and some turf grasses in these areas are grown on marginal lands under stressful environments with minimal inputs (Zhang et al., 2006). Conventional genetic improvement of *C. ciliaris* is met with reproductive and breeding barriers (i.e., its allopolyploid and apomictic nature), which limits the possibility of selecting and obtaining new promising cultivars. Hence alternative means to increase genetic variability are desirable (Flowers, 2004; Joseph et al., 2004). One system of obtaining a novel germplasm is by using non-conventional methods, as induced mutations. According to the FAO/IAEA database, there are over 2300 officially released mutant varieties in 59 countries (Ahloowalia & Maluszynski, 2001; Ahloowalia et al., 2004; Joseph et al., 2004). The most common mutagens used in enhancing genetic resources and developing improved cultivars of cereals, fruits and other crops are ethyl methane sulphonate (EMS), X-rays and gamma rays (Ahloowalia & Maluszynski, 2001; Ahloowalia et al., 2004; Joseph et al., 2004; Jain, 2005). EMS is a chemical mutagen of the alkylating group that has been widely utilized. This is because it can cause high frequency of gene mutations and low frequency of chromosome aberrations (Rocha Latado et al., 2004; Jain, 2005; Luan et al., 2007). On the other hand, the advantages of physical mutagens are accurate dosimetry and reasonable reproducibility, and high and uniform penetration of multicellular systems (Ahloowalia et al., 2004; Jain, 2005).

Induced mutation techniques provide a simple, efficient, rapid and cheap method to alter the genetic make-up and obtain desired genotypes from otherwise well-adapted genotypes. Because of the wide mutant spectrum induced by applying mutagenic agents, it is necessary to use appropriate techniques that enable to recover and characterize desirable genotypes. In the search of individuals of improved behaviour under abiotic stress, selection methods are conducted under field and/or hydroponic conditions, and are based on parameters related to production under control versus stress conditions (Munns et al., 2002; Verlues et al., 2006). The complex nature of these analyses along with the difficulty to test large populations has promoted the use of other types of methods, such as *in vitro* selection (Bajji et al., 2004; Carretero et al., 2007). It has the advantage of being a rapid and efficient technique for *screening* numerous individuals in a small space and a short period, and enables to incorporate selection agents into the culture media (Dziadczyk et al., 2003). The combined use of induced mutations with *in vitro* selection methods has great potential in breeding programs (Carretero et al., 2007; Dasgupta et al., 2008; Dörffling et al., 2009). However, there are not previous studies on the use of these techniques in *Cenchrus ciliaris* L.

Mutagenesis-induced variations can be distinguished at the morphological, physiological, biochemical and molecular levels with several techniques (Ahloowalia & Maluszynski, 2001). Random amplified polymorphic DNA (RAPD) (Williams et al., 1990) analysis has been successfully used to assess and to determine genetic diversity and/or similarity among individuals between and within species (Carvalho et al., 2004; Hofmann et al., 2004). RAPD procedures have been employed in analyzing somaclonal variation and detecting genetic variation induced by chemical and physical mutagens in several species (Hofmann et al., 2004). Therefore, the objective of this work was to evaluate whether the combination of induced mutations through chemical and physical agents and *in vitro* selection could be a useful approach, within the framework of a genetic improvement program, to increase genetic variability in *Cenchrus ciliaris* L. with emphasis on salinity and drought tolerance.

MATERIALS AND METHODS

Mutagenic treatment and *in vitro* selection. Mutagenic treatments were applied on mature seeds of *Cenchrus ciliaris* L. cv Biloela. Approximately 500 seeds were treated with a dose of 400 Gray (Gy) of X-rays at the E. A. Favret Genetic Institute, Buenos Aires, Argentina. The treatments with mutagenic agents were carried out at the Institute of Plant Pathology and Physiology, Córdoba, Argentina. Both Institutes belong to the National Institute for Agropecuarian Technology (INTA). The 500 seeds were then treated with a water solution of 5.5 mM ethyl methane sulphonate (EMS) (Sigma-Aldrich) during 24 h (semi-lethal doses were previously determined). After the chemical treatment, seeds were washed under distilled water. A sample of 100 seeds was soaked in distilled water for the same period to serve as control. Treated seeds were thereafter disinfected with 30% commercial bleach (NaClO 55 g/L) for 20 min, and placed in test tubes (15 mL) containing 4 mL of MS basal medium (Murashige & Skoog 1962), with 7 g/L of agar and 3% sucrose (w/v). The medium was sterilized by autoclaving for 20 min at 121 °C. The pH was adjusted to 5.8 with 0.1 N HCl or 0.1 N NaOH prior to autoclaving. To perform *in vitro* selection, after 7 days of EMS or X rays treatments, germinated seeds were transferred to tubes containing Murashige & Skoog medium (1962). This medium was supplemented with NaCl or mannitol to simulate salinity and drought conditions, respectively. Seedlings were sequentially subcultured every 20 days, starting from an initial concentration of 50 mM NaCl or mannitol, and increasing the dose by 50 mM in subsequent subcultures until a concentration of 200 mM. Culture conditions were 26 °C ± 2 °C, with photoperiod of 16 h light (7-11 $\mu mol/m^2s$) and 8 h darkness. Plants obtained were transplanted to pots and maintained in the greenhouse for hardening.

Molecular analysis by RAPD. Samples from young leaves of putative mutants and the original cultivar (cv Biloela) were collected for DNA analyses. Fresh leaves (100 mg) were

ground with nitrogen to perform DNA extraction following the protocol for Nucleon PhytoPure DNA Extraction Kit (GE, Healthcare). DNA was quantified with a spectrophotometer (ND1000, Nanodrop Technologies, Inc., USA). Twenty different arbitrary decamer primers of series A and B of Promega were used to explore polymorphisms in the study plants (Table 1). The reaction mixtures were conducted following Gustine et al. (1996), with modifications for *Cenchrus ciliaris*, in a final volume of 30 μL containing 25 ng of genomic DNA, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 0.1 mM of each dNTP, 0.2 mM of primer and 1 unit of Taq polymerase (GE, Healthcare). Amplification reactions were performed according to the program proposed by Promega: 1 cycle of 3 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 36 °C, with an increment of 0.6 °C/s, 2 min at 72 °C, and finally 1 cycle of 5 min at 72 °C. Three replicates of each PCR were performed for each putative mutant and the control genotype, cv Biloela. The amplification products were separated by electrophoresis in 2% agarose gel (w/v) in Tris-Borate-EDTA 1X (TBE). The gels were stained with ethidium bromide (10 mg/mL, GIBCO BRL) and visualized under ultraviolet light and analyzed with image analyzer. The differences in banding patterns generated between each putative mutant obtained from the cv Biloela and the control cv Biloela were determined by comparison on the gel using the marker 10000 bp DNA Ladder (Promega). All reactions were repeated at least twice, and only neat and reproducible bands were considered for analysis.

Table 1. Sequences of the primers, total number of amplified fragments and number of polymorphic bands generated by RAPD markers in *Cenchrus ciliaris* L. cv Biloela and selected plants (* Non-reproducible band pattern).

Tabla 1. Secuencias de los cebadores, número de fragmentos amplificados y número de bandas polimórficas amplificadas por marcadores RAPD in *Cenchrus ciliaris* L. cv Biloela y plantas seleccionadas (* Patrones de bandeo no reproducibles).

Primers	Sequence (5' to 3')	Number of amplified fragments	Number of polymorphic bands
A1	CCCAAGGGAA	4	0
A2	GGTGCGGGAA	7	4
A3	AAGACCCCTC	8	1
A4	CTTCACCCGA	4	0
A5	CACCAGGTGA	*	*
A6	GAGTCTCAGG	4	0
A7	CCCGATTCGG	*	*
A8	ACGCACAACC	8	0
A9	CTAATGCCGT	2	0
A10	ACGGCGTATG	2	3
B1	TCGAAGTCCT	*	*
B2	GCATGTCAGA	4	3
B3	ACTTCGACAA	1	0
B4	TGCCATCAGT	*	*
B5	GCGCTCACGC	*	*
B6	GTGACATCGC	*	*
B7	AGATCGAGCC	4	0
B8	TCACCACGGT	4	0
B9	ATGGCTCAGC	*	*
B10	CAGGCACTAG	8	0
Total		**60**	**11**

RESULTS AND DISCUSSION

Mutagenic treatment and *in vitro* selection. Physical and chemical mutagens have successfully been used to produce new germplasm in several cultivars (Ahloowalia et al., 2004; Jain, 2005; Luan et al., 2007; Basu et al., 2008). Likewise, mutation breeding is an effective tool in plant breeders, especially in crops, as they have a narrow genetic base. In this work, four groups of selected materials were obtained through the combined use of induced mutation techniques and *in vitro* selection procedures.

1) Ethyl methanesulfonate (EMS) – Salinity (NaCl). Seedlings obtained from seeds were exposed to EMS treatment and selected *in vitro* with NaCl. Twenty-one putative mutants that tolerated up to 200 mM NaCl were obtained.

2) Ethyl methanesulfonate – Drought (mannitol). Seedlings grown from seeds treated with 5.5 mM de EMS, that were in the selection stage with mannitol: only 5 tolerated up to 100 mM of that agent.

The 26 selected plants obtained from seeds treated with EMS were transferred to the soil in a greenhouse, and no morphological variations were observed.

3) X rays – Salinity (NaCl). Seedlings obtained from seeds were exposed to X- rays treatment and selected *in vitro* with NaCl: 20 plants that tolerated 200 mM NaCl were obtained.

4) X rays – Drought (mannitol). Seedlings grown from seeds irradiated with 400 Gy, that were in the selection stage with mannitol: only 8 tolerated a concentration of 100 mM of that agent.

In the present investigation, X-rays were found to be more effective in inducing morphological alterations. Phenotypic variations observed in plants grown from seeds treated with X rays included dwarfism-short intervals among leaves-rosetting, aberrant leaf morphology and leaf curling (Fig. 1 A, B, C). However, several authors have concluded that both physical and chemical mutagens were found to be effective in inducing a broad spectrum of viable and productive mutants (Donini & Sonnino, 1998; Ahloowalia & Maluszynski, 2001; Rekha & Langer, 2007).

Fig. 1. Phenotypic variations observed in plants grown from seeds treated with X rays. A: Short leaves and internodes forming an apical rosette. B: Aberrant morphology of the leaf lamina. C: Leaf curling.
Fig. 1 Variaciones fenotípicas observadas en plantas obtenidas a partir de semillas tratadas con rayos X. A: Hojas y entrenudos cortos formando una roseta apical. B: Morfología aberrante en hojas. C: Hojas enroscadas.

Table 2. Total number of selected plants grown from seeds of *Cenchrus ciliaris* L. cv Biloela, number of plants that exhibited polymorphism, and percentage variation observed using EMS or X-ray treatments.
Tabla 2. Número de plantas seleccionadas obtenidas a partir de semillas tratadas con EMS o rayos X de *Cenchrus ciliaris* L. cv Biloela, número de plantas que mostraron polimorfismo, y porcentaje de variación observada para los tratamientos con EMS y rayos X.

Treatment	Total number of obtained plants	Number of plants with polymorphism	Genetic variation observed (%)
EMS (NaCl and mannitol)	26	5	19.2
X rays (NaCl and mannitol)	28	5	17.8
Total	**54**	**10**	**18.5**

Molecular analysis by RAPD. The 54 selected plants, obtained after the procedures described above, and the control cv Biloela generated highly reproducible patterns, with multiple discrete bands when they were amplified with 13 of the 20 primers of the A and B series of Promega (Table 1). These 13 primers yielded a total of 60 bands with an average of 4.61 scorable bands per primer. Primer A2 amplified the largest number of 11 bands, while primer B3 amplified the lowest number of 1 band (Table 1). Of the 60 amplified bands, 11 corresponded to polymorphic fragments (18.3%) detected with the primers A2, A3, A10, and B2 (Table 1). These fragments exhibited a length that ranged between 1500 bp and 2500 bp.

Variation in the amplification patterns with respect to control cv Biloela was observed in 10 of the 54 plants analyzed, which corresponds to a genetic variation frequency of 18.5% (Table 2). Considering the type of mutagenic treatment, regardless of the selection agent used, a genetic variation of 19.2% was obtained for EMS (5 plants presented polymorphism); for X rays, genetic variation was 17.8% (Table 2). Thus, both mutagenic agents exhibited similar percentages of induced genetic variation measured through RAPD polymorphisms (Table 2). This conclusion is in agreement with some other authors (Donini & Sonnino, 1998; Ahloowalia & Maluszynski, 2001).

In two plants selected for tolerance to salinity derived from seeds exposed to EMS, the presence of an additional band for each of them was observed; this band was not present in the amplification patterns of the control, when amplified with the primer A2 (Fig. 2 A. Lanes 1 and 2). Regarding lane 1, the band present corresponded to approximately 1700 bp, whereas lane 2 was 1900 bp. Of the plants grown from seeds irradiated with 400 Gy, two of them selected for tolerance to salinity (Fig. 2 A. Lanes 7 and 9) showed a band that was not present in the control cv Biloela (T). This additional band approximately corresponded to 1900 bp. Regarding the profiles obtained with the primer A3, a plant derived from the X-ray treatment and further selection with mannitol lacked a band of approximately 2500 bp, with respect to the control cv Biloela (T). (Fig. 2 B. Lane 10). For the primer A10, in the amplification profile of plants selected for salt tolerance, grown from seeds irradiated with 400 Gy (Fig. 3 A. Lanes 4 and 5), the presence of an additional band of approximately 1500 bp was observed. In the amplification profile obtained with primer B2, absence of a band of about 2000 bp was observed with respect to the control cv Biloela (Fig. 3 B. Lanes 1, 3, and 9). These plants were obtained from seeds exposed to EMS and X rays and further selected with mannitol.

All bands that were absent in the amplification patterns with respect to control Biloela (T) corresponded to genotypes that were selected for drought tolerance (either of seeds treated with EMS or X rays). On the other hand, all the additional polymorphic fragments respect to the control corresponded to genotypes that were selected for salt tolerance, independently of the primer used. Of 10 plants (18.5% of the 54 plants analyzed) that presented discriminant polymorphisms with respect to the control cv Biloela using RAPD technique, only 5 showed morphological modifications under *ex vitro* conditions; four of them corresponding to treatments with X rays and one with EMS.

Fig. 2. A: Gel electrophoresis of RAPD fragments generated by primer A2 of putative mutants selected for tolerance to NaCl. Lanes 1 to 6: plants grown from seeds treated with EMS; lanes 7 to 10: with X rays. B: Gel electrophoresis of RAPD fragments obtained with primer A3 of putative mutants selected for tolerance to mannitol. Lanes from 1 to 5: plants grown from seeds treated with EMS; lanes from 6 to 10: with X rays. Lane T: plants cv Biloela. Lane M corresponds to the size 10000 bp DNA Ladder. The arrows indicate polymorphic bands.

Fig. 2. A: Electroforesis en gel de fragmentos RAPD obtenidos con el cebador A2 en plantas seleccionadas por tolerancia a NaCl. Líneas 1 a 6: plantas obtenidas de semillas tratadas con EMS; líneas 7 a 10: con rayos X. B: Electroforesis en gel de fragmentos RAPD obtenidos con el cebador A3 en plantas seleccionadas por tolerancia a manitol. Líneas 1 a 5: plantas obtenidas de semillas tratadas con EMS; líneas 6 a 10: con rayos X. Línea T: plantas cv Biloela. Línea M corresponde al marcador de peso molecular de 10000 pb DNA Ladder. Las flechas indican las bandas polimórficas.

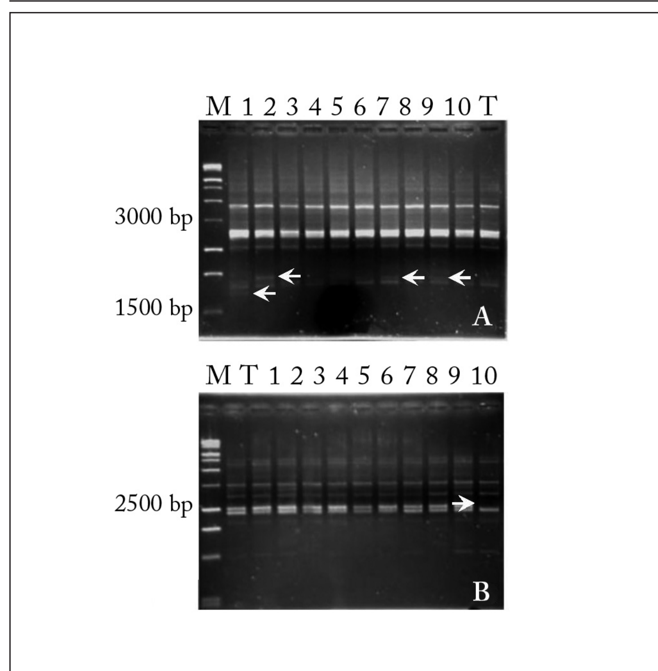
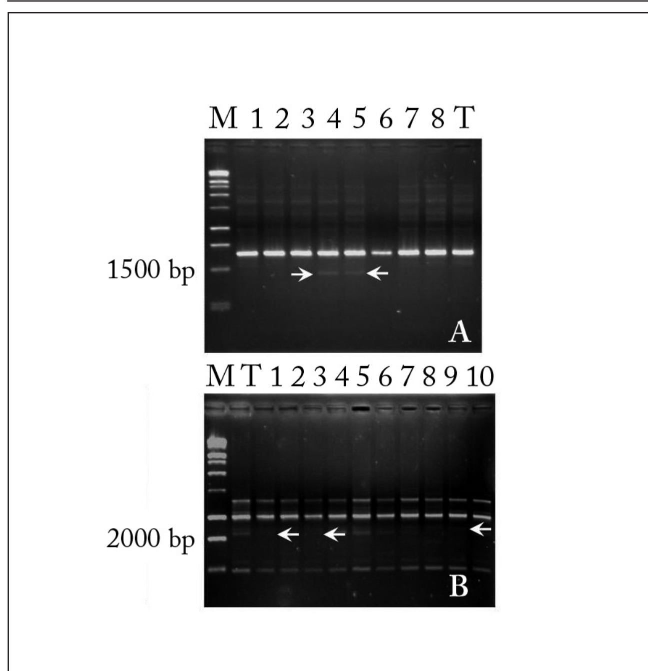


Fig. 3. A: Gel electrophoresis of RAPD fragments generated by primer A10 of putative mutants selected for tolerance to salt. Lanes from 1 to 8: plants grown from seeds treated with X rays. B: Gel electrophoresis of RAPD fragments generated by primer B2 of putative mutants selected for tolerance to mannitol. Lanes from 1 to 5: plants grown from seeds treated with EMS; Lanes from 6 to 10: with X rays. Lane T: plants cv Biloela. Lane M corresponds to the size 10000 bp DNA Ladder. The arrows indicate polymorphic bands.

Fig. 3. A: Electroforesis en gel de fragmentos RAPD obtenidos con el cebador A10 en plantas seleccionadas por tolerancia a salinidad. Líneas 1 a 8: plantas obtenidas de semillas tratadas con rayos X. B: Electroforesis en gel de fragmentos RAPD obtenidos con el cebador B2 en plantas seleccionadas por tolerancia a manitol. Líneas 1 a 5: plantas obtenidas de semillas tratadas con EMS; líneas 6 a 10: con rayos X. Línea T: plantas cv Biloela. Línea M corresponde al marcador de peso molecular de 10000 pb DNA Ladder. Las flechas indican las bandas polimórficas.



This work has demonstrated that genetic variability can be generated in *Cenchrus ciliaris* L. using mutagenic agents and *in vitro* selection. RAPD analysis showed 18.5% of genetic variation in the banding patterns of putative mutants with respect to the control cv Biloela. However, not all the plants analyzed were discriminated. Accordingly, some authors attribute the failure of RAPD to differentiate genotypes to the low portion of the genome that is covered by these markers (Carvalho et al., 2004; Hofmann et al., 2004). In addition, the large size of the *Cenchrus ciliaris* genome (1300 Mpb) (Gaut, 2002), is an important factor to consider.

The additional presence of bands in the mutants selected for tolerance to salinity, as well as their absence in plants selected for tolerance to drought, promotes an interest in recovering differential bands, cloning them and sequencing them; this will allow identification of the genome regions that have been modified as an effect of the induced mutations, the genes that are located in them (Palombi et al., 2007).

Our results encourage future works to determine whether the selection pressure used on EMS and X-ray treated plants under *in vitro* conditions was successful in obtaining mutants with desirable traits, and to test if the variation observed was the result of a true breeding and allow generation of plants of solid mutants (Nissim Amzallag, 2000; Peng & Zhang, 2009). To our knowledge, this is the first report of improvement in *Cenchrus ciliaris* through chemical and physical mutagenesis. All these obtained mutants have great potential to be incorporated in further breeding programmes for developing new cultivars with good forage productivity, quality, adaptability and/or tolerance to salinity and/or drought stress.

ACKNOWLEDGEMENTS

The author is grateful to the Instituto Nacional de Tecnología Agropecuaria (INTA) and the Fondo para la Investigación Científica y Tecnológica (FONCYT) -Agencia Nacional de Promoción Científica y Tecnológica- for their financial assistance, and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for the Senior Research Fellowship. The author is also grateful to Ing. Agr. (M. Sc.) Daniel Díaz and Dr. Liliana Di Feo for critical reading of this manuscript.

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