

Molecular characterization of accessions of crabgrass (*Digitaria nuda*) and response to ametryn

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ABSTRACT. *Digitaria* species are sugar cane crop weeds in Brazil and are being controlled with herbicides, although there are some reports of control failure, notably to the triazine group. Molecular techniques are recommended to analyze the genetic variability in weeds. RAPD (Random Amplified Polymorphic DNA), PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) and, in combination with sequencing, allow the localization of resistance genes, as well as possible mutations related to the onset of resistant individuals in some species. Thus, the objective of this work was to characterize ten accessions of *Digitaria* spp. by RAPD and PCR-RFLP markers, to sequence a conserved region of the *psbA* gene and evaluate the accessions response to ametryn. As showed by molecular analysis there was high genetic similarity among the accessions, all of them presented similar genetics profiles and were susceptible to ametryn.

Key words: *Digitaria*, *psbA* gene, RAPD, sequencing, triazine, weed.

RESUMO. Caracterização molecular de acessos de capim-colchão (*Digitaria nuda*) e resposta à ametrina. As espécies de *Digitaria* são plantas daninhas nas lavouras de cana-de-açúcar no Brasil e estão sendo controladas com herbicidas, porém há alguns relatos de falha no controle, notavelmente ao grupo das triazinas. Técnicas moleculares são recomendadas para análise da variabilidade genética em plantas daninhas, como o RAPD (DNA Polimórfico Amplificado ao Acaso) e PCR-RFLP (Reação da Polimerase em Cadeia – Polimorfismo no Comprimento de Fragmentos de Restrição) e, aliadas ao sequenciamento, permitem a localização de genes de resistência, bem como possíveis mutações relacionadas ao surgimento de indivíduos resistentes em algumas espécies. Com isso, o objetivo deste trabalho foi caracterizar dez acessos de *Digitaria* spp. por meio de marcadores RAPD e PCR-RFLP, sequenciar uma região conservada do gene *psbA* e analisar a resposta destes à ametrina. Pela análise molecular houve elevada similaridade genética entre os acessos, pois todos apresentaram perfis genéticos semelhantes e foram suscetíveis à ametrina.

Palavras-chave: *Digitaria*, gene *psbA*, RAPD, sequenciamento, triazina, planta daninha.

Introduction

The genus *Digitaria* includes approximately 300 species, distributed in tropical and subtropical regions of both hemispheres (CANTO-DOROW, 2001). Crabgrass (*Digitaria nuda*, *D. ciliaris* and *D. horizontalis*) are weeds that normally infect the sugar cane crop in Brazil, where they cause quantitative and qualitative reductions in the production. In the State of São Paulo, this genus is represented by 14 characterized species (CANTO-DOROW, 2001; DIAS et al., 2003) and the visual differentiation of these species in the field is difficult due to the morphological similarities among them (DIAS et al., 2003), which are basically differentiated by spike characteristics (DIAS et al., 2005a).

Thus, it is important that sugar cane farms make the correct identification of the species of crabgrass in its areas and use objective strategies of management for each one of the species (DIAS et al., 2007).

The management of weed in the sugar cane crop is carried out by combined chemical (application of herbicides) and mechanical (cultivation) methods. Presently, the usage of herbicides is an indispensable and common practice in sugar cane plantations, as a result of the extensiveness of the planted areas and the high cost of manual labor.

Among the registered herbicides for usage in the sugar cane crop in Brazil, those belonging to the triazine group stand out, especially ametryn, by the number of commercialized products, by the length

of time they have been commercialized and by their efficiency. Recently, escapes of *Digitaria* have been reported in cultivated areas treated with ametryn and other herbicides. Dias et al. (2005b) observed that populations of *Digitaria* spp. subjected to different treatments show tolerance indexes (GR_{50}). Thus, this plant has become one of the main weeds infecting sugar cane plantations, with chemical control becoming more problematic and uncertain, depending above all, on the *Digitaria* species that can show tolerance to triazines.

The triazines are herbicides that block the photosynthetic electron transport chain in Photosystem II by binding to the D1 protein and competing with plastoquinone Q_B for the binding site. The mechanism of resistance to triazines involves the loss in the ability of the herbicide molecule to bind to the specific D1 protein site (HOLT et al., 1993). The molecular basis of the resistance is a simple change in nucleotide pairs in the conserved region of the chloroplastic *psbA* gene that codifies the D1 protein, resulting in a substitution of the amino acid serine at position 264 for glycine, reducing the affinity of the herbicide for the binding site on the protein (TREBST, 1987; SINNING et al., 1989).

Thus, one of the objectives of integrated weed control management is the prevention of selection of biotypes resistant to herbicides. Therefore, it is vitally important that the presence of resistant biotypes to this herbicide be detected as early as possible so that management strategies can be established.

Thus, molecular biology provides an important ensemble of tools in the study of weeds. Research on genetic variability with the use of molecular markers, such as RAPD and PCR-RFLP, permit to estimate the genetic variability, among and within species, and serve as the starting point for studies to manage these plants, since, when combined with sequencing, they allow the localization of resistance genes as well as possible mutations related to the onset of resistant individuals in some species.

The detection of these molecular changes is of fundamental importance to use practical control techniques, such as chemical, since there may be differentiated responses by possible materials to the action of herbicides, increasing or decreasing the control efficiency. The aim of the present work was to characterize accessions of crabgrass (*Digitaria* spp.) by RAPD and PCR-RFLP markers, to sequence a conserved region of the *psbA* gene and evaluate the accessions response to ametryn.

Material and methods

Collection for accessions of crabgrass

Seeds of crabgrass (*Digitaria* spp.) were collected from several plants of the same population at ten different sampling points 2 km apart on average, and named 'accessions'. The harvest for accessions was made in commercial sugar cane production areas located in São Joaquim da Barra, São Paulo State, where there have been historical recordings of problems with chemical control. Subsequently, the seeds were planted in 1.5 L plastic pots containing a mixture of oxissol, sand and plant substrate (Plantimax HT, Eucatex) in a volume ratio of 3:1:1. These pots were maintained in a greenhouse for propagation, collection of leaves for molecular analysis, identification and herborization. The identification of the collected *Digitaria* plants was carried out following the analytical key proposed by Canto-Dorow (2009).

Agronomic efficacy of ametryn in the control of *Digitaria nuda*

To evaluate the susceptibility of the plants to ametryn, two assays were installed under the same conditions. Seeds of all accessions were planted in 500 mL plastic cups, containing a mixture of oxissol, sand and plant substrate (Plantimax HT, Eucatex), in a volume ratio of 3:1:1. The cups were maintained under greenhouse conditions with periodic irrigations. Thirty days after planting, when the plants reached on average four expanded leaves, thinning was carried out, leaving four plants per cup with five repetitions per accession. Two days after thinning, an application of ametryn was carried out at a dose of 2,400 g i.a. ha^{-1} , without the application of herbicide on the controls for each accession. The application was carried out with a CO_2 pressurized backpack sprayer equipped with four flare-type nozzles, 80.02 VS tips, a flow rate equivalent to 200 L ha^{-1} and a working pressure of 200 kPa.

The experimental design adopted was completely randomized with the five repetitions per treatment. The susceptibility of the plants to the herbicide was visually evaluated, and the evaluations were carried out at 7, 14, 21 and 28 days after application of the treatment (DAA).

Procedure for DNA extraction and analysis of the genetic diversity of *Digitaria nuda* by PCR-RAPD

The DNA was extracted from young leaves following the protocol described by Lodhi et al. (1994) and adapted for small scale in microcentrifuge tubes (Eppendorf type). The quantifications were carried out with a DU 640B

(Beckman) spectrophotometer and the final concentration used as working solution was 10 ng μL^{-1} . For the PCR DNA amplifications and the analysis by RAPD, the method described by Williams et al. (1990) was used. Twenty primers from 'The University of British Columbia – Nucleic Acid – Protein Service Unit' were utilized, whose collection access numbers as well as the respective sequences are described in Table 1. The amplification reactions were carried out in a volume of 20 μL , consisting of 2 μL of 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), MgCl_2 0.7 μL (1.75 mM), dNTP 0.4 μL (10 mM), Primer 3 μL (5 ng μL^{-1}), *Taq* DNA polymerase 0.4 μL (2 U), Milli-Q grade sterile water (q. s. p.) and 1 μL (10 ng) of the DNA samples. The amplification reactions were carried out in a MJ Research, Inc., PTC-100TM thermocycler and the program used for this stage of the analysis was: 2 min. at 94°C followed by 35 cycles of 1 min. at 94°C, 2 min. at 36°C, 2 min. at 72°C and, finally, 5 min. at 72°C to complete the amplification.

Table 1. Sequence of arbitrary oligonucleotide primers and their respective access numbers to collection.

Primers	Sequences	Primers	Sequences
212	GCT GCG TGA C	265	CAG CTG TTC A
231	AGG GAG TTC C	274	GTT CCC GAG T
234	TCC ACG GAC G	278	GGT TCC AGC T
239	CTG AAG CGG A	282	GGG AAA GCA G
240	ATG TTC CAG G	285	GGG CGC CTA G
249	GCA TCT ACC G	288	CCT CCT TGA C
253	CCG TGC AGT A	290	CCG CGA GCA C
254	CGC CCC CAT T	292	AAA CAG CCC G
256	TGC AGT CGA A	293	TCG TGT TGC T
258	CAG GAT ACC A	296	CCG CTG GGA G

The amplified products were separated by 1.5% agarose gel electrophoresis, visualized under UV light and stored in gel documentation system (Gel Doc 2000 Bio-Rad). The data resulting from the randomly amplified DNA was analyzed and a binary matrix was developed using the parameters 0 and 1 indicating absence and presence of the band, respectively. This binary matrix was converted into a distance matrix by the software Paup [4.0b10] (SWOFFORD, 2002) which was used for the construction of the phylogram. The phylogram was constructed by the distance method using the UPGMA (Unweighted Pair Group Method for Arithmetic Averages) algorithm with the software Mega 4.1 – Molecular Evolutionary Genetics Analysis (TAMURA et al., 2007).

Application PCR-RFLP technique for the *psbA* gene

The restriction analysis was carried out in a conserved region of the *psbA* gene, amplified with

the specific primers described by Cheung et al. (1993), with the following sequences: P1 (5' ATGAGGGTTACAGATTTGGTC 3') and P2 (5' AGATTAGCACGGTTGATGATA 3'). The amplification reactions were carried out in a volume of 20 μL , constituted of 2 μL of 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), MgCl_2 0.6 μL (1.5 mM), dNTP 0.5 μL (10 mM), primers 0.5 μL (1 pmol μL^{-1}), *Taq* DNA polymerase 0.3 μL (1.5 U), sterile water Milli-Q grade (q. s. p.) and 2 μL (20 ng) of DNA samples. The program used for this stage of the analysis was: 5 min. at 95°C followed by 40 cycles of 45 s at 94°C, 45 s at 55°C, 1 min. at 72°C and subsequently 7 min. at 72°C. To examine the success of the amplification, 5 μL of the products were taken to electrophoresis in a 1.5% agarose gel.

The restriction reactions were carried out in a volume of 10 μL , containing 4 μL (20 ng) of amplified product, 1 μL of 1X buffer (33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg mL^{-1} BSA), 0.2 μL (2 U) of the enzyme *MaeI* (Fermentas) and Milli-Q grade (q. s. p.) sterile water. The reactions were incubated for 3h at 45°C and subjected to electrophoresis in 5% polyacrylamide gel (PAGE) with 1X TEB buffer (Tris 89 mM, H_3BO_3 89 mM, EDTA 2.5 mM, pH 8.2). The gels were stained with ethidium bromide (0.5 $\mu\text{g mL}^{-1}$) and stored in gel documentation system (Gel Doc 2000 Bio-Rad). The sizes of the fragments were determined by comparison with a standard of known molecular weight (MassRulerTM DNA Ladder Low Range ready-to-use, by Fermentas).

Sequencing and analysis of the sequences

The amplified products were purified and subjected to sequencing reaction with dye-terminator and sequenced in the system ABI 3700 DNA Analyzer–Applied Biosystems. The obtained sequences were analyzed by Sequencing Analysis 3.4. The assemblies of sequences, the analysis of the chromatograms, as well as the visualization of the archives generated in FASTA format were generated by the software package 'Phred/Phrap/Consed' (GORDON et al., 1998). The alignment program ClustalW (<http://clustalw.genome.ad.jp>) was utilized to compare the generated sequences with those deposited in the GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>).

Results and discussion

Agronomic efficacy of the ametryn in the control of *Digitaria nuda*

Based on comparative morphological analysis, it was confirmed that all the accession under study

corresponded to *D. nuda*, which was reported for the first time in sugar cane cultivated areas in São Paulo State by Dias et al. (2003).

In the first bioassay, at 7 DAA, all accessions showed a control level above 90%. In the evaluation at 14 DAA, it could already be observed that accessions 10 and 14 showed replicates with re-sprouting, providing less than 50% of control, while the other accessions showed control above 90% in all replicates. At 21 and 28 DAA, this behavior was maintained, with accessions 10 and 14 showing replicates with 50% control, while the other eight accessions showed 100% control in all replicates.

In the second assay, the evaluation carried out at 7 DAA, it could be observed that all the accessions in all replicates showed a control level that varied from 80 to 100%, considered very good. At the evaluations carried out after 14 DAA, all accessions showed 100% control, not showing any re-sprouting until the end of the experiment.

For this reason, it can be inferred that ametryn at the 2,400 g i.a. ha⁻¹ provided efficient control of accessions of *D. nuda* studied in the present work. Dias et al. (2005a) tested the efficacy of herbicides in the control of *D. nuda* and found that the herbicides belonging to the triazines (ametryn), triazinones (metribuzin) and isoxazolinones (isoxaflutole) chemical groups were those that showed better levels of control.

In general, it can be affirmed that there was no tolerance in the accessions of *D. nuda* studied in the region of São Joaquim da Barra, state of São Paulo, since the replicates of the assay practically showed 100% control starting already from 14 days after application of the herbicide. The results of control escape in the first assay can be resultant of a difference of the phenological stage of the plants, surviving the oldest or in more advanced stages of development.

Analysis of the genetic similarity among the accession by RAPD

Forty-three primers were tested, but only twenty were selected for the analysis because of their reproducibility (Table 1). The band patterns generated by the RAPD reactions were clear. The twenty primers utilized allowed good amplification for all the samples of all accessions as seen in Figure 1. The fragments were amplified using the primers 249 and 290 which showed genetic similarity between the accessions, therefore, the majority of the bands obtained were monomorphic.

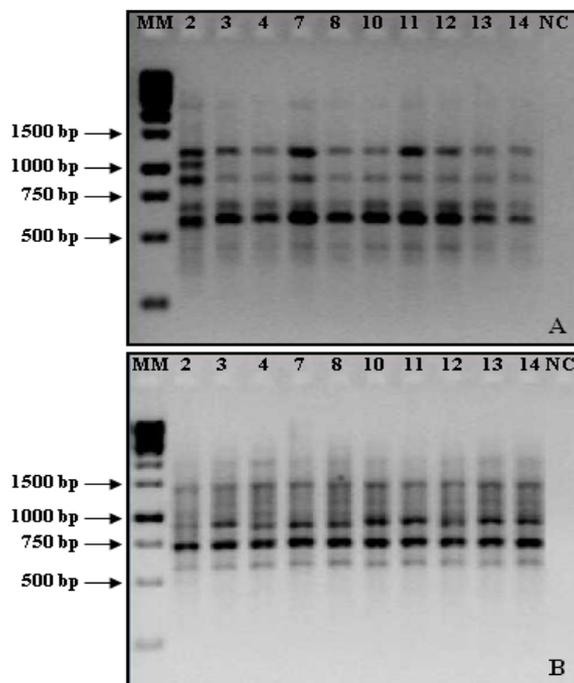


Figure 1. Amplification of the accessions of *D. nuda* with primers 249 (A) and 290 (B). MM = molecular marker (1 kb DNA Ladder); NC = negative control. The numbers above the channels refers to the accessions.

The average similarity among the accessions, inferred within the studied population of *D. nuda*, was equivalent to 98%, considered high, which is probably due to the autogamy of *Digitaria*. Similar results were also obtained in accessions of waterhyacinth (*Eichornia crassipes*) and waterlettuce (*Pistia stratiotes*), that showed high genetic similarity, due, probably to the vegetative reproduction of plants, that reduced the genetic recombination (CARDOSO et al., 2002; 2005).

From the values of genetic distance, a phylogram was obtained utilizing UPGMA as the grouping methodology (Figure 2).

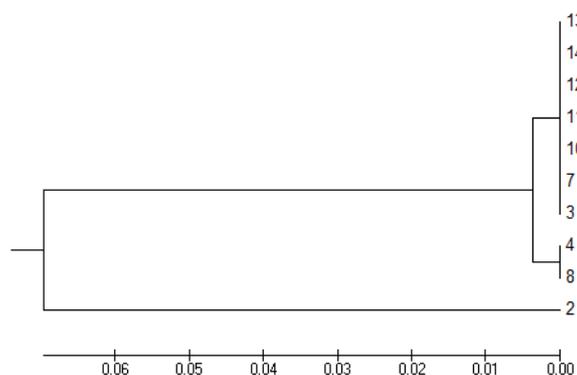


Figure 2. Phylogram of the genetic distance among the accessions of *D. nuda*, obtained by the genetic distance matrix. The numbers on the side refer to the accessions.

Two distinct groups were observed from the hierarchical grouping analysis. The first group show only one branching with Accession 2 and the second comprised of two subgroups, with one subgroup composed by Accessions 4 and 8, that have a genetic similarity of 99% with the second subgroup the other composed by seven accessions of *D. nuda*. Therefore, the analysis with molecular marker RAPD showed high genetic similarity among the accessions of *D. nuda* studied in this assay.

Analysis of the restriction fragments of the amplified products

The amplification using the primers P1 and P2 resulted in a single fragment of 267 bp (basis pair), in all of the studied accessions of *D. nuda*. The restriction of this product with the enzyme *MaeI* resulted in two fragments of DNA: 183 and 84 bp in all accessions studied (Figure 3).

In the present work, the obtained results were compared to the phenotypic response of *D. nuda* after ametryn application. The technique of the PCR-RFLP was adopted as a molecular diagnostic developed for the region of the *psbA* gene of other weed, including *Brassica napus*, *Chenopodium* spp. and *Amaranthus* spp. (CHEUNG et al., 1993).

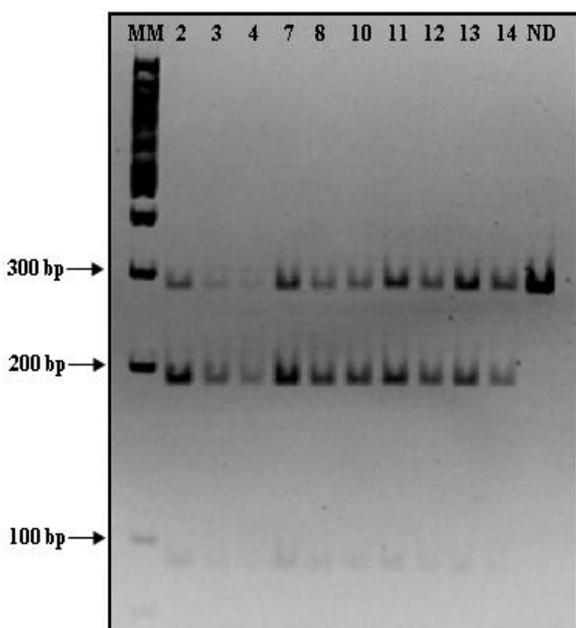


Figure 3. Product of the amplification of a conserved region of the *psbA* gene digested with the enzyme *MaeI*. MM = molecular marker (Mass Ruler™ DNA Ladder Low Range ready-to-use); ND = fragment not digested. The numbers above the channels refer to the accessions.

The restriction pattern of resistant genotypes of *Senecio vulgaris*, to which this type of analysis was also applied, differ from susceptible, since the enzyme

MaeI recognizes different restriction positions and generates fragments of different sizes (FREY et al., 1999). According to these authors, the resistance to triazine is achieved by a point mutation that eliminates a recognition site recognized by the enzyme *MaeI* at position 88, which results in two restriction fragments in individuals carrying this point mutation that confers resistance to triazine and in three fragments in those that lost this mutation.

As can be observed in Figure 3, a restriction profile of the amplified fragments for all accessions was similar to those obtained with *A. retroflexus* and *C. album* as described by Frey et al. (1999), with the presence of two fragments, 183 and 84 bp, characterizing all the accession as susceptible to the herbicide ametryn.

The results obtained in the present study with the restriction technique are in agreement with the field experiments which were carried out, since the pattern of bands generated by the restriction analysis are compatible with the susceptible phenotype, possible of being controlled by the herbicide.

Analysis of the sequenced amplicons

After PCR, the amplicons generated with the primers P1 e P2 for the ten accessions were subjected to sequencing. The obtained sequence of these amplification products was deposited in GenBank (EF633341). The amino acid sequence of the *psbA* gene of studied *D. nuda* was compared and aligned with the sequence of amino acids of the *psbA* gene of *Oryza sativa* (M36191) and *S. vulgaris* (AF061287), resulting in 100% similarity (Figure 4).

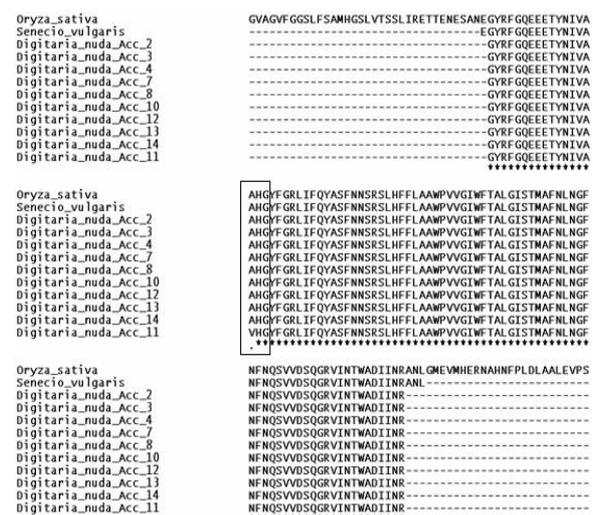


Figure 4. Alignment of the amino acid sequence of a region of the *psbA* gene of *D. nuda* having as reference the species *O. sativa* (M36191) and *S. vulgaris* (AF061287). The rectangle refers to the position where there was a change in amino acids.

An observed fact was the presence of a point mutation, that is, a SNP (Single Nucleotide Polymorphism) for Accession 11. This mutation occurred at position 48 of the nucleotides sequence of the fragment and was confirmed by four sequencing repetitions with quality 'Phred' 37 from different amplification products with the primer P2. This sequence was deposited in GenBank (EF633342).

This SNP altered the amino acid at position 16 of the fragment, substitution of one alanine codified by the codon GCT by one valine codified for GTT that underwent a base change (C-T), comparing with the amino acid sequences of other accessions (Figure 4), however, the substitution of an alanine for a valine does not alter the conformation of the protein (score zero).

Other SNPs associated with herbicide resistance were identified in weeds. Tian and Darmency (2006) identified a point mutation related to triazine resistance in higher plants, which caused a Ser₂₆₄ – Gly substitution that conferred resistance in *Setaria italica*. Park and Mallory-Smith (2006) analyzed the nucleotidic sequence in one bromoxynil-resistant *S. vulgaris* biotype and identified a point mutation which induced the amino acid substitution from asparagine to threonine at codon 266.

Therefore, the mutation observed in the accession 11, is not associated to ametryn tolerance, since it did not respond in a differentiated manner to the application of the herbicide. More detailed studies about the expression of this gene should be carried out to verify the influence of this mutation.

Conclusion

Based on the results obtained in the present study, it can be concluded that the crabgrass of the ten accessions collected in the region of São Joaquim da Barra, São Paulo State, belong to the species *Digitaria nuda*, and all presented high genetic similarity and susceptibility to ametryn at commercial rate.

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