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GENETIC VARIABILITY OF *Rottboellia cochinchinensis* POPULATIONS IN SUGARCANE FIELDS[1]

*Variabilidade Genética em Populações de **Rottboellia cochinchinensis** na Cultura da Cana-de-açúcar*

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ABSTRACT - The hypothesis assumed was the existence of biotypes within populations, which has been the cause of difficulties in itchgrass control by farmers. For that, the genetic variability of three populations of *Rottboellia cochinchinensis* in sugarcane fields in the state of São Paulo was investigated by using the Amplified Fragment Length Polymorphism (AFLP) technique. Six primers were used to obtain molecular characterization data. AFLP gels were analyzed based on marker presence (1) and absence (0). Using NTSYs (Numerical Taxonomy and Multivariate Analysis System) software, the genetic similarity was calculated by the Jaccard coefficient and, from that, a dendrogram was built through the UPGMA (Unweighted Pair Group Method Arithmetic averages) method, besides determining the isopolymorphic marks. The average genetic similarities seen in the region was 0.742 for Igarapava, 0.793 for Mococa and 0.808 for Piracicaba. Between regions it was 0.730 (Igarapava vs Mococa), 0.735 (Mococa vs Piracicaba) and 0.694 (Igarapava vs Piracicaba). In line with the dendrogram, it is possible to detect the formation of two groups, one with 8 plants from Igarapava and Mococa and the other with 21 plants from Igarapava, Mococa and Piracicaba, as well as the presence of 1 discriminant individual from Piracicaba. It can be concluded that the genetic similarity among itchgrass populations from the state of São Paulo was high (72%), which denotes that the difficulties in chemical management are not only due to different biotypes but also due to other characteristics linked to tolerance of the species to herbicides. However, biotype existence cannot be discarded because of the polymorphic marks generating 22% average genetic variability.

Keywords: *Saccharum* spp., itchgrass, chemical management, AFLP.

*RESUMO - Considerando a hipótese de que a dificuldade de controle observada pelos produtores com a espécie do gênero Rottboellia pode ser pela existência de biótipos nas populações, objetivou-se estudar a variabilidade genética entre três populações de **R. cochinchinensis** em regiões de cana-de-açúcar do Estado de São Paulo, utilizando-se da técnica Amplified Fragment Lenght Polimorfism (AFLP). Para a caracterização molecular, seis iniciadores foram utilizados para obtenção dos dados. Os géis de AFLP foram analisados com base na presença (1) e na ausência (0) de marcas. Utilizando-se o software NTSYs, foi calculada a similaridade genética pelo coeficiente de Jaccard e, a partir dele, construído o dendrograma pelo método UPGMA, além da determinação das marcas isopolimórficas. As semelhanças genéticas médias observadas nas regiões foram 0,742 para Igarapava, 0,793 para Mococa, 0,808 para Piracicaba, e entre as regiões foram 0,730 para (Igarapava x Mococa), 0,735 para (Mococa x Piracicaba), e 0,694 para (Igarapava x Piracicaba). Em consonância com o dendrograma, podemos observar a formação de três grupos, um formado por quatro indivíduos, sendo dois do local de Igarapava e de Mococa e outro pelo local Piracicaba e os demais indivíduos, com exceção do indivíduo um, de Piracicaba. Podemos concluir que a similaridade genética entre as populações de capim-camalote coletadas no Estado de São Paulo foi elevada (78%), o que evidência que o manejo realizado entre as populações estudadas devem ser similar. No entanto, não se podem descartar a presença de biótipos nas populações, como é sugerido pela presença de marcas polimórficas, detectadas pelo marcador AFLP, gerando 22% de variabilidade genética média.*

Palavras-chave: AFLP, capim-camalote, manejo, *Rottboellia cochinchinensis*, *Saccharum* spp., variabilidade.

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INTRODUCTION

Brazil is the largest producer of sugarcane, and genetic enhancement has been one of the biggest responsible for the advances in the development of superior cultivars, whether due to greater productivity, or due to adaptation to climatic changes (Landell and Bressiani, 2010). However, one of the obstacles to obtain greater productivity from the sugarcane fields is the control of weeds, which can be done correctly with the integration of several techniques, including the use of herbicides.

Rottboellia cochinchinensis, also known as Itchgrass, is a vigorous and prolific species, because a single plant can issue up to 100 tillers and produce more than 16,000 seeds (Smith et al., 2001). It is an aggressive species, with wide distribution and hard control, especially due to the lack of herbicides selective to the crop and efficient in control (Brasil, 2015).

Genetic variability is a characteristic of weed populations (Vidal et al., 2006), which, generally, can favor the appearance of new biotypes in the populations. This variability can contribute to several responses from weed populations to the application of herbicides (Circunvis et al., 2014).

The molecular techniques have facilitated and powered the genetic analysis of plants, helping the studies on genetic variability in several species. The AFLP (Amplified Fragment Length Polymorphism) technique is based on the selective amplification, through PCR, of total genomic DNA fragments generated by cleavage with restriction enzymes (Vos et al., 1995) and presents wide traceability in the detection of DNA marks amplification of several sizes by polymerase chain reaction (PCR) in the presence of thermostable enzyme 'Taq DNA polymerase' (Ferreira and Grattapaglia, 1998).

The advantages of this technique are the high degree of polymorphism and a high number of markers obtained by analyzed gel, low cost (Vos et al., 1995) and the unrequired previous knowledge of the genome of the species studied. However, the main limitation of AFLP indicators is the low content of genetic information per loco, because they have a dominant nature and the data has binary nature (Ferreira and Grattapaglia, 1998).

Therefore, biotechnology techniques can be used for the genetic variability study of weeds biotypes in order to improve management techniques. The AFLP technique has been employed to evaluate the genetic diversity of several species of plants, among which are *Amaranthus palmeri* (Chandi et al., 2013) and *Veronica hederifolia* (Wu et al., 2010). Rocha et al. (2009) studied the genetic variability of four species of *Commelina* with RAPD, and Alves et al. (2003), using the RAPD marker, identified and characterized different populations of *Rottboellia cochinchinensis*.

Thus, through reports by producers regarding infestation by itchgrass in sugarcane fields and the aggressiveness in the development of plants, the hypothesis arose that the difficulty in control observed by the farmers with the species of the *Rottboellia* genus can result from the existence of different biotypes in the populations. To check the hypothesis, we aimed to study the genetic variability among three populations of *R. cochinchinensis* collected in sugarcane crops in the state of São Paulo, using the AFLP technique.

MATERIAL AND METHODS

The collection of itchgrass leaves was done in areas of commercial production of sugarcane, between March and August/2012. The places were the cities of Igarapava, Mococa and Piracicaba, located in the state of São Paulo (Figure 1).

The young leaves of itchgrass were collected from the upper third of the plants in different sampling points, with a distance of around 10 meters between each other. Each place corresponded to the collection of 10 plants (individuals).

The leaves from each individual were conditioned in plastic bags, identified, placed in a Styrofoam box with ice in the field, and kept in an ultrafreezer (-80 °C) until the collection of all populations, to then extract the DNA in the lab.

The DNA was extracted from the leaf tissue of young leaves of each individual

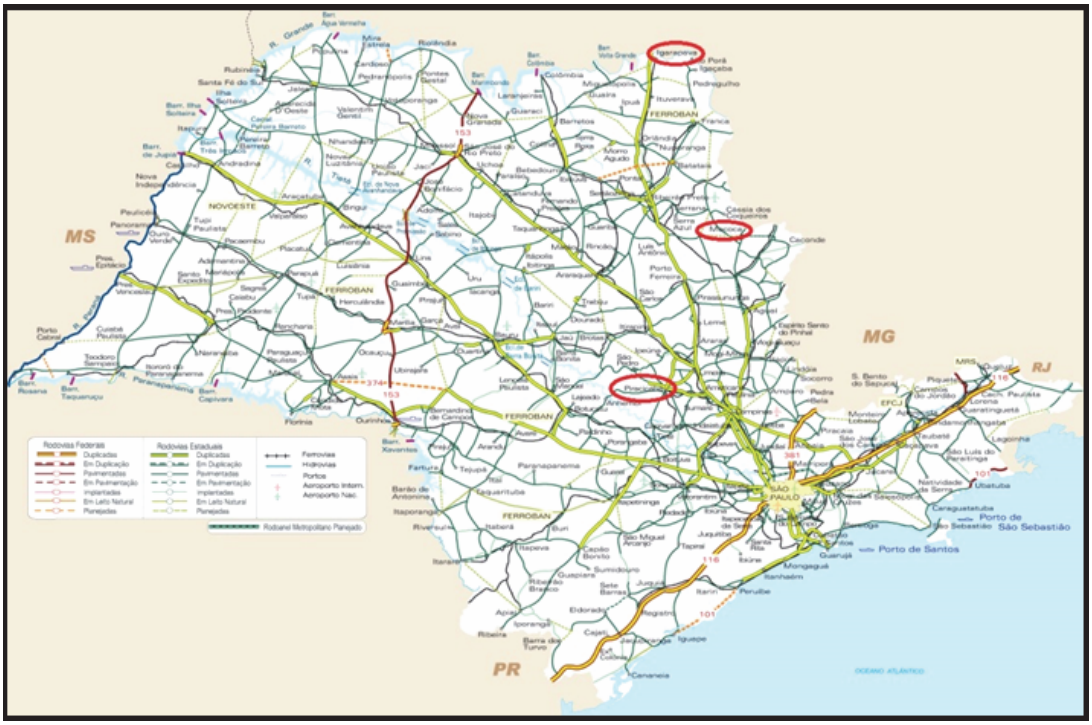


Figure 1 - Location of the collection areas in the cities of Igarapava, Mococa and Piracicaba/SP.

through the CTAB methodology, described by Al-Janabi et al. (1999). The DNA of each individual was quantified in the presence of a DNA phage standard ë of known amounts in agarose gel 0.8% (p/v) colored with ethidium bromide.

The AFLP reactions were conducted according to the protocol described by Vos et al. (1995). Summarizing, 200 ng of genomic DNA was digested with enzymes *EcoRI* and *MspI* (2.5 units each) in an OPA – One Phor All buffer (10 mM Tris.HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT), for three hours in a temperature of 37 °C and, later on, at 65 °C for five minutes to inactivate the enzyme. After digestion, the adaptors were connected, and the product from digestion/connection was diluted six times in ultrapure water to carry out the pre-amplification reaction. The pre-amplification reaction was done in a final volume of 15 µL containing 1X Buffer; 3.33 µM *EcoRI*+0; 3.33 µM *MspI*+0; 0.17 mM dNTPs; 2 mM $MgCl_{2;}$ 0.2 unit of Taq DNA polymerase; and 2.5 µL of digestion reaction/6X diluted connection. The thermocycler program consisted of 29 cycles at 94 °C for 30 seconds, 56 °C for one minute and 72 °C for one minute.

The products of pre-amplification reaction were diluted 10X with ultrapure water and used as a mold for the selective reactions, in which initiators with three selective nucleotides were used and added to the 3' end of the initiators. The selective reaction was done in a final volume of 10 µL containing 1X Buffer; 0.11 µM *EcoRI Dye* 8000; 0.25 µM *MspI*; 0.27 mM dNTPs; 2 mM $MgCl_{2;}$ 2 units of Taq DNA polymerase; and 2.5 µL of the product from the pre-amplification reaction diluted 10X. The thermocycler program consisted in a cycle at 94 °C for 30 seconds, 65 °C for 30 seconds, 72 °C for 1 minute; 12 cycles (94 °C for 30 seconds, 65 °C for 30 seconds (minus 0.7 °C per cycle), 72 °C for 1 minute); followed by 23 cycles (94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 1 minute).

The six combinations of *primers* used were: *EcoRI*ACC/*MspI*GAG, *EcoRI*ACA/*MspI*ACT, *EcoRI*ACA/*MspI*GAG, *EcoRI*ACC/*MspI*ACT, *EcoRI*AGA/*MspI*TTG and *EcoRI*AGA/*MspI*TCG. The product of the selective reaction was applied to a polyacrylamide gel at 6%. The samples were denatured at 95 °C for five minutes and immediately transferred to the gel. L*adder* of 50 - 350 base pairs (pb) was used

as molecular weight standard. The fragments were separated in an automatic sequencer (4300 DNA Analyzer - Licor). The images obtained were archived for later genotyping with the help of the Align IR software.

The genetic similarity analysis was done by using the Jaccard coefficient, which can be represented by the mathematical model $S_{ii',Jaccard} = a/(a + b + c)$, in which a is the number of concordances from type 1 1; b is the number of disagreements from type 1 0; and c is the number of disagreements from type 0 1. This coefficient was used due to its mathematical characteristics, which disregard the absence of marks as genetic similarity synonym and give different weights to the joint presence of marks (Cruz and Carneiro, 2003).

The similarity and genetic diversity relations were seen by the construction of the dendrogram by the UPGMA grouping method (Unweighted Pair Group Method with Arithmetic Mean), using the NTSYs software (Rohlf, 1992), in order to identify if there is variability among the individuals from the collected populations.

For each combination of selective *primers*, the number of marks that presented the same polymorphism pattern among the evaluated individuals (isopolymorphic marks), but in different locos, was also determined.

RESULTS AND DISCUSSION

Amplification products

The use of six combinations of selective *primers* in the evaluation of genetic variability of itchgrass (*Rottboellia cochinchinensis*) collected in three populations (cities) of the state of São Paulo generated a total of 399 marks, with an average of 66.5 marks per combination (Table 1).

All *primers* combinations used presented greater percentages of monomorphic marks, except the combination EcoRIACC/MspIACT, which had greater percentage of polymorphic marks (Table 1). The number of polymorphic marks ranged from 12 to 19, and the most polymorphic one was EcoRIACC/MspIACT (58.06%), with 31 marks. The less polymorphic combination was EcoRIACA/MspIACT (16.81%), with 113 marks.

Vieira et al. (2007), in populations of *Commelina benghalensis* identified 84 marks, from which eight had a monomorphic profile through the RAPD technique. Haldimann et al. (2003) identified 328 marks with the use of the AFLP in the species *Senecio vulgaris*, using four combinations of *primer* pairs, from which 11 were polymorphic. Using six *primer* combinations in populations of *R. cochinchinensis*, 399 marks were identified: 101 polymorphic and 298 monomorphic.

The species *Rottboellia cochinchinensis* was classified as autogamous, with two flowers: one hermaphrodite and the other male or sterile (Millhollon and Burner, 1993). Therefore, the high number of monomorphic marks (298) seen in the species *Rottboellia* can be due to their reproduction through self-pollination or apomixes, giving them the same genetic constitution as the parent plant (Dall'agnol and Schifino-Wittmann, 2005).

Analysis of the genetic similarity among the *R. cochinchinensis* individuals.

The average similarity and genetic diversity of each place or population may be observed on Table 2. The smaller average genetic similarity in the place was obtained in the population of Igarapava, next to 74%, and, consequently, greater average genetic diversity: 22.7%. The other two places had average genetic similarity and diversity of 79.3 and 14.8% for Mococa and 80.8 and 18.9% for Piracicaba, respectively. Between places, the genetic similarity was 73% for Igarapava x Mococa, 69.4% for Igarapava x Piracicaba and 73.5% for Mococa x Piracicaba (Table 2).

The average genetic similarity between the populations was approximately 78%, considered high, and we can infer that the genetic variability between populations was low. This similarity between populations, regardless of the geographic location, may have happened due to the homogenizing factors of the population, such as self-pollination, apomixes and vegetative reproduction (Vidal et al., 2006).

Table 1 - Number of marks per combination of selective *primers*, specifying the total of monomorphic and polymorphic marks, obtained with the AFLP marker – 2015

Combinations	% monomorphic marks	% polymorphic marks	Total of marks
EcoRIACC/MspIGAG	63.27 (31)	36.73 (18)	49
EcoRIACA/MspIACT	83.19 (94)	16.81 (19)	113
EcoRIAGA/MspITCG	78.67 (59)	21.33 (16)	75
EcoRIACA/MspIGAG	72.73 (48)	27.27 (18)	66
EcoRIACC/MspIACT	41.94 (13)	58.06 (18)	31
EcoRIAGA/MspITTG	81.54 (53)	18.46 (12)	65
Total	298	101	399

Table 2 - Similarity (SGm) and average genetic diversity (Gst) observed within and between collection populations of *Rottboellia cochinchinensis* – 2015

	SGm/Gst within populations		
	Igarapava	Mococa	Piracicaba
	0.742/0.227	0.793/0.148	0.808/0.189
	SGm between the populations		
Igarapava	---		
Mococa	0.730	---	0.735
Piracicaba	0.694	0.735	---

The weeds colonize environments with different characteristics. Thus, due to the survival capacity provided by genetic variability in the populations (Winkler et al., 2003), it is suggested that *Rottboellia cochinchinensis* has a narrow genetic basis due to uniformity of the bands between the populations. Therefore, the monomorphic marks can be used as plants genetic stability markers, that is, that can be due to the species founder effect. According to Deuber (1992), this species was introduced in Brazil with the rice seeds and, due to the similar size of their seeds, they were simultaneously sown.

The *Eichornia crassipes* species also had high genetic similarity. According to Cardoso et al. (2002), this can be explained by the vegetative reproduction of the species. Autogamous plants have less gene flow due to their reproduction mode, leading the species to homozygosity. Winkler et al. (2003), when studying biotypes of *Euphorbia heterophylla*, using the RAPD technique, found 40% of average genetic similarity, a value the author considers to be low. According to Kissmann and Groth (1999), the reproduction mode of the species is through the seeds, which can explain their low similarity because there is no gene flow between the plants.

With the isopolymorphic marks (Table 3), it is seen that 101 polymorphic marks may be represented by 52 isopolymorphic isolated marks or in isopolymorphic groups (Table 4). The best selective *primers* combinations were EcoRIACC/MspIGAG, EcoRIAGA/MspITCG and EcoRIAGA/MspITTG; the other combinations did not have any satisfactory results in this study due to the many determinations of isopolymorphic marks.

The combination of EcoRIAGA/MspITTG presented greater efficiency in the study

Table 3 - Marks and combinations of selective *primers* in the set of 30 individuals of *Rottboellia cochinchinensis* – 2015

Polymorphic marks	Selective primers combinations	Isopolymorphic marks	
		within combinations	with other combinations
X1-X18	EcoRIACC/MspIGAG	6/18	20/ (101-18)
X19-X37	EcoRIACA/MspIACT	17/19	20/ (101-19)
X38- X53	EcoRIAGA/MspITCG	5/16	15/ (101-16)
X54-X71	EcoRIACA/MspIGAG	12/18	19/ (101-18)
X72-X89	EcoRIACC/MspIACT	11/18	26/ (101-18)
X90-X101	EcoRIAGA/MspITTG	2/12	14/ (101-12)

Table 4 - Isopolymorphic marks in the set of 30 individuals of *Rottboellia cochinchinensis* Ribeirão Preto/SP, 2015

X1
X2 = X26 = X27
X3 = X4
X4 =X6 =X9 = X13 = X16 = X17 = X28 = X29 = X100 = X101
X5
X7 = X52 = X72 = X76 = X83
X8
X10 = X23 = X65 = X66 = X85
X11
X12 = X50
X14 = X39 = X40 = X49 = X61 = X86 = X94
X15
X16
X19 = X92
X20
X21 = X42
X22 = X12 = X53 = X85 = X85
X23 = X54 = X61 = X78 = X85 = X86 = X97
X24 = X38
X25 = X37 = X41 = X49 = X53 = X54 = X78 = X87 = X99
X27 = X47 = X84 = X92
X28 = X43 = X68 = X100 = X101
X30 = X32
X31 = X79
X33
X34
X35 = X30 = X68
X44
X45 = X95
X46
X48 = X70
X51
X55
X56
X57 = X62 = X77
X58 = X74
X59
X60
X62 = X90 = X93
X63
X64
X69
X71
X73 = X85
X75 = X76
X77
X80 = X100
X81
X82 = X83
X88
X89 = X6
X91 = X95

X = Polymorphic marks.

with the species *R. cochinchinensis* due to the low number of isopolymorphic marks (2 isopolymorphic marks/12 polymorphic marks) and the combination EcoRIACC/MspIACT, lower efficiency with the study of the species because 26 isopolymorphic marks were determined (101 polymorphic marks – 18 polymorphic marks within the combination) (Table 3).

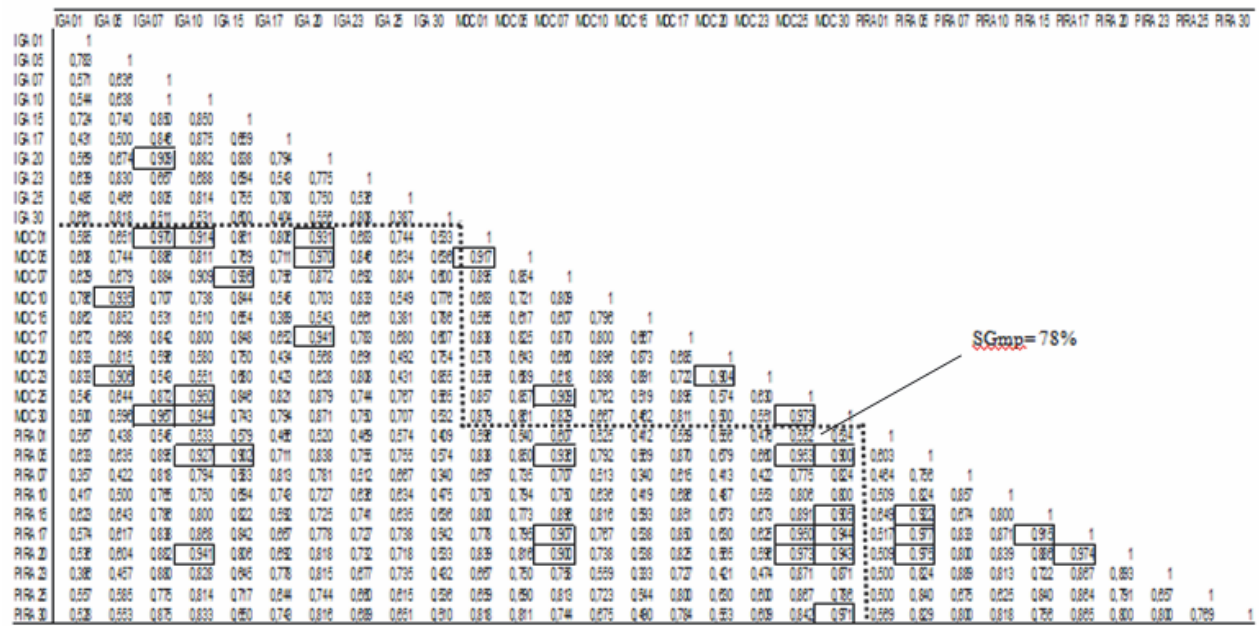
The smaller genetic similarity within each population (Table 5) was observed between individuals 25 and 30 (38.7%) from Igarapava, between individuals 15 and 30 (46.2%) from Mococa and between individuals 1 and 7 (46.4%) from Piracicaba. These values point out individuals that are more genetically distant for each population. Therefore, we cannot affirm that this is a completely autogamous species because it can have cross fertilization.

The greatest genetic similarity was found between individuals 7 and 20 (90.9%) in the Igarapava population; between individuals 1 and 5 (91,7), 7, and 95 (90.9%) and 20 and 23 (90.4%) in the Mococa population; and between individuals 5 and 15 (92.2%), 5 and 17 (97.7%), 5 and 20 (97.5%) and 17 and 20 (97.4%) within the population that covers individuals from Piracicaba (Table 5). These values point out the most similar individuals within each population, that is, a population must have the same response to factors from the environment as the other populations studied, once the environment characteristics (soil, precipitations, temperature) are similar.

Among the populations (Table 5), the most similar individuals were 5 and 10 (93.5%), 5 and 23 (90.6%), 7 and 1 (97.0%), 7 and 30 (96.7%), 10 and 1 (91.4%), 10 and 7 (90.9%), 10 and 25 (95.0%), 10 and 30 (94.4%), 15 and 7 (93.6%), 20 and 1 (93.1%), 20 and 5 (97.0%) and 20 and 17 (94.1%) for Igarapava x Mococa, 10 and 5 (92.7%), 10 and 20 (94.1%) and 15 and 25 (90.2%) for Igarapava x Piracicaba and 7 and 5 (93.6%), 7 and 17 (90.7%), 7 and 20 (90.0%), 25 and 5 (95.3%), 25 and 17 (95.0%), 25 and 20 (97.3%), 30 and 5 (90.0%), 30 and 15 (90.5%), 30 and 17 (94.4%), 30 and 20 (94.3%), 30 and 30 (97.1%) for Mococa x Piracicaba.

Alves et al. (2003) characterize six populations of itchgrass in the state of Sao Paulo and found morphological and molecular differences (RAPD technique), associating them to the number of chromosomes and their respective ploidy.

Table 5 - Genetic similarity matrix (Jaccard coefficient) between individuals of *Rottboellia cochinchinensis* in three collection places obtained with AFLP markers, 2015



IGA = Igarapava, MOC = Mococa, PIRA = Piracicaba; SGap: average genetic similarity between populations.

Through the UPGMA grouping analysis the dendrogram was obtained, in which occurred the formation of two different groups and a discriminating individual (Figure 2), with an average genetic similarity around 72% between the individuals. Group 1 can be subdivided into two subgroups, formed by individuals from Igarapava and Mococa (individuals: IGA 1 - 5, MOC 20 - 23, IGA 23 - 30) (Figure 2). Group 2 contained the other individuals from the three places analyzed (Piracicaba, Mococa and Igarapava). The formation of these groups can be due to the spread of seeds by rain, birds and man action, because they germinated close to the parent plant. Therefore, these populations are suggested to be genetically close (Circunvis et al., 2014), which shows a common ancestral.

Individual 1 from the most distant place in Piracicaba from the others in the matrix can be due to the biotypes within the populations collected, exemplified by 101 polymorphic marks observed in the gels with the AFLP marker.

Vivian et al. (2008) suggest that the individuals can receive stimuli from the environment during or after formation, which enables the alteration of their behavior from the liberation of the parent plant. Therefore, in order to manage *Rottboellia*, it is important to know the genetic diversity of its populations, because one of the factors that can contribute to the different responses of weeds to the application of herbicides is the genetic variability in them (Christoffoleti and López-Ovejero, 2003). However, it is possible that there has been simultaneous selfing in the studied areas to homogenize the individuals; therefore, the same chemical management will have a similar effect in any studied area due to the high average genetic similarity observed between the regions (78%).

With the occurrence of the *R. cochinchinensis* species in several countries (Holm et al., 1977) it is observed that this is a species with high adaptability – a characteristic that depends directly on genetic variability. However, a smaller percentage of genetic variability between the populations studied was observed, which may be explained by the founder effect. In the literature, there have been discussions about the possibility of action of the founder effect and genetic similarity among populations of weeds. The

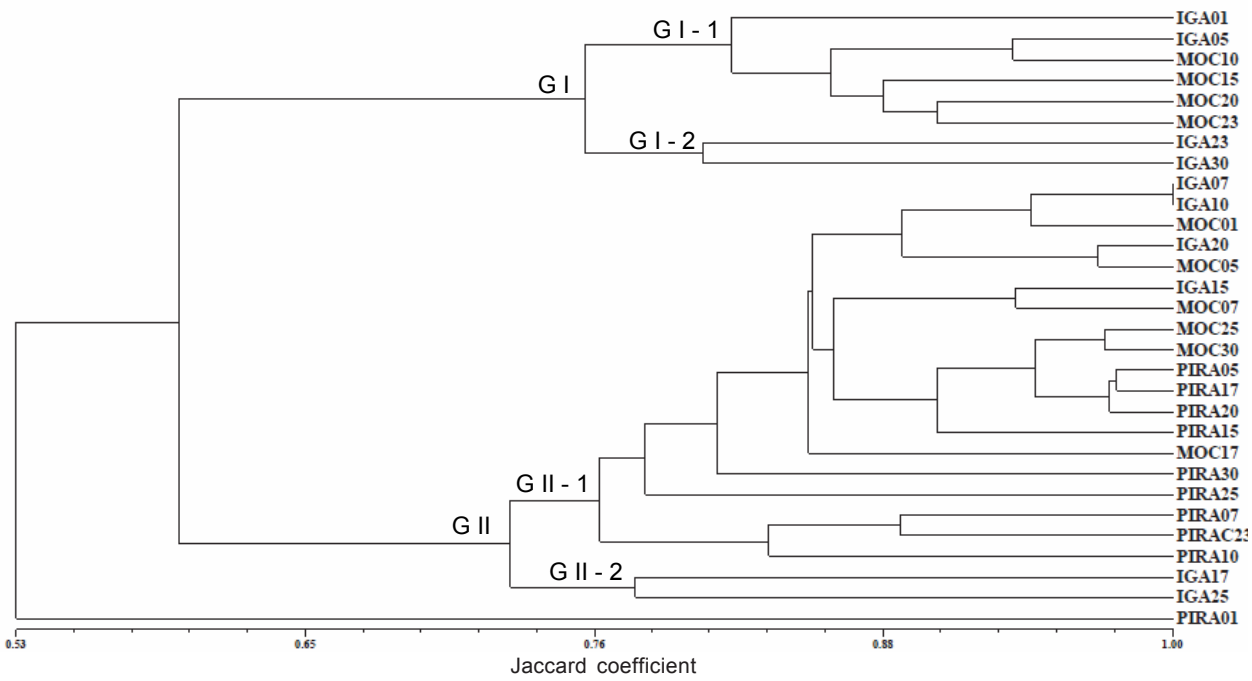
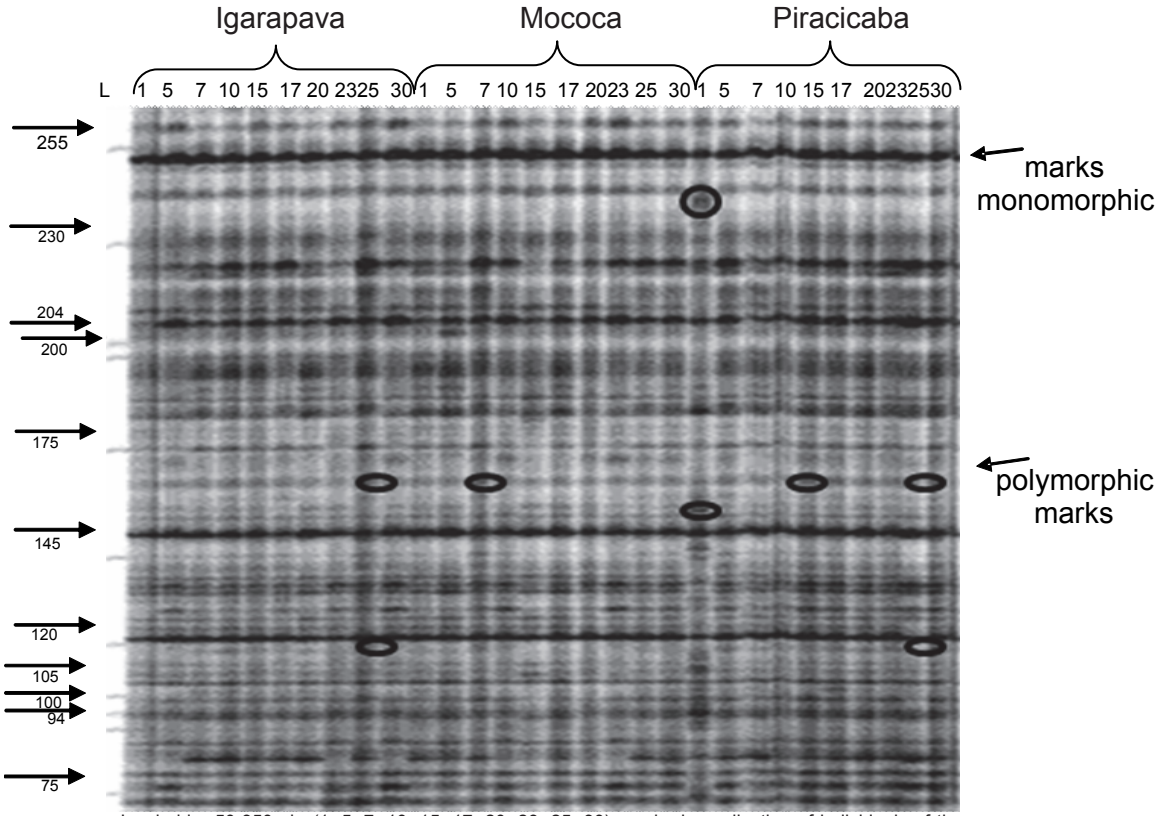


Figure 2 **-** Average genetic similarity dendrogram from the Jaccard coefficient with grouping by the UPGMA method, between the places of study (IGA=Igarapava; MOC=Mococa; PIRA=Piracicaba) – 2015.



L = ladder 50-350 pb; (1, 5, 7, 10, 15, 17, 20, 23, 25, 30) numbering collection of individuals of the populations studied; Igarapava, Mococa, Piracicaba.

Figure 3 **-** Amplification products with AFLP markers of *Rottboellia cochinchinensis* individuals with the combination EcoRIACC/MseIACT. Ribeirão Preto/SP, 2015.

founder effect is defined as the establishment of a new population by few individuals, that is, the population was formed from a single parent plant, carrying along only a small fraction of the total genetic variation of the parental population (Ridley, 2003).

Therefore, through the genetic analyses, we can conclude that the genetic similarity among the *R. cochinchinensis* populations collected in the state of São Paulo was around 72% between the individuals, which proves that difficulties in chemical management are not only due to the different biotypes, but also the other characteristics connected to the tolerance of the species to herbicides. However, we cannot disregard the presence of biotypes in the populations, as is suggested by the presence of polymorphic marks, detected by the AFLP marker, generating 22% of average genetic variability.

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