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Morphological analysis and DNA methylation in *Conyza bonariensis* L. Cronquist (Asteraceae) phenotypes

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ABSTRACT: The species *Conyza bonariensis* (L.) cause losses in agriculture due to their invasive capacity and resistance to herbicides like glyphosate. The species of this genus exhibit phenotypic plasticity, which complicates their identification and characterization. Thus, experiments were performed with 2 extreme *C. bonariensis* phenotypes (called broad leaf and narrow leaf) in greenhouse conditions and in the laboratory, in order to verify if the morphological differences among these phenotypes are a genetic character or result from environmental effects. In addition to the comparative morphological analysis, assessment of DNA methylation profile was performed to detect the occurrence, or not, of differences in the epigenetic level. The morphological characteristics evaluated were length, width, shape, margin and leaves indument; plant height and stem indument; the number of *capitula*, flowers and seeds. The Methylation Sensitive Amplified Polymorphism technique was used to investigate the methylation levels. The morphological differences of phenotypes supposed to be *C. bonariensis* are probably genetic in origin and not the result of environmental effects, since, after 6 crop cycles in a greenhouse under the same environmental conditions, these phenotypes remained with the same morphological characteristics and seed production in relation to the original phenotypes found in the collection site. The different phenotypes did not show differences corresponding to DNA methylation patterns that could indicate an epigenetic effect as the cause of the differences between the 2 phenotypes. The results of morphological analysis and methylation probably indicate that maybe they are individuals of populations from different taxa not registered yet in the literature.

Key words: phenotypic plasticity, *Conyza bonariensis*, resistance, weeds.

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INTRODUCTION

The weed *Conyza bonariensis* L. Cronquist (Asteraceae), popularly known as hairy fleabane, is a native species from South America and occurs abundantly in Argentina, Uruguay, Paraguay and Brazil. It is found mainly in the South, Southeast and Midwest regions of Brazil, being, along with *C. canadensis* and *C. sumatrensis*, the most prominent species of the genus *Conyza* as invasive in agricultural regions (Kissmann and Groth 1999). The species of *Conyza* have a great capacity of adaptability, which allows them to occur in different soil and climatic conditions (Santos et al. 2013). *C. bonarensis* is considered a species difficult to control because of its invasive potential in several crops and due to resistance to herbicides, including the glyphosate which excels. In Brazil, there are records of *C. bonariensis* (Vargas et al. 2007; Lamego and Vidal 2008), *C. canadensis* (Moreira et al. 2007) and *C. sumatrensis* (Santos et al. 2014) resistant to herbicides.

The genus *Conyza* is characterized by groups of closely-related species, some of them with a high polymorphism degree, which complicates their taxonomy (Urdampilleta et al. 2005). The chromosome variation presented by some species also contributes to this diversity, such as *C. bonariensis*, in which there are records of tetraploid (2n = 4x = 36), pentaploid (2n = 5x = 45), and hexaploid (2n = 6x = 54) (Paula and Pinto-Maglio 2015).

Phenotypic plasticity is among many characteristics that contribute to the establishment and success of weeds. It is initially defined as the change in the phenotypic expression of a genotype in response to environmental factors (Bradshaw 1965; Schlichting 1986).

The phenotypic plasticity is considered a strategy adopted by the weeds for their establishment in different environments from where they have evolved (Schlichting and Levin 1986). By changing the phenotypic characteristics, the weeds begin to compete more easily with cultivated plants for resources necessary to the growth and development of the individuals from the population (Bossdorf and Pigliucci 2009).

Some reversible or heritable changes in the genome, such as phenotypic plasticity, may occur without alterations in the nucleotide sequence of DNA. The DNA alterations in conformational order can result in morphological, physiological or structural changes in the individuals and are called, in this case, epigenetic modifications (Jablonka and Raz 2009; Johannes et al. 2009).

Epigenetic modifications may result from DNA methylation that occurs when a methyl group is added to the 5' position of the pyrimidine ring of cytosine that begins to act as a gene silencing (Law and Jacobsen 2010). Methylation can contribute to the genome maintenance, because DNA methylation patterns may be altered according to the demand of different stressful external conditions (Solis et al. 2012).

One of the methods used to detect methylation occurrence is the Methylation Sensitive Amplified Polymorphism (MSAP) technique, in which there is digestion of genomic DNA with restriction endonucleases that are sensitive to methylation, followed by amplification of restriction fragments (Yang et al. 2011).

In Brazilian agricultural areas infested with weeds of that genus, some common morphotypes of *C. bonariensis* are possible to be recognized mainly in relation to the shape and size of their leaves. The lack of morphological, physiological and genetic studies in *C. bonariensis* has limited the development of appropriate strategies for the integrated management of this species, either sensitive or resistant populations to herbicides. Given the phenotypic plasticity found in the species of the genus *Conyza*, this study aimed to verify whether the morphological differences of the 2 most common distinct morphotypes found in contrasting populations of *C. bonariensis* are due to genetic character or result from environmental effects.

MATERIAL AND METHODS

The material used consisted of seeds from plants of *C. bonariensis* populations with distinct phenotypes collected in agricultural and non-agricultural areas, i.e., areas treated and not treated with glyphosate in Campinas, São Paulo, Brazil.

Plants with narrow leaf and broad leaf phenotypes were selected. This material was called narrow leaf phenotype (NLP) and broad leaf phenotype (BLP). Those plants were subjected to chemical control with glyphosate named narrow leaf phenotype with chemical control (NLP/CC) and broad leaf phenotype with chemical control (BLP/CC).

The exsiccata relating to the individuals population evaluated in this study were deposited in the Herbarium of the Agronomic Institute of Campinas, São Paulo, Brazil) under the numbers: IAC 53451 (BLP), IAC 53452 (NLP), IAC 51013 (BLP/CC) and IAC 53450 (N - LP/CC).

Morphological evaluations of *Conyza bonariensis*

The seeds of the mentioned materials (NLP, BLP, NLP/CC and BLP/CC) were pre-imbibed in distilled water for 48 h, distributed in Petri dishes with 2 sheets for germination and then hydrated with 10 mL of distilled water. The dishes were kept in an environment with fluorescent and incandescent light for 8 h (light) and 16 h (darkness) under the temperature of 25 ± 2 °C, always keeping the germination sheet moistened.

After 15 days of sowing, the seedlings of each phenotype were transplanted to plastic pots of 2,000 mL containing vermiculite and soil in a 1:1 ratio, in a total of 2 batches of 25 pots with 3 seedlings each. This material was kept for 6 cultivation cycles in a greenhouse and, after a week from the last transplanting, the thinning was done leaving only 1 seedling per pot, totaling 25 plants per phenotype.

The height, length, width, margin shape and leaves indument of the plant were evaluated, as well as the length, width, shape of the stem and the number of capitula, flowers and seeds.

The plant height was weekly measured from the base to the apex with a millimeter ruler. The determination of length and width of the leaves, as well as their characterization (shape, margin and indument) and the stem indument were performed on 5 plants of each phenotype in the fruiting stage. A digital caliper with an accuracy of 0.01 mm was used to determine the measurements.

The capitula were quantified in weekly evaluations by counting their number in each of the 25 plants per phenotypes. The number of flowers and seeds per capitulum was quantified by counting them in 15 capitula randomly collected for each phenotype. The capitula have been preserved in 70% alcohol until the count. The number quantification of flowers and seeds was performed by using a stereomicroscope.

The number of seeds per plant was quantified by using the following formula for both phenotypes: Number of seeds = (number of capitula per plant) × (average number of seeds per capitula).

The data were submitted to the test of Shapiro and Wilk (1965) in order to assess the variance and normality of errors. The averages were compared by the Student's t-test at 5% of probability using the statistical program SISVAR (Ferreira 2011).

Assessment of DNA methylation profile of *Conyza bonariensis* by the Methylation Sensitive Amplified Polymorphism technique

Methylation levels of the 4 materials (BLP, NLP, BLP/CC and NLP/CC) were measured by the MSAP technique according to Lei et al. (2006). This technique is a modification of the Amplified Fragment Length Polymorphism (AFLP) in which the isoschizomers *HpaII* and *MspI* are used as a frequent cutting replacing *MseI* from the original protocol AFLP (Vos et al. 1995). The sequences of the adaptors are given in Table 1.

The samples were analyzed according to the presence (1) and absence (0) of bands. From this binary matrix, the genetic similarity among the materials was analyzed by adopting the similarity coefficient of Jaccard (1901). The relations of genetic similarity were visualized by the construction of a dendrogram by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using the software NTSYS, version 2.1 (Rohlf 2000).

For the percentage analysis of methylated regions in the genome of the studied phenotypes, the bands patterns or molecular profile obtained from the reaction of selective amplification arising from the digestion with enzyme →

Table 1. Sequences (5' – 3') of adaptors for analyses of Methylation Sensitive Amplified Polymorphism.

Name	Enzyme	Type	Sequence (5' – 3')
EcoA+	EcoRI	Adaptor (+)	CTCGTAGACTGCGTACC
EcoA–	EcoRI	Adaptor (–)	AATTGGTACGCAGTC
Hpa/MspA+	HpaII/MspI	Adaptor (+)	GACGATGAGTCCTGAT
Hpa/MspA–	HpaII/MspI	Adaptor (–)	CGATCAGGACTCAT

combinations *EcoRI/MspI* and *EcoRI/HpaII* were compared side by side for each phenotype. Thus, for the same phenotype, when it occurs in the respective locus marker the band presence (1) in selective amplification *EcoRI/MspI* and the band absence (0) in *EcoRI/HpaII*, it is assumed that the internal cytosine is methylated. When the opposite occurs, i.e., band presence (1) in the selective amplification *EcoRI/HpaII* and absence (0) in *EcoRI/MspI*, it is assumed that the external cytosine is methylated. When there is no change for the same phenotype, band presence (1) occurs both with *EcoRI/MspI* and *EcoRI/HpaII* combinations, and it is assumed absence of methylation (Table 2).

Table 2. Sensitivity to methylation and restriction pattern of isoschizomers.

Types	Methylation state	Restriction enzyme digestibility			
		HpaII	MspI	EcoRI/ HpaII	EcoRI/ MspI
I	CCGG GGCC	Active	Inactive	1	0
II	CCGG GGCC	Inactive	Active	0	1
III	Absence of methylations	Active	Active	1	1

The methylated cytosine is underlined; 1 = Band presence; 0 = Band absence.

Thus, by comparing the profiles (*EcoRI/MspI* and *EcoRI/HpaII*) side by side for each phenotype, the number of presence and absence of bands was computed in each locus marker. This value was used to estimate the methylation percentage of each access and compare if there are (or not) differences among the studied phenotypes (NLP, BLP, NLP/CC and BLP/CC).

The data were submitted to the Shapiro and Wilk (1965) test in order to assess the variance and normality of errors. The averages were compared by the Student's t-test at 5% probability, for the morphological variables as well as pollen viability, and by the Turkey's test at 5% probability for the results of DNA methylation by using the statistical program SISVAR (Ferreira 2011).

RESULTS AND DISCUSSION

Morphological evaluations of *Conyza bonariensis*

Even being taxonomically considered from the same species, the *C. bonariensis* evaluated in this study remained with different shapes and leaf margins after 6 cultivation cycles in the same conditions, i.e., even grown in the same environment (greenhouse), leaf differences among the phenotypes were the same as presented in the collecting from the sampled areas.

Hussain and Mahmood (2004) also identified morphological variations in 2 populations *Trifolium alexandrinum* L. cultivated in common environment. These authors reported that most of the morphological variation of these populations has happened in the environment, rather than arising from genetic variability. The authors concluded that environmental fluctuations seem to generate flexibilities of morphological responses in *T. alexandrinun*. Similar results were found by Gao et al. (2010) in the weed/ruderal *Alternanthera philoxeroides* (Mart.) Griseb in natural and handled habitats. The plants of this species not only underwent significant morphological change in the common environments of the garden, but also suffered an epigenetic reprogramming in response to different treatments used in the study.

Even though the morphological analysis is an option used to characterize the species, Oliveira et al. (2000) emphasized that these analyses may have limitations related to characters that have additive heritage, which are highly influenced by the environment, and generate difficulties in the differentiation of cultivars with a great phenotype. This fact may have occurred in the phenotypes of *C. bonariensis*.

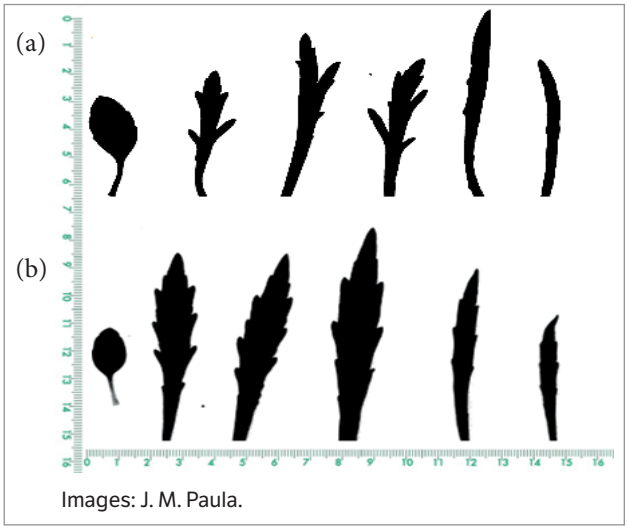
Description of narrow leaf phenotype and broad leaf phenotype morphological types

It was found in this study that, in *C. bonariensis*, besides the leaf shape is considered as a marking characteristic and easily identified on a simple observation, there are also differences in the stem and leaves indument, plant height, number of capitula, flowers and seeds.

Regarding leaf shape, NLP has a lanceolate linear shape, while the BLP is oblanceolate at the basal third and lanceolate linear at the apex and pinnatisect at the plant base. Other characteristics such as margin, width and length of the leaves also differed among the phenotypes. In the NLP, the margin is usually entire to sub-entire, with the width and length ranging from 1.5 to 4.9 mm and from 25 to 65 mm, respectively. In the BLP, the margin is strongly indented at the plant base and sub-entire at the branches apex with width and length of leaves ranging from 2.5 to 12 mm and from 22 to 70 mm, respectively (Table 3 and Figure 1).

Table 3. Variations in the morphological characteristics evaluated and in the production of flowers and seeds for both phenotypes of *Conyza bonariensis* analyzed.

Characteristics	Phenotypes of Conyza bonariensis	
	Narrow leaf phenotype	Broad leaf phenotype
Plant height (cm)	80 – 105	72 – 90
Stem indument	Hispid-scabrous, with some long trichomes and flexible along the branches.	Villous to tomentose, more strigosus in the branches.
Leaf indument	Hispid-scabrous, with some long trichomes and flexible in the margin.	Sericeous to hispid, tomentose in the young leaves.
Leaf width (mm)	1.5 – 4.9	2.5 – 12
Leaf length (mm)	25 – 65	22 – 70
Leaf shape	Lanceolate linear	Oblanceolate in the basal third to lanceolate linear at the apex, pinnatisect at the plant base.
Leaf margin	Generally entire to sub-entire	Highly indented at the plant base to sub-entire at the branches apex.
Number of flowers/capitula	208 – 385	88 – 140
Number of capitula/plant	606 – 815	326 – 354
Number of seeds/capitula	199 – 340	97 – 140
Number of seeds/plant	194,805 – 224,968	36,864 – 40,000



Images: J. M. Paula.

Figure 1. Width, length, shape and leaves margins of *Conyza bonariensis* phenotypes. (a) Narrow leaf phenotype; (b) Broad leaf phenotype.

Usually, the leaves margin is one of the characteristics used in taxonomy to differentiate species. Lorenzi (2000) used this characteristic to differentiate 2 species of the genus *Conyza*. In his description, the leaves margin *C. canadensis* are classified as dentate and the *C. bonariensis* leaves as non-serrated. Kissmann and Groth (1999) described the leaves of *C. bonariensis* as simple, alternate, sessile, being the lower of oblanceolate format with attenuated base and acute apex; the upper leaves are lanceolate with entire margin with dimensions from 6.0 to 12.0 cm × 1.5 to 2.5 cm.

When the leaf margin is taken into consideration as an evaluation criterion for the differentiation of the species *Conyza*, the BLP would not fit in this classification because, in the analysis performed, only the NLP presents the shape with non-serrated leaf margin.

Besides the leaf shape, it was observed that the phenotypes also have differences in width and length of leaf. For Engel et al. (2002), these are closely-related characteristics to the competition for light and gas exchange and both are dependent on the availability of water and nutrients. However, it was found that the differences presented in the size of the leaves of NLP and BLP phenotypes probably have no relation with the availability of water and nutrients, because both were grown in the same environment under the same growing conditions.

Other differential characteristics are those related to the stem and leaves indument, showing that the phenotypes are also different in this aspect. In the stem, the NLP indument is hispid-scabrous, with some long and flexible trichomes along the branches; in the BLP, it is villous to tormentose, but more strigosus on the branches. In the NLP leaves, the indument is characterized as hispid-scabrous, with some long and flexible trichomes on the margins, while, in the BLP, it is sericeous to hispid and tormentose in the young leaves (Table 3 and Figure 2).

The leaf represents the main organ of ruderal plants involved in the herbicide penetration, thus influencing the

intercepted and retained quantity (Hess and Falk 1990). It is evident that the absorption mechanism of herbicides can be differentiated in different phenotypes from the same species, mainly because the induments and width of the leaves are different, just as the phenotypes BLP and NLP.

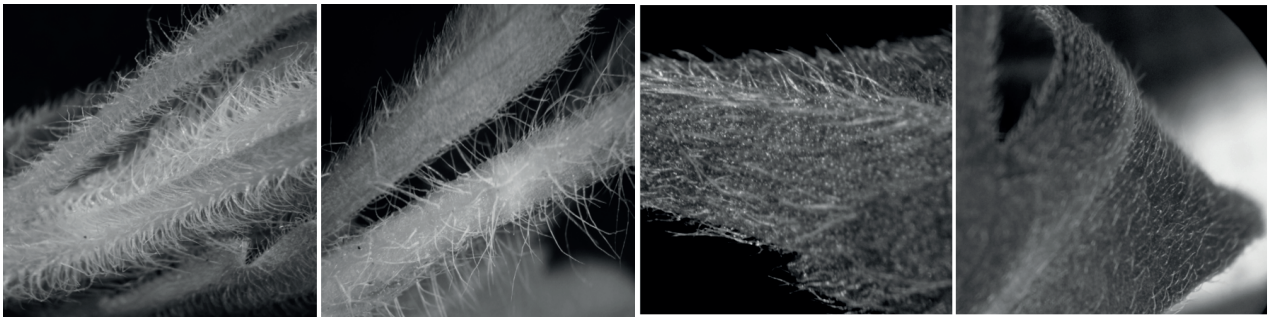
Procópio et al. (2003), by evaluating the leaves in *C. bonariensis*, identified the high trichome density indicating it, along with the large thickness of cuticle face abaxial and the low stomatal density on the adaxial face, as one of the main potential barriers detected and impositive to penetration of herbicides in this species. The characteristic of leaves with trichomes is most evident in NLP phenotype, which, in a certain way, could be a compensation for the smaller width of its leaf blade.

In addition to the relevant characteristics to leaf analyses, there was an occurrence of highly significant statistical differences in relation to the plant height, numbers of capitula, flowers and seeds (Table 4). The NLP phenotype showed higher average values to the BLP for all the characteristics shown in this table. The average difference was 11 cm in height, 144 flowers per capitulum, 378 capitula per plant, 1,486 capitula in total (evaluation in 25 plants), 141,000 seeds per plant and 644,000 seeds in the total (evaluation in 25 plants).

Regarding the NLP phenotype, it has leaves with smaller leaf area and may be related to a greater advantage when exposed to herbicides, i.e., smaller area for deposition of these pesticides, thus a greater chance of survival. This characteristic combined to the greater flowers and seeds production presented by NLP may indicate that this phenotype is the representation of a more advanced adaptation period for *C. bonariensis* or may be a different taxon of this species.

It was found that the number of seeds was higher in the NLP. This high seed production, combined with areas with presence of biotypes resistant to herbicides, is worrying from an agricultural point of view, because the herbicide resistance is a heritable characteristic. The high seed production of species of *Conyza* genus has been reported in several studies. Results by Wu and Walker (2004) estimated for *C. bonariensis* showed the average production of 110,000 seeds per plant, with an average number of seeds per capitulum from 190 to 550.

In species of *Conyza* genus, the morphological variability observed, as in *C. bonariensis* NLP and BLP phenotypes of this study, can be considered an additional advantage, because it allows the plants to exploit new niches for →



Images: J. M. Paula.

Figure 2. Stem indument and leaves of Conyza bonariensis phenotypes. Broad leaf phenotype (a,c); Narrow leaf phenotype (b,d).

Table 4. Morphological characteristics averages evaluated in *Conyza bonariensis* phenotypes.

Phenotype	Height	Capitula		Flowers	Seeds		
		Total	Plant	Capitula	Total	Plant	Capitula
NLP	93 a	3.109 a	723 a	253 a	857.974 a	205.204 a	254 a
BLP	84 b	1.623 b	345 b	109 b	213.716 b	39.129 b	113 b
Standard error	0.8	79.2	25.7	10.2	123.695	163,691	10.3
Overall average	88	2.366	534	188	535.844	122.166	189
CV (%)	8.1	7.5	10.7	21.0	13.1	7.6	21.1

Averages followed by the same letter in the column do not differ by the Student's t-test, considering the nominal value of 5%. NLP = Narrow leaf phenotype; BLP = Broad leaf phenotype; CV = Coefficient of variation.

resources and expand their distribution possibilities. This may be true if we consider the NLP phenotype is associated with a greater production of flowers and seeds in relation to the BLP (Table 4). These results may indicate the possibility that each *C. bonariensis* phenotype have an independent morphological differentiation pattern. Perhaps, this ability to modify certain characteristics presented by this species allows these plants to survive and remain in diverse environments.

Gossett and Toler (1999) emphasized that species of the same genus or family of plants have different susceptibility to the same herbicide. For Yamashita and Guimarães (2011), the species *Conyza* presented a wide ecological adaptation, which has made this plant the most important weed/ruderal in several crops in recent years. This ability to develop in adverse conditions makes it able to aggressively take the cultivation areas worldwide. Those authors suggested that researches related to ecophysiology and management of problematic species for agriculture can contribute to the development of more rational, safer and efficient practices without compromising productivity.

Evaluation of DNA methylation profile of *Conyza bonariensis*

The comparative analysis of the molecular profile obtained with MSAP technique allows to evaluate the methylation levels among different phenotypes of *C. bonariensis*, as well as the relations of genetic similarity among them, which were estimated based on the polymorphism generated from 8 combinations of selective *primers* pairs used for amplification of fragments digested with *EcoRI/MspI* and *EcoRI/HpaII* enzymes (Table 5).

On average, in all phenotypes, the selective *primers* have generated a greater number of polymorphic markes, when they were amplified in the products of digestion with *EcoRI/MspI* in relation to *EcoRI/HpaII*. However, this difference was not statistically significant, even among the corresponding phenotypes that underwent chemical treatments, suggesting that the percentage of methylation observed may reflect the methylation levels that naturally occurred in the species (Table 6).

The percentage of internal (*MspI*) and external (*HpaII*) methylation obtained for each selective combination in different types of phenotypes evaluated is shown in Table 6. For the most of selective *primers* combinations evaluated, the percentage of polymorphism varied according to the combination of enzymes used and the type of phenotype.

In BLP, digested with *EcoRI/MspI* enzymes, the selective combination ACA/TTG generated the highest percentage of polymorphism (79%). In the same phenotype (BLP), the polymorphism percentage obtained for the same selective combination *primer* was 44% when used in the amplification of the digestion products *EcoRI/HpaII*.

Similar results were also observed for the selective combination in other phenotypes. Despite that, some selective *primers* combinations produced in the same phenotype, the same polymorphism percentage or very close value, both when used in the amplification of the digestion products *EcoRI/MspI* as *EcoRI/HpaII*. The combinations ACA/ACA and ACA/TTG in the NLP group as AAG/ACT in the BLP one can be cited as examples.

Methylation levels among different organs or development stages have been observed in many species of plants (Xiong et al. 1999). From these works available in the literature, the results served to clarify the issues related mainly to changes in the morphology in phenotypes when submitted to stress. This stress can be caused by

Table 5. Internal and external DNA methylation levels in vegetal materials analyzed of *Conyza bonariensis*.

Phenotypes	External methylation	Internal methylation
NLP	51 a	58 a
BLP	55 a	69 a
Standard error	4.6	5.1
CV (%)	24	22
NLP/CC	49 a	55 a
BLP/CC	45 a	59 a
Standard error	4.1	5.9
CV (%)	24	29
BLP/CC	45 a	59 a
BLP	55 a	69 a
Standard error	4.2	5.2
CV (%)	24	23
NLP/CC	49 a	55 a
NLP	51 a	58 a
Standard error	4.4	5.7
CV (%)	25	29

Averages followed by the same letter in the column do not differ by the Student's t-test, considering the nominal value of 5%. NLP = Narrow leaf phenotype; BLP = Broad leaf phenotype; CV = Coefficient of variation; NLP/CC = Narrow leaf phenotype with chemical control; BLP/CC = Broad leaf phenotype with chemical control.

Table 6. Percentage of internal (5'-C^{m}CGG-3') and external (5'-^{m}CCGG-3') methylation of different materials of *Conyza bonariensis* using the Methylation Sensitive Amplified Polymorphism technique.

Phenotypes	BLP		BLP/CC		NLP/CC		NLP	
Enzymes combinations	*MspI** (%)	*HpaII*** (%)	*MspI** (%)	*HpaII*** (%)	*MspI** (%)	*HpaII*** (%)	*MspI** (%)	*HpaII*** (%)
AGA ACA	34 (74)	33 (72)	30 (65)	15 (33)	23 (50)	22 (48)	30 (65)	21 (46)
AGA TTG	28 (64)	32 (73)	33 (75)	23 (52)	21 (48)	29 (66)	14 (32)	15 (34)
ACA ACA	40 (75)	20 (38)	30 (57)	24 (45)	30 (57)	22 (42)	32 (60)	32 (60)
ACA TTG	41 (79)	23 (44)	12 (23)	14 (27)	16 (31)	34 (25)	16 (31)	16 (31)
AAG ACT	38 (63)	38 (62)	49 (75)	35 (45)	39 (63)	30 (56)	45 (73)	37 (62)
AAG ACA	30 (52)	31 (54)	45 (78)	39 (58)	27 (46)	27 (52)	37 (63)	30 (57)
ACG ACT	42 (77)	32 (45)	26 (53)	37 (56)	37 (70)	27 (50)	32 (59)	24 (66)
ACG ACA	38 (71)	29 (54)	36 (45)	29 (40)	39 (75)	25 (51)	42 (82)	28 (53)
Average	36 (69)	30 (55)	32 (59)	27 (45)	29 (55)	25 (49)	31 (58)	25 (51)

*Methylation of internal cytosine in the cited sequence of bases (5'-C^{m}CGG-3'); **Methylation of external cytosine in the cited sequence of bases (5'-^{m}CCGG-3'); BLP = Broad leaf phenotype; BLP/CC = Broad leaf phenotype with chemical control; NLP/CC = Narrow leaf phenotype with chemical control; NLP = Narrow leaf phenotype.

several factors, which may be climatic agents or even the management of the plant itself. When this observation is considered, it is noticeable that, even with morphological differences, the BLP/CC and NLP/CC may have shared methylations in common areas of their DNA in response to stress caused by the herbicide.

Francischini (2013), by assessing sugarcane clones, verified by MSAP technique that the most of somaclonal variations of clones were related to the epigenetic causes of DNA methylation. Scarabel et al. (2010), in their studies with weed/ruderal *Schoenoplectus mucronatus* Palla. from the Cyperaceae family, found that several Acetolactato Sintase (ALS) genes are present in the genome and are characterized by methylation of cytosine. George (1993) also noted that growth regulators, added to the culture media, served as a tool for genetic changes induction and alterations in the state of DNA methylation.

Regarding the genetic similarity of phenotypes, for the polymorphisms generated by the combination *EcoRI*/*MspI* (Table 7), the lowest value (0.394) of genetic similarity was obtained between the phenotypes NLP/CC and BLP, indicating that, for the regions sampled by MSAP markers, these 2 phenotypes would be the most different among those evaluated.

The highest value (0.500) of genetic similarity was found between NLP/CC and BLP/CC, followed by the similarity of very close value (0.490) between the phenotypes NLP and BLP, indicating that, among these phenotypes analyzed pairwise, there is a sharing around 50% of those markers obtained (Table 7). The average genetic similarity among all phenotypes for the polymorphism generated from the amplification of the digestion products *EcoRI*/*MspI* was 0.447.

Regarding the phenotypes genetic similarity for the polymorphism generated by the combination *EcoRI*/*HpaII* (Table 8), the lowest value (0.266) of genetic similarity was

Table 7. Matrix of genetic similarity* for phenotypes in relation to the polymorphism generated by the *primers* combination with 3 selective bases amplified from DNA digestion with the *EcoRI*/*MspI* enzymes.

Phenotypes	BLP	BLP/CC	NLP/CC	NLP
BLP	1.000			
BLP/CC	0.406	1.000		
NLP/CC	0.394	0.500	1.000	
NLP	0.433	0.490	0.460	1.000

*Coefficient of similarity of *Jaccard*. Matrix generated by the static program NTSYS, version 2.1. BLP = Broad leaf phenotype; BLP/CC = Broad leaf phenotype with chemical control; NLP/CC = Narrow leaf phenotype with chemical control; NLP = Narrow leaf phenotype.

Table 8. Matrix of genetic similarity* of material and phenotypes analyzed in relation to the polymorphism generated by the *primers* combination with 3 selective bases amplified from the DNA digestion with the *EcoRI*/*HpaII* enzymes .

Phenotypes	BLP	BLP/CC	NLP/CC	NLP
BLP	1.000			
BLP/CC	0.320	1.000		
NLP/CC	0.299	0.404	1.000	
NLP	0.266	0.377	0.365	1.000

*Coefficient of similarity of *Jaccard*. Matrix generated by the static program NTSYS, version 2.1. BLP = Broad leaf phenotype; BLP/CC = Broad leaf phenotype with chemical control; NLP/CC = Narrow leaf phenotype with chemical control; NLP = Narrow leaf phenotype.

obtained between the phenotypes NLP and BLP, while the highest value (0.404) was found between NLP/CC and BLP/CC. The average genetic similarity among all phenotypes for the polymorphism generated from the amplification of the digestion products *EcoRI/HpaII* was 0.338.

Comparing the average genetic similarity values for each enzyme combinations, it is noted that the highest value (0.447) was obtained with the selective *primers* amplification of the digestion products with the *EcoRI/MspI* enzymes in relation to the *EcoRI/HpaII*, whose value was 0.338. Thus, on average, the phenotypes evaluated share around 44.7% of methylations in the internal cytosine, while, for the external cytosine, the average sharing is 33.8%. These values may be considered as probable estimates of the methylation average values, respectively, internal and external of the species under studies, although a greater number of individuals of the species must be investigated, since just 1 individual of each phenotype was taken in this study.

By the dendrograms obtained with the results of similarity using *MspI* (Figure 3a) and *HpaII* (Figure 3b), 3 different groups were obtained. It is interesting to note that, in both dendrograms, the phenotypes BLP/CC and NLP/CC form the same group, despite their morphological differences regarding the leaf shape. However, these phenotypes have the same origin, i.e., they were collected from areas of intense herbicide application. This might suggest that they can share methylations in common areas of their DNA as a protection mechanism against the effects of stress suffered with the herbicide application.

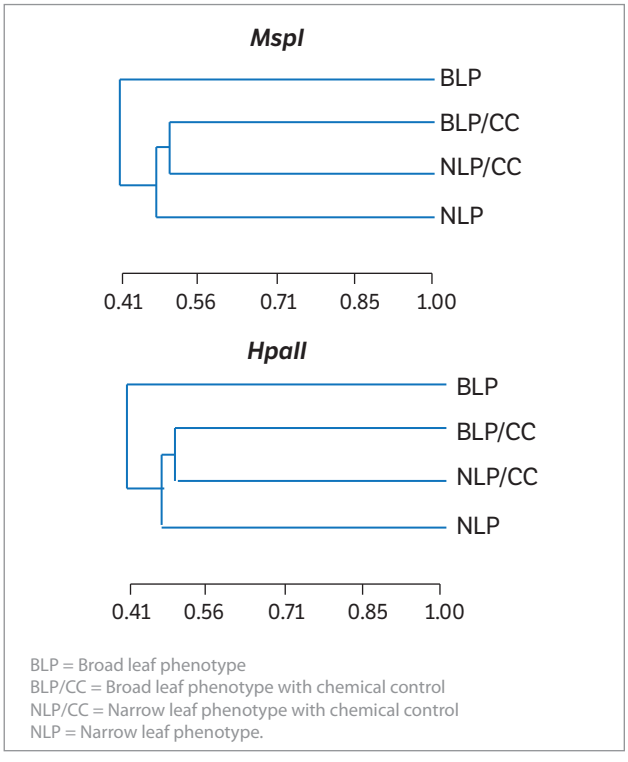


Figure 3. Dendrogram of genetic similarities among the phenotypes of *Conyza bonariensis* with Methylation Sensitive Amplified Polymorphism marker using the restriction enzymes *MspI* (a) and *HpaII* (b). Dendrogram generated by the static program NTSYS, version 2.1.

Even with little genetic diversity, the phenotypes of *C. bonariensis* showed differences in several morphological characteristics. Similar results were also found by Richards et al. (2008), in *Fallopia japonica* (Houtt.) Ronse Decr. with the weed/ruderal of Polygonaceae family, and by Marfil et al. (2009). The latter authors, in performing analyses to quantify the methylation level in wild potatoes, found that the methylation in the natural hybrid RZL (*Solanum kurtzianum* Bitter and Wittm × *S. chacoense* Bitter) could be an alternative explanation for many interespecific variations found in *S. chacoense* Bitter, *S. sparsipilum* (Bitter) Juz. and Bukasov and *S. Stoloniferum* Schltdl. and Bouché, if common to other species. Thus, plants classified as belonging to different species could be an epigenetic variation of the same species.

This study presents the first report in this area for *C. bonariensis*; however, it was not possible to relate the diversity of morphology and productions of NLP and BLP with DNA methylation levels. For Richards et al. (2008) and Loomis and Fishman (2009), knowing the sources of epigenetic variations may be particularly important in ruderal species, since many species have a good adaptation to different habitats, even with low levels of genetic variations.

The methodology of DNA methylation used did not allow associations with morphological alterations presented by the phenotypes of *C. bonariensis*. It is suggested, as a next step, the performance of molecular analyses for the identification of other specific regions, as well as the study of Quantitative Trait Loci (QTL) controllers, and methods of research mappings for the locus methylation of the underlying DNA associated with the morphological variations found in the phenotypes of *C. bonariensis*.

CONCLUSION

The fact that phenotypes NLP and BLP (a) maintain constant morphology after many cycles of cultivation →

under the same controlled conditions and (b) do not show differences between them in methylation patterns, which would conclusively prove an epigenetic effect for different morphologies, leads us to consider them as belonging to different taxa.

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