

Evidence of High Gene Flow Between Samples of Horseweed (*Conyza canadensis*) and Hairy Fleabane (*Conyza bonariensis*) as Revealed by Isozyme Polymorphisms

Alaim Anderson Fernandes Soares, Angela Maria Dalla Torre Fregonezi, Denis Bassi, Claudete Aparecida Mangolin, Sandra Aparecida de Oliviera Collet, Rubem Silvério de Oliveira Junior, and Maria de Fátima Pires da Silva Machado*

Native polyacrylamide gel electrophoresis was used to identify polymorphisms in α - and β -esterases loci and electrophoresis in starch gel to identify polymorphism in malate dehydrogenase (MDH; EC 1.1.1.37) and acid phosphatase (ACP; EC 3.1.3.2) isozymes loci in leaf tissues from samples of horseweed and hairy fleabane populations to determine genetic diversity and population structure. Similar or differential genetic divergence between the two species may guide specific use of herbicides. For samples of plants with high genetic similarity it is possible to adopt similar mechanisms and processes for their control. The proportion of polymorphic loci was 57.14, 50.0, and 53.6%, in samples of horseweed and hairy fleabane, for EST, MDH, and ACP isozymes, respectively. A comparison of the diversity parameters in the two species showed that the number of alleles is similar in the horseweed and hairy fleabane plants. The estimated heterozygosity in horseweed and hairy fleabane was also very close. A relatively low level of population differentiation was detected between horseweed and hairy fleabane ($F_{ST} = 0.0199$), which suggests a substantial genetic exchange among the two species. Accordingly, estimate of gene flow was high ($Nm = 12.3172$) for the alleles of the loci *Est*, *Mdh*, and *Acp*. The Nei's identity (*I*) values also was high ($I = 0.9561$) indicating very high similarity between the two *Conyza* species. AMOVA showed higher genetic variation within (95%) than among (5%) the two samples. The low genetic structure and high value of genetic identity was an important indication that alleles are exchanged between horseweed and hairy fleabane populations, and provides additional evidence of occurrence of outcrossing between populations or dispersion of samples of one for other site.

Nomenclature: Hairy fleabane, *Conyza bonariensis* (L.) Cronq.; horseweed, *Conyza canadensis* (L.) Cronq.

Key words: ACP isozymes, esterase isozymes, gene flow, genetic diversity, genetic structure of populations, hairy fleabane, horseweed, MDH isozymes.

The weed species horseweed and hairy fleabane occur in orchards, vineyards, corn, soybean, cotton, and also in forage crops and pastures, as well as in fallow areas (Lazaroto et al. 2008). Horseweed is an annual or biennial species, native of North America (Frankton and Mulligan 1987) and is the most widely distributed species of *Conyza* in the world (Thebaud and Abbott 1995), whereas hairy fleabane is an annual species, native to South America. The two species are frequently found in grasslands and crops in the Southern Brazil (Kissmann and Groth 1999).

Herbicide resistance in *Conyza* species has evolved in several countries with Japan reporting the first case of

resistance in 1980 for horseweed (paraquat) (Heap 2014) and Spain reporting the first case of hairy fleabane resistance (simazine) in 1987 (Prado 1987). Currently, a major concern is glyphosate resistance in *Conyza* species. Unique cases of *Conyza* sp. resistance to glyphosate account for more than 50% of the total number of resistance cases identified so far (Heap, 2014). In Brazil, glyphosate-resistant populations of horseweed and hairy fleabane have been identified in citrus orchards (Moreira et al. 2007) and glyphosate-resistant hairy fleabane has been identified in glyphosate-resistant soybean fields (Vargas et al. 2007). Glyphosate resistance was found among horseweed populations from Illinois, Indiana, Kentucky, Mississippi, Missouri, and Ohio (Main et al. 2004).

Mechanisms involved in glyphosate resistance have been widely investigated since the 1990's (Vidal and Fleck 1997). The characteristics of horseweed and hairy fleabane resistant biotypes have been investigated by many researchers (Lamego and

DOI: 10.1614/WS-D-14-00044.1

* First author: Post-graduate in Comparative Biology; second, and third authors: Graduate Students; fourth, fifth and seventh authors: Associate Professors, Department of Biotechnology, Genetics and Cell Biology; sixth author: Associate Professor, Department of Agronomy, State University of Maringá, Maringá-PR., Brazil. Corresponding author's E-mail: mfpmachado@uem.br

Vidal 2008; Main et al. 2004; Moreira et al. 2010; Penha et al. 2012; Trezzi et al. 2011; Vargas et al. 2007; Xiao-Ling et al. 2011; Yamauti et al. 2010). Different resistance mechanisms have been identified, suggesting significant variability among resistant biotypes (González-Torralva et al. 2010; Zheng et al. 2011). For modes of action such ALS inhibition, information exists regarding target-site mutations identified in resistant biotypes of horseweed (review by Zheng et al. 2011). On the other hand, far less attention has been focused on how horseweed and hairy fleabane populations are genetically structured and little is known about genetic diversity within and among populations. An understanding of genetic population structure and genetic diversity is essential for the development of effective management strategies. Genetically structured populations (with frequencies of alleles different), should be treated as different. On the other hand, not genetically structured populations may be treated as equal. If high heterozygosity is detected in weed, this can be an important indicator that the plant population has a substantial amount of adaptive genetic variation (Allendorf and Luikart 2007) and may escape the effects of a control agent that has limited the development and maintenance of the plants. Broad genetic variability is a factor that can contribute to the evolution of herbicide resistance in weeds and high genetic variability in horseweed and hairy fleabane populations may be one of the main factors contributing to the selection of resistant biotypes. Protein or enzyme electrophoresis is still the simplest and the best tool to analyze a population's genetic structure and to detect allele fixation at certain loci, the absence of heterozygotes at loci, and to suggest the presence of two or more reproductively isolated and genetically divergent populations (Allendorf and Luikart 2007). Esterase isozyme polymorphisms are co-dominant markers adequate for analysis of genetic structure. Polymorphism in α - and β -esterases loci in the weed species wild poinsettia (*Euphorbia heterophylla* L.) showed high genetic diversity and genetically structured populations (Frigo et al. 2009). Esterases (EC 3.2.1._) are often found in multigene families and a high level of polymorphism has so far been detected by polyacrylamide gel electrophoresis (PAGE) in different plant species (Carvalho et al. 2003; Carvalho et al. 2011; Frigo et al. 2009; Orasmo et al. 2007; Pereira et al. 2001; Sala et al. 2011). Other isozymes such as malate dehydrogenase (MDH; EC 1.1.1.37) and acid phosphatase (ACP; EC 3.1.3.2) are also potentially adequate biochemical markers used to

investigate genetic diversity in plants (Lopes et al. 2002; Mangolin et al. 1997).

In the current study, native PAGE was used to identify polymorphisms in α - and β -esterases loci and electrophoresis in starch gels was used to identify polymorphisms in MDH and ACP isozymes loci from samples of horseweed and hairy fleabane populations for analysis of genetic diversity and structure of populations. A high genetic variation within populations may be an obstacle to effectively controlling horseweed and hairy fleabane populations, since greater is the chance of finding alleles for resistance in a genome with high genetic diversity. Variable responses to herbicide application have been reported (Lamego and Vidal, 2008; Oliveira Neto et al. 2010). Moreover, similar or differential genetic divergence between the two species may indicate potential differential responses in relation to herbicide susceptibility.

Materials and Methods

Seeds of horseweed and hairy fleabane were obtained from AgroCosmos Produção e Serviços Rurais Ltda. (São Paulo State, Brazil). The seeds of each species (horseweed and hairy fleabane) were collected from several different plants, in two or more fields of soybean, in the rural area of Engenheiro Coelho, in the State of São Paulo (Brazil), where the company AgroCosmos provides technical assistance to producers of soybeans. The seeds of horseweed collected from several plants in two or more soybean fields were randomly mixed and recorded as samples of horseweed, while the same procedure was used for samples of hairy fleabane collected in different soybean fields although within the same perimeter rural. Seeds from each *Conyza* species were randomly distributed for germination in separated 500-ml pots containing sterile soil. Plants obtained from germinated seeds were maintained at room temperature, watered daily, and used for the experiments. Samples consisting of young leaves collected from 157 plants of horseweed and 151 plants of hairy fleabane were evaluated by electrophoresis. The seeds germination was lower in hairy fleabane than in horseweed resulting in the lowest number of plants. The young leaves were collected from plants with 15–30 days after seed germination. Leaf pieces (200 mg) were separately homogenized with a glass rod in an Eppendorf microcentrifuge tube with the use of 60 μ L extraction solution prepared with 1.0 M phosphate buffer pH 7.0, containing 5%

PVP-40, 1.0 mM EDTA, 0.5% β -mercaptoethanol, and 10% glycerol solution and maintained in an ice chamber. After homogenization, the samples were centrifuged at 25,000 rpm ($48,200 \times g$) for 30 min, at 4 C, in a Juan 23 MRi (Thermo Scientific, U.S.A.) centrifuge, and the supernatant was used for each sample.

Polyacrylamide gels (12%) were used to analyze the esterase isozymes (EST; EC 3.1.1._). The polyacrylamide gel was prepared with 0.375 M Tris-HCl, pH 8.8 as buffer (Frigo et al. 2009). A 6.2-ml volume of acrylamide/bis-acrylamide solution (30 g acrylamide and 0.8 g bis-acrylamide dissolved in 100 ml of twice-distilled water), 4.0 ml 1.5 M Tris-HCl, pH 8.0, 6.2 ml twice-distilled water, 320 ml ammonium persulfate 2%, and 16 ml TEMED were used to the gel of separation. The stacking gel was prepared with 3.0 ml acrylamide/bis-acrylamide (5 g acrylamide and 0.25 g bis-acrylamide dissolved in 50 ml twice-distilled water), 3.0 ml 0.24 M Tris-HCl, pH 6.8, 30 ml twice-distilled water, 250 ml ammonium persulfate (2%), and 3 ml TEMED.

The electrophoresis was performed for 5 h, at 4 C, at a constant voltage of 200 V. The running buffer was 0.125 M Tris/0.0959 M glycine pH 8.3.

Esterases were identified using procedures previously described by Frigo et al. (2009). Gels were soaked for 30 min in 50 ml 0.1 M sodium phosphate, pH 6.2, at room temperature. Esterase activity was visualized by placing the gels for 1 h in a staining solution prepared with 50 ml of sodium phosphate solution, 30 mg of β -naphthyl acetate, 40 mg of α -naphthyl acetate, 60 mg of Fast Blue RR salt and 5 ml of N-propanol. The polyacrylamide gels, dried as described by Frigo et al. (2009), were kept at room temperature for 60 min in a mixture of 7.5% acetic acid and 10% glycerol embedded in 5% gelatin. They were further placed between two sheets of wet cellophane paper stretched on an embroidering hoop and left to dry for 24 to 48 h.

MDH and ACP isozymes were analyzed using starch gels (16%) prepared in 0.0103 M Tris-0.0028 M citric acid, pH 7.0, and the electrode buffer consisted of 0.155 M Tris and 0.043 M citric acid, pH 7.0. Electrophoresis was carried out at 4 C for approximately 15 to 16 h, at 2.5 V cm^{-1} of gel.

MDH isozymes were stained with the reaction mixture reported by Machado et al. (1993). A staining solution containing 30 ml 0.1 M Tris/HCl buffer, pH 8.6, 300 mg malic acid, 10 mg NAD (β -nicotinamide adenine dinucleotide), 0.5 ml MTT

(thiazolyl blue; 10 mg ml^{-1}), 0.5 ml PMS (phenazinemetosulfate; 2 mg ml^{-1}), and 15 ml agar 2%, was used to visualize MDH isozymes. The gel was incubated for 30–60 min at 37 C. The ACP isozymes were visualized after 30 min on a gel incubated at 37 C with 50 ml 0.1 M sodium citrate, pH 5.0 (adjusted with acetic acid), 4 ml α -naphthyl phosphate 1% dissolved in acetone (50 ml acetone and 50 ml water), and 30 mg Fast blue RR salt ($\text{C}_{15}\text{H}_{14}\text{ClN}_3\text{O}_3 \cdot 1/2 \text{ ZnCl}_2$; Sigma-Aldrich). After staining, the gels were washed and fixed using a methanol : acetic acid : distilled water (5 : 5 : 1 v/v) solution for 3 to 5 min and finally washed with water.

The genetic variability in the samples of horseweed and hairy fleabane was analyzed with POPGENE 1.32 Computer Program (Yeh et al. 1999) for the analysis of allele frequencies, observed and expected mean heterozygosity (H_o and H_e) and mean number of alleles per locus (N_a), effective number of alleles per polymorphic locus (N_e), percentage of polymorphic loci (%P), χ^2 test for deviation from Hardy-Weinberg equilibrium. The F statistic of Sewall Wright (Wright 1965), the deficit of heterozygotes (F_{IS} and F_{IT}), and the genetic diversity between the two samples of *Conyza* species (F_{ST}) were also estimated using POPGENE 1.32. The F statistic is based on a $1/(2N)$ probability of obtaining the same allele per sampling from a finite diploid population with 100 individuals. To explore the hierarchical partitioning of genetic variation within and between the two samples of *Conyza*, we performed an Analysis of Molecular Variance (AMOVA, GenAlEx 6.2; Peakall and Smouse 2006). The genetic identity (Nei 1973) and distances among the *Conyza* samples were calculated by UPGMA grouping.

Results and Discussion

Native PAGE analysis for esterase isozymes in leaves of horseweed and hairy fleabane recorded with α -naphthyl acetate and β -naphthylacetate showed α - and β -esterases produced from seven clearly defined loci (Figure 1). The α - and β -esterases were numbered in sequence, starting from the anode, according to their decreasing negative charge. The number of loci was not identified in the case of esterase isozymes with lesser anodic migration. Two–four allelic variants were detected in the leaves of horseweed and hairy fleabane plants. Allele frequencies were analyzed for *Est-1*, *Est-2*, *Est-3*, *Est-4*, and *Est-5* loci; allele variations were not clarified for the *Est-6* and *Est-7*

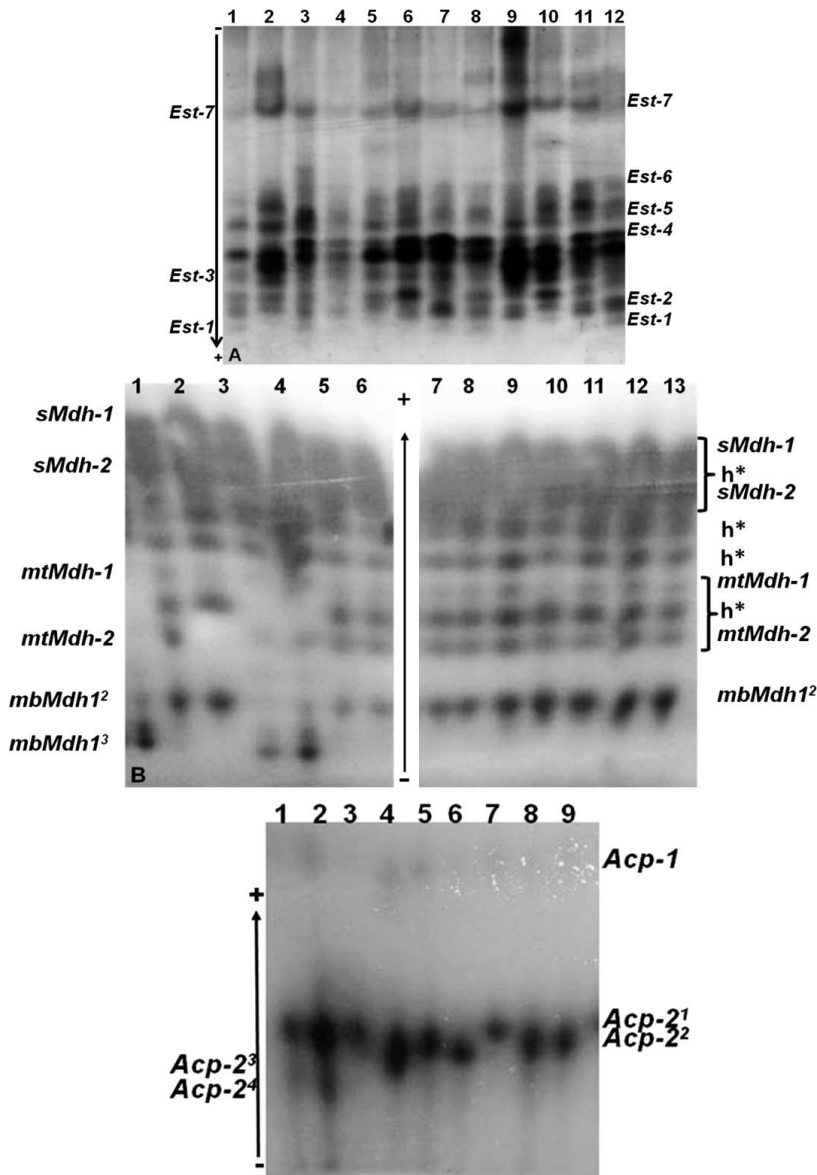


Figure 1. Polymorphism of α - and β -esterases (A), malate dehydrogenase (B), and acid phosphatase (C) isozymes detected in horseweed, produced from *Est-1*, *Est-2*, *Est-3*, *Est-4*, *Est-5*, *mbMdh-1*, and *Acp-2* loci; the different phenotypes are represented in lines: (A): 1) *Est-1*^{1/2}/*Est-2*^{1/3}/*Est-3*^{2/2}/*Est-4*^{2/2}/*Est-5*^{1/1}; 2) *Est-1*^{3/3}/*Est-2*^{1/2}/*Est-3*^{1/2}/*Est-4*^{2/2}/*Est-5*^{1/1}; 3) *Est-1*^{1/2}/*Est-2*^{1/3}/*Est-3*^{2/2}/*Est-4*^{1/2}/*Est-5*^{1/2}; 4) *Est-1*^{2/2}/*Est-2*^{3/3}/*Est-3*^{2/2}/*Est-4*^{1/1}/*Est-5*^{2/2}; 5) *Est-1*^{4/4}/*Est-2*^{1/3}/*Est-3*^{2/2}/*Est-4*^{1/2}/*Est-5*^{1/1}; 6) *Est-1*^{2/3}/*Est-2*^{2/3}/*Est-3*^{2/2}/*Est-4*^{1/2}/*Est-5*^{1/2}; 7) *Est-1*^{2/2}/*Est-2*^{3/3}/*Est-3*^{2/2}/*Est-4*^{1/1}/*Est-5*^{2/2}; 8) *Est-1*^{2/2}/*Est-2*^{1/3}/*Est-3*^{2/2}/*Est-4*^{1/1}/*Est-5*^{2/2}; 9) *Est-1*^{2/3}/*Est-2*^{3/4}/*Est-3*^{1/2}/*Est-4*^{1/2}/*Est-5*^{1/1}; 10) *Est-1*^{1/3}/*Est-2*^{2/3}/*Est-3*^{1/2}/*Est-4*^{1/2}/*Est-5*^{1/2}; 11) *Est-1*^{2/3}/*Est-2*^{3/4}/*Est-3*^{2/2}/*Est-4*^{1/1}/*Est-5*^{2/2}; 12) *Est-1*^{1/2}/*Est-2*^{3/3}/*Est-3*^{2/2}/*Est-4*^{1/1}/*Est-5*^{1/2}; (B): lines 1-2) *mbMDH-1*^{2/2}; 3-4) *mbMDH-1*^{3/3}; 5-13) *mbMDH-1*^{2/2}; (C): lines 1) *ACP-2*^{1/1}; 2) *ACP-2*^{1/4}; 3) *ACP-2*^{1/1}; 4) *ACP-2*^{2/3}; 5) *ACP-2*^{2/2}; 6) *ACP-2*^{3/3}; 7) *ACP-2*^{1/1}; 8-9) *ACP-2*^{2/2}.

loci. Polymorphism of *Est-1*, *Est-2*, *Est-3*, *Est-4*, and *Est-5* loci in the two *Conyza* species revealed 2 to 4 alleles that encode monomeric esterase forms (Figure 1A; Table 1).

MDH isozyme phenotypes revealed three anodal regions with distinct mobility (Figure 1B). The faster anodal region was considered to be the cytoplasmic MDH isozyme (sMDH); the region of intermediate activity was considered to be of mitochondrial origin (mtMDH), and the slower

region was considered to be microbody isozymes (mbMDH), in agreement with data reported for most vegetables (Jorge et al. 1997; Machado et al. 1993; Newton 1983; Pereira et al. 2006; Resende et al. 2000). Similarly to MDH isozyme pattern in maize reported by Pereira et al. (2006), the phenotype formed by three regularly spaced bands with the intermediate band being more intensely stained than the other bands seems to be a typical pattern to be expected for random binding of

Table 1. Number of alleles (Na) and number of effective alleles (Ne) per polymorphic locus, mean observed heterozygosity (Ho) and expected heterozygosity (He), in samples of hairy fleabane and horseweed plants.

	<i>Conyza bonariensis</i>					<i>Conyza canadensis</i>				
	N	Na	Ne	Ho	He	N	Na	Ne	Ho	He
<i>Est-1</i>	75	4.0	2.81	0.2667	0.6436	71	4.0	3.31	0.5070	0.6976
<i>Est-2</i>	75	4.0	3.01	0.5067	0.6680	71	4.0	3.03	0.4789	0.6700
<i>Est-3</i>	75	2.0	1.30	0.2667	0.2311	71	2.0	1.34	0.2394	0.2520
<i>Est-4</i>	75	2.0	1.96	0.5333	0.4892	71	2.0	1.79	0.3521	0.4429
<i>Est-5</i>	75	2.0	1.84	0.5733	0.4570	71	2.0	2.00	0.5775	0.5000
<i>mbMdh-1</i>	68	3.0	2.74	0.4853	0.6353	58	3.0	2.32	0.2586	0.5682
<i>Acp-2</i>	68	4.0	3.32	0.3824	0.6986	58	4.0	2.77	0.5345	0.6394
Mean	68	3.0	2.43	0.4306	0.5461	65	3.0	2.37	0.4211	0.5386

peptide chain produced by two genes (*sMdh-1* and *sMdh-2*, and *mtMdh-1* and *mtMdh-2* loci). The intergenic heterodimers between the *mtMdh-1* and *mtMdh-2* loci product (mtMDH-1/mtMDH-2 isozyme) and between the *sMdh-1* and *sMdh-2* loci products (sMDH-1/sMDH-2) have been formed. Likewise, intergenic heterodimers between the *mtMdh-2* and *sMdh-2* loci product (mtMDH-2/sMDH-2 isozyme) and between the *mtMdh-1* and *sMdh-1* loci products (mtMDH-1/sMDH-1) also have been formed. The intergenic heterodimers between the *mtMdh-1* and *sMdh-2* loci product and between *mtMdh-2* and *sMdh-1* loci product probably have coincident migration with homodimeric or heterodimeric bands. Allelic variation was detected at *sMdh-1*, *mtMdh-2*, and *mbMdh-1* loci. However, allele frequency was analyzed only for *mbMdh-1* showing three alleles (*mbMdh-1*¹, *mbMdh-1*², and *mbMdh-1*³ alleles). The definition of number and frequency of alleles for *sMdh-1*, *mtMdh-2* loci was uncertain due the inter-loci heterodimers formation and coincident migration of isozymes.

Two loci for ACP isozymes (*Acp-1* and *Acp-2*) were evident in the two *Conyza* populations. Four alleles were clearly observed for *Acp-2* locus (*Acp-2*¹, *Acp-2*², *Acp-2*³, and *Acp-2*⁴ alleles), but the definition of number and frequency of alleles for *Acp-1* locus also was uncertain since that isozymes from *Acp-1* locus were weakly stained on starch gels (Figure 1C).

A total of 15 loci for esterases (7), MDH (5), and ACP (3) isozymes were identified in samples of horseweed and hairy fleabane. The proportion of polymorphic loci was 57.14, 50.0, and 53.6%, respectively. A comparison of the diversity parameters in the two species (Table 1) shows that the number of alleles is similar in the horseweed (Na = 3.0; Ne = 2.37) and hairy fleabane (Na = 3.0; Ne = 2.43) plants. The estimated heterozygosity in horseweed (Ho = 0.4211; He = 0.5386) and hairy fleabane (Ho = 0.4306; He = 0.5461) was also very similar.

Departure from Hardy–Weinberg equilibrium was observed in the *Est-1*, *Est-2*, *Mdh-1*, and *Acp-2* loci of the horseweed samples and in the *Est-1*, *Est-2*, *Est-5*, *Mdh-1*, and *Acp-2* loci of the hairy fleabane samples, resulting from a deficiency in heterozygous plants. The fixation index (F_{IS}) was negative only in *Est-3* ($F_{IS} = -0.0475$) and *Est-5* ($F_{IS} = -0.2025$) loci indicating a deficit in homozygous plants (Table 2).

Since the observed heterozygosity was lower than the expected value in the *Est-1*, *Est-2*, *Est-3*, *Est-4*, *Mdh-1*, and *Acp-2* loci of horseweed and in the *Est-1*, *Est-2*, *Mdh-1*, and *Acp-2* loci of hairy fleabane plants (Table 1), an excess of homozygous plants occurred in both species, as is expected for species where self-pollination is predominantly the mating model.

A relatively low level of population differentiation was detected between horseweed and hairy fleabane species ($F_{ST} = 0.0199$), which suggests a substantial genetic exchange among the two species (Table 2). Accordingly, estimate of gene flow calculated from F_{ST} (Nm) was high (Nm = 12.3172) for the alleles of the loci *Est*, *Mdh*, and *Acp* (Table 2). The Nei's identity (I) values also was high (I = 0.9561) indicating very high similarity between the two *Conyza* species. AMOVA showed higher genetic variation within (95%; Sum of

Table 2. Fixation coefficients F (F_{IS} , F_{IT} , F_{ST} ; Wright 1965) and gene flow (Nm), in samples of hairy fleabane and horseweed plants.

Locus	N	F_{IS}	F_{IT}	F_{ST}	Nm
<i>Est-1</i>	146	0.4231	0.4394	0.0283	8.5949
<i>Est-2</i>	146	0.2634	0.2657	0.0031	79.8426
<i>Est-3</i>	146	-0.0475	-0.0471	0.0004	570.2336
<i>Est-4</i>	146	0.0501	0.0593	0.0097	25.4542
<i>Est-5</i>	146	-0.2025	-0.1761	0.0220	11.1219
<i>mbMdh-1</i>	126	0.3819	0.4010	0.0310	7.8090
<i>Acp-2</i>	126	0.3148	0.3354	0.0300	8.0764
Mean	140.5	0.2149	0.2306	0.0199	12.3172

Squares = 672.76; Variance Components = 4.67) than among (5%; Sum of Squares = 22.02; Variance Components = 22.02) the two samples.

Our results showed that the proportion of polymorphic loci and the mean heterozygosity values are high in both *Conyza* species. However, the observed heterozygosity was lower than the expected. The departure from Hardy–Weinberg equilibrium observed and the positive values of F_{IS} observed in the horseweed and hairy fleabane samples indicate a deficit of heterozygous plants, which could be the result of an artificial selective pressure imposed by herbicide applications in the original populations of both species. An artificial selection pressure with a reduction in population size (by death of plants) may lead to loss of alleles and consequently a reduction of heterozygous, with prevalence of homozygous phenotypes. Self-compatibility in *Conyza* species may also explain the significant proportion of homozygous phenotypes; moderate to high levels of endogamy (values of F_{IT} ; Table 2) were observed at *Est-1*, *Est-2*, *Mdh-1*, and *Acp-2* loci. *Conyza canadensis* is described as a self-compatible species (Mulligan and Findlay 1970; Weaver 2001) where the pollen is released before the capitula have fully opened, suggesting that it is primarily self-pollinated, although insects have been observed visiting open flowers (Smisek 1995). Self-pollination has also been described in hairy fleabane (Ferrer and Good-Avila 2007). However, analysis of polymorphic loci *Est-1*, *Est-2*, *Est-3*, *Est-4*, *Est-5*, *Mdh-1*, and *Acp-2* loci in horseweed and hairy fleabane plants have indicated a significant gene flow between the samples of the two species, suggesting occurrence of outcrossing between the samples (exchange of alleles) or dispersion of samples of one for other site. As the plants of horseweed and hairy fleabane were sampled from soybean fields within the same perimeter rural the genes can be moved by pollen flux or seed movement. According to Govindaraju (1989), values of $Nm > 1.0$ indicate high gene flow among populations; $Nm < 0.25$ indicate a low gene flow and values ranging from 0.25 to 0.99 indicate an intermediary gene flow.

The low level of population differentiation in the genetic structure of horseweed and hairy fleabane samples ($F_{ST} = 0.0199$) provides additional evidence of occurrence of outcrossing between the samples or mixing samples of the two species, which were erroneously identified as being of only a species; the samples of horseweed and hairy fleabane may be mixed in fields where they were

identified as being only one species. According to Wright (1978), values of F_{ST} ranging from 0.01 to 0.05 indicate a minimal difference between populations; F_{ST} values ranging from 0.05 to 0.15 indicate a moderate difference, while F_{ST} values ranging from 0.15 to 0.25 indicate a high difference among these populations. High value of genetic identity ($I = 0.95612$) also was found among the samples of horseweed and hairy fleabane. A level of genetic identity higher than 0.85 is frequently detected between populations from the same species (Thorpe and Solé-Cava 1994). These data are in agreement with the higher genetic variation within the samples of horseweed and hairy fleabane plants (95%) as compared to between (5%) the samples of the two species.

Previous studies by Thebaud and Abbott (1995) reported the occurrence of hybridization between horseweed and other species of the genus *Conyza*, especially tall fleabane (*Conyza sumatrensis* (Retz.)) E. Walker and hairy fleabane, because they often grow in associated populations. Although horseweed is diploid, with chromosome number $2n = 18$, while the other species are polyploids, the evidences from our study confirm suggestions previously reported, since high gene flow between the samples of the horseweed and hairy fleabane for *Est-1*, *Est-2*, *Est-3*, *Est-4*, *Est-5*, *Mdh-1*, and *Acp-2* loci have been shown. Differential ploidy may not be a restrictive factor for changes of alleles between *Conyza* plants. The ploidy of hairy fleabane has been investigated and considered difficult to be defined. The genus *Conyza* has been appointed as a complex polyploid. Different samples of hairy fleabane have been reported as having a diploid, tetraploid, hexaploid, and pentaploid chromosomal complement (Mendes et al. 2012; Thebaud and Abbott 1995). Guareschi et al. (2012) assumed that the tetraploid plants found native from Rio Grande do Sul State (Brazil) are originated from the nonreduced gametes of two diploid plants of hairy fleabane, which should present $2n = 18$, and then formed individuals $2n = 4x = 36$, and pentaploid plants may have originated from the natural crossing of a tetraploid plant who has formed unreduced gametes ($4n$) with a diploid plant of normal gametes (n) resulting in plants $5n$.

High genetic variation in isozyme loci observed in horseweed and hairy fleabane samples may explain the variable responses to herbicide application and may be also correlated to morphological polymorphism that has been reported for both species (González-Torralva et al. 2010; Main et al.

2004; Urdampilleta et al. 2005). The polymorphism analysis in the isozyme loci showed no specific alleles in either *Conyza* species, which could be used as markers to differentiate samples of horseweed from hairy fleabane, but was important to provide evidences of allele exchanges or of mixing samples of the two species in fields of soybean, in the rural area of Engenheiro Coelho, in the State of São Paulo, Brazil. In fields of soybean in the Southeast region where one of the *Conyza* species is predominant may have samples of the other specie, taxonomically identified as different morphotypes of the same species by agricultural technicians. Exchange of alleles is important for generating new forms of recombination, which have influence in the variability of natural populations to the adaptive process (Allendorf and Luikart 2007). Okada et al. (2013) found no difference in genetic diversity in susceptible and resistant horseweed using micro-satellite markers, but it is expected that exchange of alleles favors recombination increasing then the diversity due to recombination events. The gene flow between weed species may produce an impact on dissemination of herbicide resistance alleles or in the evolution of novel taxa with diverse “weedy” traits. Zelaya et al. (2007) have illustrated in laboratorial studies the potential for the occurrence of interspecific hybrid progenies that are vigorous and fertile, capable of transferring the R allele (glyphosate resistance allele; Zelaya et al. 2004), although the hybrid demonstrated in this research has not been specifically identified in the field. Hybrids of unknown fecundity under natural environments were reported in Spain and France, originated from crosses between horseweed and hairy fleabane, and between hairy fleabane and tall fleabane (McClintock and Marshall 1988).

Therefore, there is a possibility of crosses occurring between horseweed and hairy fleabane plants in Brazil, which may explain (1) the variable responses to herbicide applications; (2) the morphological polymorphism indicated by different authors (Guareschi et al 2012; Urdampilleta et al. 2005), (3) the high polymorphism of isozymes, and (4) high gene flow observed in our study. The evidences from the current study recommend that farmers should consider the potential for hybridization between horseweed and hairy fleabane when developing programs aimed at managing herbicide-resistant weeds. Hybridization can lead to the development of new biotypes with varying morphologies, and physiology and mechanisms of resistance differentiated of the parental species. New

biotypes may require different strategies for their control. For populations with higher identity values as the samples analyzed in our study ($I = 0.95612$) it may be possible to adopt similar mechanisms and processes for their control. However, for the future control of horseweed and hairy fleabane populations showing high level of mean heterozygosity, the use of combinations of herbicides that have different mechanisms of action is advisable. High heterozygosity has been considered an indicator that the plant population has a substantial amount of adaptive genetic variation (Allendorf and Luikart 2007) and may escape of the effects of a control agent. The association of glyphosate with different concentrations and combinations of herbicides such as 2,4-D, metsulfuron methyl, paraquat, diuron, and glufosinate of ammonium, in sequential applications has shown significant effect on reduction of the number of plants per m^2 of hairy fleabane (Paula et al., 2011; Vargas et al., 2007). The reduction of the number of plants may reduce or limit the potential for dissemination of herbicide resistance through introgressive hybridization, mitigating the evolution of the herbicide resistance mechanisms.

Acknowledgments

The authors would like to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasília DF Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for their financial support and Dr. Scott Nissen (Colorado State University) for his review of the manuscript.

Literature Cited

- Allendorf FW, Luikart G (2007) Conservation and the genetics of populations. Blackwell Publishing, Malden. 642 p
- Carvalho VM, Marochio CA, Mangolin CA, Machado MFPS (2011) The use of esterase polymorphism for analysis of the genetic diversity and structure of stevia (*Stevia rebaudiana* Bert. Bertonii) populations. *Biochem Syst Ecol* 39:594–599
- Carvalho VM, Marques RM, Lapenta AS, Machado MFPS (2003) Functional classification of esterases from leaves of *Aspidosperma polyneuron* M. Arg. (Apocynaceae). *Genet Mol Biol* 26:195–198
- Ferrer MM, Good-Avila SV (2007) Macrophylogenetic analyses of the gain and loss of self-incompatibility in the Asteraceae. *New Phytologist* 173:401–414
- Frankton C, Mulligan GA (1987) Weeds of Canada (revised). Publication 948. Ministry of Supply and Services Canada. NC Press Limited, Toronto, ON: Frankton and Mulligan. 217 p
- Frigo MJ, Mangolin CA, Oliveira Jr RS, Machado MFPS (2009) Esterase polymorphism for analysis of genetic diversity and structure of wild poinsettia (*Euphorbia heterophylla*) populations. *Weed Sci* 57:54–60

- González-Torralva F, Cruz-Hipolito H, Bastida F, Müllender N, Smeda RJ, Prado R (2010) Differential susceptibility to glyphosate among the *Conyza* weed species in Spain. *J Agric Food Chem* 58:4361–4366
- Govindaraju DR (1989) Variation in gene flow levels among predominantly self-pollinated plants. *J Evol Biol* 2:173–181
- Guareschi A, Pastori T, Kruse ND, Machado SLO, Dorow TSC, Tedesco SB (2012) Cytogenetics characterization of *Conyza bonariensis* (L.) Cronquist populations from Brazil. *Ciência e Natura* 34:39–48
- Heap I. The International Survey of Herbicide Resistant Weeds. <http://www.weedscience.org>. Accessed September 8, 2014
- Jorge IC, Mangolin CA, Machado MFPS (1997) Malate dehydrogenase isozymes (MDH; EC 1.1.1.37) in long-term callus culture of *Cereus peruvianus* (Cactaceae) exposed to sugar and temperature stress. *Biochem Genet* 35:155–164
- Kissmann KG, Groth D (1999) Plantas Infestantes e Nocivas. 2th ed. São Bernardo do Campo: Basf. Pp 152–156, 278–284
- Lamego FP, Vidal RA (2008) Resistência ao glyphosate em biótipos de *Conyza bonariensis* e *Conyza canadensis* no Estado do Rio Grande do Sul, Brasil. *Planta Daninha* 26:467–471
- Lazaroto CA, Fleck NG, Vidal RA (2008) Biologia e ecofisiologia de buva (*Conyza bonariensis* e *Conyza canadensis*). *Ciência Rural* 38:852–860
- Lopes R, Bruckner CH, Lopes MTG (2002) Polimorfismo isozimático e potencial de utilização das isozimas como marcadores genéticos em aceroleira. *Pesq Agropec Brasil* 37:151–158
- Machado MFPS, Prioli AJ, Mangolin CA (1993) Malate dehydrogenase (MDH; EC 1.1.1.37) isozymes in tissue and callus cultures of *Cereus peruvianus* (Cactaceae). *Biochem Genet* 31:167–172
- Main CL, Mueller TC, Hayes RM, Wilkerson JB (2004) Response of selected Horseweed (*Conyza canadensis* (L.) Cronq.) populations to glyphosate. *J Agric Food Chem* 52:879–883
- Mangolin CA, Prioli AJ, Machado MFPS (1997) Isozyme variability in plants regenerated from calli of *Cereus peruvianus* (Cactaceae). *Biochem Genet* 35:189–204
- McClintock D, Marshall JB (1988) On *Conyza sumatrensis* (Retz) E. Walker and certain hybrids in the genus. *Watsonia* 17:172–173
- Mendes S, Portugal J, Calha I (2012) Glyphosate resistance survey on *Conyza canadensis*. *Revista de Ciências Agrárias* 35:322–328
- Moreira MS, Melo MSC, Carvalho SJO, Nicolai M, Christoffoleti PJ (2010) Herbicidas alternativos para controle de biótipos de *Conyza bonariensis* e *C. canadensis* resistentes ao glyphosate. *Planta Daninha* 28:167–175
- Moreira MS, Nicolai M, Carvalho SJP, Christoffoleti PJ (2007) Glyphosate-resistance in *Conyza canadensis* and *C. bonariensis*. *Planta Daninha* 25:157–164
- Mulligan GA, Findlay JN (1970) Reproductive systems and colonization in Canadian weeds. *Can J Bot* 5:859–860
- Nei M (1973) Analysis of gene diversity in subdivided populations. *Proc Nat Acad Sci USA* 70:3321–3323
- Newton KJ (1983) Genetics of mitochondrial isozymes. Pages 159–176 in Tanksley SD, Orton TJ, eds. *Isozymes in Plant Genetics and Breeding, Part A*. New York: Elsevier Science
- Okada M, Hanson BD, Hembree KJ, PengY, Shrestha A, Stewart Jr CN, Wright SD, Jasieniuk M (2013) Evolution and spread of glyphosate resistance in *Conyza canadensis* in California. *Evolutionary Applications* 6:761–777
- Oliveira Neto AM, Constantin J, Oliviera Jr RS, Guerra N, Dan HA, Alonso DG, Blainski E, Santos G (2010) Winter and summer management strategies for *Conyza bonariensis* and *Bidens pilosa* control. *Planta Daninha* 28:1107–1116
- Orasmo GR, Collet SAO, Lapenta AS, Machado MFPS (2007) Biochemical and genetic polymorphisms for carboxylesterase and acetyltransferase in grape clones of *Vitis vinifera* L. (Vitaceae) cultivars. *Biochem Genet* 45:663–670
- Paula JM, Vargas L, Agostinetto D, Nohatto MA (2011) Management of glyphosate-resistant *Conyza bonariensis*. *Planta Daninha* 29:217–227
- Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6:288–295
- Penha ETS, Aparecido LEO, Souza PS, Souza CAS (2012) Aplicação de doses do herbicida *glyphosate* no controle da *Conyza bonariensis*. *Rev Agrogeoambiental* 4:1–6
- Pereira AJ, Lapenta AS, Vidigal-Filho PS, Machado MFPS (2001) Differential esterase expression in leaves of *Manihot esculenta* Crantz infected with *Xanthomonas axonopodis* pv. *Manihotis*. *Biochem Genet* 39:289–296
- Pereira LK, Mangolin CA, Scapim CA, Pacheco CAP, Bonato CM, Machado MFPS (2006) Malate dehydrogenase isozyme patterns in four cycles of half-sib selection from CMS-43 popcorn variety (*Zea mays* L.). *Maydica* 51:561–566
- Prado R (1987) Group C1/5 resistant hair fleabane (*Conyza bonariensis*). The international survey of herbicide resistant weeds. <http://www.weedscience.org>. Accessed February 20, 2014
- Resende AG, Vidigal-Filho PS, Machado MFPS (2000) Isozyme diversity in cassava cultivars (*Manihot esculenta* Crantz). *Biochem Genet* 38:203–211
- Sala J, Mangolin CA, Franzoni J, Machado MFPS (2011) Esterase polymorphism and the analysis of genetic diversity and structure in cactus populations descended from *Cereus peruvianus* plants regenerated in vitro. *Biochem Genet* 49:270–282
- Smisek AJ (1995) The evolution of resistance to paraquat in populations of *Erigeron canadensis* L. M.S. thesis. London, Ontario: University of Western Ontario. 102 p
- Thebaud C, Abbott RJ (1995) Characterization of invasive *Conyza* species (Asteraceae) in Europe: quantitative trait and isozyme analysis. *Am J Bot* 3:360–368
- Thorpe JP, Solé-Cava AM (1994) The use of allozyme electrophoresis in invertebrate systematic. *Zoologica Scripta* 23:3–18
- Trezzi MM, Vidal RA, Xavier E, Rosin D, Balbinot AA, Prates MA (2011) Resistance to glyphosate in *Conyza* spp. biotypes in Western and Southwestern Parana, Brazil. *Planta Daninha* 29:1113–1120
- Urdampilleta JD, Amat AG, Bidau C (2005) Karyotypic studies and morphological analysis of reproductive features in five species of *Conyza* (Asteraceae: Asteraceae) from Northwestern Argentina. *Bol Soc Argent Bot* 40:91–99
- Vargas L, Bianchi MA, Rizzardi MA, Agostinetto D, Dal Magro T (2007) *Conyza bonariensis* biotypes resistant to the glyphosate in Southern Brazil. *Planta Daninha* 25:573–578
- Vidal RA, Fleck NC (1997) Análise do risco da ocorrência de biótipos de plantas daninhas resistentes aos herbicidas. *Planta Daninha* 15:152–161
- Weaver SE (2001) The biology of Canadian weeds; *Conyza canadensis*. *Canadian J Plant Sci* 4:867–875
- Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution* 19:395–399

- Wright S (1978) *Evolution and the Genetics of Population, Variability Within and Among Natural Populations*. Chicago: University of Chicago Press, 590 p
- Xiao-ling S, Jia-jun W, Hong-jun Z, Sheng Q (2011) Occurrence of glyphosate-resistant horseweed (*Conyza canadensis*) population in China. *Agricult Sci China* 10:1049–1055
- Yamauti MS, Barroso AAM, Souza MC, Alves PLCA (2010) Chemical control of glyphosate-resistant horseweed (*Conyza canadensis*) and hairy fleabane (*Conyza bonariensis*) biotypes. *Rev Cienc Agron* 41:495–500
- Yeh FC, Yang R, Boyle T (1999) *Popgene* version 1.32: Microsoft Windows-based freeware for population genetic analysis. Calgary, AB: Centre for International Forestry Research, University of Alberta
- Zelaya I, Owen MDK, VanGessel MJ (2004) Inheritance of evolved glyphosate resistance in *Conyza canadensis* (L.) Cronq. *Theor Appl Gen* 110:58–70
- Zelaya IA, Owen MDK, VanGessel MJ (2007) Transfer of glyphosate resistance: evidence of hybridization in *Conyza* (Asteraceae). *Am J Bot* 94:660–673
- Zheng D, Kruger GR, Singh S, Davis VM, Tranel PJ, Weller SC, Johnson WG (2011) Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. *Pest Manag Sci* 67:1486–1492

Received March 27, 2014, and approved March 19, 2015.