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## Cytogenetic effect of *Alternanthera philoxeroides* (alligator weed) on *Agasicles hygrophila* (Coleoptera: Chrysomelidae) in its native range

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### ABSTRACT

Plant genotypic effects can alter insects' ability of utilizing their host plant. Alligator weed, *Alternanthera philoxeroides*, is a South American amphibious Amaranthaceae, which invades aquatic and terrestrial habitats. Populations with varying morphotypes and cytotypes were identified and further associated with a polyploid hybrids complex in Argentina, which probably is the centre of origin and diversification of alligator weed. The current model of the hybrid complex formation proposes that diploid ancestors gave rise to allotetraploids and allohexaploids.

*Agasicles hygrophila* is a monophagous chrysomelid beetle which successfully controls *A. philoxeroides* in regions with mild winters and where the weed grows in aquatic habitats. However, plant genetic characteristics could constrain insect development and reproduction, providing an alternative explanation for the restricted success of biological control of alligator weed with *A. hygrophila*. We investigated the effect of four *A. philoxeroides* populations with varying cytogenetical characteristics on plant–insect interaction with *A. hygrophila* in the native range of the weed. The insect's life cycle on each host population was studied. In addition, the genetic relationships among the weed populations was explored using dot blot analysis.

Plant population affected insect survival and fecundity, but not life cycle length nor third larval instar mortality, pupal mortality or female proportion. Plant internode diameter was not a restriction for insect pupation. All hexaploid populations of *A. philoxeroides*, as well as the close related *A. aquatica*, hybridized with the tetraploid population probe with similar intensity, meaning that this population is a component of their polyploid genome.

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### 1. Introduction

Successful biological control of weeds relies on natural enemies being capable of significantly reducing weed density. This achievement depends on both the insect's ability to establish a growing population as well as exerting sufficient individual damage. Factors restricting insect establishment range from physical environmental characteristics to fine-tuned matching with the host plant. The latter involves plant genotypic effects on insect performance and plant phenotypic plasticity (Lym and Carlson, 2002; Manrique et al., 2008).

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Alligator weed, *Alternanthera philoxeroides* (Martius) Grisebach, is a South American amphibious Amaranthaceae, which invades aquatic and terrestrial habitats in the United States, Australia, China and other countries (Julien et al., 1995; Buckingham, 2002; Geng et al., 2007). In its native range of Argentina it grows as an emergent plant in wetlands, but it is also recorded growing as a terrestrial plant. In its invaded ranges it may grow as a terrestrial plant (in crops, lawns and unused land), as an emergent in wetland habitats and it may develop thick, floating mats covering deep water bodies. It competes with crops, displaces native plants, disrupts water flow causing increased sedimentation, prevents drainage, causes flooding, affects navigation, limits access to water, causes reduced oxygen levels beneath the mats and increases availability of habitat for mosquitoes (Julien, 1995; Buckingham, 2002).

The genus *Alternanthera* has about 30 species in Argentina (Pedersen, 1999), which probably is the centre of origin and diversification of alligator weed. Okada (1985) recognized two different cytotypes of alligator weed within the native range;

invasive hexaploids ( $2n = \text{ca. } 102$ ) and non-invasive tetraploids ( $2n = \text{ca. } 68$ ), suggesting that the ploidy level might affect plant physiology in a way that modifies its invasiveness. Moreover, different morphotypes and cytotypes were identified and further associated with a polyploid hybrid complex within the species. The current model of the *A. philoxeroides* hybrid complex formation proposes that diploid ancestors gave rise to autotetraploids and allotetraploids. The only tetraploid for which a complete characterization of the meiotic behavior is available corresponds to an allotetraploid ( $2n = \text{ca. } 66$ ) which shows tetravalent chromosomes during meiosis, sexual reproduction and fertile seed production (Table 1). Probably due to a lack of reduction in meiosis I, these allotetraploids subsequently produced two different types of hexaploids ( $2n = \text{ca. } 100$ ). The first group of hexaploids is characterized by reproduction only through asexual means, irregular meiosis and low pollen viability. The second corresponds to hexaploids with normal meiosis and sexual reproduction. Both types of hexaploids have been found in the field (Table 1). However, it remains unknown which parents gave rise to the alligator weed hybrids (Sosa et al., 2008).

*Agasicles hygrophila* Selman and Vogt (Coleoptera: Chrysomelidae) is a monophagous insect used as a biological control agent for *A. philoxeroides*, native to the Paraná river basin, Río de la Plata and the maritime borders of Uruguay and southern Brazil (Vogt and McGuire, 1979). Females deposit an average of 32 eggs in a mass of two parallel rows on the underside of alligator weed leaves. The eggs are elongate oval with average length and width of 1.25 mm and 0.38 mm, respectively. The three larval stages and adults feed on alligator weed leaves. Pupation takes place inside the hollow stem of the plant. Prepupae chew a circular hole in the internode of the plant and then regurgitate the ingested stem to plug the entrance of the cavity. Adult females are larger than males (7 mm and 5.7 mm long, respectively). Additionally, adult males can be recognized by the conspicuous concavity on the fifth visible abdominal sternite, a secondary sexual characteristic (Maddox, 1968).

*A. hygrophila*, collected in the north-east of Buenos Aires province (Argentina), was first released in the United States in 1964 and later released in Australia and China. The success of this biological control agent is restricted to regions with mild winters (Julien et al., 1995; Sainty et al., 1998) and where the weed grows in aquatic habitats (Ding et al., 2006). In the native range, the distribution of *A. hygrophila* overlaps with the *A. philoxeroides* hybrids complex but only to latitude 36°S in the interior and 38°S around the maritime fringes (Buckingham et al., 1983). Although plant related restrictions to insect development were previously proposed, such as pupation failure due to reduced stem diameter (Vogt and McGuire, 1979; Lu et al., 2010), low temperatures (Julien et al., 1995; Stewart et al., 1996) or mineral deficiencies (Maddox and Rhyne, 1975), the potential effect of plant population structure on the insects' biology remained unexplored.

Since in its native range alligator weed is comprised of a complex of polyploid hybrids, some of which may constrain insect

development and reproduction, plant population identity could provide an alternative explanation for the restricted success of biological control of alligator weed with *A. hygrophila*. Both increased ploidy level and genomic composition could be the mechanisms implied in these constraints. The aim of this study was to explore the degree to which plant populations, with varying cytogenetical characteristics, affected plant–insect interactions in the native range of the weed. We hypothesized that the performance of *A. hygrophila* (i.e., immature stage mortality, adult sex ratio, fertility and developmental times) will depend on *A. philoxeroides* population structure, independently of climatic restrictions on *A. hygrophila* distribution (Julien et al., 1995).

## 2. Materials and methods

### 2.1. Plants and Insects

Plants of four populations of *A. philoxeroides* (a single tetraploid and three hexaploids) were collected in the field (Table 1). The cuttings were transplanted into 10 L pots for cultivation under the same conditions in a glasshouse for two years prior to the experiment (5–10 pots per cytotype). Apical portions of the branches, with 8–10 undamaged leaves, were used to feed the insects and were renewed as needed.

Approximately 20 adults of *A. hygrophila* were collected to start an insect culture from the HUR plant population at Hurlingham (Buenos Aires province), which is the closest location to the historical sites of collection of this insect for biological control purposes. The insects were raised in a rearing chamber (25 °C, 12 h light photoperiod) over the entire experiment. Egg masses laid by these adults were isolated in Petri dishes with moistened tissue paper and incubated in the rearing chamber. Once larvae hatched, they were transferred to new plastic containers (8 cm diameter, 5 cm height) with moistened tissue paper and newly-cut apical branches of alligator weed. The experiment was conducted with the second generation of insects from the laboratory culture. Since insects were only collected on HUR cytotype, the treatment involving HUR plants was considered as the control.

### 2.2. Insect performance

To test the effect of plant population on insect performance, the insect's life cycle was followed on plants of each of the four weed populations. Each replicate was started with 10 newly emerged larvae placed in a plastic container with moistened tissue paper and cut stems of alligator weed. Ten replicates were considered for each treatment (plant population). Individual survival and transition between stages were recorded daily. The pupae were removed from the stems three days after larvae entered the stems, to avoid insect mortality due to host plant putrefaction, and were placed in a plastic container (8 cm diameter, 5 cm height) with moistened tissue paper. Once insects reached the adult stage, they

**Table 1**

The locations and cytogenetical characteristics of the four *Alternanthera philoxeroides* populations used in the experiment, presence or absence of seed production and an assessment of field abundance of *Agasicles hygrophila* for each location (TAN, Tandil; STF, Santa Fe; PRE, Predelta; HUR, Hurlingham). Abundance was based on field observations made over the period 2001–2006.

Alligator weed population	Coordinates	Ploidy level	Chromosome number	Pollen viability (%)	Fruit production	<i>A. hygrophila</i> presence in the area
TAN	S 37° 10' 7.4" W 59° 25' 15.5"	Tetraploid	66	95	Yes	Absent
STF	S 29° 16' 51.3" W 59° 49' 12.8"	Hexaploid	ca. 100	50	No	Abundant
PRE	S 32° 05' 23.5" W 60° 35' 42.5"	Hexaploid	ca. 100	94	Yes	Absent
HUR	S 34° 35' 14.5" W 58° 38' 27.0"	Hexaploid	ca. 100	0	No	Abundant

were sexed and mating was allowed between insects from the same replicate to obtain an estimate of female fecundity and fertility.

### 2.3. Statistical analysis

Insect survival to the adult stage was evaluated with a generalized lineal model. The response variable was the number of insects that reached the adult stage, locality was considered as a fixed factor with four levels, the error distribution was quasipoisson and the link function was logarithmic. This analysis was conducted using the lmer function in lme4 package of R statistical software, version 2.10.1 (<http://www.r-project.org>). To evaluate if the total egg production differed between plant populations a generalized lineal model analysis was applied, with locality as fixed factor with four levels, female number as a covariate, a negative binomial error distribution and a log link function. This analysis was conducted using the glm.nb function in the MASS package of R statistical software. The best model was obtained by stepwise deletion of non-significant terms ( $p > 0.05$ ) and further comparison of LR and AKAIKE's IC. Additionally the effect of plant population on female fertility, life cycle's length, third larval stage mortality and pupae mortality were evaluated with ANOVA and deviations of sex-ratio 50:50 were tested with a Chi-square test (Infostat, 2009).

### 2.4. Cytogenetic analyses

Previous *in situ* hybridization analyses (modified from Schwarzacher and Heslop-Harrison, 2000) revealed that only one third of the chromosomes (ca. 33) from an *A. philoxeroides* hexaploid population (near Orán city, Salta Province, Argentina, S 22° 52' 23.30", W 64° 21' 49.09") and also from the close related species *A. aquatica* (Chodat) (collected in Isla del Cerrito, Chaco, Province, Argentina, S 27° 24' 36.56", W 58° 50' 39.64") were provided by the TAN genome while the other two genomes (ca. 66 chromosomes) came from an unknown progenitor.

Based on this result, it was explored if the hexaploid populations differed in their genomic composition with respect to TAN. To evaluate these relationships among polyploids, dot blot analysis was performed using DIG DNA Labelling and Detection kit by Roche and a positively charged nylon membrane. *A. philoxeroides* from Orán and *A. aquatica* from Chaco were included in this analysis.

## 3. Results

### 3.1. Insect performance

Plant population affected insect performance. However, the effect was variable in different stages of the insect's life history. Insect survival to the adult stage was reduced on all plant populations compared to HUR (Table 2, Fig. 1a). These reductions ranged from 27% (TAN, tetraploid) to 45% (PRE, hexaploid). Fecundity was reduced to 38% of reference levels (HUR) in females reared on the tetraploid population (TAN) but not reduced at all in females reared on the other two hexaploid populations (PRE and STF) (Table 3, Fig. 1b).

Plant population type did not affect life cycle length ( $F_{3,29} = 3.38$ ,  $p = 0.3164$ ,  $r^2 = 0.18$ ; Table 4) third larval instar mortality ( $F_{3,29} = 2.06$ ,  $p = 0.1277$ ,  $r^2 = 0.09$ , Table 4), pupal mortality ( $F_{3,29} = 0.93$ ,  $p = 0.4403$ ,  $r^2 = 0.00$ , Table 4) or female proportion ( $p > 0.4$  for all the cytotypes, Table 5). However, in agreement with the model's result, insect fertility was reduced by TAN cytotype ( $F_{3,29} = 3.35$ ;  $p = 0.0325$ ;  $R^2 = 0.18$ ; Table 5).

**Table 2**

Results of generalized linear model with insect survival as response variable and locality as fixed factor with four levels (AIC: 406.8).

	Estimate	Standard error	z Value	Pr (> z )
Intercept	1.5824	0.2835	5.582	2.38e-08***
PRE (6×)	-1.3787	0.4154	-3.319	0.000903***
STF (6×)	-1.0379	0.3852	-2.694	0.007055**
TAN (4×)	-1.0142	0.3767	-2.693	0.007092**

Significance codes:

\*\*  $p < 0.01$ .

\*\*\*  $p < 0.001$ .

### 3.2. Genomic composition

No effects could be attributed to the genomic composition of the polyploids. Dot blot analysis revealed that all the considered populations of *A. philoxeroides* (Orán, HUR, PRE, STF and CAN), as well as *A. aquatica* (tetraploid) hybridized with TAN probe with similar intensity, meaning that this population is a component of their polyploid genome.

## 4. Discussion

Our results show that alligator weed population origin can have a great effect on *A. hygrophila* performance. Both insect survival and fecundity were reduced by at least some of the populations included in this analysis which presumably correspond to different genotypes, based on the cytogenetical characterization available. While insect survival was lower on all of the tested populations compared to HUR, insect fecundity was only reduced on the TAN population. The number of females varied with insect fecundity. Although plant population did not affect sex-ratio, it affected insect survival. Therefore, this variation might only reflect the overall effect of plant population on insect survival. Additionally, plant population did not affect developmental time from neonate larvae to adult.

Since HUR population was the control for our experiment, a second experiment with insects collected from a different location would test for HUR effects. This would clarify the incidence of maternal effects of alligator weed on the development of the insect, as well as the effect of plant population identity. For instance, if the insect was collected on a different alligator weed population and the results were congruent with those included in this paper we would conclude that the effect of plant population is more important than plant maternal effect. Plans to perform this experiment could not be implemented because the density of *A. hygrophila* markedly diminished in the field and they could not be collected when required. Their disappearance coincided with a notable reduction of precipitation and alligator weed abundance.

A minimal plant internode diameter has been proposed as a fundamental requirement for *A. hygrophila* to pupate (Vogt and McGuire, 1979; Lu et al., 2010; Pan et al., 2010). The tetraploid and hexaploid populations utilized in our experiment had a mean stem diameter of 1.95 mm and 2.95 mm, respectively. In contrast with Lu et al. (2010) neither third larval instar mortality nor pupal mortality was affected in any of the plant populations and stem diameter was not a restriction for insect pupation in our experiment.

*A. philoxeroides* exhibits genetic differences in some invaded areas but not in others. Two biotypes were detected in the United States using isozymes; a broad-stemmed and a narrow-stemmed form of the plant (Kay and Haller, 1982; Wain et al., 1984). These biotypes resemble the tetraploid and hexaploid types described by Okada (1985), signifying that the presence of tetraploids in the United States remains plausible. In China, RAPDS (Xu et al.,

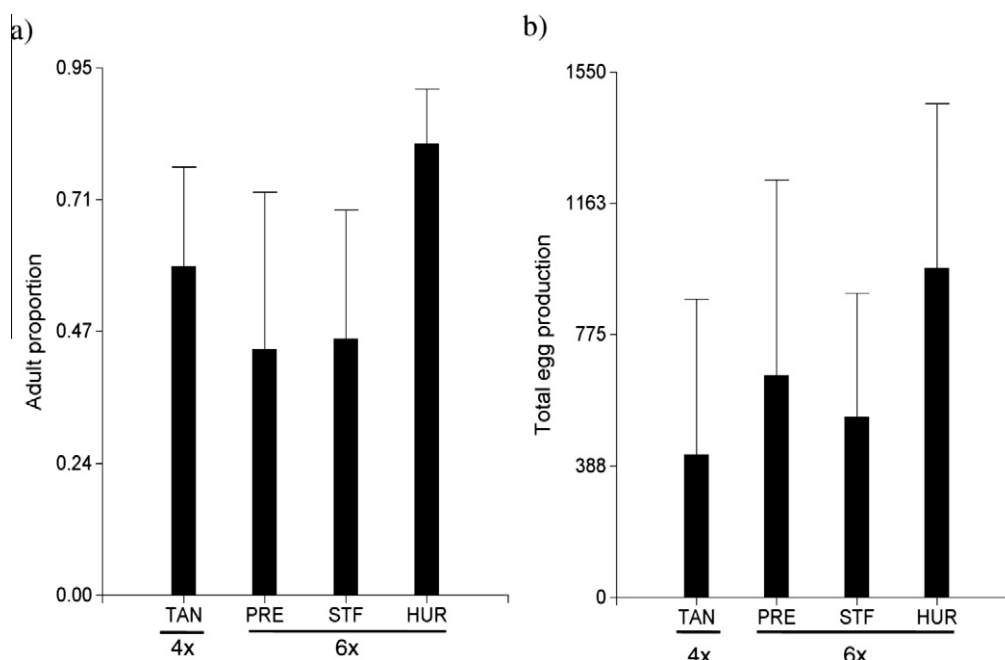


Fig. 1. Insect performance on each alligator weed population (Mean  $\pm$  SD). (a) Proportion of insects that reached the adult stage; (b) eggs produced per replicate.

Table 3

Results of generalized linear model with the total egg production as response variable, locality as fixed factor with four levels and female number as a covariate (AIC: 477.06).

	Estimate	Standard error	z Value	Pr (> z )
Intercept	5.55637	0.26399	21.047	<2e-16***
PRE (6x)	-0.04267	0.23478	-0.182	0.85578
STF (6x)	-0.29349	0.23706	-1.238	0.21570
TAN (4x)	-0.65203	0.23505	-2.774	0.00554**
Females	0.36596	0.06717	5.448	5.09e-08***

Significance codes:

\*\*  $p < 0.01$ .

\*\*\*  $p < 0.001$ .

2003) and ISSR (Li and Ye, 2006) analyses revealed only one genotype while morphological analysis suggested that the Chinese populations may have all originated from Santa Fe, central northern Argentina (Pan et al., 2006). In Australia, analyses using ISSR suggested that multiple genotypes are present (Gopurenko et al., unpublished data); however, there is no evidence of the presence of tetraploids in Australia.

We found that all the alligator weed populations included in this experiment and the close relative *A. aquaticus* share part of their genome with TAN population indicating that they all have a common ancestor and form a complex of hybrids. The use of ISSR in combination of cytogenetic methodologies may contribute to clarifying the hybridization history of alligator weed in its native range and help to establish the relationships between

Table 4

Third larval instar mortality, pupae mortality, survival to the adult stage and developmental time (days) (mean  $\pm$  SD) of *A. hygrophila* fed on different alligator weed populations. None of these variables was affected by plant cytotype.

	Third larval instar mortality	Pupae mortality	Survival to the adult stage	Developmental time (days)
TAN (4x)	0.20 $\pm$ 0.19	0.06 $\pm$ 0.12	0.59 $\pm$ 0.18	22.11 $\pm$ 2.79
PRE (6x)	0.25 $\pm$ 0.30	0.10 $\pm$ 0.14	0.44 $\pm$ 0.28	20.21 $\pm$ 3.06
STF (6x)	0.34 $\pm$ 0.22	0.13 $\pm$ 0.17	0.46 $\pm$ 0.23	22.23 $\pm$ 4.77
HUR (6x)	0.10 $\pm$ 0.08	0.03 $\pm$ 0.06	0.81 $\pm$ 0.1	19.49 $\pm$ 4.43

Table 5

Fertility and female proportion (mean  $\pm$  SD) of *A. hygrophila* fed on different alligator weed populations. Only Insect fertility was affected by plant population, different lower case letters reflect significant differences ( $p < 0.05$ ).

	Insect fertility	Female proportion
TAN (4x)	117.23 $\pm$ 96.34 <sup>a</sup>	0.44 $\pm$ 0.22
PRE (6x)	220.02 $\pm$ 178.8 <sup>b</sup>	0.55 $\pm$ 0.28
STF (6x)	198.62 $\pm$ 98.32 <sup>b</sup>	0.51 $\pm$ 0.22
HUR (6x)	304.5 $\pm$ 93.32 <sup>b</sup>	0.4 $\pm$ 0.19

plants in the native and invaded areas, as well as enhance understanding of the interactions between the plants and their natural enemies.

Previous studies show the level of attack exerted by herbivores and the patterns of visitation by pollinators can vary among plants of different ploidy levels (Thompson et al., 2004; Münzbergová, 2006; Arvanitis et al., 2007; Halverson et al., 2008). However, the evaluation of plant ploidy level effect on insect fitness remains largely unexplored. Besides the effect of plant ploidy level, hybridization among different genotypes also has the potential to influence interactions with herbivores (Manrique et al., 2008).

In conclusion, this is the first study of the effect of *A. philoxeroides*' plant characteristics on *A. hygrophila* in their native range. Plant population structure affected both insect survival and fecundity, highlighting the relevance of considering plant genotype effects on insects for biological control purposes.



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