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# Genetic diversity and flooding survival in *Aegiphila sellowiana* (Lamiaceae), a typical tree species from upland riparian forests

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**ABSTRACT.** Saplings of *Aegiphila sellowiana* were submitted to flooding and analysis of genetic diversity in order to investigate flooding tolerance as well as its genetic determination. This response is important because it means that some lines could be planted in degraded riparian areas. Leaves were sampled from each plant, and they were submitted to different flooding periods. Mortality of saplings was 40, 80, 50, 53.3, 33.3, and 33.3% in flooding for 15, 18, 25, 50, 80 days, and flooding for 50 days followed by re-aeration for 30 days, respectively. From the total number of flooded plants, 46.7% died in the first seven days of treatment, while 53.3% survived the flooding. The percentage of polymorphic loci ($P_p$), Nei's genetic diversity ($H$) and the Shannon index ($I$) were slightly higher for the group that survived the stress of flooding (surviving: $P_p$ (%) = 67.48, $H$ = 0.184, $I$ = 0.287; not surviving: $P_p$(%) = 66.67, $H$ = 0.165, $I$ = 0.261). Analysis of molecular variance showed that 5.88% of the genetic variability was due to the differences between groups of plants surviving and not surviving

 

flooding, while 94.12% was due to genetic differences between individuals within these groups. Similar results were obtained by principal coordinate analysis. Based on these results, we can assume the existence of environment-specific genotypes and the genetic determination of flooding tolerance in *A. sellowiana*. Thus, some lines of *A. sellowiana* could be used in the reforestation of riparian habitats, especially in uplands along riverbanks.

**Key words:** Genetic diversity; Flooding; Tolerance; Uplands

## INTRODUCTION

Trees from riparian habitats have an amphibian lifestyle (Braendle and Crawford, 1999) marked by temporary flooding and the subsequent return to drier conditions. They constitute an assemblage of growth habits, physiological attributes and degrees of flooding tolerance, developing into constitutive features and/or phenotypic plasticity, revealed through adaptive plant/environment interactions (Jackson et al., 2009).

The periodic changes in water tables act as a selective pressure on riparian populations for the evolution of tolerance either to soil flooding or complete submergence and re-aeration of plantlets. For some species, a quiescence strategy is selected, characterized by limited underwater growth and conservation of energy and carbohydrates. Other species use the low oxygen escape syndrome (Bailey-Serres and Voesenek, 2008), an avoidance strategy that facilitates the survival of submerged organs through the development of anatomical and morphological traits that facilitate inward diffusion of $CO_2$ and $O_2$ and reduce the resistance for internal gas diffusion, thereby improving underwater photosynthesis and aerobic metabolism. There are also species that combine those strategies in order to cope with different intensities of flooding (Silva et al., 2010).

Not all species in a riparian habitat are frequently exposed to flooding. Species distributed in upland areas do not face flooding regularly and, thus, are not expected to be adapted to flooding/re-aeration stress, like species from floodplain areas. For instance, Lenssen et al. (2004) found that populations of the clonal plant *Ranunculus reptans* from a lakeside microhabitat and a landside microhabitat differed significantly in traits related to overall fitness, indicating that flooding induced a local adaptation of the species. Peña-Fronteras et al. (2009), working with *Cyperus rotundus*, a troublesome sedge weed of rice, also found considerable differences in tuber morphology and carbohydrate metabolism when comparing ecotypes from upland and lowland sites. However, the most striking example was reported for *Himatanthus sucuuba*. Plants of this Amazonian tree, living in floodplain areas of Central Amazonia, showed a better performance in several features under experimental waterlogging and submersion than did upland plants (Ferreira et al., 2007, 2009a,b).

Two tree species from riparian forests in the State of Paraná, Brazil, with distribution in lowland and upland areas were studied before, namely *Luehea divaricata* (Carvalho et al., 2002) and *Parapiptadenia rigida* (Silva et al., 2010). The authors aimed to analyze their ability to tolerate flooding by assessing survival rates, growth, and morphological adaptations and to estimate the genetic diversity between flooded and non-flooded populations, in order to find evidence of ecotypic differentiation. They found that *L. divaricata* tolerates only moderate levels of flooding, while some individuals of *P. rigida* are able to withstand even complete submergence. Both species showed a significant genetic diversity between populations, supporting the idea that flooding selects alleles in flooded populations. Moreover, *P. rigida*

 

showed strong evidence of ecotypic differentiation, since the lowland population showed morphological attributes and survival rates that indicate a higher degree of flooding tolerance.

In this study, we addressed two new questions. Do species exclusively from uplands in riparian forests survive flooding stress? If so, is this ability genetically determined?

We chose *Aegiphila sellowiana* Cham. (Verbenaceae) for this study, since it is a tree species found in riparian forests of Paraná State, where there are no reports of its occurrence in flooded sites, but rather in more elevated adjacent areas, such as dikes along the riverbanks. In order to address the issues raised, we determined the influence of different flooding regimes on sapling mortality and analyzed the genetic diversity between surviving and non-surviving plants.

## MATERIAL AND METHODS

### Seed collection and plant growth

Seeds of *A. sellowiana*, collected in natural environments in the municipalities of Londrina, Arapongas, Nova Santa Barbara and São Jerônimo da Serra, Paraná, Brazil, from five matrices at each locality, were germinated in a nursery in tubes containing moist substrate (80% soil and 20% mixture of straw, chicken manure, lime, and coffee dregs). After six months, plants were taken to a greenhouse and transplanted in 4-L plastic pots, containing substrate made with soil and sand (3:1). The experiments were started three months after acclimation of the plants and lasted 80 days.

### Experimental design

Survival was determined using plants divided into seven groups: D - 10 plants kept in drained soil; F15 - 10 plants kept in flooded soil for 15 days; F18 - 10 plants kept in flooded soil for 18 days; F25 - 10 plants kept in flooded soil for 25 days; F50 - 15 plants kept in flooded soil for 50 days; F80 - 15 plants kept in flooded soil for 80 days; REA (re-aerated) - 15 plants kept under flooding conditions for 50 days followed by drainage starting on the 51st day and lasting for 30 days. The plants under flooding conditions were kept in soil with 2 cm water above the substrate surface. Plants in each treatment were duly numbered, and at the end of the experiments, dead individuals were recorded.

### Sampling for genetic analysis

Before the beginning of the flooding experiments, a leaf from each plant utilized in the treatments was collected, numbered and stored in a -80°C freezer. After the end of the experiments, the leaves collected were separated into two groups: 1) leaves from plants surviving the stress of flooding and 2) leaves from plants not surviving. Genetic analysis was carried out on randomly selected samples, 30 plants of each group.

### Laboratory procedure

Extraction of genomic DNA from leaves was performed based on the method described by Doyle and Doyle (1987), except that CTAB was substituted by MATAB (Sigma) in the ex-

 

traction buffer. The concentration of DNA was estimated using a fluorometer (DyNA Quant 200, Höfer-Pharmacia), in accordance with manufacturer instructions. DNA samples obtained from 30 plants of each group were adjusted to 10 ng/µL for polymerase chain reaction (PCR) use. A total of 19 primers (Operon Technologies; Table 1) were used in DNA amplifications in a volume of 15 µL containing 1X PCR buffer (75 mM Tris-HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.2 mM dNTPs, 0.4 µM primer, 0.9 U Taq DNA polymerase (Biotools), and 20 ng DNA).

DNA amplification was performed using a PTC 100 thermocycler (MJ Research), programmed for 3 min at 94°C for initial denaturation, followed by 48 cycles of 1 min at 94°C, 1 min 45 s at 38°C, and 2 min at 72°C. The last cycle was followed by 7 min of extension at 72°C. The amplification products were separated on 1.2% agarose gels immersed in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide. Electrophoretic gels were run at 120 V for 2 h. The random amplified polymorphic DNA (RAPD) profiles were visualized under UV light and photographed for data analysis.

The presence/absence of RAPD bands was recorded in a binary format statistical analysis. Only well-amplified molecular fragments were considered. Bands of similar molecular weight and migration pattern among individuals were considered to be homologous. Control samples containing all the reaction materials except DNA were run to avoid the utilization of bands produced from self-amplification or DNA contamination.

## Data analysis

The calculation of allele frequencies and genetic diversity using dominant markers can be problematic (Zhivotovsky, 1999). However, Krauss (2000) demonstrated that biased data produced due to the dominant nature of RAPD markers can be eliminated in a set of highly polymorphic data. It has been demonstrated that accurate estimates of genetic parameters of populations require the use of a large number of RAPD loci and about 30 individuals per population (Tero et al., 2003).

The bootstrap method was used with the DBOOT program version 1.1 (Coelho, 2000), for the purpose of measuring the coefficient of variation related to the number of markers used.

The percentage of polymorphic loci within populations and the Shannon index were calculated using the POPGENE program (Yeh et al., 2000). Genetic variation within and between populations was estimated by analysis of molecular variance (AMOVA), utilizing the ARLEQUIN program 2.0 (Schneider et al., 2000). Principal coordinate analysis based on genetic distances (Nei, 1978) was carried out using the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System for personal computer) software, version 2.1 (Rohlf, 2000).

## RESULTS

In total, 75 plants were submitted to different periods of soil flooding. Of these, 35 (46.7%) died (Figure 1). The treatments with the longest duration of flooding did not show a greater percentage of mortality (Figure 1). In general, the death of the plants occurred early, in the first week of flooding. Hence, those plants that had the capacity of surviving in this period continued living for the rest of the treatment period, even for the longest periods, 50 and 80 days. The surviving plants showed morphological responses to flooding, such as the presence of hypertrophied lenticels, stem fissures, and diageotropic and adventitious roots (data not shown).

 

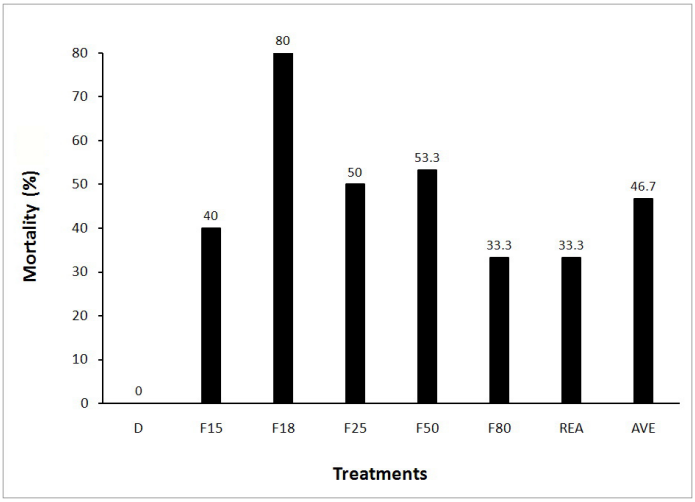


**Figure 1.** Mortality rate in young plants of *Aegiphila sellowiana* after different periods of soil flooding. D = drained; F15 = flooding for 15 days; F18 = flooding for 18 days; F25 = flooding for 25 days; F50 = flooding for 50 days; F80 = flooding for 80 days; REA = flooding for 50 days followed by re-aeration for 30 days; AVE = average mortality considering all treatments.

The 19 primers that were selected produced 123 consistent and well-defined bands. The bootstrap results showed a coefficient of variation of 4.67%, indicating that the number of markers obtained was suitable for reliable analysis of the results. A variation was observed in the percentage of polymorphic loci among the primers used. The percent of polymorphic loci was practically equal between the groups of surviving plants (67.48%) and non-surviving plants (66.67%) (Table 1). However, both indices of genetic diversity showed slightly higher values for the surviving group (Table 1).

AMOVA showed that approximately 6% of genetic variability was due to differences between groups of plants surviving and not surviving flooding, while 94% was due to genetic differences between individuals within these groups (Table 2). Principal coordinate analysis showed that the surviving plants formed a group in one of the coordinate quadrants, while the plants not surviving flooding were dispersed, where the individuals mixed with those of the surviving group (Figure 2).

**Table 1.** Percentage of polymorphic loci ($P_p$), Nei's genetic diversity (1978) (*H*) and Shannon index (*I*) for groups of plants of *Aegiphila sellowiana* surviving and not surviving flooding.

| Group | $P_p$ (%) | *H* | *I* |
|---|---|---|---|
| Surviving | 67.48 | 0.184 | 0.287 |
| Not surviving | 66.67 | 0.165 | 0.261 |

**Table 2.** Analysis of molecular variance (AMOVA) for the groups of *Aegiphila sellowiana* plants surviving and not surviving flooding.

| Source of variation | Degrees of freedom | Sum of squares | Components of variance | Percent of variation (*F*st) |
|---|---|---|---|---|
| Between populations | 1 | 25,500 | 0.55410 | 5.88** |
| Within populations | 58 | 514,867 | 8.87701 | 94.12 |

**P < 0.001; *F*st = 0.0588.

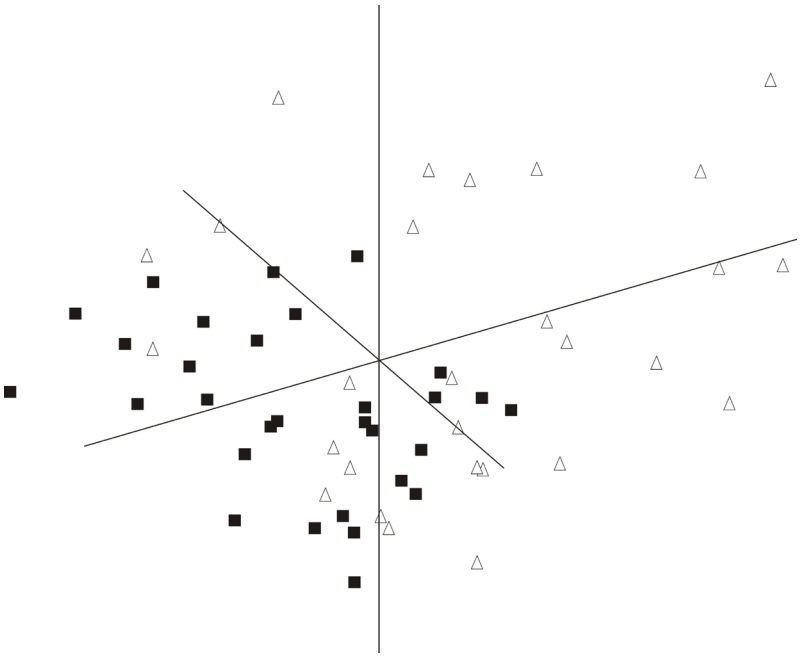

**Figure 2.** Three-dimensional plot of principle coordinate analysis showing genetic relatedness between subpopulations of surviving (squares) and non-surviving (triangles) plants of *Aegiphila sellowiana*. The first, second, and third principal coordinates explain together 30.06% of the total variation.

## DISCUSSION

Flooding is certainly an important threat to *A. sellowiana*, given the considerable mortality rate (46.7%; Figure 1) and the short time in which it occurred. However, *A. sellowiana* can be considered tolerant to this stress, since the majority of the individuals tested were able to withstand even 80 days of flooding. Hence, if the water tables rise to higher levels, reaching plant communities that are seldom challenged by flooding, we can expect a reasonable tolerance to it and the survival of a considerable portion of the population. In times of global climate changes, abrupt rises in river levels are a growing concern. According to Silva and Guetter (2003), global temperature has been rising since the 1970s, and the hydrological cycle is accompanying this change. For instance, in Paraná State, South Brazil, the enhancement of the hydrological cycle boosted the frequency and intensity of rains, the river levels, and, paradoxically, the frequency and intensity of drought. These effects are expected to be intensified as a consequence of global temperature rise, amplifying the occurrence of extreme events, such as droughts and floods.

A mortality rate similar to the one found in *A. sellowiana* was reported for *P. rigida*: 40% of the young plants died following flooding stress (Silva et al., 2010). However, *P. rigida* is not vulnerable to low-intensity waterlogging, where mortality is associated with long periods of a combination of soil flooding and complete submergence of saplings. Ferreira et al. (2007) found a higher degree of resistance in the Amazonian tree *H. sucuuba*, where seedlings from flooded habitats died only after several days of complete submergence. In another study, *L. divaricata* plants, on the other hand, were totally tolerant to one month of flooding, but were completely sus-

ceptible to an additional month of submergence. These species show different degrees of flooding tolerance, beginning with the most susceptible, *A. sellowiana*, followed by *L. divaricata*, *P. rigida*, and finally the most resistant, *H. sucuuba*. Certainly, the differences in tolerance seen among them result from a variation in the degree of historical exposure to flooding episodes.

The fact that surviving plants showed morphological responses to flooding indicates that a low oxygen escape syndrome strategy is favored in this species, leading to an improvement in oxygen supply to the tissues underwater, consequently ensuring the survival of plants (Bailey-Serres and Voesenek, 2008). The study of these adaptations to flooding is the focus of a future study.

The principal coordinate analysis results concur with the AMOVA results, which show a genetic difference of only about 6% between the groups of plants surviving and not surviving flooding (Table 2; Figure 2). Although the genetic variation between populations was much smaller than the variation within populations and the coefficient of variation and percentage of polymorphic loci were mostly the same between samples, the percentage of variation was highly significant, indicating that the two samples analyzed are different (Table 2). Similar results were obtained by Carvalho et al. (2002) for *L. divaricata* (Tiliaceae), in which AMOVA showed a difference of 10.39% between plants from areas submitted to flooding and those from areas that did not undergo flooding, and Silva et al. (2010), who found a genetic difference of 6.27% between *P. rigida* plants from areas that were flooded and not flooded.

For some species, the ability of surviving waterlogging is not widespread in the population, as we found for *A. sellowania* (Ferreira et al., 2007; Silva et al., 2010). One explanation to this assumes that tolerance is a part of the genomic plasticity of the species and that variable response is caused by microhabitat variations. Alternatively, these species coped with variable duration or pressure of waterlogging, insufficient to convert the whole population to flood-tolerant. If so, not all individuals show resistance to flooding as a constitutive feature and the population is walking the evolutionary path to becoming tolerant. In these cases, we should expect to find distinct allele frequencies as well as genetic diversity between them. The results found for *A. sellowiana* indicate that certainly the two groups possess different alleles, selected by the flooding process over time, that are expressed when the plants are submitted to stress from flooding, thereby allowing these plants to survive. Thus, we can assume that flood tolerance is genetically determined in *A. sellowiana* and that part of its population has this character as a constitutive feature.

We can conclude that *A. sellowiana* is tolerant to flooding due to genetically determined factors. However, the lack of a strong genetic differentiation between saplings surviving and not surviving flooding can be attributed to high levels of gene flow. In addition to using molecular markers to study the pattern of genetic variation of *A. sellowiana*, it would be interesting to investigate the variation in morphological and physiological characters that contribute to tolerance to soil flooding. Compared to *L. divaricata* and *P. rigida*, both tree species of riparian forests in Paraná State, *A. sellowiana* is less adapted to living on riverbanks, probably because it has not historically endured waterlogging stress as much as those species. Thus, the use of *A. sellowiana* in the reforestation of riparian habitats in Paraná State should foster its distribution in uplands.

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## REFERENCES

Bailey-Serres J and Voesenek LA (2008). Flooding stress: acclimations and genetic diversity. *Annu. Rev. Plant Biol.* 59: 313-339.

Braendle R and Crawford RMM (1999). Plants as amphibians. *Perspect*. *Plant Ecol. Evol. Syst.* 2: 56-78.

Carvalho MCCG, Silva DCG, Ruas PM, Medri ME, et al. (2002). Flooding tolerance and genetic diversity in populations of *Luehea divaricata*. *Biol. Plant.* 52: 771-774.

Coelho ASG (2000). Dboot: Avaliação dos Erros Associados a Estimativas de Distâncias/Similaridade Genéticas Através do Procedimento de Bootstrap com Número Variável de Marcadores (Software). Universidade Federal de Goiás, Goiânia.

Doyle JJ and Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15.

Ferreira CS, Piedade MTF, Junk WJ and Parolin P (2007). Floodplain and upland populations of Amazonian *Himatanthus sucuuba*: effects of flooding on germination, seedling growth and mortality. *Environ. Exp. Bot.* 60: 477-483.

Ferreira CS, Piedade MTF, Franco AC, Gonçalves JFC, et al. (2009a). Adaptive strategies to tolerate prolonged flooding in seedlings of floodplain and upland populations of *Himatanthus sucuuba*, a Central Amazon tree. *Aquatic. Bot.* 90: 246-252.

Ferreira CS, Piedade MT, Tine MA, Rossatto DR, et al. (2009b). The role of carbohydrates in seed germination and seedling establishment of *Himatanthus sucuuba*, an Amazonian tree with populations adapted to flooded and non-flooded conditions. *Ann. Bot.* 104: 1111-1119.

Jackson MB, Ishizawa K and Ito O (2009). Evolution and mechanisms of plant tolerance to flooding stress. *Ann. Bot.* 103: 137-142.

Krauss SL (2000). Accurate gene diversity estimates from amplified fragment length polymorphism (AFLP) markers. *Mol. Ecol.* 9: 1241-1245.

Lenssen JPM, Kleunen MV, Fischer M and Kroon H (2004). Local adaptation of the clonal plant *Ranunculus reptans* to flooding along a small-scale gradient. *J. Ecol.* 92: 696-706.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583-590.

Peña-Fronteras JT, Villalobos MC, Baltazar AM, Merca FE, et al. (2009). Adaptation to flooding in upland and lowland ecotypes of *Cyperus rotundus*, a troublesome sedge weed of rice: tuber morphology and carbohydrate metabolism. *Ann. Bot.* 103: 295-302.

Rohlf FJ (2000). NTSYS-pc Numerical Taxonomy and Multivariate Analysis System Version 2.1. Owner Manual. Exeter Software, New York.

Schneider S, Roessli D and Excoffier L (2000). ARLEQUIN: A Software for Population Genetic data Analysis, Version 2.0 (software). University of Geneva, Geneva.

Silva DC, Carvalho MC, Ruas PM, Ruas CF, et al. (2010). Evidence of ecotypic differentiation between populations of the tree species *Parapiptadenia rigida* due to flooding. *Genet. Mol. Res.* 9: 797-810.

Silva MES and Guetter AK (2003). Mudanças climáticas regionais observadas no estado do Paraná. *Terra Livre* 1: 111-126.

Tero N, Aspi J, Siikamaki P, Jakalaniemi A, et al. (2003). Genetic structure and gene flow in a metapopulation of an endangered plant species, *Silene tatarica*. *Mol. Ecol.* 12: 2073-2085.

Yeh FC, Yang RTJ and Xiyan JM (2000). PopGene32. Microsoft Window-based Freeware for Population Genetic Analysis, Version 1.32 (Software). University of Alberta, Edmonton.

Zhivotovsky LA (1999). Estimating population structure in diploids with multilocus dominant DNA markers. *Mol. Ecol.* 8: 907-913.