



Is a leaf life span enough to display changes on developmental instability and nitrogen after simulated herbivory?



Henrique Venâncio, Estevao Alves Silva, Jean Carlos Santos*

Instituto de Biologia, Universidade Federal de Uberlândia, Av. Pará 1720, 38400-902, Uberlândia, Minas Gerais, Brazil

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ABSTRACT

Herbivory is a main source of plant damage and injured plants might present decrements in nutrients, delayed growth and low reproductive outputs. Therefore, herbivory can be considered a stressful agent to plants. In this study, we investigated short-term changes in stress and nutrient levels of the invasive plant species *Tithonia diversifolia* (Asteraceae) after simulated herbivory. Parameters such as leaf fluctuating asymmetry (FA, a measure of plant stress, also known as developmental instability) and nitrogen levels were compared between control and treated plants reared in a greenhouse. We expected high levels of FA and significant changes in nitrogen in injured leaves. FA was assessed by measuring the right and left sides of leaves, from the leaf edge to the midrib, at the middle point of the leaves; nitrogen was evaluated through the Kjeldahl method. Though non-significant, at the end of the study, injured leaves were 22% more asymmetric than control leaves, and before-after comparisons revealed that FA increased by 33% in leaves after leaf damage. In addition, FA was positively and significantly related to leaf area loss. Nitrogen did not present changes after simulated herbivory. The control group showed non-significant changes in FA and nitrogen. FA was concluded to be a rapid estimator of plant stress following leaf damage, as during its short lifespan (45 days), leaves displayed tentatively increased developmental instability. We consider that leaf damage is a predictor of plant developmental instability and that FA can be used to detect and compare stress levels in plants after herbivory damage.

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1. Introduction

Herbivory is the main source of biotic plant damage in nature and its consequences, regardless of the plant structure that animals feed on, are well documented; plants may present delayed growth, early leaf senescence and less investment in reproduction (i.e., the production of flowers and fruits) (Maron, 1998; Stephens and Westoby, 2015). Shortly after herbivore attack, the nutritional quality of tissues may undergo several changes. Nitrogen levels, for instance, can either decrease or increase following herbivory (Casotti and Bradley, 1991; Gange and Nice, 1997; Hartley, 1998). As demonstrated by Cornelissen and Fernandes (2001a), leaf damage significantly reduced nitrogen levels in a legume tree (see also Cooke et al., 1984) and the authors attributed this factor to induced plant defenses, since low nitrogen is related to increased concentration of tannins and low susceptibility to further insect herbivores (Cornelissen and Fernandes, 2001b; Cornelissen and Stiling, 2005).

With such large detrimental effects of herbivores on plant development, one may assume that injured plants are under stress (i.e. detriments in a biological system—Hoffmann and Parsons, 1991) compared to non-injured individuals (Díaz et al., 2004). One way to assess stress in plants is through the analysis of developmental instability (DI), which is based on morphological changes in the morphometry of bilateral characters (e.g., leaves) following perturbations (Leamy and Klingenberg, 2005). DI is the inability of organisms to buffer adverse ontogenetic processes and achieve a predetermined phenotypic condition (Olofsson and Strengbom, 2000). The DI of plants subjected to different types of perturbation, including herbivory, can be statistically evaluated by an index known as fluctuating asymmetry (FA), which measures the imperfect growth of leaves (Martel et al., 1999; Telhado et al., 2010). Under normal and optimal conditions, both sides of leaves (right and left sides) should develop correspondingly, but a diverse array of biotic and abiotic stressful conditions (e.g., temperature, soil, shade, pollution, competition, parasitism, and herbivory) affects the bilateral development of leaves and gives rise to minor deviations from the perfect symmetry (reviewed in Möller and Shykoff, 1999). Since FA is a health index of populations (i.e., elevated FA levels are indicative of high stress), it can be used to compare plants

* Corresponding author.

E-mail address: jcsantos@ufu.br (J.C. Santos).

with different levels of stress within a population (Alves-Silva and Del-Claro, 2013; Cornelissen and Stiling, 2011).

Several studies report positive relationships between FA and herbivory, that is, stressed plants may attract more herbivores (Lempa et al., 2000). It occurs because stress due to abiotic factors is related to low defensive compounds and increased nitrogen concentrations, in comparison to non-stressed plants, thus benefiting herbivores (White, 1984; Cornelissen and Stiling, 2005). The competing hypothesis, i.e., that herbivores do not select stressed plants, but rather are a direct cause of FA (*herbivory-induced stress hypothesis*—Alves-Silva and Del-Claro, 2016), has scarcely been examined, with different results (Berteaux et al., 2007; Zvereva et al., 1997a). Recently, it was demonstrated that even low herbivory levels in young leaves are capable of causing significant increases in FA, an evidence for the herbivory-induced stress hypothesis (Alves-Silva and Del-Claro, 2016). In an ecological conservation context, FA can be used to anticipate changes, sometimes detrimental, in plant fitness (Siikamäki et al., 2002; Venâncio et al., 2016) and initiatives can be performed to mitigate the effect of the stressing factors. Therefore, the faster the stress is assessed, the quicker can researchers predict whether plants are under stress or not.

In this study, we experimentally assessed the impact of simulated herbivory on leaf developmental instability (FA) and nutritional quality. These tasks were evaluated in seedlings of a rapid-growing invasive species, *Tithonia diversifolia* (Hemsl) Gray (Asteraceae). The leaf life span of *T. diversifolia* lasts approximately 45 days, which might give us a rapid estimation of whether herbivory incurred in plant stress. Specifically, we compared the levels of FA and nitrogen in plants before and after leaf damage. Comparisons between control and treated groups were also performed. We expected a significant increase in FA after leaf damage, as suggested by the herbivory-induced stress hypothesis. Nitrogen levels were also expected to change after herbivory, as leaf damage can result in both increase or decrease in nitrogen (Gange and Nice, 1997; Hartley, 1998; Cornelissen and Fernandes, 2001a). By choosing a rapid-growth plant species we should be able to evaluate short-term effects of herbivory on parameters that are indicative of plant health and nutrition.

2. Material and methods

2.1. Plant species and status

T. diversifolia (also known as Mexican sunflower) is a semi-herbaceous shrub (1.5–2.5 m tall) with many branches and ramifications. Mature leaves reach up to 40 mm in width and 55 mm in length, are greenish, lobed and have small trichomes distributed evenly throughout the leaf blade. The margin is undulate, the base is acuminate, and the apex varies from acute to acuminate. Leaf lifespan lasts approximately 45 days, and senescent leaves are shed and rapidly replaced by new ones (personal observation). *T. diversifolia* presents rapid growth and is known because of its invasive capability, inhibiting the establishment of economically important crops and ornamental plants. It can reproduce both sexually and asexually, and produces over 100,000 small-sized seeds dispersed by wind, which contributes to its dispersal by colonizing every available sunny space (Muoghalu, 2008). Individuals can tolerate a wide range of environmental and edaphic conditions. Adult plants may cover large areas and restrain the movement of animals and humans (see more in Muoghalu and Chuba, 2005). *T. diversifolia* is native to North and Central America and has been successfully introduced in China and some countries of Africa, and South America (Chengdong et al., 2006; Muoghalu and Chuba, 2005; Olivares, 2003), and is a potential invader of Pacific Islands (Meyer, 2000).

2.2. Plant sampling and seed germination

Seeds of *T. diversifolia* were randomly collected in June 2012 from a population of more than a hundred individuals growing in a degraded area within a Brazilian tropical savanna (Campus Glória, 48°12'W–18°57'S) near Uberlândia city, Brazil. In January 2014, seeds were germinated in Petri dishes under controlled conditions (12L:12d, 25°C ± 1°C) in a germination chamber. Then, seedlings were individualized and transferred to pots (1.1 l; 12 cm × 10 cm) filled with a homogenized mixture of commercially available topsoil (Bioplant®), containing organic humus and nutrients. Seedlings were kept growing in a greenhouse at the Federal University of Uberlândia (18°53'S, 48°15'W), Brazil, from February to September 2014, when research on artificial herbivory commenced. At this time, all seedlings were approximately 40 cm tall and possessed fully expanded mature leaves, but our experimental procedure was conducted on the young/expanding leaves (see below). Plants at the greenhouse were evenly spaced to avoid shading and mechanically tap-watered four times a day in six-hour intervals during the entire study period. The greenhouse remained closed to avoid the entry of insect herbivores and uncontrolled herbivory, which might have interfered with the research.

2.3. Study design

For the experimental design, a total of 48 *T. diversifolia* seedlings were used. These individuals were randomly assigned to either treatment ($n = 24$) or control ($n = 24$), according to the experimental manipulation. The “treated” group contained plants that were used for the simulated herbivory tests, while the “control” group did not receive any type of damage simulation. The three most apical leaves from each plant were used for measurements ($n = 72$ leaves from each group). These leaves were 47 mm long (± 0.69 mm, mean \pm SE; range = 31.12–55.01), in contrast to fully expanded mature leaves (range = 60–69 mm in length; 63.99 ± 0.42 mm). As leaf lifespan does not last more than 45 days, we were able to observe the whole growth period of each tagged leaf. This was important because we hypothesized that simulated herbivory would affect leaf FA. This can be detected if leaves still grow following the treatment.

During the study period, some leaves became senescent and were naturally abscised from plants; the final sample size for each plant group was 65 and 68 leaves in the control and herbivory groups, respectively (2.71 ± 0.11 and 2.83 ± 0.08 leaves per plant respectively, mean \pm SE). For statistical procedures we used the average values per plant, as this is a widely used procedure in FA studies (following Alves-Silva and Del-Claro, 2016; Cornelissen and Stiling, 2011; Santos et al., 2013).

Before herbivory simulation experiments, several plant parameters were measured, namely: plant size (cm), number of leaves, leaf length (mm), leaf width (mm) and fluctuating asymmetry (mm); for plant size assessment, we used a metric tape, while for the other parameters, we used a digital caliper with 0.01 mm accuracy. All of these measurements were made in order to ensure that differences between plants of the “treated” and “control” group did not differ in these parameters before experimentation. This procedure was conducted twice in a seven-day interval, and in both occasions, no difference in any plant parameter was noticed between plant groups (see Section 3 for detailed information and statistical tests), thus discarding a possible bias in our study.

In mid-September, leaves from the “treated” group were subjected to artificial herbivory. To simulate leaf herbivory, a round portion of 28.26 mm² (6 mm diameter) was removed from tagged leaves, with the aid of standard size hole-punch equipment (Schooler et al., 2006). Each leaf had the same area removed (6 mm diameter), but the percentage of damage was different, since leaves differed in sizes (length range 34.79–59.24 mm, 47.28 ± 0.72 mm;

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