

## Grazing Scar Characteristics Impact Degree of Fungal Facilitation in *Spartina alterniflora* Leaves in a South American Salt Marsh

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### ABSTRACT

Grazing scars of burrowing crabs and Hemiptera insects were simulated on leaves of the salt marsh grass *Spartina alterniflora*. Simulations of crab feeding generated two-fold higher fungal (ergosterol) content in leaves in comparison to that generated by insect scar simulations ( $1.26 \pm 0.55$  and  $0.57 \pm 0.25$   $\mu\text{g per cm}^2$ , respectively). This study provided evidence that herbivory could facilitate microbial infection by fungi in dominant South American salt marsh plants and indicated that specific feeding mechanisms used by different herbivores might differentially impact the strength of this interaction.

**Key words:** fungi infestation, phytopathology, biotic interactions, aphids, *Neohelice granulata*

### INTRODUCTION

In many habitats generated by foundation species, consumers are key regulators of ecosystem structure and processes through top-down control. Past studies have demonstrated that consumers can affect ecosystems generated by a wide range of foundation species (*sensu* Dayton 1972), including terrestrial forests, marine kelps, and intertidal marshes (Silliman and Bertness 2002; Ellison et al. 2005; Halpern et al. 2006). Top-down control is often a direct interaction, where consumers reduce prey growth and biomass through removal of tissues (urchins on kelps: Power et al. 1996; parrotfish on algae: Nystrom and Folke 2001, Bellwood et al. 2004; wildebeest

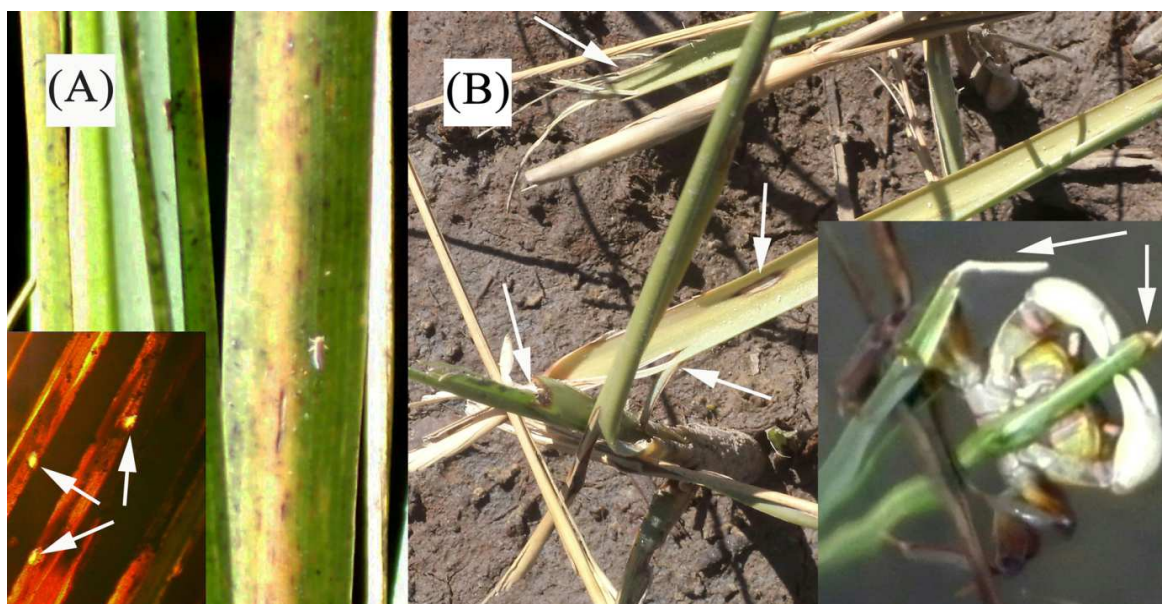
on grasses: Holdo et al. 2013) as well as photosynthetic capacity (Caldwell et al. 1981). Alternatively but less well-studied, consumer-induced tissue wounds can indirectly affect prey health by facilitating secondary infection by microbial consumers, such as fungi, bacteria, and viruses (Garcia-Guzmán and Dirzo 2001; Silliman and Newell 2003; Daleo et al. 2009). Importantly, this consumer-microbe positive interaction has been shown to lead to massive ecosystem die-off when environmental conditions simultaneously diminish the health of foundation species plants (Paine et al. 1997; Silliman et al. 2005).

Along the Atlantic coast of South America, intertidal salt marshes are dominated by the saltmarsh cordgrass, *Spartina alterniflora* Loisel.

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Several invertebrates frequently graze these plants and utilize a wide range of feeding mechanisms that result in highly variable and distinctive grazing scars. Insects of the order *Hemiptera* are common in salt marshes (Oliveira et al. 2011), and some species in this group (aphids) often exhibit high population densities in summer months (Ho and Pennings 2013). Sap-sucking insects, including aphids, use slender stylets to probe between and through plant cells in the leaf and stem until the phloem sieve is reached, producing small, round scars commonly seen on *S. alterniflora* (Fig. 1A). In contrast to sap-sucking grazing behaviors, the burrowing crab *Neohelice granulata* (Dana 1851) largely grazes *S. alterniflora* by chewing live grass leaves after climbing into the marsh canopy (Costa et al. 2003;

Alberti et al. 2007; Daleo et al. 2009; Marangoni and Costa 2012), and can also have high population densities (up to 100 individuals per m<sup>2</sup>) (D'Incao et al. 1992; Iribarne et al. 1997; Daleo et al. 2009). This crab is the dominant and most widespread herbivore that generates “chewing” feeding scars in salt marshes between southern Brazil and northeast Argentinean coast. Similar to other plants eaten by Grapsid crabs (Depledge 1989), *N. granulata* use chelae to grip *S. alterniflora* leaves and bring the leaf blade to its mandibles. With walking legs firmly standing on the ground or crossed over the leaf blade (when it is climbing the plant shoot; Fig. 1B), *N. granulata* use chelae to longitudinally rupture or scratch the leaf blade as feeding occurs.



**Figure 1** - (A) *Spartina alterniflora* leaves with aphid grazing scars. The inset depicts a magnified image of the small perforations produced by this sap-sucking feeding behavior (arrows). (B) Natural grazing scars produced by *Neohelice granulata* crabs on a *S. alterniflora* tiller (arrows highlight leaf scratches), with an inset depicting climbing and feeding behavior. In both pictures, dark areas surrounding grazing scars are visible demonstrating fungal infection.

Epiphyllic fungi are known to occur on salt marsh leaves in association with the wounds of both aphids and *N. granulata* (Fig. 1A and 1B). Fungal growth has been associated with honeydew production during aphid feeding activities (Dixon 1998; Ho and Pennings 2008). Similarly, Daleo et al. (2009) surveyed crab-induced grazing scars on marsh grasses along the northeast Argentinean coast, finding fungi infected 16-48% of wounds on

*S. alterniflora* leaves. These authors observed only the absence and presence of fungal mycelia, but no quantification of the magnitude of infestation was made. Several hundred species of fungi are known to colonize terrestrial plants, and potentially harmful pathogens can remain innocuous for some time in an epiphytic or endophytic mode culminating eventually in a pathogenic phase (Newell et al. 1989; Andrews and Harris 2000;

Silliman and Newell 2003). Since invertebrate feeding behaviors generate specific wound patterns and sizes on plant tissue, particular microsites may be created that can greatly influence the survival and growth of microbial organisms. The irregularity of plant surfaces with micro- and macro-features, such as the architecture and proximity to the epidermis of veins, can also determine successful fungal spore germination and growth (Andrews and Harris 2000). Thus, in order to begin to evaluate whether differently shaped grazing scars generated by invertebrate herbivores in South American marshes could differentially affect fungal infestation, we conducted surveys throughout the low marsh zone as well as a one-month field experiment in which different simulated grazing scars were examined for degree of fungal infection.

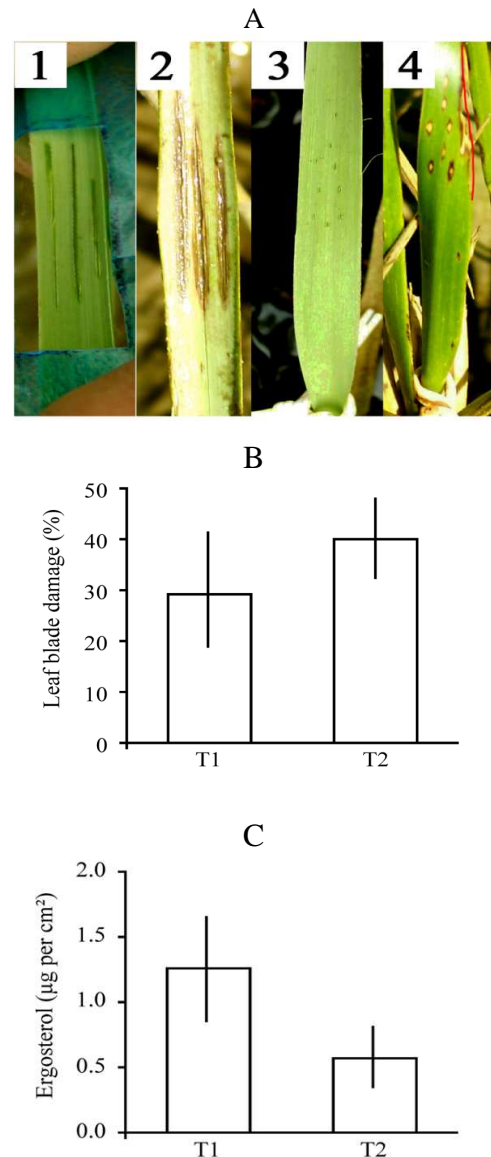
In April 2012, the number of active *N. granulata* burrows (an estimator of crab density; Iribarne et al. 1997; Alberti et al. 2007) was quantified using ten 0.25 m<sup>2</sup> quadrats randomly placed along a 6 x 30 m transect in the low marsh zone dominated by *S. alterniflora*. Plants inside the quadrats were also inspected for the presence of aphids and other sap-sucking insects. Between April and May 2012, unscarred and healthy leaves of *S. alterniflora* were wounded with simulated marks of the most common herbivore invertebrates of southern Brazil (Patos Lagoon estuary, Rio Grande, RS). Six 0.3 x 0.3 m permanent plots were placed randomly along a monospecific belt of *S. alterniflora* (mean density = 68.0 ± 7.4 tillers per plot; 755.5 ± 82.2 tillers per m<sup>2</sup>; ± standard-error). The plots were assigned to one of two treatments: crab-scar addition (T1) and aphid-scar addition (T2). Within each plot, 10 tillers were marked using colored electric wire. In T1 plots, one leaf of each marked tiller was wounded (area= 3 cm<sup>2</sup>) with a scalpel to create three longitudinal incisions parallel to leaf veins, characteristic of *N. granulata* scratch marks (Bortolus and Iribarne 1999; Alberti et al. 2007). In T2 plots, one leaf of each tiller was superficially perforated (12-15 wounds) with a needle (same leaf area, 3 cm<sup>2</sup>), mimicking sap-sucking feeding styles of aphid (Fig. 2A). Due to high tiller mortality, few artificially wounded leaves were retrieved from the permanent plots after the thirty-day experimental period (see results in the next paragraph). Because of this constraint, only one leaf per plot-replicate was collected for laboratory analysis. Additionally, a control treatment (T3) was sampled from three green leaves without

wound marks among the non-marked tillers inside T1 and T2 plots. Control leaves were the same age as scarred leaves as both types of leaves had full expanded blades, were similar in size (38-61 cm in length), and originated from summer born tillers of *S. alterniflora* (Copertino et al. 1997), suggesting that the leaves were present throughout the duration of this experiment. All leaves were transported to the laboratory using plastic bags, and the proportion of leaf area damage was estimated using a damage rating scale with six percentage classes (0, 20, 40, 60, 80 and 100%) and fungal biomass was quantified by ergosterol content (µg ergosterol per cm<sup>2</sup>). Ergosterol is the most important sterol in the cell membranes of most fungi and its quantification is considered the best method to estimate fungal biomass present in the leaves of marsh plants (Newell et al. 1989; Newell 1992; Gessner and Newell 2002). Ergosterol was extracted from wounded *S. alterniflora* blades following established methods (Newell et al. 1988). In brief, dried lipophilic extracts containing ergosterol were re-suspended in Optima grade methanol for analysis by liquid chromatography-mass spectrometry (LC-MS; Waters HPLC and UV detection hardware and a Micromass ZQ mass spectrometer). Lipophilic extracts were separated on analytical Grade C<sub>18</sub> columns using an aqueous methanol and 2% acetic acid mobile phase. Ergosterol content in *S. alterniflora* extracts was quantified against a standard curve of commercial 95% ergosterol (Sigma-Aldrich) using UV absorption peaks characteristic of ergosterol at 282 and 293 nm as well as detection of an [M + H]<sup>+</sup> ion at *m/z* 397. Differences between scar treatments on the proportion of leaf blade damage and ergosterol content were assessed using Mann-Whitney non-parametric tests.

At the beginning of the experiment, the average density of active *N. granulata* burrows was 77.0 ± 7.3 per m<sup>2</sup> in the low marsh and no evidence of sap-sucking insects was found on *S. alterniflora* leaves. The observed burrow density was among the top values recorded for *N. granulata* in Patos Lagoon estuary and other marshes in the southwestern Atlantic (D'Incao et al. 1992; Iribarne et al. 1997; Costa et al. 2003; Alberti et al. 2007). During the field experiment, the air temperature ranged between 13 and 29°C. The experimental area was flooded during 57% of the experimental period, with water salinity averaging 22.2 ± 0.9 ppt, typical conditions of the local low

marshes during the autumn (Costa et al. 2003), which benefitted crab populations (D'Incao et al. 1992; Iribarne et al. 1997). Thirty days after simulated wounding,  $83.3 \pm 17.0\%$  and  $70.0 \pm 15.5\%$  of T1 and T2 marked tillers died, likely due to intensive, natural crab grazing, respectively. These values were higher than previously reported in Pólvora Island by Marangoni and Costa (2012), who found a 35% mortality rate of *S. alterniflora* tillers due to crab wounds 30 days after grasses were planted on a low marsh during spring. This difference may be explained by reduced metabolic activities of crabs in colder temperatures during this season (D'Incao et al. 1992). Previously, Bortolus and Iribarne (1999) observed in a one-week experiment with marked leaves of *Spartina densiflora* (late summer; low marsh) that 65% of shoots died and 63% of the leaves of survived shoots were eaten by *N. granulata*.

Analysis of surviving leaves showed that although both simulated scar types covered an equal area of *S. alterniflora* leaves, wounds with small perforations typical of sap-sucking aphids tended to have a higher final average percentage of damage (T2=  $40.0 \pm 6.7\%$ ) than leaves damaged with three longitudinal incisions characteristic of crab scars (T1=  $29.2 \pm 12.4\%$ ) ( $P= 0.127$ ;  $n= 6$ ; Fig. 2B). In contrast, leaves with crab-type wounds had an average value of ergosterol two-fold higher than leaves with aphid-type wounds ( $1.26 \pm 0.39 \mu\text{g}$  ergosterol per  $\text{cm}^2$  and  $0.57 \pm 0.18 \mu\text{g}$  ergosterol per  $\text{cm}^2$ , respectively), showing a trend towards higher infestation on these scars ( $P= 0.112$ ) (Fig. 2C). These ergosterol values were similar to those reported by Silliman and Newell (2003) in *S. alterniflora* damaged by the wounds of the periwinkle snail, *Litoraria irrorata*, which ranged from 0.7-1.5  $\mu\text{g}$  ergosterol per  $\text{cm}^2$ . Ergosterol was absent in the control leaf samples (T3); however, because control grasses were not tracked during the experimental period, it was not possible to ascertain if certain leaves unscarred by grazers did not become infected by fungi on their own. In addition, results of similar studies (Silliman and Newell 2003, Daleo et al. 2009) in this system demonstrated that grazing wounds were important for initial infection of otherwise protective tissue layers. Thus, the absence of ergosterol in the control leaves was suggestive that simulated grazing scars were responsible for microbial infection in this marsh.



**Figure 2** - (A) Photographs of simulated scars of herbivorous crabs (scratches; 1 and 2) and of sap-sucking aphids (perforations; 3 and 4) on *Spartina alterniflora* leaves taken within a 30-days interval. (B) Averages ( $\pm$  standard-error) of the percentage of leaf blade damage and (C) ergosterol content of marked *S. alterniflora* tillers after 30 days of simulated invertebrate grazing. Treatments include crab-simulated scars (T1) and aphid-simulated scars (T2).

This study suggests that consumer-facilitated microbial infection may be highly context dependent upon the features and characteristics of grazing wounds specific to different herbivores.

Results also demonstrated that microbial infection of grazing wounds might occur over short temporal scales (e.g., 30 days) and across large spatial scales from Brazil to Argentina (Daleo et al. 2009). Longitudinal foliar rupture in simulated crab scratches appeared to be a highly favorable environment for the formation of fungal biomass (Fig. 2A and 2C). We speculate this feeding mechanism (e.g. leaf cutting) could promote fungal invasion and growth (Daleo et al. 2009). Crab-simulated wounds may preserve the parallel-veined vascular system next to wounds and provide better nutrition to colonizing fungi. It has been previously demonstrated that micro-scale resource patchiness of the epiphytic habitat is important for microbial growth (Andrews and Harris 2000). In salt marsh plants, fungal biomass is dependent on the quality of colonized plant material and environmental conditions such as humidity and temperature (Behalova et al. 1994; Newel 2000). Importantly, plants are known to respond to chemical signals of herbivores and produce chemical defenses in response to herbivore grazing (Caldwell et al. 1981), while fungal infection can be stimulated by sugar-rich “honeydew” chemicals excreted by aphids (Dixon 1998; Andrews and Harris 2000). As grazing simulations in the present study did not include these chemical cofactors produced by the actual herbivores, future studies using herbivores should be conducted in order to confirm the present findings.

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