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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 ± 0.55 and 0.57 ± 0.25 µg per cm², 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^2) (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 (Deplegde 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.

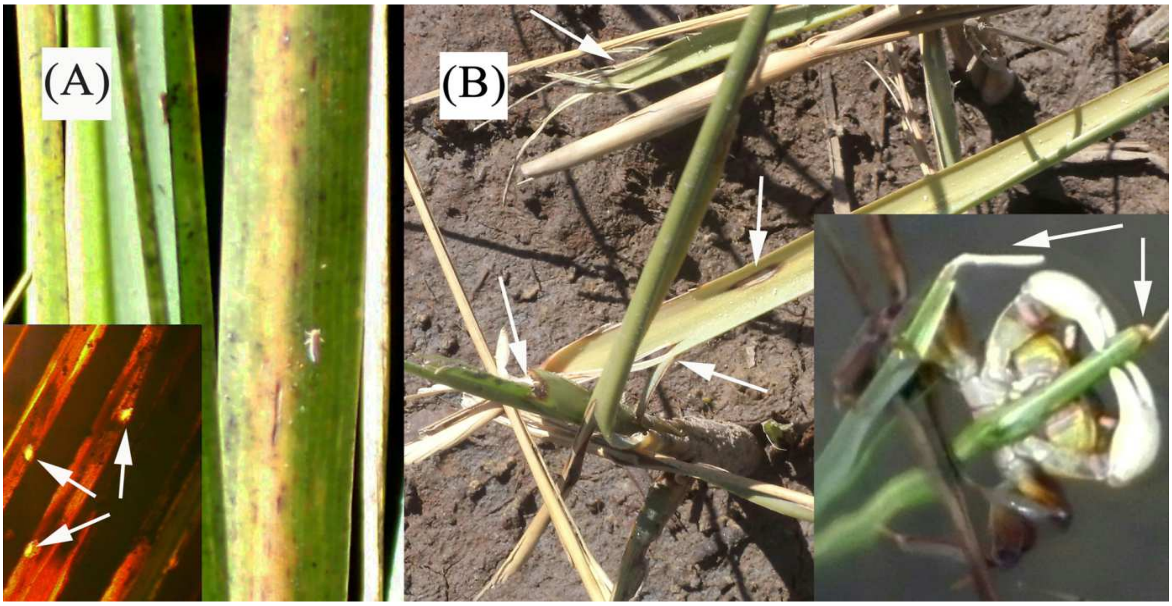

Figure 1 - (A) *Spartina alterniflora* leaves with aphid grazing scars. The inset depicts a magnified image of the small perforations produced by this sap-sucking feeding behavior (arrows). (B) Natural grazing scars produced by *Neohelice granulata* crabs on a *S. alterniflora* tiller (arrows highlight 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 m² 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 m²; ± 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^2) 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^2), 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^2). 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_{18} 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 $[M + H]^+$ 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^2 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 ± 17.0% and 70.0 ± 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 ± 6.7%) than leaves damaged with three longitudinal incisions characteristic of crab scars (T1= 29.2 ± 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 ± 0.39 µg ergosterol per cm^2 and 0.57 ± 0.18 µg ergosterol per 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 Newel (2003) in *S. alterniflora* damaged by the wounds of the periwinkle snail, *Litoraria irrorata*, which ranged from 0.7-1.5 µg ergosterol per 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.

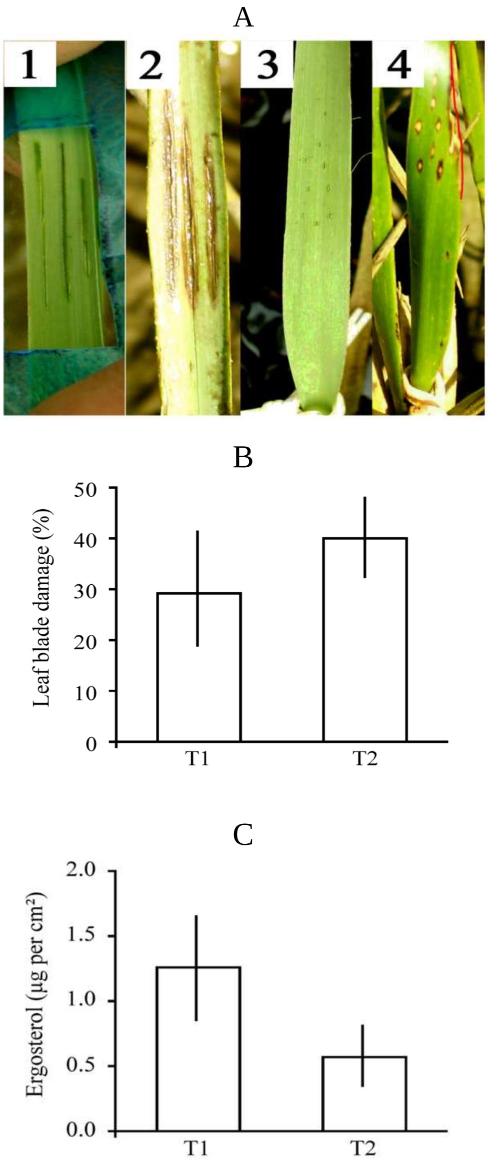

Figure 2 - (A) Photographs of simulated scars of herbivorous crabs (scratches; 1 and 2) and of sap-sucking aphids (perforations; 3 and 4) on *Spartina alterniflora* leaves taken within a 30-days interval. (B) Averages (± standard-error) of the percentage of leaf blade damage and (C) ergosterol content 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.

REFERENCES

Alberti J, Escapa M, Daleo P, Iribarne O, Silliman B, Bertness M. Local and geographic variation in grazing intensity by herbivorous crabs in SW Atlantic salt marshes. *Mar Ecol Prog Ser*. 2007; 349: 235-243.

Andrews JH, Harris RF. The ecology and biogeography of microorganisms on plant surfaces. *Ann Rev Phytopatho.* 2000; 38: 145-180.

Behalova B, Blahova M, Behal V. Regulation of sterol biosynthesis in *Saccharomyces cerevisiae. Folia Microbiol.* 1994; 39**:** 287-290.

Bellwood DR, Hughes TP, Folke C, Nyström M. Confronting the coral reef crisis. *Nature*. 2004; 429**:** 827-833.

Bortolus A, Iribarne O. Effects of the SW Atlantic burrowing crab *Chasmagnathus granulata* on a *Spartina* salt marsh. *Mar Ecol Prog Ser*. 1999; 178: 79-88.

Caldwell MM, Richards JH, Johnson DA, Nowak RS, Dzurec RS. Coping with herbivory: photosynthetic capacity and resource allocation in two semiarid *Agropyron bunchgrasses*. *Oecologia.* 1981; 50**:** 14-24.

Copertino M, Costa, CSB, Seeliger U. Dinâmica populacional de *Spartina alterniflora* em pântanos salgados do estuário da Lagoa dos Patos, Rio Grande, RS. In: *Annals of VIII Seminário Regional de Ecologia*. São Carlos (Brazil): Universidade de Federal de São Carlos; 1997. 295-312.

Costa CSB, Marangoni JC, Azevedo AMG. Plant zonation in irregularly flooded salt marshes: relative importance of stress tolerance and biological interactions. *J Ecol.* 2003; 91: 951-965.

Dayton PK. Toward an understanding of community resilience and the potential effects of enrichments to the benthos at McMurdo Sound, Antarctica. In: Parker BC. (Ed.). *Proceedings of the colloquium on conservation problems in Antarctica*; Lawrence: Allen Press; 1972. 81-96.

Depledge M.H. Observations on the feeding behaviour of *Gaetice depressus* (Grapsidae: Varuninae) with special reference to suspension feeding. Mar Biol. 1989; 100: 253-259.

D'Incao F, Ruffino ML, Silva KG, Braga AC. Responses of *Chasmagnathus granulata* Dana (Decapoda: Grapsidae) to salt-marsh environmental variations. *J Exp Mar Biol Ecol.* 1992; 161: 179-188.

Daleo P, Silliman BR, Alberti J, Escapa M, Canepuccia A. Grazer facilitation of fungal infection and the control of plant growth in south-western Atlantic salt marshes. *J Ecol.* 2009; 97: 781-787.

Dixon AFG. *Aphid ecology: an optimization approach*. 2nd ed. London: Chapman and Hall; 1998.

Ellison AM, Bank MS, Clinton BD, Colburn EA, Elliott K, Ford CR, et al. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. *Front Ecol Environ.* 2005; 9: 479-486

Garcia-Guzmán G, Dirzo R. Patterns of leaf-pathogen infection in the understory of a Mexican rain forest: incidence, spatiotemporal variation, and mechanisms of infection. *Am J Bot*. 2001; 88: 634-635.

Gessner MO, Newell SY. Biomass, growth rate, and production of filamentous fungi in plant litter. In: Hurst CJ, Crawford R., Knudsen G, McInerney M, Stetzenbach L. (Eds). *Manual of Environmental Microbiology*. Washington (DC): American Society for Microbiology; 2002. 390-408.

Halpern B, Cottenie SK, Broitman BR. Response: top-down vs. bottom-up effects in kelp forests. *Science.* 2006; 313: 1738-1739.

Ho C-K, Pennings SC. Consequences of omnivory for trophic interactions on a salt-marsh shrub. *Ecology.* 2008; 89: 1714-1722.

Ho C-K, Pennings SC. Preference and performance in plant-herbivore interactions across latitude - a study in U.S. Atlantic salt marshes. *PLoS One*. 2013; 8-e59829:1-11.

Holdo RM, Holt RD, Fryxell JM. Herbivore–vegetation feedbacks can expand the range of savanna persistence: insights from a simple theoretical model. *Oikos*. 2013; 122: 441-453.

Iribarne O, Bortolus A, Botto F. Between-habitat differences in burrow characteristics and trophic modes in the southwestern Atlantic burrowing crab *Chasmagnathus granulata*. *Mar Ecol Prog Ser*. 1997; 155: 137-145

Marangoni JC, Costa CSB. Short- and long-term vegetative propagation of two *Spartina* species on a salt marsh in southern Brazil. *Estuar Coast*. 2012; 35: 763-773.

Newell SY. Estimating fungal biomass and productivity in decomposing litter. In: Carroll GC, Wicklow DT (Eds). *The Fungal Community*. 2nd ed.; New York: Marcel-Dekker; 1992. 521-561.

Newell SY. Multiyear patterns of fungal biomass dynamics and productivity within naturally decaying smooth-cordgrass shoots. *Limnol Oceanogr*. 2001; 46: 573-583.

Newell SY, Arsuffi TL, Fallon RD. Fundamental procedures for determining ergosterol content of decaying plant material by liquid chromatography. *Appl Environ Microbiol*. 1988; 54: 1876-1879.

Newell SY, Fallon RD, Miller JD. Decomposition and microbial dynamics for standing, naturally positioned leaves of the salt marsh grass *Spartina alterniflora*. *Mar Biol*. 1989; 101: 471-481.

Nyström M, Folke C. Spatial resilience of coral reefs. *Ecosystems*. 2001; 4: 406-417.

Oliveira EA, Zardo CML, Nascimento LV. Abundância e padrão sazonal da entomofauna de restinga em uma ilha do estuário da Laguna Lagoa dos Patos, Rio Grande, RS, Brasil. *Estud Biol (Curitiba)*. 2011; 28: 27-35.

Paine TD, Raffa KF, Harrington TC. Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annu Rev Entomol*. 1997; 42**:** 179-206.

Power ME, Tilman D, Estes JA, Menge BA, Bond WJ, Mills LS, et al. Challenges in the quest of keystones. *BioScience*. 1996; 46: 609-620.

Silliman BR, Bertness MD. A trophic cascade regulates salt marsh primary production. *Proc Natl Acad Sci*. 2002; 99**:** 10500-10505.

Silliman BR, Newell SY. Fungal-farming in a snail. *Proc Natl Acad Sci*. 2003: 100: 15643-15648.

Silliman BR, Van de Koppel J, Bertness MD, Stanton LE, Mendelssohn IA. Drought, snails, and large-scale die-off of southern U.S. salt marshes. *Science*. 2005; 310: 1803-1806.

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