

LETTER

Ecosystem engineers activate mycorrhizal mutualism in salt marshes

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Abstract

Theory predicts that ecosystem engineers should have their most dramatic effects when they enable species, through habitat amelioration, to live in zones where physical and biological conditions would otherwise suppress or limit them. Mutualisms between mycorrhizal fungi and plants are key determinants of productivity and biodiversity in most terrestrial systems, but are thought to be unimportant in wetlands because anoxic sediments exclude fungal symbionts. Our field surveys revealed arbuscular mycorrhizal associations on salt marsh plant roots, but only in the presence of crabs that oxygenate soils as a by-product of burrowing. Field experiments demonstrate that fungal colonization is dependent on crab burrowing and responsible for nearly 35% of plant growth. These results highlight ecosystem engineers as ecological linchpins that can activate and maintain key mutualisms between species. Our findings align salt marshes with other important biogenic habitats whose productivity is reliant on mutualisms between the primary foundation species and micro-organisms.

Keywords

Bioturbation, ecosystem engineers, facilitation, mycorrhizal fungi, salt marshes, soil invertebrates–mycorrhizae interactions.

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INTRODUCTION

Over the past decade ecologists have begun to recognize the critical role played by ecosystem engineers in influencing the structure and function of natural communities (Jones *et al.* 1994; Bruno *et al.* 2003). This influence is because their activities can ameliorate environmental stresses and regulate levels of resources and/or abiotic factors (Bertness & Callaway 1994; Jones *et al.* 1994; Hastings *et al.* 2007). Theory predicts that ecosystem engineers should have their most dramatic effects when they enable species, through habitat amelioration, to live in zones where physical or biological conditions would otherwise suppress or limit them (Bruno *et al.* 2003; Hastings *et al.* 2007). This community-structuring effect may be especially important if engineers affect key species that, in turn, affect community function.

Over the past 400 million years, mycorrhizal fungi have evolved close associations with plants (Remy *et al.* 1994) in which fungi provide nutrients to plants, while plants provide carbon and energy to the fungi (Smith & Read 1997). Arbuscular mycorrhizal fungi (thereafter AMF) are critical in

terrestrial ecosystems, strongly affecting plant diversity, productivity, community structure and susceptibility to exotic invasion (van der Heijden *et al.* 1998a,b; Klironomos *et al.* 2000; Callaway *et al.* 2001; Hart *et al.* 2003; Callaway *et al.* 2004). In wetland systems, these symbionts are assumed to be unimportant determinants of ecosystem structure because they are obligate aerobes, and hence, are suppressed in anoxic waterlogged soils (Khan 1993; Miller & Sharitz 2000). Despite low oxygen levels in soils, AMF spores are usually abundant in these ecosystems, but fail to develop because of stressed conditions (Carvalho *et al.* 2004). Flooded, plant-dominated ecosystems (e.g. seagrasses, marshes, mangroves and freshwater wetlands); however, often harbor high densities of burrowing organisms (e.g. polychaetes, crabs and shrimps) that can oxygenate soils as a by-product of their burrowing activities (Montague 1982; Bertness 1985; Gribsholt *et al.* 2003; Gutierrez *et al.* 2006). Because of the amelioration of anoxic stress, the presence of burrows can increase aboveground plant production (Bertness 1985). This effect is thought to be direct, by increasing soil drainage, decreasing toxic compounds like sulfurs and

increasing aerobic respiration of plant roots (Montague 1982; Bertness 1985). However, these plant systems are also nutrient limited (Valiela *et al.* 1976), and their production is strongly affected by nutrient availability, particularly nitrogen (Emery *et al.* 2001). Thus, an alternative mechanism for burrowing organisms to augment wetland plant growth could be increasing nutrient supply via facilitation of AMF near constructed burrows.

Along protected shorelines of the Southwestern Atlantic Ocean, salt marshes are characterized by two dominant species: the cordgrass, *Spartina densiflora* Brongniarti, a perennial salt-tolerant grass native to South America (see Bortolus 2006) and the burrowing crab (up to 60 mm in carapace width) *Chasmagnathus granulatus* Dana (Iribarne *et al.* 1997). *Spartina densiflora* grows year around in this region and forms dense monospecific stands across the entire intertidal. Belowground, more than 80% of *S. densiflora* root biomass is found in the top 0.3 m of the soil, while its rhizomes are found primarily in the top 0.05 m of sediment. Within these *S. densiflora*-dominated marshes, *C. granulatus* commonly occurs at high densities (often $> 60 \text{ m}^{-2}$) and are active year around, primarily from early spring to late autumn (Iribarne *et al.* 1997). Their burrowing activities can transport up to $10 \text{ g day}^{-1} \text{ burrow}^{-1}$ of sediment from deeper soil layers to the surface (Gutierrez *et al.* 2006) and result in the construction of dense matrices of burrows (up to 100 mm in diameter and 1 m deep) that comprise up to 25% of the top 0.5 m of soil volume (Iribarne *et al.* 1997). This continual burrowing activity and subsequent sediment turnover greatly impacts sediment chemistry (e.g. nitrification, denitrification and organic carbon oxidation) and enhances both soil drainage and aeration (Iribarne *et al.* 1997; Botto & Iribarne 1999; Botto *et al.* 2005; Gutierrez *et al.* 2006).

Here, we examine the potential facilitative relationship between this crab and AMF in coastal Argentinean salt marshes. We experimentally tease apart their separate and interactive impacts on the production of the dominant plant (the cordgrass *S. densiflora*). Specifically, we investigated the hypothesis that burrow construction promotes AMF colonization of marsh plant roots by oxygenating waterlogged soils and, in turn, that this interaction positively affects the growth of *S. densiflora*.

METHODS

This study was performed at the Mar Chiquita coastal lagoon (Argentina, $37^{\circ}32' \text{ S}$; $57^{\circ}19' \text{ W}$), a brackish body of water affected by semi-diurnal microtides ($< 1 \text{ m}$) and characterized by mudflats and a large cord grass (*S. densiflora*) marsh (Isacch *et al.* 2006). The burrowing crab *C. granulatus* is a dominant macro-invertebrate (often $> 60 \text{ crabs m}^{-2}$; Iribarne *et al.* 1997).

To quantify the relationship between burrow density and the mycorrhizal status of *S. densiflora*, in November 2005 (mid-spring), burrow density and root samples were taken from 21 quadrats ($0.5 \times 0.5 \text{ m}$) randomly placed in the low marsh (almost daily inundated, *c.* 0.5 m above mean low tide). Burrow opening density inside each quadrat was directly counted (burrows are always open) and roots were collected from the centre of the quadrat. To collect roots, tillers were excavated to a depth of 0.4 m (more than 90% of *S. densiflora* roots are at $< 0.4 \text{ m}$ depth; P. Daleo, personal observation) and transported to the laboratory. After rinsing with tap water, three random fine roots per plant (from *c.* 0.1 m depth) were cleaned for 20 min in a 10% KOH solution at 90° C , placed in 1% HCl solution for 10 min, and then stained with lactophenol-trypan blue (0.05%) at 90° C for 20 min (Burke *et al.* 2002). Stained root samples were mounted on slides and examined at $400\times$. Quantification of root colonization by AMF was carried out by sampling a 1 mm field and determining presence or absence of mycorrhizal associations in this field. At least 30 fields per slide were observed (at least 90 per plant). In this way, the proportion of potential root tissue occupied by AMF was estimated by averaging the data obtained in the three subsamples from each quadrat. The null hypothesis of no relation between the proportion of potential root tissue occupied by AMF and burrow density was evaluated by correlation analysis (Zar 1999).

To quantify crab burrowing effects on sediment redox potential, vertical profiles of sediment redox potential (Eh) were measured at sites with ($n = 4$) and without crabs ($n = 4$) at 0–10, 10–20, 30–40, 50–60, 70–80 and 90–100 mm depth intervals. Eh was measured *in situ* by pushing a combined Pt electrode with Ag/AgCl internal reference (HI-3230B, Hanna Instruments, Woonsocket, RI, USA) into the sediment. The electrode was calibrated with a saturated quinhydrone solution at both pH 4 and 7 at 30° C and Eh measurements were corrected with respect to a reference hydrogen electrode (see Jang *et al.* 2005). The null hypothesis of no crab effect on redox potential between treatments was evaluated with two-way ANOVA with *post hoc* tests used when statistical differences were detected (Zar 1999).

To experimentally test for the effects of burrows on sediment oxygen availability, oxygen availability was measured in a 3-year crab exclusion experiment (on 10 April 2007). This experiment consisted of 27 plots ($0.7 \times 0.7 \text{ m}$). In nine plots, crabs were excluded with a plastic mesh (10 mm opening) and burrows were filled with sediment (total exclusion treatment); in nine other plots, crabs were excluded but burrows were not filled (burrows only treatment); and in the remaining nine plots, crabs were not excluded and burrows were not filled

(control treatment). Profiles of O_2 were measured by gently pushing a Clark type glass microelectrode (500 μm tip, Unisense A/S Aarhus N, Denmark) into the sediment at 10 mm intervals (Revsbech 1989). The microelectrode was positioned by a micromanipulator and the sensor current was measured with a picoammeter (PA2000, Unisense A/S). The microelectrode was calibrated with both air-saturated and oxygen-free N_2 -saturated water at the same temperature as the sediment.

A separate field experiment was conducted (11 November 2005 to 30 January 2006) to evaluate if the presence of crab burrows affected the mycorrhizal status of *S. densiflora*. Twenty 0.5×0.5 m random plots were selected in an area of the marsh with high crab burrow density (> 40 burrows m^{-2}). In 10 of these plots, burrows were filled with ambient sediment and crabs were removed and excluded with plastic mesh (10 mm opening). After 3 months, roots were collected from each plot and AMF association was quantified as described above.

To examine the hypothesis that crab burrows affect *S. densiflora* growth by affecting mycorrhizal mutualism, a factorial experiment was conducted (from 20 November 2005 to 12 May 2006). Forty-two plots (0.7×0.7 m) were established. In 14 plots, crabs were excluded with a plastic mesh (10 mm opening) and burrows were filled with sediment (total exclusion treatment); in 14 other plots crabs were excluded but burrows were not filled (burrows only treatment) and in the remaining 14 plots, crabs were not excluded and burrows were not filled (control treatment). In one half ($n = 7$) of the plots of each treatment, 20 g ($= 0.1 \text{ g kg soil}^{-1}$; Harnett & Wilson 1999) of the fungicide Benomyl (IPESA Chemical Industries, Buenos Aires, Argentina) was added every 30 days. This fungicide is the best option to suppress mycorrhizal fungi in the field (Harnett & Wilson 1999; Kahiluoto *et al.* 2000), but non-target effects cannot be excluded (see van der Putten *et al.* 1990; Callaway *et al.* 2004). Benomyl was added after being dissolved in 10 L of channel water. In the other plots ($n = 7$) of each treatment, 10 L of channel water (as control for water input) was added every 30 days. Thus, the experiment is a factorial design with two factors: (i) crab effect [with three levels: control (i.e. crabs and burrows); burrows only; total exclusion] and (ii) Benomyl effect (with two levels: control and Benomyl application). To test for possible caging artefacts, seven cage controls (cages with only two sides) were established. After 7 months, *S. densiflora* aboveground live plant material dry weight was measured in each plot by harvesting stems in a $0.15 \times 0.15 \text{ m}^2$ centered in each plot, drying them at 60°C for 48 h and then weighing each sample. The null hypotheses of no effect of crab and mycorrhizal presence on plant height, and, live and dead biomass of *S. densiflora* was assessed with two-way ANOVA (Zar 1999).

RESULTS

Field surveys revealed that the proportion of potential root tissue occupied by AMF increased with increasing burrow density ($r^2 = 0.5$, $n = 21$, $P < 0.001$; Fig. 1). Crab burrowing regulated the redox profiles of marsh sediments ($F_{5,36} = 23.24$, $P < 0.001$). In zones without crab burrows, redox potential rapidly decreased with soil depth (0–10 mm: 384 ± 27 mV; 10–20 mm: 250 ± 21 mV; 30–40 mm: 166 ± 27 mV; 50–60 mm: 170 ± 21 mV; 70–80 mm: 163 ± 22 mV; 90–100 mm: 78 ± 18 mV), while in zones with crab burrows, redox potential levels were high throughout the entire profile (0–10 mm: 415 ± 23 mV; 10–20 mm: 428 ± 30 mV; 30–40 mm: 413 ± 31 mV; 50–60 mm: 415 ± 43 mV; 70–80 mm: 415 ± 31 mV; 90–100 mm: 374 ± 25 mV).

In the experiment designed to test the effect of burrows on soil oxygenation, filling burrows and removing crabs (total exclusion treatment) led to a rapid decrease in oxygen availability throughout the soil horizon (Crab effect \times Depth effect: $F_{20,264} = 2.8$, $P < 0.05$; Fig. 2), relative to control and burrows only treatments. There was no difference in soil oxygen levels between control and burrows only treatments, except at depths of 70 and 90 mm (Tukey test; Fig. 2).

In the experiment designed to test the effect of burrows on mycorrhizal mutualism, the proportion of potential *S. densiflora* root tissue occupied by AMF was nearly 75% lower when burrows were filled ($X = 0.14$, $SD = 0.22$) compared to control plots ($X = 0.57$, $SD = 0.34$; t -test: Square root transformation, $t = 3.53$, d.f. = 18, $P = 0.0024$), indicating that burrows do facilitate mycorrhizal mutualism.

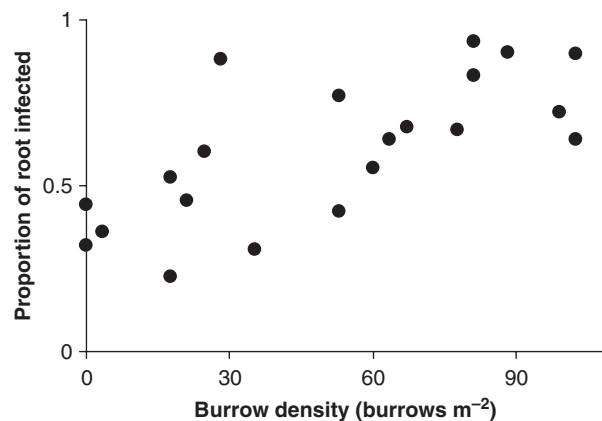


Figure 1 Relationship between the density of burrows of the crab *Chasmagnathus granulatus* and the proportion of potential root tissue occupied by mycorrhizal fungus, on roots of the salt marsh cordgrass *Spartina densiflora*.

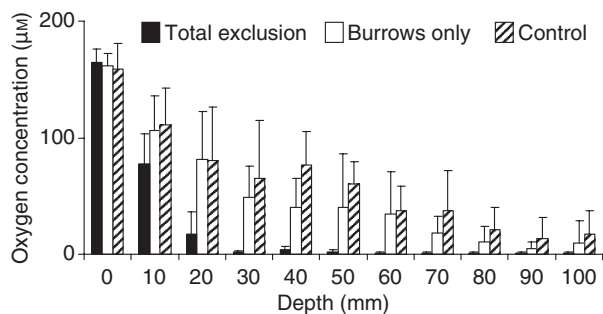


Figure 2 Effect of crab manipulation on oxygen concentration (μM) of salt marsh sediments at different depths. Measurements were made on 10 April 2007. Here and thereafter bars represent mean values and standard deviation.

In the experiment designed to examine the hypothesis that crab burrows affect *S. densiflora* growth by affecting mycorrhizal mutualism, Benomyl application led to a 92% reduction in the proportion of potential root tissue occupied by AMF ($t = 10.7$, d.f. = 12, $P < 0.001$). In all the treatments with Benomyl application, as well as in the total exclusion treatment without Benomyl application, cordgrass biomass was nearly 35% lower than in control and burrows only (without Benomyl application) treatments (Table 1, Fig. 3). The difference in biomass was due to differences in live green biomass, not in dead standing biomass (Table 1, Fig. 3). Tillers from treatments with Benomyl application, and the

Table 1 Statistical summary of ANOVA of effect of burrow presence and mycorrhizal removal on the height and biomass of *Spartina densiflora*

Source	d.f.	MS	F	P-value
Height				
Crab effect (C)	2	26 388	5.37	< 0.01
Benomyl effect (B)	1	105 102	21.4	< 0.0001
C \times B	2	21 254	4.33	< 0.01
Error	35	4910		
Total biomass				
C	2	298.9	3.87	< 0.05
B	1	655.1	8.48	< 0.01
C \times B	2	383.8	4.97	< 0.05
Error	35	77.3		
Live biomass				
C	2	215.2	14.28	< 0.0001
B	1	148.5	9.85	< 0.01
C \times B	2	62.1	4.12	< 0.05
Error	35	15.1		
Dead biomass				
C	2	9.3	0.2	> 0.05
B	1	179.8	3.85	> 0.05
C \times B	2	148.8	3.19	> 0.05
Error	35	46.7		

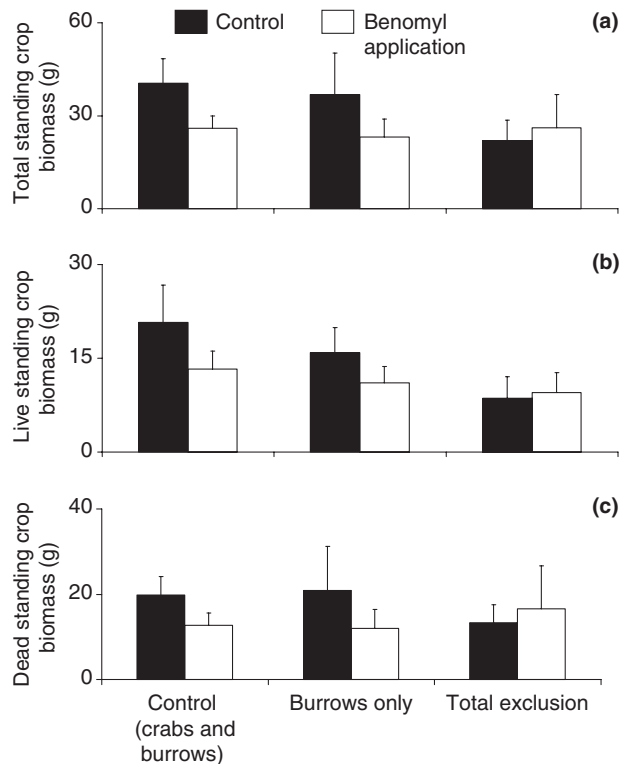


Figure 3 Effect of crab manipulation and Benomyl application on the cordgrass *Spartina densiflora*. (a) Net aboveground standing biomass; (b) live aboveground standing biomass; (c) dead aboveground standing biomass.

total exclusion treatment without Benomyl, were also smaller (in height) compared with tillers from control and only burrows (without Benomyl application) treatments (Table 1).

DISCUSSION

While burrowing organisms have been hypothesized to play a keystone role in the ecology of soils since Darwin's seminal work (Meysman *et al.* 2006; Huhta 2007), the mechanisms whereby they influence plant productivity are poorly understood. Soil invertebrates and mycorrhizal fungi co-occur in ecosystems worldwide (Gange & Brown 2002), and numerous studies have shown that earthworms, arthropods and nematodes can both positively and negatively affect mycorrhizal fungi by either increasing spore dispersion or directly eating mycorrhizal hyphae (see Gange 1993; Klironomos & Kendrick 1995; Klironomos & Moutoglis 1999; Johnson *et al.* 2005; Sjursen *et al.* 2005). Our surveys and experiments show a strong positive effect of crab burrows on soil redox potential and soil oxygen availability, the major physical factor limiting the development of AMF in wetlands (Mukerji & Mandeep 1998) and a positive association between burrows and the proportion of

S. densiflora roots associated with AMF. Field experiments demonstrate that burrow suppression leads to large decreases in AMF colonization, confirming that burrows facilitate the presence of AMF. In terrestrial systems, burrowing activity can negatively affect AMF by physical breakage of mycelium (Gange & Brown 2002) but our results reveal that crabs that burrow and thereby rework and oxygenate waterlogged soils can also activate AMF in wetland communities. Furthermore, because burrowing organisms are abundant in many aquatic ecosystems, biogenic generation of soil zones, with high oxygen availability that facilitates mycorrhizal formation, may be a general, but overlooked phenomena in systems characterized by frequently inundated soils. In this study, increases in oxygen availability may occur mainly because of burrow structures, but active ventilation and maintenance of burrows can also increase oxygen availability of soils (see Kristensen 1988; Mayer *et al.* 1995). The higher oxygen availability of controls, compared with treatments with only burrows at depths of 70 and 90 mm may be explained by active burrow maintenance and sediment turnover by crabs.

Increases in nitrogen availability can dramatically affect salt marsh community structure (e.g. Emery *et al.* 2001). Consequently, salt marshes are sensitive to interactions that affect nutrient availability (Bertness 1999). We have shown that experimental removal (both by fungicide application and crab exclusion) of AMF leads to large reductions in *S. densiflora* biomass. Burrowing crabs may also increase plant productivity by other processes, like increasing nutrient availability by deposition of faecal pellets (see Bertness 1985). Removal of crabs with burrows still present, nevertheless, did not decrease plant biomass, showing no additive effect of crabs. Increasing nitrification by sediment aeration (see Mayer *et al.* 1995) is another process that may positively affect plant growth, but fungicide application with burrows still present decreased plant biomass. Given these findings, we suggest that the primary mechanism by which crab burrowing augments marsh plant growth is the facilitation of mycorrhizal association and not by directly increasing N availability in the surrounding soils (see also Fanjul *et al.* in press).

Although Benomyl is the best option to suppress mycorrhizal fungi in the field (Harnett & Wilson 1999; Kahiluoto *et al.* 2000), its application can also affect pathogenic and detritivorous fungi (Callaway *et al.* 2004) as well as other soil organisms, like root-feeding nematodes (see van der Putten *et al.* 1990). However, (i) usually its effect on growth of host plants can be reversed by adding nutrients; (ii) there are usually no differences in plants grown on native soils with Benomyl, to those grown in pasteurized soil with other soil microflora added back and (iii) the expected result would be an increase (not a decrease) on

plant growth if it affects pathogenic more than mycorrhizal fungi (Harnett & Wilson 1999). In our experiments, although Benomyl application may have caused non-target effects, experimental application of this fungicide, as well as removal of burrows, led to a significant decrease of AMF colonization. Because both treatments negatively affected plant biomass, the results are most likely due to AMF suppression.

Our experiments reveal that nearly 35% of the vascular plant production of this salt marsh ecosystem may be dependent on mycorrhizal mutualisms and that this mutualism is strongly regulated by a burrowing organism through habitat amelioration. Given that this burrowing crab and *S. densiflora* co-occur across a large spatial scale (between southern Brazil, 28° S, and the northern part of the Argentinean Patagonia, 42° S) at similarly high densities, this interaction likely has regional ecosystem effects across SW Atlantic salt marshes. Moreover, since crabs and other burrowing and deposit feeding organisms have been shown to increase plant growth in most coastal plant communities (Montague 1982; Bertness 1985; Lee 1998), this newly uncovered mechanism may be a cryptic, critical force contributing to the high primary productivity of wetlands and the many ecosystem services they provide worldwide. Salt marsh productivity and success seems to be, as in other highly productive communities (i.e. coral reefs, terrestrial forests and grasslands), highly dependent on mutualisms between colonizers species and micro-organisms.

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