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Multiple chemical defenses produced by *Spartina alterniflora* deter farming snails and their fungal crop

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ABSTRACT: Plants are exposed to a variety of ecological threats from herbivores, pathogens, and parasites. In cases in which chemical defenses play a role in plant resistance, plants may produce a single molecule that inhibits a diverse array of enemies, or they may invest in a suite of deterrent compounds that each protect against specific threats. The snail *Littoraria irrorata* exerts substantial top-down control over smooth cordgrass *Spartina alterniflora* by culturing and grazing fungi on plant tissues. To combat fungal farming, *S. alterniflora* produces chemical defenses that inhibit fungal growth and reduce *L. irrorata* grazing. Guided by ecological assays, we isolated a fatty acid (α-dimorphecolic acid) from *S. alterniflora* that inhibited growth of *Mycosphaerella* sp., a marsh fungus commonly farmed by *L. irrorata*. *Mycosphaerella* sp. was more susceptible to the inhibitory effects of α-dimorphecolic acid than another farmed fungus, *Phaeosphaeria spartinicola*. Several phenolic compounds isolated from *S. alterniflora* deterred grazing by *L. irrorata*, of which one, the flavonoid glycoside orientin, was fully characterized. These defenses are not potent enough to completely deter fungi and snails but may slow down the negative effects caused by fungal farming. In a heavily grazed marsh, chemical defenses were constitutively expressed in *S. alterniflora* even after a month-long experiment in which exposure to fungi and herbivores was manipulated. Thus, *S. alterniflora* relies on multiple types of secondary metabolites instead of a single class of molecule to combat associated herbivores and fungi. Although α-dimorphecolic acid was not expressed in sufficient concentration on plant surfaces to prevent fungal establishment, this chemical defense may reduce fungal growth in plant tissues and increase the resistance of *S. alterniflora* to fungal farming.

KEY WORDS: *Littoraria irrorata* · Antiherbivore · Antifungal · Chemical defense · Induction · Herbivore · Pathogen

INTRODUCTION

Coastal wetlands rank among Earth's most productive ecosystems (Valiela 1995, Bertness et al. 2001) and are valuable ecological (Levin et al. 2001) and economic (Gedan et al. 2009) resources. Salt marsh primary productivity is controlled by a suite of bottom-up (Teal 1962, Deegan et al. 2012) and top-down (Srivastava & Jefferies 1996, Silliman & Zieman 2001) regulators that interact to affect overall ecosystem health (Hillebrand et al. 2007, Gruner et al. 2008). Recent evidence has suggested that the periwinkle snail *Littoraria irrorata* controls productivity of the dominant North American Atlantic marsh grass *Spartina alterniflora* by facilitating growth of fungi on plant tissues (Silliman & Zieman 2001, Silliman et al. 2005) in a rare example of grazer facilitation of fungal infection within salt marshes (but see

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Daleo et al. 2009). Traditionally viewed primarily as a detritivore (Bärlocher & Newell 1994b, Zimmer et al. 2004), *L. irrorata* establishes fungal farms on live *S. alterniflora* surfaces to gain access to a preferred fungal diet (Bärlocher & Newell 1994a, Silliman & Newell 2003). The fungi are considered facultative pathogens that exploit openings in wound sites created by *L. irrorata* but cannot easily infect living plant tissues when snails are absent (Silliman & Newell 2003). Left unchecked in the presence of compounding abiotic stressors such as drought, *L. irrorata* and their associated fungal farms can cause drastic *S. alterniflora* die-offs in salt marsh communities (Silliman et al. 2005), particularly in the absence of secondary consumers such as blue crabs (Silliman & Bertness 2002).

Since fungal farming by snails can lead to massive losses in plant biomass, salt marsh grasses including *Spartina alterniflora* should be under selective pressure to limit damage or infection due to *Littoraria irrorata* and their cultivated fungi. Marine and terrestrial autotrophs frequently employ structural and chemical defenses to deter herbivores (Hay & Steinberg 1992) and microbes (Pearce 1996, Lane & Kubanek 2008), as do salt marsh plants bordering these ecosystems (Siska et al. 2002, Hendricks et al. 2011, Sieg & Kubanek 2013). Recently, we demonstrated that *L. irrorata* prefers to establish fungal farms on *S. alterniflora* rather than on other available marsh plants, largely because *S. alterniflora* produces weaker chemical defenses against snails and fungi than do other marsh plant species (Sieg et al. 2013). However, *S. alterniflora* does possess chemical defenses: its extracts significantly inhibited fungal growth and *L. irrorata* grazing in laboratory assays relative to negative controls (Sieg et al. 2013), complementing other studies demonstrating that *S. alterniflora* produces chemical defenses against *L. irrorata* (Long et al. 2011) and other invertebrates (Siska et al. 2002).

Given that fungal establishment is often preceded by *Littoraria irrorata* herbivory, *Spartina alterniflora* could minimize allocation costs by producing a common chemical defense to deter both grazers and pathogens, similar to the way some diatoms (Ianora & Miralto 2010), macroalgae (Schmitt et al. 1995), and terrestrial plants (Krischik et al. 1991, Marak et al. 2002, Biere et al. 2004) use a single class of secondary metabolite to defend against consumers, microbes, or competitors. Alternatively, *S. alterniflora* may use a varied chemical arsenal to defend against organisms as taxonomically distinct as gastropods and fungi. Although phenolic compounds contained in *S. alterniflora* detritus are known to act as antifeedants (Valiela et al. 1979, Bärlocher & Newell 1994b), no specific chemical compounds from live salt marsh plants have previously been identified as defenses against herbivores or pathogens. Plant defenses are expected to be localized to tissues most at risk. Even if a plant appears to be chemically defended based on a high concentration of defensive compounds contained within whole tissue extracts, the plant may be susceptible to damage if grazers and pathogens first encounter other plant parts (such as blade surfaces) that are weakly defended.

Ambient *Littoraria irrorata* densities in Georgia salt marshes can exceed 600 snails m^{-2}, exerting greater top-down control of *Spartina alterniflora* production than in higher latitude marshes where herbivore densities are lower (Silliman & Bertness 2002, Silliman & Bortolus 2003, Pennings & Silliman 2005). If herbivory is intense and constant, it may be beneficial to constitutively express defenses, whereas exposure to low rates of herbivory justify minimal investment in defenses against herbivores. In cases of intermediate or cyclical herbivore pressure in which oncoming attack can be predicted by herbivore cues, many plants respond by inducing chemical or structural defenses (Karban & Baldwin 1997, Verschoor et al. 2004, Van Zandt 2007, Morrison & Hay 2011). For instance, grazing by a natural suite of herbivores in South Carolina salt marshes induced chemical defenses in *S. alterniflora* that then contributed to reduced herbivore damage (Long et al. 2011). However, chemical defenses were neither constitutively produced nor induced in *S. alterniflora* in New England marshes that contained lower ambient herbivore densities (Long et al. 2011), suggesting that *S. alterniflora* allocates resources toward defense only when herbivore grazing is substantial. Tissues and extracts from southern salt marsh plant populations also tended to be less palatable to herbivores than those from northern marshes (Pennings et al. 2001, Siska et al. 2002), and these differences in palatability persisted for multiple plant generations even when grown in common gardens (Salgado & Pennings 2005).

Although it is known that a natural suite of herbivores can influence expression of chemical defenses in some *Spartina alterniflora* populations, it has been unclear whether exposure to facultative fungal pathogens also increases allocation of resources toward defense in *S. alterniflora*. If fungi exposure changes the chemical defense profile of *S. alterniflora*, then snails that facilitate fungal pathogens

should act as a more reliable indicator of future infection than cues from other herbivore species. Using a caging experiment, we manipulated *S. alterniflora* exposure to herbivory and associated fungi for 4 wk in a Georgia salt marsh that experiences greater herbivory pressure than South Carolina sites where induction has been previously observed (Long et al. 2011). If induction of chemical defenses occurs in Georgia marshes, we hypothesized that the presence of herbivores and/or associated fungi could cause *S. alterniflora* to be more defended than plants relieved from these biotic threats, resulting in relaxation of defenses. Alternatively, if no change in chemical defenses was observed when herbivores or fungi were excluded from *S. alterniflora* stands, then we would conclude that *S. alterniflora* invests constitutively in chemical defenses or that a 4 wk timeframe is insufficient to observe significant changes in the defensive profile of the plant.

Overall, our study sought to (1) establish whether *Spartina alterniflora* allocates resources toward producing a single class of chemical defenses or a diverse suite of molecules that deter closely coupled microbes and grazers, (2) measure expression of *S. alterniflora* chemical defenses on plant surfaces and in whole tissues to assess if these defenses are likely to limit fungal farming in the field, and (3) determine whether chemical defenses of *S. alterniflora* are relaxed when herbivores and fungi are removed or if defenses are constitutively expressed.

MATERIALS AND METHODS

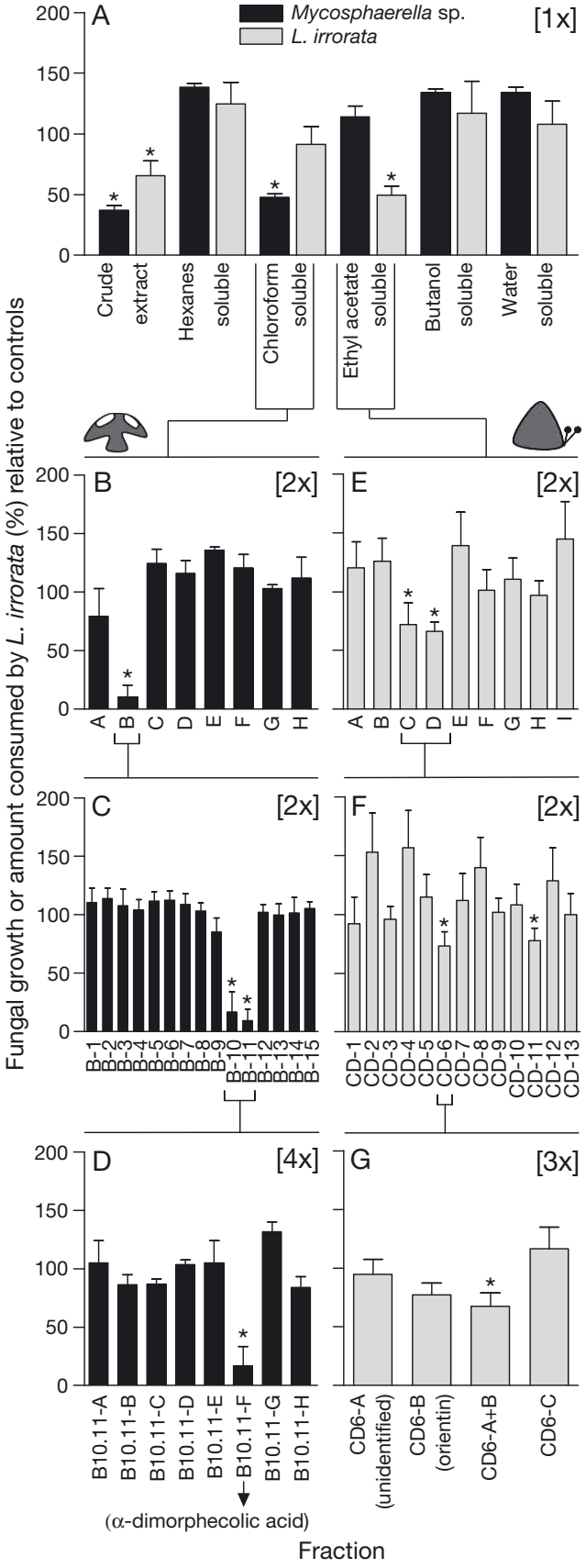
Experimental organisms

Short-form smooth cordgrass *Spartina alterniflora* was collected from mid-elevation marshes adjacent to the University of Georgia Marine Institute on Sapelo Island, Georgia, USA (31° 23′ 47″ N, 81° 17′ 00″ W) in May to July 2011. While lateral spread of *S. alterniflora* clones can vary widely (12 to 200 cm year^{-1}; Marangoni & Costa 2012), *S. alterniflora* stands on Sapelo Island are diverse, and genetically distinct ramets can be collected at 1.0 m distances (Richards et al. 2004). To maximize genetic diversity, samples were collected at least 10 m apart to accurately represent the Sapelo Island population, although we cannot exclude the possibility that some collected ramets were from the same individual. All plant samples were rinsed and stored at −20°C until extraction. A smaller suite of *S. alterniflora* samples were collected from Skidaway Island, Georgia (31° 57′ 37″ N, 81° 01′ 39″ W) in September 2012 to quantify surface and whole tissue concentrations of chemical defenses. *Littoraria irrorata* snails (average length 5.6 ± 1.9 mm) used for feeding assays were collected from Sapelo Island marshes, housed in plastic reptile cages, and fed a diet of powdered sea lettuce *Ulva lactuca* embedded in agar. Snails were checked periodically for the presence of fungal hyphae in their feces by rubbing them along an agar plate and culturing resultant microbial colonies.

We obtained 2 species of fungi, *Mycosphaerella* sp. and *Phaeosphaeria spartinicola*, from the American Type Culture Collection. These fungi (strains SAP154 and SAP136, respectively), originally isolated from Sapelo Island, Georgia, have been detected in *Spartina alterniflora* wound sites created by *Littoraria irrorata* (Newell 2001, Silliman & Newell 2003). Fungi were cultured in sterile potato dextrose broth maintained at 29°C.

Tissue extraction and extract fractionation

Prior to extraction, short-form *Spartina alterniflora* leaves were cut into 2 cm segments to maximize exposed plant surface area. We generated a crude organic extract by exhaustively extracting frozen *S. alterniflora* tissue (320 g) using methanol (MeOH) and dichloromethane (DCM). Plant tissues were extracted successively with 600 ml of 100% MeOH (1.5 h), 1:1 MeOH/DCM (1.5 h), and 1:2 MeOH/DCM (8 h at 4°C). This method was repeated 2 times, after which extracts were pooled and solvents removed by rotary evaporation. Whole *S. alterniflora* extracts were fractionated using a liquid-liquid partitioning scheme modified from Kupchan et al. (1975). In brief, the crude extract was partitioned between hexanes and 9:1 MeOH/H_2O; the aqueous extract was adjusted to 3:2 MeOH/H_2O and partitioned against chloroform. After removing MeOH by rotary evaporation, the aqueous extract was partitioned first against ethyl acetate followed by *n*-butanol (Fig. 1A). Volumes of solvent used for each step ranged from 600 to 1200 ml. The chloroform-soluble extract that was deterrent to fungi was further separated with silica gel (10 g Supelclean LC-Si solid phase extraction, SPE) using a gradient of increasing polarity from 4:1 hexanes/ethyl acetate to 4:1 MeOH/H_2O (Fig. 1B). The ethyl acetate extract that was deterrent to *Littoraria irrorata* was fractionated with C_{18} silica gel (10 g Supelclean ENVI-18 SPE) using a de-



creasing polarity gradient from 100% H_2O to 100% MeOH (Fig. 1E). Deterrent SPE fractions were separated using size-exclusion chromatography (Sephadex LH-20 resin, 1.5 × 80 cm column, 100% MeOH mobile phase, 2.4 ml min^{-1} flow rate; Fig. 1C,F). Compounds were purified using reversed-phase HPLC (Waters 515 pump and Waters 2996 photodiode array detector; Fig. 1D,G). Antifungal compounds were separated on a Zorbax Rx-C_8 silica column (9.4 × 240 mm) with a 7:3 MeOH/H_2O isocratic mobile phase (3.0 ml min^{-1}; Fig. 1D), and antigrazer compounds were separated on a Grace Alltima C_{18} silica column (10 × 250 mm) with a MeOH/H_2O gradient mobile phase (40 to 100% aqueous MeOH over 21 min, 3.0 ml min^{-1}; Fig. 1G). Nuclear magnetic resonance (NMR) spectroscopic data (^1H, ^{13}C, COSY, HSQC, and HMBC) were collected in $CDCl_3$ (for antifungal compounds) and 3:1 MeOD/D_2O (for antigrazer compounds) on a Bruker DRX-500 MHz Avance spectrometer. High-resolution mass spectra of pure compounds were obtained using an Orbitrap mass analyzer in positive and negative electrospray ionization mode, and optical rotation of compounds (in MeOH) was measured on a Jasco digital polarimeter. Structures of individual compounds were confirmed by comparing spectroscopic data with published reports of known compounds (Henry et al. 1987, Zhou et al. 2005).

Total phenolics were quantified for a subset of chromatographic fractions using the Folin-Ciocalteu assay (Folin & Ciocalteu 1927). Briefly, a 0.1 mg aliquot of each fraction was resuspended in 1.6 ml of 1:1 MeOH/H_2O, to which 100 µl of Folin and Ciocalteu phenol reagent (Sigma-Aldrich) was added. After vortexing for 10 s and allowing the sample to settle for 3 min, 300 µl of a 20% sodium carbonate solution in 1:1 MeOH/H_2O was added to each sample, followed by 1.5 h for color development. Sample ab-

Fig. 1. *Spartina alterniflora*. Isolation of antifungal and antigrazer compounds. Bars represent (A–D, black) inhibition of the fungus *Mycosphaerella* sp. or (A, E–G, gray) feeding by the snail *Littoraria irrorata* relative to paired controls. Separation methods consisted of (A) liquid-liquid partitioning, (B) silica gel column chromatography or (E) C_{18} silica gel column chromatography, (C,F) Sephadex LH-20 size-exclusion column chromatography, and (D,G) C_{18} silica HPLC. Asterisks (*) represent significant differences between treatments and paired controls (Mann-Whitney *U*-test, n = 3–5 *Mycosphaerella* sp., n = 16–20 *L. irrorata*); error bars represent 1 SE. Tested concentrations relative to natural isolated yields are denoted in square brackets in the upper right corner of each graph. Lines beneath each graph highlight pooling and subsequent separation of chromatographic fractions that significantly deterred fungal growth or snail feeding

sorbance (n = 3 per fraction) was recorded at 760 nm on a UV-visible spectrophotometer (Spectronic 21D) and compared to a tannic acid standard ranging from 0 to 2000 mg tannic acid l^{-1}.

Growth inhibition trials using marsh fungi

Extracts were tested for growth inhibition against fungi using an assay sensu Lane et al. (2009). In brief, *Spartina alterniflora* chromatographic fractions were dissolved in methanol, mixed with a sterile molten potato dextrose agar matrix, and transferred into a single well of a 24-well plate adjacent to a solvent control well (n = 3 to 5 wells per fraction). Compounds were initially embedded in agar at concentrations corresponding to the amount of extract generated from an equivalent volume of plant material, but growth inhibition was not observed at this concentration beyond the liquid-liquid partitioning step. To account for progressive losses during the fractionation process, we ran subsequent assays with extract fractions at 2 times natural volumetric concentrations during the normal-phase and size-exclusion column chromatography steps and 4 times natural volumetric concentrations for the final reversed-phase HPLC purification. Plates were incubated at 29°C after pipetting 100 µl media containing macerated fungi (*Mycosphaerella* sp. or *Phaeosphaeria spartinicola*) onto each well. After 5 d, the amount of agar surface covered by fungi was visually estimated to the nearest 10% under light microscopy using gridded visual aids as a guide. Since most, but not all, data sets were normally distributed based on a Shapiro-Wilk normality test, a Mann-Whitney *U*-test was used to compare growth of marine fungi between treatments and controls. GraphPad Prism 6 was used for statistical analyses throughout this study.

Dose-response curves were created by exposing both fungi to 9(*S*)-hydroxy-10(*E*),12(*Z*),-octadecadienoic acid (α-dimorphecolic acid; Fig. 2A) at 8 concentrations ranging from 5.0 to 850 µM using the same bioassay methods described above (n = 4 for each concentration). Each concentration was diluted from the stock sample individually, and each replicate well was paired with a solvent control. The 50% inhibition concentration (IC_{50}) for each fungus was calculated by fitting a sigmoidal dose-response curve to a plot of fungal growth inhibition against the concentration of α-dimorphecolic acid. This design simulated exposure to antifungal compounds contained within plant tissues. To mimic surface exposure to plant chemical defenses, we coated agar blocks with

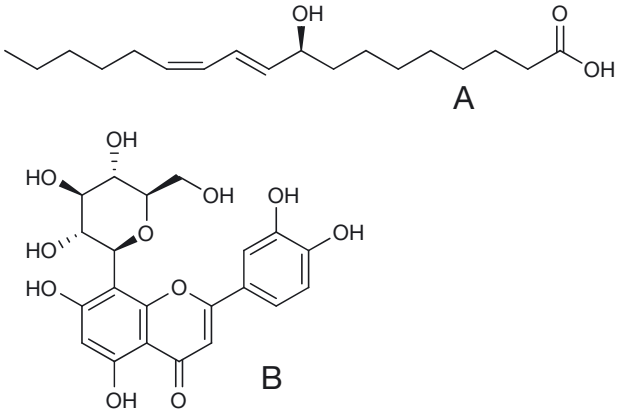


Fig. 2. *Spartina alterniflora*. Chemical defenses produced to prevent fungal farming. (A) α-dimorphecolic acid, a fatty acid that inhibits growth of the fungus *Mycosphaerella* sp.; (B) orientin, which reduces grazing by the snail *Littoraria irrorata* in conjunction with other phenolic compounds

α-dimorphecolic acid at 3 concentrations ranging from 0.5 to 5.0 µg cm^{-2}.

Concentrations of α-dimorphecolic acid were quantified in surface and whole tissue *Spartina alterniflora* extracts by separating plant extracts on a Grace Alltima C_{18} silica column attached to a Waters 2695 separations module and Waters 2996 photodiode array detector coupled to a Micromass ZQ mass spectrometer run in negative electrospray ionization mode. Surface extracts were generated by dipping individual *S. alterniflora* blades (n = 15) in hexanes for 30 s, removing non-polar chemical constituents from plant surfaces (de Nys et al. 1998). Whole tissue extracts of single *S. alterniflora* blades (n = 15) were generated by exhaustive extraction in dichloromethane and methanol. After generating a standard curve of α-dimorphecolic acid from pure compound, we integrated mass spectrometric peaks for the $[M\text{-}H]^-$ ion at the specific mass-to-charge ratio (m/z) 295 from crude extracts to determine α-dimorphecolic acid concentration relative to surface area or dry plant mass.

Feeding trials using *Littoraria irrorata*

Spartina alterniflora extracts were screened for antigrazer activity by embedding extracts in artificial diets composed of powdered *Ulva lactuca* and agar and offering treatment and control diets to *Littoraria irrorata* simultaneously sensu Sieg et al. (2013). Fractions were tested at concentrations corresponding to the amount of extract generated from a mass of plant equivalent to the mass of powdered *U. lactuca*.

Because of a loss in activity after the liquid-liquid partitioning step, fractions were tested at 2 times natural concentrations (by mass) during the SPE and size-exclusion steps, and HPLC-purified compounds were tested at 3 times natural concentrations to account for compound losses during purification. To coat artificial diets, each fraction was suspended in 2 ml MeOH with 0.6 g *U. lactuca* freeze-dried powder, while controls consisted of *U. lactuca* coated in MeOH without plant extracts. After residual MeOH was removed under vacuum, coated *U. lactuca* was mixed with 20 ml molten agar medium; 2 ml of the agar suspension was poured into 5 cm diameter Petri dishes and then allowed to cool. Artificial foods were cut in half, and control and treatment diets were paired together on single Petri dishes (n = 20 dishes per fraction) that were placed in lidded plastic containers and kept moist with a square of paper towel. Three snails were randomly added to each container, and after 48 h, the amount of control and treatment diets consumed on each plate was quantified by placing plates on a 0.3 cm^2 gridded background. Statistical differences in consumption between treatment and control portions of diet were analyzed using a Mann-Whitney *U*-test since diet consumption quantities were not normally distributed among treatments (Shapiro-Wilk normality test).

Relaxation of *Spartina alterniflora* chemical defenses

A field caging experiment was used to determine whether removal of *Littoraria irrorata* and/or fungi relaxed expression of chemical defenses in defended populations of *Spartina alterniflora*. Five sites were selected within a mid-elevation marsh zone adjacent to the University of Georgia Marine Institute (31° 23′ 47″ N, 81° 17′ 00″ W) in June 2011. Since plants had already been exposed to herbivores and fungi prior to our experiment, we could not directly test whether these factors caused *S. alterniflora* to induce chemical defenses, but our experiment was able to determine whether removal of these threats relaxed production of chemical defenses, which would indirectly suggest the potential for induction. Within each site, 40 shoots of *S. alterniflora* were haphazardly selected and cleaned of mesograzers, and each shoot was enclosed in a separate cage made from a 10 cm diameter irrigation pipe base connected to a 60 cm high tapered cylinder of window screen mesh. Our design prevented snails, grasshoppers, and other mesograzers from accessing caged plants and kept herbivores that we added to replicates within the confines of the cage. Cages were placed 1.0 m apart, forming an 8 × 5 m grid at each site. While placing cages, *S. alterniflora* rhizomes were severed using a spade to isolate caged shoots from the rest of the population, minimizing belowground signaling among *S. alterniflora* clones. Four treatments (n = 10 per site) were designed to manipulate the presence or absence of *L. irrorata* and fungi within cages. We were unable to get permission to directly infect *S. alterniflora* with cultured fungi, so we estimated the effects of *L. irrorata* or marsh fungi on production of *S. alterniflora* defenses using 3 levels of herbivory. Treatment conditions were as follows:

(1) Herbivore exclusion (ambient fungus, no *L. irrorata*): all herbivores were excluded from cages, preventing establishment of fungal farms.

(2) Artificial wounding (ambient fungus, *L. irrorata* mimic): all herbivores were excluded from cages, but a 5.0 cm scar was created on a single *S. alterniflora* blade to simulate snail grazing without chemical cues associated with *L. irrorata* herbivory or addition of fungi from snails.

(3) Addition of snails lacking fungal hyphae (ambient fungus, *L. irrorata* present): 3 laboratory-reared *L. irrorata* that had been flushed of fungal hyphae were added to each cage to measure effects of snail grazing while minimizing fungal exposure directly caused by *L. irrorata* herbivory.

(4) Addition of snails with fungal hyphae (elevated fungus, *L. irrorata* present): 3 *L. irrorata* collected from the experimental site were added to each cage, representing natural exposure to *L. irrorata* and associated fungi.

Cages were inspected twice weekly for 4 wk to ensure that snails were still present and cages were intact. The photosynthetic efficiency of the second-rank blade from each caged shoot was quantified using a pulse-amplitude modulated (PAM) fluorometer (Diving-PAM, Walz) to measure the effective quantum yield of photosystem II. Photosynthetic efficiencies of 10 uncaged plants were also measured at each site to confirm the absence of caging effects on *Spartina alterniflora* photosynthesis. After 4 wk, each caged shoot was pulled from the sediment, rinsed, and frozen at −20°C until extracted. Ten uncaged *S. alterniflora* were also collected and prepared in this manner at the same time to confirm the absence of caging effects on *S. alterniflora* chemical defenses.

Our experimental design allowed us to ask several sequential questions regarding relaxation of *Spartina alterniflora* chemical defenses. First, if extracts from *S. alterniflora* in Treatment 4 (exposure to field-

collected *Littoraria irrorata*) were significantly more deterrent to snails or fungi than those from Treatment 1 (herbivore exclusion), we could conclude that the presence of *L. irrorata* and/or associated fungi causes *S. alterniflora* to maintain production of chemical defenses. If we observed this result, then a comparison of extracts from Treatment 4 (exposure to field-collected *L. irrorata*) to Treatment 2 (artificial wounding) would allow us to assess whether mechanical damage or chemical cues from *L. irrorata*/associated fungi cause *S. alterniflora* to sustain chemical defenses. Finally, if extracts from plants in Treatment 4 (exposure to field-collected *L. irrorata*) were significantly more deterrent than those from Treatment 3 (exposure to lab-reared *L. irrorata* whose feces lacked fungi), then we could conclude that the combination of biotic cues from *L. irrorata* and fungi are required to maintain chemical defenses in *S. alterniflora.* To assess whether plant extracts significantly deterred fungi or herbivores, growth or feeding inhibition of extracts were compared to paired solvent controls. Three or 20 paired replicates (against fungi or *L. irrorata*, respectively) were averaged to generate a single estimate of deterrence for each *S. alterniflora* extract, which was then used to compare potency of extracts among treatments. Statistical differences among treatments were determined using a 2-tailed Mann-Whitney *U*-test, as not all data sets were normally distributed, and a Kruskal-Wallis test was used to confirm if sampling site had an effect on potency of *S. alterniflora* chemical defenses before we analyzed differences among treatments.

RESULTS

Chemical inhibition of fungal growth

Crude *Spartina alterniflora* extracts significantly reduced growth of farmed fungi by over 50% relative to controls ($p < 0.001$, Fig. 1A). Guided by assays with *Mycosphaerella* sp., we tracked antifungal activity through liquid-liquid partitioning (Fig. 1A), normal-phase column chromatography (Fig. 1B), size-exclusion chromatography (Fig. 1C), and reversed-phase HPLC (Fig. 1D) steps. A single lipophilic compound contained in HPLC fraction B10_11_F was responsible for fungal growth inhibition (80% reduction in growth, $p < 0.0001$, Fig. 1D). Spectroscopic analysis led to the identification of this compound as α-dimorphecolic acid (Fig. 2A, Figs. S1 to S5 in the Supplement at www.int-res.com/articles/suppl/m488p035_supp.pdf; Henry et al. 1987), isolated with a yield of 0.0036% of dry plant mass, corresponding to 36 µg g^{-1} dry plant tissue or 25 µM based on volumetric displacement of plant material. This isolated yield from bulk tissue was much lower than natural concentrations measured by liquid chromatography-mass spectrometry (LC-MS) from crude extracts of individual *S. alterniflora* plants (220 ± 31 µM, which were 320 ± 40 µg g^{-1} dry plant tissue), suggesting that almost 90% of the compound was lost during purification steps. To account for such losses, α-dimorphecolic acid was initially tested at 4 times natural volumetric concentrations (Fig. 1D). This tested concentration (100 µM) was approximately half the average natural concentration of α-dimorphecolic acid detected from individually extracted *S. alterniflora* tissue.

Using a range of α-dimorphecolic acid concentrations from 5.0 to 850 µM, we measured the sensitivity of the marsh fungi *Phaeosphaeria spartinicola* and *Mycosphaerella* sp. to this antifungal agent. *P. spartinicola* was more resistant to α-dimorphecolic acid (IC_{50} = 670 ± 4 µM, Fig. 3A) than was *Mycosphaerella* sp. (IC_{50} = 57 ± 5 µM, Fig. 3A). The minimum concentration of α-dimorphecolic acid that significantly inhibited *P. spartinicola* growth (340 µM, Fig. 3A) was equivalent to the bulk tissue concentration of α-dimorphecolic acid detected in 4 of 15 individual *Spartina alterniflora* samples but was higher than the average concentration of this compound (220 µM) across all surveyed individuals. In contrast, *Mycosphaerella* sp. was significantly inhibited by α-dimorphecolic acid concentrations as low as 10 µM (Fig. 3A), which was well within the natural concentration of this fatty acid in *S. alterniflora* tissues.

Natural concentrations of α-dimorphecolic acid on *Spartina alterniflora* surfaces were inadequate to inhibit growth of either fungus on our panel. Surface concentrations of α-dimorphecolic acid were below the limit of detection (1.1 µg cm^{-2}) of our LC-MS instrument, and neither *Mycosphaerella* sp. nor *Phaeosphaeria spartinicola* was inhibited by α-dimorphecolic acid when the compound was coated on agar surfaces at this low concentration ($p = 0.20$, Fig. 3B), suggesting that even lower α-dimorphecolic acid concentrations present on plant surfaces would have little effect on fungal growth. However, growth of *Mycosphaerella* sp. was significantly reduced at elevated α-dimorphecolic acid surface concentrations (5.0 µg cm^{-2}, 31% reduction in growth, $p = 0.044$, Fig. 3B). Since we only quantified surface concentrations of α-dimorphecolic acid from a single

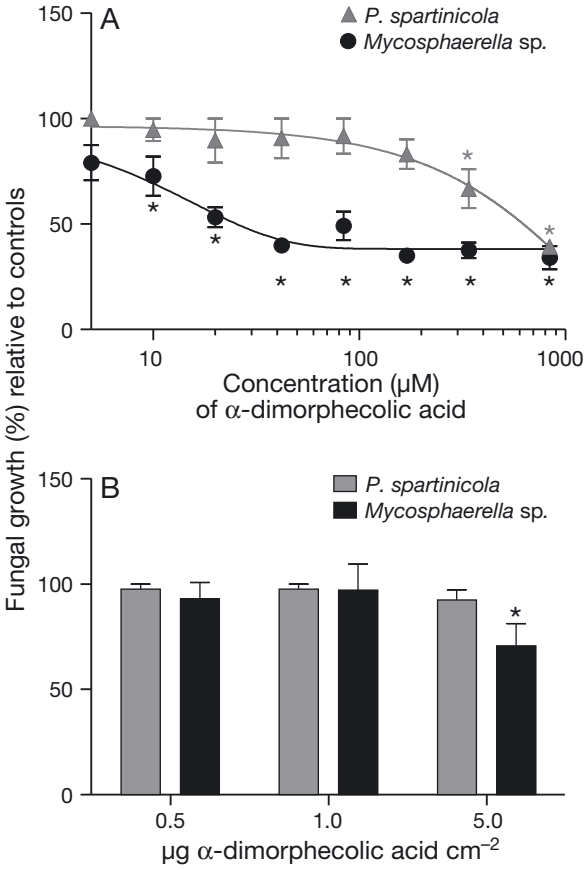


Fig. 3. Growth inhibition of α-dimorphecolic acid against the fungi *Mycosphaerella* sp. (black) and *Phaeosphaeria spartinicola* (gray) when embedded in agar representing (A) whole tissue or (B) surface concentrations. Significant differences in growth between paired treatments and controls determined by Mann-Whitney *U*-test ($p < 0.05$, $n = 4$) and denoted by asterisks. Bars represent 1 SE

site, it is possible that other *S. alterniflora* populations express this compound on surfaces at concentrations capable of preventing fungal growth.

Chemical defenses against *Littoraria irrorata*

Crude extracts generated from short-form *Spartina alterniflora* reduced *Littoraria irrorata* grazing on artificial diets by 35% relative to solvent controls ($p = 0.0016$, Fig. 1A), suggesting that *S. alterniflora* is chemically defended against herbivory. After liquid-liquid partitioning of crude extracts, chemical defenses from *S. alterniflora* that deterred *L. irrorata* were contained in the moderately polar ethyl acetate fraction (Fig. 1A). Bioassay-guided separation of this fraction using reversed-phase column chromatography (Fig. 1E) and size-exclusion chromatography (Fig. 1F) resulted in isolation of 3 compounds by HPLC that contributed to deterrence of *L. irrorata* grazing (Fig. 1F,G). Two of these compounds (CD6-A and CD6-B) had a non-significant deterrent effect on snail feeding when each was embedded into artificial diets alone at 3 times isolated concentrations (by mass) to account for progressive losses during fractionation (Fig. 1G). However, these compounds significantly reduced *L. irrorata* feeding when added to diets together at 3 times isolated concentrations, suggesting either that these compounds had an additive, deterrent effect on *L. irrorata* grazing (Fig. 1G) or that we had underestimated the loss of deterrent compounds during fractionation. Diets containing the pure compound CD6-C were not significantly deterrent unless both CD6-A and CD6-B were present (Fig. 1G). Because of progressive losses during fractionation, the elevated concentrations at which compounds were tested (3 times natural isolated yields) may actually be less than those found within live plant tissues and may be deterrent as sole compounds if tested at actual natural concentrations. Both compounds were susceptible to decomposition as fractionation progressed but were confirmed to be pure compounds instead of mixtures based on spectroscopic data. CD6-A was a moderately polar, low-molecular-weight (<500 Da) compound but was not fully characterized despite attempts to limit compound oxidation by adding antioxidants. However, spectral analysis of compound CD6-B indicated that it was the known flavonoid orientin (Fig. 2B, Figs. S6 to S10 in the Supplement; Zhou et al. 2005).

Additionally, fraction CD-11 obtained from the size-exclusion chromatography step also significantly inhibited *Littoraria irrorata* grazing (Fig. 1F) but rapidly decomposed after purification, preventing its identification or confirmation that it was a pure compound instead of a mixture of molecules. Subsequent attempts to isolate the deterrent components of CD-11 were unsuccessful, but we can conclude that CD-11 contains polar, low-molecular-weight compound(s) based on retention on silica gel and size-exclusion chromatography. The UV λ_{max} of CD6-A (225, 322 nm) and CD-11 (206 nm) are close to that of orientin (252, 345 nm), and analysis of these samples with the Folin-Ciocalteu assay suggested that both unidentified molecules contained hydroxylated aromatic functional groups (e.g. phenols), as did other palatable fractions separated by C_{18} silica gel column chromatography (data not shown).

Effect of herbivores and fungi on compound production

Among all treatments, *Spartina alterniflora* extracts generated after a month-long field experiment manipulating grazing and fungal exposure were significantly deterrent to *Littoraria irrorata* and fungi at natural concentrations (n = 9 to 15, p = 0.017 to <0.0001, Fig. 4). Sampling site did not affect the potency of chemical defenses against *L. irrorata* (Kruskal-Wallis test, p = 0.13), *Mycosphaerella* sp. (p = 0.43), or *Phaeosphaeria spartinicola* (p = 0.29). However, when tested at natural concentrations, the potency of *S. alterniflora* extracts against snails (p = 0.33) and fungi (p = 0.35 vs. *Mycosphaerella* sp., p = 0.46 vs. *P. spartinicola*) were equivalent between caged control plants or plants that had been exposed to field-collected *L. irrorata*, providing no evidence for relaxation of *S. alterniflora* chemical defenses after removal of snails and associated fungi (Fig. 4A). Differences in potency of extracts among these treatments were also non-significant when tested at half natural concentrations (Fig. 4B). Therefore, we decided it was unnecessary to draw subsequent pair-wise comparisons among treatments to determine the stimuli that maintain *S. alterniflora* chemical defenses. Furthermore, crude extracts generated from nearby non-caged *S. alterniflora* also deterred snails and fungi to a similar degree as caged plants (Kruskal-Wallis test, p = 0.27 to 0.44, data not shown). During the course of our experiment, seawater salinities surrounding experimental cages at high tide fluctuated widely from 28 to over 45 ppt. After 30 d, caging reduced the photosynthetic efficiency of *S. alterniflora* by an average of 11% relative to uncaged plants, but this effect was consistent among treatments (data not shown). Thus, photosynthesis of *S. alterniflora* appeared unaffected by snail and fungal manipulations.

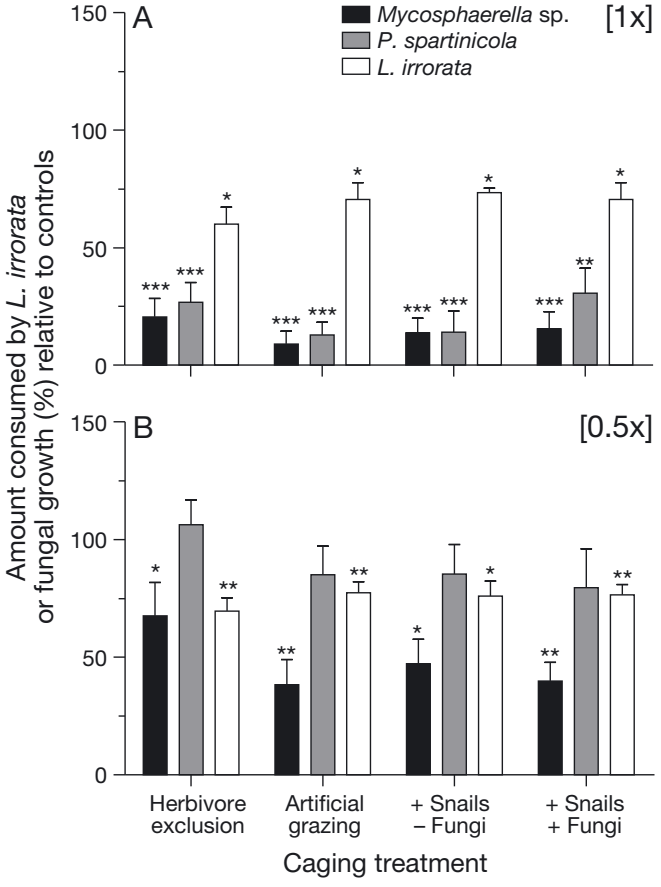


Fig. 4. *Spartina alterniflora*. Chemical defenses after cage manipulations in the field against the fungi *Mycosphaerella* sp. (black bars) and *Phaeosphaeria spartinicola* (gray bars) or the snail *Littoraria irrorata* (white bars). Caging treatments denoted on the *x*-axis. Extracts were added to agar at (A) natural or (B) half natural isolated concentrations. Significant differences between paired treatments and controls determined by Mann-Whitney *U*-test ($p < 0.05$, n = 9–15) and denoted by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Crude extracts tested at natural and half natural concentrations reduced *Littoraria irrorata* consumption of artificial diets by 25 to 40% (n = 9 to 15, $p < 0.05$ for all treatments, Fig. 4). When tested at natural volumetric concentrations, *Spartina alterniflora* extracts inhibited growth of the fungi *Mycosphaerella* sp. and *Phaeosphaeria spartinicola* by over 70% relative to controls ($p < 0.001$ for all treatments, Fig. 4A). However, when tested at half natural concentrations, *S. alterniflora* extracts were still significantly deterrent to *Mycosphaerella* sp. (25 to 60% inhibition, p = 0.001 to 0.020, Fig. 4B), whereas growth of *P. spartinicola* was statistically similar to paired controls (p = 0.092 to 0.61, Fig. 4B). The inhibitory properties of *S. alterniflora* crude extracts against *Mycosphaerella* sp. are in accordance with assays testing pure α-dimorphecolic acid (Figs. 1D & 4). In contrast, *P. spartinicola* was much more susceptible to growth inhibition by *S. alterniflora* crude extracts (containing α-dimorphecolic acid) than pure α-dimorphecolic acid at similar concentrations to that found in crude extracts (Figs. 3 & 4). Given that the caging experiment was conducted at the same sites and in the same season as harvesting of *S. alterniflora* for isolation of chemical defenses, this suggests that other presently unidentified compounds from within *S. alterniflora* tissues inhibit *P. spartinicola* growth, in addition to α-dimorphecolic acid.

DISCUSSION

Fungal farming by the periwinkle snail *Littoraria irrorata* has been detected in salt marshes along the southeastern USA (Silliman & Zieman 2001, Silliman & Newell 2003) but rarely occurs on marsh plants other than *Spartina alterniflora* (Silliman & Bertness 2002, Sieg et al. 2013). To date, evidence of grazer facilitation of fungal growth on *S. alterniflora* is limited to *L. irrorata* in the northern hemisphere (Silliman & Zieman 2001) and burrowing crabs including *Neohelice granulata* in the southern hemisphere (Daleo et al. 2009). Selection of *S. alterniflora* as a farming substrate by *L. irrorata* may be influenced by the relative potency of chemical defenses among marsh plants. In a previous study, chemical defenses produced by *S. alterniflora* were less inhibitory toward *L. irrorata* and fungi than those expressed by 4 other co-occurring marsh plants (Sieg et al. 2013). Snails also demonstrated a marked preference for *S. alterniflora* over other available plants, suggesting that *L. irrorata* assesses which plant substrates are most amenable to fungal farm establishment in selecting its habitat (Sieg et al. 2013). However, other characteristics such as plant height may also influence where snails are most likely to be found, since residing on taller plants reduces snail exposure to predators and provides relief from heat stress (Iacarella & Helmuth 2011, Hughes 2012). The current study suggests that *S. alterniflora* produces multiple polar compounds that collectively deter *L. irrorata* herbivory as well as the fatty acid α-dimorphecolic acid that inhibits the growth of the fungus *Mycosphaerella* sp. associated with *L. irrorata* farming (Fig. 1). These chemical defenses are weaker than those produced by other marsh plant species (Sieg et al. 2013), and the antifungal compound is not present on plant surfaces at concentrations adequate to prevent fungal establishment (Fig. 3B). Thus, while *S. alterniflora* is modestly chemically defended against both snail grazers and fungi, it remains a preferred target relative to other, better defended marsh plants (Sieg et al. 2013).

While several *Spartina alterniflora* compounds were isolated that additively deterred *Littoraria irrorata* grazing, we were only able to fully characterize one of these, orientin. This flavonoid glycoside is produced by a variety of plants including grasses (van de Staaij et al. 2002) and other species used as traditional medicines, such as rooibos (Bramati et al. 2002), holy basil (Devi et al. 2000), and lemongrass (Cheel et al. 2005). Orientin can function as an antioxidant (Cheel et al. 2005) and exhibits antiviral (Li et al. 2002), antibacterial (Cottiglia et al. 2001), and antifungal (De Campos et al. 2005) properties as well as a protective role against radiation injury (Devi et al. 2000). However, far less is known about the ecological role(s) of this compound, although it can act as a photoprotectant (van de Staaij et al. 2002) and defends cucumber leaves against mildew infection in conjunction with other compounds (McNally et al. 2003). Although phenolic compounds similar to orientin are often hypothesized to act as antifeedant compounds (Haribal & Renwick 1998, Renwick et al. 2001, Haviola et al. 2007), and other natural products of phenylpropanoid metabolic origin can function as plant chemical defenses (Kubanek et al. 2001, Lane & Kubanek 2006, Haviola et al. 2007), to our knowledge ours is the first study to demonstrate that orientin functions as part of a chemical defense against herbivores.

Considering that orientin did not deter *Littoraria irrorata* at the concentrations tested unless other similar compounds were also added into diets (Fig. 1), it could be that a subset of phenolic compounds produced by *Spartina alterniflora* collectively limit grazing by snails. This would support the practice of using total phenolic concentrations as a proxy for how defended a plant is against herbivory (Feeny 1976, Coley et al. 1985, Appel 1993). However, only a few of the chromatographic fractions isolated in the current study were deterrent to snail grazing (Fig. 1), whereas several non-deterrent fractions also contained phenolic compounds. Based on limited spectroscopic information, our unidentified antiherbivore molecules are also likely to be phenolic compounds, but these deterrent molecules make up only a subset of the total phenolic pool in *S. alterniflora.* Therefore, all phenolics produced by *S. alterniflora* do not act as antiherbivore compounds; instead, other phenolics might provide additional, unknown services to the plant. This explanation would be in accordance with previous studies that did not find a significant relationship between total phenolic concentrations in *S. alterniflora* and antiherbivore properties of plant extracts (Long et al. 2011, Sieg et al. 2013). However, our data suggest that *S. alterniflora* is constitutively defended against *L. irrorata* in Georgia salt marshes, which could be driven by the intense top-down control exerted by snails.

A single fatty acid compound, α-dimorphecolic acid, was responsible for antifungal activity of *Spartina alterniflora* extracts against *Mycosphaerella* sp. (Fig. 1). This compound was previously isolated from terrestrial plants (Powell et al. 1967, Henry et al. 1987, McRae et al. 2008) and cyanobacteria (Mundt et al. 2003) but has not been reported from *S. alterni-*

flora. Given the antimicrobial properties of α-dimorphecolic acid (Mundt et al. 2003, McRae et al. 2008), as well as the antifungal (Rao et al. 1991, Calvo et al. 1999, Cowley & Walters 2005) and antiherbivore (Mohri et al. 1990) properties of closely related fatty acids, it is not surprising that a compound such as α-dimorphecolic acid constitutes the moderate defense of *S. alterniflora* against the fungus *Mycosphaerella* sp.

The 2 fungi used in the current study responded differently to α-dimorphecolic acid in laboratory experiments. The average natural bulk concentration of α-dimorphecolic acid (220 µM) expressed within *Spartina alterniflora* tissues would be sufficient to protect the plant against *Mycosphaerella* sp. but not *Phaeosphaeria spartinicola* (Fig. 3A), assuming that the compound was evenly distributed in all tissues. However, use of bulk tissue concentrations may lead to an overestimate of ecologically relevant exposure of fungi to α-dimorphecolic acid, assuming that fungi first encounter *S. alterniflora* on the surface wounds created by *Littoraria irrorata*. In fact, we found natural surface concentrations of α-dimorphecolic acid to be lower than concentrations required to significantly deter fungal growth of either species (Fig. 3B), although growth of *Mycosphaerella* sp. (but not *P. spartinicola*) was significantly reduced when exposed to elevated surface concentrations of α-dimorphecolic acid (5.0 µg cm^{-2}, Fig. 3B). It appears that fungi can successfully establish on outer plant tissues where antifungal compounds are expressed at low concentrations, which could explain why *S. alterniflora* is susceptible to fungal farming by snails. However, fungal growth should be slowed as fungal hyphae extend into tissues where concentrations of α-dimorphecolic acid are higher than on plant surfaces. In a related study, *P. spartinicola* was consistently more resistant to chemical defenses of 4 other salt marsh plant species than was *Mycosphaerella* sp. (Sieg et al. 2013), which could make *P. spartinicola* the better crop for snails to cultivate and could also contribute to the prevalence of this genus on live and decaying *S. alterniflora* tissues (Newell et al. 1989, Silliman & Zieman 2001). We used *Mycosphaerella* sp. for all bioassays leading to isolation of α-dimorphecolic acid, so it is possible that we overlooked other compound(s) that *S. alterniflora* produces to inhibit *P. spartinicola* growth, which could explain why these 2 fungi were equally susceptible to *S. alterniflora* crude extracts when tested at natural concentrations (Fig. 4A).

In the current study, we found that products of multiple biosynthetic pathways are used to partially defend *Spartina alterniflora* tissues against both farming snails and their fungal crop. Mixed products of the phenylpropanoid and polyketide metabolic pathways (i.e. orientin and related flavonoids) act as chemical defenses against grazers, while a fatty acid (α-dimorphecolic acid) functions as an antifungal agent. We did not detect any overlap in antifungal or antigrazer activity within the chromatographic fractions during bioassay-guided fractionation (Fig 1A), so it is clear that α-dimorphecolic acid and orientin do not have reciprocal roles in defense against grazers or fungi, respectively. Investing in the production and maintenance of chemical defenses can impose fitness costs (see review by Koricheva 2002), but plants often employ a mixed chemical arsenal to defend against multiple threats. For instance, *Arabidopsis* uses glucosinolate hydrolysis products including nitriles and isothiocyanates to prevent caterpillar grazing (Lambrix et al. 2001) but relies on defensive proteins like defensins and thionins to defend against fungi (Epple et al. 1997). Other species rely on a single class of molecules to deter herbivores, pathogens, and competitors. The seaweed *Dictyota menstrualis* uses a suite of diterpene alcohols to prevent both fish herbivory and fouling by bryozoan larvae (Schmitt et al. 1995), and diatoms produce a blend of polyunsaturated aldehydes to defend against zooplankton grazers and to undermine competitors (see review by Ianora & Miralto 2010). Even structurally related molecules produced by the same organism can affect herbivores and pathogens differently. The iridoid glycoside catalpol is more deterrent to herbivores than its precursor molecule, aucubin, which functions as an antifungal agent in plantains (Bowers & Puttick 1988, Marak et al. 2002). Furthermore, the glycosylation status of molecules can affect their ecological targets, such that aglycones (non-glycosylated iridoids) inhibit specialist fungi, whereas iridoid glycosides deter generalist herbivores (Marak et al. 2002, Biere et al. 2004). It may be more efficient and less costly to produce generalized defenses that inhibit a range of organisms, but doing so may be disadvantageous if the targets of these defenses can adapt to or overcome defenses faster than the targeted organism can modify them.

In accordance with previous studies that found that Georgia populations of *Spartina alterniflora* were constitutively defended against herbivores (Salgado & Pennings 2005), the results of our caging experiment suggest that *S. alterniflora* produces constitutive chemical defenses and does not relax production of these defenses even if relieved from the threat of herbivory (Fig. 4). These results differ from a previous study conducted in South Carolina whereby *S.*

alterniflora exposed to a natural suite of herbivores responded by inducing more deterrent chemical defenses than those produced by plants for which herbivore pressure was removed (Long et al. 2011). Unlike the study by Long et al. (2011), we excluded all members of the salt marsh herbivore community apart from *Littoraria irrorata*. It is possible that grazing of other herbivore feeding guilds, such as planthoppers or aphids, triggers production of *S. alterniflora* chemical defenses. Furthermore, induction of chemical defenses typically occurs in a few days following initial grazing events, while relaxation of these defenses can take weeks to months (Karban 2011). In other studies, the length of our caging experiment (4 wk) was sufficient to observe changes in the chemical defense profile of established *S. alterniflora* (Long et al. 2011) and macroalgal (Pavia & Toth 2000) populations that were exposed to herbivores. We cannot exclude the possibility that plants were caged too late in the season to undergo a noticeable change in chemical defenses, since the plant population would have been exposed to intense herbivory for several months prior to initiation of our caging treatments. To account for these issues, *S. alterniflora* would ideally be exposed to caging treatments for multiple generations to assess whether *S. alterniflora* chemical defenses are induced or constitutively expressed. However, our current work and previous studies conducted on similar time scales suggest that *S. alterniflora* populations vary in their expression of chemical defenses, such that southern populations consistently exposed to intense grazing by snails (i.e. in Georgia) are constitutively defended, populations with moderate grazing pressure induce chemical defenses upon exposure to herbivore cues, and populations exposed to low herbivore densities do not invest in chemical defenses at all. Increased investment in chemical defenses because of a greater threat of herbivory may also explain why plants from southern marshes are generally less palatable to a range of herbivores than their northern counterparts (Pennings et al. 2001, Siska et al. 2002, Salgado & Pennings 2005, Long et al. 2011).

While the ambient threat of herbivory may explain when *Spartina alterniflora* populations produce chemical defenses, other factors may also influence to what extent *S. alterniflora* populations are defended. For instance, our study was conducted in a salt marsh during a period of severe to extreme drought, which could have caused *S. alterniflora* to express chemical defenses differently than the population from South Carolina studied by Long et al. (2011) where drought conditions were moderate. Reductions in freshwater inflow caused by drought can stress estuarine plants by creating hypersaline conditions and reducing nutrient input into salt marsh sediments (Alber 2002, Wetz et al. 2011). Secondary consumers of snails such as blue crabs are more susceptible to parasites in warmer, more saline waters indicative of drought conditions (Lee & Frischer 2004), which may relieve snails from predation pressure. We did not quantify nutritional quality of marsh sediments in our study and at present cannot say to what degree nutrient availability predicts *S. alterniflora* investment in chemical defenses. However, it is important to consider how abiotic environmental conditions can also explain population-level variability in *S. alterniflora* expression of chemical defenses.

In conclusion, *Spartina alterniflora* uses at least 2 classes of chemical compounds to resist establishment of fungal farms. A suite of phenolics including orientin deterred *Littoraria irrorata* herbivory, which is expected to indirectly limit fungal infection. A single fatty acid (α-dimorphecolic acid) produced by *S. alterniflora* inhibited the growth of a fungus that is commonly cultured by *L. irrorata*, although other compounds may be employed to defend against additional marsh fungi. Since fungal infection is often preceded by *L. irrorata* herbivory, *S. alterniflora* must invest in chemical defenses that deter both snail farmers and their fungal crop, requiring a more substantial investment than would be needed to inhibit one threat. However, these defenses are clearly not potent enough to completely prevent establishment of fungal farms, since *S. alterniflora* is colonized by snails (and fungi) far more frequently than other mid-elevation marsh species (Sieg et al. 2013). Chemical defenses in *S. alterniflora* were not relaxed when exposure to herbivores or fungi was relieved, suggesting that these defenses are constitutively expressed in plants from salt marshes that are likely to be damaged by tightly associated grazers and pathogens. At present, we only have a partial understanding of the abiotic and biotic factors that cause *S. alterniflora* to express chemical defenses. The chemical defenses isolated in the current study may have been sufficient to allow *S. alterniflora* to resist fungal farming in the past. However, if snail densities in the southeastern USA increase because of a loss of top-down predator control (Silliman & Bertness 2002), *S. alterniflora* could become exposed more frequently to snails and the fungi they farm. In this scenario, the weak defenses that marginally limit fungal growth and snail herbivory may no longer sufficiently protect *S. alterniflora*, which could contribute to the continued decline of *S. alterniflora*-dominated marshes.

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