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Eelgrass pathogen *Labyrinthula zosterae* synthesizes essential fatty acids

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ABSTRACT: Negative consequences of parasites and disease on hosts are usually better understood than their multifaceted ecosystem effects. The pathogen *Labyrinthula zosterae* (Lz) causes eelgrass wasting disease but has relatives that produce large quantities of nutritionally valuable long-chain polyunsaturated fatty acids (LCPUFA) such as docosahexaenoic acid (DHA). Here we quantify the fatty acids (FA) of Lz cultured on artificial media, eelgrass-based media, and eelgrass segments to investigate whether Lz may similarly produce LCPUFA. We also assess whether field-collected lesions show similar FA patterns to laboratory-inoculated eelgrass. We find that Lz produces DHA as its dominant FA along with other essential FA on both artificial and eelgrass-based media. DHA content was greater in both laboratory-inoculated and field-collected diseased eelgrass relative to their respective controls. If Lz's production scales *in situ*, it may present an unrecognized source of LCPUFA in eelgrass ecosystems.

KEY WORDS: Eelgrass · Pathogen · Fatty acids · DHA · *Labyrinthula*

1. INTRODUCTION

By definition, parasites and disease negatively affect their hosts. However, their effects, either direct or via modification of host traits or abundance, are multifaceted and relatively understudied at community and ecosystem scales (Preston et al. 2016). For example, parasites are recognized to modify biogeochemical processes (Breitbart 2012) and account for substantial biomass (Kuris et al. 2008). Thus, it should be a priority to understand potential effects of parasites beyond their hosts.

Eelgrass wasting disease (EWD), afflicting the eelgrass *Zostera marina* L., caused die-offs of Atlantic eelgrass beds in the 1930s (Renn 1934) and 1980s (Short et al. 1987). It is caused by the parasitic protist *Labyrinthula zosterae* D. Porter & Muehlst. (hereafter Lz; Muehlstein et al. 1991). With seagrasses declining worldwide (Waycott et al. 2009), EWD and related *Labyrinthula*-caused diseases are a concern, especially considering EWD's wide geographic breadth (Sullivan et al. 2013) and diverse seagrass hosts infected by labyrinthulids (Vergeer & den Hartog 1994). EWD can also be common within sites; in Washington, USA, Groner et al. (2016) found prevalences >40% at over half of their sites, with a 79% EWD prevalence at one site.

Lz belongs to the Labyrinthulomycetes, heterotrophic protists characterized by their ectoplasmic net (Raghukumar 2002). The Labyrinthulomycetes include diverse pathogens (Raghukumar 2002) but are also known for abundantly producing long-chain polyunsaturated fatty acids (LCPUFA), namely eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) (Kumon et al. 2006, Armenta & Valentine 2013). Such LCPUFA and some other fatty acids (FA), including linoleic acid (LIN, 18:2ω-6) and α-linolenic acid (ALA, 18:3ω-3), are important nutritional components for many consumers that rely on them for diverse physiological needs ranging from cell membrane fluidity to nervous system function (Parrish 2009). LIN and ALA, in particular, are con-

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sidered essential FA, because most consumers cannot synthesize them in biologically relevant amounts (Parrish 2009). DHA and EPA are also often termed essential for the same reason, although ALA can serve as a precursor for them if the organism in consideration has elongation and desaturation abilities (Parrish 2009). Eelgrass has ALA as its dominant FA but is poor in LCPUFA (Jaschinski et al. 2008, Galloway et al. 2012). While increasing evidence shows animals are more capable of synthesizing LCPUFA than recognized (Kabeya et al. 2018), increased growth and reproduction associated with greater dietary long-chain essential FA (Winder et al. 2017) indicate that exogenous sources of LCPUFA are still important. In eelgrass beds, red algae and epiphytic diatoms may supply EPA and some other LCPUFA (Jaschinski et al. 2008, Galloway et al. 2012). In contrast, DHA is uncommon in macrophytes (Galloway et al. 2012), suggesting DHA may instead enter eelgrass beds through the water column or microbial production.

Microbial bioconversion of biomolecules has been suggested as a mechanism for trophic upgrading (Klein Breteler et al. 1999) in aquatic food webs. Specifically, microorganisms consume relatively nutritionally poor organisms or substrates and then synthesize more nutritionally valuable compounds for their predators. The ability for some planktonic heterotrophic protists to produce LCPUFA and other compounds is suggested to be one means of upgrading in pelagic food webs (Chu et al. 2008, 2009). Similarly, the capacity of Labyrinthulomycetes, which are typically saprotrophs, to synthesize LCPUFA is hypothesized to be a possible upgrading mechanism for detritus (Raghukumar 2002). If so, such LCPUFA production may be an important ecological role for this taxon (Raghukumar 2002).

Here we investigate an intersection of Labyrinthulomycetes' pathogenicity and FA production. We quantify the FA of Lz on 3 substrates in the laboratory: serum seawater agar (SSA, a typical artificial medium), an eelgrass-based medium (biologically relevant substrate), and inoculated eelgrass segments (approximation of *in situ* production). We use these substrates to determine how *in vitro* FA production of Lz might vary with different resources and whether Lz FA production might be detectable in diseased host tissue. To assess whether substantial FA production may occur *in situ*, we also quantify FA of field-collected EWD-affected eelgrass. We focus particularly on ALA and DHA, hypothesizing that Lz presence will increase sample DHA at the expense of ALA, a key eelgrass FA and potential percursor for DHA.

2. MATERIALS AND METHODS

We collected EWD lesions for Lz isolation and eelgrass for experimental substrates in the South Slough in Charleston, Oregon, USA, in 2017 and 2019. We collected only eelgrass leaves (i.e. not whole turions) under Oregon Parks and Recreation Department Permit No. 008-16 and with permission of the South Slough National Estuarine Research Reserve. We performed laboratory work and FA analyses at the Oregon Institute of Marine Biology (University of Oregon, Charleston, Oregon, USA).

2.1. Substrate and sample production

We prepared 3 types of substrates in 100 mm petri dishes: SSA, eelgrass agar (EGA), and eelgrass leaf segments (hereafter segments), summarized in Table 1 and detailed in Supplement 1 at www.int-res.com/articles/suppl/d135p089_supp/. We used 9 isolates (V17–V25) of Lz, cultured from EWD lesions. We aimed to capture a greater variety of Lz FA production using multiple isolates rather than a single isolate but did not sample to specifically test isolate differences. We inoculated 4 replicate plates of each substrate with 10 µl of ~10^6 cells ml^{-1} of each Lz isolate in sterile seawater. Four replicate control plates for each substrate were treated the same but using sterile seawater as a sham inoculum. EGA did not initially yield sufficient Lz growth, so we plated pieces of Lz-colonized EGA onto new EGA plates. Isolate V22 still did not grow sufficiently and was omitted. We incubated all plates at 15.6 ± 0.34°C (mean ± SD) with 12 h light:12 h dark fluorescent lighting. We allowed dark lesions typical of EWD to develop on inoculated segments before collection. Incubation durations varied with extremely different timings of disease/degradation on segments and substantial Lz growth on agar substrates (10 d for SSA, 42 d for EGA, and 8–31 d for segments).

From SSA plates, we collected Lz-colonized agar from 1 inoculated plate per isolate (Lz-colonized substrate, n = 8) and agar from all control plates (substrate alone, n = 4). We also scraped cells from all inoculated SSA plates and pooled them by isolate (Lz cells, n = 8) to quantify FA per Lz weight and separate Lz FA from that in associated agar. From EGA plates, we collected paired colonized (Lz-colonized substrate, n = 8) and uncolonized (substrate alone, n = 8) agar. EGA samples used paired controls because another set of 4 separate control plates could not be

Table 1. Summary of *Labyrinthula zosterae* (Lz) substrates and sample types. SSA: serum seawater agar; EGA: eelgrass agar; EWD: eelgrass wasting disease; FA: fatty acids; na: not applicable

Substrate	Sample type	Eelgrass used	Other components	Sterilization	Relevance
SSA	Lz cells, Lz-colonized substrate, substrate alone	na	Horse serum, peptones, yeast extract, glucose, germanium dioxide, noble agar	Heating (dissolve agar), autoclaving	Traditional artificial medium used to culture Lz
EGA	Lz-colonized substrate, substrate alone	2nd and 3rd leaves of turion, cleaned of epibionts and blended	Filtered seawater, noble agar	Heating (dissolve agar), autoclaving	Medium plate with only eelgrass derived resources without eelgrass host response
Eelgrass	Laboratory-inoculated segments: Lz-colonized substrate, substrate alone	7 cm piece of eelgrass from 2nd and 3rd leaves of turion, cleaned of epibionts	Sterile seawater, sterile seawater agar plates	na	Controlled but realistic production of EWD-lesioned tissue
	Field-collected tissue: field diseased, field healthy	Leaves cleaned of epibionts, portions of diseased and healthy tissue separated	na	na	*In situ* FA production

produced due to the additional plating as described and limited quantities of EGA plates. Lz growth on EGA was compact enough to allow collection of distinct colonized and uncolonized samples from each plate.

Before collecting inoculated (Lz-colonized substrate) and control (substrate alone) segments, we photographed and quantified each segment's EWD severity as the percent segment area lesioned using ImageJ (Rasband 1997). We stored segments separately but pooled them for FA analyses to ensure necessary sample masses. Because pooling reduced control sample size to n = 1, we later produced a set of inoculated and control segments (n = 5 each) using larger paired segments (each pair from the same leaf) and only the high-virulence isolate V20. Methods were otherwise the same. We stored and analyzed each of the later segments individually (i.e. not pooled).

To assess whether FA of inoculated segments reflect those of EWD lesions *in situ*, we additionally collected 5 eelgrass leaves with eelgrass lesions. We excised apparently diseased (lesioned) tissue from healthy tissue, storing each separately (field diseased and field healthy, respectively, n = 5 each). We verified visual EWD diagnosis by plating small subsamples of diseased tissue on SSA.

We freeze dried and weighed all samples, obtaining dry sample weights, prior to FA extraction. We extracted and analyzed FA using gas chromatography and mass spectrometry, modifying methods of Taipale et al. (2013, 2016) as detailed in Supplement 1.

2.2. Data analysis

We analyzed data in R (version 3.5.1; see R-code in Supplement 2 at www.int-res.com/articles/suppl/d135p089_supp/). We standardized FA concentrations by sample dry weight (µg mg^{-1} DW) and calculated FA proportions on the total identified FA concentrations within a sample (%TFA). We used non-parametric methods for univariate comparisons, as data were non-normal with small sample sizes. We compared DHA and ALA concentrations and proportions among sample types within substrates. We used Wilcoxon signed rank (hereafter Wilcoxon) tests for EGA and eelgrass, as samples were paired, and Kruskal-Wallis (hereafter KW) tests for SSA. To address the specific hypothesis that Lz would increase DHA at the expense of ALA, we used 1-tailed Wilcoxon tests for eelgrass samples but also present 2-tailed tests for transparency. We did not adjust these tests for multiple comparisons because each deliberately investigated a particular FA's patterns within a substrate. We used Dunn's tests with Bonferroni corrections for post hoc pairwise comparisons following KW tests (dunn.test in package dunn.test; Dinno 2017).

We used Euclidean distances of arcsine square root transformed proportions for multivariate analyses, including FA >1% on average of FA of any substrate–sample type combination (23 FA used). We visualized FA multivariate compositions using non-metric multidimensional scaling (NMDS, metaMDS in vegan package; Oksanen et al. 2018) and compared them among substrates and sample types using permutational multivariate analysis of variance (PERMANOVA with 9999 permutations, adonis in vegan package; Oksanen et al. 2018).

We do not display or analyze the earlier segment data as the control had n = 1 due to pooling. Further, temporal and methodological differences between earlier and later segment sets discourage combining them, although they are qualitatively similar (Fig. S1 in Supplement 1).

3. RESULTS

All field-collected EWD samples produced Lz growth on SSA plates.

DHA was the dominant FA in SSA- and EGA-cultured Lz samples, followed by palmitic acid (16:0). In SSA-cultured Lz cells, DHA composed 36.6 ± 11.6% (mean ± SD, n = 8) of identified FA. Palmitic acid was the dominant FA in SSA and EGA substrate-alone samples. ALA was the dominant FA of all eelgrass samples.

On SSA, Lz cells had proportionally greater ALA than the substrate alone (Dunn's test, $p < 0.05$; Fig. 1A). In contrast, ALA concentrations were greater in Lz cells than both Lz-colonized SSA or SSA alone (Dunn's test, $p < 0.05$, Fig. 1C). For EGA, Lz-colonized substrate had greater ALA proportions and concentrations

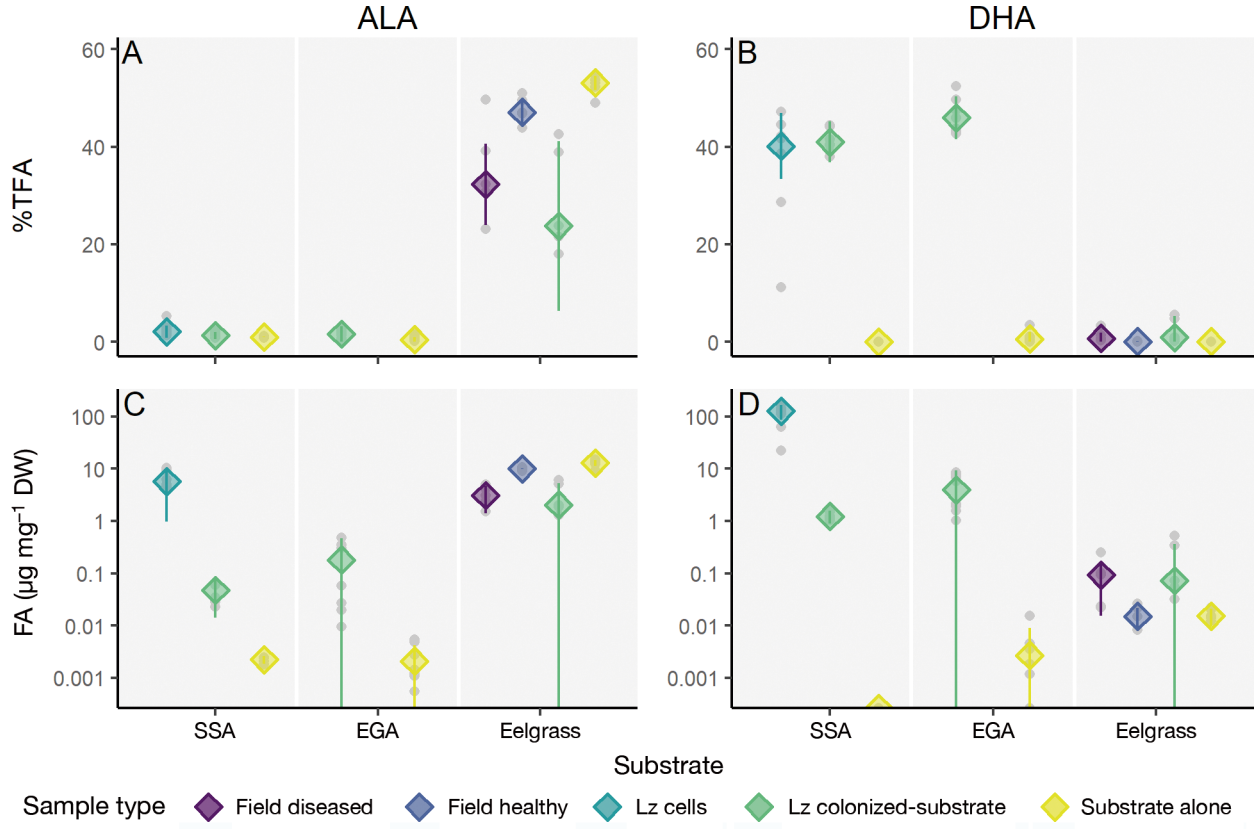

Fig. 1. (A,C) α-linolenic acid (ALA, 18:3ω-3) and (B,D) docosahexaenoic acid (DHA, 22:6ω-3) by substrate and sample type. Median (A,B) proportions (% total identified fatty acids [TFA]) and (C,D) concentrations (µg mg^{-1} sample dry weight) are shown as diamonds with error bars (±1 interquartile range). Substrates: serum seawater agar (SSA), eelgrass agar (EGA), and (eelgrass) leaf segments or tissue. Sample types: field-collected diseased eelgrass, field-collected healthy eelgrass, isolated *Labyrinthula zosterae* (Lz) cells, Lz-colonized substrate, and substrate alone. Grey points are individual analyzed samples. Sample sizes for each substrate–sample type combination are n = 5 for each eelgrass–sample type combination, n = 4 for SSA–substrate alone, and n = 8 for all other combinations. Data analyses are summarized in Tables S1 & S2 in Supplement 3 at www.int-res.com/articles/suppl/d135p089_supp/

than the respective controls (Wilcoxon, $p < 0.05$; Fig. 1A,C). In both laboratory-inoculated and field-collected eelgrass, ALA proportions and concentrations were lower in Lz-inoculated and diseased samples relative to respective controls (1-tailed Wilcoxon, $p < 0.05$, 2-tailed $p = 0.0625$, Fig. 1A,C).

DHA proportions were not different between Lz cells or Lz-colonized substrate on SSA (Dunn's test, $p = 1.0$), but both were greater than the SSA alone (Dunn's test, $p < 0.05$; Fig. 1B). DHA concentrations were greater in Lz cells than both Lz-colonized SSA and SSA alone (Dunn's test, $p < 0.05$; Fig. 1D). As with ALA, DHA proportions and concentrations were greater in Lz-colonized EGA than EGA alone (Dunn's test, $p < 0.05$; Fig. 1B,D). Eelgrass DHA showed an opposite pattern to ALA; Lz-inoculated segments and diseased tissue both had greater DHA proportions and concentrations than respective controls (1-tailed Wilcoxon, $p < 0.05$, 2-tailed $p = 0.0625$; Fig. 1B,D).

We summarize univariate comparisons of proportions and concentrations in Tables S1 & S2, respectively, in Supplement 3 at www.int-res.com/articles/suppl/d135p089_supp/.

The FA compositions of samples differed by substrate and sample type (PERMANOVA, substrate: $F_2 = 159$, partial $R^2 = 0.327$, sample type: $F_4 = 125$, partial $R^2 = 0.513$; Fig. 2).

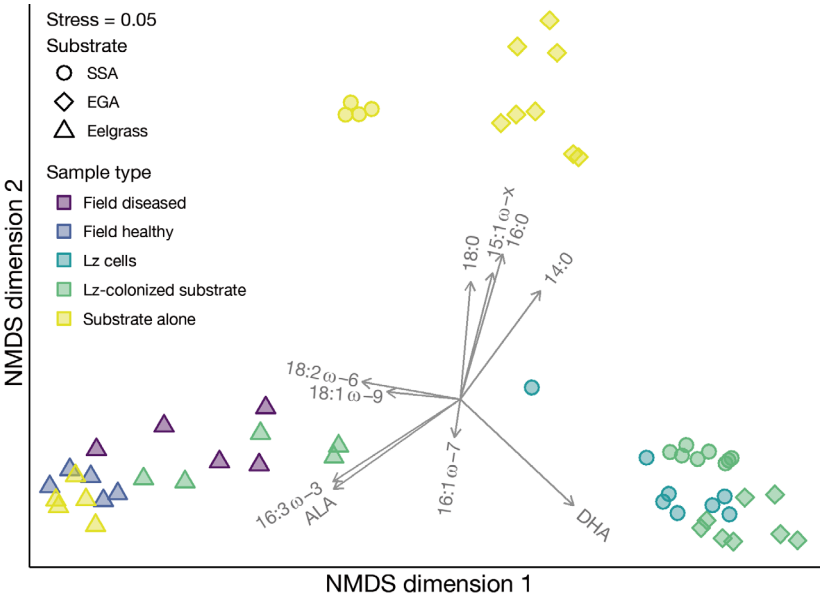

Fig. 2. Non-metric multidimensional scaling (NMDS, on Euclidean distances of arcsine-transformed fatty acid [FA] proportions) plot of FA compositions by substrate and sample type as in Fig. 1. Each point is an individual analyzed sample. Vectors show relative correlations of FA (>5% total identified FA of at least 1 substrate–sample type combination) with NMDS dimensions. In vector labels, 'x' denotes an unknown ω double bond position for the FA. SSA: serum seawater agar; EGA: eelgrass agar; Lz: *Labyrinthula zosterae*

4. DISCUSSION

Using both artificial (SSA) and eelgrass-derived (EGA) substrates, we show that pathogenic Lz produces DHA as its dominant FA. Notably, as the agar substrates provided here lack significant quantities of even precursor FA, our results indicate Lz is capable of synthesizing and elongating many FA to produce DHA. Furthermore, while substrate was a significant predictor of FA composition in PERMANOVA, sample type explained much more of the variation (partial $R^2 = 0.327$ versus 0.513, respectively) and suggests FA production of labyrinthulids on artificial substrates may qualitatively reflect the abilities of those organisms *in situ*.

Lz's DHA content far exceeds that of most primary producers. In an analysis of 40 northeast Pacific marine macrophyte species spanning 21 orders in 4 phyla, no species exceeded on average 5%TFA of DHA and only 2 had detectable DHA (Galloway et al. 2012). Field-diseased eelgrass samples found here had 1.19 ± 1.26%TFA (mean ± SD, n = 5), placing it among the top macrophyte DHA sources in the northeast Pacific. For marine phytoplankton, Galloway & Winder (2015) synthesized data for FA contents in 208 species and found the greatest DHA content in dinoflagellates (21 ± 1 µg mg^{-1} DW, 17 ± 9%TFA), exceeded by Lz cells on SSA in our study (111.9 ± 46.8 µg mg^{-1} DW, 36.6 ± 11.6%TFA). Lz's DHA content does, expectedly, fall with many other heterotrophic protists researched for industrial LCPUFA production, including Lz's relatives (5 to over 50% lipid; Kumon et al. 2006, Armenta & Valentine 2013). With greater ecological relevance, 5 planktonic heterotrophic protists studied by Chu et al. (2008, 2009) fed biologically relevant diets (bacteria or phytoplankton) contained DHA at ~5 to 17%TFA.

In eelgrass, patterns of laboratory segments and field-collected tissue were similar (Figs. 1 & 2). The culturing of Lz from field-collected lesioned eelgrass confirms our identification of EWD in the field, supporting the agreement between laboratory and field samples. The eelgrass results imply a shift in ALA and DHA with infection, with ALA being reduced and DHA being produced. A straightforward explanation could be that Lz

converts ALA to DHA, but because we did not specifically trace ALA's fate, we cannot definitively conclude this mechanism. Also, we emphasize that the loss of ALA and gain of DHA is not one for one, as ALA is likely being converted to other FA or being lost in degradation accompanying disease. Disease-associated degradation also likely explains the difference in ALA patterns between agar substrates and eelgrass: while ALA could be increased in ALA-poor agar substrates with Lz presence, the ALA could be lost in ALA-rich eelgrass though degradation.

While Lz-associated DHA increases found in both laboratory and field eelgrass were detectable, they were modest (Fig. 1B,D), which may indicate minimal relevance *in situ*. However, considering eelgrass's abundance as the foundation species in its ecosystems, the ubiquity of EWD (Sullivan et al. 2013), and the general scarcity of DHA in eelgrass bed primary producers (Kharlamenko et al. 2001, Galloway et al. 2012), even this small production may be significant for eelgrass beds. Overall, our results highlight a potential role for Lz in secondary production in its communities, but ecosystem-level effects must still be confirmed. If abundant enough, Lz could be an important upgrader (Klein Breteler et al. 1999) of LCPUFA-poor eelgrass.

Beyond considering FA production at community or ecosystem scales, investigating the potential relationship between disease severity and FA production should be a priority for future study. Specifically, investigations into the utility of FA production for pathogenic Lz may provide clues to how strain-specific virulence is modulated. Thraustochytrids, relatives of Lz, use FA like DHA for storage and the ectoplasmic net (Jain et al. 2007), an important component of infection for Lz (Muehlstein 1992). Thus, FA production may be linked to disease in mechanism, not simply pathogen load.

We here demonstrated that a marine parasite produces exceptionally large amounts of DHA, finding that Lz may be an underrecognized source of LCPUFA in eelgrass ecosystems. While unsurprising in light of its relatives (Kumon et al. 2006, Armenta & Valentine 2013), Lz's FA production is remarkable in expanding parasitism's potential ecological effects. If Lz's FA production scales *in situ*, this parasite may trophically upgrade food quality to consumers of its host via disease. This encourages studies on FA production in relation to disease in living plants to elucidate FA consequences of EWD. Future research on the trophic pathways of diseased tissue is needed to show whether Lz FA production enters higher consumers via enhanced eelgrass nutritional quality. This work demonstrates that EWD may not just be a destructive force: through producing valuable biomolecules that can regulate organismal and ecosystem production (Winder et al. 2017), Lz could be a creator as well.

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