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Moderate virulence caused by the protist *Labyrinthula zosterae* in ecosystem foundation species *Zostera marina* under nutrient limitation

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ABSTRACT: The nature of many microbe–host interactions is not static, but may shift along a continuum from mutualistic to harmful depending on the environmental conditions. In this study, we assessed the interaction between the foundation plant eelgrass *Zostera marina* and the frequently associated protist *Labyrinthula zosterae.* We tested how an important environmental factor, nutrient availability, would modulate their interaction. We experimentally infected naive eelgrass plants in combination with 2 nutrient levels (fertilized and non-fertilized). We followed *L. zosterae* infection, eelgrass growth parameters and host defense gene expression over 3 wk in large 600 l tanks. Inoculation with *L. zosterae* and nutrient limitation both reduced eelgrass growth. These effects were additive, whereas no interaction of nutrient treatment and *L. zosterae* inoculation was detected. Gene expression levels of 15 candidate genes revealed a reduced expression of photosynthesis-related genes but an increased expression of classical stress genes such as *Hsp80* in inoculated plants 2 d post-inoculation. However, we found no effects on plant mortality, and plants were able to clear high infection levels within 3 wk to ambient background levels of infection as assessed via specific RT-qPCR designed to quantify endophytic *L. zosterae*. Thus, we found no evidence that *L. zosterae* is a facultative mutualist that facilitates eelgrass growth under nutrient-limiting conditions. We suggest that the interaction between contemporary *L. zosterae* genotypes and *Z. marina* represents a mild form of parasitism in northern Europe because the damage to the plant is moderate even under nutrient limitation stress.

KEY WORDS: Seagrass · Wasting disease · Baltic Sea · Nitrogen · RT-qPCR · Gene expression

INTRODUCTION

Many host–microbe interactions may change along a continuum between parasitism and mutualism depending on the prevailing environmental conditions and life stage (Bronstein 1994, Newton et al. 2010). Environmental stressors may alter the physiology of host and microbes in different ways and thus modulate their interaction. If keystone or foundation species are involved, interactions at the host–microbe level may produce changes that affect the entire ecosystem (Harvell et al. 2002, Burge et al. 2013). It has recently been shown that host–microbial interactions may also determine ecosystem productivity and diversity, in particular in seagrass-dominated systems (Van Der Heijden et al. 2008, Mendes et al. 2013).

In this study, we focus on the foundation species *Zostera marina* L. (eelgrass). Eelgrass belongs to the seagrasses, a polyphyletic group of marine angiosperms that populate soft-bottom habitats in all climate zones except the polar regions, and provide critical ecosystem functions and services (Costanza et al. 1997, Cullen-Unsworth et al. 2014). Along with a great diversity of microorganisms such as fungi and

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bacteria (e.g. Sakayaroj et al. 2010, Garcias-Bonet et al. 2012), seagrasses are frequently colonized by endophytic net slime mold of the genus *Labyrinthula* (Vergeer & Den Hartog 1994, Garcias-Bonet et al. 2011, Bockelmann et al. 2012, 2013). *Labyrinthula* spp. live within leaf tissue of diverse seagrass species, where they may exist asymptomatically (Raghukumar 2002), or produce necrotic lesions in case of pathogenic outbreaks for which the specific triggers are still unknown. Repeated seagrass die-offs have been associated with *Labyrinthula* spp. and are collectively summarized under the somewhat unclear label of 'wasting disease' (Sullivan et al. 2013). In the 1930s, the largest ever recorded seagrass die-off was reported all across the Northern Atlantic, supposedly caused by infection with the protist *Labyrinthula zosterae* (Short et al. 1987), resulting in drastic ecological consequences such as the reduction of associated fish, shellfish and crustacean populations (Muehlstein et al. 1991).

Contrary to the situation depicted above, there is increasing evidence that *Labyrinthula* spp. may also coexist with their host without disease symptoms (Bockelmann et al. 2013, Martin et al. 2016). For example, a field survey in northern European eelgrass meadows revealed high abundances of *L. zosterae* in contemporary eelgrass meadows without any observable mortality (Bockelmann et al. 2013). Furthermore, experimental infections of eelgrass with *L. zosterae* revealed low virulence of *L. zosterae* genotypes in eelgrass populations from the Western Baltic Sea and the Wadden Sea, while inoculation even induced higher growth rates in *L. zosterae*-infected eelgrass plants when grown under ambient Western Baltic Sea conditions (Brakel et al. 2014). These experimental results demonstrate that we have still not identified the exact nature of the protist–host plant relationship, at least of contemporary *L. zosterae* genotypes.

Investigation of the host–microbe interaction while manipulating different environmental conditions may reveal insight into the continuum between parasitism and mutualism (Webster et al. 2008), and can thus lead to a better understanding of which factors influence virulence, pathogenicity and host defense. In this study, we investigated the influence of nutrient levels on the eelgrass–protist interaction. It has been shown that nutrient availability affects the interaction of several plant species to bacterial, fungal or viral pathogens by either enhancing or inhibiting infection (Hoffland et al. 2000, Snoeijers et al. 2000, Lacroix et al. 2014). Seagrasses, including our focal species *Z. marina*, may also suffer from nutrient limitation (Bulthuis & Woelkerling 1981, Reusch et al. 1994), even although eutrophication is one of the main causes for seagrass disappearance worldwide (Orth et al. 2006). The effect of nitrogen deficiency is well documented for *Z. marina*, and includes a reduction in growth rates, biomass production and shoot length (Short 1987). During summer in particular, when growth and biomass productivity are highest, nitrogen deficiency becomes substantial in shallow, nutrient-poor silicate sediments (Pedersen & Borum 1993), emphasizing the potential relevance of internal nitrogen recycling for eelgrass in the temperate zone.

The core hypothesis of this work was that degradation processes driven by *L. zosterae* will alleviate nutrient limitation in *Z. marina*, ultimately enhancing eelgrass growth and vegetative shoot production. *Labyrinthula zosterae* prefers older eelgrass leaves at the third position counting from the meristematic leaf forming zone (Bockelmann et al. 2013) while *Labyrinthula* species in general exude a wide range of enzymes enabling the degradation of organic compounds and display an absorptive mode of nutrition (Raghukumar & Damare 2011). Also, the sister group aplanochytrids are efficient degraders of mangrove litter (Bremer 1995, Leander et al. 2004). A potentially commensal or mutualistic role of *Labyrinthula* spp. has been suggested previously (Vergeer & Denhartog 1994, Raghukumar 2002), but experimental data are lacking. Alternatively, as described above, nutrient limitation is a well-described stressor and may weaken eelgrass growth and production. Therefore, our second hypothesis was that nutrient limitation enhances detrimental effects of *L. zosterae* inoculation.

In order to test our hypotheses, we designed a tank experiment that combined 2 nutrient levels with *L. zosterae*-inoculated and sham-inoculated *Z. marina* plants from the Western Baltic Sea. We measured several response variables: (1) quantification of *L. zosterae* infection by wasting disease index and *L. zosterae* abundance measurement (by RT-qPCR) in eelgrass leaves, (2) growth quantification by measuring leaf production, leaf growth rate and above- and belowground biomass and (3) host defense exploration by gene expression analysis of target defense genes.

MATERIALS AND METHODS

Zostera marina and *Labyrinthula zosterae* origin and cultivation

In order to control the infection level of our experimental plants, we raised the *Zostera marina* plants

from seeds. Seeds were collected in 2 eelgrass beds in the Western Baltic Sea near Kiel (54.39° N, 10.18° E) and Flensburg (54.75° N 9.87° E), Germany. To ensure vernalization, we incubated seeds for 12 wk at 5°C submerged within the sediment. The emerging seedlings were raised for 1.5 yr within large 600 l tanks under semi-continuous water flow with Baltic seawater (approximate salinity 15 psu) as previously described (Brakel et al. 2014).

Labyrinthula zosterae cultures were isolated from necrotic leaves of *Z. marina* plants collected at the east side of the island of Sylt, North Sea (55.04° N, 8.41° E), in August 2013. We isolated and cultivated *L. zosterae* cultures on seawater medium agar plates as described in Bockelmann et al. (2012). Isolated *L. zosterae* cultures were inspected under 100× magnification and cells were identified based on their typical spindle-shaped form. Species identity of *L. zosterae* was also confirmed by species-specific real-time qPCR, which was developed on a portion of the internally transcribed spacer (ITS) of the rDNA gene; these were 100% equal to virulent strains (GenBank accession nos.: JN121409, JN121410) (Bergmann et al. 2010). We chose not to infect healthy eelgrass plants and re-isolate *Labyrinthula* strains before the experiment according to Koch's postulate as this would have selected for the most aggressive *Labyrinthula* genotype. Instead, we wanted to maintain genetic diversity and keep cultures as short as possible in culture after isolation. We proliferated the *Labyrinthula* culture at 25°C for 2 wk to obtain sufficient material for the inoculation.

Experiment design and setup

In a 2 × 2 factorial design we combined the factors nutrient level (fertilized/unfertilized, where based on earlier studies [Reusch et al. 1994, Worm & Reusch 2000] we assume that unfertilized plants were nutrient limited) and *L. zosterae* inoculation (yes/no). The treatments were arranged in 6 tanks, 3 containing plants with high and the other 3 containing plants with low nutrient levels. Each tank was divided into 2 subareas containing either inoculated or sham-inoculated plants. Each subarea contained 6 plants, which were arranged at a distance of 40 cm to prevent leaf contact between plants. We subdivided the tanks into 2 sections to separate infected from healthy plants by installing a wall that prevented direct leaf contact. Water circulation between both sides was allowed through a 10 × 1 cm opening at the bottom of the tanks. *Zostera marina* shoots were planted individually in 6 l plastic buckets containing sandy sediment to a height of 15 cm. The sediment contained little organic material (<2%) and was collected in the vicinity of the sampling site. It was incubated overnight at 80°C before planting, to limit inadvertent microbial activity (including *L. zosterae*) in the sediment. The buckets with the plantings were submerged in 50 cm of water into 600 l tanks containing filtered Baltic seawater from Kiel Fjord, of which 300 l were exchanged every other week. Within the time of the experiment, the salinity increased from ambient 13.5 psu to 18 psu due to water evaporation, which is within the range of natural salinity variation in Kiel Fjord (Hiebenthal et al. 2012). Light was provided by 2 halogen metal vapor lamps with a light intensity of ~600 µmol photon s^{-1} m^{-1} in a 16 h light: 8 h dark cycle. Water temperature was kept at 20.7°C (±0.9°C). Salinity and temperature were measured 3 times weekly.

We fertilized the plants every third week using a mixture of 2 types of coated fertilizer (slow and immediate release 1:1; Plantacote Mix 4M, Manna) (Worm & Reusch 2000) (for concentrations, see Table 1). Fertilizer pellets were placed individually 2 cm deep in the sediment at 2 cm distance to the plants. The low nutrient treatment plants were physically handled in the same way, without adding fertilizer. Nutrient concentrations (NH_4^+, NO_2^+/NO_3^- and PO_4^{3-}) of pore water and water column were measured twice, after establishment of the plants in the sediment and before inoculation treatment of eelgrass plants (Fig. 1). About 40 ml of pore water was sampled using a syringe with a perforated tip that was pushed 5 cm deep into the sediment.

We verified that eelgrass would be nutrient limited in the treatments that received no fertilizer. The measured ammonium concentrations of pore water in the unfertilized treatments in our study of 7.5 µmol l^{-1} (SE ± 1.4 µmol l^{-1}) were shown in a previous study to be limiting in the Western Baltic Sea (Reusch et al. 1994). The ammonium levels of 41.5 µmol l^{-1} (SE ± 15.6 µmol l^{-1}) in the fertilized treatments represent natural nutrient-rich conditions, with natural ammonium concentrations measured in an eelgrass meadow in geographic vicinity ranging from 29 to 50 µmol l^{-1} and 21 to 29 µmol l^- between May and September, with and without mussels (*Mytilus edulis*), respectively (Worm & Reusch 2000).

After 7 wk of establishment in nutrient-poor or -rich sediment, we inoculated eelgrass leaves with *L. zosterae*. For inoculation, sterile gauze pieces were first placed on the surface of an agar plate covered with *L. zosterae* culture for 5 d until they were over-

Table 1. Fertilization steps and the estimated nutrient concentration by the mixed fertilizer Plantacote Mix 4M (Manna) for the high nutrient treatment. Date = dd.mm.yyyy

Date	Fertilizer type	Number of pellets per plant	Corresponds to NO_2^+/NO_3^- and NH_4^+ (mg)	Corresponds to PO_4^{3-} (mg)
15.07.2013	Coated slow releasing fertilizer	2	12.06	8.04
05.08.2013	Immediately available fertilizer	1	3.156	2.104
30.08.2013	Immediately available fertilizer	1	3.156	2.104
19.09.2013	Immediately available fertilizer	2	6.312	4.208

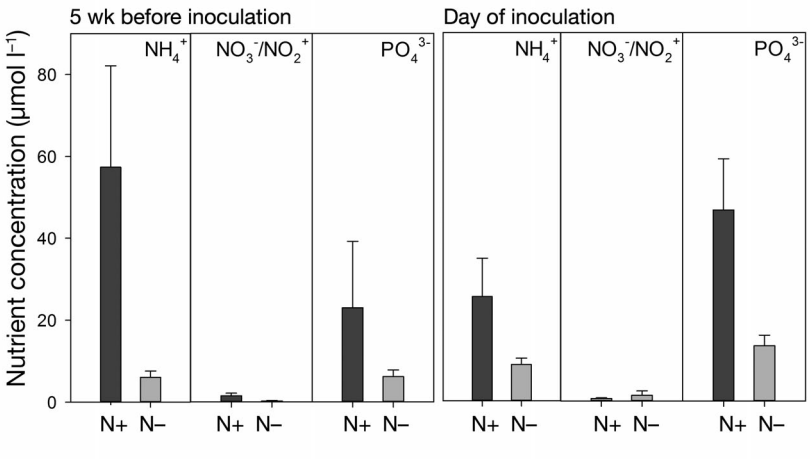

Fig. 1. Mean (+SE) nutrient concentration in sediment pore water (n = 12) measured 5 wk before inoculation and at the day of inoculation of eelgrass *Zostera marina* plants with *Labyrinthula zosterae*. N+: fertilized; N–: unfertilized

grown by *L. zosterae*. A 1 × 2 cm piece of the *L. zosterae*-infested gauze was then gently fixed onto the middle section of the 2nd and 3rd oldest eelgrass leaves for 24 h. Control treatments were treated similarly with sterile gauze pieces incubated on agar medium plates without *L. zosterae* culture. The gauze on average transferred 2.14 ± 0.197 × 10^5 (n = 6, ±1 SE) *L. zosterae* cells to the leaf surface as determined by RT-qPCR as described in the next section.

Wasting disease symptoms and *Labyrinthula zosterae* quantification

As one of the most widely observed symptoms, an *L. zosterae* infection produces black lesions covering the eelgrass leaves. We quantified lesion surface according to the wasting disease index (Burdick et al. 1993), which estimates the relative area of lesion coverage using 6 classes (0, >0–10, >10–25, >25–50, >50–75 and >75–100%). We estimated wasting disease index at 1, 2, 3, 5, 7, 9, 12, 14, 16 and 20 days post-inoculation (dpi).

We also quantified *L. zosterae* abundance in *Z. marina* leaf tissue by real-time quantitative PCR assay in accordance with Bockelmann et al. (2013), amplifying a species-diagnostic region of the ITS region of *L. zosterae*. For sampling, each harvested leaf was divided longitudinally. One section was dried for later *L. zosterae* quantification while the other half was immediately stored in RNA-later for gene expression measurements (see below). One half of each plant was harvested 2 dpi, sampling a leaf of 2nd rank, while the second half of each plant was harvested 20 dpi, sampling similarly a leaf of 2nd rank. For *L. zosterae* quantification, dried leaf pieces (3–15 mg dry weight [DW]) were ground in a ball mill with a stainless steel bead (Retsch) and DNA was extracted with Invisorb Spin DNA Extraction Kit (Stratec Molecular). One microliter of salmon sperm (Life Technologies) was added to saturate silica columns with unspecific DNA. Target DNA was purified using a one-step PCR inhibitor removal kit (Zymo Research). RT-qPCR was performed on a StepOne Plus q-PCR machine (Applied Biosystems). In a reaction, we mixed 10 µl TaqMan universal Master Mix (Life Technologies), 2.4 µl of forward and reverse primer (final concentration 40.8 nM), 2.4 µl Milli-Q H_2O, 0.8 µl fluorescently labeled probe (50 nM) and 2 µl 1:10 diluted template DNA. The thermo-cycling protocol was 2 min at 50°C and 10 min at 95°C, followed by 48 cycles at 95°C for 15 s and 1 min at 60°C. Each sample was run in technical triplicate. Cycle threshold (C_T) was calculated with a fixed threshold of 0.05. We ran on each q-PCR plate 3 standard DNA solutions of known *L. zosterae* cell numbers of 0.5 cells (C_T: 33.51 ± 0.12 SE), 15 cells (C_T: 27.75 ± 0.12) and 150 cells (C_T: 23.49 ± 0.03). C_T values above 39 were not considered. Standard devia-

tion was calculated for all samples; if it exceeded 0.5, samples were excluded from further analysis.

Eelgrass response variables

We followed leaf growth, leaf and shoot production of individually marked eelgrass shoots over 10 wk. Recognition of individual leaves was realized by pricking the tip of the respective leaf with a syringe needle (diameter 0.5 mm). We counted new leaves and novel side shoots once a week. Leaf length was measured with a ruler from the leaf tip to leaf base to the nearest 0.5 cm. We noted that leaf growth decreased with increasing leaf age. In the first week after appearance, *Z. marina* leaves showed strongest growth rates of mean 1.7 cm d^{-1}. During the second week, leaf growth strongly decreased due to age to levels of 0.6 cm d^{-1}. No growth could be detected once a leaf was older than 17 d. Therefore, we compared only leaves of the same age, irrespective of the date on which measurement were performed.

At the end of the experiment, we excavated all plants including their rhizome. We freeze-dried the material and weighed it to the nearest 1 mg.

Targeted gene expression assay

In order to assess molecular defense reaction of eelgrass plants, we measured levels of gene expression of 5 immune genes (see Table 5 for full names): *RppA*, *pl 206*, *CLT1*, *Metacasp* and *CYP73A* (Brakel et al. 2014); 4 redox and detoxification genes: *GST*, *SOD*, *APX* and *CAT* (Winters et al. 2011); and 2 general stress genes: *Hsp70* and *Hsp80* (Bergmann et al. 2010). Additionally, we included 4 genes of primary metabolism to investigate molecular physiologic response upon nutrient and inoculation treatment: *Chl_synth*, *STS*, *RuBisCO* and *FBiA* (Salo et al. 2015). Gene expression values were normalized with the housekeeping gene *eIF4A*. Gene expression was measured with a Fluidigm Biomark (HD Systems) on a 96.96 Dynamic Array IFC chip according to published protocols (Salo et al. 2015). Assays of each gene were run in 4 technical replicates.

Data analysis

All statistical analyses were performed with R version 3.1.2 (R Core Development Team 2014). To evaluate the effect of nutrient treatment, inoculation and the factor interaction on all response variables other than gene expression, we used linear mixed models of the R package lme4 (Bates et al. 2015). We ran models with both factors and their interaction and reduced the model if possible based on Akaike's information criterion (AIC). We included, according to the nested split-plot design, the terms 'tank' and 'inoculation nested in tank' as random factors to the model. If the model output revealed that the variation by 'inoculation nested in tank' was negligible ($<10^{-10}$%), we reanalyzed the dataset excluding the non-significant random term. Nevertheless, results of the full model are shown in Tables S1 and S2 in the Supplement at www.int-res.com/articles/suppl/m571p097_supp.pdf. In order to achieve variance homogeneity, cell numbers of *L. zosterae* were square root transformed and biomass data were log transformed.

To analyze gene expression values of the 15 target genes, we used a 2-step approach. First, we performed a permutational multivariate ANOVA (PERMANOVA) on $-\Delta C_T$ values for samples collected 2 and 20 dpi for each time point separately. If the PERMANOVA results revealed a significant pattern of dissimilarity, we performed univariate analyses on each single gene.

We averaged repeated measures across plants from the same split-unit, as it was not possible to include a random factor into such a model type. Tank was included as a random factor into the analysis, because inoculated and sham-inoculated plants shared the same water body (tank). A PERMANOVA was performed using the R package vegan (Oksanen et al. 2016), based on Euclidean distances and 9999 permutations. In order to illustrate the results for gene expression, a heat map (including a dendrogram based on mean values) based on average gene expression values ($-\Delta C_T$) was created within the R package gplots (Warnes et al. 2009).

All primary data have been deposited in the data repository PANGAEA under the doi: https://doi.pangaea.de/10.1594/PANGAEA.869864.

RESULTS

Wasting disease symptoms and *Labyrinthula zosterae* quantification

Characteristic symptoms for wasting disease, namely black lesions on the leaf area, were visible 24 h post-inoculation. Lesion development on the 2 leaves was highly correlated (t = 61.51, df = 402, p = <0.001). Therefore, we calculated the average index

value of leaf 2 and leaf 3 for each plant and analyzed these together. Within the first 20 dpi, black lesions did not differ significantly between nutrient-limited and fertilized plants, although there was a slight trend for nutrient-limited plants to develop symptoms faster (p = 0.10; Table 2, Fig. 2). Most inoculated leaves were 50–75% covered in necrotic lesions after 20 dpi for both nutrient treatments.

Parallel to the wasting disease index, we measured *Labyrinthula zosterae* cell abundance at 2 time points, 2 and 20 dpi. Fertilized and inoculated plants 2 dpi carried on average 12730 cells mg^{-1} eelgrass leaf DW, while the corresponding value in unfertilized plants was about double (23108 cells mg^{-1} eelgrass leaf DW). Owing to the large variance, these differences were not significant (p = 0.15; Table 2). A baseline of 41 (±9.1 SE) *L. zosterae* cells mg^{-1} eelgrass leaf DW was detected without experimental inoculation, which attained only 0.2% of values found in the inoculation treatments. After 20 dpi, *L. zosterae* cell abundance was measured in the newly grown leaf which had not been inoculated initially, but which formed at the day of measuring the 2nd rank. Measured values in these leaves did not exceed the baseline level considerably, on average 65 (±64.9 SE) *L. zosterae* cells mg^{-1} eelgrass leaf DW were detected in the inoculated, fertilized plants and 9 (±8.6 SE) cells mg^{-1} eelgrass leaf DW in inoculated, unfertilized plants (see Fig. 3).

Table 2. Results of a linear mixed model ANOVA for *Labyrinthula zosterae* concentration, wasting disease index (WDI) and eelgrass growth, based on AIC model selection. Significant results are shown in bold (p < 0.05); dpi = post inoculation, DW = dry weight

Variable	*F*	df	p	Variance	SD
Labyrinthula cells (2 dpi) (cells mg^{-1} eelgrass DW)					
Nutrient	<0.001	1,8	0.984		
Inoculation	73.608	1,4	**0.001**		
Nutrient×Inoculation	3.056	1,4	0.155		
Tank				39.2	6.261
Tank(Inoculation)				218.8	14.792
WDI – Leaf 2 & 3 (categorical index)					
Nutrient	0.020	1,60	0.888		
Inoculation	351.864	1,58	**<0.001**		
Nutrient×Inoculation	2.726	1,58	0.104		
Day	400.078	1,401	**<0.001**		
Plant ID				0.094	0.307
Leaf growth rate (cm d^{-1})					
Nutrient	1.959	1,4	0.234		
Inoculation	4.842	1,41	**0.033**		
Tank				0.035	0.189
Biomass (g)					
Nutrient	12.264	1,4	**0.025**		
Inoculation	3.589	1,65	0.063		
Tank				<0.001	<0.001
Shoot production (no. sideshoots main $shoot^{-1}$)					
Nutrient	17.105	1,4	**0.014**		
Inoculation	0.845	1,65	0.361		
Tank				<0.001	<0.001
Leaf production (no. leaves main $shoot^{-1}$)					
Nutrient	0.037	1,4	0.856		
Inoculation	0.335	1,65	0.565		
Tank				<0.001	<0.001

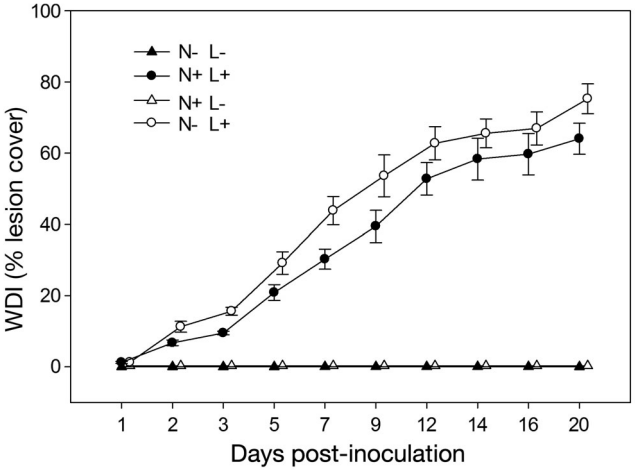

Fig. 2. Time course of the wasting disease index (WDI) estimated from the 2nd and 3rd youngest eelgrass leaves. As lesion coverage of 2nd and 3rd leaves was highly correlated (t = 61.51, df = 402, p < 0.001), we show averages of the 2nd and 3rd leaves. The WDI refers to % leaf area covered by symptomatic necrotic lesions and was estimated in 6 categories. Depicted are mean (±SE) values of estimated index data. N+: fertilized; N–: unfertilized; L+: inoculation treatment with *L. zosterae*; L–: inoculation control = sham inoculated

Eelgrass growth and biomass production

Neither inoculation with *L. zosterae* nor nutrient limitation resulted in enhanced eelgrass shoot mortality. Each plant produced on average 2.0 (±0.13 SE) side shoots throughout the experiment (Fig. 4A). The number of side shoots was increased as a result of nutrient addition (p = 0.02; Table 2) by 65% compared to non-fertilized plants. Inoculation with *L. zosterae* did not influence the production of side shoots, or the interaction of inoculation and nutrient treatment. We compared dry weight of eelgrass plants from different treatments as a proxy for biomass production. Biomass was significantly reduced

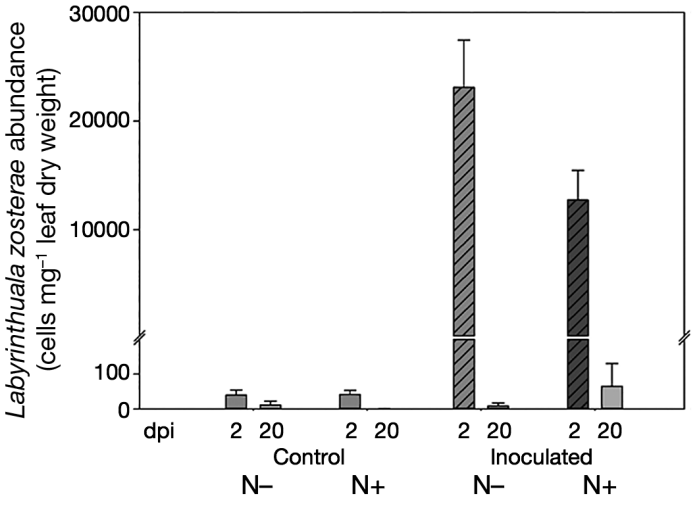

Fig. 3. Mean (+SE) *Labyrinthula zosterae* cell numbers detected via TaqMan-based RT-qPCR in *Zostera marina* leaves (2nd youngest leaf) 2 and 20 days post-inoculation (dpi) with and without *L. zosterae* inoculation. N+: fertilized; N–: unfertilized; dpi: days post inoculation

by low nutrient level (p = 0.02; Table 2), and further, there was a trend that inoculation treatment reduced biomass, though this was not significant (p = 0.06; Table 2). Biomass increased with fertilization by 25% (Fig. 4B). Biomass was not affected by the interaction of nutrient and inoculation. We compared leaf growth rates from leaves of the same age in order to correct for different leaf growth rates correlating to leaf age. Leaf growth rates were 26.7% higher (1.9 versus 1.5 cm d^{-1}) in sham-inoculated leaves compared to those inoculated with *L. zosterae* (p = 0.03; Table 2). Unfertilized and inoculated plants grew slowest, with growth rates of 1.3 cm d^{-1}, but the cumulative effect of a lack of nutrient addition along with inoculation treatment was purely additive, as no significant statistical interaction was detectable (Fig. 4C, Table 2). During 3 wk of experimental growth post-inoculation, plants had produced between 0 and 4 leaves. However, the number of newly formed leaves did not respond to fertilization, inoculation treatment or the interaction between both factors (Fig. 4D, Table 2).

Quantification of gene expression levels in 15 target genes

At 2 dpi, multivariate gene expression patterns differed strongly between inoculated and non-inoculated leaves (PERMANOVA p = 0.002; Table 3), but were unaffected by nutrient limitation. There was also no interaction detectable between inoculation and nutrient treatment (Table 3). Leaves harvested 20 dpi, containing only very few *L. zosterae* cells inside, did not differ in gene expression pattern between inoculated and sham-inoculated plants (Fig. 5, Table 3).

In the univariate analysis, at 2 dpi, 10 out of 15 genes were differentially expressed in inoculated versus sham-inoculated plants (p < 0.05). Genes that encode proteins involved in detoxification of reactive oxygen species (*CAT*, *GST*, *SOD*) were downregulated 2.0-, 3.3- and 2.9-fold, respectively, in inocu-

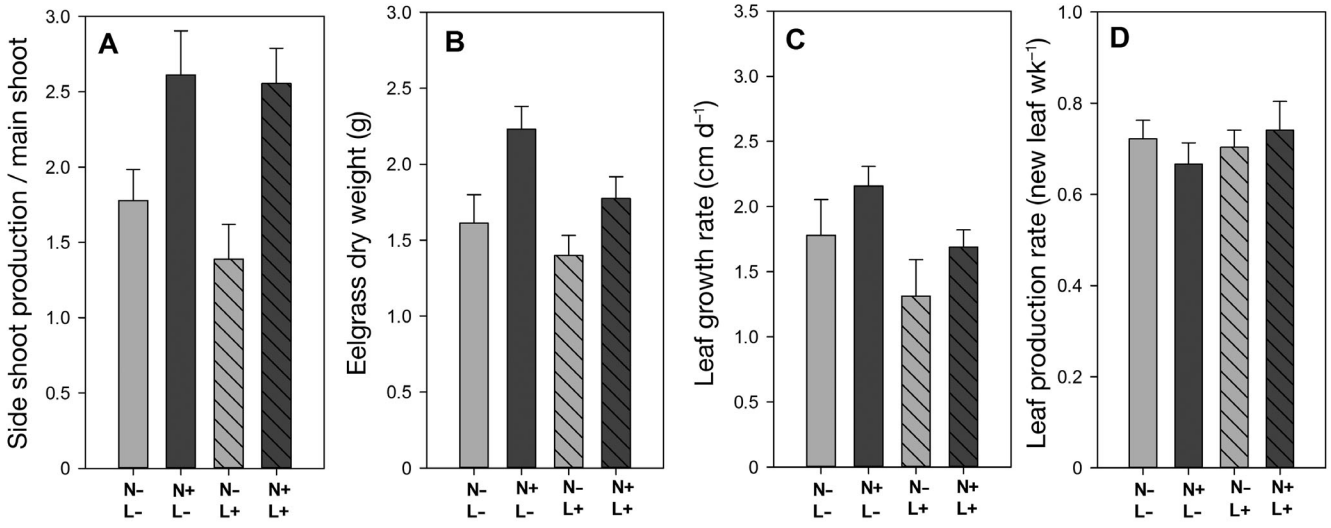

Fig. 4. *Zostera marina* growth responses to nutrient and inoculation treatment. N+: fertilized; N–: unfertilized; L+: inoculation treatment with *L. zosterae*; L–: inoculation control. (A) Mean (+SE) number of side shoots per plant sprouting from main plant 20 d post-inoculation (n = 18). (B) Biomass of *Z. marina* (dry weight) 20 d post-inoculation. (C) Leaf growth rate of youngest leaf after inoculation corrected by leaf age (mean + SE). (D) Leaf production rate post-inoculation

Table 3. Results of PERMANOVA analysis based on Euclidean distances for gene expression pattern of 15 target genes in relation to nutrient treatment, inoculation, their interaction and tank for 2 and 20 d post-inoculation (dpi). p-values are based on 9999 permutations. Significant results are shown in bold (p < 0.05)

	Variable	df	SS	*F* model	Pr (>*F*)
2 dpi	Nutrient	1	6.940	0.754	0.510
	Inoculation	1	170.221	18.486	**<0.001**
	Tank	4	38.892	1.056	0.461
	Nutrient×Inoculation	1	26.196	2.845	0.097
	Residuals	4	36.833		
20 dpi	Nutrient	1	19.864	2.041	0.161
	Inoculation	1	27.626	2.838	0.094
	Tank	4	41.780	1.073	0.459
	Nutrient×Inoculation	2	1.488	0.153	0.968
	Residuals	4	38.936		

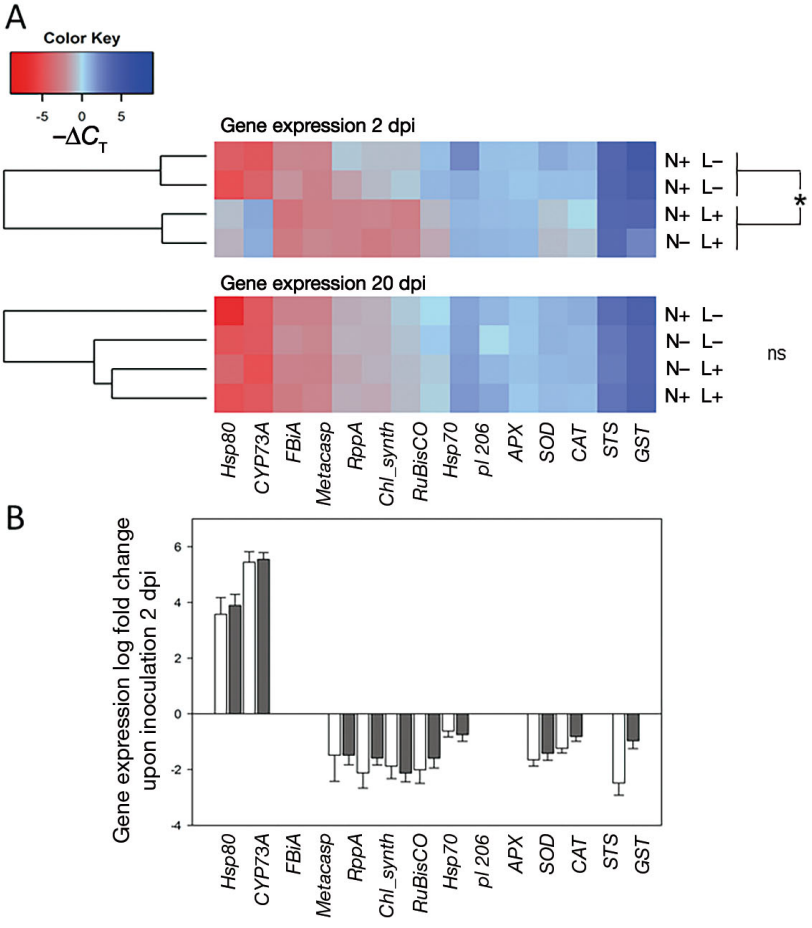

Fig. 5. (A) Mean values of relative gene expression ($-\Delta C_T$) of 15 targeted genes (see Table 5) depicted in a heat map 2 and 20 d post-inoculation (dpi). N+: fertilized; N−: unfertilized; L+: inoculation treatment with *Labyrinthula zosterae*; L−: inoculation control. *p < 0.05 in PERMANOVA (see Table 3); ns: not significant. (B) Mean (±SE) log fold change upon inoculation with *L. zosterae* 2 dpi for individual genes. Bars are only shown when fold change upon inoculation was significant (see Table 4). White bars: unfertilized plants; grey bars: fertilized plants (n = 9). C_T = cycle threshold

lated versus sham-inoculated leaves (p < 0.001, p = 0.007 and p < 0.001, respectively; Table 4). Of the known stress genes, *Hsp70* was 1.6-fold downregulated (p = 0.042, Table 4), while *Hsp80* was 13-fold upregulated (p = 0.003, Table 4). Two of 4 genes involved in primary production were downregulated as a consequence of inoculation. These were *RuBisCO* (p < 0.001; Table 4) and Chlorophyll synthase (p < 0.001). Furthermore, the immune genes Chitinase (p < 0.001; Table 4) and the receptor *RppA* (p = 0.008) were downregulated 3.4- and 2.8-fold, respectively. The highest change in gene expression was observed in *CYP73A*, which encodes the enzyme trans-cinnamate 4-monooxygenase, involved in phenol synthesis, and was upregulated 45-fold in inoculated leaves (p < 0.001; Table 4). Most genes did not show an interaction between nutrients and inoculation in their response, with the only exception of *GST*, which had the lowest expression in inoculated and nutrient-limited plants (p = 0.059; Table 4). A linear regression of *GST* gene expression values and *L. zosterae* abundance showed a significant negative correlation (R^2 = 0.379, $F_{1,29}$ = 20.777, p < 0.001) of *GST* expression and *L. zosterae* cell numbers.

DISCUSSION

In this study, we assessed how nutrient limitation affects the interaction between *Zostera marina* and *Labyrinthula zosterae*. *Labyrinthula zosterae* infection reduced eelgrass growth, as did nutrient limitation. The observed effects were purely additive, as we found no interaction among our nutrient addition treatment and *L. zosterae* inoculation. Thus our working hypothesis, namely, that rapid degradation and mineralization of decaying leaves was enhanced via the decompositional activity of *L. zostera*, which then may have alleviated nutrient limitation, was not supported. There was no evidence that *L. zosterae* is a facultative mutualist and facilitates eelgrass growth un-

Table 4. Results of linear mixed model ANOVA for gene expression of target genes 2 days post-inoculation for designated predictors by AIC model selection. For gene abbreviations, see Table 5. Significant results are shown in bold ($p < 0.05$)

Gene	Variable	*F*	df	P	Var	StdDev
SOD	Nutrient	3.811	1,13	0.072		
	Inoculation	42.390	1,27	**<0.001**		
	Nutrient×Inoculation	3.684	1,27	0.065		
	Tank				<0.001	<0.001
GST	Nutrient	0.800	1,8	0.397		
	Inoculation	25.531	1,4	**0.007**		
	Nutrient×Inoculation	6.862	1,4	0.059		
	Tank				<0.001	<0.001
	Tank(Inoculation)				0.113	0.336
APX	Nutrient	0.089	1,4	0.741		
	Inoculation	1.160	1,28	0.291		
	Tank				<0.001	<0.001
CAT	Nutrient	0.541	1,4	0.503		
	Inoculation	23.570	1,28	**<0.001**		
	Tank				<0.001	<0.001
Hsp80	Nutrient	0.117	1,4	0.729		
	Inoculation	29.574	1,5	**0.003**		
	Tank				<0.001	<0.001
	Tank(Inoculation)				0.529	0.728
Hsp70	Nutrient	3.126	1,6	0.130		
	Inoculation	8.364	1,4	**0.042**		
	Nutrient×Inoculation	2.223	1,4	0.210		
	Tank				0.245	0.138
	Tank(Inoculation)				0.019	0.138
STS	Nutrient	0.223	1,4	0.661		
	Inoculation	0.446	1,4	0.510		
	Tank				0.003	0.056
FBiA	Nutrient	<0.001	1,4	0.979		
	Inoculation	4.978	1,4	0.076		
	Tank				<0.001	<0.001
	Tank(Inoculation)				0.063	0.250
Chl_synth	Nutrient	0.058	1,4	0.822		
	Inoculation	35.551	1,29	**<0.001**		
	Tank				1.832	1.354
RuBisCO	Nutrient	0.003	1,4	0.448		
	Inoculation	26.589	1,29	**<0.001**		
	Tank				<0.001	<0.001
Metacasp	Nutrient	0.662	1,4	0.462		
	Inoculation	0.124	1,28	0.728		
	Tank				<0.001	<0.001
CTL1	Nutrient	0.707	1,4	0.448		
	Inoculation	26.589	1,28	**<0.001**		
	Tank				<0.001	<0.001
RppA	Nutrient	0.933	1,4	0.389		
	Inoculation	8.112	1,28	**0.008**		
	Tank				<0.001	<0.001
pl 206	Nutrient	0.024	1,4	0.883		
	Inoculation	0.058	1,5	0.820		
	Tank				1.385	0.883
	Tank(Inoculation)				1.385	1.177
CYP73A	Nutrient	0.412	1,4	0.557		
	Inoculation	131.918	1,26	**<0.001**		
	Tank				<0.001	<0.001

Table 5. Information about target genes

Gene code	Gene name	Function	Source
SOD	Superoxide dismutase (mitochondrial)	Antioxidant	Winters et al. 2011
GST	Glutathione S-transferase	Detoxification	Winters et al. 2011
APX	L-ascorbate peroxidase 2 (cytosolic)	Antioxidant	Winters et al. 2011
CAT	Catalase II	Antioxidant	Winters et al. 2011
Hsp80	Heat shock protein 80	Molecular chaperone	Bergmann et al. 2010
Hsp70	Heat shock protein 70	Molecular chaperone	Bergmann et al. 2010
STS	Starch synthase	Enzyme, starch biosynthesis	Salo et al. 2015
FBiA	Fructose biphosphate aldolase	Enzyme, fructose metabolism	Salo et al. 2015
Chl_synth	Chlorophyll synthase	Enzyme, chlorophyll synthesis	Salo et al. 2015
RuBisCO	Ribulose-1,5-biphosphate carboxylase/oygenase	Enzyme, photosynthesis	Salo et al. 2015
Metacasp	Metacaspase	Regulation hypersisitive response	Brakel et al. 2014
CTL1	Chitinase-like protein 1	Pathogenesis-related protein	Brakel et al. 2014
RppA	NB-ARC domain-containing disease resistance gene	Immune receptor	Brakel et al. 2014
pl 206	Disease resistance-responsive protein 206	Pathogenesis-related protein	Brakel et al. 2014
CYP73A	Trans-cinnamate 4-monooxygenase	Phenol synthesis	Brakel et al. 2014
eIF4A	Eukaryotic initiation factor	Reference gene	Ransbotyn & Reusch 2006

der nutrient-limiting conditions. In line with earlier experiments (Brakel et al. 2014), we found little evidence for enhanced plant mortality, while there were small negative effects on growth. Accordingly, plants were able to clear high inoculation levels within 3 wk to ambient background levels of infection.

Several response variables demonstrated moderate negative effects of *L. zosterae* on the eelgrass host. Eelgrass leaf growth was reduced by *L. zosterae* infection. Furthermore, we noted a 13-fold elevation of gene expression of the known stress indicator gene H*sp*80 in inoculated eelgrass plants, indicating that plants indeed suffered metabolic stress upon inoculation. Hsps not only react upon heat stress (Bergmann et al. 2010), but also play an essential role in various plant stress responses including pathogen attacks (Park & Seo 2015). The gene expression levels of Chlorophyll synthase and a subunit of *RuBisCO* were reduced in inoculated plants, which may explain why photosynthesis was reduced in infected plants. Inhibition of photosynthesis through *Labyrinthula* spp. infection has been shown before (Ralph & Short 2002, Olsen & Duarte 2015). Although we found no interactive effects of nutrient limitation and inoculation on eelgrass responses, both stressors acted in an additive way and reduced biomass production. We cannot exclude that this additive effect does not have other more long-term consequences — for example, reduced winter survival — which could not be addressed in our short-term (3 wk) experiment.

We found no evidence for a mutualistic interaction via a mechanism of enhanced nutrient recycling of *L. zosterae*, at least when assessing leaf growth rates and vegetative shoot recruitment. However, we cannot exclude that *L. zosterae* infection provides eelgrass with other fitness advantages over uninfected plants, for example, via herbivore deterrence or increased resistance against other pathogens or abiotic stressors. *Labyrinthula* spp. infection causes the accumulation of phenolic compounds surrounding the infected leaf section (Vergeer et al. 1995, Steele et al. 2005). In line with those earlier findings, we found elevated expression of the enzyme CYP73A, an essential enzyme for the phenol pathway, in this and an earlier study (Brakel et al. 2014). These phenolic compounds might reduce herbivory rates to which seagrass plants are subjected (Steele & Valentine 2015), thus infected seagrass may suffer less from grazing than uninfected plants as an indirect beneficial effect of *L. zosterae* presence.

Although *L. zosterae* cell densities were higher than measured values in the field (Bockelmann et al. 2013), this did not result in increased mortality. Survival was high in both nutrient treatments, supporting previous results that the virulence of contemporary Northern European *L. zosterae* genotypes is low (Brakel et al. 2014). It remains to be seen which environmental stressors, if any, may trigger pathogenicity and virulence on the side of the protist. In the coral–dinoflagellate symbiosis, it is well established that adverse environmental conditions, such as extreme sea surface temperature and/or ocean acidification, may turn a mutualistic relationship into a harmful one (Glynn & D'Croz 1990, Brown 1997). Further work should therefore be directed towards identifying those combinations of conditions that determine the position along the commensal–parasite gradient in the *L. zostera–Z. marina* interaction.

As an alternative explanation, the low virulence genotypes currently encountered in northern Europe may differ from the highly virulent *Labyrinthula* sp. that caused the wasting disease in the 1930s. So far, investigated *L. zosterae* strains from the East and West Atlantic, Pacific and Mediterranean show a very high similarity in ITS and 18S sequence (99.3 and 99.4 % identity, respectively), including strains that differed significantly in virulence assays (Martin et al. 2016). We speculate that there is additional hidden diversity that we cannot address with the current genetic markers because they do not address functional genes.

In conclusion, we have characterized the interaction between *L. zosterae* and its plant host under one set of varying environmental conditions (i.e. nutrients). We conclude that the interaction is rather parasitic in nature, although with a low virulence of the endophytic protist under ambient conditions. Although we did not find a mutualistic interaction, a recent report on the importance of mutualistic interactions in seagrass beds (van der Heide et al. 2012) underlines the importance of microbial interactions for the persistence of seagrass beds. Future experiments should address more realistic combinations of stressors, such as warming, light and nutrient limitation combined to further characterize the nature of the *Labyrinthula–Zostera* interaction.

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