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Seagrass–pathogen interactions: 'pseudo-induction' of turtlegrass phenolics near wasting disease lesions

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ABSTRACT: Marine protists of the genus *Labyrinthula* cause the seagrass wasting disease, which is associated with regional die-offs of eelgrass *Zostera marina* and also infects turtlegrass *Thalassia testudinum*. The ability of seagrasses to resist pathogen attack is determined by multiple factors, which are poorly understood. One factor hypothesized to influence seagrass disease resistance is the presence of (poly)phenolic natural products such as caffeic acid, which inhibits the growth of *L. zosterae* in *in vitro* laboratory bioassays. This hypothesis has been supported by reports of pathogen-induced phenolic accumulations in eelgrass *Z. marina*. To test the response of *T. testudinum* to inoculation with *Labyrinthula* sp., we conducted a series of culture experiments wherein plants were inoculated with *Labyrinthula* sp. isolated from turtlegrass beds in Perdido Bay, Florida (USA). Concentrations of phenolic acids and condensed tannins were quantified in diseased leaves as well as those treated with 5 mM salicylic acid, a signaling molecule associated with pathogen-induced responses in plants. In infection experiments, increases in the concentrations of several phenolic acids, but not condensed tannins, were observed in tissues above, but not below, microbial lesions. Salicylic acid (SA) treatments did not induce any phenolic compound, either when applied alone or in concert with the pathogen. The induction of phenolic acids above, but not below, infection sites suggests that *T. testudinum* leaves did not respond to the pathogen specifically. Instead, the pattern is consistent with the predictions of the sink/source model of plant defense, which predicts increased phenolic contents in cases where wounds disrupt plant resource allocation and cause a local overabundance of carbon-based resources. Thus, we suggest that the emergence of *Labyrinthula* sp. lesions on turtlegrass blades causes a 'pseudo-induction' of specific phenolics as carbon resources over-accumulate in tissues located above wound sites.

KEY WORDS: Turtlegrass · *Thalassia testudinum* · Wasting disease · *Labyrinthula* spp. · Pathogen · Phenolics · Induced defense

INTRODUCTION

Marine protists of the genus *Labyrinthula* are common in marine environments, occurring on seagrasses, macrophytes, and in sediment (Muehlstein et al. 1988, 1991). Some species (or 'forms') of *Labyrinthula* are associated with outbreaks of the seagrass wasting disease and periodic mass die-offs of eelgrass *Zostera marina* and, to a lesser extent, turtlegrass *Thalassia testudinum* populations (Bowles & Bell 2004). Wasting disease symptoms include brown infection sites that expand longitudinally along blades to form blackened, necrotic lesions on both young and old blades (Den Hartog 1996) and lead to the decay of the entire shoot in a period of a few days (as in eelgrass in the Dutch Wadden Sea in 1932; Den Hartog 1996), to a few weeks, (as in turtlegrass in Florida Bay in the 1980s; Robblee et al. 1991). One pathogenic 'form', *L. zosterae* Porter & Muehlstein from eelgrass, has been described (Muehlstein et al. 1988, 1991) and has been suggested

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as a causative agent in the previous large-scale outbreaks of the disease that decimated *Z. marina* beds in North America and Europe in the 1930s, in New Zealand in the 1960s, and along the eastern coasts of Canada and the United States in the 1980s (see Muelhstein et al. 1988, Short et al. 1988). Short et al. (1988) estimated that, during the 1930s event, over 95% of eelgrass beds were lost, leading to local extinction of the plant in some coastal areas. Eelgrass populations have failed to return to both the northern shore of Long Island in the United States and the Dutch Wadden Sea, even 75 yr after this event (Short et al. 1988). *Labyrinthula* spp. can cause necrotic lesions on turtlegrass blades as well. However, it remains unclear whether this is the same form that infects *Z. marina* (see Robblee et al. 1991, Vergeer & Den Hartog 1991, 1994, Zieman et al. 1999), and it remains uncertain whether or not *Labyrinthula* spp. infection leads to turtlegrass die-offs, such as those occurring in Florida Bay (Short et al. 1986, Robblee et al. 1991, Bowles & Bell 2004). Many forms of *Labyrinthula*, including those associated with wasting disease, are omnipresent and have been isolated from seagrass populations that do not show disease symptoms (Muehlstein et al. 1988). The pathogen may simply act as a secondary decomposer on senescent leaves until certain environmental conditions trigger it into a virulent pathogen (Short et al. 1988, Vergeer & Den Hartog 1994, Den Hartog 1996). However, it remains unclear what triggers disease outbreaks. Environmental conditions such as reduced irradiance, elevated temperatures, high salinities and unfavorable types of benthic substrate have been reported to increase the probability of wasting disease symptoms in eelgrass (Short et al. 1988, Buchsbaum et al. 1990, Burdick et al. 1993). In addition, the health of the plant is also an important factor, as Short et al. (1988) demonstrated that *Labyrinthula* sp. can kill stressed seagrasses, but merely weakens healthy plants.

The production of antimicrobial natural products in seagrasses, including (poly)phenolics from the shikimic acid/phenylpropenoid (SA/PP) pathways, may also affect the susceptibility of plants to the wasting disease. For example, the accumulation of phenolic substances in *Zostera marina* has been correlated with reduced microbial growth and herbivory (Harrison 1982), and with resistance to the wasting disease in mesocosm experiments (Buchsbaum et al. 1990) and in the field (Vergeer & Den Hartog 1991, 1994, Vergeer & Develi 1997). Seagrasses are a rich source of (poly)phenolics, including simple and sulfated phenolic acids and condensed (but not hydrolysable) tannins, many of which have demonstrated antimicrobial properties in other plant–pathogen interactions (Arnold & Targett 2002). One specific phenolic acid, caffeic acid, has demonstrated activity against a form of *Labyrinthula* isolated from diseased eelgrass and grown in culture (Vergeer & Develi 1997). The bioactivity of phenolics from other seagrass species, including turtlegrass, against *Labyrinthula* spp. has not been investigated.

Interestingly, many of the environmental conditions that have been linked to wasting disease outbreaks also affect levels of (poly)phenolics in seagrasses (Harrison & Durance 1989, Buchsbaum et al. 1990, Ravn et al. 1994, Vergeer et al. 1995). For example, Buchsbaum et al. (1990) found that high environmental nitrogen concentrations lead to decreased levels of phenolics in *Zostera marina* and to increased mortality due to wasting disease. Such intraspecific variation in phenolic content is often consistent with the predictions of the protein competition model for the production of SA/PP phenolics (Jones & Hartley 1999), which implies that phenolic-poor plants would predominate in high-nutrient/low-light habitats.

Seagrass phenolics are affected by *Labyrinthula* spp. as well. Vergeer et al. (1995) and Vergeer & Develi (1997) reported induced phenolic levels near disease lesions in infected blades of *Zostera marina*. In these studies, infected blade tissues were found to have elevated levels of some phenolics acids, including caffeic acid, as well as higher concentrations of Folin-reactive phenolics. While caffeic acid inhibits the growth of *L. zosterae* in culture, there is no evidence that this induced response is adaptive, i.e. that it confers pathogen resistance in nature. No studies have determined if such a response is generated from signaling pathways usually associated with plant defense responses, nor has the presence or absence of such a response been investigated in other seagrass species. In order to be considered a true induced defense, induced increases in phenolics should result in an increase in pathogen resistance, thus increasing plant fitness, and would most likely occur near an infection site (Agrawal & Karban 1999).

Our experiments were intended to determine whether or not turtlegrass possesses an induced response to infection by *Labyrinthula* spp. and, if such response were present, to evaluate the possibility that it could improve turtlegrass resistance to the seagrass wasting disease.

MATERIALS AND METHODS

A series of experiments was conducted to test the hypothesis that *Thalassia testudinum* exhibits induced accumulations of (poly)phenolic metabolites in response to infection by the wasting disease pathogen, *Labyrinthula* sp. We quantified concentrations of phenolic acids and proanthocyanins (i.e. condensed tan-

nins) in seagrass blades following inoculation with the pathogen or treatment with salicylic acid (SA), a chemical elicitor of many plant defense responses (Thaler et al. 1999).

Culture of *Thalassia testudinum*. Turtlegrass shoots were collected from the Gulf Islands National Seashore in Perdido Key, Florida, and grown in bare-root culture (without sediment) in 38 l plexiglass tanks, at a density of 10 to 15 plants/tank. Plants were illuminated using overhead VHO bulbs with Icecap™ ballasts generating a saturating irradiance of ca. 200 µmol m^{-2} s^{-1} and a day:night cycle of 12:12 h. Seawater (20 µm mesh-filtered) was obtained from the Environmental Protection Agency laboratory in Gulf Breeze, Florida, and the salinity and temperature were adjusted to match the levels observed in the field at the time that the plants were collected. To prevent the spread of the pathogen between tanks and to maintain individual tanks as true replicates for statistical analyses, there was no water flow among tanks during the experiments. Instead, tanks were aerated constantly, and a 10% seawater replacement was conducted every 4 d.

Exposure of plants to *Labyrinthula* sp. To observe their response to the pathogen, cultured plants were inoculated with *Labyrinthula* sp. using the methods of Muehlstein et al. (1988). To obtain the pathogen causing wasting disease symptoms on turtlegrass, the microbe was isolated from *Thalassia testudinum* blades collected in Perdido Bay and cultured on seawater agar (see Muehlstein et al. 1988). Microscopic evaluation of the isolated pathogen confirmed the presence of at least 1 form of *Labyrinthula* sp., which was effective at transferring disease symptoms to healthy plants, thus satisfying Koch's postulates (M. Caldwell pers. obs.). To transfer the pathogen from agar plates to experimental plants, pieces of sterile turtlegrass blades (0.5 cm long) were placed on agar plates 4 d prior to inoculation; these newly-infected leaf sections were then clamped to the outer surface of first- or second-rank leaves for 48 h using lengths of split tubing (see Muehlstein et al. 1988, their Fig. 1c). Control plants in these experiments were exposed to a 'false' inoculum, i.e. ~0.5 cm leaf sections that had been autoclaved in seawater and then clamped onto the corresponding sites on the leaves.

Collection of samples. The appropriate sampling of leaf tissues is critical, as preliminary experiments demonstrated that *Labyrinthula* sp.-induced phenolic accumulations occur only in certain tissues within leaves. For example, our preliminary experiments indicated that the response varies with proximity to the infection site, and even varies depending on whether the tissue is located above or below the infection site (also see Ralph & Short 2002). We observed that the analysis of whole leaves often fails to detect the induced accumulation of phenolics. In these experiments, tissue samples were taken from lesions and from sites located 2 cm and >2 cm above and below lesions. To test for induced changes in phenolic contents, uninfected (control) tissues were analyzed from similar sites on plants exposed to the false inoculum. All tissues harvested were frozen immediately at −80°C.

Expt 1: response to *Labyrinthula* sp. This experiment was conducted to determine whether there was an induced response in *Thalassia testudinum* leaves exposed to *Labyrinthula* sp. If induction of (poly)phenolics were to occur in response to *Labyrinthula* sp., a significant increase in these compounds would be expected in the tissue adjacent to the lesion (specifically the young, actively growing tissue below the necrotic area) in infected plants compared to uninfected controls. To test for the presence or absence of an induced response within infected leaves, leaf tissues were collected from *T. testudinum* shoots that had been inoculated with either active *Labyrinthula* sp. cultures or the false inoculum. We inoculated leaves in 2 similar experiments (Expts 1a and b) in order to harvest sufficient tissues for the analyses. In both of these experiments, 180 to 200 individual shoots were grown at a density of 10 plants per tank. Culture conditions were set to match the environmental conditions at the sites where plants were collected. In Expt 1a, shoots were held in one of 18 replicate tanks at 24 to 27°C and 30 to 32 salinity, and 1st-rank (youngest) and 2nd-rank (next youngest) leaves were harvested after 7 d. In Expt 1b, shoots were held at 31°C and 20 salinity, and 2nd-rank leaves were harvested after 5 d. Potential effects of infection were examined for condensed tannin levels, phenolic acid contents, C:N ratio and protein content.

To test the hypothesis that proximity to lesions affected the various tissue characteristics, Model I analyses of variance (ANOVA) were used to examine differences among plant tissues located adjacent to lesions, >2 cm away from lesions, and at necrotic lesions (Expt 1a), and above, below and at necrotic lesions (Expt 1b). Tukey multiple comparison tests were performed to identify the specific tissue groups among which differences occurred.

To test the hypothesis that tissues adjacent to lesions differed from healthy tissue of other, uninfected control plants, individual Student's *t*-tests were performed. This approach allowed the various tissues from infected plants to be compared to tissues from uninfected plants, which, by definition, did not possess tissue sections that could be identified as being 'above' or 'below' lesions. Specifically, Student's *t*-tests were used to determine whether differences occurred between green tissue from uninfected plants and

lesion tissue, green tissue from uninfected plants and green tissue 2 cm on both sides of the lesion, and green tissue from uninfected plants and green tissue >2 cm around the lesion from infected plants (Expt 1a only). In Expt 1b, Student's *t*-tests were used to determine whether differences occurred between green tissue from uninfected plants and lesion tissue, green tissue from uninfected plants and green tissue 2 cm above the lesion, and green tissue from uninfected plants and green tissue 2 cm below the lesion.

Expt 2: response to exogenous salicylic acid. We tested the hypothesis that phenolic metabolism in *Thalassia testudinum* leaves is inducible by the application of salicylic acid (SA), a plant signaling molecule often involved in the initiation of pathogen-induced defenses in many plants (Thaler et al. 1999). If this hypothesis were valid, an increase in the phenolic content of leaves exposed to SA would be expected compared to untreated plants. We grew 200 plants (10 per tank) in culture at 25 to 26°C and 27 to 29 salinity. Plants were laid on seawater-soaked paper towels and sprayed with either a solution of pH-adjusted (to pH 8.0) 5 mM SA in seawater, or a control solution (seawater only). Plants were allowed to sit exposed for 15 min, before being rinsed with seawater and returned to the tanks; 1st- and 2nd-rank leaves were collected after 7 d. Since there were no lesions present on leaves, leaf tissues were not dissected into regions within leaves; instead a section from the middle of the leaves was processed for analyses.

A Student's *t*-test with an α value of 0.05 was used to examine differences between control and experimental plants. This procedure was used to analyze the C:N ratio and phenolic acid level of both 1st- and 2nd-rank leaves.

Expt 3: combination of stimuli. This experiment examined the responses of *Thalassia testudinum* to treatment with SA followed by inoculation with *Labyrinthula* sp. If induction of phenolics occurs in response to treatment with SA, an increase in phenolic acids in any plants (infected or uninfected) would be expected compared to plants sprayed with seawater. If induction of phenolics occurs in response to inoculation with *Labyrinthula* sp., an increase in these compounds would be observed in the tissue below the lesions compared to uninfected controls. To determine if pre-treatment with exogenous SA affected the response of leaves to *Labyrinthula* sp., 200 plants were grown at 23°C and 25 to 27 salinty in 20 replicate tanks at a density of 10 plants per tank. Plants from 10 tanks were treated once a day for 3 d with pH-adjusted 5 mM SA, as described above, and plants from the remaining 10 tanks were sprayed with seawater only. On Day 4, the 2nd-rank leaves of plants from half of the SA-treated and seawater-only-treated tanks were inoculated with *Labyrinthula* sp. inoculum. At the same time, the 2nd-rank leaves of plants from the remaining tanks were inoculated with false inoculum. We harvested 1st- and 2nd-rank leaves 4 d after inoculation.

To test for effects of infection, SA, and the combination of factors, a 2-factor ANOVA with an α value of 0.05 was used to analyze the differences in phenolic acid content among the following groups: (1) green tissue 2 cm above a lesion from salicylic acid-treated plants, (2) green tissue 2 cm above a lesion from seawater-sprayed plants, (3) green tissue 2 cm below a lesion from salicylic acid-treated plants, (4) green tissue 2 cm below a lesion from seawater-sprayed plants, (5) green tissue from uninfected salicylic acid-treated control plants, and (6) green tissue from uninfected seawater-sprayed control plants. We used 3 separate Tukey multiple-comparison tests to identify the specific tissue groups among which differences occurred.

Statistical transformations. Since percentages and proportions often form a binomial rather than a normal distribution, all proportional data were arcsine-transformed prior to analysis (Zar 1999). Levine's homogeneity of variance tests were run to assure equal variance. An α value of 0.05 was used for all analyses.

Biochemical analyses. Proanthocyanin concentrations were quantified colorimetrically using the butanol-HCL assay, as described by Arnold & Schultz (2002).

Plant tissues (10 to 25 mg) were ground in liquid nitrogen and extracted for 24 h at 4°C in 70:30 acetone:water containing 1 mM ascorbic acid. Extracts were reacted for 1 h with a solution of 95% butanol: 5% HCl at 100°C and sample absorbances were recorded at 550 nm. Absorbances were compared to standard curves generated from condensed tannins isolated from *Thalassia testudinum* tissues. The concentrations of simple phenolic acids, including 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid, p-coumaric acid and vanillin were measured in turtlegrass tissues by RP-HPLC, using a method adapted from Ravn et al. (1994). Tissues (10 to 15 mg) were homogenized in liquid nitrogen and extracted in 50% methanol (aq.) with 2% acetic acid at 4°C. Particulates were removed by passing samples through a 0.45 µm filter. We applied 20 µl samples to a Discovery C-18 RP column (Supelco) using an isocratic solvent system of 1:1:7 methanol:2-propanol:water (with 2% acetic acid) and a flow rate of 0.8 ml min^{-1}. The peak areas measured at 254 nm were compared to commercial standards (Sigma). Tissue protein contents were determined using the Bradford-based Bio-Rad™ microassay for solubilized proteins and compared to a bovine serum-albumin standard (0 to 20.0 µg ml^{-1} in 1N NaOH). Tissue carbon:nitrogen ratios were analyzed for dried homogenized tissues using a Carlo-Erba

Particulate CNS Analyzer NA 1500 at the Dauphin Island Sea Laboratory (Dauphin Island, Alabama); 1st- and 2nd-rank leaves were treated separately in all analyses.

RESULTS

Turtlegrass shoots maintained active growth, with leaf extension rates of 0.13 to 2.18 mm d^{-1}, depending on the rank of leaves examined and culture conditions. The inoculation of healthy leaves with *Labyrinthula* sp. successfully transferred disease symptoms in >90% of the attempts. In preliminary experiments, it was possible to inoculate both 1st- and 2nd-rank leaves. In both cases, necrotic lesions formed within 4 to 7 d. Phenolic levels within turtlegrass shoots were relatively high, with roots/rhizomes and mature leaves possessing the greatest levels. For example, condensed tannin levels ranged from 57 mg g^{-1} in 1st-rank leaves to >250 mg g^{-1} in roots/rhizomes. Levels of the individual phenolic acids varied from 10.2 to 64.6 mg g^{-1}. The combined phenolic acid pool for these 4 phenolic acids, reached levels as high as 103.7 mg g^{-1}.

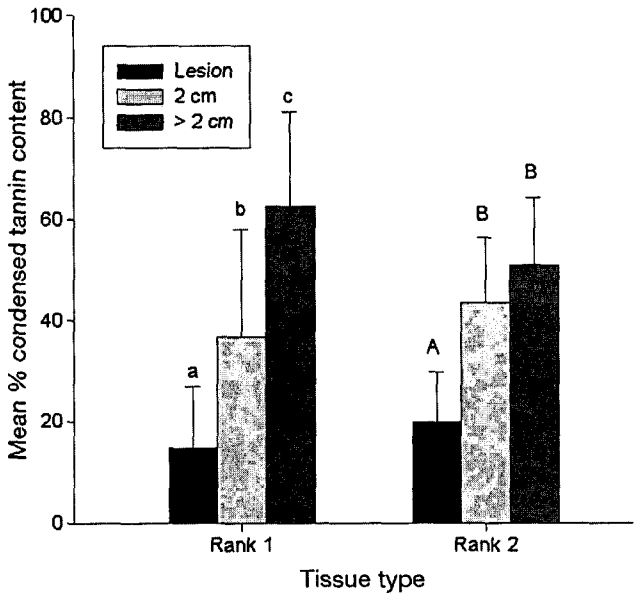

Fig. 1. *Thalassia testudinum* infected with *Labyrinthula* sp. (Expt 1a). Mean condensed tannin content of distinct tissue types in *Labyrinthula* sp. infected 1st- and 2nd-rank leaves of *T. testudinum* (lesion tissue; green tissue 2 cm on both sides of lesion; green tissue >2 cm on both sides of lesion) expressed as percentage of highest value obtained. Data bars with different letters above error bars are significantly different at $p < 0.05$. For rank 1, n = 10 for lesion, 8 for 2 cm, 12 for >2 cm; for rank 2, n = 12 for lesion, 13 for 2 cm, 20 for >2 cm

Response to *Labyrinthula* sp. (Expt 1)

Infection by *Labyrinthula* sp. resulted in changes in the concentrations of (poly)phenolics in turtlegrass leaves. The response was highly site-specific, however. Condensed tannin levels, measured in Expt 1a, were affected by leaf age (i.e. 'rank') and by infection (Fig. 1). Within leaves of a given rank, tannin levels varied with proximity to the infection sites. For example, in 1st-rank leaves, condensed tannin levels increased significantly with increasing distance from the lesion, e.g. from lesions to tissues located 2 cm from the lesion, then to those >2 cm from the lesion (Fig. 1). Tannin levels in tissues located 2 cm from the lesion and those >2 cm from the lesion were different from lesion tissue but not from each other in 2nd-rank leaves. These variations were not indicative of a pathogen-induced increase in tannin levels, as concentrations distal to lesions never exceeded those in healthy, uninfected control plants, and adjacent to lesions concentrations were lower than those in control plants. Rather, the variations suggest a decrease in tannin levels near infection sites.

In this experiment, there were no significant differences in the C:N ratios of any tissue types for 2nd-rank leaves (Table 1). However the C:N values for the tissue located 2 cm from the lesion were higher than those >2 cm from the lesion and within the lesion for 1st-rank leaves (Table 1). Tissue protein contents varied signifi-

Table 1. *Thalassia testudinum* infected with *Labyrinthula* sp. Mean C:N ratio and mean protein content (in µg protein μg^{-1} dry mass) of 3 distinct tissue types (green tissue >2 cm around lesion; lesion tissue; green tissue 2 cm around lesion) from *Labyrinthula* sp.-infected 1st- and 2nd-rank leaves of *T. testudinum*. No data were collected for protein content of 1st-rank leaf tissue from 2 cm around lesion. Values with different letters adjacent are significantly different from each other at $p < 0.05$

Tissue type	Mean C:N ratio		Mean protein content (µg protein μg^{-1} dry mass)	
	Rank 1	Rank 2	Rank 1	Rank 2
Green tissue >2 cm around lesion	17.144 ± 2.812a	20.361 ± 1.850a	0.087 ± 0.011b	0.071 ± 0.010b
Lesion tissue	18.955 ± 1.623b	20.819 ± 1.985a	0.068 ± 0.008a	0.057 ± 0.0060a
Green tissue 2 cm around lesion	19.093 ± 1.557b	22.011 ± 3.121a	–	0.073 ± 0.008b
p-value	0.049	0.153	0.014	0.005

cantly. There was enough tissue to conduct protein analyses only for lesion tissue and the tissue >2 cm from the lesion for the 1st-rank leaves. These tissues had significantly different protein values. For the 2nd-rank leaves, necrotic tissue possessed significantly lower protein concentrations (Table 1) than either tissues located 2 cm from lesions or tissue >2 cm from the lesion.

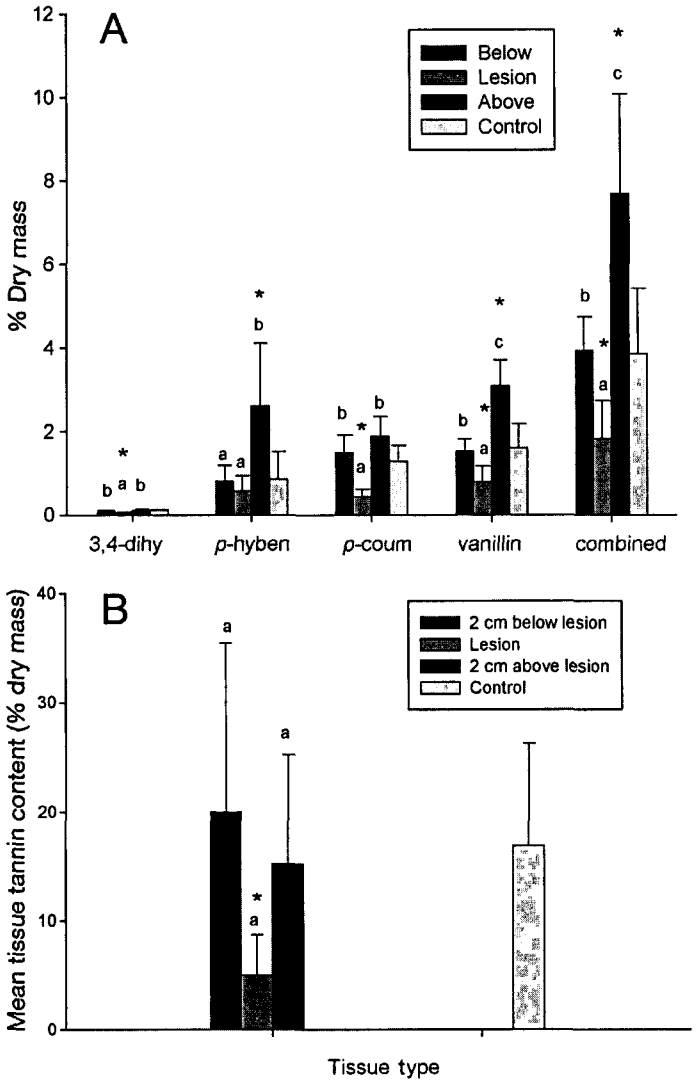

Fig. 2. *Thalassia testudinum* infected with *Labyrinthula* sp. (Expt 1b). Mean phenolic acid and condensed tannin levels of 4 distinct tissue types (lesion tissue, tissue 2 cm below lesion, tissue 2 cm above lesion, and control tissue) from *Labyrinthula* sp.-infected and uninfected 2nd-rank leaves of *T. testudinum*. (A) Phenolic acid levels for 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid, p-coumaric acid and vanillin; combined data for the 4 acids are also included; values are percentage dry mass (n = 4 for control, 5 for each of the other tissue types). (B) Condensed tannin content; values are percentage dry mass (n = 6 for below lesion, 8 for lesion, 6 for above lesion, 5 for control). Data bars with same letters above error bars indicate differences among lesion, 2 cm below lesion, and 2 cm above lesion, asterisks indicate differences from control tissue (all at $p < 0.05$)

In Expt 1b, elevated phenolic acid levels, above levels found in uninfected tissues, were observed in the infected 2nd-rank leaves. Induced increases in phenolic acid levels were detected for p-hydroxybenzoic acid, p-coumaric acid and vanillin, as well as for the total of the 4 phenolic acids in infected 2nd-rank leaves (Fig. 2A). The induced response was site-specific, however. Interestingly, tissues located below the lesion did not exhibit these induced accumulations of phenolics. Levels of condensed tannins were not induced by infection; they did not increase above levels found in healthy, uninfected tissues (Fig. 2B). However, unlike Expt 1a, tannin levels were not significantly lower in tissues located near lesions (Fig. 2B).

Response to SA (Expt 2)

Exposure to 5 mM SA did not induce an increased accumulation of individual phenolic acids in 1st- or 2nd-rank leaves. In fact, there was a 60% decrease in the overall phenolic acid content (the 4 compounds combined) of 1st-rank leaves exposed to 5 mM SA (Fig. 3). C:N ratios were not affected by SA exposure in 1st- ($p = 0.252$) or 2nd-rank leaves ($p = 0.337$).

Response to the combination of stimuli (Expt 3)

The responses of plants to the combination of infection plus SA treatment were similar to their responses in Expts 1a, 1b and 2. Infection resulted in the induced accumulation of 2 phenolic acids. Levels of p-hydroxybenzoic acid and vanillin were significantly higher in tissue located above and below infection sites compared to similar tissues in uninfected control plants that were sprayed with salicylic acid (Fig. 4). Thus, a pathogen-induced increase in the levels of various phenolic acids was detected in both infection experiments (1 and 3).

There was no significant effect of SA treatment on levels of phenolic acids (Fig. 4). In fact, on average, SA application resulted in a significant decrease in the concentrations of several compounds. Therefore, SA did not induce the accumulation of any phenolic acid or the total phenolic acid pool in either Expt 2 or 3.

In Expt 3, levels of 3, 4-dihydroxybenzoic acid and p-coumaric acid did not change in the 1st- or 2nd-rank leaves of any treatment group, in response to exogenous SA or to infection by *Labyrinthula* sp.

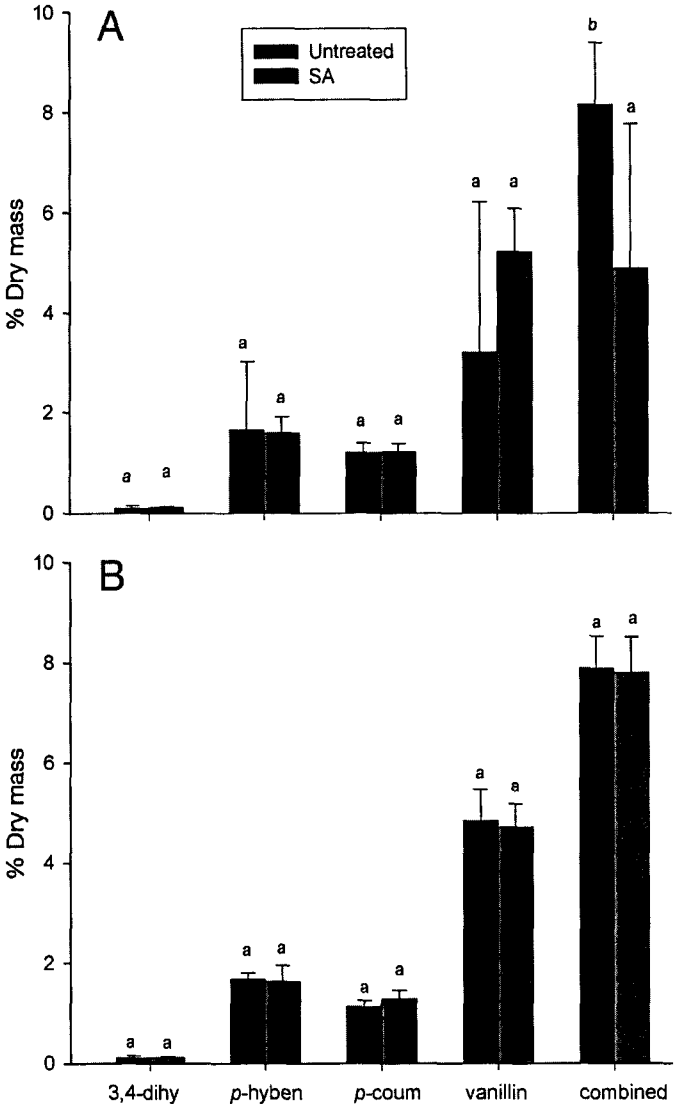

Fig. 3. *Thalassia testudinum.* Mean phenolic acid levels of untreated and 5 mM salicylic acid (SA)-treated 1st- and 2nd-rank leaves. Phenolic acid levels for 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid, p-coumaric acid and vanillin are presented; combined data for the 4 acids are also included; values are percentage dry mass. (A) Values for 1st-rank leaves (n = 5 for all samples except for untreated vanillin value and untreated combined value, where n = 3). (B) Values for 2nd-rank leaves (n = 5 except for SA vanillin value and combined value, where n = 4). Data bars with different letters above error bars are significantly different at $p < 0.05$

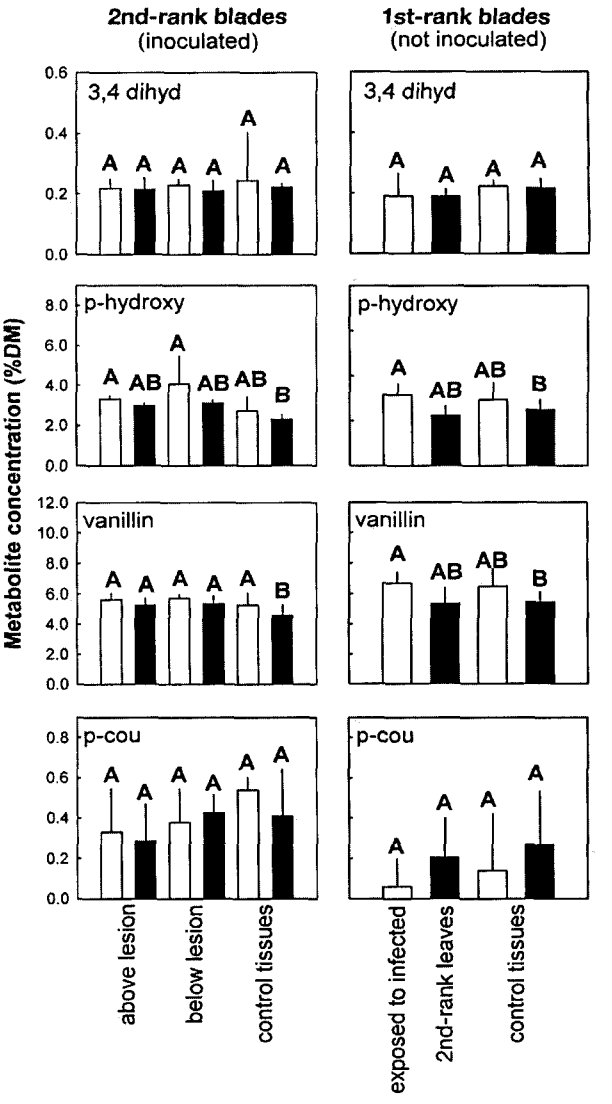

Fig. 4. *Thalassia testudinum.* Effects of *Labyrinthula* sp. infection on phenolic acid contents of inoculated 2nd-rank and adjacent 1st-rank leaves untreated (open bars) or treated with 5 mM salicylic acid (SA) (filled bars); values are percentage dry mass. In the inoculated 2nd-rank leaves, tissues 'above' or 'below' infection site are compared to similar tissues from untreated plants. Phenolic acid contents of 1st-rank leaves of same plant, which were not directly inoculated but were in physical contact with lesions, are also shown. Bars with different letters above error bars are significantly different at $p < 0.05$

DISCUSSION

Inoculations of healthy turtlegrass blades with *Labyrinthula* sp. caused wasting disease symptoms, including the emergence of necrotic lesions. Plants responded by accumulating specific phenolic compounds near infection sites; however, these responses were complex and not consistent with the view that phenolics serve as an effective induced defense against the wasting disease pathogen.

We observed pathogen-induced accumulations of 3 specific phenolic acids (p-hydroxybenzoic acid, p-coumaric acid and vanillin) as well as the combined pool of the 4 identified acids, in tissues above a lesion

site. Induced concentrations of the 4 combined phenolic acids near infection sites were ~200% of the concentrations in uninfected tissues. This pattern is similar to, but not as great as the induced accumulations of phenolic acids in infected blades of *Zostera marina* reported by Vergeer & Develi (1997), who observed increases of between 400 and 450% for caffeic and gallic acids in eelgrass tissues located near lesions. However, the response of turtlegrass blades differed from our expectations in several regards:

- Inoculations did not elicit induced condensed tannin concentrations; in fact, condensed tannin levels in 2nd-rank blades inoculated with *Labyrinthula* spp. were decreased near infection sites. This indicates that these polyphenolics do not accumulate in turtlegrass blades in response to *Labyrinthula* spp. attack. Interestingly, this differs from the response of *Thalassia testudinum* blades to general mechanical damage, as we consistently observe induced accumulations of condensed tannins within 5 to 30 d following simulated herbivory (T. Arnold & C. Tanner unpubl. data).
- The application of 5 mM SA (a chemical elicitor of many plant defense responses, including those associated with pathogen resistance) did not induce phenolic accumulations in *Thalassia testudinum*. In fact, exogenous SA resulted in a decrease in the total of the 4 acids in 1st-rank leaves. In subsequent experiments pairing SA treatments with *Labyrinthula* sp. inoculations in the same culture experiments, infected plants exhibited increased phenolic acid concentrations, but SA alone or in concert with the pathogen had no detectable effect. For these treatments, we applied 5 mM SA to emergent shoots at a higher concentration than those commonly used to elicit defense responses in terrestrial plants. We have also observed, in related experiments, that another commonly used elicitor of plant defense responses, jasmonic acid (JA), also failed to increase phenolic content in *T. testudinum* when applied as 5 mM ± JA in 10% ethanol (aq.) by spraying for 20 min (T. Arnold unpubl. data). As a result, we interpret these results as suggesting that SA-signaling pathways are not involved in activating this response.
- The response of tissues depended upon their location above or below infection sites. Induced concentrations of p-hydroxybenzoic acid, p-coumaric acid, vanillin and the 4 combined acids occurred only in tissue immediately above, but not below, infection sites. Similar uneven responses to *Labyrinthula zosterae* were observed by Ralph & Short (2002), who reported that, in eelgrass blades, the photosynthetic quantum yield of infected eelgrass shoots was significantly lower than control tissue above, but not below, necrotic lesions. These observations are not consistent with the view that *Thalassia testudinum* responds to *Labyrinthula* sp. infection specifically, with a coordinated response involving signaling cascades and/or the regulation of the SA/PP pathway. It also seems counteradaptive for a presumed antimicrobial defense to protect older leaf sections, which are generally lost as soon lesions span the entire width of the blade, instead of young leaf sections near basal meristems and the remainder of the shoot. If induction were adaptive, we would expect to see an increase in phenolics below a lesion rather than above. Based upon these results, we suggest the following alternative explanation for the accumulation of phenolics near *Labyrinthula* spp. lesions.

We propose that these responses are an example of 'pseudo-induction' of plant phenolics, that is, an accumulation of phenolics caused by simple rearrangements of resources within plant tissues. Specifically, we suggest that *Labyrinthula* spp. lesions damage vascular tissues in seagrass blades, disrupting the flow of carbohydrates within seagrass blades and resulting in accumulations of phenolics wherever carbohydrates are in overabundance, as predicted by Jones & Hartley (1999). Such responses are known from terrestrial plants, including birch, pine and poplar, and are predicted by the sink/source model of plant defense (Haukioja 1990, Tuomi et al. 1991, Honkanen et al. 1999, Arnold et al. 2004). This model predicts that 'induced' responses arise when the allocation of carbon resources among tissues acting as carbohydrate sources and sinks is altered, e.g. when herbivores remove source or sink tissues unequally or when vascular transport is disrupted.

For *Thalassia testudinum* shoots, 2nd-rank leaves have the characteristics of exporting source leaves, with a flow of carbohydrates from leaf tip to the base, and then towards nearby sink tissues (e.g. rhizomes and developing 1st-rank leaves). As a result, the emergence of necrotic lesions on 2nd-rank leaves would be expected to disrupt carbohydrate export. According to the sink/source model, this would result in the accumulation of excess carbon and phenolics above, but not below lesions, which is what we observed in inoculated plants.

The results of these culture experiments suggest that *Labyrinthula* spp.-induced accumulations of phenolics in *Thalassia testudinum* occur as a result of changing patterns of resource allocation rather than coordinated signaling events, and that such induced responses are not adaptive, i.e. are unlikely to improve plant fitness in the face of pathogen attack.

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