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Phenotypic variation in polyphenolic content of the tropical brown alga *Lobophora variegata* as a function of nitrogen availability

Thomas M. Arnold*, Christopher E. Tanner, Walter I. Hatch

St. Mary's College of Maryland, St. Mary's City, Maryland 20686, USA

ABSTRACT: Although brown algal phlorotannins have been shown to deter certain herbivores and may accumulate in response to herbivory, recent evidence has suggested that concentrations of these polyphenolic compounds are inversely related to nitrogen availability. Previous studies examining the relationship between brown algal phlorotannins and nutrient availability have been conducted in the field and, as a result, other variables may have contributed to observed variations in polyphenolic content. In order to more rigorously test the carbon-nutrient balance hypothesis, clonal isolates of the tropical brown alga *Lobophora variegata* were cultured for 4 wk in seawater media of 3 nitrogen concentrations (0.2, 1.2, 5.2 mg N l^{-1}). Total phenolic content was determined weekly by a micro-Folin-Denis assay which uses as little as 1 mg of tissue. Percent carbon and nitrogen (per dry weight algal tissue) were also determined weekly for each nutrient treatment. Significant differences (ANOVA, $p < 0.001$) in phenolic content were observed between nutrient treatments after 2 wk. Phenolic content was directly correlated with tissue C:N ratios and inversely correlated with percent tissue nitrogen. Our results support the hypothesis that nitrogen availability plays a role in determining phlorotannin concentrations in brown algae.

KEY WORDS: Polyphenols · Phloroglucinol · Phlorotannins · Intraspecific variation · Chemical defense · Carbon-nutrient balance · *Lobophora variegata* · Brown algae · Phaeophyceae

INTRODUCTION

High concentrations of polyphenolic secondary metabolites are found in many terrestrial plants, aquatic vascular plants and brown algae (Phaeophyceae), where they play a putative role in competitive plant fitness. Brown algal polyphenols (phlorotannins) are acetate-malonate derived phloroglucinol (1,3,5-trihydroxybenzene) polymers which are analogous to vascular plant tannins. Concentrations of phlorotannins as high as 25 to 40% dry weight have been recorded (Ragan & Glombitza 1986, Tugwell & Branch 1989, Targett et al. 1992, unpubl.), although the phenolic content of different tissues (Johnson & Mann 1986, Ragan & Glombitza 1986, Tugwell & Branch 1989) and individuals plants within a species (Van Alstyne 1988, Ilvessalo & Tuomi 1989, Yates & Peckol 1993) can vary considerably.

Despite biosynthetic dissimilarities (see Waterman & Mole 1994 for review), algal phlorotannins and vascular plant tannins possess similar characteristic chemical properties that form the basis for ecological activity (Sieburth & Jensen 1969, McKnight & Morel 1979, Ragan & Craigie 1980, Appel 1993). For example, polyphenolics have been observed to deter herbivory (Geiselman & McConnel 1981, Buchsbuam et al. 1984, Steinberg 1985, 1989, Targett et al. 1986), reduce digestion efficiency by binding to digestive enzymes (Swain 1979, Hagerman & Butler 1980, Targett & Targett 1990, Boettcher & Targett 1993, but see Martin et al. 1987), affect larval development (Esping 1957a, b), and inhibit epiphytic growth (Sieburth & Conover 1965, McLachlan & Craigie 1966, Glombitza & Vogels 1985, Buchsbaum et al. 1990).

*Present address: University of Delaware, Graduate College of Marine Studies, Lewes, Delaware 19958, USA

Herbivory can affect patterns of plant distribution and diversity in temperate and tropical plant communities on both ecological and evolutionary time scales (Lubchenco 1978, Cubit 1984, Coley et al. 1985, Gaines 1985, Hay & Fenical 1988). Hay & Fenical (1988) estimated that 60 to 97% of total seaweed annual production in a coral reef community may be removed by herbivores. In plant-herbivore interactions where polyphenols are influential, bioactivity is considered to be primarily, but not exclusively, dose dependent (Geiselman & McConnel 1981, Targett & McConnel 1982, Steinberg 1988, Appel 1993, Boettcher & Targett 1993). As a result, variations in plant allelochemical content may significantly affect many aspects of plant fitness, including the percentage of plant production lost to herbivores, and, therefore, influence species composition or population distribution (Feeny 1976, Rhoades & Cates 1976, Steinberg 1989). Hypotheses attempting to account for both the high concentrations and intraspecific variation of phenolic compounds originated from studies of terrestrial plants and focused on both extrinsic and intrinsic factors affecting various aspects of plant fitness. These hypotheses have been more recently applied to marine plants.

The carbon-nutrient balance hypothesis, as elucidated by Bryant et al. (1983), proposed a link between resource availability and polyphenolic content in boreal plants and, therefore, a possible explanation for observed intraspecific variation (also see Gershenzon 1984, Waterman et al. 1984, Coley et al. 1985). This hypothesis states that polyphenols, carbon-based compounds, will accumulate under conditions of nutrient limitation, when amino acid synthesis is retarded and carbon is in relative excess. Such a relationship may be advantageous because polyphenols would then accumulate under conditions of suppressed protein synthesis when tissue damage originating from herbivory is most difficult to repair. Conversely, chemical defense may be less essential when nitrogen availability is not limiting protein synthesis and tissue damage may be repaired more effectively (Bryant et al. 1983). Indeed, evidence indicates that production of polyphenolic allelochemicals in brown algae and certain species of vascular plants may vary partially or primarily as a function of the internal nutrient balance. For example, Bryant et al. (1987) observed a decrease in polyphenolic content and a related increase in herbivory in the Alaskan paper birch as a result of increased nitrogen availability and shading. Buchsbaum et al. (1990) reported that the polyphenolic content of eelgrass *Zostera marina* was inversely correlated with tissue nitrogen levels and that low-phenol plants suffered complete mortality from wasting disease. The polyphenolic content of plant cell cultures has also been observed to vary as a function of sucrose addition (Phillips & Henshaw 1977) and the C:N internal ratio (Kubek & Shuler 1980). However, Fajer et al. (1992) and Arnold & Targett (unpubl. data) found that increasing the carbon content of certain vascular species by elevating environmental CO_2 levels did not result in increased production of carbon-based allelochemicals. Ilvessalo & Tuomi (1989) and Yates & Peckol (1993) reported that variations in the polyphenolic content of *Fucus vesiculosus* were correlated with tissue nitrogen and the internal C:N ratio, but not tissue carbon.

Previous studies examining intraspecific variation of brown algal polyphenolic content in relation to nutrient availability have been conducted in the field and, as a result, other environmental and biological variables may have caused or exaggerated the observed variations in phenolic content. Observations indicate that phenotypic variations in plant phenolic content can be correlated with season (Geiselman 1980), light intensity, herbivore pressure (Hay & Fenical 1988, Van Alstyne 1988), reproductive state (Ragan & Jensen 1978), and salinity (Pedersen 1984). Also, attempts to utilize a culture system to quantify intraspecific variation have been impeded by the fact that cultured plants rarely retain high levels of phenolic allelochemicals (Ragan & Glombitza 1986).

In this study a culture isolate of the brown alga *Lobophora variegata* (Dictyotales, Dictyotaceae) was used to investigate the effects of nitrogen availability on plant phlorotannin content. *L. variegata* is a cosmopolitan tropical brown alga with several distinct growth forms (Coen & Tanner 1989), all of which have relatively high concentrations of phenolic compounds (8.33 to 13.39% of dry weight; Targett et al. 1992). The 'decumbent form' of *L. variegata* is less palatable to fish and crab herbivores than the other forms (Coen & Tanner 1989) and in some habitats has significantly higher phenolic content (Tanner unpubl. data); however, a relationship between phenolic content and palatability has not been established for the forms of *L. variegata*.

METHODS

Spores were isolated from an individual thallus of the decumbent form of *Lobophora variegata*, collected from a shallow backreef near Carrie Bow Cay, Belize, in July 1989. Cultures from these spores were grown and maintained in enriched seawater medium at 24°C, with photon irradiances of 50 to 130 µmol m^{-2} s^{-1} and a light:dark cycle of 12:12 h until the time of the experiment. In culture, plants reproduced entirely by vegetative means with blades seldom reaching a size of more than 1 cm in diameter.

Young, actively growing blades of *Lobophora variegata* were separated, rinsed with sterile seawater, and transferred to seawater that had passed through algal nutrient scrubbers of the coral reef display tank at the National Museum of Natural History, Smithsonian Institution, Washington, DC (USA). After 3 d, the blades were randomly assigned to one of 3 nitrate treatments (0.2, 1.2, and 5.2 mg N l^{-1} of seawater). Seawater scrubbed by the Smithsonian's coral reef tank (0.2 mg N l^{-1}) was used as the basal nitrogen treatment. Enriched treatments were prepared by adding sodium nitrate to the basal seawater, and all solutions were sterilized by passing through membrane filters with 0.22 µm pores. Each nitrogen treatment was assigned 35 plants, which were subdivided among five 200 ml replicate flasks each containing 150 ml of media. Cultures were agitated continuously for 4 wk in an incubator set at 24°C, 130 µmol m^{-2} s^{-1} of photon irradiance, and a light:dark cycle of 12:12 h. Culture media were replaced once, on Day 18 of the experiment.

A thallus was collected from each flask on Days 8, 15, 22, and 29 for phenolic content and carbon/nitrogen analyses. Thalli, randomly selected from each treatment, were examined microscopically, and morphological changes were noted. All samples were collected at midday to exclude daily fluctuations in plant metabolism as possible factors affecting results. Tissue for total phenolic content determination was weighed and frozen at −70°C for later extraction. Tissue not used to determine phenolic content was pooled for each treatment and dried to constant weight at 40°C for carbon/nitrogen analyses. Carbon and nitrogen content of samples were determined with a Model 440 Leeman CHN analyzer.

Total phenolic content was determined with a modified Folin-Denis assay which utilizes as little as 1 mg of tissue (Hatch & Tanner unpubl.). For Days 8, 15, and 22, 1 to 5 mg of plant tissue was macerated with a glass rod in 3 µl of 1.2 g ml^{-1} trichloroacetic acid (TCA) to precipitate proteins. This was followed by the addition of 17 µl of 50% acetone, which was found to be more efficient at extracting algal phenolics than 50 or 70% methanol (Hatch & Tanner unpubl.). Samples were vortexed and allowed to extract at room temperature for 4 h. Following extraction, samples were assayed for phenolic content as follows: 1000 µl of 1:10 Folin-Denis reagent (Association of Official Analytical Chemists 1970) was added to each sample, and immediately vortexed. After 3 min, 100 µl of saturated sodium carbonate solution was added, followed by vortexing. At the end of 1 h the samples were centrifuged at 16 000 × *g* for 10 min, and the absorbance of the supernatant was read at 725 nm. Tannic acid equivalents were calculated by comparison to a standard curve of desiccated tannic acid, diluted to 0–20 µg ml^{-1} with 2:10 parts of 1.2 g ml^{-1} TCA: 50% acetone and assayed as above. As the entire extract was used in the assay, direct determination of dry weight and the contribution of non-phenolic reactants in the Folin–Denis assay (see Yates & Peckol 1993) was not possible. Dry weights were estimated using a wet/dry weight ratio determined from samples assayed on Day 29. Specimens from this sample period were assayed with a modification of the above procedures. A sample of 9 to 16 mg of tissue from each replicate was extracted in 20 µl of 1.2 g ml^{-1} TCA and 100 µl of 50% acetone. After extracting for 4 h, samples were centrifuged for 10 min and 20 µl of each supernatant was assayed as described above. The tissue and remaining extract was dried at 60°C for 24 h and then at 110°C for up to 1 wk and weighed.

Differences in phenolic content among plants of the 3 treatments were determined by a 1-way ANOVA ($\alpha = 0.05$) for each sampling period. Correlations among culture media nitrogen concentrations and plant percent nitrogen, percent carbon, and total phenolic content were determined by a Pearson product-moment correlation ($\alpha = 0.05$).

RESULTS

By Day 6, all plants exhibited hyaline hairs, nucleated but nonpigmented sterile filaments. Although relative densities of hairs were not quantified, it was evident that plants cultured in 0.2 mg N l^{-1} media possessed a more extensive and dense corona of hyaline hairs than did plants of the 5.2 mg N l^{-1} treatment. Also, there was a noticeable difference in coloration among plants of the 3 nitrogen treatments by Day 15; plants cultured in 0.2 and 1.2 mg N l^{-1} media showed a loss of pigmentation relative to preassay plants while those of the 5.2 mg N l^{-1} media did not.

Significant differences (ANOVA, $p < 0.001$) in total phenolic content among plants of the 3 nutrient treatments were established by Day 15 (Fig. 1; Table 1). By Day 22 these differences had been disrupted, probably as a result of culture media renewal on Day 18. However, significant differences were reestablished by Day 29. *Lobophora variegata* cultured in relatively nitrogen-poor conditions consistently possessed higher total phenolic contents than did plants grown in nitrogen-enriched conditions (Table 1).

Specimen C:N ratio tended to reflect culture media nitrogen availability (Table 2) throughout the 4 wk. Plants of relatively nitrogen-rich media consistently exhibited lower C:N ratios than did plants of nitrogen-poor media. Total phenolic content of *Lobophora variegata* was inversely related to culture media nitrogen availability and, as a result, to the internal

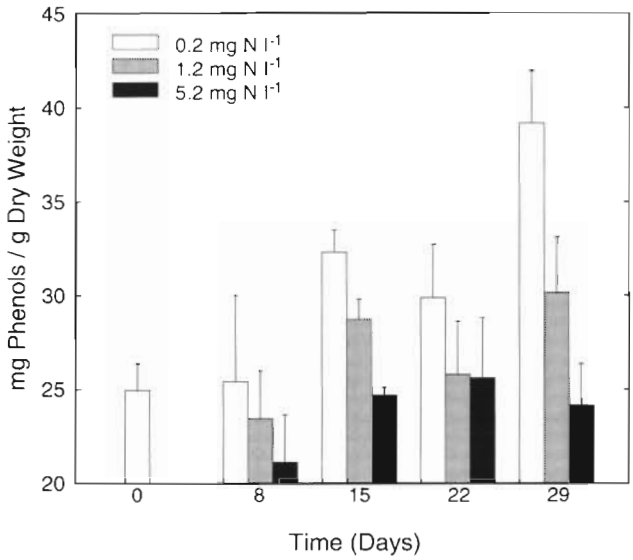

Fig. 1. *Lobophora variegata*. Phenolic content (mg phenol g^{-1} tissue) of algal isolates from the 3 nutrient treatments as a function of time (d). Day 0 represents the phenolic content of pre-treatment plants. Culture media were renewed on Day 18. Error bars are ± SE (n = 5)

C:N ratio (Fig. 2a). Overall, mean plant percent nitrogen ranged from 0.13 to 3.02% and was significantly correlated with total phenolic content (Fig. 2b; Table 2). On the other hand, there was no relationship between total phenolic content and plant percent carbon (Fig. 2c).

Table 1. *Lobophora variegata*. Statistical analysis (1-way ANOVA) of observed differences in phenolic content (mg phenol g^{-1} tissue) among plants of the 3 nutrient treatments over the 4 wk culture period

Time interval	Treatment (mg N l^{-1})	N	Phenolic content (mg^{-1}) Mean	SE	p
Day 8	0.2	5	25.396	4.59	0.675
Day 8	1.2	5	23.436	2.55	
Day 8	5.2	5	21.108	2.53	
Day 15	0.2	5	32.314	1.20	<0.001[a]
Day 15	1.2	5	28.756	1.08	
Day 15	5.2	5	24.668	0.43	
Day 22	0.2	5	29.878	2.84	0.533
Day 22	1.2	5	25.794	2.84	
Day 22	5.2	5	25.594	3.21	
Day 29	0.2	5	39.214	2.76	0.006[b]
Day 29	1.2	5	30.144	2.97	
Day 29	5.2	5	24.144	2.21	

[a]Pairwise multiple comparisons (Student-Newman-Keuls method) indicate that phenolic contents of plants of all 3 treatments are statistically different ($p < 0.05$)

[b]Phenolic contents of plants of the 0.2 vs 1.2 mg N l^{-1} and 0.2 vs 5.2 mg N l^{-1} treatments are significantly different ($p < 0.05$); plants of the 1.2 vs 5.2 mg N l^{-1} treatments are not ($p > 0.05$)

DISCUSSION

Our data indicate that the phenolic content of *Lobophora variegata* varies inversely as a function of nitrogen availability and, therefore, support the carbon/nutrient balance hypothesis as presented by Bryant et al. (1983) for terrestrial systems and applied to marine systems by Ilvessalo & Tuomi (1989) and Yates & Peckol (1993). Our data also suggest that intraspecific variation in brown algal polyphenolic content caused by rearrangement of the resource budget may be sufficient in magnitude to influence plant fitness, at least for *L. variegata*.

The significant correlation that developed between culture media nitrate levels and plant percent nitrogen by Day 7 is indicative of nutrient availability and uptake. Renewal of the culture media on Day 18 caused an observable interference in plant percent nitrogen, underscoring the sensitivity of nitrogen uptake. As expected, plants cultured in 5.2 mg N l^{-1} media exhibited the highest percent nitrogen contents during all 4 wk, followed by plants of the 1.2 mg N l^{-1} and 0.2 mg N l^{-1} treatments, respectively. The emergence of hyaline hairs on Day 6 is consistent with studies that implicate these sterile filaments in increased nutrient absorption. In fact, DeBoer & Whoriskey (1983) observed that ambient ammonium concentrations below 0.5 µM stimulated the growth of hyaline filaments in *Ceramium rubrum* while concentrations above 20 µM inhibited growth, and they proposed that such a response may allow plants to take advantage of intermittent bursts of nutrients by increasing surface area (see also O'Connor & West 1991, Hurd et al. 1993). Although precise quantification was impractical, it was evident that plants cultured in 0.2 mg N l^{-1} media possessed a more extensive and dense growth of hairs than did plants of the 5.2 mg N l^{-1} treatment. This suggests that increments of nitrogen availability can influence the density of hyaline hairs.

As predicted by the carbon/nutrient balance hypothesis, the total phenolic content of *Lobophora variegata* was inversely correlated with the internal C:N ratio. Like Ilvessalo & Tuomi (1989) and Peckol & Yates (1993) we observed that, while the phenolic content of brown algae was also correlated with plant percent nitrogen, it was not correlated with variations in plant percent carbon. Several studies of vascular plant tannin contents indicate that an increased carbon content, as supplied by either glucose addition

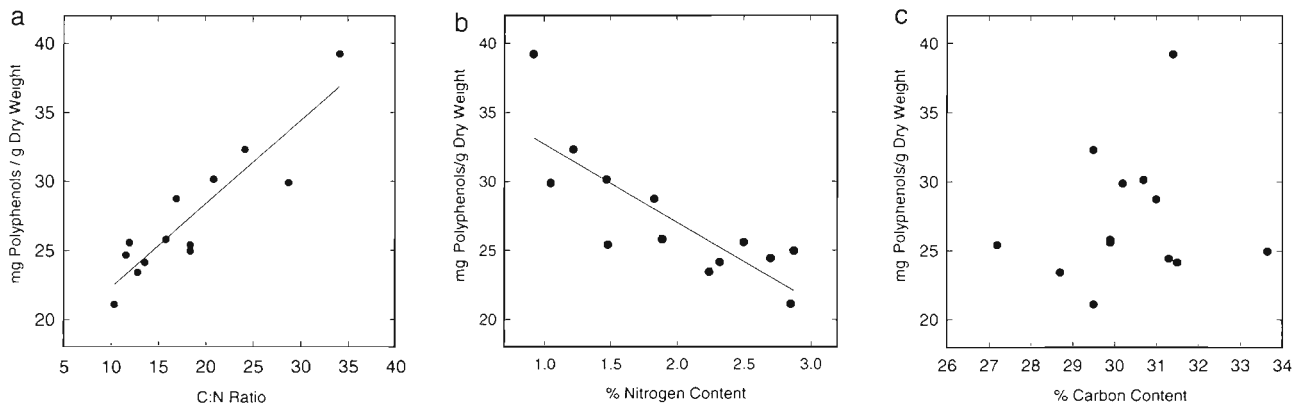

Fig. 2. *Lobophora variegata*. Culture media nitrogen concentrations affect the internal nutrient balance and the total phenolic content of algal isolates. (a) Phenolic content of *L. variegata* was directly correlated with internal C:N ratio ($p < 0.001$). Specifically, plant phenolic content was correlated with internal nitrogen content (b) but not internal carbon content (c). Points represent the mean of 5 plants and are grouped irrespective of sampling time. Linear regressions (solid line) and 95% confidence ranges (dotted line) are provided where applicable

(Kubek & Shuler 1980) or atmospheric CO_2 enrichment (Fajer et al. 1992), is often insufficient to cause an increase in carbon-based allelochemicals. Renewal of culture media seems to have affected plant percent nitrogen and total phenolic contents as determined 96 h later. These data are consistent with a nitrogen-limited system where carbon is in relative excess and suggest that under such conditions, which are common in tropical marine habitats, relatively slight variations in nitrogen availability can cause significant changes in plant phenolic content. We observed significant variation in the total phenolic content of *L. variegata* within a 2 wk period. Interestingly, this time period is similar to that in which Van Alstyne (1988) observed an induced increase in *Fucus distichus* phenolic content due to simulated herbivore damage (but see Steinberg 1994). Other examinations of resource-dependent variations in brown algal phenolic content (Ilvessalo & Tuomi 1989, Yates & Peckol 1993) have focused upon seasonal and monthly variations within field plants.

Previous attempts to manipulate brown algal phenolic content in culture have been hindered by the phenomenon that cultured plants seldom retain significant levels of polyphenols (Ragan & Glombitza 1986). The phenolic content of cultured *Lobophora variegata* isolates reported here is considerably lower than that of field plants from Belize (Targett et al. 1992), although phenolic contents of all plants remained above 20 mg phenol g^{-1}, which has been considered by some to be the general lower limit for deterring herbivory. This may be a direct result of the high nutrient levels of commonly employed culture media or of artificially low irradiances, which would affect the internal resource balance. Lapointe et al. (1987) reported substantially

Table 2. *Lobophora variegata*. Summary of correlations (Pearson product-moment correlation) between phenolic content, C:N ratio, and carbon and nitrogen availability. Pairs with negative coefficients and p below 0.05 have an inverse relationship. Those with positive coefficients and p below 0.05 tend to increase together

		C:N ratio	% carbon	% nitrogen	Treatment N
Phenolic content	Coefficient	0.919	0.169	−0.817	−0.556
	p	<0.001	0.581	<0.001	0.048
	N	13	13	13	13
C:N ratio	Coefficient		−0.029	−0.928	−0.623
	p		0.925	<0.001	0.023
	N		13	13	13
% carbon	Coefficient			0.283	0.074
	p			0.348	0.811
	N			13	13
% nitrogen	Coefficient				0.682
	p				0.010
	N				13

lower nitrogen levels ($NH_4^+ + NO_3^- = 0.005$ to 0.008 mg N l^{-1}) at the site from which *L. variegata* was initially isolated for this study. Clearly, culture conditions can greatly affect the phenolic content of brown algae. Our data also suggest that *in situ* variations in nitrogen availability can cause significant changes in phenolic content in a relatively short period of time. It is unclear whether the variations in plant phenolic content which we observed are of sufficient magnitude to imply ecological significance. However, we observed no leveling off in the trend of increasing phenolic contents, especially for plants of nitrogen-poor media. Therefore, it is possible that plant phenolic contents may continue to increase and may reach concentrations which have been demonstrated to have ecological consequences (Sieburth & Conover 1965, Steinberg 1985, 1988, 1989, Johnson & Mann 1986) under such conditions. Such variation in the allelochemical content of the decumbent form of *L. variegata* in relation to nutrient availability may help to explain its predominance in low-nutrient, high-herbivory habitats (Coen & Tanner 1989).

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