Bacterial Leaf Nodule Symbiosis in *Ardisia* (Myrsinaceae): Does it Contribute to Seedling Growth Capacity?

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Abstract: **Numerous species of *Ardisia* (shrubs in the Myrsinaceae) possess conspicuous bacterial nodules in their leaf margins. This is an obligate, life-cycle symbiosis: the bacteria are maintained in the bud, and re-infect each new leaf primordium, as well as flowers and seeds, and are transmitted vertically to the next generation. Previous studies have shown that treatments which kill the bacteria in the buds lead to death of the plant. This study is the first to test for a net cost or benefit of the nodules in seedling growth capacity. A net benefit of the symbiosis would be expected from the elaborate nodule structure, and also from evolutionary theory. Seedlings of two symbiotic species *(A. crenata* and *A. virens)* and two non-symbiotic species *(A. elliptica* and *A. sieboldii)* were grown comparatively. For the symbiotic species, performance was assessed for intact plants, for plants with nodules clipped off, and for control plants in which the lamina was clipped between the nodules. The nodules did not contribute to, or detract from, seedling performance in high resource supply. Although plants increased *ca.* 4- to 6-fold in dry mass, nodule removal had no significant impact on plant growth, gas exchange, biomass allocation, or on foliar concentrations of chlorophyll or of 11 nutrients. No significant advantage was observed for the two symbiotic species over the two non-symbiotic species. The nodules might contribute to growth capacity during other life stages, during resource shortage, or during exposure to specific herbivores or pathogens.**

Key words: **Endophyte, growth analysis, mutualism, photosynthetic rate, stomatal conductance, vertical transmission.**

Introduction

Symbiosis, the intimate and protracted association between two or more different species, has a central role in biological systems (Bronstein, 2001). Symbioses are generally viewed as "biological markets," in which organisms trade substances or services (Noë and Hammerstein, 1994), though costs and benefits may be distributed asymmetrically (Frank, 1997; Faeth, 2002; Saikkonen et al., 2004).

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Leaf bacterial nodules are an exceptional though still relatively obscure form of symbiosis. The symbiosis exists in about 400 species within the Dioscoreaceae, Myrsinaceae, and Rubiaceae (Lersten and Horner, 1976; Miller, 1990). In symbiotic *Ardisia* species (Myrsinaceae) there are *ca.* 10 – 35 ellipsoid nodules per leaf, up to 500 µm in cross-section, within marginal crenations (Fig. **1**; Miller, 1990; Lebard and Belin-Depoux, 2003). Leaf nodulation in *Ardisia* is an obligate, whole life-cycle symbiosis. Bacterial colonies in the shoot buds infect each developing leaf through specialized marginal pores, and infect reproductive parts and embryos, thus transferring to the next generation in the seed (Miller, 1990; Lebard and Belin-Depoux, 2003). The bacteria have often been isolated, though their classification remains contentious (Miller, 1990; Lebard and Belin-Depoux, 2003). Killing the bacteria causes the apical bud to degenerate to callus, and the plant dies if it does not recover the bacteria (reviewed in Miller, 1990; see Discussion). Some have proposed that the bacteria produce cytokinin (Miller, 1990). Several tests have found no N-fixing capacity in either Myrsinaceous plants or in isolated nodule bacteria (reviewed in Miller, 1990).

Additionally, some have hypothesized that the nodules stimulate plant growth (Miehe, 1919; Miller, 1990). Such a benefit for the plant would be consistent with its notable investment in nodule structure, replete with transfer surfaces, and extensive vascularization (Miller et al., 1983; Miller, 1990). Evolutionary theory also predicts that a vertically transmitted symbiont would provide a benefit, as its fitness is linked with that of the host (Faeth, 2002). This is the first study to investigate a role of leaf bacterial nodules in plant growth capacity. Seedling growth was investigated because it is a critical stage in plant establishment (Grubb, 1977), when benefits conferred by a symbiont are likely to be effective (Faeth, 2002). Symbiotic and non-symbiotic *Ardisia* species were grown comparatively, and treatments applied to determine the effects of nodule removal.

Materials and Methods

Species, seedling source, and greenhouse

Studies were made of four *Ardisia* species – two with leaf bacterial nodules *(A. crenata* and *A. virens)* and two without (*A. elliptica* and *A. sieboldii;* nomenclature follows Zheng-yi and Raven, 1994). These species are evergreen shrubs, native to for-

Fig. 1 A seedling leaf of *Ardisia crenata,* 15 cm^2 in area, with bacterial leaf nodule circled.

ests of Asia and grown ornamentally; they have attracted special concern due to being both shade-tolerant and invasive in Hawai'i and the southern USA (Wagner et al., 1999; Bray et al., 2003). On 23 Jan. to 4 Feb. 2004, naturally occurring seedlings < 1 y old on the grounds of the University of Hawai'i's Lyon Arboretum were carefully excavated and transplanted to 1-L pots containing potting soil (Big R, Cascade Forest Products, Carson, California, USA) in a greenhouse. The greenhouse microclimate was assessed on five days for three points across the area occupied by seedlings, by making spot measurements between 1230 and 1500 h (using a LI-COR 1600 porometer; LI-COR, Lincoln, Nebraska). Mean values ± SE for photosynthetically active irradiance, temperature, and relative humidity were 260 ± 46 µmol m^{-2} s^{-1}, 26.8 ± 0.3 °C, and 86.5 ± 1.8 %.

Experimental design and treatments

The growth experiment ran from initial harvest on 24 May to final harvest on 28 July 2004. All plants were fertilized at the time of initial harvest with a slow release N, P, K fertilizer (Osmocote 19-6-12, Scotts-Sierra Horticultural Products Co., Marysville, Ohio, USA; 4 ml per pot) so that the impacts of nodules on growth in high soil resource supply could be assessed. All pots were watered to field capacity each second day for the duration of the experiment.

On the first day of the experiment, seedlings of *A. crenata* and *A. virens* were randomly allocated to three treatment groups. In the first treatment, bacterial nodules were clipped off each leaf, using a 3-mm diameter hole puncher, leaving a half disk of missing marginal lamina where the nodule had been. In the second treatment, a "clipping control", the leaf margin was clipped *between* the nodules. In the third treatment, plants were left intact. In the clipping treatments, typically 2 – 5 % of leaf area was removed. Along with intact plants of non-symbiotic species *A. elliptica* and *A. sieboldii,* the plants were randomly sorted into blocks of one plant per species per treatment, across the greenhouse. The clipping treatments were re-applied to each new leaf, once unrolled. Midway through the experiment, the location of plants within blocks was re-randomized. After initial harvest, there were 5 – 8 plants per species per treatment.

Harvest measurements

Initial and final harvests were made respectively on 24 – 27 May and 26 – 28 July 2004. At initial harvest, six individuals of each species were randomly selected, and at final harvest all plants were harvested. Measurements were made of total leaf chlorophyll per area (using a Minolta SPAD meter; Spectrum Technologies Inc., Plainfield, Illinois, USA; in SPAD units, which correlate with extractable chlorophyll a + b per leaf area; Marquard and Tipton, 1987), shoot height, and leaf area (using Epson 3170 scanner, Epson America, Long Beach, California, USA). Roots, stems (including petioles), and leaf blades were separated and placed in an oven at > 70 °C for at least 72 h before dry mass determination. Biomass allocation variables were calculated for the final harvest; specific leaf area (SLA) as lamina area/lamina dry mass; leaf area ratio (LAR) as lamina area/plant dry mass; leaf and root mass fraction (LMF and RMF) as, respectively, leaf and root dry mass/plant dry mass. For the growth interval, relative growth rate (RGR) was determined as (ln [final mass] – ln [initial mass])/growth time, LAR as the mean of values for the harvests, and unit leaf rate (ULR) as the quotient of RGR and LAR for the interval (Hoffmann and Poorter, 2002).

Foliar nutrient concentrations were determined for four subsamples of mature leaves from 1 – 2 plants of each species and treatment. Samples were analyzed for total N (LECO CN2000; LECO Corp., St. Joseph, MI, USA), and for B, Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn (by plasma emission spectroscopy; Isaac and Johnson, 1985).

Gas exchange measurements

On 23 July 2004, stomatal conductance *(g)* and light-saturated net photosynthetic rate (A_{max}) were measured for young mature leaves of all individuals (LI-COR 6400 Photosynthesis System; LI-COR, Lincoln, Nebraska). Cuvette irradiance was set at 1200 µmol m^{-2} s^{-1}, and CO_2 reference concentration at 400 µmol mol^{-1}. Relative humidity ranged from 57 – 66 %, and cuvette temperature from 27 – 33 °C. Plants were measured block by block, thus alternating plants from each treatment.

Statistical analyses

Tests were first made of the impact of the treatments applied to the symbiotic species. Two-way ANOVAs were applied to data for final mass, height, and chlorophyll per area, testing for species and treatment effects and their interaction. Two-way MANOVAs were applied to data for biomass allocation variables together (RMF, LMF, SLA, and LAR), to data for *g* and A_{max} together, and to data for foliar concentrations of the 11 elements together (Zar, 1999). Testing these variables singly using two-way ANOVAs gave the same results. Tests were also made for differences among the four species. One-way MANOVAs and one-way ANOVAs were used for variables as above. All analyses were performed with Minitab Release 14.

Results

Plants of the four species grew substantially, increasing 3.6- to 6.1-fold in dry mass, and 1.5- to 1.9-fold in height (Fig. **2**). The nodules did not contribute a measurable cost or benefit to seedling growth. For symbiotic species *A. crenata* and *A. virens*

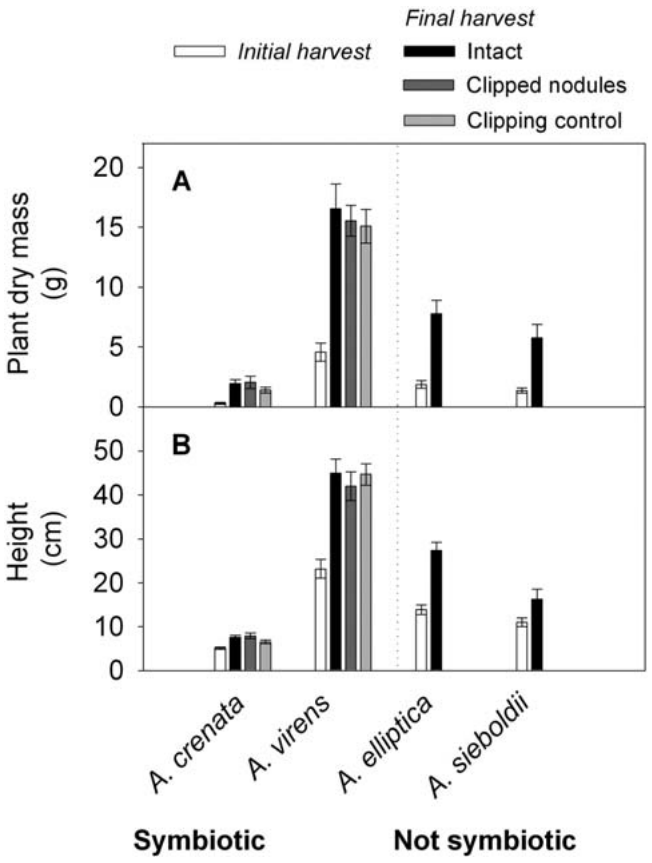

Fig. 2 Mean values ± SE for plant dry mass (**A**) and shoot height (**B**) for two symbiotic and two non-symbiotic *Ardisia* species, with applied treatments, at initial and final harvest (n = 5 – 8).

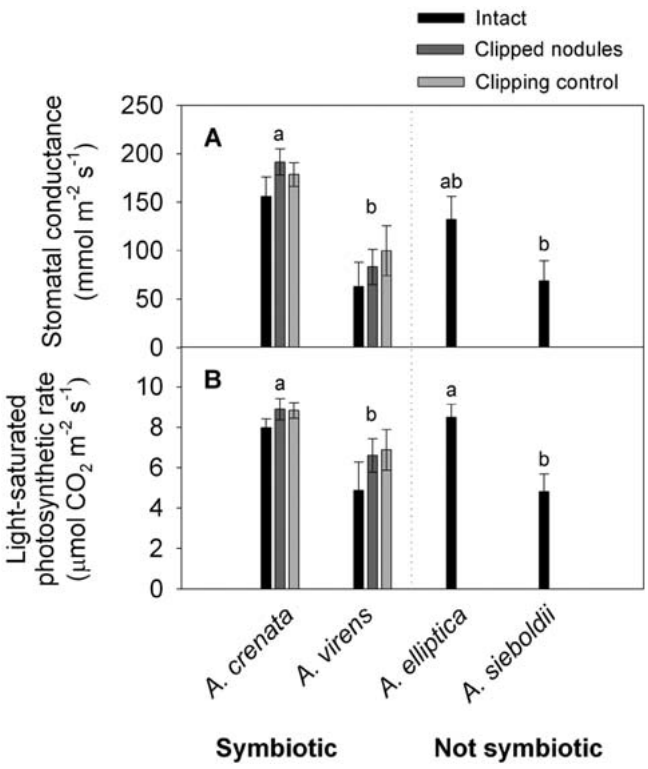

Fig. 3 Mean values ± SE for stomatal conductance (**A**) and light-saturated net photosynthetic rate (**B**) for two symbiotic and two non-symbiotic *Ardisia* species, with applied treatments (n = 5 – 8). Different letters indicate differences *among species* ($p < 0.05$, Tukey tests).

the removal of nodules, or of leaf margin between the secondary veins, did not impact significantly on final height or dry mass (Figs. **2 A, B**; $p > 0.69$ for treatment in two-way ANOVAs; $p < 0.001$ for species differences; $p > 0.49$ for the interaction). Similarly, leaf nodules had no significant impact on A_{max} and g ($p > 0.26$ for treatment in two-way MANOVA; $p < 0.001$ for species; $p > 0.97$ for the interaction; Figs. **3 A, B**), biomass allocation ($p > 0.92$ for treatment effects in two-way MANOVA; $p < 0.001$ for species; $p > 0.3$ for the interaction; Table **1**), leaf nutrient concentrations ($p > 0.47$ for treatment differences in two-way MANOVA, $p < 0.001$ for species; $p > 0.90$ for the interaction; Table **1** and Fig. **4**) or for total chlorophyll per area ($p = 0.32$ for treatment differences in two-way ANOVA; $p < 0.001$ for species differences; $p = 0.44$ for the species-treatment interaction; Table **1**).

The four *Ardisia* species differed significantly in growth, in A_{max} and g, and in biomass allocation variables RMF, LMF, SLA, and LAR ($p < 0.001$ in one-way MANOVAs; $p < 0.001$ in one-way ANOVAs for each variable alone; for the symbiotic species, treatment data were combined; Table **1** and Figs. **2, 3**). Species also differed in foliar nutrient concentrations ($p < 0.001$; one-way MANOVA), due to differences in N, P, K, and in B, Ca, Fe, Mg, Mn, Na, and Zn (p values ranged from < 0.001 to < 0.05; one-way ANOVAs), but not in Cu ($p = 0.15$; one-way ANOVA; Table **1** and Fig. **4**).

Of all variables considered, including growth, gas exchange, biomass allocation, and foliar chlorophyll and nutrient concentrations, no advantage was found for the two symbiotic species over the two non-symbiotic species. The only systematic difference was a lower foliar Mg concentration in the two symbiotic species (Table **1** and Figs. **2 – 5**). Across the four species, LAR was a stronger determinant of RGR than unit leaf rate (ULR). The symbiotic species had both the highest and lowest RGRs (Figs. **5 A, B**).

Discussion

Bacterial leaf nodules had no clear cost or benefit in *Ardisia* seedling growth capacity. The symbiotic species performed equally, whether intact, or with their nodules clipped, or with clipped margins. The lack of a detrimental effect of clipping *per se,* relative to the control plants left intact, would have been due to the small proportion of lamina removed, and an effective wounding response (Bloch, 1952; Davis et al., 1991). Additionally, the two symbiotic species had no advantage over the two non-symbiotic species in growth, gas exchange, or foliar chlorophyll or nutrient concentrations. A more comprehensive sampling would be necessary to generalize for the whole genus (*ca.* 30 out of *ca.* 250 spp. are symbiotic; Lersten and Horner, 1976; Miller, 1990). However, the fact that the nodulated species possessed both the highest and lowest RGR indicates no strong inherent contribution of nodules to species differences. Species differences in RGR were related to leaf area ratio, as often found for sets of species of young woody seedlings (e.g., Kitajima, 1994; Sack, 2004).

Table 1 Mean values ± SE for biomass allocation variables and for foliar concentrations of nutrients and chlorophyll for four species of *Ardisia* at final harvest. Abbreviations RMF, LMF, SLA, LAR, and Chl/A represent respectively root and leaf mass fraction, specific leaf area, leaf area ratio, and total foliar chlorophyll concentration per area. For the symbiotic species, data are presented for intact plants, for plants that had nodules clipped from the leaves throughout the experiment ("Clipped nodules"), and for plants that had margins clipped between the nodules ("Clipping control"). For symbiotic species, no significant differences were found among treatments. Different letters indicate significant differences *among species* for a given variable ($p < 0.05$, Tukey tests). For N, P, and K concentrations, n = 4 per treatment. For other indices, n = 5 – 8 per treatment

Species	RMF ($g\ g^{-1}$)	LMF ($g\ g^{-1}$)	SLA ($cm^2\ g^{-1}$)	LAR ($cm^2\ g^{-1}$)	N ($mg\ g^{-1}$)	P ($mg\ g^{-1}$)	K ($mg\ g^{-1}$)	Chl/A (SPAD)
Symbiotic species								
A. crenata								
Intact	0.299 ± 0.013 *a*	0.602 ± 0.020 *a*	211 ± 5.7 *a*	127 ± 6.2 *a*	22.7 ± 0.027 *a*	4.75 ± 0.194 *a*	37.3 ± 1.18 *a*	50.2 ± 2.4 *a*
Clipped nodules	0.280 ± 0.009	0.617 ± 0.010	223 ± 9.5	138 ± 7.9	21.7 ± 0.102	5.90 ± 1.153	36.4 ± 1.81	49.2 ± 1.7
Clipping control	0.293 ± 0.008	0.613 ± 0.005	228 ± 8.1	140 ± 5.2	22.2 ± 0.037	4.13 ± 0.755	37.3 ± 1.40	51.0 ± 1.5
A. virens								
Intact	0.157 ± 0.008 *b*	0.486 ± 0.017 *b*	211 ± 11.6 *a*	103 ± 8.1 *b*	24.3 ± 0.312 *b*	2.03 ± 0.197 *b*	24.0 ± 7.11 *b*	56.7 ± 1.2 *b*
Clipped nodules	0.180 ± 0.011	0.473 ± 0.015	216 ± 6.5	103 ± 6.3	25.2 ± 0.213	2.20 ± 0.168	24.3 ± 4.91	60.0 ± 1.6
Clipping control	0.154 ± 0.006	0.495 ± 0.010	217 ± 9.2	103 ± 6.2	28.2 ± 0.156	2.33 ± 0.165	26.8 ± 2.40	61.2 ± 1.8
Non-symbiotic species								
A. elliptica	0.241 ± 0.016 *c*	0.495 ± 0.029 *b*	216 ± 7.6 *a*	108 ± 9.4 *b*	22.1 ± 0.109 *ab*	1.93 ± 0.111 *b*	51.1 ± 4.24 *c*	54.9 ± 2.3 *ab*
A. sieboldii	0.296 ± 0.018 *a*	0.513 ± 0.028 *b*	166 ± 7.0 *b*	85 ± 5.6 *b*	17.5 ± 0.043 *a*	1.33 ± 0.048 *b*	36.3 ± 1.33 *a*	59.4 ± 3.4 *b*

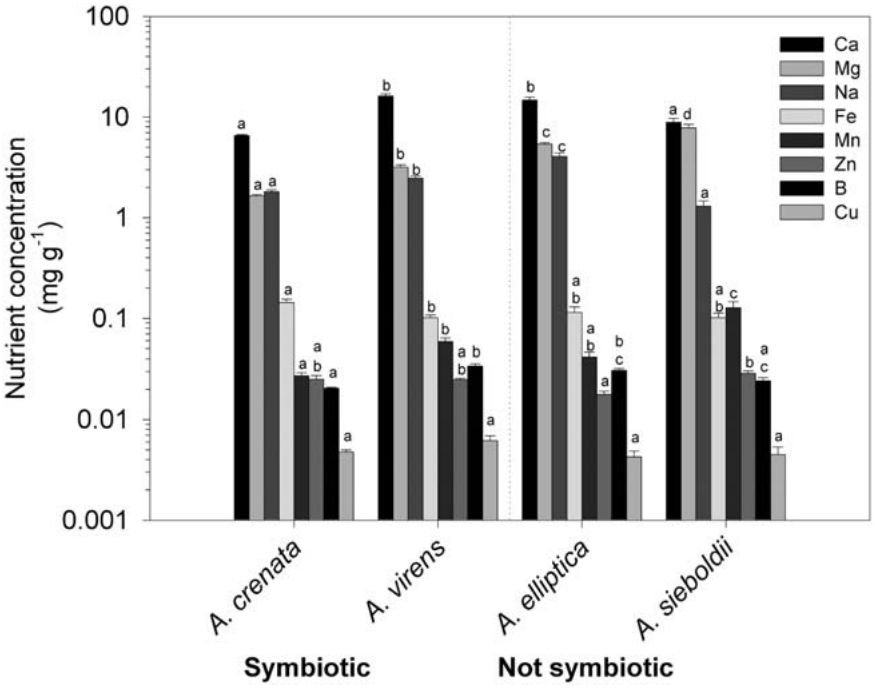

Fig. 4 Mean values ± SE for foliar nutrient concentrations for two symbiotic and two non-symbiotic *Ardisia* species. Different letters indicate differences *among species* ($p < 0.05$, Tukey tests). For non-symbiotic species, n = 4 for each nutrient concentration; for symbiotic species, treatment data were combined, and n = 12 for each nutrient concentration.

Our expectation was that the bacteria would have contributed a growth benefit for the symbiotic species. In *Ardisia,* living bacteria in the mature nodule are developed into pleomorphic forms, surrounded by a vascularized nodule apparently specialized for transfer of metabolites between bacteria and plant (Miller et al., 1983; Miller, 1990). Previous work indicated that symbiotic species require bacteria for a viable apical meristem and for seedling growth (Yamada, 1953, 1954, 1955; Hofstra and Koch-Bosma, 1970; Lersten and Horner, 1976; Miller, 1990). For instance, when the bacteria were killed by treating the apical bud of a newly germinated seedling with antibiotics for four weeks, the bud degenerated to callus (Yamada, 1955).

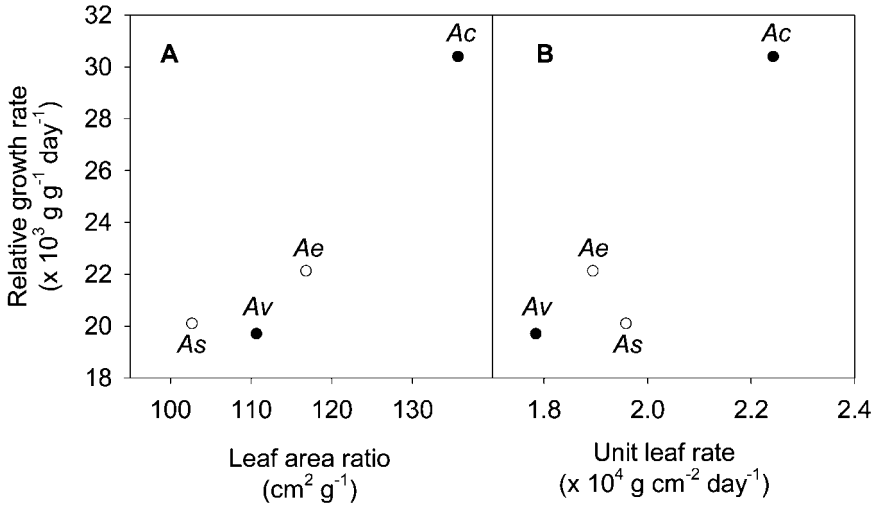

Fig. 5 Mean values for dry mass relative growth rate (RGR) plotted against leaf area ratio (**A**) and unit leaf rate (**B**) for the growth interval. Filled circles represent symbiotic species (Ac = *Ardisia crenata;* Av = *A. virens*); open circles represent non-symbiotic species (Ae = *A. elliptica;* As = *A. sieboldii*).

The so-called "cripples" survive up to three years without new leaf production before dying (Miller, 1990). Cripples have also been produced by immersing seeds in antibiotics for 3 – 7 days, or by heating seeds at 45 – 50 °C for 5 to 60 min; seedlings that emerged produced only a few leaves before the apical bud degenerated to callus (Yamada, 1953, 1954; Miller, 1990). By contrast, in this study, clipping the nodules from mature seedling leaves had no detrimental effect. One explanation is that the bacterial symbiosis might not be as important to the host as indicated previously, but rather the heat and antibiotic treatments produced other detrimental effects. However, those same treatments reportedly had no negative effect on at least one non-symbiotic *Ardisia* species (Yamada, 1955). From evolutionary theory, one would also expect an essential role for the nodules. An obligate, vertically transmitted symbiont would be expected to contribute to the performance of its host, as it subsists on the host's resource (Frank, 1997; Saikkonen et al., 2004). If a vertically transmitted symbiont were selectively neutral, then it would disappear from a plant population, being lost due to (a) random loss of viability due to mutations, and (b) occasional defective transmission to seed (Faeth, 2002).

Our findings allow us to propose three remaining alternative hypotheses for a critical role of the bacteria. First, the clipping treatments may have had no effect because sufficient bacteria to fulfill the essential function were present outside of the elaborate nodules, e.g., in the stem apex or the developing leaves or elsewhere in the plant. Second, the nodules in mature leaves might confer no benefit to seedling growth, but instead the nodules might play a beneficial role during another stage, e.g., early after germination, or during leaf development. In that case, a benefit that is limited to one or several specific life stages is sufficient for preservation of the symbiosis through the entire life cycle. Such a pattern would be analogous to grass-fungus symbioses in which costs and benefits to the plant can occur in separate life stages, punctuated by harmonious periods (Saikkonen et al., 2004). A third hypothesis is that the symbiosis is not important under high resource supply as in this study, but rather under resource shortage, or when plants are exposed to herbivores or pathogens. That is the case for certain fungal endophytes (Faeth, 2002; Saikkonen et al., 2004). We note that even under any of these scenarios, the bacteria might not stimulate net plant growth substantially. Some have suggested that the plant relies on the bacteria only for a minimum essential requirement for baseline function, such as a vitamin or plant growth substance (Miller, 1990). In that case, the symbiosis might never even have provided an ultimate benefit, but rather the plant may have evolved dependence on the bacteria, and is now effectively held as a slave (De Mazancourt et al., 2005). That situation would contrast with most described obligate microbe symbioses, in which the microbe becomes domesticated, and provides net benefit to the host (Frank, 1997; Saikkonen et al., 2004).

Thus, further work is required to test for a role of the bacteria during resource shortage, and during other life stages. The symbiosis in *Ardisia* may also be a valuable model for molecular evolutionary biology, as a case of vertically transmitted symbiosis in a long-lived plant (cf. Frank, 1997; Saikkonen et al., 2004). In *Ardisia,* expectations from models may be tested, e.g., whether the plant will select bacteria to be genetically uniform, optimally producing the substance(s) that confer a benefit or simply fulfill an essential requirement (Frank, 1997). In turn, the bacteria would be expected to exert selection on the host, restricting possible variation to that which will not disturb the symbiosis. In this context, the diversification of symbiotic *Ardisia* species and their bacteria merits investigation. The cyclic symbiosis in *Ardisia* makes it a model system for investigation at an exceptionally wide range of scales – for transfer of materials between plant and bacteria, for the interactive development and ecophysiology of plant-bacteria symbiosis, and for testing evolutionary theory.

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