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Host plant cyanotype determines degree of rhizobial symbiosis

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Abstract. Plants with nitrogen-fixing bacteria, such as legumes with rhizobia, can tap the atmospheric nitrogen pool to obtain resources for defense compounds. Cyanogenesis, a nitrogen-based plant defense against herbivores, increases in response to rhizobial colonization, but depends on plant genotype. Here, we tested whether genotypic differences in host plant cyanogenesis influence symbiotic reliance on nitrogen-fixing rhizobia. Using thin, clear soil containers, we counted nodules on live root systems of distinct high (HC) and low (LC) lima bean (*Phaseolus lunatus*) cyanotypes across the duration of an eight-week study. We measured changes in cyanogenic potential (HCNp) and protein content to reveal quantitative interactions between nodule number and both leaf traits. High cyanogenic plants maintained consistently twice as many nodules as LC plants. Including both cyanotypes, nodule number correlated positively with HCNp, but negatively with foliar protein content. However, within-cyanotype interactions between nodule number and plant traits were not significant except for foliar protein in HC plants, which decreased with increasing nodule number. Our results imply that while genotypes with higher levels of nitrogen-based defense invest more in the rhizobial partner, the costs involved in maintaining the symbiosis may cause resource allocation constraints in the plants' primary nitrogen metabolism.

Key words: cyanogenesis; genotype; lima bean; mutualism; nitrogen fixation; *Phaseolus lunatus;* plant defense; rhizobia; symbiosis.

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INTRODUCTION

Plant defenses emerged from a long coevolutionary history with herbivores and pathogens (Ehrlich and Raven 1964), but also by coevolving alongside beneficial symbiotic partners that influence plant–herbivore interactions (Weber and Agrawal 2014). Mutualists that aid plants in defense include predators, but also nutrient-provisioning microbes that enhance the availability of certain resources for plant growth and defense (Herms and Mattson 1992, Stamp 2003, Thamer et al. 2011). In particular, under widespread nitrogen-limited terrestrial conditions (Vitousek et al. 2002), microbes facilitating resource acquisition can mediate plant–insect interactions (Pineda et al. 2010, Thamer et al. 2011). Symbioses between plants and nitrogen-fixing microbes range from loose associations with rhizobacteria in the soil (Dean et al. 2009, 2014, Pineda et al. 2010, Algar et al. 2014, Pangesti et al. 2015) to highly regulated interactions within root nodules. Nodule-based endosymbiosis involves substantial resource exchange and has evolved in multiple taxa of plants and microbes (Vessey et al. 2004, Kempel et al. 2009). Receiving symbiotic nitrogen in exchange for photoassimilates can enable plants to increase growth and defense traits simultaneously, even for relatively costly nitrogen-based defenses (Thamer et al. 2011, Ballhorn et al. 2017). Access to nitrogen is a significant advantage as producing such costly defense compounds can reduce plant–plant competitive ability and fitness (Herms and Mattson 1992, Marak et al. 2003).

Cyanogenesis, the release of toxic hydrogen cyanide from wounded cells, is an example of a costly nitrogen-based defense. In several species, including lima bean (*Phaseolus lunatus*), the total amount of cyanide-containing precursors in a given tissue (cyanogenic potential [HCNp]) is mostly constitutive and varies by genotype. Cyanogenic genotypes (cyanotypes) produce consistent levels of HCNp that range from high to low HCNp (Ballhorn et al. 2013, Kautz et al. 2014). High expression can incur both biochemical and ecological costs (Ballhorn et al. 2008, 2010, Kautz et al. 2017). Biochemical costs include producing cyanide-containing compounds (glucosides) from proteinogenic amino acids, as well as two enzymes, β-glucosidases and hydroxynitrile lyases. In various plant species, these enzymes work sequentially to efficiently release cyanide from these precursors (Frehner and Conn 1987, Kakes 1990, Poulton 1990, Vetter 2000, Gleadow and Møller 2014). Further resources are required to transport and store vacuolar cyanogenic glucosides, spatially separating them from apoplastic β-glucosidases to prevent autotoxicity (Frehner and Conn 1987). Cyanogenesis is ecologically costly because free cyanide interferes with the function of metal-containing enzymes, including enzymes critically involved in resistance to pathogens (Ballhorn et al. 2010). Consequently, highly cyanogenic plants are generally well defended against herbivores (Ballhorn et al. 2005), but weakly defended against pathogens (Lieberei et al. 1989, Ballhorn et al. 2008, 2010). Taken together, the associated ecological consequences and biochemical mechanism involved in releasing cyanide make cyanogenesis a relatively costly plant defense. Differences in defense costs imply potential for high cyanogenic (HC) and low cyanogenic (LC) cyanotypes to have different nitrogen requirements. However, cyanotype-driven differences in symbiotic investment to obtain fixed nitrogen have not been previously explored.

While rhizobia may alleviate nitrogen-related costs of defense, maintaining this beneficial relationship introduces another set of physiological and ecological costs. Rhizobia can consume 16–30% of a plant's total photosynthate pool (Peoples et al. 1986, Kaschuk et al. 2009) and require plant-synthesized essential amino acids for their own metabolism and nitrogen fixation (Lodwig et al. 2003). The cost for plants to increase nodule numbers includes nodule biomass and metabolic demand to maintain nitrogenase activity, both of which demand carbohydrates. Carbohydrate demands serve as carbon sinks that stimulate photosynthetic rates (Kaschuk et al. 2009). Yet despite increased photosynthetic rates, carbon-rich traits such as indirect defense via extrafloral production and predator recruitment can be influenced by rhizobial carbon demands (Godschalx et al. 2015). Mutualistic carbon sinks play an important role in terrestrial carbon cycles (Pringle 2015), implying that increasing degree of rhizobia colonization to obtain nitrogen is not trivial.

Despite greater carbon sink and ecological costs, plant cyanotypes with constitutively high levels of cyanogenesis that require large inputs of nitrogen for defense could exert a pressure for plants to form more nodules. Here, we tested for quantitative differences in rhizobia colonization between HC and LC lima bean cyanotypes and differential responses in leaf chemical phenotype, including HCNp and soluble protein content to assess overall leaf quality. We collected all three metrics weekly—nodule number, HCNp, and protein content—over the course of a two-month study using a nondestructive method for nodule counting through clear, thin soil containers. If costs of direct chemical defense impose demand for stronger rhizobial association (measured as nodule number), we expected more nodules would form on roots of HC compared with LC plants. If leaf phenotype is directly influenced by nitrogen available from symbiosis, we would expect a positive correlation between nodule number and both nitrogen-containing traits. While nitrogen-fixing symbioses benefit plant productivity and provide a competitive edge in costly defense investment, understanding the factors facilitating or limiting the degree to which plants engage in this symbiotic exchange remains limited. Here, we used two different cyanotypes of the same plant species to test the impact of investment into nitrogen-based defense on the legume–rhizobia relationship.

Materials and Methods

Experimental setup

To determine rhizobia colonization differences between plant cyanotypes, we created two treatments by inoculating low and high cyanotypes

with rhizobia. We used lima bean (Fabaceae: *Phaseolus lunatus* L.) genotypes previously established as HC or LC cyanotypes based on consistent HCNp (Ballhorn et al. 2008). These accessions, HC_8078 and LC_8071, were provided by the Institute of Plant Genetics and Crop Plant Research in Gatersleben, Germany. Six seeds per cyanotype were germinated on moist paper towels. Once germinated, lima bean plants were individually planted 0.5 cm below the substrate surface level (greenhouse mix #3; SunGro Horticulture, Bellevue, Washington, USA) in soil containers that were custom-designed to facilitate rhizobia nodule counts on the intact root system. Soil containers (15 × 20 × 1.25 cm) were clear plastic containers wrapped in sheets of aluminum foil to block light, thus simulating belowground conditions. Plants were arranged randomly, and positions were rotated bi-weekly to account for potential position effects. Plants were watered daily with no additional nutrient solutions and cultivated under greenhouse conditions according to Ballhorn et al. (2014) at Portland State University (Portland, Oregon, USA) from March to April 2015.

Rhizobia inoculation

To identify rhizobia, several nodules were surface-sterilized, lysed, and plated to isolate colonies before the 16S gene was sequenced using 27f/1492r(I) primers. Using Geneious software, colonies were identified as *Bradyrhizobium elkanii* (Accession DJB1033-Ballhorn Lab; Portland State University). Inoculum was prepared by grinding 10 nodules, 0.5–5 mm in diameter, with a micropestle in a 1.5-mL centrifuge tube, and suspending the slurry in 600 mL H_2O. Both cyanotypes were inoculated with rhizobia once seedlings developed at least two true leaves by pouring 50 mL of rhizobia inoculum on the soil at the base of each seedling. Two weeks after inoculation, all plants showed root nodules.

Plant trait analysis

Nodulation was quantified as total nodule number per root system display. All five surfaces of the clear, thin soil containers were included in the root system display, including both sides, both narrow edges, and the narrow base, enabling most of the plant's root system to be included in the survey. Nodule assessments took place weekly for 8 weeks. The same collection schedule was followed for leaf trait determination in order to relate rhizobia nodule counts to the quantitative expression of chemical leaf traits. Cyanogenic potential was quantified using the Spectroquant cyanide test (Ballhorn et al. 2005). Briefly, leaves were removed, and three leaf punches from each individual leaf were weighed to the nearest 0.001 g, ground with a mortar and pestle at 4°C in 2 mL ice-cold Na_2HPO_4 buffer, and centrifuged. Samples were analyzed for HCNp through enzymatically hydrolyzing cyanogenic precursors in gas-tight glass Thunberg vessels and spectrophotometrically assaying released cyanide at 585 nm (Ballhorn et al. 2005, 2013). Foliar soluble protein was quantified from the same leaf extracts; using Bradford's reagent and a calibration curve from 50 μg/mg to 1000 μg/mg bovine serum albumin (Amresco, Solon, Ohio, USA), soluble protein was measured spectrophotometrically at 595 nm (Bradford 1976).

Statistical analysis

Weekly quantified nodulation, HCNp, and protein content were all analyzed with repeated-measures ANOVAs with cyanotype and time as factors. All within-cyanotype data met assumptions of ANOVA and were not transformed. Relationships among trait means in response to nodule number means were analyzed with a linear model to determine significant relationships and Pearson's coefficients. All analyses were conducted using the software R (version 3.0.2; R Core Team, 2016).

Results

Rhizobia colonization varied greatly by cyanotype in repeated nodule counts throughout an experimental period of 2 months. On average, HC plants formed 60% more nodules than LC plants ($F_{1,10} = 21.27$, $P < 0.001$; Fig. 1A). For any given sampling date, HC plants maintained consistently higher numbers of nodules ($F_{6,60} = 20.72$, $P < 0.001$), with this lead ranging from 56 to 143 more mean nodules than LC plants. Nodule numbers varied by a significant interaction between cyanotype and sampling date ($F_{6,60} = 2.64$, $P < 0.05$).

To test the effects of rhizobia colonization on aboveground plant traits, we measured HCNp

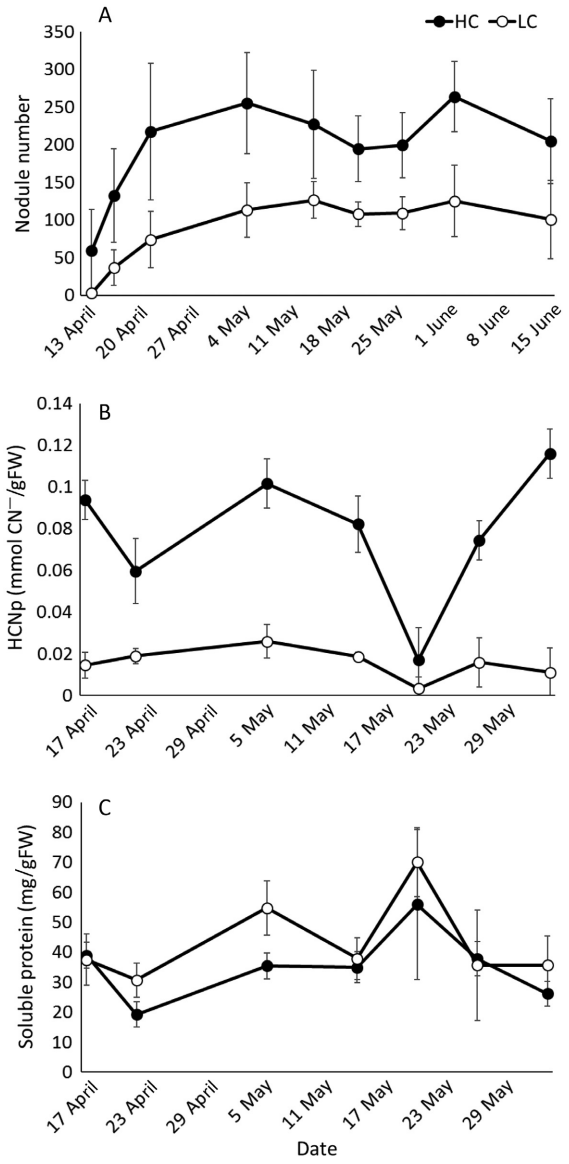

Fig. 1. Nodulation and leaf traits differences between cyanotypes across time. Low cyanogenic plants (LC; white circles) and high cyanogenic plants (HC; black circles) measured repeatedly across a two-month span to determine (A) extent of nodule colonization, (B) cyanogenic potential (HCNp), and (C) soluble protein content as a nutritive trait. Points show mean values, and bars represent standard deviation of the mean.

and soluble protein content in leaves of a defined developmental stage from HC and LC plants. HC leaves produced an average of 77% higher HCNp than LC leaves ($F_{1,10} = 329.4$, $P < 0.001$; Fig. 1B); however, HC leaves contained 23% less average protein than LC leaves ($F_{1,10} = 13.44$, $P < 0.05$; Fig. 1C). Sampling date significantly affected both HCNp ($F_{6,60} = 46.09$, $P < 0.001$) and soluble protein ($F_{6,60} = 15.461$, $P < 0.001$). Further, significant interaction effects between sampling date and cyanotype affected HCNp ($F_{6,60} = 29.06$, $P < 0.001$), but not protein content ($F_{6,60} = 1.739$, $P = 0.128$). Both cyanotypes were flowering on 17th May, eight weeks after being planted, which corresponded with varying expression of plant traits (Fig. 1).

To test for quantitative relationships among leaf chemistry in relation to nodule number across and within cyanotype, we regressed chemical trait averages for each plant replicate against nodule number averages for that same plant individual to test for significant correlations. We found a significant positive relationship between nodule number and HCNp when we included both cyanotypes ($F_{1,10} = 15.2$, $P = 0.003$, adjusted $R^2 = 0.564$; Fig. 2A). This positive correlation between nodule number and HCNp did not hold true for within-cyanotype correlations. Within HC plants only, HCNp did not form a significant correlation with nodule number, and the slope of the trendline was slightly negative ($F_{1,4} = 0.834$, $P = 0.413$, adjusted $R^2 = -0.034$). Within LC plants, the positive trendline was not significant ($F_{1,4} = 3.817$, $P = 0.122$, adjusted $R^2 = 0.337$).

Foliar protein content also responded to increasing nodulation. Including both HC and LC plants, as plants formed greater numbers of nodules, protein content significantly decreased ($F_{1,10} = 21.67$, $P < 0.001$, adjusted $R^2 = 0.653$; Fig. 2B). Unlike HCNp, which did not form a significant correlation within either cyanotype, a significant negative correlation between nodule number and leaf protein was present within HC plants ($F_{1,4} = 20.25$, $P = 0.01$). By contrast, such correlation was not significant within LC plants, which showed a positive trendline between nodules and protein content ($F_{1,4} = 3.542$, $P = 0.133$, adjusted $R^2 = 0.337$).

Discussion

Incorporating symbiotic interactions into plant secondary metabolism patterns has been an important challenge because advantages afforded by symbioses can drastically influence plant

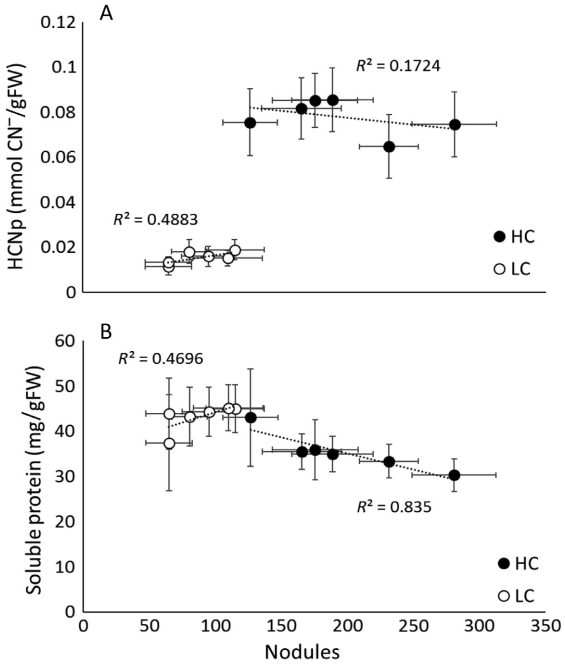

Fig. 2. Quantitative relationships between nodule number and variation in plant traits. Plant trait values including (A) cyanogenic potential (HCNp) and (B) soluble protein content were averaged across the duration of the time series experiment and regressed against nodulation means to assess putative correlations. Low cyanogenic plants (LC) and high cyanogenic plants (HC) are represented by white and black circles, respectively. Points show mean values from repeated assays and nodule counts for each plant across the time series; bars represent standard error of the mean.

resource allocation patterns (Kempel et al. 2009, Heath et al. 2014). Here, we show how the legume–rhizobia symbiosis interacts with leaf trait expression quantitatively in HC and LC cyanotypes of lima bean. While HC plants formed more root nodules and produced constitutively higher HCNp than LC plants, this positive relationship between nodulation and cyanogenesis did not result in quantitatively higher HCNp within either cyanotype. These data support our hypothesis that cyanotype may have played a role in selecting for the degree of rhizobial colonization based on constitutive nitrogen demands inherent in cyanogenesis. Surprisingly, our data do not support degree of nodulation quantitatively benefitting defense phenotype. While nodule number did not translate into an increase in HCNp, HC plants expressed quantitatively less soluble protein in plants with greater nodule numbers. By contrast, LC plants formed fewer nodules overall and foliar protein was not constrained by a negative correlation with nodule number. Our findings suggest that symbiotic investment plays a role in plant defense and nutritive phenotype, but also that genotypic defense levels may simultaneously shape the plant's obligatory investment in maintaining the symbiosis.

Genotypic nitrogen requirements and nodule formation

Our hypothesis that HC plants require more fixed nitrogen for cyanogenesis and would therefore form more nodules than LC plants was supported by our nodulation data. In another system with polymorphic cyanogenesis, *Trifolium repens*, acyanogenic strains did not form more nodules than cyanogenic strains (Kempel et al. 2009). However, the nature of cyanogenesis in *T. repens* is qualitative with presence or absence of either cyanogenic glucosides or β-glucosidases, which may impose different resource demands compared to the quantitative variation in the lima bean system, with LC plants that are cyanogenic but at lower levels than HC plants (Ballhorn et al. 2005). If degree of colonization depends on plant nitrogen availability and demand, one potential mechanism for differential nodulation could involve the autoregulated negative feedback loop inhibiting further nodulation. Autoregulation of nodulation involves an interplay of root- and shoot-derived signals in the presence of excess soil nitrate (Oka-Kira and Kawaguchi 2006). While the chemical nature of these signals is still largely unknown (Kouchi et al. 2010), our data may present evidence for shoot-derived signals to be differentially regulated in high and low cyanotypes based on nitrogen requirements.

Putative mechanisms for nodule differences between cyanotypes

Nodule formation and regulation is a highly controlled process, involving crosstalk of several plant hormones, plant signaling molecules, and bacterial Nod factors (Sun et al. 2006). Therefore, cyanotypic differences in nodule numbers may be connected to cyanotype-specific biology. In addition to a myriad of traits regulated

differentially between cyanotypes (Ballhorn et al. 2013), the most obvious difference between cyanotypes would be the presence of high concentrations of cyanogenic glucosides and potentially free cyanide surrounding damaged leaf cells. However, regulation of nodulation is not likely a product of direct cyanide exposure because free cyanide, which is released in the ethylene biosynthesis pathway, acts as a positive feedback loop for further ethylene synthesis (Smith et al. 2000), and ethylene is well known to inhibit nodulation (Penmetsa and Cook 1997). Inhibiting or reducing ethylene synthesis could be one way HC plants enable more nodules to form if cyanotypes differentially regulate this pathway, although this remains to be tested.

Is there a nodule number optimum? Nitrogen benefit vs. cost of maintaining nodules

If HC plants form more nodules to attain sufficient nitrogen for cyanogenesis, we would expect HCNp to correlate positively with nodule number. We see this effect across both genotypes, as HCNp increased with increasing nodule number. However, once these data are examined within cyanotype, plants do not produce more cyanide with higher nodule numbers. Interestingly, foliar protein concentration also correlated with nodule number overall, but in the opposite direction, decreasing as plants formed more nodules, which could point to the cost of maintaining symbiosis. In a study comparing symbiotic plants against nitrogen-fertilized plants, plants with rhizobia had lower protein levels, along with tannins and overall biomass (Briggs 1990). Our within-cyanotype data support this notion as HC plants, which likely have tighter allocation budgets, had quantitatively reduced protein levels as colonization intensity grew—as opposed to LC plants, in which nodule number and protein levels show a positive trend. If neither cyanotype's HCNp responded to rhizobia colonization, but protein trends correlated with nodules in opposite directions, HC plants may allocate more of the total symbiotic nitrogen pool to HCNp, reducing overall soluble protein levels. Alternatively, the number of nodules HC plants formed may have passed a threshold from which plants quantitatively benefit from greater colonization, contributing to why plants regulate nodulation (Oka-Kira and Kawaguchi 2006). Although it remains to be tested, HC plants may have selected for relaxed autoregulation in order to attain nitrogen for constitutive cyanogenic levels, which resulted in resource allocation constraints as the cost of maintaining higher numbers of nodules may limit this additional colonization from directly benefitting leaves.

In conclusion, cyanotype influences HCNp more strongly than input of nitrogen from increased nodulation. This finding is consistent with previous work in which nitrogen treatments increased both foliar nitrogen and cyanogenesis in *Eucalyptus cladocalyx*, but cyanogenic levels were restricted within genetically determined constraints (Simon et al. 2010). Despite nitrogen benefits, providing carbohydrates (Kaschuk et al. 2009) and specific amino acids (Prell et al. 2009) to nodules, our plant trait data demonstrate how symbiotic maintenance contributes to plant resource allocation challenges (Herms and Mattson 1992). Additionally, because high and low cyanotypes differentially engage in symbiosis, our data show the potential for plant defense schemes to influence the degree of symbiotic resource exchange.

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