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Symbiotic nitrogen fixation strategies relate to phenotypic plasticity but not invasion risk

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Abstract Many invasive plant species are symbiotic N-fixers that can have strong impacts on ecosystem processes. Nitrogen-fixing plants use a diversity of strategies to regulate the degree of N-fixation, each well suited for specific environmental conditions. However, little is known about whether fixation strategies are related to invasiveness. Weed risk assessment scores were used as an index of invasiveness for eight non-native N-fixing tree species (four high-risk and four low-risk for invasiveness) in Hawai'i. In a shade house experiment using an isotopic tracer, we found that species varied in their growth, biomass allocation, and N-fixing traits in response to three levels of nitrogen fertilization. Species sorted into distinct fixation strategies with three species displaying a facultative strategy, four species displaying an incomplete downregulation strategy, and one species displaying either a facultative or incomplete downregulation strategy. Fixation strategies were associated with the trait plasticity of each species, but not related to risk assessment scores for invasiveness in Hawai'i. Facultative fixers had the highest trait plasticity and were able to regulate symbiotic nitrogen fixation with the greatest magnitude. Collectively, our results suggest that species growth traits are better predictors of N fixation strategy than weed risk assessment scores, suggesting that the link between invasiveness and N fixation strategy is tenuous.

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Introduction

Nitrogen (N) availability is often the limiting factor for primary production in terrestrial ecosystems (LeBauer and Treseder 2008). Symbiotic partnerships between N-fixing bacteria and plants provide one of the most efficient systems for reducing, or fixing, atmospheric N needed in the biosphere. The relationship between N-fixing bacteria and host plants is mutualistic in nature: bacteria provide reduced N for the plant and the plant provides reduced carbon (C)

and other metabolic elements for bacteria (Udvardi and Poole 2013). This is costly for the plant, but having access to fixed N is a strong competitive advantage under conditions of low soil N availability (Rastetter et al. 2001).

Some plants regulate the amount of N derived from fixation depending on what is available in the soil and what the plant needs. Symbiotic N-fixing plant species use fixation strategies ranging from obligate to facultative, resulting in different amounts and rates of N fixation (Barron et al. 2011; Batterman et al. 2013; Drake 2011; Menge et al. 2023). Obligate fixation occurs at a constant rate per plant biomass unit, independent of the amount of available soil N; whereas facultative fixation adjusts the rate per plant biomass unit with changes in the amount of available soil N to meet the plant's needs (Hedin et al. 2009; Menge et al. 2009). In this respect, obligate fixers devote energy and resources to fixing atmospheric N regardless of soil N availability, whereas facultative fixers that regulate fixation are able to allocate energy and resources to growth if there is adequate available N in the soil (Menge et al. 2015; Pearson and Vitousek 2001). Thus, a facultative strategy provides a competitive advantage over an obligate strategy under high N conditions. A survey of eight co-occurring herbaceous legumes in California identified a range of fixation strategies, from obligate to facultative, which are likely to have vastly different effects on ecosystem-level nitrogen cycling (Menge et al. 2015).

Invasive plant species, non-native species that naturalize and increase rapidly outside their home range (Daehler 2003; Richardson et al. 2000), can possess increased growth rates, greater leaf areas, and higher fitness compared to non-invasive species (van Kleunen et al. 2010). Invasive species can also have lower construction costs compared to natives (Daehler 2003). These traits allow invasive plants to outcompete natives and non-invasive species, particularly in areas with high resource availability such as disturbed environments (Davis et al. 2000). However, invasiveness is not only related to individual traits, but the plasticity of those traits (Baker 1965). Invasive plant species can display higher trait plasticity than non-invasive plant species (Davidson et al. 2011; Richards et al. 2006), even in low resource environments (Funk 2008). High phenotypic plasticity may allow a species to invade a wide range of habitats, take advantage of temporal or spatial variation in

resource availability, or persist in a new environment until the species can adapt via genetic changes (Fox et al. 2019; Goldberg and Price 2022). Yet, not all plasticity is adaptive and differences in plasticity between native and invasive species may depend on the trait in question and the stage of invasion (van Kleunen et al. 2018; Zettlemoyer et al. 2019).

Many studies have attempted to identify the mechanisms for invasiveness, focusing on traits associated with physiology, growth rates, and allocation of resources (van Kleunen et al. 2010). Studies have tied the ability to fix N with invasion (Castro-Díez et al. 2014), but these studies typically focus on plants that are known to be aggressive invaders. There are many non-native N-fixers that do not become invasive. It is possible that invasive N-fixers have more efficient fixation strategies than non-invasive N-fixers. If fixation is well regulated (i.e., facultative strategy), N-fixers can adjust their investment in N fixation and allocate more resources to growth, dependent on how limiting N availability is in a particular environment. Conversely, an obligate strategy may give invasive species an advantage if the environment fluctuates rapidly and facultative fixers up or downregulate fixation too slowly relative to the environment or if there is a cost to turning fixation on and off (Menge et al. 2009). Thus, the strategy used for regulating N fixation, rather than the ability to fix N, may explain the differential success of invasive N-fixing species. Despite the potential importance of fixation strategies in explaining mechanisms of invasion, the relationship between N-fixation strategy and invasiveness has not been examined.

In Hawai'i, N-fixing invasive plants have been shown to transform ecosystems (Allison et al. 2006; Vitousek et al. 1987). For example, the invasive symbiotic N-fixing tree *Morella faya* has altered forest development on young volcanic substrate by increasing inputs of biologically available N with substantial impacts on N cycling in these N-limited habitats where no native symbiotic N-fixing plants are present (Vitousek et al. 1987). Hawai'i has adopted a risk assessment system based on the Australian and New Zealand Weed Risk Assessment system (Daehler et al. 2004) called the Hawai'i Pacific Weed Risk Assessment (HPWRA, https://plantpono.org/risk-assessment/). However, the assessment for N fixation is simply a question of whether or not the plant can fix N and does not consider the strategy used for N

fixation which, as hypothesized above, may differentiate invasive and non-invasive N-fixers. Filling this gap in research may contribute to a more informative assessment system for plants being introduced to Hawai'i and other regions.

This research addresses the following questions: Do growth rates, allocation traits, and N-fixation traits vary among non-native N-fixing woody plant species that have a range of assessment scores using the HPWRA? To what extent do growth rates, allocation traits, and N fixation traits change with N addition? Are N-fixing strategies and trait plasticity associated with HPWRA scores? Do species with different N-fixation strategies share similar growth traits and levels of trait plasticity? We grouped species into two categories for risk of invasion based on their HPWRA score: high risk and low risk. We hypothesized that high-risk species would have higher growth rates and trait plasticity, and would respond to N addition by decreasing N fixation rates and nodule density, supporting a facultative N-fixation strategy. In contrast, we expected that low-risk species would have lower growth rates and trait plasticity, and would respond to N addition with little or no change in N-fixation rates and little or no change in nodule density, supporting an obligate strategy.

Methods

Experimental design

In a shade house experiment, we grew eight N-fixing tree species within the family Fabaceae (Table 1), all of which have been evaluated using the HPWRA. Greenhouse studies allow for environmental factors, such as temperature, light, and precipitation, to remain constant. With these factors constant we can isolate N-fixing plants' responses to N availability by providing N-limited and N-saturated conditions while keeping other nutrients constant and in adequate supply. Risk scores and evaluations are derived from the 49 questions on the HPWRA followed by interviews of experts with personal field experience for each plant (Online Resource 1; Daehler et al. 2004). We selected four high-risk (HR) fixers, four low-risk (LR) fixers, and one HR non-fixing Fabaceae species for experimentation. Daehler et al. (2004) compared HPWRA output with opinions from local botanists, and demonstrated that the system was accurate ($>85\%$) at identifying major pest and non-pest species. Species with a HPWRA score greater than 6 are deemed likely to become a pest, and this threshold was used for our HR category (>6). Species in our LR category were those with scores ≤ 6. N-fixing species included *Erythrina variegata* (ERVA), *Sesbania grandiflora* (SEGR), *Pterocarpus indicus* (PTIN), *Samanea saman* (SASA), *Albizia lebbeck* (ALLE), *Falcataria moluccana* (FAMO), *Acacia confusa* (ACCO), and *Pithecellobium dulce* (PIDU). Data from the non-fixing HR species, *Senna siamea*, were used to calculate % N_{dfa} in fixing plants. A LR non-fixing species was attempted, but due to high mortality was not considered for comparison in analyses. Three levels of N addition were given for each species, beginning with 16 replicates each (12 for *E. variegata*). For natural history of these species, see Online Resource 2.

Species	HPWRA Score
Low risk	
Erythrina variegata Lam. (ERVA)	-2
Sesbania grandiflora (L.) Pers. (SEGR)	2
Pterocarpus indicus Willd. (PTIN)	4
Samanea saman (Jacq.) Merr. (SASA)	4
High risk	
Albizia lebbeck (L.) Benth. (ALLE)	7
Falcataria moluccana (Miq.) Barneby & J.W. Grimes (FAMO)	8
Acacia confusa Merr. (ACCO)	10
Senna siamea (Lam.) H.S. Irwin & Barneby* (SESI)	11
Pithecellobium dulce (Roxb.) Benth. (PIDU)	14

Table 1 Nitrogen-fixing study species (family Fabaceae) were chosen based on a range of HPWRA scores

*Non-fixing reference species grown at the same time, under the same conditions

Scores greater than six denote high risk of invasion. Four letter species codes are included in parentheses

Seed and soil collection, and growing conditions

Seeds were collected in the field on Hawai'i Island, except for ERVA and SEGR which were purchased from online vendors. Seeds (10–60) of each species were dried at 70 °C and weighed to run composite samples for % N to quantify % N in the seeds. Seeds (~ 100 per species) were scarified in hot water, imbibed for 10–24 h, and germinated on trays according to the needs of each species. Seedlings were planted in a 1:1:1 mixture of perlite (Pahroc Giant #2, Wilkin Mining & Trucking, Inc. Caliente, NV, USA), vermiculite (#2 coarse, Therm-O-Rock West, Inc. Chandler, AZ, USA), and volcanic cinder (naturally occurring on Hawai'i Island, sterilized) in 4″ × 4″ × 14″ sapling pots (Stuewe & Sons, Inc. Tangent, OR, USA). While N content of the soil media was not measured, we assumed that initial levels were quite low.

Seedlings were inoculated with a similar amount of slurry containing a small quantity of crushed nodules and rhizosphere soil collected beneath naturally occurring trees for each species (Menge et al. 2015; Wolf et al. 2017). Soil was collected from near the roots of the mature tree unless seedlings were present, in which case entire seedlings were collected for their root nodules. All seeds, soil, and seedling samples were placed in Ziploc bags, stored in a cooler for transport, then stored in a refrigerator until germination. When possible, seedlings were grown ahead of time for nodule harvest to include in the slurry (Sprent and Sprent 1990); however, the number of microbial cells added to each seedling was not controlled.

Plants were watered adequately with overhead sprinkler irrigation depending on seasonal needs (three to six times daily). Saplings grew for approximately 4 months after the first fertilization treatment. Plants were randomly arranged in three blocks per species and rotated around the shade house by block every 2 weeks to account for variation in light. All plants were grown between January 2018 and November 2019 in Kea'au, Hawai'i (19.55733°N, −154.97638°W). Average daily temperature was 72.5 °F (22.5 °C). Light measurements were taken at three locations inside and two locations outside the shade house, three times per day, every few weeks. Light inside the shade house averaged 73% of light outside the shade house.

Fertilization

Each plant received a standard Hoagland N-free fertilizer corresponding to 0.252 g P m^{-2} y^{-1} for phosphorus, a level that should make plant growth limited by non-nitrogen soil nutrients at high N levels. This assumes there was no P in the growing medium, which we did not measure. Three levels of nitrogen addition were used for each species: 0.3 g N m^{-2} y^{-1}, 6 g N m^{-2} y^{-1}, and 20 g N m^{-2} y^{-1} (low, medium, and high, respectively). Our low N treatment mimics a N-limited environment similar to young substrates in Hawai'i (Vitousek and Sanford 1986), the medium N treatment mimics a non-N-limited environment similar to old substrates (Vitousek and Sanford 1986), and the high N treatment mimics a N-saturated environment (Menge et al. 2015). Labelled ^{15}N fertilizer (Cambridge Isotope Labs, Tewksbury, MA, USA) was added with a 7 atom % ^{15}N, making it distinguishable from atmospheric N (0.359–0.377 atom % ^{15}N). Fertilizers were added every other week by micropipette (Menge et al. 2015) beginning when true leaves had emerged and were open.

Growth measurements

Height and stem diameter at base were collected for each plant shortly before harvesting. All components of the plants were harvested after approximately four months of growth. Leaf area was measured on fresh leaves using a LI 3100 Area Meter (Licor, Inc. Lincoln, NE). Root nodules, roots, stems, and leaves were dried separately at 70 ºC and weighed. Specific leaf area was calculated using the combined area and dry weight of all leaves on a plant. Because plants were grown for different periods of time (115–132 days), measurements for height, stem diameter, total biomass, nodule biomass, root biomass, stem biomass, leaf biomass, and leaf area were converted to growth rates (growth yr^{-1}) to account for variation in growth periods (growth rate $= \frac{\text{biomass measure}}{\text{growth period (yr)}}$). Plant fractions (e.g., roots, leaves) were then pooled for each individual for % N and ^{15}N analysis. Plant biomass is an indicator of the effects of N-limitation on plant productivity; root nodule mass is a measure of

structural allocation to N fixation; % N_{dfa} indicates the percentage of N that is derived from N fixation versus soil N over the lifetime of the plant; and N fixed per biomass is our primary indicator of N fixation.

Quantifying nitrogen fixation

The percentage of a plant's nitrogen from fixed N (% N_{dfa}) was quantified by comparing ^{15}N enrichment in the potentially N-fixing plant tissues to atmospheric N_2 and the non-fixing reference species. Non-fixing plant values were used as a baseline for comparison, as is standard (Shearer and Kohl 1986). Plant tissues were ground, and whole plant subsamples were analyzed at the UC Davis Stable Isotope Facility (Davis, CA, USA) using a PDZ Europa ANCA-GSL elemental analyzer coupled with a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Seed N was a small percentage of total N for these plants (0.004–0.18%); thus, the contribution of seed N to fixation was not accounted for in our model. Isotopic values were converted to atom % and standard atmospheric ^{15}N value was subtracted to give atom % excess (AE). Tissue ^{15}N values of the non-fixing reference individuals were averaged per treatment before calculating % N_{dfa} using the following equation (Yelenik et al. 2013):

$$\% \ N_{dfa} = \frac{^{15}N \ AE_{non\text{-}fixer} - {}^{15}N_{fixer}}{^{15}N \ AE_{non\text{-}fixer}} \times 100$$

These % N_{dfa} values were multiplied by total plant N (total biomass multiplied by the % N of composited tissue) for each potentially N-fixing plant to calculate the total N fixed. To express total N fixed per biomass, we divided total N fixed by total biomass for each potentially N-fixing plant.

Statistical analysis

To investigate differences in species means for growth rates, allocation traits, and N-fixation traits, linear models (two-way ANOVAs) were performed on two factors, species and N treatments, followed by Tukey's honest significant difference (HSD) post hoc comparison test. Each reponse variable was analyzed separately for individual species. Following Moran (2003), corrections for multiple statistical tests were not conducted; instead, all *P*-values are reported. Main response variables included biomass growth rate and leaf area growth rate to represent growth; SLA and root-to-shoot ratio to represent resource allocation; and nodule density, % N in tissue, % N_{dfa}, and total N fixed per biomass to represent N fixation traits. Height, stem diameter, total biomass, root-to-shoot ratio, and leaf area data were log transformed to achieve normality. FAMO and SESI are missing data for leaf biomass and are excluded from statistical tests for leaf biomass and SLA.

To determine fixation strategies for each species, biomass and total N fixed per biomass were compared across N addition treatments (Menge et al. 2015). If fixation drops to zero in the medium or high N levels, that is consistent with a facultative strategy. If fixation drops in the medium or high treatments but not to zero, and N limitation has been overcome (biomass is the same in medium and high N), this is consistent with incomplete downregulation. If fixation drops in the medium or high treatment but not to zero and N limitation has not been overcome (biomass is higher in high N than medium N), then the species has either a facultative or incomplete downregulation strategy. If fixation stays the same across all three N addition treatments and N limitation has been overcome, this is consistent with an obligate strategy because N fixation is insensitive to N availability. To assess whether N-fixing strategies were associated with HPWRA scores, we calculated species averages of total N fixed per biomass (our indicator of fixation) in the high N treatment and compared this metric across low- and high-risk species groups using a linear model.

To investigate differences in trait values and trait plasticity among species, a principal components analysis (PCA) was conducted on all response variable means (biomass, height, stem diameter, and leaf area growth rates, root-to-shoot ratio, nodule density, % N in tissue, % N_{dfa}). Plasticity indices were calculated for each trait of every species using the coefficient of variation (standard deviation/mean) because of its simplicity and ease in comparing phenotypic variability within and between environments (Valladares et al. 2006). We ran a second PCA using species-level plasticity indices on growth rates (biomass, height, stem diameter, leaf area, root-to-shoot ratio, and % N in tissue). We used linear regression to determine if trait plasticity (PC 1) predicted N fixation ability (high N treatment means of nodule density, %

N_{dfa}, N fixed per biomass) or HPWRA scores. Statistical analyses were performed using RStudio1.2.5033 (Team [2015]) and PRIMER v6 (Clarke and Gorley [2006]).

Results

All growth measurements, allocation traits, and N-fixation traits varied among species (Table [2]). ERVA had the highest biomass growth rate (g yr^{-1}), leaf area growth rate (cm^2 yr^{-1}), % N_{dfa}, and second highest total N fixed per biomass (Fig. [1]; Online Resource 3). SEGR had the lowest nodule density (g g^{-1}), % N in tissue, % N_{dfa}, and N fixed per biomass. While SASA had the lowest biomass and leaf area growth rates, it had the highest specific leaf area (SLA, cm^2 g^{-1}), nodule density, % N in tissue, and had among the highest root-to-shoot ratio, % N_{dfa}, and N fixed per biomass (Figs. [1], [2]; Online Resource 3). ALLE had the lowest SLA and the highest root-to-shoot ratio. ACCO had the lowest root-to-shoot ratio and among the highest % N_{dfa}. PTIN, FAMO, and PIDU were intermediates among growth, allocation, and N-fixation traits (Figs. [1], [2]; Online Resource 3). The non-fixing species, SESI, did not nodulate, as expected.

Growth measurements, allocation traits, and N-fixation traits were affected by N addition treatments as well, with the exception of SLA (Table [2]). Generally, N addition enhanced growth and reduced investment in roots and nodules (Fig. [2]). No significant difference was found between low and medium treatments for % N in tissue.

Strong evidence for interaction effects of the two factors (species and N treatments) was found for all response variables except root-to-shoot ratio (Table [2]). SEGR, FAMO, ACCO, and PIDU had statistically significant increases in means with N addition for biomass and leaf area growth rates, while SASA had statistically significant decreases (Online Resource 3). SEGR and PIDU had statistically significant differences in means across N treatments for SLA. SEGR had highest mean SLA with medium N addition, while PIDU had lowest mean SLA with medium N addition (Online Resource 3). ERVA, SEGR, FAMO, and PIDU had statistically significant decreases in means across N treatments for root-to-shoot ratio, although PIDU increased with medium N addition (Fig. [2]). ERVA, PTIN, ALLE, FAMO, ACCO, and PIDU had statistically significant decreases in means across N treatments for nodule density, although ACCO increased with medium N addition. ERVA, SEGR, SASA, FAMO, and PIDU

Table 2 Results of two-way ANOVA for N-fixers by factors species and N treatment

Variable	Species			N			Species x N		
	P value	F value	Df	P value	F value	Df	P value	F value	Df
Growth rates									
Biomass	<0.001	100.13	7	<0.001	74.03	2	<0.001	19.18	14
Height	<0.001	118.43	7	<0.001	93.83	2	<0.001	17.14	14
Stem diameter	<0.001	437.24	7	<0.001	53.09	2	<0.001	11.53	14
Nodule biomass	<0.001	164.84	7	<0.001	7.91	2	<0.001	4.74	14
Root biomass	<0.001	140.90	7	<0.001	10.08	2	<0.001	4.12	14
Stem biomass	<0.001	166.66	6	<0.001	17.22	2	<0.001	4.77	12
Leaf biomass	<0.001	106.04	6	<0.001	32.24	2	<0.001	7.67	12
Leaf area	<0.001	65.51	7	<0.001	56.06	2	<0.001	11.31	14
Allocation traits									
SLA	<0.001	138.90	6	0.638	0.45	2	<0.001	2.57	12
Root-to-shoot ratio	<0.001	115.69	7	<0.001	18.35	2	0.022	1.94	14
N-fixation traits									
Nodule density	<0.001	68.50	7	<0.001	44.13	2	<0.001	3.47	14
% N in tissue	<0.001	90.57	7	<0.001	11.45	2	<0.001	4.34	14
% N_{dfa}	<0.001	199.23	7	<0.001	640.99	2	<0.001	14.31	14
N fixed per biomass	<0.001	213.22	7	<0.001	230.06	2	<0.001	4.60	14

F. moluccana is not included in statistical tests for stem biomass, leaf biomass, or specific leaf area due to missing data. *S. siamea* is a non-fixer and was not included in statistical tests. *P*-values, *F*-values, and degrees of freedom given with α = 0.05.

Fig. 1 Indicators of N limitation (biomass) and N fixation (N fixed per biomass) for each species ordered from left to right by HPWRA score (low to high) for eight N-fixers and one non-fixer (SESI). Data are means and standard error. Significant differences among N addition treatments within each species are denoted by asterisks (***$p < 0.001$). Species codes are provided in the text
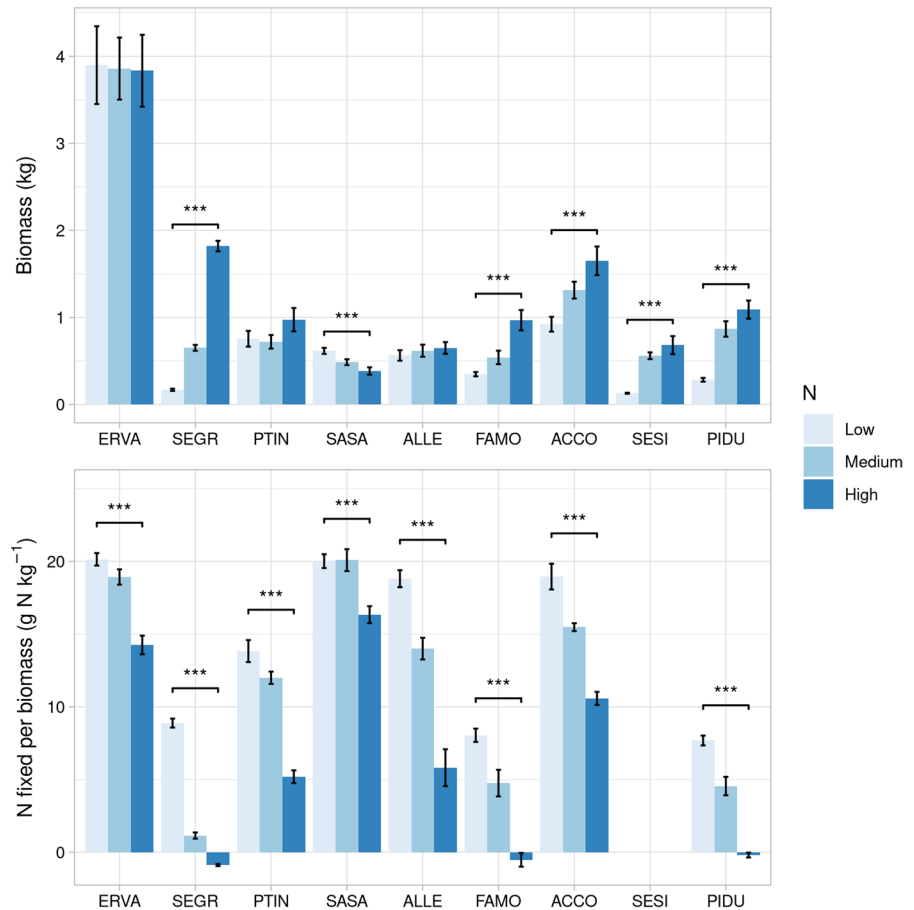
had statistically significant differences in means across N treatments for % N in tissue, ERVA and SEGR had variable responses while SASA, FAMO, and PIDU increased with N addition. All N fixers had statistically significant decreases in means across N treatments for % N_{dfa} and N fixed per biomass (Fig. 1; Online Resource 3). These interactions for the different traits demonstrate plastic responses of species, dependent on environmental conditions.

Fixation strategies were determined for each species using biomass as an indicator of N limitation and total N fixed per biomass as an indicator of fixation (Fig. 1). Biomass growth rate showed the same pattern across treatments as total biomass; thus, both metrics provided similar determinations of N-fixation strategies. Three species were found to be facultative (FAMO, PIDU, and SEGR) and four species diplayed incomplete downregulation (ALLE, ERVA, PTIN, SASA). ACCO downregulated fixation with N addition but not to zero (its growth was still limited at

high N); thus, this species could have either a facultative or incomplete downregulation strategy. No species displayed consistent fixation across N treatments; thus, no species exhibited an obligate strategy. While there was a trend towards lower N fixed per biomass at high N (e.g., more facultative) in high-risk species (3.92 mg N g^{-1}) compared to low-risk species (8.72 mg N g^{-1}), this trend was not statistically significant (Online Resource 4).

A PCA on all species traits showed species grouping by growth rates and N-fixation traits (Online Resource 5). PC1 was strongly associated with growth (driven by biomass, height, stem diameter, and leaf area growth rates, and root-to-shoot ratio), explaining 49% of the variation among species groups. PC2 was strongly associated with N-fixation traits (driven by nodule density, % N in tissue, and % N_{dfa}), explaining 32% of the variation. SASA had high measures of nodule density, % N in tissue, and % N_{dfa}, across all three N treatments, showing that it had little ability

Fig. 2 Effects of N addition on height growth rate, nodule density, and root to shoot biomass ratio for each species ordered from left to right by HPWRA score (low to high) for eight N-fixers and one non-fixer (SESI). Data are means and standard error. Significant differences among N addition treatments within each species are denoted by asterisks ($*p < 0.05$, $***p < 0.001$). Species codes are provided in the text
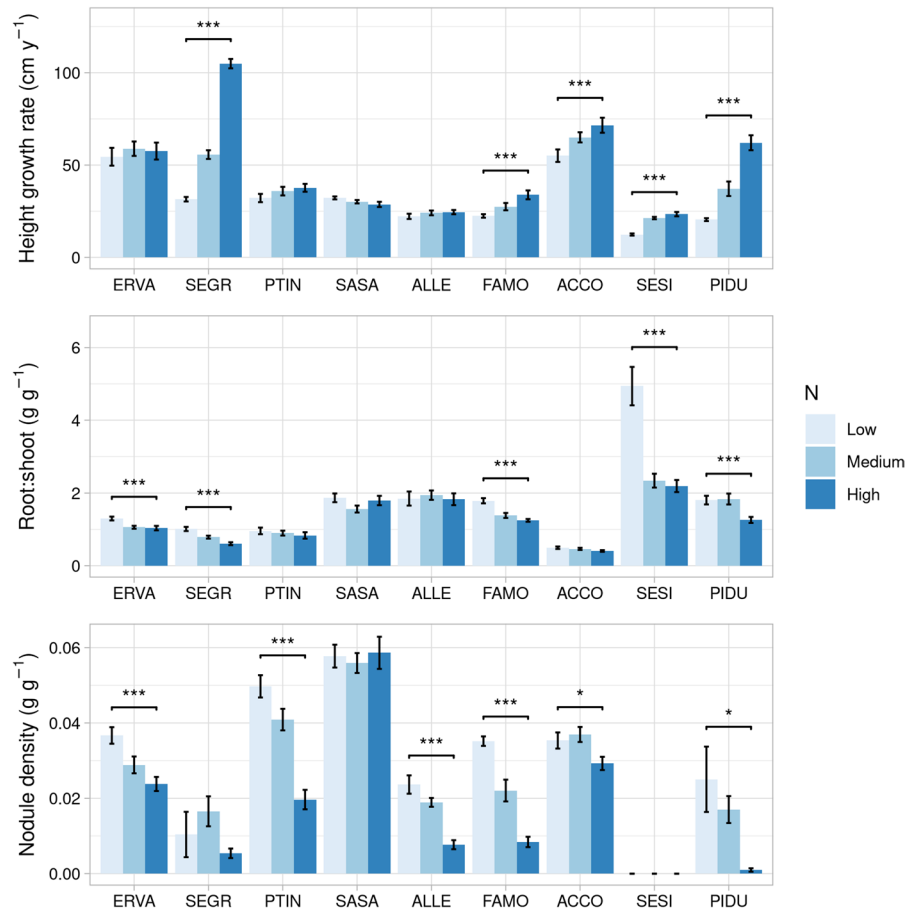
to regulate fixation under varying nutrient conditions. SASA was intermediate for growth overall, a potential trade-off for contributing so much energy to fixation. ERVA had the most aggressive growth, but intermediate for N-fixation traits. SEGR, FAMO, and PIDU had low measures of nodule density and % N_{dfa} at the highest N treatment. Growth for these species was low compared to other species, possibly due to N limitation. ERVA, PTIN, ALLE, and ACCO were moderate in their N-fixation traits compared to other species and varied in growth compared to each other.

For the PCA on plasticity indices, PC score 1 explained 83.7% of the variation among species with strong loadings of biomass, biomass N, and leaf area growth plasticity. PC1 from this analysis was used as an integrated plasticity index for each species. This plasticity index was strongly correlated with N-fixation traits. An increase in plasticity was associated with an increase in fixation regulation via reduction in nodule biomass, % N_{dfa}, and N fixed per biomass

(Fig. 3). The three facultative fixers (SEGR, FAMO, and PIDU) had the most trait plasticity and were able to regulate their N fixation with the greatest magnitude. Plasticity was not correlated with weed risk assessment score ($F_{1.6} = 0.51$, $p = 0.50$).

Discussion

The eight N-fixing species varied in their growth, allocation, and N-fixing traits, and displayed either a facultative or incomplete downregulation strategy. Thus, the results of this experiment support the idea that many Fabaceae species are facultative fixers (Barron et al. 2011; Menge et al. 2015; Pearson and Vitousek 2001) and that the obligate strategy may be rare. While fixation strategies were associated with the trait plasticity of each species, they were not related to weed risk assessment score, our proxy for invasiveness. In addition, trait plasticity was not
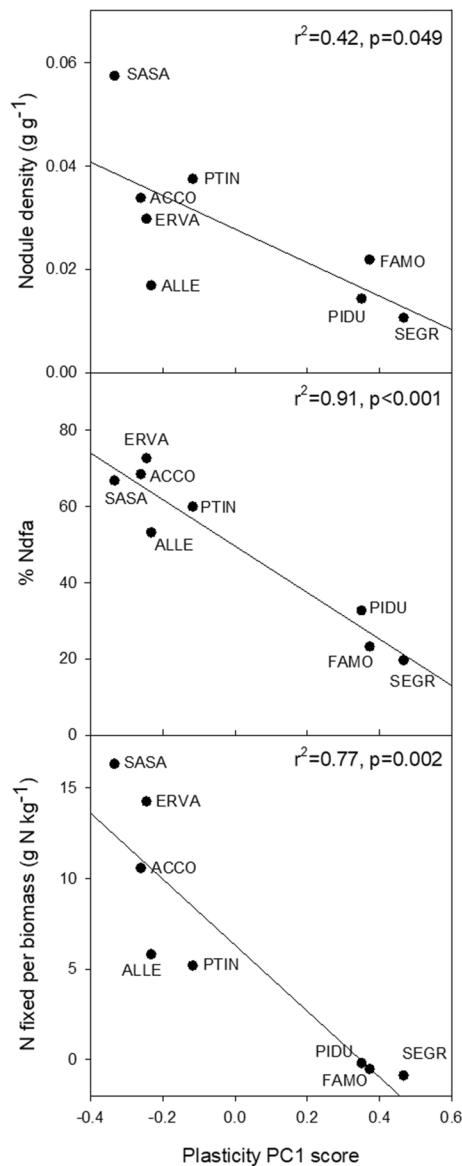
Fig. 3 Plasticity indices (PC score 1) regressed with species means for nodule density, % N_{dfa}, and N fixed per biomass in the high N treatment. The three species with a facultative fixation strategy (FAMO, PIDU, SEGR) cluster away from the others. A higher PC score means greater plasticity. Species codes are provided in the text

related to HPWRA score, despite good evidence that such a relationship is common (Davidson et al. 2011).

Species with a facultative strategy downregulate fixation depending on soil N availability to meet the needs of the plant, and this should result in a competitive advantage relative to obligate fixers under conditions of high N. Specifically, facultative fixers can decrease resource expenditures to fixation at high N availability and allocate them to growth resulting in increased aboveground biomass and leaf area (Pearson and Vitousek 2001). Three of our N fixers displayed a facultative strategy and these were the species that showed the highest growth advantage with fertilization (Fig. 1). On average, the three facultative species increased growth fivefold in the high versus low N treatments (range 2.4 to 10-fold). In contrast, the four species with an incomplete downregulation strategy displayed much smaller increases (0 to 0.5-fold) or negative growth (SASA). Our non-fixing control (SESI), which is not burdened by fixation costs at high N also showed a fivefold growth advantage with fertilization. Thus, our data support the idea that a facultative strategy benefits plants under high N conditions.

We found no support for an obligate strategy. Obligate fixers tend to do poorly in tropical ecosystems, which are typically not N limited, because the investment of resources into long-lasting nodules that fix N at a constant rate is too costly to compete with coexisting non-fixers (Menge et al. 2009). However, young volcanic substrates in Hawai'i are N limited (Vitousek and Sanford 1986) and may provide a habitat suitable for N-fixers with an obligate strategy. Data from a field experiment in Hawai'i suggests that a few woody species have an obligate strategy, at least in open-grown field conditions (Menge et al. 2023). Obligate fixers may do well in tropical ecosystems during early succession if N availability is low, but after enriching the soil via fixation and litterfall they will likely be outcompeted by non-fixers (Menge et al. 2014).

The most striking result in this study was that species with a greater ability to regulate N fixation displayed higher trait plasticity. While not all trait plasticity is adaptive, high plasticity may allow a species to invade a wide range of habitats or persist in a new environment until the species can adapt via genetic changes (Fox et al. 2019; Goldberg and Price 2022), and high plasticity has been found for many invasive plant species (Davidson et al. 2011). Trait plasticity is easy to measure, and our approach could be used in other studies as a way to predict either the relative invasiveness of N fixers or, with an expanded data set including more species, the N-fixation strategy of a given species. SASA had the least trait plasticity

and, while not characterized by an obligate strategy, showed the weakest regulation of fixation under varying nutrient conditions. Collectively, these results suggest that SASA has little capacity to adapt to changing or new environments and would not likely be an aggressive invader, but may have more success colonizing younger substrates based on its fixation strategy. In support of this idea, SASA (native to northern South America) has naturalized throughout the tropics, but is only considered invasive in Fiji and Vanuatu (Online Resource 1). Our three facultative fixers (SEGR, FAMO, and PIDU) had the most trait plasticity and the largest growth increase across the soil N gradient. Collectively, these data suggest that facultative fixers have the capacity to adapt to changing environments and would very likely coexist with or outcompete natives adapted to the N-poor local environment while also thriving under nutrient-rich conditions. FAMO and PIDU both appear to fall into this specific situation: they were found to have high trait plasticity, utilized an efficient facultative fixation strategy, and have successfully invaded (coexisted and outcompeted natives) N-poor and N-rich environments in Hawaiʻi (Hughes and Denslow 2005; Zimmerman et al. 2008).

Although we found a diversity of fixation strategies in plants with a wide range of HPWRA scores, we did not find an association between fixation strategies and risk; thus, this particular hypothesis was not supported. Several reasons may contribute to this finding. First, HPWRA scores are not computed from quantitative metrics of plant function on a continuous scale (see Online Resource 1). Rather, it asks a series of "yes" or "no" questions about the plant's life-history traits, native geographic and climate range, and history of invasiveness outside its native range and generates an additive score (Daehler et al. 2004). Answers to the questions are derived from scientific literature and experts in the field. Assessment scores using this system should ultimately be considered a prediction of whether an introduced plant is likely to become invasive in Hawaiʻi (yes/no), rather than as a scale for how invasive a species is likely to become if introduced. Second, there is only one question on the HPWRA that asks whether or not a species can fix N. Nitrogen fixation itself does not determine whether or not a species will be invasive, but the strategy used for fixation may carry more weight than just one "yes" or "no" answer. Finally, it is possible that N-fixation strategy in conjunction with other invasive traits contribute to invasiveness in Hawaiʻi. However, in comparing HPWRA datasheets for each species (Online Resource 1) there is no clear pattern between responses and N-fixation strategies. In other words, the three facultative fixers (SEGR, FAMO, and PIDU) in this study do not share a set of HPWRA responses that is set apart from the rest of the species' responses and HPWRA is likely not a good method for predicting invasiveness for N-fixing plants.

Our study is the first to determine N-fixation strategies for invasive and non-invasive non-native plants intending to answer the question of whether fixation strategy is related to invasiveness. Our results suggest that fixation strategies are associated with trait plasticity, a known characteristic of invasive species, but are not associated with weed risk assessment scores using the HPWRA. Additionally, we found that species identity and N addition had strong effects on growth and fixation traits, which is consistent with other recent studies (Batterman et al. 2013; Menge et al. 2023; Wurzburger and Hedin 2016). Fixation rates vary across species and ontogenetic stages and only eight species were observed in this study. Thus, additional studies that focus on older plants across a larger diversity of species (e.g., actinorhizal fixers) are needed to conclusively answer this question. Finally, the identity and host compatibility of rhizobia strongly influence invasive species establishment and performance (Le Roux et al. 2017). Our study used bacteria collected in the invaded range (Hawaii); thus, N-fixation rates and growth traits may have differed if a broader inoculum was used.

Author contributions ASK, RO, DMLN and JLF conceived the ideas and designed methodology; ASK collected and analyzed the data and led writing of the manuscript. All authors contributed to drafts and gave final approval for publication.

Data availability Data are available as Online Resource 6.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

References

Allison SD, Nielsen C, Hughes RF (2006) Elevated enzyme activities in soils under the invasive nitrogen-fixing tree *Falcataria moluccana*. Soil Biol Biochem 38:1537–1544

Baker HG (1965) Characteristics and modes of origin of weeds. In: Baker HG, Stebbins GL (eds) The Genetics of colonizing species. Academic Press, New York, pp 147–169

Barron AR, Purves DW, Hedin LO (2011) Facultative nitrogen fixation by canopy legumes in a lowland tropical forest. Oecologia 165:511–520

Batterman SA, Hedin LO, van Breugel M et al (2013) Key role of symbiotic dinitrogen fixation in tropical forest secondary succession. Nature 502:224–227

Castro-Díez P, Godoy O, Alonso A et al (2014) What explains variation in the impacts of exotic plant invasions on the nitrogen cycle? A meta-analysis. Ecol Lett 17:1–12

Clarke KR, Gorley RN (2006) PRIMER v6. PRIMER-E, Plymouth

Daehler CC (2003) Performance comparisons of co-occurring native and alien invasive plants: implications for conservation and restoration. Annu Rev Ecol Evol Syst 34:183–211

Daehler CC, Denslow JS, Ansari S et al (2004) A risk-assessment system for screening out invasive pest plants from Hawaii and other Pacific Islands. Conserv Biol 18:360–368

Davidson AM, Jennions M, Nicotra AB (2011) Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. Ecol Lett 14:419–431

Davis MA, Grime JP, Thompson K (2000) Fluctuating resources in plant communities: a general theory of invasibility. J Ecol 88:528–534

Drake D (2011) Invasive legumes fix N_2 at high rates in riparian areas of an N-saturated, agricultural catchment. J Ecol 99:515–523

Fox RJ, Donelson JM, Schunter C et al (2019) Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. Philos Trans R Soc B Biol Sci 374:20180174

Funk JL (2008) Differences in plasticity between invasive and native plants from a low resource environment. J Ecol 96:1162–1174

Goldberg EE, Price TD (2022) Effects of plasticity on elevational range size and species richness. Am Nat 200:316–329

Hedin LO, Jack Brookshire ENJ, Menge DNL et al (2009) The nitrogen paradox in tropical forest ecosystems. Annu Rev Ecol Evol Syst 40:613–635

Hughes RF, Denslow JS (2005) Invasion by a N_2-fixing tree alters function and structure in wet lowland forests of Hawaii. Ecol Appl 15:1615–1628

Le Roux JJ, Hui C, Keet J-H et al (2017) Co-introduction vs ecological fitting as pathways to the establishment of effective mutualisms during biological invasions. New Phytol 215:1354–1360

LeBauer DS, Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. Ecology 89:371–379

Menge DNL, Levin SA, Hedin LO (2009) Facultative versus obligate nitrogen fixation strategies and their ecosystem consequences. Am Nat 174:465–477

Menge DNL, Lichstein JW, Ángeles-Pérez G (2014) Nitrogen fixation strategies can explain the latitudinal shift in nitrogen-fixing tree abundance. Ecology 95:2236–2245

Menge DNL, Wolf AA, Funk JL (2015) Diversity of nitrogen fixation strategies in Mediterranean legumes. Nat Plants 1:15064

Menge DNL, Wolf AA, Funk JL, Perakis SS, Akana PR, Arkebauer R, Bytnerowicz TA et al (2023) Tree symbioses sustain nitrogen fixation despite excess nitrogen supply. Ecol Monogr e1562. https://doi.org/10.1002/ecm.1562

Moran MD (2003) Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos 100:403–405

Pearson HL, Vitousek PM (2001) Stand dynamics, nitrogen accumulation, and symbiotic nitrogen fixation in regenerating stands of *Acacia koa*. Ecol Appl 11:1381–1394

Rastetter EB, Vitousek PM, Field C et al (2001) Resource optimization and symbiotic nitrogen fixation. Ecosystems 4:369–388

Richards CL, Bossdorf O, Muth NZ et al (2006) Jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. Ecol Lett 9:981–993

Richardson DM, Pysek P, Rejmanek M et al (2000) Naturalization and invasion of alien plants: concepts and definitions. Divers Distrib 6:93–107

Shearer G, Kohl DH (1986) N_2-fixation in field settings: estimations based on natural ^{15}N abundance. Aust J Plant Physiol 13:699–756

Sprent JI, Sprent P (1990) Nitrogen fixing organisms: pure and applied aspects, 1st edn. Chapman and Hall, London

Team R (2015) RStudio: integrated development for R. http://www.rstudio.com/

Udvardi M, Poole PS (2013) Transport and metabolism in legume–rhizobia symbioses. Annu Rev Plant Biol 64:781–805

Valladares F, Sanchez-Gomez D, Zavala MA (2006) Quantitative estimation of phenotypic plasticity: bridging the gap between the evolutionary concept and its ecological applications. J Ecol 94:1103–1116

van Kleunen M, Weber E, Fischer M (2010) A meta-analysis of trait differences between invasive and non-invasive plant species. Ecol Lett 13:235–245

van Kleunen M, Bossdorf O, Dawson W (2018) The ecology and evolution of alien plants. Annu Rev Ecol Evol Syst 49:25–47

Vitousek PM, Sanford RJ (1986) Nutrient cycling in moist tropical forest. Annu Rev Ecol Syst 17:137–167

Vitousek PM, Walker LR, Whiteaker LD et al (1987) Biological invasion by *Myrica faya* alters ecosystem development in Hawaii. Science 238:802–804

Wolf AA, Funk JL, Menge DNL (2017) The symbionts made me do it: legumes are not hardwired for high nitrogen concentrations but incorporate more nitrogen in the presence of bacterial partners. New Phytol 213:690–699

Wurzburger N, Hedin LO (2016) Taxonomic identity determines N_2 fixation by canopy trees across lowland tropical forests. Ecol Lett 19:62–70

Yelenik S, Perakis S, Hibbs D (2013) Regional constraints to biological nitrogen fixation in post-fire forest communities. Ecology 94:739–750

Zettlemoyer MA, Schultheis EH, Lau JA (2019) Phenology in a warming world: differences between native and non-native plant species. Ecol Lett 22:1253–1263

Zimmerman N, Flint Hughes R, Cordell S et al (2008) Patterns of primary succession of native and introduced plants in lowland wet forests in eastern Hawai'i. Biotropica 40:277–284