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Nutrient Availability Has a Greater Influence than Pot Host on Seedling Development of Hemiparasitic Hawaiian Sandalwood (*Santalum paniculatum* Hook. and Arn.)

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Abstract: Sandalwood (*Santalum* spp.) has been overharvested throughout its range, including the Hawaiian Islands, where 6 of the 19 species *Santalum* spp. are endemic. As hemiparasitic plant species, Hawaiian sandalwoods require a host plant for optimal forest establishment, yet the importance of a host during seedling development is unclear. Furthermore, understanding interactions between pot hosts and nutrient availability on sandalwood seedling development during nursery culture will help to promote the production of high-quality sandalwood seedlings for restoration and commercial purposes. We evaluated the effects of controlled-release fertilizer (CRF), chelated Fe treatments, and two pot host species (*Acacia koa* and *Dodonaea viscosa)* on the seedling development of Hawaiian sandalwood (*Santalum paniculatum*). Increased nutrient availability (CRF) led to increased dry mass, root collar diameter, shoot height, chlorophyll index, and nutrient status values, confirming that the hemiparasitic *S. paniculatum* can be successfully grown in early stages of cultivation by providing adequate mineral fertilizers. There was a significant interaction between the nutrient availability and chelated iron treatments associated with increased height, root collar diameter, dry mass, chlorophyll index, Fe concentration, and Fe content when chelated Fe was applied (vs. not) in a nutrient-limiting environment. The pot host treatment did not affect any growth metrics, but it did affect the total count of haustoria, with *A. koa*-hosted seedlings developing 60.3% more haustoria than *D. viscosa*-hosted and control seedlings. Our results demonstrate that high-quality *S. paniculatum* seedlings can be grown in containers by providing adequate nutrition and that *S. paniculatum* in a nutrient-limiting growing environment may benefit from chelated iron fertilizers.

Keywords: *Santalum paniculatum*; hemiparasite; fertilizer; pot host; nursery culture; controlled-release fertilizer; chelated iron fertilizer; *Acacia koa*; *Dodonaea viscosa*

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1. Introduction

Members of the *Santalum* L. genus are root hemiparasitic woody shrubs or trees that produce aromatic oil-rich heartwood and are collectively referred to as sandalwoods [1]. Indian sandalwood (*Santalum album* L.) has been coveted for its high-quality heartwood and has been traded and used for 2500 years in China and 3200 years in India [2]. Indian sandalwood was historically burned as incense in Hindu and Buddhist ceremonies, carved into deities' sculptures, and used as fuel for funeral pyres for revered religious figures such as Mahatma Gandhi [1]. Sandalwood species grow naturally in India, Australia, Indonesia, and numerous islands throughout the Pacific Ocean [3,4]. The high value of the heartwood has led to overharvesting and the exploitation of the genus throughout its range, including the Hawaiian Islands, where six of the 19 sandalwood species are endemic [5,6].

In Hawaii, sandalwood is known locally as 'iliahi and was used for medicines and to add scent to plant fiber cloths [7]. A Hawaiian sandalwood trade occurred from 1790 to 1840 and ultimately extirpated Hawaiian sandalwood species from an estimated 90% of their natural range [7,8]. All known Hawaiian sandalwood species are still extant, although

they occur in much smaller populations, and one variety, *Santalum freycinetianum* var. *lanaiense* Rock, is endangered [6,9]. Historic distribution models show that sandalwood populations covered a broad elevational gradient (0–2550 m) and grew in most Hawaii forest types before being overharvested [10,11]. After the collapse of the sandalwood trade, forests that once hosted sandalwood populations were converted to agricultural use, predominantly for sugar cane and cattle grazing [7]. The introduction of invasive species associated with the conversion of native forests to an agricultural landscape contributed to the displacement and eventual extinction of unknown numbers of native plant and animal species in Hawaii, ultimately leading to the deterioration of and the disruption of ecosystem services [12–16]. Except for the coastal species, *Santalum ellipticum* Gaudich., remnant populations of Hawaiian sandalwood primarily grow in the high-elevation regions on six of the main Hawaiian Islands [5,17]. Remnant populations persist across a patchwork of public and private lands, with varying levels of disturbance and management intensity [18–20].

Many areas that once hosted Hawaiian sandalwood forests are actively targeted for restoration planting, and there is also a burgeoning interest in practicing commercial sandalwood forestry in Hawaii [18,21]. The historical uses for sandalwood persist in the modern day, while new uses are evolving in the essential oil and pharmaceutical industry [1,22,23]. The global demand for sandalwood has steadily outpaced the supply, causing prices to soar and creating an opportunity for savvy sandalwood foresters [24]. The Hawaiian Islands are uniquely poised to host a burgeoning sandalwood industry that could restore ecological, economic, and cultural value to a landscape that has historically been overexploited for its natural resources. The Hawaii Island endemic, *Santalum paniculatum* Hook. and Arn., produces commercial grade heartwood oil and is of particular commercial interest [25].

Commercial production and restoration planting will require reliable propagation protocols to produce high-quality seedlings. The seedling quality is closely linked to survival and performance at outplanting and can be influenced by nursery culture practices, including but not limited to container selection, fertilization, irrigation, and competition reduction [26]. Therefore, understanding how different cultural practices affect specific aspects of seedling quality is imperative to designing effective and efficient propagation protocols. Such protocols are established for other sandalwood species outside of Hawaii, such as *S. album*. The literature has shown that co-planting *S. album* seedlings with a pot host enhances growth during the nursery grow-out phase and after field planting [27,28]. Additionally, providing essential nutrients improves the growth of *S. album* seedlings [29–31]. However, Hawaiian sandalwood propagators cannot adopt many of the established methods because of differences in sandalwood species physiology, available host species, and growing environments [5,27,28,32–36]. Seed germination protocols developed for *S. album* have been successfully adapted for Hawaiian species, but protocols for growing seedlings to planting maturity require further development [37]. The nursery propagation of sandalwood is complicated by its hemiparasitic nature and the resulting uncertainty concerning best practices for integrating hosts and fertilizers into nursery culture practices to produce the highest quality seedlings. Understanding how nutrient availability and co-planted hosts should be utilized to produce high-quality *S. paniculatum* seedlings is the first of many steps in developing comprehensive propagation protocols.

As root hemiparasites, members of the *Santalum* genus are capable of autotrophic carbon gain through photosynthesis and heterotrophic carbon gain through parasitic attachments to the root xylem tissues of neighboring host plants [38,39]. In addition to carbohydrates, sandalwoods can acquire water, mineral nutrients, amino acids, and other organic xylem solutes from host plants [40,41]. Although sandalwood can grow without a host, attachment to a host increases its growth rates and capacity to grow competitively [32]. Nitrogen-fixing plants were initially thought to be superior hosts, although growth trials with *S. album* have since shown varying levels of benefit from different N-fixing species, with some providing less benefit than some non-N-fixing host species [28,42,43]. Sandalwood trees use a specialized organ called haustorium to attach to

the host roots, penetrate the root epidermis, and gain access to the host root xylem, where they extract resources [31,40,43]. The host-derived acquisition of resources is reliant on haustoria connections, and there is potential for the host to negatively affect the sandalwood through competition if they do not become attached [28,40].

Controlled-release fertilizer (CRF) has been used to produce high-quality seedlings with Hawaiian sandalwood species [37,44,45]. These are water-soluble fertilizers encased in a semi-permeable polymer coat that allows mineral ions to leach through at a controlled rate [46]. The regulated release of nutrients may lead to reduced leaching and improved fertilizer use efficiency compared to conventional non-encapsulated fertilizers [47,48]. Controlled-release fertilizers have been used effectively for agricultural crops and forest tree species, although the effect of CRF on Hawaiian sandalwood has not been quantified [46,49–51]. In addition to CRF, propagators of Hawaiian sandalwood have found that chelated iron treatments increase the growth and correct commonly occurring iron deficiencies of Hawaiian sandalwood seedlings [37,44,45]. Chelated iron fertilizers are composed of a Fe^{2+} ion bound to a synthetic chelating agent such as ethylenediamine di-(o-hydroxyphenyl acetic) acid (EDDHA) [52]. Chelated forms of iron remain water-soluble and available for plants in calcareous soils, whereas unchelated forms of iron typically do not [53]. Chelated iron is provided to the plant in a form that is readily absorbed by the plant roots or leaf tissue and rapidly corrects iron deficiencies in numerous crops and Hawaiian sandalwood [45,54,55]. Pot hosts and fertilizers have been used to successfully increase the seedling quality of *S. album*, although further investigation is required to determine their effects on Hawaiian sandalwood seedlings [3,23,28,31,56]. We conducted an experiment to quantify the independent and interacting effects of CRF, chelated iron, and pot host species on the seedling quality of Hawaiian sandalwood (*S. paniculatum*).

Our experiment utilized *S. paniculatum*, which has the most extensive potential range and the largest remnant population of the Hawaiian sandalwoods [10,17,57]. *S. paniculatum* is endemic to Hawaii Island, the largest of the main Hawaiian Islands and it is the only Hawaiian species that is commercially cultivated or harvested. The oil extracted from *S. paniculatum* heartwood is high in santalols, increasing the value of the oil to be comparable to Indian sandalwood oil [25]. It is a broadleaf evergreen tree that forms a single bole trunk and can reach heights of 13 m to 20 m when mature [11]. It grows in moderately wet to dry forests, although remnant populations primarily grow in high-elevation mesic and dry forests on the west side of Hawaii Island [5].

Specifically, we hypothesize that (i) nutrients available from CRF will improve the quality of *S. paniculatum* seedlings through increased nutrient acquisition, decreasing the chance that nutrients will be a limiting factor to growth; (ii) the interaction between the CRF and chelated iron will have a significant effect on seedling quality because the chelated Fe will ensure adequate Fe concentration; and (iii) the host treatment will improve the seedling quality by providing a host to parasitize and extract resources to support growth. Specifically, the nitrogen-fixing *Acacia koa* A. Gray, host will provide the greatest benefit to sandalwood seedling quality.

2. Materials and Methods

2.1. Experimental Site

The experiment was conducted at the Hāloa ʻĀina Reforestation Project (HARP) native plant nursery (N 19°32′16.550″, W 155°48′22.101″, elevation: 1462 m) in Kealakekua on Hawaii Island. The HARP manages degraded sandalwood forests on 1164 hectares of privately owned land and 435 hectares of lease land in the montane regions of Kealakekua. The experiment occurred in a 7 m × 8 m shade house with a 50% shade film roof and walls of 70% shade cloth. The top was 4 m tall on the east end and tapered down to 3 m on the west. We recorded the temperature and humidity at a weather station installed 30 m from the experiment site. The average annual temperature and relative humidity of the growing area during the experiment (9 August 2020–5 August 2021) were 18.3 °C and 70% RH, respectively, and the minimum and maximum temperatures were 3.3 °C and 27.7 °C, respectively.

2.2. Experimental Design and Treatments

The experiment was a randomized complete block design with a full factorial combination of CRF (two levels; applied, control), chelated iron fertilizer (two levels; applied, control), and host species (three levels; *A. koa*, *Dodonaea viscosa* Jacq., control) as the predicting factors. We defined twelve unique treatments by combining the three predicting factors. The experiment had four blocks containing one representation of each of the twelve treatments. Each treatment was represented by a tray of 32 seedlings subsamples that received the same combination of CRF, chelated iron, and host species. We randomly selected twelve of the 32 seedlings from each treatment for destructive sampling at the end of the grow-out period. We grew 1536 (32 * 12 * 4) seedlings and randomly selected 576 (12 * 12 * 4) for destructive sampling. The treatment trays were randomly distributed within each block and were rearranged within the block every three months to maintain independence between treatments and limit edge effects.

S. paniculatum seeds were germinated by first soaking them for 24 h in a solution of 400 ppm gibberellic acid (GA3) (ProGibb T and O®, Valent BioSciences, Libertyville, IL, USA) and distilled water. After the GA3 solution was rinsed from seeds, they were sown into a bed of vermiculite one inch from the surface and watered top-down twice per week. We transplanted the *S. paniculatum* seedlings from the vermiculite bed into grow-out containers at the 4–6 true leaf stage on 6–7 August 2020. The mean height and root collar diameter (RCD) of the *S. paniculatum* seedlings at the time of transplantation was 8.6 cm (±0.23) and 3.01 mm (±0.07), respectively. The seedlings were grown in a 1540 mL container (MT49BT; 10 cm × 10 cm × 23 cm depth) (Stuewe and Sons Inc., Tangent, OR, USA) in media composed of equal parts of PRO-MIX MP MYCORRHIZAE ORGANIK® potting soil (Premier Tech Horticulture®, Quakertown, PA, USA), perlite, and fine black cinder. The host seedlings were transplanted into designated growing containers with *S. paniculatum* seedlings on 16 December 2020. The mean shoot height and RCD of the hosts when transplanted was 10.3 cm (±3.1) and 2.1 mm (±0.18) for *A. koa* (n = 25) and 8.8 cm (±1.6) and 0.9 mm (±0.01) for *D. viscosa* (n = 25), respectively. The hosts were co-planted when the *S. paniculatum* seedlings were four months old, which provided a competitive advantage to the sandalwood. We replaced host seedlings that died until 20 January 2021, and excluded subsamples with dead hosts in the random sampling. The host seedlings were not pruned.

Controlled-release fertilizer was applied to designated treatments by incorporating Osmocote plus® (5–6-month release, at 21.1 °C)(Scotts Co.® Marysville, OH, USA) into the soil at medium bag rates (6.2 kg/m^3), which was then reapplied at six months by mixing 9.2 g per container into the surface of the soil. Osmocote plus® is composed of 15% total nitrogen, 9% available phosphate, 12% soluble potash, 1.3% Mg, 6.0% S, 0.02% B, 0.05% Cu, 0.46 % Fe (0.01% chelated), 0.06% Mn, 0.02% Mo, and 0.05% Zn by weight. The fertilizer and soil were mixed thoroughly with a shovel until the fertilizer was considered evenly distributed via visual inspection. Once the grow-out container trays were filled with growing medium, the treatments were randomly assigned to trays. Chelated iron was applied to the designated treatments one month after we transplanted *S. paniculatum* seedlings to growth containers. The chelated iron was applied by dusting 4.8 g of fertilome® EDDHA chelated iron (6% water soluble iron) (Voluntary Purchasing Groups®, Bonham, TX, USA) onto the soil and watering it through until all powder was dissolved from the surface. The chelated iron was first applied on 14 September 2020, then reapplied with the same method on 28 December 2021 and 12 April 2021.

Each container received approximately 265 mL of tap water applied top-down with a hand watering wand once per week for the first six months, then twice per week for the remaining duration of the experiment. The same volume (265 mL) of a dilute solution (374 ppm) of Miracle-gro® water-soluble fertilizer (18% N, 18% P, 21% K, 0.5% Mg, 0.02% B, 0.05% Cu, 0.1% Fe (0.1% chelated Fe), 0.05% Mn, 0.0005% Mo, 0.05% Zn) (Scotts Co.® Marysville, OH, USA) was applied to every plant in place of irrigation water one day every

other week to ensure the control treatment plants did not die and grew large enough to provide leaf samples for the nutrient analysis.

2.3. Plant Material

S. paniculatum seeds were collected from a site 37.6 km northeast of the experiment site (N 19°48′22.6″, W 155°36′12.8″, elevation of 2025 m) in an ecoregion similar to the experiment site. *Acacia koa* A. Gray, a nitrogen-fixing tree, and *D. viscosa*, a non-nitrogen-fixing tree shrub, were chosen as the species for the host treatment. They are commonly associated with *S. paniculatum* in the surrounding forest and are frequently used in restoration planting practices in Hawaii [58–60]. *A. koa* is endemic to Hawaii, and while *D. viscosa* is a native Hawaiian species, it is widespread and also associated with *S. album* in India [11,61]. All host seedlings are grown from seeds collected within 2 km of the experiment site. The *A. koa* seeds were scarified by soaking them in 95 °C water for 24 h as they cooled, then sown into a tray of vermiculite 7 cm deep. The *D. viscosa* seeds were soaked in distilled water for 24 h and sown with the same method. All seedlings were inoculated with rhizobium collected from potted *A. koa* seedlings from the Hāloa ʻĀina nursery. A 124 mL slurry of *A. koa* nitrogen-fixing nodules was diluted into 190 L of water and applied to all pots with a watering wand.

2.4. Measurements

Destructive sampling occurred by block starting on 24 June 2021 and lasting until 5 August 2021, which spread the age at sampling from 322 to 364 days. Thirty-four seedlings were girdled by rats or slugs and were not included in the sampling and analysis. Chlorophyll index measurements were collected using an atLEAF+® chlorophyll meter. The atLEAF+® chlorophyll meter measures the greenness of a leaf sample to produce a chlorophyll index value similar to a SPAD® meter [62]. The chlorophyll index generated by the atLEAF+ and SPAD® meters correlates with the chlorophyll concentration of the measured leaf [62]. We used the mean of five readings from each seedling for the analysis. Chlorophyll readings were taken from the newest pair of fully mature leaves on the terminal shoot, at two-thirds the distance from the leaf base to the apex [63]. The leaves used for chlorophyll readings were collected, dried, and ground for the tissue nutrient analysis for the concentrations of N (Total), P, K, S, Mg, Ca, Na, B, Zn, Mn, Fe, Cu, and Cl [64]. Sample leaves for the nutrient analysis were collected from each of the twelve subsamples to form a composite sample representing each treatment in a block. We calculated the foliar N and Fe contents for each seedling by multiplying the treatment's nutrient concentration by the seedling's dry mass.

The growing medium was washed from seedlings using a water bath. The removed soil was filtered to catch severed root fragments to be incorporated into the root dry mass (g) measurement. We measured the shoot length (cm) from the root collar to the last node on the terminal shoot using a meter stick, and we measured the RCD (mm) using dial calipers. The shoot and root were severed at the root collar and dried in a convection drying oven at 70 °C for 72 h to achieve a constant mass [65]. The dry mass (g) of the shoots, roots, and leaves were measured using a Mettler Toledo® AB104-S analytical balance. We calculated the shoot/root ratio for each seedling by dividing the shoot dry mass by the root dry mass. We visually counted the number of haustoria on the roots of each sandalwood seedling and the number of haustoria attached to the host for paired seedlings.

2.5. Statistical Analysis

The response variables (RCD, height, dry mass, shoot: root, chlorophyll index, haustoria count, foliar N concentration, foliar N content, foliar Fe concentration, and foliar Fe content) were analyzed separately using a linear mixed effects model with the block and treatment ID as a random factor. The treatment ID was added as a random factor to eliminate pseudo-replication in the model from subsampling within a treatment replicate. The samples for foliar nutrient concentrations (N and Fe) were composites of the treat-

ment, meaning only the block was included as a random factor in the analysis. We used a three-way analysis of variance (ANOVA), and a type III sum of squares was calculated on each model to examine comparisons defined in the a priori hypotheses. We evaluated the residual values of each model to ensure the assumptions of normality and homogeneity of variance were met before the analysis. The response variables that did not meet the assumptions of the linear model were log-transformed to satisfy the model assumptions. A comparison of the estimated marginal means comparison (emmeans) with Tukey's *p*-value adjustment was used to determine significant differences ($\alpha = 0.05$) among treatments when detected with the ANOVA ($p < 0.05$). All statistical analyses were performed with R software (R Version 3.2.4, the R Foundation for Statistical Computing Platform).

3. Results

3.1. Plant Morphology

The mean dry mass of *S. paniculatum* seedlings was significantly affected by the CRF ($F_{1,33.17} = 439.58$, $p < 0.001$) and chelated iron ($F_{1,3.19} = 50.47$, $p < 0.001$), but the host treatment had no effect (Table 1). However, there was a significant interaction between the CRF and chelated iron treatments ($F_{1,33.20} = 34.46$, $p < 0.001$) (Table 1), which was associated with an increase in mean dry mass when we applied chelated iron without CRF, but not when applied with CRF (Figures 1 and 2). The mean dry mass of the seedlings that received chelated iron only was 3.09 g (±0.26) compared to 1.51 g (±0.26) for the seedlings that did not receive CRF or chelated iron (Figure 1).

Table 1. The *p* values resulting from a three-way ANOVA performed on mixed effect models fitted for each response variable. CRF = controlled-release fertilizer; Fe = chelated iron fertilizer; S/R = shoot/root ratio; Chl. = chlorophyll index.

	Dry Mass	Collar Diameter	Shoot Height	S/R	Chl.	N Cont.	N Conc.	Fe Cont.	Fe Conc.	Total Haustoria
CRF	**<0.001**	**<0.001**	**<0.001**	0.442	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.211
Fe	**<0.001**	**<0.001**	**<0.001**	0.400	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.832
Host	0.145	0.291	0.640	0.249	**0.004**	0.198	0.876	0.435	**0.014**	**0.015**
CRF × Fe	**<0.001**	**0.032**	**0.002**	0.951	**<0.001**	**0.016**	**<0.001**	**<0.001**	0.703	0.319
CRF × Host	0.656	0.845	0.452	0.533	0.227	0.178	0.268	0.166	0.090	0.663
Fe × Host	0.214	0.389	0.658	0.708	0.120	0.170	0.575	0.634	0.528	0.905
CRF × Fe × Host	0.701	0.532	0.884	0.506	0.138	0.156	0.270	0.856	0.736	0.592

Bolded values represent a significant effect on the response variable.

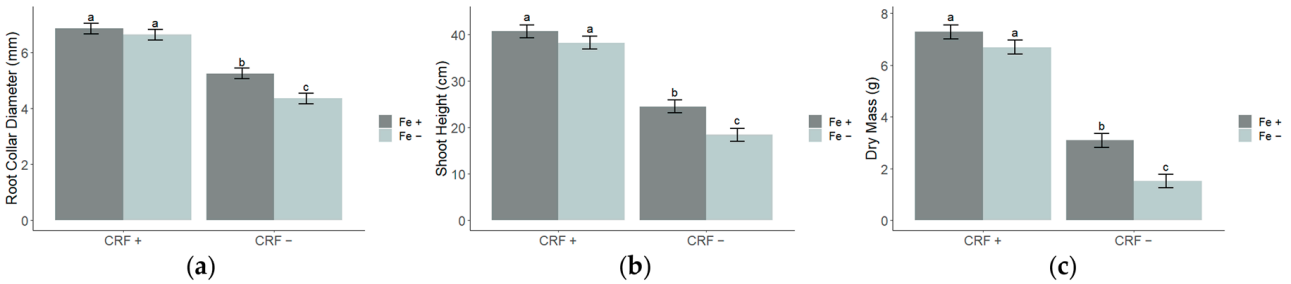

Figure 1. The mean (±SE) (**a**) dry mass, (**b**) root collar diameter, and (**c**) shoot height values of *S. paniculatum* seedlings at the end of the experiment. Different letters indicate significant differences among treatments ($\alpha = 0.05$). CRF improved growth in all treatments, although chelated iron only improved growth when applied without CRF.

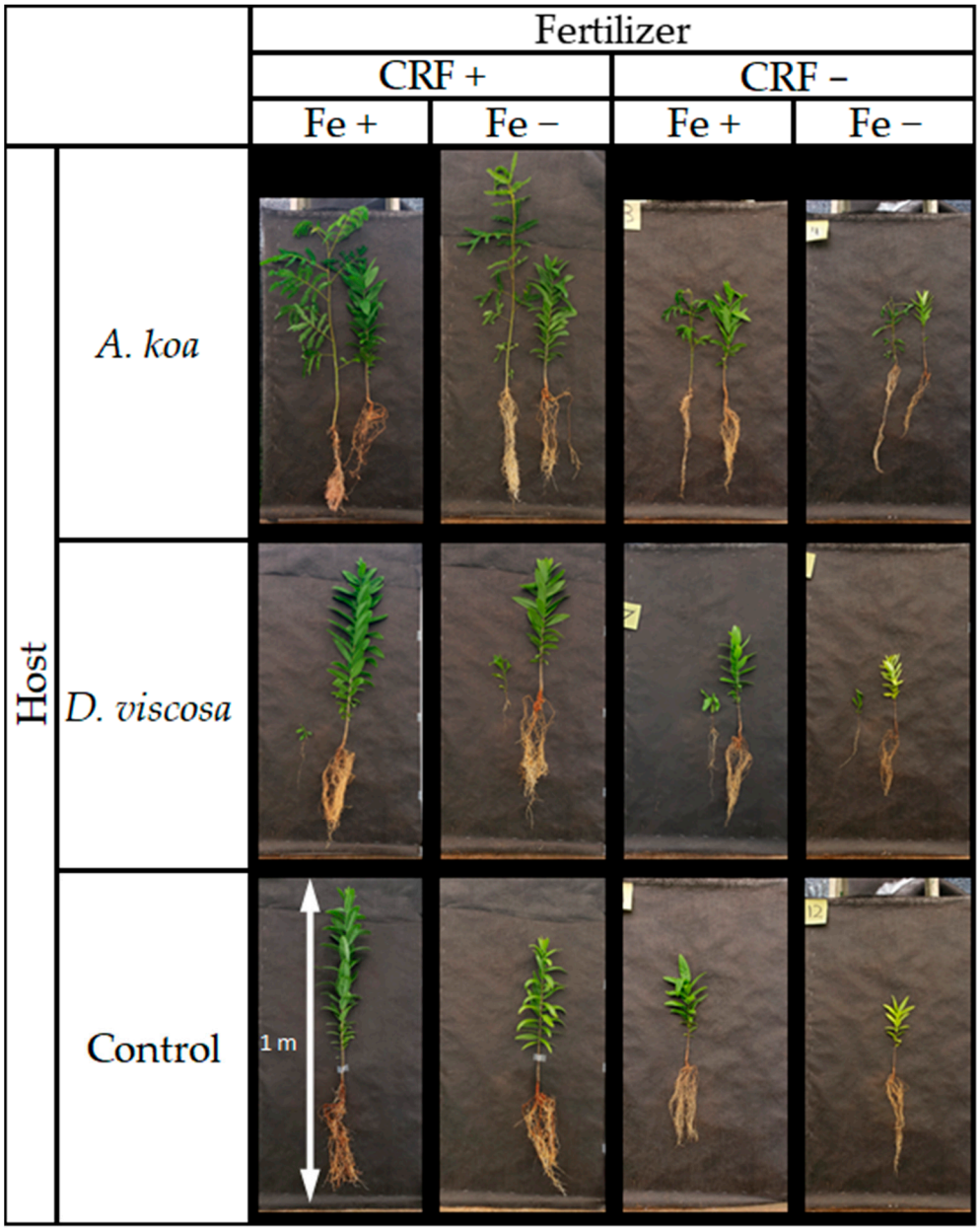

Figure 2. Pictures of representative seedlings from each of the twelve unique treatments show that seedlings that received CRF were significantly larger than seedlings that did not receive CRF. The *D. viscosa* hosts were much smaller than the *A. koa* hosts at the end of the experiment. Fe+ = chelated Fe applied; Fe− = control; CRF+ = CRF applied; CRF− = control.

The mean root collar diameter was significantly affected by CRF ($F_{1,33.72}$ = 165.64, $p < 0.001$) and chelated iron ($F_{1,33.73}$ = 13.62, $p < 0.001$) but was not affected by the host treatment (Table 1). There was a significant interaction between CRF and chelated iron ($F_{1,33.20}$ = 5.0, $p = 0.032$) (Table 1) that was associated with an increase in RCD when we applied chelated iron without CRF but not when applied with CRF (Figures 1 and 2). The mean root collar diameter of treatments that only received chelated iron was 5.25 mm (±0.18) compared to a mean of 4.34 mm (±0.18) in treatments that did not receive chelated iron or CRF (Figure 1).

There was a significant effect of CRF ($F_{1,32.97}$ = 285.79, $p < 0.001$) and chelated iron ($F_{1,32.98}$ = 24.41, $p < 0.001$) on the seedling dry mass, but the effect of the host treatment was not significant (Table 1). The interaction between CRF and chelated Fe had a significant effect ($F_{1,32.99}$ = 11.14, $p = 0.002$) (Table 1) associated with an increase in dry mass when we

applied chelated iron without CRF but not when applied with CRF (Figures 1 and 2). The treatment plants that only received chelated iron had a significantly greater mean shoot height of 24.05 cm (±1.38) compared to 18.4 cm (±1.38) from those that did not receive CRF or chelated iron (Figure 1). The mean shoot root ratio was not significantly affected by CRF ($F_{1,33.05} = 1.00$, $p = 0.324$), chelated iron ($F_{1,33.05} = 1.74$, $p = 0.196$), or the host ($F_{1,33.05} = 1.57$, $p = 0.223$).

3.2. Chlorophyll Index

The mean chlorophyll index was significantly affected by the CRF ($F_{1,21.69} = 1263.8$, $p < 0.001$), chelated iron ($F_{1,21.71} = 157.52$, $p < 0.001$), and host ($F_{1,21.81} = 157.52$, $p = 0.004$) treatments (Table 1). The mean chlorophyll index values of *A. koa* and control (49.4 ± 0.7) plants were greater than for the treatment plants that received *D. viscosa* (47.0 ± 0.7) (Figure 3). The interaction between the CRF and chelated iron had a significant effect ($F_{1, 21.69} = 59.60$, $p < 0.001$) (Table 1) on the chlorophyll index, which was associated with an increase in the mean chlorophyll index when we applied chelated iron without CRF, but not when CRF was applied (Figure 3).

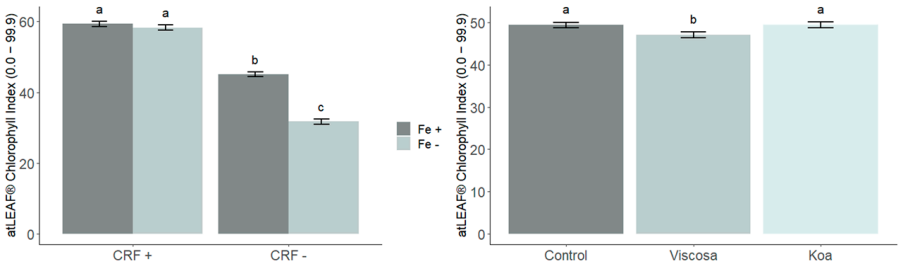

Figure 3. Mean (± SE) chlorophyll index values. (**a**) The interaction between CRF and chelated iron significantly affected the chlorophyll index, causing chelated iron to have an effect only when applied without CRF. (**b**) Seedlings paired with *D. viscosa* had a lower chlorophyll index value than *A. koa*-paired and control seedlings. Different letters indicate significant differences among treatments ($\alpha = 0.05$). Fe+ = chelated Fe applied; Fe− = control; CRF+ = CRF applied; CRF− = control.

3.3. Foliar Nitrogen and Iron

The mean nitrogen concentration was significantly affected by the CRF ($F_{1,33} = 315.38$, $p < 0.001$) and the chelated iron ($F_{1,33} = 10.26$, $p = 0.003$), but it was not affected by the host treatment (Table 1). The interaction between the CRF and chelated iron had a significant effect ($F_{1,33} = 9.77$, $p < 0.001$) (Table 1) associated with an increase in the mean N concentration when we applied chelated iron without CRF but not when applied with CRF (Figure 4). The mean N concentration of the seedlings that received only chelated Fe was 1.70% (±0.16) compared to 2.44% (±0.16) for the treatments that did not receive CRF or chelated iron (Figure 4).

The mean nitrogen content of the sandalwood seedlings was significantly affected by the CRF ($F_{1,33} = 315.38$, $p < 0.001$) and chelated iron ($F_{1,33} = 10.26$, $p = 0.003$) but was not affected by the host treatment (Table 1). The interaction between the CRF and chelated iron had a significant effect on the mean N content ($F_{1,32.73} = 6.48$, $p < 0.001$) (Table 1) associated with an increase in mean nitrogen content when we applied chelated iron without CRF but not when applied with CRF (Figure 4). The mean N content of seedlings that only received chelated Fe was 21.9 mg (±5.8) compared to 15.1 mg (±5.8) for seedlings that did not receive CRF or chelated iron (Figure 4).

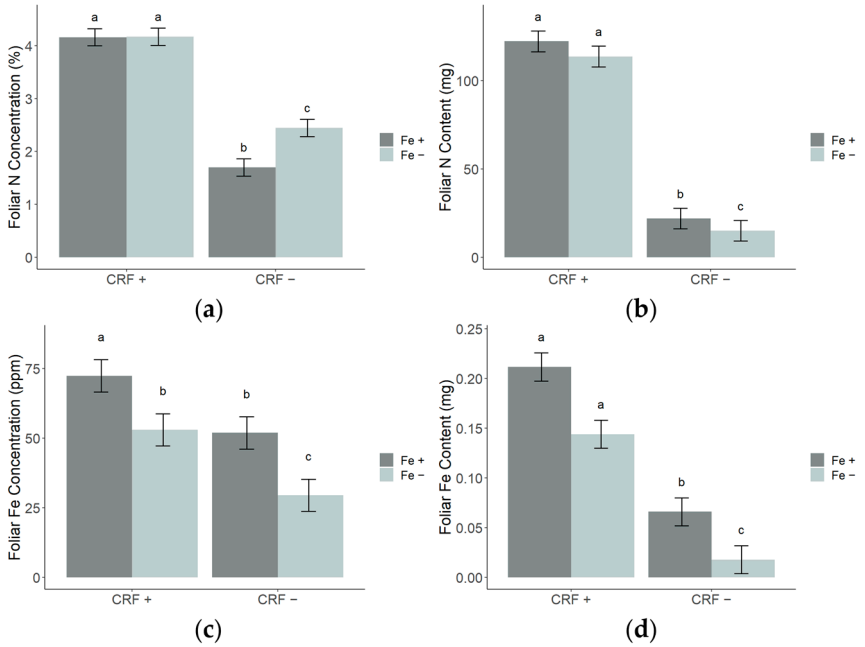

Figure 4. The mean (±SE) foliar nitrogen (N) (concentration (%) and content (mg)) and foliar iron (Fe) (concentration (ppm) and content (mg)) values of *S. paniculatum* seedlings. (**a**) The N concentration (%), (**b**) N content (mg), and (**d**) Fe content was increased in all plants that received CRF. The chelated iron had a significant effect when applied without CRF, decreasing the N concentration, but increasing the N content and Fe content. (**c**) The foliar Fe concentration was the same in treatments that only received CRF and treatments that only received chelated iron. Different letters indicate significant differences among treatments ($\alpha = 0.05$). Fe+ = chelated Fe applied; Fe− = control; CRF+ = CRF applied; CRF− = control.

The iron concentration (ppm) was significantly affected by CRF ($F_{1,33} = 31.80$, $p < 0.001$), chelated iron ($F_{1,33} = 28.74$, $p < 0.001$), and the host ($F_{2,33} = 4.80$, $p = 0.015$) treatment (Table 1). The mean Fe concentration of treatments that received CRF was 62.7 ppm (±5.1) compared to 40.7 ppm (±5.1) for those that did not. The mean Fe concentration of treatments that received chelated iron was 62.2 (±5.1) compared to 41.2% (±5.1) for those that did not. The mean Fe concentration of seedlings that received the *A. koa* host treatment (60.2 (±5.46)) was significantly greater than the mean for *D. viscosa* (46.6 (±5.35) and control (48.4 (±5.46)) (Figure 4). None of the interactions between predictors significantly affected the mean Fe concentration (Table 1)

3.4. Haustoria Abundance

The host treatment significantly affected the mean total haustoria present on a sandalwood seedling ($F_{2,32.39} = 4.83$, $p = 0.015$) (Table 1). The mean number of haustoria on *A. koa*-hosted sandalwood seedlings (19.5 (±2.53)) was greater compared to the mean number of haustoria found on the *D. viscosa*-hosted (12.5 (±2.53)) and control (11.0 (±2.53)) seedlings. There was no difference between the *D. viscosa*-hosted and control treatment levels. The count of the attached haustorium revealed that 54.7% (±5.8) of the sandalwood seedlings paired with *A. koa* were attached and 14.4% (±3.8) of the seedlings paired with *D. viscosa* were attached.

4. Discussion

Our results confirm that CRF, chelated Fe, and pot host species significantly affect several aspects of *S. paniculatum* seedling quality, including the height, collar, dry mass, chlorophyll concentration, and nutrient status. Although each predictor affected the seedling quality differently, the nutrient availability provided by CRF had the most significant effect. In support of our hypothesis (i), we found that nutrients provided by CRF significantly

increased in shoot height, RCD, dry mass, chlorophyll index, and foliar nutrient (N and Fe) concentrations and contents relative to the non-fertilized control. Increased macronutrient availability resulting from applying CRF has been shown to improve growth in other Hawaiian forest species [51,66]. Similar to studies with other sandalwood species, our findings demonstrate that nutrient availability is a limiting factor for growth and that simply providing nutrients to the hemiparasitic sandalwood can produce high-quality seedlings [29,31]. In further support of our findings, a study that controlled nutrient levels through fertigation demonstrated that providing macronutrients (N, P, K, S, Ca) increases the shoot height and dry mass in nursery-grown *S. album* seedlings independent of a host [31]. The nutrient availability unexpectedly did not affect the shoot root ratio or the total count of haustoria, which was contradictory to what Barret and Fox (1997) found with *S. album*.

In support of our hypothesis (ii), we found that the interaction between the nutrient availability and chelated iron significantly increased the shoot height, RCD, dry mass, and chlorophyll index and improved the nutrient status, although the mechanism of effect was different from what was predicted. Rather than chelated iron improving the seedling quality when applied with CRF, it only enhanced the seedling quality in a nutrient-limiting growing environment that did not receive CRF. The foliar Fe concentration was the same for treatments that only received CRF (53.00 ppm ($\pm$5.79)) and for treatments that only received chelated iron (51.92 ppm ($\pm$5.79)), implying that both fertilizers provided adequate levels of Fe, within the range required to avoid deficiency and toxicity symptoms (44–250 ppm) [67]. The nitrogen content increased but the N concentration decreased when chelated Fe was applied in a nutrient-limiting environment. The increased growth and dry mass associated with applying chelated iron in a nutrient-limiting environment resulted in increased N content values. However, the nutrients are diluted across more tissue, which can cause a decrease in the foliar concentration [68,69]. The opposite trend was observed for the Fe concentration, likely because the chelated Fe fertilizer provided sufficient Fe to maintain an increase in Fe concentration despite the increase in dry mass.

The chelated iron treatment only provided a benefit to growth in a nutrient-limiting environment, suggesting that iron may be a limiting factor for growth under these conditions. Fe is required by plants in the greatest quantity of any micronutrient and is necessary for fundamental cellular processes, including chlorophyll biosynthesis, electron transport, and vital enzymatic reactions [53,70]. Fe is the second most abundant mineral in the earth's crust and rarely deficient in soils, although calcareous soil conditions convert plant-available ferrous iron (Fe^{2+}) to the more unavailable form of ferric iron (Fe^{3+}) [69,71,72]. Plants can secrete organic acid compounds and phyto-chelators into the soil to increase Fe availability in calcareous Fe-limiting environments [67,71]. Applying synthetic chelated Fe emulates the natural secretion of phyto-chelates and more efficiently corrects iron deficiencies compared to Fe-compound and natural Fe-complex fertilizers [73]. Chelated iron is widely used in industrial agriculture to correct iron deficiencies and improve yields, although its application in forest restoration systems is largely unstudied [74,75]. Considering that deforestation can decrease the abundance of natural iron chelators in the soil, further investigation into the effects of synthetic iron chelators in silvicultural systems is warranted [76]. Our findings show that chelated iron provided a benefit to growth in a nutrient-limiting nursery environment, although further research is required to see if this effect persists in a field setting. It should be noted that the growing medium that was used (PRO-MIX MP MYCORRHIZAE ORGANIK®) contains endomycorrhizal inoculum PTB297, and endomycorrhiza has been shown to increase the iron uptake by promoting the production of plant-derived iron chelators [77].

The evidence for the influence of the pot host on the seedling quality (hypothesis iii) was largely unsupported by our findings. We found that the pot host only affected the haustoria abundance and Fe concentration, contradicting results from growth trials that demonstrated that co-planting pot hosts of numerous species enhanced the growth of co-planted sandalwood seedlings [28,43]. It is likely that the growth of our sandalwood seedlings did not benefit from the pot host treatment, because of the poor rates of attachment

that resulted from the pairings. Although only 54.7% (±5.8) of *A. koa*-paired seedlings and 14.4% (±3.8) of *D. viscosa*-paired seedlings were attached by the end of the experiment, we assumed those that were attached were receiving nutrition from their hosts. Haustoria formation is initiated by chemical signals from the host plant in many hemiparasitic plants, including sandalwood [43,78–80]. Chemical signals from different host plants can provide differential benefits for haustoria formation and root growth prior to being attached to the sandalwood [81]. The suitability and resulting influence of the pot host varies by species, and both the N-fixing and non-N-fixing species have been found to be suitable hosts for *S. album* [28,43,82]. Like studies with other species of sandalwood, we found that a nitrogen-fixing host species produced greater haustoria abundance compared to a non-N-fixing host species, although both had relatively low rates of attachment to the sandalwood [78,83,84]. The duration of our experiment was determined by the prevailing methodology for raising *S. paniculatum* in the nursery, although considering the low percentage of plants that attached to their host, this rearing period may not allow enough time for the sandalwood to establish haustoria connections with the host. A longer rearing time may allow more haustoria connections to form, but may not be practical for nursery cultivation, where an increased rearing time translates to increased costs. Further research is required to elucidate the mechanisms of the haustoria attachment in Hawaiian sandalwood and to determine whether culture practices could be developed to enhance the rate of attachment in nursery propagation. Considering the pot host did not negatively affect the sandalwood development, using a pot host may still be advisable if the host benefits sandalwood field planting performance similar to *S. album*. More research is required to determine the effect of the co-planted nursery host on the field planting success of Hawaiian sandalwood.

5. Conclusions

Hemiparasitic *Santalum* spp. are known to acquire carbon and mineral nutrients autotrophically and heterotrophically. We demonstrated that applying fertilizers significantly improved the growth of *S. paniculatum*, while pairing with a pot host did not affect the growth. The increased nutrient availability from the CRF application consistently improved the growth; however, chelated iron fertilizers only improved the growth in a nutrient-limiting environment where CRF was not applied, indicating iron availability may be a limiting factor to *S. paniculatum* growth in nutrient-poor environments. Applying CRF is an effective and cost-efficient method for improving sandalwood seedling growth and should be integrated into propagation protocols for Hawaiian sandalwood species and would likely enhance the growth of all sandalwood species. The pot host treatment did not affect the growth of the *S. paniculatum* seedlings during nursery propagation. However, the *A. koa*-paired seedlings had more haustoria, so the pot host may provide a benefit following field planting [27]. We suggest that *S. paniculatum* should still be planted with a pot host in the nursery, although further research is required to improve this treatment for Hawaiian sandalwood species. Although our study focused on nursery culture practices specific to the Hawaiian endemic *S. paniculatum*, our results contribute to the scientific knowledge base of propagation practices for the commercially valuable *Santalum* genus and other hemiparasitic species.

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