Improving Cacao Based Reforestation through Whole Soil Microbial Inoculation.

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Made in dedication to Will Malicote, a great brother, friend, student and entrepreneur.

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Abstract

Deforestation is one of the greatest greenhouse gas sources, releasing more greenhouse gas than all the world's cars, trucks and trains combined. Since deforestation rates are highest in the tropics reforestation efforts in this region are greatly needed. In particular, regenerative agroforestry would not only increase tree cover but also provide farmers with a livelihood outside of the agriculture associated with deforestation. Regenerative Agroforestry creates a matrix that is valuable to wildlife and the people who most depend on the land to survive. In Ecuador, where my study took place, Cacao is a native tree valuable to smallholder farmers, but depending on the variety and conditions, may or may not be ideal for reforestation. A technique that could improve reforestation efforts is inoculation with mycorrhizal fungus taken from a nearby intact ecosystems. Therefore, in this study I ask: 1) do whole soil inoculants taken from nearby secondary forest increase arbuscular mycorrhizal fungi (AMF) in planted cacao trees? 2) Does percent canopy cover and cacao cultivar influence the beneficial effects of whole soil inoculants on plant growth? And, 3) Is the level of AMF in plant roots associated with plant growth? My results suggests that whole-soil inoculations did increase AMF colonization in cacao seedlings in terms of easily extractible glomalin-related soil protein and relative quantity AMF DNA but not total glomalin-related soil protein and percent root length colonization. Data from 480 cacao seedlings over 1 year suggests whole soil inoculations resulted in an increase in seedling growth. In addition soil inoculations resulted in a decrease in herbivory in both cacao cultivars at 2 months. Furthermore, full sun had a negative effect on cacao growth (a predicted 79% decrease) which was reversed when whole soil inoculations were used resulting in higher predicted growth under full sun. Given the low costs, I recommend introducing nearby intact forest soil for ongoing reforestation efforts in order to combat climate change with carbon sequestration and increase small holder farmer's adaptation to a changing climate with regenerative agroforestry.

Background

The tropics face the highest deforestation rates in the world (Hansen et al. 2013, González-Jaramillo et al. 2016). Furthermore, the natural recovery of abandoned land, which makes up about 62 million hectares, is greatly impeded in the tropics because of high levels of degradation (Daily 1995, Mendoza 2007, Aguirre et al. 2011, Poore 2016). Still, tropical reforestation is critical, not only to slow down the rapid rate of biodiversity loss in this biome, but also to counter the carbon emissions associated with tropical deforestation (van der Werf et al. 2009, Ipcc 2014, Jantz et al. 2014, Bastin 2019).

The land cover change of tropical forests to pastures have turned productive and diverse ecosystems into an unrecognizable field of mono-dominant exotic grasses (Mendoza 2007). This is in part due to the fact that cattle greatly increase soil compaction (Mendoza 2007), and once pasture land is abandoned, the degraded soils stall succession to forest (Daily 1995, Mendoza 2007). Without intervention, it can take up to 100 years for forest regrowth in abandoned pasture (Daily 1995). Under such circumstances, natural successional processes might need to be jumpstarted via active restoration. However, for most rural tropical communities resources for restoration are limited. Still, reforestation practices incorporating agroforestry could turn abandoned pasture into a forested ecosystem, while also providing a livelihood to locals struggling with poverty (Verchot et al. 2007).

Agroforestry practices often involve a valuable crop, such as cacao or coffee, and intercropped fruit, nut, or timber shade tree. Often the shade trees are nitrogen-fixing legumes. The resulting plant community contributes to the development and regeneration of soil resources (Burrows and Pfleger 2011). In places like Latin America there is the opportunity to plant a high value tree, cacao, which is also a native species and therefore valuable to habitat restoration (Loor Solorzano et al. 2012, 2015). However, one of the main barriers to the wide-spread adoption of this practice is the harsh abiotic and biotic conditions for growing young seedlings in former pasture land (Aguirre et al. 2011). These harsh conditions include

high soil compaction, high heat, low water availability and high below and aboveground competition with exotic grasses (Mendoza 2007).

In this case, a thorough understanding of the ecological processes that promote plant establishment in degraded land could facilitate the implementation of reforestation practices at a relatively low cost. One such component is the symbiosis between mycorrhizal fungi and plants, which could play a critical role in promoting plant establishment and growth under adverse conditions (Urgiles et al. 2009, Hartley and Gange 2009, Maltz and Treseder 2015). Arbuscular mycorrhizal fungi (AMF), the mycorrhizal fungi most common in tropical forests (Allen et al. 1995), grows within and around the rhizosphere acting as foraging extensions to the plant's roots. AMF are so critical that many tree species cannot survive let alone colonize cleared land without them (Janos 1980, Perry et al. 1987). In exchange for foraging soil nutrients, the plant will give up to a sixth of its photosynthetic sugars to the fungus (Kaschuk et al. 2009). There is also strong evidence for AMF improving plant defenses (Jung et al. 2012, Delavaux et al. 2017) including in *T. cacao* (Herre et al. 2007). Furthermore, there is potential for AMF to confer defense benefits against herbivores through plant wide activation of defense related genes, but these benefits are context dependent (Jung et al. 2012). When deforestation occurs, AMF species are often lost from the immediate area (Maltz and Treseder 2015), which may further contribute to the perpetuation of the degraded ecosystem. This AMF disturbance phenomenon is particularly understudied in the tropics where deforestation rates and AMF alpha diversity are the highest globally (Alexander and Selosse 2009, Davison et al. 2015).

An additional benefit of AMF is related to their production of glomalin, a hard to degrade glyco-protein. Glomalin is so abundant and influential that AMF have been termed an ecosystem engineer because of it (Rillig and Steinberg 2002). Glomalin stores carbon in the soil for long periods of time (Rillig et al. 2001a) and also contributes to the creation of soil aggregates, which are critical for soil aeration and retention of water and nutrients (Wilson et al. 2009). Glomalin is commonly measured as glomalin-related soil protein when extracted from soil samples. In the

tropics, glomalin-related soil protein can make up as much as 12% of the total soil carbon pool (Rillig et al. 2001a, Lovelock et al. 2004). This is especially noteworthy given that the world's soil carbon pool has been estimated to be even greater than aboveground terrestrial carbon pool (Lal 2004). There, the introduction of AMF to degraded ecosystems could not only increase plant productivity but also contribute to soil development and recovery (Wilson et al. 2009, Maltz and Treseder 2015). Glomalin has been found to be a good general proxy for more complex measures of soil restoration and has been called an ecosystem engineer for its profound beneficial effect on soil ecosystems (Purin and Rillig 2007, Vasconcellos et al. 2016).

A past meta-analysis showed that the most effective way of restoring mycorrhizal fungi in an ecosystem is inoculating with local species rather than commercial isolates that often have exotic AMF species (Maltz and Treseder 2015). Using whole soil inoculate of nearby secondary forest soil is then a scalable and, economical way of restoring land with native mycorrhizal fungus (Maltz and Treseder 2015, Wubs et al. 2016, Symanczik et al. 2017). Still, the impact of these inoculations might vary with both the plants being affected and the environmental context of the inoculation. In terms of environmental context, high phosphorus levels often make mycorrhizal fungus commensal or even parasitic (Johnson et al. 1997, Grman 2012). Low light levels have mixed evidence of effecting AMF's relationship's with plants (Johnson et al. 1997, Hodge and Fitter 2010, Ibáñez and McCarthy-Neumann 2015, Nagata et al. 2015, Zhang et al. 2015). Thus, understanding the role of AMF inoculation as a function of context, i.e., soil fertility, and light, is critical for the development of effective management practices during restoration.

To fully assess the affectivity of AMF inoculation during restoration, this study was undertaken in a recently abandoned pasture in Coastal Ecuador. We conducted an experiment in which we inoculated cacao plants with nearby forest soil and planted them together with a shade tree in an abandoned pasture land. We planted two cacao varieties, a commercially grown hybrid and a native cultivar, in case local AMF inoculations might be more effective on native varieties (Keller-Pearson et al.

2020). With our experiment, we addressed these questions: 1) do whole soil inoculants taken from nearby secondary forest increase arbuscular mycorrhizal fungi in planted cacao trees? 2) Does percent canopy cover and cacao cultivar influence whole soil inoculants' beneficial effect on plant growth and pest resistance? And, 3) Is the level of arbuscular mycorrhizal fungi in plant roots associated with plant growth? There is a growing understanding that reforestation, especially in the tropics, is one of the greatest tools humanity has to stop the climate and biodiversity crisis's (Bastin 2019). Investigating these questions further develops our understanding of how to do reforestation in a way that is most effective and sustainable economically and environmentally.

Methods

Site Description

Our field site was located in the ecotone between tropical dry forest and pre-montane humid forest (0º 6' 31.7756" **S** 80º 7' 48.8666" **W** WGS 84, 265 ± 15 m a.s.l.), in the Ecuadorian province of Manabí. The dry season starts in July and lasts until November during which almost no rain falls. Dry season temperatures range between 21-34 °C, and precipitation is less than 120 mm. The wet season is warmer (22-35 °C) and with an average precipitation of 1000 mm. Differences in canopy cover cause drastic differences in microclimate with low shade sites reaching up to 34 °C and high shade sites experiencing highs of only 28 °C. Furthermore during the wet season, the low shade sites air average 23% air relative humidity while the higher shade sites average 71%.

The field sites have been clear cut and burnt at least one time in recent history and planted with the aggressive exotic grass, *Megathyrsus maximus*, for cattle and horse pasture. This is a 3 m tall grass with allelopathic properties that grows in monoculture and creates a thick mass of dead grass (Kanife et al. 2012). After being converted to pasture, our field site was purchased by a small local conservation organization, Third Millennium Alliance, in 2007 and left to fallow. However, natural regeneration and previous plantings have been largely impeded by a myriad of harsh abiotic and biotic factors which include: high heat and solar radiation, steep slope, severe soil compaction, and competition by *Megathyrsus*.

Experimental Design

Our restoration plan to aid cacao cultivation focused on improving soil growing conditions, reducing exotic grass crowding and creating shade, through microbial inoculation, mechanical removal, and planting trees. Microbial inoculation with native AMF species and its subsequent generation of hyphae and glomalin seeks to address soil compaction through increasing soil aggregation and micro-tunneling (Rillig 2004, Rillig and Mummey 2006, Miransari et al. 2009). Mechanical removal of the pasture exotic grass that was introduced by cattle farmers sought to

remediate aboveground competition for light. It should be noted that belowground removal of *Megathyrsus maximus* through tilling was not possible due to the steepness of the sites, although this would have been highly desirable to decrease belowground crowding and release of plant root allelopathic chemicals. We sought to remediate high solar radiation through the planting of two species of shade trees, *Tabebuia donnell-smithii,* and *Gmelina arboreaI*. These shade trees also aid to reduce competition by *Megathyrsus* as the pasture grass is moderately shade intolerant.

Cacao Cultivar

In this study we used two cultivars of *Theobroma cacao*. One was a hybrid variety with Nacional (native variety) and foreign germoplasm, which is more disease-resistant to witches' broom (*Moniliophthora perniciosa*) and frosty pod rot (*Moniliophthora roreri*). This hybrid variety was called "Jehova" by the local commercial nursery that sourced it. It was reported to be roughly 50% Nacional. The second variety was heirloom Nacional which was sourced from the adjacent Jama Coaque Ecological Reserve. Heirloom Nacional is 95% comprised of the nearly extinct Nacional variety giving its superior flavor to the mass produced hybrid varieties like CCN-51 which can be chalky, bitter and astringent (Boza et al. 2014, Engeseth and Ac Pangan 2018, Rottiers et al. 2019).

Experimental Restoration

On March 12th of 2017 ten 27 x 27 m sites were selected. Sites were spread across a south facing hill above a seasonal stream. Site edges were spaced between 9 and 250 m. Sites edges are also between 9 and 30 m in their distance to secondary forest. Design included multiple species in a way that balanced restoration, experimental quality and viability to small-holder farmers. Hybrid variety cacao plants, 288 seedlings, were sourced from a local nursery and were approximate 4-12 months old and 28.2 cm tall on average. Nacional cacao variety plants, 192 seedlings, were sourced from the Jama- Coaque Reserve forest and cultivated in the nursery onsite. The Nacional variety seedlings were also 4- 12 months old and 27.3 cm tall on average. In total, 400 *Gmelina* were planted at least

3 m from the outer perimeter of cacao plants, 160 *Tabeduia* were planted within the cacao blocks. In addition to the tree species mentioned *Musa sp.*, commonly known as plantain and *Triplaris cumingiana* locally known as fernan sanchez were also planted in the 9 m margin to increase shading for ecological restoration and provide increased value to local agroforestry owners.

At each site, we planted within four blocks separated by 9 m (**Figure 1**), two of which received whole soil inoculation and two were left as control (C). Whole soil inoculation consisted on adding 250 ml of top soil per plant from a nearby secondary forest. This clumped inoculation pattern was chosen given mycorrhizal networks ability to disperse underground and connect plants (Giovannetti et al. 2004).

Figure 1
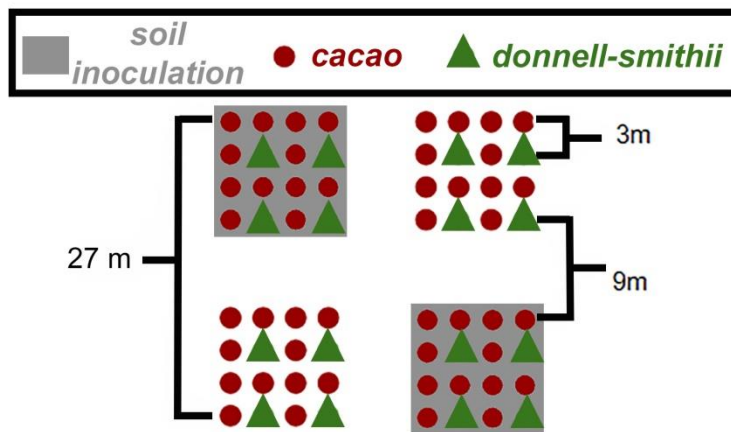
Figure 1. Graphical representation of the planting arrangement and inoculation at each of the sites.

Plant Measurements

Growth Measurements - Plant height and diameter was recorded 4 times over a year after planting, near the end of the rainy season (May 2017), in the dry season (July 2017), near the end of the dry season (September 2017), and again in the dry season (July 2018). Plant height was measured along the longest stem from the top

node of the apical meristem to the ground at the base of the seedling. Plant diameter was measured at approximately 7.5 cm (1 hand) above the ground along the thinnest part of the longest stem. There were some rare cases of dieback of green woody material. We recorded these as negative values within the growth datasets as they were ecologically relevant to quantifying the productivity of our cacao seedlings. Any future mention of growth is shorthand for net change in size.

Leaf Herbivory Measurements - Two months after planting, presence/absence of leaf herbivory was recorded on all cacao plants by searching all leaves for missing leaf surface area. Leaf herbivory was distinguished from discoloration herbivory and leaf disease by not having brown, yellow or orangish discoloration around the missing leaf spots.

Mycorrhizal Measurements

Percent Root Length Colonization - At two months, newly-grown fine white root tissue was harvested evenly across a subset of cacao seedlings' root systems and stored in 70% ethanol before being cleared and stained. A total of 40 seedlings were sampled one from each block, four seedlings per site, two per soil inoculation treatment. Percentage of root length colonized by arbuscular mycorrhizal fungi was assessed following standard methods by Vierheilig et al. (1998).

Real-time PCR (qPCR) and Glomalin-related Soil Protein- Fine roots were removed from a different subset of 40 cacao seedlings, one per block. To prevent cross-contamination we used a set of tweezers and scalpels for inoculated samples and another set for control; equipment and latex gloves were wiped with 70% ethanol for sterilization. We sampled all the visible lateral non-lignified root growth.

We also sampled rhizosphere soil from these samples for glomalin-related soil protein assays by shaking off the dirt from these rootlets into a paper envelope. Due to the lack of access to refrigeration at our field research station we stored the samples with silica desiccants following Öpik et al. (2013) for 4 days before they were stored at – 20 °C. 30-100 mg of dried roots were then weighed following Öpik et al. (2013).

DNA samples were extracted using a Mo Bio PowerSoil DNA Isolation Kit. We acquired a DNA export permit (MAE-DNB-CM-2018-0085) from the Ecuadorian Ministerio Del Ambiente. In March 2019, at the University of Michigan Advanced Genomics Core we ran a Quantitive PCR (qPCR) on our samples. We used AMV4.5Nf AMDGr primers because of their high specificity to AMF (Sato et al. 2005), short target region (300 bp) necessary for high efficiency PCR and past success in use with SYBR green qPCR (Dai et al. 2013, Liang et al. 2016). A Qubit 2.0 Flourometer was used to measure DNA concentrations of test samples as well as 2 positive controls that previously successfully amplified using our AMF specific primers, as well as 4 negative controls of samples collected during the dry season when mycorrhizae were not present due to seasonality. Samples were then diluted with DNA free water to 0.1 ng DNA per µl and 5 ul of sample was used per 20 µl reaction of Applied Biosystems Powerup SYBR Green Master Mix following the master mixes instructions. Five µl of DNA extraction product was used in qPCR for negative control samples that had no detectable DNA on the Qubit. The positive control samples were 10:1 serial diluted from 1000 to 1 pg to verify high PCR efficiency. In addition to template DNA, 10 µl of SYBR Green MasterMix, and 12.5 µM primers was added.   An Applied Biosystems 7900 HT was used with a thermal profile set to 2 min at 50 ºC and 2 min at 95 ºC and 40 cycles each of 95 ºC for 15 s, 55 ºC for 15 s, and 72 ºC for 1 min followed by a default dissociation curve. The threshold cycle (C_t) was calculate by the 7900 HT as the threshold where SYBR Green fluorescence from PCR amplification went significantly above background noise. The final AMF 18s rDNA concentrations were calculated as relative values and weighted for total extracted DNA and dried mass of roots. We used the following formula where R is relative quantity of DNA per mg dried root and C_t is the PCR cycle that the target DNA was detectable with $C_{t_{max}}$ being the maximum of the dataset and C_{t_i} being each individual sample; furthermore, in the following formula M is the mass of dried root sample tissue used in DNA extraction and Q is the dilution ratio used to create PCR samples from DNA extraction product:

$$R = \frac{2^{C_{t_{max}} - C_{t_i}}}{M} \times Q$$

Glomalin-related soil protein was extracted with sodium citrate at 121 ºC from rhizosphere soil collected at 2 months from 40 cacao seedlings one from each block. Protein concentration of both easily extractable glomalin-related soil protein(EE-GRSP and total glomalin-related soil protein (T-GRSP) was measured using Bradford reagent and 96 well plate spectroscopy. All steps followed standard procedures described in Lovelock et al. (2004).

Environmental Data:

Soil Nutrients and Organic Matter - Thirty three of the rhizosphere soil samples described within the glomalin section of our methods had enough remaining soil mass to be sent for soil nutrient analyses to the Laboratoria De Suelos, Foliares Y Aguas within the government agency of Agrocalidad in Quito. Soil organic matter was assessed volumetrically by the Walkley-Black method (Walkley and Black 1934). Available phosphorus was measured using the Olsen method (Olsen et al. 1954).

Distance to Forest - As distance to forest edge may have affected AMF root colonization via propagule availability, we estimated distance to forest edge using satellite imagery from 2013 Worldview 2 imagery Maxar Technologies accessed through Google Earth software. QGIS 3.4 was then used to calculate distance from forest edge to each plant.

Percent Canopy - Orthomosaic drone imagery with a 0.43 m pixel size was provided by Third Millennium Alliance. Tree canopy was manually digitized in QGIS 3.4.7 Considering a canopy tree height averaging about 9 m we choose to calculate the percent canopy for a 9-m radius around the seedlings, thus their light environment and direct exposure to solar radiation. This would yield the most ecologically informative percent canopy cover as a smaller radius would not pick up shading lasting half of the day and a larger radius would be too sensitive to trees that may only provide partial shade at dusk and dawn.

Statistical Analysis

We performed exploratory data analysis and developed models that directly addressed our research questions but were also ecologically relevant and parsimonious.

1) Do whole soil inoculants taken from nearby secondary forest increase arbuscular mycorrhizal fungi in planted cacao trees?

To address our first question we analyzed percent AMF root colonization via four proxies: percent root length colonization, quantity of barcode DNA, concentration of easily extractible glomalin-related soil protein and concentration of total glomalin-related soil protein. These proxies were analyzed using 4 separate linear models in R version 3.3.2 (R Core Team 2020). In these models, we investigated which other variables might have contributed to these 4 mycorrhizal proxies. We ran general linear models that not only included an inoculation treatment effect but also environmental data relevant to plant growth and mycorrhizal colonization. These environmental data were percent canopy and distance to forest.

2) Does percent canopy cover and cacao cultivar influence whole soil inoculants' beneficial effect on plant growth and pest resistance?

First, we ran a Welch's t-test to compare inoculation and control groups within the Nacional and Hybrid cultivars for growth. We assessed if there were differences between inoculation and control groups not only in their amount of mycorrhizal colonization, but also on their growth, height and stem diameter at two, four and twelve months as well as the amount of herbivory plants experienced. Tests were run in R version 3.3.2.

Also in R version 3.3.2 we analyzed the differences in growth between inoculation and control plants and how these may depend on canopy cover. Our hypothesis is that the positive effects of mycorrhizal fungi may be higher at high light when the plant can take full advantage of the symbionts without jeopardizing its carbon reserves.

We ran the following model with the LME4 package in R version 4.0.2 to analyze the inoculation effect in context of cultivar, percent canopy and their interactions. We also included site random effects (SRE) in the model due to high site level variance.

$$\Delta Height_i = \alpha_{Treatment(i)} + \beta_{Treatment(i)}\, Percent\ Canopy_i + \gamma_{Cultivar(i)}$$
$$+ \delta_{i_{Cultivar(i)}}\, Percent\ Canopy_i + \mu\, Percent\ Canopy_i + SRE_{site(i)}$$

Parameters 95% confidence intervals (CI) were used to test if a predictor had a statistically significant effect, intervals that do not overlap with zero were considered statistically significant.

3) Is the level of arbuscular mycorrhizal fungi in plant roots associated with plant growth?

To answer this question we analyzed changes in plant height, including negative changes due to dieback, as a function of mycorrhizal colonization, light availability, soil nutrients and initial plant size (a proxy for maternal effects). We also included site random effects (SRE).

$$\Delta Height_i = \alpha + \beta\, Percent\ Colonization_{block(j)} + \gamma_1\, Percent\ Canopy_i$$
$$+ \gamma_2\, Soil\ Phosphorus_{block(j)} + \gamma_3\, Planting\ Height_i + SRE_{site(i)}$$

Due to sampling limitations, percent colonization and phosphorus was extrapolated from the one value measured per block. There were four blocks per each of the 10 sites. Sites were included as a random effect. For fixed effects, a lack of overlap with zero in the parameters 95% confidence intervals (CI) was used to test if a predictor had a statistically significant effect.

To help us evaluate our models we calculated the R^2 value for each general linear model and we used the conditional R^2 for mixed models (Nakagawa and Schielzeth 2013).

Results

Generally, plant growth was stagnated (only 0.8 cm per month) in the first 4 months during the end of the wet season and dry season, then after the wet season plant growth increased to 2 cm per month (**table SM-8**). Similarly mortality was high in the first 4 months (30%) and decreased to only 8% in the next 6 months of the wet season, showing the stress that the dry season had on the young plants (**table SM-8)**. This also indicates that percent canopy and subsequently lower vapor deficits and higher moisture levels will likely be important for plant growth. Percent canopy coverages varied between full shade and near full sun (100% and 12%) with an average of partial shade (62%). The standard deviation of percent canopy was 22% for all samples.

1) Do whole soil inoculants taken from nearby secondary forest increase arbuscular mycorrhizal fungi in planted cacao trees?

Cacao roots ranged from having low to high AMF percent root length colonization rates (0-86%). However, easily extractable gomalin-related soil protein and total glomalin related soil protein values were low (0.17 and 0.24 mg/g, respectively). This indicates low glomalin-related soil protein abundance and concomitantly AMF abundance in the rhizosphere soil's recent history.

Percent Root Length Colonization

The linear model of percent root length colonization data showed no statistically significant difference in percent root length colonization between inoculation and control groups (p = 0.68) (**Table SM-1**).

Quantity of barcoding DNA (QPCR)

As expected, arbuscular mycorrhizal fungi DNA per mg of root tissue was greater with inoculation. Inoculated samples' mean was 235% greater than control mean (p = 0.025).
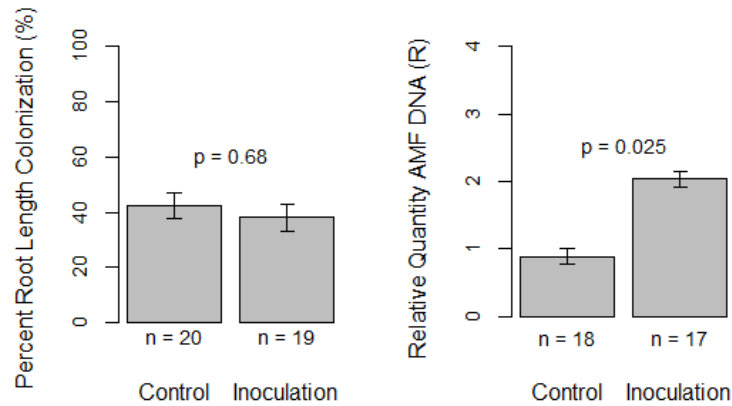
Figure 2 The right graph's y-axis depicts R, a unit-less proportion based on C_t per mg root and is fully defined in the methods. Bars depict means with standard errors as the error bars. P-value is from linear model described in methods.

Glomalin

Easily extractable glomalin related soil protein (EE-GRSP) values were significantly higher in the inoculation group than the control group (inoculation samples' mean was 38% more; Fig. 2; p value = 0.008) supporting the hypothesis that inoculation increased AMF abundance.

Figure 3 Bars depict means with standard errors as the error bars. P-value is from linear model described in methods.

As opposed to easily extractible glomalin-related soil protein, total glomalin-related soil protein (T-GRSP) values were only sligh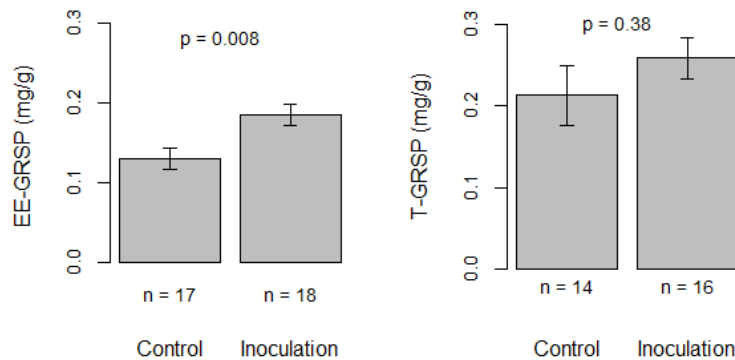tly more with inoculation and the difference was not statistically significant (**Figure 3**). Although this does not support our hypothesis that inoculants increase AMF abundance, this might be due to total glomalin being a proxy for older AMF byproducts which haven't had sufficient time to form since inoculation. In total inoculation generally increased arbuscular mycorrhizal fungi.

2) Does percent canopy cover and cacao cultivar influence whole soil inoculants' beneficial effect on plant growth and pest resistance?

Inoculation, Percent Canopy and Cultivar's Effect on Cacao Growth

When incorporating percent canopy, cultivar and their interactions, inoculation significantly increased cacao growth in height at two (p=0.0004), four (p=0.00001) and one year (p=0.06), supporting our hypothesis that intact forest soil inoculation increases cacao growth (**Figure 4**). Furthermore percent canopy (shade) significantly negatively interacted with inoculation at two months (p=0.001), four months (p=0.00002) and one year (p=0.02) (**Figure 5, Figure SM-1**). After two months and also one year percent canopy was found to be positively correlated with cacao growth across both cultivars (two months p=0.02; one year p=0.01). This suggests that, although shade makes inoculations less effective, shade also has an overall beneficial effect on cacao. However, this is further complicated because, after one year, there was a negative interaction effect between percent canopy and cultivar (p=0.02) with the Nacional cultivar benefiting from shade and the Hybrid cultivar doing just slightly worse with shade. This supports our expectation that Hybrid cultivars are less shade tolerant than Nacional cultivars. We list the full results of the previous models in **Table SM-6.** The conditional correlation coefficient (R^2) of the model's predicted vs. observed was 0.14 for 2 months, 0.18 for 4 months and 0.33 for 1 year.
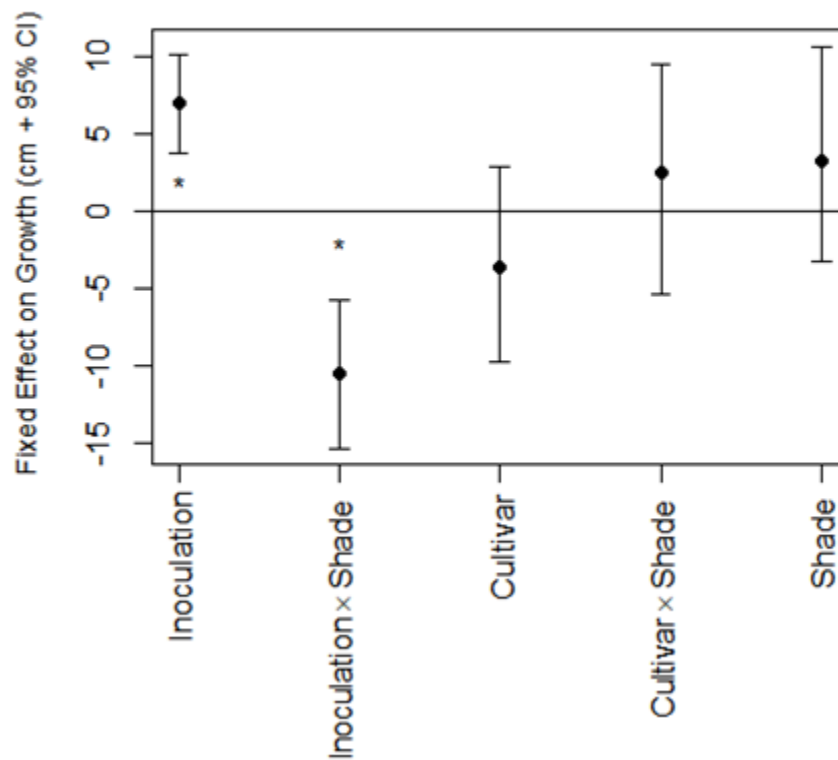
Figure 4 shows the estimated effect size of inoculation and cultivar on height growth after 4 months. Four months was used because it shows the peak effect of the dry season on our samples. **Figure 4** also shows the interaction of those categorical factors with percent canopy (which is labeled the shorthand, "shade"). Mean parameter estimate is shown along with 95% confidence interval (CI). When the 95% confidence interval doesn't overlap with 0 it is deemed to have a significant effect and marked with an asterisk.
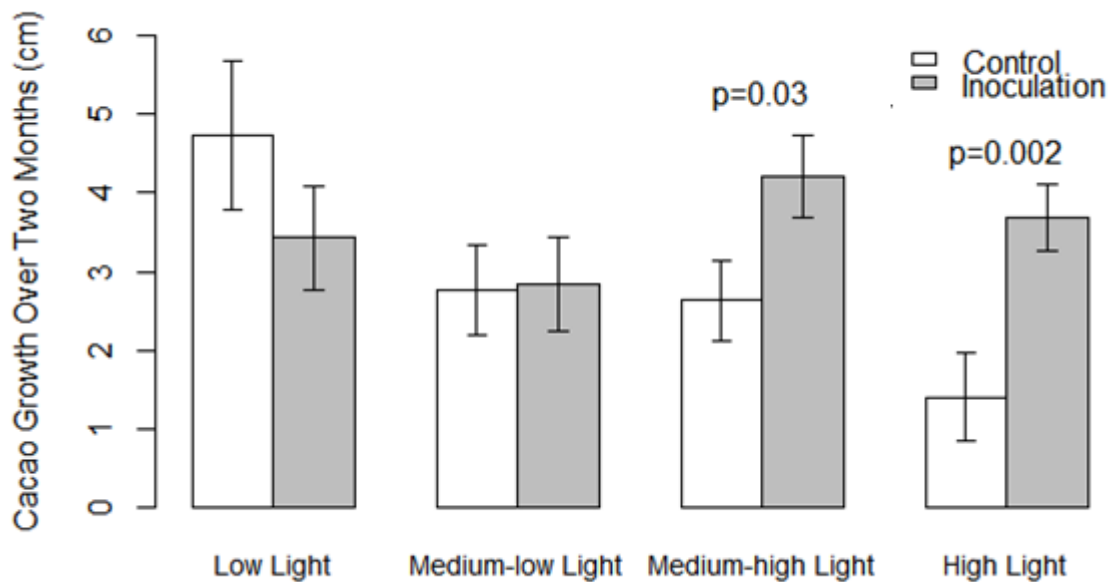
Figure 5 Percent canopy values were divided into quartiles and subsequently grouped into 4 categories (low light, medium-low light, medium-high light and high light). Bars depict means and error bars represent one standard error. P-values based on a post-hoc Welch's t-test between control and inoculation within a light level.

Inoculation's Beneficial Effect on Nacional vs. Hybrid Cultivar's Growths

For Nacional, control and inoculated groups were not significantly different. However, plants of the Hybrid cultivar that were inoculated with forest soil grew significantly more in height after 2 months than those left as controls (**Figure 6**). The same trend was found after 4 months (inoculated samples' mean was 42% more than controls' mean, p= 0.02). However
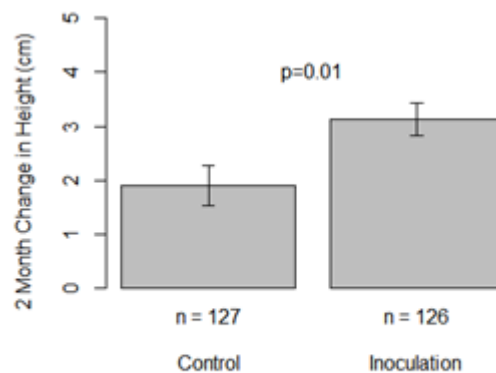
Figure 6 Bars represent means and error bars are one standard error. P-value is from Welch's t-test.

after one year there was no significant difference in the growths (inoculated samples' mean was 1% less, p=.98). This supports our hypothesis that intact forest soil inoculation increased cacao growth at two and four months but not one year.

Inoculation's Effect on Herbivory

As expected, plant herbivory was lower on plants with forest soil inoculations but only with marginal significance (p=0.064).

3) Is the level of mycorrhizal fungi in plant roots associated with plant growth?

Our multivariate model showed that AMF colonization had a marginally significant effect on cacao plant growth (**Figure 7**). This model predicts that AMF colonization may be responsible for a large portion of cacao growth (a predicted 44% of growth at the measured mycorrhizal colonization levels). Although the model estimates mycorrhizal colonization being very important to growth, the 95% confidence interval of this estimate varies substantially. The 95% confidence interval implies that percent root length colonization of AMF predicted anywhere from 3% to 86% of total growth at the measure mycorrhizal colonization levels. The overall model had low fit with a conditional R^2 of just 0.05. In total, this model suggests that arbuscular mycorrhizal fungi were at least partially responsible for plant growth.

Figure 7 shows the estimated effect size of percent colonization and covariates on height growth after 4 months. Four months was used because it shows the peak effect of the dry season on our samples. Mean parameter estimate is shown along with 95% confidence interval (CI). When the 95% confidence interval does not overlap with 0 it is deemed to have a significant effect and marked with an asterisk.
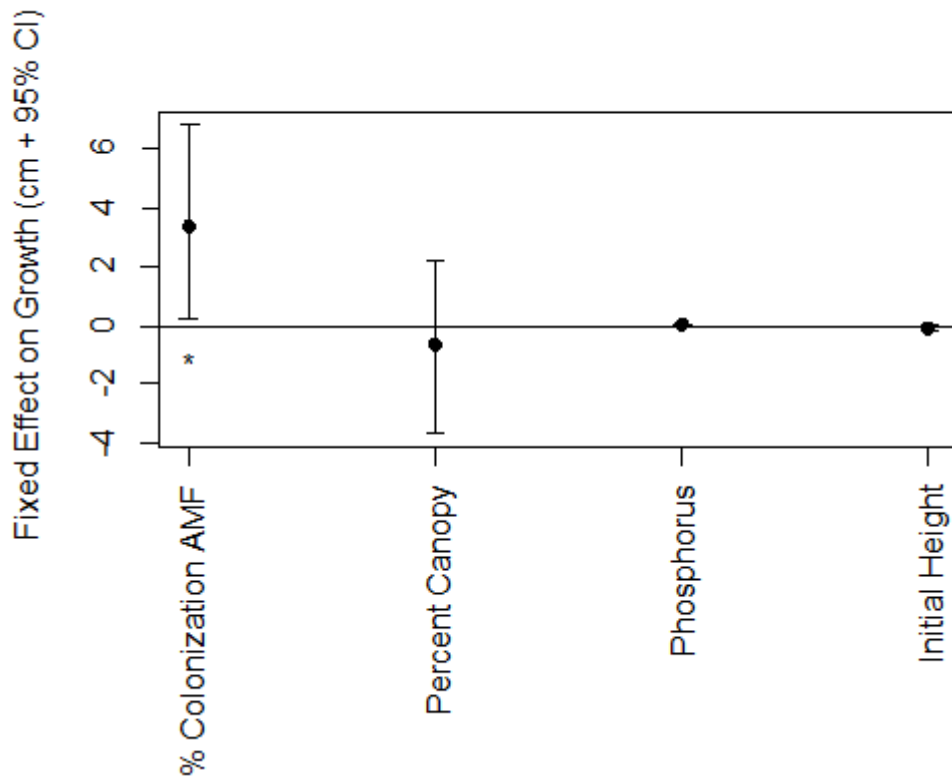
Discussion

Researching soil restoration techniques for regenerative agroforestry (agroforestation) is critical to ensure agroforestation lives up to its' potential as a socially just tools for mitigating the extinction and climate crises (Rillig et al. 2001a, Montagnini and Nair 2004, Verchot et al. 2007, Knoke et al. 2009, Bastin 2019). We ran an experimental cacao agroforestation project in recently deforested cattle pastures in the highly threatened forests of coastal Ecuador. Our experimental whole-soil inoculations were found to increase arbuscular mycorrhizal fungi in the soil, which had an overall positive effect on cacao plant growth during the first 4 months. However, in heavily shaded areas of our study, we found that soil inoculations had a negative effect during the first 4 months, perhaps due to a potential parasitic effect of mycorrhizal fungus that can occur when plants grow in low light conditions (Ibáñez and McCarthy-Neumann 2015, Hoeksema and Bruna 2015).

1) Do whole soil inoculants taken from nearby secondary forest increase arbuscular mycorrhizal fungi in planted cacao trees?

Although this process has been shown to be generally true (Maltz and Treseder 2015), site specific details such as level of soil degradation, seral stage of inoculant source soil and fungi host specificity may all affect AMF colonization rates (Janos 1980, Johnson et al. 1991, Allen et al. 2003). In our experiment, 2 of 4 measurements (EE-GRSP and quantity AMF DNA) showed evidence that AMF quantity had increased two months after inoculating soil of planted seedlings with a small amount of soil from regenerating secondary forest. We might interpret these results with caution as measurements were done only 2 months after inoculation and the passing of more time may increase or decrease the colonization levels. However, there we believe these results generally support the large body of literature which suggests that whole soil inoculation is an effective method for increasingly arbuscular mycorrhizal fungi's colonization (Allen et al. 2003, Maltz and Treseder 2015).

Although, percent root length colonization values of AMF were not higher in inoculated samples than control samples, multiple measurements give a fuller picture of AMF's relationship with our study plants. For percent root length colonization, a plant's mycorrhizal status is typically assessed in ten 1 cm segments of roots. Because mycorrhizal colonization is highly time and space dependent, there is always the possibility of a sampling bias when only 10 small root fragments per sample are used. Percent root length colonization microscopy is also very time intensive making it difficult to analyze more subsamples per individual without sacrificing on total number of samples included.

This subsampling effect can be overcome with the qPCR genetic analyses since tens of thousands of microscopic pieces of root from up to 100 mg of dried root tissue can be used. We were able to collect all the visible new lateral root growth from our young cacao plants and stay within this weight limitation. In cases with more extensive root systems it is possible to lyophilize and mix the roots fragments as in Öpik et al. (2013) which would avoid subsampling bias.

Other studies also found no observable link between physical quantifications of AMF and quantity of AMF DNA. Despite this, the qPCR quantifications were found to be verifiable and relevant ecologically in terms of correlating with measurements of plant growth and soil phosphorus levels (Jansa et al. 2008, Dai et al. 2013). A lack of correlation with other measures of mycorrhizal colonization is believed to be because ribosomal DNA is more heterogeneous in hyphae than in spores (Gamper et al. 2008). For this reason, qPCR values are thought to be better correlated to spore count than percent root length colonization.

Our findings reflect these complex relationships between different AMF measurements and give further proof that getting different results from similar but different arbuscular mycorrhizal fungi assays is reasonable. These seemingly contradictory results show the complexity of quantifying these microscopic relationships. Despite the complexity it is important to improve our understanding of these relationships because of AMF's macroscopic consequences on ecosystems and climate.

Glomalin-related Soil Protein

Global forest restoration has recently been gaining international attention as one of the most effective tools for combating climate change (Bastin 2019). This is due to the fact that trees' biomass could sequester 2/3rd of humanity's carbon released to date (Bastin 2019). This vast sequestration potential is not even considering reforestations ability to augment belowground carbon pools (i.e. through glomalin, soil aggregation, etc. (Rillig et al. 2007)). Our glomalin-related soil protein values were low (0.02-0.31 mg/g) compared to other researchers' surveys (>100 mg/g) of more pristine tropical ecosystems indicating that our sites, abandoned tropical pasture, has the potential to store more carbon through glomalin (Rillig et al. 2001b, Lovelock et al. 2004). Research has shown that root-associating fungi are more responsible for sequestering carbon than previously thought (Clemmensen et al. 2013). Therefore, our findings that soil inoculations increased glomalin-related soil protein values by 38% are hugely consequential not only on soil restoration but the ability to sequester carbon via restoration efforts in the tropics. Also, this increase in glomalin-related soil protein with inoculation provided further evidence for whole soil inoculations efficacy to restoring arbuscular mycorrhizal fungi communities.

Glomalin is recalcitrant and can remain in the soil up to 40 years (Rillig et al. 2001a). Previously it was thought that easily extractible glomalin-related soil protein (EE-GRSP) was correlated to recently produced glomalin and total glomalin-related soil protein (T-GRSP) was the glomalin fraction which has persisted longer in the soil (Wright and Upadhyaya 1998). Therefore, our results seem to indicate that recently generated glomalin levels were significantly different due to soil inoculations, while more recalcitrant glomalin values were the same across experimental groups. This might therefore suggest the inoculation treatment caused a spike of AMF biomass visible in the EE-GRSP that was undetectable in terms of the total accumulated GRSP. However there is mixed evidence for these previously held conceptions about EE-GRSP and T-GRSP representing short-term vs. long-term glomalin pools (Steinberg and Rillig 2003, Koide and Peoples 2013).

Although there is mounting evidence that glomalin is of critical importance to soils, it is still uncertain why AMF produces glomalin at all (Driver et al. 2005). Additionally there has been evidence suggesting glomalin- related soil protein assays are measuring substantial quantities of proteins of nonmycorrhizal origin (Purin and Rillig 2007, Singh et al. 2013). Despite this more recent research including studies on larger scales and longer terms suggests glomalin-related soil protein is in fact a robust measure of glomalin from arbuscular mycorrhizal fungi (Wilson et al. 2009, Koide and Peoples 2013, Li et al. 2020).

High light levels have been found to cause a chemical cascade within plants that results in higher AMF colonization (Nagata et al. 2015). Potentially in agreement with this, our study found that high light plants (low percent canopy) experienced a greater benefit from whole soil mycorrhizal inoculation. However, in contradiction with this physiological mechanism, higher percent canopy correlated with higher EE-GRSP despite it being a byproduct of AMF colonization (**Figure SM-2 and Table SM-2**). We believe that this might be due to higher baseline rates of AMF in the samples with full canopy cover as well as higher plant productivity from surrounding canopy tree roots in the rhizosphere soil we sampled. We found that high canopy cover was positively correlated with higher organic matter. This confirmed field observations that high canopy cover soil was less compact and seemingly more hospitable to seedlings. Furthermore, in high canopy cover areas *Megathyrus maximus* grass was less dominant. Since *Megathyrus maximus* grass releases anti-fungal root defense compounds, there might be higher baseline AMF rates in areas where this grass is less dominant (Kanife et al. 2012).

2) Does percent canopy cover and cacao cultivar influence whole soil inoculants beneficial effect on plant growth and pest resistance?

Our study site varied widely in the level of sunlight reaching the cacao seedlings, from 12 to 100%. Not only does low sunlight levels have the potential to limit photosynthesis and in turn the plants carbon resources but high sun-light can increase temperature and vapor-pressure deficit causing drought. The latter is especially relevant at our site which lied at the lower edge of the elevation gradient

in coastal Ecuador in which cacao can be easily grown without irrigation. This environment is due to higher temperatures and lower precipitation levels at the lower edge. The importance of percent canopy on cacao growth was made even more explicit by the use of two cultivars of cacao in our experiment. One cultivar was a hybrid full-sun variety and the other is the Nacional variety which is highly accustomed to growing in the forest understory.

It seems plausible that inoculation might have further increased high control levels of mycorrhizae of rhizosphere under higher canopy cover (**Figure SM-2**). Although the levels of mycorrhizae in the control were found to be higher in high canopy cover, the effect of the inoculation was negative for cacao growth under intense canopy cover (both in terms of height, diameter and volume) at 2 months (**Figure 5**). Height growth was also negative from the inoculation treatment at 4 months. This indicates field evidence for a relationship with mycorrhizal fungus that approaches parasitism at low light levels (**Figure SM-1**). These findings are supported by other greenhouse, laboratory experiments and meta-analyses (Gehring 2003, Olsson et al. 2010, Hoeksema and Bruna 2015, Ibáñez and McCarthy-Neumann 2015, Nagata et al. 2015, Konvalinková and Jansa 2016, Bitterlich et al. 2019, Veresoglou et al. 2019). Despite this rapidly growing field of research a literature review failed to find any other field-based verifications of shade induced parasitism from mycorrhizal fungus. Only one other study tested for these same effects and they found that shade did not induce mycorrhizal fungal parasitism (Friede et al. 2016).

Our results also show that control cacao plants do better in the shade than in the sun (**Figures 4 and 5**). This is likely because the two month measurement corresponded with the end of the transition to the dry season. The next two months before the four month measurement were dry enough that high light and subsequent vapor pressure deficit would be a disadvantage to cacao. This would help explain the low values for high light control in **Figure 5.** It appears the inoculation treatment ameliorated the high light disadvantage that existed in the control, and turned higher

light levels into a net benefit for the cacao plants. It has been found that AMF infer significant drought resistance benefits which could be especially important during the dry season to allow photosynthesis to continue to occur (Delavaux et al. 2017). These trends continued into one year's time although they were more diminished and more marginally statistically significant. This could be because the wet season's return gave the non-inoculated plants the chance to catch back up in growth with the more drought-tolerant inoculated plants. It is also possible that the AMF mycorrhizal inoculate naturally spread from inoculation treatment samples to control samples via underground hyphae or burrowing animals. This long-term tapering off of the benefit of AMF inoculations is seen across many inoculation studies (Maltz and Treseder 2015). For these reasons, the further exploring how long the benefits of AMF inoculations last remains an open research topic important for reforestation and restoration efforts.

3) Is the level of arbuscular mycorrhizal fungi in plant roots associated with plant growth?

While arbuscular mycorrhizal fungi are likely a strong driver of the benefit of the whole soil inoculants it is important to note that our observations are correlational and that a whole host of beneficial soil organisms could be benefiting cacao plants. Even beyond other individual types of beneficial soil organisms, soil microbiome diversity in itself is thought to increase plant performance (Chaparro et al. 2012). Furthermore, the soil microbiome undergoes succession alongside the plant community (Cline and Zak 2015). Therefore it is feasible that using whole soil inoculants could help facilitate this invisible successional process if coupled with the tree planting required to bring a shift in the leaf matter that is thought to bring about microbial succession (Cline and Zak 2015). These considerations of microbial community suggest caution for concluding that arbuscular mycorrhizal fungus alone played a significant role in facilitating cacao plant growth (**Figure 7**), they further support a growing consensus that whole soil inoculants are not only a superior form of mycorrhizal inoculation but also better for reaching more general soil restoration goals.

Soil ecosystems are pivotal to reforesting degraded land and therefore combating climate change (Maltz and Treseder 2015, Bastin 2019). However, not all degraded ecosystems are deficient in the quantity and diversity of their belowground communities. Furthermore, even with robust and diverse underground communities, abiotic conditions like light and soil nutrients preclude beneficial relationships. Even AMF can become parasitic at extremely low levels and at high soil nutrient levels (Grman 2012, Ibáñez and McCarthy-Neumann 2015). Therefore, meticulously engineering soil to have the right beneficial microorganisms to aid in reforestation may be nearly impossible. However, the effectiveness of whole forest soil inoculations suggest we may be able to help undo human caused degradation by facilitating the return of later successional stages of the native soil microbiome.

Using forest soil inoculations to increase arbuscular mycorrhizal abundance and glomalin could be a powerful tool for soil restoration. This is especially the case in tropical pasture where soil compaction is extremely high from high clay content soils that have been heavily grazed by cattle (Mendoza 2007, Urgiles et al. 2009, Krüger 2013). Ultimately our research adds to a growing body of work which suggests soil restoration coupled with ecologically informed agroforestation is a powerful tool for both mitigating and adapting to climate change (Mendoza 2007, Verchot et al. 2007, Knoke et al. 2009, Urgiles et al. 2009, Vasconcellos et al. 2016, Bastin 2019).

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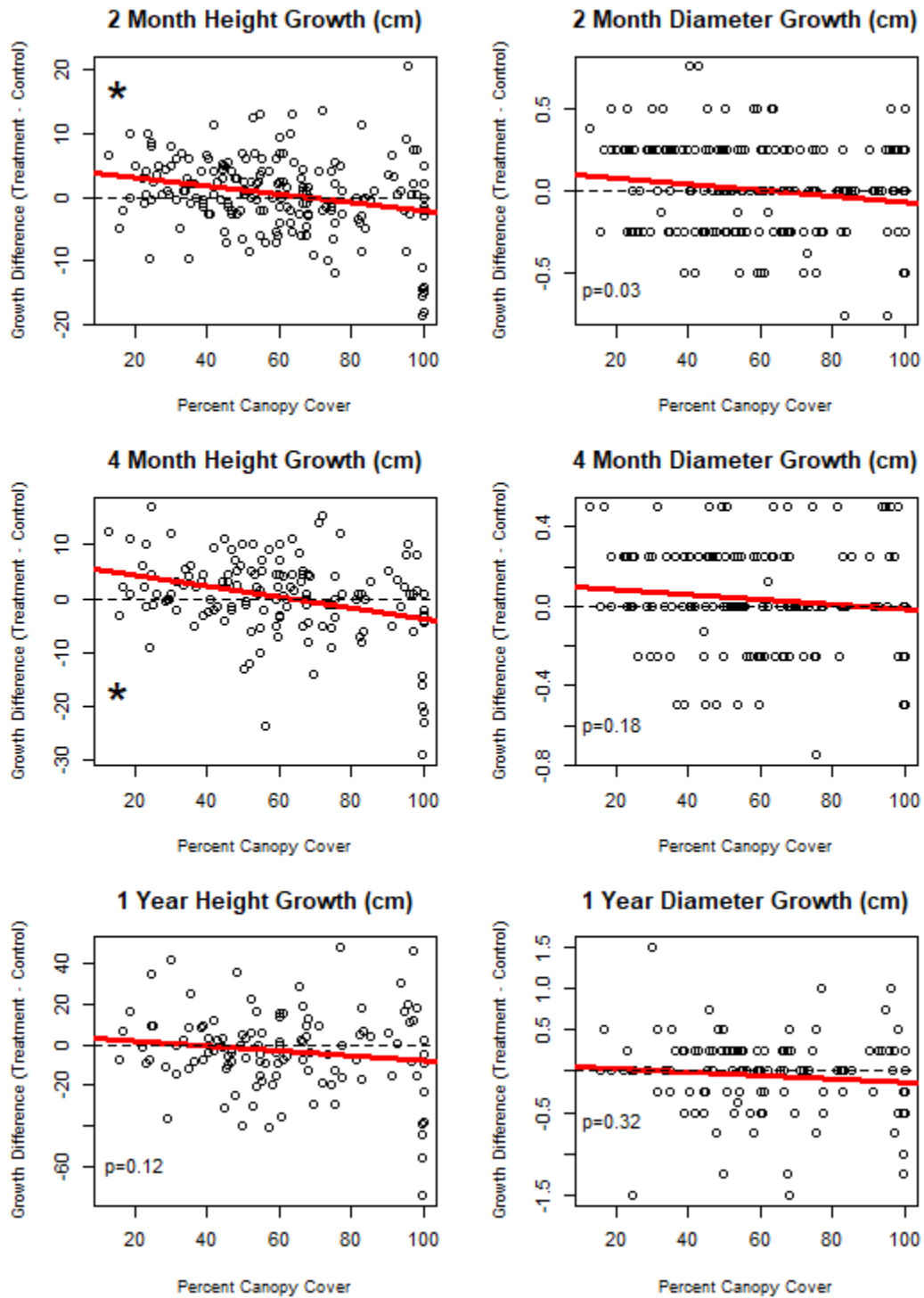
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Supplement

Figure SM-1

2 Month Volume Growth (cm^3)

4 Month Volume Growth (cm^3)

1 Year Volume Growth (cm^3)
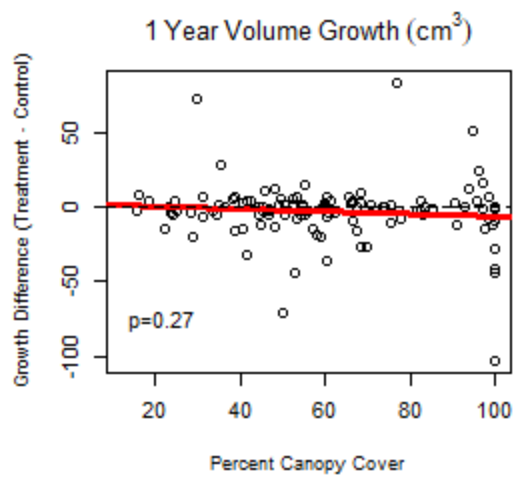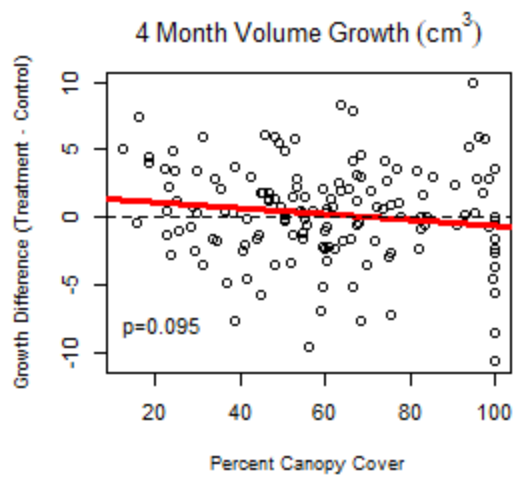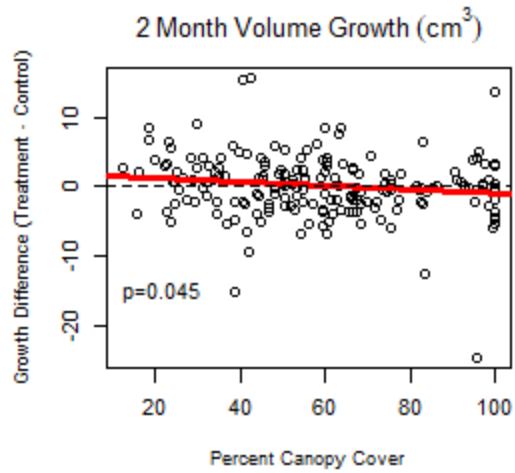
Figure SM-1 Samples were ordered by their canopy cover quantile so that we could compare growth levels of inoculated samples and control samples. Dotted line is a slope and intercept of zero. A negative slope indicates a trend of a positive inoculation effect in high light (low canopy cover) and a negative inoculation effect in low light (high canopy cover). An asterisk indicates a slope with statistical significance of $p < 0.0005$ from a univariate linear model. We listed out linear model results in more detail in table SM-5.

Figure SM-2

Percent Root Length Colonization (PRLC) AMF and Canopy Cover
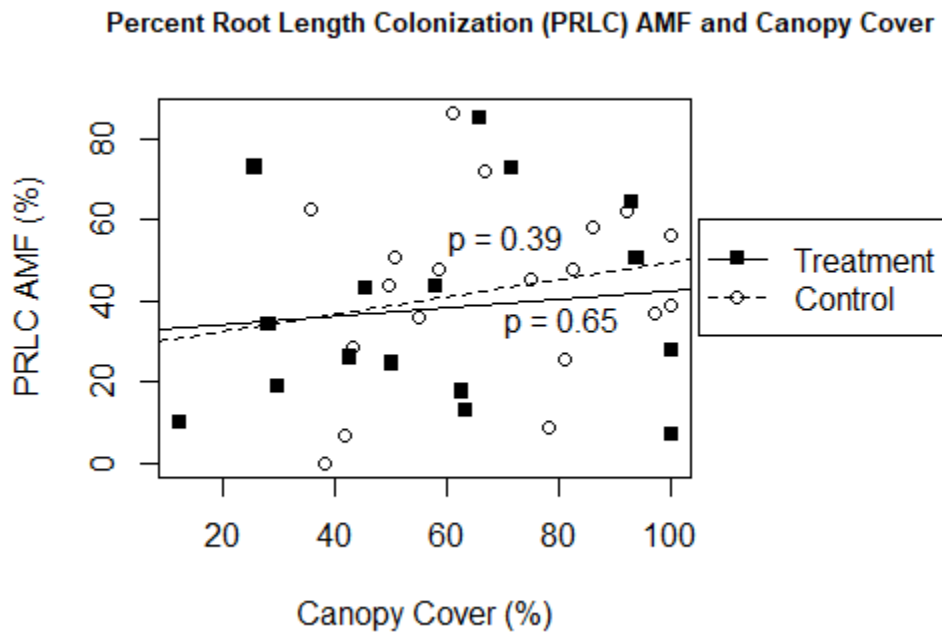
The line and p-values are from two univariate models between percent root length colonization and canopy cover, one for inoculation samples and one for control samples. P-values are for percent canopy's correlation with PRLC.

Glomalin and Canopy Cover
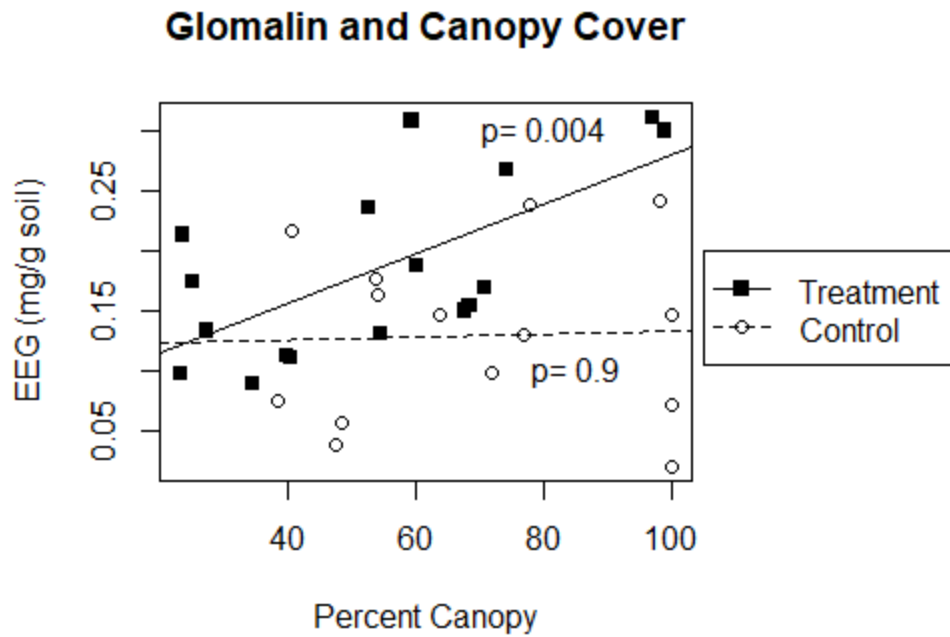
The line and p-values are from two univariate models between easily extractable glomalin-related soil protein (EEG) and canopy cover, one for inoculation samples and one for control samples. P-values are for percent canopy's correlation with EEG.

AMF DNA Quantity and Percent Canopy
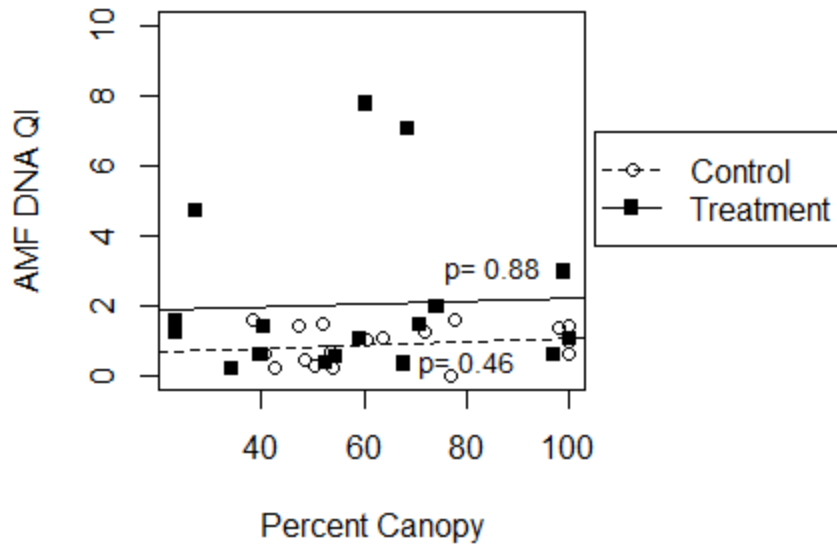
The line and p-values are from two univariate models between relative quantity AMF DNA and canopy cover, one for inoculation samples and one for control samples. P-values are for percent canopy's correlation with relative quantity AMF DNA.

Table SM-1   Results of Percent Colonization Multivariate Model

	Estimate	Standard Error	T value	P-value (* for $p < 0.05$)
(Intercept)	0.43	0.22	2.0	0.056
Inoculation	-0.34	0.81	-0.41	0.68
Percent Canopy	0.14	0.16	0.83	0.41
Distance to Forest	-0.0013	0.0023	-0.59	0.56

Table SM-2   **Results of EEG Multivariate Model**

	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	-0.082	0.068	-1.2	0.24
Inoculation	0.073	0.025	2.9	0.0080*
Percent Canopy	0.11	0.053	2.2	0.039*
Distance to Forest	0.0013	0.00077	1.7	0.11

Table SM-3   **Results of TG Multivariate Model**

	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	0.17	0.12	1.4	0.18
Inoculation	0.042	0.047	0.90	0.38
Percent Canopy	-0.017	0.095	-0.18	0.86
Distance to Forest	0.00029	0.0013	0.22	0.83

Table SM-4 **Results of Relative DNA quantity Multivariate Model**

	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	1.7	1.2	1.5	0.15
Inoculation	1.3	0.57	2.4	0.025
Percent Canopy	0.75	1.2	0.62	0.54
Distance to Forest	-0.03	0.019	-1.6	0.11

Table SM-5 Model Results from Canopy Cover Rank Comparison Between Inoculation and Control

2 month	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	4.4	1.2	3.8	0.00011*
Inoculation	-6.6	1.8	-3.6	0.00032*

4 month	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	6.4	1.6	4.0	0.00011*
Inoculation	-10.2	2.5	-4.1	0.000078*

1 year	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	4.2	5.1	0.83	0.41
Inoculation	-12	7.8	-1.5	0.13

Table SM-6 Results of Percent Canopy and Inoculation Interaction Model

Two Months

	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	0.93	1.9	0.48	0.63
Inoculation	4.4	1.2	3.4	0.0005*
Percent Canopy	5	2.2	2.2	0.03*
Cultivar	0.18	2.00	0.09	0.93
Inoculation and Percent Canopy Interaction	-6.20	1.9	-3.2	0.0012*
Cultivar and Percent Canopy Interaction	-3.6	2.5	-1.4	0.15

Four Months

	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	2.1	3.1	0.67	0.51
Inoculation	7.1	1.6	4.3	0.000014*
Percent Canopy	3.3	3.5	0.95	0.33
Cultivar	-3.6	3.3	-1.1	0.26
Inoculation and Percent Canopy Interaction	-11	2.5	-4.3	0.000018*
Cultivar and Percent Canopy Interaction	2.5	3.7	0.67	0.50

One Year

	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	4.3	7.5	0.58	0.56
Inoculation	7.9	4.2	1.9	0.063*
Percent Canopy	20.0	7.9	2.5	0.011
Cultivar	6.0	8.3	0.73	0.47
Inoculation and Percent Canopy Interaction	-15	6.4	-2.3	0.019*
Cultivar and Percent Canopy Interaction	-18	8.8	-2.0	0.04*

Table SM-7 Results of Mycorrhizal Colonization Effect on Growth Model

	Estimate	Standard Error	T value	P-value (* for p <0.05)
(Intercept)	2.6	1.7	1.6	0.11
Percent Colonization	3.4	1.7	2.0	0.046 *
Percent Canopy	-0.63	1.6	-0.40	0.69
Phosphorus	0.038	0.026	1.5	0.14
Initial Height	-0.056	0.042	-1.3	0.19

Table SM-8 General Statistics of Plant Growth Measurements

	Mean Plant Height (cm)	Plant Height SD (cm)	Plant Height Range (min-max cm)	Plant Survival	Tropical Season
First measurement	28.2	7.7	9-57	83%	Wet
2 months	31.5	8.5	10-68	79%	End of Wet
4 months	31.3	9	10-70	70%	Dry
1 year	43.0	16.6	11-111	62%	End of Wet

Table SM-9 General Statistics for Arbuscular Mycorrhizal Colonization Measurements

	Mean	SD	Range (min - max)	N
Percent Root Length Colonization	40%	22%	0-86%	39
Easily Extractable GRSP (mg/g soil)	0.17	0.08	0.02-0.31	35
Total GRSP (mg/g soil)	0.24	0.11	0.03-0.44	30
Relative Quantity AMF DNA	1.3	1.7	0-7.8	39