N_2 fixation of three legume species in an agroforestry system

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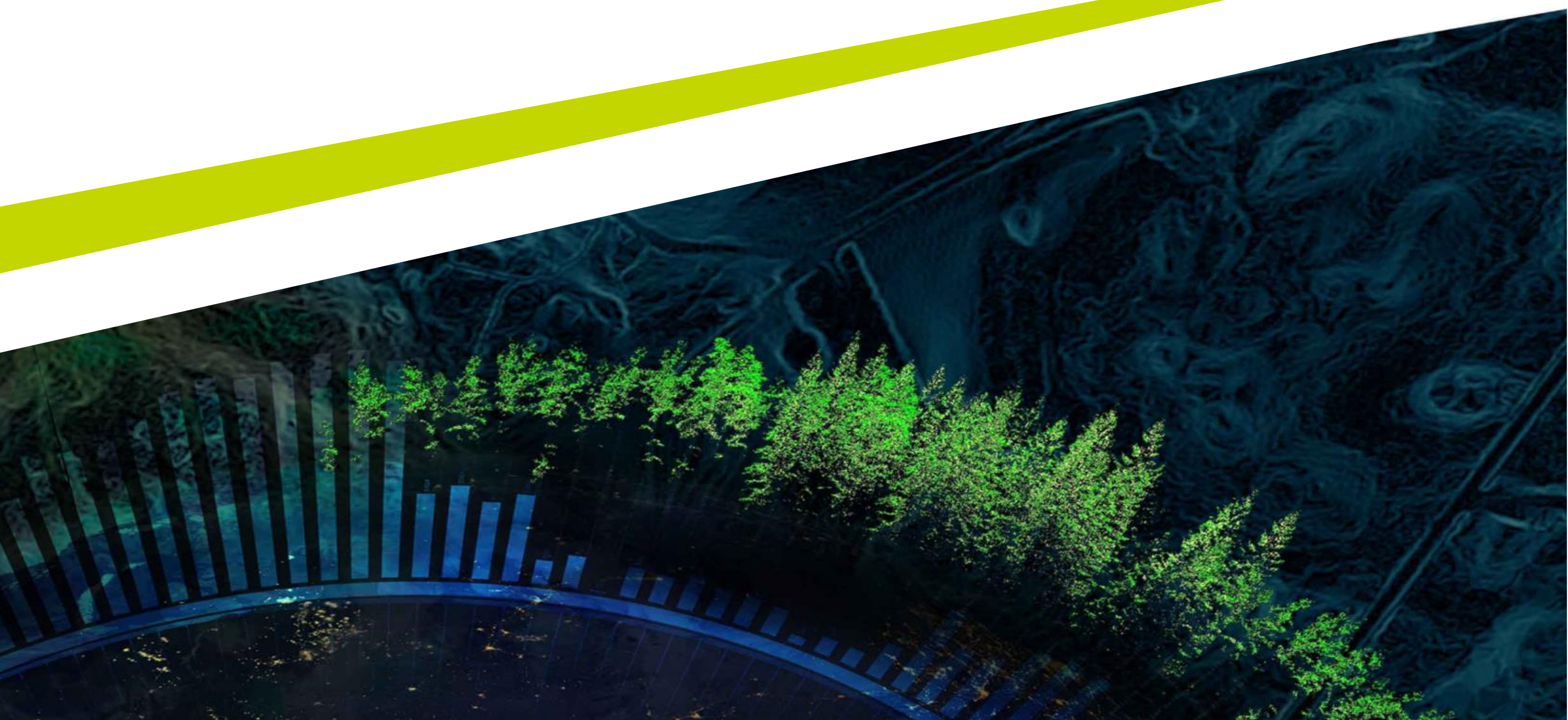

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Keywords: nitrogen, dinitrogen fixation, legumes, groundnut, stylo, pinto peanut, understorey crop

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Abstract

Due to the strong soil erosion caused by agricultural activities and sloping landscape, agroforestry has been introduced as a potentially sustainable cropping practice in the mountainous NW Vietnam. Using legumes as understorey crops in these systems has a potential to limit erosion and smother weeds, as well as provide additional nitrogen (N) to the system. The approximate amounts of N added through dinitrogen fixation serves as an essential information for the comprehensive evaluation of agroforestry practices. Three factors, legume species (*Arachis hypogaea*, *Arachis pintoi* and *Stylo guianensis*), harvest time and trial, were evaluated regarding to the estimated N_2 fixation from air (kg/ha). Results were obtained through field sampling and analysis of total N concentration and ^{15}N abundance. Plants inoculated with rhizobia from the fields were grown in the lab in order to obtain data reflecting ^{15}N discrimination during N_2 fixation (B values). However, the data indicated N contamination, so two other sets of B value were used in the calculation of %Ndfa. Similar total N concentrations (2.56-3.09 %) were found in all legume species. Total N concentration (%) and %Ndfa were dependent on harvest time, with total N concentration higher at harvest 1 than harvest 2, while the %Ndfa of *A. hypogaea* on the contrary was higher at harvest 2. Even though all legumes showed N fixation from air (%Ndfa), *A. hypogaea* had the lowest proportion. Perennial legumes, *Stylosanthes guianensis* and *A. pintoi*, contained much higher amount of N than *A. hypogaea*. Between the two harvests in the same growing season, legume shoots had a significant decrease of total N concentration, but no significant difference in total N amount, which arose from the drastic difference of biomass. Estimated N amount in *A. pintoi* and *S. guianensis* were higher than previous studies. Using the two sets of B values in the calculations produced somewhat different values of %Ndfa, but both indicated the same trend. No difference was found between the three trials and could be considered as a random variable in further study. Some factors caused the uncertainty of the study, lack of local B values and the limited amount of reference for B value of *A. pintoi*. Besides, the different sampling time of N samples and biomass, as well as the limited amount of reference for B value of *A. pintoi* lead to the decrease of accuracy. Nevertheless, due to the consistent findings obtained from the two B values, this study offers an indication of the N supply to the agroforestry system by these different understorey legumes.

Keywords: nitrogen, dinitrogen fixation, legumes, groundnut, stylo, pinto peanut, understorey crop.

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Abbreviations

AF	agroforestry
A. hypogaea	*Arachis hypogaea*
A. pintoi	*Arachis pintoi*
B. pilosa	*Bidens pilosa*
B value	Background value
N	Nitrogen
N_2	Dinitrogen
NW Vietnam	Northwest Vietnam
P	Phosphors
S. guianensis	*Stylosanthes guianensis*
Z. mays	*Zea mays*

1. Introduction

Northwest Vietnam is a mountainous region with the lowest average income in Vietnam. Minority ethnic groups are the highland farmers who experience the most income inequality, hence, this poverty households primarily rely on agriculture income (Tuyen, 2016). To the farmers, crop income is a more equitable source of income which provides them a better stability. Coffee (*Coffea arabica* L.) and maize (*Zea mays* L.) cropping is widespread in this region, and the common practice is monocropping results in intensive management, leading to significant soil degradation (Nguyen et al., 2020, Zimmer et al., 2017). The landscape is also challenging for field management; heavy machinery is impossible to operate on the upland farms, so the field management, especially weeding without herbicides, requires intense labour. Furthermore, the mountainous landscape with steep slopes as well as agriculture activity has a great potential of soil degradation. Hoang et al. (2017) showed that *Coffea arabica* (L.) (coffee) was the major cash crop in this region, while sole income from *Coffea arabica* (L.) made farmers suffer from the potential of price variation. Especially with the monocropping system of *Coffea arabica* (L.), bare soil makes soil erosion even more severe and required attention.

Soil erosion and the following nutrient loss are the major concerns. The agroforestry system demonstrated the ability to preserve soil and was suitable for coffee plantation (Rigal et al., 2020, Tumwebaze and Byakagaba, 2016). Agroforestry has been introduced to NW Vietnam and farmers are aware of its benefits, and monocropping farmers have expressed interest in adopting agroforestry (Nguyen et al., 2020, Zimmer et al., 2017). Previous research from the same region and farming practice demonstrated that soil erosion could be reduced by introducing grass strips along the contour in the field (Do et al., 2023). Meanwhile, in different agroforestry system, the complexity arises due to the coexistence of different plants, making it more complex than in monoculture settings. Weed competes with crops and trees and adaptation of weed management in agroforestry shall consider the presence of multiple species. Depending on the crop combination, reducing herbicide is often recommended. But the reduction of herbicide usage is likely to amplify the demand for manual weeding especially in these hilly environments. However, this increase in manual labour can be challenging because labour resources are generally constrained.

Many legumes are known for their symbiosis with rhizobia and the ability of this for N_2 fixation. As aforementioned, the soil erosion is a major challenge and understorey crops proven to reduce soil erosion (Atangana et al., 2014). Selecting legumes as understorey crops has the potential to control weeds and reduces the amount of labour input. Common legumes as understorey crop are *Glycine max* (soybean) and *Arachis hypogaea* (peanut), which can also be another source of income. Comparing to weed as understorey crop, utilising legumes and the N_2 fixing characteristic as understorey crops hold the potential to benefit both the growth of the cash crop and mitigate soil erosion in the agroforestry system. However, the amounts of N_2 fixed by these crops can differ strongly due to various reasons.

Given that crop income is the primary source of revenue for farmers, the utilization of legumes has the potential to provide economic and sociological advantages, specifically through the sale of legumes as an additional income. Also, selecting legumes as understorey crop can be a type of weed control, which can reduce the labour input during weeding and the usage of herbicide. Moreover, legumes are likely to benefit soil quality by reducing soil erosion and increasing N input. This study aims to assess the effect of using legumes as understorey crop to the N cycle in the coffee agroforestry system.

2. Aim and Objective

The aim of the thesis was to estimate the effect of using three different species of legumes as understorey crops to the N cycle in an agroforestry system, by estimating the relative dependency of the crops on N_2 fixation and the amounts of N_2 fixed. Three replicated trials with similar crop history were considered as the same agroforestry practice and revisited and used for this study. The estimation of N_2-fixation ability of legumes was based on the natural abundance technology. To fulfil the requirement for estimation, plant shoots were sampled in the field and plants were grown in the lab to support the isotope-based calculations.

Specific objectives were to,

1) To estimate the dependence of the legume on N_2 fixation (%Ndfa) for their N nutrition through ^{15}N abundance in each species.
2) To evaluate the total amount of N fixed by the legumes from the estimation of %Ndfa and the dry biomass.
3) To compare the dependence on N_2 fixation at different times and the amount of N derived from air in shoots of the different legumes, as well as the variation between trials.
4) To gain a better insight of the impact of legumes in this agroforestry system based on the estimation of fixed N.

3. Literature review

3.1 Agroforestry system

Soil degradation, resulting from soil erosion and nutrient loss, is a common issue of highland arable land. In Tuan Giao, NW Vietnam, the dominating production of annual monocrop system is found unsustainable because of the land degradation. To establish a sustainable agricultural system, agroforestry is one of the alternatives. The definition of agroforestry from FAO is, “agroforestry is a collective name for land-use systems and technologies where woody perennials (trees, shrubs, palms, bamboos, etc.) are deliberately used on the same land-management units as agricultural crops and/or animals, in some form of spatial arrangement or temporal sequence”. As a combination of agriculture with trees, agroforestry serves multiple purposes, soil preservation, biodiversity improvement and hydrogeological protection, as well as a potential to increase income._Compared to sole-cropped coffee, agroforestry system has been shown to benefit the soil nutrient accumulation of coffee plantation (Notaro et al., 2014, Munroe et al., 2015). Hence, it indicated more microbial activity and higher soil carbon. The slow release of N from plant residue could have long-term benefit to the cash crop. In addition, agroforestry system applied in coffee plantation proved to reduce soil erosion (Sepúlveda and Carrillo, 2015, Do et al., 2023). Grass strips demonstrated a positive effect to soil conservation when grown as an intercrop in an agroforestry system with *Coffea arabica.*

Both woody and herbaceous legumes have been used in agroforestry system. N_2-fixing shade trees had shown to accumulate nitrogen (N) and increase soil microbial population in agroforestry systems with coffee (Munroe et al., 2015). However, the effect of N_2 fixation only occurred in the rhizosphere near the N_2-fixing shade trees and the competition between species resulted in lower coffee yield compared to monoculture systems. The effect of N fixation in an agroforestry system had a lot of uncertainty, which required further investigation. Other than serving as understorey crop, herbaceous legume could be used as food or green manure; *Medicago sativa* (Alfalfa) and *Trifolium repens* (white clover) are often used as green manure, while common grain legumes are *A. hypogaea*, *Cajanus cajan* (pigeonpea), *Glycine max* (soybean) and *Vigna unguiculata* (cowpea). Legumes used as green manure in agroforestry coffee systems showed to release nutrient, including N mineralization (Balota and Chaves, 2011, Matos et al., 2008).

Agroforestry system not only benefited the soil, but also often showed positive economic impact. In a broad aspect, it contributed to food security and resilience climate change (Mbow et al., 2014). To farmers, agroforestry compared to sole-cropped system provided better resistance to climate change and a more stable income, as well as lower reliance on chemical fertilizer (Kerr, 2012, Penot et al., 2017). The reduce of weeding from understorey crop required lower the manpower for maintenance (Olorunmaiye, 2010).

3.1.1 Agroforestry system of coffee with legume as understorey crop

Legumes had been used as green manure for coffee plantation to increase the nutrient level. Study had found that *A. pintoi* and *S. guianensis* intercropped with coffee can release N to soil, and both species released N and P which has positive effect as green manure in the agroforestry system (Matos et al., 2011). N_2 fixation ability of legumes varied due to various factors, genetic, environment, soil N amount and activity of rhizobia (Herridge et al., 2008). Although the impact of intercropped *A. hypogaea* on soil in coffee plantations remained unknown, it has been shown to contribute to N availability and increase yield of *Zea mays* (maize) (Okito et al., 2004, Senaratne et al., 1995). Using legume as understorey crop had economical potential. For example, *A. hypogaea* intercropped in agroforestry system created extra profit and benefit in economical and sociological aspect (Karmini et al., 2017)

3.1.2 Legume species in the agroforestry system

A wide range of legumes had been used as understorey crop, e.g. *A. hypogaea* (L.), *Glycine max* ((L.) Merr.) (soybean) and *Canavalia ensiformis* ((L.) DC.) (jack bean). Beside serving as green manure to benefit soil fertility, legumes commonly served another purpose, as grain legume. By the characteristic, grain legume could be a source of food and extra income.

Arachis hypogaea (L.)

Commonly known as groundnut or peanut, it is an annual plant and matured between 84-105 days, depending on the species (Olayinka and Etejere, 2015). *Arachis hypogaea* is both grain legume and green manure, but also exhibits outstanding dinitrogen fixation ability compared to most other legumes (Herridge et al., 2008). Also, agroforestry system with *A. hypogaea* contributes to soil conservation (Sarminah et al., 2018). *Arachis hypogaea* in agroforestry system provides additional economical potential to small-scale farmer (Adinya et al., 2010).

Arachis pintoi (Krapov. & W.C.Gregory)

Also known as pinto peanut, *Arachis pintoi* is a perennial legume with a plant height of 20-50 cm, and the creeping characteristic allowed a good coverage. *Arachis pintoi* has been used as pasture plant and green manure to benefit soil nutrients. Using *A. pintoi* as green manure in *Coffea arabica* plantation resulted in higher plantation height than ammonium sulphate fertilizer (Vilela et al., 2011). The intercrop of *A. pintoi* has a potential to benefit the nutrient cycle in the rainy season of tropical climate (Oliveira et al., 2003). As an understorey crop, *A. pintoi* contributes to N_2 fixation and potential to mitigate nitrous oxide emissions in subtropical coffee plantations (Rose et al., 2019a).

Stylosanthes guianensis ((Aubl.) Sw.)

Stylosanthes guianensis, also known as stylo, was a perennial legume that is commonly found in tropical climate. It can reach a plant height to 1-1.5 m. *Stylosanthes guianensis* had a long history in conservation agricultural practices aimed at enhancing soil fertility (Tarawali et al., 1999), and intercropped has demonstrated an economical potential by increasing the yield of cash crop (Ahmad et al., 2022). In coffee agroforestry system, *S. guianensis* has been observed to release N (Matos et al., 2011). Also, *S. guianensis* showed a good growth among common tropical legumes in acid soil (Tilki and Fisher, 1998) .

3.2 N_2 fixation process

The symbiosis between rhizobia and legume has been shown to be the most important source of biologically fixed N in the agricultural system (Herridge et al., 2008). The process of N_2 fixation involves the reduction of atmospheric N_2 into NH_3 by the enzyme nitrogenase:

$$N_2 + 8H^+ + 16ATP \xrightarrow{nitrogenase} 2NH_3 + H_2 + 16ADP + 16P_i$$

This process involves energy consumption by reducing ATP, and legumes act as the source of the energy. The amount of N fixed is regulated by various factors, including infection of bacteria species, soil N amount, soil pH, sunlight, and drought. Every combination of legume and rhizobia has an optimum temperature of infection, mostly between 25-40 ℃. Lower population level of rhizobia has been found in drought conditions (Mohammadi et al., 2012). Due to the energy consumption of the process, legumes are less likely to establish symbiosis with rhizobia or will down-regulate the fixation activity at higher N availability in the soil. In temperate forests, the N_2 fixation was higher at higher CO_2 concentration and low available N in soil (Rastetter et al., 2001).

3.2.1 Determination of N_2 fixation from air

N_2 fixation was difficult to measure, different approaches have been used to estimate the amounts of N fixed by legumes, including the N balance method, the N difference method, the ^{15}N dilution method and the ^{15}N natural abundance method. N_2 fixation from air is commonly studied through the tracer approach of ^{15}N by utilising the characteristic of isotope. The principles of the ^{15}N natural abundance method will be presented here as that was selected for the present study.

Stable isotope, such as ^{15}N, have different abundances depending on the origin of the sample, and e.g., the geological and biological processes that have occurred. The relative concentration of a particular isotope compares to other isotopes of the same element is the natural abundance, and the natural abundance of ^{15}N refers to the relative concentration of ^{15}N compared to ^{14}N in the environment. The atmospheric abundance of ^{15}N is 0.3676%, and is used as the standard for $d^{15}N$ (Ehleringer and Rundel, 1989). Different plant species, soils and air contains different ^{15}N abundance. These differences can be used to estimate proportion of N in an N-fixing plants that is derived from air, and from the soil, respectively. Shearer and Kohl (1986) thus established a method to estimate the percentage of legume N_2 fixation from air by comparing the ^{15}N abundance of a targeted legume, a neighbouring non-N_2-fixing plants, and targeted legume grown in non-N medium. The ^{15}N abundance from microbial activity in N_2 fixation is usually lower than other source of N, which creates a statistically significant fraction that impact the total ^{15}N abundance. Eliminate the impact from microbial activity from N_2 fixation and N from soil, the percentage of ^{15}N abundance can reflect the percentage of N from air. The equation to determination N_2 fixation from air (%Ndfa) is:

$$(\%)Ndfa = \frac{(d^{15}N_o - d^{15}N_t)}{(d^{15}N_o - d^{15}N_a)} \times 100(\%)$$

where $d^{15}N_t$ is the $d^{15}N$ value of the targeted legume from the field value, which has both soil and air as the source of ^{15}N. The $d^{15}N$ value of the reference plant is the $d^{15}N_o$, i.e., a plant species without N_2 fixation ability but growing under the same condition as targeted legume, which represented the N uptake from soil. In this study, the weed *Bidens pilosa* (L.) (black-jack), was chosen as the reference plant due to the similar root structure to the legumes and the presence in every plot of all trials. Finally, $d^{15}N_a$ is the background value (B value) of the targeted legume when grown solely with a N free medium. This value serves to correct for the degree of discrimination of the 15N atoms that occurs during the fixation process. If hydroponic data are unavailable, proxy B values of the legumes can be derived by two approaches by 1) Unkovich et al. (2008) and 2) Eriksen and Høgh-Jensen (1998). Unkovich et al. (2008) published an approach indicating that under the condition without local hydroponic data as B value, the mean of previous published B value can be used. Eriksen and Høgh-Jensen (1998) showed that the lowest value of field data, $d^{15}N_t$, was similar to the B value from hydroponics, so the lowest value of field data could be taken as B value for the same research.

4. Materials and Methods

The percentage and amount of N derived from air were estimated for three understorey legumes in three field trials in Tuan Giao, NW Vietnam. The estimation method of N_2 fixation from Shearer and Kohl (1986) was chosen to estimate the N_2 fixation of the legumes during growing season. To this end, shoots were collected twice during the growing season from the legumes and reference plant. Shoot samples was milled and packed for EA-IRMS and elemental analysis.

4.1 Study site

Tuan Giao District is located at Northwest region of Vietnam (Fig. 1). Mountainous region with steep slope. *Zea mays and Coffea arabica* (L.) (coffee) was the major cash crop in this region (Hoang et al., 2017).

The experimental site is above 800 meters altitude, and the cash crop at the site was *Coffea arabica*. Most farmers come from the minority ethnic group, the Hmong people. The topsoil is an acid sandy loam (Table 1). Rainfall is concentrated from May to August (Fig. 2), which is the main growing season. Except for *Coffea arabica*, intercropped system of temperate fruit trees and vegetable can also be found in this region. Lack of rainfall from October to December makes the accessibility of water a concern and is a limitation to the crop growth.

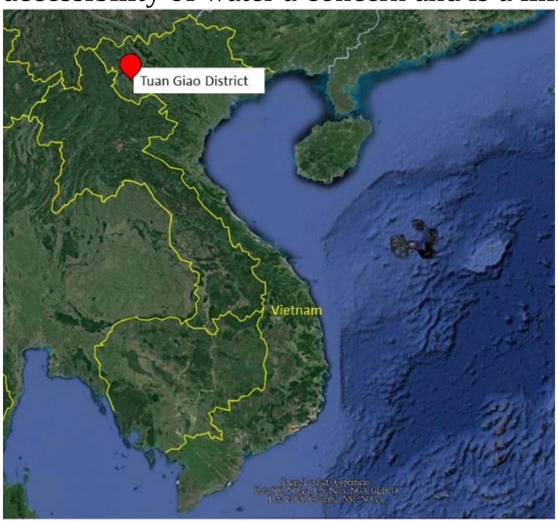

Figure 1. Study site location (Google Earth Pro, 2023)

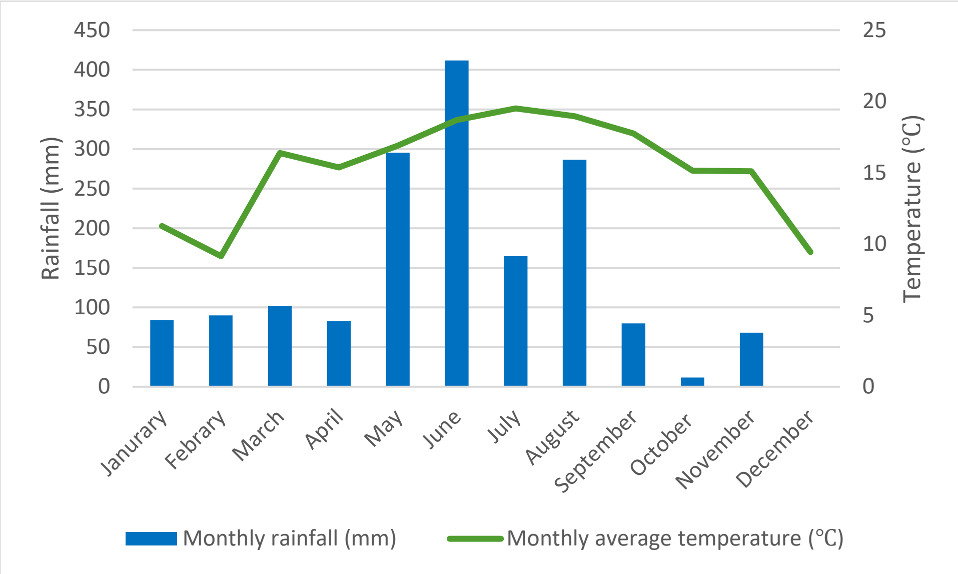

Figure 2. Rainfall and temperature of 2022.

Table 1. Soil chemical and physical properties of Tuan Giao.

Depth (cm)	Bulk density (g/cm)	Porosity (%)	Total organic C (%)	Total N (%)	pH (H_2O)	CEC (meq/100g)
0-20	1.24	51.67	0.71	0.13	5.22	12.44
20-45	1.23	51.18	0.40	0.08	4.99	9.08

4.2 Trial design and management

The study site consisted of three trials which were a short distance apart (Fig. 3). *Coffea arabica* and *Prunus salicina* (L.) (plum) were the cash crops. The average slope of trial 1 to 3 were 28°, 29° and 34°, respectively.

Each trial consisted of four blocks, with each block further divided into four parallel plots assigned to specific understorey crop treatments, *A. hypogaea*, *A. pintoi*, *S. guianensis*, and a control treatment without any understorey crop, which were randomly assigned within each block. The reference plant, *Bidens pilosa* (L.) (black-jack), was sampled in the control treatment. Each plot consisted of 8 rows of *Coffea arabica* bush, trees of *Prunus salicina* (L.) and grass strips (*Megathyrsus maximus*), as well as legumes as understorey crop (Fig. 4).

Arachis hypogaea was sowed on May 3rd, 2022. The perennial legumes, *A. pintoi* and *S. guianensis*, had been on the site since 2020. Harvest time of the *A. hypogaea* was September 6th, 126 days after sowing. Fertiliser was only applied near the roots of *Coffea arabica* and the plum trees, whereas no fertilization was made of grass strips and legumes.

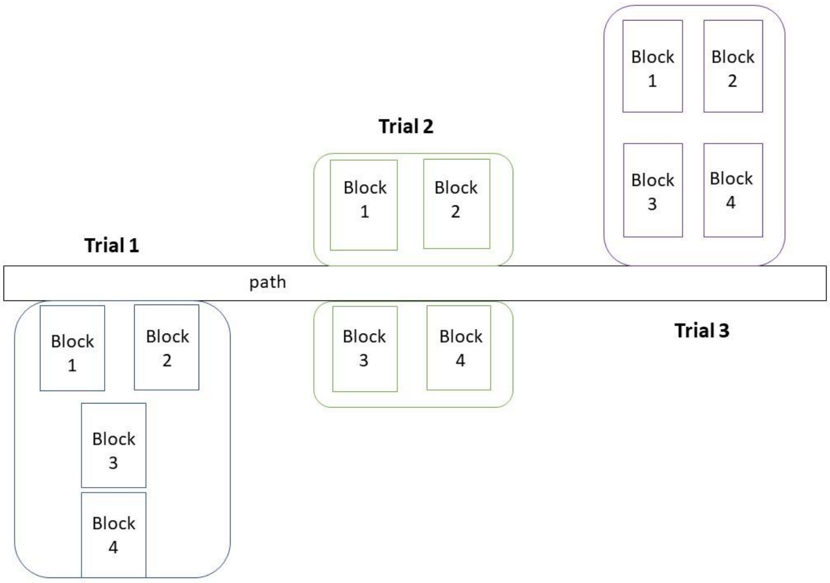

Figure 3. Schematic presentation of location of trials and blocks; length of path and block did not reflect the true distances.

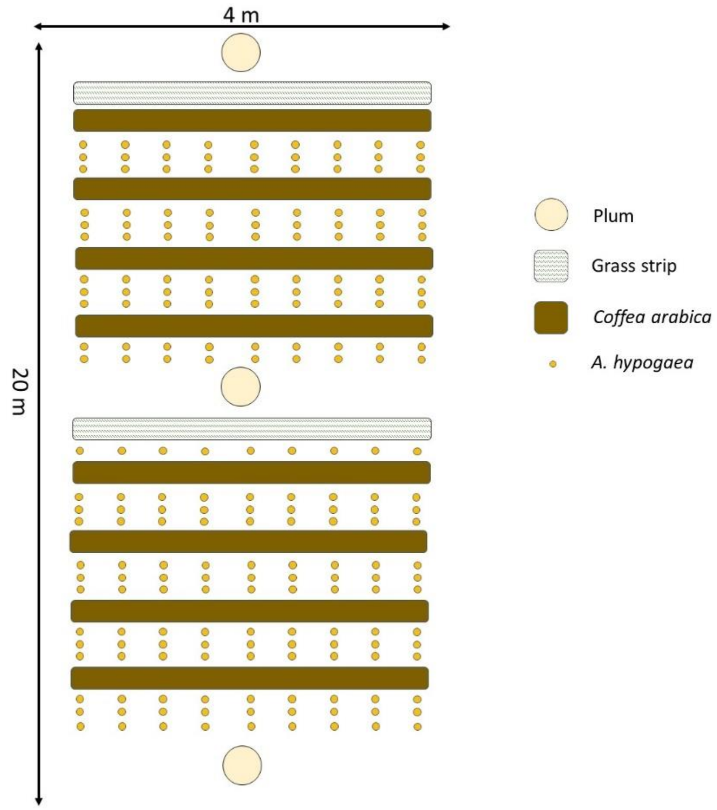

Figure 4. Schematic presentation of an *A. hypogaea* plot design. The overall design was the same for the other legume treatments, although the legumes had spread across rows, especially in the *A. pintoi* plots.

4.3 Field sampling

Legume shoots were collected twice in 2022. The first sampling occurred on May 27th and the second occurred on July 25th and 26th. During the first sampling, the collected length of shoots was 10 - 15 cm corresponded to the spring (re)growth. For the next sampling, the shoot length collected was based on previous field work which showed the growth of legumes between two months; *B. pilosa*, *A. hypogaea* and *A. pintoi* were collected 10 cm from the shoot tip, and *S. guianensis* was collected 20 cm.

To ensure the representativeness of the sample collection, at least ten shoots were collected from each plot, each originating from a different legume row within the plot. The shoot had to come from the middle of the plot to mitigate the impact from contamination from fertilization of *Coffea arabica* and *Prunus salicina* (L.), as well as neighbouring plots.

4.4 B value of legumes

To take into account the degree of discrimination by the *in situ* rhizobia during the N_2 fixation process Plants were grown to produce background values (B values). To this end, a first generation of each species was germinated or rooted with rhizobia derived from root nodules collected from each species in the field. These were then cultivated in a controlled situation without N provided.

Due to the distinctive characteristic of each species, different reproduction methods were employed. The B value plants of *A. hypogaea* and *S. guianensis* were sprouted from the same batch of commercial seeds used for the field trials. Ten seeds of *A. hypogaea* and 20 seeds of *S. guianensis* were germinated, and the five best performing plants of each were retained as the background value sample. The plant material for *A. pintoi* was collected as on-site cuttings of the parental generation. 20 fresh shoots were collected on-site and brought back to the lab. Five shoots with the best root development were kept as the test group after rooting in water. All B value plants had started sprouting on July 24th.

Rhizobia were introduced twice to the roots, once while still in Vietnam and once after arrival in Sweden. After each species had initiated roots, nodules were crushed and mixed with pure water to extract the rhizobia. The rhizobia extracts were added directly to the plant roots. Right after the plants were planted in pots and started growing in the lab, new extracts of rhizobia were added to the pots. Legumes were grown in a combination substrate from perlite, vermiculite and sand to ensure the drainage. Hoagland solution without N were added as the source of nutrient, and plants were watered with Milli-Q® water. Nodules were found on roots while harvesting for analysis, confirming successful inoculation. To imitate the field condition, room temperature was controlled between 18-27 ℃ and extra light of 5000 lm for 12 hours was provided due to the insufficient sunlight during winter. All B value plants were harvested on November 8^{th}.

4.5 Sample processing and N analysis

Samples were air-dried in room temperature and were milled to approximately 1-2 mm with a knife mill (Retsch® GM 200), then mixed well and a subsample ground to powder in a ball mill (Retsch® MM2). Isotope ratio mass spectrometer (EA-IRMS) was used for the ^{15}N abundance and elemental analyser for total N analysis. To increase the precision, subsamples weighed into tin capsules were 3-5 mg as recommended by the lab for 0.5-5 % of N concentration. Plant samples in tin capsules were sent to SLU Umeå for EA-IRMS (Thermo Fisher Scientific® DeltaV) and elemental analysis (Thermo Fisher Scientific® Flash EA 2000).

4.6 Dry biomass of legume species

To determine the amount of total N fixed (kg/ha), total aboveground biomass data from the growing season were required. The biomass was collected by Thuong Pham Huu, ICRAF Vietnam, who provided the dry matter data. Biomass of *A. hypogaea* was collected once at the end of the growing season, on September 6^{th}, 2022, 126 days after sowing. *Stylo guianensis* biomass was collected twice in the growing season, April 24^{th} and July 4^{th}, which corresponded to the two harvests of shoots made in the project hosting this study. These dry matter data were taken as approximations for the two occasions of N analysis in this study. *Arachis pintoi* biomass was collected on March 23^{rd}, 2023 and the interval of biomass data was representing the growth of a year.

4.7 Calculations

To estimate the N_2 fixation ability from legume species, equation from Shearer and Kohl (1986) was used for calculation.

$$(\%)Ndfa = \frac{(d^{15}N_o - d^{15}N_t)}{(d^{15}N_o - d^{15}N_a)} \times 100(\%)$$

$d^{15}N_o$ was the $d^{15}N$ value of *Bidens pilosa* that was grow in the same area. $d^{15}N_t$ was the $d^{15}N$ value of *A. hypogaea*, *S. guianesis* and *A. pintoi*. $d^{15}N_a$ was the background value of legumes which grow solely by pot experiment with N free medium. Two approaches were used to determine the background value of legumes. 1) Unkovich et al. (2008) suggested that if no local B value exist, the average of previously published B value can be used. 2) Research from Eriksen and Høgh-Jensen (1998) showed that the lowest value from field data can be used as B value.

Total N amount (kg/ha) was calculated by multiplying the N concentration (%) and dry biomass (kg/ha). To evaluate the N fixed by air, total N amount (kg/ha) was multiplied by %Ndfa of the respective biomass. As mentioned previously, the sampling date for total N concentration and determination of dry biomass (kg/ha) were not the same. Nevertheless, the total N concentrations and ^{15}N abundances were taken as approximations of those at the time of biomass harvesting.

4.8 Statistical analysis

To understand the factors affecting the N fixed from air (%Ndfa and kg N/ha), the difference between the two harvests, three trials and three legume species were considered. Three-way ANOVA (with 95% confidence interval) was used to determine the significance of harvest time, legume species and trials on the total N concentration (%), N fixation from air (%Ndfa). Tukey's Honest Significant Difference (Tukey's HSD) test with a variance of 95% confidence interval was used to evaluate the significance between means for legume species and trials. JMP® Pro 17 (2023) and Microsoft® Excel were used for three-way ANOVA, data distribution and box plot design.

5. Result

The field sampling worked out well, but the ^{15}N abundance of the lab-grown *A. pintoi* and *S. guianensis* showed extremely high values suggesting N contamination from another source. The data could thus not be used as B values in the calculations of %Ndfa. References provided two substitutional approaches of B value; Unkovich et al. (2008) and Eriksen and Høgh-Jensen (1998) which were used.

5.1 Total N concentration

Total N concentration in shoot samples was significantly affected by harvest time and species. *Arachis pintoi* (2.56%) showed significantly lower concentration than *A. hypogaea* (2.96%) and *S. guianensis* (3.09%). Harvest 1 showed significantly higher concentration (3.18%) than harvest 2 (2.87%).

The annual growth characteristic of *A. hypogaea* made the seed N concentration one of the potential factors to its high total N amount (Table 2). The seed of *A. hypogaea* had a higher N concentration than the shoots.

Table 2. N amount of *A. hypogaea*.

Species	$d^{15}N$ (‰)	Average weight of seed (g)	Average N concentration (%)	Average N in each seed (mg)
A. hypogaea	1.6	0.49	4.5	22.7

5.2 N_2 fixation from air

5.2.1 B value approach from Unkovich et al. (2008)

Following the recommendation of Unkovich et al. (2008), the average value obtained from previous published B values (Table 3).

Table 3. B values of legumes from the literature

Species	$d^{15}N$ / ‰
A. hypogaea	-1.33[a]
A. pintoi	-0.20[b]
S. guianensis	-1.15[c]

[a] Average of Okito et al. (2004), Peoples et al. (1992, unpublished data) and Nyemba and Dakora (2010)

[b] Rose et al. (2019a)

[c] Average of Yoneyama et al. (1986), Nguluu et al. (2002), People (unpublished data) and Zemek et al. (2018)

The three-way ANOVA indicated that species and harvest time significantly affected %Ndfa (Fig. 5), and that there was an interaction between species and harvest time. No significant differences were found in the %Ndfa between the three trials.

Among three species, *A. hypogaea* showed significantly lower %Ndfa compared to the other two (Fig. 5). The average %Ndfa through the growing season of *A. hypogaea*, *A. pintoi* and *S. guianensis* was 48%, 80% and 86%, respectively. The %Ndfa showed significant overall increase between harvest 1 and 2.

In the case between the interaction of harvest and species, %Ndfa was only significant for *A. hypogaea*. Although the average %Ndfa of *A. pintoi* and *S. guianensis* was slightly higher in harvest two, there was no significant difference. *Arachis hypogaea* in harvest 1 has significantly lowest value of 39% compared to others, while *A. pintoi* in harvest 2 has the highest average of 86%.

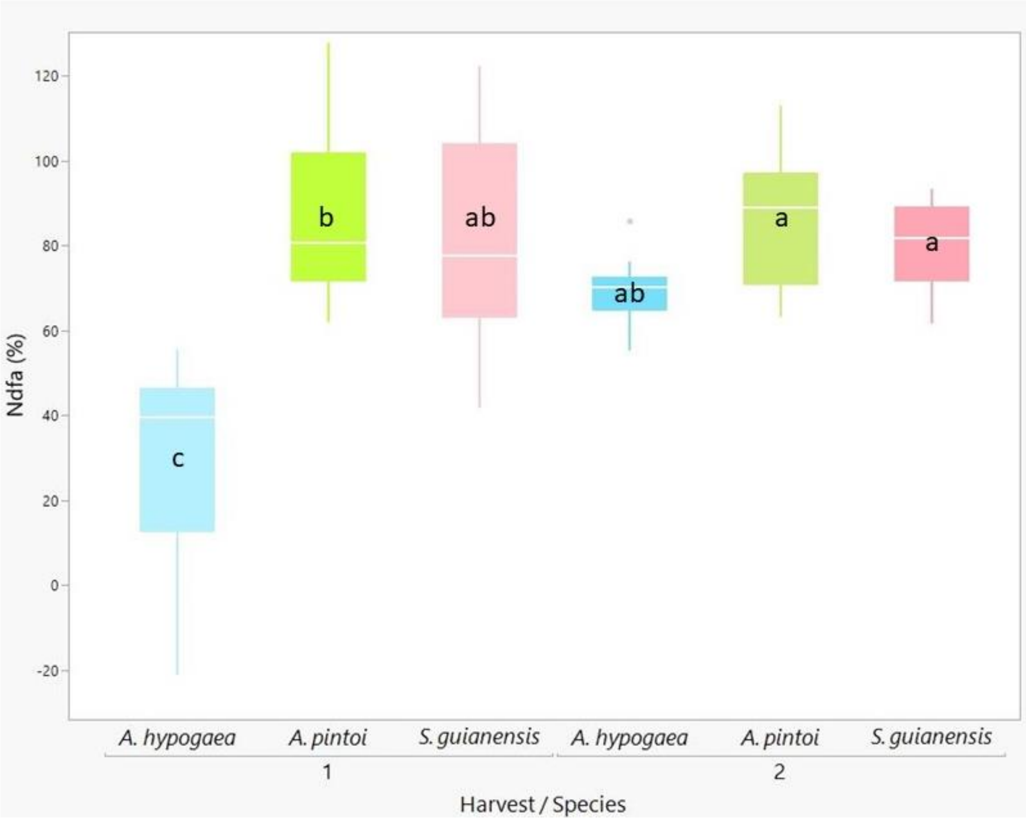

Figure 5. %Ndfa of different species and harvest time based on the B value approach of Unkovich et al. (2008).

5.2.2 B value approach from Eriksen and Høgh-Jensen (1998)

Eriksen and Høgh-Jensen (1998) reported that the B value derived from pot-grown plants fully dependent on N from air was similar to the lowest value from plant materials collected in the field (excluding outliners) and concluded that the lowest value from field experiment could be used as a proxy of the B value. The lowest $d^{15}N$ value of each legume species in these field experiments was thus used as a proxy of a B value (Table 4). Compared to the B value from Unkovich et al. (2008), the difference in B value between species were lesser in with this approach.

Table 4. B values of legumes from the field experiments

Species	$d^{15}N$ / ‰
A. hypogaea	-0.33
A. pintoi	-0.84
S. guianensis	-1.82

Results revealed that only harvest occasion and the interaction between harvest and species had significant effects on %Ndfa, while no significant differences were observed between trials. The average %Ndfa through across the two harvests were 58%, 73% and 71% for *A. hypogaea*, *A. pintoi* and *S. guianensis*, respectively, but in contrast to the estimates based on B values from the literature, these did not differ significantly.

Furthermore, harvest 2 exhibited significantly higher values than harvest 1, suggesting that N_2 fixation took place during the growing season even in *A. hypogaea*. Regarding the interaction between harvest occasion and species, *A. hypogaea* resulted in significant increase between harvests, while other species had no significant pattern. *Arachis hypogaea* in harvest 1 had significantly lower %Ndfa values compared to the other species and harvest. o, *A. hypogaea* in harvest 2 had the highest average value of 81%, while *A. hypogaea* in harvest 1 had the lowest average value of 47% (Fig. 6).

Comparing the two sets of the %Ndfa, both were dependent on harvest and the interaction between harvest, while only Unkovich et al. (2008) had species as a significant main factor. Both approaches had *A. hypogaea* in harvest 1 as the lowest %Ndfa, while the highest %Ndfa from Unkovich et al. (2008) appeared at *A. pintoi* in harvest 2, but Eriksen and Høgh-Jensen (1998) had it at *A. hypogaea* in harvest 2.

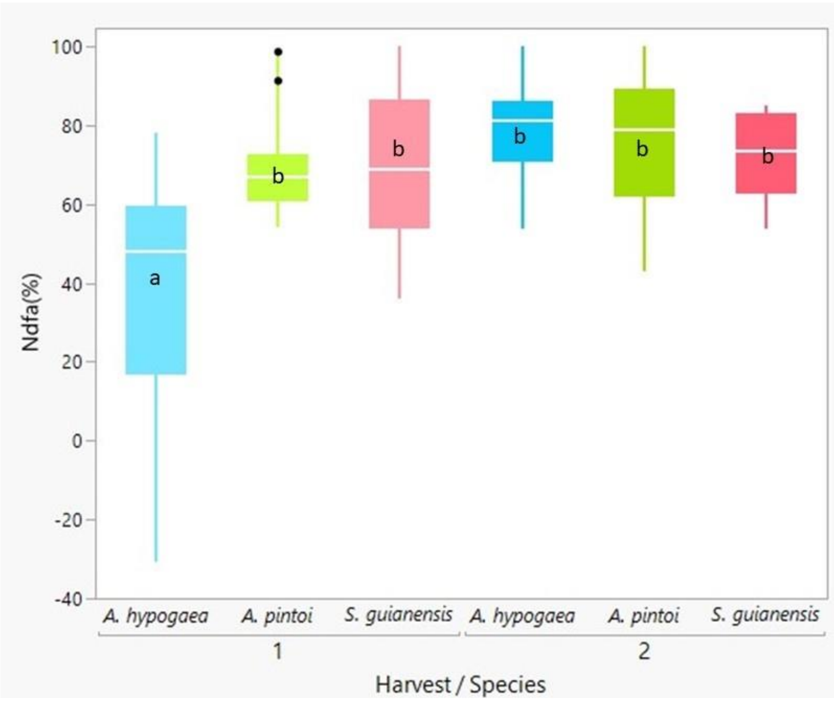

Figure 6. %Ndfa of different species and harvest time based on the B value approach of Eriksen and Høgh-Jensen (1998).

5.3 Estimation of total N amount

Average dry biomass of *A. hypogaea* accumulated in the growing season was 343 (kg/ha). Average dry biomass of *S. guianensis* from harvest 1 and 2 were 1546 and

1802, respectively. Biomass of *A. pintoi* was collected on a different time, March 11th, 2023, and the average dry weight was 1915 (kg/ha).

Average of total N amount in shoots of *A. hypogaea* was 10 kg/ha over the growing season. *Stylo guianensis* was estimated by each harvests, and similar N amount were found in both harvests. N amount of *S. guianensis* was 51 kg/ha in harvest 1 and 50 kg/ha in harvest 2. Using the %Ndfa of harvest 2 to estimate the annual N amount of *A. pintoi* suggested a total amount of N at 49 kg/ha. Comparing the three species, the annual amount of *S. guianensis* was at least two times higher than *A. pintoi*, and *A. hypogaea* has much less than both perennial legumes (Fig. 7).

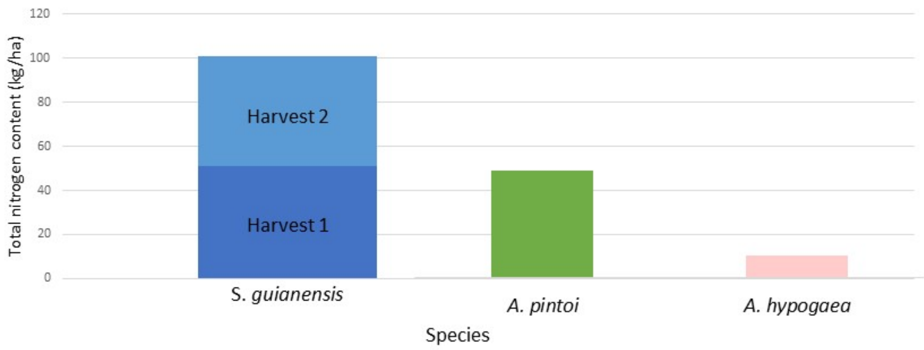

Figure 7. Estimated total N amount (kg/ha) of legumes; *S. guianensis* was estimated at both harvests, *A. pintoi* was estimated for the entire year, and *A. hypogaea* was estimated for one growth cycle.

5.4 Estimated amounts of fixed N in aboveground biomass

When combining the %Ndfa and estimating the N amount (kg/ha), the total N fixation from air for *A. hypogaea* was estimated to be 5 and 6 (kg/ha) in a year, using the B values from Unkovich et al. (2008) and Eriksen and Høgh-Jensen (1998), respectively (Fig. 8). For *S. guianensis*, using the two B values, the expected N fixation from air was 39 and 36 (kg/ha) in harvest 1, as well as 39 and 36 (kg/ha) in harvest 2. *Arachis pintoi* was as to have 43 and 36 (kg/ha) annually.

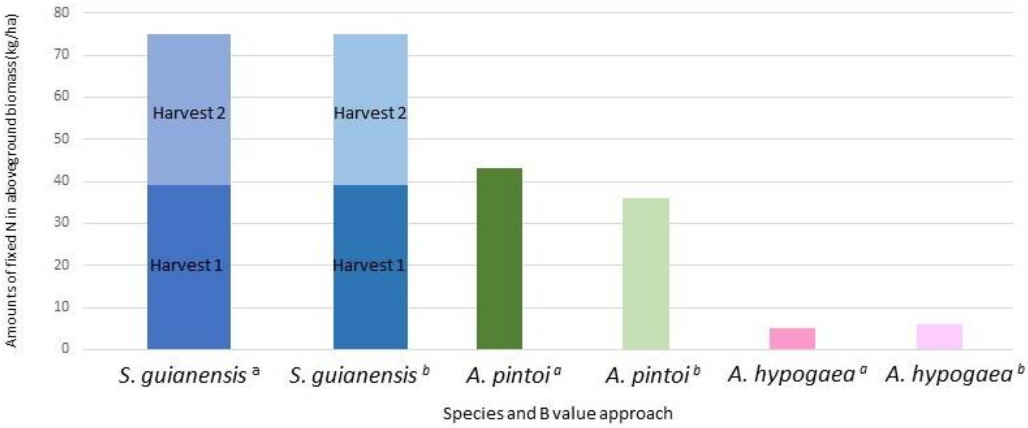

Figure 8. Estimated amounts of fixed N in aboveground biomass (kg/ha). *S. guianensis* was estimated at both harvests, *A. pintoi* was estimated for the entire year, and *A. hypogaea* was estimated for one growth cycle; a. was estimated with the B value approach from Zemek et al. (2018) and b. was estimated with the B value approach from Eriksen and Høgh-Jensen (1998).

6. Discussion

6.1 Total N concentrations and amounts of N in aboveground biomass

The N concentrations (%) of *A. pintoi*, and *S. guianesis* in this study were lower than those reported by Mendonça et al. (2017) who found them to be 2.72% and 3.20%, respectively. While N concentration might be affected by multiple reasons, both Mendonça et al. (2017) and this study demonstrated the same trend of higher N concentration (%) in *S. guianesis* compared to *A. pintoi*.

The perennial legumes indicated a higher accumulation than the annual *A. hypogaea*. Between the harvests, there was no evidence showing that perennial legume species depended on N_2 fixation to different extent at different times of the year. The average N concentrations (%) were similar for the three species, while the annual accumulated total N (kg/ha) of *S. guianensis* was at least two times higher than *A. pintoi*, and *A. pintoi* were five times higher than *A. hypogaea*. Since the total N amount was derived as the N concentration multiplied by the dry biomass, the dry biomass of each species was clearly the major factor explaining the difference in total N amount. Notaro et al. (2014) also indicated that the realise of the N amount varied among the coverage of legume litters. Overall, total amount of N fixed from air were found in all three legumes. All three species benefit the accumulation of nitrogen.

6.2 The dependence of the legume on N_2 fixation and amounts of N fixed

Of the three species, *A. pintoi* and *S. guianensis* showed no significant difference were found between the speceis in %Ndfa. However, research from Mendonça et al. (2017) reported that the *S. guianensis* had a significant higher N_2 fixation from biological activity than *A. pintoi*.

In similar growing system, Rose et al. (2019b) showed that the average %Ndfa for *A. pintoi* was 66%, which was lower than the %Ndfa based on both B values in this study. However, the estimation of annual N fixation from air was 95 kg/ha (Rose et al., 2019b), which was higher than this study. Rose et al. (2019b) carried out their research through the entire year using *A. pintoi* as understorey crop in subtropical coffee agroforestry system and found that %Ndfa was higher during the warmer months, which led to most N being fixated in the warm months. The main growing season at the site of this study took part from May to September, which coincides with the warm and moist season in North Vietnam. If year-round N_2 fixation were extrapolated from this study, N levels might be overestimated. Also, *A. pintoi* in Mendonça et al. (2017) showed lower total N amount and N fixed from air: 38% of N derived from fixation in *A. pintoi*, and 14 kg/ha of N fixed over 150 days.

Research with *A. hypogaea* as understorey crop in *Coffea arabica* agroforestry system was not found. On the other hand, Senaratne et al. (1995) presented data for an intercrop of *A. hypogaea* and *Zea mays* and sampled at the end of the growing season, in which *A. hypogaea* derived 85% of the N from air. Intercrop of *Z. mays* showed similar N fixed from air. Preferrable growth environment of *Zea mays* and *Coffea arabica* were similar, both preferred well-drained soils, while *Coffea arabica* had a taller plant height than *Z. mays*.

Stylo guianensis showed 46% of N derived from air (Mendonça et al., 2017), which was much lower than in this study. The dry biomass was 2070 kg/ha within 150 days, with 28 kg/ha of N fixed from air. In that case, the lower total amount of N fixed was a result from both lower dry biomass and proportion of N derived from air.

6.3 Uncertainty of this study

The ^{15}N abundance produced by lab cultivation were higher than the field values and could not be utilized as B value of the legumes. The higher B value was indicating N contamination during the cultivation. After further experimentation, vermiculite was found to contain nitrogen. The lack of B value from lab made the %Ndfa less accurate, due to the different amount of N_2 fixation from rhizobia species and strains.

Among three species, *A. hypogaea* was the only one that had seed planted in the same year. With the large size of each seed and high N concentration, the amount of N in seeds was very likely to affect the total N amount of the shoots. Each plot was planted with 640 seeds, and the estimated total N from seeds was 2 kg/ha. As aforementioned, seed N accommodated 18% of total N amount from *A. hypogaea*. Taking the high amount of N from seeds into consideration, it was likely to reduce N_2 fixation.

Arachis hypogaea was sown on May 3rd 2022, and the shoots for the second N analysis were collected on July 25th 2022 (83 days), which was the reproductive stage in a growth cycle. The dry biomass data were collected on September 6th, 2022 (126 days), at the maturity stage and the only harvest for the entire growing season. To estimate the N_2 fixation, dry biomass data and total N concentration from different dates were thus used, which created uncertainty to the estimation. Loganathan and Krishnamoorthy (1977) has showed that maturity stage had much higher N presented in shoots than the reproductive stage. This study using total N concentration from the reproductive stage to estimate the N_2 fixation of the entire growing season was therefore likely to underestimate the amounts of N fixed.

Kyei-Boahen et al. (2005) indicated that the strain of rhizobia inoculated in *A. pintoi* did not create a difference in field studies. Also, nodules from a field study had a great diversity of rhizobia (Nygren et al., 2012). Due to the diversity of rhizobia that may inoculate on this trial, the potential effect from rhizobial strain was not considered in this study.

Legumes in artificial substrate without source of N were done in the lab, but the $d^{15}N$ of N free legumes ($d^{15}N_a$) were above the $d^{15}N$ of field value ($d^{15}N_t$). Without hydroponic data, methods from Unkovich et al. (2008) and Eriksen and Høgh-Jensen (1998) were used to determine the B value. In the case of Unkovich et al. (2008), the average B value of previous published data was used for all the legume species. *Stylo guianensis* and *A. hypogaea* had found more multiple references, while *A. pintoi* had only one reference. The lack of reference for *A. pintoi* increases the uncertainty to determine %Ndfa.

6.4 Choice of B value approach

Comparing the two approaches of B value, Unkovich et al. (2008) had higher B values for *A. pintoi* and *S. guianensis,* while the approach of Eriksen and Høgh-Jensen (1998) had a higher B value for *A. hypogaea*. *Arachis hypogaea* in harvest 1 had the lowest average value of %Ndfa irrespective of the approach used. On the other hand, the variation in B values resulted in different highest values of %Ndfa in the study. Additionally, following the approach of Eriksen and Høgh-Jensen (1998) produced somewhat smaller differences between species in the average %Ndfa; as a result, Unkovich et al. (2008) indicated a significant impact, whereas Eriksen and Høgh-Jensen (1998) did not. Despite these differences, both approaches indicated similar results and both approaches indicated that there was a significant increase in %Ndfa between harvest 1 and 2.

6.5 Discussion for further research

This study found that N_2 fixation occurred with legumes as understorey crop in the growing *Coffea arabica*, with substantially different amounts of N added to the systems. However, how much of this N that enters the soil and can be utilised by *Coffea arabica* and *Prunus salicina* (L.) are still unknown. Also, the potential to reduce soil erosion by legume is uncertain. The different legume species appeared to have greatly different biomass and harvest method, which may be reflected on the efficiency of legume for soil erosion mitigation. Further research on soil erosion and N use efficiency could better identify the impact of legume in this agroforestry system.

7. Conclusion

The dependence of perennial legumes on fixation was similar between the harvest occassions and also similar to one another; *A. hypogaea* showed relatively low dependence on fixation at the start of the growing season but this later increased to approximately the same level as the perennial legumes. N_2 fixation was found in all three species, even though the dependence on N_2 fixation and the amounts of N fixed from air differed greatly between the species and harvest times. Although the increase percentage of %Ndfa from different B value approach appeared were different, the significant factors and general trends were similar. *S. guianensis* was estimated to fix 51 kg/ha in harvest 1 and 50 kg/ha in harvest 2, *A. pintoi* was estimated to fix 50 kg/ha annually, and *A. hypogaea* 10 kg/ha over its growing season. The legumes fixed different amounts of N from the air which in may partially be provide to the cash crops (plum trees and coffee shrubs). Whether the N_2 fixation from legumes in reality benefits the growth of the plum trees and coffee shrubs requires further research.

Popular science summary

Soil erosion has been a long-term issue to arable land in mountainous regions. Tuan Giao is located in Northwest Vietnam with high altitude mountains and steep slopes. Coffee is one of the most common cash crops in this region. Due to the steep slope, agriculture practices are highly labour consuming. To provide an easier weed control and reduce soil erosion, agroforestry has been introduced in Tuan Giao. Agroforestry is a sustainable agriculture system planted with trees or shrubs and can reduce soil degradation and increase biodiversity. Legumes have been included in agroforestry systems for their ability to utilise N in the air through a symbiosis with specific soil bacteria, the so-called rhizobia. Introducing agroforestry with legumes as understory crops serves multiple additional purposes: increasing plant coverage to reduce soil erosion, (potentially) decrease labour input for weed control and obtain extra income from the legumes.

The amount of N input from different legumes is uncertain. This study focused on estimating the amount of N fixed from the atmosphere by three different legume species as understory crops in coffee fields and plum trees located in Tuan Giao, NW Vietnam. The amount of N derived from air were evaluated for three legume species at two times, in three trials. After field sampling and lab analysis, all three legumes were found to fix N from the atmosphere, but groundnut had the lowest proportion of its N from air (%Ndfa) and also lowest total amount of fixed N (kg N/ha). Stylo bean fixed more than five times as much N as groundnut, and the amount of fixed N in pinto peanut was intermediate. Several uncertainties, including different sampling time and lack of true background values needed for the calculations, may have led to reduced accuracy. Compared to other research, this study showed a higher amounts of N concentration (%). This study provided an insight of the impact of legumes in the coffee agroforestry system.

Keywords: nitrogen, dinitrogen fixation, legumes, groundnut, stylo, pinto peanut, agroforestry, understorey crop.

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Farmers from Tuan Giao were the most hospitality people I’ve ever met. They gave unforgettable experiences.

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