The diversity of microbial communities associated with rubber tree plantations in North-East Thailand

By Laetitia Herrmann

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Deakin University, April 2017
The diversity of microbial communities associated with rubber tree plantations in North-East Thailand

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Abstract

Thailand is the world leading producer of rubber and a high number of farmers rely on the culture of rubber trees. The area under rubber tree plantation is currently strongly expanding in Thailand and particularly in the north-eastern part of the country, where the soils are poor and unsuitable for alternate crop plants. However, the impact of these plantations on soil fertility is unknown. Restoring and maintaining soil fertility is a critical issue in this region and alternative practices are currently being promoted to achieve this goal in a cost-effective way. Biochar is the product of the pyrolysis of biomass under low oxygen conditions. Its application as a soil amendment has received increasing attention and it is now promoted in Thailand as an alternative tool for carbon sequestration and crop growth promotion. The role of the soil microbial communities in sustaining soil fertility is widely acknowledged although poorly understood in the case of rubber trees in Thailand.

In this study, the taxonomic and functional diversity of the microbial communities was assessed along a chronosequence of rubber trees including three age groups: 3, 6 and 16 year-old plantations, as well as in cassava plots (no rubber tree history plots). Total bacterial and fungal communities, as well as arbuscular mycorrhizal fungi (AMF) communities were studied by 454 sequencing. Functional diversity of bacterial communities was assessed by qPCR and Biolog plates. In addition, the impact of increasing doses of biochar application on the microbial diversity was evaluated in a field trial including 2 soil types and 4 doses of biochar, 18 and 28 months after application.

Bacterial taxonomic diversity was more strongly affected by the age of the trees than the total fungal diversity. However, the diversity and the structure of the AMF communities differed along the chronosequence. The functional bacterial diversity was less impacted although a
gradual shift seemed to occur over time for some functions. Microbial communities were significantly affected by the application of biochar with a stronger effect observed on fungal than on bacterial communities. Bacterial and fungal communities’ composition was significantly impacted by both soil type and biochar addition (biochar vs no-biochar), but no effect of the biochar dose was observed.

Our results highlight the importance of taking the microbial communities into account in the promotion of alternative management practices for a more sustainable management of rubber tree plantations in Thailand. A better characterization of the relationships between the microbial communities, the environmental characteristics and the tree growth and latex yield is of major importance to allow the sustainable development of rubber production in northeast Thailand.
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1. CHAPTER 1: General introduction

This chapter was written by Laetitia Herrmann, with guidance from Dr Lambert Bräu and Dr Didier Lesueur.

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See the “Authorship statement – chapter 1” (appendix 1) for details.
1.1. The rubber tree in Thailand

The rubber tree (*Hevea brasiliensis* Müll. Arg.) is a fast-growing upright tropical tree mainly cultivated for its production of latex, a milky plant liquid which serves as a basis for various rubber products including tires, pipes and hoses. Rubber tree wood was initially considered a secondary product, however, due to recent improvements in wood technology, rubber tree wood is becoming an increasingly important source of timber (Evans and Turnball 2004). Moreover, it benefits from an environmentally friendly reputation as it can substitute timber from natural forests when grown in renewable plantations.

The rubber tree belongs to the Euphorbiaceae family and is native to Amazonia. However, it is an important crop in South East (SE) Asia and in Thailand in particular. Thailand is the world’s leading producer of latex (approximately 3.2 million t per year, representing more than 25% of the world’s total production) and exports 90% of its production overseas (http://faostat.fao.org). In 2009, rubber plantations in Thailand covered about 2.7 million hectares. These plantations are predominantly found in southern and eastern regions, however, there is scope for increasing the area under cultivation and improvement to the capacity of production (Saengruksawong et al. 2012). The establishment of rubber tree plantations in new areas, especially in North East (NE) Thailand, has represented a major potential for increased production during the past two decades. Furthermore, the latter has been strongly supported by the Royal Thai Government initiative to assist farmers with technology and production inputs such as seedlings, land preparation and fertilizers (Joshi, 2005).

In contrast with other countries in SE Asia, most of the Thai rubber is produced in small, family-owned plantations of less than eight hectares in size, with an average area of only two
hectares. Since a large part of the Thai population relies on rubber plantations as a source of income, improving the productivity of rubber agroforestry would have a significant impact on the incomes of subsistence farmers in Thailand, particularly in the north-eastern region where soil fertility is low and soils are unsuitable for growing many of the other cash crops.

1.2. Soil fertility management in rubber tree plantations in NE Thailand

The rubber tree is known to grow best in regions where rainfall is heavy and dry seasons do not exist. The most suitable soil types for rubber tree growth in Thailand have been identified in a study conducted by the Land Development Department (LDD) of Thailand in 2005 (unpublished data). Optimal soil properties such as soil depth, pH, water retention capacity, and moisture were shown to be critical factors to determine the suitability of a growing site. Well drained, clayey and deep clay soils with a pH of 5-6 are the best conditions for growth, even though trees can withstand physical conditions from stiff clay with poor drainage to well drained sandy loam. While they grow best in a modest level of soil nutrients they have been shown to be able to grow in a wide range of soils, even in very poor soils (sandy soils, low fertility, subject to erosion and leaching of applied fertilizers). According to Saengruksawong et al. (2012), only 102,000 hectares in Thailand have suitable soil characteristics, representing less than 4% of the planted surfaces. In NE Thailand, not only are the soils particularly poor, but the annual rainfall is less than optimal and the dry season lasts for about six months, leading to reduced plant growth (Chantuma et al. 2005).

Soil fertility, temperature and the distribution of rainfall (and a prolonged dry period, in particular) significantly affect both rubber tree growth and latex yield (Akpan et al. 2007; Rao et al. 1998). In addition, the monoculture of tree crop species often results in a considerable
depletion of soil fertility (Akpan et al. 2007). Thus, plantation management is a key issue to ensure the sustainability of the plantation while maintaining or restoring the soil quality. The effects of rubber tree plantations on soil physical and chemical characteristics have been increasingly studied during the last decade and several studies have shown that soil fertility (soil organic matter, total N, available P and available K) decreases with time (Akpan et al. 2007; Cheng et al. 2007; Murbach et al. 2003; Orimoloye et al. 2010; Zhang et al. 2007).

To counteract the low level of soil fertility and to replace nutrients’ loss, mineral fertilizers are widely applied in NE Thailand. Although rubber trees usually require modest levels of soil nutrients – in comparison to coffee, tea, coconut or oil palm – high rates of mineral fertilizers are applied, especially during the first five years of growth, since the nutrient requirements of young trees are the highest. It is acknowledged that the extensive use of fertilizers may lead to even more severe soil fertility depletion (Akpan et al. 2007; Orimoloye et al. 2010), therefore, identification and promotion of alternative management practices is of primary importance to reduce the use of mineral fertilizers and to maintain and restore soil fertility in rubber tree plantations.

While the importance of the soil biotic compartment in soil fertility has been recognized generally, it has been poorly investigated in the case of rubber trees in SE Asia. A study on macro-fauna conducted in Ivory Coast rubber tree plantations indicated that soil fauna diversity and density vary with rubber production and time (Gilot et al. 1995). Biological activities were maintained at a high level for up to 20 years but, in older plantations (30 years’ old), exhaustion of the resources (biomass, associated legumes) resulted in a dramatic drop in soil biological activities. Chauduri et al. (2008) studied rubber plantations in several Indian districts and showed that even with a good leaf litter input, some anecic and epigeic earthworm species were absent or rarely present. To date, no studies on the impact of rubber
trees on soil microbial communities have been undertaken, and the role of microbial communities in enhancing soil fertility and rubber tree growth are yet to be understood.

1.3. Plant-microbe interactions

Soils are complex matrices harboring the greatest biological diversity on Earth. Soil microorganisms are abundant, diverse, and show multiple metabolic activities (Kirk et al. 2004; Torsvik et al. 1990; Torsvik and Øvreås 2002). They are a key component of both natural and managed terrestrial ecosystems through their crucial roles in different biogeochemical cycles and fundamental processes, including organic matter decomposition, water movement and nutrient turnover. Microorganisms are thus recognized as having an undoubted role in maintaining soil quality ((Bertini et al. 2014; Paz-Ferreiro and Fu 2016). It is known for many agricultural and horticultural systems that a healthy microbial population in soils leads to increased yields and healthier plants and that there is an intricate relationship between plants, soil microbes and their environment. The plant rhizosphere (area of soils that surrounds the roots of the plants) is a unique environment and its associated soil microbial community is of central importance for plant nutrition, health and quality (Berg and Smalla 2009). Plant roots encounter an enormous variety of organisms in the rhizosphere (Roesch et al. 2007); they involve highly complex communities that function in very heterogeneous environments (Giri et al. 2005) and their interactions are ubiquitous across various trophic levels and are essential components of ecosystem functions.

Groups of microorganisms of particular interest for plant growth promotion, such as the symbiotic microorganisms or microorganisms generally designated as Plant-Growth-Promoting Rhizobacteria (PGPR) have received extensive attention. These microorganisms
are a significant component in cycling/recycling of mineral nutrients and carbon in soils (Leake et al. 2006; Morgan et al. 2005). Through their activities, nutrients can become more (through solubilization, mineralization) or less (through adsorption, immobilization) available to plants. Microorganisms establishing a symbiotic relationship with plants usually trade plant carbon for mineral nutrients gathered in the soil (e.g. phosphorus (P) and zinc (Zn), which normally have low diffusion rates in the soil) or nitrogen (N) fixed from the atmospheric N pool (Gao et al. 2012; Lendenmann et al. 2011; Smith and Smith 2012). Their mutualistic interactions involve intimate and sometimes obligate interactions with a more or less restricted range of host plants. Among the microbial symbionts, the rhizobia have undoubtedly been the most studied microorganisms but interest in Arbuscular mycorrhizal fungi (AMF) has been growing over the last two decades.

In a similar way, it is now acknowledged that there is a huge potential among the free-living PGPR strains in soils to promote plant growth, health or productivity. PGPR are widely represented within the rhizo-microbiome and as a result must be highly competitive in order to successfully colonize the root zone. Although contradictory reports are available, the motility of bacteria is considered an important factor for root colonization and can potentially enhance rhizosphere competence in terms of movements, both from bulk soil to root and along the roots (Dutta and Podile 2010), leading to a heterogeneous colonization of the host plant roots by the PGPR (Compant et al. 2010). After colonization of the root surface, PGPR can stimulate the growth of the plant through several mechanisms impacting plant nutrition or by increasing the plant’s resistance to pathogens (Pérez-Montaño et al. 2014). Plant growth stimulation can occur either: (i) indirectly through the production of phytohormones or plant growth regulators (PGRs), leading to a root surface increase; or (ii) directly by an influencing nutrient, such as P availability in the soil and ultimately the uptake by plants (Richardson et
PGPR strains can modify the root system architecture and the structure of root tissues, mainly through their ability to interfere with the plant hormonal balance (Vacheron et al. 2013). According to Dodd et al. (2010), PGPR strains can directly influence the uptake and efflux of phytohormones that they have synthesized (or their precursors) and alter root-to-shoot long-distance signaling to mediate shoot hormonal status.

The increased understanding of the role of root- or rhizosphere-associated microorganisms in nutrition and/or growth, biomass accumulation and fitness of plants in general, and yield of agricultural crops in particular, has resulted in their increased promotion for use in agricultural production as alternatives or supplements to mineral or organic fertilizers (Adesemoye and Kloeper 2009; Azcón-Aguilar and Barea 1997).

The influence of the plant in the plant-microorganism relationship should not be neglected and different plant species are often associated with different microbial communities. A number of studies reported specific indigenous microbial (both bacterial and fungal) communities in the rhizosphere of different plant species in a range of edaphic conditions (Berg and Smalla 2009; Ladygina and Hedlund 2010; Landesman et al. 2014; Prescott and Grayston 2013; Schweitzer et al. 2008).

Different mechanisms have been described to explain the influence of the plant on its associated microbial community which may be influenced by the quantity and nature of root exudates which vary from plant to plant and under different environmental conditions. Root exudates are comprised of carbohydrates, amino acids, aliphatc and aromatic acids, fatty acids, enzymes and hormones and provide soil microbes with the main source of carbon and nitrogen (Prescott and Grayston 2013; Prescott and Vesterdal 2013). Thus, they are likely to be a driving force in the structure and function of the microbial community (Prescott and Grayston 2013; Xue and Huang 2014). Soil type and soil pH in particular have also been
reported as the dominant factors influencing the microbial community in a number of studies (Bastida et al. 2008; Fierer and Jackson 2006; Jeanbille et al. 2016; Landesman et al. 2014).

Moreover, a multitude of other biotic and abiotic factors — including the age of the plant or its growing stage, production of litter, leaching of dissolved organic materials and nutrients or the alteration of the plant microclimate and of soil physical structure by roots and water flow — have been suggested to influence the structure, the diversity and the functional diversity of the soil microbial community (Marschner et al. 2002; Prescott and Grayston 2013; Xue and Huang 2014).

The impact of the rubber tree plantation on the soil biotic compartment has received limited attention to date (Ahrends et al. 2015; Sodhi et al. 2010). Some studies showed that the conversion of rainforest to rubber tree plantation in SE Asia resulted in a dramatic loss of biodiversity, but these studies mainly focused on mammals, plants, birds and invertebrates and didn’t take soil microorganisms into account (Sodhi et al. 2010; Warren-Thomas et al. 2015). Available information on the microbial communities associated with rubber trees is scarce and often contradictory. For example, Nurulita et al. (2015) observed a significant increase in bacterial biomass and Shannon diversity following the conversion of the natural forest to rubber plantation, whereas a decrease of total bacterial phospholipid-derived fatty acids (PLFA) was reported in a different study (Krashevska et al. 2015). The abundance of specific groups of bacteria such as Gram-negative or Gram-positive bacteria was particularly influenced by the rubber tree plantations whereas the fungal communities were not affected (total fungal PLFA, saprophytic fungi and AMF) (Krashevska et al. 2015). However, negative impacts on mushroom diversity and fungal richness were reported in other studies (reviewed by Monkai et al. 2017).
The functional diversity of the microbial communities was also affected by the rubber trees. Soil bacterial communities associated with rubber trees were generally able to use a higher variety of C substrates when compared to forests and oil palm tree plantations (Nurulita et al. 2015). However, there was a shift in the substrate utilization profile with time, suggesting that this functional diversity may be negatively affected by the age of the plantations.

There is a real need of more information and for long-term studies on the microbial communities existing in the rhizosphere of rubber trees in SE Asia and in Thailand in particular. A better understanding of the microbial community/rubber tree interactions and of their relation with soil fertility over time is critical to improve the management of rubber trees plantations. A better understanding of the diversity, the structure, and the activities of these communities could assist in the development of cost effective and environment-friendly alternatives to increase tree growth, reduce the use of inorganic fertilizers and improve and sustain soil fertility in rubber tree plantations in northeast Thailand.

1.4. Arbuscular mycorrhizal fungi (AMF)

Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil microorganisms of particular interest because they play a key role in plant nutrition, are found in most ecosystems (van der Heijden et al. 2015) and are of primary importance as they interact with the physical, chemical, and biological properties of soils. AMF belong to the phylum Glomeromycota and are obligate symbionts in nature: they are completely dependent on plant carbon and are unable to complete their life cycle without association with their host plant (Nakano et al. 1999). They are known to associate with a wide majority of the plants (including most commercial crops and agroforestry trees) (van der Heijden et al. 2015), forming a novel composite organ which
is the site of nutrient and carbon transfers between the plant and the AMF. AMF receive carbon from their plant hosts and offer a range of benefits in return. They notably increase the uptake of macro- and micro-nutrients. The improvement of P nutrition of plants (inorganic P in particular) has been the most recognized beneficial effect of AMF, but improved nutrition of other macronutrients such as N were also reported. They play this important role in plant nutrition through a wider exploration of the soils (Cardoso and Kuyper 2006; Schwob et al. 1998). Mycorrhizal hyphae make a substantial contribution to increasing the volume of the rhizosphere, sometimes called the mycorrhizosphere (Jansa et al. 2005), to access resources that would otherwise be unavailable to roots. This is of particular relevance for poorly mobile nutrients such as P (Hinsinger et al. 2011). While root-hair length is around 1 mm, AMF hyphae can extend several centimeters from the root surface (Jakobsen et al. 1992) and result in a network amounting up to 100 cm of hyphae per millimeter of root length (Allen, 2007).

In addition, fungal hyphae are more than two orders of magnitude smaller in diameter than are fine roots. Their small diameter allows mycorrhizal fungi to penetrate smaller soil pores than can roots or root hairs, increasing the volume of soil explored.

AMF symbiosis can also improve plant water use efficiency and alleviation of drought stress (Augé, 2001; Yang et al. 2014). Mycorrhizal plants contain more water and leaf chlorophyll than non-mycorrhizal plants (Colla et al. 2008; Srivastava et al. 2002), resulting in improved plant growth. This is usually attributed to effective water extraction by the extraradical AMF mycelium giving access to tightly held soil water (Yang et al. 2014) while, under drought conditions, AMF may contribute to increasing the capacity of soil to store water (Augé, 2004). In addition, AMF contribute to soil formation, soil aggregation and its subsequent stability thanks to the development of external hyphae in soil that create a skeletal structure that holds the soil particles together (Cardoso and Kuyper 2006; Rillig et al. 2015).
Mycorrhizal roots promote the creation of micro-aggregates and the aggregation of soil particles, resulting in improved soil porosity, water infiltration and storage (Augé, 2004; Cardoso and Kuyper 2006; Yang et al. 2014).

AMF have the potential to strongly contribute to protection against pathogens and reduce the damage caused by soil-borne fungi, nematodes, or bacteria (Cardoso and Kuyper 2006; Lewandowski et al. 2013; Sikes et al. 2009). A variety of mechanisms, with direct and indirect effects on the plant, have been proposed to explain the protective role of AMF against pathogens. Direct effects include the activation of plant defense systems, changes in exudation patterns and competition for photosynthates and space for colonization and infection sites. Indirect effects mainly relate to nutritional mechanisms, because plants with good nutrient status will be stronger and less sensitive to pathogen damage, but AMF were also shown to compensate for the loss of root biomass or function caused by pathogens (Azcón-Aguilar and Barea 1996; Cardoso and Kuyper 2006). Some metal tolerant AMF may have a role in facilitating heavy metal phytoremediation of contaminated sites through the production of siderophores and glomalin (Jeffries et al. 2003; Rajkumar et al. 2012).

AMF inoculation has been used for several decades in a wide range of edaphic conditions and on a variety of crops, with variable results. Successful results on field application of AMF on different crop growth, nutrient status or yields were reported in many studies (summarized in Adhohleya et al. 2005; Cardoso and Kuyper 2006; Dodd et al. 2002; Jansa et al. 2011; Siqueira et al. 1998; Strack et al. 2003). However, very low mycorrhization levels were recorded after inoculation with a number of nonspecific commercial products, resulting in no or limited effect on the targeted crops (Berruti et al. 2013; Tarbell and Koske 2007). Several factors were reported to influence the success of inoculation under field conditions: the crop species involved, the nature of the inoculants (species and their specificity, density, quality),
the abundance and effectiveness of indigenous AMF communities, the soil characteristics (nutrient status, physical properties) and cultural practices. These factors determine the AMF establishment (leading to alternative stable communities) and their persistence in soils over several growing seasons (Adholeya et al. 2005; Verbruggen et al. 2013).

The relationships between AMF and rubber trees have been poorly investigated. To date, studies mainly focused on the inoculation of seedlings in vitro or at the nursery stage and resulted in inconsistent results. AMF were shown to improve growth and P uptake by rubber tree plantlets to a certain degree, although the effects were site-specific (Ikram et al. 1992; Ikram et al. 1994; Schwob et al. 1998), and to induce a defence-like response in rubber tree plantlets (Schwob et al. 2000). Their effectiveness under field conditions is yet to be determined.

In many tropical areas, including the North-East province of Thailand, rubber tree growth is mostly limited by the poor fertility level of soils. AMF could play a beneficial role in improving the nutrient status of the tree, resulting in improved latex yields. Thus, a better understanding of the indigenous AMF communities associated with rubber trees is a topic that deserves attention.

Although AMF have physiological benefits and ecological importance, the actual number of AMF species is still unknown. AMF are usually considered as having a low specificity towards their hosts (Cuenca and Meneses 1996), although it is not clear how many fungal partners can be involved in the symbiosis with a single plant host. This may vary with a range of factors including the nature of the host plant, climate and soil physical and chemical characteristics. However, a high AMF diversity is generally associated with more benefits for the host plant than a low diversity. Investigations that have estimated the biodiversity and
distribution of AMF in various ecosystems showed that communities of AMF occurring in different ecosystems have different species composition (Liu et al. 2009).

The conversion of natural ecosystems into agro-ecosystems and into long-term mono-cultures in particular led to a pronounced decrease in AMF diversity (Oehl et al. 2009; Verbruggen and Kiers 2010). In addition, extensive applications of mineral fertilizers and pesticides and land use intensification may significantly affect AMF richness and diversity both positively and negatively (Cardoso and Kuyper 2006; Hassan et al. 2013; van der Heijden et al. 2015; Van Geel et al. 2015). There is evidence that plant species can strongly affect AMF diversity and community composition (Johnson et al. 2003; Johnson et al. 2005; Öpik et al. 2009).

Conversely, the composition of AMF in soils is an important driver of plant productivity and diversity (van der Heijden et al. 2008) and may have a strong impact on the aboveground host species composition and succession (Koske and Gemma 1997; Liu et al. 2009). An understanding of community dynamics is essential for managing landscapes over the long term (Hart et al. 2014). However, the factors driving the AMF community composition and diversity in a range of ecosystems and management systems, and in tropical soils in particular, require more investigations. In particular, little is known about changes in AM communities over the long term in tree monocultures. Cuenca and Meneses (1996) showed that the AMF diversity was maintained after many years of continuous cocoa cultivation, but the number of spores and the intensity of root colonization were affected. Similar results were obtained by Liu et al. (2009) on plantations of Caragana korshinskii. Surprisingly, Hart et al. (2014) found that that AM fungal species richness in roots increased with tree age in a breadfruit chronosequence and older trees were associated with communities that were compositionally different from younger trees. These results suggest that AMF successions occur in long-lived perennial species but this is yet to be studied in the case of rubber trees.
1.5. Biochar

Alternative management strategies have been proposed to reduce the use of mineral fertilizers, and to restore and maintain soil fertility in the rubber tree plantations at a reasonable cost for the farmers. These strategies include the application of organic fertilizers and the promotion of cover crops (Akpan et al. 2007; Orimoloye et al. 2010). The use of biochar is also currently widely promoted in Thailand as a tool for the improvement of soil quality and crop yields. Biochar is the charred end-product of the pyrolysis of C-based biomass (often referred to as feedstock). Pyrolysis is the chemical decomposition of an organic substance by heating to temperatures between 300 and 1000°C in a low (preferably zero) oxygen environment. The definition adopted by the International Biochar Initiative (IBI) furthermore specifies the intent to deliberately apply to soils to sustainably sequester carbon and improve soil properties or obtain agricultural and environmental gain (based on Lehmann et al. 2006). Biochar is a fine-grained, porous and highly aromatic, and thus very stable substance, similar in its appearance to charcoal. While charcoal is produced for other reasons (heating, cooking etc), biochar is produced for application to soils.

Biochar application is considered primarily as a mean to sequester atmospheric CO₂ (and other greenhouse gases) into soils while concurrently producing secondary agronomic benefits and improving soil functions (Lehmann et al. 2011). Biochar’s potential to form an effective C sink is mainly due to the fact that it is made of a very highly stable form of C, which is highly recalcitrant in soils, with residence times estimated from hundreds to thousands of years. A meta-analysis done by Verheijen et al. (2009) showed that, globally, biochar application resulted in a small but significant positive effect on plant growth, soil
nutrient status and crop productivity, although the intensity of this effect was dependent on the biochar type, the nature of the soils and the crop type. The enhanced environmental performances were mainly attributed to a liming effect, an improved soil structure, as well as an improved water and nutrient retention. However, the underlying mechanisms involved are not yet well understood.

The inspiration for the use of biochar as soil amendment comes from the observations of patches of anthropogenic deep black soils, called terra preta, initially found in the Brazilian Amazon. Similar soils were since found in other parts of the world, including Ecuador, Peru, Benin, Liberia and South Africa. These black soils are characterized by high levels of soil fertility and are very popular among local farmers for growing cash crops (Glaser et al. 2001). They contain enhanced levels of soil organic matter (SOM), as well as other nutrients such as N, P, K and Ca, and a higher microbial biomass and diversity. This particularly high fertility is generally attributed to the high charcoal content of the soils (Glaser et al. 2001). Biochar found in the terra preta was likely produced by the ancient local population practices, but it can also be produced in the event of natural fires. Different techniques for the industrial production of biochar have been developed, but the choice of the biomass and of the pyrolysis conditions are essential parameters. As a result, there is a wide variety of biochar products, of various qualities. The pyrolysis process transforms feedstock (biomass) into three different components: gas, liquid and solid. The remaining solid component after pyrolysis is biochar. The proportions of the different end products depend upon both the nature of the organic material and the pyrolysis conditions.

Many different organic materials can be used as feedstocks. The most popular feedstocks include wood, grain husks, nut shells, manure and crop residues, although biowaste (sewage sludge, municipal waste, chicken litter) and compost have also been proposed as suitable
materials. The suitability of a particular feedstock depends on a range of chemical, physical, as well as economic and logistical factors. The operating conditions during pyrolysis can also greatly affect biochar physical structure, quality and its potential value in terms of C sequestration and environmental performances (Downie et al. 2009; Lei and Zhang 2013). Temperature and furnace residence time have been shown to be of particular importance. The term biochar was originally associated with the slow pyrolysis process (at low temperatures) but is has since been extended to products of higher temperatures pyrolysis at (i.e., fast pyrolysis) and novel techniques such as microwave conversion. For instance, Tanaka (1963) showed that the biochar C content is inversely related to biochar yield. Increasing pyrolysis temperature decreases the yield of biochar but increases the C content of the end product. These results indicate that with regard to the use of biochar as a soil amendment, slow pyrolysis at low temperatures would be preferable since it maximizes the biochar yield.

Although some features show little variability between different types of biochar — such as the high C content, strongly aromatic structure or the neutral or basic pH — other characteristics including chemical composition, surface chemistry, particle and pore size distribution are highly heterogeneous and are greatly affected by both the biomass type and the pyrolysis conditions. These properties have a significant impact on the interactions of biochar with its environment and, as a result, drive its suitability for a site-specific application; i.e., its effects on soil functions and agronomic benefits (Downie et al. 2009). To date, the relative contribution of each of these factors has been poorly assessed.

After addition to soils, biochar interacts with a wide range of factors. Soil physical properties such as texture, structure, pore size distribution and density can be affected, with many implications for the various soil functions (Downie et al. 2009). For instance, the overall soil bulk density may be reduced since the biochar has a lower density than most of the mineral
soils. This generally implies an improved soil organic matter (SOM) content and, therefore, a possibly reduced amount of applied fertilizers. The presence of biochar in soil may darken its color, depending on the applied rate and type of soils. Since dark soils absorb a higher quantity of solar energy, they may display higher temperatures, affecting a number of functions including the rates of various metabolic processes and/or nutrient cycling. However, this is mitigated by the presence and the nature of plant cover, as well as the soil water content. As a result, the warming effect is generally considered to be relatively small.

Many studies reported a positive effect of biochar application on soil water-holding capacity and cited this moisture retention as a key factor in the observed agronomic benefits (Biederman and Harpole 2013; Bruun et al. 2014; Ding et al. 2016; Obia et al. 2016). Water retention of soils is largely determined by the soil texture: i.e., the distribution and connectivity of the pores of different sizes. The direct effect of biochar application is therefore related to its porous structure. Water will fill the small pores of biochar and be retained for a long time, mitigating leaching potential and being available for plants as the soils dry (Novak et al. 2012). This improved water-holding capacity may be of higher importance in the sandy soils, since clay soils usually retain more water due to the presence of a higher proportion of micropores. Because the soil water regime is modified, a number of other processes may be positively or negatively affected, including the cycling of various nutrients, and as a result, the production of greenhouse gases such as CH\(_4\) or N\(_2\)O. This, in addition to the soil sequestration of CO\(_2\) in a highly stable and recalcitrant form, may significantly participate in the mitigation of global warming.

The stabilization and the sequestration of SOM into soils are likely to affect the turnover of other forms of indigenous carbon. SOM is generally associated with agronomic benefits (improved plant growth and crop yield), although the understanding of the influence of the
different factors (environmental conditions, crop types) on this relationship and the underlying mechanisms are still poorly understood. The accessibility of SOM to microorganisms and enzymes might be positively or negatively modified, depending on several mechanisms such as changes in pH or nutrient availability, or changes in pore structure and water regime. The occurrence of a priming effect has also been proposed (Hamer et al. 2004; Zimmerman et al. 2011). The priming effect has been defined as “the short-term acceleration of SOM decomposition due to fresh SOM inputs to soils” (Fontaine et al. 2004). The priming effect is related to increases in the soil microbial biomass and changes in microbial community composition and activity.

Several studies have recently reported an effect of biochar on microbial communities (Domene et al. 2014; Grossman et al. 2010; Noyce et al. 2015; Prayogo et al. 2014; Su et al. 2017), although the mechanisms of their interactions with plants and nutrient cycling are highly complex and yet poorly understood. The highly porous structure of biochar provides a secure refuge for small organisms (including beneficial microorganisms such as AMF) (Ding et al. 2016; Gul et al. 2015; Sohi et al. 2010) since larger organisms cannot access those small pores to prey on them. It may represent a physical niche for proliferation or sporulation. In addition to this secure environment, biochar (especially if produced at low temperatures) provides a wide range of compounds on its surface layer that may be more or less easily metabolized by microorganisms (such as sugars and aldehydes which are turned over quickly) or may have bactericidal and fungicidal properties (Prendergast-Miller et al. 2014; Sohi et al. 2010). The nature and the quantity of these compounds result from the pyrolysis process and thus depend upon the biomass type and the pyrolysis conditions. However, the residence time of these substrates is likely to be short (a couple of growing seasons). Since biochar itself is a recalcitrant form of C and cannot be used as a C source for respiration, long-term effects on
the soil biota are unlikely related to this mechanism (Biederman and Harpole 2013). Biochar materials, regardless of the type, have a neutral or basic pH. Its application to soil is therefore considered as having a liming effect, in particular in the acidic soil types. It is acknowledged that higher pH values in soils lead to increases cation exchange capacity (CEC). CEC is a measure to assess how the cations nutrients are bound to the soils, thus being available for plant uptake and prevented from leaching (Mia et al. 2017). Biochar’s CEC is consistently higher than that of bulk soils, clays or SOM and it has been shown to increase with residence time in soils. As a result, liming effect is considered as one of the key mechanisms for the observed long lasting effects on plant growth and crop yield. This effect of pH, combined with the biochar effect on water regime is also likely to affect the soil’s microorganisms as it is well acknowledged that the latter quickly react to changes in their environment (Fierer and Jackson 2006; Yuan et al. 2015). However, there is surprisingly scarce information on the interactions of biochar with bacterial and saprophytic fungal communities. Considering the diverse saprophytic abilities of fungi, it is probable that they affect both the stability and longevity of biochar in soils. Similarly, due to its effects on the physical and chemical properties of soils, biochar is likely to induce changes in the balance of microbial activity between different functional groups engaged in food webs and nutrient cycling. There has been a particular scientific interest on the relationship between AMF and biochar, as reviewed by Warnock et al. (2007). For instance, it has been demonstrated that the interaction between biochar and inoculated AMF led to positive effects on yield, while the sole application of biochar did not produce significant results (Hammer et al. 2015). A combined effect on the nutrients, water and CEC of soils was considered as the most probable mechanism to explain these positive interactions.
There are, to our knowledge, no published studies on the effect of biochar application on rubber tree plantations. Because of the high variability of effects that depend on several factors — including the biochar used, the soil type and the targeted crop — a case-by-case evaluation of each biochar type prior to its incorporation in a particular situation is mandatory. Interest in biochar research is still in its relative infancy and, as such, more data are required to draw robust conclusions regarding its effects across a range of soil, environmental and agronomic situations. Additionally, the irreversibility of the biochar application and its long residence time into soils is an essential element to consider before policies are written and large scale deployment is promoted.

1.6. Techniques to assess microorganism diversity in soil

Despite the key role of the microorganisms in providing soil ecosystem services, our general understanding of their composition, diversity and functions in soils is limited, partly because of the limits imposed by the analysis techniques.

Culture techniques have been largely used to describe the microorganisms living in the soils or in symbiosis with the plants. Culture media have been developed to grow particular groups of microorganisms depending on their morphological, physiological and biochemical characteristics allowing for the isolation of individual strains for further study. This is of particularly interest in the case of PGPR and symbiotic bacteria, to obtain a better understanding of the mechanisms involved in the plant growth promotion processes. However, these techniques give an overview of a very limited portion of the microorganisms present in the soils, since it is generally considered that about 99% of the microorganisms cannot be cultivated using current available techniques.
The development of molecular biology techniques has offered great opportunities to access the uncultured fraction of the soil microorganisms. They are based on the study of the nucleic acids (DNA or RNA) of all microorganisms present in a sample. A particular fragment of the DNA (or cDNA if RNA was initially targeted) is generally amplified by PCR (Polymerase Chain Reaction) to obtain a sufficient quantity of DNA for further analyses. PCR-based techniques have provided greater insights in the structure of particular communities and include a wide range of techniques, such as the fingerprinting or the high throughput sequencing technologies.

The fingerprinting methods, such as the PCR-DGGE (Denaturing Gradient Gel Electrophoresis), allow the study of the structure of a community and the comparison of several samples on a single electrophoresis gel. They are generally considered as cheap and fast methods to observe the changes in a particular community in different environments or conditions (management practices, soil types) or over time. The PCR-DGGE is based on the separation the different PCR amplification products of same length but with different base-pair sequences by electrophoresis under increasingly denaturing conditions (Muyzer et al. 1993). The denaturing gradient affects the migration of the PCR fragments based on their sequence, and on their G-C content in particular. The denaturants cause the melting of the DNA fragments in discrete domains, negatively affecting the speed of migration of the fragments in the gel. Therefore, two fragments of different sequences will stop their migration at different positions and hence can be separated in the DGGE gel and theoretically, fragments differing only by one base pair can be discriminated by DGGE. The profiles obtained for different samples can then be analyzed to assess their structure similarity or their diversity. The isolated bands can be excised and purified for sequencing analysis and identification. However, only short fragments (>500 bp) can be separated by DGGE and this
may limit the quality of the identification of the excised bands in some cases. In addition, when targeting large communities (i.e., 16S rDNA or 18S and 28S rDNA for total bacterial and fungal communities, respectively), the number of bands can be very high and the analysis of the gel (separation of the different bands and comparison of the different profiles) may be more difficult.

More recently, the development of high-throughput sequencing (or next-generation sequencing) technologies has revolutionized the field of genomics, allowing the creation and the analysis of increasingly large datasets, leading to a deeper understanding of the different microbial communities present in a range of environments. Several technologies have now out-performed the older Sanger-sequencing technology in terms of throughput, cost and quantity of labor. Technologies are now fully automated and allow the analysis of a high number of samples in one run. The Roche/454 GS FLX Titanium sequencing technique (so-called 454 sequencing) is based on the pyrosequencing approach which is based on the iterative completion of single strands while simultaneously reading out the signal emitted from the nucleotide being incorporated (Kircher and Kelso 2010) and requires the preparation of a particular library for each individual sample. Template DNA strands are amplified inside water droplets in an oil solution (emulsion PCR) and adapters are included for the identification of the sample. Eventually, each droplet contains a single strand DNA template attached to a particular primer-adapter fragment. The sequencing plate contains about two million wells, each of them able to contain a template sequence amplified during the emulsion PCR. During the pyrosequencing process, one of the four nucleotides (A, T, C or G) is sequentially washed over the strands to be analyzed. If one (or more) nucleotide is complementary to the template, it is incorporated by the polymerase. One pyrophosphate per inserted nucleotide is then released and converted to ATP which is used by a luciferase to
produce a light signal that is linear to the number of nucleotides incorporated. This signal is captured and computed to generate the sequence read-out. One of the major drawbacks of the 454 sequencing technology is the error rate which is higher than that of Sanger sequencing. In particular, errors arise from homopolymer length inaccuracy (fragment constituted of a single nucleotide type), or insertions of base-pairs caused by the noise threshold. The number of errors increases with the position in the fragment, so the technology is considered reliable for intermediate read length only (300-500 bp) (Kircher and Kelso 2010).

The Illumina sequencing technology (initially called Solexa), released in 2007, employs a reversible terminator technology that is based on a sequencing-by-synthesis concept similar to that used in Sanger sequencing. However, as in 454 sequencing, the preparation of a sequencing library is required before the sequencing step can be initiated. DNA strands, primers and adapters are first bound on a slide and amplified by bridge amplification: the single DNA strand bends over and attaches to the oligonucleotides complementary to the adapter on the plate, forming a bridge. The reverse strand is then synthesized and the two complementary strands are released and straighten. The result is a cluster of DNA forward and reverse strand clones. The bridge amplification is repeated until about one thousand copies of each fragment of DNA (both forward and reverse strands) are present in very close proximity to each other, forming micro colonies or clusters. Before sequencing, one of the strands (generally the forward strand) is removed by cleavage of the oligonucleotides on the plate. As a result, each cluster consists of single-stranded, identically oriented copies of the same sequence, ready for sequencing. Primers are attached to the forward strands and a mix of the four fluorescently labeled nucleotides is added (each nucleotide having a unique emission). A reversible terminator is attached to every nucleotide so that the incorporation reaction is stopped after each base. The label of the incorporated base is read, the terminator is
chemically removed and the next base can be incorporated. The sequencing of the reverse DNA strands is generally made once the sequencing of the forward strands is completed. The synthesized sequence is chemically washed away and a new bridge amplification takes place. The resulting forward strands are then cleaved and the sequencing process repeats for the forward strands. This paired-end sequencing allows the quality control of the obtained sequences and the detection of errors, as the reverse and forward strands should be complementary to each other.

Although the sequencing data provide very useful information on the composition, structure and diversity of the microbial communities in soils, they do not provide information on the functions supported by these communities. The functional diversity of the microbial communities in soils is also a very important parameter to study, as it reflects the ecological processes that are driven by these communities and, as a result, the ecosystem productivity. Functional diversity of a community results not only from its genetic diversity but also from environmental effects on gene expression and ecological interactions among taxa (Chodak et al. 2015). It has been suggested that restoring the functional diversity of an ecosystem and of degraded lands in particular, may be more crucial than returning soil microbial diversity per se (Banning et al. 2012).

The real-time polymerase chain reaction (or quantitative PCR, qPCR) can be used as a proxy to assess potential functional diversity of the soil communities. The technique measures the number of copies of a particular DNA fragment, using specific primers. It is thus possible to quantify the number of copies of genes of interest, involved in specific soil functions such as N fixation or denitrification. The method is based on the general principle of the conventional end-point PCR, but the accumulation of PCR product (double-stranded DNA) is monitored in real time throughout the amplification by the use of a DNA-binding dye. A double-stranded
DNA dye, such as SYBR Green, is generally used because it binds specifically to double-stranded DNA fragments. Once bound to DNA, the SYBR Green dye produces a fluorescent signal that can be continuously measured. The analyses of the reaction kinetics and that of reference samples (of which the gene concentration is known) enable the calculation of the number of targeted genes initially present in the studied sample. This method presents the advantage of keeping the costs and sample handling down. However, the dye can bind to any kind of double-stranded DNA, including dimers of primers. This may result in a non-specific increase of the fluorescent signal.

Another common approach to assess the microbial functional diversity of a microbial community in soils is to measure its metabolic activity (i.e., the respiration response of a community to a range of C substrates) to produce a Community Level Physiological Profile (CLPP). This method measures potential rather than real activities (Preston-Mafham et al. 2002) but it can successfully detect changes in functional ability in response to soil disturbance or to various management practices (Campbell et al. 2008; Classen et al. 2003; Rutgers et al. 2016; Stefanowicz, 2006). One of the widely used CLPP methods involves the inoculation of environmental samples into Biolog Ecoplates containing 31 substrates that vary in structural complexity and are known to be plant root exudates or highly discriminatory among soil communities (Insam and Goberna 2004). The substrates are bound to a redox dye that produces a purple product after degradation of the substrate. Therefore, the utilization of a particular substrate can be monitored by spectrometric detection during incubation. However, it is important to note that fungal species are not able to decompose the substrate-dye complex. As a result, this technique only focuses on bacterial community activities. The main biases of the method are related to bacterial extraction from soil and inoculum density.
standardization. As it is not a culture-independent technique, biases toward fast-growing and easily cultivable members of the community may occur (Insam and Goberna 2004).

1.7. Thesis Aims and how the chapters below go about meeting the thesis aims

Thailand is the world’s leading producer of latex, and the area covered by rubber tree plantations is increasing throughout the country, particularly in NE Thailand. This is despite the fact that many of the newly planted areas possess unsuitable soils and/or climate for the growth of rubber trees. Under these circumstances, both tree growth and latex production are significantly reduced and the level of soil fertility is also negatively affected. To counteract this and optimize tree growth and latex production (thus ensuring higher incomes), farmers currently apply high quantities of mineral fertilizers, which results in a stronger depletion of soil fertility. To sustain rubber tree plantation in NE Thailand, the restoration and maintenance of soil fertility is of great importance.

The role of the soil biotic compartment has been widely recognized but yet poorly studied and understood in the case of rubber tree plantations in low fertility soils such as present in NE Thailand. The relationship between soil microorganisms (including both fungi and bacteria) and plants is intricate and results in a wide range of direct or indirect effects. For instance, symbiotic microorganisms, such as AMF, provide nutrients to the plants in exchange of carbon substrates. Other PGPR are able to provide hormones to the plants as well as cause or reduce pathogen attack. A better understanding of the diversity and functions of soil microorganisms associated with rubber trees is needed and could assist in promoting alternative and more sustainable management practices, thus reducing the extensive use of mineral fertilizers.
Biochar has been recently promoted in NE Thailand by the national authorities as an alternative tool to promote tree growth and to reduce the amount of applied fertilizers. Biochar is the result of the pyrolysis of biomass under low oxygen concentration and has received increasing attention for the past decade as a soil amendment for C sequestration and plant growth promotion. The results of scientific studies show contradictory results with both positive and neutral effects and the implied mechanisms are yet to be determined. It has been suggested that biochar may directly or indirectly affect soil microorganisms but very few studies are available and there has been to date no such study on rubber tree.

Therefore, this project will first assess the taxonomic and functional diversity of total bacterial and fungal communities present in rubber tree plantations of different ages (chapter 2). Colonization and diversity of Arbuscular mycorrhizal fungi (AMF) associated with rubber trees along the same chronosequence will be evaluated in chapters 3 and 4. The effect of biochar application on the soil microbial communities will be assessed in an eight year-old rubber tree plantation, 18 and 28 months after application (chapter 5 and 6).
2. CHAPTER 2: Effect of rubber tree plantations on soil microbial taxonomic and functional diversity

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2.1. Introduction

Soils are complex matrices harboring great biological diversity. Soil microorganisms are abundant, diverse, and play key roles in fundamental biogeochemical processes including nutrient cycling, decomposition, mineralization and humification of organic matter, water movement and soil aeration and aggregation (Kirk et al. 2004; Torsvik et al. 1990; Zak et al. 2003). They perform a linking role between plants and ecosystem functions and thus have an undisputed role in maintaining soil health and quality (van der Heijden et al. 2008; Vitali et al. 2016). Despite their importance, the general understanding of the environmental factors shaping soil microbial community abundance, diversity and functions, as well as their responses to ecosystem perturbations are still unclear (Constancias et al. 2015).

Microorganism structure, activity and diversity are known to react quickly to changes in their soil environment including changes in pH (Fierer and Jackson 2006; Jeanbille et al. 2016; Mendes et al. 2015), soil water and nutrient content (Bossio and Scow 1998; Drenovsky et al. 2004), soil type (Berg and Smalla 2009) and plant community composition (Berg and Smalla 2009; Carney and Matson 2006; Stefanowicz, 2006). However, different microbial groups are not influenced by the same parameters and may respond differently to alterations in their environment (Thomson et al. 2015). As a result, changes in microbial biomass, metabolic activity or community structure and diversity may be monitored and used as early signals of perturbations and for assessing the sustainability of an ecosystem (Stefanowicz, 2006).

South East (SE) Asia has experienced one of the highest rates of deforestation in the tropics due to agricultural expansion (Sodhi et al. 2010) which has become a significant agent of ecosystem disturbance. Large areas of tropical forest and fallow land have been converted to cash and food crops (Li and Fox 2012), resulting in major land use changes, and extensive environmental alterations (Ahrends et al. 2015; Nurulita et al. 2015). Intensive agricultural
practices represent a very intense form of ecosystem disturbance and have been reported to cause adverse effects on soil quality and fertility such as soil erosion, nutrient depletion and plant diversity reduction (Cao et al. 2010; Nurulita et al. 2015). In particular, the conversion of agricultural land to perennial tree plantations such as rubber tree or oil palm tree has been a controversial issue and generates criticism due to its potential impacts on soil fertility and biodiversity (Cao et al. 2010; Li et al. 2012).

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is a fast-growing upright tropical tree (Euphorbiaceae) mainly cultivated for its production of latex, a milky plant liquid which serves as a basis for various rubber products. Thailand is the world’s leading producer and exporter of rubber with a production capacity of about 3.2 million t per year (more than 25% of the world’s total production) (http://faostat.fao.org) and in 2009, rubber plantations in Thailand covered about 2.70 million ha (Saengruksawong et al. 2012). While it grows best in areas characterized by high soil fertility, adequate rainfall distribution (short dry season) and even temperature distribution (Rao et al. 1998), rubber tree has been shown to be able to grow in a wide range of edaphic conditions, including low annual rainfall, long dry seasons and very poor soils (sandy soils, low fertility, subject to erosion and leaching of applied fertilizers) such as those found in the North East province of Thailand. In this region, which was previously largely planted with cassava (*Manihot esculenta*), another Euphorbiaceae species, the establishment of rubber tree plantations represents a major potential for increased production. The impact of the conversion of annual crop fields (such as cassava) into rubber tree plantations and the impact of the plantations over time on the soil biotic compartment has been poorly investigated in SE Asia. Soil macro-fauna diversity and density has been shown to vary with rubber production and age of the trees (Chaudhuri et al. 2008; Gilot et al. 1995) and recent studies showed that the Arbuscular mycorrhizal fungi (AMF) communities were
affected by the establishment of the rubber tree plantations and by the age of the trees (Herrmann et al. 2016a; Herrmann et al. 2016b). However, to date, the role of bacterial communities in relation to tree growth and soil fertility in rubber tree plantations are yet to be understood. A better understanding of the microbial community-rubber tree interactions would help in developing the best practices for sustainable management of rubber tree plantations.

The objectives of this study were to describe the structural and functional diversity of the bacterial communities associated with rubber trees and to assess the impact of both field conversion and tree age on those communities along a chronosequence of rubber tree plantations.

2.2.Materials and methods

2.2.1. Site information and soil sampling

A chronosequence of rubber tree plantations was identified in Baan Don Chang area (Khon Kaen district, North East province of Thailand). Khon Kaen district has a tropical savanna climate with long dry winters and rainy seasons that can last up to 6 months. Temperature and precipitation data were monitored for several years in the Khon Kaen region. According to these records, temperature throughout the year varies between 18°C and 35°C, with average daily high and low temperature of 34°C and 22°C, respectively, and a mean annual temperature of 30°C. Rainfall occurs from April to October, with mean annual precipitation of 1250 mm. The area is predominantly covered by croplands (95%), with cassava, rice and sugar cane being the major crops.
The chronosequence included 9 rubber tree plantations of three ages: 3 plantations of 3 year-old trees, 3 plantations of 6 year-old trees and 3 plantations of 16 year-old trees. The different plantation ages were selected to represent the different stages of the rubber cultivation.

Rubber trees of 3 year-old are considered as young and immature trees. Six year-old trees usually start producing latex and the canopy closes. The 16 year-old trees are considered as old and mature trees, in the final stage of the plantation exploitation. Rubber trees are usually grown for 20 to 25 years before being cut because the production of latex decreases after 20 years. Three nearby cassava fields with no history of rubber tree cultivation were included for comparison. Cassava is one of the major crops in the region, and was the previous cultivated crop before the establishment of all the selected rubber plantations. Notably, both cassava and rubber tree belong to the family Euphorbiaceae.

The same clone (Rubber Research Institute of Malaysia [RRIM] 600) was used in all the rubber tree plantations and the sites were selected in a small area to reduce the soil variability. The distances between sites were less than 500 m, the total area was about 800 x 1400 m and the elevation varied from 184 to 201 m above sea level (Figure 1, Table 1). The rubber tree plantations had been cultivated with cassava and jute (Corchorus capsularis) for 20 to 40 years prior to rubber tree plantation. No cover crops were associated with the trees at the early stages of the plantations. All plantations had been tilled between the tree lines twice a year (at the beginning and at the end of the rainy season) during the first 5 years of rubber tree growth. Chemical (N:P:K, 20:10:12) and organic fertilizers (manure) had been applied during the first 5 years, depending on availability.
Figure 1. Map of the study sites in Baan Don Chang, Khon Kaen district, Thailand. 3A, 3B, 3C: 3 year-old plantations; 6A, 6B, 6C: 6 year-old plantations; 16A, 16B, 16C: 16 year-old plantations; CA, CB, CC: Cassava fields. Yellow circles indicate the centroid of the 9 sampled rubber trees.
Table 1. Coordinates, elevation and area of the study sites in Baan Don Chang.

<table>
<thead>
<tr>
<th>Site</th>
<th>Easting (d°)</th>
<th>Northing (d°)</th>
<th>Elevation (m)</th>
<th>Area (ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A</td>
<td>259 939</td>
<td>1 810 040</td>
<td>190</td>
<td>1.19</td>
</tr>
<tr>
<td>3B</td>
<td>260 319</td>
<td>1 810 461</td>
<td>191</td>
<td>0.46</td>
</tr>
<tr>
<td>3C</td>
<td>260 698</td>
<td>1 810 358</td>
<td>193</td>
<td>1.53</td>
</tr>
<tr>
<td>6A</td>
<td>260 270</td>
<td>1 810 366</td>
<td>191</td>
<td>1.79</td>
</tr>
<tr>
<td>6B</td>
<td>260 041</td>
<td>1 810 525</td>
<td>184</td>
<td>1.79</td>
</tr>
<tr>
<td>6C</td>
<td>260 248</td>
<td>1 809 908</td>
<td>198</td>
<td>1.06</td>
</tr>
<tr>
<td>16A</td>
<td>260 226</td>
<td>1 809 854</td>
<td>198</td>
<td>3.19</td>
</tr>
<tr>
<td>16B</td>
<td>259 953</td>
<td>1 809 259</td>
<td>201</td>
<td>1.82</td>
</tr>
<tr>
<td>16C</td>
<td>260 502</td>
<td>1 810 243</td>
<td>196</td>
<td>1.05</td>
</tr>
<tr>
<td>CassA</td>
<td>260 470</td>
<td>1 810 415</td>
<td>195</td>
<td>0.59</td>
</tr>
<tr>
<td>CassB</td>
<td>260 290</td>
<td>1 810 296</td>
<td>193</td>
<td>0.43</td>
</tr>
<tr>
<td>CassC</td>
<td>260 605</td>
<td>1 810 611</td>
<td>196</td>
<td>0.53</td>
</tr>
</tbody>
</table>

3A, 3B, 3C: 3 years-old rubber tree plantations; 6A, 6B, 6C: 6 years-old rubber tree plantations; 16A, 16B, 16C: 16 years-old rubber tree plantations; CassA, CassB, CassC: cassava fields.
In August 2013, soils (0-30 cm deep) were collected from 9 trees selected in the middle of each rubber tree plantation. Soil samples were collected 40 to 60 cm away from the trunk of each tree, on two opposite sides of the trunk. Soil samples collected from the rhizosphere of the same tree were pooled, sieved to pass a 1 mm mesh and kept at 4°C for microbiology analysis, -20°C for molecular biology analyses or air dried at room temperature for physical and chemical analysis. Nine soil samples were also collected in each of the cassava sites using the same protocol.

2.2.2. Physical and chemical soil analyses

Chemical analyses were performed on five of the nine samples for each site. Soil analyses included pH (in H₂O), organic matter (Walkley and Black 1934), P (Bray and Kurtz 1945, Bray II), N (Bremner, 1965), K (ammonium acetate pH 7), Ca and Mg (ammonium acetate pH 7) contents (Thomas, 1982), and texture (Kilmer and Alexander 1949).

2.2.3. DNA extraction

Total genomic DNA was extracted following the method described by Plassart et al. (2012). Briefly, 1 g of soil was mixed with a solution containing 1 M Tris (pH=8), 0.5 M EDTA (pH=8), 1 M NaCl and 20% SDS in tubes containing 2 g of silica beads (0.1 mm diameter), 2.5 g of ceramic beads (1.4 mm diameter) and 4 glass beads (4 mm diameter). Tubes were homogenized in a FastPrep beadbeater (MPBiomedicals, NY, USA) for 3 x 30 sec at 4 m/sec, incubated at 70°C for 2x15 min and centrifuged for 5 min at 7000 g. Protein precipitation was done with a 3 M K-acetate solution (10 min, 4°C) and DNA was precipitated with 1 volume of ice-cold isopropanol (30 min, -20°C). DNA pellet obtained after centrifugation (30 min, 4°C, 13000 rpm) was cleaned with cold 70% ethanol, resuspended in micropure water and
kept at 4°C. DNA was further purified using polyvinylpolypyrrolidone (PVPP) Microbiospin minicolumns (BIORAD, Marnes-la-Coquette, France) and GeneClean Turbo kit (MP-Biomedicals, NY, USA). Purified DNA concentrations were assessed using the PicoGreen staining kit (Molecular Probes, Paris, France), according to the manufacturer’s instructions.

2.2.4. Sequencing of bacterial and fungal communities

DNA extracts were diluted to a final concentration of 1 ng/µl and the 9 replicates of the same site were pooled to form one composite sample per site. Primer pairs F479 (5’-CAGCMGCYGCNGTAANAC-3’)/R888 (5’-CCGYCAATTCMTTTRAGT-3’) and FR1 (5’-ANCCATTCAATCGGTANT-3’)/FF390 (5’-CGATAACGAACGAGACCT-3’) were used to amplify a fragment of the bacterial 16S and fungal 18S rDNA, respectively (Terrat et al. 2015). PCR reactions were done in a 25 µl final volume containing 5 ng of template DNA, 12.5 µM of each primer, 5 mM of each dNTP, 1.5 U of Pfu Taq polymerase, 75 mM of MgCl₂ and 0.625 µg of T4 gene 32. Amplification conditions of 16S rDNA were: initial denaturation 2 min, 94°C, 35 cycles of denaturation (30 sec at 94°C), annealing (30 sec, 52°C), elongation (1 min 72°C), and final elongation of 7 min, 72°C. Amplification conditions of 18S rDNA were: initial denaturation 3 min, 94°C, 35 cycles of denaturation (30 sec at 94°C), annealing (1 min, 52°C), elongation (1 min 72°C), and final elongation of 5 min, 72°C. For both PCR, a second PCR amplification of 7 cycles was done using 2 ng of DNA to include the adaptors A and B and a unique multiplex identifier (MID) tag to each sample. After amplification, DNA was purified using the MinElute PCR purification kit (Qiagen, Manchester, UK) and quantified using the PicoGreen procedure (Molecular Probes, Paris, France). Amplicons were pooled at equimolar concentrations to reach a final 2 mg of DNA for each library.
Pyrosequencing of the amplicons was performed on the GS-FLX 454 Titanium platform (Roche, Basel, Switzerland) by the Genosol platform (INRA, Dijon, France).

2.2.5. Bioinformatics and phylogenetic analysis

Sequencing datasets were analyzed using the GnS-PIPE pipeline (version v1.1.11) described by Terrat et al. (2015). Briefly, reads were first sorted according to the MID sequences and quality checked based on their length, number of ambiguities and primer sequences. Reads were retained only if they carried the correct MID and primer sequences, had an average quality score $>20$ and were $\geq 300$ bp long. Rigorous dereplication (i.e. clustering of strictly identical sequences) was done using a PERL program and the dereplicated reads were then aligned using INFERNAL alignment and clustered into OTU using a 5% dissimilarity threshold. Taxonomic assignment was done using the Silva reference database. Singletons (reads detected only once and not clustered) were retained based on the quality of their taxonomic assignment. The retained high quality reads were used for further taxonomy and diversity analyses.

2.2.6. qPCR

Quantitative PCR (qPCR) was used to quantify the abundance of different functional genes. To overcome inhibitory effects, DNA extracts were diluted to a final concentration of 1.25 ng$\mu$l$^{-1}$ prior to analysis. Seven replicates per site were randomly selected to allow the analysis of the different treatments on a single plate, thus limiting the variability occurring between different plates. qPCR assays were run in duplicate using a Biorad CFX96 Real-time PCR System with a SYBRGreen detection and the results were analyzed with the Software Bio-
Rad CFX Manager 2.0. Mean values of the duplicates were retained for analysis. Details on the primers used as well as qPCR conditions are given in Table 2. Initial DNA quantity was 2.5 ng and targeted fragments were amplified in a total reaction volume of 10 µl containing 1X of Sso advancedTM SYBR Green supermix (BioRad, Hercules, CA, USA) and 0.5 µM of each primer. Two negative controls were included in every assay. Standard curves were obtained using tenfold serial dilutions of a linearized plasmid pGEM-T Easy Vector (10^2 to 10^8 copies) containing the targeted gene for each qPCR assays. The reliability of the standard curves were controlled by verifying reproducibility of the Ct values, the quality of the dilutions series and ensuring that the r² value was constantly higher than 0.98. To verify the specificity of the primers, melting curves were generated after amplification by increasing the temperature from 55°C to 95°C (+0.5°C per increment). Purity of the amplified products was checked by observation of a single melting peak and the presence of a single band of the expected length after horizontal electrophoresis on 1% agarose gel.
Table 2. PCR primers and amplification conditions used in qPCR assays.

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Primers</th>
<th>Sequence</th>
<th>Fragment length</th>
<th>Amplification conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoA (AOA)</td>
<td>Crenamo23F</td>
<td>AGGTCTGGGCTWAGACG</td>
<td>491 pb</td>
<td>98°C, 2 min, 1 cycle, 98°C 5s, 60°C 45s, 72°C 30 sec, 39 cycles</td>
<td>Nicol et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Crenamo616R</td>
<td>GCCATCCATCTGTATGCTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AmoA1F</td>
<td>GGGTTTACTCTGCTGCTGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amoB (AOB)</td>
<td>AmoA2R</td>
<td>CCCCTCKGSAAGCCTTCTTC</td>
<td>624 pb</td>
<td>98°C, 2 min, 1 cycle, 98°C 5s, 55°C 30s, 72°C 30 sec, 39 cycles</td>
<td>Rotthauwe et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>PolF</td>
<td>TGCGAYCCSAAR GCGBACTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nifH</td>
<td>PolR</td>
<td>ATSGCCATCATYTNRCCGGA</td>
<td>359 pb</td>
<td>98°C, 2 min, 1 cycle, 98°C 5s, 60°C 30s, 72°C 30 sec, 39 cycles</td>
<td>Poly et al. (2001)</td>
</tr>
</tbody>
</table>
2.2.7. Community Level Physiological Profile (CLPP) by Biolog Ecoplates

Biolog Ecoplates (Biolog Inc., USA) containing 31 carbon substrates (and a control without substrate) were used to assess the CLPP of the bacterial community from each sample. The substrates are classified into 6 substrate groups: 9 carboxylic acids (D-Glucosaminic Acid, D-Galactonic Acid γ-Lactone, G-Galacturonic Acid, 2-Hydroxy Benzoic Acid, 4-Hydroxy Benzoic Acid, γ-Hydroxybutyric Acid, Itaconic Acid, α-Ketobutyric Acid, D-Malic Acid), 7 carbohydrates (D-Cellulobiose, α-D-Lactose, β-Methyl-D-Glucoside, D-Xylose, i-Erythrol, D-Mannitol, Glucose-1-Phosphate), 6 amino acids (L-Arginine, L-Asparagine, L-Phenylalanine, L-Serine, L-Threonine, Glycyl-L-Glutamic Acid), 4 polymers (Tween 40, Tween 80, α-Cyclodextrin, Glycogen), 3 amines (N-Acetyl-D-Glucosamine, Phenylethylamine, Putrescine) and 2 esters (Pyruvic Acid Methyl Ester, D-L-α-Glycerol Phosphate).

A soil suspension was prepared using 10 g of fresh soils mixed with 90 ml of sterile saline solution (NaCl 9g/l). The soil suspension was shaken for 1h at 200 rpm, and let for decantation for 30 min at room temperature. Supernatant was diluted in sterile saline solution to a final concentration of 10⁸ cells/ml and an aliquot (150 µl) was inoculated in each of wells of the Biolog Ecoplate. Plates were incubated at 28°C (+/-1°C) for 7 days. The ability of the bacterial community to utilize the particular carbon sources were assessed colorimetrically at a wavelength of 590 nm. The OD₅₉₀ was read immediately after inoculation and every 24h for 7 days.
2.2.8. Statistical analyses

2.2.8.1. 454 sequencing data
Sampling efficacy was assessed with rarefaction analysis of the sequencing datasets, using the function rarefy() from the R package Vegan (Oksanen et al. 2012). Singletons were eliminated and sequencing depth per sample was standardized by retaining a random selection of reads in each sample, where the number retained corresponded to the minimum read count across all samples (5961 and 10137 reads for bacterial and fungal datasets, respectively).

Linear mixed-effects models (LME) (Pinheiro et al. 2013) were used to test for differences in richness and diversity indices (Shannon, Simpson and Evenness) in the different groups of plantations. The Shannon index ($H'$) was exponentially transformed resulting in a variable ($\text{exp}H'$). That, in contrast to the untransformed index, satisfies the replication principle and can be considered a linear representation of diversity (Jost, 2006). Site was included in models as a random effect.

Analysis of bacterial and fungal community structure was performed using quantitative data, where proportions of reads representing different taxa were used as a proxy for the relative abundance of the taxa in a sample (Moora et al. 2014). Differences in communities associated with different host species (cassava vs rubber tree) or age groups (3, 6 and 16 year-old rubber plantations) were tested using a nested PERMANOVA, with the nested.npmanova() function (999 permutations) form the BiodiversityR package (Kindt and Coe 2005), to account for the nested study design.

NMDS (function metaMDS() from R package Vegan, 50 iterations) was used to explore the community composition variation in a two-dimensional solution. Ellipses representing
standard deviations around group centroids were defined using the \texttt{ordiellipse()} function from the R package \textit{Vegan}.

Soil chemical and physical variables were ln-transformed before they were submitted to linear mixed-effects models (LME) (Pinheiro et al. 2013) to test for differences between the different site groups. The effect on community structure of soil variables was tested using redundancy analysis (RDA). Data were chord transformed (Legendre and Gallagher 2001), and all explanatory soil variables were entered into the model in the order determined by their incorporation into a stepwise selection procedure (Blanchet et al. 2008). The significance of effects was determined using sequential ANOVA and restricted permutation (999 iterations) following the block-permutation procedure described above.

### 2.2.8.2. Network analysis

In total the abundances of approximately 6540 Operational taxonomic units (OTU’s) were measured across all the conditions. For further analysis, those OTU’s that were not detected in either of the conditions (abundance value equals to 0) were disregarded. This filtering approach yielded a set of 99 OTU’s common to all the conditions. The abundance values of the selected OTU’s was used to perform Pearson correlation analysis, and a correlation network was generated for each condition, by taking into consideration only those correlation values between -0.95 and + 0.95. Hence, this process resulted in generating a network (containing 99 OTU’s) for each condition. Pearson correlation analysis and network generation was performed using \texttt{ExpressionCorrelation} plugin (v1.1.0, Saito et al. 2012) in Cytoscape environment (v 3.4.0). Each network was treated as an undirected network, and Cytoscape’s inbuilt network analysis function was used to calculate parameters such as Closeness centrality, clustering coefficient and neighborhood connectivity for each of the
networks. Based on the values, the R package ggplot2 was used to develop boxplots for each parameter, so as to enable comparison of these values between conditions specifically for each phyla (Wickham, 2011). Furthermore, an ANOVA analysis was performed in R (v 3.3.1) to identify significant differences in network parameter values between conditions for each phyla. Distribution of the response variable (network parameter values) was checked, and other diagnostic checks were made, prior to conducting ANOVA. A sub-cluster identification plugin MCODE in Cytoscape (v 3.4) was used to identify sub-clusters in each network using default parameters (Bader and Hogue 2003). In order to measure phyla composition and diversity in each of these sub-clusters, Simpson’s diversity index was calculated, and the distribution of these values for each condition were presented as histograms to enable inter-condition comparisons, using Vegan package in R (v 3.3.1).

2.2.8.3.qPCR

Linear mixed-effects models (LME) (Pinheiro et al. 2013) were used to test for differences in abundance of the different targeted genes in the different groups of sites.

2.2.8.4.Community Level Physiological Profile (CLPP)

The OD of the 31 substrate wells were standardized by subtracting the OD value of the control well and negative values were set to zero. The average well-color development (AWCD) represents the utilization rates of various carbon sources (or group of substrates) and was calculated for each sample using the formula: \( \text{AWCD} = \frac{\sum \text{OD}_i}{31} \); with \( \text{OD}_i \) representing the optical density of a particular well, after correction by blank well value subtraction. To diminish biases by different inoculum densities, the standardized data were
normalized by dividing each well OD by the AWCD (Weber et al. 2007). For further statistical analysis, OD values measured 72h after inoculation were retained. The average color well development (ACWD) was 0.501 per sample and a maximum of individual OD values were < 2 (Stefanowicz 2006).

Analysis of carbon source utilization was performed using quantitative data, where proportions of OD values representing different carbon sources were used as a proxy for the relative utilization of the carbon source in a particular sample. A heatmap was constructed based on the proportional use of group of substrates using the XLStat software (v2017.2).

2.3. Results

2.3.1. Soil characteristics

Rubber plantations of different ages and cassava fields were selected in a small area (less than 2km²) to reduce the soil variability. Soils were mainly sandy (above 80% of sand), however cassava fields tended to contain less sand and more silt than rubber tree plantations (P = 0.070 and 0.060, respectively, Table 3).

Soils from all sites had similar chemical characteristics (acidic pH, low organic matter and nutrient contents). Soils were more acidic in the 6 year-old plantations (P = 0.035) and contained less Ca and Mg than the plantations of different ages (P = 0.043 and 0.066, respectively). The level of P and N was higher in the youngest plantations while the organic matter content showed tendency to increase with the age of the plantations, although these results were not significant (Table 3).
Table 3. Soil characteristics of the groups of study sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Chemical characteristics</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH (H₂O)</td>
<td>OM (%)</td>
</tr>
<tr>
<td>Cassava</td>
<td>0.34 ± 0.12</td>
<td>9.87 ± 7.78</td>
</tr>
<tr>
<td>3 year-old</td>
<td>0.32 ± 0.13</td>
<td>25.07 ± 23.11</td>
</tr>
<tr>
<td>6 year-old</td>
<td>0.34 ± 0.11</td>
<td>12.13 ± 6.07</td>
</tr>
<tr>
<td>16 year-old</td>
<td>0.45 ± 0.07</td>
<td>6.53 ± 5.76</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site</th>
<th>Clay (%)</th>
<th>Silt (%)</th>
<th>Sand (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava</td>
<td>3.87 ± 1.19</td>
<td>12.15 ± 3.42</td>
<td>83.99 ± 3.81</td>
</tr>
<tr>
<td>3 year-old</td>
<td>4.53 ± 1.27</td>
<td>10.34 ± 1.40</td>
<td>85.13 ± 1.62</td>
</tr>
<tr>
<td>6 year-old</td>
<td>3.30 ± 0.41</td>
<td>10.18 ± 0.94</td>
<td>86.52 ± 0.93</td>
</tr>
<tr>
<td>16 year-old</td>
<td>4.30 ± 0.92</td>
<td>9.05 ± 1.45</td>
<td>86.65 ± 1.89</td>
</tr>
</tbody>
</table>

Mean ± standard error values (15 observations) of each age group. Values followed by different letters are significantly different at the level α= 0.05 (pairwise comparisons using the Tukey (HSD) test).
2.3.1. Bacterial communities structure and diversity

2.3.1.1. Bacterial 16S rDNA 454 sequencing data

454 sequencing generated 77669 quality-checked sequences. After removal of singletons and standardization, 71532 sequences were retained, grouped into 2767 OTU which were taxonomically assigned to 401 genera, 163 families and 18 phyla.

Rarefaction curves showed that sequencing depth per sample was generally sufficient to describe the bacterial diversity in our study sites. A limited proportion of the sequences could not be affiliated to any bacterial groups, which tends to indicate that the stringency of the cleaning and denoising of the dataset was sufficient.

OTU richness per sample varied from 751 to 1110, and showed tendency to decrease with the age of the plantations. Cassava sites showed similar OTU richness to the youngest plantations (Table 4).
Table 4. Richness and diversity indices of the bacterial communities per study site type.

<table>
<thead>
<tr>
<th>Site</th>
<th>Richness</th>
<th>Shannon (expH')</th>
<th>Simpson (1/D)</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava</td>
<td>926.33</td>
<td>162.20</td>
<td>32.31</td>
<td>0.74</td>
</tr>
<tr>
<td>3 year-old</td>
<td>957.67</td>
<td>228.88</td>
<td>58.17</td>
<td>0.79</td>
</tr>
<tr>
<td>6 year-old</td>
<td>877.33</td>
<td>175.25</td>
<td>43.04</td>
<td>0.76</td>
</tr>
<tr>
<td>16 year-old</td>
<td>889.00</td>
<td>149.76</td>
<td>31.90</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Pairwise comparisons of the means using the Tukey (HSD) test did not show any significant difference at \( P \leq 0.05 \).
The bacterial community was dominated by Proteobacteria in all sites, representing 40% of the total sequences, followed by Firmicutes (20%), Actinobacteria (13%) and Acidobacteria (9%). The other phyla represented less than 7% of the total reads of the dataset (Figure 2A). Both Proteobacteria and Acidobacteria phyla were significantly affected by the site types ($P = 0.028$ and 0.048, respectively). Abundance of Proteobacteria decreased from Cassava sites to 3 year-old plantations (change of land use from arable to plantation), but increased with the age of the trees to reach a similar level to cassava after 16 years of plantation.

Betaproteobacteria seemed to be the most affected class showing a similar trend ($P = 0.18$) with a significant decrease from Cassava to 3 year-old sites, and a gradual increase from 3 to 16 year-old sites (Figure 2B). Firmicutes tended to show an opposite pattern, that is an increased abundance in Cassava sites compared to rubber tree plantations ($P = 0.044$) and a gradual decrease from 3 to 16 year-old plantations, although this decrease was not significant (Figure 2A). Similarly, Acidobacteria were significantly reduced in the 16 year-old plantations as compared to the 6 year-old sites ($P = 0.050$).

Populations of several families were affected by the age of the trees, mainly belonging to the Betaproteobacteria phylum. Burkholderiaceae and Methylophilaceae abundances decreased over time ($P = 0.65$ and 0.005, respectively), while the Oxalobacteraceae were negatively affected by the conversion of arable land to rubber tree plantations, but positively affected by the age of the plantations ($P = 0.146$) (data not shown).

Only 15 out of the 2767 OTU represented >1% of the total reads. They belonged to 5 different phyla (7 Proteobacteria, 3 Actinobacteria, 2 Acidobacteria, 2 Firmicutes and 1 Bacteroidetes) and mainly included members of Bacilliales, Burkholderiales and Holophagales. These highly abundant OTU were more frequently affected by the age of the trees than by the conversion of cassava to rubber plantations (data not shown).
Figure 2. Composition of the soil bacterial community at the phylum level (A) and composition of the Proteobacteria at the class level (B) in the different site types.

Within each bacterial phyla, means accompanied by the same letter do not differ significantly at P ≤ 0.05 (pairwise comparisons using the Tukey (HSD) test).

Pairwise comparisons of the bacterial class means using the Tukey (HSD) test did not show any significant difference at P ≤ 0.05.
Conversion of cassava fields to rubber tree plantations tended to result in the increase of the OTU richness and diversity indices. These parameters showed tendency to decrease with the age of the plantations, although this was not significant (P>0.2) (Table 4).

2.3.1.2. Community patterns

The NMDS solution showed a clear separation between the four site groups (Figure 3). PERMANOVA analysis demonstrated that the bacterial communities associated with trees of different ages were significantly different (P = 0.017), in particular between the youngest and the oldest plantations (P = 0.0014). Communities associated with cassava sites did not differ from any of the rubber plantations.

Redundancy analysis (RDA) of the main phyla (each representing more than 1% of the total number of sequences) with axes constrained by soil parameters (constrained inertia = 85%) showed a similar separation of the different sites groups along the two first axes (Figure 4). Cassava sites grouped together with the oldest plantations (16 year-old) while the sites of different ages were well separated along axis 1 (for the 3 year-old sites) and axis 2 (for 6 and 16 year-old sites). Firmicutes was the only phylum that significantly influenced the ordination (based on its position on the ordination equilibrium circle (Legendre and Legendre 1998)) and was strongly correlated with the young plantations (3 and 6 year-old sites). Cassava and 16 year-old plantation samples correlated significantly with the level of soil organic matter whereas samples from 3 year-old plantations correlated with higher pH and higher clay content.
Figure 3. Non-metric multi-dimensional scaling (NMDS) plots displaying the soil bacterial communities (OTU level) associated with cassava and rubber tree sites.

Ellipses indicate one standard deviation around the centroid position of each site group.
Figure 4. Redundancy analysis (RDA) of bacterial communities associated with cassava and rubber tree sites constrained by variables describing soil chemistry and texture. Ellipses indicate one standard deviation around the centroid position of each rubber tree age group or cassava. The direction of maximum correlation between environmental variables and (linear combination) ordination scores is shown by biplot arrows. Variables and arrows shown in red were significant contributors to the constrained ordination ($P < 0.1$) in a sequential permutation test, where the order of terms in the model was determined by a stepwise model selection procedure (function ordistep in R package Vegan). The species scores of phyla falling outside the equilibrium circle were marked in red.
2.3.1.3. Network analysis

The correlation based network obtained from 99 nodes (>1 sequence per sample) are presented Figure 5. Only inter-node interactions with Pearson correlation values between -0.95 and +0.95 were included, and this resulted in 1017 interactions for network associated the rubber plantations of 3 year-old, 1031 interactions for the 6 year-old, 1087 interactions for the 16 year-old and 942 interactions for the cassava sites (Table 5). Numbers of total correlations and of positive correlations were lower in the cassava sites than in the rubber plantations regardless of the age. Number of negative correlations was decreased after conversion of cassava to rubber tree plantation, but increased with the age of the plantations.

The analysis of the abundance of the different OTU in the different sites didn't lead to the identification of specialist OTU (highly abundant in a reduced number of sites only). Most of the OTU were identified as widely generalist (similar abundance in a majority of sites).

However, there were very few correlations that were commonly found in all the sites, suggesting that the relationships between the different OTU strongly differ in the different site types. Only 10.6% of the correlations occurred in both cassava and 3 year-old sites, suggesting a strong impact of the land-use conversion on the interactions between the different bacterial community members. The age of the plantations also strongly affected the networks, since only 11.6 and 11.0% of the significant correlations were found to occur from 3 to 6 and from 6 to 16 year-old sites, respectively. Only 2.1% of the correlations occurred in all rubber plantations (data not shown).
Figure 5. Interaction networks of bacterial communities associated with cassava and rubber trees.

Thick lines represent the positive correlations (p>0.95) and fine lines the negative correlations (p < 0.95).

Only modules grouping more than 10 nodes were presented. Round, triangle, parallelogram, and arrow shaped symbols represent the different modules. Square shaped symbols represent the unclustered nodes or node belonging to modules grouping less than 10 nodes.
Table 5. Parameters of the interaction networks of the bacterial communities associated with cassava and rubber trees.

<table>
<thead>
<tr>
<th>Site</th>
<th>No of correlations</th>
<th>No of positive correlations</th>
<th>No of negative correlations</th>
<th>No of modules</th>
<th>Average path length</th>
<th>Average clustering coefficient</th>
<th>Average degree</th>
<th>Closeness centrality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava</td>
<td>942</td>
<td>470</td>
<td>472</td>
<td>5</td>
<td>3.24</td>
<td>0.73</td>
<td>19.03</td>
<td>0.31</td>
</tr>
<tr>
<td>3 year-old</td>
<td>1017</td>
<td>616</td>
<td>401</td>
<td>8</td>
<td>3.16</td>
<td>0.77</td>
<td>20.55</td>
<td>0.32</td>
</tr>
<tr>
<td>6 year-old</td>
<td>1031</td>
<td>522</td>
<td>509</td>
<td>5</td>
<td>3.08</td>
<td>0.75</td>
<td>20.83</td>
<td>0.33</td>
</tr>
<tr>
<td>16 year-old</td>
<td>1087</td>
<td>622</td>
<td>465</td>
<td>9</td>
<td>3.03</td>
<td>0.73</td>
<td>21.96</td>
<td>0.34</td>
</tr>
</tbody>
</table>
The number of modules (identified using MCODE) varied from 5 to 9 and only 2 to 4 modules per age were found to include at least 10 nodes. Cassava and 6 year-old site networks were composed of 5 modules while networks of 3 and 16 year-old sites were composed of 8 and 9 clusters, respectively (Table 5). Cassava contained the highest proportion of unclustered nodes (11 nodes) while 16 year-old contained the lowest proportion (3 nodes). Unclustered nodes were exclusively related to Actinobacteria and Proteobacteria except in the cassava fields where they were also related to Acidobacteria, Firmicutes and Planctomycetes phyla.

The average degree (number of interactions per node) and closeness centrality slightly increased from cassava sites to 3 year-old and with the age of the trees. Closeness centrality is the reciprocal of the average path length and measures how fast the information spreads from a given node to other reachable nodes in the network. The closer to 1, the faster the information spreads (Table 5).

Further analysis of these networks revealed that nodes associated with Planctomycetes phyla in plantations of 3 year-old and cassava sites had a significantly lower closeness centrality score in comparison to the same nodes in networks for 6 and 16 year-old rubber tree plantations (one way ANOVA, P < 0.05). In contrast, nodes associated with Actinobacteria phyla had significantly higher closeness centrality score in comparison to the Actinobacteria associated nodes in other conditions (one way ANOVA, P < 0.05). Similarly, nodes belonging to Acidobacteria also obtained significantly higher closeness centrality score in network belonging to 16 year-old plantation in comparison to those present in other networks (data not shown).

Average clustering coefficient increased from cassava to 3 year-old but decreased with the age of the trees. Clustering coefficient relates to the proportions of occurring interactions with regards to the total number of potential interactions or how nodes are embedded in their
neighborhood, and thus the degree to which they tend to cluster together (Table 5). Nodes associated with Acidobacteria phyla had significantly higher clustering coefficient scores in network belonging to 3 year-old plantations in comparison to the Acidobacteria nodes associated with networks obtained from plantations of 16 year-old and cassava sites (one way ANOVA, \( P < 0.05 \)). Nodes associated with Planctomycetes had significantly higher neighborhood connectivity score in network associated with plantations of 16 year-old in comparison to those in networks obtained from younger plantations and cassava (one way ANOVA, \( P < 0.05 \)).

Compositional analysis of the modules identified in each of these networks using MCODE revealed that diversity values (Simpson index) of most of the modules ranged from 0.6 to 0.8. However, this analysis also revealed the presence of a non-diverse cluster (cluster 5, Simpson score of 0) in the network associated with the 6 year-old plantation, and a highly diverse cluster (cluster 1, Simpson's score above 0.8) in the network associated with Cassava. Proteobacteria and Firmicutes tended to cluster with members of their own phyla: about 50% of the OTU belonging to these phyla were clustered in the same module, particularly in cassava and older plantations (6 and 16 year-old). In the youngest plantations (3 year-old), members of the different phyla seemed to be more widely distributed across the different modules. Bacteroidetes were mainly clustered in a single module, whatever the site type (data not shown).

2.3.2. Fungal communities structure and diversity

The fungal dataset contained 124,908 quality-filtered sequences and a total of 2876 OTU were found in the samples. They were grouped into 431 genera, 204 families, and 8 phyla.
Richness and diversity indices were not affected either by the species or the age of the trees. OTU richness varied between 827 and 965, with the maximum reached in the youngest plantations (896 OTU). Richness slightly decreased with the age of the plantations, and the minimum richness was found in the cassava fields (873 OTU). Diversity indices were higher in the 3 year-old plantations as compared to 6 year-old plantations, although this was not significant (Table 6).

The composition of the fungal community was highly stable over time and didn’t differ between the rubber tree and the cassava fields (Figures 6 and 7). Basidiomycota and Ascomycota sequences represented more than 75% of the reads in all studied groups. None of the major identified phyla or classes was affected by the species (i.e. rubber vs cassava) or by the age of the rubber tree plantation and only 10 out of the 204 families were affected by the species or by the age of the trees.

2.3.3. Functional gene abundance

The measure of the abundance of the different genes showed a diminution of the \textit{nifH} and \textit{amoA} genes abundance from cassava to 3 year-old sites. The abundance of \textit{amoA} and \textit{amoB} genes tended to increase with the age of the plantation, while the highest abundance of \textit{nifH} was measured in the 6 year-old plantation (Figure 8). However, there was no clear differentiation of the sites based on the abundance of these genes.
Table 6. Richness and diversity indices of the fungal communities per study site and site group.

<table>
<thead>
<tr>
<th>Site</th>
<th>Richness</th>
<th>Shannon (expH’)</th>
<th>Simpson (1/D)</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassava</td>
<td>873.00</td>
<td>66.10</td>
<td>12.17</td>
<td>0.62</td>
</tr>
<tr>
<td>3 year-old</td>
<td>906.33</td>
<td>68.50</td>
<td>11.89</td>
<td>0.62</td>
</tr>
<tr>
<td>6 year-old</td>
<td>893.67</td>
<td>63.81</td>
<td>11.50</td>
<td>0.61</td>
</tr>
<tr>
<td>16 year-old</td>
<td>887.00</td>
<td>65.59</td>
<td>12.00</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Pairwise comparisons of the means using the Tukey (HSD) test did not show any significant difference at $P \leq 0.05$. 
Figure 6. Composition of the soil fungal community at the phyla level in the different site types.

Pairwise comparisons of the means using the Tukey (HSD) test did not show any significant difference at $P \leq 0.05$. 
Figure 7. Non-metric multi-dimensional scaling (NMDS) plots displaying the soil fungal communities (OTU level) associated with cassava and rubber trees.
Figure 8. Mean microbial molecular abundance of archaeal AOA \textit{amoA}, bacterial AOB \textit{amoB}, and \textit{nifH} genes (copies.ng$^{-1}$ of DNA) in the different site types.

Pairwise comparisons of the means using the Tukey (HSD) test did not show any significant difference at $P \leq 0.05$. 
2.3.1. Community Level Physiological Profile (CLPP)

The heatmap analysis showed a separation of the samples based on the species (rubber and cassava) but the samples issued of plantations of different ages could not be separated. Two main clusters were identified: the first cluster was mainly related to the use of carbohydrates, esters and polymers while the second cluster was related to the use of amino acids, carboxylic acids and amines (Figure 9A). Samples originating from the cassava sites were mainly found in the cluster 1 (65.4%) while the rubber tree samples were mostly found in the cluster 2 (60.5%), in particular samples from the 6 and 16 year-old sites (66.7 and 63.0%, respectively). Samples from the youngest plantations were found in both clusters (Figure 9B). This suggests a gradual shift from cassava to rubber in the preferential use of carbon substrates by the bacterial community.

There was no clear different the plantations of different ages. However, communities associated with 6 year-old plantations showed a higher utilization of polymers while samples originating from the 16 year-old plantations were associated with a higher use of carbohydrates (data not shown).
Figure 9. Heatmap of the utilization of the carbon substrates (Biolog) for rubber tree and cassava samples (A) and repartition of the samples per cluster (B).
2.4. Discussion

2.4.1. Bacterial community composition

The bacterial community was dominated by proteobacteria, regardless of the site type but the abundance of proteobacteria was affected by both the conversion of cassava fields and the age of the trees. In particular, Betaproteobacteria were affected by both the conversion of agricultural field to rubber tree plantation and the age of the trees.

Proteobacteria have been shown to have ubiquitous distributions and to be highly abundant across a range of soil ecosystems (Jeanbille et al. 2016; Mendes et al. 2015; Xue and Huang 2014). They have been shown to be enriched in acidic and nutrient poor soils, as those found in the studied chronosequence (Jeanbille et al. 2016). They are also known as to presumptive initial degraders of OM (Sarr et al. 2017), which may explain their higher abundance in the oldest plantations, where the presence of litter results in a higher soil OM content.

Betaproteobacteria have been described as copiotroph (Fierer and Jackson 2006). The higher levels of OM and nutrients (such as Ca and K) in the oldest plantations may explain the increase of the Betaproteobacteria in these sites.

Firmicutes were the second most abundant phyla and were more affected by the conversion than by the age of the trees. Firmicutes have been shown to be abundant in different land uses and to be resistant to environmental variations such as high soil temperatures (Jangid et al. 2008; Mendes et al. 2015). They were however shown to be positively affected by increases in pH and nutrient contents (Mendes et al. 2015) and this may explain the variations observed in the different studied sites.

Acidobacteria abundance decreased in the oldest plantations, while the other phyla were not affected by the conversion nor the age of the trees. Acidobacteria are frequently found in
studies focusing on soil bacterial communities, in particular in acidic environments (Jeanbille et al. 2016; Sait et al. 2006; Tripathi et al. 2012). They have been classified as oligotrophic and slow growing and are of particular interest since they are presumed to be involved in C and N cycles, with important roles in various soil functions (Fierer et al. 2007; Sarr et al. 2017). Abundance of Acidobacteria was decreased in the 3 and 16 year-old rubber plantations, which have less acidic pH and higher nutrient contents than the other site types. This is in accordance with other studies that showed that abundance of Acidobacteria was negatively correlated with increases in pH and other soil properties including P, K, Ca, Mg contents (Jangid et al. 2008; Jeanbille et al. 2016). It is possible that members of this phylum were able to outcompete other bacterial groups in sites where they were exposed to stronger environmental stress such as resource limitation.

2.4.2. Bacterial community diversity and structure

Bacterial community diversity and structure was not significantly affected by the conversion of cassava field into rubber plantation. Land use changes and deforestation are expected to result in dramatic effects on soil biodiversity (Sodhi et al. 2010). However, in the case of rubber tree plantations in SE Asia, studies reported contradictory effects of the conversion of forest (primary or secondary) on the soil microbial compartment. Kerfahi et al. (2016) showed that conversion of rainforest to rubber plantations did not result in consistent effect on alpha or beta diversity of bacteria but significant differences in community composition occurred. A recent study on rainforest transformation into jungle rubber in Indonesia reported that only some bacteria groups were detrimentally affected by the conversion of rainforest to rubber plantations and suggested that microbial communities were remarkably resistant to conversion of rainforest into agricultural production systems (Krashevska et al. 2015). Schneider et al.
(2015) found that both bacterial and archaeal community composition and diversity were affected by the transformation of rain forest soils to managed soils.

Natural forests have almost disappeared in NE Thailand and most of the rubber tree plantations in this region were set up on agricultural fields (mainly grown to cassava). Our results however, are in accordance with those obtained in the previously mentioned studies and showed a limited impact of land-use conversion on the bacterial and fungal community diversity and composition. All the studied rubber tree plantations had been established on former cassava fields, and cassava and rubber tree both belong to the Euphorbiaceae family. The large proportion of shared bacterial populations is likely to also reflect the former land use. Although slight changes in diversity and composition were observed, our results suggest that the rubber plantation environment is not as drastically detrimental to diversity as it has been presumed.

Bacterial community was affected by the age of the plantations. Richness tended to decrease but composition was significantly affected. The effects of the age of the plantation on soils have been less extensively studied than impact of forest conversion. Cheng et al. (2007) reported a continuous decrease in soil fertility with the increase of stand age of rubber plantation (up to 30 year-old). In a chronosequence of 11 rubber plantations ranging in age from 5 to 46 years, carbon stocks declined drastically during the first 5 years since deforestation (up to 20%) and reached a steady state (around 30%) after 20 years (de Blécourt et al. 2013). However, the consequences of these effects on the soil microbial communities are yet to be studied.

Plant age has been shown to be an important factor affecting microbial community structure (Marschner et al. 2002) but its role has been poorly investigated in tree plantations. Changes in the composition of soil microbial community were observed after 5 years in a Eucalyptus
plantation (Wu et al. 2013). A study on tree peony reported that changes observed in microbial community structure were more related to planting years than to tree cultivars (Xue and Huang 2014). Changes were often attributed to differences in root exudation. Through the root development and the production of roots exudates, soil chemical properties are modified and this may participate in the selection of specific microorganisms (Berg and Smalla 2009; Wu et al. 2013).

To our knowledge, our study is the first report on the effects of the rubber tree plantation age on the associated microbial communities. Our results showed that bacterial diversity tended to decrease with the age of the plantation and that the community composition was particularly affected. These changes were associated to changes in soil parameters, particularly to pH, and OM and Ca contents which may partially result from changes in root exudates.

Plantations of different ages are also subject to different fertilization treatments. Young plantations usually receive high doses of NPK fertilizers while plantations of 6 year-old do not receive any fertilizers. The direct effects of changes in nutrient contents on soil microbial communities have been widely acknowledged and have been reported to be more dramatic than either land use or season (Cao et al. 2010; Jangid et al. 2008; Jeanbille et al. 2016; Kerfahi et al. 2016; Wu et al. 2013). Fertilization has been shown to be a driver of soil acidifications and could affect microbial community structure indirectly by influencing soil pH which is known as the most important driver of microbial community composition (Berg and Smalla 2009; Girvan et al. 2003; Mendes et al. 2015; Yuan et al. 2015). The greater quantity of litter found in the old plantation (after the closure of the canopy) represents a source of OM and nutrients to the soil underneath. The resulting nutrient allocation to soil directly affects soil microbial community (Bamminger et al. 2014; Fanin et al. 2014; Pfeiffer et al. 2013).
2.4.3. Fungal community diversity and composition

Fungal communities were not affected by the conversion of plantation nor by the age of the trees. Our results are in accordance with those of other studies as no change was observed in fungal diversity after conversion of forest to rubber tree plantation (Kerfahi et al. 2016; Krashevska et al. 2015) or to conifer planted forest (Purahong et al. 2014). Similar fungal community composition was observed with increasing tree age in a Eucalyptus chronosequence (Wu et al. 2013). Fungi populations have been suggested to be more different over space but more stable over time (Vitali et al. 2016). Our results support this hypothesis as the different sites were selected in a small area and all presented similar soil type.

2.4.4. Interaction networks

Interactions between the principal OTU were highly site type dependent. Network models provide tools to explore complex microbial assemblages in various environments in order to obtain a better understanding of how microbiomes influence and are influenced by a variety of factors (Poudel et al. 2016; Shi et al. 2016). Documenting interactions between bacterial groups across complex communities may help to ascertain the functional roles or environmental niches occupied by specific microorganisms (Barberán et al. 2012).

In our study, although the bacterial diversity and the community composition was not significantly affected, important differences were observed in the interaction networks built on a set of principal OTU in each of the site type. Overall, soil microbial networks were highly connected (average degree >19 and closeness centrality >0.3 regardless of the site type) with a modular structure (>5 modules per network). The comparison of these network
structural properties enables a quick analysis of complex datasets in order to get a better understanding of how certain environmental conditions influence the assembly of microbial communities (Barberán et al. 2012).

Interestingly, there was a very low proportion of interactions that was shared by the different site types. In addition, OTU from the same phyla tended to co-occur more with members of their own phyla than with OTU related to other phyla. This may indicate non-random assembly patterns in these different conditions and that soil microorganisms tended to co-occur more than expected by chance. Similar results were obtained in other studies and it has been proposed that non-random community assembly may be a general characteristic across all life domains (Barberán et al. 2012; Cardinale et al. 2015; Horner-Devine et al. 2007). The large number of co-occurrences within particular phyla may also reflect non-overlapping niches (environmental preferences) or common life history strategy of particular phyla and inter-phyla competition for ecological niches or nutrients (Barberán et al. 2012; Poudel et al. 2016). A more detailed analysis of the node interactions may provide additional information on the structure of the bacterial communities, such as keystone taxa which are critical in maintaining the various soil functions among different land-uses (cassava vs rubber) or across time.

2.4.5. Abundance of functional genes involved in the N cycle

Abundances of \textit{nifH}, \textit{amoA} and \textit{amoB} genes were not significantly affected by the land-use change or by the age of the plantations. The three targeted genes were related to N cycle functions (nitrogen fixation for \textit{nifH} and the first (and potentially limiting) step of nitrification for \textit{amoA} and \textit{amoB} (Rotthauwe et al. 1997). Recent studies demonstrated that AOA are
generally more abundant than AOB in acidic soil (Petersen et al. 2012; Ribbons et al. 2016) and our results are in accordance with these findings.

None of the gene abundances was affected by the conversion of arable land into rubber tree plantation, although all genes tended to be negatively affected by the conversion. The effect of plant species on functional gene abundance was reported in studies on several non-leguminous plant species (Bremer et al. 2007; Briones et al. 2002; Ribbons et al. 2016). However, the N status of the soil and the C:N ratio in particular were shown to have a predominant and a greater effect than the tree species (Högberg et al. 2007; Högberg et al. 2013; Ribbons et al. 2016). Total N content was very low in all our studied sites, which may explain the absence of significant differences in the abundance of the different targeted genes.

It has long been suggested that changes in taxonomic diversity may lead to a decrease or even loss of functional redundancy and consequently affect ecosystem functions, production and nutrient cycling (Loreau and de Mazancourt 2013; Mendes et al. 2015). Differences existed in the community structure between the rubber plantations of different ages. However, there was no significant differences in the abundances of the targeted genes. This may be due to the fact that all major phyla including groups noted for mutualisms or N fixation were present in all sites and that functional redundancy was maintained.

2.4.6. Bacterial community level physiological profiles (CLPP)

Surprisingly, although there was no significant difference in bacterial community composition, patterns of C sources utilization were affected by the conversion of cassava sites into rubber tree plantations. It seems that the small differences observed in the bacterial
community structure and composition were sufficient to induce significant changes in soil functions linked to the C cycle.

Communities associated with rubber showed higher use of amino acids, carboxylic acids and amines while those found in cassava sites were associated with a higher utilization of carbohydrates, ester and polymers. The latter are more complex substrates, and communities associated with cassava are likely to include specific populations able to degrade this type of complex substrates. Similar results were obtained by Jeanbille et al. (2016) on soil sequences having different properties, and of pH in particular. Cassava soils were more acidic than those of 3 year-old plantations and this may have affected the metabolic potentials. In addition, soils from the 3 year-old plantations also tended to contain higher levels of soil nutrients (P, N, Ca, Mg) which is likely to have contributed to the changes in the preferential use of substrates in those sites.

CLPP were not strongly affected by the age of the plantations. As hypothesized for the abundances of genes involved in the N cycle, changes in community composition associated with the plantations of different ages are unlikely to have affected the functional redundancy, resulting in a certain degree of stability with regards to metabolic potentials of C substrates.

2.5. Conclusions

Our results showed that conversion of cassava fields into rubber tree plantations did not drastically affect the bacterial and fungal communities’ diversity and structure but affected some of the soil functions linked to the use of various carbon sources by soil bacteria. Bacterial community composition was significantly affected by the age of the rubber trees but functional diversity was maintained. Changes in microbial communities were associated with
differences in soil parameters, mainly pH and organic matter content. Complementary in-depth studies of the microbial communities in relation with soil functions and soil properties may provide new insights in the role of the biological compartment in maintaining soil fertility in rubber tree plantations in SE Asia.
3. CHAPTER 3: High colonization by native arbuscular mycorrhizal fungi (AMF) of rubber trees in small-holder plantations on low fertility soils in North East Thailand

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See the “Authorship statement – chapter 3” (appendix 2) for details.
3.1. Abstract

Rubber tree is a very important crop in Thailand, representing an essential source of income for farmers. In the past two decades, rubber tree plantations have been greatly expanding in unfavorable areas, where climate conditions are difficult and soil fertility is very poor. To optimize latex yields, mineral fertilizers have been widely used. A better understanding of the roles of the biological compartment in soil fertility is essential to determine alternative management practices to sustain soil fertility and optimize latex yields. Arbuscular mycorrhizal fungi (AMF) are widely recognized as beneficial for plants, mainly through their role in improving plant nutrient uptake. The objective of this study was to assess the AMF populations in rubber tree plantations and the impact of both soil characteristics and plantation age on these communities. Our results showed that all rubber trees were highly colonized, regardless of the soil structure and nutrient contents. AMF colonization was not affected by the age of the trees, suggesting that maintaining the symbiosis could be beneficial at all stages. A better understanding and management of the microbial communities would contribute to maintaining or restoring soil fertility, leading to a better tree growth and optimized latex yield.

3.2. Introduction

The rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important crop in South East (SE) Asia and particularly for smallholder farmers in Thailand due to its ability to produce latex and a large part of the population relies on rubber plantations as a source of income. Thailand is the world leading producer and exporter of rubber with a production capacity of about 3.2 million tons per year, of which about 90% is exported to foreign markets (http://faostat.fao.org).
Rubber trees have traditionally been cultivated in areas characterized by rainfall higher than 1300 mm/year well distributed year-round as well as average monthly temperature around 23°C. Deviation from these optimal conditions may have a significant influence on the latex yield (Rao et al. 1998). Optimal soil properties such as soil depth, pH, water retention capacity, and moisture were shown to be critical factors to determine the suitability of a growing site. Well drained, deep clayey soils with a slightly acidic pH ranging between 5 and 6 are the best conditions for growth, even though trees can withstand physical conditions from stiff clay with poor drainage to well drained sandy loam. According to Saengruksawong et al. (2012), while only 102,000 hectares in Thailand have suitable soil characteristics, rubber plantations covered about 2.7 million hectares in 2009. In the last 20 years, the plantations have been greatly expanding in less favorable regions of the country, particularly, in the North East regions, where the fertility of soils is particularly low and rainfall is less than optimal with a dry season lasting for about 6 months, leading to reduced plant growth (Li and Fox 2012).

It is acknowledged that the soil physico-chemical characteristics influences latex production (Akpan et al. 2007). In addition, the monoculture of tree crop species often results in a considerable depletion of fertility. To counteract this, mineral fertilizers have been widely applied in Thailand, but the level of soil fertility is still decreasing and remains dramatically low in some regions. The effects of rubber tree plantations on soil characteristics have been increasingly studied during the last decade (Cheng et al. 2007; Murbach et al. 2003; Zhang et al. 2007) and alternative management strategies have been proposed, such as the use of organic fertilizers or the promotion of cover crops (Akpan et al. 2007; Orimoloye et al. 2010). While the importance of the soil biotic compartment in soil fertility has been recognized generally, it has been poorly investigated in the case of rubber trees in SE Asia.
Plant roots encounter an enormous variety of organisms in the rhizosphere and they interact with the physical, chemical, and biological properties of soils. Extensive interactions of roots with soil microorganisms affect plant nutrition either directly by influencing mineral nutrient availability, or indirectly through plant (root) growth promotion enhancing uptake efficiency (Richardson et al. 2009). Arbuscular mycorrhizal fungi (AMF) are known to be associated with a wide variety of plants and to play a key role in plant nutrition (and P uptake in particular) through a wider exploration of the soils and thus a better nutrient availability to the plants (Cardoso and Kuyper 2006; Schwob et al. 1998).

Many studies have shown the positive impact of AMF symbiosis on plants (Cardoso and Kuyper 2006; Strack et al. 2003). The occurrence of AMF in rubber tree roots was first reported by D’Angremond and Van Hell (1939) but has been poorly investigated since. To date, studies have mainly focused on the AMF communities associated with rubber trees in South America (Feldmann et al. 2000; Schwob et al. 1999; Sosa-Rodriguez et al. 2014) and little is known about the potential of native populations of AMF associated with mature rubber trees in the context of low fertility soils in SE Asia. The objective of this study was to assess the existence and importance of native AMF populations able to colonize mature rubber tree roots under unfavorable conditions (adverse soils and climate) in Thailand.

3.3. Material and methods

3.3.1. Site information and sampling

Nine plantations were selected in Ban Don Chang village, in Khon Kaen district, Northeastern (NE) Thailand. The same clone (Rubber Research Institute of Malaysia [RRIM] 600) was used in all the plantations and the sites were selected in a small area (about 2 square km) to
reduce the soil variability. More details on the site can be found in chapter 2, section 2.1. The plantations were 3, 6, and 16 year-old (3 plantations for each age) and no cover crops were associated with the trees. Soil and root samples were collected in August 2013 following the protocol described in Chapter 2, section 2.1. Fine rubber tree roots were collected from the sieve and dried at 45°C before analysis.

3.3.2. Soil analyses
Soil chemical (pH, organic matter, available P, total N, K, Ca, and Mg) and physical (texture) characteristics were determined on five soil samples per plantation.

Soil characteristics were determined on five soil samples per plantation and included pH (in H$_2$O), organic matter (Walkley and Black 1934), available P (Bray and Kurtz 1945, Bray II), total N (Bremner, 1965), K, Ca and Mg (ammonium acetate pH7) contents (Thomas, 1982), and texture (Kilmer and Alexander 1949).

3.3.3. Root staining and AMF colonization assessment
Fine roots were selected (less than 1 mm in diameter) and AMF staining in root tissue was performed following the ink and vinegar technique (Vierheilig et al. 1998) with an additional tissue bleaching step as described by Koske and Gemma (1989). Fifteen roots fragments of 1 cm were observed per sample (x40) and the presence and the intensity (scored from 0 to 5) of colonization in each fragment were recorded to assess the frequency (F%) and the intensity (M%) of AMF colonization (Trouvelot, 1986).
3.3.4. Statistical analysis

AMF colonization and soil characteristics data were ln(x) transformed and verification of the normal distribution was performed before the analysis of variance (P < 0.05). A principal component analysis (PCA) was performed on ln(x) transformed data to assess the correlations between the soil characteristics, the age of the plantations and the mycorrhization parameters.

3.4. Results and discussion

Approximately 1250 mm rain fell in the Khon Kaen district in 2013, distributed from June to October. The rainfalls in August were lower than expected, with only 80 mm while more than 400 mm were received in both July and September. The daily temperatures ranged from 9 to 45°C, with an average of 30°C throughout the year. Highest temperatures were observed from April to September, with an average of 31°C in August (data not provided). Conditions associated with optimum latex yields are maximum temperature of 30.4°C and 72 mm of rainfall per week. Limited water availability and strong variations of temperature are often causes of yield loss (Rao et al. 1998).

Table 7 presents the physical and chemical characteristics of the soils collected in the different sites. Soils were mostly sandy in texture (>80%), acidic and very poor in organic matter (less than 0.5%) and macro elements (N, P, K, Ca, and Mg). The total N and available P values were often too low to fully support growth of rubber trees, which could result in lower latex yield (Akpan et al. 2007). Soil sand fraction was also above the desirable range of 50–70% as defined for rubber plant. Same authors have mentioned that higher values may imply higher porosity, higher nutrient leaching and reduced water holding capacity of the soils.
Table 7. Soils chemical and physical characteristics and AMF root colonization frequency (F%) and intensity (M%) of the rubber plantations and cassava fields.

<table>
<thead>
<tr>
<th>Site</th>
<th>Clay (%)</th>
<th>Silt (%)</th>
<th>Texture (USDA classification)</th>
<th>Chemical characteristics</th>
<th>AMF colonization</th>
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<td></td>
<td></td>
<td></td>
<td>Very fine (%&lt;br&gt;Medium (%)&lt;br&gt;Coarse (%)&lt;br&gt;Very coarse (%)&lt;br&gt;Total (%)</td>
<td>pH (H₂O) (H)&lt;br&gt;OM (%)&lt;br&gt;P (Bray II) (mg/kg)&lt;br&gt;Total N (%)&lt;br&gt;K (mg/kg)&lt;br&gt;Ca (mg/kg)&lt;br&gt;Mg (mg/kg)&lt;br&gt;F%&lt;br&gt;M%</td>
<td></td>
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<tr>
<td>3 year-old</td>
<td>4.5&lt;sup&gt;b&lt;/sup&gt; (1.3)&lt;br&gt;10.4&lt;sup&gt;b&lt;/sup&gt; (1.4)&lt;br&gt;37.25&lt;sup&gt;a&lt;/sup&gt; (3.07)&lt;br&gt;43.38&lt;sup&gt;a&lt;/sup&gt; (1.46)&lt;br&gt;4.21&lt;sup&gt;a&lt;/sup&gt; (1.00)&lt;br&gt;0.26&lt;sup&gt;a&lt;/sup&gt; (0.10)&lt;br&gt;0.027&lt;sup&gt;a&lt;/sup&gt; (0.06)&lt;br&gt;85.1&lt;sup&gt;a&lt;/sup&gt; (1.6)</td>
<td>5.54&lt;sup&gt;b&lt;/sup&gt; (0.48)&lt;br&gt;0.32&lt;sup&gt;a&lt;/sup&gt; (0.13)&lt;br&gt;25.07&lt;sup&gt;b&lt;/sup&gt; (23.11)&lt;br&gt;0.054&lt;sup&gt;a&lt;/sup&gt; (0.02)&lt;br&gt;20.93&lt;sup&gt;a&lt;/sup&gt; (6.81)&lt;br&gt;117.07&lt;sup&gt;b&lt;/sup&gt; (58.47)&lt;br&gt;36.0&lt;sup&gt;c&lt;/sup&gt; (24.0)</td>
<td>100&lt;sup&gt;a&lt;/sup&gt; (0)&lt;br&gt;73.8&lt;sup&gt;a&lt;/sup&gt; (18.5)</td>
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<td>6 year-old</td>
<td>3.3&lt;sup&gt;a&lt;/sup&gt; (0.4)&lt;br&gt;10.2&lt;sup&gt;b&lt;/sup&gt; (0.9)&lt;br&gt;38.57&lt;sup&gt;b&lt;/sup&gt; (2.12)&lt;br&gt;43.75&lt;sup&gt;a&lt;/sup&gt; (1.86)&lt;br&gt;3.99&lt;sup&gt;a&lt;/sup&gt; (0.64)&lt;br&gt;0.21&lt;sup&gt;a&lt;/sup&gt; (0.09)&lt;br&gt;0.007&lt;sup&gt;a&lt;/sup&gt; (0.03)&lt;br&gt;86.5&lt;sup&gt;a&lt;/sup&gt; (0.9)</td>
<td>4.71&lt;sup&gt;a&lt;/sup&gt; (0.19)&lt;br&gt;0.34&lt;sup&gt;a&lt;/sup&gt; (0.11)&lt;br&gt;12.13&lt;sup&gt;b&lt;/sup&gt; (6.07)&lt;br&gt;0.042&lt;sup&gt;a&lt;/sup&gt; (0.01)&lt;br&gt;20.13&lt;sup&gt;a&lt;/sup&gt; (3.70)&lt;br&gt;59.53&lt;sup&gt;a&lt;/sup&gt; (22.58)&lt;br&gt;36.0&lt;sup&gt;c&lt;/sup&gt; (3.2)</td>
<td>100&lt;sup&gt;a&lt;/sup&gt; (0)&lt;br&gt;76.1&lt;sup&gt;a&lt;/sup&gt; (18.3)</td>
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<td>16 year-old</td>
<td>4.3&lt;sup&gt;b&lt;/sup&gt; (0.9)&lt;br&gt;9.1&lt;sup&gt;a&lt;/sup&gt; (1.5)&lt;br&gt;36.09&lt;sup&gt;a&lt;/sup&gt; (1.84)&lt;br&gt;46.01&lt;sup&gt;b&lt;/sup&gt; (1.95)&lt;br&gt;4.36&lt;sup&gt;a&lt;/sup&gt; (0.84)&lt;br&gt;0.17&lt;sup&gt;a&lt;/sup&gt; (0.03)&lt;br&gt;0.007&lt;sup&gt;a&lt;/sup&gt; (0.03)&lt;br&gt;86.6&lt;sup&gt;a&lt;/sup&gt; (1.9)</td>
<td>5.51&lt;sup&gt;b&lt;/sup&gt; (0.45)&lt;br&gt;0.45&lt;sup&gt;b&lt;/sup&gt; (0.07)&lt;br&gt;6.53&lt;sup&gt;a&lt;/sup&gt; (5.76)&lt;br&gt;0.042&lt;sup&gt;a&lt;/sup&gt; (0.02)&lt;br&gt;28.73&lt;sup&gt;b&lt;/sup&gt; (11.20)&lt;br&gt;227.93&lt;sup&gt;b&lt;/sup&gt; (140.0)&lt;br&gt;14.2&lt;sup&gt;b&lt;/sup&gt; (7.1)</td>
<td>99.7&lt;sup&gt;a&lt;/sup&gt; (1.5)&lt;br&gt;77.9&lt;sup&gt;a&lt;/sup&gt; (11.9)</td>
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<tr>
<td>Cassava</td>
<td>3.9&lt;sup&gt;ab&lt;/sup&gt; (1.2)&lt;br&gt;12.1&lt;sup&gt;b&lt;/sup&gt; (3.4)&lt;br&gt;34.89&lt;sup&gt;b&lt;/sup&gt; (2.69)&lt;br&gt;43.57&lt;sup&gt;a&lt;/sup&gt; (2.59)&lt;br&gt;5.21&lt;sup&gt;b&lt;/sup&gt; (0.89)&lt;br&gt;0.29&lt;sup&gt;b&lt;/sup&gt; (0.05)&lt;br&gt;0.027&lt;sup&gt;a&lt;/sup&gt; (0.05)&lt;br&gt;84.0&lt;sup&gt;a&lt;/sup&gt; (3.8)</td>
<td>4.91&lt;sup&gt;a&lt;/sup&gt; (0.22)&lt;br&gt;0.34&lt;sup&gt;a&lt;/sup&gt; (0.12)&lt;br&gt;9.87&lt;sup&gt;a&lt;/sup&gt; (7.78)&lt;br&gt;0.037&lt;sup&gt;a&lt;/sup&gt; (0.01)&lt;br&gt;22.40&lt;sup&gt;ab&lt;/sup&gt; (8.24)&lt;br&gt;99.40&lt;sup&gt;b&lt;/sup&gt; (52.66)&lt;br&gt;11.27&lt;sup&gt;ab&lt;/sup&gt; (6.3)</td>
<td>98.0&lt;sup&gt;a&lt;/sup&gt; (6.8)&lt;br&gt;72.7&lt;sup&gt;a&lt;/sup&gt; (16.1)</td>
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F%: Frequency of mycorrhization; M%: Intensity of mycorrhization.
Average values for 15 and 21 samples per site group for soil characteristics and AMF assessment respectively. For each variable, values followed by different letters are significantly different at P < 0.05 according to the Tukey test. Standard deviation values are given in brackets.
Soils collected in the 3 year-old plantations contained generally more P than the other plantations. That can be explained by the management practices of the plantations, with a regular application of variable rates of P fertilizers during the early stages of the plantation (twice a year, for approximately 5 years after planting) to promote the growth of the trees. One of the 3 year-old plantations had received higher rates of P fertilizers (30 kg P/ha) while the two others 3 year-old plantations had received less than 10 kg P/ha, which explains the high standard deviation value observed for the P content in soils. The 6 year-old plantations contained the lowest levels of K, Ca, and Mg, probably because the trees at this stage are still rapidly growing in the absence of extra applied fertilizer.

Aweto et al. (1987) reported that during the first 10 years of growth, the rate of nutrient uptake by rubber trees greatly exceeded that of nutrient return to the soil through litter fall during the rapid phase of growth. On the other hand, soils collected in the 16 year-old plantations were significantly richer in organic matter, K, Ca, and Mg. In addition to a slower growth, this can be attributed to the presence of a higher quantity of litter in the old plantations than in the younger plantations in which the trees are much smaller and the canopy has not closed yet.

In our study, all root samples were highly colonized (Table 7) and no site without mycorrhizae was found. The frequency of AMF occurrence (F%) was close to 100% in all fields, regardless of plant, age of the trees or soil characteristics. These results are consistent with those obtained by Dhar and Mridha (2006) and Schwob et al. (1999) who observed a high frequency of colonization on rubber trees growing in other tropical soils (Brazil and Bangladesh, respectively). The intensity of colonization (M%) was also high in all sites with a minimum of 72.7% in cassava roots. Surprisingly, AMF colonization of roots in the rubber tree plantations was as high as in the cassava fields with an intensity of colonization ranging
from 73.8% to 77.9% with no significant impact of the age of the plantations (Table 7). This is not in accordance with results obtained in previous studies that found that tree monocultures can reduce populations of AMF in soils (Muleta et al. 2008; Pagano et al. 2009).

AMF are known to play a significant role in the alleviation of drought stress in many plants through mechanisms including an increase of the mobilization of nutrients in dry soils, a better access to tightly held soil water or an improvement of soil porosity, water infiltration and storage (Yang et al. 2014). Schwob et al. (1999) studied the impact of climatic factors on the AMF colonization rate in several rubber tree plantations in Brazil and observed no seasonal effect at any site. This is in accordance with our results since all the sites were highly colonized even if the rainfall was particularly low at the sampling time (less than 80 mm in one month) and demonstrates the ability of AMF to establish the symbiosis with rubber trees under low humidity conditions, and highlights the high potential of AMF even in low rainfall areas. The root colonization intensity of rubber trees was previously shown to vary greatly with site location, type of soils and management practices. Under controlled conditions (greenhouse studies) reported colonization was generally low and reached 31 to 64% even after inoculation with different AMF species (Ikram et al. 1992; Jayaratne et al. 1984; Schwob et al. 1998). Colonization rates in natural stands (forests) or plantations in South America were higher and ranged from 36 to 89%, depending on soil type and fertilizer applications (Feldmann, 1994; Feldmann et al. 2000; Schwob et al. 1999).

However to our knowledge, there is scarce information about the effect of the age of the trees on AMF colonization of roots. Furthermore, the age of the trees is often not indicated in studies working on natural stands or mature plantations. Our results indicate that AMF established high levels of colonization with the rubber trees regardless of their age, and as
previously reported, positive interactions are more likely to emerge and be maintained in poor soils (Verbruggen and Kiers 2010). This suggests that the interactions between the rubber trees and the fungi are likely to be beneficial and essential for the trees at all stages (growth, latex production). AMF have received increasing attention in recent years and have been described as the most important but poorly understood resource for plant growth through their role in maintaining soil structure and improving nutrient acquisition (Cardoso and Kuyper 2006). Their role in improvement of P nutrition in particular is no longer disputed, but acquisition of other nutrients such as N or K may also be improved, particularly in ecosystems with reduced soil nutrient availability (Cardoso and Kuyper 2006; Cavagnaro et al. 2015; Clark and Zeto 2000; van der Heijden et al. 2015).

Figure 10A and 10B show the relations between the soil physical and chemical characteristics and the mycorrhization parameters. The two first axes (F1 and F2) accounted for approximately 50% of cumulated variability. The first axis (F1) was strongly linked to the soil nutrients characteristics (mainly Mg, Ca, and OM) while the second axis (F2) was more linked to the texture of the soils (sand and silt contents). The P and N content variables were strongly correlated to the third axis (F3) (0.657 and 0.459, respectively) which accounted for 12.47% of the variability. The mycorrhization parameters (both F and M%) were not significantly correlated with any of the measured soil characteristics.
Figure 10. Principal Component Analysis (PCA) of the soils characteristics and AMF colonization of the roots collected in the different rubber plantations and cassava fields.

A) and B): variables correlations with F1-F2 and F1-F3 axes, respectively. C) and D): ordination of samples along F1-F2 and F1-F3 axes, respectively. Ellipses indicate 95% confidence intervals.
Interestingly, it was not possible to distinguish the cassava fields and the rubber plantations along the three main axes (Figure 10C and 10D). In addition, the distribution of the observations on the F1 and F2 axes did not show strong correlations between the age of the trees and the soil characteristics, even though plantations of different ages were better distributed along the F1 axis, implying a stronger effect of the age on the chemical characteristics than of the soil texture (Figure 10C), as expected. Aweto (1987) observed that rubber trees did not adversely affect soil physical status over time. However, he also observed a significant decrease in soil nutrient contents, Ca and Mg in particular. Cheng et al. (2007) also observed a significant decrease of nutrient concentrations with time in rubber plantations in China. We observed a similar decrease of soil nutrient status in the 6 year-old plantations compared to the younger sites, although this could also be attributed to the changes in fertilization management of the plantations. Moreover, this nutrient content decrease was not observed in the 16 year-old plantations, which showed significantly higher Ca, K, and OM contents than all the other sites. The plantations of different ages were also well distributed along the F3 axis, showing that the levels of P and N decreased over time (Figure 10D). P in particular is known to significantly affect the mycorrhization of plants. The application of P fertilizers has been shown to decrease or increase the AMF colonization, depending on the applied rate (Cardoso and Kuyper 2006; Reynolds et al. 2006; Boerner 1986). Increases of AMF colonization were presumably explained by improved plant vigor (Sieverding and Howeler 1985) while decreases were observed in many studies, explained mainly by the fact that the plants found the necessary elements in the environment and thus the symbiosis with AMF was less profitable (Cardoso and Kuyper 2006; Feldmann et al. 2000; Ikram et al. 1992). Surprisingly, our results showed no correlation between the mycorrhization parameters and the P content of the soils. The level of P in the soils from our study was very low, even
after application of P fertilizers in the young plantations (3 year-old). It is possible that the addition of P fertilizers in the young plantations was not sufficient to generate any effect on the colonization of the roots and that the trees were still strongly relying on AMF after fertilization to improve their P nutrition. It could also be hypothesized that the effect of the treatment was masked by the high abundance and a maximized colonization by AMF in low-input systems compared to other systems.

3.5. Conclusions

This study highlights the importance of native AMF populations able to colonize both mature rubber trees and cassava in very poor soils and under adverse climatic conditions (low rainfall, high temperatures, and long dry season). These preliminary results emphasize the high need of research to better understand and use the potential for improving soil fertility using the AMF communities naturally existing in tropical soils, and the importance of maintaining or restoring these communities, especially in low fertility soils such as in NE Thailand. Complementary studies of the roles of the biological components in rubber tree plantations would assist in determining and recommending the best management practices for increased and more sustainable latex yields in regions where rubber tree plantations are an important source of income.
4. CHAPTER 4: Diversity of root-associated arbuscular mycorrhizal fungal communities in a rubber tree plantation chronosequence in North-East Thailand

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See the “Authorship statement – chapter 4” (appendix 3) for details.
4.1. Abstract

Rubber tree (*Hevea brasiliensis*) is of major economic importance in Southeast Asia and for small land holders in Thailand in particular. Due to the high value of latex, plantations are expanding into unsuitable areas, such as the northeast province of Thailand where soil fertility is very low and therefore appropriate management practices are of primary importance. Arbuscular mycorrhizal fungi (AMF) contribute to plant growth through a range of mechanisms and could play a key role in a more sustainable management of the rubber plantations. We described the diversity of AMF associated with rubber tree roots in Northeast Thailand in relation to tree age and soil parameters along a chronosequence of rubber tree plantations. Cassava fields were included for comparison. Rubber tree and cassava roots harbored high diversity of AMF (111 Virtual Taxa, VT), including 20 novel VT. AMF VT richness per sample was consistently high (per site mean 16 to 21 VT per sample) along the chronosequence and was not related to soil properties. The composition of AMF communities differed between cassava and rubber tree plantations and was influenced by soil texture and nutrient content (sand, K, P, Ca). AMF community composition gradually shifted with the age of the trees. Our results suggest that the high diversity of AMF in this region is potentially significant for maintaining high functionality of AMF communities.

4.2. Introduction

Rubber tree (*Hevea brasiliensis*, Euphorbiaceae) is grown mainly for its ability to produce latex, which is used to manufacture a wide range of products, including tires, pipes, and hoses. It has been traditionally cultivated in the equatorial belt as well as in more humid tropical and monsoonal zones (Rao and Vijayakumar 1992). Although it grows well in a wide
range of edaphic and climatic conditions, areas of optimal growth and latex production are
classified by high soil fertility, adequate rainfall distribution (short dry season), and even
temperature distribution, with minor year-round fluctuation (Rao et al. 1998).

Thailand is the world’s leading producer of latex (approximately 3.2 million tons per year,
representing more than 25% of the world’s total production) and exports 90% of its
production overseas (http://faostat.fao.org). While rubber tree plantations covered about 2.7
million hectares in 2009, only 102,000 ha (less than 4%) had optimal soil properties
(Saengruksawong et al. 2012). Due to the high value of latex, plantations are expanding into
less suitable areas, such as the northeast province of Thailand, which used to be
predominantly covered by cassava (*Manihot esculenta*), another Euphorbiaceae species. In
this region, soils are mostly sandy and nutrient poor and the dry season can last up to 6
months.

Soil fertility, temperature, and the distribution of rainfall (and prolonged dry period in
particular) greatly affect both rubber tree growth and latex yield variability (Akpan et al.
2007; Rao et al. 1998). Moreover, monoculture of industrial crop species results in a
considerable depletion of soil fertility (Akpan et al. 2007) which is counteracted by
application of mineral fertilizers especially in the young rubber tree plantations when the
nutrient requirements of the trees are highest. High rates of mineral fertilizers are also applied
to increase the yields of other crops in the region, including cassava, but the extensive use of
fertilizers may lead to even more severe soil fertility depletion (Akpan et al. 2007; Orimoloye
et al. 2010). Identification and adoption of appropriate soil management practices to reduce
the use of mineral fertilizers and to maintain soil fertility in rubber tree plantations is therefore
of primary importance. In this context, soil microorganisms including arbuscular mycorrhizal
fungi (AMF) may play a key role, since they influence various important ecosystem functions in agricultural systems (Gianinazzi et al. 2010).

AMF may also provide non-nutritional benefits to the rubber trees such as protection from drought stress during the long dry season. There is experimental evidence for differential plant growth effects by AMF differently adapted to drought conditions (Symanczik et al. 2015). Furthermore, AMF communities in seasonally dry tropical forests show season-related shifts (Guadarrama et al. 2014). Thus, diversity of AMF in rubber plantations might confer different functional benefits to the host depending on water availability.

The occurrence of AMF in rubber tree roots has been reported in several field studies (Ikram et al. 1992; Pereira et al. 2014; Schwob et al. 1999; Tawaraya et al. 2003). Furthermore, rubber tree growth and leaf nutrient content respond positively to AMF inoculation (Ikram et al. 1992; Jayaratne et al. 1984; Sosa-Rodriguez et al. 2013). Rubber trees can exhibit high levels of AMF colonization regardless of soil conditions (Feldmann, 1994; Herrmann et al. 2016a), with little variation in relation to seasonal or climatic factors (Schwob et al. 1999).

The conversion of natural ecosystems into agro-ecosystems, especially long-term monocultures may reduce AMF diversity (Oehl et al. 2009; Verbruggen and Kiers 2010). As a part of this process, excessive use of mineral fertilizers or pesticides and land use intensification can significantly affect (both positively and negatively) AMF richness and diversity (Cardoso and Kuyper 2006; Hassan et al. 2013; van der Heijden et al. 2015; Van Geel et al. 2015). Similarly, rubber plantations (monocultures) have been shown to harbor lower AMF spore diversity than natural rubber tree stands, possibly due to management practices applied in the plantations, such as the use of pesticides or extensive weeding (Feldmann et al. 2000).
The diversity and dynamics of AMF communities in rubber tree plantations, through the plantation cycle, is poorly understood. Rubber trees are usually grown for 20 to 25 years before plantations are replaced. Changes in soil physical characteristics (soil bulk density and total porosity decrease) and nutrient content (soil exchangeable calcium, magnesium, and potassium decline) occur in plantations of different ages (Aweto, 1987), which are likely to influence soil microbial communities, including root-associated AMF communities.

Therefore, the objectives of our study were (i) to describe the diversity of AMF naturally present in mature rubber tree roots in Northeast Thailand, (ii) to compare rubber-associated AMF diversity with that present in a regionally abundant food crop cassava, and (iii) to assess the impact of tree age and soil parameters on AMF communities along a chronosequence of rubber tree plantations.

4.3. Material and methods

4.3.1. Site information

Roots and soils were sampled from a chronosequence of rubber tree plantations in the Baan Don Chang area (Khon Kaen District, NE Thailand). Cassava fields were sampled for comparison. Cassava is one of the major crops in the region and was the previously cultivated crop before the establishment of all the selected rubber plantations. Notably, both cassava and rubber tree belong to the family Euphorbiaceae. Khon Kaen District has a tropical savanna climate with long dry winters and rainy seasons that can last up to 6 months. Temperature and precipitation data were monitored for several years in the Khon Kaen Region. According to these records, temperature throughout the year varies between 18 and 35 °C, with average daily high and low temperatures of 34 and 22 °C, respectively, and a mean annual
temperature of 30 °C. Rainfall occurs from April to October, with mean annual precipitation of 1250 mm. The area is predominantly covered by croplands (95 %), with cassava, rice, and sugarcane being the major crops.

The rubber plantation chronosequence included plantations of three ages (3, 6, and 16 years old). In addition, nearby cassava fields with no history of rubber tree cultivation were sampled. Three plantations or fields were sampled for each site group. Thus, a total of 12 sites (nine rubber tree plantations and three cassava sites) were sampled. The distances between sites were less than 500 m, the total area was about 800 × 1400 m and the elevation varied from 184 to 201 m above sea level (Figure 1, Table 1). The rubber tree plantations had been cultivated with cassava and jute (*Corchorus capsularis*) for 20 to 40 years prior to rubber tree plantation. All plantations had been tilled between the tree lines twice a year (at the beginning and at the end of the rainy season) during the first 5 years of rubber tree growth. Chemical (N:P:K, 20:10:12) and organic fertilizers (manure) had been applied during the first 5 years, depending on availability.

4.3.2. Sampling

Nine trees of similar size were selected in the middle of each plantation. In August 2013, soils (0–30 cm deep) were collected 40 to 60 cm away from the trunk of each tree, on two opposite sides of the trunk. Soil samples collected from the rhizosphere of the same tree were pooled, sieved to pass a 1-mm mesh, air-dried, and stored at room temperature until analysis. The finest rubber tree roots were collected from the sieve, dried at 45 °C for a week, and then stored dry in a plastic bag with silica gel until analysis. Nine samples of roots and soils were also collected in each of the cassava sites using the same protocol.
4.3.3. Soil analyses

Chemical analyses were performed on five of the nine samples for each site. Soil analyses included pH (in H2O), organic matter (Walkley and Black 1934), P (Bray and Kurtz 1945, Bray II), N (Bremner, 1965), K, Ca and Mg (ammonium acetate pH 7) contents (Thomas, 1982), and texture (Kilmer and Alexander 1949).

4.3.4. DNA extraction from root samples

DNA was extracted from 70 mg (±5 mg) of dry roots using the MoBio PowerSoil (MO BIO Laboratories, Inc., Carlsbad, CA, USA) kit with modifications following Öpik et al. (2014) and Saks et al. (2014). Briefly, prior to the extraction, roots were milled in 2 ml centrifuge tubes with four tungsten carbide beads for 3 runs of 2 min. Bead solution (750 µl) was added to the tubes and the mixture was transferred to the bead-plate. To increase the DNA yield, bead-plates were incubated at 60°C for 10 min under agitation (150 rpm) to allow mechanical cell lysis. To maintain DNA concentration but increase DNA yield, the final elution was performed twice with 75 µl of elution buffer.

4.3.5. 454 sequencing

Based on the results of the DNA extraction (yield and quality), 6 of the 9 root DNA samples for each site were selected for AM fungal identification via 454 sequencing (total of 72 samples).

Glomeromycota sequences were amplified from root DNA extracts using the small-subunit (SSU) rRNA gene primers NS31 (5’-TTGGAGGGCAAGTCTGGTGCC-3’) and AML2 (5’-GAACCCAAACACTTTGGTTTCC-3’) (Lee et al. 2008; Simon et al. 1992), linked to 454-
sequencing adaptors A and B, respectively. To identify sequences originating from individual samples, a set of 8 bp barcodes designed following Parameswaran et al. (2007) was used. The barcode sequences were inserted between each adaptor and its associated primer sequence. The composite forward primer was 5’-GTCTCCGACTCAG (NNNNNNNNN) TTGGAGGCAAGTCTGGTGCC-3’ and the reverse primer was 5’-TTGGCAGTCTCAG (NNNNNNNNN) GAACCCAAACACTTTGGTTCC-3’, where the A and B adaptors are underlined, the barcode is indicated by Ns in parentheses, and the specific primers NS31 and AML2 are shown in italics. The amplification was done using a two-step PCR, in a final volume of 10 µl containing 1 µl of template DNA, 0.2 µM of each primer and 5 µl of Qiagen HotStarTaq Master Mix (Qiagen Gmbh, Hilden, Germany). During the first PCR, primers with truncated adaptors were used to optimize amplification. The 10x diluted products of the first PCR reaction were used in the second PCR with A and B adaptors as primers. The reactions were run on a thermal cycler (2720; Applied Biosystems, Foster City, California, USA) under the following conditions: 95°C for 15 min, five cycles of 42°C for 30 s, 72°C for 90 s, 92°C for 45 s; 35 cycles (first PCR) or 20 cycles (second PCR) of 65°C for 30 s, 72°C for 90 s, 92°C for 45 s; followed by 65°C for 30 s and 72°C for 10 min. Sample preparation for sequencing reactions was performed by BiotaP. Ltd (Tallinn, Estonia). PCR products were separated by electrophoresis on 1.5% agarose gel in 0.5× TBE, purified from the gel using the Qiagen QIAquick Gel Extraction kit (Qiagen GmbH) and quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). A total of 2 µg of the resulting DNA mix was sequenced on a Genome Sequencer FLX System using Titanium Series reagents (Roche Applied Science, Mannheim, Germany) at Microsynth AG (Switzerland).
4.3.6. Bioinformatics and phylogenetic analysis

454-sequencing reads were retained only if they carried the correct barcode and forward primer sequences, had an average quality score >25 and were ≥170 and ≤520 bp long, excluding the barcode and primer sequences (Davison et al. 2012). Chimeric sequences were detected and removed using UCHIME (Edgar et al. 2011) in reference database mode (using the MaarjAM database – see below) using the default settings.

After stripping the barcode and primer sequences, obtained reads were assigned to virtual taxa (VT) via BLAST against the MaarjAM database (version April 2014, which contained 5264 sequences and 348 virtual taxa) of Glomeromycota SSU rRNA gene sequences (Öpik et al. 2010) using an open reference operational taxonomic unit picking approach (Bik et al. 2012). The following criteria were used for BLAST search: sequence similarity ≥ 97%; the alignment length ≥95% of the shorter of the query (pyrosequencing read) and subject (reference database sequence) sequences; and a BLAST e-value < 1e-50. The best hit on the basis of the BLAST score was recorded for each read. Sequence reads not receiving a match against the MaarjAM database were subjected to a further BLAST search against the non-redundant International Nucleotide Sequence Database (INSD) using lowered thresholds: sequence similarity ≥90% and alignment length ≥90% of the shorter of the sequences.

Phylogenetic analyses were performed to determine whether putatively Glomeromycotan sequences identified in the INSD BLAST constituted novel VT (Öpik et al. 2010). Such sequences were clustered with 99% similarity using BLASTclust (Altschul et al. 1990), and up to four sequences from each cluster were aligned with all sequences available in MaarjAM using the MAFFT multiple sequence alignment web service implemented in JALVIEW version 2.8 (Waterhouse et al. 2009). Neighbor-joining phylogenetic analysis was implemented in TOPALi v2.5 (Milne et al. 2009). The longest high-quality sequence
(containing no ambiguous nucleotides) of each novel VT was chosen as the representative sequence and added to the reference sequence dataset. Finally, a second BLAST with the original full read set was performed against this updated reference sequence set. A set of representative sequence reads (longest high-quality sequences) has been deposited in the EMBL nucleotide collection (accession numbers LT216621-LT217529).

Read filtering, removal of primer and barcode sequences and parsing of BLAST output was carried out using a series of Python and Java scripts developed at the Department of Botany, University of Tartu, as in Davison et al. (2012). The Glomeromycota nomenclature used here follows Redecker et al. (2013) except that genus *Glomus* is treated sensu lato for the reasons provided in Öpik et al. (2014). Global singletons (12 VT represented once in the data set) and samples yielding fewer than 100 sequences were omitted from the final dataset, leaving a data matrix consisting of 57 samples and 138733 sequences.

4.3.7. Statistical analyses

Sampling efficacy was assessed with rarefaction analysis and species accumulation curves, using the functions `rarefy` and `specaccum` from the R package Vegan (Oksanen et al. 2012). For subsequent analyses, sequencing depth per sample was standardized by retaining a random selection of reads in each sample, where the number retained corresponded to the median read count across all samples (2644 reads). This approach has been shown to represent an optimal approach for reducing bias due to differences in sample size while retaining information (de Cárcer et al. 2011).

Linear mixed-effects models (LME) (Pinheiro et al. 2013) were used to test for differences in AMF richness and diversity indices (Shannon, Simpson and Evenness) in the different groups.
of plantations: cassava vs rubber tree; and between the three different age groups in rubber. The Shannon index ($H'$) was exponentially transformed, resulting in a variable (exp$H'$) that, in contrast to the untransformed index, satisfies the replication principle and can be considered a linear representation of diversity (Jost, 2006). Plantation identity was included in models as a random effect.

Analysis of AMF community structure was performed using quantitative data, where proportions of reads representing different VT were used as a proxy for the relative abundance of the AMF taxa in a sample (Moora et al. 2014). Differences in AMF communities associated with different host species (cassava vs rubber tree) or age groups (3, 6 and 16 year-old rubber plantations) were tested using a nested PERMANOVA, with the nested.npmanova() function (999 permutations) from the BiodiversityR package (Kindt and Coe 2005), to account for the nested study design. Two dimensional NMDS (function metaMDS from R package Vegan, 50 iterations) was used to explore variation in AMF community composition. Ellipses representing standard deviations around group centroids were defined using the ordiellipse function from the R package Vegan.

Indicator species analysis was used to investigate the strength of pairwise associations between plant species and AMF VT (function indval from R package labdsv; (Robert, 2012)). To test the significance of observed indicator values, we recalculated 999 values following permutation of blocks of samples corresponding to entire plantations. Significant VT with an indicator value of at least 0.25 were considered as good indicator taxa.

Soil chemical and physical variables were ln transformed before they were included in linear mixed-effects models (LME) (Pinheiro et al. 2013) to test for differences between the different species and plantation ages. Pearson’s correlation was conducted on ln transformed data to assess whether AMF diversity indices were related to soil parameters. The effect on
AMF community structure of soil variables was tested using redundancy analysis (RDA). AMF community data were chord transformed (Legendre and Gallagher 2001), and all explanatory soil variables were entered into the model in the order determined by a stepwise selection procedure (Blanchet et al. 2008). The significance of individual soil variables was determined using sequential ANOVA and restricted permutation (999 iterations) following the block-permutation procedure described above.

4.4. Results

4.4.1. Soil characteristics

Soils in all sites were mostly sandy in texture (>80%), acidic (pH 4.7-5.5) and had low organic matter (< 0.5%) and macro element (N, P, K, Ca, Mg) content (Table 8). Cassava soils contained more silt (P = 0.02) and less sand (P = 0.04) than the rubber plantations but otherwise had similar chemical characteristics. The 3 year-old rubber tree plantations tended to have higher P and N content than the other site types (P = 0.12 and 0.16, respectively). Soils in 6 year-old plantations had lower pH (P = 0.04) and Ca (P = 0.05) levels and tended to have lower Mg levels (P = 0.09) than those sampled from the 3 and 16 year-old plantations. The 16 year-old plantations tended to contain a higher level of organic matter than the other site types (P = 0.18).
Table 8. Soil characteristics of the groups of study sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>pH (H$_2$O) (± standard error)</th>
<th>OM (%) (± standard error)</th>
<th>P (Bray II) (mg/kg) (± standard error)</th>
<th>Total N (%) (± standard error)</th>
<th>K (mg/kg) (± standard error)</th>
<th>Ca (mg/kg) (± standard error)</th>
<th>Mg (mg/kg) (± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubber</td>
<td>5.20 ± 0.54</td>
<td>0.36 ± 0.11</td>
<td>14.20 ± 15.85</td>
<td>0.045 ± 0.02</td>
<td>23.92 ± 10.30</td>
<td>129.16 ± 124.88</td>
<td>16.40 ± 16.84</td>
</tr>
<tr>
<td>Cassava</td>
<td>4.94 ± 0.25</td>
<td>0.35 ± 0.14</td>
<td>10.18 ± 8.35</td>
<td>0.037 ± 0.01</td>
<td>23.09 ± 9.17</td>
<td>106.00 ± 60.38</td>
<td>11.82 ± 7.28</td>
</tr>
<tr>
<td>3 year-old</td>
<td>5.51 ± 0.44</td>
<td>0.28 ± 0.05</td>
<td>25.43 ± 25.57</td>
<td>0.059 ± 0.03</td>
<td>22.29 ± 8.32</td>
<td>93.86 ± 52.05</td>
<td>30.57 ± 25.31</td>
</tr>
<tr>
<td>6 year-old</td>
<td>4.71 ± 0.21</td>
<td>0.34 ± 0.12</td>
<td>11.90 ± 6.47</td>
<td>0.043 ± 0.01</td>
<td>19.60 ± 3.86</td>
<td>58.70 ± 23.54</td>
<td>6.60 ± 3.60</td>
</tr>
<tr>
<td>16 year-old</td>
<td>5.55 ± 0.45</td>
<td>0.45 ± 0.07</td>
<td>7.25 ± 7.63</td>
<td>0.036 ± 0.02</td>
<td>30.75 ± 14.23</td>
<td>248.13 ± 160.99</td>
<td>16.25 ± 8.28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site</th>
<th>Clay (%) (± standard error)</th>
<th>Silt (%) (± standard error)</th>
<th>Sand (USDA classification)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Very fine (%)</td>
<td>Fine (%)</td>
<td>Medium (%)</td>
</tr>
<tr>
<td>Rubber</td>
<td>3.94 ± 1.21</td>
<td>9.93 ± 1.27</td>
<td>37.41 ± 2.81</td>
</tr>
<tr>
<td>Cassava</td>
<td>3.91 ± 1.36</td>
<td>12.45 ± 3.94</td>
<td>34.25 ± 2.24</td>
</tr>
<tr>
<td>3 year-old</td>
<td>4.43 ± 1.77</td>
<td>9.90 ± 1.54</td>
<td>37.66 ± 3.72</td>
</tr>
<tr>
<td>6 year-old</td>
<td>3.30 ± 0.35</td>
<td>10.14 ± 1.12</td>
<td>38.50 ± 2.47</td>
</tr>
<tr>
<td>16 year-old</td>
<td>4.31 ± 1.10</td>
<td>9.69 ± 1.33</td>
<td>35.84 ± 1.66</td>
</tr>
</tbody>
</table>

Mean ± standard error values for the 3 sites (7 to 11 observations) of each age group. Plantations of different age were combined (9 sites, 25 observations) to compare rubber and cassava species.

Means accompanied by the same letter do not differ significantly $P \leq 0.05$ (pairwise comparisons using the Tukey (HSD) test).
4.4.2. AMF sequencing data
A total of 277,796 quality filtered reads were obtained from 69 samples. After removal of chimeras (1.5% of reads), singleton VT and samples with low numbers of reads (< 100 reads), a total of 57 samples and 138,733 Glomeromycota reads were retained. These were classified into 111 AMF VT in ten genera and eight families, including 20 novel VT (Table 9, Figure S1 presented in Appendix 4). Novel VT were mainly *Glomus* taxa (9), but VT belonging to other genera (*3 Acaulospora, 3 Archaeospora, 2 Scutellospora, 2 Paraglomus*, and 1 *Claroideoglomus*) were also identified. Novel VT represented 18% of the VT and 1.8% of reads. They were found in all plantation types, but in a larger proportion in cassava fields (11 of 20 VT) and young plantations (9 and 8 of 20 VT in the 3 and 6 year-old plantations, respectively). Only 4 of the 20 novel VT were found in the 16 year-old plantations (data not shown).

Rarefaction curves showed that sequencing depth per sample was generally sufficient to describe the AMF diversity in our study sites but that additional sampling might detect some more taxa within rubber tree age or species groups (Figure S2 presented in Appendix 5). After standardization to the median number of sequences per sample (2644 reads), at least 4 samples per site were retained for the downstream analyses.

4.4.3. AMF richness and diversity
A minimum of 4 and a maximum of 38 AMF VT were found per sample, with an average of 18 VT per sample across all sites (Table 9). The richness of VT per sample did not differ significantly among site types (P = 0.19); however, highest mean AMF richness was found in the cassava sites (21 VT per sample) and the lowest in the 16 year-old plantations (average of 16 VT per sample, Table 10).
Table 9. Details of AMF sequence reads and numbers of virtual taxa (VT) obtained from the study sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Species</th>
<th>Age</th>
<th>No of samples</th>
<th>Average no of AMF reads per sample</th>
<th>Total no of AMF reads</th>
<th>Average no of AMF reads per sample</th>
<th>Total no of AMF reads</th>
<th>Total no of VT per site</th>
<th>Average no of VT per sample</th>
<th>Min no of VT per sample</th>
<th>Max no of VT per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A</td>
<td>Rubber</td>
<td>3</td>
<td>5</td>
<td>2250</td>
<td>11248</td>
<td>1848</td>
<td>9240</td>
<td>42</td>
<td>14</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>3B</td>
<td>Rubber</td>
<td>3</td>
<td>5</td>
<td>3902</td>
<td>19512</td>
<td>2672</td>
<td>13360</td>
<td>49</td>
<td>21</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>3C</td>
<td>Rubber</td>
<td>3</td>
<td>4</td>
<td>2205</td>
<td>8820</td>
<td>1527</td>
<td>6108</td>
<td>23</td>
<td>13</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>6A</td>
<td>Rubber</td>
<td>6</td>
<td>4</td>
<td>1911</td>
<td>7644</td>
<td>1828</td>
<td>7312</td>
<td>40</td>
<td>21</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>6B</td>
<td>Rubber</td>
<td>6</td>
<td>5</td>
<td>1620</td>
<td>8098</td>
<td>1588</td>
<td>7942</td>
<td>60</td>
<td>25</td>
<td>15</td>
<td>38</td>
</tr>
<tr>
<td>6C</td>
<td>Rubber</td>
<td>6</td>
<td>4</td>
<td>1057</td>
<td>4229</td>
<td>817</td>
<td>3269</td>
<td>31</td>
<td>11</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>16A</td>
<td>Rubber</td>
<td>16</td>
<td>4</td>
<td>1288</td>
<td>5153</td>
<td>1197</td>
<td>4789</td>
<td>39</td>
<td>17</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>16B</td>
<td>Rubber</td>
<td>16</td>
<td>4</td>
<td>1655</td>
<td>6619</td>
<td>1629</td>
<td>6514</td>
<td>48</td>
<td>19</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>16C</td>
<td>Rubber</td>
<td>16</td>
<td>4</td>
<td>1588</td>
<td>6351</td>
<td>886</td>
<td>3542</td>
<td>26</td>
<td>11</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>CassA</td>
<td>Cassava</td>
<td>-</td>
<td>6</td>
<td>3949</td>
<td>23694</td>
<td>2538</td>
<td>15230</td>
<td>58</td>
<td>24</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>CassB</td>
<td>Cassava</td>
<td>-</td>
<td>6</td>
<td>2915</td>
<td>17487</td>
<td>1902</td>
<td>11412</td>
<td>53</td>
<td>24</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>CassC</td>
<td>Cassava</td>
<td>-</td>
<td>6</td>
<td>3313</td>
<td>19878</td>
<td>2355</td>
<td>14128</td>
<td>50</td>
<td>20</td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>57</td>
<td>2434</td>
<td>138733</td>
<td>1804</td>
<td>102846</td>
<td>111</td>
<td>18</td>
<td>4</td>
<td>38</td>
</tr>
</tbody>
</table>

Standardization by retaining a random selection of reads in each sample, where the number retained corresponds to the median read count across all samples (2644 reads)
Table 10. Measures of AMF VT diversity indices by crop species and rubber tree age groups.

<table>
<thead>
<tr>
<th>Species</th>
<th>Age</th>
<th>Richness</th>
<th>Exponential Shannon $\text{exp}(H')$</th>
<th>Inverse Simpson $1/D$</th>
<th>Equitability (Evenness-Piérou)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubber</td>
<td>-</td>
<td>17.03 ±7.97</td>
<td>4.27 ±2.47</td>
<td>2.97 ±1.60</td>
<td>0.47 ±0.21</td>
</tr>
<tr>
<td>Cassava</td>
<td>-</td>
<td>21.06 ±9.18</td>
<td>3.57 ±1.81</td>
<td>2.45 ±1.29</td>
<td>0.39 ±0.16</td>
</tr>
<tr>
<td>Rubber</td>
<td>3</td>
<td>16.21 ±7.50</td>
<td>3.35 ±1.36</td>
<td>2.42 ±1.04</td>
<td>0.42 ±0.18</td>
</tr>
<tr>
<td>Rubber</td>
<td>6</td>
<td>19.15 ±9.97</td>
<td>5.18 ±2.98</td>
<td>3.46 ±1.77</td>
<td>0.50 ±0.22</td>
</tr>
<tr>
<td>Rubber</td>
<td>16</td>
<td>15.67 ±6.04</td>
<td>4.36 ±2.67</td>
<td>3.06 ±1.87</td>
<td>0.49 ±0.25</td>
</tr>
</tbody>
</table>

Means ±standard error values for 12 to 18 samples of each group. Plantations of different age were combined to compare rubber and cassava species. Pairwise comparisons of the means using the Tukey (HSD) test did not show any significant difference at $P \leq 0.05$. 

The total number of VT per site ranged from 23 to 60 (Table 9). Among the 111 VT recovered from plant roots, 95 and 87 were found in rubber tree and cassava samples, respectively. A large number of VT (71 VT, 96.8% of reads) colonized both rubber and cassava roots and 30 VT (89.5% of reads) were found in samples from all studied groups (Figure 11). Root AMF communities were dominated by *Glomus* species (65-96% of reads) in all sites, followed by *Acaulospora* (11.3% of reads in total). Other genera, such as *Scutellospora*, *Gigaspora* and *Diversispora*, constituted on average 2% of the total number of reads (Figure 12).

AMF diversity indices did not differ significantly between crop species or in relation to rubber tree age (Table 10). The inverse Simpson index (1/D), exponential Shannon index (exp H’) and evenness were strongly negatively correlated with pH (P < 0.01), and more weakly negatively correlated with the Ca and Mg content of soil (P < 0.05). Richness tended to be negatively correlated to soil P content (r = -0.31, P = 0.07) while the exponential Shannon (exp H’) and inverse Simpson (1/D) indices tended to be negatively correlated to the clay content in soils (r = -0.30 and -0.32, and P = 0.08 and 0.06, respectively) (Table 11).

4.4.4. AMF community patterns

PERMANOVA indicated that the AMF communities associated with rubber trees and cassava plants differed significantly (P = 0.03), but that there were no significant differences between the communities retrieved from rubber plantations of different ages (P = 0.17). NMDS solution suggested a gradual shift in AMF community composition along the first axis, from cassava fields to the oldest rubber plantations (Figure 13).
Figure 11. Venn diagram showing numbers of VT shared between the 3 ages of rubber tree plantation (A) and between cassava and rubber (B).
Figure 12. Composition of AMF communities (proportions of VT) at genus (A and B) and family (C and D) level in the roots of rubber tree and cassava in different study sites (A and C) and site types (B and D).
Figure 13. Non metric multi-dimensional scaling (NMDS) plots displaying AMF communities detected in the roots of rubber tree or cassava roots.

Ellipses indicate one standard deviation around the centroid position of each rubber tree age group or cassava.
Table 11. Pearson’s correlation (r values) between the soil characteristics and AMF diversity and richness indices.

<table>
<thead>
<tr>
<th>r values</th>
<th>ln pH</th>
<th>ln OM</th>
<th>ln P</th>
<th>ln K</th>
<th>ln Ca</th>
<th>ln Mg</th>
<th>ln N</th>
<th>ln Sand</th>
<th>ln Silt</th>
<th>ln Clay</th>
<th>Richness</th>
<th>I/D</th>
<th>exp(H’)</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln pH</td>
<td>0.758</td>
<td>0.557</td>
<td>0.416</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.485</td>
<td>0.191</td>
<td>0.034</td>
<td>0.252</td>
<td>0.178</td>
<td>0.003</td>
<td>0.005</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>ln OM</td>
<td>-0.05</td>
<td>0.869</td>
<td>0.064</td>
<td>0.001</td>
<td>0.056</td>
<td>0.657</td>
<td>0.009</td>
<td>0.019</td>
<td>0.352</td>
<td>0.133</td>
<td>0.676</td>
<td>0.865</td>
<td>0.325</td>
<td></td>
</tr>
<tr>
<td>ln P</td>
<td>0.10</td>
<td>-0.03</td>
<td>0.210</td>
<td>0.851</td>
<td>0.627</td>
<td>0.442</td>
<td>0.915</td>
<td>0.781</td>
<td>0.449</td>
<td>0.071</td>
<td>0.728</td>
<td>0.465</td>
<td>0.868</td>
<td></td>
</tr>
<tr>
<td>ln K</td>
<td>0.14</td>
<td>0.31</td>
<td>0.21</td>
<td>0.036</td>
<td>0.007</td>
<td>0.221</td>
<td>0.275</td>
<td>0.282</td>
<td>0.532</td>
<td>0.238</td>
<td>0.343</td>
<td>0.348</td>
<td>0.356</td>
<td></td>
</tr>
<tr>
<td>ln Ca</td>
<td><strong>0.66</strong></td>
<td><strong>0.53</strong></td>
<td>-0.03</td>
<td><strong>0.35</strong></td>
<td>0.001</td>
<td>0.171</td>
<td>0.408</td>
<td>0.994</td>
<td>0.153</td>
<td>0.109</td>
<td>0.027</td>
<td>0.037</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>ln Mg</td>
<td><strong>0.51</strong></td>
<td>0.32</td>
<td>0.08</td>
<td><strong>0.44</strong></td>
<td><strong>0.54</strong></td>
<td>0.179</td>
<td>0.297</td>
<td>0.329</td>
<td>0.641</td>
<td>0.132</td>
<td>0.041</td>
<td>0.042</td>
<td>0.044</td>
<td></td>
</tr>
<tr>
<td>ln N</td>
<td>0.12</td>
<td>-0.08</td>
<td>0.13</td>
<td>-0.21</td>
<td>-0.23</td>
<td>0.23</td>
<td>0.140</td>
<td>0.469</td>
<td>0.020</td>
<td>0.486</td>
<td>0.784</td>
<td>0.679</td>
<td>0.955</td>
<td></td>
</tr>
<tr>
<td>ln Sand</td>
<td>0.22</td>
<td>-<strong>0.43</strong></td>
<td>0.02</td>
<td>-0.19</td>
<td>-0.14</td>
<td>-0.18</td>
<td>0.25</td>
<td>&lt;0.001</td>
<td>0.009</td>
<td>0.371</td>
<td>0.219</td>
<td>0.190</td>
<td>0.721</td>
<td></td>
</tr>
<tr>
<td>ln Silt</td>
<td>-<strong>0.35</strong></td>
<td><strong>0.39</strong></td>
<td>-0.05</td>
<td>0.18</td>
<td>-0.00</td>
<td>0.17</td>
<td>-0.12</td>
<td><strong>-0.87</strong></td>
<td>0.951</td>
<td>0.769</td>
<td>0.577</td>
<td>0.467</td>
<td>0.722</td>
<td></td>
</tr>
<tr>
<td>ln Clay</td>
<td><strong>0.20</strong></td>
<td>0.16</td>
<td>0.13</td>
<td>0.11</td>
<td>0.24</td>
<td>0.08</td>
<td><strong>-0.39</strong></td>
<td><strong>-0.43</strong></td>
<td>-0.01</td>
<td>0.391</td>
<td>0.061</td>
<td>0.081</td>
<td>0.484</td>
<td></td>
</tr>
<tr>
<td>ln Clay</td>
<td><strong>-0.23</strong></td>
<td>-0.26</td>
<td>-0.31</td>
<td>-0.20</td>
<td>-0.27</td>
<td>-0.26</td>
<td>-0.12</td>
<td>0.15</td>
<td>-0.05</td>
<td>-0.15</td>
<td>0.011</td>
<td>&lt;0.001</td>
<td>0.464</td>
<td></td>
</tr>
<tr>
<td>ln Clay</td>
<td><strong>-0.48</strong></td>
<td>0.07</td>
<td>-0.06</td>
<td>-0.16</td>
<td><strong>-0.37</strong></td>
<td><strong>-0.34</strong></td>
<td>-0.05</td>
<td>0.21</td>
<td>-0.10</td>
<td>-0.32</td>
<td><strong>0.42</strong></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>ln Clay</td>
<td><strong>-0.46</strong></td>
<td>0.03</td>
<td>-0.13</td>
<td>-0.16</td>
<td><strong>-0.35</strong></td>
<td><strong>-0.34</strong></td>
<td>-0.07</td>
<td>0.22</td>
<td>-0.13</td>
<td>-0.30</td>
<td><strong>0.57</strong></td>
<td><strong>0.96</strong></td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>ln Clay</td>
<td><strong>-0.47</strong></td>
<td>0.17</td>
<td>0.03</td>
<td>-0.16</td>
<td>-0.32</td>
<td><strong>-0.34</strong></td>
<td>-0.01</td>
<td>0.06</td>
<td>-0.06</td>
<td>-0.12</td>
<td>0.13</td>
<td><strong>0.87</strong></td>
<td><strong>0.78</strong></td>
<td></td>
</tr>
</tbody>
</table>

Significant correlations are shown in bold. *: different from 0 with a significance level α = 0.05; **: different from 0 with a significance level α = 0.01.

r and p values are indicated in the lower and upper halves of the table, respectively.
Redundancy analysis (RDA) of AMF communities with axes constrained by soil parameters (constrained inertia = 42%) showed a clear separation of rubber tree plantations of different ages along the first axis; and of cassava sites and rubber plantations along the second axis (Figure 14). Cassava samples were associated with high silt content, while rubber tree samples correlated with higher soil nutrient concentrations (except K) and higher sand and organic matter content. Rubber plantation age was correlated with increasing K and decreasing clay content. Two VT (Glomus VT403 and Glomus VT280) had a strong influence on the ordination according to their position in relation to the ordination equilibrium circle (Legendre and Legendre 1998). One of these, Glomus VT403, correlated strongly with cassava.

Indicator species analysis identified 5 and 8 VT as indicators for the different groups of rubber tree and cassava plantations, respectively. Indicator VT were also identified for the 3 and 16 year-old plantations (Table 12). Most of the indicator VT were Glomus (17 out of 24). Non-Glomus VT were identified as Scutellospora (3 VT), Acaulospora (3 VT) and Claroideoglomus (1 VT). All Scutellospora VT were found to be the best 3 indicators of the 3 year-old. Three the novel VT were indicators for either rubber tree or cassava sites (Table 12).
Figure 14. Redundancy analysis (RDA) of AMF communities associating with rubber tree and cassava roots, constrained by variables describing soil chemistry and texture.

Ellipses indicate one standard deviation around the centroid position of each rubber tree age group or cassava.

The direction of maximum correlation between environmental variables and (linear combination) ordination scores is shown by biplot arrows.

Variables and arrows shown in red and marked with an asterisk were significant contributors to the constrained ordination (P < 0.1) in a sequential permutation test, where the order of terms in the model was determined by a stepwise model selection procedure (function ordistep in R package Vegan).

The species scores of VT falling outside the equilibrium circle were marked in blue.
Table 12. AMF indicator species (VT) for study site groups.

<table>
<thead>
<tr>
<th>Species</th>
<th>Age</th>
<th>Virtual Taxon</th>
<th>Identity</th>
<th>Indicator value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubber</td>
<td>-</td>
<td>VT399</td>
<td><em>Glomus sp.</em></td>
<td>0.584</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT227</td>
<td><em>Acaulospora sp.</em></td>
<td>0.513</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT90</td>
<td><em>Glomus clarum, manihotis</em></td>
<td>0.505</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT124</td>
<td><em>Glomus sp.</em></td>
<td>0.382</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT99</td>
<td><em>Glomus proliferum</em></td>
<td>0.382</td>
<td>0.031</td>
</tr>
<tr>
<td>Cassava</td>
<td>-</td>
<td>VT28</td>
<td><em>Acaulospora sp.</em></td>
<td>0.787</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT403</td>
<td><em>Glomus sp.</em></td>
<td>0.718</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT312</td>
<td><em>Glomus sp.</em></td>
<td>0.643</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT248</td>
<td><em>Glomus sp.</em></td>
<td>0.525</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT108</td>
<td><em>Glomus sp.</em></td>
<td>0.511</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glomus.LH.Gl09</td>
<td><em>Glomus sp.</em></td>
<td>0.461</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT146</td>
<td><em>Glomus sp.</em></td>
<td>0.389</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT247</td>
<td><em>Glomus sp.</em></td>
<td>0.369</td>
<td>0.011</td>
</tr>
<tr>
<td>Rubber</td>
<td>3</td>
<td>VT255</td>
<td><em>Scutellospora dipapillosa, heterogama, reticulata</em></td>
<td>0.504</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT41</td>
<td><em>Scutellospora castanea, fulgida, gilmorei, gregaria, persica, weresubiae</em></td>
<td>0.457</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT96</td>
<td>Scutellospora.LH.Sc01</td>
<td><em>Scutellospora sp.</em></td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT80</td>
<td><em>Glomus sp.</em></td>
<td>0.411</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT222</td>
<td><em>Glomus sp.</em></td>
<td>0.328</td>
<td>0.008</td>
</tr>
<tr>
<td>Rubber</td>
<td>6</td>
<td>VT270</td>
<td><em>Glomus sp.</em></td>
<td>0.511</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glomus.LH.Gl07</td>
<td><em>Glomus sp.</em></td>
<td>0.492</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT76</td>
<td><em>Glomus sp.</em></td>
<td>0.455</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT96</td>
<td><em>Glomus sp.</em></td>
<td>0.421</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT28</td>
<td><em>Acaulospora sp.</em></td>
<td>0.343</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VT193</td>
<td><em>Claroideoglomus claroideum, etunicatum, lamellosum, luteum, viscosum</em></td>
<td>0.302</td>
<td>0.041</td>
</tr>
</tbody>
</table>
4.5. Discussion

Our results showed that rubber tree plantations and cassava fields can harbor highly diverse AMF communities (total 111 VT, 23-60 VT per site, including 20 novel VT), dominated by *Glomus* and *Acaulospora* taxa. A large proportion of the identified AMF colonized both cassava and rubber tree roots (64%), and 27% of the VT were found in all site types. AMF richness and diversity did not differ along the rubber tree chronosequence and were influenced by soil parameters to a small degree. However, AMF communities differed between cassava and rubber trees and were influenced by soil characteristics (sand, P, Ca, K and N contents). The composition of the AMF communities gradually shifted with the age of the rubber trees, and was probably mostly affected by the change in edaphic conditions along the chronosequence.

4.5.1. High diversity of AMF, including 20 novel VT

A total of 111 VT were found in our study, which is a relatively high number of AMF species per study system. Similar total species number was recorded in temperate apple orchards (Van Geel et al. 2015), but only 36 OTUs were found to be associated with coffee (De Beenhouwer et al. 2015) and a maximum of 66 VT were obtained in a study conducted on a chronosequence of breadfruit (Hart et al. 2014). In our study, average richness per sample was 18 VT, and average richness per site was 43, which is in a similar range to those recorded from other tree-focused studies (Hart et al. 2014; Van Geel et al. 2015). Only 30 AMF morphospecies were found in a rubber tree plantation in Brazil (Pereira et al. 2014) but this is likely an underestimate of the actual AMF richness of the system, because of the omission of species currently not sporulating.
Of the 111 VT found in our sites, 18% had not been previously described. Most research on the diversity and distribution of Glomeromycota has been conducted in Europe and North America (Öpik et al. 2013); other regions, including South East Asia, have been less well studied and are prone to harbour yet undescribed Glomeromycota species. Recent AMF studies from Thailand reported several novel AMF VT (Chaiyasen et al. 2014; Watanarojanaporn et al. 2013), but to our knowledge no other studies have investigated the diversity of root-associated AMF in rubber tree plantations and cassava fields in SE Asia. These results, together with those from our study suggest that further novel AMF species probably remain to be described in this region.

A large proportion of recorded AMF VT were found in both rubber tree plantations and cassava fields (64%). AMF are often considered predominantly generalists with some AMF communities shown to be more responsive to land use and soil type than to plant species composition in some systems (Oehl et al. 2010). Furthermore, geographic distance (dispersal limitation) is an important driver of AMF communities at some spatial scales (Davison et al. 2015). The high number of VT shared by rubber trees and cassava plants in this study may thus reflect the fact that the 12 sites of this study were located in a limited area (less than 2 km²) on similar soils. Rubber tree plantations had been established on former cassava fields. The large proportion of shared AMF can also reflect the earlier land use and suggest that shift from cassava cropping to rubber plantation had a moderate effect on the AMF assemblages, maintaining a large proportion of the earlier AMF diversity.

4.5.2. Glomus VT were the most dominant, followed by Acaulospora

*Glomus* taxa dominated in all samples. This is in accordance with other studies in different ecosystems and disturbance levels (De Beenhouwer et al. 2015; Hart et al. 2014; Öpik et al. 2013).
In addition, Glomeraceae taxa are thought to be resistant to many kinds of disturbance (Chagnon et al. 2013) and, as a result, are common in tropical and arid areas (Camenzind et al. 2014; Stutz et al. 2000).

Acaulosporaceae was the second most dominant family, while VT from other families, such as Diversisporaceae or Gigasporaceae, were almost absent in all studied sites. These observations are consistent with results obtained in a rubber tree plantation in Brazil (Pereira et al. 2014) as well as in other tree plantations in tropical regions (Cuenca and Meneses 1996; De Beenhouwer et al. 2015; Hart et al. 2014; Liu et al. 2009). Glomus and Acaulospora species have been shown to be more competitive in the root infection process because they are equally able to colonize roots from spores, root fragments, or hyphae, whereas root colonization by Gigaspora or Scutellospora is only successful from spores (Klironomos and Hart 2002). Acaulospora taxa also establish better in acidic soils, as are present in the Baan Don Chang region (Pereira et al. 2014; Veresoglou et al. 2013).

4.5.3. Richness and diversity indices

Sample- and site-based AMF richness did not change significantly with the plantation age and was only slightly correlated with P content. Richness was consistently high despite the higher levels of fertilizer application at the establishment of the plantations. This is somewhat surprising since several studies have recorded significant reductions in AMF richness in response to increased soil fertility (Camenzind et al. 2014; Van Geel et al. 2015). Our result could be related to the low realized soil fertility and overall relatively small differences in soil parameters. However, the constantly high richness is noteworthy and may also reflect a high number of AMF species that have adapted to the mechanical disturbance levels and fertilizer loads used in the region for long periods of time.
Diversity indices were strongly negatively correlated with soil pH and Ca and Mg content whereas there was no correlation with P and N content. High P and N levels in soils have often been found detrimental for AMF diversity (Alguacil et al. 2010; Borriello et al. 2015; Liu et al. 2012) but neutral effects have also been reported (Van Geel et al. 2015). A recent study conducted on these same sites showed a similar neutral relationship between the nutrient content of soils and the intensity of mycorrhization, possibly because the levels of P and N in soils remained too low to influence colonization of plant roots by mycorrhizal fungi even after application of fertilizers (Herrmann et al. 2016a). Soil Ca and Mg contents have been earlier reported to affect AMF positively (Gryndler et al. 1992) or negatively (Jarstfer et al. 1998). It has been suggested that Ca and Mg ions could suppress germination of spores and growth due to their role in regulation of osmotic pressure and pH.

High AMF richness early after the establishment of the rubber plantations shows that the change in land use had only moderate effect on AMF. Secondary succession, and in particular land use change has been suggested to bring changes in AMF communities constrained by the arrival of AMF propagules (Zobel and Öpik 2014). Apparently in this system, the propagule pools were not destroyed, and/or the AMF were able to disperse and establish quickly from the neighborhood. Fast arrival of AMF, within a year after disturbance, has been recently reported (García de León et al. 2016).

Persistent AMF diversity levels in plantation chronosequence have also been reported from long term cocoa cultivation (Cuenca and Meneses 1996) and plantations of Caragana korshinskii (Liu et al. 2009). However, while maintaining the same number of species, shifts in AMF communities can occur during the different plant growth stages (Cavaglieri et al. 2009; Tscherko et al. 2004; Yu et al. 2012) due to shifts in relative abundance and/or species replacement. We found particular VT more abundant (indicators) in the youngest (3 year-old)
and the oldest (16 year-old) plantations, but not the 6 year-old plantations, which is consistent with a gradual shift in abundances of particular AMF through time. Interestingly, *Scutellospora* VT were identified as the best indicators in the young plantations. It is possible that the rapid growth of the young trees restricts or promotes the growth of particular soil microbes – in this case *Scutellospora* VT - either due to nutrient competition or phyto-chemical inhibition (Cao et al. 2010).

A further aspect that may drive the gradual replacement of AMF in the chronosequence is the change in host root biomass and carbon allocation within the tree as the rubber trees age. When the root space available for AMF colonization increases, an increase in total AMF biomass is expected (Treseder and Cross 2006). This may result in increase in AMF richness, but that was not observed in our study. Alternatively, changing carbon availability for the symbionts would drive the shift of AMF species with differing demand on the host’s photosynthates. *Scutellospora* species, here identified as young plantation indicators, may incur higher carbon cost for the host than Glomeraceae and Claroideoglomeraceae, indicators of 16-year old plantations.

4.5.4. Cassava and rubber tree AMF communities

AMF communities associating with rubber trees and cassava clearly differed in composition, despite the high number of VT in common. The cassava sites also harbored a higher AMF richness per sample than the rubber tree plantations, regardless of their age. Furthermore, several VT were identified as indicators for one of the two hosts, suggesting a certain level of host preference or responses to environmental conditions accompanying shift of cropping with the different host (Pereira et al. 2014). The potential role of host identity in structuring AMF communities is still debated (Davison et al. 2015; Davison et al. 2016; Helgason and
Fitter 2009) possibly depending on host ecological properties rather than taxonomic identity (Öpik et al. 2009).

Differences in AMF communities may also be explained by the different management practices occurring in these two types of land-use. Management practices such as tillage and chemical inputs influence AMF communities (Alguacil et al. 2010; Mathimaran et al. 2007; Oehl et al. 2004). Cassava fields in North-East Thailand are cultivated by intense mechanical disturbance of the soils during planting every year whereas the level of mechanical disturbance is considerably lower after rubber tree plantation establishment. Varying levels of disturbance possibly contribute to the differences in AMF richness and diversity in differently managed habitats (Moora et al. 2014; Pereira et al. 2014; Verbruggen et al. 2010). The relative contributions of host, edaphic and management effects in shaping AMF communities of cassava and rubber tree sites in this study require further investigation.

4.5.5. Shift in the AMF community composition in chronosequence

The different rubber tree age groups were well separated along the first two axes of an RDA effectively constrained by soil variables, including nutrient content and texture. This suggests that AMF community shift was probably mostly driven by the changing edaphic conditions in the rubber tree plantation chronosequence.

Mineral fertilizers (P and N fertilizers in particular) are regularly applied to the young stages of rubber plantation (up to 5 year-old), to fulfill the nutrient requirements of the trees. This explains the higher average level of P in the 3 year-old plantations compared to the older sites. However, P content varied to a large degree among sites of the same age group, which could be caused by variation in management practices. The older rubber plantations no longer
received high doses of fertilizers, and this is reflected in the lower average soil nutrient concentration in the 6 year-old plantations, when the trees are still growing rapidly. In the 16 year-old plantations, the trees are mature and the canopy is closed, potentially allowing more nutrients to return to the soils in the form of litter. This may explain the high organic matter, K, and Ca contents in such soils (Table 8).

P and N application affects the AMF communities associating with various host plants (Bünemann et al. 2006; Chen et al. 2014; Van Geel et al. 2015). When the level of soil nutrients is high, plants may reduce or cease resource allocation to their roots, resulting in stronger competition between the symbiotic partners (Verbruggen and Kiers 2010). These conditions could become detrimental to sporulation, root colonization or survival of sensitive AMF species and as a result, the establishment of more competitive AMF might be favored, at the expense of other symbionts (Alguacil et al. 2010; Camenzind et al. 2014; Hassan et al. 2013; Verbruggen and Kiers 2010).

During the first 10 years after plantation establishment (or until the trees are tapped), rubber trees grow rapidly and their nutrient requirements are high (Aweto, 1987). In suboptimal environmental conditions such as those found in NE Thailand, and despite the applied fertilizers, young trees may be more dependent on mycorrhizal symbiosis than older trees and thus more likely to favor symbiotic partners that provide nutritional benefits (Verbruggen and Kiers 2010). Due to tapping, the presence of litter and canopy closure, the nutrient requirements of trees in the older plantations (16 years-old) presumably differ to those of younger trees. Thus, the depletion of fertilizers added initially, changing nutrient requirements of rubber trees of different ages and concurrent changing input of organic litter shape the edaphic conditions in the rubber tree chronosequence and accordingly bring about a gradual shift of AMF communities as observed in this study.
4.6. Conclusions

Our results show that establishment of rubber tree plantations on fields previously planted with cassava bring about a significant shift in AMF community composition, but not in richness. Furthermore, AMF communities showed gradual changes as the plantations aged and in relation to soil physical and chemical characteristics. However, the sample- and site-based richness of AMF remained high even at high initial fertilizer loads, suggesting that the long-term moderate cropping has sustained a high diversity of AMF in this region, which is potentially significant for maintaining high functionality of AMF communities.
5. CHAPTER 5: Relevance of taking into account the fine scale soil variability to assess the effects of agricultural inputs on soil characteristics and soil microbial communities: a case study of biochar application in a rubber plantation in North East Thailand

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See the “Authorship statement – chapter 5” (appendix 6) for details.
5.1. Abstract

In agricultural studies, estimating and testing differences between treatment regimes in a designed experiment usually determines the subsequent success or failure of inputs or management practices. Fine-scale soil variability of the experimental site can reduce statistical power and should be determined in order to optimize the experiment design. Although randomization is often performed, there is a probability that such randomization does not cover for fine scale soil variability. To determine the effect of this fine scale soil variability, a well characterized test system comprising of biochar’s effect on soil microbial community was studied, in conjunction with Electrical Resistivity Tomography (ERT) to identify the potential influence of fine scale soil variability on biochar’s differential interaction with soil microbial communities. Two main soil profiles were identified by the ERT survey and soil characteristics and soil microbial communities were differently affected by the biochar application with regards to soil profile. Fungal communities (mycorrhization intensity and fungal richness in particular) seemed to be more strongly affected than bacterial communities, however, these effects didn’t show a similar trend in between the two studied soil profiles. Consequently, most of the effects of biochar application were masked by the soil variability of the experimental site when analysed in a nested design with randomization. Our results support the relevance of taking fine scale soil variability into account prior to the establishment of a field trial to optimize experimental design.

5.2. Introduction

Estimating and testing differences between treatments in the field is usually based on the principle of the randomized block design (CRB): the experimental area is divided into blocks
that are internally as uniform as possible and treatments are randomly assigned to plots within blocks. The between-block variation is therefore removed from the residual in the data analysis, increasing the statistical power of the experiment. However, to optimize the experimental design, prior knowledge of the variation of the experimental site is required, but this information is rarely available (Doncaster and Davey 2007). Moreover, the growing interest in mapping soil variability at field scale has shown that soil variability is not randomly organized, even at small scale (Ge et al. 2011; Stadler et al. 2015; Takagi and Lin 2011).

Agricultural practices such as soil amendments are known to affect soil fertility but their effects depend on multiple parameters including the physico-chemical characteristics of soils (e.g. texture, porosity, permeability, water holding capacity, acidity, organic matter (OM) and major nutrient content). These parameters have been acknowledged as being important drivers of soil health, predominantly through their strong effects on the microbial communities involved in key biogeochemical processes (Gobat et al. 2013). Although the importance of the soil biota in soil fertility has been widely recognized, the impact of the interactions of fine scale soil variability and amendments on microbial communities remains largely unstudied. As a consequence, in experiments in which soil variability is not accounted for, results may be biased and lead to falsely non significant effects and misinterpreted conclusions.

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is a fast-growing upright tropical tree mainly cultivated for its production of latex. Thailand is the world’s leading producer and exporter of rubber with a production capacity of 3.2 million t per year (http://faostat.fao.org). While most of the latex is produced in the south and the centre of the country, the establishment of rubber tree plantations is currently expanding in less suitable areas, especially in North East (NE) Thailand where soil fertility is low, dry seasons last up to six months and soils are unsuitable
for growing most other cash crops. This expansion of rubber tree plantations is strongly supported by the Royal Thai Government initiative that is assisting farmers with technology and production inputs such as seedlings or fertilizers. As an alternative to the application of high doses of chemical inputs, biochar applications have been recommended for the establishment of new rubber plantations in non-optimal environmental conditions (Joshi, 2005).

Biochar is a carbon-rich product obtained when biomass such as wood, manure or leaves is heated in a closed container with little or no air. It is distinguished from charcoal by its use as a soil amendment (Lehmann and Joseph 2009). The scientific interest in biochar has been growing during the past decade because it appears to be a convenient strategy for carbon sequestration as it remains in soils for hundreds or thousands of years. Indeed, as the carbon stored in biochar is incorporated into soil and cannot be released easily by changes in land management practices (Lehmann, 2007). Keith et al. (2015) showed that the presence of biochar may partially offset the positive rhizosphere priming effect, or reduce it further where it is already negative, thereby contributing to long-term C storage in soils. Other advantages of biochar applications on soil properties, processes and functions include water infiltration and percolation through soil, pollutant removal, reduction of greenhouse gas emissions and the modification of microbial communities. The effects observed on soil fertility have been mainly explained by pH increases in acid soils or by improved nutrient retention corresponding to cations adsorption enhanced by the high specific surface of biochar. Biochar has also been shown to affect soil biological community composition and abundance, possibly resulting in an effect on nutrient cycles, soil structure and indirect effects on plant growth.

Biochar can be produced from many different materials including feedstocks such as wood, crop residues or manures. The suitability of a particular feedstock depends on a number of
chemical, physical, environmental, technical, economical and logistical factors and consequently, biochar characteristics and effects may be highly variable. (see the following reviews for detailed analysis of these: Brassard et al. (2016); Ding et al. (2016); Gul and Whalen (2016); Luo et al. (2016); Schmalenberger and Fox (2016); Zhang et al. (2016); Amini et al. (2016); Ajayi and Horn, (2017); Amendola et al. (2017); Nguyen et al. (2017); Zhou et al. (2017). However, the benefits of biochar have been subject to controversy and neutral and negative effects have also been reported in a number of studies (see the review papers cited above). Despite a large and growing number of scientific studies on the effects of biochar addition to soil conducted over a wide range of laboratory, field and greenhouse conditions, there is still insufficient information to understand the underlying mechanisms of the observed effects after application. A better understanding of the relationships between agricultural practices such as biochar application, soil biota and fine scale soil variability in the case of low soil fertility in NE Thailand would lead to more adapted management practices for the sustainable cultivation of rubber trees.

The utilization of non-invasive geophysical techniques such as ERT to determine agronomical or biological parameters could allow the optimization of trial design through the monitoring of the spatial variability of soils at the experiment scale and a better understanding of the observed effect (or lack thereof) of the studied factor(s). ERT has been widely used in geological body prospecting, in evaluating the performances of subsurface structures and in mapping solute and fluid movement in porous media. According to the literature, ERT is appropriate for monitoring the 3-D spatial and temporal variation of soils water content in the field (Kemna et al. 2002; Michot et al. 2003; Zhou et al. 2001), for studying the transport processes in the subsurface (Slater et al. 2002; Vanderborght et al. 2005), but also for determining soil characteristics (Gambetta et al. 2011; Sudha et al. 2009), monitoring
mountain permafrost evolution (Hilbich et al. 2008) and for agronomical management by identifying areas of excessive compaction or soil horizon thickness and bedrock depth (Samouëlian et al. 2005).

To assess the importance of the fine scale soil variability on the effect of agricultural inputs on soil microbial communities, a trial was set up in a rubber tree plantation in North East Thailand. Increasing doses of biochar were used as treatments. The objectives of our study were: i) to characterize the fine scale soil variability of an established rubber plantation in North-eastern Thailand using ERT, ii) to assess whether the soil type interferes with the effect of biochar applications on soil microbial communities and iii) to investigate whether the characterization of the fine scale soil variability could assist in designing an experiment to assess the impact of biochar on the soil characteristics and soil microbial communities.

5.3. Materials and Methods

5.3.1. Experimental design and biochar application

Northeast Thailand is a square shaped plateau with mainly sandy, acidic and infertile soils associated to highly weathered parent material (Wada, 2005). Destruction of natural vegetation has led to further depletion of soil OM and nutrients.

The trial was set up in May 2013 in a 1.28 ha seven-year old rubber tree plantation in Phu Wiang (Khon Kaen district - N16°38.544’ E102°16.864’), in NE Thailand. Soils in the Khon Kaen district were shown to be mostly sandy in texture (>80 %), acidic (pH 4.7–5.5), with low OM and macrobelement (N, P, K, Ca, Mg) contents (Herrmann et al. 2016b). The average annual rainfall is about 1000 mm (distributed from June to October) with an average temperature of 30°C throughout the year.
The trial consisted in four treatments corresponding to 4 doses of biochar: no biochar (T1), 5 tons/ha (T2), 10 tons/ha (T3) and 20 tons/ha (T4). Each treatment was replicated 4 times in a randomized design and each plot contained 12 trees (Figure 15).

The biochar was made from bamboo following a slow pyrolysis process (350 to 400°C for 8 hours). Its physicochemical characteristics are presented in Table 13. Biochar was mixed with the top soil (0-30 cm) layer in a ring around each tree, 40 cm from the trunk to optimize the presence of tree roots.

5.3.2. Electrical Resistivity Tomography (ERT) measurements

The Electrical resistivity tomography survey (Griffiths and Barker 1993) was carried out in July 2014. The measurements were made along 9 parallel rectilinear profiles of lengths between 173 m and 143 m. The distance between the parallel profiles was between 6 and 9m in order to fit with the layout of the trial. The determination of the profiles and the measurement of elevations were performed with a total station Nikon DTM-332.

Along each profile, electrodes were driven into the soil with a constant spacing of 1 m between adjacent electrodes. The electrodes were connected with a multi-wire cable to a multi-channel resistivity-meter system (SYSCAL-Pro switch 72 from Iris Instruments, France). The following acquisition parameters were used: injection of square shaped alternating current with 500 ms half-period, output voltage of 200 V, stacking number ranging from 3 to 9, dispersion of measurements lower than 0.5%. The measurements that had dispersion higher than 1% were discarded from the data set. The measurements were carried out automatically using a programmed sequence of connexions corresponding to 972 different quadrupoles selected among 72 electrodes installed.
Figure 15. Layout of the Phu Wyang rubber trees plantation.
Table 13. Physicochemical characteristics of the biochar applied in the Phu Wiang rubber tree plantation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>9.5</td>
</tr>
<tr>
<td>Mean of iodine number (mg/g)</td>
<td>312.3</td>
</tr>
<tr>
<td>Heavy metal content (mg/kg)</td>
<td></td>
</tr>
<tr>
<td>- Cu</td>
<td>6.8</td>
</tr>
<tr>
<td>- Pb</td>
<td>5.4</td>
</tr>
<tr>
<td>- Zn</td>
<td>1214.4</td>
</tr>
<tr>
<td>EC (dS/m)</td>
<td>1.8</td>
</tr>
<tr>
<td>Moisture content (% fresh weight)</td>
<td>4.8</td>
</tr>
<tr>
<td>Ash content (% dry weight)</td>
<td>6.4</td>
</tr>
<tr>
<td>Volatile matter content (% dry weight)</td>
<td>20.8</td>
</tr>
<tr>
<td>Fixed carbon (% dry weight)</td>
<td>72.8</td>
</tr>
<tr>
<td>Carbon content C (% dry weight)</td>
<td>76.9</td>
</tr>
<tr>
<td>Hydrogen content H (% dry weight)</td>
<td>3.28</td>
</tr>
<tr>
<td>Nitrogen content N (% dry weight)</td>
<td>0.38</td>
</tr>
<tr>
<td>Oxygen content O (% dry weight)</td>
<td>13.0</td>
</tr>
<tr>
<td>C:N ratio</td>
<td>202.4</td>
</tr>
</tbody>
</table>
The apparent resistivity of the ground is calculated as:

\[
\rho_a = K \cdot \frac{\Delta V_{R-P}}{I_{C_1-C_2}}
\]

with

\[
K = \frac{2\pi}{1 + \frac{1}{C_1P_1} - \frac{1}{C_1P_2} - \frac{1}{C_2P_1} + \frac{1}{C_2P_2}}
\]

K (unit: m) is a geometrical coefficient depending only on electrode separations \(C_iP_j\) (for reciprocal Wenner-Schlumberger configuration \(K = \pi \cdot n \cdot (n+1) \cdot \alpha\) with \(P_1C_1 = C_2P_2 = n \cdot \alpha\) and \(C_1C_2 = \alpha\));

\(I\) (unit: A) is the intensity of the current injected between electrodes \(C_1\) and \(C_2\); \(\Delta V\) (unit: V) is the electrical potential measured between electrodes \(P_1\) and \(P_2\).

A typology of the principal types of vertical geo-electrical profiles was then established using successively Principal Component Analysis (PCA) and Ascendant Hierarchical Classification (AHC) (Pagès, 2010). This typology allowed the selection of samples individually according to a quantitative characterization of soil variability within each replicate of the trial in order to test the influence of soil variability.

5.3.3. Soil and root sampling

Soil and root samples were collected in November 2014 at the end of the rainy season, 18 months after biochar application.

Soil samples were collected from eight trees in each identified soil class and treatment (2 classes of soil x 8 replicates x 4 treatments) avoiding trees located at the edge of plots or with suboptimal soil class characterization. For every selected tree, soils were sampled from two opposing sites (within the ring were the biochar was applied, 0–30 cm deep) and pooled to
form a composite sample. Soils were then passed through a 2 mm sieve and stored at -20°C (for molecular biology analyses) or air-dried (for chemical analyses). The finest roots were collected from the sieve and dried at 45°C.

5.3.4. Soil chemical characteristics

Soil chemical analyses were performed on 5 samples per plot. The pH (H₂O) was determined following the protocol of Mc Lean (1982). The percentage of OM was assessed according to the protocol of Walkley and Black (1934) and the phosphorus (P) content was measured by the method of Bray and Kurtz (1945).

5.3.5. AMF roots colonization

Fine roots were selected (less than 1 mm in diameter) and AMF staining in root tissue was performed following the ink and vinegar technique (Vierheilig et al. 1998) with an additional tissue bleaching step as described by Koske and Gemma (1989). Fifteen roots fragments of 1 cm were observed per sample (x40) and the presence and the intensity of colorization were scored (from 0 to 5) to assess the frequency (F%) and the intensity (M%) of AMF colonization (Trouvelot, 1986).

5.3.6. Bacterial and fungal community structure and diversity assessed by denaturing gradient gel electrophoresis (DGGE)

DNA was extracted using the commercial kit MP116004-500 Fast DNA Spin kit for soil, MP Biomedical, Santa Anna, CA) according to Tournier et al. (2015). PCR amplifications targeted a fragment of bacterial 16S rDNA using the specific primers GC-338F (5′
and of fungal 18S rDNA using the specific primers GC-FR1 (5’
CGCCCGCCGCGCGCGGCGGGCGGGGCACGGGGGGACTCCACGACTCCT
ACGGGAGGCAG3’) and FF390 (5’- ATTACCGCGGCTGCTGG -3’)
(Vainio and Hantula 2000). Reactions were carried out in a 25 µL reaction volume containing 12.5 µL of 2X Master Mix
solution (iNtRON Biotechnology, Korea), 10 pmol of each primer and 2 µL of total DNA
extract.

PCR products were loaded into 8% acrylamide/bis gel (40% solution) containing a linear
denaturation gradient of 45% to 60% for bacteria and 40% to 60% for fungi. The 100%
denaturing solution contained 7 M of urea and 40% formamide. Denaturing electrophoresis
were run with an Ingeny DGGE system in Tris Acetate EDTA (TAE) 1X buffer for 17h at
100V and a constant temperature of 60°C (Muyzer et al. 1993).

DGGE patterns were analysed using Lab Phoretix 1D software (v11.5): similarity of the
patterns was assessed using similarity Dice index and dendrograms were built using UPGMA
method (Un-weighted Pair Group Mathematical Averages). Shannon Weaver (H’), Piélou (J’)
and Simpson (D) indices were calculated using the following formulas:

\[ H’ = \left( -\sum_{i=1}^{S} p_i \log p_i \right) \]

With \( p_i = \frac{V_i}{V_t} \) (\( V_i \) = volume of the I band; \( V_t \) = total volume of all bands seen on the profile
and \( p_i \) is the relative abundance of each community)

\[ J’ = \frac{H’}{\log S} \]

With \( S \) = richness and \( H’ \) = Shannon-Weaver index
\[ D = \sum \frac{V_i (V_i - 1)}{V_t (V_t - 1)} \]

In this study, the exponential of Shannon-Weaver exp (H’) and the reciprocal of Simpson 1/D were used, in order to satisfy the replication principle and to be considered a linear representation of diversity (Jost, 2006).

5.3.7. Statistical analyses

Data were ln-transformed when needed to satisfy the normal distribution before analysis of variance (Shapiro-Wilk test; \( P < 0.05 \)). Differences among the means of AMF root colonization, soil chemical characteristics and diversity indices were analysed by an analysis of variance (ANOVA) followed by a Tukey (HSD) test of comparison by pairs (\( P < 0.05 \)). All the statistical analyses were run with XLSTAT software (Version 2015.4.01.22209) on individual soil class and on the combination of both soil classes.

5.4. Results and Discussion

5.4.1. Stratification of the samples using soil variability

The analysis of the variability of soils within the experimental site studied resulted in the identification of 4 main types of vertical resistivity profiles which may be considered as a quantitative signature of soil layering (Figure 16).
Figure 16. Typology of vertical profiles of electrical resistivity (left) - Dendrogram of the typology (surfaces of the circles are proportional to the size of the groups) (right).
• “soil profile C1” with 4 layers: a thin low resistive layer (about 500 W.m) at top soil, overlaying with a sharp transition a lower resistive layer (150 W.m) then a more resistive layer (700 W.m) and at depth a layer with resistivity decreasing progressively until about 200 W.m.

• “soil profile C2” with 3 layers: a thin resistive layer (about 1000 W.m) at top soil, overlaying with a smooth transition a moderately resistive layer (700 W.m) and at depth a layer with resistivity decreasing progressively until about 60 W.m.

• “soil profile C3” with 4 layers: a thin resistive layer (about 2000 W.m) at top soil, overlaying with a smooth transition a moderately resistive layer (700 W.m) then a slightly more resistive layer (800 W.m) and at depth a layer with resistivity decreasing progressively until about 200 W.m.

• “soil profile C4” with 3 layers: a thin resistive layer (about 3000-4000 W.m) at top soil, overlaying a very resistive layer (4000-5000 W.m) and then a layer with resistivity decreasing progressively until about 100 W.m.

The variability of soil within a circle of 1.5 m diameter around each sampled tree was used to build the map of the soil class distribution (Figure 17). This shows that the proportion of the different types of soil differed between treatments. In particular soil profiles C1 and C2 were under represented for most of the treatments. The observed variability of soil within the trial led to adopt a posteriori a specific sampling scheme: samples for which no dominant soil profile existed and soil profiles for which the 4 doses were not represented were discarded. This allowed the comparison of samples from the 4 biochar doses in 2 major soil classes corresponding to the respective soil profiles C3 and C4.
Figure 17. Map of soil class distribution within the Phu Wyang rubber trees plantation.
5.4.2. Biochar and soil characteristics

The chemical characteristics of soils are presented in Table 14 for the two dominant soil classes (C3 and C4). Soils in all treatments were acidic (pH ≤ 5.5) and contained low levels of OM and P. However, soils of class C4 tended to be more acidic and to contain more OM and P than those of class C3. The effects of biochar applications on soil characteristics depended on both biochar dose and soil class.

Interestingly, no effect of biochar on pH was observed when the soil classes C3 and C4 were combined (P = 0.122, Table 15) but a significant increase of pH was observed in the class C4 (P = 0.012), in particular for a biochar dose above 10t/ha (Table 14). Other studies showed that biochar application resulted in soil pH increases through the negative charge on the surface that buffers acidity in soils, with a stronger effect in both moderate acidic and neutral soils than in extreme acidic soils (pH < 5) (Gul et al. 2015; Jeffery et al. 2011; Van Zwieten et al. 2010). Our results are in accordance with these findings since soils from the class C3 in absence of biochar (T1) were less acidic than those from the class C4.

Soil P content was significantly affected by the biochar application when the analysis was performed on the combination of the C3 and C4 soil classes. The analysis of the individual soil classes showed that the observed effect is actually significant in soil class C4 only (P ≤ 0.001, Table 15). Biochar effects have been shown to depend on several factors in other studies. For instance, Soinne et al. (2014) showed that biochar did not have the affinity to sorb phosphate in clayey soil, while Xu et al. (2014) showed that biochar amendment sharply increased the Ca-bounded P but slightly enhanced the Al-retained P. It may be implied that the biochar effects are likely to be dependent on soil acidity. In our study, soil pH was affected by biochar dose in soil class C4 only, which may explain the absence of biochar effect in soil class C3 (Table 14).
Table 14. Soil chemical characteristics of the Phu Wiang rubber plantation.

<table>
<thead>
<tr>
<th>Soil class</th>
<th>Treatment</th>
<th>pH (H₂O)</th>
<th>OM (%)</th>
<th>P (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>T1</td>
<td>5.49±0.34a</td>
<td>0.50±0.04ab</td>
<td>4.63±2.17a</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>5.41±0.40a</td>
<td>0.65±0.08b</td>
<td>8.50±9.91a</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>5.53±0.17a</td>
<td>0.45±0.10a</td>
<td>4.75±2.30a</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>5.5±0.15a</td>
<td>0.63±0.13b</td>
<td>9.90±9.64a</td>
</tr>
<tr>
<td>C4</td>
<td>T1</td>
<td>4.93±0.36a</td>
<td>0.65±0.09ab</td>
<td>7.40±4.62a</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>4.99±0.27a</td>
<td>0.64±0.08b</td>
<td>14.07±7.45ab</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>5.24±0.18ab</td>
<td>0.57±0.06ab</td>
<td>6.00±3.98a</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>5.41±0.27b</td>
<td>0.52±0.09a</td>
<td>20.28±5.83b</td>
</tr>
</tbody>
</table>

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha;

Means ± standard deviation;

Within the same class, means followed by different letters are significantly different according to the Tukey test (α = 0.05).
Table 15. Results of the analyses of variance (p values) of soil characteristics, root mycorrhization and soil diversity indices of the Phu Wiang plantation for both soil classes C3 and C4 (individually or combined) according to the dose of biochar applied.

<table>
<thead>
<tr>
<th>Soil class</th>
<th>pH (H₂O)</th>
<th>OM (%)</th>
<th>P (mg/kg)</th>
<th>M (%)</th>
<th>Richness 16s</th>
<th>Richness 18s</th>
<th>Exp (H') 16s</th>
<th>Exp (H') 18s</th>
<th>1/D 16S</th>
<th>1/D 18S</th>
<th>J' 16s</th>
<th>J' 18s</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3 + C4</td>
<td>0.122</td>
<td>0.010***</td>
<td>&lt;0.001***</td>
<td>0.138</td>
<td>0.665</td>
<td>0.634</td>
<td>0.903</td>
<td>0.676</td>
<td>0.894</td>
<td>0.523</td>
<td>0.741</td>
<td>0.326</td>
</tr>
<tr>
<td>C3</td>
<td>0.897</td>
<td>0.008***</td>
<td>0.439</td>
<td>0.909</td>
<td>0.360</td>
<td>0.321</td>
<td>0.230</td>
<td>0.368</td>
<td>0.169</td>
<td>0.234</td>
<td>0.405</td>
<td>0.263</td>
</tr>
<tr>
<td>C4</td>
<td>0.012**</td>
<td>0.022**</td>
<td>0.001***</td>
<td>0.020**</td>
<td>0.480</td>
<td>0.098*</td>
<td>0.749</td>
<td>0.335</td>
<td>0.557</td>
<td>0.880</td>
<td>0.960</td>
<td>0.863</td>
</tr>
</tbody>
</table>

C3 and C4: soil classes

OM: Organic matter H’: Shannon index; D: Simpson index; J’: Evenness; M: intensity of mycorrhization

*: P < 0.1; **: P < 0.05; ***: P < 0.01
Soil OM content was affected by the biochar amendment when C3 and C4 soil classes were combined for analysis ($P = 0.010$, Table 15). Contrary to pH and P, changes in OM contents were significantly affected by the biochar dose regardless of the soil class, although the effect was more strongly significant in C3 than in C4 soil class ($P = 0.008$ and 0.022, respectively, Table 15). However, effects of biochar applications on OM contents were not consistent for the two soil classes. The highest dose of biochar (T4) resulted in an increase of OM content in C3 soil class while it caused a decrease in C4 soil class as compared with the controls (Table 14).

Zimmerman et al. (2011) also observed both positive and negative priming effects of biochar in soils depending on biochar type, soil type and time. Keith et al. (2011) showed that there are strong and complex interactions between biochar, carbon and the decomposition of labile OM, which may be partially related to changes in physical properties of biochar with time.

5.4.3. Biochar impact on rubber root colonization by native AMF

All the root samples were infected by AMF, regardless of soil class and biochar dose ($F\%=100\%$; data not shown). M (intensity of colonization) varied from 79 to 91.5% with the maximum of colonization found in the C3 soil class after application of 10 t/ha of biochar (Table 16). Rubber trees have previously been shown to be naturally highly colonized in Bangladesh (Dhar and Mridha 2006) and Thailand (Herrmann et al. 2016a). This suggests the importance for rubber trees to establish a symbiotic relationship with native AMF to grow in poor soils since AMF are particularly known to play a significant role in nutrient and water uptake and to improve soil porosity (Cardoso and Kuyper 2006; Lenoir et al. 2016; Varma, 2008).
Table 16. Soil diversity indices of the Phu Wiang plantation for both soil classes according to the rate of biochar applied.

<table>
<thead>
<tr>
<th>Soil class</th>
<th>Treatment</th>
<th>Richness 16s</th>
<th>Richness 18s</th>
<th>Exp (H’) 16s</th>
<th>Exp (H’) 18s</th>
<th>J’ 16s</th>
<th>J’ 18s</th>
<th>1/D 16s</th>
<th>1/D 18s</th>
<th>M%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>48.8 ± 3.9 a</td>
<td>42.7 ± 2.8 a</td>
<td>5.2 ± 0.2 a</td>
<td>4.0 ± 0.2 a</td>
<td>0.98 ± 0.01 a</td>
<td>0.84 ± 0.03 a</td>
<td>42.6 ± 3.2 a</td>
<td>14.4 ± 2.0 a</td>
<td>82.5 ± 10.8 a</td>
</tr>
<tr>
<td>C3</td>
<td>T1</td>
<td>49.3 ± 1.4 a</td>
<td>40.8 ± 5.8 a</td>
<td>5.2 ± 0.2 a</td>
<td>4.1 ± 0.4 a</td>
<td>0.97 ± 0.01 a</td>
<td>0.87 ± 0.03 a</td>
<td>41.3 ± 4.0 a</td>
<td>19.7 ± 5.0 a</td>
<td>88.2 ± 5.9 a</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>47.2 ± 1.9 a</td>
<td>37.7 ± 6.0 a</td>
<td>5.1 ± 0.1 a</td>
<td>3.7 ± 0.6 a</td>
<td>0.97 ± 0.01 a</td>
<td>0.82 ± 0.07 a</td>
<td>40.0 ± 1.6 a</td>
<td>13.8 ± 7.8 a</td>
<td>91.5 ± 1.9 a</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>47.5 ± 1.4 a</td>
<td>39.2 ± 3.5 a</td>
<td>5.1 ± 0.1 a</td>
<td>3.9 ± 0.2 a</td>
<td>0.97 ± 0.01 a</td>
<td>0.86 ± 0.04 a</td>
<td>39.0 ± 3.1 a</td>
<td>15.7 ± 4.3 a</td>
<td>85.9 ± 5.7 a</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>41.2 ± 3.7 a</td>
<td>44.8 ± 2.1 a</td>
<td>4.6 ± 0.1 a</td>
<td>4.3 ± 0.1 a</td>
<td>0.95 ± 0.01 a</td>
<td>0.88 ± 0.02 a</td>
<td>29.3 ± 1.8 a</td>
<td>21.8 ± 1.4 a</td>
<td>79.0 ± 10.8 a</td>
</tr>
<tr>
<td>C4</td>
<td>T1</td>
<td>43.0 ± 1.4 a</td>
<td>46.7 ± 4.9 a</td>
<td>4.7 ± 0.1 a</td>
<td>4.4 ± 0.2 a</td>
<td>0.94 ± 0.01 a</td>
<td>0.89 ± 0.01 a</td>
<td>29.4 ± 1.3 a</td>
<td>22.6 ± 3.2 a</td>
<td>89.4 ± 5.1 b</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>41.0 ± 2.6 a</td>
<td>49.2 ± 2.3 a</td>
<td>4.6 ± 0.1 a</td>
<td>4.5 ± 0.2 a</td>
<td>0.95 ± 0.01 a</td>
<td>0.89 ± 0.01 a</td>
<td>29.1 ± 1.9 a</td>
<td>23.1 ± 4.4 a</td>
<td>87.9 ± 5.0 b</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>42.3 ± 1.8 a</td>
<td>43.3 ± 5.3 a</td>
<td>4.7 ± 0.1 a</td>
<td>4.3 ± 0.3 a</td>
<td>0.95 ± 0.01 a</td>
<td>0.89 ± 0.01 a</td>
<td>29.7 ± 2.2 a</td>
<td>21.5 ± 4.4 a</td>
<td>88.0 ± 3.8 b</td>
</tr>
</tbody>
</table>

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha; H’: Shannon index; D: Simpson index; J’: Evenness; M%: intensity of mycorrhization; Means ± standard deviation. Within the same class, means followed by different letters are significantly different according to the Tukey test (α = 0.05).
Biochar application increased the intensity of root colonization of rubber trees (M), regardless of the applied dose. When studied at the plantation scale (C3 and C4 soil classes combined), a non-significant increase was observed ($P = 0.138$, Tables 15 and 16). However, when analysed separately, this increase (up to 13%) was actually significant in soil class C4 only ($P = 0.020$, Table 15).

As reviewed by Warnock et al. (2007) and Lehmann et al. (2011), AM fungi are affected by the addition of biochar in soils. Decreases in AM fungi abundance have been sometime observed after addition of biochar. These decreases may be explained by a reduced requirement for mycorrhizal symbiosis due to increased nutrient and water availability to plants; changes in soil conditions due to modification of pH or water relations and direct negative effects from high contents of mineral elements or organic compounds detrimental to the fungi, such as high salt or heavy metal contents.

Usually however, biochar applications significantly enhance the mycorrhizal response through an increase in overall plant P nutrition and/or an increase in plant pathogen resistance and/or an increase plant drought resistance (Amendola et al. 2017). Additionally, these effects are also attributed to alteration of soil physical-chemical properties, but also to changes in other soil microorganism communities interacting with AMF, detoxification action beneficial to soil biota or the creation of a niche where fungi are more protected from grazers. Changes in soil chemical properties were observed in our study (pH, P and OM) in soil class C4 in particular, and these changes may have affected positively the mycorrhization of the rubber trees.
Rillig and Mummey (2006) hypothesized that secretion of mycorrhizal mycelium and polysaccharides might stabilize biochar with the formation of stable OM aggregates, a form of protected OM. Our results support this hypothesis since we observed a positive impact of the biochar application on OM content in soil class C3 (Table 14). The available P in soil usually drives plant root mycorrhizal infection and determines if the plants try to form root symbioses with AM fungi in order to enhance P access. The colonization process is clearly described by Bonfante and Desiro (2015) and it shows how sensitive these mechanisms are to available P content in soil. However, the root mycorrhization of the rubber trees in Phu Wiang was not negatively affected by the increase of P content in the soil with biochar application regardless of soil classes (even for the soil class C4 where the P content was about 7.40 mg kg\(^{-1}\) of soil for the control and 20.28 mg kg\(^{-1}\) of soil for the treatment with 20 t of biochar ha\(^{-1}\)). It is likely that these P soil contents remained too low for inducing any negative impact on the root mycorrhizal infection by native AM fungi.

5.4.4. Biochar impact on the structure and diversity of the microbial communities

The investigated fungal and bacterial communities were not strongly affected by the biochar application, regardless of the soil class. However, as shown on Figure 18, the structure of the communities seemed to be affected to some extent by the biochar dose, as T1 and T4 treatments formed separate clusters.

Bacterial and fungal diversity indices were not affected by any of the biochar treatment, regardless of the soil class (Tables 15 and 16). However, fungal richness was affected by biochar application for soil class C4 (P = 0.098) while for soil class C3 and combined soil classes P values remained high (P = 0.321 and P = 0.634 respectively, Table 15).
Figure 18. Dendrogram of similarity of amplified fungal 18S (A) and bacterial 16S (B) rDNA from the C3 soil class for the 4 biochar doses.
Recent studies described the effects of biochar applications on microbial community structure. For instance, Muhammad et al. (2014) showed that feedstock type, biochar type and application rate differently affected soil microbial communities. Tian et al. (2016) highlighted the relationship between the effects of biochar applications on microbial community structure and the mineral fertilization status. It was also demonstrated that the water status of the soils interacts with biochar with direct and indirect effects on the microbial communities (Xu et al. 2016). Our preliminary results support these findings, as the biochar effects seemed to be dependent on the soil physical and chemical characteristics. According to Tian et al. (2016), soil OM cycling was the main driver of the observed changes: microorganisms mine N from the soil OM to compensate for high C:N ratios caused by biochar application, which consequently accelerate cycling of stable N. Our findings support this hypothesis, although soil OM was very limited in our study site, and we can assume that this has limited the impact of biochar application on soil microbial communities.

Effects of biochar application on soil microbial communities obtained in other studies have varied and shown to be either negative or positive (Ameloot et al. 2013; Gul et al. 2015; Lehmann et al. 2011; Zhang et al. 2016). The nature of material and its source, the pyrolysis temperature, the application rate and soil characteristics were shown to differently affect microbial biomass, colony forming units and community structure diversity (Gul et al. 2015). Singh and Cowie (2014) and Wang et al. (2014b) obtained similar findings with biochar produced from *Eucalyptus saligna* but applied at different rates.

Thus the interactions between biochar type, soil characteristics and microbial communities should not be ignored. In our study, biochar was produced from bamboo using a low pyrolysis temperature (450°C) process but the literature on the effects of biochar produced from bamboo wood is limited. Demisie et al. (2014) described the effect of bamboo biochar in clay
loam soil on the microbial biomass carbon and observed stimulation at a rate of 0.5% but a reduction at a rate of 2% as compared to the control. Watanabe and Sato (2015) studied the priming effect of bamboo biochar produced at 400°C in soils cropped with legumes and found a reduced priming capability after biochar application. The authors suggested that this was the direct consequence of changes in physicochemical and biological properties of soil during the duration of the experiment. The changes in soil parameters that occurred in our study may have directly or indirectly affected the soil microbial communities in the different soil classes.

5.5. Conclusion

The present study supports the scientific relevance of the assessment of the fine scale soil variability at an experimental site by using ERT before running biological analyses. The ERT survey allowed the identification of two main soil classes distributed within the four studied treatments (T1 to T4). The effects of biochar application on soil microbial communities could thus be observed in relation with the nature of soils. When results from both soil classes C3 and C4 were combined for statistical analysis, most of the effects of the biochar application were masked by the soil variability within the trial. We showed that biochar affected the microbial communities differently (fungal richness and AMF in particular) with regards to the soil class. Our results suggest that paying attention to the fine scale soil variability before the establishment of a field trial as described by Rudolph et al. (2016) who showed that soil apparent conductivity (ECa) could be used in the planning phase of an experiment to achieve efficiencies by improved blocking.
6. CHAPTER 6: Impact of increasing doses of biochar application on microbial communities associated with rubber trees in NE Thailand

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This chapter was written by Laetitia Herrmann with guidance from Dr Lambert Bräu and Dr Didier Lesueur.

Lab work apart from soil chemical and physical analysis that was performed by Dr Wanpen Wiriyakitoneekul, data analysis and data interpretation were conducted by Laetitia Herrmann with guidance from Dr Lambert Bräu and Dr Didier Lesueur.
6.1. Introduction

Biochar is the product of the pyrolysis of C-based biomass under low or zero oxygen conditions. It has received increasing attention for the past decades as an efficient tool to mitigate climate change by enhancing C sequestration after addition to soils (Lehmann et al. 2006; Sohi et al. 2010). Its use as a soil amendment has concurrently resulted in the improvement of soil properties and functions and ecosystem productivity (based on Lehmann and Joseph 2009). However, the biochar effectiveness as a soil amendment has been a controversial subject and both negative and positive results have been reported (Amini et al. 2016; Brassard et al. 2016; Ding et al. 2016; Sohi et al. 2010; Verheijen et al. 2009). The nature and the intensity of the effects are likely to depend on several factors including soil type and nutrient contents, cultivated species and management practices, biochar properties etc. (see chapter 1 section 1.5 for details).

Biochar’s effects on soils and crops have been attributed to changes in physical, chemical and biological soil properties, including modification of pH, water regime and nutrient cycling. The occurrence of a priming effect has also been proposed (Hamer et al. 2004). It has been shown to affect soil microorganisms through both direct and indirect effects (Ameloot et al. 2013; Quilliam et al. 2013), provide favorable sites for microorganisms to grow while protected from the predatory soil fauna and desiccation (Warnock et al. 2007) and may represent a food source despite its recalcitrant nature.

Thailand is the world leading producer of natural rubber, and rubber tree plantations are expanding in unsuitable areas such as the NE regions, where soils are mainly sandy and very poor in nutrients. Biochar application to soils has been widely promoted in NE Thailand as a means of enhancing rubber tree growth and productivity while reducing the negative impacts of extensive mineral fertilizer application. While the role of the soil biota in soil fertility has
been largely recognized, there is, however, no information on the impact of biochar application on the soil microbial communities’ structure and diversity associated with rubber trees in Thailand. A better understanding of their interactions with soil amendments such as biochar is of primary importance as it could assist in the development of effective alternatives for rubber tree plantation management.

The objective of this study was to assess the impact of application of increasing doses of biochar on soil bacterial and fungal communities associated with rubber trees in NE Thailand.

6.2. Material and methods

6.2.1. Site description, experimental design and sampling

The trial was set up in a rubber tree plantation located in Phu Wiang, Khon Kaen district, in NE Thailand (N16°38.544’ E102°16.864’) as described in the Chapter 5, section 5.3.1. Detailed information on the biochar production and application can also be found in this section.

Results from the Electrical Resistivity Tomography (ERT) survey carried out in July 2014 indicated the presence of two main soil classes (C3 and C4) distributed along the four biochar dose treatments (see Chapter 5 for details). In order to include the impact of soil classes on the effects of increasing doses of biochar on the microbial communities, only trees belonging to these two soil classes were sampled and included for analysis. A total of 6 trees were sampled per treatment (dose-soil class combination) following the protocol described in section 5.3.1. A total of 48 soil samples were collected in August 2016 (28 months after application), sieved to pass a 1mm mesh and stored at -20°C for molecular analyses or dried at room temperature.
for chemical analyses. The finest rubber tree roots were collected from the sieve and dried at 45°C until analysis.

6.2.2. Soil analysis

Soil analyses were performed on 5 samples per treatment/soil class combination and included pH (in H₂O), organic matter (Walkley and Black 1934), available P (Bray and Kurtz 1945, Bray II), total N (Bremner, 1965) and texture (Kilmer and Alexander 1949). Soil water content was assessed after drying at 100°C for 48 hours.

6.2.3. DNA extraction and sequencing

DNA was extracted from 0.5 g of soils using the MP116004-500 Fast DNA Spin kit for soil, MP Biomedical, Santa Anna, CA) according to Tournier et al. (2015). Fragments of the V4 variable region of the 16S rRNA gene and of the ITS region were amplified by PCR using the primer pairs 341F (5’- CCTACGGGNGGCWGCAG -3’)/785R (5’- GACTACHVGGGTATCTAATCC -3’) and ITS1F (5’- CTTGGTCATTTAGAGGAAGTAA -3’)/ITS2 (5’- GCTGCGTTCTTCATCGATGC -3’) for the bacterial and fungal communities, respectively, and a barcode was included on the forward primers. Samples were pooled together in equal DNA concentrations and purified using calibrated Ampure XP beads. Purified DNA was used for the preparation of the DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq instrument following the manufacturer’s guidelines.
6.2.4. Sequencing data processing

Sequences (5’-3’ and 3’-5’) were joined and barcodes were removed. Sequences were retained only if they carried the correct primer sequences and were >200 bp. Sequences with ambiguous bases and homopolymers exceeding 6bp were also removed from the dataset. Operational taxonomic units (OUT) were defined by clustering at 97% similarity, and singletons and chimeras were eliminated (Dowd et al. 2008). Final OTUs were taxonomically assigned using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu). Sequences that were not assigned to bacteria or fungi were removed from the dataset.

Sampling efficacy was assessed with rarefaction analysis using the functions rarefy from the R package Vegan (Oksanen et al. 2012). For subsequent analyses, sequencing depth per sample was standardized by retaining a random selection of reads in each sample, where the number retained corresponded to the minimum read count across all samples (52061 and 40784 reads for bacterial and fungal datasets, respectively).

6.2.5. AMF colonization assessment

AMF presence and colonization intensity were determined on fine roots (less than 1 mm in diameter) and AMF staining in root tissue was performed following the ink and vinegar technique (Vierheilig et al. 1998) with an additional tissue bleaching step as described by Koske and Gemma (1989). Fifteen roots fragments of 1 cm were observed per sample (x40) and the presence and the intensity (scored from 0 to 5) of colonization in each fragment were recorded to assess the frequency (F%) and the intensity (M%) of AMF colonization (Trouvelot, 1986).
6.2.6. Statistical analyses

Linear mixed-effects models (LME) (Pinheiro et al. 2013) were used to test for differences in richness and diversity indices (Shannon, Simpson and Evenness) in the different treatments. The Shannon index ($H'$) was exponentially transformed, resulting in a variable ($\text{expH}'$) that, in contrast to the untransformed index, satisfies the replication principle and can be considered a linear representation of diversity (Jost, 2006).

Analysis of bacterial and fungal community structure was performed using quantitative data, where proportions of reads representing different species were used as a proxy for the relative abundance of the species in a sample (Moora et al. 2014). Differences in communities associated with different treatments were tested using a PERMANOVA, with the `adonis` function from R package Vegan. Two dimensional NMDS (function `metaMDS` from R package Vegan, 50 iterations) was used to explore variation in community composition. Ellipses representing standard deviations around group centroids were defined using the `ordiellipse` function from the R package Vegan.

Soil chemical and physical variables were ln-transformed before they were included in linear mixed-effects models (LME) (Pinheiro et al. 2013) to test for differences between the different doses and soil classes.

6.3. Results

6.3.1. Soil characteristics

Soil application of biochar differently affected the soil texture in the two soil classes (Table 17). In soils of class C3, high rate of biochar (T4, 20 tons/ha) led to a lower sand content ($P =$
0.003) and a higher silt content (P = 0.001). The opposite was found in the C4 soils, with soils receiving the highest dose of biochar having the lowest silt and highest sand contents (P = 0.013 and 0.007, respectively). Clay content decreased with the application of biochar regardless of the dose in C4 soils only (P = 0.007), but no change was observed in the C3 soils (P = 0.956). However, the general texture of the soils remained unchanged, being mainly sandy.

Soil chemical characteristics were also affected by the biochar application in the two soil classes. Soil pH tended to increase with the application of biochar regardless of the soil class, but the increase was significant in C3 soils only (P = 0.019). Soil OM showed inconsistent results in the two soil classes: it tended to increase in C3 soils and to decrease in the C4 soils after biochar application. Soil N content significantly decreased after biochar application in C3 soils only, regardless of the dose (P < 0.0001). Biochar application resulted in an increase of soil P content in both C3 and C4 soils, but the results were significant in C4 soils only (P = 0.027). Other nutrient levels (Ca, Mg, K) all showed tendency to increase with the application of biochar in both soil classes (Table 17).

Soils from class C3 had a significantly higher water content than soils from class C4 (P < 0.0001), regardless of the biochar application treatment (data not shown). Soil water content was affected by the biochar application in C3 soils (P = 0.082) but not in C4 soils (P = 0.632). The soil water content was decreased after the addition of biochar, in particular for the smallest dose (T2, 5 ton/ha, P = 0.054, Table 17).
Table 17. Soil characteristics for the different biochar rates in the two soil classes.

<table>
<thead>
<tr>
<th>Soil class</th>
<th>Biochar dose</th>
<th>Soil texture</th>
<th>Chemical characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sand (%).</td>
<td>Silt (%).</td>
</tr>
<tr>
<td>C3</td>
<td>T1</td>
<td>83.4a</td>
<td>13.8b</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>83.4a</td>
<td>13.9b</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>83.0a</td>
<td>14.4b</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>79.7b</td>
<td>17.7a</td>
</tr>
<tr>
<td>C4</td>
<td>T1</td>
<td>78.9b</td>
<td>18.5a</td>
</tr>
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<td></td>
<td>T2</td>
<td>83.9a</td>
<td>14.0b</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>83.2a</td>
<td>14.9b</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>84.6a</td>
<td>14.5b</td>
</tr>
</tbody>
</table>

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha;
Means accompanied by the same letter do not differ significantly P ≤ 0.05 (pairwise comparisons using the Tukey (HSD) test).
6.3.2. Bacterial and fungal sequencing data

A total of 3,401,226 and 4,490,456 quality filtered reads were retained for the bacterial and fungal datasets, respectively. Bacterial sequences were classified into 32,742 OTU, in 239 families and 29 phyla, while fungal sequences were classified into 10,709 OTU, in 562 families and 31 phyla. Rarefaction curves showed that sequencing depth per sample was sufficient to describe both bacterial and fungal diversity across all treatments in our experimental site (Figure 19).

6.3.2.1. Bacterial community composition, richness and diversity

Bacterial community was dominated by Proteobacteria (> 30%), followed by Acidobacteria (> 20%), Actinobacteria (> 14%) and Firmicutes (> 10%) in all biochar dose-soil class combinations. The most abundant class was Alphaproteobacteria, which represented about 17% of the sequences regardless of the treatment and soil class (Figure 20).

Most of the bacterial phyla and classes detected in the different samples (8 of the 9 phyla and 12 out of the 15 classes) were affected by the soil class, regardless of the biochar dose (data not shown). However, when soil classes were studied separately, biochar application had a limited and variable effect on the abundance of the main phyla and classes. In C3 soils, Planctomycetes decreased with the application of biochar (P = 0.027), while an opposite trend was observed for Gemmatimonadetes (P = 0.036). Actinobacteria also tended to decrease after application of high rates of biochar (T4, P = 0.081). In C4 soils, Acidobacteria were significantly decreased after application of biochar dose above 10 tons/ha (P = 0.003) while Chloroflexi and Verrumicrobia showed increased and decreased abundances after application of 5 tons/ha (T2), respectively (Figure 20).
Figure 19. Detection of bacterial (A) and fungal (B) genus in rubber tree and cassava soils: rarefaction curves showing estimated genus richness in relation to sequencing depth per sample.

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha;
Figure 20. Composition of the soil bacterial community at the phylum level (A) and class level (B) in the different site types.

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha;

Within each soil class (C3 and C4), means accompanied by the same letter do not differ significantly at \( P \leq 0.05 \) (pairwise comparisons using the Tukey (HSD) test).
Proteobacteria classes were strongly affected by biochar application in C3 but not C4 soils. Alphaproteobacteria and Betaproteobacteria respectively increased and decreased after application of biochar, regardless of the dose (P = 0.001 and 0.020, respectively). Deltaproteobacteria also tended to increase in abundancy after application of biochar dose, although this result was not significant (P = 0.086).

Species richness was affected by the soil class (P = 0.002) but not by the biochar dose (P = 0.523). However, richness tended to increase with the biochar rate (especially above 10 tons/ha) in C4 but not in C3 soils. A decreasing trend was observed for the diversity indices (expH’ and 1/D). However, it is interesting to note that the application of a low dose of biochar (T2, 5 tons/ha) resulted in a decrease of bacterial diversity in both soils, while higher doses (T3 and T4, 10 and 20 tons/ha, respectively) caused an increase of the bacterial diversity in C4 soils, but not in C3 soils (Table 18).
<table>
<thead>
<tr>
<th>Soil class</th>
<th>Biochar dose</th>
<th>Bacterial communities</th>
<th>Fungal communities</th>
<th>Intensity of AMF colonization (M%)</th>
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<td></td>
<td></td>
<td>richness</td>
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<td></td>
<td>T4</td>
<td>1037</td>
<td>133.90</td>
<td>53.20</td>
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C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha; Pairwise comparisons of the means using the Tukey (HSD) test did not show any significant difference at P ≤ 0.05
PERMANOVA indicated that the bacterial communities associated with both biochar dose and soil class differed significantly, but the effect of the soil class was stronger than that of biochar dose ($P = 0.001$ and $0.015$, respectively). Results of the ADONIS test showed that the effect of biochar was only significant for the soil belonging to the class C4, especially for the doses above 10 tons/ha (T3 and T4) when compared to the plots with no biochar addition. No significant difference was found between the different biochar rates. In C3 soils, biochar application showed no effect on the bacterial community structure.

The NMDS solution confirmed that the community composition was not affected by the biochar application in C3 soils, regardless of the dose (Figure 21). However, the samples from the control plots (T1) of the C4 soils clustered separately suggesting a shift in the composition of the bacterial community after application of biochar in these soils, even at the smallest rate (5 tons/ha). The samples of different biochar doses did not form separate clusters, suggesting that there was no biochar dose effect on the bacterial communities.

6.3.2.2. Fungal community composition, richness and diversity

Fungal communities were dominated by Ascomycota and Basidiomycota (means of 50% and 42% respectively, across the treatments, Figure 22). The most abundant classes were Agaricomycetes and Dothideomycetes which represented more than 40% and 20% of the sequences from all treatment combinations, respectively.

Both Ascomycota and Basidiomycota were affected by the soil class ($P = 0.005$ and $0.008$, respectively), regardless of the biochar treatment. Similar results were obtained at the fungal class level, with 6 out of the 15 classes detected in the samples that were significantly affected by the soil class (data not shown).
Figure 21. Non-metric multi-dimensional scaling (NMDS) plots displaying bacterial communities detected in the soils associated with rubber trees after application of different rates of biochar in two soil classes.

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha;

Ellipses indicate one standard deviation around the centroid position of each biochar dose-soil class combination.
Figure 22. Composition of the soil fungal community at the phylum level (A) and class level (B) in the different site types.

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha;

Pairwise comparisons of the fungal phyla means using the Tukey (HSD) test did not show any significant difference at P ≤ 0.05

Within each soil class (C3 and C4), fungal class means accompanied by the same letter do not differ significantly at P ≤ 0.05 (pairwise comparisons using the Tukey (HSD) test).
None of the detected fungal phyla was affected by the addition of biochar, in both C3 and C4 classes, regardless of the dose (Figure 22). At the fungal class taxonomic level, however, fungal communities were differently affected in C3 and C4 soils. Kickxellomycotina and Eurotiomycetes were affected by the biochar dose in C3 and C4 soils, respectively (P = 0.0002 and 0.017, respectively). Both classes strongly increased in abundance after the application of a biochar dose of 5 tons/ha (T2), in comparison with the treatment without biochar (T1), but were not significantly affected by the higher doses.

Fungal communities were differently affected in C3 and C4 soils (Table 18). In C3 soils, both richness and diversity indices tended to be negatively affected by the application of biochar, in particular for the highest dose of biochar (T4, 20 tons/ha). In C4 soils, biochar soil application had a strong effect on the fungal diversity (P = 0.058 for the 1/D index) and, to a small extent, on fungal richness (P = 0.191). Biochar addition tended to increase the fungal species richness and diversity in comparison to the controls although, surprisingly, there was no effect in the T3 treatment (10 tons/ha) (Table 18).

PERMANOVA showed that both soil class and biochar application had a significant effect on the fungal community (P = 0.001 and 0.019, respectively). ADONIS results showed that the fungal communities were significant affected by the rate of biochar, regardless of the soil class. Application of 10 tons/ha and 20 tons/ha had the strongest effect as compared the control in both C3 (P = 0.051 and 0.022 for T3 and T4, respectively) and C4 soils (P = 0.047 and 0.020 for T3 and T4, respectively).

The NMDS solution showed that the fungal communities associated to different soil classes clustered separately. Samples from T1 (controls, receiving no biochar) formed separate clusters from other biochar treatments, particularly in C3 soils. However, there was no clear
differences between the communities after application of the different doses of biochar, although the biochar dose effect was more evident in C3 than in C4 soils (Figure 23).

6.3.1. AMF colonization

Roots collected in all treatments were highly colonized regardless of biochar dose and soil class (M ≥ 53%) and no fragment was found with no colonization (F = 100%). Biochar application didn’t significantly affect the intensity of mycorrhization in both soil classes (P = 0.160 and 0.122 in C3 and C4 soils, respectively). However, application of biochar at a rate above 10 tons/ha resulted in an increased colonization intensity for both soil classes. Surprisingly, application of a lower dose (T2) caused a decrease of mycorrhization in C4 soils while an increase was observed in C3 soils (Table 18).
Figure 23. Non-metric multi-dimensional scaling (NMDS) plots displaying fungal communities detected in the soils associated with rubber trees after application of different rates of biochar in two soil classes.

C3 and C4: soil classes, T1=0 t of biochar/ha, T2=5 t of biochar/ha, T3=10 t of biochar/ha, T4=20 t of biochar/ha;

Ellipses indicate one standard deviation around the centroid position of each dose-soil class combination.
6.4. Discussion

6.4.1. Importance of the soil variability

The results obtained from all the analyses showed a great effect of the soil class on the different parameters. More importantly, the effects of biochar application were strongly related to the nature of the soil class, both in terms of nature (positive or negative) and intensity. It has been previously reported that biochar’s effects depend strongly on soil characteristics (among other factors), but the variability within a single field trial is rarely taken into account. This supports the preliminary results obtained after 18 months of applications on the soil characteristics and microbial structure (assessed by DGGE) and confirms the importance of performing the analysis of C3 and C4 soil classes separately (see Chapter 5 for details).

6.4.2. Biochar and soil water content

Biochar did not improve the water content in C4 soils which was quite low (< 7%) and surprisingly had a negative effect on the soil water content in C3 soils. Neutral results on soil moisture characteristics were also reported elsewhere (Domene et al. 2014; Noyce et al. 2015). Biochar’s ability to improve water holding capacity, water retention, aggregation and permeability is widely recognized and has been often reported as the main explanation for other effects, such as changes in soil biota (Biederman and Harpole 2013; Ding et al. 2016; Laird et al. 2010).
6.4.3. Biochar and pH

Increases in soil pH following biochar application have been frequently reported (Biederman and Harpole 2013; Ding et al. 2016; Domene et al. 2014) but this liming effect has been shown to be a function of both initial soil and biochar pH. Application of alkaline biochar in acidic soils was shown to result in greater positive responses to biochar than alkaline soils (Biederman and Harpole 2013). However, other factors including the biochar dose were shown to affect soil pH changes (Domene et al. 2014) and long-term studies suggested that pH responses may be transient (Jones et al. 2012; Quilliam et al. 2012). In our study, pH slightly increased with the application of biochar, although the results were significant in C3 soils only.

6.4.4. Biochar and soil nutrient contents

Nutrient contents (apart from N) tended to increase with the biochar rate, regardless of the soil class. Variable biochar-induced changes in soil nutrient contents were previously reported such as increased soil P, N, K, Ca, Mg and Na contents (Biederman and Harpole 2013; Jones et al. 2012; Noyce et al. 2015; Wang et al. 2014a). Neutral effects were also found (Ding et al. 2016; Noyce et al. 2015). Various mechanisms may explain the observed changes. Nutrients may be introduced to the soils through labile organic compounds associated with biochar and become available as these compounds weather (Sohi et al. 2010). However, these effects are presumed to be short-lived and, thus, are unlikely to explain the soil nutrient increase observed in our study (Biederman and Harpole 2013). Long-term effects of biochar addition may be related to changes in soil pH. Increase of soil pH could change the form of some nutrients (and of P in particular) and make them more available for plants or microorganisms (Ding et al. 2016). In low pH soils, P can be adsorbed onto iron oxides, therefore being less
available to plants. Biochar liming effects reduce the concentration of iron and aluminum in soils, releasing P which then becomes available to plants or microorganisms (Biederman and Harpole 2013). Reduction of nutrient leaching due to biochar’s physicochemical properties (porous structure, large surface area, and negative surface charge) is another way biochar may affect soil nutrients on a long-term basis through an increased adsorption to biochar’s surface and consequently reduced leaching (Biederman and Harpole 2013; Laird et al. 2010). In contrast to the other nutrients, N content decreased with the application of biochar, in both soil classes. It has been suggested that N compounds could be retained on biochar due to its sorption properties, thereby affecting soil N content (Brassard et al. 2016). However, effects of soil type, biochar’s characteristics, as well as biochar’s age should not be neglected (Brassard et al. 2016). For example, the aging of biochar changes its physiochemical properties, and these changes may have significant consequences for the bioavailability and transport of nutrients, although the underlying mechanisms are still poorly understood (Mia et al. 2017).

6.4.5. Biochar and soil organic matter

Soil OM content showed contradictory results related to the soil class and tended to increase and decrease in C3 and C4 soils, respectively. Biochar has been shown to interact with various forms of organic matter and both increases and decreases in mineralization of native soil OM were also reported in other studies (Brassard et al. 2016; Maestrini et al. 2014; Zimmerman et al. 2011; Zimmermann et al. 2012). Different conclusions may be due to a variety of factors, including soil type and period over which measurements were made. As SOM content is linked to the pH buffering capacity of soils, long-term effects of biochar on soil pH may also result in long-term changes in OM (Gul et al. 2015).
6.4.6. Effect of biochar on soil bacterial and fungal communities

Many studies have reported effects of biochar on microbial biomass, respiration or community structure using molecular tools such as qPCR, DGGE or T-RFLP (Ding et al. 2016; Domene et al. 2014; Noyce et al. 2015; Prayogo et al. 2014). Further sequencing of the obtained bands is possible to obtain data on community composition and diversity but provides a generally low sequencing depth as compared to high throughput sequencing techniques. To date, studies showing the impact of biochar on microbial community composition and diversity using high throughput sequencing methods are scarce.

In our study site, the bacterial communities were dominated by Proteobacteria (Alphaproteobacteria in particular) followed by Acidobacteria, Actinobacteria and Firmicutes, regardless of the treatment and soil class. However, differences in community composition were found and abundances of phyla and classes were differently affected in C3 and C4 soils. Grossman et al. (2010) showed that the structure of the bacterial community in Brazilian anthrosol soils (naturally containing a high proportion of biochar) differed from that of adjacent soils containing no biochar but having similar mineralogy. Proteobacteria were negatively affected by the presence of biochar while Verrucomicrobia were more specifically found in the anthrosol soils. Similar differences in community composition were found in experiments with soils amended with biochar (Kolton et al. 2017; Luo et al. 2016; Nielsen et al. 2014; Noyce et al. 2015) and differences were related to various phyla including Proteobacteria, Planctomycetes, Verrucomicrobia, Bacteroidetes and Acidobacteria (Chen et al. 2013; Imparato et al. 2016; Nielsen et al. 2014; Su et al. 2017). This is in accordance with our results since we found that biochar addition mainly affected the abundances of several Proteobacteria classes (Alphaproteobacteria, Betaproteobacteria and Deltaproteobacteria) and
Planctomycetes in C3 soils and Acidobacteria and Verrucomicrobia in C4 soils. Interestingly, Nielsen et al. (2014) highlighted the occurrence of associations between members of the phyla Acidobacteria and Verrucomicrobia in the presence of biochar.

Fungal communities were affected by the biochar addition in both soil classes, although the composition was not strongly affected when looking at the high taxonomic levels (phyla and classes). Similar results were obtained by Su et al. (2017) who showed that fungal communities were strongly dominated by Ascomycota and Basidiomycota regardless of the treatment. It could be argued that rare OTU are more affected than abundant ones, thus resulting in community composition changes while maintaining the general abundance of particular phylum or class. Similar observations were made on the changes observed in the bacterial community associated with corn roots after addition of biochar (Nielsen et al. 2014).

Fungal communities have generally been shown to be negatively affected by biochar addition (Chen et al. 2013; Gul et al. 2015; Noyce et al. 2015; Su et al. 2017) and this may also have affected the structure and the composition of the fungal communities. Arbuscular mycorrhizal fungi (AMF) colonization was not strongly affected by the biochar application, although it tended to increase with the biochar dose, regardless of the soil class. Similar positive results were reported by others (as reviewed by Warnock et al. (2007) and Biederman and Harpole (2013).

Several mechanisms have been proposed to explain the effect of biochar on microbial communities and include i) a better protection against predators or competitors by exploring the pores of biochar, thus creating an expanded niche for both bacteria and fungi, ii) the initial addition of soluble nutrients contained in the biochar and an initial pulse in C and mineralization of the labile fraction of biochar itself, associated with the release of numerous volatile and biologically active compounds, iii) reduced nutrient leaching and adsorption of
toxic compounds and iv) the improvement of the water status of the soils through changes in soil aggregation and porosity (Biederman and Harpole 2013; Ding et al. 2016; Domene et al. 2014; Gul and Whalen 2016; Laird et al. 2010; Sohi et al. 2010). The initial status of the soils may affect the direction and the magnitude of each of the mechanisms, which may partly explain the different observations in the two soil classes identified in our study site. Changes in soil characteristics may have explained the observed changes in microbial community, both directly and indirectly. In particular, changes in soil pH and nutrient availability are likely to dictate major changes in microbial community. pH is widely recognized to result in changes in microbial community composition. The increases in pH observed in our study may have positively affected bacteria while reducing growth of fungi as found elsewhere (Domene et al. 2014; Noyce et al. 2015; Prayogo et al. 2014; Rousk et al. 2010). Modification of nutrient availability, and of P and N in particular, may enhance or limit growth and activity of specific groups of microorganisms, such as P-solubilizing bacteria, AMF or bacteria involved in the N cycle process such as nitrification or denitrification process (Biederman and Harpole 2013; Brassard et al. 2016; Ding et al. 2016). Consequently, the changes in the activity of soil organisms can potentially translate to changes in nutrient availability and cause further cascading effects on other microbial groups and trophic relationships.

However, it is important to note that some of these effects may be short lived. A majority of studies were conducted over a short-term period of time (< 1 year) but it is acknowledged that properties of biochar (and thus its consequent effects) change with time (Gul et al. 2015). Sackett et al. (2015) found that most nutrient effects observed within 2 to 6 weeks after addition were no longer present by the end of the first year, and observed changes may fade or opposite effects become apparent. As the residence time of biochar in soil ranges from hundreds to thousands of years, the biochar aging effect should not be ignored. Depending on
their nature and magnitude, changes in microbial community may be transient (due to the resilience ability of the community) or persist for an extensive period of time (Gul et al. 2015; Noyce et al. 2015). Although changes were observed in both bacterial and fungal communities, they did not seem to be related to the applied biochar dose. Similarly, a meta-analysis conducted on 371 biochar studies highlighted the absence of an obvious threshold or trend with increasing application rates (Biederman and Harpole 2013).

6.5. Conclusions

This study showed that biochar has a significant effect on soil characteristics and on bacterial and fungal communities associated with the rubber trees, 28 months after application, regardless of the soil class and of the applied dose. However, the nature and the magnitude of the observed changes differed in the two soil classes. This highlights the importance of the soil variability even within a single trial experiment and the importance of the underlying mechanisms that are still poorly understood, especially over the long-term.

Biochar is generally considered as either beneficial or not toxic to the soils, microorganisms and plants. However, only few studies have been conducted on perennial plants, and our study is the first report on the medium-term effect of biochar on microbial communities associated with rubber trees. More research is of primary importance to assess the long-term effect of biochar application on perennial plantations, including its impact on ecosystem functions and plant productivity.
Rubber tree and latex production is of primary importance in SE Asia, and in Thailand in particular, as it represents an important source of income for the local population. It is believed that rubber tree plantations are often associated with a decrease in soil fertility, however, the real impact on the soil compartment is still largely unknown. In particular, the role of the soil biota, in relation to soil characteristics and soil functions, is still poorly understood. Soils in NE Thailand are mainly sandy and contain very low concentrations of nutrients and organic matter, and are considered unsuitable for the cultivation of many crops. As rubber trees require a moderate level of soil fertility and are able to grow in a wide range of environments (including poor sandy soils), plantations are expanding widely in NE Thailand at the expense of other crops, such as cassava. In order to better manage and sustain soil fertility in this region, alternative management practices have been widely promoted, without strong scientific evidence of their effect on the soils. One such management strategy that has received increased attention in recent decades is the application of biochar. Biochar is the result of the pyrolysis of biomass under a low- or zero-oxygen environment, and is being used as a means for sequestrating carbon into soils while producing secondary agronomic benefits and improving soil functions. However, few studies focused on the long-term effect of biochar into soils and there is, to our knowledge, no such study on rubber tree plantations.

A better understanding of the role of the soil biological compartment in managing soil fertility in rubber tree plantations is thus mandatory. In this study, the soil bacterial and fungal communities were studied along a chronosequence of 3, 6 and 16 year-old rubber tree plantations and compared to those of nearby cassava fields. Arbuscular mycorrhizal fungi (AMF) communities were also included in the study. To assess the effect of biochar
application on the soil bacterial and fungal communities associated with rubber trees, an experiment was set up in a 7 year-old plantation and included four doses of biochar (0, 5, 10 and 20 tons/ha). Both bacterial and fungal communities were characterized 18 and 28 months after biochar application while taking fine-scale soil variability into consideration.

Our results showed that microbial community structure and diversity were more strongly affected by the age of the plantation itself than by the conversion of cassava fields into rubber tree plantations. The opposite trend was observed for the functional microbial diversity, which was more affected by the conversion of the cassava fields than by the age of the trees. Changes were particularly found in functions linked to the use of various carbon sources by soil bacteria, while functions linked to the N cycles remained unaffected. Fungal community was not affected by either the conversion from cassava or the age of the trees. While AMF community composition gradually shifted with the age of the plantation, the richness and the intensity of colonization remained high in all the studied sites. Changes in microbial communities were strongly related to soil physical and chemical characteristics. pH was the main driver of the changes in the bacterial community, while soil P content was more related to the AMF community changes.

This study provided the first results on the microbial communities associated with rubber trees of different ages in poor tropical soils. Despite the fact that rubber tree plantations may not be as detrimental to the soil fertility as may have been expected, our results highlight the need for complimentary research to better understand the underlying mechanisms and the potential roles of the native microbial communities in managing soil fertility of perennial plantations in NE Thailand.

The characterization of the biochar experimental site resulted in the identification of two main soil classes, presenting different soil layering and soil characteristics. Biochar application
differently affected the microbial community with regards to the soil class, both after 18 and 28 months. These results highlight the importance of the soil variability, even at the fine-scale. Fungal communities were generally more severely affected than bacterial communities and no dose-effect from biochar was observed in any of the soil classes and changes were related with changes to the chemical characteristics and the water status of the soils. Although biochar is generally considered as either neutral or beneficial with regards to soil fertility, more research is needed to ensure that the changes observed in the microbial communities are not responsible for changes in soil functions or other ecosystem services.

Results from such research may be of benefit to the national and local authorities that are responsible for plantation management policies. Appropriate use of alternative management practices, such as biochar addition, could thus be promoted with regards to the local environmental conditions of the plantations. This would assist in the maintenance and restoration of soil fertility while limiting the use of mineral fertilizers that are expensive for the local growers.

Subsequent studies should focus on improving the understanding of the underlying mechanisms of the relationships between soil microbial communities and other ecosystem services, including tree nutrition and latex production, both under conventional management and after the application of biochar. Other types of biochar, together with various application strategies (dose, depth, location, period and frequency of applications…) may also result in different results and this deserves further investigation. Since bacteria and fungi are important elements of complex soil food webs, other groups of organisms (including nematodes and other higher trophic levels of fauna) may also be significantly affected and result in substantial changes in soil functioning, but this has been poorly investigated to date. The durability of the changes is also yet to be assessed. The impact of several rotations of rubber
trees on the same site, for instance, is unknown. In the case of biochar addition, as its residence time in soils is very long (hundreds to thousands of years), even small changes may result in long-lasting or irreversible consequences for soil functions and services but there is no information to answer to this question at this stage. In addition, biochar was shown to result in sometimes opposite effects with regards to the soil class, and more research is also needed to better understand the relationships between the effect of the biochar and the soil class (with different layering, having a direct effect on the water behavior in the different soil layers) and soil properties in general before its widespread application in a range of soils and conditions.

It is important that we better understand the role of the microbial communities in relation to other aspects of rubber tree plantation management and in different soil types to ensure that suitable and sustainable management practices are promoted in order to maintain and possibly restore the soil fertility of strongly depleted soils in tropical regions. More sustainable rubber tree management practices would also benefit the local populations through a reduced use of expensive inputs (mineral fertilizers in particular) and an increased source of income (higher latex yield).

The approaches developed in our study may be also useful in improving the management of other perennial plantations in the region, including oil palm and coffee tree plantations. Indeed, the roles of the microbial compartment in soil fertility in these agricultural systems are poorly understood and this deserves deeper investigation. As with rubber trees, sustainable economic benefits may result from improved management practices through the restoration of soil fertility.
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Appendix 1

Authorship statement – Chapter 1

1. Details of publication and executive author

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<tr>
<td>Philippe Hinsinger</td>
<td>INRA, UMR Eco&amp;Sols, Montpellier, France</td>
<td><a href="mailto:philippe.hinsinger@supagro.inra.fr">philippe.hinsinger@supagro.inra.fr</a></td>
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2. Inclusion of publication in a thesis

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<td>School of Life and Environmental Sciences</td>
<td>The diversity of microbial communities associated with rubber tree plantations in North-East Thailand</td>
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If there are multiple authors, give a full description of HDR thesis author’s contribution to the publication (for example, how much did you contribute to the conception of the project, the design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)

Drafting part of the manuscript, revising the manuscript for submission

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

Signature and date: April 12th, 2017

4. Description of all author contributions

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<th>Name and affiliation of author</th>
<th>Contribution(s) (for example, conception of the project, design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)</th>
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<tr>
<td>Philippe Hinsinger</td>
<td>Drafting the manuscript, reviewing the manuscript</td>
</tr>
<tr>
<td>Didier Lesueur</td>
<td>Drafting part of the manuscript, revising the manuscript</td>
</tr>
<tr>
<td>Agnès Robin</td>
<td>Drafting part of the manuscript, revising the manuscript</td>
</tr>
<tr>
<td>Jean Trap</td>
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<tr>
<td>Kittima Waithaisong</td>
<td>Drafting part of the manuscript, revising the manuscript</td>
</tr>
<tr>
<td>Claude Plassard</td>
<td>Drafting part of the manuscript, revising the manuscript</td>
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5. Author Declarations

I agree to be named as one of the authors of this work, and confirm:

i. that I have met the authorship criteria set out in the Deakin University Research Conduct Policy,

ii. that there are no other authors according to these criteria,

iii. that the description in Section 4 of my contribution(s) to this publication is accurate,

iv. that the data on which these findings are based are stored as set out in Section 7 below.

If this work is to form part of an HDR thesis as described in Sections 2 and 3, I further

v. consent to the incorporation of the publication into the candidate’s HDR thesis submitted to

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<table>
<thead>
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<th>Name of author</th>
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<td>Philippe Hinsinger</td>
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<td>Jean Trap</td>
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<tr>
<td>Kittima Waihaisong</td>
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<td>20/04/2017</td>
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<tr>
<td>Claude Plassard</td>
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<td>14 04 2017</td>
</tr>
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</table>
6. Other contributor declarations

I agree to be named as a non-author contributor to this work.

<table>
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<th>Name and affiliation of contributor</th>
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This form must be retained by the executive author, within the school or institute in which they are based.

If the publication is to be included as part of an HDR thesis, a copy of this form must be included in the thesis with the publication.
Appendix 2

Authorship statement – Chapter 3

1. Details of publication and executive author

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<th>Title of Publication</th>
<th>Publication details</th>
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<tr>
<td>High colonization by native arbuscular mycorrhizal fungi (AMF) of rubber trees in small-holder plantations on low fertility soils in North East Thailand</td>
<td>ARCHIVES OF AGRONOMY AND SOIL SCIENCE, 2016 VOL. 62, NO. 7, 1041–1048 DOI: 10.1080/03650340.2015.1110238</td>
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<table>
<thead>
<tr>
<th>Name of executive author</th>
<th>School/Institute/Division if based at Deakin; Organisation and address if non-Deakin</th>
<th>Email or phone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laetitia Herrmann</td>
<td>School of Life and Environmental Sciences</td>
<td><a href="mailto:lherrman@deakin.edu.au">lherrman@deakin.edu.au</a></td>
</tr>
</tbody>
</table>

2. Inclusion of publication in a thesis

<table>
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<tr>
<th>Is it intended to include this publication in a higher degree by research (HDR) thesis?</th>
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3. HDR thesis author’s declaration

<table>
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<tr>
<th>Name of HDR thesis author if different from above. (If the same, write “as above”)</th>
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If there are multiple authors, give a full description of HDR thesis author’s contribution to the publication (for example, how much did you contribute to the conception of the project, the design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)

Conception of the project, sample collection, lab and data analyses, drafting the manuscript, revising the manuscript for submission

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

<table>
<thead>
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<th>Signature and date</th>
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</table>
4. Description of all author contributions

<table>
<thead>
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<th>Name and affiliation of author</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Lambert Bräu</td>
<td>Conception of the project, revising the manuscript</td>
</tr>
<tr>
<td>Agnès Robin</td>
<td>Conception of the project, revising the manuscript</td>
</tr>
<tr>
<td>Henri Robin</td>
<td>Conception of the project, assistance with the statistical analysis, revising the manuscript</td>
</tr>
<tr>
<td>Wanpen Wiriyakittleekul</td>
<td>Contribution in the lab analysis, revising the manuscript</td>
</tr>
<tr>
<td>Didier Lesueuer</td>
<td>Conception of the project, sample collection, revising the manuscript</td>
</tr>
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5. Author Declarations

I agree to be named as one of the authors of this work, and confirm:

vi. that I have met the authorship criteria set out in the Deakin University Research Conduct Policy,

vii. that there are no other authors according to these criteria,

viii. that the description in Section 4 of my contribution(s) to this publication is accurate,

ix. that the data on which these findings are based are stored as set out in Section 7 below.

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Appendix 3

Authorship statement – Chapter 4

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<th>Name of HDR thesis author if different from above. (If the same, write “as above”)</th>
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Conception of the project, sample collection, lab and data analyses, drafting the manuscript, revising the manuscript for submission

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

Signature and date: April 12th 2017

Signature Redacted by Library

4. Description of all author contributions

<table>
<thead>
<tr>
<th>Name and affiliation of author</th>
<th>Contribution(s) (for example, conception of the project, design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)</th>
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<tbody>
<tr>
<td>Didier Lesueur</td>
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</tr>
<tr>
<td>John Davison</td>
<td>Statistical analysis, revising the manuscript</td>
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<tr>
<td>Teele Jairus</td>
<td>Lab analysis, revising the manuscript</td>
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<tr>
<td>Henri Robain</td>
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<tr>
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<td>Lab analysis, revising the manuscript</td>
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<tr>
<td>Maarja Öpik</td>
<td>Conception of the project, data analysis, revising the manuscript</td>
</tr>
</tbody>
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207
5. Author Declarations

I agree to be named as one of the authors of this work, and confirm:

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xiv. that the data on which these findings are based are stored as set out in Section 7 below.

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Appendix 4

Figure S1: Phylogenetic tree (NJ tree, Bootstrap n=100) of SSU rRNA gene sequences of earlier described and novel AMF VT detected in rubber tree and cassava roots. Sequences obtained in this study are indicated in black and type sequences of AMF VT from MaarjAM database are indicated in red. Novel VT are indicated in green.
Appendix 5

Figure S2: Detection of AMF virtual taxa (VT) in rubber tree and cassava roots using 454 sequencing of SSU rRNA gene fragments: (a) rarefaction curves showing estimated VT richness in relation to sequencing depth per sample; and (b) species accumulation curves showing estimated VT richness in relation to sample size per site type.
Appendix 6

Authorship statement – Chapter 5

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<td>Relevance of taking into account the fine scale soil variability to assess the effects of agricultural inputs on soil characteristics and soil microbial communities: a case study of biochar application in a rubber plantation in North East Thailand</td>
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Signature and date: April 25th 2017

Signature Redacted by Library

4. Description of all author contributions

<table>
<thead>
<tr>
<th>Name and affiliation of author</th>
<th>Contribution(s) (for example, conception of the project, design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marie Elodie Le Guen</td>
<td>Lab and data analysis, revising the manuscript</td>
</tr>
<tr>
<td>Henri Robain</td>
<td>Conception of the project, revising the manuscript</td>
</tr>
<tr>
<td>Wanpen Wiriyakhitateekul</td>
<td>Lab analysis, revising the manuscript</td>
</tr>
<tr>
<td>Tatiana de Oliveira</td>
<td>Student supervisor (ME Le Guen)</td>
</tr>
<tr>
<td>Agnès Robin</td>
<td>Conception of the project, revising the manuscript</td>
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<tr>
<td>Prapaipit Srimawong</td>
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<td>Conception of the project, revising the manuscript</td>
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