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Diversiteit en functie van arbusculaire mycorrhiza van Vicia faba in de tropische hooglanden van Ethiopië

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List of symbols and abbreviations

AEM anion exchange membranes
AIC Akaike information criterion
Al Aluminium
AMF arbuscular mycorrhizal fungi
ATP adenosine triphosphate
BNF biological nitrogen fixation
Bp base pairs
C Carbon
Ca Calcium
DI distilled water
DNGE denature gradient gel electrophoresis
dw Dry weight
Fe Iron
K potassium
m.a.s.l. meters above sea level
MS mother solution
N nitrogen
N₂ atmospheric nitrogen or dinitrogen
NH₃ ammonia
NMDS non-metrical multidimensional scaling
OTU Operational Taxonomic Units
P phosphorus
PCR polymerase-chain reaction
pNP para-nitrophenol
pNPP para-nitrophenylphosphate
PUE phosphor use efficiency
Summary

Knowledge about the presence and diversity of arbuscular mycorrhizal fungi (AMF) is essential due to their potential role in sustainable intensification of P limiting areas. Additionally, determining which factors drive the diversity of AMF and how this diversity effects the plant, is important in understanding the role of these micro-organisms. In this study, conducted in the highlands of Ethiopia, we used 454-pyrosequencing on 75 root hairs of *Vicia faba* distributed on 15 fields along an altitude range between 2155 and 2306 meters above sea level. We aimed at identifying factors like total soil P, available soil P, soil pH and altitude that affect the AMF diversity and community. Furthermore we aimed to assess the effects of this AMF diversity on the phosphatase activity in the soil and the P content in the plant. Additionally we investigated the interaction of AMF and N$_2$-fixation. In total, 81 OTU’s were detected, of which the majority belonged to the Glomeraceae (36). On average, 18.6 ± 1.1 AMF taxa per plant were detected and the AMF species *Claroideoglomeraceae Claroideoglomus sp.* was detected in 92% of the analysed samples. Furthermore, we showed that the community was significantly determined by the factor field. This indicates that the community is determined by local biotic and abiotic factors. We also found that the diversity was significantly positive correlated with the total soil P content, while it was significantly negative correlated with the pH. Additionally, we found that the phosphatase activity was significantly positive correlated with AMF richness, however no correlation between AMF diversity or richness and the P content of the plant was observed. Finally, we observed a significantly negative correlation between the N$_2$ fixed per g plant and the AMF diversity and AMF richness, suggesting a negative interaction between *Rhizobium* and AMF colonization. Overall we conclude AMF communities are present in the agricultural system in the highlands of Ethiopia, and plants can benefit from their diversity. Secondly, under photosynthate limiting conditions, AMF and *Rhizobia* may compete for the same resources.
Samenvatting

Arbusculaire mycorrhiza (AM) kunnen een belangrijke rol vervullen in duurzame intensivering van de landbouw in fosfor (P) limiterende regio’s. Daarom is het belangrijk om kennis te vergaren over hun aanwezigheid en diversiteit. Bovendien, is het belangrijk om de factoren die de diversiteit van deze AM bepalen, te identificeren. Net zoals het belangrijk is om de effecten van deze diversiteit op de plant te bepalen. In deze thesis, hebben we 454-pyrosequencing toegepast op de wortelharen van 75 Vicia faba stalen die verdeeld waren over 15 velden langs een helling in de hooglanden van Ethiopië tussen 2155 en 2306 meter boven zeeniveau. Het was de bedoeling om de factoren die de diversiteit bepalen te identificeren. We hebben daarom de totale bodem P concentratie, beschikbare P, pH en de hoogte boven zeeniveau gemeten voor elk staal. Daarnaast hebben we ook het effect van de AM diversiteit op het P gehalte van de plant en de fosfatase activiteit in de bodem onderzocht. Als laatste was het ook de bedoeling om de interactie tussen AM en N\textsubscript{2}-fixerende bacteriën te onderzoeken.

In dit onderzoek, vonden we 81 OTU’s, waarvan 36 geclasseificeerd werden bij de Glomeraceae. Gemiddeld werden er 18.6 ± 1.1 verschillende taxa gedetecteerd per plant en in 92 % van de gevallen werd de plant gekoloniseerd door de soort Claroideoglomeraceae Claroideoglomus sp. Bovendien toonden we aan dat de AM gemeenschap voornamelijk bepaald werd door de parameter ‘Veld’. Daarnaast was de diversiteit significant positief gecorreleerd met de totale bodem P concentratie en negatief gecorreleerd met de pH. Bovendien, vonden we geen correlatie tussen de AM diversiteit en het P gehalte in de plant, alhoewel we een positieve correlatie vonden met de soortenrijkdom van AM met de fosfatase activiteit. Finaal observeerden we een negatieve correlatie tussen de soortenrijkdom van AM en de hoeveelheid stikstof gefixeerd per gram plant. Dit laatste lijkt aan te tonen dat er negatieve interacties zijn tussen AM en Rhizobium kolonisatie. In het algemeen besluiten we dat AM aanwezig is in de landbouwsystemen van de hooglanden van Ethiopië, en dat de diversiteit van gemeenschap de plant positief kan beïnvloeden. Daarnaast vonden we ook dat er onder fotosynthese limiterende omstandigheden competitie kan optreden tussen AM en Rhizobia bacteriën.
1. Introduction

The highlands of Ethiopia face a growing agricultural problem. Population growth, due to immigration from other regions in Ethiopia, causes an increasing food demand and the reduction of farm sizes (Nebiyu, 2014). Increased production can supply the growing food demand. However, agricultural intensification is inhibited by land degradation, and low access and plant response to fertilizer. A more sustainable and feasible solution seems the introduction of N$_2$-fixing and P efficient grain legumes in the traditional cropping systems (Belane & Dakora, 2009).

*Vicia faba* has the potential to enhance N and P nutrition of cereals (Habtemichial et al., 2007; Jia et al., 2004; Nuruzzaman et al., 2005). Additionally, the legume is of great importance in legume-cereal production systems where it is used as break crop for cereals (Amanuel et al., 2000). The key is that faba bean forms a symbiosis with nitrogen fixing bacteria and thus introduces N into the agricultural system.

Furthermore, *Vicia faba* form a second symbiosis with arbuscular mycorrhizal fungi (AMF). These AMF enhance the plant water and nutrient uptake, in exchange for carbon (Smith & Read, 2010). Especially, P uptake is enhanced and thence it is of importance in P limiting areas, like the highlands of Ethiopia (Smith & Smith, 2011).

The diversity of AMF tends to be negatively correlated with the land use intensity and available soil P content (Van Geel et al., 2015; Oehl et al., 2003). Therefore we can expect a high diversity of AMF species in the highlands of Ethiopia. The effect of AMF diversity on the P uptake, and the N$_2$-fixation is less known. Also the circumstances that effect the diversity like pH, total soil P, bioavailable P, and altitude are less investigated, in particular with next generation sequencing methods. Thus, the aim of this thesis is to observe these effects and help us understand the role of the diversity of AMF in Vicia faba in the tropical highlands of Ethiopia.

1.1 Description of the study area

Ethiopia is located in east Africa in the sub Saharan region between ca. 3°N and 15°N latitudes, and 33°E and 48°E longitudes (Figure 1). It covers an area of 1.1 million km$^2$ and has a population of nearly 97 million inhabitants which makes it the worlds most populated landlocked country (The World Bank, 2015). There’s a considerable variation of climate due to the wide altitude which ranges from 155 meters below sea level to 4620 meters above sea level (Billi, 2015). The Ethiopian landmass consists of a large,
elevated plateau bisected by the Rift Valley into the north-western and the south-eastern highlands (Figure 1).

The Ethiopian population has grown from 76 million in 2000 till ca. 100 million in 2016. The yearly increase of inhabitants was 2.48 % between 2015 and 2016 and it is estimated that Ethiopia will have around 190 million inhabitants by the year 2050 (Worldometers, 2015). This growing population will inevitable increase food demand. In the highlands, this population pressure also reduces the farm size. Some farms are already smaller than 1 ha (Nebiyu, 2014).

The Ethiopian highlands is the main agricultural production area. It covers 50% of the country’s area but hold 90% of its total population, over 95% of its cropped lands and about two thirds of its livestock population. This region has become one of the most severely affected by land degradation in the sub-Saharan region. This is due to poor resource management, poor governance, institutional instability, lack of appropriate policy and to the lack of participation of communities in the decision-making process in the past (Zeleke, 2003).

The Gilgel-Gibe catchment (Figure 2) is located in the south-western highlands. This catchment has an area of 4225 km² with an altitude range of 1689-3018 meters above sea level (Nebiyu, 2014). Three points will be discussed further more in detail, i.e. soil, climate, and the agricultural system.

1.1.1 Soil characteristics

Nitisols are the most common soils in the highlands of the humid tropics (WRB, 2015). Compared to other red tropical soils, Nitisols are far more productive. The soil has good workability, internal drainage, and water holding properties. Nevertheless, low yields often occur due to the high P sorption of the soil, leading to P deficiencies (WRB, 2015).

The Gilgel-Gibe catchment is vulnerable to landslides. This is caused by the three main reasons: heavy annual rainfall, cultivation of most of the area, and in lesser extend steep slopes. These phenomena occur increasingly due to land use change of forest into agricultural land (Broothaerts et al., 2012). Figure 3 shows an example of a landslide observed near the study area.

Depletion of soil nutrients is one of the fundamental causes for declining food production per capita in Ethiopia. At the national level, a depletion rate of 122 kg N ha⁻¹ yr⁻¹, 13 kg P ha⁻¹ yr⁻¹ and 82 kg K ha⁻¹ yr⁻¹ is reported (Haileslassie et al., 2005). In the highlands of Ethiopia a depletion rate was calculated between 53-58 kg N ha⁻¹ yr⁻¹, 8-11 kg P ha⁻¹ yr⁻¹, and 48 – 53 kg K ha⁻¹ yr⁻¹ (Aticho, Eyasu, & Diels, 2011). Furthermore, soil erosion contributes respectively 70, 80, and 63 % of the total N, P, and K depletion. Though, this flux showed a significant variability.

Generally, nutrient losses under permanent and vegetable cropping were caused by the removal of residues and harvested products, while losses under cereals were dominated by erosion (Haileslassie et
al., 2005). However, this correlation doesn’t always hold and the magnitude of nutrient fluxes and their degree of imbalance are greatly influenced by the environmental condition, farming system (e.g. choice of crop), access to resources (e.g. land, livestock and fertilizer) and smallholders’ source of off farm income (Haileslassie et al., 2007).

1.1.2 Climate

The climate of Ethiopia is mainly controlled by the inter tropical convergence zone and the complex topography, leading to considerable variations in the country (Billi, 2015). The highlands encounter both a temperate as a tropical climate with an annual temperature ranging between 10 and 20°C.

In Jimma, the main city of the Gilgel Gibe catchment, the annual mean temperature is 19.7°C and the average annual precipitation is 1500 mm. The main rain season lasts from April to September with two peaks in precipitation in June and September (Figure 4).

1.1.3 Agricultural system

The agricultural system in the highlands of Ethiopia is characterized by subsistence crop-livestock mixed farming dominated by cereal-based cropping systems (Getachew Agegnehu & Yirga, 2009). Plants are cultivated on small plots ranging in size from <1 ha to over 3 ha. Most abundant crops are cereals (wheat, teff, and barley) and legumes (faba bean and field peas) (Nebiyu, 2014). Another important crop in the highlands is enset (*Ensete vertricosum*), an indigenous banana-like perennial crop. It’s usually planted around the homesteads and used for its starchy pseudostems. The starch is fermented into a drink. Mostly nutrients are allocated to the high demanding enset gardens, therefore nutrient accumulations occur in the enset garden areas and depletions occur outfields (Figure 5) (Haileslassie et al., 2005).

Low productivity, poor response of crops to chemical fertilizers, and very high cost of chemical fertilizers are the key constraints of the local agriculture (Nebiyu, 2014). Mostly more N is removed from the soil than replenished causing depletion of soil nutrients and land degradation (Cocking, 2009). Additionally, a growing populations increases the pressure (CSA, 2007; Worldometers, 2015). This calls for a sustainable agricultural intensification.
Figure 1 The main geomorphological regions of Ethiopia (Billi, 2015)

Figure 2 Map of the Gilgel Gibe catchment and the study area (based on Ranst et al., 2011)
Figure 3 Photograph of a landslide, taken close to one of the fields in the study.

Figure 4 Average rainfall, average maximum temperature (tmax) and average minimum temperature (tmin) per month of 2012-2013 in Jimma, based on climatic data from 2012-2013 received from the National Meteorological Agency.

Figure 5 Illustration of an enset garden in the back and a faba bean field in front
2. Literature review

The aim of this thesis is to understand the role of the diversity of Arbuscular Mycorrhizal fungi (AMF) associated with *Vicia faba* in Ethiopian Agriculture. Therefore we set up three objectives.

Our first objective is to assess AMF richness and diversity and investigate if there’s an effect of environmental parameters like pH, altitude, total soil P and available soil P. In particular, we believe soil P is a key determinant and that P limiting conditions increase the AMF diversity. Furthermore we want to determine the factors of the AMF communities.

The second objective is to assess the effect of the observed diversity on the plant performance, and in particular the P uptake and phosphatase activity. The effects of specific species of AMF have been observed before, but less investigation went to the effect of a divers AMF community.

And the last objective is to investigate the tripartite symbiosis of AMF, *rhizobia* bacteria, and *Vicia faba*. In particular we’ll investigate the effect of the diversity on BNF and the amount of nodules.

To accomplish this we use 454 pyrosequencing on 75 root samples of *Vicia faba* from 15 fields located along an altitude gradient between 2155 and 2306 m.a.s.l. But first, the four main aspects of this study i.e. Vicia Faba, AMF, BNF, and soil phosphorus are further explained in this literature review. The emphasis will lay on the interaction between these aspects.

2.1 Faba bean

2.1.1 Description

Faba bean, also known as broad bean, horse bean or fava bean (Figure 6) is a crop belonging to the genus of *Vicia* L., which is family of the *Fabaceae* (Smýkal et al., 2014). This open pollinator can grow up to 2 m tall and its tap root can grow down to 1,5 m underground. The white flowers with purple
markings grow in the axils of the leaves in clusters of 1 to 5. The pods are greenish to black and can grow 8 to 20 cm and contain 3 to 4 seeds.

Figure 6 Illustration of Vicia faba (Hallier, 1886)

The grain legume, faba bean, is grown world-wide as a source of protein for food and feed and is commonly included in the diet of Ethiopians and Chinese (Jensen et al., 2010). The world cultivated area was estimated at 2.4 million hectares in 2014 (FAOSTAT, 2015) of which China cultivates the most, followed by Ethiopia, Morocco and Australia (Table 1). Since 1999, production of faba bean in Ethiopia has steadily increased (Figure 7) which resulted in an annual production of more than 800 kton in 2014. The Ethiopian average grain yield (1.9 ton ha\(^{-1}\)) is comparable to the world’s average of 1.8 ton ha\(^{-1}\). Although, beans are considered of good nutritional value due to its high protein (294 g/kg) and starch (443 g/kg) content (Crépon et al., 2010; Sauvant, Perez, & Tran, 2002). The seeds can contain tannins, vicine and convicine, which have been demonstrated to have anti-nutritional effects on monogastric animals (Crépon et al., 2010; Olaboro et al., 1981).

Table 1 Production statistics in the four countries with the biggest faba bean cultivated area (FAOSTAT, 2015)

<table>
<thead>
<tr>
<th>Unit</th>
<th>Area 1000 ha</th>
<th>Area %</th>
<th>Yield ton ha(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>925</td>
<td>39%</td>
<td>1,7</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>443</td>
<td>19%</td>
<td>1,9</td>
</tr>
<tr>
<td>Morocco</td>
<td>191</td>
<td>8%</td>
<td>0,9</td>
</tr>
<tr>
<td>Australia</td>
<td>142</td>
<td>6%</td>
<td>2,2</td>
</tr>
<tr>
<td>World</td>
<td>2395</td>
<td>100%</td>
<td>1,8</td>
</tr>
</tbody>
</table>
Faba beans can grow in diverse conditions. In subtropical areas, they are mostly grown as a winter annual plant. As the crop is late maturing, it benefits from longer growing seasons. Furthermore, faba beans grow best on rich loams, but tolerate nearly any soil type. In particular, the plant is more tolerant to acid soil conditions than other legumes. Its optimum lays between pH of 4.5-8.3 (Duke, 2012). Additionally, optimal growth is established in the temperature range from 18 to 27 °C, but excessive heat in the growing season is undesirable (Jensen et al., 2010).

2.1.2 Faba bean for sustainable intensification

According to Jensen et al. (2010), faba beans contribute to the sustainability of cropping systems via: (1) its ability to contribute nitrogen (N) to the system due to biological N₂ fixation, (2) diversification of systems leading to decreased disease, pest and weed build-up and potentially increased biodiversity, and (3) providing food and feed rich in protein. They form symbioses with the soil bacteria *Rhizobium leguminosarum* bv. *viciae* and with arbuscular mycorrhizal fungi (AMF). As most soils contain population of indigenous rhizobia and mycorrhiza, inoculation is usually not needed. These rhizobia fix atmospheric nitrogen (N₂) into ammonia (NH₃), which is available for the plant. AMF can enhance water and nutrient uptake for the plant in exchange for carbon. Partly due to this symbiosis, faba bean is an option for sustainable agricultural intensification in regions like the highlands of Ethiopia (Nebiyu, 2014).

Growing the legumes and incorporating the residues can significantly enhance yields of subsequent crops such as cereal or other crops (Amanuel et al., 2000; Wani et al., 1994). Growing faba bean before wheat or barley could induce respectively a yield increase of 20 and 21 % (Kirkegaard et al. 2008;
Wright 2011). In Ethiopia, a wheat grain yield increase of 36% was established in faba bean- wheat rotation compared to a barley-wheat rotation (Habtemichial et al., 2007). In the region around Jimma, a grain yield increase of 65% and a total biomass increase of 112% compared to a wheat-wheat rotation was established using the faba bean variety Moti instead of a local variety (Nebiyu et al., 2014). In the following section, the pre-crop effects will be subdivided in nitrogen, phosphorus, and non-nutrient effects.

2.1.3 Nitrogen effects

Faba bean have the largest N₂ fixing capacity compared to pea (Pisum sativum), chickpea (Cicer arietinum), and lentill (Lens culinaris). The world overall mean amounts 154 kg shoot N ha⁻¹ (Jensen et al., 2010). The cultivation of faba bean before cereals can save up to 100-200 kg N ha⁻¹ of N-fertilizers (Jensen et al., 2010; López-Bellido & López-Bellido, 2001), However, other researchers suggest a much lower net effect if the soil N balance is taken in account (< 27 kg N ha⁻¹) (Evans et al., 1991). Furthermore, in some studies there is still a yield enhancing effect on the second and third crop following faba bean, nonetheless the effect is the most remarkable in the first crop following faba bean (Paré et al. 1993; wright 2011).

In the research of Nebiyu (2014) in the region of Jimma, the beneficial effects of the crop rotation faba bean-wheat are confirmed. Compared to the rotation wheat-wheat, a total plant N yield increase of 84% occurred in wheat following the variety Moti and a total grain N yield increase of 92% occurred in wheat following the faba bean variety Obse. In both Moti and Obse rotations, there was a higher plant and grain N yield effect compared to the rotation of a local variety (Table 2).

It is important to take the net N balance of the soil into account. N₂-fixation brings N into the system but harvesting legumes and using crop residues removes N from the system (M. Peoples & Craswell, 1992). The net balance of N to the system will depend on the amount of residues staying in the system. Furthermore, legumes take up N from the soil and thus not all N is derived from fixation. Therefore, the net N balance will depend on the percentage N derived from atmospheric N (%Ndfa) in the legume and it’s residues (M. B. Peoples et al., 2001). It has been shown that in low-input systems, that on top of harvesting the grains, also using the crop residues as animal feed or source of energy, can sharply reduce the net N balance to a neutral or even negative balance (Nebiyu, 2014). Also, as there is a gap between incorporating residues and uptake of N by the following crop, there is a risk for N losses via nitrate leaching or denitrification (Jensen et al., 2010). These plants residues are the most important transfer of N to the following crop but have additional benefits like adding organic matter and P. And therefore, farmers have to be convinced that they benefit more from plant residues used as a source of nutrients (Bakht et al., 2009; Giller, 2001).
2.1.4 Phosphorus effects

Introducing faba bean into the crop rotation improves the P availability for the subsequent wheat and thereby better growth in soils where P availability was the main growth limiting factor, especially when the faba bean has received P fertilizer (Nebiyu, Vandorpe, et al., 2014; Nuruzzaman et al., 2005). Nebiyu et al. (2014) found that compared to a wheat-wheat rotation, total plant P yield increased 117% in wheat following the variety Moti and total grain P yield increased 95% in wheat following the variety Obse (Table 2).

The key is that faba beans are efficient in P uptake and thereby improve the P availability for the next plant. Only a small proportion of the soil P is available for plants, due to chemical precipitation and physical adsorption (Barrow, 1980). Therefore improving P acquisition is essential for sustainable cropping systems (Lynch, 1998; Otani et al., 1996). Mostly, the acquisition is enhanced by increased root density, length, and surface area as movement of P is the most limiting factor for the P acquisition (Barber, 1995; Lambers et al., 1998). Nuruzzaman et al. (2005) have observed that this enhanced root biomass is the reason why plant P content has been observed higher in Vicia faba than in wheat plants at the same soil P level. Furthermore, faba beans improve the bioavailability of P for the following crop. This is proven as both growth and P concentration increase in the following crop. If the beneficial processes other than increased P availability would take place, growth would increase but P content would stay constant or decrease.

This increased P bioavailability for the following crop can be explained by three processes taking place in Vicia faba. First, as mentioned before, P uptake in faba beans is high because of their big root system (Nuruzzaman et al., 2005), but also the symbiosis with AMF increase the P acquisition in faba beans (Smith & Read, 2010). These two effects increase the P content in the plant in general. Roots mostly stay behind for the next season, but sometimes shoot biomass too. The recycling of these plant residues produce available phosphor for the following crop (Horst et al., 2001). Secondly exudation of carboxylates produced by legumes can improve P bioavailability. In general, P can form strong complexes with aluminum and iron oxides in the soil, but carboxylates like malate and citrate compete with phosphates on these binding places, liberating P in the soil solution. However, this is more characterizing for legumes like white lupin and pigeon pea, but it is not excluded that Vicia faba produce other active compounds like organic anions or phenolics that have the same effect as citrate and malate (Nuruzzaman et al., 2005). However, it is unlikely that exuded carboxylates have a direct effect on the subsequent crop, since carboxylates are subject to rapid turnover in soil, depending on temperature and soil type (Jones et al., 2003; Jones, 1998).

Finally, it has been observed that N2-fixing faba beans absorbs more cations than anions. Therefore, to balance the plant’s internal charge, protons are released, acidifying the rhizosphere (Köpke & Nemecek, 2010). A pH of 6.5 is optimal for P availability and further acidification increases the solubility of
aluminium and iron cations which form very strong bonds with P, unavailable for plant uptake (Hinsinger, 2001).

Table 2 N and P yield of rotational wheat and the per cent increase over the wheat–wheat rotation as affected by pre-crop faba bean varieties and wheat. The N and P yield measurements are based on the dry yield. The values are means ± SE. (+) indicates that crop residues were retained and (-) shows they were removed (Nebiyu, Vandorpe, et al., 2014)

<table>
<thead>
<tr>
<th>Pre-crop</th>
<th>Grain</th>
<th>% increase over wheat(%)</th>
<th>Total plant</th>
<th>% increase over wheat(%)</th>
<th>Grain</th>
<th>% increase over wheat(%)</th>
<th>Total plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degaga(+)</td>
<td>38.3 ±5.1</td>
<td>27</td>
<td>48.6 ±7.7</td>
<td>15</td>
<td>4.0 ±1.3</td>
<td>8</td>
<td>6.7 ±0.9</td>
</tr>
<tr>
<td>Motl(+)</td>
<td>52.4 ±5.4</td>
<td>74</td>
<td>77.3 ±6.7</td>
<td>84</td>
<td>5.5 ±1.6</td>
<td>49</td>
<td>11.7 ±1.1</td>
</tr>
<tr>
<td>Obse(+)</td>
<td>57.8 ±6.8</td>
<td>92</td>
<td>55.9 ±12.7</td>
<td>33</td>
<td>7.2 ±1.3</td>
<td>95</td>
<td>8.2 ±2.3</td>
</tr>
<tr>
<td>F-test</td>
<td>*</td>
<td>*</td>
<td>ns</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD&lt;sub&gt;0.05&lt;/sub&gt;</td>
<td>14.2</td>
<td>32.2</td>
<td>3.7</td>
<td>5.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reference
crop (control)

Local faba(-) 39.0 ±0.4 29 48.6 ±15.1 15 4.5 ±0.8 22 6.4 ±1.5 19
Wheat(-) 30.1 ±1.1 - 42.1 ±7.9 - 3.7 ±3.7 - 5.4 ±0.3 -

* = significant at P <0.05; ns = not significant

2.1.5 Non-nutrient effects

By introducing faba bean, the monoculture of cereals is broken, therefore it has the possibility to suppress soil-borne diseases such as Gaeumannomyces graminis and other diseases or pests. Besides this, faba beans also reduce the incidence of grassy weeds. Incorporating the residues also introduces carbon to the soil, which improves soil structure and stimulates microbial activity. All this mentioned before, improves the yield (J. McEwen et al., 1990; Jensen et al., 2010; Kirkegaard et al., 2008; Rochester et al., 2001).

2.1.6 Limits of faba bean

The unstable grain yields, the susceptibility to a range of foliar fungal diseases, and the occurrence of parasitic weeds (Orobanche crenata) in the Mediterranean area make faba beans less popular for farmers than other crops (Jensen et al., 2010; Pérez-de-Luque et al., 2010; Stoddard et al., 2010). Diseases that occur frequently are chocolate spot (Botrytis fabae), Ascochyta blight (Ascochyta fabae), Cercospora leaf spot (Cercospora zonata), and Downy mildew (Perenospora viciae).

2.2 Arbuscular mycorrhizal fungi

All AMF are asexual obligate symbiotic organisms that belong to the phylum Glomeromycota (Schüßler et al., 2001). The phylum consists of one class i.e. Glomeromycetes, which is divided in the four orders Glomerales, Diversisporales, Archeosporales, and Paraglomerales. There are in total ten families and the most abundant are Diversisporaceae, Acaulosporaceae, Gigasporaceae, Claroideoglomeraceae and
Archeosporaceae (Redecker et al., 2013). Despite that arbuscular mycorrhiza are part of a genetic distinguishable phylum, they form together with Ecto-, Ericoid, Orchid, Arbutoid, and Monotropoid mycorrhiza the group of mycorrhizal fungi (Cannon & Kirk, 2007). Figure 8 shows the different categories of mycorrhiza and how they interact with the plant root.

Typically, arbuscular mycorrhizal symbiosis consists of three components: the plant root, an extensive extraradical mycelium that scavenges nutrients and the fungal structures within and between the root cells involved in nutrient transfer (Smith & Smith, 2011). A characterizing trait of the fungus is the arbuscule (Figure 9), a treelike haustorial organ formed in the cortical cells of many plant roots and in some mycothalli. Together with storage vesicles (Figure 10), these structures are considered diagnostic for AMF. Nevertheless, not all species have arbuscules and only 80% produce vesicles (Smith & Read, 2010). Since these hyphal structures allow identification to the family level at best (Merryweather & Fitter, 1998), molecular techniques have become the standard to identify AMF species (Gorzelak et al., 2012).

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**Figure 8** Illustration of the different categories of mycorrhiza (van der Heijden & Sanders, 2002)

**Figure 9** An arbuscule of the Glomus mosseae in leek roots x1700 (Brundrett et al., 1984)
2.2.1 Benefits from AMF colonization

AMF symbiosis enhances plant productivity the most in P limiting conditions, nevertheless growth depressions in colonized plants can still occur (Smith & Smith, 2011). The effect of AMF colonization also depends greatly of the host plant, for example wheat shows low responsiveness to colonization, while faba bean shows a significant response (Qiao et al., 2015). Additionally, environmental factors determine the success of colonization. Especially a high content of available soil P seems to limit the colonization (Gosling et al., 2013).

Enhanced P uptake is one of the most important reasons explaining the enhanced productivity of AMF plants (Smith & Read, 2010; Watts-Williams et al., 2013). Experiments with single plants have shown that AMF can contribute up to 90% of plant P uptake (van der Heijden, Bardgett, & van Straalen, 2008).

The enhanced P uptake by mycorrhizal plants, not particularly arbuscular, has been explained by various mechanisms. The three main mechanisms are the exploration of larger soil volume, faster movement of P into mycorrhizal hyphae, and solubilisation of soil phosphorus. The first, exploration of larger soil volume, is achieved by decreasing the distance that P ions must diffuse to plant roots and by increasing the surface area for absorption. The second, faster movement of P into mycorrhizal hyphae, is achieved by increasing the affinity for P ions and by decreasing the threshold concentration required for absorption of P. The last, solubilisation of soil P, is achieved by the release of organic acids and phosphatase enzymes (Bolan, 1991). It has been shown that the acid phosphatase activity is much higher in AMF colonized plants (Tarafdar & Marschner, 1994). This enzyme mineralises unavailable organic P, allowing plant uptake. However, Joner et al. (1995) found no increased phosphatase activity in similar experiments. In some studies it has been shown that the plant uptake of poorly soluble P sources, such as Fe and Al phosphate, and rock phosphate, increases. However, studies using radioactive $^{32}$P indicated that both mycorrhizal and non-mycorrhizal plants utilise the same P sources (Bolan, 1991; Mosse et al., 1973).
Furthermore, increased P supply due to AMF colonization increases the N accumulation, N productivity and N use efficiency. Direct effects of N uptake are rather contested, as AMF lack the saprotrophic capability to enable N mineralisation (Read & Perez-Moreno, 2003). However, AMF might be involved in decomposition and in the capture of the less mobile amino-acids or ammonium ions (Hodge, 2001; Mader et al., 2000).

Next to increased nutrient uptake, a major benefit of the mycorrhizal symbiosis is that it can protect plants from below-ground enemies, such as pathogens (Sikes, Cottenie, & Klironomos, 2009). Additionally, AMF increases salt stress tolerance by enhancing nutrient acquisition of P, N, Mg and Ca, maintenance of the K:Na ratio, and biochemical, physiological, molecular and ultra-structural changes (Evelin, Kapoor, & Giri, 2009).

2.2.2 The effects of AMF Diversity

Previous mentioned advantages of AMF are colonization effects, however the diversity and abundance of some AMF species might determine the degree of effect (Rillig & Mummey, 2006; van der Heijden et al., 2008). It is known that AMF diversity determines plant biodiversity, ecosystems variability and productivity in a controlled environment (van der Heijden et al., 1998). Furthermore, AMF can improve soil structure by promoting soil aggregation in both agricultural as non-agricultural ecosystems (Rillig & Mummey, 2006). Nevertheless, less is known about the effect of the diversity or abundance of some AMF species in an uncontrolled agriculture environment, and especially in low input farming in the tropics.

In the research of (van der Heijden et al., 1998), the effect of AMF species richness on the ecosystem was tested. Therefore 70 macrocosms containing 15 plant species were inoculated with 1 kg AMF inoculum containing 1,2,4,8, or 14 species from a pool of 23 species. The results are shown in Figure 11. It’s clear that the root and shoot biomass, and the hyphal length increase with an increasing species richness until a maximum is reached around 8-14 species. Furthermore, it is observed that the more species, the more P disappears from the soil. And last, the richness has a positive linear effect on the P uptake of the plants in the ecosystem. This effect can be explained as followed. Different AMF species have a different effect on the nutrient uptake, growth, and gene expression (Burleigh, 2002) and the bigger the species richness the higher the chance the most effective AMF species is included (van der Heijden et al., 1998). For example, the influence on the root expression of MtPT2 and Mt4 from *Medicago truncatula* varied widely between the seven AMF species tested in Burleigh (2002). At one extreme, *Glomus mosseae* colonization resulted in the greatest reduction in MtPT2 and Mt4 gene expression and the highest level of P uptake and growth, while at the other extreme, *Gigaspora rosea* colonization resulted in the highest levels of MtPT2 and Mt4 gene expression and the lowest P uptake.
and growth. Additionally, a higher diversity leads to a more efficient exploration of different resources (van der Heijden et al., 1998).

![Figure 11](image)

**Figure 11**: Effects AMF species richness on: a) Simpson’s diversity index (fitted curve is \( y = 0.271 + 0.077x - 0.003x^2; r^2 = 0.63; P \text{ less than or equal to } 0.0001 \)); b) shoot biomass (\( y = -0.334x^2 + 8.129x + 72.754; r^2 = 0.69; P \text{ less than or equal to } 0.0001 \)); c) root biomass (\( y = -0.265x^2 + 6.772x + 96.141; r^2 = 0.55; P \text{ less than or equal to } 0.0001 \)); d) length of external mycorrhizal hyphae in soil (\( y = 0.001x^3 - 0.046x^2 + 0.756x + 2.979; r^2 = 0.60; P \text{ less than or equal to } 0.0001 \)); e) soil phosphorus concentration (\( y = 0.065x^2 - 1.593x + 14.252; r^2 = 0.67; P \text{ less than or equal to } 0.0001 \)); and f) total plant phosphorus content (linear relationship; \( y = 61.537x + 115.281; r^2 = 0.48; P \text{ less than or equal to } 0.001 \)), in macrocosms simulating North American old-field ecosystems. Squares represent means (copied from van der Heijden et al., 1998).

The diversity, species richness and the community of AMF is affected by numerous environmental effects. For example, positive correlations have been shown between AMF colonisation and soil properties related with biological activity (soil organic matter content, soluble carbohydrate content, and microbial biomass content) (Alguacil et al., 2014). In particular, the addition of oats straw appeared to enhance the AMF diversity in the Mediterranean region. This could be the consequence of the high cellulose content of oat straws, which is an easily available compound for micro-organisms. Other research has found positive correlations between AMF sporulation and organic matter content (Gryndler et al., 2005; Oehl et al., 2009). Additionally, different management practices appear to have an impact on the diversity. For example, practices like ploughing greatly alter the composition of the AMF communities, but it does not significantly decrease diversity (Alguacil et al., 2014). Furthermore, organic farming increases the AMF richness (Van Geel et al., 2015; Oehl et al., 2009). This could be because of the lower amount of chemical biocides, which have negative effect on AMF, in organic farming (Schreiner & Bethlenfalvay, 1996). Or, this could be caused by the higher amount of weeds in organic agriculture. It has been suggested that AMF communities follow the weed communities (Zobel & Öpik, 2014).
Other edaphic parameters like soil pH or altitude can have an effect on the AMF population. In a Canadian study of AMF in *Acer saccharum*, researchers found that acidification decreases the AMF diversity but there was no decline in AMF population observed (Coughlan & Dalpé, 2000). In the contrary, Wang et al. (2008) found in their research on AMF in agricultural soils in Sichuan province that increasing pH had a significant but small negative effect on the colonization of AMF. Moreover, they found no effects of altitude (1000-1200 m.a.s.l) or soil organic matter (0.5-4.4%). However, it’s widely accepted that the major plant-fungal in high altitude and polar regions are ectomycorrhizal rather than arbuscular mycorrhizal (Gao & Yang, 2010; Liu et al., 2011; Newsham et al., 2009) this could be the result of less compatible hosts in these regions. This does not mean that there’s no AMF symbiosis on higher altitude. There have been AMF symbioses reported up to an altitude of 5200 m.a.s.l. (Gai et al., 2009). However, in a study of AMF in the South American Puna Grasslands, researchers found a negative correlation between AMF species richness and altitude, when the altitude ranged between 3320-3850 m.a.s.l. (Lugo et al., 2008).

2.3 Biological Nitrogen Fixation

Biological nitrogen fixation has high potential for low-input systems. In those system, mostly more N is removed from the soil than replenished causing depletion of soil nutrients (Cocking, 2009). Additionally, smallholder farmers in developing countries don’t have easy access to the high priced mineral fertilizers (Amanuel et al., 2000). Legumes can introduce N into the agro-ecosystem due to their symbiosis with a gram-negative *Proteobacteria* (*Rhizobium leguminosarum* bv. *viciae*). The Rhizobia fixes atmospheric nitrogen (*N₂*) into ammonia (*NH₃*) which is available for the plant, in return for carbohydrates such as glucose and sucrose (Strodtman & Emerich, 2009). The reaction which is catalysed by the enzyme nitrogenase is shown below:

\[ N₂ + 8H₂ + 16ATP \rightarrow 2NH₃ + H₂ 16ADP + 16P_i \]  
*(Equation 1)*

Nodulation and eventually fixation, is reduced by excessive tillage, long-term fallows, application of fertilizer N and extended legume rotation. Generally, high soil nitrate concentrations suppress fixation (Peoples et al., 2001; Schwenke et al., 1998).

2.3.1 Interaction between AMF colonization and nodulation

AMF colonization can decline significantly in the presence of *Rhizobium*. Although, nodulation is not affected by the presence of AMF and in general the tripartite symbiotic association of *Rhizobium*, AMF and faba bean increases plant productivity (Jia et al., 2004). Ossler (2015) found in his research that *Rhizobium* and AMF colonisation of partridge pea are generally positive correlated. However, heavily
nodulated plants in controlled experiments showed to have a negative impact on the AMF hyphal colonization and spore production.

There’s still uncertainty about the explanation of this negative correlation between nodulation and AMF colonization. Caetano-Anolles & Gresshoff (1991) explains this phenomenon as follows, “Host negative feedback systems that regulate symbiont densities have been presumed to be an adaptation to prevent over-colonization and thus wasted photosynthate”. Hence, at some point of saturation, where the plant can’t produce enough photosynthates, a trade-off between AMF and Rhizobium colonization occurs. The effect of this trade-off will depend on the environmental conditions like P and other nutrient availability, light availability, or even priority effects such as rhizobium saturation prior to AMF colonization according to Werner & Kiers (2015) and Ossler (2015). In the experiment of Ossler (2015), it was argued that in the situation of heavily nodulated plants, N was the most limiting nutrient and therefore Rhizobium inoculation was favoured. In contrast, Sakamoto et al. (2013) argue that the carbon (C) cost is more determining than the nutritional advantage of a symbiosis. Rhizobial and arbuscular mycorrhizal symbioses can each consume 4-16% of recently photosynthetically fixed C of legumes to maintain their growth, activity, and reserves (Kaschuk et al., 2010). However, nodules return 21-52% of that C to the plant in the form of organic N, ureides, or amides (Minchin & Summerfield, 1981). While AMF do not have a physiological system to return assimilated C into the host plant. Thus, in the perspective of C economy, nodulation will be preferred by host plants irrespectively to the environmental conditions in photosynthates scarcity (Kaschuk et al., 2009; Kaschuk et al., 2010; Sakamoto et al., 2013). A last explanation is that rhizobia generally colonise a host prior to AMF (this is called the priority effect), and therefore have priority for assimilated Carbon (Sakamoto et al., 2013; Werner & Kiers, 2015). This effect can be strengthened by the production of Nod factors of the rhizobia, which actively suppress the AMF colonization (Catford & Staehelin, 2003).

2.4 Soil phosphorus

Phosphorus is an essential macronutrient for plants. Phospholipids, nucleotides, nucleic acids, and certain proteins contain phosphorus. P also is crucial in the energy metabolism in the form of adenosine triphosphate (ATP). Usually, P is concentrated in the fast-growing parts of the plant, particularly in the root tips. It speeds up the maturation of crops and is found in large quantities in seeds and fruits. It benefits root development and especially early in the life of a plant, it is important for the development of the reproductive parts (Olaitan & Lombin, 1984).

Phosphorus deficiency leads to slow growth, slender stalks, delayed maturity and low yields. There may be a darkening of the leaves and in some plants like maize, leaves colour purple (Olaitan & Lombin, 1984).
2.4.1 Phosphorus pools and dynamics

The P cycle, illustrated in Figure 12, consists of many chemical and microbial reactions. In all terrestrial environments, phosphorus primarily originates from weathered soil minerals and other stable geological materials. By weathering P solubilises and soil P solution can be accumulated by plants and animals, reverted to stable organic forms, or eroded from soils and deposited as sediments in fresh waters, estuaries, or oceans. Biological accumulated P (food or feed) can be recycled into the ecosphere by human or animal wastes (Pierzynski, Sims, & Vance, 2005). In this thesis we investigate the role of AMF in the uptake of phosphorus in the plant. However, understanding the overview of soil P dynamics is important. Therefore, the inorganic and organic soil phosphorus pools, and their main transformations will be discussed further in detail.

![Figure 12 The soil P cycle. An overview of the processes controlling the availability of P to plants and P transport by runoff or leaching (Gachon, 1969)](image)

3.4.2.1 Inorganic soil phosphorus
Weathering P minerals release P into the soil solution. There exist more than 150 mineral forms of P in the lithosphere. The minerals present in a soil depend on the time and soil development (Figure 13). In the case of un-weathered or moderately weathered soil, the dominant minerals are calcium (Ca) phosphates like apatites. In areas of intense weathering, like in this study area, Ca and other basic minerals leach from soils, decreasing the pH. At lower pH, iron (Fe) and aluminium (Al) dissolve, enabling precipitates of Fe, Al and P which become the main mineral forms of P. Amorphous oxides of Fe and Al are also common in these soils and they act as sinks and sources of P by sorption and desorption (Pierzynski et al., 2005).
Typically, all P minerals in soils have a low solubility. The soil solution concentration of P ranges from <0.01 to 1 mg P/L (ppm), although after fertilization, concentrations as high as 6 to 8 mg P/L have been observed. The available P is largely controlled by the soil pH as can be seen in Figure 14.

The four main transformations of inorganic soil P are sorption, desorption, precipitation and dissolution. Dissolution means the weathering of primary or secondary minerals containing P, which slowly releases orthophosphate into the soil solution. Precipitation is the opposite, where a discrete insoluble compound is formed. Common secondary P minerals are the products of reaction between soluble P, which is an anion and cations like Ca, Al, or Fe. Sorption reaction include all nonspecific adsorption and chemisorption reactions, and the formation of a chemical bond between phosphate anions and soil colloids. Desorption is the opposite and occurs, in the contrary of sorption, at low soluble P concentration. Once P is sorbed, it is not readily desorbed. This effect is called hysteresis (Pierzynski et al., 2005). All these adsorption/desorption and precipitation/dissolution equilibria control the concentration of P in the soil solution (Hinsinger, 2001).

In a mesocosm study in the area around Jimma, mostly acidic clay soils were found (Nebiyu, 2014). In general, acidic and high in clay or Fe- and Al-oxides, particularly amorphous oxides, soils have the greatest P adsorption capacities. Hence, the soils in our study are less susceptible to leaching of P but P plant uptake is also limited (Pierzynski, Sims, & Vance, 2000).

![Diagram of soil P forms](image)

*Figure 13 changes in soil P form as affected by time of soil development* (Pierzynski et al., 2005)
3.4.1.2 Organic soil phosphorus

Inorganic phosphorus is taken up by soil microbial biomass and plants where it is converted to organic phosphorus. This incorporation of P into biomass is called demineralisation. The P can be recycled by microbial decomposition of organic P into soluble P. This process is called mineralisation and it occurs if the C:P ratio of the organic matter is <200:1. Demineralisation occurs if the C:P ratio is >300:1.
3. Material and methods

3.1 Description of the project

The field experiment was conducted on 15 fields close to Dedo. These fields were concentrated in three locations across a mountain side near Dedo, i.e. Dedo I, Dedo II, Dedo III. The altitude of Dedo I and Dedo II ranges between 2155 and 2184 meters above sea level (m.a.s.l) and that of Dedo III ranges between 2261 and 2306 m.a.s.l. Each location had 5 fields. IA, IB, IC, ID, and IE are respectively fields A, B, C, D, and E on location Dedo I, the same applies for Dedo II (IIA, IIB, IIC, IID, IIE) and Dedo III (IIIA, IIIB, IIIC, IIID, IIIE). On each field five plants were sampled. The assigned codes for the five plants on field A at Dedo I will be IA1, IA2, IA3, IA4, and IA5.

Table 3 Elevation of the different fields and the averages per location in meter above sea level (m.a.s.l)

<table>
<thead>
<tr>
<th>Field</th>
<th>Dedo I</th>
<th>Dedo II</th>
<th>Dedo III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Elevation (masl)</td>
<td>2182,5</td>
<td>2173</td>
<td>2178</td>
</tr>
<tr>
<td>mean elevation (m.a.s.l)</td>
<td></td>
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</tbody>
</table>

On each field, faba bean variety Moti was cultivated by a local farmer. The plants were planted in the first half of July. During first flowering, 5 plants of each field were collected. This was around the first week of September 2015. Consequently of each of the 75 plants, the upper biomass was oven dried at 40°C, the nodules were counted, around 25 g of rhizosphere soil was collected in a jar, and the root hairs were collected in a paper bag and dried with silicagel. In order to determine the BNF of the faba beans, a reference non N-fixing crop, wheat, was collected nearby each field. All faba bean samples were manually grinded through a 0.5 mm sieve using a mortar and transported to Belgium. Steps of the procedure are shown in Figures 15, 16, and 17.

As the fields were managed by local farmers, there’s lots of variability between management practices. Most of the fields were the previous year cultivated by cereals (i.e. wheat, teff, and barley). However, at one field the preceding crop was faba bean. In general, faba bean is cultivated once every two years. Fields IC, IIB, and IIIC were located close to a homestead. Due to better management, plant growth
was remarkably higher on these fields. Variability between fields was also determined by the high variability of field slope.

Figure 15 A faba bean at first flowering, notice the emerging white flowers

Figure 16 counted nodules on the right and soil rhizosphere in the jar of the third plant on field A of location Dedo III
3.2 Soil rhizosphere parameters

3.2.1 Phosphatase enzyme activity

AMF have the ability to produce phosphatase enzymes and therefore make organic phosphorus available for plant uptake. The effect of AMF diversity on the enzyme activity is less investigated. Therefore the phosphatase activity was measured from the rhizosphere of each plant using a modification of the method of Tabatabai & Bremner (1969).

The principle of the method is based on the ability of the phosphatase enzyme to convert colourless para-nitrophenylphosphate (pNPP) to the yellow coloured para-nitrophenol (pNP), and phosphate. This applies for both alkaline and acid phosphatase activities but as the examined soil had an average pH of 5.5, only acid phosphatase was measured.

For the analysis, half a gram of rhizosphere soil was weighed and solved in 0.125 ml of toluene, 0.5 ml of 100 mM pNPP solution, and 2 ml of modified universal buffer – prepared as described by (Skujins, 1967). The buffer’s pH was adjusted to 5.5 by adding concentrated NaOH (1M). After incubation of half an hour on 37°C, 0.5 ml of 2M CaCl₂ and 2 ml of 0.5M NaOH was added to stop
the reaction. CaCl$_2$ addition is necessary to prevent dispersion of clay and to extract soil organic matter during the treatment with NaOH. It is necessary to increase the pH and so inactivating the enzyme and therefore stopping the reaction. The next step was to add 45 ml of distilled water (DI) and the filter the solution through a whatman no.2 filter. The absorbance of the filtrate was measured using a UV-VIS at 400 nm. Finally, the absorbance was fitted to a calibration curve to obtain the enzyme activity.

The calibration curve was made by solving 0.5 g para-nitrophenol into 500 ml DI. This solution was diluted to the mother solution (MS) with a concentration of 100 µg pNP ml$^{-1}$ MS. Now a set of solutions ranging from 0-8 µg/ml was made by pipetting 0-8 ml in steps of nine into a 100 ml volumetric flask and adding up the volume with DI. Thereafter, from all solutions 0.5 ml was pipetted and 0.125 ml of toluene, 0.5 ml of 100 mM pNPP solution, and 2 ml of modified universal buffer was added in succession. Subsequently, the solution was filtered and 2M CaCl$_2$, 2 ml of 0.5M NaOH and 45 ml of DI was added before measuring the absorbance at 400 nm. This was based on the method described in Schneider et al. (2000).

3.2.2 Olsen-P

To estimate the plant available P, the Olsen P method can be used (Sims, 2000). The method is based on the use of HCO$_3^-$, CO$_3^{2-}$ and OH$^-$ to precipitate soluble Ca$^{2+}$ as CaCO$_3$ and soluble Al$^{3+}$ and Fe$^{3+}$ by formation of Al and Fe oxyhydroxides, by adding 0.5M NaHCO$_3$ solution with a pH of 8.5. Additionally, high pH levels increase surface negative charges and decrease the number of sorption sites on Fe and Al oxide surfaces. Thus the P solubility increases.

Normally an Olsen P value of 10 mg P/kg soil is considered as optimal for plant growth. Nevertheless comparing P availability in soils with large differences in P chemistry should be done cautiously (Sims, 2000).

The first step is to prepare the extraction solution (0.5 M NaHCO$_3$, pH 8.5) by dissolving 84 g sodium bicarbonate (NaCO$_3$) in 2 L of milli-Q® water (MerckMillipore, Darmstadt, Germany). A magnetic stirrer is used to dissolve the NaHCO$_3$ completely. The pH is adjusted to 8.5 by using 50% sodium hydroxide (NaOH). Note that there’s a little adjustment to the protocol of Sims (2000). Using ultrapure water instead of distilled water, decreases the P concentration in the extraction solution, enabling a better estimate of the available P in the soil.

The second step is to extract the P from the soil. This is done by weighing 1 g of soil into a 50 mL plastic jar and adding 20 ml of the extraction solution. The closed jar is shaken for 30 minutes at room temperature at 200 epm. The solution is filtered through a P-free Whatman No. 5 filter, which enables a better estimate of the P availability. The filtrate was frozen in before analysing P concentration to prevent microbial interaction.
Phosphor analysis was done by an auto-analyser (Autoanalyzer AA3, Bran+Luebbe, Norderstedt, Germany). The analyser works as followed. First all the samples are sucked in a tube one by one and separated by an air bubble, resulting in a continuous flow of fluid and air. Subsequently, the flow enters the reaction segment where the ortho-phosphate in the sample reacts with molybdate and ascorbic acid into a blue compound. Subsequently, the absorbance is measured with a colorimeter at 660 nm. The colorimeter is connected with a computer which stores the data. By analysing a series of known concentrations of potassium dihydrogen phosphate, a calibration is made.

As the flow entering the colorimeters is an alternation of air and samples, a typical output of the auto-analyser is a series of peaks as shown in Figure 19. Four stadia are identifiable. The first stage is an increasing absorbance according to the transition between air and sample. In the second stage the sample enters the colorimeter resulting in a steady state. In this stage the absorbance is measured. The third stage is a decreasing absorbance according to the wash out of the sample by air and the last stage accords to the air bubble between two samples.

![Figure 19 Illustration of a typical output from the auto-analyser. Absorbance is plotted against time. Four stages are identified: (1) a transition between air and sample, (2) only sample is measured, (3) transition between sample and air, and (4) only air passed the colorimeter.](image)

3.2.3 Total P

Total soil phosphorus is a parameter which represents all P in the soil. This is important as it is known that AMF symbiosis enhances plant productivity the most in P limiting conditions (Smith & Read, 2010). The method was carried out by the lab of forest and nature of UGent in Gontrode. To determine total P, soil samples were treated with HClO₄, HNO₃ and H₂SO₄ for 4 hours at 150°C, after which phosphate concentrations were measured colorimetrically with malachite green (Varian Cary50, Agilent Technologies, US; Lajtha et al., 1999).
3.2.4 pH

The pH was measured potentiometrically in the supernatant suspension of a 1:2.5 soil-water mixture (van Reeuwijk, 2002). Due to scarcity of soil sample, the 5 samples of each field were pooled into one sample by weighing 1 gram into a jar. To that 5 g of soil, water was added (12.5 ml) and the solution was shaken for 2 hours. Before opening the bottle the sample was shaken manually. Thereafter, the pH was measured using a potentiometer.

3.2.5 Resin P

The resin P method enables the extraction of biological available forms of inorganic P (Pansu & Gautheyrou, 2007). The result gives an indication of the P immediately available for plants (Quesada et al., 2010).

Extraction methods like Olsen P have been contested in some soil types. In some cases, researchers have reported that the competing ion used in these methods can extract forms of P unavailable to plants (Menon et al., 2008). The resin method using anion exchange membranes (AEM) have been proven better than chemical extraction methods in some cases (Mason et al., 2008).

Before starting the method, it is very important to clean the AEM strips. Therefore, the strips are washed thoroughly 3 times with milli-Q® water (MerckMillipore, Darmstadt, Germany), making sure all residual P has been removed. Subsequently, the strips are regenerated into the bicarbonate form by placing the strips into 0.5 M NaHCO₃ (pH 8.5). Now the strips are activated

To measure the available P, 1 g of soil is weighed into a plastic jar containing 2 activated AEM strips and 30 ml of milli-Q® water. This jar is shaken end-over-end for 16 hours at room temperature. Consequently, the membranes are removed, cleaned with as little milli-Q® water as possible to remove soil particles, placed into another plastic jar with 20 ml of 0.5 M HCL, and shaken for another 16 hours. Subsequently, the strips are removed and the HCL desorption solution is ready for P analysis.

As the desorption solution is too acid to measure with the auto-analysers, a phosphate colorimetric kit (Sigma-Aldrich) was used, following the instruction of the manufacturer.

3.2.6 E-value

The quantity of isotopically exchanged P after a time of isotopic exchange is called the E-value and it is an equivalent to the available soil P (IAEA, 2001). If measurements of isotopically exchangeable phosphate ions in soil solution systems have been carried out in steady state than the E-value can be calculated based on the assumption that the specific activity, i.e. the ratio between the quantity of the
radioactivity and the quantity of P as phosphate ions, is the same for phosphate ions of the soil solution and all isotopically exchanged phosphate ions in the system and thus (IAEA, 2001):

\[ \frac{R}{E_t} = \frac{r_t}{q_s} \]

And therefore:

\[ E_t = q_s \frac{R}{r_t} \]

The following parameters should be measured to calculate the E-value, i.e. the radioactivity of the mother solution (R), the radioactivity of the samples at a certain time (r), and the phosphate concentration at the same moment (q).

This is done by, first preparing the mother solution. Caution is advised in this protocol. Keep as much distance from the radioactive material, use gloves and eye protection. The jars are best kept into a lead cover and behind PVC glass. A certain quantity of labelled carrier free \(^{32}\)P is dissolved into 90 ml of milli-Q® water. This solution is filtered through a 0.2µm filter to eliminate other than \(^{32}\)P labelled ions. The filtrate is called the mother solution.

Secondly the radioactivity of the mother solution is counted by dissolving 1 ml of the mother solution into a 100 ml Erlenmeyer. Add 25 ml of a 1 mg P\(^{1-}\) KH\(_2\)PO\(_4\)-solution to avoid fixation of the radioactive phosphate ions to the glass wall. Next, add distilled water until the mark. From this solution 4 replicates were subsampled of 1 ml. To each subsample, 9 ml of LSC cocktail was added and the activity was measured by a scintillator. R is 100 times the radioactivity of the measured value because of the dilution described before.

To measure the activity (r) and concentration (q) of the samples, 2 g of soil was weighed into a jar. Then, 19 ml of milli-Q and 2 drops of toluene were added and this solution was shaken for 8 hours. Subsequently, 1 ml of mother solution was added and the result was shaken for another 5 days. The samples were aerated by opening each jar for a minute daily. After these 5 days, the samples were centrifuged and filtered through a 0.2 µm filter. The activity (r) was counted by subsequently, pipetting 1 ml of filtrate, adding 9 ml of LSC cocktail, and measuring the activity. The P concentration (q) of the filtrate was measured using a phosphate colorimetric kit (Sigma-Aldrich) following the instruction of the manufacturer. In brief, 1 ml of filtrate was pipetted into a cuvette and 150 µl of phosphate reagents was added. After half an hour with occasional shaking, the absorbance was measured with an UV-VIS and fitted to a calibration curve to estimate the concentration.

Measuring the activity of one sample could last up until half an hour, thus there’s a considerable amount of time between the measurement of the first and last sample. Therefore all the activity was recalculated to the time of the first measurement using the formula of radioactive decay (Domenico et al., 1998):
$$A = A_0 \cdot e^{\left(\frac{\ln(2) \cdot T_{1/2}}{T}\right)}$$

With,

- \(A_0\) and \(A\), the activity at the beginning of the experiment and at the time of measurement
- \(T_{1/2}\), the half-life time of a \(^{32}\)P isotope which amounts (Brown, 1971).

### 3.3 Plant and AMF parameters

#### 3.3.1 AMF diversity

The root hairs of each sample were collected separately and dried with silicagel in a paper bag. The in total 75 samples were transported to Belgium and stored until measurement. The DNA was extracted from the root hairs following the instruction of the PowerSoil®DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA). This kit is intended to extract DNA from soil samples, nevertheless experience has taught it is also recommended for root hairs. After isolation, DNA was multiplied using a polymerase-chain reaction (PCR) with as forward primer AMV4.5NF (5’-AAG CTC GTA GTT GAA TTT CG-3’) and as reverse primer AMDGR (5’-CCC AAC TAT CCC TAT TAA TCA T-3’) (Sato, Suyama, Saito, & Sugawara, 2005). The result of this PCR is a 300 base pairs (bp) long fragment of the small sub unit (SSU) of 18S rDNA. This primer pair has been proven the most successful for AMF in apple orchards (Van Geel et al., 2014).

PCR reactions were performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, CA, USA) in a reaction volume of 20 µl, containing 0.15 mM of each dNTP, 0.5 µM of each primer, 1x Titanium Taq PCR buffer, 1U Titanium Taq DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), and 1µl genomic DNA. First, the DNA samples were denatured for 2 min at 95°C. Thereafter, 35 cycles were run, consisting of 45 s at 94°C, 45 s at 65°C, and 45 s at 72°C, followed by a final elongation of 10 min at 72°C.

The PCR is followed by a denature gradient gel electrophoresis (DNGE). The resolved DNA within the appropriate size (ca. 300 bp) was cut from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Hamburg, Germany). Next, the purified samples are quantified using the Qubit fluorometer (Invitrogen, Gent, Belgium) and pooled into equimolar quantities, resulting in two amplicon libraries. The quality of these amplicon libraries was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). Finally, pyrosequencing was performed by Macrogen (Seoul, Rep. of Korea) using a GS-FLX Titanium sequencer.
Clustering and mapping of the reads were performed using the Uparse algorithm, following the recommended pipeline (Edgar, 2013). This means that all non-biological sequences (i.e. barcodes, primers, and adapter), and singletons are removed. Also, the quality of the reads is filtered with the ‘fastq-filter’ command allowing a maximum expected error of 0.5 for individual sequences. In order to maximize the number and length of retained sequences from the shorter amplicons, the reads are truncated to 230 bp.

The filtered reads are next blasted against GenBank and the Maarjam data bank. Only operational taxonomic units (OTU) that were allocated to the phylum of the Glomeromycota in both data banks and with a bitscore ≥ 223 according to the Maarjam data bank were preserved. The databank which resulted in the highest bitscore was assumed to be correct.

Usually the reads are rarefied to a certain minimum to do further analysis, nevertheless it is decided not to. Rarefaction curves helps determining the threshold to rarefy. These curves are created by randomly re-sampling the pool of N samples multiple times and then plotting the average number of species found in each sample. Thus, rarefaction can be used to determine the expected number of species in a random sample (Gotelli & Colwell, 2001). Unfortunately, the amount of reads per sample are low and only in rare cases a more or less asymptotic curve was obtained. Pooling the samples per field gave better rarefaction curves but it resulted in only 8 sample points. Therefore it was opted to not rarefy. Nevertheless, all samples with less than 10 reads were discarded from further analysis.

On the retained samples, diversity indices were calculated, using the “summary.single” function. The calculated indices are: Chao, Ace, Jackknife, Shannon, npShannon, and the Simpson index. In literature the expH index is also often used, which is the exponential function of the Shannon. The meaning of the indices is given in Table 4. The difference between richness and diversity is that richness only takes the observed number of species into account, while diversity additionally takes the abundance of the observed species into account.

Additionally, a non-metrical multidimensional scaling (NMDS) was used to compare the environmental distance of different sample communities. This was done using Bray-Curtis distances and performed in R (Oksanen et al., 2007). It is important to run the script several times to obtain the global solution as sometimes the program gets stuck in local optima.
Table 4 Overview of the calculated richness and diversity indices (retrieved from the Mothur forum (2016))

<table>
<thead>
<tr>
<th>Index</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sobs</td>
<td>Observed number of species</td>
</tr>
<tr>
<td>Chao</td>
<td>Estimated richness</td>
</tr>
<tr>
<td>Ace</td>
<td>Estimated richness</td>
</tr>
<tr>
<td>Jackknife</td>
<td>Estimated richness</td>
</tr>
<tr>
<td>Shannon</td>
<td>Diversity estimator</td>
</tr>
<tr>
<td>npShannon</td>
<td>Not parametrical diversity estimator</td>
</tr>
<tr>
<td>expH</td>
<td>Exponential of the Shannon index: diversity</td>
</tr>
<tr>
<td>Simpson</td>
<td>Invers of diversity</td>
</tr>
</tbody>
</table>

3.3.2 N/P ratio

The N/P ratio, the ratio between the N content and the P content of the plant, reflects the relative availability of N and P to plants and may indicate the degree of N or P deficiency experienced by a plant population. The parameter can be used to investigate how the relative availability of N and P influences various ecological processes and how it is affected by human impacts or management (Güsewell et al., 2003). To calculate the N/P ratios, P content and N content from the plant is needed.

The procedure to determine the P concentration in the plant material is that of Chapman and Pratt (1961), by measuring the P content colorimetrically in the dissolved ash of the plant material. (Ryan et al., 2001).

First 0.25 g of manually grinded plant material is weighed in a porcelain crucible. Subsequently, the crucibles are placed into a cool muffle furnace, and the temperature increases gradually to 550°C. This temperature is maintained for 5 hours before the furnace is shot down and the opened for rapid cooling. When cool, 5 ml of 2N hydrochloric acid (HCl) is added. Now the content is transferred to a volumetric flask of 25 ml. After 15 – 20 minutes the volume is made up to 25 ml using milli-Q ®. The solution is mixed and filtered through a P-free Whatman filter. The concentration is measured using an auto-analyser (Autoanalyzer AA3, Bran+Luebbe, Norderstedt, Germany) as is described in the section about Olsen P.

Plant N concentration was analysed with Elemental Analyser Isotope Ratio Mass Spectrometry (EA-IRMS) (20-20, SerCon, Crewe, UK). In contrary of the determination of plant P, the manually grinded samples weren’t sufficient to analyse. Therefore the faba bean samples were grinded using a Retsch ZM-200 centrifugal mill (Düsseldorf, Germany) with a sieve of 0.5 mm. In the EA-IRMS the plant
material is consequently combusted, and reduced to \( \text{CO}_2 \) and \( \text{N}_2 \) by copper wires. The gasses are afterwards analysed in a mass spectrometer. The data is analyses automatically and the percentage N content (N\%) can be used to calculate the total nitrogen content of the plant samples as followed, Total N yield (g N plant\(^{-1}\)) = dry matter yield (g plant\(^{-1}\)) x N\%/100.

### 3.3.3 BNF

To estimate the amount of fixated N in the legumes, the \(^{15}\text{N}\) abundance method is used. This method is based isotopic fractionation. This occurs because different isotopes have a small difference in mass which can lead to different reaction kinetics and equilibriums. Heavier isotopes like \(^{15}\text{N}\) have slightly lower velocities, form stronger bonds and are less reactive than lighter isotopes like \(^{14}\text{N}\). Therefore natural abundance of \(^{15}\text{N}\) isotopes will differ between the soil and atmosphere. The \(^{15}\text{N}\) content of a non-fixing plant will approach the \(^{15}\text{N}\) content of the soil while the content of a N-fixing plant will approach that of the air (Knowles & Blackburn, 1993).

To determine the fixated N amount following formulas are used (Nebiyu et al., 2014):

\[
\%N_{df\alpha} = 100 \times \frac{\delta^{15}N_{ref} - \delta^{15}N_{fix}}{\delta^{15}N_{ref} - Bvalue}
\]

with,

- \%N\text{df}a, Percentage Nitrogen derived from atmosphere
- \( \delta^{15}N_{ref} \), the natural abundance of \(^{15}\text{N}\) in the reference non-fixing plant per million
- \( \delta^{15}N_{fix} \), the natural abundance of \(^{15}\text{N}\) in the N-fixing plant per million
- Bvalue, a correction value for N coming from the seed and differences between plant varieties.

The natural abundance of both the fixing as non-fixing plants can be calculated with the formula (Högberg et al., 1994):

\[
\delta^{15}N(\%) = \frac{^{15}N_s - ^{15}N_{air}}{^{15}N_{air} / N_{air}} \times 1000
\]

With,

- \(^{15}N_s\) and \(^{15}N_{air}\), the amount of the \(^{15}\text{N}\) isotopes of the sample and in the air
- \( N_s \) and \( N_{air} \), the total amount of the N found in the samples and in the air.
The abundance of $^{15}$N in the air and the Bvalue used in these functions can be found in literature. As the atmosphere is a very large pool of N, the natural abundance of $^{15}$N is less likely to change. In literature a standard of 0.3663% of the total N is taken as the natural abundance of $^{15}$N in the atmosphere (Mariotti et al., 1981). The Bvalue variates significant between varieties and the Bvalue should take into account the N coming from the seed. Especially in faba bean, as these seeds are big and have a significant influence on the $^{15}$N content of the plant. The plants in this study are of the variety Moti and therefore a Bvalue of 0.5 was taken (Nebiyu et al., 2014).

The total amount of $^{15}$N and $^{14}$N isotopes of the plants can be measured with a EA-IRMS as explained in the section about the N/P ratio. The 75 plant samples (5 on each field) were first grinded and measured. Additionally, 15 reference plants, for each field 1 plant, were grinded and analysed. The reference plant, wheat, was taken from fields abutting the faba fields that were sampled. Additionally, with the %Ndfa it is easy to calculate the amount of N fixed per gram plant dry weight, by multiplying the %Ndfa with the total amount of N per gram sample, which is measured by the EA-IRMS.

3.3.4 Nodules

To get an indication of the rhizobium colonization and biological nitrogen fixation, the nodules were counted one by one at plants first flowering stage.

3.3.5 Above ground biomass

The plants were taken from the field in first flowering stage and weighted before and after oven drying using a balance. The plant samples were dried at 40°C with mild convection until considered dry. As the plants weren’t fully mature, the result doesn’t indicate yield effects. However, the results are inevitable for the processing of data.

3.4 Data analysis

All statistical analysis were carried out using RStudio R 3.2.3 (R Core Team, 2014). First all calculated parameters are summarized using the function `summary`. Next, the data was divided in three groups according to the location. All parameters were tested for normality by descriptive statistics and Levene’s test was employed to test for differences in variances between different locations. When differences in variances were observed, the use of ANOVA was excluded and a nonparametric test was performed instead. First the non-parametric Kruskal-Wallis was applied. If significant differences in means were observed, the Mann-Whitney-U test, was applied to determine which locations showed
significant differences. Thereafter, Pearson’s correlation analysis was conducted to determine relationships between all measured parameters.

To assess objective one, the response of AMF diversity on environmental variation, all the diversity indices were analysed using a linear mixed-effect model. Location (Dedo I, Dedo II, and Dedo III) was set as random effect while pH, altitude, total soil P and available soil P (Resin P, Olsen P, and E-value) were set as fixed effects. Additionally, the effect of P limiting conditions were tested by using the N/P ratio. Mixed-effect modelling was preferred as graphical analyses suggested an unwanted effect of location and did not exclude heteroscedasticity amongst different locations. Moreover, the location and altitude were not completely independent. The regression was performed stepwise and evaluated by the AIC (Akaike information criterion). The lower the AIC the better the model fits the data. For this regression in particular, the packages Matrix and lme4 were used. The assumptions of the model were checked by graphical methods: the normality of the residuals was checked through a QQ plot and homoscedasticity was of the residuals was checked by boxplots and the modified levene test. Additionally, the NMDS graphs show the determining factors that shape the AMF communities.

To test objective two, the effect of the diversity on P uptake and phosphatase activity, the Pearson correlation matrix was consulted. Additionally, interacting effects were removed using a linear mixed-effects model. For both models the parameters location, altitude, pH, Total P, Olsen P, Resin P and E-value were set as random effects, because these parameters might have an effects on the plant P and may hide the effect of AMF diversity.

To test the third objective, the interactions in the tripartite symbiosis, the same method as in objective two was used. The difference is that the dependent variables in the linear mixed-effects model were set to nodules and %Ndfa.
4. Results

4.1 AMF identification

In total, 81 OTU’s were retained from the databank. It is assumed that each OTU coincides with a different taxa of the phylum Glomeromycota. Eight different families were identified, i.e. 36 *Glomeraceae*, 14 *Acaulosporaceae*, 10 *Claroideoglomeraceae*, 7 *Gigasporaceae*, 6 *Diversisporaceae*, 5 *Archaeosporaceae*, 2 *Paraglomeraceae*, and 1 *Pacisporaceae* strain(s). Additionally, 27 different species were distinguished, which means there are still different taxa distinguished within species. The most abundant AMF was *Claroideoglomeraceae Claroideoglomus sp.* that was observed in 56 of the 61 of the successful analysed samples. The second two most observed species were *Acaulosporaceae Acaulospora sp.* and *Glomeraceae Glomus sp.* which were found in 53 and 43 samples, respectively. An identification table can be found in the appendix. Additionally, 22% of all OTU reads was this *Claroideoglomeraceae Claroideoglomus sp.*, while 13% of the read OTU’s were this *Acaulosporaceae Acaulospora sp.* and 7% of the read OTU’s were *Glomeraceae Glomus sp.*

4.2 Rarefaction curves

Figure 20 shows all the rarefaction curves of the 15 sampled fields. All curves reach their asymptotic phase, thus ideal sample size is, around 500-1000 reads per field. For 8 fields the curve more or less reaches the asymptotic phase, i.e. IA, IB, IC, IIA, IID, IIIB, IIID, and IIIE. The species richness can only be compared in the asymptotic phase, therefore we can see that the richness is the highest in the fields IIA, IIIB, IB, and IA. On these fields, there have been 54, 48, 45 and 45 different AMF species observed, respectively. These curves aren’t representative for the diversity as abundancy of species isn’t taken into account.
Figure 20 Rarefaction curves of the Vicia faba colonizing AMF species pooled per field. IA, IB, IC, ID, IE are the five fields in Dedo I; IIA, IIB, IIC, IID, IIE are the five fields in Dedo II; and IIIA, IIIB, IIIC, IIID, IIIE are the five fields in Dedo III.

4.3 Summary of collected data

On average, 18.6 ± 1.1 distinct OTU’s were identified per sample, excluding the 14 samples of which an insufficient amount of reads were observed. The succeeded samples ranged from 5-37 OTU’s per sample. The average reads per sample were 255 ± 51, omitting the same 14 samples. Table 5 shows the average diversity and richness indices measured on all the fields. Additionally all edaphic and plant parameters were summarized in Tables 6 and 7. The Pearson correlation matrix Table 8.

Soil pH varied from 5.4 to 6.4. The total soil phosphorus varied between 563 and 2409 mg P kg\(^{-1}\) soil. The available soil P was measured as Olsen P (1.6-111.28 mg P kg\(^{-1}\) soil), Resin P (1.0-28.5 mg P kg\(^{-1}\) soil) and E value (1.7-63.0 mg P kg\(^{-1}\) soil). This is around 1.1-1.2% of the total soil P. The observed N/P ratio in the plant ranged from 11.7 to 95.2 which means that mostly P limiting (N/P >16%) conditions are observed (Garrish et al., 2010). More precise, 3 plant samples (IIA5, IIIC1, and IIIE1) were situated in N limiting conditions and in 2 plant samples (IB1, and IIB4) did not show a limiting nutrient while all other 70 samples were situated in P limiting conditions. The P content in the plant
samples varied between 484.6 and 4485.2 mg P kg\(^{-1}\) plant. The phosphatase activity in the soil was observed between 1.72 and 8.11 µmol g\(^{-1}\) soil h\(^{-1}\). On the plant roots, between 10 and 300 nodules were counted. Finally, the dry weight of the upper biomass of the plant samples varied between 2.3 and 32 g.

Table 5 Average of the diversity indices measured on all samples with more than 10 reads for the three locations Dedo I (2171 m.a.s.l.), Dedo II (2177 m.a.s.l.), and Dedo III (2283 m.a.s.l.). Thus, 14 samples were excluded (significance code, all parameters with an equal letter show no significance differences).

<table>
<thead>
<tr>
<th>Location</th>
<th>Sobs</th>
<th>Chao</th>
<th>Ace</th>
<th>Jackknife</th>
<th>Shannon</th>
<th>npshannon</th>
<th>expH</th>
<th>Simpson</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedo I</td>
<td>18.59 ± 3.12a</td>
<td>25.97 ± 3.42a</td>
<td>33.69 ± 7.18a</td>
<td>92.1 ± 119.1a</td>
<td>2.17 ± 0.16a</td>
<td>2.39 ± 0.16a</td>
<td>11.71 ± 1.8a</td>
<td>0.17 ± 0.04a</td>
</tr>
<tr>
<td>Dedo II</td>
<td>16.33 ± 3.75a</td>
<td>22.18 ± 5.51a</td>
<td>25.54 ± 6.31a</td>
<td>20.32 ± 8.6a</td>
<td>1.9 ± 0.22a</td>
<td>2.06 ± 0.21ab</td>
<td>8.64 ± 1.75a</td>
<td>0.24 ± 0.06a</td>
</tr>
<tr>
<td>Dedo III</td>
<td>19.8 ± 3.9a</td>
<td>24.24 ± 4.72a</td>
<td>26.6 ± 5.01a</td>
<td>22.45 ± 6.94a</td>
<td>2.2 ± 0.19a</td>
<td>2.32 ± 0.19a</td>
<td>11.09 ± 1.86a</td>
<td>0.17 ± 0.04a</td>
</tr>
<tr>
<td>Overall mean</td>
<td>18.34 ± 2.07</td>
<td>24.25 ± 2.59</td>
<td>28.84 ± 3.69</td>
<td>46.94 ± 43.32</td>
<td>2.1 ± 0.11</td>
<td>2.27 ± 0.11</td>
<td>10.6 ± 1.08</td>
<td>0.19 ± 0.03</td>
</tr>
</tbody>
</table>

Table 6 Averages of the edaphic parameters measured in all 75 sample’s rhizospheres for the three locations (significance code, all parameters with an equal letter show no significance differences).

<table>
<thead>
<tr>
<th>Location</th>
<th>altitude (m.a.s.l.)</th>
<th>pH</th>
<th>Total P (mg P kg(^{-1}) soil)</th>
<th>Resin P (mg P kg(^{-1}) soil)</th>
<th>Olsen P (mg P kg(^{-1}) soil)</th>
<th>E-value (mg P kg(^{-1}) soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedo I</td>
<td>2171.9 ± 3.74a</td>
<td>5.56 ± 0.01a</td>
<td>1053.22 ± 68.43a</td>
<td>17.44 ± 2.23a</td>
<td>13.04 ± 2.76a</td>
<td>11.89 ± 3.41a</td>
</tr>
<tr>
<td>Dedo II</td>
<td>2176.6 ± 5.13a</td>
<td>5.62 ± 0.07b</td>
<td>1097.4 ± 101.47a</td>
<td>11.32 ± 2.71b</td>
<td>9.90 ± 4.50a</td>
<td>11.98 ± 3.26a</td>
</tr>
<tr>
<td>Dedo III</td>
<td>2282.9 ± 6.39b</td>
<td>5.79 ± 0.08c</td>
<td>1393.07 ± 161.72b</td>
<td>11.08 ± 3.42b</td>
<td>20.49 ± 10.6a</td>
<td>19.63 ± 5.61b</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>2210.47 ± 12.05</td>
<td>5.66 ± 0.04</td>
<td>1181.23 ± 75.02</td>
<td>13.31 ± 1.74</td>
<td>14.46 ± 4.02</td>
<td>14.5 ± 2.55</td>
</tr>
</tbody>
</table>

Table 7 Averages of all plant related parameters and also the phosphatase activity measured in the plant rhizosphere in all 75 samples for the three locations (significance code, all parameters with an equal letter show no significance differences).

<table>
<thead>
<tr>
<th>Location</th>
<th>P plant (ppm)</th>
<th>Phosphatase activity (µmol g(^{-1}) h(^{-1}))</th>
<th>Nodules</th>
<th>Dry weight (g)</th>
<th>N/P Ratio</th>
<th>%Ndfa (%)</th>
<th>N fixed (mg N g(^{-1}) dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedo I</td>
<td>(2172 m.a.s.l.)</td>
<td>1536.06 ± 265.71a</td>
<td>99.76 ± 20.61a</td>
<td>8.72 ± 1.3a</td>
<td>37.85 ± 7.25a</td>
<td>77.39 ± 7.08a</td>
<td>313.9 ± 52.3 a</td>
</tr>
<tr>
<td>Dedo II</td>
<td>(2177 m.a.s.l.)</td>
<td>1638.83 ± 314.41a</td>
<td>80.04 ± 26.24a</td>
<td>12.16 ± 2.7a</td>
<td>32.26 ± 4.61a</td>
<td>67.92 ± 8.43ab</td>
<td>402.7 ± 109.3a</td>
</tr>
<tr>
<td>Dedo III</td>
<td>(2283 m.a.s.l.)</td>
<td>1813.96 ± 350.22a</td>
<td>3.5 ± 0.35b</td>
<td>87.12 ± 25.45a</td>
<td>8.42 ± 2.6a</td>
<td>30.84 ± 5.76a</td>
<td>53.14 ± 11.28b</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>1662.95 ± 179.62</td>
<td>3.99 ± 0.27</td>
<td>88.97 ± 13.92</td>
<td>9.77 ± 1.36</td>
<td>33.65 ± 3.47</td>
<td>66.15 ± 5.66</td>
<td>325.8 ± 54.06</td>
</tr>
</tbody>
</table>
Table 8 Correlation matrix of all significant (P < 0.05) Pearson correlations (r) between the collected data (’/’ means that the correlation isn't significant (P>0.05). The darker the the higher the value. Significance codes (P < 0.01 ‘*' and <0.001 ‘**').

<table>
<thead>
<tr>
<th></th>
<th>Jackknife</th>
<th>shannon</th>
<th>epshannon</th>
<th>expol</th>
<th>simpson</th>
<th>altitude</th>
<th>pH</th>
<th>TotalP</th>
<th>ResinP</th>
<th>chlorP</th>
<th>evale</th>
<th>phosphates</th>
<th>nodules</th>
<th>N ratio</th>
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<td>-0.34*</td>
<td>-0.42**</td>
<td>-0.38*</td>
<td>0.41**</td>
<td>0.28</td>
<td>0.45**</td>
<td>0.36*</td>
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<td>0.45**</td>
<td>0.36*</td>
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<td>0.36</td>
<td>0.39</td>
<td>dry weigh</td>
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</tr>
<tr>
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<td>-0.36*</td>
<td>0.35**</td>
<td>0.28</td>
<td>0.45**</td>
<td>0.36*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.26</td>
<td>0.36</td>
<td>0.39</td>
<td>dry weigh</td>
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</tr>
<tr>
<td>-0.36</td>
<td>-0.26</td>
<td>-0.45**</td>
<td>-0.38*</td>
<td>0.44**</td>
<td>0.35*</td>
<td>-0.36*</td>
<td></td>
<td></td>
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<td></td>
<td>0.32**</td>
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<td>0.93**</td>
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</tr>
<tr>
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<td>-0.26</td>
<td>-0.45**</td>
<td>-0.38*</td>
<td>0.44**</td>
<td>0.35*</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.32**</td>
<td>0.93**</td>
<td>0.93**</td>
<td>N fixated</td>
<td></td>
</tr>
</tbody>
</table>
4.4 Non-metrical multidimensional scaling (NMDS)

Figure 21 shows the non-metrical multidimensional scaling of the AMF community composition of the 75 plant samples. The closer two sample points are on the figure, the more similar the AMF community composition is of these two samples. Furthermore, it was tested if environmental factors influence the nestedness of AMF communities. The field has a significant effect (P < 0.05) on the nesteness of the AMF communities, which is illustrated in Figure 21 and Table 9. It is clear that points from the same field are situated close to each other. Table 9 also shows no significant effect of the location. This means that the AMF communities are not nested between fields on one location. Additionally, olsen P value, and pH of the soil have no significant effect (P > 0.05) on the nestedness of the AMF communities, however the pH of the soil has a significant effect on a significance level of 0.10 (Table 9). The E-value and Resin P were discarded from the analyses as they show to have a lower significance level than Olsen P.

Table 9 Results of the permutation tests of the nonmetric multidimensional scaling coordinates testing for significant relationships between AMF community composition and the location, field, pH and Olsen P. Significance codes: 0 ‘***’, 0.001 ‘**’, 0.01 ‘*’, 0.05 ‘.’, 0.1 ‘ ’.

<table>
<thead>
<tr>
<th></th>
<th>R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>0.028</td>
<td>0.498</td>
</tr>
<tr>
<td>Field</td>
<td>0.4605</td>
<td>0.002 **</td>
</tr>
<tr>
<td>pH</td>
<td>0.0899</td>
<td>0.079</td>
</tr>
<tr>
<td>Olsen P</td>
<td>0.0625</td>
<td>0.154</td>
</tr>
</tbody>
</table>
Figure 21 Non-metric multidimensional scaling of AMF communities taken from the 75 plant samples, calculated using Bray-Curtis distances. The samples are categorized by field. IA, IB, IC, ID, IE are the five fields in Dedo I; IIA, IIB, IIC, IID, IIE are the five fields in Dedo II; and IIIA, IIIB, IIIC, IIID, IIIE are the five fields in Dedo III.

4.5 Effect of edaphic parameters on AMF diversity

A stepwise general linear mixed-effect regression model was performed in R and the Akaike information criterion (AIC) is given in Table 10. A general rule is, the lower the AIC, the better the model and negative values are better than positive. Hence, the Simpson diversity index is selected. The coefficients of this model are given in Table 11. Total soil P and pH have a significant effect (P < 0.05) on the diversity, but Olsen P hasn’t. Nevertheless, Olsen P should not be discarded according to the AIC rule. The results are interpreted as followed. A low Simpson value means a high diversity. Therefore a high total soil P concentration, low pH and Olsen P value result in a high Simpson index and thus a low diversity and vice versa. The variance explained by the fixed effects is 12.3% of the total variance and 15.2 % of the total variance is explained by both the fixed as random effects.
Table 10 Akaike information criterion (AIC) and model output received from a stepwise linear regression in both directions for each diversity index. Interaction effects are excluded.

<table>
<thead>
<tr>
<th>Diversity index</th>
<th>AIC</th>
<th>Retained parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chao</td>
<td>454.304</td>
<td>Chao ~ TotalP + ResinP + altitude</td>
</tr>
<tr>
<td>Ace</td>
<td>430.8569</td>
<td>Ace ~ TotalP + ResinP</td>
</tr>
<tr>
<td>Jackknife</td>
<td>794.6099</td>
<td>Jackknife ~ TotalP + ResinP</td>
</tr>
<tr>
<td>Shannon</td>
<td>77.70949</td>
<td>Shannon ~ TotalP + pH + altitude + ResinP</td>
</tr>
<tr>
<td>NpShannon</td>
<td>73.75272</td>
<td>NpShannon ~ TotalP + pH</td>
</tr>
<tr>
<td>ExpH</td>
<td>348.2478</td>
<td>ExpH ~ TotalP + pH</td>
</tr>
<tr>
<td>Simpson</td>
<td>-88.3326</td>
<td>Simpson ~ TotalP + OlsenP + pH</td>
</tr>
</tbody>
</table>

Table 11 Coefficients from the linear mixed-effect regression model (Simpson ~ TotalP + OlsenP + pH with location as random effect) without interaction effects (significance codes: 0 ‘***’, 0.001 ‘**’, 0.01 ‘*’, 0.05 ‘.’, 0.1 ‘ ’).

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std.Error</th>
<th>t-value</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1.0499</td>
<td>0.447</td>
<td>2.349</td>
<td>115.245</td>
</tr>
<tr>
<td>TotalP</td>
<td>0.0002</td>
<td>0.000</td>
<td>2.115</td>
<td>0.722</td>
</tr>
<tr>
<td>Olsen P</td>
<td>-0.0009</td>
<td>0.001</td>
<td>-1.589</td>
<td>3.844</td>
</tr>
<tr>
<td>pH</td>
<td>-0.1785</td>
<td>0.081</td>
<td>-2.208</td>
<td>3.611</td>
</tr>
</tbody>
</table>

If interaction is allowed, the model can be improved. A three way interaction effect of pH, Olsen P and Total P on the Simpson diversity improves the AIC to -83.78. The new coefficients are given in Table 12. Notice that there are no significant effects (P>0.1). It can be considered that the residuals are distributed normally and homoscedastic, which is confirmed by the modified levene test (P>0.05) and

Figure 22 output of the residual analysis of the mixed-effect regression model (Simpson ~ TotalP + OlsenP + pH with location as random variable). On the left a boxplot of the residuals are given for each location and on the right the residuals are plotted into a Q-Q plot.
shown in Figure 22. However, as all parameters are now not significant, we prefer the model without interaction.

Table 12 Coefficients from the linear mixed-effect regression model (Simpson ~ TotalP * OlsenP * pH with location as random effect) and interaction of variables is allowed (significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1).

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std.Error</th>
<th>t-value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-4.7600</td>
<td>3.5400</td>
<td>-1.345</td>
</tr>
<tr>
<td>OlsenP</td>
<td>0.0511</td>
<td>0.0742</td>
<td>0.689</td>
</tr>
<tr>
<td>pH</td>
<td>0.8360</td>
<td>0.6220</td>
<td>1.344</td>
</tr>
<tr>
<td>TotalP</td>
<td>0.0058</td>
<td>0.0035</td>
<td>1.655</td>
</tr>
<tr>
<td>OlsenP:pH</td>
<td>-0.0084</td>
<td>0.0128</td>
<td>-0.657</td>
</tr>
<tr>
<td>OlsenP:TotalP</td>
<td>-0.0001</td>
<td>0.0001</td>
<td>-1.002</td>
</tr>
<tr>
<td>pH:TotalP</td>
<td>-0.0010</td>
<td>0.0006</td>
<td>-1.606</td>
</tr>
<tr>
<td>OlsenP:pH:TotalP</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.993</td>
</tr>
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</table>

Additionally the effect of the environment on the AMF richness is investigated. The major explanatory factor which effects the species richness (sobs) is plant N/P ratio. Regression output is given in Table 13. It indicates that P limiting conditions is positively correlated with the species richness. However, this is not significant. The variance explained by the fixed effects only, and the fixed and random effects, are both 5.3 % of the total variance.

Table 13 Output of the general linear mixed-effect model of the species richness (sobs) explained by N/P ratio (significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1).

<table>
<thead>
<tr>
<th>variable</th>
<th>Coefficient</th>
<th>Std.Error</th>
<th>t-value</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>14.34</td>
<td>2.46</td>
<td>5.82</td>
<td>314.45</td>
<td>0.000 ***</td>
</tr>
<tr>
<td>N/P ratio</td>
<td>0.12</td>
<td>0.07</td>
<td>1.82</td>
<td>3.47</td>
<td>0.068</td>
</tr>
</tbody>
</table>

4.6 Effect of AMF diversity on plant P content and phosphatase activity

According to the correlation matrix shown in Table 8, no significant correlations are visible between the richness and diversity parameters and the plant P. When a mixed-effect model was used to remove the effects of Olsen P, Resin P, E-value, Total P, and pH, still no significant (P > 0.05) effect was observed.

The phosphatase activity is significantly correlated with the Ace index, which is an index for species richness. A mixed-effect model removed the effects of Olsen P, Resin P, E-value, Total P, pH, and altitude and now a significant (P < 0.05) positive effect was found for the species richness indices: sobs, Ace, Chao. The coefficient and the significance level are shown in Table 14. Additionally, the phosphatase activity was plotted against the species richness parameter (Ace) because this is the only richness index that also showed a significant correlation with the phosphatase activity in the correlation matrix (Table 8).
Table 14 The coefficients and their significance level of the diversity indices on the P content in the plant and the phosphatase activity (significance codes: 0 ‘***’, 0.001 ‘**’, 0.01 ‘*’, 0.05 ‘.’, 0.1 ‘ ’)

<table>
<thead>
<tr>
<th>index</th>
<th>coefficient</th>
<th>P</th>
<th>coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>sobs</td>
<td>-5.63</td>
<td>0.599</td>
<td>0.039</td>
<td>0.016</td>
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<tr>
<td>Chao</td>
<td>-3.09</td>
<td>0.727</td>
<td>0.029</td>
<td>0.024</td>
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<tr>
<td>Ace</td>
<td>-1.72</td>
<td>0.777</td>
<td>0.021</td>
<td>0.020</td>
</tr>
<tr>
<td>Jackknife</td>
<td>-0.45</td>
<td>0.377</td>
<td>0.001</td>
<td>0.267</td>
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<tr>
<td>Shannon</td>
<td>69.96</td>
<td>0.724</td>
<td>0.282</td>
<td>0.357</td>
</tr>
<tr>
<td>npShannon</td>
<td>89.83</td>
<td>0.652</td>
<td>0.311</td>
<td>0.311</td>
</tr>
<tr>
<td>expH</td>
<td>8.18</td>
<td>0.689</td>
<td>0.032</td>
<td>0.302</td>
</tr>
<tr>
<td>Simpson</td>
<td>-132.23</td>
<td>0.865</td>
<td>-0.466</td>
<td>0.697</td>
</tr>
</tbody>
</table>

Figure 23 The phosphatase activity in function of the species richness (Ace)

4.7 Interaction between nodules and AMF colonization

According to the correlation matrix shown in Table 8, no significant correlations are observed between richness and diversity parameters, and the amount of nodules. When a mixed-effect model was used to remove the effects of Olsen P, Resin P, E-value, Total P, pH, and altitude still no significant (P > 0.05) effect was observed.

There is a negative correlation found between the Shannon diversity index and the %Ndfa in the correlation matrix. When mixed-effect models was used to remove the effects of Olsen P, Resin P, E-value, Total P, pH, and altitude, still only the Shannon index showed a significant (P < 0.05) negative correlation. The coefficients and significance levels are shown in Table 15. Additionally a scatterplot
was made for a visual interpretation of the correlation between the amount of reads per sample and the nodule count on the one hand and the correlation between %Ndfa and the Shannon diversity on the other hand (Figure 24).

The most important finding is that the sobs, Shannon and Simson index are all significant (P<0.01) correlated with the N fixed in mg N per gram dry weight of the plant. The trend is that richness and diversity are negatively correlated with the N fixed. The trend is visualised in Figure 25. As mentioned before, the Simpson index is the inverse of the diversity. A mixed-effect model was used to remove the effects of Olsen P, Resin P, E-value, Total P, pH, and altitude. The results are shown in Table 15 and the sobs, Shannon, and npShannon are significantly (P < 0.05) negative correlated with the N fixed, while the Simpson is significantly (P < 0.05) positive correlated with the N fixed.

Table 15 The coefficients and their significance level of the diversity indices on nodule number, %Ndfa, and N fixed (mg N g\(^{-1}\) dw) resulted from a linear mixed effect model with all edaphic parameters as random effect (significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ')

<table>
<thead>
<tr>
<th>Index</th>
<th>Coefficient</th>
<th>P</th>
<th>Coefficient</th>
<th>P</th>
<th>Coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>#nodules</td>
<td>-0.48</td>
<td>0.568</td>
<td>-7.293</td>
<td>0.072</td>
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<td>0.0476</td>
</tr>
<tr>
<td>Chao</td>
<td>-0.1088</td>
<td>0.87</td>
<td>-4.509</td>
<td>0.396</td>
<td>-2.099</td>
<td>0.332</td>
</tr>
<tr>
<td>ace</td>
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<td>0.936</td>
<td>-1.364</td>
<td>0.3618</td>
</tr>
<tr>
<td>jackknife</td>
<td>0.02</td>
<td>0.519</td>
<td>66.57</td>
<td>0.453</td>
<td>0.009</td>
<td>0.9459</td>
</tr>
<tr>
<td>Shannon</td>
<td>-5.08</td>
<td>0.741</td>
<td>-0.506</td>
<td>0.023</td>
<td>-154.496</td>
<td>0.0021</td>
</tr>
<tr>
<td>npShannon</td>
<td>1.52</td>
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<td>-126.398</td>
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<td>Simpson</td>
<td>46.23</td>
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<td>0.067</td>
<td>0.277</td>
<td>626.491</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

Figure 24 The percentage Nitrogen derived from atmospheric nitrogen (%Ndfa) plotted against the Shannon index of the 75 samples.
Figure 25 scatterplot of the most significant correlations between the AMF richness and diversity indices and the amount of N fixed per g plant dry weight for all 75 plant samples. In the tree plot the N fixated is shown in function of (left) the species observed; (middle) AMF diversity as Shannon; (right) inverse of AMF diversity as Simpson.
5. Discussion

This discussion will start with the evaluation of the values measured during the research. The focus will lay on the low amount of OTU’s read per sampled plant. Further, the hypotheses will be evaluated step by step.

5.1 Evaluation of collected data
5.1.1 Diversity indices

As can be deduced from the rarefaction curves (Figure 20), most of the samples taken don’t cover all AMF species present in the plant roots. Only for 8 field, the rarefaction curves reach their asymptotic phase. Therefore, on the other 7 fields more species are expected than observed but as the sample size (the amount of different OTU’s reads) isn’t large enough, there are more species expected on these fields. Additionally, these rarefaction curves are pooled per field and the sample sizes of the individual plants are even smaller. Hence, little derived diversity indexes are representative for the samples. It is important that all conclusion based on these data have to be nuanced. The small reads per sample also increases the variation in the dataset and therefore it’s more difficult to show effects. Nevertheless, it was preferred to work further with more but less reliable data point so that statistical analysis was possible.

In our research an average of 18 OTU’s per sample was observed. This is a similar result to that found in the research of Van Geel et al. (2015) with AMF in roots of apple in Belgium, i.e. 22 OTU’s per sample in organic and 19 OTU’s per sample in conventional agriculture. However, the rarefaction curves indicate an underestimation of the species richness in these samples. Additionally a richness up to 54 species per field was observed. Furthermore, the diversity in our research in terms of the exponent of the Shannon diversity amounts 10.59±0.55 which is approximately the double of the values observed in the same research of Van Geel et al. (2015), i.e. 5.76 in organic and 4.51 in conventional agriculture. This means that the evenness of the AMF community in our research is higher. Additionally, in this thesis a quite high diversity and species richness was observed compared to cowpea in Benin and different leguminous crops in the Sichuan Province of mainland China. In Benin the calculated indices were Simpson (3.3±0.83), Chao (14-15), and jackknife (14-17)(Johnson, Houngnandan, & Kane, 2013). In China 14 AMF species were distinguished per sample (Wang et al., 2008). This is because in these research, AMF was characterized on morphology. In our study, next generation sequencing technologies like 454 pyrosequencing were used, allowing to distinguish similar morphotypes better (Kivlin et al., 2011; Ópik et al., 2009).
But how come we observe this low amount of OTU’s reads per sample, while high AMF richness and diversity is observed? Two explanations will be given: decreased colonization due to competition between Rhizobium bacteria and AMF for photosynthates, and errors in the sampling and lab analysis.

First, in highly nodulated plants there’s a negative correlation between nodulation and AMF colonization (Jia et al., 2004; Ossler, 2015). This can be explained by competition for photosynthates between nodules and AMF. As July and August of 2015 were remarkably dry, slow growth of the Vicia faba plants was observed, however this climatic data could was not be received from the Ethiopian National Meteorological Agency. Nevertheless, expert advice told that plant growth was delayed and it is therefore likely that competition for photosynthates occurred between AMF and Rhizobium.

In the situations where plants produce insufficient photosynthates, it is argued that host plants give assimilates to the symbiont they prefer depending on the nutrient and light availability (Werner & Kiers, 2015). In the research of Ossler (2015), they argue that P is a key variable in differentiation plant assimilates to the AMF or Rhizobium bacteria. We saw in our research that the average N/P ratio in the plant amounts 33.7 ±1.8, which indicates P limiting conditions (Garrish et al., 2010). This would mean that AMF colonization would be enhanced and Rhizobium colonization inhibited. However, compared to other research (Agegnehu et al., 2006; Ghizaw et al., 1999), nodulation has been observed high, indicating other processes take place. It is hard to explain the observed effect with other environmental conditions like nutrients or light availability as these observation were not made except for the high temperatures and low rainfall. However, plants seem to profit from AMF colonization under drought, heat and salt stress (Augé, 2004; Porras-Soriano, 2009; Rillig et al., 2002) and therefore it’s more likely AMF colonization benefitted from plant assimilates due to dry weather conditions.

As the latter theory is unsatisfying in explaining our results, we argue that the Rhizobium bacteria are more competitive than AMF. It has been observed that nodulation isn’t correlated with high AMF colonization but AMF colonization decreases significantly under highly nodulated conditions (Jia et al., 2004). The higher competitiveness might be caused by the priority effect, which means that the first organisms infecting the plant has a competitive advantage. Under glasshouse conditions, AMF colonization typically occurs within 3 to 12 days after inoculations, depending on the AMF and host species, and environmental factors. Furthermore, it is believed that under natural conditions colonization is less effective (Afek & Rinaldelli, 1990). Nodulation of Rhizobia typically occurs within 2-4 days after inoculation in rice (Perrine-Walker et al., 2007) and within 3 days in Medicago truncatula (Journet, 2006). Thus, rhizobia infect the plant generally faster than AMF (Sakamoto et al., 2013). This head start allows Rhizobia to take up the majority of the root and photosynthates, leaving less for AMF. In general, a trade-off doesn’t occur and AMF colonization is still possible (Ossler, 2015) but due to dry weather conditions photosynthates were perhaps limited. Although, plants tend to
stimulate the symbiosis they need the most, it’s likely these priority haven’t evened out as the sampling took place around 4 months after sowing. Especially in annuals, priority effect can be important (Werner & Kiers, 2015). It would be interesting to investigate how the interaction between the nodulation and AMF colonization evolves through the season, cause it is likely that AMF colonization increases with time. Perhaps Rhizobia also actively suppress the colonization of AMF during photosynthate limiting conditions. Catford & Staehelin (2003) confirm this theory in their research. They tested AMF colonization by inoculation of one side of a split-root system of alfalfa plants with Sinorhizobium meliloti or with Nod factor compounds. In both situation, AMF root colonization on the other side of the split-root system reduced drastically. They stated that Nod factors produced by rhizobia trigger a secondary signal in the host plant which initiates suppression of AMF colonization.

The negative correlation between nodulation and AMF colonization can also be explained from the perspective of C economy (Sakamoto et al., 2013). Rhizobial and arbuscular mycorrhizal symbioses can each consume 4-16% of recently photosynthetically fixed C to maintain their growth, activity, and reserves (Kaschuk et al., 2010). However, nodules return 21-52% of that C to the plant in the form of organic N, ureides, or amides (Minchin & Summerfield, 1981), while AM fungi do not have a physiological system to return the C assimilated in the mycorrhiza to the host plant. Thus, nodulation is more carbon efficient than AMF symbioses and therefore preferred by the host under photosynthate limiting conditions (Kaschuk et al., 2009; Kaschuk et al., 2010; Sakamoto et al., 2013).

The dataset supports the theory that there’s low colonization because of high nodulation but does not prove it. First of all, there is a significant (P < 0.05) negative correlation between the richness and diversity parameters, and the amount of N fixed by the plant (mg N g⁻¹ dw) (see Table 15 and Figure 25). Secondly, the Shannon index correlates with a decreasing amount of N₂-fixation (%Ndfa) (see Table 15 and Figure 24). Nevertheless there was no significant correlation found between the amount of nodules and the species richness or diversity. This is in slight contrast with what is found before, as it is not the Rhizobium colonization that interacts with the AMF colonization but it is the Rhizobium activity which interacts with AMF richness and diversity. Additionally it should be remarked that we do not have quantitative data of AMF colonization, therefore a quantitative PCR or microscopic observations should have been made. However, we conclude from our observations that there is a negative interaction between the two symbionts in our dataset.

An additional remark is that both negative as positive interactions between AMF and Rhizobia bacteria can take place in the dataset because, there’s no certainty if any of the tripartite symbioses are situated in photosynthate limiting conditions. If photosynthates are not limited, AMF colonization has been shown to enhance nodulation and N₂ fixation (Chalk et al., 2006; Ossler, 2015; Sakamoto et al., 2013). This is caused by increased P supply to the nodules through AMF (Ossler, 2015; Wang et al., 2011).
Moreover, the production of specific flavonoids produced by AMF increase nodulation (Antunes et al., 2006; Antunes & Varennes, 2006).

A second reason that explains the low amount of reads per sample is an insufficient laboratory processing. For example, during analysis some powerbeads from the PowerSoil®DNA Isolation Kit were overloaded with root hairs. Normally, 0.25 g of soil is added to the power beads tubes, containing power beads and extraction solution. Next, these tubes are closed and vortexed severely until all cell structures are demolished (around 10 minutes). In this study, the beads were filled with root hairs to extract the DNA of the AMF colonizing the faba beans. In cases of overloading the tubes, root hairs and remaining soil formed a paste preventing the beads to move during vortexing. Removing root hairs afterwards solved the problem in a few cases but mostly too much extraction solution was removed in the process, decreasing the concentration of DNA in the further analysis. Additionally, impurities like organic acids in the solution can inhibit DNA polymerase during PCR. Furthermore, we chose primer pair AMV4.5NF-AMDGR as it was showed the best for 454 pyrosequencing of AMF communities from apple tree roots in Flanders (Van Geel et al., 2014). However, efficiency of a primer pair depends on the situation, so ideally a preliminary evaluation of their performance is recommended (Gamper et al., 2009; Mummey & Rillig, 2007). Moreover, this primer pair have been suggested to favour amplification of Glomeraceae sequences at the expense of Ambisporaceae, Claroideoglomeraceae and Paraglomeraceae sequences (Van Geel et al., 2014). These reasons may explain some of the failure measurements however most of the samples were processed as they should and gave clear DNA bands.

5.1.2 Soil parameters

In Table 6, altitude, pH, Total P, Resin P, Olsen P, and E-values are given.

The pH was not normal distributed among the samples and differs for every location significantly (P<0.05). Nevertheless, all soils showed a lower pH than 6.5, which is considered as the lower bound of the optimal range of pH for faba beans (Jensen et al., 2010), however another study shows that the optimal pH for faba bean ranges between 4.5 and 8.3 (Duke, 2012).

The total P, available P and pH values in this study are similar to values observed in a study of Nebiyu (2014) near to Dedo. On a field with an altitude of 2160 m.a.s.l., a total soil P concentration of 1156 ± 18 mg P kg⁻¹ soil, and available P concentration of 10.7 ± 2.5 mg P kg⁻¹ soil, and a pH of 5.6 ± 0.1 was observed. The Bray-II P method was used in Nebiyu’s study therefore comparing the data should be done with caution. Because in general it is believed that the Bray method is better in acid soils. Nevertheless, we conclude our data is not exceptional.

Furthermore, an Olsen P value of 10 mg P kg⁻¹ soil is considered as an optimal value for plant growth (Sims, 2000), so this would mean that on average all location optimal P conditions are established.
Additionally, soil pH is quasi optimal. Nevertheless P limiting conditions have been observed according to the N/P ratio (Garrish et al., 2010), which is remarkable. The discrepancy between the available P and N/P ratio can be explained as follows. It was observed that each location had 1 or 2 fields with an extremely high available P due to management. For example, at the third location field C had an average Olsen P of 49 mg P kg⁻¹ soil. Thus, the average Olsen P increased much. These observed high values can be explained by better management practices, as this is a field next to the homestead of the farmer. Therefore the increased P content will probably coincide with an increase N content and therefore the N/P ratio will decrease relatively less.

5.1.3 N₂-fixation parameters

Comparing %Ndfa, number of nodules, plant P and dry weight with results from other studies is hard as most data is not available for the first flowering stage.

Fan et al. (2006) found a decrease in %Ndfa from 64% at 0 kg N ha⁻¹ to 40% at 120 kg N fertilizer ha⁻¹. In our study, an average %Ndfa of 66.0±2.0 was found, which indicates that our plants were taken on soils with little amount of N. Nebiyu, (2014) found in similar conditions as those in our thesis, that the %Ndfa depended on the Faba bean variety and that the variety Moti had the highest (84 ± 4.5%) %Ndfa. The variety used in our study was also Moti, therefore we conclude the %Ndfa observed is reduced. However, this could be because the samples were taken after first flowering while the samples from Nebiyu were taken at late flowering. It takes time for the nodules to become active and indeed, inactive nodules were observed on the plant roots. Additionally our plants were samples after a dry rainy season and there has been a delayed growth observed of the plants.

Nodulation numbers are considered quite high, because in a study of (Agegnehu et al., 2006) the amount of nodules counted in a mixed cropping system of faba bean and barley in the central highlands of Ethiopia, never exceeded 30 nodules per plant during 3 years of research. This threshold of 30 nodules a plant is considered as good nodulation (Ghizaw et al., 1999). However, our results are not exceptional as Mengel et al. (1974) had observed between 200 and 300 nodules per faba bean plant in a research. Furthermore, in the research of Nebiyu (2014), a nodule number of 165 ± 8.7 was observed in Dedo soils. So we conclude that nodulation is reduced. This could be explained by water stress caused by the dry season, which have been shown to reduce nodulation in legumes (Ramos, 1999) and in faba bean (Wahab & Abd-Alla, 1995). Additionally, nodule number was counted at the first flowering stage in this thesis while Nebiyu (2014) counted the nodule number at late flowering, which also could explain the nodule reduction.
5.2 Hypotheses

5.2.1 The response of AMF community, richness, and diversity to edaphic factors.

5.2.1.1 AMF community
The NMDS figure (Figure 21) shows that field is the major determining factor of the AMF community. Furthermore, analysis indicate that soil characteristics like pH and Olsen P have no significant influence on the AMF community composition (Table 9). In literature, especially pH and rainfall have been observed as significant abiotic parameters determining the AMF community composition (Fitzsimons et al., 2008; Hazard et al., 2013). There’s still uncertainty about the effect of available P on AMF community composition. On the one hand, Jansa et al. (2014) found that available P levels in the soil had no effect on AMF community composition. On the other hand, Van Geel et al. (2015) observed a significant effect of plant availability (Olsen P) on the AMF community and diversity was in Belgian apple orchards. Moreover, no specific research on the effect of available P on the AMF community in Vicia faba was found. Additionally, other determining factors found in literature are host plant, soil type and agricultural management (reviewed by Chaudhary et al., 2008), but these factors were not taken into account in this thesis.

As field has been observed as the most determining parameter on the AMF community, it can be argued that the Baas-Becking hypothesis doesn’t apply but that a dispersal limit shapes the community. Nevertheless, it is more likely that this is not the case. The parameter field is intensely correlated with the abiotic and biotic variables. For example each sample on a field has the same altitude, and similar management. Therefore it is expected that abiotic factors like pH and soil P content are similar on a field. Hence, the result supports the Baas-Becking hypothesis, which says that everything is everywhere, but the environment selects (Baas-Becking, 1934). Additionally, no significant differences are found between the 3 location and geographical distances were perhaps not large enough to find effects as all the fields were located within a radius of approximately 15 km. Because, dispersal limitation may only become more important than environmental factors at regional scales (250 km) (Gast & Gosling, 2011; Martiny & Eisen, 2011)

5.2.1.2 AMF richness and diversity
Mixed-effect linear modelling shows that the species richness is most determined by the degree of P limitation. The higher the N/P ratio and therefore the more limiting P was, the higher the species observed in a sample (sobs). However the effect was not significant (p = 0.68) on a 5% significance level and additionally only 5.3% of the variance was explained by the model. We conclude that no real observation can be made.

Mixed-effect linear modelling of the diversity indices shown that the Simpson index fitted best the data. pH and Total P have a significant effect on a 5% significance level. Furthermore, Olsen P was not discarded to minimize the AIC. The final model explained 15.2% of the total variance if both fixed
and random effects are included, which is very low. The results are interpreted as follows. A low Simpson value means a higher diversity. Therefore a high total soil P concentration, low pH and Olsen P value result in a high Simpson index and thus a low diversity and vice versa.

The results contradict what is found mostly in literature. In general AMF richness and diversity is negatively correlated with the available P (Van Geel et al., 2015; Johnson et al., 2013) and the pH (Hazard et al., 2013; Wang et al., 2008). Additionally, researchers found in the South American Puna grassland a negative correlation between AMF diversity and altitude within a range of 3320 and 3850 m.a.s.l. (Lugo et al., 2008), and in our model altitude is discarded. The only parameter in line with what is found in literature is the total soil P. As plants seem to allocate more photosynthates to the structures that best garner the most limiting resources and therefore a low soil P content will favour AMF richness and diversity (Ossler, 2015; Werner & Kiers, 2015). However, the latter may not be the case in photosynthate limiting conditions. Furthermore, Coughlan & Dalpé (2000) found also a negative effect between pH and AMF colonization under sugar maple. They argue that Ca" levels tend to be lower at decreasing pH, and it has been shown that Ca can be critical for AMF colonization (Jarstfer et al., 1998).

The regression models do not explain much of the observed variance. Although parameters like altitude, pH, total P, available P (Olsen P, resin P and E-value), amount of nodules, and the N/P ratio were fitted in the regression, most of them were discarded. This means that there’s a lot of external variance not taken into account. Farm management or distance to the homestead might be an explanatory variable that could improve the model (Van Geel et al., 2015; Johnson et al., 2013; Schneider et al., 2015). Furthermore, CEC and organic carbon have been proven to influence the AMF colonization (Alguacil et al., 2014; Porras-Soriano, 2009). Additionally, most of the variability might be originated from random effects in the fields and variability during sample processing.

5.2.2 Effect of diversity and richness on the plant P uptake and phosphatase activity

The positive correlation between AMF diversity and plant P uptake found in van der Heijden et al. (1998) was not observed in our study (Table 8). However, this could be explained by the effects of pH, total soil P and available soil P. As these factors have a high effect on the soil P it might hide the effect of the AMF diversity on plant P content. However, removing the effect of available P in mixed models doesn’t show a better correlation. We conclude that, based on our dataset, the effect of AMF diversity or richness on the P content in the plant is not observable.

Nevertheless, positive correlation has been observed between the AMF richness and the phosphatase activity. In literature effects both positive and no effects were observed (Joner et al., 1995; Tarafdar & Marschner, 1994). Different AMF taxa have different abilities (Burleigh, 2002), thus perhaps some produce phosphatase and others not. Therefore a high AMF species richness increases the chance a
phosphatase producing AMF is present causing the positive effect on the phosphatase activity (based on: van der Heijden et al., 1998).

As mentioned before, most of the taken samples don’t cover all the possible reads per sample, therefore it could be interesting to test if this correlation still hold when it is tested on those data points where there’s more certainty all reads are covered. Therefore we calculated all the diversity indices on field level and discarded all the fields that didn’t reach the asymptotic phase in the rarefaction curves. The result is given in Figure 26, and it is clear that although there are few data points, there is a strong indication positive correlation between the richness and the phosphatase activity. Additionally, linear regression of this data results in a significant interaction.

![Figure 26 Phosphatase activity in function of the species richness. We pooled richness index on field level and discarded all the fields where the rarefaction curve didn’t reach the asymptotic phase.](image)

5.2.3 Interaction within the tripartite symbiosis of AMF, Rhizobia and Vicia faba

In this study no correlation between nodule count and AMF diversity or richness have been found, however the N₂-fixation seems to decrease with an increasing AMF diversity. A detailed discussion has been conducted already.

The dry weight of the *Vicia faba* plants seems to be negatively correlated with the AMF diversity, which can be explained as followed. As is seen in the correlation matrix, the plants on the soils with the lowest total P, available P (Olsen P and resin P), and most P limiting conditions (N/P ratio), have the lowest dry weight. And, these soils select for a higher AMF diversity (van der Heijden et al., 1998). It has also been observed that an increasing amount of nodules increases the plant weight (Strodtman & Emerich, 2009).
6. Conclusion

We observed that the community of arbuscular mycorrhizal fungi in our study was dominated by species of the family *Glomeraceae* because 36 of the 81 found species were classified in this family. Three generalists, species that occur in most of the situations, were found, i.e. *Claroideoglomeraceae* *Claroideoglomus sp.*, *Acaulosporaceae* *Acaulospora sp.*, and *Glomeraceae* *Glomus sp.*. Furthermore, a high AMF diversity was observed, however the richness was not exceptionally high.

Our first objective, to assess the determining factors of the AMF community, richness and diversity, was not performed adequate to make strong conclusions. Nevertheless, it has been shown that the factor field significantly determined the AMF community composition, and not location. Thus we conclude that the AMF community is determined by local abiotic and biotic factors, more than dispersal limitation. Our results indicate that especially pH and in less extent available phosphorus were the determining factors of the AMF community, although not significantly. The latter two factors also influenced the diversity, together with the total soil P. In particular, we found that high total soil P concentration, low pH and low Olsen P values result in low diversity and vice versa. Additionally, P limiting conditions seemed to coincide with AMF richness.

The second objective, to investigate the effect of the AMF diversity on plant P content and phosphatase activity gave promising results. Although no significant correlation between AMF and plant P content was observed, a high richness coincided with higher phosphatase activity. However, we also observed that P availability had a bigger impact on the plant growth than AMF diversity. As we observed low plant dry weight in soils with a low P content, however the richness of AMF tended to be bigger in these soils.

Finally, we conclude from our last objective that there’s an indication of a negative interaction between *Rhizobium* bacteria and AMF colonization. Although, nodulation and AMF diversity are not significantly correlated, %Ndfa is negatively correlated with AMF diversity and more importantly, the N fixed (mg N g⁻¹ dw) is significantly negatively correlated with the AMF richness and diversity. Therefore we argue that the negative effects of Rhizobium colonization on AMF colonization may occur under dry conditions where photosynthates are limited.
References


Nebiyu, A. (2014). *Role of faba bean (Vicia faba L.) for intensification of cereal-based cropping systems in the humid highlands of Ethiopia*. Gent University.


c


## Appendix

<table>
<thead>
<tr>
<th>OTU</th>
<th>Familie</th>
<th>genus</th>
<th>species</th>
<th>Bit-score</th>
<th>Identities</th>
<th>ID%</th>
<th>relative occurrence</th>
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<td>Claroideoglomus</td>
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<td>456</td>
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<td>100.00%</td>
<td>92%</td>
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<td>87%</td>
</tr>
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<td>sp.</td>
<td>456</td>
<td>230</td>
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<td>70%</td>
</tr>
<tr>
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<td>sp.</td>
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<td>100.00%</td>
<td>69%</td>
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<td>67%</td>
</tr>
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<td>Glomus</td>
<td>sp.</td>
<td>456</td>
<td>230</td>
<td>100.00%</td>
<td>66%</td>
</tr>
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<td>sp.</td>
<td>426</td>
<td>228</td>
<td>98.70%</td>
<td>66%</td>
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<td>Diversisporaceae</td>
<td>Diversispora</td>
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<td>227</td>
<td>98.70%</td>
<td>64%</td>
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<td>97.83%</td>
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<td>98.26%</td>
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<td>424</td>
<td>226</td>
<td>98.26%</td>
<td>36%</td>
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</tr>
<tr>
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</tr>
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<td>sp.</td>
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</tr>
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</tr>
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<td>21%</td>
</tr>
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<td>18%</td>
</tr>
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</tr>
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</tr>
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</tr>
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</tr>
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