ANGELA YOMAIRA BENAVIDES MARTINEZ

NATURAL DISTRIBUTION OF THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA* IN A COFFEE CROP

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

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APROVADA: 16 de março de 2016.

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Ricardo Ribeiro de Castro Solar Arnoldus Rudolf Maria Janssen

_____

Simon Luke Elliot
(Orientador)

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RESUMO

BENAVIDES MARTINEZ, Angela Yomaira M.Sc., Universidade Federal de Viçosa, Março de 2016. **Distribuição natural do fungo entomopatógeno *Beauveria* no cultivo de café.** Orientador: Simon Luke Elliot.

O cultivo de café manejado agroflorestalmente apresenta potencial e uma grande oportunidade em termos de diversidade de componentes enquanto inimigos naturais quando geridos sob este sistema. Os fungos entomopatógenos que pertecem à ordem Hypocreales são alguns destes inimigos. Dentre eles, o mais conhecido e utilizado como biopesticida tem sido o *Beauveria*, principalmente para o controle de broca do café *Hypothenemus hampei*. Recentemente, este fungo revelou seu desempenho em outros papéis na natureza, a exemplo de seu hábito endofílico, no entanto pouco se sabe realmente sobre sua ecologia e como se dá sua ocorrência natural nos cultivos agroflorestais do trópico. Desta forma, avaliou-se a distribuição natural das populações de *Beauveria* (UFC) antes e depois da colheita de café. Entre maio e setembro de 2015, foram coletadas amostras em diferentes compartimentos (solo, raiz, frutos caídos e frutos da árvore) e o *Beauveria* foi isolado pelo meio seletivo para entomopatógenos. Determinou-se a ocorrência endofílica e epifílica em diferentes níveis de posição da folha. As populações de *Beauveria* não apresentaram um padrão de distribuição nos diversos compartimentos, foram encontradas diferenças antes e depois da colheita, além dos niveis distintos das folhas. As populações encontradas na raiz e nos frutos foram abundantes, ademais nenhuma ocorrência endofílica foi descoberta nas folhas, mas de forma epifílica com uma alta frequência nas folhas basais. Assim, os resultados deste trabalho demonstram que o *Beauveria* estão ocupando vários compartimentos com abundante presença nas regiões da raíz, dos frutos caídos e do solo.

ABSTRACT

BENAVIDES MARTINEZ, Angela Yomaira M.Sc., Universidade Federal de Viçosa, March, 2016. **Natural distribution of the entomopathogenic fungus *Beauveria* in a coffee crop.** Adviser: Simon Luke Elliot.

Coffee agroforestry crops present a great opportunity and a potential in terms of diversity of components and their natural enemies when they are handled under this system. Entomopathogenic fungi are some of these enemies that belong to the order Hypocreales. Among them the best known and used as biopesticide it has been *Beauveria*, mainly for control of *Hypothenemus hampei*. This fungus has recently shown to play other roles in nature as their endophytic habit but little is really known about its ecology and as is their natural occurrence in tropical agroforestry crops. Therefore, it evaluated the natural distribution of populations of *Beauveria* (CFU) before and after coffee harvest. Between May and September 2015, samples in different compartments (soil, root, fallen fruit and fruit tree) were collected and *Beauveria* was isolated using selective medium for entomopathogenic. Endophytic and epiphytic occurrence was determined in leaves at different levels on the bush. *Beauveria* populations had significant differences in samples before and after harvest. This study found that roots and fallen fruits *Beauveria* populations were abundant. No endophytic occurrence was found in leaves but it found epiphytic occurrence with high frequency in basal leaves. The results of this study show that *Beauveria* is occupying several compartments with abundance presence in the root zone, fallen fruit and soil.

1. INTRODUCTION

For some tropical countries, coffee is the most valuable export crop in the international market (Duke and Backer, 2003). World coffee production for 2015 was around 143,371 sacks (1 sack = 60kg), of which approximately 30% was produced by Brazil, followed by Vietnam and Colombia and then other countries (International Coffee Organization, 2015). As Brazil is the world's largest producer and exporter of coffee, the economical importance of this crop is beyond question.

For all crops, pests and diseases are of great importance as they can adversely affect quality and yield. In coffee, *Hypotenemus hampei* (Coleoptera: Scolytinae) known as the Coffee Berry Borer (CBB) is considered to be the most important pest in many producing countries around the world (De la Rosa et al 2000; Neves and Hirose, 2005; Bustillo, A., 2006).

In Brazil, a key component of the management of this pest has been biological control with entomopathogenic fungi, principally the genus *Beauveria* within a system of integrated pest management. Isolates of this fungus are used as the active ingredient in biopesticides and as such these are the main type of biopesticide used in control of this pest (Bustillo, 2006; Vega et al, 2009a; Vega et al, 2009b). *Beauveria bassiana* (Balsamo) Vuillemin is a cosmopolitan and generalist entomopathogenic fungus of the order Hypocreales (Ascomycota) and is a natural enemy of a wide range of insects (Meyling and Eilenberg, 2007). Within natural ecosystems considerable populations of these fungi are found to occur naturally, i.e., without being applied by growers (Monzón et al. 2008).

Although *B. bassiana* is best known as the principal ingredient of entomopathogenic biopesticides, the ecology of this fungus is being unraveled and it is known to be able to occur endophytically (Bing and Lewis, 1991; Posada and Vega, 2006; Meyling and Eilenberg, 2006; Vega et. al. 2008a; Meyling et al.2009;

Vega et al. 2009a; Saucedo et al. 2014; Behie et al. 2015). Endophytes are organisms that, during a part of their life cycle cause asymptomatic infections in plant tissues (Wilson, 1995). This ability for endophytic colonization has aroused great interest from researchers due to the possibility of its use in plant protection from pest attack (Backman and Sikora, 2008; Schneider et al. 2013). While endophytic *B. bassiana* has been reported naturally in some instances, investigations of its endophytic capacity mostly consider artificial inoculations into a diverse range of crop plants, including maize (Bing and Lewis, 1991), cocoa (Vega et. al. 2008), pine (Reay et al. 2010), opium (Quesada Moraga et al. 2014) and coffee (Posada and Vega, 2006; Posada et al. 2007).

This endophytic capacity is an important example of the potential of entomopathogenic fungi like *Beauveria* and its hypocrealean relative, *Metarhizium*, to establish symbiotic associations with plants (Meyling and Eilenberg, 2007). Reports on *M. robertsii* show that it fungi can supply nitrogen from dead insects to the plant (Behie and Bidochka, 2014) and such associations might be expected in *Beauveria*. However, understanding of these associations is still at an early stage as there is limited information on the natural occurrence of fungus populations. In examining, the fungus-plant relationship and the fungus' natural population distribution, we could gain insights into harnessing the potential of these fungi for field applications with biopesticides, inoculation of crop plants or conservative biocontrol.

Most studies of the occurrence and diversity of entomopathogenic fungi have focused on comparisons of managed habitats, principally agro-ecosystems (e.g. areas with conventional management systems, semi-natural habitats or organic systems) (Keller et al 2003; Khudhair et al 2014; Tkaczuk et al 2014.). In these studies relatively few soil samples were collected at each location and only once because the studies were conducted at regional or national scales. But to

understand in more detail the ecology of natural populations of fungi, studies should be carried out with isolates collected at a more local spatial scale and in different compartments of the ecosystem (e.g. soil, fallen fruits and different parts of the plant) in order to obtain details on prevalence (and fungus diversity) at a more local scale (Meyling and Eilenberg, 2007).

Meyling and Eilenberg, (2007) consider the ecology of these entomopathogens in a compartmentalized system (below ground and above ground, within insects, on leaf surfaces, etc.) in which they consider how the fungus might disperse between these compartments. They emphasize the potential importance of these fungi in conservative biological control if their natural occurrence were better understood. However, the authors leave open a number of unanswered questions, notably whether *Beauveria* is to be found as an endophyte in a given system. Furthermore, such studies have been conducted almost exclusively in temperate habitats (Keller et al 2003; Meyling and Eilenberg, 2006; Meyling and Eilenberg, 2007), so very little is known from the tropics. Moreira, (2012) undertook such a study in coffee systems in southeastern Brazil. She found that survival of bait insects was lower when they were held on soils from agroforestry systems compared to soils from a full sun coffee system, due to entomopathogenic fungi, and that these fungi were also present in higher numbers and diversity in the first of these systems. An interesting aspect of this study was that, in one of study areas the frequency of *B. bassiana* was much greater after the harvest period (for both farming systems), opening the possibility that harvest might be influencing the populations of the fungus.

Based on the above, the principal objective of this study was to determine the natural of *Beauveria* populations in a compartmentalized system (soil, fallen fruits and different parts of plant) through time (so before and after harvest).

2. MATERIAL AND METHODS

2.1 Field Sampling

Our study was conducted in a single field of coffee (*Coffea arabica* L.), cultivar “Catuai” located in the Zona da Mata in the municipality of Araponga, in the Viçosa micro-region, Minas Gerais, in southeastern Brazil (20º40’01’’S 42º31’15’’W). This is an important coffee producing zone in the state of Minas Gerais with mean temperature and precipitation of 18ºC and 1,500mm. The landscape can be characterized by steep slopes ranging from 20% to 45%, and altitudes between 200-1800 m. The vegetation of the region was originally Atlantic Forest but has largely been replaced by coffee plantations. This study was conducted in a single organic agroforestry field in which no sprayed pesticides are used, and that has organic fertilizer management.

Thirty coffee bushes were chosen and labeled in a grid of 5 plants per row, spaced ca. 20 m from one another along the rows, in 6 rows, alternating every three rows (spacings of ca. 5 m per row, so 15 mts between rows). The sampled bushes were similar in height (approx. 2 m). Samples were taken repeatedly from the same plants and from soil adjacent to each of these plants, from May to September from 2015. This period includes the coffee harvest (in July and August).

Sampling, and subsequent fungal isolation and densities estimates, was conducted in three separated fashions (as sampling techniques were quite different, we feel that it would be misleading to treat the three together: 1. Determination of populations of *Beauveria* in roots, fruits and soil (coffee berries on plants, coffee berries on soil surface, root and soil); 2. Determination the presence or absence of epiphytic *Beauveria* in three levels of leaves (apical, middle and basal) and 3. Attempted detection of endophytic *Beauveria* (from leaf samples). As stated above,

the three types of sampling are not directly comparable; for this reason the data were divided into three groups.

Samples of plant material consisted of: (a) five leaves with petioles, from three levels (basal, middle and apical), giving a total of 15 leaves per plant (b) five coffee berries still on the plant, (c) five fallen berries and (d) root samples. The samples of leaves and fruits were selected at random from different branches at the three levels. We sampled fruits because this is where *H. hampei* is most commonly found. The root samples from each bush were collected with the aid of trowels to a depth of 20 cm. The trowels were partially disinfected with 70% alcohol between each sample. The fine roots emerging from lignified roots were sampled. All samples were placed in plastic bags and labeled and were then transported to the laboratory where they were kept at 4°C until processing.

For each soil sample, ca. 200 g of soil were taken from a single point adjacent to each coffee bush. Samples were taken of topsoil at a depth of 0-20 cm from the base of the bushes, beneath the canopy but ca. 75 cm from the plant trunks using a core sampler and trowels. The core sampler and trowels were partially disinfected with 70% alcohol between each sample to reduce cross-contamination between samples. The soil samples were placed directly into plastic bags before being taken to the laboratory.

2.2 Isolation from root, fruit and soil samples

For root samples, 0.5 g of coffee roots (see above) were placed in a sterile mortar with 15 ml of sterile dd H_2O with 0.01% Tween 80. Samples were macerated until homogeneous mixtures were obtained. For fruits (fallen and/or on the bush, collected separately) we used 5 coffee beans plus 20 ml H_2O with 0.01%Tween 80 in a sterile mortar and crushed the samples, applying manual pressure until homogeneous mixture were obtained. All samples were placed in a

shaker for 1 hour and after this 0.1 ml of each was plated in 90 mm diameter x 15 mm of depth Petri dishes in duplicate on selective culture medium described by Posadas et al. (2012) (protocol modified). The selective media was Saboreaud Dextrose Agar (SDA: 10 g of peptone, 20 g of dextrose, 20 g of agar and 1L water, supplemented with antibiotics 0.6 g of streptomycin,0.05 g of cycloheximide, 0.05 g of tetracycline and a fungicide 0.35 g of Cetil trimethyl ammonium bromide (CTAB)) in Petri dishes. The incubation of plates was at 26 ± 2 ° C for 20-30 days and *Beauveria* colonies were counted, isolated and subcultured on Potato Dextrose Agar (PDA: SIGMA) in Petri dishes in 60 mm diameter x 15 mm depth.

In the laboratory, 5 g of soil were weighed for each sample and put in a sterile Falcon tube with 40 ml of water with 0.01%Tween 80. Samples were put in a shaker for 1 hour. After this, 100 µl subsamples were plated on to Petri dishes (in duplicate) on selective culture medium. Plates were kept at 26 ± 2°C for 20 to 30 days and *Beauveria* colonies were counted, isolated and subcultured on PDA media.

2.3 Epiphytic isolation

The initial intention was to use the same counting and isolate method in all samples (colony forming units or CFU`s from direct plating). In the first month, however, there was no growth from epiphytic samples. We switched, therefore, to printing technic described by Wraight et al. (2007). We cut with a sterile scalpel leaf pieces approximately 1 cm^2 for each sample. The leaf pieces were pressed against the surface of an entomopathogenic selective culture medium (see above) in five different points each and the position of the leaf was marked. The plates were incubated at 26 ± 2° C for 20-30 days and resultant *Beauveria* colonies were isolates and subcultured on PDA media for confirmation of identification.

2.4 Endophytic isolation

Isolation of endophytic fungi was performed, beginning with a previously published method for surface-sterilization of coffee leaves (Posada & Vega, 2006; Saucedo et al. 2014, protocol modified). With a sterile scalpel, we cut leaf pieces approximately 2-3 cm^2 for each sample. The petioles were processed separately. Samples were dipped in 2% sodium hypochlorite for 3 min., then 70% ethanol for 2 min., and were then rinsed twice in sterile distilled water. After sterilization, pieces of 1 cm diameter were cut with a sterile scalpel on sterile filter paper. These pieces were plated on entomopathogenic selective culture medium (see above). The last rinse (100 µl) was plated to check the process of surface sterilization (these controls were negative; no growth of any fungus was detected on the plates). Incubation was at 26 ± 2°C for 20-30 days. All *Beauveria* isolates collected monthly were subcultured on PDA and classified by compartment where these were isolated.

2.5 Identification and quantification of fungi

From the samples taken (see above), colony forming units (CFU) of *Beauveria* were quantified monthly. *Beauveria* colonies were identified by observing the growth, color and texture (Appendix 1), while other fungi were identified as they were found. From each plate where *Beauveria* was found, a single subculture was taken on to PDA medium and this was kept refrigerated at 4°C until identification. The identification of fungi was facilitated by a light microscope and characteristics such as the morphology and origin of conidiophores, size and shape of conidia, size and completion of phialides were used. Confirmation of fungal genera was based on keys by Humber (1998). After identification *Beauveria* isolates were stored in vials with sterile Glycerol at freezer – 4ºC.

2.6 Data Analysis

Abundance data (*Beauveria* CFU densities) were analyzed to compare means. The data were evaluated by compartments (root, soil, soil fruit and tree fruit) and evaluated by plant (individual).The data by month and compartment were not formally analyzed statistically (see Results for explanation). A specific test was conducted to compare the months before and after harvest (July and August respectively) a Shapiro Wilk test showed non - normality of the data so this comparison was made using. Wilcoxon sing rank tests for each compartment separately (with paired samples) in SPSS v.22.

The occurrence epiphytic *Beauveria* on leaves was considered as binomial data (presence or absence). A Chi square test was performed for to determine significant differences among level of the leaves (basal, middle and apical) and presence or absence of *Beauveria* by month. For all statistical tests, $\alpha=0.05$ was used to reject or accept null hypotheses. The statistical software R (version 3.2.3) and SPSS Version 22 were used.

3. RESULTS

3.1 Isolation from root, fruit and soil samples

3.1.1 Abundance and distribution of *Beauveria* populations

In this study, abundances of *Beauveria* (as densities of colony-forming unities, or CFU, in sampled material) were examined in several compartments (leaves, root, soil and fruits) of field-planted coffee. This was done monthly, between May and September 2015, a period that included the harvest of coffee berries. While it appeared that *Beauveria* was most abundant in the soil and then root samples (Fig.1), initial statistical analyses did not support this observation. Indeed, there were no consistent differences in *Beauveria* abundance between the compartments. As can be seen in fig. 1, means were highly variable and errors were large, showing that little pattern could be discerned in this study, conducted in this fashion.

Regarding efforts at statistical analyses of the data, there was no consistent pattern through time in any of the compartments and the variability in the data was very high. Preliminary analyses also indicated that there was no pattern through time and for these reasons, no formal statistical analyses were conducted on these data to look for such patterns.

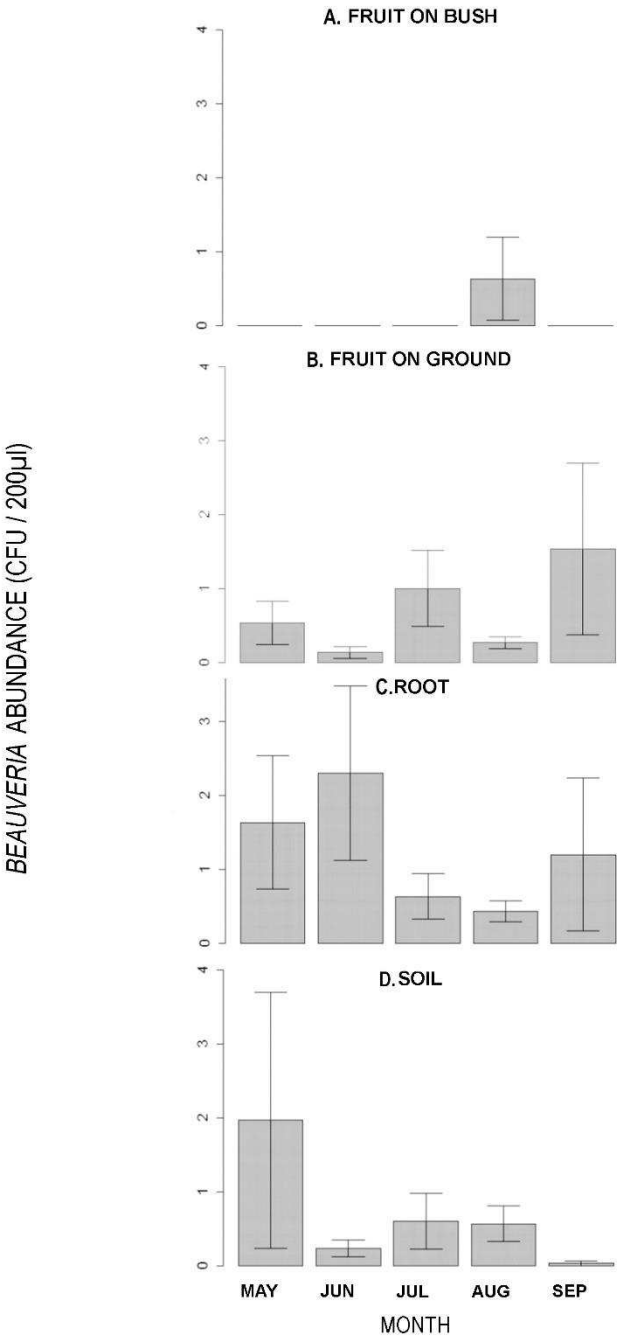

Fig.1. Means of *Beauveria* abundance (CFU/200µl of sample on SDA media) when isolated from field samples on from four compartments: A. Fruit on bush, B. Fruit on ground, C. Root and D. Soil (n = 150 per compartment) between May and September 2015 in a coffee field in Araponga, Minas Gerais, Southeastern Brazil.

3.1.2 Before and after harvest analysis

We wished to compare densities of *Beauveria* before and after harvest period. As mentioned above, a Shapiro Wilk test showed that data were not normally distributed (data not shown), so a non-parametric test was used. The Wilcoxon signed-rank test uses pairwise comparisons of data to generate a series of signs (positive or negative). Here, data were examined immediately before and immediately after harvest (i.e. the months July and August respectively). *Beauveria* densities were found to be higher before harvest than after, for all four of the compartments examined (Fruit on bush $Z_{[150]}$=-5.842; P=2.57x10^{-9}; Fruit on ground $Z_{[150]}$=-5.775; P=3.84x10^{-9}; Root $Z_{[150]}$=-2.818; P=0.005; Soil $Z_{[150]}$=-5.842; P=2.57x10^{-9} (μ_2-μ_1<0). It is worth noting that *Beauveria* densities were minimal in the vast majority of samples, and so it was the infrequent cases of >8 CFU/ 200 µl that most affected the distribution of the data (Fig.2).

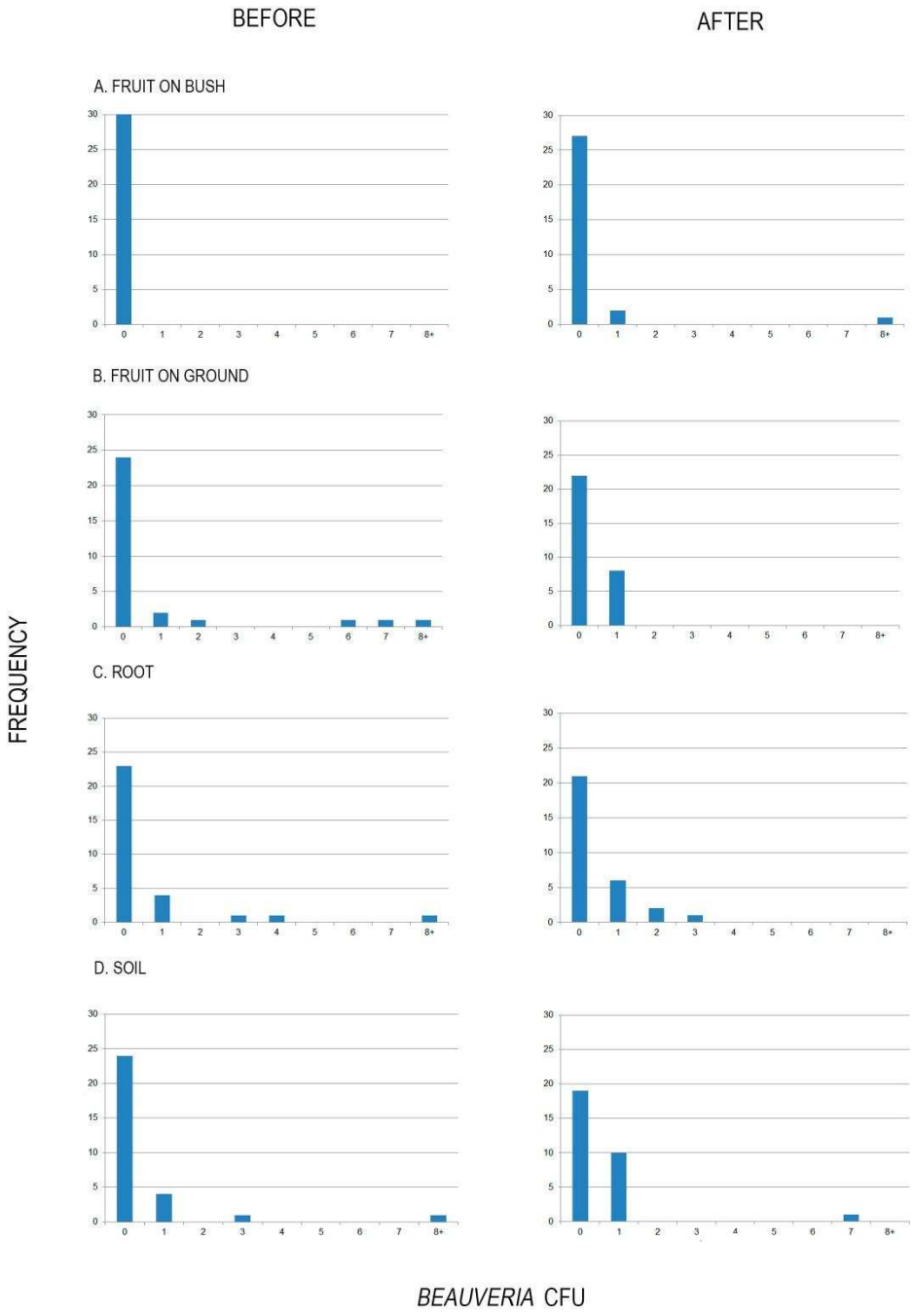

Fig.2. *Beauveria* Frequency (CFU/ 200µl of sample on SDA media) found before and after harvest (July – August) 2015 on samples of fruit on bush, fruit on ground, root and soil in a coffee field in Araponga, Minas Gerais, Southeastern Brazil.

3.2 Epiphytic Isolation

Data on the isolation of *Beauveria* from leaves phylloplanes were considered binomially (presence or absence of *Beauveria*) and each month was considered in isolation. Chi-square (X^2) tests were used to look for differences in the distribution of observed levels of *Beauveria* abundance from the expected, according to leaf level (basal, middle and apical). Significance differences were found between leaves only for June ($X^2_{[2]}$=13.199;*P*=0.001) - *Beauveria* was most abundant in basal and then middle leaves (Fig. 3) - but not for subsequent months: July ($X^2_{[2]}$=3.567;*P*=0.168), August ($X^2_{[2]}$=2.069;*P*=0.355) and September ($X^2_{[2]}$=2.965;*P*=0.227).

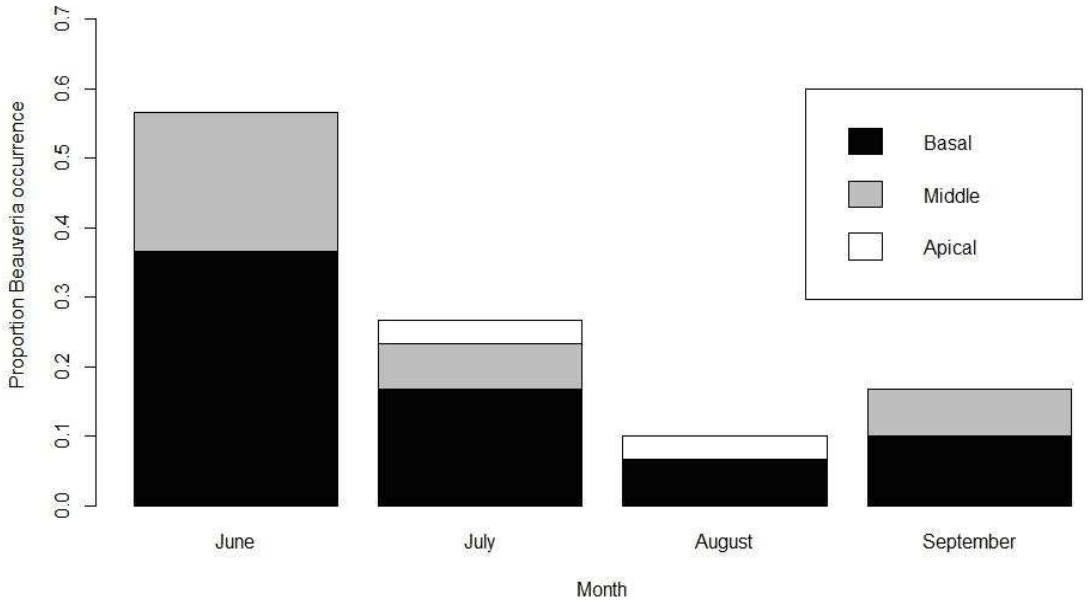

Fig.3. Proportion of occurrence of epiphytic *Beauveria* isolated on SDA media on samples leaves in different levels (Basal, Middle and Apical) from coffee plants between June and September 2015. Araponga, Minas Gerais, Southeastern Brazil.

3.3 Endophytic isolation

In a screening of 900 leaves and petiole samples we found three genera of endophytic fungi that could be differentiated based on their morphology and coloration. No entomopathogenic fungi were found (this naturally includes *Beauveria*). Many of the fungi found presented sterile mycelial growth and for this reason their identification could not be carried out (Appendix 2).

4. DISCUSSION

4.1 Isolation from root, fruit and soil samples

This study was aimed at a documentation of the distribution of *Beauveria* populations in coffee considered a compartmentalized system and how this population might vary over time in a sampling period that included harvest (July-August). It was found that abundance (CFU densities) obtained through time showed active populations of the fungus but without any consistent pattern. CFU counts showed great variability between compartments and between individual plants. *Beauveria* populations were found to be quiet abundant. Behie et al., (2015) demonstrated like *Beauveria bassiana* showed preferential localization in roots when it was inoculated in *Phaseolus vulgaris* (haricot beans) compared with other plant tissues. In root samples there may have been a slight decline in July and August. It is possible that lower temperatures in these months (according to data from the weather station - Appendix 3) limit the photosynthesis, leading to reduce exudate production by the plants that indirectly influences the abundance of microorganisms. Besides, conditions of insolation and luminosity, indicate that conidia present in the aerial part, could show higher percentages of germination, which could explain the higher number of isolates recovered in this area for these months (Jaronski, 2010). Also, It is possible that physiological conditions of the plant or the environment around where fungus is growing could change over time and influence populations of *Beauveria* It has been shown that the abundance and diversity of entomopathogenic fungi vary in a particular habitat (Meyling et al., 2009).

Abundance and recovery of isolates in root, soil and soil fruit shows the importance of these compartments to hold the fungus and indicate that its distribution is concentrated mainly on the ground and the surface. Some dispersal

pathways showed in theoretical models have been proposed for understanding ecology of fungal entomopathogens and show how they occupy different compartments in an ecosystem with high diversity (Meyling and Eilenberg, 2007; Hesketh et al. 2010). These models, based in temperate zones, show that *Beauveria* is moving mainly in the aerial part of the plant and above ground; although Ormond et al. (2010) observed that a large population of the fungus remains active in the below ground (soil and roots), as in our study.

The rhizosphere and the soil are areas that provide nutrients and space for colonization of entomopathogenic fungi, besides serving as reservoirs and protection from unfavorable conditions (St. Leger, 2008; Vega et al., 2009; Jaronsky, 2010). Probably, production of root exudates, low luminosity (sunlight) or higher humidity conditions in the lower part of plant may stimulate the increase the *Beauveria* population and its activity. Perhaps, sunlight and humidity conditions are unfavorable at the top of the plant and may be influencing in the appearance of *Beauveria* populations in tree fruit. The presence of *Beauveria* in fruits on bush only in August (after harvest) may have arisen by contamination because of the movement of the trees by machinery or staff to do the harvest. It is possible that this effort disperses spores of the fungus.

It could be considered that agricultural practices such as harvesting of fallen fruits after the harvest period could harm the populations of the fungus, because a larger number of isolates was recovered in soil fruit (Appendix 4). Reports in Colombia have shown the permanence of the fungus on fallen fruits, probably because of the presence of the insect host (Bustillo et al., 1999; Damon, 2000). Thus, it could be that leaving part of the fallen fruit could help improve the establishment of *Beauveria* as a natural enemy of coffee berry borer (Bustillo et al. 1999), working as conservative biological control, but the total volume of soil is likely to be far greater than that of fruits so this effect could be reduced. With such

strategies in the management of crop pests, it could help the implementation of a more efficient sustainable agriculture and better management of natural resources.

It is worth reconsidering the agricultural practices of “repase” (passing over again), that involves the collection of every coffee berry of all stages from both the bushes and the ground, once, immediately after harvest. This is aimed at removing any remaining individuals of coffee berry borer but could also potentially affect *Beauveria*.

4.2 Epiphytic Isolation

In this study, we located epiphytic *Beauveria* on leaf samples. Several studies have previously reported *B. bassiana* species present in phylloplanes and other parts of plants in natural and agricultural systems (Meyling and Eilenberg, 2006; Garrido et al., 2015). The level of leaves (apical, middle and basal) within the plants is important in determining the occurrence of epiphytic *Beauveria*. It is probable that, sunlight is unfavorable at the top of the plant, leaving larger populations of *Beauveria* in the middle and apical leaves. Sunlight is known to influence germination and viability in entomopathogenic fungi (Braga et al., 2001; Ottati-de-Lima et al., 2014). In southern California Jaronski (2010) investigated the persistence of *Beauveria* sp. applied to the lower and upper surfaces of melon (*Cucumis melo* L.) leaves, where conidial viability on leaf undersides decreased approximately 9-11% per day while on upper surfaces viability dropped by 47% per day. Meyling and Eilenberg (2006a) show that percentages of *B. bassiana* in leaf surfaces can vary from 9% to 40% between upper and lower leaves in hedgerow vegetation. According to Bustillo (2005) the position of the branch in the coffee tree (upper, middle and lower) can influence the average mortality of coffee berry borer (32%, 73% and 84% respectively) when *Beauveria* is sprayed as a biopesticides, probably due to effects of solar radiation. This would be of great importance to take

into account when making recommendations of field applications with these microorganisms. Meteorological data (Appendix 3) show a continual decline in mean relative humidities through the study period, which could be reflected in the decreasing proportions of occurrence of epiphytic *Beauveria* on leaves. The exception is September where epiphytic *Beauveria* levels increase but this may be an effect of temperature or coffee berry borer populations (Appendix 3).

The existence of a natural *Beauveria* occurrence on leaves would be important to consider in studies where applications with products based on these fungi are evaluated, e.g. spore permanence, as spore count made after these applications could come from natural populations and not from applications (treatments), perhaps generating false results.

4.3 Endophytic isolation

Initially, we expected to find endophytic *Beauveria* in leaves, but this did not happen. The majority of reports citing endophytic *Beauveria* sp. have followed inoculations into plants (Posada & Vega, 2006; Posada et al., 2007). However, studies of endophytic coffee diversity in tropical areas (Puerto Rico and Brazil) do not report the presence of *Beauveria* sp. (Santamaría and Bayman, 2005; Oliveira et al., 2014). Only a few studies have reported *Beauveria* naturally occurring endophytically in coffee, but with low frequencies and / or in zones where it is not clear whether biopesticides applications had occurred (Saucedo et al., 2014; Vega et al., 2010). Here, other genera of endophytic fungi were found; although their identification was not possible because they only produced mycelium, they are unlikely to be known entomopathogens. It is known that many fungi with sterile mycelium are commonly found as endophytes (Guo et al., 2000; Santamaria and Bayman, 2005).

The absence of endophytic *Beauveria* in this study can be atributed to several factors. First, it can represent an antagonism between *Beauveria* and other endophytic fungi, as most of fungi are known for their antimicrobial activity, while production of secondary metabolites is an important tool for these organisms in competition and the establishment in the hosts (Shields et al., 1981; Ownley et al., 2008; Kusari, et al., 2013; Oliveira et. al., 2014; Nair and Padmavathy, 2014). Some reports show that endophytic colonization and persistence of microorganisms could be influencing by communities (endo and epiphytic) and their interactions (Santamaria and Bayman, 2005). Secondly, *Beauveria* isolates of coffee plants sampled did not have capacity to infect endophytically; genetic differences between species could influence the role in the ecosystem. Campos et. al 2010, showed that strains of same genus of entomopathogenic fungi affecting ticks failed to infect plants endophytically, some of those strains did not have this capacity despite being of the same genus. Third, it has been reported that abiotic factors and fitness of the plant can also influence the persistence of endophytes (Davitt et al., 2011; Gundel et al., 2011). It would be particularly interesting to verify which of these factors could be related to the natural endophytic establishment of *Beauveria* in coffee plants.

In general, results and the richness of isolates (percentages) recovered of different compartments show that *Beauveria* is occupying several niches (Appendix 4). However, more studies are needed to elucidate how real fungus dispersal between compartments is and to obtain a more real distribution. It would be important to know if the same strains of *Beauveria* are present on the ground and on the plant, possibly, if enzootics infections or epizootics are occurring. The next step in this study would be to conduct molecular studies to elucidate if the isolates obtained from different compartments (lower and upper part of the plant) are from the same strains. Furthermore, the study with these isolates could also make known as diversity in a small compartmentalized system. Besides, expand the sampling to

ascertain if *Beauveria* could be reaching the aerial part as naturally endophytic fungus. It is anticipated that multiple factors, whether abiotic (temperature, humidity, sunlight) or biotic (hosts, niches) and agronomic practices affect the establishment of *Beauveria* endophytically and epiphytically, which it is suggest be considered in future studies. Finally, these studies as a tool for knowledge about the ecology of entomopathogenic fungi can be used to increase its success and potential use as well as to change the perspective about "disappointing results" respect to action and effectiveness of biological control with microorganisms.

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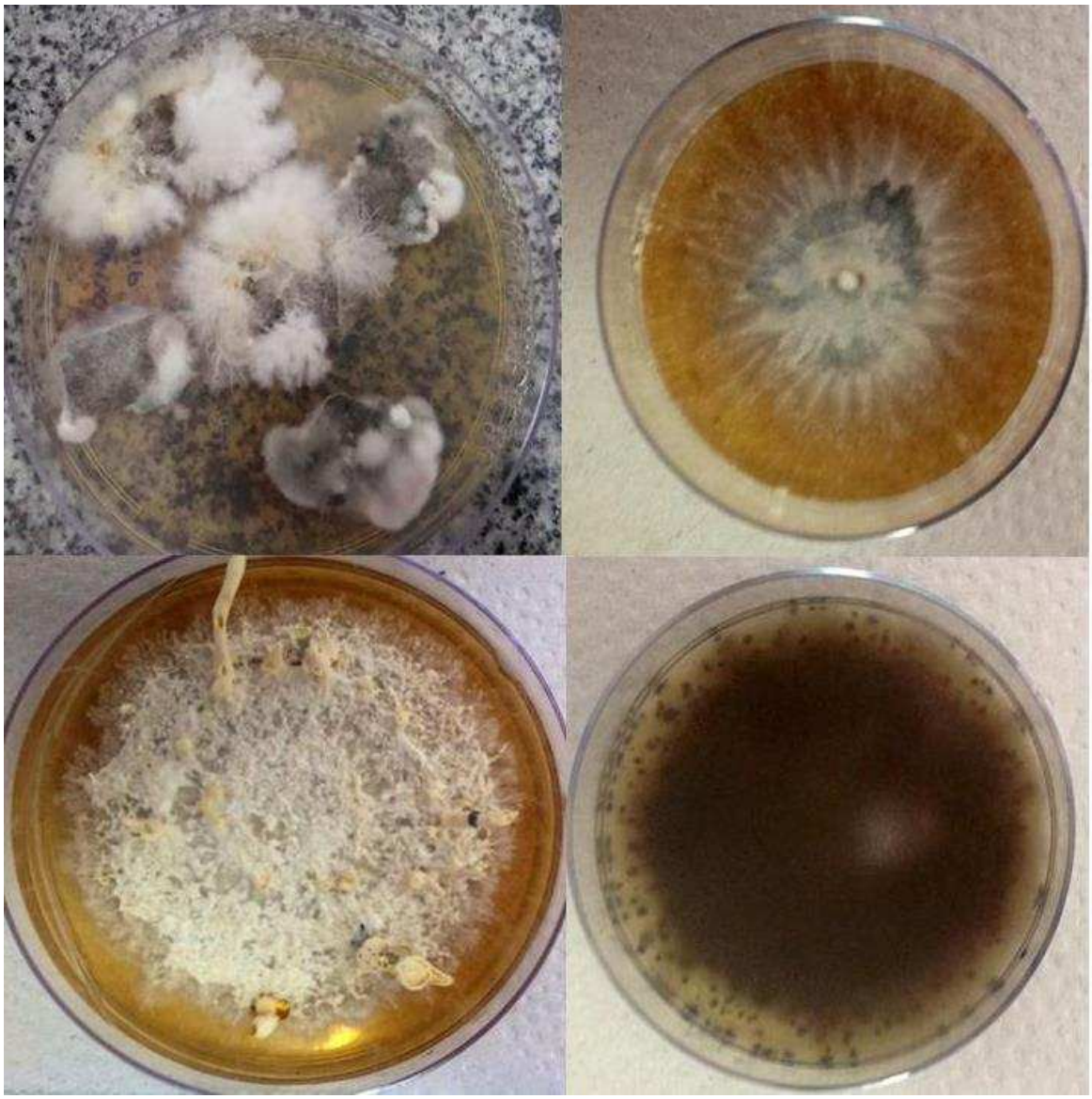
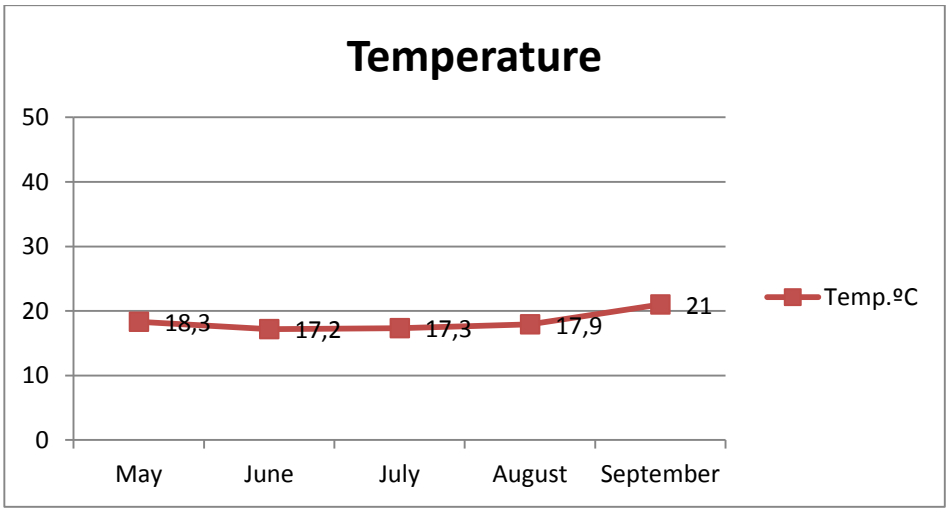
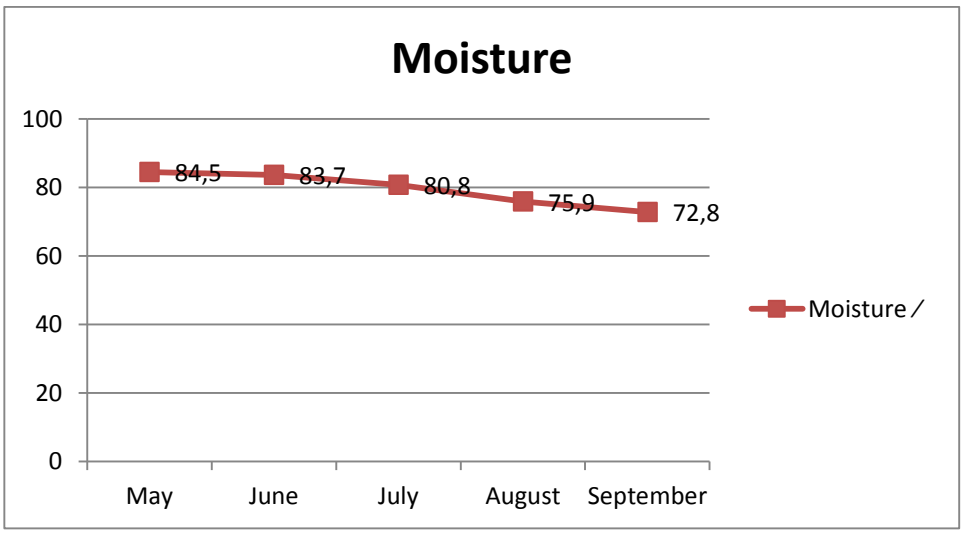
B.

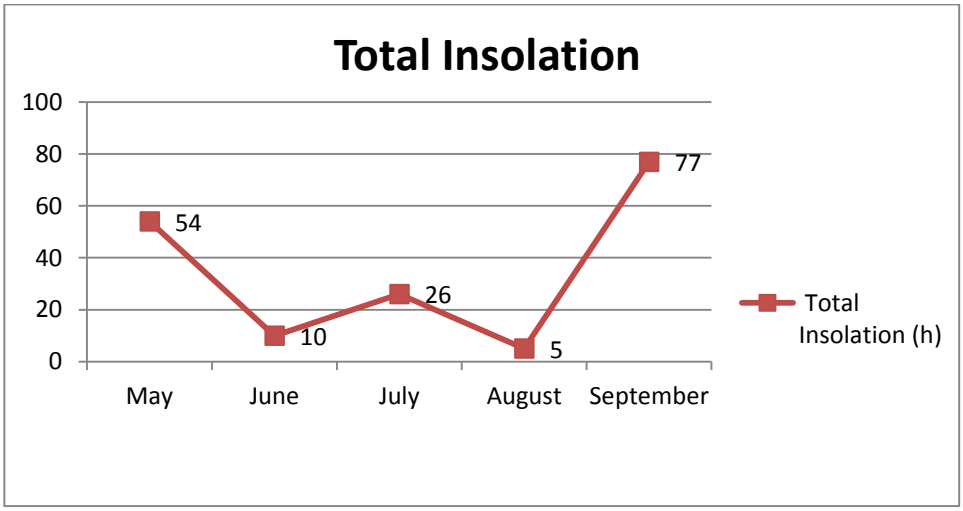
Appendix1. *Beauveria* colonies. A. Colonies from soil sample in selective media for entomopathogenic fungi, detail arrows. B. Isolates recovered in PDA, detail different morphologies.



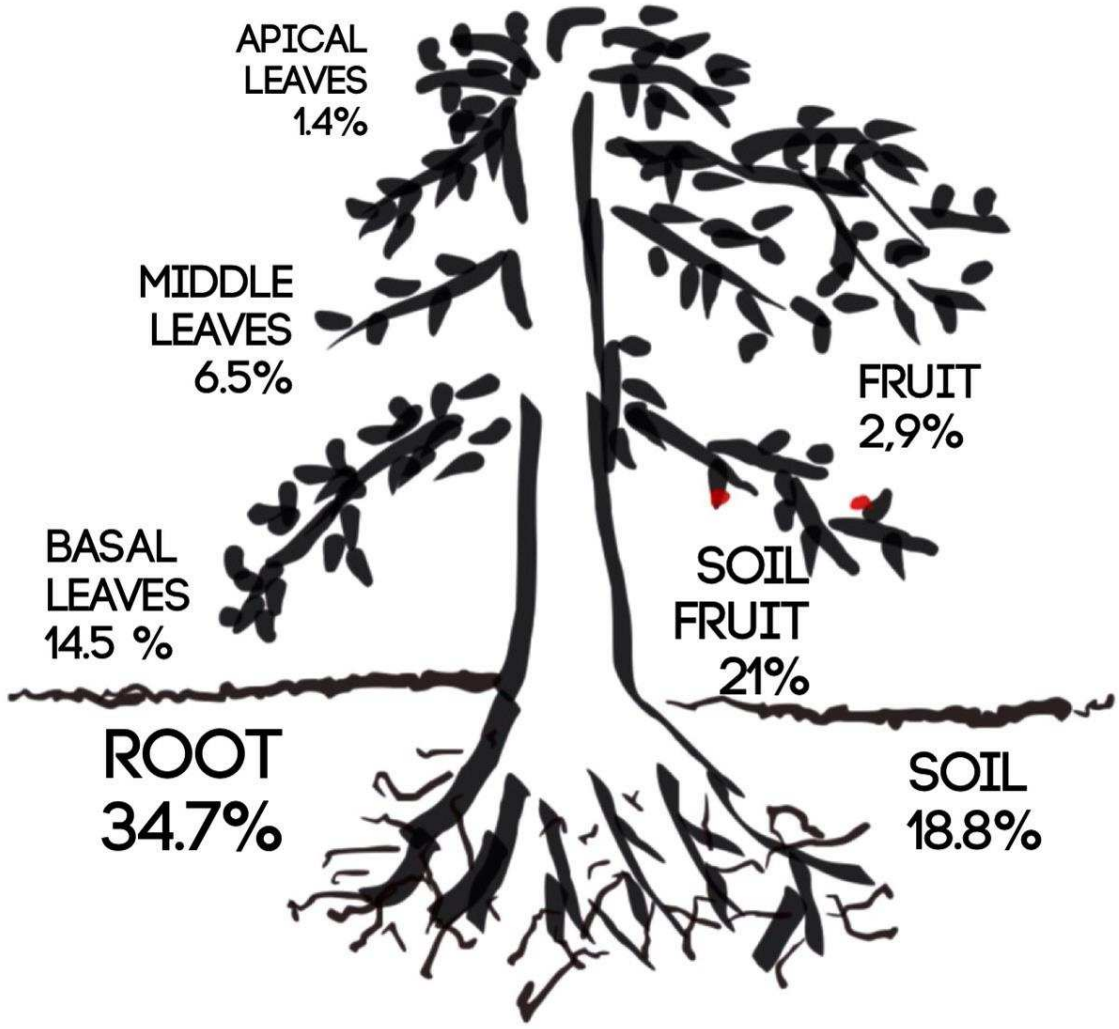
Appendix2. Endophytic fungi isolated from leaves in a coffee agroforestry crop. Araponga. Minas Gerais. Brazil.







Appendix3. Meteorological data of temperature, moisture and total insolation in the region of Viçosa (MG) between May and September 2015.



Appendix4. Richness of *Beauveria* spp. isolates. Total percentages found from May-September 2015 in different compartments in a coffee agroforestry field in Araponga, Minas Gerais, Southeastern Brazil. Although the method of insolation was different between leaves (basal, middle and apical) and root, soil and berries (soil fruit and tree fruit), this design gives a general idea of where are located *Beauveria* populations.