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RESEARCH ARTICLE
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Incidence of leaf diseases in the agroforestry systems at Yurimaguas, Peru

Incidencia de las enfermedades foliares en los sistemas agroforestales de Yurimaguas, Perú

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Abstract

An agroforestry system (SAF) is characterized by having a diversity of components, such as timber and non-timber forest species and crops, pastures, or a livestock production system. This diversity of components in the system reduces the intensity of diseases, e.g. foliar diseases. This study aimed to detect the fungal microorganisms associated with the leaf spots of plant species that are part of the agroforestry production systems of the Peruvian farmers from Yurimaguas and to determine the level of incidence of the leaf spots in the systems of agroforestry production. Different land cropping systems were implemented in the farms, such as palm (*Bactris gasipaes*) to produce palm hearts, cocoa (*Theobroma cacao*), and plantain (*Mussa sp*.). Also, silvopastoral systems with fast and slow growing timber species and cattle with pastures for grazing and reforestation in areas of secondary forests in a state of degradation. Prospecting, collecting, and determining the incidence of diseases in each farm were carried out and later they were identified with molecular methods using the primers ITS 1 and ITS 4. The symptoms predominantly observed were, leaf spots in cocoa (rootstock), palm, and plantain. Symptoms like wilting, decline, or rot were not observed. The incidence was evaluated in two collection periods (2018 and 2019). The fungi isolated from the leaf spots were *Pestalotiopsis sydowiana* and *Colletotrichum siamense* as causative agents of leaf spots on palm and cocoa, and *Mycosphaerella fijiensis* on plantain. When determining the incidence from April 2018 to October 2019, a decrease in this parameter (incidence) was observed for farms with palm, especially in those where the production system was improved by the use of fertilizants as a requirement of the crop. It was concluded that the highest intensity of foliar diseases occurred in agricultural systems with monoculture of palm with 100 % at the beginning of the evaluation, and for agroforestry systems in the silvopastoral prototype, it was only detected in a range of 0 % to 25 %.

Keywords: *Agroforestry Systems, Leaf spots, palm*

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Resumen

Un sistema agroforestal (SAF) se caracteriza por tener una diversidad de componentes como especies forestales maderables y no maderables, así como cultivos, pastos o un sistema de producción ganadera. Esta diversidad de componentes en el sistema reduce la intensidad de las enfermedades, por ejemplo, las foliares.

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Los objetivos fueron detectar los microorganismos fúngicos asociados a las manchas foliares de las especies vegetales que forman parte de los sistemas de producción agroforestal de los agricultores de Yurimaguas (Loreto) que participaron en el SLA y determinar el nivel de incidencia de las manchas foliares en los sistemas de producción agroforestal. En estas fincas se implementaron diferentes sistemas de cultivo de la tierra, como la palma (*Bactris gasipaes*) para producir palmitos y cacao (*Theobroma cacao*), incluyendo el plátano (*Mussa* sp.). También, sistemas silvopastoriles con especies maderables de crecimiento rápido y lento y ganado con pastos para el pastoreo y la reforestación en áreas de bosques secundarios en estado de degradación. Se realizó la prospección, recolección y determinación de la incidencia de las enfermedades en cada finca, y posteriormente se identificaron con métodos moleculares utilizando los cebadores ITS 1 e ITS 4. Los síntomas que se observaron, predominantemente, fueron manchas foliares en cacao (patrón), palma y plátano. No se observaron síntomas de marchitamiento, decaimiento o podredumbre. La incidencia se evaluó en dos periodos de recolección (2018 y 2019). Los hongos aislados de las manchas foliares fueron *Pestalotiopsis sydowiana* y *Colletotrichum siamense* como agentes causantes de las manchas foliares en palma y cacao, y *Mycosphaerella fijiensis* en plátano. Al determinar la incidencia desde abril de 2018 hasta octubre de 2019, se observó una disminución de este parámetro para las fincas con palma, especialmente en aquellas donde la implementación consistió en mejorar el sistema de producción a través de la fertilización con base en los requerimientos del cultivo. Se concluyó que la mayor intensidad de enfermedades foliares se presentó en los sistemas agrícolas con monocultivo de palma con un 100 % al inicio de la evaluación, y para los sistemas agroforestales en el prototipo silvopastoril, solo se detectó en un rango de 0 % a 25 %.

Palabras clave: Sistemas agroforestales, manchas foliares, palma

1. Introduction

An agroforestry system is production in which, unlike a monoculture, at least two or more plant species coexist; or plants and animals; but one of them must be a perennial tree species (Nair, 2014). This greater diversity of species makes the existing ecology more complex than if it were a monoculture.

From a phytopathological point of view, their development and productivity can affect commercial production fields. In addition, phytopathogens can affect the quality of the harvested product generating economic losses. These phytopathogens can be fungi, bacteria, viruses, or nematodes, among others. Favorable conditions for the pathogen growth, such as temperature, relative humidity, optimal rainfall, etc. Together with the host susceptibility reaction, produce high levels of disease intensity, generating epidemics (Agrios 2005). Greater activity of these behaviors is observed in intensive monoculture systems. But, under an agroforestry system, in which there are different plant species, the behavior of phytopathogens is expected to be different because of the coexistence of a greater diversity of plant species. Banerjee et al (2015) found that agroforestry systems were characterized by having a greater diversity of bacterial communities in their biofilm, such as bacteria of the genera *Arthrobacter, Acidobacteria*_Gp16, *Burkholderia, Rhodanobacter*, and *Rhizobium* at the level of the rhizosphere. Müller et al (2006) reported the role of microorganisms in the phyllosphere of plant species in forests, a role related to nutrient transformation processes. Additionally, the action of these microorganisms is attributed to changes in environmental biotic behavior, such as the increase in the population density of certain groups of microorganisms, which would impact their metabolic activity. Díddier and Castro (2017) reported that, in the experience of the organic banana agroforestry system in Costa Rica, there is coexistence with Sigatoka (whose causal agent is *Mycosphaerella fijiensis*); under a condition of enough leaves and clusters; with fruits that satisfy the quality standards for the national and export markets. They also showed that nematicide applications were not carried out because the nematode populations were below the damage thresholds, and the increase in biodiversity in the system explained this. Mosquera-Mena (2013) mentioned that small producers in the Urabá region from Antioquia, Colombia, reported a favorable phytosanitary balance in agroforestry systems because these systems favored a greater diversity of microorganisms.

According to the experience of the Project to Recover Natural Ecosystems in the Caquetaño Foothills in Ecuador (1998), one advantage of agroforestry systems was the reduction of pest and disease problems through sanitary pruning practices. Montagnini et al. (2015) analyzed the effect of output under shade in the decrease or increase of pests and diseases in the agroforestry system of coffee production. For example, in *Colletotrichum kahawai*, the presence of trees reduced the spread of pathogen propagules by reducing the impact of rain. Under a shady environment, the activity of biocontrol fungi, such as *Beauveria bassiana* and *Lecanicillium lecanii* on CBB and rust, respectively, were favored. Based on these behaviors in the different multifunctional systems, the following objectives were proposed in the present work; detect fungal microorganisms associated with leaf diseases such as leaf spots of plant species that are part of the agroforestry production systems of farmers from Yurimaguas (Loreto) who participated in the Sustainable Landscapes for the Amazon Project, and determine the level of incidence of leaf diseases in agroforestry production systems as a comparative parameter between the different multifunctional systems.

2. Material and Methods

Location

The study was carried out at the Yurimaguas district, Alto Amazonas province, Loreto Region, Per**ú** (Figure 1). This zone was the development area of the “Sustainable Landscapes for the Amazon project” funded by CIAT (Centro Internacional de Agricultura Tropical) – UNALM (Universidad Nacional Agraria La Molina). Eighteen farmers participated in the project committed certain areas for implementation or conservation purposes (Table 1). In this study, the farmers expressed their interest in two types of strategies for the sustainable use of the forest. On the one hand, there is the implementation of systems with different components, among which the Agro-Forestry Systems (SAF) stand out, followed by the Silvopastoral Systems (SSP) with natural pastures or paddocks; Forest Enrichment Systems (SEF), and finally Reforestation (R). There are different SAF´s: Palm Heart Implementation, Cocoa Implementation, Palm crop Improvement, and Cocoa crop Improvement. Forest enrichment (SEF) corresponds to forest areas with more tree species installed. In Reforestation (R), there are pasture areas with tree species established (Table 1).

Detection of fungal microorganisms associated with leaf spots of plant species in the agroforestry production systems

Phytopathological sampling

A disease evaluation was made for each agroforestry system (18 in total), after the diseased plants be collected, following the methodology reported by French & Hebert (1982). Plant organs showing any disease symptom were collected in propylene plastic bags, at least ten units per type of symptom, and were kept under refrigeration conditions, because of the high temperatures in Yurimaguas, to later be transported to the UNALM plant pathology laboratory (French & Herbert 1982).

Isolation and maintenance

The samples were analyzed at the Plant Pathology Laboratory from the UNALM. The collected samples were washed with plain water to remove traces of soil or dust. They were then disinfected with ethanol (70%) for one minute and finally rinsed with sterilized distilled water for the same period. The diseased tissue sections of the disinfected samples were placed in Petri dishes containing PDAA (Acidified Potato Dextrose Agar) culture medium. The plates were incubated at 25 °C in the dark. After five days, the developed cultures were checked (French & Hebert, 1982). The purified isolates were kept in tubes containing PDA medium under refrigeration (10 °C).

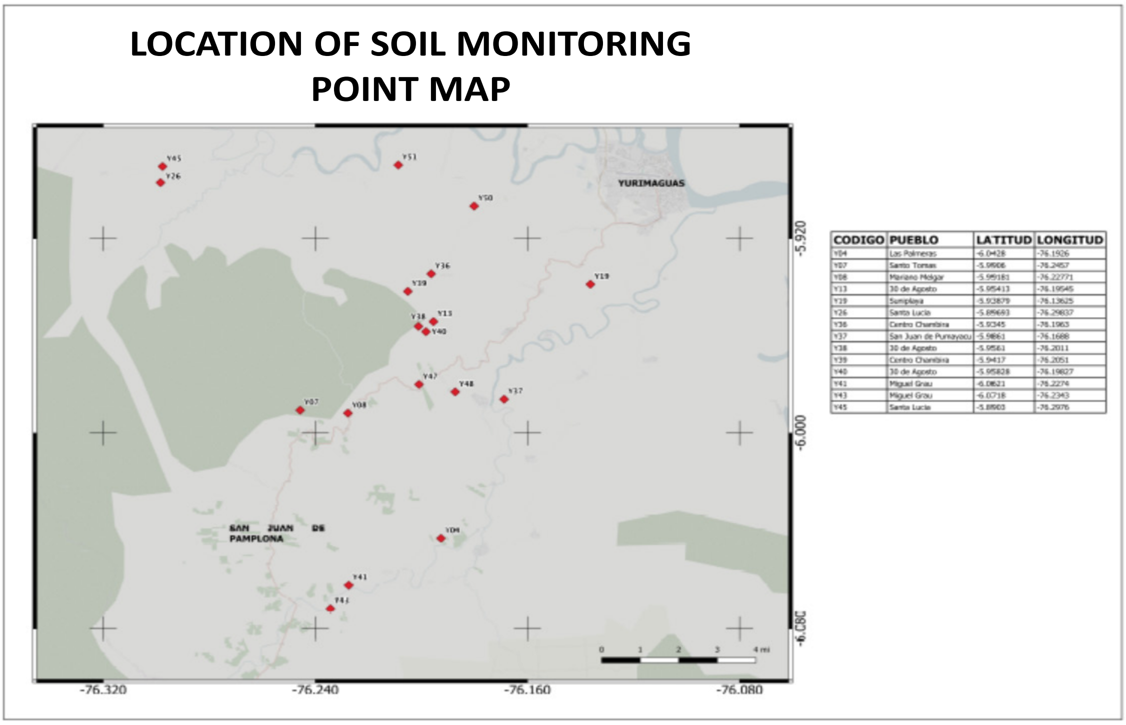

Figure 1. Location map of the farms in the district of Yurimaguas (Loreto) that participated in the Sustainable Landscapes for the Amazon project, according to geo-referencing *(elaboration: Bach. André Mauricio Valderrama Espinoza)*.

Identification of isolates

Morphological identification. The preparations of the fungal structures were made on slides and coverslips. The propagative structures were visualized through the compound microscope. Barnett's (1999) identification key was used for identification at the gender level.

Molecular identification. The fungi were cultivated in PDA for 3 to 5 days at 25 °C to obtain the extraction material. The DNA extraction methodology was carried out from agar described by Saitoh et al. (2006) modified by Huarhua et al. (2020). A 5 to 10 mm mycelium block was used with agar, which was placed in a 1.5 ml Eppendorf tube to which 500 microliters of lysis buffer (100 mM Tris HCl, 10 mM ethylene diamine tetraacetic acid [EDTA], 1M KCl; pH 8.0). The mixture was kept at room temperature for 10 minutes, then it was triturated with micropistils, then phenol-chloroform iso-amyl-alcohol (24: 24: 1) was applied, and it was centrifuged for 10 minutes at 12000 rpm, it was recovered the upper aqueous phase, and a similar volume was added to the iso-propanol collection to be incubated at -20 °C for 15 minutes. After this time, it was centrifuged at 12000 rpm for 10 minutes at room temperature, and the supernatant was discarded; then, a wash was carried out with 1 ml of 70% ethanol and centrifuged for 5 minutes. After that time, the ethanol was removed, and it was left to dry for 2 hours. Finally, the DNA was resuspended in 30 µl of ultra-pure water and stored at -20 °C. The concentration and quality of DNA were determined using the Nanodrop 2000 spectrophotometer and by 1% agarose gel electrophoresis, which was stained with hydragreen and run at 90 V for 30 minutes. Genomic DNA was used as the template strand for PCR to amplify the internal transcribed space (ITS) of the ribosomal DNA region, including 5.8S rDNA and partial regions of the 18S and 25S ribosomal subunits for which the primers were used. ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) as described by White et al. (1990).

Table 1. Result of the phytopathological analyzes and molecular identification, as well as the incidence observed in the 2018 and 2019 sampling of the Farms (each one corresponds to a farmer) of Yurimaguas (Loreto) of the Sustainable Landscapes Project for the Amazon.

ID_Soils	Implementation of:	Area (ha)	Symptons	Pathogen	Incidence (April, 2018)	Incidence (October, 2019)
Y04	Palm heart Improvement	0.6	Leaf spots	*Colletotrichum siamense, Neopestalotiopsis foedans*	100%	60%
	Implementation of Cocoa in Agroforestry	2.1	Leaf spots	*Cercospora sp*	20%	no
Y07	Palm heart Implementation	1	Leaf spots	*Neopestalotiopsis foedans Colletotrichum siamense*	100%	90%
	Reforestation	1	Without leaf spots		no	no
Y08	Improvement Palm heart	2.9	Leaf spots	*Colletotrichum siamense, Neopestalotiopsis foedans*	50%	10%
Y13	Reforestation	2.7	without symptons		no	no
Y19	Silvopastoral Systems	3	without symptons		no	no
	Reforestation	1.9	without symptons		no	no
Y26	Cocoa Improvement in Agroforesty	1.4	Sigatoka	*Paracercospora fijiensis*	80%	70%
	Reforestation	2.3	without leaf spots		no	no
Y36	Silvopastoral Systems	2.6	without symptons		no	no
	Reforestation	2.7	without symptons		no	no
Y37	Silvopastoral Systems	4.2	Leaf spots in Paliperro	*Cercospora sp*	10%	5%
Y38	Implementation of Cocoa in Agroforestry	0.9	without symptons		no	no
	Reforestation	1.3	without symptons		no	no
Y39	Silvopastoral Systems	3	without symptons		no	no
Y40	Reforestation	2.5	without symptons		no	no
Y41	Implementation of Cocoa in Agroforestry	1.8	sigatoka (banano); leaf spot (Cocoa)	*Paracercospora fijiensis (banano); Colletotrichum siamense (Cacao)*	80% 25%	80% 25%
	Forest Enrichment	2	without symptons		no	no
Y43	Cocoa Improvement in Agroforesty	1.5	die-back	*Lasiodiplodia theobromae*	20%	20%
	Implementation of Cocoa in Agroforestry	2	without symptons		no	no
Y45	Implementation of Cocoa in Agroforestry	1	Poor development (cocoa) Sigatoka (plantain)	abiotic, unsuitable weather (cocoa) *Paracercospora fijiensis*	100% 100%	100% 100%
	Silvopastoral Systems	2.7	without symptons		no	no
Y47	Implementation of Cocoa in Agroforestry	1.25	without symptons		no	no
	Reforestation	0.8	without symptons		no	no
Y48	Palm heart Implementation	1.4	Leaf spots	*Colletotrichum siamense, Neopestalotiopsis foedans*	60%	40%
	Implementation of Cocoa in Agroforestry	0.9	without symptons		no	no
	Reforestation	2.2	without symptons		no	no
Y50	Silvopastoral Systems	2.8	without symptons		no	no
	Forest Enrichment	3	without symptons		no	no
Y51	Silvopastoral Systems	3.6	without symptons		no	no

The total PCR reaction mix (25 µl) contained 1 µl of genomic DNA (50 to 100 ng / µl), 4 µl of 10X PCR Buffer (ACTaq ™), 2 out of 2.5 mM dNTPs (ACTaq ™), 0.125 µl (5 U) of the enzyme Taq DNA polymerase (ACTaqTM) and 0.5 µl of each primer at a concentration of 20 Mm. The amplification was carried out with a thermal cycler (Thermo Scientific), following the next reaction cycles: initial denaturation of 94 °C for 2 minutes; followed by 35 cycles of denaturation at 98 °C for 1 min, a hybridization temperature at 58 °C for 1 min, elongation of 72

Figure 2. Leaf spots on palm heart. The small, blackish spots correspond to *Colletotrichum siamense* and the straw-colored spots to *Neopestalotiopsis foedans*.

Figure 3. Banana leaf spots caused by *Mycosphaerella fijiensis*; the causative agent of black leaf streak disease.

°C for 2 min, and final elongation temperature of 72 °C for 10 min (Ferrer et al., 2001; Kumar & Shukla, 2005; Huarhua et al., 2018; Rep et al., 2004; Inami et al., 2014). The PCR products were separated by 2% agarose gel electrophoresis (0.5 X TAE buffer) containing Hidragreen and run at 90V for 30 minutes. For the visualization of the fragments, the ultraviolet light transilluminator (UVP brand) was used, the 100bp Ladder (PROMEGA) was used as a marker. The PCR amplified fragments were sequenced in both directions. For sequencing, the eluted products were sent to MACROGEN Korea for processing and delivery of the chromatograms, and these were "cleaned" of indeterminacies with the MEGA 7 program (Kumar et al., 2016), thus obtaining the sequences that were compared using BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine similarities in GenBank.

Determination of the level of incidence of leaf spots in agroforestry production systems

Incidence assessment

Two visits were made to assess the incidence of diseases in the plant species in each of the agroforestry systems. Incidence assessment was calculated by the percentage of affected plants concerning the total, one for each year during the duration of the project (2018, 2019) (French & Hebert, 1982). It should be noted that in 2016 a preliminary survey trip was made and in 2017 the evaluation of diseases in each agroforestry production system was done.

3. Results and Discussion

Between 2018 and 2019, the fields of the 18 farmers who participated in the Sustainable Landscapes for the Amazon Project (CIAT - UNALM) were visited. The production systems were characterized by having different agroforestry systems: cocoa implementation systems, palm implementation to produce palm heart, cocoa maintenance, palm maintenance, silvopastoral design, forest enrichment, and reforestation (Table 1). Symptoms of leaf spots were observed predominantly in systems with less diversity of species such as those for the implementation or maintenance of palm, and only in an agroforestry system for implementing cocoa (it was found in the installation of the rootstocks): as well as in plantain as part of the cocoa implementation system. *Lasiodiplodia theobromae*, which was causing regressive death in a cocoa holding system, was isolated but it was a rare case. This fungus behaves as an endophyte, and under stress conditions, its behavior turns as a phytopathogen; therefore, it is an indicator of a stress condition in plants. The farmer reported that the focus of plants in which this phytopathogen was detected corresponded to a flood zone accentuated with problems in fertilization.

Detection of fungal microorganisms associated with leaf spots of plant species in the agroforestry production systems

Table 1 shows the results of the phytopathological analyzes of each of the farms in which leaf spots were detected. The species identified morphologically and molecularly corresponded to the species *Pestalotiopsis sydowiana* and *Colletotrichum siamense* as causal agents of leaf spots in palm and cocoa; and *Mycosphaerella fijiensis* on plantain. Figures 2 and 3 show the characteristic symptoms and those caused by the isolated pathogens. No necrotic lesions were detected in pastures or tree species located between farms, nor in the forest species situated in the reforestation or forest strengthening systems in the silvopastoral systems.

Molecular identification

The method used to get the genomic DNA of the leaf spot isolates from the farms is described by Saitoh et al. (2006). Genomic DNA was evaluated to determine purity and sufficient concentration to perform the polymerase chain reaction (PCR). The concentration of the evaluated samples was found between 90.1 ng / µL and 428 ng / µL.

The samples' PCR amplification for the ITS region of rDNA, generated products of 500 to 600 base pairs (bp) with the primers ITS 1 and ITS 4. The analysis of the sequences compared with the NCBI database -BLAST showed a percent identity of 100%. The identification results were: *Pestalotiopsis sydowiana* (Y04, Y07, Y08, Y41, and Y48), *Lasiodiplodia theobromae* (Y43). In the case of other isolates from the same farms Y04, Y07, Y08, Y41, and Y48, it was possible to determine based on the ITS region of the isolates that the *Colletotrichum* species had 100% similarity of their sequences with the *Colletotrichum siamense*, *Colletotrichum gloesporioides*, *Colletotrichum karsti* (Table 2).

Belisário et al. (2020) reported *N. foedans* as a causal agent of leaf spots in *Licuala grandis* (totuma, ornamental palm). Maharachchikumbura et al. (2016) identified the new genera *Neopestalotiopsis* and *Pseudopestalotiopsis* (from the genus *Pestalotiopsis*) based on the regions of the genome that encode the internal transcribed space (ITS), partial β-tubulin (TUB), and partial translation of elongation factor 1 alpha (TEF); such new genera do not show differences with the morphological structures of the genus *Pestalotiopsis*; this is also reported by Norphanphoun et al. (2019) for which *Pestalotiopsis sp* is recognized as a cryptic species. Morsbach et al. (1998) said *Colletotrichum* is a causal agent of necrotic lesions in the nursery stage or the first phenological stages of the palm crop. Arroyo et al. (2004) and Peña (1996) reported *Colletotrichum sp* as a causal agent of leaf spots, which they call black leaf spot; which appear as small black spots surrounded by a

Table 2. Results of molecular ID of the isolates (from the plant samples with symptoms of leaf spots and regressive death) compared with the NCBI.

MU	Species	Max score	Query cover	% Identity	ID NCBI
Y48	*Pestalotiopsis sydowiana*	963	100%	100%	MN856236.1
Y04	*Pestalotiopsis sydowiana*	965	100%	100%	MN856236.1
Y48	*Colletotrichum siamense* / *Colletotrichum gloeosporioides*	961	100%	100%	MK184442.1 MN548460.1
Y04	*Colletotrichum siamense*/ *Colletotrichum karsti* / *Colletotrichum gloeosporioides*	1007	100%	100%	MN635698.1 MN486559.1 MH700456.1
Y43	*Lasiodiplodia theobromae*	950	100%	100%	MT103324.1

small chlorotic circular halo; as it was observed in the palm plants of the farms of Yurimaguas. Weir et al. (2012) determined *Colletotrichum gloeosporioides* as a species complex and, due to the identification of ITS genes, several species of *Colletotrichum* (such as *C. siamense*) are included. James et al. (2014) reported *Colletotrichum siamense* in cacao, as it was found in the molecular identification of isolates from cacao from the Yurimaguas farms.

Determination of the incidence of leaf spots in agroforestry production systems

Table 1 also shows the incidence percentages determined during the visits to the farms. A decrease in the incidence from 2018 to 2019 is distinguished for the improvement of cocoa, cocoa implementation, implementation of palm, and improvement of palm plantations. In plantain, that were part of the cocoa implementation and improvement systems, the reduction was minimal, or there was no incidence decrease.

The causative agents of leaf spots have a necrotrophic behavior; this means that, according to their physiology of parasitism, they cause the death of plant living tissue mainly through toxins. The pathogen feeds on dead tissue as a consequence of the action of these metabolites. With plants stressed by low fertilization levels or extreme cases of fertilizer deficiencies, they become more susceptible to the action of necrotrophic pathogens. This is supported by Pornsuriya et al (2020), who showed that *Neopestalotiopsis* species (*Pestalotiopsis*) are weak and secondary pathogens. Alfenas et al., 2009, also mentioned the opportunistic behavior of *Pestalotiopsis spp* agent, the causant of Eucalyptus leaf spots, as well as the fact of infecting physiologically weak plants. When determining the incidence from April 2018 to October 2019, a decrease in this parameter could be observed for farms with palm, especially in those which the typology of the palm trees was improved through an increase in the fertilization of the farms according to the crop. By having plants with adequate nutrient requirements, susceptibility to necrotrophs was reduced. Bovi (1993), cited by Morsbach et al. (1998), also refers to the fact that the action of *Colletotrichum* can be minimized with good nutrition. Arroyo et al. (2004) show that *Colletotrichum sp* occurs mainly during the first six months of the crop or in the first two-thirds of the leaves that differentiate leaflets; as it could be seen in the plantations of the Yurimaguas farms, especially those in which the fertilization levels were not adequate.

A constant harvest characterized the palm heart crop, therefore fertilization of macroelements and microelements is highly required. In the case of banana, based on the concept of the disease triangle; the banana variety was susceptible to Sigatoka, the environmental conditions favored that pathogen, and the inoculum source existed in the production system, so the disease development was clear. In this case, no incidence reduction was observed, because no palliative measurements had been implemented, since the aim of the plantain installation was to generate shade for the Cacao rootstocks that were later grafted with a commercial variety, so there was no requirement for the bananas crop fertilization. Although the fertilization practice was improved by reducing susceptibility of the palm trees in the systems implementation and maintenance, the influence of the diversity of microorganisms action, existing in the biofilm of the phyllosphere, was not ruled out (Müller et al, 2006). It could be corroborated through the microbiome study which it was not considered as part of the CIAT project. This reduction can also be explained by the induction of systemic resistance generated by the microbial load existing in the rhizosphere (Banerjee et al, 2015).

4. Conclusions

The fungi *Pestalotiopsis sydowiana*, *Colletotrichum siamense,* and *Mycosphaerella fijiensis* were isolated and identified as the causative agents of leaf spots on palm, cocoa, and plantain leaves, respectively.

They reported the lowest diseases incidence in the silvopastoral systems, and the highest occurred in monocultures. Therefore, a greater diversity of plant species within a production system under the Yurimaguas' farms conditions leads to a reduce development of leaf diseases.

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Conflicts of interest

The signing authors of this research work declare that they have no potential conflict of personal or economic interest with other people or organizations that could unduly influence this manuscript.

Author contributions

Elaboration and execution, Development of methodology, Conception and design; Editing of articles and supervision of the study have involved all authors.

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