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Monitoring of Black Pod Rot Disease and Identification of the Causal Agent in the Bengamisa Cocoa zone in the Democratic Republic of Congo (DRC)

Limba K. mukwa Kibuka Gaston[1], Onautshu Didy[2], Monde Godefroid[3], Mukandama Jean-Pierre[4], Kikukama Kezile Laurent[1], Bahati David [1]and Wembonyama Shako François[1]

[1]Institut National pour l'Etude et la Recherche Agronomiques, Programme National de Recherche sur le cacao INERA Yangambi, Kisangani, RD Congo.

[2]Université de Kisangani, Faculté des Sciences, Département de Microbiologie et Phytopathologie. Kisangani RD Congo

[3]Institut Facultaire des Sciences Agronomiques de Yangambi, Département de Phytotechnie, Kisangani, RD Congo.

[4]Université de Kisangani, Faculté de Gestion des Ressources Naturelles Renouvelables, Département de l'Amélioration des

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ABSTRACT

The regular maintenance of the fields and harvesting of the pods had in itself made it possible to maintain a good sanitary state of the Congolese plantations. Black rot and mirid diseases were still under control at that time. The plantations managed by the planters under the supervision of the "CABEN" Company, Cacaoyère de Bengamisa, were well maintained and monitored. However, in recent years, farmers, especially those in the Bengamisa cocoa zone, have observed their production to be declining.In order to accurately determine the level of this disease in this zone, where abnormal production losses have been reported, observations were carried out in the cocoa farms at the Kisangani, Kapalata and Yangambi posts in the Banalia Territory in the Democratic Republic of Congo "DRC".Weekly passages during which pods affected by black rot and healthy pods were counted revealed very high and increasing levels of infection over two successive harvest seasons (2018 and 2019) (i.e. a growth rate of 17.5 in Kisangani, 14.08 in Kapalata and 9.25 in Yangambi). These rates are similar to those recorded in countries where Phytophtora megakarya, a species known for its aggressiveness, regularly occurs. This serious disease becomes more important in 2019, which evokes the evolution of the latter from year to year. However, it remains understood that the environmental conditions created by the demotivation of coffee and cocoa growers from 1980 onwards, combined with the lack of maintenance of orchards, would have favoured a rapid emergence of black rot. *Lasiodiplodia theobromae* is becoming a major constraint to cocoa production in DRC. This study is the first to report the effects of *L. theobromae* causing the decline of cocoa pods in DRC.

Introduction

The cocoa tree (theobroma cacao L) is a persistent perennial crop in tropical regions with a production period sufficiently spread out over the year. As a result, the cocoa tree offers pathogens conditions for survival without any real break in its life cycle.

In the Bengamisa cocoa zone, apart from attacks by pests such as Mirides, which can be easily controlled with conventional insecticides, there were no major diseases in cocoa farming at the time when farmers were still supported by the Bengamisa Cocoa Farming Company "CABEN". This is a Congolese state enterprise established in 1978, thanks to the financing of the Development Bank of Africa "BAD" and whose mission was to develop cocoa cultivation in DRC. Pod rot diseases could be controlled by sanitary crops alone, whereas in Cameroon and Ghana, for several years now, Brasier and Griffin, 1970; Zentmyer, 1988; Opoku, 1997 reported that the species known as aggressive, in this case *Phytophthora megakarya*, was endemic.

Curiously, in recent years in the Bengamisa cocoa zone, there has been an increase in the incidence of black pod rot in cocoa pods, leading to significant production losses, especially in the Bengamisa Cocoa Industrial Block "CABEN", (Limba 2013). The summary observations made at the level of farmers' plantations and those of CABEN had suggested black pod rot. The literature indicates that this disease is quite widespread. However, the damage it causes is generally much less than that caused by P. palmivora (INEAC, 1962). However, once it has established itself in the pod, the black pod rot fungus can destroy the pod completely, leading to enormous losses in production and thus economic losses (INEAC, 1962).

According to Limba's estimates, during his working visit in 2013 to the CABEN Industrial Block, more than 60% of production had shown symptoms of black pod rot disease. However, apart from these estimates, so far no quantitative data on the extent of this disease in this cocoa zone and even in the whole country is available. This situation makes it impossible to establish statistics to verify the exact extent of this disease in the area and even throughout the country with a view to considering possible solutions to reduce, if not contain this disease.

Tele:
E-mail address: gastonlimba49@gmail.com

In this study, we proposed to observe village plantations in different localities of the Bengamisa cocoa zone in order to assess the level of infection of cocoa pods by the black pod rot disease and to know the agent responsible for the disease.

Material And Method

Study environment

Our study area was the Bengamisa cocoa zone shown in figure 1 below.

The Bengamisa cocoa zone is located in the administrative post of Bengamisa. Bengamisa is located in Banalia territory between 0°57' and 10°00' N latitude; 24°11' and 25°30' E longitude (Lokombe, 2005). It is located in the Central Basin. It has a hot and humid climate of the Af type of KOPEN classification.

The rainfall regime in the region has a double periodicity: with the main and secondary maxima being respectively in October (237 mm) and May (178 mm), and the minima in January (46.8 mm) and July (154 mm) (Lokombe, 2005). Different soil types are found in the Bengamisa region. In general, the soil is heavy clayey, with 50 to 70% clay. However, some series represent a clay content of less than 30%. In the Bengamisa administrative post, the vegetation is of the grassy fallow type along the roads and as one moves away, there is a dominance of dryland rainforests (CABEN, 1990).

Materials and methods

The study materials consisted of all the pods collected by passage. To do this, we adopted the working method used by Pocet Jean et al in 2013 on the study of the aggressiveness of brown pod rot in Côte d'Ivoire. In our case, observations took place from September to November 2018 and the same period in 2019 in village plantations made up of seedlings from the "CABEN". In its organization, CABEN had grouped the plantations in managerial posts that it had named: Kisangani post, Kapalata post, Yangambi post and Yuma post. Each management post had as many village cocoa plantations within it. We had worked in three out of four posts, namely KISANGANI post, Baombi 2 and Bagunguzi 1 villages, KAPALATA post, Bayakyadu and Badile villages and YANGAMBi post, Bangole 2 and Basolombi 2 villages. In these posts, the plantations are maintained differently and do not undergo any pesticide treatment. The areas of these village plantations generally vary from 1 to 1.5 hectares and some of them are in an almost abandoned state since the farmers were no longer supported and were demotivated by prices. Observations were made on 2 plantations (repetitions) per post. The experimental system is therefore made up of 2 elementary plots (plantations) which constitute repetitions per locality, i.e. 6 plots for all the observations. The number of replicates was limited because of the reluctance of some farmers to collaborate with us and because of the unavailability of the manpower needed to monitor the harvest. This shortfall was compensated by repeating observations in two seasons, in 2018 and 2019. The harvest consisted of weekly passages during which the healthy pods and those affected by black rot were counted.

Macroscopic observations

Symptoms are fairly comparable to those caused by P. palmivora (Fig.2).

The determination of the disease was made on the basis of symptoms already described by other authors. The symptoms on the fruit are brown spots at the beginning, which later become blackish.

Fig 2. Symptom development on pods.

Careful observations on the disease spots were made on pods with an age ranging from 3 to 5 months, and the diseased pods set aside. This was done to avoid re-infections from these infected pods, which are potential sources of the fungus inoculum. The results are then transcribed in an Exel page opened for this purpose.

Microscopic obsevations

These microscopic observations were direct Fruiting bodies on infected pods are carefully scraped off with a sterile needle, mounted in 2% lactic acid on a glass slide, covered with a slide and examined under an optical microscope (Nikon 50i eclipse, Japan) equipped with a camera (Nikon DSFi1, Japan).

The fungus was characterized by intense aerial activity and rapidly growing mycelium filling a 90 mm diameter petri dish in 3 days at 25°C. The mycelium darkened with age, with black globular pycnidia developing singly or in groups, with or without stroma, with central ostioles which were observed after 14 days incubation at 25°C. The morphology of the fungus assessed in this study closely matched that described for L. theobromae by Burgess et al. (2006) Taylor et al. (2005) and Sutton (1980). Lasiodiplodia theobromae was identified on the basis of morphological data described by Punithalingam (1976). These included a colony and dark green or black conidia and conidia spread with dark brown, striated, ellipsoidal, uniseptate and produced in ascostromatic pycnidia.

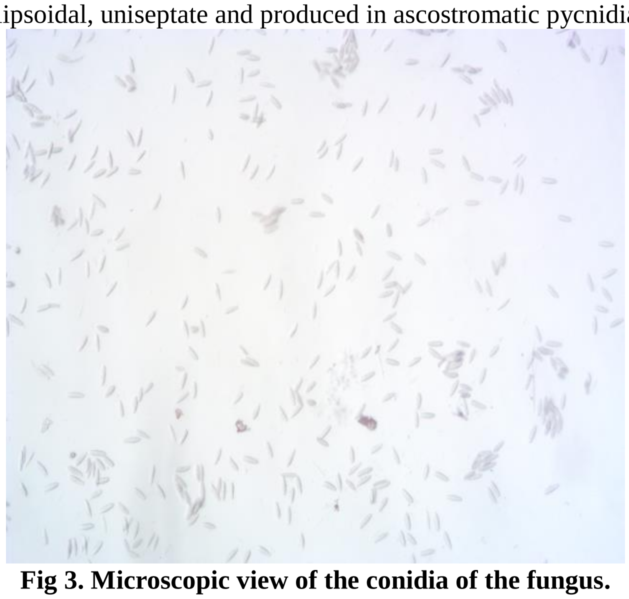

Fig 3. Microscopic view of the conidia of the fungus.

Although the morphological characteristics of sporocysts give indications on species according to the descriptions of Brasier et al. in 1981, it is not always obvious as other species may also attack cocoa. Hence the need to resort to molecular analysis.

Molecular analysis

Despite all these observations, only molecular analysis gives the precise species of the fungus in question. For example, fruiting bodies from infected pods had been collected with a lanceolate needle and grown in a Petri dish and sent to specialized laboratories for identification of the causal agent (Fig. 4).

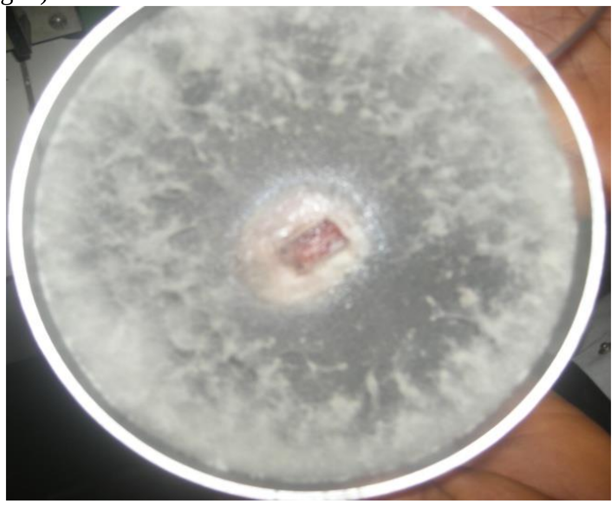

Fig 4. Fruiting of the mushroom

All 10 isolates collected from the field had to be purified for sequencing. It was necessary to manufacture a spore by UV light for 5 days and inoculate the diluent so that only a few spores developed. The Biomolecular Analysis had consisted of obtaining the monospore strains, liquid PBS plating, DNA extraction and quantification, normal PCR, electrophoresis, and finally sample preparation for sequencing performed in Germany for the sample sent to the University of Ghent.

The monospores were sent for sequencing in Sanger and gave qualitative and consistent results.

Indeed, two batches of samples had been constituted. One batch was sent to the laboratory of the University of Aberdeen in Scotland and the other batch to the laboratory of the University of Ghent in Belgium for molecular analyses.

Counting infected pods

Trials of all fruits showing symptoms were carried out with teams initially trained in this field. This operation consisted in counting the diseased pods separately from the apparently healthy ones Fig.3

Figure 5. Pod sorting.

The determination of pod production had consisted of counting all the pods harvested ripe, healthy and diseased, as well as cherelles at least three months old that had shown symptoms of the disease.

The total production is equal to the sum of apparently healthy pods and pods showing symptoms of black rot disease.

Infection levels are calculated as the percentage of pods affected by black rot in relation to the total number of pods observed.

The ANOVA tests and the comparison of the slopes of the trend curves by using dummy variables on StatAdvisor software, allowed to evaluate and compare the different levels of the disease during the campaign .

At each weekly run, after counting the collected pods, the results were recorded in the observation notebooks for each site from September to November, a period of high production in the area, for two seasons. The detailed observations of the disease spots were thus carried out on pods aged from 3 to 5 months and the diseased pods set aside. This was done to avoid reinfections from these infected pods, which are potential sources of the fungus inoculum. The results are then transcribed in an Exel page opened for this purpose.

Results

Rainfall data for the 2018 and 2019 observation period

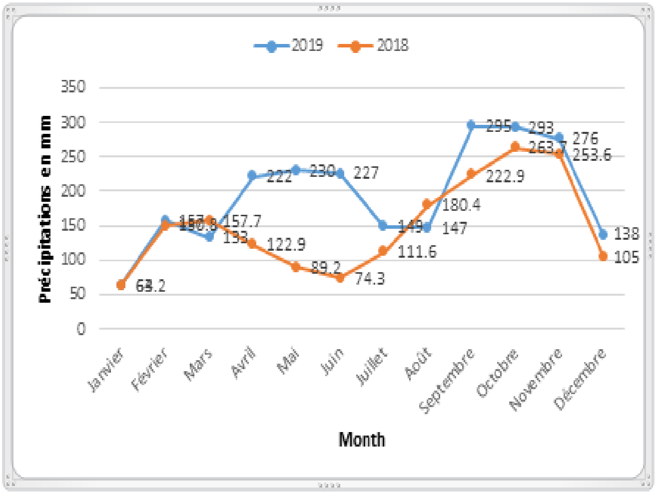

Figure 6. Average monthly precipitation in 2018 and 2019.

In 2018, the level of monthly rainfall is highly variable during the year. It experiences three significant bending levels in March (157.7), June (74.3) and October when it peaks at 263.7 before falling in November.

During the study period, September, October and November, the rate was lowest in September (222.9) and the peak in October (263.7), declining slightly in November (253.6) before falling sharply after this period.

In 2019, the monthly rainfall for the period corresponding to this study observations (September, October to November) appears to be the highest of the year, unlike last year 2018 (Figure 6).

Level of disease progression.

From observational data, we were able to plot the evolution of the rates of black pod rot disease of cocoa pods caused by Lasiotheobroma sp as a function of time as shown in Figures 6 and 7.Pod black rot curves for both years, 2018 and 2019, all show an irregular pattern with localized peaks.

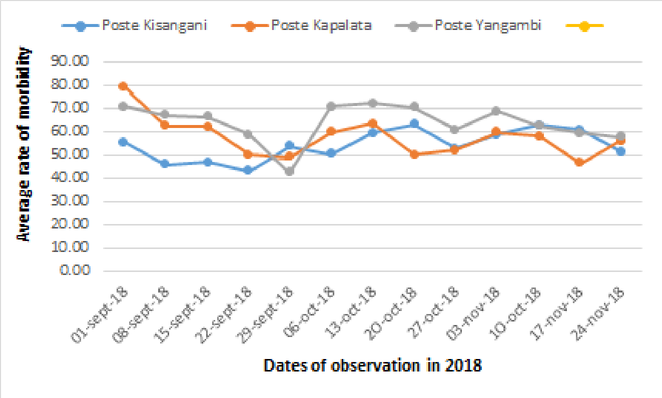

Figure 7. Evolution of the average morbidity rate in 2018.

For the year 2018, at the beginning of observations in September, the Kapalata post recorded a higher morbidity rate than other posts, followed by the Yangambi post and then the Kisangani post. However, afterwards, the other harvests recorded very variable peaks.

The results of the analysis of variance are shown in Table 1.

Table 1. Analysis of variance.

Source	Sum of squares	dl	Mean square	F-Ratio	Meaning
Between groups	1224.48	2	612.242	6.01	0.0038
Inter-group	7644.0	75	101.92		
Total	8868,49	77			

The analysis of variance confirms the observed difference between the mean morbidity rate per shift using F-ratio. The probability p0,0038<0,05, shows that there is a significant difference in morbidity rates between the different positions. However, this analysis does not yet reveal how this difference is presented, although the direction of the difference seems to be given in Table 1.

Monitoring the progress of the disease in 2019

The average morbidity rates calculated in 2019 for this study observations yielded results shown in Figure 2.

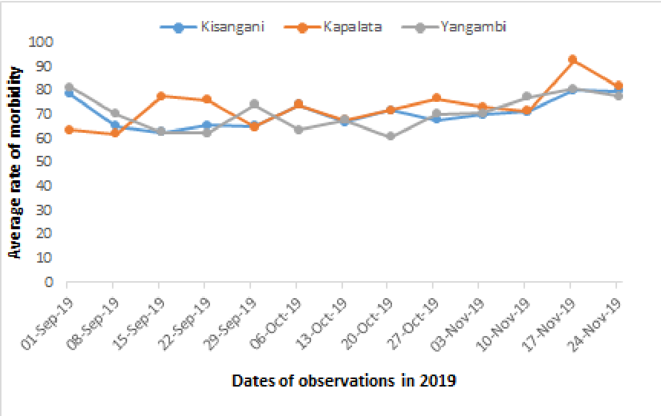

Figure 8. Evolution of the average morbidity rate in 2019.

Contrary to what was observed last year at the first harvest, the morbidity rate observed in Kapalata station was the lowest. This was offset by the highest peaks in November.

The one-factor Analysis of Variance was performed and the result is reported in Table 4.

Table 2. Analysis of variance.

Source	Sum of squares	dl	Mean square	F-Ratio	Meaning
Between groups	15, 3692	2	7, 68462	0, 13	0, 8780
Inter-group	4420,16	75	58,9355		
Total	4435,53	77			

The analysis of variance by its estimated F-ratio of 0.13 is tested non-significant. Therefore, it does not allow for a difference in morbidity rates between the 3 positions. With a predicted probability of p= 0.8780>0.05, there is no significance. The results from the three observation sites are identical. However, non-significant differences were observed.

Evaluation of morbidity rates in the posts studied.

Morbidity rates were calculated as the percentage of the number of diseased cocoa beans in relation to the total number of pods harvested in each station. The results of two years of observations are summarized in Table 3 below.

Poste	**Morbidité (%)**		**Taux d'accroissement de la maladie**
Poste	2018	2019	2018 – 2019
Kisangani	54,20±8,16b	71,79±7, 08 a	17,59
Kapalata	57,19±10,52ab	71, 08± - 8,00a	13,89
Yangambi	63,69 ±10,94a	72,15 ± 7,90a	8,46
Signification	0, 003 xx	0,8780NS	

Means with the same letters: no significant difference for a probability (P>0.05) according to Tukey's test.

- P= Probability
- xx= Highly significant difference
- NS= No significant difference

The average disease rate in the three observation posts ranges from 54.2% (Kisangani) to 63.0% (Yangambi) to 57.0% (Kapalata) for the year 2018. The dispersion of the data observed around their respective averages is high, confirming the non-homogeneity of the distributions. The result shows that the morbidity rate is variable with a more pronounced trend at the Yangambi post and less pronounced at the Kisanagani post.

Compared to the year 2018, the morbidity rate in 2019 varied from 71.08 to 72.15. It seems to be higher in Yangambi post than in the other sites.

Statistical analysis of Tukey's test for comparing morbidity rates.

The Tukey test showed the significance of the difference in the rates of the diseases observed between the different observation posts. The results are shown in Table 3.

The magnitude of the morbidity rates in the study sites.

It was necessary to highlight the magnitude of the pod morbidity rates as shown in Table 3. This had been calculated by the difference in morbidity rates observed between the two observation periods. This magnitude reflects the growth rate of the disease from one year to the next. It can be seen that there is a different growth in the average morbidity rate in the different positions between the years 2018 and 2019. This rate is 17.59 per cent in Kisangani post, 13.89 per cent in Kapalata post and 8.46 per cent in Yangambi post.

Identification of the Agent Responsible for Black Pod Rot Disease in DRC

Macroscopic and microscopic observations made at the level of the diseased pods were not sufficient to give the identity of the fungus involved in the black rot disease of cocoa pods. However, the diagnosis made at the level of the genus of the fungus was the same as that made by molecular analysis. It was indeed the genus Lasiodiplodia that was involved in this disease. In fact, several healthy organisms of this genus and even outside of it present identical symptoms. It was therefore necessary to resort to molecular analysis.

Molecular analyses indicated that all samples contained an STI region specific to *Lasiodiplodia theobromae*(Patt), Maure and (Maubl). The results from the University of Ghent corroborated with those found by the University of Aberden in Scotland where the same samples had been shipped.

Discussion

The peak rainfall was not the same during two observation periods. This peak was in September for the year 2018 and in October for the year 2019. This had a small impact on the morbidity rate of the pods. Charts 6 and 7 clearly indicate that there was an increase in the morbidity rate in September for the 2018 observation year while the morbidity rate was higher in October 2019. In general, the incidence of black pod rot disease was higher during this period than the rest. Indeed, rainfall favours the establishment and development of Botryosphaeriaceae pod rot such as black pod rot due to Lasiodiplodia sp. To this end, it creates favourable conditions for their reproduction and the dissemination of their propagules. Water appears to be the indispensable element, especially in the initiation and maintenance of the epidemic (Brasier and Hansen, 1992). It is by no means a limiting factor for cryptogamic diseases in the humid tropics (Babacauh, 1980). The months of September, October and November, which correspond to the period of this study, recorded the highest rainfall according to data from the INERA Climatological Section (2018 and 2019). The water conditions were therefore favourable for the development of black pod rot in cocoa pods during this period.

Nevertheless, the difference in morbidity rates was observed in the different positions. Despite this difference from one post to another, the level of the disease is now abnormally high in all the plantations observed in the Bengamisa cocoa zone. In the past, although figures are not available, sanitary crops alone could contain the disease.

This "observations" study shows that the infection rates of the disease are currently above 50%, of our hypothesis. The results are close to those obtained in other countries on brown pod rot disease due to Phytophtora megakarya, for example in Côte d'Ivoire 62.82 ± 6.75%, 69.92 ± 4.63% and 77.60 ± 4.77% (Pocet Jean et al in 2013). In our case, the average morbidity rate was 54.20±8.66, 57.00±10.52 and 63.00±10.94 respectively for Kisangani, Kapalata and Yangambi posts in 2018 and 71.79±7.08, 71.08 ±8.00 and 72.15±7.90 for the same posts in 2019. Looking at the results obtained in 2018, it was found that their distribution was not homogeneous, the coefficient of variation was less than 0.15. This reflects the dispersion observed around their averages. This dispersion shows that the Yangambi post with a rate of 63% had a higher morbidity rate than the other posts without being statistically different from the Kapalata post 57% but very significantly different from the Kisangani post 54.20%. Kisangani 54.20% did not show a morbidity rate significantly different from Kapalata. This situation can be explained by the proximity of the latter two management positions to the product consumption environment. Indeed, as the Kisangani and Kapalata posts are close to the town of Kisangani, merchant cocoa is very popular, and the pods are harvested as they ripen, either by the farmers themselves or by thieves without being dragged over the trees. Unlike the Yangambi post far from the trading centre, the town of Kisangani. Also, the first two posts in 2018 had not been strictly adhered to the research harvest schedule. The times planters were harvesting without waiting for the program, either because they had a buyer or they had found that the pods were mature and could not wait. In contrast to the Yangambi post far from Kisangani, a trading centre, the demand for purchase was low. Hence the sick pods could still be found in large numbers compared to the last two posts.

In 2019, the morbidity rate was almost homogeneous in all posts. This was confirmed by statistical calculations which showed that there were no significant differences between the results of different posts. This situation can be explained by the fact that strategies had been changed by making the harvesters responsible for monitoring fields against predators and passing the pod count every time the plantation owner had to remove the pods from his field before the planned passage period. As a result, the production losses experienced in 2018 were reduced in 2019. The result for that year was that production per shift had increased and the disease had almost equally spread to all shifts. Finally, it was observed that the morbidity rate is increasing in each shift. The morbidity rate is 17.59% in Kisangani, 13.89% in Kapalata and 8.46% in Yangambi.

This increase is greatest at Kisangani post, followed by Kapalata post and finally Yangambi post. This situation confirms the explanations given in the 2018 results that crop control had escaped the observation team, which resulted in fewer sick pods in the first two posts compared to Yangambi post. Now that harvest strategies have been changed, the actual number of diseased pods has been recorded. There is therefore a big gap between the 2019 and 2018 results in Kisangani and Kapalata posts.

The introduction of the Lasiodiplodia Theobromae species into the Bengamis cocoa zone would be made possible by importing plant material from Côte d'Ivoire where the seeds came from. Indeed, the disease was first observed in 2013 in the Bengamisa cocoa block, three years after the orchard started production. The disease was first localized in plots 5 and 6 of the Enterprise's Industrial Block, before spreading to several plots. Later, the disease was observed in the fields of the Family Block belonging to individual growers.

However, it must be recognized that the cocoa orchards in the Bengamisa cocoa zone have lacked maintenance as a result of the lack of funding from CABEN due to the drastic fall in world commodity prices and the disappearance of a minimum stabilized price. The decrease in the purchasing power of cocoa farmers has forced them to turn to other speculations, particularly food crops, abandoning cocoa trees without pesticide treatment, land clearance and sanitary harvesting. All these conditions would predispose the cocoa tree to black rot which is currently at an infection rate of between 54.20 and 72.15% according to this study observations. The emergence of this pod rot disease had called on government authorities so that, following the experts' report, the central government released a sum of $223,500 to purchase phytosanitary products to combat this disease in this cocoa zone (Limba, 2013). Despite this government intervention, the disease persists and is far from being eradicated. This is because this amount was not only insufficient to cover all the costs of the CABEN Industrial Block (±400 ha) but also did not concern the family block (±600 ha), which is made up of farmers' plantations, even though they are supervised by CABEN. The disease continues to spread and is far from being eradicated. This situation is worrying and there is a risk that it could spread throughout the country if security measures are not taken in good time. Indeed, accumulations of various Lasiodiplodia propagules would have taken place over the years and would have contributed to the severity of the attacks. It is known in plant pathology that the more propagules there are, the more regular the infections are and the greater the disease (Shafer And Heagle, 1985; Simmonds, 1988; Carisse and Kushila, 1989).

In any case, cocoa production is under threat in the DRC and a joint effort by researchers, development workers and policy makers is needed to address this.

Conclusion

It emerges from this study that cocoa farms in the Bengamisa cocoa zone are under threat. Attack levels are high everywhere and similar to those recorded in countries where the aggressive Phytophthora megakarya species is prevalent in the absence of fungicide applications. In addition, as the fungus was first reported in Cameroon in 1895, where it has since caused black pod rot symptoms, similar severe decline has been described on other crops such as mango in other countries (Khanzada et al., 2004. Kao et al.; 2004). It cannot be excluded that this is or has already occurred on crops other than cocoa in the DRC. Lasiodiplodia theobromae with this important cocoa disease that is increasing year by year is becoming a major constraint for cocoa production in the DRC. The situation calls for urgent and adequate intervention by decision-makers with regard to the control strategy to be implemented against the black rot which has become aggressive. Given that pesticides are rare and their use expensive in the environment, the use of resistant materials would be an economically profitable and ecologically acceptable solution to preserve the environment. High hopes should therefore be placed on the selection and multiplication of cocoa varieties resistant to black rot. These varieties would come from the INERA Yangambi Research Centre. However, the first essential precaution is to avoid injuring the developing pods, which is not always certain. The ripe fruit must also be harvested in good time. However, with this new development of severe dieback, similar to that recently described on other crops such as mango and kumquat in other countries (Khanzada et al., 2004; Ko et al., 2004), Lasiodiplodia theobromae is becoming a major constraint to cocoa production in DRC. This study is the first report of Lasiodiplodia theobromae causing cocoa pod dieback in the Democratic Republic of Congo.

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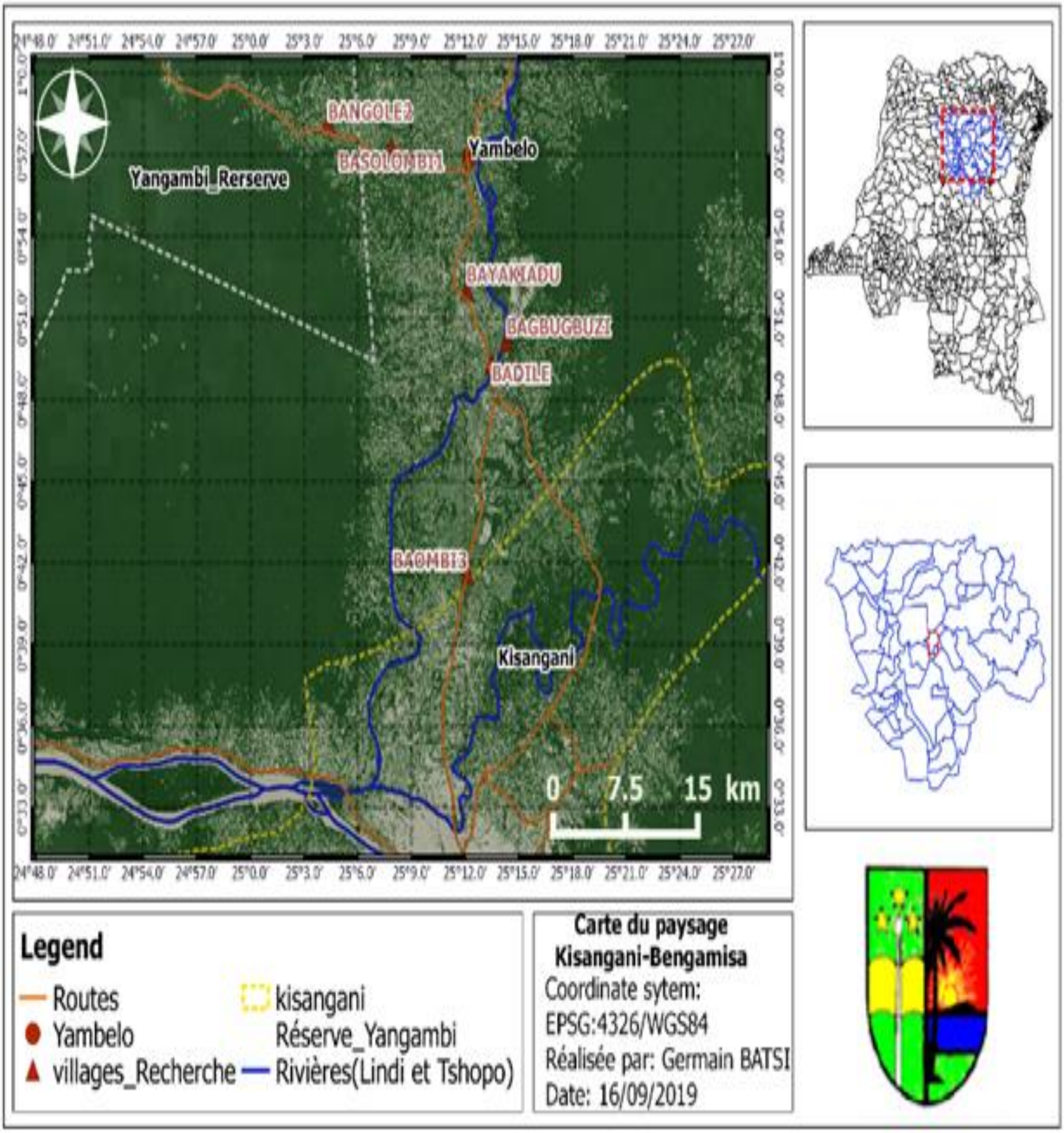

Figure 1. The Bengamisa cocoa zone.