Impact of mirids and fungal infestation on dieback of cocoa in Cameroon

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Abstract
A study was carried out between March 2016 and April 2017 with the aim to determine the relationship between mirid infestations and dieback process of cocoa plants due to fungal infestations. Three infestation types on different cocoa organs were studied in farm and in the laboratory as well as the fungal diversity of the cocoa organs and interaction between these fungi and dieback. It emerges from this work that *Sahlbergella singularis* presented a developmental cycle of 28.35 ± 5.10 days in the laboratory and 42.53 ± 6.70 days in the farm. As for fungal diversity, *Lasiodiplodia sp.* (34%) had the highest abundance followed by *Fusarium* sp. (23%), then *Asperillus* sp. (16%). These fungi became opportunistic due to mirid attack, leading to dieback of the host plant. This clearly shows the combined actions of mirids and fungi in dieback processing.

Keywords: *Lasiodiplodia sp.*, Dieback, Cocoa, *Sahlbergella singularis*, Infestation

1. Introduction
Cocoa (*Theobroma cacao* L.) occupies one of the most important place in the national economy of Cameroon as it represents close to 28% of the non-petroleum exports and 40% exports of the primary sector [1]. The revenues from this culture are estimated to more than 100 billion franc CFA per year. However, for many decades, the cocoa sector is a prey to great difficulties provoked by many factors amongst which, diseases and insect pests which significantly reduce the yield in farms. Losses related to these factors are estimated between 30 and 100% in case of non-adapted treatment of the host plant [2, 3]. Among the factors responsible for these losses, are insects pests such as mirids, especially the species *Sahlbergella singularis* Haglund, 1895 which is considered as the most important insect pest [4, 5, 6, 7, 8]. In general, these insects affect all the aerial parts of the tree especially the pods and the foliage (the young shoots notably the terminal extremities of branches and twigs) [9]. During feeding on the host plant, mirids inject saliva which contain homolylational properties in the targeted tissues. The direct consequences is the appearance of lesions which evolve generally to wounds, thus fragilizing the tree physiologically. The infection of the host plant by opportunist fungi such as *Lasiodiplodia theobromae* (Berk and Brome) are indirect consequences of these bites [10]. Mirid attack and/or the combined action of mirids and opportunist fungi are usually at the origin of drying out and/or the rapid decaying of affected organs (cherelles, branches and leaves, etc) or the whole host plant [11]. However, there is a paucity of information which permits to clearly define or determine the nature of the relationship which exist between the mirid attack and the decay observed in the host plant on a large scale. Furthermore, a good knowledge on the impact of the infestation of cocoa by mirids on the epidemiological profile of decaying in the latter can significantly ameliorate the program of integrated management of pests of the cocoa-based agrosystems of Cameroon. To this effect, the general objective of this study was to determine the relationship between mirid and fungal infestations on dieback process of different cocoa genotypes.

2. Materials and Methods
2.1 Study Area
The present study was carried out between March 2016 and April 2017 at the experimental cocoa farm of the multipurpose Research Station of Nkoémvone (2°90 of latitude North to 12°2 of longitude East), South region of Cameroon. Mirids were collected from local cocoa farmers in Ayos and Okola localities in the Center region. These two localities are found in the

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agroecological zone II, humid forest zone with bimodal pluviometry. This zone is located between latitude 2°6” to 4°54”/5°48” North and longitude 10°30” and 16°12” East [12]. It covers almost all the Southern Cameroon plateau between 500 and 1000 m of altitude and integrated the Center, South and East regions for a total surface of 22.5 million hectares [12]. The climate is of equatorial guinean type characterized by a high temperature with an annual average of 25 °C and a rainfall of 1500-2000 mm per year [13]. The mired rearing and observations were done in the Central Laboratory of Entomology and the Phytopathology Laboratory of IRAD, Nkolbisson, Centre region.

2.2 Collection of mirids from the natural environment
The method adopted in this work was that of capturing live individuals according to the technique of Babin et al. [14] from local cocoa farms of the localities of Ayos and Okola. Capture was done between 6 a.m and 7 a.m, reason being that these organisms are more active at that time [15]. The captured specimens were conserved in a cylindrical polystyrene container and brought to the Central Laboratory of Entomology of IRAD; Nkolbisson for rearing.

2.2.1 Rearing of mirids
One hundred and forty (140) individuals were captured with a sex ratio of 1:1. After determining the sex ratio of the studied specimens, 70 couples were randomly formed. Thirty couples were reared in the Central Entomology laboratory of IRAD and 40 couples in the experimental plot of IRAD Nkoémvone or the mass production of individuals which shall later serve for the experimentation.

2.2.2 Rearing in the laboratory
After disinfection of the insectarium and the rearing containers with alcohol, the containers were drained with the aid of absorbing paper. The mirid couples (30) were put in the containers containing pods or twigs. Observations were done daily and the food source made of cocoa fruit renewed every 72 hours. The larvae issued from these couples were counted and monitored to maturity. Adults obtained from the F1 permitted to obtain F2 then F3 and F4 and counted.

2.2.3 Rearing in the farm
In the farm, each mirid couple was deposited on the pods and covered with a gauze cape (Fig 1). The confection of the transparent gauze cape consisted of the use of a white cloth of fine mesh permitting in one part the aeration of the content and on the other part the protection from external aggression (Fig 1). The dimensions of the gauze cape varied with respect to the usage: dimension 40 x 30 cm were used for mature pods and dimension 5 x 10 cm for cherelles. The mirid couples deposited on the pods were observed daily until the obtention of L1 to L5 larvae. The fertility of the females expressed in number of larva hatched per day was determine and recorded in an excel table. Observations were made right up to imagoes (adults) stages for F1, F2 etc.

2.2.4 Evaluation of the impact of mirids on dieback
The impact on dieback of cocoa by mirids was evaluated in the experimental plots. For so doing, 2 types of infestations and a negative control were realized. The first type of infestation consisted of infecting the young shoots, pods at all stages (i.e. cherelles, young pods, mature pods and ripe pods) with mirids (which were starved for 48 hrs) and the second type consisting of manually puncturing the organs using fines needles (10 bites of a two-cm depths per infested organ). In order to avoid all bias in the sampling, each organ from the infested cocoa tree was covered with a gauze cape. For each type of infestation, a total of 30 pods per stage of development and 30 young shoots were infested.

2.2.5 Isolation and characterization of parasitic fungi
Isolation and characterization of cocoa plant parasitic fungi was realized at the Phytopathology Laboratory of IRAD. For so doing, the twigs from the three types of infestation were rinsed with tap water for 10 minutes to get rid of impurities and debris on their surface [16, 17], then they were immerged in 70 °C ethanol for 1 minute, then in sodium hypochlorite (NaOCl) (2.5%) for 4 minutes. The twigs were put again in ethanol for 30 seconds [18] and rinsed thrice with distilled water for 1 minute and dried on sterile absorbing paper [17, 18]. The twigs were later cut into small fragments and placed in petri dishes containing Potato Dextrose Agar (PDA) previously autoclaved at 121 °C for 15 minutes and supplemented with 1 mg/l of chloramphenicol to inhibit bacteria growth, and they were finally incubated at the laboratory temperature (Fig 2).

Fig 1: On-farm experimental setup in IRAD Nkoémvone.

Fig 2: Isolation of fungi in the phytopathology laboratory
2.3 Statistical analysis
Diversity and daily relative abundances were calculated using the Generalized Linear Model (GLM) procedure which involved a linear regression analysis and variance analysis (ANOVA). Then the correction of Poisson was applied for counting data. The mean numbers of eggs laid were compared between mating system using the ANOVA (GLM proc) test with associated Tukey HSD test corrected by sequential Bonferroni procedure for Pairwise comparisons. The analyses were conducted with the R software (Version 3.0.2, 2013) and the results were appreciated at the 5% confidence level.

3. Results and Discussion
3.1 Rearing of mirids
In the farm, females laid 30 to 50 eggs all through their life in the farm with an average of 4 eggs per day whereas in the laboratory, 20 to 30 eggs were laid with a daily average of 2 eggs (Table 1). However, there is no significant difference between the lay in the farm and the laboratory (F<sub>1,99</sub> = 4.5; P > 10–5).

The results differ from those of Babin et al. [14] who obtained 30 to 70 eggs for an average of 14.5 eggs per female for the same species. The difference can be due to the experimental protocol and the environmental conditions or the host plant. The sex ratio in the farm tend towards 1:1. This result is close to that of Samaila et al. [19] who obtained a sex ratio of 1:1 while working in natural conditions on live pest (Rhyparochromus littoralis Dist) of groundnut in the Adamawa state in Nigeria. Normally, insects and other animals living under favorable conditions generally have an equilibrated sex ratio [20]. On the other hand, in the laboratory, females out-numbered males. This can be explained by the fact that in the laboratory animals are stressed due to confinement and multiple manipulations. In order to assure their survival, genetic modification took place [21] which led to the production of a great number of females on one part [22], and on the other part the males sacrificed themselves to make place for the females who will later on lay more eggs. This is also what happens with the praying mantises, where the male is devoured by the female after mating [23, 24], thus serving as a source of proteins necessary for the proper development of pregnancy. This is the ultimate sacrifice for the survival of his offspring and the species [24].

<table>
<thead>
<tr>
<th>Experimental site</th>
<th>Couples</th>
<th>Eggs</th>
<th>Larvae</th>
<th>Adults</th>
<th>Females</th>
<th>Males</th>
<th>Sex-ratio</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm</td>
<td>40</td>
<td>2800</td>
<td>2100</td>
<td>1850</td>
<td>930</td>
<td>920</td>
<td>1.01</td>
<td>1850</td>
</tr>
<tr>
<td>Laboratory</td>
<td>30</td>
<td>1400</td>
<td>950</td>
<td>450</td>
<td>235</td>
<td>215</td>
<td>1.09</td>
<td>450</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>4200</td>
<td>3050</td>
<td>2300</td>
<td>1180</td>
<td>1120</td>
<td>1.05</td>
<td>2300</td>
</tr>
</tbody>
</table>

3.2 Development cycle of S. singularis
The average duration of the development cycle was significantly (F<sub>1,99</sub> = 4.7; P = P > 10–3) higher in the farm reared mirids than in the laboratory reared ones with a sexual maturity period of 6 days in females (Table 2). These results differ from those of Babin et al. [5] who obtained an average pre-embryonal cycle duration of 46 days while working on the biology of S. singularis. The difference thus observed could be due to the size of the sample and the duration of the experiment given that they worked on a period of 4 years while the present study was done for two consecutive years.

<table>
<thead>
<tr>
<th>Development cycle</th>
<th>Stage Durations (in days)</th>
<th>In the laboratory</th>
<th>In the farm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>Mean</td>
</tr>
<tr>
<td>Egg-L1</td>
<td>2</td>
<td>7</td>
<td>3.68</td>
</tr>
<tr>
<td>L1-L2</td>
<td>3</td>
<td>8</td>
<td>4.50</td>
</tr>
<tr>
<td>L2-L3</td>
<td>5</td>
<td>10</td>
<td>6.13</td>
</tr>
<tr>
<td>L3-L4</td>
<td>4</td>
<td>9</td>
<td>5.31</td>
</tr>
<tr>
<td>L4-L5</td>
<td>2</td>
<td>7</td>
<td>3.70</td>
</tr>
<tr>
<td>L5-imagoes</td>
<td>4</td>
<td>8</td>
<td>4.93</td>
</tr>
<tr>
<td>Pre-imaginal development</td>
<td>20</td>
<td>49</td>
<td>28.35</td>
</tr>
</tbody>
</table>

3.3 fungal Diversity
The fungal diversity deriving from the mirid infestation of the studied cocoa plant organs reared in the farm is illustrated in Fig 3. The pie chart showed an important specific fungal diversity with Lasiodiplodia sp presenting the highest abundance followed by Fusarium sp, Asperillus sp and then unidentified others.

Most of these fungi are endophytes and causes no problem to the plant in its normal physiological conditions. For this reason, they are qualified as opportunists in pathogenicity situation. Anikwe and Otuonye [23] in Nigeria working on the question on Dieback of cocoa identified more than five species of fungi (L. theobromae, Fusarium species, Asperillus species, Rhizoctonia species and others unidentified) at the proportions practically similar to the present results and whose role was clearly defined in this process. Mvondo-Nganti et al. [10] on the other hand in Cameroon isolated the species L. theobromae and Fusarium sp from roots of infected cocoa tree plants. These results were less diversified but complimentary to the present study given these authors worked on the underground parts of the plant whereas in the present study, experiment was carried out on the aerial parts of the plant which are more sensitive to attacks due to climatic variation (rain and wind) factors of dissemination of microscopic fungal spores.

Table 1: Production of mirids in the farm and the laboratory

Table 2: Development cycle of mirids
3.4 Evaluation of dying of cocoa trees

The impact of type of infestations on the targeted organs with respect to dying rates is presented in Table 3. Physical observations showed that only organs bitten by mirids went through the dying process while organs injected with fines needles and the control were spared because in the latter type of infestations, the parasite (endophytic fungi) is in its latent state. Results showed that cherelles infested by mirids had 100% dying rates as they all died within one month. On the other hand, dying rates were progressive for young shoots and twigs when infested by mirids.

Table 3: Evaluation of dying rates (%) of targeted organs with respect to type of infestation

<table>
<thead>
<tr>
<th>Organs</th>
<th>Control</th>
<th>fines needles</th>
<th>Mirids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cherelles</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Twigs</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Young Shoots</td>
<td>0</td>
<td>0</td>
<td>75</td>
</tr>
</tbody>
</table>

The fact that mirid infested organs exhibited dying agrees with the works of Anikwe and Otuonye [25] who observed that *S. singularis* like all other mirids cause necrosis of organs due to the injection of haemolytic substances contained in their saliva which create an important stress to the plant leading to the development of diseases. Moreover, this suction action favors the passage of *Lasiodiplodia* sp and other fungi from non-pathogenic stage to acute pathogenicity stage [25] thereby provoking the death of targeted organs of the whole plant. Anikwe and Otuonye [25] and N’Guessan et al. [26] demonstrated that the conjugated action between mirids and endophytic fungi which became opportunists.

The highest dying rate observed in cherelles could be justified by the fact that these organs which are young pods have not yet acquired the necessary physiological capacities needed to resist mirids bites. Moreover, it was observed that mirids clustered around the cherelles and bite them several times on the same spot thereby accelerating the process of dieback. The progressive dying rates observed in twigs and young shoots could be due to the fact that they offered many sites of suction to the mirids. For a long time, works on the impact of mirids on cocoa have been documented on the underground parts [10] or the whole plant [25]. This study for the first time shows how mirids infest young organs (cherelles, twigs and young shoots) of cocoa trees.

3.5 Evaluation of dying rates of organs with respect to type of infestation and genotypes of host plants

Fig 4 illustrates the impact of the 3 infestation types (mirid sting and entomological sting) on the twigs with respect to host plant genotype. Results showed that mirid infestation exhibited the highest dying rate for the genotype SNK181 followed by the Hybrid then T79/501 crossing while entomological sting exhibited no dying rate irrespective of the host plant genotype.

The studied genotypes are usually cultivated in the locality of Nkoemvone and its environs due to their high agronomical performance. They have a high genetic potential and are resistant to diseases as illustrated by Kamga et al. [27] and Dibog et al. [4].

The genotype SNK 181 was in general most sensitive to the process of dieback (about 50%), followed by T79/501 (Ghanaian genotype) and finally the hybrid. This could be justified by the fact that the genotype T79/501 which is made of Trinitario (T79) a Latin American exotic variety contains genes permitting them to survive in harsh environmental conditions. This corroborates the observations of Hall [28] who reported that Amazonian high clones such as UPA 143, and those belonging to the Trinitario group such as T79/501 are more efficient during cuttings [27].
3.6 Interaction between fungi and cocoa dieback
Fungi were present in varying proportions in the medium (Table 4) and played a significant role in the dieback process. The most important being Lasiodiplodia sp whose presence is omnipresent in cocoa farms and other speculations such as Mangifera indica as demonstrated respectively by Mbenoun et al. [29] and by Ismail et al. [30].

The latter has long been incriminated by many authors [10, 25] as the main opportunistic fungi involved in the process of dieback overshadowing the actions of other fungi such as Fusarium, Aspergillus ... [10, 25]. However, the combination of several factors is necessary for the activation of this dieback including stress that can be hydric or nutritional (poor soil), the action of opportunistic fungi and finally the catalytic activity of mirids that inject haemolytic substances into the plant tissues [31, 32]. All these factors together constitute a complex responsible for the death of the host plant.

### Table 4: Role of the different observed fungi in the decaying process of cocoa tree.

<table>
<thead>
<tr>
<th>Fungi species</th>
<th>Control</th>
<th>Entomological needle</th>
<th>Mirids</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lasiodiplodia sp</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Fusarium sp</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Aspergillus sp</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Others</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Necrosis</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td></td>
</tr>
</tbody>
</table>

(++++) highly abundant, (+++) abundant, (+) averagely abundant,
(+/-) least abundant, (-) absence of necrosis.

4. Conclusion
This present study conclude that mirids (which are the main insect pest of cocoa) associated with the activities of fungi especially Lasiodiplodia sp could be responsible for die back of cocoa. The former through their suction activity create favorable physiological (stress) conditions for the development of opportunistic fungi which cause necrosis and consequently death. This death was shown to vary with host plant genotypes with the genotype SNK181 having the highest death rate when infested by mirids. It is important to note that these fungi generally present and live in harmony with the host plant. This study provides more added information to the comprehension of Dieback and its control on cocoa trees.

5. Acknowledgement
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6. References


