PHYTOPHTHORA PALMIVORA IN FLOWER CUSHIONS, OLD INFECTED PODS AND LEAVES OF CACAO PLANTS

by

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Thesis

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To my parents
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BIOGRAPHY

The author was born in Guaira, "São Paulo" State, "Brasil", in 1938.

He studied at the "Escola Nacional de Agronomia" of the "Universidade Rural do Brasil, Rio de Janeiro" State, from 1959 to 1962. In 1963 he began to work in the "Comissão Executiva do Plano de Recuperação Econômica Rural da Lavoura Cacaueira", and today is in the "Centro de Pesquisas de Cacao" (CEPEC) of this "Comissão" in Itabuna–Ilheus, Bahía, Brasil.

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INTRODUCTION

Efficient control of many fungus diseases involves studies of different aspects of the disease cycle such as location of the sources of inoculum, manner of inoculum dispersal, length of incubation period of inoculum and the environmental factors that influence the progress of the disease. On the Cacao Black Pod rot, caused by Phytophthora palmivora Butl., studies have been made on dispersal of spores, inoculum potential, correlation between environmental conditions and disease occurrence, certain aspects of host-parasite relations, and the existence of physiologic strains.

Referring to the source of inoculum in relation to the disease cycle it is said that the soil, and pods systemically infected from diseased cushions are primary sources, and sporangia produced on pods are the propagators of the disease. But, there are indications that the canopy of the trees has some importance as a source of inoculum amplifying the amount of the disease. In Costa Rica there are no records of the prevalence of cushion cankers nor of survival of the fungus in old infected pods remaining in the trees, both of which are considered to be primary sources of inoculum. The peak of incidence of the disease has been correlated with the biannual flushings of cacao trees (which occur more or less in January and September). The present thesis is concerned with some information on survival of the fungus in flower cushion-cankers and in old infected pods on the trees, and the role that the canopy of the trees plays on the incidence of the disease. Cushions and pods were artificially infected to study the first two aspects. In the third, the study was oriented to prove that the fungus
was present on leaves, and to detect the structures of the fungus which were present in the canopy and could be a source of inoculum. The formation of sporangia during the night was observed under field conditions. The temperature and relative humidities which determine sporangia formation were studied more precisely in the laboratory. The study was done from October 1964 to August 1965, at "La Lola" Farm, in the eastern coastal plains of Costa Rica with a warm and humid climate.
REVIEW OF THE LITERATURE

The Black Pod rot disease (also known as Brown Pod rot) of cacao is one of the most important in the cacao growing areas of the world. It attacks pods, flowers, leaves, twigs, cushions and trunks.

Rorer (6) states that in Trinidad rotting of cacao pods from the stem end was a sure sign of cushion cankers in the tree. Dade (2) affirms that the fungus can survive the dry "harmattan" wind season (December to January) in the Gold Coast (Ghana) in cushions, and estimates that 17 of the total of 40 percent pod infection was due to infected cushions. Ciferri (1) in Venezuela found that basal pod infection was greater on the purple and foreign Venezuelan cacao than on the native white. Thorold (9) affirms that in Nigeria the occurrence of pod infection from diseased cushions is less than one percent. In West Africa, Wharton (10) reported in 1954 that 19 percent of the cushions were diseased, and indicated that the spread of infection could take place from cushions to pods or vice-versa. He reported in 1958 (11) that the infection of pods from cushions was 3 to 4.3 percent of total pods, or 9 to 13 percent of the diseased pods or 13 to 18 percent of all proximal infections.

The first hint of the importance of chlamydospores in infected pods was made by Rorer (6) who stated that, "in addition to the spores on the surface of the pods, the resting chlamydospores, which are formed within the rotted tissue, also aid in the distribution of the fungus and tide it over long periods of drought". Wright (13) found that the fungus was viable in husks for six months. Thorold (9) proved that the fungus could remain in old piles of husks for two years.
In 1953 Orellana (5) reported that the records of pod rot incidence at "La Lola" indicate that infections reach their annual maxima during or after the biannual flushings. He stated that young succulent leaves, formed when the trees are in active flushing, are more susceptible to *P. palmivora* infection than mature leaves. Thorold (9) inoculated young chupons with a sporangial suspension and brown lesions developed on the stems and leaves, five days after inoculation. Later a sparse production of sporangia took place. Thorold declared that no evidence of natural infection of leaves and radial branches had been observed in Nigeria. But in 1960 Wharton and Turner (11), in Ghana, reported *P. palmivora* infection on branches and leaves, with free sporulation.
MATERIAL, METHODS AND RESULTS

I. Survival of the fungus in cushions of cacao trees

1. The fungus in cushions

At "La Lola" Farm, with a warm and humid climate (mean temperature of 25.3° C, with a mean daily range of 9.3° C, and a mean annual rainfall of 5028 mm or 125.7 inches) (7), pod infection from cushions took place naturally in many instances observed. To prove that these cushions were diseased, part of them were brought to the laboratory, and planted in healthy cacao pods. The resulting rotted tissue from these cacao pods was plated out on agar plus 5 percent pulp juice of ripe pods and the growing fungus transferred to test tubes. The remaining infected tissue in the trees was examined for sporangia production for a period of four days. Bits of tissue were dissected out and pieces were cut with a scalpel for microscopic examination. The little pieces of material were mounted in a drop of water on a slide under a cover glass.

Under the microscope the mounted cuts showed mycelium and chlamydo-sporas. But the remaining tissue did not produce asexual spores, under field conditions. The fungus thus isolated from cushions was P. palmivora. The trees showing this type of infection were from clones UF 221, 650, 667 and the common cacao "Matina". The dissection of diseased cushions showed that the infected tissue was more or less confined either to the vascular tissue in the trunk, which extends into the peduncle, or to cushion tissue or to the peduncle. In the pods the advancing margin of the diseased tissue forms a straight line across the long axis of the pod. This line is thus different from that surrounding infected areas which arise in any other way.
2. **Infection of cushions to verify the survival of the fungus**

In order to see how many cushions get infected through the peduncle of diseased pods, 200 hanging pods were inoculated with an aqueous suspension of zoospores and zoosporangia of *P. palmivora*. The cushions were on 50 year old "Matina" trees and on 10 year old UF 221, 613 and 667 trees. The point of infection was located approximately at the middle of each pod. The cushions eventually became infected by fungus growth from the outside of the peduncle. The trees were not sprayed with fungicides after inoculation.

When the fungus apparently reached the cushions a collection of pieces of each cushion was begun using the following technique. A piece of each cushion, approximately 0.5 cm in length, was removed with a sterile knife, then immersed in alcohol, and flamed. Then each piece was transferred to sterile water in a test tube. On bringing to the laboratory, each piece was later used to inoculate a healthy cacao pod. The healthy cacao pods were collected in the field from clones UF 221 and 667, and transported to the laboratory in polyethylene bags, where they were kept in quarantine for three days. Those which began to show rotting were discarded. The remainder, free from *P. palmivora* were washed with soap and water, followed by one percent sodium hypochlorite solution, and again with water. After one week, the pods inoculated with those bits of cushion tissue were examined for discoloration and sporangia formation of *P. palmivora*.

Two collections of pieces of the same cushions were made, one during the 2nd and 3rd month after inoculation and another nine months after inoculation. Flowers were observed on those cushions.
The progress of the disease was variable from the 200 pod inoculations described above. The pods themselves rotted from 10 to 20 days on the trees. From observation of the discolored vascular elements of several peduncles, it appeared that the fungus grew into the cushions in from 30 to 45 days. In some cases it did not reach the cushions at all. Of the first collection of 200 pieces of presumably diseased tissue, 16 of them were taken from peduncles. The fungus had not reached the cushions, and pieces of 14 of the peduncle tissue were not capable of infecting healthy cacao pods, presumably because the fungus was dead. In 177 cases, from the discoloration of tissue the fungus evidently had reached the cushions and, in 78 cases the fungus was alive as shown by the ability of cushion tissue to infect healthy cacao pods. The other 99 were negative in their tests. Of the 7 remaining, of the entire 200, the fungus reached the trunk in approximately two months. Seven pieces of the trunk tissue were tested and 4 of these infected healthy cacao pods.

In the second collection of pieces taken from the same cushions nine months after inoculation, only 106 were tested. The others unfortunately were lost during pruning operations. This second collection included 8 pieces from cushions where the fungus, by time of the first test, had not gone beyond the peduncle and in 7 of them had been considered dead. The one having contained living fungus still did. But interestingly enough 5 of the 7 now gave positive infections on pods, indicating possible natural reinfection during the 6 months interval between sampling. There is also the possibility that the fungus did not survive the alcohol flaming process. In 96 of the collections of cushion tissue, made
from 30 that had proved positive and 66 negative in the first test, 11 of the 30 were still able to infect pods on inoculation and interestingly enough, 14 of the 66 were now able to give positive pod infections. So 9 months after inoculation of pods on trees the fungus was re-isolated from 32 out of 106 cushions left by the pruner. Of these 32, in 13 the fungus remained alive from the time it infected the cushions. These cushion sites were distributed throughout the trees. In general the cushions produced flowers although no counts were made of the relative numbers produced by infected and disease-free cushions.

II. Survival of the fungus in old infected pods on trees

About 200 pods were inoculated with an aqueous suspension of zoospores and zoosporangia of *P. palmivora*. They were labeled and remained in the field hanging on the trees. Each month 20 of those pods were collected, brought to the laboratory and dried for three days. Half of each pod was cut in little pieces and macerated. The other half of each pod was held in the laboratory. The macerated portions were put in polyethylene bags with sterile water, to which were added healthy pods previously held, washed and disinfected as described earlier. The controls consisted of old pods naturally rotted in the laboratory but without *P. palmivora*. They received the same treatment as the Phytophthora rotted pods. After 10 days the bags were opened and the pods examined for the presence of *P. palmivora* spores. Samples of the macerated tissue were examined microscopically for chlamydospores and their germination.
According to the test method employed in this experiment, the fungus survived in old infected pods held in the field on the trees for seven months. The structure which remained inside the pods were chlamydo-spores. (Figure 1). These were observed to germinate by germ tubes and sporangia. Generally the two types of germination took place from the same spore. (Figure 2).

III. Phytophthora palmivora on cacao leaves

1. Phytophthora palmivora in diseased leaves in the field

Diseased leaves collected in the field in batches of ten were brought to the laboratory during November and December where each leaf was washed with soap and water, followed by one percent sodium hypochlorite solution, and again washed with water. Each leaf was placed in a polyethylene bag with water and a healthy cacao pod, previously washed and surface disinfected as previously described. Another group of leaves apparently healthy, were washed like the diseased ones and served as control. One week later the pods were examined to see if infection had taken place as evidenced by the production of P. palmivora spores on their surface.

These collections of diseased leaves from the field demonstrated that P. palmivora was sometimes present in them in viable condition. Of 80 diseased leaves, 28 of them infected healthy cacao pods, on which spores of P. palmivora soon developed, while checks remained healthy.

2. Demonstration of sources of inoculum in the upper parts of trees

a. One hundred eighty pods, on living trees, were enveloped in polyethylene bags. The bags were fixed by nylon fish line knotted at
the peduncle of the pods in such a manner as to permit the entrance of rain water. Unbagged pods which became infected were removed from the trees every three days to eliminate such sources of inoculum. Thirty days after, the enclosed pods were examined for *P. palmivora*. During the test period (November to December) the trees were not sprayed with fungicides.

This test demonstrated that in the canopy of trees there is a source of inoculum for cacao pods, because 42 out of the 180 enveloped pods became rotted due to *P. palmivora*.

b. Every 14 days, on 8 year old trees of UF 677, *Phytophthora* rotted pods were counted at two heights: one below 1.2 m and one above this height. This was done in June and July. The trees were sprayed with fungicide at monthly intervals.

The data presented in Table 1, shows that of the total rotten pods, one third were found on the upper trunk and in the canopy of the trees.

3. *Phytophthora palmivora* sporulation on leaves

To demonstrate sporulation of *P. palmivora* on leaves, a number of "Matina" cacao seedlings growing in a wooden box were inoculated with a suspension of zoospores and zoosporangia of the fungus. After the appearance of the first symptoms of infection on the leaves, they were sprayed with water to wash the old sporangia off. The box walls and the soil surface in the box were covered with plastic, and the box was placed in a compartment of a plant propagator, where water was continuously dropping and a hygrothermograph showed the humidity to be 100 percent.

After one week, when the leaf lesion had grown and presumably the remainder of the sporangia from the infecting suspension had been removed
or were dead, regular sampling for P. palmivora sporangia from the leaves was performed at 8 hour intervals. Each time 5 samples were taken at random. All 25 samples were taken by sucking up with a Pasteur's pipette a thin film of water on the infected leaf spots. Petri dishes were poured with a solid agar-water containing pimaricin and Na penicilin according to Eckert and Tsao (3). The outside of the dishes was marked with circles of 1 cm diameter. Those marks indicated the site where a drop of the water from each leaf was placed. The plates were then brought to the laboratory and observed under a microscope. To further prove that the spores collected were of P. palmivora, three days later small pieces of agar were removed from the encircled points and transferred to healthy cacao pods. Those pods were then placed in plastic bags with sterile water and held at room temperature for 5 days, after which time they were examined for Black Pod rot and P. palmivora sporulation. One month later, another sampling was performed from the same seedlings. Slides covered with solid water-agar with pimaricin and Na penicilin were used for microscope observation. From each sample, one part was placed on a slide and another on a Petri dish. The other steps were similar to those already described. In both tests, the same number of water droplets collected from non-infected leaves served as control.

In the first sampling, 9 out of 25 droplets as seen under the microscope contained sporangia of Phytophthora. When plated on agar and then inoculated into healthy cacao pods, 4 of them caused positive infection. In the second sampling 12 out of 80 samples contained sporangia of which 4 infected healthy cacao pods on inoculation. No sporangia were found in the control samples and no infection of healthy cacao pods resulted when drops were tested for viable spores.
4. **Sampling of Phytophthora palmivora** diseased leaves under field conditions to detect asexual spores

Typical *P. palmivora* diseased leaves from trees in the field were brought to the laboratory, where a small drop of water was placed on each leaf spot or part of it. The water was then drawn up by suction with a Pasteur's pipette and dropped on a slide for microscope observation. In the course of five days, during the month of April, 10 leaves were collected from which a total of 72 samples were examined, 62 from infected parts and 10 from healthy parts.

From the 62 samples from infected leaves, 44 showed presence of sporangia of *P. palmivora*. On the remaining 18 no sporangia were observed. None was found in the check samples. In the 44 samples referred to above a total of 742 sporangia were observed. Measurement of 512 of them showed that they varied between 18 to 41.5 by 21 to 59.9 micra.

5. **Time required for sporangial formation on leaves under field conditions**

During eight nights sporangial formation was observed on leaves previously inoculated. About 60 leaves were infected, and observations began at 11 p.m. and followed at one hour intervals. Five samples were taken each hour in different spots as described earlier. The spots anteriorly observed were not sampled again.

It became evident that the fungus began to produce spores at 1 a.m. Fruiting structures large enough to be measured (7 to 11 by 7 to 16 micra) formed by 2 o'clock. During the following hours the size increased and by 6 a.m. the sporangia varied between 7 to 39 by 10 to 53 micra. The largest ones looked mature. The conditions of temperature and the
relative humidity, at the cacao plantation during the nights of the experiment (from 6 p.m. to 6 a.m.) varied from 19° to 25° C in temperature, and from 95 to 100 percent relative humidity. No rainfall occurred.

6. *Phytophthora palmivora* sporulation on leaves at different temperatures

Diseased leaves were collected in the field. Round discs approximately 9 cm in diameter were cut from them. These were washed and the spores removed by rubbing them with a piece of cotton. Then the discs were placed on absorbent paper for three hours in order to remove the excess of water on the discs surface. Petri dishes were provided with water absorbent paper to act as moist chambers. To the moist chambers were added drops of water and one leaf disc upper side down. They were incubated at the following temperatures: 15°, 20°, 21°, 22°, 23°, 24°, 25° and 30° C in constant temperature electric incubators. Twenty four hours later, the surface of each leaf disc was examined for *P. palmivora* sporangia using a stereoscopic microscope at a magnification of 120 x. The field covered approximately 2 square millimeters of the material. Three observations were made on each disc and the sporangia counted on 24 leaf discs.

The data are presented in Table 2. It is evident that the optimum temperature for sporulation was 22° C. (significant at P of 0.05). However, good sporulation also took place at 23°, 21°, 24° and 20° C. It fell off at 25°, 15° and 30° C. A graph is presented in figure 3.
7. Phytophthora palmivora sporulation on cacao leaves at different relative humidities

Six-inch Fruhling and Schulz desiccators were prepared to maintain 90, 95 and 100 percent relative humidities by using 100 ml of different sodium hidroxide solutions, according to Lange (3). Discs from diseased leaves were prepared as previously described and placed in the desiccators on a support made of plastic screen. The desiccators were closed and placed at 22° C in constant temperature electric incubators. Twenty four hours later the 20 leaf pieces were examined as in the preceding test and the data tabulated. (Table 3).

The data show that in a 24 hour period, a reduction from 100 percent humidity to 95 percent decreased sporulation by one half; a further reduction to 90 percent RH decreased sporulation 6 times, thus indicating how dependent upon high humidity this fungus is for a rapid reproduction. (See figure 4)
TABLE 1. Incidence of Black Pod rot infection at two heights in the trees. Data taken at "La Lola" Farm.

<table>
<thead>
<tr>
<th>Replications</th>
<th>Number of pods examined</th>
<th>Number of infected pods</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Below 1.2 m</td>
<td>Above 1.2 m</td>
</tr>
<tr>
<td>I</td>
<td>106</td>
<td>31</td>
<td>16</td>
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<td>38</td>
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<tr>
<td>II</td>
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<td>III</td>
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<td>Percent</td>
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TABLE 2. Effect of temperature on sporangia production of *Phytophthora palmivora* on 24 cacao leaves. Number of sporangia per 2 mm² leaf area of the lower surface, after 24 hours incubation, mean of three observations for each sample.

<table>
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<th>Temperature</th>
<th>15°C</th>
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Total 176 322 713 1,228 861 683 214 58

Means 7.33 13.41 29.70 51.16 38.87 28.45 8.91 2.41
TABLE 3. Sporangia production of *Phytophthora palmivora* on 20 cacao leaves at three different relative humidities. Number of sporangia per 2 mm² leaf area of the lower surface, after 24 hours incubation. Mean of three observations for each sample.

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<td>Total</td>
<td>129</td>
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Fig. 1. *Phytophthora palmivora* chlamydospores in cacao pod tissue.

Drawing from photomicrographies.

Fig. 2. *Phytophthora palmivora* chlamydospores germinating by germ tubes and sporangia

Drawing from photomicrographies.
Fig. 3. Graphic representation of *Phytophthora palmivora*
sporulation at different temperatures on cacao leaves

Fig. 4. Graphic representation of *Phytophthora palmivora*
sporulation at different humidities on cacao leaves
DISCUSSION AND CONCLUSIONS

It has been demonstrated, at "La Lola" Farm, that cacao pods may become infected with Black Pod from diseased flower cushions by fungus growth through the peduncle. This would be of importance if it can be proved that such infected cushions fail to bear fruit, or that the diseased cushions themselves bear P. palmivora sporangia, or that the fruits arising from them become infected through the peduncle and so act as sources of inoculum.

Artificial infection of cushions for detection of fungus survival in them resulted in great variation in this respect. It was shown that the fungus can survive in cushions and trunk for at least 8 months. The cushions can be infected by fungus growth from the pods or by natural infection of the remaining parts of cut peduncles. Thereafter, the fungus can live as mycelium and chlamydospores in cushions without direct influence on flowering, except in those cases where the fungus destroys the cushions. Pods becoming infected from diseased cushions in the field are sources of inoculum. The formation of sporangia in cut cushions did not occur, but the fungus may destroy the cushions and be of importance in lowering production in older trees.

The fungus remained for seven months in the field in old infected pods hanging on trees, as was demonstrated by monthly tests. This is, therefore, another source of inoculum in plantations where old diseased pods are left on the trees.

Observations by the writer show that the above part of trees appear to have considerable importance in Cacao Black Pod rot incidence. He was impelled to verify the origin of this phase of the disease. First
it was conclusively proved that \textit{P. palmivora} was present in leaves in the field. It was shown that in the canopy of trees there is a source of inoculum for Black Pod rot infection under field conditions at "La Lola", and rain water is the carrier of the inoculum. Following this, it became in order to determine the structures which were involved in this phenomenon. One possibility would be sporangia formed on leaves and their dissemination by rain water; another might be sporulation on cankers; and a third might be insects as carriers of sporangia from infected pods. The third as far as we know, has never been proved and it remains a subject of speculation. The second did not occur. But the first possibility was proved at "La Lola" under field conditions. It was supported by examination of diseased leaves from the field, by observation of sporangia formation during the nights, and by laboratory tests indicating fungus requirements for sporangia production on leaves with respect to temperature and relative humidity. Moreover, it was found that an average of 24 sporangia per square millimeter of leaf could develop in 24 hours, under optimum conditions of temperature and relative humidity in laboratory tests. The work reported here indicates that the sporangia formed on leaves play an important role as source of inoculum for Black Pod rot of cacao, and rain water is the carrier of infecting sporangia.

Certain aspects need to be studied to clarify the role played by infected cushions, such as the following:

1. The number of cushions which are infected on a young and on an old tree;

2. The effect of diseased cushions on flower production;
3. The effect of dry, warm or cool weather on the activity and survival of the fungus in cushions;

4. The possibility of a therapeutic or systemic agent to be employed with success to eliminate cushion cankers that are infected with *P. palmivora*.

With reference to fungus survival in old infected pods it will be wise to find out:

1. The effect on disease control of a well carried out program of diseased pod removal, or sanitation program.

Concerning the asexual reproduction of *P. palmivora* on leaves as sources of inoculum it is necessary to determine:

1. Their abundance throughout the year;

2. Whether young succulent leaves can produce more sporangia than older ones;

3. The possibility of insect dispersal of the spores;

4. Rain dispersal of the fungus versus aerial dispersal as proved to occur by Thorold (8) in Nigeria.

The finding of so many sporangia on leaves in the upper canopy seems to point to the wisdom of spraying to keep leaves and pods covered with a protection covering during all rainy periods.
SUMMARY

The survival of *Phytophthora palmivora* in flower cushions in old infected pods hanging on the trees, the asexual reproduction of the fungus on leaves, its importance to pod infection together with the environmental factors affecting sporangia formation, were studied at "La Lola" Farm, Costa Rica.

It was found that cacao pods can become infected from diseased cushions. The cushions were shown to become infected by fungus growth from inoculated pods or from cut peduncles. The fungus was found to remain in cushions as mycelium and chlamydospores.

The fungus in old infected pods hanging on trees was viable for seven months.

Sporangia formation on leaves was demonstrated. Their formation during the nights were observed at hourly intervals. Their importance as source of inoculum was proved. The environmental requirements of temperature and relative humidities for sporangia development on leaves were determined, with optimum of 22°C and 100 percent respectively. An average of 24 sporangia per square millimeter of the lower surface of infected leaves for a period of 24 hours was calculated.
RESUMEN

Fueron estudiados la sobrevivencia de Phytophthora palmivora en cojines florales en mazorcas infectadas viejas, la reproducción asexual del hongo, su importancia en la incidencia del Podredumbre Negro del cacao, y las condiciones ambientales que influyen en su formación, en La Finca La Lola, Costa Rica.

Se ha demostrado la ocurrencia de la infección de frutos de cacao originados de los cojines florales. Los cojines pueden ser infectados por crecimiento del hongo desde el fruto o por infección de pedúnculos cortados, y que el hongo puede permanecer en los cojines en forma de micelio o clamidosporas.

Las clamidosporas pueden sobrevivir en frutos infectados viejos colgados en los arboles por siete meses.

La formación de esporángios en hojas fue demostrada. Su formación durante la noche fue observada. Su importancia como fuente de inóculo para el Podredumbre Negro de los frutos de cacao fue evidenciada. Las condiciones de temperaturas y humedades relativas que afectan su producción fueron determinadas, con el óptimo de 22°C y 100 porciento, respectivamente. Un promedio de 24 esporángios por milímetro cuadrado de área foliar del envez fue calculado para un período de 24 horas.
BIBLIOGRAPHY


