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ISOLATION OF PHYTOPLASMA DNA FROM THE COCONUT PALMS (*Cocos nucifera* L.) COLLECTED FROM GHANA

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ABSTRACT

This study aimed to verify the presence of the causative agent of Lethal Yellowing which is phytoplasma in samples provided from infected coconut trees. Study was carried out by using various samples like zygotic embryo, young leaves and immature & mature inflorescences. These materials were collected from trees at the stage 1 and 2 of the disease development.. Stage 1 of disease development is characterized by leaf yellowing and the start of the falling nuts while at the stage 2 of disease development, the trees has not bear nuts longer. From infected material, DNA was extracted by three different processes and isolated DNA was amplified by PCR. 16S rRNA gene was amplified by two specific primers of phytoplama viz P1/P2 and Ghana 813/AKSR. Among the various tested materials presence of phytoplasma was reported from the mature inflorescences while the presence of the phytoplasma was not reported from the leaves and embryos of the coconut.

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1 Introduction

Coconut is a perennial crop for which genetic resource conservation is mainly based on field collections because of its seed size (one of the largest of the plant kingdom) and physiology (no dormancy and recalcitrant to storage) (Assy Bah et al., 1987). Though field conservation of plant genetic resources is possible but during this type of storage, plams faced various problems caused by pests, natural calamities and diseases (Withers & Engels 1990). Among various coconut diseases, the most dreadful disease is the Lethal Yellowing Type Syndromes (LYTS).

Lethal Yellowing is caused by a phytoplasma, formerly known as mycoplasma-like organisms (MLOs) with small genomes localized in the phloem (Beakbane et al., 1972; Dollet & Giannotti, 1976; Firrao et al., 2007; Bertaccini & Duduk, 2010). Phytoplasmas are transmitted to plants during feeding activity by their vectors, primarily leafhoppers, planthoppers, and psyllids (Weintraub and Beanland, 2006). The disease is known by different names in various countries like in Ghana it is called Cape Saint Paul Wilt Disease (CSPW) while in Togo, it is known by Kaïncopé disease, similarly in Nigeria it is Awka and in Cameroon it is known by the name of Kribi disease.

Lethal Yellowing led to the loss of many hundreds or thousands hectares of coconut palms worldwide, it is causing rapid death of trees and the majority of the available accessions are susceptible (Oropeza & Zizumbo, 1997). This disease is now the gateway to the world's largest collection of coconut localized in Côte d'Ivoire (Konan et al., 2013).

It is believed that embryos is containing a fully differentiated vascular system, and can be sources of Lethal Yellowing propagation or transmission (Harrison et al., 1995; Cordova et al., 2003). The exchange of coconut germplasm, usually done by embryos, becomes difficult, especially when embryos provided from areas where the disease occurs (Jones et al., 1999). The objective of this work is to detect the presence of phytoplasma from the embryo and surrounding materials. This work helps in developing the understanding of phytoplasma dissemination and identification of Lethal Yellowing (Dollet et al., 2009) threatens in global coconut collections.

2 Materials and Methods

2.1 Plant material

The plant material consisted of young leaves, immature (row 1 to 4) and mature inflorescences (row 5 to 10) were collected from infected plants at the first and the second stage of disease development. Furthermore mature embryos (10 to 11 months) were collected at the first stage of disease development. The samples of west African Tall (WAT) accession were supplied by Research Station of Sekondi- Takoradi in Ghana in the form of endosperm cylinders containing zygotic embryos, young leaves and inflorescences. The row of inflorescences is defined from the youngest inflorescence which is still in the husk and represent as e row 0. The material has identified and marked tree by tree for all the sampling trees. For each tree, a total of 5 to 7 embryos were collected. After receipt, the leaves and inflorescence were frozen in liquid nitrogen at -196°C for 5 to 20 minutes before being stored at -80°C. For embryos, the samples were supplied in the form of endosperms cylinders containing embryos. After receipt, the embryos are isolated and stored without disinfection at the same way like the leaves. DNA from these samples, were extracted by several methods.

2.2 DNA extraction

DNA which used as a template in the polymerase chain reaction (PCR) was extracted from fresh sample tissues and purified as process described by Doyle & Doyle, 1990, Daire et al., 1997 and process proposed by Qiagen Kit (Dneasy® plant mini kit 2000).

2.3 PCR Amplification

Two pairs of oligonucleotides viz phytoplasma universal primer pairs P1 / P7 (Deng & Hiruki, 1991; Smart et al., 1996.) and primer pairs Ghana 813 / Awka SR (AKSR) which is specific to the phytoplasma responsible of Lethal Yellowing disease in Ghana and Nigeria (West Africa) were used for the amplification of ribosomal RNA gene (rRNA) by direct PCR. The gene 16S rRNA of Aster Yellow were also used as a control because these genes show strong similarities with the sequence 16S rRNA of phytoplasma (Harrison et al., 1992 Harrison et al., 1994a). *Acheloplasma* which is a mollicute (Tymon et al., 1998) was also used as an out group.

PCR was carried out in 50 µl volume reaction mixture. The reaction mixture contained for each PCR 1µl DNA from each sample, 5µl of 10 X PCR buffer, 2µl of MgCl2 (5 mM), 1µl of dNTP (8mm), 1µl of primer "forward" (sense), 1µl of primer "reverse" (antisense), 0.25µl of Taq DNA polymerase, 0.25µl of Qiagen Kit and 38,75µl of sterilized distilled water. The PCR started with the first denaturing of DNA which was performed at 94°C for 90 seconds, a denaturing at 94ºC for 30 seconds, a primer annealing performed on the matrix at temperature lower than the reverse primer for 50 seconds. Elongation occurs at 72 ° C for 30 seconds. The final extension was performed at 72 ° C during 10 minutes, PCR is launched for 35 cycles. The amplified material is stored at 4 ° C for during several minutes to several hours or overnight.

The sequences of the corresponding primers are:

P1: AAGAGTTTGATCCTGGCTCAGGATT specific gene 16S RNA 5 'end, TM = 56
P7: CGTCCTTCATCGGCTCTT specific RNA 16S 3' end, TM = 56
Ghana 813: CTAAGTGTCGGGGGTTTCC specific gene 16S RNA 5 'end, TM = 60

AKSR: TTGAATAAGAGGAATGTGG specific to the sequence of the spacer 16-23S, TM = 52

2.4 Electrophoresis

The PCR products were fractionated on 1% agarose gel by using 1X TAE buffer containing 5 µg/ml ethidium bromide. Electrophoresis is carried out with agarose gel (Seaken LP) 1%. The migration is initiated between 100 or 125 volts for 45 mn to 1 hour. PCR products (850 and 1650 kb) of phytoplasma 16S rDNA sequence, amplified with the two primers pairs P1/P2 and Ghana 813/ASKR were visualized on agarose.

3 Results

Result of the study revealed the presence or absence of phytoplasma associated with Lethal Yellowing disease by the PCR amplification of 16S rRNA gene in the embryos, leaves and inflorescences (mature and immature).

3.1 16S rRNA gene of Phytoplasma from the leaves

Leaves harvested from infected trees at early stage of diseased development (stage 1) have not showing any amplification of the corresponding 16S rRNA gene of the phytoplasma by using the two pairs of primers P1/ P7 and Ghana G813 / AKSR.

3.2 16S rRNA gene of Phytoplasma from the inflorescence

In case of inflorescences DNA amplification, desired segments were reported in the older inflorescence with stage 2 of disease development. As shown in Figure 1 and 2, lane 3 the presence of phytoplasma DNA and it is not depending on the process of DNA extraction and primers used. While the DNA extracted from young inflorescence (row 5, 6, 7 and 8) was not showing the amplification. Two bands of 1650 kb and 850 kb were obtained respectively form primers pairs P1/P2 and Ghana G813/ASKR with two processes of DNA extraction (Qiagen method and the Daire et al. 1997). Figures 1 and 2 show the results of three PCR performed by using DNA extracted from inflorescences collected at stage 2 of 2 trees identified L12-10 and L19-4 with the two pairs primers P1/ P7 and G813 / AKSR.

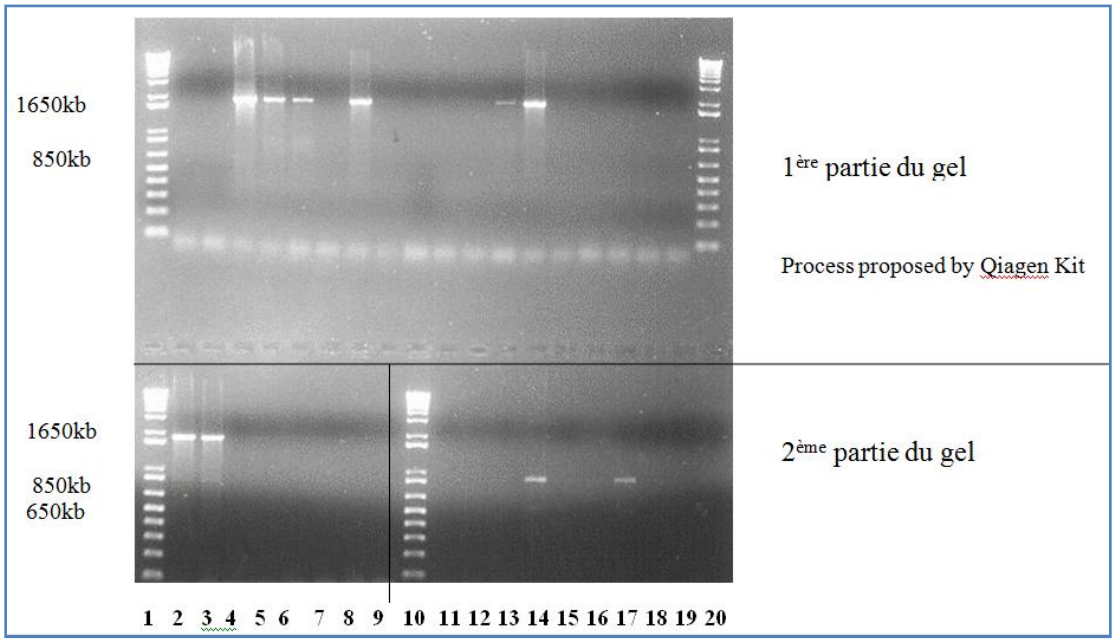

Figure 1 First part: PCR results of DNA of inflorescences taken from two infected trees (L12-10 and L19-4) at the second stage of disease.

[The pair primers P1/P7 was used in the first part and in the second part to lane 1 to lane 10. **First part of the gel**: DNA extraction by process proposed by QIAGEN. 1: 1kb marker size. 2: water. 3: Aster yellow. 4: *Acholeplasma*. 5: phytosplame Mozambique. 6: Inflorescence at stage two of disease. 7: inflorescence taken from healthy plant. 8: inflorescence rank 9 of tree L12-10. 9, 10, 11, 12, inflorescences rank 8, 7, 6 and 5 of tree L12-10; 13 inflorescences rank 4 of tree L12-10. 14: inflorescence rank 10 of tree L19-4. 15, 16, 17, 18, 19 : inflorescences rank 8, 7, 6, 5 and 4 of tree L19-4. 20: size marker and **Second part of the gel**: DNA extraction by Daire et al (1997) process. 1:1kb marker size. 2, 3: inflorescences of rank 10 and 9 of tree 10 L-12-10. 4, 5, 6, 7, 8, 9: inflorescences rank 7 5 L12-10, 8, 6, 4 and 5 of L19-4.]

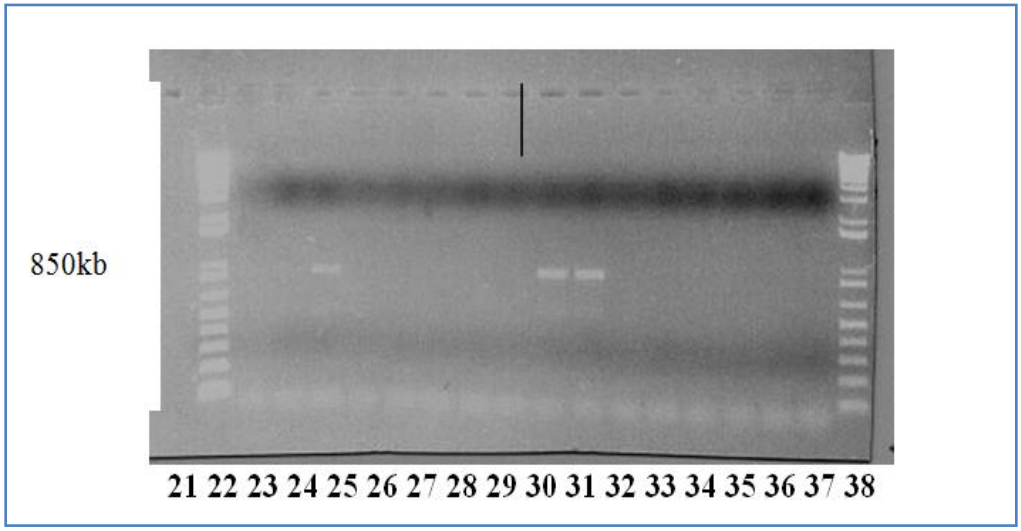

Figure 2 Second Part of PCR results of DNA from inflorescences collected from two infected trees at the second stage of disease. (L12-10 and L19-4).

[The primers used in the second part of the 10 to 20 and in Figure 2 are primers G813/AKSR. **DNA extraction by process proposed by QIAGEN Kit.** 21: 1KB size marker; 22, 23 inflorescences rank 5 and 4 of tree L12-10. 24: inflorescence rank 10 of tree L19-4. 25, 26, 27, 28 and 29: inflorescence rank 8, 7, 6, 5 and 4 of tree L19-4. **DNA extraction by Daire *et al.*** (1997) process. 30, 31: inflorescence rank 9 and 10 of tree L12-10. 32 and 33: inflorescence rank 7 and 5 of tree L12-10. 34, 35, 36 and 37 inflorescences rank 8, 6, 5 and 4 of tree L19-4. 38: 1kb size marker. **Second part of the gel: DNA extraction by process proposed by QIAGEN**. 10: size marker. 11: water. 12: *Acholeplasma*. 13: Aster yellow. 14: Inflorescence rank 9 at the second stage of disease. 15: Q3; 16: inflorescence row 9 L12-10. 17: inflorescence rank 8 of tree L12-10; 18, 19, 20 inflorescence rank 8, 7 and 6 of tree L12-10.]

3.3 16S rRNA gene of Phytoplasma from the embryos

For embryos, which were taken from early diseased trees (stage 1), not reported any band of the desired fragment after amplified by PCR.

Discussions

For screening the presence of phytoplasma which caused coconut Lethal Yellowing disease in coconut, amplification of 16S rRNA (ribosomal operon) was done with the help of PCR. Among the various screen plant parts and their DNA the desired fragment corresponding to the gene 16S rRNA of the phytoplasma was amplified in none of the young inflorescences, leaves and embryos collected from trees at early disease development (stage 1). The presence of the desired segments was reported from the older inflorescence only.

For the embryo, various studies have focused on the amplification of the gene 16S rRNA of Lethal Yellowing phytoplasma. Only the work of Harrison et al. (1995) and Cordova et al. (2003) showed the possible presence of phytoplasma in the embryo. Recently, work of Nipah et al. (2007) was also reported the presence of phytoplasma from the zygotic embryos while they were working on West African Tall (GOA). Cordova et al. (2003) conducted the amplification of gene 16S rRNA of the phytoplasma in the embryo by PCR while works of Harrison et al. (1995) has been performed with 40 cycles of PCR on samples collected from Florida that require other primers as those specific to the West African phytoplasma. Nipah et al. (2007) used the Nested PCR.

All the above said researches have contradictory opinion than the present study. These researchers reported the presence of phytoplasma DNA from the coconut embryo during the PCR amplification which is not reported during present study. According to above said authors, pytoplasma DNA is available in embryo at very low concentration, so for the detection of phytoplasma gene from embryo large number of embryo should used during PCR amplification. Harrison et al (1995) already suggested that several ultracentrifugation cycles can be helpful for amplification of 16S rRNA gene of phytoplasma. The use of ultracentrifugation is difficult to implement with the embryo because this process requires many quantities of samples, which is not be possible with embryos.

This work carried out with several samples taken from various plant tissues (leaves, inflorescences, embryos) helped highlight the unequal distribution of the phytoplasma in the plant (Harrison et al., 1992; Harrison et al., 1995; Escamilla et al., 1995). The unequal distribution would be a handicap for the detection of the phytoplasma in all tissues. Considering all the samples tested, it appears that for the same stage of the disease,

the desired fragment corresponding to the 16S gene was amplified or not depending on the sample, it's age and of the tree from which the sample has been collected. Similar types of trends was reported during this study and amplification of the desired fragment will vary depending on the development stage of the disease for the same tissue. This could justify the none amplification of the gene of phytoplasma in embryos of the samples used in this study and detection of phytopplame by Nipah et al. (2007) in embryos of the GOA. No detection of phyoplasma in embryos could be due to a unequal distribution of phytoplasma in the various parts of the plant (Harrison et al., 1992; Harrison et al., 1994b, Harrison et al., 1995, Tymon et al., 1998). None amplification of the gene 16S rRNA of the phytoplasma cannot be attributed to a faulty method because all the samples have been submitted to the same process and amplification of the gene has been done with matures inflorescences. The use of samples at the second stage of disease aimed to ensure and verify the reliability of the process used in this study. The presence of phytoplasma in the embryo shown by some authors not means necessary that the embryos can be a way of Lethal Yellowing disease transmission. The nut fall before the appearance of the first symptoms showed that the presence of phytoplasma in the embryos may be at very low level. According to McCoy et al. (1983) and Cousin (2001), transmitting of phytoplasma by the embryo is in contradiction with biological principles which certify that the seeds do not transmit phytoplasma. Similar type of findings was reported by Nipah et al. (2007); Myrie et al. (2011) who's have shown that *in vitro* culture of embryos from infected plants leads to healthy plants.

Conclusion

Maturity of the tissue can make it a target tissue of phytoplasma gene amplificationn, but in addition, the accumulation of the phytoplasma can be variable from one tissue to another. The absence of PCR amplification of the corresponding gene 16S rRNA in the embryo could be due to an absence or a low concentration of the phytoplasma in the embryo. However, these studies have confirmed the unequal distribution of the phytoplasma in different parts of the plant.

The studies failed to show the presence of phytoplasma in the embryo. However given the fact that the nuts fall even before the onset of symptoms and the *in vitro* regeneration of embryos generate healthy plants, the use of the embryo for the exchange of materials can make but some precautions must be taken. The most problem is the eradication of this disease. Further studies to learn more about the phytoplasma so to eradicate it would be the best way to preserve the existing collections.

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