

Review of Coconut “Lethal Yellowing type diseases” Diversity, variability and diagnosis.

Revue sur les maladies de type « Jaunissement mortel » du cocotier. Diversité, variabilité et diagnostic

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Abstract:

Coconut palms (*Cocos nucifera* L.) can be affected by several types of Lethal Yellowing (LY) diseases worldwide. Some of the syndromes are caused by phytoplasmas, small bacteria that are impossible to detect by light microscopy. Amplification of a given gene of the phytoplasmas by Polymerase Chain Reaction (PCR) is the most convenient diagnosis method. The problem is that there are at least 28 "groups" of phytoplasmas and only one pair of primers -P1/P7- commonly used for PCR. As these primers belong to a very conserved gene, false positives are frequent. Consequently, alternative primers specific to one "strain" (or subgroup) have to be used, such as LY-F/LY-R for the Caribbean LY, Rohde primers for LD Tanzania. Such specific primers are sometimes restrictive. Indeed, there is variability within each strain and the sequence of the primers has to be adapted to that variability. There are at least 5 LY subgroups. The subgroups can only be identified by RFLP or sequencing. In Africa, two subgroups of LY phytoplasmas have been identified so far.

Keywords: Coconut, Lethal Yellowing, Cape Saint Paul Wilt, Diversity, Variability, Phytoplasma, PCR, 16S rDNA, Syndrome, Ghana, Mozambique, Tanzania.

Résumé:

De par le monde, les cocotiers (*Cocos nucifera* L.) sont affectés par divers types de jaunissements mortels (JM). Certains de ces syndromes sont causés par des phytoplasmes, petites bactéries impossibles à détecter en microscopie optique. L'amplification d'un gène donné des phytoplasmes par Polymerase Chain Reaction (PCR) est le moyen le plus commode de faire le diagnostic. Le problème, c'est qu'il y a au moins 28 « groupes » de phytoplasmes et une seule paire d'amorces -P1/P7- fréquemment utilisée pour la PCR. Comme ces séquences appartiennent à un gène très conservé, les faux positifs sont fréquents. Il faut donc également utiliser d'autres amorces spécifiques d'une « souche » (ou sous-groupe) - par exemple, LY-F/LY-R pour le JM des Caraïbes, les amorces de Rohde pour la « LD Tanzania ». De telles amorces spécifiques sont parfois restrictives. En effet il existe une variabilité au sein de chaque souche et les séquences des amorces doivent être adaptées à cette variabilité. On connaît à ce jour au moins 5 sous-groupes de JM. Ces sous-groupes sont identifiables par RFLP ou séquençage. En Afrique deux sous-groupes de phytoplasmes associés à des JM ont été identifiés.

Resumen: Revista de las enfermedades del tipo « Amarillamiento Letal » del cocotero. Diversidad, variabilidad y diagnóstico.

En todas partes, los cocoteros (*cocos nucifera* L.) son atacados por diversos tipos de amarillamientos letales (ALC). Ciertos de esos síndromes son causados por phytoplasmas, pequeñas bacterias imposible de detectar en microscopía óptica. La amplificación de un gene dado de los phytoplasmas por “Polymerase Chain Reaction (PCR) es el medio más cómodo de hacer el diagnóstico. El problema es que hay por lo menos 28 “grupos” de phytoplasmas y solo un par de iniciadores -P1/P7- a menudo utilizada para la PCR. Como estas secuencias pertenecen a un gene muy conservado, los falsos positivos son frecuentes. Entonces es preciso utilizar otros iniciadores específicos de una “cepa” (o subgrupo) – por ejemplo, LY-F/LY-R para el ALC del Caribe, los iniciadores de Rohde para la “LD Tanzania”. Tales iniciadores específicos son a veces restrictivos. En efecto una variabilidad existe dentro de cada cepa y las secuencias de los iniciadores deben de ser adaptadas a esta variabilidad. Se conocen actualmente por lo menos 5 subgrupos de ALC. Estos subgrupos son identificables por RFLP o por análisis de secuencia. En África dos cepas de phytoplasmas asociadas a los ALC fueron identificadas.

Introduction

In the 1830s, a disease resembling what is known today in the Caribbean as Coconut Lethal Yellowing (LY) was rife in the Cayman Islands (Martyn, 1945; 1949). Since then, this type of Lethal Yellowing (LY) disease has spread in the Caribbean region and central America. The same type of disease was observed on coconut palms in Tanzania, in East Africa, at the beginning of the 20th century (Morstatt, 1911; 1920), then in West Africa, in Togo and Ghana (Meiffren, 1951). The Caribbean, from Florida to Honduras, and tropical Africa, are currently the zones affected by these Lethal Yellowing type Syndromes (LYTS). Indeed, one has to refer to a syndrome — development of successive symptoms — to describe these diseases. Like many other monocots, the coconut palm only displays a limited number of symptoms in response to biotic and abiotic stresses. Seeing a coconut palm with yellow fronds during a one-off inspection does not necessarily mean Lethal Yellowing. Many mineral deficiencies cause fronds to turn yellow. Likewise, the existence of coconut stems that have lost all their fronds can have various causes. Lastly, it needs to be known that, in the Caribbean zone, two other coconut diseases, Hartrot caused by a trypanosomatid – *Phytomonas* sp.– (Parthasarathy *et al.*, 1976; Dollet, 1984) and Red Ring, caused by the nematode *Bursinaphelencus cocophilus* Cobb. (Kastelein *et al.*, 1985) can be confused with LY, even after observation of the syndrome.

In this review, we shall focus on the various LYS to which phytoplasmas (ex MLO) have been linked, either under the electron microscope, or by PCR (Polymerase Chain Reaction).

1. Symptomatology

The very first symptom in LYS involving phytoplasmas, commonly called Lethal Yellowing (LY) in most cases, is for all the nuts to fall, both ripe and unripe. The existence of nuts of all sizes and all ages at the foot of a coconut palm is the first alarm signal. It is only once the nuts have fallen that leaflets start to turn yellow at the tip of a lower frond among the oldest. The yellowing spreads towards the stem. Right from the yellowing lower frond stage, blackening can be seen – necrosis – on the rachillas of a newly opened inflorescence. If the next inflorescence is collected, still wrapped in its spathe, and opened, browning can be seen on some or all of the male flowers, which may be detached from their rachillas, and on the tips of the rachillas themselves.

Rotting of the spear (f0), then of the immediately younger fronds (f-1, f-2) occurs more or less rapidly, before the yellowing reaches the youngest fronds. The yellow fronds then turn brown, dry out, hang down the stem, and eventually fall. In the end, only the stem remains, terminating in 5 to 6 young yellow fronds that are smaller than normal. This type of "tuft" eventually snaps due to the rot gradually spreading throughout the meristem zone after the spear has shown signs of necrosis, or following a gust of wind.

It should be noted that the Hartrot syndrome in the Caribbean zone exhibits exactly the same stages. The reason probably lies in the fact that each of these diseases is caused by a micro-organism that multiplies in the sap, in the phloem sieve tubes.

2. Distribution of LY type syndromes

2.1. Caribbean – America

It is in the Caribbean — the West Indies — that Lethal Yellowing Type Syndromes were reported for the first time at the end of the 20th century (Martyn, 1945; 1949). It can be imagined that the disease spread naturally from island to island in that zone: Cayman Islands-Jamaica-Cuba-Haiti-Dominican Republic, as those islands are close to each other. It may also be that humans played a role, as short distances are conducive to trade. In 1969, LY was detected in Key Largo — northern part of the Florida Keys archipelago — then it spread step by step as far as Palm Beach county¹. However, it may be that LY reached Florida via the southern tip of the Keys (Key-West) near to Cuba as early as the 1930s (Howard and Harisson, 1999).

The west coast of Florida has remained relatively unscathed, mainly due to serious epidemiological monitoring, combined with rapid containment measures (felling, insecticide treatments, antibiotic treatments). It was then not until the very beginning of the 1980s that LY took a real leap to the tip of the Yucatan peninsula in Mexico. In this case, the hypothesis most often put forward is human introduction

¹. Northern limit of LY spread (26.76N – 80.14 W).

of infectious vectors from Florida. In fact, that period corresponds to the hotel development of the future seaside resort of Cancun. Uncontrolled imports of planting material from Florida (palms and grasses for golf course greens – grasses that are propitious to vector development- may have enabled the introduction of infectious vectors. From the Yucatan tip, the disease gradually decimated the coconut plantings as far as Vera Cruz. Southwards it spread to Belize. It was not until the beginning of the 1990s that LY was identified on the island of Roatan located off La Ceiba in Honduras, central America (Ashburner *et al.*, 1996). The disease, which rapidly passed over to the continent, spread as far as Guatemala to the West and to Colon in the East where it stopped spreading eastwards. For the moment, it has not affected Nicaragua or El Salvador. Two explanations have been put forward for this other leap by LY: either it was a similar process to that assumed for Mexico, or it was by the passive transportation of infectious vectors on the winds of a cyclone.

The latest focus, the most unexpected, occurred this time east of the original core, on the island of Nevis in 2005-2006 (Myrie *et al.*, 2006). The strain of the LY etiological agent on Nevis is very similar to that in Florida, and the hypothesis of a scenario identical to that at Cancun seems to be the most plausible. As Nevis is surrounded by numerous very nearby islands, the Caribbean Arc southeast of Nevis, from Antigua-and-Barbuda to Trinidad, is worried about the possible spread of LY from North to South.

2.2. Africa

A Lethal Yellowing type syndrome would appear to have been reported in Africa for the first time by Stein (1905) in Tanzania. However, according to Schuiling *et al.* (1992), the disease could have been present before 1900, i.e. at the same time as the first reports of LY in the Caribbean. It was then described in Mozambique (Carvalho, 1958 quoted by Santana Quadros, 1972) and Kenya (Dowson, 1921; Bock *et al.*, 1970).

In West Africa, the first LYS to be described would seem to have been in Nigeria, where it was called Awka disease (Bull, 1955). At the beginning of the 1930s, some LYS were observed simultaneously in Togo (Kaincope disease), Ghana (Cape Saint Paul Wilt) (Meiffren, 1951) and in Cameroon (Kribi disease) (Heim and Chevaugnon, 1948; Grimaldi and Monveiller, 1965). A LYS also exists in Equatorial Guinea but, curiously, Benin, which is separated from Togo by a river, has never been affected by this type of disease.

2.3. Asia

In Indonesia, lethal diseases of the coconut palm with frond yellowing exist on the island of Natuna (Natuna wilt) (Sitepu *et al.*, 1989) and in Kalimantan (Kalimantan wilt) (Sitepu *et al.*, 1988). However, although phytoplasmas have been reported to be the etiological agents of those wilts, they do not belong to the 16S rDNA group containing the phytoplasmas of coconut LYTS in Africa and the Caribbean (Group 16S rDNA IV).

3. Ecological diversity

The LYTS of the Caribbean and Africa are all similar in their symptomatology. Yet, their environments differ considerably. The insect life and flora are variable. For instance, the LY vector in Florida, *Haplaxius crudus*-ex. *Myndus*- (Howard, 1983) does not exist in Africa. In addition, *H. crudus* has never been identified in Haiti or the Dominican Republic so far. Another insect of the same family – Cixiidae – *Myndus adiopodoumeensis* – was long suspected of being the vector of Cape Saint Paul Wilt in Ghana (CSPW) (Philippe, 2007). That species does not exist in Tanzania.

As everyone can imagine, the flora on the northern coast of Zambesia is different from that on the northern coast of Jamaica or Yucatan. Consequently, the reservoirs of vectors and inoculum, be it native palms or any other plant, will probably be different. As LYTS are diseases comprising three components, host-vector-parasite, influenced by the environment, a large number of situations exist (figure 1). Even on an island or in a single country, there can be several "strains" of phytoplasmas depending on the regions, spreading at varying speeds and causing different degrees of damage. For example, in Tanzania, the impacts of the disease to the north and to the south Dar es Salaam are different (Schuiling *et al.*, 1992b.).

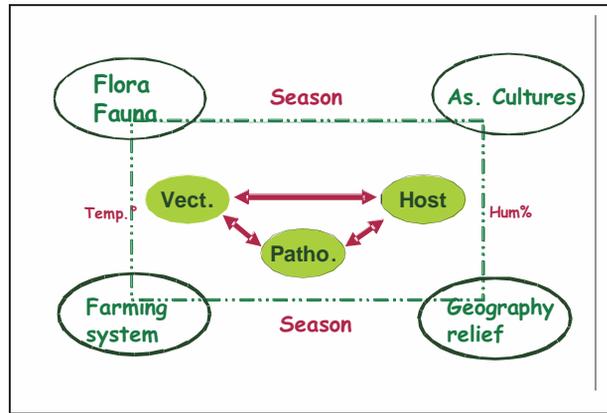


Figure 1. Specific host-vector-pathogen relations for diseases involving phloem-restricted micro-organisms transmitted by insect vectors.

4. Phytoplasma diversity

Phytoplasmas (ex. Mycoplasma-like-organisms or MLO) are specifically associated with various LYTS in the Caribbean and Africa. They were firstly discovered under the electron microscope (Plavsic Banjac *et al.*, 1972; Beakbane *et al.*, 1972; Dollet and Giannotti, 1976; Dabek *et al.*, 1976; Dollet *et al.*, 1977; Nienhaus *et al.*, 1982). As phytoplasmas cannot currently be grown *in vitro*, it is impossible to demonstrate their etiological role in LYTS based on Koch's postulates. However, the remission of some diseased palms has been obtained after treatment with tetracycline (McCoy, 1972), which supports the thesis of a phytoplasma-based aetiology. With the development of molecular biology, Polymerase-Chain-Reaction (PCR) has replaced lengthy searches under the microscope — fixing, embedding, cross-sections and examination under the microscope — (Rhode *et al.*, 1993; Harrison *et al.*, 1994).

Consequently, today, most phytoplasmosis diagnoses are carried out by PCR using DNA extracts from diseased coconut palms. The phytoplasma gene amplified by PCR is that of ribosomal RNA (rRNA). That gene is conserved and it is the one that has been most widely used to carry out all kinds of phylogeny and evolution studies (Seemüller *et al.*, 1994; Lee, 1998; Wang *et al.*, 2003; Wei *et al.*, 2007).

4.1. Ribosomal RNA

As phytoplasmas are small wall-less bacteria (mollicutes), the sequence of their rRNA is fairly similar to that of bacteria, and the rRNAs of the various phytoplasmas causing several hundred syndromes display considerable sequence homology. However, an analysis of the PCR products by RFLP (Restriction Fragment Length Polymorphism) has led to the delimitation of 15 to 28 "groups" — 16s rDNA — of phytoplasmas (Bertaccini, 2007; Wei *et al.*, 2007). Some of these different groups contain subgroups. For instance, group I would appear to contain 11 subgroups (Bertaccini, 2007).

The phytoplasmas associated with LYS all belong to group IV called Coconut Lethal Yellows Group (Tymon *et al.* 1998; Bertaccini, 2007; Wei *et al.*, 2007). That group also contains some sequences of phytoplasmas from other palms, which, whilst not always displaying a "Lethal Yellowing Type Syndrome" (in particular, not yellowing but browning, are often considered to be species "susceptible to Lethal Yellowing" (the LY disease) in the Caribbean (Howard and Harrison, 2007).

Group IV contains several subgroups (table I). By studying RFLPs, Tymon *et al.* (1997) had already shown that the phytoplasmas of coconut palms in Africa were different from those in the Caribbean. Moreover, the phytoplasmas of Tanzania or Kenya were different from those of Ghana and Nigeria, which led to a distinction being made between at least three subgroups associated with the various LYS.

It should be noted that in this group, near the phytoplasmas associated with LY in the Caribbean, there is the phytoplasma associated with the Yucatan Lethal Decline (LDY) syndrome, which stands out well from that of LYTS. A syndrome similar to that of LDY has been identified, associated with the same phytoplasma, on the Pacific coast of Mexico. It has been called Coconut Leaf Yellowing, to clearly distinguish its difference from Lethal Yellowing.

Table I. Subgroup 16s rDNA IV– Coconut Lethal Yellows group (according to Bertaccini, 2007).

Strain name	Code	Origin
Coconut Lethal Yellowing Ca.P. <i>palmae</i>	16s rIV — A	Florida
Yucatan coconut Lethal Yellowing	16s rIV — B	Mexico
Lethal disease Tanzania	16s rIV — C	Tanzania
<i>Cardulovica palmata</i> yellowing	16s rIV — D	Mexico, Texas
Walnut witches broom Ca.P. <i>casteneae</i>	16s rIV — E	Korea

Another phytoplasma, very close to that of LDY, has been identified in diseased coconut palms and *Acrocomia aculeata* (different syndrome from that of LY) in Honduras (Roca *et al.*, 2006). In addition, it has recently been shown that another subgroup exists that is associated with a coconut Lethal Yellowing disease in the Dominican Republic (Martinez *et al.*, 2007) and a new subgroup associated with a disease assimilated to LY on *Washingtonia robusta* in Florida (Harrison *et al.*, 2008). This new palm phytoplasma also exists in mixed infections with the LY phytoplasma in *Poenix dactylifera* (Harrison *et al.*, 2008).

There therefore exist different phytoplasmas associated with different palm pathologies, involving or not a yellowing stage, and generally ending up more or less rapidly with a crownless stem.

4.2. Non-ribosomal sequences

Another gene, not belonging to the ribosomal operon, is also used for PCR diagnosis of LY in Florida and the Caribbean. The PCR primers are known as LYF/LYR (Harrison *et al.*, 1994). However, those two primers do not give any amplification with certain LYTS in Cuba or the Dominican Republic (unpublished results), or with coconut wilt diseases in the centre of Honduras (Roca *et al.*, 2006). This is further proof of the diversity of the phytoplasmas found in coconut.

5. Phytoplasma variability

A comparative analysis of the sequences obtained with the primers used to amplify the ribosomal sequence (P1/P7) reveals some highly conserved regions, notably in the 16S rRNA gene, and some more variable regions which correspond, among other things, to the spacer between the 16S and the tRNA^{ile} gene on the one hand, and between the tRNA gene and the 23S gene on the other hand (figure 2).

After alignment of the sequences specific to each strain (subgroup), some specific primers for PCR were constructed, to amplify a specific LYTS. This was the case for primers G813/AKSR assumed to diagnose CSPW in Ghana (Tymon *et al.*, 1998). We have used those primers in our research programme on CSPW, and we found that we sometimes obtained negative PCRs for palms that were exhibiting typical CSPW symptoms. In order to find the origin of those false negatives, we sequenced the PCR products obtained with primers P1/P7 since primers G813/AKSR are inside the P1-P7 sequence (figure 2).

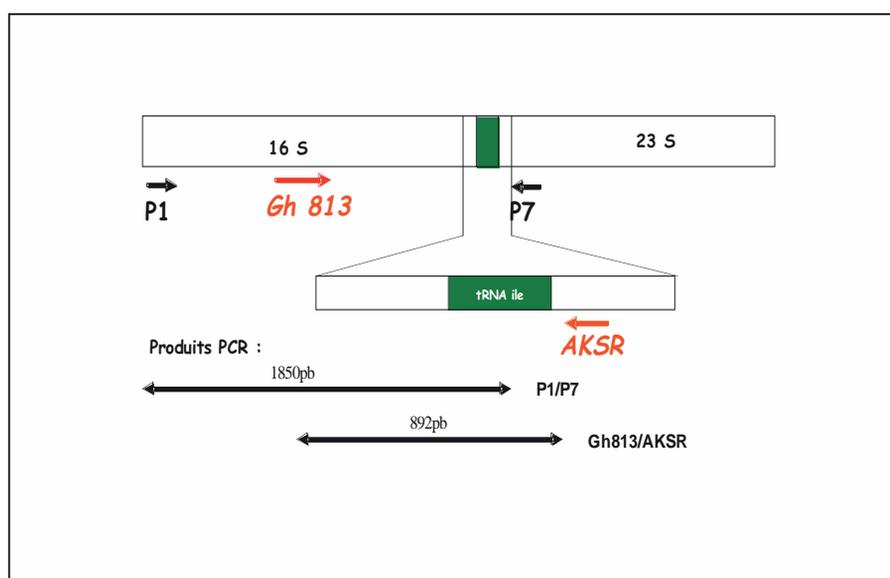


Figure 2.

Regions of

the ribosomal operon amplified by PCR for the diagnosis.

We found variability in the 16S/23S sequence of isolates collected in Ghana and those collected in Mozambique (Dollet *et al.*, 2006). In all, for all the isolates, there were around forty mutations in this sequence. It turns out that a mutation affects the sequence of a primer –AKSR– said to be specific to Ghana. Whilst the sequence of part 3' of that primer is AATGTGG, the sequence found in the majority of CSPW sequences is AATATGG. We therefore constructed a new primer with that sequence. It seems to enable the diagnosis of all CSPW cases (Dollet *et al.*, 2006).

In Ghana and Mozambique, LYTS do not occur in the same way everywhere. For example, in Mozambique the disease in Zambezia province develops in large foci, in which from around ten to around thirty palms can be found at different stages of the disease. In Cabo Delgado province, further to the North, only small foci of 3 to 4 diseased or dying palms were found; the disease does not seem to have the same epidemic potential there as in Zambezia (unpublished).

An exhaustive study of the 16S/23S sequences in Ghana and Mozambique is currently under way and should soon enable us to specify this variability of the phytoplasmas associated with the LYTS in those two countries.

6. Variability for resistance

The diversity and variability of LYTS is also reflected in the resistance of coconut varieties to these different pathological syndromes.

For instance, the Vanuatu Tall variety (VTT) — ex New Hebrides Tall — is highly susceptible to LY in Jamaica, whereas the Malayan Yellow Dwarf (MYD) displayed a degree of resistance in the 1960s to 1980s (Been, 1981). In Ghana, after several trials were set up to test how coconut varieties reacted to CSPW between 1981 and 2007, it turned out that, on the contrary, the VTT is fairly resistant to CSPW and the MYD highly susceptible (Dery *et al.*, 2008). In addition, whereas "Local Tall" coconut palms (West African Tall or WAT) be they from Ghana or Benin, are highly susceptible to CSPW, there are several "Local Tall" ecotypes (East African Tall) that display a degree of resistance to LDT (Mpunami, pers. com.).

7. Conclusion

In the Caribbean, Africa and Asia, coconut plantings can be found that are affected by a yellowing which can evolve more or less rapidly towards palm death. In most cases, such symptoms are reported as being cases of "Lethal Yellowing". However, coconut palms can display yellowing in response to various types of biotic or abiotic stress. For instance, a prolonged drought can cause nuts to fall, inflorescence necrosis and more or less marked yellowing. In the Caribbean zone, the same Lethal Yellowing syndrome may be due to a trypanosome or a phytoplasma. In fact, most LYTS in the Caribbean and Africa are associated with the existence of phytoplasmas, and we speak of "Lethal Yellowing", as if the disease were one and the same. However, the term phytoplasma was created to designate all phloem-restricted mollicutes, transmitted by vector insects and impossible to grow *in vitro* (Tully, 1979). There are at least 28 groups of phytoplasmas, and in those groups there are several subgroups which might be as many species; for example, 4 subgroups, 4 species possible for group 16S rX (Wei *et al.*, 2007). It can therefore be said that there exists a substantial diversity of LYTS and for each of those LYTS there is variability. It is important to take these data into account, particularly for PCR analysis, to search for vectors, and for genetic control.

Acknowledgements

The authors benefited from the French Ministry of Foreign Affairs Priority Solidarity Fund project FSP No. 2004-34, "Agricultural research on tree and food crop cultivation in Ghana" (2005-2008).

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