Identification d'insectes vecteurs "potentiels" du Cape Saint Paul Wilt sur le cocotier au Ghana par PCR

Identification of potential insect vectors of the Cape Saint Paul Wilt of coconut in Ghana by PCR

Fabian Pilet[1], René Philippe[1], Simon Reignard[1], Sophie Descamps[1], Robert Quaicoe[2,] Joe Nkansa Poku[2] , Sandrine Fabre[3] and Michel Dollet[3].

(1) CIRAD - Department BIOS - UPR29, OPRI Coconut program , Sekondi – Ghana
(2) OPRI – Coconut Programme - P.O. Box 245 – Sekondi – Ghana
(3) CIRAD - Department BIOS - UPR29, 4398 Montpellier - France

Résumé:
Le vecteur du phytoplasme responsable du jaunissement mortel du cocotier au Ghana est inconnu à ce jour. Il est cependant admis que les phytoplasmes sont transmis par des insectes piqueurs-suceurs, ces insectes étant supposés être les seuls à avoir la capacité d'injecter les phytoplasmes dans les tubes criblés du phloème.
Bien que la présence de phytoplasmes dans un insecte ne prouve sa capacité à transmettre la maladie, nous avons testés une grande quantité d'insectes pour la présence de phytoplasmes par PCR (directPCR et NestedPCR), en utilisant des amorces spécifiques des phytoplasmes en général, et du phytoplasme responsable du jaunissement mortel du cocotier en particulier. En effet, la mise en évidence d'une ou plusieurs espèces d'insectes porteurs du phytoplasme pourrait orienter nos recherches pour les essais de transmission en cages par insectes spécifiques.

Abstract:
The vector of the phytoplasma responsible for the coconut lethal yellowing disease in West Africa is unknown to date. However, it is known that phytoplasmas are transmitted by leafhoppers and plant hoppers, which are supposed to be the only ones able to inject the phytoplasma in the phloem.
Whereas the presence of phytoplasma in the insect does not prove its capacity to transmit the disease. We have tested a large number of insects for the presence of phytoplasmas by PCR (directPCR and NestedPCR) using both primer pairs specific for all phytoplasmas and those specific for the coconut lethal yellowing disease phytoplasma. In effect the evidence of one or several species carrying the phytoplasma would direct us on the insects to focus on in our transmission cages trials.

Resumen: Identificación insectos vectores potenciales del Cape Saint Paul Wilt en Ghana por PCR
El vector del fitoplasma responsable del amarillamiento letal del cocotero en Ghana sigue desconocido hasta ahora. Se reconoce sin embargo que los fitoplasmas se transmiten por insectos de aparato bucal picador-chupador (leafhoppers, planthoppers), ya que se admite que estos insectos son los únicos que tienen la capacidad de inyectar fitoplasmas en los tubos del floema.
Mismo si la presencia de fitoplasmas dentro de un insecto no corrobora su capacidad a transmitir la enfermedad, buscamos esta presencia por PCR (directPCR y NestedPCR) en una grande cantidad de insectos, utilizando iniciadores específicas de los fitoplasmas en general, y del fitoplasma responsable del amarillamiento letal del cocotero en particular. De hecho, si se comprueba que una o varias especies de insectos son portadoras del fitoplasma, podríamos modificar la orientación de nuestras investigaciones en los ensayos de transmisión en jaulas por insectos determinados.

Introduction

Lethal yellowing is the most damaging coconut disease in West Africa (Ghana, Nigeria and Togo) (Dery *et al.*, 1997). The disease was first observed in Ghana in 1932 and is locally called Cape Saint Paul Wilt (CSPW). As other coconut lethal yellowing diseases around the world, the CSPW disease is caused by a phytoplasma, cell wall-less bacteria which inhabit the phloem sieve elements. This location inside the plant and the obligate host status of the *Phytoplasma* implies they can be transmitted and spread mainly by insect vectors that are leafhoppers and planthoppers. In fact, all the known phytoplasmas insect vectors are *Auchenorrhyncha* family members to date.

Myndus crudus (*Homoptera*: *Cixiidae*) has been identified as the vector of the coconut LY in Florida (Howard *et al.*, 1983). Because of the similitude between the LY and the CSPW disease, and the presence of one insect of the same genera very common on coconut in Ghana, *Myndus adiopodoumeensis* has been suspected to be the vector in Ghana (Dery *et al.*, 1996). However, transmission trials by introducing numbers of *Myndus adiopodoumeensis* in cages have not resulted in the production of the disease in coconut plants to date (Philippe *et al.*, 2007). Introductions of other common species on coconut (mainly Derbidae) in transmission cages have not also reproduce the disease in palms.
Phytoplasma can be detected in the insect vector by direct PCR (Maixner *et al.*, 1995; Mpunami *et al.*, 2000) or Nested PCR. However, presence of phytoplasma in one insect does not prove that it is the vector. The phytoplasmas can be ingested by the insect during feeding, but fail to be acquired (i.e. passage through the intestinal wall into the haemolymph, multiplication and then accumulation in the salivary glands) for transmission. However, detection of the phytoplasma can give some important indication about the status of the insect towards the phytoplasma.
The aim of this study was to check the presence of phytoplasma i. in the insect species that have been introduced in the transmission cages, ii. in all planthoppers and leafhoppers found in and around coconut plot.

1. Materials and Methods

1.1. Collection of insects.

Insects were collected from a plot of Malayan Yellow Dwarf x Vanuatu Tall hybrid coconut planted in June 2001 at Asebu, Ghana, where coconut trees showing lethal yellowing disease symptoms can be observed since April 2005. Three series of collections have been realized.
The first collection, which corresponded mainly to the same insects species introduced into transmission cages, was done on coconut leaves of both healthy and diseased coconut trees. The most common species were bulked in tubes of five insects per species, whereas the rarer species were bulked in tubes of one to five insects according their size.
The second series consisted of collection of insects by 'sweeping the grasses' in the plot with sweeping net. Insects of each species were bulked into specific tubes of one to five insects according their size and their frequencies.
The third series consisted of collection of all Auchenorrhyncha, during both the day and the night on diseased coconut, and other crops such Citrus and Oil Palm growing around the plot as exhaustive.
Some *Aleurodidae, Aphididae* and *Pseudococcidae* were collected from coconut trees too.

1.2. Extraction of insect DNA.

DNA was extracted from insects according to the protocol of Maixner *et al.* (1995) with the following minor modification. Insects were ground in 400 l of extraction buffer [100 mM Tris–HCl at pH 8.0, 2% cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA) and 2% polyvinyl pyrrolidone], and the slurry was incubated for 60 min at 65°C. After incubation, an equal volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged for 20 min at 12 000 g. The supernatant was collected and the nucleic acid precipitated with an equal volume of isopropanol. Following a 30 min incubation at 4°C, the DNA was pelleted at 12 000 g for 20 min and the pellet washed with 70% ethanol and resuspended in 25 to 100 l of TE. (pH 8.0).

1.3. PCR analyses.

The detection of phytoplasmas in insect DNA was performed using direct PCR with the phytoplasma universal primers P1(5'-AAGAGTTTGATCCTGGCTCA GGATT-3') / P7 (5'-CGTCCTTCATCGGCTCTT-3') derived from 16SrDNA (Smart *et al.*, 1996). A sample of 2 l of template DNA solution was used in a PCR reaction mixture (25 l). Positive samples using P1 / P7 were checked using the specific CSPWD primers (Tymon *et al.*, 1998) G813 (5'-CTAAGTGTCGGGGGTTTCC-3') / GAKSR (5'-TTGAATAAGAGGAATATGG-3'), corresponding in the primer AKSR modified (Dollet *et al.*, 2006) whereas some negative samples were controlled by nested PCR. For nested PCR assays, 2 l of direct PCR P1/P7 product were used as template DNAs and the PCR performed using the specific CSPWD primers G813/GAKSR. The PCR products were analyzed

by electrophoresis through 0.8% agarose gel and stained with ethidium bromide and exposed to ultraviolet light. The size of the PCR products was estimated by the GelPilot 1 Kb Plus Ladder (Qiagen) as standard marker.

1.4. Data analyses.

Any sample**s** showing one visible band around the expected size was considered positive. With P1/P7 a primer, that is expected to give a product of 1750 bp, samples showing one band from 1600 to 1900 bp have been marked as positive. Both G813/GAKSR and nested PCR products showing one band in the 800-1000 bp range (expected size of 900 bp) were considered positive.

2. Results

A total of 12549 insects representing 2157 batches, and distributed among 204 species of 19 families were collected (Table 1). To date, 1683 of those batches have been already checked by PCR P1/P7 and 126 of them have shown one band closed to the expected size. However, none of those positive samples was positive using the specific CSPW primers (Table1).
Half of the tested insects were part of the species which were introduced into the transmission cages (Philippe *et al.*, 2007) and correspond mainly to the most common species observed on coconut, and are detailed in the Table 2. Some bands were observed with P1/P7 for some of the species screened such as *Diostrombus mayumbensis* and *Metaphenice stellulata* both *Derbidae*, but no band was observed for the candidate *Myndus adiopodoumeensis*, whatever the primer-pair used. Among the positive samples, none turned out positive by using the CSPW primers. However, one tube among 174 (862 insects tested) of *Diostrombus mayumbensis* was tested positive by nested PCR.
While most of the P1/.P7 PCR products were of low intensity, three samples were remarkable because of the high intensity of the bands observed. These three samples were one batch (22B 1347) of large *Cicadellidae* (LGC), containing 4 specimens of *Goniagnathus obesus obesus* (*Deltocephalinae*:*Cicadellidae*) and one undetermined *Cicadellidae* (C11), one batch (C32-1 0108) among 42 (representing 175 insects) of *Recilia canga* (*Cicadellidae*:*Deltocephalinae*), and one batch of *Numicia damocles* (*Tropiduchidae*).

Table 1: Number of batches (N B), Number of insects collected (NI) and number of positive batches (presence of band) of insects on the number of batches of insect tested for the PCR P1/P7, PCR G813/GAKSR and nested PCR for each family and subfamily collected in the field. (LGC= Large Cicadellidae; SMC= Small Cicadellidae; ND= Not Determinated).

Family	Subfamily	Species	N B	N I	PCR P1/P7	PCR G813/GAKSR	Nested PCR
Achilidae		2	3	1149	1 / 2	0 / 2	0 / 1
Aleurodidae	*Aphidinae*	1	65	643	0 / 65	0 / 2	0 / 8
Aphididae	*Hormaphidina*	1	57	2843	9 / 57	0 / 19	0 / 3
Aphrophoridae		2	87	101	0 / 29		0 / 8
Cercopidae		2	43	44	0 / 33		0 / 0
Cicadellidae	*Achilidae*	1	2	3	0 / 1		0 / 1
	Agalliinae	3	12	40	0 / 12	0 / 2	0 / 2
	Cicadellinae	2	55	171	2 / 55	0 / 2	0 / 7
	Deltocephalin	18	163	577	4 / 160	0 / 28	0 / 63
	Gyponinae	1	35	83	1 / 9	0 / 4	0 / 4
	Hecalinae	1	78	232	0 / 77		0 / 4
	Paraboloponin	1	1	3	0 / 1	0 / 1	0 / 0
	Typhlocybinae	2	11	40	0 / 11		0 / 2
	LGC	ND	46	222	5 / 44	0 / 4	0 / 4
	SMC	ND	35	171	0 / 32	0 / 2	0 / 4
	Undertermine	95	172	403	0 / 130	0 / 6	0 / 116
Cixiidae		5	141	665	0 / 83	0 / 2	0 / 11
Delphacidae		19	58	189	3 / 47	0 / 7	0 / 22
Derbidae		18	848	4266	94 / 668	0 / 61	1 / 267
Dictyopharidae		1	1	1	0 / 1		0 / 1
Flatidae		1	23	43	0 / 7		0 / 4
Lophopidae		1	7	31	0 / 1		
Menoplidae		5	74	336	0 / 60		0 / 51
Pentatomidae		13	86	87	6 / 45	0 / 3	0 / 14
Pseudococcidae		2	5	19	0 / 4	1 / 3	0 / 1
Ricaniidae		2	5	21	0 / 5	0 / 4	0 / 1
Thripidae		1	28	1267	0 / 28		
Tingidae		1	3	3	0 / 3	0 / 1	0 / 2
Tropiduchidae		2	13	34	1 / 13		0 / 13
Total		**204**	**2157**	**12549**	**126 / 1683**	**1 / 153**	**1 / 614**

Table 2: Number of batches (N B), Number of insects collected (NI) and number of positive batches (presence of band) of insects on the number of batches of insect tested for the PCR P1/P7, PCR G813/GAKSR and nested PCR for the most common species observed on coconut at Asebu, Ghana. (LGC= Large Cicadellidae; SMC= Small Cicadellidae).

Family	Species	N B	N I	N I/B	PCR P1P7	PCR G813/GAKSR	Nested PCR
Cixiidae	*Myndus adiopodoumeensis*	131	645	4,9	0 / 80 (394)	0 / 2 (10)	0 / 8 (39)
Cicadellidae	LGC	46	222	4,8	5 / 44 (212)	0 / 4	0 / 4
	SMC	35	171	3,3	0 / 32 (107)	0 / 2	0 / 4
Derbidae	*Diostrombus annetti*	43	212	4,9	0 / 23 (113)	0 / 1 (5)	0 / 23 (113)
	Diostrombus dilattatus	98	479	4,9	6 / 97 (474)	0 / 5 (25)	0 / 8 (39)
	Diostrombus luteus	24	88	3,7	1 / 24 (88)	0 / 2 (7)	0 / 4 (15)
	Diostrombus mayumbensis	250	1239	5,0	35 / 184 (912)	0 / 9 (45)	1 / 174 (862)
	Diostrombus nitida	79	388	4,9	15 / 79 (388)	0 / 12 (59)	0 / 9 (44)
	Metaphenice stellulata	139	683	4,9	23 / 88 (432)	0 / 19 (93)	0 / 13 (64)
	Patara armara	141	698	5,0	8 / 98 (485)	0 / 6 (30)	0 / 10 (50)
	Metaphenice stellulata larve	38	339	8,9	6 / 43 (384)		
	Proutista fritillaris	24	112	4,7	0 / 20 (93)	0 / 7 (33)	0 / 18 (84)
Menoplidae	*Nibia nervosa*	24	114	4,8	0 / 15 (71)		0 / 11 (52)
Total		**1048**	**5276**		**99 / 812**	**0 / 69**	**1 / 355**

Table 3: Number of batches (N B), Number of insects collected (NI) and number of positive batches (presence of band) of insects on the number of batches of insect tested for the PCR P1/P7, PCR G813/GAKSR and nested PCR for the *Cicadellidae*: *Deltocephalinae.*

Species	N B	N I	N I/B	PCR P1/P7	PCR G813/GAKSR	Nested PCR
Balclutha aff. dufela	1	5	5,0	0 / 1 (5,)	0 / 1 (5)	0 / 1 (5)
Balclutha dufela	6	22	3,7	1 / 6 (22)	0 / 1 (4)	0 / 1 (4)
Balclutha incisa	15	68	4,5	0 / 15 (68)	0 / 14 (6)	0 / 13 (59)
Balclutha sp	3	10	3,3	1 / 3 (10)	0 / 1 (3)	
C11	1	3	3,0	0 / 1 (3)		0 / 1 (3)
C15	4	15	3,8	0 / 4 (15)		
C26	1	5	5,0	0 / 1 (5)		
C30	4	9	2,3	0 / 2 (4)		
C7	1	2	2,0	0 / 1 (2)		
C92	4	19	4,8	0 / 4 (19)	0 / 4 (19)	0 / 1 (5)
C93	3	8	2,7	0 / 3 (8)		0 / 3 (8)
Exitianus occidentalis	20	59	3,0	0 / 20 (59)		0 / 1 (3)
Exitianus sp.	1	3	3,0	0 / 1 (3)	0 / 1 (3)	
Goniagnathus obesus obesus	26	52	2,0	0 / 26 (52)		0 / 13 (26)
Recilia canga	42	175	4,2	2 / 42 (175)	0 / 5 (21)	0 / 27 (113)
Recilia lactipennis	24	108	4,5	0 / 24 (108)	0 / 1 (5)	0 / 2 (9)
Exitianus capicola	6	13	2,2	0 / 6 (13)		
Cicadulina mbila	1	1	1,0			

3. Discussion

The very high diversity of Auchenorrhycha observed at Asebu, Ghana, confirm the necessity of this study. In fact, to date mainly the common species have been introduced in transmission cages but introduction of all the 208 species met cannot be envisaged.

The first important result consists of the absence of phytoplasma in *Myndus adiopodoumeensis*. Introduction of this species in transmission cage did not result in the transmission of the disease (Philippe *et al.*, 2007). While *Myndus adiopodoumeensis* has been the main suspected vector of the CSPW (Dery *et al.*, 1995), those two results do not seem to support this hypothesis.

Because nested PCR is a very sensitive tool, it is difficult to conclude about the presence of the CSPW phytoplasma in one *Diostrombus mayumbensis*. The fact that it has been detected only by nested PCR reveal a low concentration of phytoplasma. This low concentration can just correspond to the ingestion of phytoplasma during feeding and does not give any indication about the capacity of the phytoplasma to multiply inside this insect. This result needs more investigation by using other techniques to evaluate the exact concentration of phytoplasma in the insect.

One of the three samples showing a band of high intensity by direct PCR using P1/P7 primers contains *Recilia canga*. Because *Recilia canga* belongs to the sub-family of *Deltocephalinae* (*Cicadellidae*), which contains the highest number of known phytoplasma vectors (Weintraub and Beanland, 2006), and *Recilia mica* is the vector of the blast disease of oil palm nurseries (de Chenon, 1979), this positive result is probably due to a phytoplasma. However, the negative result using G813/GAKSR exclude the LY phytoplasma, as for the tube containing *Goniagnathus obesus obesus* (*Deltocephalinae*) and *Numicia damocles* (*Tropiduchidae*). These three samples will be sequenced.

To date, no CSPW phytoplasma has been detected by direct PCR with both the P1/P7 and G813/GAKSR primers pair even in Derbidae or Meenoplidae as observed by Mpunami *et al*. (2000), even though those insects were mainly collected on coconut. It is not the case of some *Cicadellidae* sub-families like the *Deltocephalinae* which were captured by sweeping or 'light-attraction'. The percentage of those insects which have fed on coconut and moreover on diseased coconut is unknown. Also, some species of this sub-family have so far been collected in low numbers as presented in Table 3. Because the known vectors of phytoplasma are predominantly among the *Deltocephalinae* sub-family (Weintraud and Beanland, 2006), the investigation must be continued.
While bands have been observed by direct PCR in some insects, it is still necessary to check by nested PCR. Some samples of insects were composed of only one very small insect, in which case DNA yield would be low during the extraction, or if the insect was collected before the process of acquisition of the pathogen was completed, then only the nested PCR would be able to detect the phytoplasma, even if the result has to be considered with caution.

Acknowledgement

This research was funding by the French Ministry of Foreign Affairs through the FSP 2004-34 (Agronomical research in Ghana in the area of perennial and food crop cultivation). We thank Jacques Bonfils, Thierry Mateille (IRD) and Jean-François Germain (LNPV) for determining all the species collected.

References

- Dery, S.K., Philippe, R., and Mariau D. 1996. Auchenorrhyncha (Homoptera), suspected vectors of coconut lethal yellowing disease in Ghana. *Plantations, Recherche, Developpement* 3:355-363.
- Dery, S.K., N'Cho, Y.P., Sangare, A., and Arkhurst, E.D. 1997. Cape Saint Paul Wilt Disease of coconut in Ghana. In Eden-Green, S.J., and Ofori, F. (Eds.). Proceedings of an International Workshop on lethal yellowing-like disease of coconut, Elmina, Ghana, November 1995. Chatman, UK: Natural Resources Institute, pp 147-151.
- de Chenon RD. 1979. Demonstration of the role of *Recilia mica* Kramer (Homoptera, Cicadellidae, Deltocephalinae) in the blast disease of oil palm nurseries in the Ivory Coast. *Oleagineux* 34:113--15
- Dollet M., Fabre S., Pilet F., Quaicoe R., Mugini J.A., Rassaby L. (2006) - Variability and diagnosis of coconut lethal yellowing Syndromes in Africa. 16th International Congress International Organization for Mycoplasmology (IOM) - Cambridge, United Kingdom. 9-14 July 2006.
- Howard, F.W., Norris, R.C., and Thomas, D.L. 1983. Evidence of transmission of palm lethal yellowing agent by a planthopper, *Myndus crudus* (Homoptera, Cixiidae). *Tropical Agriculture* 60:168-171.
- Lee, I., Gundersen, R.D., and Bertaccini, A.1998. Phytoplasma: ecology and genomic diversity. *Phytopathology* 88:1359-1366.
- Maixner, M., Ahrens, U., and Seemuller, E. 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology* 101:241-250.
- Mpunami, A., Tymon, A., Jones, P. and Dickinson, M.J. 2000. Identification of potential vectors of the coconut lethal disease phytoplasma. *Plant Pathology* 49:355-361.
- Philippe, R., Nkansah, J.P., Fabre, S., Quaicoe, R., Pilet, F., Dollet, M. 2007. Search for the vector of Cape Saint Paul wilt (coconut lethal yellowing) in Ghana. *Bulletin of Insectology* 60 (2):179-180.
- Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., and Harrison, N.A. 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNa spacer region. *Applied and environmental microbiology* 62:2988-2993
- Weintraub, P.G., and Beanland, L. 2006. Insect vectors of phytoplasmas. *Annual review of entomology* 51:91-111.