Search for alternate hosts of the coconut Cape St. Paul Wilt disease pathogen

Recherche d'Hôtes Alternatifs de l'Agent Pathogène de la Maladie de Cape Saint-Paul du Cocotier

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Abstract:
Lethal yellowing disease locally called Cape St. Paul Wilt disease (CSPW) is the bane of the coconut industry in Ghana and is caused by a phytoplasma. In Ghana, there are areas where the disease has re-infected in re-plantings long after decimating all the palms in the area. This brings to the fore the possibility of alternate hosts in the spread of the disease since the pathogen is an obligate parasite. In this work a number of plants were screened for their host status to the CSPW pathogen. The presence of phytoplasmas in these plants was tested by PCR analysis using universal phytoplasma primers P1/P7 and CSPW specific primers G813/GAKSR. Although *Desmodium adscendeus* tested positive to the CSPWD specific primers, cloning and sequencing did not confirm it as an alternate host. The identification of alternate hosts will help us to evolve sound control strategies against the spread of the disease.

Résumé:
Le jaunissement mortel, localement appelé Cape Saint Paul Wilt Disease (CSPW), est le fléau de l'industrie du cocotier au Ghana et est causé par un phytoplasme.
Au Ghana existe des endroits ou la maladie est réapparue sur des replantations longtemps après qu'elle y ait décimé l'ensemble des cocotiers. Ceci suppose la possibilité d'hôtes alternatifs qui participeraient à la propagation de la maladie, puisque l'agent pathogène est un parasite obligatoire. Pour ces travaux, de nombreuses plantes ont été testées pour leur statut d'hôte au CSPW. La présence de phytoplasmes dans ces plantes a été testée par analyse PCR en utilisant les amorces spécifiques des phytoplasmas P1/P7, et les amorces spécifiques du CSPWD G813/GAKSR. Bien que *Desmodium adscendeus* fut testé positivement par une analyse PCR avec une amorce spécifique, le séquençage de l'ADN n'a pas permi de confirmer son role de plante hôte. L'identification d'hôte alternatif pourra nous aider à définir des stratégies de contrôle contre la dispersion de la maladie.

Resumen: "Búsqueda de Huéspedes Alternos del Agente Patógeno del Marchite Cape Saint Paul del Cocotero"
La enfermedad de amarillamiento mortal llamada localmente enfermedad de marchite de Cape Saint Paul (CSPW) es el azote de la industria del cocotero en Ghana y es causada por un fitoplasma. En Ghana, hay zonas en las que la enfermedad ha resurgido en repoblaciones mucho después de diezmar todas las palmeras de la zona. Esto añade a lo anterior la posibilidad de la existencia de huéspedes alternos que participan en la propagación de la enfermedad puesto que el agente patógeno es un parásito obligatorio. La presencia de fitoplasmas en estas plantas se corroboró por análisis PCR utilizando cebos universales de fitoplasma P1/P7 y cebos específicos CSPW G813/GAKSR. La identificación de huéspedes alternos nos ayudará a desarrollar estrategias sólidas de control contra la propagación de la enfermedad.

Introduction

The coconut palm (*Cocos nucifera* L.) is considered the most important crop along the coastal belt of West Africa and can be grown (with minimal capital outlay) in poor sandy salt loaded soils where very few or no other crop would survive (Ofori and Nkansah, 1997; Bourdeix *et., al*,2005). It is reported that about 4.2 percent of Ghana's population depend on coconut for their livelihood (Adam *et.al*., 1996). The cultivation, processing and marketing of the crop supports the livelihoods of many poor coastal people and help sustain

the environment (Ayirebi – Acquah, 1997). In 1932, a disease called lethal yellowing (LY) was detected among some palms in the Volta Region of Ghana and ever since the fortunes of the coconut industry has been on the decline.

Lethal yellowing is a highly destructive and fast spreading disease affecting coconut palm and at least 35 other palm species in the Americas (Harrison *et. al* 1999).The disease has brought great distress to several rural coastal communities engaged in the coconut industry in Ghana; leaving them without a sustainable source of livelihood (Dery *et.al*, 1997). The destruction of the coconut palms has environmental repercussions too as previously covered lands become exposed leaving these areas prone to degradation.

LY disease (LYD) are caused by pytoplasmas which are essentially cell wall-less prokaryotes belonging to the class Mollicutes. Plant to plant transmission of these pathogens are carried out by insect vectors (Plant hoppers and leaf hoppers), through vegetative propagation of infected plant materials or by graft inoculation (Kirkpatrick, 1992). Phytoplasmas cannot be cultured in vitro, a phenomenon due probably to the lack of essential genes and functions (Razin, 2007)

After devastation by the LYD in 1964 at Cape Three Points in the Western region of Ghana, in 1977 the Crops Research Institute of the Council for Scientific and Industrial Research (CSIR-CRI) put in some varieties to evaluate their resistance to the disease. All the varieties succumbed to the disease (Dery *et al*., 1997). Then in 1981, the Ministry of Agriculture under the France-Ghana- Cote d'Ivoire coconut project also brought in some varieties from outside the country to screen for their resistance to the disease. The disease decimated these palms as well with the exception of the Sri – Lanka Green Dwarf variety (SGD) (Dery *et al*., 1997). In 1995, the CSIR- Oil Palm Research Institute, Coconut Research Programme under the EEC STD III Project also imported some varieties from Cote d'Ivoire for resistance trials. Yet again the disease has cleared almost all the palms (62.3%) (Anon, 2006). It is in the light of this history that the possibility of the presence of alternate hosts of the CSPWD phytoplasma was investigated. The hypothesis being that the phytoplasma, an obligate parasite needed another host to survive in the periods when their primary hosts, the coconuts were unavailable due to death from the disease. In the Americas, LY phytoplasma is known to have other hosts other than the coconut. This situation may hold true for other areas affected by similar lethal yellowing – type coconut diseases, like CSPWD.

In choosing the species to sample, priority was given to plants related to known hosts of the coconut lethal yellowing phytoplasma. *Emelia fosbergii* and *Synedrella nodiflora* were reported to be alternate hosts in Jamaica (Brown *et al*., 2007), so *Emelia sonchifolia* and *Synedrella nodiflora* were sampled. Plants which have been reported to host any type of phytoplasma were also sampled. As a result pepper, tomato, potato, cassava, sugar cane, *Solanum* spp. and *Euphorbia* spp which are reported to host phytoplasmas (Montano *et. al*., 2007; Gungoosingh- Bunwaree *et. al*., 2007; Nasir *et. al*., 2007; Lebsky and Poghosyan, 2007) were sampled. Plants showing any of the general symptoms of phytoplasma infection such as stunting, yellowing, withering, witches' broom (proliferating shoots) (Lee *et. al*., 2000) as well as some asymptomatic plants were also selected.

1. Materials and methods

1.1. Sampling Sites

Sampling of plant species was carried out from two locations; Cape Three Points in the Western Region (WR) and Asebu in the Central Region (CR). Cape Three Points is approximately 66 Km South-west from Sekondi. Asebu is about 100 Km North-east from Sekondi, both active disease foci.

1.2. Sampling Period

Sampling was done twice in a year, one in the rainy season and the other in the dry season. This was done to take account of the effect of changes in environmental conditions on the dynamics of plants and insect life. For example, some plants which cannot withstand drought die out during the dry season and this may be true

for the insect vector population fluctuation as well. The implication is that some potential hosts of the pathogen can be missed if sampling is not done in the two seasons. The rainy season sampling took place from July – December 2007 and that of the dry season was done from March to April 2008.

1.3. Sampling Methodology

The sampled species were mainly grasses, a few shrubs and a few tree plants. Fifteen species were sampled per location in each of the seasons. A total of 57 species were sampled during the period. The plants were sampled within and around the two trial fields as well as from some coconut farms surrounding our trial plots. For every species 30 individual plants were picked. A few, however, due to their rarity did not meet this number.

1.4. Total DNA Extraction from Plant Tissue

For each species, 2 individual plants were pooled together to give 1 sample. Hence 15 DNA samples were prepared for each species. One (1) gram of each sample was ground in 5ml CTAB (Cetyl trimethyl ammonium bromide) and the DNA extracted according to the protocol of Daire *et al*., 1997.

1.5. PCR & Gel electrophoresis

The DNA samples were assayed for the presence of phytoplasmas using phytoplasma universal primer pair P1 (5' – AAG AGT TTG ATC CTG GCT CAG GAT T- 3') (Deng and Hiruki, 1991) and P7 (5'- CGT CCT TCA TCG GCT CTT - 3') (Smart *et. al.*, 1996). Positives from this test were screened for the CSPWD phytoplasma employing primers G813f (5' – CTA AGT GTC GGG GGT TTC C- 3') and GAKSR (5' – TTG AAT AAG AGG AAT GTG G- 3') (Tymon *et.al*., 1998). The PCR was performed using a PTC – 100 Programmable Thermal Controller manufactured by MJ Research Inc. The PCR products were analysed and visualized by Gel electrophoresis on a 0.8% agarose gel prepared using Tris Borate buffer (TBE) (1x) and stained with ethidium bromide.A 1 kb ladder (Invitrogen / Quiagen) was loaded in either the first or last lane. The gel was visualized and photographed under UV light. Negative controls consisted of reaction mixtures with DNA template from healthy coconut palms. DNA templates from CSPWD infected palms were used for the positive controls and the blanks had the DNA templates substituted with water.

1.6. Restriction Fragment Length Polymorphism (RFLP)

Samples which tested positive to the CSPWD specific primers were digested with restriction endonucleases. The enzymes used were RSA 1, EcoR1, Hind III, and Alu 1. The reaction was incubated in a water bath at 37^{o}C for 9 hours. Analysis of digestion product was done by electrophoresis on a 2% agarose gel.

2. Results

Table 1: Results of assay of rainy season samples

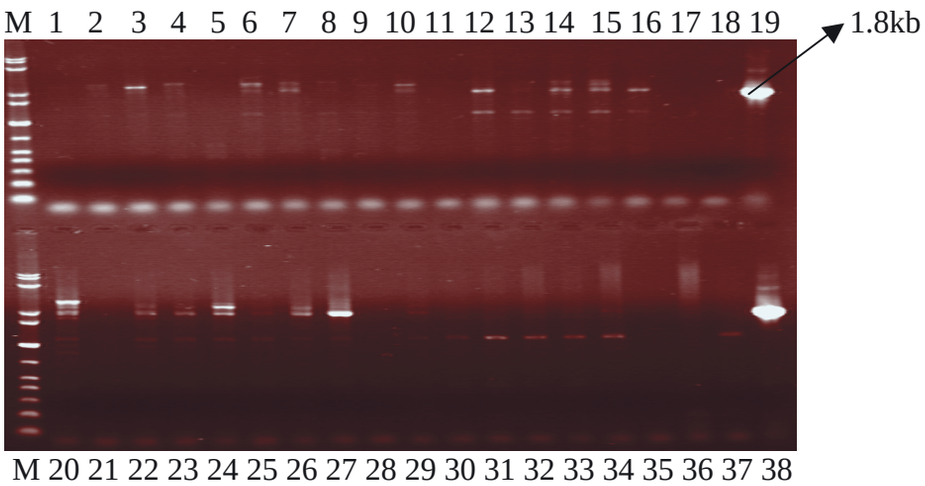
	Primers					
	P1P7			G813/GAKSR		
	N° of samples	N° of positives	%+	N° of samples	N° of positives	%+
Plant species(Asebu)						
Commelina benghalensis	15	7	46.7	7	0	0
Solanum torvum	15	0	0	0	0	0
Calopogon mucunoides	15	2	13.3	2	0	0
Desmodium adscendeus	**15**	**13**	**86.7**	**13**	**13**	**100**
Malacantha alnifolia	15	4	26.7	4	0	0
Panicum maximum	15	1	6.7	1	0	0
Citrus limon	15	3	20	3	0	0
Nephrolepis bisserata	15	0	0	0	0	0
Sida acuta	15	0	0	0	0	0
Tridax procumbens	15	0	0	0	0	0
Pennisetum purpureum	15	0	0	0	0	0

Ficus exasperate	15	0	0	0	0	0
Oxythenanthera abyssinica	15	0	0	0	0	0
Chromolaena odorata	15	0	0	0	0	0
Rauvolfia vomitoria	15	0	0	0	0	0
Plant species (Cape 3 Points)						
Setaria megaphylla	15	0	0	0	0	0
Mimosa pudica	15	0	0	0	0	0
Stachytarpheta indica	15	0	0	0	0	0
Aspilia africana	15	0	0	0	0	0
Borreria scabra	15	0	0	0	0	0
Saccharum officinale	15	0	0	0	0	0
Dissotis rotundifolia	15	0	0	0	0	0
Asystasia gangetia	15	0	0	0	0	0
Ananas sativa	15	0	0	0	0	0
Rauvolfia vomitoria	15	0	0	0	0	0
Flagellaria guineese	15	0	0	0	0	0
Pueraria phaseoloides	15	0	0	0	0	0
Manihot esculenta	15	0	0	0	0	0
Voacanga Africana	15	0	0	0	0	0

Table 2: Results of assay of dry season samples

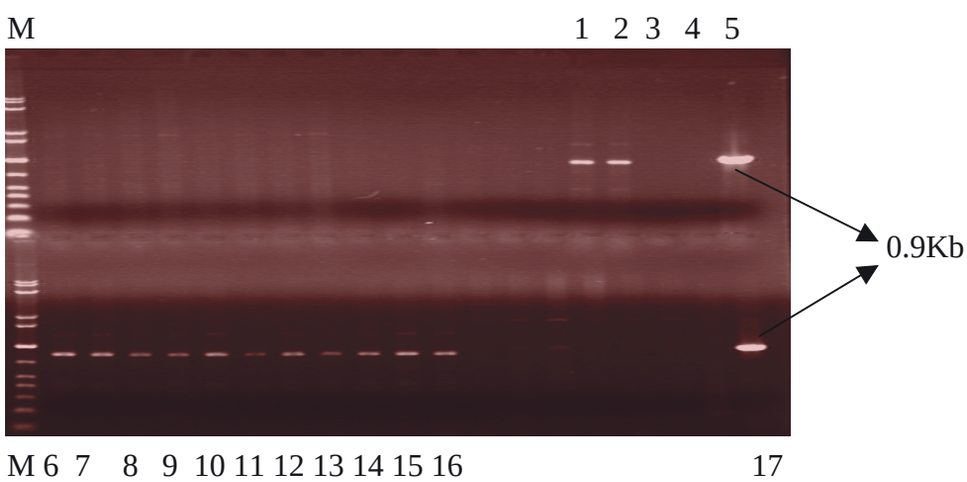
	Primers					
	P1P7			G813/GAKSR		
	N° of samples	N° of positives	%+	N° of samples	N° of positives	%+
Plant species(Asebu)						
Stachytarheta indica	15	0	0	0	0	0
Desmodium adscendeus	15	0	0	0	0	0
Synedrella nodiflora	10	0	0	0	0	0
Ipomoea involucrate	15	0	0	0	0	0
Lypersicon esculentum	4	0	0	0	0	0
Jussiae spp.	15	0	0	0	0	0
Sporobolus pyramidalis	15	0	0	0	0	0
Brachiara deflexa	15	0	0	0	0	0
Cymbopogon citratus	15	0	0	0	0	0
Capsicum annuum	15	0	0	0	0	0
Emelia sonchifolia	15	0	0	0	0	0
Eleusine indica	15	0	0	0	0	0
Digitaria adscendens	15	0	0	0	0	0
Euphorbia heterophylla	15	0	0	0	0	0
Solanum tuberosum	15	0	0	0	0	0
Plant species (Cape 3 Points)						
Paspalum scrobiculatum	15	0	0	0	0	0
Panicum laxum	15	0	0	0	0	0
Cassytha filiformis	15	0	0	0	0	0
Lantana camara	15	0	0	0	0	0
Clerodendrom capitatum	15	0	0	0	0	0
Morinda lucida	15	0	0	0	0	0
Chassalia kolly	15	0	0	0	0	0
Rottboellia exaltata	15	0	0	0	0	0
Cyperus spp.	15	0	0	0	0	0
Alchornea cordifolia	15	0	0	0	0	0
Sanseviera liberica	15	4	26.7	0	0	0
Pennisetum pedicellatum	15	0	0	0	0	0
Spigelia anthelmia	15	0	0	0	0	0
Phyllanthus amarus	15	0	0	0	0	0
Justica flava	15	0	0	0	0	0

Fig 1. Amplification of samples with primers P1P7.



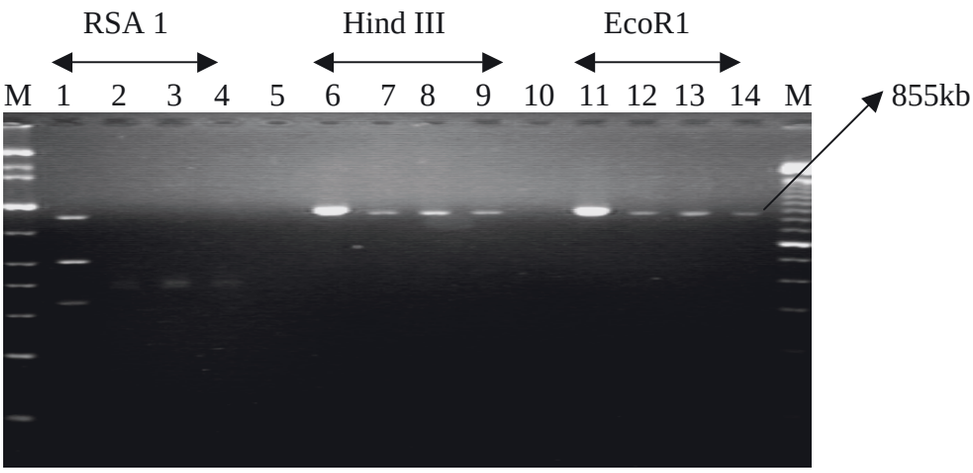
Legend: M: marker; lane 17- Blank, lane 18- Negative control; lane 19& 38 – positive controls
Lanes 1-11: *Calopogon mucunoides* (Calopo) samples; Lanes 12-16, 20-29 : *Desmodium adscendeus* samples
Lanes 30- 37 : *Malacantha alnifolia* samples

Fig 2: Amplification of *Desmodium adscendeus* samples with G813/GAKSR



M: Marker, lane 3 – Blank; lane 4- negative control; lanes 5 & 17- positive control. lanes 1,2 ,6-16: *Desmodium adscendeus* samples

Fig 3. Restriction Fragment Length Polymorphism (RFLP) analysis of 3 samples of *Desmodium adscendeus* (G813/ GAKSR products) using 3 restriction endonucleases



Samples from plants such as *Calopogon mucunoides* (Calapo) and *Desmodium adscendeus* were amplified at the expected size of 1.8kb when primers P1P7 were applied (Fig 1). When the specific primers G813/GAKSR were applied to the positive samples from the P1P7 test, only the *Desmodium adscendeus* samples were amplified at the expected size of 0.9kb (Fig 2).when restriction digestion was carried out on G813/GAKSR products of *D. adscendeus* samples, RSA 1 did not produce the expected fragment sizes of 498, 311 and 21 kb, however Hind III and EcoR1 produced fragments corresponding to the profile of the CSPWD phytoplasma of the expected size 855kb.

2.1. Cloning and Sequencing

A sample of *Desmodium adscendeus* was cloned and sequenced by Cogenics society (France). DNA sequences from the sample were determined to be related to *Bacillus megatorium* and *Rhodobacter sphaeroides.*

Discussion

The search failed to identify any alternate hosts of the CSPWD phytoplasma. However we cannot conclude that there are no alternate hosts in the two areas since only a few plants were screened from the hundreds of plant species that can be found at the two places. The results imply that the presence of any of the screened species in coconut plantations will not pose any threat to the palms vis-a-vis the spread of CSPWD. Whilst plants like cassava, sugar cane, *Solanum spp, Stachytarphta spp* have been reported host at least one type of phytoplasma elsewhere, this was not the case in this work as no amplifications were obtained from these species when the phytoplasma universal primers P1P7 was applied. This means that the phytoplasmas infecting these species are not in the environment of the two sampling sites. As far as we are concerned this work is the first major attempt at finding alternate hosts of the CSPWD phytoplasma and the search needs to continue with more species being screened

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