SHORT COMMUNICATION

A PHYTOPLASMA IS ASSOCIATED WITH THE WELIGAMA COCONUT LEAF WILT DISEASE IN SRI LANKA

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SUMMARY

Coconut palm (*Cocos nucifera*), is a major and economically important plantation crop widely cultivated in Sri Lanka. A sudden outbreak of a rapidly spreading non-lethal syndrome was first reported in coconut palms from southern Sri Lanka in 2006. This syndrome was named Weligama coconut leaf wilt disease (WCLWD) as it occurred in the Weligama area. WCLWD symptoms are flaccidity and marginal necrosis of leaflets and intense yellowing of the fronds, similar to the Kerala wilt disease of coconut in India. As the disease progresses the crown becomes smaller, the trunk begins to taper and the palm becomes unproductive. Abiotic factors and extra-cellular pathogens were discarded as the cause of WCLWD. Considering that a phytoplasma was previously associated with Kerala wilt disease, the possible phytoplasma aetiology of WCLWD was investigated. DNA extracted from midribs of spear leaves was subjected to nested PCR with phytoplasma universal primers R16F2n/R16R2 and R16mF2/R16R2 nested with fU5/rU3; P1/P7 nested with Chrfor/rU3; and direct PCR with Pc399/P1694. PCR products of expected sizes were obtained from diseased but not from healthy palms from a disease-free area. The sequences generated from the PCR products were submitted to similarity search (BlastN) in the NCBI database which confirmed that a phytoplasma belonging to the 16SrXI '*Candidatus* Phytoplasma oryzae' group is associated with WCLWD. The phytoplasma was found to be highly similar but not identical to Sugarcane white leaf phytoplasma (99%), Sugarcane grassy shoot phytoplasma (99%) and Kerala wilt phytoplasma (99%).

Key words: *Cocos nucifera*, nested PCR, sequencing, detection.

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The coconut palm (*Cocos nucifera*) is a major, economically important plantation crop widely cultivated in Sri Lanka. Coconut is cultivated in many parts of the island with large scale plantations located in the areas covering the Gampaha, Kurunegala, and Puttalam districts, which is termed the "Coconut Triangle" (Fig. 1). However, many mid to small scale coconut plantations and several home coconut gardens are found in southern Sri Lanka.

During late 2006, an unusual yellowing and flaccidity of leaflets on coconut palms were observed in the Weligama area in the Matara district in the southern part of the country (Wijesekara *et al*., 2008; Perera *et al*., 2010) and the syndrome was named "Weligama coconut leaf wilt disease" (WCLWD). WCLWD is now prevalent in the divisional secretariat regions Akuressa, Athuraliya, Devinuwara, Dickwella, Hakmana, Kamburupitiya, Kirinda-Puhulwella, Malimboda, Matara, Pitabeddera, Thihagoda, Weligama and Welipitiya of Matara district, Galle and Habaraduwa of Galle district and Beliatta, Ookewela, Tangalle and Walasmulla of Hambantota districts in southern Sri Lanka. The syndrome is prevalent in all type of soils from flat coastal areas to undulating lands inland, and the affected palms are distributed in pockets, sometimes several kilometres away from each other.

The earliest and most striking symptom of the disease is the flattening and downward bending of leaflets giving to the frond a ribbed or flaccid appearance (Fig. 2A). Crowns of such palms appear dark green in colour. This symptom is first seen in the younger leaves and becomes more prominent when the fronds are fully opened. The degree of flaccidity of leaflets varies among the fronds in a single palm, and is not readily recognizable to an untrained eye. The intense yellowing of lower whorls of fronds, which is more prominent just after the rainy season up to about the 12th frond (Fig. 2B), is also a specific symptom of the disease. Occasionally, yellowing of mid whorls of fronds is seen in some palms. In such cases, yellowing is restricted to about 6 to 8 fronds in the middle whorl. Subsequently, drying up of the leaflets starts from the margins of the affected fronds and dried fronds hang in the crown for some time before falling from some severely affected palms, the fronds also curl down-

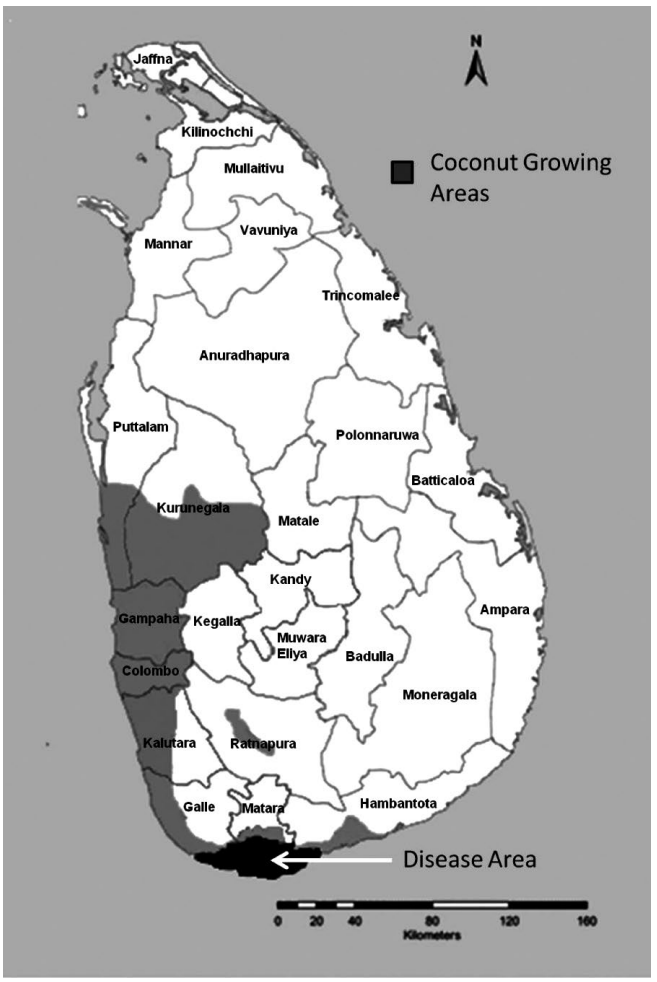

Fig. 1. Map of Sri Lanka showing major coconut growing areas and the disease area.

ward giving a ragged appearance to the crown. The tips of fronds become twisted or break and hang down in some palms. Unopened bud leaves lose their rigidity and bend downwards in severely affected palms. With the reduction in the number of fronds, the crown becomes smaller and the trunk begins to taper. As the disease progresses, female flower production declines and the palm becomes unproductive. The majority of the WCLWD-affected palms that were observed initially were at the bearing stage, while a few were pre-bearing. However palms of any stage have subsequently been found to be susceptible to the disease, with palms over 3 years of age most commonly affected. Furthermore, WCLWD seems to predispose the palms to another disease condition called leaf rot disease with which a complex of fungi is associated (unpublished observations). Flaccidity of leaflets is evident on seedlings younger than 3 years of age, whereas yellowing is always seen only in older palms. WCLWD symptoms were found to be very similar to those of the Kerala wilt phytoplasma disease in Kerala, India, which is reported to be caused by a phytoplasma (Manimekalai *et al.*, 2010).

A multi-disciplinary research approach that included analysis of the soil, environment, plant nutrition, physiological malformation and extra-cellular pathogens as possible factors in WCLWD aetiology failed to identify the cause of the syndrome (unpublished information). However, tetracycline treatment applied to selected diseased palms induced a clear symptom remission. Symptoms reappearance 4 months after treatment discontinuity (unpublished information) suggestested that phytoplasmas might be involved. Similar observations had

Fig. 2. WCLWD affected coconut palms showing flaccidity (A) and intense yellowing symptoms (B).

been reported for coconut lethal yellowing disease (McCoy, 1982) and its variants, such as Cape Saint Paul wilt and Kaincopé diseases, which were later confirmed to be associated with phytoplasmas (Dabek *et al.*, 1976). A number of phytoplasmas-induced coconut diseases are known in the USA, the Caribbean, Central America, Africa (Lethal yellowing and Lethal yellowing-like diseases) (Harrison *et al.*, 1999), southeast Asia, such as Kalimantan wilt in Indonesia (Warokka *et al.*, 2006), Malayan yellow decline in Malaysia (Nejat *et al.*, 2009) and in south Asia, e.g. Kerala wilt in India (Manimekalai *et al.*, 2010). Therefore the involvement of a phytoplasma in WCLWD was hypothesized and investigated in this study.

PCR assay employing primer pairs based upon 16S rRNA gene sequences were first used to detect and identify phytoplasmas (Ahrens and Seemüller, 1992; Deng and Hiruki, 1991). Other assays incorporating primer pairs derived from conserved rRNA operon sequences (Gundersen and Lee, 1996; Smart *et al.*, 1996), the *secA* gene (Hodgetts *et al.*, 2008), *secY* gene (Lee *et al.*, 2010) or various ribosomal protein genes (Martini *et al.*, 2007) have since been developed.

An extensive survey was carried out in the WCLWD-affected area to prepare a "Disease Index" (unpublished information) based on the degree of severity of the phenotypic symptoms (i.e. flaccidity, yellowing and necrosis). Accordingly, diseased palms were classified into three categories: mild, moderate and severe. Coconut spear leaves were surveyed in the more severely WCLWD-affected areas covering all symptom categories. In total, samples were collected from 56 palms, 50 exhibiting mild, moderate and severe WCLWD symptoms, and 6 coming from apparently healthy palms adjacent to WCLWD-affected palms. All samples were transferred to the molecular biology laboratory of the Coconut Research Institute at Lunuvila (Sri Lanka), wrapped with aluminium foil and placed in polythene bags to prevent drying and avoid any possible movements of vectors to disease-free area. Samples collected from 10 additional palms at Bandirippuwa estate located outside the disease epidemic provided healthy controls. Sugarcane white leaf phytoplasma disease samples collected from a sugarcane plantation in the Udawalawa area (Sri Lanka) were used as positive controls. DNA was extracted from midribs of spear leaves using the CTAB protocol for coconut DNA extraction as described by Perera *et al.* (1999). Representative DNA extracts and plant tissues were also sent to the University of Nottingham (UK) for further analysis.

The CTAB extraction protocol from host plant material yielded non-degraded high quality nucleic acids as visualized in a 1% agarose gel and a high quantity of DNA in the range of 100-150 µg/µl. Therefore, to identify the presence of any phytoplasmas, amplification of 16S rDNA was performed using both direct PCR and nested PCR assays. Amplifications were performed in 20 µl reactions with appropriate forward and reverse primers. The universal primers used in this study were R16mF2/R16mR1 (Gundersen and Lee, 1996), fU5/rU3 (Lorenz *et al.*, 1995), Pc399/P1694 (Skrzeczkowski *et al.*, 2001) and Chrfor (Christensen *et al.*, 2004). PCR products obtained from each amplification were analyzed in 1% agarose gels stained with ethidium bromide

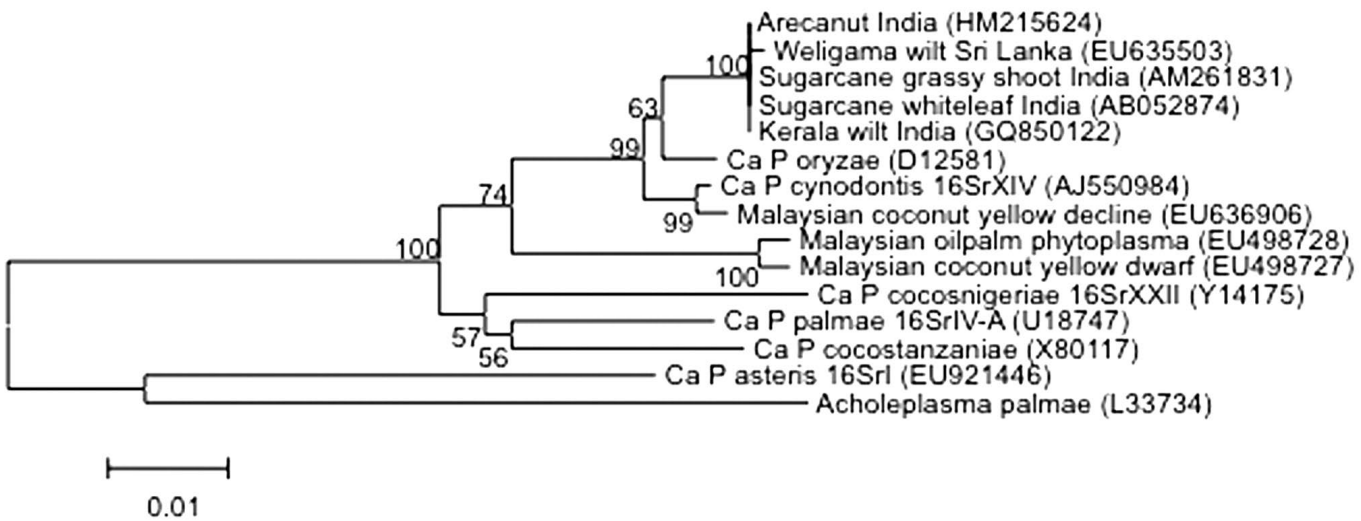

Fig. 3. Dendrogram constructed by the Neighbor-Joining method, showing the phylogenetic relationships amongst coconut and sugarcane phytoplasmas, '*Candidatus* Phytoplasma asteris', '*Candidatus* Phytoplasma oryzae', '*Candidatus* Phytoplasma cynodontis' and *Acholeplasma laidlawii* based on DNA sequences of the 369 bp to 1249 bp region of the 16S rRNA gene. GenBank accession numbers for sequences are shown in parenthesis alongside the names of the phytoplasmas. Bootstrap values greater than 50% (expressed as percentages of 1000 replications) are shown, and branch lengths are proportional to the number of inferred character state transformations. Bar – number of substitutions per base.

and visualized under UV trans-illuminator.

The universal phytoplasma primers, R16mF2/R16mR1 nested with fU5/rU3, R16F2n/R16R2 nested with fU5/rU3, P1 (Deng and Hiruki, 1991)/P7 (Smart *et al.*, 1996) nested with Chrfor/rU3 as well as Pc399/P1694, yielded PCR products of expected sizes (880 bp for fU5/rU3, 350 bp for Chrfor/rU3 and 1290 bp for Pc399/P1694) from 27 of 50 (54%) diseased palms and four symptomless palms (apparently healthy), as well as from Sugarcane white leaf phytoplasma (positive control). All primers gave consistent results with positive palms. A moderate disease condition was found to be the most appropriate stage for PCR detection. No PCR products were amplified from any of the healthy controls. It was later found that the reason for detecting only 54% of positives from diseased palms was the low titre of phytoplasma in the WCLWD-affected palms. Thus, detection was improved using phytoplasma enriched DNA extraction procedures.

The amplified fragments were excised carefully from gels and purified using the Wizard SV Gel purification system (Promega, USA). Direct sequencing of purified product was carried by Macrogen (Korea) and at the University Nottingham (UK). Sequence similarity was scored by BlastN option in the NCBI database. The amplified fragments from nested PCR with fU5/rU3 and Chrfor/rU3 were 773 bp and 350 bp in size, respectively. The fU5/rU3 and Chrfor/rU3 sequences were similar but not identical to the Sugarcane white leaf phytoplasma (99%) (GQ121046), Sugarcane grassy shoot phytoplasma (99%) (AM261831.1) (Ariyarathne *et al.*, 2007) and the Kerala wilt phytoplasma (GQ850122) (Manimekalai *et al.*, 2010) (Fig. 3). These sequences showed 98% sequence identity to Coconut yellowing decline phytoplasma from Malaysia (Nejat *et al.*, 2009) and also to Kalimantan wilt from Indonesia (Warokka *et al.*, 2006). However, the WCLWD sequence (EU635503) was very much different from that of coconut lethal yellowing (LY) (Harrison *et al.,* 1999) and other LY-like phytoplasma that have devastated coconut in the Caribbean and Africa. Based on 99% sequence identity with the Sugarcane white leaf phytoplasma, WCLWD was identified as a new member of 16SrXI '*Candidatus* Phytoplasma oryzae', former Rice yellow dwarf group (Jung *et al.,* 2003; Firrao *et al.,* 2004). The fU5/rU3 and Chrofor/rU3 sequences of the WCLWD phytoplasma were deposited in the NCBI database with accession numbers EU635503 and GQ121047, respectively.

A previous study has indicated that the phytoplasma associated with Kalimantan wilt disease of coconut in Indonesia (Warokka *et al.,* 2006) is also a member of the Rice yellow dwarf (16SrXI) group. In addition, because 16SrXI and 16SrXIV group phytoplasmas are closely related taxonomically, the agent of WCLWD was found to be more closely related to the phytoplasma associated with the Coconut yellowing decline in Malaysia (Nejat *et al.*, 2009), which is in the 16SrXIV group ('*Candidatus* Phytoplasma cynodontis), than to the phytoplasmas associated with coconut lethal yellowing type diseases in the Caribbean (16SrIV) or west Africa (16SrXXII) (Harrison and Oropeza, 2008; Martini *et al.*, 2007; Wei *et al.*, 2007). It therefore appears that coconut phytoplasmas causing the lethal yellow type diseases are phylogenetically quite distinct from those causing the less aggressive diseases in south and southeast Asia, which are much more similar to the grass-type phytoplasmas.

Collectively, symptoms of WCLWD are most similar to those described for Kerala wilt disease, according to a phytopathologist from southern India who visited and inspected the WCLWD affected area. It may be that the Kerala wilt phytoplasma was inadvertently moved from Kerala to Sri Lanka as the Kerala wilt disease has been prevalent in Kerala for the past 130 years or more and, during this period, plenty of opportunities may have existed for some infected planting material or vectors reaching Sri Lanka. However, the WCLWD phytoplasma 16S rRNA sequence (369 bp to 1249 bp region) shows 3 bp differences with the identical region of the Kerala wilt phytoplasma sequence archived in NCBI GenBank (GQ850122). This suggests that the WCLWD phytoplasma of Sri Lanka may not have originated from southern India. Alternatively, WCLWD may have arisen in Sri Lanka as a result of vector dispersal of a mutated sugarcane phytoplasma from sugarcane plantations to nearby coconut plantations. However, not enough evidence to support this hypothesis is available. Similarly, it may be that WCWLD has always been present in Sri Lanka and that the reason for its emergence as a prominent problem might be a result of global climate change and its influence on insect vector abundance. Perhaps climate changes have influenced vector population dynamics and/or abundance of alternative host plants over time which, in turn, has provided a larger inoculum pool and appearance of WCLWD on a much larger numbers of coconut palms. Ongoing sequencing of genes other than 16S rRNA gene of WCLWD phytoplasmas and its comparison with other phytoplasmas present in Asia along with identification of their host ranges (including wild grasses) and vectors should help to precisely establish where they have originated from and whether they can pass to alternate hosts.

REFERENCES

Ahrens U., Seemüller E., 1992. Detection of DNA of plant pathogenic mycoplasma like organisms by polymerase chain reaction that amplifies a sequence of 16S rRNA gene. *Phytopathology* **82**: 828-832.

Ariyarathne H.A.C.K., Everard J.M.D.T., Karunanayake E.H., 2007. Diseased sugarcane in Sri Lanka is infected with sugarcane grassy shoot and/or sugarcane white-leaf phytoplasma. *Australian Plant Disease Notes* **2**: 123-125.

Christensen N.M., Nicolaisen M., Hansen M., Schulz A., 2004. Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant Microbe Interactions* **17**: 1175-1184.

Dabek A.J., Johnson C.G., Harries H.C., 1976. Mycoplasma-like organisms associated with Kaincope and Cape St. Paul wilt disease of coconut palms in West Africa. *Pest Articles News Summaries* **22**: 354-358.

Deng S., Hiruki C., 1991. Amplification of 16S rRNA genes from culturable and non culturable molecutes. *Journal of Microbiological Methods* **14**: 53-61.

Firrao G., Marcone C., Bertaccini A., 2004. Phytoplasma classification. *Journal of Plant Pathology* **86**: 299.

Gundersen D.E., Lee M.I., 1996. Ultrasensitive detection of phytoplasmas by nested PCR using two universal primers. *Phytopathologia Mediterranea* **35**: 144-151.

Harrison N.A., Cordova I., Richardson P.A., Dibonito R., 1999. Detection and diagnosis of lethal yellowing. In: Oropeza C., Verdeil C.L., Ashburner G.R., Cardena R., Santamaria J.M. (eds). Current Advances in Coconut Biotechnology, pp. 183-196. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Harrison N.A., Oropeza C., 2008. Coconut lethal yellowing: In: Harrison N.A., Rao G.P., Macron C. (eds). Characterization Diagnosis and Management of Phytoplasmas, pp. 219-248. Studium Press LLC, Houston, TX, USA.

Hodgetts J., Boonham N., Mumford R., Harrison N., Dickinson M., 2008. Phytoplasma phylogenetics based on analysis of *secA* and 23S rRNA gene sequences for improved resolution of candidate species of 'Candidatus Phytoplasma'. *International Journal of Systematics and Evolutionary Microbiology* **58**: 1826-1837.

Jung H.Y., Sawayanagi T., Wongkaew P., Kakizawa S., Nishigawa H., Wei W., Oshima K., Miyata S., Ugaki M., Hibi T., Namba S., 2003. '*Candidatus* Phytoplasma oryzae', a novel phytoplasma taxon associated with rice yellow dwarf disease. *International Journal of Systematic and Evolutionary Microbiology* **53**: 1925-1929.

Lee I.M., Davis R.E., Gundersen D.E., 2000. Phytoplasma: Phytopathogenic mollicutes. *Annual Review of Microbiology* **54**: 221-255.

Lee I.M., Bottner-Parker K.D., Zhao Y., Davis R.E., Harrison N.A., 2010. Phylogenetic analysis and delineation of phytoplasmas based on *secY* gene sequences. *International Journal of Systematic and Evolutionary Microbiology* **60**: 2887-2897.

Lorenz K-H., Schneider B., Ahrens U., Seemüller E., 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. *Phytopathology* **85**: 771-776.

Manimekalai R., Soumya V.P., Kumar R.S, Selvarajan R., Reddy K., Thomas G.V., Sasikala M., Rajeev G., Baranwal V.K., 2010. Molecular detection of 16SrXI group phytoplasma associated with root (wilt) disease of coconut (*Cocos nucifera*) in India. *Plant Disease* **94**: 636.

McCoy R.E., 1982. Use of tetracycline antibiotics to control yellows diseases. *Plant Disease* **66**: 539-542.

Martini M., Lee I.M., Bottner K.D., Zhao Y., Botti S., Bertaccini A., Harrison N.A., Carraro L., Marcone C., Khan A.J., Osler R., 2007. Ribosomal protein gene-based phylogeny for finer differentiation and classification of phytoplasmas. *International Journal of Systematics and Evolutionary Microbiology* **57**: 2037-2051.

Nejat N., Sijam K., Abdullah S.N.A., Vadamalai G., Dickinson M., 2009. Phytoplasmas associated with disease of coconut in Malaysia are from two different phylogenetic groups and occur in other plant species. *Plant Pathology* **58**: 1152-1160.

Perera L., Russell J.R., Provan J., Powell W., 1999. Identification and characterization of microsatellites in coconut (*Cocos nucifera* L.) and analysis of coconut population in Sri Lanka. *Molecular Ecology* **8**: 344-346.

Perera L., Meegahakumbura M.K., Wijesekara H.T.R., Kalani N.G.A, Munasing C.E., Fernando W.B.S., Dickinson M.J., 2010. Detection of Weligama coconut leaf wilt disease in Sri Lanka by polymerase chain reaction. In: George V.T., Krishnakumar V., Augustine Jerard B., Niral, V., Josephrajkumar A. (eds). Proceedings of the International Conference on Coconut Biodiversity for Prosperity, pp. 146. Niseema Printers and Publishers Kochi-18, India.

Skrzeczkowski L.J., Howell W.E., Eastwell K.C., Cavileer T.D., 2001. Bacterial sequences interfering in detection of phytoplasma by PCR using primers derived from the Ribosomal RNA Operon. *Acta Horticulturae* **550**: 417-424.

Smart C.D., Schneider B., Blomquist C.L., Guerra L.J., Harrison N.A., Ahrens U., Lorenz K.H., Seemüller E., Kirkpatrick B.C., 1996. Phytoplasma-specific PCR primers based on sequences of the 16-23 rRNA spacer region. *Applied and Evolutionary Microbiology* **62**: 2988-2993.

Warokka W.A., Jones P., Dickinson M., 2006. Detection of phytoplasmas associated with Kalimanthan wilt disease of coconut by Polymerase chain reaction. *Jurnal Littri* **12**: 154-160.

Wei W., Davis R.E., Lee I.M., Zhao Y., 2007. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *International Journal of Systematics and Evolutionary Microbiology* **57**: 1855-1867.

Wijesekara H.R.T., Perera L., Wikramananda I., Herath I., Meegahakumbura M.K., Fernando W.B.S., de Silva P.H.P.R., 2008. Preliminary investigation on Weligama coconut leaf wilt disease: A new disease in southern part of Sri Lanka. In: Nainanayake N.P.A.D., Everard J.M.D.T. (eds). Proceedings of the Second Plantation Crop Symposium, pp. 336-341. Samayawardana Printers, Colombo, Sri Lanka.

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