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Sugarcane White Leaf Disease Characterization, Diagnosis Development, and Control Strategies

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ABSTRACT

Sugarcane white leaf (SCWL) is the most destructive disease of sugarcane in Thailand where major cane and sugar production of the world are situated. The severity degree and symptom variance are depended on soil fertility, temperature, cane sette quality, cultural practice and the phloem-colonized phytoplasma amount. The leafhopper *Matsumuratettix hiriglyphicus* is the first known vector that transmits SCWL phytoplasma. Recently at least other six leafhoppers have been found containing SCWL phytoplasma DNA. However, only *Yamatotettix flavovitatus* has been closely investigated and found its transmissibility but with lesser extent. Outbreak of SCWL disease is mainly activated by transportation and propagation of endemic cane setts. This SCWL phytoplasma is closely related to Thailand sugarcane green grassy shoot (SCGGS) and India sugarcane grassy shoot (SCGS) with 96-98% similarity in their 16S-23S rDNA sequences. Some diversity among SCWL phytoplasma isolates from different sources and growing locations has also been indicated within the range of 89-98% similarity by the 16S-23S rDNA sequence analyses. In addition to existed conventional diagnosis methods, the electrochemical DNA sensors have been proposed as a new tool for reliable routine practice. At present, control of the SCWL disease is difficult due to their systemic nature and the lack of desirable resistant variety. Application of insecticide is ineffective either. The disease control measures thus are mostly emphasized on the other strategies such as physical and chemical treatment of the cane setts, disease free plant production via tissue cultures, sanitation and crop rotations, and regulatory quarantine. Cooperative assistance among growers and involving association is strongly needed to accomplish effective control.

Keywords: control measures, disease outbreak, diversity, electrochemical DNA sensors, transmission
Abbreviations: AFM, atomic force microscope; **CV**, cyclic voltammetry; **DAPI**, 4-6-diamidino-2-phenylindole; **DPV**, differential pulse voltammetry; **ELISA**, enzyme linked immunosorbent assay; **PCR**, polymerase chain reaction; **TEM**, transmission electron microscope

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INTRODUCTION

In Thailand, approximately three million acres are belong to sugarcane field which makes this amount to be the fifth biggest planting area of the world and almost half of them are located in the Northeast of the country. Although this huge planting area can push Thailand to become the second exporter of the world but the exact yield capability is still as low as 20-25 tons per acre. One of the most serious factors of such low productivity is sugarcane white leaf disease. This sugarcane white leaf (SCWL) disease has been considered as the most important disease of sugarcane in Thailand since the year 1962 (Kusalwong 1980). According to the 2010 year report from Thailand Office of The Cane and Sugar Board, over 30 million US dollars has been correspondent for the losses in Thai sugarcane industry each year due to this disease. An economic threshold of SCWL has also been reported in Taiwan, Bangladesh and Sri

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Invited Review

Lanka (Leu 1983; Kumarrasingh and Jones 2001). The common name sugarcane white leaf disease has been given from dominant appearance of the complete white throughout the whole leaf of severely affected sugarcane. The evidence of SCWL disease was found on cv. 'NCo 421' grown in North Thailand at Kao Ka district of Lampang Province by the first sugarcane industry, Thai Lampang Sugar Industry Company and it was first reported epidemic with at least 50% yield losses in the year 1962-1964 to the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand under a research program for Thailand Sugar Industry (Chen 1974; Kusolwong 1980). Then the disease was rapidly distributed to several provinces in the North, the Central and especially in the East of Thailand by the year 1974 to 1983 until all canes at the East were burnt out from the fields (Kusalwong and Ouvanich 1993). The main planting area has then shifted to the Northeast that is even more suitable for SCWL disease development by its sandy soil type. Serious destructive epidemic in this area was pronounced in 1989 with endemic cv. 'F-154'. Intensive disease control program under a cooperation between Ministry of Agriculture and Cooperatives and Ministry of Industry established from 1989-1991 has demonstrated that the losses could be effectively reduced by replacing the cv. 'F-154' with a new cv. 'Phil 58-260' coupled with green manure crop rotations (Kusalwong and Ouvanich 1993). However, a few years later the disease reoccurred at much higher incident and subsequently occupies all growing areas throughout the country owing to the lack of disease free cane setts by a rapid swift demand of sugarcane production (Wongkaew *et al.* 1999; Wongkaew and Fletcher 2004).

The SCWL disease was also discovered in the south of Taiwan by the year 1958 on cv. 'NCo 310' at Tainan, Kaochiung and Pingtung districts. The disease was further spread to nearby districts and readily covering the island by the use of infected cane setts for transplanting propagation (Ling and Chaung-Yang 1962). But the crisis was substantially declined later by effective integrated control campaigns (Richi and Chen 1989). In Japan, this disease was recognized in 1986 at Tanegashima island of Kagoshima prefecture by Arai and Ujihara (1989), but all the infected plants were eradicated which resulted in the disease disappearance ever since (Nakashima *et al.* 1999). Similar type of the disease has been simultaneously observed in Bangladesh, India, Malaysia, Nepal, Pakistan and Sri Lanka. Indeed, it has been first recorded by Barber in 1947 at Belapur district of Bombay, Maharashtra in India (Vasudeva 1955). The disease caused 10-40% economic losses and subsequently proceeded to Panjab, Uttar Pradesh, Haryana, Bihar, West Bengal, Madhya Pradesh, Andhra Pradesh, Karnataka and Tamilnadu (Agnihotri 1983). This disease is now a great economic concern to both farmers and sugar industry in India, Which the yield losses in ratoons reach their maximum in crops and up to 70% or higher incidence has been arisen resulting in 100% yield losses (Rao *et al.* 2008). In these countries, at least 6 different names were formerly called such as albino, bunchy, grassy shoot, leafy tuft, new chlorotic and yellowing disease. But the name "sugarcane grassy shoot (SCGS)" was then accepted as a common name according to a compilation study by Rane and Dakshindas (1962). The symptom appearances of this SCGS disease are rather resembled the Taiwan and Thailand SCWL than the sugarcane green grassy shoot (SCGGS) disease investigated in Central Thailand that all the affected leaves of bunchy cane in the latter case remain their normal green (Lairungreung *et al.* 1995; Wongkaew *et al.* 1997). Although it has been notified that these so-called SCWL, SCGS, and SCGGS disease are likely caused by closely related unculturable phytoplasmas from their 16S rDNA and 16S-23S rDNA sequencing analysis (Wongkaew *et al.* 1997; Jung *et al.* 2003; Rao *et al.* 2008), still there are arguments on their true identity and their variability in different sources, cultural practice condition and geographical attitudes.

SCWL DISEASE CONFIGURATION

Disease symptoms

The typical characteristics symptoms of the disease caused by SCWL phytoplasma are bunchy white leaf proliferation on the dwarfed shoots. The disease symptoms can be pronounced in every growth stages of sugarcane plants from the earliest seed cane or cane sett germination until the latest maturity. Variation among diseased plants is frequently exhibited in different plant stages and conditions including the disease development stages. There are subsistent levels of leaf chlorosis that vary degrees of the basic white color which lead to the appearances such as pale green, pale yellow, yellow plus white, pale white and yellow, cream, and lastly pure white throughout all leaves of the whole plants. Pathological effects of the disease on green components of the leaf had been investigated by Wu *et al.* (1969) and it has been concluded that the symptom expression of white leaf is considered to be directly related to the development of chloroplast and chlorophyll biosynthesis. Quantity of the causal phytoplasma inside affected plants was also shown to rule on symptoms extent by an experiment on the relationship between chlorophyll losses and SCWL phytoplasma amount (Nakashima *et al.* 1994). The leaf color appearance is sometimes variegation and becomes green again in certain environment. Profuse budding and shooting with bushy slim white leaves are arisen in severely infected young plants. These affected plants are soon dried as a result of heavy chlorosis and die before they can produce desirable sugar juicy stems. Climate and temperature effects have been reported in Taiwan that the symptoms were masked in winter and rapidly developed severe white leaves in warmer season (Leu 1983). Observation on white leaf disease incidence in various locations and preliminary screening for disease resistance indicated different scale of the disease symptoms that were depended on the soil fertility and sugarcane cultivars (Ouvanich *et al.* 1990; Ouvanich and Kusalwong 1993). Thus the disease severity is influenced by several factors such as the cane sett quality, sugarcane vigor, cultivation practice, sugarcane cultivar, soil type and fertilization, and the containing phytoplasma quantity.

Disease transmission

Transmission of SCWL phytoplasma to healthy plants has been assumed to be possible in the tests with two leafhoppers, *Matsumuratettix hiroglyphicus* Matsumura (Matsumoto *et al.* 1969) and *Yamatotettix flavovittatus* Matsumura (Hanboosong *et al.* 2006). Grafting experiment with sugarcane plantlets and periwinkle (*Catharanthus roseus* L.) could also transmit the phytoplasma from infected sugarcane to each grafted plant (Wongkaew and Fletcher 2004). While the attempts using mechanical injurie and dodder (*Cuscuta* spp.) feeding were failed to transmit the SCWL phytoplasma (Richi and Chen 1989; Sarindu and Clark 1993). However, a natural transmission in Taiwan and Thailand is likely happened via the most effective insect vector *M. hiroglyphicus* according to its feeding preference, transmission efficiency from 55-100% and its population dynamics accompanying to monthly disease incidence (Matsumoto *et al.* 1969; Yang and Pan 1969; Pisitkul *et al.* 1989; Hongspluk *et al.* 1993; Wongkaew 1999). Transovarian transmission has been found in *M. hiroglyphicus* which declare it to be a reservoir of SCWL phytoplasma (Hanboonsong *et al.* 2002). On the other hand, although the phytoplasma could be detected by nested PCR and 16S-23S rDNA gene sequencing in some other leafhoppers such as *Balcluta* sp., *Bhatia olivacea*, *Exitianus indicus*, *Hecalus prasinus* and *Recilia* sp. (**Fig. 1**), their transmissibility have not yet been intensively investigated. While the leafhopper *Y. flavittatus* has been ascertained it's capable to transmit the SCWL phytoplasma but with less efficiency. Their population also fluctuates differently from the disease inci-

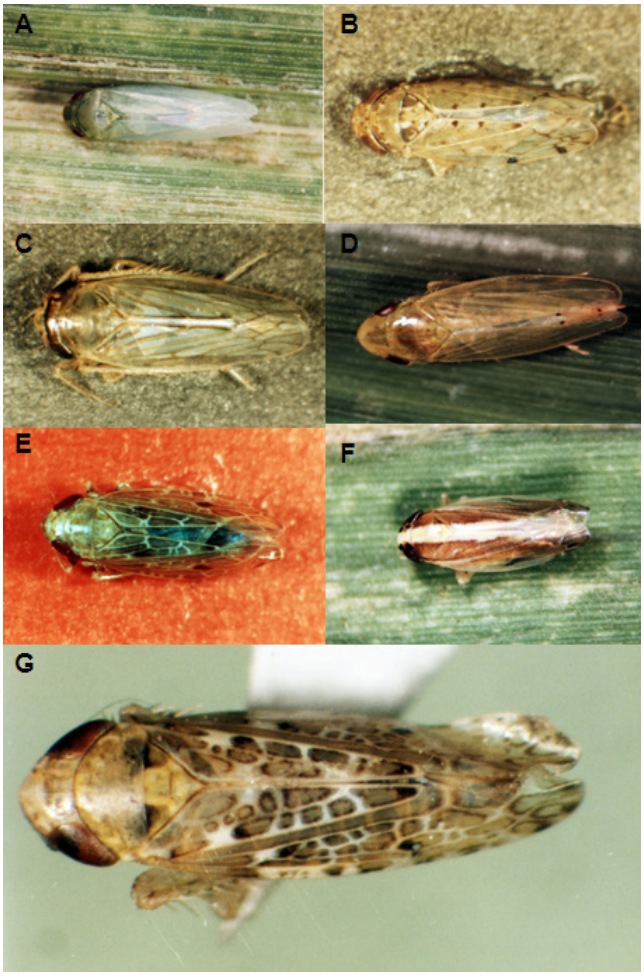


Fig. 1 Sugarcane white leaf phytoplasma-containing leafhoppers detected by nested PCR and 16S-23S rDNA sequencing. (**A**) *Balcluta* sp.; (**B**) *Bhatia olivacia*; (**C**) *Exitianus indicus*; (**D**) *Hecalus prasinus*; (**E**) *Recilia* sp.; (**F**) *Yamatotettix flavovittatus*; (**G**) the first known insect vector, *Matsumuratettix hiroglyphicus*. Reprinted from **Wongkaew P** (1999) *Sugarcane White Leaf Disease Management*, Thailand Research Fund, Pimpatana Press, Khon Kaen, Thailand, 228 pp, with kind permission from the publisher.

dence recorded throughout the year (Hanboonsong *et al.* 2006; Wongkaew 1999).

The transmission by *M. hiroglyphicus* requires at least 3 h acquisition feeding on the diseased plant and 30 min inoculation on the target plant (Chen 1973; Pisitkul *et al.* 1989). Efficiency in disease transmission by female adults is 65.6% while the transmission by males is 45.8% (Chen 1973). Incubation period of SCWL phytoplasma is 4-5 weeks in the insect body and 2-4 weeks in sugarcane plant (Matsumoto *et al.* 1969). A life span of *M. hiroglyphicus* at 14°C is 92 days and at 35°C is 29.6 days. Hence its life span observed in Taiwan is generally 42 days since egg immergence including 25 days of the 5th instar development, while the life span in Thailand is 30 days including 12-15 days of instar development as shown in **Fig. 2** (Yang and Pan 1969; Wongkaew 1999). The optimum condition for its ovulation is an environment of sandy loam soil texture with 10% relative humidity at 30-35°C which as much as 200 eggs with at least 72% hatching capability by each female can be produced. Eggs can be found in soil near the base of sugarcane plant throughout the year but peaks of their population are usually seen in February to March, May to June, and September to October (Yang 1972; Pisitkul *et al.* 1989). While the adult population is abundant during June to October and reaches the highest peak in August along with the peak of SCWL disease incidence (Yang and Pan 1969; Pisitkul *et al.* 1989; Hongspluk *et al.* 1993; Wongkaew 1999).

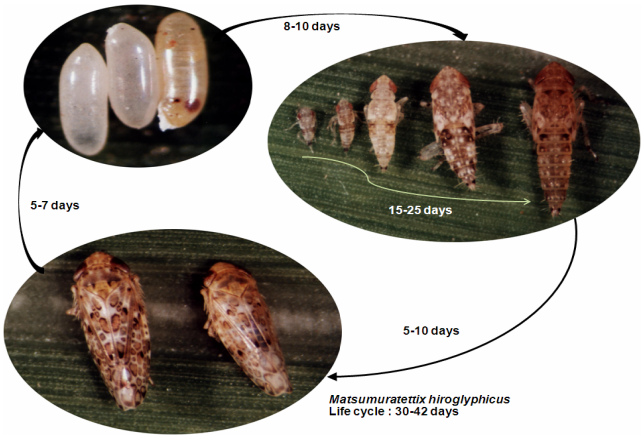


Fig. 2 Life cycle of the sugarcane white leaf phytoplasma's preference insect vector, *Matsumuratettix hiroglyphicus*. Reprinted from **Wongkaew P** (1999) *Sugarcane White Leaf Disease Management*, Thailand Research Fund, Pimpatana Press, Khon Kaen, Thailand, 228 pp, with kind permis-sion from the publisher.

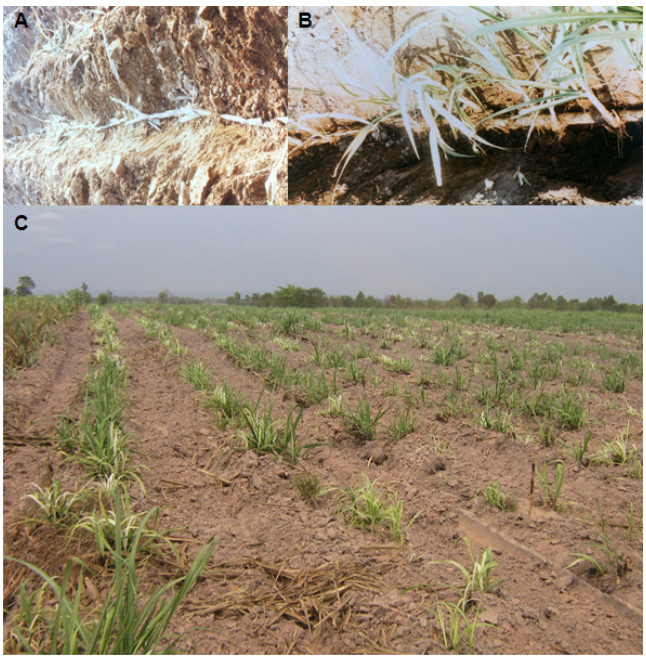


Fig. 3 Epidemic spread of sugarcane white leaf disease following the transportation and propagation of causal phytoplasma-embedded cane setts. (**A**) Cane setts planting; (**B**) explicit disease symptoms since the first immergence of sugarcane shoots; (**C**) severely affected sugarcane fields.

Epidemic spread of the disease

As the disease is systemic due to a colonization of the fastidious phytoplasma in sugarcane phloem, thus it can easily spread via vegetative propagation by cane setts. The transportation and the use of endemic cane setts for sugarcane production are considered to be the most effective means responsible for severe epidemic outbreak. An ignorance on quality control of the cane setts prior to their growing in large plantations is the main reason that execute the rapid spread and extreme disease incidence. The typical white leaf symptoms can be obviously seen since the first immergence of sugarcane shoots by this transplanting of the phytoplama embedded cane setts. Secondary spread within sugarcane field and nearby location is then accelerated by the vector-leafhopper that rapidly distributes SCWL phytoplasma further on (**Fig. 3**). It has been confirmed that the yearly population dynamic of the insect vector *M. hirogly-phicus* is correlated to the advance of the disease within the

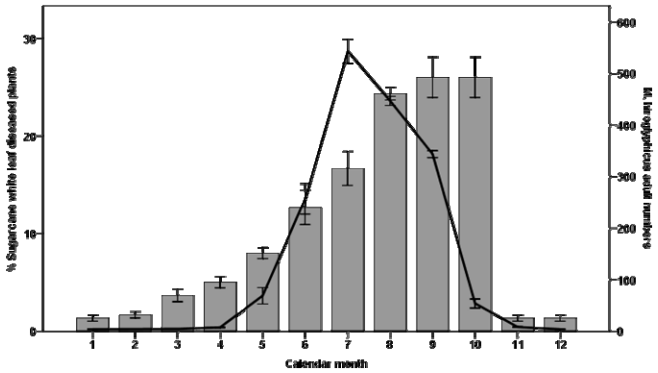


Fig. 4 ***Matsumuratettix hiroglyphicus*** **leafhopper population dynamic and sugarcane white leaf disease incident percentage in growing areas of Udornthani Province from monthly observations throughout 1996-1998.** Reprinted from **Wongkaew P** (1999) *Sugarcane White Leaf Disease Management*, Thailand Research Fund, Pimpatana Press, Khon Kaen, Thailand, 228 pp, with kind permission from the publisher.

fields as shown in **Fig. 4** (Yang and Pan 1969; Wongkaew 1999).

A wild species of cane *Saccharum spontaneum* has been suggested to be an alternate host for SCWL phytoplasma from the transmissibility test with *M. hiroglyphicus*. Several other grass species have also been investigated but most of them could not attract this insect feeding. Survival after rearing from egg to the 5th instar of this insect were 13.2% on burmuda grass (*Cynodon dactylon* L.), 3.7% on *Euphorbia hirta* L. and 2.7% on *Cyperus roduntus* L. but no adult could be developed (Yang 1972). Among 90 species of sugarcane weeds that were checked for an existence of SCWL phytoplasma, only burmuda grass, brachiaria grass (*Brachiaria distachya* L.), and crowfoot grass (*Dactyloctenium aegyptium* L.) showed their containing phytoplasmas which closely related to SCWL phytoplasma in the 16S rDNA restriction fragment polymorphism and the 16S-23S rDNA sequence (Wongkaew *et al.* 1997; Wongkaew 1999; Jung *et al.* 2003). Still, the alternate host capacity of these grasses needs to be re-examined although the vector transmission feeding tests remain unsuccessful at present.

The causal SCWL phytoplasma and recent molecular characterization

SCWL phytoplasma is localized within phloem sieve element of the diseased plant. An ultrastructural observation under electron microscope has revealed its typical mollicute cell structure that lacks cell and is surrounded by only a soft cell membrane unit. Pleomorphic shapes of the phytoplasma in sugarcane phloem are often seen with different sizes in their diameter from 80-900 nm. Although it is not possible to culture this phytoplasma *in vitro* but the progress of molecular biology nowadays has allowed an opportunity to analyze comparable genetic information involving its genotypic characterization. Roughly, the SCWL phytoplasma has been classified into the Class Mollicutes of the Tenericutes Division under Prokaryotes Kingdom. Recent genomic analysis concerning the 16S rDNA sequence has categorized its position into the phylogenetic SCWL group that the memberships of this group as shown in **Table 1** include each representative causal phytoplasma of SCWL in Thailand, SCGS in India, rice yellow dwarf (RYD) in Japan and Thailand, nepier grass stunt in Kenya and Uganda, kalimantan coconut wilt in Indonesia, waligama coconut wilt in Sri Lanka, coconut root wilt and areca palm yellow leaf (YLD) in India, burmuda or cynodon grass white leaf (BGWL) in Thailand, Australia, China, Italy and India, Delhi grass white leaf (DicWL) in India, chlorotic streak and white leaf of *Oplismenus burmannii* and *Digitaria sanguinalis* grasses in India, coconut yellow decline (CYD) in Malaysia, brachiaria grass white leaf (BraWL) in China and Thailand, sorghum grassy shoot (SGS) in Australia, BVK phytoplasma from *Psammotettix cephalotes* leafhopper, galactia little leaf (GaLL) in Australia, and *Circium arvense* phyllody (CirP) in Germany. The 16S rDNA sequence similarity among the phytoplasmas in Gramineae plants are 97% up and the SCWL phytoplasma shows its similarity percentage as high as 97.5-98.8 to SGS (Rao *et al.* 2008), 98 to Kenya and Uganda Napier grass stunt (Jones *et al.* 2004; Nielsen *et al.* 2007); 97.8-98.4 to BGWL (Jung *et al.* 2003; Marcone

Table 1 The 16S rDNA sequence similarity percentages between SCWL phytoplasma from Udornthani, Thailand (SCWL-Ud) with other phytoplasmas within closely related group.

Phytoplasma	Associated plant disease	Origin	% Similarity to SCWL-Ud	Reference/Investigator
SCWL-T	Sugarcane white leaf	Central Thailand	99.8	Jung *et al.* 2003
SCGS	Sugarcane grassy shoot	India	97.5-98.8	Rao *et al.* 2008
Candidatus Phytoplasma oryzae	Rice yellow dwarf (RYD)	Japan, Thailand	97.6	Jung *et al.* 2003
	Nepier grass stunt	Kenya, Uganda	98	Jones *et al.* 2004; Nielsen *et al.* 2007
	Kalimantan wilt disese of coconut	Indonesia	98	Warokka 2005
	Waligama wilt disese of coconut	Sri Lanka	98	Nejat *et al.* 2009; Nejat and Vadamalai 2010
	Root wilt disese of coconut	India	99	Manimekalai *et al.* 2010b
	Yellow leaf disease (YLD) of areca palms	India	99	Manimekalai *et al.* 2010a
Candidatus Phytoplasma cynodontis	Burmuda grass white leaf (BGWL)	Khon Kaen and Central Thailand, Australia, China, Italy, Iran	97.8-98.4	Jung *et al.* 2003; Marcone *et al.* 2004; Salehi *et al.* 2009
		India		Rao *et al.* 2007b; Snehi *et al.* 2008
	Delhi grass white leaf (DicWL)	India	97.9	Nasare *et al.* 2007; Rao *et al.* 2009
	Chlorotic streaks and white leaf disease of *Oplismenus burmannii* and *Digitaria sanguinalis* grasses	India	97	Rao *et al.* 2010
	Coconut yellow decline (CYD) diseases in cv. 'Malayan Tall' and 'Malayan Red'	Malaysia	98	Nejat *et al.* 2009; Nejat and Vadamalai 2010
BraWL	Brachiaria grass white leaf	Thailand, China	97.9-98.4	Jung *et al.* 2003; Marcone *et al.* 2004
SGS	Sorghum grassy shoot	Australia	97.1	Blanche *et al.* 2003
BVK	Phytoplasma from *Psammotettix cephalotes*	Germany	96.1	Seemüller *et al.* 1998; Rao *et al.* 2008, 2009
GaLL	Galactia little leaf	Australia	95.5	Jung *et al.* 2003
CirP	Circium phyllody	Germany	94.6	Jung *et al.* 2003; Rao *et al.* 2008, 2009

Table 2 The 16S-23S rDNA gene spacer sequence similarity percentages between sugarcane white leaf phytoplasma from Udornthani (SCWL-Ud) with other SCWL from different sources and with some related grass species.

Phytoplasma isolates	Associated disease	Origin	% Similarity to SCWL-Ud	No. bases sequenced	Reference/ Investigator
SCWL-Kk	Sugarcane white leaf	Khon Kaen, Thailand	98-100	210, 810	Wongkaew 1999
SCWL infected *M. hiroglyphicus* leafhopper	SCWL-Insect vector	Udornthani, Thailand	97	810	Wongkaew 1999
SCWL-TC	Sugarcane white leaf	Tissue cultured plantlets, Khon Kaen, Thailand	93	810	Wongkaew 1999
SCWL-Nr	Sugarcane white leaf	Nakornrachasima, Thailand	89	810	Wongkaew 1999
SCWL-Sk	Sugarcane white leaf	Sra Kaew, Thailand	89	810	Wongkaew 1999
SCGGS	Sugarcane green grassy shoot	Suphanburi, Thailand	96-98	210, 810	Wongkaew *et al.* 1997; Wongkaew 1999
SCGGS	Sugarcane green grassy shoot	Southern Thailand	87	452	Sadudee 2004
SCGS	Sugarcane grassy shoot	Sri Lanka	98	452	Ariyarathna *et al.* 2007
SCGS	Sugarcane grassy shoot	India	86.9	240	Rao *et al.* 2007a
			97.9	210	Rao *et al.* 2008
SGS	Sorghum grassy shoot	Australia	86.9	452	Ariyarathna *et al*. 2007
Candidatus Phytoplasma cynodontis	Burmuda grass white leaf (BGWL)	Australia, India, Italy, Thailand	83-87	210, 250, 810	Wongkaew 1999; Marcone *et al*. 2004; Rao *et al.* 2007b, 2008
	Firouzabud burmuda grass white leaf (FBGWL)	Iran	90.8	210	Salehi *et al.* 2009
	Juyom burmuda grass white leaf (JBGWL)		91.2		
BraWL	Brachiaria grass white leaf	China, Thailand	83-87	210, 810	Wongkaew 1999; Rao *et al.* 2008
Candidatus Phytoplasma oryzae	Rice yellow dwarf (RYD)	Japan, Thailand	79.8	210	Rao *et al.* 2008

et al. 2004; Rao *et al*. 2007; Snehi *et al.* 2008; Salehi *et al.* 2009), 97.9-98.4 to BraWL (Jung *et al.* 2003; Marcone *et al.* 2004), 97.9 to DicWL (Nasare *et al.* 2007; Rao *et al.* 2009), 97.1 to SGS (Blanche *et al.* 2003) and 97 to *O. burmannii* and *D. sanguinalis* chlorotic streak and white leaf (Rao *et al.* 2010). Moreover, the analyses of 16S rDNA sequences of phytoplasmas affected coconut and other palm species in some Asian countries have revealed their close relationship to SCWL phytoplasma with as high as 98-99% similarities such as the phytoplasma in kalimantan coconut wilt (Warokka 2005), waligama coconut wilt (Nejat *et al*. 2009; Nejat and Vadamalai 2010), coconut root wilt and areca palm YLD (Manimekalai *et al*. 2010) and coconut CYD (Nejat *et al*. 2009; Nejat and Vadamalai 2010).

Determination of 16S-23S rDNA spacer region sequence of SCWL phytoplasma and its closely related strains provide similar trend of their relationship. The similarity percentages in reference to the SCWL-Ud from Udornthani Province, Thailand are summarized in **Table 2** on the basis of the sequenced 210 bases (bp) level (Wongkaew *et al.* 1997; Wongkaew 1999; Rao *et al.* 2008, 2009; Salehi *et al.* 2009), 240 bp level (Rao *et al.* 2007a), 250 bp level (Rao *et al.* 2007b), 452 bp level (Ariyarathna *et al.* 2007) and 810 bp level (Wongkaew 1999, 2000). The SCWL phytoplasmas from Udornthani and adjacent Khon Kaen Province of Thailand exhibit an identical or almost identical sequence with 98-100% similarity in 210-810 bp level sequencing. A very close relationship has also obtained with the India and Sri Lanka SCGS at 97.9-98% in 210-452 bp level and with the SCGGS from central Thailand at 96-98% in 210-810 bp levels. The relationships of SCWL-Ud with Southern Thailand-SCGGS, SGS, RYD, BGWL and BraWL have been shown up to be 87, 86.7, 79.8, and 83-87% of similarity, respectively. It is interesting that there is also diversity among the SCWL phytoplasma 16S-23S rDNA sequenced from its insect vector and different sources or locations of the SCWL-diseased sugarcane collected. As the sequence similarity between SCWL-Ud and SCWL from other source such as SCWL-Khon Kaen, SCWL in *M. hiroglyphicus*, SCWL in infected tissue culture plantlets, SCWL-Nakornrachsima Province, and SCWL-Srakaew Province have been found to be 98, 97, 93, 89 and 89%, respectively. Thus, it seems that this diversity may possibly caused by cultural environment, host species or cultivars and geographical differences. However, further details such as their cross interaction, the whole genome sequencing, environmental effects and other biological behaviors are still necessary to justify the true classification and effective disease control.

SCWL DISEASE DIAGNOSIS DEVELOPMENT

Conventional disease diagnosis

1. Diagnosis based on symptoms

The disease can be easily distinguished by its predominant symptoms of thorough white leaf expression and bunchy stunt, although some variations from white to green color are presented within the scope of classical symptoms. Physical finding in this type of diagnosis are based on close speculations following the progress of disease development in growing cane and suspected mask symptom accounts. In this scheme the cane setts prepared from suspected stems are transplanted separately into each sandy soil filling pot in greenhouse condition and the growing plants are investigated daily to notify the development of the disease. Typical symptoms of SCWL disease usually arise since the sprout of their first leaf and are obviously displayed within 2-4 weeks from the infected cane setts. On the other hand, diagnosis can also be done by observing the remission of SCWL disease symptoms of cane plants after infiltration of the tetracycline antibiotic into main stems (Wongkaew and Fletcher 2004). This method depends on factors such as the extent of accompanying phytoplasma, indigenous cane sett health, planting season, and other environmental circumstances. Thus, diagnosis by the symptom basis is considered less efficient; however it still can be used to ensure the visual characterization of the disease.

2. Microscopy techniques

Microscopic methods that have been used to detect phytoplasma in the infected sugarcane plants include observations using light microscope, fluorescence microscope and transmission electron microscope (TEM). In light microscopy, the phloem cells of sugarcane infected with phytoplasma exhibit a distinguish deep blue coloration (**Fig. 5A**) that is absent in healthy sieve tissues (**Fig. 5B**) after staining

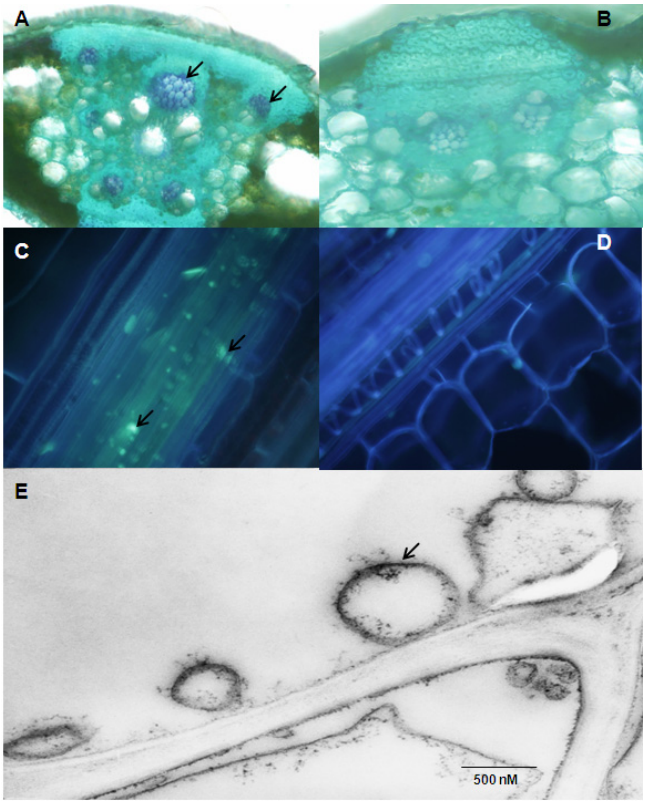


Fig. 5 Observation for the existence of sugarcane white leaf phytoplasma within plant tissues by microscopic techniques. (**A**) Dienes' staining-light microscopy of infected tissue; (**B**) Dienes' staining-light microscopy of healthy tissue; (**C**) DAPI staining-fluorescence microscopy of infected tissue; (**D**) DAPI staining-fluorescence microscopy of healthy tissue; (**E**) ultrastructural micrograph of the phytoplasma cells localized in sugarcane phloem sieve cells revealed by transmission electron microscopy. Arrows indicate the presence of phytoplasma.

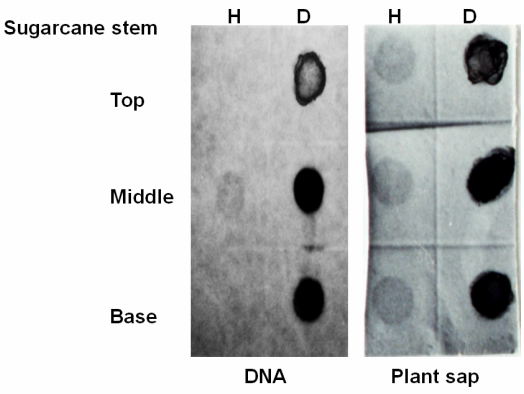


Fig. 6 Detection of sugarcane phytoplasma containing in the top, the middle and the base of sugarcane stem by dot blot hybridization using 16S-23S rDNA probe. Simplified detection using direct plant sap (on the right) produces similar dot blot reaction as the use of DNA extract (on the left), the H columns represent healthy plant and the D columns represent diseased plant. Reprinted from **Wongkaew P** (1999) *Sugarcane White Leaf Disease Management*, Thailand Research Fund, Pimpatana Press, Khon Kaen, Thailand, 228 pp, with kind permission from the publisher.

the tissue sections with Dienes' stain proposed by Deeley *et al.* (1979). Florescence microscopy in combination with DNA-specific fluorochrome, 4-6-diamidino-2-phenylindole (DAPI) staining of tissue sections is a sensitive and reliable technique for rapid and precise localization of phytoplasma in phloem sieve of the infected sugarcane. By this technique, the infected phloem cells show a diffuse fluorescence brighter than the one typical of the nuclei of parenchyma cells and these bright spots are not visible in healthy tissues (**Fig. 5C, 5D**). In case of transmission electron microscopy (TEM), it reveals the presence of phytoplasma cells in sieve and neighboring cells of the infected plants (**Fig. 5E**). The ultrastructural characteristics of phytoplasma and host cell modifications induced by phytoplasma can be demonstrated by this technique (Nakashima *et al.* 1999; Wongkaew and Fletcher 2004). It has been shown by this TEM illustration that the SCWL phytoplasma shapes are pleomorphic due to the lack of cell wall and are variable in size ranging from 80-900 nM in diameters. Although these traditional microscopy techniques have permitted only morphological characters that are indistinguishable among phytoplasma strains, but their performances remain valuable for preliminary diagnosis and cytological investigation on host-parasite interactions.

3. Serology-based techniques

A serological method using conventional polyclonal antibodies has been tested for the detection of SCWL phytoplasma in the last decade (Sarindu and Clark 1993; Wongkaew 1999). The antisera rose up to 1:100 titers that were adequate for an indirect enzyme linked immunosorbent assay (ELISA). This method can differentiate the phytoplasma in diseased gramineae plants such as white leaf disease and green grassy shoot disease of sugarcane and the white leaf disease of grasses from other phytoplasma in diseased dicots such as cowpea phyllody and sesame phyllody. But the antisera also showed high cross reaction signal and low sensitivity which limited their use for desirable diagnosis. Recently, monoclonal antibodies have been introduced to improve detection efficiency and the SCWL phytoplasma detection kit has then been developed commercially for field diagnosis by a collaborative program of National Center for Genetic Engineering and Biotechnology, Thailand (Patent No. 9582). However, it has not yet widely accepted because of a considerable high cost due to its complicate preparation and high investment.

4. Dot blot-DNA hybridization techniques

Detection of the hybridization between complementary DNA by dot blot technique has been employed for SCWL disease diagnosis in the last decade. The DNA probes used in the assays include randomly cloned fragments of chromosomal and extrachromosomal DNA of SCWL phytoplasma and the complementary ribosomal DNA (Klinkong and Seemüller 1993; Nakashima *et al.* 1994; Wongkaew *et al.* 1995; Wongkaew 1999). The resulted signal color intensity is highest with the cloned extrachromosomal DNA in both peroxidase-labeled gene detection system and digoxigenin colorimetric gene assay, while the faintest signal is from 16S-23S rDNA probe. All probes are capable to rank the phytoplasma amount in various parts of sugarcane stem approximately by their reaction color intensity. The cloned DNA probe detects phytoplasma DNA in SCWL diseased plants and the insect vector *M. hiroglyphicus* as well as phytoplasma DNA from other white leaf diseased Gramineae plants such as brachiaria grass, burmuda grass, crowfoot grass and yellow dwarf diseased rice plants. Dot blot-DNA hybridization techniques have been successfully simplified for field survey detection with sugarcane sap stream from freshly cutting as displayed in **Fig. 6** (Wongkaew *et al.* 1998). Diagnosis of SCWL disease has been done by this dot blot-DNA hybridization for several years, but recently it is rarely used because of its complexity and time consuming process in comparison to a subsequently developed polymerase chain reaction technique.

5. Polymerase chain reaction based techniques

Polymerase chain reaction (PCR) has distinct advantages over dot blot-DNA hybridization by its higher sensitivity and specificity. The technique requires less processing time and complicates operation. A single copy of a target DNA

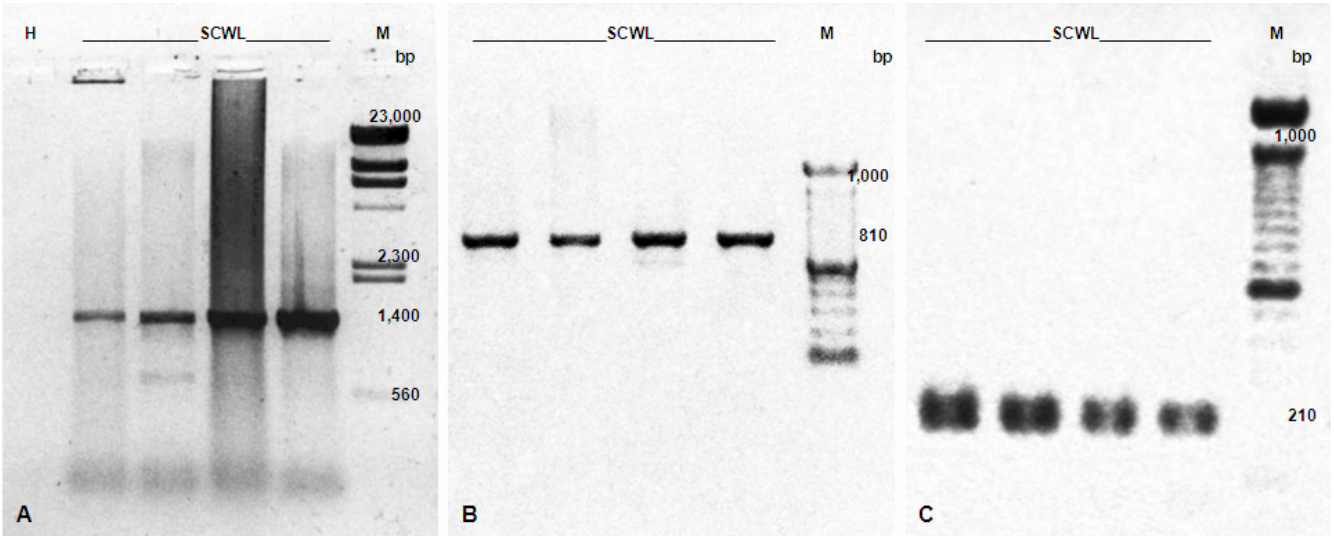


Fig. 7 Electrophoresis gel expression in the detection of sugarcane white leaf phytoplasma by traditional polymerase chain reaction and nested polymerase chain reaction techniques. (**A**) indication of the phytoplasma DNA in samples by the appearance of 16S rDNA at 1,400 bp; (**B**) indication of the phytoplasma DNA in samples by the appearance of 16S-23S rDNA at 810 bp; (**C**) nested PCR indication of the phytoplasma DNA in samples by the appearance of 16S-23S rDNA at 210 bp. Reprinted from **Wongkaew P** (1999) *Sugarcane White Leaf Disease Management*, Thailand Research Fund, Pimpatana Press, Khon Kaen, Thailand, 228 pp, with kind permission from the publisher.

sequence can be amplified one million fold within an hour prior to an electrophoresis illustration. Thus, PCR has been widely used for SCWL phytoplasma detection in the concerning laboratories (Nakashima *et al.* 1996; Wongkaew *et al.* 1997; Sdoodee *et al.* 1999; Kumarasinghe and Jones 2001). A variety of primer pairs have been designed according to the ribosomal gene alignment including the universal phytoplasma primers from 16S rDNA and the narrowed group specific primers from 16S-23S rDNA sequences. The universal primers produce a DNA band size of about 1.4 kb that indicates the presence of non-specific phytoplasma 16S rDNA in sample. While the group specific primers are capable to demonstrate the phytoplasma in sugarcane and gramineae white leaf diseased samples which the DNA band at either 210 or 810 bp can be seen depending on the selected primers (**Fig. 7**). Detection of SCWL phytoplasma usually employs a common one-round PCR cycle using any pair of those universal or more specific primers for the target plant samples and a nested PCR which re-amplifies the first round PCR with an internal primers specific to the phytoplasma for the target insect vector and suspected-very low amount of phytoplasma containing samples. In spite of a powerful capability, these techniques need special skills to carry out an operation and are too much expensive for routine detection in sugarcane industry. The techniques thus are being used only for certain confirmation in laboratories and research activities.

INNOVATIVE DNA BIOSENSORS IN SCWL PHYTOPLASMA DETECTION AND THEIR PERSPECTIVE

The term "biosensor" implies for an analytical system that integrates a biological component with a physicochemical detector to yield a measurable electric signal. It generally consists of three components: a biological sensing element, a transducer or detector element and a signal processor. This system takes advantage of the ability of a biomolecule to specifically recognize the target substance. The biological sensing element can be created from biological materials such as enzymes, antibodies, nucleic acid, cell organelles, microorganisms, biomimics, and etc. that specifically recognize the target in the analyzing condition and produce a signal related to the concentration. The transducer or detector element can be each of or combination of physicochemical techniques such as optical, thermometric, piezoelectric, magnetic, micromechanical and electrochemical techniques

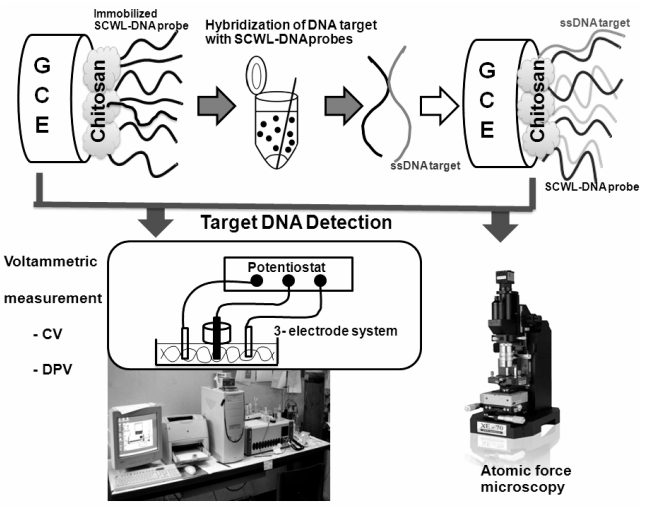


Fig. 8 Schematic diagram of DNA biosensor for sugarcane white leaf phytoplasma detection using voltammetry method and visualization of the hybridization event by atomic force microscopy.

that transform the recognizing reaction into recordable signal for electronic processor to interpret and display the output result. Biosensors have been widely applied in various types of research and commercial industry for monitoring the target or bioterrorists in an environmental atmosphere such as food, medicine, metabolite, and pathogenic infection. Among several types of biosensors, the DNA biosensor in combination of an electrochemical method has provided the utmost desirable tool for several biological analyses including disease diagnosis because of its simplicity, rapidity, low cost and ability to work with turbid samples. Techniques mainly used in electrochemical transduction include amperometric, conductometric, impedimetric, and potentiometric that based on corresponding signal measurement as current, conductance, impedance and potential, respectively. The specificity and unlimited sensitivity of this biosensor type can be easily enhanced in coupled with a modification of biosensing platform and other biosensing techniques (Teles and Fonseca 2008). The electrochemical DNA biosensors have been successfully developed following the rule of DNA hybridization for rapid detection of several microorganisms such as hepatitis B virus (Erdem *et al.* 1999, 2000; Ye and Ju 2003; Guo *et al.* 2007), *Micro-*

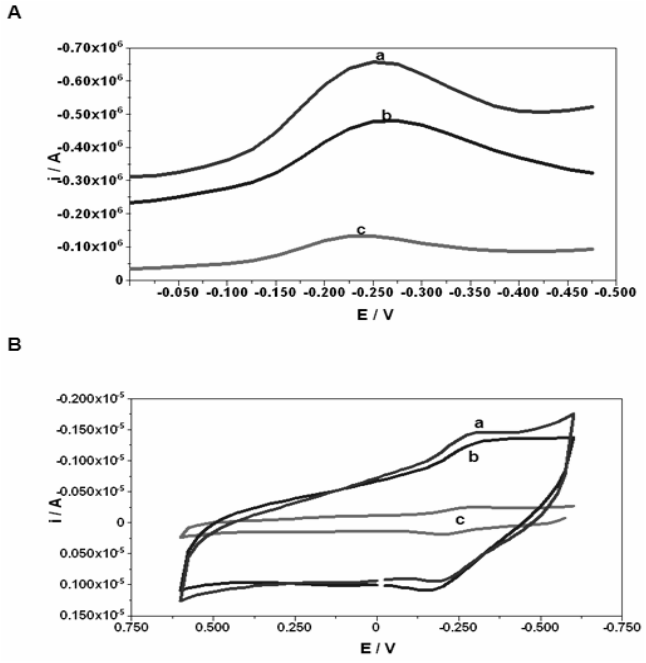


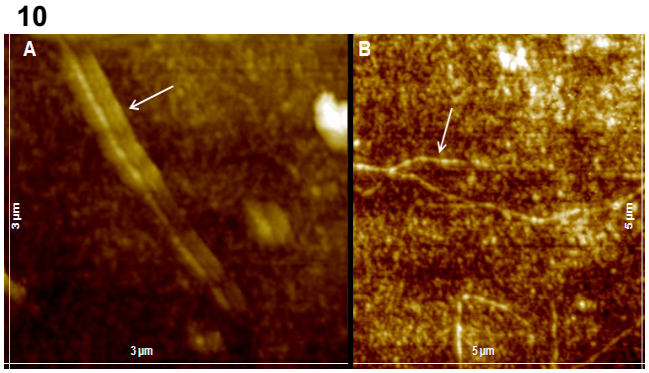
Fig. 9 Detection of sugarcane white leaf phytoplasma DNA from sugarcane plants using SCWL-ssDNA probe by differential pulse (A) and cyclic (B) voltammograms. (a) Hybridization with ssDNA from healthy plant, (b) SCWL-ssDNA probe only, (c) hybridization with ssDNA from diseased plant. The signal current of an intercalator methylene blue was determined in 20 mM Tris buffered saline (pH 7.0) at 100 mVs^{-1} scan rate.

cystis spp. (Erdem *et al.* 2002), native yeast (Ju *et al.* 2004), human immunodeficiency virus (Zhang *et al.* 2010), *Yersinia enterocolitica* (Sun *et al.* 2010) and several food-borne pathogenic bacteria (Velusamy *et al.* 2010).

The electrochemical DNA biosensor has been applied in the detection of SCWL phytoplasma (**Fig. 8**). It has been tried first with a capacitance measurement of the hybridization reaction using a pair of 21 bp each known sequence from phytoplasma 16S rDNA designed by Namba *et al.* (1993) as complementary DNA probes and oligochitosan-modified glassy carbon electrode as conductive platform. The charging current signal and total capacitance obtained from hybridization reaction between phytoplasma 16S rDNA probes with ssDNA of SCWL diseases plant target have shown to be obviously higher than the reaction with its noncomplementary ssDNA of healthy sugarcane plant (Wongkaew and Poosittisak 2008). Subsequently, a label-free DNA biosensor using the whole chromosomal ssDNA of SCWL phytoplasma that immobilized on chitosan-modified glassy carbon electrode (GCE) has been developed in couple with methylene blue intercalator for specific indication of the SCWL-DNA hybridization. Progressive detection capability can be achieved by cyclic and differential pulse voltammetry through the three electrode system potentiostat controlled with general purpose electrochemical system software (**Fig. 9**). Quantitative determination of SCWL phytoplasma DNA in the target sample could be operated in these experiments with a detection limit of 0.1 nM. The corresponding DNA hybridization event has also been visible through atomic force microscopic (AFM) observation as shown in **Fig. 10** (Wongkaew and Poosittisak 2010). These performances thus allow a feasibility of the DNA biosensor for practical use in SCWL phytoplasma detection and encourage further studied on the efficiency enhancement and optimization for real time field monitoring by a reliable, cost effective and convenient portable device.

DISEASE CONTROL STRATEGIES

Several control strategies have been introduced including



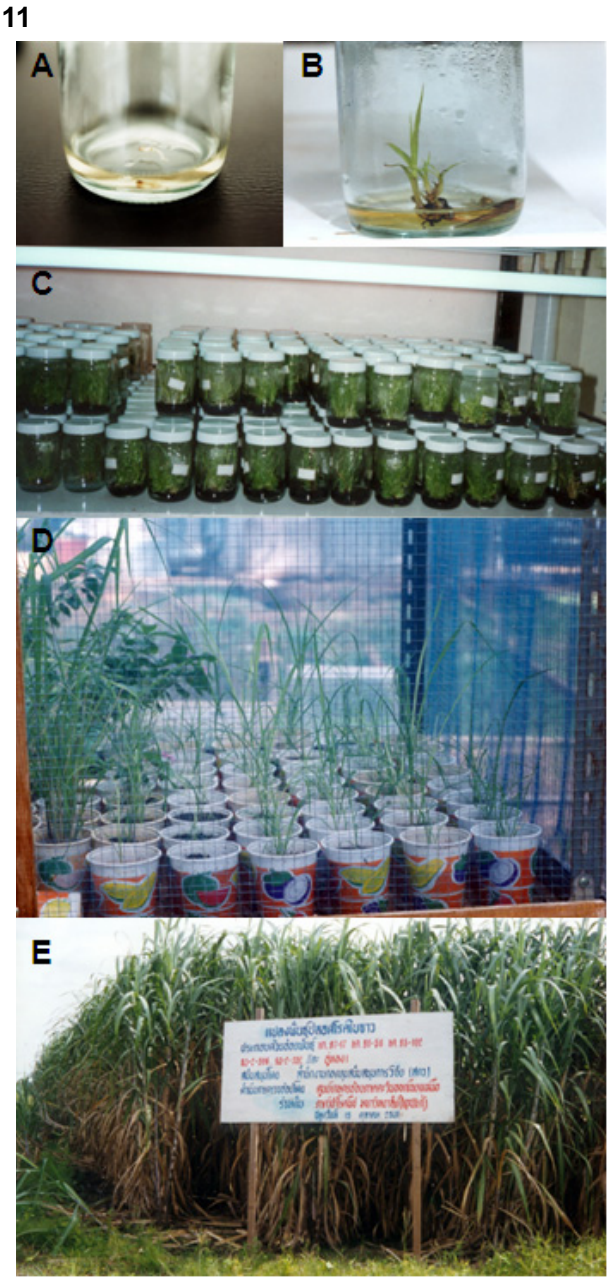


Fig. 10 Atomic force microscopic observation of the hybridization event directed by sugarcane white leaf DNA biosensor. (**A**) Hybridization with complementary phytoplasma DNA from diseased plant; (**B**) reaction with non-complementary DNA from healthy plant. Arrows indicate observed DNA strands. **Fig. 11** Production of disease-free sugarcane plants and cane setts from apical meristem tissue cultures. (**A**) Cultured apical meristem section; (**B**) shooting derivation; (**C**) developing plantlet cultures; (**D**) acclimatization of plantlets transplanting; (**E**) field-grown plants intensively prepared for disease cane setts production. Reprinted from **Wongkaew P** (1999) *Sugarcane White Leaf Disease Management*, Thailand Research Fund, Pimpatana Press, Khon Kaen, Thailand, 228 pp, with kind permission from the publisher.

eradication of the phytoplasma in sugarcane setts by hot water and tetracycline treatment, disease free plant production and propagation, sanitation, crop rotation, insecticide application, regulatory quarantine, and disease resistance.

Hot water and tetracycline treatments

It has been reported that treatment of sugarcane setts with hot water only could not eliminate SCWL phytoplasma (Ling and Chaung-Yang 1963a; Liu *et al.* 1963). Unsuccessful therapy has been indicated from the heat treatment studies by dipping infected cane setts into hot water at 50°C for 2-4 h and at 53°C for 1 h. Somewhat curing effect could be seen when the cane setts were treated with hot water at 52°C for 8 h and 54°C for 40 min, but most of the cane setts could not withstand nor produce regular shoot buds following such high temperature in that given times. Better results have been achieved in the treatment with tetracycline antibiotic. Shigata *et al.* (1969) reported that treatment of cutting infected sugarcane with tetracycline for 72 h could reduce SCWL phytoplasma multiplication and the growing shoots remained free of white leaf disease symptoms for three months. Similarly, temporary reduction of the disease symptoms by tetracycline has been confirmed in an investigation performed by Mongkolsuk and Sutrabutra (1976) in Thailand. In this case curing effect was shown up within 2 weeks after dipping the cutting in tetracycline solution at 200-500 ppm for 24 h. The leaves became completely green in the 4th week and stayed green for 8 weeks. Remission of SCWL symptoms has also been demonstrated in tissue culture system that about 70-100% of plantlets sustained the green appearance through 5-8 subcultures or at least 8 months of sequential serial transfers from their mother plantlets cultures that were grown in 200-500 ppm of oxytetracycline (Wongkaew and Fletcher 2004).

Disease-free plant production and propagation

The strategy of control through disease-free plants is considered to be one of the most effective methods for controlling a pathogen that has systemically colonized the plant such as the case of SCWL phytoplasma. Intensive production programs by conventional selection and tissue culture technique carried out in Taiwan have proved to be highly success in breaking off the disease epidemic (Leu 1978; Liu 1981). In Thailand, the disease free plants could be extensively produced from tissue culture of meristem tip as well (Wongkaew and Fletcher 2004). An extension in the field use of disease free tissue culture plants and their derived cane setts has been established during 1996-1999 by sugarane white leaf management research project as concluded in **Fig. 11** (Wonkaew 1999). Selves' preparation of disease free healthy cane setts by growers have also intensively persuaded although this propaganda has not yet fully accepted for their routine practicing.

Sanitation and crop rotation

The main activity for sanitation to eliminate the re-infective risk of SCWL phytoplasma is concentrated on removal and disposal of infected plants and debris from the fields. Crop rotation with other plant species seemed to be unnecessary in an adequate water supply land of Taiwan which other effective plans have been occupied. According to the lower disease occurrence record during spring season, the sugarcane growers have been suggested to change their planting time from ordinary autumn to the planting in spring time. As the population of *M. hiroglyphicus* insect vector and the multiplication of SCWL phytoplasma are limited by low temperature during winter season. This situation, thus deteriorate the infection efficacy in the following spring (Ling and Chaung-Yang 1963b; Pan and Yang 1970). However, in unavailable water supply areas, a substitution of sugarcane planting with green manure plants from season to season is strongly recommended. Combining these actions with the use of disease free cane setts strategies have made Taiwan completely control the disease to a harmless condition ever since (Leu and Kusalwong 2000).

In tropical country such as Thailand which there is not much different in year round temperature, it is insignificant for planting time differences. The most appropriate strategies are likely the sanitation, the disease free cane setts utilization and the crop rotation with economic green manure plants. As there has been an evidence that the SCWL disease could be successfully controlled in a defined research area at Udornthani Province of Northeast Thailand during 1989-1991 using pigeon pea (*Cajanus canja* L.) and sword bean (*Canavalia gladiata* Jacq.) as green manures for crop rotation and healthy new cane variety for a replacement of the weak old variety (Kusalwong and Ouvanich 1993). Five years later, the intensive control program has to be repeatedly performed during 1996-1999 against a higher epidemic incidence of SCWL disease reoccurrence due to an ignorance of such proper cultural practices (Wongkaew 1999). Although the losses were effectively reduced through this latter program until the end of the project period, however, the SCWL disease has resettled soon after that by similar former reasons.

Insecticide application

Application of insecticides to kill the insect vector in the field infested with SCWL disease has proved ineffective. The attempt on spraying an insecticide malathion since the young stage of sugarcane plant has failed to control the vector and the disease incidence (Leu, 1974). In the sugarcane white leaf management project, over 3.2 hectares of each sugarcane plantation were thoroughly treated with carbosulfan insecticide every two weeks from April to June before the insect vector population could reach its regular peak in July and August. But neither the insect vector nor the disease has been successfully controlled by this insecticide application (Wongkaew 1999).

Regulatory quarantine

While sugarcane is a prohibit material by plant quarantine regulation, a strict inspection is still needed for the local SCWL disease to limit further distribution of the disease. This cooperative practice has contributed successful control of SCWL disease in Taiwan (Leu 1983). However, regular inspection of cane setts and growing plants in the fields including mother plants for propagation has not yet widely applied in Thailand, thus sustains yearly serious epidemic spread and economic losses by this disease.

Disease resistance

Although the use of disease resistant varieties is the most desirable mean for growers, however it is unavailable in sugarcane to the white leaf disease. The screening test for SCWL disease resistance from at least 400 varieties including a wild cane *Saccharum spontaneum* L. by insectary method has displayed inopportunity in obtaining the required resistant character (Leu 1974). Another test with 158 sugarcane varieties and 24 hybrids in Thailand in 1988-1991 has also shown susceptible response in all populations (Ouvanich and Kusalwong 1993; Ouvanich *et al.* 1988). Recent observations on sugarcane varieties including their hybrids and wild canes in Thailand during 2004-2007 have confirmed the unavailability of resistance or tolerance to this SCWL disease (Sa-Nguanrangsirikul *et al.* 2007). Subsequent investigations by natural selection screening and breeding programs have still proceeded by various associations involve such as the sugarcane research experiment stations of the Ministry of Industry and the Ministry of Agriculture and Cooperatives including private sugarcane associations and universities, but so far there is no report on the evidence of SCWL disease resistance up to the present.

CONCLUSIONS

SCWL disease is named after the complete white color developed appearance of the whole leaf in severely affected plants in Taiwan and Thailand. The disease has also been reported for its sporadically occurrence in Bangladesh, Japan, Pakistan and Sri Lanka. Variation within the white leaf symptoms and their severity degree are often shown up due to several factors such as soil fertility, temperature, cane sett quality, cultural practice and the causal phytoplasma amount. Transmission of this phloem sieve-colonized phytoplasma by insect vector has been confirmed firstly with *M. hiroglyphicus* leafhopper (Matsumoto *et al.* 1969; Yang and Pan 1969; Pisitkul *et al.* 1991; Wongkaew and Fletcher 2004). The SCWL phytoplasma DNA has also been detected in at least six other leafhoppers but at present only *Y. flavovittatus* has been experimentally found to posses SCWL phytoplasma transmissibility (Wongkaew 1999; Hanboonsong *et al.* 2006). Epidemic outbreak of SCWL disease is primarily activated by transportation and the use of endemic cane setts for sugarcane production and secondary spread within growing fields and nearby areas is then accelerated by insect vectors. The causal phytoplasma has been placed into phylogenetic group of SCWL concerning the 16S rDNA sequence. The reports on 16S-23S rDNA sequence analyses have indicated that SCWL and SCGGS of Thailand and SCGS of India are closely related with 96-98% similarity. While some diversity according to the 16S-23S rDNA sequences has also been found among SCWL phytoplasma isolates from different sources and locations within 89-98% similarity range (Wongkaew 1999, 2000). The evidence of this diverseness thus remains to be clarified.

The SCWL disease has formerly diagnosed by its symptoms expression. Microscopic techniques include light microscopy with Dienes' staining, fluorescence microscopy with DAPI staining and transmission electron microscopy have then and still been used for morphological identification and investigation on cytological interaction during pathogenesis. Diagnosis of the disease can be done by other conventional methods such as serology-based techniques, dot blot DNA hybridization and PCR but they need high investment and special skill with laborious production and detection process. Recently, preliminary experiments on electrochemical analyses of SCWL-DNA biosensors for specific DNA hybridization have proved their advantages as new desirable diagnostic tool. As significance differences in charging current signals and total capacitances have been displayed in the reaction after hybridization of the SCWL-16S rDNA probe with healthy and diseased plant DNA (Wongkaew and Poosittisak 2008). Discrimination between DNA extracts from healthy and SCWL diseased plants has also been pronounced by cyclic and differential pulse voltammetry using the whole chromosomal DNA probe for specific DNA biosensor. This difference in hybridization reaction event has similarly revealed by visible images under atomic force microscopy (Wongkaew and Poosittisak 2010). These results hence provide the feasibility to construct the new rapid, specific, quantifiable, simple and cost effective portable tool for routine use in early warning and control of the SCWL disease.

The strategies for SCWL disease control are mostly relied on cultural methods because disease resistant cultivars are unavailable and physico-chemical therapy including insecticide application are ineffective. Excellent control of SCWL disease has been achieved in Taiwan by means of field sanitation and disease-free cane sett transplanting in spring time instead of an ordinary planting season in autumn (Pan and Yang 1970). Integration of disease disinfestations, green manure crop rotation and re-transplanting with disease free cane setts have also proved effectively control SCWL disease in Thailand during the intensive disease management programs (Kusalwong and Ouvanich 1993; Wongkaew 1999). But these strategies have been neglected and hence result in persisting serious epidemic spread throughout the country. Cooperative assistances among involving associations and intensive researches for innovative tools should be emphasized in coupled with a proper subsidy to accomplish the successful disease control.

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