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Sugarcane white leaf phytoplasma in tissue culture: long-term maintenance, transmission, and oxytetracycline remission

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Abstract Sugarcane white leaf (SCWL)-diseased sugarcane plants collected from Udonthani Province, in northeastern Thailand, were the source for tissue culture experiments. Explants from axillary buds, meristem tips, and leaves grew optimally in Murashige-Skoog medium containing 0.5 mg/l α -naphthaleneacetic acid, 0.5 mg/l 6-benzylaminopurine, and 15% coconut water. Callus development and shoot/root proliferation were more rapid in cultures from diseased than from healthy plants. Disease symptoms continued for 6 years after culture initiation, and SCWL phytoplasma persisted, as confirmed by polymerase chain reaction using both 16S rDNA and 16S-23S rDNA primers. Phytoplasmas in the cultured plantlets were transmissible by grafting to sugarcane and periwinkle, and by feeding of the leafhopper vector *Matsumuratettix hiroglyphicus* to sugarcane. Although 50% of the plantlets were killed by oxytetracycline at 500 mg/ml, 70–100% of plantlets grown with 200–500 mg/ml oxytetracycline showed symptom remission through 5–8 subcultures. Typical phytoplasma-like bodies, visible by electron microscopy in sieve tubes of untreated diseased plantlets, were absent in antibiotic-treated plantlets. Thus, tissue culture provides a convenient and reliable *in vivo* system for investigation of SCWL phytoplasma.

Keywords Sugarcane tissue culture · SCWL phytoplasma · Persistence · Graft transmission · Insect transmission · Oxytetracycline treatment

Introduction

Sugarcane white leaf (SCWL) disease, considered the most important disease of sugarcane in Thailand (Wongkaew et al. 1999), is caused by a phytoplasma transmitted naturally by the leafhopper vector *Matsumuratettix hiroglyphicus*. Symptoms on sugarcane (*Saccharum officinarum* L.) include complete loss of green pigment in the leaf, which becomes completely white, and profuse tillering (Fig. 1a). Knowledge of the SCWL phytoplasma has been limited by the inability to culture it, or any phytoplasma, *in vitro*. Plant material collected from sugarcane fields as a phytoplasma source is useful for some studies but does not allow detailed study of the causal phytoplasma. Field collection and nursery or greenhouse propagation of the cane plants is time consuming and tedious.

Tissue culture has been used for propagation of diseased plants *in vitro* as a more constant source of phytoplasmas (Sears and Klomparens 1989; Davies and Clark 1994; Wongkaew et al. 1995; Jarausch et al. 1996). Diseased plant tissue cultures have been effectively exploited for several studies of other phytoplasmas such as the characterization of phytoplasmal DNA and the development of detection protocols (Jarausch et al. 1994a,b). In this paper we describe the establishment of SCWL-diseased plant tissue cultures for long-term maintenance of the pathogenic white leaf phytoplasma as well as for evaluation of the possibility that the phytoplasma could be eliminated from diseased plant tissues by oxytetracycline treatment.

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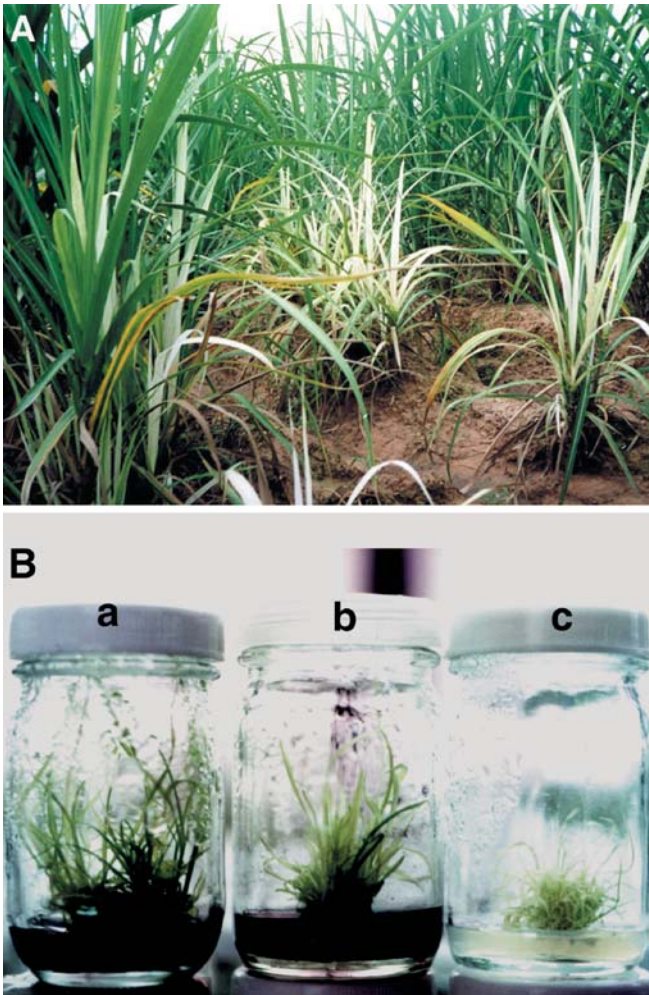


Fig. 1 **a** Sugarcane plants in the field showing typical symptoms of white leaf and profuse tillering and **b** profuse white shoot tiller proliferation following tissue culture of parts of sugarcane white leaf (SCWL)-diseased explants: *a* axillary bud, *b* meristem tip, *c* leaf tissue

Materials and methods

Plant sources

Healthy and white leaf diseased sugarcane plants were collected from sugarcane-growing areas in Udonthani Provinces, north-eastern Thailand and directly subjected to tissue culture preparations.

Tissue culture of sugarcane

Materials for tissue culture experiments were selected from 4 to 5-month-old SCWL-diseased and healthy plants collected in a sugarcane field in Udonthani Province. Tissue explants were excised from three separate parts of the plants: the axillary bud, apical meristem tip, and young leaf tissue next to the apical meristem dome. The explant materials were surface sterilized in 10% (v/v) alcohol for 5 min and then in 10% sodium hypochlorite for 10–15 min followed by rinsing with sterile distilled water. Sterile explants were placed on agar-solidified Murashige-Skoog (MS) medium containing mineral salts including vitamins and sucrose

(Murashige and Skoog 1969) and supplemented with 15% coconut water. Coconut water, the liquid contained in the core of fresh coconuts, is a common additive for the very profitable orchid culture industry in Thailand. It was collected from a number of fresh coconuts, mixed together in a single batch, stored in aliquots at -80°C , and used in all experiments. The medium was adjusted to pH 5.7 before autoclaving for 20 min at 121°C . The following growth regulator combinations and additives were used to optimize the culture conditions: 0.5 mg/l α -naphthylacetic acid (NAA) and 0.5 mg/l 6-benzylaminopurine (BAP); 3.0 mg/l NAA; 0.01 mg/l indole butyric acid (IBA) and 0.1 mg/l gibberellic acid (GA) plus 100 mg/l citric acid; 0.1 mg/l kinetin and 0.2 mg/l BAP; 3.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 1.0 mg/l BAP and 1.0 mg/l IBA; and 3.0 mg/l NAA with 20 mg/l yeast extract. Activated charcoal (Fluka, Buchs, Switzerland) was added at 1 g/l in some cases for prolonged subcultures. For long-term maintenance observation, the tissue cultures were transferred onto fresh medium every 4–6 weeks for at least 6 years and maintained at $25\text{--}28^{\circ}\text{C}$ under 16 h/day light at 3,000 lx.

Oxytetracycline antibiotic treatments

SCWL-diseased sugarcane tissue cultures derived from axillary buds were treated with oxytetracycline (Fluka) to examine the phytoplasmal elimination efficiency. Antibiotic was added at 0, 100, 200, 300, and 500 mg/l to the tissue culture medium, which was supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP. At least 100 well-established tissue cultured shoot clusters were subcultured onto medium containing each oxytetracycline concentration. The shoot clusters were maintained on these media for 4–6 weeks and then transferred to antibiotic-free medium for three subsequent serial transfers. Phytoplasma presence in the subcultures was assessed by the polymerase chain reaction (PCR) and electron microscopy.

Insect transmission test

M. hiroglyphicus leafhoppers were collected from sugarcane fields at Udonthani Province and maintained in the greenhouse in $40\times 50\times 75\text{ cm}^3$ nylon-mesh insect-proof cages containing healthy young sugarcane plants. White leaf phytoplasma-free leafhoppers were maintained by serial feeding transfers on healthy sugarcane plants. Their pathogen-free status was confirmed by the fact that control plants exposed to such leafhoppers as negative controls in these experiments never became diseased. Leafhopper acquisition of phytoplasma from diseased sugarcane tissue cultures was accomplished by confining female adult leafhoppers in groups of four with diseased or healthy tissue cultures for 3 days. An incubation period of 1 week was provided by transferring the insects into cages containing healthy sugarcane seedlings. For inoculation access, insects were placed in groups of five on 3-week-old healthy sugarcane seedling in each cage for 5 days. For each treatment, a total of 50 insects was tested in groups of five on a total of ten test plants. After the inoculation access period, test plants were maintained in the greenhouse at $25\text{--}28^{\circ}\text{C}$ and 14–16 h daylength for 6 weeks and observed daily for symptom development.

Grafting transmission test

Young sugarcanes and periwinkle (*Catharanthus roseus* L.) seedlings used for grafting transmission tests were grown in an insect-proof greenhouse. The terminal growing shoots, approximately 5–7 cm in length, of 4-week-old tissue cultured sugarcane plantlet, about 0.1–0.2 cm in diameter, were excised from culture bottles for use as scions. For sugarcane-sugarcane transmission, a side-graft approach was employed. A second sugarcane plant of about the same size and stem diameter as the scion was selected to serve as the stock. For sugarcane-periwinkle transmission, a wedge-grafting procedure was used. A healthy, 3- to 4-week-old periwinkle seed-

ling of about 0.2–0.3 cm stem or branch diameter was selected as the stock. All graft junctions were wrapped with stretched Parafilm, and grafted plants were enclosed for 3–4 days in a plastic bag to ensure a moist environment. The grafted plants were maintained in the greenhouse at 25–28°C and 14–16 h daylength and observed daily for symptom development. At least five plants of each stock species were grafted with sugarcane plantlet scions from each of 50 culture bottles, for a total of 250 plants in each experiment. Fifty stock plants were grafted with healthy scions as controls. All grafting was carried out in the month of October or November, the season most conducive for grafting success for many crops in Thailand, and always in the early morning or late evening, when sunlight is less direct and the temperature cooler.

Electron microscopy

Leaves from plantlets in at least five tissue culture bottles from each treatment (healthy and oxytetracycline-treated, and untreated) of diseased sugarcane tissue cultures were examined for the presence of SCWL phytoplasma by electron microscopy. Leaves were cut into 1×2 mm pieces and pre-fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 16 h at 4°C. The specimens were rinsed with 0.1 M phosphate buffer containing 0.5% sucrose and were post-fixed with 1% osmium tetroxide for 2 h at 4°C. Afterward the specimens were dehydrated in a graded series 20, 50, 70, 90, 100, 100, 100% of acetone for 30 min in each grade and embedded in Spurr's resin (Spurr 1969). Ultra-thin (70 µm) sections, prepared using a LKB Bromma-8800 ultramicrotome, were stained with lead nitrate and uranyl acetate and observed at a magnification of 20,000× using a Hitachi Hu-12A transmission electron microscope at 75 kV.

Detection of SCWL phytoplasma by PCR amplification

Total DNA from SCWL-diseased sugarcane tissues was isolated by the CTAB method (Kollar et al. 1990; Nakashima et al. 1991). DNA from healthy sugarcane plants and tissue cultures were extracted by the same procedure for comparison. These DNA extract fractions were used as templates for PCR. Two sets of oligonucleotide primers were used as a double check for the presence of SCWL phytoplasma. The first set was a pair of universal 16S rDNA primers: 5'-GTT TGA TCC TGG CTC AGG ATT-3' and 5'-AAC CCC GAG AAC GTA TTC ACC-3', based on the 16S rRNA of various members of the *Mollicutes* (Namba et al. 1993; Wongkaew et al. 1997). PCR amplification using the universal primers was performed in a 25 µl reaction mixture containing 25 ng DNA template; 0.2 mM each of dATP, dGTP, dCTP, dTTP; 0.25 µM each primer and 1 U *Taq* polymerase (Promega, Madison, Wis.) in 1×PCR reaction buffer (supplied by the manufacturer) with 1.5 mM MgCl₂. Thirty PCR cycles were conducted in an Autogene thermocycler (Grant Instruments, Cambridge, UK). Each cycle consisted of 1 min denaturation at 94°C, 30 s annealing at 55°C and 1 min extension at 72°C in the continuing cycles and extension for 3 min at 72°C in the final cycle. The second primer set was based on sequences matching the 16S-23S rRNA region of phytoplasmas (Wongkaew et al. 1997; Hanboonsong et al. 2002). The oligonucleotides sequences of these two primers, MLO-X and MLO-Y are: 5'-GTT AGG TTA AGT CCT AAA ACG AGC-3', and 5'-GTG CCA AGG CAT CCA CTG TAT GCC-3'. Previous work (Wongkaew et al. 1997; Hanboonsong et al. 2002) showed that MLO-X and MLO-Y were specific for a small group of monocot-infecting phytoplasmas including SCWL. PCR amplification was performed in a 20 µl reaction mixture containing 25 ng DNA template; 0.2 mM each of dATP, dGTP, dCTP, dTTP; 0.25 µM each primer and 1 U *Taq* polymerase in 1×PCR reaction buffer with 1.5 mM MgCl₂. PCR was carried out in 37 cycles with the following conditions: 5 min denaturation at 92°C for the first cycle and 1 min denaturation at 92°C for the continuing cycles, 30 s annealing at 60°C, 90 s extension at 72°C and 10 min extension in the last cycle.

The PCR products obtained from each amplification were examined by 1% agarose gel electrophoresis at 100 V and 0.5 µg/ml ethidium bromide in Tris-borate-EDTA pH 8.0 buffer staining. Presence of the phytoplasma in sugarcane tissues was indicated by amplification of a 1.4 kb DNA product when using the universal 16S rDNA primers, or a 0.64 kb band when using the 16S-23S rDNA gene spacer MLO-X,Y primers.

Results

Axillary bud culture

Sprouts and numerous shoots grew from the excised sugarcane axillary buds within 3–5 weeks on MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BAP and 15% coconut water. Abundant roots could be induced by subculturing of these shoots onto fresh MS medium supplemented with 3.0 mg/l NAA and 15% coconut water. However, MS medium with 0.5 mg/l NAA, 0.5 mg/l BAP and 15% coconut water was most suitable for culture maintenance and for subsequent serial transfers. On the latter medium, diseased bud cultures developed a clump of numerous small shoots and hairy roots within 2 weeks of culture, while healthy bud cultures did so only after 4–6 weeks. Thus, more rapid sprouting and shoot proliferation was observed in cultures from SCWL-diseased buds. However, the shoots derived from diseased explants displayed abnormal shoot proliferation, absence of leaf pigment (white leaf), and reduced leaf size (Fig. 1b), while those from healthy bud cultures were much larger and had leaves of normal size and color. Healthy cultures had fewer roots, but the roots were larger than those of diseased samples. The shoot proliferation characteristic of white leaf disease was apparent in every replicate of the diseased bud-derived cultures and in every subsequent serial subculture. There was no spontaneous recovery of disease-free shoots or plantlets from the axillary bud cultures. The presence of SCWL phytoplasma in shoots and plantlets derived from the diseased bud cultures was confirmed in 20 cases by PCR detection using both universal 16S rDNA primers and 16S-23S rDNA primers.

Meristem tip culture

In the meristem tip cultures, elongation and shoot bud development were best induced on MS medium supplemented with 0.01 mg/l IBA, 0.1 mg/l GA and 100 mg/l citric acid. Abundant shoot clumps were produced after transfer to MS medium supplemented with 0.1 mg/l kinetin, 0.2 mg/l BAP and 15% coconut water. Complete plantlets with entire shoot and root systems were obtained on the next transfer, which was to MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BAP and 15% coconut water. This latter formula of the MS medium was therefore used for serial maintenance. In the diseased meristem tip culture, leafy shoot development was visible within 2 weeks, about twice as rapidly as in the healthy meristem tip culture, in which it occurred only 4–6 weeks

Table 1 Apparently healthy and PCR-negative plantlet clumps obtained in cultures derived from sugarcane white leaf (SCWL)-diseased explants

Explant source	Number of explants initiated	Number of symptomatic explants	Number of disease-free explants ^a	% Disease-free explants
Axillary bud	215	215	0	0
Meristem tip	150	144	6 ^b	4
Leaf	150	138	12 ^b	8

^a Number of subcultures counted after transfer of the first initiated shoot stage to fresh medium

^b Absence of SCWL phytoplasma in asymptomatic cultures was confirmed by PCR using both the 16S rDNA universal primers and 16S-23S rDNA SCWL-group specific primers

after culture. Approximately 4% of the plantlets obtained following the first transfer of diseased cultures were asymptomatic and apparently healthy (Table 1). The spontaneous remission of SCWL symptoms was accompanied by the significant reduction or elimination of phytoplasmas as shown by negative PCR results using both the universal 16S rDNA and the 16S-23S rDNA primers.

Young leaf tissue culture

Development of calli was induced by culture of explants from the youngest sugarcane leaf that was located next to the apical meristem dome on MS medium supplemented with 3 mg/l 2,4-D, 1 mg/l BAP, 1 mg/l IBA and 15% coconut water. Shoot buds were initiated in the next transfer, which was to fresh MS medium supplemented with 3 mg/l NAA, 20 mg/l yeast extract and 15% coconut water. Proliferation of shoots and roots was then obtained after transfer to MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BAP and 15% coconut water, and this medium formula was thus chosen for subsequent serial transfers and maintenance of the callus-derived plantlets. As with the meristem tip cultures, differentiation of the diseased explants was visible within 2 weeks, twice as rapidly as in the explants of healthy origin. About 8% of the plantlet clumps in subcultures of shoots derived from diseased tissue were asymptomatic, and there was no trace of detectable phytoplasma in these plantlet clumps using the universal 16S rDNA and the 16S-23S rDNA primers (Table 1).

Pathogenicity and transmissibility of phytoplasmas from the diseased tissue cultures

Typical white leaf symptoms were apparent in all 50 sugarcane plants inoculated by adults of the insect vector *M. hiroglyphicus* that were previously caged on diseased plantlets. No symptoms were seen on control plants caged with non-inoculated leafhoppers. Inoculation by grafting stocks of healthy sugarcane or periwinkle seedlings with scions of diseased plantlet cultures was also successful, despite the gradual decline of the grafted scions over a period of 2–3 weeks. Typical SCWL symptoms were produced in all 250 grafted sugarcane seedlings, but not in

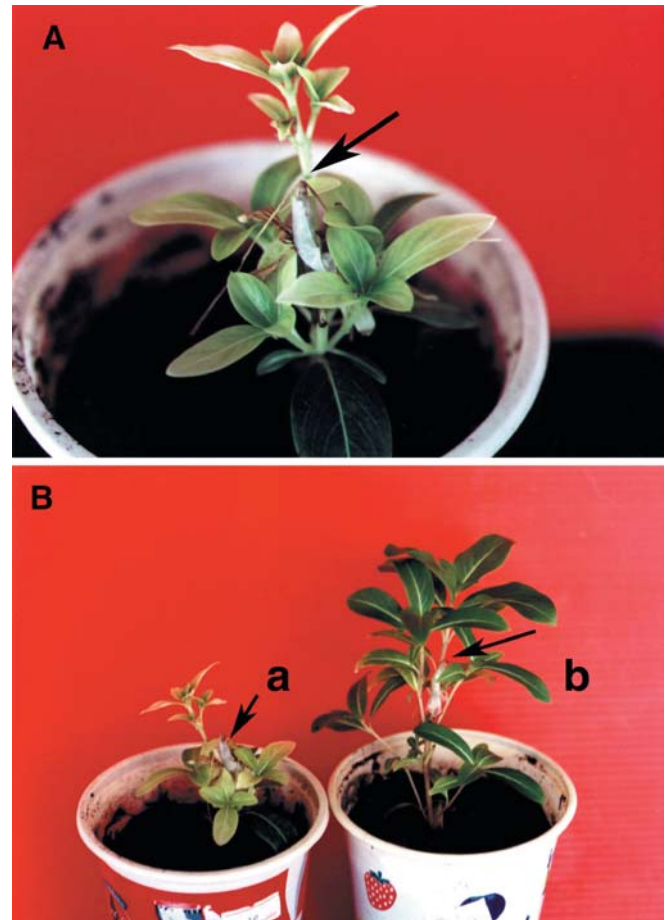


Fig. 2 **a** Periwinkle showing chlorosis development following graft transmission of SCWL phytoplasma from diseased tissue culture derived plantlets and **b** comparison of periwinkle plants after grafting with SCWL-diseased plantlet (*a*) and healthy sugarcane plantlet (*b*). Arrow Sugarcane plantlet collected directly from the axillary bud explant-derived subculture bottle

control plants, within 4 weeks. After grafting of diseased sugarcane plantlet scions onto periwinkle plants, leaf chlorosis and proliferation of shortened branches were observed within 3–4 weeks on all 250 plants grafted with diseased scions (Fig. 2a,b-a), while all 50 control periwinkle plants grafted with healthy scions remained symptom-free (Fig. 2b-b). PCR detection using both 16S and 16S-23S rDNA primers following the insect feeding inoculation and grafting experiments confirmed the

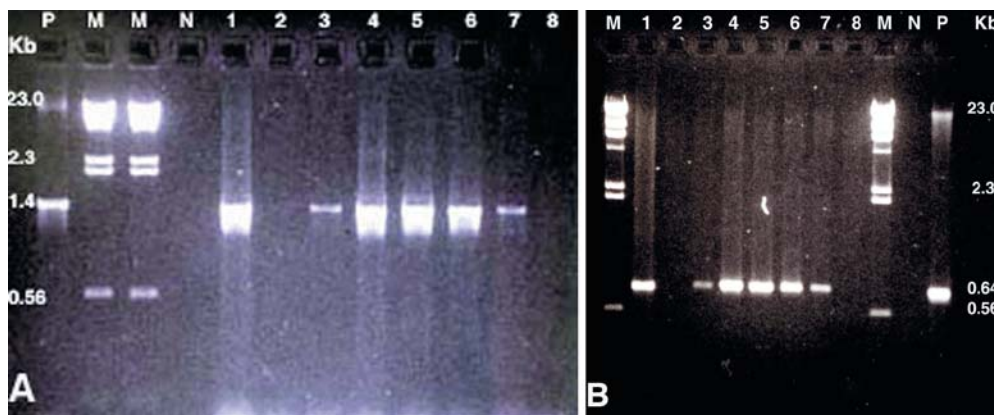


Fig. 3 Agarose gel electrophoresis of the PCR amplicons obtained using **a** phytoplasma 16S rDNA universal primers (the expected PCR product of about 1.4 kb indicates the presence of phytoplasma in the sample), and **b** 16S-23S rDNA primers (the expected PCR product of about 0.64 kb of 16S-23S rDNA indicates the presence of phytoplasma in the sample). Lanes: *M* Molecular-weight marker

(λ DNA cleaved with *Hind*III), *P* SCWL-diseased plant from Udonthani Province, *N* healthy sugarcane, *1* SCWL-diseased axillary bud tissue culture, *2* healthy sugarcane tissue culture, *3* sugarcane grafted with SCWL diseased axillary bud tissue culture, *4-7* periwinkle grafted with SCWL-diseased axillary bud tissue culture, *8* healthy periwinkle

presence of SCWL phytoplasma in these recipient sugarcane and periwinkle plants (Fig. 3).

Identification of SCWL phytoplasma in tissue culture

PCR testing of diseased tissue culture shoots and plantlets using 16S rDNA universal primers resulted in amplification of a band of about 1.4 kb, the size expected for the 16S rDNA fragment of the phytoplasma (Fig. 3a). Use of SCWL group-specific primers also yielded a band of expected size, the 0.64 kb product of the 16S-23S rDNA fragment (Fig. 3b). No PCR amplification was obtained with either set of primers from healthy sugarcane tissue cultures or plants.

Longevity of phytoplasma in tissue culture

Shoots or plantlets were transferred onto fresh medium every 6 weeks to prevent culture deterioration, which would otherwise have occurred within 6–9 weeks. White leaf symptoms persisted in long-term serial subcultures of the shoots and plantlets derived from diseased explants; to date they remain symptomatic, 8 years after the experiments began in 1996. The presence of the SCWL phytoplasma was confirmed by PCR in randomly-selected plantlets, and recent grafting and insect vector feeding experiments showed that the phytoplasmas in such plantlets are still graft-transmissible and pathogenic to sugarcane.

Electron microscopy

Structures having typical phytoplasma size and cell morphology (Doi et al. 1967; Kirkpatrick 1989) were observed in the sieve tube elements of diseased sugarcane

field plants and in tissue culture plantlets derived from such diseased plants (Fig. 4a), but not in healthy field plants or tissue cultures derived from them.

Effect of treatment with the antibiotic oxytetracycline

Addition of the antibiotic oxytetracycline to the culture medium at 0–100 mg/l had no apparent effect on the phytoplasma, and cultures retained the white leafy shoot clumps typical of disease. A degree of white leaf disease remission was gradually achieved by increasing the concentration of the antibiotic to 200–500 mg/l, although plantlet survival was also reduced following these treatments (Tables 2, 3). For example, the leafy shoots became green and began to produce leaves of normal size within 3 weeks at an oxytetracycline concentration of 200–300 mg/l. From 70% to 85% of the subcultures made on fresh medium without oxytetracycline following antibiotic treatment at these concentrations yielded symptom-free plantlets, which tested negative for SCWL phytoplasma by PCR. However, disease symptoms reappeared after five subsequent serial transfers. Repeated serial transfers to medium containing 200 mg/l or 300 mg/l oxytetracycline showed that the antibiotic was strongly phytotoxic at this level and did not induce disease remission. On medium containing 500 mg/ml oxytetracycline about half of the shoot cultures became necrotic, but those that survived were symptom-free. The shoot clumps gradually recovered from the phytotoxic effect after the next transfer to fresh medium without oxytetracycline, and the subcultures remained asymptomatic through as many as five to eight subsequent serial transfers.

Electron microscopic examination of SCWL-diseased tissue cultured sugarcane plantlets revealed the presence of typical phytoplasma-like bodies in the sieve tubes of

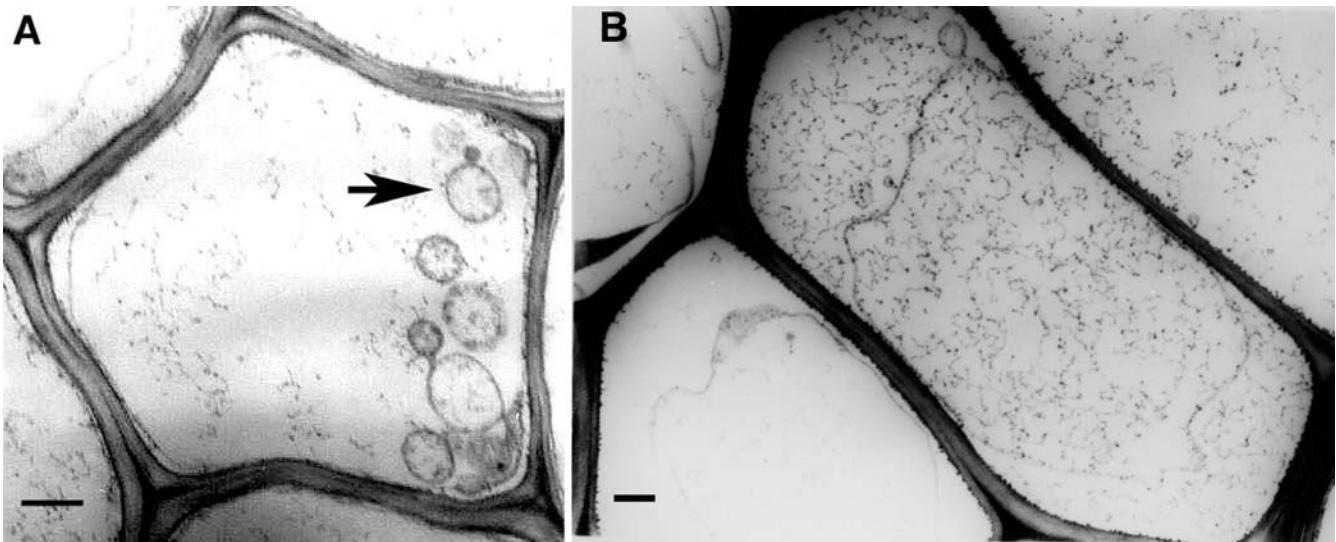


Fig. 4 a Electron micrographs showing SCWL phytoplasma (arrow) in sieve tubes of diseased plantlet subcultures grown on Murashige-Skoog (MS) medium supplemented with 0.5 mg/l α -naphthylacetic acid (NAA), 0.5 mg/l 6-benzylaminopurine (BAP)

and 15% coconut water. **b** No recognizable phytoplasma cells were seen in SCWL-diseased plantlet subcultures grown on the same formula of MS medium but amended with 300 mg/l oxytetracycline. Bar 0.5 μ m

Table 2 Effect of oxytetracycline on survival of SCWL-diseased axillary bud-derived plantlet cultures after transfer onto medium amended with the antibiotic^a

Oxytetracycline (mg/ml)	Original plantlet cultures		
	Total	Number surviving ^b	% Survival
0	240	236	98.3
100	240	238	98.2
200	240	240	100
300	240	194	80.8
500	240	90	37.5

^a Basal Murashige-Skoog (MS) medium supplemented with 0.5 mg/l α -naphthylacetic acid (NAA), 0.5 mg/l 6-benzylaminopurine (BAP) and 15% coconut water

^b Counted 4–6 weeks following oxytetracycline treatment

each of the five untreated plantlets (Fig. 4a). No recognizable phytoplasmas were seen in any of the originally infected plantlets after treatment with 300–500 mg/l oxytetracycline (Fig. 4b).

Discussion

Establishment of tissue culture plantlets from explants of SCWL phytoplasma-infected plant tissues was successful using previously reported medium constituents (Wongkaew et al. 1995). When diseased axillary buds were the source of explants for tissue culture, white leaf symptoms were consistently produced throughout subsequent serial subculturing with no spontaneous remission. Initial calli of diseased material differentiated into shoots and roots about twice as rapidly as did control healthy tissue cultures. However, when explants were taken from meristem tips or leaves, 38% of the serial subcultures were symptom-free, and the absence of phytoplasma in such cultures was confirmed by PCR. Therefore, axillary bud culture is the most suitable indirect culture system for long-term maintenance of SCWL phytoplasma. Use of meristem tip and leaf tissue culture systems, on the contrary, could be a strategy for the production of disease-free plants from originally diseased sugarcane.

Table 3 Apparently disease-free subcultures derived from SCWL-diseased axillary bud cultures surviving after oxytetracycline treatment

Oxytetracycline (mg/ml)	First serial subcultures ^a			Fifth serial subcultures ^b		
	Total	Number disease free ^c	% Disease free	Total	Number disease free ^c	% Disease free
0	100	0	0	100	2	2
100	100	20	20	100	19	19
200	100	70	70	100	27	27
300	100	85	85	100	45	45
500	50	50	100	50	32	64

^a Subcultures from SCWL-diseased parent tissue cultures surviving oxytetracycline treatment, made in fresh MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BAP and 15% coconut water without oxytetracycline

^b Subcultures from phytoplasma-free parent cultures made in fresh MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BAP and 15% coconut water without oxytetracycline

^c Detected by PCR with 16S-23S rDNA, SCWL-group specific primers

Indirect culture of phytoplasmas in diseased plant tissue culture has been reported previously in several cases. Paulownia witches' broom phytoplasmas multiplied more rapidly in plant tissue cultures than in field plants and the phytoplasma titer increased 5-fold after subculture (Wang et al. 1994). In general, cultures originating from axillary buds or shoot buds retained their phytoplasma populations more stably than did cultures from other parent plant tissues. A phytoplasma infecting *Populus alba* could be maintained through plantlet subculture (Cousin et al. 1990) as could other phytoplasmas in infected shoot cultures of *Oenothera*, *Catharanthus*, *Chrysanthemum*, *Gladiolus*, *Hydrangea*, *Rubus*, *Populus*, *Solanum*, *Prunus*, *Pyrus*, and *Malus* (Sears and Klomparens 1989; Cousin et al. 1990; Bertaccini et al. 1992; Raj Bhansali and Ramawat 1993; Davies and Clark 1994; Jarausch et al. 1994a,b, 1996). Long-term maintenance of the phytoplasmas was achieved in those cases; at least 3 years in *Oenothera* leaf tip cultures (Sears and Klomparens 1989), 3 years in *Populus alba* micropropagation (Cousin et al. 1990), 5 years in *Prunus marianna* (Jarausch et al. 1994a), and 10 years in *Malus pumila* (Jarausch et al. 1996). Although survival of SCWL phytoplasma for up to 2 years in shoot tip cultures of diseased-sugarcane was demonstrated previously (Wongkaew et al. 1995), the current work confirms the persistence of the pathogen for at least 6 years (the length of the study), especially in cultures derived from axillary buds. The white leaf phytoplasma in such cultures retained pathogenicity and transmissibility over the entire period, as shown by *M. hiroglyphicus* transmission tests and direct grafting inoculation experiments. Transmissions to healthy sugarcane and to the index plant *C. roseus* were confirmed by PCR detection using universal or SCWL group-specific primers. Thus, diseased tissue cultures provide a convenient and reliable source of SCWL phytoplasma for further research. The exchange of phytoplasma strains among researchers throughout the world could also be facilitated by such cultures.

A number of subcultures from meristem tip and young leaf tissue culture, but not from axillary bud culture, showed spontaneous recovery from phytoplasma disease. Because of the general assumption that the titer of small pathogens such as viruses and phytoplasmas is very low in the growing points of the plants, the meristem tip is usually selected as the starting material for disease-free plantlet production by tissue culture. Similarly, although the SCWL phytoplasma could parasitize phloem in all parts of diseased plants, its concentration was lowest in the stem apex (Nakashima et al. 1994, 1999; Wongkaew 1999). It is possible that alterations induced by some growth factors during development also may contribute to spontaneous curing. Sustained, gradual degeneration and disappearance of phytoplasmas within 80 days of explant establishment were demonstrated in carrot and tobacco callus cultures infected with aster yellows phytoplasma (Jacoli 1978a,b). Sears and Klomparens (1989) reported that a side-shoot arising from *Oenothera* callus displayed spontaneous curing from phytoplasma disease. Others

have also observed similar spontaneous elimination of phytoplasmas in cultures of carrot, tobacco and potato (Mitsuhashi and Maramorosch 1964; Jacoli and Ronald 1974; Petru and Ulrychova 1975; Moellers and Sarkar 1989). Phytoplasma-free plants were also obtained at a high percentage through stem culture of dwarf diseased mulberry, *Morus alba* (Dai et al. 1997).

The fact that phytoplasmas primarily colonize phloem sieve tube elements may explain the reliable maintenance of phytoplasmas in axillary bud cultures as compared to the meristem tip- and leaf-derived cultures, as the vascular tissue is continuously maintained throughout development of cultures from axillary buds. Some evidence suggests that the relative ratios of internal growth regulators influence the development of phytoplasma disease symptoms. Chen et al. (1991) proposed that symptoms of witches' broom in jujube plants are the result of an increase in the ratio of cytokinin to auxin (C/A) in the infected plants. Cytokinin plays an important role in nutrient distribution, thus contributing to the intercellular movement of the phytoplasmas. An alteration of the internal hormone balance that resulted in a decrease of the C/A ratio would lower the concentration of essential nutrients and possibly induce phytoplasma degeneration. This could be another explanation for the spontaneous curing in our meristem tip and young leaf tissue cultures.

Graft transmission of SCWL phytoplasma from a sugarcane host plant is an important achievement; successful grafting of monocots has generally been considered unlikely due to the scattered nature of their vascular bundles and their lack of cambial tissue. However, grafting between heterologous dicot species has been reported; grapevine phytoplasmas were graft-transmitted to the common phytoplasma host species, periwinkle (Tanne and Orenstein 1997). The fact that grafting of various crop and ornamental species is common in Thailand, led us to attempt the experiments. The transmission of SCWL phytoplasmas from sugarcane to sugarcane (monocot to monocot) by stem grafting is, to our knowledge, the first successful grafting of this monocot species. The phytoplasma transfer from sugarcane to periwinkle is also significant, with respect to both host species (heterologous, and monocot to dicot) and phytoplasma transmission. A previous attempt to transmit phytoplasmas from Bermuda-grass (monocot) to periwinkle (dicot) using a parasitic plant, dodder, were unsuccessful (Sarindu and Clark 1993).

Phytoplasma infection in grafted sugarcane or periwinkle stocks was determined initially by symptomatology, and confirmed by PCR using a universal 16S rDNA primer set and a 16S-23S rDNA primer set specific to a small group of closely related monocot-infecting phytoplasmas (Wongkaew et al. 1997; Hanboonsong et al. 2002). The fact that all 50 of the control stocks in each experiment grafted with healthy scions remained symptom-free and PCR negative, removes the possibility that the stocks were infected by some other means. Our work, which represents the first introduction of SCWL phytoplasma into a dicotyledonous species, resulted in the es-

establishment of periwinkle as a convenient experimental system and as a long-term plant host for SCWL phytoplasma.

The addition of the antibiotic tetracycline or its derivatives has been suggested to provide more effective phytoplasma disease curing. Remission of phytoplasma-induced symptoms in *Oenothera* leaf tip cultures was accomplished with tetracycline treatment. However, significant curing required several months of exposure to the antibiotic. If the plantlets were removed after only short exposure, symptoms recurred at high frequency (Sears and Klomparens 1989). In one study, oxytetracycline treatment led to permanent elimination of a causal phytoplasma. Davies and Clark (1994) reported complete elimination of the pear decline phytoplasma from pear micropropagates by growing them on oxytetracycline-amended medium for 4 weeks. In this study, electron microscopy showed degeneration of sugarcane phytoplasmas following oxytetracycline treatment at 300–500 mg/l, but the elimination was temporary. However, the SCWL phytoplasma was suppressed over 5–8 subculture generations or at least 8 months of sequential serial transfers.

In summary, we have demonstrated that diseased sugarcane axillary bud culture is a useful and convenient method for long-term maintenance of, and experimentation with, SCWL phytoplasma for at least 6 years, and that diseased sugarcane meristem tip and young leaf explant culture are suitable methods for obtaining disease-free sugarcane plantlets.

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