

Full Length Research Paper

Effective methods of preserving SCWL-diseased sugarcane leaves for genomic DNA extraction and molecular detection of phytoplasma

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Simple and economical methods to preserve sugarcane leaf tissues infected with sugarcane white leaf (SCWL) phytoplasma for later detection of SCWL using nested-PCR analysis were described. Diseased leaf tissues were preserved by air-drying and stored at room temperature or stored fresh at 4 or -20°C in two types of bags: brown paper bags and plastic bags with punctured holes. Leaf materials were stored for up to 32 days prior to DNA extraction. The best storage condition was placing fresh leaves at -20°C and air-drying leaves in paper bags at room temperature. High molecular weight plant genomic DNA bands were detected in all stored infected tissues. Genomic DNAs were used as templates for nested-PCR which showed a single band of 210 bp in the 16S-23S intergenic spacer region specific to SCWL phytoplasma. Nested-PCR was also performed on genomic DNA extracted from diseased leaves heat-treated in a microwave oven and the SCWL-specific bands were effectively detected. Therefore, treating diseased sugarcane leaves by air-drying, freezing, refrigeration or microwave heat treatment were all effective for molecular detection of SCWL phytoplasma. However, air-drying and storage in paper bags was the most economical and practical, especially when large numbers of samples are to be stored or transported from the fields to a distant laboratory or exchanged among laboratories.

Key words: Sugarcane white leaf phytoplasma, nested-polymerase chain reaction (PCR), preservation, 16S-23S intergenic spacer region.

INTRODUCTION

Phytoplasmas are wall-less bacteria inhabiting the phloem sieve elements of infected plants. They cause several plant diseases worldwide, including many diseases of economically important plants (Lee and Davis, 1992). Phytoplasmas have been reported to be associated with two major sugarcane diseases: sugarcane grassy shoot (SCGS) and sugarcane white leaf disease (SCWL). Both diseases occur only in Asia,

especially Thailand, India, Pakistan and Sri Lanka (Nakashima and Murata, 1993).

Phytoplasmas cannot be isolated in culture, and therefore, conventional methods by which most bacteria can be detected and identified are not suitable for use with phytoplasmas. The polymerase chain reaction (PCR) is the most versatile tool for detecting phytoplasmas in their host (Lee et al., 1993; Liu et al., 1994). Recently, the nested polymerase chain reaction assay was found to be highly sensitive and more efficient to detect phytoplasmas from sugarcane (Hanboonsong et al., 2005; Srivastava et al., 2006; Rao et al., 2008). The importance of molecular diagnostics using PCR protocols is the preparation of high-quality DNA (Heinrich et al., 2001; Boben et al., 2007; Aldaghi et al. 2009). The efficiency of molecular detection can also be affected by the possible presence of PCR inhibitors in phytoplasma-

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Abbreviations: SCWL, Sugarcane white leaf; SCGS, sugarcane grassy shoot; RT, room temperature; PP, paper bag; PH, punctured plastic bag; AR, air-dried.

infected plant materials (Lepka et al., 1999; Musetti et al., 2000; Heinrich et al., 2001). In plants tissues, phytoplasma usually occur at very low titres which varies according to the season, variety, age and storage of the infected plant (Seemüller et al., 1998; Berges et al., 2000). Several methods have been reported for the preservation of phytoplasmas, including maintenance in plant tissue cultures, living host plants, leafhopper vectors under -70°C and freeze drying as traditional method for preservation. These methods, sometime, are not suitable for storage of large number of the infected plant samples (Wang and Hiruki, 1998).

Early protocols for isolation of DNA resulted to the use of liquid nitrogen to assist in the grinding of plant material for DNA extraction. The use of liquid nitrogen presented some problems. It was imperative that the tissue was not allowed to thaw before extraction (Tai and Tanksley, 1990). If large samples of plants are to be extracted, the tissue would have to be stored frozen or processed and then stored in freezer. Storage of tissue in freezers may lead to problems involving degradation of DNA. Therefore, it is necessary to find a more practical method to preserve large number of the infected plant samples under laboratory conditions.

In a previous study, DNA was isolated from plant tissues after dehydration by food dehydrator. The quality of DNA was equal with that obtained from fresh frozen tissues (Tai and Tanksley, 1990). Heat treatment of infected periwinkle plant tissues in microwave oven was used to preserve clover proliferation (CP) phytoplasma DNA. The DNA extracted was effectively used to amplify a prominent band which matched the size of the 16S rRNA gene specific to CP phytoplasma (Khadhair, 1995).

SCWL is one of the most important diseases occurring throughout Thailand. Molecular diagnosis of infected plants is routinely carried out in only a few laboratories. Infected plant materials often need to be transported across a long distance from an infected field to the laboratory where numerous samples need to be stored and the lack of freezer space is a common problem. To date, there is not yet a suitable method available for preservation of large quantity of infected tissues. This paper reports a suitable method which offers many advantages in preserving genomic DNA from phytoplasma infected sugarcane tissues for molecular detection of SCWL.

MATERIALS AND METHODS

Source of phytoplasma and preservation treatments

Diseased sugarcane plants showing typical white leaf symptoms were collected from sugarcane plantation in Udonthani, a province situated in Northeast Thailand during March to April, 2010. The fourth leaf from the top was used for the experiment based on a preliminary test which showed that the third and fourth leaves of a diseased plant contained the highest relative concentration of SCWL on leaf fresh weight basis. Two types of preservation methods were performed either by storing the freshly harvested leaf

at low temperature or drying the leaves before storing at room temperature. For low temperature storage, leaf tissues without midribs (1 g) were stored immediately in paper bag (PP) or punctured plastic bag (PH) at 4 or -20°C . After 7, 15, 25 and 32 days, samples were retrieved for DNA extraction. For drying, two dehydration treatments were performed on 1 g leaf samples including: (1) air-drying overnight at room temperature (RT); (2) heat-treatment in a microwave oven (Model MB1026,2450 MHz, 800 W) set at 480 W for 1, 2, 4, 6 and 8 min, and 600 W for 1 and 2 min. Air-dried (AR) samples were stored in PP or PH at room temperature for 7, 15, 25 and 32 days before used for DNA extraction. Microwave-treated samples were immediately used for DNA extraction.

DNA extraction

Total genomic DNA from various preservative methods and fresh leaves was extracted by the method of Li and Midmore (1999) with some modifications. Each sample was ground in liquid nitrogen in a mortar. The powder was transferred to 800 μl of DNA extraction buffer [2% cetyl trimethylammonium bromide (CTAB), 1.4 M NaCl, 0.1 M Tris-HCl (pH 8.0), 0.02 M EDTA (pH 8.0), 1% polyvinylpyrrolidone (PVP)]. The samples were incubated at 60°C for 30 min and then centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with 500 μl of chloroform : isoamyl alcohol (24:1 v/v). After centrifugation at 12,000 rpm for 10 min, the DNA was precipitated by the addition of 500 μl of ice-cold isopropanol, incubated at -20°C for 15 min and then centrifuged at 12,000 rpm for 2 min. The pellet was washed two times with ice-cold 70% ethanol and then centrifuged at 12,000 rpm for 2 min, dried at room temperature and dissolved in 180 μl of distilled sterile water, 20 μl of 5 M NaCl, 200 μl of 95% ethanol and gently mixed. This mixture was centrifuged at 12,000 rpm for 2 min; the pellet was washed with ice-cold 70% ethanol and then centrifuged at 12,000 rpm for 2 min, dried at room temperature and finally resuspended in 40 μl of TE buffer. The DNA was quantified using UV-Vis spectrophotometer and 2- μl aliquots of genomic DNA were verified by 1% agarose gel electrophoresis.

PCR amplification

Two sets of primers were used to amplify phytoplasma DNA in PCR that comprised of two steps, that is, direct-PCR followed by nested-PCR. Direct-PCR amplification was performed using universal phytoplasma primer pair MLO-X (5'- GTT AGG TTA AGT CCT AAA AGC AGC-3')/MLO-Y (5'- GTG CCA AGG CAT CCA CTG TAT GCC-3') that amplifies a fragment of approximately 700 bp in length extending from 5' end of the 16S rRNA gene to the 5' region of the 23S rRNA gene (Hanboonsong et al., 2005). The reaction mixture (15 μl) contained 30 ng DNA template, 0.5 μM of each primer, 1x PCR buffer, 1.5 mM MgCl_2 , 0.2 mM dNTP, 0.1 U Tag polymerase (Fermentas, USA). Reaction mixtures containing distilled water in the place of DNA served as negative controls. Cycling was performed in a thermal cycler (Perkin-Elmer, Norwalk, CT, USA) as follows: pre-denaturation at 95°C for 2 min, 24 cycles of 1 min denaturation at 95°C , 30 s annealing at 55°C and 1 min extension at 72°C .

Each direct-PCR product was diluted 1:100 and then 3 μl of the diluted samples were re-amplified by nested-PCR. The components of nested-PCR mixtures were the same as those described for direct-PCR, except for the primers and the concentration of MgCl_2 (1 mM). The second set of primers, P1 (5'- GTC GTA ACA AGG TAT CCC TAC CGG -3')/P2 (5'- GGT GGG CCT AAA TGG ACT TGA ACC -3') were used. The nested-PCR amplified a fragment of approximately 210 bp in the 16S-23S intergenic spacer region (Hanboonsong et al., 2005). A total of 25 cycles were performed

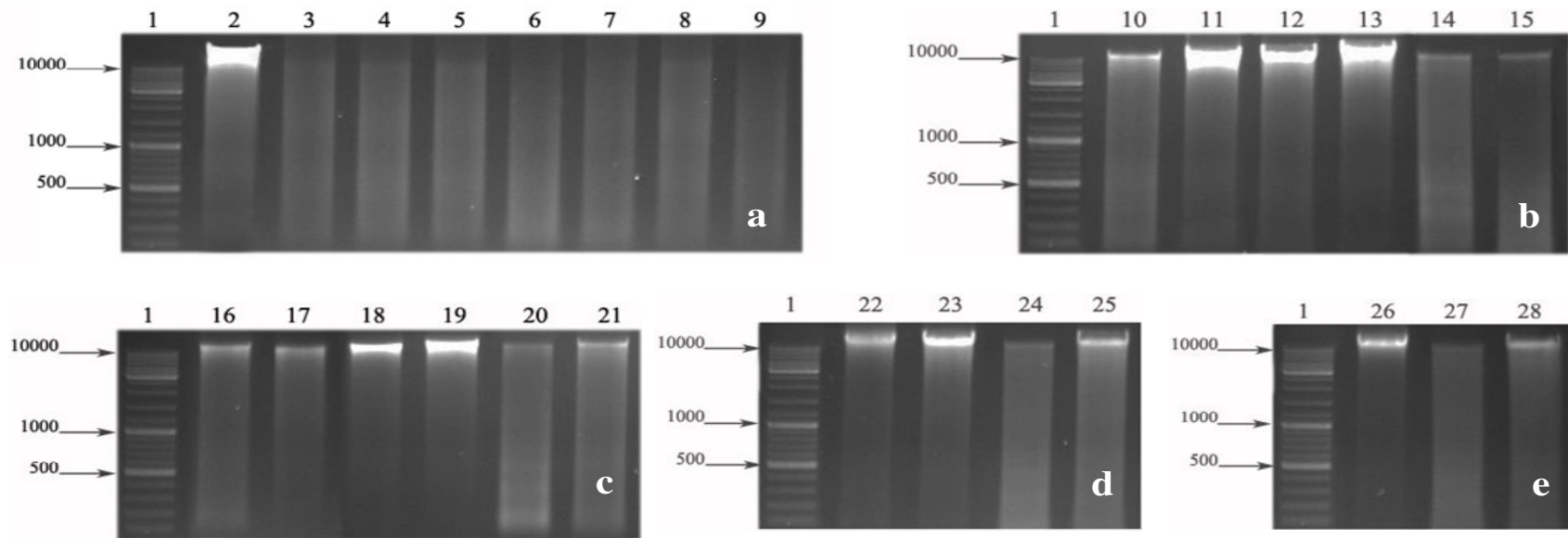


Figure 1. (a) The total DNA samples extracted from fresh sugarcane leaves (lane 2), leaves dried in microwave oven at 480 W for 1, 2, 4, , and 8 min (lanes 3 to 7) and 600 W for 1 and 2 min (lanes 8 to 9); (b) DNA samples after 7 days storage: air-dried leaves stored at RT in PH (lane 10) and PP (lane 11); fresh leaves stored at 4°C in PH (lane 12) and PP (lane 13) or -20°C in PH (lane 14) and PP (lane 15); (c) DNA samples after 15 days storage: air-dried leaves stored at RT in PH (lane 16) and PP (lane 17); fresh leaves stored at 4°C in PH (lane 18) and PP (lane 19) or -20°C in PH (lane 20) and PP (lane 21); (d) DNA samples after 25 days storage: air-dried leaves in PP stored at RT (lane 22); fresh leaves stored at 4°C in PP (lane 23); fresh leaves stored at -20°C in PH (lane 24) and PP (lane 25); (e) DNA samples after 32 days storage: air-dried leaves in PP at RT (lane 26); fresh leaves stored at -20°C in PH (lane 27) and PP (lane 28). Lane 1: DNA 100-bp ladder.

with denaturation at 94°C for 1 min, annealing at 60°C for 30 s and extension at 72°C. PCR products were analyzed by gel electrophoresis in 1% agarose gel stained with SYBR® Gold (Invitrogen, USA) and DNA bands were visualized by using UV transillumination.

RESULTS AND DISCUSSION

Several methods of preservation of infected sugarcane leaf tissues were evaluated to find the most suitable storage conditions for later molecular detection of SWCL phytoplasma using nested PCR analysis. Two types of preservation methods were performed either by storing the

freshly harvested leaf at low temperatures (4 and -20°C) or air-drying the leaves before storing at room temperature, in two types of bags for up to 32 days. The intactness of the phytoplasma was also evaluated by heat treatment of infected leaves in a microwave oven.

The period of time in which leaf materials can be kept depends largely on temperature treatments and packaging materials in which the samples are stored. Fresh leaves stored at -20°C in both PP and PH were still in perfect conditions after 32 days storage. On the other hand, storage life of fresh leaves at 4°C was only 15 days in PH and 25 days in PP. Samples stored at 4°C for

longer periods suffered from fungal infection and could not be used for DNA analysis. Air-dried leaf samples in PP could be kept for as long as 32 days at RT without fungal infection. However, air-dried samples in PH were kept for only 15 days after which they were infected by fungi. Microwave-treated leaves appeared completely burnt and therefore were used immediately for DNA extraction without storage. Total genomic DNAs extracted from microwave-treated leaves, low temperature-stored and RT-stored air-dried leaves which were not infected by fungi during storage are shown in Figure 1. The total genomic DNA bands were not detectable in 1% agarose

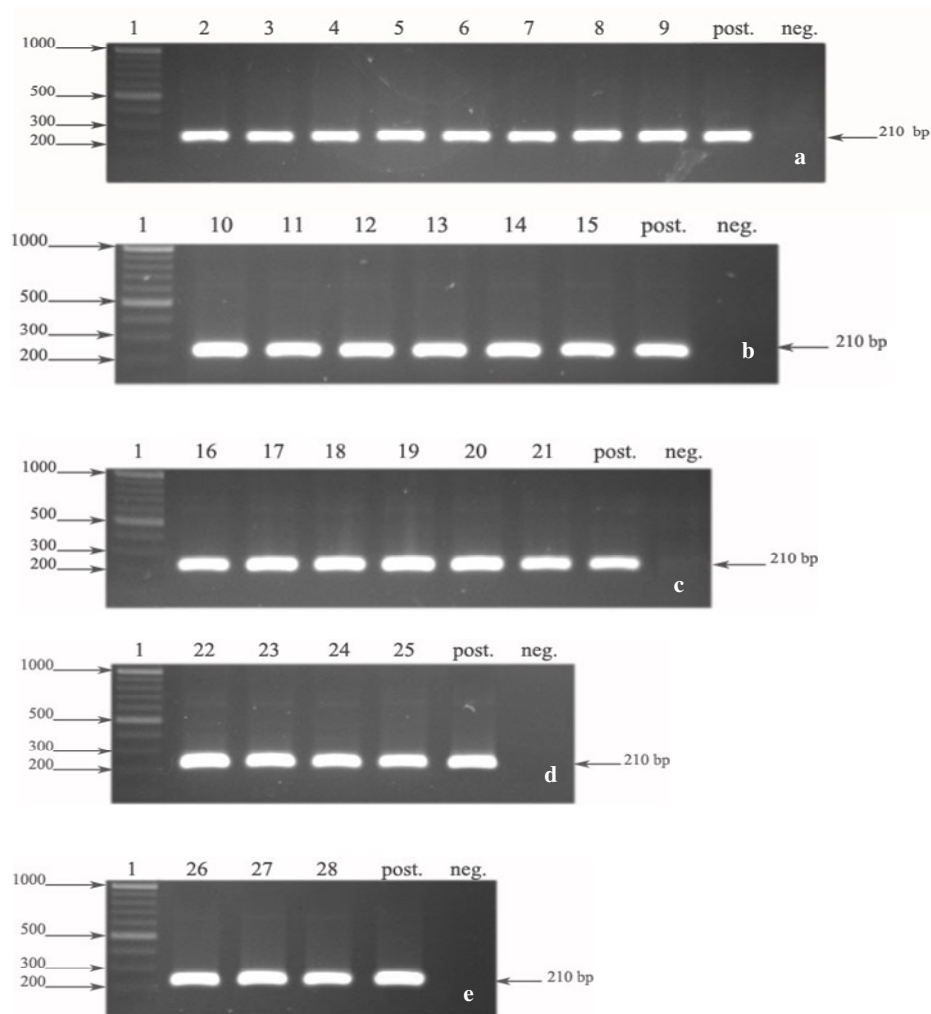


Figure 2. The 210-bp fragment in the 16S-23S rDNA intergenic spacer region (16-23S ITS) specific to SCWL amplified by nested-PCR using genomic DNA extracted from (a) fresh sugarcane leaves (lane 2), leaves dried in microwave oven at 480 W for 1, 2, 4, 6 and 8 min (lane 3-7) and 600 W for 1 and 2 min (lanes 8 to 9); (b) leaf samples after 7 days storage: air-dried leaves stored at RT in PH (lane 10) and PP (lane 11); fresh leaves stored at 4°C in PH (lane 12) and PP (lane 13) or -20°C in PH (lane 14) and PP (lane 15); (c) leaf samples after 15 days storage: air-dried leaves stored at RT in PH (lane 16) and PP (lane 17); fresh leaves stored at 4°C in PH (lane 18) and PP (lane 19) or -20°C in PH (lane 20) and PP (lane 21); (d) leaf samples after 25 days storage: air-dried leaves in PP stored at RT (lane 22); fresh leaves stored at 4°C in PP (lane 23); fresh leaves stored at -20°C in PH (lane 24) and PP (lane 25); (e) leaf samples after 32 days storage: air-dried leaves in PP at RT (lane 26); fresh leaves stored at -20°C in PH (lane 27) and PP (lane 28). Lane 1: DNA 100-bp ladder. post. = SCWL confirmed diseased plant, neg.= water control.

gel when samples were extracted from microwave-treated leaves (Figure 1a). After 7 days storage, the air-dried samples kept in PP (Figure 1b, lane 11) gave high molecular weight genomic DNA of high quality as compared to that of fresh leaf (Figure 1a, lane 2). Whereas, the air-dried sample kept in PH was badly degraded (Figure 1b, lane 10). Storage at 4°C gave better quality genomic DNA (Figure 1b, lanes 12 and 13) than freezing at -20°C (Figure 1b, lanes 14 and 15). After 15 days storage, the intensity of high molecular weight

genomic DNA of all samples was considerably reduced (Figure 1c). For longer storage period, only air-dried samples kept in PP gave good quality DNA until 32 days (Figure 1e, lane 26).

All genomic DNA samples presented in Figure 1 were used as templates to successfully amplify 210 bp fragments in the 16S-23S rDNA intergenic spacer region (16-23S ITS) specific to SCWL using nested PCR (Figure 2). The 210-bp band was also amplified from the positive control DNA previously extracted from confirmed

diseased leaves (Figure 2a to e; lane 'post.')

and DNA from fresh diseased leaf (Figure 2a, lane 2). This nested PCR method was also used to detect SCWL-specific fragment from SCWL-diseased sugarcane (Wongkaew and Fletcher, 2004; Hanboonsong et al., 2005). Although, microwave heat treatment resulted in total degradation of high molecular weight DNA of plants (Figure 1a, lanes 3 to 9), it did not change gross structure of the phytoplasma DNA as demonstrated by prominent 210 bp bands from all microwave-treated samples (Figure 2a, lanes 3 to 9). Figure 2b to e shows the 210 bp PCR products amplified from the corresponding genomic DNA samples illustrated in Fig 1b to e, respectively. Although, different temperature treatments, storage conditions and length of storage obviously affected the quality of high molecular weight DNA as seen in Figure 1b to e, the phytoplasma DNA was still intact leading to efficient amplification of the 210 bp 16-23S ITS fragments from all genomic DNA samples.

Several conditions were suitable for preservation of sugarcane diseased leaves for later use in DNA extraction. Air-drying and storing in PP at RT was the best preservation method due to its low cost and long storage life. The genomic DNA extracted from air-dried samples in PP kept for 32 days at RT was of greater quality than that from frozen tissues stored at -20°C. Storage of frozen tissues at -20°C was also effective but not as practical in the case of large number of samples. In the case where the samples need to be transported from the field to a distant laboratory, air-drying is obviously more advantageous. It seems that air-drying caused less damage to genomic DNA than freeze-thawing. On the contrary, Bainard et al. (2010) observing plant roots colonized by arbuscular mycorrhizal (AM) fungus found that plant and AM fungal DNAs were much better preserved in hydrated (frozen at -20 and -80°C) than in dehydrated condition (silica-gel drying). Nilda et al. (2010), however, found that silica-gel drying was effective for preserving infected plant materials for detection of phytoplasma using nested PCR. Another method for preservation of dried plant tissues for later PCR detection of pathogens was heating virus-infected plant tissues at 65°C for 2 days and storing at 4°C (Sipahioglu et al., 2006). It was also found in this study that paper bag was a more suitable packaging material for long term preservation of diseased plant tissues in both dried and hydrated conditions.

Phytoplasma DNA proved to be highly stable even under extremely harsh conditions of microwave heat treatment. The PCR detection of phytoplasma DNA after microwave heat treatment was earlier demonstrated in diseased tissues of periwinkle (Khadhair et al., 1995) and paulownia (Wang and Hiruki, 1998). These authors suggested that drying with microwave is a more economical and practical method for long term preservation than the earlier methods using lyophilizer and food dehydrator (Tai and Tanksley, 1990). The total dryness

and non-infectivity of microwave-treated sample made it suitable for international exchange of phytoplasma specimens for molecular genetic studies among different laboratories (Wang and Hiruki, 1998).

It can be concluded that three methods of preserving SCWL-diseased sugarcane leaves for later molecular detection of phytoplasma: air-drying and storing in paper bags, storing frozen tissues in -20°C in paper or plastic bags and heating in microwave oven gave rise to satisfactory detection of phytoplasma using nested PCR. However, for approximately one month storage, air-drying and storing in paper bags proved to be the most simple and economical, especially when large number of samples have to be handled or when transportation or exchange of plant materials among laboratories is involved.

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REFERENCES

- Aldaghi M, Massart S, Dutrecq O, Bertaccini A, Jijakli MH, Lepoivre P (2009). A simple and rapid protocol of crude DNA extraction from apple trees for PCR and real time PCR detection of *Candidatus* Phytoplasma mali. *J. Virol. Methods*, 156: 96-101.
- Bainard LD, Kironomos JN, Hart MM (2010). Differential effect of sample preservation methods on plant and arbuscular mycorrhizal fungal DNA. *J. Microbiol. Methods*, 82: 124-130.
- Berges R, Rott M, Seemüller E (2000). Range of Phytoplasma concentration in various plant hosts as determined by competitive polymerase chain reaction. *Phytopathology*, 90: 1145-1152.
- Boben J, Mehle N, Ravnkar M (2007). Optimization of extraction procedure can improve phytoplasma diagnostics. *B. Insectol.* 60(2): 249-250.
- Hanboonsong Y, Ritthison W, Choosai C (2005). Molecular detection and transmission of sugarcane white leaf phytoplasma in leafhoppers. *KKU. Res. J.* 10(1): 13-21.
- Heinrich M, Botti S, Caprara L, Arthofer W, Strommer S, Hanzer V, Katinger H, Bertaccini A, Laimer M (2001). Improved detection methods for fruit tree phytoplasmas. *Plant Mol. Biol. Rep.* 19: 169-179.
- Khadhair AH, Momani F, Hiruki C (1995). Molecular stability of clover proliferation phytoplasma DNA in periwinkle plant tissues after thermal treatment under microwave conditions. *Proc. Jpn. Acad.* 71: 265-268.
- Lee IM, Davis RE (1992). Mycoplasma which infect plants and insects. In McElhaney RN, Finch LR, Baseman JB, Maniloff J, eds. *Mycoplasma Molecular Biology and Pathogenesis*. Washington, DC: Am. Soc. Microbiol. pp. 379-390.
- Lee IM, Hammond RW, Davis RE, Gunderson DE (1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organism. *Phytopathology*, 83: 834-842.
- Lepka P, Stitt M, Moll E, Seemüller E (1999). Effect of phytoplasmal infection on concentration and translocation of carbohydrates and amino acid in periwinkle and tobacco. *Physiol. Mol. Plant Pathol.* 55: 59-68.
- Li M, Midmore DJ (1999). Estimating the genetic relationships of Chinese water chestnut (*E. dulcis* (Burm.f.) Hensch) cultivated in Australia, using RAPDs. *J. Hort. Biotech.* 74(2): 224-231.
- Liu HW, Goodwin PH, Kuske CR (1994). Quantitation of DNA from the

- aster yellow mycoplasma like organism in aster leafhoppers (*Macrostelus fascifrons* Stal) by a competitive polymerase chain reaction. *Syst. Appl. Microbiol.* 17: 274-280.
- Musetti R, Favalli MA, Pressacco L (2000). Histopathology and polyphenol content in plants infected by phytoplasmas. *Cytobios*, 102: 133-147.
- Nakashima K, Murata N (1993). Destructive plant diseases caused by mycoplasma like organisms in Asia. *Outlook on Agric.* 22(1): 53-58.
- Nilda Z, Jiménez A, Helena A, Montano G (2010). Detection of phytoplasma in desiccated tissue of *Momoradica charantia*, *Catharanthus roseus* and *Sechium edule*. *Trop. Plant Pathol.* 36(6): 381-384.
- Rao GP, Srivastava S, Gupta PS, Sharma SR, Singh A, Singh S, Singh M, Marcone C (2008). Detection of sugarcane grassy shoot phytoplasma infecting sugarcane in India and its phylogenetic relationships to closely related phytoplasmas. *Sugar Tech.* 10(1): 74-80.
- Seemüller E, Stolz E, Kison H (1998). Persistence of European stone fruit yellow phytoplasma in aerial parts of *Prunus* taxa during the dormant season. *J. Phytopathol.* 146: 407-410.
- Sipahioglu HM, Usta M, Ocak M (2006). Use of dried high phenolic laden host leaves for virus and viroid preservation and detection by PCR method. *J. Virol. Methods*, 137: 120-124.
- Srivastava S, Singh V, Gupta PS, Sinha OK, Baitha A (2006). Nested PCR assay for detection of sugarcane grassy shoot phytoplasma in the leafhopper vector *Deltocephallus vulgaris*. a first report. *Plant Pathol.* 55: 25-28.
- Tai TH, Tanksley SD (1990). A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. *Plant Mol. Biol. Rep.* 8(4): 297-303.
- Wang K, Hiruki C (1998). The molecular stability of genomic DNA of phytoplasma in the witches broom affected paulownia tissues after microwave heat treatment. *J. Microbiol. Methods*, 33: 263-268.
- Wongkaew P, Fletcher J (2004). Sugarcane white leaf phytoplasma in tissue culture culture long term maintenance, transmission, and oxytetracycline remission. *Plant Cell Rep.* 23: 426-434.