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Full Length Article

Detection and Molecular Characterization of Bermuda Grass (*Cynodon dactylon*) White Leaf Phytoplasma from Turkey

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Abstract

Bermuda grass [*Cynodon dactylon* (L.) Pers.] plants exhibiting phytoplasma-like symptoms, (light green to yellow streaks on the leaves, bushy growth and stunting) were observed in different regions of Turkey. All diseased Bermuda grass plants were positive when 16SrDNA-amplifying primers were used in nested PCR. It was noted that Bermuda grass white leaf-phytoplasma (BGWL-phytoplasma), which belongs to the 16SrXIV group, was consistently associated with symptomatic plants and was present in more than one province of Turkey such as Aydin, Antalya, Mersin and Adana. RFLP assays conducted on the 16S primed-DNA of seven BGWL-phytoplasma PCR-positive samples yielded a uniform profile from all samples, indicating that a single strain of the BGWL-phytoplasma was affecting all the diseased Bermuda grass plants. This information was further confirmed by sequence and phylogeny analyses. All Turkish isolates of BGWL-phytoplasma shared 96-99% sequence homology among them, and all grouped in one cluster close to BGWL isolates from Iran and Italy. Presumably, this phylogenetic allocation is related to the geographical origin of the identified isolates. To our knowledge, this is the first report of BGWL disease and of its associated phytoplasma in Turkey.

Keywords: Bermuda grass; Phytoplasma; PCR; RFLP; Sequence; Phylogeny analysis

Introduction

Bermuda grass [*Cynodon dactylon* (L.) Pers.], family Poaceae, is a perennial plant native to the Mediterranean area, north and east Africa, Asia, Australia and southern Europe. In Turkey this grass is common to public gardens, forests, uncultivated areas, and in areas given over to forage production. This species is affected by a destructive phytoplasmal disease, Bermuda grass white leaf (BGWL), first reported from Taiwan (Chen *et al*., 1972), known to occur in several Asian countries (Lee *et al*., 2000; Jung *et al*., 2003; Rao *et al*., 2007), from Africa (Dafalla and Cousin, 1988), Australia (Padovan *et al*., 1999; Tran-Nguyen *et al*., 2000), Europe (Marcone *et al*., 2004; Nejat *et al*., 2009), Cuba (Arocha *et al*., 2005) and lately reported also from Iran (Salehi *et al*., 2009). The disease causes light green to yellow streaks on the leaves, extensive chlorosis and white discolourations, proliferation of axillary shoots, bushy growth, small leaves, shortened stolons and rhizomes, stunting, and, eventually, death of plants. The BGWL-associated phytoplasma, together with the agents of Brachiaria grass and annual blue grass white leaf diseases are classified in the BGWL phytoplasma 16SrXIV-A subgroup (Lee *et al*., 1998, 2000; Marcone *et al*., 2004).

In July 2011, during phytosanitary surveys conducted in specialized fields for strawberry, tomato and pepper production in Aydin, Antalya and Mersin regions, respectively, Bermuda grass plants exhibiting yellowing, whitening, shortened stolons and stunting were observed. Similar symptoms were also observed in some forests of Adana district. Phytoplasma etiology being suspected, leaf samples from diseased and healthy plants were taken for laboratory assays. Accordingly, in this study we report the first detection of BGWL-phytoplasma on Bermuda grass in Turkey and give information on its phylogenetic relatedness to other BGWL-isolates already reported in the Mediterranean region.

Materials and Methods

Source of Plant Material

Twenty samples from Bermuda grass plants showing typical yellowing and whitening of leaves (Fig. 1), and samples from symptomless plants of the same species were collected in the above mentioned areas (Aydın, Antalya, Mersin and Adana). In addition, 8-10 individuals of potential phytoplasmas vectors i.e., *Exitianus taeniaticeps* (males and females), *Recilia schmidtgeni* (males and females), *Euscelis lineolatus*, (males), *Cicadula frontalis* (males and females), *Psammotettix alienus* (males), were also collected from symptomatic Bermuda grass plants in Adana district.

DNA Extraction and PCR Amplification

DNA was extracted according to Ahrens and Seemüller

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(1992) from fresh leaves of diseased and healthy Bermuda grass plants. Tissue samples (1 g) were homogenized in 4 mL of CTAB buffer (2% w/v cetyltrimethylammonium bromide, 1.4 M NaCl, 0.2% 2-β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, 2% polyvinylpyrrolydone, pH 8.0) and 1.5 mL aliquots of the extract were incubated at 65°C for 30 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the lysis buffer (CTAB) vigorously mixed for 1 min and centrifuged at 12,000 rpm for 10 min. This step was repeated twice. The aqueous nucleic acid layer was precipitated overnight at -20°C with 0.6 volume of isopropanol. The pellet obtained after centrifugation at 8,000 rpm for 10 min was washed with 70% ethanol, vacuum-dried and suspended in 50 µL sterile water. Then it was used as DNA template for direct PCR amplification. DNA was also extracted from hoppers using the procedure described by Doyle and Doyle (1990).

The universal phytoplasma primer pair R16F1/R16R0 (5'-AAGACGAGGATAACAGTTGG-3'/5'-GGATACCTTGTTACGACTTAACCCC-3') (Lee *et al*., 1994; Davis *et al*., 1997) was used in one step PCR for amplifying a 1.8 kbp fragment of ribosomal operon consisting of the 16SrRNA gene, the 16S-23S intergenic spacer region (SR) and a portion of the 5' region of 23SrRNA gene. A 1:100 dilution of the single step PCR product amplified by the R16F1/R16R0 primer pair was used as template for a second nested PCR round, utilizing the primer pair R16F2n/R16R2 (5'-ACGACTGCTAAGACTGG-3'/5'-TGACGGGCGGTGTGTACAAACCCCG-3'), which amplify an internal DNA fragment of 1,250 bp from the 16SrRNA gene (Gunderson and Lee, 1996).

For single step PCR, amplification was performed in 50 reaction mixtures, each containing 100 ng of extracted DNA from diseased and healthy plants, 1.25 µL dNTPs (10 m*M*), 1 µL forward and reverse primers (10 pmol), 10 µL of 5X Crimson Ta reaction buffer, 3 µL $MgCl_2$ (25 m*M*) and 0.25 µL Crimson Taq DNA polymerase (5U/µL) (BioLabs, USA). PCR was conducted in a Techne TC 4000 apparatus using the following parameters: 35 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C. PCR conditions for the second round (nested PCR) were the same, except for the annealing temperature that was at 58°C. An extension cycle consisting of 10 min at 72°C was used for both PCRs. 10 µL of PCR products primed with R16F2n/R16R2 were electrophoresed in 1% agarose gel in 1xTBE buffer (67 m*M* Tris-HCl, 22 m*M* boric acid, 10 m*M* EDTA, pH 0.8) together with 1 kb DNA marker (Fermentas, Life Science, Milan, Italy), stained with ethidium bromide and photographed on a UV transilluminator.

Restriction RFLP Analysis

Sequences of the R16F2n/R16R2-primed PCR products obtained from diseased Bermuda grass plants were subjected to RFLP analysis. Two µL of each amplicon were digested with *Eco*RI, *Alu*I, *Hha*I and *Taq*I endonucleases, according to the manufacturers' instruction (Promega, USA) at 37°C (65°C for *Taq*I) overnight. The *Eco*RI restriction enzyme was first used to discriminate phytoplasma from host DNA (Nejat *et al*., 2009). The products of digestions were then analyzed by electrophoresis on a 2.5% agarose gel, stained with ethidium bromide and the DNA bands were visualized on UV transilluminator.

Cloning, Sequencing and Phylogenetic Analysis

The R16F2n/R16R2 primed-16S rDNA PCR products obtained from seven positive samples to BGWL-phytoplasma were excised from agarose gel, washed and eluted by centrifugation through siliconized glass wool, as described by Gromadka (1995). The eluted DNAs were sequenced from both directions using M13 forward and reverse sequencing-primers. DNA fragments were subjected to automated sequencing (ABI 3130xl Genetic Analyzer, Applied Bio. REFGEN Gen Araştırmaları ve Biyoteknoloji Ltd. Şti., Ankara, Turkey).

Computer-assisted analysis of nucleotide sequences was assembled using the Strider 1.1 program (Marck, 1988). 16S-23S rDNA sequences of Turkish BGWL isolates with similar reference phytoplasmas were separately aligned using Clustal X 1.81 (Thompson *et al*., 1997). Phylogenetic tree was constructed using the NJ plot and Boostrap analysis with 1000 replicates using the NEIGHBOR, SEQBOOT, PROTDIST and CONSENSE methods of the PHYLIP package (Felsenstein, 1989).

Results

Phytoplasma Detection and RFLP Analysis

Nested PCR performed with R16F2n/R16R2 primers on the single-step PCR products generated 10 amplicons 1,250 bp in size, from symptomatic but not from symptomless Bermuda grass (Fig. 2). The use of 1/10 and 1/100 dilutions for single-step and nested-PCR, respectively, gave better amplifications than undiluted samples. No DNA bands were obtained from single-step and nested-PCR conducted on extracts from any of the leafhoppers collected from Bermuda white leaf-diseased fields.

Based on the RFLP analysis, the presence of one single *Eco*RI restriction site in the 16F2n/R16R2 primed PCR products (1250 bp), that generated two DNA fragments (750 bp and 500 bp), ascertained the phytoplasmal nature of the nested-PCR amplicon (Fig. 3a). Performing separate digestions of PCR products with different endonucleases, all samples collected from different regions of Turkey showed identical restriction profiles with each enzyme used (Fig. 3a-d). This was retained as an indication that the Bermuda grass white leaf disease observed in Turkey was caused by a single phytoplasma isolate, hereafter referred to as "BGWL-Tu-M" isolate.

Fig. 1: (a) light green to yellow streaks on the leaves, yellowing and whitening symptoms on BGWL phytoplasma-affected Bermuda grass (left); healthy Bermuda grass plant is on the right, (b) whitening and small leaves, bushy growing habit occurrence on BGWL phytoplasma-affected Bermuda grass

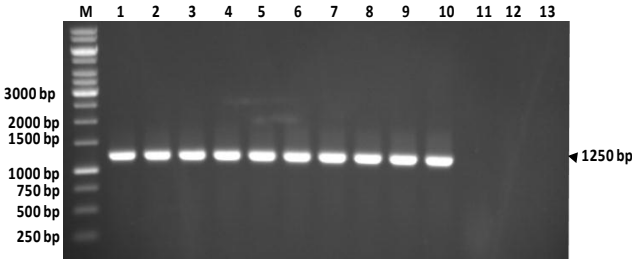

Fig. 2: Electropherogram of 16SrDNA nested-PCR products amplified with R16F2n/R16R2 from Turkish Bermuda grass samples (Tu). M: 1 kb DNA marker; lanes 1-10: diseased-Bermuda grass plants; lanes 11-12: negative controls; lane 13: PCR mix control

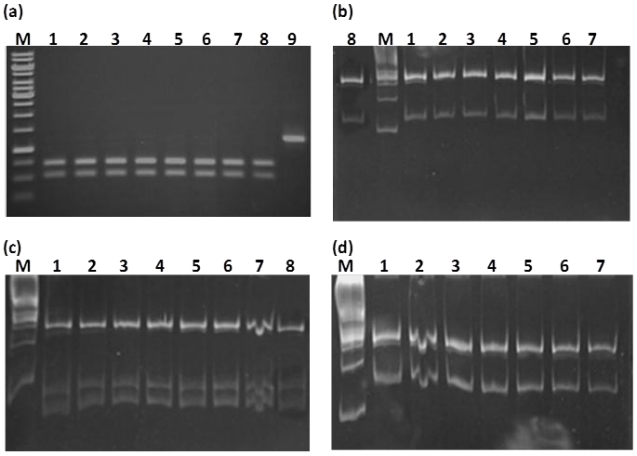

Fig. 3: Restriction Fragment Length Polymorphism analyses of 16SrDNA amplified by nested PCR from seven Bermuda grass white leaf phytoplasmas from Turkey using four restriction enzymes (a) *Eco*RI, (b) *Hha*I, (c) *Alu*I and (d) *Taq*I. M: 1 kb DNA marker; lane 1: Tu-M1; lane 2: Tu-M3; lane 3: Tu-M4; lane 4: Tu-M5; lane 5: Tu-M6; lane 6: Tu-M7; lane 7: Tu-M8; lanes 8a, b, c: positive controls of BGWL-phytoplasma; lane 9: undigested DNA used as control

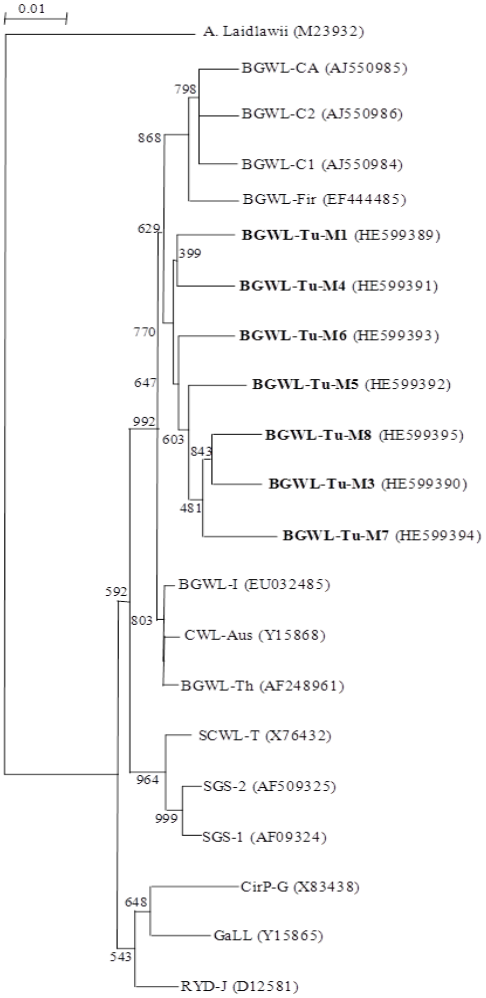

Fig. 4: Phylogenetic tree constructed with 16SrDNA sequences of BGWL-phytoplasma isolates from Turkey (BGWL-Tu-M1 to M8), Italy (BGWL-C1, BGWL-C2, BGWL-CA), Thailand (BGWL-Th), Iran (BWGL-Fir), India (BGWL-I), and BWGL-related agents: Cynodon white leaf (CWL-Aus) from Australia, Sugarcane white leaf (SCWL-T) from Thailand, Sorghum grassy shoot (SGS1, SGS2) from Australia, Cirsium phyllody (CirP-G) from Germany, Galactia little leaf (GaLL) from Australia and Rice yellow dwarf (RYD-J) from Japan. *Acholeplasma laidlawii* was used as an out-group. Accessions numbers of sequences used are reported between brackets

Sequence Analysis

BLAST and sequence analysis conducted on seven 16S-primed PCR products, revealed that the phytoplasma from Turkish Bermuda grass is nearly identical to isolates of the BGWL agent of group 16SrXIV (*Candidatus* Phytoplasma cynodontis) whose 16SrDNA sequences are available in GenBank database (accession No. AJ550984, AJ550986, AJ550985, EF444485) and share with them a sequence

Table 1: Nucleotides similarity among BGWL-phytoplasma from Turkey (Tu-M1, Tu-M3-8) and other related isolates from the same group from Italy (C1, C2, CA) and Iran (Fir) as determined by analyzing the 16SrDNA sequences

Isolates	Tu-M1	Tu-M3	Tu-M4	Tu-M5	Tu-M6	Tu-M7	Tu-M8	C1	C2	CA
Tu-M3	99.1									
Tu-M4	98.7	99.4								
Tu-M5	98.9	99.6	99.5							
Tu-M6	98.8	99.4	99.7	99.8						
Tu-M7	96.1	96.9	96.9	96.8	96.9					
Tu-M8	99.0	99.8	99.6	99.7	99.7	96.9				
C1	98.5	99.2	99.3	99.3	99.4	96.8	99.3			
C2	98.3	99.9	99.1	99.1	99.2	96.6	99.1	99.8		
CA	98.4	99.1	99.2	99.2	99.3	96.7	99.2	99.9	99.7	
Fir	98.5	99.2	99.3	99.3	99.4	96.8	99.3	99.9	99.8	99.9

similarity rannging from 96% to 99% (Table 1). The identity at the nucleotide level among Turkish isolates ranged from 98 to 99% i.e., a divergence (2%). Only one isolate (BGWL-Tu-M7) unexpectedly showed a higher nucleotide divergence (3.9%) from the other isolates under study.

Phylogenetic Tree

The phylogenetic tree constructed with 16SrDNA sequences of the BGWL-phytoplasma from Turkey, together with members of 16SrXIV-related group, confirmed the RFLP results, hence placed all the BGWL Turkish isolates in one sub-clade, closely related to one containing strains from Iran (BGWL-Fir) and Italia (BGWL-C1, C2, CA), but far from other BGWL-related agents.

Discussion

The molecular investigation conducted on the affected Bermuda grass plants from Turkey showed that the infection is of phytoplasmal origin, and that Bermuda grass white leaf phytoplasma is the causal agent, to which the name "*Candidatus* Phytoplasma Cynodontis" has been assigned. The molecular analysis showed that one strain of BGWL-phytoplasma is occurring in the Turkish samples, since the four restriction enzymes used in the RFLP assay were not able to individuate divergent profiles. This result is in harmony with previous studies on BGWL-phytoplasmas from different geographical areas (Marcone *et al*., 1997; Lee *et al*., 1998; Tran-Nguyen *et al*., 2000; Wongkaew *et al*., 2004), according to which no polymorphism was registered, and showed that BGWL-phytoplasmas are largely identical and represent a distinct taxonomic entity. Besides, the level of nucleotide divergence found among the sequenced Turkish isolates (2%) was in line with that previously reported for members of the 16SrXIV group (Marcone *et al*., 1997), except for one single Bermuda grass sample (BGWL-Tu-M7) that unexpectedly showed a more marked nucleotides variance (3.9%) if compared to the other BGWL-Turkish isolates. Although, this level of sequence divergence is higher than the recommended threshold of 2.5% deemed sufficient by the International Research Program on Comparative Mycoplasmology (IRPCM, 2000) for defining the status of novel "*Candidatus*" phytoplasma specie, there is not enough supporting evidence that the agent detected in this sample could be recognized as a novel putative specie, and\or belonging to another subgroup of the 16SrXIV group. Further RFLP analyses using additional endonucleases are needed for a better understanding of the significance of this molecular variation. The fact remains that: (i) the RFLP pattern of this isolate is identical to those of the other Turkish isolates, (ii) all isolates investigated in this study showed a homogenous phylogeny, gathering in the same sub-clade, close to the BGWL-Fir isolate from Iran and Italy (BGWL-C1, C2, CA) but far from other BGWL-related agents (Fig. 4).

The possible vector of BGWL-phytoplasma is still unknown for none of the six leafhoppers species collected from BGWL-affected plants in the Adana district was PCR-positive. However, this investigation should be extended to other species, considering that *Exitianus capicola, a* putative vector of BGWL-P-cynodontis recently reported from Iran (Salehi *et al*., 2009), occurs also in Turkey (Weintraub *et al*., 2004). Nonetheless, this is the first report of BGWL phytoplasma in Turkey; and its presence represents a potential threat for *C. dactylon* cultivation which is widely used for forage and turf production.

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