J. Turk. Phytopath., Vol. 40 No. 1-3, 51-56, 2011 ISSN 0378 - 8024

# Experimentally Transmission of Bermuda Grass (*Cynodon dactylon* (L.) Pers.) White Leaf Phytoplasma to Wheat Plants by Root-Bridge Modality

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*Accepted for publication May 17, 2013*

## ABSTRACT

Bermuda grass white leaf phytoplasma (BGWL) was previously reported in Turkey. In the study, transmission of agents from Bermuda grass to wheat (*Triticum* spp.) plants was experimentally investigated by a root-bridge system in August 2012, Adana. Twelve Bermuda grass plants, infected with *Candidatus* Phytoplasma cynodontis, strain M6 from Turkey and showing symptoms of light green to yellow streaks on the leaves and bushy growth, were taken out from soil separately and transplanted to two plastic pots. Two hundred of wheat seeds were sown in to each pot between diseased Bermuda grass plants. After two months under net screen, wheat plants in the pots started to turn into yellow color, compared with healthy control plants. All infected wheat plants were positive when 16S rDNA-amplifying primers were used in nested PCR, contrarily to the healthy ones used as negative controls. Restriction fragment length polymorphism (RFLP) assays conducted on the 16S primed-DNA of BGWL-phytoplasma PCR-positive samples yielded a uniform profile and similar to the strain M6 of the BGWL-phytoplasma used as an inoculum. This information was further confirmed by sequence analyses. To our knowledge, this is the first report of transmission of BGWL disease to another host, different from Bermuda grass plants, by root-bridge.

**Keywords:** Transmission, Root-bridge system, 16S rDNA, PCR, RFLP

## INTRODUCTION

Bermuda grass [*Cynodon dactylon* (L.) Pers.] 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*., 1997), Cuba (Arocha *et al*., 2005), Iran (Salehi *et al*., 2009) and lately reported also from Turkey (Çağlar *et al*., 2013). The disease causes light green to yellow streaks on the leaves, extensive chlorosis and white discolorations, proliferation of axillary shoots, bushy growing habit, small leaves, shortened stolons and rhizomes, stunting, and death of the plants. (Marcone *et al*., 1997). The BGWL-associated phytoplasma, together with the agents of phytoplasmas related to the *Candidatus* Phytoplasma cynodontis (16SrXIV) group was found on coconut (Nejat *et al*., 2009a), date palms (*Phoenix dactylifera*) (Cronje´*et al*., 2000), annual blue grass (*Poa annua*) and brachiaria grass (*Brachiaria distachya*) (Lee *et al*., 1997; Marcone *et al*., 2004 ), carpet grass (*Axonopus compressus*) (Padovan *et al*., 1999), phytoplasma also found on sugarcane, rice and sorghum. In addition to monocotyledon, BGWL-related phytoplasmas have been identified in *Cirsium arvensis* (Canada thistle) and *Galactia tenuifolia* as

dicotyledons (Seemüller et al., 1994; Schneideret al., 1997). However, there is no report on the possible transmission of BGWL-phytoplasma to wheat (*Triticum* spp.).

In nature, BGWL phytoplasma is transmitted between Bermuda grass by leafhopper *Exitianus capicola* (Stal 1855) *cicadellidae*, while *E. capicola* was first time reported as the natural and experimental vector of Bermuda grass white leaf agent in Iran (Salehi *et al*., 2009).

In August 2012, Adana, the transmission of BGWL-phytoplasma from Bermuda grass to wheat plants was conducted in experimental condition through a root-bridge procedure, for which the results are hereafter reported.

## MATERIALS AND METHODS

### Source of Material and Installation of the Experiment

Twelve samples of Bermuda grass plants infected with phytoplasma (*Candidatus* Phytoplasma cynodontis, strain M6 from Turkey, Accession number HE599393) showing symptoms of light green to yellow streaks on the leaves, bushy growth and stunting from Adana province were taken out from soil and six plants were separately transplanted to two plastic pots (39 cm diameter and 30 cm height) including mixture of soil; sand; animal compost in the rate of 1:1:1. The same procedure was followed using instead healthy Bermuda grass plants. All plants were fertilized regularly in two mounts to grow and multiply in pots and used it as source BGWL phytoplasma for experimental transmission. Two hundred of wheat seeds were sown in to each pot between Bermuda grass plants and watered. The seeds started to germinate in 20 days and plants started to grow. All the experiment was conducted under net screen and insecticides were applied to plants to prevent possible transmission of BGWL-phytoplasma to wheat by vectors.

### DNA Extraction

DNA was extracted according to Ahrens and Seemüller (1992) from fresh leaves of diseased and healthy wheat plants. Leaf samples (1 g) were homogenized in 4 ml of CTAB buffer (2% w/v cetyltrimethylammonium bromide, 1.4 M NaCl, 0.2% 2-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.

### PCR Amplification

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

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 MgCl2 (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 1 X TBE 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 infected wheat plants were subjected to RFLP analysis. Five µl of each amplicon were digested with *Eco*RI, *Taq*I, *Hha*I and *Alu*I endonucleases, at different temperatures, according to the manufacturers' instruction (Fermentas, Life Science). The *Eco*RI restriction enzyme was used to discriminate phytoplasma from host DNA (Nejat *et al*., 2009b). 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 Computer-assisted Analysis**

The R16F2n/R16R2 primed-16 S rDNA PCR products obtained from positive sample 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 from wheat plants 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

**Symptoms Appearance**

After two months, more than 30% of wheat plants in the pot of BGWL-infected plants started to turn into yellow color comparing with negative control plants (Fig. 1), indicating the transmission of BGWL phytoplasma to wheat by a root-bridge modality. The presence of BGWL-phytoplasma in the affected wheat plants was also confirmed by PCR/nested-PCR.

**Figure 1**. Two plastic pots showing diseased wheat plants experimentally infected, by a root-bridge modality, with BGWL-phytoplasma.

### Phytoplasma Detection and RFLP

Nested-PCR performed with R16F2n/R16R2 primers on the single-step PCR products generated DNA amplicons of 1,250 bp in size (Fig. 2), from six plants of wheat chosen for their symptomatic aspects. Based on the RFLP analysis, the presence of one single *EcoR*I restriction site in the 16F2n/R16R2 primed PCR products (1,250 bp), generated two DNA fragments (750 bp and 500 bp) and ascertained the phytoplasmal nature of the nested-PCR amplicon (Fig. 2). Performing separate digestions of PCR products with different endonucleases, the samples of experimentally infected wheat plants showed identical restriction profiles with each enzyme used (Fig. 3).

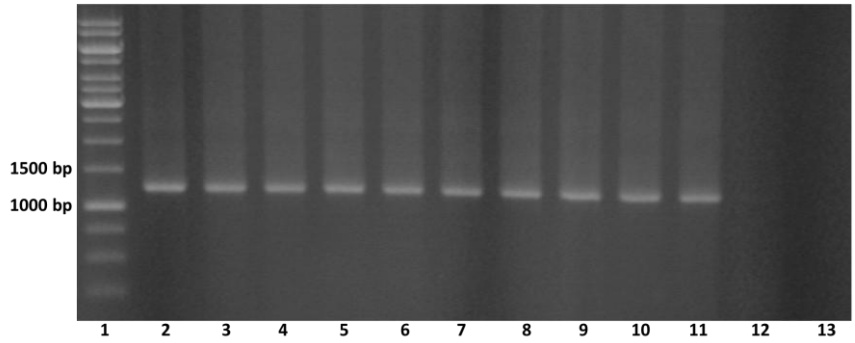


**Figure 2**. Electropherogram showing R16F2n/R16R2 primed-16S rDNA PCR amplicons obtained from infected wheat plants (lanes 2-10). Lane 11: BGWL-phytoplasma used as positive control; lane 12: healthy wheat plant; lane 13: water control. Lane 1: 1kb DNA ladder.

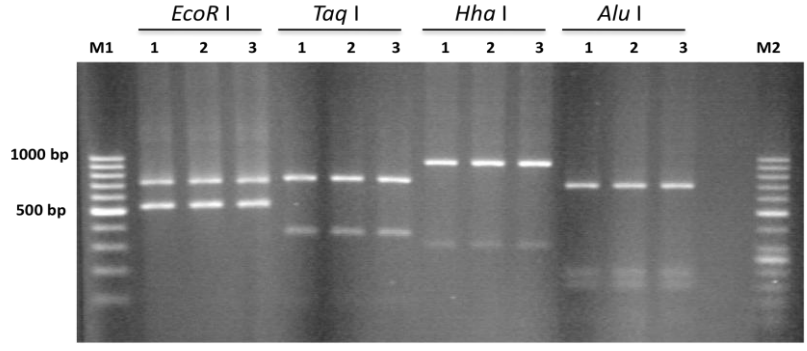


**Figure 3**. RFLP pattern generated from the use of four endonucleases (above lines) on nested-PCR products of wheat plants (lanes 1 and 2), infected with the BGWL-phytoplasma through a root-bridge transmission. Lanes 3: BGWL-phytoplasma strain M6 used as a positive control; M1: 100 bp DNA ladder; M2: 50 bp DNA ladder.

### Sequence and Phylogenetic Analyses

Alignment of nucleotide sequences of six nested-PCR amplicons (1250 bp), obtained from the phytoplasma present in the affected wheat plants, showed complete identity with the homolgue region of BGWL-phytoplasma strain M6 from Turkey. Moreover, the genetic similarity of 16S rDNA of the phytoplasma infecting wheat plants with that strain was also confirmed by the phylogenetic analysis (data not shown).

## DISCUSSION AND CONCLUSION

In this study, the transmission of Bermuda grass white leaf phytoplasma by the root-bridge modality to another host was experimentally demonstrated for the first time. The percentage of transmission for this agent was found relatively high (30%) and this phenomenon lead to suppose that natural anastomoses can play an important role in spreading of the disease in wheat fields, specially that Bermuda grass is considered as an invasive plants frequently present where wheat is grown. Accordingly, this transmission could aggravate on the short distances

between infected Bermuda grass plants and wheat in the field. Taking BGWL phytoplasma causes light green to yellow streaks on the leaves, extensive chlorosis and white discolorations, proliferation of axillary shoots, bushy growing habit, small leaves, shortened stolons and rhizomes, stunting, and death of the plants (Marcone *et al*., 1997) and transmits between Bermuda grass by leafhopper *Exitianus capicola* (Stal 1855) C*icadellidae* (Salehi *et al*., 2009) into consideration, this could become a serious problem for wheat cultivation since the planting distances in commercial fields are very reduced. However, further investigations are necessary to clarify the possible role of BGWL-phytoplasma spread, naturally, by root bridges in wheat plantations.

**ÖZET**

**KÖPEK DİŞİ AYRIĞI YAPRAK BEYAZLAŞMA FİTOPLAZMASI (BGWL)'NIN KÖPEK DİŞİ AYRIK OTUNDAN KÖK KAYNAŞMASI SİSTEMİ YOLUYLA BUĞDAY BİTKİLERİNE (*TRITICUM SPP.*) DENEYSEL OLARAK TAŞINMASI**

Köpek dişi ayrığı yaprak beyazlaşma fitoplazması (BGWL) daha önce Türkiye'de rapor edilmiştir. Ağustos 2012, Adana'da yapılan çalışmada etmenin köpek dişi ayrık otundan kök kaynaşması sistemi yoluyla buğday bitkilerine (*Triticum spp*.) taşınması araştırılmıştır. Etmenin Türkiye deki M6 ırkı ile bulaşık, yaprakta açık yeşil, sarı çizgiler ve bodurlaşma simptomu gösteren köpek dişi ayrık otları topraktan kökleriyle birlikte sökülmüş ve 12' şer adet bulaşık bitki plastik saksılara dikilmiştir.. Daha sonra her bir plastik saksıdaki hastalıklı köpek dişi ayrık otlarının arasına ikiyüz adet buğday tohumu ekilmiştir.. Tül kafes içinde iki ay sonra, sağlıklı kontrollere göre karşılaştırıldığında, plastik saksılardaki buğday bitkilerinde sararma başlamıştır. 16S rDNA'nın nested PCR (Polimeraz Zincir Reaksiyonu) yöntemi ile çoğaltılması sonucunda, negatif kontrol olarak kullanılan sağlıklı bitkilerin aksine, hastalık belirtisi gösteren bulaşık bitkilerin tamamı pozitif bulunmuştur. Pozitif sonuç alınan buğday bitki örneklerine ait PCR ürünleri restriksiyon enzimleri kullanılarak RFLP çalışmalarına tabi tutulmuştur. Elde edilen RFLP profili ile inokulum kaynağı olarak kullanılan *Candidatus* Phytoplasma cynodontis'in M6 ırkı ile aynı olduğu ortaya konulmuştur. Sonuçlar DNA dizileme ile desteklenmiştir. Bilgilerimize göre bu bulgu, BGWL-fitoplazmasının köpek dişi ayrığı bitkisinden kök kaynaşması yoluyla farklı bir konukçuya taşındığına dair ilk rapordur.

**Anahtar Kelimeler:** Taşınma, Kök-kaynaşma sistemi, 16S rDNA, PCR, RFLP

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