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Molecular identification of some turfgrass cultivars and their resistance to *Fusarium graminearum*

A. Al-Humaid[1], G. H. Ibrahim[2] and M.I. Motawei[1*]

[1]Plant Production and Protection Dept., College of Agriculture and Veterinary Medicine, Al-Qassim University, Saudi Arabia
[2]Plant Pathology Research Institute, ARC, Giza, Egypt

***Corresponding author: rumotawei@hotmail.com**

Abstract

Ten turfgrass cultivars (three cultivars of bermudagrass (*Cynodon dactylon*) hybrids, Common bermudagrass, Paspalum, Saint-Augustine, El-Toro (*Zoysia japonica*), Korean Velvetgrass (*Zoysia tenuifolia*), SI 2000, and Mahar cultivar were investigated using ISSR fingerprinting as genetic marker to assess genetic diversity. Analysis of the six ISSR primers among the turfgrass cultivars in this study generated 51 bands. Over 96% (49 bands) were found to be polymorphic in the turfgrass cultivars. The genetic similarity coefficients among all turfgrasses ranged from 0.43 to 0.88. The dendogram constructed with UPGMA analysis revealed two main clusters. Also, SCAR marker was performed on DNA extracts of rhizome of turfgrass cultivars for detection of the fungus *F. graminearum*. St. Augustine and Mahar cultivars were the most susceptible cultivars to infection *F. graminearum*, whereas the other turfgrass cultivars were resistant cultivars.

Keywords: Turfgrass, ISSR markers, SCAR marker, cultivars, cluster, Fusarium crown and root rot resistance.
Abbreviations: ISSR - Inter-Simple Sequence Repeats, SCAR - sequence-characterization RAPD fragments, UPGMA- Unweighted Pair-Group Method with Arithmetical Averages, TD- Tifsport; TW- Tifway 419(sample 1), OTW - Tifway 419(sample 2), TG – Tifgreen, CB - Common bermudagrass, El - El-Toro, KV - Korean Velvetgrass, SI - SI 2000, Pa – Paspalum, St - St. Augustin, Ma - Mahar cultivar.

Introduction

Turfgrasses are used for a range of purposes, including residential lawns, general grounds, sports fields, golf courses, and native areas. Each turfgrass species has a number of cultivars, varieties or hybrids, each of which is genetically different (Pound and Street, 2001). Bermudagrass species such as *Cynodon dactylon* and *C. transvaalensis* and their derived hybrids (Tifway, Tifgreen, and Tifdwarf) are most commonly used in turf grass areas for various purposes. Bermudagrass are well known for their vigor and heat and drought tolerance (Rodrigues *et al*., 2002). However, different problems have been noticed such as newly planted springs lack of mature roots (Rodrigues *et al*., 2002), exhibition of a mosaic of patches of different kinds of bermudas (off-type grasses), and a variation of plants caused by repeated growing on the same area, which may be due to mutations or contaminations by seeds from other places. In Kingdom of Saudi Arabia and Arab countries, turfgrasses are spread commercially depending on public markets. With the increased development and release of new turfgrass cultivar lines each year, cultivar identification becomes critical to control germplasm quality and protect ownership rights and the rights of the buying public. Some of turfgrasses such as bermudagrass cultivars (Al-Humaid and Motawei, 2004) and zoysiagrass (*Zoysia spp*.) cultivars (Cai *et al*., 2005) are difficult to be distinguished morphologically. DNA fingerprinting is one of the most precise techniques available for comparing biological samples. DNA fingerprinting technologies have been applied in genetic studies in a wide range of plant species, including turf-type bermudagrass (Cato and Richardsin, 1996; Caetano-Annolles *et al*., 1997; Arcade *et al.,* 2000; Karaca *et al.,* 2002). Several PCR-based DNA fingerprinting techniques, including random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and amplified fragment length polymorphism (AFLP) are available for detecting genetic differences within and among cultivars (Chai and Sticklen, 1998). Among these, simple sequence repeat (SSR) markers are efficient, cost-effective and can detect a significantly higher degree of polymorphism in turf species such as seashore paspalum (*Paspalum vainatum* Swartz) (Brown *et al.,* 1997). They are ideal for genetic diversity studies and intensive genetic mapping. Cai *et al*., (2005) concluded that SSR markers should be a valuable tool for identification, estimation of genetic diversity and construction of genetic linkage maps in *Zoysia* species. An alternative method to SSR, called inter-SSR (ISSR)-PCR (Nagaoka and Ogihara, 1997), has also been used to fingerprint the different plant species and cultivars (Nagaoka and Ogihara, 1997; Levi and Rowland, 1997; Wolf *et al.,* 1998; Nagaraju *et al*., 2002; Al-Humaid *et al.,* 2004). In Al-Qassim region (Central Saudi Arabia), one of the main diseases affecting turfgrass is Fusarium crown and root rot (FCRR), caused by the fungus *Fusarium graminearum*. This fungus is a common pathogen on many gramineae crops in Al-Qassim region (Motawei and Abdalla, 2003). It is a warm season disease that is also referred to in some references as Fusarium blight of turfgrass (Smiley *et al.,* 1992). It is characterized by rotting of roots, crowns, stolons and rhizomes. In California, this disease can be so severe that it has been referred to as the summer obliteration disease of turfgrass (Endo *et al.,* 1973). In several instances in Al-Qassim region, FCRR has become damaging only a few months after establishment. The problem often becomes

progressively more severe after the turf stand is two or more years old. Therefore, the ability of the sod purchasers to detect the presence of Fusarium infection even in healthy looking sod, along with identifying Fusarium resistant turfgrass genotypes may greatly improve the long-term survival of turfgrass in Al-Qassim region. Recently, the utilization of molecular markers for species specific detection assays has become very popular (Henson and French, 1993). Based on polymerase chain reaction (PCR), highly sensitive diagnostic assays have been implemented successfully for the identification and detection of the major fungi (Doohan *et al*., 1998). Wiglesworth *et al.*(1994) isolated a distinct fragment of randomly amplified polymorphic DNA (RAPD) of *Peronospora tabacina* representing a repetitive sequence. Using specific primers, the amplification of this sequence enabled the detection of minute amounts of fungal DNA in plant tissue. This approach of sequence-characterization RAPD fragments (SCARs) was first applied by Paran and Michelmore (1993) to mark downy mildew resistance genes in lettuce. Motawei and Abdalla (2003) concluded that specific-PCR represents a valuable new tool for diagnosis and screening of plant materials for resistance to *Fusarium graminearum*.The objectives of this study were to (1) determine the usefulness of ISSR markers to distinguish between the different turfgrass cultivars through fingerprinting, (2) study the genetic variation within cultivar Tifway '419' (3) estimate the genetic relationship between turfgrasses based on ISSR markers, and (4) detect the presence of *Fusarium graminearum* in infected turfgrass plants using SCARs technique.

Results and discussion

DNA amplification fingerprinting

Ten turfgrass cultivars (Hybrid bermudagrass: "Tifsport, Tifway419 (two samples), Tifgreen", Common bermudagrass, El-Toro, Korean Velvetgrass, SI 2000, Paspalum, St. Augustin, and Mahar) were screened for ISSR markers (Table 1). The number of amplification bands per primer varied between 0 and 10. The trinucleotide repeats $(CAC)_n$ and $(CTC)_n$ primer had more bands than dinucleotide repeats $(GA)_n$ and $(CA)_n$ primers (Table 2), probably because of its greater abundance in turfgrass genome. The repeats $(GA)_n$ and $(CA)_n$ were the most abundant in rice (Nagaraju *et al*., 2002) and date palm (Trifi *et al.,* 2000) genomes. On the other hand, tetranucleotide repeats $(ATCG)_n$ did not amplify with DNA of turfgrass genotypes. This might indicate that di- and trinucleotide-based ISSR-PCR markers could provide potential markers in the turfgrass genome (Al-Humaid *et al.,* 2004). Kamps *et al.* (2007) enhanced turfgrass variety identification by developing an SSR based marker system. Analysis of the six ISSR primers among the turfgrass cultivars in this study generated 51 bands. Over 96% (49 bands) were found to be polymorphic in the turfgrass cultivars using ISSR, indicating that the bands were present in at least one cultivar but were not observed in others (Yerramsetty *et al.,* 2005). Figure 1 shows the amplification profiles generated by $(CAC)_n$ and $(CA)_n$ primers across the tested turfgrass genotypes. The two samples of cultivar Tifway419 were distinguished by a 400 bp amplification fragment produced by primer D14 (Fig.1). The genetic differences between the two samples of Tifway419 could be, possibly, explained by mutations resulting from repeated growing on the same area or contaminations by seeds from other places (Rodrigues *et al.,* 2002). The 500-bp amplification fragment produced by primer D24, which distinguished cultivar Tifgreen, was absent in the other bermudagrass hybrids. The cultivar El-Toro was distinguished by a 700-bp amplification fragment produced by primer D14, which was absent in cultivar Korean Velvetgrass (Fig. 1).Therefore, the molecular marker at 700-bp band produced by primer D14 can be used to distinguish the two *Zoysia* species. Cai *et al*. (2005) developed SSR markers for zoysiagrass identification. Also, Kamps *et al*. (2007) found that PCR amplification resulted in SSR marker profiles having up to 17 unique bands to discern tested bermudagrass genotypes. Therefore, ISSR represents an excellent technique for bermudagrass cultivar identification, varietal protection, and for the identification of mistakes in plantings, mislabeled plant materials, and contamination or substitutions of sod fields (Caetano-Annolles *et al.,* 1995).

Cluster analysis

The pair-wise genetic distance estimates of the turfgrass cultivars in this study were analyzed and are given in Table 3. The genetic similarity coefficients among all turfgrasses ranged from 0.43 to 0.88. Maximum similarity was observed between the two samples of Tifway419 (0.88). Cultivar Tifsport was more closely related to cultivar Tifway 419 than cultivar Tifgreen. Cultivar SI 2000 was quite distinct from other turfgrasses. Cluster analysis was conducted to generate a dendogram elucidating for relationships among turfgrass cultivars. The dendogram constructed with UPGMA analysis revealed two main clusters (Fig. 2). The first cluster divided into three sub-clusters comprising: (i) Saint-Augustin grass, Mahar, and Paspalum, (ii) common bermudagrass and the bermudagrass hybrids (Tifway, and Tifsport), and (iii) the bermudagrass hybrid Tifgreen. The second cluster consisted of cultivars El-Toro, Korean Velvetgrass, and SI 2000. Within the second cluster cultivar SI 2000 was separated from the zoysiagrass cultivars. As revealed by ISSR analysis, cultivars Saint Augustine, Mahar, and Paspalm were quit distinct from both common bermudagrass and the bermudagrass hybrids. Similar results were reported by Al-Humaid and Motawei (2004) using RAPD markers. ISSR primer sequences are designed from microsatellite regions and the annealing temperatures used are higher than those used for RAPD markers (Wolf *et al.,* 1998). Extremely high variability and high mapping density as compared with RFLP and RAPD data make these new dominant, microsatellite-based molecular markers ideal for producing genetic maps of individual species (Karaca *et al.,* 2002).These features, combined with greater robustness in repeatability of experiments and less prone to changing band patterns with changes in constituent or DNA template concentrations, make them superior to other readily available marker systems in investigations of genetic variation among very closely related individuals and in crop cultivar classification (Fang and Roose, 1997; Karaca *et al.,* 2002). The results clearly demonstrate that a methodology based on ISSR markers can be used to identify and fingerprint turfgrass cultivars.

Evaluation of turfgrass genotypes for Fusarium graminearum resistance

Various turfgrass cultivars reacted differently to infection with *F. graminearum*. Specific-PCR analysis was employed using SCAR-85 to detect the presence of *F. graminearum* in infected turfgrass genotypes (Fig. 3). Primer UBC 85 amplified a fragment of 410 bp that was unique to *F. graminearum* according to Schilling *et al.* (1996). Short random primers used in RAPD analysis usually anneal with

Table 1. List of turfgrass cultivars used in this study.

Turfgrass cultivars	Scientific name
Hybrid bermudagrass:	*Cynodon dactylon* X *C. transvaalensis*
*Tifsport (TD)	
*Tifway 419(sample 1) (TW)	
*Tifway 419(sample 2) (OTW)	
*Tifgreen (TG)	
Common bermudagrass (CB)	*Cynodon dactylon* (L) Pers.
El-Toro (El)	*Zoysia japonica*
Korean Velvetgrass (KV)	*Zoysia tenuifolia*
SI 2000 (SI)	*Paspalum vagantum*
Paspalum (Pa)	*Paspalum vagantum*
St. Augustin (St)	*Stenotaphrum secundatum* (Walt.) Kuntze
Mahar cultivar (Ma)	*Paspalum vagantum* X *Cynodon dactylon*

Table 2. ISSR primers with the number of amplified products and polymorphic fragments.

Primers	Sequence 5` to 3`	Amplified products	Polymorphic fragments
P02	$(ATCG)_4$	0	0
D12	$(GA)_6CG$	7	7
D14	$(CAC)_3GC$	10	9
D24	$(CA)_6CG$	8	8
HB 13	$(GAG)_3GC$	7	7
HB 14	$(CTC)_3GC$	10	9
HB 15	$(GTG)_3GC$	9	9

multiple sites at different regions of the genome so that several genetic loci are amplified. Moreover, poor reproducibility can occur in RAPD analysis. To overcome these problems, RAPD markers need to be converted into SCAR markers (Cao *et al.,* 2001). Amplification of SCAR-85 revealed the presence of *F. graminearum* in all infected turfgrass cultivars St. Augustine and Mahar. Whereas, the other turfgrass cultivars were resistant cultivars. It should be noted here that disease severity data were recorded only 32 days after infection. This might indicate that specific-PCR can be very reliable in detecting infection with *F. graminearum* directly in extracts of infected plant tissue at various degrees of disease severity (Motawei and Abdalla 2003; Wiglesworth *et al.,* 1994). Schilling *et al.* (1996) found that a minimum amount of 5pg of *F. graminearum* total genomic DNA was sufficient for specific and reliable amplification of SCAR85 that was visible in the gel. Moreover, the PCR assay was capable of identifying the particular species *in vivo* in weakly infected plant tissues and at early stages of disease with barely visible symptoms. In contrast, the reliability and precision of enzymatic and immunological test systems can be affected by various factors such as sample having high protein content (Schilling *et al.*, 1996; Taylor 1993).

Materials and methods

Greenhouse experiments

Autoclaved sandy loam soil (pH 6.3) was placed in plastic boxes (10 cm x 10 cm x 5 cm) and evenly sown with turfgrass seeds. Ten cultivars of turfgrass (Table 1) were used, each cultivar was sown in three different boxes. The soil boxes were incubated in the greenhouse at 25±3°C with relative humidity varying between 60 and 80%, durational light conditions (12 h of dark per 12 h of light) maintained at 6000 lx and watered daily. After 27 days, healthy-looking seedlings of each cultivar were collected and rinsed in distilled water to check for any root discoloration. Seedlings with discolored roots were discarded and then fifty healthy-looking seedlings were placed into pots (6 cm x 25 cm), ten seedlings per pot. The pots contained a steamed soil: perlite:sand mixture (1: 1: 1 vol./vol./vol.). Five pots of each turfgrass entry were then inoculated by inserting 3 g of oats infested with *F. graminearum* per pot into the soil surrounding roots. Oat inoculum was prepared as described by Tissart *et al*. (1989). Soil in five additional pots of each turfgrass cultivar was amended with 3 g of sterile oats as checks. Pots were placed in the greenhouse under the same previous conditions and watered daily. Pots were arranged in a randomized block design. After 32 days, plants were gently removed from soil and washed thoroughly in tap water.

Plant materials and DNA extraction

Bulk leaf samples from 25 plants of each of turfgrass cultivars (Table 1), bermudagrass hybrids "Tifsport, Tifway 419 (sample 1), Tifway 419 (sample 2) and Tifgreen", Common bermudagrass, Paspalum, Saint-Augestin, El-Toro (*Zoysia japonica*), Korean Velvetgrass (*Zoysia tenuifolia*), SI 2000, and Mahar were used. The bulk sample of leaves was first ground into fine powder with liquid nitrogen. The DNA extraction was done using CTAB method. The DNA concentration was assessed spectrophotometrically at 260 nm, and quality was assessed by the 260/280 ratio (Sambrook et al. 1989). The DNA was suspended to a final concentration of 10 ng/µl in 0.5X TE and stored at 4ºC.

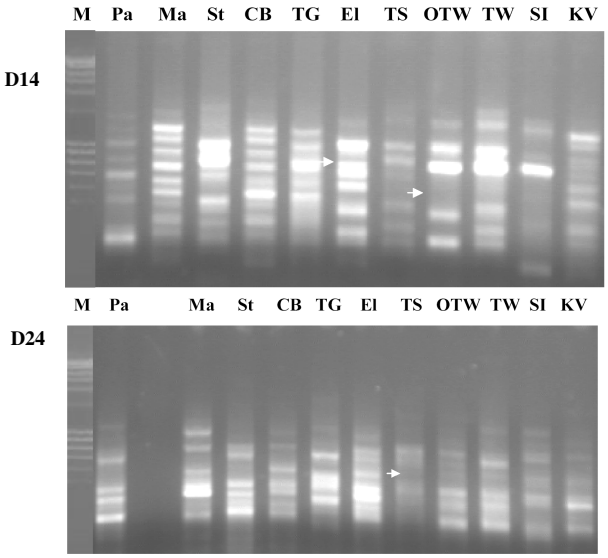

Fig 1. Polymorphism revealed using primer D14 and D24 to amplify genomic DNA purified from the tested turfgrass cultivars. M lane is 1 kbp ladder DNA marker. Arrow shows polymorphic band at 700 bp produced by primer D14, which was present in cultivar El-Toro and was absent in cultivar Korean Velvetgrass. Also, three arrows show amplification fragments produced by primer D14, which distinguished the two samples of cultivar Tifway419. Primer D24, arrow shows a 500-bp amplification fragment, which distinguished cultivar Tifgreen, was absent in the other bermudagrass hybrids.

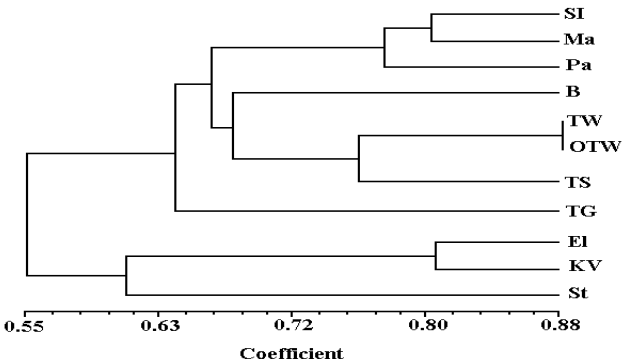

Fig 2. Dendrogram constructed from similarity coefficients and showing the clustering of the tested turfgrass cultivars.

ISSR assay:

The ISSR-PCR method was carried out according to Negaoka and Ogihara, (1997). Amplification were carried out in 25 µl reaction volumes, containing 1X Taq polymerase buffer (50 mM KCl, 10mM Tris, pH 7.5, 1.5 mM $MgCl_2$) and 1 unit of Taq polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each dNTPs (Pharmacia Biotech, Germany), 50 pmol of ISSR primers (Table 2), and 50 ng of total genomic DNA. Amplification was performed in a thermal cycler (Thermolyne Amplitron) programmed for 1 cycle of 2 min at 94˚C; and 35 cycles of 30 secs at 94˚C, 45 secs at 44˚C, and 1.3 min at 72˚C; followed by 20 min at 72˚C. After completion of PCR, samples were cooled immediately to 10˚C and stored at 4˚C until gel separation. A gel-loading solution (5 µl) was added, and 10 µl of the total product volume was resolved in 1.5% agarose in 1X TAE buffer for 2 h aside with a 100- bp ladder (Pharmacia, Germany) as the size standard. Gels were stained in ethedium bromide and images were recorded.

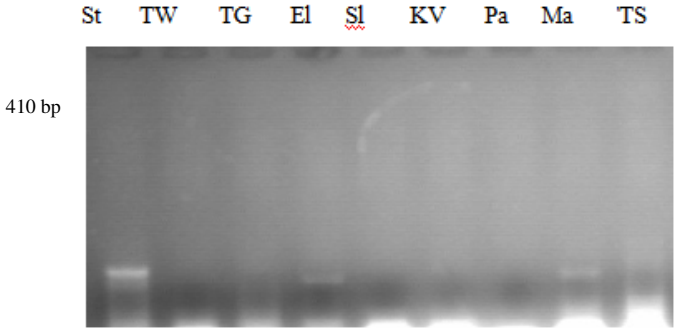

Fig 3. Detection of *F. graminearum* in turfgrass cultivars using specific primer (SCAR 85). Specific primers (SCAR 85) amplified with turfgrass cultivars St. Augustine and Mahar. Arrows show the one polymorphic band (410 bp).

Statistical analysis

Data of ISSR analysis were scored for computer analysis on the basis of the presence or absence of the amplified products for each ISSR primer. If a product was present in a cultivar, it was designated "1", if absent it was designated "0". Pair-wise comparisons of cultivars, based on the presence or absence of unique and shared polymorphic products, were used to generate similarity coefficients based on SIMQUAL module. The similarity coefficients were then used to construct a dendogram by UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) using NTSYS-PC software version 2.0 (Exeter Software, New York) (Rohlf, 2000).

Detection of the presence of Fusarium graminearum in infected turfgrass

DNA of *Fusarium* was extracted from infected rhizome sections of freeze-dried plant samples using the protocol of Doohan *et al.* (1998). Specific PCR was performed using SCAR primers which were synthesized by Pharmacia Biotech, Roosendaal, Netherlands. The primer sequences for *F. graminearum* was (UBC 85 F/R: GCAGGGTTT-GAATCCGAGAC / AGAATGGAGCTACCAACGGC) (Schiling *et al*., 1996). PCR amplification was conducted in 25 µL reaction volumes containing 1X Taq polymerase buffer (50 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM MgCl2) and 1 unit of Taq polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each dNTP (Pharmacia, Germany), 25 ρmol of each forward and reverse primer and 50 mg of total genomic DNA. Cycling profiles consisted of 30 cycles of 1 min. at 94°C, 1 min. at 61°C and 2 min. at 72°C. At the beginning of the cycling profile, reactions were held for 2 min. at 94°C to denature the genomic DNA templates, and the final cycle was extended to 5 min. at 72°C. After completion of PCR, samples were immediately cooled to 10 °C and stored at 4 °C until gel separation. A gel-loading solution (5ul) was added, and 10 µl of the total product volume was resolved in 1.5 agarose in 1x TAE buffer for 2 h.

Table 3. Simple matching coefficients of similarity determined from analysis using 51 ISSR loci .

Turfgrass cultivars	St	Pa	Ma	CB	TG	TW	TS	OTW	El	SI	KV
St. Augustin (St)	1.00										
Paspalum (Pa)	0.78	1.00									
Mahar (Ma)	0.80	0.78	1.00								
Common bermudagrass (CB)	0.55	0.73	0.65	1.00							
Tifgreen (TG)											
Tifway-1 (TW)	0.67	0.65	0.65	0.57	1.00						
Tifsport (TS)	0.71	0.57	0.65	0.61	0.65	1.00					
Tifway-2 (OTW)	0.73	0.67	0.67	0.67	0.63	0.75	1.00				
El-Toro (El)	0.67	0.65	0.65	0.73	0.69	0.88	0.84	1.00			
SI 2000 (SI)	0.53	0.47	0.55	0.51	0.59	0.59	0.59	0.53	1.00		
Korean (KV)	0.43	0.49	0.49	0.57	0.57	0.57	0.57	0.63	0.43	1.00	
	0.57	0.47	0.55	0.55	0.63	0.67	0.67	0.61	0.80	0.59	1.00

Gels were stained in ethedium bromide and images were recorded.

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