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Genome polymorphism markers and stress genes expression for identifying turf species

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Reactive oxygen species (ROS) are produced in both stressed and unstressed cells. Superoxide dismutase (SOD) and phenylalanine ammonia lyase (PAL) play an important role in the defense against ROS. Eight different turf grass species were used in order to detect their ability to withstand environmental stress through investigating *SOD* and *PAL* gene expression and also the genetic relationship among them using random amplified polymorphic DNA (RAPD) molecular markers. The levels of expression of *PAL*, *SOD* genes and mRNA varied with the type of turf; both PAL and SOD gene expressions were low in cold season turf grasses (kentucky blue grass and fine fescue), moderate for bermuda hybrids (tifgreen and tifway) and high in *Paspalum vaginatum*. Primer 3 (UBC-245) can be used to distinguish between *Paspalum* species, also between common burmuda (*Cynodon dactylon*) and burmuda hybrids. It was concluded that hot season genera can withstand environmental stress more than cold season ones since they have more *SOD* and *PAL* gene expressions. Also, DNA markers can be used to differentiate between different turf genera which are hard to be differentiated morphologically.

Key words: Turf, superoxide dismutase, phenylalanine ammonia lyase, RAPD markers, oligonucleotide primers, burmuda hybrids.

INTRODUCTION

Reactive oxygen species (ROS) are produced in both stressed and unstressed cells. Plants have developed a defense system against ROS for limiting the formation of ROS as well as organizing its removal. In the cell, the superoxide dismutase (SOD) constitute the first line of defense against ROS. SOD is found in plant cell wall, mitochondria, chloroplast, microsomes, gloxysomes, apoplast and cytosol (Ruth et al., 2002). Phenylalanine ammonia lyase (PAL) is the enzyme at the entry-point of the phenylpropanoid pathway, which yields a variety of phenolic compounds, salsilic acid and lignin with structural and defense-related functions. PAL activity has been considered to be part of a defense mechanism operating in stress-afflicted cells (Dixon and Pavia, 1995; Yang and Shetty, 1998). Identifying turf grass phenotypes based on morphological traits involves a lengthy survey of plant growth that is labor intensive and vulnerable to environmental conditions (Lin and Hong, 1994), also

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Abbreviations: SOD, Superoxide dismutase; **PAL,** phenylalanine ammonia lyase; **ROS,** reactive oxygen species.

identifying cultivar breeding lines is critical for turfgrass industries to control germplasm quality and protect their rights (Lin and Hong, 1994).

DNA polymorphisms amplified by oligonucleotide primers, 9 or 10 nucleotides in length or longer, were used as genetic markers for fingerprinting (Gustave et al., 1991; Hu and Quiros, 1991). Isoenzyme and protein electrophoresis method that have been developed to identify turfgrass has disadvantages such as the limited amount of polymorphism that can be detected among closely related genotypes, the quality and quantity of isoenzymes and proteins may be subject to variation due to environmental conditions during plant growth and development therefore DNA-based procedures and genetic markers have been proposed for improving turfgrass identification (Lin and Hong, 1994). DNA markers are not typically influenced by environmental conditions and therefore can be used to describe patterns of genetic variation among plant populations (Zabeau and Vos, 1993). The randomly amplified polymorphic DNA (RAPD) markers techniques is quick, easy and requires no prior sequence information; it detects nucleotide sequence polymorphisms using single primer of arbitrary nucleotide sequence (Williams et al., 1990).

Since the identification and differentiation of different turf grass phenotypes based on morphological traits is very difficult and also the mixing of different turf varieties in landscape uses, therefore the main aim of this research was to study the genetic diversity between eight commercial turf grasses in order to distinguish between and to detect the genetic relationship among them using RAPD markers and also to compare between the eight turf grasses in their ability to withstand abiotic and environmental stress through investigating the SOD and PAL gene expressions thus enhancing the selection of drought-tolerant cultivars for landscape uses.

MATERIALS AND METHODS

Plant material

Eight different turf grass species were brought from a private nursery and planted in the greenhouse and lab. of the Horticulture department of the Faculty of Agriculture Alexandria University in November 2013. In 20 cm pots, the grasses were irrigated as necessary to prevent drought stress, mowed twice a week and fertilized weekly with water soluble fertilizer of 20:20:20 (N:P:K). The eight turf species were: Bermuda grass (*Cynodon dactylon*), Tifway (*C. dactylon* x *Cynodon transvalensis*), Tifgreen (*C. dactylon* x *C. transvalensis*), *Paspalum vaginatum*, *Paspalum dilatatum*, St. Augustine grass (*Stenotaphrum secundatum*), fine fescue (*Festuca rubra*) and Kentucky bluegrass (*Poa pratensis*).

RNA extraction and purification for SOD and PAL gene expression

Fresh leaf tissues (100 mg), from the eight turf species, were used for the extraction of total RNA. Total RNA extraction was done using GeneJET RNA Purification Kit (Fermentas) according to the manufacturer's instructions.

The first strand cDNA synthesis

According to the manufacturer's instructions, reverse transcription of the purified RNA was performed by first strand cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Fermentas).

Primer design for the Mn SOD and the PAL gene expression

The sequence of the Mn SOD gene was taken from *Oryza sativa* a related plant from the same family (graminae). The sequence was obtained from the gene bank (www.ncbi.gov) under accession number GQ848046. The primer was designed using www.ncbi.nlm.nih.gov/tools/primer-blast tool. The sequence of the forward primer for the Mn SOD was GTCGCCAACTACAACAAGGC and the reverse primer was TGCAGGTAGTACGCATGCTC with product length of 413 bp. While, the forward primer used for the PAL gene was GGGTCTCTCTACCAGGTGTTAT and the reverse primer sequence was GATCACGTCCTTCATTACGACC, each of forward and the reverse primers were used to amplify about 350 bp.

DNA extraction and purification for the RAPD analysis

Total DNA was extracted from the plants using gene jet TM plant genomic DNA purification Mini Kit # K0791, # K0792 from Fermentas. 100 mg of young leaves were taken from each plant and thoroughly washed with water then ethanol to remove dust and other contaminants and then milled under liquid nitrogen. The DNA was extracted using Fermentas plant tissue DNA purification kit according to the manufacturer's instructions. DNA existence was tested using electrophoresis on 1.2% agarose gels. DNA was stored at -20°C for further work.

Primers design for the RAPD analysis

Six oligonucleotide primers, 10 nucleotides in length were brought from Bioneer Korea and used for RAPD analysis for the eight turf grasses (Table 1). Two PCR reactions were performed one for the gene expression and one for the RAPD analysis, each PCR reaction was repeated twice to insure precision. Master Mix (Dream Taq TM green PCR Master Mix (2x)) containing (DNA polymerase + optimized green buffer + $MgCl_2$ and dNTPs.) from Thermo-Scientific was used. For the RAPD analysis, the PCR amplification was performed with initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 30 s extension at 72°C for 30 s, and a final extension at 72°C for 5 min and storage at 4°C using thermal cycler Techne Endudrance TC 3000 while, for the Mn SOD and PAL gene expression, the PCR reaction was initial denaturation at 94°C for 3 min, 35 cycles of denaturation 94°C for 30 s, annealing at 55°C for 30 s extension at 72°C for 30 s, and a final extension at 72°C for 5 min and storage at 4°C.

Gel electrophoresis

PCR amplified products were subjected to electrophoresis in a 1.5% agarose gel containing Ethidiumbromide in (1x) TBE buffer at 100 V for 40 min using Cleaver submarine electrophoresis unit. Gene ruler 100 bp plus DNA ladder from Fermentas was used to identify the DNA amplified bands.

Data analysis

Gelquant program for quantification of protein, DNA and RNA gel (version 1.8.2) was used for the quantification of bands for the Mn

Table 1. Primers used in RAPD-PCR reactions.

S/N	Primer	Sequence (5'-3')
P1	OPD-05	TGAGCGGACA
P2	OPH-20	GGGAGACATC
P3	UBC-245	CGCGTGCCAG
P4	UBC-261	CTGGCGTGAC
P5	OPC-12	TGTCATCCCC
P6	UBC-231	AGGGAGTTCC

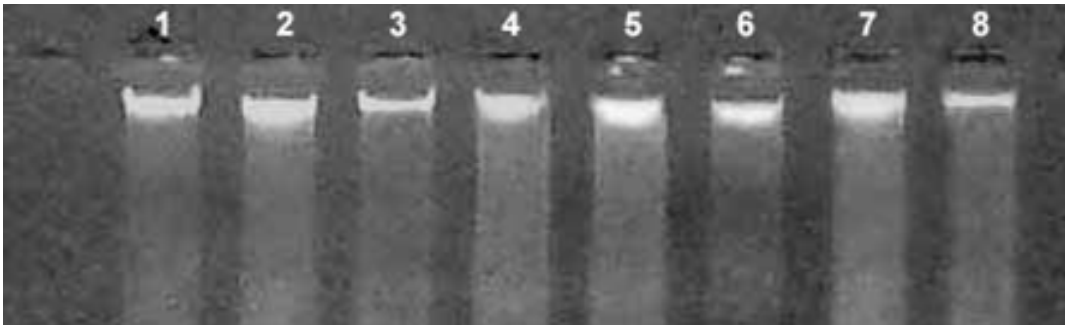


Figure 1. Gel electrophoresis of the DNA extracted from leaf samples of the eight turf species. 1, Kentucky bluegrass; 2, fine fescue; 3, St. Augustine; 4, *P. vaginatum*; 5, *P. dilatatum*; 6, Tifgreen; 7, Tifway; 8, common bermudagrass.

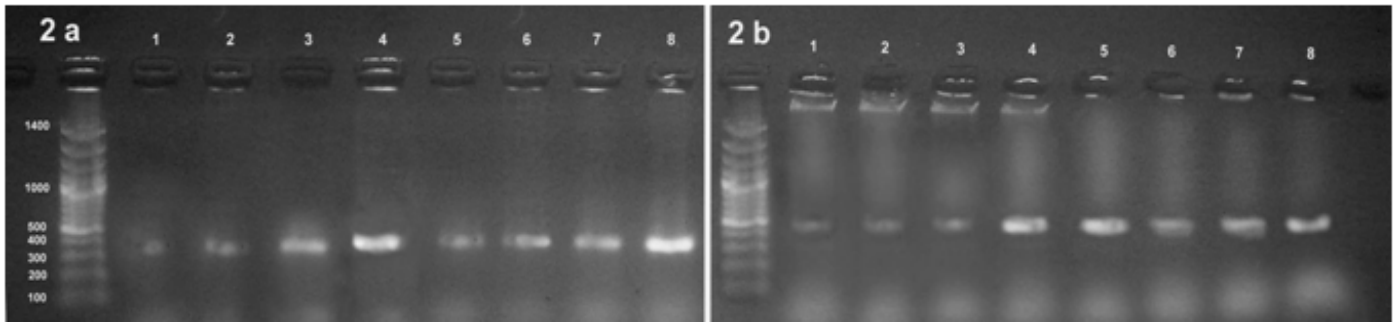


Figure 2. (a) Gel electrophoresis of PCR reaction showing bands amplified between 300 and 400 bp for the PAL gene with different band intensity. **(b)** Gel electrophoresis showing bands between 400 and 500 bp for the MnSOD gene. 1, Kentucky bluegrass; 2, fine fescue; 3, St. Augustine; 4, *P. vaginatum*; 5, *P. dilatatum*; 6, Tifgreen; 7, Tifway; 8, common bermudagrass.

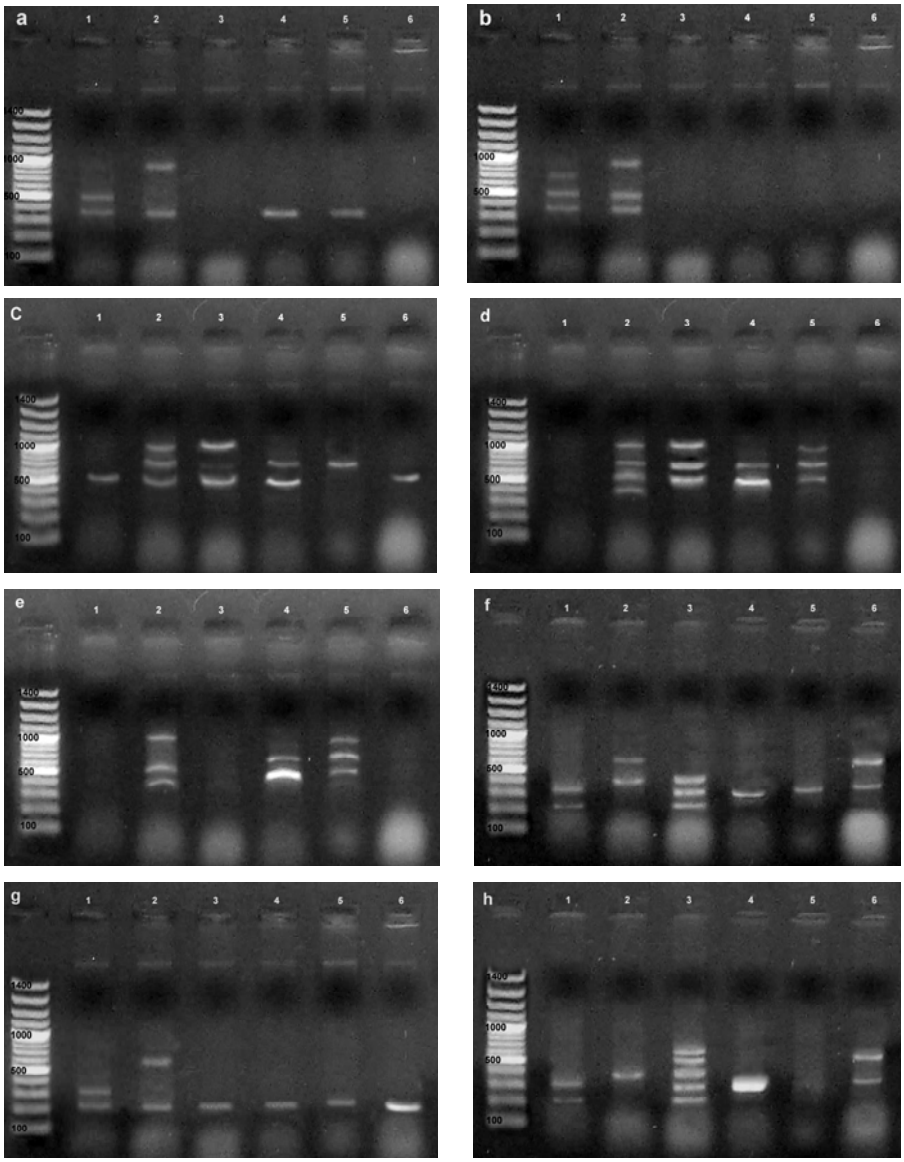
SOD and PAL gene expression. The cluster analysis and polymorphic tree was done by PyElph software system for gel image analysis and phylogenetics (version 2.6.5).

RESULTS

Highly purified DNA extracts of the eight species were used as templates for RAPD-PCR analysis. In all species evaluated, DNA extracted from leaf samples using the described protocol was successfully amplified (Figure 1). The results show bands between 300 and 400 bp which is consistent with the 341 bp of the PAL gene primers (Figure 2a). Also, bands between 400 and 500 bp were consistent with the 413 bp of the SOD gene primers (Figure 2b). The PAL gene expression was low for Kentucky bluegrass, fine fescue and *P. dilatatum*, moderate for St. Augustine, tifgreen and tifway and high for *C. dactylon* followed by *P. vaginatum* (Figure 2a). For the SOD gene expression, it was low in cold season turf grass (kentucky bluegrass and fine fescue), moderate for the Bermuda cultivars and high in both *Paspalum* species (Figure 2b). Data in Table 2 and Figures 3 shows that the number of amplified fragments differed with different primers. The phylogenetic tree (Figure 4) of the eight turf

Table 2. The number of amplified fragments using different primers for the RAPD analysis.

Primer	Number of fragments							
	Kentucky	**Fine fescue**	**St Augustine**	***P. vaginatum***	***P. diltatum***	**Tifway**	**tifgreen**	***C. dactylon***
OPD-05	3	2	1	0	0	2	2	2
OPH-20	3	2	3	3	4	2	2	1
UBC-245	0	0	3	0	3	3	1	5
UBC-261	0	1	2	2	2	1	1	1
OPC-12	0	1	1	4	3	1	1	0
UBC-231	0	0	1	0	0	2	1	2
Total number of bands	6	6	11	9	12	11	8	11



Figures 3. Gel electrophoresis of RAPD-PCR reaction of the eight turf species. a, Kentucky bluegrass; b, fine fescue; c, St. Augustine; d, *P. vaginatum*; e, *P. dilatatum*, f. Tifgreen; g, Tifway; h, common bermudagrass. Lanes from 1 to 6 indicates primers OPD-05, OPH-20, UBC-245, UBC-261, OPC-12, UBC-231 respectively.

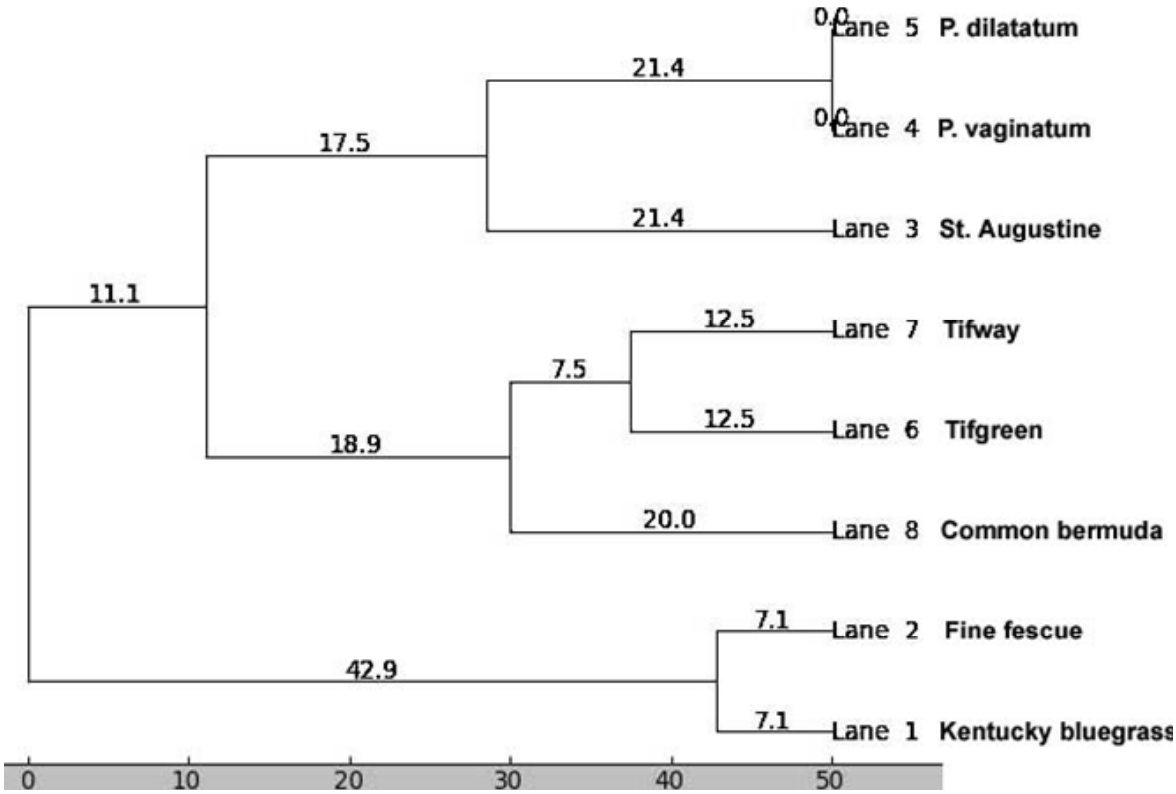


Figure 4. The phylogenetic tree of the eight turf species lanes 1-8 represents. 1, Kentucky bluegrass; 2, fine fescue; 3, St. Augustine; 4, *P. vaginatum*; 5, *P. dilatatum*; 6, Tifgreen; 7, Tifway; 8, common bermudagrass.

species showed that the first cluster included Kentucky and fine fescue (cold season) while the second cluster included tifway, tifgreen, *C. dactylon*, *P. diltatum*, *P. vaginatum* and St. Augustine (warm season). Also, lanes 6 and 7 (tifway and tifgreen) were genetically close to each other. St. Augustine is genetically closer to *Paspalum* more than to common burmuda and burmuda hybrids.

DISCUSSION

The coordinative control and regulation of activity and gene expressions of antioxidant enzymes may be important to plant survival from drought stress (Shaomin and Yiwei, 2009). The increase and decrease in antioxidant enzyme activities in the leaves of grass indicate a different antioxidant metabolism in response to stress and recovery (Shaomin and Yiwei, 2009). PAL is the enzyme at the entry-point of the phenylpropanoid pathway, which yields a variety of phenolic compounds with structural and defense-related functions. Environmental stresses, cause oxidative stress and thus injury to plants through over production of reactive oxygen species (ROS) such as super oxide radical, hydrogen peroxide, hydroxyl radical and singlet oxygen (Dat et al., 2000). SOD, the first enzyme in the detoxifying process, converts superoxide radicals (O_2-) to hydrogen peroxide (H_2O_2), and APX reduces H_2O_2 to water (Asada, 1992, Foyer et al., 1994, Asada, 1999). Antioxidant enzymes, including superoxide dismutase convert O_2- to hydrogen peroxide (H_2O_2) and molecular oxygen in the cell (Luna et al., 2008).

In this study, we investigated the levels of expression of two genes in eight turf species. The levels of expression of PAL and SOD genes varied with the type of turf. Based on the differences in band intensity as a measure of gene expression, it was found that the PAL gene expression was low for kentucky bluegrass, fine fescue and *P. diltatum* and moderate levels of expression for St. Augustine, tifgreen and tifway while the highest level of expression was for *C. dactylon* followed by *P. vaginatum* (Figure 2a) while for the SOD gene expression, it was low in cold season turf grasses (kentucky blue grass and fine fescue), moderate for the bermuda hybrids and high in both *Paspalum* species (Figure 2b).

This findings are inconsistent with previous studies (Mittova et al., 2004), mentioning that tolerant cultivars generally have an enhanced or higher constitutive antioxidant enzyme activity under stress when compared with the sensitive cultivars. Also, Mane et al. (2011) found that the active involvement of the free radical scavenging

enzymes such as SOD is related to induced oxidative stress tolerance in grass. Dombrowski et al. (2008) mentioned that the plants can increase level of tolerance by reprogramming the expression of endogenous genes. Higher level of these antioxidant enzyme activities is considered as one of the tolerance mechanism in most plants (Ashraf, 2009). Bermuda grass species are classified as semi-tolerant to tolerant species to drought stress (Kamal et al., 2012). Common Bermuda grass tolerates drought and salinity stresses by increasing the activity of antioxidant enzymes and could be grown under moderate combined drought and salinity stresses without considerable damage to plants at the physiological or biochemical level. Therefore, common bermuda grass can be recommended for culture in semi-arid areas with limited water resources and areas with semi-saline water resources (Reza and Hassan, 2014). SOD gene expression was low in cold season varieties and this was also found by Zhang and Schmidt (1999) who mentioned that drought stress also had no effect on leaf SOD activity in Kentucky bluegrass.

RAPD is a multiplex marker system that conventionally uses single-primer PCR to amplify random DNA fragments (Kumar et al., 2009). The fragment size produced by primers ranged from 200 to 1000 BPs. The number and size of DNA fragments found in the RAPD profiles varied among turf species. A number of unique DNA fragments were found between the two paspalum species among primer 3 (Figure 3d and e) can be used to distinguish between the two paspalum species. To distinguish between tifway and tifgreen, two extra bands were found in tifway using primer 3 while the same primer gave 5 bands with *C. dactylon* (Figure 3f, g and h). This difference may be due to the difference in the amount of genetic variation that exists between the different species. The primers and conditions for DNA amplification in this study produced reasonably RAPD markers that can be used to identify different turf cultivar. More researches are needed to be done to establish a more broadly applicable DNA fingerprint for identifying different turf grasses.

Conclusion

In general, hot season species have SOD and PAL gene expressions more than cold season ones. Also, oligonucleotide primers can be used to differentiate between different turf varieties which are hard to differentiate morphologically. Primer 3 (UBC-245) can be used to distinguish between *Paspalum* species and also between *C. dactylon* and burmuda hybrids.

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