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Within approximately ten years of the release of Tifgreen, the first "off-types" of bermudagrass were documented. Researchers at Mississippi State University, Auburn University, and University of Missouri investigated whether the use of a commonly used family of pre-emergence herbicides, dinitroanilines, could result in off-types of six bermudagrass cultivars used for southern putting greens.

Volume 2, Number 10
May 15, 2003

PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 215 projects at a cost of \$21 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

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The Genetic Stability of Six Bermudagrasses as Affected by Chronic Exposure to Dinitroaniline Herbicides

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SUMMARY

Off-type bermudagrasses continue to be a major concern on southern United States golf greens due to the disruption of surface uniformity and playability. The origin of off-types is widely debated, with some off-types believed to be a result of contamination and others likely arising from mutation. Glasshouse research was conducted to evaluate the genetic stability of six bermudagrasses ('Champion', 'Floradwarf', 'MS-Supreme', 'Tifdwarf', 'Tifeagle', and 'Tifgreen') commonly used on putting greens in response to chronic exposure to two dinitroaniline herbicides (pendimethalin and oryzalin). Through five repeated cycles of grass grow-in and chemical treatment, findings include:

- Both herbicides caused visible and sustained growth reduction in all grasses.
- No off-types were detected in any chemically-untreated grass.
- Four off-types were recovered during the five experimental cycles from one cultivar treated with herbicides.
- All off-types recovered were larger than the cultivar from which they arose.
- DNA analyses confirmed the off-types were genetically distinct from other cultivars.

Bermudagrass is the predominant turf of southern United States golf courses from tee to green. The release of 'Tifgreen' bermudagrass, a sterile triploid ($2n=3x=27$) hybrid of *Cynodon dactylon* and *Cynodon transvaalensis* developed by Dr. Glen Burton in 1951, revolutionized southern golf putting green turf. Tifgreen greatly improved playability characteristics, as well as much-needed uniformity by way of planting a single-source cultivar.

However, within approximately ten years

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of the release of Tifgreen, the first "off-types" of bermudagrass were documented, and one of those became the very popular cultivar 'Tifdwarf'. The perception of the term "off-type" is generally negative. The term is associated with an undesirable grass that likely differs in growth habit and color, and disrupts uniformity and playability of the turf. Off-types are particularly difficult to manage on golf putting greens where trueness of ball roll is of the utmost importance.

However, it is important to consider that of all the latest generation of bermudagrasses for putting green turf except for Tifeagle (often referred to as the "ultradwarfs", including cultivars such as 'Champion', 'Floradwarf', 'Mini-Verde', 'MS-Supreme', and 'Tifeagle'), were selected from off-types that arose either from Tifgreen or Tifdwarf.

Why do the off-types develop? Off-types are likely attributed to either of two causes: (1) genetic instability (commonly referred to as "mutation") that is a result of an induced or randomly occurring shift in the grass genome, or (2) by contamination of the desirable bermudagrass planting material with a foreign bermudagrass that is unknowingly established with the desirable bermudagrass at planting or is unintentionally introduced into the desirable turf at a later date.

Using DNA amplification fingerprinting, Caetano-Anollés et al. (2) reported that off-types of bermudagrass found in golf turf fairways and athletic fields vegetatively established with the sterile triploid cultivar 'Tifway' were so genetically dissimilar that they most likely originated through contamination and not as a result of somatic mutation of 'Tifway'.

Dr. Phil Busey and colleagues at the University of Florida found evidence of both contamination and mutations on Florida golf courses (<http://grove.ufl.edu/~turf/dna/>), and reported that DNA fingerprinting procedures were repeatable.



Figure 1. Off-type (OT3) that emerged in an orzalyin-treated flat of 'Champion' bermudagrass during the fourth growth cycle.

However, the origin of the off-types of bermudagrasses in putting greens continues to be a subject of much debate, and the interest in off-type emergence will likely only increase as new triploid bermudagrasses become available.

This research project examined the likelihood of somatic mutation of six bermudagrasses commonly used on putting greens: 'Tifgreen', 'Tifdwarf', 'Tifeagle', 'Champion', 'Floradwarf', and 'MS-Supreme'. Somatic mutations (often referred to as "vegetative" mutations to distinguish them from reproductive mutations) occur during cell division and include single gene changes or possibly aneuploidy, the addition or loss of a chromosome (5, 8).

The mutation pressure in our research was supplied by chronic exposure to emulsifiable concentrate formulations of dinitroaniline herbicides that are often applied as preemergence herbicides

in turf management programs: pendimethalin {N-(1-ethylpropyl)-3,4-dimethyl-2, 6-dinitrobenzenamine} and oryzalin (3,5-dinitro-N4N4-dipropylsulfanilamide). These herbicides are classified as dinitroanilines, and their mode of action is the disruption of normal microtubule formation during mitosis (7, 8).

Because dinitroanilines persist in the root-zone for weeks to months depending on soil pH, moisture, temperature, and soil type (4), there would seem to be at least a possibility of somatic mutation from the exposure to these chemicals. Fishel and Coats (3) reported that some dinitroanilines, present in low part per billion (ppb) concentrations, were effective at reducing 'Tifgreen' root biomass. Emulsifiable concentrates (EC), a very popular formulation for dinitroaniline class herbicides, contain organic solvents which are known to pass readily through

plant cell membranes, further increasing the chance that the herbicide could immediately enter the triploid bermudagrass plants and cause microtubule disruptions.

Recommended rates for pre-emergence herbicide treatments are high enough to completely inhibit mitosis and terminate growth of germinating seed. However, this rate constitutes a low, sub-optimal dose of herbicide in relation to established vegetative material. When such a low dose is delivered to plant tissue over time, it frequently causes a localized disruption of microtubules, with the end result being a potential somatic mutation. Our research efforts investigated the potential for a commonly used family of herbicides to induce bermudagrass off-types in a greenhouse dose-response experiment.

The project objectives were to: (1) Observe the phenotypic stability of six cultivars during repeated cycles of grow-in that involved chronic exposure to the herbicides (as well as untreated standards); (2) quantify any morphological changes of apparent off-types by measuring leaf blade length, leaf blade width, internode diameter, and internode length; and (3) verify genotypic changes in apparent off-types by subjecting them, as well as the six original cultivars, to amplified fragment length polymorphism (AFLP) techniques.

Materials and Methods

Samples of each grass were obtained from foundation sources and used to plant nine trays (30 cm wide x 60 cm long x 10 cm deep) filled with a sterilized 80% sand:20% Pro-mix potting soil (v/v) growing medium. Twelve stolons of each cultivar were planted into each tray with cultivar and herbicide treatment combinations replicated three times. The trays were fertilized with Hoagland's solution and watered as necessary to encourage complete coverage of the flats. They were regularly mowed at 1.3 cm.

After reaching 100% coverage, the grasses were treated with one liter of water alone or with a water-based suspension containing either pendimethalin or oryzalin at 6.8 kg ai/ha. This

level corresponds to twice the maximum turf application level according to the chemical label. Each treatment cycle was observed unmowed for a period of six months for the development of apparent morphological off-types.

After the six-month period, normal appearing stem samples were randomly removed from each tray to provide planting material for the next cycle. Five cycles were completed from 1998 through 2002 (i.e. for completion of one entire cycle, a minimum of 9-10 months were required for grow-in, treatment, and observation for off-types).

Any apparent off-types were isolated, removed and propagated as previously described. Stem samples from the untreated control cultivars and the off-types were planted in commercial potting soil (void of the herbicide treatments) in 15-cm diameter pots, and grown to maturity. These pots were then subjected to morphological measurements detailing leaf blade length, leaf blade width, internode diameter, and internode length as well as DNA evaluation. Means of the morphological measurements were calculated and statistical comparisons of phenotypic data were made using accepted statistical procedures (6).

Molecular analyses using the amplified fragment length polymorphism (AFLP) technique were conducted on all suspected off-types for comparisons to the standard grasses. Primers selected according to the method described by



Figure 2. Visual comparisons of 'Champion' off-types (OTs 1-4) and 'Tifgreen' bermudagrasses. All off-types arose from dinitroaniline herbicide-treated 'Champion' bermudagrass.

Zhang et al. (9). Genetic relationships between off-types and standard cultivars of bermudagrass were made by calculating genetic similarity coefficients. These data were used in the development of a phenogram using the Unweighted Pair Group Mean Average Method (UPGMA). Flow cytometry was used to determine the ploidy level of each genotype as described by Arumuganathan et al. (1).

Results

All herbicide treatments caused a noticeable reduction in growth of sprigs in each tray, with the suppression being observed for at least six to eight weeks. Off-types appeared after growth resumed, the first off-types being discovered in cycle three (Figure 1). After five cycles of growth over a four-year period, no bermudagrass off-types were discovered in control (chemically untreated) trays. Four off-types were recovered from trays of 'Champion' bermudagrass treated with the dinitroaniline herbicides (Figure 2).

Morphology

In general, off-type 3 was the largest plant, while off-types 1, 2 and 4 are similar to 'Tifgreen'. Specifically, leaf blades of offtypes 1, 2, and 4 were similar in length to 'Tifgreen' and significantly longer than 'Tifdwarf' and all the ultradwarf cultivars, including 'Champion' (Table 1). Off-type 3 had longer leaves than any other genotype included. Leaf blades of off-type 3 were significantly wider than any other genotype. Leaf blade widths of other off-types were not significantly different from 'Champion'.

Internodes of off-types 1, 2, and 4 were significantly longer than 'Champion' and 'Tifdwarf', but were not significantly different from 'Tifgreen'. Internodes of off-type 3 were longer than all other genotypes measured. The diameter of internodes of off-types 2, 3, and 4 was similar to 'Tifgreen' and significantly larger than 'Champion'.

| Cultivar | Leaf Blade Width (mm) | Leaf Blade Length (mm) | Internode Diameter (mm) | Internode Length (mm) |
|------------|-----------------------|------------------------|-------------------------|-----------------------|
| Off-type 3 | 2.1 | 52.9 | 0.52 | 35.8 |
| Off-type 4 | 1.5 | 44.8 | 0.47 | 30.6 |
| Off-type 2 | 1.4 | 44.3 | 0.46 | 28.9 |
| Tifgreen | 1.5 | 42.7 | 0.48 | 28.3 |
| Off-type 1 | 1.6 | 42.3 | 0.44 | 30.5 |
| Tifdwarf | 1.7 | 33.9 | 0.41 | 24.8 |
| TifEagle | 1.7 | 29.3 | 0.45 | 27.7 |
| MS-Supreme | 1.7 | 23.8 | 0.38 | 27.9 |
| Floradwarf | 1.6 | 21.8 | 0.44 | 25.6 |
| Champion | 1.6 | 18.4 | 0.37 | 23.8 |
| LSD (0.05) | 0.2 | 7.8 | 0.09 | 6.3 |

Table 1. Morphological measurements of bermudagrass cultivars and off-types.

DNA testing

A phylogenetic tree provides evidence of genetic relationships, with grasses that have very similar DNA being closely linked to each other (Figure 3). The AFLP analyses reported here indicate that OT 1, 2, and 3 (OT 4 was not included in these analyses) are genetically distinct from each other and the standard cultivars, including 'Champion', the grass from which they arose.

Zhang et al. (9) previously reported that

AFLP analyses were very successful in differentiating between 27 bermudagrass cultivars and introductions. In our research, OT3 is very different from the other bermudagrasses, as it is distinctly separated in its linkage. Hence, we will pursue further analysis regarding its genetic composition. The phylogenetic tree also indicates that the off-types are distinct from Tifgreen, the cultivar that the off-types were most similar to in morphology.

These primers were selected based on the

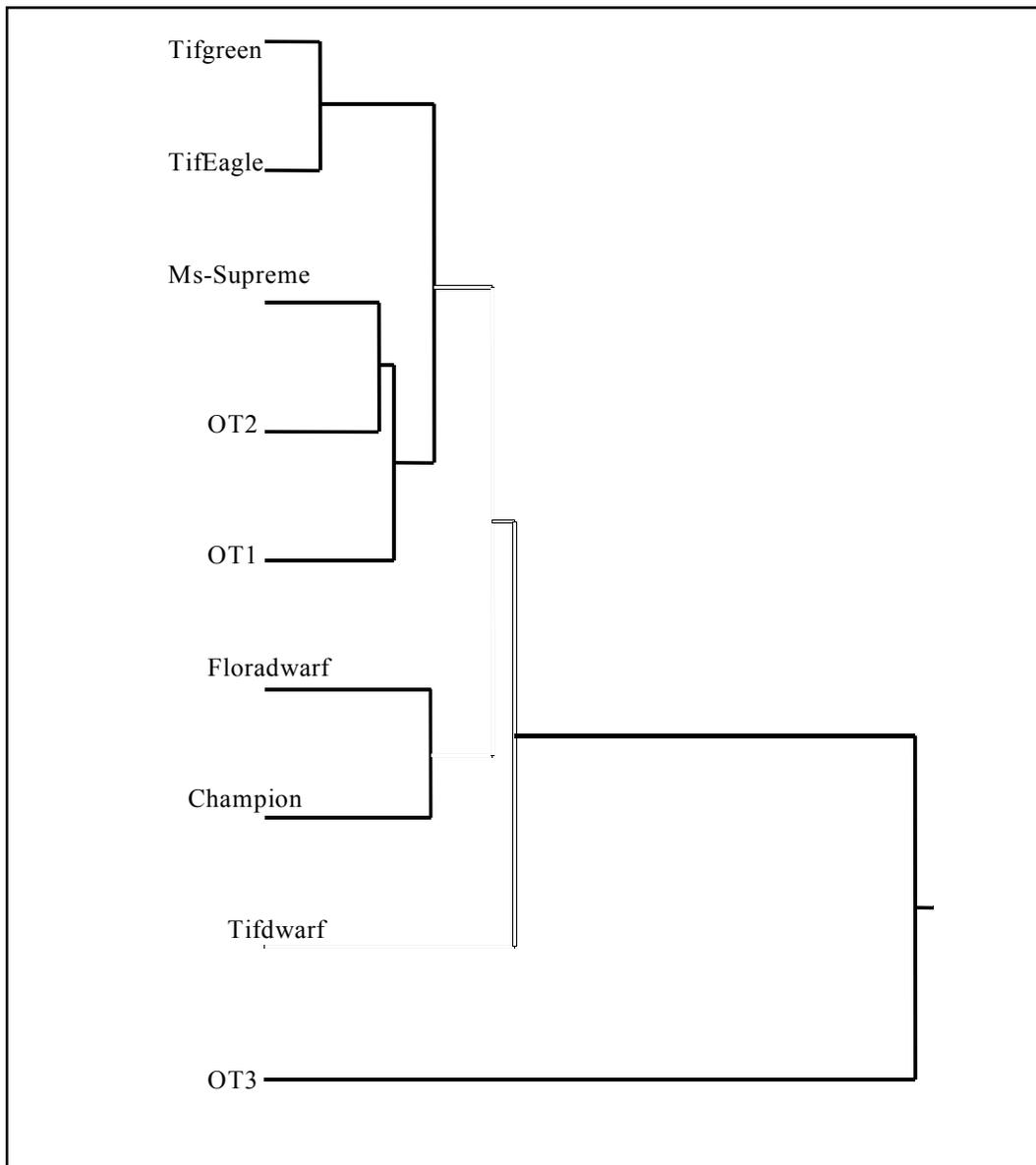


Figure 3. Phylogenetic tree of nine bermudagrass genotypes analyzed by UPGMA cluster analysis based on AFLP marker obtained with five primer combinations. This tree does not include off-type 4 (OT4)

work of Zhang et al. (9), and similar to their research findings, these primer combinations distinguished between the standard bermudagrasses and the off-types. However, it is important to understand that we had to visually detect the off-types before we could confirm that they were different from the standards by AFLP analyses. Random DNA sampling within the trays probably would not have revealed any differences and would simply confirm how closely related these bermudagrasses generally are (with the possible exception of OT3).

Chromosome numbers

Flow cytometry results indicated that off-types 1, 2, and 4 are triploids ($2n=3x=27$) that most likely resulted from gene mutation. Off-type 3 contains more than 27 chromosomes and could possibly be an aneuploid. A conventional chromosome count is needed to determine its exact number. If it should turn out to be an even tetraploid ($2n=4x=36$), then we would conclude that off-type 3 most likely originated from a contaminant seed. At this time, off-type 3 has not displayed the characteristics of a typical fertile tetraploid plant.

Conclusions

From these results we conclude that chronic exposure to dinitroaniline herbicides can potentially cause genetic change in triploid bermudagrasses. To our knowledge, these results represent the first time that a bermudagrass mutation at the whole plant level has been confirmed in a controlled environment.

We were successful in recovering four off-types in a controlled environment, with three of these being apparently newly discovered triploids (off-types 1, 2, and 4), and one off-type (OT3) requiring further evaluation to determine if it is a contaminant or an aneuploid. The mutations that were collected were morphologically distinct from Champion, the cultivar from which they arose. The off-type plants were significantly larger (i.e. longer leaf blades, longer internode spacing, etc.) than Champion. The off-types were

much more similar in morphology to 'Tifgreen'. Other changes may have occurred that were not visually detected by our research methods.

This research utilized five successive periods of herbicide exposure and subsequent regrowth cycles on the bermudagrasses, and approximately four years were required to complete even this limited amount of work. Although we only found changes in one cultivar, we feel it is important to not draw conclusions about mutation frequency or the genetic instability of a particular cultivar. When one considers the vast number of cell divisions that are occurring at any point in time with bermudagrasses, the frequency of mutations is actually very small.

This research does, however, support the contention that somatic mutations are a contributor to the occurrence of off-types in the golf turf industry. Our findings support the theory that Tifdwarf and other dwarf cultivars resulted from natural mutation and were not likely the result of chance hybridization.

Acknowledgements

The authors wish to thank the USGA Turfgrass and Environmental Research Program for financial support of this project.

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