PROGRESS REPORT: May 1, 1994 to October 31, 1994

INVESTIGATOR(S): Wm. Vance Baird and Landon C. Miller

INSTITUTION: Clemson University

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SPONSOR: United States Golf Association, Green Section

PROJECT: Low temperature and drought regulated gene expression in bermudagrass

We have focused our current efforts on two bermudagrass "cultivars", 'Midiron' and 'U3'. Dr. Jiyu Yan, post-doctoral research associate, has initiated the characterizing of the fatty acid composition of cellular membrane lipids. She has optimized extraction and purification procedures on leaf, root and crown material of greenhouse (ambient) and growth chamber (8/2 °C, 14 hr photoperiod, 300 umol/s/m^2) grown plants. Basically, our experimental design paralleled that of Anderson, Kenna and Taliaferro (HortScience 23:748, 1988), were it was shown that following a three to four week exposure to moderately low temperatures, certain bermudagrass cultivars, in particular 'Midiron', were able to survive exposures to markedly lower temperatures than the could have without the "acclimation" period.

Dr. Yan's findings indicate that there is an overall increase in total membrane lipid fatty acids (TFA) during the four week exposure. Basically, total lipids are extracted from the plant tissue by organic solvents. Then the polar fraction (membrane phospholipids) is separated from other lipids and free fatty acids by thin layer chromatography. The phospholipids are then hydrolyzed (e.g., saponification) to release the individual fatty acid components and these are derivatized (e.g., methylated) prior to analyzing on a gas chromatograph and/or a mass spectrometer. Quantification is done by integration of the peak volumes, and qualitative fatty acid identification is accomplished by the use of a microbial identification database (Microbial ID, Inc.) interfaced to the GC.

Measurable changes in TFA were observed as early as six days following transfer to the growth chamber. When the three main organ systems were compared individually, it was noted that this increase in TFA was maintained for the crowns; whereas the roots and leaves showed only a transitory increase, peaking after 12 days (see for example Figure 1 in the Appendix). Interestingly, even these two organs differed in their long term response. The increase in root TFA leveled off (e.g., Figure 1B); whereas, that of the leaf actually dropped, back down to baseline (control)

EXECUTIVE SUMMARY

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Cellular membranes have been considered a primary site of freezing injury, and alterations of membrane composition correlate with cold acclimation processes that allow plants to tolerate freezing temperatures. As major components in membrane bilayers, the polar lipid fatty acids could directly regulate membrane structure, and therefore membrane function, through the alterations of acyl chain length (number of carbon atoms) and/or unsaturation (number of double bonds). Alterations in plant membrane lipid fatty acids can be induced by many physiological and environmental factors, and these changes could play an important role in adaptation to low temperature. Bermudagrass (*Cynodon dactylon*) shows an increased tolerance to cold after a period of exposure to moderately low temperatures. However, whether this cold acclimation correlates with cell membrane alterations, and how the membrane lipid fatty acids (MLFA) respond to low temperature are unknown for bermudagrass.

Bermudagrass total MLFA (ug), per unit of total lipids (mg), increased in crown tissues, but not roots or leaves, over the 4-week exposure to moderately low temperatures (8°C d/ 3°C n, 14 hr photoperiod). The major fatty acids in bermudagrass were determined to be palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2), and linolenic acid (C18:3). These four made up 95% of the total MLFA. In crown tissues the concentration of shorter chain and saturated fatty acids (e.g., C16:0, C18:0) declined significantly during the cold treatment, while the concentration of the longer chain, unsaturated fatty acids (e.g., C18:3) increased. As a result, the double bond index increased in crown tissues over this same four week period. These changes increase the fluidity of membranes, and therefore, could reduce cold-induced membrane leakage and freezing injury.

mRNA profiling/differential display techniques are being refined and employed in our efforts to characterize genetic polymorhisms between bermudagrass cultivars differing in levels of cold tolerance. These PCR based methods allow for the relatively rapid identification and cloning of gene sequences differentially expressed in response to a particular environmental stimulus such as low temperature, drought or chemical applications. The reproducibility of this technique and the identification of the appropriate oligonucleotide primers has been the focus of our current efforts.

levels (e.g., Figure 1C). To a first approximation, these findings are consistent with the regenerative capacity of the bermudagrass plant; where one might predict that if alterations in membrane lipids/membrane fluidity are important to surviving low temperatures, then those parts of the plant that will over-winter (i.e., the crown) should undergo those changes, and the parts which do not survive (i.e., the leaves) may not respond appropriately.

A similar pattern was observed when the amounts of the individual fatty acid components of the membrane lipids were measured. First, from our analysis we have determined that ninety-five percent (95%) of the bermudagrass TFA is made up of four components: palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:1) and linolenic acid (C18:2). In the crown tissues, we observed that over the four week cold treatment the concentration of the shorter-chained and saturated fatty acid components decreased (e.g., C16:0, Figure 2A; C18:0, Figure 2B), while the longer-chained unsaturated fatty acids increased (e.g., C18:3; Figure 2D). This overall change in the level of membrane lipid fatty acid saturation is quantified as a change in the double bond index [DBI = Σ (% of each FA x # of double bonds in that FA)]. Over the four week period there was a significant increase in the DBI (e.g., Figure 3). Again, these changes in the profile of the membrane lipid fatty acids is consistent with increasing membrane fluidity, thus minimizing damage due to low temperature exposures. It is interesting to note that, when comparing 'U3" to 'Midiron', although similar changes in membrane lipid fatty acids are seen, 'U3' tends to lag behind 'Midiron' in a number of instances (e.g., starting later, or never reaching the same level). This may be related to the accepted idea that 'U3' has a reduced capacity to survive low temperature exposures than does 'Midiron' (e.g., Figure 2 and Figure 3). And this is consistent with our earlier findings that there exists DNA polymorphisms between 'Midiron' and 'U3' with respect to certain gene-related sequences found to be differentially regulated in other plant species in response to low temperature.

These changes must be the result of differential activity of enzyme involved in fatty acid biosynthesis, and provide us with some insight into which genes [e.g., fatty acyl desaturases (Δ^9 or Δ^{12}) and elongases], and their spatial or temporal expression, may be important to surviving cold stress through mitigation of the effects of membrane water loss, which accompany exposure to low temperatures.

The fatty acid isolation and analysis experiments are scheduled to be repeated, with modifications, in late fall. One major addition will be to see what effects the exogenous application of abscisic acid (ABA) has on the compliment of fatty acids in the crowns of bermudagrass. If changes, similar in magnitude and duration to those induced by low temperature, are observed then in additional experiments we may monitor the levels of

endogenous ABA in the crowns during low temperature exposures to correlate temperature, ABA and fatty acid alterations..

John Wells, Research Technician/Ag. Sci. Assoc. III, has focused his efforts on screening for differences in gene expression during the acclimation period. He is optimizing the parameters of "differential mRNA display" for use in his analysis. This method uses the polymerase chain reaction, and pairs of oligonucleotide primers of known sequence, to visualize subsets (i.e. one twelfth) of all the expressed genes within a given population of transcripts (i.e., mRNA). By using RNA isolated from different cultivars and/or different experimental treatments (e.g., acclimated and non-acclimated) as a template for cDNA synthesis in the presence of reverse transcriptase and one of the (anchor, $T_{11}N_1N_2$) primers, followed by PCR amplification using an "arbitrary" 10-mer, he can determine if there are any genes being expressed in one situation and not in the other. The uniquely expressed sequences thus identified can be cloned by excising the band/fragment from the acrylamide sequencing gel and reamplifying this PCR product using the same primer pair.

John has been very successful in his initial experiments aimed at optimizing the biochemical conditions for reproducibility. This ability coupled with our knowledge that significant physiological and biochemical changes occur in the crowns as early as six days after exposure to low temperatures provides John with a developmental window within which to focus his efforts. As his experimental material, John will have pools of total RNA isolated from the two bermudagrass cultivars at 12 hour intervals post-treatment, over a six day period; as well as from control (non-cold acclimated) plants.

As you may know, Clemson University and the South Carolina Agriculture Experiment Station have placed turfgrass research and education as its number one priority. This is manifested as a Turfgrass Initiative with both recurring and non-recurring funds from the State. The Initiative is in its first year, and has just recently provided my program with $42,000 for the purchase of a DNA and protein computer imaging and analysis system, and a thermal cycler (PCR machine). The Initiative has also provided for a new Horticulture faculty position in Turfgrass Physiology, with an emphasis on environmental issues (a search committee, of which I am a member, is currently reviewing applications to try and fill this position by early next year).

The renovations to my laboratory, initiated in early May through a biotechnology bond project, were finally completed this month, and we have been allowed to "re-occupy" our research area. We should be completely moved in by the end of this month. The "new" space is vastly improved ergonomically (to use a popular term) as well as esthetically. However, the fact the project essentially doubled in length of down-time was an expensive tradeoff.

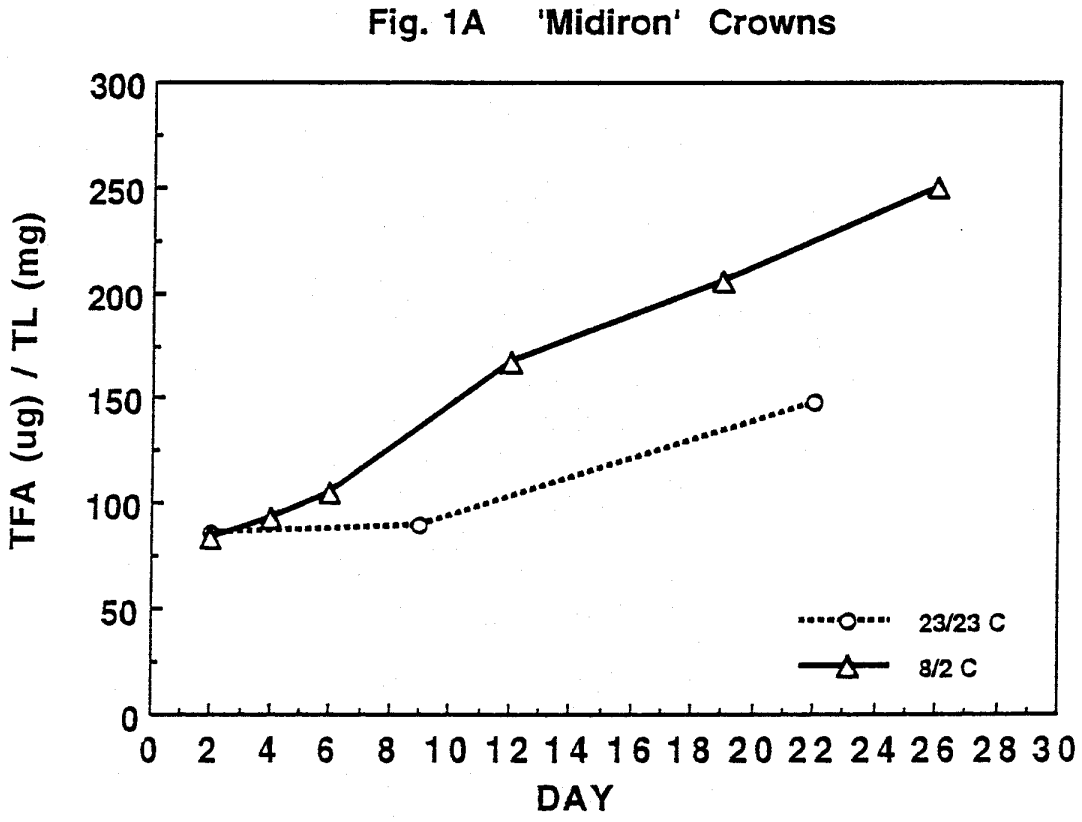
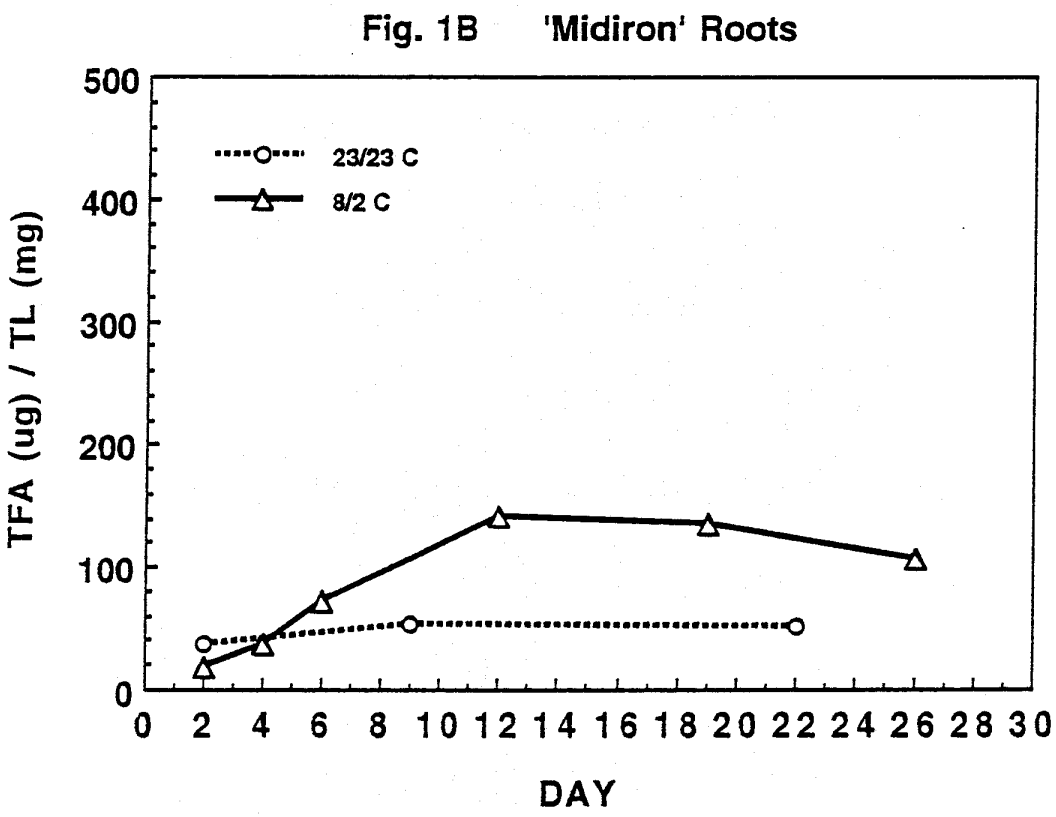


Fig. 1 Ratio of total membrane lipid fatty acids to total lipids [TFA (ug) / TL (mg)] in bermudagrass exposed at 23/23 C and 8/2 C.



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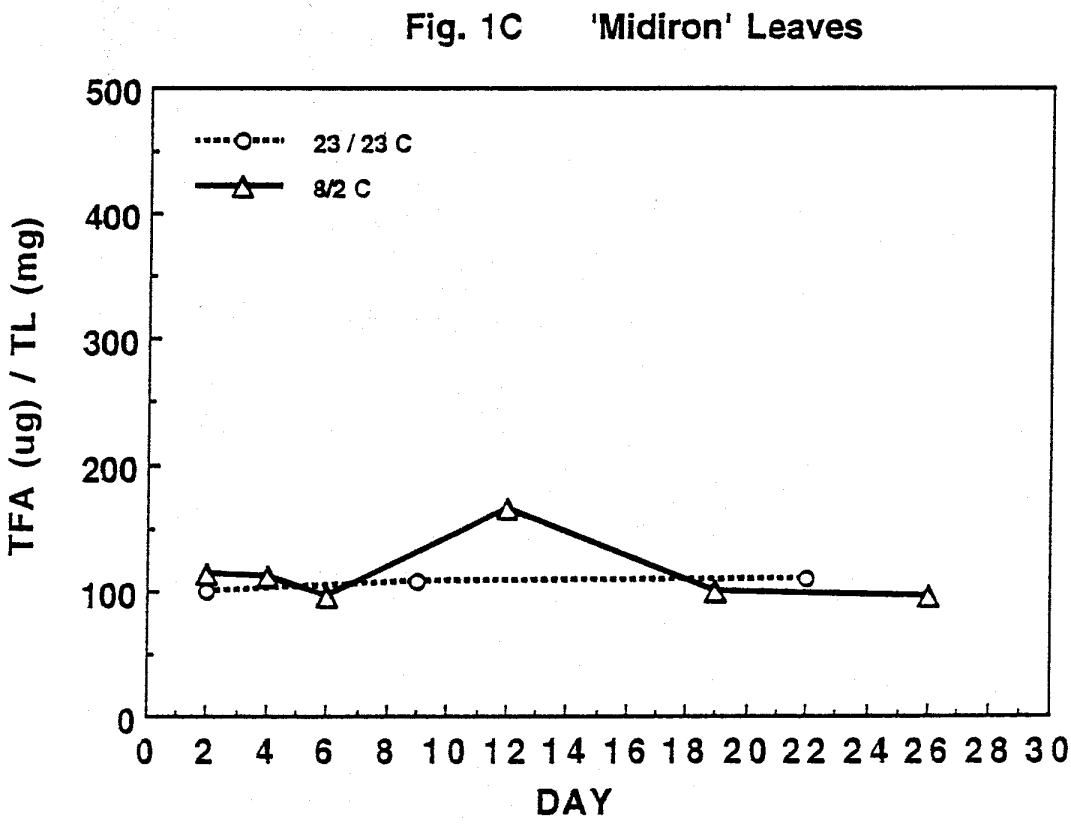

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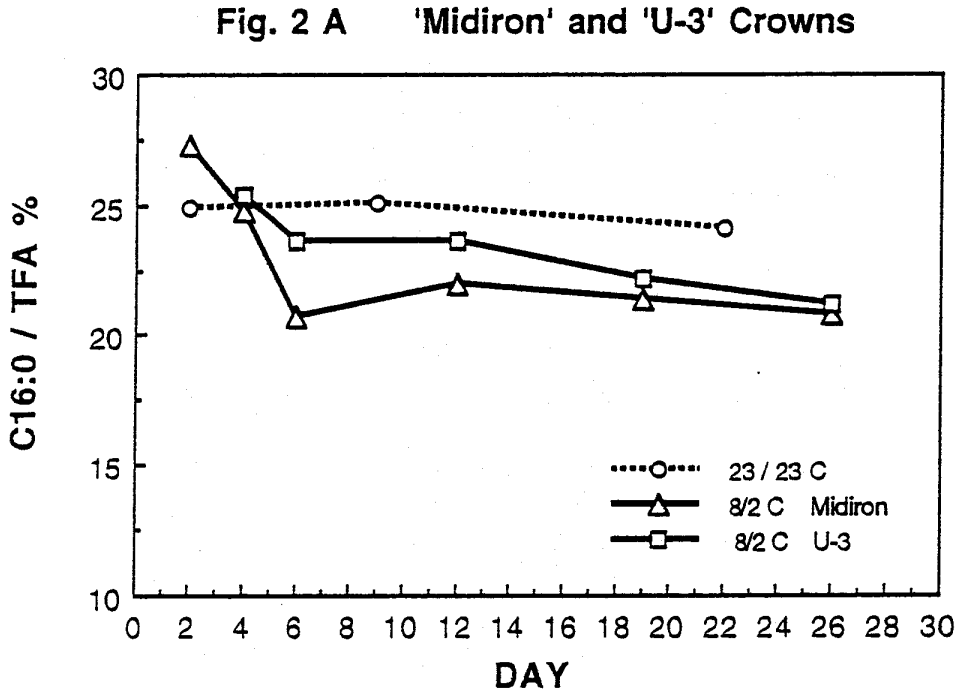
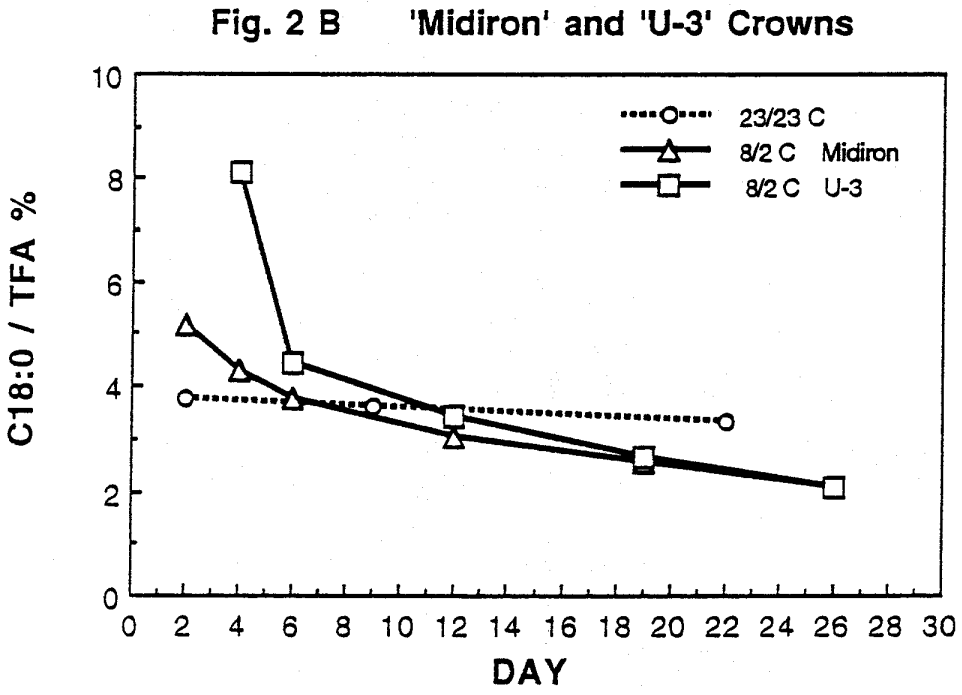


Fig. 2 Concentration of major membrane lipid fatty acids in crown tissues of bermudagrass exposed at 23/23 C and 8/2 C.



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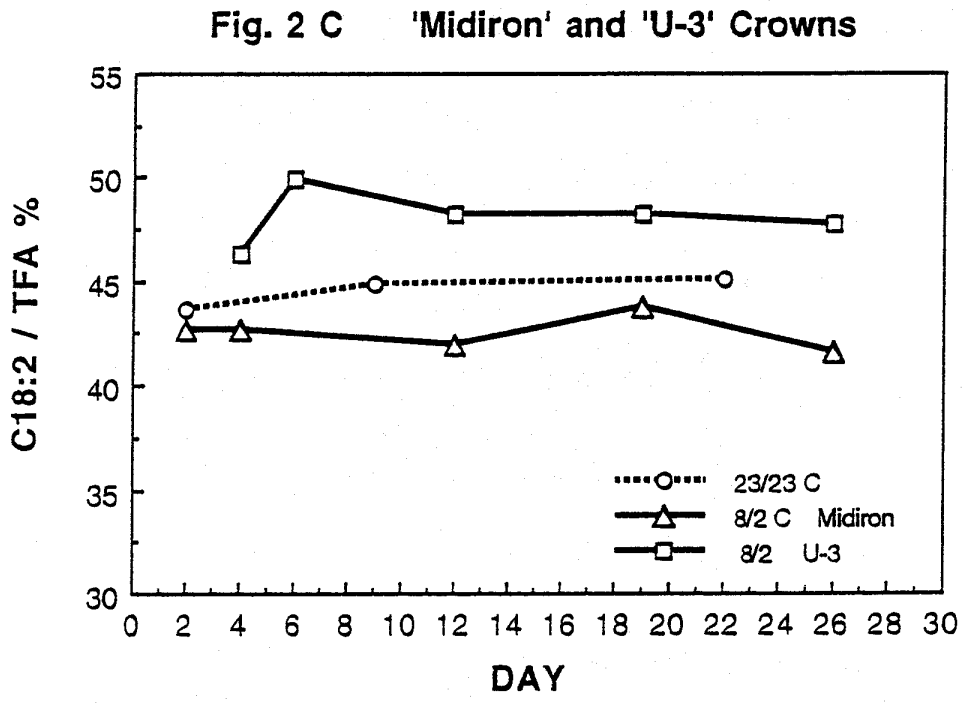

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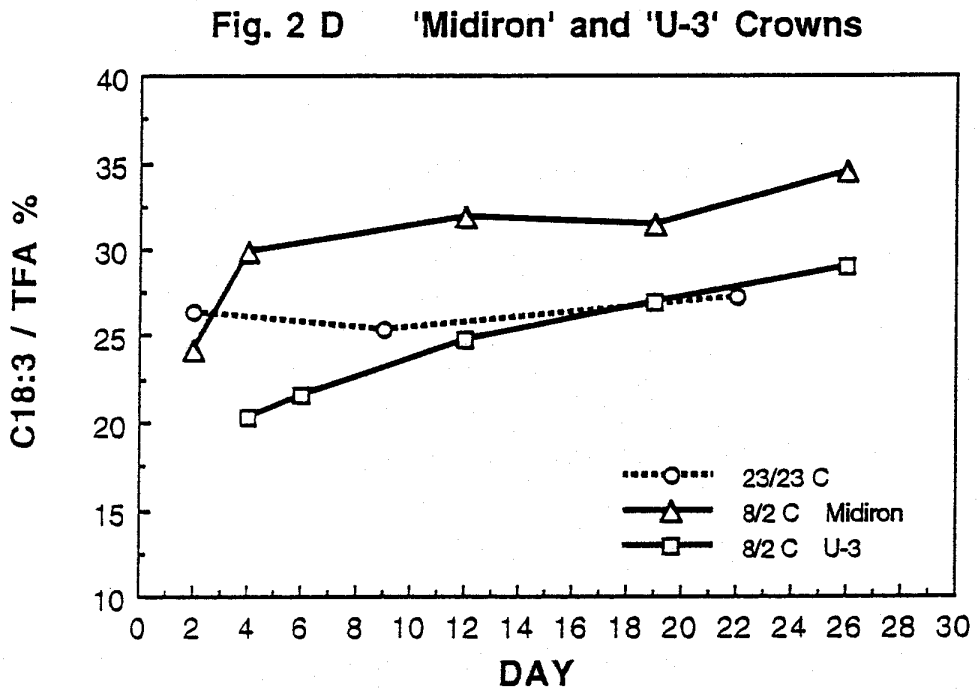

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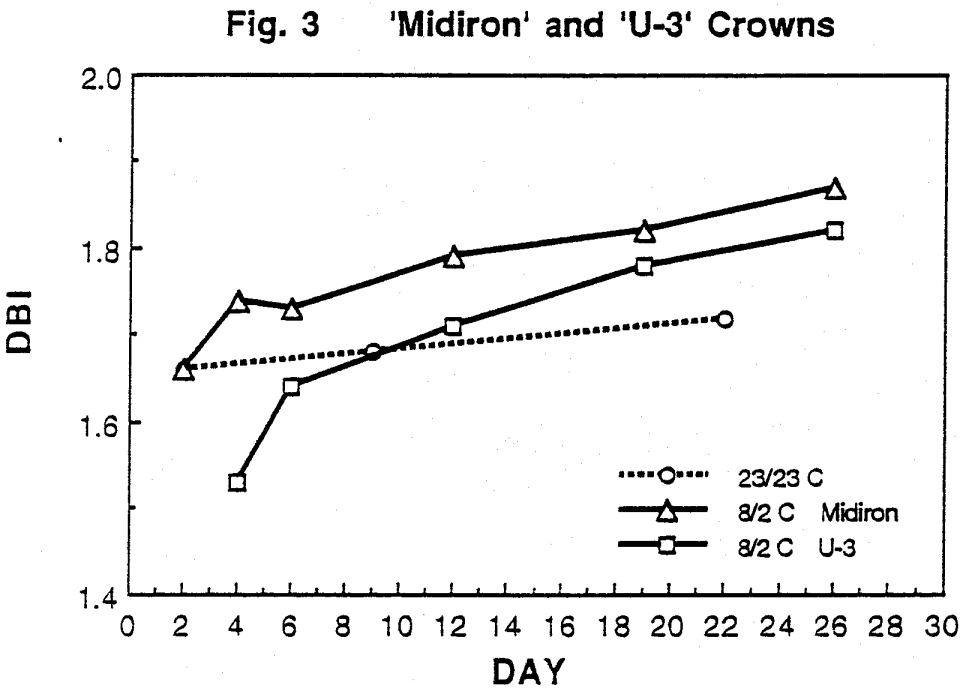

Fig. 3 Double bond index (DBI) of membrane lipid fatty acids in crown tissues of bermudagrass exposed at 23/23 C and 8/2 C.