INFLUENCE OF NITROGEN, REDUCED IRRADIANCE AND
BIOSTIMULANTS ON TURFGRASS GROWTH, SUPEROXIDE DISMUTASE
CONTENT AND CHLOROPHYLL FLUORESCENCE

by

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APPROVED:

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(ABSTRACT)

Biostimulants (BIOS) are non-mineral substances that, when exogenously applied in very small quantities, stimulate the metabolic activity of plants.

Past research with biostimulants has not included differing levels of nitrogen fertility or shade as variables. The research presented was designed to explore some interactions between biostimulants and nitrogen fertility on turfgrass grown under several light conditions.

Field experiments were conducted on Kentucky bluegrass using shade cloth and seasonal variations to reduce to amount of light to the turfgrass. The nitrogen fertility was supplied by urea (46-0-0) in all the experiments. The biostimulants used in the experiments were a seaweed extract, a humic acid extract, a combination of both and a commercial blend of biostimulants and iron.

In summer and fall seasons the increases that the biostimulants produced over the N only treatments were greater under low to medium N inputs and under reduced light (summer shade and full sun in the autumn).
Shade decreased rooting while increasing top growth.

Several experiments were performed to explore the potential use of superoxide dismutase (SOD) content and chlorophyll fluorescence activity of turfgrass leaves to determine the responses to nitrogen and biostimulant treatments. In these experiments, a general increase in SOD content occurred in the leaves with additional N or biostimulant treatments. Additional nitrogen fertility increased the chlorophyll content while reducing the maximum level of fluorescence (Fmax).
ACKNOWLEDGMENTS

The Good Lord with whom all things are possible.

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Most of all my loving and very patient partner, Janet, who has had the tougher end of the graduate student life and who has always been my foundation.

This paper is dedicated to our children, Sammy and Katie.
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CHAPTER ONE

The Influence of Biostimulants and Nitrogen Fertility on Kentucky Bluegrass Growth Under Ambient Sunlight and Reduced Irradiance

INTRODUCTION

Biostimulants have been researched since the idea of hormonal control in plants was envisioned in the 1930’s. Trewavas 1981 put forth the idea that growth substances are "integrating agents in development" that act as buffers to environmental and nutrient changes. These substances help the plant respond to various stresses in an organized manner. Plants respond to environmental stress by altering their hormonal balance (e.g.; more abscisic acid and less cytokinins) (Chapin et al., 1988). Past research with growth enhancing materials or biostimulants has not included differing levels of nitrogen fertility or shade as variables. The research presented was designed to explore some interactions between biostimulants and nitrogen fertility on turfgrass grown under several environmental conditions.
LITERATURE REVIEW

Nitrogen

Excluding O₂, H and C, N is required in larger amounts than any of the other essential nutrients for optimum turfgrass growth. Most soils rarely contain sufficient N levels to meet the turfgrass’s needs (Turner and Hummel 1992). Therefore, N fertilizer is needed in order to maintain a healthy and visually appealing turf.

The ultimate source of N used by plants is the atmosphere which is composed of 78% N gas (Tisdale et al., 1985). This gaseous N can enter the soil-plant continuum by four methods: it can be fixed in the manufacture of chemical fertilizers (major source), fixed (used) by free soil bacteria, fixed by symbiotic bacteria that live on host plants, fixed as oxides via lightning (minor contribution). Non-gaseous forms of N can enter the system via plant or animal residues and through dissolved N in precipitation.

The cycling of N includes many pathways that remove the N from the soil-plant system. Plants use N and then are eaten by animals or harvested. Some N is lost to the atmosphere when it is volatilized (changed to ammonia gas) or denitrification occurs (anaerobically changed to nitrous oxide or N₂). The plant-available-nitrogen can be immobilized by microbes and put in a non-usable form. The N can be removed
from the system by leaching.

Nitrogen is essential to the production of amino acids which are incorporated into
nucleic acids and proteins and is a key element in the chlorophyll molecule. Moderate
amounts of N made available to the plant at the proper time will stimulate both root
growth and shoot growth (Juska et al., 1955; Canaway, 1984; Yust et al., 1984;
Schmidt and Snyder, 1984). However, high levels of available N at times favorable to
top growth may suppress root growth (Harrison, 1934; Juska et al., 1955; Sills and
Carrow, 1983) and reduces non-structural carbohydrates (Schmidt and Blaser, 1967;
Watschke and Waddington, 1975).

Nitrogen applications improve turfgrass wear-tolerance up to a point, above which
more N reduces the turf quality. This point was reported to be between 200 - 300 kg
Turfgrass responses to N are influenced by many factors including soil type, soil
moisture, temperature, light and even species and cultivars. Researchers have found
that N applications improved turfgrass recovery from drought (Schmidt and
Breuninger, 1981; Watschke and Waddington, 1975), increased net photosynthesis
(Goatley and Schmidt, 1990; Schmidt and Snyder, 1984; Pakeman and Lee, 1991)
and increased the bulk leaf osmotic potential (Carrol and Petrovic, 1991). Higher N
fertility increased the incidence of several fungal diseases on turfgrass such as
melting-out, Helminthosporium, Pythium blight, Fusarium blight, Rhizoctonia blight,
Cercospora leaf spot and others (Couch, 1995; Muse, 1974; Smiley, 1983; Huber et
al., 1968). However, the incidence and severity of Dollar spot and leaf rust were diminished under high N fertility (Couch and Joyner, 1976).

**Biostimulants**

Biostimulants (BIOS) are non-mineral substances that, when exogenously applied in very small quantities, stimulate the metabolic activity of plants. Since very small quantities are needed to produce plant response, this eliminates the argument that the BIOS materials supply enough mineral nutrition to fully explain the growth effects. Past research with several commercial BIOS and plant hormones supports the idea that a hormonal process is the chief stimuli for the growth and response to stresses (Crouch and Van Staden, 1993; Chapin, 1991).

This thesis will primarily focus on two BIOS and two combinations of these materials (seaweed extract and a humic acid).

**Seaweed**

Seaweed has been used as a fertilizer and soil amendment for over a thousand years, but in recent years seaweed and its effects have been studied more extensively.
Like any plant material, variables like species collected, timing of the harvest, processing method (liquid, meal or dried) can influence the final product quality and efficacy (Crouch, 1991; Nelson and Van Staden, 1984). This can add to the variability in results. Many rates have been applied and different application methods have been used (soil drench, plant dip or foliar spray) on a wide variety of plants (turfgrass, lettuce, tomatoes, rice, botanical trees and others). Results vary, but generally these experiments have shown improved growth responses. The precise mechanism which causes the growth responses are not fully understood. The small amount of mineral nutrients in commercial seaweed materials cannot account for the magnitude of the responses (Abeiz, P., 1980; Crouch and van Staden, 1993; Blunden, 1977). Seaweed extracts contain vitamins and polyamines (Jensen, 1969; Kanazawa, 1963) as well as five important plant hormones: cytokinins, auxins, gibberellins, abscisic acid and an ethylene precursor (Crouch et al., 1992a, 1993; Mooney and Van Staden, 1986; Brain et al., 1973; Sanderson et al., 1987; Taylor and Wilkerson, 1977; Wildgoose et al., 1978; Kingsman and Moore, 1982). Seaweed extract applications to plants have: increased the chlorophyll content in leaves (Featonby-Smith and van Staden, 1984; Beckett et al., 1994), improved absorption of trace elements (Aitken and Senn, 1965; Senn and Kingman, 1978; Crouch et al., 1990; Becket et al., 1994), increased the alpha tocopherol and other anti-oxidant levels (Schmidt, unpublished data), enhanced top growth (Nabati et al., 1994; Goatley and Schmidt, 1990; Atzmon and Van Staden, 1994), improved root growth (Blunden
and Wildgoose, 1977; Featonby-Smith and van Staden, 1984; Nabati et al., 1994; Goatley and Schmidt, 1990; Atzmon and Van Staden, 1994), reduced salinity stress (Nabati et al., 1994), improved germination (Goh, 1971; Button and Noyes, 1964) and delayed senescence (Goatley and Schmidt, 1990).

**Humic acid**

Humic substances are defined as the principal organic components in soil and water by Schnitzer (1978). Organic matter is a mix of animal and plant materials in various degrees of decay. The organic matter is divided in two groups: nonhumic substances and humic substances (Schnitzer and Kahn, 1972). The nonhumic material can contain recognizable compounds such as proteins, amino acids, lipids, peptides, carbohydrates and other short lived compounds that can be used by micro-organisms.

The majority of the organic matter consists of humic material. These substances have molecular weights ranging from several hundred to tens of thousands. The humic fraction is further divided into three parts based on their solubility in acid or base. Fulvic acid and humic acid are soluble, but the humic fraction cannot be extracted by acid or alkali (Schnitzer and Kahn, 1972). Dr. T.L. Senn defined humic acids as the most biochemically active and important group of soil organic materials and humates as the salts of these acids (Senn, 1991).
Many commercial humate products are a result of an alkaline extraction of leonardite, an oxidized form of lignitic coal. The amount of the humic acids, as a percent of the total, will vary from site to site, which adds to the variability factor. Humates have been used on teak seedlings, tomatoes, corn, soybeans, peanuts and other crops (Fagbenro and Agboola, 1993; Tan and Nopamornbodi, 1979; Guminski et al., 1977). The results have shown increases in nutrient uptake (Maggioni et al., 1987; Preston et al., 1981; Piccolo et al., 1992; Fagbenro and Agboola, 1993), root and shoot growth (Tan and Tantiwiramanond, 1983; Poapst and Schnitzer, 1981) and anti-oxidant compounds (Schmidt, unpublished data).

Reduced irradiance

Shade or reduced irradiance is generally viewed as a stress factor on maintained turfgrass areas. Beard, 1973 estimated that 20 to 25% of turf areas are kept up under some form of shade. The amount of photosynthetically active radiation (PAR) available will change daily but more importantly it changes seasonally. Reduced irradiance causes some adaptive turfgrass responses: increased the shoot weight to root weight along with a decrease in root and rhizome production, increased leaf area, thinner leaves, lower specific leaf weight, elongation of the stems, (Harrison, 1934, Burton et al., 1959, Beard, 1973, Patterson, 1980, Kephart and Buxton, 1989, Allard
et al., 1991a,). These adaptations result in a plant with a more fragile leaf structure and a weaker root system than plants in full sun. Some other adaptations include decreased photosynthesis rates and carbohydrate reserves along with a general decline in growth.

The addition of liberal N fertility will reduce the root and rhizome development in shaded areas (Harrison, 1934, Watkins, 1940, Schmidt and Blaser, 1967, Burton et al., 1959, Wilkinson and Beard, 1975). Some disease incidences are more common under shaded conditions include; *E. graminus*, *Fusarium* spp., *Pythium* spp. *Rhizoctonia* spp. (Couch, 1995; Gilbert and Dipaola, 1985; Beard, 1965). This can be partially attributed the changes in the microenvironment such as increased humidity and leaf wetness and partially due to the increased stress on the turf plant and its ability to withstand the disease pressure.

The turfgrasses used in these experiments were Kentucky bluegrass (*Poa pratensis* L.) and Creeping bentgrass (*Agrostis palustris* Huds.). Kentucky bluegrass is a important turfgrass used in athletic fields, lawns and lower maintenance areas of golf courses in the transition zone and cooler regions. Kentucky bluegrass is rated as one of the less shade tolerant cool season grasses by Beard (1973) and Harivandi et al., (1984). Creeping bentgrass is used primarily for highly maintained putting and lawn bowling surfaces. The first section of this research examined interactions of shade, nitrogen and biostimulants.
OBJECTIVES

The objective of this research was to determine the influence of biostimulants and nitrogen fertility on Kentucky bluegrass growth responses under full and reduced light.
METHODS AND MATERIALS

Experiment 1.1 - Influences of nitrogen and biostimulants on the root and shoot growth of Kentucky bluegrass under greenhouse conditions in the winter of 1994-1995

In Early December 1994, 15.3 cm diameter sod plugs were taken from an area of one-year-old Kentucky bluegrass (Poa pratensis L. var. 'Plush') at the Virginia Tech Turfgrass Research Center in Blacksburg, Virginia. Plugs selected for visual uniformity were cut to a soil thickness of 3.8 cm and all soil then was washed from the turf plug. The washed plugs were placed into rings made of PVC pipe 4.0 cm high with course hardware cloth attached to the bottom. The hardware cloth allowed the turfgrass roots to grow into the soil. Plastic pots (4.7 L) were filled with a 75/25 (V/V) mix of course sand and a Groseclose silt loam soil (a clayey, Koalinitic, mixed Typic Hapludult). Soil test results of the mix reported a pH of 6.1 with the following ppm; $P_2O_5 = 13$, $K_2O = 17$, $Ca = 216$, $Mg = 32$, $Zn = 1.5$ and $Mn = 4.4$. The pots were heavily watered several times over 5 days and allowed to drain 24 hours before the rings were placed in them.

Treatments consisted of four $N$ rates with a seaweed extract and a humic acid treatments applied individually and in a half rate combination. Treatment application rates and products are shown on Table 1.1.1.

On 18 January 1995 the biostimulant (BIOS)treatments were foliarly applied to the
plugs with a compressed air sprayer that delivered 374 L ha\(^{-1}\) of liquid at a pressure of 276 kPa. The leaves were allowed to dry and the rings were randomly placed in the pots. The pots then were placed in a climate controlled greenhouse with an average temperature of 24\(^{\circ}\) C. Experimental units received only ambient winter sunlight which created natural low light conditions. Total nitrogen rates of 0.0, 12.3, 24.5 or 49.0 kg ha\(^{-1}\) from Urea (46-0-0) were split into six weekly treatments and applied with 230 ml water, starting on 26 January (1 WAT) and ending on 3 March (6 WAT). After fertilization ended, the turf received only 230 ml of tap water per week unless significant moisture stress symptoms appeared. The pots all received on extra 230 ml of water after wilting was noticed. The 230 ml is the equivalent of 1/2 acre inch of water per week per container. This protocol was intended to create a mild water stress on the turfgrass.
Table 1.1.1: Amounts of biostimulants per hectare applied to Kentucky bluegrass sod in Experiments 1.1, 1.2 and 1.3.

<table>
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<tr>
<th>Biostimulants</th>
<th>Amount ha⁻¹ used by experiment</th>
<th>Exp. #1.1</th>
<th>Exp. #1.2</th>
<th>Exp. #1.3</th>
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<tr>
<td>Seaweed extract (grams)</td>
<td></td>
<td>320¹</td>
<td>640¹</td>
<td>640¹</td>
</tr>
<tr>
<td>Humic Acid (liters)</td>
<td></td>
<td>4.9²</td>
<td>47.4³</td>
<td>47.4³</td>
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<tr>
<td><strong>Combination</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seaweed extract (grams)</td>
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<td>160¹</td>
<td>640¹</td>
<td>640¹</td>
</tr>
<tr>
<td>Humate extract (liters)</td>
<td></td>
<td>2.5²</td>
<td>47.4³</td>
<td>47.4³</td>
</tr>
<tr>
<td>Iron (kg)</td>
<td></td>
<td>None</td>
<td>11.2⁴</td>
<td>11.2⁴</td>
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<tr>
<td><strong>Commercial Product</strong></td>
<td></td>
<td>9.3⁵</td>
<td>9.3⁵</td>
<td>9.3⁵</td>
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¹Low heat and low pressure extract of Ascophyllum nodosum supplied by Caudill Seed Company. Rates based on previous research.

²A 25% humic acid solution supplied by Caudill Seed Company. Rates based on previous research.

³A 2.9% humic acid solution from Actisol™. Rate is based on manufactures directions.

⁴A 10% chelated iron product from Sequestene™. The rate is equal to 1 lbs. of actual iron per acre.

⁵From 3DTM a commercial product that is a proprietary blend of fortified seaweed, humic acid and iron. The rate is equal to 1 gallon per acre.
The turf was clipped 3 times during the experiment with hand clippers to a height of 3.5 cm and the clippings weighed. On 10 March a vertical pull method similar to Schmidt et al. (1986) was used to measure the relative root mass of each treatment.

Four replications of treatments were arranged in a completely randomized design with the pots randomly moved every 7 days.

The data were subjected to an ANOVA and the means separated using Duncan’s Multiple Range test. A p value of 0.05 was used. The analyses were computed using SAS Institute’s procedures for the personal computer, version 6.11 (1995).

Experiment 1.2 - Influences of nitrogen and biostimulants on the root and shoot growth of Kentucky bluegrass grown under full sun and reduced irradiance during a summer growth period and treated with biostimulants and different rates of nitrogen.

An area of Kentucky bluegrass (*Poa Pratensis* L. var. 'Plush') at the Virginia Tech Turfgrass Research Center in Blacksburg, Virginia was selected as the site for this experiment. This one year old sod was established on a Groseclose silt loam soil (a clayey, Koalinitic, mixed Typic Hapludult) with a pH of 5.9 and 31 ppm $P_2O_5$, 64 ppm $K_2O$, 420 ppm Ca and 72 ppm Mg. The area received 49 kg ha$^{-1}$ of N, $P_2O_5$ and $K_2O$ from a 10-10-10 fertilizer in early April 1995. Mowing height was maintained weekly at 4.5 cm and area was irrigated as needed to prevent severe wilting. Individual treatment plots were 1.2 by 1.2 meter and arranged in a randomized
complete block design with five replications. The treatments consisted of five BIOS applied to turf fertilized with three rates of N (0.0, 36.8 and 73.5 kg ha\(^{-1}\)) supplied by urea (46%-N). Biostimulant treatments included: seaweed extract (SE) and a humic acid extract (HE) alone, and a combination (SE+HE), Iron (Iron), a commercial product of growth enhancers (CP), and a N only control. The CP\(^1\) was a commercial mixture of seaweed, humic acids and iron that has shown good results in previous research. All treatments were foliarly applied on 6 June 1995 using a compressed air boom sprayer that delivered 374 L ha\(^{-1}\) of liquid at a pressure of 276 kPa. The amounts of the treatments applied are listed in Table 1.1.1.

Two 15.2 cm (diameter) plugs were taken from each plot 4 weeks after treatment. Each plug was cut to a uniform thickness of 3.8 cm and the remaining soil was washed from the sod. The plugs were transferred to 15.2 cm (diameter) PVC pipe sections (4.0 cm-height) with hardware cloth fixed to the bottom. This allowed the turfgrass roots to grow through the hardware cloth into the soil following transplanting. The pipe sections (rings) were placed in the same bare soil field, which received no fertilizer, either under a 73% shade cloth shelter or placed in an adjacent full sun area. The top of the shade cloth was 1.25 meters above the soil surface with the sides extending down to within 0.3 meters of the soil surface. Additional vertical screens of shade cloth were placed on the east and west sides about 1.5 meters away from the shelter to prevent any direct early morning or late afternoon sunlight from

\(^1\)D supplied by Plant-Wise Biostimulant Co.
reaching the plugs but permitting natural air circulation. The plugs were irrigated as needed to prevent any wilting and were not mowed after transplanting.

Rooting measurements were taken on 7 Aug., which is 8 WAT (weeks after treatment), using a vertical lift method which measured the force required to lift the rings from the soil. This was a modification of the method described by Schmidt et al. (1986). Hooks were pushed into pre-drilled holes in the rings and attached to a hand held scale (John Chatillon & Sons - model DPPH-100) which was used to record the vertical force (in kgs) required to separate the rings from the soil. Significant correlation between the vertical force required and the root mass was reported by Schmidt et al. (1986).

Measurements of blade height were taken with a device consisting of a 15.2 cm PVC ring with two plastic rulers attached to opposite sides and a disk of construction paper cut to allow it to freely move down the rulers. This device was placed over the plugs and after the construction paper settled on the leaves, the average height of the construction paper along the two rulers was recorded to determine the height of the turf.

The data were subjected to an ANOVA and the means separated using Duncan's Multiple Range test at a p value of 0.05. The analyses were computed using SAS Institute's procedures as outlined in SAS/STAT User's Guide version 6 and run on a personal computer using SAS version 6.11 (1995).
Experiment 1.3 - Influences of nitrogen and biostimulants on the root and shoot growth of Kentucky bluegrass grown under full sun and reduced irradiance during an autumn growth period and treated with biostimulants and different rates of nitrogen.

The same field plots used for Experiment 1.2 were used for this experiment. All the plots except those receiving iron and the combination applications were retreated on 28 August 1995. Refer to Table 1.1.1 for the exact amounts. The methods, materials and rates were identical to Experiment 1.2. The total N applied to separate plots in 1995 (spring fertilization + Experiment 1.2 + Experiment 1.3) was 49.0, 122.5 and 196.0 kg N ha\(^{-1}\) (Table 1.1.2).

Two 15.2 cm plugs taken from each plot four weeks after treatment, using the same procedures as before, were put into the PVC rings and placed in a prepared soil area. One ring from each treatment was placed under a 27% ambient sunlight (AS) cloth shelter and the other put into an adjacent full sun area.

Rooting estimates were ascertained by the vertical lift technique on 6 December 1995 (14 WAT which is 10 weeks after transplanting) and the statistical procedures were identical to the analysis performed on the August data (Experiment 1.2).
Table 1.1.2: Amounts of nitrogen per hectare applied to the Kentucky bluegrass field plots in Experiments 1.2 and 1.3 as supplied by an urea solution.

<table>
<thead>
<tr>
<th>Application Dates</th>
<th>April</th>
<th>June</th>
<th>August</th>
<th>Yearly totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>49.0</td>
<td>0.0</td>
<td>0.0</td>
<td>49.0</td>
</tr>
<tr>
<td>Medium</td>
<td>49.0</td>
<td>36.8</td>
<td>36.8</td>
<td>122.5</td>
</tr>
<tr>
<td>High</td>
<td>49.0</td>
<td>73.5</td>
<td>73.5</td>
<td>196.9</td>
</tr>
</tbody>
</table>

Nitrogen source was Urea (46-0-0) in solution

Experiment 1.2 was applied in June

Experiment 1.3 was applied in August

English conversion for 49.0 N Kg ha\(^{-1}\) is 1 lb N/1000 ft\(^2\).
RESULTS AND DISCUSSION

Experiment 1.1 Kentucky bluegrass greenhouse experiment using biostimulants and several rates of nitrogen.

Rooting strength

The BIOS-treated turfgrass had significantly more root strength than the non-BIOS-treated plants at each level of N fertility (Table 1.1.3). The average rooting mean of the seaweed treated turf was 20% greater than the average N only turf.

The addition of N fertility did not improve rooting and the 24.5 kg N ha\(^{-1}\) regime significantly reduced rooting below the 0.0 kg N ha\(^{-1}\) level. A reduction in Kentucky bluegrass rooting as N increased has been demonstrated in previous experiments by Harrison, (1934), Juska et al., (1955); Sills and Carrow, (1983) and Adams et al. (1974).

Clipping weight

When averaged over the four N regimes, the BIOS-treated turfgrass produced significantly more clipping weight than the non-BIOS-treated grass (Table 1.1.4).
Table 1.1.3: Rooting strength of Kentucky bluegrass grown under three N regimes following either an application of nitrogen and a seaweed extract, a humic acid extract or a combination of both at half rate on 18 Jan. 1995. Measurements were taken on 10 March 1995. Only ambient light was used which created natural low light conditions.

<table>
<thead>
<tr>
<th>Kg of Nitrogen ha(^{-1}) (total N applied)</th>
<th>0.0</th>
<th>12.3</th>
<th>24.5</th>
<th>49.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOS</td>
<td>-----</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>SE</td>
<td>10.8</td>
<td>9.4</td>
<td>9.0</td>
<td>9.6</td>
</tr>
<tr>
<td>HE</td>
<td>9.6</td>
<td>9.8</td>
<td>9.0</td>
<td>9.5</td>
</tr>
<tr>
<td>1/2 SE + 1/2 HE</td>
<td>9.5</td>
<td>8.9</td>
<td>8.9</td>
<td>9.2</td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>8.4</td>
<td>8.2</td>
<td>7.6</td>
<td>8.4</td>
</tr>
<tr>
<td>Means</td>
<td>9.6 x</td>
<td>9.0 xy</td>
<td>8.2 y</td>
<td>9.2 xy</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not significantly differ at the 5% level of probability using Duncan's Multiple Range Test.

Capital letters represent differences within a column (ABC).
Small letters represent differences within a row (xyz).

Means are the average of 4 replications in a completely randomized design.

Significance levels from the ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-N-LEVEL</td>
<td>0.041</td>
</tr>
<tr>
<td>BIO</td>
<td>0.009</td>
</tr>
<tr>
<td>N-LEVEL * BIO</td>
<td>0.137</td>
</tr>
</tbody>
</table>
Although not statistically significant, each additional level of N increased top growth. This additional top growth was produced to the detriment of the root growth (Table 1.1.3) and demonstrates that environments favorable for turfgrass foliar growth often have less photosynthate energy transmitted for root development (Bushby et al., 1992; Schmidt and Blaser 1967).

The seaweed and humic acid-treated turf produced more leaf weight than the control (N only) at each level of fertility. The mean leaf weight of the seaweed and the humic acid-treated turf, when averaged over three yields, was 36% above the control mean.

The BIOS-treated turf produced an increase in rooting and top growth beyond the N only-treated grass.

This experiment created questions about the role of irradiance and the interaction of biostimulants and N on the growth of turfgrass.
Table 1.1.4: Clipping weight (average of 3 clipping dates) of Kentucky Bluegrass grown under three N regimes following either a single application of a seaweed extract, a humic acid extract or a combination of half rates of both on 18 Jan. 1995. Clippings were taken on 8 Feb., 20 Feb. and 5 Mar. 1995. Only ambient light was used which created natural low light conditions.

<table>
<thead>
<tr>
<th>BIOS</th>
<th>0.0</th>
<th>12.3</th>
<th>24.5</th>
<th>49.0</th>
<th>MEANS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>grams fresh weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>3.3</td>
<td>3.4</td>
<td>4.2</td>
<td>4.2</td>
<td>3.8 A</td>
</tr>
<tr>
<td>HE</td>
<td>3.4</td>
<td>3.8</td>
<td>3.8</td>
<td>4.1</td>
<td>3.8 A</td>
</tr>
<tr>
<td>1/2 SE+1/2 HE</td>
<td>2.7</td>
<td>3.5</td>
<td>3.5</td>
<td>3.6</td>
<td>3.3 B</td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>2.4</td>
<td>2.9</td>
<td>2.8</td>
<td>3.2</td>
<td>2.8 C</td>
</tr>
<tr>
<td>Means</td>
<td>2.9</td>
<td>3.4</td>
<td>3.6</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

Values followed by the same letter do not significantly differ at the 5% level of probability using Duncan’s Multiple Range Test.

Capital letters represent differences within a column (ABC). Small letters represent differences within a row (xyz).

Means are the average of 4 replications in a completely randomized design.

Significance levels from the ANOVA

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr &gt; F</th>
<th>N-LEVEL</th>
<th>NS</th>
<th>BIO</th>
<th>0.003</th>
<th>N-LEVEL * BIO</th>
<th>0.444</th>
</tr>
</thead>
</table>

21
Experiment 1.2 - Summer growth period. Kentucky bluegrass treated with biostimulants and several rates of nitrogen and grown under 100% ambient sunlight.

In Experiments 1.2 and 1.3, the experimental procedures of treating the sod then transplanting it four weeks later on to a bare soil, that did not receive any fertilizer, allowed the assessment of the preconditioning influences of the treatments.

Rooting strength

Biostimulant and iron treatments enhanced post transplant-rooting of turf receiving the 49.0 kg N ha\(^{-1}\) regime (Table 1.2.1). The rooting did not differ between the BIOS-treated turf at the 85.5kg N ha\(^{-1}\) level; however, at the highest level of fertility, only the seaweed-treated turf produced greater root strength/mass than the non-BIOS treatments (N only).

Generally, the addition of N improved the root mass of the transplanted sod except for the iron and the blend (which contains iron) treatments (Table 1.2.1). Under conditions of this study, the enhancement of new root development with iron treatments decreased as N fertilization increased. Goatley (1988) reported that an application of iron during the summer reduced the rooting of Kentucky bluegrass sod.
TABLE: 1.2.1 : Four-week post-transplant rooting strength of a one year old Kentucky bluegrass sod as influenced by pretransplant biostimulants and nitrogen regimes grown under 100% ambient sunlight (AS) in summer.

<table>
<thead>
<tr>
<th>BIOS</th>
<th>Kg of Nitrogen ha⁻¹</th>
<th>g of vertical force per 182 cm²</th>
<th>MEANS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE</td>
<td>23.8 A y</td>
<td>26.8 A y</td>
<td>32.6 A x</td>
</tr>
<tr>
<td>HE</td>
<td>22.8 A y</td>
<td>26.4 A x</td>
<td>28.0 AB x</td>
</tr>
<tr>
<td>SE + HE</td>
<td>23.6 A x</td>
<td>23.6 A x</td>
<td>27.2 AB x</td>
</tr>
<tr>
<td>CP</td>
<td>25.2 A x</td>
<td>22.4 A x</td>
<td>24.0 B x</td>
</tr>
<tr>
<td>Iron</td>
<td>25.8 A x</td>
<td>22.6 A xy</td>
<td>21.4 B y</td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>16.2 B y</td>
<td>23.4 A x</td>
<td>24.0 B x</td>
</tr>
<tr>
<td>Means</td>
<td>22.9</td>
<td>24.2</td>
<td>26.2</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not significantly differ at the 5% level of probability using Duncan's Multiple Range Test.

Capital letters represent differences within a column (ABC). Small letters represent differences within a row (xyz).

Means are the average of 5 replications in a completely randomized design.

Significance levels from the ANOVA

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-LEVEL</td>
<td>0.048</td>
</tr>
<tr>
<td>BIO</td>
<td>0.029</td>
</tr>
<tr>
<td>N-LEVEL * BIO</td>
<td>0.005</td>
</tr>
</tbody>
</table>
The N only and the humic acid (HE) treatments increased the rooting 44 and 16% respectively, between the 49.0 kg ha\(^{-1}\) and 85.8 kg ha\(^{-1}\) levels. The seaweed (SE) and combination (SE+HE) treatments increased rooting by 22 and 15%, respectively, between the 85.8 and 122.5 kg ha\(^{-1}\) levels. This indicated that the response of different BIOS treatments varied with the level of fertility.

Generally, more N was required to generate equivalent root mass by the N only treated turfgrass than the BIOS-treated turf. Possibly, under these field conditions (summer) the biostimulant treatments enhanced hormonal development within the turfgrass.

Canopy heights

Kentucky bluegrass treated with seaweed or humic acid alone or in combination produced greater canopy heights than the N only at the 49.0 kg N ha\(^{-1}\) regime (Table 1.2.2). Heights of turfgrass grown with 85.8 kg N ha\(^{-1}\) were all similar regardless of the treatments applied. At the highest N level, the grass treated with humic acid had lowered canopy growth than the non-biostimulant-treated grass as well as the SE+HE, iron and CP-treated turf.
Table 1.2.2: Four-week post-transplant canopy height of a one year old Kentucky bluegrass sod grown under 100% ambient sunlight (AS) in summer as influenced by pretransplant biostimulants and nitrogen regimes.

<table>
<thead>
<tr>
<th>Kg of Nitrogen ha⁻¹</th>
<th>49.0</th>
<th>85.8</th>
<th>122.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOS</td>
<td>height in cm</td>
<td>MEANS</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>5.7 A x</td>
<td>5.2 A y</td>
<td>5.8 AB x</td>
</tr>
<tr>
<td>HE</td>
<td>5.6 AB x</td>
<td>5.6 A x</td>
<td>5.2 B x</td>
</tr>
<tr>
<td>SE + HE</td>
<td>5.5 AB y</td>
<td>5.4 A y</td>
<td>6.3 A x</td>
</tr>
<tr>
<td>CP</td>
<td>5.2 BC y</td>
<td>5.6 A y</td>
<td>6.1 A x</td>
</tr>
<tr>
<td>Iron</td>
<td>5.1 BC y</td>
<td>5.9 A x</td>
<td>5.9 x</td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>4.9 c z</td>
<td>5.3 A y</td>
<td>6.0 A x</td>
</tr>
<tr>
<td>Means</td>
<td>5.3</td>
<td>5.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not significantly differ at the 5% level of probability using Duncan's Multiple Range Test.

Capital letters represent differences within a column (ABC).
Small letters represent differences within a row (xyz).

Means are the average of 5 replications using a randomized block design.

Probability values for the main effects:

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-LEVEL</td>
<td>0.0017</td>
</tr>
<tr>
<td>BIO</td>
<td>0.658</td>
</tr>
<tr>
<td>N-level * BIO</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
Experiment 1.2 - Summer growth period Kentucky bluegrass treated with biostimulants and several rates of nitrogen and grown under 27% ambient sunlight.

Rooting strength

The commercial product (CP) and the SE+HE-treated grass produced superior root strength/development compared to the N only treatments when the results are averaged over all N levels (Table 1.2.3). It should be noted that the CP contains SE and HE.

Increasing levels of N did not significantly improve rooting under shade when averaged over all the treatments. In contrast, the root mass produced under 100% AS (Table 1.2.1) increased in root development as the N level increased.

The BIOS-treated turf did not demonstrate a clear pattern of improved root mass with the increasing N. There were no differences in root mass between any of the treated turf under the two higher N regimes, which were applied in June. Apparently, the increase in root development of the non-BIOS-treated turf with the increasing N regime lessened the difference between biostimulant-treated and non-biostimulant-treated grass. The commercial product-treated plugs at the lower N levels produced root mass similar to the best non-BIOS treated (N only) turf's rooting at high N fertility (122.5 kg N ha⁻¹).
Table 1.2.3: Four-week post-transplant root strength of a one year old Kentucky bluegrass sod as influenced by pretransplant biostimulants and nitrogen regimes grown under 27% ambient sunlight (AS) in summer.

<table>
<thead>
<tr>
<th>Kg of Nitrogen ha⁻¹</th>
<th>49.0</th>
<th>85.8</th>
<th>122.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>17.0</td>
<td>20.6</td>
<td>20.0</td>
</tr>
<tr>
<td>HE</td>
<td>18.6</td>
<td>16.8</td>
<td>18.2</td>
</tr>
<tr>
<td>SE + HE</td>
<td>18.8</td>
<td>18.6</td>
<td>22.2</td>
</tr>
<tr>
<td>CP</td>
<td>21.0</td>
<td>20.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Iron</td>
<td>18.6</td>
<td>15.8</td>
<td>17.2</td>
</tr>
<tr>
<td>Nitrogen only</td>
<td>13.2</td>
<td>17.8</td>
<td>19.2</td>
</tr>
<tr>
<td>Means</td>
<td>17.8 x</td>
<td>18.3 x</td>
<td>19.6 x</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not significantly differ at the 5% level of probability using Duncan’s Multiple Range Test.

Capital letters represent differences within a column (ABC). Small letters represent differences within a row (xyz).

Means are averages of 5 replications in a randomized block design.

Probability values for the main effects:

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>N- LEVEL</td>
<td>0.55</td>
</tr>
<tr>
<td>BIO</td>
<td>0.04</td>
</tr>
<tr>
<td>N-LEVEL * BIO</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Over all N levels, the commercial product (CP) and the combination (SE+HE) treated-turf produced greater root development than the other treated grass. The average root mass produced under the shade averaged 75% of comparable treatments in an adjacent full sunlight (100% AS) area. This would support research of Schmidt and Blaser 1967 and Bushby et al., 1992, which showed a reduction in turfgrass rooting under shade even with additional N fertility. This reduction may be linked to the reduced carbohydrate production associated with the reduced irradiance.

**Canopy height**

Each additional level of N fertility resulted only in a slight increase in average canopy height (Table 1.2.4). Hull, 1992 demonstrated that the natural depression of cool season grass leaf production in mid-summer cannot be eliminated with increased N fertilization. However, the biostimulant-treated turf was generally able to produce about the same top growth with less N than the non-biostimulant-treated turf.
Table 1.2.4: Four-week post-transplant canopy height of a one year old Kentucky bluegrass sod grown under 27% ambient sunlight (AS) in summer as influenced by pretransplant biostimulants and nitrogen regimes.

<table>
<thead>
<tr>
<th>BIOS</th>
<th>Kg of Nitrogen ha⁻¹ applied this year</th>
<th>height in cm</th>
<th>MEANS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>49.0</td>
<td>85.8</td>
<td>122.5</td>
</tr>
<tr>
<td>SE</td>
<td>9.5</td>
<td>10.4</td>
<td>10.5</td>
</tr>
<tr>
<td>HE</td>
<td>9.6</td>
<td>9.4</td>
<td>10.5</td>
</tr>
<tr>
<td>SE + HE</td>
<td>9.8</td>
<td>9.7</td>
<td>10.5</td>
</tr>
<tr>
<td>CP</td>
<td>9.9</td>
<td>10.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Iron</td>
<td>9.2</td>
<td>9.4</td>
<td>10.1</td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>8.8</td>
<td>9.4</td>
<td>10.2</td>
</tr>
<tr>
<td><strong>Means</strong></td>
<td><strong>9.7</strong></td>
<td><strong>9.8</strong></td>
<td><strong>10.5</strong></td>
</tr>
</tbody>
</table>

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Means are the average of 5 replications using a randomized block design.

Probability values for the main effects:
- SOURCE: Pr>F
  - N-LEVEL: 0.0709
  - BIO: 0.022
  - N-LEVEL * BIO: 0.97
The BIOS treatments within each N regime did not differ in canopy height from the N only treatments. However, when averaged over all nitrogen regimes, canopy heights of the commercial product was significantly greater than the average of the non-BIOS-treated turf. This also corresponded to the commercial product-treated grass producing the best average root development under shade.

Although not statistically comparable, the canopy heights under 27% AS were about 80% taller than the grass grown under 100% AS. This elongation of the leaf blades demonstrates the adaptive morphology of leaf blades in shade as discussed by Beard 1973, Wilkinson and Beard 1975, and Boardman 1977. These authors reported that leaf expansion as influenced by the low light intensity can reflect a correspondingly lower root development. This shoot to root preference again demonstrates the partitioning of the energy transfer in turfgrass under reduced irradiance.
**Experiment 1.3 - Autumn growth period.** Kentucky bluegrass treated with biostimulants and several rates of nitrogen grown under 100% ambient sunlight.

**Rooting strength**

The rooting of the Kentucky bluegrass treated with BIOS was generally greater than the non-biostimulant controls regardless of the N level (Table 1.3.1). The one exception was the rooting increase associated with the humic acid treated turf grown under the 122.5 kg N ha\(^{-1}\) level. Although the root mass of the humate treated sod was 13% greater than the N only-treated turf, the root mass measurements were not statistically different.

All treatments showed an increase in root development with increasing N fertility. This increase was more pronounced than during the summer growth period under full sun (Table 1.2.1). Although not statistically comparable, the rooting in December sampling was 40 to 50% of the rooting taken in August (both full sun). This confirms the findings of Allard et al., (1991a) and Kephart and Buxton (1993), that show changes in morphological and photosynthate changes may allow a change in carbohydrate partitioning and an alteration in shoot/root growth ratios.
Table 1.3.1: Ten-week post-transplant root strength of a one year old Kentucky bluegrass sod grown under 100% ambient sunlight in autumn as influenced by pre-transplant biostimulants and nitrogen regimes

<table>
<thead>
<tr>
<th>BIOS</th>
<th>49.0</th>
<th>122.5</th>
<th>196.0</th>
<th>MEANS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kg of force per 182 cm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>11.5 A y</td>
<td>12.9 A x</td>
<td>13.3 AB x</td>
<td>12.6</td>
</tr>
<tr>
<td>HE</td>
<td>10.0 AB y</td>
<td>10.4 B y</td>
<td>12.3 B x</td>
<td>10.9</td>
</tr>
<tr>
<td>CP</td>
<td>10.6 AB y</td>
<td>12.9 A y</td>
<td>13.8 A x</td>
<td>12.4</td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>6.8 C x</td>
<td>9.2 B x</td>
<td>10.1 C x</td>
<td>8.7</td>
</tr>
<tr>
<td>Means</td>
<td>9.7</td>
<td>11.4</td>
<td>12.4</td>
<td></td>
</tr>
</tbody>
</table>

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Capital letters represent differences within a column (ABC).

Small letters represent differences within a row (xyz).

Means are the averages of 5 replications using a random block design.

Probability values for the main effects:

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-LEVEL</td>
<td>0.001</td>
</tr>
<tr>
<td>BIO</td>
<td>0.0001</td>
</tr>
<tr>
<td>N-LEVEL * BIO</td>
<td>0.0165</td>
</tr>
</tbody>
</table>
Only the commercial product-treated grass showed a significant increase with each increase in N level. The commercial product-treated grass produced superior rooting in the autumn sunlight and in the shade during the summer (Table 1.2.1). This may indicate that a composite mix of biostimulants perform better under certain reduced ambient light conditions.

**Experiment 1.3 - Autumn growth period.** Kentucky bluegrass treated with biostimulants and several rates of nitrogen and grown under 27% ambient sunlight.

**Rooting mass**

The increased N fertilization reduced the average rooting of the treated turf (Table 1.3.2).

The rooting of all the BIO treated-plants at the 49.0 kg N ha\(^{-1}\) level was significantly greater than the rooting of the N only-treated turf. However, at the 122.5 kg N ha\(^{-1}\) level only the humic acid and the commercial product-treated (which contained humic acid) turf were superior to the N only treated turf. Rooting of all the BIOS treated turf under the 196.0 kg N ha\(^{-1}\) regime was similar and generally lower than grasses grown under lower N regimes. This result roughly parallels the rooting results of the greenhouse experiment (Table 1.1.3), where additional N caused a
decrease in rooting. Turfgrass in both of these experiments was subjected to low levels of Photosynthetically Available Radiation (PAR) and would indicate that light was a limiting factor, impacted by high N availability, to the growth of the Kentucky bluegrass roots.

The autumn rooting of the treated turfgrass under the shade was 40 to 50% of comparably treated turfgrass grown in the sun. This reduction in average rooting as N increased is a change from the summer shade treatment (Table 1.2.3) in which N, at amount applied, was not a significant factor in average root production. The large reduction of ambient light duration caused by the approaching winter solstice in combination with the shade treatment greatly reduced the PAR reaching the plants and could be a factor in the root mass decline. Researchers have shown that under low light, turfgrass will increase the shoot to root ratio which results in more top growth at the expense of root production (Burton et al., 1959; Eriksen and Whitney, 1981 and Wong and Wilson, 1980). High levels of available N at times favorable to top growth, have suppressed root growth (Juska et al., 1955; Sills and Carrow, 1983) and reduced non-structural carbohydrates (Schmidt and Blaser, 1967, Watschke and Waddington, 1975).
Table 1.3.2. Ten-week post-transplant root strength of a one year old Kentucky bluegrass sod as influenced by pre-transplant biostimulants and nitrogen regimes grown under 27% ambient sunlight in autumn.

<table>
<thead>
<tr>
<th>Kg of Nitrogen ha(^{-1}) (applied this year)</th>
<th>49.0</th>
<th>122.5</th>
<th>196.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>6.3 A x</td>
<td>4.7 B y</td>
<td>4.6 A y</td>
</tr>
<tr>
<td>HE</td>
<td>6.1 A x</td>
<td>6.2 A x</td>
<td>4.6 A y</td>
</tr>
<tr>
<td>CP</td>
<td>6.1 A x</td>
<td>6.0 A x</td>
<td>4.6 A y</td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>4.5 B x</td>
<td>4.8 B x</td>
<td>4.6 A x</td>
</tr>
<tr>
<td>Means</td>
<td>5.8</td>
<td>5.4</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Values followed by the same letter do not significantly differ at the 5% level of probability using Duncan's Multiple Range Test.

Capital letters represent differences within a column (ABC). Small letters represent differences within a row (xyz).

Means are the average of 5 replications in a randomized block design.

Probability values for the main effects:

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-LEVEL</td>
<td>0.001</td>
</tr>
<tr>
<td>BIO</td>
<td>0.099</td>
</tr>
<tr>
<td>N-LEVEL * BIO</td>
<td>0.022</td>
</tr>
</tbody>
</table>
The low initial root mass of the N only-treated turf did not change as the N levels increased. The seaweed treated-grass realized a significant reduction in rooting between the 49.0 and the 122.5 kg N ha\(^{-1}\) levels, but the humate and the commercial product-treated turf did not have a significant reduction until the 196/0 N kg ha\(^{-1}\) level. This may indicate that the humate and the commercial product (which has a humate component) helps the turf utilize this level of N more efficiently under these experimental conditions.
SUMMARY OF RESULTS FROM CHAPTER ONE

Nitrogen fertility under the greenhouse conditions of low winter light (PAR) tended to reduce the rooting and stimulate the top growth. The BIOS-treated turfgrass under winter light conditions produced more root mass and clipping weight than the non-BIO controls overall regardless of N regime.

In full summer sun, the post-transplant rooting mass was enhanced with each increase in N fertility used in this study. However, the BIOS-treated Kentucky bluegrass produced greater rooting response with less N. Generally, turfgrass treated with either seaweed or humic acid generated an increase of 31 and 21%, respectively, in root development enhancement over the N only treated turf. The grass treated with iron showed a trend of reduced root mass as the N fertility increased.

The canopy heights of the N only-treated turf increased as the N level rose. The seaweed and humic acid-treated plants had the tallest canopies at the low N level. The BIOS treated Kentucky bluegrass within the higher two N levels did not have taller blades that the N only turf.

The seaweed and humic acid treated turf also produced the best overall root development under these summer conditions possibly in association with controlled top growth.
Under the summer shade, N fertility did influence the root production when averaged over all treatments. Again the BIOS-treated turf at the lowest level (49.0 kg N ha\(^{-1}\)), except seaweed, generated more root mass than the N only at the medium level (85.8 kg N ha\(^{-1}\)). The commercial product-treated turf fertilized at the lowest N level (49.0 kg N ha\(^{-1}\)) produced more rooting than the N only fertilized with the highest level (122.5 kg N ha\(^{-1}\)). The turf treated with the composite mixes (commercial product (CP) and combination (SE+HE)) produced better rooting under reduced irradiance than the single component treatments. The reverse was true for the 100% ambient sunlight experiment, in which the single component-treated turf produced better rooting. This may indicate that light intensities can influence biostimulant activity.

Increasing fertility enhanced rooting of the turf during the full autumn sun (100% AS). All of the biostimulant-treated turf at the 49.0 and 122.5 kg N ha\(^{-1}\) regimes had rooting equal to or better than the non-biostimulant control at the 196.0 kg N ha\(^{-1}\) level. All treatments except humic acid caused the Kentucky bluegrass to have the largest increases in root mass of turf grown under the 49.0 and the 122.5 kg N ha\(^{-1}\) levels.

The root mass of the turfgrass grown under the autumn shade (27% AS) was reduced as the N fertility was increased. The humic acid and the commercial product-treated turf produced the best average root mass. This may indicate that the humic acid component was able to offset some of the negative effects of the very low light,
possible through increasing auxin action.

Under summer and fall experimental conditions, the BIOS-treated Kentucky bluegrass was able to produce more root and top growth with less N fertility than the N only-treated turfgrass. The influence of the BIOS in these experiments appeared to increase with the seasonal reduction in available light. This may indicate that the BIOS treatments have the greatest root development effect by enhancing the partitioning of photosynthates toward the roots.

The root to shoot growth of these experiments agrees with previous research that under light limiting conditions the turfgrass will preferentially produce shoot growth at the expense of root production.

In summer and fall seasons, the increases that the biostimulants produced over the N only treatments were greater under low to medium N inputs and under reduced light (summer shade and full sun in the autumn).

More research on the specific plant mechanisms and interactions effected by BIOS is needed. The dissimilarities in performance by several of the biostimulants and combinations of biostimulants under different light conditions could be explored. Physiological reasons for the heightened influences by BIOS under reduced light and lower N should be investigated. Answers to these and other question will help the turfgrass managers to use of (biostimulants) in the most efficient manner.
CONCLUSIONS

This research has shown that the exogenous application of BIOS can improve endogenous mechanisms in the turfgrass which can help root development and top growth while lowering the required N inputs. These experiments demonstrate the potential of biostimulants in enhancing growth beyond the traditional fertility program.
CHAPTER TWO

The Effects of Nitrogen Fertility and Biostimulants on the Superoxide Content and Chlorophyll Fluorescence of Turfgrass Leaves

INTRODUCTION

Oxidation occurs in all aerobic organisms and excess reduced oxygen species (forms of oxidants) can cause serious damage. Plants and animals have developed an array of defense mechanisms against oxidative stress. Superoxide dismutases (SOD) are metal containing enzymes that catalyze the scavenging of superoxide radicals to oxygen and hydrogen peroxide which plays a major role in the defense the against reduced oxygen radicals. Superoxide dismutase is reported to be a key part of plant stress tolerance (Bowler et al., 1994, Mishra et al., 1993, Madamanchi and Alscher, 1994). The literature contains little material about the effects of nitrogen fertility and biostimulants on the antioxidant content of turfgrass leaves. These experiments were conducted to ascertain the effects of N fertility and biostimulants on the SOD content of turfgrass. The information obtained may help explain an aspect of the turfgrass’s increased stress tolerance associated with exogenous treatments of biostimulants.
Chlorophyll fluorescence has been used in plant research for over twenty years, but little has been published on the use of this technology applied to turfgrass. Chlorophyll fluorescence measurements are usually done on a leaf disc or individual leaves on a specific plant. The narrow width of most turfgrass blades does not allow examination of individual leaves. Some modifications to the manufacturers' suggested sampling techniques were made in order to gather data from an area of the turfgrass canopy rather than from individual blades.

The first experiment discussed (Experiment 2.1) was conducted in a field during the autumn growth period in which two biostimulants and three rates of N fertility were applied. This experiment explored the relationships between SOD and N fertility and biostimulant applied to Kentucky bluegrass. The next experiment (2.2) was performed in a greenhouse and focused on the influence of N on SOD content, fluorescence, rooting and top growth of Kentucky bluegrass. The last experiment described (2.3) was conducted on bentgrass field plots and includes data on SOD and fluorescence as influenced by N fertility.
LITERATURE REVIEW

Superoxide dismutase

Superoxide dismutases were originally discovered by McCord and Fridovich in 1969. Superoxide dismutases are metal containing enzymes that catalyze the scavenging of superoxide species (radicals) to oxygen and hydrogen peroxide. This is an enzymatic process and an important step in the plants’ defense against the damage caused by the reduced oxygen radicals. Non-enzymatic defense mechanisms include antioxidants as ascorbate and alpha tocopherol.

There are many types of reduced oxygen species. Superoxide (O$_2^-$) and singlet oxygen (¹O$_2$) are produced by photoreduction of oxygen and energy transfer from excited (triplet) chlorophyll. Hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH·) can form as a result of reactions with superoxide (Smirnoff, 1993).

The most common radical is the superoxide radical (O$_2^-$), the first product of O$_2$ reduction (Halliwell, 1984). Superoxide radicals are formed when electrons are donated to oxygen. This reaction can happen in mitochondrial electron transport system, chloroplasts and the photosystems. When the protective systems’ abilities to scavenge excess superoxide is inadequate, the superoxide can interact with hydrogen peroxide to form hydroxyl radicals (Smirnoff, 1993, Dodge, 1994). This reaction is
known as the Haber-Weiss reaction: $\text{O}_2^- + \text{H}_2\text{O}_2^- \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2$.

A variety of stresses can increase the oxygen radical concentrations in a plant. Stresses such as high light, low temperatures, drought, air pollutants or herbicides have been associated with increased superoxide radicals in plants (Bowler et al., 1994, Demmings-Adams and Adams, 1994, Smirnoff, 1993). Stress conditions will increase the concentrations of reduced oxygen species through a combination of decreased capacity to scavenge and/or the increase of production of reduced oxygen.

In studies by Walker et al. (1991), chilling resistance is thought to be related to the ability to decrease the potential for free radical production by regulation of electron transport within the chloroplast. They showed that as temperature declined the rate of electron transport declined. Wise and Naylor (1987) used a 100% $\text{N}_2$ atmosphere and a photosynthetic electron transport inhibitor to demonstrate that the production of oxygen radicals plays an important part in chilling-enhanced photooxidation. Low temperature exposed maize leaves were found to have increased SOD levels indicating a response by the plant to stress (Massacci et al., 1995).

Atmospheric pollution, primarily $\text{O}_3$, $\text{SO}_2$, PAN and $\text{NO}_2$, have a deleterious effect on the carotenoid composition. Ozone may have an added effect by raising the levels of active oxygen within the plant cells. Different carotenoids seem to have different levels of destruction due to the various pollutants. Chlorophyll a is more susceptible to PAN than chlorophyll b, but the opposite is true for exposure to NO$_2$. Ozone will degrade both chlorophylls at about the same rate (Pallett and Young, 1993).
and Mudd 1975). Ozone has been shown to inhibit the ETS and to impair the
functioning's of amino acids. Exposure to ozone has increased the SOD content in
(insensitive to oxidation) pea cultivar demonstrated a rapid and significant increase in
SOD where the sensitive cultivar did not. This could be the mechanical basis for
resistance in plants tolerant to oxidative stress (Madamanchi and Alscher, 1991).

In general, as the temperatures of the chloroplasts rise over an optimum (25°C
to 30°C in C₃ plants and > 35°C in C₄ plants) the photosynthetic rate declines with
the inhibition of enzyme activity. This slowing down can also relate to the slowing of
phytotoxin scavenging and, therefore, allow the buildup of reduced oxygen
components to accumulate and lead to photooxidation.

Plants have natural defenses to combat excesses in radical oxygen species.
These defenses can be divided into two categories (Smirnoff, 1993). The first system
reacts with the active oxygen species and maintains them at less than destructive
levels. The second system involve the regeneration of antioxidants via ascorbate and
glutathione (GSH). The best defense is to prevent the reduced oxygen species
formation by efficient scavenging of hydrogen peroxide and superoxide (Bowler et al.,
1994). This chapter will focus on the first system and particularly the production of
superoxide dismutase.

Madamanchi and Alscher, 1994; Krause, 1994, and Schmidt (unpublished
data), showed that a rapid increase in SOD will provide a significant level of
tolerance from oxidative stress. Several experiments using transgenic tobacco plants with enhanced SOD expression have shown that the transformed plants have improved tolerance of oxidative stress (Bowler et al., 1991; Gupta et al., 1993).

**Chlorophyll fluorescence**

In recent years, non-destructive tests have been developed to measure plants responses to various stressors, particularly environmental stresses. One test has gained wider interest than most; this test involves the measurement of chlorophyll fluorescence. Chlorophyll fluorescence analysis is an important tool in plant stress physiology because of the speed and the non-destructive nature of the tests.

Under optimum conditions, only 85% of the light intercepted by a plant leaf is absorbed by photosynthetic pigments (Wilson and Greaves, 1990). Light energy in the photosynthetic process has several paths it can travel. The energy can be used in the electron transport chain for the reduction of carbon (photochemical reaction) or the excess energy can be reemitted as fluorescence, transferred to a neighboring (ground state) molecule or radiated as heat (non-radiative dissipation) (Guilbault, 1967; Krause and Weis, 1984; Ireland et al., 1984).

Chlorophyll fluorescence generates primarily from photosystem II (PSII) and reflects changes in the PSII activity and can serve as an indicator of rate of the
electron transport system (ETS) under many conditions (Wilson and Greaves, 1990; Demming-Adams and Adams, 1994). The fluorescence yield is only about 3% in optimum functioning living cells (Guilbault, 1967) and will be increased as the inhibition of ETS and the photochemical reaction increases (Demming-Adams and Adams, 1994; Smillie and Hetherington, 1990).

Chlorophyll fluorescence emissions produce peaks at 690 nm and 730 nm which are generated by the two photosystems' antenna complexes (Guilbault, 1967; Krause and Weis, 1984).

The typical chlorophyll fluorescence curve is shown in Figure 1.

The Fo represents the nonvariable fluorescence and is not considered to be responsive to physicochemical events in the chloroplasts. The Fv is the variable fluorescence and originates in the chlorophyll of PSII and is responsive to physicochemical events in the chloroplasts. The Fm is the maximum fluorescence value and is responsive to changes in the ETS and chlorophyll (Wilson and Greaves, 1990; Krause and Weis, 1984).
Figure 1: Typical chlorophyll fluorescence induction curve for a plant leaf. Fo represents the initial fluorescence. Fm is the maximum fluorescence and Fv is the variable fluorescence.
The change in Fv and Fm are the usual parameters for measuring the impact of environmental stresses because these values will decline as PSII becomes inhibited. In recent studies, the ratio of Fv to Fm has been used to test chilling tolerance in rice, corn, conifers and other field crops (Bjorkman and Demming, 1987; Verheul et al., 1995; Westin et al., 1995).

Fluorescence will increase as the plant's ability to use the photosynthetically active radiation decreases due to a variety of stresses which results from the decreased capacity of the ETS and quantum yield of photochemistry in PSII (Bjorkman and Demming, 1987).

It has been shown that fluorescence is a valid indicator of in vivo photosynthetic carbon assimilation (Ireland et al., 1984). The ratio of Fm 690 to Fm 730 has been used to indicate the chlorophyll content of spruce needles and other trees leaves and significant correlation has been found between the ratio the Fm of both wavelengths to the chlorophyll content (Lichtenthaler et al., 1990; Hak et al., 1990). This ratio has been proposed as a suitable method to be used in remote sensing (Lichtenthaler, 1988).

Fluorescence has been used to screen for cold tolerance in maize, rice, wheat, potato and other plants (Smillie et al., 1987, Wilson and Greaves, 1990, Sthapit et al., 1995). The susceptibility of plants to herbicide injury has been quantified by the use of chlorophyll fluorescence (Willard et al., 1990; Dodge, 1994).
Changes in chlorophyll fluorescence alone cannot determine the actual processes affected by stress, but they can be an early indicator of plant response to stress (Ireland et al., 1986; Krause and Weis, 1984; Demming-Adams and Adams, 1994).

Antioxidant research has shown that it may be possible to precondition turfgrass photosystems before a stress period in order to enable the plants to maintain normal functioning during a future stress time. Havaux, 1992, demonstrated that exposure to water stress enhanced resistance of photosystem II to subsequent heat and high light stress.

**OBJECTIVES**

The purpose of these experiments was to explore the potential of using superoxide dismutase (SOD) extractions and chlorophyll fluorescence to gather information on the responses of N and biostimulants treatments.
METHODS AND MATERIALS

Experiment 2.1 - Superoxide dismutase extractions of field-grown Kentucky bluegrass leaves as influenced by N and BIOS treatments.

Leaf tissue from the Kentucky bluegrass (*Poa pratensis* var. Plush) field plots used in field Experiment 1.3 (Chapter one) was sampled for superoxide dismutase (SOD) analysis six weeks after the 28 August application of treatments. This one year-old sod was grown on a Groseclose silt loam soil (a clayey, Koalinitic, mixed Typic Hapludult) and received 49 kg ha⁻¹ of N, P₂O₅ and K₂O from a 10-10-10 fertilizer in early April 1995. This was prior to subsequent fertilizer treatment applications. Mowing height of the turf was 4.5 cm. Plots were irrigated as needed to prevent severe wilting.

Two biostimulants (BIOS) were applied to the turf plots which were fertilized with three different N regimes two times during the experiment. Both applications of the treatments (BIOS and N) were applied as a foliar spray with a compressed air boom sprayer that delivered 374 L ha⁻¹ of liquid at a pressure of 276 kPa. The applications were made on 6 June and on 28 August 1995 for a total N application of 0.0, 73.5 and 147.0 kg ha⁻¹ as supplied by 46-0-0 urea. BIOS were applied on the same dates to separate plots of each N regime. The BIOS treatments were a seaweed extract (SE) and a humic acid extract (HE) (Table 2.1.1). Individual treatment plots
were 1.2 by 1.2 meter and arranged in an randomized complete block design with five replications. Three replications were utilized in conducting this experiment.

The plots had been mowed four times at a height of 4.5 cm before the sampling and the leaf samples were clipped using hand shears on 9 October 1995 six weeks after the second application of biostimulants and N. The clippings were placed in plastic bags and liquid N was immediately poured over the leaves. They were then placed in an insulated container. This procedure was done in the field to freeze the samples quickly and therefore stop all metabolic activity. The samples were then stored in a freezer (-20°C) until the extraction process for SOD analysis could be performed.

The original extraction and analytical methods were developed for use on corn, peas and oats (Giannopolitis, and Ries; 1977). These procedures were modified for turfgrass and the modifications have been verified using pure superoxide dismutase from bovine erythrocytes from Sigma Chemical Company.

The extraction solution is a mixture of three parts: (1) 39 ml of a 0.2 M solution of NaH₂PO₄ (FW 141.96 @99%). (2) 61 ml of a 0.2 M solution of Na₂HPO₄ (FW 137.98 @98%) and (3) 100 ml H₂O. This mixture was further diluted to a 0.05 M solution of NaH₂PO₄ / Na₂HPO₄ (pH 7.0).
Table 2.1.1: Amounts of biostimulants per hectare applied to Kentucky bluegrass sod in Experiments 1.1, 1.2, and 1.3.

<table>
<thead>
<tr>
<th>Biostimulants</th>
<th>Amount ha(^{-1}) used by experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. #2.1</td>
</tr>
<tr>
<td>Seaweed extract (grams)</td>
<td>560(^1)</td>
</tr>
<tr>
<td>Humic acid (liters)</td>
<td>7.8(^2)</td>
</tr>
<tr>
<td>Nitrogen (kg N ha(^{-1}))</td>
<td>49.0</td>
</tr>
<tr>
<td></td>
<td>122.5</td>
</tr>
<tr>
<td></td>
<td>196.0</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Low heat extract of *Ascophyllum nodosum* supplied by Caudill Seed Company.

\(^2\) A 2.9% humic acid solution from Actisol™.

Rates of the seaweed and humic acid treatments are rates that have produced superior results in previous experiments.
The turfgrass modifications started with the extraction procedures by adding 0.6 grams of finely chopped frozen leaves to 10 ml of extraction solution in a 60 ml french square bottle placed in an ice water bath. This mixture was homogenized using a Brinkman Polytron PT 3000 at 28,000 rpm for two 45 second intervals. The homogenates were filtered through 4 layers of cheesecloth slightly wetted with 0.5 ml of the extraction solution. The homogenates were kept on ice until they were centrifuged at 4º C for 20 minutes at 15,000 x g. The supernatants were decanted and stored in a refrigerator at 15º C.

Two identical (color and thickness) test tubes for each sample and two extra tubes to serve as calibration blanks were used for the assay. Six reagents were added to each test tube in a set order. The first was 0.3 ml of a 0.5 M sodium carbonate solution (pH 10.2), next was 0.3 ml of 130 mM methionine and then 1.75 ml of deionized distilled water (used to bring total volume of assay solution up to 3.0 ml). An extra 50ul of water was added to the blank test tubes to compensate for the missing supernatant extract volume. The fourth reagent that was added was 0.3 of a 630 uM nitro blue tetrazolium (NBT), followed by 50 ul of the extract into two tubes per sample and finally 0.3 ml of a 13 uM riboflavin. The adding of the reagents was executed under low light conditions because the assay is light sensitive.

One tube from each extract and reagent solution was placed into separate 1000 ml beakers partially filled with 25º C water. One beaker was exposed to light while the other was kept in the dark. The 1000 ml beaker exposed to light had a 200 ml
beaker placed in the center so the test tubes were uniformly distributed around the inside of the larger beaker to form a single test tube circle. To uniformly expose the test tubes to light, the light was generated by a circular florescent lamp (Sylvania, FC 12 T 10-CW-RS) that surrounded the 1000 ml beaker that was installed in a large insulated container.

The light reaction was conducted by switching the light on for ten minutes. The reaction was measured by the increase of light absorbance of the extraction at 560 nm using a spectrophotometer in the following manner. After the spectrophotometer was set to zero, a reading from the blank not exposed to the light (reagents only - no extract) was taken and the spectrophotometer was adjusted to 100% using this reading. Then a reading from the other blank exposed to light was recorded the reading as decimal (ie 79% is 0.79). Readings from the light exposed and the dark exposed solutions for each sample (A) were obtained. The negative log for each observation was calculated (-LOG A) and the following formula was used:

V1 = the blank exposed to light, V2 = the blank - no light, v1 = extract exposed to light and v2 = the extract - no light. The 1 refers to one SOD unit defined, as the amount of the SOD present that is required to inhibit 50% of the NBT reaction. The (16.67 X 60) equals the dilution factor which is based on the 0.6 g leaves to 10 ml extraction solution (16.67) and the 0.05 ml supernatant to 3.0 ml total volume (60).
The following example illustrates the formula:

\[
\text{Transmission} = (A) \quad \text{Absorption} = (-\log A)
\]

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>0.795</td>
<td>0.0996</td>
</tr>
<tr>
<td>V2</td>
<td>1.000</td>
<td>0.0000</td>
</tr>
<tr>
<td>v1</td>
<td>0.660</td>
<td>0.1805</td>
</tr>
<tr>
<td>v2</td>
<td>0.705</td>
<td>0.1518</td>
</tr>
</tbody>
</table>

SOD activity =
\[\{[(0.0996-0.0000)/(0.1805-0.1518)] - 1\} \times (16.76 \times 60) =\]

2470.38 SOD units

The data were subjected to an ANOVA and the means separated using Tukey’s Studentized Range at a p value of 0.05. The analyses were computed using SAS Institute’s procedures as outlined in SAS/STAT User’s Guide version 6 and run on a personal computer using SAS version 6.11.

The SOD methodology use in this thesis is based on fresh weight leaf samples and ample soil moisture. Dry weight and low moisture would tend to concentrate the SOD and produce different results.

Experiment 2.2 - SOD content and chlorophyll fluorescence of Kentucky bluegrass leaves as influenced by only N treatments.

On 13 December, 1995, 10.2 cm diameter sod plugs were taken from a 14-month old Kentucky bluegrass 'Plush' sod at the Virginia Tech Turfgrass Research
Center. The plugs were trimmed to a uniform soil thickness of 3.8 cm and the soil
was washed from the plugs. The plugs then were placed in 750 ml plastic containers
filled with a 75/25 (v/v) mix of course sand and a Groseclose silt loam soil (a
clayey, Koalinitic, mixed Typic Hapludult). A soil test revealed a pH of 6.1 and 29
ppm P₂O₅, 58 ppm K₂O, 390 ppm Ca and 65 ppm Mg. The containers with the turf
plugs were placed in a greenhouse and watered to saturation for three days and then
allowed the drain for three days before the N treatments were applied. The total N
applied to the containers during the experimental period was 0.0, 24.5, 49.0, 73.5 or
98.0 kg N ha⁻¹ as supplied by urea. The N was applied daily (18 Dec. to 21 Dec.) to
supply 24.5 kg N ha⁻¹ as a 25 ml solution until the desired rate was obtained. The
containers that did not receive any N on a given day received 25 ml of distilled water.
There was no leaching of water from the containers. Twelve hours per day of
supplemental lighting was supplied by a 1000 watt Sylvania Metal Arc lamp supplying
approximately 625 um m⁻²s⁻¹ as measured by a quantum sensor, model Li-185 made
by Li-Cor Inc. Full summer sunlight reading on 9 June 1996 was 2700 um m⁻²s⁻¹,
using the same meter. The experiment was a completely random design with four
replications. The sod was maintained at a height of 5.1 cm.

Forty days after treatment the turf was clipped to a height of 3.8 cm and the
leaves were weighed and frozen in liquid N for SOD analysis. Superoxide extraction
procedures were those described to experiment 2.1. Roots were washed free of most
soil and ashed in order to get a true weight of the roots. This procedure was done
forty-eight days after treatment.

The chlorophyll fluorescence induction readings were measured in the 690 and 730 nm regions with a portable two-wavelength continuous excitation chlorophyll fluorometer (Opti-Sciences model OS-50). The excitation source is a solid state 635 nm source with an intensity of 400 uE and a time of 6 seconds. The dark acclimation period was 45 seconds.

Measurements of chlorophyll fluorescence were taken on 27 January. Modifications to the manufactures’ suggested methods allowed the fluorometer to gather readings from an area rather than an individual leaf. These modifications included placing the light sampling probe inside a small black container which was open at one end. This setup allowed the dark acclimation of a larger area and prevented any light from entering the sample area. The sample area was further narrowed by placing a black rubber hose over the end of the probe and allowing a 32 mm gap between the end of the probe and the end of the hose. These modifications were necessitated by the narrow width of individual turfgrass blades. The resulting data collected were, therefore, an average of the blades in the sampled area. Readings were taken twice on each turfgrass ring and each treatment was replicated four times.

The Fm 690 or maximum chlorophyll fluorescence at 690 nm was used as an indicator of the relative status of the electron transport system (ETS) and photosystem II (PSII) and, therefore, the level of stress of the turfgrass. The higher the value the more inhibited the ETS and the more stress on the photosystem.
The ratio of maximum fluorescence of 730 nm to 690 nm was used as an indication the chlorophyll content of the sample area. This is based on work by Hak et al., 1990 and Lichtenhaler et al., (1990).

Experiment 2.3 - Chlorophyll fluorescence and SOD levels of bentgrass leaves as influenced by only N treatments.

Four-year-old creeping bentgrass turf (*Agrostis palustris* var. 'Penncross') growing on a clayey, Koalinitic, mixed Typic Hapludult soil with a pH of 5.9 was used for this experiment. The 3.4 m² field plots were mowed three times per week at a height of 62.5 mm and irrigated to prevent drought. The turf was treated 3 times with two levels of N (urea) to supply 162.1 and 74.4 kg N/ha (yearly totals)(Table 2.3.1). N applications treatments were applied with a compressed CO₂ boom sprayer that delivered 374 L ha⁻¹ of liquid at a pressure of 276 kPa. The initial N application was made on 20 May and subsequent applications were made on 20 June and 17 July 1996. On 20 May and 17 July, granular applications of P₂O₅ and K₂O at rates of 6.5 kg/ha and 13.0 kg/ha, respectively, were made. The leaf samples and fluorescence readings were collected on 30 July. The leaf samples were cut using a right angle grinder that had a modified housing and blade which allowed the mowing and collection of turfgrass blades on microplots. An area of visually uniform turfgrass approximately 1 meter square was sampled from each plot. The clippings were placed
in plastic bags and liquid N was immediately poured over the leaves. They were then placed in an insulated container. This procedure was done in the field to freeze the samples quickly and therefore stopping all metabolic activity. The samples were then stored in a freezer (-20° C) until the extraction process could be performed.

Superoxide dismutase extraction and quantification procedures used are identical to Methods and Materials described in Experiment 2.1.
RESULTS AND DISCUSSION

Experiment 2.1 - Superoxide dismutase extractions of field-grown Kentucky bluegrass leaves as influenced by N and BIOS treatments.

The amount of SOD in the leaves significantly increased with each additional level of N (Table 2.1.2). The mean SOD of both biostimulant-treated turf were significantly greater than the mean of the control turf; therefore, the combination of high N and BIOS produced the largest SOD concentrations.

Top growth as measured by clipping weights increased with each level of N (Table 2.1.3). The BIOS-treated turf produced more clippings than the N only when averaged over all N treatments. This increased top growth of Table 2.1.3 corresponded with the increased SOD in Table 2.1.2.

Turf in this experiment apparently experienced little to no stress. This conclusion is based on the observations of increasing root mass (Table 1.3.1, in Chapter One) and the simultaneous top growth expansion of the Kentucky bluegrass, yet the endogenous levels of SOD were significantly increased with exogenous applications of BIOS. The BIOS increase was almost twice as much as by N fertility. This indicates that Kentucky bluegrass can be stimulated to develop SOD during a pre-stress period.
Research with transgenic plants has shown that genetically enhanced production of SOD can provide increased stress tolerance. In transgenic tobacco plants, the enhanced plants demonstrated superior tolerance to oxidative stress (Gupta et al., 1993, Bowler et al., 1991). Transgenic alfalfa with enhanced SOD activity had increased tolerance to herbicide injury and increased regrowth after freezing stress (McKersie et al., 1993).

A stimulated increase in SOD may help condition the plants to tolerate subsequent stresses, that produce an increase in oxygen radicals (Massacci et al., 1995; Madamanchi and Alscher, 1991; Gupta et al., 1993).
Table 2.1.2: Superoxide dismutase found in one year old Kentucky bluegrass leaves 6 weeks after the last treatment and fertilized with urea, to supply 0.0, 36.8 and 73.5 kg nitrogen per hectare during each application, and BIOS on 6 June and 28 August.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Means by Treatment (SOD Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nitrogen fertilization</strong></td>
<td></td>
</tr>
<tr>
<td>0.0 kg N/ha</td>
<td>2.03 C</td>
</tr>
<tr>
<td>73.5 kg N/ha</td>
<td>2.50 B</td>
</tr>
<tr>
<td>147.0 kg N/ha</td>
<td>3.48 A</td>
</tr>
<tr>
<td><strong>Biostimulants</strong></td>
<td></td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>2.07 B</td>
</tr>
<tr>
<td>Seaweed</td>
<td>2.79 A</td>
</tr>
<tr>
<td>Humic acid</td>
<td>2.84 A</td>
</tr>
</tbody>
</table>

Values followed by the same letter, in the same column, do not significantly differ at the 5% level of probability using Tukey's Studentized Range (HSD).

Means are the average of 3 replications using a randomized block design.

Probability values for the main effects:

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr &lt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLEVEL</td>
<td>0.0001</td>
</tr>
<tr>
<td>BIOS</td>
<td>0.0008</td>
</tr>
<tr>
<td>N-LEVEL * BIO</td>
<td>0.0769</td>
</tr>
</tbody>
</table>
Table 2.1.3: Fresh clipping weight generated by one year old Kentucky bluegrass that had been fertilized with urea and BIOS on 6 June and 28 August to supply 0.0, 36.8 and 73.5 kg N ha\(^{-1}\) during each application. The total nitrogen applied on the plots to 0.0, 73.5 and 147.0 kg N ha\(^{-1}\).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Means by Treatment (Clipping yield (grams))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nitrogen fertilization</strong></td>
<td></td>
</tr>
<tr>
<td>0.0 kg N/ha</td>
<td>20.3 C</td>
</tr>
<tr>
<td>73.5 kg N/ha</td>
<td>47.5 B</td>
</tr>
<tr>
<td>147.0 kg N/ha</td>
<td>66.5 A</td>
</tr>
<tr>
<td><strong>Biostimulants</strong></td>
<td></td>
</tr>
<tr>
<td>Nitrogen Only</td>
<td>37.5 B</td>
</tr>
<tr>
<td>Seaweed</td>
<td>45.0 A</td>
</tr>
<tr>
<td>Humic acid</td>
<td>43.7 A</td>
</tr>
</tbody>
</table>

Values followed by the same letter, in the same column, do not significantly differ at the 5% level of probability using Tukey’s Studentized Range (HSD).

Means are the average of 3 replications using a randomized block design.

Probability values for the main effects:

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Pr (&lt; F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLEVEL</td>
<td>0.0001</td>
</tr>
<tr>
<td>BIOS</td>
<td>0.0015</td>
</tr>
<tr>
<td>NLEVEL*BIO</td>
<td>0.0856</td>
</tr>
</tbody>
</table>
Experiment 2.2 - SOD content and chlorophyll fluorescence of Kentucky bluegrass leaves as influenced by only N treatments.

Superoxide dismutase

The higher levels of N significantly increased the SOD content of the Kentucky bluegrass leaves (Table 2.2.1). This increase in SOD with increased N fertility was also shown in Experiment 2.1. The rise in SOD levels may be stimulated by the additional fertility.

Leaf weights became greater as N was added. Additional N fertility increased the chlorophyll content, increased the canopy height and increased SOD content of the turfgrass leaves.

However, the higher N inhibited root development in this experiment. The combination of seasonal reduction in photosynthetically active radiation (PAR) and seasonal partitioning of carbohydrates (Hull, 1992) may have contributed to the rooting decline in this experiment. Possibly, additional BIOS applications, as in Experiment 2.1, would boost the SOD levels and therefore increase rooting. It is possible that higher SOD content may have been required to increase carbohydrate partitioning to the roots. White and Schmidt, 1989, indicated that applications of a biostimulant increased carbohydrate partitioning in bermudagrass.
Although Experiment 2.2 corroborated Experiment 2.1's results that showed SOD content increases with increased N fertility, it is possible that sufficient SOD was not generated with N to stimulate significant root development in this experiment.

**Chlorophyll fluorescence**

Under the experimental conditions, as the N levels increased, the chlorophyll fluorescence (FM) decreased (Table 2.2.2). This would indicate that the turfgrass's electron transport system was not inhibited by the increased level of N.

The chlorophyll content, as measured by ratio chlorophyll fluorescence measurement at 690 and 720 nm, increased as the N fertility increased (Lichtenthaler, 1990; Hak et al., 1990). Rooting did not increase as the N levels increased but top growth did significantly increase (Table 2.2.1). The partitioning of the photosynthates to the leaves instead of the roots may be responsible for the increased chlorophyll content and increasing chlorophyll efficiency (FM 690).
Table 2.2.1: Rooting weight, leaf weights and SOD samples of 10.2 cm plugs (diameter.) of Kentucky bluegrass following a single application of several rates of Nitrogen on 19 Dec. 1995. The turf received (in addition to ambient sunlight) 12 hours of artificial light from a 1000 W metalarc lamp in a temperature controlled greenhouse. Clipping weights and SOD samples were taken on 28 Jan. 1996. Root weights (ashed) were taken on 5 Feb. 1996.

<table>
<thead>
<tr>
<th>Kg N ha⁻¹</th>
<th>Leaf (grams)</th>
<th>Root (grams)</th>
<th>SOD (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>98.0</td>
<td>3.8 A</td>
<td>1.07 B</td>
<td>3.01 A</td>
</tr>
<tr>
<td>73.5</td>
<td>3.8 A</td>
<td>1.08 B</td>
<td>2.70 A</td>
</tr>
<tr>
<td>49.0</td>
<td>2.5 B</td>
<td>1.15 AB</td>
<td>2.06 B</td>
</tr>
<tr>
<td>24.5</td>
<td>2.0 BC</td>
<td>1.19 A</td>
<td>2.05 B</td>
</tr>
<tr>
<td>0.0</td>
<td>1.6 C</td>
<td>1.13 AB</td>
<td>2.09 B</td>
</tr>
</tbody>
</table>

Values followed by the same letter, in the same column, do not significantly differ at the 5% level of probability using Duncan’s Multiple Range Test.

Means are the average of 4 replications in a completely randomized design.

Probability values for each main effect:

ROOT WEIGHT  NITROGEN LEVEL = 0.0371
LEAF WEIGHT   NITROGEN LEVEL = 0.0003
SOD UNITS     NITROGEN LEVEL = 0.0031
Table 2.2.2: Chlorophyll fluorescence of Kentucky bluegrass 10 weeks after treatment, following a single application of several rates of Nitrogen on 19 Dec. 1995. The turf received (in addition to ambient sunlight) 12 hours of artificial light from a 1000 W metalarc lamp in a temperature controlled greenhouse.

<table>
<thead>
<tr>
<th>Kg N ha⁻¹</th>
<th>FM 690²</th>
<th>FM730/FM 690</th>
<th>SOD (units)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>98.0</td>
<td>533 C</td>
<td>1.66 A</td>
<td>3.01 A</td>
</tr>
<tr>
<td>73.5</td>
<td>547 C</td>
<td>1.61 AB</td>
<td>2.70 A</td>
</tr>
<tr>
<td>49.0</td>
<td>556 BC</td>
<td>1.60 AB</td>
<td>2.06 B</td>
</tr>
<tr>
<td>24.5</td>
<td>580 AB</td>
<td>1.52 BC</td>
<td>2.05 B</td>
</tr>
<tr>
<td>0.0</td>
<td>590 A</td>
<td>1.43 C</td>
<td>2.09 B</td>
</tr>
</tbody>
</table>

Values followed by the same letter, in the same column, do not significantly differ at the 5% level of probability using Duncan's Multiple Range Test.

Means are the average of 4 replications in a completely randomized design.

Probability values for each main effect:
- FM 690: NITROGEN LEVEL = 0.0002
- FM 730/Fm690: NITROGEN LEVEL = 0.0019
- SOD UNITS: NITROGEN LEVEL = 0.0031

²The lower the value for Fm, the lower the inhibition of the photochemical system.

³SOD data taken from table 2.2.1.
**Experiment 2.3** - Chlorophyll fluorescence and SOD levels of bentgrass leaves as influenced by only N treatments, grown on a simulated putting green.

The SOD content of the bentgrass leaves increased with additional N fertility (Table 2.3.1). The SOD content of the turf’s leaves rose 22% as the N fertility increased. This increase in SOD content with increased N fertility was also demonstrated in Experiments 2.1 and 2.2 (Tables 2.1.2 and 2.2.1).

Moyafar (1994) reviewed research that indicates that antioxidants will tend to increase with rates of N up to a maximum then decrease as additional N is applied. Schmidt and Zhang (unpublished data) have shown that, in turfgrass, as SOD levels increase the levels of other antioxidants also increase. These experiments apparently did not have sufficient N levels or the combination of environmental stress and N to produce a reduction in SOD levels. This would indicate that the experiments presented did not include any turfgrass that was under a serious stress.

An increase in leaf SOD does not necessarily indicate the health of a plant. In fact, it could show that the plant is under a stress (Massacci et al., 1995). Superoxide dismutase increases were used to indicated an increase in hydrogen peroxide activity (Franck et al., 1995).

If the plant SOD / antioxidant content can be increased beyond what is required by a stress factor by pre-conditioning the plant with BIOS (biostimulants) or N, then that plant would be better able to tolerant the stressful period and maintain
normal growth. Much work remains to answer questions about interactions of antioxidants, hormones and mineral nutrition on stress resistance/tolerance.

**Chlorophyll fluorescence**

The bentgrass treated with the higher N showed reduced Fm 690 readings which are related to a reduced levels of stress on the photochemical processes (Smillie et al., 1987; Ireland et al., 1984, Wilson and Greaves, 1990) (Table 2.3.1). Even though the data were collected in the middle of the summer (30 July), the turfgrass had not been through the "normal" summer heat stress. The summer of 1996 was noted for exceptionally mild temperatures with the day time high temperatures only reaching the lower 90's a few times. Heat stress is known to reduce shoot growth, root size and stand density. These problems were not evident on the bentgrass plots. The lower Fm readings indicate that, under the experimental conditions, the level of stress of the photochemical reactions was reduced with the additional N.

The chlorophyll content index (Fm730/Fm690) of the bentgrass treated with higher N did increase slightly (14%) above the low N control but it was not statistically significant.

The Kentucky bluegrass experiment in the green house (Experiment 2.2) produced similar results with reduced Fm 690, increased chlorophyll index and
greater SOD content as N fertility increased.
Table 2.3.1: Chlorophyll fluorescence and SOD readings of Penncross creeping bentgrass following two rates of nitrogen. Samples and readings were taken on 30 July.

<table>
<thead>
<tr>
<th>N level (kg N/ha)</th>
<th>Yearly totals</th>
<th>FM 690$^4$</th>
<th>FM730/FM 690</th>
<th>SOD (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>162.1</td>
<td>116 B</td>
<td>0.82 A</td>
<td>2.75 A</td>
<td></td>
</tr>
<tr>
<td>74.8</td>
<td>180 A</td>
<td>0.72 A</td>
<td>1.94 B</td>
<td></td>
</tr>
</tbody>
</table>

Values followed by the same letter, in the same column, do not significantly differ at the 5% level of probability using Duncan's Multiple Range Test.

Means are the average of 4 replications in a split plot design

Probability values for the main effect ( Treatments):

<table>
<thead>
<tr>
<th></th>
<th>FM 690</th>
<th>FM730/FM 690</th>
<th>SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N level</td>
<td>0.0305</td>
<td>NS</td>
<td>0.0059</td>
</tr>
</tbody>
</table>

$^4$The lower the number, the lower the inhibition of the photochemical process.
SUMMARY OF RESULTS FROM CHAPTER TWO

The SOD content of the turfgrass leaves increased in all three of Chapter Two's experiments as N fertility increased. The addition of BIOS also increased the SOD content in the one experiment that included BIOS (2.1). Leaf weights also became greater as N was added in Experiments 2.1 and 2.2. In these experiments, additional N fertility increased the chlorophyll content, increased the canopy height and increased SOD content of the turfgrass leaves.

Experiments 2.2 and 2.3 both demonstrated that, under these experimental conditions, additional N reduced the maximum fluorescence (FM) and increased the chlorophyll content index (FM730/FM690). This is further evidence that the turfgrass was not placed under stress by the additional N fertility and the increase in SOD was the result of the N and BIOS applications.

CONCLUSIONS

Many different factors combine to produce resistance or tolerance to stress in plants. The experiments in Chapter Two have focused on SOD and chlorophyll fluorescence as an indicator of increased stress tolerance as influenced by N fertility and biostimulants. The experiments demonstrated a general increase in SOD content
in turfgrass leaves with N or BIOS treatments. The chlorophyll instrument used showed a decrease in the FM and an increase in chlorophyll content as N increased, under the experimental conditions.

The methodology used in this chapter is exploratory in nature, however, it was able to produce significant differences between different treatments. These results prove the potential value of SOD extractions and chlorophyll fluorescence in turfgrass research.

More research is needed to understand the cyclic nature of antioxidants and hormones in plants and to discover when is the best time to exogenously apply materials to increase to endogenous concentrations in order to increase tolerance to environmental stresses and postpone senescence.
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VITA

Samuel O. Doak

The author is the son of Emily O. and Samuel L. Doak (Capt. USN ret.) and was born in Portsmouth Naval Hospital Portsmouth, Va with a twin brother, Ralph. The early years were spent in typical military family style moving every 18 months or so. I graduated from Ithaca High school, Ithaca, NY and attended Illinois Institute of Tech. in Chicago but received my BS in business from Old Dominion University (1979). Work before attending VPI included production planning, supervising and operating a small construction firm. Janet, my patient better half, our son Sammy and I moved to Christiansburg VA in 1993 to start my masters in CSES (agronomy). Our daughter Arcadia 'Katie' was born in 1994 while I attended VPI. The author is employed by Plant-Wise Biostimulant Company in Louisville, KY.

[Signature]

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