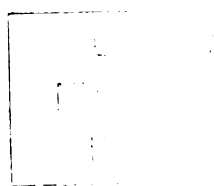

ABSTRACT

THE INFLUENCE OF TEMPERATURE AND OTHER ENVIRONMENTAL FACTORS ON NITRATE REDUCTASE ACTIVITY OF *AGROSTIS PALUSTRIS* HUDS. AND *CYNODON DACTYLON* L.

By

John E. Kaufmann

The influence of temperature on nitrate reductase activity was studied to determine if nitrate reductase is associated with shoot growth inhibition at super- and sub-optimal temperatures. The investigations were conducted in controlled environment chambers with temperature variables of 10°, 15°, 20°, 25°, 30°, 35°, and 40°C. Toronto bentgrass and Tifgreen bermudagrass, the cool season and warm season grass respectively, were chosen because of these distinctly different temperature optimums. The bermudagrass was omitted from the 10°C temperature treatment, and the bentgrass was omitted from the 40°C treatment because of extremely adverse conditions for shoot growth.

The results indicated that nitrate reductase activity did not decrease significantly under temperature stress conditions until observed shoot growth inhibition

occurred at 35°C and up for bentgrass and 15°C and below for bermudagrass. At high temperatures (35°C to 40°C) samples of bentgrass exhibited an inhibition of growth and nitrate reductase activity while bermudagrass was relatively unaffected. This reduction of activity could be either an inhibition of enzyme synthesis or inhibition of the activity *per se*. It was found that the activity *per se* of the bentgrass enzyme preparation was also inhibited by these same temperatures, suggesting that nitrate reductase inhibition may be a factor causing high temperature growth inhibition in this species.

The investigation also indicated that when bentgrass is grown at 15°C and bermudagrass at 30°C, the nitrate reductase activity was higher in the leaf blade tissue than in stem tissue, was higher following application of a nitrate nutrient solution, increased with increasing light intensity, and increased with length of light exposure.

THE INFLUENCE OF TEMPERATURE AND OTHER ENVIRONMENTAL FACTORS ON NITRATE REDUCTASE ACTIVITY OF *AGROSTIS PALUSTRIS* HUDS. AND *CYNODON DACTYLON* L.

By

John E. Kaufmann

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TABLE OF CONTENTS

	Page
LIST OF TABLES	iv
LIST OF FIGURES	v
INTRODUCTION	1
LITERATURE REVIEW	3
Source and Assimilation of Nitrogen	3
Pathway of Nitrate Reduction	4
Effects of Inhibitors and Stimulators	6
Properties and Functions of the Enzyme and Cofactors	7
Effect of Hereditary Factors	9
Physiological Responses of Turfgrasses to Temperature	9
MATERIALS AND METHODS	14
Establishment Procedures	14
Growth Conditions for Temperature Studies	16
Preparation of Nitrate Reductase	17
Assay Procedure	18
RESULTS AND DISCUSSION	21
Effect of Tissue Sampling on Nitrate Reductase	21
Effect of Nutrients on Nitrate Reductase	23
Effect of Light on Nitrate Reductase	24
Effect of Temperature on Nitrate Reduction	26
Effect of Temperature on Enzyme Activity	29
CONCLUSIONS	33
BIBLIOGRAPHY	35
APPENDIX	41

LIST OF TABLES

Table		Page
1.	The effect of tissue sampling on nitrate reductase activity	22
2.	The effect of nutrient application on nitrate reductase activity	23
3.	The effect of light intensity on nitrate reductase activity	25
4.	The effect of length of light exposure on nitrate reductase activity	25

LIST OF FIGURES

Figure Page

1. The effect of temperature on the induction of nitrate reductase of creeping bentgrass and bermudagrass 27

2. The effect of temperature on the activity of nitrate reductase of creeping bentgrass and bermudagrass 30

INTRODUCTION

Toronto creeping bentgrass, Agrostis palustris Huds., is one of the most widely used, vegetatively established creeping bentgrasses on greens in the northern cool-humid climatic regions of the United States. It is commonly subject to growth inhibition at high temperatures, especially under the cultural conditions practiced on greens.

Tifgreen bermudagrass, Cynodon dactylon L., is the counterpart of bentgrass for greens in the warm-humid climatic regions. Bermudagrass exhibits growth inhibition more commonly at suboptimal temperatures.

The temperatures at which growth inhibition occurs are common in the regions where each grass is cultivated. Under prolonged periods of temperature stress high temperature results in bentgrass kill, and low temperature in bermudagrass dormancy.

Nitrate reductase is an important enzyme in the pathway which provides nitrogen to the plant in a form which is readily available for the synthesis of essential nitrogen compounds such as amino acids. It is considered by Hageman (20, 21) and others to be a limiting factor in plant growth.

The objectives of this investigation were to determine the effect of certain major environmental factors on nitrate reductase, and to study whether the influence of temperature on nitrate reductase activity of these species is related to growth inhibition under temperature stress.

LITERATURE REVIEW

In some of the early studies concerning the occurrence of nitrate (NO_3) in plants Berthelot (as reported by Anderson (2)) found that the NO_3 content may vary greatly during the life cycle of the plant. Also, NO_3 levels were localized in various parts of the plant, and were dependent on the availability of NO_3 in the soil. Anderson further reports the studies of (a) Kastle and Elove and (b) Haas and Hill, as indicating a mechanism for nitrate reduction in potato sap and milk respectively. Conclusions from this work indicate the probability that an oxidizable substance is involved rather than an enzyme system.

Source and Assimilation of Nitrogen

NO_3 assimilation has been studied through measurement of the loss of NO_3 from nutrient solutions. The assimilation of NO_3 was absent in darkness and increased with increasing light intensity (8). NO_3 assimilation paralleled CO_2 assimilation and at the same time utilized no carbohydrates. Ammonium (NH_4) assimilation required carbohydrates at all times and was assimilated both in the light and dark. However, addition of glucose stimulated nitrate assimilation in Chlorella vulgaris (54).

Through the use of nitrogen (^{15}N), assimilation rates of NO_3 and NH_4 were measured. Light and dark did not influence on the total amount of ^{15}N assimilated (12). Tomato plants fed $^{15}NH_4$ had a high concentration of ^{15}N in the roots while plants fed $^{15}NO_3$ accumulated ^{15}N in the leaves (31). Studies with Chlorella vulgaris show that NH_4 is assimilated four times as fast as NO_3 in nitrogen-starved cells (55), and that NO_3 assimilation is inhibited during NH_4 assimilation (56). Morton (34) reports that NH_4 inhibits the reduction of nitrate in fungi because of direct ammonia (NH_3) assimilation.

Fertilization with NO_3 has been shown superior to NH_4 in supporting better shoot growth and rhizome development in Poa pratensis L. (23), and has been shown to be effective over a wider range of soil pH and temperature (11). Recent work on Lolium multiflorum Lam. indicated that at cool temperatures high rates of NO_3 were more effective in promoting growth than high rates of NH_4. At high temperatures, NO_3 was again more effective than NH_4 at both high and low levels of nutrition (51).

Pathway of Nitrate Reduction

Nitrate assimilation involves a pathway of nitrate reduction to provide the plant with the nitrogen form necessary for protein synthesis. Many pathways from NO_3 to NH_3 have been proposed and reviewed (9, 27, 31, 35, 59,

60). However, Burstrom (9) lists the possible intermediates of nitrate reduction:

$H\ NO_3$	Nitric acid
$H\ NO_2$	Nitrous acid
$(H\ NO)_2$	Hyponitrous acid
$H\ NO\ H_2$	Hydroxylamine
$H\ N\ H_2$	Ammonia

Of these five intermediates, hyponitrous acid has never been shown to exist in the plant. The presence of nitric acid, nitrous acid, and ammonia in the plant is easily detected. Hydroxylamine has been shown to exist in the leaves of higher plants following the addition of NO_3.

According to Webster (60), Zucker and Nason have actually isolated a hydroxylamine reductase enzyme in neurospora. Betts and Hewitt (6) indicate the chloroplasts of higher plants are the sites for photochemical reduction of nitrite and hydroxylamine. They suggest that both compounds are reduced by the same or closely related enzymes, indicating that hydroxylamine is not a true intermediate in the pathway.

This pathway of nitrate reduction is a series of endothermic reactions (8, 9). All steps which have been shown to occur require a reductase enzyme and a series of electron donors referred to as reaction cofactors. The following discussion of the enzyme and cofactors will be limited to the first step of reduction, namely NO_3 to NO_2.

Effects of Inhibitors and Stimulators

In Chlorella vulgaris NO_3 assimilation appears to be inhibited during NH_4 assimilation, perhaps due to the inhibition of the nitrate reductase enzyme (52). Further study indicates that carbamyl phosphate when in alkaline solution, produces a cyanate which inhibits nitrate reduction. Carbamyl phosphate may be the primary product of NH_4 assimilation (33).

The presence of a metal constituent in the nitrate reductase system is shown by the effect of cyanate, a known metal binding inhibitor (16, 47). The presence of sulfhydryl groups on the enzyme is substantiated by the inhibitory effect of p-chloromercuribenzoate. The inhibition is easily reversed through the addition of a known sulfhydryl-group containing compound such as cysteine (16, 47).

Carbon monoxide (CO) inhibits nitrate reductase in the dark only, and is reversed by light. This suggests that a specific cytochrome of photosynthesis is utilized in the electron transport system of nitrate reductase. Benzylviologen also reverses the inhibitory effect of CO apparently through by-passing the cytochrome (47). A more recent investigation of a purified nitrate reductase preparation shows benzylviologen as a capable substitute for the natural flavin cofactors (41).

Studies of the effect of 2,4-D on nitrate reductase activity shows an increase in Zea mays L. and a decrease in cucumbers (4). It was speculated that 2,4-D causes an intramolecular change in the protein sulfhydryl groups.

Simazine has been shown to stimulate the assimilation of NO_3, however, the study was conducted at sub-optimal conditions of low NO_3 and low temperature levels (57).

Most of the amino acids present at specific concentrations not uncommon to the cell, have an effect on nitrate reductase activity (18). Of two categories described, repressors and derepressors, methionine and alanine are repressors while arginine and lysine are derepressors.

Properties and Functions of the Enzyme and Cofactors

The nitrate reductase enzyme is thought to be a sulfhydryl-metallo-flavoprotein (16, 17, 35, 47). The sulfhydryl group is considered to be the site on the enzyme which binds the unreduced cofactor or electron donor. Many electron donors are shown to be involved in nitrate reduction. The most widely recognized being the flavin requirement of either FMN or FAD and either NAD, or NADP, or both. In some studies the enzyme system was specific for NAD (14, 46, 50), while another was NADP specific (28).

The metal constituents may vary with plant species. Molybdenum, a minor element, has been shown to be a cofactor for nitrate reduction (37). It has been found to serve as an electron donor in bacteria (17), fungi (35, 39, 40), and in higher plants (24, 27) with specific studies in tomatoes (58) and soybeans (15, 38).

The role of phosphate in nitrate reduction is more indirect than molybdenum. Nicholas and Nason (38) indicate that nitrate reductase in soybeans has no requirement for inorganic phosphate, while Spencer (50) shows a need for phosphate in a nitrate reductase system of germinating wheat embryos. Further study suggests that phosphate either binds molybdenum into a phosphomolybdenum complex (28), or serves in binding molybdenum to an apoenzyme of nitrate reductase (27).

The role of another metal is considered part of the system through the fact that light influences nitrate reduction. The electron transport system of photosynthesis requires iron and in this sense has been reported as a requirement for nitrate reductase (17). Another investigation, reviewed by Nason and Takahashi (36) found that the copper ion may substitute for iron. Extensive work has been done to formulate the series of cofactors responsible for providing electrons for the nitrate reduction system. Extreme variation between species of proposed pathways makes it impossible to record a general form. Most pathways

include (a) grana of the chloroplast (29, 16) or chlorophyll (45), (b) one of the many cytochromes (17, 28, 36, 37, 47), (c) the flavins, (d) NAD or NADP, and (e) the heavy metals previously discussed.

Effect of Hereditary Factors

Nitrate reductase activity is known to vary among the genetic lines in corn (Zea mays L.) (61). Hybrid crosses from an inbred line of high nitrate reductase activity and an inbred line of low activity proved to exhibit enzyme activity intermediate between the two parental inbreds (22, 48). With these hereditary factors in mind, a hybrid could be developed with a nitrate reduction system which would compliment the other improved factors of the hybrid.

Physiological Responses of Turfgrasses to Temperature

Cool and warm season grasses exhibit different growth responses when placed under various temperature regimes. Early observations on colonial bentgrass (Agrostis tenuis Sibth.) indicate that at a temperature of 90°F (32.2°C) as compared to 60°F (15.5°C) the seeds germinated more rapidly, the plants grew more rapidly but lacked vigor, and 4 weeks later chlorosis occurred under the 90°F (32.3°C) treatment. At 80°F (26.6°C) the topgrowth tended toward a

horizontal position, and the roots were white, fleshy, and unbranched (52).

Kentucky bluegrass is described as producing succulent, bushy shoot-growth at 15°C while the shoot growth at 35°C is less succulent, shorter, and the shoot density is severely thinned (11). After a 40 day exposure to day-night temperatures of 90-80°F (32.2-26.6°C) respectively, the shoot growth of perennial ryegrass (Lolium perenne L.) was severely stunted, the leaves were spindly and dark green in color, and the roots were fibrous and discolored (53).

Early work by Brown (7) showed no reduction in shoot growth as temperatures were increased from 80°F (26.6°C) to 100°F (37.7°C), whereas a reduction was observed in cool season grasses. The curve of temperature and leaf matter production of bermudagrass indicates that growth steadily increases from 40°F (4.4°C) to 100°F (37.7°C). Maximum shoot growth occurred at 100°F (37.7°C). A growth curve for perennial ryegrass indicates that growth peaked at 65°F (12.7°C) with a slight reduction in growth at 45°F (7.2°C) and total inhibition of growth at 95°F (35°C) (32). In a recent and comprehensive study of temperature effects on Toronto creeping bentgrass, Duff (13) provides a growth curve reported as dry matter production per week. The temperatures were day-night regimes of 20-10°, 25-15°, 30-20°, 35-25°, and 40-30°C. A gradual decrease in shoot growth was noted from 20-10°C to 30-20°C. A sharp reduction

occurred between 30-20°C and 35-25°C, and at 40-30°C growth cessation occurred. Duff states that increasing temperature increased the percentage dry weight of the tissue produced, and decreased the leaf width.

Another factor is interaction of nutrition and temperature levels. Harrison (23) concludes that the high levels of nitrogen are undesirable at high temperatures. Pellett and Roberts (42) state that turf grown on low levels of nitrogen is more heat tolerant than turf grown on high nitrogen levels. They propose an apparent upset of growth-differentiation balance where at high nitrogen levels, the carbohydrates are utilized for growth and are not available for differentiation.

In the case of the cool season grasses such as Toronto creeping bentgrass, temperatures of 80°F tend to exert an adverse effect on carbohydrate reserves (19). Sullivan and Sprague (53) state that high temperatures cause a rapid dissipation of reserve carbohydrates and a possible toxic formation of nitrogen compounds. Hewitt and Curtis (25) contend that high temperatures increase respirational and translocational losses of carbohydrates. At extremely high temperatures respiration of transportable materials occurs.

Recent work, however, refutes the theory that carbohydrate loss causes growth stoppage at high temperatures. While the loss of carbohydrate reserves from the root system

may take place, Duff (13) clearly states that leaf tissue of Toronto creeping bentgrass actually exhibits an approximate 50% increase of both water soluble and 85% ethanol soluble carbohydrates when grown at super-optimal temperatures. Photosynthesis and respiration both increased as temperatures were increased. The chlorophyll content dropped severely at these same temperatures although the leaves were visibly darker green.

In further study of high temperature stress, Petinov and Molotkovskii (44) propose four mechanisms: (a) When respiration is inhibited, the tissue exhibits less tolerance to heat; (b) Proteolysis is intensified and ammonia accumulates; (c) Amino acid synthesis ceases and abnormal amino acid metabolism occurs; and (d) Respiration is concluded to be the source of active metabolites and energy for resynthesis of proteins destroyed by high temperature. In a second study, Petinov and Molotkovskii (43) describe the organic acids as the active metabolites responsible for resynthesis of proteins. They state that plants with high temperature resistance form organic acids during the respiratory process which combine with ammonia (reducing ammonia toxicity) to form amides and eventually new proteins. They further state that the accumulation of ammonia is the mechanism which triggers the defense reactions forming the organic acids.

The quantities of various nitrogen compounds in leaf tissue of bentgrass and bermudagrass, over a range of four constant temperatures (50°, 60°, 70°, and 80°F), have been reported by Beard (3). Total nitrogen content increased in both species with increasing temperature. Non-protein nitrogen increased with increasing temperature in bentgrass but decreased with increasing temperature in bermudagrass. The free ammonia level increased from 50° to 70°F and decreased at 80°F in both species. The amide level in bermudagrass decreased with increasing temperature. A continuing reduction in amide levels is noted with increasing temperature in bentgrass. Bermudagrass, when grown at 80°F, appears to have a mechanism to reverse this downward trend.

MATERIALS AND METHODS

Establishment Procedures

Mature sod of Toronto creeping bentgrass was obtained from the Michigan State University experimental turfgrass field laboratory where it had been maintained at 0.25 inch cutting height. Circular plugs (3.5 inch diameter by 3 inch depth) were removed with a cup cutter and taken to the greenhouse for preparation.

The Tifgreen bermudagrass, also maintained at 0.25 inch cutting height, was obtained from the Plantation Field Laboratory, Ft. Lauderdale, Florida, and was mailed in sod strips (2' by 2') to Michigan State University. The sod pieces were plugged and prepared for establishment.

A soil mix of 50% sand and 50% sandy loam topsoil was poured into waxed cups (3.5 inch diameter by 6 inch depth) to a height 1.5 inch from the rim. The sod plug, which had been trimmed to a 1 inch soil depth, was firmly pressed on top of the soil mix. This procedure allowed 0.5 inch of shoot growth to reach the cup rim.

Thirty six cups of each species were prepared in this manner. Drainage holes were punched in the bottom of the cups which were placed in the greenhouse under an

automatic watering system for 2 weeks. Following the establishment period the plugs were placed in controlled environment chambers and watered twice daily with a full nutrient solution.

This nutrient solution was similar to Hoaglands (26) solution, with modification of N, P, and K to a 4:1:2 ratio respectively. Minor elements were added according to Hoagland. Two-thirds of the nitrogen was applied as NO_3 and one-third as NH_4. The concentration of the nutrient solution was adjusted to apply an approximate rate of 4 lbs. N/1000 sq. ft./month. The bentgrass was placed in a 15°C chamber, and the bermudagrass was placed in a 30°C chamber. The temperatures chosen corresponded to the optimum growth rate of each species. Light intensity was adjusted at 2,000 foot candles, and the daylength was set at 16 hours, with day-night temperatures held constant. Dyrene, a turf fungicide, was applied every other week for disease prevention, and an occasional dusting with malathion was necessary for insect control.

The harvest consisted of a daily clipping of the grass to a 0.5 inch height in each cup. The sample was composed primarily of leaf tissue with very little stem tissue collected. The cup was held at a 45° angle and a scissors was used in such a motion as to cause the clippings to fall on a piece of paper. The clippings were then immediately transferred to a 4 ounce bottle of ice cold

distilled water. The bottle containing the sample was immediately placed in an ice bucket and kept near 0°C.

Growth Conditions for Temperature Studies

In the experiment designed to determine the induction or availability of the nitrate reductase enzyme at various temperature levels, two growth chambers were used with environmental factors carefully controlled in each chamber.

The two chambers were first adjusted to 30°C and 20°C. Eighteen plugs of each species were placed in each chamber. The following week the chambers were adjusted to 35°C and 15°C. Six plugs of bentgrass were removed from 15°C and placed in 35°C, and six plugs of bermudagrass were transferred from 35°C to 15°C in order to provide the necessary one gram (fresh weight) tissue per day as the grasses approached temperature stress. The third temperature levels were 10°C and 40°C. At these temperatures all the bentgrass was placed in the cool chamber and all the bermudagrass was placed in the warm chamber. The turf was allowed to adjust to each temperature level for one week and harvests, consisting of one composite sample from all cups of each species, were taken on the 5th, 6th, and 7th day of each week.

The final temperature was 25°C. All of the turf was placed in the same chamber. Twelve cups of corn seedlings grown with 4 seedlings per cup, were placed in the chamber and watered with the same nutrient solution. At the three leaf stage, leaf tissue was harvested and analyzed. The corn variety (Hy2 x OH7), was used to provide a reference point to the work done by Hageman (20).

In the experiment designed to determine the actual activity of the nitrate reductase enzyme system over a range of temperatures, the turf was maintained at 25°C for 2 weeks prior to harvesting. One large sample was taken of each species. The four temperatures at which the enzyme reaction was determined, were 10°, 20°, 30°, and 40°C.

Preparation of Nitrate Reductase

In the laboratory the entire sample was removed from the bottle with the aid of a hooked glass rod. The sample was blotted dry in paper toweling, cut into small pieces, and weighed. Exactly one gram was removed and placed in a blending cup.

The extraction proceeded according to the methods of Hageman and Flesher (20) with few modifications. The extraction medium, consisting of 0.1 M Tris buffer, 0.01 M cysteine, and 0.0003 M EDTA, was added to the sample in the ratio of 15 ml per gram of tissue. Cysteine was added to the medium to protect the sulfhydryl-groups of the enzyme.

EDTA, a metal cheleting agent was used to bind excessive amounts of heavy metal ions. The pH was adjusted to 7.2 with HCl prior to each extraction.

A model "45" Virtis homogenizer was used to grind the sample; one minute at slow speed to cut the tissue, and two minutes at maximum speed to blend the sample. Due to excellent blending it was not necessary to press the homogenate through cheese cloth before centrifuging for 20 minutes at 20,000 x g. The supernatant was carefully decanted into test tubes. The temperature of the enzyme preparation was maintained at 0 to 3°C throughout the entire extraction procedure.

Assay Procedure

The assay procedure was based on the method of Evans and Nason (16), and was similar to the procedure of Hageman and Flesher (20). The assay mixture included 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.0), 0.5 ml of 0.1 M KNO_3, 1.0 ml of 1.36×10^{-3} M $NADH_2$, and 1.0 ml of the nitrate reductase enzyme preparation. Potassium phosphate and KNO_3 were pipetted into test tubes and allowed to adjust to the incubation temperature of 28°C. The reaction was started with the addition of $NADH_2$, followed immediately by the enzyme extract. Exactly 12 minutes later the reaction was stopped by adding 2.0 ml of 1% w/v sulfanilamide in 1.5 N HCl. N-(1-napthyl) ethylene-diamine-dihydrochloride

reagent (2.0 ml of 0.0228% w/v) was immediately added and the entire volume of 8 ml was thoroughly mixed.

Following the procedure reported by Snell and Snell (49) the color was allowed to develop for 10 minutes. The absorbancy was determined by reading each sample against its own blank (complete except for NADH) in a Beckman DU spectrophotometer at 540 mμ. Samples were drawn from the bottom of the tube and analyzed quickly and accurately with the aid of a flow-through cell.

The samples were completely analyzed within two hours of harvest. Enzyme extracts that were heated for five minutes in boiling water were inactive. The activity of the nitrate reductase was expressed as micromolar potassium nitrite (μM KNO_2) formed per gram fresh weight per hour.

The final expressed activity was the result of the average absorbancy of two blanks subtracted from the average of three determinations for each sample, and transposed, through the use of a predetermined standard curve, into μM KNO_2. Replication was achieved by repeating the experiment on the following day again using fresh plant material.

In measuring the temperature stability of the enzyme, the above procedure was slightly modified. The incubation temperature normally at 28°C was adjusted to 10°, 20°, 30°, and 40°C, with the aid of 4 water baths.

Also, following the final step, the reaction mixtures were immediately removed from the water baths to avoid possible differentiation in breakdown of the red color compound.

RESULTS AND DISCUSSION

In order to determine optimum conditions for enzyme activity, it was necessary to study various factors which have been reported to have an effect on nitrate reductase activity. Each environmental factor was controlled as described in the plant establishment procedures unless it was being examined in the specific study.

Effect of Tissue Sampling on Nitrate Reductase

Six plugs of bermudagrass were allowed to grow to a 4 inch height, while another six were maintained at a 0.5 inch height. The grass was allowed to adjust to these conditions for one week. At that time 0.5 inch of tissue was harvested from the shorter grass and 2 inches was harvested from the longer grass. In the latter, the 3 to 4 inch increment and the 2 to 3 inch increment were harvested as separate samples. The 2 to 3 inch increment consisted of a stemmy tissue, being about 50% leaf sheath. The samples were analyzed for nitrate reductase activity. The greatest activity was found in the grass maintained at the 0.5 inch height (Table 1). Also the leaf blade tissue exhibits more nitrate reductase activity than tissue containing 50% leaf blade and 50% leaf sheath tissue.

Table 1.--The effect of tissue sampling on nitrate reductase activity.

Bermudagrass 30°C

Tissue Length	Activity μM KNO_2/g.F.wt./Hr.		
	I	II	Average
1" to 0.5"	48.0	45.0	46.5
4" to 3"	11.0	7.0	9.0
3" to 2"	6.0	5.0	5.5

According to other studies, nitrate reduction takes place primarily in the leaf tissue. In working with cauliflower, Candella, et. al. (10) reported greater nitrate reduction in the leaves than in the petioles or roots, and that mature leaves yielded extracts with maximum activity. Wallace and Pate (59) report in their work with peas that enzyme induction occurs most readily in actively growing tissues; hence the newly expanded leaf exhibited the highest activity. Hageman and Flesher (20), using corn seedlings state that the activity of nitrate reductase was 80% lower in root extracts as compared to extracts of shoots and leaves.

Since each cup was filled with soil to within 0.5 inch of the rim, accurate and reproducible harvest methods allowed the grass to grow 0.5 inch which was then clipped back to the rim of the cup. At optimum temperatures for growth, 0.5 inch of shoot growth was attained daily.

Effect of Nutrients on Nitrate Reductase

Twelve plugs of bentgrass were clipped daily and watered with tap water for one week. At the end of the week the plugs were harvested and the nutrient solution was immediately applied. A complete nutrient program was maintained and harvests were again made at 24 and 48 hours following the initial harvest. The samples were analyzed for nitrate reductase activity. Within 24 hours after nutrient application, the enzyme activity was induced to a maximum rate (Table 2). A slight drop in activity was noted at 48 hours.

Table 2.--The effect of nutrient application on nitrate reductase activity.

Bentgrass 15°C

Nutrient Application	Activity μM KNO_2/g.F.wt./Hr.		
	I	II	Average
0 Hrs.	0.0	0.0	0.0
24 Hrs.	85.0	72.0	78.5
48 Hrs.	72.0	75.0	73.5

Related studies show that NO_3 is the principle form of nitrogen absorbed by higher plants and induces higher nitrate reductase activity than NH_4 (10). Low fertility levels result in low activity of nitrate reductase (30). The nitrate reductase activity in corn seedlings increased

with increasing concentrations of NO_3 (20). Beevers, et. al. (5) reported that the induction of nitrate reductase is proportional to, and dependent on the amount of NO_3 in the tissue. Relative amounts of NO_2 or NH_3 had no effect. The availability of NH_4 did not inhibit the induction of the nitrate reductase enzyme.

The time of application of nutrients had an effect on enzyme activity. However, this could not be separated from a moisture stress effect since the plugs received no water other than the full nutrient solution. Nutrients were applied twice daily to overcome moisture stress at high temperatures.

Effect of Light on Nitrate Reductase

Twelve cups of bermudagrass were placed in a 30°C chamber at a light intensity of 2,000 foot candles. After a one week establishment period, six were moved to a 30°C chamber with new bulbs producing 2,400 foot candles. After 48 hours, each set of six cups was harvested and analyzed for nitrate reductase activity. An increase in light intensity increases the activity of the enzyme (Table 3).

The time of day of harvest also had an effect on nitrate reductase activity. Six plugs of bentgrass were harvested at 8:00 a.m. (after 3 hours of daylight), and six plugs were harvested at 8:00 p.m. (after 15 hours of

daylight). Each of the samples were analyzed for enzyme activity (Table 4). The higher activity at 8:00 p.m. indicated that a period of light exposure was necessary to obtain maximum activity.

Table 3.--The effect of light intensity on nitrate reductase activity.

Bermudagrass 30°C

Light Intensity	Activity μM KNO_2/g.F.wt./Hr.		
	I	II	Average
2,000 F.C.	42.0	45.0	43.5
2,400 F.C.	49.0	53.0	51.0

Table 4.--The effect of the length of light exposure on nitrate reductase activity.

Bentgrass 15°C

Length of Light Exposure	Activity μM KNO_2/g.F.wt./Hr.		
	I	II	Average
3 Hours	51.0	50.0	50.5
15 Hours	65.0	78.0	71.5

Light is known to be a very important, and perhaps essential factor in nitrate reduction through the capability of the photosynthetic process to donate the necessary electrons for reduction of NO_3. Hageman and Flesher (20) have extensively studied the various effects of light;

including a reduction in enzyme activity due to partial shading and total darkness, and a subsequent increase upon light restoration. Diurnal variation was such that a sampling made at dawn had approximately 50% the activity level of a sample taken at midday. The enzyme activity of plants under artificial shade also showed a reduction when sampled in early morning. However, the difference between shade and full sun was much greater in the midday sample (21).

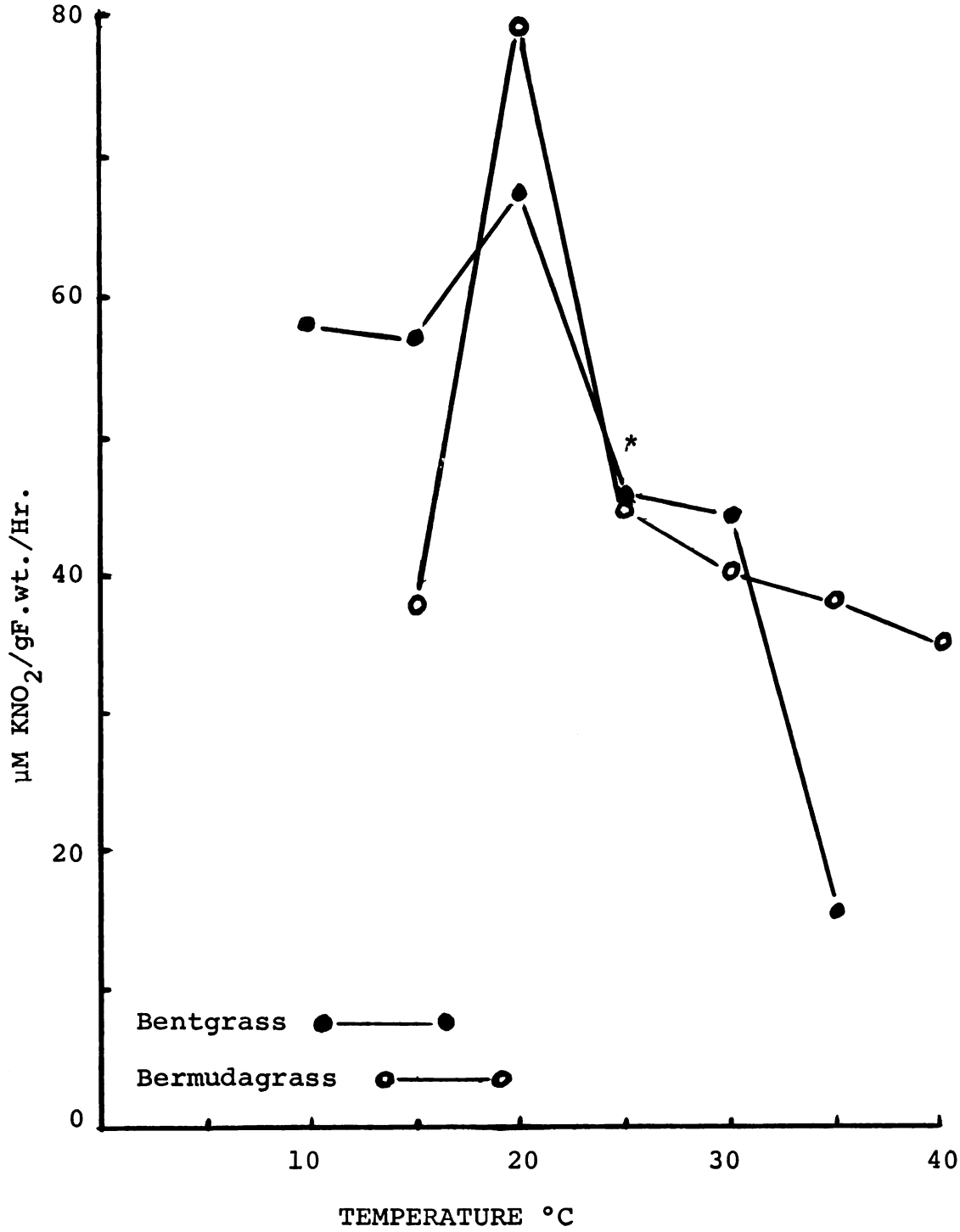
It is impossible to maintain the growth chambers at 2,400 foot candles for any length of time, so the light intensity was maintained at 2,000 foot candles and was checked bi-weekly with a light meter. All harvests were carried out at 12:00 noon (after 7 hours daylight), with analysis of the enzyme following immediately.

Effect of Temperature on Nitrate Reduction

In determining the effect of temperature on nitrate reductase activities one species with, and one without high temperature resistance was used. The temperature effect on the apparent availability and actual activity of the nitrate reductase enzyme was studied.

Nitrate reductase appeared to be induced at 20°C in both species, with warmer temperatures reducing the activity that could be isolated (Fig. 1). Following the peak of the

Figure 1.--The effect of temperature on induction of nitrate reductase of creeping bentgrass and bermudagrass.



*Nitrate reductase activity of corn (cultivar Hy2 x OH7) to provide a point of reference to work done by Hageman (20).

curve at 20°C, the activity of the enzyme dropped significantly, but seemed to be maintained at an adequate level of growth for both species through 30°C.

The activity level of the corn seedlings grown at 25°C was comparable to that of both bentgrass and bermudagrass. Variations in the technique, as compared to Hageman (20) are probably responsible for the difference in the reported levels of μM KNO_2 per gram fresh weight per hour.

The level of nitrate reductase in bermudagrass seemed adequate for growth at 15°C. However the turf exhibited little growth at this temperature, indicating that under these conditions nitrate reductase activity was not limiting growth.

At high temperatures, bermudagrass exhibited only slight reductions of enzyme activity from 25° to 45°C. These levels of activity found for 25°, 30°, 35° and 40°C, did not greatly differ from the activity noted at 15°C.

The nitrate reductase activity of bentgrass was greater at 10°C than at 25°C or higher temperatures, correlating with the cool season growth pattern of this species. The peak of the curve was noted at 20°C. High temperature stress of this species, noted at 35°C, sharply reduced both the activity of the nitrate reductase enzyme (Fig. 1) and growth (13).

At temperatures of 35°C and higher, the amount of nitrate reductase activity that could be isolated from the

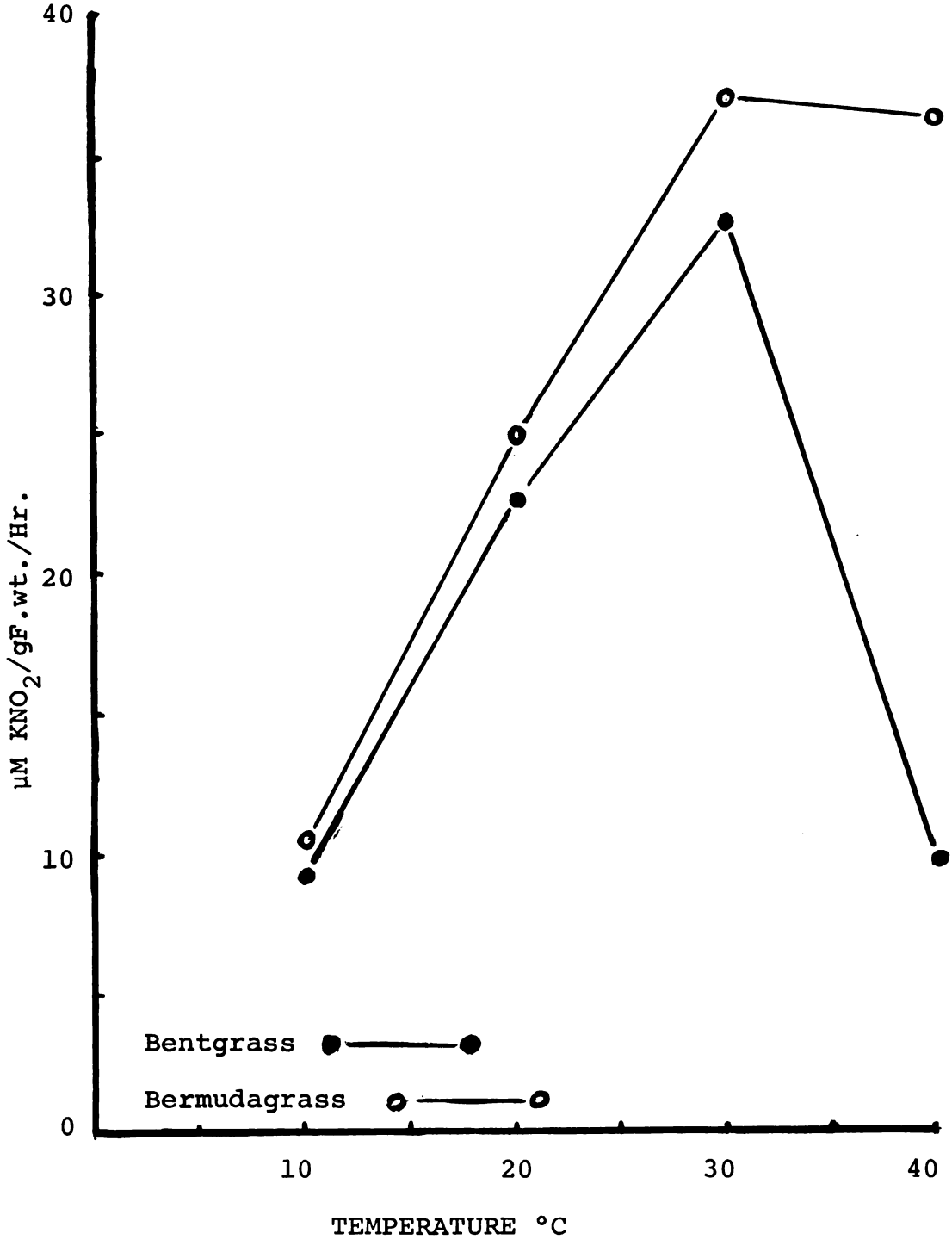
bentgrass was much lower than for the bermudagrass. Either there was less enzyme present in the bentgrass at high temperatures, or the enzyme was present but inactive. Subsequent studies were initiated to study the latter possibility.

Effect of Temperature on Enzyme Activity

A differential effect of temperature on the activity *per se* of nitrate reductase was observed between the two species (Fig. 2). From 10° to 30°C both species exhibited normal enzyme-temperature curves of increased temperature yielding increased activity. In bermudagrass the activity at 40°C remains at the same level found at 30°C, while in bentgrass the activity at 40°C drops to approximately the level found at 10°C.

Temperature is an important factor influencing both nitrate reductase induction and activity. The induction of nitrate reductase is temperature dependent, however, some of the effect may be due to the temperature effect on increased uptake of NO_3 (5). Afridi and Hewitt (1) report that when NO_3 is added at various temperatures, increasing enzyme activity was noted with increasing temperature up to a critical temperature. Mattas and Pauli (30) point out that it is difficult to distinguish between temperature and moisture stress. They examined this temperature-moisture stress combination and found that a reduction in nitrate

Figure 2.--The effect of temperature on the activity of nitrate reductase of creeping bentgrass and bermudagrass.



reductase activity occurred before the stress was measurable through relative turgidity.

The temperature for inactivation of bentgrass growth (13), and observed nitrate reduction (Figs. 1 & 2) is between 30°C and 40°C. In this same temperature range, bermudagrass exhibits adequate growth and nitrate reduction. The difference between these two species may be described as the difference in adaptation to environment through evolutionary processes. But this approach does not answer the question of why bentgrass lacks heat resistance. Several proposals have previously been suggested to answer this question.

It was first thought that increased respiration at high temperatures depleted the carbohydrates in the plant. Duff (13) clearly shows that this is not the case in bentgrass. Ammonia toxicity was also proposed as a mechanism of high temperature growth stoppage in the plant. As an end product of nitrate reduction, ammonia could become concentrated in leaf tissue causing the lack of heat tolerance. However, the high temperature inactivation of nitrate reductase in bentgrass leaf tissue shown in Fig. 2 seems to indicate that if a toxic level of ammonia exists at high temperatures, it is not produced by the pathway of nitrate reduction. High temperatures can also increase proteolysis, which may increase NH_3 levels. If, in this breakdown of protein, NH_3 is not utilized through protein

resynthesis, toxic levels of NH_3 could eventually occur and possibly inhibit nitrate reductase synthesis or activity.

Figure 1 indicates that the enzyme activity in bentgrass is reduced when the sample is harvested under high temperature conditions. This reduction is due to either high temperature inhibition of the enzyme synthesis or high temperature inactivation of the enzyme. While both of these activity reductions may occur in bentgrass at high temperature, Figure 2 indicates that high temperature inactivation of the enzyme is responsible for at least part of the reduced activity found in Figure 1.

CONCLUSIONS

1. As reported in studies using other species, the nitrate reductase activity of Toronto creeping bentgrass and Tifgreen bermudagrass is found to respond to these factors.
   a. The activity of nitrate reductase is higher in the leaf blade tissue than in stem tissue.
   b. The activity of nitrate reductase increases upon the addition of a nitrate nutrient solution.
   c. The activity of the enzyme increases with increasing light intensity and increasing periods of light exposure.
2. Temperature has a differential effect on nitrate reductase according to species. The enzyme system of both species appears to be induced at 20°C but at high temperatures induction may be inhibited in bentgrass while it remains adequate in bermudagrass.

   Temperature also has a differential effect on inactivation of the enzyme between the two species. Increasing temperature increases the activity of the enzyme through 30°C. At 40°C the enzyme of bentgrass is inactivated while no inactivation is found in bermudagrass.

If nitrate reductase is a limiting factor of growth, the inactivation of the enzyme *per se* of bentgrass grown at high temperatures may explain the high temperature growth inhibition of this species.

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APPENDIX

Appendix Table 1.--The nitrate reductase activity of bentgrass expressed as μM KNO_2 per gram fresh weight per day.

Temperature Treatment	Day 5th	6th	7th	Average	
10°C	51.0	54.0	70.0	58.3	a b
15°C	73.5	53.5	43.5	56.8	a b
20°C	73.0	63.0	63.5	66.8	a
25°C	44.5	47.5	44.0	45.3	b
30°C	52.0	42.0	39.0	44.3	b
35°C	13.5	13.0	20.0	15.5	c

Those values containing the same letter are not significantly different at the 1% level (Duncans multiple range test).

Appendix Table 2.--The nitrate reductase activity of bermudagrass expressed as μM KNO_2 per gram fresh weight per hour.

Temperature Treatment	Day 5th	6th	7th	Average	
15°C	49.0	36.0	28.5	37.8	b
20°C	81.5	79.5	75.0	78.7	a
25°C	46.5	44.5	42.0	44.3	b
30°C	51.0	36.5	33.5	40.3	b
35°C	27.5	39.0	49.5	38.7	b
40°C	36.0	37.0	34.0	35.7	b

Those values containing the same letter are not significantly different at the 1% level (Duncans multiple range test).

Appendix Table 3.--The nitrate reductase activity per se of bentgrass and bermudagrass expressed as μM KNO_2 per gram fresh weight per hour.

Temperature Treatment	Bentgrass Samples I	II	Average	Bermudagrass Samples I	II	Average
10°C	9	9.5	9.25	11.5	9.5	10.5
20°C	23.5	21.5	22.5	26.0	23.5	24.75
30°C	35.5	29.0	32.5	38.0	35.5	36.75
40°C	10	9.5	9.75*	36.5	36.0	36.25*

*Statistically significant difference [LSD (p = .01) = 20.18] between species is found at this temperature.