CHAPTER II

QUALITATIVE BLACK LAYER RESEARCH

Abstract. Black layer formation in turfgrass soils was hypothesized to result from accumulations of metal sulfides produced by SO_4^{-2} reduction in response to low redox potential. Qualitative greenhouse research was initiated to determine whether black layer could be experimentally reproduced and whether S addition or moisture content would influence development. In subsequent studies the effect of possible controls was determined as was the influence of free H_2S on 'Penncross' creeping bentgrass. Addition of S at 48 kg S ha^{-1} resulted in production of black layer when soil moisture was excessive. Increasing the level of S to 244 kg ha^{-1} intensified the condition. Without S addition minimal black layer formed. Addition of 0.1 M MoO_4^{-2} or NO_3^- solution prevented black layer formation. It was also shown that H_2S at 1000 ug ml^{-1} killed turfgrass. It was concluded that S was the key ingredient in black layer formation and that S reduction was responsible. Soil moisture was considered very important. Further, H_2S could realistically be involved in the turfgrass decline. Finally, adequate NO_3^- fertilization of turf was proposed as a black layer control.

Black layer has been called the number one malady on bentgrass greens and tees today (12). Black layer was reported to begin with a noticeable drop in percolation followed by the formation

of a black, offensive smelling layer somewhere in the upper soil profile (12). Affected turf shows symptoms of bronzing, often followed by a decline, thinning or outright loss (12).

Many hypotheses regarding black layer formation have been proposed (2, 11). Initially, these ranged from the algae hypothesis (11) to blatant mismanagement (9). Work in our laboratory suggested that black layer formation was related to dissimilatory SO_4^{-2} reduction. In this respiratory process soil bacteria use SO_4^{-2} as the terminal electron acceptor in place of O_2 (1). It happens only where O_2 is absent. The reaction produces H_2S as an end product, which is very reactive with divalent soil metals such as Fe^{+2} (1). Upon reaction between H_2S and soil metals, black metal S^{-2} precipitates such as FeS are formed. These precipitates flocculate on organic debris and in soil pore space giving the profile a blackened appearance . It was suggested that an accumulation of such precipitates physically composed the black layer, probably in response to low redox potential.

If not enough active metal is available in the system to bind H_2S as e.g. FeS, free H_2S may be evolved (7). Hydrogen sulfide is well known as a respiratory poison inhibiting electron transport (16). It has also been shown to interfere with the oxidative power of rice (Oryza spp.) roots (16). Thus, it was thought that H_2S could be at least partially involved in the observed turfgrass decline.

Addition of NO_3^- was reported to keep the redox potential of rice paddy soils high enough to bypass S^{-2} formation and thus alleviate rice suffocation disease (5). The effect of the NO_3^-

was to act as an alternate electron acceptor as O_2 became depleted (15). The objective of this research was to qualitatively determine whether black layer formation could be related to SO_4^{-2} reduction and S addition, and whether H_2S could produce a turfgrass decline. The use of fertilizer NO_3^- as a black layer control was also evaluated.

MATERIALS AND METHODS

Experiments with Sulfur. This experiment was conducted in a glasshouse facility. The objective was to determine whether S addition would contribute to black layer formation, and whether soil moisture would exert an influence. Two liter plastic buckets having 3 small bottom drainage holes (i.e., 6 mm) were used in the study. Buckets were 19 cm high by 15 cm diameter at the top. Bottoms were lined with 3 cm of pea gravel. Buckets were then packed with 1 L of either washed Lake Michigan dune sand (99% > 0.1 mm and 84.5% > 0.25 mm, 0.0125% om), or dune sand plus peat as an 90:10 mix. A 2 cm layer of washed mortar sand (81% > 0.1 mm and 35.1% > 0.25 mm) was then placed over the dune sand in each unit. This was done to create a perched water table. Bulk density of the dune sand was 1.7 g cm^{-3}.

Packed buckets were uniformly wetted then treated with 200 mls of 0.01 M lactate amended with flowable 52% S. Sulfur was applied to the units at levels of 0.0, 48.8 or 244 kg S ha^{-1}, and Fe^{+2} was applied as $FeSO_4$ at 6.2 kg ha^{-1}. Additionally, N as $(NH_4)_2SO_4$ was applied at 48.8 kg N ha^{-1}. Fifty mls of crude lactate enriched cultures of sulfate-reducing bacteria was used

to inoculate each bucket. Bacteria were obtained from a wet area of Baker Woods in East Lansing. One half of the treated units of each sand type (i.e., sand or sand + peat) were then immersed in tap water contained in an aluminum pan. This was done to waterlog the units via capillarity. Pans were filled daily to keep the moisture content constant. The other buckets were arranged so that free drainage would occur and units would not be water-logged. A hundred mls of water were added periodically to keep the soil moist. Bucket units were fitted with lids to prevent evaporation and to reduce O_2 diffusion. Units were then incubated for 33 days at ambient temperature. There were 4 replications of each treatment. After 33 days each bucket was disassembled and scored for the presence or absence of any black layering.

In additional experiments buckets were prepared in much the same way except that re-precipitated colloidal S was used instead of flowable S. Two thirds of the sand was placed in the buckets prior to treatment with S. Then S was applied uniformly to the sand surface at levels of 96 or 488 kg ha^{-1}. Remaining sand was then placed over the S to create a sandwich effect. Units were treated with 50 mls of sulfate-reducer inoculum, 200 mls 0.01 M lactate, and 48.8 kg ha^{-1} N, then uniformly wetted. The lids were placed and the units were water-logged as described. No peat was added to the sand in this portion of the study and all units were flooded for 33 days. After 33 days the buckets were disassembled and scored for the presence or absence of any black layering.

Experiments with Inhibitors. These experiments used sand buckets identical to the ones previously described. Both flowable and colloidal S were used. The objective was to determine whether black layer could be prevented or reversed by chemical addition. No $(NH_4)_2SO_4$ N was applied, but N as either $Ca(NO_3)_2$, KNO_3 or $NaNO_3$ was added as 200 mls of 0.1 M solution. In addition, other experimental units also received 200 mls of 0.1 M Cl^- bleach, NH_4MoO_4, or NaN_3. Chemicals were added at the time of S treatment or after 21 days when formation of black layer was evident. One set was left as a check. Experimental units were then inoculated with sulfate-reducers and lactate, fitted with lids and water-logged for 21 days. At the end of 21 days units treated with the oxidants were disassembled and scored for the presence or absence of any black layering. After 21 days, units in which black layer was active were treated with the oxidants and observations were made immediately.

Experiments with Hydrogen Sulfide. This portion of the experiment sought to determine whether H_2S would be toxic to turfgrass. Cores were constructed from 10.8 cm diameter x 15 cm PVC. The PVC had been previously cured at 60 C for 48 hrs to drive off ethylene. Plexiglas was glued to the bottom of each core so that no leakage would occur. One 6 mm hole was drilled 2.5 cm from the bottom of the core and one hole was drilled 2.5 cm from the top. Plastic nipples were glued into each hole for attachment of tygon tubing. The holes served as solution ports. Covers for each core were constructed from a 5 cm ring of PVC to which plexiglas had been glued.

Each core was filled to within 2.5 cm of the top with equal weights of sterile 3, 4, and 5 mm Pyrex glass beads. The glass beads served as a soil matrix which would allow for movement of solution through the core and would allow for ample root generation. Next, a 10.8 cm diameter plug of 'Penncross' creeping bentgrass (Agrostis palustris Huds.) consisting of only turf and thatch (approximately 1.5 cm thick) was transplanted into each core. Cores were fertilized with 20-20-20 at a rate of 48.8 kg N ha^{-1} and incubated in a growth chamber for two weeks. Water was added as needed to prevent wilting.

At the end of two weeks each core was attached from the bottom port to a peristaltic pump connected to a 4 L anoxic water reservoir. Each reservoir was subsequently connected to a pryogallol trap which minimized resovoir exposure to O_2 as water was withdrawn by the pump. For each run of the experiment one resovoir contained only freshly boiled water while another contained water amended with H_2S at 1000 ug ml^{-1}. Water was amended by sparging with gaseous H_2S and periodically checking concentration by the method of Cord-Ruswich (6). The pH of the fresh water was adjusted to correspond to the pH of the S^{-2} amended water (i.e., pH = 3). Cores were then capped with the lids and sealed with tape, and the pump switched on at a speed equivalent to pumping 0.6 L day^{-1}. Capping prevented undue atmospheric exposure to the core during the pumping but still allowed light to reach the turf. Cores were incubated in a fume hood under a plant gro lite (G.E.) at ambient temperature. Solution was circulated through each core for 7 days. As the cores became filled the solution excess drained from the upper

port. In this way the transplanted turfgrass was constantly exposed to only water or water plus H_2S with minimal atmospheric O_2 exposure. Also, since glass was the soil matrix no interfering ions (e.g., Fe^{+2}) were available to bind the H_2S as a precipitate and render it inert.

RESULTS AND DISCUSSION

Experiments with Sulfur. Where no S was applied no blackening occurred regardless of soil type or moisture status, even after 33 days. However, a slight darkening of both the soils did occur where water was in excess. The darkening appeared to be greater in the peat mix but the visual difference was minimal. Where soil was freely drained no color change appeared.

Where S was applied at 48.8 kg ha^{-1} to freely drained soil, again no black layer formed regardless of soil type. A distinct blackening of the entire profile did take place when S was added at 48.8 kg ha^{-1} and soil moisture was in excess. This was considered a type I layer (4). No difference in black layer formation between sand or sand + peat was observed. When level of S increased to 244 kg ha^{-1} the blackening became more intense in all replicates. Again, no differences between soils was evident. Sulfur applied at 244 kg ha^{-1} to freely drained soil produced some blackening as "pockets" in 75% of the replicates. This blackening occurred near the bottom of the bucket and did not encompass the entire profile. The blackened soil reacted positively to S^{-2} spot testing (8). Soil also lost the blackening upon exposure to atmosphere with time. Units

possessing black layer also had a distinct foul odor. Thus, blackening was considered an accumulation of S^{-2} in response to water-logging, probably produced by bacterial SO_4^{-2} reduction.

The blackening of saturated sand was more intense in the mortar sand layer compared to the dune sand profile. This was attributed to the mortar sand having a wider range of particle diameters with a greater percentage of fine particles compared to the dune sand, hence smaller pore spaces (10). This implied that perched water tables created by textural interfaces may be areas of the profile most conducive to formation of intense black layering. Alternatively, it could be that a majority of the added S was effectively retained by the finer soil impeding its movement into the profile. However, this topic was not addressed in the research.

It appeared that capping the bucket and flooding the soil reduced O_2 diffusion (10) to the point where S or SO_4^{-2} reduction readily occurred. Adding large quantities of S probably also helped to reduce available O_2 in response to S binding O_2 upon oxidation, as evidenced by pocket black layer formation where S was applied at 244 kg ha^{-1}. Lactate addition also undoubtedly stimulated microbial respiration contributing to O_2 consumption. It also appeared that S was limiting for intense black layer formation in the experimental sand, as evidenced by lack of black layer where no S was applied and where lactate and excess water was added.

Algae also appeared on the surface of all experimental units except those not receiving S. Algal invasion was secondary to black layer formation. Evidence of black layer appeared in 4-5

days while the algae proliferation did not appear to begin until near day 15. No algae growth was noticed below the soil surface. Algae were probably indigenous to the sand but some were undoubtedly added with the sulfate-reducer inoculum. In this experiment black layer formation was attributed to presence of S and induced anaerobiosis through waterlogging, not algal proliferation as suggested by Hodges (11).

When sandwiched units were examined black layer had readily formed. In these units however, the black layer was observed to be in a distinct banding as opposed to coloring the whole profile. Thus, in this experiment a type II layer was produced (4). This would tend to suggest that the colloidal S was relatively immobile in the soil compared to the flowable S. The 488 kg level of S produced a darker banding that did the 96 kg ha^{-1} level.

Experiments with Inhibitors. The compounds $Ca(NO_3)_2$, KNO_3, $NaNO_3$, $NaMoO_4$, Cl^- bleach, and NaN_3 all prevented black layer formation when added concurrently with the S treatment. In addition the bleach and NO_3^- also oxidized existing black layer at the experimental concentration (i.e., 0.1 M) but only when exposed to atmospheric O_2. The effect of the NO_3^- was to act as an alternate electron acceptor in the absence of O_2. This in effect probably kept the redox potential from falling below a critical level necessary for S^{-2} formation, but this was not demonstrated. The effect of the $NaMoO_4$ was to act as a competitive inhibitor of sulfate-reducing bacteria. This meant the K_m value (i.e., Michaelis constant) was increased by addition of the MoO_4 but V_{max} was similar (13). The configuration of the

MoO_4 is stereochemically similar to SO_4^{-2} and had been demonstrated to inhibit ATP-sulfurylase, the first enzyme in the SO_4^{-2} reduction pathway (15). The effect of the NaN_3 was to act as a sterilizing agent killing the sulfate-reducers. Addition of azide proved that black layer formation was biological in nature while molybdate suggested SO_4^{-2} reduction was responsible.

In a practical aspect these results imply addition of fertilizer NO_3^- could be a successful control measure for preventing black layer, but probably not for reversing the condition unless coupled with core aerification where atmospheric O_2 is not limiting. Although N is routinely applied to golf greens, in this modern age many turf managers have strayed from using NO_3^- to protect the groundwater and environment. Slow release organic fertilizers have largely replaced the NO_3^- for golf course use. These materials are acceptable if the soil is aerobic enough to permit nitrification, but if O_2 status in soil is diminished, nitrification becomes a significant O_2 sink further enhancing the depression of redox potential, possibly contributing to black layering.

Experiments with Hydrogen Sulfide. After 7 days incubation in the presence of H_2S, 'Penncross' creeping bentgrass was killed. The turfgrass turned from deep green to mottled straw brown in only 3 days and to straw brown in 7. Turf aerial tissue was visibly stunted after only 12 hours exposure. Exposure to only water had no observable deleterious effect. Root tissue of the turf exposed to H_2S was visibly stunted and necrotic compared to

the untreated turf. In addition, the thatch and roots exposed to H_2S took on a blackened appearance characteristic of black layering.

The effect of the H_2S was to act as a respiratory toxin blocking electron transport at cytochrome C + C3 by binding to divalent metals such as Fe^{+2} (1).

CONCLUSIONS

This qualitative research has demonstrated a relationship between the addition of S to soil and formation of black layering, especially when soil moisture was in excess. The black layering was shown to be prevented by addition of fertilizer NO_3^-. The biological activity of black layer formation as S^{-2} production was demonstrated using MoO_4, a specific inhibitor of sulfate-reducing bacteria, and with NaN_3, a universal toxin. The black layer reacted positively to spot testing, confirming the presence of S^{-2} in the experimentally produced black layer. Finally, it was shown that H_2S was toxic to creeping bentgrass. From these results we may conclude that black layering is biological in nature and involves the anaerobic chemistry of S. Further, it is possible that H_2S may be involved in the decline of turfgrass which frequently accompanies black layer. Finally, an acceptable control measure in the field may be as simple as applying adequate fertilizer NO_3^-.

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