Elemental Sulfur Reduces to Sulfide in Black Layer Soil

William L. Berndt¹ and Joseph M. Vargas, Jr.

Division of Professional and Technical Studies, Edison College, 8099 College Parkway S.W., P.O. Box 60210, Building C-101, Fort Myers, FL 33906-6210; and the Department of Botany and Plant Pathology, 102 Pesticide Research Center, Michigan State University, East Lansing, MI 48824

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Abstract. Black layer (BL) has reduced the quality of many putting greens since the 1980s. Initially, the nature of BL was unknown. Research established that BL was sulfide (S^{2-}) formed in response to low redox. Its formation was linked to dissimilative sulfate reduction using labeled sulfate ($^{35}SO_4^{2-}$). The objective of this study was to see if elemental sulfur (S^0) reduced to S^{2-} . When labeled sulfur ($^{35}S^0$) with a specific activity of 3.7×10^4 Bq·mg⁻¹ was added to soil from a green with BL in a reaction vessel kept at a low redox potential, it reduced at a per-minute rate of 5.3 nmol·cm⁻³, resulting in accrual of labeled hydrogen sulfide ($H_2^{35}S$) and acid-soluble sulfide ($AS^{35}S$). Nearly 32% of the $^{35}S^0$ reduced to labeled sulfide ($^{35}S^{2-}$) in 24 h. Adding S^0 to greens with low redox may result in rapid formation of S^{2-} and an accelerated rate of BL development. Avoiding this requires limiting the input of S^0 or encouraging high soil redox through chemical or physical means such as fertilizing with nitrate (NO_3^-) and aerifying. This is the first report implicating S^0 reduction as a source of BL development in putting green soil.

Root-zone blackening in putting greens was termed black layer (BL) (Scott, 1986). Sometimes BL appears as a horizontal band of variable thickness, whereas other times the entire profile is blackened. A decline in turf quality usually accompanies BL. Because of its impacts on turf quality, BL can be a major problem of putting greens. It was referred to as the number one malady of creeping bentgrass (*Agrostis palustris* Huds.) putting greens in the late 1980s (Scott, 1986).

Black layer was determined to be an accumulation of sulfidic (S^{2-}) precipitates such as iron sulfide (FeS) within the soil and plant root matrix (Berndt et al., 1987):

This was confirmed by spot testing BL from greens at 26 golf courses in Michigan and Ohio with a solution of azide (NaN_3) and iodine (I_2) (Berndt, 1990; Fiegl, 1972). Metal sulfides (MeS) such as FeS were present if bubbling was observed on introducing BL soil to solution. Bubbling occurred because MeS catalyzes production of NaI with release of N_2 from solution:

Samples of BL at all 26 courses tested positive for the presence of MeS.

One source of sulfide (S^{2-}) in putting green soil containing BL was dissimilative reduction of SO_4^{2-} by sulfate-reducing bac-

teria (SRBs) in response to low redox (Berndt and Vargas, 1987, 2006):

This was established using labeled sulfate ($^{35}SO_4^{2-}$) (Berndt and Vargas, 2006). When 10^{-3} M $^{35}SO_4^{2-}$ with a specific activity (SA) of 1.554×10^5 Bq·mg⁻¹ was injected into intact microcores taken from a BL in a 'Penncross' creeping bentgrass green, it reduced at a mean per-minute rate of 4.9×10^{-3} nmol·cm⁻³ (Berndt and Vargas, 2006). Labeled hydrogen sulfide ($H_2^{35}S$) and labeled acid-soluble sulfide ($AS^{35}S$) were produced. Adding either azide (N_3^-) or molybdate (MoO_4^{2-}) with the label decreased the rate to 2×10^{-5} and 7×10^{-6} nmol·cm⁻³, respectively, which proved $^{35}SO_4^{2-}$ reduction was biological and SRBs were involved (Berndt and Vargas, 2006).

Although tracers proved bacterial reduction of $^{35}SO_4^{2-}$ occurred, other research suggested elemental sulfur (S^0) was reduced to S^{2-} (Berndt, 1990; Berndt and Vargas, 1992, 1996). For example, when anoxic sand was treated with S at 48 kg·ha⁻¹ using S^0 or SO_4^{2-} , there was ≈ 4.7 times more S^{2-} produced where S^0 was applied (Table 1). Differences in S^{2-} concentration occurred because eight electrons (e^-) are needed to reduce a molecule of SO_4^{2-} to S^{2-} , whereas only two are needed to reduce a molecule of S^0 to S^{2-} (Lindsay, 1979):

This means four times more S^{2-} results from reduction of S^0 for a given level of reducing

equivalents such as soil organic matter. This in turn implies that adding S^0 to putting greens having conditions favorable for BL development, like low soil redox, may result in a rapid accumulation of sulfidic precipitates, hence an accelerated rate of BL formation.

Reduction of S^0 occurs in many environments, and greater than 90 species of bacteria reduce S^0 through enzymes like sulfur reductase (Hao and Ma, 2003; Hedderich et al., 1999; Stetter and Gaag, 1983). However, information on the reduction of S^0 in soils supporting putting greens is needed, because this topic is absent in the turf literature. The objective of the research described in this article was to determine if $^{35}S^0$ reduces to $^{35}S^{2-}$ in soil from a creeping bentgrass green affected by BL.

Materials and Methods

Experimental soil. Soil with BL was collected from a 'Penncross' creeping bentgrass green at the Robert Hancock Turfgrass Research Center at Michigan State University in East Lansing. The concentration of S^0 in soil was 0.19 mg·cm⁻³ (Tabatabai, 1974), and most probable number estimates were 3×10^3 to 7×10^5 SRBs/g soil (Alexander, 1982; Berndt and Vargas, 2006). The per minute rate of $^{35}SO_4^{2-}$ reduction was 4.9×10^{-3} nmol·cm⁻³ for intact cores (Berndt and Vargas, 2006) and 36.6×10^{-3} nmols·cm⁻³ for disturbed soil (Berndt, 1990).

Sulfide still. Recovering $^{35}S^{2-}$ required an anaerobic still and S^{2-} trapping train, similar to the one used by Berndt and Vargas (2006) (Fig. 1). The reaction vessel was a 125-mL Wheaton serum bottle (Wheaton Science Products, Millville, NJ) linked to an upstream source of O_2 -free N_2 and a downstream series of $^{35}S^{2-}$ traps. Oxygen was removed from the N_2 stream by sparging it over hot Cu^{2+} filings in a Hungate apparatus (Kaspar and Tiedje, 1982). Sulfide traps were 20-mL glass scintillation vials containing 3 mL 2% cadmium chloride ($CdCl_2$).

Radioactive sulfur. Elemental $^{35}S^0$ with a SA of 3.7×10^4 Bq·mg⁻¹ (Perkin-Elmer, Waltham, MA) was dissolved in benzene, producing a solution with a SA of 4.8×10^5

Table 1. Influence of sulfur on production of free hydrogen sulfide (H_2S), acid-soluble sulfide (ASS^{2-}), and redox potential as $pe + pH^2$.

	Means (mg·kg ⁻¹)		Means $pe + pH^2$
	H_2S	ASS^{2-}	
Control	0.0	5.1	6.9
Sulfur (S^0)	8.0	53.2	6.3
Sulfur (S^0) + nitrate (NO_3^-)	2.8	1.6	9.9
Sulfate (SO_4^{2-})	5.2	7.8	6.2
LSD $P = 0.05$	4.4	16.3	0.6

²Sulfur as S^0 and SO_4^{2-} was applied to Lake Michigan dune sand at 48 kg·ha⁻¹ at Michigan State University in East Lansing. Nitrate as calcium nitrate [$Ca(NO_3)_2$] was applied to provide N at 48 kg·ha⁻¹ (from Berndt, 1990).

³Redox potential as sum of measured pe and pH .

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¹To whom reprint requests should be addressed; e-mail lberndt@edison.edu

Fig. 1. Schematic of the sulfide still and trapping train used to distill labeled sulfide released from soil from a creeping bentgrass putting green affected by black layer at Michigan State University in East Lansing.

Bq·mL⁻¹. The SA of this solution was verified by liquid scintillation counting (LSC) using a Beckman LS 8100 LSC instrument (Beckman Coulter, Fullerton, CA).

Reduction of elemental sulfur. One milliliter of the ³⁵S-benzene solution was injected into the empty reaction vessel. Benzene was evaporated by purging the bottle with O₂-free N₂ at a flow rate of 100 cm³·min⁻¹ for several minutes, leaving ³⁵S⁰ adsorbed to the inside of the glass reaction vessel.

As purging was continued, 18 cm³ of BL soil was put into the vessel along with 50 mL of boiled, distilled water cooled to room temperature under N₂. The water was amended with cysteine to establish low redox (i.e., *E_h* ≈ -340 mV) in the reaction vessel (Kaspar and Tiedje, 1982). Resazurin indicator was used to verify existence of low redox (Kaspar and Tiedje, 1982). The vessel opening was then plugged with butyl rubber, crimped with an aluminum seal, and the vessel was attached to both the upstream O₂-free N₂ gas stream and the downstream trapping train through the butyl rubber using syringe needles and Teflon tubing. Traps containing 3 mL 2% CdCl₂ were installed into the trapping train, and the flow rate of the O₂-free N₂ was adjusted to 100 cm³·min⁻¹.

If the ³⁵S⁰ was reduced by the soil in the reaction vessel, then AS³⁵S precipitates (i.e., Fe³⁵S) and free H₂³⁵S would be produced. Any free H₂³⁵S gas that evolved from the soil was swept from the reaction vessel into the trapping train with the gas stream, where it reacted with the Cd solution contained in the traps forming a Cd³⁵S precipitate (Lindsay, 1979):

Radioactivity contained in each of the traps as Cd³⁵S was then counted with a Beckman LS 8100 liquid scintillation counter (Beckman Coulter). Counting required addition of counting cocktail for aqueous solutions (Research Products International, Mt. Prospect, IL). All counted samples were corrected for quenching by the H# method (Beckman Coulter). Traps were changed and Cd³⁵S was counted at 60, 120, 180, 360, 1080, and 1440 min.

After the trap change at 1440 min, 3 mL 10% w/v NaN₃ and 2 mL anoxic 37% HCl were injected into the reaction vessel. Azide sterilized soil, which stopped reduction of ³⁵S⁰, and the HCl lowered vessel solution pH to ≈1, which released AS³⁵S from soil as

free H₂³⁵S gas. The flow rate of O₂-free N₂ was once again adjusted to 100 cm³·min⁻¹, and the distillation was continued. Traps were changed as necessary until levels of radioactivity being swept into the trapping train with the gas stream were near background.

Calculating sulfur reduction. The per-minute rate of S⁰ reduction was calculated using the following formula (Sorokin, 1962):

$$\frac{(S)(a)(1.06)}{(A)(V)(t)} = \text{nmol} \cdot \text{cm}^{-3}$$

The (S) was the concentration of S⁰ in the reaction vessel in nmol, (a) was Bq of ³⁵S²⁻ recovered, 1.06 was an isotope correction factor, (A) was Bq added, (V) was soil volume (cm³), and (t) was the incubation time in minutes. The mean residence time (*t_{mrt}*) of the S⁰ pool was calculated by

dividing (S) by the calculated rate of reduction, and the residence half-life of the ³⁵S-label (*t_{rh}*) was (A) ÷ (a)(2) (Berndt and Vargas, 2006).

Other calculations, graphics, and statistics. The concentration of ³⁵S²⁻ recovered (nmol·cm⁻³) was plotted versus time to depict the release pattern of H₂³⁵S over 1440 min and accrual of AS³⁵S in the reaction vessel during the 1440 min. From these measurements, a first-order rate constant (*k*) was calculated (Segel, 1976):

$$2.3 \log \frac{[S]_0}{[S]} = kt$$

In this equation, [S]₀ was the original concentration of ³⁵S⁰ added as a percent (i.e., 100%), [S] was the percentage of ³⁵S⁰ remaining at 1440 min (*t*), and *k* was the rate constant min⁻¹.

Fig. 2. (A) Time course of appearance of H₂³⁵S and acid-soluble ³⁵S²⁻ in soil from a creeping bentgrass putting green affected by black layer at Michigan State University in East Lansing. (B) Calculated velocity (*v*) of the reduction of ³⁵S⁰ over time.

After k was estimated, $[S]$ was calculated for times of 60, 120, 180, 360, and 1080 min using:

$$2.3 \log \frac{100}{[S]} = kt$$

Subtracting $[S]$ from $[S]_0$ for each time gave the amount of $^{35}\text{S}^{2-}$ released ($\text{nmol}\cdot\text{cm}^{-3}$), which was plotted versus time. The $[S]$ at 1440 min was determined experimentally.

The velocity (v) of the reduction of $^{35}\text{S}^0$ per minute ($\text{nmol}\cdot\text{cm}^{-3}$) was estimated by $v = k[S]$ and plotted versus time. A plot of v versus the concentration of $^{35}\text{S}^0$ and corresponding double reciprocal plot (Segel, 1976) were developed to show the relationship between v and $^{35}\text{S}^0$.

Regression analysis was used to describe accrual of $^{35}\text{S}^{2-}$ versus time, velocity of reduction versus time, and other velocity curves. Graphics and regressions were developed using SigmaPlot 10 (Steel and Torrie, 1980; Systat Software, 2006).

Results

Radioactive $^{35}\text{S}^{2-}$ was trapped with no apparent lag time (Fig. 2A). Approximately 31.5% of $^{35}\text{S}^0$ was recovered as $^{35}\text{S}^{2-}$ with the ratio of total $^{35}\text{S}^{2-}$ trapped to H_2^{35}S trapped being 4.95. The H_2^{35}S trapped over 1440 min was 6.4% of the added label, or 20.2% of trapped $^{35}\text{S}^{2-}$. The AS^{35}S liberated by HCl was 25.2% of the added label, or 79.8% of trapped $^{35}\text{S}^{2-}$. The per-minute rate of S^0 reduction was $5.3 \text{ nmol}\cdot\text{cm}^{-3}$. The t_{mrt} of the S^0 pool was 77,900 min and the t_{rhl} of the label was 2304 min.

The k at 1440 min was 8.1×10^{-4} per min. Velocity (v) of the reduction of $^{35}\text{S}^0$ decreased with time in curvilinear style ($R^2 = 0.9998$; $\hat{Y} = 18.4 - 0.137X + 3.0 \times 10^{-6}X^2$) (Fig. 2B). The per-minute v at 1440 min of $5.77 \text{ nmol}\cdot\text{cm}^{-3}$ was very close to the rate of $5.33 \text{ nmol}\cdot\text{cm}^{-3}$ calculated using Sorokin's (1962) formula. When v was plotted versus concentration of $^{35}\text{S}^0$ (Fig. 3A), the curve was linear ($R^2 = 1.0$; $\hat{Y} = -0.0023 + 0.0008X$), which meant v was proportional to concentration of $^{35}\text{S}^0$. When plotted as a Lineweaver-Burk plot (Fig. 3B), the curve intercepted both axes too closely to the origin to determine either maximum velocity (i.e., V_{max}) or the Michaelis constant (i.e., K_m). This meant that enzyme saturation did not occur, V_{max} and K_m appeared infinite, and the reaction was first order.

Discussion

Understanding the reduction of S^0 is important, because S^0 is recommended for turf in a variety of publications (Beard, 1973; Landschoot, 2007; Vargas, 2005; Voight et al., 2007). Sulfur can be used to help mitigate alkaline soil conditions and offset S deficiency (Beard, 1973; Vargas, 2005). It has also been used to try to prevent encroachment of annual bluegrass (*Poa annua* L.) into greens (Joseph Vargas, personal communication). Conversely, the negative consequence

Fig. 3. (A) Velocity of reduction of $^{35}\text{S}^0$ as a function of its concentration in soil from a creeping bentgrass putting green affected by black layer at Michigan State University in East Lansing. (B) Data presented as a Lineweaver-Burk double reciprocal plot.

of applying S^0 , especially in relation to its reduction, has not been adequately addressed in the turfgrass management literature.

Applying S^0 can help to generate and perpetuate favorable environmental conditions for release of S^{2-} to soil, hence BL formation (Berndt and Vargas, 1992). These include low redox and abundant S. Consider that applying S^0 to soil increases acidity through microbial oxidation of S^0 to H_2SO_4 :

This reaction is widely known, but what has not been emphasized is this process consumes O_2 and increases H^+ thereby reducing redox potential as pe + pH (Berndt and Vargas, 1992).

Oxidation of S^0 is obviously not the only source of low redox in soils. It is well known that water-logging and the presence of restrictive layers in soil can exclude diffusion of O_2 and in turn generate low redox. Excessive microbial respiration can also cause low redox. The point is, however, that once low redox exists, and provided S^0 is abundant, as when it is applied to greens, the formation of S^{2-} through S^0 reduction may occur rapidly, and the rate of BL formation and development may be accelerated. In the current research introducing S^0 and cysteine into the reaction vessel and continually purging it with O_2 -free N_2 generated favorable environmental conditions for formation of S^{2-} , a redox potential of $\approx -340 \text{ mV}$ (Kaspar

and Tiedje, 1982), and plenty of S^0 to be reduced.

Avoiding an accelerated rate of BL formation in putting greens requires limiting input of S^0 and sustaining high soil redox potential through chemical or physical means such as fertilizing with nitrate (NO_3^-) and aerifying.

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