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SORGOLEONE FROM ROOT EXUDATE INHIBITS MITOCHONDRIAL FUNCTIONS[1]

JAMES A. RASMUSSEN,[2,*] ANGELA M. HEJL,[3] FRANK A. EINHELLIG,[3] and JOHN A. THOMAS[4]

[2]*Department of Biology*
Southern Arkansas University
Magnolia, Arkansas 71753
[3]*Department of Biology*
[4]*Department of Molecular Biology and Biochemistry*
University of South Dakota
Vermillion, South Dakota 57069

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Abstract—The aim of this investigation was to determine if sorgoleone (SGL), a hydrophobic compound in *Sorghum bicolor* (L.) Moench root exudate, interferes with mitochondrial functions. Tests were conducted on mitochondria isolated from etiolated soybean [*Glycine max* (L.) Merr.] and corn (*Zea mays* L.) seedlings. The data show SGL is a potent inhibitor of state 3 and state 4 respiration rates in both soybean and corn. Using either NADH, succinate, or malate as substrate, the I_{50} was about 0.5 μM SGL for state 3 and 5.0 μM for state 4 based on 0.3–0.5 mg mitochondrial protein. Absorption spectra indicate SGL blocks electron transport at the b-c_1 complex. These data show that disruption of mitochondrial function may be a mechanism of SGL-mediated growth inhibition previously reported and demonstrate a probable role of SGL in *Sorghum* allelopathy.

Key Words—Sorgoleone, mitochondria, inhibitor, allelochemical, allelopathy, root exudate, *Sorghum bicolor*, electron transport.

INTRODUCTION

Cultivated *Sorghum* species have a reputation for suppressing weed growth (Overland, 1966; Putnam et al., 1983; Putnam and DeFrank, 1983; Forney et

*To whom correspondence should be addressed.
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al., 1985; Einhellig and Rasmussen, 1989). Roots of *Sorghum* are known to exude several biological products, both hydrophilic and hydrophobic, that exhibit allelochemical activity (Panasiuk et al., 1986; Netzly and Butler, 1986; Einhellig and Souza, 1992). Some of the hydrophilic exudates include phenols, protein, and 3-deoxanthocyaninidin derivatives (Netzly and Butler, 1986). A major component of the hydrophobic exudate of grain sorghum [*Sorghum bicolor* (L.) Moench] roots is a compound identified as 2-hydroxy-5-methoxy-3-[(8′Z,11′Z)-8′,11′,14′-pentadecatriene]-*p*-hydroquinone, which oxidizes readily into the quinone form (Figure 1), and three minor *p*-benzoquinones that are structurally similar (Netzly and Butler, 1986; Chang et al., 1986; Netzly et al., 1988). The unstable dihydroquinone form of this compound was shown to be a potent germination stimulant for witchweed [*Striga asiatica* (L.) Kuntz. #STRLU]. The stable quinone form, which we will refer to as sorgoleone (SGL), causes inhibition of root elongation in lettuce and some weed species (Netzly et al., 1988). Recent studies in our laboratory indicate that SGL inhibits the growth of several weed species at micromolar concentrations (Einhellig and Souza, 1992).

The mechanisms through which allelopathic chemicals cause their effects remain obscure. Most of the literature reflects quantitative effects of individual allelochemicals on plant growth parameters, with only a limited number of investigations directed toward determining the actual physiological role of an inhibitor that leads to the growth reduction response. Specific allelochemicals have been shown to interfere with phytohormones, plant–water relations, mineral nutrition, stomatal function, carbon fixation and distribution, and respiration (Einhellig, 1986). It is unlikely that the mode of action of the large diversity of individual inhibitors is the same. However, understanding the specific physiological action of a compound is valuable information for potential applications

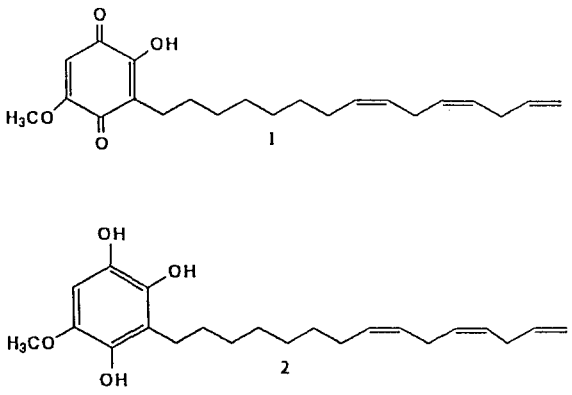

FIG. 1. Structures of **(1)** sorgoleone and **(2)** dihydroquinone of sorgoleone (Chang et al., 1986).

in agroecosystems. This information is also important because allelopathy is generally not the isolated effect of a single compound, and it is affected by the abiotic environment (Rice, 1984; Einhellig, 1986, 1989).

Several allelopathic compounds, as well as some synthetic herbicides, show an effect on respiration and oxidative phosphorylation (Stenlid, 1968, 1970; Koeppe, 1972; Demos et al., 1975; Moreland and Huber, 1979; Jacobson and Jacobson, 1980; Moreland and Novitzky, 1987). Koeppe (1972) found that juglone, a naphthoquinone, is a potent inhibitor of corn mitochondrial respiration. The lipophilicity of SGL suggested it would readily dissolve in plant membranes, and we suspected that SGL might interfere with mitochondrial respiration. The structural similarity between SGL and coenzyme Q, an intermediate in the mitochondrial electron transport chain, was another rationale to investigate possible effects of SGL on respiration.

The initial intent of this investigation was to determine if SGL impacts on oxygen uptake in isolated plant mitochondria. If this occurred, the aim was to determine if the site of inhibition was located in the electron transport pathway. The third objective was to see if response differences exist between mitochondria isolated from monocots and dicots.

METHODS AND MATERIALS

Extraction and Preparation of Sorgoleone. Sorghum root exudate was collected from the roots of grain sorghum seedlings [*Sorghum bicolor* (L.) Moench., Dekalb Hybrid DK 28] in a procedure modified from Netzly et al. (1988). The seeds were rinsed with distilled water and surface sterilized by soaking for 10 min in a 20% bleach solution. They were then grown on moistened filter paper in 9-cm petri dishes (10 seeds per dish) at approximately 27°C for five to seven days. The roots were individually dipped for 1 sec in an extraction solution consisting of 20 ml of methylene chloride acidified with 50 μl of glacial acetic acid, and the extract was filtered through Whatman No. 1 filter paper in a syringe–filtration system. The extract was then evaporated to dryness, resulting in 12–15 mg of residue per 1000 roots. Fate et al. (1990) indicated that the exudate collected in this way is remarkably clean and contains only two major components: sorgoleone and its dihydroquinone. The dihyroquinone readily oxidizes into the quinone. Analysis of this exudate by high-pressure liquid chromatography (HPLC) (Spectra Physics, model SP8800) using a Spherisorb 5 μM ODS2 column (4.6 $\times$ 250 mm) showed over 90% correlated to the SGL peak published by Netzly and Butler (1986) and Netzly et al. (1988). Since no commercial SGL is available, Dr. Larry Butler (Purdue University) provided the initial sample for comparison. This exudate, which we will call SGL, was stored at -4°C until used.

Isolation of Mitochondria. Mitochondria were isolated from 4- to 5-day-old dark-grown, etiolated corn (*Zea mays* L., Pioneer #3615) coleoptiles or soybean [*Glycine max* (L.) Merr., Pioneer #9202] hypocotyls grown in 10^{-4} M $CaCl_2$-saturated vermiculite at 25°C according to procedures modified from Miller et al. (1970). The isolation procedures consisted of grinding tissue in a defined aqueous medium followed by a series of refrigerated centrifugations (1–4°C). Approximately 80 g of tissue were placed in 100 ml of chilled grind mix (0–4°C) containing 0.3 M mannitol, 0.1% bovine serum albumin (BSA), 50 mM HEPES-KOH (pH 7.5), 4 mM L-cysteine, and 5 mM EDTA. Coleoptiles or hypocotyls were homogenized with a chilled mortar and pestle. The homogenate was strained through four layers of cheesecloth. The liquid suspension was placed in each of four, 50-ml tubes and centrifuged (Sorvall RC-5, SS-34 rotor) at 1500*g* for 12 min. The supernatant was reserved and centrifuged at 28,000*g* for 6 min to isolate the mitochondria in the pellet. Pellets were resuspended in chilled, 0.4 M sucrose using a soft camel-hair brush to gently loosen the pellet, and this suspension was centrifuged at 1500*g* for 12 min. The supernatant was reserved, underlain with 0.6 M sucrose, and centrifuged at 17,500*g* for 18 min. The supernatant was carefully aspirated to avoid disruption of the pellet containing the mitochondria. The pellets were resuspended for a final time with enough of a 0.4 M sucrose solution to yield a suspension that contained optimal concentrations for study.

The final mitochondrial suspension used for oxygen uptake studies contained 7–10 mg protein/ml and that used for cytochrome studies was 10–15 mg protein/ml. Mitochondrial suspensions were kept on ice until used. A small amount of suspension was frozen and saved for protein analysis using the Bio-Rad Protein Analysis Method (Bio-Rad Chemical Division) with BSA used as the protein standard.

Experimentation. Oxygen utilization was measured polarographically with a Hansatech instrument (King's Lynn, Norfolk, England), which has a Clark-type oxygen electrode. The 2-ml, water-thermostated reaction chamber was maintained at 25°C. The mitochondrial reaction medium consisted of: 2 mM KH_2PO_4, 10 mM TES (pH 7.6), 5 mM $MgCl_2$, 0.3 M mannitol, and either 10 mM succinate, 33 mM malate, or 1 mM NADH as substrate. Preliminary experiments included 0.1% BSA in this reaction medium. BSA was subsequently found unnecessary, and it was deleted from the reaction medium. A mitochondrial concentration of 0.3–0.5 mg was maintained in 2 ml of reaction medium. For certain tests, 1 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was employed as an uncoupling agent.

SGL was dissolved in EtOH for use in mitochondrial studies. The concentration of SGL was adjusted so no more than 20 μl of EtOH were injected into the 2-ml reaction chamber. Controls run with 20 μl of EtOH had no impact on mitochondrial respiration rates.

The ADP-stimulated rate of respiration will be referred to as state 3, and the ADP-limited rate will be referred to as state 4 (Chance and Williams, 1955). A respiratory control ratio (RCR) and ADP/O ratio were determined at the onset of each set of experimental trials. The RCR was computed as state 3/state 4. The ADP/O ratio was computed from the moles of ADP utilized per moles of oxygen used in state 3 respiration. Percentage of inhibition was determined as: (control rate minus rate at inhibition)/control rate. An I_{50} is defined as the concentration required to inhibit respiratory rates by 50%.

Spectrographic methodology was used to determine if restricted electron flow occurred at specific cytochrome sites in the electron transport chain. A dilution of approximately two parts mitochondrial suspension (10–15 mg protein/ml) and one part aerated reaction medium was prepared in a 1-ml cuvette. The sample was scanned (Beckman DU70) from 500 to 650 nm using sodium dithionite to reduce all cytochromes, allowing definition of the region of the *b* (560–557) and *c* (554–550) peaks (Douce, 1985; Keightley, 1991). A similar mitochondrial preparation without sodium dithionite was scanned following amendments of SGL. The scans were compared for evidence of location of inhibition of electron transport.

RESULTS AND DISCUSSION

Oxidation of Substrates. Representative polarographic traces of NADH, succinate, and malate oxidation by mitochondria containing approximately 0.3–0.5 mg protein are depicted in Figure 2A–C. Corn mitochondria had average RCR values of 2.5, 2.3, and 2.4 for NADH, succinate, and malate, respectively. The ADP/O ratios for these substrates were 1.2, 1.3, and 1.9. The RCR and ADP/O values for soybean mitochondria were similar. Addition of 1 μM FCCP during state 4 elicited an increased rate of oxygen utilization that persisted until anaerobiosis, indicating the electron transport system could be uncoupled from its requirement for ADP (Douce, 1985).

Sorgoleone Inhibition. Numerous tests were conducted with SGL additions to the reaction medium during state 3 and state 4 mitochondrial respiration after a linear slope was established (Figure 2D and E). As illustrated, SGL is a strong inhibitor of oxygen uptake, and this inhibition is not overcome by FCCP. Although soybean was generally more affected, there were no trend differences between the responses of isolated corn and soybean mitochondria to the addition of SGL. In an average of six or more trials, the I_{50} for NADH substrate state 3 respiration for corn and soybean mitochondria was between 0.5 and 0.7 μM (Figure 3). This lack of differential action between effects on monocot and dicot mitochondria corresponds with our previous study, which showed that SGL strongly inhibited growth in both broadleaf and grass weeds (Einhellig and

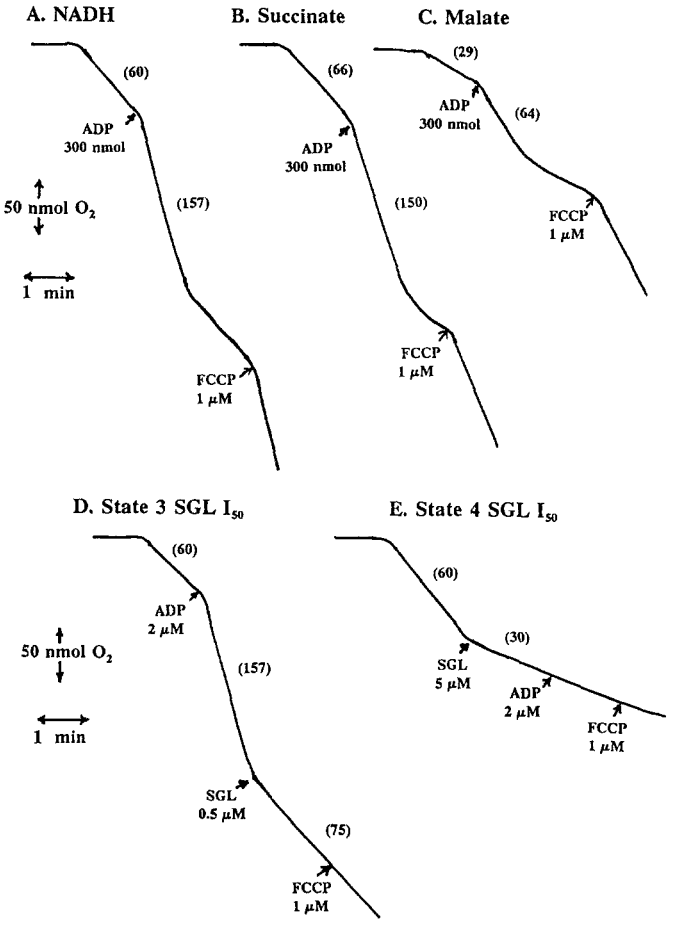

FIG. 2. Representative polarographic traces of oxygen utilization by soybean mitochondria. (A) oxidation of NADH; (B) oxidation of succinate; and (C) oxidation of malate. Traces D and E show sorgoleone inhibition of state 3 and state 4 respiration, respectively.

Souza, 1992). Hence, it appears that the action of SGL on mitochondria can not explain the differences that we found in broadleaf and grass weed abundance following a crop of *Sorghum bicolor* (Einhellig and Rasmussen, 1989).

SGL is a strong inhibitor of mitochondrial oxidation for all three substrates tested in these experiments (Figure 4). NADH, succinate, and malate oxidation were similarly inhibited by SGL concentrations between 0.1 and 0.5 μM, with the latter being the I_{50}. However, SGL approaches maximum inhibition of state-3 succinate oxidation at lower concentrations (0.8–1.0 μM) than when NADH and malate are the substrates. Perhaps this is because succinate provides elec-

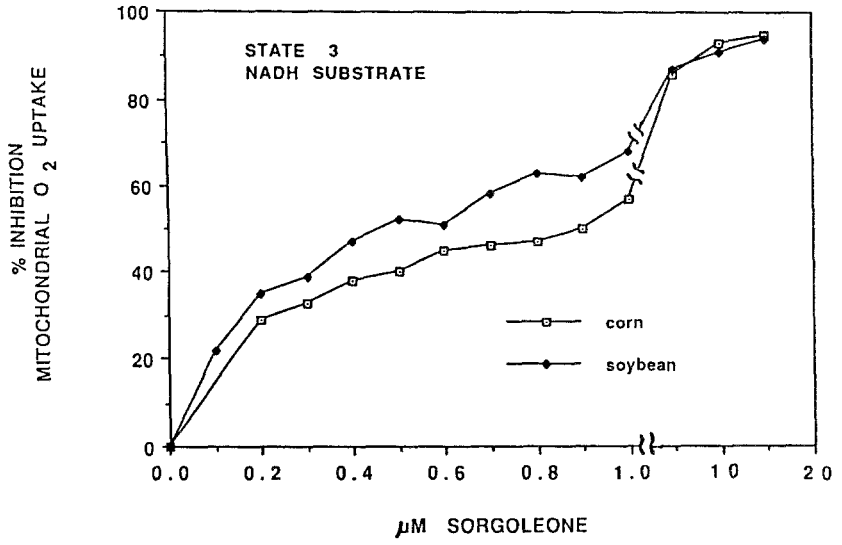

FIG. 3. Effects of sorgoleone on NADH state 3 oxygen uptake of isolated soybean and corn mitochondria, approximately 0.4 mg protein.

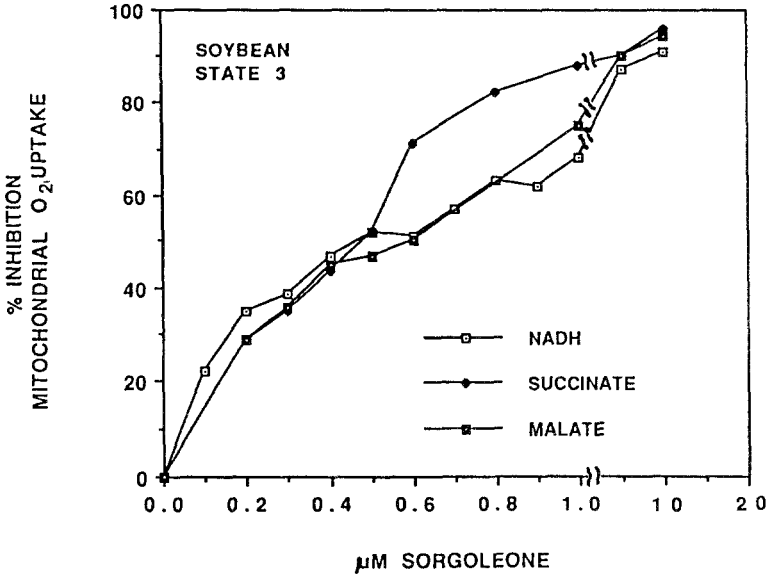

FIG. 4. Effects of sorgoleone on NADH, succinate, and malate state 3 oxygen uptake of isolated soybean mitochondria, approximately 0.4 mg protein.

trons to the electron transport system via complex II, whereas NADH and malate feed electrons through complex I (Douce, 1985). The maximum SGL inhibition of oxidation for each of the three substrates is approximately 90% even when the SGL concentration is raised 10-fold to 10 μM (Figure 4).

The extreme toxicity of SGL to state 3 mitochondrial respiration is highlighted by comparisons to other compounds reported in the literature (Table 1). It is in the same inhibitory range as rotenone, a commonly used mitochondrial respiratory inhibitor. These comparisons also show SGL is effective at several orders of magnitude below other reported allelochemicals.

State 4 respiration is also inhibited by SGL, but it is much less sensitive to the effects of this compound (Figure 5). The state 4 I_{50} was approximately 5.0 μM, which is 10 times the I_{50} of state 3 (Figures 4 and 5). It should be noted that SGL inhibition of state 4 soybean respiration is essentially identical with all three substrates. After the injection of SGL during state 4, state 3 respiration could not be elicited by the addition of ADP (Figure 2E). This is logical given the high concentrations required to inhibit state 4. As in all previous work, there was no material difference between effects of SGL on corn and soybean mitochondrial functions.

In both state 3 and state 4 respiration studies, the introduction of SGL elicited an immediate inhibitory response that persisted during the course of the experiment. The strongly hydrophobic nature of SGL with its long nonpolar, hydrocarbon chain (Figure 1) makes it very lipid soluble. Hence, it should readily solubilize in the mitochondrial membrane, providing a mechanism for immediate and persistent interaction with the site of electron transport.

TABLE 1. COMPARISON OF SORGOLEONE AND OTHER INHIBITORY COMPOUNDS ON STATE 3 PLANT MITOCHONDRIAL RESPIRATION[a]

Compound	Malate oxidation (μM) I_{50}	Reference[b]
Sorgoleone	0.5	
Rotenone	0.5	1
2,6-Dinitroaniline Herbicides	12 to 43	1
Selected Allelochemicals:		
Quercetin	20	2
Naringenin	110	2
Umbelliferone	1,180	2
Vanillin	4,030	2
Ferulic Acid	4,530	2
Vanillic Acid	17,670	2

[a] Mitochondria containing approximately 0.4 mg protein.
[b] 1 = Moreland and Huber (1979); 2 = Moreland and Novitzky (1987).

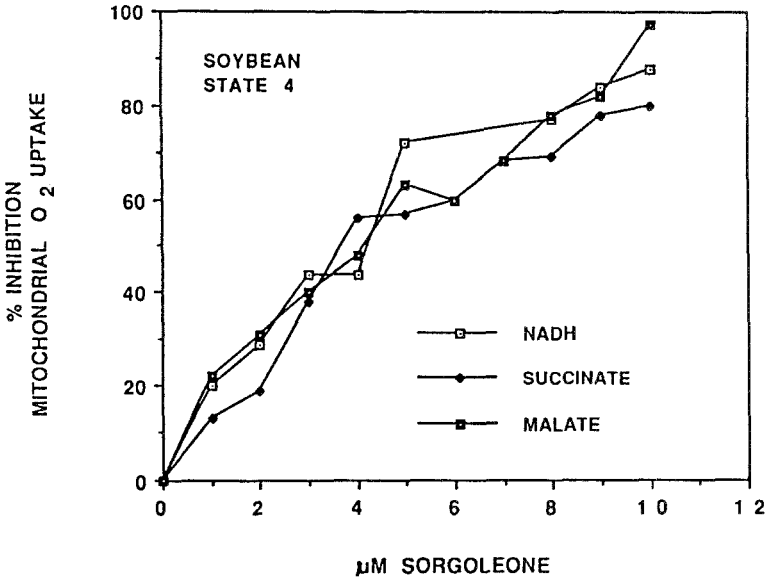

FIG. 5. Effects of sorgoleone on NADH, succinate, and malate state 4 oxygen uptake of isolated soybean mitochondria, approximately 0.4 mg protein.

The long hydrocarbon chain of SGL probably had another impact in the preliminary stages of the experiments when BSA was used in the reaction medium. In our early experiments BSA was utilized because Douce (1985) had reported its ability to bind free fatty acids. However, we found the presence of BSA in the reaction medium caused a profound effect on the I_{50} of SGL. With BSA in the reaction medium, the I_{50} for state 3 respiration of NADH in soybeans was 75 μM SGL compared to 0.5 μM SGL without BSA. It seems very probable that BSA in the medium binds to SGL, limiting the activity of SGL.

Site of Inhibition. The failure of FCCP to circumvent inhibition of state 3 and state 4 respiration by SGL (Figure 2D and E) suggests that SGL acts primarily as an electron-transport inhibitor (Moreland and Novitzky, 1987). When sodium dithionite was used to reduce the cytochromes of isolated mitochondria, characteristic peaks of the cytochrome *b* and *c* regions were revealed (Figure 6). The *b*-type cytochromes are reduced with the addition of SGL, but there is no evidence of a cytochrome $c_{1\text{-}554}$ peak. Since details of the cytochrome *b* complex are elusive, we can conclude that SGL blocks electron flow between the cytochrome *b* and c_1 complex. Several inhibitors of animal mitochondrial electron transport, including hydroquinone analogs, are known to block in this region (Von Jagow and Link, 1986).

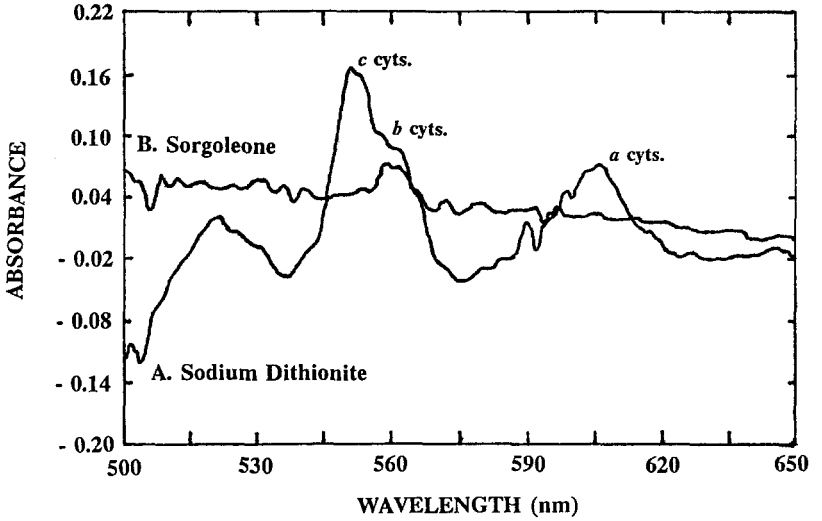

FIG. 6. Spectrophotometric traces of mitochondrial cytochromes (cyts.) after amendments of sodium dithionite (A) and sorgoleone (B).

CONCLUSION

The data demonstrate that SGL, a recently reported *Sorghum bicolor* root exudate, is a powerful inhibitor of respiration in isolated soybean and corn mitochondria. Spectrophotometric studies suggested one mechanism of SGL inhibition is a blockage of electron flow between the cytochrome *b* and c_1 complex.

The effects of SGL on mitochondrial respiration may be an important mechanism responsible for causing the inhibition of growth of several weed species that we have reported (Einhellig and Souza, 1992). This mitochondrial work, plus the evidence that SGL is abundant in *S. bicolor* root exudate, has been isolated from soil, and can persist for a substantial period in soil (Netzly et al., 1988), demonstrates SGL has a role in *Sorghum* allelopathy.

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