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RESEARCH PAPER

Dynamic root exudation of sorgoleone and its *in planta* mechanism of action

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Abstract

The oily droplets exuded from the root hairs of sorghum are composed of a 1:1 ratio of sorgoleone and its lipid resorcinol analogue. The production of these droplets appears to be suppressed when *c*. 20 μg of exudate mg^{-1} root dry weight accumulates at the tip of the root hairs. However, more exudate is produced following gentle washing of the roots with water, suggesting that the biosynthesis of lipid benzoquinones and resorcinols is a dynamic process. Sorgoleone interferes with several molecular target sites, including photosynthetic electron transport, in *in vitro* assays. However, the *in planta* mechanism of action of sorgoleone remains controversial because it is not clear whether this lipid benzoquinone exuding from the roots of sorghum is taken up by roots of the receiving plants and translocated to their foliage where it must enter the chloroplast and inhibit PSII in the thylakoid membrane. Experiments designed to test the *in planta* mode of action of sorgoleone demonstrated that it has no effect on the photosynthesis of older plants, but inhibits photosynthesis in germinating seedlings. Sorgoleone is not translocated acropetally in older plants, but can be absorbed through the hypocotyl and cotyledonary tissues. Therefore, the mode of action of sorgoleone may be the result of inhibition of photosynthesis in young seedlings in concert with inhibition of its other molecular target sites in older plants.

Key words: Allelochemical, allelopathy, lipid resorcinols, mode of action, sorghum, sorgoleone.

Introduction

Sorghum (*Sorghum bicolor* L. Moench) is an allelopathic species that represses the growth of weeds and even injures crops grown in the same field the following year (Breazeale, 1924; Putnam *et al.*, 1983; Einhellig and Rasmussen, 1989; Overland, 1966). Sorghum is now planted as a green manure or as a cover crop to suppress weed populations in integrated pest management systems (Weston, 1996) or as a crop residue in no-tillage farming. Small-seeded weed species are the most affected by sorghum and sorgoleone (Netzly and Butler, 1986; Panasiuk *et al.*, 1986; Einhellig and Souza, 1992; de Souza *et al.*, 1999; de Almeida Barbosa *et al.*, 2001).

The allelopathic potential of sorghum has been associated with phytotoxic lipophilic exudates released by the roots. This exudate consists of sorgoleone, a lipid benzoquinone (Netzly *et al.*, 1988; Czarnota *et al.*, 2003*b*; Dayan *et al.*, 2003), and a resorcinol analogue (Erickson *et al.*, 2001) along with several other congeners, but in much lower quantities (Kagan *et al.*, 2003) (Fig. 1).

The biosynthesis of sorgoleone has been elucidated using retrobiosynthetic NMR analysis (Fate and Lynn, 1996; Dayan *et al.*, 2003), and mature sorghum root hairs contain the entire genetic material and biochemical machinery required for the production of this bioactive benzoquinone (Czarnota *et al.*, 2003*a*; Dayan *et al.*, 2007; Pan *et al.*, 2007; Baerson *et al.*, 2008). The amount exuded from the roots is sensitive to temperature, suggesting that the overall allelopathic potential of sorghum may be optimum between 25 °C and 35 °C (Dayan, 2006).

Sorgoleone is a soil-active lipophilic compound that is phytotoxic to a wide range of plant species, causing

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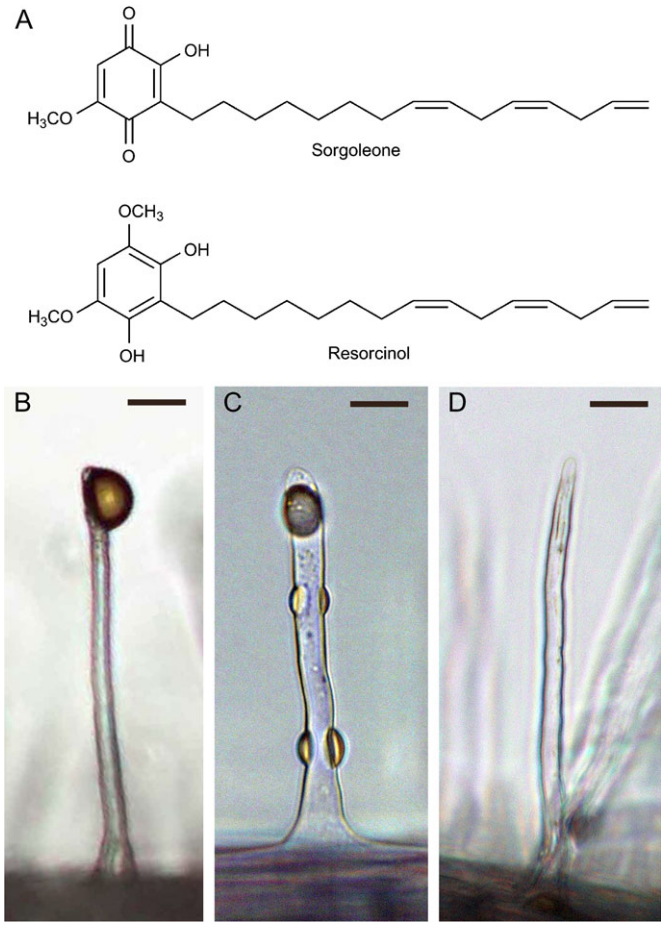

Fig. 1. (A) Structure of sorgoleone and a related lipid resorcinol present in the oily droplets exuding from the root hairs of sorghum. (B) Oily droplet exuding from sorghum root hair. (C) Rehydrated sorghum root hair where most of the oily droplet at the tip has been washed off and additional oil exudes along the shaft of the hair. (D) Sorghum root hair free of sorgoleone after several subsequent washes with water. Bars in (B), (C), and (D) represent 10 μm.

a reduction in shoot growth, with little or no effect on root growth (Weston *et al.*, 1997). Sorgoleone applied to soil is easily recovered within 1 h of application (85%). However, the recovery decreases over time, although low levels of sorgoleone are extractable after 6 weeks. Sorgoleone appears to degrade slowly to as yet uncharacterized metabolites (Weston *et al.*, 1997).

The molecular target sites affected by sorgoleone include photosynthetic and mitochondrial electron transport (Rasmussen *et al.*, 1992; Einhellig *et al.*, 1993; Nimbal *et al.*, 1996; Gonzalez *et al.*, 1997; Rimando *et al.*, 1998) and the enzyme *p*-hydroxyphenylpyruvate dioxygenase (Meazza *et al.*, 2002). While sorgoleone is a potent inhibitor of PSII in isolated chloroplasts, Hejl and Koster (2004) have shown that photosynthesis of 7–10-d-old plants does not appear to be affected by this lipid benzoquinone. This group instead suggested that the mode of action of sorgoleone involves the inhibition of root H^+-ATPase activity and water uptake. Furthermore, they correctly pointed out that it remains to be established whether this highly lipophilic natural herbicide is actually taken up by roots and translocated to the foliage where it must enter the chloroplast and inhibit PSII in the thylakoid membrane (Hejl and Koster, 2004).

Therefore, while sorgoleone interferes with several physiological and biochemical processes *in vitro*, its primary mechanism of action *in planta* remains unclear. In particular, the problems posed by the spatial separation between the location of sorgoleone exudation (soil) and its putative site of action (foliage) as a PSII inhibitor have not been addressed to date. This paper aims to bridge this gap by determining whether the production of sorgoleone by sorghum root hairs is a dynamic process and whether sorgoleone can interfere with photosynthetic electron transport *in planta*.

Materials and methods

Plant materials

Seeds of the sorghum cultivar SX17 (*S. bicolor*×*S. sudanense*) were purchased from Dekalb Genetics (Dekalb, IL). Velvetleaf (*Abutilon theophrasti* Medic.) seeds were purchased from Azlin Seed Service, Leland, MS. Wild-type and triazine-resistant redroot pigweed (*Amaranthus retroplexus* L.) seeds were purchased from Herbiseed (Twyford, UK).

Large-scale sorgoleone production

Sorghum seeds were surface-sterilized by soaking for 10 min in 10% bleach and rinsing with deionized water. Seeds were grown in the dark on a capillary mat system as described previously, except that the heating element was omitted, and seeds were placed directly on the screen (Czarnota *et al.*, 2001). Roots were harvested 6–7 d after planting by excising the root sections extending below the screen.

Biosynthesis of ^{14}C-ring labelled sorgoleone

Seeds were grown in the presence of U-^{14}C-acetate (100 mCi mmol^{-1}) (American Radiolabelled Chemicals, Inc., St Louis, MO) for labelling sorgoleone. The procedure was similar to that used to obtain ^{13}C-labelled sorgoleone (Dayan *et al.*, 2003), except that 50 μCi of U-^{14}C-acetate was added to each plate. The dishes were sealed and incubated in the dark at 25 °C in an E30LED3 plant growth chamber (Percival Scientific Inc. Perry, Iowa 50220 USA). All labelling procedures were done under low-intensity green light to prevent the formation of anthocyanins by sorghum roots.

Extraction and purification of sorgoleone

Sorghum roots were immersed in $CHCl_3$ for 3 min, and the extract was then decanted through a fluted glass funnel lined with Whatman No. 1 filter paper to remove root debris. The crude sorgoleone extract from the mat system (100 mg) was applied to 20×20 cm silica F_{254} glass-backed

preparative plates (Analtech, Newark, DE) and developed in hexane-isopropanol (9:1, v/v). The band containing sorgoleone (R_F=0.35) was scraped off the plates and eluted with $CHCl_3$-MeOH (19:1, v/v). The sample was concentrated under N_2 flow, yielding 30–40 mg purified sorgoleone per large batch. This standard was stable for several months when stored at 4 °C. ^{14}C-ring labelled sorgoleone was purified using the same method.

Composition and dynamism of individual oily droplets released by sorghum root hairs

Sorghum seedlings were grown in Petri dishes in darkness for 4 d or 10 d. The oily droplets accumulating at the tips of root hairs located in a region between 2–4 cm distal from the seed were collected using an SPME (solid phase microextraction) 100 μm polydimethylsiloxane (PDMS) probe (Supelco, Bellefonte, PA) (Fig. 2). The probe was conditioned at 250 °C for 30 min prior to collecting the exudate. The probe was attached to a MN-151 micromanipulator (Narishige International USA, Inc., East Meadow, NY) and all manipulations were done using an Olympus SZX12 microscope (Olympus America, Inc., Melville, NY) equipped with a Q-Color 5 camera. For each treatment, 200 droplets were collected per fibre. The content of each fibre was analysed by GC-MS following the methods of Erickson *et al.* (2001). The GC-MS system consisted of an Agilent 6890 gas chromatograph and an Agilent 5975 quadrupole mass spectrometer. An HP-5MS column (J&W Scientific), 30 m×0.25 mm ID×0.25 μm film thickness was used. SPME fibres were desorbed manually in a 250 °C injection port for 2 min in splitless mode. The initial oven temperature was 70 °C for 2 min, increased by 20 °C min^{-1} to 250 °C, then by 5 °C min^{-1} to 300 °C, and held there for 6 min. The retention times of sorgoleone and its resorcinol analogue were 17.3 min and 18.1 min, respectively, under these conditions. Relative amounts of these compounds were quantified based on a comparison of the total ion chromatogram peak areas.

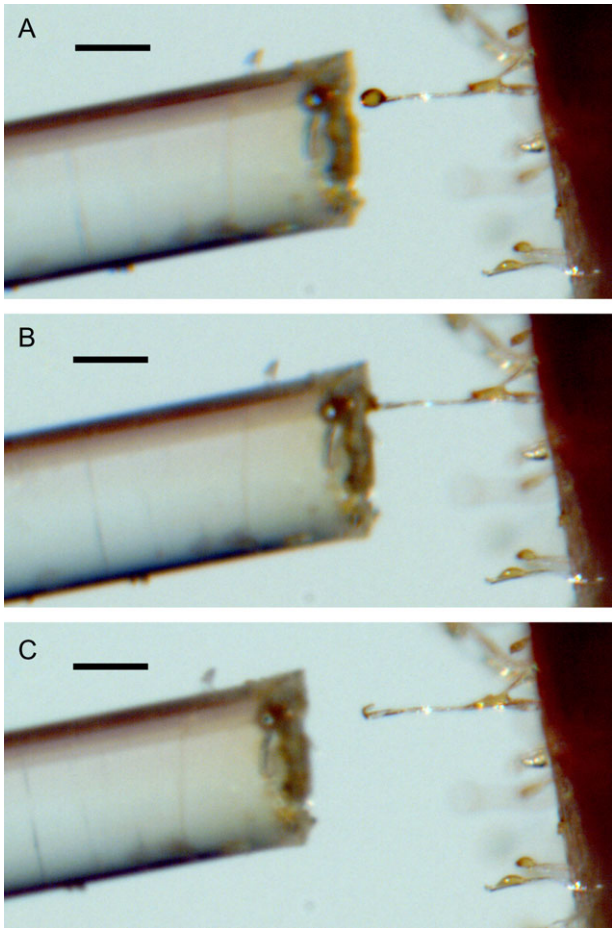

Fig. 2. Solid-phase microextraction of the oily droplets accumulating at the tip of sorghum root hairs using a PDMS probe mounted on a micromanipulator. (A) Before contact with root hair with oily droplet still present; (B) Probe in contact with the root hair; (C) After contact with the root hair showing that the oily droplet was collected by the PDMS fibre. Bars in (A), (B), and (C) represent 20 μm.

The ability of root hair to produce sorgoleone over time was tested by measuring the amount of exudate released over time after washing the roots of 4-d-old seedlings with water. Measurements were made immediately after washing and at set times for up to 7 d. The exudate was collected from sets of 15 root sections with $CHCl_3$ as described above.

Photosynthetic efficiency measurement by chlorophyll fluorescence

Velvetleaf seeds were surface-sterilized in 10% bleach for 20 min and rinsed three times with sterile deionized water before scarification for 30 s using a Model 6K030G seed scarifier (Forsberg, Inc., Thief River Falls, MN). Seeds were germinated in a peat-lite soilless medium at 25 °C with 16 h d at 200 μmol m^{-2} s^{-1}.

Ten millimetre leaf discs were cut from 7–10-d-old leaves and placed in a solution of 0.01% Tween-20 containing atrazine or sorgoleone at concentrations of 0 (solvent control=1.0% DMSO), 1, 3, 10, 33, or 100 μM. Three leaf discs were placed on their adaxial side in each solution in 60×15 mm culture dishes and placed in the dark with gentle rocking. After 5.5 h, the plates were transferred to red light for 30 min before measurements were made.

Photosynthetic quantum yield (Y) and electron transport rate (ETR) were measured using a pulse-modulated fluorometer (Opti-Science, Model OS5-FL, Tyngsboro, MA). The instrument was set on Kinetic Mode and adjusted so that the initial Ft (instantaneous fluorescence signal) value in the control samples was approximately 210. Quantum yield was determined by the following light treatment: each cycle consisted of a 0.8 s pulse of saturating light generated with a laser diode actinic source to saturate PSII, followed by a 1 s far-red light pulse used to re-oxidize PSII, and a 20 s delay to allow PSII to regain steady-state conditions. A total of eight cycles were performed for each sample.

A time-course experiment was performed by incubating the leaf discs of young leaves on a solution of 0.01% Tween-20

containing 1.0% DMSO, 100 μM sorgoleone or 30 μM atrazine in 100×20 mm culture dishes. Samples were placed in the dark with gentle rocking. Three discs from each treatment were measured at 0, 1, 2, 3, 4, 5, and 6 h post-treatment. *ETR* was measured as described above.

Effect of leaf age on phytotoxicity of sorgoleone

Velvetleaf seeds were germinated as described for the dose–response experiments. Cotyledonary or true leaf discs were obtained from plants at different developmental stages. The discs were exposed to either 100 μM sorgoleone or 33 μM atrazine prior to fluorescence analysis as described above.

Alternatively, velvetleaf seeds were planted in trays containing a Metro-Mix 350 potting soil (Sun Gro Horticulture; Bellevue, WA 98008) and allowed to germinate in a growth chamber at 25 °C with 16/8 h light/dark cycle and 150 μmol m^{-2} s^{-1} light. Sorgoleone was dissolved in acetone and applied (10–60 μg) with a micropipette directly to the hypocotyls and cotyledons of the seedlings as they emerged from the soil. The plants were grown under the same conditions for an additional 3 weeks after treatment. At that time, the seedlings were harvested and dried at 60 °C for several days before recording the dry weights (dw). A similar experiment was designed where 20 μg of sorgoleone or 5 μg of atrazine were applied to emerging hypocotyls and the photosynthetic *ETR* was measured at different times from 0–50 h post-treatment.

Uptake and translocation of sorgoleone by measuring chlorophyll fluorescence and monitoring movement of ^{14}C-ring labelled sorgoleone

Velvetleaf seeds were surface-sterilized and germinated in Petri plates containing 2 ml of sterile deionized water. Individual 7-d-old seedlings were transferred to 25×100 mm flat-bottomed culture tubes containing 5 ml of Hoagland's solution and placed in a CU-32L plant growth chamber (Percival Scientific Inc. Perry, Iowa 50220 USA) set at 25 °C and 16/8 light/dark cycle for a 7 d acclimation period. The medium solution was replenished as needed during the experiment. After the period of acclimation, culture media containing either 100 μM sorgoleone or 33 μM atrazine was placed in the tubes and photosynthetic electron transport was monitored over the next 24 h. Fluorescence analysis was as described above, except that the probe was positioned at 60° angle over the leaf still attached to the plant using a clamp with a 5 mm diameter opening exposing part of the leaf tissue.

Uptake and translocation of radiolabelled sorgoleone was done on seedlings grown as described above, except that the seedlings were transferred to fresh nutrient solutions containing 5 μCi of ^{14}C-ring labelled sorgoleone. The seedlings were removed from the labelled solution 3 d later and exposed to a phosphoscreen (Perkin-Elmer, Downers Grove, IL 60515) for 24 h. The autoradiograms were visualized using a Cyclone Plus phosphoimager (Perkin-Elmer, Downers Grove, IL 60515).

In vivo *effect of sorghum density on the growth of velvetleaf, and wild-type and triazine-resistant redroot pigweed (*Amaranthus retroflexus*)*

The effect of sorghum density was tested on the growth of velvetleaf, wild-type and triazine-resistant pigweed. Plastic pots (12 cm diameter) were filled with coarse builder's sand and placed in 17 cm saucers with Miracloth lining the bottom of the pots to prevent loss of medium. Each pot received a total of nine plants in the following weed:sorghum ratios: 9:0, 6:3, 3:6, and 0:9. Pots were watered by overhead irrigation daily, with the addition of Hoagland's Modified Basal salt mixture supplemented with additional iron twice a week. Plants were grown for 30 d and their photosynthetic efficiency was measured as described above. Each individual plant height was measured prior to harvesting the shoots for dw measurement.

*Effect of sorgoleone and atrazine on photosynthetic oxygen evolution of isolated chloroplasts of wild-type and triazine-resistant redroot pigweed (*Amaranthus retroflexus*)*

Chloroplasts of wild-type and triazine-resistant redroot pigweed were obtained as published before (Kagan *et al.*, 2003), except that the chloroplasts were further purified by centrifugation on a 30:52% sucrose step gradient at 30 000 *g* for 1 h at 4 °C (Dayan *et al.*, 1998). These chloroplast preparations were incubated with 0–10 μM of either sorgoleone or atrazine, and photosynthetic oxygen evolution was measured using a DW1 oxygen probe (Hansatech Instruments Ltd, Norfolk, UK) as described previously (Kagan *et al.*, 2003).

Statistical analysis

All statistical analyses were performed using the SAS statistical software program (SAS, 2004). Where appropriate, experiments were analysed using the dose–response curve module (Ritz and Streibig, 2005) of R version 2.2.1 (R-Development-Core-Team, 2005). Means and standard deviations were obtained using the untransformed data.

Results

Composition and exudation of sorghum oily droplets

Collection of 200 droplets with the PDMS probes (collected individually and pooled) provided ample material for GC-MS analysis (Fig. 2). The composition of the oily droplets consists of a 1:1 ratio of sorgoleone and its dimethylated resorcinol analogue in both 4-d-old and 10-d-old roots (Table 1).

The dynamism of sorgoleone production was studied by comparing the exudate recovered from sections of 4-d-old roots to the amount released over time from thoroughly washed roots (Table 2). Nearly all lipophilic exudate was removed by the wash. The release of newly synthesized sorgoleone is noticeable within 24 h of the wash. However, the amount of exudates returned to typical levels within 7 d.

Table 1. Microanalysis of 200 oily droplets collected from a similar region of the 4-d-old and 10-d-old sorghum roots

Data represent means and SD.

Root age	Composition (%)[a]	
	Sorgoleone	**Resorcinol**
4-d-old	48.9±9.5 a	51.1±8.5 a
10-d-old	47.1±2.8 a	52.9±3.5 a

[a] Numbers in columns followed by the same letter are not different at $P < 0.05$ according to Duncan's multiple range test.

Table 2. Amount of root exudate extracted from sorghum root segments before and after washing with water

Numbers represent the mean of three replications followed by standard deviation.

Tissue	Exudate[a](μg mg^{-1} root dw)
Unwashed	15.8±10.1 ab
Washed	1.9±1.8 c
Days after wash	
0.5	0 c
1	5.6±3.2 bc
2	7.2±1.4 bc
4	9.1±5.4 bc
7	24.1±8.0 a

[a] Numbers in columns followed by the same letter are not different at $P < 0.05$ according to Duncan's multiple range test.

Effect of sorgoleone on photosynthetic efficiency of velvetleaf leaf discs

Exposing leaf discs of 7-d-old velvetleaf seedlings to increasing concentrations of sorgoleone resulted in a dose-dependent inhibition of *ETR* (Fig. 3A). However, 100 μM sorgoleone caused only a 50% reduction of *ETR*, whereas atrazine resulted in 100% inhibition. A subsequent time-course experiment showed that the effect of sorgoleone on PSII activity was slower than that of atrazine (Fig. 3B).

The potency of sorgoleone is greatly affected by leaf age. Indeed, *ETR* of 3-d-old and 4-d-old cotyledon discs was completely inhibited by 100 μM sorgoleone (Fig. 4). However, leaf discs from 7-d-old plants were significantly less sensitive, and sorgoleone has no effect on older leaves (Fig. 4). By contrast, 33 μM atrazine completely inhibited *ETR* in leaves of all ages.

Direct application of 20 μg or more of sorgoleone to the hypocotyls and cotyledons of velvetleaf emerging from the soil was also phytotoxic to the seedlings (Fig. 5A). This was accompanied with a time-dependent inhibition of *ETR*. The effect was not as strong as that obtained with 5 μg atrazine (Fig. 5B).

Uptake and translocation of sorgoleone by measuring chlorophyll fluorescence and monitoring movement of ^{14}C-ring labelled sorgoleone

Incubating the roots of velvetleaf seedlings in 100 μM sorgoleone solution did not affect the photosynthetic *ETR*

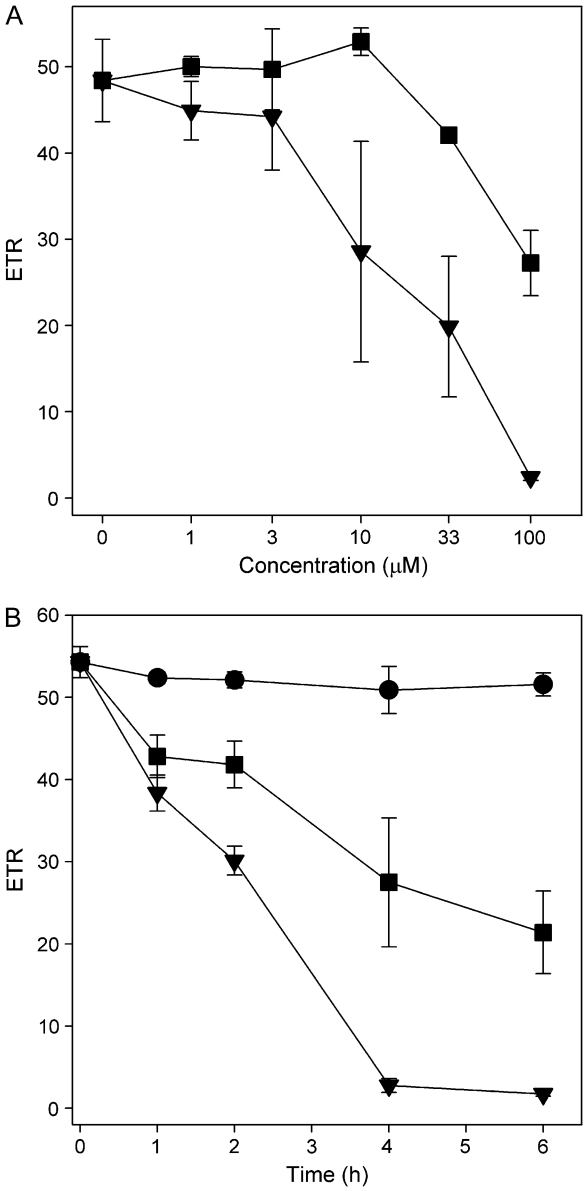

Fig. 3. (A) Dose–response curves showing the effect of sorgoleone (filled squares) or atrazine (filled inverted triangles) on photosynthetic electron transport rate (*ETR*) after 4 h incubation. (B) Time-course illustrating the effect of 100 μM sorgoleone (filled squares) or 33 μM atrazine (filled inverted triangles) on *ETR*, relative to controls (filled circles).

in the foliage (Fig. 6A). On the other hand, *ETR* of plants exposed to 33 μM atrazine decreased very rapidly, with 100% inhibition after 3 h.

In order to monitor the movement of sorgoleone in plant tissue, 55 mg of ^{14}C-ring labelled sorgoleone (specific activity, 196 μCi mmol^{-1}) was generated by growing sorghum seedlings in the presence of ^{14}C-acetate. ^{14}C-ring labelled sorgoleone exposed to roots of velvetleaf seedings did not translocate to the foliage (Fig. 6B, C), which is consistent with the previous observation that photosynthetic *ETR* was not affected in leaves of velvetleaf plants.

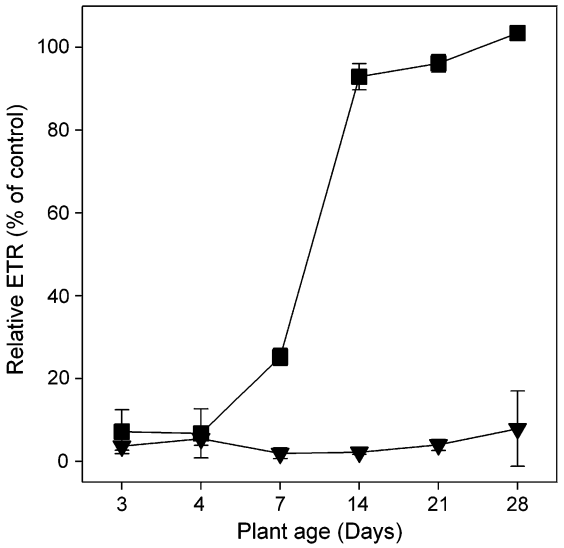

Fig. 4. Effect of sorgoleone (filled squares) and atrazine (filled inverted triangles) on photosynthetic electron rate of velvetleaf leaf tissues of different age. Measurements at 4-d-old and 7-d-old plants were done on cotyledon discs. All other time points were done on leaf discs. The samples were incubated for 6 h on 100 μM sorgoleone or 33 μM atrazine prior to analysis.

In vivo *effect of sorghum density on the growth of velvetleaf, and wild-type and triazine-resistant redroot pigweed (*Amaranthus retroflexus*)*

The allelopathic effect of sorghum was tested by monitoring the height and dw of velvetleaf, and wild-type and resistant pigweed plants grown in the presence of sorghum. Both wild-type and atrazine-resistant pigweed biotypes were more sensitive to the presence of sorghum than velvetleaf (Table 3). The plant heights of wild-type and atrazine-resistant pigweed biotypes decreased by 50–60% when three sorghum plants were present, relative to the sorghum-free pots. On the other hand, the height of velvetleaf did not change under these conditions. The velvetleaf plants were only 30% shorter when grown in the presence of six sorghum plants, relative to controls. The density effect of sorghum plants was even greater on the dw of the weeds (Table 3), with up to 90% reduction of biomass when pigweed was grown in the presence of six sorghum plants. As with plant height, the plant dry weight of velvetleaf seedlings was less affected by the presence of sorghum. There was no difference in F_v/F_m between weed seedlings grown alone or in the presence of sorghum plants, except for a slight reduction of the wild-type pigweed in 6:3 ratio, relative to control.

*Effect of sorgoleone and atrazine on photosynthetic oxygen evolution of isolated chloroplasts of wild-type and triazine-resistant redroot pigweed (*Amaranthus retroflexus*)*

The potency of sorgoleone and atrazine were tested on isolated chloroplasts of wild-type and triazine-resistant redroot pigweed in order to confirm that the seedlings

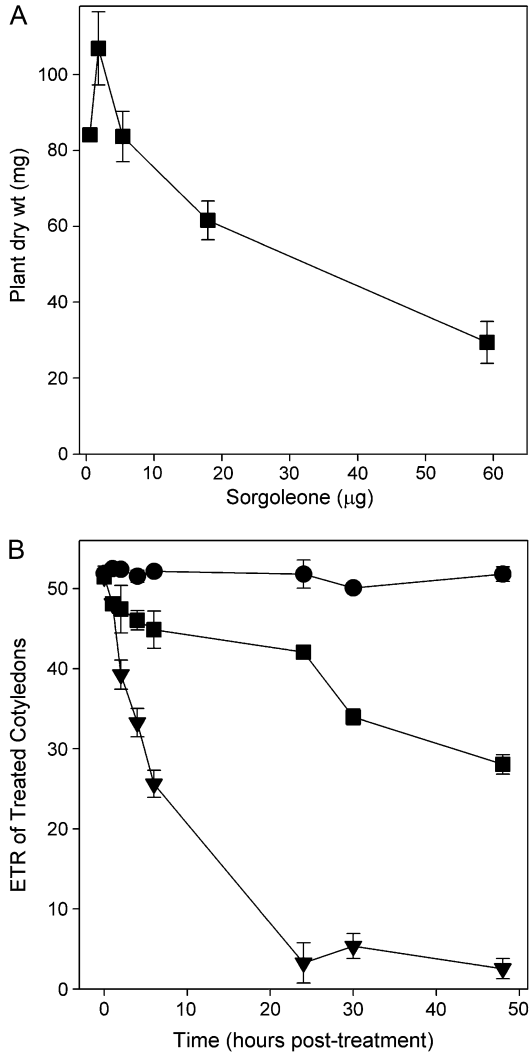

Fig. 5. (A) Effect of sorgoleone dissolved in acetone on emerging velvetleaf hypocotyls. Biomass measurements were made three weeks after treatment. (B) Effect of 20 μg sorgoleone (filled squares) and 5 μg atrazine (filled inverted triangles) on photosynthetic electron rate when applied directly on the surface of velvetleaf cotyledons, relative to solvent control (filled circles).

obtained from Herbiseed were indeed triazine resistant. Dose–response curves confirmed that the triazine-resistant biotype of pigweed was at least 80 times more resistant to atrazine than the wild-type (Fig. 7). Interestingly, resistance to atrazine did not correlate with resistance to sorgoleone, with chloroplast preparations from both biotypes showing similar sensitivity to this lipid benzoquinone.

Discussion

Sorghum species exude an array of lipid quinones and resorcinols from their roots (Netzly and Butler, 1986; Rimando *et al.*, 1998; Erickson *et al.*, 2001; Czarnota *et al.*, 2003*b*; Kagan *et al.*, 2003; Rimando *et al.*, 2003). Sorgoleone (Fig. 1) is one of the main components of that exudate. This lipid benzoquinone is phytotoxic and is able

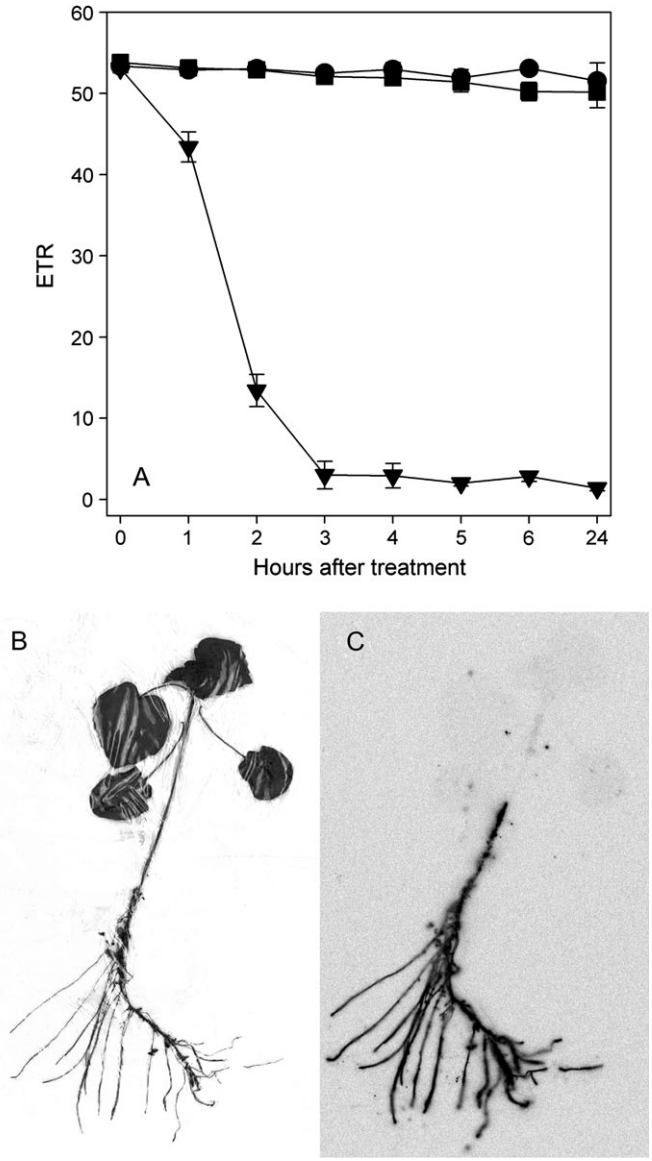

Fig. 6. Effect of 100 μM sorgoleone (filled squares) or 33 μM atrazine (filled inverted triangles) applied to the roots on photosynthetic electron transport rate (*ETR*) of velvetleaf, relative to solvent controls (filled circles) on 3-week-old seedlings. (B) Picture of a representative velvetleaf seedling used in the time-course shown in (A), as well as those used for ^{14}C-ring labelled sorgoleone uptake experiment. (C) Autoradiogram of a velvetleaf seedling exposed to ^{14}C-ring labelled sorgoleone through the roots.

to interfere with a number of physiological processes *in vitro*.

Extraction of the exudate has traditionally been achieved by dipping the roots in neutral or acidified $CHCl_3$ or CH_2Cl_2 for a few minutes. This method has proved very efficacious for the extraction of a large amount of sorgoleone and has permitted the discovery of a host of sorgoleone and resorcinol analogues (Netzly and Butler, 1986; Rimando *et al.*, 1998; Czarnota *et al.*, 2003*b*; Kagan *et al.*, 2003). However, this approach does not discriminate between the oily droplet accumulating at the tip of the root hairs and other components adhering to the root epidermis.

Table 3. *Effect of sorghum density on height, dry weight and photosynthesis of velvetleaf, wild-type* A. retroflexus *and triazine-resistant* A. retroflexus

Means are followed by standard deviation.

Weed:sorghum ratio	Weed species		
	Velvetleaf	**WT-pigweed**	**R-pigweed**
	Plant height (cm)[a]		
9:0	25.8±1.0 a	16.9±1.4 a	13.7±0.8 a
6:3	25.7±3.6 a	6.8±1.1 b	6.9±2.1 b
3:6	19.0±3.2 b	5.8±1.0 b	5.7±1.4 b
	Plant dry weight (mg)[a]		
9:0	464±81 a	250±22 a	256±71 a
6:3	382±46 b	39±9 b	73±28 b
3:6	286±26 c	26±16 b	44±16 b
	Photosynthesis (F_v/F_m)[a]		
9:0	0.826±0.002 a	0.609±0.016 a	0.584±0.062 a
6:3	0.826±0.015 a	0.532±0.036 b	0.479±0.108 a
3:6	0.830±0.017 a	0.565±0.034 ab	0.504±0.078 a

[a] Numbers in columns followed by the same letter are not different at $P < 0.05$ according to Duncan's multiple range test.

Therefore, the actual composition of the individual droplets of exudate has remained unknown. A new method was developed to analyse the composition of individual droplets exuding at the tip of sorghum root hair using SPME fibres (Fig. 2). The exudate collected at the tip of 4-d-old and 10-d old root hair consists of a 1:1 ratio of sorgoleone and its dimethylated analogue (Table 1), which is similar to that reported with an acidified CH_2Cl_2 extract of roots (Erickson *et al.*, 2001). The production of sorgoleone is dependent on the presence of root hairs (Yang *et al.*, 2004) and is mostly constitutive and proportional to the root biomass (Dayan, 2006). Analysis of individual droplets indicates that the 1:1 ratio does not change over time.

Previous work reported that the amount of exudate produced by sorghum root hairs is constant over time, reaching approximately 20 μg of sorgoleone mg^{-1} dw of root (Dayan, 2006). The more detailed experiments used in this study suggests that the relatively constant amount of sorgoleone produced per root dry weight may be due to a feed-back inhibition mechanism regulating the production of this bioactive natural product. Exudation of lipid quinones and resorcinols apparently stops once droplets (approximately 20 μg mg^{-1} root dw) accumulate at the root tip (Table 2). However, gentle removal of the exudate by washing the roots with water releases the inhibition and exudation resumes until approximately 20–25 μg of exudate mg^{-1} dw of root is released (Table 2). This suggests that sorghum roots have the potential continuously to exude lipophilic benzoquinones and resorcinols in the soil as droplets of exudates are released into the soil and the soil solution surrounding root hairs.

The terminology used to describe work done on sorgoleone requires some clarification. Indeed, the term sorgoleone refers specifically to 2-hydroxy-5-methoxy-3-[(Z,Z)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone (Fig. 1), but it has also

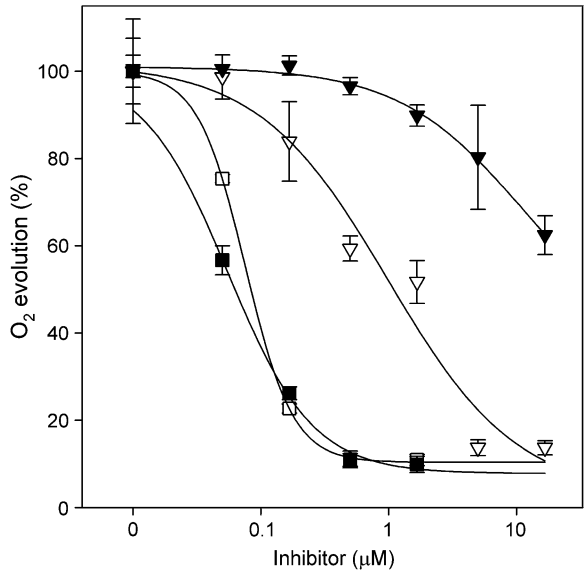

Fig. 7. Effect of sorgoleone on oxygen evolution from thylakoid membranes isolated from wild-type and triazine-resistant redroot pigweed (*Amaranthus retroflexus*). (Open inverted triangles), wild-type with atrazine; (filled inverted triangles), resistant with atrazine; (open squares), sensitive with sorgoleone; (filled squares), resistant with sorgoleone.

been used to describe the group of structurally related lipophilic *p*-benzoquinones also present in small amounts in the oily droplets exuding from the root hairs of sorghum (Chang *et al.*, 1986; Netzly *et al.*, 1988; Kagan *et al.*, 2003). The situation is now further confounded by the fact that the exudate also contains an equivalent amount of the resorcinol analogues (Erickson *et al.*, 2001). Therefore, the subsequent experiments in this report were carried out with purified sorgoleone, which is 90% or more of the lipid benzoquinone shown in Fig. 1.

Sorgoleone has been tested on several molecular target sites. This lipid benzoquinone can interrupt photosynthetic and mitochondrial electron transport by mimicking the natural electron acceptors plastoquinones and ubiquinone, respectively (Rasmussen *et al.*, 1992; Einhellig *et al.*, 1993; Nimbal *et al.*, 1996; Gonzalez *et al.*, 1997; Rimando *et al.*, 1998) and inhibit the activity of *p*-hydroxyphenylpyruvate dioxygenase, a key enzyme in plastoquinone biosynthesis (Meazza *et al.*, 2002). In addition, sorgoleone can inhibit root H^+-ATPase activity and water uptake (Hejl and Koster, 2004).

Since sorgoleone inhibits photosynthesis of isolated chloroplasts at submicromolar concentrations, it has been postulated that the herbicidal activity of sorgoleone is associated with inhibition of PSII. However, Hejl and Koster (2004) have shown that photosynthesis is not affected by this lipid benzoquinone in 7–10-d-old plants grown hydroponically in solutions containing sorgoleone. Consequently, they questioned whether this highly lipophilic natural herbicide can actually be taken up by roots and translocated to the foliage where it must enter the chloroplast and inhibit PSII in the thylakoid membranes (Trebst and Draber, 1986; Sobolev and Edelman, 1995).

A series of experiments was designed to understand better the absorption and mobility of sorgoleone in plants. Since sorgoleone is non-polar, with a log*P* of 6.1 (Trezzi *et al.*, 2006), preliminary studies eliminated most physiological barriers between root uptake and translocation to the foliage by floating leaf discs of velvetleaf on sorgoleone solutions. Velvetleaf was selected as a dicotyledonous species known to be sensitive to sorgoleone (Einhellig and Souza, 1992). This system showed that sorgoleone can be absorbed through the cuticle and epidermis of young plants and reach its molecular target site in the thylakoid membranes. As expected, this process was concentration and time dependent (Fig. 3A, B). Although sorgoleone is a stronger inhibitor of photosynthesis than atrazine in isolated chloroplast membranes (Rimando *et al.*, 1998), the opposite was observed in this experiment, suggesting that absorption of this lipophilic benzoquinone is a limiting factor on the efficacy of exogenously applied sorgoleone.

The inhibitory activity of sorgoleone on photosynthesis was strongly dependent on the age of the leaf tissue, with complete inhibition of *ETR* of cotyledonary tissues (3–4-d-old) exposed to 100 μM sorgoleone (Fig. 4). However, much less inhibition was measured on the very young first leaves (7-d-old), and no inhibition at all on tissues 14 d or older. By contrast, inhibition with atrazine was strong on tissues of all ages.

Since the leaf discs assays determined that sorgoleone could inhibit *ETR* on young photosynthetic tissues, the allelochemical was then applied directly to the hypocotyls and cotyledons of velvetleaf emerging from the soil. An application of 20 μg or more proved to be phytotoxic to the seedlings (Fig. 5A). The tissues showed signs of necrosis and the phytotoxicity appears to be associated with an inhibition of *ETR*, confirming that sorgoleone can inhibit photosynthesis in very young tissues (Fig. 5B). However, inhibition increased slowly over time (Fig. 5B), further suggesting that leaf penetration and/or membrane partitioning of this highly lipophilic molecule limits the amount reaching its molecular target site (Donovan, 2007).

Having demonstrated that sorgoleone can inhibit photosynthesis in very young plants via absorption through hypocotyl and cotyledonary tissues, other experiments evaluating root uptake and translocation of sorgoleone were performed. Exposing the roots of 3-week-old velvetleaf seedlings to sorgoleone for 24 h had no effect on photosynthesis, suggesting that the molecule was not translocated to the foliage (Fig. 6A). This was confirmed on the autoradiograms of velvetleaf seedlings exposed to ^{14}C-ring labelled sorgoleone. None of the radioactivity could be detected in the foliage, confirming that sorgoleone is not translocated to the foliage through the transpiration stream. This is not unexpected, since lipophilic molecules (compounds with log*P* values greater than 4) have no xylem systemicity (Sicbaldi *et al.*, 1997; Briggs *et al.*, 1982; Donovan, 2007). The radioactivity on the roots could not be washed off, suggesting that sorgoleone had entered the roots. Although this process can be the result of uptake into the aqueous phase in roots for weak acids and water-soluble

compounds, the absorption of sorgoleone is most likely the result of partitioning in lipophilic root solids, as typically observed with molecules with log$P$ >4 (Briggs *et al.*, 1982; Trapp, 2000).

Sorgoleone can act as a pre-emergence herbicide affecting photosynthesis in very young seedlings (Figs 4, 5). Uptake of sorgoleone may occur when the hypocotyls and cotyledons of developing seedlings come in contact with the root exudate of sorghum as they attempt to emerge from the soil. This is similar to that observed with some lipophilic preemergence herbicides such as the dinitroanilines. These compounds (i.e. oryzalin and trifluralin) partition into emerging plant shoots and roots as the plants germinate in the soil (Upadhyaya and Noodén, 1980). Once absorbed, these lipophilic compounds have little activity beyond the root (Penner, 1971; Fedtke, 1993).

In our progression from simple systems with least variations to more complex ones, our last set of experiments tested the effect of sorghum density on the growth of velvetleaf, and wild-type and resistant pigweed seedlings and whether inhibition of photosynthesis was a factor. The presence of sorghum plants reduced the growth of both wild-type and atrazine-resistant pigweed biotypes (Table 3). Velvetleaf seedlings were affected to a lesser degree. In all, plant biomass (dw) was a more sensitive biometric parameter than plant heights. The dw of either pigweed biotypes decreased by 90%, whereas their shoot heights were 50–60% shorter than the plants grown in sorghum-free pots. However, the F_v/F_m of any of the seedlings was essentially not affected by the presence of sorghum plants (Table 3), which suggests that *ETR* of photosystem II is not inhibited (Peterson *et al.*, 1988; Gleiter and Renger, 1993). This observation is consistent with that reported by others (Hejl and Koster, 2004), and in agreement with our data showing that sorgoleone is not translocated from the roots to the shoots of 3-week-old seedlings (Fig. 6A, B).

It should also be noted that, while sorgoleone is known to compete with atrazine for the same Q_B binding site on photosystem II (Nimbal *et al.*, 1996; Gonzalez *et al.*, 1997), mutations resulting in resistance to atrazine do not lead to cross-resistance to sorgoleone (Fig. 7). This is due to the fact that atrazine belongs to the Ser_{264} family of photosystem II inhibitors (also called the classical family) whereas sorgoleone is from the His_{215} family (also called the quinone or phenolic family). Mutation of Ser_{264} to Gly or Ala causes resistance to triazines, but not to the quinone inhibitors (Oettmeier *et al.*, 1982). Therefore, the fact that both the wild-type and atrazine-resistant pigweed biotypes had similar reductions in growth in the presence of sorghum should not be misinterpreted as sorgoleone having no effect on photosynthesis on young tissues. However, the lack of effect on foliar F_v/F_m confirms that sorgoleone does not affect photosynthesis on older plants.

Finally, the interpretation of the effect of sorgoleone on photosynthesis must be understood as the cumulative contribution of the lipid benzoquinone and resorcinol analogues present in the extract (Table 1). Fortunately, the potency of these analogues on photosynthetic electron transport rate is similar (Kagan *et al.*, 2003; Rimando *et al.*, 2003), therefore, they all contribute equally towards the total activity. On the other hand, the lipid resorcinol analogue is more phytotoxic than sorgoleone (Kagan *et al.*, 2003), so it is thus difficult to determine the respective contributions of the components of the exudate in the sorghum density experiments. These results suggest that the lipid resorcinol analogue deserves greater attention in investigations of the allelopathic effects of sorghum.

In conclusion, the oily exudate is composed of a 1:1 ratio of sorgoleone and its lipid resorcinol analogue. Exudation of these products is modulated by the amount accumulating at the tips of the root hairs. As the phytotoxic exudate is released directly in the soil, its action is similar to a pre-plant incorporated herbicide. One factor that may prolong the persistence of sorgoleone in soil is the fact that sorgoleone may be released continually from the roots during the growing season of sorghum. This 'slow release' of *de novo* synthesized sorgoleone may sustain its concentration in soil over a much longer time than that typically resulting from a single application of a herbicide. Sorgoleone has no effect on the photosynthesis of older plants, but it can cause *in planta* inhibition of photosynthesis in germinating seedlings. Therefore, the mode of action of sorgoleone may be the result of inhibition of photosynthesis in young seedlings in concert with inhibition of its other molecular target sites in older plants.

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