GC-MS Analysis of Hydrophobic Root Exudates of *Sorghum* and Implications on the Parasitic Plant *Striga asiatica*

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Striga asiatica is a parasitic angiosperm that responds to germination stimulants produced by host plants, including many grasses. GC-MS analyses of hydrophobic root exudates of sorghum revealed the root exudates to be composed of fatty acids, resorcinol, and a series of structurally related hydroquinones, three of which were previously unknown. High yields of resorcinol and the hydroquinone series were detected in sorghum. At least one of the hydroquinones induces germination in *Striga*, and the resorcinol is thought to stabilize the hydroquinones in the soil. The previously unknown series of hydroquinones offers insight into the possible biosynthesis of the components of the exudate and their possible importance in initiating *Striga* germination.

Keywords: *Striga; resorcinol; SXSg; sorgoleone; sorgolactone; germination; parasite*

INTRODUCTION

Striga asiatica, witchweed, is an obligate parasitic angiosperm that causes enormous economic damage, food shortages, and human suffering in Africa and Asia (*1*). Its host range includes corn, *Sorghum*, and millet as well as other grasses (*2*, *3*). Its life cycle has at least two recognition events that are important. First, *Striga* seeds germinate in the presence of a host root exuded germination stimulant (*4*, *5*). Next, after the root meristem of the new *Striga* seedling makes contact with a host root, it begins haustorial formation. This results in the eventual connection of the parasite to the host through the xylem (*6*). Water and nutrients are thus rerouted from host to parasite even though the parasite is photosynthetically capable after emergence. The haustorial formation process begins with the recognition of 2,6-dimethoxy-*p*-benzoquinone, which is derived from the host root (*4*, *7*). Without successful recognition of either the germination stimulant or the haustorial inducer, *Striga* cannot begin/complete its lifecycle. The germination stimulant and haustorial inducer therefore play a central role in the successful propagation of the species.

The search for germination stimulants has yielded at least two compounds capable of initiating *Striga* seed germination (Figure 1). SXSg, **1**, was isolated from the hydrophobic exudate of sorghum roots (*4*). Sorgolactone, **2**, was isolated from the aqueous extracts of sorghum roots (*8*, *9*) and its structural similarity to strigol, **3**, a *Striga* germination stimulant isolated from cotton (*10*), a nonhost, was noted. Recently, there has been considerable debate about which of the host-isolated compounds, SXSg or sorgolactone, is "the" germination stimulant important in soil systems in nature (*11*).

Each of the proposed germination stimulants offers explanations for observed phenomena associated with *Striga* seed germination. For example, SXSg, a labile hydroquinone, oxidizes readily to the corresponding quinone, which is not capable of inducing *Striga* germination (*12*). This concentration gradient may explain observations that *Striga* seeds must be within 5−7 mm of the host root to germinate. Lynn and Fate report that

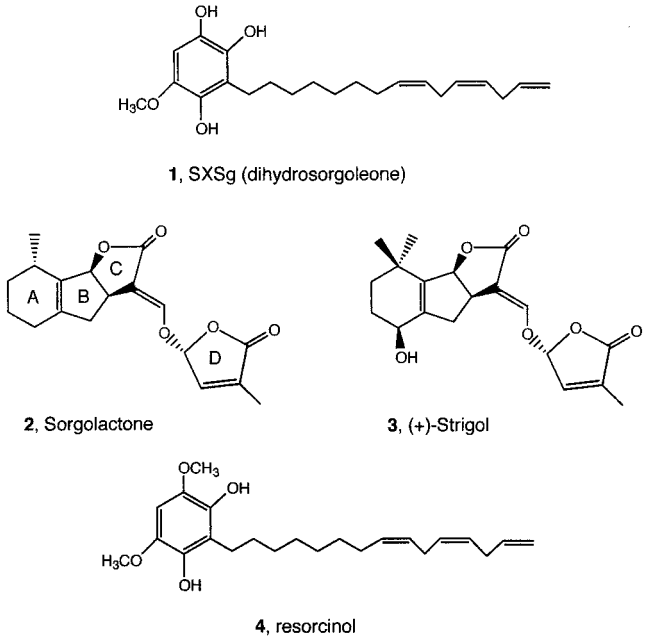

Figure 1. Structures of various compounds implicated in the germination of *Striga* spp.

SXSg is produced along with a closely related resorcinol compound, **4**, that may act to stabilize SXSg, thus enabling sufficient concentrations of SXSg to build up in the soil to induce germination (*13*).

The other compound, sorgolactone, **2**, induces *Striga* seed germination as well (*8*). It may not be labile in some nonsoil systems, as strigol was noted to have stability in agar systems (*12*), but evidence has been presented that it may be labile in soil systems (*14*, *15*). These studies cited soil moisture content and pH as affecting the germination rates of *Striga* plants in response to synthetic strigol analogues. These studies, however, did not directly measure the concentration or monitor for the continued presence of the analogue in the soil, only an expected biological response (germination), and therefore they cannot show conclusively the fate of the

10.1021/jf0111099 CCC: $20.00
Published on Web 10/27/2001

sorgolactone. There could be other reasons for the lack of a measured biological response that are unrelated to the stability, or lack thereof, of sorgolactone.

A criticism recently leveled against SXSg as "the" true germination stimulant is that the concentration needed to produce half-maximal activity is several orders of magnitude higher than that for sorgolactone (*11*). Obvious differences exist between the seed sources and methodology for the studies implicating either compound as the germination stimulant. An important point to note here, however, is that the maximal level of germination SXSg produces is typically 90−100% (*12*), as opposed to levels approaching only 60−70% with sorgolactone (*16*, *17*). Thus, the comparisons of half-maximal activity are not equivalent levels of germination. Whether this is an artifact of the seeds and methodology employed or of the compounds themselves cannot be discerned.

Additionally, the yields of sorgolactone and related compounds from demonstrated hosts thus far has been very low, typically <0.1% from the crude aqueous exudate (*8*, *9*). Furthermore, yields from the plants have not been reported directly, but inferences can be drawn from the papers that the yield of sorgolactone is quite low, judging from the huge numbers of plants grown and harvested during the isolation schemes. The yields for the SXSg from the hydrophobic exudate have not previously been reported, but NMR spectral data suggested it was a major component of the hydrophobic exudate. Although the two sets of exudates are not equivalent, these data appear to question the notion that sorgolactone should be the natural germination stimulant simply because its ED_{50} is lower.

During the course of work looking into SXSg and resorcinol production by resistant and susceptible sorghum hosts, we began to accumulate data that appeared to be inconsistent with the previously proposed structure of SXSg (*7*). This paper describes a more detailed analysis of the hydrophobic root exudates of sorghum plants and will propose that the first described host germination stimulant, SXSg, is actually composed of at least four closely related compounds. Additionally, the data support the notion that the resorcinol compound does not exhibit this same degree of structural variability. The possible implications of these structures on *Striga* germination and on a recently proposed biosynthetic pathway for SXSg and resorcinol (*13*) will also be discussed.

MATERIALS AND METHODS

General Methods. For each experiment, 2 g of seeds (~80 seeds) of *Sorghum sudanense* (Piper) Stapf (L. L. Olds Seed Co., Madison, WI) was surface sterilized with a solution of 1% sodium hypochlorite (100 mL) for 10 min and then washed three times with sterile, distilled H_2O (100 mL). The seeds were transferred aseptically onto moist filter paper in foil-lined aluminum trays, and the trays were covered with foil. Seedlings were allowed to grow in the dark at 27 °C for 4 days before harvesting and dipping.

The roots (~3−4 in.) were dipped in 0.5% $HOAc/CH_2Cl_2$ (50 mL) for 2 s, and the extract was evaporated in vacuo to give the crude exudate. For nonderivitized samples, this crude exudate was dissolved in a small amount of CH_2Cl_2 (typically 1000 μL), and an aliquot of this solution was immediately subjected to GC-MS analysis on a Hewlett-Packard 5890 gas chromatograph and a Hewlett-Packard series 5871 or 5872 mass detector. Injection volumes were typically 1 μL. Chromatography was accomplished by a Hewlett-Packard HP-5 column under the following oven conditions: an initial oven temperature of 70 °C for 2 min, a ramp of 20 °C/min up to a temperature of 250 °C, and a ramp of 5 °C/min to the final temperature of 300 °C, which was held for 6 min. Total run time was 27 min. An alternate oven program used for further separation started with an initial oven temperature of 70 °C for 2 min and proceeded to a ramp of 20 °C/min up to a temperature of 200 °C, followed by a ramp of 5 °C/min to the final temperature of 300 °C, which was held for 6 min. Total run time was 34.5 min.

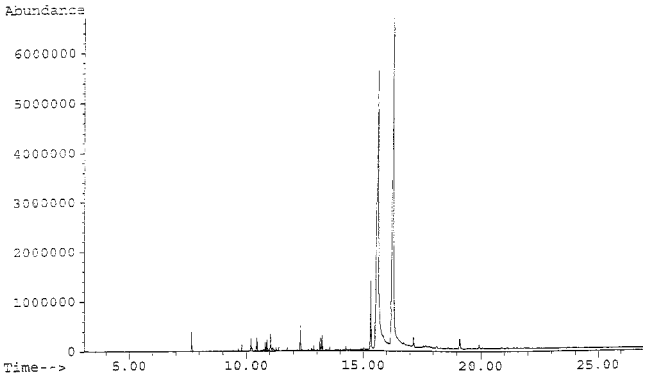

Figure 2. Representative GC trace for the nonderivatized crude hydrophobic exudate from *Sorghum*.

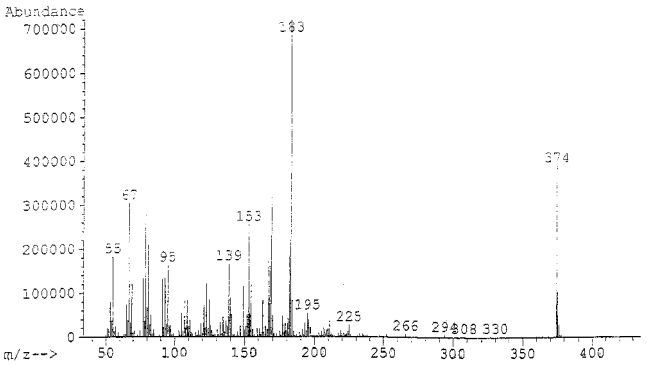

Figure 3. Mass spectrum of the hydrophobic exudate compound eluting at 16.5 min.

Derivatization of Exudates. In the derivatization experiments, the crude exudate was silylated using *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (*18*). The crude exudate was evaporated under N_2 in a small screw-cap vial, and 100 μL of BSTFA was added under an N_2 atmosphere. The vial was sealed and placed in an oven at 125 °C for 1−2 h. After cooling, 1 μL aliquots of the reaction mixture were injected directly into the gas chromatograph, operating under the following conditions: an initial oven temperature of 120 °C for 2 min, a ramp of 20 °C/min to a final temperature of 300 °c, which was held for 5 min. Total run time was 16 min.

RESULTS

The hydrophobic extracts of sorghum roots consisted primarily of two compounds by GC-MS (see Figure 2). The compounds comprising these two peaks typically accounted for 85−95% of the total overall ion abundance.

On the basis of the mass spectra (Figure 3) and structural assignments previously reported for hydrophobic sorghum exudates, it appeared that the compound with a retention time of 16.5 min was the previously discovered resorcinol, **4**. The mass spectrum of this peak had a molecular ion of *m*/*z* 374 and the major retro-ene fragment of *m*/*z* 183 (base peak).

The mass spectrum of the compound(s) at 15.6 min (Figure 4), however, did not appear to be supportive of the structure previously assigned to SXSg. The molec-

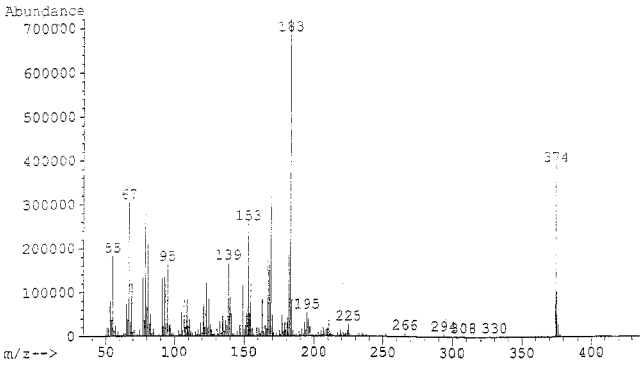

Figure 4. Mass spectrum of the hydrophobic exudate compound(s) eluting at 15.6 min.

ular ion (358) previously reported for the quinone version of SXSg was represented in the mass spectrum of the peak at 15.6 min. However, additional significant ions were present at 359, 360, 362, 363, 364, 365, and 366, indicating the possible presence of more than one closely related compound in addition to the expected hydroquinone. The ion of *m/z* 362 was in fact the most prominent ion of this series and is not consistent with either the quinone (358) or hydroquinone (360) versions of SXSg. Upon sitting exposed to the air for extended periods, the abundance of the *m/z* 362 ion significantly dropped, and this drop was accompanied by an increase of the ion at *m/z* 360, indicating a possible hydroquinone to quinone oxidation had occurred. Base ions for the compound(s) at 15.6 min were *m/z* 168 and 169, consistent with the major benzylic cleavage reported for SXSg (*5*, *13*) and corresponding to QH^+ and QH_2^+, respectively. Other lower molecular weight ions noted by Fate and Lynn (*13*) , that is, *m/z* 141 and 126, were of significant abundance in the mass spectrum for the compound eluting at 15.6 min.

Silylation of the exudate was undertaken to verify the various molecular ions for the compounds eluting at 15.6 and 16.5 min. GC-MS of the silylated samples confirmed a number of things. The hydroquinone and resorcinol structures again comprised the majority of the exudate (85−95% by ion abundance), and the other components were fatty acids of various chain lengths and degrees of unsaturation (data not shown).

The resorcinol compound, upon silylation, produced a compound having a molecular ion at *m/z* 518 (Figure 5), consistent with the double silylation of the previously assigned resorcinol structure, **4**. The ion at *m/z* 518 was accompanied by ions at 519 (40.6% relative to *m/z* 518) and 520 (17.7% relative to *m/z* 518), with no ion visible at *m/z* 521 (see Figure 6). Similar isotopic groupings and ratios were seen for ions at *m/z* 503 (M − 15, accompanied by ions at 504 and 505), *m/z* 488 (M − 30, accompanied by ions at 489 and 490), and *m/z* 458 (M− 60, accompanied by ions at 459 and 460).

The mass spectrum for the silylated hydroquinone, however, was supportive of the notion that the peak present previously at 15.6 min was in fact a mixture of closely related compounds. The triple silylation of SXSg would be expected to produce a compound having a molecular ion at *m/z* 576. This is in fact observed in the spectrum (see Figure 7) and, alhtough it is the most prominent ion in this mass range, it is accompanied by a series of ions. Additional ions (see Figure 8) are present at *m/z* 577 (50.6% relative to 576), 578 (24%), 579 (8%), 580 (30.6%), 581 (14.6%), 582 (6.7%), 583 (2.6%), and 584 (0.6%). Fragment ions at 561 (M − 15) and 546 (M − 30) are accompanied by similar ion groupings and ratios. The base ion for this spectrum is at *m/z* 73 (TMS group). The relative abundances of these groupings of ions are not explained by the mere presence of three A+2 silicon atoms and appear in fact to represent distinct molecular ions for hydroquinones that have three (*m/z* 576), two (*m/z* 578), one (*m/z* 580), or zero (*m/z* 582) double bonds in their C15 side chains.

In an attempt to verify that the peak eluting at 15.6 min was indeed a mixture, further chromatographic resolution was undertaken with a new HP-5 column and the alternate oven temperature program. This resolved the peak previously eluting at 15.6 min into four peaks (Figure 9). Mass spectral analysis of the resulting peaks confirmed the presence of the four related isomers. The mass spectra of the compounds eluting at 27.45 (MW 578), 27.64 (MW 582), 27.9 (MW 576), and 28.30 (MW 580) were supportive of the proposed respective structures, each showing M − 15, M − 30, M − 60, etc., from the molecular ion (indicative of silylation with TMS groups), whereas lower molecular weight ions remained similar for all four compounds. Isotopic effects of the silicon atoms were clearly visible in higher molecular weight ions. The base ion for all four compounds was *m/z* 73. Attempts to further resolve the peak at 16.5 min under similar chromatographic conditions were not successful, again supportive of a single resorcinol compound.

DISCUSSION

The data presented here indicate that sorghum roots exude two major classes of hydrophobic compounds (besides fatty acids). The first class appears to comprise a single compound, resorcinol, **4**. The mass spectrum of the nonderivatized compound is consistent with a single compound. Silylation of this compound produced a compound having a molecular ion consistent with the presence of two A+2 elements (silicon). The molecular ion of *m/z* 518 offers confirmation of the nonderivatized sample's molecular ion at *m/z* 374.

The other class, the SXSg series or hydroquinones, comprises at least four different compounds. The mass spectrum of the nonderivatized sample is consistent with the presence of compounds with differing degrees of unsaturation in the side chain. Hydroquinones containing three, two, one, and zero double bonds in the C15 side chain are represented. Three predominant facts support the assumption that the molecular weight differences result from the side chain. First, the major benzylic (or retro-ene) cleavage of *m/z* 168/169 indicates that upon the loss of the side chain, molecular weights for the fragment ring portions are identical. Second, upon exposure to air the *m/z* 362 ion decreases in relative intensity and the *m/z* 360 ion increases in relative intensity. This is consistent with at least one of the hydroquinones undergoing oxidation to the corresponding quinone. Third, the ion groupings apparent around the molecular ion in the nonderivatized sample are not present in fragments (i.e., the retro-ene cleavage, etc.) where the loss of the alkyl side chain has occurred.

The silylated version of this compound shows a similar ion grouping around the molecular ion and higher molecular weight fragments, supporting the presence of hydroquinones with differing side chains. Resolution of the initial mixture clearly shows the presence of the four derivatized hydroquinones having masses and fragmentation consistent with variations in

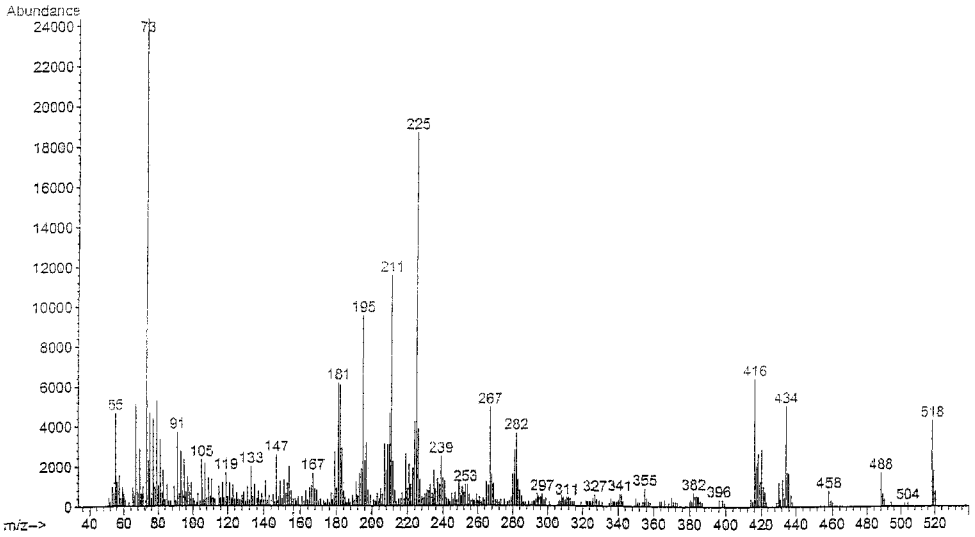

Figure 5. Mass spectrum of the TMS derivative of the compound eluting at 16.5 min, identified as resorcinol, **4**.

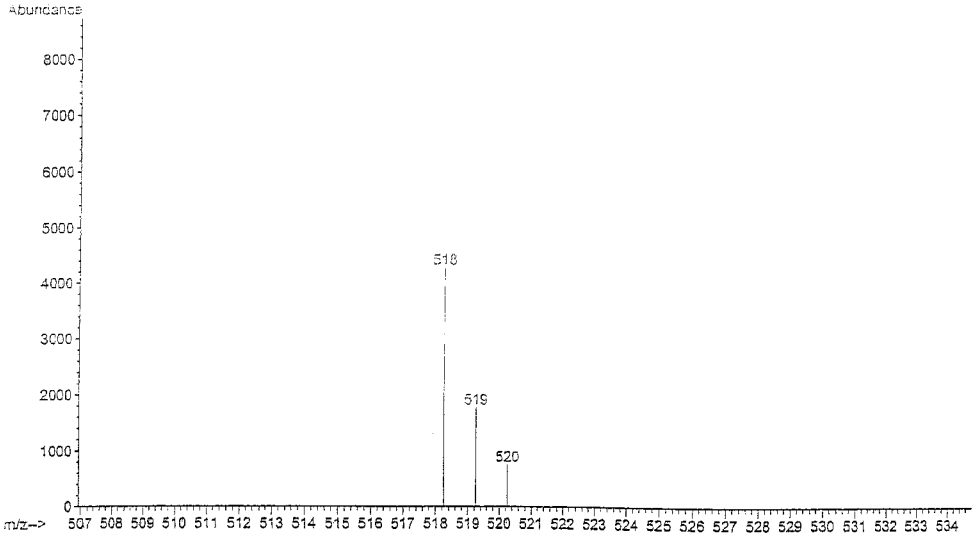

Figure 6. Detail of the molecular ion area, *m*/*z* 518, of Figure 7.

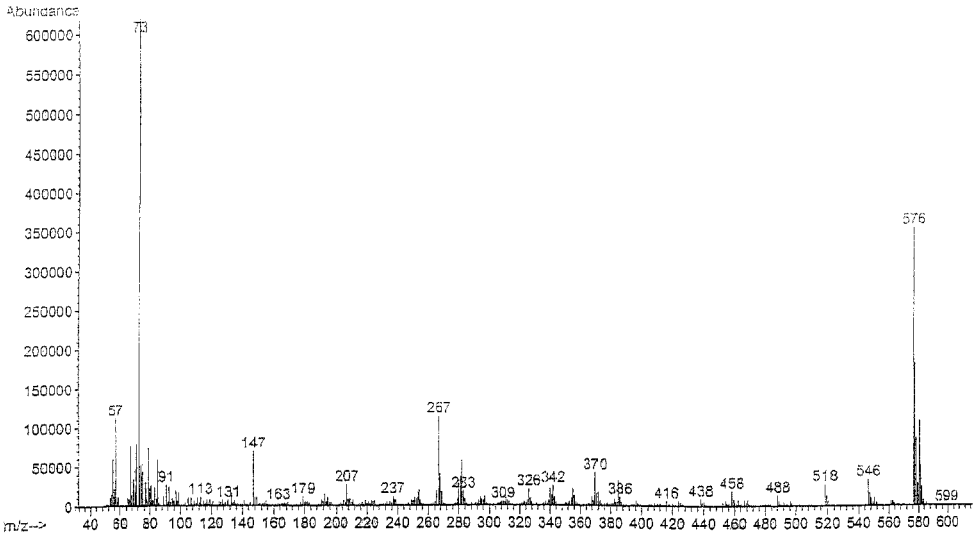

Figure 7. Mass spectrum of the TMS derivative of the compound(s) eluting at 15.6 min.

side-chain saturation. Interestingly, this variation in side chains does not appear to exist for the related resorcinol compound.

It should be noted that one of the C15 side-chain hydroquinones is capable of intitiating high levels of seed germination in *Striga asiatica*. It now seems that

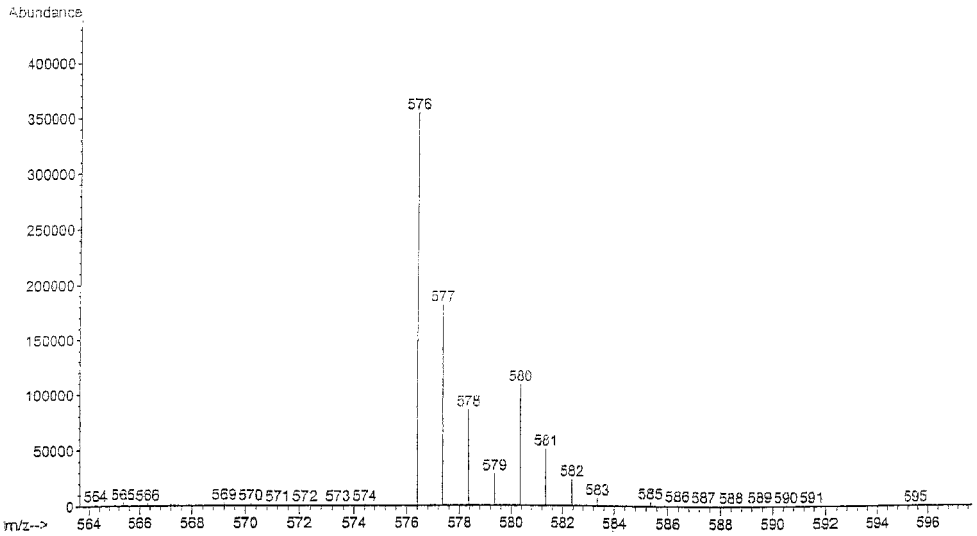

Figure 8. Detail of the molecular ion area, *m*/*z* 576, of Figure 9.

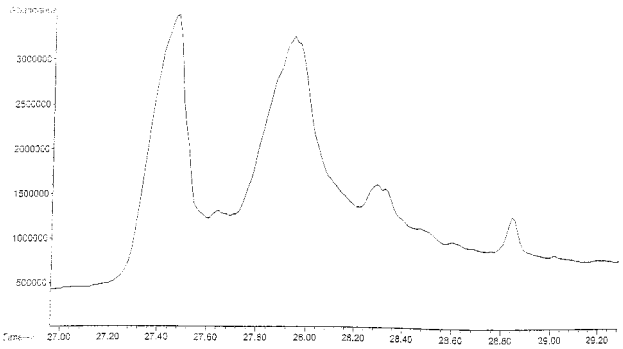

Figure 9. Further chromatographic resolution of the peak eluting previously at 15.6 min.

four compounds comprise this class from typical sorghum varieties. Whether the variation in the side chains affects germination rates in *Striga* is not known at this time. Fate (unpublished data) attempted hydrogenation of the side chain to examine the effect of saturation upon germination levels, but these experiments proved to be inconclusive. The side-chain variation could have significant consequences for binding of these compounds to a parasite receptor to initiate the germination process, and thus they may differ in activity. The overall three-dimensional structures of these hydroquinones are likely to be very different as the structures of saturated versus unsaturated fatty acids are quite different (*19*). Permeability of the *Striga* seed coat to these four compounds may also be different and could result in different germination activities.

Previous work noted that three different sorghum varieties, Piper Sudan Grass, *Sorghum* cv. Dabar, and Sudan Grass Hybrid, each had differing responses to exudate in terms of the concentration needed to induce half-maximal activity (*13*). They noted a correlation between lower ED_{50} concentrations and higher resorcinol to SXSg ratios. What was not known at that time is that instead of being a single compound, SXSg appears to be produced along with closely related hydroquinones. Another possible explanation for the observed differences in germination response among the hosts could potentially be related to the differing SXSg compounds in the exudates of different grasses. The compositions of various sorghum exudates are currently being explored.

A possible biosynthetic pathway for the resorcinol and SXSg has also been proposed (*13*). In this pathway, the aromatic nucleus of SXSg and the resorcinol could be formed by the polyketide pathway, and eventual cyclization of a 21-carbon acyclic precursor would form the basic aromatic ring and side chain of SXSg. Under this scheme, the resorcinol compound would be formed by later methylation after production of a basic SXSg pool. Clearly, both SXSg and the resorcinol would be expected to have identical C15 side chains. The data within this paper demonstrate that the resorcinol and SXSgs have differences in their side chains that would not be accounted for in this scheme. It appears to be unlikely that differing rates of degradation for resorcinol compounds with other C15 tails (had a series similar to that of SXSg been produced) would explain the observed lack of variation in the resorcinol side chain. Therefore, possible explanations for the observed difference are that either an SXSg with the triple unsaturation in its chain is preferentially methylated to form resorcinol or that the biosynthetic pathways for these two classes of compounds differ at an earlier point. Indeed, the presence of four different side chains in SXSgs may support the view (*13*) that the quinone could be added biosynthetically onto a pre-existing 16-carbon fatty acid. The addition of two inhibitors, haloxyflop and fluoroacetate, diminished the production of resorcinol and SXSg but did not alter the ratio of the two (*13*). These inhibitors may have reduced fatty acid availability for the biosynthesis or directly inhibited the synthase involved in ring construction, possibilities both noted by Fate and Lynn. The data presented here support the notion that fatty acid availability may be reduced. Indeed, Houser and Olson (*20*) noted that considerable side-chain variation did not stop the methylation of ubiquinone by mitochondrial methyltransferases, again supporting an earlier branch point for the biosynthesis of SXSg and resorcinol.

There has been considerable debate as of late as to the nature of the "natural" *Striga* germination stimulant from sorghum. It is clear from the GC-MS data [which support previous NMR observations (*5*)] that the SXSg series and resorcinol compounds make up the overwhelming majority of the hydrophobic exudate from sorghum. The dry weight of seedlings typically used for each study averaged 1.24 g (fresh weights averaged 9.5

g), and the weights of hydrophobic exudates from various varieties of sorghum seedlings (4 days old, root length typically 10−12 cm) typically ranged from 1 to 4 mg. The GC-MS data indicate yields of the SXSgs/resorcinol mixture from the exudate are in the range of 85−95%. Thus, yield of the SXSgs/resorcinol from dry weight of seedling is in the range of 0.006−0.003%, and from fresh weight the yield ranged from 0.009 to 0.04%. Yields of sorgolactone, by comparison, are 0.1% from crude exudate, and yields from dry or fresh weight of seedlings, although not specifically reported, must be low to require thousands of plants in the isolation scheme. The demonstrated overall yield of the SXSgs/resorcinol mixture from seedlings is thus significantly higher than that for sorgolactone and related compounds. Even if the previously mentioned differences in germination rates for the SXSg series versus sorgolactone are more related to the compound than to the seeds or methodology used in the various studies, the significantly greater yields of the SXSg series appear to offset any possible differences in ED_{50} values.

In the past, there have been attempts to correlate resistance to *Striga* with SXSg (sorgoleone) production by various sorghum varieties. Hess et al. (*21*) concluded that there was no correlation between resistance and SXSg production. Levels of SXSg were measured by UV−vis at 286 nm. This conclusion was used to support the hypothesis that other stimulants (at the time unidentified but later identified as the aqueous derived sorgolactone) are more important to *Striga* germination than SXSg. Their study failed to take into account that SXSg was already known to be accompanied by resorcinol production, and it is expected that monitoring at 286 nm alone would not result in an accurate assessment of SXSg levels. Additionally, what is now clear is that SXSg production is accompanied by the production of several related hydroquinones of unknown activity, and again these compounds would not show appreciable differences by monitoring at 286 nm. Studies continue here as to whether resistance can be better correlated to new differences in the hydrophobic exudates better observable by GC-MS and whether the newly discovered compounds have differing activities.

ACKNOWLEDGMENT

We thank Gebisa Ejeta of Purdue University for the gift of sorghum seeds, some of which were used in these studies.

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Received for review August 4, 2001. Revised manuscript received August 12, 2001. Accepted August 15, 2001. We gratefully acknowledge research support from Ursinus College.

JF0111099