Striga hermonthica (Del.) Benth Germination Stimulant(s) from *Menispermum dauricum* (DC.) Root Culture

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Germination of *Striga hermonthica*, an important root parasitic weed on cereals, is induced by stimulants exuded by roots of host and some nonhost plants. Efforts to isolate active compounds have focused on root exudates from intact plants. In this paper, a much easier and less laborious novel approach, based on root cultures, is described. *Menispermum dauricum* roots cultured in a modified B5 medium produced and exuded into the medium compounds with activity comparable to that of the synthetic strigol analogue GR24. The active components stimulate germination by induction of ethylene biosynthesis. Their accumulation in roots and culture medium, as indicated by activity, was at a maximum 6−8 weeks after subculturing and was positively correlated with root growth ($r = 0.76-0.92$). HPLC and TLC of the culture filtrate revealed at least two or three compounds, none of which had chromatographic properties consistent with those of strigol. The results indicate the potentials of root cultures as a possible source of novel *Striga* germination stimulants.

Keywords: *Germination stimulant; root culture; Striga hermonthica*

INTRODUCTION

Striga hermonthica (Del.) Benth is a root parasitic weed that reduces the yield of many important cereals in the semiarid tropics in Africa. Vulnerable crops include sorghum [*Sorghum bicolor* (L.) Moench], maize (*Zea mays* L.), millet [*Penisetum glaucum* (L) R. Br.], and rice (*Oryza sativa* L.). Yield losses from damage by the parasite are often significant, and complete crop failure is not uncommon (Ejeta *et al.*, 1993). The life cycle of the parasite is closely linked to its host. In order to germinate, a *Striga* seed has to be in a warm moist environment for several days (conditioning) prior to exposure to an exogenous stimulant (Worsham, 1987). For successful attachment and parasitism, germination has to occur in close proximity to a host root. Induction of germination in the absence of, or away from a host root, suicidal germination has been considered to have great potential for *Striga* control (Worsham, 1987).

Several germination stimulants were identified from host and nonhost plants (Butler, 1993), and many of their analogues have been synthesized and proved to be effective (Johnson *et al.*, 1976; Butler, 1993). However, further work revealed their extreme instability in soils and limited usefulness under practical field conditions (Babiker *et al.*, 1987; Vail *et al.*, 1990). On the basis of circumstantial evidence and experimentation, several workers concluded that germination stimulants from susceptible hosts and their synthetic mimics are likely to be unstable (Babiker *et al.*, 1987; Fate *et al.*, 1990; Siame *et al.*, 1993). The need to probe nonhost plants for potent, more stable stimulants or leading compounds from which more effective and stable derivatives can be synthesized has been emphasized (Joel *et al.*, 1995).

In this paper, we report on *S. hermonthica* germination stimulants from root cultures of *Menispermum dauricum* (DC.), a nonhost broad-leaved herbaceous plant renowned for production of biologically active compounds (Sugimoto *et al.*, 1994). The culture was selected on the basis of preliminary screening of several root cultures, which were established and grown in our laboratory. The report includes studies on activity, production, extraction, and chromatographic properties. The latter was compared to that of strigol, a natural *Striga* germination stimulant isolated from several plants, including host and nonhost species (Siame *et al.*, 1993).

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MATERIALS AND METHODS

Source of Plant Materials and Chemicals. Seeds of *S. hermonthica* (Del.) Benth were collected in 1992/93, from under sorghum, at the Gezira Research Station, Sudan. *M. dauricum* roots were obtained from established cultures (Sugimoto *et al.*, 1994). Standard (±)-strigol was provided by Dr. K. Yoneyama, Utsunomiya University, Japan. 1-[(Aminoethoxy)-vinyl]glycine (AVG) and 1-aminocyclopropane-1-carboxylic acid (ACC) were purchased from Sigma.

Surface Sterilization and Conditioning of *Striga* Seeds. Seeds were surface sterilized by immersion in ethanol and sonication for 3 min with occasional swirling followed by through washing with sterilized distilled water. The seeds, unless mentioned otherwise, were pretreated (conditioned) in distilled water for 12−15 days on 8 mm disks of glass fiber filter paper as described by Parker *et al.* (1977).

Germination Assay and Statistical Analysis. Aqueous solutions and extracts were assayed directly, by applying 20 μL aliquots of the respective test solution to conditioned *Striga* seeds on 8 mm disks of glass fiber filter paper, placed in petri dishes. For solutions and extracts containing organic solvents, aliquots (20 μL each) of the test solution were applied to 8 mm disks of glass fiber filter paper. The disks were allowed to dry. A disk of conditioned *Striga* seeds was placed on top of each and moistened with 40 μL of distilled water. The treated seeds were incubated at 30 °C and examined for germination 24 h later. Culture medium, in which no roots were grown, and distilled water controls were included where appropriate. Individual treatments were replicated four to six times. Germination data were transformed to arcsin, examined by analysis of variance, and then back-transformed.

Activity of *M. dauricum* Culture Filtrate on *S. hermonthica* Seeds. Three experiments were undertaken. In

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the first experiment, *Striga* seeds were conditioned in distilled water for varying periods (0−17 days). Eight-week-old *M. dauricum* cultures were filtered through Whatman No. 1 filter paper. The seeds, treated with culture filtrate or the strigol analogue GR24 at 0.1 ppm, were incubated and examined for germination.

In the second experiment, a C18 Sep-Pak (Waters) preparatory column (1 g), conditioned as described by Weerasuriya *et al.* (1993), was loaded with 10 mL of culture filtrate. The column was washed with 10 mL of water followed by elution with 10 mL of methanol. The methanol eluate, which contained the active components, was then evaporated to dryness at 40 °C. The residue, henceforth referred to as the column eluate, was dissolved in 10 mL of distilled water. *Striga* seeds, spread on 8 mm disks, were placed in petri dishes lined with glass fiber filter papers. The seeds were then moistened with 5 mL of distilled water or 1.5 mL filter-sterilized column eulate diluted to 5 mL with distilled water, henceforth referred to as the culture filtrate solution. The seeds, incubated at 30 °C, were periodically sampled, treated with GR24, reincubated, and examined for germination.

In the third experiment, conditioned *Striga* seeds were treated with various dilutions of *M. dauricum* culture filtrate, each alone or in mixtures with 1-[(aminoethoxy)vinyl]glycine (AVG) or AVG plus 1-aminocyclopropane-1-carboxylic acid (ACC). The seeds were incubated and examined for germination.

Culture Period and Stimulant Production. Excised roots of *M. dauricum* were cultured for 1−8 weeks on a rotary shaker (70 rpm), supplemented with 1 μM 1-naphthaleneacetic acid (NAA) in the dark at 27 °C in a B5 medium as described by Sugimoto *et al.* (1994). At harvest, the culture medium was filtered. The roots were thoroughly washed with distilled water, freeze-dried, weighed, and powdered. Samples of the powder (10 mg each) were placed in plastic vials. Water (1 mL) was added to each vial. The samples were sonicated for 15 min and centrifuged. Culture filtrates and supernatants from root extracts were assayed for activity.

Extraction and Chromatographic Properties of *Striga* Germination Stimulant(s) from *M. dauricum* Root Cultures. Filtrates (300 mL) from 8-week-old cultures were extracted with 4 × 100 mL of ethyl acetate. The individual ethyl acetate extracts were assayed for activity and then combined. The combined ethyl acetate extract was evaporated to dryness at 35 °C. The residue was dissolved and brought to 300 mL using distilled water. For thin layer chromatography (TLC), the dried residue was taken up in 1 mL of methanol. Aliquots (50 μL each) of the resulting solution or 20 μL of a methanolic solution of strigol (1.8 mg/mL) was applied to TLC plates. The plates were developed with ethyl acetate/hexane (9:1 v/v) and marked into 1 cm horizontal strips. The coating on each strip was scraped and packed into a small plastic vial. Methanol (1 mL) was added to each vial. The vials were sonicated for 5 min and centrifuged, and the supernatant was bioassayed for activity.

For high-performance liquid chromatography (HPLC), residue from ethyl acetate extract of culture filtrate was dissolved in 20 mL of distilled water. The resulting solution was loaded onto a C18 Sep-Pak (Waters) preparative column (10 g) previously conditioned as described by Weerasuriya *et al.* (1993). The column was washed with 60 mL of water followed by successive washes with a series of methanol/water mixtures in which the methanol content was increased, stepwise, from 40 to 100%. Fractions (15 mL each) were collected and assayed for activity. Fractions showing activity were combined and evaporated to dryness, and the residue was taken up in methanol (0.5 mL). An aliquot (20 μL) of the resulting solution or 10 μL of a methanolic solution of strigol (1.8 mg/mL) was loaded onto a Develosil ODS-HG-5 (150 × 4.6 cm) analytical HPLC column. The column was eluted with aqueous methanol (60% v/v) at a flow rate of 0.5 mL/min. UV absorption of the column effluents was monitored at 215 and 245 nm for the culture extract and strigol, respectively. Fractions were collected at 1 min intervals and bioassayed for activity.

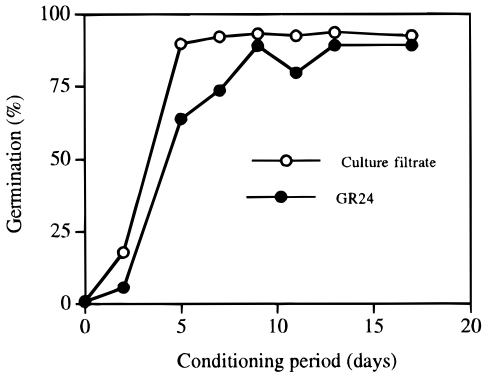

Figure 1. Activity of *M. dauricum* culture filtrate and GR24 on *S. hermonthica* seeds: effects of conditioning period.

RESULTS

Activity and Production of Stimulant. The modified B5 medium, used in this study, and aqueous controls did not stimulate *Striga* seed germination. Unconditioned *Striga* seeds did not respond to *M. dauricum* culture filtrate or GR24. Seeds conditioned for 2 days displayed little germination (6−18%) in response to both stimulants. Seeds conditioned for 5 and 9 days prior to treatment with GR24 displayed 64 and 89% germination, respectively. Extension of the conditioning period to 17 days had no further influence on germination (Figure 1). Seeds conditioned for 5 days and then treated with *M. dauricum* culture filtrate displayed 89% germination with no further significant improvement on prolonged conditioning (Figure 1).

Seeds conditioned in distilled water displayed a higher response to subsequent treatments with GR24 than those conditioned in *M. dauricum* culture filtrate solution. Seeds conditioned in distilled water and treated with GR24 at 0.001 ppm displayed a consistent increase in germination with conditioning (Figure 2A). Seeds pretreated for 6 and 12 days exhibited 40 and 73% germination, respectively. Corresponding germination figures for the same seeds, when treated with GR24 at 10-fold the initial rate, were 66 and 75% (Figure 2B). Increasing GR24 to 100-fold increased germination of the 6-day-pretreated seeds to 85%. However, the germination of seeds pretreated for more than 6 days showed no significant improvement (Figure 2C).

Seed pretreated in *M. dauricum* culture filtrate solution displayed little and inconsistent germination (1−14%) in response to GR24 at 0.001 ppm (Figure 2A). GR24 at 10-fold the initial rate increased germination to between 13 and 51% (Figure 2B). Increasing GR24 to 100-fold induced a high response. Germination increased, almost linearly, from 32−78% at 6 and 12 days of pretreatment, respectively. However, no further increase in germination was attained on extension of the conditioning period to 15 days (Figure 2C).

M. dauricum culture filtrate-induced germination was inhibited considerably by AVG. The culture filtrate, at 1:2, 1:4, and 1:8 dilutions, elicited 73−95% germination. AVG, applied simultaneously with the culture filtrate, reduced germination to between 40 and 50%. Addition of ACC to the stimulant/AVG mixtures increased germination to between 48 and 77% (Table 1).

Undiluted extracts from roots cultured for 1 week elicited 86% germination. A 5- and 10-fold dilution of the extracts induced moderate and negligible germination, respectively. Extracts from roots cultured for 2 and 3 weeks induced 73 and 43% germination, respectively. The activity of the extracts was reduced to 10 and 8%

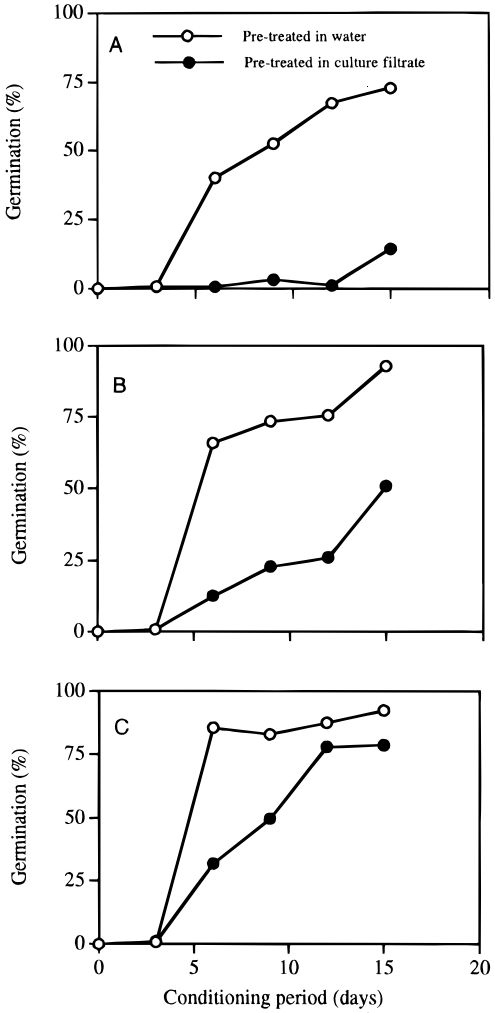

Figure 2. Effects of pretreatment of *S. hermonthica* seeds *in M. dauricum* culture filtrate on response to subsequent treatments with GR24. *S. hermonthica* seeds were pretreated in *M. dauricum* culture filtrate or distilled water for varying periods of time prior to treatment with GR24: (A) 0.001 ppm, (B) 0.01 ppm, and (C) 0.1 ppm.

Table 1. Inhibition of *M. dauricum* Culture Filtrate-Induced *Striga* Germination by 1-[(Aminoethoxy)vinyl]glycine (AVG) and Its Partial Reversal by 1-Aminocyclopropane-1-carboxylic Acid (ACC)[a]

dilution of original filtrate (x)	AVG (μM)	ACC (μM)	germination (%)
1:2	0	0	(77.4) 95[b]
1:2	200	0	(45.1) 50
1:2	200	100	(52.4) 63
1:2	200	150	(59.3) 74
1:4	0	0	(72.5) 91
1:4	200	0	(41.7) 44
1:4	200	100	(47.1) 54
1:4	200	150	(61.1) 77
1:8	0	0	(58.7) 73
1:8	200	0	(39.3) 40
1:8	200	100	(43.9) 48
1:8	200	150	(44.5) 49
			SE = ±3.39

[a] Conditioned *S. hermonthica* seeds were treated with *M. dauricum* culture filtrate alone or mixtures with AVG or AVG plus ACC and then incubated and examined for germination 24 h later. [b] ( ) arcsin-transformed.

on 5-fold dilutions and was completely lost on 10-fold dilutions. Extracts from roots cultured for 4−6 weeks displayed high activity which was considerably reduced on 5- and 10-fold dilutions. Root extracts from 7- and 8-week-old cultures displayed high activity which was largely maintained on further dilutions (Figure 3A).

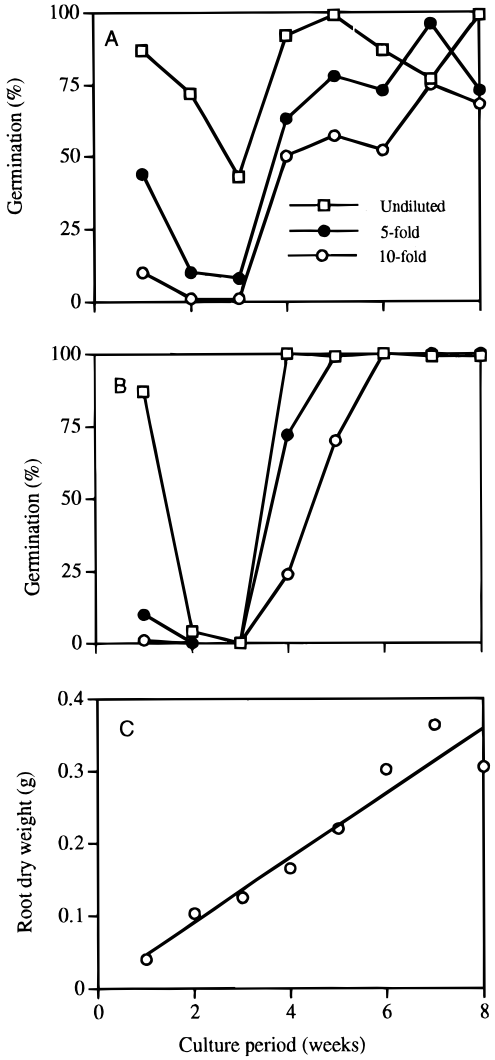

Figure 3. Effects of culture period on root growth and production of *Striga* germination stimulants by *M. dauricum* root cultures: (A) root extract, (B) culture filtrate, and (C) root growth.

Activity of the culture filtrates was consistent with that of the root extracts. Filtrate from 1-week-old cultures induced 87% germination. However, a complete loss of activity was displayed on a 5-fold dilution. Filtrate from 2- and 3-week-old cultures, irrespective of dilution, elicited negligible germination. Filtrates from 4-week-old cultures demonstrated high activity which was largely maintained at a 5-fold but not at a 10-fold dilution. Cultures maintained for 6−8 weeks yielded filtrates with high activity. The displayed activity was not influenced by 5- or 10-fold dilution (Figure 3B).

Root dry weight progressively increased with time up to 8 weeks (Figure 3C). The activity of culture filtrates and root extracts at 10-fold dilutions was positively correlated with root dry weight ($r = 0.76$ and 0.92 for culture filtrate and root extract, respectively; $P \leq 0.05$).

Extraction and Chromatographic Properties. The culture filtrate before extraction displayed very high activity (100% germination). Germination was reduced to 95 and 51% following the first and second ethyl acetate extractions, respectively (Table 2). After four successive extractions, the aqueous phase promoted

Table 2. Efficiency of Extracting Stimulants into Ethyl Acetate (EtAC) from *M. dauricum* Culture Filtrate (Aqueous Phase)

	germination (%)		
	dilution		
temp	*x*	1:5	1:10
before EtAC ext[a]	(86.9) 100[b]	(73.6) 92	(68.4) 86
after first EtAC ext	(76.9) 95	(65.2) 82	(42.2) 45
after second EtAC ext	(45.6) 51	(24.9) 18	(17.7) 9
after third EtAC ext	(22.2) 14	(10.1) 3	(7.3) 2
after fourth EtAC ext	(9.9) 3	(6.9) 1	(5.4) 1
		SE = ±3.58	

[a] ext = extract. [b] ( ) arc sin-transformed.

Table 3. Efficiency of Extracting Stimulants into Ethyl Acetate (EtAC) from *M. dauricum* Culture Filtrate (Ethyl Acetate Phase)

	germination (%)		
	dilution		
temp	*x*	1:5	1:10
first EtAC	(81.0) 98[a]	(84.9) 99	(70.4) 89
second EtAC	(77.1) 95	(66.7) 84	(63.7) 81
third EtAC	(69.8) 88	(43.6) 48	(28.1) 22
fourth EtAC	(42.5) 46	(15.6) 7	(11.5) 4
combined EtAC	(86.3) 100	(72.9) 91	(62.6) 79
		SE = ±3.58	

[a] ( ) arc sin-transformed.

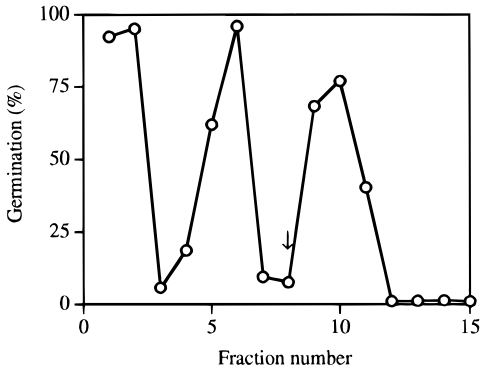

Figure 4. TLC chromatogram of ethyl acetate extract from *M. dauricum* culture filtrate. The arrow shows the position of strigol in the chromatogram.

negligible germination (3%) and all activity was recovered in the ethyl acetate extractions (Tables 2 and 3).

TLC of the extract from *M. dauricum* culture filtrate revealed three zones of activity with maximum germination, 95, 96, and 77%, corresponding to R_f values of 0.13, 0.4, and 0.73, respectively. TLC of standard strigol displayed one zone of activity showing maximum germination (79%) at an R_f of 0.47 (Figure 4).

Water and aqueous methanol (20−40%) washes of a C18 Sep-Pak column, subsequent to loading of culture filtrate, did not induce *Striga* germination. Most of the activity was eluted in four successive fractions at methanol concentrations of 50−70% (v/v). The highest germination (91%) was induced by fraction number 15 (Figure 5A). On combination and further separation of the active fractions from the C18 Sep-Pak column on HPLC, two major zones of activity were revealed. Maximum germination, 50 and 93% in zones 1 and 2, respectively, coincided with fractions having retention times (t_R) of 7 and 15 min. The latter had the highest activity (Figure 5B). UV monitoring of the column effluent did not yield useful absorption properties. The activity of strigol was eluted in six successive fractions

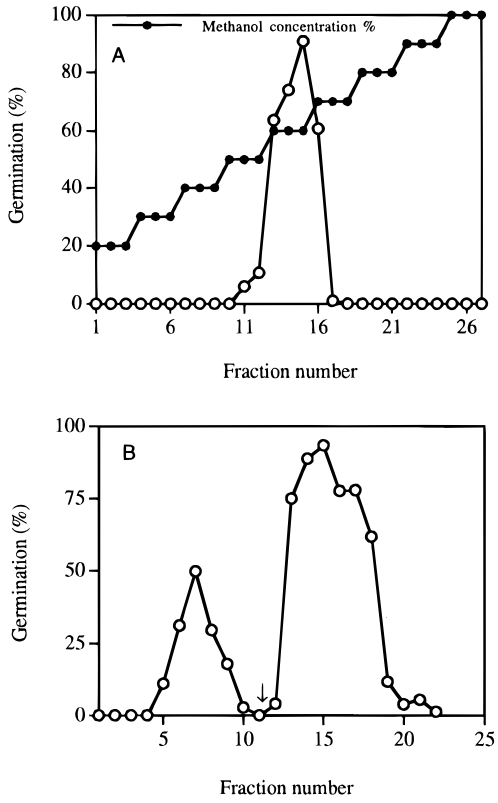

Figure 5. Stimulant activity of *M. dauricum* culture filtrate: (A) C18 Sep-Pak elution profile and (B) HPLC chromatogram. The arrow shows the position of strigol in the chromatogram.

(t_R 8−13 min). The highest activity (67% germination), which coincided with maximum UV absorption, was exhibited by the fraction with a t_R of 11 min (Figure 5B).

DISCUSSION

It is evident from the results (Figures 1−3) that cultured roots of *M. dauricum*, a nonhost of *S. hermonthica*, produce and exude into the culture medium active substances that induce the parasite germination. The relatively high initial activity displayed by both culture filtrate and root extract of *M. dauricum* 1 week after subculturing, the decline of activity at 2−3 weeks, and the progressive increase in activity at 4−8 weeks (Figure 3) suggest that accumulation of the active substances is a function of production and utilization. This suggestion is substantiated by the observation that filtrate from *M. dauricum* root cultures did not lose activity on storage at 27 °C for more than 10 weeks (A. G. T. Babiker, Y. Q. Ma, Y. Sugimoto, and S. Inanaga, unpublished).

The negligible response of unconditioned seeds or those conditioned for 2 days and the surge in germination on prolonged conditioning together with the decrease in response to GR24 following pretreatment in *M. dauricum* culture filtrate (Figures 1 and 2) are consistent with several reports on *Striga* germination stimulants (Worsham, 1987). The observed consistency suggests a common mechanism of action. Germination of several *Striga* species is induced by endogenous ethylene, the biosynthesis of which is triggered by exogenously applied stimulants (Jackson and Parker, 1991; Logan and Stewart, 1991; Babiker *et al.*, 1993).

Unconditioned seeds and those conditioned for 2−3 days are neither capable of producing ethylene (Babiker

et al., 1993) nor highly sensitive to the phytohormone (Egley, 1972). Seeds conditioned for 5 or more days are more responsive and produce more ethylene when treated with germination stimulants (Babiker *et al*., 1993).

Involvement of ethylene biosynthesis in *M. dauricum* root culture filtrate-induced *Striga* germination is further substantiated by the inhibitory effect of AVG, an ACC synthase inhibitor, and by the counteraction of the inhibitory effect of AVG by ACC, the immediate ethylene precursor. Like sorghum root exudate, active substances from *M. dauricum* root cultures seem to induce ACC synthase activity. The latter is a limiting enzyme in ethylene biosynthesis in *Striga* seeds (Logan and Stewart, 1991).

On the basis of R_f values on TLC and t_R values on HPLC, none of the active substances from *M. dauricum* root culture seems to have chromatographic properties consistent with those of strigol (Figures 4 and 5). The discrepancy in the number of active substances shown on TLC and HPLC may be due to breakdown products on the TLC plate. Siame *et al.* (1993) working on *Striga* germination stimulants from sorghum demonstrated breakdown and loss of activity on silica gel columns.

In recent years, much attention has been focused on isolation and identification of *Striga* germination stimulants from root exudates of intact plants. Strigol and strigol-like molecules were identified in root exudates of phylogenetically divergent species (Hauck *et al*., 1992; Siame *et al*., 1993). Apart from a few attempts (Cai *et al*., 1993), the potential of root cultures for production of *Striga* germination stimulants remains largely unexploited. The present work demonstrates the potential of tissue culture techniques in furthering research on *Striga* germination stimulants. Tissue cultures are less laborious, require less space, are easy to handle, and may provide novel compounds with higher activity and/or stability compared to those from intact plants or their synthetic mimics. Furthermore, it should be possible through manipulation of culture conditions to establish high-stimulant-producing cultures. It is worth mentioning that in this study root growth and stimulant activity are positively correlated and seem to be compatible. Further work is now in progress on isolation and identification of the active compounds as well as their activity and stability in soils. The ability of intact plants to produce and exude the compounds in soils is also under investigation.

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