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(+)-Strigol, a witchweed seed germination stimulant, from *Menispermum dauricum* root culture

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Abstract

(+)-Strigol was isolated from *Menispermum dauricum* root culture filtrate. Its identity was confirmed by HPLC, ^{1}H NMR, UV and MS, and on the basis of its CD spectrum. This is the first report on isolation of strigolactone from aseptic plant culture.

Keywords: *Menispermum dauricum*; Menispermaceae; Moonseed; *Striga hermonthica*; Scrophulariaceae; Witchweed; Root culture; Seed germination stimulant; (+)-Strigol

1. Introduction

Parasitic angiosperms are at least in part dependent on their host species for the supply of carbon, nutrients and water, and, where the host is of agricultural importance, can seriously constrain food production. Witchweed (*Striga hermonthica* [Del.] Benth.) (Scrophulariaceae) is an economically important root parasitic weed of the Poaceae in semiarid Sub-Saharan Africa. Vulnerable crops include sorghum (*Sorghum bicolor* [L.] Moench), maize (*Zea mays* L.) and pearl millet (*Pennisetum glaucum* [L.] R. Br.). Yield losses caused by this parasite are often extremely large, reaching 100% in heavily infested soil (Ejeta et al., 1993). To germinate, seeds of *Striga* and other parasitic species require after-ripening pretreatment (conditioning) in a moist warm environment for 2–14 days and subsequent exposure to an exogenous stimulant. The natural germination stimulants so far reported are thought to be sesquiterpene lactones, and are collectively named strigolactones (Butler, 1995). The molecules are labile and active at concentrations as low as picomolar, and thus stimulate germination of seeds only in close proximity to potential host roots. Understanding the structure–function relations of the germination stimulants has been hindered by their low rates of production, laborious isolation procedures and complex stereochemistry. However, the CD part of these molecules is primarily responsible for germination stimulation (Mangnus and Zwanenburg, 1992) and their activity appears to be more dependent on the precise three-dimensional structure than the specific nature of functional groups on the molecule (Sugimoto et al., 1998). Induction of germination in the absence of or away from a host root, termed suicidal germination, had been considered to have great potential for *Striga* control (Worsham, 1987). However, further work revealed that strigolactones were extremely unstable in soils and their usefulness was limited under practical field conditions (Babiker et al., 1987).

Plant tissue culture with high productivity of a target molecule is useful for investigating the biosynthetic pathway of the plant metabolite because the culture is kept aseptic and cultural parameters are easily manipulated. Since all the naturally occurring germination stimulants so far isolated are analogous (Cock et al., 1972; Hauck et al., 1992; Müller et al., 1992; Yokota et al., 1998), germination stimulants produced by tissue cultures of other plants, if any, could be also strigolactones.

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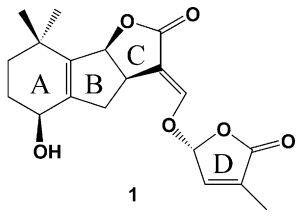
Preliminary screening of plant tissue cultures in our laboratory (Sugimoto et al., 2001) revealed that root culture of *Menispermum dauricum* DC. (Menispermaceae), a broad-leaved herbaceous plant (Sugimoto, 1999), was most potent in inducing germination of conditioned *Striga hermonthica* seeds. This paper reports on the isolation and identification of the major germination stimulant, (+)-strigol (**1**), from *M. dauricum* root culture filtrate.



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2. Results and discussion

M. dauricum root culture filtrate was subjected to solvent partitioning to give a neutral EtOAc fraction. This was separated by Sephadex LH-20 column chromatography using a mixed solvent of MeOH–$CHCl_3$ (4:1) (Fig. 1A). Residue obtained from active fractions 27–42 was subjected to further Sephadex LH-20 column chromatography using MeOH–H_2O (1:1) (Fig. 1B). Activity was found in fractions 13–32, the first half (13–18) of which showed strong UV absorption. On the other hand, the second half (19–32) demonstrated much weaker UV absorption. In a separate experiment, where the both groups of the active fractions were further purified independently by preparative HPLC, as mentioned below, activity was detected in the same fractions. The active fractions 13–32 in the second chromatography were combined, MeOH was removed, then active ingredients were extracted with EtOAc from the aq. phase. After removing EtOAc, the residue was purified further by preparative HPLC, resulting in the separation of a potent stimulant at 36 min (Fig. 2). The active substance was subjected to analytical HPLC connected with a mass spectrometer in the ESI mode as well as a UV detector. The active substance was eluted at 18.5 min (Fig. 3A), ESI-MS analysis of which gave rise to two major signals at m/z 715 and 369 (Fig. 3B), suggesting that the molecular mass of the active substance is 346. This was confirmed by EI-MS, where almost the same fragmentations as those of (±)-strigol were observed (see Section 3). Yokota et al. (1998) demonstrated that the fragment ions are the same in both strigol and alectrol but these germination stimulants are distinguishable from each other on the basis of relative intensities of the fragment ions. The ^1H NMR spectrum of the active substance showed major peaks identical with those found in authentic (±)-strigol. Chromatographic behaviour on the preparative and analytical HPLC as well as the UV spectrum of the active substance was also identical to those of (±)-strigol. A CD spectrum of the active substance showed positive and negative cotton effects at 234 nm and 206 nm, respectively, which are consistent with those reported for (+)-strigol (Reizelman et al., 2000). Based on the results mentioned above, the major germination stimulant produced by *M. dauricum* root culture was unambiguously identified as (+)-strigol (**1**).

(+)-Strigol was first isolated from the root exudate of false host cotton (*Gossypium hirsutum* L.) and its structure

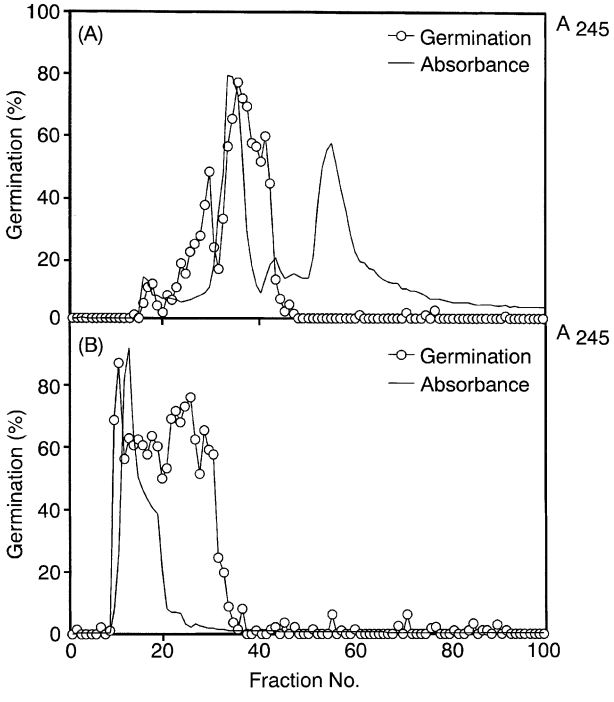

Fig. 1. Separation of germination stimulant, extracted from *M. dauricum* root culture filtrate, by LH-20 chromatography. MeOH–$CHCl_3$ (4:1) (A) and MeOH–H_2O (1:1) (B) were used as mobile phase.

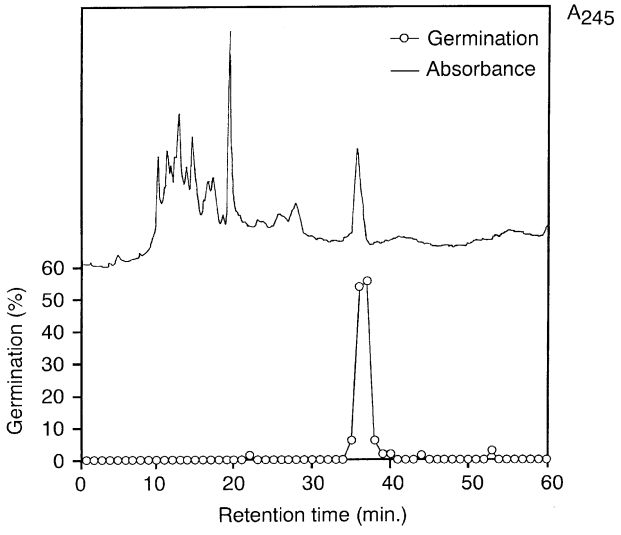

Fig. 2. Subsequent separation of germination stimulant by preparative HPLC.

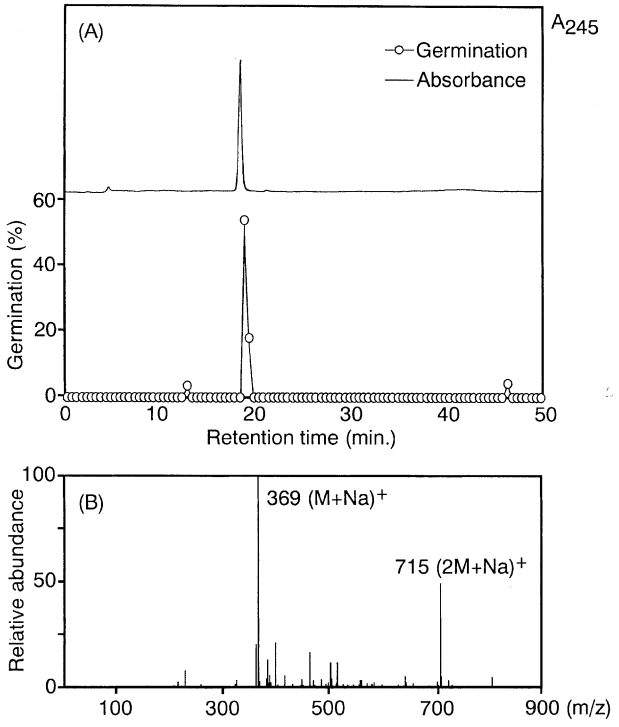

Fig. 3. LC-ESI-MS analysis of germination stimulant isolated from *M. dauricum* root culture filtrate. Elution profile by analytical HPLC (A) and mass spectrum (B) of the stimulant.

was elucidated by Cock et al. (1966, 1972). The absolute configuration was established by Brooks et al. (1985a). Identification of strigol from root exudates of maize, sorghum and proso millet was also reported, based on its *R*ts in HPLC and negative ion chemical ionization mass spectrometry (Siame et al., 1993). However, as Yokota et al. (1998) suggested, these parameters may be insufficient to specify the compound. So far there have been four reports on isolation of strigolactones. The isolated amounts of sorgolactone and alectrol were 5 μg from drainage water of 300 000 sorghum plants and 300 μg from that of 75 000 cowpea plants, respectively (Hauck et al., 1992; Müller et al., 1992). Although as much as 1700 l of aquaculture filtrate of 300 000 red clover plants were used to isolate orobanchol, the amount obtained was not sufficient to reach an unambiguous conclusion regarding its structure (Yokota et al., 1998). In all the cases, it is difficult to ensure the sterility of the media. Therefore whether these plants are genuine producers of the strigolactones has been questioned (Boone et al., 1995). In the present experiments, (+)-strigol was, for the first time, isolated from aseptic culture, which unambiguously demonstrates that the strigolactone is of plant origin. We isolated 140 μg of (+)-strigol from 6 l of *M. dauricum* root culture filtrate, on the basis of peak area monitored in the preparative HPLC. Assuming that the activity found in the culture filtrate is attributable only to (+)-strigol, its concentration is roughly 1 mg/l. This indicates that the recovery rate of the stimulant in the present experiments was as low as 2%. Removal of MeOH from aq. MeOH solution and the subsequent extraction of the active substance from the aq. phase were considered to be the most critical steps. Highly end-capped, silica-based, reversed phase columns were selected for HPLC because the activity was lost by treatment with silica gel.

Since germination stimulants have been believed to be specific for host plants (Müller et al., 1992), the significance of strigol was long uncertain until the isolation of sorgolactone and alectrol, close analogues of strigol, from genuine host plants. We found that root culture filtrate of *Stephania cepharantha* (Menispermaceae), obtained from established cultures (Sugimoto, 2002), were also active in inducing germination of conditioned *Striga hermonthica* seeds. The *Stephania* culture filtrate was subjected to the same purification procedures employed for the present study. As a result, an active ingredient was separated whose chromatographic behaviour on the preparative and analytical HPLC and molecular mass are identical with those of authentic strigol (data not shown) though further identification was not conducted because of the small amount available. Preliminary experiments showed that conditioned *Striga hermonthica* seeds germinated and formed haustoria in proximity to *M. dauricum* and *Stephania cepharantha* roots. However, the seedlings appeared to parasitize neither of these roots. This observation suggests wider distribution of strigolactones in the plant kingdom than would be expected from the conventional idea that they are host-specific. Though seed germination and haustorial initiation are critical developmental events for parasitic weed seeds, more elaborate mechanisms might be involved in establishing the parasitic association with the host.

Strigolactones have been considered to be sesquiterpenes, based on their structures. However, there has been no experimental proof which supports this hypothesis. *M. dauricum* root culture is the first material which produces strigolactone in vitro. Investigation of (+)-strigol biosynthesis in the *Menispermum* roots is in progress.

3. Experimental

3.1. General experimental procedures

^{1}H NMR spectra were recorded in $CDCl_3$ on a JEOL Lambda 400 spectrometer. MS spectra were obtained on a JEOL JMS-700 mass spectrometer in the EI or ESI mode. UV and CD spectra were recorded in MeOH–H_2O (3:2) on a Hitachi U-2000 UV spectrometer and a JASCO J-720 spectrometer, respectively.

3.2. Chemicals

GR24 was supplied by Professor B. Zwanenburg, University of Nijmegen, The Netherlands. Authentic (±)-strigol was synthesized as described by Brooks et al. (1985b) and by Dailey (1987).

3.3. Plant materials

Striga hermonthica (Del.) Benth. seeds were collected in 1998/1999, from under sorghum, at the Gezira Research Station, Sudan, and kindly supplied by Professor A.G.T. Babiker, Agricultural Research Corporation, Sudan. *Menispermum dauricum* DC. roots were obtained from established cultures (Sugimoto et al., 1994).

3.4. Bioassay

The seeds were surface sterilized by immersion in 0.5% (w/v) NaOCl, containing a few drops of Tween 20, and sonication for 3 min in an ultrasonic cleaner. After having been rinsed three times with distilled water and surface-dried at 27 °C in a laminar hood, the seeds were pretreated (conditioned) with distilled water for 12–15 days on 8 mm glass fiber filter paper disks (ca. 50 seeds each) placed on water-saturated filter paper. For each bioassay, distilled water and 0.34 μM GR24 were included as negative and positive controls, respectively. Dilution series were prepared for each sample. Aq. solutions and extracts were assayed directly, by applying 20 μl aliquots of the respective test solution to conditioned *Striga hermonthica* seeds on 8 mm disks. 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, which were then allowed to dry for 1 h at room temp. A disk with conditioned *Striga* seeds was placed on top of each treated disk and moistened with 40 μl of distilled water. The treated seeds were incubated at 30 °C and microscopically evaluated for germination (radicle protrusion) 24 h later. Distilled water and 0.34 μM GR24 induced negligible and 50–60% germination, respectively.

3.5. Root culture

Roots were cultured in a modified B5 medium containing 3% sucrose and 1 μM NAA as described previously (Sugimoto et al., 1994). The roots, placed in 200-ml flasks containing 50 ml of the medium, were cultured in the dark at 27 °C on a rotary shaker at 70 rpm. Activity in the culture filtrate reached maximum after 5 to 6 weeks of culture, as reported previously (Babiker et al., 1996).

3.6. Isolation of (+)-strigol from the root culture filtrate

All separation steps were monitored by UV absorption at 245 nm and bioassays, where fractions were diluted 1:10 repeatedly and tested on conditioned *Striga hermonthica* seeds as mentioned above. At the end of each LH-20 and HPLC procedure, the column was washed with methanol in order to recover all applied materials.

The culture filtrate (6 l) was collected and treated with hexane (3 l × 2) and then with EtOAc (3 l × 4). Activity was exclusively detected in EtOAc solution. After being washed with 0.2 M K_2HPO_4 (pH 9), the EtOAc solution was dried over Na_2SO_4 and concentrated under reduced pressure to give a neutral fraction. The residue was dissolved in MeOH and chromatographed on a Sephadex LH-20 column (600 × 35 mm) using MeOH–$CHCl_3$ (4:1) as a mobile phase. Active fractions (27–42) were combined and evaporated to dryness. The residue was dissolved in MeOH and subjected to further Sephadex LH-20 column chromatography (450 × 20 mm) using MeOH–H_2O (1:1) as a mobile phase. In both phases of the LH-20 chromatography, fractions were collected every 5 ml. In the second phase of the chromatography, activity was detected in fractions from 13 to 32. These fractions were combined, MeOH was evaporated at room temp., and active ingredients were extracted from the aq. phase with EtOAc. After evaporating EtOAc at room temp., the residue was dissolved in MeOH and subjected to preparative HPLC on a Develosil ODS-UG-5 column (250 × 20 mm) using MeOH-H_2O (3:2) as a mobile phase at a flow rate of 6 ml/min, giving rise to an active fraction at 36 min. This fraction was used directly for measurement of UV and CD spectra, and for measurement of EIMS and NMR spectra after removing the solvent. This fraction was also subjected to analytical HPLC on a Develosil CN-UG-5 column (250 × 4.6 mm), using MeOH–H_2O (1:1) as a mobile phase at a flow rate of 0.5 ml/min. The column effluents were divided into two, one portion subjected to a UV detector and another to a mass spectrometer in the ESI mode. A single peak showing activity, was detected in the chromatogram at 18.5 min. Effluents from the UV detector were collected every 1 min and 30 sec in the preparative and analytical HPLC, respectively, and used for bioassay.

3.6.1. (+)-Strigol

^{1}H NMR (400 MHz) δ : 1.10 (3H, *s*, 8-Me), 1.18 (3H, *s*, 8-Me), 1.45 (1H, *m*, 7-H), 1.62 (1H, *m*, 6-H), 2.00 (1H, *m*, 6-H′), 2.03 (3H, *s*, 4′-Me), 2.72 (2H, *m*, 4-CH_2), 3.65 (1H, *m*, 3a-H), 5.51 (1H, *d*, J = 7.8, 8b-H), 6.13 (1H, *m*, 2′-H), 6.92 (1H, *m*, 3′-H), 7.45 (1H, *d*, J = 2.68, 9-H). 7-H′ (δ 1.5) and 5-H (δ 4.1) signals were hindered by

those of contaminants. UV λ_{max} 237.5 nm. CD $\Delta\varepsilon_{206}$ −21.8, $\Delta\epsilon_{234}$ +29.0 (based on the concentration determined using the molar absorptivity of (±)-strigol reported by MacAlpine et al., 1976). ESI-MS *m*/*z* (rel. int.) 715 $[2M+Na]^+$ (70), 369 $[M+Na]^+$(100). EIMS *m*/*z* (rel. int.) 346 $[M]^+$ (0.27), 328 (4.2), 249 (15.7), 231 (78.6), 203 (25.6), 97 (100), 69 (6.1).

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