

New Sesquiterpene Lactones from Sunflower Root Exudate as Germination Stimulants for *Orobanche cumana*

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ABSTRACT: *Orobanche cumana* is a serious threat for cultivation of sunflower in Europe and Asia. Germination of the parasite is induced by metabolites released from the host root system. The first germination stimulant from sunflower root exudate was recently identified as dehydrocostus lactone, a sesquiterpene lactone. Bioassay-guided fractionation of root exudates now showed the release of additional sesquiterpene lactones. Besides dehydrocostus lactone, costunolide, tomentosin, and 8-epixanthatin were purified and identified spectroscopically. All four compounds induced germination of *O. cumana* at nano- to micromolar concentrations. Costunolide and dehydrocostus lactone concentrations above 1 μM reduced the activity, and application of 100 μM inhibited germination irreversibly. Seeds of *Phelipanche ramosa* could not be induced with costunolide. *O. cumana* seeds also germinated with GR24, a synthetic strigolactone. No bioactive fraction of sunflower contained compounds of this type. This supports previous findings that sesquiterpene lactones instead of strigolactones trigger the sunflower/*O. cumana* interaction.

KEYWORDS: broomrape, *Helianthus*, *Orobanche*, sunflower, strigolactone, sesquiterpene lactone

INTRODUCTION

Parasitic weeds of the genera *Striga*, *Orobanche*, and *Phelipanche* are a serious threat for many crops, particularly in Poaceae, Fabaceae, and Solanaceae.¹ A common feature of these parasites is that germination of their tiny seeds is induced by chemical signals released from potential host plants into the rhizosphere.² This mechanism prohibits seed germination in the absence of a host and is crucial, because the embryo will die if its radicle does not reach the host root within a distance of only a few millimeters. Since the identification of strigol, a germination activator for seeds of *Striga lutea*,³ numerous derivatives with a similar four-ring terpenoid structure have been identified from plant root extracts or exudates. They were functionally analyzed with respect to their germination inductive capacity toward *Orobanche* and *Phelipanche* species.⁴ After studies on the structure–activity relationships had unraveled the involvement of a nucleophilic addition of the stimulant to the receptor site,⁵ synthetic strigolactone analogues were designed.^{6,7} Expectation was fuelled that their use in the field prior to crop sowing could lead to suicidal germination of the parasitic seed bank in the soil. GR24 is such a derivative⁸ and is currently used in most scientific studies as a control for the germination viability of seeds of Orobanchaceae.

Several other non-strigolactone compounds have so far been identified to have germination stimulating activity on broomrapes or witchweeds.^{5,9,10} Among them, the sesquiterpene lactone 11 β ,13-dihydroparthenolide gained attraction due to its effects on the witchweeds *Striga asiatica*¹¹ and *Striga hermonthica*,¹² although the unusual activity range to 10^{−20} M as described for the latter case was never confirmed. Later, a structure–activity investigation with different sesquiterpene lactones was performed and showed strong germination-inducing effects on seeds of *Orobanche cumana*, whereas *O. crenata* and *Phelipanche ramosa* did not react.¹³ This result was in line with the facts that Asteraceae in general and sunflower in particular are rich sources of sesquiterpene lactones^{14–16} and

that *O. cumana* is the only broomrape species parasitizing sunflower. However, no sesquiterpene lactones were reported from roots of sunflower until Joel et al.¹⁷ identified dehydrocostus lactone (**1**; Figure 1) as a natural root metabolite of *Helianthus annuus* and showed its stimulating activity on seed germination of *O. cumana*. This guaianolide type sesquiterpene lactone is a well-known constituent of costus root oil, a drug from *Saussurea lappa* (Asteraceae) with interesting pharmacological activities,¹⁸ but it was not previously detected in sunflower. Interestingly, **1** does not occur in the STL-rich capitate glandular trichomes of sunflower leaves and flowering parts, which had been studied intensively with respect to the biosynthesis of sesquiterpene lactones.¹⁹ Therefore, we were interested to see whether **1** is the only product of a trichome-independent sesquiterpene lactone biosynthesis in sunflower roots and thus the only stimulant for the host recognition by *O. cumana*. We here report the bioassay-guided fractionation of sunflower root exudates and spectroscopic identification of new sesquiterpene lactones with germination-stimulating activity.

MATERIALS AND METHODS

Plant Materials and Reagents. Seeds of *O. cumana* Wallr., kindly provided by D. Rubiales (CSIC, Cordoba, Spain), were collected in 2005 in Cordoba (Spain). Seeds of *P. ramosa* (L.) Pomel were collected in 2007 from tobacco fields in the Baden region in Germany. Both seed samples were stored in darkness at room temperature until use. Sunflower (*H. annuus* L.) seeds of different commercially available cultivars were used as host for the bioassays. Lines HA89 and HA300 served as control, but no differences were observed with respect to the stimulation of broomrape seeds.

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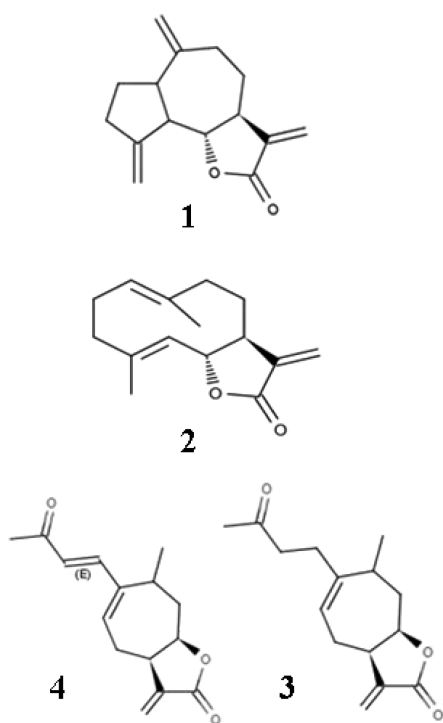


Figure 1. Bioactive sesquiterpene lactones isolated from the root exudate of sunflower, *Helianthus annuus*: dehydrocostus lactone (1); costunolide (2); tomentosin (3); 8-epixanthatin (4).

The synthetic strigolactone GR24 was purchased from B. Zwaneburg, Department of Organic Chemistry, Radboud University Nijmegen, Nijmegen, The Netherlands. Costunolide (2, Figure 1), dehydrocostus lactone (1), tomentosin (3), and 8-epixanthatin (4) were extracted from *H. annuus* roots and isolated from exudates as described below. All sesquiterpene lactone samples were checked for impurities by ^1H NMR measurements. An additional sample of costunolide was purchased from Selleck, batch S131901 (Selleck Chemicals LLC, Houston, TX, USA). Additional dehydrocostus lactone was extracted from costus root oil (Roland Jahn, Alambic, Leutenbach, Germany).

Conditioning of Broomrape Seeds and Germination Test. Seeds of *Orobanche* or *Phelipanche* were surface sterilized with sodium hypochloride (1%, sonicated for 2 min) and washed with deionized water. Afterward, they were placed on small squares of moist filter paper (ca. 1 cm², Whatman filter type GF/A) with a density of approximately 150 seeds per square. The squares were stored on wet filter paper in sealed (para film) 9 cm Petri dishes in darkness at 16 °C for at least one week. This method of conditioning has been reported to improve the ratio and speed of germination.²⁰ Before use, all filter papers were sterilized in dry heat at 150 °C for 2 h, to prevent mold formation.

The germination test was carried out in separate Petri dishes of 3.5 cm in diameter. On top of a filter paper (3.5 cm in diameter) was placed a square of a Whatman filter paper (ca. 1 cm²), and sample solutions with putative germination stimulants were added in the center. After evaporation of the solvent, a square with conditioned seeds of *O. cumana* (or other broomrape species) was placed on top of it. The filter paper was moistened with 250 μL of double-distilled H₂O. The Petri dishes were sealed with parafilm and stored at 16 °C in darkness for 1 week, before the germination of the seeds was counted under a stereomicroscope. Samples with 250 μL of double-distilled H₂O were used for negative control. Samples with 1 μM GR24 served as positive control for the viability of broomrape seeds and their readiness to germinate. ED₅₀ values for the sesquiterpene lactones were calculated using the maximum germination obtained with 1 μM GR24 as 100% of inducible seeds.

Sunflower Seedling Test. To test the site of stimulant release, 6-day-old sunflower seedlings, raised on wet filter paper, were placed horizontally on water agar (1%) in Petri dishes. To prevent external diffusion of compounds, the agar was trenched between the root, hypocotyl, and cotyledon area. Strips of Whatman filter paper with conditioned seeds of *O. cumana* were placed on the plant surface for 24 h and then stored for 6 days in darkness on wet filter paper, before the ratio of germinated seeds was counted under a stereomicroscope.

Statistics. Germination tests were statistically analyzed using the program R.²¹ Differences were considered as significant if $P < 0.05$ in the Welch test. All tests were carried out at least three times, and germination rate was calculated as mean value with standard deviation.

Exudate Generation. To test the natural release of germination stimulants from roots, sunflower seedlings were raised on wet filter paper for ca. 5 days and then transferred to hydroponic cultivation in tap water (50 seedlings per 1 L; room temperature, 14 h light with 145 $\mu\text{M m}^{-2} \text{s}^{-1}$ photosynthetic photon flux). The hydroponic solution was circulated (600 mL/h) with a peristaltic pump (Heidolph Pumpdrive 5001, SP quick, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) over a column (3 \times 1 cm) filled with 2 g of Amberlite XAD-4 resin (Sigma, Taufkirchen, Germany). After 24 h of circulation, the water was replaced and the absorbed compounds were extracted from the column with acetone. The extracted exudate samples were dried, dissolved in 400 μL of 50% aqueous MeOH, and tested with the bioassay for stimulating activity as described before. Seedlings used for the collection of exudates for structure identification were in the age of ca. 10–25 days post germination.

For the quantitation of sesquiterpene lactones released with 24 h, 21-day-old seedlings were used. After 24 h of exudation, the roots of the seedlings were harvested and dried. Quantitation of the sesquiterpene lactones in the exudate was performed using HPLC calibration curves with reference samples of the respective sesquiterpene lactone. The daily exudated amount was calculated per gram dry weight of roots.

Purification of Stimulants. A Shimadzu HPLC (Prominence UFLC with a diode array detector SPD-M10AVP) was used for the first purification step of the stimulants. Exudate samples of sunflower roots were separated on an RP18 Kromasil column (250 \times 4 mm, 5 μm particle size, MZ-Analysentechnik, Mainz, Germany) in a MeOH gradient (50–65% MeOH in 20 min, followed by 65–100% in 15 min) at a flow of 1 mL/min. Fractions (starting after 4 min) of ca. 1 mL (or peak separated) were sampled, dried in a vacuum concentrator, redissolved in MeOH, and used in a germination test with seeds of *O. cumana* as described above. Fractions with germination-stimulating activity were rechromatographed on a Dionex HPLC (P580 pump system with a UVD340S diode array detector) with an RP18 Gromsil column (150 \times 4.6 mm, 3 μm particle size, Alltech GROM GmbH, Worms, Germany), subfractionated if necessary, and tested again in the bioassay with *O. cumana*. A gradient of 30–50% acetonitrile in 20 min was used for the purification of 3 and 4 from fractions A and B, respectively, whereas 2 and 1 were rechromatographed in 66% acetonitrile to confirm their purity. Pure compounds from numerous runs were pooled to reach ca. 1 mg for NMR spectroscopy and structure elucidation. The retention times of the compounds relative to 2,5-dimethylphenol (used as internal standard) were 0.718 for 4, 0.922 for 3, 1.922 for 2, and 2.007 for 1 in separation system 1 and 1.036 for 4 and 1.246 for 3 in separation system 2.

Root Extraction. For the bioassays, additional purification of the sunflower sesquiterpene lactones was necessary. The roots (15–20 g FW) of sunflower seedlings (surface sterilized before germination and cultivated on wet filter paper) were chopped with a razor blade and extracted with acetone (3 \times 100 mL, 10 min). The extract was dried under reduced pressure, resuspended in CHCl₃, and partitioned three times against the same volume of water. The chloroform fraction was dried, resuspended in a mixture of pentane, methanol, and water (3:3:1), and partitioned again. The polar fraction was dried, redissolved in MeOH (50%), and used for compound purification in semipreparative HPLC. All steps were monitored for stimulation of *O. cumana* germination by bioassay as described above.

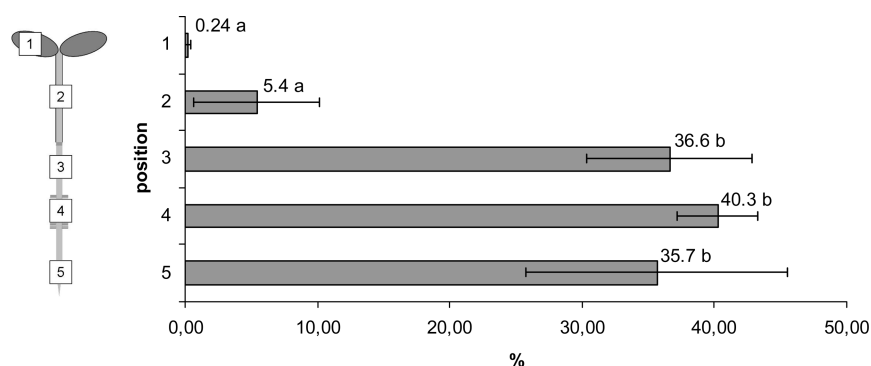


Figure 2. Localization of the exudation area of germination stimulants from a sunflower seedling. Seeds of *O. cumana* on wet filter paper disks were placed for 24 h on the surface of sunflower seedling at five different areas (1, cotyledon; 2, hypocotyl; 3, older root part; 4 hairy root zone; 5, root tip) and then placed in Petri dishes for germination. Values with the same letter are not significantly different according to the Welch test ($P = 0.05$), $n = 5$; positive control (GR24) = 55%, germination of *O. cumana*.

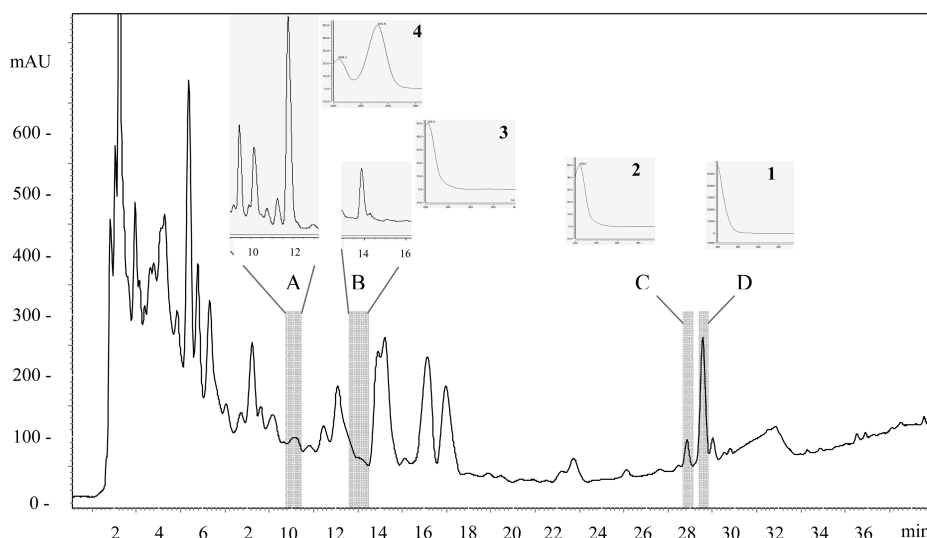


Figure 3. HPLC chromatogram of sunflower root exudate in a MeOH gradient at 210 nm. The four fractions (A–D) showed germination-stimulating activity on *O. cumana* seeds. Fractions C and D contained the sesquiterpene lactones costunolide and dehydrocostus lactone, respectively, whereas fractions A and B afforded a compound mixture from which 8-epixanthatin and tomentosin, respectively, after reperification in acetonitrile–water gradient on Gromsil RP18 (see insets A* and B*). Insets 1–4 show the spectra of the four sesquiterpene lactones, with maxima at 209 and 281 nm for 8-epixanthatin, 205 nm for tomentosin, 210 nm for costunolide, and 200 nm for dehydrocostus lactone in the acetonitrile–water system.

Quantitation of Sesquiterpene Lactones by HPLC. Different amounts of the purified sesquiterpene lactones were injected in the two HPLC systems (RP18 Kromasil; Gromsil RP18) used for the exudate separation (see above). All samples were run in triplicate, and integrals of the peaks at 210 nm detection wavelength were used for calibration. As the separation of the xanthanolides required fractionation in the MeOH system and rechromatography in CH_3CN , the recovering efficacy was determined and was found to be 40%. The quantitation of sesquiterpene lactones in the exudates was performed in the CH_3CN system with regard to the recovering efficacy at 210 nm for 1–3 and 280 nm for 4.

Spectroscopy. NMR experiments were performed in a Varian Unity Inova spectrometer at 500 MHz. ^1H chemical shifts were referenced to residual solvent signals at $\delta_{\text{H/C}}$ 7.27 (CDCl_3). HRMS-EI data were obtained from measurements on a JEOL JMS-700.

1, dehydrocostus lactone: EI MS m/z 230.1305 $[\text{M}]^+$ ($\text{C}_{15}\text{H}_{18}\text{O}_2$); ^1H NMR (500 MHz, CDCl_3) δ 6.22 (1H d, $J = 3.3$ Hz, H-13a), 5.48 (1H dd, $J = 3.2$ Hz, H-13b), 5.27 (1H d, $J = 1.7$ Hz, H-15a), 5.07 (1H d, $J = 1.7$ Hz, H-15b), 4.89 (1H s, H-14a), 4.81 (1H s, H-14b), 3.97 (1H t, $J = 9.6$ Hz, H-6), 2.80–2.95 (3H, H-1, H-5, H-7), 2.42–2.58 (4H, H-3a,b, H-9a/b), 2.1–2.3 (2H, H-8a/b), 1.82–2.02 (2H, H-2a/b).

2, costunolide: EI MS m/z 232.1459 $[\text{M}]^+$ ($\text{C}_{15}\text{H}_{20}\text{O}_2$); ^1H NMR (500 MHz, CDCl_3) δ 6.26 (1H d, $J = 3.5$ Hz, H-13a), 5.52 (1H d, $J = 3.2$ Hz, H-13b), 4.84 (1H bdd, $J = 11$ and 4 Hz, H-1), 4.73 (1H bd, $J = 9.6$ Hz, H-5), 4.56 (1H t, $J = 8.9$, H-6), 2.56 (1H m, H-7), 1.70–2.43 (8H, H-2a/b, H-3a/b, H-8a/b, H-9a/b), 1.69 (3H s, H-15), 1.42 (3H s, H-14).

3, tomentosin: EI MS m/z 248.1484 $[\text{M}]^+$ ($\text{C}_{15}\text{H}_{20}\text{O}_3$); ^1H NMR (500 MHz, CDCl_3) δ 6.26 (1H d, $J = 3.4$ Hz, H-13a), 5.52 (1H d, $J = 2.8$ Hz, H-13b), 5.44 (1H dd, $J = 8.8$ and 5.3 Hz, H-5), 4.64 (1H ddd, $J = 12$, 8.4, and 2.8 Hz, H-8), 3.32 (1H m, H-7), 2.57 (1H ddd, $J = 16.7$, 7.8, and 7.5 Hz, H-3a), 2.47 (1H ddd, $J = 16.7$, 7.7, and 7.4 Hz, H-3b), 2.44 (1H ddd, $J = 14.3$, 5, and 1.5 Hz, H-6a), 2.35 (1H m, H-10), δ 2.25 (2H m, H-2a,b), 2.19 (1H m, H-6b), 2.16 (3H s, H-15), 2.02 (1H m, H-9a), 1.90 (1H m, H-9b), 1.14 (3H d, $J = 6.9$ Hz, H-14).

4, 8-epixanthatin: EI MS m/z 246.1215 $[\text{M}]^+$ ($\text{C}_{15}\text{H}_{18}\text{O}_3$); ^1H NMR (500 MHz, CDCl_3) δ 6.99 (1H d, $J = 16.2$ Hz, H-2), 6.33 (1H d, $J = 3.3$ Hz, H-13a), 6.21 (1H dd, $J = 9.0$ and 6.4 Hz, H-5), 6.15 (1H d, $J = 16.2$ Hz, H-3), 5.80 (1H d, $J = 3.0$ Hz, H-13b), 4.66 (1H ddd, $J = 12$, 8.4, and 2.6 Hz, H-8), 3.42 (1H m, H-7), 2.83 (1H m, H-10), 2.60 (1H ddd, $J = 14$, 6.5, and 5 Hz, H-6a), 2.52 (1H ddd, $J = 14$, 6.5, and 5.1 Hz, H-6b), 2.30 (3H s, H-15), 2.18 (1H ddd, $J = 14.1$, 6, and

2.5 Hz, H-9a), 1.93 (1H ddd, $J = 14.1, 12.1,$ and 12 Hz, H-9b), 1.18 (3H d, $J = 7$ Hz, H-14).

RESULTS

Site of Stimulant Release. To test the sites of release of germination stimulants from sunflower seedlings, small strips of moist filter paper with conditioned seeds of *O. cumana* were placed for 24 h on the surface of 6-day-old seedlings and then stored for an additional 6 days in moisturized Petri dishes. The broomrape seeds readily germinated when they had been in liquid contact with the root zone (Figure 2). Samples of the three areas of the root (root tip, hairy root zone, older root part) showed high rates of germination with mean values between 35.7 and 40.3%. The differences were not significant ($P = 0.05$ in the Welch test), and the means reached the range of the positive control achieved with GR24 (55%). In contrast, significantly lower germination activity (5.4 and 0.24%) was found after contact of the seeds with the hypocotyl and cotyledon surface.

Guided by this observation, root exudates were collected from hydroponic cultures of sunflower seedlings and tested for bioactivity. Exudates showed strong, but sometimes highly variable, activity in comparison to controls tested with GR24. In samples with weak activity, the effects often decreased when higher concentrations were applied, but rose when a 1:10 or 1:100 dilution was applied. This indicated that the stimulating activity could be covered by inhibiting effects. This had to be taken into account and complicated the search for the responsible compounds in bioassay-guided fractionation.

Isolation and Structure Identification of Stimulants. HPLC analysis of the root exudate on Kromasil RP18 in a methanol–water gradient showed a broad array of hydrophilic to hydrophobic compounds. The four fractions A–D (Figure 3), which showed stimulating activity on seeds of *O. cumana*, were subjected to final purification (A, B) or were directly used for spectroscopic analysis (C, D).

LC-MS/MS measurements (data not shown) were carried out to check the presence of strigol-type compounds in the bioactive fractions. However, unlike in the control sample of GR24, in our active samples we could not detect the m/z 97.028 mass fragment of the D-ring, which is typical for strigol-type compounds.^{22,23} In HPLC, peak spiking with a reference sample and comparison of the UV spectrum indicated a potential peak of **1** in the most hydrophobic fraction, D. MS and ¹H NMR spectroscopic measurements of the purified compound confirmed the structural identity with **1**.^{24,25} In fraction C, a second active compound was detected, and its spectroscopic data were identical with those of **2** (peak maximum at 210 nm in acetonitrile–water).²⁶ This germacranolide, although recently synthesized in yeast cells after transformation with sunflower enzymes involved in sesquiterpene lactone biosynthesis,²⁷ has not been identified from *H. annuus* before. The more polar fractions B and A were subfractionated with HPLC on Gromsil RP18 in an acetonitrile–water gradient (see insets A* and B* in Figure 3). Fraction B contained a bioactive compound with UV maximum at 205 nm in acetonitrile–water. Its MS and ¹H NMR spectroscopic signals were identical to those of **3**, a *cis*-7,8-STL of the xanthanolide type.²⁸ The bioactive compound in fraction A showed UV maxima at 209 and 281 nm. The ¹H NMR signals were similar to those of **3**, but the replacement of four aliphatic by two olefinic signals in combination with the molecular weight of 246 (instead of 248) indicated the

presence of a double bond at C-2 and C-3. This corroborated the spectroscopic data of **4**,²⁹ a xanthanolide that was previously found in shoots of sunflower.³⁰

The daily amount of released sesquiterpene lactones in the sunflower root exudate of 3-week-old plants was quantified by HPLC. Within 24 h of exudation, the average amount per gram DW of root tissue was 1.0 μg for **1** ($n = 4, \text{SD} = 0.7$), 0.5 μg for **2** ($n = 2, \text{SD} = 0.19$), 37.2 μg for **3** ($n = 3, \text{SD} = 8.7$), and 7.8 μg for **4** ($n = 3, \text{SD} = 1.4$).

Bioactivity of the Sesquiterpene Lactones. Compounds **1** and **2** stimulated germination of *O. cumana* seeds in a similar range of concentrations. Starting at 0.1–1 nM concentration, the stimulation reached a maximum at 0.1–1 μM (Figure 4). The ED_{50} values (relative to the maximum stimulation with 1 μM GR24) were 3×10^{-8} M for **1** and 2×10^{-8} M for **2**, both isolated from sunflower.

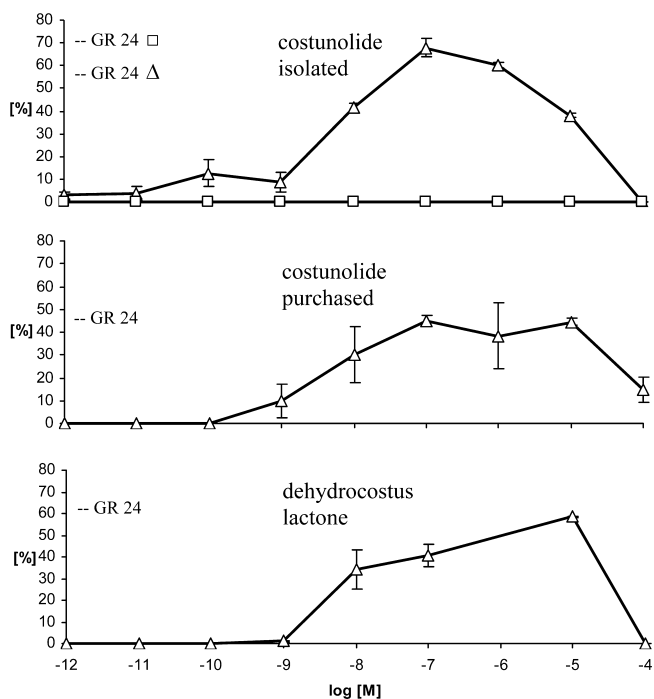


Figure 4. Influence of isolated and purchased sesquiterpene lactones on germination of *O. cumana* (Δ) and *P. ramosa* (\square). The germination of seeds obtained with 1 μM GR24 is marked separately for each experiment (mean, $n = 3$). All tests were carried out in triplicate. Controls with pure water showed no germination.

The germination decreased rapidly above the 10 μM level and went to zero at 0.1 mM in both cases. The absence of germination after treatment of seeds with 0.1 mM **2** could not be compensated when the seeds were washed with H_2O and subsequently treated with GR24. The germination was blocked irreversibly. Tests with commercially available **2** showed a similar correlation with increasing concentrations. However, contrary to **2** isolated from sunflower, no germination was found below 1 nM and 0.1 mM did not completely inhibit the seeds. Seeds of *P. ramosa* treated with **2** showed no stimulation of germination at all in the range from 1 pM to 0.1 mM, thus indicating high specificity of *O. cumana* for this compound.

The amount isolated of the two xanthanolides was insufficient to complete the bioassays with *O. cumana* in micromolar concentrations and with sufficient replications. However, the results for **3** (three replicates) showed that

stimulation started already at 0.01 nM and had a maximum (reaching ca. 60% of the GR24 control) at 0.1 nM. Concentrations of 1 and 10 nM resulted in only weak stimulation of seed germination. Compound **4** induced germination at concentrations from 100 nM to 10 μ M (reaching 100% of the GR24 control), but the results varied considerably from one experiment to the next, and the amount of pure compound fell short of accomplishing the experiment with sufficient repetitions for statistical evaluation.

DISCUSSION

For more than a century, broomrape has been a serious problem in sunflower cultivation in Russia and later became a serious threat to sunflower production in many other European countries. The fast development of new parasitic races competed with breeding of resistant host lines, and the currently prevailing populations of *O. cumana* cannot be controlled by sunflower cultivars bearing the known resistance genes ORI–ORS.³¹ Host specificity is an important feature in which *O. cumana* differs from most broomrape species, and none of the other economically important taxa are known to depend on a single host.^{1,32} Besides resistance reactions of the attacked plant, recognition of host-released rhizosphere metabolites is likely to account for the specialization.³² Previous germination studies with seeds of *O. cumana* showed positive reaction with sesquiterpene lactones, but the compounds were not host-derived and the seeds were similarly stimulated by the strigolactone GR24.³³ No natural stimulant from sunflower was identified before Joel et al.¹⁷ isolated **1** from roots and traced the presence of the guaianolide in the exudate indirectly by cochromatography with the reference. As in many other host–parasite interactions, host recognition is seldom based on a single compound.^{34,35} Therefore, it was not surprising to find three additional sesquiterpene lactones with germination-stimulating activity when we tested root exudates from hydroponic sunflower cultures. Interestingly, the identified compounds belong to three different skeletal types (germacranolides, guaianolides, xanthanolides), which are rare or not represented in the sesquiterpene lactone profile known from sunflower trichomes (mostly harbouring heliangolides).¹⁵

Structure elucidation of all four sesquiterpene lactones was performed from extracts of the hydroponic solution to show that the compounds were secreted into the rhizosphere in a non-root-destructive manner. The bioassays showed that the stimulating activity was largely restricted to the root surface, whereas contact to the epidermis of the hypocotyl and cotyledons of sunflower had only weak effects on *O. cumana* seeds. In contrast to the root, the hypocotyl and cotyledons are covered by a relatively thick cuticle, which prohibits diffusion of compounds from the inner tissues. In addition, both organs are glabrous, and it is noteworthy that none of the four identified compounds has yet been found in glandular trichomes of true leaves and flowering parts of *H. annuus*, where STL are sequestered in large amounts.^{15,16} The small amounts of **1**, previously reported from leaves of sunflower³⁶ and from stem and cotyledon extracts of young seedlings,¹⁷ apparently derive from inner tissues of the plant, because, in the first case, the abundant trichome-based sesquiterpene lactones known from true leaves were entirely missing and, in the latter case, predominantly trichome-less cotyledons and hypocotyls were extracted when using 5–6-day-old seedlings. Obviously, independent pathways exist for the formation of sesquiterpene lactone in the surface-located glandular trichomes of aerial parts

as well as in inner tissues of sunflower. Bioassays with trichome-derived sesquiterpene lactones from leaf leachates of sunflower indicated that they have also germination-stimulating activity on seeds of *O. cumana* (data not shown), but their primary function in the plant is most likely protective activity against herbivory.

One of the exudate compounds, 8-epixanthatin (**4**), was previously found in shoots of sunflower and was suggested to play an important role in the phototropic reaction of the plant.³⁷ This could be a hint for a possible hormonal function of STL in the inner tissues, where low concentration appears to be necessary for regulatory effects related to auxin-mediated elongation growth. As recently suggested for **1**,¹⁷ this would parallel the hormonal activity reported for strigolactones.^{38,39} In contrast, high concentrations of sesquiterpene lactones are cytotoxic due to the nucleophilic reactivity of the exocyclic methylene group of the lactone moiety. The latter effect explains the gradual and irreversible loss of the stimulating activity when *O. cumana* seeds were treated with sesquiterpene lactone concentrations above 10 μ M. On the other hand, structure–activity studies for strigolactones by Zwanenburg et al.⁵ have shown that the nucleophilic addition to a receptor is essential for the stimulation of *Orobanch*e. This is supported by the experiments of Joel et al.,¹⁷ who showed a drastic loss of activity when the methylene function of **1** had been reduced to a methyl group.

No strigolactones were found in the bioactive fractions of sunflower root exudates. This indicates that *O. cumana* may recognize its natural host exclusively by sesquiterpene lactones or by compounds of yet unknown chemical nature. Similarly, Auger et al.⁹ recently reported germination of *O. cumana* in concentrated root exudates of the nonhost *Brassica napus*, where they failed to detect strigolactones even at a range of picograms. This appears contradictory to a report on strigolactones exuded by Asteracean plants, in which LC-MS/MS measurements identified a putative fragment of orobanchyl acetate and 5-deoxystrigol in sunflower.⁴⁰ However, contrary to all other taxa tested in the study, no germination-stimulating activity in the bioassay with *Orobanch*e minor was found with the exudate of sunflower, although *O. minor* is highly sensitive to strigolactones. Despite the high sensitivity of *O. cumana* for the sesquiterpene lactones released from its natural host, the seeds of sunflower broomrape also react on strigolactones such as the synthetic compound GR24 and to a lesser degree on the natural apocarotenoid strigol.⁴¹ According to Fernandez-Aparicio et al.,³² this could be indicative for the relatively recent speciation of *O. cumana*.

Our bioassays with *P. ramosa* and **2** showed no induction of seed germination, even at micromolar concentrations. This corroborates results reported from a structure–activity study with various sesquiterpene lactones (including **1**), which had specific effects on *O. cumana*, but none on *Orobanch*e crenata, *P. ramosa*, and *Phelipanche aegyptiaca*.³³ In contrast, Joel et al.¹⁷ reported significant stimulation when seeds of *P. aegyptiaca* were treated with 0.1–1 μ M **1**. Fernandez-Aparicio et al.³² found that *O. cumana* was the only of seven species of *Orobanch*e that was stimulated by sunflower exudate, whereas in both tested *Phelipanche* species, *P. aegyptiaca* and *P. ramosa*, seeds germinated readily when treated with the same exudate. In the face of these somehow contradictory results, it appears possible that different ecotypes of broomrape species could vary in their reaction as was shown by Joel et al.¹⁵ for different samples of *O. cumana* with sunflower exudates. Additional tests

will be necessary to unravel the specificity of stimulants and the structural modifications in the receptors of broomrape species.

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Notes

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ABBREVIATIONS USED

DW, dry weight; FW, fresh weight; SD, standard deviation

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