Strigolactones Stimulate Arbuscular Mycorrhizal Fungi by Activating Mitochondria

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The association of arbuscular mycorrhizal (AM) fungi with plant roots is the oldest and ecologically most important symbiotic relationship between higher plants and microorganisms, yet the mechanism by which these fungi detect the presence of a plant host is poorly understood. Previous studies have shown that roots secrete a branching factor (BF) that strongly stimulates branching of hyphae during germination of the spores of AM fungi. In the BF of Lotus, a strigolactone was found to be the active molecule. Strigolactones are known as germination stimulants of the parasitic plants Striga and Orobanche. In this paper, we show that the BF of a monocotyledonous plant, Sorghum, also contains a strigolactone. Strigolactones strongly and rapidly stimulated cell proliferation of the AM fungus G. rosea at concentrations as low as 10^{-13} M. This effect was not found with other sesquiterpenes lactones known as germination stimulants of parasitic weeds. Within 1 h of treatment, the density of mitochondria in the fungal cells increased, and their shape and movement changed dramatically. Strigolactones stimulated spore germination of two other phylogenetically distant AM fungi, G. intraradices and G. claroideum. This was also associated with a rapid increase of mitochondrial density and respiration as shown with G. intraradices. We conclude that strigolactones are important rhizospheric plant signals involved in stimulating both the pre-symbiotic growth of AM fungi and the germination of parasitic plants.

Introduction

In the immediate area surrounding plant roots, called the rhizosphere, roots are in active contact with soil-borne microorganisms. Besides the beneficial or competitive interactions between roots and microorganisms that involve microbial nutrient cycling, plant supply of carbon substrates, and competition for nutrient sources, plant roots and soil microbes also interact more intimately. Roots exude not only large quantities of amino acids, organic acids, sugars, vitamins, purines, nucleosides, enzymes, hormones, inorganic ions, and CO₂, but also small amounts of a variety of flavonoids, terpenoids, and other secondary metabolites [1] that serve as early chemical mediators of more specific plant-microbe interactions [2]. Some of them act as chemotactants [3,4], as antibiotics that confer resistance to bacterial pathogens [5], or as root signals that specifically induce microbial genes necessary for the establishment of compatible associations with plants [6].

About 80% of all plant species form intimate symbiotic associations with a class of ubiquitous soil microorganisms called arbuscular mycorrhizal (AM) fungi, yet little is known about how these fungi detect the presence of their plant hosts. As obligate biotrophs, the survival of these fungi depends on their ability to associate rapidly with plant roots and to set up symbiotic relationships with them. Spores of AM fungi can germinate in the absence of a host (non-symbiotic growth); however, further growth and branching of germinating hyphae prior to root infection (pre-symbiotic growth) require the presence of compounds released by roots [7]. Among the diversity of molecules present in root exudates and active in hyphal branching, flavonoids have often been proposed to switch AM fungi from non- to pre-symbiotic growth [8,9]; micromolar concentrations of some of them can stimulate growth of AM fungi. However, the roots of chalcone synthase mutants of maize, which are deficient in flavonoid synthesis, are colonised normally by AM fungi, demonstrating that flavonoids are not essential for the process [10].

In earlier studies, a branching factor (BF) from hairy root cultures of D. carota was discovered that strongly stimulates branching of the germinating hyphae of G. rosea spp. [11,12]—a response similar to that typically observed when hyphae of AM fungi grow in the presence of living roots. BF induces mitosis in the hyphae [12], which is necessary to form new hyphal branches and increase root-fungus contacts. We found this BF also in several other mycorrhizal plants [12]. BF first induces expression of genes related to mitochondrial activity in the fungus (after 1 h), then

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Table 1. Quantitative Hyphal Branching Response of Gi. rosea Stimulated by Various Concentrations of Germination Stimulants of Striga and Orobanche

| Germination Stimulants | Compound Concentrations (M) | 0 (Control) | $10^{-15}$ | $10^{-14}$ | $10^{-13}$ | $10^{-11}$ | $10^{-9}$ | $10^{-7}$ | $10^{-5}$ |
|------------------------|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| GR7                    | 1                           | ND          | ND          | ND          | 1.04        | 1.19        | 1.68        | 2.74        |
| GR24                   | 1                           | ND          | ND          | 1.55        | 1.58        | 2.81        | 3.31        | ND          |
| Sorgolactone           | 1                           | 1.31        | 1.42        | 1.91        | 2.01        | 2.86        | 3.81        | 5.37        |
| Artemisinin            | 1                           | ND          | ND          | 1.06        | 0.79        | 0.90        | 0.93        | 1.01        |
| Parthenolide           | 1                           | ND          | ND          | ND          | ND          | 0.57        | 0.35        | 0.30        |

The means of the apex numbers (from three independent experiments) are presented as ratios of control mean values.

*Significant one-way Anova test ($p < 0.05$).

ND, not determined.

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increases the rate of respiration (after 1 h 30 min) and mitochondrial reorganization (after 4 h) before stimulating fungal ramification (after 24 h) [13], suggesting that the branching response is the result of a metabolic switch.

Preliminary investigations of BF of D. carota indicated that the active component is a low-molecular-weight lipophilic molecule present in root exudates at extremely low concentration [12]. Among candidate molecules in root exudates that share these characteristics and are already known to be involved in plant–soil organism interactions are the strigolactones—potent germination stimulants of parasitic weeds such as Striga and Orobanche [14] (Figure S1). Like AM fungi, these parasitic plants are obligate biotrophs whose survival depends on rigorous host recognition [15]. Recently Akiyama et al. [16] demonstrated that the BF of the dicotyledenous (dicot) plant Lotus japonicus contains the strigolactone 5-deoxy-strigol. This report also showed that 5-deoxy-strigol, strigol, sorgolactone, and the strigolactone analogue GR24 stimulate hyphal branching of the AM fungus Gi. margarita at subnanomolar concentrations.

To substantiate the hypothesis of strigolactones as important stimulants of AM fungi, our aim was to examine whether: i) the BF isolated from a monocotyledenous (monocot) plant species contains strigolactones; ii) among known stimulants of Striga and Orobanche seed germination, strigolactones specifically stimulate AM fungi [15]; iii) strigolactones not only stimulate hyphal branching of germinating spores of AM fungi but also are the molecules responsible for activating mitochondria, the typical response to BF [13]; iv) strigolactones are active on more than one AM fungal species.

Our data show that strigolactones must indeed be a widespread class of rhizospheric stimulants of AM fungi produced by dicots as well as monocots. They also demonstrate that these molecules provoke a rapid and strong cellular response targeted on mitochondrial apparatus in the fungus.

Results

The BF of D. carota Hairy Roots Stimulates not only AM Fungi but also Seed Germination of Orobanche

We first tested whether BF isolated from D. carota (carrot) hairy roots, which stimulates branching of the hyphae of AM fungi, would also stimulate germination of the seeds of the parasitic weed Orobanche. The percentage of germinating seeds was 6.2- to 8.9-fold higher for BF-treated seeds compared to controls. A positive control with GR24 ($10^{-7}$ M), a strigolactone analogue that induces germination of parasitic weeds, increased germination an average of 6.3-fold compared to the control, showing that, in our experimental conditions, the carrot BF and GR24 had similar stimulatory activity on Orobanche seed germination.

Seed Germination Stimulants of Parasitic Weeds other than Strigolactone Have No Effect on Hyphal Branching of Gigaspora rosea

Several molecules have been identified as potent stimulants of Striga and Orobanche germination: strigolactones [17] (from which the synthetic analogue GR24 is derived [18]), sesquiterpene lactones such as parthenolide and artemisinin [19], and dihydrosorgoleone [20] (Figure S1). To see whether AM fungi respond to one or more of these compounds, we tested the effect of GR24, parthenolide, artemisinin, and dihydrosorgoleone on hyphal branching of Gi. rosea. We used the very sensitive bioassay of branching described by Bué et al. [12]. Parthenolide, artemisinin, GR24, and dihydrosorgoleone were tested at concentrations known to activate parasitic weed germination; GR24 was the only compound that gave a positive response (Table 1).

Sorghum BF Contains the Strigolactone Sorgolactone

5-deoxy-strigol was found to be the active substance in the BF of L. japonicus [16]. For generalisation purposes, it is essential to determine whether the BF of other plants, particularly in divergent ones like monocots, also contains strigolactones. Therefore, we analysed the BF of Sorghum because the strigolactones of this plant species have already been characterized. Sorghum contains at least two strigolactones: sorgolactone and, in smaller amounts, strigol [21]. We collected root exudates from Sorghum seedlings that had germinated for 5–30 d. The organic content of the exudates was extracted in ethyl acetate and separated using C18 fractionation. All fractions were tested for activity in the fungal branching assay. 24 h after its application, the most hydrophobic fraction (fraction 6) could be seen to stimulate hyphal branching of Gi. rosea. Sorgolactone was identified in this fraction by using ESI-MS/MS from the ($M + Na^+$) $339 \text{ m/z}$ ion showing the loss of the specific D ring of strigolactones (daughter ion at 242 m/z) (Figure 1). Fraction 6 was further separated using C18 high-performance liquid chromatogra-
phy (HPLC), and the subfraction that eluted in the same position as a synthetic sorgolactone marker was collected. This fraction also stimulated hyphal branching.

The same protocol was applied to crushed roots and leaves from *Sorghum* seedlings grown for 4 wk. Sorgolactone was detected in the same C18 column fraction 6 as in the separation of root exudates (100% acetonitrile; unpublished data). This rapid method for detecting strigolactones directly in plant tissue extracts has not previously been reported in the literature and may provide an important tool for exploring their occurrence in the plant kingdom.

**Strigolactones Act on *Gi. rosea* at Very Low Concentrations**

We carried out a quantitative dose-response analysis of the effect of GR24, GR7 (another strigolactone analogue that contains only the BCD rings; Figure S1), and synthetic sorgolactone on *Gi. rosea* hyphal branching (Table 1). GR7 stimulated branching at concentrations above 10⁻⁷ M, whereas GR24 and sorgolactone were still significantly active at 10⁻¹¹ M and 10⁻¹³ M, respectively. This shows that GR24 and sorgolactone are more potent branching stimulants than GR7, and thus that the A ring, absent from the GR7 molecule, is probably required for a maximum activity.

To determine whether the stimulation of hyphal branching by strigolactone observed after 24 h was also significant after several days, hyphal branching and hyphal length were measured up to 2 wk after stimulation with 10⁻² M GR7 (Figure 2). GR7 was used for this assay because a large amount was needed and it was available commercially. Stimulation of hyphal branching by GR7 was maintained throughout the 2-wk period. At the end of the experiment, the treated spores had produced 2.8-fold more hyphal branches than control spores. The hyphae that grew from the treated spores were also longer. Hyphal branching, therefore, seems to be a durable effect, suggesting that the stimulated fungus enters a new morphogenetic program, preceding the symbiotic stage.
The Strigolactone Analogue GR24 Induces a Rapid Mitochondrial Response in the Fungus

Tamasloukht et al. [13] showed that before *Gi. rosea* cells began to branch in response to BF isolated from carrot hairy roots, they produced more mitochondria and their respiratory rate increased. These important fungal responses involve the oxidative catabolism of lipids—the main form of carbon storage in AM fungi—and the production of ATP. This raises the possibility that strigolactones in BF are responsible for stimulating fungal respiration and lipid catabolism. To address this question, we tested the effect of GR24 on mitochondrial density, shape, and movement in *Gi. rosea*. Living hyphae were stained with Mitotracker Green, a fluorescent vital stain specific for mitochondria [22], and observed by fluorescence microscopy [13]. After only 1 h of GR24 treatment, the mitochondrial density increased by 23% (Figure 3). This phenomenon was more pronounced after 5 h of treatment (32%; Figure 3). To substantiate these data, we used monoclonal antibodies against the yeast mitochondrial cytochrome oxidase (COX) subunit I and against the yeast mitochondrial porin protein to observe the mitochondria by immunofluorescence microscopy in fixed fungal hyphae (Figure S2). Double-labelling with the two antibodies showed clearly co-localised staining (Figure S3). We calculated the fluorescence intensity of fungal cells labelled with each antibody before and after exposure of the fungus to GR24. Consistent with the Mitotracker Green test, we found a significant increase in mitochondrial density with the anti-porin antibody as well as with the anti-COX antibody (Figure 3).

Observations of living cells stained with Mitotracker Green showed that the shape and distribution of the organelles was also remarkably modified. In control cells, the mitochondria appeared spherical and randomly distributed, whereas in treated cells they adopted a thread-like structure oriented parallel to the hyphal axis (Figures 4 and 5). Their motility was also affected: in control hyphae the mitochondria made circular movements and moved short distances, whereas in the GR24-treated hyphae the mitochondria moved faster and in an oriented fashion (Videos S1–S4). These changes in shape and movement might reflect increased fission and fusion of the mitochondria, typical of active organelle biogenesis; they suggest that strigolactones are the BF molecules responsible for this response [13].

**Figure 3.** Cellular Response of Germinating Spores of *Gi. rosea* to GR24 after 1 h or 5 h of Treatment with GR24

Hyphae were stained with MitoTracker Green and treated with 0.001% acetone (control) or 30 nM GR24. Mitochondrial density is significantly higher in treated samples than in control at 1 h (p < 0.001, n = 26; and p < 0.001, n = 42, respectively). For porin and COX immunolabelling, hyphae were treated with acetone or GR24 (as above) and incubated with antibodies as described in Materials and Methods. The fluorescence density of porin and COX immunolabelling is significantly higher in treated hyphae than in controls both at 1 h (p < 0.001, n = 52; and p < 0.05, n = 60, respectively) and at 5 h (p < 0.05, n = 28; and p < 0.001, n = 29, respectively). Error bars show SEM. DOI: 10.1371/journal.pbio.0040226.g003

**Figure 4.** Effect of GR24 on Mitochondrial Shape and Density in Hyphae of *Gi. rosea* Stained with Mitotracker Green

Hyphae were treated with 30 nM GR24 (up) or with 0.001% acetone (bottom) for 1 h. Bar = 10 μm. DOI: 10.1371/journal.pbio.0040226.g004

Strigolactone Analogues GR7 and GR24 Are Active on Phylogenetically Distant AM Fungi

Buée et al. [12] reported that the BF of carrot hairy roots was active not only on several *Gigaspora* species but also on the phylogenetically distant AM fungus *Gl. intraradices*. Similarly, Tamasloukht et al. [13] verified that the higher mitochondrial activity and rate of respiration seen in *Gi. rosea* in response to carrot BF was also seen in *Gl. intraradices*. The fact that *Gl. intraradices* responds in the same way as *Gigaspora* to BF indicates that the molecular mechanism is widespread among AM fungi. This is important also because the genome of *Gl. intraradices* is currently being sequenced, so this fungus is likely to become the model AM fungus of choice for further investigations.

The branching response of *Gl. intraradices* is difficult to assess due to its thin, multiple germ tubes; therefore, we replaced this test with a germination test. We observed that 10⁻⁷ M GR7 and 10⁻⁷ M GR24 increased the percentage of
spores that germinated by 55% and 50% (300 spores tested per treatment), respectively, compared to their respective controls. We then tested the effects of GR7 and GR24 on the rate of respiration of *Gl. intraradices* spores. *Gl. intraradices* is a suitable species for quantitative measurements of its oxygen consumption in a polarograph due to the small size of its spores and hyphal tubes. We found an average increase of 35% after 5 h in the presence of 10⁻⁷ M GR7 or GR24. This 5-h treatment was too short for the spores to produce new hyphal growth; therefore, the higher rate of respiration in response to GR7 and GR 24 must have been an intrinsic response from pre-existing material rather than a simple cellular growth response. To test the hypothesis that the cellular basis for these physiological responses is mitochondrial as in *Gi. rosea*, we labelled hyphae of *Gl. intraradices* germinated spores with Mitotracker Green. After 1 h of treatment with 30 nM GR24, an increase of 30% fluorescence density was obtained (*P*, 0.05). Moreover, as in *Gi. rosea*, mitochondria of *Gl. intraradices* appeared more often with a threadlike structure in stimulated hyphae (Figure 6). Mitochondria of *Gl. intraradices* appeared to move faster than *Gi. rosea* mitochondria, making it impossible to appreciate differences in movement between treated and control mitochondria.

To further validate the status of strigolactones as general AM fungi stimulants, we tested GR7 10⁻⁷ M on a third AM fungal species, *Gl. claroideum*. This species belongs to group B of Glomerales, whereas *Gl. intraradices* belongs to group A [25]. Spore germination of *Gl. claroideum* was stimulated in the presence of GR7. The stimulated spores germinated faster, and a higher percent germination was obtained after 4 d of incubation (Figure S4). These data were validated with a χ² test (23.78; *P* < 0.05).

**Discussion**

We have shown that the BF produced by roots of the monocot *Sorghum* contains a strigolactone and that strigolactone analogues induce strong physiological effects on three diverse AM fungi, as does authentic BF. We have shown also that the BF of the dicot *D. carota* stimulates seed germination of *Orobanche*, as do strigolactones. Akiyama et al. [16] showed that the active ingredient of the BF of *L. japonicus*, another dicot, is a strigolactone. Taken together, these data indicate that strigolactones, previously defined as stimulants of germination of parasitic weeds, are also involved in early AM fungus–host-root interactions in a wide range of plant and fungus species.
The fact that many plant species are hosts to AM fungi [26] and/or to parasitic weeds suggests that strigolactones are widespread in the plant kingdom. Yet, due to their very low concentrations and the lack of easy analytical methods to detect them, these small lipophilic molecules have been detected in only a few higher plants [27]. Since the discovery of strigol, the first strigolactone to be characterised (in cotton in 1966 [28]), only five other natural strigolactones have been described: strigyl acetate, isolated from cotton [29]; sorgolactone, from *Sorghum* and maize; alectrol, from *cowpea*; orobanchol, from red clover [15]; and 5-deoxy-strigol, from *L. japonicus* [16]. Recently, an ESI-MS/MS method was developed using multiple reaction monitoring that was suitable for the detection and quantification of several strigolactones in the same sample [29]. We also used the ESI-MS/MS but in the enhanced product ion (EPI) mode to be more confident in molecular identification. With this type of analysis, all daughter ions and their respective abundance are visible after collision. This method proved very sensitive in our hands (with a limit of detection of $10^{-9}$ M for standard sorgolactone), enabling us to detect sorgolactone not only in *Sorghum* root exudates but, more directly, in roots and shoots. If strigolactones are also present in shoots of maize, this may explain the presence in maize stem of a non-identified germination stimulant of *Striga hermonthica* seeds [30]. For now, nothing is known about the biosynthesis pathway of strigolactones; however, Matusova et al. [31] suggested that strigolactones may be derived from the carotenoid pathway. This hypothesis is consistent with the presence of strigolactones in shoots.

Plants produce thousands of chemicals, many of them with strong biological activities. It would not be surprising, therefore, if AM fungi had learned to interact with many of these chemicals during the course of evolution. Therefore, strigolactones may not be the only one class of molecule in plant roots that stimulates hyphal growth. None of the other stimulants of parasitic weed germination we tested had any activity on *Gi. rosea*, indicating the structural specificity of strigolactones as AM fungal stimulants. Moreover, the very high activity of strigolactones and the fact that they are active not only on *Gigaspora* species: *G. margarita* [16], *G. rosen* (this study), and *G. gigantea* (unpublished data) but also on two *Glomus* species (this study) considered to be phylogenetically distant from the Gigasporaceae [25], argue in favour of an important role for strigolactones in hyphal growth. Studies with strigolactone mutants or ecotypes of plants that produce less or no strigolactones—ideally in model plants such as *Medicago truncatula* and *L. japonicus*—will be crucial to establish whether or not strigolactones are essential for establishing the mycorrhizal symbiosis.

Sorgolactone was active on germinating *Gi. rosea* hyphae at concentrations as low as $10^{-15}$ M, or even lower (considering the dilution effect in the assay; see Materials and Methods). Such high sensitivity has never been reported before in AM symbiosis. The lowest concentrations of flavonoids active in AM fungal growth are in the micromolar range [9]. The fact that strigolactones are active at these very low concentrations and that rapid and extensive cellular reorganisation occurs in the fungus suggests that strigolactones act through a signalling pathway. The high turnover of strigolactones in soil [32] is also consistent with a signalling role.

The rapid and strong induction of mitochondrial activity in the fungus in response to strigolactones, we believe, illustrates the most significant effect of strigolactone on AM fungi. This mitochondrial response was found in *Gi. rosea* and *Gl. intraradices*, two distant AM fungi. It was correlated to a higher respiration rate with *Gl. intraradices*. We hypothesize that hyphal branching in *Gi. rosea* and activation of spore germination in *Gl. intraradices* and *Gl. claroideum* are physiologically significant. Activation of the mitochondria leads to oxidation of lipids—the main form of carbon storage in AM fungi. From their observations of fungal spore germination in the absence of roots and in the presence of root exudates, Bécard and Piché [33] proposed that a mechanism prevents AM fungal spores from using their carbon reserves until they encounter some specific root exudates. Strigolactones may be that crucial component of root exudates that switches on lipid catabolism in the pre-symbiotic stage of the fungus, which is characterised by the capacity of the fungus to use its full energy potential.

Interestingly, two types of unrelated organisms, AM fungi and parasitic weeds, respond to the same molecules, the strigolactones. These organisms have in common the fact that they are root obligate biotrophs, that their survival relies on efficient host recognition, and that they respond with their germinative structures. The production of strigolactones by plants may well have given them a selective advantage during evolution by encouraging their advantageous association with AM fungi. The parasitic weeds, which appeared much later in evolution than AM fungi, may then have exploited the production of strigolactones by roots for the purpose of colonising their hosts. Thus, the signal transduction systems through which AM fungi and parasitic weeds detect strigolactones may have emerged independently by convergent evolution.

A similar evolutionary mechanism might have given rise, from the ancestral mycorrhizal (Myc) signalling pathway, to the more recent nodulation (Nod) signalling pathway, activated by Nod factors [34]. Myc factors have not been isolated and characterised yet, but they are suspected to be essential symbiotic signals produced by AM fungi that activate the plant’s symbiotic program. In the legume *M. truncatula*, gene expression and root development respond to (a) diffusible compound(s) produced by AM fungi [35–37]. It will be interesting to investigate whether the strigolactone stimulation of AM fungi is strictly required for Myc factor production, as is the case for Nod factor production by *Rhizobium* in response to plant flavonoids [38]. Whether directly or indirectly, we expect that strigolactone stimulation of fungal respiration will increase production of Myc factors, and will help us to isolate and characterise these fungal symbiotic signals.

**Materials and Methods**

**Plant materials.** Roots of carrot (*D. carota* L.) transformed by the Ri T-DNA of *Agrobacterium rhizogenes* were routinely cultivated according to Bécard and Fortin [39] on minimal (M) medium solidified with 0.4% Gelman gum (*Phytagel*, Sigma, Steinheim, Germany). The carrot RF was extracted according to Buret et al. [12]. Briefly, 500 mg of hairy roots were incubated for 2 d in 100 ml of sterile water in the dark. Crude exudates were filtered and fractionated with ethyl acetate: water (1:1). The lipophilic fraction was dried and re-dissolved in 1 ml of methanol. Insoluble matter was removed by centrifugation, and the methanol phase was diluted with one volume of water. Purification was carried out by HPLC (Spectraphysics, Newport, United States) with a C18 column (Hypurity Elite C18, 250 × 5 × 5 μm, Hypersil, Thermo Electron, Cergy-Pontoise, France). The samples...
were separated using a gradient from 80:20 (v/v) water/acetonitrile (AcN) to 100% AcN at a constant flow rate of 400 µl/min⁻¹, and fractions were collected every 2 min. The absorbancy of the eluates at 300 nm was monitored with a model Spectra 100 detector (Spectraphysics), and the data were integrated with Borwin 1.5 chromatography software (Borwin, Varian, Les Ulis, France). The fractions were treated with 10% C₅Cl₃, and the sorgoleone analogues (GR24, GR7) and synthetic sorgolactone. Hyphal elongation was measured with a 2-mm square grid. Mean values were compared using the one-way Anova test (p < 0.05) and SPSS software. Chi-squared tests were used to determine the significance of values was validated with a one-way Anova test (p < 0.05).

Germination test on *Gl. intraradices*. Solid M medium containing GR7 (10⁻⁷ M) was poured into 25-well petri dishes. Ten spores of *Gl. intraradices* were added to each well. After 6 d, germination was checked under the stereomicroscope. A total of 300 spores were used per treatment, and the experiment was repeated three times. Variations in germination rates were calculated by comparison to control. Unpaired t-tests were used to determine the significance of values was validated with a one-way Anova test (p < 0.05).

Germination test on *Gl. clavicipitum*. Solid M medium containing GR7 (10⁻⁷ M) was poured as above and poured in small petri dishes (3-cm diameter). Four spores were added per plate. Germination was checked under the stereomicroscope every day after the third day of incubation. A total of three spores were used in each experiment, and the experiment was repeated twice. The significance of values was validated with a χ² test (p < 0.05).

Polarography. The respiration rate of *Gl. intraradices* in the presence or absence of strigolactone analogues GR7 or GR24 was measured according to Tamasloukht et al. [13]. Four hundred spores were germinated in 1 ml of liquid M medium for 6 d in Falcon petri dishes (3-cm diameter). Then, GR7 or GR24 was added to a final concentration of 10⁻⁷ M. Control spores were treated with 30% methanol or with 0.1% acetone. After 5 h treatment, the spores were transferred to a Clark electrode inserted into a water-jacketed sealed glass chamber (Hansatech, Norfolk, United Kingdom). The oxygen electrode was connected to a chart recorder calibrated between 0% and 100% with atmospheric oxygen. Spores were added to the chamber and the temperature was maintained at 30°C using a circulating water bath. Relative differences of O₂ consumption were read directly for 15 min from the chart recording. Increased O₂ consumption in treated spores was calculated by comparison of the slope with the control slope. Three assays and three controls were performed for each experiment. The experiment was repeated three times.

Cytology. Mitochondria in *Gi. rosea* and *Gl. intraradices* hyphae were determined after staining mitochondria with the fluorescent probe MitoTracker Green (Molecular Probes, Leiden, Netherlands). After 5–6 d of germination in liquid M medium, germinating spores of either fungal species (ten spores per treatment) were incubated with the dye (1 µM) for 5 h (Gi. rosea) or 1 h (Gl. intraradices) at 28°C in the dark under 2% CO₂. During the staining process, germinated spores were treated with 30 nM GR24 (final concentration of 0.001% v/v acetone), or 0.001% v/v acetone (control) for 1 h (Gi. rosea and Gl. intraradices) or 5 h (Gi. rosea). After treatment, germinated spores were washed with M medium, mounted on glass slides, and observed under an inverted microscope (Leica DMIRBE, Rueil-Malmaison, France) (Excitation, 450–490 nm; Emission, 515nm). Images were acquired with a CCD camera (Color Coolview, Photonic Science, Robertsbridge, United Kingdom) using a 40× immersion oil objective. An average of 35 (Gi. rosea) or 65 (Gl. intraradices) hyphal segments, randomly selected among either treated or control germinated spores, were accumulated in three independent experiments and processed by image analysis (Image Pro Plus, Media Cybernetics, Silver Spring, Maryland, United States). In each hyphal segment, mitochondria were counted, and the number of mitochrondria per μm² was calculated as an estimate of mitochondrial density. Sub-sampled images of treated and control Gi. rosea and Gl. intraradices hyphal segments were randomly selected for the analysis of the shape of mitochondria. The calculation of mitochondrial length-to-width ratios was made on 140–150 mitochondria per hyphal segment (Gi. rosea) or 65–150 mitochondria per hyphal segment (Gl. intraradices). In addition, time-lapse (2 sec) observation of treated and control Gi. rosea living hyphae was carried out to observe mitochondrial movements.
For immunolabelling, 20 germinated spores were prepared and treated for 1 h or 5 h with 30 nM GR24 or 0.001% acetone, as above. Hyphae were then fixed with 1% (v/v) paraformaldehyde in water. The fungal cell wall was partially digested with a lytic enzyme mix from Trichoderma harzianum (Acros Organics, Noisy le Grand, France) at a concentration of 12.5 mg/mL in phosphate-buffered saline (PBS, pH 7.4). After digestion, hyphae were blocked with 0.1% bovine serum albumin (BSA) in PBS (pH 7.4) at 1:100 and 1:20 v/v, respectively. After five washes, rabbit anti-mouse antibody coupled to rhodamine or FITC (Molecular Probes) was added diluted 1:50 or 1:100 in PBS (pH 7.4), respectively. Hyphae were incubated for 2 h at 28 °C in the dark with secondary antibodies. Samples were then washed, mounted on glass slides, and observed with an inverted microscope with a 100× immersion oil objective (Excitation 490 nm, Emission 515 nm for FITC; Excitation 515–560 nm, Emission 590 nm for rhodamine). Thirty images of hyphal segments randomly selected among the treated and control germinating spores and labelled with anti-COX1 and anti-porine, in three to five independent experiments, were acquired and processed as above to evaluate mitochondrial density. Statistical analyses of the Mitotracker Green and immunolabelling data were performed using the standard Student t-test and SPSS software.

Sorgolactone production, extraction, and characterisation. Sorghum seeds (around 80) were transferred to a sterilised funnel culture system: a 15-cm diameter filter unit packed with glass wool moistened with 100 mL of sterile distilled water and placed on top of a 500-mL vacuum flask. Each culture unit was incubated in a growth chamber under a day/night cycle of 16/8 h, at 25 °C and 70% relative humidity. At 24 h intervals, root exudates were collected by suction. Every 24 h, the glass wool was washed with 150 mL of sterile distilled water. Root exudate samples were extracted three times with 50 mL of ethyl acetate. The ethyl acetate extracts were dried in a rotary evaporator, redissolved in 5 mL of water:AcN (70:30, v:v) and chromatographed on a 6-ml C18 column Mega Bond Elut (Varian) using increasing concentrations of AcN (0:100) fraction 6, the fractions were dried in a rotary evaporator, and the crude material was redissolved in 2 mL of methanol and stored at −20 °C. They were then pooled and dried in a rotary evaporator, redissolved in 5 mL of water:AcN (70:30, v:v) and chromatographed on a 6-mL C18 column Mega Bond Elut (Varian) using increasing concentrations of AcN, as the mobile phase: (80:20) water:AcN fraction 1, (70:30) fraction 2, (60:40) fraction 3, (50:50) fraction 4, (40:60) fraction 5, and (0:100) fraction 6. The fractions were dried in a rotary evaporator and purified by HPLC. 50-μL samples were injected into a 25-cm × 3-mm Hypurpetyl Elite C18 reverse-phase column (Hypersil) with a 5-μm pore size. A P8900 Ternary HPLC Pump (Spectraphysics) was used. The samples were separated using a gradient from 70:30 water:AcN to 100% AcN at a constant flow rate of 400 μL/min, and fractions were collected every 2 min. The absorbance of the eluent was monitored at 239 nm (λmax) of synthetic sorgolactone as determined with a UV-Vs spectrophotometer; Cary 100 Scan, Varian. The ESI-MS/MS analyses of 250 amu/s in the positive ion mode. A standard solution of synthetic sorgolactone was used for the optimisation procedure. The nebuliser gas flow was 10 L/min and monitored in the EPI scan mode at a scan rate of 250 amu/s in the positive ion mode. A standard solution of synthetic sorgolactone at 10−7 M in AcN:water:HCOOH (90:10:0.1) was used for the optimisation procedure. The nebuliser gas flow was 40 psi, and the capillary voltage was 4400 V. Fragmentation was performed by collision induced dissociation with nitrogen at a collision gas pressure of 40 psi, and the capillary voltage was 4400 V. Fragmentation was performed by collision induced dissociation with nitrogen at a collision gas pressure of 40 psi, and the capillary voltage was 4400 V.

Statistical analyses of the Mitotracker Green and immunolabelling data were performed using the standard Student t-test and SPSS software.

Supporting Information

Figure S1. Molecular Structure of Various Stimulants of Seed Germination of Parasitic Weeds

(S) sorgolactone, (B) GR24, (C) GR7, (D) parthenolide, (E) artemisinin, (F) dihydroartemisinone.

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