

Strigolactones as Germination Stimulants for Root Parasitic Plants

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Witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) are the two most devastating root parasitic plants belonging to the family Orobanchaceae and are causing enormous crop losses throughout the world. Seeds of these root parasites will not germinate unless they are exposed to chemical stimuli, 'germination stimulants' produced by and released from plant roots. Most of the germination stimulants identified so far are strigolactones (SLs), which also function as host recognition signals for arbuscular mycorrhizal fungi and a novel class of plant hormones inhibiting shoot branching. In this review, we focus on SLs as germination stimulants for root parasitic plants. In addition, we discuss how quantitative and qualitative differences in SL exudation among sorghum cultivars influence their susceptibility to *Striga*.

Keywords: Germination stimulant • *Orobanche* • *Phelipanche* • Root parasitic plants • *Striga* • Strigolactone

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; SL, strigolactone.

Introduction

Among the parasitic angiosperms, witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) in the family Orobanchaceae are the two most devastating weeds that parasitize roots of host plants (Parker and Riches 1993, Joel et al. 2007, Parker 2009). *Striga* species are hemi-parasites as they have functional chloroplasts. However, their photosynthesis cannot support their survival without connecting to host roots and thus they are obligate parasites. *Striga* spp. are serious weeds of important food crops including sorghum, maize, millet and rice, in sub-Saharan Africa, the Middle East and Asia. Their effects are severe, and complete loss of harvest is not uncommon in heavily infested areas.

Orobanche (and *Phelipanche*) species are Chl-lacking holoparasites, which attack economically important dicotyledonous crops, including tomato, tobacco, carrot, clover, cucumber, sunflower and legumes.

Seeds of these root parasites are extremely small (0.2–0.4 mm, about half the size of *Arabidopsis* seed) and composed of a relatively small number of cells (Joel et al. 1995). Although non-parasitic plants, in general, produce several hundred to several thousand seeds, a single plant of *Striga* or *Orobanche* can produce up to half a million seeds which remain viable in the soil for many years. Large numbers of long-lived seeds ensure that these root parasites adapt to changes in host availability, resistance, etc., and consequently make them difficult to control (Joel et al. 1995, Joel et al. 2007).

The life cycles of *Striga* and *Orobanche* are very similar, and a number of mechanisms ensure the co-ordination of the parasites' life cycles to that of their hosts (Fig. 1). *Striga* or *Orobanche* seeds germinate after a pre-incubation period of moist and suitable temperatures (Phase I), and only when they perceive host-derived chemicals, termed 'germination stimulants', released from plant roots (Phase II), ensuring that only seeds within the host rhizosphere will germinate (Joel et al. 1995, Joel et al. 2007). The parasite seedling radicle grows only a few millimeters and must reach a host root, within a few days, before exhausting the resources in the tiny seed. Upon contact with the host root (Phase III), the radicle develops a specialized organ, the haustorium, which adheres to the root, penetrates the epidermis and cortex tissues of the root (Phase IV) and ultimately establishes connections to the host vascular system. Through this connection the parasite draws water and its nutritional needs from the host (Phase V). The parasite tubercles grow underground for several weeks (*Striga* spp.) to several months (*Orobanche* spp.) (Phase VI) and then produce aboveground flowering shoots (Phase VII).

So far, three different types of compounds have been identified as germination stimulants for root parasitic plants;

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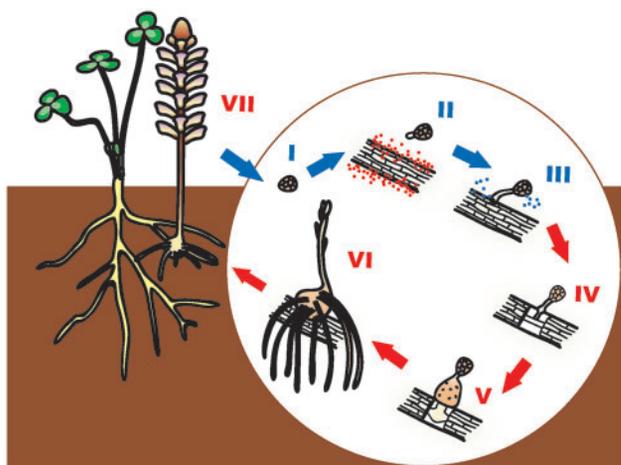


Fig. 1 Life cycle of a root parasitic plant, *Orobanche minor*. (I) Seed becomes responsive after a pre-incubation period of moist and suitable temperatures. (II) Seed germination is induced by host-derived stimulants including strigolactones. (III) The growing radicle attaches to the host root and develops a haustorium. (IV) The parasite penetrates the host epidermis and cortex tissues and connects with the vascular system. (V) The parasite sucks water, minerals and photosynthates from the host. (VI) The parasite tubercles grow underground for several weeks or several months before emergence of the flowering shoots. (VII) The parasite produces a large number of seeds which remain viable for many years in the soil. I–III (in blue) and IV–VII (in red) are pre-parasitic and parasitic phases, respectively.

dihydroquinones, sesquiterpene lactones and strigolactones (SLs) (Bouwmeester et al. 2003). Among them, SLs are the most potent stimulants, inducing germination at ≤ 10 pM (Kim et al. 2010), and also acting as host recognition signals for symbiotic arbuscular mycorrhizal fungi (Akiyama et al. 2005, Besserer et al. 2006). In addition to these functions as rhizosphere signaling chemicals, another function of SLs as plant hormones inhibiting shoot branching has been unveiled (Gomez-Roldan et al. 2008, Umehara et al. 2008, Xie et al. 2010). In this review, we focus on SLs as germination stimulants for root parasitic plants. In addition, we discuss how quantitative and qualitative differences in SL exudation among sorghum cultivars influence their susceptibility to *Striga*.

Strigolactones as Germination Stimulants for Root Parasitic Plants

It was a well-known phenomenon that seeds of root parasitic plants germinate in the close vicinity of a host root. Several attempts to identify chemicals inducing germination of root parasitic plants were reported early in the 20th century. Although Brown et al. reported assay techniques, germination stimulation activity of root exudates from various plant species, and partial purification of *O. minor* and *S. hermonthica* germination stimulants in the early 1950s (Brown et al. 1949, Brown et al. 1951a, Brown et al. 1951b, Brown et al. 1952a,

Brown et al. 1952b), it took nearly 20 years until the isolation and characterization of *S. lutea* (synonym *S. asiatica*) germination stimulants. Strigol and strigyl acetate, the first described SLs, were isolated from root exudates of cotton, a false host of *Striga* (Cook et al. 1966, Cook et al. 1972). Later, Siame et al. (1993) identified strigol in the root exudates of genuine hosts of *Striga*, sorghum, maize and proso millet. Then two germination stimulants structurally related to strigol, sorgolactone (Hauck et al. 1992) and alectrol (Müller et al. 1992), were isolated from root exudates of sorghum and cowpea, respectively. Butler (1995) named these strigol-related compounds 'strigolactones'. Isolation of orobanchol (Yokota et al. 1998), the first *Orobanche* germination stimulant, clearly demonstrated that both *Striga* and *Orobanche* spp. utilize SLs as germination signals. To date, >14 SLs have been detected in root exudates of various plant species (Fig. 2) (Yoneyama et al. 2009). The structure of solanacol has recently been revised, as shown in Fig. 2 (Takikawa et al. 2009).

All natural SLs identified so far contain a tricyclic ring system (ABC part) connected to a butenolide (D ring) via an enol ether bridge (Fig. 2). Extensive studies on the structure–activity relationships of SLs in germination stimulation of parasitic plant seeds have revealed that the C–D ring moiety is the essential structure for exhibiting germination stimulation activity (Zwanenburg et al. 2009). Indeed, all natural SLs contain this essential structure, and have different substituents on the A ring and/or B ring. These substitutions affect not only the germination stimulation activity but also the stability of SLs.

The natural SLs shown in Fig. 2 induce >80% germination of *O. minor* seeds at ≤ 1 nM (Kim et al. 2010). GR24, a synthetic analog, elicits >60% germination at 100 nM and thus is 100-fold less active than the natural SLs. Among the natural SLs, >100-fold differences exist in their germination stimulation activity on *O. minor* seeds (Kim et al. 2010). The three monohydroxy-SLs, 2'-epiorobanchol (Xie et al. 2007), orobanchol and sorgomol (Xie et al. 2008), are the most active germination stimulants that induce >80% germination of *O. minor* seeds at 10 pM. Strigol and solanacol (Xie et al. 2007) are slightly less active than these three monohydroxy-SLs and display >80% germination at 100 pM. Among hydroxy-SLs, 7-oxoorobanchol is the weakest stimulant, eliciting high germination (>80%) at 1 nM, probably because of its instability (Xie et al. 2009b). The acetylation of the hydroxyl group of this SL results in a 10-fold increase in the germination stimulation activity (Xie et al. 2009b). Sorgolactone and 5-deoxystrigol, rather lipophilic SLs without oxygen-containing substituents on the A/B-ring moiety, are less active germination stimulants on *O. minor* seeds and induce >80% germination at 1 nM. Therefore, both the stability and lipophilicity of the molecules influence in vitro germination stimulation activity of SLs on the seeds of a root parasitic plant *O. minor*. Similar structure–activity relationship of SLs in germination stimulation may be observed with other root parasitic plants.

Most of the natural SLs have the C-2'-(R)-configuration which has been reported to be an important structural

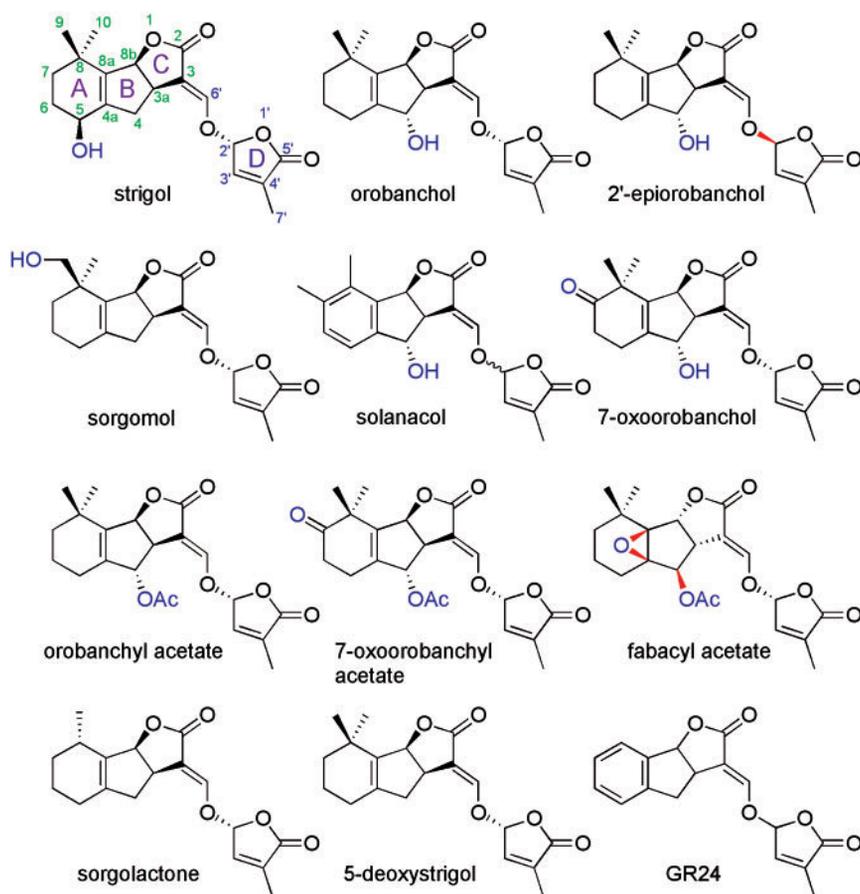


Fig. 2 Structures of natural strigolactones and the synthetic analog GR24.

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feature for exhibiting high germination stimulation activity. Among the stereoisomers of strigol (Reizelman et al. 2000), sorgolactone (Sugimoto et al. 1997) and GR24 (Thuring et al. 1997), C-2'-(*R*)-isomers are more active than their (*S*)-isomers. 2'-Epiorobanchol is an exception as it has a C-2'-(*S*)-configuration and is slightly more active than orobanchol (Xie et al. 2007). This is probably due to the positive effect of the 4-hydroxyl group on the germination stimulation activity. A similar effect is also observed with solanacol, 7,8-dimethyl-4 α -hydroxy-GR24, which is about 1,000-fold more active than GR24 (Xie et al. 2007). Production of both 2'-epiorobanchol and orobanchol suggests that coupling of the ABC part with the D-ring moiety, if it occurs in the later steps of SL biosynthesis (Rani et al. 2008), is not a stereoselective process, at least in tobacco plants. Furthermore, isolation of the first *ent*-SL, fabacyl acetate (Xie et al. 2009a), in which the configuration of the ABC part is opposite to that of known SLs implies that plants may produce all stereoisomers. Recent progress in chromatography and mass spectrometry has enabled rapid and sensitive detection of these natural SLs. However, germination assays are

at least 100-fold more sensitive than mass spectrometry for the detection of SLs. Therefore, it is likely that many novel SLs remain to be characterized.

Strigolactone Exudation of Sorghum Cultivars and Their Susceptibility to *Striga*

Striga hermonthica and *S. asiatica* cause serious yield losses mainly to sorghum and pearl millet in the semi-arid tropics. One of the most promising approaches to minimizing these losses is breeding of resistant genotypes (Ejeta and Gressel 2007). The existence in sorghum of varietal differences in resistance to *S. asiatica* or *S. hermonthica* has been reported (Doggett 1965). For many years, a number of research programs have attempted to identify sorghum varieties resistant to *S. hermonthica* and to transfer this resistance to high-yielding, well-adapted varieties (Ejeta and Gressel 2007).

Among possible mechanisms of resistance to *Striga*, reduced production of *Striga* seed germination stimulants is the best characterized. Sorghum cultivars with this trait are expected to

be resistant because only a few *Striga* seeds located very close to the host roots germinate.

Selection of sorghum genotypes with reduced or no stimulant production was done by comparing the germination stimulation activity of root exudates of individual genotypes (Olivier *et al.* 1991, Hess *et al.* 1992). However, it is not clear if these differences in germination stimulation activity are due to quantitative or qualitative differences in the germination stimulants or the germination inhibitors, because the degree of germination stimulation was examined only with a bioassay. Therefore, *Striga*-resistant and *Striga*-susceptible sorghum cultivars were examined for the production of germination stimulants including SLs.

Germination stimulation activity of root exudates from *Striga*-resistant and *Striga*-susceptible sorghum cultivars

Seedlings of six sorghum cultivars comprising three which were *Striga* resistant (Nagade, Tetron and SRN39) and three which were susceptible (Tabat, Wad El Mubark and Korakollow) were grown in containers filled with perlite in growth chambers at 34/28°C with an 18h photoperiod ($\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$). The water loss was replenished every day with tap water to keep the perlite wet. After 7 d the three healthiest seedlings from each cultivar were carefully removed and the perlite was cleaned off. Plants were transferred to 20 ml test tubes filled with 18 ml of sterilized distilled water. The tubes were covered with aluminum foil to exclude light and returned to the incubator to collect the root exudates. After 20 h, root exudates were collected, and the root fresh weight of each seedling was determined. The root exudates were extracted with ethyl acetate and the extracts were examined for their germination stimulation of *O. minor* and *S. hermonthica* seeds.

Many traits may be different among sorghum cultivars irrespective of susceptibility to *Striga* infection. In general, the plant heights of *Striga*-resistant cultivars with low stimulant production are lower and they produce smaller grains; resistance is often associated with low yield potential and/or poor quality of sorghum (Ramaiah *et al.* 1990). Therefore, to compare germination stimulation activity of root exudates from different sorghum cultivars, concentrations of root exudates were normalized based on the root fresh weights (Matusova *et al.* 2005). Among the sorghum cultivars examined, Nagade was the shortest in stature and had the smallest root fresh weight: Nagade, 53 ± 6 mg; Tetron, 108 ± 13 mg; SRN39, 78 ± 8 mg; Tabat, 105 ± 14 mg; Wad El Mubark, 72 ± 6 mg; Korakollow, 134 ± 16 mg (mean \pm SE, $n = 3$).

Germination stimulation activities of root exudates from different sorghum cultivars at an equal concentration based on root fresh weights, $10 \text{ mg root FW ml}^{-1}$, are shown in Fig. 3. There are some differences in germination stimulation activities of the root exudates from the six sorghum cultivars, but no distinct differences were observed in germination stimulation activity between *Striga*-resistant (Nagade, Tetron and SRN39) and *Striga*-susceptible (Tabat, Wad El Mubark

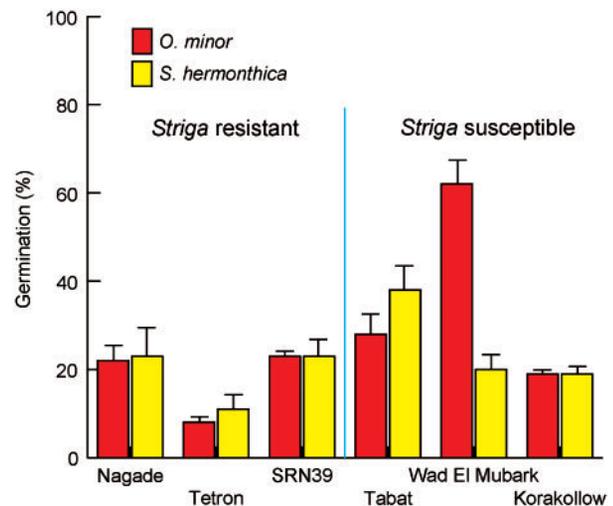


Fig. 3 *Orobancha minor* and *Striga hermonthica* seed germination induced by root exudates from *Striga*-resistant (Nagade, Tetron and SRN39) and *Striga*-susceptible (Tabat, Wad El Mubark and Korakollow) sorghum cultivars. The root exudates were collected from three seedlings for 20 h. The concentrations of root exudates were equalized for differences in root fresh weight. GR24 at $1 \mu\text{M}$ induced 78 and 72% germination in *O. minor* and *S. hermonthica*, respectively, and no germination was observed for the seeds treated with distilled water. Data are presented as means \pm SE ($n = 3$).

and Korakollow) cultivars, except that the root exudates of the resistant cultivar Tetron elicited low germination. In addition, the root exudates induced similar levels of germination in both *O. minor* and *S. hermonthica* seeds. Thus, when the root exudates were collected for a short period of 20 h, both the *Striga*-resistant and the *Striga*-susceptible sorghum cultivars seem to produce similar levels of germination stimulants.

Approximately 500 seedlings each of the two representative cultivars, *Striga*-resistant SRN39 and *Striga*-susceptible Tabat, were grown in containers and root exudates were collected daily for 7 d. The root exudates were immediately extracted with ethyl acetate, and the ethyl acetate extracts were combined. Each of the extracts of the root exudates from the two sorghum cultivars grown hydroponically was dissolved in methanol and examined for germination stimulation activities at concentrations of 1/100 and 1/1,000.

As shown in Fig. 4, the root exudates from the resistant sorghum cultivar SRN39 induced 44 and 76% germination at concentrations of 10^{-3} and 10^{-2} , respectively. Similar levels of germination, 29 and 73%, were observed when seeds were treated with the root exudates from the susceptible cultivar Tabat. Thus, root exudates from these two cultivars contain similar levels of germination stimulation activity. In other words, *Striga* resistance in SRN39 may not be due to low stimulant production compared with the susceptible cultivar Tabat.

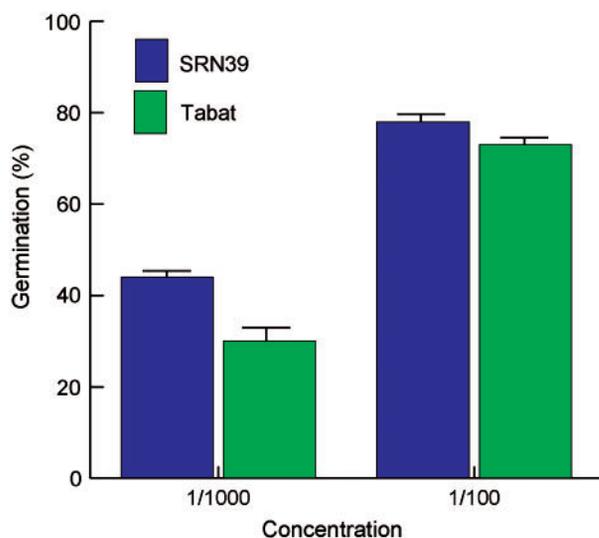


Fig. 4 *Orobanchae minor* seed germination induced by root exudates from *Striga*-resistant SRN39 and *Striga*-susceptible Tabat sorghum cultivars at two different concentrations. The root exudates were collected daily from approximately 500 seedlings for 7 d. The germination rate of seeds treated with 1 μ M GR24 was 75% and distilled water induced no or negligible germination. Data are presented as means \pm SE ($n = 3$).

Quantitative and qualitative differences in strigolactone exudation between sorghum cultivars SRN39 and Tabat

There were no distinct differences in germination stimulation activity between *Striga*-resistant and *Striga*-susceptible sorghum cultivars as shown in Figs. 3 and 4. Therefore, SLs in root exudates of the two sorghum cultivars, *Striga*-resistant SRN39 and *Striga*-susceptible Tabat, were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) to examine if there are any quantitative and/or qualitative differences in SL exudation (Sato et al. 2003). For LC–MS/MS analyses of sorghum SRN39 and Tabat root exudates, a multichannel MRM (multiple reaction monitoring) method was employed to quantify major SLs produced by sorghum, sorgomol and/or strigol, sorgolactone and 5-deoxystrigol (Awad et al. 2006).

Fig. 5 shows MRM chromatograms of root exudates from *Striga*-susceptible Tabat (Fig. 5A) and *Striga*-resistant SRN39 (Fig. 5B), respectively. In both chromatograms, the upper channels are for monitoring the transition of m/z 369 > 272 for the detection of strigol isomers including sorgomol. The mid and lower channels are for monitoring the transitions of m/z 339 > 242 and m/z 353 > 256, respectively, for the detection of sorgolactone and 5-deoxystrigol. In both MRM chromatograms, sorgomol, sorgolactone and 5-deoxystrigol can be identified.

Unfortunately, the amount of sorgomol in the Tabat root exudate (Fig. 5A) could not be quantified because its peak overlapped with peaks of impurities or its isomers, and thus sorgomol was not quantified. The other two major SLs,

sorgolactone and 5-deoxystrigol, in the root exudates of SRN39 and Tabat were estimated. During 7 d after germination, a single seedling of *Striga*-resistant SRN39 exudes 0.48 pg of sorgolactone and 0.08 pg of 5-deoxystrigol, while *Striga*-susceptible Tabat exudes 0.37 pg of sorgolactone and 3.53 pg of 5-deoxystrigol. These estimated amounts of SLs are only for known SLs and thus each sorghum cultivar may produce unknown SLs as well.

Although *Striga*-resistant cultivars were reported to induce low germination, there are no distinct differences in germination stimulation activity on *O. minor* and *S. hermonthica* seeds between the root exudates from the resistant cultivars and those from the susceptible ones (Figs. 3, 4). This discrepancy may be due in part to the exclusion of hydrophilic inhibitors by solvent extraction.

Approximately 4% of >20,000 sorghum lines, mainly screened by the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), have been found to have a low stimulation of germination. Hess et al. (1992), using a simple agar assay, selected one sorghum line, SRN39, as the most resistant to *Striga*. However, root exudate of the *Striga*-resistant line SRN39 is as active as that of the susceptible cultivar Tabat in germination stimulation of *O. minor* seeds, and SRN39 does exude sorgomol, sorgolactone and 5-deoxystrigol.

In addition to the possible exclusion of hydrophilic inhibitors by solvent extraction, the stability of SLs may affect the results of bioassays, as discussed before. In general, hydroxy-SLs, such as strigol, orobanchol and sorgomol, are less stable than non-hydroxy-SLs such as sorgolactone and 5-deoxystrigol. In our in vitro germination tests, germination would be elicited within a few hours and thus relatively unstable compounds can exhibit their activities. In contrast, in the agar assay conducted by Hess et al. (1992) and, in particular under field conditions, it is likely that unstable hydroxy-SLs hardly contribute to germination stimulation of parasite seeds. It should be noted that the amount of 5-deoxystrigol in the root exudate of the susceptible cultivar Tabat is 35-fold greater than that of SRN39. In addition, the *Striga*-sensitive cultivar Swarna was found to exude very large amounts of 5-deoxystrigol; 200 pg per seedling in 5 d (Awad et al. 2006). Therefore, susceptibility of sorghum cultivars to *Striga* may be related in part to the production and/or exudation of more stable non-hydroxy-SLs. Furthermore, resistant mechanisms include low stimulation of parasite seed germination, unsuccessful penetration of host roots, delay in post-attachment tubercle development and necrosis of the attached tubercles. Consequently, resistance is the result of several mechanisms acting at different stages of the infection process (Olivier et al. 1991, Hess et al. 1992, Pérez-de-Luque et al. 2005a, Pérez-de-Luque et al. 2005b, Kubo et al. 2009, Pérez-de-Luque et al. 2009, Yoshida and Shirasu 2009).

Plant roots continuously produce and secrete primary and secondary metabolites into their immediate rhizosphere. However, the mechanisms that drive and regulate root secretion of secondary metabolites are not fully understood. Walker et al. (2003) conducted metabolic profiling of root

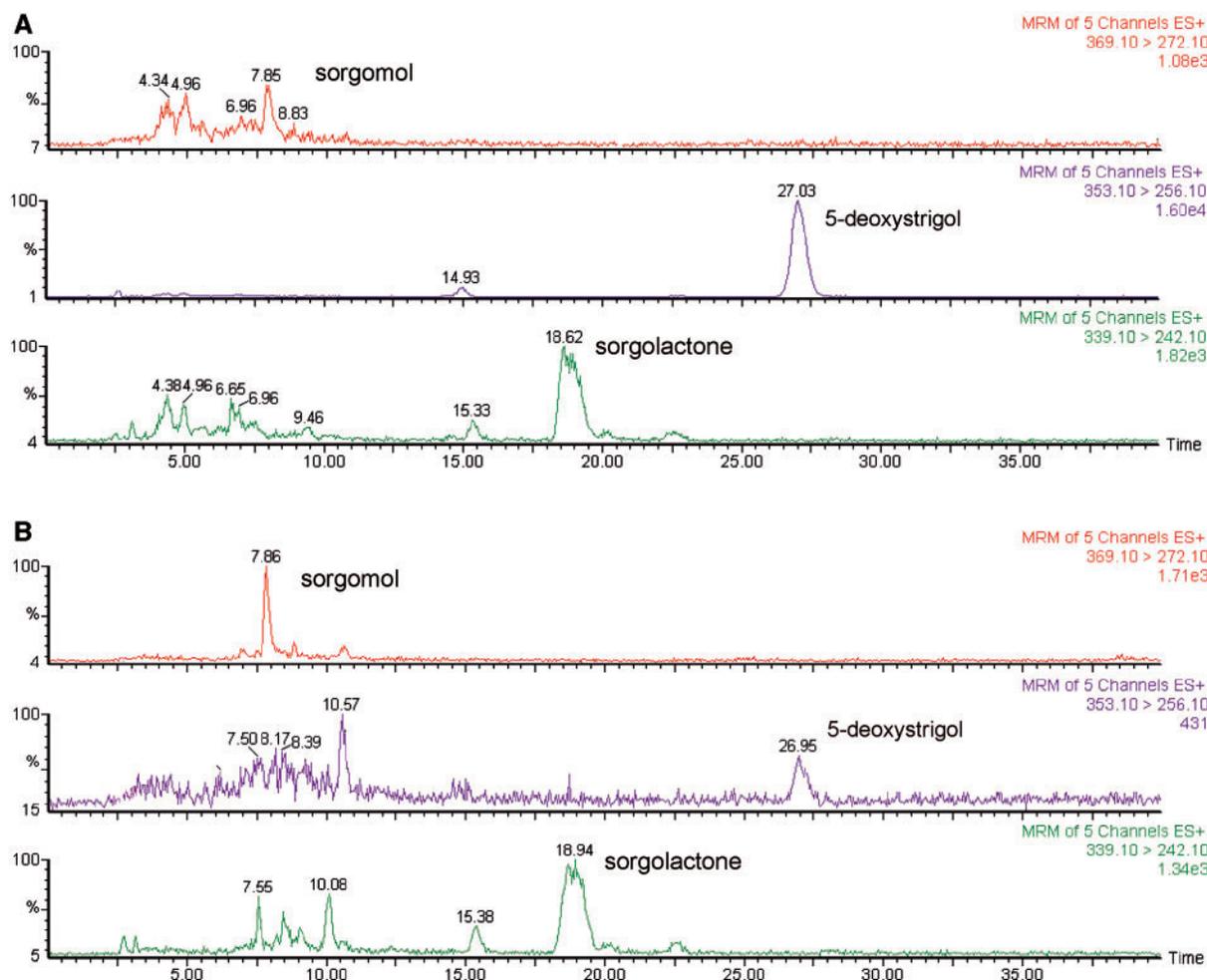


Fig. 5 LC-MS/MS analyses of strigolactones in root exudates. (A) MRM chromatogram of root exudate from the *Striga*-susceptible sorghum cultivar Tabat. (B) MRM chromatogram of root exudate from the *Striga*-resistant sorghum cultivar SRN39. The transitions monitored were m/z 369>272 for sorgomol, m/z 339>242 for sorgolactone and m/z 353>256 for 5-deoxystrigol.

exudates of the model plant *Arabidopsis thaliana* which had been elicited with salicylic acid, jasmonic acid, chitosan or fungal cell wall elicitors, and quantified a total of 289 secondary metabolites; 68 of them were found in non-elicited controls. The total number of compounds present in the whole plant kingdom approaches 200,000, and nearly 5,000 can be found in *Arabidopsis* which can serve as a host of *Orobanchae* spp. (Goldwasser et al. 2000, Westwood 2000) and exudes germination stimulants; one of the major stimulants appears to be orobanchol (Goldwasser et al. 2008). The mechanisms of regulation of production and exudation of SLs are not clear, but environmental factors such as temperature, humidity, day length and nutrient availability are known to affect stimulant exudation (Weerasuriya et al. 1993). For example, fertilizer applications in general reduce crop damage caused by root parasites (Raju et al. 1990, Gworgwor and Weber 1991, Cechin and Press 1993a, Cechin and Press 1993b, Mumera and Below 1993, Parker and Riches 1993). In addition, root exudates from plants grown under phosphate-limited conditions were

reported to be more active in hyphal branching of arbuscular mycorrhizal fungi than those from plants with sufficient phosphate nutrition (Nagahashi and Douds 2000), indicating that exudation of SLs is promoted under phosphate deficiency. In fact, in the case of red clover, phosphate deficiency significantly enhances exudation of orobanchol (Yoneyama et al. 2007a). Similar enhancement of SL exudation under phosphate starvation was reported for tomato (López-Ráez et al. 2008). In sorghum, not only phosphate deficiency but also nitrogen deficiency enhances exudation of 5-deoxystrigol (Yoneyama et al. 2007b).

So far, there has been no report on the identification of SLs in the rhizosphere. Furthermore, for example, sorghum may exude different SLs under different growth conditions. Therefore, at the moment, we do not know which SL is actually involved in germination stimulation of root parasites in the field. Under these circumstances, it is important to clarify the effects of environmental factors, including nutrient availability, on production and exudation of SLs in a defined model

experiment and then to understand the correlation between the model and the field experiments.

As mentioned earlier, root parasitic plants took advantage of the communication cues that are released by plants to attract symbiotic arbuscular mycorrhizal fungi, and developed the ability to detect the adjacent and living host roots by sensing the SLs. An important point that arises from the results described here is that crop cultivars resistant to parasitic weeds due to lower stimulant production may have lower productivity due to poor communication with essential symbiotic arbuscular mycorrhizal fungi. Breeding and genetic engineering of crops for resistance to parasitic weeds by reducing production of SLs may therefore fail to produce high-yielding cultivars. In contrast, plants with high SL production could be utilized in rehabilitation or phytoremediation of wastelands and would be high-yielding trap crops which effectively induce 'suicidal germination' of parasite seeds (Worsham 1987).

There are other germination stimulants structurally unrelated to SLs (Bouwmeester et al. 2003). For example, the fungal metabolites cotylenins and fusicoccins (Yoneyama et al. 1998b, Evidente et al. 2006) and the plant hormone jasmonate (Yoneyama et al. 1998a) induce germination of root parasites. In addition, for example, a medicinal plant, *Houttuynia cordata* Thumb., produces and exudes germination stimulants which are stable in soil (Ma et al. 2005). Therefore, it may be possible to identify novel germination stimulants and develop practical germination stimulants based on their structures.

To date, >14 SLs have been identified mainly from plant root exudates. In addition to these known SLs, at least 10 novel SLs remain to be characterized. Plants appear to produce these SLs at different levels and release mixtures of SLs into the rhizosphere. Further studies are needed in order to clarify how and why plants produce many different SLs and how different SL mixtures contribute to the host recognition by root parasitic plants and by arbuscular mycorrhizal fungi, and to the regulation of shoot branching.

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