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TOWARDS UNDERSTANDING *OROBANCHE* HOST-SPECIFICITY

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ABSTRACT

For the first time the strigolactone composition in root exudates of host plants of rare *Orobanche* spp. occurring in the spontaneous flora have been investigated by LC-MS/MS analysis. The obtained results were discussed in the context with host specificity of *Orobanche* spp. The results show host plant specific qualitative differences in the composition of strigolactones. Known structures have been found, but there are also indications for related compounds, whose structures are not yet revealed. Evidence is seen for the assumption that the composition of strigolactone "bouquets" may determine the host specificity.

Key words: wheat, variety, grain number per spike, fungicide.

INTRODUCTION

Orobanche spp. (*Orobanchaceae*) are angiospermic holoparasites living on the roots of other higher plants. A few of the nearly 200 species and subspecies (taxa) are devastating parasitic weeds, which can destroy whole crops. Most species, however, are found in natural ecosystems, often in protected areas; they are rare and some of them belong to the endangered species.

The host ranges appear very different. Generally the few weedy *Orobanche* spp. parasitize a wide variety of hosts. *Orobanche crenata* attacks almost all leguminoses. *Phelipanche* (*Orobanche) ramosa* and *Orobanche minor* grow on hosts from various plant families. An interesting exception is *Orobanche cumana*, which exclusively parasitizes *Helianthus annuus*. A transition form between non-weedy to weedy behaviour is *Orobanche foetida*, which in South Spain lives on hosts of the wild flora, while it attacks crops in Tunisia (Kharrat et al., 1992) and Morocco (Rubiales et al., 2005).

Fernández-Aparicio (2008) has carried out systematic germination experiments in 9 *Orobanche* spp. with root exudates from 38 crop and fodder plant species, which belong to 12 botanical families. Her results showed different sensitivity of *Orobanche* spp. to the root exudates; the seeds of most *Orobanche* spp. germinated with many, but not with all exudates. While *O. densiflora*, *O. gracilis* and *O. hederae* only responded to the exudate of their specific host, *O. aegyptiaca*, *O. minor* and *P. ramosa* responded to almost all exudates; *O. crenata*, *O. cumana* and *O. foetida* germinated with some, but not all exudates.

With *P. ramosa*, *O. minor*, *O. foetida* (Román et al., 2007a) and *O. gracilis* (Román et al., 2007b) races or subspecies with distinct host preferences were found.

In contrast to weedy *Orobanche* spp., seeds of some *Orobanche* spp. from the wild flora germinate only with a few or one specific hosts, *O. hederae* with *Hedera helix*, *O. lucorum* with *Berberis vulgaris*, *O. rapum-genistae* with *Genista* spp.

A character of *Orobanche* spp. is that their tiny seeds need a conditioning phase under humid warm conditions in the soil, and the subsequent induction of germination by a chemical stimulant exuded by the host root. Close physiological binding of the parasite to the host plant is the consequence. Early researchers had recognized already the significance of a host root for *Orobanche* seed germination, e.g. Caspary (1854). Koch

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(1883) was the first to assume a chemical compound involved in the germination stimulation. Cook et al. (1966) could isolate a stimulant from hydroponic culture of cotton-plants, which they named strigol, because the substance stimulated the germination of *Striga lutea* seeds, which is a related root parasite. Cook et al. (1972) revealed the chemical structure of strigol, and also that of strigyl acetate, an accompanying compound. Cotton, nevertheless, is not the host of any of the root parasites.

Since the chemical synthesis of strigol appeared difficult, Johnson et al. (1981) synthesized structural analogues with the intention to apply such compounds in agriculture for the induction of suicidal germination of parasite seeds, in order to reduce the seed bank in the soil (Johnson et al., 1976). This goal has not been achieved, but one of the synthetic strigol-analogues, GR 24, is still used in germination tests as a standard.

More naturally occurring strigol relatives have been isolated and their structures revealed. Hauck in Schildknecht's group at Heidelberg in his PhD Thesis (1990) has revealed the structure of sorgolactone in the root exudate of *Sorghum bicolor*, which had been isolated by Visser at Stellenbosch, South Africa (Hauck & Schildknecht, 1990). In the same group Müller (1991) has isolated and proposed the structure of alectrol from the root exudate of *Vigna unguiculata*, a host plant of *Alectra vogelii* (Müller et al., 1992). *Alectra* also is a root parasite. Butler, in 1995, has proposed the term strigolactones for these closely related chemical compounds.

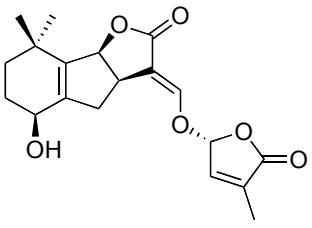
Host roots (but also non-host roots) exude several structurally related strigolactones. In all the studies to isolate germination stimulants, several active compounds have been observed (Brown et al., 1951, 1952; Sunderland, 1960; Visser & Botha, 1974; Visser, 1975), however, for practical reasons only the most active peak has been isolated. The strigolactones show their biological activity at concentrations of 10^{-10} to 10^{-14} M. Therefore their isolation is difficult.

Stereoisomeric forms of the strigolactones exhibit different biological activity. Before the stereoselective chemical synthesis of (+) and (–)-strigol was developed, Hauck and Schildknecht (1990) separated the synthetic strigol racemate on cellulose triacetate and tested both the enantiomers for their germination stimulating activity with three root parasites, *S. asiatica*, *O. aegyptiaca* and *A. vogelii*. The (+)-strigol with *S. asiatica* and *O. aegyptiaca* was two magnitudes more active than (–)-strigol. In contrast, for *A. vogelii* (–)-strigol was more active.

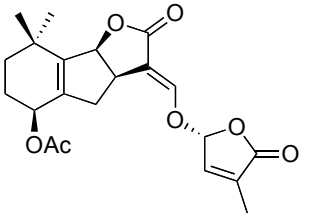
Later numerous structural analogs were synthesized in order to reveal the structure-activity relations (Zwanenburg et al., 1994). Welzel's group has been successful with the synthesis of stereoisomeric forms of strigol, epi-strigol and several derivatives. Wegmann's group studied the structure-activity relations with these compounds (Bergmann et al., 1993; Welzel et al., 1994), however, not with *Orobanche* spp. from the spontaneous flora.

Significant progress in the analysis of strigolactones in root exudates has been achieved by applying a combination of high-performance-liquid chromatography (HPLC) with tandem mass spectrometry (Yoneyama et al., 2004). This method allows the identification and quantification of strigolactones in root exudates. Since then, much knowledge of strigolactones in root exudates has been collected.

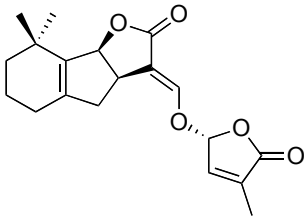
Up to date the following strigolactones are known:



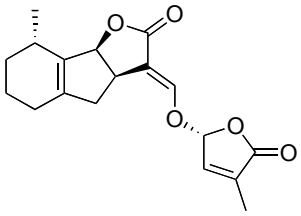
Strigol
Gossypium hirsutum (Cook et al., 1972)
Pennisetum glaucum, Zea mays,
Sorghum bicolor (Siame et al., 1993)
Menispermum dauricum (Yasuda et al., 2003)



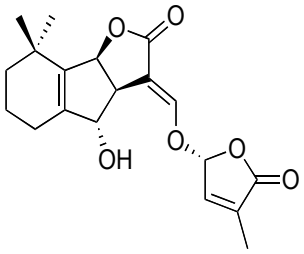
Strigyl acetate
Gossypium hirsutum (Cook et al., 1972)



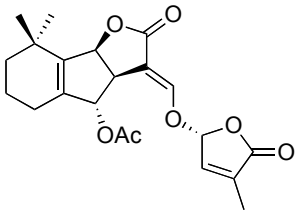
5-Deoxystrigol
Sorghum bicolor, Zea mays, Pennisetum typhoideum (Awad et al., 2006)
Arachis hypogaea, Astragalus sinicus, Cicer arietinum, Glycine max, Lupinus albus, Medicago sativa, Phaseolus vulgaris, Pisum sativum, Psophocarpus tetragonolobus, Vicia faba, Vigna angularis (Yoneyama et al., 2008), *Lotus japonicus* (Sugimoto & Ueyama, 2008)



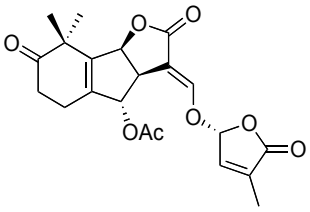
Sorgolactone
Sorghum bicolor (Hauck, 1990; Hauck & Schildknecht, 1990)



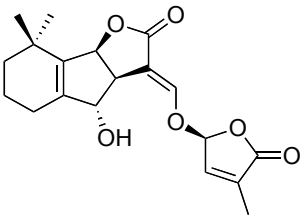
Orobanchol
Trifolium pratense (Yokota et al., 1998)
Lupinus albus (Yoneyama et al., 2008)
Centaurea scabiosa, Carduus personata, Hedera helix, Galium verum (Höniges, 2009)



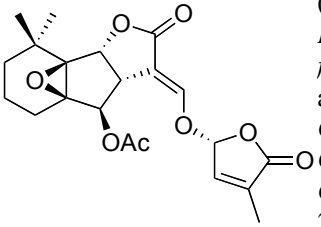
Orobanchyl acetate
(formerly Alectrol)
Arachis hypogaea, Astragalus sinicus, Cicer arietinum, Glycine max, Lupinus albus, Medicago sativa, Phaseolus vulgaris, Pisum sativum, Psophocarpus tetragonolobus, Trifolium incarnatum, Vicia faba, Vigna angularis (Yoneyama et al., 2008)
Cirsium vulgare, Carduus personata, Centaurea scabiosa (Höniges, 2009)



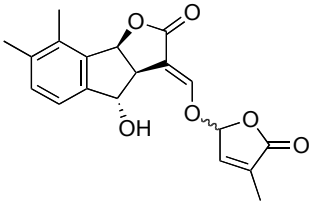
7-Oxo-orobanchyl acetate
Linum usitatissimum, Cucumis sativus (Yoneyama et al., 2008)



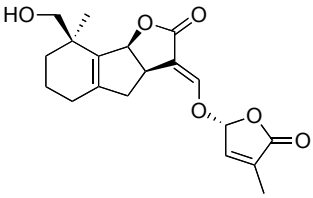
2'-epi-Orobanchol
Nicotiana tabacum (Xie et al., 2007)



Fabacyl acetate
(pea strigolactone)
Pisum sativum, Vicia faba (Yoneyama et al. 2008)
Cirsium vulgare, Carduus personata, *Galium verum* (Höniges, 2009)



Solanacol
Nicotiana tabacum (Xie et al., 2007)
Trifolium incarnatum (Yoneyama et al., 2008)



Sorgomol
Nicotiana tabacum, Solanum lycopersicum, Sorghum bicolor, Zea mays (Awad et al., 2006; Xie et al., 2008)

The structures of didehydro-orobanchol isomers are not yet fully revealed.

Didehydro-orobanchol isomers
Poaceae, *Nicotiana tabacum, Solanum lycopersicum* (Sato et al., 2003; Xie et al., 2007)
Lupinus albus, Pisum sativum, Astragalus sinicus, Cicer arietinum (Yoneyama et al., 2008)
Centaurea scabiosa, Hedera helix, Galium verum (Höniges, 2009)

Because of the findings that root exudates contain several active compounds, and that the enantiomeric forms of strigolactones show different activities, Wegmann (1996, 2005, 2006) developed the hypothesis, that each *Orobanche* species responds to a mixture of various strigolactones, what would explain the different, sometimes very narrow host specificity. This is regarded in analogy to the insect pheromones, which only in specific bouquets, enantiomeric ratios of components included, induce the specific behaviour of insects, e.g. attraction of the mating partner.

In the present work, for the first time, root exudates of the host plants of rare *Orobanche* spp. occurring in the wild flora, were analysed for their strigolactone composition.

MATERIAL AND METHODS

Carduus personata, *Centaurea scabiosa*, *Cirsium vulgare*, *Galium verum* and *Hedera helix* seeds were germinated and grown in vermiculite under greenhouse conditions, then transferred into hydroponic culture. Since strigolactones are exuded in small amounts only, they need to be concentrated until their concentration is high enough for LC-MS/MS analysis. Therefore the water from the rhizosphere was collected on three subsequent days, filtered, extracted with ethyl acetate and concentrated.

100 mL each were shaken twice with 400 mL ethyl acetate in a separation funnel. 400 mL of the ethyl acetate extract were washed with 100 mL 0.2 M aqueous Na_2HPO_4 solution, then dried over anhydrous $MgSO_4$.

The dried ethyl acetate extract was concentrated in a Büchi Rotavapor R-124 equipped with a Büchi Waterbath B-480. Vacuum was – 0.95 Torr, cooling temperature was 10°C, the water bath was set to 30°C. The final volume of 2 mL were transferred into a small vial and dried off in a nitrogen stream. The dry matter was 6 mg (*Carduus personata*), 12 mg (*Centauerea scabiosa*), 5 mg (*Cirsium vulgare*), 8 mg (*Galium verum*) and 12 mg (*Hedera helix*). The samples were dissolved in 60% methanol, filtered, and injected onto a reversed-phase (C_{18}) HPLC column that was connected to a MS/MS spectrometer.

A U980 HPLC instrument (Jasco, Tokyo, Japan) equipped with an ODS (C_{18}) column (Mightysil RP-18, 2 x 250 mm, 5 µm, Kanto Chemicals) was used. The mobile phase was 60 % methanol in H_2O (v/v). 30 min. after injection of the sample the mobile phase was changed to 100% methanol. The flow rate was 0.2 mL min^{-1}. The column temperature was set to 40°C.

Mass spectrometry was performed with a Quattro LC mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray source. Both the drying and nebulising gas was nitrogen generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). The nebulizer gas flow was set to approximately 100 L h^{-1}, and the desolvation gas flow to 500 L h^{-1}. The interface temperature was set to 400°C, and the source temperature to 150°C. MS/MS experiments were conducted by using argon as the collision gas and the collision energy was set to 16 eV. The collision gas pressure was 0.15 Pa. Data acquisition and analysis were performed with the MassLynx software (ver. 4.1).

Strigolactones were determined by the multiple reaction monitoring (MRM) method. Seven transitions of *m/z*, namely 339 > 242, 353 > 256, 365 > 268, 367 > 270, 369 > 272, 411 > 254 and 427 > 270 were monitored for 5-deoxystrigol, sorgolactone, tetradehydro-orobanchol isomer (solanacol), didehydro-orobanchol isomer, orobanchol (strigol), orobanchyl acetate, and fabacyl acetate (pea strigolactone).

RESULTS

The results of the LC-MS/MS analyses are shown in Figures 1-5. The abscissa shows the time course of the HPLC separation, the ordinates show the signals for the specific mass transitions (multiple reaction monitoring, MRM), characteristic for known strigolactone structures. Seven mass transitions were investigated.

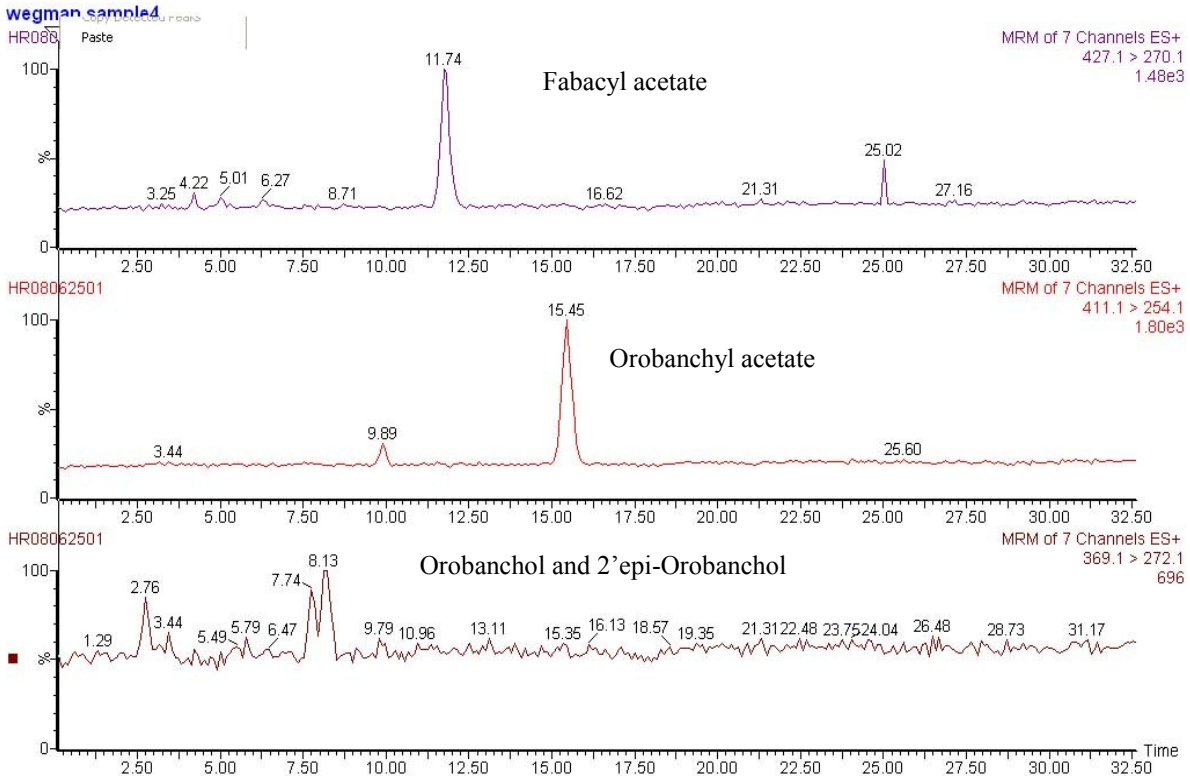


Figure 1. Strigolactones in the root exudate of *Cirsium vulgare*, host for *Orobanche reticulata*

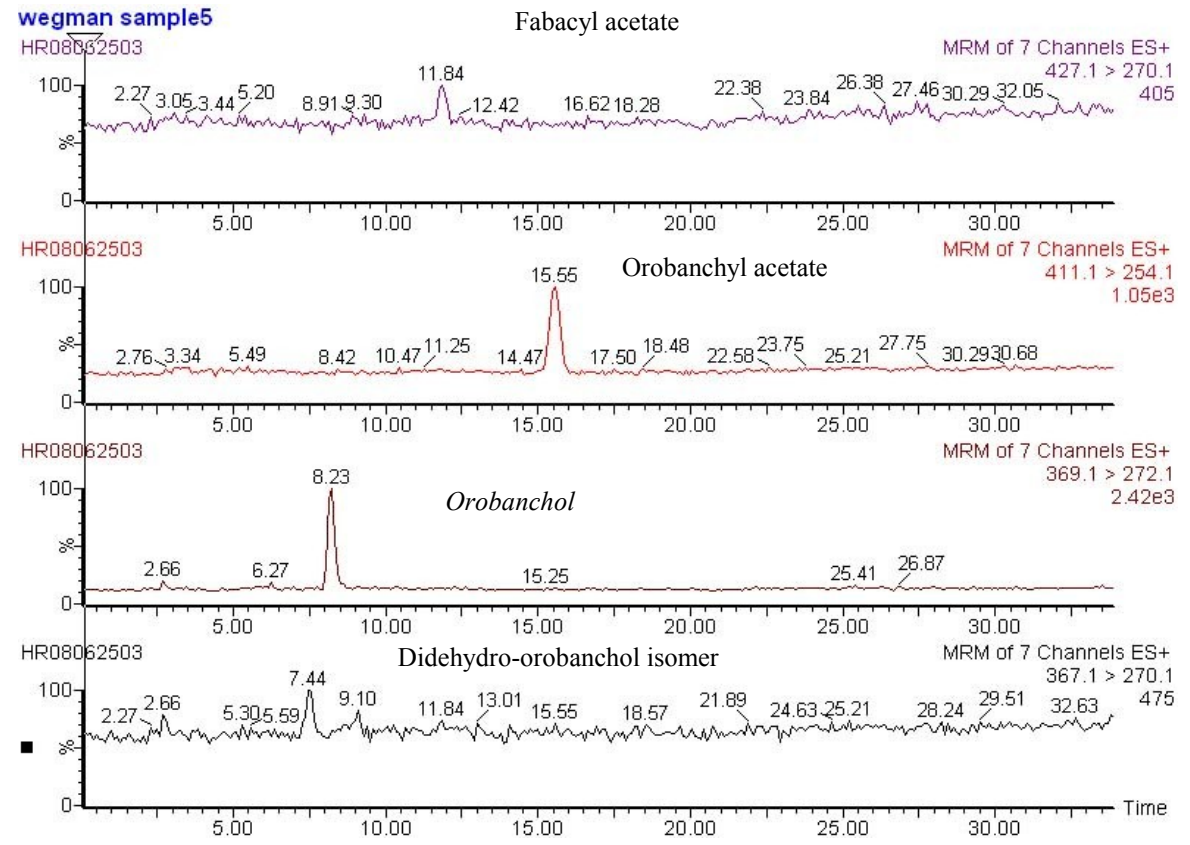


Figure 2. Strigolactones in the root exudate of *Centaurea scabiosa*, host for *Orobanche elatior*

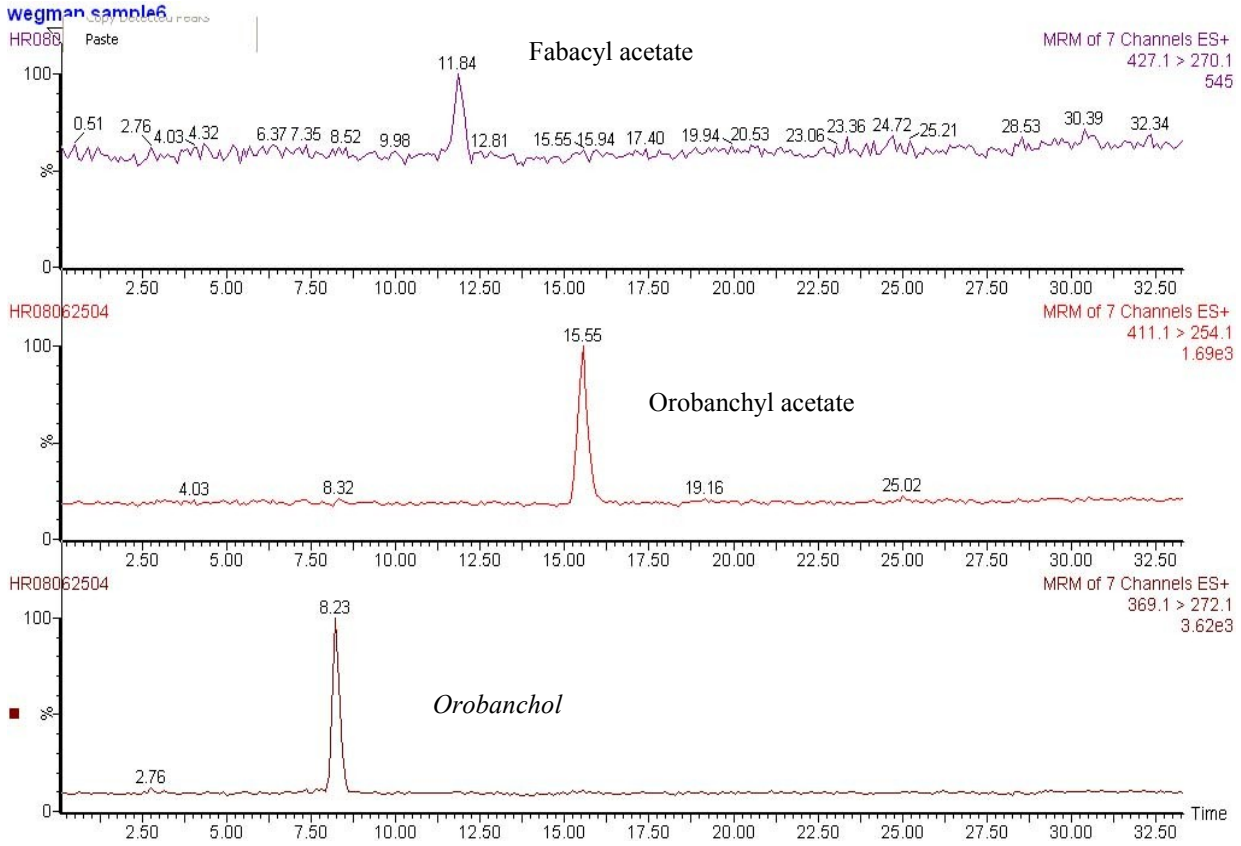


Figure 3. Strigolactones in the root exudate of *Carduus personata*, host for *Orobanche reticulata*

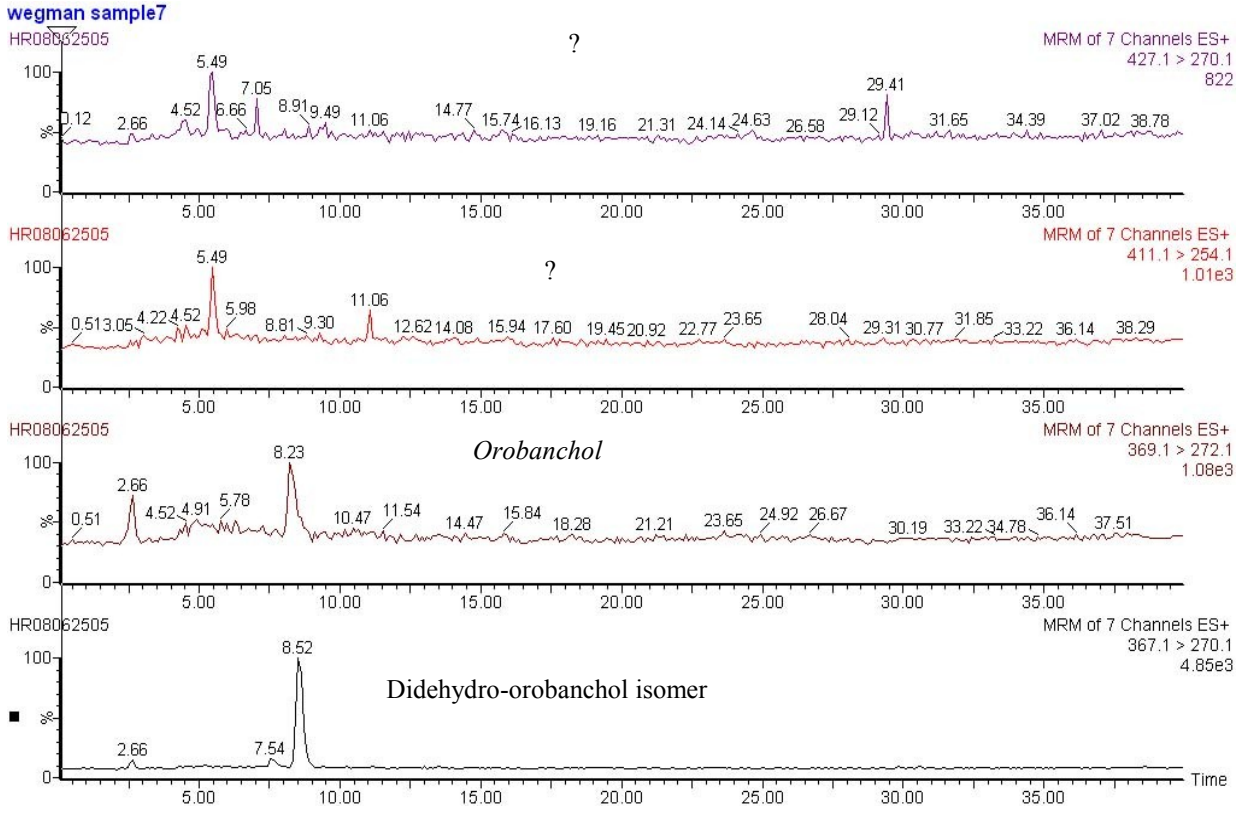


Figure 4. Strigolactones in the root exudate of *Hedera helix*, host for *Orobanche hederae*

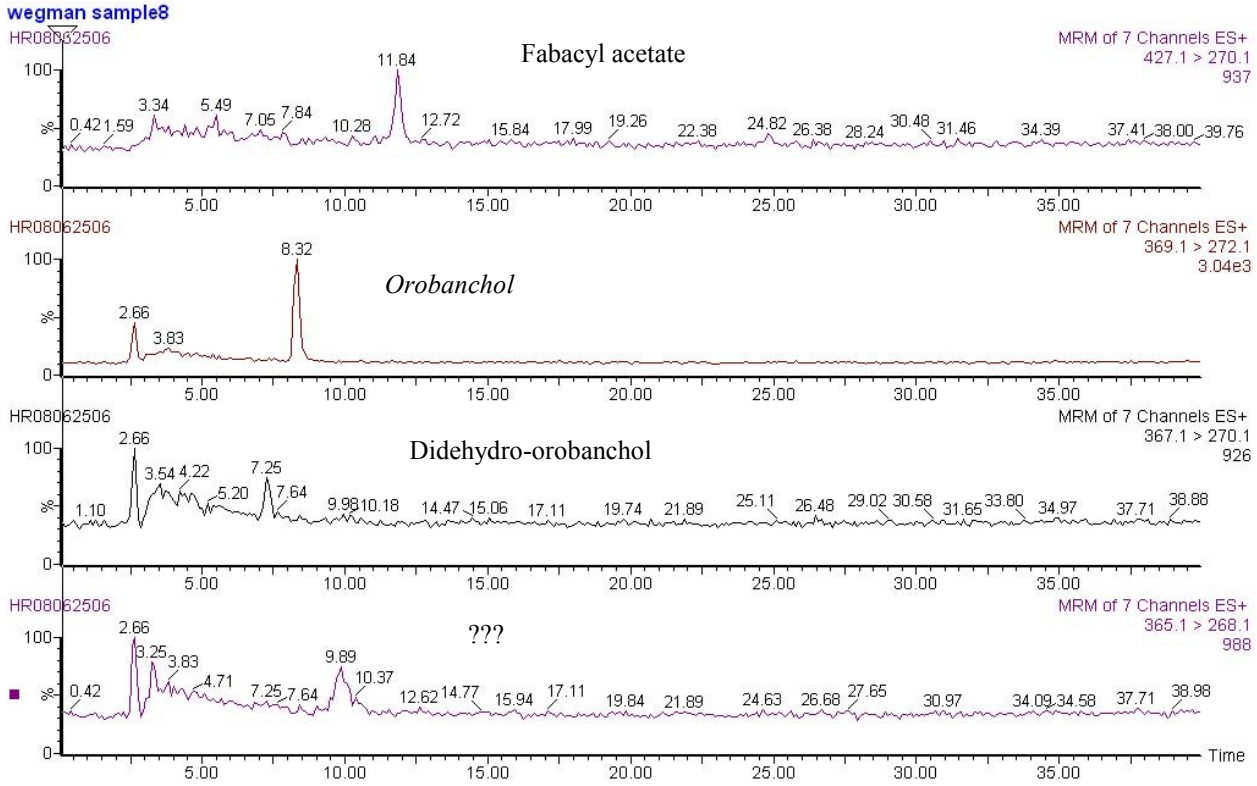


Figure 5. Strigolactones in the root exudate of *Galium verum*, host for *Orobanche caryophyllacea*

The results of the analyses of strigolactones are summarized in Table 1.

The strigolactone components in the root exudates are qualitatively differently composed. The root exudates would contain unknown strigolactones, whose structures have not yet been determined, as in the case of didehydro-orobanchol isomers.

Table 1. Composition of strigolactones in the exudates of some host roots of rare *Orobanche* spp. occuring in the spontaneous flora

Host plant	Orobanchol	epi-Orobanchol	Orobanchyl-acetate	Fabacyl-acetate	Didehydro-orobanchol isomers
Cirsium vulgare	+	+	+	+	nd
Centaurea scabiosa	+	nd	+	+	+
Carduus personata	+	nd	+	+	nd
Hedera helix	+	nd	nd	nd	nd
Galium verum	+	nd	nd	+	+

The investigated plants are hosts for different *Orobanche* species:

- *Cirsium vulgare* and *Carduus personata* for *Orobanche reticulate*;
- *Centaurea scabiosa* for *O. Elatior*;
- *Hedera helix* for *O. Hederae;*
- *Galium verum* for *O. caryophyllacea.*

DISCUSSION

The results support the hypothesis that the qualitative (and quantitative) composition of strigolactones in the root exudates of plants determines the recognition of a suitable host plant by the seeds of a defined *Orobanche* species, which shows a narrow host range. A defined "bouquet" only, specific for that *Orobanche* species, stimulates its seeds germination.

If all components in sufficient amounts will be synthesized or isolated, systematic germination experiments with defined mixtures of strigolactones would prove the hypothesis. However, (stereospecific)

strigolactone synthesis is difficult, preparative isolation is laborious and time-consuming; therefore most components are still unavailable.

Strigolactones are exuded by many higher plants roots, independent whether they are hosts for root parasites or not. This became understandable since the discovery by Akiyama et al. (2005), see also Akiyama & Hayashi (2006) and Akiyama (2007), that strigolactones induce arbuscular mycorrhizal *(Gigaspora margarita*) hyphae branching. Thus, strigolactones play an important role for mycorrhiza development. Besserer et al. (2006) could show that strigolactones in a concentration of 10-13 M activated the mitochondrial activity of the AM fungi Gigaspora rosea, Glomus intraradices and G. claroideum. In contrast inhibition sprout branching of higher plants was recently described in Nature by two independent research groups (Gomez-Roldan et al., 2008; Umehara et al., 2008). Strigolactones may be regarded as a new class of phytohormones.

Compounds exhibiting specific action at such low concentrations, like hormones or pheromones, as a rule are bound to a specific membrane-bound receptor, which then causes a cascade of physical and biochemical reactions. While the odorant receptors of animals are well known by the pioneer work of Buck & Axel (1991), awarded with the Nobel prize in 2004, as also for insects (Nakagawa et al. 2005), strigolactone receptors from *Orobanche* spp. are under investigation (Zwanenburg & Reizelman, 2001; Reizelman et al. 2003). Due to different specificities a family of related receptors is to be expected.

Receptors can mutate, what may alter their specificity, and hence the host specificity of *Orobanche* could change. Up to date the discussion is going on, whether *Orobanche cernua* with a wide host spectrum has mutated to the specific sunflower parasite *Orobanche* cumana with sunflower as the only host. *Orobanche cumana* seeds are stimulated by sesquiterpene lactones other than strigolactones (Macías et al., 2006).

Some Orobanche spp. seeds *(O. crinita, O. densiflora, O. elatior, O. foetida* var. *broteri, O. foetida var. foetida, O. hederae, O. lutea*) are not stimulated by GR 24. This may be interpreted by the lack or the loss of a GR 24 sensitive receptor.

Since receptors as proteins are genetically coded and inherited, by hybridisation host-specificity may change. Schuchardt et al. (1998) described an example: *Orobanche lavendulacea* by hybridization with *Orobanche ramosa* became a tobacco parasite in Bulgaria. In these self-pollinators, hybridisation is a rare event, but it can happen. If hybrids inherit the receptors (host specificity) of both parents, the host spectrum becomes broader. *Orobanche crenata* has a very broad host spectrum, comprising almost all legumes. As a cross-pollinator and with numerous mutations in this species, *O. crenata* is a mixture of different phenotypes and genotypes, forming a big seed potential in the soil. The broad host spectrum might be mimicked by the availability of suitable seeds fitting to any legume crop planted.

The findings provoke another question. Strigolactones are sensitive compounds, which rapidly decompose in the soil. Biologically this makes sense, because durable stimulants would cause the germination of the whole *Orobanche* seed bank in the absence of hosts, thus eliminating the parasite by suicide germination. However, what will happen, when a strigolactone structure is modified by the biochemical activities of the soil microflora? According to the discussion above the host specificity could change.

Acknowledgement

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Besserer, A., Puech-Page, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., Portais, J.-C., Roux, C., Bécard, G. & Sejalon-Delmas, N., 2006. *Strigolactones at a concentration of 10^{-13} M activate mitochondrial activity in Gigaspora rosea, Glomus intraradices and Gl. claroideum.* PLoS Biol., 4: 1239-1247.

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