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SHORT COMMUNICATION

# The role of strigolactones in host specificity of *Orobanche* and *Phelipanche* seed germination

**Mónica Fernández-Aparicio[1,2], Koichi Yoneyama[3] and Diego Rubiales[1]***

[1]Institute for Sustainable Agriculture, CSIC, Apdo. 4084, E-14080 Córdoba, Spain; [2]Virginia Tech, Department of Plant Pathology, Physiology and Weed Science, Blacksburg, Virginia 24061, USA; [3]Weed Science Center, Utsunomiya University, 350 Mine-machi, Utsunomiya 321-8505, Japan

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## Abstract

Strigolactones are apocarotenoids regulating shoot branching. They are also known to be exuded by plant roots at very low concentrations, stimulating hyphal branching of arbuscular mycorrhizal fungi and germination of root parasitic weed seeds. We show that strigolactones play a major role in host specificity of *Orobanche* and *Phelipanche* (the broomrapes) seed germination. This observation confirms that host-derived germination stimulants are an important component determining the host specificity of these parasitic plants. Weedy broomrape species were less specialized in germination requirements than the non-weedy species except for *O. cumana* and *O. foetida* var. *broteri*. Similar results were obtained with the root exudates. Some species, such as *P. aegyptiaca* and *O. minor*, showed a broad spectrum of host specificity in terms of seed germination, which was stimulated by exudates from the majority of species tested, whereas others, such as *O. cumana*, *O. hederae* and *O. densiflora*, were highly specific. Some species, such as *O. minor*, *P. aegyptiaca* and *P. nana*, were responsive to the three strigolactones studied, whereas others were induced by only one of them, or did not respond to them at all. The synthetic strigolactone analogue GR24, generally used as a standard for germination tests, was not effective on some *Orobanche* and *Phelipanche* species. Seeds of some species that did not respond to GR24 were induced to germinate in the presence of fabacyl acetate or strigol, confirming the role of strigolactones in host specificity.

*Correspondence
Fax: + 34 957499252
Email: diego.rubiales@ias.csic.es

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## Introduction

Parasitic plants have evolved from non-parasitic or autotrophic ancestors by synchronizing their life cycles to the host species that they parasitized. This is achieved by coordinating early developmental stages with chemical signals from the hosts (Smith *et al.*, 1990). Most of the species belonging to the holoparasitic genera *Orobanche* and *Phelipanche* (broomrapes) have a narrow host spectrum and grow exclusively on perennial host plants (Schneeweiss, 2007). A number of *Orobanche* and *Phelipanche* species have evolved to parasitize a wide range of crops in agricultural ecosystems, thus becoming noxious weeds (Joel *et al.*, 2007; Parker, 2009).

Strigolactones are apocarotenoids that regulate shoot branching (Gómez-Roldán *et al.*, 2008; Umehara *et al.*, 2008) and are exuded by host roots, serving in mycorrhizal symbiosis to branch the mycelium toward the host root (Akiyama *et al.*, 2005). *Orobanche* and *Phelipanche* seeds germinate mainly in response to strigolactones (Yoneyama *et al.*, 2008). They have evolved a complex process of parasitization that is mediated primarily by host-derived chemical signals controlling germination of the parasitic seeds. It is thus possible that the specificity of germination stimulants plays a major role in host specialization; however, there is insufficient evidence to support this hypothesis (Yoneyama *et al.*, 2009). This work aims to investigate the degree of specificity in the inductor potential of strigolactones to stimulate germination of parasitic seeds of *Orobanche* and *Phelipanche* species. We studied germination responses of broomrape seeds to root

exudates and to three selected strigolactones: fabacyl acetate, a strigolactone first isolated from pea root exudates (Xie *et al.*, 2009) and considered to be typical of legumes (Yoneyama *et al.*, 2008); strigol, first identified in cotton (*Gossypium hirsutum*) and known to be typically produced by cereals, such as maize (*Zea mays*), pear millet (*Penisetum glaucum*) and sorghum (*Sorghum bicolor*) (Cook *et al.*, 1972; Siame *et al.*, 1993); and the strigolactone synthetic analogue GR24, commonly used as a standard in broomrape germination studies (Johnson *et al.*, 1976).

## Materials and methods

### Plant materials

Germination was tested of seeds of 16 broomrapes belonging to 15 species in the *Orobanche* or *Phelipanche* genera (Table 1) collected from dry inflorescences using a 0.6 mm mesh-size sieve (Filtra, Barcelona, Spain) and stored dry in the dark at 4°C until use. Viability of these seeds was tested using triplicates of 100 seeds per broomrape with the 2,3,5-triphenyl tetrazolium chloride (TTC) method (Aalders and Pieters, 1985). Seeds were imbibed in 1% (w/v) TTC (Sigma-Aldrich, St. Louis, Missouri, USA) solution and incubated at 37°C for 3 d. TTC solution was eliminated and seeds were immersed in a solution of 50% (w/v) sodium hypochlorite for 2–3 min, to clear the testa. Seeds were observed under a stereoscopic microscope at 30× magnification to determine the percentage of viable seeds. Seeds were considered viable when their embryos were stained pink to red. Root exudates released by several host or non-host plants (Table 2) were collected and tested for parasitic seed germination.

### Strigolactones

Two natural strigolactones, fabacyl acetate (provided by Xiaonan Xie, Utsunomiya University, Japan) and strigol (provided by Kenji Mori, The University of Tokyo, Japan) and the synthetic strigolactone GR24 (provided by Binne Zwanenburg, Radboud University, The Netherlands) were used in this work.

### Collection of root exudates

Twelve seeds per host or non-host plant species (Table 2) were surface sterilized with 2% (w/v) sodium hypochlorite solution containing 0.02% (v/v) Tween 20 for 5 min and then rinsed thoroughly with sterile distilled water. Seeds were grown in sterile expanded perlite (Europelita, Barcelona, Spain) for 2–3 weeks [20°C, 12/12 h dark/light (200 $\mu$mol m$^{-2}$ s$^{-1}$) photoperiod]. To collect exudates, plants were removed from the perlite and the roots were carefully washed and immersed in distilled sterile water contained in flasks (4 plants per flask) for 2 d, allowing them to release the root exudates. To perform accurate comparisons, root exudates contained in each flask were diluted with sterile distilled water to adjust the concentrations equivalent to 0.06 g root (fresh weight basis) per millilitre water.

**Table 1.** *Orobanche* and *Phelipanche* species used in this study and viability of seeds

| Broomrape species | Host | Collection site | Seed viability (mean ± SE) |
|---|---|---|---|
| Weedy | | | |
| *P. aegyptiaca* (Pers.) Pomel (syn. *O. aegyptiaca* Pers.) | Chickpea (*Cicer arietinum* L.) | Israel | 94.3 ± 0.9 |
| *O. crenata* Forsk. | Faba bean (*Vicia faba* L.) | Spain | 75.3 ± 2.6 |
| *O. cumana* Wallr. | Sunflower (*Helianthus annuus* L.) | Spain | 94.7 ± 1.5 |
| *O. foetida* var. *broteri* (J.A. Guim.) Merino | Faba bean (*Vicia faba* L.) | Tunisia | 86.0 ± 2.7 |
| *O. minor* Sm. | Red clover (*Trifolium pratense* L.) | Chile | 95.7 ± 1.5 |
| *P. ramosa* (L.) Pomel (syn. *O. ramosa* L.) | Tobacco (*Nicotiana tabacum* L.) | Spain | 66.3 ± 3.2 |
| Non-weedy | | | |
| *O. alba* Steph. ex Willd. | *Thymus* sp. | Spain | 73.0 ± 4.2 |
| *O. ballotae* A. Pujadas | *Ballota hirsuta* Benth. | Spain | 90.0 ± 3.8 |
| *O. cernua* Loefl. | *Artemisia* sp. | Spain | 86.7 ± 3.9 |
| *O. crinita* Viv. | *Lotus* sp. | Tunisia | 94.7 ± 1.5 |
| *O. densiflora* Reut. | *Lotus creticus* L. | Spain | 86.3 ± 2.4 |
| *O. foetida* Poir. var. *foetida* | *Astragalus lusitanicus* Lam. | Spain | 88.0 ± 1.5 |
| *O. hederae* Duby | *Hedera helix* L. | France | 83.0 ± 2.0 |
| *O. santolinae* Loscos and Pardo | *Santolina* sp. | Spain | 88.3 ± 1.2 |
| *P. schultzii* (Mutel) Pomel (syn. *O. Schultzii* Mutel) | *Pimpinella villosa* Schousb. | Spain | 84.7 ± 0.7 |
| *P. nana* (Reut.) Soják (syn. *O. nana* (Reut.) Beck) | *Oxalis* sp. | Greece | 88.0 ± 1.5 |

**Table 2.** Plant materials used to obtain root exudates for broomrape seed germination tests

| Family | Plant species | Cultivar | Described interaction with parasitic species |
|---|---|---|---|
| Fabaceae | Pea (*Pisum sativum* L.) | Messire | *O. crenata* |
| | *Lotus japonicus* Regel. | GiFu | *O. densiflora, O. crinita* and *O. minor* |
| | Faba bean (*Vicia faba* L.) | Prothabon | *P. aegyptiaca, O. crenata* and *O. foetida* |
| | Cowpea (*Vigna unguiculata* L.) | Egyptian landrace | *Alectra vogelii* and *Striga gesnerioides* |
| Poaceae | Maize (*Zea mays* L.) | Pioneer | *Striga hermonthica* |
| | Durum wheat (*Triticum durum* L.) | Meridiano | None |
| Araliaceae | Ivy (*Hedera helix* L.) | Leafy stem cuttings of local landrace | *O. hederae* |
| Asteraceae | Sunflower (*Helianthus annuus* L.) | Peredovic | *O. cumana* |

### *Conditioning of broomrape seeds*

*Orobanche* and *Phelipanche* seeds were surface sterilized with formaldehyde 0.2% (w/v) and 0.02% (v/v) of Tween 20, rinsed thoroughly with sterile distilled water and dried for 60 min in a laminar air flow cabinet. Approximately 100 seeds of each species were placed on 1.5-cm diameter glass fibre filter paper (GFFP; Whatman International Ltd., Brentford, UK) moistened with 120 μl of sterile distilled water. Thirty separate discs containing seeds were placed in a 10-cm sterile Petri dish and incubated in the dark at 20°C for 11 d for warm stratification to promote germination (Fernández-Aparicio *et al.*, 2009).

### *Germination tests*

The effects of strigol and fabacyl acetate on seed germination were tested at concentrations of $10^{-6}$, $10^{-7}$ and $10^{-8}$ M. GR24 was tested at $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ M. After 11 d of conditioning, the 1.5-cm diameter GFFP discs containing the warm-stratified broomrape seeds were transferred to a sterile sheet of paper for 4–5 s to remove the water and transferred to a new 10-cm sterile Petri dish. Each strigolactone was dissolved in acetone and diluted with sterile Milli-Q (Millipore, Billerica, Massachusetts, USA) water to a final concentration of 0.7% acetone (119.6 μl Milli-Q water/0.84 μl acetone). Aliquots of 120 μl of strigol, fabacyl acetate or GR24 at each concentration, or aliquots of 120 μl of each root exudate, were applied to GFFP containing the conditioned seeds. Petri dishes were sealed with Parafilm (Pechiney, Chicago, Illinois, USA) and incubated in the dark at 20°C for 7 d. Milli-Q water (0.7% acetone) was used as a negative control. Germination was assessed as the percentage of seeds with an emerged radicle. The germination percentages were calibrated with the percentages of viable seeds (Table 2) (Rubiales *et al.*, 2006). That is, the germination percentages expressed in the figures indicate the percentage of viable seeds that germinated in each treatment. Germination tests were performed in triplicate.

### *Statistical analysis*

Experiments were performed using a completely randomized design. Percentage data were approximated to normal frequency distribution by means of angular transformation ($180/\pi \times$ arcsine [sqrt (%/100)] and subjected to analysis of variance (ANOVA) using SPSS software for Windows, version 15.0 (SPSS Inc., Chicago, Illinois, USA), after which residual plots were inspected to confirm data conformed to normality. The null hypothesis was rejected at the level of 0.05.

## Results

The effects of strigolactone on broomrape seed germination were significant ($P < 0.001$) (Fig. 1). Induction of seed germination by each strigolactone was concentration dependent ($P < 0.001$). In addition, there were significant interactions between the concentration tested × broomrape species ($P < 0.001$) and concentration tested × compound ($P < 0.001$). Reduction of germination at lower strigolactone concentration was not linear for all broomrape species. Even the opposite was observed for some species, such as *O. crinita* and *O. densiflora*, in which lower concentrations were more effective in the induction of seed germination.

Seeds of *O. minor*, *P. aegyptiaca* and *P. nana* were highly responsive to all strigolactones tested (Fig. 1), exhibiting more than 70% germination for all inducers. *Orobanche minor* and *P. aegyptiaca* were also responsive to root exudates of the plant species studied (Fig. 2).

Germination of *O. ballotae*, *O. crenata* and *O. cernua* seeds was significantly dependent on the concentration of strigolactones ($P < 0.001$). The germination of *O. ballotae* decreased markedly from 30 to 0%; from 90 to 23%, and from 81 to 58% when the concentrations of fabacyl acetate, strigol and GR24 were decreased from $10^{-6}$ M to $10^{-8}$ M, respectively (Fig. 1). Similarly, germination of *O. crenata* decreased from 56 to 7% and

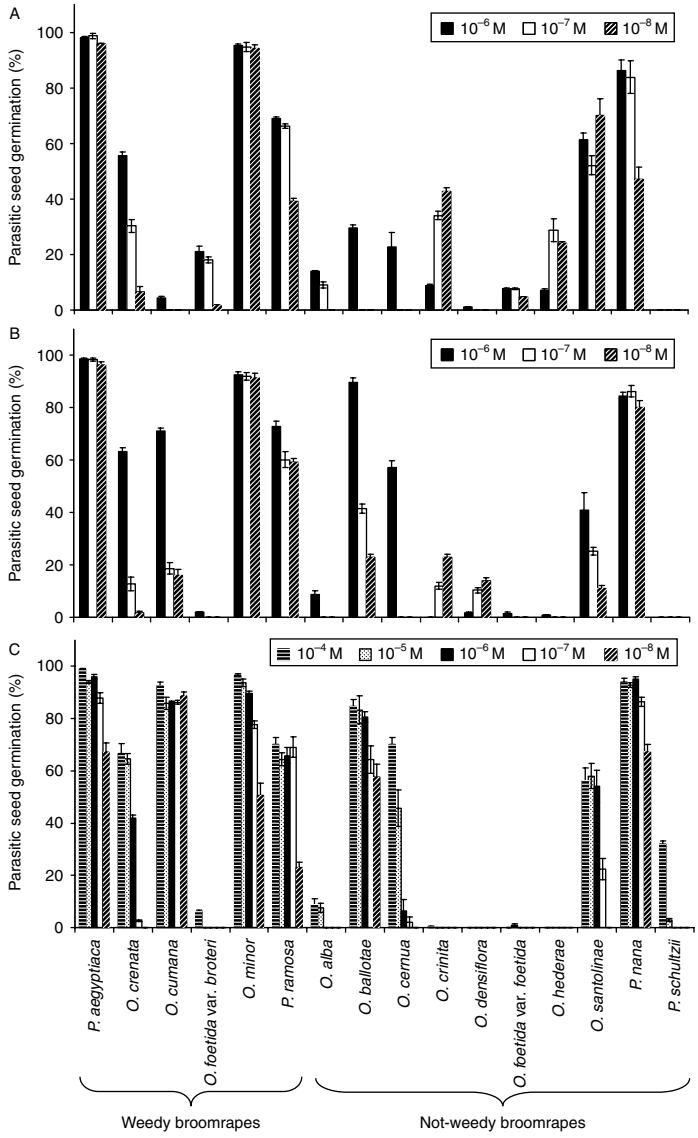


**Figure 1.** Effects of fabacyl acetate (A), strigol (B) and GR24 (C) on seed germination of *Orobanche* and *Phelipanche* species. Strigolactones were tested at $10^{-6}$, $10^{-7}$ and $10^{-8}$ M. Note that higher concentrations $10^{-4}$ and $10^{-5}$ M were also tested for GR24 (C). Each data point indicates mean ± SE.

from 63 to 2% when the concentrations of fabacyl acetate and strigol were decreased from $10^{-6}$ M to $10^{-8}$ M, respectively, and from 67 to 0% when the concentration of GR24 was decreased from $10^{-4}$ M to $10^{-8}$ M. Germination of *O. cernua* decreased from 23 to 0% and from 57 to 0% when the concentrations of fabacyl acetate and strigol were decreased from $10^{-6}$ M to $10^{-8}$ M, and from 70 to 0% when the concentration of GR24 was decreased from $10^{-4}$ M to $10^{-8}$ M.

For some species germination was activated by one strigolactone but not by the others. Examples were *O. cumana* and *O. densiflora* seeds, in which germination was stimulated only by strigol and not by fabacyl acetate. In contrast, seed germination of *O. foetida* and *O. hederae* was stimulated by fabacyl acetate but not by strigol. Of these four species, only *O. cumana* was stimulated by GR24.

*Orobanche crinita* seeds were induced by both strigol and fabacyl acetate, but not by GR24 (Fig. 1). *Orobanche alba* responded to all strigolactones tested only at low levels. *Orobanche alba* seeds were induced by GR24 at $10^{-4}$ and $10^{-5}$ M but not at lower concentrations. *Phelipanche schultzii* seeds germinated only at the highest concentration of GR24 tested ($10^{-4}$ M) but did not germinate in any concentrations of the strigol or fabacyl acetate tested (Fig. 1).

Average germination induced by all strigolactones was significantly higher ($P < 0.001$) for the weedy species (*O. minor*, *P. aegyptiaca*, *P. ramosa* and *O. crenata*) than for the non-weedy, with the exception of

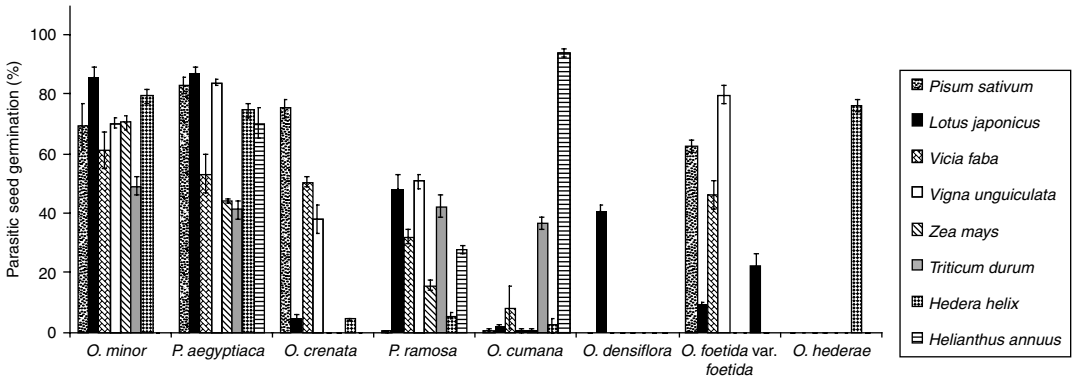


**Figure 2.** Germination of weedy broomrape seeds in the presence of root exudates from several host and non-host plants. *Pisum sativum* host of *O. crenata*; *Lotus japonicus* host of *O. crinita*, *O. densiflora* and *O. minor*; *Vicia faba* host of *P. aegyptiaca*, *O. crenata* and *O. foetida*; *Vigna unguiculata* host of *Alectra vogelii* and *Striga gesnerioides*; *Zea mays* host of *Striga hermonthica*; *Hedera helix* host of *O. hederae*; and *Helianthus annuus* host of *O. cumana*. In addition, *Triticum durum* was included as a cereal non-host of parasitic plants. Each data point indicates mean ± SE.

*O. cumana* and *O. foetida* var. *broteri*. Conversely, non-weedy species generally had more specific germination stimuli, with the exception of *P. nana* and *O. santolinae* (Fig. 1).

Similar results were obtained with the root exudates. For example, seed germination of some broomrape species was induced by the exudates from the majority of species tested (e.g. *P. aegyptiaca* and *O. minor*) while seed germination responses in other broomrape species were highly specific to only a few plant species tested (e.g. *O. cumana*, *O. densiflora* and *O. hederae*). *Orobanche cumana* is known to infect only sunflower (Joel *et al.*, 2007). Consistent with previous reports, the exudate from sunflower roots induced the highest level of *O. cumana* germination (94%), but exudates from non-hosts such as faba bean and wheat were also able induce some, albeit low, *O. cumana* germination. The non-weedy species *O. densiflora* germinated only in the presence of root exudates from its host *L. japonicus* (35%). *Orobanche hederae* germinated in the presence of root exudates from its host, ivy (65%).

*Orobanche crenata* and *O. foetida* germinated in the presence of root exudates from the legumes pea, faba bean and cowpea, but not in the presence of those from *L. japonicus*. Root exudates from ivy induced moderate germination of *O. foetida* (18%) and low germination of *O. crenata* (3%). The other species included in this work did not show any stimulatory activity on *O. crenata* and *O. foetida*.

## Discussion

The results of the present study are consistent with previous findings (Fernández-Aparicio *et al.*, 2009) and support the idea that weedy broomrape species are less specialized in germination requirements than the non-weedy species, with the exception of *O. cumana* and *O. foetida* var. *broteri*. A possible explanation for the atypical host specialization of these two species is that they evolved as weedy parasitic plants relatively recently. *Orobanche cumana*, parasitizing sunflower exclusively, was an autochthonous species from Eastern Europe and Central Asia growing on *Artemisia* spp. With the introduction of sunflower as a new crop in Eastern Europe in the 19th century, *O. cumana* encountered the new host and became a parasitic weedy species and spread to other areas with sunflower crops (Pujadas-Salvá and Velasco, 2000). *Orobanche foetida* is widely distributed in the western Mediterranean as a non-weedy species (Pujadas-Salvá *et al.*, 2003), but only recently has been reported as weedy, infecting faba bean or vetch crops (Kharrat *et al.*, 1992; Rubiales *et al.*, 2005). A host specialization process, which is still in progress, has been described for *O. foetida* (Román *et al.*, 2007; Vaz Patto *et al.*, 2008). Specificity of these two species has been related to their sensitivity to non-strigolactone compounds, such as sesquiterpene lactones for *O. cumana* (Macías *et al.*, 2009) and peagol and a polyphenol for *O. foetida* (Evidente *et al.*, 2009, 2010). However, here we show that these two species also respond to strigolactones. *Orobanche cumana* responds to GR24, at levels similar to those observed for most weedy species, but also, to a minor extent, to strigol and fabacyl acetate. *Orobanche foetida* does not respond to GR24 but does respond to fabacyl acetate.

GR24, generally used as standard for germination tests (Mangnus *et al.*, 1992) was not effective on some *Orobanche* and *Phelipanche* species. This is in agreement with recent reports (Fernández-Aparicio *et al.*, 2008b, 2009; Thorogood *et al.*, 2009) in which a number of non-weedy species were reported not to respond to GR24. Here, we found more non-weedy species that did not respond to GR24. We also describe non-weedy species

that responded to GR24, such as *P. nana*, *O. santolinae*, *O. ballotae* and the non-weedy population of *O. cernua* used in this study. Thorogood *et al.* (2009) suggested that responsiveness to GR24 might be related to a broader host range. However, here we show that not only the broomrapes with a broad host range, but also host-specific broomrapes, such as *O. cumana* or *O. ballotae*, are responsive to GR24. Seeds of some of the species that are not responsive to GR24 were capable of germinating in the presence of fabacyl acetate (*O. crinita*, *O. foetida* and *O. hederae*) or strigol (*O. densiflora*). Therefore, it is difficult to relate patterns of the responsiveness to GR24 with the weedy or non-weedy status or the level of host specialization of broomrapes. GR24 is a synthetic strigolactone, which has proven to be very useful for germination testing (Mangnus *et al.*, 1992; Rubiales *et al.*, 2004) but is not present in root exudates of any plant species. It is the presence or absence of natural strigolactones, or their combination with other possible metabolites, having synergistic or antagonistic activity to strigolactones, that plays a role in host specialization.

*Orobanche ballotae* is a non-weedy parasitic plant which has only recently been separated from *O. minor* (Pujadas-Salvá, 1997). Both *O. ballotae* and *O. minor* responded to GR24 strongly. However, they responded differently to fabacyl acetate. Similarly, Thorogood *et al.* (2009) found that *O. minor* subsp. *maritima* differed from *O. minor* in capacity to respond to GR24. This suggests that host specificity in terms of seed germination may occur even between different taxa within a single species.

*Phelipanche schultzii* and *O. alba* are non-weedy species with a narrow host spectrum that responded slightly to, and only at the higher concentrations of, the three strigolactones studied here. Similarly, recent results in our laboratory suggest that other non-weedy, host-specific species, such as *O. clausonis*, *O. gracilis* and *O. rapum-genistae*, do not geminate in the presence of any of these strigolactones (Fernández-Aparicio, unpublished). It seems that the recognition requirements in these non-weedy species have evolved specifically for other stimulants not identified to date.

The mechanism of host recognition by each broomrape species, which is required for eliciting seed germination, might have been specialized to different combinations of strigolactones and their concentrations present in each host root exudate. Roots might exude a mixture of substances, some being stimulative and others being inhibitory to seed germination (Whitney, 1978; El-Halmouch *et al.*, 2006). To further substantiate this, various new germination stimulants differentially affecting germination of seeds of different *Orobanche* species have been isolated from pea root exudates (Evidente *et al.*, 2009, 2010), some of which might play a significant role in host specialization in addition to fabacyl acetate, previously reported in pea exudates (Xie *et al.*, 2009). Similarly, recent studies resulted in identification of both stimulants and inhibitors of *Orobanche* and *Phelipanche* seeds in separate chromatographic fractions of fenugreek root exudates (Evidente *et al.*, 2007; Fernández-Aparicio *et al.*, 2008a). Specialization could therefore be mediated by unique combinations and concentrations of signalling chemicals that are synergistic or antagonistic to strigolactone action.

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