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Stimulation of Seed Germination of *Orobanche* Species by Ophiobolin A and Fusicoccin Derivatives

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Various *Orobanche* species (broomrapes) are serious weed problems and cause severe reduction on yields in many important crops. Seeds of these parasitic weeds may remain dormant in the soil for many years until germination is stimulated by the release of a chemical signal by roots of a host plant. Some fungal metabolites, such as ophiobolin A and fusicoccin derivatives, were assayed to determine their capacity to stimulate the seed germination of several *Orobanche* species. The results obtained showed that the stimulation of seed germination is species-dependent and also affected by the concentration of the stimulant. Among ophiobolin A, fusicoccin, and its seven derivatives, tested in the concentration range of $10^{-4}-10^{-7}$ M, the highest stimulatory effect was observed for ophiobolin A and the hexacetyl and pentacetyl isomers of 16-*O*-demethyl-de-*tert*-pentenylfusicoccin prepared by chemical modification of the fusicoccin, while the other fusicoccin derivatives appeared to be practically inactive. The most sensitive species appeared to be *O. aegyptica*, *O. cumana*, *O. minor*, and to a lesser extent, *O. ramosa*.

KEYWORDS: Broomrape; *Orobanche*; seed germination; ophiobolin A; fusicoccin derivatives

INTRODUCTION

*Orobanche*spp. (broomrapes) are holoparasitic plants that have lost their autotrophic way of life. This genera comprises together 170 species distributed predominantly in the Northern Hemisphere (*1*) and has adapted to obtain its organic and inorganic resources by parasitizing the roots of a range of plant species mainly in wild ecosystems. They are responsible for major losses to vegetable, legume, and sunflower crops by interfering with water and mineral intake and affecting photosynthate partioning (*2, 3*). *Orobanche* species vary in their host specificity. Most species have a rather narrow host range, as *O. densiflora* Salzm. ex Reut., *O. gracilis* Sm., and *O. hederae* Duby., which are highly specialized, parasitizing few wild species in nature. However, a few species of broomrapes have become weedy, adapting to parasitize crops in agricultural environments. These are usually more generalists. Species such as *O. aegyptiaca* (Pers.) (syn. *Phelipanche aegyptiaca*), *O. crenata* Forsk., *O. minor* Sm, and *O. ramosa* (L.) Pomel (syn. *P. ramosa*) are know to parasitize a wide range of crops since antiquity (*4, 5*). However, others are far more specific, such as *O. cumana* Wallr., parasitizing only sunflower (*2, 3*), and *O. fetida* Poir that parasitizes many wild species of Leguminosae (*6*) and only recently has been reported as weedy on fava bean (*7*) and vetch (*8*).

Because of its unusual life cycle and the total dependence by the host, traditional control methods very often are impractical. The use of herbicides is not easy because of their economical or ecological unfeasibility or lack of tolerance to the herbicides in some crops, which might be overcome by the use of transgenic crops with target-site herbicide resistance.

Biological control is considered an attractive approach for broomrape control. Plant pathogens have also been proposed as a source of natural herbicides because they produce many toxic metabolites (*9, 10*).

Considering that the seed germination of parasitic plants depends upon the presence of stimulating exudates produced by the roots of the host plant, an alternative approach for the management of parasitic host plants is the so-called "suicidal germination". This latter consists in the induction of seeds germination by the application of a germination stimulant to the soil, in the absence of host. The parasite seeds germinate but, in the absence of the host, will die in few days, resulting in a reduction of seed bank.

Much attention has therefore been focused on the isolation and identification of germination stimulants (*11*), including the metabolites recently isolated from the fenugreek (*Trigonella foenum-graecum* L.) root exudates (*12, 13*).

Among several fungal metabolites tested with the aim of finding new natural stimulants, Yoneama and co-authors (*14*) reported that cotylenins and fusicoccins (FCs) induced high seed germination (>50%) of *S. hermonthica* (Del.) Benth and *O. minor*, at concentrations as low as 10^{-5} M.

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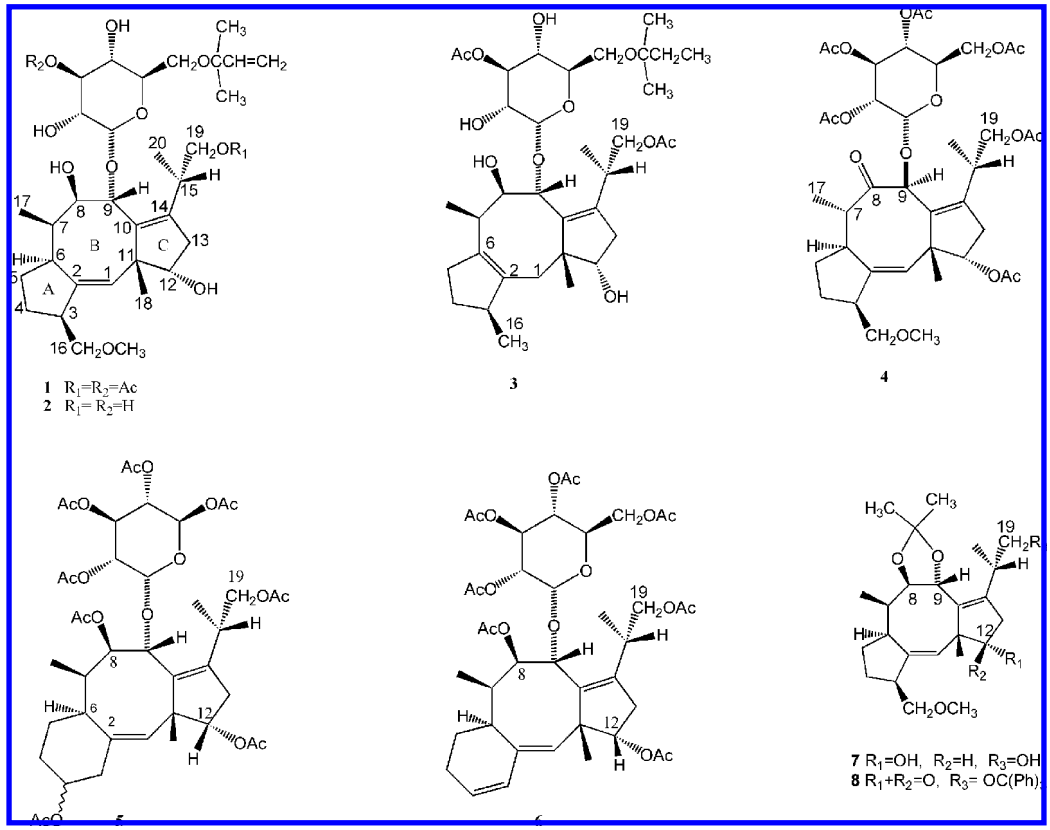

Figure 1. Structures of fusicoccin (**1**), some of its derivatives (**2**−**6**), and fusicoccin deacetyl aglycone derivatives (**7** and **8**).

FC is the major carbotricyclic phytotoxic diterpenoid produced by *Fusicoccum amygdali* Delacr., the causative fungal agent of peach and almond canker, isolated in 1962 and structurally described in 1968. Many studies were carried out on the chemical, biosynthetic, and biological properties of this toxin and structure−activity relationships (SARs) (*15*). Ophiobolins are sesterterpenoid phytotoxins closely related to fusicoccins and cotylenins and are produced by the pathogenic fungi *Bipolaris* species, which usually infect rice, maize, and sorghum. Recently, ophiobolin A, 6-*epi*-ophiobolin A, 3-anhydro-6-*epi*-ophiobolin, and ophiobolin I (*16*) were isolated together with ophiobolins B and J and the new ohphiobolins E and 8-*epi*-ophiobolin J (*17*) as phytotoxic metabolites with potential herbicide activity from the liquid culture of *Drechslera gigantea*, proposed as a potential mycorbicide of large crabgrass weed (*Digitaria sanguinalis*). Many studies were carried out on the chemical and biological properties of various ophiobolins (*18*).

The efficacy of fusicoccin in stimulating seed germination of parasitic plants was previously reported (*15, 19*), and considering the availability of several derivatives and natural analogues of fusicoccin and its aglycone, as well as cotylenol, because of previous works on the purification and identification of those compounds in our laboratory, we decided to carry out a structure−activity study using the seeds of another parasitic plant species, *O. ramosa*, which proved to be useful in a preliminary screening. Some of the compounds, tested at a concentration of 10^{-4} and 10^{-5} M, proved to be highly active, with 8,9-isopropylidene of the corresponding fusicoccin aglycone and dideacetyl derivative being the most active FC derivatives. In both groups of glucosides and aglycones (including cotylenol), the most important structural feature to impart activity appears to be the presence of the hydroxyl group at C-19 (*20*).

Considering these results and that of the FC efficacy in stimulating seed germination of parasitic plant could be species-dependent, we decided to carry out a study testing the effect of some FC derivatives and ophiobolin A on seed germination of different *Orobanche* species, namely, *O. aegyptiaca*, *O. ramosa*, *O. crenata*, *O. cumana*, *O. densiflora*, *O. fetida*, *O. gracilis*, *O. hederae*, and *O. minor*.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 600 MHz in $CDCl_3$, on a Bruker spectrometer of the Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy. The same solvent was used as an internal standard. Analytical and preparative thin-layer chromatography (TLC) were performed on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) plates; the spots were visualized by exposure to UV radiation and/or by spraying with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in methanol, followed by heating at 110 °C for 10 min. Column chromatography was carried out on silica gel (Merck, Kieselgel 60, 0.063−0.20 mm).

Plant Material. The weedy species assayed were *O. aegyptiaca*, whose seeds were collected from plants parasitizing chickpea in Israel, *O. crenata* collected on fava bean in Spain, *O. cumana* collected on sunflower in Spain, *O. fetida* collected on fava bean in Tunisia, *O. minor* collected on red clover in Chile, and *O. ramosa* collected on tobacco in Spain. Additionally, some nonweedy species were included for comparisons: *O. densiflora* collected on *Lotus creticus* in Spain, *O. gracilis* collected on *Retama monogyna* in Spain, and *O. hederae* collected on ivy in France.

Capsules were air-dried and opened, allowing for seed extrusion. The material was then sifted through thin sieves to separate seeds from other vegetable residues, and finally clean seeds were collected and stored in plastic vials at 5 °C until their use.

Chemical. Fusicoccin (**1**, **Figure 1**) was produced by *Fusicoccum amygdali* as reported by Ballio et al. (*21*). The crystalline sample of **1** obtained as previously reported (*22*) preserved at −20 °C under dark for about 26 years showed by TLC [eluent $CHCl_3$/*iso*-PrOH (9:1)] and ^{1}H NMR analyses the presence of some minor alteration products, which probably are the well-known isomer formed by the shift of the acetyl group from the C-3 to C-2 and C-4 of the glucosyl residue,

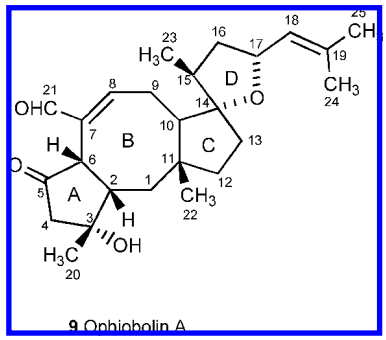

Figure 2. Structure of ophiobolin A (**9**).

respectively (*allo*- and *iso*-FC) (*23*), of the sugar moiety. The sample was purified by column chromatography [eluent $CHCl_3$/*iso*-PrOH (9:1)]. The corresponding dideacetyl derivative (**2**, **Figure 1**) was prepared by alkaline hydrolysis of **1** according to the procedure previously reported (*24*) and purified by preparative TLC [eluent $CHCl_3$/*iso*-PrOH (4:1)]. The sample purity of **1** and **2** were checked by TLC and ^{1}H NMR analysis.

The other FC derivatives and analogues, whose purity was ascertained by TLC and ^{1}H NMR, were prepared according to previous methods, as follow: **3** (*25*), **4** (*26*), **5**, **6** (*27*), **7** (*22*), and **8** (*28*) (**Figure 1**). Ophiobolin A (**9**, **Figure 2**) was obtained from the purification of the ethyl acetate extract of *Drechslera gigantea*. The crude ophiobolin A obtained from the chromatographic fraction column was crystallized 3 times with ethyl acetate/*n*-hexane (1:5) and gave the main metabolite (25 mg/L) as a white crystal, as recently reported (*16*) The sample purity was checked by TLC and ^{1}H NMR analysis.

Seed Germination Tests. The stimulatory activity of ophiobolin, fusicoccin, and its derivatives on the germination of nine broomrape species was tested *in vitro* at concentrations of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M. Seeds of nine bromrapes, *O. aegyptiaca*, *O. crenata*, *O. cumana*, *O. densiflora*, *O. fetida*, *O. gracilis*, *O. hederae*, *O. minor*, and *O. ramosa*, were sterilized with formaldehyde and spread over a 2 cm diameter disk of glass fiber filter paper (GFFP, Whatman GF/A) at a density of 50 seeds/cm^2 (*13*). Three replicate discs per compound were prepared with each *Orobanche* species. The GFFP discs containing the seeds were individually placed in small Petri dishes (6 cm in diameter) and moistened with 250 μL of sterile distilled water. The dishes were placed in the dark at 20 EC for 10 days to break the dormancy of broomrape seeds.

For bioassays, each compound was dissolved in methanol, then solution-diluted with sterile distilled water to a final concentration of the alcohol at 0.7%, and applied to each GFFP disk carrying the conditioned seeds of *Orobanche*. The synthetic germination stimulant GR24 (*29*) was used as a positive control at 10 ppm. To allow valid comparisons, 0.7% methanol was also added to GR24 dilution. As a negative control, a solution of methanol at 0.7% in sterile distilled water was included in the experiment.

After treatment, dishes containing the discs were maintained in the dark at 20 EC for 7 days. At this stage, 100 broomrape seeds per disk were studied under a stereoscopic microscope at 30× magnification to determine the percentage of germination. Seeds with an emerged radicle were scored as germinated.

Statistical Analysis. Data were approximated to normal frequency distribution by means of angular transformation, and analysis of variance (ANOVA) was conducted using SPSS 15.0 on the percentage of broomrape germination observed, with broomrape species, the inductor effect performed by each compound, concentration at which each compound was applied, and their interaction as factors.

RESULTS AND DISCUSSION

In this SAR study, a total of nine compounds were used, with six of them fusicoccin glucosides, two fusicoccin aglycones, and ophiobolin A (**9**, **Figure 2**). In particular, beside fusicoccin (**1**, **Figure 1**), the following compounds were tested for their capacity to stimulate the seed germination of *Orobanche* species: the glucoside derivatives **2**−**6** (**Figure 1**) prepared from fusicoccin by "ad hoc" chemical modification, the 8,9-isopropylidene derivative of fusicoccin deacetyl aglycone (**7**, **Figure 1**), which was prepared by chemical degradation of the sugar moiety of **1** and its 19-*O*-trytil-12-oxo derivative (**8**, **Figure 1**), and ophiobolin A isolated from liquid culture filtrates of *Drechslera gigantea*.

The *Orobanche* species used were *O. aegyptica*, *O. crenata*, *O. cumana*, *O. densiflora*, *O. fetida*, *O. gracilis*, *O. hederae*, *O. minor*, and *O. ramosa*. A positive germination control was obtained by stimulating the seed germination of all of the species using the synthetic stimulant GR24 (*29*) and adding 0.7% of methanol, which is the final concentration of this solvent present in the solution of the compound tested. A negative control using sterile distilled water with 0.7% methanol was also used.

The results reported in **Figure 3** showed that GR24 at the concentrations tested has a high stimulatory activity (inducing 55−90% seed germination) of *O. crenata*, *O. cumana*, *O. minor*, *O. aegyptiaca*, and *O. ramosa* but not of *O. densiflora*, *O. fetida*, *O. gracilis*, or *O. hederae*. As expected with the negative control, practically all of the species did not show any germination.

The compounds were tested in the concentration range of 10^{-4}−10^{-7} M. The results of the bioassays are reported in **Figure 4**. There were significant differences in the broomrape germination because of the broomrape species tested (ANOVA, $p < 0.001$) to the compound tested (ANOVA, $p < 0.001$) and to the concentration used (ANOVA, $p < 0.001$) and their second- and third-order interactions (ANOVA, $p < 0.001$).

Practically, only *O. aegyptiaca*, *O. ramosa*, *O. cumana*, and *O. minor* were stimulated up to 50% in the range of the concentration tested but only by the fusicoccin derivatives **5** and **6** and ophiobolin A (**9**). The other fusicoccin derivatives were inactive, except the 8,9-isopropylidene derivative of fusicoccin deacetyl aglycone (**Figure 4A**) that showed a relatively high stimulation on *O. minor* of about 35%. Compound **7** was assayed at a higher concentration (10^{-7} M) and on the same species, and other ones were practically inactive, inducing at maximum at 10% of stimulation against the 55% of stimulation previously observed on *O. ramosa* (*20*).

Our results on response of *O. minor* and *O. ramosa* to fusicoccin and its derivatives differed slightly to previous reports (*14, 20*). We found fusicoccin to be a little active on *O. minor* and *O. ramosa*, whereas Yoneyama et al. (*14*) found high stimulation (56−86%) when assayed at 10^{-5} and 10^{-4} M on *O. minor* and medium (37 and 25%) on *O. ramosa* (*20*).

The most active fusicoccin derivatives **5** and **6**, which are differently acetylated isomers of the 16-*O*-demethyl-de-*tert*-pentylfusicoccin, were prepared by chemical modification of fusicoccin by the reaction with a Fritz and Shenck reagent normally used for the acetylation of the highly hindered hydroxy group (*30*). The structural modification induced by this reaction was essentially the cleavage of the ether bond and the expansion of the cyclopentane ring A to the cyclohexane or cyclohexene ring as can be observed in **5** and **6**, respectively. Derivative **5** (**Figure 4C**) showed the same stimulatory effect (about 20%) on *O. aegyptiaca*, *O. cumana*, and *O minor* in the concentration range of 10^{-4}−10^{-6} M, which rapidly decreased at 10^{-7} M. *O. ramosa* showed a lesser extent of germination with a maximum of <10% at 10^{-5} M, which agree with the results previously observed (*20*). Derivative **6** (**Figure 4D**) showed a differentiate activity that is species- and concentration-dependent. The highest stimulatory effect was observed on *O. aegyptiaca*, whose germination increased from 20% at 10^{-4} M at up to <50% at 10^{-5} M and rapidly decreased with the decrease of the

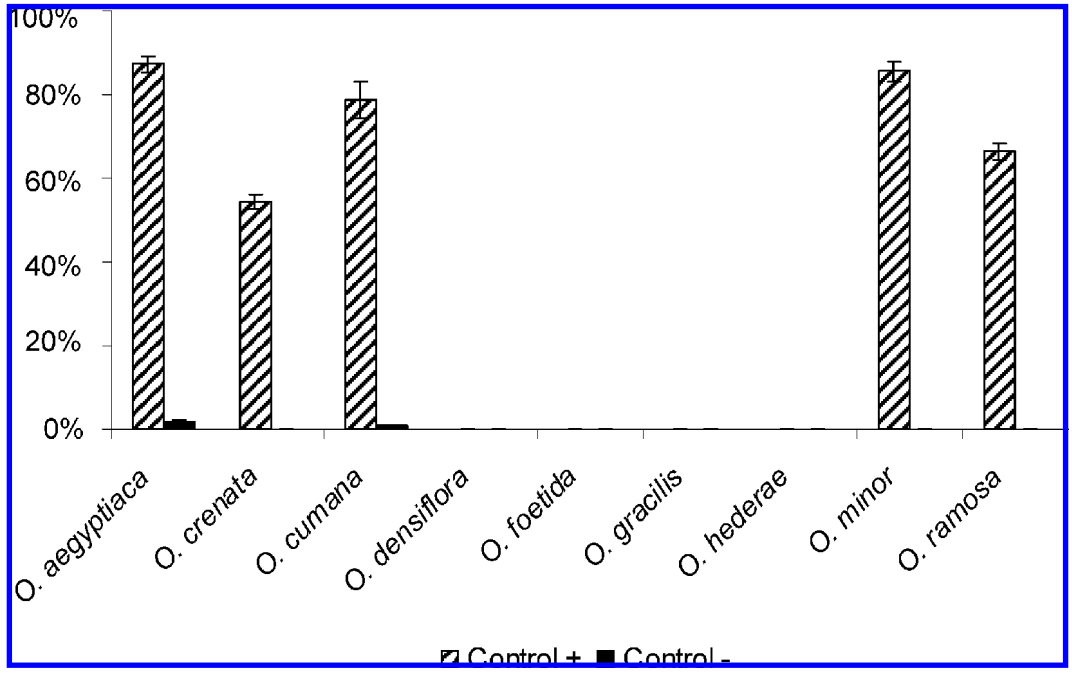

Figure 3. Percentage of germination of *O. aegyptiaca*, *O. crenata*, *O. cumana*, *O. densiflora*, *O. fetida*, *O. gracilis*, *O. hederae*, *O. minor*, and *O. ramosa* seeds induced by the positive treatment control (GR24) and the negative treatment control (sterile distilled water). Error bars represent ±2 standard error (SE).

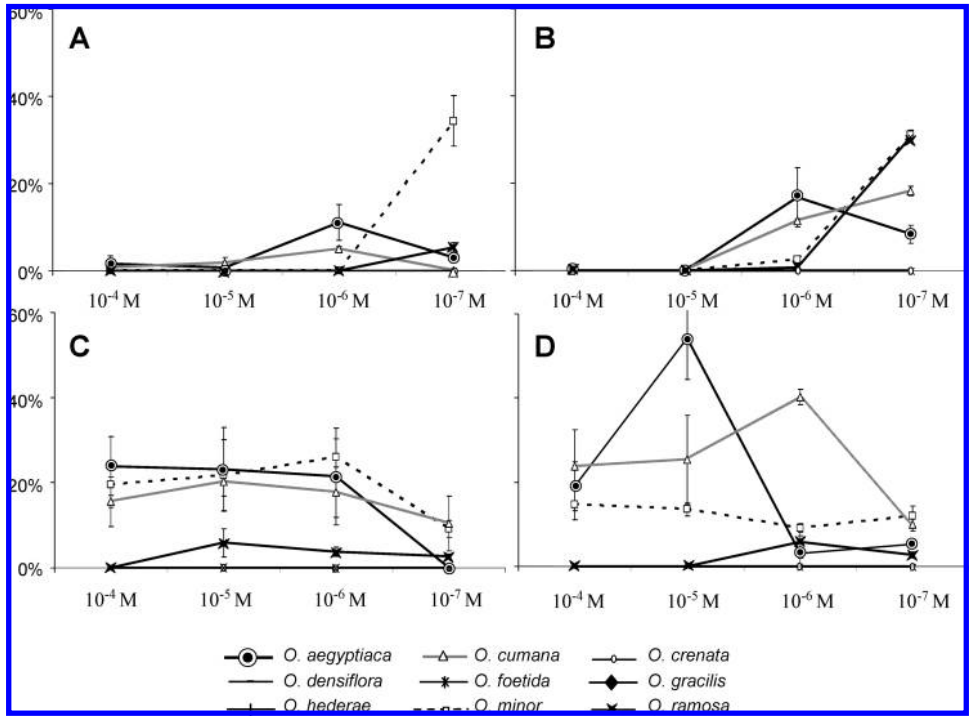

Figure 4. Percentage of germination of *O. aegyptiaca*, *O. crenata*, *O. cumana*, *O. densiflora*, *O. fetida*, *O. gracilis*, *O. hederae*, *O. minor*, and *O. ramosa* seeds induced by (A) 8,9-isopropylidene derivative of FC aglycone, (B) ophiobolin A (**9**), (C) FC derivative **5**, and (D) FC derivative **6**. Error bars represent ±2 SE.

concentration. *O. cumana* showed a similar 10% of germination at 10^{-4} M and increased up to 40% at 10^{-6} M and then rapidly decreased with the decrease of the concentration. *O. minor* showed a similar 10−17% germination at the concentrations assayed. Finally, *O. ramosa* was practically not stimulated up to 10^{-5} M and a little stimulated (<10%) at higher concentrations. In agreement with this result, a low stimulatory effect (10%) was also previously observed, testing **6** on the same species.

The results observed with both **5** and **6** are remarkable because the chemical modification induced on the cyclopentane ring A consequently determines a strong modification of the conformation of the carbotricyclic ring, which is an important feature to impart activity to fusicoccin as previously demonstrated in some SAR studies (*15, 31, 32*).

Similarly, ophiobolin A (**9**) induced (**Figure 4B**) a stimulation depending upon the broomrape species and the concentration. No stimulation was observed at a concentration less than 10^{-5} M for *O. aegyptiaca* and *O. cumana*, while for the other two species *O. minor* and *O. ramosa*, the stimulation started at 10^{-6} M. For the first two species, the stimulation increased up to 10^{-6} M and then rapidly decreased with the decrease of the concentration for *O. aegyptiaca*, while for *O. cumana*, a linear

increasing was inversely observed with respect to the concentration. For *O. minor* and *O. ramosa*, the stimulation rapidly increased with the decrease of the concentration.

The results obtained with ophiobolin A did not surprise us, because it is a sesterterpene structurally close to fusicoccin sharing the same carbotricyclic ring, although differently functionalized. The biological activity of ophiobolin A (*18*) resembles in part that of FC essentially for the phytotoxicity, but at relatively low concentrations (10^{-6}−10^{-8} M), it could have, as fusicoccin, a hormone-like activity (*15*).

In conclusion, the bioactive metabolites fusicocin and ophiobolin A appeared to have a different biological activity when tested at different concentrations. *Orobanche* species showed a different sensitivity toward the different compounds and the concentrations tested. The fusicoccin derivatives **5** and **6** and ophiobolin A could represent a potential herbicide in view of their practical application in agriculture for the biocontrol of parasitic *Orobanche* species. It could also be interesting to assay some natural analogues and derivatives of ophiobolin A, prepared by chemical modification of **9**, to carry out a SAR study aimed to find a compound with an increased and modulated stimulant activity against the different *Orobanche* species but essentially with the ability to stimulate those ones that showed resistance to natural and synthetic stimulants.

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