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# Induction of Haustorium Development by Sphaeropsidones in <sup>2</sup> Radicles of the Parasitic Weeds Striga and Orobanche. A Structure-**Activity Relationship Study**

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12 ABSTRACT: Crop attack by parasitic weeds such as Striga and Orobanche occurs through developmental processes triggered by 13 host chemodetection. Seeds of those weed species remain dormant in the soil until germination is triggered by host root exudates. The development of haustorium, a parasitic plant organ that invades the host to withdraw its nutrients, is also initiated 14 in Orobanchaceae by host molecular cues. The induction of haustorium development by exogenous signals has previously been 15 reported for Striga but not for Orobanche species. In this work, we demonstrate that sphaeropsidone and epi-sphaeropsidone, two 16 phytotoxic cyclohexene oxides isolated from the fungus Diplodia cupressi, a causal agent of cypress canker, induce haustorium 17 development in radicles of the parasitic weeds Striga hermonthica, Orobanche crenata, and Orobanche cumana. This is the first 18 report of chemical stimulation of haustorium development in radicles of Orobanche in the absence of host. In addition, SAR 19 studies were carried out by testing the haustorium-inducing activity of the natural cyclohexene oxides, seven already known and 20 four new hemisynthetic derivatives, in O. cumana, O. crenata, and S. hermonthica, to find a molecular specificity model required 21 22 for haustorium induction. The results suggested that the haustorium-inducing activity is due to the possibility to convert the natural sphaeropsidone and natural and hemisynthetic derivatives in the corresponding 4-methoxyquinone and that the 23 stereochemistry at C-5 also seems to affect this activity. 24

KEYWORDS: Striga hermonthica, Orobanche crenata, Orobanche cumana, cyclohexene oxides, attachment organ, 25

26 haustorium-inducing factor

#### 27 INTRODUCTION

28 Root parasitic plants have evolved to exploit another plant root 29 system to fulfill their water and nutrient requirements. Two 30 main types of parasitic plants can be recognized regarding their 31 photosynthetic status: hemiparasitic plants perform some 32 degree of photosynthesis but are dependent on the host mainly 33 for water and inorganic nutrients, while holoparasitic plants lack 34 of photosynthetic machinery and in the nutrient diversion from 35 the host they include all their required reduced carbon. 36 Hemiparasitic plants are also classified in obligated or 37 facultative parasites depending on their ability to develop an 38 autotrophic way of life in the absence of a suitable host.<sup>1</sup> Plants 39 from up to 28 dicotyledonous families use the plant-parasitic 40 strategy to obtain competitive advantage from neighboring host 41 plants.<sup>2</sup> In all of them, the core feature of their parasitic strategy 42 is a specialized host-invasive organ called haustorium that 43 develops sequential functions of host attachment, penetration, 44 and connection with host vascular tissues.<sup>3,4</sup> At least in one 45 family of parasitic plants, the Orobanchaceae, the haustorium 46 development is initiated by host-derived metabolites.<sup>5</sup> Some 47 members of this family, i.e., the hemiparasitic plants Striga spp. 48 and the holoparasitic plants Orobanche spp., are weeds to which 49 despite their high negative economic impact in agriculture there

is not effective and or practicable control and thus the 50 development of innovative control strategies is urgent.

In vitro screenings of host-derived factors that initiate 52 developmental processes in parasitic plants are essential in 53 breeding programs for parasitic plant resistance.<sup>6</sup> Striga 54 haustorium can be monitored in vitro by exposing Striga 55 seedlings to inducing factors. Upon detection of haustorium- 56 inducing factors, the Striga seedling initiates haustorium 57 development and undergoes quick morphological changes 58 that prepare it for its first step in the host invasion process: 59 attachment to host root. Long haustorial hairs with functions of 60 adhesion and attachment develop from elongation of epidermal 61 cells surrounding the haustorium apex.<sup>4</sup> Despite the fact that 62 Striga and Orobanche haustoria have a common evolutionary 63 origin, fundamental differences exist at their host-preattached 64 haustorial stage. Morphologically, Orobanche haustorium is by 65 far less defined. It lacks haustorial hairs but rather it develops 66

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67 short secretory papillae with function of the adhesion surface.<sup>8</sup> 68 Another fundamental difference with *Striga* is that in *Orobanche* 69 species, the chemistry of haustorium initiation is completely 70 unknown. Several authors have suggested that *Orobanche* 71 species do not require host chemical factors for haustorium 72 development, which would make *Orobanche* the notable 73 exception in the Orobanchaceae.<sup>8–10</sup> In consequence, in vitro 74 assays of haustorium-inducing factors or haustorium-inducing 75 root exudates have been unfeasible in *Orobanche*, hampering 76 the screening for low-inducer genotypes in crop germplasm 77 collections.

78 Several phenols, flavonoids, and quinones have been 79 identified as factors inducing both terminal and lateral haustoria 80 in obligated hemiparasitic plants, i.e., *Striga hermonthica* and 81 facultative hemiparasitic plants, i.e., *Triphysaria versicolor*.<sup>4,13–15</sup>

<sup>82</sup> The Atsatt's hypothesis<sup>16</sup> that parasitic plants evolved to use <sup>83</sup> defense metabolites as host recognition signals is supported by <sup>84</sup> the fact that many haustorium-inducing factors are structurally <sup>85</sup> similar to phytotoxins produced by allelopathic plants. There is <sup>86</sup> a similar gene expression in parasitic roots when they are <sup>87</sup> exposed to host contact or allelochemicals.<sup>5,16</sup> The haustorium <sup>88</sup> development in hemiparasitic Orobanchaceae is under redox <sup>89</sup> control mediated by cycling between the reduced and oxidized <sup>90</sup> states of the haustorial-inducing compounds.<sup>17</sup> Similar models <sup>91</sup> in the quinone-mediated actions of both phytotoxic allelopathy <sup>92</sup> and haustorium initiation are proposed.<sup>5,10,18</sup>

93 Some fungal metabolites are able to interfere with weed 94 parasitism either by phytotoxic action on germination or 95 growth inhibition or by triggering developmental programs in a 96 suicidal fashion, i.e., in the absence of a host. These new 97 compounds constitute alternative candidate ingredients for the 98 development of new herbicides. Parasitic weeds that can be 99 targeted include species of high economic important members 100 of *Orobanche, Phelipanche,* and *Striga* genera.<sup>11,12,19–22</sup>

f1

<sup>101</sup> Sphaeropsidone and *epi-sphaeropsidone* (1 and 2, Figure 1) <sup>102</sup> are two cyclohexene oxides isolated together, several



Figure 1. Structures of sphaeropsidone and *epi*-sphaeropsidone (1 and 2) and chlorosphaeropsidone and epichlorosphaeropsidone (3 and 4).

103 sphaeropsidins<sup>23</sup> as the main phytotoxins produced from 104 *Diplodia cupressi*, the causal agent of cypress (*Cupressus* 105 *sempervirens* L.) canker in the Mediterranean basin.<sup>24</sup> 106 Successively, two chlorinated cyclohexenenones, closely related 107 to 1 and 2 and named chlorosphaeropsidone and epichlorop-108 sphaeropsidone (3 and 4, Figure 1), were isolated from the 109 same fungal culture filtrates.<sup>25</sup> They have phytotoxic activity 110 causing necrosis and wilting in *Quercus* species and in tomato 111 and antifungal activity by inhibiting mycelial growth in species 112 of *Phytophthora*.<sup>26</sup>

III3 In a previous study, among fungal and plant phytotoxins, III4 sphaeropsidones were identified as metabolites able to induce III5 morphological changes in the *Orobanche* radicle that resemble 131

the attachment organ.<sup>11</sup> Because of the nuanced morphology of 116 Orobanche preattached haustorium and to the fact that no 117 chemicals with haustorium-inducing activity have been 118 previously identified for any Orobanche species, we have 119 included in the present work S. hermonthica as a living marker 120 for haustorium induction due to its very distinct haustorium 121 morphology. The present article confirms the haustorium- 122 inducing nature of sphaeropsidone activity and that it acts 123 across the parasitic weed genera Orobanche and Striga. 124 Haustorium-inducing assays of sphaeropsidones were per- 125 formed in O. cumana, O. crenata, and S. hermonthica. In 126 addition, structure-activity relationships were carried out by 127 assaying two natural analogues (3 and 4), seven already known 128 and four new sphaeropsidones derivatives<sup>26</sup> prepared by 129 chemical transformation of 1 and 2. 130

#### MATERIALS AND METHODS

General Experimental Procedures. IR spectra were recorded as 132 deposit glass film on a 5700 FT-IR spectrometer (Thermo Electron 133 Corp. Nicolet, Madison, WI, USA), and UV spectra were measured in 134 MeCN on a V-530 spectrophotometer (Jasco, Tokyo, Japan). <sup>1</sup>H and 135 <sup>13</sup>C NMR spectra were recorded at 400/100 or 500/125 MHz in 136 CDCl<sub>3</sub> on Bruker spectrometers (Karlsruhe, Germany). The same 137 solvent was used as internal standard. Carbon multiplicities were 138 determined by DEPT spectra.<sup>27</sup> ESI spectra were recorded on a 6230 139 TOF LC/MS instrument (Agilent Technologies, Milan, Italy). 140 Analytical and preparative TLC were performed on silica gel (Kieselgel 141 60, F<sub>254</sub>, 0.25 and 0.5 mm, respectively) plates (Merck, Darmstadt, 142 Germany). The spots were visualized by exposure to UV radiation 143 (253) or by spraying first with 10% H<sub>2</sub>SO<sub>4</sub> in MeOH and then with 144 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 145 10 min. Column chromatography was performed using silica gel 146 (Kieselgel 60, 0.063-0.200 mm) (Merck).

**Fungal Strains.** The *D. cupressi* strain used in this study was 148 purchased from Centraalbureau voor Schimmelcultures of Baarn 149 (Netherland). Pure culture was maintained on potato dextrose agar 150 (PDA) (Fluka, Sigma-Aldrich Chemic GmbH, Buchs, Switzerland) 151 and stored at 4  $^{\circ}$ C in the collection of Dipartimento di Agraria, 152 Università di Sassari, Italy, as 261.85 CBS. The fungus was grown as 153 previously reported<sup>26</sup> 154

Extraction and Isolation of Sphaeropsidones. One-week-old 155 colonies of the fungus on PDA in 9 cm Petri dish were cut off and 156 blended for 30 s with 100 mL of sterile distilled water to prepare the 157 inoculum for liquid fermentation. Each 2 L Erlenmeyer flask 158 containing 400 mL of modified Czapek medium supplemented with 159 2% corn meal (pH 5.7) was seeded with 5 mL of the mycelial 160 suspension and then incubated at 25 °C for 4 weeks in darkness. The 161 culture filtrates (15 L) were acidified and extracted exhaustively with 162 EtOAc as previously reported.<sup>24</sup> The organic extract, obtained as a 163 brown-red oil (9.2 g), was chromatographed on a silica gel column 164 (110 cm  $\times$  5 cm) eluted with 3 L of CHCl<sub>3</sub>/*i*-PrOH (9:1, v/v), 165 affording nine groups of homogeneous fractions. The residues (3.6 g) 166 of fractions 4-7 were combined and further purified by a silica gel 167 column (90 cm  $\times$  4 cm) eluted with 2.5 L of CHCl<sub>3</sub>/*i*-PrOH (9:1, v/ 168 v), yielding six groups of homogeneous fractions. The residue of 169 fraction 3 was crystallized from EtOAc/n-hexane (1:5, v/v), yielding 170 sphaeropsidone, 1 (R<sub>f</sub> 0.40, 2.3 g, 153.3 mg/L) (Figure 1), as white 171 needles. The mother liquors were further purified by column 172 chromatography on silica gel (75 cm  $\times$  3 cm) eluted with 1 L of 173 petroleum ether-Me<sub>2</sub>CO (7:3, v/v), affording epi-sphaeropsidone, 2 174  $(R_{\rm f} 0.53, 725 \text{ mg}, 48.3 \text{ mg/L})$  (Figure 1), as a homogeneous oil. 175 Chlorosphaeropsidone, 3, and epichlorosphaeropsidone, 4 (Figure 1), 176 were purified from the same culture filtrate, and the purification 177 procedures for these compounds have been described previously.<sup>25</sup>

**Preparation of Sphaeropsidones Derivatives (5–11).** The 5- 179 O-acetyl, 5, 2,4,5-triacetylanisole, 6, 1,4-dione, 7, bromohydrin, 8, 1,4- 180 diol, 9, and 2,4-dihydroxycyclohexanone, 10, derivatives of sphaerop- 181 sidone, 1 (Scheme 1) as well as the 5-O-acetylderivative, 11, of epi- 182 s1s2 Scheme 1. Synthesis of Sphaeropsidone Derivatives  $(5-10, 12, \text{ and } 14)^a$ 



"Reagents and conditions: (a) Ac<sub>2</sub>O, pyridine, 80 °C; (b) MnO<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>, rt; (c) Li<sub>2</sub>NiBr<sub>4</sub>, THF, rt; (d) NaBH<sub>4</sub>, MeOH, rt; (e) H<sub>2</sub>, Pd 10%, MeOH, rt; (f) CH<sub>3</sub>CN, DMAP, BrC<sub>6</sub>H<sub>4</sub>COCl, rt; (g) CH<sub>2</sub>Cl<sub>2</sub>, pyridine, DCC, C<sub>5</sub>H<sub>9</sub>N<sub>3</sub>O<sub>2</sub>, 0 °C 1 h and rt 2 h.

183 sphaeropsidone, **2** (Scheme 2), were prepared according to the 184 procedures previously reported.<sup>26</sup>

s2

Scheme 2. Synthesis of *epi*-Sphaeropsidone Derivatives (11, 13, and 15)<sup>*a*</sup>



<sup>*a*</sup>Reagents and conditions: (a) Ac<sub>2</sub>O, AcONa, 80 °C; (b) NaBH<sub>4</sub>, MeOH, rt; (c) CH<sub>2</sub>Cl<sub>2</sub>, pyridine, DCC, C<sub>3</sub>H<sub>9</sub>N<sub>3</sub>O<sub>2</sub>, 0 °C 1 h and rt 2 h..

**5-O-p-Bromobenzoyl Ester of Sphaeropsidone (12).** Sphaer-186 opsidone, 1 (5 mg), was dissolved in CH<sub>3</sub>CN (0.5 mL), and DMAP 187 (4-dimethylaminopyridine) (10 mg) and *p*-bromobenzoyl chloride (10 188 mg) were added. The reaction mixture was stirred at room 189 temperature for 4 h and then evaporated under reduced pressure. 190 The residue (10.0 mg) was purified by TLC on silica gel eluted with 191 CHCl<sub>3</sub>/*i*-PrOH (95:5, v/v), giving derivatives **12** (3.65 mg, *R*<sub>f</sub> 0.78) 192 (Scheme 1, as uncolored oil. **12** had: UV λ<sub>max</sub> (log ε) 250 (4.07) nm. 193 IR ν<sub>max</sub> 1728, 1670, 1618, 1590 cm<sup>-1</sup>. <sup>1</sup>H NMR, δ: 8.01 (d, *J* = 8.7 Hz, H-2' and H-6'), 7.63 (d, J = 8.7 Hz, H-3' and H-5'), 6.19 (d, J = 2.5 194 Hz, H-5), 5.35 (br s, H-3), 3.87 (dd, J = 4.3 and 2.5, H-6), 3.71 (s, 195 OMe), 3.54 (dd, J = 4.3 and 1.7, H-1). ESIMS (+) m/z 364 [M + 2 + 196 Na]<sup>+</sup>, 362 [M + Na]<sup>+</sup>, 342 [M + 2 + H]<sup>+</sup>, 340 [M + H]<sup>+</sup>. 197

**5-O-5'**-**Azidopentanoyl** Ester of Sphaeropsidone (14). To 198 compound 1 (5.0 mg) dissolved in anhydrous  $CH_2Cl_2$  (4.5 mL) and 199 pyridine (100 mL) were added DCC (N,N'-dicyclohexylcarbodiimide) 200 (5 mg) and 5-azidopentanoic acid (20  $\mu$ L). The reaction was left at 0 201 °C for 1 h and then at room temperature for 2 h. The reaction was 202 stopped by evaporation under N<sub>2</sub>. The residue (10 mg) was purified 203 by TLC on silica gel eluted with CHCl<sub>3</sub>/*i*-PrOH (95:5, v/v), giving 204 derivatives 14 (7.90 mg,  $R_f$  0.74) (Scheme 1), as uncolored oil. 14 had: 205 UV  $\lambda_{max}$  (log  $\varepsilon$ ) 253 (3.51) nm. IR  $\nu_{max}$  2100, 1746, 1672, 1618 cm<sup>-1</sup>. 206 <sup>1</sup>H NMR, 5.97 (d, J = 2.9 Hz, H-5), 5.30 (br s, H-3), 3.76 (dd, J = 4.0 207 and 2.9, H-6), 3.70 (s, OMe), 3.50 (dd, J = 4.0 and 1.4, H-1), 3.32 (t, J 208 = 6.5 Hz, CH<sub>2</sub>-5'), 2.53 (t, J = 7.3 Hz, CH<sub>2</sub>-2'), 1.83–1.76 (m, CH<sub>2</sub>- 209 3'), 1.71–1.64 (m, CH<sub>2</sub>-4'). ESIMS (+) m/z 304 [M + Na]<sup>+</sup>, 282 [M 210 + H]<sup>+</sup>.

**NaBH**<sub>4</sub> **Reduction of** *epi-Sphaeropsidone* (13). To *epi-* 212 Sphaeropsidone, 2 (5 mg), dissolved in MeOH (15 mL), was added 213 NaBH<sub>4</sub> (5 mg) under stirring at room temperature for 30 min. The 214 mixture was neutralized with 0.1 M HCl, extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 215 30 mL) and dried (Na<sub>2</sub>SO<sub>4</sub>). The oily residue was purified by 216 preparative TLC, using CHCl<sub>3</sub>–*i*-PrOH (9:1) for elution to give the 217 derivative 13 as white needles (3.45 mg,  $R_f$  0.37) (Scheme 2). 13 had: 218 UV  $\lambda_{max}$  (log  $\varepsilon$ ) < 220 nm. IR  $\nu_{max}$  3413, 1671 cm<sup>-1</sup>. <sup>1</sup>H NMR,  $\delta$ : 4.64 219 (br d, J = 10.4 Hz, H-2), 4.57 (br s, H-3), 4.39 (br s, H-5), 3.57 (s, 220 OMe), 3.53 (br s, H-6), 3.50 (br s, H-1), 2.25 (d, J = 4.4 Hz, HO- 221 C(5)), 1.84 (d, J = 10.4 Hz, HO-C(2)). ESIMS (+) *m*/*z* 181 [M + 222 Na]<sup>+</sup>, 159 [M + H]<sup>+</sup>.

**5-O-5'-Azidopentanoyl Ester of** *epi*-**Sphaeropsidone (15).** To 224 compound **2** (5.0 mg) dissolved in anhydrous  $CH_2Cl_2$  (4.5 mL) and 225 pyridine (100 mL) were added DCC (5.0 mg) and 5-azidopentanoic 226 acid (20  $\mu$ L). The reaction was left at 0 °C for 1 h and then at room 227 temperature for 2 h. The reaction was stopped by evaporation under 228 N<sub>2</sub>. The residue (10.0 mg) was purified by TLC on silica gel eluted 229 with CHCl<sub>3</sub>/*i*-PrOH (95:5, v/v) giving derivatives **15** (7.90 mg,  $R_f$  230

231 0.74) (Scheme 2) as uncolored oil. **15** had: UV  $\lambda_{max}$  (log  $\varepsilon$ ) 249 (3.48) 232 nm. IR  $\nu_{max}$  2098, 1749, 1671, 1624 cm<sup>-1</sup>. <sup>1</sup>H NMR, 5.92 (br s, H-5), 233 5.36 (br s, H-3), 3.70 (s, OMe), 3.59 (br s, H-6), 3.45 (br s, H-1), 3.31 234 (t, J = 6.5 Hz, CH<sub>2</sub>-5'), 2.45 (t, J = 7.2 Hz, CH<sub>2</sub>-2'), 1.80–1.72 (m, 235 CH<sub>2</sub>-3'), 1.68–1.71 (m, CH<sub>2</sub>-4'). ESIMS (+) m/z 304 [M + Na]<sup>+</sup>, 236 282 [M + H]<sup>+</sup>.

237 Seed Conditioning. The haustorium-inducing activity of sphaer-238 opsidones was tested on parasitic seedlings, and the starting material for the haustorium bioassays were parasitic seeds. Striga and Orobanche 239 240 seeds require chemical stimulation to germinate but prior they become 241 responsible to germination-inducing factors these seeds require a 242 period of warm stratification called conditioning.<sup>28</sup> To achieve that, O. 243 crenata, O. cumana, and S. hermonthica seeds were surface sterilized by immersion in 0.5% (w/v) NaOCl and 0.02% (v/v) Tween 20, were 244 sonicated for 2 min, rinsed thoroughly with sterile distilled water, and 245 dried in a laminar air flow cabinet. To allow seed conditioning, 246 247 approximately 100 seeds of each species were placed separately in 9 248 mm diameter glass fiber filter paper disks (GFFP) moistened with 50 249  $\mu$ L of sterile distilled water and placed inside Petri dishes in incubators 250 during 14 days at 30 °C for S. hermonthica and 10 days at 22 °C for O. 251 crenata and O. cumana.

Haustorium-Inducing Assay. A solution of the germination 252 253 stimulant  $GR24^{29}$  was prepared at  $10^{-6}$  M in sterile distilled water. 254 Immediately before use, stock solutions in methanol of the candidate 255 haustorial-inducing compounds as sphaeropsidone, 1, epi-sphaerop-256 sidone, 2, their natural analogues, 3 and 4, and hemisynthetic derivatives, 5-15, were diluted in the GR24 solution at five decreasing 2.57 concentrations of sphaeropsidone (100, 10, 1, 0.1, 0.01  $\mu$ M) on Striga 258 hermontica and three decreasing concentrations of sphaeropsidone 259 (100, 10, 1  $\mu$ M) on Orobanche spp. but keeping constant the 260 261 concentration of GR24 and methanol in order to allow comparisons. 262 Final concentration of methanol was adjusted to 0.70% (v/v). In these 263 conditions, the solutions of natural sphaeropsidones and derivatives 264 had a pH value of 6.5. GFFP disks containing the conditioned Striga 265 and Orobanche seeds were transferred inside a laminar flow cabinet to 266 sterile filter paper in order to remove the excess of water used for seed 267 conditioning and then transferred to new 10 cm sterile Petri dishes. Then, each disk was treated with a 50  $\mu$ L alignot of the respective test 268 269 solution. GR24 solution (containing 0.70% methanol) was used as 270 negative control. Seeds were incubated in the dark at 30 °C (S. hermonthica seeds) or at 22 °C (O. crenata and O. cumana seeds). 271 Because of the faster germination of Striga when compared with 272 Orobanche species, haustorium formation was examined at 3 days in S. 273 hermonthica and 6 days in O. crenata and O. cumana. Using a 274 275 stereoscopic microscope at 30× magnification, the percentage of 276 haustorium formation in emerged radicles of each species was 277 established.

**Statistical Analysis.** The bioassays were performed twice with three replicates. Percentage data were approximated to normal requency 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 21.0 (SPSS Inc., Chicago, Illinois, USA). The significant of mean differences between each treatment against the negative control was evaluated by the two-sided Dunnett test. Null hypothesis was rejected at the level of 0.05.

#### 286 RESULTS AND DISCUSSION

 $f_2$ 

287 Radicles of *O. crenata* and *O. cumana* did not develop 288 haustorium when treated with the negative control test solution 289 GR24 (Figure 2C,E). This finding is in agreement with 290 observations of radicles made by Cimmino et al.  $(2014)^{21}$  but 291 in contradiction with the generalized view suggesting that 292 radicle of *Orobanche* seedlings spontaneously initiate typical 293 attachment organs in the absence of exogenous signals.<sup>8–10</sup> *S.* 294 *hermonthica* radicles exposed to negative control also lacked 295 haustorial development (Figure 2A). The spontaneous develop-296 ment of terminal haustorium does not make biological sense for 297 obligated parasitic weeds such as *Orobanche* and *Striga* as it will



**Figure 2.** Haustorium formation in (A) *Striga hermonthica* seedlings exposed to negative control, (B) *S. hermonthica* seedlings exposed to sphaeropsidone, (C) *Orobanche crenata* seedlings exposed to negative control, (D) *O. crenata* seedlings exposed to sphaeropsidone, (E) *Orobanche cumana* seedlings exposed to negative control, (F) *O. cumana* seedlings exposed to sphaeropsidone.

threaten the success of host attack. Because the root meristem 298 does not resume its normal growth after terminal haustorium 299 development, a haustorium initiated before host contact 300 impedes successful host attack, leading to the death of the 301 parasitic seedling. In this work instead of spontaneous 302 haustorium development, specificity for the chemistry that 303 initiates terminal haustorium development was expected and 304 was confirmed for *Orobanche* as well as is the case for *Striga.*<sup>30</sup> 305

Sphaeropsidone and *epi*-sphaeropsidone, phytotoxins pro-  $_{306}$  duced by *D. cupressi*,<sup>26</sup> were strong inducers of haustoria in *S.*  $_{307}$  *hermonthica* radicles. Recognition of sphaeropsidones by  $_{308}$  radicles of *S. hermonthica* promotes a cessation in the growth  $_{309}$  of the parasitic radicle and the development of attachment  $_{310}$  organ at the radicle tips that is in agreement with the  $_{311}$  description of terminal haustorium made by Riopel and Timko  $_{312}$  (1995).<sup>4</sup> More than 80% of *S. hermonthica* radicles developed  $_{313}$  haustorium when exposed to sphaeropsidone at concentrations  $_{314}$  between 1 and 0.1  $\mu$ M or to *epi*-sphaeropsidone at  $_{315}$  concentration between 10 and 1  $\mu$ M (Figures 2B and 3). For  $_{316}$  figures 2B and 3).



Figure 3. Induction of *S. hermonthica* haustorium formation by sphaeropsidone, 1, *epi*-sphaeropsidone, 2, two natural analogues, 3 and 4, eight sphaeropsidones derivatives, 5–10, 12, and 14, and three *epi*-sphaeropsidone derivatives, 11, 13, and 15, tested in the range 0.01–100  $\mu$ M. \*Indicates differences at the 0.05 level compared with the control.



Figure 4. Inhibition of *S. hermonthica* germination by sphaeropsidone, 1, *epi*-sphaeropsidone, 2, two natural analogues, 3 and 4, eight sphaeropsidones derivatives, 5–10, 12, and 14, and three *epi*-sphaeropsidone derivatives, 11, 13, and 15, tested in the range 0.01–100  $\mu$ M. \*Indicates differences at the 0.05 level compared with the control.

317 each compound, decreased concentrations did not induce *S*. 318 *hermonthica* haustorium while higher concentrations showed 319 inhibition activity of *S. hermonthica* germination (Figure 4).

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Necrosis was not detected in S. hermonthica radicles during 320 the length of the experiment at any concentration. Both 32.1 phytotoxic and organogenic activities in radicles of parasitic 322 weeds has been previously observed for haustorium-inducing 323 factors.<sup>31</sup> Both active concentrations of sphaeropsidone, 1 and 324 0.1  $\mu$ M, induced similar rate of haustorium induction (85% of 325 326 radicles developed haustorium) (Figure 3). However, the 327 radicles grew longer before detecting the haustorium-inducing 328 signal at 0.1 than at 1  $\mu$ M, visualized by longer radicles 329 terminated in haustorium (data not shown). The same phenomenon was observed with epi-sphaeropsidone as S. 330 hermonthica radicles terminated in haustorium were longer 331 when treated at 1  $\mu$ M than at 10  $\mu$ M. This could be indicating 332 that the required time of exposure for signal detection and 333 haustorium development was indirectly related with the 334 compound concentration. Parasitic radicle responding later to 335 decreased concentrations of haustorium-inducing signal could 336 constitute a mechanism aimed to measure distance for host 337 338 attachment.<sup>30</sup> Several phenolic derivatives of plant origin have 339 been previously identified as haustorium-inducing factors for 340 Striga.<sup>4,15,30</sup> Many of those phytochemicals are commonly

found in the rhizosphere and play roles in chemical 341 communication with microbial organisms. Fungal phytotoxins 342 have been described previously with potential to trigger host- 343 dependent developmental programs in parasitic plants such as 344 germination<sup>20,21</sup> but never were related before to the chemistry 345 of haustorium initiation. Active concentrations of **1** and **2** 346 where similar to those performed by previously known 347 haustorium-inducing factors such as xenognosin A and B, 2,6- 348 dimethoxybenzoquinone, ferulic, vanillic, sinapic, and syringic 349 acids.<sup>15,30,32</sup> 350

Radicles of *O. crenata* and *O. cumana* responded to 351 sphaeropsidone and *epi*-sphaeropsidone with a cessation of 352 radicle elongation, the radicle tip became spherical, and crowns 353 of cell extensions in the form of papillae were observed at the 354 outer surface around the apex (Figure 2D,F). This observation 355 is in agreement with the description of anchoring device of 356 *Orobanche* haustoria made by Joel and Losner-Goshen (1994).<sup>8</sup> 357 According with *S. hermonthica* results, *epi*-sphaeropsidone was a 358 weaker *Orobanche* haustorium-inducer than sphaeropsidone. 359 These results may suggest that the haustorial activity of 360 sphaeropsidones is mediated by a similar mechanism in *Striga* 361 and *Orobanche*. The activity of both sphaeropsidones in 362 *Orobanche* radicles was slightly weaker than that observed in 363 *Striga* radicles. Haustorium was observed in *Orobanche* radicles 364

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Figure 5. Induction of *O. crenata* haustorium formation by sphaeropsidone, 1, *epi*-sphaeropsidone, 2, two natural analogues, 3 and 4, eight sphaeropsidones derivatives, 5–10, 12, and 14, and three *epi*-sphaeropsidone derivatives, 11, 13, and 15, tested in the range 1–100  $\mu$ M.. \*Indicates differences at the 0.05 level compared with the control.



Figure 6. Induction of *O. cumana* haustorium formation by sphaeropsidone, 1, *epi*-sphaeropsidone, 2, two natural analogues, 3 and 4, eight sphaeropsidones derivatives, 5–10, 12, and 14, and three *epi*-sphaeropsidone derivatives, 11, 13, and 15, tested in the range 1–100  $\mu$ M. \*Indicates differences at the 0.05 level compared with the control.

365 treated with sphaeropsidone at concentrations between 10 and  $\mu$ M and with *epi*-sphaeropsidone at concentrations between 366 1  $_{367}$  100 and 10  $\mu$ M (Figures 5 and 6). The induction of haustorium development by exogenous signals has never been documented 368 for Orobanche, and therefore our results constitute a break-369 through against the general belief that Orobanche does not 370 respond to exogenous stimulation in order to develop 371 haustoria.<sup>8–10</sup> Sphaeropsidone at 100  $\mu$ M did not inhibit 372 Orobanche germination but it was phytotoxic, inhibiting 373 Orobanche radicle development leading to haustorium inhib-374 ition (Figures 5 and 6). The inhibitory activity developed by 375 sphaeropsidones at high concentrations differentially targeted 376 the processes of germination in Striga and radicle development 377 in Orobanche. Phytotoxicity developed by haustorium inducing 378 factors has been previously associated with a common 379 metabolic pathway shared by germination and root growth 380 processes. 381

These results prompted to prepare some derivatives of both and **2** to elucidate patterns in the molecular specificity of haustorial induction in *S. hermonthica*, *O. crenata*, and *O.* ses *cumana. epi*-Sphaeropsidone, **2** (Figure 1), is a natural isomer of 1 (Figure 1), being its epimer at C-5, and the chlorosphaer- <sup>386</sup> opsidones 3 and 4 (Figure 1) are two C-6 epimeric natural <sup>387</sup> analogues of sphaeropsidones showing the opening of the <sup>388</sup> epoxy ring and its conversion in the corresponding chloridrin, <sup>389</sup> with the chlorine at C-6 and the hydroxy group at C-5. <sup>390</sup>

Six already known derivatives of sphaeropsidone (5-10, 391)Scheme 1) and one of epi-sphaeropsidone (11, Scheme 2) were 392 hemisynthesized and showed the same spectroscopic properties 393 previously reported.<sup>26</sup> Derivative 5 showed the reversible 394 acetylation of the hydroxy group at C-5, while the 2,4,5- 395 acetoxyanisole, 6, obtained from the same acetylation reaction, 396 is a tetrasubstituted benzene derivative and thus also the 397 aromatization of the cyclohexenone ring. The same hydroxy 398 group at C-5 was converted by oxidation to carbonyl group, 399 yielding the corresponding 2,3-epoxyhemiquinone 7, while the 400 reduction of the carbonyl group at C-2 generated the 401 corresponding 3-methoxy-cyclohex-3-ene-2,5-diol 9. 1 was 402 also converted into the corresponding bromydrin 8, which 403 differs from the epichlorosphaeropsidone 4 for the halogen at 404 C-1, which has a bromine instead of chlorine, and for the 405 opposite stereochemistry of the hydroxy group at C-5. The 406

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407 catalytic hydrogenation of **1** yielded 2,4-dihydroxy-5-methox-408 ycyclohexanone **10**, which showed the saturation of the double 409 bond between C-2 and C-3 and the reductive opening of the 410 epoxy ring. *epi*-Sphaeropsidone, **2**, was converted into the 411 corresponding acetyl derivative **11**, which, although epimer at 412 C-5, showed as **5** the reversible esterification of the hydroxy 413 group at the same carbon.

Sphaeropsidone, 1 was converted into the new esters 12 and 414 415 14 (Scheme 1), which showed always the reversible 416 modification of the hydroxy group at C-5 although the acyl 417 group is different, being a p-Br-benzoyl in 12 and 5-418 azidopentanoyl in 14. Both derivatives 12 and 14 showed 419 spectroscopic properties consistent with their structures. 420 Significantly, their IR spectra did not show bands for hydroxy 421 groups.<sup>33</sup> In particular, the <sup>1</sup>H NMR spectrum of 12 differed <sup>422</sup> from that of 1 for the expected downfield shift ( $\Delta\delta$  0.96) of H-423 5 appearing as a doublet (J = 2.5 Hz) and for the couple of two 424 doublets (J = 8.7 Hz) of the protons of the para-substituted 425 benzene ring resonating at  $\delta$  8.01 and 7.63 for H-2',6' and H-426 3',5', respectively. Its ESIMS spectrum showed the character-427 istic isotopic peaks of bromine for both sodium clusters [M + 2] $428 + Na^{+}$  and  $[M + Na^{+}]$  and the pseudomolecular ion [M + 2 + 2]429 H]<sup>+</sup> and  $[M + H]^+$  at m/z 364 and 362, and 342 and 340. 430 respectively. The <sup>1</sup>H NMR spectrum of **14** differed from that of 431 1 for the expected downfield shift ( $\Delta\delta$  0.74) of H-5, which 432 resonated as a doublet (J = 2.9 Hz) at  $\delta$  5.97, and for the 433 presence of the signals of the 5-azidopentanoyl group appearing 434 as two triplets (I = 6.5 and 7.3 Hz, respectively) at  $\delta$  3.32 and 435 2.53 for the methylene protons, respectively, linked to the azido 436 and the carboxyl group. The other two methylene groups 437 appeared as multiplets at 1.83-1.76 (H<sub>2</sub>C-3') and 1.71-1.64 438 (H<sub>2</sub>C-4'). Its ESIMS spectrum showed the sodium cluster [M  $(439 + Na]^+$  and the pseudomolecular ion  $[M + H]^+$  at m/z 304 and 440 282, respectively.

epi-Spheropsidone,  $\overline{p_{z_1}}$  was chemically reduced into the 441 442 corresponding 3-methoxy-cyclohex-3-ene-2,5-diol, 13 (Scheme 443 2), which is an epimer at C-5 of 9, similarly obtained from 1. 444 The <sup>1</sup>H NMR spectrum of 13 differed from that of 2 for the 445 significant presence of the broad doublet (J = 10.4 Hz) of H-2 446 and for the expected upfield shift ( $\Delta\delta$  0.66) of H-3, which 447 appeared as a broad singlet at  $\delta$  4.57 instead of a doublet. Its 448 ESIMS spectrum showed the sodium cluster  $[M + Na]^+$  and 449 the pseudomolecular ion  $[M + H]^+$  at m/z 182 and 159, 450 respectively. As 1 was converted into 14, 2 was similarly 451 esterified into the corresponding 5-azidopentanoyl derivative 452 15 (Scheme 2). Its IR spectrum did not show a band for a 453 hydroxy group. The <sup>1</sup>H NMR spectrum of **15** differed from that 454 of **2** for the expected downfield shift ( $\Delta\delta$  1.28) of H-5, which 455 appeared as a broad singlet at  $\delta$  5.92 and for the presence of the 456 signals of the 5-azidopentanoyl group appearing as two triplets  $_{457}$  (*I* = 6.5 and 7.2 Hz, respectively) at  $\delta$  3.31 and 2.45 for the 458 methylene protons, respectively, linked to the azido and the 459 carboxyl group. The other two methylene protons appeared 460 both as multiplets at  $\delta$  1.80–1.72 (H2C-3') and 1.71–1.68 461 (H2C-4'). Its ESIMS spectrum showed the sodium cluster [M  $(462 + Na]^+$  and the pseudomolecular ion  $[M + H]^+$  at m/z 304 and 463 282, respectively.

464 The haustorium-inducing activity in *S. hermonthica* of 465 sphaeropsidones (1-4) and their derivatives (5-15) is 466 shown in Figure 3. The *S. hermonthica* assays indicated that 467 conversion of 1 in derivatives 6 and 10 led to a severe reduction 468 in the haustorium-inducing activity being only significant at 100 469  $\mu$ M, while conversion in derivatives 4, 7, 8, and 9 led to loss at 0.1  $\mu$ M of haustorium-inducing activity that characterizes the 470 parent compound. Structural modifications in **3**, **5**, **12**, and **14** 471 did not affect the haustorial activity, inducing high rates of *S*. 472 *hermonthica* haustoria at 1 and 0.1  $\mu$ M comparable with 473 sphaeropsidone, **1**. 474

Esterification at C-5 in **11** and **15** increased haustorium- 475 inducing activity on *S. hermonthica* seedlings in comparison 476 with **2**, and in derivative **14** in comparison with **1**, while the 477 modifications at C-5 made in **3** and **12** maintained the strong 478 activity observed in **1** up to 0.1  $\mu$ M. Acetylation of **1** in 479 derivatives **5** did not strongly affect the haustorial activity as **1** 480 and **5** are strong inducers, although a slight reduction in activity 481 was found at 0.1  $\mu$ M in derivative **5** in comparison with **1**. It is 482 interesting to note that **2** and derivative **11** are weaker 483 haustorial inducers when compared with their respective 484 epimers of **1** and **5**. These results allow us to hypothesize 485 that the stereochemistry at C-5, although not strongly, play an 486 important role in impart this activity.

The epoxy ring by itself was not essentially associated with 488 haustorial inducing activity as some derivatives lacking the 489 epoxy ring, i.e., the analogue **3**, had strong haustorial activity, 490 however, when the selective reductive opening of the epoxy 491 ring was associated with the reduction of the olefinic double 492 bond in **10** or with the aromatization of the cyclohexenone ring 493 in **6**, the haustorial-inducing activity was strongly affected. The 494 reduction of the C-2 carbonyl group in **9** and **13** led to 495 reduction of haustorial activity in comparison with their parent 496 compounds **1** and **2** in seedlings.

These results could be explained, except for 7, in the easy 498 conversion of all derivatives at physiological pH into 499 sphaeropsidone and then into the corresponding above 500 hypothesized 2,3-epoxy-5-methoxyquinone. In fact, 3, having 501 the requested stereochemistry of the hydroxy and chlorine 502 groups at C-5 and C-6, according to the well-known 503 intramolecular nucleophilic substitution could give 1. This 504 reaction is less easy for the other chloridrin 4, probably for the 505 opposite not suitable stereochemistry of chlorine at C-6.<sup>34</sup> 506 Furthermore, derivatives 5, 12, and 14 being ester of 1 at 507 physiological pH, according to the well-known lethal 508 metabolism,<sup>35</sup> probably were hydrolyzed into 1. The slight 509 minor activity of derivative 8 is due to the same reasons above- 510 reported for 4 having the bromine in 8 the same stereo- 511 chemistry of chlorine in 4. The reduced activity of 9 could be 512 explained from different oxidation stage in respect to 1 being 9, 513 a 3-methoxy-cyclohex-3-ene-2,5-diol, and 1, a 2,3-epoxy-5- 514 methoxyhemiquinone. The noteworthy reduction of the activity 515 of derivatives  $\overline{6}$  and 10 was due to the inability to convert their  $_{516}$ benzene and the cyclohexanone rings into 2,3-epoxyhemiqui- 517 none ring of sphaeropsidone and then in the corresponding 518 2,3-epoxy-5-methoxyyquinone.

The haustorium-inducing activity in *O. crenata* and *O.* 520 cumana of sphaeropsidones (1-4) and their derivatives (5-521**15**), is shown in Figures 5 and 6, respectively. SAR results 522 indicated the haustorium-inducing activity of the parent 523 compound was affected by the structural modifications in a 524 slightly different way for *Striga* and *Orobanche* species, being 525 those differences more obvious between *Striga* and *O. cumana*. 526 The reduction of C-2 carbonyl group in **9** and **13** led to 527 reduction of haustorial activity at 1  $\mu$ M in *O. crenata* seedlings, 528 but it induced a strong loss of activity at all concentrations in *O.* 529 cumana. A marked loss of haustorial-inducing activity was 530 observed in *O. crenata* for compounds **6** and **10** when 531 compared with **1**, as it was observed for *S. hermonthica*, while 532

533 the activity was completely lost for O. cumana. For O. crenata, 534 structural modifications in compounds 5 and 12 maintained 535 nearly the same levels of haustorial activity than 1, as it 536 happened for S. hermonthica, however, activity of compound 5 s37 was lost at 1  $\mu$ M for O. cumana. The activity of compounds 4 538 and 8 was observed to be very high for O. crenata, being the 539 haustorial papillae especially well developed by compound 4 in 540 both Orobanche species. Differential effects caused by structural 541 modifications across hemiparasitic genera have been previously 542 suggested. Steffens et al. (1982)<sup>32°</sup> found that for the system 543 Agalinis purpurea-xenognosin A, haustorium was initiated 544 when the molecular inducer meet two structural features: (i) 545 hydroxy and methoxy groups were present and both groups 546 occupying a meta-position and (ii) an alkyl branching ortho to 547 the methoxy substituent. MacQueen (1984)<sup>15</sup> studied the 548 activity of phenolic compounds on S. hermonthica and 549 demonstrated that the activity was also initiated when the 550 hydroxy group was occupying a para-position and the methoxy 551 group a meta-position while the alkyl branching ortho to the 552 methoxy substituent was not required for activity in Striga.

Regarding germination inhibition, the activity of the two 553 554 natural analogues (3 and 4), eight sphaeropsidones derivatives 555 (5-10, 12, and 14), and three epi-sphaeropsidone derivatives 556 (11, 13, and 15) in comparison with sphaeropsidone, 1, and 557 epi-sphaeropsidone, 2, was tested on S. hermonthica (Figure 4). The germination inhibitory activity was reduced in 6, 8, 9, and 558 559 10 and increased in 5, 7, 12, and 14 in comparison with 560 sphaeropsidone, 1, while it was reduced in 13 and increased in 561 11 and 15 in comparison with epi-sphaeropsidone, 2. The 562 increased germination inhibitory activity in 5, 11, 12, 14, and 563 15 can be explained by their increased lipophilicity which could 564 facilitate cell membrane permeability.<sup>35</sup> The reduced germina-565 tion inhibitory activity in 9 and 13 could be explained for the s66 absence of the  $\alpha_{\mu}\beta$ -unsaturated ketone due to the reduction of 567 the carbonyl group at C-2, and in 6 and 10 for the 568 aromatization of the cyclohexenone ring, and for the reduction 569 of the double bond between C-4 and C-5 and for the reductive 570 opening of the epoxy ring.

The SAR study results suggested that the ability to initiate 571 572 haustorium development in S. hermonthica is probably due to 573 the conversion of the natural sphaeropsidones, their analogues, 574 and hemisynthetic derivatives in the corresponding 4-575 methoxyquinone. This conversion could occur more easily in 576 the analogue 3 and derivatives 5, 8, 12, and 14 of sphaeropsidone and derivatives 11 and 15 of epi-sphaerop-577 sidones. In fact, as above cited the esters 5, 11, 12, and 14, by 578 579 hydrolysis, could be converted into the parent compounds as 580 well as the analogue 3 and derivative 8, by nucleophilic elimination.<sup>36</sup> A successive oxidation step could be converted 581 both 1 and 2 into the 2,3-epoxy-4-methoxyquinone, and this 582 583 finally, by reductive opening of the epoxy group followed by water nucleophilic elimination into the 4-methoxyquinone. 584 This hypothesis on the mode of action of sphaeropsidone is in 585 full agreement with the results obtained using natural and 586 synthetic quinones as sorghum xenognosin and dimethox-587 ybenzoquinones, the latter very closely related to the 4-588 589 methoxyquinone, which as above explained could be generated 590 by oxidation of sphaeropsidones in the studies carried out on 591 haustoria and the chemistry in host recognition ion parasitic 592 angiosperms. Quinone/hydroquinone structures serve as 593 cofactors in many metabolic pathways, playing critical chemical 594 roles in oxidation/reduction processes.<sup>31,36</sup> This mode of action 595 could also operate in the haustorium-induction in Orobanche.

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The slight ference observed between *Orobanche* and 596 *Orobanche* is not surprising as differences between the species 597 in their ability to recognize and respond to the same 598 haustorium-inducing factors has been reported.<sup>31</sup> The presence 599 of the epoxy ring and the  $\alpha,\beta$ -unsaturated ketone group could 600 be the responsible for the inhibition of *S. hermonthica* seed 601 germination. 602

The induction of haustorium is a little researched area in 603 parasitic weeds, especially in Orobanche species. Our work has 604 identified for the first time natural occurring compounds with 605 haustorium-inducing activity for Orobanche species, which 606 opens several research directions aimed either to understand 607 the chemistry of haustorium initiation or toward the develop- 608 ment of alternatives to conventional control. It is now possible 609 to envision research in which host-encoded haustorium- 610 inducing factors are identified through bioactivity-guided 611 chromatographic purification of host root extracts. In addition, 612 Orobanche transgenic seedlings in which candidate genes for 613 haustorium initiation are silenced could be easily phenotyped 614 by the in vitro application of haustorium inducers. It could be 615 possible, in addition, once host-encoded haustorium factors are 616 identified for Orobanche, to select crop cultivars with low 617 haustorium-induction activity in their roots. In addition, this 618 work also opens the way of the development of new 619 agrochemicals with a novel mode of action based on disturbing 620 the fine-tuned process of host recognition. Compounds such as 621 sphaeropsidones with the ability of triggering haustorium 622 induction in a suicidal fashion are good candidates for such 623 strategy. Current research in our laboratory is starting to 624 explore the above-mentioned alternatives for Orobanche weed 625 management. 626

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