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# Host–guest complexation of phthalimide-derived strigolactone mimics with cyclodextrins. Application in agriculture against parasitic weeds†

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Parasitic weeds are noxious plants that damage crops of economic relevance, especially in Mediterranean and African countries. The strategy of suicidal germination was proposed to deal with this plague by using seed germination inducers that work as a pre-emergence herbicide and reduce the parasitic seed load before sowing. *N*-Substituted phthalimides with a furanone ring were found to be efficient in inducing the germination of *Phelipanche ramosa* and *Orobanche cumana*, two of the most problematic parasitic weeds of crops. However, the solubility of these compounds in water is low. A strategy for enhancing their aqueous solubility is the synthesis of host–guest complexes with cyclodextrins. Three bioactive phthalimide-lactones (**PL01**, **PL04**, and **PL07**) were selected and studied to form complexes of increased water solubility with  $\alpha$ -,  $\beta$ -, HP- $\beta$ -, and  $\gamma$ -cyclodextrin. The complexes obtained by the coprecipitation method, with increased aqueous solubility (up to 3.8 times), were studied for their bioactivity and they showed similar or slightly higher bioactivity than free phthalimide-lactones, even without the addition of organic solvents. A theoretical study using semiempirical calculations of molecular models including a solvation system confirmed the physicochemical empirical results. These results demonstrated that cyclodextrins can be used to improve the physicochemical and biological properties of parasitic seed germination inducers.

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## Introduction

Strigolactones are phytohormones involved in the root/branch architecture, development and stress resistance of plants.<sup>1–3</sup> However, these compounds exhibit an undesired secondary role as signaling compounds for the germination of parasitic plants. Even though strigolactones are released in very small quantities into the soil (around 15 pg per plant per day),<sup>4</sup> this amount is high enough to initiate the life cycle of parasitic plants. This is the case for the seeds of the genera *Orobanche*, *Phelipanche*, and *Striga*, which start germination by recogniz-

ing concentrations as low as  $10^{-12}$  M.<sup>5</sup> Parasitic plants are a serious problem, causing crop losses amounting to billions of dollars around the world and, especially, in Africa and Mediterranean countries.<sup>6</sup> In the case of obligate parasites, a suicidal germination strategy (recently, also named honeypot strategy<sup>7,8</sup>) was proposed to deal with these weeds, where a low concentration of strigolactones can be employed as pre-emergence treatment of the soil before the cultivation of crops to cause the death of parasitic plants by starvation.<sup>9</sup>

Stability and availability are the two major problems associated with the practical application of the aforementioned strategy. The isolation of strigolactones from natural sources provides a very low yield and takes a lot of effort, making it impractical for large scale purposes. For example, up to 0.35 mg and 0.15 mg of (+)-2'-*epi*-orobanchol and (+)-orobanchol, respectively, can be isolated from 5000 seedlings of 5-month grown tobacco plants.<sup>10</sup> Strigolactones like solanacol or solanacyl acetate are fully degraded after 4 to 9 days in water, and only 50% remain after 2 to 4 days, respectively.<sup>11</sup>

In order to successfully apply the suicidal germination strategy, strigolactone mimics with better stability and low cost have been proposed. More than 15 synthesis steps have to be performed to obtain low amounts of nature identical strigolac-

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tones, while simple mimics such as debranones require fewer steps and possess a high biological activity, comparable to that of the widely used strigolactone analogue GR24.<sup>12,13</sup> Other mimics, such as auxin-lactone, are synthesized in 3 steps and are more stable than GR24 under variable pH conditions. These mimics based on auxins are highly active against *P. ramosa* and exhibit a similar activity against *S. hermonthica* and *O. minor* when compared to strigolactones.<sup>14</sup> Lastly, there are stimulators of germination based on a phthalimide backbone, such as Nijmegen-1 and phthalimide-lactones (PLs), as the main representatives. Among the PL collection previously reported, some bioactivity profiles were similar to those of GR24 against *O. minor*, *P. aegyptiaca*, and *P. ramosa*. This is the case for **PL01**, **PL04**, **PL06**, **PL07**, or **PL14** (Figure Fig. 1), according to the results shown by Cala *et al.*<sup>15</sup> On the other hand, Nijmegen-1 has been tested in field trials, reducing the *S. hermonthica* population by ~65% in sorghum crops with a concentration of 1  $\mu\text{M}$ .<sup>16</sup>

One of the major problems for the practical application of suicidal germination is the limited availability in soil. These compounds have poor water solubility due to their low polarity, which limits their application for large scale agrochemical purposes. There are different strategies to overcome this problem, but encapsulation is one of the most promising strategies with several advantages: no need to modify the bioactive structure, low-cost reagents and affordable simple synthesis methods, and a possibility for controlled release. We had success in previous studies with the synthesis of polymeric organic nanoparticles for the encapsulation of **PL01**, improving its water solubility more than twenty-five times.<sup>7</sup> However, a more direct and cheaper approach would be the use of cyclodextrins (CDs). Cyclodextrins are natural compounds with a cyclic structure of 6 ( $\alpha$ -), 7 ( $\beta$ -), or 8 ( $\gamma$ -) units of  $\alpha$ -D-glucose, and have a hydrophilic outer surface and a hydrophobic cavity. This cavity can be used to host organic molecules inside and obtain organic complexes with enhanced water solubility compared with the guest.<sup>17,18</sup> These complexes, like the  $\beta$ -cyclodextrin inuloxin complex, have been applied successfully in the past, and showed similar activity values to free inuloxin against *P. ramosa* without the addition of organic solvent.<sup>19</sup> We reported in a previous study the application of CDs with sesquiterpene lactones, which are germination indu-

cers of *O. cumana* and *P. ramosa*. Water solubility and bioactivity were improved with this method.<sup>20</sup> In addition, CDs are authorized for human consumption by the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA)<sup>21</sup> and are considered environmentally friendly.

In this work, we have employed the natural  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD, as well as 2-hydroxypropyl- $\beta$ -cyclodextrin (**HP- $\beta$ -CD**), which is one of the most used non-natural CDs, to study the host-guest complexes generated with three different phthalimide-lactones (**PL01**, **PL04**, and **PL07**). They have been chosen among the most active compounds against *P. ramosa* and other parasitic plants. Water solubility studies, bioactivity profiles, calorimetry analysis, complexation experiments, and semi-empirical *in silico* simulations have been carried out as part of the study to evaluate the potential of CDs for the encapsulation of strigolactone mimics.

## Results and discussion

### Phase-solubility diagrams

Calibration curves for quantification were established by following a previously reported procedure,<sup>20</sup> using **PL01**, **PL04**, and **PL07** dissolved in MeOH as calibrators. Quantification was carried out by RP-HPLC with DAD (wavelength range 200–381 nm; see the Experimental section). Chromatograms exhibit two or more peaks (Fig. 2, top chromatograms), indicating a partial degradation of the compounds **PL01** and **PL04** in MeOH. The peak intensity of degradation products increased over time, so MeOH was discarded as a suitable solvent for the preparation of the standard solutions. Therefore, MeCN was selected as a solvent for standard solution preparation, due to its similar polarity to other organic solvents. In this case, the samples were stable for a very long time and only one peak was observed in the chromatograms even when the samples were injected days later (Fig. 2, bottom chromatograms), indicating suitable stability. However, the use of MeCN as sample solvent resulted in a slightly modified retention time as shown in the chromatograms in Fig. 2.

After calibration, the host-guest interaction of each compound with  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD as well as **HP- $\beta$ -CD** was studied using a modified Higuchi and Connors method that was reported recently.<sup>20</sup> The concentration of each phthalimide-lactone was measured for every experiment carried out with variations in the concentration of cyclodextrins by following the standard solubility diagram procedure. The **PL04** signal was not detected (LOD 0.025 mg mL<sup>-1</sup>), neither in the cyclodextrin solutions nor in deionized water, indicating that the solubility was marginal even after interaction with cyclodextrins. Therefore, this compound was excluded from further studies. A reason for these results could be related to the different reactivities of **PL01** and **PL07**, when compared with **PL04**, which bears a nitro group at the aromatic ring. Polarity and charge distribution of the molecule are involved in the lack of supramolecular interaction of this phthalimide.<sup>15</sup>

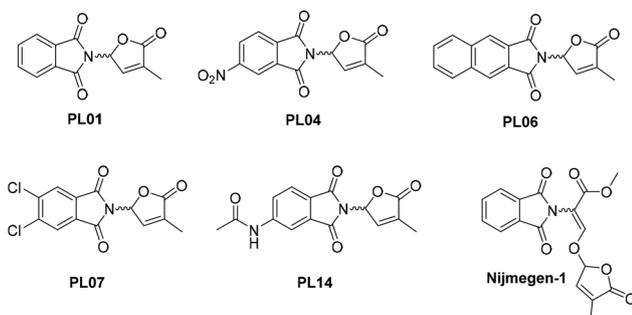


Fig. 1 Relevant structures of phthalimide-derived strigolactone mimics.



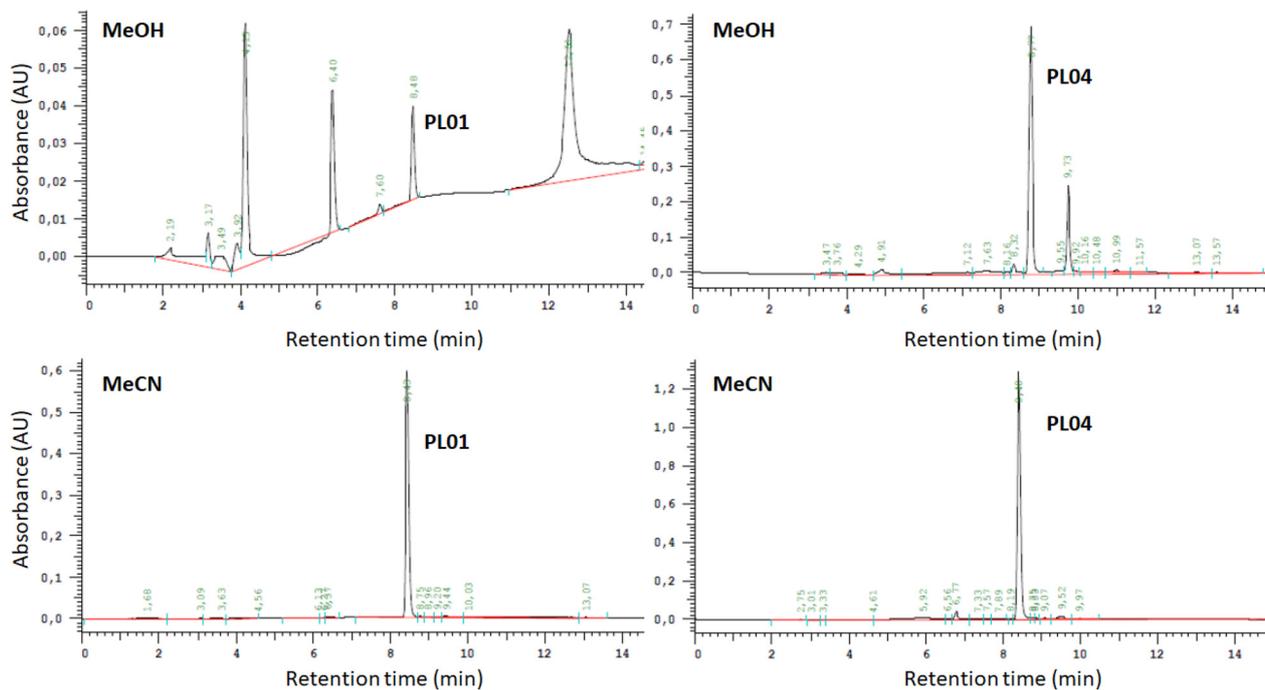


Fig. 2 Chromatograms obtained by RP-HPLC analysis (acquisition wavelength range: 200–381 nm) for samples dissolved in MeOH (PL01 top left, PL04 top right) and MeCN (PL01 bottom left, PL04 bottom right). Degradation peaks were observed in samples dissolved in MeOH. Retention time changes slightly when changing sample solvent.

**Diagrams for  $\alpha$ -CD with PL01 and PL07.** Fig. 3 shows the phase solubility diagram for PL01 and PL07. In the first case,  $\alpha$ -CD barely interacted with any of the compounds. Concentrations of PL01 changed almost imperceptibly with the increasing concentration of  $\alpha$ -CD. The results obtained were fitted to a trendline, obtaining the following equation:

$$[\text{PL01}] = 9 \times 10^{-4}[\alpha\text{-CD}] + 0.111 \quad (1)$$

This adjustment produced  $R^2 = 0.0413$ , with a slope close to zero, indicating that no complex was formed. This could be associated with a  $B_S$ -type Higuchi–Connors diagram indicating that CD preferably forms self-assembled aggregates and the solubility of CD gradually increases even in the presence of the drug. An example of this behavior has been already reported by Schönbeck *et al.* in the formulation of hydrocortisone.<sup>23</sup> This is usually observed with really poorly-soluble drugs.

When using PL07, compound peaks were observed under only two conditions, both out of the calibration range (LOD  $0.017 \text{ mg mL}^{-1}$  and LOQ  $0.052 \text{ mg mL}^{-1}$ ). These conditions were the values of 0 and 12 mM, and the estimated concentration values were very similar for both ( $2 \times 10^{-3}$  and  $3 \times 10^{-3} \text{ mg mL}^{-1}$ , respectively). No equation was reported in this case since only two unreliable data were obtained. Again, results did not hint at complex formation. In addition, this could also be explained by B-type diagrams.

**Diagrams for  $\beta$ -CD with PL01 and PL07.** In the case of  $\beta$ -CD-PL01, a linear trend was observed, indicating that the solubility increased linearly with a positive slope falling between 0 and 1 in the range of 0–10 mM  $\beta$ -CD, and these

data were fitted to a trendline, obtaining  $R^2 = 0.9542$  and the following equation:

$$[\text{PL01}] = 2.44 \times 10^{-2}[\beta\text{-CD}] + 0.113 \quad (2)$$

The final data of guest concentration at 12 mM were lower than the previous data at 10 mM, which may occur when the precipitation of a second less-soluble complex is produced at that concentration. The data fit to an  $A_L$  type diagram, indicating a 1:1 type complex in the straight slope. In these cases, as reported previously,<sup>20</sup> the complex constant ( $K_{1:1}$ ) and the complexation efficiency (CE) were determined using the following equations:

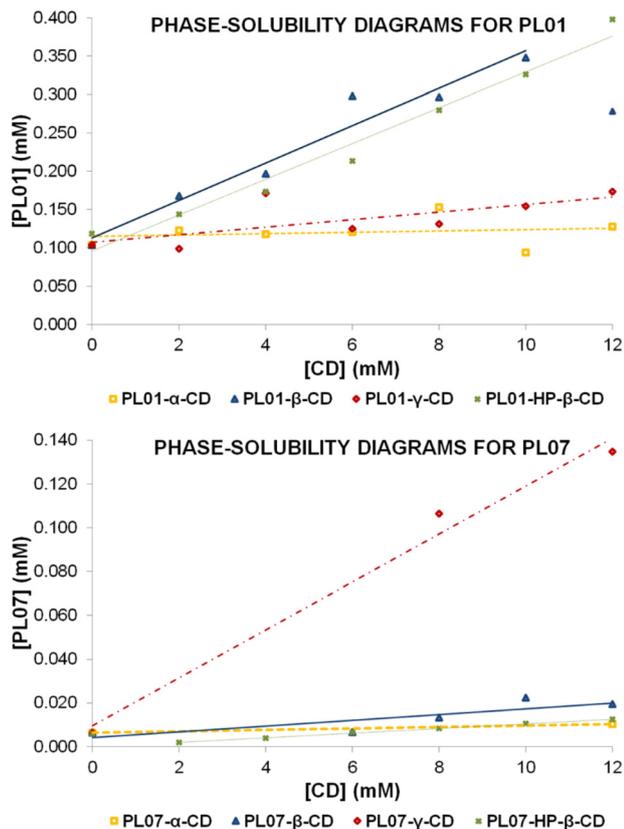
$$K_{1:1} = \frac{\text{Slope}}{S_0 \cdot (1 - \text{Slope})} \quad (3)$$

$$\text{CE} = S_0 \cdot K_{1:1} = \frac{\text{Slope}}{1 - \text{Slope}} \quad (4)$$

where  $S_0$  is the solubility of each PL in  $\text{H}_2\text{O}$  at 0 mM [CD]. In this case, by applying eqn (3) and (4),  $S_0 = 0.104 \text{ mM}$ ,  $K_{1:1} = 240 \text{ M}^{-1}$  and  $\text{CE} = 0.025$ . The complexation constant is similar to that obtained for (–)- $\alpha$ -santonin with previously reported  $\beta$ -CD,<sup>20</sup> although the CE indicates a low efficiency in the complexation process, thus predicting a low increase of solubility of the solid complex when compared with the free PL01 in water.

Regarding PL07, the concentrations detected were not much higher than in the case of  $\alpha$ -CD, and still out of the calibration range. This could be explained by the higher molecular





**Fig. 3** Phase-solubility diagrams obtained by following the Higuchi and Connors method<sup>20,22</sup> for PL01 (top) and PL07 (bottom) with 4 different cyclodextrins:  $\alpha$ - (orange),  $\beta$ - (blue),  $\gamma$ - (red), and HP- $\beta$ -CD (green). Concentration of each sample was measured after treatment with CD in a range of 0–12 mM.

volume exhibited because of two chlorine atoms that are substituted in the aromatic ring. Data are fitted using the following equation:

$$[\text{PL07}] = 1.30 \times 10^{-3}[\beta\text{-CD}] + 0.004 \quad (5)$$

Fig. 3 seems to show a linearly increasing tendency. However, the slope was very low and the goodness of the adjustment value was only  $R^2 = 0.7033$ . It can be considered that the interactions of PL07 with  $\beta$ -CD are stronger than those with  $\alpha$ -CD, but still weak. The observed data suggest the formation of a 1 : 1 complex, although the results were not satisfactory enough for a confident calculation of  $K_{1:1}$  and CE.

**Diagrams for  $\gamma$ -CD with PL01 and PL07.** The phase solubility diagram for PL01 with  $\gamma$ -CD indicated again a linear increase of solubility with the concentration of CD, with a positive slope falling between 0 and 1 and  $R^2 = 0.9184$ , obtaining the following equation:

$$[\text{PL01}] = 5.90 \times 10^{-3}[\gamma\text{-CD}] + 0.093 \quad (6)$$

The slope in this case is higher than that in the experiment with  $\alpha$ -CD, implicating stronger interactions with  $\gamma$ -CD. However, the slope is still low when compared with the value

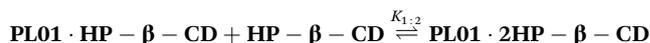
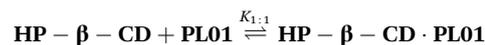
for  $\beta$ -CD. By applying eqn (3) and (4),  $S_0 = 0.104$  mM,  $K_{1:1} = 57$   $\text{M}^{-1}$  and CE = 0.006, which, as expected by observing the shape of the diagram, indicates a lower efficiency of the complexation process, which will cause a small increment in the solubility of the complex when compared with free PL01.

As in the previous two cases, complexation with PL07 worked worse than that with PL01 when using  $\gamma$ -CD, and also the measurements were out of the calibration range, obtaining only three valid measurements. Nevertheless, in this experiment, when using 8 and 12 mM  $\gamma$ -CD, the concentration of PL07 increased in a higher degree, as observed in the areas of the peaks. As in the case with  $\beta$ -CD, a trendline was obtained to get conclusions:

$$[\text{PL07}] = 1.09 \times 10^{-2}[\gamma\text{-CD}] + 0.096 \quad (7)$$

This equation has  $R^2 = 0.985$ , indicating a linear increase of the solubility, still very low, but with a higher slope than in the previous cases, suggesting stronger interactions of PL07 with this CD. By observing the data, a 1 : 1 complex could be hypothesized, although the results were not satisfactory enough for a confident calculation of  $K_{1:1}$  and CE.

**Diagrams for HP- $\beta$ -CD with PL01 and PL07.** The solubility diagram developed with HP- $\beta$ -CD and PL01 presents a different tendency, away from a linear diagram  $A_L$  (Fig. 4a). In this case, a positive deviation from linearity ( $A_P$  type) can be observed, suggesting the formation of a higher-order complex with respect to CD.<sup>24</sup> According to the data, two cyclodextrins host one molecule of PL01.



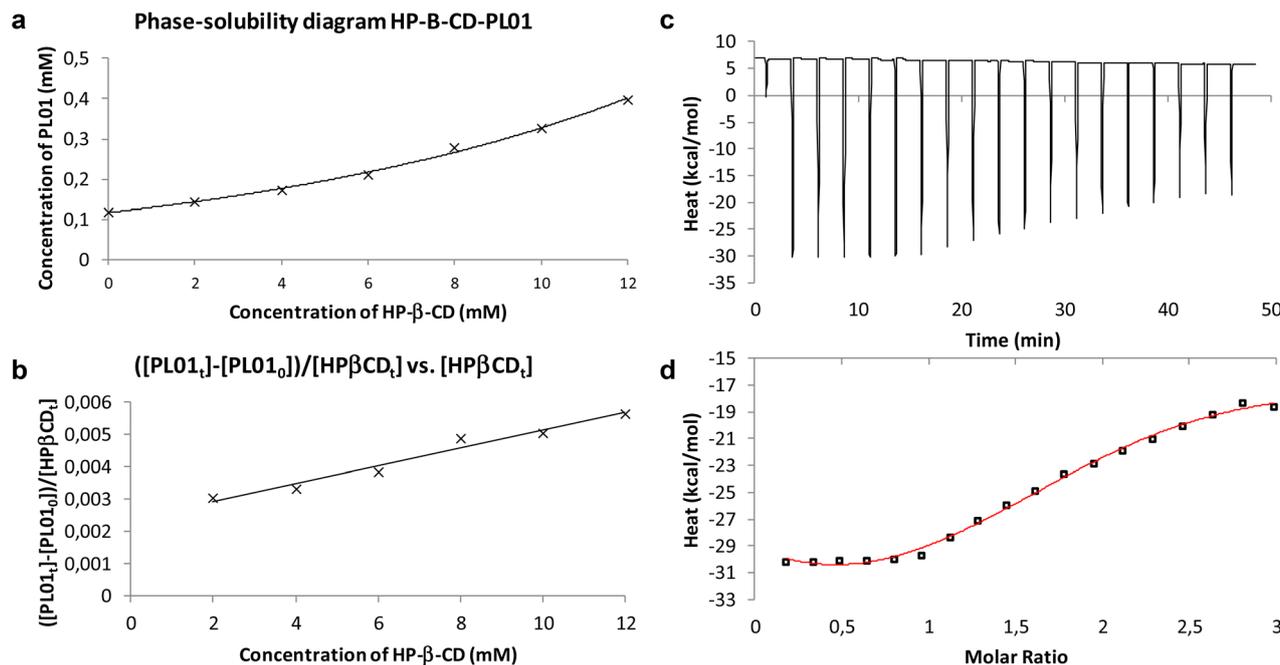
The system settled out in equations allows us to get the binding constants of Higuchi–Connors diagrams by:

$$\frac{[\text{PL01}]_t - [\text{PL01}]_0}{[\text{HP} - \beta - \text{CD}]_t} = K_{1:1} \cdot [\text{PL01}]_0 + K_{1:1} \cdot K_{1:2} \cdot [\text{PL01}]_0 \cdot [\text{HP} - \beta - \text{CD}]_t \quad (8)$$

Table 1 shows binding constant values calculated from experimental data. This result differs from those of other PL solubility studies. Different assays carried out with  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD showed 1 : 1 type complexation. In addition to that, no other PLs presented a different complex but the 1 : 1 host-guest. Comparing inner cavity sizes,  $\gamma$ -CD presents a portal diameter between 7.5 and 8.3 Å, while HP- $\beta$ -CD presents values between 6.0 and 6.4 Å.<sup>25</sup> Simplicity of PL01, without voluminous functional groups in the main structure, does not help to understand the formation of the postulated 1 : 2 complex. Due to that, isothermal titration calorimetry (ITC) was developed to confirm the results (Fig. 4c and d).

The performed ITC experiment confirmed the Higuchi–Connors results. Fig. 4d shows that the stoichiometry of the system is  $N = 2$ . Furthermore, Table 1 shows that  $K_{\text{obs}}$  values calculated with both approaches are quite similar.





**Fig. 4** (a) Higuchi–Connors solubility diagram between HP-β-CD and PL01. (b) Representation of the PL01/HP-β-CD ratio to calculate binding constants. (c) Titration calorimetry diagram between HP-β-CD ( $8.00 \times 10^{-4}$  M) and PL01 ( $5.00 \times 10^{-5}$  M) in deionized water. (d) Representation of thermodynamic values obtained from titration calorimetry studies.

**Table 1** Comparison of Higuchi–Connors vs. ITC binding constants

Method	$K_{1:1}, K_{1:2}$ ( $M^{-1}$ )	$K_{obs}$ ( $M^{-1}$ )
Higuchi–Connors	$8.276 \times 10^{-4}, 1.250 \times 10^{-4}$	$10.34 \times 10^{-5}$
ITC	—	$(6.63 \pm 1.88) \times 10^{-5}$

Experimental enthalpy obtained from Fig. 4d is  $\Delta H = -323.0 \pm 48.7$  kcal mol<sup>-1</sup> and  $-T\Delta S = 318.0$  kcal mol<sup>-1</sup>, so 1:2 host-guest complexation is spontaneous with  $\Delta G = -5.71$  kcal mol<sup>-1</sup>. From Fig. 4a, it can be observed that the water solubility of PL01 when HP-β-CD was applied was enhanced around 3 times. Taking into account the results of the performed experiments, it is suggested that the small structure of PL01 makes the approach of a new cyclodextrin molecule to establish intermolecular forces among branched functional groups (2-hydroxypropyls) possible, generating a capsule-like complex of PL01. This differs from PL07 because its functional groups already would have established interactions with hydroxypropyl groups, without the possibility of a further cyclodextrin coordination. In fact, the interactions of PL07 were still weaker than those with the other CDs, indicating that there is not a huge difference with the inclusion of the hydroxypropyl groups. In this case, the increase of solubility was similar to that of the β-CD diagram (with a similar slope), following a linear trend with the increase of CD concentration. However, the values were out of the calibration range and the concentration of PL07 decreased at lower concentrations of CD to increase later to a higher concentration than that at 0 mM. This indicates two linear trends. An adjustment of the data in

the second trend (starting at 2 mM) produced the following equation:

$$[\text{PL07}] = 1.1 \times 10^{-3}[\text{HP-}\beta\text{-CD}] + 0.001 \quad (9)$$

with a very good value of  $R^2 = 0.9989$ , demonstrating clearly the difference between PL01 and PL07 interactions with this CD, and suggesting a 1:1 complex, although the results were not satisfactory enough for a confident calculation of  $K_{1:1}$  and CE.

### Preparation of the solid complexes

The complexes were prepared by following the co-precipitation method according to our previous findings,<sup>20</sup> where precipitation of the corresponding complexes (as a white precipitate) was observed shortly after adding the dissolved guests into the solution of cyclodextrin for the cases of PL01 and PL07 with β-, γ-, and HP-β-CD. The amount of the observed precipitate increased significantly as the complexation reaction was approaching the end. This is expected, since the CD-complexes are expected to be less soluble in water than the free CDs, and the concentrations were adjusted to be close to the solubility limits of free CDs. After that, the solutions were concentrated and dried to recover as much precipitate as possible. However, a low amount of precipitate was obtained for α-CD, which was expected, since almost negligible interaction was observed in the phase-solubility diagrams. Nevertheless, the reaction was treated in the same way, in order to obtain as much complex as possible and analyze the sample in the bioassays and the solubility measurements. Compound PL04 was not employed,



since the phase-solubility diagram did not show evidence of interaction with the CDs employed and the peaks for this compound were not found in the chromatograms. Stoichiometry was employed as the phase-solubility diagram suggested, and therefore 1 : 1 was considered for all the cases, except for **PL01** and **HP- $\beta$ -CD** where 1 : 2 was employed instead. The employed concentration of  **$\beta$ -CD** was lower than the rest of the cases since it is the least water soluble among all the CDs employed, although, the relative molar amounts of the guest and host were adjusted to maintain the expected stoichiometry (see the Experimental section).

### Solubility studies

The aliquots of saturated solutions of each CD complex and free **PL** were injected into the RP-HPLC system by following the method reported in the Experimental section and the areas of the peaks were used to calculate the concentration of each original sample. Solubility ratios were calculated using eqn (10), to compare the efficiency of the different cyclodextrins to increase the solubility of the compounds (Table 2).

$$\text{Solubility ratio} = \frac{[\text{Compound in the complex}]}{[\text{Free compound}]} \quad (10)$$

At a first glance of the results, it was appreciated that they were in agreement with the phase-solubility diagrams: weak interactions with the CDs translated into low increments of the solubility in water. By comparison of the different solubility ratios, the best results when comparing the different natural CDs were obtained with  **$\beta$ -CD** for both compounds, with an increase of solubility of almost 3 times the original in the case of **PL01**, and a discrete increase in the case of **PL07**. Though this CD is the least soluble among the three, its interactions and cavity size may have played a significant role.<sup>26</sup> When comparing both  **$\beta$ -CD** and **HP- $\beta$ -CD**, the increases of solubility are even higher for **PL01**, with almost quadruple of the initial solubility in water, and in the case of **PL07**, this increase doubles. These findings indicate that even when a weaker interaction with CDs is produced (the case of **PL07**), the selection of a more soluble CD with an appropriate cavity size may help to obtain a higher solubility. On the other hand,

although the solubility increases obtained for  **$\alpha$ -CD** are comparable to those of  **$\gamma$ -CD**, the lack of interactions suggested by the solubility diagrams may imply that these solubility increases are caused by an adjuvant or micellar effect rather than proper complexation.<sup>27,28</sup>

### Molecular modelling

Semiempirical optimizations (PM3 calculation level) were carried out to compare the results of the experiments with the processes happening at a molecular level. They were carried out using Hyperchem® and more details about the conditions can be found in the Experimental section.

In the first case, it was observed that both **PL01** and **PL07** did not fit inside the  **$\alpha$ -CD** cavity, thus explaining the weak interactions with this CD. The geometry optimization was then carried out with  **$\beta$ -**,  **$\gamma$ -**, and **HP- $\beta$ -CD**, with bigger cavity sizes. The calculated energies of the geometry optimizations are shown in Table 3, while Fig. 5 shows the images of the most stable complexes shaded in gray in the table. The images of the least stable complexes and the separated model are shown in the ESI (S1–S56†).

The most stable models were **PL01- $\beta$ -CD (C)**, **PL01- $\gamma$ -CD (E)**, **PL01-HP- $\beta$ -CD (D)**, **PL01-HP- $\beta$ -CD (B4)**, **PL07- $\beta$ -CD (A)**, **PL07- $\gamma$ -CD (A)**, and **PL07-HP- $\beta$ -CD (D)**. In all the cases, **PL01** and **PL07** fitted at least partially inside the cavity of the CDs, although **PL01** was hosted completely in the case of **PL01- $\gamma$ -CD (E)**.

Regarding **PL01**, the furanone ring inside the cavity was the most stable geometry in the case of  **$\beta$ -CD (PL01- $\beta$ -CD (C))**, while for  **$\gamma$ -CD (PL01- $\gamma$ -CD (E))**, the molecule was fully hosted (Fig. 5). None of the complexes of **PL01- $\beta$ -CD** were more stable than the separate model as indicated for  $\Delta E_{\text{PM3}} > 0$ , which is the opposite case for **PL01- $\gamma$ -CD**, therefore indicating a higher stabilization of the structure when surrounded by the CD, which can be observed again in the models with **HP- $\beta$ -CD (PL01-HP- $\beta$ -CD (D) and PL01-HP- $\beta$ -CD (B4))**. The highest stabilization was obtained for the **HP- $\beta$ -CD complexes**, where the energy stabilization was higher (in absolute terms) for the 1 : 1 model ( $-361.78 \text{ kJ mol}^{-1}$ ) than the 1 : 2 model ( $-292.80 \text{ kJ mol}^{-1}$ ). However, these values are in the same order of magnitude and this discrepancy is caused by the change in con-

**Table 2** Solubility ratios of cyclodextrin (CD) complexes

Compound	mg mL <sup>-1</sup>	mM	Solub. (0 mM CD)	Solub. ratio $\pm$ SD
<b>PL01-<math>\alpha</math>-CD</b>	0.060	0.247	0.104	2.41 $\pm$ 0.06
<b>PL01-<math>\beta</math>-CD</b>	0.073	0.300		2.86 $\pm$ 0.04
<b>PL01-<math>\gamma</math>-CD</b>	0.055	0.225		2.25 $\pm$ 0.15
<b>PL01-HP-<math>\beta</math>-CD</b>	0.097	0.398		3.82 $\pm$ 0.01
Compound	Mean area <sup>a</sup> (complex)	Mean area <sup>a</sup> (0 mM CD)	Solub. ratio <sup>b</sup> $\pm$ SD	
<b>PL07-<math>\alpha</math>-CD</b>	12 869	14 128	0.91 $\pm$ 0.62	
<b>PL07-<math>\beta</math>-CD</b>	16 848		1.26 $\pm$ 0.12	
<b>PL07-<math>\gamma</math>-CD</b>	14 560.5		1.03 $\pm$ 0.12	
<b>PL07-HP-<math>\beta</math>-CD</b>	27 496		1.95 $\pm$ 0.01	

<sup>a</sup> For those with values out of the calibration range, mean areas of the peaks instead of concentrations were employed. <sup>b</sup> Increase of solubility was obtained from mean area quotient.



**Table 3** Complexation stabilization energy values ( $\text{kJ mol}^{-1}$ ) after geometry optimization of the complexes and the separate molecules with the PM3 calculation level including 250 (PL01- $\beta$ -CD, PL01- $\gamma$ -CD, PL01-HP- $\beta$ -CD, PL07- $\beta$ -CD, and PL07- $\gamma$ -CD) or 222 (PL01-HP- $\beta$ -CD (1:2) and PL07-HP- $\beta$ -CD (1:1)) molecules of  $\text{H}_2\text{O}$ . Four different orientations of the PLs were used, except for PL01-HP- $\beta$ -CD (Fig. 5 and ESI S1–S56<sup>†</sup>), the most stable is shaded in gray. The complexation stabilization values for  $\Delta E_{\text{PM3}}$  were calculated as  $E_{\text{complex}} - E_{\text{separate}}$

Complex	$E_{\text{PM3}}$ ( $\text{kJ mol}^{-1}$ )	$\Delta E_{\text{PM3}}$ ( $\text{kJ mol}^{-1}$ )	Complex	$E_{\text{PM3}}$ ( $\text{kJ mol}^{-1}$ )	$\Delta E_{\text{PM3}}$ ( $\text{kJ mol}^{-1}$ )
<b>Separate PL01 and <math>\beta</math>-CD</b>	–304 260.90	—	<b>Separate PL07 and <math>\beta</math>-CD</b>	–304 035.17	—
PL01- $\beta$ -CD (A)	–304 022.01	238.89	PL07- $\beta$ -CD (A)	–303 989.81	45.35
PL01- $\beta$ -CD (B)	–304 009.19	251.71	PL07- $\beta$ -CD (B)	–303 866.00	169.16
PL01- $\beta$ -CD (C)	–304 147.37	113.53	PL07- $\beta$ -CD (C)	–303 751.73	283.43
PL01- $\beta$ -CD (D)	–304 044.06	216.83	PL07- $\beta$ -CD (D)	–303 774.74	260.43
<b>Separate PL01 and <math>\gamma</math>-CD</b>	–312 689.03	—	<b>Separate PL07 and <math>\gamma</math>-CD</b>	–312 614.77	—
PL01- $\gamma$ -CD (A)	–312 641.74	47.29	PL07- $\gamma$ -CD (A)	–312 620.80	–6.03
PL01- $\gamma$ -CD (B)	–312 580.88	108.14	PL07- $\gamma$ -CD (B)	–312 512.25	102.52
PL01- $\gamma$ -CD (C)	–312 455.16	233.87	PL07- $\gamma$ -CD (C)	–312 336.61	278.17
PL01- $\gamma$ -CD (D)	–312 500.68	188.34	PL07- $\gamma$ -CD (D)	–312 336.79	277.98
PL01- $\gamma$ -CD (E)	–312 747.26	–58.24	PL07- $\gamma$ -CD (E)	–312 539.93	74.85
<b>Separate PL01 and HP-<math>\beta</math>-CD</b>	–334 304.35	—	<b>Separate PL07 and HP-<math>\beta</math>-CD</b>	–334 030.39	—
PL01-HP- $\beta$ -CD (A)	–334 232.04	–201.66	PL07-HP- $\beta$ -CD (A)	–334 127.13	–96.74
PL01-HP- $\beta$ -CD (B)	–334 235.39	–205.01	PL07-HP- $\beta$ -CD (B)	–334 091.23	–60.84
PL01-HP- $\beta$ -CD (C)	–334 162.83	–132.45	PL07-HP- $\beta$ -CD (C)	–334 229.54	–199.16
PL01-HP- $\beta$ -CD (D)	–334 392.16	–361.78	PL07-HP- $\beta$ -CD (D)	–334 297.84	–267.45
<b>Separate PL01 and HP-<math>\beta</math>-CD</b>	–395 306.97	—	<b>PL01-HP-<math>\beta</math>-CD (C1)</b>	–395 001.03	305.95
PL01-HP- $\beta$ -CD (A1)	–395 252.57	54.40	PL01-HP- $\beta$ -CD (C2)	–395 135.77	171.20
PL01-HP- $\beta$ -CD (A2)	–395 386.26	–79.28	PL01-HP- $\beta$ -CD (C3)	–395 214.73	92.24
PL01-HP- $\beta$ -CD (A3)	–395 328.48	–21.51	PL01-HP- $\beta$ -CD (C4)	–395 115.85	191.13
PL01-HP- $\beta$ -CD (A4)	–395 342.55	–35.57	PL01-HP- $\beta$ -CD (C5)	–395 114.58	192.39
PL01-HP- $\beta$ -CD (A5)	–395 244.44	62.53	PL01-HP- $\beta$ -CD (C6)	–395 179.94	127.03
PL01-HP- $\beta$ -CD (A6)	–395 239.05	67.93	PL01-HP- $\beta$ -CD (D1)	–395 190.16	116.81
PL01-HP- $\beta$ -CD (B1)	–395 392.53	–85.56	PL01-HP- $\beta$ -CD (D2)	–395 238.79	68.18
PL01-HP- $\beta$ -CD (B2)	–395 376.07	–69.10	PL01-HP- $\beta$ -CD (D3)	–395 326.65	–19.68
PL01-HP- $\beta$ -CD (B3)	–395 553.86	–246.89	PL01-HP- $\beta$ -CD (D4)	–395 203.05	103.92
PL01-HP- $\beta$ -CD (B4)	–395 599.77	–292.80	PL01-HP- $\beta$ -CD (D5)	–395 090.49	216.49
PL01-HP- $\beta$ -CD (B5)	–395 394.30	–87.33	PL01-HP- $\beta$ -CD (D6)	–395 117.69	189.28
PL01-HP- $\beta$ -CD (B6)	–395 400.09	–93.12			

ditions, since more water molecules were employed in this model than in the 1:2 models due to the limitations of software. It is expected that by including a higher number of water molecules the energy values will become lower for the 1:2 complex. In addition, it has been stated before<sup>29</sup> that complexation with CDs is an equilibrium where more than one species can co-exist in solution, suggesting the co-existence of both complexes in solution. The highest stabilization for HP- $\beta$ -CD when compared with the other two CDs explains the higher increases in the solubility of the obtained complexes, the higher slopes and the better bioactivity results.

On the other hand, results for stabilization energy for the models of PL07 were very discrete for  $\beta$ -CD (PL07- $\beta$ -CD (A) = 45.35  $\text{kJ mol}^{-1}$ ) and  $\gamma$ -CD (PL07- $\gamma$ -CD (A) = –6.03  $\text{kJ mol}^{-1}$ ), which were positive and slightly negative, respectively. This correlates with the small solubility ratios for this compound, which were only improved by HP- $\beta$ -CD, which presents the highest stability for this compound (PL07-HP- $\beta$ -CD (D) = –267.45  $\text{kJ mol}^{-1}$ ).

In this case, the geometry of this model shows that PL07 is completely surrounded by the CD and the furanone ring interacts with the isopropyl residues. This interaction could be crucial for the increase in stability since the slightly more

soluble  $\gamma$ -CD complex presents a model where this ring is close to the  $-\text{CH}_2\text{OH}$  fragments (hydrophilic outer ring), while in the  $\beta$ -CD model, the phthalimide ring was located in this part. The weaker interaction of the CDs with PL07 than with PL01 also explains the slightly lower bioactivity of PL07-complexes vs. free-PL07.

### Bioassays on parasitic weeds

The induction of germination of *P. ramosa* seeds was tested for the PLs, CDs and their complexes by following the procedure already described in the literature.<sup>30</sup> The free compounds PL01 and PL07 were predissolved in acetone (1% v/v) and then diluted with water. On the other hand, the complexes were directly dissolved in water without a co-solvent. In all the cases, complexes with natural CDs ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -) showed similar levels of bioactivity in comparison with the bioactivity of the PLs without CDs, even without the use of an organic co-solvent (Fig. 6A). An increase of bioactivity was observed at the third dilution (1  $\mu\text{M}$ ) when comparing PL01- $\alpha$ -CD and PL01- $\beta$ -CD with PL01, while a decrease was observed for PL01- $\gamma$ -CD. On the other hand, the bioactivity decreased at the fourth dilution (0.1  $\mu\text{M}$ ) for all the PL01 complexes. Regarding PL07, it was observed that the bioactivity profile was slightly impaired



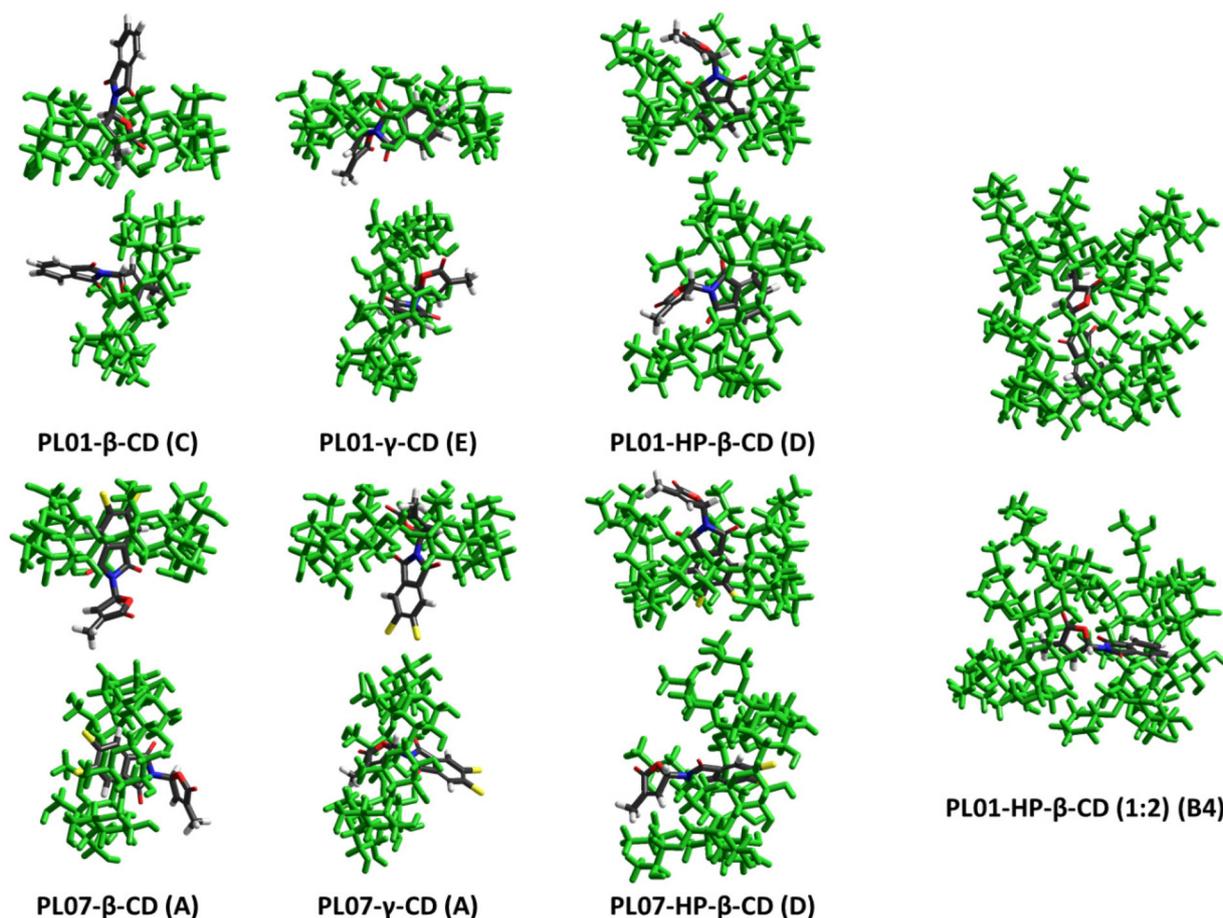


Fig. 5 Images of the geometrically optimized theoretical complexes of PL01 and PL07 with  $\beta$ -,  $\gamma$ -, and HP- $\beta$ -CD with the most stable orientations of the complexes. H<sub>2</sub>O molecules are hidden for the sake of clarity. Least stable optimizations, as well as high quality images for the stable complexes, are available in the ESI (S1–S56†).

for the complexes, starting at the third dilution and, especially, at the fourth dilution, the bioactivity dropped dramatically. This is correlated with theoretical simulations, which exhibited weak interactions of PL07 with the three CDs and therefore a lower increase of solubility of the complex. Nevertheless, these results imply that these compounds can be applied in a formulation for the fight against parasitic plants without organic solvents. In the case of HP- $\beta$ -CD (Fig. 6B), the bioactivity was, in general, increased when compared with the free PLs even at the lowest concentration. The bioactivity of PL01-HP- $\beta$ -CD was increased for the complex in correlation with the phase-solubility diagrams, where strong host-guest interactions were observed. The complex PL07-HP- $\beta$ -CD showed high water solubility, and its bioactivity was slightly better than PL07- $\beta$ -CD, although both complexes present similar interaction energy. It is likely that cyclodextrins exert an influence on bioavailability and not only on the water solubility.

Activity on *O. cumana* was also tested, finding only activity at the highest concentration tested (100  $\mu$ M), although the effect of treatment with cyclodextrins on the bioactivity was similar (similar or slight increase of germination). Compounds tested did not induce germination of *O. crenata* and the posi-

tive control GR24 only induced moderate germination. A deviation in the bioactivity results compared with previous studies<sup>7,15</sup> is expected for this bioassay because of the season, age of the seeds and other environmental factors.<sup>31</sup> In general, it was observed that encapsulation with cyclodextrins not only preserved the bioactivity of the pure compounds but also increased it in some cases. This bioactivity was also observed using completely aqueous test solutions.

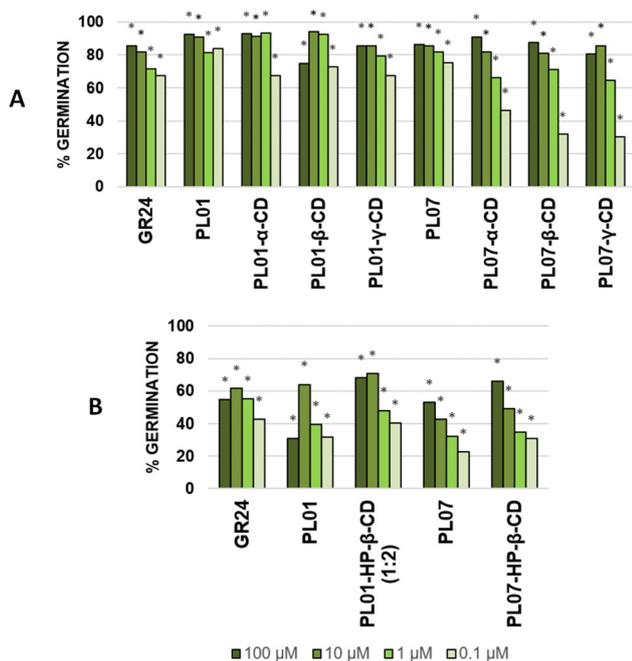
Avoiding the use of organic co-solvents, such as acetone or DMSO that are common in this kind of bioassays, by formulation with cyclodextrins is not only a greener choice, but also decreases the possibilities of precipitate formation by the evaporation of the prior, improving the potential for practical use in preparations as future commercial herbicides.

## Experimental

### General experimental procedures

Complexation reactions were carried out on a Heidolph Synthesis 1 at a controlled temperature and constant stirring. RP-HPLC analysis was carried out using a VWR Hitachi





**Fig. 6** Activity on the germination of *P. ramosa* seeds. Germination (%) was calculated by comparison with the germination rate of negative control (water). Significance of the data is indicated with an asterisk (\*) over each significant value assessed by Dunnett's test ( $p$  0.05). Each assay was performed with a different batch of seeds; (A) results for  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD and (B) results for HP- $\beta$ -CD.

Chromaster equipped with a DAD model 5430 and a Phenomenex Gemini C<sub>18</sub> 250 × 4.6 mm column with a 5 μm particle diameter and 110 Å pore size in gradient mode. HPLC gradient quality solvents were supplied by Avantor (Pennsylvania, USA). Sigma-Aldrich Co. (St Louis, Missouri), Merck (Darmstadt, Germany), Alfa Aesar (Ward Hill, Massachusetts) or TCI (Oxford, UK) supplied the reagents. Parasitic weed seeds were kindly provided by professors Leonardo Velasco (CSIC, Córdoba, Spain) and Maurizio Vurro (National Research Council – Institute of Sciences of Food Production, Bari, Italy).

### Synthesis of phthalimide-lactones

**PL01**, **PL04**, and **PL07** were synthesized using the procedure described in the literature.<sup>15</sup>

### Calibration of phthalimide-lactones in RP-HPLC

Eight standard solutions of each compound **PL01**, **PL04**, and **PL07** (0.025–1 mg mL<sup>-1</sup>) were used to calibrate the RP-HPLC system. The method consisted a 15 min gradient at room temperature starting at 7 : 3 H<sub>2</sub>O : MeCN (see Table 4), a flow of

**Table 4** Conditions for the PL analysis method by RP-HPLC at a flow of 1 mg mL<sup>-1</sup>

Time (min)	0	5	7	9	11	13	15
% MeCN	30	70	70	100	100	30	30

1 mL min<sup>-1</sup>, a re-equilibration time of 4 min and a 10 μL injection volume. Due to the low solubility of PLs in H<sub>2</sub>O, samples were dissolved in MeCN (or at the beginning in MeOH) and filtered through a 0.45 μm nylon syringe filter (25 mm, Scharlau NYL2545200) prior to injection. The chromatograms were acquired in triplicate using a DAD set in the wavelength range of 200–381 nm, using the integrated chromatogram setting to measure the areas below the peaks. Retention times for each compound were 8.18 (±0.10), 8.33 (±0.07) and 9.71 (±0.03) minutes for **PL01**, **PL04** and **PL07**, respectively.

The correlations between the mean areas of the peaks in the chromatograms and the concentrations of each phthalimide-lactone were established using two ranges, the first one including the lower concentrations (0.025–0.250 mg mL<sup>-1</sup>) and the second for the higher concentrations (0.250–1.000 mg mL<sup>-1</sup>). In both ranges, the coefficients were  $R^2 = 0.9991$  (**PL01**), 0.9946 (**PL04**), and 0.9973 (**PL07**) for the lower range; and  $R^2 = 0.9802$  (**PL01**), 0.9799 (**PL04**), and 0.9767 (**PL07**) for the higher range. The correlations were considered good enough to be used in the next experiments, by employing the resulting eqn (1)–(6). LOD and LOQ were as follows: 0.010 mg mL<sup>-1</sup> and 0.030 mg mL<sup>-1</sup> (**PL01**), 0.025 mg mL<sup>-1</sup> and 0.075 mg mL<sup>-1</sup> (**PL04**), and 0.017 mg mL<sup>-1</sup> and 0.052 mg mL<sup>-1</sup> (**PL07**).

$$C(\mathbf{PL01}) = \frac{\text{Area} - 41\,709}{6 \times 10^6} \text{ mM} \quad (11)$$

$$C(\mathbf{PL01}) = \frac{\text{Area} - 1\,000\,000}{2 \times 10^6} \text{ mM} \quad (12)$$

$$C(\mathbf{PL04}) = \frac{\text{Area} - 33\,917}{7 \times 10^6} \text{ mM} \quad (13)$$

$$C(\mathbf{PL04}) = \frac{\text{Area} - 930\,509}{4 \times 10^6} \text{ mM} \quad (14)$$

$$C(\mathbf{PL07}) = \frac{\text{Area} - 57\,742}{7 \times 10^6} \text{ mM} \quad (15)$$

$$C(\mathbf{PL07}) = \frac{\text{Area} - 1\,000\,000}{3 \times 10^6} \text{ mM} \quad (16)$$

### Phase-solubility diagrams for the complexation of phthalimide-lactones by HPLC

Solubility studies were carried out by following a modified Higuchi and Connors method<sup>22</sup> which was reported previously.<sup>20</sup> An excess of **PL01**, **PL04**, or **PL07**, above their solubility in H<sub>2</sub>O (2–3 mg) was added to aliquots of 5 mL of each CD in a concentration range of 0–12 mM in each tube. The concentrations of the compounds in these aliquots were evaluated by RP-HPLC using the methods and the equations described previously for calibration curve establishment. As previously reported for sesquiterpene lactones,<sup>20</sup> the retention times for the peaks of phthalimide-lactones changed slightly in comparison with the standard depending on the CD concentration (by less than one minute).

### ITC

Isothermal titration calorimetry was employed to confirm the 1 : 2 complex formation between **PL01** and **HP-β-CD** deduced



from the phase-solubility diagrams. MicroCal PEAQ-ITC was kindly supplied by Malvern® to carry out the experiment. **PL01** was placed in the cell of the ITC with a concentration of  $5.00 \times 10^{-5}$  M and 2-hydroxypropyl- $\beta$ -cyclodextrin was continuously added ( $8.00 \times 10^{-4}$  M) during 20 injections at 25.3 °C (2.00  $\mu$ L per injection). The experiment was performed in Milli-Q water and stirred at 750 rpm.

### Preparation of the solid complexes

Based on our previous findings with sesquiterpene lactones,<sup>20</sup> the co-precipitation method was chosen and the following conditions were used: 5.4 mg of **PL01** (0.022 mmol) or 6.9 mg **PL07** (0.022 mmol) were dissolved in 500  $\mu$ L MeCN and added dropwise to 1.25 mL of a  $\beta$ -CD solution (17.6 mM) in deionized H<sub>2</sub>O. The same amount of either **PL01** or **PL07** was dissolved in 200  $\mu$ L or 400  $\mu$ L MeCN and added dropwise to 500  $\mu$ L of  $\alpha$ -CD (44 mM) or  $\gamma$ -CD (44 mM) aqueous solutions to prepare the corresponding complexes of  $\alpha$ - and  $\gamma$ -CD, respectively. In the case of **HP- $\beta$ -CD**, 0.022 mmol of **PL01** were dissolved in 200  $\mu$ L MeCN and added dropwise to 500  $\mu$ L of **HP- $\beta$ -CD** (88 mM) and 0.025 mmol of **PL07** were dissolved in 446  $\mu$ L MeCN and added dropwise to 500  $\mu$ L of **HP- $\beta$ -CD** (49 mM). Additions were carried out while stirring at room temperature for 72 h, then the solvents were removed on a rotary evaporator at 50 °C and dried overnight in a vacuum at 37 °C to remove any remaining H<sub>2</sub>O.

### Solubility measurements

Each complex and each phthalimide-lactone evaluated (**PL01**, **PL04**, and **PL07**) were separately added in excess to tubes containing 5 mL deionized H<sub>2</sub>O and stirred at 25 °C until no more solid dissolved. Each tube was centrifuged at 4400 rpm, the supernatant filtered through a 0.45  $\mu$ m nylon syringe filter and an aliquot of 1 mL was analyzed by RP-HPLC. In order to minimize the measurement error, samples of complexes were analyzed in triplicate and samples of phthalimide-lactones in quintuplicate. Concentration was obtained using the calibration equations reported above to calculate the solubility of each compound.

### Molecular modelling

Computational studies were carried out by using the software HyperChem v7.5 for Windows (Hypercube, Inc.) to predict the most stable geometries of the hypothetical complexes in H<sub>2</sub>O. Periodic boxes were built by including the guest (**PL01** or **PL07**), the host ( $\beta$ -,  $\gamma$ -, or **HP- $\beta$ -CD**) and a solvation shell of H<sub>2</sub>O molecules. Compound **PL04** was not included among the calculations since no interaction was found in the phase-solubility diagrams. Since the interaction with  $\alpha$ -CD was almost negligible and assumed to be related to a micellar effect rather than a complexation phenomenon, this CD was also excluded. Two kinds of aggregates were designed: separate host and guest, and guest inserted into the host. The size of the periodic boxes was initially set to fit 250 molecules of H<sub>2</sub>O as reported previously;<sup>20</sup> however, the high use of RAM memory (over 1 GB) when optimizing the geometry of the complexes with **HP- $\beta$ -CD** caused an error of exhausted memory after the 10th iteration (first cycle) due to limitations of the software. In order to

avoid this problem, the periodic box sizes for this CD were adjusted to contain a total of 222 H<sub>2</sub>O molecules (in random positions in the box), which were manageable for the software in all the cases without errors. The model for **HP- $\beta$ -CD** was based on PDB 3CGT and built as reported previously,<sup>32</sup> the models for the other CDs were already reported,<sup>20</sup> and the **PL**s were built on ChemDraw v20.1 (PerkinElmer) and their geometry was pre-optimized in a vacuum using PM3, which is the same level of calculation for the host-guest-solvation water complex. Since the employed **PL**s possess a stereochemical center, the same enantiomer, with *S*-stereochemistry (**S-PL01** and **S-PL07**), was built in the three cases ( $\beta$ -,  $\gamma$ -, or **HP- $\beta$ -CD**) to allow comparison. Geometry optimizations for the supramolecular systems were carried out using PM3. Four possible insertions (A–D) of the guests into the hosts and a last additional calculation with separate guests and hosts were considered for the 1 : 1 complexes with  $\beta$ -CD, considering that the **PL** could approach, either through the lactone or the phthalimide backbone, the hydrophilic or lipophilic part of the CD. A fifth possible insertion was also considered in the bigger  $\gamma$ -CD (**E**) where the molecules fit inside the cavity in the horizontal position. Since the complex of **PL01** with **HP- $\beta$ -CD** was found to be a 1 : 2 type complex, this was also taken into consideration and the system was built accordingly, increasing the amount of possible insertions to 24 by combining the different orientations of **PL01** with the possibilities of the **PL** approaching both CDs in a 90° position adding to the original insertions already considered in the other CDs (for a total of 6 possibilities, models with numbers 1–6) and 4 possible orientations of the two CD molecules (confronting the CDs on both their HP-residues, and then rotating 180° each CD at a time, or both, models with letters A–D). The models for the **PL01-HP- $\beta$ -CD** 1 : 1 type complex were also studied to confront with the other CDs, by including 250 molecules of H<sub>2</sub>O this time, like in  $\beta$ - and  $\gamma$ -CD. For both systems, the model for the separate host and guest was also prepared and optimized. In summary, a total of 57 models were carried out for each different guest (**PL01** and **PL07**)-host ( $\beta$ -,  $\gamma$ - and **HP- $\beta$ -CD**) system and the 5–24 geometric possibilities. The mean calculation time was 20–30 days, depending on the number of atoms, on an average computer with an Intel Core i7-6900K CPU at 3.20 GHz, 3201 MHz, 8 core, 16 logic processors, using 64 GB RAM and running on Microsoft Windows 10 Enterprise.

### Bioassays on parasitic weeds

Free CDs, **PL01**, **PL04** and **PL07** and their complexes were tested on the germination of seeds of *O. cumana*, *P. ramosa*, and *O. crenata* in the concentration range of 100–0.1  $\mu$ M by following the previously reported method.<sup>30</sup>

## Conclusions

The complexation of phthalimide-lactones with cyclodextrins allowed us to increase their solubility to different degrees depending on the presence of functional group(s) at the aro-



matic ring and the cyclodextrins employed. The best results were obtained with **PL01**, with an increase of water solubility almost 4 times.

The titration calorimetry employed in this study allowed us to verify the results with the experiment designed by Higuchi and Connors, but using a significantly smaller amount of the sample and experiment time, thus determining correctly the stoichiometry of the complex **PL01-HP- $\beta$ -CD** formed as 1:2. The molecular modelling supported theoretically the experimental results of the phase-solubility diagram, the solubility and the bioactivity experiments, thereby allowing us to explain and predict the behavior of phthalimide-lactones in water. In fact, the lowest energies were obtained for **PL-HP- $\beta$ -CD** aggregates, which produced the most soluble complexes.

All the results above demonstrated that  **$\beta$ -CD** and its derivative **HP- $\beta$ -CD** are the best choices amongst the studied **CDs** for the complexation of **PLs**. The bioactivity results demonstrated that without the addition of an organic co-solvent, similar levels of bioactivity can be achieved after treatment with cyclodextrins. This bioactivity can even be increased, as such was the case of **HP- $\beta$ -CD**.

This work concludes that future studies on germination inducers chemically similar to these compounds should be carried out with these cyclodextrins or their derivatives. The use of cyclodextrins would allow us to maintain or even increase the bioactivity of germination inducers without using organic co-solvents as a greener and safer alternative to traditional preparations.

## Author contributions

Conceptualization: A. C. P., F. J. R. M., J. A., and J. M. G. M.; data acquisition: A. C. P., F. J. R. M., and C. R.; data curation: A. C. P., F. J. R. M., J. A., and C. R.; writing—original draft preparation: A. C. P., F. J. R. M., and C. R.; writing—review and editing: A. C. P., F. J. R. M., J. A., C. R., J. M. G. M., J. A. A., S. S., and F. A. M.; supervision: J. M. G. M., and F. A. M.; funding acquisition: J. M. G. M., and F. A. M. All authors have read and agreed to the published version of the manuscript.

## Conflicts of interest

There are no conflicts to declare.

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