Research Article

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On the formulation of disulfide herbicides based on aminophenoxazinones: polymeric nanoparticle formulation and cyclodextrin complexation to combat crop yield losses

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Abstract

Background: The resistance of weeds to herbicides is a significant issue in ensuring future food supply. Specific examples are *Plantago lanceolata, Portulaca oleracea* and *Lolium rigidum*, which mainly infect rice, wheat, barley and pastures, and cause high yield losses every year. In this regard, natural products and their mimics have provided new hope as a result of their different modes-of-action, activity at low concentrations and reduced pollution effects relative to conventional herbicides. However, the poor water solubility and physicochemical properties of these compounds limit their broad application. These problems can be addressed by formulation techniques, and encapsulation appears to be of great interest.

Results: Disulfide herbicides inspired by aminophenoxazinones have been formulated with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), γ-CD and polymeric nanoparticles (NPs). *In silico* studies were employed to identify which complexes would be generated and complex formation was confirmed by nuclear magnetic resonance spectroscopy. Solubility diagrams were generated to assess any improvement in water solubility, which was enhanced 2–13-fold. Scanning electron microscopy and energy-dispersive X-ray spectra confirmed the success of the formulation process for the nanoparticles. Formulated compounds were evaluated in an *in vitro* wheat coleoptile bioassay, with almost 100% elongation inhibition achieved using only water for the bioassay. Specific *in vitro* testing on weed phytotoxicity showed that the application of core/shell NPs is highly effective in the fight against *P. lanceolata* seed germination.

Conclusions: The formulation of disulfide herbicides with CD complexes and NPs led to an enhancement in water solubility and bioactivity. These systems can be applied in pre-emergent mode against *P. lanceolata*, using only water to prepare the sample, and they showed better activity than the positive controls.

Supporting information may be found in the online version of this article.

Keywords: encapsulation; formulation; aminophenoxazinones; nanoparticles; Plantago

1 INTRODUCTION

Food production must be protected and improved in order to guarantee sustainable development. The population of Europe has reached >700 million and the supply of vegetables must be ensured without uncertainty.[1] Mediterranean crops play a key role in this supply chain, but every year their production is adversely affected by weeds that compete for the main resources in the soil. Among these weeds, common purslane (*Portulaca oleracea*), ribwort plantain (*Plantago lanceolata*) and annual ryegrass (*Lolium rigidum*) are highly problematic as a result of their abundance around several key crops, namely wheat, tomatoes and beans.[2–4]

Commonly used postemergent herbicides have been invaluable in the fight against these weeds, but only once they have germinated and they have grown sufficiently to be visible. 2,4-D, paraquat and flumetsulam are the most common herbicides applied to control these weeds.[5] Nevertheless, resistance phenomena have been observed and these are due to continuous exposure to these agrochemicals, even to the point where biotypes have developed that cannot be affected by the modes-of-

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action of the aforementioned herbicides. The first report of this resistance phenomenon appeared in 2012, but the Weed Science Society of America (WSSA) reiterated the seriousness of this issue in 2020.[6] Furthermore, European Union policies are focused on reducing soil pollution caused by the application of the aforementioned 'traditional' herbicides in order to promote ecologically sound agriculture.[7,8]

As a consequence of this green shift, natural products and their mimics have emerged as an alternative approach to solve problems such as resistance to current commercial herbicides. In particular, 2,2′-disulfanediyldianiline (DiS-NH_2) is a compound inspired by natural structures such as aminophenoxazinones [e.g. 2-amino-3*H*-phenoxazin-3-one (APO)] and benzoxazinones [e.g. 2-hydroxy-7-methoxy-2*H*-benzo[*b*][1,4]oxazin-3(4*H*)-one (DIMBOA)], which have similar activity profiles and structural features (Fig. 1). In addition, these compounds previously have provided interesting results in agriculture as possible herbicides against *L. rigidum*, *Echinochloa crus-galli* and *Urochloa decumbens*.[9] The main drawback with these compounds is their poor water solubility, which limits their broad application and scale-up process to become new bioherbicides.

Techniques such as complexation with cyclodextrins (CDs) have been applied to solve the problem of low solubility. The lipophilic inner cavity of the macrocycle promotes complex formation, with the molecule located inside the toroid and thus avoiding any interaction with the surrounding solvent. The application of CDs previously has provided interesting results in agriculture by allowing the complexation of sesquiterpene lactones. The compounds encapsulated include dehydrocostuslactone, costunolide, (−)-α-santonin and inuloxin, and their water solubility was enhanced 1–46-fold depending on the CDs used. These systems presented strong activity toward parasitic plants.[10,11] There are other encapsulation procedures that can enhance the physicochemical properties of compounds and these include fully organic core-shell nanoparticles (NPs). These suprastructures also have shown impressive results in improving solubility. Mejías *et al.* applied these techniques to encapsulate strigolactone mimics that presented activity against parasitic plants, with increases in water solubility by a factor of 27 achieved for the encapsulated compound.[12]

In the work described here, the complexation and encapsulation of DiS-NH_2 was carried out with four different CDs and polymeric NPs to enhance water solubility and activity against weeds. Solubility diagrams were generated and complexes were synthesized with α-, β-, γ- and 2-hydroxypropyl-β (HP-β-) CDs. The synthesis and characterization by electron microscopy were carried out for the fully organic NPs. In both cases, the encapsulation percentage was evaluated by high performance liquid chromatography (HPLC) to achieve the appropriate concentration in bioassays against *P. oleracea, P. lanceolata* and *L. rigidum*.

2 EXPERIMENTAL

2.1 General information

β-Cyclodextrin [molecular weight (MW) 1134.99 amu] and γ-CD (MW 1297.13 amu) were obtained from TCI (Tokyo, Japan). 2-Hydroxypropyl-β-cyclodextrin (mean MW 1469.40 amu) and polyvinyl alcohol (PVA) were obtained from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). Pluronic F-127® was purchased from Sigma-Aldrich (St Louis, MO, USA). The ^{1}H NMR encapsulation study was carried out at 25 °C using D_2O as solvent with an INOVA spectrometer (Agilent, Santa Clara, CA, USA) at 499.719 MHz. The residual peak for water was referenced to δ 4.79 ppm.

Samples for scanning electron microscopy (SEM) were prepared by depositing a small amount of the NPs directly onto a lacey-carbon coated 200 mesh copper grid. The areas shown in the images were recorded by SEM on an Nova NanoSEM 450 (FEI, Hillsboro, OR, USA). Bright-field (BF), dark-field (DF), high-angle annular dark-field (HAADF) and secondary electron (SE) images were recorded using the annular-type detector installed on the microscope. Images were recorded at voltages of 30 kV using a working distance in the range 6.5–6.6 mm.

Aqueous solubility and solubility diagrams were generated by HPLC techniques on a VWR Hitachi Chromaster (VWR International, Radnor, PA, USA) with a model 5430 DAD, model 5310 column oven, model 5260 autosampler and model 5110 solvent pump. A Rever® C18 column (25 × 4.6 mm; Phenomenex, Macclesfield, UK)) with a 5-µm pore size was employed. Solvents were purchased from VWR and filtered through a Durapore® 0.4-µm filter membrane (3M, Saint Paul, MN, USA) before use in the HPLC apparatus.

2.2 Synthesis of DiS-NH_2

The method developed by Oliveira *et al.*[9] was followed to synthesize DiS-NH_2.

2.3 Complexation with CDs

Solid complexes were obtained by co-precipitation. DiS-NH_2 (20 mg, 8.0 mM) was dissolved in ethanol (10 mL). In a different vessel, CD (8.0 mM) was dissolved in milli-Q water and the solution was added dropwise to the first solution. The mixtures were stirred for 1 h, frozen in liquid nitrogen (N_2) and lyophilized to obtain the solid complexes.

Density functional theory (DFT) calculations were performed using Gaussian v16 at a B3LYP/Lanl2dz level of calculation. In addition, a continuum polarizable model was applied to simulate water as a solvent during the development of the calculation. ΔG was evaluated to check the spontaneous formation of the complex.

A 400 MHz spectrometer (Bruker, Billerica, MA, USA) was used for ^{1}H nuclear magnetic resonance (NMR) experiments to evaluate complex formation. D_2O was used as deuterated solvent, referenced at 4.79 ppm.

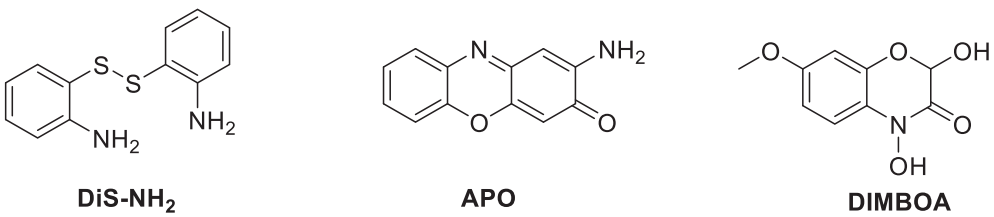

Figure 1. Structures of the disulfide compound DiS-NH_2, aminophenoxazinone APO and benzoxazinone DIMBOA.

2.3.1 Solubility diagrams

The aqueous solubility and complexation were analyzed by the solubility diagram technique, as described by Cala *et al.*[10] Seven aqueous solutions with different concentrations of CDs were prepared (0–12 mM) and 5-mL aliquots (H_2O milli-Q quality) were added to assay tubes (21 in total). DiS-NH_2 (5 mg, 1.2 mM) also was added to each assay tube and the mixtures were stirred for 5 days at 25 °C and 550 rpm using a Synthesis 1® system (Heidolph Instruments GmbH & Co., Schwabach, Germany). Once the time had elapsed, the samples were centrifuged to collect the supernatant which was analyzed by HPLC. Each aliquot was filtered through a 0.45-µm nylon membrane. Experiments were carried out in sextuplicate and data represented in diagrams are the mean of the area analyzed by HPLC.

2.4 Encapsulation with polymeric NPs

Encapsulation with Pluronic F-127® was carried out by the method reported by Mejias *et al.*[12] DiS-NH_2 (0.5 g) was dissolved in pure ethanol (20 mL). In a different vessel, Pluronic F-127® (1.5 g) was dissolved in milli-Q water (120 mL). A solution of PVA (10 mg) in water (2.5 mL) was prepared separately.

The Pluronic F-127® solution was cooled in ice as it was stirred. When dissolution was complete, the DiS-NH_2 solution was added dropwise through a 0.45-µm nylon membrane and the solution was stirred for 5 min. Finally, the PVA solution was added dropwise and the sample was frozen in liquid N_2 to avoid excessive growth of the NPs. The solution with the functionalized NPs was dried by lyophilization. A fine yellow powder was obtained. The percentage of encapsulation was assessed by dissolving 1 mg of the NPs in 1 mL acetonitrile. Vigorous shaking was carried out in order to break the particles and release DiS-NH_2. The sample was filtered through a 0.22-µm nylon membrane and the solution was analyzed by HPLC to determine the concentration of DiS-NH_2. UV–visible absorbance was the detection mode at 260 nm, a wavelength that no other compounds present in the sample absorbed. The retention time of DiS-NH_2 was 4.67 min in isocratic mode (30% MeOH: H_2O). The areas of the peaks were converted to concentrations with a calibration curve ([DiS-NH_2] (ppm) = $2{\cdot}10^{-4}{\cdot}$area + 0.4139, R^2 = 0.9985) (Supporting information, Figure S1).

2.5 *In vitro* etiolated wheat coleoptile bioassay

The experiments were carried out according to a procedure from the literature[13] with some modifications. DiS-NH_2 molecules encapsulated within NPs and CDs were added without being predissolved in dimethyl sulfoxide (DMSO), but instead diluted in an aqueous phosphate-buffered saline solution (PBS) containing 2% sucrose at pH 5.6 and the corresponding concentrations of the tested compound (10, 30, 100, 300 and 1000 µM). The concentrations of the bioactive compound encapsulated within the NPs were recalculated by applying the encapsulation percentage. A 10 mM citric acid/potassium phosphate buffer solution was used to avoid osmotic stress. The commercial herbicides Stomp® Aqua and Logran® were employed as positive controls. They are based on pendimethalin and triasulfuron, two classic herbicides with halogens and nitrous functional groups that are not ecofriendly.

The half maximal inhibitory concentration (IC_{50}) values were obtained using the Prism v5.00 software package (GraphPad, San Diego, CA, USA). The data then were adjusted to a sigmoidal dose–response model (constant slope), where possible, and goodness-of-fit was described by the regression coefficient (R^2).

2.6 *In vitro* phytotoxicity bioassays

The selection of target plants was based on an optimization process – with some modifications –carried out in our investigation into weed species.[14] The monocotyledon *L. rigidum* and the dicotyledons *P. oleracea* and *P. lanceolate* were selected. Bioassays were conducted using Petri dishes (50-mm diameter) with one sheet of Whatman No. 1 filter paper (Cytiva, Marlborough, MA, USA) as a support. Germination and growth were conducted in aqueous solutions at controlled pH by using 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH (pH 6.0). The compounds to be assayed were dissolved in water only or water +0.5% DMSO, depending on whether they were encapsulated or not, and these solutions were diluted with buffer so that test concentrations for each compound (1000, 333, 100, 33 and 10 µM) were achieved. Four replicates were used for each weed species, each containing 20 seeds. Treatment, control or internal reference solution (1 mL) was added to each Petri dish. After adding the seeds and aqueous solutions, Petri dishes were sealed with Parafilm® (VWR) to ensure closed-system models. Seeds were further incubated at 25 °C in a ICE 700 (Memmert GmbH & Co.KG, Schwabach, Germany) controlled environment growth chamber. The photoperiod was 16 h:8 h, light:dark for each weed. Bioassays took 8 days. After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. Evaluated parameters (germination rate, root length and shoot length) were recorded using Fitomed software, which allowed automatic data acquisition and statistical analysis. Data were analyzed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percentage differences from the control. Zero represents control, positive values represent growth stimulation and negative values represent inhibition. The commercial herbicide Stomp® Aqua was employed as positive control.

3 RESULTS AND DISCUSSION

Solubility diagrams provide relevant information about not only the increase in the water solubility of DiS-NH_2, but also the number of molecules that fit inside of the cavity of the CDs. Furthermore, it is possible to obtain information about the binding constants of the complexes and their behavior in solution. In this work it was planned to use the natural CDs (α, β and γ) together with the highly soluble HP-β-CD. However, before carrying out the experiments DFT calculations were performed in order to assess whether the disulfide could fit inside the toroidal cavity.

According to simulation studies at 298.15 K and 1.0 atm, only the complex between the disulfide and γ-CD will be generated spontaneously from the solutions (Figs S2–S6). In the cases of α-CD, β-CD and HP-β-CD, the dimensions of the inner cavities are 0.57 (the diameter for A-CD) and 0.78 nm (for B-CD and HP-B-CD), respectively, whereas DiS-NH_2 has a diameter of 0.58 nm for the aromatic ring. Although the disulfide bond, which is the middle part of the molecule, is small, it is still necessary for the aromatic unit to cross into the toroidal cavity first. This could explain the high positive Gibbs' free energy for the α-CD complex as this CD has the smallest cavity (Fig. S2). In the cases of β-CD and HP-β-CD, the cavity is bigger than DiS-NH_2 (Figs S3, S4). The process to fit the DiS-NH_2 inside these CD would demand a lot of energy, and could happen at high temperature or/and high pressure. We would need to strain the CD bonds, thus increasing the internal energy, because the cavity of the CD and the molecular volume of DiS-NH_2 are quite similar. However, the branched substituted groups found in HP-β-CD allow the easy generation of an

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exclusion complex by coordination of a guest and 2-hydroxypropyl groups. This phenomenon has been reported several times with small organic molecules, as in the case of diloxanide furoate reported by Aloisio *et al.*[15–17] The largest natural CD, γ-CD, is the only one that gave a negative ΔG value, not only with a 1:1 but also a 1:2 complex (Figs S5, S6). The calculated relative Gibbs free energies and formation enthalpy with α-CD, β-CD and HP-β-CD are shown in Table 1; positive values would preclude the formation of the complexes in solution.

The calculations were performed at the B3LYP/Lanl2dz level and water solvent was simulated by a continuum polarizable model. The thermochemical values (Table 1) also showed that the enthalpies of complexes generated with β-CD and γ-CD (both 1:1 and 1:2 complexes) are exothermic, whereas the formation of HP-β-CD and α-CD are endothermic processes. The arrangements in the complexes are shown in Figs S5 and S6, where the disulfide bond is allocated mainly in the center of the toroidal void. The complexation of two guests in the same host increases the relative Gibbs free energy of formation when compared to a 1:1 complex, but double complexation is highly favored in terms of absolute values. Specifically, DFT calculations show π–π staking of the aromatic rings of DIS-NH_2 in the inner cavity of the CD, which also would help to stabilize the complex.

On the basis of the results obtained by modelling the complexes, only γ-CD and HP-β-CD were selected to generate the solubility diagrams. In the case of the latter, the decision was made based on the possibility of generating the exclusion complex with high water solubility.[18] The solubility diagram for DiS-NH_2 with the two aforementioned CDs is shown in Fig. 2. In the case of DiS-NH_2·HP-β-CD, a linear increase of the disulfide solubility in water can be observed as the concentration of the CD increases. A maximum solubility was reached close to 12 mM, after which the linearity deviates negatively. This system is an A_N-type model according to Higuchi–Connors,[19] who suggested the formation of higher-order complexes with a ratio >1:1. The slope of the fitting function ([DiS-NH_2] = 0.1333 + 0.1698·[HP-β-CD]) is not close to unity and this means that disulfides must be present around one of the CDs and establish interactions with the outer part of the structure.[16] The γ-CD solubility diagram is consistent with a B_S-type model in which the complex has water solubility, albeit limited.[20,21] In the initial linear region (from 0 to 2 mM) the addition of the disulfide did not affect the CD solubility, but in the plateau region (from 2 to 6 mM) a complex formed that had limited solubility. In this region of the diagram the addition of the γ-CD did lead to an increase in the water solubility for DiS-NH_2 and the generation of new complexes began. Finally, in the region >6 mM the complex generated start to precipitate, and from 10 to 12 mM a new complex with low solubility was the most abundant in the solution. The stoichiometry of the complex increased to higher-order – possibly a 2:1 DiS-NH2: γ-CD.[22]

Table 1. Thermodynamic values for complexes generated between DiS-NH_2 and different CDs

Complexes	ΔG formation	ΔH formation
AlphaCD·DiS-NH_2	39.491085	28.767737
BetaCD·DiS-NH_2	9.987289	−6.760685
GammaCD·DiS-NH_2	−120.807688	−122.769247
GammaCD·2DiS-NH_2	−105.343305	−111.754927
HP-BetaCD·DiS-NH_2	17.224875	2.526315

The values observed in the graphical representation allow a calculation of the increase in DiS-NH_2 solubility in water in the presence of different CDs. Regarding the last concentration of HP-β-CD added before the negative deviation from linearity, it is possible to estimate the new aqueous solubility of the DiS-NH_2·HP-β-CD exclusion complex. Free disulfide in water has a solubility of 0.148 mM, whereas the addition of HP-β-CD increases the solubility to 1.974 mM. Thus, addition of this non-natural CD led to an increase in the solubility by 1333%. In the case of γ-CD, it was decided to evaluate the increase in solubility in the plateau region alone before the formation of complexes of higher order, which are less soluble than the first complex. A total of 0.152 mM DiS-NH_2 can be solubilized after encapsulation with γ-CD and this represents a solubility increase of 195%.

The complexes were studied by ^{1}H NMR spectroscopy to assess how the new chemical environment affected the encapsulated molecules. In this experiment a solution of 1.2 mM DiS-NH_2 in 5 mL D_2O was prepared and HP-β-CD or γ-CD was added to give a 6 mM concentration. The mixture was stirred for 1 h and was then filtered before recording the ^{1}H NMR spectra. The NMR spectrum of the free disulfide in deuterated water is shown in Fig. 3

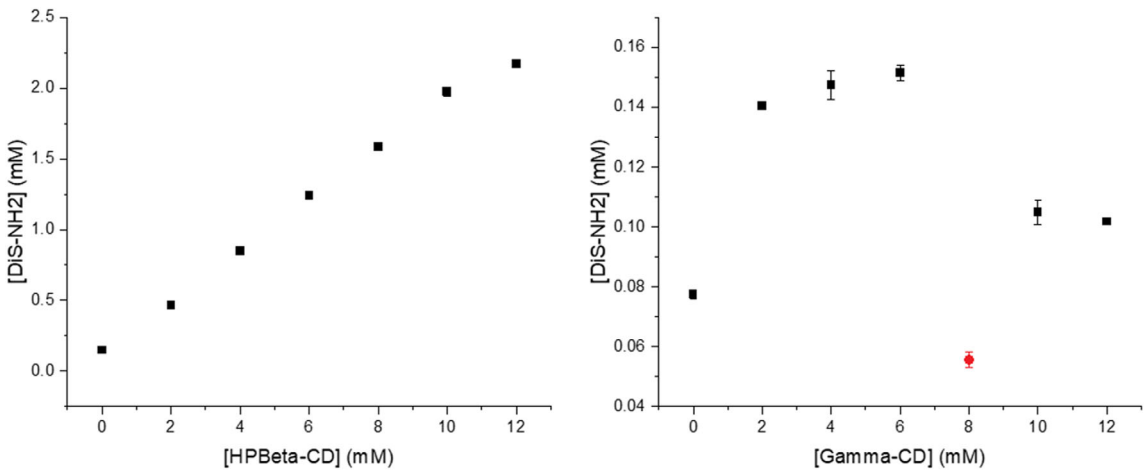

Figure 2. Solubility diagrams of DiS-NH_2 with (left) HP-β-CD and (right) γ-CD.

along with those of the possible complexes with γ-CD and HP-β-CD. The signal:noise ratio in pure DiS-NH_2 is due to the low water solubility, and this ratio was decreased in both cases after complexation. Regarding HP-β-CD, it was observed that the best signal:noise ratio is consistent with the marked increase in solubility seen in the solubility diagrams. The chemical shifts for this complex are very similar to those of free DiS-NH_2, an observation that also is in agreement with the results of DFT studies and suggests the presence of the exclusion complex. In this respect, most of the interactions will be with the solvent and only the stabilization with the branched substituents (2-hydroxypropyl groups) slightly modifies the chemical shifts. By contrast, γ-CD complexation leads to a different chemical microenvironment for the DiS-NH_2 due to the formation of the inclusion complex. The multiplicities of the signals are not defined and these signals are broader as a consequence of the nonhomogeneous chemical environment. On considering the Mulliken charges of the DiS-NH_2 atoms (Table S1) it is apparent that the charge nucleus density is modified and this affects the part of the molecule located within the CD. This situation explains the pronounced change in the shifts in the ^{1}H NMR signals. In this case the formation of the 1:1 complex (DiS-NH_2·γ-CD) alone is evidenced by the presence of one set of signals. Furthermore, a solution was prepared to select only the simple complex and this was filtered to avoid the presence of the less soluble high-order complex expected according to the solubility diagram.

In the search for a formulation that would provide a higher water solubility, encapsulation with polymeric NPs was investigated. Pluronic F-127® was selected to generate a core/shell system with DiS-NH_2 present in the inner cavity. The methodology described by Mejias *et al.*[12] was employed to encapsulate the DiS-NH_2 with Pluronic F-127® and this polymer was functionalized with PVA at the surface. The temperature of the system was decreased rapidly with liquid N_2 to avoid growth of the NPs. The solvent was evaporated by lyophilization. The resulting solid was a pale yellow powder that was analyzed first by SEM. It can be seen from Fig. 4 that the PVA generated a matrix in which the NPs are embedded. The matrix is >1 μm in diameter but it can be seen in Fig. 4(C) that there are small particles located inside it. According to Fig.S7, NP·DiS-NH_2 particles have a mean particle size of 14.1 nm and this is consistent with previous results obtained for this kind of particle. It was important to determine the elemental composition of the particles to confirm the presence of the disulfide within the nanostructures. EDX analysis allows an evaluation of the energy loss of electrons in the different atomic shells and these values can be unequivocally assigned to different atoms [Fig. 4(B)]. Exposure of NP·DiS-NH_2 to the electron beam in the area marked by the red square [Fig. 4(A)] led to an increase in a signal with a value of ≈2.31 KeV in the spectra [Fig. 4(B)]. This peak correlates to the K-edge value for sulfur (S) and this atom is present only in the aminophenoxazinone mimic (DiS-NH_2). EDX analysis of empty NPs [Fig. 4(D)] did not give rise to this signal. Furthermore, the S signal was observed only on evaluating the specific area of the matrix with the NP·DiS-NH_2 and not in other parts of the lacey-carbon/copper grid. Carbon, nitrogen and oxygen signals were observed in both samples (empty NP and NP·DiS-NH_2) due to the composition of the polymer, PVA and herbicide, but the S signal was only observed when DiS-NH_2 was present in the sample, thus confirming the presence of the herbicide in the NPs.

Before performing bioassays to evaluate the formulations, it was necessary to analyze the percentage of DiS-NH_2 inside the NPs.

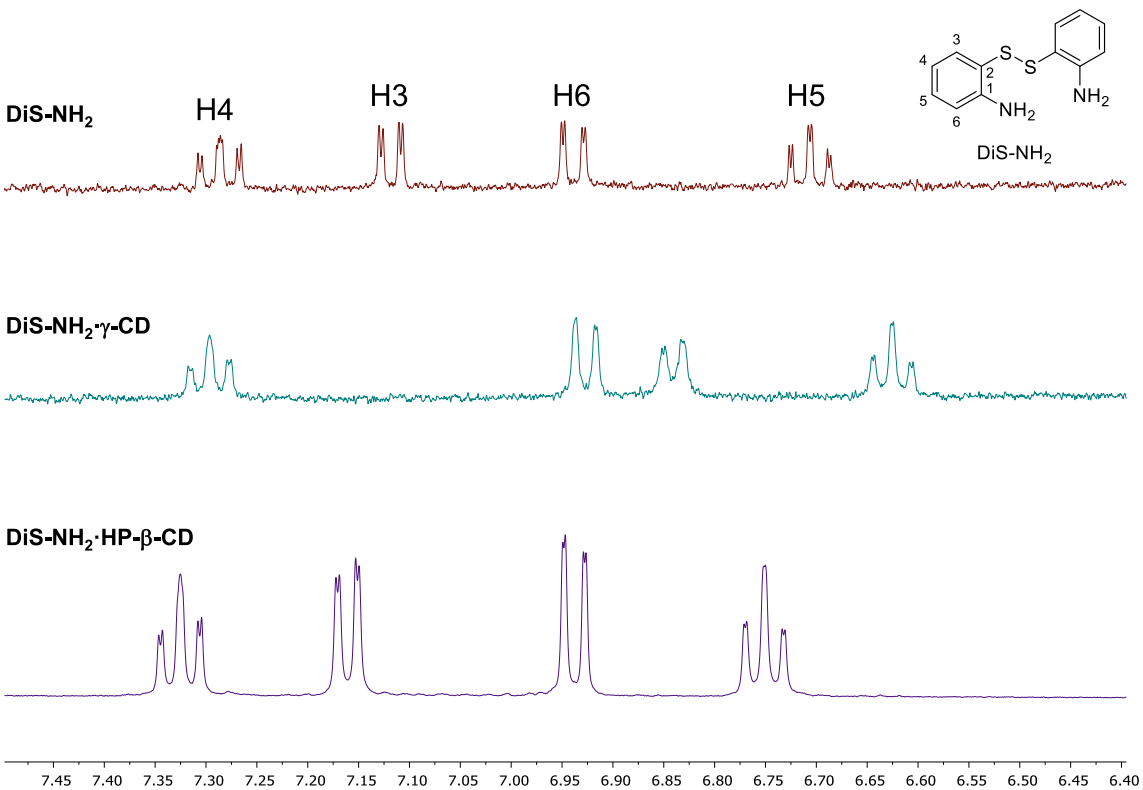

Figure 3. (Top) ^{1}H NMR spectrum of DiS-NH_2 in D_2O. (Middle) ^{1}H NMR spectrum of DiS-NH_2·γ-CD in D_2O. (Bottom) ^{1}H NMR spectrum of DiS-NH_2·HP-β-CD in D_2O.

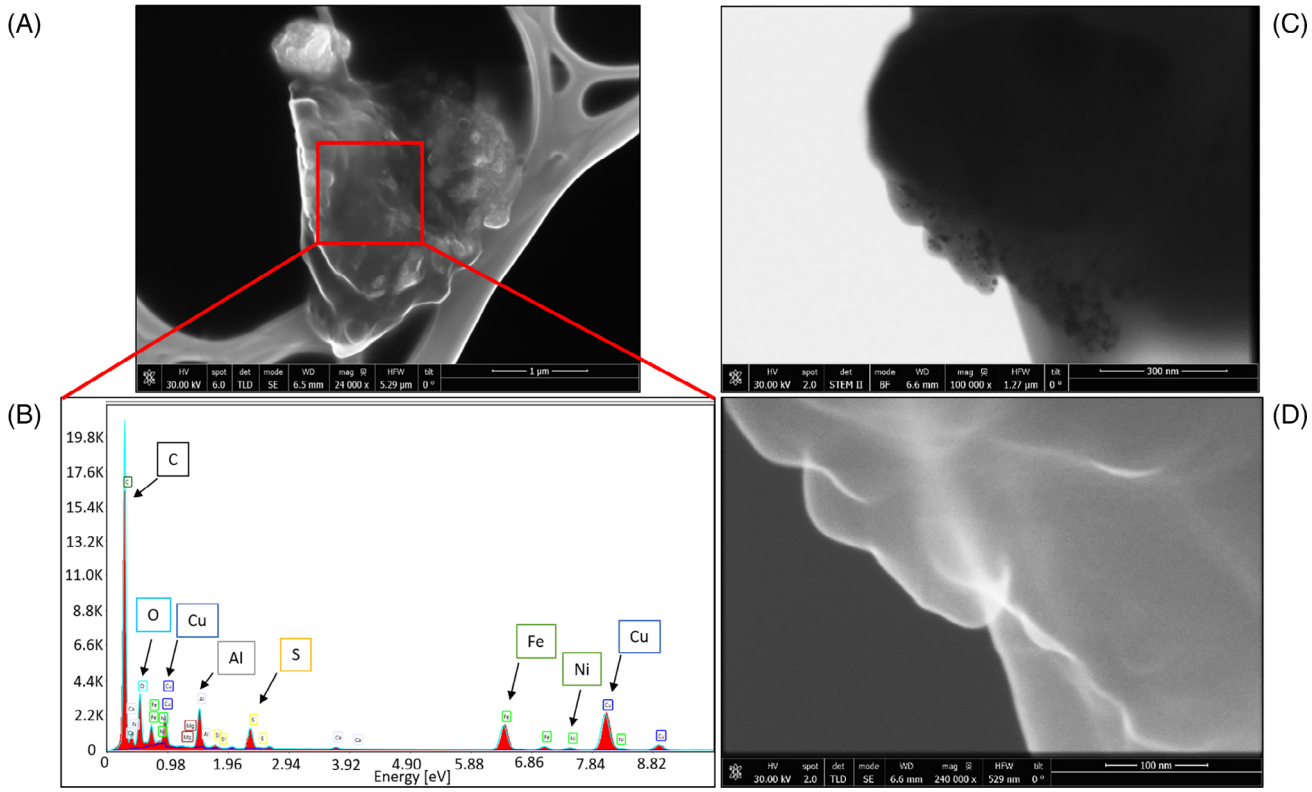

Figure 4. (A) SEM images recorded in secondary electrons mode of the PVA matrix with Pluronic F-127® NPs with embedded DiS-NH_2. (B) EDX spectra of elements found in the sample NP·DiS-NH_2. Copper, nickel, iron, aluminum and silicon signals belong to the materials of the grid, the detector and the electron microscope itself. (C) SEM image recorded in bright field mode to show the presence of the NPs filled with DiS-NH_2 embedded in the PVA matrix. (D) SEM image recorded in the secondary electrons mode of empty NP (i.e. without DiS-NH_2).

This information would allow a reasonable comparison of the improvement in activity between the encapsulated sample and the free compound at comparable concentrations. According to the procedure described in Section 2, the NPs were dispersed in acetonitrile to break them up and release the DiS-NH_2 from within. It can be seen from the results in Table S2 that the percentage of encapsulation was ≈21%. This amount can be fully solubilized when it is encapsulated, so therefore it is possible to increase the water solubility of the disulfide to 4.22 mM. In comparison with the solubility of free disulfide in water (0.148 mM), this represents a 28.5-fold improvement (2850%). This finding is consistent with a previous solubility enhancement reported in the literature.[12]

A general phytotoxicity bioassay was carried out *in vitro* on etiolated coleoptiles obtained from wheat (var. Burgos). In this experiment the response of plant cells to DiS-NH_2 can be assessed when it is complexed with CDs or encapsulated inside the NPs. The main results of this bioassay are represented in Fig. 5, which includes the activity of the herbicide tested in buffer solution, using organic solvent and in the encapsulated form only with the buffer solution. Values in negative represent the inhibition of the elongation respect to the control, and positive values represent the stimulation of the wheat coleoptile. The highest concentrations tested (1000 μM) of DiS-NH_2 (water) and DiS-NH_2 (DMSO) displayed similar bioactivity, although lower concentrations have dramatically reduced inhibition when just buffer is employed for dilutions.

All of the host molecules (CDs and NP polymer) tested without the guest were completely harmless according to Fig. 5. A comparison of the activity at 300 μM shows that encapsulation of DiS-NH_2 allowed an increment of the activity. Furthermore, inhibition values at 100 μM also are very good in comparison with free DiS-NH_2 in water. This demonstrates that we have improved the water solubility making the compound available to interact with the coleoptile.

In the case of DiS-NH_2·HP-β-CD a high inhibition of the elongation was observed at maximum concentration, but a rapid reduction of the activity occurred when a 100 μM sample was tested. This behavior was not observed in the case of DiS-NH_2·γ-CD, which showed inhibition of the plant cells at each concentration tested and retained 60% of the inhibition at 100 μM. This observation can be explained by differences in the inclusion complex (DiS-NH_2·γ-CD) and exclusion complex (DiS-NH_2·HP-β-CD) that was already demonstrated by NMR experiments. In the former case, the guest molecule is trapped inside the CD and this makes it easy for the recognition process by the plant cell wall. Most of the exo-proteins of the cell could easily recognize the sugar fragments of the CD to incorporate them into the cytosol. By contrast, the exclusion complex will not offer any advantage in passing through the cell wall. This kind of complex is easier to break down if some proteins interact with the CD. Even dilution processes could affect the stability, as observed with DiS-NH_2·HP-β-CD in Fig. 5. Encapsulation with NPs led to the highest inhibition of the elongation of wheat coleoptiles and the result is very similar to that of the positive control Logran®. This activity is quite remarkable, especially in the case of the 30 μM dose, which gave an inhibition of 40%. The achievement of phytotoxicity with low concentrations is desired in ecofriendly applications to avoid soil pollution after repeated applications. The enhancement of water

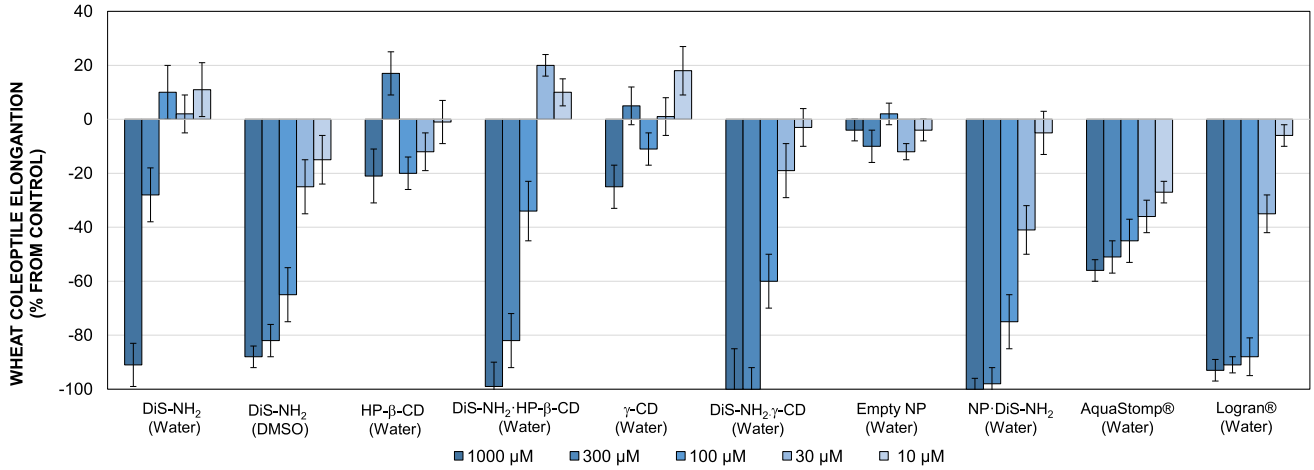

Figure 5. *In vitro* wheat coleoptile bioassay to evaluate the general phytotoxicity of the encapsulated compounds and host molecules. Solvents employed to prepare the dilutions are shown below the name of the sample. In the case of water, this means buffer solution to preserve the osmotic pressure. DMSO means 0.5% DMSO:Buffer. Stomp® Aqua and Logran® were tested as positive controls.

Table 2. IC_{50} values from the coleoptile bioassay

Compounds	IC_{50} (µM)	R^2
DiS-NH_2 (water)	421.50	0.9998
DiS-NH_2 (DMSO)	62.91	0.9910
HP-β-CD	–	–
DiS-NH_2·HP-β-CD	138.5	0.9990
γ-CD	–	–
DiS-NH_2·γ-CD	77.94	0.9956
Empty NP	–	–
NP·DiS-NH_2	41.58	0.9953
Stomp® Aqua	560.3	0.9973
Logran®	37.73	0.9962

solubility could explain the impressive activity of NP·DiS-NH_2 as this is the main physicochemical property that limits the large-scale application of this natural mimic herbicide in the field. Specifically, γ-CD complexation and NP encapsulation display better phytotoxic profiles using water as the medium than free disulfide solubilized in organic solvent.

The IC_{50} values show the enhancement of the inhibition when DiS-NH_2 was complexed with CDs (Table 2). DiS-NH_2·γ-CD had an IC_{50} value that is half that of DiS-NH_2·HP-β-CD and this correlates with the activity profile observed in Fig. 5. Although the water solubility increased, together with the activity of the disulfide, the encapsulation process was not sufficient to preclude the need for organic solvent. However, in the case of DiS-NH_2·γ-CD the activity increased more than five-fold if the IC_{50} values are compared with that of DiS-NH_2 applied only with water. The same happened with NP·DiS-NH_2, which displayed an IC_{50} value comparable to the positive control Logran® (Table 2), even when this standard is applied in DMSO and our formulation is not. This result demonstrates the utility of encapsulation for formulation purposes.

Specific bioassays on weeds that infect important crops also were carried out (Fig. 6). *Plantago lanceolata*, or ribwort plantain, is a problematic weed that causes yield losses in alfalfa crops and pastures for cattle. Furthermore, ribwort has demonstrated increased resistance to classical herbicides since 2018. It is therefore important to find new herbicides to control this weed in the near future.[6,23] Common purslane (*P. oleracea*) also is part of the eudicot clade and it primarily infects rice crops.[24] Ryegrass (*L. rigidum*) is a monocot that mainly affects barley and wheat production,[25] and it also shows resistance phenomena to herbicides such as glyphosate.[26]

In vitro testing on these seeds allowed us to evaluate the inhibition/stimulation of germination, root formation and shoot length. Figure S8 displays the bioassay on *L. rigidum*, showing that DiS-NH_2 and its encapsulated forms do not show high inhibition of this weed. Germination and shoot length parameters seem to be unaffected by the action of DiS-NH_2, regardless of whether the compound is encapsulated with NPs or CDs. In the case of root evaluation, it seems that there is stimulation of the root length when the compound is complexed with γ-CD. This value is really similar to that when DMSO solvent was employed, and it happens only a low concentration. However, they are residual values and DiS-NH_2 remains inactive against *L. rigidum*.

A similar profile of poor activity was displayed when compounds were tested on *Portulaca oleracea* (Fig. S9). Germination is not affected by DiS-NH_2 and shoot length presents an inhibition of 40% at 1000 mM when the bioactive compound is encapsulated inside NPs. Nevertheless, high inhibition values were obtained on root growth. Specifically, the 1000 µM concentration of DiS-NH_2 encapsulated in NPs gave test results similar to those of the positive control. When the disulfide was complexed with CDs, similar inhibition values were obtained to those for DiS-NH_2 dissolved in organic solvent. These findings reinforce the enhancement of the water solubility and physicochemical properties when compounds are formulated using these approaches. Specific inhibition on root length suggests a possible application as a postemergent herbicide directly in the soil.

Plantago lanceolata is the most susceptible weed to the aminophenoxazinone mimic DiS-NH_2 according to the results displayed in Fig. 6. High inhibition values for every parameter tested can be observed, with results comparable, at some concentrations, to that of the positive control Stomp® Aqua. It is interesting that free DiS-NH_2 solubilized in water is quite active at 1000 and 333 µM. In any case, the activity improved markedly after complexation/encapsulation with all formulation strategies tested. Root and

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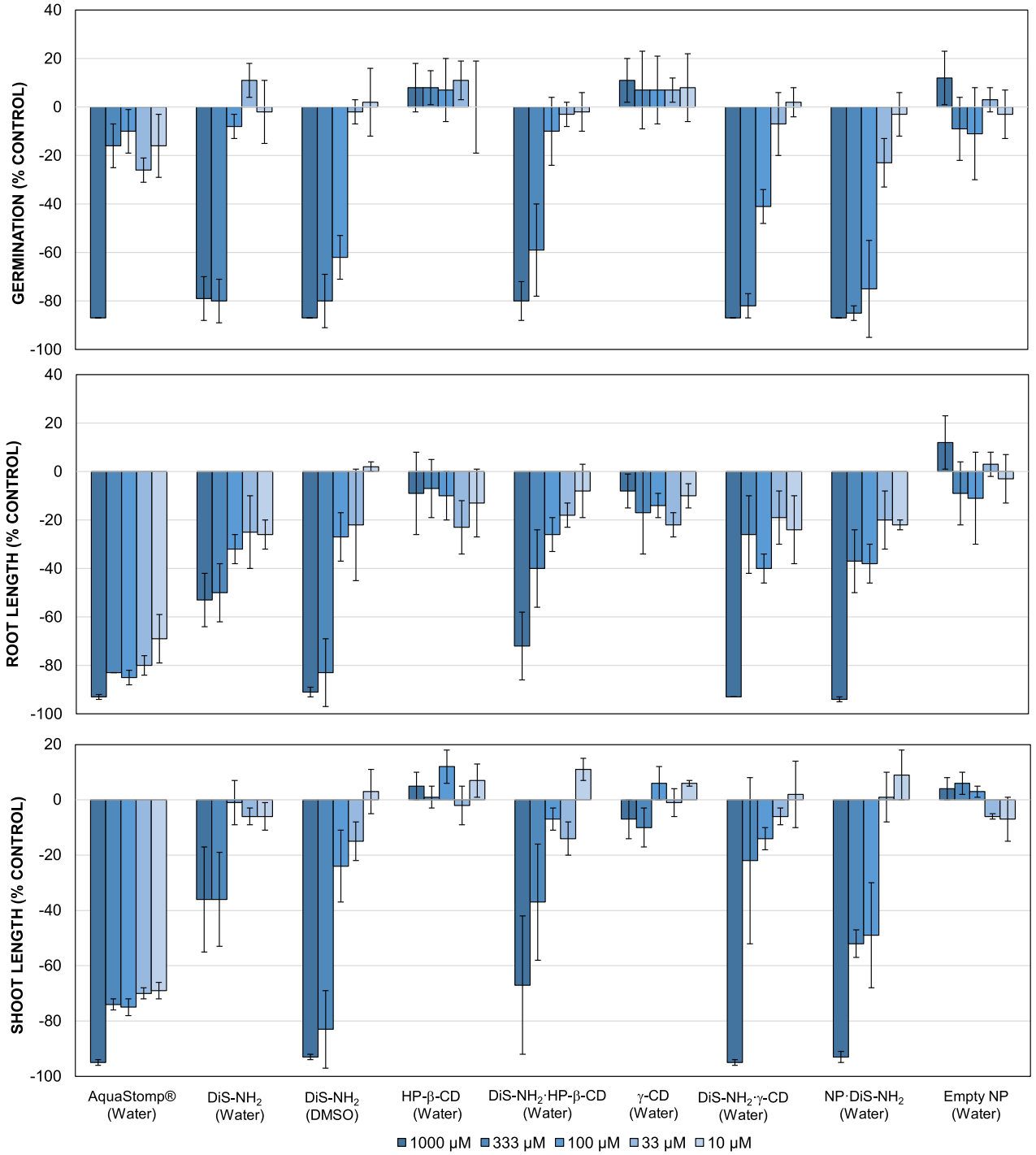

Figure 6. *In vitro* phytotoxicity bioassay on *P. lanceolata*. Different parameters evaluated were germination (top), root length (middle) and shoot length (bottom). The results are referred to negative control. Stomp® Aqua is the positive control. Positive values express stimulation and negative values express inhibition. IC_{50} values from *P. lanceolata* bioassay (germination): DiS-NH_2 (water) 179.7 µM, $R^2 = 0.9821$; DiS-NH_2 (DMSO) 77.05 µM, $R^2 = 0.9847$; DiS-NH_2·HP-β-CD 257.1 µM, $R^2 = 0.9879$; DiS-NH_2·γ-CD 115.20 µM, $R^2 = 0.9945$; NP·DiS-NH_2 50.43 µM, $R^2 = 0.9900$; Stomp® Aqua 662.8 µM, $R^2 = 0.9628$. IC_{50} values from *P. lanceolata* bioassay (root length): DiS-NH_2 (water) 871.80 µM, $R^2 = 0.9872$; DiS-NH_2 (DMSO) 155.20 µM, $R^2 = 0.9725$; DiS-NH_2·HP-β-CD 420.90 µM, $R^2 = 0.9908$; DiS-NH_2·γ-CD 564.30 µM, $R^2 = 0.9081$; NP·DiS-NH_2 455.40 µM, $R^2 = 0.9214$; Stomp® Aqua 0.5969 µM, $R^2 = 0.9945$. IC_{50} values from *P. lanceolata* bioassay (shoot length): DiS-NH_2 (DMSO) 364.60 µM, $R^2 = 0.9943$; DiS-NH_2·HP-β-CD 894.30 µM, $R^2 = 0.9985$; DiS-NH_2·γ-CD 476.30 µM, $R^2 = 0.9912$; NP·DiS-NH_2 238.90 µM, $R^2 = 0.9922$; Stomp® Aqua 1.134 µMM, $R^2 = 0.9693$.

shoot lengths present better inhibition than the positive control for DiS-NH_2·γ-CD, DiS-NH_2·HP-β-CD and NP·DiS-NH_2, albeit only at the maximum concentration tested. Most of the systems display a rapid reduction in efficacy upon dilution. Nevertheless, the compounds prepared seem to be very specific in inhibiting *P. lanceolata* seed germination. In all cases where DiS-NH_2 was complexed with CD or encapsulated in NPs, the inhibition surpassed 50% of the seed bank germination, thus exceeding the

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results with the positive control. IC_{50} values for *P. lanceolata* germination for DiS-NH_2·γ-CD, DiS-NH_2·HP-β-CD and NP·DiS-NH_2 were 115.20, 257.1 and 50.43 μM, respectively. Regarding NP·DiS-NH_2, its inhibition values were ≈80% at low concentration (100 μM), and thus eight-fold higher than that of the positive control and 13-fold better when IC_{50} values are compared.

Pre-emergent application would be the best strategy to deal with *P. lanceolata* infections using NP-DiS-NH_2. The application of this compound to the soil before starting alfalfa/pasture sowing would reduce the yield losses associated with ribwort plantain.

4 CONCLUSIONS

A number of different herbicide formulations were generated using exclusion and inclusion complexes with CDs and core/shell NP encapsulation. DiS-NH_2 is a synthetic disulfide inspired by natural aminophenoxazinones and this was formulated to improve its poor water solubility and physicochemical properties.

DFT calculations demonstrated that only γ-CD can generate an inclusion complex, mainly with a 1:1 host–guest ratio, although a 1:2 complex also is thermodynamically favored. In the case of HP-β-CD, the Gibbs free energy limits the spontaneous formation of the inclusion complex, but the exclusion complex is generated by coordination with branched substituents on the CDs. Solubility diagrams confirmed this hypothesis and allowed the calculation of the water solubility enhancement with these formulation techniques. γ-CD increased the water solubility by a factor of two, in comparison with the free compound, whereas HP-β-CD improved this value to a factor of 13. NMR confirmed the formation of the expected complexes and new chemical shifts were observed after modification of the chemical environment once DiS-NH_2 was complexed.

Polymer NPs also were generated to evaluate a core/shell encapsulation system. Pluronic F-127® was employed to generate the NPs and these were functionalized with PVA at their surface. This formulation was evaluated by SEM and EDX spectroscopy to confirm the presence of DiS-NH_2 in the particles generated. The particles were ≈14.1 nm mean size with a pseudospherical shape. HPLC studies showed that the water solubility had been improved by a factor of 28 in comparison with the free compound.

The results of an *in vitro* wheat coleoptile bioassay showed that these formulation techniques have great potential to improve phytotoxicity. The activity observed was better than that of the free compound dissolved in organic solvent for every encapsulation/complexation system generated. A specific phytotoxicity bioassay on three different weeds was performed to evaluate the real application on a large scale. *Plantago lanceolata, P. oleracea* and *L. rigidum* were selected as a consequence of their problematic elimination in important crops. Among them, the application of NP·DiS-NH_2 as a postemergent herbicide is an option in the fight against *P. oleracea* due to the effect of this compound on this plant root. However, the highest activity was displayed against *P. lanceolata*, where DiS-NH_2·γ-CD, DiS-NH_2·HP-β-CD and NP·DiS-NH_2 surpassed the activity of the positive control against seed germination.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in RODIN at https://rodin.uca.es.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 United Nations Department of Economic and Social Affairs, World Population Prospects—Population Division, 2019. population.un.org. Accessed 9 November 2019.
2 Kumar S, Rana S and Chander N, Efficacy of Carfentrazone and Pinoxaden combinations against mixed weed Flora in wheat. *Pestic Res J* **25**:128–134 (2013).
3 Qasem JR, Nutrient accumulation by weeds and their associated vegetable crops. *J Hortic Sci* **67**:189–195 (1992).
4 Zhang SZ, Li YH, Kong CH and Xu XH, Interference of allelopathic wheat with different weeds. *Pest Manag Sci* **72**:172–178 (2016).
5 Gawn TL, Harrington KC and Matthew C, Weed control in establishing mixed swards of clover plantain and chicory. *N Z Plant Prot* **65**:59–63 (2012).
6 Russell TR, Lulis TT, Aynardi BA, Tang KT and Kaminski JE, Buckhorn plantain (Plantago lanceolata) resistant to 2,4-D in Pennsylvania and alternative control options. *Weed Technol* **35**:297–303 (2021).
7 Law EU, Commission decision of 10 march 2004 concerning the non-inclusion of atrazine in annex I to council directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing this active substance. Official Journal of the European Union, 2004. https://eur-lex.europa.eu/eli/dec/2004/248/oj.
8 European Commission Commission Staff Working Document Impact Assessment Report. Accompanying the document proposal for a regulation of the European Parliament and of the council on the sustainable use of plant protection products and amending regulation (EU) 2021/2115. 2021. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52022SC0170&qid=1663580565339. Accessed 19 September 2022.
9 Oliveira SCC, Andrade CKZ, Varela RM, Molinillo JMG and Macías FA, Phytotoxicity study of ortho-disubstituted disulfides and their acyl derivatives. *ACS Omega* **4**:2362–2368 (2019).
10 Cala A, Molinillo JMGG, Fernández-Aparicio M, Ayuso J, Álvarez JA, Rubiales D *et al.*, Complexation of sesquiterpene lactones with cyclodextrins: synthesis and effects on their activities on parasitic weeds. *Org Biomol Chem* **15**:6500–6510 (2017).
11 Moeini A, Masi M, Zonno MC, Boari A, Cimmino A, Tarallo O *et al.*, Encapsulation of inuloxin a, a plant germacrane sesquiterpene with potential herbicidal activity, in β-cyclodextrins. *Org Biomol Chem* **17**:2508–2515 (2019).
12 Mejías FJR, López-Haro M, Gontard LC, Cala A, Fernández-Aparicio M, Molinillo JMG *et al.*, A novel electron microscopic characterization of Core/Shell Nanobiostimulator against parasitic plants. *ACS Appl Mater Interfaces* **10**:2354–2359 (2018).
13 Rial C, Novaes P, Varela RM, Molinillo JMG and Macias FA, Phytotoxicity of cardoon (Cynara cardunculus) allelochemicals on standard target species and weeds. *J Agric Food Chem* **62**:6699–6706 (2014).

14 Macías FA, Castellano D and Molinillo JMG, Search for a standard phytotoxic bioassay for allelochemicals, selection of standard target species. *J Agric Food Chem* **48**:2512–2521 (2000).
15 Aloisio C and Longhi M, Diloxanide furoate binary complexes with β-, methyl-β-, and hydroxypropyl-β-cyclodextrins: inclusion mode, characterization in solution and in solid state and *in vitro* dissolution studies. *Pharm Dev Technol* **23**:723–731 (2018).
16 Chen Y, Zuo Z, Dai X, Xiao P, Fang X, Wang X *et al*., Gas-phase complexation of α–/β-cyclodextrin with amino acids studied by ion mobility-mass spectrometry and molecular dynamics simulations. *Talanta* **186**:1–7 (2018).
17 Lee J, Lee S-S, Lee S and Oh HB, Noncovalent complexes of cyclodextrin with small organic molecules: applications and insights into host–guest interactions in the gas phase and condensed phase. *Molecules* **25**:4048 (2020).
18 Ghosh I and Nau WM, The strategic use of supramolecular pKa shifts to enhance the bioavailability of drugs. *Adv Drug Deliv Rev* **64**:764–783 (2012).
19 Saokham P, Muankaew C, Jansook P and Loftsson T, Solubility of cyclodextrins and drug/cyclodextrin complexes. *Molecules* **23**:1161 (2018).
20 Jansook P, Moya-Ortega MD and Loftsson T, Effect of self-aggregation of γ-cyclodextrin on drug solubilization. *J Incl Phenom Macrocycl Chem* **68**:229–236 (2010).
21 Saokham P and Loftsson T, A new approach for quantitative determination of γ-cyclodextrin in aqueous solutions: application in aggregate determinations and solubility in hydrocortisone/γ-cyclodextrin inclusion complex. *J Pharm Sci* **104**:3925–3933 (2015).
22 Schönbeck C, Madsen TL, Peters GH, Holm R and Loftsson T, Soluble 1:1 complexes and insoluble 3:2 complexes–understanding the phase-solubility diagram of hydrocortisone and γ-cyclodextrin. *Int J Pharm* **531**:504–511 (2017).
23 Patton AJ, Weisenberger DV and Schortgen GP, 2,4-D–Resistant Buckhorn Plantain (*Plantago lanceolata*) in Managed Turf. *Weed Technol* **32**:182–189 (2018).
24 Tabaglio V, Gavazzi C, Schulz M and Marocco A, Alternative weed control using the allelopathic effect of natural benzoxazinoids from rye mulch. *Agron Sustain Dev* **28**:397–401 (2008).
25 Macías FA, Marín D, Oliveros-Bastidas A, Castellano D, Simonet AM and Molinillo JMG, Structure-activity relationship (SAR) studies of benzoxazinones, their degradation products, and analogues. Phytotoxicity on problematic weeds Avena fatua L and Lolium rigidum gaud. *J Agric Food Chem* **54**:1040–1048 (2006).
26 Yanniccari M, Vila-Aiub M, Istilart C, Acciaresi H and Castro AM, Glyphosate resistance in perennial ryegrass (Lolium perenne L.) is associated with a fitness penalty. *Weed Sci* **64**:71–79 (2016).

