Research Article

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Field efficacy of a bioherbicide mimic (DiS-NH_2) in a nanoparticle formulation for weed control in durum wheat

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Abstract

BACKGROUND: Weed-resistance phenomena have increased dramatically in recent years. Bioherbicides can offer a sustainable alternative to chemical weed control but they often have low water solubility and therefore low efficacy in the field. The research reported here represents the first study on the field efficacy against weeds of a nanoencapsulated bioherbicide mimic of aminophenoxazinones, namely DiS-NH_2 (2,2′-disulphanediyldianiline). Field experiments were carried out across three different locations to evaluate the bioherbicide disulphide mimic at standard (T1, 0.75 g m^{-2}) and double (T2, 1.5 g m^{-2}) doses when compared to no weed control (NC) and chemical weed controlled (PC) in durum wheat.

RESULTS: The nanoencapsulated bioherbicide displayed better soil permeability than the free compound and also showed lower ecotoxicity on comparing the toxic doses on the *Caenorhabditis elegans* nematode model. It was found that T2 gave the best performance in terms of phytotoxicity (−57% weed biomass when compared with NC) and crop yield enhancement (3.2 *versus* 2.2 Mg ha^{-1} grain yield), while T1 showed comparable results to PC. T1 and T2 did not cause shifts in weed communities and this is consistent with a broad spectrum of phytotoxicity. Moreover, the nanoparticle formulation tested in this study provided stable results across all three locations.

CONCLUSION: It is reported here for the first time that a nanoencapsulated DiS-NH_2 bioherbicide mimic provided an efficient post-emergence and contact bioherbicide that can control a wide range of weed species in durum wheat without damaging the crop. The mimic also has low ecotoxicity and improved soil permeability.

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

Keywords: weed management; bioherbicides; encapsulation; nanoparticles; weed associations; crop safety

1 INTRODUCTION

Modern agriculture is facing increasing risks related to weed resistance, herbicide persistence in the environment and the development of specialised weed flora. Herbicides represent the largest share of the pesticides used worldwide, with over three million tonnes produced per year.[1] The globally observed rapid increase in herbicide-resistant weed species has resulted in higher herbicide application rates and also the use of active principles with longer environmental persistence.[2] Hulme[3] proposed that the numbers of herbicide-resistant weeds are probably underestimated and agronomic drivers suggest that many countries will probably experience an increase in the future.

Given the situation outlined earlier, the search for alternative and eco-friendly weed management strategies has become a prerequisite. The use of natural products of biological origin, derived either from living organisms or their secondary metabolites, to suppress target weed populations without harming the

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environment represents the future in crop enhancement.[4] The benefits of bioherbicides include their environmentally friendly chemical structures, negligible impact on the environment, new modes of action against weeds and public acceptance.[5] However, several drawbacks still limit the use of such compounds under natural conditions and these include low water solubility, rapid biodegradation in the environment and high costs of synthesis and isolation.[6] According to Roberts *et al.*,[7] the successful incorporation of bioherbicides within integrated weed management systems requires them to have suitable formulations, economic sustainability, a high mortality rate on target plants and very limited or zero impact on the surrounding natural environment and human health. Current examples of such formulations include the encapsulation of phytotoxic sesquiterpene lactones in organic nanotubes, which show *in vitro* inhibitory activity against the weeds *Phalaris arundinacea* L., *Lolium perenne* L. and *Portulaca oleracea* L., and monoterpenes encapsulated in organoclays in order to prevent rapid volatilisation of the active species.[8,9] The release rate of the active substances is another important factor and, in general, larger structures facilitate the gradual and prolonged release of the active species whereas smaller particles allow a more homogeneous dispersion, increase the release rate and facilitate the transport and absorption of the substances. The latter situation results in a controlled release of the active substance and, for this reason, among others, different technologies have been developed to achieve increasingly smaller encapsulation sizes.[10]

Inspired by aminophenoxazinone and benzoxazinone natural scaffolds, *ortho*-substituted disulphides have been synthesised due to their similar bioactivity profiles and positive *in silico* evaluation of their mode of action. These mimics have been reported to inhibit germination and root and shoot elongation in *Lolium rigidum* and *Echinochloa crus-galli* (L.) P.Beauv.[11] In addition, formulation methods applied to one of the most promising disulphides (DiS-NH_2) have also been tested *in vitro* against *Portulaca oleracea*, *Plantago lanceolata* L. and *L. rigidum* and improved phytotoxic profiles were achieved. This enhancement was observed after encapsulation in fully organic nanoparticles and it was attributed to higher water solubility and bioavailability of the active compound.[12] Similar compounds have recently been tested in the field to evaluate how they affect wheat parameters such as crop height, spikes and quality of the kernels, with positive effects observed on the crop.[13]

Given the promising results obtained *in vitro*, we report here the application of organic formulations in which an aminophenoxazinone mimic, namely 2,2′-disulphanediyldianiline (DiS-NH_2), was encapsulated within polymeric nanoparticles to provide a post-emergence herbicide on durum wheat across three locations. The goals were to evaluate the effects of the nanoparticles on weed abundance, species composition and diversity, as well as their soil stability for ecotoxicological risk assessment.

2 MATERIALS AND METHODS

2.1 Composition of DiS-NH_2 and preparation of the nanoparticle formulation

The method used for the synthesis of DiS-NH_2 is similar to that reported by Oliveira *et al.*[11] The formulation of the compound in organic nanoparticles (NP@DiS-NH_2) was reported by Mejías *et al.*[12] and Scavo *et al.*[13]

2.2 Degradation experiments in soil

Degradation experiments in crop soil were carried out to evaluate the soil residence times of DiS-NH_2 and NP@DiS-NH_2 and to identify the degradation products after soil and water interactions. Given that the degradation experiments were started before the three locations for field trials were selected and, with the aim of obtaining cross-sectional biodegradability data, the degradation experiment was performed on a standard crop soil used for wheat. A chromatography column was filled with natural soil obtained from 41°11′42.36″ N, 0°34′42.24″ E to give a soil column with a length of 20 cm and diameter of 2.3 cm. DiS-NH_2 or NP@DiS-NH_2 were placed on the top of the soil column and deionised water (83 mL) was added to simulate a watering process. Samples of water that had passed through the soil were collected in vials every 15 min. Samples were analysed by high-performance liquid chromatography (HPLC) to determine the soil retention time of the compound and identify possible degradation products that could be formed after soil interaction. A LiChrospher® 100 RP-8 endcapped (5 μm) column (25 cm × 4 mm) was used and the method was as follows: flow rate 1 mL min^{-1}, 30 °C, 0–5 min 35:65 water/methanol, 5–10 min 20:80 water/methanol, 10–15 min 10:90 water/methanol, 15–17 min water/methanol 100% methanol, 17–22 min 35:65 water/methanol. Ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) data were obtained for each sample using a BEH C18 ACQUITY 1.7 μm column set to 50 °C with a flow of 0.3 mL min^{-1} in isocratic mode (0–6.0 min 40:60 water/methanol). Samples were filtered through a 0.45 μm PTFE 13 mm syringe filter to prepare each sample. A XEVO-G2S-QTOF mass spectrometer was used with ES+ polarity and mass filtering from 50 to 1200 Da. The gas temperature was set to 450 °C, the drying gas flow was 850 L h^{-1} and the nebuliser pressure was 1294 Torr. The sample injection volume was 5 μL.

2.3 Culturing, synchronisation and bioassay of *Caenorhabditis elegans* worms

Nematode growth medium (NGM) was created in a sterile environment according to established formulations: NGN contained (per litre) 3 g sodium chloride (NaCl), 2.5 g peptone, 17 g agar, 1 mL of 5 mg mL^{-1} cholesterol in ethanol, 1 mL of 1 M calcium chloride ($CaCl_2$), 1 mL of 1 M magnesium sulphate ($MgSO_4$) and 25 mL of 1 M dipotassium phosphate/potassium dihydrogen phosphate (K_2HPO_4/KH_2PO_4, pH 6.0). The medium was inoculated with *Escherichia coli* (strain OP50–1). Worm populations were synchronised using an alkaline bleaching method with slight modifications.[14] A mixture of worms at various stages (adults/larvae/eggs) was collected from NGM culture plates by rinsing with sterile water (V_{Total} = 8 mL) into a Falcon tube. After allowing the worms/eggs to settle, a portion of the supernatant (7.5 mL) was removed after 20 min. A fresh alkaline sodium hypochlorite solution [consisting of 3.5 mL sterile water, 1 mL of 4% bleach and 0.5 mL of 5 M sodium hydroxide (NaOH)] (3 mL) was added. The suspension was gently vortexed for 2 min until dissolved worms were no longer visible under a light microscope. The suspension was then centrifuged for 2 min at 200 × *g* and the bleach was discarded. The remaining eggs were subjected to two additional rounds of washing and centrifugation (200 × *g* for 2 min each) with sterile water (V_{Total} = 5 mL), followed by a final centrifugation at 300 × *g* and a wash with S-medium (V_{Total} = 5 mL). The eggs were finally resuspended in S-medium (V_{Total} = 20 mL) and placed on an orbital shaker at 20 °C overnight to allow hatching into L1 larvae. After 12 h of shaking the L1 larvae were transferred

to fresh NGM plates seeded with *Escherichia coli* OP50−1 and incubated at 20 °C, where they developed into young adult worms within 48 h.

Standard *Caenorhabditis elegans* N2 ancestral nematodes were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, MN, USA) and kept at the University of Innsbruck, Research Institute for Biomedical Ageing Research. In preparation for anthelmintic screening, the worms were cultivated on NGM in Petri dishes coated with a layer of *Escherichia coli* OP50−1 bacteria. The bacterial culture and NGM were prepared under sterile conditions according to established methods.[15] Synchronised groups of adult worms were obtained using the alkaline bleaching methods described earlier.

The anthelmintic screening test was carried out in a 96-well microplate (flat-bottom, Nunclon®, Sigma-Aldrich, St Louis, MO, USA). Synchronised adult worms were gathered in S-medium (V = 8–12 mL) and the volume of the suspension was adjusted until the desired average number of worms per drop was achieved (10–15 worms 10 μL^{-1}). A volume of 100 µL was added to each well to give approximately 100 worms per well. The microplate was shaken for 12 min and was then examined with a WMicroTracker instrument (Phylumtech, Sunchales, Argentina) (t = 30 min) to measure basal worm motility. DiS-NH_2 and NP@DiS-NH_2 solutions were prepared in dimethyl sulphoxide (DMSO). Each test solution or control was then added to the desired well (V = 1 µL each) to give final concentrations of 1000, 300 and 30 µM, with 1% DMSO in the well. Ivermectin (c = 30 µM) and albendazole (c = 30 µM) were used as positive controls and the vehicle negative control was DMSO (1%). The 96-well microplate was shaken (t = 2 min) and then placed in the WMicroTracker apparatus and incubated (up to t = 12 h, T = 24 °C). Worm motility (WMT) within each well was measured every 30 min and motility was recorded by the WMicroTracker as activity counts per defined time interval. The relative WMT anthelmintic activity values for test samples were estimated by normalising the values to their respective basal activity value and they are expressed as a percentage of the DMSO (1%) control. Each reported result was obtained from three independent biological replicates with three internal replicates each.

The egg hatching effect was studied by following the same method but with eggs added to the 96-well plate instead of the full adult worm. An average of 100 worms per well were introduced (V = 100 µL) together with 1 µL of the DiS-NH_2 or NP@DiS-NH_2 test solutions (final concentrations of 1000, 300 and 30 µM). The relative WMT was recorded to evaluate the number of eggs that hatched in comparison with the negative control (S-medium + 1% DMSO). Each reported result was obtained from three independent biological replicates with three internal replicates each.

2.4 Field experimental set-up and locations

The efficacy of the nanoencapsulated DiS-NH_2 (i.e., NP@DiS-NH_2) on natural weed infestations was evaluated by carrying out field experiments during the 2021/2022 growing season across three different locations (hereafter referred to as locations I, II and III) sited in central Sicily, southern Italy. The locations are displaced in an approximately 40 km range falling within the territory of Butera (Caltanissetta, location I) and Piazza Armerina (Enna, locations II and III) – an area devoted to durum wheat cultivation. All locations presented Regosoils, Typic Xerorthensis or Xerochrepts,[16] with a clayey texture and alkaline reaction. The zone is subjected to a typic semi-arid Mediterranean climate that is characterised by mild-wet winters, springs with rising temperatures and with most of the rainfall during the autumn/early winter period. The three locations showed similar meteorological trends during the wheat biological cycle (see Supporting Information Fig. Figure S1). More specifically, among the total rainfall experienced in locations I (276 mm), II and III (282 mm), the highest levels were detected in January (99 and 131 mm, respectively). In contrast with the normal trend for the zone in question, spring was particularly wet, with 47% of the total rainfall that fell between March and May in location I, and 43% in locations II and III. The air temperatures were appropriate for durum wheat growth although location I showed an average temperature that was +3 °C higher than those in locations II and III throughout the experimental period.

2.5 Field design and cultural practices

In each location, four treatments were factorially combined according to a randomised block design (RBD) with three replications. Treatments included the application of a standard dose of the NP@DiS-NH_2 bioherbicide mimic (T1), a double dose of the NP@DiS-NH_2 bioherbicide mimic (T2), a negative control (NC) with the absence of weeding and a positive control (PC) that involved chemical weeding. In location I chemical weed control involved spraying Atlantis® WG (mesosulfuron-methyl + iodosulfuron-methyl-Na + mefenpyr-diethyl) and Buctril® Universal (bromoxynil + 2,4-D), respectively, at 0.5 kg ha^{-1} (equivalent to 0.05 g m^{-2}) and 1 L ha^{-1} (equivalent to 1×10^{-4} L m^{-2}) during wheat tillering (growth stage 23 according to the BBCH scale of Lancashire *et al.*[17]) using a knapsack hand sprayer. In locations II and III, Traxos® Pronto 60 (pinoxaden + clodinafop-propargyl + cloquintocet-mexyl) and Manta® Gold (fluroxypyr + clopyralid + MCPA) at 2×10^{-4} L m^{-2} were mixed with Amadeus® Top (thifensulfuron-methyl + tribenuron-methyl) at 3×10^{-3} g m^{-2} during BBCH growth stage 23 of the wheat. These doses were selected according to supplier recommendations, with a volume of 500 L water ha^{-1}. The amounts of NP@DiS-NH_2 bioherbicide mimic were 0.75 g m^{-2} for the standard dose (T1) and 1.5 g m^{-2} for the double dose (T2), both of which were applied at wheat emergence (28 February in location I and 7 March in locations II and III) with a knapsack hand sprayer and with a volume of 500 L water ha^{-1}. Given that the NP@DiS-NH_2 active ingredient is estimated to be present at only 21% (*w/w*), with the remainder represented by formulation excipients, it can be considered that the bioactive ingredient doses are 0.1575 g m^{-2} for T1 and 0.315 g m^{-2} for T2. These rates are consistent with the *in vitro* findings reported by Oliveira *et al.*[11] and Mejías *et al.*[12] Each plot size was 4 m^{-2}, thus giving a net plot size of 48 m^{-2} per location (4 treatments × 3 replicates = 12 plots), with a 2 m border between PC and the other treatments.

Durum wheat [*Triticum turgidum* subsp. *durum* (Desf.) Husn.] cv. 'Core' (Platani/Gianni), a modern variety with medium-early maturity and well-adapted to varying environmental conditions, was hand-sown in the second half of December at a rate of 200 kg seeds ha^{-1}. All experimental fields were managed according to the local agricultural practices.[18] The seedbed was prepared by ploughing in autumn at a soil depth of 20 cm followed by disc-harrowing. Fertilisation consisted of the application of 120 kg ha^{-1} diammonium phosphate (18% N–46% P_2O_5) before sowing, followed by 60 kg N ha^{-1} as ammonium nitrate (27%) and 50 kg ha^{-1} as diammonium phosphate in post-emergence. Fungal disease was controlled by spraying Mirador® SC (azoxystrobin) in all plots at the flowering stage at 1 L ha^{-1}.

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2.6 Weed monitoring, sampling and analysis

Prior to weed assessments, a field scouting was conducted on each experimental field to assess the weed spatial distribution and positions of the sampling units. Three 1.0 m^2 quadrats were positioned for each treatment and these were kept fixed during the crop cycle, excluding the borders of each plot and the non-representative areas. Two sampling times were selected (i.e., April and June before crop harvest) to monitor the autumn–winter and spring–summer weed flora, respectively. Therefore, a total of 72 quadrats (4 treatments × 3 replicates × 2 sampling times × 3 locations) were monitored. Weed root biomass was not considered due to the difficulties in measuring this parameter in open-field conditions, especially in our case where the soils had a clayey texture. However, the effects on root length were previously tested *in vitro* by Oliveira *et al.*[11] and Mejías *et al.*[12] The results of these previous studies, combined with those on soil degradation, indicate that the availability of the compounds and their effect should not be limited once in contact with the roots in a similar way to other herbicides. In the present study, weed assessments involved the analysis of weed abundance and weed diversity, in accordance with Nkoa *et al.*[19] and Scavo *et al.*[20] Abundance was evaluated by measuring the weed above-ground biomass (WAB), the relative density (RD), the relative frequency (RF) and the relative abundance index (RAI). The method of Scavo *et al.*[21] was used to obtain WAB by clipping the weeds at the soil surface and drying the samples in an oven at 55 °C to obtain a pooled weight at the quadrat level. WAB was also expressed as the percentage of weed inhibition, which was calculated as (1 − treatment/NC) × %. The RD is the ratio between the number of individuals for each weed species and the total number of weeds within the quadrat; the RF is the absolute frequency of a species divided by the total absolute frequency. The RAI [(RD + RF)/2] is a powerful parameter that expresses both the density and the evenness of weed communities.[22]

Diversity was analysed by taking into account the weed community structure, the weed species richness (WSR, i.e., the number of species per quadrat) and two α-biodiversity indices.[23] As far as the former is concerned, weed species or genera were classified by botanical family, life cycle (annuals, biennials or perennials), ecophysiological group (emergence seasonality) and biological group (life-form category considering the Raunkiaer system).[24] The α-biodiversity was characterised from four 0.25 m^2 patches per quadrat through the Shannon diversity index (*H*) and Pielou's evenness index (*J*),[25] both computed as described by Scavo *et al.*[26]

2.7 Determination of wheat grain yield

In all locations the grain yield (Gy) was measured by hand harvesting the whole plot after grain ripening. This process was carried out on 12 June in location I and 15 June in locations II and III. The grain from each plot was weighed, referred to the unit area and the results were adjusted to a 13% moisture content.[27]

2.8 Statistical analysis

Univariate and multivariate techniques were applied to analyse the data statistically. The former technique consisted of a three-way analysis of variance (ANOVA) factorial model with Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$ for means separation, considering '3 locations', '4 treatments' and '2 sampling times' as the main factors. Given the $P \leq 0.001$ significance of 'location', data were then processed according to a generalised mixed model (GMM) with 'location' as a random factor, as suggested by Gomez and Gomez.[28] One-way ANOVA was also applied to pooled data to test the effect of the main factors and also at each location to assess the average effect of 'treatment' and 'sampling time'. Gy was analysed through a two-way 'location × treatment' factorial model and correlated to WAB through the Pearson product moment correlation coefficient (*r*) on mean values. Confirmation that all assumptions of ANOVA had been met was achieved by checking the homogeneity of error variances with Bartlett's test and the normality through a graphical inspection of the residuals. The following transformations were applied[26]: $\log_{10}(x + 1)$ for WAB, square root for *H*, arcsine-square root (Bliss) for RAI and logit for *J*, whereas species richness data did not show any violation.

Multivariate statistics were used to analyse the species composition. For each location, the effects of 'treatment', 'sampling time' and their interaction were tested by a multivariate analysis of variance (MANOVA) based on Bliss-transformed RD data with the Wilks' criterion.[26] The effects of MANOVA were then graphically generated on 'distance' biplots resulting from a principal component analysis (PCA) on major weeds or genera (those with RD ≥ 7%) for each location.[29] The PCAs were performed on the correlation matrix of Bliss-transformed RD data and the biplots were displayed through the means for each 'treatment × sampling time' combination.[30] The statistical packages employed for data analysis were CoStat® 6.003 (CoHort Software, Monterey, CA, USA) for univariate and Minitab® 16 (Minitab Inc., State College, PA, USA) for multivariate statistics.

3 RESULTS AND DISCUSSION

3.1 Polymeric nanoparticle encapsulation

This particular formulation was developed based on prior specifications and results reported by Mejías *et al.*[31] Pluronic F-127® nanoparticles functionalised with polyvinyl alcohol (PVA) have already provided promising results in inhibiting the germination of parasitic plants such as *Phelipanche aegyptiaca* (Pers.) Pomel and *Phelipanche ramosa*. Furthermore, these materials have previously been tested *in vitro* on seed germination and seedling growth of common purslane (*Portulaca oleracea*), ribwort plantain (*Plantago lanceolata*) and annual ryegrass (*L. rigidum*), which are all herbicide-resistant weeds that infect several key crops such as wheat, tomatoes and beans.[12,32] An important aspect of these formulations is the innocuous nature of the empty nanoparticles and the independent ingredients (Pluronic F-127® and PVA) employed in their synthesis. Indeed, the results of *in vitro* studies demonstrated the absence of toxicity for the target plants after treatment with the individual components. Furthermore, previous reported studies by Lee *et al.*[33,34] showed a lack of toxicity in humans.

The mechanism of encapsulation is based on the generation of a shell around the bioactive compound (DiS-NH_2) while it is dispersed in the medium. The synthetic process to obtain the dispersed nanoparticles in the PVA matrix is represented in Fig. 1. The synthesis method provided approximately 4.0 g of material with the expected specification according to nuclear magnetic resonance (NMR) spectroscopy and LC–MS (Fig. S2). The results obtained by comparison with the calibration curve showed that a level of encapsulation of 21.0 ± 1.2% for the bioactive compound. The synthesis procedure was repeated until 67.5 g of the material had been obtained. This is the amount required for the experimental design and the composition of

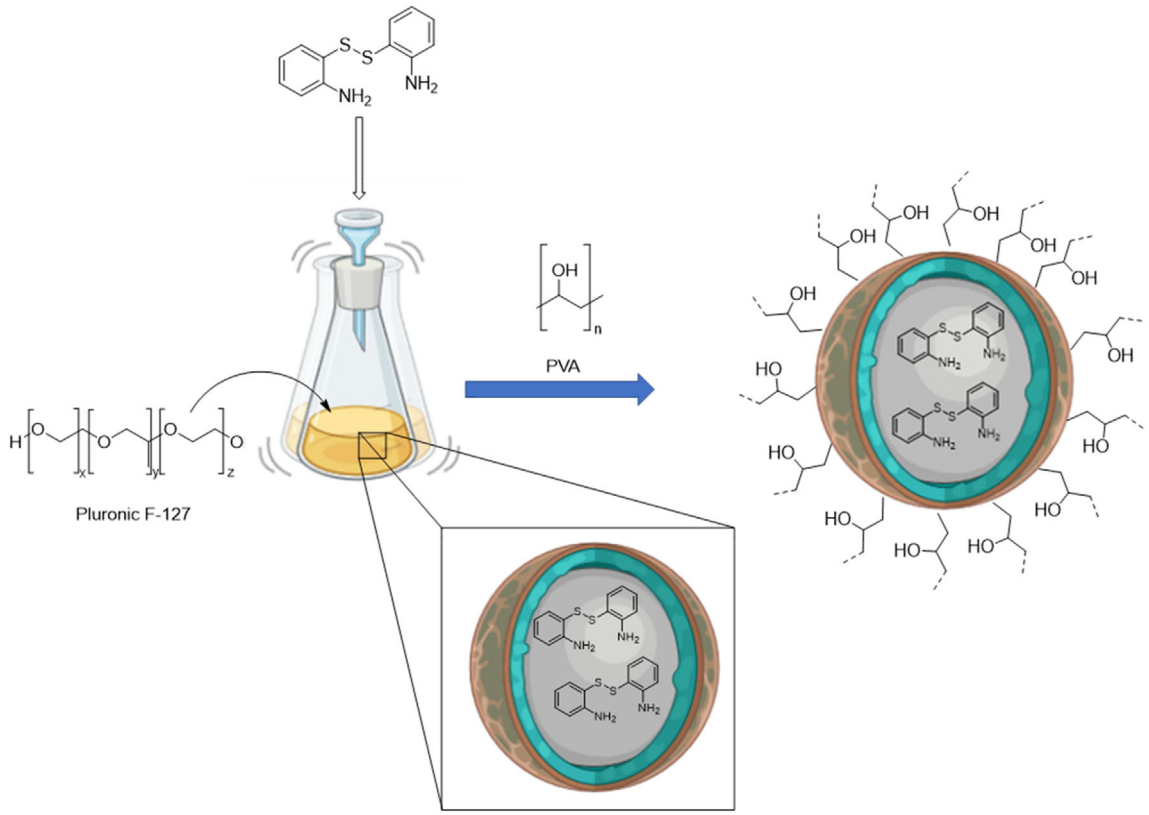

Figure 1. Scheme of the synthetic procedure to understand the mechanism of encapsulation.

the material employed was: NP@DiS-NH_2 (21.0 ± 1.2% DiS-NH_2, 74.6 ± 2.4% Pluronic F127® and 0.5 ± 0.3% PVA).

The material was characterised by transmission electron microscopy (TEM) in HAADF and BF mode. Low magnification was employed to observe the generated PVA matrix and the nanoparticles embedded in it. A matrix covering more than 1 μm^2 with nanoparticles containing DiS-NH_2 that are less than 100 nm in diameter (average size 51.6 ± 17.1 nm) is shown in Fig. S3. These structures present a multi-cavity arrangement rather than the single core commonly observed in metallic particles. The elemental composition of the structures was analysed by energy-dispersive X-ray spectroscopy (EDXS) and the results are represented in Fig. S4. It was observed that the composition of the nanoparticle incorporates a signal for sulphur due to the presence of the encapsulated bioactive compound. The sulphur signal was not observed on analysing empty nanoparticles. The authors hypothesise that the DiS-NH_2 is located within the cavity generated by the Pluronic F-127® nanoparticles.

3.2 Soil permeability studies

Degradation and soil persistence experiments were carried out to analyse the possible ecotoxicological implications of the compounds. DiS-NH_2 and NP@DiS-NH_2 were eluted through crop soil (L = 20 cm, $\varnothing$ = 2.3 cm). A solid sample of each compound (30 mg) was placed on the top of the soil to simulate direct application to the crop. Water was subsequently introduced from the top of the column to simulate a watering process. In the case of DiS-NH_2, which has very low water solubility, it was observed that the compound simply remained on the top of the soil. The portionwise addition of water did not solubilise and elute the compound through the column. This situation led to the absence of signals in the HPLC traces of the aqueous samples collected and highlighted the limitations of this compound as an agrochemical since it is insoluble and cannot be assimilated by target plants. In contrast, the NP@DiS-NH_2 applied to the top of the soil column dissolved upon the portionwise addition of water. HPLC analysis of the resulting aqueous fractions (Fig. S5) showed the appearance of the bioactive compound beginning at 240 min and finishing at 300 min. In contrast with the non-formulated compound, NP@DiS-NH_2 was able to penetrate to a depth of 20 cm of soil in 4 h. This finding demonstrates the advantageous effect of nanoparticles to provide a formulation that can be applied on seeds located at a soil depth of 0–20 cm in a short time period. Degradation of the compound was not observed after 72 h, thus meaning that the formulation will remain active during the soil crossing process.

3.3 *In vivo* assay of *Caenorhabditis elegans* worms

In an effort to establish and validate the worm motility assay, *Caenorhabditis elegans* adult worms were treated with 1% *v/v* DMSO and the anthelmintic ivermectin (10 μM). In the case of the egg hatching study, 1% *v/v* DMSO was employed along with the anti-hatching agent albendazole (10 μM). In the setting described here, worms exposed to 1% DMSO retained motility and had visibly bending and motile bodies when observed by microscopy after the experiment. A similar effect was observed for the eggs in that those exposed to 1% DMSO hatched with the same statical significance as the eggs dispersed in NGM buffer. In the case of ivermectin, the 10 μM concentration led to reduction in adult motility by 95% within 12 h (Fig. S6). A similar effect was observed for albendazole and the hatching effect, with a reduction in hatching by 90% after 16 h.

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An *in vivo* test was carried out to compare the effect on the worm motility phenotype of free DiS-NH_2 and nanoparticle-encapsulated (NP@DiS-NH_2) compound on applying different concentrations (30, 300 and 1000 μM). The motility parameter is an indirect measure of survival rate, since the bioassay is set up to supply sufficient food and provide adequate living conditions for the worms. The preliminary worm motility results (Fig. S6) revealed that NP@DiS-NH_2 gave a lower motility reduction than the non-formulated bioactive compound. Comparison of the two compounds at 300 μM shows a variation of ±50% motility, which indicates that the formulated compound had lower toxicity against nematodes. The results thus demonstrate that the application of encapsulated DiS-NH_2 is safer for fully adult nematodes. In order to evaluate whether this effect was applicable to other nematode stages that may be found in soil and cohabit with plants, the effects of the two compounds on hatching were evaluated (Fig. S7). The study revealed a similar activity profile to that found in the *Caenorhabditis elegans* adult study, with higher motility values observed for the encapsulated DiS-NH_2 than for the free compound. The motility values in this bioassay relate to the percentage of eggs that hatch in comparison to the control. The findings indicate the lower toxicity of NP@DiS-NH_2 for egg development when compared to the neat active compound. In this respect it is worth highlighting the dose of 30 μM of encapsulated agent, which led to the retention of 94.6 ± 11.2% of the motility. The literature data for *in vitro* phytotoxicity studies published by Mejías *et al.*[12] indicate an activity of 75–40% inhibition for etiolated wheat coleoptiles for concentrations in the range 100–30 μM. These two results, together with those of the full adult nematode test, imply that the application of NP@DiS-NH_2 as a bioherbicide is safer than DiS-NH_2 for microorganisms in the soil. Even the highest concentration evaluated (1000 μM) gave a difference of 50% motility for the encapsulated compound. The authors hypothesise that the lower permeability of the nanoparticles, due to their larger size in comparison with the free compounds, has an effect on egg hatching.

3.4 Effects on the weed aboveground biomass (WAB)

Based on observations from soil permeability studies, along with results of *in vivo* tests with soil model organisms and previously reported *in vitro* plant cell assays,[12] NP@DiS-NH_2 was selected for the subsequent field experiments and the evaluation of non-encapsulated DiS-NH_2 was not pursued. The formulated compound in organic nanoparticles (NP@DiS-NH_2) exhibits higher water solubility and has a lower ecotoxicological impact, greater soil permeability and maintains the same *in vitro* phytotoxicity profile, thus making it the optimal choice for agricultural applications. Concerning the application rates of NP@DiS-NH_2, Oliveira *et al.*[11] and Mejías *et al.*[12] indicated that concentrations below 300 μM are harmless to the growth of crops such as wheat when applied *in vitro* directly on the coleoptile. Hence, considering that the bioherbicide was applied with a knapsack hand sprayer at the wheat BBCH growth stage 23, the concentration was increased to 0.315 g m^{-2} of active ingredient to ensure a phytotoxic effect, notwithstanding the principles of bioaccumulation and bioavailability. This approach was supported by the results obtained on soil permeability and ecotoxicity. Despite the higher application rates of NP@DiS-NH_2 than synthetic herbicides, it should be highlighted that the amounts of biological products in organic farming are commonly much higher than synthetic products (for instance the rates of organic *versus* mineral fertilisers), but the former are environmentally safer than the latter. Moreover, Oliveira *et al.*[11] and Mejías *et al.*[12] indicated that the synthesis of the final bioherbicide is affordable and it can be produced on a multigram scale at the laboratory level.

The three-way ANOVA (Supporting Information Table Figure S1) results showed that 'location' contributed the most to variance, so this was considered as a random factor for the GMM. In all locations 'treatment' showed a $P \leq 0.001$ significance and the 'treatment × sampling time' interaction was not significant, while in location III the 'sampling time' made the highest contribution (Table 1). In locations I and III, T1 showed similar results to PC in terms of WAB reduction, with decreases of 55.5% and 24.1%, respectively, compared to NC (Fig. 2). Interestingly, the disulphide bioherbicide mimic applied at double dose (T2) did not differ significantly from the standard dose (T1) in locations I and II, which means that the dose applied did not affect the weed control efficacy. In location II, T2 had a markedly lower WAB compared to NC (−56.2%), whereas PC showed the highest value [126.6 g dry weight (DW) m^{-2}]. Pooling over locations and sampling times (Fig. S8) highlighted the trend NC > PC > T1 > T2, with T1 and T2 reducing the WAB by 40.9% and 51.3%, respectively, with respect to NC. Overall, T2 performed better than PC (69.2 *versus* 96.3 g DW m^{-2}). These results confirmed the conclusions of our previous study on wheat coleoptile elongation under controlled conditions.[12] The preliminary weed-suppressive ability of the disulphide bioherbicide mimic was also evaluated in our previous study,[13] although that was mainly focused on wheat crops. The low weed control efficacy of PC could be related to the application of synthetic herbicides over previous years. Although farmers have not reported weed resistance in this area, it is reasonable to assume that resistance could effectively evolve over time. Despite this possibility, it was decided to perform our experiments across the three chosen locations as it was strongly believed that on-farm field experiments offer a more realistic evaluation of agrochemicals than experimental farms. Hasan *et al.*[35,36] obtained similar results to ours under field conditions on comparing the weed-suppressive ability of the bioherbicide WeedLock (whose active principle is formulated 2-undecanone = EGX-101™, a natural compound of *Solanum habrochaites* S. Knapp & D.M. Spooner and *Solanum nigrum*), glyphosate isopropyl-amine and glufosinate-ammonium. The authors reported that WeedLock showed a similar weed control efficacy to commercial herbicides, especially between 1 and 7 days after application. To the best of our knowledge, other reports on the application of bio-based formulations for weed control in agroecosystems have not been published. In open fields, the weed-suppressive ability of bioherbicides is closely influenced by climatic conditions, soil properties and application methods.[37] However, in this study the efficacy of the disulphide bioherbicide mimic was almost constant across the three locations, thus demonstrating the role of the nanoparticle formulation in maintaining good weed control performance under varying pedo-climatic conditions. Nevertheless, except for location III, differences in the WAB were not observed between April and June (Fig. S8).

According to recent studies, histone deacetylases (HDACs) are the main target of aminophenoxazinones. HDAC enzymes (mainly HDA6 and HDA2) are involved in the regulation of gene expression. Chromatin modification provides a means for rapid cellular responses to changing conditions; by modulating chromatin composition and accessibility, histone acetylation and deacetylation constitute both on–off switches and rheostats for gene expression. Ultimately, this will cause a slowdown in growth and development.[37] The authors suggest a similar mode of action

TABLE 1. The *F*-values as absolute value of main factors and their interaction resulting from two-way analysis of variance (ANOVA) on weed above-ground biomass (WAB), weed species richness (WSR), Shannon–Wiener (*H*) and Pielou's (*J*) biodiversity indices

		Source of variation		
	CV	Treatment (T)	Sampling time (S)	(T) × (S)
df		3	1	3
Location I				
WAB	4.4	20.95***	0.14 ns	0.46 ns
WSR	25.0	8.3**	0.6 ns	0.6 ns
H	7.7	11.4***	0.3 ns	0.6 ns
J	8.7	3.8*	0.1 ns	1.4 ns
Location II				
WAB	5.8	15.79***	2.21 ns	4.23 ns
WSR	23.0	5.5*	13.1**	7.7**
H	8.9	2.6 ns	16.7***	16.4***
J	16.0	2.0 ns	0.3 ns	4.0*
Location III				
WAB	5.2	6.50**	39.59***	3.23 ns
WSR	23.1	1.8 ns	60.3***	1.8 ns
H	15.0	4.8*	73.6***	5.7**
J	9.6	4.3*	150.3***	6.5**

*$P \leq 0.005$.
** $P \leq 0.01$.
*** $P \leq 0.001$.
Abbreviations: CV, coefficient of variation (%); ns, not significant; df, degrees of freedom.

for DiS-NH_2 on the basis of the macroscopical effects observed and considering the structural similarities with 2-amino-3*H*-phenoxazin-3-one. Moreover, according to studies published by Macías *et al.*,[38] compounds from the aminophenoxazine family and their derivatives, such as the bioherbicide mimic DiS-NH_2, would not affect Poaceae members or non-pathogenic associated organisms. These considerations highlight NP@DiS-NH_2 as a bioherbicide with great potential to protect Poaceae crops, which have the highest global economic importance.[39]

3.5 Effects on species richness

The effects on species richness varied from location to location (Table 1). In location I, the lowest species richness was found in PC and T2 (3.0 and 3.3 weeds m^{-2}, respectively) whereas 5.7 weeds m^{-2} were counted in NC (Fig. 2). In location II the effect of 'treatment' was sampling time-dependent: in April only T2 showed a significant reduction in species richness compared to NC (2.3 *versus* 6.0 weeds m^{-2}), while relevant differences between treatments were not observed in June, except for PC (2.0 *versus* 4.3 weeds m^{-2} of NC); in location III the 'treatment' was not significant. Averaged over locations and sampling times, the effects on species richness were in line with those obtained for WAB (Fig. S8). Indeed, T2 and PC caused the lowest species richness (3.0 and 3.3 weeds m^{-2}, respectively), whereas T1 did not differ significantly from NC. Deligios *et al.*[40] found that non-weeded and underdosed herbicide treatments significantly lowered species richness when compared to high-dosed herbicides in Mediterranean climates. The trend observed for the WAB was location I > location II > location III. In contrast to the WAB, pooling over treatments and locations, the species richness was 43.4% greater in April than in June – probably due to the reduction of treatment efficacy on the one hand and to the increasingly unfavourable climatic conditions (high temperature and low rainfall) on the other hand (Fig. S8). This effect was exacerbated in location III (Fig. 2). The reduction in species richness, which is not always accompanied by a decrease in weed density, is an important aspect within integrated weed management systems, since it allows a focus on specific control methods for a smaller spectrum of weeds.[41] It should be highlighted, however, that the weed control efficacy of the bioherbicide mimic discussed here may be further improved if combined with other weeding practices.

3.6 Effects on weed community structure and species composition

Weed structure was somewhat similar in all three locations. In total, 14, 14 and 10 taxa were detected respectively in locations I, II and III (Tables 2–4). Most of these taxa belonged to the Asteraceae family (36%, 29% and 30%) followed by Poaceae (29%, 21% and 20%). In common to similar agroecosystems under semi-arid conditions,[20,42] the weed communities were dominated by annual therophytes (79%, 79% and 70%) and spring–summer species. The major weeds, that is, those contributing more than 7% to the total density, were (in decreasing order): *Centaurea napifolia* L., *Avena fatua* L., *Glebionis coronaria* (L.) Cass. ex Spach, *Fumaria officinalis* L., *Sinapis arvensis* L. and *Lolium multiflorum* Lam. in location I; *G. coronaria*, *Sinapis arvensis*, *Papaver rhoeas* L., *Dactylis glomerata* L. and *Anethum graveolens* L. in location II; *L. perenne*, *Medicago polymorpha* L., *Avena fatua* and *Sinapis arvensis* in location III. Altogether, these weeds accounted for 76.9%, 80.1% and 89.9% of the total weed density in locations I, II and III, respectively. These results are consistent with those reported by Cirujeda *et al.*,[43] who carried out a long-term survey of 138 winter cereal fields in ten survey areas where winter cereals were the main crops. It was found that only 26 species, out of the total 175 species, were found in more than 10% of the surveyed fields and that four species (namely *Papaver rhoeas*, *L. rigidum*,

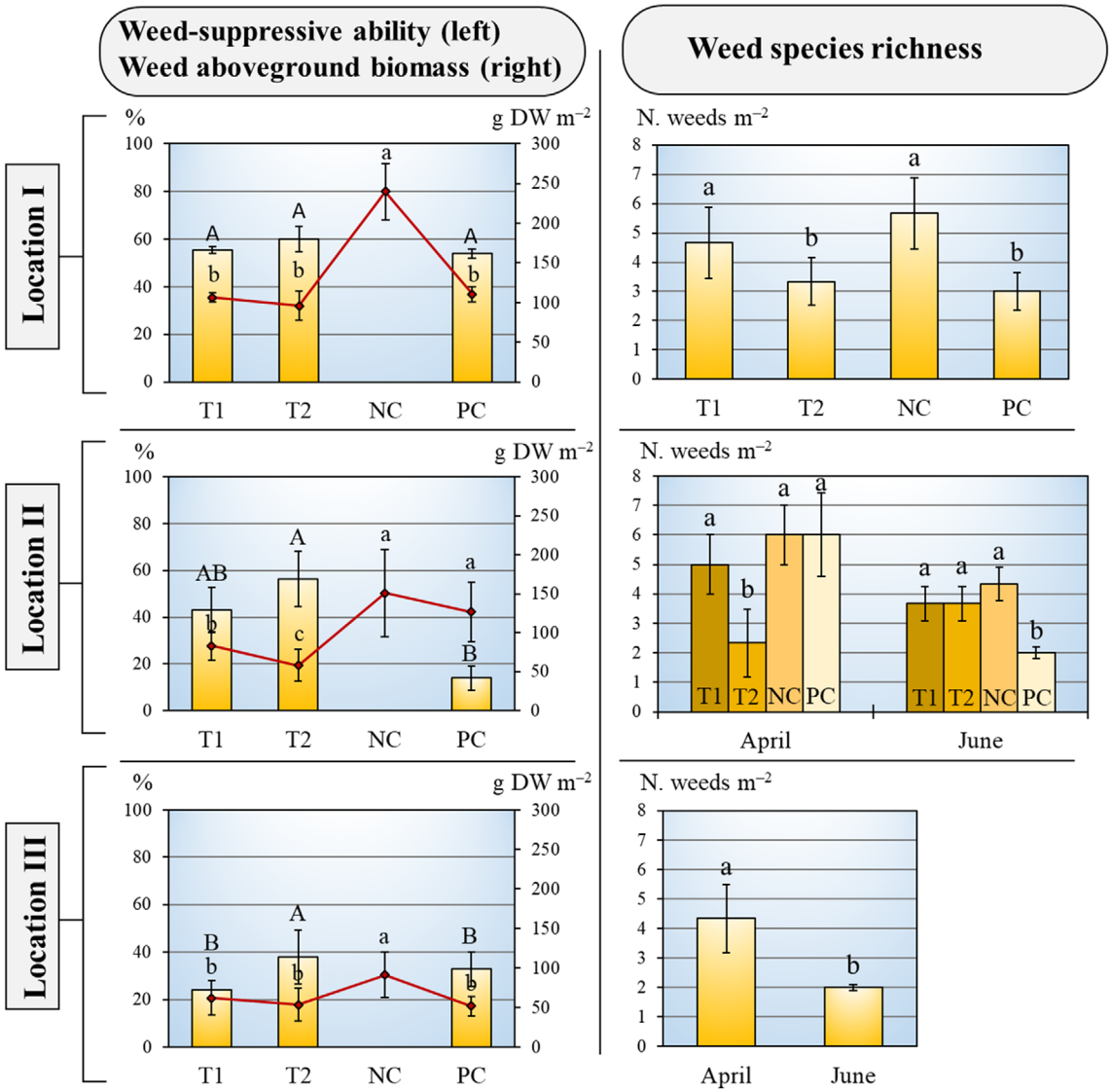

Figure 2. Weed-suppressive ability (left axis, histograms, capital letters), weed aboveground dry biomass (right axis, line, lowercase letters) and weed species richness over three different locations derived from the two-way analysis of variance shown in Table 1. Bars are standard deviation ($n = 3$). Different letters indicate statistical significance at $P \leq 0.05$ (Fisher's protected LSD test). T1, DiS-NH_2 at standard dose; T2, DiS-NH_2 at double dose; NC, negative control (no weeding); PC, positive control (chemical weeding).

Avena sterilis and *Convolvulus arvensis* L.) were the most frequent and abundant related to dryland areas. According to Riba and Recasens,[44] *L. rigidum* and *Papaver rhoeas* are recognised as the most important grass and broad-leaved weeds, respectively, that infest winter cereals in north-eastern Spain. In addition, a strong association of *Glebionis* spp. with southern Italian areas was reported by Fanfarillo *et al.*[45] Deligios *et al.*[40] studied the influence of herbicide underdosage on the composition and diversity of weeds in oilseed rape (*Brassica napus* L. var. *oleifera* D.C.) in Mediterranean fields and found that *Raphanus raphanistrum*, *Sinapis arvensis*, *G. coronaria* and Papaveraceae species (*Papaver rhoeas* and *F. officinalis*) are relatively the most abundant weeds. Interestingly, in the study reported here only two species (namely *Avena fatua* and *Sinapis arvensis*) were observed in June in location III (Table 4). However, the ANOVA carried out on RAI data did not show significant differences between treatments or sampling times (data not shown).

In addition to species richness, the species composition was analysed in terms of diversity (*H*) and evenness (*J*). Both indices were highly affected by 'location' (Table Figure S1), with the former more dependent on 'treatment' and the latter on 'sampling time' (Table 1). Regardless of 'location' and 'sampling time', T2 was characterised by the lowest *H* (1.22) and, at the same time, the highest *J* (0.86). These findings indicate that the weed communities had shifted towards fewer species present at higher density in a similar way to the chemical control ($H = 1.25$ and $J = 0.83$). This finding is not surprising since cropping systems subjected to continuous disturbance, such as herbicide applications or tillage, are characterised by low diversity and increased dominance of particular weeds.[46] It should be highlighted, however, that in this study these indices were strongly affected by the 'treatment × sampling time' interaction in locations II and III (Fig. 3). In fact, they performed differently from April to June. Overall, both diversity and evenness showed slightly higher

TABLE 2. Binomial names, botanical families, life cycle, ecophysiological (EG) and biological groups (BG), mean relative abundance values, and mean relative densities (RDs) of the weed communities under four weeding treatments and two sampling times in location I

Binomial name	Botanical family	Life cycle	EG	BG[†]	April	June	RD (%)[‡]
Avena fatua L.	Poaceae	Annual	Spring–summer	T	0.12	0.13	14.3
Calendula arvensis (Vaill.) L.	Asteraceae	Annual	Autumn–winter	T	0.10	—	5.3
Centaurea napifolia L.	Asteraceae	Annual	Spring–summer	T	0.24	0.20	22.6
Convolvulus arvensis L.	Convolvulaceae	Perennial	Indifferent	G	—	0.07	4.0
Fumaria officinalis L.	Fumariacee	Annual	Indifferent	T	0.14	0.08	11.8
Glebionis coronaria (L.) Cass. ex Spach	Asteraceae	Annual	Spring–summer	T	0.16	0.10	12.5
Helminthotheca echioides (L.) Holub	Asteraceae	Annual	Summer–autumn	T	0.07	0.01	3.4
Lolium multiflorum Lam.	Poaceae	Perennial	Spring–summer	H	—	0.13	7.2
Papaver rhoeas L.	Papaveraceae	Annual	Winter	T	—	0.06	2.3
Phalaris paradoxa L.	Poaceae	Annual	Spring	T	—	0.10	3.4
Poa annua L.	Poaceae	Annual	Indifferent	T	—	0.03	0.8
Silene fuscata Brot.	Caryophyllaceae	Annual	Spring–summer	T	0.07	—	3.1
Sinapis arvensis L.	Brassicaceae	Annual	Spring	T	0.06	0.10	8.5
Sonchus asper (L.) Hill	Asteraceae	Biennial	Indifferent	H	0.02	—	0.7

[†] T, therophytes; H, hemicryptophytes; G, geophytes.
[‡] Averaged over all treatments and sampling times.

TABLE 3. Binomial names, botanical families, life cycle, ecophysiological (EG) and biological groups (BG), mean relative abundance values, and mean relative densities (RDs) of the weed communities under f our weeding treatments and two sampling times in location II

Binomial name	Botanical family	Life cycle	EG	BG[†]	April	June	RD (%)[‡]
Anethum graveolens L.	Apiaceae	Annual	Spring–summer	T	—	0.16	7.7
Avena fatua L.	Poaceae	Annual	Spring–summer	T	0.03	—	1.2
Calendula arvensis (Vaill.) L.	Asteraceae	Annual	Autumn–winter	T	0.07	—	3.4
Dactylis glomerata L.	Poaceae	Perennial	Autumn–winter	H	0.04	0.14	9.2
Diplotaxis erucoides (L.) DC.	Brassicaceae	Annual	Indifferent	T	0.01	—	0.5
Euphorbia sp.	Euphorbiaceae	Annual	Summer–autumn	T	0.01	—	0.1
Galium aparine L.	Rubiaceae	Annual	Spring–summer	T	0.04	0.08	5.9
Glebionis coronaria (L.) Cass. ex Spach	Asteraceae	Annual	Spring–summer	T	0.23	0.36	32.4
Lolium perenne L.	Poaceae	Perennial	Autumn–winter	H	—	0.05	2.3
Medicago polymorpha L.	Fabaceae	Annual	Spring	T	0.10	—	5.4
Papaver rhoeas L.	Papaveraceae	Annual	Winter	T	0.19	0.08	13.7
Sinapis arvensis L.	Brassicaceae	Annual	Spring	T	0.23	0.13	17.1
Sonchus oleraceus L.	Asteraceae	Biennial	Indifferent	H	0.04	—	1.0
Verbesina sp.	Asteraceae	Annual	Spring–summer	T	0.02	—	0.4

[†] T, therophytes; H, hemicryptophytes; G, geophytes.
[‡] Averaged over all treatments and sampling times.

values than those widely reported in the literature. This situation is corroborated by Fanfarillo *et al.*,[45] who indicated particularly high levels of species richness and Shannon diversity in a traditionally managed agroecosystem of southern Italy.

The MANOVA results highlighted a $P \leq 0.001$ significance of the main factors ('treatment' and 'sampling time') and their interaction on weed species composition in all three locations (Table S2). In particular, 'sampling time' was the largest source of variation in locations I (80.5%), II (62.6%) and III (90.7%). This result is consistent with the findings of Scavo *et al.*,[26] where sampling time contributed more to variance than cover cropping on the composition of the soil weed seedbank. The two-way interactions for each location were analysed by PCAs on major weeds and are represented in ordination biplots that show the means for each 'treatment × sampling time' combination (Fig. 4). The eigenanalysis indicated that the cumulative variance explained by the first two eigenvalues was 72.1% for location I, 85.0% for location II and 98.0% for location III (Table S3). These percentages are sufficiently acceptable to interpret associations and discriminations and, as a consequence, principal component one (PC1) and principal component two (PC2) were considered. The weeds *Centaurea napifolia*, *F. officinalis* and *G. coronaria* captured 63% of the variance for PC1 in location I, while *Avena fatua* and *Sinapis arvensis* added 58% in PC2. In location II, *Anethum graveolens*, *Dactylis glomerata* and *Sinapis arvensis* jointly accounted for 86% of the variance for PC1, and a further 81% was added by *G. coronaria* and *Papaver rhoeas* for PC2. In location III, *Avena fatua* and *L. perenne* showed the majority of variance (52%) in PC1, while *M. polymorpha* and *Sinapis arvensis* added an additional 86% in PC2. PC1 showed the largest power of discrimination for

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TABLE 4. Binomial names, botanical families, life cycle, ecophysiological (EG) and biological groups (BG), mean relative abundance values, and mean relative densities (RDs) of the weed communities under four weeding treatments and two sampling times in location III

Binomial name	Botanical family	Life cycle	EG	BG[†]	April	June	RD (%)[‡]
Avena fatua L.	Poaceae	Annual	Spring–summer	T	0.04	0.45	20.8
Crepis foetida L.	Asteraceae	Biennial	Summer–autumn	H	0.03	—	0.4
Galium aparine L.	Rubiaceae	Annual	Spring–summer	T	0.08	—	4.4
Lolium perenne L.	Poaceae	Perennial	Autumn–winter	H	0.05	0.55	30.8
Medicago polymorpha L.	Fabaceae	Annual	Spring	T	0.36	—	26.9
Papaver rhoeas L.	Papaveraceae	Annual	Winter	T	0.05	—	1.3
Polygonum aviculare L.	Polygonaceae	Annual	Indifferent	T	0.07	—	2.4
Sinapis arvensis L.	Brassicaceae	Annual	Spring	T	0.23	—	11.4
Sonchus oleraceus L.	Asteraceae	Biennial	Indifferent	H	0.09	—	1.6
Verbesina sp.	Asteraceae	Annual	Spring–summer	T	0.01	—	0.1

[†] T: therophytes; H: hemicryptophytes; G: geophytes.
[‡] Averaged over all treatments and sampling times.

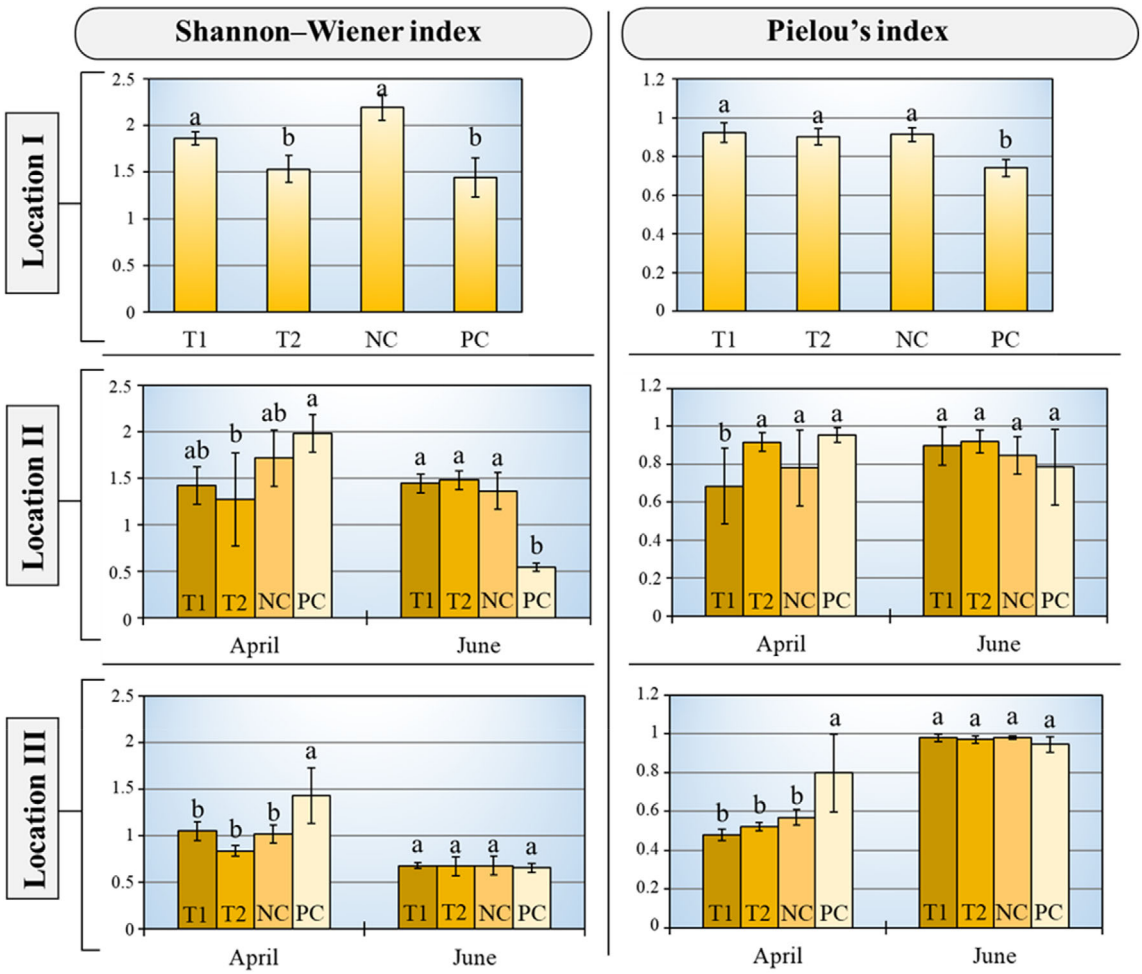

Figure 3. Shannon–Wiener (*H*) and Pielou's (*J*) biodiversity indices over three different locations derived from the two-way analysis of variance shown in Table 1. Bars are standard deviation ($n = 3$). Different letters indicate statistical significance at $P \leq 0.05$ (Fisher's protected LSD test). T1, DiS-NH_2 at standard dose; T2, DiS-NH_2 at double dose; NC, negative control (no weeding); PC, positive control (chemical weeding).

all three locations. Consistently with the MANOVA, it can be clearly seen from Fig. 4 that sampling time was discriminated along PC1, with April (green symbols) positioned on the right-hand side of the biplots and June (red labelling) on the left-hand side. As far as treatments are concerned, PC, both in April and in June, was not associated with any species except for *Papaver rhoeas* in April for location II. The bioherbicide mimic at double dose (T2) was positively associated with *G. coronaria*, *F. officinalis*

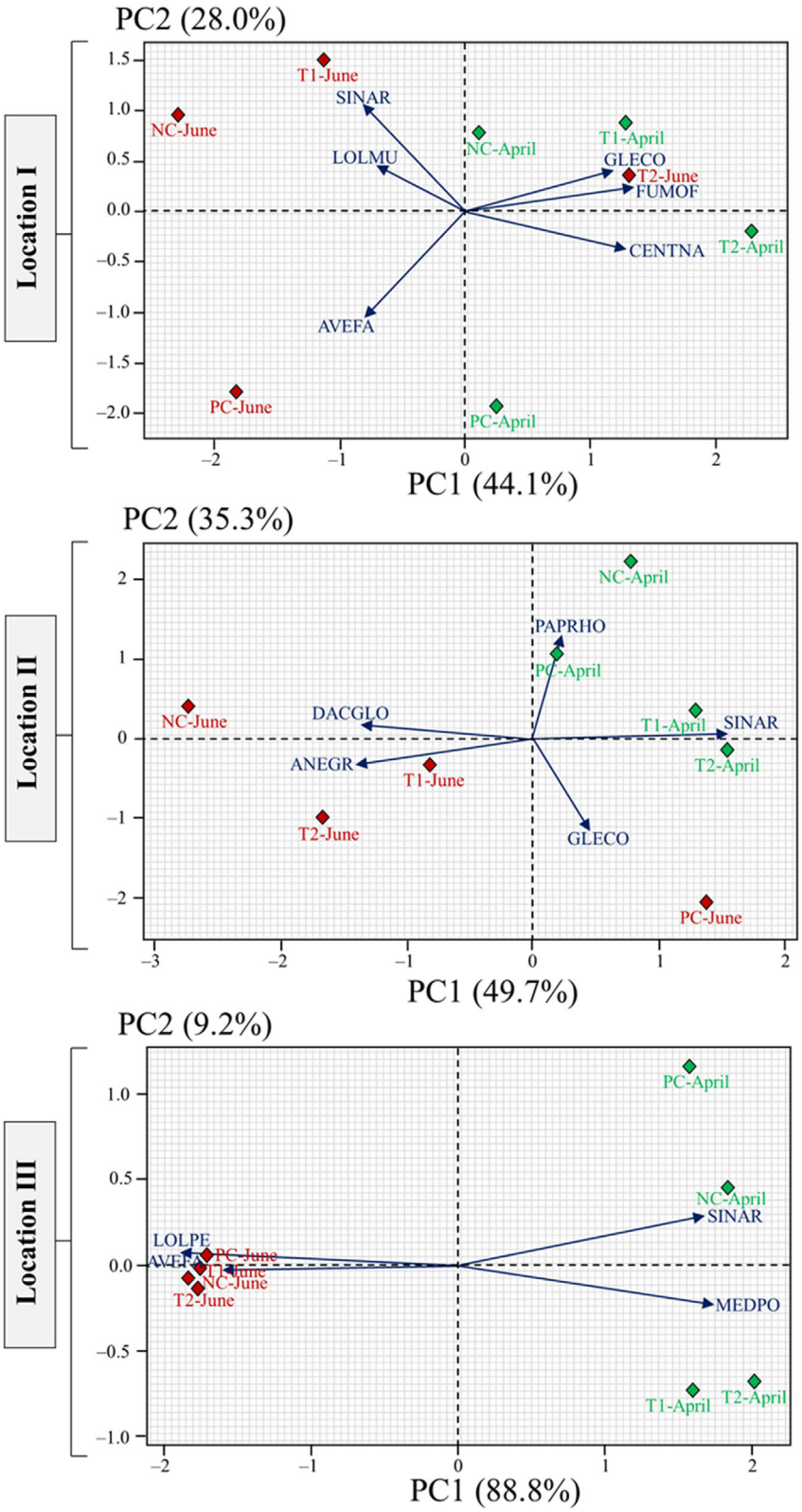

Figure 4. Principal components analysis (PCA) ordination biplots from the correlation matrix with major weeds across the three locations under study. Arrows highlight the discrimination of weeds along the principal components. Treatments: T1, DiS-NH_2 at standard dose; T2, DiS-NH_2 at double dose; NC, negative control (no weeding); PC, positive control (chemical weeding) Atlantis® WG (mesosulfuron-methyl + iodosulfuron-methyl-Na + mefenpir-diethyl) and Buctril® Universal (bromoxynil + 2,4-D). Weeds: ANEGR (*Anethum graveolens*); AVEFA (*Avena fatua*); CENTNA (*Centaurea napifolia*); DACGLO (*Dactylis glomerata*); FUMOF (*Fumaria officinalis*); GLECO (*Glebionis coronaria*); LOLMU (*Lolium multiflorum*); LOLPE (*Lolium perenne*); MEDPO (*Medicago polymorpha*); PAPRHO (*Papaver rhoeas*); SINAR (*Sinapis arvensis*).

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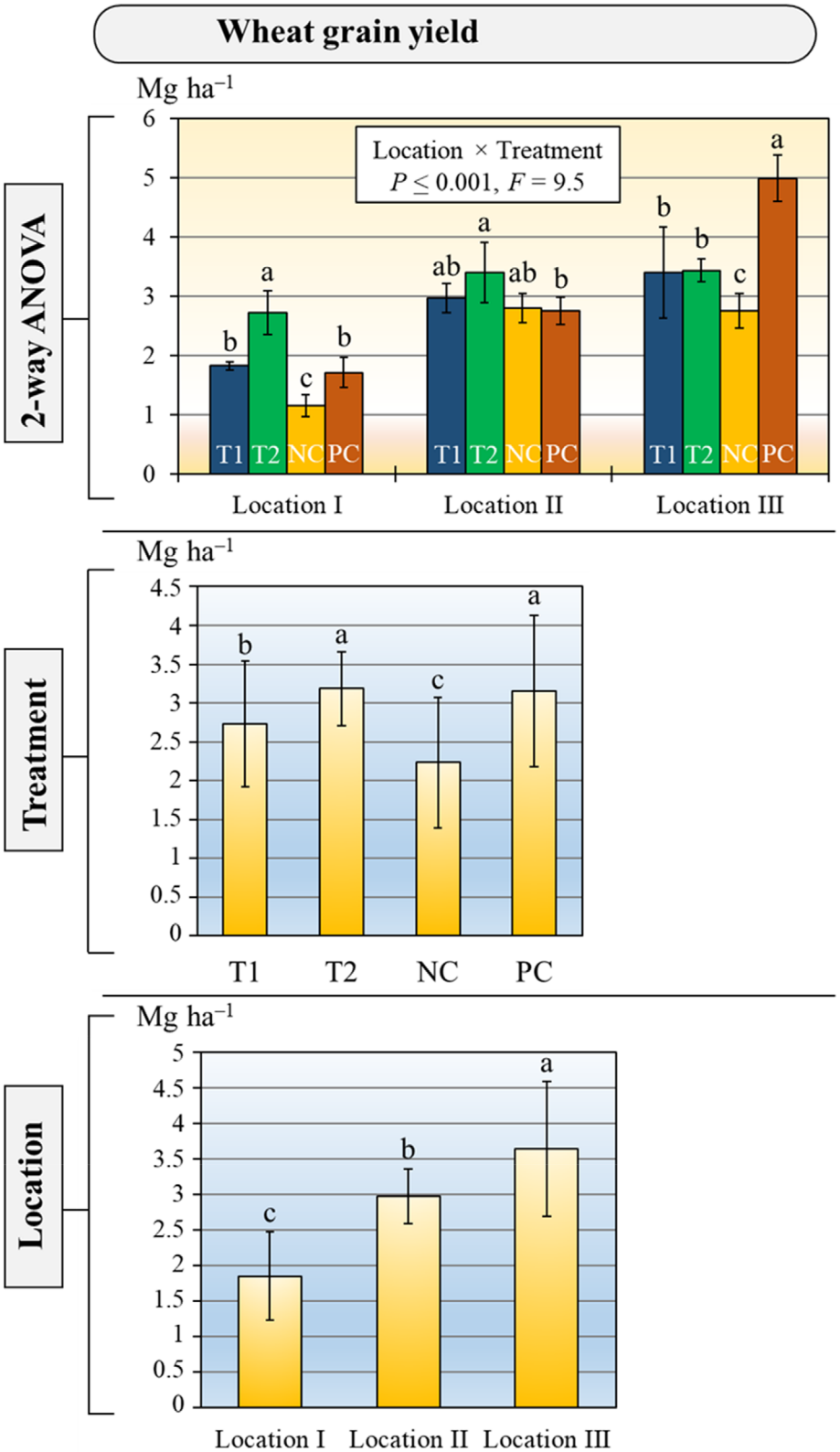

Figure 5. Two-way analysis of variance (ANOVA) and effects of treatment and location on durum wheat grain yield. Bars are standard deviation ($n = 3$). Different letters indicate statistical significance at $P \leq 0.05$ (Fisher's protected LSD test). T1, DiS-NH_2 at standard dose; T2, DiS-NH_2 at double dose; NC, negative control (no weeding); PC, positive control (chemical weeding).

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and *Centaurea napifolia* in location I, and negatively with *L. multiflorum* and *Sinapis arvensis*. In location II, T2 had a positive association with *Sinapis arvensis* in April and a negative one with *Anethum graveolens*, whereas the opposite trend was found in June. In location III, all of the treatments correlated positively with *L. perenne* and *Avena fatua* in June, but specific associations for T1 and T2 were not observed in April. In summary, the disulphide bioherbicide mimic under study did not create clear shifts in weed communities and this denotes a broad spectrum of phytotoxicity.

3.7 Effects on wheat grain yield

The two-way ANOVA showed that Gy was significantly affected by 'location' ($F = 76.7$, $P \leq 0.001$), 'treatment' ($F = 13.9$, $P \leq 0.001$) and their interaction ($F = 9.5$, $P \leq 0.001$). Compared to NC, T2 increased Gy by 57.6%, 17.6% and 19.8%, respectively, in locations I, II and III (Fig. 5). Similarly, T1 enhanced Gy by 36.7%, 5.8% and 19.0%, respectively, albeit not always significantly with respect to NC. The double bioherbicide mimic dosage (T2) only differed statistically from the standard dosage (T1) in location I and, except for location III, both led to higher Gy values of durum wheat than the chemical control (PC). In terms of the average values, the Gy of T2 (3.19 Mg ha^{-1}) was comparable with PC (3.15 Mg ha^{-1}), while the lowest value was found in NC (2.24 Mg ha^{-1}). In support of this trend, a significant and negative correlation was found between WAB and Gy ($r = -0.848$, $P = 0.0152$), thus demonstrating that the lower the weed biomass, the higher the Gy. In our previous study[32] it was found that, compared to NC, T2 enhanced the number of spikes m^{-2} (+19%) and the number of kernel $spikes^{-1}$ (+26%), while at the same time reducing the non-vitreous kernels (−40%). Regardless of treatments, the trend location III > location II > location I was found and this corroborates the results for the WAB. Similar Gy values to ours have been documented by CREA-CI of Foggia (~2.6–3.0 Mg ha^{-1}) in the absence of chemical input management systems. The mean Gy values reported here are slightly lower than the normal trend for the zone, probably due to the adverse climatic conditions (Fig. Figure S1). In particular, it is worth highlighting the high levels of rainfall in the early winter, which postponed wheat sowing, and in spring.

4 CONCLUSION

The results of the study reported here demonstrate, for the first time, the field efficacy of a disulphide bioherbicide mimic DiS-NH_2 in a nanoparticle formulation for weed management in durum wheat. Although this formulation is not yet available on the market, the NP@DiS-NH_2 bioherbicide mimic proved to be an efficient post-emergence and contact herbicide that can control a wide range of weed species in durum wheat without damaging the crop. The formulation proved to be safer for the *Caenorhabditis elegans* soil organism model, with the toxicity decreased by 50% in adults and eggs, in comparison with the non-encapsulated bioactive compound. This encapsulated bioherbicide displayed higher soil permeability in crop soil 72 h after application.

The proposed formulation provided stable phytotoxicity, in terms of weed biomass and species richness reduction, across three different locations and for the whole crop biological cycle. The best performance in terms of weed control efficacy and crop yield enhancement was obtained with the bioherbicide mimic applied at double dose (1.5 g m^{-2}), which decreased WAB by 57% and increased wheat Gy by 42% when compared with the untreated control. The standard dose (0.75 g m^{-2}), however, showed results comparable to those obtained with chemical weeding. The proposed bioherbicide mimic can therefore be suggested as a promising product for the sustainable management of weeds in wheat agroecosystems to reduce the need for chemical herbicides. Further investigations are required to improve the solubility and stability of the formulation and to test the product on a wider range of crops and weeds under different pedo-climatic conditions. Specifically, future studies should be conducted on how this new bioherbicide, NP@DiS-NH_2, could affect aquatic organisms. Analyses of potential bioaccumulation and acute toxicity should be carried out in aquatic invertebrates such as *Daphnia magna* and microalgae such as *Chlorella vulgaris*, which are fundamental to the trophic chain. Concerning formulation, future studies should analyse the stability of the bioherbicide during raining seasons and high temperature seasons to assure the efficacy in extreme conditions. Another possibility for further study is the evaluation of different doses and application times to evaluate dose–response in the field.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

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

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