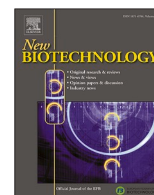
New BIOTECHNOLOGY 72 (2022) 48–57

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com/locate/nbt



Automatable downstream purification of the benzohydroxamic acid D-DIBOA from a biocatalytic synthesis

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A R T I C L E I N F O

Keywords:
D-DIBOA
Green chemistry
Whole-cell biocatalyst
Downstream processes
Chromatography
Ecological herbicides

A B S T R A C T

Herbicides play a vital role in agriculture, contributing to increased crop productivity by minimizing weed growth, but their low degradability presents a threat to the environment and human health. Allelochemicals, such as DIBOA (2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4 H)-one), are secondary metabolites released by certain plants that affect the survival or growth of other organisms. Although these metabolites have an attractive potential for use as herbicides, their low natural production is a critical hurdle. Previously, the synthesis of the biologically active analog D-DIBOA (4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one) was achieved, using an engineered *E. coli* strain as a whole-cell biocatalyst, capable of transforming a precursor compound into D-DIBOA and exporting it into the culture medium, although it cannot be directly applied to crops. Here a chromatographic method to purify D-DIBOA from this cell culture medium without producing organic solvent wastes is described. The purification of D-DIBOA from a filtered culture medium to the pure compound could also be automated. Biological tests with the purified compound on weed models showed that it has virtually the same activity than the chemically synthesized D-DIBOA.

Introduction

Herbicides are useful and potent tools for weed control that accounted for 51.9 % of the whole pesticide market in 2019 and present a growing trend [1]. However, agriculture is currently facing certain harmful effects due to the application of these chemicals, including a growing number of herbicide-resistant weeds, the emergence of substituting weed flora and the contamination of food and the environment derived from an improper use of synthetic herbicides. For these reasons, the EU is promoting the marketing of pesticides that contain biological active substances and has shortened the procedure for the authorization by Member States for new pesticides [2]. An alternative and environmentally-friendly methodology for weed management consists of the implementation of allelopathic mechanisms, a strategy that has attracted increasing interest according to the many articles published in the field over the last decade, half of them focused on weed control [3]. Allelopathy is a biochemical phenomenon with ecological implications that describes the effect that one plant (donor) has on another (target) through the production of certain chemical compounds (allelochemicals) released into the environment [4]. Allelochemicals are biodegradable defensive secondary metabolites that affect the survival or growth of other organisms and comprise a very wide range of chemical classes, including phenolics, terpenoids and nitrogen containing compounds, such as benzoxazinoids [5].

Abbreviations: DIBOA, 2,4-dihydroxy-(2H)-1,4-benzoxazin-3-(4H)one; D-DIBOA, 4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one, or 2-deoxy-DIBOA; wcb-D-DIBOA, D-DIBOA obtained by whole-cell biocatalysis; cs-D-DIBOA, D-DIBOA obtained by chemical synthesis; PC, ethyl-2-(2′-nitrophenoxy)acetate (D-DIBOA precursor); FT, flow-through fractions; W, wash fractions; E, elution fractions; IMAC, immobilized-metal affinity chromatography; IEX, ion-exchange chromatography; Mono Q, anion exchange column; Mono S, cation exchange column; RPLC, Reversed Phase Liquid Chromatography.

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https://doi.org/10.1016/j.nbt.2022.09.001
Received 28 April 2022; Received in revised form 16 September 2022; Accepted 18 September 2022
Available online 19 September 2022
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The incorporation of allelochemicals as new herbicides into agricultural management would reduce the use of synthetics and minimize the negative environmental impact of the latter [6–8]. However, their scarce natural production is a critical hurdle for their large-scale application [9]. As an alternative, they could be produced by chemical synthesis, which would provide high yields [10], but this strategy often implies the use of harmful chemicals under critical conditions that are associated with the generation of undesirable by-products [11]. The development of biocatalysts is a green chemistry approach that would mitigate the negative impacts of traditional chemical synthesis. For instance, the hydroxamic acid DIBOA (Supplementary Fig. S1a) is an allelochemical produced by certain plant species from the Poaceae family and is considered a herbicide model [9]. DIBOA is a secondary metabolite synthesized in a branch of the tryptophan biosynthesis pathway comprising 5 reactions catalyzed by the BX enzymes. In this pathway, indole-3-glycerolphosphate is converted into indole by BX1. The remaining reactions consist of the conversion of indole into DIBOA by introduction of 4 oxygen atoms catalyzed by the 4 cytochrome-P450-dependent monooxygenases BX2 to BX5 [12]. The chemical synthesis of the biologically active analogous compound D-DIBOA (Supplementary Fig S1a) comprises two steps. The first is a reaction that uses 2-nitrophenol and ethyl bromoacetate as starting materials to produce ethyl-2-(2′-nitrophenoxy)acetate, the D-DIBOA precursor (PC), by a nucleophilic substitution. This chemical reaction has been optimized to reach a 99 % molar yield [10]. However, the second step (Supplementary Fig. S1b), which consists of the reduction of a nitro group of the PC followed by a cyclization with the acetate radical, is a hindrance for the scaling-up of the process. This reaction requires an expensive Pd/C catalyst and involves the use of hazardous or harmful reagents such as dioxane or $NaBH_4$ in an exothermic reaction with a potentially dangerous release of H_2. The use of a genetically engineered *Escherichia coli* strain overexpressing the autologous nitroreductase NfsB as a whole-cell biocatalyst can overcome these drawbacks [13]. Indeed, the *E. coli* strain is capable of catalyzing the conversion of the PC into D-DIBOA and exporting it into the culture medium with molar yields similar to those achieved by chemical synthesis (70 %). Furthermore, the optimization of the mutant background (Δ*lapA*Δ*fliQ*/pBAD-NfsB strain), the use of a specifically adjusted medium, and the addition of several loads of PC at different times made it possible to produce up to 5.01 mM D-DIBOA or 100 % molar yield at a lower concentration (4.4 mM) [14]. In a recent report, the monitoring of D-DIBOA concentration in real-time allowed a controlled PC addition that increased D-DIBOA production up to 7.17 mM [15]. Since the use of D-DIBOA in biological tests is within the μM range, its production using *E. coli* as a whole-cell biocatalyst is a feasible possibility.

Nevertheless, an obstacle remains to enabling the production of biological D-DIBOA on an industrial scale: the culture medium cannot be applied directly to plant crops because it is a product derived from a genetically modified organism with a relatively high salt content. Therefore, the development of a D-DIBOA purification method that avoids the use of harmful chemicals and allows pure D-DIBOA to be obtained for its further formulation as a herbicide is still crucial. Such downstream processes are challenging and often represent the bottleneck for whole-cell biocatalysis applications.

Here, a chromatographic method to purify the D-DIBOA found in a cell culture medium without generating any organic solvent wastes is described. In addition, the method could be automated. The purified compound has been tested in biological assays using weed models and the results have been compared with those obtained using chemically synthesized D-DIBOA, with virtually the same results being achieved in either case.

Material and methods

Reagents and standard solutions

All the reagents used were of the highest purity available (Panreac Quimica, Barcelona, Spain). The medium used for the standard solutions and for the biocatalysis process was M9 minimal medium, containing (g/L): 0.24 $MgSO_4$, 0.01 $CaCl_2$, 11.178 Na_2HPO_4, 3.00 KH_2PO_4, 0.50 NaCl, 1.00 NH_4Cl, 4.00 glucose. D-DIBOA and PC stock solutions (200 mM) in methanol were used to prepare an equimolar concentration standard solution (2 mM) of both D-DIBOA and PC in M9 fresh medium. The chemically synthesized D-DIBOA and the PC were obtained following the method described by [10]. The samples of D-DIBOA produced by whole-cell biocatalysis were obtained following the procedure previously described [14]. The *E. coli ΔlapAΔfliQ*/pBAD-NfsB strain, registered in the Spanish Type Culture Collection (CECT 9760), was used as whole-cell-biocatalyst.

Chromatographic techniques

An EP-1 Econo Pump (Bio-Rad, Hercules, CA, USA) peristaltic pump was used for quantitative liquid transfer. Small-scale and preparative chromatography was carried out as follows.

Small-scale chromatography.

Minicolumns with several stationary phases (below) were used for low-volume tests at 1 mL/min flow rate. To test D-DIBOA and PC binding capability, the minicolumns were equilibrated with 5 mL of M9 medium, loaded with 5 mL of M9 containing 2 mM D-DIBOA and 2 mM PC and washed with 2–5 mL of M9. Elution was carried out using 100 % isopropanol or methanol. The following samples were collected in 1 mL fractions: flow-through (FT), wash (W) and elution fractions (E). All the collected samples were analyzed following the analytical methods described in the *Analytical Techniques* section.

Affinity chromatography: Immobilized-Metal Affinity Chromatography (IMAC): Five 1 mL Chelating HiTrap columns (7 mm × 25 mm) (GE Healthcare, Chicago, IL, USA) were charged with Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} or Co^{2+}, following the supplier's instructions, using the following solutions: 0.2 M $NiSO_4$, $CuSO_4$, $ZnSO_4$, $FeCl_3$ or $CoCl_2$.

Ion-Exchange Chromatography (IEX): IEX chromatography was tested with 1 mL (5.6 mm × 40 mm) cartridges (Bio-Rad) containing an anion exchanger (Bio-Scale™ Mini-Prep[R] High Q) or a cation exchanger (Bio-Scale™ Mini-Prep[R] High S).

Reversed Phase Liquid Chromatography (RPLC): Reversed phase was studied using a Discovery[R] DSC-18 6 mL SPE cartridge (Merck, Darmstadt, Germany), containing 1 g of solid phase.

Preparative chromatography

In order to scale up the chromatographic processes, two 2.5 × 10 cm Econo-column[R] Chromatography columns (Bio-Rad) were packed with 50 mL of Chelating Sepharose™ Fast Flow (GE Healthcare) and charged with Cu^{2+} or 50 mL of Macro-Prep[R] High S Media (Bio-Rad). For RPLC, a 5 × 10 cm Econo-column[R] Chromatography column (Bio-Rad) was packed with 1 g (20 mL) of LiChroprep[R] RP-18 (Merck). The columns were then equilibrated using 3 column volumes (cv) of the appropriate buffer, loaded and eluted as described in the Results section. Elution was carried out by real-time detection of the fractions containing D-DIBOA employing the colorimetric method described below. The flow rates set for each column were 3 mL/min for IMAC-Cu^{2+}, 4 mL/min for Mono S and 6 mL/min for C18. The elution fractions were pooled and evaporated to dryness using a rotary evaporator.

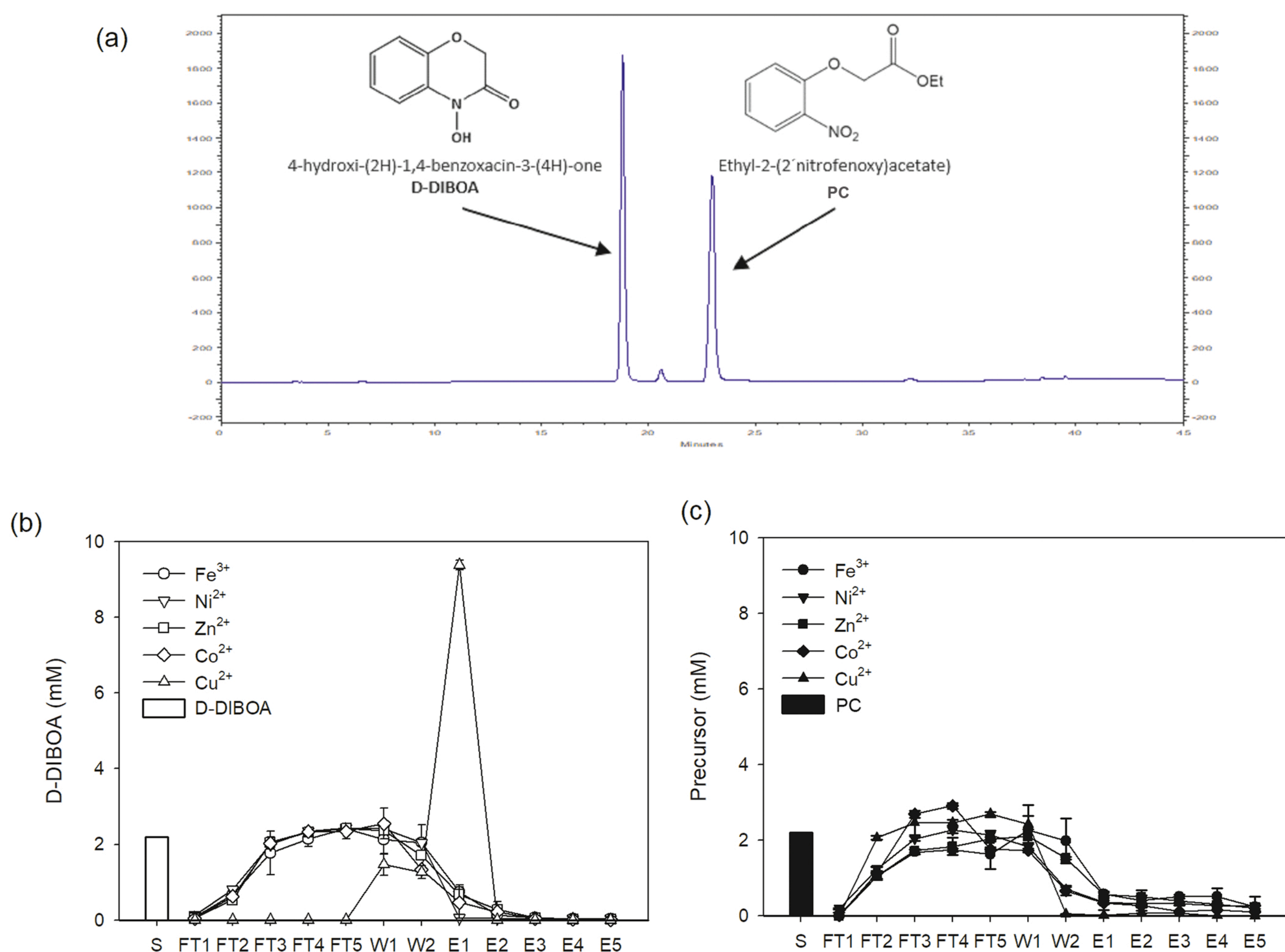

Fig. 1. Binding tests for D-DIBOA and PC to several IMAC coupled cations. (a) HPLC chromatogram of the sample used for the test consisting of M9 medium containing an equimolar concentration of PC and D-DIBOA (2 mM) and the corresponding chemical structures. 5 mL of the sample was applied to each of the five 1 mL HiTrap mini-columns to which Fe^{3+}, Ni^{2+}, Zn^{2+}, Co^{2+}, or Cu^{2+} were previously coupled. The minicolumns were equilibrated with 5 mL of M9 medium, loaded, washed with 2 mL of M9 medium and eluted by an isocratic method with 5 mL of 100% isopropanol. 1 mL fractions were collected during the column loading (flow-through fractions FT1 to FT5), the washing (W1 and W2), and the elution (E1 to E5). D-DIBOA (b) and PC (c) concentrations in the fractions were monitored by HPLC. Only the IMAC-Cu^{2+} column was capable of retaining D-DIBOA (white triangle). None of the columns showed binding capacity for the PC.

Analytical techniques

D-DIBOA and PC were quantified by reverse-phase High Performance Liquid Chromatography using a Merck HITACHI HPLC system equipped with a Phenomenex Gemini C18 4.6 × 250 mm column and the method previously described [16]. For those samples that only required D-DIBOA quantification, the spectrophotometric method based on the generation of blue Fe^{3+}-$(D\text{-}DIBOA)_3$ complexes, as recently described [15], was employed. D-DIBOA concentration was calculated by measuring its absorbance at 750 nm in a microtiter reader plate (Thermo Fisher Scientific, Waltham, MA, USA). Before quantification, all the samples were filtered through 0.22 µm nylon filters (VWR International, Radnor, PA, USA).

Nuclear Magnetic Resonance (NMR) and mass spectrometry procedures

The structural elucidation of compounds was carried out using equipment available at the University of Cadiz. An Agilent (Santa Clara, CA, USA) spectrometer 500/125 MHz was used to record NMR spectra. $CDCl_3$ (Prolabo™, VWR) was the solvent for samples and the residual peak was used as internal reference at δ 7.26 in ^1H and δ 77.0 in ^{13}C NMR. The exact masses of compounds were measured on a UPLC-QTOF ESI (Waters Synapt G2, Manchester, UK) high-resolution spectrometer (HRTOFESIMS), with mass spectra recorded by the negative- or positive-ion method in the *m/z* range 100–2000, mass resolution of 20,000 and an acceleration voltage of 0.7 kV. A Perkin-Elmer Spectrum TWO IR spectrophotometer was used to obtain FTIR spectra.

Phytotoxicity bioassay on seeds

The selection of target plants was based on an optimization process carried out in our search for a standard phytotoxicity bioassay [17]. Several Standard Target Species were proposed, including the monocotyledon *Allium cepa* L. and the dicotyledons *Solanum lycopersicum* Will., *Lepidium sativum* L., and *Lactuca sativa* L. In addition, four weed species were selected because they belong to important families of agricultural weeds: barnyard grass (*E. crus-galli*, *Poaceae*), slender amaranth (*A. viridis*, *Amaranthaceae*), guinea grass (*M. maximus*, *Poaceae*) and annual ryegrass (*L. rigidum*, *Poaceae*).

The phytotoxic activity of the compounds on seeds was assessed following the procedure previously described [18]. The bioassays were conducted using Petri dishes (50 mm diameter) with one sheet of Whatman No. 1 filter paper 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 solutions containing the compounds were tested at the same

concentrations reported above. All the D-DIBOA and control solutions had a constant concentration of DMSO (5 μL/mL). The buffer solution lacking the tested compounds was used as a negative control and the herbicide Logran Extra 60 WG (Syngenta Basel, Switzerland) as a positive control, as in the coleoptile bioassay.

The experimental design was completely randomized and contained four replicates of 20 seeds for each concentration. After adding the seeds and the aqueous solutions, the Petri dishes were sealed with Parafilm to ensure closed-system models. The seeds were further incubated at 25 °C in the dark in an ICE 700 growth chamber (Memmert, Schwabach, Germany). The bioassays took 4 days for *L. sativum*, 7 days for *S. lycopersicum*, *L. sativa*, *A. cepa* and *L. rigidum* and 11 days for *A. viridis*, *E. crus-galli* and *M. maximus*. After growth, the plants were frozen at − 10 °C for 24 h. Their germination ratios, root lengths, and shoot lengths were recorded using a Fitomed system [19]. The data were analyzed statistically using Welch's test, with significance set at 0.01 and 0.05. The germination ratios, root lengths, and shoot lengths were presented as the percentage difference with respect to the control sample. Zero represents the control sample, while the positive values represent stimulation, and the negative values represent inhibition.

Results and discussion

D-DIBOA purification from M9 medium.

The production of D-DIBOA by whole-cell biocatalysis using genetically modified strains of *E. coli* was carried out under mild conditions, without use of organic solvents (methanol was used only at a low concentration as a vehicle for the addition of the PC into the cell culture medium, M9 defined medium). Bacteria take up the PC, transform it into D-DIBOA at a high yield and export it into the culture medium. This strategy has produced up to 7.17 mM D-DIBOA, transforming 89.90 % of the PC in 24 h [15] However, recovering D-DIBOA from the medium is a difficult challenge since it is present together with the remaining untransformed PC, other bacterial products and the inorganic compounds contained in the M9 medium. The product obtained from the biocatalysis cannot benefit from the methodology applied for the purification of the chemically synthesized compound because, in this case both, PC and D-DIBOA are in an organic matrix and mixed with very different by-products. Several chromatographic principles were therefore investigated for isolation of D-DIBOA from the PC present in M9 culture medium.

Immobilized-Metal Affinity Chromatography (IMAC)

Affinity chromatography is very selective and provides high resolution with an intermediate to high sample loading capacity. Hydroxamic acids are capable of binding iron (III) with high affinity [20]. It was hypothesized that a complexation reaction of D-DIBOA with Fe^{3+} or other cations could be used as an affinity binding principle because the PC used to synthesize D-DIBOA does not contain a hydroxylamine moiety and would presumably not bind to these ions. This hypothesis was tested by applying IMAC, in which a sepharose matrix is coupled with iminodiacetic acid, to allow the matrix to be charged with a suitable metal ion. Several metal ions can be used for this purpose, i.e. Ni^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} and Co^{2+}. However, it has not been tested to purify hydroxamic acids.

For this purpose, five 1 mL IMAC minicolumns were prepared containing each of the above cations, the columns were equilibrated with 5 mL of M9 culture medium and 5 mL of the same solution containing an equimolar concentration of PC and D-DIBOA (2 mM) (Fig. 1a) was loaded, then washed with 2 mL of M9 culture medium, and eluted isocratically with 100 % isopropanol. Only the column coupled with Cu^{2+} was capable of binding D-DIBOA (Fig. 1b). Although a small fraction of D-DIBOA was detected during washing with M9 medium (Fig. 1b), most was eluted in the first fraction (1 mL), increasing its concentration by 3.9 times with respect to the sample concentration. As expected, the PC did not bind to Cu^{2+} or to any of the other tested cations (Fig. 1c).

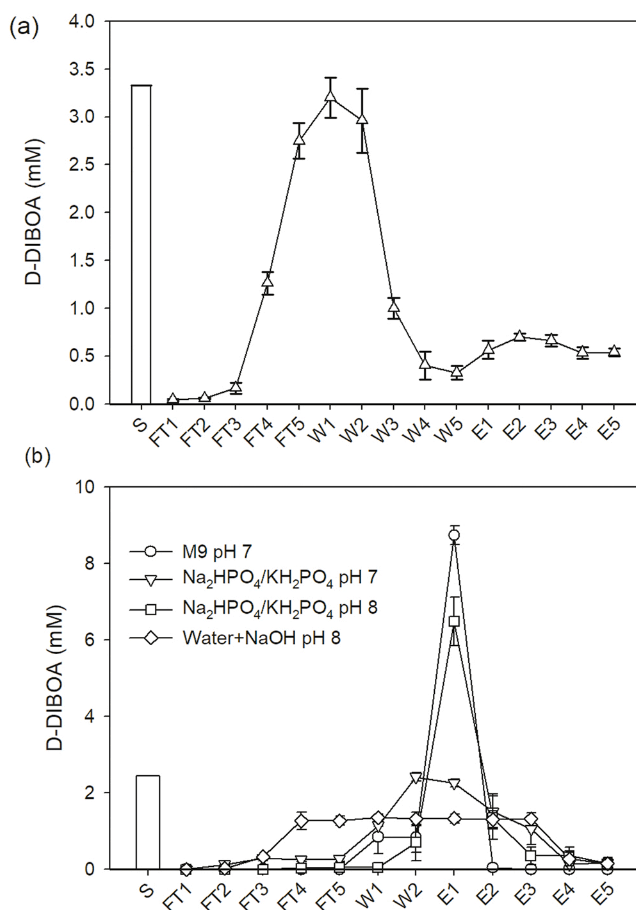

Fig. 2. Optimization of D-DIBOA binding buffer to IMAC-Cu^{2+} Analysis of D-DIBOA binding to an IMAC-Cu^{2+} 1 mL minicolumn using a real-time colorimetric D-DIBOA quantification method (a) D-DIBOA from a 5 mL biotransformation sample did not bind to IMAC-Cu^{2+}. (b) Optimization of loading buffer for IMAC-Cu^{2+}. Several solutions containing 2.2 mM D-DIBOA were tested for IMAC-Cu^{2+} binding capability. A 100 mM phosphate solution, pH 8 was sufficient for D-DIBOA binding to IMAC-Cu^{2+}.

The product was then assayed of a biotransformation sample from a whole-cell biocatalysis carried out following an experimental design previously reported [14]. For this, 24 h after the addition of the PC, the cells were removed from the culture medium by centrifugation and filtration, and 5 mL of the cell-free medium were loaded into the IMAC-Cu^{2+} minicolumn and eluted under similar chromatographic conditions to those of the abiotic test. To determine D-DIBOA concentration, a quick and reliable colorimetric test was used as previously reported [15]. Unexpectedly, D-DIBOA did not bind to the Cu^{2+} matrix in this case (Fig. 2a) and the compound was found in the flow-through as well as the washing fractions. This was probably because *E. coli* had modified the composition of the initial M9 culture medium by consuming and/or excreting compounds. Indeed, the pH at the end of the transformation (24 h after the addition of the PC to the cell culture) had fallen from pH 7.10 to pH 5.25. Several solutions containing single components of the M9 medium were therefore tested in order to find out which of those in the M9 medium were responsible for the D-DIBOA binding. These tests revealed that the most relevant features were phosphate salts and pH. A 100 mM Na_2HPO_4/KH_2PO_4 solution pH 8 was sufficient for D-DIBOA to bind the IMAC-Cu^{2+} matrix (Fig. 2b). Nevertheless, attempts to adjust the biotransformation solution to pH 8 by addition of phosphates or NaOH did not facilitate D-DIBOA binding to the matrix (data not shown), probably because they increased the saline concentration of the solution. For this reason, another type of

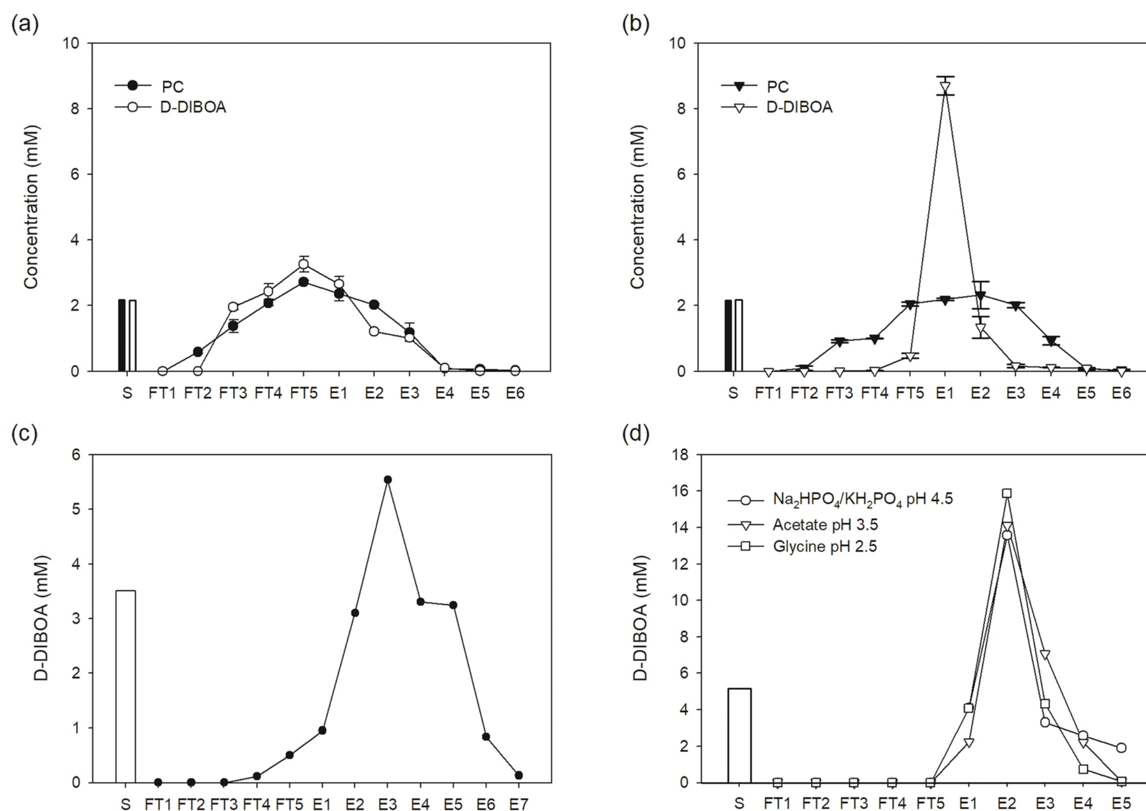

Fig. 3. IEX binding tests and their application as a buffer exchanger. For (a) and (b), abiotic M9 culture medium containing 2 mM PC and D-DIBOA was tested with anion and cation exchanger stationary phases using an isopropanol isocratic elution method. (a) A Mono Q anion exchanger did not retain PC or D-DIBOA. (b) D-DIBOA bound to Mono S cation exchanger, although PC also was slightly retained. (c) D-DIBOA from a biotransformation bound to Mono S and eluted with 100 mM phosphate solution, pH 8. (d) IMAC-Cu^{2+} chromatography applying the pool of the eluted fractions from Mono S in (c). D-DIBOA bound to the stationary phase and was successfully eluted by lowering pH with phosphate (pH 4.5), acetate (pH 3.5) and glycine (pH 2.5) solutions. PC and D-DIBOA were quantified by HPLC in (a) and (b). In (c) and (d) D-DIBOA was quantified colorimetrically.

chromatography was attempted with the aim of changing the biotransformation solution into a buffer suitable for the application of IMAC-Cu^{2+}.

Ion-Exchange (IEX) chromatography

To test if IEX could be a suitable strategy to change the D-DIBOA biotransformation matrix solution and/or for its purification, an anion exchange Q-Sepharose and a cation exchange S-Sepharose minicolumns loaded with 5 mL of M9 abiotic medium containing 2 mM of D-DIBOA and PC were tested. While neither the PC nor the D-DIBOA bound to the Q-sepharose resin (Fig. 3a), D-DIBOA was retained by the S-Sepharose column (Mono S), although the PC also showed a low binding capacity (Fig. 3b). Mono S chromatography therefore could be used to enrich the D-DIBOA concentration, although not sufficient to completely purify the target molecule. Nevertheless, it was reasoned that it could be helpful as a buffer interchanger to have the D-DIBOA diluted in a suitable solution for later application to IMAC-Cu^{2+}.

In order to test this possibility, 5 mL of a cell-free biotransformation sample was loaded onto a 1 mL Mono S minicolumn equilibrated with M9 medium and eluted with 5 mL of a 100 mM Na_2HPO_4/KH_2PO_4, pH 8 solution, which is a suitable buffer for IMAC-Cu^{2+} application. It was found that the D-DIBOA from the biotransformation solution also bound to Mono S and was also successfully eluted (Fig. 3c). The elution fractions were pooled and 5 mL were loaded onto an IMAC-Cu^{2+} minicolumn and, as predicted, the D-DIBOA was retained under these conditions. The washing step was omitted in order to avoid any losses of D-DIBOA. Different buffered solutions with pH under 7 for IMAC-Cu^{2+} elution were also explored, with the aim of avoiding organic solvents such as isopropanol, since it was known that D-DIBOA did not bind the stationary phase at acidic pH. Indeed, phosphate (100 mM) pH 4.5; acetate (100 mM) pH 3.5 or glycine (100 mM) pH 2.5 buffers eluted D-DIBOA (Fig. 3d). Acetate pH 3.5 was selected for further experiments, as the least expensive of the three tested.

Reversed Phase Liquid Chromatography (RPLC)

Both PC and D-DIBOA, being aromatic hydrophobic molecules, would be able to bind to a hydrophobic matrix. Their binding capacity was tested in a 1 g C-18 cartridge by loading 6 mL of an equimolar sample in M9 (Fig. 4a) or 6 mL of a real biotransformation sample (Fig. 4b). Both PC and D-DIBOA bound strongly to the stationary phase in both cases and were eluted using isocratic 100 % methanol. The strong D-DIBOA binding allowed an exhaustive washing of the loaded column with water before proceeding with the elution, which would help to eliminate the saline and polar compounds from any of the solutions containing D-DIBOA.

Integration of the IEX, IMAC-Cu^{2+} and RLPC in a purification scheme

The complete purification of a compound of interest usually involves several chromatographic steps where the target molecule increases its concentration while the rest of the compounds are progressively discarded. On the other hand, HPLC, a very resolving chromatography, cannot often be applied to biotransformations due to the high salt content and the large volume of the solutions in which the target molecules are produced. The main impurity found in biosynthetic D-DIBOA, once the cells have been removed, is unreacted PC. Although biocatalysis yields are close to 100 %, the remaining PC must be eliminated because of its toxicity.

In this work a three-step chromatographic downstream process was

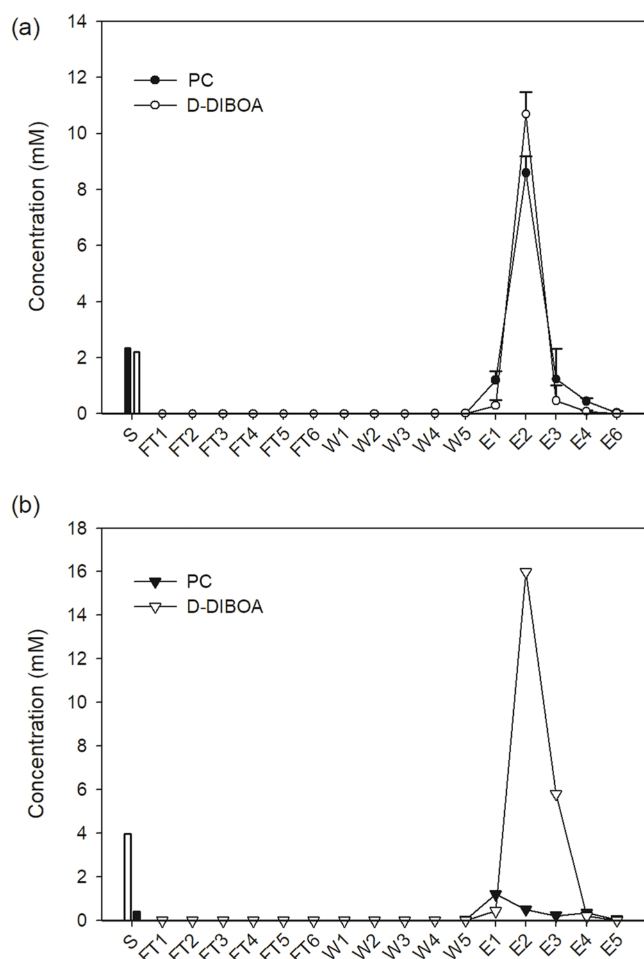

Fig. 4. RPLC binding tests. (a) D-DIBOA (white) and PC (black) in M9 medium bound strongly to C18 minicolumn but co-eluted with 100% methanol. (b) D-DIBOA in biotransformation culture edium bound to C18 and also co-eluted with PC.

designed with the aim of obtaining highly purified D-DIBOA but also to minimize toxic wastes and the use of organic solvents. In this sequence of operations, an IEX chromatography (Mono S) was first applied, followed by an affinity chromatography (IMAC-Cu^{2+}) and finally an RPLC (C18) using an isocratic elution for each (Fig. 5). The aim of applying Mono S as the first step (Fig. 5a) was not only the partial purification of the product of interest but, above all, to change the culture medium into one more suitable for the loading of the IMAC-Cu^{2+} column, thereby allowing the application of the same buffer for Mono S elution and IMAC-Cu^{2+} equilibration and loading (phosphate buffer, pH 8). The key role of IMAC-Cu^{2+} in this purification scheme is the elimination of the unreacted PC (Fig. 5b). The elution of this column with an inexpensive, non-toxic acetate solution (pH 4.5) was also compatible with the loading of the C-18 column (RPLC), which is capable of strongly binding D-DIBOA but does not retain ions or polar molecules contained in the elution of the second step (Fig. 5c). To avoid D-DIBOA losses, the washing steps after loading the samples into the Mono S and IMAC-Cu^{2+} columns were omitted, but the C18 column was washed with water to remove all non-specifically bound molecules. The elution of this column with methanol would allow the separation of D-DIBOA as a solid compound through the evaporation of the organic solvent.

In order to put this sequence of operations to the test, the volume of the columns was scaled up as described in the Material and Methods section to purify D-DIBOA from 400 mL of a biotransformation solution. This experiment was carried out in triplicate using three different biotransformation solutions (Fig. 5). Fractions of 2 mL were collected in all the steps and the D-DIBOA concentration in each fraction monitored by applying the colorimetric method [15] that allowed measurements in real time. The eluted fractions containing D-DIBOA were pooled and loaded into the next column (Mono S and IMAC-Cu^{2+}) or evaporated (C18) to obtain finally a solid product. The biotransformation solution and the pools from each elution were analyzed by HPLC to determine the concentrations of PC, D-DIBOA and other unidentified compounds. The degree of purification was estimated as the percentage of the areas of the peaks detected in the HPLC chromatograms, since the actual concentration of the unidentified components could not be calculated (Fig. 5d). It is worth noting that the HPLC analysis of the final pool eluted from the C18 column detected highly purified D-DIBOA. The final C18 eluate was dried byrotary evaporation to give a solid product and analyzed by NMR. The spectroscopic data of both the solid D-DIBOA previously obtained [10] and that obtained by the present biotransformation (data not shown), were identical and consistent with those described in the relevant literature [21]. Quantification of the different D-DIBOA concentrations along the process showed a recovery yield close to 100 % (Fig. 5d).

Prototype design for automatic D-DIBOA purification

The purification scheme designed here allows the equilibration of the second (IMAC-Cu^{2+}) and third (C18) columns with the same elution buffer used in the previous step. The pools eluted from Mono S and IMAC-Cu^{2+} can therefore be loaded onto the next column without further treatment. This process can be easily automated by employing three pumps with solenoids that would allow the automatic equilibration of each column before loading the eluted pool from the previous one (Fig. 6). This prototype would allow the purification of D-DIBOA from the cell-free biotransformation solution to the final C-18 pool without manual intervention. The evaporation of this pool would separate the solid compound from the methanol, which can be recovered and used again for the C18 elution, thus avoiding waste of the only toxic solvent used in this design. This system could also be used to purify other benzoxazinoids produced by biocatalysis, such as the chlorinate derivatives of D-DIBOA [22].

Comparison of the biological activities of the D-DIBOA obtained by whole-cell biocatalysis with D-DIBOA produced by chemical synthesis

In order to verify if the final biotransfomation product (wcb-D-DIBOA) was functionally comparable to that of chemically synthesized D-DIBOA (cs-D-DIBOA), the biological activities of both were determined. The compounds were tested in the etiolated wheat coleoptile bioassay, a rapid and sensitive test to evaluate the phytotoxic activity of a product [23]. The results revealed that both D-DIBOA products have very similar inhibitory activity in the coleoptile bioassay with values close to 50 % inhibition of coleoptile growth (46 % for wcb-D-DIBOA and 49 % for cs-D-DIBOA 1000 μM). This inhibitory activity decreased with dilution (Fig. 7). D-DIBOA is characterized by having a high phytotoxic activity [24–26], hence the importance of developing new, simpler, more ecological and affordable procedures such as those described in this work. It showed a marked inhibition of root and stem growth of the four model seeds tested, the so-called STS (Standard Target Species) [17]. *Allium cepa* was the most sensitive species with a root and shoot growth inhibition of around 80 % or even higher with the first concentration ratio (81 % for wcb-D-DIBOA and 78 % for cs-D-DIBOA in shoot growth inhibition and 90 % for wcb-D-DIBOA and 85 % for cs-D-DIBOA in root growth inhibition) and, in the case of the root with the second concentration ratio tested (75 % for either wcb-D-DIBOA or cs-D-DIBOA). The next most affected seed was *Lepidium sativum*, followed by *Lactuca sativa*, and finally *Solanum lycopersicum* (Fig. 8a).

This inhibitory activity by D-DIBOA on the four weed seeds tested is highly significant from an agriculture production point of view, since its phytotoxic activity was greater than that exhibited by the commercial herbicide Logran. *Amaranthus viridis* seeds were the most sensitive to this

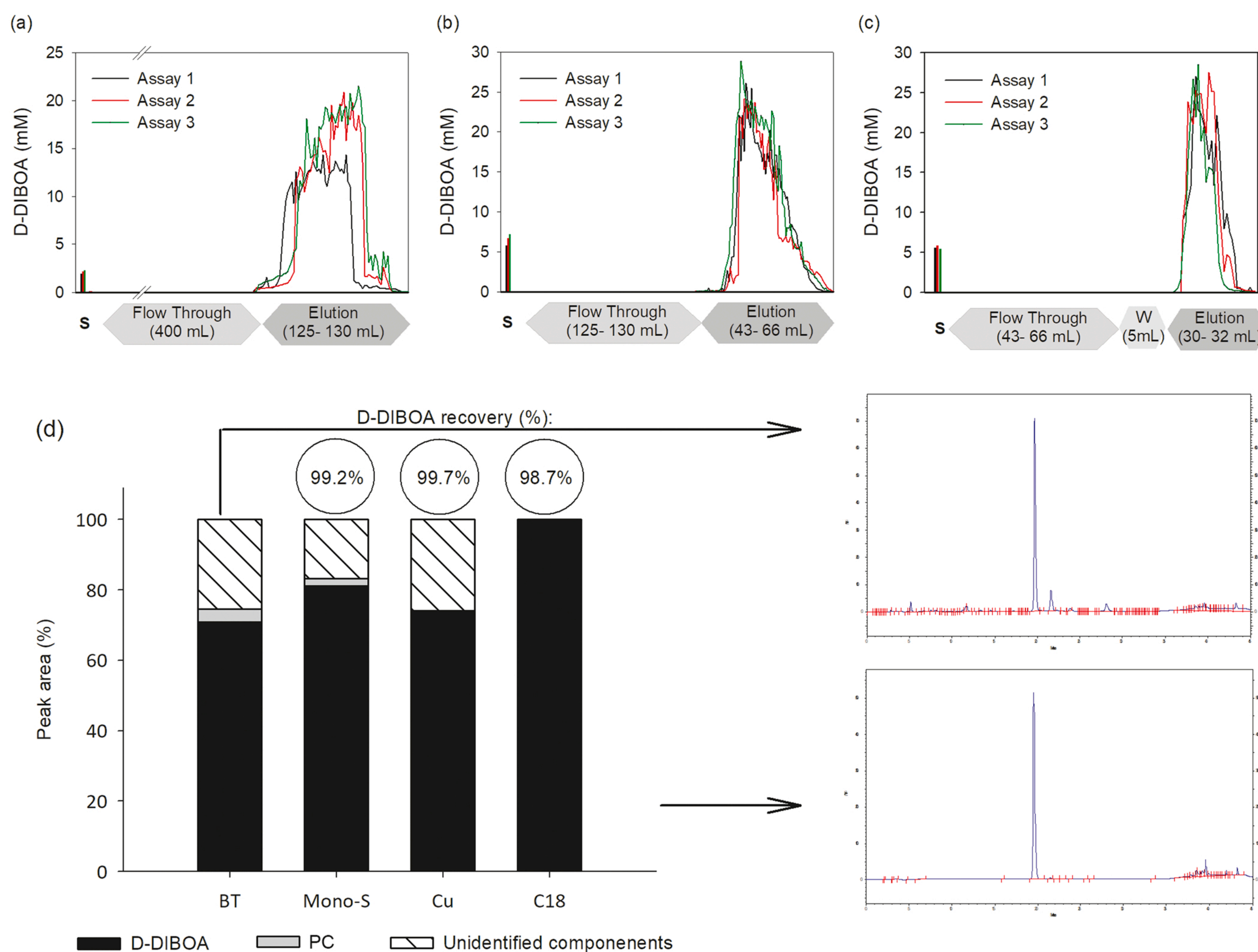

Fig. 5. Scheme for the purification of D-DIBOA from a biocatalysis-based synthesis.
The stationary phases and the column supports were scaled for D-DIBOA purification from 400 mL of a biocatalysis in three different assays. (a) IEX chromatography: 400 mL of biocatalysis were loaded on a 50 mL Mono S column and eluted with a 100 mM phosphate solution, pH 8. (b) The eluted fractions were pooled (125–130 mL) and loaded in a 50 mL IMAC-Cu^{2+} column and eluted with a 100 mM acetate solution, pH 3.5. (c) A pool of the former elution (150 mL) was loaded in a 20 mL (10 g) C-18 column, washed with water and eluted with methanol 100 %. D-DIBOA real-time concentration in each fraction was monitored using a colorimetric method. (d) To estimate the purity of the process, the pools from each elution were analyzed by HPLC and the relative areas of D-DIBOA, PC, and unidentified compounds peaks were calculated as the percentage of the total area detected. The chromatogram from the C18 pools showed a highly-purified D-DIBOA (lower chromatogram) compared with the biotransformation initial sample (upper chromatogram).

high phytotoxic activity, with inhibition values of 95 % for the root and shoot at 300 μM and 1000 μM concentrations of either wcb-D-DIBOA or cs-D-DIBOA, 82 % for wcb-D-DIBOA and 90 % for cs-D-DIBOA in shoot inhibition, and 85 % for wcb-D-DIBOA and 90 % for cs-D-DIBOA in root inhibition at 100 mM. Similarly, it also showed 76 % of germination inhibition up to the third concentration. The next most sensitive seed to the phytotoxic effects of this compound was *Megathyrsus maximus*, followed by *Echinochloa crus galli* and lastly *Lolium rigidum* (Fig. 8). In view of these results, it can be concluded that the compounds obtained by both procedures have the same physical and spectroscopic properties, as well as the same phytotoxic activities.

Conclusions

In order to achieve the objective of developing an economically feasible whole-cell biocatalysis process for D-DIBOA, in addition to the rational design of whole-cell biocatalysts, the entire production process must be optimized for economic viability. Energy requirements, reagents and labor costs are critical factors that have a financial impact on the entire process [27]. Whole-cell biocatalytic processes must, by definition, involve a bacterial growth process and a substrate conversion process under low temperature and pressure levels that do not require costly abiotic catalysts. Nevertheless, the downstream purification of the compound of interest must be taken into account as a key factor for the scale up of the procedure to an industrial level. In this work, a three-step chromatographic downstream process was designed that (1) achieves highly purified D-DIBOA yields, (2) uses inexpensive solvents, (3) minimizes organic solvents, thus avoiding the generation of toxic wastes, and (4) can be fully automated, once the cells have been removed from the culture media. The biological tests of the purified compound have confirmed that its herbicide activity is virtually the same as that of chemically synthesized D-DIBOA.

CRediT authorship contribution statement

MEDLC, TL and RMV carried out the experimental procedure. JB, Conceptualization. MEDLC, GC, TL, RMV, JMGM, DC, AV and JB carried out the analysis and interpretation of the data; DC, Funding acquisition; JB, MEDLC wrote the initial draft and MEDLC, GC, RMV and JB wrote the final version of the manuscript. All the authors have read and

Fig. 6. Scheme of a prototype for an automatic purification of D-DIBOA from a cell-free biotransformed medium. After the biotransformation process in a bioreactor (1), cells are removed by centrifugation (2) and/or filtration (3). The cell-free medium is then loaded in the Mono S column equilibrated with M9 medium (4). Flow-through is discarded and the phosphate elution is pooled (5) and loaded (6) in the IMAC-Cu^{2+} column equilibrated with the same phosphate buffer (6). The IMAC-Cu^{2+} flow-through is discarded (7) and the acetate, pH 3.5 elution pool is loaded in the C18 column equilibrated with the same buffer (8). The C18 flow-through and the washing is discarded (9) and D-DIBOA in the methanol elution is finally purified by evaporation (10) and methanol recovered by condensation (11).

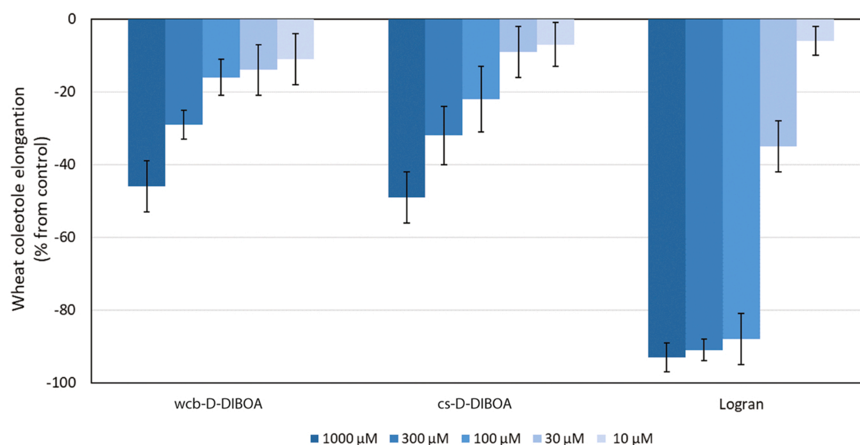

Fig. 7. Effect of the herbicide Logran and the compounds obtained on wheat coleoptile elongation. Values are expressed as percentage difference with respect to the control. Each bar is the mean ± standard deviation.

approved the final version of this manuscript.

Funding

This work was supported by the Council for Innovation, Science and Enterprise of the Regional Government of Andalusia through the Project for Excellence D.P12-TEP725, and the Andalusian Research Groups (PAIDI) BIO-367 and TEP-105.

Declaration of Competing Interest

The authors declare that they have no known competing financial

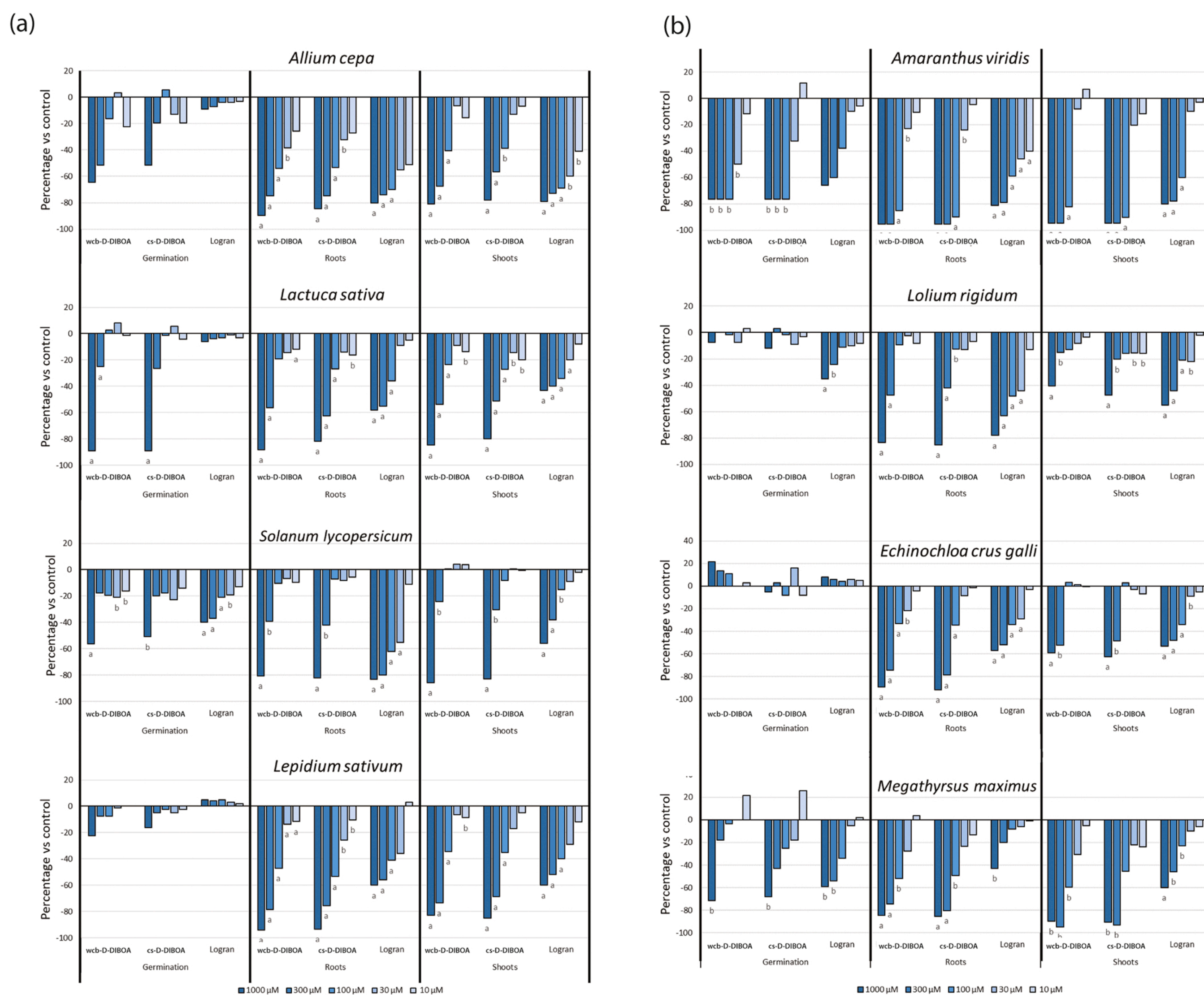

Fig. 8. Effect of wcb-D-DIBOA, cs-D-DIBOA, and the herbicide Logran on germination and plant growth. (a) Effect on Standard Target Species seeds. (b) Effect on four weed species. Values are expressed as percentage difference with respect to the control. Significance levels $p < 0.01$ are labeled with “a” or “b” for $0.01 < p < 0.05$.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.09.001.

References

[1] Gao Y, Zhou Z, Chen X, Tian Y, Li Y, Wang H, et al. Controlled release of herbicides by 2,4-D-, MCPA-, and bromoxynil-intercalated hydrotalcite nanosheets. Green Chem 2021;23:4560–6. https://doi.org/10.1039/D1GC01349A.

[2] Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions, Document 52020DC0381 (⟨https://eur-lex.europa.eu/legal-content/en/txt/?uri=celex:52020dc0381⟩).

[3] Scavo A, Mauromicale G. Crop allelopathy for sustainable weed management in agroecosystems: knowing the present with a view to the future. Agron 2021;11:2104. https://doi.org/10.3390/AGRONOMY11112104.

[4] Rice EL. Allelopathy. 2nd ed. Orlando: Elsevier Science; 1984.

[5] Macías FA, Mejías FJR, Molinillo JMG. Recent advances in allelopathy for weed control: from knowledge to applications. Pest Manag Sci 2019;75:2413–36. https://doi.org/10.1002/PS.5355.

[6] Chou C-H. Roles of allelopathy in plant biodiversity and sustainable agriculture. CRC Crit Rev Plant Sci 1999;18:609–36. https://doi.org/10.1080/07352689991309414.

[7] Duke SO, Dayan FE, Romagni JG, Rimando AM. Natural products as sources of herbicides: current status and future trends. Weed Res 2000;40:99–111. https://doi.org/10.1046/j.1365-3180.2000.00161.x.

[8] Macías FA, Molinillo JMG, Galindo JCG, Varela RM, Simonet AM, Castellano D. The use of allelopathic studies in the search for natural herbicides. J Crops Prod 2001;4:237–55. https://doi.org/10.1300/J144v04n02_08.

[9] Macías FA, Marín D, Oliveros-Bastidas A, Chinchilla D, Simonet AM, Molinillo JMG. Isolation and synthesis of allelochemicals from gramineae: benzoxazinones and related compounds. J Agric Food Chem 2006;54:991–1000. https://doi.org/10.1021/JF050896X.

[10] Macías FA, De Siqueira JM, Chinchilla N, Marín D, Varela RM, Molinillo JMG. New herbicide models from benzoxazinones: aromatic ring functionalization effects. J Agric Food Chem 2006;54:9843–51. https://doi.org/10.1021/jf062709g.

[11] Macías FA, Marín D, Oliveros-Bastidas A, Molinillo JMG. Optimization of benzoxazinones as natural herbicide models by lipophilicity enhancement. J Agric Food Chem 2006;54:9357–65. https://doi.org/10.1021/jf062168v.

[12] Frey M, Schullehner K, Dick R, Fiesselmann A, Gierl A. Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. Phytochemistry 2009;70:1645–51. https://doi.org/10.1016/J.PHYTOCHEM.2009.05.012.

[13] Valle A, Le Borgne S, Bolívar J, Cabrera G, Cantero D. Study of the role played by NfsA, NfsB nitroreductase and NemA flavin reductase from *Escherichia coli* in the conversion of ethyl 2-(2`-nitrophenoxy)acetate to 4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one (D-DIBOA), a benzohydroxamic acid with interesting biological properties. Appl Microbiol Biotechnol 2012;94:163–71. https://doi.org/10.1007/s00253-011-3787-0.

[14] de la Calle ME, Cabrera G, Cantero D, Valle A, Bolivar J. A genetically engineered *Escherichia coli* strain overexpressing the nitroreductase NfsB is capable of producing the herbicide D-DIBOA with 100 % molar yield. Microb Cell Fact 2019;18:86. https://doi.org/10.1186/s12934-019-1135-8.
[15] Cabrera G, Linares T, de la Calle ME, Cantero D, Valle A, Bolivar J. optimization of the biocatalysis for D-DIBOA synthesis using a quick and sensitive new spectrophotometric quantification method. Int J Mol Sci 2020;21:8523. https://doi.org/10.3390/ijms21228523.
[16] Valle A, Cabrera G, Molinillo JMG, Gómez JM, Macías FA, Cantero D. Biotransformation of ethyl 2-(2`-nitrophenoxy)acetate to benzohydroxamic acid (D-DIBOA) by *Escherichia coli*. Process Biochem 2011;46:358–64. https://doi.org/10.1016/j.procbio.2010.09.011.
[17] Macías FA, Castellano D, Molinillo JM. Search for a standard phytotoxic bioassay for allelochemicals. Selection of standard target species. J Agric Food Chem 2000;48:2512–21. https://doi.org/10.1021/jf9903051.
[18] Rial C, Varela RM, Molinillo JMG, Bautista E, Hernández AO, Macías FA. Phytotoxicity evaluation of sesquiterpene lactones and diterpenes from species of the *Decachaeta*, *Salvia* and *Podachaenium* genera. Phytochem Lett 2016;18:68–76. https://doi.org/10.1016/J.PHYTOL.2016.09.002.
[19] Castellano D, Macías FA, Molinilo JM, Cambronero R. FITOMED (automated system for measurement of variable lengths). Spain Pat P9901565; 2001. ⟨https://consultas2.oepm.es/InvenesWeb/detalle?referencia=P9901565⟩.
[20] Tipton CL, Buell EL. Ferric iron complexes of hydroxamic acids from maize. Phytochemistry 1970;9:1215–7. https://doi.org/10.1016/S0031-9422(00)85311-X.
[21] Atkinson J, Morand P, Arnason JT, Niemeyer HM, Bravo HR. Analogues of the cyclic hidroxamic acid 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one: decomposition to benzoxazolinones and reaction with β-mercaptoethanol. J Org Chem 1991;56(5):1788–800. https://doi.org/10.1021/jo00005a025.
[22] de la Calle ME, Cabrera G, Cantero D, Valle A, Bolivar J. Overexpression of the nitroreductase NfsB in an *E. coli* strain as a whole-cell biocatalyst for the production of chlorinated analogues of the natural herbicide DIBOA. New Biotechnol 2019;50:9–19. https://doi.org/10.1016/J.NBT.2019.01.002.
[23] Cutler SJ, Hoagland RE, Cutler HG. Evaluation of selected pharmaceuticals as potential herbicides: bridging the gap between agrochemicals and pharmaceuticals. In: Narwal SS, Hoagland RE, Dilday RH, Reigosa MJ (eds.), Allelopathy in ecological agriculture and forestry. Dordrecht: Springer. ⟨https://doi.org/10.1007/978-94-011-4173-4_9⟩.
[24] Macías FA, Chinchilla N, Varela RM, Oliveros-Bastidas A, Marín D, Molinillo JMG. Structure-activity relationship studies of benzoxazinones and related compounds. Phytotoxicity on *Echinochloa crus-galli* (L.) P. Beauv. J Agric Food Chem 2005;53:4373–80. https://doi.org/10.1021/jf0502911.
[25] Macías FA, Marín D, Oliveros-Bastidas A, Castellano D, Simonet AM, 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 2006;54:1040–8. https://doi.org/10.1021/jf050903h.
[26] Macías FA, Marín D, Oliveros-Bastidas A, Castellano D, Simonet AM, Molinillo JMG. Structure-activity relationships (SAR) studies of benzoxazinones, their degradation products and analogues. Phytotoxicity on standard target species (STS). J Agric Food Chem 2005;53:538–48. https://doi.org/10.1021/jf0484071.
[27] Crater JS, Lievense JC. Scale-up of industrial microbial processes. FEMS Microbiol Lett 2018;365(13):fny138. https://doi.org/10.1093/femsle/fny138.