Supplementary materials and methods

2.2 Evaluation of the heavy metal tolerance, plant growth promotion-associated traits, and antioxidant activity of *Acinetobacter* sp. ME1

2.2.1 Heavy metal tolerance

To assess the tolerance of strain ME1 to various types and concentrations of heavy metals (Long et al., 2013), stock solutions of $CdCl_2 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Pb(NO_3)_2$, $Cr_2K_2O_7$, and $NiCl_2$ were prepared to a final concentration of 10,000 mg/L using reagents obtained from Duksan (South Korea). The heavy metal stock solutions were sterilized by filtration through a 0.45-µm filter membrane and then silanized. After sterilization at 120 °C for 15 min, 1/10 LB was cooled to around 60 °C and Cd, Zn, Pb, or Cr stock solutions were added to achieve final concentrations ranging from 0 to 1000 mg/L, while Cu or Ni stock solutions were added to achieve final concentrations ranging from 0 to 100 mg/L. Then, the test solutions were thoroughly mixed. The bacterial suspensions of strain ME1 were inoculated into the LB test solutions (1% v/v), and they were incubated at 35 °C for 24 h with shaking at 140 r/min. The growth of bacteria was quantitatively evaluated by measuring the absorbance of the culture broth at a wavelength of 600 nm.

2.2.2 Nitrogen-fixing ability

To assess the nitrogen fixation ability of the ME1 strain, we applied a modified version of a nitrogen fixation assay method used in previous studies (Kim et al., 2012; de Oliveira et al., 2018). Nitrogen-free bromothymol blue (NFB) agar medium was prepared, adjusting the pH to 7.0 using KOH and NaOH. The NFB agar medium was made by combining 5.0 g malic acid, 0.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.01 g $MnSO_4$, 0.05 g $MgSO_4$, 0.02 g NaCl, 0.002 g Na_2MoO_4, 2 mL bromothymol blue (0.5% (w/v) in alcohol), and 1.75 g agar in 1 L of distilled water. The NFB agar medium was dispensed into 15 mL test tubes (5 mL per tube) and sterilized under high pressure. Each strain's bacterial suspension was inoculated into the medium (1% v/v), and the tubes were then incubated at 20°C for 48 h. After incubation, the absorbance ($OD_{630\ nm}$) of the culture broth was measured using a spectrophotometer at a wavelength of 630 nm.

2.2.3 Indole-3-acetic acid (IAA) production ability

To evaluate the IAA production ability of strain ME1, the bacterial suspension was inoculated into LB agar medium supplemented with 0.1% (w/v) L-tryptophan (1% v/v). The inoculated plates were then incubated at 35°C for 24 h. After incubation, the culture broth was mixed with Salkowski reagent (98 mL of 35% (v/v) $HClO_4$ and 2 mL of 0.2 mol/L $FeCl_3 \cdot 6H_2O$) at a ratio of 800 µL to 1 mL and allowed to react in the dark for 30 min. The mixture was then centrifuged at 13 200 × *g* for 1 min, and the absorbance of the supernatant was measured at a wavelength of 535 nm (Lee et al., 2021).

2.2.4 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase production ability

The activity of ACC deaminase is a key indicator of environmental stress resistance mechanisms (Dell'Amico et al., 2005; Grobelak et al., 2018). Bacterial suspensions of the selected strain were inoculated into Dworkin and Foster (DF) agar medium containing ACC instead of $(NH_4)_2SO_4$ as the nitrogen source (1% v/v) and incubated at 35 °C with shaking at 200 r/min for 48 h. The DF medium was a 5.8 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.25 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 0.15% (w/v) glucose and 0.3 mg/L biotin solution. The ACC (98%, Sigma-Aldrich, Israel) was prepared as a 0.5 mol/L stock solution and sterilized by filtration using a sterile filter (Minisart® Syringe filter, 0.2 µm). It was then added to the DF agar medium to a final concentration of 3 mmol/L. For the control group, bacterial suspensions of the selected strain were inoculated into DF agar medium without the addition of a nitrogen source and incubated at 35 °C with shaking at 200 r/min for 10 d. The absorbance of both experimental and control groups was measured at 600 nm, and an increase in turbidity indicated the presence of ACC deaminase activity. The ACC deaminase activity of the selected strain was evaluated by subtracting the absorbance value of the control group from that of the experimental group.

2.2.5 Phosphate solubilization ability

To assess the phosphate solubilization ability of strain ME1, Pikovaskaya's agar medium containing 5 g/L tricalcium phosphate (calcium phosphate tribasic, $Ca_3(PO_4)_2$) was utilized (Kim et al., 2012). The Pikovaskaya's agar medium contained 10.0 g/L glucose, 0.5 g/L $(NH_4)_2SO_4$, 0.2 g/L KCl, 0.1 g/L $MgSO_4$, 0.002 g/L $MnSO_4$, 0.002 g/L $FeSO_4$, yeast extract 0.5 g/L yeast extract, and 20 g/L agar. After sterilization, the medium was dispensed into petri dishes (10 mL per dish) and allowed to solidify. Paper discs with a 6-mm diameter were placed in the center of each plate, and 6 µL of bacterial suspension for each strain was inoculated onto the paper discs. The plates were then incubated at 35°C for 14 d. After incubation, the formation of clear zones around the paper discs was observed, and the diameter (mm) of the zones was measured to assess the extent of phosphate solubilization.

2.2.6 Siderophore production ability

The siderophore production of the ME1 strain was assessed using chrome azurol S (CAS) blue agar medium (Kannahi and Senbagam, 2014; Louden et al., 2011; Srimathi anf Suji, 2018). To prepare the CAS blue dye solution, 60 mg of CAS (Sigma Aldrich Co.) was dissolved in 50 mL of distilled water, 2.7 mg of $FeCl_3 \cdot 6H_2O$ (Sigma Aldrich Co.) was dissolved in 10 mL of 10 mmol/L HCl solution, and 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA, Sigma Aldrich Co.) was dissolved in 40 mL of distilled water. These three solutions were mixed together in a light-free environment to prepare the blue dye solution, which was then sterilized under high pressure (121 °C, 15 min).

To prepare the CAS agar, a mixture of 32.24 g of 1,4-piperazinediethanesulfonic acid (PIPES, Sigma Aldrich Co.) and 15 g of agar in 750 mL of distilled water (pH 6.8) was sterilized under high pressure and allowed to cool to an appropriate temperature. Then, 30 mL of 10% (w/v) casamino acid (Difco^{TM})

and 10 mL of 20% (w/v) glucose were added, followed by roughly 110 mL of distilled to top the volume off at 900 mL. Finally, 100 mL of the previously prepared blue dye solution was added to the CAS agar to make a total volume of 1 L. The CAS agar medium containing the blue dye was then dispensed into Petri dishes to create CAS-agar plates.

A 6-mm paper disc was placed in the center of each plate, and 6 μL of each bacterial suspension was inoculated onto the paper discs. The plates were then incubated at 35°C for 14 d. After incubation, the formation of an orange circular halo zone around the paper discs was observed, and the diameter (mm) of the zone was measured to assess siderophore production ability.

2.2.7 Protease activity

We analyzed the activity of protease, an enzyme present in cells, in strain ME1 (Ahn et al., 2006; Rejsek et al., 2008; Greenfield et al., 2021). The bacterial suspension (1 mL) was mixed with 1 mL of 0.2 mol/L Tris buffer (pH 8.0) in a 15 mL test tube (1:1, v/v). Then, 1 mL of 2% (w/v) sodium caseinate was added and thoroughly mixed in. The test tubes were sealed with a lid and parafilm to prevent evaporation and incubated in a water bath at 50 °C for 2 h to allow the reaction to proceed.

After incubation, 1 mL of 10% (w/v) trichloroacetic acid was added to the test tubes to precipitate the proteins, and the mixture was incubated at 35 °C for 10 min. The sample was then centrifuged at 13,000 r/min for 1 min, and 0.5 mL of the supernatant was transferred to a new 15 mL test tube. To this, 0.75 mL of 1.4 mol/L Na_2CO_3 and 0.25 mL of 33% (v/v) Folin–Ciocalteu's phenol reagent (Sigma Aldrich Co.) were added. After incubating the mixture at room temperature for 10 min, the absorbance was measured at 650 nm.

Protease activity was calculated using a standard curve prepared by quantifying tyrosine (Sigma-Aldrich Co.). The protease activity of the selected strains was compared by calculating the rate of tyrosine production per unit dry cell weight per h (μmol tyrosine/g-DCW/h). The dry cell weight was determined by taking 100 mL of the culture broth, centrifuging it (5000*g*, 5 min), drying the resulting cell pellet at 105 °C for 4 h, and measuring the weight until a constant weight was obtained at room temperature.

2.2.8 Evaluation of antioxidant activities

2.2.8.1 Catalase activity

The activity of the antioxidant enzyme catalase was evaluated for strain ME1 (Iwase et al., 2013). Standard solutions of catalase were prepared by diluting catalase powder (2950 units/mg, product No. C1345, Sigma-Aldrich) in third distilled water to obtain standard solutions ranging from 0 to 60 units. To assess catalase activity, 100 μL of each catalase standard solution and 100 μL of the ME1 strain bacterial suspension were added to separate glass test tubes. Then, 100 μL of 1% (v/v) Triton X-100 solution and 100 μL of 30% (v/v) hydrogen peroxide were added to each, and the reaction was allowed to proceed at room temperature for 15 min. The height of the bubbles formed during the reaction of the standard solutions was measured, and the relationship between catalase activity and bubble height was utilized to create the standard curve. For the bacterial suspension, the height of the bubbles within the test tubes was compared to the standard curve to assess the catalase activity.

2.2.8.2 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity

To evaluate the antioxidant activity of ME1 against the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), a DPPH assay procedure was performed (Hwang et al., 2013; Shehata et al., 2019). One mL of bacterial suspension was mixed with an equal volume of a 0.2 mmol/L DPPH solution. The mixture was then incubated at 30°C in the dark for 30 min. After incubation, the absorbance of the reaction mixture was measured at 517 nm. As a control, 99.9% (v/v) ethanol (EtOH) was used instead of the bacterial suspension. The DPPH radical scavenging activity was quantitatively evaluated using the formula

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{(A-B)}{A}\right) \times 100 \qquad (1)$$

where A represents the absorbance value at 517 nm for the control (ethanol) and B represents the absorbance value at 517 nm for the experimental group after adding the DPPH solution.

Table S1 Factors that were studied using the Taguchi approach and the corresponding levels

Factor	**Level**		
	1	**2**	**3**
Temperature (°C)	25	30	35
pH	6	7	8
Inoculation (%)	1	10	

Table S2 EE and *I* constants used for the calculation of in vitro SPF

Wavelength (λ nm)	**EE × *I* (normalized)**
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
Total	**1**

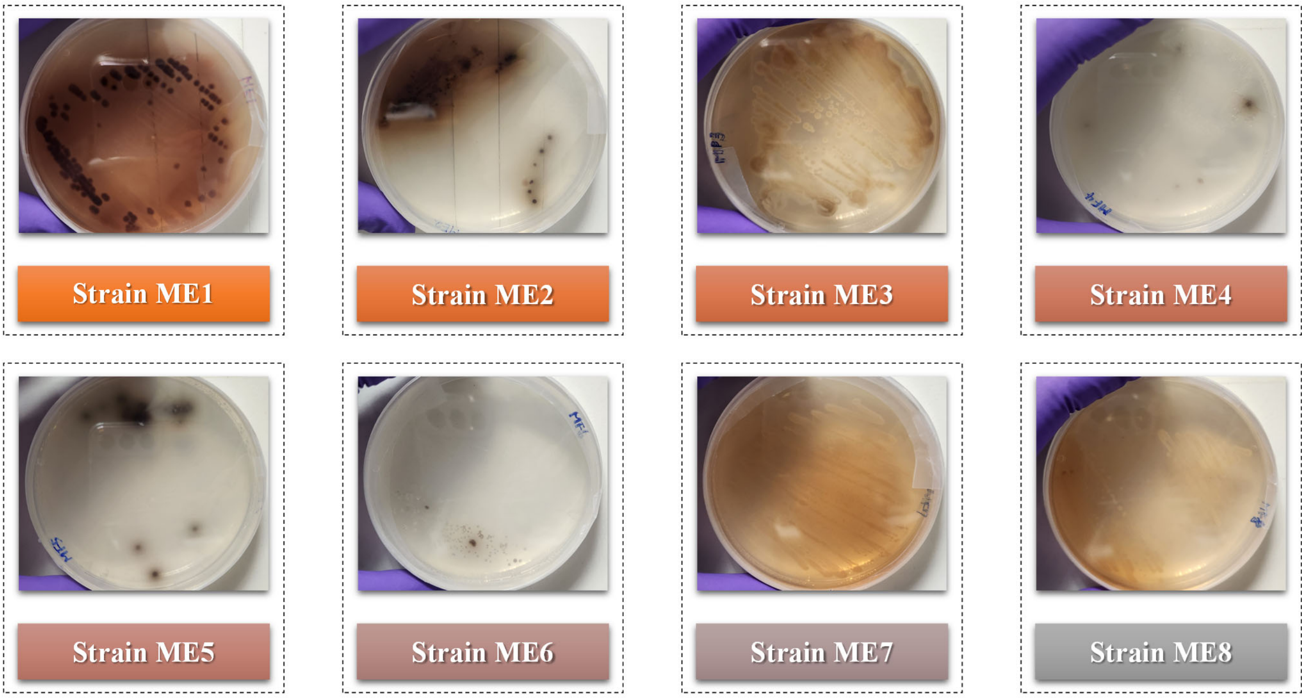

Fig. S1 Representative images of melanin producing bacteria (strains ME1–ME8) on a solid medium supplemented with tyrosine.

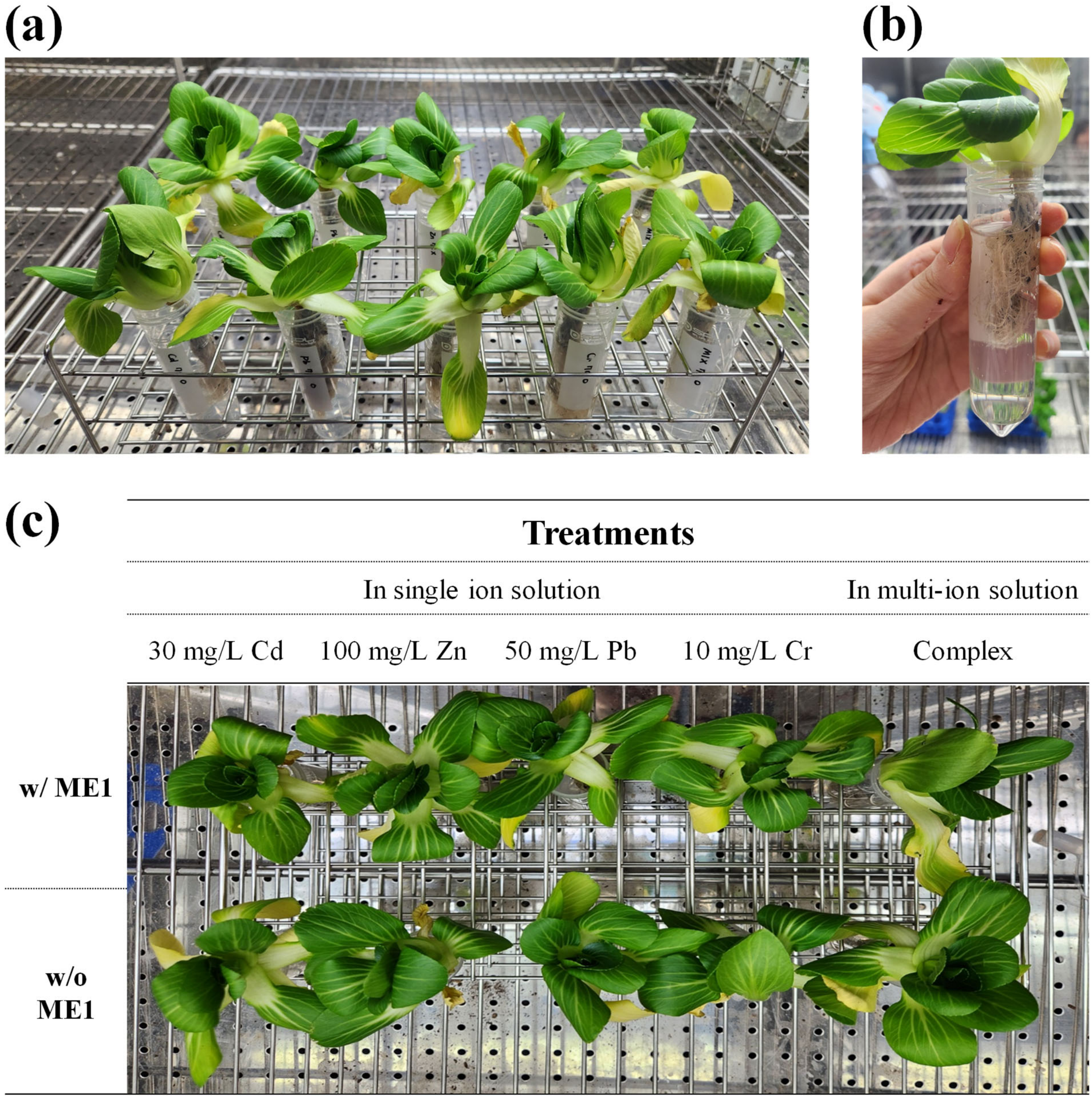

Fig. S2 Experimental setup for evaluating heavy metal removal efficiency in hydroponic cultivation with *Brassica chinensis*. (a) Multiple hydroponic setups of *B. chinensis* in 50-mL test tubes. (b) Close-up view of an individual *B. chinensis* in hydroponic cultivation, and (c) Comparison of *B. chinensis* growth with ME1 (w/ ME1) and without ME1 (w/o ME1) treatment under various heavy metal stress conditions (single heavy metal (Cd 30, Zn 100, Pb 50, or Cr 10 mg/L) and multiple heavy metals (Cd+Zn+Pb+Cr)).

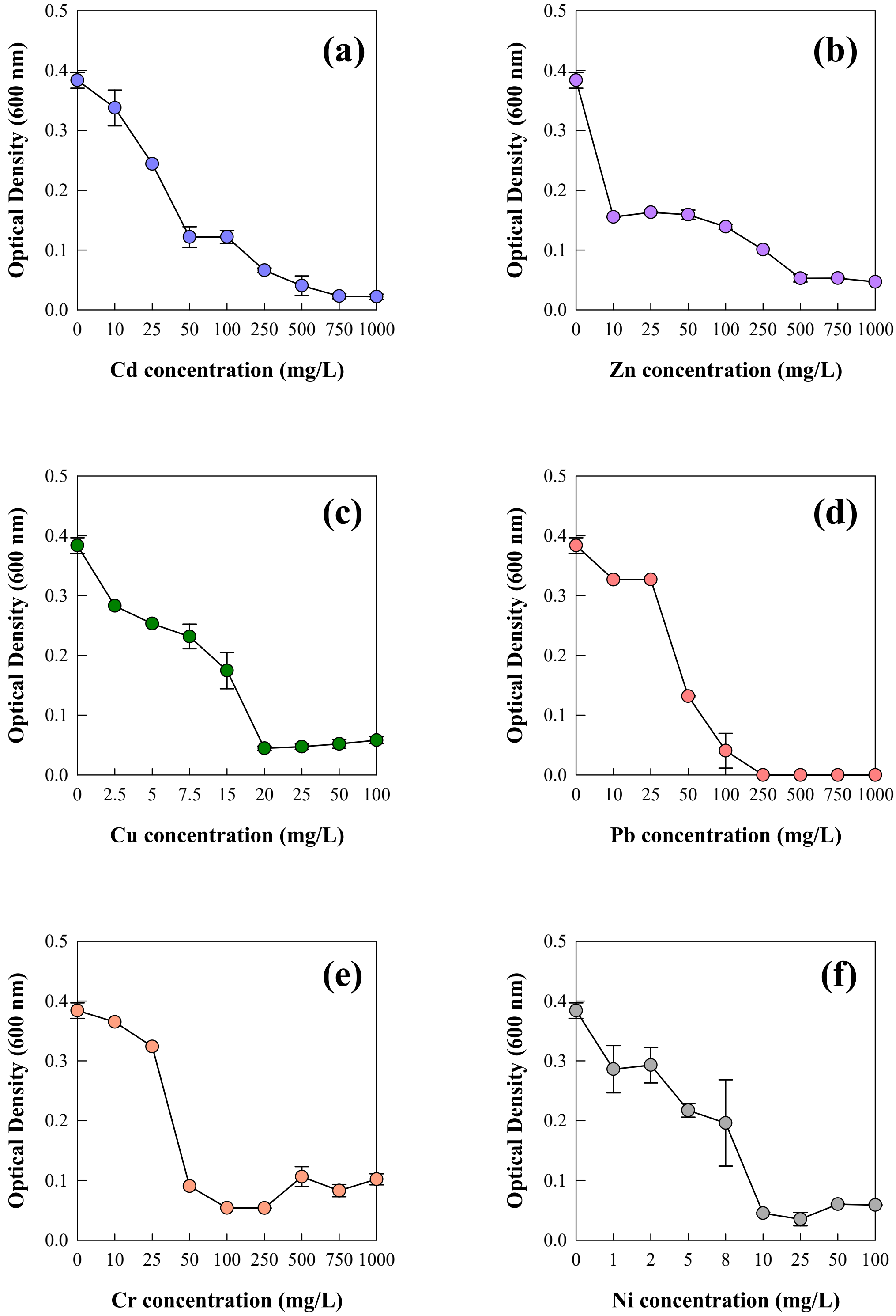

Fig. S3 Growth of heavy metal-resistant bacterial isolate ME1 in 1/10 Luria–Bertani medium supplemented with different heavy metals at varying concentrations: (a) Cd, (b) Zn, (c) Cu, (d) Pb, (e) Cr and (f) Ni. Points represent mean optical densities and error bars represent the standard deviation (n–1).

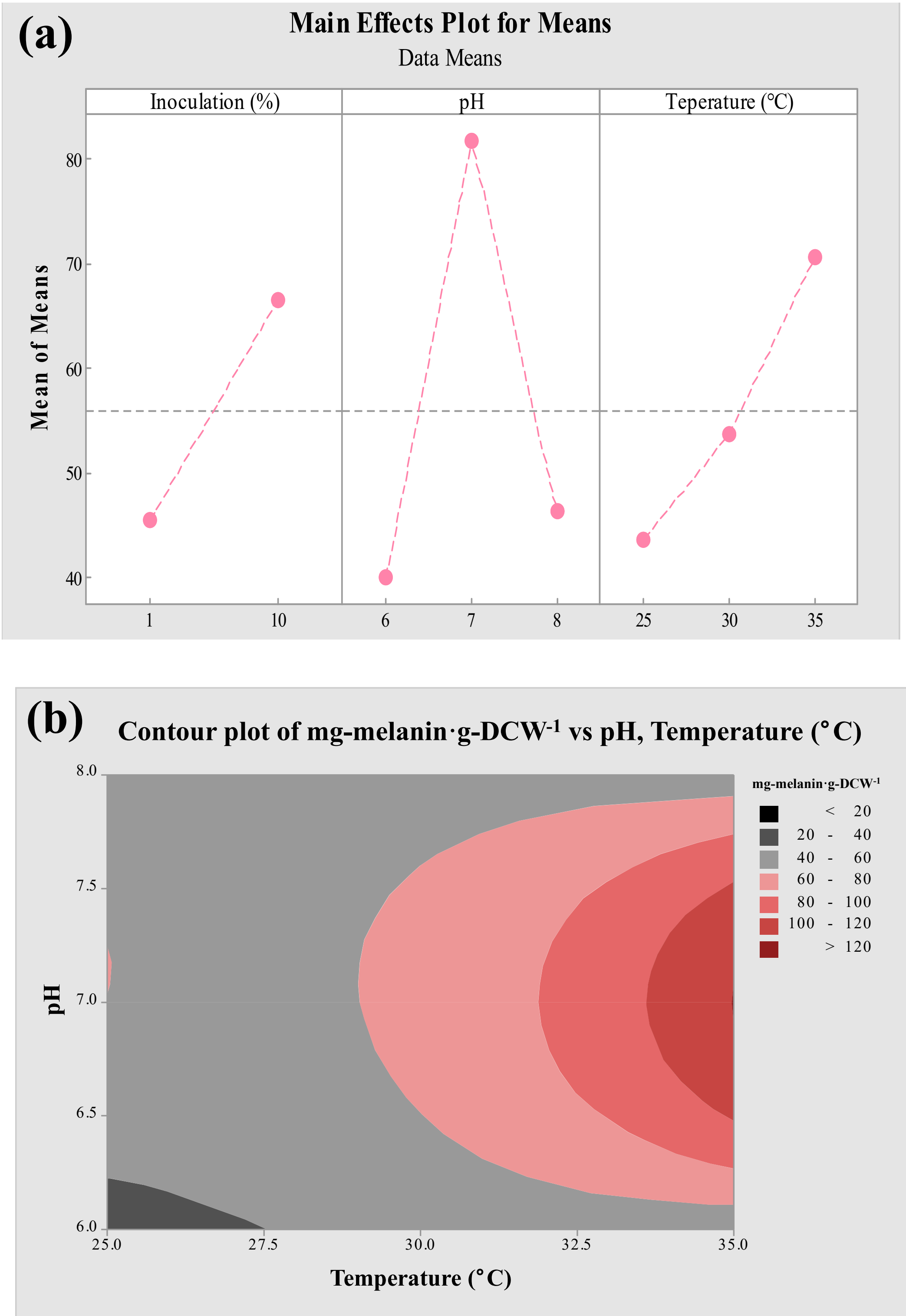

Fig. S4 (a) Main effects of three factors showing the average for each value (mg-melanin/g-DCW) averaged across the other factors. For descriptions of the effect "levels" refer to Table 1. (b) A three-dimensional response contour plot showing the effects of interactions between pH and temperature on melanin yield.

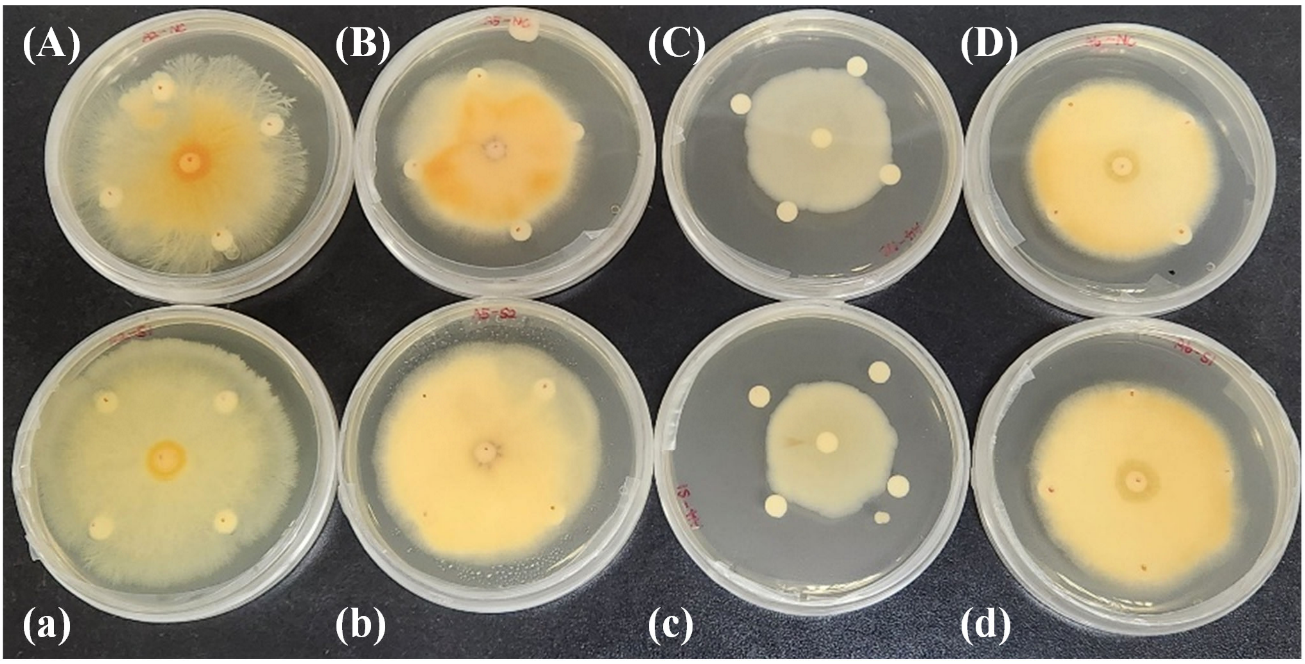

Fig. S5 Images showing the antimicrobial acitivity assays using purified melanin with several plant pathogens: (A & a) *Rhizoctonia solani* AG-4, (B & b) *Fusarium fujikuroi*, (C & c) *Xanthomonas campestris*, and (D & d) *Botrytis cinerea*. Upper and lowercase panel leters indicate whether the melanin is a commercialy available product (A–D; positive control; top) or the melanin biosynthesised by strain ME1 and extracted for this study (a–d; bottom).

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