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Indole-acyl esters improve the effect of nitrogen and phosphorous fertilization by mitigating the phytotoxicity and concentrations of cadmium and lead in *Jatropha curcas* L. in contaminated soils

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Abstract: The effects of indole-acyl esters (ID), NH_4NO_3 (N), and KH_2PO_4 (P), on the mitigation of the toxic effects of Cd and Pb and their concentration in *Jatropha curcas* L. from contaminated soils was investigated. The concentrations of ID, N, and P were optimized (0.1 mL·L^{-1}, 7 mM, and 2.5 mM, respectively) and they were applied in various combinations to the contaminated soils of potted plants of *J. curcas*. The results showed that ID together with the N and P fertilizers, increased plant biomass and improved the mitigating effects of the N-P treatments on Cd and Pb toxicity. Plants growing under ID-N-IP treatments had high whole plant biomasses, high concentrations of P, N, Pb and Cd in whole plants, as well as enhanced activities of superoxide dismutase (SOD) and peroxidase (POD). These results point to the phytoremediation ability of *J. curcas*. We propose a new methodology that can be utilized to study the effects and interactions of multiple factors on plant growth.

Keywords: *Jatropha curcas* L.; Pb/Cd stress; indole-acyl esters; nitrogen; phosphorus

INTRODUCTION

Cadmium (Cd) and lead (Pb) can be detrimental to soil, plants and human health. These metals are released worldwide into the environment via industrial processes and agricultural practices, which makes them a global problem [1,2]. Their uptake by plants can lead to successive accumulation in human tissues and biomagnification throughout the food chain [1]. Several applicable and cost-effective methods have been proposed for the remediation of contaminated soils. Phytoremediation represents a cost-effective, environmentally friendly, and *in situ* applicable technology for cleaning metal-polluted soils. Plants that are utilized for the phytoremediation of heavy metal-polluted soils should have rapid growth, high biomass and high concentrations of heavy metals in their tissues [3,4].

Jatropha curcas L. (Euphorbiaceae), commonly known as the physic nut, is a deciduous perennial shrub distributed in the tropical and subtropical regions of America, Africa and Asia. Its seeds contain an oil (30-35%) that can be converted into a good quality and eco-friendly biofuel [5-7]. *J. curcas* is also a very adaptive plant, as it is tolerant of drought and can be grown in arid and semi-arid regions (rock gaps, degraded lands, etc.); it is a pest-tolerant plant, and unpalatable by animals [8]. In addition, *J. curcas* can be used as a phytoremediator of heavy metals in contaminated soils [8-11]. However, about 70~150 mg·kg^{-1} total Cd and about 500~1000 mg·kg^{-1} total Pb had toxic effect on plant growth, even for those plants used for phytoremediation [12]. For *J. curcas*, high concentrations of Cd and Pb in soils, such as 50 mg Cd kg^{-1} (Cd50) and 500 mg Pb kg^{-1} (Pb500), respectively, can decrease the concentrations of photosynthetic pigments, damage chloroplast structures, reduce biomass, and ultimately slow plant growth [13], which substantially reduces its phytoremediation efficiency.

Cd and Pb can induce oxidative stress by generating free radicals and reactive oxygen species (ROS) that can damage major cell macromolecules (proteins, lipids, and DNA) in plants [14,15]. Malondialdehyde (MDA) is the final product of the peroxidation of polyunsaturated fatty acids in the cells, and is commonly used as an indicator of oxidative damage of cell plasma membranes [16]. The tolerance of plants to heavy metals depends on the presence of strong antioxidant defense systems [17,18] that are mainly composed of metabolites and antioxidant enzymes, i.e., superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), which are capable of scavenging free radicals in order to protect cells from oxidative stress [19-23].

The exogenous applications of nitrogen (N) [24,25], phosphorus (P)[26], low doses of indole acetic acid (IAA) [27,28] or acyl esters (DA-6) [29,30] could mitigate Pb and Cd toxicity to plants: Response surface methodology is a tool for modeling and analyzing problems in which the response of interest is influenced by several variables [31]. The Box-Behnken partial factorial design for three factors and three levels for each factor produces 10 runs to be tested, meaning that a three-factor analysis can feasibly be investigated with real plants grown in pots. The runs from other factorial designs would be too large to investigate using real plants grown in pots. In our study, Box-Behnken three-variable partial factorial designs were used to obtain the optimum ratio of indole-acyl esters IAA+DA-6 (ID), NH_4NO_3 (N) and KH_2PO_4 (P) on whole plant biomass of *J. curcas* under Cd50-Pb500 stress. The growth and some biochemical parameters were evaluated in *J. curcas* in order to explore the effects of their combinations on its phytoremediation ability and mitigating the Cd and Pb toxicity.

MATERIALS AND METHODS

Soil collection and treatment

The soil used for this study was collected from the surface layer (0-15 cm) of a forest at Xichang College in Liangshan Prefecture, Sichuan Province, China. All soil samples were semi-dried and the stones and coarse plant roots or residues were removed. The soil samples were transported to the laboratory and air-dried until they could be crumbled to pass through a 2-mm-mesh sieve for the pot experiments. The physical and chemical properties of the soil were: pH_{water}=6.10; available P=6.29 mg·kg^{-1} soil; hydrolysable N=170.84 mg·kg^{-1}; available K=787.16 mg·kg^{-1}; organic matter=12.34 mg·kg^{-1}; Pb=49.7 mg·kg^{-1}; Cd=0.94 mg·kg^{-1}. Pb and Cd were added to the sieved soil as aqueous mixtures at a rate of 500 mg kg^{-1} soil and 50 mg kg^{-1} soil using Pb $(NO_2)_2$ and $CdCl_2 \cdot 2.5H_2O$, respectively. The prepared soil samples were incubated for a minimum of two weeks at room temperature prior to being transferred to plastic pots.

Pot experiments

Seeds of *J. curcas* were obtained from native habitats at Liangshan Prefecture, Sichuan Province, China. They were surface sterilized with 1% potassium permanganate for 30 min, and then thoroughly washed with distilled water and soaked in water at a room temperature for 2 h. Then the seeds were sown in plastic pots filled with nursery substrate (peat, perlite, and vermiculite according to 5:1:1, v/v). Seed germination, seedling growth and pot experiments were performed in growth chambers under conditions of 16 h daylight at 30°C, 8 h night at 25°C and relative humidity of 50%. Uniform seedlings with 4-5 leaves were selected and transplanted into pots filled with treated soil. Each treatment consisted of 6 replicates and each replicate had 3 seedlings. Pots were arranged randomly, and each pot was irrigated weekly with 200 mL of the designed solution.

Response surface methodology for optimizing the concentrations of ID, N, and P

The effects of three independent variables (ID (x_1, 0.1-1.1 mL·L^{-1}), N (x_2, 2-12 mM) and P (x_3, 0.5-2.5 mM)) on whole plant dry biomass (y) were investigated. Ten runs (9 runs+1 center point) per block were generated using Box-Behnken 3**(3-1) fractional factorial designs [31]. Seedlings of *J. curcas* were transplanted into Pb- and Cd-contaminated soils that were irrigated weekly using ten different solutions in ten different runs, respectively:(i) 0.1 mL·L^{-1} ID+2 mM N+0.5 mM P; (ii) 0.1 mL·L^{-1} ID+7 mM N+2.5 mM P; (iii) 0.1 mL·L^{-1} ID+12 mM N+1.5 mM P; (iv) 0.6 mL·L^{-1}+2 mM+2.5 mM P; (v) 0.6 mL·L^{-1} ID+7 mM N+1.5 mM P; (vi) 0.6 mL·L^{-1} ID+12 mM N+0.5 mM P; (vii) 1.1 mL·L^{-1} ID+2 mM N+1.5 mM P; (viii) 1.1 mL·L^{-1}

ID+7 mM N+0.5 mM P; (ix) 1.1 mL·L^{-1} ID+12 mM-1 N+2.5 mM P; (x) 0.6 mL·L^{-1} ID+7 mM N+1.5 mM P. Plants were harvested after 42 days of growth, and five uniform plants from each treatment were thoroughly washed with distilled water and used to investigate the whole plant dry biomass (y). A multiple regression equation was used to predict whole plant biomass with the proc glm and proc rereg functions of SAS 8.0. The figure for the response surface was plotted using g3grid and g3d SAS 8.0. The calculations for the optimized ratios of ID, N, and P were executed with the ridge max function of SAS 8.0. The optimized ratios of ID, N and P were selected for the following experiments in this investigation.

Single factor experiments with different combinations of ID, N, and P

Single factor experiments of six treatments using different combinations of N, P and ID were conducted. Seedlings of *J. curcas* that were transplanted into Pb- and Cd-contaminated soils were irrigated weekly using five different solutions as five different treatments: (i) CK1 (only H_2O), (ii) N-P (NH_4NO_3 and KH_2PO_4), (iii) N-ID (NH_4NO_3 and ID), (iv) P-ID (KH_2PO_4 and ID), and (v) N-P-ID (NH_4NO_3, KH_2PO_4 and ID). Soil samples that added no extra $Pb(NO_2)_2$ and $CdCl_2{\cdot}2.5H_2O$ were used for the control (CK2) and were irrigated weekly with H_2O only. Plants were harvested after 30 days of growth, the plants were thoroughly washed with distilled water and separated into roots, leaves and stems. The following parameters were investigated: leaf, stem and root fresh weight, and leaf, stem and root dry weight. Six plants in each treatment were randomly selected and divided into three groups to determine the parameters. The dried roots, leaves and stems of the six plants from each treatment were pulverized into powder to pass through a 0.25-mm-mesh sieve for the analysis of N, P, Pb and Cd concentrations. The remaining 12 plants in each treatment were used to analyze the concentration of MDA and antioxidant activity.

Determination of the concentrations of N, P, Pb and Cd in plants

Dried and ground plant samples were digested with a mixture of H_2SO_4 and H_2O_2, and the digests were used to determine the concentrations of N and P in the plants. The total P in the digests was reacted with Mo-Sb reagent solution to obtain a phosphoantimonylmolybdenum complex that was detected at 880 nm according to Li [32]. The total N concentrations in the digests were reacted with the Nessler's reagent in an alkaline solution to obtain a yellowish-brown colloidal compound that was detected at 425 nm according to McDonald [33].

For estimation of Pb and Cd, approximately 0.2 g of dry ground samples was digested with a concentrated HNO_3-$HClO_4$ mixture. The digested material was washed into a 15-mL flask that was made up to a volume of 15 mL using distilled water. The total Pb and Cd concentrations were analyzed using the atomic absorption spectrum (AAS)[34].

Determination of lipid peroxidation and antioxidant activity

MDA as a lipid peroxidation marker, was determined by using the methods of Li [35] with the following formula:

$$\text{MDA concentration} = (6.45(A_{532}-A_{600})-0.56A_{450})xV/m \quad (1)$$

where the MDA concentration was nmol·g^{-1}, A_{532}, A_{600} and A_{450} were absorbance values at 532, 600 and 450 nm, respectively, M was the fresh biomass of leaf, V was the volume of the extraction.

The activity of SOD (EC 1.15.1.1) was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to Beauchamp and Fridovich [36]. One unit of SOD activity (U) was defined as the amount of enzyme that was required for 50% inhibition of the initial reaction rate of the NBT reduction at 560 nm. POD (EC 1.11.1.7) activity was determined with guaiacol by a microplate reader according to Lagrimini [37]. In the presence of H_2O_2, POD catalyzed the oxidation of guaiacol to tetraguaiacol and the absorbance was read at 470 nm. One unit of POD activity was an increase of 0.01 at A_{470} nm in 1 min. CAT (EC 1.11.1.6) activity was assayed according to the procedures of Aebi [38]; one unit of enzyme activity was the reduction of 0.01 at A_{240} nm in 1 min.

Statistical analysis

All values reported were the means of three independent replications. The data were analyzed by using the statistical software package SPSS 19.0, and differences among the treatments were separated using the least significant difference (LSD) test at $P \leq 0.05$.

RESULTS

The effect of ID (x_1), N (x_2) and P (x_3) on plant biomass (y)

The results for the Box-Behnken three variable fractional factorial designs are shown in Table 1. A multiple regression analysis was performed with the experimental data and the results were incorporated into a 2nd order polynomial equation; the regression model predictions of the whole plant biomasses are shown in Table 2, and the corresponding equation is:

$$y=0.372-0.266x_1+0.035x_2+0.206x_3+0.306x_1^2 +0.009x_2^2-0.077x_1x_2-0.234x_1x_3 \quad (2),$$

where y represents the whole plant dry biomass and x_1, x_2 and x_3 were ID, N and P, respectively. Model adequacy was checked using regression R^2 and the F-test. The R^2 and *F*-value of the regression model were 0.9985 and 258.35, respectively, Pr>|f|:0.0039(≤0.01) (Table 2), with the lack of fit 54.82, Pr>|f|:0.0855(>0.05), suggesting that the regression model was a significant fit. In the corresponding equation, the *P*-values of the intercept, two linear coefficients (x_1,x_3) and the quadratic coefficients (x_1^2, x_1x_3) were significant, and items containing x_2, such as the quadratic coefficients x_2x_3 and x_2^2, were not significant (Table 2). The predicted values for the optimal concentration ratios of ID, N, and P were obtained using the above equation. The predictive optimal concentration ratios of ID, N, and P were 0.1 mL·L^{-1},12 mM, and 2.5 mM, respectively.

The effects of ID (x_1), N (x_2) and P (x_3) on plant biomass (y) are shown in Fig. 1. The effect of x_1(ID) and x_2(N) on the dry biomass y of *J. curcas* when setting x_3 =-0.5(p=1 mM) are shown in Fig 1A. When $x_1 \geq 0$(ID≥0.6 mL·L^{-1}), the increase in x_2(N) had little effect on y. However, when $x_1<0$(ID<0.6 mL·L^{-1}), and especially when it was less than -0.5(ID<0.35 mM), x_2(N) was significantly positively related to y and the lower the dosage of x_1 (ID), the stronger the positive effect on x_2 (N). This suggested that the low dosage of x_1 (ID) and the high dosage of x_2(N) promoted the growth of *J. curcas*. Similar results were found for x_1(ID) and x_3(P) (Fig. 1B); the lower dosage of x_1(ID) had a stronger positive effect on x_3(P). Fig. 1C points to the effect of x_2(N) and x_3(P) on the dry biomass of *J. curcas* plants when x_1=0 (ID=0.6 mL·L^{-1}). The increase in x_3(P) significantly linearly increased y, and x_2(N) had a small effect on x_3(P).

Table 1. Box-Behnken three-variable fractional factorial designs (N=5).

Run	Block	Natural variables			Coded variables			Biomass y(g)
		ID(mL·L^{-1})	N(mM)	P(mM)	x_1	x_2	x_3	
1	1	0.1	2	0.5	-1	-1	-1	0.4005
2	1	0.1	7	2.5	-1	0	1	1.4042
3	1	0.1	12	1.5	-1	1	0	1.0512
4	1	0.6	2	2.5	0	-1	1	0.5352
5	1	0.6	7	1.5	0	0	0	0.3764
6	1	0.6	12	0.5	0	1	-1	0.2268
7	1	1.1	2	1.5	1	-1	0	0.4802
8	1	1.1	7	0.5	1	0	-1	0.4222
9	1	1.1	12	2.5	1	1	1	0.3518
10	1	0.6	7	1.5	0	0	0	0.3684

Box-Behnken design 3**(3-1) fractional factorial designs, 1block, 9 runs+1 center point per block.
x_1 – Indole-acyl esters (N) 0.1-1.1 mL·L^{-1}; x_2 – NH_4NO_3 (N) 2-12 mM; x_3 – KH_2PO_4 (P) 0.5-2.5 mM.
Factor levels were coded as 1 (low), 0 (central point) and 1 (high); y – the whole plant biomass (g).

Table 2. Regression model for the prediction of whole plant biomass.

Parameter	Coefficient Estimate	Standard Error	t Value	Pr>|t|
Intercept	0.3724	0.0211	17.62	0.0032**
x_1	-0.2670	0.0122	-21.88	0.0021**
x_2	0.0357	0.0122	2.92	0.0998
x_3	0.2069	0.0122	16.96	0.0035**
x_1^2	0.3069	0.0199	15.40	0.0042**
x_1x_2	-0.0778	0.0199	-3.91	0.0597
x_2^2	0.0086	0.0299	0.29	0.8001
x_1x_3	-0.2339	0.0264	-8.88	0.0125*
Regression R^2: 0.9985, F:258.35Pr>|f|:0.0039**				
Lack of fit:54.82, Pr>|f|:0.0855				

ANOVA *,** – correlation of significance values of *P*≤ 0.05 and 0.01, respectively;
p≤0.05 values were considered to be significant;
p≤0.01 values were considered to be very significant.

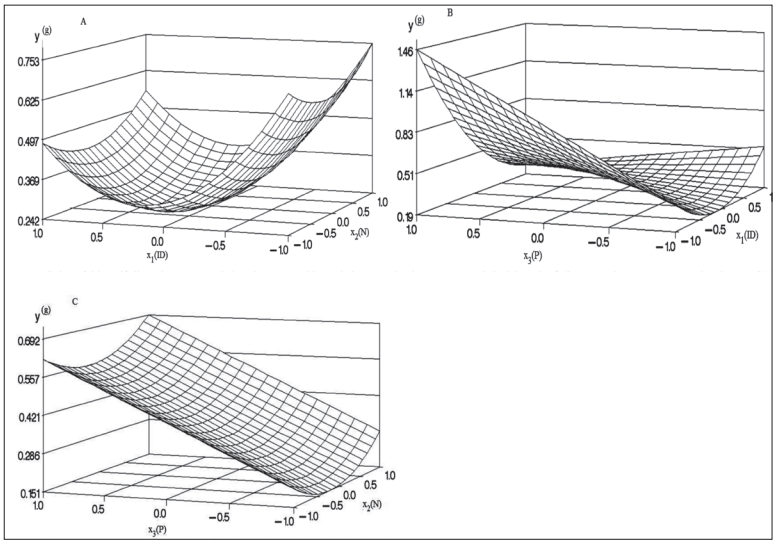

Fig. 1. The effect of x_1 (ID), x_2 (N), and x_3 (P) on plant biomass (y). **A** – Effects of x_1 (ID) and x_2 (N) on plant biomass (y). **B** – Effects of x_1 (ID) and x_3 (P) on plant biomass (y). **C** – Effects of x_2 (N) and x_3 (P) on plant biomass (y). [x_1 – indole-acyl esters (ID) 0.1-1.1 mL·L^{-1}; x_2 – NH_4NO_3(N) 2-12 mM; x_3 – KH_2PO_4(P) 0.5-2.5 mM]; the factor levels were coded as −1 (low), 0 (central point) and 1 (high); y indicates the whole plant biomass (g).

Table 3. Biomass of *J. curcas* growing under different treatments after 30 days of growth (Cd50-Pb500 stress).

Parameters	Tissue	Treatment					
		CK1	N-P	N-ID	P-ID	N-P-ID	CK2
Fresh biomass	Root (g)	0.54a	1.37c	0.69a	0.97b	2.06e	1.64d
	Stem (g)	5.52a	8.11cd	6.44b	7.44c	10.22e	8.40d
	Leaf (g)	1.77a	3.57b	1.92a	3.18b	4.80c	3.55b
	Whole plant (g)	7.80a	13.06cd	9.05b	11.60c	17.09e	13.59d
Dry biomass	Root (g)	0.10a	0.20c	0.13a	0.17b	0.32e	0.28d
	Stem (g)	0.54a	0.80c	0.68b	0.74bc	0.96d	0.88d
	Leaf (g)	0.29a	0.51cd	0.31a	0.48c	0.72e	0.58d
	Whole plant (g)	0.93a	1.51c	1.11a	1.37b	1.99e	1.74d

CK1 (only H_2O), N-P (combination of NH_4NO_3 and KH_2PO_4), N-ID (combination of NH_4NO_3 and indole-acyl esters), P-ID (combination of KH_2PO_4 and indole-acyl esters), N-P-ID (combination of NH_4NO_3, KH_2PO_4, indole-acyl esters), CK2 (only H_2O, no contaminated soil).
Means with the same lowercase letters are not significantly different from each other in one row (LSD test, $P \leq 0.05$).

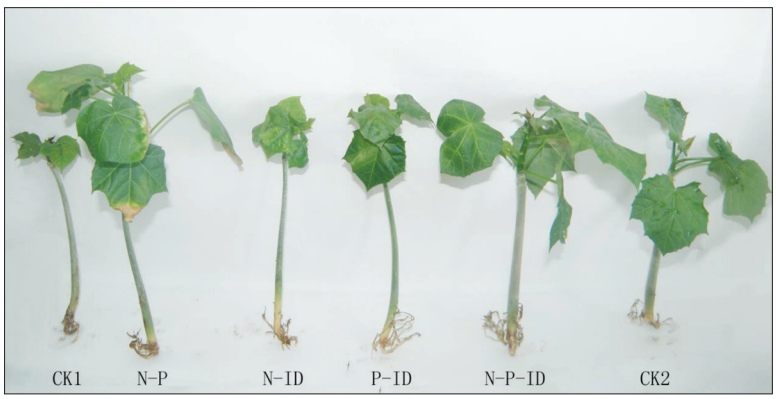

Fig. 2. *J. curcas* after 30 days of growth under Cd50-Pb500 stress. CK1 (only H_2O), N-P (combination of NH_4NO_3 and KH_2PO_4), N-ID (combination of NH_4NO_3 and indole-acyl esters), P-ID (combination of KH_2PO_4 and indole-acyl esters), N-P-ID (combination of NH_4NO_3, KH_2PO_4, indole-acyl esters), CK2 (only H_2O, no contaminated soil).

J. curcas after 30 days of growth under Cd50-Pb500 stress

The fresh and dry biomass of the plants under different treatments are shown in Table 3. Compared with the CK2 treatment, the fresh and dry biomasses of the roots, stems and leaves from the CK1 treatment with the Cd50-Pb500 stress, were significantly lower (Table 3). The Cd50-Pb500 stress caused decreases in the root, stem and leaf dry (64.3, 37.5 and 50%, respectively) and fresh biomasses (67.1, 34.5 and 50.1%, respectively) in comparison with CK2. Compared with the stems and leaves, the fresh and dry biomasses of the roots showed a larger reduction, indicating that the highest phytotoxic effects were in the roots.

All four treatments had significant effects on the fresh and dry biomass of *J. curcas* in comparison to the CK1 treatment. The fresh and dry biomass of the whole plants increased significantly with N-P, N-ID, P-ID and N-P-ID treatments in comparison to CK1; the order of the four treatments based on their increases in dry biomass were as follows: N-P-ID>N-P>P-ID>N-ID (Table 3, Fig. 2). The N-P treatment recovered total plant fresh biomass to the normal levels in CK2, whereas ID improved the effects of the N-P treatments (Table 3). ID combined with N-P enhanced the dry biomass to a normal level in the contaminated soils (Table 3).

The roots were more sensitive to Cd and Pb than the stems and leaves

Table 4. N and P concentrations of *Jatropha curcas* growing under different treatments after 30 days of growth (Cd50-Pb500 stress).

Parameters	Tissue	Treatment					
		CK1	N-P	N-ID	P-ID	N-P-ID	CK2
N concentration	Root ($mg \cdot g^{-1}$)	20.93a	23.56b	22.33ab	20.62a	23.49b	25.72c
	Stem ($mg \cdot g^{-1}$)	16.60a	18.38bc	17.70ab	17.42ab	18.42bc	19.40c
	Leaf ($mg \cdot g^{-1}$)	31.77a	36.35c	34.00b	34.86b	40.33cd	50.00d
	Whole plant ($mg \cdot plant^{-1}$)	18.79a	32.97c	25.30b	28.68bc	44.63d	49.58e
P concentration	Root ($mg \cdot g^{-1}$)	3.73a	5.42c	4.20b	4.22b	5.30c	4.44b
	Stem ($mg \cdot g^{-1}$)	3.88a	6.74bc	6.24b	6.81c	6.83c	6.84c
	Leaf ($mg \cdot g^{-1}$)	6.21a	8.78c	7.17b	7.10b	8.53c	8.30c
	Whole plant ($mg \cdot plant^{-1}$)	4.25a	10.94d	6.94b	9.03c	14.33f	12.10e

CK1 (only H_2O), N-P (combination of NH_4NO_3 and KH_2PO_4), N-ID (combination of NH_4NO_3 and indole-acyl esters), P-ID (combination of KH_2PO_4 and Indole-acyl esters), N-P-ID (combination of NH_4NO_3, KH_2PO_4, indole-acyl esters), CK2 (only H_2O, no contaminated soil). Means with the same lowercase letters are not significantly different from each other in one row (LSD test, $P \leq 0.05$).

Table 5. Concentrations of Pb and Cd in *J. curcas* growing under different treatments after 30 days of growth (Cd50-Pb500 stress).

Parameters	Tissue	Treatment					
		CK1	N-P	N-ID	P-ID	N-P-ID	CK2
Pb concentration	Root ($\mu g \cdot g^{-1}$)	96.55b	136.72c	356.49f	225.46e	176.12d	22.90a
	Stem ($\mu g \cdot g^{-1}$)	27.71b	53.79d	53.69d	89.14e	47.24c	8.11a
	Leaf ($\mu g \cdot g^{-1}$)	21.32b	22.56b	22.10b	22.01b	23.29b	4.14a
	Whole plant ($\mu g \cdot plant^{-1}$)	30.81b	81.86c	89.70c	114.85d	118.47d	9.81a
Cd concentration	Root ($\mu g \cdot g^{-1}$)	75.71b	108.66c	235.80f	159.30e	119.38d	0a
	Stem ($\mu g \cdot g^{-1}$)	14.72b	22.75d	16.40c	27.41e	17.00c	0a
	Leaf ($\mu g \cdot g^{-1}$)	6.31b	6.85c	7.28c	7.25c	7.67c	0a
	Whole plant ($\mu g \cdot Plant^{-1}$)	17.34b	43.42c	44.06c	50.84d	60.04e	0a

CK1 (only H_2O), N-P (combination of NH_4NO_3 and KH_2PO_4), N-ID (combination of NH_4NO_3 and indole-acyl esters), P-ID (combination of KH_2PO_4 and indole-acyl esters), N-P-ID (combination of NH_4NO_3, KH_2PO_4, Indole-acyl esters), CK2 (only H_2O, no contaminated soil). Means with the same lowercase letters are not significantly different from each other in one row (LSD test, $P \leq 0.05$).

as they had direct contact with the soil. ID combined with the N-P treatments caused increases of 3.8- and 3.2-fold in the fresh and dry weights of the roots, compared to the CK1 treatment (Table 3). The fresh and dry weights of the roots from plants receiving the N-P-ID treatment were greater than the dry and fresh weights of the stems and leaves when compared to the CK1 treatment. Similar results were observed for the other treatments (Table 3). N-P-ID treatments stimulated lateral root production and increased the ability of *J. curcas* to grow in the contaminated soils (Fig. 2).

Concentrations of N and P in plants

The N and P concentrations in the different tissues with the different treatments are shown in Table 4; significant differences were observed between the different treatments. The N concentration in the leaves decreased from 50.00 $mg \cdot g^{-1}$(CK2) to 31.77 $mg \cdot g^{-1}$(CK1) when exposed to the Cd and Pb stress; the 36.5% decrease in N concentrations in the leaves was more than that found in the roots and stems. The concentration of P in the stems under Pb and Cd stress in the CK1 treatment decreased by 43.3% in comparison to the CK2 treatment, and it was more than that found in the roots and leaves.

Compared to the CK2 treatment, the concentration of N in the whole plant under Pb and Cd stress in the CK1 treatment decreased by 62.1%, and the concentration of P decreased by 64.9%. (Table 4). All four treatments significantly increased the concentrations of N and P in the whole plants exposed to Cd and Pb stress. Compared to the CK2 treatment, the concentration of N in the whole plant under Pb and Cd stress in the N-P-ID treatment decreased by 10.0%, whereas the concentration of P increased by 18.4% (Table 4). The concentrations of N and P in whole plants exposed to N-P and N-P-ID treatments were significantly higher than those exposed to N-ID and P-ID treatments. These results demonstrated that the simultaneous use of N and P fertilizers could improve

Table 6. Malondialdehyde (MDA) concentration and activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in *J. curcas* growing under different treatments after 30 days of growth (Cd50-Pb500 stress).

Treatment	MDA ($nmol \cdot g^{-1}$)	SOD ($U \cdot g^{-1}FW$)	POD ($U \cdot min^{-1}g^{-1}FW$)	CAT ($U \cdot min^{-1}g^{-}$ FW)
CK1	49.92c	127.81b	425.05b	14.16b
N-P	35.71b	202.17e	638.71d	26.19e
N-ID	42.73bc	148.67c	459.83b	16.20c
P-ID	39.55b	171.88d	511.28c	20.95d
N-P-ID	24.48a	233.93f	790.12e	27.24e
CK2	22.54a	103.26a	377.90a	9.94a

CK1 (only H_2O), N-P (combination of NH_4NO_3 and KH_2PO_4), N-ID (combination of NH_4NO_3 and indole-acyl esters), P-ID (combination of KH_2PO_4 and indole-acyl esters), N-P-ID (combination of NH_4NO_3, KH_2PO_4, Indole-acyl esters), CK2 (only H_2O, no contaminated soil). Means with the same lowercase letters are not significantly different from each other in one row (LSD test, $P \leq 0.05$).

the uptake of N and P in Cd- and Pb-contaminated soils. The concentrations of N and P in whole plants exposed to N-P-ID treatment were significantly higher than those exposed to N-P treatment, demonstrating that ID improved the uptake of N and P.

Concentrations of Cd and Pb in plants

The concentrations of Cd and Pb in the roots, stems and leaves of plants under different treatments were significantly higher than those in CK2. The concentrations of Cd and Pb in the roots were about 17-80- and 4-10-fold higher than those in the stems and leaves (except Cd in CK2), respectively (Table 5), meaning that the maximum phytotoxic effects were exerted on the roots (CK1 and CK2 in Table 3).The concentrations of Cd and Pb in different tissues were root>stem>leaf (Table 5). There were no significant differences between the concentrations of Cd and Pb in the leaves after N-P, N-ID, P-ID and N-P-ID treatments. In contrast, treatments N-ID, P-ID, N-P, and N-P-ID significantly increased the concentrations of Cd and Pb in the roots and stems as compared to the CK1 treatment. The largest increases in Cd and Pb concentrations in stems were recorded after the P-ID treatment, while the smallest were observed after the N-P-ID treatment (except for Cd under N-ID treatment) (Table 5). The concentrations of Cd and Pb in whole plants under different treatments were significantly higher than those after the CK2 treatment. The N-P-ID treatment resulted in the largest Pb and Cd concentrations in the whole plants (Table 5).

MDA concentrations and activity of enzymatic antioxidants in *J. curcas*

The concentrations of MDA increased more than 2-fold when the plants were exposed to Cd50-Pb500 stress without any treatments (Table 6). The following treatments could be classified into three groups according to the concentration of MDA: (i) N-P-ID, (ii) N-P and P-ID, and (iii) N-ID. No significant difference was observed between group (ii) and group (iii). The N-P, N-ID, P-ID and N-P-ID treatments reduced MDA by 28.45, 14.40, 20.77 and 50.96%, respectively, when compared to the CK1 treatment. The N-P-ID treatment reduced the concentration of MDA to the levels observed in the CK2 treatment (Table 6).

MDA concentrations were negatively associated with enzymatic antioxidants such SOD, POD and CAT (Table 5). The activities of SO, POD and CAT increased significantly with N-P, N-ID, P-ID and N-P-ID treatments when compared to the CK1 treatment; the order of the four treatments based on their increased activities was: N-P-ID>N-P>P-ID>N-ID (Table 6). Compared to CK1, the N-P-ID, N-P, P-ID and N-ID treatments increased the activities of CAT by 92.42, 85.03, 48.00 and 14.43%, respectively; the activities of POD by 85.89, 50.27, 20.29 and 8.18%, respectively; and the activities of SOD by 83.03, 58.19, 34.49 and 16.33%, respectively.

DISCUSSION

In this study, a mixture of Cd and Pb in the soil (50 mg kg^{-1} soil and 500 mg kg^{-1}, respectively) caused 34.3 and 67.1 % decreases in root, stem and leaf biomass when compared to the CK2 treatment. These findings are in agreement with the results of Li et al. [12] and Lianget al.[13]. High concentrations of Cd and Pb in the roots decreased their biomass, showing that it had stronger phytotoxic effects on the roots than on the stems in *J. curcas*, which is in accordance with previous research [39-42].

Exogenous application of N [25], P [26] and low doses of IAA [27,28] or DA-6 [29,30] could mitigate Cd/Pb stress. The results of response surface method-

ology analysis indicated that the order in which these three factors can improve the growth of *J. curcas* under Pb and Cd stress was: $x_1(ID)>x_3(P)>>x_2(N)$. The increase in ID concentration had a negative effect on the growth of *J. curcas*, especially when the concentration of ID was greater than 0.5 $mL \cdot L^{-1}$. The main components of ID are IAA and DA-6, which can promote the growth of *J. curcas* at low concentrations and inhibit it at high concentrations. Bashri et al. (2015) confirmed that Cd alone and together with high concentrations of IAA can induce oxidative stress, while combinations of Cd and low doses of IAA can decrease it and attenuate Cd toxicity [28]. The toxic effects of Pb and Cd on *J. curcas* can be increased with applications of high-concentration ID. Applications of low-concentration ID, N and P showed synergistic effects on *J. curcas* growth. The predicted optimal concentration ratios of ID, N and P were 0.1 $mL \cdot L^{-1}$, 12 mM and 2.5 mM, respectively. Combined treatments, such as N-P, N-ID, P-ID and N-P-ID, significantly mitigated Pb/Cd phytotoxicity, but the mitigation efficiencies significantly differed between the treatments.

Fresh and dry biomass from the N-P treatment significantly increased in comparison to the N-ID and P-ID treatments, showing that the simultaneous use of N and P fertilization was necessary to mitigate the Cd and Pb phytotoxicity on *J. curcas*. The optimum levels of both N and P can be used to improve seedling growth [43] due to the different roles N and P have in a plant's energy metabolism, involving enzymes associated with ROS metabolism, such as CAT, SOD and POD [44]. In the present study, the N-P treatment produced much higher activity of SOD, POD and CAT when compared with single N or P treatments, even when these were combined with ID.

Only the N-P-ID treatment caused significant increases in both fresh and dry biomass compared to the CK2 treatment (the normal level), suggesting that ID improved the mitigating effects of the N and P fertilizers on Cd and Pb phytotoxicity. IAA can stimulate lateral root production with higher root dry biomass [27,45]. Compared to the N-P treatment, the N-P-ID-treated plants retained more Pb and Cd in their roots, as ID decreased the translocation of the metal to the leaves [30,46]. The higher activities of SOD and POD enzymes in the plants exposed to the N-P-ID treatment can be explained by the fact that ID is capable of upregulating their activity [47] and the ascorbate-glutathione cycle [48], alleviating oxidative stress [49,50].

Our results showed that the concentrations of N and P in the roots, stems, leaves and whole plants after the CK1 treatment were decreased as compared to the CK2 treatment. Cd and Pb can disturb the uptake and distribution of N and P, which agrees with the results of Ramon et al. [51], Kibria et al. [52] and Farouk et al. [53]. The N-P-ID and N-P treatments under Cd50-Pb500 stress can elevate N and P when compared with the CK1 treatment. A significant difference was observed between the N-P and N-P-ID treatments, suggesting that ID could improve the uptake and translocation of N and P [54, 55].

J. curcas could accumulate Cd and Pb from contaminated soils [8-11]. Phosphate [56,57], IAA and DA-6 increase the concentrations of metal in the roots and stems [27,30]. In the present study, the concentrations of Cd and Pb were significantly increased in the roots, stems and whole plants after the N-ID, P-ID, N-P and N-P-ID treatments when compared with the CK1 treatment. Plants after the N-P-ID treatment had higher Pb and Cd concentrations in the roots and whole plant when compared with the N-P treatment, which indicates that ID improved the phytoremediation ability. ID can affect Pb and Cd concentrations in plants by depositing more metal in the cell walls [27], increasing Pb and Cd concentrations in the roots [46], increasing the root surface areas [30], promoting a higher transpiration rate, and by regulating the levels of nutrient elements [58]. Phosphate can increase Cd and Pb concentrations in plants by forming insoluble Cd/Pb phosphate complexes [56,57]. However, in this study, the applications of N and P were important for mitigating Cd and Pb toxicity in *J. curcas* by increasing the biomass and the activities of enzymatic antioxidants, which in turn can increase metal uptake and protect plants from oxidative stress. ID can improve the effect of the N-P treatments by enhancing the mitigation of toxic effects and concentrations of cadmium and lead in *J. curcas* from contaminated soils.

CONCLUSIONS

ID improved the mitigation effects of N-P fertilizers and the phytoremediation ability of *J. curcas* by in-

creasing root biomass, increasing the concentration of N and P, increasing the concentration of Cd and Pb in the roots, and by promoting the activities of SOD and POD in the plants. The Box-Behnken three-variable partial factorial design with the response surface methodology modeling technique was verified as a reliable methodology that can be used to study the effects and interactions of multiple factors on plants.

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