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Influence of Relative Humidity on Germination and Metal Accumulation in *Vigna radiata* Exposed to Metal-based Nanoparticles

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Abstract: The effect of single (0.1, 1, and 10 mg L^{-1}) and binary mixtures (0.1 + 0.1, 1 + 1, and 10 + 10 mg L^{-1}) of metal-based nanoparticles (CdO and CuO) on the germination of *Vigna radiata* was studied under two humidity ranges (70% and 80%). Filter paper-based tests were conducted. The surface-sterilized seeds were exposed to CdO and CuO under controlled environmental conditions (70% and 80% humidity at 35 °C). Germination rates were scored after 24 h and 48 h. The accumulation of metals was tested in seedlings after 48 h using inductively coupled plasma mass spectrometry. Compared with 70% humidity, the germination rate was higher under 80% humidity in all tested conditions. The germination rate of the CdO + CuO treatment was less than that of the single metal exposure under both humidities (70% and 80%) at 48 h. By two-way analysis of variance (ANOVA), we found that germination was greatly influenced by humidity. The accumulation of metal was higher in the CuO test than in the CdO test. Metal accumulation was concentration and humidity dependent, except for Cd accumulation in the CdO + CuO treatment. Here we show that the germination of seeds depends on the humidity and concentration of metal oxide nanoparticles. Understanding these strategies in seeds might help to avoid environmental and chemical stress and improve crop yield.

Keywords: metal-based nanoparticle; germination; accumulation; CuO; CdO

1. Introduction

The use of metal-based nanoparticles (M-NPs) in many sectors (such as textile, electronics, medical, cosmetics, and environmental treatment processes) leads to their release into water and soil environments. Nanoparticles are just 1 to 100 nm in diameter and this small size might induce undesired effects in soil and water, affecting animals and plants. Furthermore, due to their minute size, nanoparticles can penetrate the plant cell wall and disrupt cellular functions. Based on their specific properties (size, surface characteristics, reactivity, and optical sensitivity) [1], many toxic effects of nanoparticles have been recorded in the literature, including tissue inflammation [2] and altered cellular oxidative metabolism [3], which can lead to cell damage and death [4]. In toxicity experiments, researchers must choose the test organism, the test endpoint, and the sensitivity of the organisms. The typical endpoints in plant ecotoxicology tests are seed germination, growth, biomass, and enzyme activity, among others. Many previous studies have investigated these aspects using metal-based nanoparticles [5], typically using the endpoint seed germination in phytotoxicity assay, where the seeds are exposed to the M-NPs throughout germination [6].

Seeds are protected against various stresses; however, they become stress-sensitive during the vegetative development stages. Therefore, many toxicity studies on seed germination have reported the effect of M-NPs as well as accumulation in biomass [5,6]. There have been reports of the inhibition of seed germination, seedling growth, and root elongation of various plant species by the nanoparticles CdS [7–9], CuS [8], CuO [9,10], ZnO [11], as well as rare earth nanoparticles [12]. However, to better understand these effects, studies are needed that apply a mixture of metals. We identified two such studies in the literature. Ko et al. [13,14] have studied the seed germination of Brassica, and another study addressed the cell count and chlorophyll content of algae. In both studies, Brassica seeds and algae were exposed to individual and binary mixtures of various metal oxide nanoparticles (e.g., CuO, NiO, ZnO, TiO_2, Fe_2O_3, and Co_3O_4). In addition, in plant ecology, environmental factors (e.g., temperature, humidity, and light) are important parameters for the growth of plants. High relative humidity enhances the uptake of metals from the air by leaves due to hydration of the cuticle [15]. In a controlled environment, without any chemical stress, the optimum temperature and relative humidity for germination of black gram seeds were 35 °C and 80%, respectively [16]. Furthermore, there are no reports available in the literature on the influence of relative humidity on the sprouting of seeds when exposed to a single and binary mixture of nanoparticles. Therefore, this study uses metal oxide nanoparticles (CuO and CdO) as pollutants exposed to *Vigna radiata*. Additionally, two studies tested the germination of *Lathyrus sativus* L [9] and *Coriandrum sativum* [17] seeds pretreated with M-NPs (CuS and CdO) for 2 to 6 h. Binary mixtures of these M-NPs and the humidity effect was not tested in these studies. Therefore, these two aspects are considered in this study.

2. Materials and Methods

2.1. Chemicals and Seeds

The CdO (CAS No. 7740-50-8, 99.9% purity) and CuO (CAS No. 1306-19-0, 98.9% purity) NP powders (partially passivated with oxygen by the manufacturer) were purchased and used in this study (American elements, USA). The properties of the nanoparticles are given on the manufacturer's website, and the compilation of some of the properties is listed in Table 1. The stock solutions (1000 ppm) were prepared by mixing 1 g of Cd and Cu in Millipore water. For optimum uniform suspensions, both M-NPs were sonicated (Hwashin Tech, Powersonic 610) for 1 h and stirred using a magnetic stir plate. In single effect treatments, the concentrations were 0, 0.1, 1, and 10 mg L^{-1} CuO or CdO and the binary mixture treatments were 0, 0.1 + 0.1, 1 + 1, and 10 + 10 mg L^{-1} of CdO + CuO prepared from stock solutions. To avoid aggregation during exposure, the suspensions of single and combined metal solutions were stirred using the method described previously. The certified seeds of *Vigna radiata* were purchased from the local seed store. The uniform size, color, and weight of the seeds were chosen for this study. The seeds were surface sterilized with 0.1% mercuric chloride for 2 min, washed thoroughly with tap water, and then by Millipore water for 30 min.

Table 1. Physicochemical characteristics of the nanoparticles used in this study.

Physicochemical Property	CdO	CuO
Molecular mass	128.4	79.55
Appearance	White	Black to brown
Melting point (°C)	900–1000	1201
Boiling point (°C)	1559	2000
Density (g/cm^3)	8.15	6.31
Exact mass (g/mol)	129.9	78.9
Particle Size (nm)	20–80	1–30
Specific surface area (m^2/g)	10–50	100–200

2.2. Germination Experiment

The seed germination test was carried out on a moist filter paper (ISTA, 1997). The surface-sterilized seeds were placed on two sheets of filter paper (Whatman No. 1) contained in 90 mm sterile Petri dishes. Each Petri plate was moistened uniformly with 5 mL of a single and binary mixture of CdO and CuO concentrations, as mentioned above. Control seeds were irrigated with distilled water. All concentrations were carried out in triplicate (each containing ten seeds). No additional solution was added during this test. The experiment was conducted in a growth chamber (LabTech, LGC-4201, South Korea) at two relative humidities (70% and 80% ± 1%) at a constant temperature of 35 ± 0.1 °C under a 16 h light (2200 lux)/8 h dark cycle for 48 h. Cumulative germination percentage (%) was recorded at 24 and 48 h after exposure. The emergence of the radicle from the seed was considered as germinated. The results are expressed as the percent germination and relative germination rate (after 48 h):

Percentage germination (%) = (Number of germinated seeds × 100) / total number of seeds.
Relative germination rate = Germination rate in treatment / germination rate in control.

Finally, the sprouted plants were carefully removed using forceps at the end of the experiment for the accumulation test.

2.3. Metal Accumulation

At the end of the experiment (48 h), all of the sprouted plant samples were washed with running tap water and then immediately rinsed several times with Milli-Q water (Puris, Expe-UP, South Korea) to remove the M-NPs associated on the plant surface. Prior to the estimation of dry weight and accumulation of Cd and Cu, the seedling samples were air-dried for 2 days and then dried in an oven at 60–70 °C for 12 h. The known weight of previously ground and dried plant samples were digested with HNO_3 (65%) and HCl (37%) at a ratio of 3:1. The mixtures were boiled at 100 °C until the sample had completely dissolved (maximum 4–5 h). The digested samples were then cooled and made up to 50 mL using Milli-Q water. All samples were stored at 4 °C until further analysis. The plant digestives were analyzed for elemental concentration using inductively coupled plasma mass spectrometry (ICP-MS, Leemans Labs, USA).

2.4. Statistical Analysis

Duncan's multiple range test was performed to determine the significance of results ($p < 0.05$) when comparing the control and M-NP treatments. A two-way ANOVA was performed to compare the exposure concentrations under two humidities and M-NP mixtures. SPSS software (Version 25) was used for all analyses.

3. Results

The percent germination (24 and 48 h) and relative germination rates of *V. radiata* are shown in Table 2 and Figure 1a–c. At 80% humidity, at an early stage (24 h), the germination rate significantly differed ($p < 0.05$) between control and single and binary mixtures. However, when we compared the germination percentage at the end of the experiment, we found no difference between seeds that were exposed to single M-NPs (nano CdO and nano CuO) and the control. This significant difference ($p < 0.05$) was maintained in all concentrations of binary mixtures at the end of the experiment (48 h). All control seeds (100%) germinated within 24 h, but none of the treatments achieved this value except, CuO 0.1 mg L^{-1}. The lowest and highest germination percentages for CdO exposure were 93% (10 mg L^{-1}) and 100% (0.1 and 1 mg L^{-1}); these values for CuO exposure were 96.7% (10 mg L^{-1}) and 100% (0.1 and 1 mg L^{-1}); and 80% (10 CdO + 10 CuO mg L^{-1}) and 93.3% (1 CdO + 1 CuO mg L^{-1}) for CdO + CuO exposure.

Table 2. The effect of a single and binary mixture of CdO and CuO on seed germination (%) (after 24 and 48 h) in filter paper tests at two humidity conditions. Results are given as mean (* $p < 0.05$ vs. control) and means following with different letters (a to d) in each column are significant at 5% level of probability.

Concentration (mg L $^{-1}$)		Germination (%)			
		80% humidity		70% humidity	
		24 h	48 h	24 h	48 h
Control		100.0 ^{a}	100.0 ^{a}	66.7 ^{a}	70.0 ^{a}
CdO	0.1	93.3 ^{b}	100.0 ^{a}	60.0 ^{a}	63.3 ^{a}
	1	80.0 ^{c}	100.0 ^{a}	40.0 ^{ab}	43.3 ^{ab}
	10	70.0 ^{d}	93.3 ^{a}	23.3 ^{b}	23.3 ^{b}
CuO	0.1	100.0 ^{a}	100.0 ^{a}	63.3 ^{a}	66.7 ^{a}
	1	90.0 ^{b}	100.0 ^{a}	60.0 ^{a}	66.7 ^{a}
	10	53.3 ^{c}	96.7 ^{a}	60.0 ^{a}	63.3 ^{a}
CdO + CuO	0.1 + 0.1	70.0 ^{b}	90.0 ^{ab}	56.7 ^{a}	60.0 ^{a}
	1 + 1	70.0 ^{b}	93.3 ^{a}	56.7 ^{a}	60.0 ^{a}
	10 + 10	50.0 ^{c}	80.0 ^{c}	30.0 ^{b}	30.0 ^{b}

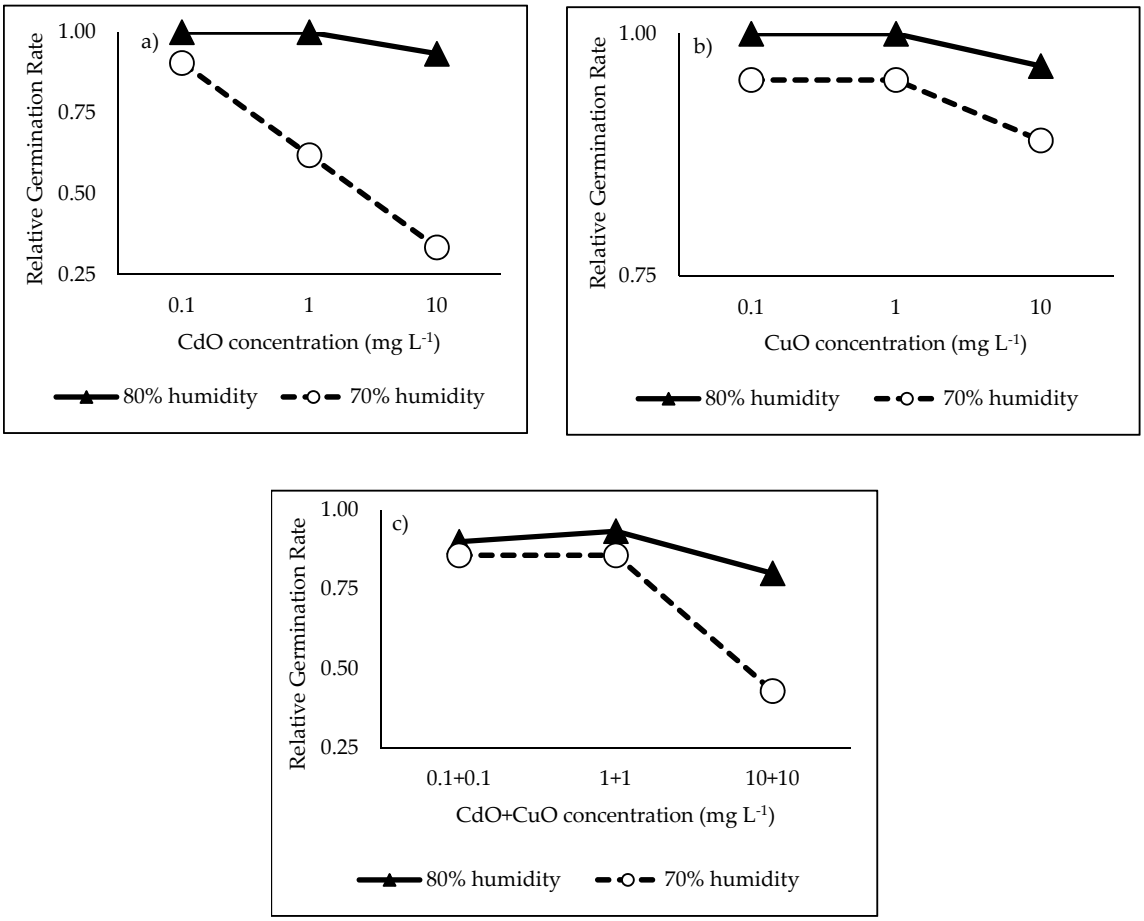

Figure 1. Relative germination rate (after 48 h) (germination rate in treatment / germination rate in control) of *V. radiata* seeds exposed to (**a**) CdO, (**b**) CuO, and (**c**) CdO + CuO at two huminities.

At 70% humidity, none of the treatments (CdO, CuO, and CdO + CuO) achieved 100% within 48 h. The maximum germination rate (70%) was achieved in the control. The maximum germination in CdO exposure was 63.3% (0.1 mg L^{-1}), CuO exposure was 66.7% (0.1 and 1 mg L^{-1}), and the binary mixture

of metal (CdO + CuO) was 60% (0.1 CdO + 0.1 CuO and 1 CdO + 1 CuO) at the end of the experiment (48 h). At the highest exposure concentrations of CdO (10 mg L^{-1}) and CdO + CuO (10 + 10 mg L^{-1}), we detected a significant ($p < 0.05$) difference compared with the control after 48 h of exposure.

By two-way ANOVA (Table 3), we found that the humidity was the major factor influencing the germination of seeds either exposed to single M-NPs or exposed to binary M-NPs. Similarly, the CdO and CdO + CuO concentrations also showed a significant effect but not for the case of CuO. Furthermore, the significant interactive effects of concentration and humidity on percent germination were detected only in the CdO treatment.

Table 3. Results of two-way ANOVA on the interaction between concentration (CdO, CuO, and CdO + CuO) and humidity on the germination of *V. radiate* seeds. (Df – degrees of freedom, F- F statistic and P – *p* value).

Exposure	Source of Variation	Df	F	P
CdO	Concentration	3	6.667	0.004
	Humidity	1	108.516	0.000
	Concentration vs humidity	3	3.914	0.028
CuO	Concentration	3	0.253	0.858
	Humidity	1	60.840	0.000
	Concentration vs humidity	3	0.040	0.989
CdO + CuO	Concentration	3	10.591	0.000
	Humidity	1	84.045	0.000
	Concentration vs humidity	3	1.500	0.253

The relative germination rate data are summarized in Figure 1. The relative germination rate did not show any difference at 80% humidity test in all single (CdO and CuO) and binary (CdO + CuO) exposures (Figure 1a–c). Similarly, at 70% humidity, CuO exposure had no significant inhibitory effect (Figure 1b). However, at 70% humidity, the results clearly showed that the inhibition began from 1 mg L^{-1} of CdO (Figure 1a) and 10 + 10 mg L^{-1} of a binary mixture of CdO + CuO exposure (Figure 1c).

Compared with the control condition, under both humidities, the accumulation of Cd and Cu was significantly increased ($p < 0.05$) with increasing (concentration) metal exposure (Table 4). Our two-way ANOVA clearly showed that the accumulation was significantly influenced by the humidity and M-NPs exposure (both single and binary mixture) (Table 5).

Table 4. Metal accumulation in seedlings of *V. radiata* exposed to CdO, CuO, and CdO + CuO over 48 h (mg kg^{-1} d.w.). Results are given as mean (* $p < 0.05$ vs. control) and means following with different letters (a to d) in each column are significant at 5% level of probability.

Concentration (mg L^{-1})		80% humidity		70% humidity	
		CdO	CuO	CdO	CuO
Control		37.67 ± 2.8 ^{a}	-	19.70 ± 1.65 ^{a}	-
CdO	0.1	83.13 ± 12.1 ^{b}	-	51.47 ± 5.50 ^{b}	-
	1	100.9 ± 7.90 ^{c}	-	84.29 ± 10.04 ^{c}	-
	10	193.3 ± 8.02 ^{d}	-	126.47 ± 13.42 ^{d}	-
Control		-	46.0 ± 8.00 ^{a}	-	34.6 ± 9.45 ^{a}
CuO	0.1	-	208.5 ± 0.50 ^{b}	-	175.1 ± 6.92 ^{b}
	1	-	260.3 ± 19.9 ^{c}	-	203.7 ± 6.86 ^{c}
	10	-	317.0 ± 10.15 ^{d}	-	206.4 ± 22.6 ^{c}
Control		37.67 ± 2.8 ^{a}	46.0 ± 8.00 ^{a}	19.70 ± 1.65 ^{a}	34.6 ± 9.45 ^{a}
CdO + CuO	0.1 + 0.1	78.8 ± 13.58 ^{b}	171.0 ± 13.58 ^{c}	72.97 ± 7.46 ^{b}	118.7 ± 6.25 ^{b}
	1 + 1	82.17 ± 8.14 ^{b}	91.83 ± 17.32 ^{b}	47.67 ± 15.8 ^{c}	86.14 ± 8.47 ^{c}
	10 + 10	111.3 ± 13.05 ^{c}	96.66 ± 7.15 ^{b}	91.43 ± 3.98 ^{d}	57.66 ± 7.53 ^{d}

Table 5. Results of two-way ANOVA on the interaction between concentration (CdO, CuO, and CdO + CuO) and humidity (80% and 70%) on the accumulation of Cd and Cu in *V. radiata* biomass. (Df—degrees of freedom, F—F statistic and P—*p* value).

Exposure / Accumulation	Source of Variation	Df	F	P
CdO / Cd accumulation	Concentration	3	245.01	0.000
	Humidity	1	89.18	0.000
	Concentration vs humidity	3	11.00	0.000
CuO / Cu accumulation	Concentration	3	368.28	0.000
	Humidity	1	107.12	0.000
	Concentration vs humidity	3	17.35	0.000
CdO + CuO / Cd accumulation	Concentration	3	59.57	0.000
	Humidity	1	25.02	0.000
	Concentration vs humidity	3	2.25	0.121
CdO + CuO / Cu accumulation	Concentration	3	131.38	0.000
	Humidity	1	50.86	0.000
	Concentration vs humidity	3	8.78	0.001

4. Discussion

Germination is a seed development process that might be influenced by environmental factors such as temperature, humidity, and contaminants in the environment [17,18]. Therefore, a germination test was conducted under these environmental factors stress to determine the viability of the seeds [19]. The optimum temperature and humidity for the germination of black gram were reported as 35 °C and 80% respectively [16]. Therefore, we selected this temperature (35 °C) and humidity (80%), as well as 70% humidity, as our test conditions. Without M-NPs stress (in control), the germination rate was 100% at 80% humidity and 70% at 70% humidity. These results are consistent with earlier reports, which have reported a reduction in germination in black gram at ≤70% humidity [16] and the germination in *Sesamum indicum* at ≤70% humidity [20]. This might be due to the 80% of humidity ameliorated seed growth and development, which might involve the activities of various plant enzymes and hormones [16,21].

Similar to humidity, the concentration-dependent reduction in seed germination when exposed to M-NPs has been reported by many studies [9,13]. There was no inhibition of germination under an M-NPs exposure level of 0.1 to 10 mg L^{-1} at 80% humidity test, indicating that *V. radiata* tolerates

this range of exposure. Similarly, at 70% humidity, there was no significant difference between control and exposure (CdO and CuO), except for a high concentration of CdO. However, the germination rate at 70% humidity was less than at 80% humidity in all concentrations (after 48 h). The similarity of germination between control and exposure was indicated by the value 1 in the calculation of relative germination rate (germination in exposure/germination in control) (Figure 1a–c). The relative germination of <1 in this study indicated the negative influence of CdO (1 and 10 mg L^{-1}) and CdO + CuO (10 + 10 mg L^{-1}) exposure on seed germination.

The seed coat is a barrier and can have selective permeability, which might protect the embryo from external toxic chemicals and environmental stresses such as temperature and humidity. Many studies have reported the inhibitory effect of M-NPs on plant shoot and root length; however, this may not inhibit the germination rate since they cannot pass through seed coats [22–24]. During germination, the dry seed expands its size due to the imbibition process. This process culminates in the rupture of the seed coat, which might create the opportunity to direct contact with the nanoparticles in the media. This might account for the reduction of the development of seed in all exposures in this study. We detected a considerably lower germination rate under the 70% humidity condition. This finding was compatible with previous study results [16], where the experiment was conducted without any metal stress.

Even though seed coats offer some protection, in this study, the accumulation of M-NPs in seedlings was increased with increased exposure concentration (Table 4). The accumulation was greatly influenced by the presence of other metals, resulting in a reduction in bioaccumulation under both humidities (80% and 70%) in the binary mixture of M-NPs (Table 4). Meanwhile, the accumulation of metals at 70% humidity was less than that at 80% humidity. In general, the size of the CuO nanoparticle (1–30 nm) was less than that of the CdO nanoparticle (20-80 nm) (Table 1). In addition, the solubility of CdO in water was 4.8 mg L^{-1}. Therefore, the solubility was higher in the exposed concentration of 0.1 and 1 mg L^{-1}. Meanwhile, the nature of CuO is insoluble in water. Therefore, some of the CuO NPs might have been able to penetrate the cell wall (cell wall pore size ranged from 1.6 to 4.6 nm) and enter the cell as a result, affecting the biological process of the seed. This might account for the higher accumulation of Cu than Cd (Table 4). In the case of CdO, since their size (20–80 nm) was greater than the plant cell pore size (1.6 to 4.6 nm), initially, CdO might not have entered or entered slowly. The rupture of the seed coat then allowed free entry of the CdO ions. This might account for the lower accumulation of Cd than Cu (Table 4). Later, they entered into seeds through intracellular spaces of parenchymatous tissue, which facilitated its diffusion to cotyledons [25,26]. However, the exact mechanism of CdO toxicity on germination needs to be further investigated. No other reports are available in the literature that addresses the toxic effect of M-NPs under different humidity conditions.

5. Conclusions

The single and binary mixtures of metal-based nanoparticles (CdO and CuO) on the germination of *V. radiata* were tested at 70% and 80% humidity. Germination percent, relative germination rate, and the metal accumulations were the parameters considered in this study after 48 h. The germination rate was lower in binary mixtures than the single metal exposure in both humidity ranges. The relative germination rate did not change by 80% humidity in all concentrations tested. Meanwhile, the inhibition of the germination rate began from 1 mg L^{-1} of CdO and 10 + 10 mg L^{-1} of CdO + CuO exposures at a 70% humidity test. The results of two-way ANOVA showed that the accumulation was concentration and humidity dependent. These preliminary results reported here clearly show that the germination rate was controlled by humidity and single and binary mixture of M-NPs (CdO, CuO, and CdO + CuO) exposure. To confirm these findings, further studies are now needed in a pot culture experiment.

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project administration, S.S.; funding acquisition, S.S. All authors have read and agreed to the published version of the manuscript.

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