Note

Effect of hymexazole (3-hydroxy-5-methylisoxazole) on cadmium stress and accumulation in Japanese millet (*Echinochloa frumentacea* Link)

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The mitigating effects of hymexazole (HMI), choline chloride, indolebutyric acid, ethephon and isoprothiolane on cadmium (Cd) stress and accumulation in Japanese millet were evaluated under aquatic conditions. Among five PGRs, HMI showed the highest mitigating effect; however, in this experiment, almost no effect of calcium (Ca), which has been demonstrated to be a Cd mitigator, on Cd accumulation was observed and the mitigating effect of Ca on Cd stress was less than that of HMI. © Pesticide Science Society of Japan

Keywords: phytoremediation, calcium, cadmium, hymexazole, plant growth regulators.

Introduction

Cadmium (Cd) contamination of soil is a serious problem in agricultural fields, mine sites, residential districts and factory sites in cities. In recent years, soil remediation technologies using plants, also known as phytoremediation, have attracted attention as techniques for solving this problem.¹⁾ Until now, phytoremediation studies have mainly focused on crops such as radish (*Raphanus sativus* L.), mustard (*Brassica juncea* L.) and rice (*Oryza sativa* L.)^{2–4)}; however, studies on the usefulness of weeds, which are highly adaptable to undesirable environmental growth conditions, are limited with the exception of *Thlaspi caerulescens* and *Athyrium yokoscense*.^{5,6)} Previously, we examined the Cd sensitivity of 186 weed species and Cd contents of 93 weed species. It was found that purslane (*Portulaca oleracea* L. var. *sativa* (Haw.) DC.) and pokeweed (*Phytolacca americana* L.) were highly tolerant to Cd,^{7,8)} and slender amaranth (*Amaranthus viridis* L.), hairy beggartick (*Bidens pilosa* L.) and devil's beggartick (*Bidens fron-*

dosa L.) accumulated a high content of Cd in their shoots.⁹⁾ These results indicate that the weed species mentioned above may be useful for Cd phytoremediation; however, to enhance the practicability of phytoremediation with weeds, their Cd tolerance and Cd accumulation abilities need to be further improved. In particular, for phytoextraction, high biomass and high Cd accumulation in shoots were required in the candidate weeds.

Moreover, several mitigating effects of plant hormones on Cd stress have been reported; reduction of chlorophyll content caused by Cd was suppressed by abscisic acid in *Brassica napus* L.,¹⁰⁾ and growth suppression caused by Cd was also mitigated by 28-homobrassinolide in chickpea (*Cicer arietinum* L.).¹¹⁾ These reports suggest that certain plant hormones are able to promote Cd tolerance and Cd accumulation in weeds. PGRs, whose physiological actions are similar to those of plant hormones, have wide horticultural applications, such as promoting the parthenocarp of grapes, and flowering and fruit thinning in mandarins and apples. In addition to these physiological actions, PGRs are known to mitigate certain growth stresses of plants; e.g., crop injuries caused by herbicides and salinity were mitigated by hymexazole (3-hydroxy-5-methylisoxazole) and aminolevulinic acid.^{12–14)}

In bioassays utilizing soil culture with nutrient supplements, the effects of PGRs on the Cd stress of plants may be difficult to measure with precision because of the soil adsorption of PGRs and also the effects of plant nutrients on growth. Therefore, water culture, in which plants can be cultured without soils and nutrients, and Japanese millet (*Echinochloa frumentacea* Link), belonging to the same family as barnyardgrass (*Echinochloa crus-galli* (L.) Beauv. var. *crus-galli*), the most representative paddy weed, were used in the present study.

Materials and Methods

Experiment 1. Screening of 5 PGRs, hymexazole (HMI), choline chloride, indolebutyric acid (IBA), isoprothiolane, and ethephon in terms of their mitigating effect on Cd stress in Japanese millet

For this experiment, HMI (30% a.i., water-soluble liquid; Sankyo Agro Ltd., Japan), choline chloride (30% a.i., water-soluble liquid; Agro Kanesho Ltd., Japan), IBA (0.4% a.i., water-soluble liquid; Bayer Crop Science Ltd., Japan), isoprothiolane (40% a.i., emulsifiable concentrate; Nihon Noyaku Ltd., Japan), ethephon (10% a.i., water-soluble liquid; Nissan Chemical Industries Ltd., Japan) and Cd (cadmium standard solution for atomic absorption spectrometry 1000; Cd-HNO₃; Kanto Chemical Ltd., Japan) were used. Two milliliters of distilled water and/or aqueous solutions of Cd, Cd+HMI, Cd+choline chloride, Cd+IBA, Cd+isoprothiolane and Cd+ ethephon were added to a glass vial (3 cm internal diameter and 6.5 cm depth) and then 6 newly germinated Japanese millet seeds (cv. Shiro-hie; Snow Brand Seed Ltd., Japan) were sown in the vial. The plant were then grown in an incubator (BIOTRON LH200; NK-SYSTEM Ltd., Japan) maintained at 25°C with a 24 hr daylight length of 1.5 klx for 7 days,

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and shoot and root lengths were measured.

Experiment 2. Mitigating effects of HMI and Calcium (Ca)

Two milliliters of distilled water and/or aqueous solutions of Cd, HMI, Ca (calcium standard solution for atomic absorption spectrometry 100; $\text{CaCO}_3\text{--HNO}_3$; Kanto Chemical Ltd., Japan), Cd + HMI and Cd + Ca were added to the glass vial and then 6 just-germinated Japanese millet seeds were sown in the vial. The plants were grown for 7 days in the incubator under the same conditions as mentioned above, and then shoot and root lengths were measured.

Experiment 3. Effects of HMI and Ca on Cd accumulation

Eight milliliters of distilled water and/or aqueous solutions of Cd, Cd + HMI and Cd + Ca were added to a glass vial (6 cm internal diameter and 6 cm depth) and then 20 just-germinated Japanese millet seeds were sown in the vial. The plants were grown for 10 days under the same conditions as mentioned above. After the dry weight of the shoots and roots was measured, Cd content ($\mu\text{g/plant}$) was measured by the following method. Twenty shoot and root samples were digested with 10 ml HNO_3 (Guaranteed reagent; Kanto Chemical Ltd., Japan) and filled up to 10 ml with distilled water. Then, the Cd concentration of the digested solutions was analyzed by atomic absorption spectrometry (AA-670; Shimadzu Ltd., Japan). Finally, Cd content was calculated by the following equation. Cd content ($\mu\text{g/plant}$) = Cd conc. ($\mu\text{g/ml}$) $\times$ 10 (ml)/20 (plants).

Statistical Analyses

All experiments were replicated three times and significant differences among treated plots were determined using the Tukey–Kramer test.

Results and Discussion

Experiment 1. Screening of 5 PGRs in terms of the mitigating effect

Shoot and root lengths (% of control) at $4.4 \times 10 \mu\text{mol/l}$ of Cd alone were 43.9% and 4.0%, respectively. With a combination of Cd + HMI ($4.4 \times 10 + 1.0 \times 10^2$, 5.0×10^2 , $1.0 \times 10^3 \mu\text{mol/l}$), suppression of shoot and root length recovered from 57.6 to 84.9% and from 5.0 to 18.4%, respectively, as % of the control basis. However, at the highest HMI concentration ($2.5 \times 10^3 \mu\text{mol/l}$), shoot and root lengths were to 70.5% and 11.4%, respectively, and were lower than $4.4 + 1.0 \times 10^3 \mu\text{mol/l}$. For the combination of Cd + choline chloride ($4.4 \times 10 + 7.1 \times 10$, $3.6 \times 10^2 \mu\text{mol/l}$), although shoot length recovered slightly to 46.9% and 47.6%, no obvious mitigating effect was observed in root length. For Cd + IBA, Cd + isoprothiolane and Cd + ethephon, no and/or little mitigating effect was observed and instead the shoot and root growth of the plant was suppressed with an increase in the rate of PGRs (Table 1).

Experiment 2. Mitigating effects of HMI and Ca

From Experiment 1, it was found that HMI showed the highest mitigating effect on Cd stress of Japanese millet; therefore, the

Table 1. Shoot and root length of *Echinochloa frumentacea* exposed to Cd and/or PGRs.

Cd ($\mu\text{mol/l}$)	PGRs ($\mu\text{mol/l}$)	% of control	
		Shoot length	Root length
Hymexazole (HMI)			
4.4×10	—	43.9 a	4.0 a
4.4×10	1.0×10 ²	57.6 b	5.0 ab
4.4×10	5.0×10 ²	61.8 c	6.0 b
4.4×10	1.0×10 ³	84.9 e	18.4 d
4.4×10	2.5×10 ³	70.5 d	11.4 c
Choline chloride			
4.4×10	—	43.9 bc	4.0 b
4.4×10	7.1	42.6 ab	4.8 c
4.4×10	7.1×10	46.9 cd	4.0 b
4.4×10	3.6×10 ²	47.6 d	3.8 ab
4.4×10	7.1×10 ²	40.6 a	3.2 a
Indolebutyric acid (IBA)			
4.4×10	—	43.9 bc	4.0 bc
4.4×10	4.9×10 ^{−2}	45.1 c	4.0 bc
4.4×10	4.9×10 ^{−1}	44.3 bc	4.1 c
4.4×10	4.9	41.3 ab	3.6 b
4.4×10	4.9×10	38.5 a	2.9 a
Isoprothiolane			
4.4×10	—	43.9 d	4.0 c
4.4×10	3.4×10 ^{−2}	42.0 cd	3.7 bc
4.4×10	3.4×10 ^{−1}	37.5 bc	3.4 b
4.4×10	3.4	35.7 b	3.5 b
4.4×10	3.4×10	26.6 a	2.8 a
Ethephon			
4.4×10	—	43.9 bc	4.0 b
4.4×10	6.9×10 ^{−1}	45.0 c	4.5 b
4.4×10	6.9	41.1 b	4.4 b
4.4×10	6.9×10	37.7 a	3.3 a
4.4×10	6.9×10 ²	38.0 a	2.8 a

Data are presented as the means ($n=3$). The means followed by the same letter within a column are not significantly different by Tukey–Kramer test ($p < 0.05$).

mitigating effect was evaluated in detail and was compared to that of Ca, which has been demonstrated to be a Cd mitigator.^{15,16} Shoot lengths at 2.2×10 and $4.4 \times 10 \mu\text{mol/l}$ of Cd alone were 73.0% and 48.5%, respectively. In contrast, although shoot

length at the highest rate of HMI ($5.0 \times 10^3 \mu\text{mol/l}$) alone was suppressed to 74.1%, it recovered at 1.0×10^2 , 5.0×10^2 , 1.0×10^3 and $2.5 \times 10^3 \mu\text{mol/l}$ HMI compared to that of the untreated control; however, with a combination of Cd+HMI ($2.2 \times 10 + 1.0 \times 10^2$, 5.0×10^2 , 1.0×10^3 , 2.5×10^3 , $5.0 \times 10^3 \mu\text{mol/l}$), shoot length exceeded the value of Cd alone ($2.2 \times 10 \mu\text{mol/l}$). In particular, for the combination of Cd+HMI ($2.2 \times 10 + 1.0 \times 10^3 \mu\text{mol/l}$), shoot suppression caused by $2.2 \times 10 \mu\text{mol/l}$ of Cd was considerably mitigated to 92.5%. Furthermore, when the plants were treated with a high concentration of Cd ($4.4 \times 10 \mu\text{mol/l}$), inhibition of shoot length was clearly mitigated by addition of HMI ranging from 1.0×10^2 to $5.0 \times 10^3 \mu\text{mol/l}$, and the greatest mitigating effect was observed in combination with $1.0 \times 10^3 \mu\text{mol/l}$ of HMI.

On the other hand, root lengths at 2.2×10 and $4.4 \times 10 \mu\text{mol/l}$ of Cd alone were 46.3% and 4.2%, respectively. Even though root lengths at 1.0×10^2 and $5.0 \times 10^3 \mu\text{mol/l}$ of HMI alone were greater than that of the untreated control, root length was suppressed at 2.5×10^3 and $5.0 \times 10^3 \mu\text{mol/l}$ of HMI alone. In contrast, with the combination of Cd+HMI ($2.2 \times 10 + 1.0 \times 10^2$, 5.0×10^2 , $1.0 \times 10^3 \mu\text{mol/l}$), root lengths exceeded the values of Cd alone. When the plant was exposed to a high concentration of

Cd ($4.4 \times 10 \mu\text{mol/l}$), root length with Cd alone (4.2%) increased to 23.9% by the addition of $1.0 \times 10^3 \mu\text{mol/l}$ HMI (Table 2).

Regarding the effect of Ca on Cd stress, shown as follows, shoot lengths increased to 113.4% at $1.3 \times 10^2 \mu\text{mol/l}$, but no significant effects were observed at 1.3×10 , 2.5×10 , and $2.5 \times 10^2 \mu\text{mol/l}$. In contrast to the shoot length, root growth suppression was observed from a Ca concentration as low as $1.3 \times 10 \mu\text{mol/l}$ and was a remarkably only 44.4% of the control at $2.5 \times 10^2 \mu\text{mol/l}$. Shoot length with Cd+Ca ($2.2 \times 10 + 1.3 \times 10$, $1.3 \times 10^2 \mu\text{mol/l}$) and root length with Cd+Ca ($2.2 \times 10 + 1.3 \times 10$, 2.5×10 and $1.3 \times 10^2 \mu\text{mol/l}$) exceeded the values of Cd alone. For the combination of Cd+Ca ($4.4 \times 10 + 1.3 \times 10^2 \mu\text{mol/l}$), shoot suppression caused by Cd was mitigated, but no mitigation effect was observed for root lengths in any combination (Table 3).

Experiment 3. Comparison of the effects of HMI and Ca on Cd accumulation

(1) *Effects of HMI and Ca on dry weight of Japanese millet*
Dry weights of shoots and roots (mg/plant; mean $\pm$ SD) were 1.15 ± 0.13 and 1.05 ± 0.09 for the untreated control and 0.85 ± 0.13 and 0.87 ± 0.07 for $2.2 \times 10 \mu\text{mol/l}$ Cd alone. When the plant was treated with Cd+HMI ($2.2 \times 10 + 1.0 \times 10^3$, $2.5 \times 10^3 \mu\text{mol/l}$), the dry weights of shoots were 0.87 ± 0.10 and 0.98 ± 0.08 and dry weights of roots were 1.08 ± 0.14 and 0.95 ± 0.05 , respec-

Table 2. Shoot and root length of *Echinochloa frumentacea* exposed to Cd and/or HMI.

Cd ($\mu\text{mol/l}$)	HMI ($\mu\text{mol/l}$)	Shoot length (cm)	Root length (cm)
—	—	2.93 ± 0.12 (100%) b	2.59 ± 0.35 (100%) c
—	1.0×10^2	3.23 ± 0.06 (110.2%) b	2.87 ± 0.32 (110.8%) c
—	5.0×10^2	3.22 ± 0.48 (109.9%) b	2.75 ± 0.31 (106.2%) c
—	1.0×10^3	3.04 ± 0.18 (103.8%) b	1.46 ± 0.17 (56.4%) b
—	2.5×10^3	3.22 ± 0.37 (109.9%) b	0.64 ± 0.15 (24.7%) a
—	5.0×10^3	2.17 ± 0.14 (74.1%) a	0.17 ± 0.03 (6.6%) a
2.2×10	—	2.14 ± 0.30 (73.0%) a	1.20 ± 0.17 (46.3%) b
2.2×10	1.0×10^2	2.24 ± 0.10 (76.5%) ab	1.66 ± 0.17 (64.1%) b
2.2×10	5.0×10^2	2.60 ± 0.11 (88.7%) ab	2.29 ± 0.22 (88.4%) c
2.2×10	1.0×10^3	2.71 ± 0.20 (92.5%) b	1.68 ± 0.29 (64.9%) b
2.2×10	2.5×10^3	2.66 ± 0.11 (90.8%) b	1.14 ± 0.24 (44.0%) b
2.2×10	5.0×10^3	2.47 ± 0.19 (84.3%) ab	0.23 ± 0.02 (8.9%) a
4.4×10	—	1.42 ± 0.13 (48.5%) a	0.11 ± 0.01 (4.2%) a
4.4×10	1.0×10^2	1.82 ± 0.13 (62.0%) ab	0.12 ± 0.02 (4.6%) a
4.4×10	5.0×10^2	2.25 ± 0.16 (76.8%) bc	0.19 ± 0.06 (7.3%) a
4.4×10	1.0×10^3	2.42 ± 0.03 (82.6%) c	0.62 ± 0.22 (23.9%) b
4.4×10	2.5×10^3	2.34 ± 0.28 (79.9%) c	0.30 ± 0.03 (11.6%) a
4.4×10	5.0×10^3	2.39 ± 0.13 (81.6%) c	0.16 ± 0.01 (6.2%) a

Data are presented as the means $\pm$ standard deviation ($n=3$). The means followed by the same letter within a column are not significantly different by Tukey–Kramer test ($p < 0.05$).

Table 3. Shoot and root length of *Echinochloa frumentacea* exposed to Cd and/or Ca.

Cd ($\mu\text{mol/l}$)	Ca ($\mu\text{mol/l}$)	Shoot length (cm)	Root length (cm)
—	—	2.93 ± 0.12 (100%) a	2.59 ± 0.35 (100%) a
—	1.3×10	2.81 ± 0.26 (95.9%) a	2.20 ± 0.48 (84.9%) a
—	2.5×10	2.68 ± 0.39 (91.5%) a	2.12 ± 0.78 (81.9%) a
—	1.3×10^2	3.29 ± 0.10 (113.4%) a	2.34 ± 1.01 (90.3%) a
—	2.5×10^2	2.89 ± 0.34 (98.6%) a	1.15 ± 0.31 (44.4%) a
—	1.3×10^3	0.00 ± 0.00 (0.00%) b	0.00 ± 0.00 (0.00%) b
2.2×10	—	2.14 ± 0.30 (73.0%) a	1.20 ± 0.17 (46.3%) a
2.2×10	1.3×10	2.34 ± 0.45 (79.9%) a	1.75 ± 0.93 (67.6%) a
2.2×10	2.5×10	2.16 ± 0.11 (73.7%) a	1.54 ± 0.78 (59.5%) a
2.2×10	1.3×10^2	2.41 ± 0.14 (82.3%) a	1.82 ± 0.18 (70.3%) a
2.2×10	2.5×10^2	2.12 ± 0.16 (72.4%) a	1.20 ± 0.28 (46.3%) a
4.4×10	—	1.42 ± 0.13 (48.5%) a	0.11 ± 0.01 (4.2%) b
4.4×10	1.3×10	1.40 ± 0.25 (47.8%) a	0.11 ± 0.00 (4.2%) a
4.4×10	2.5×10	1.61 ± 0.12 (54.9%) a	0.11 ± 0.00 (4.2%) a
4.4×10	1.3×10^2	1.80 ± 0.10 (61.4%) a	0.11 ± 0.01 (4.2%) ab
4.4×10	2.5×10^2	1.51 ± 0.22 (51.5%) a	0.11 ± 0.00 (4.2%) a

Data are presented as the means $\pm$ standard deviation ($n=3$). The means followed by the same letter within a column are not significantly different by Tukey–Kramer test ($p < 0.05$).

tively. In the case of $4.4 \times 10 \mu\text{mol/l}$ of Cd, this tendency became clearer, e.g., the dry weight of roots increased to 0.89 ± 0.06 by the addition of $2.5 \times 10^3 \mu\text{mol/l}$ HMI, which was 2.3 times the value of Cd alone; however, the dry weights of shoots and roots at Cd+Ca ($4.4 \times 10 + 2.5 \times 10 \mu\text{mol/l}$) were similar to or higher than that of Cd alone (Fig. 1).

(2) Effects of HMI and Ca on Cd accumulation in Japanese millet

Cd contents ($\mu\text{g/plant}$; mean $\pm$ SD) in shoots and roots at $2.2 \times 10 \mu\text{mol/l}$ of Cd alone were 0.19 ± 0.02 and 0.30 ± 0.02 , respectively. For the combination of Cd+HMI ($2.2 \times 10 + 2.5 \times 10^3 \mu\text{mol/l}$), Cd content in shoots decreased to 0.09 ± 0.01 but almost no changes in Cd content in roots were observed; however, for Cd+Ca ($2.2 \times 10 + 2.5 \times 10 \mu\text{mol/l}$), there were no marked changes in Cd content in either shoots or roots (Fig. 2).

From these results, it was found that HMI mitigates Cd stress and affects Cd accumulation in Japanese millet. HMI is known to be metabolized to 3-(β -D-glucopyranosyloxy)-5-methylisoxazole (*O*- β -glucoside) and 2-(β -D-glucopyranosyl)-5-methyl-4-isoxazoline-3-one (*N*- β -glucoside), and the former shows fungicidal

effects and the latter promotes the root growth of plants.¹⁵⁾ Thus, there is a possibility that the mitigating effect of HMI on Cd stress depends on *N*- β -glucoside. Moreover, it is supposed that HMI mitigates Cd stress by enhancing the biosynthesis and translocation of indole acetic acid in plants.¹²⁾

With regard to the mitigating effects of calcium, Harada *et al.*¹⁶⁾ reported that when wild tobacco (*Nicotiana tabacum* L.) was grown for 3 weeks in a medium containing 0.2 mM Cd and 30 mM Ca, the shoot length increased to 2.3 times more than with Cd alone, and Noguchi *et al.*¹⁷⁾ reported that when rice seeds (*O. sativa* cv. Kosihikari) were soaked for 1 hr in mixed aqueous solutions of 2×10^{-5} to 2×10^{-1} eq/l Cd and 2×10^{-6} , 2×10^{-4} and 2×10^{-2} eq/l Ca, suppression of root respiration was mitigated. However, in contrast to the results mentioned above, no definite mitigating effects were observed for Ca in the present experiment.

Moreover, the ratio of Cd contents in shoots and roots (S/R) was measured in order to understand the translocation of Cd from roots to shoots in Japanese millet. The S/R values under Cd alone ($2.2 \times 10 \mu\text{mol/l}$) and at Cd+HMI ($2.2 \times 10 + 1.0 \times 10^3$, $2.5 \times 10^3 \mu\text{mol/l}$) were 0.63, 0.50, and 0.35, respectively. Similarly, the S/R values for Cd alone ($4.4 \times 10 \mu\text{mol/l}$) and Cd+HMI

Fig. 1. Dry weight (mg/plant) of *Echinochloa frumentacea* exposed to Cd, HMI and/or Ca. Error bars represent the standard deviation of the mean ($n=3$). ^{a)} $\mu\text{M} = \mu\text{mol/l}$.

Fig. 2. Shoot and root cadmium content ($\mu\text{g/plant}$) of *Echinochloa frumentacea* exposed to cadmium. Error bars represent the standard deviation of the mean ($n=3$). Means followed by the same letter within a column were not significantly different by Tukey–Kramer test ($p < 0.05$). ^{a)} $\mu\text{M} = \mu\text{mol/l}$.

($4.4 \times 10 + 1.0 \times 10^3$, $2.5 \times 10^3 \mu\text{mol/l}$) were 1.54, 0.58, and 0.53, showing that HMI clearly reduced S/R values. An S/R value of Cd content <1.0 indicated that HMI may suppress the translocation of Cd from roots to stems and leaves. With regard to the physiological effects of HMI on plants, it has been reported that HMI increases the oxidation-reduction capability in roots of rice (*O. sativa* cv. Nipponbare) and promotes the accumulation of compounds such as calcium (CaO), magnesium (MgO), and silicates (SiO_2)¹²⁾; however, the reason for HMI suppression of Cd translocation from the roots to shoots in Japanese millet is unclear. In contrast to HMI, the S/R value of Cd+Ca was almost the same as that of Cd alone; hence, Ca probably has almost no effect on Cd translocation.

Additional studies comparing of different Cd forms and Cd metabolism are necessary for further understanding of the mechanisms of the mitigating effects of HMI.

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