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Cadmium Tolerance and Accumulation in a Halophyte *Salicornia europaea* as a New Candidate for Phytoremediation of Saline Soils

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Abstract

The ability of a halophyte *Salicornia europaea* to accumulate cadmium (Cd) in the shoots was examined in laboratory conditions. Aseptically growing seedlings were transferred at 7 days after sowing onto a sterilized nutrient medium solidified with 0.2% Gellan Gum, and cultivated for 21 days in a growth chamber. In the presence of 0.3 M NaCl in the medium, the growth of the shoots was not influenced by the addition of Cd to the medium at the concentrations up to 0.8 mM. At the addition of 0.2 and 0.8 mM $CdCl_2$, the amount of Cd in the plant shoots was 2.2 ± 0.1 and 7.2 ± 0.8 μmol Cd g^{-1} dry weight, respectively. The more NaCl in the range of 0.1 to 0.4 M was added to the culture medium, the significantly less amount of Cd per g dry weight was accumulated in the shoots, though the total amount of Cd accumulated in a plant shoot was not affected except at 0.4 M NaCl addition. If the plants were grown for 5 weeks on a sterilized saline soil instead of the nutrient medium, they accumulated Cd in the shoots at the concentration of roughly half of those in the shoots cultured on the nutrient medium when compared at the same doses. We propose here that *S. europaea* is a new candidate plant species for using in effective phytoremediation of Cd-contaminated saline soils.

Key words: cadmium, glasswort, halophyte, phytoremediation, *Salicornia*

INTRODUCTION

Cadmium (Cd) is one of the toxic heavy metals for humans causing severe illness such as Itai-itai disease in Japan (Inaba *et al*. 2005). Soils naturally contain a slight amount of Cd, but could be heavily contaminated through leakage from mines and refineries of metals, industrial wastes, and phosphorus fertilizers (McLaughlin *et al.* 1996). As Cd coexists in several ores, soils contaminated with Cd have been widely distributed near the lower reaches of rivers where zinc, copper, or silver mines were located. Consequently, salt marshes and seaside sediments on estuaries tend to act as sinks for the derived Cd (Caçador *et al.* 2000, Wang & Liu 2003). Marine products, such as seaweeds, shellfishes, and fishes grown at the contaminated sites would take and eventually accumulate Cd. Codex Alimentarius Commission of FAO/WHO adopted in 2006 that maximum standards of Cd concentrations in polished rice and marine shellfish were 0.4 mg kg^{-1} and 2.0 mg kg^{-1}, respectively (Codex 2006).

One of the countermeasures against excessive accumulation of Cd in food is to reduce the concentration of Cd in the contaminated sites. Using the natural capacity of particular species of plants to absorb Cd through the roots and accumulate it in aerial parts is an attractive way for soil rehabilitation, because the phytoextraction technique is suitable for large area, inexpensive, and not destructive to environment, compared with physicochemical and engineering techniques (Makino *et al*. 2006). Several

species of plants have been reported to hyperaccumulate heavy metals (Baker *et al*. 1999).

These hyperaccumulators, however, are sensitive to high salinity and waterlogged conditions. They cannot be used for phytoextraction of Cd from salt marshes or seaside sediments at estuaries on industrialized sites where heavy metals tend to be contaminated. Successful performance of phytoextraction depends on selecting plant species that is well adapted to specific contaminated sites.

Halophytes are adapted to the highly salty environments. Most of them develop vacuoles in cell where metals are accumulated to be segregated from cytosol. *Sesuvium portulacastrum* and *Atriplex halimus* have been reported to accumulate 200 – 300 μg and 800 μg Cd per g dry matter from nutrient media containing 100 μM Cd, respectively (Ghnaya *et al*. 2005, Lutts *et al*. 2004). However, Cd severely inhibited the growth of these halophytes even at 100 μM Cd in culture medium. And they are not adapted to waterlogged soils.

A glasswort, *Salicornia europaea*, is an annual halophyte species belonging to the Chenopodiaceae and is adapted to the salt marshes at the seashore (Fig. 1). The previous study on the salt tolerance of *S. europaea* showed that this halophyte is tolerant to high concentration of NaCl up to 0.4 mol L^{-1} in the nutrient medium and has the great capacity to accumulate Na^+, K^+, Mg^{2+}, and Ca^{2+} in the shoot (Ozawa *et al*. 2007). These metals seem to be sequestered in the well-developed vacuoles, which could be a good reservoir for heavy metals. The present study aims at estimating the capacity of this halophyte to accumulate Cd in the shoot from nutrient media and soils supplemented with Cd.

Fig. 1. *Salicornia europaea* at the seashore in the Seto Inland Sea at August 28, 2002. After germinating in April, the plant of the species reaches 30 cm tall in August.

MATERIALS AND METHODS

Plant material and germination

The seeds of *Salicornia europaea* were soaked in tap water for 6 h, and sterilized in 200 g L^{-1} calcium hypochlorite for 30 min. They were sowed on Murashige-Skoog agar plates (Murashige & Skoog 1962) modified with nitrogen concentration of 6 mM (1.88 mM KNO_3 and 2.06 mM NH_4NO_3) and supplemented with 0.3 M NaCl. Glycine, nicotinic acid, pyridoxine, thiamine, and *myo*-inositol were excluded from the medium. The pH of the medium was adjusted to 5.8 with KOH. The medium was designated as MSM-6N-Na in this study.

Plant cultivation

Seeds on the MSM-6N-Na agar plates were germinated for 7 days in a growth chamber (26° C; 140 μmol m^{-2} s^{-1}, 16 h light-8 h dark). Four 7-d old seedlings were transferred onto 70 ml of MSM-6N-Na solidified with 0.2% (w/v) Gellan Gum in a plastic pot ("AGRIPOT", Iwaki Glass, Tokyo, Japan, 6 cm in diameter, 11 cm in height). For cultivation on soil, the 7-d old seedlings were transferred onto 70 g of a gardening soil ("Koushitsu-Akadama, Small grain", Kanuma Kohsan, Tochigi, Japan) in the plastic pot (8 seedlings per pot). The soil was supplied with 40 mL of artificial seawater (ASW; Wako Pure Chemical Industries, Inc., Osaka, Japan) supplemented with 0.94 mM KNO_3 and 1.03 mM NH_4NO_3, and then autoclaved before transplanting. No more water was supplied to the soil. Plants on MSM-6N-Na and soil were grown in the growth chamber for 28 and 42 days after sowing, respectively.

Measurements of growth and metal concentrations

At harvest, the fresh and dry weights of plant shoots were measured, and the dried samples were decomposed for metal determination in 5:3 (v/v) HNO_3-H_2O_2 at 130°C for 2 h. Na^+, K^+, Mg^{2+}, Ca^{2+}, and Cd^{2+} in the digest were determined by using a flame or a graphite furnace atomic absorption spectrometer (Z-8270; Hitachi High-Tech Co, Japan). Cl^- and SO_4^{2-} in the ASW were determined by using a multi-channel capillary electrophoresis measuring system (CAPI-3300; Otsuka Electronics Co., Ltd, Osaka, Japan). The other metals and As were determined by using ICP optical emission spectrometer (SPS5000, Seiko Instruments Inc., Japan). Chlorophylls in the shoots were determined by the method of Mackinney (Mackinney 1941). After pulling the plants out from the media, roots were washed by dipping into distilled water, the length of tap root was measured on a paper towel, and then the roots were dried for metal determination. After growing plants, the pH of the MSM-6N-Na medium was obtained by measurement with a Horiba Model pH meter. The pH of the soils was obtained by the glass electrode method after mixing 20 g of soil with 50 ml of distilled water.

Computational procedure

The speciation of Cd in the MSM-6N-Na and the ASW was calculated using PHREEQC version 2 (Parkhurst & Appelo 1999).

RESULTS AND DISCUSSION

To examine the toxicities of heavy metals on *S. europaea*, we transferred the 7 d-old seedlings onto the MSM-6N-Na pots supplemented with NH_4VO_3, $CrCl_3$-$6H_2O$, $MnCl_2$-$4H_2O$, $FeSO_4$-$7H_2O$, $CoSO_4$-$7H_2O$, $NiCl_2$-$6H_2O$, $CuSO_4$-$5H_2O$, $ZnSO_4$-$7H_2O$, As_2O_3, Na_2MoO_4, $CdCl_2$-$2.5H_2O$, $InCl_3$-$4H_2O$, Na_2WO_4, $HgCl_2$, or $PbCl_2$, and then cultured them for further 21 days. Approximate concentrations at which V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Mo, Cd, In, W, Hg, and Pb caused 50% inhibition of growth in the fresh weight of *S. europaea* were 0.1, 1, 8<, 0.2, 1.5, 0.5, 0.2, 2, 0.05, 1, 2, 0.3, 0.5, 0.01, and 1 mM, respectively. *S. europaea* was particularly sensitive to As and Hg, compared with the other metals examined here. Manganese did not inhibit the plant growth even at 8 mM. The effect of higher concentration of Mn was not examined, as the MSM-6N-Na supplemented with more than 10 mM Mn was not solidified with 0.2% Gellan Gum. Cadmium, though more toxic than Zn, did not significantly influence the fresh weight and dry weight of the shoots at the concentrations up to 0.8 mM (Fig. 2). Root growth was more sensitive to

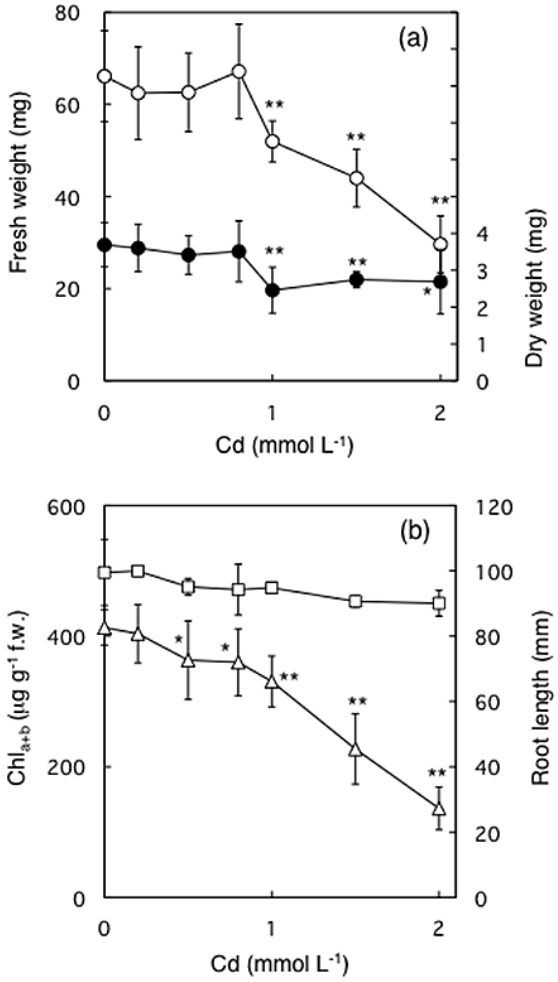

Fig. 2. Effect of the addition of $CdCl_2$ to nutrient media on the growth characteristics, (a) fresh weight (○) and dry weight (●) of shoot and (b) chlorophyll concentration in shoot (□) and length of tap root (△), of *S. europaea*. The 7-d old seedlings were transferred to the MSM-6N-Na media in plastic pots supplemented with $CdCl_2$, and plants at 21 days after transplanting were harvested for measuring the growth parameters. The values are the means ± SE (vertical bars) from 8 plants. Asterisks indicate significant differences with each control according to Student's *t*-test (*: $P < 0.05$, **: $P < 0.01$).

Cd than shoot growth. The elongation of taproots was retarded by the addition of over 0.5 mM Cd. Chlorophyll concentration in the shoots did not significantly changed in the range of 0 to 2.0 mM Cd.

Accumulation of Cd in the shoots of *S. europaea* grown on the MSM-6N-Na was examined at 21 days after the addition of $CdCl_2$ to the culture medium (Fig. 3). The amount of Cd in the shoots depended on the concentration of Cd in the medium. At the addition of 0.2 and 0.8 mM $CdCl_2$, the amount of Cd in the plant shoots was 2.2 ± 0.1 and 7.2 ± 0.8 μmol Cd g^{-1} d.w., respectively, and it reached 12.1 ± 2.4 μmol Cd g^{-1} d.w. at the addition of 1.5 mM $CdCl_2$. At the dose of 1 mM, significantly more Cd was accumulated in the roots (29.0 ± 5.9 μmol g^{-1} d.w.) than in the shoots. The Cd concentrations in shoot reached approximately 9 μmol Cd g^{-1} d.w. after 2 weeks of exposure to 1 mM Cd, and gradually increased to reach the maximum level during further 5 weeks of cultivation (Fig. 4). Eventually, approximately 15 μg of Cd were accumulated into a shoot after 7 weeks of exposure in the MSM-6N-Na medium.

Plants that can accumulate more than 0.9 μ mol Cd g^{-1} d.w. are considered as hyperaccumulators for Cd (Brooks 1998). The results in this study show that *S. europaea* is a hyperaccumulator for Cd. *Sesuvium portulacastrum* and *Atriplex halimus* have been reported to accumulate 1.8 – 2.7 μmol and 7.1 μmol Cd per g dry matter from nutrient media containing 100 μM Cd, respectively (Ghnaya *et al*. 2005, Lutts *et al*. 2004). However, Cd severely inhibited the growth of these halophytes even at 100 μM Cd in culture medium. And they are not adapted to waterlogged soils. *S. europaea* is a new candidate plant for phytoremediation of salt marshes contaminated with Cd.

Previous report shows that *S. europaea* is a euhalophyte growing optimally in the artificial medium supplemented with NaCl ranging in concentration from 0.1 to 0.3 M (Ozawa *et al*. 2007). When supplied with 1 mM $CdCl_2$, however, the optimal range of NaCl concentrations

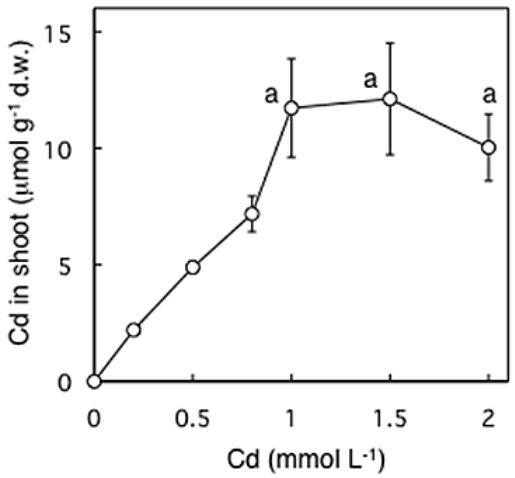

Fig. 3. Effect of the addition of $CdCl_2$ to nutrient media on the accumulation of Cd in the shoots of *S. europaea*. The 7-d old seedlings were transferred to the MSM-6N-Na media in plastic pots supplemented with $CdCl_2$, and plants at 21 days after transplanting were harvested for measuring the Cd concentrations. The values are the means ± SE (vertical bars) from 8 plants. Means followed by the same letter are not significantly different at $P < 0.05$ according to Student's *t*-test.

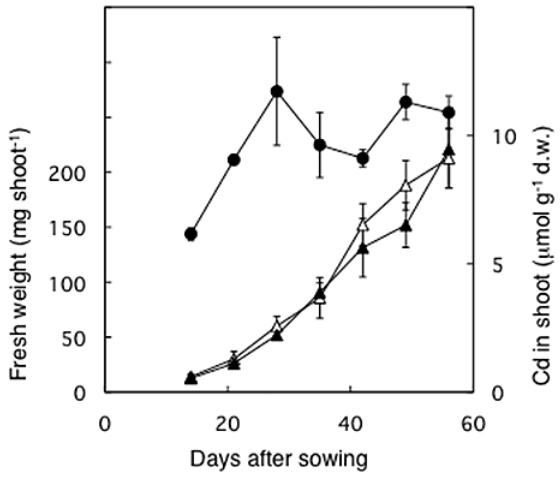

Fig. 4. Concentration of Cd in the shoots of *S. europaea* over 8 weeks of cultivation on the MSM-6N-Na medium. Fresh weight of shoot when not supplied with Cd (△), and supplied with 1 mM $CdCl_2$ at 7 days after sowing (▲). ●, concentration of Cd in the plant shoots when supplied with 1 mM $CdCl_2$ at 7 days after sowing. The values are the means ± SE (vertical bars) from 8 plants.

for growth became narrow to be from 0.2 to 0.3 M (Fig. 5). As the medium without NaCl was not solidified with 0.2% Gellun Gum when supplemented with 1 mM $CdCl_2$, we were not able to know in this study what amount the plants accumulated Cd from MSM-6N without NaCl. In the MSM-6N with NaCl, however, the Cd concentration in the shoots depended on the concentration of NaCl supplied to the medium. Other investigations with different salt-tolerant or salt-sensitive species plants have shown that increased salinity enhances the Cd concentrations in the plant shoots (Smolders *et al*. 1998; McLaughlin *et al*. 1994; Ghnaya *et al*. 2007). Cd concentrations in the shoots of *S. europaea*, in contrast, did not show such a correlation with external NaCl concentrations (Fig. 5). The more NaCl in the range of 0.1 to 0.4 M was supplied to the MSM-6N, the significantly less amount of Cd per g dry weight was accumulated in the shoots, though the total amount of Cd accumulated in a plant shoot were not affected except at 0.4 M NaCl. The lower amount of Cd in a plant shoot grown at 0.4 M NaCl may be due to the decrease in water content of the shoot by the salt stress.

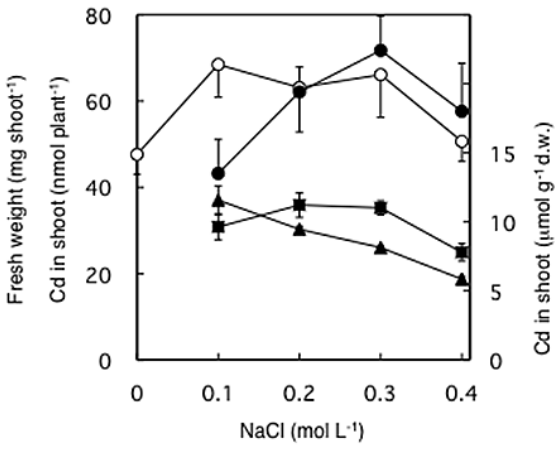

Fig. 5. Effect of the concentration of NaCl in the nutrient medium on the fresh weight of shoot and the Cd concentration in shoots of *S. europaea*. Plants at 28 days after sowing were harvested for measuring the fresh weight of shoot: ○, plants not supplied with Cd; ●, plants supplied with 1 mM $CdCl_2$ at 7 days after sowing. Concentrations of Cd in shoot supplied with 1 mM $CdCl_2$ at 7 days after sowing were expressed with two different units: ▲, mmol g^{-1} d.w.; ■, nmol $plant^{-1}$. The values are the means ± SE (vertical bars) from 8 plants.

Cd^{2+} forms chloride complexes with Cl^- in aqueous solutions, $CdCl^+$, $CdCl_2^\circ$, $CdCl_3^-$, and $CdCl_4^{2-}$, as the following equations (Lindsay 1979).

$Cd^{2+} + Cl^- \rightleftarrows CdCl^+$ ($\log K^\circ = 1.98$)
$Cd^{2+} + 2\ Cl^- \rightleftarrows CdCl_2^\circ$ ($\log K^\circ = 2.60$)
$Cd^{2+} + 3\ Cl^- \rightleftarrows CdCl_3^-$ ($\log K^\circ = 2.40$)
$Cd^{2+} + 4\ Cl^- \rightleftarrows CdCl_4^{2-}$ ($\log K^\circ = 2.50$)

These equilibrium constants indicate that, in the presence of 0.3 M NaCl, the concentrations of Cd^{2+}, $CdCl^+$, $CdCl_2^\circ$, $CdCl_3^-$, and $CdCl_4^{2-}$ in water supplemented with 1 mM $CdCl_2$ are estimated at 0.013, 0.383, 0.479, 0.091, and 0.034 mM, respectively. As the concentrations of the other anions that make complexes with Cd^{2+} are negligibly low compared with Cl^-, we can obtain similar estimates of concentrations of the chemical species of Cd in MSM-6N-Na. When the speciation of Cd in the MSM-6N-Na was calculated using PHREEQC version 2 (Parkhurst & Appelo 1999), the concentrations of Cd^{2+}, $CdCl^+$, $CdCl_2^\circ$, and $CdCl_3^-$ were estimated at 0.020, 0.406, 0.354, and 0.047 mM (activity), respectively. Plants uptake metals mainly in the form of cations into the cells. The complexation of Cd^{2+} and Cl^- may cause the decrease in Cd concentration in plant shoots grown at higher concentrations of NaCl.

Regardless of Cd supplementation, the initial pH ranging from 4 to 7 of the MSM-6N-Na medium did not affect the growth of *S. europaea* with respect to the fresh weight of plant shoots (Table 1). The root growth, however, was significantly inhibited by the Cd supplementation especially at the initial pH of 6 or above. This may explain that the maximum Cd accumulation in the plant shoots was achieved at the initial pH below 6. When the seedlings were transferred to MSM-6N-Na media adjusted to higher than 6, the amount of Cd accumulated in the plant shoots during 3 weeks of cultivation, as well as Zn, decreased depending on the initial pH of the media. The similarity of accumulation of Cd and Zn may be due to a presence of any common transport systems in the plant cell membrane (Papoyan & Kochian 2004).

The concentrations in the shoots of the other

Table 1. Effect of initial pH of the MSM-6N-Na on the growth of *S. europaea* and metal contents of the shoots.
Seven d-old seedlings were transferred onto MSM-6N-Na media whose pH was adjusted before preparation. Plants were harvested at 28 days after sowing. Each value is the mean ± standard error (n = 4). Differences between the values followed by the same letter for a parameter of growth and metal content are not significant as determined by Student's *t*-test ($P < 0.05$).

	Cd addition	Initial pH of the medium				
		4.2	5.3	6.0	7.1	8.1
pH of media at 28 DAS	- Cd	3.8	3.8	3.9	5.6	6.1
	+ Cd	3.7	3.7	3.7	4.7	6.0
f. w. (mg shoot^{-1})	- Cd	66±16 a	64±9 a	63±7 a	59±7 a	45±7
	+ Cd	64±9 a	61±11 a	57±8 ab	62±9 a	53±6 b
Root length (mm shoot^{-1})	- Cd	63±7 ab	82±11 d	100±8 e	114±10	95±15 de
	+ Cd	54±10 f	55±9 acf	68±8 b	61±4 a	55±4 cf
Metal in the shoot						
Na (mmol g^{-1} d. w.)	- Cd	9.42±1.18 ab	8.66±0.02 b	8.63±0.31 b	7.75±0.75 ab	7.46±0.06 a
	+ Cd	9.19±0.22 b	8.94±0.12 b	8.96±0.33 b	7.26±0.96 ab	8.73±1.01 ab
K (mmol g^{-1} d. w.)	- Cd	0.22±0.02 b	0.28±0.03 ab	0.30±0.03 abd	0.45±0.03 c	0.40±0.03 cd
	+ Cd	0.32±0.14 abc	0.30±0.01 a	0.47±0.07 ac	0.39±0.07 abc	0.34±0.06 abc
Mg (mmol g^{-1} d. w.)	- Cd	61.6±3.0 a	72.1±1.3 c	87.5±7.2 cd	104.8±10.9 ce	91.9±4.6 de
	+ Cd	49.2±2.2 b	56.5±4.9 a	59.4±3.7 ab	55.6±4.4 ab	63.3±3.1 a
Ca (mmol g^{-1} d. w.)	- Cd	39.2±1.8 a	34.7±1.0 a	33.9±4.5 ab	28.5±0.4 b	33.3±2.2 ab
	+ Cd	36.4±2.9 ab	35.1±2.1 ac	33.6±7.1 ab	29.1±0.3 bc	35.8±3.2 ab
Zn (mmol g^{-1} d. w.)	- Cd	1.5±0.2 ab	1.5±0.1 a	1.7±0.2 ac	1.6±0.1 a	1.4±0.2 ab
	+ Cd	2.5±1.1 ab	2.3±0.6 ab	2.0±0.1 c	0.9±0.1 b	1.0±0.2 b
Cd (mmol g^{-1} d. w.)	+ Cd	11.8±1.9 a	13.5±0.5 a	10.6±1.4 a	7.3±1.0 b	4.5±0.5 b

metals examined in this study, Na, K, and Ca, were virtually unaltered in the initial pH range, while the Mg accumulation was significantly inhibited by the Cd supplementation to the media especially at higher pHs. In the MSM-6N-Na medium, divalent cations form insoluble complexes such as carbonates and phosphates whose amounts in the medium are expected higher at more alkaline pHs except for magnesium salts (Lindsay 1979). Besides the retardation of root growth, increase in the insoluble forms of Cd would occur in alkaline environment. However, as all of the media were acidified during 3 weeks of plant cultivation, and the pH of the media reached to below 6, formation of these insoluble complexes should not practically occur.

S. europaea seedlings were more tolerant to Cd when cultured on soils compared to MSM-6N-Na (Fig. 6). The supplementation of Cd in ASW at the concentration up to 4 mM did not significantly affect the growth of plant shoots. It may be partly because of lower amount of Cd in the plants. In spite of the longer period of cultivation, Cd concentrations in the shoots were roughly half of those in the shoots cultured on MSM-6N-Na when compared at the same doses.

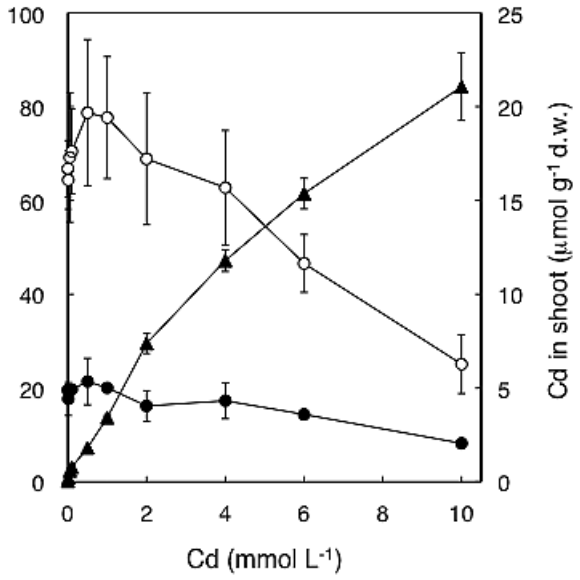

Fig. 6. Growth and Cd accumulation of *S. europaea* grown on soils added with $CdCl_2$. The 7-d old seedlings germinated on the MSM-6N-Na agar plates were transferred to the soils in plastic pots supplemented with $CdCl_2$, and plants at 35 days after transplanting were harvested for measuring weight and Cd concentration. ○, fresh weight of shoot; ●, dry weight of shoot; ▲, Cd concentration in shoot. The values are the means ± SE (vertical bars) from 8 plants.

Since the redox potential of cadmium (E° (Cd^{2+}/Cd) = - 0.402 V) is sufficiently low, Cd(s) is not expected in the MSM-6N-Na medium and the soil used in this study. The ASW used in this study contained Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and SO_4^{2-} at the concentrations of 390, 12, 42, 8, 428, and 23 mM, respectively. As described above, most of Cd is expected to exist in the forms of $CdCl^+$ and $CdCl_2^\circ$ in ASW as well as in MSM-6N-Na. Calculation using by the PHREEQC version 2 revealed that major species of Cd in the ASW were $CdCl_2^\circ$ (42.7 %), $CdCl^+$ (36.6 %), and $CdCl_3^-$ (7.6 %) besides minor ones such as Cd^{2+} (1.4 %) and $CdSO_4^\circ$ (0.32 %).

The solubility of Cd in soil solution and its availability for plants can be affected by soil pH (Street *et al*. 1978). The pH(H_2O) of the soil used in this study was 6.7 at the start of cultivation, and decreased to 5.9 during 5 weeks of cultivation. Acidification of such levels would not be enough to solubilize the adsorbed Cd (Makino *et al*. 2006).

This study shows that *S. europaea* is able to accumulate as much amount of Cd as the other hyperaccumulators ever reported, and is tolerant to Cd up to the concentration of 0.8 mM in a culture medium. In addition, its upright form of shoot would facilitate a dense cultivation in a field as well as the subsequent harvest procedures. In a preliminary experiment, we cultivated 4,000 plants of *S. europaea* per m^2 of a salt marsh uncontaminated with Cd. Dry weight of the shoots reached 4 kg per m^2 during 3 months of cultivation. These results along with the results in this study indicate that we may be able to recover approximately 100 mg of Cd from the salty field of 1 m^2 contaminated with Cd at 10 ppm. We propose here that *S. europaea* is a new candidate plant species for using in effective phytoremediation of Cd-contaminated salty soils, though the increase in the availability to plants of Cd^{2+} and $CdCl^+$ adsorbed to the soil is the subject for a future study.

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