Cadmiun Uptake by *Pinus resinosa* Ait. Pollen and the Effect on Cation Release and Membrane Permeability

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**ABSTRACT**

Cadmium uptake by red pine (*Pinus resinosa* Ait.) pollen from a graded series of Cd²⁺ solutions (0 to 2.88 microequivalents per 50 milligrams pollen) and its effect on membrane integrity were examined by atomic absorption spectroscopy. Uptake was strongly dependent on Cd²⁺ concentration and was limited to adsorption and cation exchange in pollen walls during a 3-hour measurement period. Good correlation between measured Cd²⁺ uptake and that predicted by the Langmuir and Freundlich isotherm equations indicated the adsorptive nature of Cd²⁺ uptake. While substantial quantities of Ca²⁺ and Mg²⁺ were released by exchange mechanisms concurrent with Cd²⁺ uptake, there was no evidence for leakage of cations due to membrane impairment as indicated by a poor correlation between Cd²⁺ uptake and K⁺ efflux. Virtually all Cd²⁺ removed from solution was freely exchangeable with 0.5 millimolar CaCl₂ and demonstrated that Cd²⁺ did not readily enter pine pollen but was adsorbed on the pollen wall. Ultraviolet transmission spectra of treatment solutions and analyses of phosphate and reducing sugar efflux also indicated that the potential toxicity of Cd²⁺ to pollen germination and germ tube elongation was not the result of membrane damage.

Studies have shown that Cd²⁺ can increase membrane permeability (12, 13). However, the effects of Cd²⁺ and other heavy metals show considerable variation in their influence on membrane integrity. For example, Miller and McCallan (8) showed that Ag⁺ and Cu²⁺ caused phosphate leakage from *Aspergillus niger* conidia, but that Hg²⁺, Zn²⁺ and Cd²⁺ had little effect.

Preliminary experiments have shown Cd²⁺ inhibition of pollen germination (1). However, the mechanism of this toxicity is not known, and the study reported here was initiated to investigate Cd²⁺ uptake and its effect on pollen membrane permeability with the objective of determining the nature of Cd²⁺ toxicity to pine pollen.

**MATERIALS AND METHODS**

Cd²⁺ Sorption and Desorption. Fifty mg of red pine (*Pinus resinosa* Ait.) pollen, which had been stored for 9 months at 4°C, were weighed into each of 21 modified 12-ml disposable poly-ethylene syringes. The syringe tips were reamed to fit the hollow plastic shaft of a cotton swab snugly. One end of the swab was removed just behind the cotton tip and the shaft of the swab was put through the syringe tip so that the remaining cotton tip was on the inside of the syringe. The shaft of the swab and the syringe tip were sealed with Parafilm. The combination of syringe and cotton swab was an efficient filter to keep pollen in the syringe and to allow for rapid changing of solutions used to suspend the pollen. The dry pollen was equilibrated in the syringes at 29°C for 20 min in a growth chamber before subsequent treatment to reduce variation in germinating ability and respiratory activity (3, 10). After temperature equilibration, each syringe was filled at 50-s intervals with 10 ml of glass-redistilled H₂O. The syringes were placed horizontally on a reciprocating shaker and oscillated approximately 60 strokes/min at 29°C. The initial water was washed to include removal of microorganisms, which are plentiful on stored pollen (4, 10), and to remove contaminating materials released from both viable and nonviable pollen during storage.

After 20 min the water was removed from the syringes and replaced with a Cd²⁺ solution. A completely random design with seven Cd²⁺ treatments and three replications was used. Six treatments used live pollen exposed to either 0.00, 0.18, 0.36, 0.72, 1.44, or 2.88 μeq Cd²⁺/50 mg pollen prepared from CdCl₂-2.5 H₂O and glass-redistilled H₂O. These concentrations were selected because they were detectable by atomic absorption spectroscopy and covered a range known to moderately and completely inhibit red pine pollen germination. The seventh treatment was 1.44 μeq Cd²⁺/50 mg pollen killed by heating at 80°C for 20 min. Emptying and refilling syringes was performed at 20-min intervals for 3 h. After 3 h, pollen in each syringe was rinsed twice with 5 ml of redistilled H₂O and the syringe filled with 10 ml of 0.5 mM CaCl₂. Pollen was treated three times for 20 min each with the Ca²⁺ solution to determine if the Cd²⁺ removed from solution by pollen was exchangeable with a competing cation. Solutions collected for each treatment-time combination were stored in acid-washed polyethylene test tubes for subsequent analysis of Cd²⁺, Ca²⁺, Mg²⁺, and K⁺ by atomic absorption spectroscopy using standard techniques. Ca²⁺, Mg²⁺, and K⁺ were chosen for analysis because: (a) they were likely inorganic cations to be exchanged during Cd²⁺ uptake by pine pollen; (b) they comprise the majority of inorganic cations found in pine pollen (6, 11); and (c) increases in the release of these cations would suggest membrane damage from Cd²⁺ treatment. Ca²⁺, Mg²⁺, and Cd²⁺ contents were determined for all samples collected during both the uptake and desorption portions of this study. K⁺ content was measured for the first 80 min of the uptake period because during subsequent time periods K⁺ concentrations were too low to determine accurately.
The UV transmission spectrum of at least one replicate for each Cd²⁺ concentration-time combination was determined from 180 to 370 nm with a Perkin-Elmer model 270 double beam spectrophotometer. The spectrophotometer was blanked at 370 nm using redistilled H₂O. Samples examined in the visible region (370–750 nm) showed little variation in transmission spectra except near the UV.

P content of each Cd²⁺-time combination sample was determined by the method of Murphy and Riley (9). Four ml of sample were thoroughly mixed with 1 ml of Murphy-Riley reducing solution and allowed to stand for 15 min. A was read at 885 nm. P content was expressed as μg P released/ml of solution. KH₂PO₄ was used to construct a standard curve.

Reducing sugar content of each solution was determined by the orcinol method (5). One ml of each sample was added to 2.5 ml of concentrated H₂SO₄ and 0.5 ml of 2% (w/v) orcinol in 20% (v/v) H₂SO₄. Samples were thoroughly mixed and heated for 30 min at 80 C in a forced-air oven. A was determined at 540 nm. Glucose was used to construct a standard curve, and all A readings were converted to glucose equivalents released/ml of sample.

The pH of all solutions collected was determined with a Beckman Zeromatic pH meter equipped with a combination electrode. Measurement of pH was performed after atomic absorption analysis to prevent K⁺ contamination of the samples from the pH electrode.

Significant differences among treatment means were examined by the Student-Newman-Keuls sequential range test using the P < 0.05 level of significance.

Cd²⁺ uptake and cation release for each sample time also were used to determine the constants of the Freundlich and Langmuir adsorption isotherm equations to determine if uptake could be described by an adsorptive mechanism. The Freundlich equation is empirically derived and takes the form M = Kcⁿ, where M = the amount adsorbed, K and n are constants, and c = the equilibrium concentration of absorbate present in solution (2). The Langmuir isotherm is expressed as M = abp/(1 + ap), where M = amount adsorbed, a and b are constants, and p = the equilibrium concentration of the absorbate (14).

While both equations classically use the equilibrium concentration of adsorbed material as the independent variable (14), equations using the applied Cd²⁺ concentrations as the independent variables also were calculated. The constants for the Freundlich equation were calculated from the slope and intercept of logarithmic plots of either Cd²⁺ remaining in solution or the applied Cd²⁺ concentration. The constants for the Langmuir equation were calculated in the same manner using reciprocal plots. All data were expressed as μeq/50 mg pollen and only data points for live pollen having values greater than zero were included in the calculations.

Germination. Fifty mg of pollen, after equilibration to room conditions and washing with distilled H₂O, were suspended in 10 ml of each Cd²⁺ concentration used in the uptake study. Each of these suspensions was used to obtain three samples of 3 ml each. These samples were germinated in sealed glass shell-vials (15 x 75 mm), with constant shaking in a growth chamber at 29 C. After 48 h, two counts of 100 pollen grains each from each sample were used to estimate Cd²⁺ inhibition of germination. A pollen grain was considered germinated when the germ tube extended 1 mm or more.

RESULTS

Cd²⁺ Uptake. It is evident that Cd²⁺ uptake by live pollen was dependent on both the applied concentration and the duration of exposure (Fig. 1). The higher the Cd²⁺ treatment the more rapid the initial accumulation and the faster saturation or equilibrium occurred. Total Cd²⁺ uptake expressed as a percentage of total exposure was 81.5, 61.0, 35.9, 19.0, 13.8, and 29.7 for the 0.18, 0.36, 0.72, 1.44, 2.88, and 1.44 D² treatments, respectively.

Adsorption Isotherms and Cd²⁺ Uptake. Both Freundlich and Langmuir equations were applicable to Cd²⁺ uptake by red pine pollen, and predicted Cd²⁺ uptake values very similar to those measured (Fig. 1). The correlation coefficients were all between 0.96 and 0.99 and highly significant (P < 0.01). Over-all, the Langmuir equation predictions showed the best correlations with measured uptake values although uptake was overestimated in the 1.44 μeq/50 mg pollen treatment and underestimated in the 2.88-μeq treatment. The Freundlich equation tended to overestimate uptake in the 0.36- and 0.72-μeq treatments. Application of these equations indicated adsorption to external structures was a major factor in Cd²⁺ accumulation by pine pollen.

2 Abbreviation: D: dead.
Cation Release. The initial 20-min distilled-H₂O wash removed 1.38, 0.08, and 0.07 μeq/50 mg pollen of K⁺, Ca²⁺, and Mg²⁺, respectively. This cation release was attributed to surface washing of physiologically intact pollen (capable of germination) and leaching from defective pollen (incapable of germination). Heat-treated pollen released 4.15, 0.19, and 0.76 μeq/50 mg pollen of K⁺, Ca²⁺, and Mg²⁺, respectively.

The pH of the initial distilled H₂O wash for the live pollen was 5.4 to 5.6 and varied less than 0.2 pH units, regardless of treatment, during the entire study. The pH of the distilled H₂O wash for the dead pollen averaged 5.1, but was similar to the live pollen after 20-min exposure to Cd²⁺. There were no clear relationships between Cd²⁺ treatment, time, and pH change since replicates within a given Cd²⁺-time combination often showed the maximum variation in pH.

The μeq of Cd²⁺ accumulation associated with Ca²⁺ release (Fig. 2) after subtraction of Ca²⁺ release from the control was 3.1, 30.1, 34.1, 40.1, 55.1, and 18.7% for the 0.18, 0.36, 0.72, 1.44, 2.88 and 1.44 D treatments, respectively. Ca²⁺ release was highly correlated with both Cd²⁺ uptake and the applied Cd²⁺ concentration during all sampling periods.

In contrast to Ca²⁺, Mg²⁺ (Fig. 3) was more easily released from the 1.44 D treatment, exceeding by 87% the μeq of Ca²⁺ released in response to the 2.88 μeq/50 mg pollen treatment. There was a close relationship between Mg²⁺ release and Cd²⁺ uptake by dead pollen (Figs. 1 and 3). After correction for release from the control, Mg²⁺ release explained 12.6, 22.4, 34.9, 38.6, 29.0, and 59.0% of Cd²⁺ uptake in 0.18, 0.36, 0.72, 1.44, 2.88 and 1.44 D treatments, respectively. As found for Ca²⁺, maximum Mg²⁺ release in response to the various Cd²⁺ treatments was related to time and Cd²⁺ uptake.

In contrast to Ca²⁺ and Mg²⁺, there was no lag in the release of K⁺ (Fig. 4). This suggested that washing was the principal mechanism of K⁺ release. K⁺ release accounted for 1.4, 6.5, 1.8, 1.1, 4.1, and 22.4% of Cd²⁺ uptake by pollen in the 0.18, 0.36, 0.72, 1.44, 2.88, and 1.44 D treatments, respectively.

Total cation release (μeq Ca²⁺ + μeq Mg²⁺ + μeq K⁺) in response to Cd²⁺ treatment is shown in Figure 5. Most of total cation release occurred in the first 100 min concurrent with the most Cd²⁺ uptake. The amount of Cd²⁺ uptake explained by total cation release, after adjustment for the control, for the 0.00, 0.18, 0.36, 0.72, 1.44, 2.88, and 1.44 D treatments was 18.8, 62.0, 58.0, 56.8, 71.1, 69.3, and 89.3%, respectively. Cation release from the

1.44 D treatment showed that more cations were available in the live pollen than were actually released.

Release of P. The release of P from live pollen treatments was detected only in the initial distilled H₂O wash, 6 μg P/50 mg pollen, and the first 20-min incubation period, 2.5 μg P/50 mg pollen. The heat-treated pollen released 11.0 μg P/50 mg pollen in the distilled H₂O wash and small (<0.5 μg P/50 mg pollen) but detectable amounts for the next 60 min. While heat treatment increased the release of P there was no relationship between phosphorus release and Cd²⁺ treatment. Separate analysis of pollen from the same lot used in this study for P content by digestion in H₂SO₄ showed a total P content of 110.0 μg P/50 mg pollen.

Release of Reducing Sugars. There was no evident relationship between Cd²⁺ treatment and the release of reducing sugars. Heat-treated pollen released greater amounts of reducing groups than live pollen and the initial distilled H₂O wash removed greater amounts of reducing groups than subsequent periods of incubation. For example, the initial water washes removed 28 glucose
equivalents/ml from 50 mg of live pollen and 224 glucose equivalents/ml from heat-treated pollen. After 20-min exposure to 2.88 μeq Cd²⁺ live pollen solutions contained approximately 8.3 glucose equivalents/ml whereas the 1.44 D treatment solutions contained two times as much (16.7 glucose equivalents/ml).

Release of UV Absorbing Materials. No differences in release of UV absorbing materials could be associated with Cd²⁺ treatment. While the spectra in Figure 6 are more evenly spaced than those found for some of the other treatments, the shape and relative position of the spectra for live pollen treatments remained similar. The spectra at 0 time represents transmission of a 20-min water wash prior to treatment with 0.72 μeq Cd²⁺. The family of spectra for the 1.44 D treatment (the 20-min tracing is included in Fig. 6) also showed increasing transmission with time, but had greater over-all absorption than that for live pollen treatments.

Cd²⁺ Desorption. Table 1 summarizes data from three 20-min washes with 0.5 mM CaCl₂ (10 μeq Ca²⁺/50 mg pollen). K release is not presented since the concentrations released were too low for detection with the techniques used.

Approximately 60% of the accumulated Cd²⁺ was exchanged by Ca²⁺ during the first 20-min wash with decreasing concentrations released during subsequent washes (Table I). The 0.18, 0.36, 0.72, 1.44, 2.88, and 1.44 D treatments exchanged 98.4, 86.6, 93.9, 104.5, 94.5, and 86.7%, respectively, of the total Cd²⁺ accumulated after 60-min exposure to Cd²⁺. Cd²⁺ not accounted for by Ca²⁺ exchange may represent Cd²⁺ that entered the pollen grain. It is also possible that small amounts of Cd²⁺ were removed in the two short distilled H₂O washes performed before desorption began, or perhaps the Cd²⁺ not desorbed would have been released if additional or longer Ca²⁺ treatments were included.

Further evidence for association of both Cd²⁺ and Ca²⁺ uptake with cation exchange is shown by Mg²⁺ release during the Ca²⁺ washes (Table I). The lower the applied Cd²⁺ level the more Mg²⁺ was available for release by Ca²⁺. For example, Mg²⁺ release from the control was significantly lower than release from the 0.18 μeq Cd²⁺ treatment at 3 h (Fig. 3) and therefore more Mg²⁺ was available in the control pollen for exchange in the Ca²⁺ washes. Table I also shows that significant amounts of Mg²⁺ were available for Ca²⁺ exchange in the higher Cd²⁺ treatments. This suggests that Cd²⁺ accumulation and cation release in the uptake period were due to an equilibrium between the Cd²⁺ that could be bound at a given concentration and the cations already present.

Germination. Pollen germination in the absence of Cd²⁺ was 60%. Treatment with 0.18 μeq Cd²⁺/50 mg pollen reduced germination to 28% and no pollen germinated at higher Cd²⁺ concentrations.

The amount of Cd²⁺ causing complete inhibition of germination, approximately 0.36 μeq/50 mg pollen, was accumulated during the first 20-min uptake period (Fig. 1). There apparently is little relation between the amount of Cd²⁺ pollen can remove from solution and the inhibition of germination.

Table 1. Release of Cd²⁺ and Mg²⁺ and Uptake of Ca²⁺ (μeq/50 mg Pollen) during Desorption in 0.5 mM CaCl₂ by Pollen Previously Exposed to Various Concentrations of Cd²⁺ for 180 Min

<table>
<thead>
<tr>
<th>Desorption Time</th>
<th>Previous Cd²⁺ Treatment (μeq/50 mg Pollen)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>min</td>
<td></td>
</tr>
<tr>
<td>Cd²⁺ released</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0 a(a)</td>
</tr>
<tr>
<td>40</td>
<td>0 a(a)</td>
</tr>
<tr>
<td>60</td>
<td>0 a(a)</td>
</tr>
<tr>
<td>Mg²⁺ released</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.773 a(a)</td>
</tr>
<tr>
<td>40</td>
<td>1.055 a(b)</td>
</tr>
<tr>
<td>60</td>
<td>1.176 a(b)</td>
</tr>
<tr>
<td>Ca²⁺ uptake</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2.645 a(a)</td>
</tr>
<tr>
<td>40</td>
<td>3.593 a(a)</td>
</tr>
<tr>
<td>60</td>
<td>4.092 a(a)</td>
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</tbody>
</table>

FIG. 6. Representative UV transmission spectrum of 0.72 μeq Cd²⁺ solutions added to 50 mg red pine pollen at 20-min intervals for 180 min. Time is indicated on the graph. The 1.44 D (20 min) spectrum is for a 1.44-μeq Cd²⁺ solution added to heat-killed pollen 20 min after initiation of the experiment.
DISCUSSION

There was a relationship between time, Cd²⁺ concentration, and Ca²⁺ release not readily apparent from Figure 2 that is consistent with Ca²⁺ being released by exchange. While Ca²⁺ release in the 0.00 and 0.18 μeq/50 mg pollen treatments was relatively constant throughout the study, the maximum μeq of Ca²⁺ released within a time period for the other treatments was associated with the applied Cd²⁺ concentration. For the 0.36 μeq treatment, maximum Ca²⁺ release occurred during the 60- and 80-min sample periods, while for the 0.72-, 1.4-, and 2.88-μeq treatments maximum release was in the 40-, 40-, and 20-min sample periods, respectively. These results suggest that Cd²⁺ does not exchange with Ca²⁺ until more available sites are filled.

Cd²⁺ uptake not associated with total cation release (Fig. 5) can be accounted for by one or more of the following considerations: (a) Cd²⁺ may have entered the pollen; (b) other inorganic cations known to be present in pine pollen (i.e., Na⁺, Mn²⁺, Zn²⁺) but in lesser amounts than Ca²⁺, Mg²⁺, or K⁺ may have been exchanged (6); (c) uptake may have been associated with the release of organic cations from the exterior or interior of the pollen; or (d) Cd²⁺ was bound by Van der Waals forces. The most likely explanation for the remainder of the Cd²⁺ uptake is Van der Waals attraction between Cd²⁺ and the extensive pollen wall. McWilliam (7) found that pine pollen had an inherent negative charge. The percentage of Cd²⁺ uptake unexplained by exchange for other cations in the 0.18-μeq treatment is 81.2 which means that 1.06 μeq Cd²⁺/50 mg pollen was bound by other than an exchange mechanism. If this is assumed to have been physically bound and if it is assumed that 1.06 μeq Cd²⁺/50 mg pollen also was physically adsorbed by pollen in the other Cd²⁺ treatments, an additional 54.1, 46.1, 43.5, 30.0, and 27.6% of uptake in the 0.36, 0.72, 1.44, 2.88, and 1.44 D treatments, respectively. Uptake greater than 100% was experimental error.

Accumulated Cd²⁺ was freely exchangeable with 10 μeq Ca²⁺, except in the 2.88-μeq treatment. Further treatment with Ca²⁺ probably would have desorbed more Cd²⁺ from pollen in this treatment. Mg²⁺ also was released by desorption with Ca²⁺ and this release was inversely related to the concentration of Cd²⁺ in the uptake treatment. These results indicate that Cd²⁺ was adsorbed to pollen walls with no detectable entry into the pollen.

During this short term experiment there was no indication of membrane damage by Cd²⁺. While the elevated release of Ca²⁺ and Mg²⁺ could be associated with Cd²⁺ uptake and cation exchange, the efflux of K⁺, P compounds, reducing sugars, and UV adsorbing materials was not related to Cd²⁺ treatment and was probably the result of surface washing and leaching.

Heat-killed pollen treated with 1.44 μeq Cd²⁺ showed greater Cd²⁺ accumulation and greater release of cations (except Ca²⁺), P compounds, reducing sugars, and UV adsorbing materials than live pollen. Most of the accumulated Cd²⁺ was exchangeable by Ca²⁺. Results with dead pollen indicated that total disruption of the pollen membrane did not occur; however, the lack of Ca²⁺ release and the increased release of UV absorbing materials suggested some wall modification occurred.

Germination of pollen in Cd²⁺ solutions revealed no correlation between toxicity and the quantity of Cd²⁺ adsorbed. Since membrane damage was not associated with Cd²⁺ toxicity, perhaps minute quantities of Cd²⁺ entered the pollen and were toxic. Cadmium may have interfered with membrane integrity at a later stage of the germination process. i.e. after germ tube initiation. These possibilities warrant further study.

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