



Article

# Ectomycorrhizal Fungal Strains Facilitate Cd<sup>2+</sup> Enrichment in a Woody Hyperaccumulator under Co-Existing Stress of Cadmium and Salt

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**Abstract:** Cadmium (Cd<sup>2+</sup>) pollution occurring in salt-affected soils has become an increasing environmental concern in the world. Fast-growing poplars have been widely utilized for phytoremediation of soil contaminating heavy metals (HMs). However, the woody Cd<sup>2+</sup>-hyperaccumulator, *Populus × canescens*, is relatively salt-sensitive and therefore cannot be directly used to remediate HMs from salt-affected soils. The aim of the present study was to testify whether colonization of *P. × canescens* with ectomycorrhizal (EM) fungi, a strategy known to enhance salt tolerance, provides an opportunity for affordable remediation of Cd<sup>2+</sup>-polluted saline soils. Ectomycorrhization with *Paxillus involutus* strains facilitated Cd<sup>2+</sup> enrichment in *P. × canescens* upon CdCl<sub>2</sub> exposures (50 μM, 30 min to 24 h). The fungus-stimulated Cd<sup>2+</sup> in roots was significantly restricted by inhibitors of plasmalemma H<sup>+</sup>-ATPases and Ca<sup>2+</sup>-permeable channels (CaPCs), but stimulated by an activator of plasmalemma H<sup>+</sup>-ATPases. NaCl (100 mM) lowered the transient and steady-state Cd<sup>2+</sup> influx in roots and fungal mycelia. Noteworthy, *P. involutus* colonization partly reverted the salt suppression of Cd<sup>2+</sup> uptake in poplar roots. EM fungus colonization upregulated transcription of plasmalemma H<sup>+</sup>-ATPases (*PcHA4*, 8, 11) and annexins (*PcANN1*, 2, 4), which might mediate Cd<sup>2+</sup> conductance through CaPCs. EM roots retained relatively highly expressed *PcHAs* and *PcANNs*, thus facilitating Cd<sup>2+</sup> enrichment under co-occurring stress of cadmium and salinity. We conclude that ectomycorrhization of woody hyperaccumulator species such as poplar could improve phytoremediation of Cd<sup>2+</sup> in salt-affected areas.

**Keywords:** annexins; calcium-permeable channels; Cd flux; MAJ; NaCl; NAU; *Paxillus involutus*; *Populus × canescens*; PM H<sup>+</sup>-ATPase

## 1. Introduction

Cadmium ( $\text{Cd}^{2+}$ ) pollution presents a critical threat to ecological environment and human life [1–5]. The  $\text{Cd}^{2+}$  contamination occurring in salt-affected soils has become an increasing environmental concern in recent years [6–17]. Coastal areas are polluted by  $\text{Cd}^{2+}$  due to rapid urbanization and industrialization. Cadmium is mainly derived from wastewater discharged by electroplating, mining, smelting, fuel, battery and chemical industry [18]. In some coastal saline zones, soil heavy metal pollution also comes from sludge and sewage irrigation [19]. Mining activities cause the release and spread of both hazardous heavy metals (HMs) and soluble salts in inland regions [11]. The  $\text{Cd}^{2+}$  contamination in salt-affected soils complicates remediation processes [6,7]. Naturally occurring halophytes may be potentially useful for remediation and phytomanagement [6,20–23]. However, halophytic species are commonly characterized by slow growth and therefore low biomass production [24]. Poplar trees have been widely utilized for phytoremediation of soils and water resources contaminated with HMs, because of their fast-growth, large biomass and remarkable  $\text{Cd}^{2+}$  accumulation in shoots and below-ground [25–31]. Moreover, several poplars, e.g., *Populus tremula*, *P. × canescens*, are known  $\text{Cd}^{2+}$  hyperaccumulators [32,33] in terms of the buildup of heavy metals in aerial parts (i.e., 100 times higher than non-accumulators) [34–37]. However, despite its high ability to tolerate  $\text{Cd}^{2+}$  stress [29,33,38], *P. × canescens* is relatively salt-sensitive [39] and therefore cannot be directly utilized to remediate HMs from salt-affected soils. The use of salt-resistant poplar, *P. euphratica*, is also hindered because this species is relatively susceptible to  $\text{Cd}^{2+}$  stress [40–43]. Therefore, efficient phytomanagement of heavy metal-contaminated salt soils with fast-growing poplars requires increased abilities of the plants to deal with the ionic stress situations produced by heavy metals and salts [6].

Ectomycorrhization offers great potential and feasibility for remediation of cadmium-contaminated soils [44–50]. Ectomycorrhization is the formation of symbiosis of a soil fungus with plant roots, whereby the root tip is completely ensheathed by the fungal hyphae. The plant benefits from this interaction by improved mineral nutrition and health [51]. Colonization of roots of *P. × canescens* with *Paxillus involutus*, an ectomycorrhizal (EM) fungus, has been repeatedly shown to improve  $\text{Cd}^{2+}$  uptake and tolerance [48,52]. The association of *Populus canadensis* with *P. involutus* leads to a highly significant increase of  $\text{Cd}^{2+}$  uptake and root-to-shoot transport, thus enhancing the total  $\text{Cd}^{2+}$  extraction by *P. canadensis* [44]. *P. involutus* ameliorates the negative effects of  $\text{Cd}^{2+}$  on shoot and root growth and chlorophyll content of old needles in Norway spruce seedlings (*Picea abies*) [53]. A protective effect against  $\text{Cd}^{2+}$  toxicity in the host was observed in *Pinus sylvestris* colonized with *P. involutus* [54,55]. *P. involutus* strains have also been used for phytoremediation of other heavy metals. Inoculation with a lead ( $\text{Pb}^{2+}$ )-tolerant strain of *P. involutus* improves growth and  $\text{Pb}^{2+}$  tolerance of *P. × canescens* [56,57]. *P. involutus* decreases  $\text{Pb}^{2+}$  in roots and the translocation from the roots to the stems in Norway spruce (*Picea abies*) [58,59]. Similarly, *P. involutus* fungi act as a safety net that can immobilize large amounts of zinc, thus preventing transport to the host plant, *Pinus sylvestris* [60]. Moreover, ectomycorrhization of *P. × canescens* with *P. involutus* increases salt tolerance by maintaining nutrient uptake of  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{NO}_3^-$ , and improves  $\text{Na}^+$  homeostasis in the symbiotic associations [61–66]. Thus, it can be hypothesized that *P. involutus* could increase plant ability for  $\text{Cd}^{2+}$  enrichment in salt-affected soils. Arbuscular mycorrhizal fungi are able to enhance growth of pigeonpea (*Cajanus cajan*) by lowering  $\text{Cd}^{2+}$  content and strengthening antioxidant defense under NaCl and Cd stress [67]. Whether the ectomycorrhizal fungus *P. involutus* can mediate  $\text{Cd}^{2+}$  uptake under co-existing stress of NaCl and cadmium needs to be clarified by further experimental investigations.

Under cadmium stress, the *P. involutus*-facilitated  $\text{Cd}^{2+}$  influx is stimulated by plasma membrane (PM)  $\text{H}^+$ -ATPases in EM roots [48]. Upregulated transcription of the PM  $\text{H}^+$ -ATPase genes (*HA2.1* and *AHA10.1*) results in accelerated  $\text{Cd}^{2+}$  transport into roots of transgenic [38] and EM poplars [52]. Increased proton pumping activity and transcription of  $\text{H}^+$ -ATPases have also been observed in EM *P. × canescens* under salt stress [66].

H<sup>+</sup>-ATPases maintain a proton gradient across PM to drive the entry of Cd<sup>2+</sup> [38,48] and nutrient elements, such as K<sup>+</sup>, Ca<sup>2+</sup>, and NO<sub>3</sub><sup>-</sup>, in addition to promotion of Na<sup>+</sup>/H<sup>+</sup> antiport [64–66]. Moreover, the *P. involutus*-activated H<sup>+</sup>-pumps hyperpolarize the membrane potential, facilitating Cd<sup>2+</sup> influx via hyperpolarization-activated Ca<sup>2+</sup>-permeable channels (CaPCs) [48]. Although the *P. involutus*-stimulated H<sup>+</sup>-ATPase enhances Cd<sup>2+</sup> uptake under single stress of cadmium [48,52], little is known whether the fungi-activated H<sup>+</sup>-ATPase could improve Cd<sup>2+</sup> enrichment in combined stress of CdCl<sub>2</sub> and NaCl.

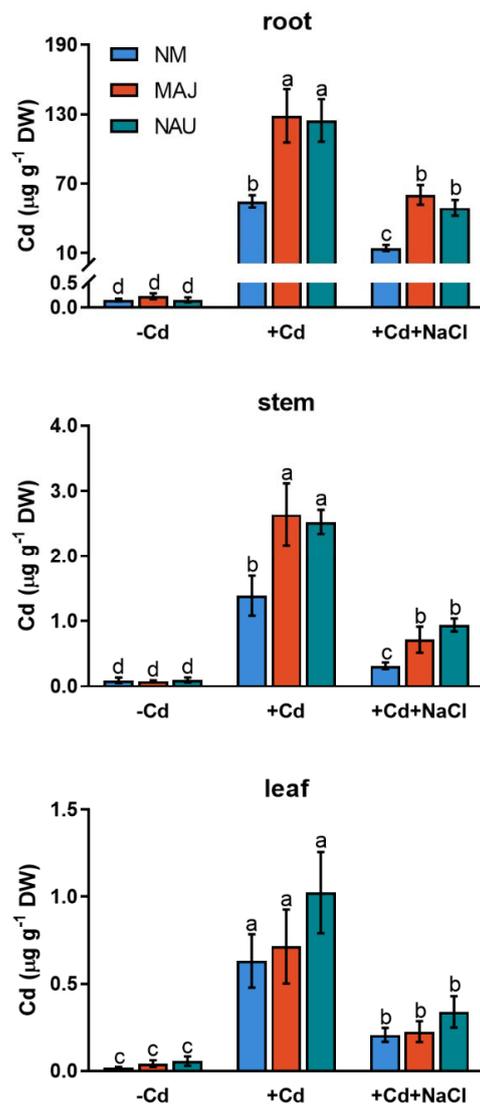
Cellular uptake of Cd<sup>2+</sup> also involves the PM CaPCs, as demonstrated for various species [38,41,48,68]. Plant annexins (ANNs) might serve as channels to allow the entry of Ca<sup>2+</sup> [69–76] or indirectly mediate Ca<sup>2+</sup> conductance [77,78]. Chen et al. suggested that OsANN4 mediates the transmembrane Cd<sup>2+</sup> influx along rice roots [73]. The *P. euphratica* annexin ANN1 facilitates Cd<sup>2+</sup> enrichment through CaPCs in roots of transgenic Arabidopsis [79]. *P. × canescens* colonization with *P. involutus* leads to Cd<sup>2+</sup> enrichment [52] due to stimulation of Cd<sup>2+</sup> influx via CaPCs [48]. Cadmium treatment results in increased transcript levels of annexins in maize (*ZmAnx9*, [80]), peanut (*ANNAh3*, [81]), and rice (*ANN4*, [73]). Whether *P. × canescens* annexins are affected by cadmium and contribute to Cd<sup>2+</sup> enrichment in *P. involutus* ectomycorrhizal associations needs to be investigated. Under sodium chloride salinity, competition between Na<sup>+</sup> and Cd<sup>2+</sup> for Ca<sup>2+</sup> ion channels reduced Cd<sup>2+</sup> uptake in *Amaranthus mangostanus* [82]. The salt effects on annexin-mediated Ca<sup>2+</sup> channels remain unclear in ectomycorrhizal roots under co-existing stress conditions of Cd<sup>2+</sup> and NaCl.

In this study, we examined the impact of ectomycorrhizal fungi on root Cd<sup>2+</sup> uptake under combined stress of salt and cadmium, aiming to elucidate the underlying mechanisms. We used two different *P. involutus* isolates, MAJ and NAU, for this study. Strain MAJ forms a complete ectomycorrhiza composed of a thick hyphal mantle ensheathing root tip and a typical Hartig net structure inside the roots for nutrient exchange, while strain NAU forms only the outer mantle [83]. We studied Cd<sup>2+</sup> uptake in the presence and absence of NaCl and analyzed gene expression of annexins because previous studies show that PeANN1 facilitates Cd<sup>2+</sup> enrichment through CaPCs [79]. *P. involutus* activates H<sup>+</sup>-pumps and hyperpolarizes membrane potential in EM roots [48,64,65]. Therefore, the PM H<sup>+</sup>-ATPases-promoted Cd<sup>2+</sup> flux was also verified in EM roots under salt stress. Our data reveal that *P. involutus* inoculation stimulates Cd<sup>2+</sup> influx under salt stress, resulting from the upregulated H<sup>+</sup>-ATPases and annexins in the ectomycorrhizal roots. Both MAJ and NAU conserved the Cd<sup>2+</sup> uptake capacities under co-occurring stresses of cadmium and salinity, regardless of the formation of Hartig net in the ectomycorrhizal symbioses.

## 2. Results

### 2.1. Cd<sup>2+</sup> Concentrations in Roots and Shoots of Ectomycorrhizal Poplars under NaCl Stress

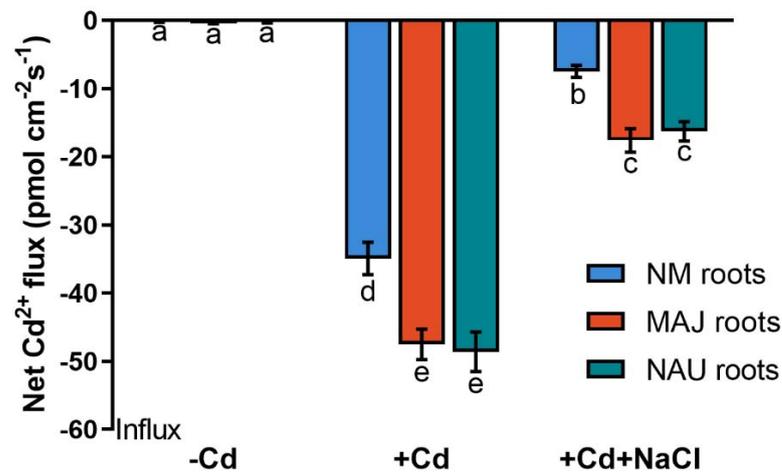
Cd<sup>2+</sup> concentrations were analyzed in roots, stems and leaves of NM and EM *P. × canescens* after 24 h exposure to CdCl<sub>2</sub> (50 μM) or combined stress of CdCl<sub>2</sub> and NaCl (100 mM). Under CdCl<sub>2</sub> stress, non-mycorrhizal (NM) roots displayed remarkably higher Cd<sup>2+</sup> concentrations than stem and leaves (Figure 1). Compared to NM plants, Cd<sup>2+</sup> concentrations were 0.8- to 1.4-fold higher in roots and stems of poplars colonized with *P. involutus* isolates, MAJ and NAU (Figure 1). However, the addition of NaCl (100 mM) significantly decreased Cd<sup>2+</sup> accumulation in roots and shoots of both NM- and EM-plants (Figure 1). Of note, EM-plants retained significantly higher Cd<sup>2+</sup> concentrations in roots and stems than NM poplars under salt stress (Figure 1). Therefore, EM fungi enhanced Cd<sup>2+</sup> enrichment in both root and aerial parts of *P. × canescens* under co-occurring stresses of cadmium and salinity.



**Figure 1.**  $\text{Cd}^{2+}$  concentrations in roots, stems and leaves of non-mycorrhizal (NM) and ectomycorrhizal (EM) *Populus × canescens* under cadmium and salt stress. Poplar plantlets inoculated with or without *Paxillus involutus* isolates (MAJ or NAU, 30 d), were hydroponically acclimated and subjected to 24 h of  $\text{CdCl}_2$  (0 or 50  $\mu\text{M}$ ) in combination with NaCl (0 or 100 mM). Mean values of  $\text{Cd}^{2+}$  concentrations in control (–Cd),  $\text{CdCl}_2$  stress (+Cd), and combined stress of  $\text{CdCl}_2$  and NaCl (+Cd + NaCl) are shown. Each column is mean  $\pm$  SD obtained from 3 individual plants. Statistically significant differences ( $p < 0.05$ ) among treatments are indicated with different letters (a–d).

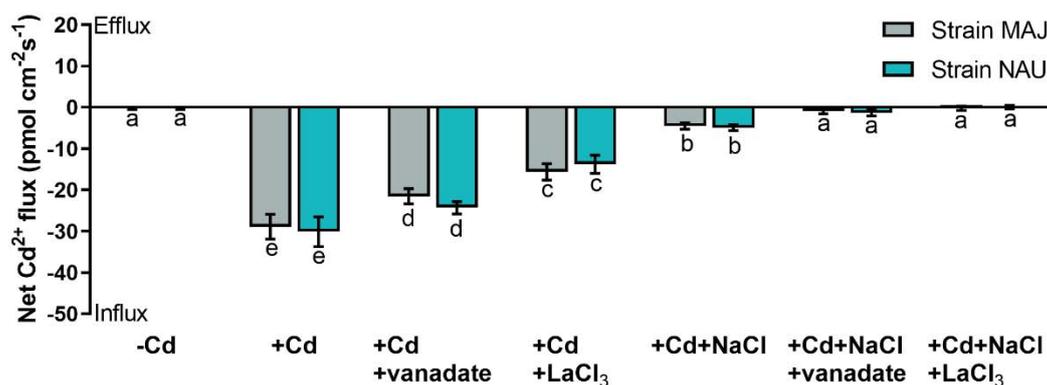
## 2.2. Steady-State $\text{Cd}^{2+}$ Influx in Ectomycorrhizal Poplar Roots and Fungal Mycelia under NaCl Stress

To determine whether the  $\text{Cd}^{2+}$  enrichment in EM *P. × canescens* resulted from the *P. involutus*-stimulated uptake,  $\text{Cd}^{2+}$  fluxes were examined in NM-, EM-roots and fungal mycelia under  $\text{CdCl}_2$  and NaCl stress.  $\text{CdCl}_2$  exposure (50  $\mu\text{M}$ , 24 h) resulted in an apparent  $\text{Cd}^{2+}$  uptake, 34.9  $\text{pmol cm}^{-2} \text{s}^{-1}$ , along NM-roots of the hyperaccumulator, *Populus × canescens* (Figures 2 and S1). EM-roots exhibited 36% to 39% higher  $\text{Cd}^{2+}$  fluxes than the NM-roots (Figure 2). The presence of NaCl (100 mM) significantly decreased the flux rates in both NM- and EM-roots but the EM-roots still exhibited 1.2–1.4-fold greater  $\text{Cd}^{2+}$  uptake than the NM-roots (Figures 2 and S1). The effect of NaCl on root  $\text{Cd}^{2+}$  fluxes resembles the trend of  $\text{Cd}^{2+}$  accumulation in salinized NM- and EM-roots (Figures 1 and 2).



**Figure 2.** Steady-state  $\text{Cd}^{2+}$  fluxes in non-mycorrhizal (NM) *Populus × canescens* and ectomycorrhizal (EM) roots under cadmium and salt stress. Poplar plantlets inoculated with or without *Paxillus involutus* isolates (MAJ or NAU, 30 d), were hydroponically acclimated and subjected to 24 h of  $\text{CdCl}_2$  (0 or 50  $\mu\text{M}$ ) in combination with NaCl (0 or 100 mM). Root tips were excised from EM- and NM-poplars and equilibrated for 30 min in measuring solution. Net fluxes of  $\text{Cd}^{2+}$  along root axis (100 to 2300  $\mu\text{m}$ ) were monitored at an interval of 200–300  $\mu\text{m}$  (Figure S1). Mean values of  $\text{Cd}^{2+}$  fluxes in control (–Cd),  $\text{CdCl}_2$  stress (+Cd), and combined stress of  $\text{CdCl}_2$  and NaCl (+Cd + NaCl) are shown.  $\text{Cd}^{2+}$  flux was not detectable in salt controls that were treated without  $\text{CdCl}_2$ . Each column is mean  $\pm$  SD obtained from 5 individual plants. Statistically significant differences ( $p < 0.05$ ) among treatments are indicated with different letters (a–e).

Fungal hyphae of the two tested *P. involutus* isolates, MAJ and NAU, showed a drastic  $\text{Cd}^{2+}$  influx, 28.9–30.1  $\text{pmol cm}^{-2} \text{s}^{-1}$ , under  $\text{CdCl}_2$  treatment (50  $\mu\text{M}$ , 24 h, Figure 3). NaCl reduced the  $\text{Cd}^{2+}$  influx by 84–85% in the mycelia (Figure 3), which is similar to the reduction in EM-roots upon salinity stress (Figure 2).

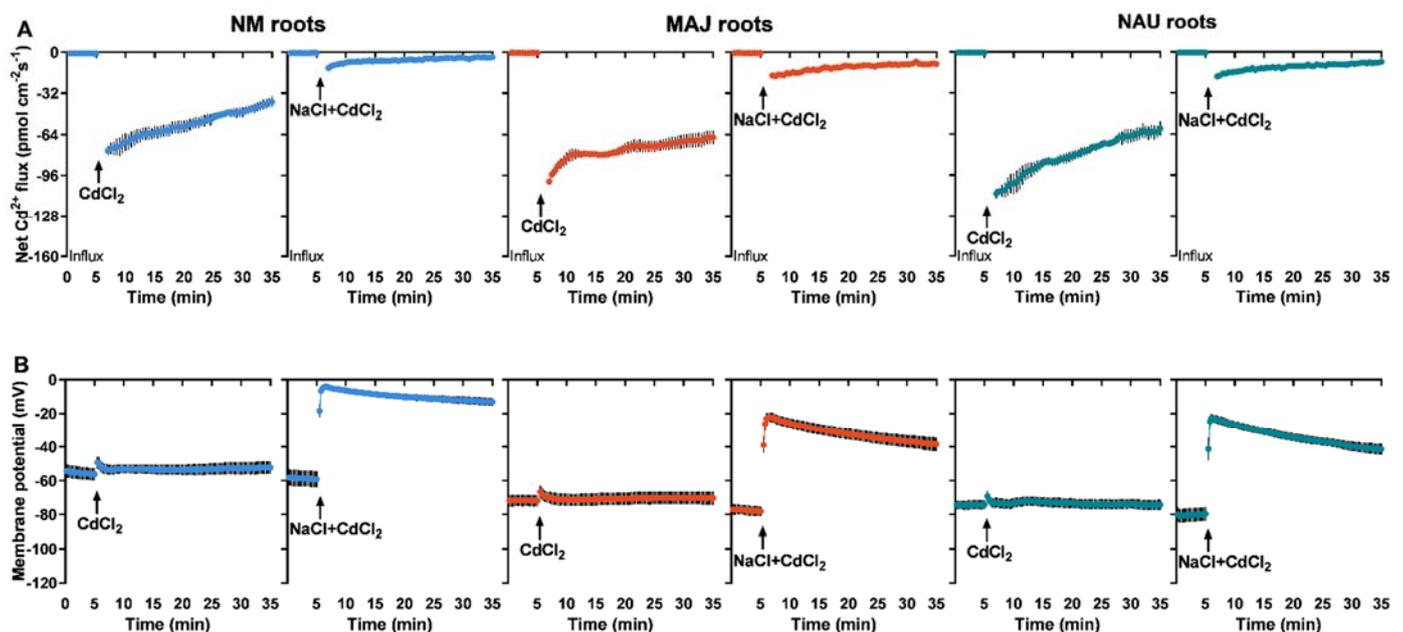


**Figure 3.** Net  $\text{Cd}^{2+}$  fluxes in fungal hyphae of *Paxillus involutus* isolates (MAJ and NAU) under cadmium, salt, and inhibitor treatments. MAJ and NAU mycelia (the youngest and active hyphae) were hydroponically acclimated and subjected to 24 h of  $\text{CdCl}_2$  (0 or 50  $\mu\text{M}$ ) in combination with NaCl (0 or 100 mM). The short-term Cd- and Cd + NaCl-stressed fungal mycelia were treated with an inhibitor of plasmalemma  $\text{H}^+$ -ATPase (sodium orthovanadate, 0 or 500  $\mu\text{M}$ ) or an inhibitor of  $\text{Ca}^{2+}$ -permeable channels ( $\text{LaCl}_3$ , 0 or 5 mM) for 30 min. Following 30 min equilibration in measuring solutions,  $\text{Cd}^{2+}$  flux recordings were continued for 15 min on the surface of pelleted hyphae. Mean values of  $\text{Cd}^{2+}$  fluxes in control (–Cd),  $\text{CdCl}_2$  stress (+Cd), and combined stress of  $\text{CdCl}_2$  and NaCl (+Cd + NaCl) in the presence and absence of inhibitors are shown.  $\text{Cd}^{2+}$  flux was not detectable in salt controls that were treated without  $\text{CdCl}_2$ . Each column is mean  $\pm$  SD obtained from 5 fungal cultures. Statistically significant differences ( $p < 0.05$ ) among treatments are indicated with different letters (a–e).

### 2.3. Transient $\text{Cd}^{2+}$ Kinetics and Membrane Potential upon Salt Shock

$\text{CdCl}_2$  shock (50  $\mu\text{M}$ ) created a transient  $\text{Cd}^{2+}$  influx in roots of NM *P. × canescens*, although the flux gradually decreased with prolonged exposure time (Figure 4A). EM-roots exhibited a pattern similar to NM-roots but with typically higher influx rates (Figure 4A). The  $\text{Cd}^{2+}$  influxes in both NM- and EM-roots were markedly reduced upon the NaCl addition (Figure 4A), similar to reduction found for the steady-state  $\text{Cd}^{2+}$  influx in salinized roots (Figure 2). Compared with the EM-roots, the restriction effect of NaCl was more pronounced in NM-roots (Figure 4A).

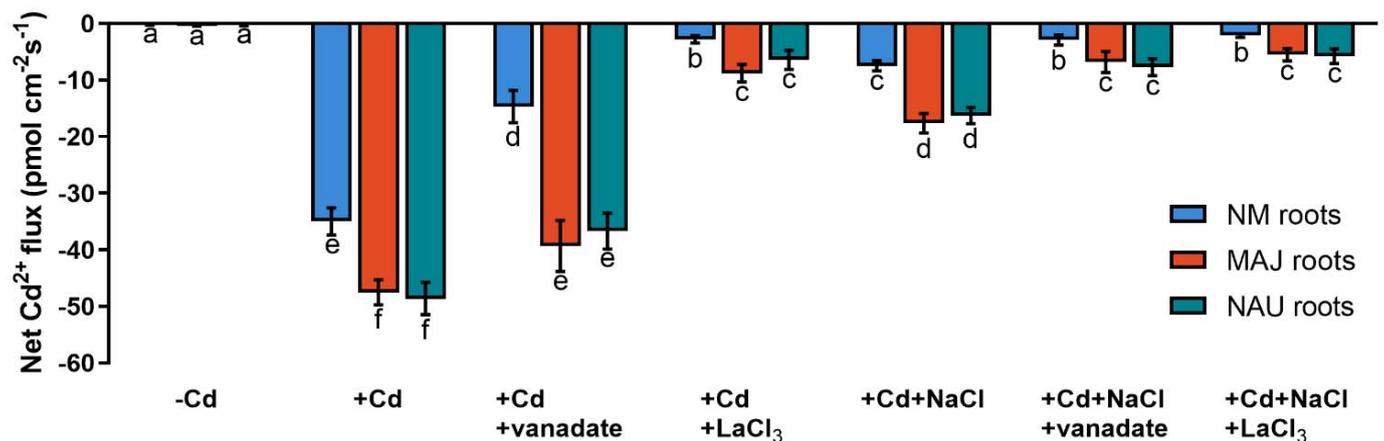
Transient kinetics of membrane potential upon  $\text{CdCl}_2$  (50  $\mu\text{M}$ ) and NaCl (100 mM) shocks were compared between roots of NM- and EM-poplars because the membrane potential indicates activity of PM  $\text{H}^+$ -ATPase [66]. NMT recordings showed that the resting membrane potential ranged from  $-54.4$  to  $-59.2$  mV in NM-roots under control conditions (Figure 4B). EM-roots had a more strongly hyperpolarized PM, with a membrane potential ranging from  $-71.7$  to  $-80.8$  mV (Figure 4B).  $\text{CdCl}_2$  shock exerted no significant effects on the membrane potential in NM- and EM-roots, although a marginal rise (5.0–6.1 mV) was observed after the onset of  $\text{CdCl}_2$  addition, which returned to the pretreatment level 1–2 min after  $\text{Cd}^{2+}$  addition (Figure 4B). However, the addition of NaCl together with  $\text{CdCl}_2$  caused an immediate and substantial depolarization of the membrane potential in NM- and EM-roots, although the PM tended to be rehyperpolarized during prolonged exposure to NaCl +  $\text{CdCl}_2$  (Figure 4B). In comparison, the membrane potential in EM-roots was less depolarized ( $-22.2$  to  $-41.4$  mV) after the onset of  $\text{CdCl}_2$  + NaCl shock as compared to NM-roots ( $-4.1$  to  $-13.0$  mV, Figure 4B).



**Figure 4.**  $\text{CdCl}_2$  and NaCl shock-altered  $\text{Cd}^{2+}$  kinetics and membrane potential in non-mycorrhizal (NM) *Populus × canescens* and ectomycorrhizal (EM) roots. (A)  $\text{Cd}^{2+}$  flux kinetics. (B) Membrane potential. Poplar plantlets were inoculated with or without *Paxillus involutus* isolates (MAJ or NAU) for 30 d. Root tips were excised from EM- and NM-poplars and equilibrated for 30 min in  $\text{Cd}^{2+}$  or  $\text{H}^+$  measuring solution. At the apical zones  $\text{Cd}^{2+}$  fluxes and membrane potential were recorded before and after the addition of  $\text{CdCl}_2$  (100  $\mu\text{M}$ ) or a combined solution of  $\text{CdCl}_2$  (50  $\mu\text{M}$ ) and NaCl (100 mM). The recordings continued respectively for 5 and 30 min before and after the cadmium and salt shock. Each data point is mean  $\pm$  SD obtained from 5 individual plants.

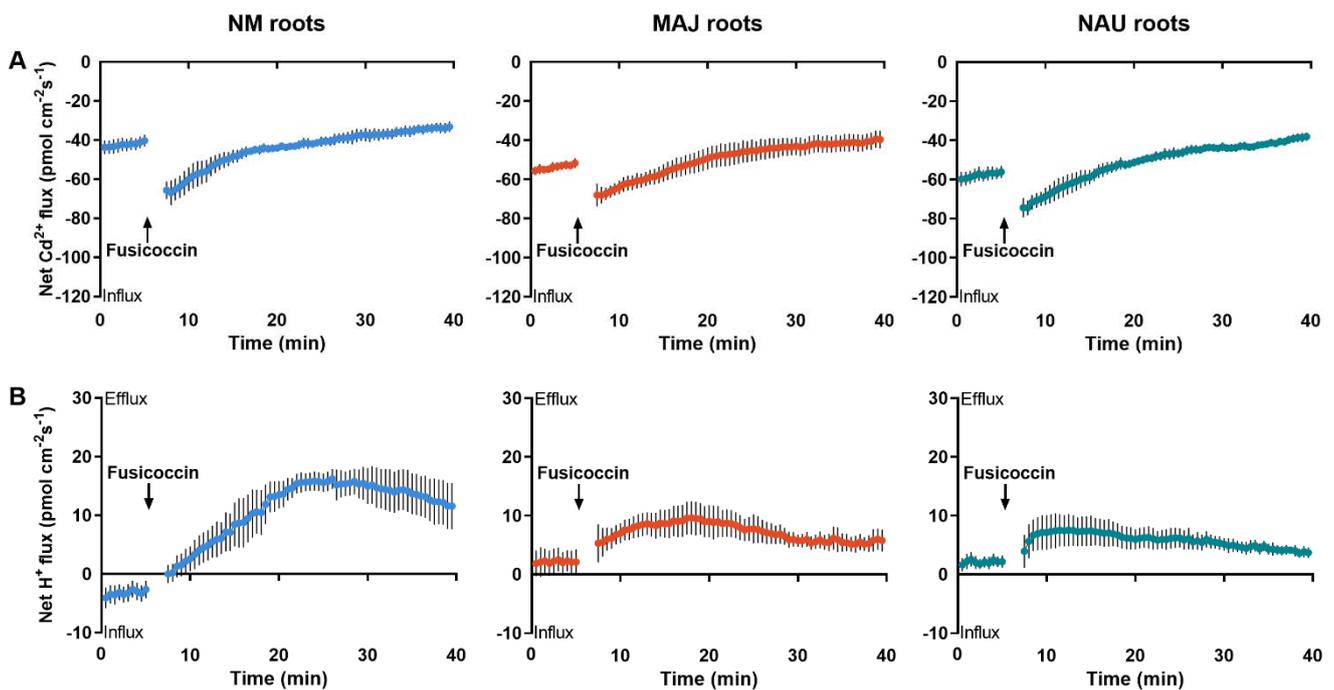
#### 2.4. Effects of PM H<sup>+</sup>-ATPase Inhibitor and Activator on Cd<sup>2+</sup> Uptake

Cd<sup>2+</sup> transport in poplar trees is accelerated by the PM H<sup>+</sup>-ATPase [38,48,52]. An H<sup>+</sup>-pump inhibitor, orthovanadate, was used to testify the crucial role of H<sup>+</sup>-pumps for Cd<sup>2+</sup> uptake in NM-, EM-roots, and fungal hyphae under CdCl<sub>2</sub> and salt stress. In NM-roots, orthovanadate decreased the Cd<sup>2+</sup> influx approximately two-fold, while in EM-roots only 17–25% decreases were found (Figures 5 and S2). In mycelia, vanadate also caused moderately reduced Cd<sup>2+</sup> influx (Figure 3). In the presence of NaCl, the inhibition of orthovanadate was evident in the fungus and roots, although the Cd<sup>2+</sup> influx had been significantly lowered by the salt treatment (Figures 3, 5 and S2).



**Figure 5.** Net Cd<sup>2+</sup> fluxes in non-mycorrhizal (NM) *Populus × canescens* and ectomycorrhizal (EM) roots under cadmium, salt, and inhibitor treatments. Poplar plantlets inoculated with or without *Paxillus involutus* isolates (MAJ or NAU, 30 d), were hydroponically acclimated and subjected to 24 h of CdCl<sub>2</sub> (0 or 50 μM) in combination with NaCl (0 or 100 mM). Root tips were excised from EM- and NM-poplars and subjected to an inhibitor of plasmalemma H<sup>+</sup>-ATPase (sodium orthovanadate, 0 or 500 μM) or an inhibitor of Ca<sup>2+</sup>-permeable channels (LaCl<sub>3</sub>, 0 or 5 mM) for 30 min. Following 30 min equilibration in measuring solutions, net fluxes of Cd along root axis (100 to 2300 μm) were monitored at an interval of 200–300 μm (Figures S2 and S3). Mean values of Cd<sup>2+</sup> fluxes in control (–Cd), CdCl<sub>2</sub> stress (+Cd), and combined stress of CdCl<sub>2</sub> and NaCl (+Cd + NaCl) in the presence and absence of inhibitors are shown. Cd<sup>2+</sup> flux was not detectable in salt controls that were treated without CdCl<sub>2</sub>. Each column is mean ± SD obtained from 5 individual plants. Statistically significant differences ( $p < 0.05$ ) among treatments are indicated with different letters (a–f).

Furthermore, the activator of PM H<sup>+</sup>-ATPase, fusicoccin (FC), was used to test the effect of H<sup>+</sup> pumping on Cd<sup>2+</sup> uptake in short-term stressed roots. Following the CdCl<sub>2</sub> treatment (50 μM, 24 h), roots of NM- and EM-poplars were subjected to FC activation. Immediately after the onset of FC addition, a stimulation of Cd<sup>2+</sup> influxes was observed at the surface of NM- and EM-roots (Figure 6A). H<sup>+</sup> efflux was correspondingly increased in FC-treated NM- and EM-roots (Figure 6B), indicating that H<sup>+</sup> pumps were transiently activated [84–87]. The observation that the increase in H<sup>+</sup> efflux corresponded to the Cd<sup>2+</sup> influx in *P. × canescens* roots suggests that the uptake of Cd<sup>2+</sup> was promoted by the H<sup>+</sup>-ATPases in the PM.

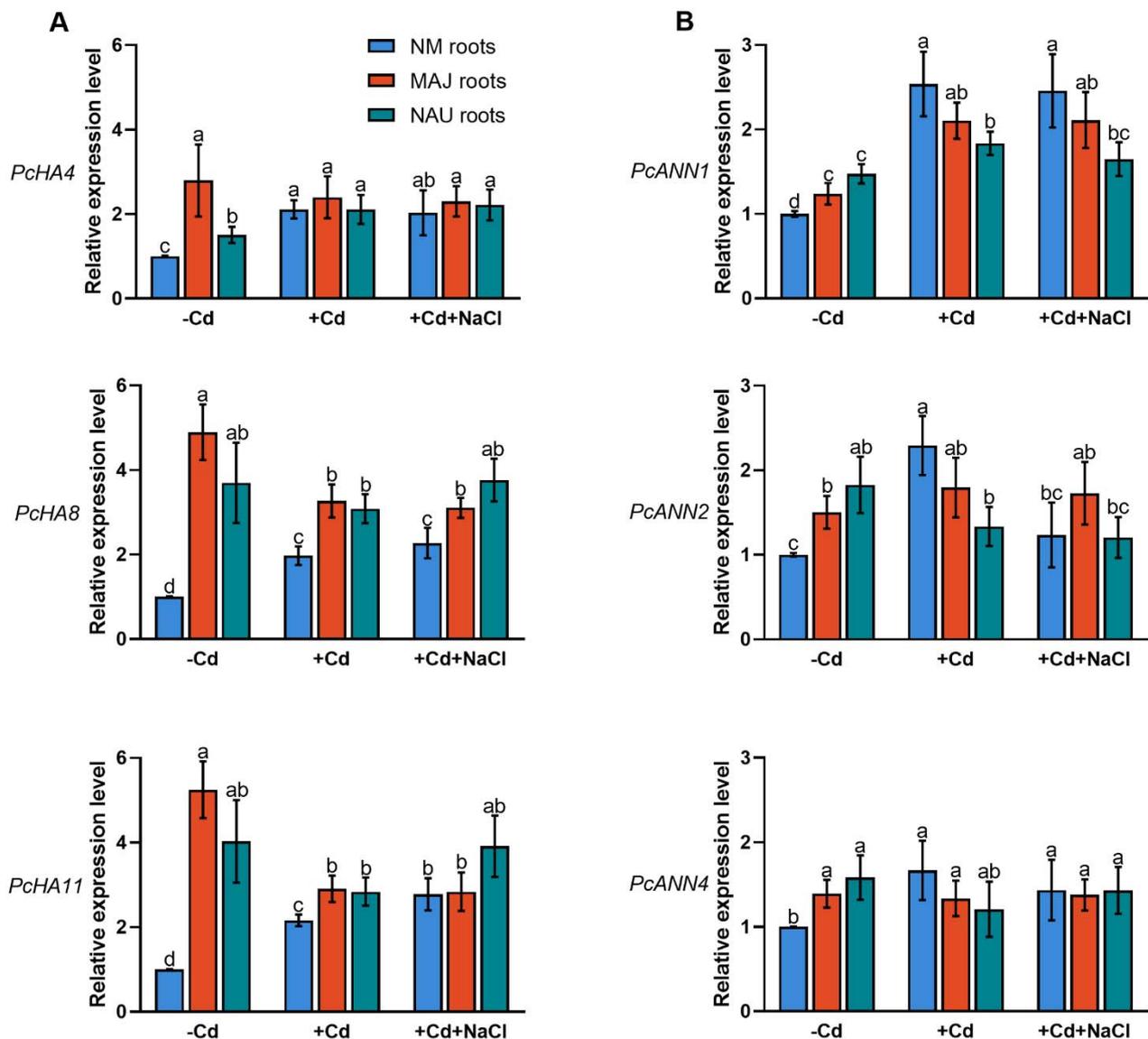


**Figure 6.** Fusicoccin shock-altered Cd<sup>2+</sup> and H<sup>+</sup> kinetics in non-mycorrhizal (NM) *Populus × canescens* and ectomycorrhizal (EM) roots. (A) Cd<sup>2+</sup> flux kinetics. (B) H<sup>+</sup> flux kinetics. Poplar plantlets inoculated with or without *Paxillus involutus* isolates (MAJ or NAU, 30 d) were hydroponically acclimated and subjected to 24 h of CdCl<sub>2</sub> (50 μM). Root tips were excised from EM- and NM-poplars and equilibrated for 30 min in Cd<sup>2+</sup> or H<sup>+</sup> measuring solution. At the apical zones, Cd<sup>2+</sup> and H<sup>+</sup> fluxes were recorded before and after the addition of fusicoccin (10 μM). The recordings continued, respectively, for 5 and 35 min before and after fusicoccin shock. Each data point is mean ± SD obtained from 5 individual plants.

### 2.5. Transcriptional Activation of H<sup>+</sup>-ATPase in Ectomycorrhizal *P. × canescens*

Transcript levels of the PM H<sup>+</sup>-ATPase-encoding genes, *PcHA4*, *PcHA8* and *PcHA11*, were examined in NM and EM roots since these three *PcHAs* were previously shown to be differently expressed under control and Na<sup>+</sup> stress conditions [66]. EM-roots showed significantly higher (0.5–4.2 fold) transcript levels of *PcHA4*, *PcHA8* and *PcHA11* than NM-roots (Figure 7A). This observation agrees with Sa et al. (2019) [66]. Cadmium treatment (50 μM CdCl<sub>2</sub>, 24 h) resulted in upregulation of *PcHA4*, *PcHA8*, and *PcHA11* in NM-roots (Figure 7A). In contrast, Cd<sup>2+</sup> caused a 14–45% decline of *PcHAs* in EM-roots, with the exception of *PcHA4* in NAU roots (Figure 7A). It is notable that the transcript levels, in particular those of *PcHA8*, and *PcHA11*, still remained higher in the EM- than in NM-roots, despite the decline caused by Cd<sup>2+</sup> stress (Figure 7A).

NaCl treatment (100 mM, 24 h) lowers the transcript levels of *PcHAs* (4, 8, 11) in NM-roots [66]. Here, exposure to NaCl of the Cd<sup>2+</sup>-treated roots did not result in decreased *PcHA4* and *PcHA8* transcript levels and an increase of *PcHA11* was observed (Figure 7A). Similarly, NaCl did not significantly change *PcHAs* transcription in EM-roots in the presence of Cd<sup>2+</sup> (Figure 7A). We noticed that EM-roots retained overall higher transcript levels of *PcHAs* than NM-roots under co-occurring stresses of cadmium and salinity.



**Figure 7.** Effects of CdCl<sub>2</sub> as single stress factor or in combination with NaCl on transcriptional profiles of plasmalemma H<sup>+</sup>-ATPase (*PchAs*) and annexins (*PcANNs*) in roots of non-mycorrhizal or ectomycorrhizal (EM) *Populus × canescens*. (A) *PchA4*, 8, 11. (B) *PcANN1*, 2, 4. Poplar plantlets inoculated with or without *Paxillus involutus* isolates (MAJ or NAU, 30 d), were hydroponically acclimated and subjected to 24 h of CdCl<sub>2</sub> (0 or 50 μM) in combination with NaCl (0 or 100 mM). Roots were harvested from EM- and NM-poplars and used for total RNA isolation and RT-qPCR. *18S rRNA* was used as a reference gene. Specific primers designed to target *PchA4*, 8, 11, *PcANN1*, 2, 4 and *18S rRNA* are shown in Table S1. Mean values of *PchAs* and *PcANNs* relative transcript levels in control (−Cd), CdCl<sub>2</sub> stress (+Cd), and combined CdCl<sub>2</sub> and NaCl stress (+Cd + NaCl) are shown. Each column is mean ± SD obtained from 3 independent experiments. Statistically significant differences ( $p < 0.05$ ) among treatments are indicated with different letters (a–d).

## 2.6. Calcium Channel Inhibitor Blocks Cd<sup>2+</sup> Fluxes

Cadmium ions enter the plasma membrane through CaPCs in plant cells [48,79,88]. To determine whether CaPCs contributed to the mediation of Cd<sup>2+</sup> influx under combined CdCl<sub>2</sub> and NaCl stress, LaCl<sub>3</sub> was used to block Ca<sup>2+</sup>-channels in the roots of NM- and EM-poplars. The inhibitor significantly decreased root Cd<sup>2+</sup> uptake in the presence and absence of NaCl, although NaCl treatment reduced the apparent Cd<sup>2+</sup> influx under coexisting stress (Figures 5 and S3). Similarly, the LaCl<sub>3</sub> significantly reduced Cd<sup>2+</sup> uptake in fungal hyphae regardless of the NaCl addition (Figure 3).

### 2.7. Transcript Levels of Annexin Genes in Ectomycorrhizal *P. × canescens*

Plant annexins (ANNs), such as ANN1, ANN2, ANN4, function as Ca<sup>2+</sup>-permeable channels in higher plants [70–76,79,89]. We have shown that *P. euphratica* PeANN1 facilitates cadmium enrichment by regulation of calcium-permeable channels [79]. Here, we examined the *P. × canescens* orthologs *PcANN1*, *PcANN2* and *PcANN4* in NM- and EM-roots. In the absence of Cd and salt, *PcANN1*, *PcANN2* and *PcANN4* showed significantly higher transcripts in EM-roots than in the NM (Figure 7B). This observation is in accord with previous findings that EM-roots retain typically higher influx of Ca<sup>2+</sup> than NM-roots [64,65]. Short-term cadmium exposure (50 μM, 24 h) caused significant increases of *PcANN* transcript levels in NM roots (Figure 7B), supporting Cd<sup>2+</sup> enrichment in the woody hyperaccumulator [29,33,38,52]. The Cd<sup>2+</sup> stimulation of annexin transcript levels was less pronounced in EM-roots (Figure 7B). For example, *PcANN1* levels which increased by 25–70% in MAJ and NAU roots under Cd<sup>2+</sup> treatment were still lower than those in CdCl<sub>2</sub>-treated NM-roots (Figure 7B). The *PcANN2* responded differently to short-term cadmium exposure in the EM-roots colonized with the strain MAJ (increase) and the strain NAU (decrease) (Figure 7B). Cadmium exposure also slightly decreased *PcANN4* in EM-roots (4–24%, Figure 7B). In CdCl<sub>2</sub>-stressed NM roots, NaCl lowered the transcripts of *PcANNs* by 3–46% (Figure 7B). As a result, the cadmium stimulation of annexin genes (with the exception of *PcANN1*) was lost by the addition of NaCl (Figure 7B). Compared to NM-roots, *PcANNs* was either less (*PcANN1*, *PcANN2*) or not reduced (*PcANN4*) by NaCl in EM-roots under cadmium treatment (Figure 7B).

## 3. Discussion

### 3.1. The *P. involutus*-Activated PM H<sup>+</sup>-ATPase Contributes to Cd<sup>2+</sup> Enrichment in EM Roots

Our data show that the woody hyperaccumulator, *P. × canescens*, exhibited strong Cd<sup>2+</sup> uptake and accumulation in root and shoots, which is further enhanced by colonizing with EM-fungus *P. involutus* (Figure 1). These findings are similar to previous reports in long-term studies [29,33,48,52]. The root flux recordings confirmed that the enhanced Cd<sup>2+</sup> entry in *P. × canescens* roots was due to the colonization with MAJ and NAU isolates, which were characterized by a remarkable Cd<sup>2+</sup> enrichment in the hyphae (Figures 2, 3 and S1) [48,90]. However, we observed that salt stress caused by NaCl reduced the Cd<sup>2+</sup> influx in roots and fungus (Figures 2, 3 and S1). Similarly, NaCl reduced root cadmium uptake and translocation in the halophyte *Carpobrotus rossii* [7,8] and *Atriplex halimus* [91]. An important novel result was that the *P. involutus* could alleviate the salt suppression of Cd<sup>2+</sup> uptake in *P. × canescens* roots (Figures 2, 4 and S1). To obtain a mechanistic understanding of the underlying processes, we inhibited and stimulated the Cd<sup>2+</sup> fluxes with pharmacological agents. The entry of Cd<sup>2+</sup> in the roots and fungal hyphae declined when the plasmalemma H<sup>+</sup>-ATPase was inhibited by vanadate (Figures 3, 5 and S2) [48] and increased when the plasmalemma H<sup>+</sup>-ATPase was stimulated by FC (Figure 6). These data suggest that Cd<sup>2+</sup> uptake required a proton gradient [48,52]. Moreover, *P. involutus* colonization resulted in a higher H<sup>+</sup> efflux and correspondingly a more negative membrane potential (Figure 6), indicating that the PM H<sup>+</sup>-ATPases were activated by the ectomycorrhiza [48,64,66]. This is similar to the enhanced proton-ATPase in arbuscular-mycorrhizal symbiosis [92,93]. The highly activated H<sup>+</sup>-pumps hyperpolarize the PM, thereby facilitating Cd<sup>2+</sup> influx via hyperpolarization-activated CaPCs [48,73]. In accordance with our flux analyses, transcript levels of the PM H<sup>+</sup>-ATPase-encoding genes, *PcHA4*, *PcHA8*, *PcHA11*, generally remained at higher levels in ectomycorrhizal roots under control and CdCl<sub>2</sub> stress compared to NM *P. × canescens* roots, although two or three of the tested *PcHAs* were down-regulated by CdCl<sub>2</sub> in MAJ and NAU roots (Figure 7). Of note, EM-roots maintained higher transcripts of *PcHA4*, 8, 11 than non-colonized roots under combined stress of CdCl<sub>2</sub> and NaCl (Figure 7). Similarly, Sa et al. showed that both MAJ and NAU roots retain higher transcript levels of *PcHA4* and/or *PcHA8* than NM-roots under control and NaCl stress conditions [66]. Increased abundances of PM H<sup>+</sup>-ATPase transcripts are expected to contribute to the activated H<sup>+</sup>-pumps because the plasmalemma H<sup>+</sup>-ATPases are transcriptionally regulated

in poplars [85,86,94]. Thus, the retained H<sup>+</sup>-pumping activity resulted in less depolarization of membrane potential under NaCl stress (Figure 4) [66], thereby upkeeping Cd<sup>2+</sup> influx into the EM-roots. This result concurs with those of Ma et al. (2014), who found that upregulation of *HA2.1* and *AHA10.1* leads to Cd<sup>2+</sup> uptake in EM poplar roots [52].

### 3.2. The Fungus-Elicited Annexins Mediated Cd<sup>2+</sup> Uptake in EM Roots

Since LaCl<sub>3</sub> inhibited Cd<sup>2+</sup> uptake into roots and fungal hyphae, our results support that Cd<sup>2+</sup> uptake involves CaPCs in the PM (Figures 3, 5 and S3) [41,48,68,73,88]. Plant annexins, in particular ANN1, ANN2 and ANN4, have been shown to function as CaPCs in Arabidopsis, maize and rice [70–73,75,76]. Zhang et al. suggested that PeANN1 facilitates the flow of cadmium ions through CaPCs [79]. CdCl<sub>2</sub> treatment upregulated transcripts of *PcANN1*, *PcANN2* and *PcANN4* in roots of NM *P. × canescens* (Figure 7), similar to the findings in crop species, such as maize, peanut and rice [73,80,81]. Accordingly, the cadmium-elicited annexins might mediate root Cd<sup>2+</sup> inflow through CaPCs in the poplar, contributing to its hyperaccumulator character [33]. Noteworthy, *PcANN1*, *PcANN2* and *PcANN4* showed remarkably higher transcripts in EM-roots than in the non-colonized under control conditions; CdCl<sub>2</sub> treatment caused a further increase in *PcANN1* in EM-roots and *PcANN2* was specifically increased in MAJ-colonized roots (Figure 7). The arbuscular mycorrhiza-stimulated transcription of *GmAnn1a* was observed in soybean roots [95]. In addition, annexin proteins also showed enhanced accumulation in arbuscular mycorrhizal roots of *Medicago sativa* and *M. truncatula* following cadmium application [96,97]. Therefore, the fungus-induced annexins might have collectively contributed to the CaPCs-mediated Cd<sup>2+</sup> enrichment in root cells of the poplar [73,79]. In accordance with this notion, we have previously shown that *Paxillus*-colonized roots showed higher Ca<sup>2+</sup> and Cd<sup>2+</sup> influxes than NM-roots [48,64,65]. We noticed that the transcripts of *PcANN1*, 2, 4 in EM-roots exhibited lower levels than non-colonized roots under CdCl<sub>2</sub> stress (Figure 7). However, root Cd<sup>2+</sup> influx remained higher in EM than in NM (Figures 2 and 4). Thus, it can be inferred that the annexin-mediated uptake of Cd<sup>2+</sup> was mainly promoted by the electrochemical gradient across the PM that was established by H<sup>+</sup>-ATPases. NaCl decreased *PcANN2* and *PcANN4* in NM-roots, but the transcript levels of *PcANN1*, 2, 4 were less reduced by NaCl in EM-roots (Figure 7). It is worth noting that in these ectomycorrhizal roots, transcription of *PcHAs* was retained at high levels under cadmium and salinity stress (Figure 7). Taken together, this suggests that the fungus-stimulated transcription of *annexins* contributed to Cd<sup>2+</sup> enrichment in EM-roots under combined stresses of cadmium and salt.

## 4. Materials and Methods

### 4.1. Fungal Inoculation with *Populus × canescens*

The two isolates of EM fungus *P. involutus* (MAJ and NAU) from Büsgen-Institute: Forest Botany and Tree Physiology (Göttingen University, Büsgenweg 2, Göttingen, Germany) were cultured on modified Melin Norkrans medium [83]. *P. × canescens* plantlets were micropropagated and rooted in modified Murashige and Skoog (MMS) medium [98]. Uniform and healthy plantlets were inoculated with MAJ or NAU for 30 d using a Petri-dish culture system [99].

### 4.2. Cadmium and NaCl Treatment

The agar plugs with hyphae, and plants colonized with or without EM fungus, were hydroponically acclimated in MMS nutrient solution for 2–3 d [66]. Then fungal mycelia, NM- and EM-plants were treated with CdCl<sub>2</sub> (0 or 50 µM) in combination with NaCl (0 or 100 mM) in MMS solution. Following 24 h of CdCl<sub>2</sub> treatment and combined stress of CdCl<sub>2</sub> and NaCl, steady-state Cd<sup>2+</sup> fluxes were recorded in fungal mycelia, NM- and EM-roots. Transcript levels of genes encoding annexins (*PcANN1*, 2, 4) and PM H<sup>+</sup>-ATPases (*PcHA4*, 8, 11) were examined in control and stressed roots.

#### 4.3. Inhibitor and Activator Treatment

The fungal mycelia, NM-, and EM-roots pretreated with short-term CdCl<sub>2</sub> or CdCl<sub>2</sub> + NaCl were exposed to inhibitors of Ca<sup>2+</sup> channels (LaCl<sub>3</sub>, 0 or 5 mM) [48,100] or PM H<sup>+</sup>-ATPases (sodium orthovanadate, 0 or 500 μM) [100,101] for 30 min. Steady-state Cd<sup>2+</sup> fluxes were recorded on the surface of roots and pelleted hyphae, respectively [48].

After 24 h exposure to 50 μM CdCl<sub>2</sub>, roots from NM- and EM-poplars were subjected to an activator of PM H<sup>+</sup>-ATPase, Fusicoccin (FC). FC produced by *Fusicoccum amygdali*, has the function of activating H<sup>+</sup>-ATPase in the PM [102,103]. Cd<sup>2+</sup> and H<sup>+</sup> transient kinetics were continuously recorded for 35 min after FC (10 μM) were added to measuring solutions.

#### 4.4. Assessed of Cd<sup>2+</sup> Concentrations

After 24 h exposure to CdCl<sub>2</sub> (0 or 50 μM) in combination with NaCl (0 or 100 mM), roots, stems and leaves of NM- and EM-poplars were sampled and oven dried at 70–80 °C for 5 d. Dried samples was weighed 0.1 g and digested in 5 mL of concentrated HNO<sub>3</sub> and 2 mL 30% H<sub>2</sub>O<sub>2</sub> in a microwave accelerated reaction system (Titan MPS Microwave Sample Preparation System, Perkin-Elmer, Waltham, MA, USA). Concentrations of Cd<sup>2+</sup> were assessed by a PerkinElmer Optima 8000 ICP-OES Spectrometer (Perkin-Elmer, Waltham, MA, USA).

#### 4.5. Flux Recordings of Cd<sup>2+</sup> and H<sup>+</sup>

##### 4.5.1. Microelectrodes Preparation and Calibration

Cd<sup>2+</sup> and H<sup>+</sup> flux profiles were recorded using an NMT system (NMT-YG-100, Younger USA LLC, Amherst, MA, USA). The glass microelectrodes were prepared as previously described [42,43,48,84,104]. Prior to flux recordings, the calibration of Cd<sup>2+</sup>- and H<sup>+</sup>-selective microelectrodes were carried out in the following standards (concentrations in mM):

- (a) H<sup>+</sup> microelectrodes: 0.1 NaCl, 0.1 CaCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, and 0.5 KCl, pH 4.5, 5.5, and 6.5 (pH was adjusted to 5.3 during H<sup>+</sup> flux recordings); and
- (b) Cd<sup>2+</sup> microelectrodes: 0.05 CaCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, 0.5 KCl, 0 or 100 NaCl, and CdCl<sub>2</sub> series (0.01, 0.05, and 0.1), pH 5.3 (Cd<sup>2+</sup> concentration was 0.05 mM during Cd<sup>2+</sup> flux recordings).

After calibration, the microelectrodes that showed Nernstian slopes of 58 ± 6 mV/decade (H<sup>+</sup>) and 29 ± 4 mV/decade (Cd<sup>2+</sup>) were used in our NMT recordings.

##### 4.5.2. Steady-State Cd<sup>2+</sup> Flux Recordings

After 24 h exposure to CdCl<sub>2</sub> (0 or 50 μM) in combination with NaCl (0 or 100 mM), sodium orthovanadate (0 or 500 μM), and LaCl<sub>3</sub> (0 or 5 mM), fungal mycelia and root tips excised from NM- and EM-poplars were subjected to 30 min equilibration in the following measuring solutions (concentrations in mM), respectively:

- (i) Control (–Cd): 0.05 CaCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, 0.5 KCl, pH 5.3;
- (ii) +Cd: 0.05 CaCl<sub>2</sub>, 0.05 CdCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, 0.5 KCl, pH 5.3; and
- (iii) Cd+NaCl: 0.05 CaCl<sub>2</sub>, 0.05 CdCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, 0.5 KCl, 100 NaCl, pH 5.3.

Following equilibration, net fluxes of Cd<sup>2+</sup> along root axis (100 to 2300 μm) were monitored at an interval of 200–300 μm. The flux recording at each point was continued for 6–8 min [41,64,101,105]. For the fungal mycelia, Cd<sup>2+</sup> flux recording of pelleted hyphae was continued 15 min [48]. Cd<sup>2+</sup> fluxes were recorded from at least five individual plants or fungal cultures for each treatment. The flux oscillations in EM fungus and poplars are not so pronounced as that observed in crop seedlings [48,106].

#### 4.5.3. Transient Recordings of Cd<sup>2+</sup>, H<sup>+</sup> Flux and Membrane Potential

##### Transient Cd<sup>2+</sup> Kinetics and Membrane Potential.

NM- and EM-roots were incubated in basic solutions of Cd<sup>2+</sup> (concentration in mM: 0.05 CaCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, 0.5 KCl, pH 5.3) and H<sup>+</sup> (0.1 CaCl<sub>2</sub>, 0.1 MgCl<sub>2</sub>, 0.1 NaCl, 0.5 KCl, pH 5.3) for 30 min. Cd<sup>2+</sup> fluxes and membrane potentials at apical regions were recorded for 5 min prior to CdCl<sub>2</sub> and NaCl shocks. Membrane potential was measured using Ag/AgCl microelectrodes (XY-CGQ03; Xuyue (Beijing) Sci and Tech Co. Ltd., Suzhou street 49, Haidian District, Beijing, China) as previously described [66]. Then, CdCl<sub>2</sub> (100 μM) stock, or a combined stock solution of CdCl<sub>2</sub> (100 μM) and NaCl (200 mM) was added slowly to reach final concentrations of 50 μM (CdCl<sub>2</sub>) and 100 mM (NaCl). Kinetics of membrane potential and Cd<sup>2+</sup> uptake were recorded up to 30 min in NM- and EM-roots. Cd<sup>2+</sup> fluxes and membrane potentials were recorded from at least five individual plants for each treatment.

##### Transient Kinetics of Cd<sup>2+</sup> and H<sup>+</sup> upon FC.

The NM- and EM-roots pretreated with CdCl<sub>2</sub> (50 μM, 24 h) were excised and equilibrated in measuring solutions of Cd<sup>2+</sup> or H<sup>+</sup> for 30 min. Fluxes of Cd<sup>2+</sup> and H<sup>+</sup> at apical regions were recorded for 5 min before the addition of FC (Sigma-Aldrich, St. Louis, MO, USA). Then, FC stock solution (dissolved in DMSO) was added to Cd<sup>2+</sup> and H<sup>+</sup> measuring solutions, reaching a final concentration of 10 μM [103]. Cd<sup>2+</sup> and H<sup>+</sup> transient kinetics in FC-treated roots were further recorded for 35 min. Fluxes of Cd<sup>2+</sup> and H<sup>+</sup> were recorded from at least five individual plants for NM-, MAJ- and NAU-roots.

#### 4.6. Determination of Gene Expression of Annexins and PM H<sup>+</sup>-ATPases

After 24 h exposure to CdCl<sub>2</sub> (0 or 50 μM), or to CdCl<sub>2</sub> (50 μM) in combination with NaCl (100 mM), total RNA was isolated from NM and fungus-colonized roots and used for real-time quantitative PCR (RT-qPCR) [66]. The primer sequences for annexins (*PcANN1*, 2, 4) [79], plasmalemma H<sup>+</sup> ATPase (*PcHAs*, *PcHA4*, 8, 11) [66], and reference genes (*18S rRNA*) [107], are shown in Table S1. The RT-qPCR amplification was performed as previously described [66,79,86]. Expression profiles for *PcANNs* and *PcHAs* were normalized to the transcripts of *18S rRNA* [108]. The RT-qPCR experiment was repeated three times.

#### 4.7. Data Analysis

The calculations of flux rate and membrane potential were processed using JCal V3.2.1 program (Xuyue (Beijing) Sci and Tech Co. Ltd., Suzhou street 49, Haidian District, Beijing, China, Available online: <http://www.xuyue.net/>, accessed on 12 March 2021). All experimental data were subjected to SPSS version 19.0 (IBM Corporation, Armonk, NY, USA). Differences between means were considered significant at  $p < 0.05$ .

## 5. Conclusions

Our data provide further evidence that cadmium can be enriched in ectomycorrhizal poplars under co-existing stress conditions of Cd<sup>2+</sup> and NaCl. *P. involutus* stimulated Cd<sup>2+</sup> influx through CaPCs in ectomycorrhizal *P. × canescens* roots, depending on the plasmalemma H<sup>+</sup>-ATPase. NaCl lowered the uptake of Cd<sup>2+</sup> in poplar roots, which was alleviated by ectomycorrhization with *P. involutus*. Ectomycorrhizal fungus colonization upregulated transcription of PM H<sup>+</sup>-ATPases (*PcHA4*, 8, 11) and increased transcripts of annexins (*PcANN1*, 2, 4), which might mediate Cd<sup>2+</sup> conductance through PM CaPCs. NaCl-treated EM-roots retained relatively highly expressed *PcHAs* and *PcANNs*. We hypothesize that the sustained transcription of *PcHAs* resulted in H<sup>+</sup> pumping activity and PM hyperpolarization in the ectomycorrhiza, thus promoting Cd<sup>2+</sup> enrichment through the *PcANNs*-mediated Ca<sup>2+</sup> channels in EM-roots under co-occurring stresses of cadmium and salinity. Although the colonization of MAJ and NAU varies with regard to the formation of intraradical hyphae, i.e., the Hartig net, both strains conserved higher Cd<sup>2+</sup> uptake under salt stress than NM-roots. We propose that *P. involutus* strains, which have been repeatedly

shown to improve salt tolerance, may be applied as beneficial microbes to improve plant phytoremediation for cadmium in salt-affected areas.

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**Author Contributions:** C.D.: Investigation, Data curation, Visualization, Writing-original draft preparation. Z.Z., Investigation, Data curation, Visualization, Writing-original draft preparation. J.L.: Investigation, Data curation, Visualization. Y.Z. (Ying Zhang): Investigation, Visualization, Data curation. Y.Z. (Yinan Zhang): Investigation, Visualization. D.Y.: Visualization, Validation. S.H.: Investigation, Data curation. Y.Z. (Yanli Zhang): Software, Validation. J.Y.: Methodology, Software. H.Z.: Methodology, Software. N.Z.: Methodology, Visualization. G.S.: Visualization, Validation. Y.Z. (Yuhong Zhang): Visualization, Validation. X.M.: Visualization. R.Z.: Methodology, Software. A.P.: Conceptualization, Resources, Writing-reviewing & editing, Project administration. S.C.: Conceptualization, Supervision, Writing-reviewing & editing, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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