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RESEARCH

Inter- and intra-specific metal tolerance variation in ectomycorrhizal fungal *Suillus* species

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Abstract

Soil metal contamination can affect growth, metabolism, and reproduction of organisms, and can lead to death. However, some fungi have evolved metal tolerance and are able to live in contaminated soils. Species in the ectomycorrhizal genus *Suillus* from Europe and Asia display variation in metal tolerance, yet it is unknown whether this is a widespread trait in the genus and whether it occurs in North America. Here we investigate cadmium (Cd) and zinc (Zn) tolerance in *S. brevipes* and *S. tomentosus* isolates collected from sites in the Rocky Mountains of Colorado displaying different metal content. In line with previous findings for other *Suillus* species, we hypothesized (1) *S. brevipes* and *S. tomentosus* to display intraspecific metal tolerance variation, (2) Zn and Cd tolerance to be correlated to soil metal content, and (3) tolerant isolates to show lower metal tissue content compared to sensitive isolates (due to increased metal exclusion). We found ample intra- and inter-specific Zn and Cd tolerance variation in both *S. brevipes* and *S. tomentosus*, but no correlation between soil metal content and tolerance. There was a negative correlation between tolerance level and Zn uptake, indicating an exclusion-based Zn tolerance strategy. Sensitive and tolerant isolates showed no difference in Cd accumulation, indicating that Cd tolerance in these species is likely not dependent on exclusion. Our study sets the groundwork for further investigation into the genetic basis of *Suillus* metal tolerance and whether and how it impacts pine mycorrhizal partners.

Keywords *Suillus* · Heavy metals · Ectomycorrhizal adaptation

Introduction

Soil metal contamination is widespread and high metal levels can be toxic and interfere with growth, metabolism, and reproduction (Wuana and Okieimen 2011). While some metals are essential micronutrients but toxic in high concentrations, such as zinc (Zn), others have no biological function within the cell and are toxic at even low concentrations. This is the case for cadmium (Cd) that has no known biological function (but see Lane and Morel (2000) for an interesting exception). Metal toxicity responses include the buildup of reactive oxygen species (ROS) in cells, which can lead to protein inactivation and DNA damage, along with deficient nutrient acquisition, leading to reductions in cell growth and reproduction that can be lethal (Singh et al. 2016).

Metal tolerance is well known in fungi, with several examples of species and isolates able to withstand and even thrive in high metal environments. For example, isolates of *Penicillium janthinellum* tolerate high Zn concentrations through increased Zn compartmentalization and inactivation combined with antioxidant responses that mitigate toxicity (Teng et al. 2018). Cadmium tolerance has been documented in a *Penicillium chrysogenum* strain isolated from Cd polluted soil, with biosorption and immobilization mechanisms being implicated in tolerance (Din et al. 2022). There are also examples of fungal tolerance to multiple metals, including *Paecilomyces variotii* (Eurotiales) isolates showing tolerance to five metals derived from the transfer of a large transposable element carrying genes involved in metal homeostasis (Urquhart et al. 2022). Metal tolerance has also been documented in ectomycorrhizal fungi. Examples include Zn and Cd tolerance in *Hebeloma mesophaeum* derived from sequestration within the cells and metal chelation (Sácky et al. 2014), and Cd tolerance in *Paxillus*

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involutus through transport and cellular compartmentation allowing it to persist in soils rich in Cd (Blaudez et al. 2000).

The genus *Suillus* is known to show metal tolerance, including to Zn and Cd. This widespread temperate ectomycorrhizal genus associates with pine trees, providing nutrients and receiving carbohydrates in return (Lofgren et al. 2021, 2024). Studies on European *S. bovinus* and *S. luteus* documented intra-specific variation in metal tolerance, with isolates displaying different levels of Zn and Cd tolerance that is mostly (but not always) correlated with soil of origin contamination (Colpaert et al. 2000, 2004; Colpaert and Vanassche 1992; Colpaert and van Assche 1987; Krznaric et al. 2009). Previous experimental, genetic, and genomic studies showed *Suillus* metal tolerance is rooted in metal exclusion, immobilization, and detoxification (Bazzicalupo et al. 2020; Branco et al. 2022; Smith et al. 2023). Notably, *S. luteus* Zn and Cd tolerant isolates accumulate less metal than their sensitive counterparts when exposed to the same metal concentrations, while also being able to tolerate a much higher concentration of intracellular metal (Krznaric et al. 2009; Colpaert et al. 2005). Candidate genes for metal tolerance in this species include transmembrane transporters, chelators, and detoxifying antioxidants including metallothioneins (Nguyen et al. 2017; Ruytinx et al. 2017; Coninx et al. 2017, 2019; Bazzicalupo et al. 2020). *Suillus* can also moderate the effects of metal toxicity on pine partners. For example, Zn tolerant *S. bovinus* colonizing *Pinus sylvestris* roots improve pine growth under high Zn concentrations (Colpaert et al. 2004). Similarly, metal tolerant *S. luteus* can prevent the accumulation of Zn in *P. sylvestris* roots (Zhang et al. 2021) and improve pine growth under Cd stress, with Cd-tolerant isolates significantly improving pine nutrient uptake and reducing Cd needle content in high Cd concentrations (Krznaric et al. 2009). Similar trends have been found in *S. luteus* from Asia, that reduces the toxic effects of high soil aluminum concentrations for pine partners (Liu et al. 2021). These effects make metal tolerant *Suillus* isolates good candidates for assisting in recovering degraded sites, for example by priming seedlings for transplantation into metal contaminated soils (Branco et al. 2022).

The long history of mining in the American West from the 1800s on has led to widespread metal soil contamination (Cubley et al. 2022), making fungal metal tolerance likely to evolve. However, it is unknown whether American *Suillus* species display metal tolerance or not. Here, we investigate Zn and Cd tolerance in *S. brevipes* and *S. tomentosus* from native conifer forests in Colorado along a range of soil Cd and Zn concentrations. Based on previous research, we hypothesized (1) the presence of intra-specific metal tolerance variation in both species, (2) Zn and Cd tolerance to be correlated to soil Zn and Cd content, and (3) *Suillus* metal tolerant isolates to show lower metal tissue accumulation compared to sensitive isolates. We performed in vitro growth assays in Zn and Cd gradients and found both inter- and intra-specific metal tolerance variation, but no correlation between soil content and metal tolerance. In addition, Zn tissue content was negatively correlated with tolerance, but Cd accumulation was not. These results contrast with previous findings and point to overall metal tolerance across *Suillus* species resulting from distinct mechanisms.

Materials and methods

Field sites and isolate collection

We obtained 73 *S. brevipes* and 58 *S. tomentosus* isolates between July 2021 and September 2022 from 23 native conifer forests dominated by *Pinus contorta* and *P. ponderosa* in Colorado (USA) with a range of soil Zn and Cd levels (Table S1). Sites were selected based on proximity to disused mines and government Superfund sites. Sites with disused mines were initially disturbed in the 1800s and likely experienced contamination through mining activity and leaching over time. We collected a soil sample from each site by clearing the leaf litter layer from the ground and sampling about 6 inches into the organic soil layer. Around 100 g of soil was sent for chemical analyses for each site, including soil Cd and Zn content (Ward Labs, Kearny, NE, USA, Table S2). Soil was digested using a wet ash method and analyzed with inductively coupled plasma spectroscopy (EPA method 6010 C). In house controls were used by Ward Labs. Soil pH and soluble salts (electrical conductivity) were determined with a Ross Sure-Flow reference electrode using a 1:1 soil water ratio method. When soil pH was 6.5 or less the modified Woodruff Buffer solution was added to the sample to determine the amount of lime needed to neutralize the soil acidity (McLean 1982; Watson and Brown 1998).

We obtained pure cultures for each fungal species from fruitbodies collected at least 10 m apart to increase the chance of sampling different genets. Isolates were initially identified in the field based on morphology and later confirmed by ITS sequencing (see below). We cultured the isolates in Fries medium amended with streptomycin and chloramphenicol at 23 °C in the dark for 2 weeks (Lofgren et al. 2024). Successful isolates were then transferred to plain Fries medium, grown at 23 °C in the dark for 2 weeks, and stored at 4 °C. We dried the remaining fruitbodies and deposited the vouchers at the Denver Botanic Garden Sam Mitchel Herbarium of Fungi (Table S1).

Isolate molecular identification

To confirm species identification, we sequenced and analyzed the Internal Transcribed Spacer (ITS) region for each isolate. We extracted DNA using the Sigma Extract-N-Amp kit (MilliporeSigma, Burlington, MA, USA), according to the manufacturer's instructions and amplified the ITS region using the ITS-1 F and ITS4 primers (Gardes and Bruns 1993). We confirmed PCR products using gel electrophoresis, cleaned successful products with the DNA Clean and Concentrator − 5 kit (Zymo Research, Irvine, CA) and obtained Sanger sequences (Genewiz, Azenta Life Sciences, Burlington, MA) for each isolate. We used BLAST (Altschul et al. 1990) to confirm all sequences belonged to the genus *Suillus* and deposited them in GenBank under accession numbers OR665177 to OR665292 (Table S1). We then computed a phylogenetic tree to assess placement of all isolates within respective *Suillus* clades. We used our ITS sequences along with available *Suillus* sequence data from GenBank. These included *S. brevipes* and *S. tomentosus* ITS sequences, as well as outgroups (*S. elbenisis*, *S. glandulosipes*, *S. kaibabiensis*, *S. lakei*, and *S. pungens*, Table S3). Sequences were first aligned in AliView (Larsson 2014) using Muscle (Edgar 2004) and edited manually. Aligned sequences were trimmed to the first and last informative base. The aligned sequence matrix was analyzed in CIPRES Web Portal (Miller et al. 2010) and we conducted maximum likelihood analyses using RaxML (Stamatakis 2014). We selected the best tree from 1000 bootstrap replicates and edited in FigTree v1.4.4 (Rambaut 2010).

Metal tolerance assays

We investigated *S. brevipes* and *S. tomentosus* Zn and Cd tolerance by setting up plate assays using Fries medium amended with increasing metal concentrations (Fig. 1A). Specifically, we added individual metals as their sulfate salts ($ZnSO_4 \cdot 7H_2O$ and $CdSO_4$) to the Fries media from stock solutions, and adjusted pH to 4.5 before autoclaving. We used 2.5, 5, 10, 15 and 20 mM Zn concentrations, and 5, 50, 200, 800 and 1600 µM Cd concentrations. Some isolates did not grow on 2.5 mM Zn. For these, we performed the assay with an additional concentration of 1 mM Zn. Assays were plated in triplicate and grown in the dark at 23 °C. After two weeks, we quantified growth from each replicate by measuring the distance from the original inoculation to the edge of mycelial growth along four axes per plate. The total 12 measurements were averaged into a radial growth measurement. We calculated the daily growth rate of each isolate on non-amended media by dividing the radial growth number by 14 (days). We generated Zn and Cd dose response curves for each isolate and determined EC_{50} values using the drc package (v3.0-1) in RStudio (R version 4.2.1, Ritz et al. 2015; Team 2021). The EC_{50} value is the concentration of metal at which 50% of growth is inhibited compared to the control. We plotted the EC_{50} using the ggplot2 package (v3.4.1) and performed t-tests using the ggpval package (v0.2.5) to investigate metal tolerance differences across the two species as the data met the test assumptions. To determine correlations between metal tolerance and soil metal content, Zn and Cd tolerance, and metal tolerance and growth rate, we used the ggscatter function within the ggpubr package (v0.4.0), using the stat_cor function to generate regression lines and respective equations, R^2 values, and *p* values.

Tissue metal content analysis

To determine fungal tissue metal content, we quantified Zn and Cd in mycelia and sporocarps using ICP-MS analysis (Green Analytical, Durango, CO). We analyzed 100 mg of dried sporocarp cap tissue from 3 Zn tolerant isolates, 3 Zn sensitive, 3 Cd tolerant and 3 Cd sensitive isolates from each species (12 samples x 2 species = 24 samples; Table S1). To assess for differences in mycelial metal content, we grew the same isolates in Fries medium amended with 0.1 mM and 2.5 mM Zn and 0.2 µM and 5 µM Cd. We selected relatively low metal concentrations to allow sensitive isolates to persist. The media was covered with disks of sterile cellophane to allow collection of tissue. Tissue was collected after 14 days and dried in an oven at 60 °C. We analyzed 100 mg of dried mycelium through ICP-MS analyses utilizing in-house certified reference controls (Green Analytical, Durango, CO). Samples were digested according to EPA3050B. We used the ggplot2, ggpval, ggpubr packages in R to plot and analyze the data. We compared mycelium metal content across groups using ANOVA (using log10 transformed data to meet test assumptions) followed by post-hoc Tukey tests to determine significant differences. We used ggscatter to determine if mycelial metal content was correlated to isolate EC_{50}. To determine if sporocarp metal content was correlated to soil metal content, we compared sporocarp metal concentration against soil metal level at the site of collection, as described above.

Results

Isolates belonged to *S. brevipes* and *S. tomentosus* and showed different growth rates

The phylogenetic analyses showed all 131 isolates belonged to *Suillus*, with 73 *S. brevipes* and 58 *S. tomentosus* (Supplementary Fig. 1, Table S1). We compared the growth rates

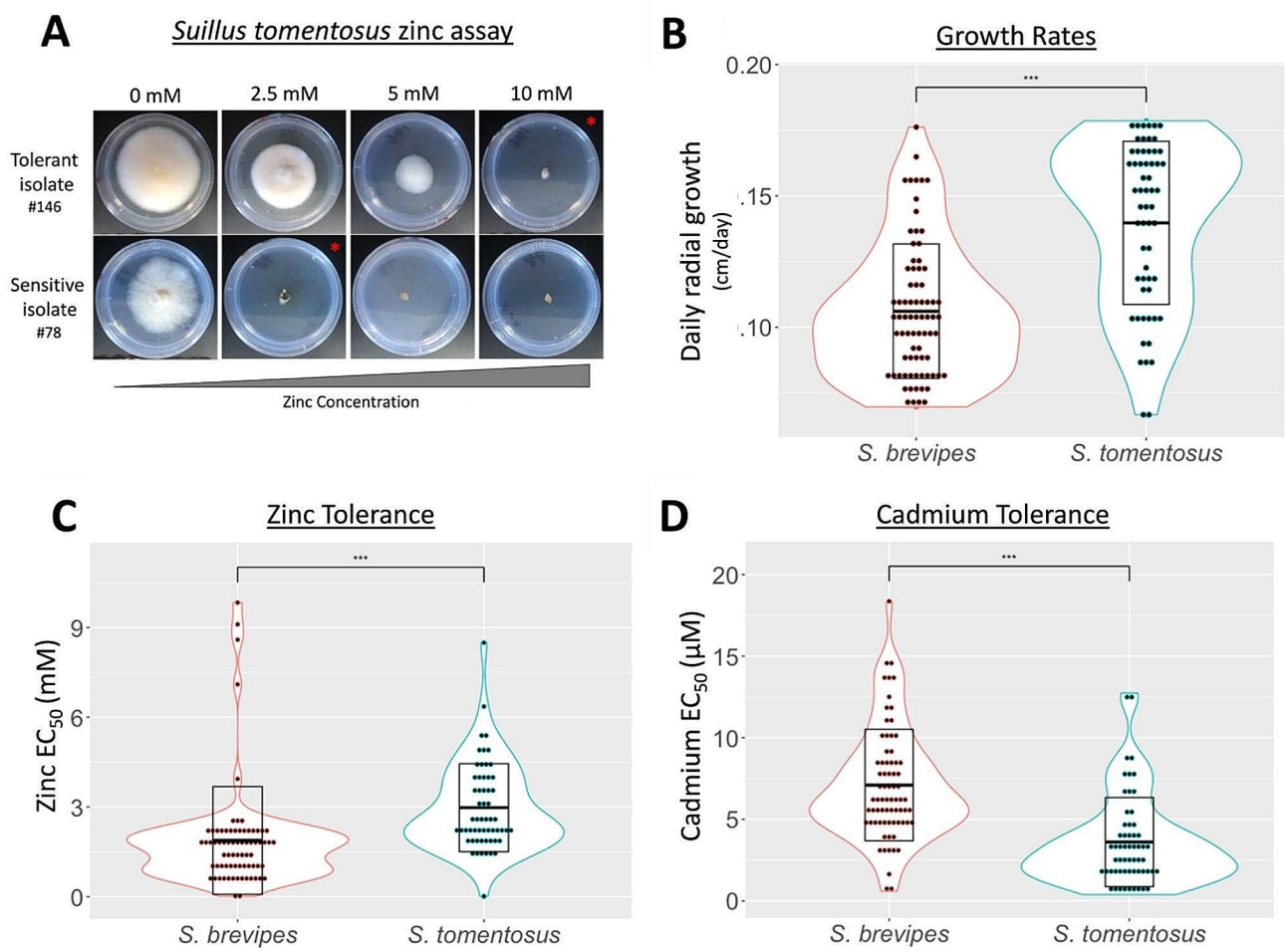

Fig. 1 *Suillus brevipes* and *S. tomentosus* growth and metal tolerance. **A** Example of Zn tolerance in *S. tomentosus*, showing a Zn tolerant (top row) and a Zn sensitive (bottom row) isolate grown on a zinc gradient (0, 2.5, 5, and 10 mM Zn in Fries agar) for 14 days. The red asterisk indicates the highest Zn concentration that each isolate grew on. **B** *S. brevipes* and *S. tomentosus* growth rate (average isolate daily growth rate in cm/day grown on plain Fries agar for 14 days). Black boxes indicate mean and standard deviation. Using a two-sample t-test comparing mean growth rates by species, we found that *S. tomentosus* (mean = 0.140 cm/day, range = 0.067–0.179 cm/day, SD = 0.031) grows significantly faster ($p = 1.2e^{-9}$) than *S. brevipes* (mean = 0.106 cm/day, range = 0.070–0.176 cm/day, SD = 0.025). **C** *Suillus brevipes* and *S. tomentosus* isolate Zn EC_{50}. Using a two-sample t-test comparing mean EC_{50} by species, we found that *S. brevipes* isolates (mean = 1.88 mM, range = 0.01–9.83 mM, SD = 1.80) were significantly less Zn tolerant ($p = 0.0002$) than *S. tomentosus* isolates (mean = 2.98 mM, range = 0.01–8.49 mM, SD = 1.47). **D** *Suillus brevipes* and *S. tomentosus* isolate Cd EC_{50} values. Using a two-sample t-test comparing mean EC_{50} by species, we found that *Suillus brevipes* isolates are significantly ($p = 1.6e^{-9}$) more Cd tolerant (mean = 7.10 µM, range = 0.59–18.36 µM, SD = 3.42) than *S. tomentosus* isolates (mean = 3.61 µM, range = 0.39–12.74 µM, SD = 2.73)

of each isolate (Fig. 1B, Table S1) and found high growth variation across isolates within species, with some isolates growing much faster than others in plain Fries medium. We also found *S. tomentosus* grows significantly faster than *S. brevipes* ($p = 1.2e^{-9}$; see Fig. 1B for details).

Inter- and intra-specific Zn and Cd tolerance

We found both inter- and intraspecific variation in Zn and Cd tolerance across *Suillus* isolates (Fig. 1A, C, D; see Table S1 for isolate Zn and Cd EC_{50} values). *Suillus tomentosus* was significantly more tolerant to Zn than *S. brevipes*, but significantly less tolerant to Cd. There was large within species metal tolerance variation, with isolates in both species showing a two to three order of magnitude difference in Zn and Cd tolerance (see Fig. 1C, D for mean values, range, standard deviation, and results of statistical tests).

No correlation between metal tolerance and soil metal content

There was no correlation between soil metal content and metal tolerance for neither *S. brevipes* nor *S. tomentosus* (Fig. 2A, B). Notably, we recovered metal sensitive and

tolerant isolates from high and low metal sites in both species and for both metals. For example, isolates from the most Zn and Cd rich site (California Gulch; 2611 mg/kg Zn and 44 mg/kg Cd), displayed a wide range of tolerance to Zn (EC_{50} values ranging from 0.63 to 8.592 mM Zn) and to Cd (EC_{50} values ranging from 1.035 to 14.636 µM Cd) and isolates collected from Niwot Ridge MRS, a site with low soil metals (2 mg/kg Zn and > 1 mg/kg Cd), also displayed a wide range of tolerance (EC_{50} values ranging from 0.762 to 4.109 mM Zn and 1.008 to 10.786 µM Cd). There was also no correlation between Zn tolerance and growth rate for either species (Fig. 2C-E), showing that Zn tolerance is decoupled from growth. However, we did find a significant negative correlation between growth rate and Cd tolerance in *S. tomentosus* (Fig. 2D), indicating that in this species faster growing isolates are more sensitive to Cd. There was no correlation between Cd and Zn tolerance in *S. brevipes* or *S. tomentosus*, (Fig. 2E).

Suillus brevipes sporocarp cd content was correlated with soil cd

Contrary to our expectations, we found few differences in sporocarp Zn and Cd content between sensitive and tolerant isolates. The sporocarp Zn content was not different across species or levels of Zn tolerance. However, *S. tomentosus* sporocarps showed significantly higher Cd content than *S. brevipes* sporocarps, regardless of tolerance phenotype (see Fig. 3A, B and Table S4 for details). Sporocarp Zn content was not correlated with site Zn level for either species (Fig. 3C) Sporocarp Cd content was significantly positively correlated with site Cd level in *S. brevipes*, but not in *S. tomentosus* ($p = 0.042$; Fig. 3D).

Zn tolerance may be rooted in metal exclusion

We found mycelium metal content increased with metal concentrations (Fig. 4A, B and Table S5). Not surprisingly, sensitive isolates were unable to grow at the highest metal concentrations, while the tolerant isolates persisted and showed an increase in mycelium metals. There was a negative correlation between Zn content and tolerance for *S. brevipes* when isolates were grown at 0.1 mM Zn ($p = 0.011$, Fig. 4C), indicating Zn tolerant isolates exclude the metal when growing in low Zn concentrations. *S. tomentosus* isolates grown at 2.5 mM Zn also showed a negative correlation between Zn content and tolerance (Fig. 4D). We did not find any significant correlation between Cd content and tolerance (Fig. 4E, F).

Discussion

We found pronounced Zn and Cd tolerance variation in North American *S. brevipes* and *S. tomentosus*. We also found evidence of Zn tolerance associating with metal exclusion, but surprisingly there was no correlation between soil metal content and metal tolerance.

Inter- and intraspecific variation in metal tolerance are well-known in *Suillus* and highlight the diversity of metal tolerance within the genus. *Suillus* is known to be tolerant to Zn and Cd and to show marked differences across species and isolates. For example, *S. bovinus* displays Zn tolerance intraspecific variation (Colpaert and Vanassche 1992), while *S. luteus*, *S. granulatus* and *S. variegatus* isolates show differences in Zn and Cd tolerance (*S. granulatus* is more Cd tolerant, and *S. variegatus* is more Zn tolerant; Colpaert et al. 2004; Blaudez et al. 2000a, b; Hartley et al. 1997). Our results expand the already documented widespread *Suillus* metal tolerance variation to American species, showing there are consistent metal tolerance differences across and within *Suillus*, with closely related isolates showing very different abilities to withstand metal toxicity.

Suillus brevipes and *S. tomentosus* from Colorado are much less tolerant to Cd and Zn than other species in the genus. Specifically, the highest detected Cd EC_{50} values are less than half of those seen in *S. luteus* from Europe (Krznaric et al. 2009; Colpaert et al. 2000). In addition, the EC_{50} values of our most Cd tolerant isolates match those of the most sensitive European isolates. Furthermore, *S. brevipes* and *S. tomentosus* Zn sensitive isolates are much more sensitive to Zn than Zn sensitive European *Suillus* isolates (Colpaert et al. 2000). These findings corroborate the differences across the genus *Suillus*, with some species showing much higher tolerance than others. These differences can stem from distinct soil metal levels, as the Colorado sites display much lower Zn levels compared to previously studied European sites (our most metal rich site contained 260 mg/kg Zn compared to up to 1750 mg/kg in Belgium; Colpaert et al. 2000). However, low soil metal content does not explain the differences in Cd tolerance, as our sites displayed higher levels of Cd (the most Cd rich site had 45 mg/kg Cd, compared to 14 mg/kg in Belgium; Colpaert et al. 2000).

Previous research in Belgian *S. luteus* found that isolates from geographically distinct but relatively close sites (~20 km apart) display marked differences in their ability to tolerate soil metal content, generally correlating with metal content at the site of origin (Colpaert et al. 2000). Interestingly, even though these tolerant and sensitive isolates show genetic differentiation at genes involved in metal homeostasis, whole genome analyses indicated that they all belong to the same population, with differences in metal

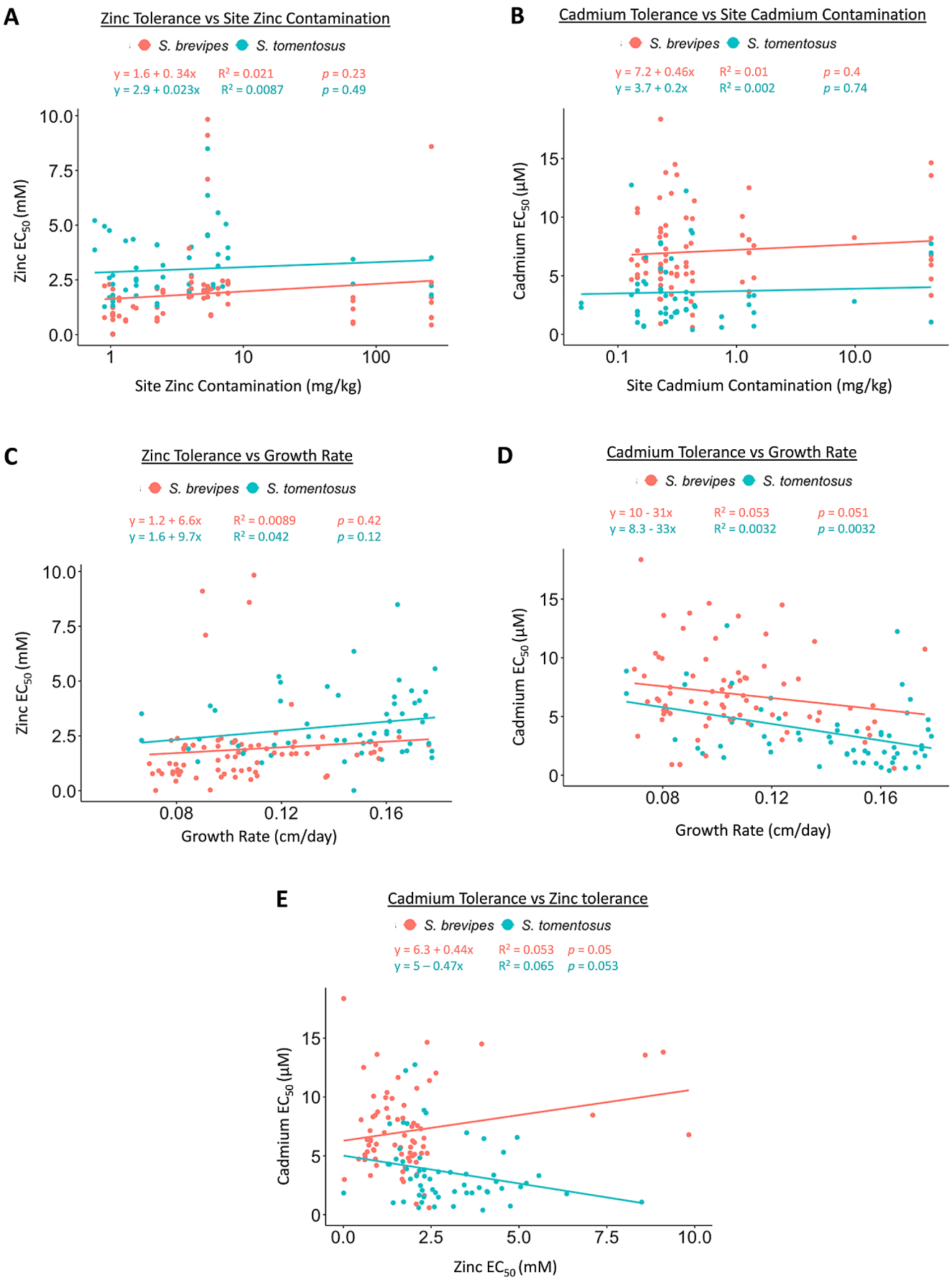
A
Zinc Tolerance vs Site Zinc Contamination
S. brevipes
S. tomentosus
y = 1.6 + 0. 34x
R² = 0.021
p = 0.23
y = 2.9 + 0.023x
R² = 0.0087
p = 0.49
Zinc EC50 (mM)
Site Zinc Contamination (mg/kg)
B
Cadmium Tolerance vs Site Cadmium Contamination
S. brevipes
S. tomentosus
y = 7.2 + 0.46x
R² = 0.01
p = 0.4
y = 3.7 + 0.2x
R² = 0.002
p = 0.74
Cadmium EC50 (µM)
Site Cadmium Contamination (mg/kg)
C
Zinc Tolerance vs Growth Rate
S. brevipes
S. tomentosus
y = 1.2 + 6.6x
R² = 0.0089
p = 0.42
y = 1.6 + 9.7x
R² = 0.042
p = 0.12
Zinc EC50 (mM)
Growth Rate (cm/day)
D
Cadmium Tolerance vs Growth Rate
S. brevipes
S. tomentosus
y = 10 - 31x
R² = 0.053
p = 0.051
y = 8.3 - 33x
R² = 0.0032
p = 0.0032
Cadmium EC50 (µM)
Growth Rate (cm/day)
E
Cadmium Tolerance vs Zinc tolerance
S. brevipes
S. tomentosus
y = 6.3 + 0.44x
R² = 0.053
p = 0.05
y = 5 – 0.47x
R² = 0.065
p = 0.053
Cadmium EC50 (µM)
Zinc EC50 (mM)

Fig. 2 Metal tolerance correlation analyses, including regression lines and equations, R^2 values and *p* values for *S. brevipes* (salmon) and *S. tomentosus* (blue). **A** No correlation between Zn EC_{50} (mM) and soil Zn soil content (mg/kg). **B** No correlation between Cd EC_{50} (μM) and soil Cd content (mg/kg). **C** Zn tolerance (EC_{50}) is not correlated with growth rate in *S. brevipes* or *S. tomentosus*. **D** Cd tolerance (EC_{50} in μM) is not correlated with growth rate in *S. brevipes* or *S. tomentosus*. **E** Cd tolerance (EC_{50} in μM) is not significantly correlated with Zn tolerance (EC_{50} in mM) in both species

tolerance thought to be a result of standing genetic variation (Bazzicalupo et al. 2020). Along with genomic variation, transcriptomic control of efflux pumps, chelators, and ROS detoxification systems seem to play an important role in *S. luteus* Zn tolerance. Specifically, metal tolerant isolates show very different gene expression patterns compared to sensitive isolates and are likely constitutively metal tolerant due to differential gene expression (Smith et al. 2023). It is yet unclear whether the *Suillus* isolates from Colorado follow a similar pattern of population structure, with one population of *S. brevipes* and another of *S. tomentosus* including sensitive and tolerant isolates, or metal tolerance mechanisms. Further research will shed light on these matters.

We were surprised by the lack of correlation between soil metal content and isolate metal tolerance. This comes in contrast with other *Suillus* studies, that document the majority of isolates from metal-contaminated sites showing higher metal tolerance (Colpaert et al. 2000, 2004; Colpaert and Vanassche 1992; Colpaert and van Assche 1987). However, these reports also detected metal sensitive *Suillus* isolates inhabiting contaminated soils, possibly due to soil heterogeneity and the potential existence of pockets with little to no metal content, and/or low metal bioavailability, allowing metal sensitive isolates to persist in polluted sites (Fomina et al. 2005; Colpaert et al. 2004; Blaudez et al. 2000a, b; Adriaensen et al. 2005). In addition, metal tolerance has been suggested to have little to no fitness costs in uncontaminated environments, which could explain the presence of tolerant isolates in non-contaminated soils (Bazzicalupo et al. 2020; Colpaert et al. 2004). We found no correlation between Zn and Cd tolerance in *S. brevipes* and *S. tomentosus*, a result also documented in *S. luteus* (Colpaert et al. 2000). One possible explanation is that tolerance to the two metals is achieved through mechanisms involving different genes and pathways. Follow up genomic and genetic investigations will contribute to clarify this possibility.

Zn tolerance in *S. brevipes* and *S. tomentosus* is rooted in exclusion, with tolerant isolates showing lower mycelium Zn compared to sensitive isolates. However, this is not reflected in sporocarp Zn content with sensitive and tolerant isolates showing similar fruitbody Zn. This suggests that *Suillus* might regulate Zn transport to the fruitbody, independently of soil Zn. Zinc exclusion was documented in *S. luteus*, with tolerant isolates taking up less metal than sensitive isolates when grown on the same concentration of metal (Colpaert et al. 2005). *Suillus luteus* sporocarp Zn content was also not correlated with soil Zn and were comparable to our results (Colpaert et al. 2000).

Previous research also showed *S. luteus* Cd tolerant isolates take up less Cd than sensitive isolates (Krznaric et al. 2009) and a correlation between soil Cd levels and sporocarp Cd content (Colpaert et al. 2000). Our results do not corroborate the former finding, however *S. brevipes* sporocarps from soils with high Cd content had more Cd in their tissue. Interestingly, *S. tomentosus* sporocarps contained significantly more Cd than *S. brevipes* sporocarps independently of the soil Cd content or Cd tolerance. This can be explained by differences in the ability to either control Cd uptake from the soil, or to sequester and manipulate Cd mobility intracellularly. Potential reasons for these differences include the presence and high efficiency of efflux pumps or more stringent uptake mechanisms. Again, deeper investigation into the genomics, transcriptomics and internal metal mobility of each species could help us understand the basis for these interspecific differences in metal tolerance. Notably, faster growing *S. tomentosus* isolates are more sensitive to Cd toxicity. This suggests that cellular metal load may be a limiting factor for tolerance. It is likely that in the absence of effective exclusion mechanisms, isolates growing more quickly may be taking up more metal from the environment, increasing cell toxicity as Cd serves no known nutritional function and is toxic at low levels (De Oliveira and Tibbett 2018). It is likely that *S. brevipes* and *S. tomentosus* rely on distinct mechanisms to achieve Cd tolerance compared to *S. luteus*, perhaps depending more on internal metal sequestration and/or detoxification instead of exclusion to persist in high metal environments. It is also possible that Cd tolerant *S. brevipes* and *S. tomentosus* have efficient extra-cellular chelation, rendering metals immobilized in the outer cell wall. Given we did not wash the mycelium before measuring, we are not able to assess whether Zn and Cd are sequestrated within the cell or on the cell wall. Washing the mycelium before determining metal content may allow to distinguish between intracellular metal and extracellularly sequestered metal. Further work focusing on metal cell localization in *S. tomentosus* and *S. brevipes* is needed to explain our results.

In summary, our investigation into Zn and Cd tolerance in North American *Suillus brevipes* and *S. tomentosus* unveils substantial diversity in metal tolerance within the genus. The observed inter- and intraspecific metal tolerance variation, coupled with comparisons to the better studied *Suillus luteus*, emphasize the complexity of metal stress responses and indicate the existence of diverse metal tolerance mechanisms within the genus. The lack of relationship between site metal level and metal tolerance challenges previous ideas of

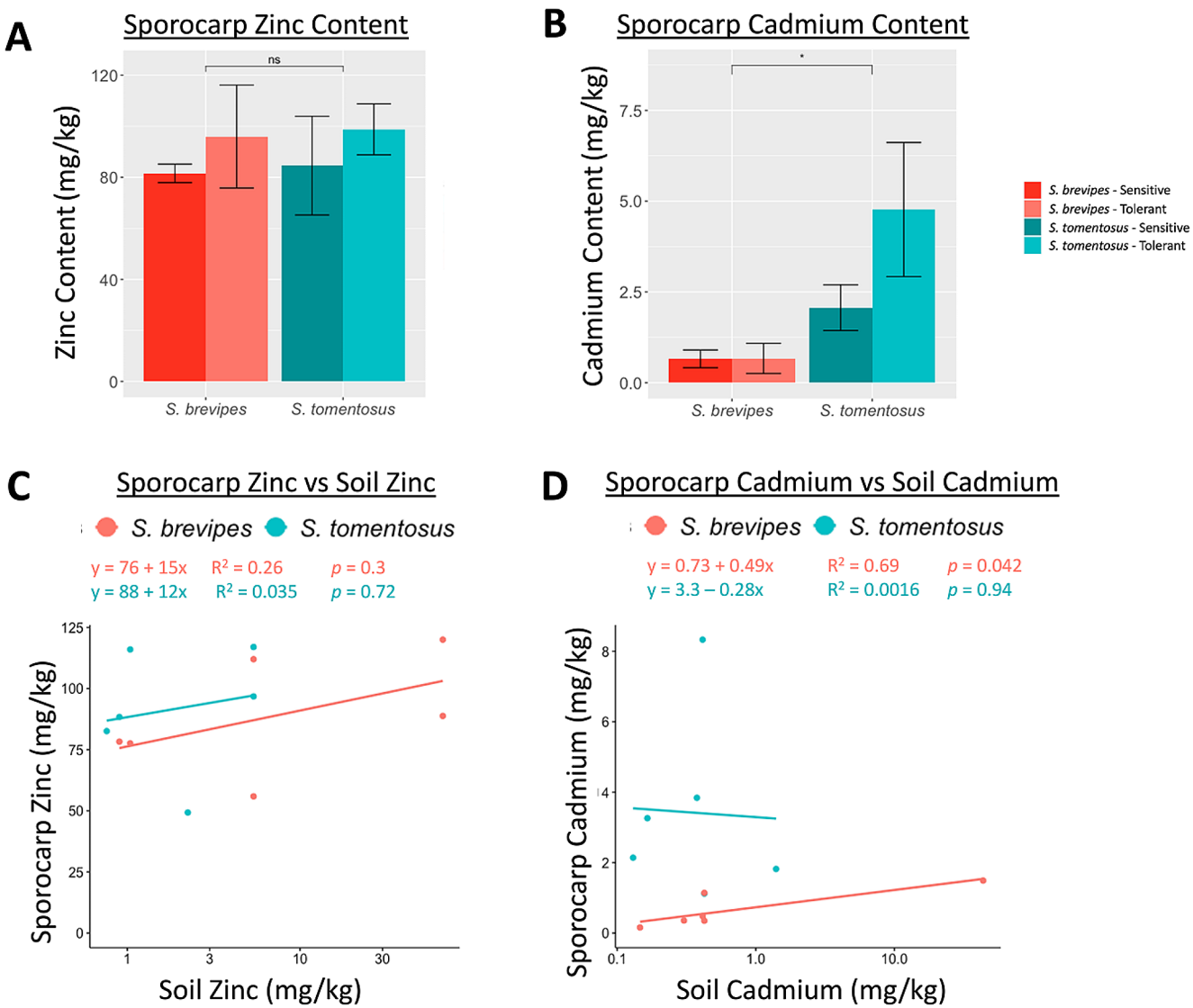

Fig. 3 *Suillus brevipes* (salmon) and *S. tomentosus* (blue) sporocarp Zn and Cd content. **A** Sporocarp Zn content was not different between *S. brevipes* (mean = 88.8 mg/kg, SD = 23.8) and *S. tomentosus* (mean = 91.7 mg/kg, SD = 25.1, $p = 0.85$) or between sensitive and tolerant isolates ($p = 0.366$). **B** Sporocarp Cd content was significantly higher in *S. tomentosus* isolates (mean = 3.42 mg/kg, SD = 2.6) compared to *S. brevipes* isolates (mean = 0.66 mg/kg, SD = 0.528$p = 0.0253$). We found no significant difference between sensitive and tolerant isolates ($p = 0.214$). **C** Sporocarp Zn content (mg/kg) was not correlated to site Zn level (mg/kg). **D** Sporocarp Cd content (mg/kg) was not correlated to site Cd level (mg/kg)

environmental adaptation at the population level and warrants deeper investigation. Moving forward, exploring the genetic basis of metal tolerance, such as exclusion, internal metal sequestration and metal detoxification, will be essential. These insights contribute not only to our understanding of *Suillus* ecology but also have broader implications for predicting fungal contributions to forest ecosystems, especially in metal contaminated sites in North America.

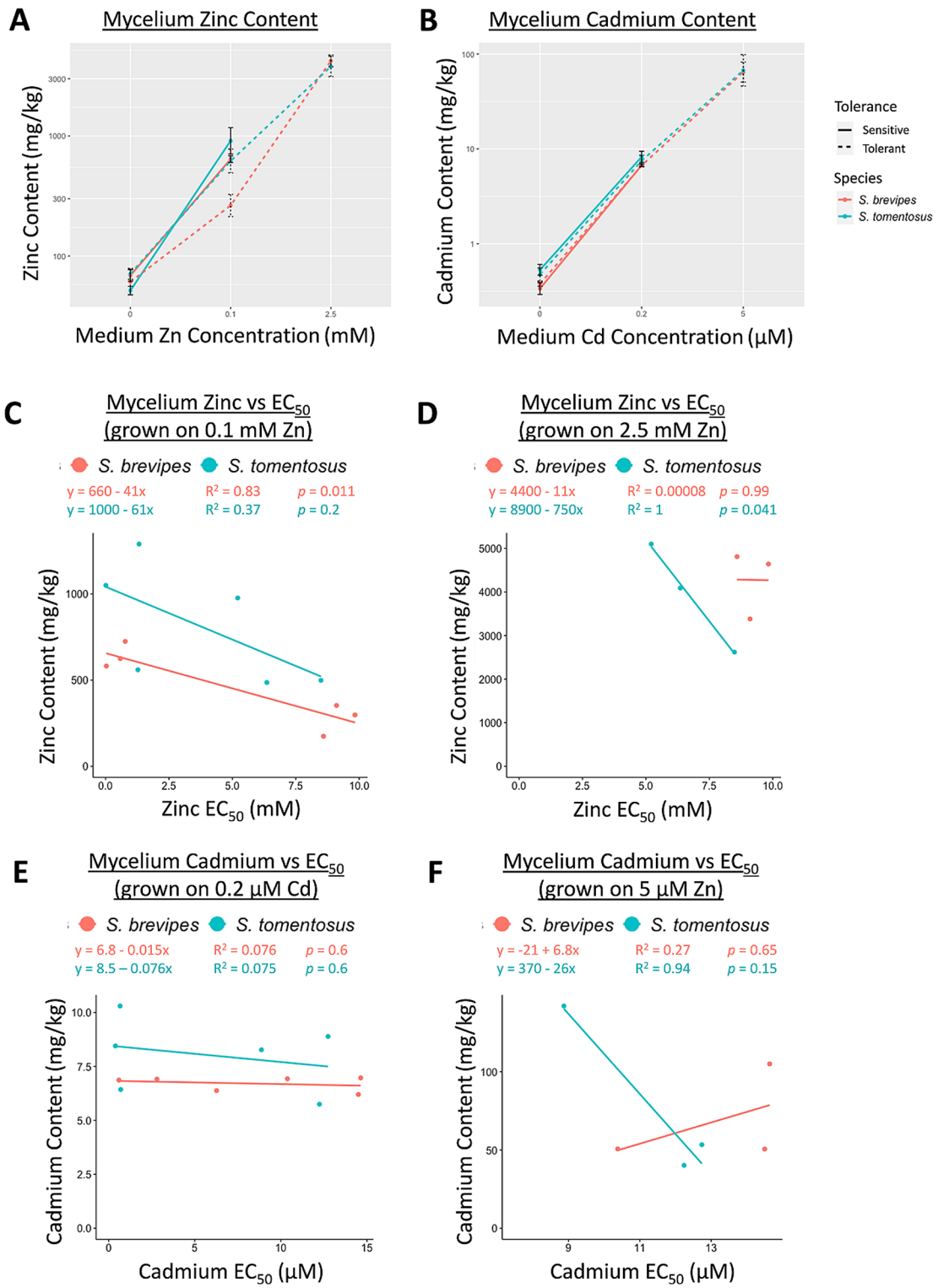

Fig. 4 *Suillus brevipes* (salmon) and *S. tomentosus* (blue) mycelium Zn and Cd content. **A** Mycelial content of isolates grown on 0, 0.1 and 2.5 mM Zn. Sensitive isolates did not grow on the highest Zn concentration. **B** Mycelial content of isolates grown on 0, 0.2, and 5 µM Cd. The sensitive isolates did not grow on the highest cadmium concentration. **C** Significant negative correlation between mycelium Zn content (mg/kg) of each isolate grown on 0.1 mM Zn agar versus isolate Zn EC_{50} (mM) *in S. brevipes* but not *S. tomentosus***D** found Significant negative correlation between mycelium Zn content (mg/kg) of each isolate grown on 2.5 mM Zn agar versus isolate Zn EC_{50} (mM) in *S. tomentosus*, but not *S. brevipes*. **E** No correlation between mycelium Cd content (mg/kg) of each isolate grown on 0.2 µM Cd agar versus isolate Cd EC_{50} (µM) for either species. **F** Significant negative correlation between mycelium Cd content (mg/kg) of each isolate grown on 5 µM Cd agar versus isolate Cd EC_{50} (µM) for *S. tomentosus*, but not *S. brevipes*

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Data availability Sequence data that support species identification in this study have been deposited in GenBank under accession numbers OR665177 to OR665292.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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