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Dark septate endophytes isolated from non-hyperaccumulator plants can increase phytoextraction of Cd and Zn by the hyperaccumulator *Noccaea caerulescens*

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Abstract

Dark septate endophytes (DSEs) can improve plant stress tolerance by promoting growth and affecting element accumulation. Due to its ability to accumulate high Cd, Zn and Ni concentrations in its shoots, *Noccaea caerulescens* is considered a promising candidate for phytoextraction in the field. However, the ability of DSEs to improve trace element (TE) phytoextraction with *N. caerulescens* has not yet been studied. The aim of this study was therefore to determine the ability of five DSE strains, previously isolated from poplar roots collected at different TE-contaminated sites, to improve plant development, mineral nutrient status and metal accumulation by *N. caerulescens* during a pot experiment using two soils differing in their level of TE contamination. Microscopic observations revealed that the tested DSE strains effectively colonized the roots of *N. caerulescens*. In the highly contaminated (HC) soil, a threefold increase in root biomass was found in plants inoculated with the *Leptodontidium* sp. Pr30 strain compared to that in the non-inoculated condition; however, the plant nutrient status was not affected. In contrast, the two strains *Phialophora mustea* Pr27 and *Leptodontidium* sp. Me07 had positive effects on the mineral nutrient status of plants without significantly modifying their biomass. Compared to non-inoculated plants cultivated on HC soil, Pr27- and Pr30-inoculated plants extracted more Zn (+30%) and Cd (+90%), respectively. In conclusion, we demonstrated that the responses of *N. caerulescens* to DSE inoculation ranged from neutral to beneficial and we identified two strains (*i.e.*, *Leptodontidium* sp. (Pr30) and *Phialophora mustea* (Pr27)) isolated from poplar that appeared promising as they increased the amounts of Zn and Cd extracted by improving plant growth and/or TE accumulation by *N. caerulescens*. These results generate interest in further characterizing the DSEs that naturally colonize *N. caerulescens* and testing their ability to improve phytoextraction.

Keywords: Fungal endophytes, Hyperaccumulation, Mineral nutrition, Phytoextraction, Plant growth, Trace elements

Introduction

Noccaea caerulescens (J. & C. Presl) F.K. Mey. (Brassicaceae) is a European pseudometallophyte that is known to hyperaccumulate trace elements (TEs) and is able to accumulate $>20,000$ mg Zn kg⁻¹, 2000 mg Cd kg⁻¹ and 10,000 mg Ni kg⁻¹ in its shoots. As a model species, it has considerably expanded our knowledge of metal hyperaccumulation and tolerance mechanisms (Assunção et al., 2018; Krämer, 2010; Peng et al., 2020; Verbruggen et al., 2018). Due to its abilities, *N. caerulescens* is also considered a promising candidate for field phytoextraction of Cd and/or Zn in moderately contaminated soils (Hammer and Keller, 2003; Jacobs et al., 2017, 2018a; Rosenfeld et al., 2018; Schwartz et al., 2003; Simmons et al., 2015; Sterckeman and Puschenreiter, 2018). Recent studies have been carried out to improve the efficiency of metal extraction by *N. caerulescens*, which depends on both shoot trace element concentrations and plant biomass, by (i) understanding the influence of edaphic conditions on the growth and accumulation of TEs (Jacobs et al., 2019; Wang et al., 2006; Yanai et al., 2006), (ii) initiating the selection of cultivars for field applications (Sterckeman et al., 2019), and (iii) testing and improving cultural practices (Jacobs et al., 2018b; Luo et al., 2019a, 2019b, 2020), including organic and inorganic fertilization (Jacobs et al., 2018a; Maxted et al., 2007a; Rees et al., 2020).

Recent approaches to the optimization of phytoextraction systems noted the importance of cooperation between hyperaccumulators and microorganisms that show plant growth-promoting activity and/or metal tolerance and accumulation (Khan, 2005). Among these microorganisms, plant growth-promoting rhizobacteria (Hansda et al., 2014) and endophytic bacteria (Ma et al., 2016), arbuscular mycorrhizal fungi (Miransari, 2017), ectomycorrhizal fungi (Gil-Martínez et al., 2018) and dark septate endophytes (DSEs) (Deng and Cao, 2017) are known as major drivers of plant growth and tolerance to abiotic stress and resistance to phytopathogens (Kong and Glick, 2017; Otero-Blanca et al., 2018). The positive effects of these strains on plant growth and metal bioavailability can markedly improve metal accumulation in plant tissues, thus positively influencing phytoextraction at metal-polluted sites (Ma et al., 2019; Phanthavongsa et al., 2017; Rajkumar et al., 2012).

The diversity of these auxiliary microorganisms associated with *N. caerulescens* has been poorly investigated (Visioli et al., 2015a). Only the belowground bacterial diversity associated with *N. caerulescens* has been recently investigated through 16S rDNA profiling (Visioli et al., 2019). Additionally, culture-dependent techniques have enabled the isolation and characterization of several promising strains, such as metal-resistant endophytic bacteria (Fones et al., 2016; Visioli et al., 2014) or bacterial endophytes with plant growth-promoting traits (Burgess et al., 2017), and the testing of their potential to improve biomass production and metal accumulation (Burgess et al., 2017; Visioli et al., 2015b). The latter studies showed that the tested strains, which belonged to *Arthrobacter*, *Microbacterium*, *Variovorax* and *Micrococcus*, were efficient in promoting plant growth, soil Ni removal and translocation (Visioli et al., 2015b) or improving the physiological status of host plants by increasing their content of chlorophylls and carotenoids (Burgess et al., 2017). As *N. caerulescens* is a non-mycorrhizal plant (Cosme et al., 2018), the *N. caerulescens* fungal microbiota has been poorly studied (Coles et al., 1999; David et al., 2000; Kidd et al., 2018). However, DSEs, which are abundant in stress-inducing environments, can colonize the roots of non-mycorrhizal plants (Liu et al., 2017) and induce a wide range of host plant responses to fungal colonization (Berthelot et al., 2019; Wang et al., 2016). In numerous cases, DSEs can be considered mutualistic fungi (Newsham, 2011) that may also be beneficial for their host plant (Berthelot

et al., 2019). They are known to facilitate P, N, and C uptake (Vergara et al., 2018; Yakti et al., 2018), release phytohormones such as auxin (Berthelot et al., 2016; Khan et al., 2011), protect plants against biotic and abiotic stress (Likar and Regvar 2013; Wang et al., 2016) and improve the phytoextraction of TEs from soils (Lindblom et al., 2017; Shi et al., 2017). To our knowledge, the only reports focusing on the effect of DSEs on the development and phytoextraction potential of *N. caerulescens* deal on the Ni phytoextraction using serpentine ecotype of the species (Kidd et al. 2018; Rozpadek et al. 2017). Thus, there is no data reporting on the effect of DSEs on Cd and Zn phytoextraction using metallicolous ecotype of *N. caerulescens*.

Based on DSE strains that were previously isolated from poplar roots collected at TE-contaminated sites, our work aimed to (i) check their ability to colonize the roots of the non-host hyperaccumulating plant *N. caerulescens*, (ii) study their effects on overall plant growth, leaf pigment contents and mineral nutrient status, and (iii) determine their impacts on the accumulation and phytoextraction of TEs.

Materials and Methods

Soil and plant materials

Pot experiments were conducted with soils collected from the upper horizons at two TE-contaminated sites. The first soil, called LC soil for “low contamination”, originated from the Pierrelaye–Bessancourt plain (located approximately 25 km northwest of Paris, France). This 1,200 ha agricultural plain was historically irrigated for a century by wastewater from Paris (Baize et al., 2002), resulting in an increase in the Cu, Zn, Pb and Cd contents in soils; however, the topsoil concentrations do not exceed three times the local background concentrations (Table 1, Saby et al. (2020)). The second soil, called HC soil for “high contamination”, originated from the town of Aubry (northern France). It was highly contaminated due to metal emissions from the smelters of local metallurgic plants, resulting in concentrations of Zn, Pb and Cd that are 250, 70 and 50 times higher than the local background concentrations, respectively (Table 1, Saby et al. (2020)). The physico-chemical characterization of the soils was performed by the INRAE LAS Laboratory according to international standard methods. The results are summarized in Table 1. Once collected, the soils were homogenized, sieved to <2 mm, dried at ambient temperature, sterilized (two runs of autoclaving) and finally divided into 6 aliquots. The local background values were obtained from spatial predictions of the total trace element content in France (Saby et al., 2020).

For this study, the *N. caerulescens* seeds originated from the well-studied metallicolous Ganges population that developed on a highly contaminated mining site (Southern France, 43°56'10.98"N 03°40'19.88"E) (Gonneau et al. 2014; Sterckeman et al. 2017). The seeds were sown in a sterilized mix of commercial garden compost (75%, v/v) and sand (25%, v/v) and placed in a growth chamber. Seven weeks after germination, plants at the four-to-six leaf stage that had a similar developmental phenotype were selected for the pot experiment.

Table 1. Physico-chemical characteristics and total concentrations of trace and major elements in a composite soil sample of the LC and HC soils used for this study and the local background of each of the studied soils.

	pH	C _{org}	N _{org}	C/N	CaCO ₃ tot	CEC	K ⁺	Ca ²⁺	Mg ²⁺	Na ⁺	Cd	Cu	Ni	Pb	Zn	
		%	%		%		cmol+/kg					mg/kg				
LC soil	8.1	10.0	0.87	12	3.3	7.5	0.6	31	0.73	0.1	0.67	27	11	55	117	
background											0.23	11	16	33	40	
HC soil	6.0	3.2	0.13	26	<1	12.9	0.6	7.7	1.26	0.1	18.2	96	24	902	3290	
background											0.36	13	17	34	61	

Soil inoculation and experimental design

In the present study, we tested five fungal strains of DSE, which were isolated from poplar roots collected at Cd/Zn-contaminated sites (Berthelot, 2017). The Pr27 (*Phialophora mustea* Neerg., 1942), Pr28 (*Phialocephala fortinii* C.J.K. Wang & H.E. Wilcox, 1985) and Pr30 (*Leptodontidium* sp.) strains originated from the Pierrelaye–Bessancourt site (Berthelot et al., 2016). Me07 (*Leptodontidium* sp.) originated from the Auby site (Berthelot et al., 2016). Epn (*Epicoccum nigrum* Link, accession number MT295307) was isolated from Jeandelaincourt (Meurthe-et-Moselle, France). Prior to the inoculation procedure, the five fungal strains were individually grown on malt-agar extract medium at 24°C for three weeks. For each strain, 20 agar plugs of 1 cm² with fungal mycelium were transferred to sterilized glass flasks of 500 mL containing 75% perlite and 25% liquid malt extract medium (v/v). Then, the flasks were stored in a growth chamber at 24°C for two weeks and shaken three times a week to ensure homogenous growth of the fungus throughout the substrate. The five fungal inocula were individually mixed (10%, v/v) with previously sterilized aliquots of LC and HC soils. As a control condition (CTRL), we mixed one aliquot of each soil with perlite containing fungus-free agar plugs. Five pots were established for each treatment.

After clearing the roots to remove residual compost, the *N. caerulescens* seedlings were transplanted into 60 plastic pots (2 soils x 6 treatments x 5 replicates) of 500 mL (8.6 cm of diameter) previously loaded with 400 g of LC or HC soil. To prevent nutrient leaching through drainage and soil slumping due to the high soil water content, water was supplied to the pot from a 10 L plastic box filled with deionised water through a 15 mm diameter fibreglass wick passed through a central hole at the base of each pot. Each plastic box containing 12 randomized pots was then placed in a growth chamber with 16 hours of light at 22°C and 8 hours of darkness at 18°C, 70% relative humidity and a photon flux density of 196 μmol photons m⁻² s⁻¹ in the PAR range.

Estimation of NBI and pigment indices

Leaf pigments (*i.e.*, chlorophylls, flavonoids and anthocyanins) and the nitrogen balance index (NBI) were quantified using a DUALEX® SCIENTIFIC+ dual excitation fluorimeter (Force-A, Orsay, France). The chlorophyll level was estimated from 710 nm light

transmittance, corrected for interference from other leaf structures by dividing the transmittance at a reference wavelength of 850 nm. The amount of polyphenols was estimated from the difference between the chlorophyll fluorescence and the fluorescence induced by UV (360 nm) for flavonoids and red light (650 nm) for anthocyanins (Cerovic et al., 2012). The NBI index, corresponding to the chlorophyll/flavonoid ratio, provides information about the leaf nitrogen status of plants. These non-destructive measurements were carried out on five mature leaves per plant, representing 25 measurements per treatment.

Plant harvesting, biomass determination and verification of fungal colonization

After 12 weeks of growth, aboveground and belowground plant parts were harvested and separated. Shoots were thoroughly washed with tap water and rinsed with deionized water. Roots were carefully separated from the soil, washed with tap water and immersed in a shaken tetrasodium diphosphate (37.6 mM) bath for 16 hours to remove the most persistent soil particles. For each plant, a known weight of fresh root samples was stored in 70% (v/v) ethanol for microscopic observations of fungal structures. To confirm the colonization of plant roots by the inoculated strains, selected root segments were cut into 10 mm pieces and stained with Trypan blue 0.5% (v/v) (Trouvelot et al. 1986) or labelled with 20 µg/ml WGA-AF®488 (Invitrogen, France) (Berthelot et al. 2016) and observed by optical or fluorescence microscopy, respectively. The remaining root and shoot samples were then dried at 70°C for 48 hours. Once dried, they were carefully weighed using a Mettler AE 163 analytical balance.

Analyses of trace and major elements in plant samples

Before the analyses, dried plant samples were ground into a homogenous powder in an MM40 mixer mill (Retsch, Eragny sur Oise, France) at 30 Hz for 5 min. For each sample, 0.5 g of plant material was digested in a 50 mL tube using 2.5 mL of HNO₃ and 5 mL of H₂O₂ (DigiPREP® system; SCP Science, Baie-d'Urfé, QC, Canada) and allowed to stand at ambient temperature for 16 hours. Tubes were then heated to 95°C for two hours. The digested samples were filtered to 0.45 µm and brought to 25 ml with ultra-pure water. Trace (*i.e.*, Cd, Cu, Pb, Ni and Zn) and major (*i.e.*, Al, Ca, Fe, K, Mg, Na, P and S) elements were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, iCap 600, Thermo Fischer Scientific, Inc., Pittsburgh, USA). Control samples from *N. caerulea* with known compositions (internal analyses carried out by INRA-USRAVE, Villenave d'Ornon, France), as well as a certified solution (EU-H-2, SCP Science, Courtaboeuf, France), were included in all analyses as quality controls.

Statistical analyses

Statistical analyses were performed with R software v.3.5.1 (R Development Core Team, 2013). All statistical tests were considered significant at $P \leq 0.05$. For each soil, a principal component analysis (PCA) was used to evaluate the influence of the five tested strains on i) plant shoot and root biomass production, ii) leaf pigments and NBI and iii) concentrations of trace and major elements in roots and shoots. The latter variables and the amount of extracted TEs (concentration of TEs in leaves x biomass) were then analysed by

comparing the CTRL condition with the five fungal treatments using an analysis of variance (ANOVA) followed by a Dunnett test in the “multcomp” package. The adequacy of the data from each measurement with respect to the ANOVA models was verified by examining the residuals for their independence (Durbin-Watson test, “car” package), normality (Shapiro test) and homogeneity (Levene test). If the latter assumptions were not validated after the “sqrt” or “log” transformation of the data, a Wilcoxon pairwise test with a Holm correction of the p-value was used. For all the DSE treatments together, as well as for each treatment individually, Pearson’s correlation coefficients were calculated to determine the relationships between the studied parameters, with no distinction between the soils.

Results

Observation of fungal structures within *N. caerulescens* roots

After the inoculation of the *N. caerulescens* plants with the five DSE strains (EPN, Me07, Pr27, Pr28 and Pr30), the presence of melanized fungal structures inside the stained root fragments was observed (**Fig. 1b-c**). Typical dark septate hyphae and microsclerotia were observed for all fungal treatments (**Fig. 1**). WGA labelling enabled a better detection of twisting hyphae (**Fig. 1a-d**). All the fungal structures were detected in the cortical cells of inoculated plants. No fungal structures were found in the non-inoculated condition (CTRL).

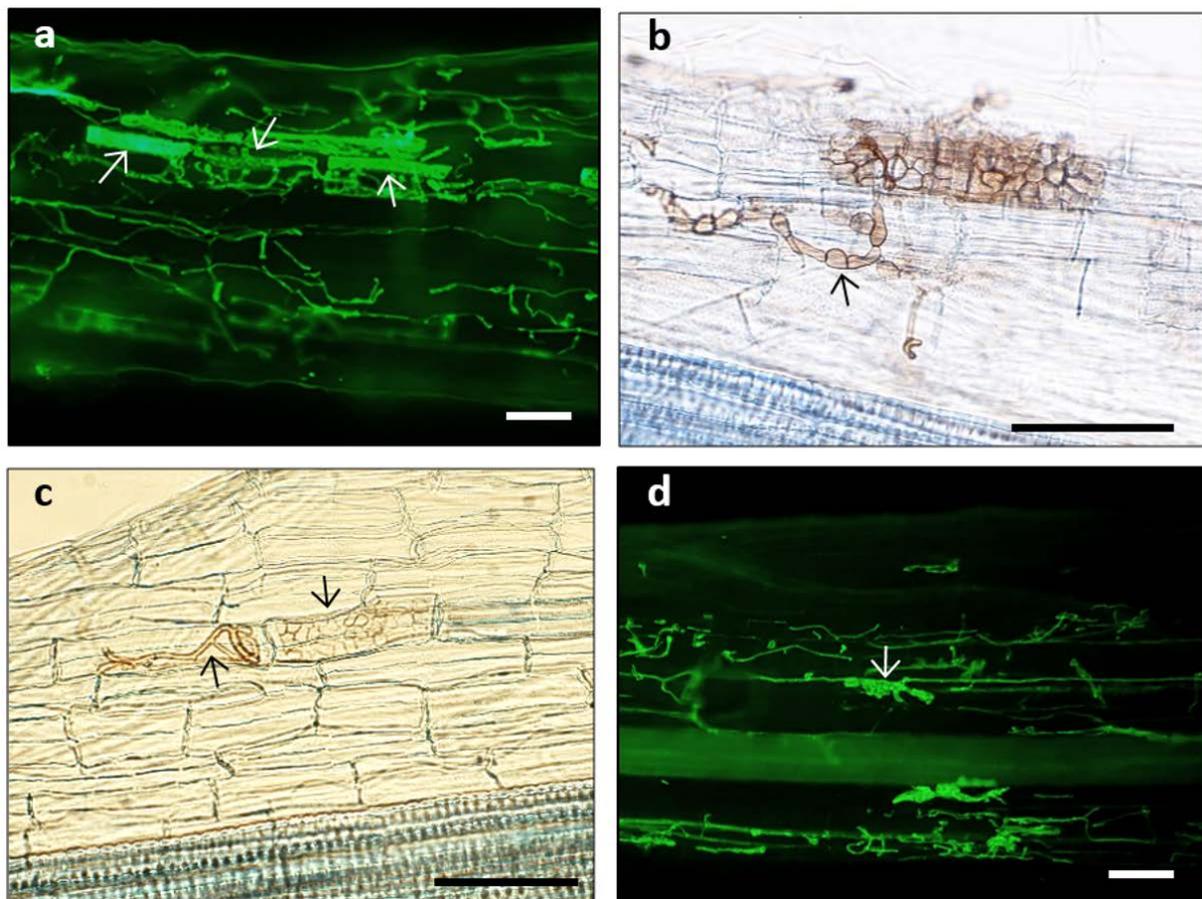


Fig. 1 Microscopic observations (x200) of fungal structures within cortical cells of *N. caerulescens* inoculated with DSE. Trypan blue-stained roots were observed by optical microscopy (b-c), whereas fungal structures labelled with WGA-AF488 were observed by confocal microscopy (a-d). For most of the strains, typical hyphae (a-b-c-d), loosely packed

(b-d) and fully packed (a-c) microsclerotia were observed in the cortical cells of inoculated plants. Scales bars = 25 μ m.

Effect of fungal strains on the growth of *N. caerulescens*

At the end of cultivation, the *N. caerulescens* biomass ranged between 0.6 and 2.6 g DW for roots and between 1.1 and 2.5 g DW for leaves (Fig. 2). The biomass parameters were not influenced by the TE level in the soil. As suggested by the PCA for the HC soil, the contribution of variables related to growth parameters (*i.e.*, dry shoot biomass (DSB) and dry root biomass (DRB)) to the variability was low (Fig. 3a). Only Pr30-inoculated plants grown in the HC soil showed a significant effect on biomass production, with a threefold increase in the mean root biomass compared to that in the CTRL condition (Fig. 2a). This effect was not observed in plants grown on the LC soil. Other strains, regardless of the soil, did not significantly influence root growth. For both soils, plants inoculated with Me07 and Pr27 tended to produce more root biomass; however, the substantial inter-sample variability did not allow us to confirm this result statistically (Fig. 2a). Compared to the CTRL condition, the DSE strain treatments did not significantly influence the shoot biomass (Fig. 2b).

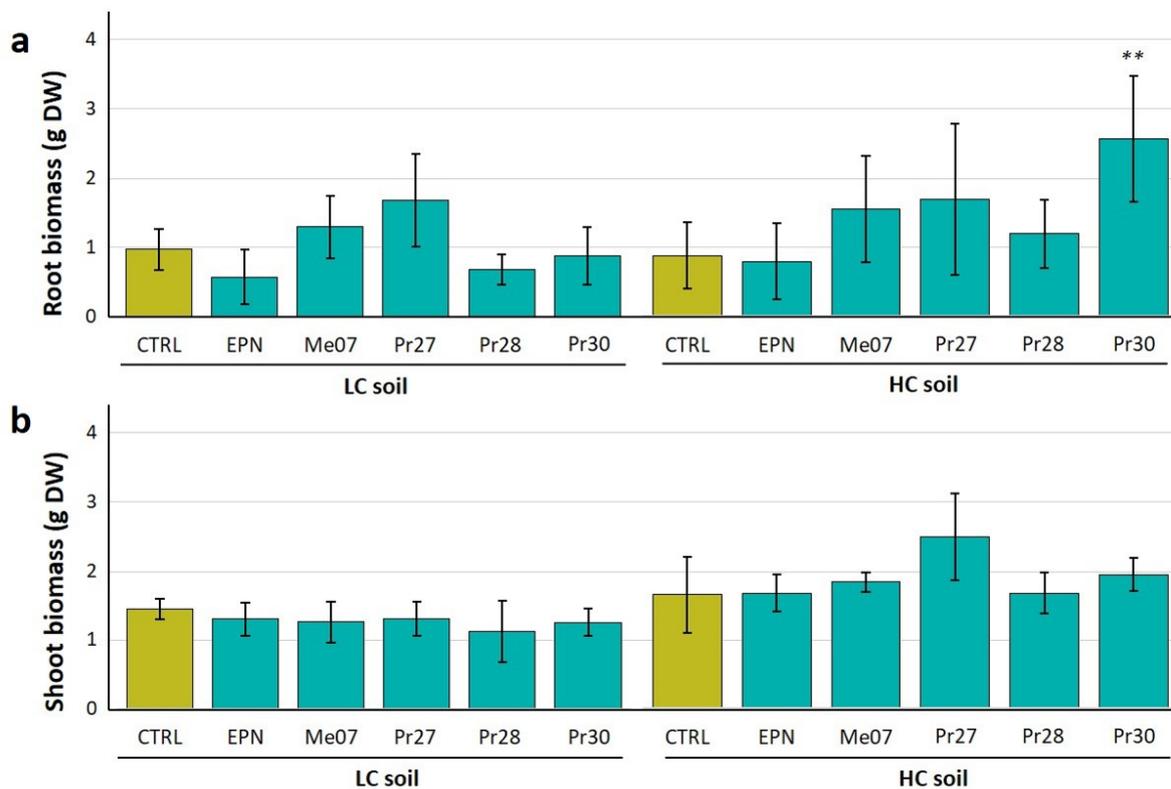


Fig. 2 Effect of DSE strains on root (a) and shoot (b) biomass production in *N. caerulescens*. Plants were grown for 12 weeks in soils with either low (LC) or high (HC) metal contamination and were either inoculated with a DSE strain or mock-inoculated (CTRL). Data are the means \pm SDs ($n = 5$). Significant differences (ANOVA and Dunnett's test) between the CTRL condition and the fungal treatments are represented with asterisks ($P < 0.01$).

Effect of fungal strains on the content of leaf pigments and the NBI of *N. caerulescens*

Due to the high inter-sample variability, no significant influence of the DSE strains on the content of leaf pigments or on the NBI could be found (**Fig. 3**). However, when compared to the non-inoculated condition, inoculation with the five strains tended to decrease the chlorophyll index and to increase the anthocyanin index of plants grown in the LC soil. The flavonoid index did not show any particular trend (**Fig. 3**).

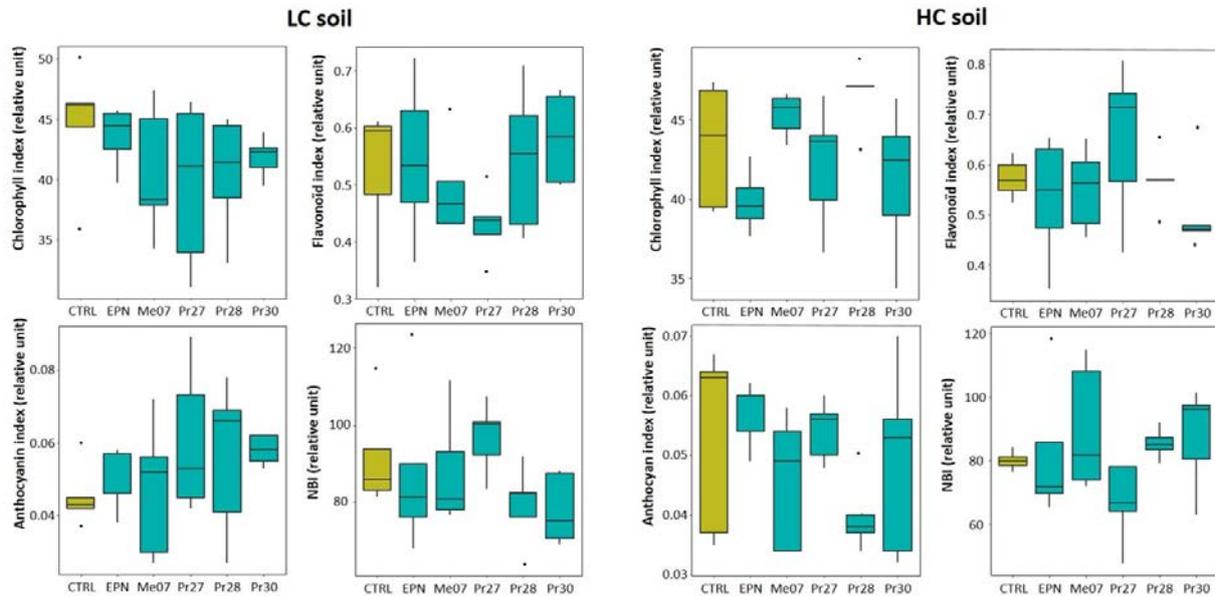


Fig. 3 Effect of DSE strains on the leaf pigment contents and nitrogen balance index (NBI) of *N. caerulescens*. Plants were grown in soils with either low (LC) or high (HC) metal contamination and were either inoculated with a DSE strain or mock-inoculated (CTRL). Data are the means \pm SDs ($n = 5$). Statistical analyses (ANOVA and Dunnett or Wilcoxon pairwise tests) revealed no significant difference ($P > 0.05$) between CTRL and the fungal treatments regardless of the parameter studied.

Effect of fungal strains on the mineral nutrients in *N. caerulescens*

According to the PCA, the group corresponding to plants inoculated with Me07 differed from the other groups due to its different nutritional status (**Fig. 4**). Indeed, for plants grown on the HC soil, inoculation with Me07 significantly increased the root concentrations of K (94%), Mg (22%) and S (30%) but reduced that of Ca (16%) compared with the control condition (**Table S1**). Other tested strains did not significantly modify the concentration of macroelements in the roots of plants grown on the HC soil. For plants grown on the LC soil, Pr27 significantly modified the content of nutrients by increasing the Ca (+46%) and decreasing the K (-51%) concentrations in roots (**Table S1**). In the latter treatment, much higher concentrations of Fe (+314%) than in the mock-inoculated treatment were also found (**Table S1**). Pr28-inoculated roots had a higher Ca (+22%) concentration than those in the mock-inoculated treatment. The previous trend, suggesting that Me07, Pr27 and Pr28 modified the concentrations of macroelements in roots, was not observed in leaves. For the LC soil, fungal inoculation did not affect the concentration of macroelements in leaves (**Table S1**). Concerning the HC soil, the EPN strain was the sole strain that significantly affected the mineral nutrient status of *N. caerulescens* by reducing the concentration of Ca in leaves by 38%.

When considering both the mock-inoculated condition and all the DSE treatments together, the concentrations of most of the macroelements, particularly Ca, Fe, Mg and Na, were positively correlated with each other ($P < 0.05$) in the roots of *N. caerulea* (**Fig. 5a-b**). In leaves, comparable results were observed for the concentrations of K, S and P, although only in the fungal treatments (**Fig. 5a-b**). The dry shoot biomass was negatively correlated with Ca ($R = -0.37$), Mg ($R = -0.35$) and Na ($R = -0.53$) concentrations in the roots of DSE-inoculated plants (**Fig. 5b**), but we did not detect such a correlation for the mock-inoculated condition (**Fig. 5a**).

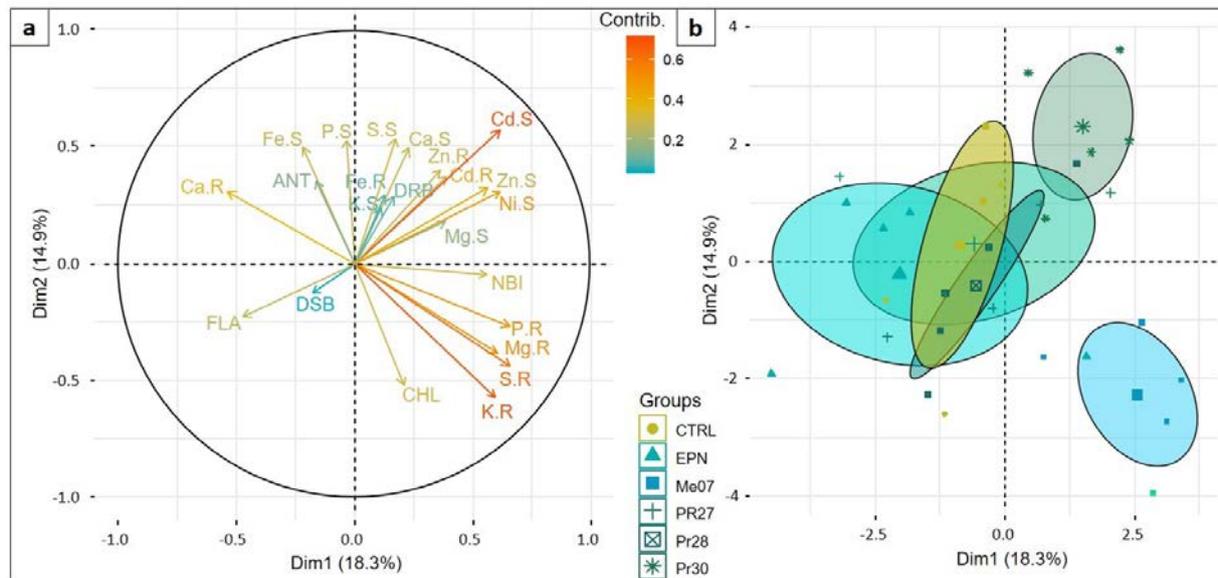


Fig. 4 Principal component analysis including plant biomass (*i.e.*, dry shoot biomass (DSB) and dry root biomass (DRB)), concentrations of trace and major elements in roots (.R) and shoots (.S), pigment indices (*i.e.*, anthocyanins (ANT, chlorophyll (CHL) and flavonoids (FLA)) and NBI variables for *N. caerulea* grown in the high-contamination soil. a) Projections of element vectors on principal components 1 and 2 with the degree of contribution indicated by the colour. b) Projection of the groups (*i.e.*, plants inoculated with fungal strains and mock-inoculated (CTRL) plants) on principal components 1 and 2, with confidence ellipses (b).

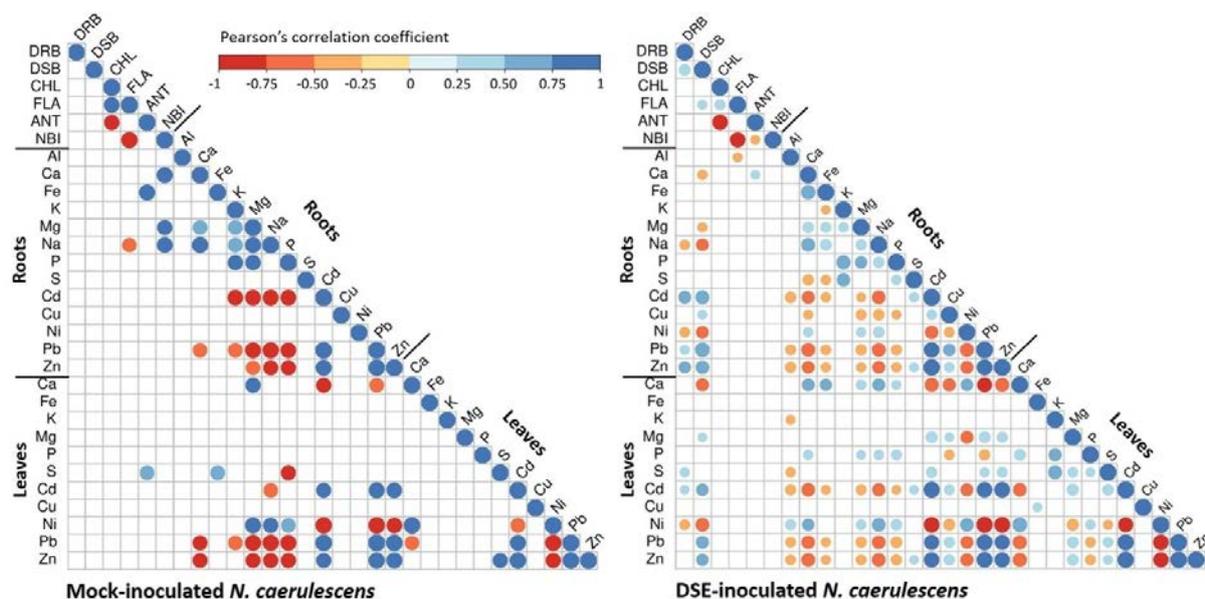


Fig. 5 Correlograms based on Pearson's correlations. The data include the plant biomass (*i.e.*, dry shoot biomass (DSB) and dry root biomass (DRB)), concentrations of trace and major elements in roots and shoots, pigment indices (*i.e.*, anthocyanins (ANT), chlorophyll (CHL) and flavonoids (FLA)) and NBI variables for the mock-inoculated (a) and all DSEs combined (b) conditions. Significant correlations ($P < 0.05$) were represented by circles with colours corresponding to the value of the Pearson's correlation coefficient (*i.e.*, R).

Effect of fungal strains on the accumulation and extraction of TEs by *N. caerulescens*

The Cd and Zn concentrations in the above- and belowground tissues of *N. caerulescens* were significantly (3- to 7-fold) higher in plants grown on the HC soil than those in plants grown on the LC soil (**Fig. 6**). For plants grown on the HC soil, the mean concentration of Cd in roots and shoots ranged from 285 to 590 mg/kg DW and from 132 to 216 mg/kg DW, respectively, which corresponds to values 8- to 33-fold higher than those found in the soil. The Zn concentrations in roots and shoots varied from 2487 to 3895 mg/kg DW and from 3243 to 3784 mg/kg DW, respectively, and these concentrations were similar to those in the soil. Unlike Cd and Zn, and despite its higher concentration in the HC soil, Ni accumulated more in plants grown on the LC soil, with concentrations in tissues up to 3-fold higher than those in the soil (**Table S1**). High concentrations of Pb, ranging from 500 to 700 mg/kg DW, were recorded in the roots of plants grown on the HC soil. These values are approximately 30-fold those recorded in the leaves. Cu also mainly accumulated in roots, with comparable values for plants grown in both soils (**Table S1**). When considering all the treatments, the Cd, Pb and Zn concentrations were strongly correlated in both roots and leaves ($R > 0.75$) (**Fig. 4a-b**).

The fungal treatments did not affect the TE concentrations in either roots or shoots of plants grown on the LC soil (**Fig. 6**). When considering the HC soil dataset, and according to the PCA, the group corresponding to plants inoculated with Pr30 tended to differ from the other groups due to differences in their TE accumulation (**Fig. 3**). Indeed, the Cd (590 mg/kg DW) and Zn (3895 mg/kg DW) concentrations in roots of Pr30-inoculated plants grown on the HC soil were 2.1- and 1.6-fold higher, respectively, than those measured in plants in the non-inoculated condition (**Fig. 6a**). Compared to those in the mock-inoculated condition, higher concentrations of Ni were measured in the leaves of Pr30-inoculated plants (**Table**

S1). Me07, Pr27 and Pr28 also tended to increase the Cd concentration in the roots of plants grown in the HC soil, but the increase was only significant in the Me07 treatment (**Fig. 6**). Positive correlations were found between the TE concentrations and the plant biomass of fungal-inoculated plants (**Fig. 5b**), whereas such correlations were not found in the control treatment (**Fig. 5a**), suggesting a positive influence of the fungal strain on the TE extraction. These positive correlations were the most pronounced in Pr30-inoculated plants (**Fig. S1**).

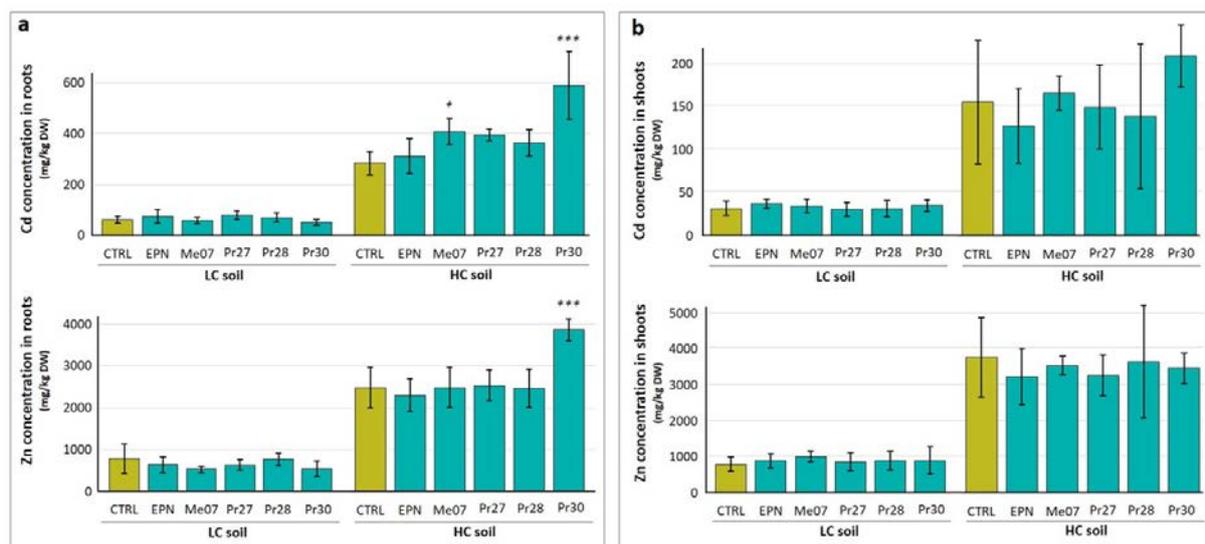


Fig. 6 Effect of DSE strains on Cd and Zn concentrations in roots (a) and shoots (b) of *N. caerulescens*. Plants were grown for 12 weeks in soils with either low (LC) or high (HC) metal contamination and were either inoculated with a DSE strain or mock-inoculated (CTRL). Data are the means \pm SDs ($n = 5$). Significant differences (ANOVA and Dunnett or Wilcoxon pairwise tests) between the CTRL condition and the fungal treatments are represented with the following symbol: $P < 0.001$ (***)).

N. caerulescens extracted more TEs when grown on the HC soil, except for Ni, which accumulated more in plants grown on the LC soil. Pr27-inoculated plants grown on the HC soil extracted 1.6- and 1.3-fold higher amounts of Cd (not significant) and Zn ($P < 0.05$), respectively, than the mock-inoculated plants (**Fig. 7b**). In the same soil, Pr30 significantly improved the extraction of Cd by 1.9-fold compared to that in the non-inoculated condition (**Fig. 7a**). This result is corroborated by strong positive correlations between Cd and Zn concentrations and biomass for Pr30-inoculated plants (**Fig. S1**). Pr30 and Pr27 also significantly increased the extraction of Ni in plants grown on the HC soil, for which the Ni concentrations in plant tissues were low (**Table S2**).

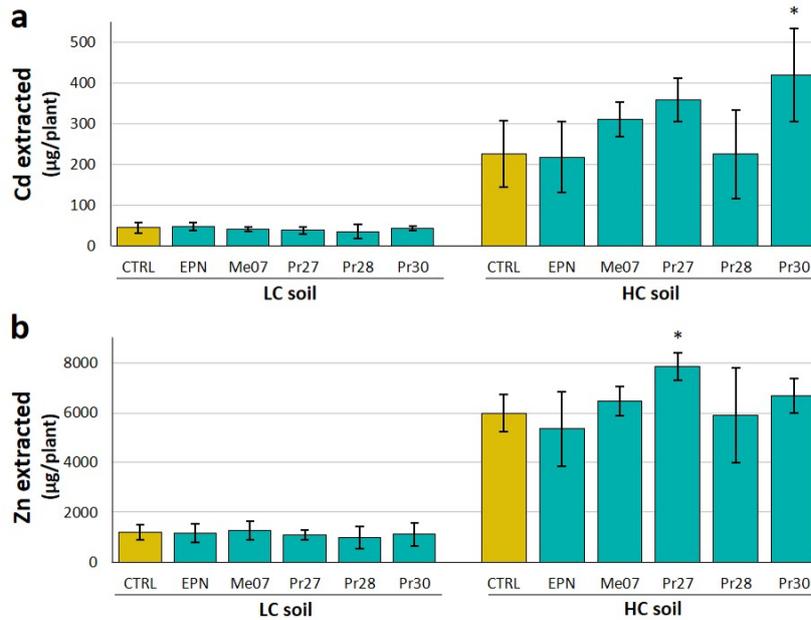


Fig. 7 Effect of DSE strains on the amount of Cd (a) and Zn (b) extracted by leaves of *N. caerulescens* from the two studied soils. Plants were grown for 12 weeks in soils with either low (LC) or high (HC) metal contamination and were either inoculated with a DSE strain or mock-inoculated (CTRL). Data are the means \pm SDs ($n = 5$). The results from the statistical comparisons (ANOVA and Dunnett or Wilcoxon pairwise tests) between the CTRL condition and the fungal treatments are represented with the following symbol: $P < 0.05$ (*).

Discussion

In the present study, we tested the effect of five DSE strains isolated from non-hyperaccumulator plants on the growth, mineral nutrient status and TE accumulation of the hyperaccumulator *N. caerulescens*. Several studies have highlighted the positive effect of DSEs on plant growth (Li et al., 2019; Suroño and Narisawa, 2017; Vergara et al., 2017) and on the alleviation of heavy metal stress (Diene et al., 2014; Wang et al., 2016). *Leptodontidium* sp. (Pr27 and Pr30), *Phialophora mustea* (Pr27) and *Phialocephala fortinii* (Pr28) belong to the Helotiales. They are known colonizers of tree roots (Berthelot et al., 2017; Chu et al., 2019; Fernando and Currah, 1996; Likar and Regvar, 2013; Stroheker et al., 2018) and, to a lesser extent, of herbaceous plants (Berthelot et al., 2016; Suroño and Narisawa, 2018; Węzowicz et al., 2017; Zhu et al., 2018). Pr27, Pr28, Pr30 and Me07 have been previously demonstrated to be tolerant to As, Cd, Mn and Zn (Berthelot et al., 2016). *Epicoccum nigrum* (EPN, Pleosporales) is commonly present in crops (Camatti-Sartori et al., 2005; Martini et al., 2009; Musetti et al., 2011) and has been employed as a biocontrol agent in the management of plant diseases caused by pathogenic fungi (Cal et al., 2009; Li et al., 2013; Mari et al., 2007).

By microscopic observations, we confirmed that the inoculated strains successfully colonized the roots of *N. caerulescens*, forming typical DSE structures such as looping and twisting hyphae and microsclerotia, easily visible after WGA labelling. Two types of microsclerotia were observed in roots i.e. fully packed and loosely packed. Loosely packed microsclerotia were the most abundant structure across all treatments. Our results regarding

mineral nutrient status and biomass production suggest that the responses of the host plant to colonization by the tested DSEs ranged from neutral to beneficial. According to the “mutualism-parasitism continuum” paradigm (Mandyam and Jumpponen, 2015), plant-DSE associations induce host responses ranging from mutualistic interactions, in which DSEs are beneficial to the host plant, to parasitic interactions, which lead to a deleterious impact for the host plant (Berthelot et al., 2019; Schulz and Boyle, 2005). Several studies have highlighted that the success of inoculation is thought to be mainly determined by the host-symbiont combination of the plant and the fungal species (Berthelot et al., 2016, 2017; Fernando and Currah, 1996; Mayerhofer et al., 2013; Newsham, 2011). Indeed, endophytic fungal communities generally demonstrate relative host specificity at the plant species level (Deng and Cao, 2017).

DSE inoculation induced minor changes in the leaf pigment composition

According to our results, the five tested strains could induce changes in the metabolism of *N. caerulescens*, as they tended to reduce the chlorophyll content while increasing the anthocyanin content, although not significantly. Anthocyanins are pigments synthesized in a branch of the flavonoid pathway that is related to secondary metabolism. These compounds are particularly biosynthesized in plants exposed to oxidative stressors, metals and/or metalloids or nutrient deficiency (Landi et al., 2015). The promotion of secondary metabolism in plants has also been associated with plant-DSE mutualistic associations under drought stress (Nascimento and Fett-Neto, 2010). In the case of *N. caerulescens*, the authors reported that the production of anthocyanins was enhanced in leaves experiencing thrips attacks or foliar damage (Asad et al., 2014). Similarly, but much more pronounced, the enhancement of secondary metabolism was reported in DSE-inoculated sorghum (Zhang et al., 2017) and ryegrass (Dupont et al., 2015). For the latter, these observations were confirmed by a transcriptomic analysis revealing that endophyte infection favoured ryegrass secondary metabolism at a cost to primary metabolism (Dupont et al., 2015). However, the promotion of primary metabolism detected through an increase in chlorophyll content in the shoots of DSE-inoculated plants has also been reported (Ban et al., 2017; Likar and Regvar, 2013), confirming the somewhat unpredictable outcomes of host-DSE interactions.

***Leptodontidium* sp. Pr30 had a positive effect on *N. caerulescens* root growth**

The tested DSE strains did not influence the biomass of shoots, suggesting their neutral effect on the development of aerial tissues in *N. caerulescens*. In contrast, Pr30 (*Leptodontidium* sp.) had a beneficial effect on the root biomass when inoculated in plants growing in the HC soil. A similar, but less pronounced, tendency was observed for Me07, which belongs to the same genus, for the two levels of soil contamination. In a previous study (Berthelot et al., 2017), the same Pr30 strain also promoted root biomass in birch grown in slightly and moderately TE-contaminated soil. Consequently, this strain possesses plant growth-promoting capacities that are compatible with both herbaceous plants and trees grown in TE-contaminated soils. In the present study, we noted that Pr27 tended to have a slight positive effect on the root biomass of *N. caerulescens*, while in Berthelot et al. (2017), Pr27 had a strong positive effect on the growth of both roots and shoots of poplar and birch. In our study, EPN (*E. nigrum*) did not influence any of the studied parameters, suggesting that it has no effect on the growth of *N. caerulescens*. Few data about the direct influence of *E. nigrum* on plant growth are available. However, Fávvaro et al. (2012) highlighted a root

biomass increase in *E. nigrum*-inoculated sugarcane. In our study, the polluted soils were sterilized and reinoculated with a single strain to highlight any direct effect on the host. As a promising biocontrol agent against pathogenic fungi, the EPN strain would probably be more suitable for indirect plant growth promotion in non-sterilized soil with native fungi.

DSEs enhanced nutrient acquisition by *N. caerulea*

It is now well known that the plant growth enhancement by DSEs is partially related to an improvement in plant nutrition through better nutrient uptake (Likar and Regvar, 2013; Vergara et al., 2017, 2018; Yakti et al., 2018). Several studies reported evident increases in P, K and/or microelement concentrations in DSE-inoculated *Carex* (Haselwandter and Read, 1982), *Pinus contorta* (Jumpponen and Trappe, 1998) and *Clethra barbinervis* (Yamaji et al., 2016). In the present study, Me07 improved the nutrient status of *N. caerulea* when grown in the HC soil, and Pr27 had a comparable effect in the case of the LC soil. Despite these positive effects on nutrient status, there was no subsequent influence on plant biomass. Indeed, strains (*i.e.*, Me07 and Pr27) that significantly improved plant nutrition only slightly influenced plant biomass but not significantly. Conversely, in the case of Pr30, which significantly improved the growth of *N. caerulea*, the nutrient status of the inoculated plants did not change. This is consistent with De Maria et al. (2011) who showed that although *Cadophora finlandica* had no effect on the growth of willow in Cd/Zn-contaminated soil, it indirectly enhanced the mineral nutrient status of willow. In the absence of a specialized plant-fungus interface for nutrient transfer, several studies hypothesized that DSEs could favour plant growth by an indirect acquisition of nutrients related to a saprophytic mode of nutrition (*i.e.*, mineralization of organic compounds or solubilization of insoluble minerals) (Caldwell et al., 2000; Mandyam and Jumpponen, 2005; Newsham, 2011). More specifically, our results corroborated those obtained by Berthelot et al. (2019), which showed that alfalfa nutrition was also improved after inoculation with *Leptodontidium sp.* (Me07) and *P. mustea* (Pr27).

PR30 and PR27 improved the phytoextraction potential of *N. caerulea*

N. caerulea is considered a suitable candidate for Cd phytoextraction in moderately contaminated soils (Martínez-Alcalá et al., 2016; Maxted et al., 2007b; Sterckeman et al., 2019). Among the populations of *N. caerulea* that naturally colonize metalcolous sites, the present study focused on the Ganges population (calamine group). Compared to other populations, the Ganges population is considered the most efficient at tolerating and hyperaccumulating high amounts of Cd and Zn (Gonneau et al., 2014) and is therefore adapted for cultivation on the studied soils. Our study showed that *N. caerulea* grown on HC soil accumulated more than 3000 mg/kg Zn in its leaves, corresponding to the hyperaccumulation threshold suggested by van der Ent et al. (2013). These Zn concentrations in leaves were 9-fold higher than the soil Zn concentration. With concentrations exceeding 100 mg/kg, Cd was hyperaccumulated in plants grown on HC soil (Baker et al., 2000), although the accumulation factors were higher in the LC soil (40- to 100-fold the LC soil concentration vs 7- to 12-fold the HC soil concentration). These accumulation levels were similar for Cd but lower for Pb than those obtained in the Ganges population grown in 24 field TE-contaminated soils for 20 weeks (Jacobs et al., 2019), for which concentrations in leaves ranged from 10- to 100-fold the soil concentrations. These inconsistencies are not likely related to the bioavailability of the TEs, since the pH was comparable between the two studies, but rather to the nitrogen amendments that were applied weekly and the longer growing time in Jacobs et al. (2019).

In terms of micro- and macronutrients, DSEs can increase TE bioavailability by producing multiple extracellular enzymes that break down complex forms of carbon, nitrogen and phosphorus. Coles et al. (1999) demonstrated that 17 fungal strains isolated from various tissues of *N. caerulescens* were able to solubilize zinc oxide and zinc phosphate. Similarly, Pr27 and Me07 had the ability to solubilize Zn oxide and Zn phosphate (Berthelot et al., 2019). Previous studies also showed that DSEs can improve the TE tolerance of host plants and subsequently induce changes in metal accumulation in plant tissues (De Maria et al., 2011; Dos Santos Utmazian et al., 2008; Li et al., 2011; Likar and Regvar, 2013; Sharma et al., 2019). However, the direction (*i.e.*, decrease or increase in TEs) of these changes remained plant-DSE dependent (Berthelot et al., 2017, 2019). The present study highlighted that the Pr30 strain significantly enhanced the accumulation of Zn and Cd in the roots of inoculated *N. caerulescens*. Additionally, Me07, which belongs to the same genus as Pr30 (*Leptodontidium*), also increased the accumulation of Cd in roots, although to a lesser extent. Furthermore, inoculation only significantly impacted the uptake of TEs from soil to roots and had little effect on their translocation to the shoots. For these two *Leptodontidium* strains, the correlations between biomass and TE concentrations, which were not found for the non-inoculated plants, suggest that inoculation would alleviate the deleterious effects of excessive TE inputs. These results are consistent with the findings of Wang et al. (2016), who demonstrated that DSE inoculation in maize significantly increased Cd accumulation on the cell walls and the conversion of Cd into inactive forms by roots to limit its translocation.

We finally determined the phytoextraction potential of DSE-inoculated plants by calculating the TE extraction capacity (shoot biomass x accumulation of TEs in shoots). Pr30 and Pr27 significantly enhanced the amount of Cd and Zn extracted by *N. caerulescens*, but only in the HC soil. These results support the suitability of *N. caerulescens* for phytoextraction applications in soils contaminated with high concentrations of Cd and Zn. Unlike plant growth-promoting endophytic bacteria that were efficient for Ni phytoextraction only when they were used in combination with each other (Visioli et al., 2015b), two of the tested individual endophytic fungi were effective in improving the phytoextraction of Cd and Zn by *N. caerulescens*. However, a further challenge will be testing DSE consortia in a similar context.

Conclusion

In conclusion, we demonstrated that *N. caerulescens*, a TE hyperaccumulator obtained from metal-contaminated sites, could be colonized by DSE strains isolated from poplar. The host plant responses to fungal inoculation ranged from neutral to beneficial. Depending on the strain, we detected significant effects of inoculation on the root biomass (Pr30), macroelement contents in roots (Pr27 and Me07) and accumulation of Cd (Me07 and Pr30) and Zn (Pr30). Among the five tested strains, *Leptodontidium* sp. (Pr30) and *Phialophora mustea* (Pr27) exhibited the most promising ability to enhance the phytoextraction potential of *N. caerulescens*, as they increased the amounts of Zn and Cd extracted by improving plant growth and/or TE accumulation. It would be interesting to conduct a similar study with DSE strains isolated from *N. caerulescens* roots collected from TE-contaminated soils.

Declarations

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Ethical approval

We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

Authors contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Loïc Yung, Nicolas Maurice and Antonin Azou-Barré. The first draft of the manuscript was written by Loïc Yung and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent to participate

Not applicable

Consent to publish

Not applicable

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ASSOCIATED CONTENT

Table S1. Effect of DSE strains on the concentrations of elements in roots and shoots of *N. caerulescens*. Plants were grown for 12 weeks in soils with either low (LC) or high (HC) metal contamination and were either inoculated with a DSE strain or mock-inoculated (CTRL). Data are the means \pm SDs (n = 5). The results of the statistical comparisons (ANOVA and Dunnett or Wilcoxon pairwise tests) between the CTRL condition and the fungal treatments are represented with the following symbols: $P < 0.001$ (***) ; $P < 0.05$ (*). LQ: limit of quantification.

		Ca	Na	K	Mg	P	S	Fe	Al	Cu	Pb	Ni
Concentrations in roots (mg/kg DW)	CTRL	7625 \pm 811	987 \pm 139	12107 \pm 3873	1399 \pm 109	4216 \pm 532	6968 \pm 758	1406 \pm 430	1310 \pm 566	210 \pm 48	662 \pm 132	4.9 \pm 6.6
	EPN	8326 \pm 499	1102 \pm 237	11469 \pm 4899	1447 \pm 141	3748 \pm 770	7032 \pm 1597	1078 \pm 153	1392 \pm 220	242 \pm 47	550 \pm 113	7.8 \pm 2.1
	HC Me07 soil	6374 \pm 363 *	1011 \pm 69	23436 \pm 4546 ***	1711 \pm 171 *	5392 \pm 805	9041 \pm 1536 *	1058 \pm 283	1630 \pm 484	203 \pm 20	639 \pm 74	<2.0
	Pr27	8366 \pm 563	856 \pm 65	12618 \pm 2572	1482 \pm 194	4492 \pm 1126	7545 \pm 398	1270 \pm 362	1368 \pm 355	205 \pm 34	707 \pm 60	<2.0
	Pr28	8033 \pm 979	856 \pm 229	9883 \pm 1889	1559 \pm 174	3776 \pm 509	6931 \pm 779	1260 \pm 164	1370 \pm 415	192 \pm 32	500 \pm 55*	7.9 \pm 4.1
	Pr30	7911 \pm 886	1103 \pm 279	12129 \pm 3610	1525 \pm 133	4559 \pm 1540	7468 \pm 911	1048 \pm 160	877 \pm 194	157 \pm 22	541 \pm 112	4.3 \pm 2.2
	LC Me07 soil	8971 \pm 929	2100 \pm 490	20089 \pm 2753	1766 \pm 284	5484 \pm 531	6997 \pm 239	952 \pm 322	1302 \pm 460	164 \pm 55	15 \pm 4	12.4 \pm 1.2
EPN	9480 \pm 802	1447 \pm 157*	16770 \pm 2080	1900 \pm 313	5851 \pm 806	6890 \pm 1077	1703 \pm 442	1894 \pm 377	126 \pm 15	21 \pm 4	18.8 \pm 10.5	
Me07	8428 \pm 646	1329 \pm 150**	16981 \pm 3458	1535 \pm 136	5166 \pm 534	7259 \pm 1119	1090 \pm 467	4038 \pm 1171***	145 \pm 11	39 \pm 7**	13.7 \pm 3.9	
Pr27	13101 \pm 780 ***	1889 \pm 777	10187 \pm 2248 ***	1862 \pm 224	4346 \pm 319	5894 \pm 804	2991 \pm 786 ***	2714 \pm 1076	140 \pm 23	26 \pm 6*	14.3 \pm 5.2	
Pr28	10985 \pm 1789*	2087 \pm 711	19208 \pm 2986	1773 \pm 207	5918 \pm 616	7892 \pm 665	1560 \pm 230	1726 \pm 1258	191 \pm 81	20 \pm 6	13.6 \pm 3.3	
Pr30	9837 \pm 1696	2184 \pm 422	17846 \pm 1838	1767 \pm 188	6245 \pm 1734	6836 \pm 854	1208 \pm 420	1134 \pm 487	94 \pm 41	18 \pm 3	19.1 \pm 16.2	
Concentrations in leaves (mg/kg DW)	CTRL	4250 \pm 777	<4.6	18887 \pm 2245	766 \pm 136	1568 \pm 111	3543 \pm 1179	40 \pm 7	< 4	3.9 \pm 0.9	19 \pm 11	3.6 \pm 0.7
	EPN	3045 \pm 438 *	<4.6	18367 \pm 1452	612 \pm 179	1571 \pm 164	2390 \pm 389	82 \pm 66	< 4	5.2 \pm 2.4	28 \pm 24	3.7 \pm 0.9
	HC Me07 soil	3533 \pm 728	<4.6	18176 \pm 2267	935 \pm 262	1381 \pm 209	2697 \pm 663	28 \pm 5	< 4	4.1 \pm 2.1	17 \pm 4	4.7 \pm 0.4
	Pr27	3938 \pm 658	<4.6	19357 \pm 3560	835 \pm 112	1511 \pm 235	3955 \pm 1023	37 \pm 5	< 4	5.1 \pm 0.7	20 \pm 3	4.5 \pm 0.6
	Pr28	3856 \pm 217	<4.6	19291 \pm 1449	857 \pm 207	1490 \pm 195	3147 \pm 487	38 \pm 10	< 4	5.7 \pm 4.5	20 \pm 11	3.8 \pm 0.7
	Pr30	3958 \pm 1080	<4.6	19914 \pm 1488	807 \pm 273	1840 \pm 441	3973 \pm 797	73 \pm 49	< 4	5.4 \pm 1.4	18 \pm 5	4.8 \pm 0.6 *
	LC Me07 soil	5594 \pm 1084	<4.6	18402 \pm 2400	646 \pm 149	1635 \pm 144	3085 \pm 666	43 \pm 10	< 4	5.0 \pm 1.4	< 0.7	32.9 \pm 8.9
EPN	5503 \pm 502	<4.6	18943 \pm 1931	489 \pm 235	1873 \pm 277	2440 \pm 516	41 \pm 17	< 4	4.8 \pm 2.1	< 0.7	27.7 \pm 6.7	
Me07	5853 \pm 498	<4.6	15708 \pm 2341	607 \pm 152	1693 \pm 202	2335 \pm 516	60 \pm 29	< 4	7.6 \pm 5.5	< 0.7	29.6 \pm 5.2	
Pr27	6282 \pm 492	<4.6	18759 \pm 2087	667 \pm 143	1806 \pm 391	3182 \pm 718	37 \pm 11	< 4	7.5 \pm 4.7	< 0.7	23.5 \pm 5.6	
Pr28	5378 \pm 1075	<4.6	20432 \pm 7236	710 \pm 154	1883 \pm 584	2903 \pm 1195	43 \pm 9	< 4	5.8 \pm 4.1	< 0.7	23.9 \pm 4.0	
Pr30	5983 \pm 1081	<4.6	19033 \pm 6182	537 \pm 90	1847 \pm 504	2835 \pm 1017	43 \pm 20	< 4	5.1 \pm 2.5	< 0.7	29.0 \pm 3.9	

Table S2. Effect of DSE strains on the amount of Cu, Pb and Ni extracted by shoots of *N. caerulescens* from the two studied soils. Plants were grown for 12 weeks in soils with either low (LC) or high (HC) metal contamination and were either inoculated with a DSE strain or mock-inoculated (CTRL). Data are the means \pm SDs (n = 5). The results of the statistical comparisons (ANOVA and Dunnett or Wilcoxon pairwise tests) between the CTRL condition and the fungal treatments are represented with the following symbols: $P < 0.001$ (***) ; $P < 0.05$ (*).

		Cu	Pb	Ni	
Amount extracted ($\mu\text{g/plant}$)	HC soil	CTRL	6.6 \pm 3.7	40.1 \pm 6.1	6.1 \pm 2.3
		EPN	8.6 \pm 4.4	29.0 \pm 13.5	6.0 \pm 1.6
		Me07	7.6 \pm 4.2	31.2 \pm 8.7	8.5 \pm 1.3
		Pr27	12.7 \pm 3.6	47.4 \pm 9.1	10.9 \pm 2.2 ***
		Pr28	9.0 \pm 5.9	32.0 \pm 14.2	6.2 \pm 1.1
		Pr30	10.3 \pm 2.6	33.4 \pm 7.3	9.1 \pm 0.9 *
	LC soil	CTRL	7.3 \pm 2.5	1.0 \pm 0.1	47.0 \pm 16.3
		EPN	6.4 \pm 3.7	0.9 \pm 0.2	35.5 \pm 10.1
		Me07	9.2 \pm 5.9	0.9 \pm 0.2	37.8 \pm 13.7
		Pr27	9.2 \pm 4.8	0.9 \pm 0.2	29.8 \pm 6.0
		Pr28	6.2 \pm 3.9	0.8 \pm 0.3	27.8 \pm 13.2
		Pr30	6.4 \pm 3.2	0.9 \pm 0.1	36.2 \pm 6.5

Fig. S1 Correlograms for the five tested DSE strains, based on Pearson's correlations, including the plant biomass (*i.e.*, dry shoot biomass (DSB) and dry root biomass (DRB)), concentrations of trace and major elements in roots and shoot pigment indices (*i.e.*, anthocyanins (ANT), chlorophyll (CHL) and flavonoids (FLA)) and NBI variables (with no distinction between the two soils). Significant correlations ($P < 0.05$) were represented by circles with colours corresponding to the value of the Pearson's correlation coefficient (*i.e.*, R).

