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Interactions between the Hyperaccumulator *Noccaea caerulescens* and *Brassica juncea* or *Lupinus albus* for Phytoextraction

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Abstract: Trace-element-contaminated soils cause environmental concern and represent a source of contamination for surrounding areas. Phytoremediation uses plants to diminish the environmental risks associated with this contamination. When the final aim is the extraction of the pollutants, this technique requires the use of plants that are able to accumulate high concentrations of the target elements in their aerial part, while producing high plant biomass. Here, pot experiments were carried out in order to determine the interaction between a hyperaccumulator (*Nocaea caerulescens*) and a metal excluder (*Lupinus albus*) or an accumulator (*Brassica juncea*) species regarding their trace element accumulation/exclusion capacity when sharing the rhizosphere. The plants were grown alone or were cocultivated in soils with different levels of trace element contamination. The Zn concentration in *N. caerulescens* plants was lower in cocultivation with *B. juncea* than when they were grown alone, indicating competition between the two species for Zn uptake. Contrastingly, when grown with *L. albus*, the Zn concentrations in *N. caerulescens* plants were higher than when grown alone. Therefore, under climatic conditions adequate for *N. caerulescens* growth, cocultivation with *L. albus* could favor Zn phytoextraction, while in the case of *B. juncea*, crop rotation rather than cocultivation is recommended for efficient phytoextraction.

Keywords: phytoremediation; trace elements; soil contamination; cocultivation; rhizosphere

1. Introduction

Soil pollution by trace elements (TEs; heavy metals and metalloids) is a global environmental problem. In the 1990s, the International Soil Reference and Information Centre (ISRIC) and the United Nations Environment Programme (UNEP) estimated that 22 million hectares of soil are affected by soil pollution [1]. The Food and Agriculture Organization of the United Nations (FAO) was unable to obtain an exact assessment of polluted soil since there were no precise data from low- and middle-income countries [2].

Phytoremediation techniques have been extensively used to remediate various contaminated soils, with good results being obtained for heavy metals and metalloids, radionuclides, and persistent organic pollutants in affected soils [3]. Specific plant characteristics are taken advantage of to remove, degrade, or stabilize different potentially toxic TEs and organic compounds occurring in polluted soils and waters. This gives rise to the techniques of phytoextraction, phytovolatilization, phytodegradation, phytostabilization, and rhizofiltration, among others [4].

Many members of the Brassicaceae family are known to be hyperaccumulators of TEs because they accumulate metals/metalloids in their aboveground tissues without showing any toxicity symptoms [5].

Noccaea caerulescens (previously named *Thlaspi caerulescens*), a member of the Brassicaceae, is the best-documented Zn hyperaccumulator; some lines can also be considered Cd hyperaccumulators [6–8]. Plants of this species can take up and accumulate large amounts of Zn and Cd (up to 40,000 μg g^{-1} of Zn and 380 μg g^{-1} of Cd, on a dry weight basis) in their aboveground parts without showing toxicity symptoms [9,10]. In addition, they can tolerate high concentrations of Ni [11] and Pb [12] in the xylem sap. However, high concentrations of Cu in the growing medium reduce Zn accumulation [13], which can limit the phytoextraction potential of *N. caerulescens* at multicontaminated sites. The scarce aboveground biomass, the slow growth rate, and the shallow root system of *N. caerulescens* are the main constraints on its phytoremediation efficiency [14].

Indian mustard (*Brassica juncea*), also a member of the Brassicaceae, is a species adapted to the Mediterranean climate [15] and is known for its capacity to bioaccumulate heavy metals such as Cd (>400 μg g^{-1} dry weight in its shoots) [16], Pb [17], Se [18], or Zn, Cr, Cu, and Au [19]. However, its phytoextraction efficiency is low in pluricontaminated soils [20], probably due to the interaction between the different TEs for their uptake by roots [21]. Nevertheless, this plant species presents several advantages for phytoremediation, such as fast growth, low water requirements, and relatively high biomass production [22].

White lupin (*Lupinus albus*), a member of the Fabaceae, is adapted to the Mediterranean climate and is tolerant of heavy metals through their exclusion, although it is able to accumulate large amounts of Mn in its aerial part [23]. This species induces changes in its rhizosphere (iron plaque formation, pH, Eh, water-soluble organic-C) that lead to TE immobilization [23–27]. Additionally, its proteoid roots release chelating agents in root exudates to mobilize soil Fe, Mn, P, and Zn when these elements are deficient [25].

Plants induce changes in the rhizosphere through the release of exudates, adsorption and desorption of elements, water uptake, and other physicochemical processes [14,28]. These changes can be directed, in the case of hyperaccumulator species, to improve their phytoextraction potential. For example, Wu et al. [29] used a combination of the metal hyperaccumulator plants *N. caerulescens* and *Sedum alfredii* and the low-accumulating but highly mycorrhizal-dependent corn (*Zea mays*) to increase the efficiency of phytoextraction of Zn from contaminated sewage sludge. Similarly, intercropping oat (*Avena sativa*) with white lupin (which represented 11% of the plants) enhanced the mobility of TEs relevant for phytoremediation (Pb, Th) or phytomining (La, Nd, Sc) in a contaminated soil [30]. Gove et al. [31] found that the Cd concentration in *Hordeum vulgare* increased when grown with *N. caerulescens*, and they also found increased concentrations of Cd, Zn, Cu, Pb, and Ni in *N. caerulescens* when grown with *H. vulgare* and *Lepidium heterophyllum*. They suggested that *N. caerulescens* may alter the conditions in shared rhizospheres, possibly affecting the availability of certain metals to neighboring plants. Nonetheless, studies using cocultivation or intercropping for phytoremediation purposes are scarce.

Therefore, it can be hypothesized that the Zn accumulation capacity of the hyperaccumulator *N. caerulescens* is affected differently according to whether it shares a rhizosphere with an accumulator or with an excluder species in cocultivation. In the present work, *N. caerulescens* was cultivated in soils with high TE concentrations and different pH values, in combination with either the accumulator *B. juncea* or the excluder *L. albus*, to study the interaction of these cocultivated plant species regarding its Zn accumulation, which will ultimately affect the efficiency of phytoremediation.

2. Materials and Methods

2.1. Soil Characteristics

Two composite soil samples were taken from two different plots within the same site (37°26′21″ N, 06°13′00″ W), which had been contaminated as a consequence of the toxic spillage of the Aznalcóllar mine (Seville, Spain) in 1998 [20]. The two soils differed in their pH and metal concentrations (Table 1), but both were noncalcareous with a loamy texture (19.7% clay, 34.3% silt, and 46.0% sand), and were

classified as Typic Xerofluvent (American Soil Taxonomy). Phyllosilicates (principally illite and kaolinite) and jarosite were the main minerals found (X-ray diffraction; [20]) in the clay fraction (particles <2 μm in diameter) of the soils. Quartz minerals and feldspars (anorthite, albite) were the main components of the coarser fractions [20]. Soil samples taken at 0–20 cm depth were air-dried, sieved to <2 mm, and used in the pot experiments.

Table 1. Characteristics of the soils (mean ± standard error).

	Soil 1	**Soil 2**
pH	5.02 ± 0.05	6.00 ± 0.04
EC (dS m^{-1})	2.33 ± 0.00	1.90 ± 0.01
$CaCO_3$ (%)	<0.5	<0.5
CEC (cmolc kg^{-1})	16.0 ± 0.5	16.0 ± 0.5
OM (%)	1.93 ± 0.09	3.35 ± 0.12
TOC (g kg^{-1})	11.1 ± 0.50	19.5 ± 0.12
Total-N (g kg^{-1})	1.16 ± 0.10	3.63 ± 0.02
Fe (g kg^{-1})	40.5 ± 0.02	46.2 ± 0.03
Mn (μg g^{-1})	1024 ± 0.05	1377 ± 44.6
Cu (μg g^{-1}) [1]	196 ± 4.81	207 ± 11.2
Zn (μg g^{-1}) [1]	549 ± 16.0	781 ± 41.9
Cd (μg g^{-1}) [1]	2.00 ± 0.00	1.37 ± 0.00
Pb (μg g^{-1}) [1]	311 ± 1.04	473 ± 29.4

[1] EU limit (μg g^{-1}) for agricultural soils (pH 6–7): Cu 50–140; Zn 150–300; Cd 1–3; Pb 50–300 [32].

2.2. Plant Species

Seeds of *N. caerulescens* (J. Presl & C. Presl) F.K. Mey were collected at a metalliferous site (High Tor, Derbyshire, UK) [13], *B. juncea* L. Czern. cv. 0814–79 seeds were obtained from a germplasm bank collection of Crucifers and provided by Dr. Gómez-Campo (E.T.S.I. Agrónomos Universidad Politécnica, Madrid, Spain) [33], and *L. albus* L. cv. Marta seeds were acquired commercially.

The seeds were sterilized with 10% HClO for 30 min, washed three times with distilled water, and germinated in sand at 28 °C: for 3 days for *L. albus*, 4 days for *B. juncea*, and 16 days for *N. caerulescens*. Then, the plantlets were transferred to pots (surface area 100 cm^2) containing 100 g of sand at the base and 500 g of soil on top.

2.3. Experimental Design

Two experiments were run (Table 2):

Table 2. The different soil/plant combinations used in the experiments.

	Soils	**Plants (n° of Plants per Pot)**
Experiment 1		
D1-Nc	D1: Soil 1 at pH 5.5	*N. caerulescens* (6)
D1-Bj		*B. juncea* (2)
D1-cocultivation		*N. caerulescens* (6) + *B. juncea* (2)
D2-Nc	D2: Soil 1 at pH 6.4	*N. caerulescens* (6)
D2-Bj		*B. juncea* (2)
D2-cocultivation		*N. caerulescens* (6) + *B. juncea* (2)
Experiment 2		
S1-Nc	S1: Soil 1 at pH 5.0	*N. caerulescens* (5)
S1-La		*L. albus* (1)
S1-co-cultivation		*N. caerulescens* (5) + *L. albus* (1)
S2-Nc	S2: Soil 2 at pH 6.1	*N. caerulescens* (5)
S2-La		*L. albus* (1)
S2-cocultivation		*N. caerulescens* (5) + *L. albus* (1)

Experiment 1. Two plants of *B. juncea* per pot and six plants of *N. caerulescens* per pot were grown alone or mixed in the same pot (84 days for *N. caerulescens* and 70 days for *B. juncea*) in Soil 1, with two rates of $CaCO_3$ (1.3 and 3.0 g kg^{-1}) to increase soil pH to 5.5 (D1) and 6.4 (D2), respectively. The experiment lasted 80 days.

Experiment 2: One plant of *L. albus* per pot and five plants of *N. caerulescens* per pot were grown alone or mixed in the same pot (168 days for *N. caerulescens* and 63 days for *L. albus*) in the two soils (S1 and S2). In this experiment, pots of soil without plants were used as controls. The experiment lasted 105 days.

In Experiment 1, *N. caerulescens* plantlets were transplanted 14 days before *B. juncea*, while in Experiment 2, *N. caerulescens* plantlets were transplanted 105 days before *L. albus*. This was to let the slower-developing *N. caerulescens* plantlets grow and develop in the pots before planting the second species with greater biomass. Harvesting was performed at the same time for both species. The different biomass expected per pot, according to the species, was the rationale for having different numbers of plants in the two experiments. Different doses of $CaCO_3$ were added to the soil in each experiment to evaluate the influence of soil pH on the phytoremediation capacity.

Four replicates (pots) were used per treatment in both experiments. The plants were maintained in a growth chamber with a light/dark regime of 16/8 h, temperature of 25/17 °C (day/night), and relative humidity of 70% until harvest. The pots were watered manually every 3–7 days (according to the plants' water requirements) from the bottom of the pot, using tap water. The plants were harvested by cutting the aerial part about 1 cm above the soil surface and then were washed twice with distilled water and dried at 60 °C for 48 h. The plant weight and total TE concentrations in the plants were determined. After harvesting, soil from each pot was sampled for chemical analysis by carefully emptying the content of the pots (without taking out the sand at the bottom), homogenizing, removing any visual roots, and collecting samples of at least 100 g. Plant roots were not isolated and analyzed as it was not possible to separate the roots of the different cocultivated plant species.

2.4. Analytical Procedures

The soil samples were air-dried, passed through a 2-mm sieve, and homogenized before analysis. Soil pH was determined in water-saturated pastes using a pH meter (CRISON BasiC 20), and electrical conductivity (EC) was measured in 1:5 (*w/v*) soil/water extracts using a conductivity meter (CRISON GLP 31). Total organic C (TOC) and total N (TN) were measured in an automatic microanalyzer (EA3000, EuroVector, Pavia, Italy). Soil pseudototal TEs, after digestion of the soil with *aqua regia* in a microwave digester (Ethos 1, Milestone Srl, Sorisole, Italy) [34], and $CaCl_2$-extractable metals (0.1 M $CaCl_2$ 1:10 *w/v*, 16 h) [35] were determined by inductively coupled plasma–optical emission spectrometry (ICP-OES; Iris Intrepid II XDL, Thermo Scientific, Waltham, MA, USA). The analytical accuracy was checked with certified reference material (SRM 2711 Montana Soil). All the concentrations were adjusted to values for oven-dried soil (12 h at 105 °C).

Aerial biomass production was determined as the fresh and dry (60 °C for 48 h) weight per pot for each plant species, and heavy metal concentrations in the plant material were determined by ICP-OES after microwave digestion with H_2O_2/HNO_3 (1:4 *v/v*) [34]. The bioconcentration factor (BCF) was calculated as the concentration of a certain element in the aerial part of the plant with respect to its total concentration in the soil.

2.5. Statistical Analysis

Statistical analyses were performed with the software IBM SPSS Statistics Version 24.0 (IBM Corporation, Armonk, NY, USA). The results were subjected to a two-way ANOVA, considering the soil and the cultivation method (each species grown individually or in cocultivation) as factors, and differences between means were determined using Tukey's test. Before the ANOVA, the data were tested for normality using the Kolmogorov–Smirnov test and log-transformed when necessary. The data

from each plant species in Experiment 2 were evaluated by principal component analysis (PCA) in order to highlight general tendencies.

3. Results and Discussion

3.1. Experiment 1: Noccaea caerulescens and Brassica juncea

3.1.1. Plant Growth and Metal Accumulation

The dry weight of *B. juncea* was significantly higher in soil D2, with the highest pH value, than in D1 (Figure 1). Contrastingly, *N. caerulescens* plants had higher dry weight in the soil of lower pH, D1. For this species, the growth conditions have been reported to be optimal at a pH value of about 5.1 [36]. However, according to Broadhurst et al. [37], *N. caerulescens* plants do not exhibit phytotoxicity symptoms over a soil pH range of 5.5–7.0. In addition, in soil D1, the yield of *B. juncea* plants was very low, and the determination of their elemental composition was not possible when cocultivated with *N. caerulescens*. In fact, *B. juncea* plants have low survival in soils with pH <4 [20].

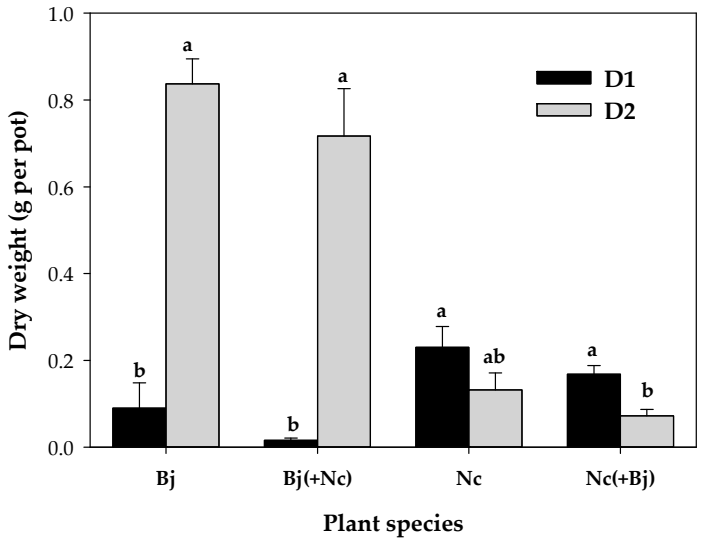

Figure 1. Plant dry weight in the different soils and types of cultivation (g DW pot^{-1} ± standard error). For each species, bars marked with different letters indicate significant differences at $p < 0.05$, according to Tukey's test. Bj = *B. juncea*; Nc = *N. caerulescens*.

The concentrations of Fe found in *B. juncea* plants, grown in both soils, were similar to those found in other studies (e.g., 91.0 μg g^{-1}) [38] and generally within the range considered deficient in plants (50–150 μg g^{-1}) [39]. This was also the case for *N. caerulescens* plants grown in D1, while plants from D2 had slightly higher Fe concentrations (Table 3). Interestingly, the Fe concentration found by Walker and Bernal [13] in the same line of *N. caerulescens* grown in a nutrient solution (103 μg g^{-1}) was similar to that found in the present experiment.

The concentrations of Cu in *B. juncea* plants grown in D1 were near the threshold considered for plant toxicity of 20 μg g^{-1} [40]. The Cu concentrations were lower in D2, but not significantly so (Table 3). The highest Cu concentration in *N. caerulescens* was 15 μg g^{-1} for plants grown alone in D2. This value is of the same magnitude as that obtained by Walker and Bernal [13] in contaminated soil (7 μg g^{-1}) and is in the interval considered normal for plants (5–30 μg g^{-1}) [40]; no significant differences were observed between soils D1 and D2

Table 3. Heavy metal concentrations in the plants from Experiment 1 (μg g^{-1} DW), *B. juncea* (Bj) and *N. caerulescens* (Nc), grown individually and under cocultivation ($n = 4$).

Soils		Fe		Cu		Mn		Zn		Cd	
		Bj	**Nc**	**Bj**	**Nc**	**Bj**	**Nc**	**Bj**	**Nc**	**Bj**	**Nc**
D1	Individual	137 ± 37	76.9 ± 2.2 b	18.3 ± 7.5	5.66 ± 0.04 ab	61.4 ± 36.7 a	11.9 ± 4.3 ab	3600 ± 575 a	6025 ± 1205 ab	6.22 ± 0.74	1.09 ± 0.67 b
	Cocultivation	-	88.4 ± 7.8 b	-	4.91 ± 0.85 ab	-	20.3 ± 1.7 a	-	6814 ± 808 a	-	2.06 ± 1.01 ab
D2	Individual	47.7 ± 3.5	251 ± 120 a	10.7 ± 0.7	14.9 ± 6.7 a	16.5 ± 0.3 b	22.8 ± 3.4 a	220 ± 48 b	6367 ± 1536 ab	0.45 ± 0.45	23.1 ± 10.2 a
	Cocultivation	98.5 ± 37.7	133 ± 5 ab	7.28 ± 1.26	1.00 ± 0.57 b	16.0 ± 2.5 b	4.08 ± 1.82 b	210 ± 53 b	2303 ± 319 b	bdl	3.66 ± 1.52 ab
ANOVA	Cultivation	ns	ns	ns	**	ns	ns	ns	ns	-	ns
	Soil	ns	**	ns	ns	*	ns	***	ns	*	*
	CxS	-	ns	-	*	-	**	-	*	-	ns

ns, *, **, and ***: not significant and significant at $p < 0.05$, 0.01, and 0.001, respectively. Values followed by the same letter in each column do not differ significantly according to Tukey's test ($p < 0.05$).bdl = below detection limit. Samples of *B. juncea* grown in D1 under cocultivation were not analyzed due to the low weight obtained.

The *B. juncea* plants had their highest concentration of Mn when grown in D1 (Table 3), this value being similar to the results of Feigl et al. [38] (61.4 μg g^{-1}). The concentrations of Mn in cocultivated *N. caerulescens* plants grown in D1 were significantly higher than in soil D2. The values generally were lower than in previous reports for this species in a nutrient solution (e.g., 156 μg g^{-1} [13] and 178 μg g^{-1} [41]), but were similar to the results of McGrath et al. [42] (35.5 μg g^{-1}) in contaminated soils.

The concentration of Zn in *B. juncea* differed significantly between the soils, with the highest concentrations in soil D1, of low pH. In a field experiment conducted in the area of Aznalcóllar from which our soil samples were taken, high concentrations of Zn in the aerial part of *B. juncea* (2029 μg g^{-1}) were also observed [20]. Del Rio et al. [15] also found increasing Zn concentrations in *B. juncea* plants, from 37.5 μg g^{-1} in uncontaminated soil to 94 μg g^{-1} in soil with 462 μg g^{-1} of total Zn, demonstrating a clear accumulator behavior. BCF found in this species ranged from 0.3 to 6.0, the latter when *B. juncea* grew alone in D1. This BCF is much higher than that (1.52) found by Ali and Chaudhury [43] in plants grown for 3 months in a noncontaminated soil.

The levels of Zn in *N. caerulescens* did not reach hyperaccumulation (10,000 μg g^{-1}), neither in soil nor in the growth conditions, perhaps due to the multielemental contamination of the soils (discussed below). The highest concentrations of Zn in *N. caerulescens* occurred when grown alone (Table 3), while the lowest values were found in cocultivation with *B. juncea* in soil D2 (pH 6.4). Zn BCF in the hyperaccumulator was approximately 12 when grown in D1 or alone in D2 but fell to 4.2 when grown with *B. juncea* in D2. All these BCF values were lower than those found by McGrath et al. [42] in a pot experiment using different contaminated soils. In soil D1, *B. juncea* plants did not grow well and produced little biomass (Figure 1), a clear symptom of toxicity. Therefore, competition/interaction between *B. juncea* and *N. caerulescens* for Zn uptake was not found in this soil.

The results indicate that the presence of *N. caerulescens* did not improve the accumulation of Zn in the accumulator species (*B. juncea*). In contrast, the presence of the latter reduced the hyperaccumulating potential (yield and Zn concentration) of the former, which may indicate competition between the two species for the uptake of nutrients and the bioavailable Zn fraction in soil D2. The level of Zn in these soils is not extremely high (although it is greater than the limits for agricultural soils [32]), and Zn is an essential micronutrient, which is taken up from the same soil fractions by the roots of the hyperaccumulator and other plant species [44]. *N. caerulescens* is able to bioaccumulate Zn even in soils with low or moderate concentrations [45] due to its highly efficient transport system [46]. According to Whiting et al. [47], the absence of a large-scale active mechanism of Zn mobilization in the roots of *N. caerulescens* can severely limit the rate of uptake from forms poorly available in the soil and the subsequent accumulation of Zn in the plant. This may have occurred in the high-pH soil D2, with low 0.1 M $CaCl_2$-extractable concentrations of Zn (discussed later; Figure 2). Thus, in soil D2, the presence of high-biomass *B. juncea* plants could have limited the development of the roots of the hyperaccumulator species, preventing them from exploring areas of the soil rich in Zn that were not dominated by the roots of *B. juncea*. This factor is critical for *N. caerulescens* to maintain its high accumulation of Zn [47].

In fact, in soil D2, *B. juncea* showed a mean Zn phytoextraction value of 121 μg pot^{-1}, similar to *N. caerulescens* (174 μg pot^{-1}) when grown together. However, *N. caerulescens* reached the greatest Zn phytoextraction value when growing alone (1844 ± 964 μg pot^{-1}).

For *B. juncea*, the concentration of Cd was below the detection limit (0.01 μg g^{-1}) when grown combined with the hyperaccumulator species in soil D2, while its maximum Cd concentration occurred in D1 when grown alone (6.22 μg g^{-1}; Table 3). Contrastingly, the Cd concentration in *N. caerulescens* was highest (23.1 μg g^{-1}) when grown alone in D2, which resulted in a Cd BCF of 11.6, similar to that reported by Martínez-Alcalá et al. [48] for *N. caerulescens* plants grown in TE-contaminated soil (around 13).

As with Zn, the low biomass of *B. juncea* in D1 precluded any effect on the Cd uptake by the hyperaccumulator, but its presence in soil D2 greatly reduced the Cd concentration in *N. caerulescens*. All the Cd concentrations found were below the minimum value (100 μg g^{-1}) considered to represent

hyperaccumulation, confirming the report of Walker and Bernal [13] that this line of *N. caerulescens* is not a Cd hyperaccumulator.

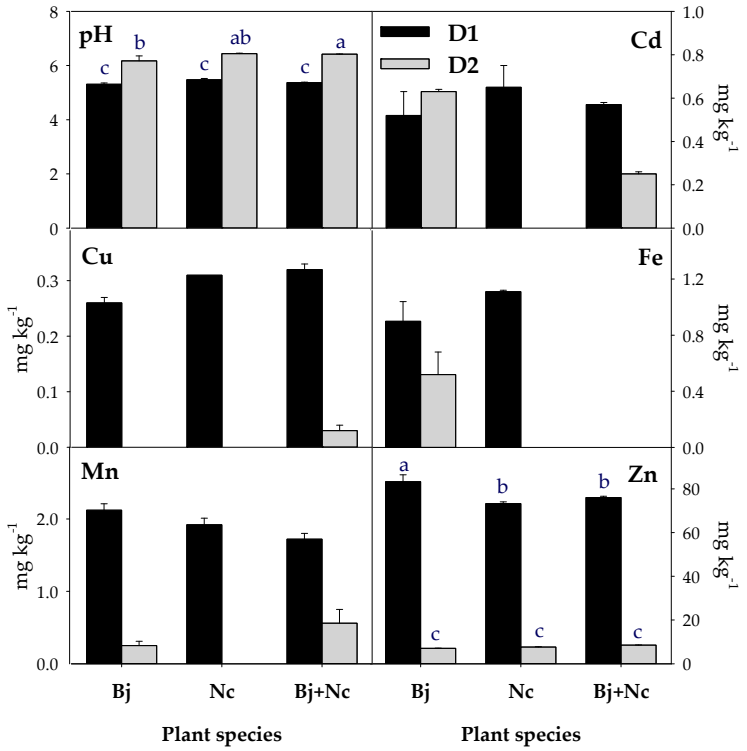

Figure 2. Soil pH values and concentrations of metals extracted with 0.1 M $CaCl_2$ solution in the soils where *B. juncea* (Bj), *N. caerulescens* (Nc), or *B. juncea* and *N. caerulescens* (Bj + Nc) were grown (mean values (dry soil basis) ± standard error). For each soil, bars marked with different letters indicate significant differences at $p < 0.05$, according to Tukey's test. Bars without letters indicate that statistically significant differences were not found ($p > 0.05$) for the same soil.

3.1.2. Soil Properties

The soil pH values at the end of the experiment were slightly acidic in soil D1, and there was no significant effect of the plant species grown in the soil or of the cocultivation of the two species (Figure 2). Soil D2 showed, as expected, higher pH values that were, in addition, higher with cocultivation. Kim et al. [49] found that the pH of the soil solution extracted from the rhizosphere of *B. juncea* plants was higher than that of the bulk soil solution (<6.5). This effect was not observed in our experiment, as the pH values did not change compared to the initial values of soils D1 and D2. Although the rhizosphere had not been separated from the bulk soil, the abundance of visually detected roots, which completely covered the soil, could imply a relevant contribution of the rhizosphere in the soil samples. Martínez-Alcalá et al. [48] found no significant changes in the pH of the rhizosphere of *N. caerulescens* plants, with respect to the bulk soil, in soils with different pH. In fact, the mechanism of metal mobilization by a hyperaccumulator species does not necessarily involve a pH reduction in the rhizosphere [42,50].

In general, soil D1 had higher concentrations of extractable metals (0.1 M $CaCl_2$-extractable) than soil D2 as a consequence of its lower pH (Figure 2). No significant differences were found between the extractable Cd concentrations in the soils where *B. juncea* or *N. caerulescens* plants were grown, whether in monoculture or together (Figure 2). This was also true for Cu, Fe, and Mn in both soils.

The extractable Zn concentrations in soil D1 were highest when *B. juncea* was grown individually (Figure 2), likely because of the scarce plant biomass of *B. juncea* (Figure 1) and the strong Zn uptake by *N. caerulescens* (Table 2) in this soil. The uptake of Zn by the hyperaccumulator species likely first

involves soluble and exchangeable forms (extractable in 0.1 M $CaCl_2$). In fact, active soil mobilization by hyperaccumulator species appears to be less important than their highly active and efficient metal uptake systems [48,51], which results in a depletion of the concentration of highly soluble forms of the metals in the soil. Moreover, the rate of replenishment of soluble forms of Zn in the soil solution from less labile forms has been found to be slower than the rate of metal uptake by *N. caerulescens* [52]. Contrastingly, extractable Zn concentrations in soil D2 were much lower than in D1 and were not altered in the soils with *N. caerulescens* with respect to the other species. Hammer and Keller [53] found that EDTA extraction was more suitable to assess the uptake of Zn by *N. caerulescens* in acidic soils than extraction with a neutral salt (0.1 M $NaNO_3$), which can only extract the easily soluble Zn fraction, quickly replaced from less-soluble forms at acidic pH.

3.1.3. Correlations

Simple linear correlations between soil and plant parameters for *B. juncea* and *N. caerulescens* were calculated. Highly significant negative correlations were found between soil pH and the extractable concentrations of Cd, Cu, Mn, and Zn in the soil, which confirms that pH is the key factor influencing the solubility of these metals in the soil (Table 4). Soil pH was correlated positively with the dry weight of *B. juncea* plants and negatively with the Cd and Zn concentrations in this species. Several authors have observed the difficulty of this species to grow in acidic soils [20,54]. Negative correlations between the extractable concentrations of Cu, Mn, and Zn and the plant dry weight indicate that high concentrations of these elements in easily available forms may be toxic for *B. juncea*.

Table 4. Correlation matrix between the soil characteristics and the biomass and metal concentrations of the plants (*B. juncea* and *N. caerulescens*) from Experiment 1.

	pH	Dry Weight	Cu $CaCl_2$	Mn $CaCl_2$	Zn $CaCl_2$	Cd $CaCl_2$
			B. juncea			
pH	-	0.867 ***	−0.947 ***	−0.921 ***	−0.966 ***	−0.581 *
Dry weight	0.867 ***	-	−0.912 **	−0.931 ***	−0.923 ***	ns
Cd_{plant}	−0.968 ***	−0.825 **	0.982 **	0.920 **	0.974 ***	ns
Fe_{plant}	ns	−0.783 **	ns	ns	ns	ns
Zn_{plant}	−0.939 **	−0.919 ***	0.932 *	0.958 ***	0.957 ***	ns
			N. caerulescens			
pH	-	−0.675 *	−0.992 ***	−0.921 ***	−0.997 ***	−0.831 **
Dry weight	−0.675 *	-	0.794 *	0.768 **	0.730 **	0.860 ***
Cd_{plant}	0.561 *	ns	−0.726 *	−0.763 *	ns	ns
Fe_{plant}	ns	ns	−0.869 **	−0.908 ***	ns	−0.839 **
Cu_{plant}	ns	ns	0.802 *	0.834 **	ns	0.819 **
Mn_{plant}	ns	ns	0.787 *	ns	ns	ns
Zn_{plant}	ns	ns	0.854 **	0.737 *	ns	0.730 *

***, **, and *: significant at $p < 0.001$, 0.01, and 0.05, respectively; ns: not significant.

In agreement with this, the Cd and Zn concentrations in *B. juncea* were negatively correlated with plant dry weight. The positive correlation between the concentrations of Zn in the soil (0.1 M $CaCl_2$-extractable) and in the plants reflects the Zn accumulator character of *B. juncea* and its potential to serve as an indicator of Zn availability in soil [20].

In *N. caerulescens*, a negative correlation between soil pH and plant biomass was found (Table 4), which indicates that this species grows better in the slightly acidic soils. As a consequence, plant dry weight was positively correlated with extractable metal concentrations in the soil. The extractable concentrations of Cu correlated positively with the corresponding concentrations in *N. caerulescens*, which may indicate that the Cu concentration in the soils was not high enough to cause toxicity in this species [12], with Cu acting only as an essential micronutrient.

3.2. Experiment 2: Noccaea caerulescens and Lupinus albus

3.2.1. Plant Growth and Metal Accumulation

The *L. albus* plants showed no significant differences in dry weight (on a per pot basis), whether in monoculture or combined with *N. caerulescens* (Figure 3). However, this species grew better in soil S2, with higher pH, than in soil S1. Martínez-Alcalá et al. [25] also found that even though *L. albus* can grow in acid soils, the species performs better in neutral soils. In contrast, *N. caerulescens* grew better in S1 than in S2, independent of the presence or not of *L. albus*, in agreement with the well-known preference of this species for soil pH values of 5–6 [36]. The dry weight of the *N. caerulescens* plants was higher in Experiment 2 than in Experiment 1 due to the longer growing period of the former (an attempt to achieve a plant coverage similar to that of the *L. albus* plants).

There were no significant differences in the concentrations of Cd, Fe, or Mn in *L. albus* between the two soils or the two cultivation methods (Table 5). The Cd concentrations in *L. albus* were very low (<1 μg g^{-1}), regardless of the soil or cultivation method, indicating an exclusion behavior of this species for this element, which is retained mainly in the roots [55]. Kerley [56] reported similar Fe concentrations in *L. albus* (80–150 μg g^{-1} in the aerial part) grown in a range of soils of different pH. These concentrations are above the Fe sufficiency threshold (50 μg g^{-1}) [39].

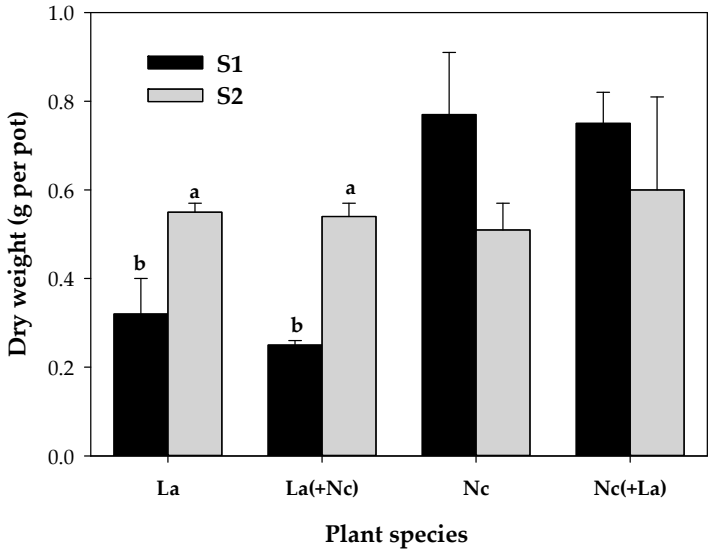

Figure 3. Plant dry weight in the different soils and treatments (g DW pot^{-1} ± standard error). For each species, bars marked with different letters indicate significant differences at $p < 0.05$, according to Tukey's test. La = *L. albus*; Nc = *N. caerulescens*. Bars without letters indicate that no significant differences were found ($p > 0.05$) between those samples.

The concentrations of Mn in *L. albus* ranged from 480 to 783 μg g^{-1}, considered normal for this plant species [25], as it can accumulate large amounts of Mn in its aerial part [25,56]. The Cu concentrations in *L. albus* were within the same range as those obtained by Martínez-Alcalá et al. [25] in soils of the same area and increased significantly under cocultivation with *N. caerulescens* (Table 5). The Zn concentrations in *L. albus* were significantly higher in soil S1 than in soil S2, while only in soil S1 were they significantly higher in cocultivation with *N. caerulescens* than when grown individually (Table 5). The activity of *N. caerulescens* roots may have induced Zn uptake by *L. albus* in the most acidic soils, with a high proportion of soluble Zn. However, in a similar experiment, where plants of *Thlaspi arvense* or *Festuca rubra* were cocultivated with *N. caerulescens* in a slightly acidic soil containing 150 μg g^{-1} Zn, the hyperaccumulator did not actively mobilize this element and did not promote its uptake by the other plant species [47], as observed in soil S2.

Table 5. Heavy metal concentrations in the plants (aerial part) from Experiment 2 (μg g^{-1} DW), *L. albus* (La) and *N. caerulescens* (Nc), under individual cultivation and cocultivation (n = 4).

Soils		Fe		Cu		Mn		Zn		Cd	
		La	**Nc**	**La**	**Nc**	**La**	**Nc**	**La**	**Nc**	**La**	**Nc**
S1	Individual	57.0 ± 5.3	70.0 ± 10.8 b	6.29 ± 0.58	8.10 ± 1.42	480 ± 44	502 ± 47 a	437 ± 18 b	5713 ± 491 ab	0.50 ± 0.06	4.54 ± 0.45 ab
	Cocultivation	70.3 ± 14.7	170 ± 38 ab	8.54 ± 0.74	8.58 ± 0.77	707 ± 46	543 ± 19 a	636 ± 71 a	7387 ± 472 a	0.63 ± 0.04	5.49 ± 0.15 a
S2	Individual	148 ± 51	162 ± 28 ab	7.29 ± 0.51	7.96 ± 1.28	624 ± 119	281 ± 58 b	222 ± 22 c	1998 ± 310 c	0.14 ± 0.08	3.23 ± 0.31 b
	Cocultivation	84.8 ± 15.0	472 ± 196 a	8.49 ± 0.73	7.89 ± 1.31	783 ± 179	172 ± 43 b	197 ± 13 c	4161 ± 633 b	0.69 ± 0.52	4.97 ± 0.49 a
ANOVA	Cultivation	ns	*	*	ns	ns	ns	ns	**	ns	**
	Soil	ns	*	ns	ns	ns	***	***	***	ns	*
	CxS	ns	ns	ns	ns	ns	ns	*	ns	ns	ns

ns, *, **, and ***: not significant and significant at $p < 0.05$, 0.01, and 0.001, respectively. Values followed by the same letter in each column do not differ significantly according to Tukey's test ($p < 0.05$).

The concentrations of Zn in *L. albus* were above the range considered normal for plants (20–150 μg g^{-1}) [40] and, in S1, even exceeded the threshold considered as toxic (300 μg g^{-1}) [39], which impaired plant growth (Figure 3). High Zn concentrations were previously found in this species when it was grown in metal-contaminated soils [25].

Plants of *N. caerulescens* had significantly higher Fe concentrations in soil S2 than in soil S1, and higher concentrations when cocultivated than when grown individually (Table 5). These concentrations are lower than those previously reported for this element in *N. caerulescens* (674 μg g^{-1}) [41]. The Cu concentrations were similarly low in *N. caerulescens* plants, and there were no significant effects of soil or cultivation type. The Mn concentrations in *N. caerulescens* were lower than for *L. albus* and, as expected, were significantly higher in the plants from the soil with the lower pH (S1, pH 5.0) than in those from soil S2 (pH 6.1).

Uptake and accumulation of Zn in *N. caerulescens* were significantly higher in soil S1 and significantly higher in cocultivation with *L. albus* (Table 5). *Lupinus albus* has a tolerance mechanism based on metal exclusion and so did not compete with *N. caerulescens* for the uptake of Zn. There was some apparent interaction with *L. albus* in the rhizosphere that promoted Zn accumulation in *N. caerulescens* in both soils. The ability of *L. albus* to produce changes in the rhizosphere (like a decrease in soil pH or an increase in water-soluble C concentrations) in order to mobilize soil nutrients has been previously tested [23,25,57]. In fact, it can develop proteoid roots, on which clusters of rootlets exude chelating agents (organic anions and enzymes such as phosphatase and probably phytase) and hydrogen ions in order to improve the acquisition of nutrients (P, Fe, Mn, and Zn) under conditions of deficiency [58]. Dessureault-Rompré et al. [44] suggested that the maintenance of high biological activity in the soil could help to increase the efficiency of metal extraction by hyperaccumulator species. Therefore, the production of root exudates by *L. albus* and enhanced microbial activity in the rhizosphere [25] may be responsible for the increased accumulation of Zn in *N. caerulescens* plants. The calculated BCFs for Zn in *N. caerulescens*, when grown in combination with *L. albus*, were 13.4 and 2.49 for soils S1 and S2, respectively. These values were in a much narrower range than those reported by McGrath et al. [40] (6 to 34) and Robinson et al. [59] (0.3 to 104). These authors utilized soils with higher pH values and wider Zn concentration ranges, which may account for the differences from the current results.

The concentrations of Cd in *N. caerulescen*s were highest with cocultivation in both soils (Table 5), with values of 10-times those of *L. albus*, but still far below those considered as hyperaccumulation.

3.2.2. Soil Properties

No significant differences in soil pH were produced when *L. albus* plants grew alone or in combination with *N. caerulescens* in soil S1, nor when grown individually in soil S2, with respect to the control soil without plants (Figure 4). However, in S2, the pH was significantly decreased in the presence of *N. caerulescens* (both alone and cocultivated) compared to the soil with *L. albus* alone and the control soil, likely as a consequence of the changes in the rhizosphere produced by root exudates [48,60]. Similarly, a reduction of 0.2 to 0.3 pH units in the rhizosphere of *N. caerulescens*, with respect to the bulk soil, was previously reported [42], these changes being related to the excess uptake of cations over anions.

The extractable concentrations of Fe in the soils did not vary with the different cultivation methods, and only minor changes were found for Cu in each soil (Figure 4). The extractable concentrations of Mn and Zn were, overall, higher in soil S1 than in soil S2, and only the extractable Zn concentrations in S2 were significantly higher in the soils with *N. caerulescens* (for both individual cultivation and cocultivation) than in the control soil (without plants; Figure 4). The strong uptake of Zn by *N. caerulescens* could have induced its mobilization from less soluble soil fractions through changes in the rhizosphere [48,52], like the observed pH reduction.

It is noteworthy that the two soils (S1 and S2) with *N. caerulescens* alone showed similar concentrations of extractable Cd, Mn, and Zn, associated with their similar soil pH values at the end of the experiment (Figure 4). This effect may also explain why the differences found between the

cocultivated S1 and S2 soils, regarding the soluble concentrations of Cd, Mn, and Zn, were smaller than those between the two soils with *L. albus* cultivated individually.

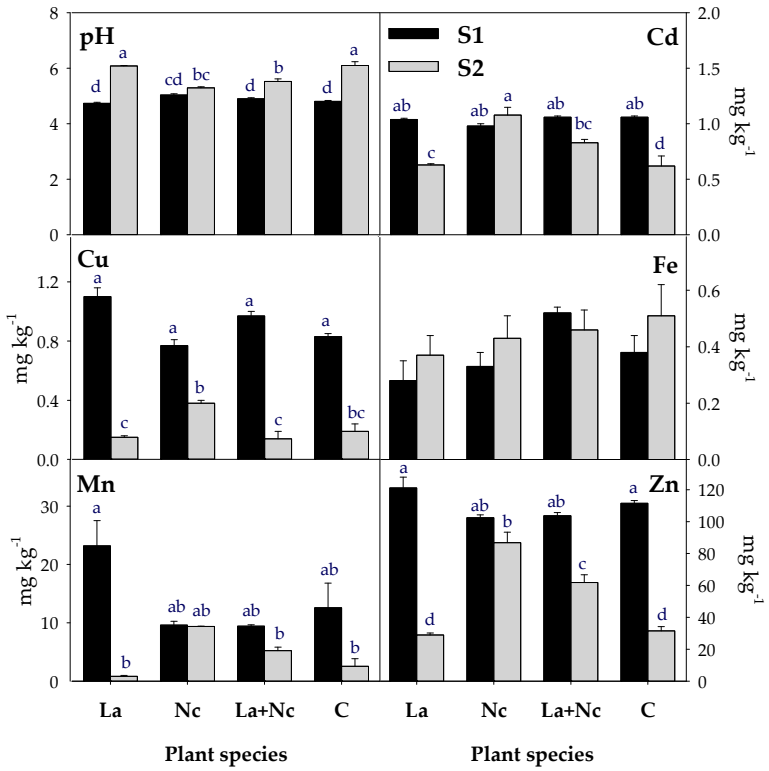

Figure 4. Concentrations of metals extracted with 0.1 M $CaCl_2$ in the soils where *L. albus* (La), *N. caerulescens* (Nc), or *L. albus* and *N. caerulescens* (La + Nc) were grown and in the control (C) soils (with no plants) (mean values (DW) ± standard error). Bars marked with different letters indicate significant differences at $p < 0.05$, according to Tukey's test. Bars without letters indicate that no significant differences were found ($p > 0.05$) between those samples.

3.2.3. Correlations and Principal Component Analysis (PCA)

Highly significant negative correlations were found between soil pH and the extractable concentrations of Cd, Cu, Mn, and Zn, which indicates that pH was key for the solubility of these metals in the soils (Table 6). There were also higher Zn concentrations in the plants at lower soil pH; this was also observed for Mn in *N. caerulescens*.

Table 6. Correlation matrix between the soil characteristics and the biomass and metal concentrations in the plants (*L. albus* and *N. caerulescens*) from Experiment 2.

	pH	Cu $CaCl_2$	Mn $CaCl_2$	Zn $CaCl_2$	Cd $CaCl_2$
			L. albus		
pH		−0.905 ***	−0.751 ***	−0.804 ***	−0.924 ***
Dry weight	0.776 **	−0.802 ***	ns	−0.726 **	−0.748 **
Fe_{plant}	0.570 *	ns	ns	ns	ns
Zn_{plant}	−0.705 **	0.796 ***	ns	0.726 **	0.787 **
			N. caerulescens		
pH		−0.854 ***	−0.715 ***	−0.707 ***	−0.806 ***
Fe_{plant}	0.679 **	−0.543 *	ns	−0.515 *	ns
Mn_{plant}	−0743 **	0.822 ***	0.763 **	0.787 ***	ns
Zn_{plant}	−0.711 **	0.741 **	ns	ns	ns

***, **, and *: significant at $p < 0.001$, 0.01, and 0.05, respectively; ns: not significant.

There was a positive correlation between soil pH and *L. albus* dry weight (Table 6), which confirms that this species grows better in soils with a neutral or alkaline pH [23,25]. The extractable concentrations of Cd, Cu, and Zn were correlated negatively with *L. albus* dry weight, which suggests that metal toxicity could have limited the growth of this species in these soils, as indicated by the negative correlation between plant dry weight and Zn concentration in *L. albus* ($r = -0.755$, $p < 0.01$). The positive correlation of the extractable concentration of Zn in the soils with the Zn concentration in *L. albus* plants suggests the potential for this species to serve as an indicator plant for soil Zn availability.

For *N. caerulescens*, soil pH correlated negatively with the extractable concentrations of Cd, Cu, Mn, and Zn in the soil and with the plant Mn and Zn concentrations (Table 6). Only for Mn was there a positive correlation between the extractable concentration in the soil and the concentration in the plants. There was no correlation between plant dry weight and any parameter.

All these relationships and effects were confirmed by PCA for *L. albus* and *N. caerulescens* (Figure 5). Plant dry weight of *L. albus* was associated positively with soil pH (loadings >0.5; Table S1, Supplementary Materials) and negatively with the extractable metal concentrations in the soil and the plant Zn concentration in La-PC1 (51.2% of the variance). This component clearly separated the soils (Figure S1, Supplementary Materials). The second component showed the relationship between the plant metal concentrations (La-PC2, 27.8% of the variance) and separated the monoculture from the cocultivation (Figure S1, Supplementary Materials). The PCA for *N. caerulescens* also related the soil pH and soil extractable-metal concentrations negatively in Nc-PC1 (51.3% of the variance) and the plant Cd and Zn concentrations positively in Nc-PC2 (17.5% of the variance). Plant dry weight was negatively related to the Cu concentration in the *N. caerulescens* plants in a third component (13.9% of the variance; Table S2, Supplementary Materials), in agreement with the previously reported sensitivity of this species to this element [13]. The component Nc-PC1 separated soil S1 from S2, while the separation of individually cultivated plants from cocultivated ones was not so evident for this species (Figure S1, Supplementary Materials).

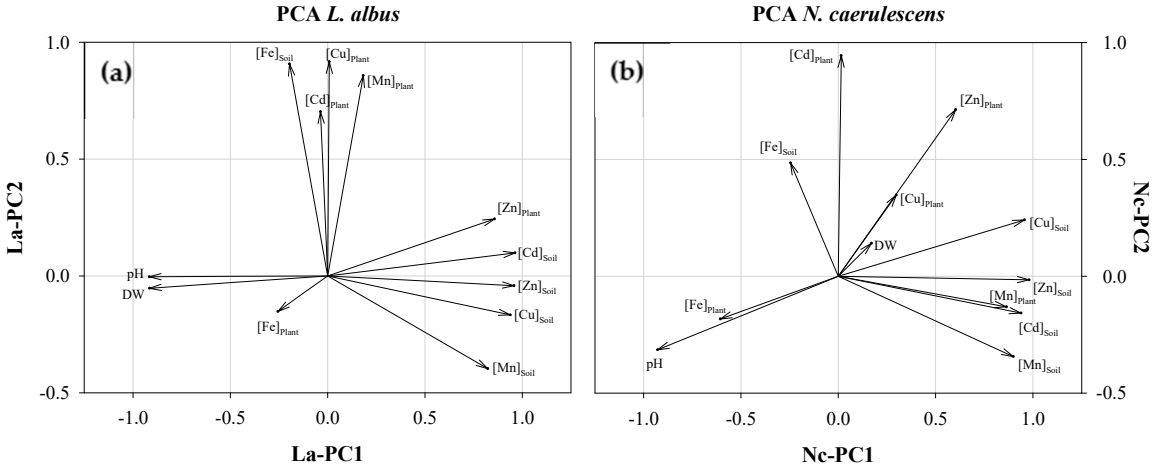

Figure 5. Principal component analysis of the data corresponding to the individual cultivation and cocultivation of *L. albus* (**a**) and *N. caerulescens* (**b**) plants in Experiment 2.

4. Conclusions

The uptake and accumulation of Zn by *N. caerulescens* diminished in the presence of *B. juncea*, especially at circum-neutral soil pH, indicating competition between these species for the uptake of the available Zn forms in the soil. Nevertheless, the plants of *B. juncea* did not grow well in soils with acid pH and were sensitive to the soluble metal concentrations in the soil and to high Cu and Zn accumulation in the aerial part of the plants. Contrastingly, *N. caerulescens* grew better in the acid soil than in the circum-neutral one and, generally, was not negatively influenced by the elevated presence of metals in the soil and the plants; the exception was Cu, which is toxic to this species to a

certain extent, in both experiments. The excluder species *L. albus* was also negatively affected by low pH values, high metal availability in the soils, and high Zn accumulation in the plants, but showed potential as an indicator of Zn availability. The changes provoked by this species in the root-affected soil apparently favored the accumulation of Zn in *N. caerulescens* plants when they were cocultivated.

For practical application and extending the results to climatic conditions where *N. caerulescens* can be appropriately cultivated (temperate and medium–high rainfall areas in northern and central Europe), this species may be cocultivated with *L. albus* for Zn phytoextraction. In the case of *B. juncea*, alternating cultivation (successive crops or alternate rows) with *N. caerulescens* would be appropriate for Zn phytoextraction, provided that the relevant agronomic conditions (pH, fertilizer, planting density) are optimized for plant growth and Zn accumulation and an appropriate *B. juncea* line with high accumulation capacity is selected.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/9/1367/s1. Figure S1: Treatment loadings from the PCAs run with data for each plant species in Experiment 2. Table S1: Results of the PCA run with data for *L. albus* plants from Experiment 2. Table S2: Results of the PCA run with data for *N. caerulescens* plants from Experiment 2.

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