Stress Response and Phytoextraction Potential of Two Noccaea caerulescens Populations in Multicontaminated Soil

Mohammad Chafik Sherri
Catherine SIRGUEY
catherine.sirguey@univ-lorraine.fr

Universite de Lorraine https://orcid.org/0000-0002-3181-9014
Ali Kanso
Kassem Hamze
Stéphanie Ouvrard

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Abstract

Background and Aims

Multi-contamination of soils by various organic and inorganic pollutants is considered an obstacle for the development of hyperaccumulator plants and phytoextraction of metals. The aim of this study was to investigate the effect of polycyclic aromatic hydrocarbons (PAHs) in combination with trace elements on the antioxidant response and phytoextraction efficiency of the Ganges and Chavignée populations of the hyperaccumulator *Noccaea caerulescens*.

Methods

Plants were grown in soil containing some heavy metals at moderate concentrations under phenanthrene (PHE), a model PAH stress condition, for 17 days.

Results

In general, exposure to PHE resulted in a reduction of growth parameters, along with the upregulation of antioxidant enzymes and compounds and limitations in nutrient uptake and heavy metal extraction in *N. caerulescens*. Variations were observed in the magnitude of enzymatic activities and the amount of extracted metals between the two studied populations. Chavignée plants exhibited a slightly more tolerant response to stress than Ganges.

Conclusion

The presence of PHE in the soil proved to be highly toxic for *N. caerulescens*. Nevertheless, to some extent, growth, metals extraction, and antioxidant defense responses differed slightly between the studied populations, suggesting that the difference in defense capacity might ensue different tolerance. This distinction may be related to the adaptations acquired by each population depending on the soil type it originated from.

Introduction

The anthropogenic industry and waste disposal activities have resulted in large-scale contamination of soils (Balseiro-Romero and Baveye 2018). These soils may present multicontamination including trace elements (*e.g* cadmium (Cd), nickel (Ni), zinc (Zn)) and/or persistent organic pollutants (*e.g.* polycyclic aromatic hydrocarbons (PAH)). Past anthropic activities have also resulted in significant alteration of soil physico-chemical properties resulting in additional abiotic stresses such as soil salinity and decreased water retention capacity leading to hydric stress. These are known to limit plant growth and development through multiple orders of magnitude and to trigger perturbations in the metabolism of plants (Godoy et al. 2021; Haider et al. 2021; Jogawat et al. 2021; Molina Lázaro and Segura Ana 2021).

Phytoremediation is a plant-based method that requires the use of plants to extract and eliminate pollutants or reduce their bioavailability in soil (Yan et al. 2020). This technology involves various techniques including phytostabilization, phytoextraction, phytovolatilization and rhizodegradation (Ernst 2005; Marques et al. 2009). In the case of trace elements, phytoextraction consists of the use of hyperaccumulator plants that have the ability to absorb contaminants from soil, transfer, and accumulate them in their aerial parts. Among the various hyperaccumulators, *Noccaea caerulescens* (Brassicaceae) is documented to be a good hyperaccumulator of Zn or Cd depending on the population (Assunção et al. 2003; Gonneau et al. 2017). However, under abiotic stress conditions (multi-contamination), the establishment and development of *N. caerulescens* can sometimes be impossible which therefore inhibits phytoextraction process (Ouvrard et al. 2011; Bauddh and Singh 2012; Sirguey and Ouvrard 2013).

PAHs are widespread, toxic, environmentally persistent, and carcinogenic by-products of the combustion of carbon-based fuels. These compounds have relatively low solubility due to their chemical structure based on non-functionalized benzene rings and medium to high molecular weight. They mostly interact with plant through adsorption processes on the root system or within the first internal cellular layers (Dupuy et al. 2016). Plant transfer via water uptake is quantitatively limited but may still strongly affect plant metabolism (Wilcke 2000). Previously, plant studies revealed that PAHs induce oxidative stress, reduce growth, and cause leaf deformation as well as tissue necrosis (Alkio et al. 2005; Meudec et al. 2007; Liu et al. 2009). A similarity between the effect of PAHs and water stress has also been demonstrated (Dupuy et al., 2015; Kummerová et al., 2013; Pašková et al. 2006). The more documented drought stress is reported to limit the growth of several plants via inducing biochemical and physiological perturbations (Zia et al., 2021). It is known to inhibit photosynthesis, induce stomatal closure, reduce leaf area, increase the amount of osmolytes leading to a decrease in water potential, and induce oxidative damage to plant cells (Ibrahim et al. 2019).

Oxidative stress is the consequence of the overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H2O2), superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals (HO•), of which H_2O_2 can be the most harmful. ROS play a dual role in plant biology, acting as essential signaling molecules and as toxic by-products of aerobic metabolism in response to various stressors (Mittler et al. 2004). ROS are highly bioactive and can cause damage to DNA, lipids and proteins, resulting in cell membrane peroxidation (Liu et al. 2009; Sharma et al. 2012). Studies have reported visible symptoms such as chlorosis, white spots along with necrosis when plants are exposed to PAHs (Vollenweider and Günthardt-Goerg 2005; Liu et al. 2009; Oguntimehin et al. 2010).

However, to scavenge ROS, plants have developed a well-equipped antioxidant defense system that includes enzymatic and non-enzymatic antioxidants (Vardhini and Anjum 2015a). Non-enzymatic antioxidants include flavonoids, anthocyanins, tocopherols, and various phenolic compounds (Munné-Bosch 2005; Rai et al. 2013). Enzymatic antioxidants include catalase (CAT), guaiacol peroxidase (GPOX), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and other enzymes that detoxify ROS (Gill and Tuteja 2010). Upregulation of antioxidants has been observed in stressed plants. This suggests an important role of antioxidants in alleviating stress (Song et al. 2011; Spinedi et al. 2021). hus, knowledge of the defense mechanisms and stress responses involved at the molecular level is necessary for understanding plant tolerance to abiotic stresses.

Our research aims to study the effects of PAHs on the antioxidative response and phytoextraction efficiency of two populations of the hyperaccumulator *N. caerulescens*. These correspond to two edaphic types: metalliferous (Ganges population) and non-metalliferous (Chavignée population) habitats. Our results should provide a comprehensive and comparative knowledge of stress responses, both in terms of phytoextraction and plant tolerance to PAHs in a multi-contamination situation.

Materials and Methods

Plant material and growth conditions

The experiment was conducted with a sandy-loamy soil taken from the upper horizon of an agricultural soil (Gleyic Luvisol, IUSS 2016) located at Chenevières (Grand Est, France) with aged artificial contamination. A batch of 2 mm sieved top horizon was spiked in 2013 with an artificial solution of Cd, Cu, Ni, Pb and Zn and used for the cultivation of the hyperaccumulator plant *N. caerulescens* over 3 years (Sterckeman et al., 2017, 2019). This aged artificial polluted soil was homogenized and passed through a 2-mm sieve. Its main physio-chemical properties are presented in Table 1. The seeds of *N. caerulescens* J & C Presl. originated from two populations, a metallicolous population collected from a mining site located in Ganges (Occitanie, France) and a non-metallicolous population collected in Chavignée (Bourgogne-Franche-Comté, France) (Gonneau et al., 2014).

Table 1
Soil main properties

Particle size distribution (%)				
Clay	Fine silt	Coarse silt	Fine sand	Coarse sand
10.3	19.1	13.9	15.2	41.5
Agronomic parameters				
pH	Organic C (%)	Total N (%)	Total $CaCO_3$ (%)	Effective CEC ($cmol^+ kg^{-1}$)
6.45	0.974	0.105	0.12	6.12
Aqua Regia trace elements ($mg\ kg^{-1}$)				
Cd	Cu	Ni	Pb	Zn
3.63	28.4	120	60.2	649

In the preculture phase, seeds were placed on water-soaked filter papers and cotton and incubated at 4°C in the dark. They were then sown in multi-cell trays (cell size: 8 × 4 × 4 cm) filled with moistened sowing substrate (Klasmann-Deilmann 5, Germany). These trays were placed in a climatic chamber with a photoperiod of 16 h and a temperature of 23°C/18°C day/night for a period of 42 days.

During the culture phase, a phenanthrene stress was applied. An aliquot of metal contaminated soil (10% in mass) was spiked with highly pure phenanthrene (PHE) (Sigma-Aldrich, USA, purity-98%) dissolved in acetone. After acetone was evaporated, the treated soil was diluted by mixing it with the rest of the soil (90%) to obtain a final concentration of 200 mg PHE kg^{-1} soil. This spiking procedure insures a homogenous distribution of PHE crystals in the soil. Both soil treatments (control (Ctrl) and PHE) were conducted in 14 replicates distributed evenly between both populations Ganges (Ga) and Chavignée (Ch). Thus, twenty-eight homogeneous seedlings were transferred into 750 cm^3 pots filled with 800 g soil and placed in the climatic chamber in a randomized block design.

Plant harvest

After 17 days of cultivation under stress, plants were removed from their pots. The shoots were rinsed with deionized water and the roots were thoroughly cleaned from all adhering soil with tap water before measurement. Fresh weight of aerial parts and root systems was then measured. Dry weight of root systems was measured after oven drying at 60°C for 48 hours. Aerial parts were frozen in liquid nitrogen and then divided into two parts; the first was used for dry weight and elemental measurements after freeze-drying, and the second was stored at -80°C for further biochemical analysis.

Assessment of plant element content and leaf pigment indices

Total flavonoids, anthocyanins, chlorophyll concentration and nitrogen balance index (NBI®) were measured in the leaves of the plants before harvest using a non-destructive DUALEX Scientific™ portable instrument. Measurements were taken on the adaxial side of the youngest upper fully developed leaves of the plants. Measurements were taken from 2 leaves per plant. (Goulas et al. 2004; Cartelat et al. 2005; Cerovic et al. 2012).

Freeze-dried aerial parts and oven-dried root systems of plant material were finely ground using an agate mortar before successive digestion of 50 mg with 8 ml 65% HNO_3 for 12 h and 4 ml 30% H_2O_2 for 3.5 h at 95°C using the DigiPREP® system (SCP Science, Baie-d'Urfé, QC, Canada). The samples were then adjusted to 25 mL with ultrapure water (18 MΩ) after filtration of the extract at 0.45 µm. The concentrations of elements (Cd, Ni, Zn) in the plant extracts were analyzed by induced coupled plasma emission spectrometry (ICP-AES, iCAP 6000 series, Thermo Scientific, Cambridge, UK). To check the validity of the results, a blank and a control material of *N. caerulescens* with known composition (internal analyses carried out by INRAE-USRAVE, Villenave d'Ornon, France), as well as a certified solution (EU-H3, SCP Science, Courtaboeuf, France), were periodically inserted into the batches to be analyzed.

Carbon and nitrogen contents of shoot and root samples were determined using an elemental analyzer (Vario Micro, Elementar). Plant material (5 mg) was placed in 6×4 mm tin capsules. For quality control, standard plant samples and sulfanilamide standard were periodically included in the batches to be analyzed.

Assessment of the antioxidant response

Lipid peroxidation assessment was applied to plant material stored at -80°C (100 mg) after homogenization in 0.1% (w/v) cold trichloroacetic acid, followed by centrifugation of the homogenate for 15 min at 15,000 × g. The supernatant obtained was used for measuring the concentration of malondialdehyde (MDA), a product of unsaturated fatty acid peroxidation, according to the method described by (Senthilkumar et al. 2021).

Antioxidant enzyme activities were evaluated on enzyme extracts from plant leaves. Protein extraction was applied to plant material samples (100 mg) stored at -80°C after homogenization in 0.1 *M* phosphate extraction buffer (pH 7.6) containing 1 *mM* EDTA, 1 *mM* ascorbate, 0.5% w/v polyvinylpolypyrrolidone and 1 *mM* phenylmethylsulfonyl, followed by centrifugation at 15000 × g for 20 min at 4°C. Protein concentration in the extracts was determined by the method of Bradford (1976), using bovine serum albumin as a standard and the Bio-Rad commercial reagent. Catalase (CAT, EC 1.11.1.6) activity assay was adapted from Aebi (1984). The reaction mixture was prepared by mixing 20 µL of H_2O_2 (40 *mM*), 930 µL of 0.1 *M* phosphate buffer (1 *mM* EDTA, pH 7.6) and 50 µL of enzyme extracts from plant leaves. The decrease in absorbance was monitored at 240 nm for 1 min. The activity of the catalase enzyme was estimated from the amount of decomposed H_2O_2. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined based on the method of Nakano & Asada (1981). The reaction mixture was prepared by mixing 10 µL of H_2O_2 (20 *mM*), 890 µL of 0.1 *M* phosphate buffer (1 *mM* EDTA, pH 7.6), 50 µL of ascorbate (10 *mM*) and 50 µL of enzyme extracts from plant leaves. The decrease of absorbance was monitored spectrophotometrically at 290 nm for 2 min. Guaiacol peroxidase (GPOX, EC 1.11.1.7) activity was determined by the method modified from Castillo et al. (1984). The reaction mixture was prepared by mixing 50 µL of enzyme extracts from plant leaves with 950 µL of reacting solution containing 0.2% guaiacol (16 *mM*), 0.1 M phosphate buffer (1 *mM* EDTA, pH 7.0) and H_2O_2 (2 *mM*). The increase of absorbance, due to the formation of tetra-guaiacol, was monitored at 470 nm. Finally, dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was determined essentially as described by Noctor et al. (2016) with some modifications. The reaction mixture was prepared by mixing 905 µL of 0.1 *M* phosphate buffer (1 *mM* EDTA, pH 7.0), 50 µL of DHA (4 *mM*), 25 µl of reduced glutathione (100 *mM*) and 20 µL of enzyme extracts from plant leaves. The change of absorbance was monitored spectrophotometrically at 265 nm for 2 min.

Statistical analysis

All tests were performed with R software v 4.1.1 (R Development Core Team, 2015). Two-way ANOVA was performed, followed by Tukey's HSD test from the “multcomp” package in parametric conditions to evaluate the effect of treatment, population and their interaction. The adequacy of the data with respect to the ANOVA models was verified by examining the residuals for independence (Durbin-Watson test, “car” package), normality (Shapiro test) and homogeneity (Levene test). If the latter assumptions were not validated after “sqrt” or “log” transformation of the data, a Wilcoxon pairwise test with a Holm correction of the p-value was used. The relationships between the variables describing i) plant growth, ii) nutrient uptake iii) metal uptake and iv) stress response were assessed through a multiple-factor analysis (MFA) with the “FactomineR” package.

Results

Growth indicators: plant development and nutritive status

Although the stress level was chosen according to previous results to avoid excessive damage (i.e. plant death), plant survival was limited in the PHE treatment with 43% survival for both populations (3 plants out of 7). In the control treatment, 100% and 70% of plants survived in the Chavignée and Ganges populations, respectively (5 plants out of 7). Of the surviving plants from the Ga-PHE treatment, some produced too little biomass (< 500 mg FW) to perform all the analyses. As a result, measurements of water content (WC), C, N, and metal concentrations could only be made on the largest individual. However, the dry biomass produced by the two smallest Ga-PHE plants was estimated by considering the mean WC of all PHE-stressed plants. Dead plants are not part of any measurement.

At the 10% level, only root fresh biomass production was significantly affected by PHE stress ($F_{(1,13)} = 5.09$, $p = 0.061$, Table 2). On average, fresh biomass decreased by a factor of 1.5 and 2.6 for shoots and roots respectively. Consistently, we observed a strong significant effect of PHE stress on both shoot and root water content (shoot: $F_{(1,12)} = 17.0$, $p = 0.0014$; root: $F_{(1,13)} = 27.8$, $p < 0.001$). Shoot N and P contents and root K contents were all significantly reduced when plants were exposed to PHE stress (shoot N: $F_{(1,12)} = 7.74$, $p = 0.017$; shoot P: $F_{(1,12)} = 3.77$, $p = 0.076$; root K: $F_{(1,13)} = 5.41$, $p = 0.036$)

Table 2
Shoot and root growth parameters and nutrient content of two *N. caerulescens* populations in response to PHE stress. At the organ level, different letters significant differences ($p < 0.05$)

Organ	Population	Treatment	Fresh weight (g)		Dry weight (mg)		Water content (%)		C ($g\ kg^{-1}$)		N ($g\ kg^{-1}$)		P ($g\ kg^{-1}$)		S ($g\ kg^{-1}$)		K ($g\ kg^{-1}$)		Na ($g\ kg^{-1}$)		
Shoot	Chavignée	CTRL	1.011	a	169	a	82.4	b	363	a	41.9	a	2.61	a	7.78	a	18.2	a	17.2	b	
		PHE	0.837	a	249	a	70.1	a	374	a	31.1	a	1.76	a	7.26	a	19.8	a	16.2	ab	
	Ganges	CTRL	1.198	a	230	a	78.3	ab	390	a	33.1	a	2.46	a	7.53	a	20.7	a	9.48	a	
		PHE	0.626	a	175	a	71.6	ab	404	*	25.6	*	1.71	*	5.47	*	11.6	*	9.9	*	
	Chavignée		0.959	a	193	a	78.7	a	367	a	38.6	b	2.36	a	7.62	a	18.7	a	16.9	b	
	Ganges		0.984	a	209	a	75.8	a	392	b	31.9	a	2.34	a	7.19	a	19.2	a	9.54	a	
		CTRL	1.089	a	195	a	80.7	b	375	a	38.2	b	2.55	b	7.68	a	19.2	a	14.1	a	
		PHE	0.732	a	212	a	70.8	a	382	a	29.7	a	1.74	a	6.82	a	17.8	a	16.3	a	
Root	Chavignée	CTRL	0.160	ab	42,1	a	72.9	b	448	a	28.7	a	2.54	a	6.58	a	8.66	ab	3.58	a	
		PHE	0.061	a	36,6	a	37.3	a	455	a	23.9	a	1.91	a	5.69	a	4.75	a	3.2	a	
	Ganges	CTRL	0.261	b	48,9	a	80.5	b	430	a	29.6	a	3.12	a	8.56	a	10.3	b	3.35	a	
		PHE	0.090	ab	23,7	a	72.7	b	448	a	30.8	a	1.87	a	8.01	a	7.80	ab	3.65	a	
	Chavignée		0.116	a	40,3	a	61.0	a	450	a	27.1	a	2.33	a	6.28	a	7.35	a	3.45	a	
	Ganges		0.197	b	39,4	a	77.6	a	437	a	30.1	a	2.65	a	8.36	a	9.35	a	3.46	a	
		CTRL	0.197	a	45,2	a	76.3	b	440	a	29.1	a	2.81	a	7.48	a	9.40	b	3.47	a	
		PHE	0.075	a	30,2	a	55.0	a	451	a	27.4	a	1.89	a	6.85	a	6.27	a	3.21	a	

*only one measurement

Trace elements uptake

At the population level, Chavignée plants concentrated 1.3 and 1.9 times more Ni and Zn, respectively, than Ganges plants (Table 3). The effect was significant at the 10% level only for Zn. Overall, trace element concentrations in both roots and shoots were strongly reduced when plants were exposed to PHE stress, with a significant effect at the 5% level. The effect was greatest in shoots with a 4-, 2-, and 5-fold decrease for Cd, Ni, and Zn, respectively. Irrespective of the element considered, the effect was also higher in Chavignée than in Ganges plants. For example, shoot Zn content decreased by a factor of 6 and 3 in Chavignée and Ganges plants, respectively.

Table 3
Trace element concentrations in shoots and roots of two *N. caerulescens* populations in response to PHE stress. At the organ level, different letters indicate significant differences ($p < 0.05$)

Organ	Population	Treatment	Cd (mg/kg)		Ni (mg/kg)		Zn (mg/kg)	
Shoot	Chavignée	CTRL	27.6	a	72.6	a	2240	a
		PHE	6.87	a	31.3	a	386	a
	Ganges	CTRL	25.2	a	49.8	a	1015	a
		PHE	7.24	*	24.0	*	318	*
	Chavignée		21.4	a	60.2	a	1684	a
	Ganges		22.2	a	45.5	a	899	a
		CTRL	26.6	b	63.1	b	1729	b
		PHE	6.96	a	29.5	a	369	a
Root	Chavignée	CTRL	18.4	a	68.1	a	1058	b
		PHE	6.03	a	60.4	a	270	a
	Ganges	CTRL	22.4	a	58.0	a	795	ab
		PHE	10.2	a	64.6	a	430	ab
	Chavignée		14.3	a	65.6	a	795	a
	Ganges		17.8	a	60.5	a	658	a
		CTRL	20.2	b	63.5	a	938	b
		PHE	8.11	a	62.5	a	350	a
*only one measurement								

Translocation factor (TF) was calculated as the ratio between shoot and root concentrations (Table 4). Although not significant, TF values also tended to decrease in the Ganges and Chavignée populations upon exposure to PHE. The Ganges population seemed to be the most affected with a 50% decrease in TF values regardless of the element considered. The amount of metal extracted by the plant was calculated as the product of shoot biomass and metal shoot concentration (Table 5). Although no significant differences were observed, PHE-stressed plants had a poor ability to extract metals. For example, Chavignée plants extracted 2.6, 1.5, and 3.9 times less Cd, Ni, and Zn, respectively, when subjected to PHE stress. Similarly, Ganges plants extracted 3.4, 1.8, and 3.1 times less Cd, Ni, and Zn, respectively, when subjected to PHE stress. In comparison, the Chavignée population was more efficient in extracting Zn and the Ganges population was more efficient in extracting Cd, both with and without PHE stress.

Table 4
Shoot/root translocation factors (TF) of Cd, Ni and Zn for two *N. caerulescens* populations in response to PHE stress. Different letters indicate significant differences ($p < 0.05$)

Population	Treatment	TF Cd		TF Ni		TF Zn	
Chavignée	CTRL	1.46	a	1.10	a	2.05	a
	PHE	1.48	a	0.60	a	1.56	a
Ganges	CTRL	1.03	a	0.90	a	1.23	a
	PHE	0.55	*	0.45	*	0.59	*
Chavignée		1.47	a	0.94	a	1.89	a
Ganges		0.95	a	0.83	a	1.12	a
	CTRL	1.26	a	1.01	a	1.68	a
	PHE	1.25	a	0.56	a	1.32	a
*only one measurement							

Table 5

Quantity (µg plant^{-1}) of Cd, Ni and Zn extracted by shoots for two *N. caerulescens* populations in response to PHE stress. Different letters indicate significant differences ($p < 0.05$)

Population	Treatment	Cd		Ni		Zn	
Chavignée	CTRL	4.63	a	12.4	a	388	a
	PHE	1.80	a	8.28	a	98.1	a
Ganges	CTRL	7.15	a	13.0	a	287	a
	PHE	2.09	*	6.93	*	92.0	*
Chavignée		3.69	a	11.0	a	292	a
Ganges		6.31	a	11.9	a	254	a
	CTRL	5.78	a	12.6	a	342	a
	PHE	1.87	a	7.94	a	96.6	a
*only one measurement							

Plant stress indicators

The response to the stress induced by PHE application was evaluated by non-destructive measurement of leaf pigments and by measurement of enzymatic activities involved in the antioxidant response (Table 6 and Table 7).

Regarding chlorophyll concentration (CHL), a non-significant decrease from 39.2 to 32.8 µg cm^{-2} was observed under PHE stress in the Ganges population (Table 6). At the population level, Ganges plants had a significantly higher CHL content than Chavignée plants ($F_{(1,14)} = 16.3$, $p = 0.0012$; Table 6). For both populations combined, PHE stress resulted in a significant increase (at the 5% level) by a factor of 1.4 to 1.9 in anthocyanin (ANT) and flavonoid (FLA) indices and in malondialdehyde (MDA) content. Furthermore, we observed that the response was much stronger in plants from the Ganges population with respect to MDA production, with an increase by a factor of 3.2 compared to 1.4 for the Chavignée plants. Overall, we did not observe any difference in the production of stress molecules between the two populations.

The Nitrogen Balance Index (NBI) was calculated as the ratio of the CHL content to the FLA index. Similarly, although the NBI decreased by a factor of 1.5 when PHE treatment was applied to replicates, no significance was obtained. The behavior of the two populations was similar.

Table 6

Leaf pigment indices and malondialdehyde content of two *N. caerulescens* populations in response to PHE stress. ANT: anthocyanin index, CHL: epidermal chlorophyll (µg cm^{-2}), FLA: flavonoids index, NBI: Nitrogen balance index, MDA: Malondialdehyde concentration (nmol g^{-1} FW). Different letters indicate significant differences ($p < 0.05$)

Population	Treatment	ANT		CHL		FLA		NBI		MDA	
Chavignée	CTRL	0.110	a	26.9	a	0.653	a	37.0	a	31.5	ab
	PHE	0.194	b	25.8	a	0.923	ab	24.7	a	43.2	ab
Ganges	CTRL	0.096	a	39.0	b	0.807	a	44.7	a	16.4	a
	PHE	0.211	b	32.8	ab	1.192	b	29.6	a	52.4	b
Chavignée		0.135	a	26.6	a	0.734	a	33.3	a	36.5	a
Ganges		0.139	a	36.7	b	0.952	a	39.0	a	34.4	a
	CTRL	0.104	a	32.0	a	0.717	a	40.2	a	25.0	a
	PHE	0.202	b	29.3	a	1.057	b	27.2	a	47.8	b

When comparing the Ganges and Chavignée populations, we observed no difference in guaiacol peroxidase (GPOX) and ascorbate peroxidase (APX) activities, but strong differences in catalase (CAT) and dehydroascorbate reductase (DHAR) activities (Table 5), with values 2 and 1.5 higher, respectively, in Ganges plants than in Chavignée plants (CAT: $F_{(1,11)} = 3.5$, $p = 0.088$; DHAR: $F_{(1,9)} = 5.54$, p 0.043).

Globally, PHE stress resulted in an increase in all enzyme activities by a factor varying from 1.5 to 2.4, the difference being significant at the 5% level for APX and DHAR. Interestingly, we observed an interaction effect between population and PHE treatments for CAT ($F_{(1,11)} = 3.33$, $p = 0.095$), APX ($F_{(1,10)} = 3.68$, $p = 0.084$) and DHAR ($F_{(1,9)} = 3.42$, $p = 0.097$). Thus, DHAR activity in Ganges plants was not affected by PHE stress, whereas it increased from 4.6 (µmol min^{-}1 mg^{-}1 protein) in Ch-Ctrl to 12.9 (µmol min^{-}1 mg^{-}1 protein) in Ch-PHE. An opposite behavior was observed for CAT activity, with an increase by a factor of 1.7 in Ganges plants under PHE stress, whereas no effect was observed in Chavignée plants. Regarding APX and GPOX activities, both increased under PHE stress for both populations. However, the intensity of the response varied according to the population. In fact, the level of increase was 1.4 times higher for GPOX in Ganges and 1.8 times higher for APX in Chavignée.

Table 7
Antioxidant activity in shoots of two N. caerulescens populations in response to PHE stress. CAT: catalase, GPOX: guaiacol peroxidase, APX: ascorbate peroxidase, DHAR: dehydroascorbate reductase. Activities are expressed in µmol min^{-1} mg^{-1} protein. Different letters indicate significant differences ($p < 0.05$)

Population	Treatment	CAT		GPOX		APX		DHAR	
Chavignée	CTRL	0.189	a	0.030	a	0.076	a	4.63	a
	PHE	0.205	a	0.044	ab	0.237	b	12.92	a
Ganges	CTRL	0.311	ab	0.026	a	0.095	a	12.29	a
	PHE	0.515	b	0.054	b	0.160	ab	12.46	a
Chavignée		0.195	a	0.036	a	0.136	a	8.18	a
Ganges		0.398	a	0.040	a	0.128	a	12.38	b
	CTRL	0.243	a	0.029	a	0.083	a	7.91	a
	PHE	0.360	a	0.049	a	0.198	b	12.69	b

Multivariate Analysis

A multi-factor analysis was used to assess the relationships between four groups of variables describing plant growth, nutrient uptake, metal uptake and stress response (Fig. 1 and Fig. 2).

The first 2 axes of the analysis express 56% of the total inertia of the data set; this means that 56% of the total variability of the cloud of individuals (or variables) is represented in this plane. This is a fairly large percentage, and so the first level adequately represents the variability contained in a large portion of the active dataset. This value is significantly higher than the base value of 38.59%, so the variability explained by this design is highly significant.

The Wilks test indicates that the best qualitative variable to illustrate the distances between individuals on the plane is the treatment variable (Fig. 1). The graph of individuals on the factorial design shows that dimension 1 pits the PHE treatment (on the left side of the graph, characterized by a strongly negative coordinate on the axis) against the CTRL treatment (in the center-right of the graph, characterized by a slightly positive coordinate on the axis). The confidence ellipses and the Wilks test indicate a significant treatment effect ($p = 0.0125$). Dimension 2 contrasts the Ganges population (at the top of the graph, characterized by a strongly positive coordinate on the axis) with the Chavignée population (at the bottom of the graph, characterized by a slightly negative coordinate on the axis). Again, the confidence ellipses and the Wilks test indicate a significant treatment effect ($p = 0.0243$).

Regarding the representation of the variables on the factorial plane (Fig. 2), dimension 1 contrasts two groups of strongly contributing variables ($|R|min = 0.61$; $p.value < 0.0261$). The variables DHAR, APX and ANT are characterized by a strongly negative coordinate on the axis. These three variables all belong to the group of stress indicators. The variables Zn.R, Cd.S, Cd.R, NBI, Ni.S, P.S, Zn.S, WC.S, WC.R, N.S are characterized by a strongly positive coordinate on the axis. In this group, 5 of the 10 variables belong to the metal uptake group, the other 5 belong to the nutrient uptake and growth indicators groups. Dimension 2 contrasts two groups of strongly contributing variables ($|R|min = 0.57$; $p.value < 0.0412$). On the one hand, the variables N.S, C.R and S.S are characterized by a strongly negative coordinate on the axis and, on the other hand, the variables S.R, C.S, CHL and FLA are characterized by a strongly positive coordinate on the axis. With the exception of FLA, which belongs to the Stress Indicators group, all variables belong to the Growth Indicators and Nutrient Uptake groups.

Discussion

The present work begins to unravel the antioxidative responses of two populations of *N. caerulescens* (Ganges and Chavignée), with a particular focus on growth performance, antioxidant enzyme activities, photosynthesis as well as the efficiency of phytoextraction.

PHE reduces plant growth performance with a more pronounced effect for the metalicolous population

For both populations, PHE resulted in high mortality and has the potential to significantly suppress the growth of the remaining plant by reducing shoot and root fresh and dry weights. These observations were consistent with previous work on PAH phytotoxicity (Liu et al. 2009; Ahammed et al. 2012a). This study also revealed a significant decrease in the relative water content of shoots and roots of both populations, with a more pronounced significant decrease in Chavignée roots (Table 2). The greater reduction in water content of roots compared to shoots highlights a barrier effect of PHE on water flux at the root level. This is consistent with the study by Zelko et al. (2017) which shows that PHE affects the development of the peri-endodermal layer of roots at the stage with well-developed suberin lamellae. Additionally, the same study showed that suberin lamellar endoderm cells appeared closer to the root apex of *N. caerulescens* exposed to PHE, likely due to the decrease in mean root diameter and inhibition of the elongation of cells and root hairs. Overall, these symptoms resembled a response to water deficit. Moreover, PAHs caused the reduction of plant water content, which subsequently resulted in the inhibition of PAH transport and led to a greater accumulation of PAHs in the root than in the shoot (Li et al., 2008). Huang et al. (2004) also suggested that it may be more beneficial for plants to retain the PAHs in the roots than in the shoots, where photosynthesis takes place.

The carbon to nitrogen (C/N) ratio is one of the key factors controlling plant metabolism, development and growth (Coruzzi and Zhou 2001; Martin et al. 2002). In this study, the C/N ratio was increased in PHE treatments (Table 2). The C/N imbalance under stress might be due to sugar accumulation and nitrogen deficiency in both populations (Table 2), which are commonly associated with leaf senescence under plant stress (Chen et al. 2015; Zhu et al. 2015). Several studies reported that various nutrient elements, such as N, decreased in the aerial part of some plant species under stress conditions (Nathawat et al. 2007; Larsen et al. 2011; Sun et al. 2017). The nitrogen balance index (NBI) indicates the ratio of both the chlorophyll and flavonoids in leaves. This parameter reflects the nitrogen status and health of the plant. The obtained results showed that PHE decreased the NBI under PHE treatment. This result is evidence of the lack of nitrogen content and low nitrogen use efficiency (Ramamoorthy et al. 2022).

Regarding chlorophyll content, it was found to be slightly decreased only in the leaves of Ganges plants (Table 3). This could also be attributed to different adaptation mechanisms of the two plant populations in response to PHE addition. Similar to the results observed in Ganges plants, Liu et al., (2009) also found decreased leaf pigments after PHE application in *Arabidopsis thaliana*. This could be due to the overproduction of abscisic acid (ABA) together with ROS in plants, which promotes chlorophyll degradation (Liu et al. 2009; Váňová et al. 2009). In addition, a low K content in Ganges plants was observed in the presence of PHE, similar to the observation of Dupuy et al. (2015). This decrease in K content may reduce its resistance to stress and affect photosynthesis (Tsay et al. 2011; Wang et al. 2013a) with a consequent decrease in CO_2 fixation and utilization (Waraich et al. 2012). This explains why the decrease in dry biomass observed in this study was greater in Ganges plants than in Chavignée plants. However, for the Chavignée population, the chlorophyll content was unchanged perhaps due also to the K content in the shoots of stressed plants, which was similar to that of the control. This is consistent with how tolerant plants respond to abiotic stress (Chen et al. 2007; Wang et al. 2013a). In fact, plant mineral nutrition represents the key factor for plant stress resistance (Wang et al. 2013b; Mateus et al. 2021).

PHE reduces phytoextraction efficiency

Metal concentrations measured in aerial parts and roots of both populations of *N. caerulescens* showed that the presence of PHE in the soil decreased both the amount of extracted metals (Table 4) and their translocation factor from roots to aerial parts (Table 5). These results are in full agreement with previous research which showed that PHE and pyrene (PYR) induced negative effects on the phytoextraction of Cd from Cd-spiked soil by the hyperaccumulator *Sedum alfredii* (Wang et al., 2012). Similarly, it has been reported that Cd accumulation in stems of *Medicago sativa* was reduced after soil contamination with PHE, which was probably caused by the accumulation of organic pollutants in roots, which hindered Cd transport from roots to aboveground parts (Li et al. 2020). Lin et al. (2008) also found that co-contamination of soil with PYR and Cu decreased the Cu phytoextraction efficiency and translocation factor in *Zea mays*. The different results suggest that a combination of metal and organic contaminants may affect bioaccumulation patterns. The exact mechanism by which phenanthrene affects metals uptake is not fully understood. In addition to its passive transport, the active transport of PHE by symporters coupled to H^+ involve changes in the function of plasma membrane H^+-ATPase (PM H^+-ATPase) and cytosolic and intracellular pH (Zhan et al. 2012; Yin et al. 2015). In addition, some studies suggest that the consumption of H^+ by PHE uptake can increase the pH of the hydroponic solution (Zhan et al. 2010; Liang et al. 2012). Thus, metals uptake capacity may be reduced due to the increased in pH. In addition, studies have shown that exposure to PHE stimulates PM H^+-ATPase activity due to high H^+ flux into root cells, suggesting that PM H^+-ATPase plays an essential role in PHE uptake (Yin et al. 2015). This could be also a factor affecting metal uptake, especially since Dalir et al. (2017) showed a negative correlation between PM H^+-ATPase activity and Ni uptake in wheat cultivar under Ni deficiency stress. Sun et al. (2011) studied the interactions of arsenic (As) and PHE on plant uptake and antioxidative response of *Pteris vitatta*. They observed that PHE degradation products can compete with As for reduced glutathione, a molecule known to play a key role in As(V) reduction in the plant and subsequent As(III) compartmentation in fronds. This resulted in a dramatic reduction of As(III) in the fronds. Thus, PHE could indirectly affect metal accumulation in aerial plant parts by diverting the metabolic pathway for metal sequestration to PHE degradation.

A global up-regulation of stress biomarkers and antioxidant enzymes in PHE-treated plants, but with different signatures depending on the metallic or non-metallic origin of the populations studied.

Phenolic compounds are crucially involved in the detoxification of oxidative metabolites and can therefore protect the cell from oxidative damage (Kováčik et al. 2009). Anthocyanins in the vacuoles of mesophyll cells have been shown to act as scavengers of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) (Kytridis and Manetas 2006). A remarkable increase in the production of anthocyanins and flavonoids, which are known to be hallmarks of plant stress in leaves, was observed in the present study for both the populations studied, especially for the Ganges population (Table 3). These results are similar to those obtained by Tarigholizadeh et al. (2021), who found that PHE induced a significant increase in anthocyanins and total phenolic compounds in shoots of *Panicum miliaceum* L. when treated with different concentrations of PHE. Such an increase in anthocyanin levels has already been observed in *N. caerulescens* when subjected to herbivory stress (Asad et al. 2015). Furthermore, Eryılmaz (2006) reported a high accumulation of anthocyanins in two plants belonging to Solanaceae and Brassicaceae when subjected to salinity stress.

Membrane lipid peroxidation is a biomarker of damage commonly expressed as MDA content. MDA accumulation in plants treated with PHE has been previously reported in *Arabidopsis thaliana* (Liu et al. 2009) and in *Triticum aestivum L.*, *Medicago sativa* L. and *Helianthus annus L.* (Salehi-Lisar and Deljoo 2015). In this study, the MDA value for the control treatment was higher in the non-metalicolous Chavignée population than in the metalicolous Ganges population. In fact, it has been shown that MDA levels can also increase in *N. caerulescens* under the effect of Cd, although this element is not considered a stressor for this hyperaccumulating plant (Wang et al. 2008). Thus, the higher level of MDA in the control plants of the non-metalicolous Chavignée population could be a consequence of the presence of Cd in the soil used.

When treated with PHE, MDA content in plants increased in both populations tested, with the increase being significant and more intense in the Ganges population (Table 3). The increased MDA levels indicate a lipid peroxidation and even cell damage in response to PHE, supporting the idea of ROS production and oxidative stress (Zhang et al. 2011). This suggests that PHE stress resulted in more oxidative damage in plants from the Ganges population. A clear

physiological difference was observed between the two populations in response to PHE. The exposure to PHE increased the Na/K molar ratio in the shoot of Ganges plants by 86%, which was also associated with a disruption of the metal balance (Ca, K, Fe) (Table 2). In fact, the strong decrease in K content in shoot and root was associated with an accumulation of Na in both parts. Inhibition of protein synthesis and cytosolic enzyme activities occurs when the Na/K ratio increases significantly (Shabala and Cuin 2008). Therefore, the alteration of Na/K ratio in Ganges population may be an explanation for its low resistance to PHE-induced stress. In contrast, the Na/K molar ratio was slightly decreased in the shoot of the Chavignée population under PHE treatment. This work showed that the translocation of K from roots to shoots was enhanced in the Chavignée population, as the shoot/root ratio of K was consistently higher in PHE-treated plants. This explains why the Na/K ratio was reduced in the shoot as a strategy to prevent disruption of various enzymatic processes and inhibition of protein synthesis (Cai and Gao 2020; Huang et al. 2020).

The presence of PAHs can disrupt the biomembrane structure and cause damage to plant cells as a result of the formation of ROS induced by these molecules and their derivatives (Tukaj and Aksmann 2007). Both enzymatic and non-enzymatic antioxidants play an essential role in neutralizing ROS to prevent oxidative damage. GPOX defends cells against harmful concentrations of hydroperoxides and reduces H_2O_2 to H_2O using various electron donors present in cells (Mittler et al. 2004). In addition, GPOX also plays an important role in the inactivation and oxidation of xenobiotics (Farkas et al. 2007). Therefore, improved GPOX activity may enhance the degradation of PHE by plants. APX is another important enzyme involved in the ascorbate–glutathione cycle, whose increased activity quenches excess ROS via enzymatic reduction to water and oxidizes electron rich buffers such as ascorbate (Apel and Hirt 2004). In this study, APX and GPOX activities increased in both the Ganges and Chavignée plants under PHE stress (Table 6). These results are similar to those observed in non-accumulating plants. Indeed, Mei et al. 2009 reported that GPOX activity was slightly increased in fresh tea leaves exposed to PHE. A concentration dependent increase in GPOX and APX activities was also reported after exposure of tomato root to PHE (Ahammed et al. 2012b).

In plants, CAT is mainly located in peroxisomes where it is mainly involved in the removal of H_2O_2 generated during β-oxidation or photorespiration of fatty acids in glyoxysomes (Foyer and Noctor 2005). In this study, PHE did not affect CAT activity in Chavignée plants, but increased its activity in Ganges ones (Table 6). This could also be attributed to different adaptive mechanisms of the two plant populations in response to PHE. For the Ganges population, the increase in CAT activity under stress conditions may help to minimize PHE-induced H_2O_2 generation (Ahammed et al. 2012a). However, it is clear that CAT activities were not affected in Chavignée plants under PHE stress conditions.

DHAR is a key enzyme involved in the recycling process of ascorbate, which catalyzes the glutathione (GSH)-dependent reduction of oxidized ascorbate (dehydroascorbate, DHA). Consequently, DHAR regenerates a pool of reduced ascorbate and deactivates ROS (Do et al. 2016). DHAR was not affected by PHE in Ganges plants, while its activity was increased in Chavignée ones (Table 6). It has been commonly reported that DHAR activity increases under various stresses (Vardhini and Anjum 2015b; Zahra et al. 2021). However, recent studies reported that DHAR was the only antioxidative enzyme whose activity remained unchanged in hydroponic culture with PAHs. This is due to the uncoupled Halliwell-Asada-Foyer cycle. In fact, since DHAR uses Glutathione (GSH) as a substrate and produces oxidized GSH (GSSG) in the uncoupled cycle (González et al. 2020, 2021), it is possible to say that the unchanged DHAR activity in the Ganges population is due to the high consumption of GSH by other enzymes.

The variance in K level, Na/K ratio, chlorophyll content, MDA and some antioxidant enzymes between the two studied populations revealed a difference in PHE tolerance

Several recent studies have demonstrated that the ability of various plant tissues to maintain an adequate plant K content and a low Na/K ratio under stress is an important trait for plant resistance (Chakraborty et al. 2016; Gao et al. 2016; Lu et al. 2016; Wang et al. 2016; Wu et al. 2018). A sufficient level of K in stressed plants can protect the photosynthesis and then CO_2 fixation and transport, then inhibit the transfer of photosynthetic electrons to O_2, thus reducing ROS production (Cakmak 2005).This is the case of the studied Chavignée population under PHE stress and that may have induced lower production of ROS and mainly H_2O_2 with less oxidative damage. In addition, the low ROS level in this case could be involved in the stress-signaling pathway by triggering stress defense/acclimation responses (Dat et al. 2000). However, the reduction of photosynthesis can disturb the balance between ROS production and antioxidant defense resulting in ROS accumulation (Cruz de Carvalho 2008). In this case, ROS became extremely damaging to cell membranes and other cellular components when their concentrations reached the point of phytotoxicity (Dat et al. 2000). This suggests that the Chavignée population may be slightly more tolerant to PHE than the Ganges population.

Moreover, both the treatment and population effects were significant in this study. A clear effect was obtained for the PHE treatment over the control one, in addition to the effect of the Ganges population over the Chavignée one (Fig. 1). The overall antioxidant response to PHE stress was almost similar for both populations of *N. caerulescens*, but there were some differences in the response of certain parameters, where the Chavignée plants seemed to be slightly more tolerant to PHE stress. These differences may be related to the adaptations acquired in the soil from which each of the two populations originated, in terms of detoxification strategies of pollutants and regulation of the antioxidant system. A similar trend was observed by Visioli et al. (2010), when antioxidant compounds such as glutathione S-transferase (GST), thioredoxin and cytochrome P450 were found to increase in abundance at 10 *µM* Ni in *N. caerulescens* plants originating from a metalliferous soil, while their levels in the non-metalliferous population didn't change so much among treatments. Furthermore, low competitive ability and growth rate of metal-tolerant plants have been observed in previous experiments (Macnair 1981) and have often been attributed to a potential cost of constitutive metal tolerance due to a trade-off with growth or reproductive allocation (Ernst et al. 1990). Similarly, Martellini et al. (2014) observed an overproduction of phytoalexins (molecules produced in response to abiotic and biotic stresses) in the copper-tolerant metalliferous population of the plant *Silene paradoxa* compared to the non-metalliferous population, under biotic stress. This led them to suggest that, in certain cases, the adaptation to metalliferous environments can effectively affect the plant response to stress. Furthermore, *N. caerulescens* Ganges from metalicolous soils has adapted to uptake cations and then allocate more energy due to metabolic (transport, sequestration, delocalization, and concentration) and genetic adaptations (Zemanová et al. 2016; Zhang et al. 2023). Thus, these plants require a substantial amount of ATP to reduce the toxicity of accumulated metals (Maestri et al. 2010; Zhang et al. 2023). However, these plants can offset this cost by reducing energy investments in other processes (Farinati et al. 2009) or by dampening of ROS signals generated by another stress (Fones and Preston 2013).

Conclusion

Taken together, it can be concluded that PHE stress induced the production of harmful ROS, thereby causing oxidative stress to *N. caerulescens*. ROS generation reduced phytoextraction efficiency and ultimately reduced biomass. With some exceptions, the antioxidant defense responses consisting of up-regulation of enzymes and antioxidant compounds were roughly similar for the Ganges and Chavignée populations under PHE stress. The difference in response and growth may be constitutional and related to the type of soil (metalliferous or not) and habitat from which each of the two populations originated.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors (M. Chafik Sherri, C. Sirguey, A. Kanso, K. Hamze, S. Ouvrard) designed the study. Material preparation, data collection, and analysis were performed by M. Chafik Sherri with the participation of C. Sirguey and S. Ouvrard. Data treatment was performed by M. Chafik Sherri and C. Sirguey. M. Chafik Sherri wrote the original draft. All authors read, improved and approved the final manuscript. C. Sirguey, A. Kanso, K. Hamze, S. Ouvrard were involved in funding acquisition.

Data Availability

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

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Figures

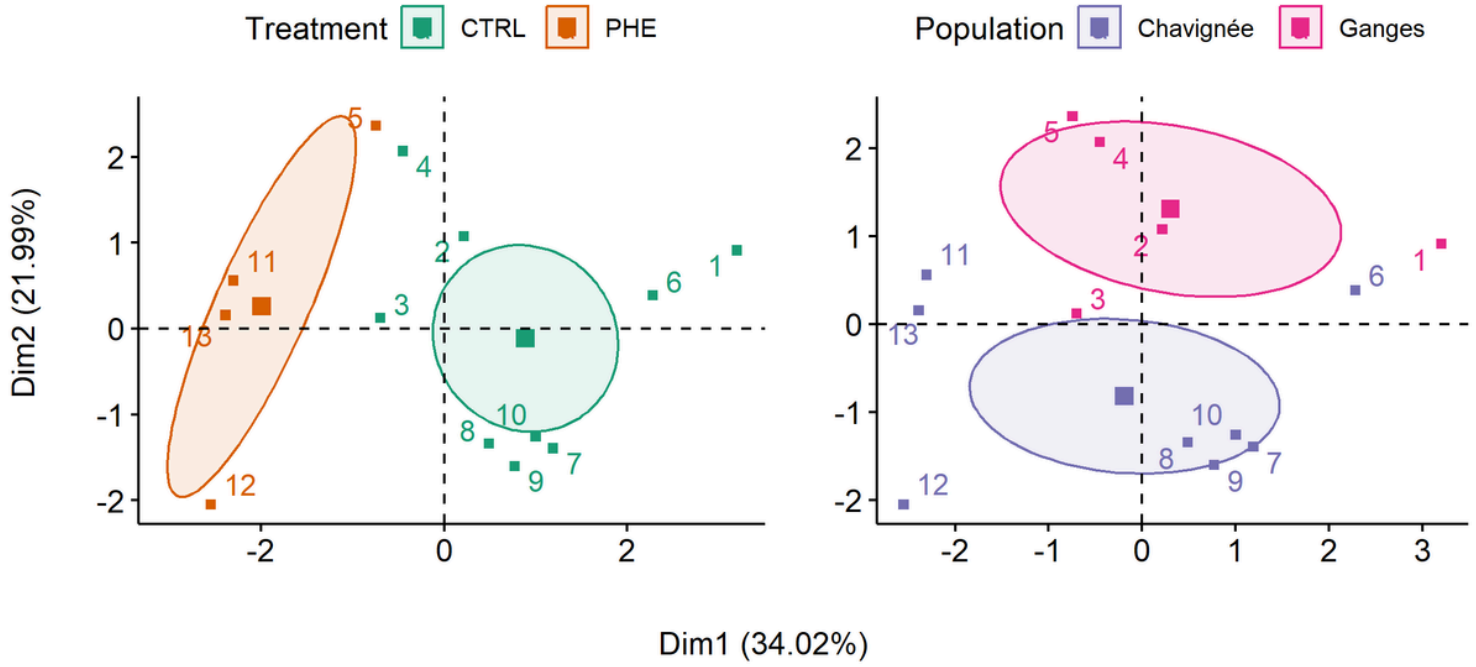

Figure 1

Map of individuals (MFA). The labeled individuals are those best represented on the map. The numbers (in %) next to each axis represent the variance explained by the respective principal components. Labeled individuals are those with the greater contribution to the construction of the plane. Individuals are colored according to their membership in modalities of the variables “Treatment” (left) and “Population” (right)

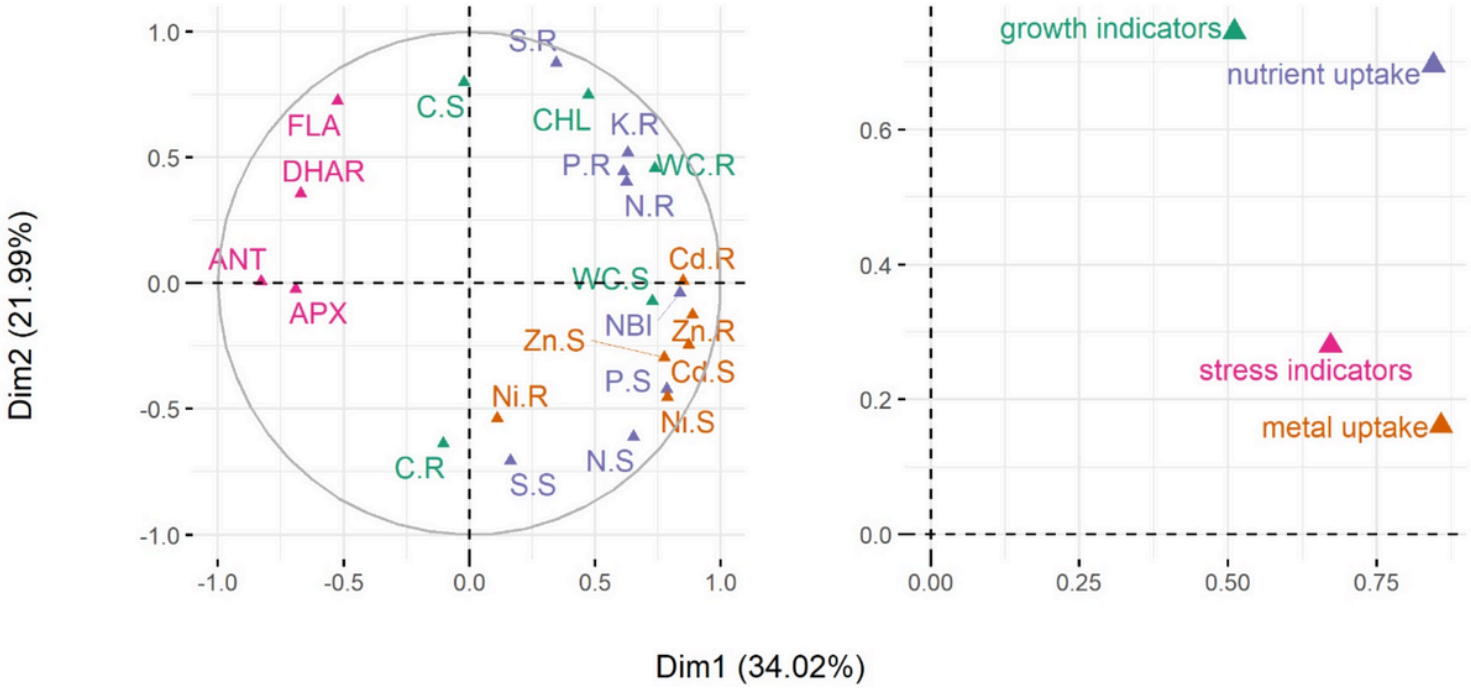

Figure 2

Representation of the variables in the Factorial Design (MFA). The labeled variables are those that are best represented on the map. The variables are colored according to their membership in the variable groups.