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Management of the antioxidant response of the hyperaccumulator plant *Noccaea caerulescens* to abiotic stress

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Résumé Général

Au cours des dernières décennies, l'augmentation des activités humaines, y compris les développements urbains, industriels et agricoles intensifs, a élargi le champ des sols fortement impactés. En conséquence, d'immenses étendues de terres fertiles sont désormais considérablement dégradées, posant des défis à la capacité mondiale de production alimentaire. À l'heure actuelle, environ 2 milliards d'hectares, soit 15 % de la surface terrestre, sont qualifiés de dégradés (PNUE 2006).

Dans des contextes tels que l'agriculture, l'urbanisation et l'industrie, le niveau d'interférence humaine est étroitement lié aux niveaux de pollution (Satterthwaite et al. 2010). Ainsi, les sols avec une plus grande influence humaine contiennent souvent de hauts niveaux de polluants organiques persistants (POP) et d'éléments traces métalliques (ETM), conduisant à des problèmes de contamination combinée (Lindh et Lemenkova 2022).

Les ETM existent naturellement dans les sols en quantités minimales. Certains, comme le cuivre (Cu), le manganèse (Mn) et le zinc (Zn), sont bénéfiques pour les organismes en petites quantités mais deviennent nocifs à des concentrations élevées. D'autres, notamment l'arsenic (As), le cadmium (Cd), le chrome (Cr), le mercure (Hg), le nickel (Ni) et le plomb (Pb), sont nocifs même à de faibles niveaux (Jaishankar et al. 2014). Ces ETM ont un impact négatif sur les écosystèmes et la santé humaine en s'insérant dans la chaîne alimentaire (Mitra et al. 2022). Parmi les polluants organiques persistants (POP), les hydrocarbures aromatiques polycycliques (HAP) sont particulièrement préoccupants en raison de leurs risques sanitaires et environnementaux. Parmi ceux-ci, les 16 HAP identifiés par l'US EPA sont les plus dangereux en fonction de leur toxicité et de leur prévalence dans l'environnement (Patel et al. 2020).

Bien qu'il y ait eu des efforts mondiaux pour atténuer les effets des activités humaines nuisibles sur la santé et l'écologie, de nombreux lieux restent largement pollués par un mélange de ETM

potentiellement toxiques et de POP. Les sites de friches industrielles illustrent ce problème, portant les stigmates de perturbations significatives et d'une contamination industrielle persistante. Aborder la contamination multifactorielle de ces sols, souvent à des niveaux élevés, en conjonction avec d'autres facteurs abiotiques limitants tels que la rareté de l'eau, la salinité du sol et la faible fertilité, devient complexe (Azeez et al. 2014). Historiquement en Europe, la solution principale était d'excaver le sol pollué pour un stockage en décharge (Brand et al. 2018). Cependant, avec des réglementations plus strictes concernant les décharges et une conscience environnementale accrue, les stratégies de remédiation sur site et hors site gagnent du terrain. La phytoremédiation attire de plus en plus d'attention en raison de ses avantages environnementaux et économiques. Cette méthode consiste à utiliser des plantes en association avec des micro-organismes du sol pour extraire (phytoextraction), dégrader (rhizodégradation) ou confiner (phytostabilisation) les polluants du sol (Ali et al. 2013; Yan et al. 2020). Bien que marginalement mise en œuvre sur le terrain, elle offre des avantages environnementaux et économiques justifiant un intérêt croissant pour son développement. Cependant, l'applicabilité dans le contexte de multi-contamination a rarement été envisagée, même si la présence des deux catégories de polluants est fréquemment rencontrée sur des sites contaminés. En effet, le développement d'un couvert végétal sur des sols multi-contaminés est souvent limité par diverses contraintes liées à la présence de POP (HAP) et de ETM (métaux et métalloïdes) et la faible qualité physicochimique de ces sols.

Certaines plantes peuvent tolérer et même accumuler des quantités substantielles d’ETM, tels que Cd, Co, Cu, Ni, Pb et Zn, en particulier lorsqu'elles sont cultivées dans des sols riches en métaux. Ces plantes uniques sont appelées "hyperaccumulateurs" (Baker et Brooks 1989). La culture de ces hyperaccumulateurs a des implications pratiques dans divers domaines. Par exemple, ils peuvent être utilisés pour nettoyer les sols contaminés par des ETM, un processus appelé phytoextraction (Trakal et al. 2015). De plus, ils peuvent être utilisés sur des sites riches

en métaux et des déchets miniers, permettant la récupération d'éléments d'intérêt pour la production de métaux ou la création de catalyseurs multi-métaux (Sheoran et al. 2013).

Dans des applications réelles, les plantes peuvent être confrontées à d'autres défis que l'exposition aux métaux, tels que les co-polluants organiques, une salinité élevée ou la sécheresse. L'efficacité de la phytoremédiation dans les sols contaminés par plusieurs polluants dépend de la capacité de l'espèce végétale choisie à croître, prospérer et produire une biomasse importante face aux stress abiotiques. Ces plantes doivent résister aux effets des stress abiotiques tout en extrayant simultanément les ETM. En France, les efforts en matière de phytoextraction se sont orientés vers l'espèce *Noccaea caerulescens* (Brassicaceae). Cette espèce est reconnue pour sa capacité exceptionnelle à hyperaccumuler les éléments tes que le Ni, le Zn et plus particulièrement le Cd (Gonneau et al. 2014). Malgré cela, la recherche sur les mécanismes de résistance de ces plantes au stress oxydatif, notamment chez *N. caerulescens*, reste limitée. Les études existantes se concentrent principalement sur la manière dont l'hyperaccumulation affecte les réponses antioxydantes de la plante (Souri et al. 2017; Balafrej et al. 2020). Lorsqu'elles sont exposées à de fortes concentrations de polluants, les plantes peuvent subir des effets phytotoxiques, rendant les efforts de phytoremédiation inefficaces. Cela a été observé lors d'essais sur le terrain impliquant l'hyperaccumulateur *N. caerulescens* sur des terrains dégaradés par des activités de cokéfaction et métallurgiques antérieures (Ouvrard et al. 2011). Il a été établi dans ce contexte que la salinité du sol entravait la germination, mais la contamination organique était le principal obstacle à la croissance, en particulier la croissance des racines (Sirguey et Ouvrard 2013; Zelko et al. 2017).

En raison des impacts du changement climatique, divers facteurs environnementaux, tels que les températures extrêmes, les fluctuations de salinité, les radiations UV, les polluants organiques et les métaux lourds, menacent les plantes. En réponse à ces stress variés, les plantes produisent de grandes quantités d'espèces réactives de l'oxygène (ROS) dans différentes parties

de leurs cellules. Ces ROS peuvent oxyder des macromolécules essentielles, notamment l'ADN, les protéines et les lipides, entraînant des dommages cellulaires et physiologiques potentiellement irréversibles (Das et Roychoudhury 2014; Chele et al. 2021). Pour combattre ce stress oxydatif, les plantes ont développé des mécanismes de défense. Ces défenses impliquent souvent des systèmes antioxydants, composés d'enzymes tels que les catalases (CAT), la superoxyde dismutase (SOD) et les peroxydases (APX, GPOD), parmi d'autres composés, qui aident à neutraliser les ROS (Noctor et al. 2016).

Des recherches antérieures ont montré que la supplémentation des plantes en hormones peut améliorer leur capacité à tolérer et à détoxifier les contaminants (Kozlova et al. 2017). Les objectifs de l'application de phytohormones aux plantes sont multiples : ils visent à renforcer l'établissement et la croissance des plantes sous divers stress abiotiques, à diminuer le stress oxydatif de la plante, et pour les plantes hyperaccumulatrices, à augmenter l'absorption des métaux dans les parties aériennes (El Sabagh et al. 2022). Les phytohormones, ou hormones végétales, sont des petites molécules que les plantes produisent en réponse à des déclencheurs externes et en tandem avec leurs processus de croissance et de développement. Ces hormones sont classées en dix groupes principaux : auxines (IAA), brassinostéroïdes (BR), acide gibbérellique (GA), cytokinines (CK), éthylène (ET), acide jasmonique (JA), acide abscissique (ABA), acide salicylique (SA), oxyde nitrique, et strigolactones, ainsi que les hormones peptidiques végétales moins étudiées (Kudoyarova 2022). Par conséquent, pour améliorer la phytoremédiation dans un environnement multi-stress, il semble essentiel d'évaluer la réponse de la plante modèle hyperaccumulatrice *N. caerulescens* face à une gamme de stress abiotiques et d'examiner l'impact de l'application de phytohormones qui pourrait alors permettre d’optimiser l'efficacité de la phytoextraction.

Par conséquent, pour évaluer pleinement la réponse antioxydante de *N. caerulescens* face à plusieurs stress abiotiques et proposer une approche d’optimisation de la phytoextraction par

application de phytohormone cette étude s'est fixé deux grands objectifs scientifiques : i. Explorer la réponse antioxydante de phytoextraction de deux populations de *N. caerulescens*, l'une provenant d'un site non métallifère (Chavignée) et l'autre d'un site métallifère (Ganges), lorsqu'elles sont exposées à plusieurs stress abiotiques. ii. Examiner l'impact de l'application de phytohormones sur la réponse antioxydante et la phytoextraction de *N. caerulescens* dans un environnement multi-stress, dans le but de développer des stratégies pour protéger les plantes pour la mise en œuvre de la phytoextraction.

Pour aborder ces objectifs, cette thèse est structurée en cinq chapitres. Le Chapitre I correspond à l'état de l'art, qui présente le stade le plus récent de la recherche dans les domaines couverts par ce travail, en mettant l'accent sur le potentiel de phytoextraction et la réponse antioxydante des hyperaccumulateurs dans un environnement multi-stress. Le Chapitre II étudie le fonctionnement de la phytoextraction et la réponse antioxydante de *N. caerulescens* lorsqu'elle est soumise à un stress abiotique standard (PAH). Le Chapitre III traite de l'effet de la sécheresse, de la salinité et des stress HAP sur la réponse de *N. caerulescens* tandis que le Chapitre IV examine l'effet de l'application foliaire de IAA et de 24-épibrassinolide (EBR) sur le fonctionnement et la phytoextraction des métaux lourds par *N. caerulescens*. Enfin, le Chapitre V approfondit la réponse antioxydante et la phytoextraction de *N. caerulescens* sous la sécheresse, la salinité, et les stress PAH, accompagnée du traitement avec les phytohormones IAA et EBR.

En résumé, les résultats obtenus montrent que le stress dû au PHE a entraîné la génération d'espèces réactives de l'oxygène (ERO) nuisibles, qui à leur tour ont provoqué un stress oxydatif chez *N. caerulescens*. Ce stress a diminué les capacités de phytoextraction de la plante et a entraîné une diminution de la biomasse. Les populations de Ganges et de Chavignée ont montré des réactions défensives antioxydantes assez comparables sous stress de PHE. Cependant, les plantes de la population Chavignée semblaient être légèrement plus tolérantes

que celles de la population Ganges face à ce stress. De plus, l'exposition à des conditions de sécheresse, de salinité et de PHE a également induit la production d'ERO nuisibles, provoquant un stress oxydatif chez *N. caerulescens*. Ce défi oxydatif a eu un impact négatif sur la capacité de phytoextraction et a conduit à une baisse de la production de biomasse. Chaque population, Ganges et Chavignée, a présenté des mécanismes spécifiques d'adaptation au stress. Les disparités dans leur croissance et leurs réponses peuvent être attribuées à des facteurs tels que la nature de leur sol d'origine (riche en métaux ou non) et leurs environnements natifs.

L'application foliaire d'IAA et d'EBR, seuls ou en association, a été plus bénéfique pour améliorer la phytoextraction chez la population Ganges par comparaison à la population Chavignée. La population Ganges a manifesté une réponse antioxydante plus robuste à ces traitements hormonaux. De plus, elle a connu une augmentation des activités des enzymes antioxydantes avec chacun des traitements hormonaux, tandis que la plus forte réponse antioxydante de la population Chavignée a été significative uniquement avec l'apport d'EBR et le traitement hormonal combiné. Dans l'ensemble, l'application du mélange d'hormones a présenté les meilleurs résultats toutes populations confondues. Nos travaux ont également mis en évidence que les deux populations de *N. caerulescens* examinées avaient des réponses variées en fonction du type de stress et d'exposition hormonale. La population Chavignée avait des attributs de croissance supérieurs, tandis que celle de Ganges présentait des fonctions antioxydantes élevées. Le traitement hormonal combiné était le plus bénéfique pour tous les scénarios de stress. Dans le cas du stress hydrique, l'EBR a également aidé la population Chavignée à atténuer les effets du stress. Face au stress organique (PHE), la population Chavignée a réagi positivement à tous les traitements hormonaux, contrairement à la population Ganges, qui a répondu principalement au traitement d'hormones combinées. Pour les conditions de stress hydrique, l'IAA a amélioré l'efficacité de la phytoextraction de la population Chavignée. L'application d'EBR seul ou associé à l'IAA a également augmenté la

performance de phytoextraction des deux populations lors de l'exposition au PHE. La croissance différentielle et les réponses pourraient être intrinsèques, liées à la nature du sol de leur site d'origine (riche en métaux ou non) et à leurs environnements d'origine respectifs.

Ainsi, les recherches futures pourraient privilégier l'examen des métabolites afin d'obtenir une compréhension approfondie de la manière dont *N. caerulescens* réagit aux stress combinés, en parallèle avec les phytohormones. De plus, expérimenter avec un éventail plus large de phytohormones et leurs combinaisons sur *N. caerulescens* pourrait offrir des perspectives plus approfondies sur les meilleures méthodes pour améliorer la phytoextraction.

General Introduction

Over the past few decades, the rise in human-driven activities, including urban, industrial, and intensive agricultural developments, has expanded the scope of heavily impacted soils. As a result, vast tracts of fertile land are now significantly degraded, posing challenges to global food production capacities. At present, about 2 billion hectares, or 15% of the Earth's surface, are labeled as degraded (UNEP 2006).

In contexts like farming, urbanization, and industry, the level of human interference is closely tied to pollution levels (Satterthwaite et al. 2010). So, soils with greater human influence often contain high levels of persistent organic pollutants (POPs) and trace metal elements (TMEs), leading to combined contamination issues (Lindh and Lemenkova 2022).

TMEs naturally exist in soils in minimal amounts. Some, like copper (Cu), manganese (Mn), and zinc (Zn), are beneficial to organisms in small quantities but turn harmful at elevated concentrations. Others, including arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg), nickel (Ni), and lead (Pb), are harmful even at minute levels (Jaishankar et al. 2014). These TMEs negatively impact ecosystems and human health by infiltrating the food chain (Mitra et al. 2022). Among the persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs) are especially concerning due to their health and environmental risks. Of these, the 16 PAHs identified by the US EPA are the most hazardous based on their toxicity and prevalence in the environment (Patel et al. 2020).

While there have been global endeavors to mitigate the effects of harmful human activities on health and ecology, numerous locations remain extensively polluted with a mix of potentially toxic TMEs and POPs. Ruined industrial sites represent this issue, bearing the scars of significant disturbances and enduring industrial contamination. Addressing the multifaceted

contamination of these soils, often at high levels, along with other limiting abiotic factors such as water scarcity, soil salinity, and low fertility, becomes complex (Azeez et al. 2014). Historically in Europe, the primary solution was to excavate the polluted soil and discard it in landfills (Brand et al. 2018). However, with stricter landfill regulations and heightened environmental consciousness, both on-site and off-site remediation strategies are gaining traction. Phytoremediation is drawing increasing attention due to its environmental and economic benefits. This method consists of using plants in association with soil microorganisms to extract (phytoextraction), degrade (rhizodegradation), or confine (phytostabilization) soil pollutants (Ali et al. 2013; Yan et al. 2020). Although marginally implemented on the ground, it offers both environmental and economic advantages justifying growing interest in its development. However, the applicability in the context of multi-contamination has rarely been considered, even if the occurrence of both categories of pollutants is frequently encountered on contaminated sites, as we mentioned previously. Indeed, the development of a plant cover on multi-contaminated soils is often limited by various constraints linked to the presence of POPs (PAHs) and TMEs (metals and metalloids) and the low physicochemical quality of these floors.

Interestingly, certain plants can tolerate and even accumulate substantial amounts of TMEs, such as Cd, Co, Cu, Ni, Pb, and Zn, especially when grown in metal-rich soils. These unique plants are termed "hyperaccumulators" (Baker and Brooks 1989). Growing these hyperaccumulators has practical implications in various areas. For instance, they can be used to clean up soils contaminated with TMEs, a process called phytoextraction (Trakal et al. 2015). Additionally, they can be used to reclaim metal-rich sites and mining waste, allowing for metal production or the creation of multi-metal catalysts (Sheoran et al. 2013).

In real-world applications, plants might face other challenges besides metal exposure, such as organic co-pollutants, elevated salinity, or drought. The effectiveness of phytoremediation in

soils contaminated with multiple pollutants depends on the ability of the selected plant species to grow, thrive, and produce substantial biomass in response to abiotic stresses. Such plants need to withstand the effects of abiotic stresses while simultaneously extracting TEMs. In France, efforts towards phytoextraction have leaned towards the species *Noccaea caerulescens* (Brassicaceae). This species is recognized for its exceptional ability to hyperaccumulate substances like Ni, Zn, and particularly Cd (Gonneau et al. 2014). Despite this, research into the mechanisms of these plants' resistance to oxidative stress, especially in *N. caerulescens*, remains limited. Existing studies mainly focus on how hyperaccumulation affects the plant's antioxidant responses (Souri et al. 2017; Balafrej et al. 2020). When exposed to high pollutant concentrations, plants can experience phytotoxic effects, which could render phytoremediation efforts ineffective. This was observed in field trials involving the hyperaccumulator *N. caerulescens* on lands tainted by previous coking and metallurgical activities (Ouvrard et al. 2011). It was determined that soil salinity hindered germination, but organic contamination was the primary impediment to growth, especially root growth (Sirguey and Ouvrard 2013; Zelko et al. 2017).

Due to the impacts of climate change, various environmental factors, such as extreme temperatures, salinity fluctuations, UV radiation, organic pollutants, and heavy metals, pose threats to plants. In response to these diverse stressors, plants produce large amounts of reactive oxygen species (ROS) in different parts of their cells. These ROS can oxidize crucial macromolecules, including DNA, proteins, and lipids, leading to potentially irreversible cell and physiological harm (Das and Roychoudhury 2014; Chele et al. 2021). To combat this oxidative stress, plants have evolved defense mechanisms. These defenses often involve antioxidant systems, composed of enzymes such as catalases (CAT), superoxide dismutase (SOD), and peroxidases (APX, GPOD), among other compounds, that help neutralize ROS (Noctor et al. 2016).

Prior research has shown that supplementing plants with hormones can enhance their ability to tolerate and detoxify contaminants (Kozlova et al. 2017). The goals of applying phytohormones to plants are multifaceted: they aim to boost plant establishment and growth under diverse abiotic stresses, diminish the plant's oxidative stress, and for hyperaccumulating plants, increase metal uptake in the above-ground parts (El Sabagh et al. 2022). Phytohormones, or plant hormones, are distinct small molecules that plants produce in response to external triggers and in tandem with their growth and development processes. These hormones are sorted into ten main groups: auxins (IAA), brassinosteroids (BR), gibberellic acid (GA), cytokinins (CK), ethylene (ET), jasmonic acid (JA), abscisic acid (ABA), salicylic acid (SA), nitric oxide, and strigolactones, along with the lesser-researched plant peptide hormones (Kudoyarova 2022). Consequently, to improve phytoremediation in a multi-stress environment, it seems essential to assess the response of the model hyperaccumulator plant *N. caerulescens* under a range of abiotic stresses and examine the impact of phytohormone application. This would help anticipate and optimize the efficacy of the phytoextraction procedure.

Chapter I

Phytoextraction potential and antioxidative response of hyperaccumulators in multi-stress environments: *Noccaea caerulescens* as a model plant

This chapter aims to provide a state of the art on our knowledge of the general concept of phytoextraction and the response of plants to abiotic stresses and mainly the cases of multi-stressors. It first proposes to define and describe the process of phytoremediation along with the mechanism of metal hyperaccumulation especially in *N. caerulescens*. The second part concerns the cases of multi-stresses and their impact on the phytoextraction process and the antioxidative response of plants in general and hyperaccumulators in particular. The last part is a description of phytohormones and their ability to alleviate abiotic stresses and enhance phytoextraction.

1 Phytoremediation and phytotechnologies for contaminated soil remediation

1.1 Soil pollution

The increasing demand for agricultural products has caused widespread farming. To ensure the quality and amount of these goods, it's essential to use fertilizers, pesticides, and herbicides. Yet, overusing these chemicals can lead to environmental issues, like their buildup in the soil and uptake by plants (Aktar et al., 2009). For instance, using phosphate fertilizers has raised the levels of Cd, Cu, Zn, and As in soils (Liao et al., 2019). Additionally, since the industrial revolution began, the contamination of our environment with heavy metals has risen significantly and is now a serious concern. This type of pollution is a major threat to our soil, water, and health (Tchounwou et al., 2012). The soils have seen a notable rise in toxic metal contamination like Cd, Pb, Cr, Zn, Ni, and Cu due to global industrial growth (Simon, 2016). Excessive pesticide use in farming and byproducts from soil de-acidification also contribute to this pollution (Alengebawy et al., 2021). Polluting the environment with organic xenobiotics, including pesticides, drugs, and petroleum products, is similarly a worldwide issue (Štefanac et al., 2021). Thus, it's crucial to innovate ways to clean affected areas. Various methods, whether physical, chemical, or biological, can address polluted sites (Akhtar et al., 2020; Sharma, 2021). However, phytoremediation stands out as a cost-efficient way to cleanse polluted soil and water (Alsafran et al., 2022; Mocek-Płóciniak et al., 2023a).

1.2 Phytoextraction: a green soil remediation technique

Over the past 30 years, various technologies such as bioremediation, phytoremediation, soil washing, thermal desorption, and stabilization have been crafted to address soil contamination. However, many of these methods are constrained by specific contaminants or site conditions. While some technologies can handle mixed contaminations, they come with significant

drawbacks (Balseiro-Romero and Baveye, 2018a). For instance, certain approaches involve chemicals, such as soil washing or in-situ flushing (Ramadan et al., 2018). Others can drastically alter the soil's physical and chemical attributes, like its pH or organic content (O'Brien et al., 2018). Methods like stabilization, bioremediation of metals, or vitrification don't eliminate contaminants but merely stabilize them, raising concerns about future re-contamination. Furthermore, many of these methods consume considerable energy, making them costly (Evanko et al., 1997; Khalid et al., 2017).

Phytoremediation arises as a promising, cost-effective solution for sites with complex contamination. It offers an in-situ, eco-friendly means to purify and rejuvenate polluted sites while preserving the soil's biology and structure. This green approach, which harnesses plants and solar energy, is considered more sustainable than traditional cleanup methods (Bhat et al., 2022). Literature showcases various phytotechnologies aimed at rehabilitating polluted sites, encompassing techniques like rhizofiltration, phytostabilization, phytodegradation, and phytoextraction which will be discussed below (Sharma et al., 2023).

Phytoextraction, a subset of phytoremediation, is defined as the process of using plants to absorb, concentrate, and remove heavy metals or other pollutants from soil or water through their roots and store them in the above-ground parts, which are then harvested and removed. This method stands out as an economical and appealing approach for removing heavy metals from the environment (Mocek-Płóciniak et al., 2023) (Figure 1). Leveraging certain plants, this technique not only detoxifies contaminated sites but also enhances environmental health (Garbisu and Alkorta, 2001; Suman et al., 2018). Phytoextraction shares the advantages of its broader field: it's eco-friendly, visually pleasing, and effective at removing contaminants (Da Silva et al., 2019). This method can address a wide range of metal pollutants, offers scalability, is cost-effective, and can integrate with other remediation strategies. Furthermore, it can strengthen agriculture and mining sectors by fortifying soils and pausing the spread of metal

pollutants (Ayangbenro and Babalola, 2017). An integral aspect of successful phytoextraction is choosing the right hyperaccumulator plants that can absorb high amounts of metals without toxicity (Skuza et al., 2022).

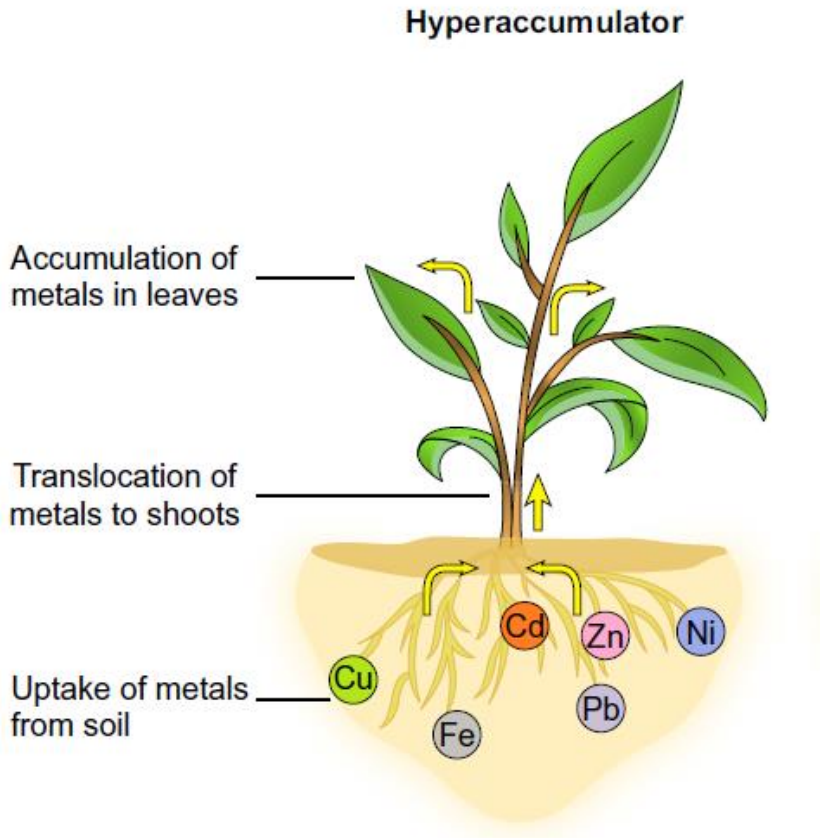

Figure 1. The process of phytoextraction

1.3 Hyperaccumulator plants

Plants exhibit varying degrees of tolerance to heavy metal toxicity, with some species being extremely sensitive while others demonstrate hypertolerance (Singh et al., 2016). For instance, lettuce (*Lattuca sativa L.*) is highly susceptible, radish (*Raphanus sativus L.*) is sensitive, cauliflower (*Brassica oleracea L.*) exhibits tolerance, and corn (*Zea mays L.*) shows significant tolerance (Castillo-Michel et al., 2009; Chaney, 1983). Meanwhile, *N. caerulescens* demonstrates an extreme tolerance, or hypertolerance to Cd, Zn, and Ni (Halimaa et al., 2014a). Hypertolerant species tend to either store metals mainly in their roots (known as excluders) or

accumulate large amounts in their shoots (referred to as hyperaccumulators) in comparison to most other plants. Aside from excluders, those plants that can tolerate and accumulate high amounts of metals in their shoots are termed hyperaccumulators. These plants are potential assets for extracting heavy metals from soil using phytoextraction (Leitenmaier and Küpper, 2013; Pasricha et al., 2021; Sterckeman and Thomine, 2020).

Currently, more than 500 plant species spanning over 30 families are classified as hyperaccumulators (Purwadi et al., 2021). The Brassicaceae family includes the most hyperaccumulator species (Zeremski et al., 2021a). Nearly half of these are nickel hyperaccumulators, possibly due to the prevalence of Ni-rich soils known as ultramafic or serpentine (van der Ent et al., 2013). Other significant groups include Cu hyperaccumulators (35 species), Mn (30), Co (26), Zn (18), Pb (14), and Cd (8) (Fernando et al., 2009; Küpper et al., 2000; Verbruggen et al., 2009). Notably, *N. caerulescens* stands out as a model hyperaccumulator, tolerating and amassing Zn, Cd, and Ni (Halimaa et al., 2014a). Other well-studied hyperaccumulators include Cd-Zn hyperaccumulators like *Sedum alfredii* (Xv et al., 2020) and *Arabidopsis halleri* (Cosio et al., 2004), and As hyperaccumulators from the *Pteris* genus (Poynton et al., 2004).

1.4 Mechanisms of metal tolerance in hyperaccumulators

Plants that exhibit tolerance to heavy metals have developed intricate strategies, involving both physiological and biochemical processes, to manage the metal's toxicity. Hyperaccumulators, a subset of these tolerant plants, possess distinct tolerance mechanisms enabling them to absorb and store heavy metals in their shoots at particularly high levels (Viehweger, 2014). Essentially, there are two core mechanisms: compartmentalization and detoxification. The compartmentalization involves the reallocation of heavy metals to less sensitive parts of the plant, such as trichomes, cell walls, and vacuoles (Ejaz et al., 2023). As evidence, the highest

concentration of Cd was found in epidermal cells of species like *N. caerulescens* and *Noccaea praecox* (Callahan et al., 2016; Vogel-Mikuš et al., 2008), in the trichomes of *A. halleri* (Küpper et al., 2000), and in the parenchyma tissue of *Sedum alfredii* (Tian et al., 2011). In contrast, for some Mn hyperaccumulators like *Gossia sp*., Mn mainly accumulates in photosynthetic cells (Fernando et al., 2013).

The detoxification mechanism, on the other hand, involves the formation of less harmful complexes with organic or inorganic compounds, which can include thiols (like glutathione, phytochelatins, and metallothioneins) and non-thiols (like histidine and nicotianamine) as well as phosphates (Callahan et al., 2006; Emamverdian et al., 2015). For instance, Kentucky bluegrass and tall fescue demonstrated significant accumulation of Cd in their shoots without displaying toxic symptoms, possibly due to the formation of insoluble Cd phosphates in their leaves (Xu and Wang, 2013). It's important to note that hyperaccumulators exhibit selective binding between the metals they accumulate and those they don't. An example is the way Cd is primarily bound to weak oxygen ligands in the *N. caerulescens*, whereas non-accumulated Cd binds to stronger ligands, like sulfur ones, in non-hyperaccumulators (Leitenmaier and Küpper, 2013).

1.5 Defense trade-offs in metal hyperaccumulators

Three key insights emerge from the study of metal accumulation in plants. Firstly, plants that hyperaccumulate metals often require a higher baseline level of these elements compared to non-accumulating species (Fones and Preston, 2013a). Such hyperaccumulators may find themselves at a disadvantage in soils lacking heavy metals, facing higher mortality and reduced reproductive success when grown in 'normal' soils, unlike their performance in metal-rich environments where some can show up to a 75% increase in biomass with Cd supplementation (Halimaa et al., 2014; Roosens et al., 2003). Essential metals like Zn, Mn, or Ni are crucial for

processes including oxidative stress management, enzyme functions, and nitrogen metabolism (Yruela, 2013). A shortfall in these metals can negatively affect the vitality of hyperaccumulating plants in less metal-concentrated soils.

Secondly, when grown in metal-rich substrates, hyperaccumulators can gather larger amounts of metals, which may synergistically interact with each other and with organic defense molecules, effectively lowering the threshold concentrations needed for defense against biological threats. This synergy has been termed the joint-effects hypothesis by Boyd (2012).

Thirdly, it appears that the accumulation of metals may compromise the mechanisms plants use to defend against pathogens, an effect outlined by the trade-off hypothesis (Farinati et al., 2009a; Fones and Preston, 2013a). The process of hyperaccumulation involves metabolic and genetic changes that allow plants to absorb and store high concentrations of metals that could be toxic, such as the increase in antioxidant levels, the upregulation of genes responsible for metal uptake, and the enhanced movement and storage of metals within the vacuoles. These adaptations are energetically costly, leading hyperaccumulating species to possibly reduce the energy allocated to their pathogen defense systems (Maestri et al., 2010a). Proteomic studies have highlighted this phenomenon in *A. halleri* shoots, where exposure to Cd and Zn resulted in the downregulation of signaling pathways crucial for defense against herbivores, insects, and diseases (Farinati et al., 2009a). Additionally, a balance has been observed between the accumulation of Zn and the presence of glucosinolates in *N. caerulescens*, as well as between Ni and glucosinolates in *Streptanthus polygaloides*, indicating a trade-off between metal accumulation and organic defense mechanisms (Tolrà et al., 2001). Notably, in the case of the latter, such trade-offs between organic defenses and Ni-based defenses seem to be inherent, showing no variation with changes in soil composition (Davis and Boyd, 2000).

The development of hyperaccumulating plants should be understood within the framework of their natural habitats, where they have evolved. This environment allows for a range of combined effects and compromises between various defense strategies, which are constantly evolving and adapting to their surroundings.

1.6 *Noccaea caerulescens*: A model hyperaccumulator plant

N. caerulescens, previously identified as *Thlaspi caerulescens*, is renowned for its ability to hyperaccumulate Cd, Zn, and Ni (Figure 2). This plant, which can be biennial, perennial, or annual, is part of the Brassicaceae family (Halimaa et al., 2014a). In its shoots, it can tolerate and accumulate Zn levels reaching 40000 mg kg^{-1} DW, Cd levels up to 14000 mg kg^{-1} DW, and Ni levels surpassing 4000 mg kg^{-1} DW (Jacobs et al., 2018; Schwartz et al., 2006). In contrast, the ideal Zn and Cd concentrations for plants that don't hyperaccumulate are between 20–100 mg kg^{-1} and 1–10 mg kg^{-1}, respectively (Mengel and Kirkby, 2004). This species has an exciting ability: its roots gravitate towards zones in metal-polluted soils rich in Cd or Zn, even when these soils present scarce bioavailable metal concentrations (Whiting et al., 2001). What's more, the hairy roots of *N. caerulescens* were found to hyperaccumulate heavy metals even in cultures lacking shoots (Boominathan and Doran, 2003). Due to these unique attributes, *N. caerulescens* serves as an ideal model for delving into the processes of metal tolerance, accumulation, and uptake (Papoyan and Kochian, 2004), and it holds promise for application in cleansing sites polluted with heavy metals.

Across Europe, *N. caerulescens* thrives in three distinct soil-based environments, classifying into three primary ecological or edaphic groups, which are frequently referred to as ecotypes (Sterckeman et al., 2017). These include two groups that inhabit metal-rich soils: the calamine group, which is found in soils heavily loaded with Zn, Pb, and Cd typically around mining or smelting areas, and the serpentine group, which is adapted to Ni-rich soils formed primarily

from serpentinite ultramafic rocks. The third group, known as the non-metalliferous group, consists of populations growing in soils without significant metal content, located in low mountain regions like the Ardennes, Vosges, Jura, Alps, Pyrenees, and Massif Central (Gonneau et al. 2014, 2016).

The diversity in responses of *N. caerulescens* to metals across its populations is markedly evident, with the evolutionary dynamics of this variation still under preliminary exploration. Studies have documented distinct responses in terms of tolerance and accumulation of metals like Zn, Cd, and Ni across populations from different soil backgrounds, including non-metallicolous, calamine, and serpentine origins (Assunção et al., 2003a; Escarré et al., 2013; Gonneau et al., 2014). Notably, adaptations concerning root-to-shoot metal transfer, elemental homeostasis, and traits impacting survival and reproduction indicate the evolution of varied strategies for coping with metal stress among populations from diverse soil types (Assunção et al., 2003; Gonneau et al., 2014). Despite the significant phenotypic variability, the genetic architecture of *N. caerulescens* seems to maintain consistency at the species level, suggesting that these behavioral differences are not a result of major genomic alterations (Mandáková et al., 2015).

Figure 2. *Noccaea caerulescens* plant (Brassica)

2 Plant response to stress

2.1 General response traits to abiotic stresses

2.1.1 Salt response

Salinity's influence on plant growth occurs in two phases. In the initial phase, growth is relatively unaffected, as the salts entering the xylem are stored in vacuoles, and growth centers continue to function through the phloem. However, in the subsequent phase, as salt accumulates, cells can't store them in vacuoles anymore, leading to enzyme inhibition due to increased cytoplasmic concentration (Acosta-Motos et al., 2017). Salinity can also diminish photosynthesis because of reduced plant water potential and hindered chlorophyll production. Chlorophyll production, for example, can be disrupted by chlorine, with specific concentrations leading to reduced crop yields (Hnilickova et al., 2021). Salinity can upset a plant's nutritional balance, making nutrient absorption more challenging (Chele et al., 2021a). For example, it can limit nitrogen uptake due to interference from sodium and chloride. Other elements like

calcium, potassium, magnesium, and phosphate can also be affected by high salinity levels (Farooq et al., 2015).

Like other stressors, high salinity can cause oxidative stress in plants. This happens because stomatal closure, a response to high salinity, leads to an energy overflow which is then diverted to oxygen. As a result, various reactive oxygen species like superoxide, hydrogen peroxide, and hydroxyl radicals form, leading to further stress (AbdElgawad et al., 2016).

2.1.2 Drought response

Cultivation under drought conditions brings about molecular, biochemical, physiological, morphological, and ecological changes in plants (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Plants adjust their stomatal aperture to modulate water loss and optimize CO_2 intake, preventing photosynthesis inhibition, and thus resisting water stress. Stomatal closure serves as an initial adaptive response, counteracting the reduction in xylem water potential and preventing severe xylem embolism when water becomes scarce (Li et al., 2021; Peng et al., 2022). Moreover, through osmotic regulation, plants reduce osmotic potential to combat the adverse effects of water stress, sustaining stomatal conductance and mitigating water deficits.

Plants also accumulate proline as a defensive measure against drought stress (Furlan et al., 2020) and gather signaling molecules like abscisic acid (ABA), calcium (Ca^{2+}), inositol-1, 4, 5-triphosphate (IP3), and cyclic adenosine 50- ribose-diphosphate (cADPR) in response to water stress (Bharath et al., 2021; Yang et al., 2021). Drought stress also modulates enzyme activity, impacting antioxidant activities as a result of oxidative stress and carbon metabolism (Chaves et al., 2002).

2.1.3 PAH response

Environmental contamination by PAHs has deleterious effects on plant growth and development (Afegbua and Batty, 2018). It stunts the growth of plant parts and disrupts

processes like germination and flowering. On the cellular level, the presence of PAHs can lead to the breakdown of chlorophyll and other cellular deformities (Alkio et al., 2005a). Sunlight exacerbates the harmful effects of PAHs on both land and aquatic plants. Under sunlit conditions, PAHs become more toxic due to photo-induced changes and heightened photosensitivity. Such heightened sensitivity often stems from the generation of harmful reactive oxygen species, predominantly oxygen molecules (Lampi et al., 2006). These reactive molecules further oxidize PAHs, producing even more toxic derivatives. These compounds can inflict oxidative damage on critical biological molecules like proteins, DNA, and lipids, similar to the toxic effects triggered by certain metabolic pathways in living organisms (Molina and Segura, 2021a).

2.2 Oxidative stress biochemistry in plants

Plants maintain a redox balance through a delicate equilibrium between reactive oxygen species (ROS) production and the action of antioxidant enzymes. This balance ensures that ROS levels remain neither too low (which would hinder cellular activities) nor too high (which could be harmful). It's crucial for optimal redox signaling in cells, held in check by the balance between ROS production and their neutralization (Das and Roychoudhury, 2014; Huang et al., 2019). Thus, the term “redox biology” has been coined by researchers to describe how ROS function as signaling molecules, regulating standard plant physiological processes (Milkovic et al., 2019). However, when this balance is disrupted, typically due to a shortfall in antioxidant activity, it can lead to an excessive buildup of ROS, causing oxidative stress under various environmental stressors (Miller et al., 2010). Such stress can damage cell components, including causing lipid peroxidation, harming nucleic acids and proteins, and altering carbohydrate metabolism (Figure 3). This, in turn, can result in cellular malfunctions and death (Mansoor et al., 2022; Taylor et al., 2004).

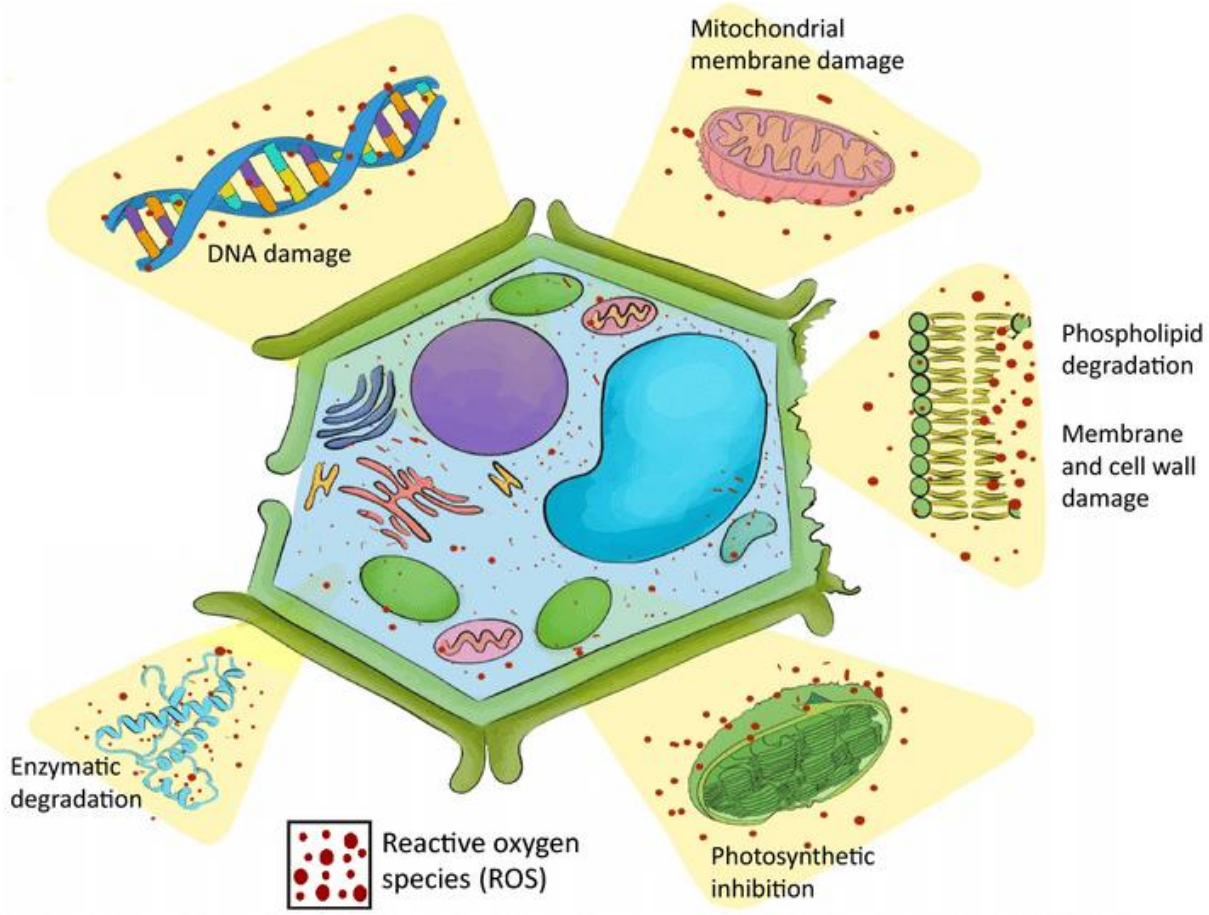

Figure 3. Oxidative stress damage caused by unbalanced reactive oxygen species (ROS) in a plant

Atmospheric O_2 exists in its ground state as triplet oxygen ($^3O_2$), characterized by two unpaired electrons with parallel spins, making it relatively unreactive. Yet, with added energy from specific biochemical processes, electron transport chains, ultraviolet-B rays, and ionizing radiation, $^3O_2$ can overcome this spin constraint, turning it into ROS (Khorobrykh et al., 2020).

Within plant cells, ROS formation occurs in various regions, including the chloroplasts, mitochondria, peroxisomes, and the plasma membrane (Janku et al., 2019). In chloroplasts, chlorophyll pigments capture light and are excited to their triplet state. If not efficiently neutralized, this can result in a charge recombination that excites $^3O_2$ to form $^1O_2$ (Santabarbara et al., 2007). Even though $^1O_2$ has a brief existence (lasting between 3.1 to 3.9 μs) and covers a limited diffusion range (about 190 nm), it can still move beyond the chloroplast

to affect areas like the plasma membrane, tonoplast, or cytosolic signaling processes (Fischer et al., 2013). $3O_2$ can also take electrons from the electron transport chain or through the action of NADPH oxidase, forming $O_2•^-$, which persists for 1–1000 μs (Panday et al., 2015). $O_2•^-$ further reacts with H^+ to produce $HO_2•^-$, which is notably more reactive, stable, and can penetrate biological membranes. H_2O_2 can also emerge from the conversion of $O_2•^-/HO_2•^-$, facilitated by SOD isoforms, NADPH oxidases, and heme-containing class III peroxidases (Andrés et al., 2022; Nita and Grzybowski, 2016). Chemically, H_2O_2 behaves as a mild acid, which is both diffusible and stable (Wagner et al., 2022).

Cellular ROS encompasses both radicals and non-radicals. Among radicals, common ones are $O_2•^-$, OH•, RO•, and the peroxyl radical (ROO•), whereas non-radicals include H_2O_2, $1O_2$, and ozone (O_3) (Khan et al., 2023). Some other non-radical ROS observed in plants comprises hypochlorous acid (HOCl), hydroperoxides (ROOH), and excited carbonyls (RO*) (Hasanuzzaman et al., 2020). Reactive oxygen intermediates (ROI) are also termed oxygen molecules formed due to the incomplete reduction of O_2; thus, ROS encompasses all ROI varieties along with O3 and $1O_2$. Additionally, various acids like hypobromous acid (HOBr), hypoiodous acid (HOI), and HOCl, as well as radicals such as the carbonate radical ($CO_3•^-$) and semiquinone ($SQ•^-$), are also categorized as ROS (Gill and Tuteja, 2010a; Hasanuzzaman et al., 2021).

Plants possess various strategies to counteract the excessive production of ROS due to abiotic stresses, which involve activating both enzymatic and non-enzymatic antioxidants.

2.2.1 Enzymatic antioxidants

Plant cells combat oxidative harm by employing antioxidant enzyme defense mechanisms that target ROS in various cellular compartments. Several of these enzymatic antioxidants include guaiacol peroxidase (GPOD, EC 1.11.1.7), glutathione reductase (GR, E.C 1.6.4.2), catalase

(CAT, EC 1.11.1.6), superoxide dismutase (SOD, E.C.:1.15.1.1), ascorbate peroxidase (APX, E.C.:1.11.1.11), dehydroascorbate reductase (DHAR, EC.:1.8.5.1), and monodehydroascorbate reductase (MDHAR, EC.:1.6.5.4) (Rajput et al., 2021).

Guaiacol peroxidases (GPOD). Peroxidases can interact with H_2O_2, with their oxidized state being renewed by specific potent reductases. Often, the concentration of enzymatic antioxidants is notably diminished in mitochondria and chloroplasts, while their oxidized versions primarily amass in vacuoles and apoplasts (Hasanuzzaman et al., 2019).

Glutathione reductase (GR). GR is located in various cellular compartments such as mitochondria, cytosol, peroxisomes, and predominantly in chloroplasts, which account for 70-80% of its activity (Romero-Puertas et al., 2006). This enzyme is a type of flavoprotein oxidoreductase, and its functionality is influenced by the levels of glutathione disulfide (GSSG), NADPH, and pH (Vašková et al., 2023). GR is pivotal in catalyzing the conversion of oxidized GSH (GSSG) back to its reduced form, GSH. Additionally, it maintains the equilibrium between the GSH/GSSG ratio, crucial for neutralizing H_2O_2 (Hasanuzzaman et al., 2019).

Superoxide dismutase (SOD). There are three types of SODs: Cu/Zn-SOD, Fe-SOD, and Mn-SOD. Cu/Zn-SOD can be found in the cytosol, mitochondria, and apoplast; Fe-SOD is located in chloroplasts, mitochondria, and peroxisomes; while Mn-SOD is present in peroxisomes, mitochondria, and vascular structures. Serving as the initial line of defense against ROS, SOD reduces the formation of harmful $O_2^{\bullet -}$ and OH• concentrations (Alscher, 2002; Wang et al., 2018).

Ascorbate peroxidase (APX). APX stands out as a crucial enzymatic antioxidant in plants. It plays an active role in the ascorbate glutathione (ASH/GSH) cycle (Figure 4), using ascorbate (AsA) to capture and transform H_2O_2 into water. In this process, two ascorbic acid molecules

are consumed to convert H_2O_2 into water, yielding two MDHA molecules (Caverzan et al., 2012). Numerous studies have highlighted APX's importance, noting its superior activity compared to other antioxidant enzymes, especially in neutralizing H_2O_2 under various stress conditions like cold exposure, drought, pesticide exposure, metal toxicity, and salt stress (Sofo et al., 2015).

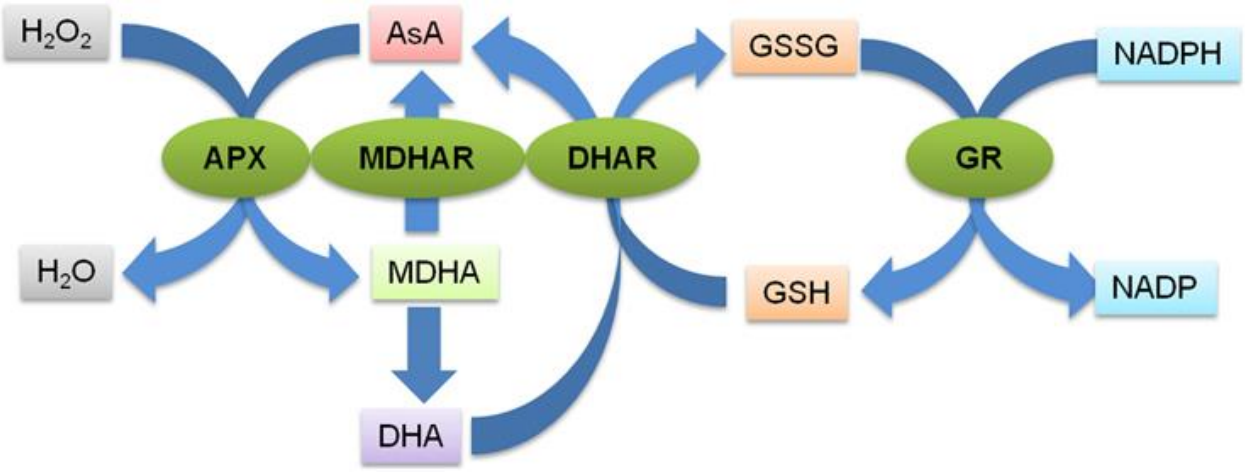

Figure 4. Ascorbate-glutathione (AsA-GSH) cycle in plants. This cycle consists of a series of redox-coupled reactions whose main function is the scavenging of H_2O_2 and the recycling to the reduced forms of ascorbate and glutathione

Catalase (CAT). Catalase is an important antioxidant enzyme that plays a pivotal role in neutralizing ROS under diverse stress conditions (Nandi et al., 2019), converting H_2O_2 and other substances like methanol, formaldehyde, ethanol, and formic acid through dismutation. Factors of abiotic stress, such as salinity and lack of water, stimulate the expression of genes encoding for catalase in various plants (Abdelaal et al., 2022).

2.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants encompass a diverse range of chemical substances including carotenoids, proline, phenolics, and flavonoids. These compounds are essential in neutralizing ROS under different stress conditions (Kasote et al., 2015).

Carotenoids are essential pigments in numerous plant species (Figure 5), working alongside chlorophylls. They are also present in algae and photosynthetic bacteria, imparting vibrant yellow and orange hues to plants, fruits, and vegetables. These pigments not only attract pollinators and aid in seed dispersion but are also involved in the xanthophyll cycle, influencing light absorption in photosystem II (Maoka, 2020). They help counter the adverse effects of intense light, which can cause oxidative harm, and contribute to maintaining the thylakoid's function by preventing over-stimulation of photosystem II through O_2 scavenging (Gammone et al., 2015). Carotenoids play a pivotal role in helping plants adapt to environmental signals by interacting with ROS and generating various oxidized compounds like aldehydes, ketones, lactones, and the volatile b–cyclocitral. These compounds are bioactive and can stimulate gene expression alterations, enhancing the plant's resilience to stress (Havaux, 2014).

Proline is a crucial amino acid that serves as a prevalent osmolyte, functioning as a non-enzymatic antioxidant. It helps neutralize ROS in various stress conditions. Many plants elevate their proline levels when faced with drought or salinity, aiding in water retention, and osmotic balance, and enhancing certain physiological functions (Hayat et al., 2012). Proline accumulates mainly in the cytosol and vacuole, shielding plant cells from harm caused by superoxide and hydrogen peroxide during stress periods (Cakmak, 2005). Additionally, proline can bind with redox-reactive metal ions, safeguarding cellular components against oxidative stress by neutralizing $1O_2$ and directly counteracting HO•. This action helps in preserving proteins, DNA, and the plasma membrane (Ghosh et al., 2022). Proline's role as an osmolyte is vital in defending plant cells from heightened ROS production during stressful times (Yoshiba et al., 1997).

Phenolics and flavonoids are biologically active substances produced through pathways involving malonic and shikimic acids. They serve as secondary metabolites in plants, functioning as non-enzymatic antioxidants. These compounds also play a role in modulating

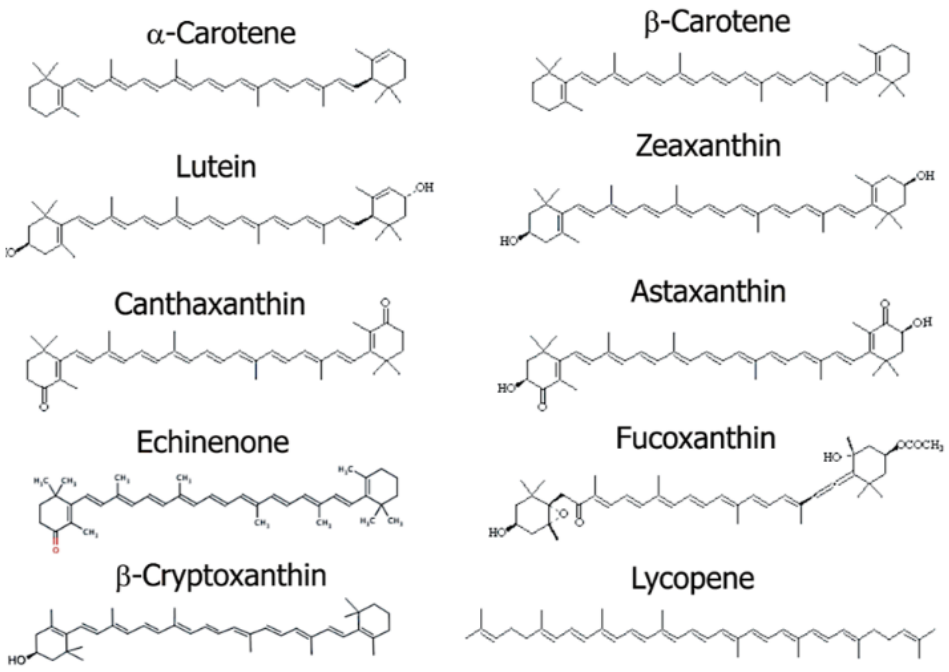

Figure 5. Chemical structures of carotenoids in plants

plant growth and response under environmental stressors (Mandal et al., 2010; Sagita et al., 2021). The levels of phenols have been observed to rise in various plants when faced with abiotic stress (Ramakrishna and Ravishankar, 2011). Furthermore, the heightened presence of flavonoids and phenolics, acting as antioxidants, may be linked to changes in leaf morphology and metabolism, which collectively work to reduce oxidative stress in plants during drought conditions (Agati et al., 2020).

3 Phytohormones as a way to alleviate abiotic stresses

Phytohormones are organic compounds naturally synthesized by plants, playing critical roles in various plant physiological functions, and are known as plant growth regulators (PGRs). They are released throughout the plant's life cycle under regular conditions and in reaction to environmental triggers. Even though present in minute amounts, these signaling molecules partake in numerous biochemical reactions (EL Sabagh et al., 2022). Specific hormones like ethylene, jasmonic acid, and salicylic acid are involved in tolerance to biotic stresses, while

abscisic acid, auxins, and brassinosteroids are pivotal in adapting to abiotic stresses. Notably, certain phytohormones, such as jasmonates and salicylic acid, enhance plant resilience against both biotic and abiotic environmental challenges (Figure 6) (Khan et al., 2020; Rasool, 2022). These hormones influence nearly all aspects of the plant's lifecycle and are crucial in defending against external stresses, both living and non-living (Wang and Irving, 2011). A hormone's efficacy hinges on its appropriate concentration, its timely and localized production, and its interaction with specific receptors. Depending on their type and levels, phytohormones can either promote or inhibit plant growth and maturation. They operate on target cells by binding to transmembrane receptors and can be modulated by both positive and negative feedback mechanisms (Mukherjee et al., 2022).

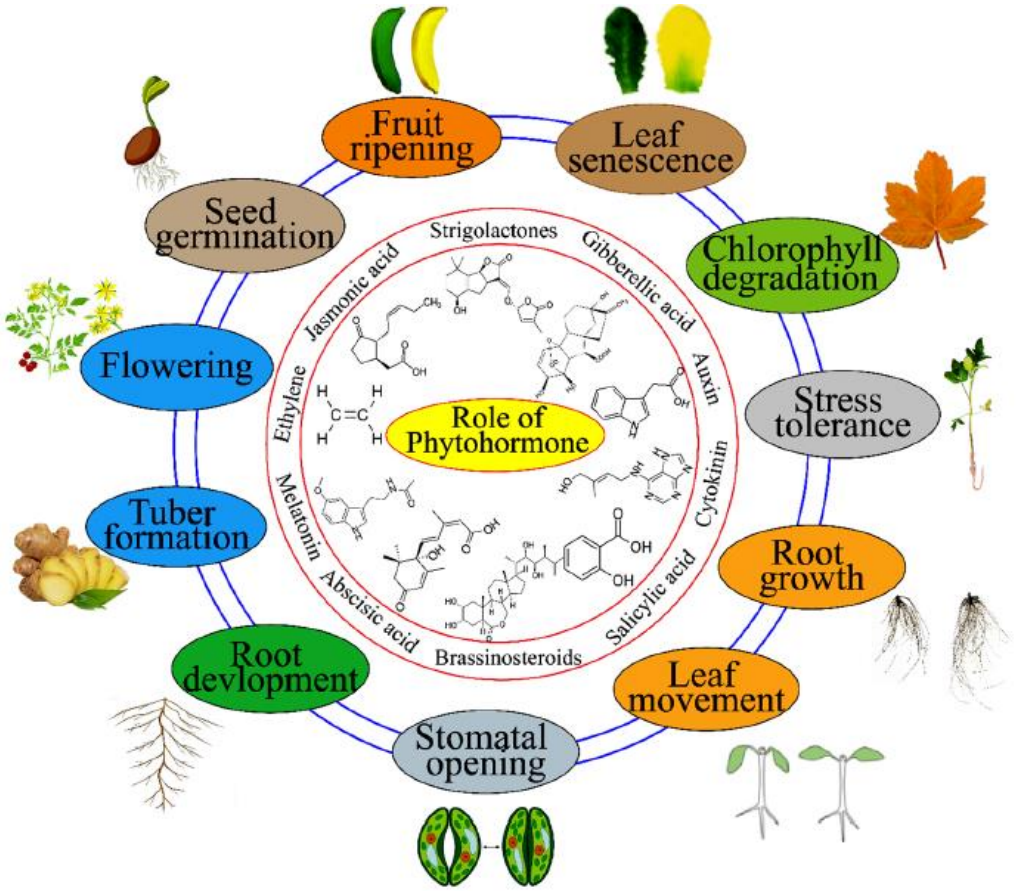

Figure 6. Roles of ten important phytohormones in plants

3.1 Auxin

Despite over a century of research on auxin, the intricacies of its biosynthesis, transport, and signaling remain elusive (Ma et al., 2018). Nonetheless, suggestions have been made about its biosynthesis in plants, pointing to four pathways dependent on tryptophan (Trp) and one that isn't (Bing Wang et al., 2015). IAA (**Error! Reference source not found.**), a versatile phytohormone, is crucial not only for typical plant growth and development but also for modulating plant growth under stressful scenarios (Fu et al., 2015). Although we've recently gained more insight into how auxin influences plant growth and development, its capacity to regulate stress responses is yet to be fully grasped. Notably, emerging research suggests that IAA is central to how plants adapt to stress (Emenecker and Strader, 2020).

IAA, when applied exogenously, triggers an intrinsic antioxidant defense mechanism within plants. This involves the enhanced activation of key antioxidant enzymes, notably superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which work synergistically to detoxify ROS, thus shielding the cellular machinery from oxidative damage (Lecube et al., 2014). Concurrently, IAA plays an instrumental role in osmoregulation. It fosters the accumulation of osmoprotectants, such as proline and soluble sugars, which help maintain cell turgor and membrane integrity under osmotic stress conditions (Sharma et al., 2019). Moreover, the hormone influences root morphogenesis, driving root elongation and enhancing lateral root formation (Jing and Strader, 2019). This architectural adaptation is of paramount importance, especially under drought or nutrient-deficient conditions, as it enables plants to explore a greater volume of soil, accessing deeper water reserves and essential nutrients (Kou et al., 2022). Thus, through these intricate, interconnected mechanisms, IAA emerges as a crucial agent in supporting plant resilience against the different challenges posed by abiotic and oxidative stressors.

3.2 Brassinosteroids

Brassinosteroids (BRs) (Figure 8) are a newer class of polyhydroxy steroidal hormones in plants, known for their significant impact on growth and development (Rajewska et al., 2016). Initially discovered in the pollen of the rape plant (Brassica napus), over 70 BRs have been identified in various plants. Of these, brassinolide, 28-homobrassinolide, and 24-epibrassinolide are the three most biologically active and are frequently utilized in research (Xu et al., 2019). These BRs are found throughout the plant, from pollen and buds to leaves and roots. They are crucial for multiple growth processes like stem and root elongation, as well as the development of flowers and fruits (Figure 11) (Hu et al., 2021). Newer research has indicated that BRs and related compounds help plants combat various environmental stresses, such as extreme temperatures, salinity, light conditions, drought, flooding, metal exposure, and organic pollutants (Vardhini and Anjum, 2015a; C. Wu et al., 2019).

Recent studies highlight the significant capability of BRs and related compounds in adjusting the antioxidant defense system in plants to combat oxidative bursts caused by abiotic stresses (Chaudhuri et al., 2022). When plants are treated with 24-epibrassinolide (EBR), there is a notable enhancement in their internal antioxidant defense mechanisms. This is evidenced by the augmented activity of crucial enzymes like ascorbate peroxidase (APX), glutathione reductase (GR), and guaiacol peroxidase (GPX), which act in tandem to scavenge ROS and maintain cellular redox homeostasis (Guo et al., 2022). Alongside these enzymatic defenses, EBR application also stimulates the production of non-enzymatic antioxidants such as glutathione and ascorbate, fortifying cellular defense further (Jan et al., 2018). Additionally, 24-epibrassinolide plays a role in the modulation of stress-responsive signaling pathways and the activation of specific stress-related genes, empowering plants to better acclimate to harsh environmental scenarios (Yu et al., 2023). By fine-tuning cellular responses and organizing

multifaceted defense mechanisms, 24-epibrassinolide proves instrumental in enhancing plant resilience against the ever-present challenges of abiotic and oxidative stress.

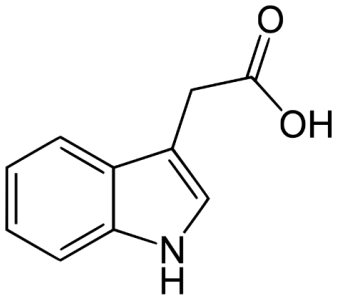

Figure 7. Chemical structure of indole-3-acetic acid hormone

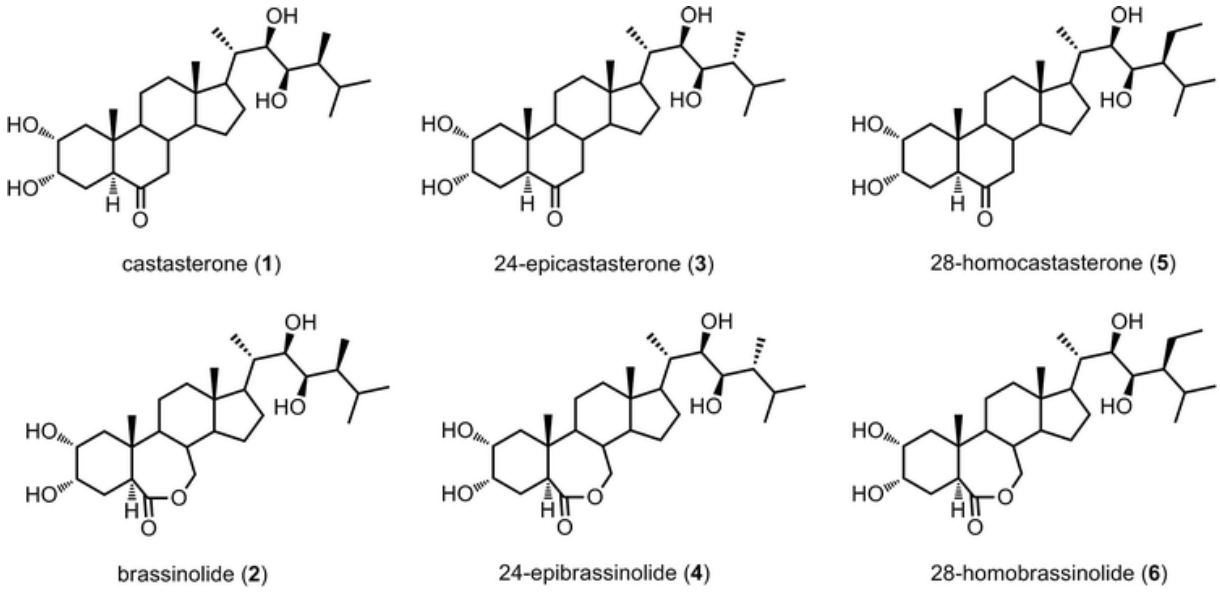

Figure 8. Chemical structure of natural Brassinosteroids hormones in plants

4 Metals hyperaccumulation in a multi-stress environment

4.1 General response traits of hyperaccumulators to abiotic stresses

Heavy metal pollution, coupled with climate change, profoundly impacts the environment, human beings, and plant life. While there are instances where climate change can promote plant

growth, such as enhanced plant growth due to increased CO_2 levels in the atmosphere, these benefits are often offset by other environmental changes. For example, rising temperatures or inconsistent rainfall patterns can counteract the positive effects. One of the notable consequences of climate change is reduced rainfall, which has been linked to increased drought conditions worldwide (Cinner et al., 2022). Decreased precipitation is considered to be a significant impact of climate change, triggering drought conditions globally (Seleiman et al., 2021). Furthermore, soil salinity, which results from natural processes like mineral weathering and is intensified by human activities such as improper irrigation methods and the utilization of saline water resources (Chele et al., 2021b), presents a complex situation when combined with phytoextraction efforts. In phytoextraction, metal hyperaccumulator plants are employed to remove heavy metals from contaminated soils (Ali et al., 2013a), leading to an intricate interplay between salinity stress and metal uptake efficiency. The heightened salt concentrations in soil can dramatically impede the plant's ability to uptake and sequester heavy metals, as the plants are engulfed in a simultaneous struggle to mitigate the osmotic and ionic stresses imposed by salinity (Schück and Greger, 2023). This dual stress scenario can severely limit the efficiency of heavy metal phytoextraction, as the plant metabolic machinery diverts vital resources to combat salt stress, thereby constraining the physiological and biochemical processes essential for metal uptake and detoxification. Thus, soil salinity not only imposes physiological stress onto these plants but also blocks the potential advancements in the field of phytoextraction, making it an intricate antagonist in the battle against soil pollution (Ievinsh et al., 2022).

Moreover, in many mining and metallurgy industry sites, organic contaminants like PAHs are commonly co-present with heavy metals like cadmium (Cd). Their combined presence would influence the soil properties differently, which would affect their fates due to the interaction among different compounds and soil biota (Gran-Scheuch et al., 2020). Therefore, it is

necessary to consider heavy metal co-existence when investigating PAH's influence on the soil ecosystem in the context of phytoextraction.

As a result of these compounded stress scenarios, the metal hyperaccumulator plants experience significant physiological stress. Their natural mechanisms, which usually facilitate the uptake and sequestration of heavy metals, are overwhelmed by the multifaceted stress conditions, limiting the effectiveness of phytoextraction as a remediation strategy. Furthermore, these unfavorable conditions that will be discussed below, potentially induce oxidative stress in hyperaccumulator plants, leading to damage to important cellular components.

4.1.1 Salinity

Soil salinity is a significant environmental stressor that severely impacts plant growth and metabolism (AbdElgawad et al., 2016). It imposes two types of stress on plants. Firstly, a quick osmotic stress is caused by reduced water potential, leading to diminished water uptake. Secondly, a slower ionic imbalance is caused by the harmful accumulation of Na^+ in plant tissues, which affects nutrient absorption over time. This buildup of Na^+ in the shoots can trigger leaf aging and hinder photosynthesis, affecting various processes like the photosynthetic rate and competing with K^+ transport and enzyme reactions (Balasubramaniam et al., 2023). One major outcome is increased oxidative stress in plants (Carrasco-Ríos and Pinto, 2014). As a result, using phytoremediation in saline soils with metal contamination becomes challenging, since hyperaccumulator plants must manage both the stresses of heavy metals and salinity. Leblebici et al. (2011) found that increased salinity reduced the uptake of Cd and Ni in *Spirodela polyrrhiza*. Similarly, *Atropa belladonna*, when grown with 100 mM NaCl and 150 µM $NiCl_2$, showed reduced Ni accumulation (Stetsenko et al., 2017). On the other hand, *Helianthus annuus* plants, when exposed to combined Cu and salt stress, accumulated more Cu than when only facing Cu alone, as observed by (Silambarasan et al., 2020). This was possibly

due to salinity changing the rhizosphere's features, such as increasing organic acid excretion from plant roots, which led to lower soil pH in the rhizosphere and increased metal availability (Ma et al., 2019).

4.1.2 Drought

Drought, a major abiotic stress, arises when decreased water potential and turgor pressure impede standard metabolic activities and hinder the reproductive capabilities of plants (Yang et al., 2021). This phenomenon is considered one of the gravest environmental challenges confronting the global population today (Vicente-Serrano et al., 2020). Anticipated to intensify and expand due to climate change-induced alterations in precipitation and evaporation rates, drought is becoming more common (Mohammed and Scholz, 2017). Unpredictable rainfall patterns, a byproduct of global warming, have also led to more frequent and prolonged drought conditions globally (Kumar, 2012). Persistent drought conditions substantially hinder plant development, delaying growth, disrupting physiological processes, and impairing reproduction (Anjum et al., 2017; Bijalwan et al., 2022).

Consequently, drought can modify soil pollutant behaviors, negatively affecting plant growth and reducing the efficiency of phytoremediation (Seleiman et al., 2021). The combination of metal and drought stresses reduced *Trifolium arvense*'s growth more than each stress individually. Moreover, drought conditions notably decreased Cu and Ni accumulation (Ma et al., 2017), possibly due to inhibited root growth or changes in root structure, which limits metal absorption by plants (Remans et al., 2012). Furthermore, it has been shown that managing agronomic aspects of the soil, such as irrigation and improving soil texture, is essential for phytoremediation practices, particularly true for plants like *N. caerulescens* (Angle et al., 2003). In hyperaccumulator *Brassica napus L*., combined Cd and drought conditions reduced biomass production and metal uptake (Kniuipytė et al., 2023). Additionally, introducing drought stress notably impacted biomass, root structure, and Cd absorption in several wheat

varieties in the presence of Cd (Xiao et al., 2023). (Rahmany-Samani et al., 2023) indicated that soil water content influences Cd accumulation and extraction from the soil. Also, the moisture content of the soil not only determines contaminant availability but also their transport to the above-ground parts of plants.

4.1.3 PAH

Sites contaminated with trace metals often have an array of organic pollutants as well. One of the common contaminants in coastal regions, including estuaries, are petroleum derivatives, such as polycyclic aromatic hydrocarbons (PAHs) (Patel et al., 2020). PAHs originate from various sources: run-off and erosion, wastewater discharge, atmospheric fall-out, natural biological processes, and incidents like oil spills and shipping activities (Panwar and Mathur, 2023). The co-existence of heavy metals and PAHs can influence phytoremediation because these compounds might interact with one another, the plants, and the microorganisms in the rhizosphere (Ali et al., 2022).

Studies have shown both antagonistic and synergistic effects of metals and PAHs on plant growth and their ability to accumulate contaminants. For example, certain levels of pyrene could counteract the inhibitory effects of Cu on maize (*Z. mays*) (Lin et al., 2008), but when paired with Cd, it couldn't lessen the Cd's impact on the same plant (Zhang et al., 2009). Such interactive impacts aren't limited to just PAHs; they've been noticed with other organic pollutants combined with metals as well. For instance, the growth of *Lolium perenne* and *Raphanus sativus* improved when exposed to a low concentration of pentachlorophenol (PCP) with Cu in soils. In contrast, a higher concentration of PCP combined with Cu led to additive harmful effects (Lin et al., 2006). These findings indicate that the interaction between metals and organic pollutants can be either beneficial or detrimental to plant growth and functionality. Several factors influence this interaction, including the type of plant, its growth phase, pollutant concentrations and characteristics, and various soil properties like pH and organic content.

Specifically, PAHs have been found to affect the solubility of Cu, increasing its uptake in plants like *Halimione portulacoides* (Almeida et al., 2008). Prior studies have also shown that phenanthrene (PHE) and pyrene (PYR) negatively affected the uptake of Cd from soil contaminated with Cd by the hyperaccumulator *Sedum alfredii* (Wang et al., 2012). Similarly, Cd accumulation in the stems of *Medicago sativa* decreased upon soil contamination with PHE. This reduction might result from the organic pollutants accumulating in the roots, which in turn blocks Cd's movement from the roots to the above-ground parts (Li et al., 2020).

4.2 Antioxidative response of hyperaccumulators to multi-stresses

While *N. caerulescens* can survive in highly contaminated soils without showing apparent signs of plant toxicity, the functionality of its antioxidant enzymes and the levels of MDA in its tissues can fluctuate based on the contamination intensity of the soil (Bayçu et al., 2017). This suggests that metals, particularly those not hyperaccumulated, can adversely affect the plant's physiological processes, especially when combined with other stress factors. Such adverse effects could compromise the long-term efficacy of phytoremediation. Over time, this might result in successive generations of *N. caerulescens* exhibiting diminishing phytoremediation abilities at the same polluted location (Sterckeman et al., 2019).

Although *N. caerulescens* has not been frequently studied about metal phytoextraction in multi-contaminated scenarios with other abiotic factors, there's evidence that this plant produces glucosinolates and anthocyanin when experiencing herbivory stress during Zn phytoextraction (Asad et al., 2015). Additionally, the Ni hyperaccumulator *Cleome heratensis*, under combined Ni and drought conditions, exhibited increased lipid peroxidation, heightened activities of antioxidant enzymes SOD and CAT, and a rise in osmoprotectant accumulation (Salehi Eskandari et al., 2017). Furthermore, in *Matthiola* (Brassicaceae) exposed to both Pb and drought, there was an increase in phenolic compounds as well as enhanced CAT and APX

enzyme activities. These responses are likely to coordinate to combat various stresses. Also, there was a noticeable increase in the activities of enzymes like Glutathione s-transferase and glutathione peroxidase (GPOX), both crucial for coping with heavy metal and drought stress (Salehi-Eskandari et al., 2022).

Moreover, in the As hyperaccumulator plant *Pteris vittata L*., when exposed to both PHE and As, there was an observed increase in GSH content and SOD activities. This suggests that while the combined contaminants intensified plant toxicity, the elevated levels of antioxidants GSH and SOD in the leaves helped maintain their physiological functioning (Sun et al., 2011). In addition, when examining *Oudemansiella radicata* in the context of Cd phytoextraction, the presence of both Cd and pyrene caused significant alterations in antioxidant enzyme activities, particularly superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (R. Chen et al., 2015).

4.3 Phytohormones and hyperaccumulators

PGRs (plant growth regulators) have been eyed as potential enhancers for phytoremediation techniques like phytoextraction due to their capacity to enhance plant growth and alleviate abiotic stress (Barbafieri and Tassi, 2011; Cabello-Conejo et al., 2013). Various research shows that using IAA (Indole-3-acetic acid) can lead to a boost in metal accumulation in plant shoots, making it more effective in achieving the main goal of phytoextraction: increased metal removal. For instance, Hadi et al. (2010) noted that a foliar spray of IAA (0.175 mg L^{-1}) considerably upped the Pb accumulation in plants. Similarly, Liphadzi et al. (2006) treated *Helianthus annuus* with IAA, witnessing a rise in Pb and Cd accumulation in the leaves. Also, experiments have delved into the effects of PGRs on Ni phytoextraction, especially in hyperaccumulator plants of the *Alyssum* genus (Qiu et al., 2009). For instance, (Cassina et al., 2011) found that using cytokinins on *A. murale* in serpentine soils enhanced Ni

phytoextraction, attributed to the augmented biomass of the treated plants when compared to untreated ones. Similarly, (Cabello-Conejo et al., 2013) showed an increase in biomass and Ni phytoextraction efficiency in *A. corsicum* when treated with a mix of gibberellins and cytokinins. These findings emphasize the need for research into PGRs, including IAA and Brassinosteroids, to explore their viability in phytoextraction. It's crucial to identify the most effective application techniques, particularly when dealing with multicontamination, to mitigate potential stresses and ensure the optimal growth and functioning of hyperaccumulator plants.

5 Conclusion and research objectives

Given the ability of *N. caerulescens* to hyperaccumulate heavy metals from polluted soils, along with its ecotypes that originate from both calamine (MET) and ultramafic (SER) metalliferous and non-metalliferous (NMET) environments, it stands as a notable example of phytoremediation potential. However, the plant faces challenges in actual multi-stress conditions, where heavy metals, along with salinity, PAHs, or drought, can induce oxidative stress, reducing the plant's growth and, consequently, its capacity for phytoextraction. To mitigate these stresses and enhance phytoextraction capabilities, the application of phytohormones like indole-3-acetic acid (IAA) and 24-epibrassinolide (EBR) has been identified as an effective strategy.

Therefore, to fully evaluate the antioxidant response of *N. caerulescens* to different abiotic stresses, two main objectives were studied. The first one consisted of the understanding of the antioxidant response of two ecotypes, one from an NMET site (Chavignée) and the other from a MET one (Ganges), when exposed to different abiotic stresses. The second one consisted of the assessment of the effect of phytohormone application on the antioxidant response and phytoextraction yield of *N. caerulescens* in a multi-stress environment, to develop strategies to

protect plants during phytoextraction. These objectives will be addressed as follows: Chapter II studies the phytoextraction yield and antioxidant response of *N. caerulescens* when subjected to standard abiotic stress (PAHs), Chapter III compares different stresses (salt, salinity, drought, and PAH) on the plant response, Chapter IV examines the effect of the foliar application of IAA and 24-epibrassinolide (EBR) on *N. caerulescens* functioning and phytoextraction yield, and Chapter V delves into the antioxidative response and phytoextraction of *N. caerulescens* under drought, salinity, and PAHs stresses along with the phytohormones treatment.

Chapter II

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

This chapter focuses on the antioxidant responses and phytoextraction capabilities of the Ganges and Chavignée populations of *N. caerulescens* when exposed to environments contaminated with heavy metals combined with standard abiotic stress, PAH. It provides an understanding of the concentrations, amounts, and translocation factors of heavy metals within these populations. Furthermore, the chapter sheds light on the stress biomarkers and the activity of antioxidant enzymes in plants due to the imposed stress conditions.

This work has been presented at the international conference on *Serpentine Ecology* and published in the international journal *Ecological Research* in a special issue.

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Abstract

Multi-contamination of soils by various organic and inorganic pollutants is considered an obstacle to the development of hyperaccumulator plants and the phytoextraction of metals. This study aimed to investigate the effect of polycyclic aromatic hydrocarbons (PAHs) in combination with trace elements on the antioxidant response and phytoextraction efficiency of two populations of the hyperaccumulator *Noccaea caerulescens* from either a metalliferous (Ganges) or a non-metalliferous (Chavignée) site. Plants were grown for 17 days in soil containing moderate concentrations of trace elements and under the effect of phenanthrene (PHE), a PAH stress model. In general, exposure to PHE resulted in a reduction of growth parameters, together 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 extent of enzymatic activities and the amount of metals extracted between the two populations studied. Plants from Chavignée exhibited a slightly more tolerant response to PHE stress than those from Ganges. The presence of PHE in the soil proved highly toxic to *N. caerulescens*, resulting in low numbers of survivors. Nevertheless, the differences observed between the two populations concerning growth, metal extraction, and antioxidant defense responses suggest that the difference in defense capacity may ensure different tolerance. This difference may be linked to adaptations acquired by each population according to the soil type from which it originates. However, these results need to be confirmed by further experiments.

Keywords: hyperaccumulator, PAH, abiotic stress, phytoextraction, antioxidant

1 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 and Segura, 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., 2020a). 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 can absorb contaminants from soil, and transfer and accumulate them in their aerial parts. Interest in hyperaccumulators has stimulated research into various facets of the biota inhabiting metalliferous soils (Reeves 2024). The search for new examples of hyperaccumulators continues, facilitated in part by non-destructive X-ray fluorescence scanning of herbarium specimens, which was previously used to provide minute fragments for meticulous but damaging analysis (Zeremski et al., 2021). Among the various hyperaccumulators, *Noccaea caerulescens* (Brassicaceae) is documented to be a good hyperaccumulator of Cd, Ni, or Zn depending on the population (Assunção et al., 2003; Gonneau et al., 2017a). While Schwartz (1997) demonstrated that *N. caerulescens* was able to grow on a former coking plant soil co-contaminated with PAHs and metals, other recent studies showed that under abiotic stress conditions, the establishment and development of *N.*

caerulescens can sometimes be impossible which therefore inhibits phytoextraction process (Bauddh and Singh, 2012; Ouvrard et al., 2011; 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 plants through adsorption processes on the root system or within the first internal cellular layers (Dupuy et al., 2015). Their 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., 2005b; Liu et al., 2009a; Meudec et al., 2007). A similarity between the effect of PAHs and water stress has also been demonstrated (Dupuy et al., 2015a; Pašková et al., 2006a). The more documented drought stress is reported to limit the growth of several plants via inducing biochemical and physiological perturbations (Kamran et al., 2020a; Rajput et al., 2017a; Zia et al., 2021a). 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 (H_2O_2), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radicals ($HO^{\bullet}$). 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., 2009a; Sharma et al., 2012). Studies have reported visible symptoms such as chlorosis, white spots along with necrosis when plants are exposed to PAHs (Liu et al., 2009a; Oguntimehin et al., 2010; Vollenweider and Günthardt-Goerg, 2005).

However, to scavenge ROS, plants have developed a well-equipped antioxidant defense system that includes enzymatic and non-enzymatic antioxidants (Vardhini and Anjum, 2015b). 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), superoxide dismutase (SOD), 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). Thus, 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.

2 Materials and Methods

2.1 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., 2019, 2017). 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., 2014a).

Table 1. Soil main properties. CEC: cationic exchange capacity.

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 (Figure 9), seeds were placed on water-soaked filter papers and cotton and incubated at 4°C in the dark for three days to lift dormancy. Seeds 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.

Figure 9. *N. caerulescens* seedlings in germination trays during the preculture phase

During the culture phase, a phenanthrene stress was applied. The PHE concentration tested was chosen based on a previous study in which maize cultivated on pure sand was exposed to a range of PHE concentrations from 50 to 750 mg kg^{-1} (Dupuy et al., 2015). Despite a similar survival rate (mean 75%), a threshold effect was observed at 50 mg PHE kg^{-1} with a strong loss of biomass (mean 90%) and strong morphological changes for all higher concentrations. Given this threshold effect and the fact that PHE tends to adsorb more strongly to metal-contaminated soils (Saison et al. 2004), an intermediate concentration of 200 mg PHE kg^{-1} was chosen. 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 ensures 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 about three weeks old were

transferred into 750 cm^3 pots filled with 800 g soil and placed in the climatic chamber in a randomized block design (Figure 10).

Figure 10. Illustration of the experimental design one week after transplantation

2.2 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. The fresh weight of aerial parts and root systems was then measured. The 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.

2.3 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 (Cartelat et al., 2005; Cerovic et al., 2012; Goulas et al., 2004).

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.

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 standards were periodically included in the batches to be analyzed.

2.4 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 and 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.

2.5 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. Due to a high mortality rate, particularly in the Ganges population, a one-sided Student's t-test was also used to test the treatment effect (CTRL and PHE) for each population under the hypothesis that PHE stress has a negative effect on plant growth indicators and metal uptake and a positive effect on stress indicators and antioxidant enzyme activities.

The relationships between the variables describing i) plant growth, ii) nutrient uptake iii) metal uptake, and iv) stress response (Table 2) were assessed through a multiple-factor analysis (MFA) with the “FactomineR” package (Pagès 2013). This multicanonical analysis was developed for addressing relationships between several data sets (typically more than two) with a particular focus on efficient normalization of the individual data sets (Abdi et al. 2013). Compared to the Principal Component Analysis, this method aims to balance the groups in an overall analysis by weighting the variables. MFA was successfully applied to sensory, ecological, and omic data (Escofier and Pagès 1994; Dumas et al. 2005; De Tayrac et al. 2009; Bernier and Gillet 2012). The Wilks’ lambda test from the “rrcov” package was then used to evaluate which qualitative variable best illustrates the distances between individuals on the factorial plane. The significance was evaluated with a F-test.

Table 2. Allocation by groups of variables used in the multi-factor analysis (MFA)

Growth indicators		Metal uptake	
DW.S	Aerial dry weight (mg)	Cd.S	Shoot cadmium content (g kg^{-1})
DW.R	Root dry weight (mg)	Ni.S	Shoot nickel content (g kg^{-1})
WC.S	Shoot water content %	Zn.S	Shoot zinc content (g kg^{-1})
WC.R	Root water content %	Cd.R	Root cadmium content (g kg^{-1})
C.S	Shoot carbon content (g kg^{-1})	Ni.R	Root nickel content (g kg^{-1})
C.R	Root carbon content (g kg^{-1})	Zn.R	Root zinc content (g kg^{-1})
CHL	Epidermal chlorophyll content (µg/cm^2)		
Nutrient uptake		**Stress indicators**	
NBI	Nitrogen balance index	ANT	Epidermal flavonol index
N.S	Shoot nitrogen content (g kg^{-1})	FLA	Epidermal anthocyan index
K.S	Shoot potassium content (g kg^{-1})	CAT	Catalase activity (units min^{-1} g^{-1} FW)
P.S	Shoot phosphorus content (g kg^{-1})	GPOX	Guaiacol peroxidase (units min^{-1} g^{-1} FW)
S.S	Shoot sulfur content (g kg^{-1})	APX	Ascorbate peroxidase (units min^{-1} g^{-1} FW)
N.R	Root nitrogen content (g kg^{-1})	DHAR	Dehydroascorbate reductase (units min^{-1} g^{-1} FW)
K.R	Root potassium content (g kg^{-1})	MDA	Malondialdehyde (nmol g^{-1} FW)
P.R	Root phosphorus content (g kg^{-1})		
S.R	Root sulfur content (g kg^{-1})		

3 Results

3.1 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 ($F_{(1,13)} = 5.09$, $p = 0.061$, Figure 11). 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: $F_{(1,13)} = 27.8$, $p < 0.001$, Figure 11). Shoot N and P contents) and root K contents (Figure 11) 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: $F_{(1,13)} = 5.41$, $p = 0.036$).

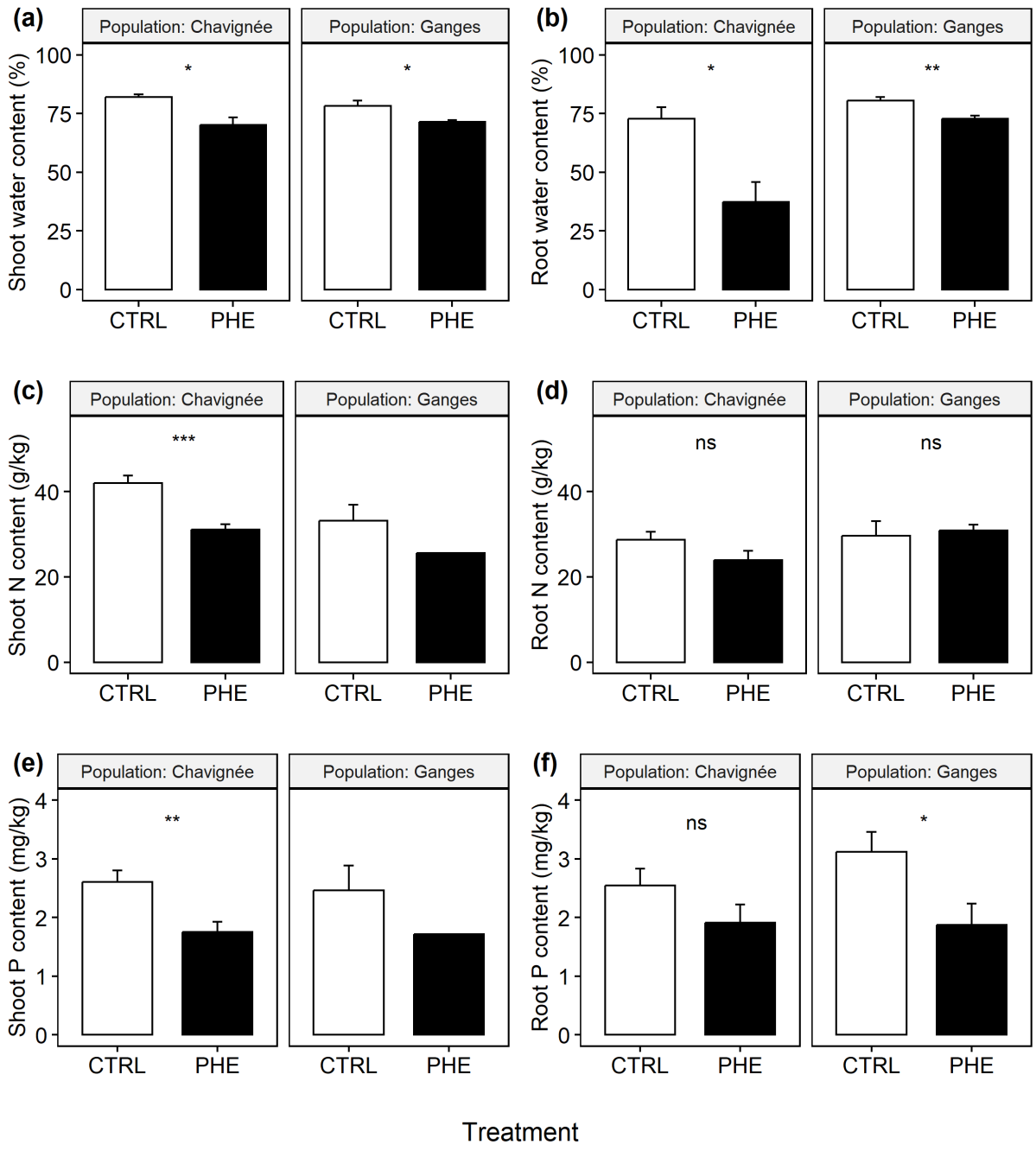

Figure 11. Shoot and root water (a, b), nitrogen (c, d), and phosphorus (e, f) contents of two *Noccaea caerulescens* populations in response to phenanthrene (PHE) stress. For each population, results are presented as means with standard errors and the one-sided Student's t-Test significant level for the treatment effect (ns non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For the Ganges population, no statistical marker will appear if only one replicate has been used for the measurement of the parameter.

3.2 Trace elements uptake

At the population level, Chavignée plants concentrated 1.3 and 1.9 times more Ni and Zn, respectively, than Ganges plants. 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 on Chavignée plants (Figure 12). The effect was greatest in shoots with a 4-, 2-, and 5-fold decrease for Cd, Ni, and Zn, respectively. Whatever the element considered, the effect is also higher in the Chavignée than in the Ganges plants, even if the comparison between the two populations is limited by the high mortality in the Ganges population. For example, shoot Zn content decreased by a factor of 6 and 3 in the Chavignée and Ganges plants, respectively. For the latter, a significant decrease at the 10% level was observed for Cd and Zn concentrations in the roots.

The translocation factor (TF) was calculated as the ratio between shoot and root concentrations (Figure 13). In the Chavignée population, only shoot to root Ni ratio tended to decrease under the effect of PHE stress ($p < 0.1$). 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 (Figure 13). Chavignée plants extracted 2.6, 1.5, and 3.9 times less Cd, Ni, and Zn, respectively, when subjected to PHE stress. This negative effect was significant at the 5% and 10% levels for Cd and Zn yields, respectively. Similarly, Ganges plants extracted 3.4, 1.8, and 3.1 times less Cd, Ni, and Zn, respectively, when subjected to PHE stress. However, these results should be treated with caution due to the low number of survivors in the Ganges population following 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.

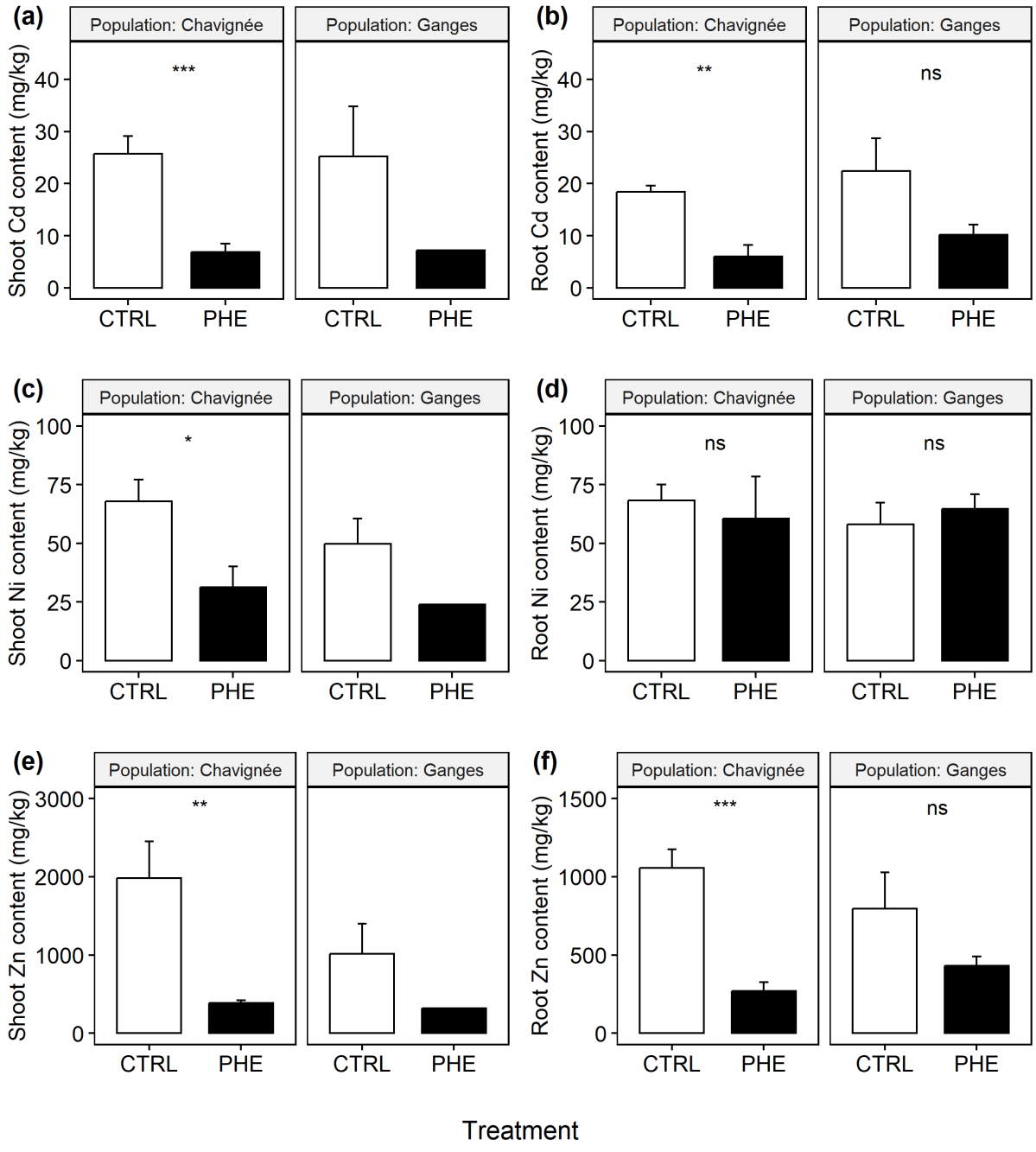

Figure 12. Shoot and root Cd (a, b), Ni (c, d), and Zn (e, f) concentrations of two *N. caerulescens* populations in response to PHE stress. For each population, results are presented as means with standard errors and the one-sided Student's t-Test significant level for the treatment effect (ns non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For the Ganges population, no statistical marker will appear if only one replicate has been used for the measurement of the parameter.

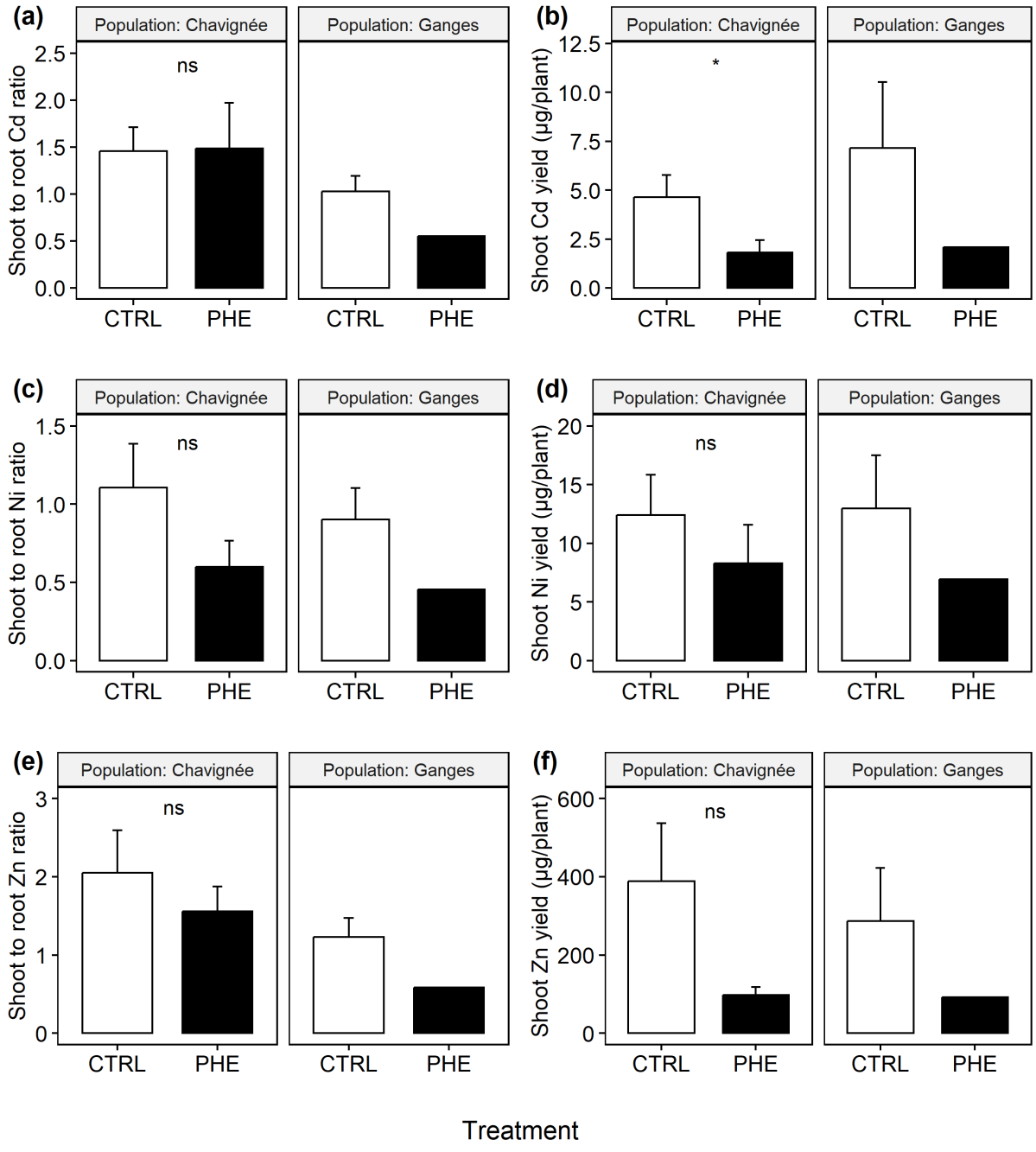

Figure 13. Shoot/root translocation factors and shoot metal yields (µg plant^{-1}) of Cd (a, b), Ni (c, d), and Zn (e, f) for two *N. caerulescens* populations in response to PHE stress. For each population, results are presented as means with standard errors and the one-sided Student's t-Test significant level for the treatment effect (ns non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For the Ganges population, no statistical marker will appear if only one replicate has been used for the measurement of the parameter.

3.3 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 (Figure 14 and Figure 15).

Regarding chlorophyll concentration (CHL), a significant decrease at the 10% level from 39.2 to 32.8 µg cm^{-2} was observed under PHE stress in the Ganges population (Figure 14). At the population level, Ganges plants had a significantly higher CHL content than Chavignée plants ($F_{(1,14)} = 16.3$, $p = 0.0012$). 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 concerning 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. The NBI decreased by a factor of 1.5 when PHE treatment was applied with a significant effect at the 5% and 10% levels for Chavignée and Ganges respectively.

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 (Figure 15), 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).

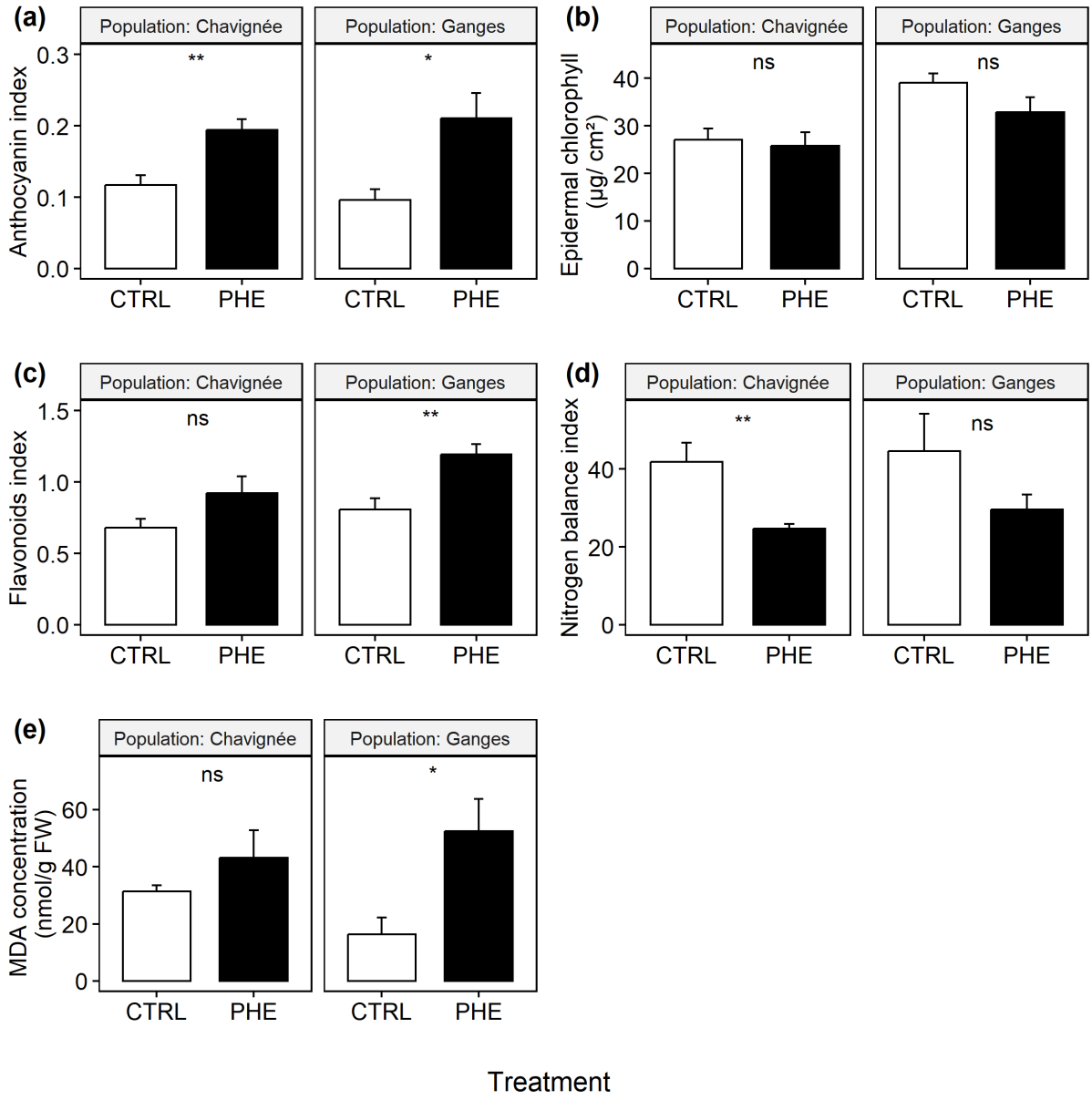

Figure 14. Leaf pigment indices (a, b, c, d) and malondialdehyde (MDA) content (e) of two *N. caerulescens* populations in response to PHE stress. For each population, results are presented as means with standard errors and the one-sided Student's t-Test significant level for the treatment effect (ns non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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 significantly

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 (p = 0.02). An opposite behavior was observed for CAT activity, with a significant increase by a factor of 1.7 in Ganges plants under PHE stress (p = 0.026), whereas no effect was observed in Chavignée plants. Regarding APX and GPOX activities, both significantly 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.

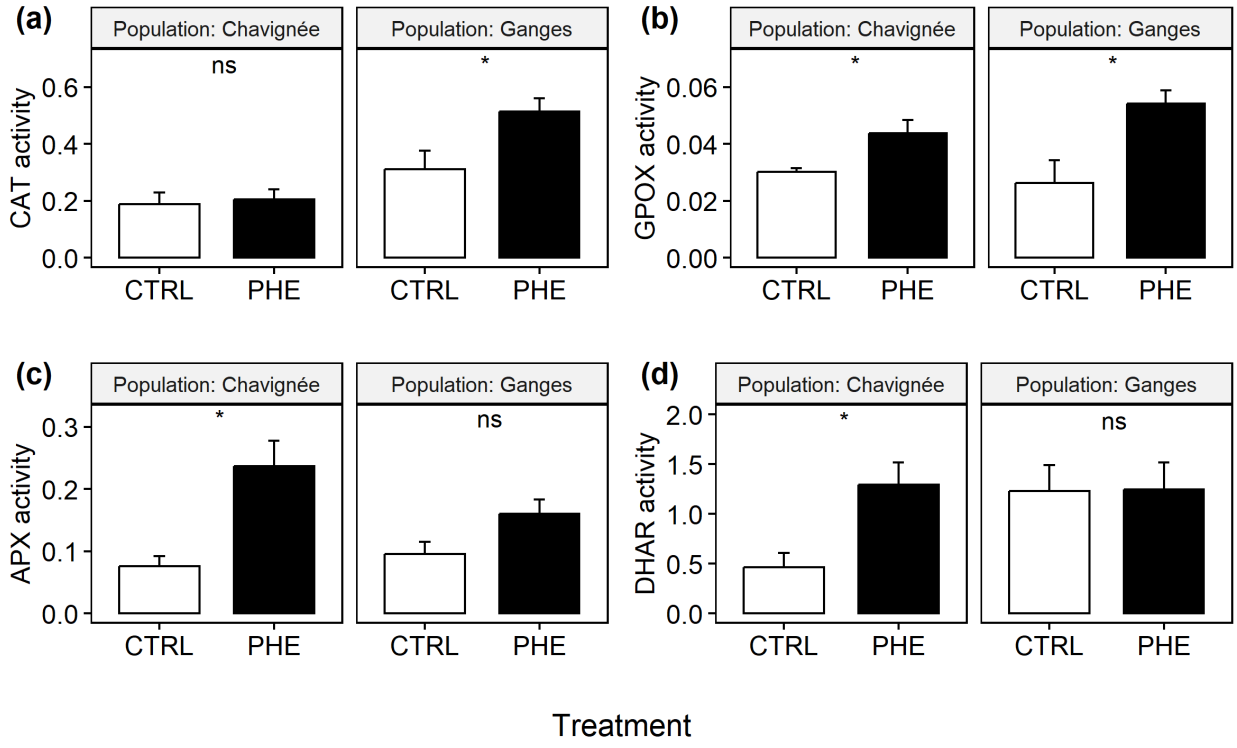

Figure 15. Antioxidant activity in shoots of two *N. caerulescens* populations in response to PHE stress. CAT: catalase (a), GPOX: guaiacol peroxidase (b), APX: ascorbate peroxidase (c), DHAR: dehydroascorbate reductase (d). Activities are expressed in µmol min^{-1} mg^{-1} protein. For each population, results are presented as means with standard errors and the one-sided Student's t-Test significativity for treatment effect (ns non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4 Multivariate analysis

A multi-factor analysis was used to assess the relationships between four groups of variables (Table 2) describing plant growth, nutrient uptake, metal uptake, and stress response (Figure 16). 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 (Figure 16a). 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) (Figure 16b). 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 (Figure 16c), 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, and 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, and 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. Except for FLA, which belongs to the Stress Indicators group, all variables belong to the Growth Indicators and Nutrient Uptake groups.

When considering the groups of variables (Figure 16d), dimension 1 is highly explained by the metal uptake and stress response groups, whereas dimension 2 is highly explained by the plant growth and nutrient uptake groups. The findings show that, on the first factorial plan, the groups of variables discriminate between the population and treatment factors. In fact, the application of PHE stress leads to a significant change in the behavior of *N. caerulescens*, resulting in lower metal uptake and a stronger antioxidant response in the stressed plants. The differences observed between the two populations, Ganges and Chavignée, were independent of the application of PHE stress and were characterized by differences in terms of growth and nutrient uptake. These results point in the same direction as the significant statistical tests presented above.

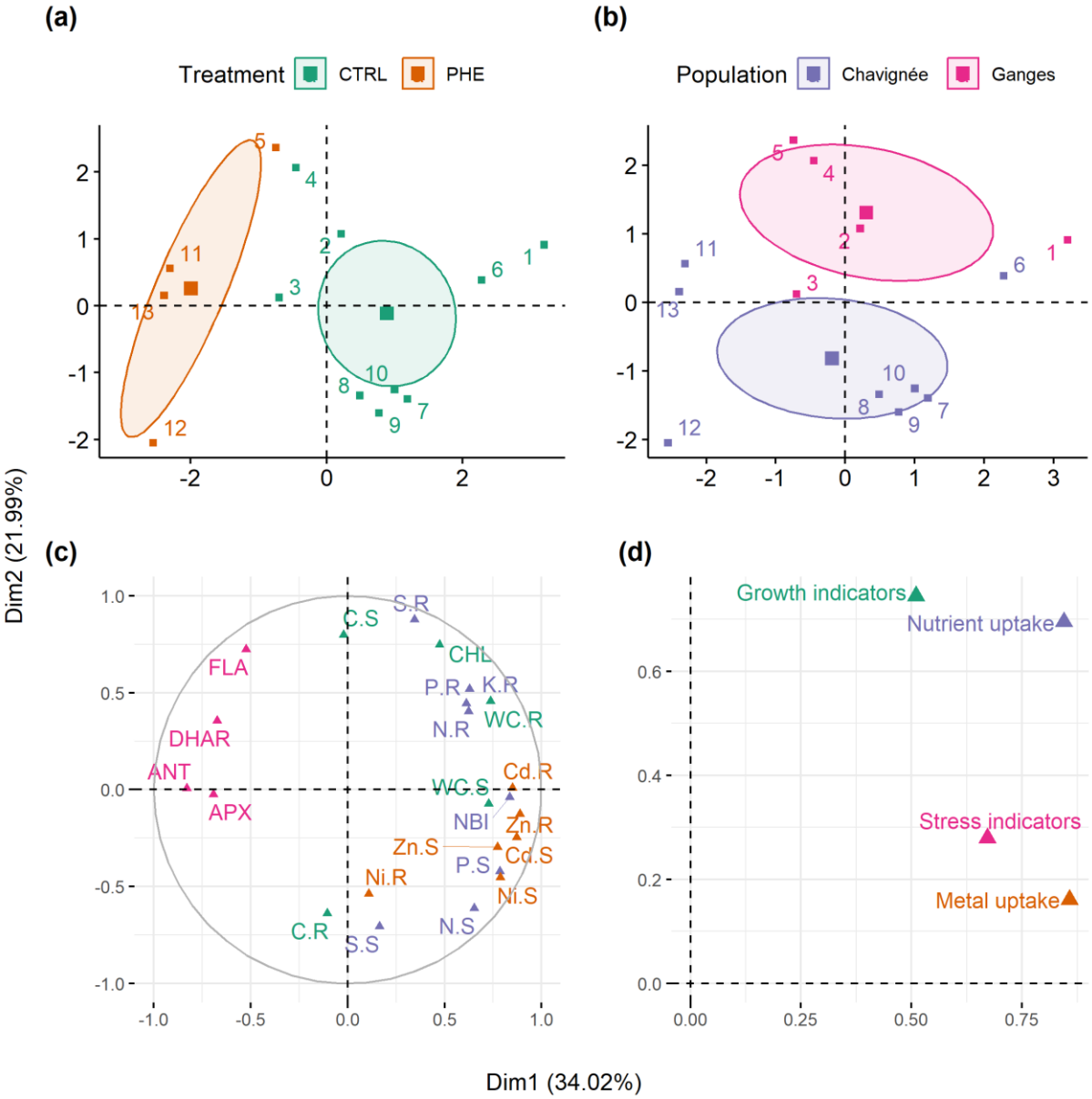

Figure 16. First factorial map from multiple factorial analysis with four groups of variables. The numbers (in %) next to each axis represent the variance explained by the respective principal components. At the top, is the map of individuals. The labeled individuals are those best represented on the map. Labeled individuals are those with the largest contribution to the construction of the plane. Individuals are colored according to their membership in the modalities of the variables "Treatment" (a) and "Population" (b). At the bottom, is the representation of the variables (c) and the group of variables (d) in the factorial design. The labeled variables are those that are best represented on the map. The variables are colored according to their membership in the variable groups (Table 2).

4 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.

4.1 PHE reduces plant growth performance with a more pronounced effect on the metallicolous 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 (Ahammed et al., 2012b; Liu et al., 2009b). 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 (Figure 11). 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. These results are similar to those of Naz et al. (2015) who also reported a decrease in root hair elongation of the drought-tolerant *Lasiurus scindicus* subjected to high salt levels. 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 (Appendix 1). The C/N imbalance under stress might be due to sugar accumulation and nitrogen deficiency in both populations, which are commonly associated with leaf senescence under plant stress (D. 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 (Larsen et al., 2011; Nathawat et al., 2007; Sun et al., 2017). The nitrogen balance index (NBI) indicates the ratio of both 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 (Figure 14). 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 (Figure 14). 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., 2009b)) 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., 2009b; 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., 2015b). 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 (Mateus et al., 2021; Wang et al., 2013b).

4.2 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 shoot concentration and the amount of all extracted metals (Figure 13). 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 (Liang et al., 2012; Zhan et al., 2010). 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.

4.3 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 (Figure 15). 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., 2009b) 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-metallicolous Chavignée population than in the metallicolous 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-metallicolous 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 (Figure 14). 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 (Liu et al., 2009b; 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) (Appendix 1). 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., 2004b). 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, 2004a). In this study, APX and GPOX activities increased in both the Ganges and Chavignée plants under PHE stress (Figure 15). 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., 2012c).

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 (Figure 15). 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 (Figure 15). It has been commonly reported that DHAR activity increases under various stresses (Vardhini and Anjum, 2015a; 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., 2021, 2020), 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.

4.4 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 (Figure 16). 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 metallicolous 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., 2010b; Zhang et al., 2023). However, these plants can offset this cost by reducing energy investments in other processes (Farinati et al., 2009b) or by dampening of ROS signals generated by another stress (Fones and Preston, 2013b).

5 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. From these first results, it seems that the effectiveness of co-remediation with hyperaccumulators depends on the selection of populations that are tolerant to organic pollutants. However, due to the low number of replicates inherent to the high PHE toxicity observed in this study, these results need to be confirmed by further experiments. Other levers for removing oxidative stress also need to be studied, such as the use of plant growth-promoting micro-organisms or phytohormones.

Chapter III

Do *Noccaea caerulescens* populations have similar responses under different abiotic stresses?

This chapter explores the antioxidant responses and the potential for phytoextraction of the Ganges and Chavignée populations of *N. caerulescens* when subjected to environments loaded with heavy metals and accompanied by multiple abiotic stresses, including drought, salinity, or PAH. It provides insights into the levels, amounts, and movement patterns of heavy metals among these populations. Additionally, the chapter highlights the stress indicators and the functions of antioxidative enzymes in the plants as a result of the prevailing stress conditions.

Abstract

Noccaea caerulescens represents one of the hyperaccumulator plants used in phytoextraction of trace metals from contaminated soils. However, the implementation of treatment processes based on its extraction capacities is challenged by different abiotic stresses present in the multi-contaminated environments. This study aimed to explore the phytoextraction efficiency and antioxidant response of *N. caerulescens* when subjected to different abiotic stresses. For that, plants from two populations of *N. caerulescens* originating from metallicolous (Ganges) and non-metallicolous (Chavignée) sites were cultivated in hydroponics. They were first exposed to moderate trace metal concentration for 8 weeks and then various stress treatments were applied for 3 weeks: salinity (NaCl at 40 mM), drought (polyethylene glycol at 5%), and organic contaminants (phenanthrene at 200 µM). The exposure to stresses resulted in a reduction in the growth parameters, along with the upregulation of antioxidant compounds and enzymes, together with limitations in nutrient uptake and root-to-shoot translocation and heavy metals extraction in *N. caerulescens*. Differences were observed in the activity levels of enzymes and the amount of extracted metals between the two populations. There were variations in growth, metal extraction, and antioxidant defense responses between the two populations, indicating that their differing defense capacities might contribute to their tolerance levels. These variations could be attributed to adaptations acquired by each population based on the type of soil they originated from. The Chavignée plants exhibited a slightly higher tolerance to stress compared to the Ganges plants.

1 Introduction

Climate change, industrial development, and intensive agriculture are expected to increase the frequency, intensity, and coexisting of various environmental abiotic stresses, with harmful effects on the plants and soil health. Salinity and drought are destructive abiotic stresses that

are reported to restrict the growth of various plants by inducing biochemical and physiological disturbances (Kamran et al., 2020b; Rajput et al., 2017b; Zia et al., 2021b). They also restrict plant productivity as a result of nutritional imbalance and osmotic stress (Shahid et al., 2020). Salt stress leads to the accumulation of salt ions in the cytosol, ultimately causing significant damage to the plant cell (Rajput et al., 2016). As for drought stress, it is known to induce stomatal closure, inhibit photosynthesis, reduce the leaf area, decrease water potential and increase the quantity of osmolytes (Ibrahim et al., 2019b). Moreover, polycyclic aromatic hydrocarbons (PAHs) are harmful substances that result from burning carbon-based fuels. They are environmentally persistent, toxic, and carcinogenic. When it comes to plants, PAHs primarily affect them by attaching to the root system or penetrating the initial layers of cells through adsorption processes (Dupuy et al. 2016).

The coexistence of several stressors has drawn attention worldwide (Wang et al., 2020; S. Wu et al., 2019). This is the case for example of soils contaminated with heavy metals in addition to drought conditions, high soil salinity or persistent organic pollutants (Islam and Sandhi, 2022; Jeelani et al., 2018; Kholodova et al., 2010). However, the combined effect of heavy metals and other stresses on plant functioning stays poorly studied, although heavy metal stress has been explored extensively on several plant species (Sperdouli, 2022).

To remediate polluted soils, different phytoremediation techniques have been exploited. They are considered as energy and cost-effective approaches to restore metal-contaminated environments in contrast to traditional remediation technologies (Bhat et al., 2022; Sabreena et al., 2022). Phytoextraction represents one of the methods used in the case of soils contaminated by heavy metals, and is based mainly on the use of hyperaccumulator plants capable of absorbing these contaminants from the soil, transferring and concentrating them in their harvestable parts (Pasricha et al., 2021; Yan et al., 2020b). Among the various hyperaccumulator plants, *Noccaea caerulescens* (Brassicaceae) is one of the most model

species for studying the physiological and molecular aspects of these plants. It can hyperaccumulate Cd, Zn and Ni, though, the accumulating capacity of each metal is population specific (Gonneau et al., 2017b; Mohtadi et al., 2012). However, the establishment and development of *N. caerulescens* can sometimes be limited, which is the case in soils highly affected by the presence of abiotic stress factors such as the co-contamination with both organic and inorganic contaminants (Ouvrard et al., 2011b; Sirguey and Ouvrard, 2013b).

Abiotic stresses trigger the accumulation and generation of excess reactive oxygen species (ROS) inside cells. ROS are mainly by-products of metabolic phenomena acting as essential molecules tissue homeostasis and cell signaling. However, high ROS levels threaten plants and may disrupt morphological, physiological, and metabolic processes, thus causing oxidative damage (Sachdev et al., 2021). Superoxide anion (O_2^-), hydroxyl radical (OH^-), singlet oxygen (O_2) and hydrogen peroxide (H_2O_2) are the major ROS where each has an oxidizing potential and a distinctive half-life (Apel and Hirt, 2004b; Juan et al., 2021; Miller et al., 2010). These molecules can lead to damage of cellular and molecular components due to the oxidation of main biomolecules such as carbohydrates, lipid, proteins and DNA, thus leading to programmed cell death (PCD) (Qamer et al., 2021; Sachdev et al., 2021). To maintain plant growth and development, plants respond to oxidative stress by stimulating specific molecular and physiological responses. The well-equipped antioxidant machinery in plants include both enzymatic and non-enzymatic compounds that function in concert to regulate cascades of uncontrolled oxidation (Gutiérrez-Martínez et al., 2020). Non-enzymatic antioxidants include glutathione, ascorbate, and various polyphenolic compounds such as flavonoids. Additionally, several enzymes including catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPOD), enzymes of ascorbate-glutathione (AsA-GSH) cycle including ascorbate peroxidase (APX), dehydroascorbate reductase, (DHAR) and glutathione reductase (GR), act vitally in the plant defense antioxidant system (Ali et al., 2019; Noctor and Foyer, 1998; Rajput et al., 2021).

The upregulation of antioxidants that has been witnessed in stressed plants, points to an important role of antioxidants in alleviating stress (Kusvuran et al., 2016). Given the nature of plant response to abiotic stresses a better understanding of the specific answers of hyperaccumulator species such as *Noccaea caerulescens* is crucial for the development of phytoremediation processes. However, defense mechanisms and antioxidant response are still little studied in these very unusual plants

Our study aims to explore the effects of most common abiotic stresses encountered in contaminated soils, PAHs, salt, and drought stresses on the phytoextraction efficiency and antioxidative response of two populations of the hyperaccumulator *N. caerulescens*. These are two edaphic types: non-metalliferous (Chavignée population) and metalliferous (Ganges population) habitat. The obtained results should offer a thorough and comparative understanding of how plants respond to stress, encompassing their ability to extract contaminants from the environment and their mechanisms for tolerating abiotic stresses.

2 Materials and Methods

2.1 Plant cultivation

N. caerulescens seeds (J. & C. Presl) were collected from two different populations, the first corresponds to a metallicolous population found in a mining site in Ganges (Occitanie, France) and the second is a non-metallicolous population located in Chavignée (Bourgogne-Franche-Comté, France) (Gonneau et al., 2014). In the germination phase, seeds were placed on water-soaked filter papers and cotton inside petri dishes and incubated at 4 °C in the dark. They were then transferred to a climatic growth chamber with a photoperiod of 16 h and a temperature of 23 °C/18 °C day/night, a photon flux density of 196 µmol photons m^{-2} s^{-1} in the

photosynthetically active radiation (PAR) range and 70% humidity for a period of 14 days (Figure 17).

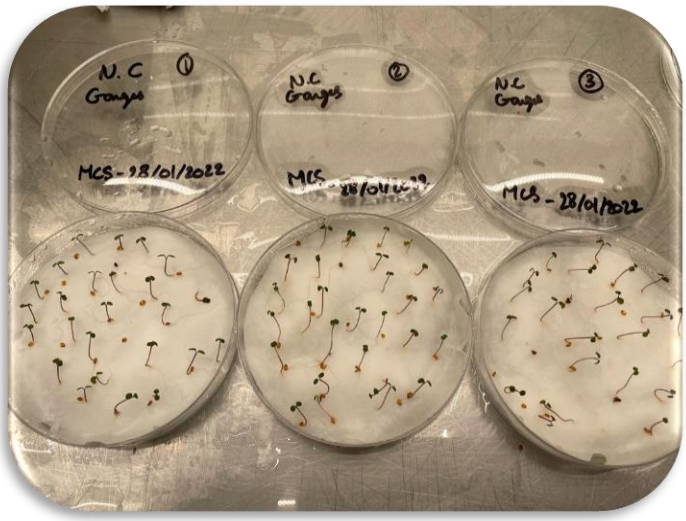

Figure 17. *N. caerulescens* seedlings during the germination phase in the petri-dishes

The preculture phase (56 days) consisted of two equal consecutive periods. In the first period, plants of both populations were grown in hydroponics and randomly distributed in two polyethylene trays of 36 cm length, 26 cm width and 10 cm depth, filled with 10 L nutritive solution. Then, during the second period, plants were placed in two other trays that had the same length and width, but a greater depth of 21 cm and containing 21 L solution, to avoid mechanical constraints on the roots. In each tray, the cover plate contained 24 plants put in holes enclosing a growing basket with sponge utilized as a support for the plants (Figure 18). Adapted from (Küpper et al., 2007), the nutrient solution was composed of: 1 mM $Ca(NO_3)_2$, 1 mM KNO_3, 0.1 mM KH_2PO_4, 50 µM KCl, 0.5 mM $MgSO_4$, 20 µM NaFe (III) EDTA, 10 µM H_3BO_3, 1 µM $MnCl_2$, 10 µM $ZnSO_4$, 0.7 µM $NiSO_4$, 0.2 µM $CuSO_4$, 0.2 µM Na_2MoO_4 dissolved in deionized water. The solution was buffered by the addition of 2 mM 2-(Nmorpholino) ethanesulfonic acid (MES) buffer, adjusting the pH at 5.5 via the addition of 0.2 M KOH. Cadmium was added to the nutrient solution starting from the day 21 in the form of $Cd(NO_3)_2$ at a moderate concentration of 0.25 mM. In each tray, capillary tubes were placed

through each tray and immersed in the nutrient solution, to allow the aeration of the solution by bubbling it with filtered air. The nutrient solution was renewed once a week.

Figure 18. *N. caerulescens* seedlings during the preculture phase in the plastic hydroponic trays

During the culture phase, plants were grown in the same controlled conditions in the presence of stresses. The 56 days old plants were distributed evenly over 8 stainless steel trays, each filled with 8.3 L nutrient solution. The used solutions in all trays had the same composition as that used in the preculture phase, in addition to a stressor depending on the treatment. The experiment consisted of 4 treatments as follows:

i) Control treatment (Ctrl); no stress.

ii) Drought treatment (PEG); adding polyethylene glycol (PEG 6000) at 5% in increments.

iii) Salinity treatment (Sal), adding sodium chloride (NaCl) at 40 mM in increments.

iv) Phenanthrene treatment (PHE), adding PHE (a model PAH) at 200 µM.

For each of the two populations Ganges (Ga) and Chavignée (Ch), treatments were conducted in 5 replicates, with a total of 40 plants in the experiment (Figure 19).

Figure 19. *N. caerulescens* seedlings during the culture phase in the stainless-steel hydroponic trays

2.2 Plant harvest

After 21 days of cultivation under stress, roots and shoots were separated at the crown. The WinRhizo® software (Regent Instruments Inc., Canada) was utilized to assess the architecture of the root systems by measuring the total root length (TRL), total root surface (TRS) and mean root diameter (MRD). Root dry weight was measured after oven drying at 70 °C for 48 hours.Fresh shoots were frozen and crushed in liquid nitrogen after measuring their wights, and then divided into two portions; the first was freeze-dried and used for dry weight measurement in addition to the elemental and biochemical analysis, and the second was kept in storage at a temperature of -80 °C for further enzymatic analysis.

2.3 Assessment of plant element content

Oven dried root systems and freeze-dried shoots of the plant material were ground finely into fine particle with pre-cooled agate mortar. Then, 50 mg of the plant material were digested successively with 8 ml of 65% HNO_3 for 12 hours and 4 ml of 30% H_2O_2 for 3.5 hours at 95

°C using the DigiPREP® system (SCP Science, Baie-d'Urfé, QC, Canada). After filtering the extracts at 0.45 µm, the samples were adjusted to 25 mL with ultrapure water (18 MΩ). The concentrations of major and minor elements in the plant extracts were analyzed by induced coupled plasma emission spectrometry (ICP-AES, iCAP 6000 series, Thermo Scientific, Cambridge, UK). The validity of results was checked by periodically adding a blank and a control material of *N. caerulescens* with a known composition (internal analyses made by INRAE-USRAVE, Villenave d'Ornon, France), along with a certified solution (EU-H3, SCP Science, Courtaboeuf, France), to the batches to be analyzed.

2.4 Assessment of leaf pigments, metabolites, and stress biomarkers

Photosynthetic pigments, metabolites and stress biomarkers were quantified in aboveground parts of the plants. Freeze dried samples (10 mg) were extracted with 80% (v/v) cold ethanol, and the resulting crude extract was centrifuged (López-Hidalgo et al., 2021). The supernatant was collected and utilized to quantify photosynthetic pigments (chlorophyll A and B, and carotenoids), metabolites (total phenolic compounds, and total flavonoids) and stress biomarkers (proline, malondialdehyde (MDA)) using a microplate reader (Safas Xenius, Monaco). Chlorophyll A, chlorophyll B and carotenoid contents were measured and calculated following (López-Hidalgo et al., 2021). Total phenolics were determined according to (Ainsworth and Gillespie, 2007) by mixing the supernatant thoroughly with 10% Folin–Ciocalteu reagent followed by adding 700 mM sodium carbonate, then measuring the absorbance at 720 nm using gallic acid as a standard to generate the calibration curve. Total flavonoids were measured by mixing the plant extract with 10% aluminum chloride, 1 M potassium acetate and methanol, then measuring the absorbance at 415 nm using quercetin as a standard (Huang et al., 2018). Proline content was assayed using the method of (López-Hidalgo et al., 2021) by adding a commercial ninhydrin reagent to the supernatant and

measuring the absorbance at 450 nm, using proline standard for the calibration curve. Lipid peroxidation, estimated by the amount of malondialdehyde (MDA), was assayed following the description of (Hodges et al., 1999) and (Landi, 2017).

2.5 Antioxidant enzymes assays

Protein extraction was done by the method of (Murshed et al., 2008). Samples of the aboveground parts stored at –80 °C (0.20 to 0.4 g) were mixed with 1 ml of 50 mM MES/KOH buffer (pH 6.0), containing 40 mM KCl, 2 mM $CaCl_2$, and 1 mM AsA and homogenized. The obtained extracts were centrifuged at 4 °C for 15 min at 16000g, and the supernatants were immediately subjected to analysis of the enzymes' activities. Protein content was measured following Bradford's method (Bradford, 1976b).

All enzyme activities were measured by kinetic reactions done in volume of 200 µL at 25 °C using a microplate reader (Safas Xenius, Monaco). APX, DHAR, and GR activities were determined following the method described by (Murshed et al., 2008). SOD activity was measured using a method modified from that of (Dhindsa et al., 1981) in a 1 ml reaction mixture containing 50 mM potassium phosphate (pH 7.8) buffer, 13 mM methionine, 0.1 mM EDTA, 75 µM nitro blue tetrazolium (NBT), 2 µM riboflavin and 10 µL of sample supernatant. After vortexing tubes briefly, 200 µL aliquots from each tube, were added into 96-well plates. A standard curve was generated using commercial SOD of 0.6, 1.2, 1.8, 2.4 and 3 units. The plates were placed for 5 min under white light and the absorbance was then read at 560 nm. CAT activity was measured in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 20 µL of sample supernatant, according to the method of (Aebi, 1984b). The activity was calculated by monitoring the decrease in the reaction rate at 240 nm. Finally, GPOD activity was assayed by following the H_2O_2 dependent oxidation of guaiacol at 470 nm

according to the method downscaled for the microplate and modified from that of (Castillo et al., 1984b).

2.6 Statistical analysis

The statistics were conducted using R software version 4.3.1. To evaluate the impact of treatment, population, and their interaction, a two-way ANOVA was performed, followed by Tukey's HSD test using the "multcomp" package under parametric conditions. The suitability of the data for ANOVA models was assessed by examining normality (Shapiro test) and homogeneity (Levene test). To investigate the relationships between variables related to i) plant growth, ii) nutrient uptake, iii) metal uptake, and iv) stress response, a multiple-factor analysis (MFA) was conducted using the "FactomineR" package.

3 Results

3.1 Growth indicators: nutritive status and plant development

In general, there were few significant differences between the two populations regarding plants growth indicators and mineral concentration in shoot and root (Table 3). Despite that, the biomass of both populations Ganges and Chavignée tended to decrease after plants were exposed to salinity and drought stresses. Conversely, the root biomass of both populations increased under salinity. In contrast with Ganges population, Chavignée showed an increase in the dry biomass of shoots and roots when exposed to PHE.

In general, minerals concentrations in the shoots were significantly higher in the Chavignée plants by 2 to 4 folds, when compared to the Ganges. Nevertheless, the concentrations of potassium (K), phosphorous (P) and sulfur (S) decreased under all stresses except under drought conditions, where their concentrations increased in both populations. Calcium (Ca) and magnesium (Mg) decreased under stress in shoots and roots of both populations, except of

drought and PHE where they increased in shoots of Ganges plants. As a main consequence of salinity, the concentration of sodium (Na) increased significantly in the shoot and root of both studied populations, where the increase was more pronounced in the Ganges population when compared to its controls. Then, the Na/K ratio increased significantly in the shoots and roots of both populations under salt stress. However, the Na/K ratio was lower in the roots of the Chavignée when compared to that of Ganges's.

3.2 Root architecture and leaf pigments

Overall, in

Table 4, all stresses affected root architecture parameters in the studied populations, with a decrease in the TRL and TRS. The effect was more pronounced in Ganges plants mainly under drought and salt stress. Nevertheless, MRD increased significantly in the Ganges roots under salinity from 0.43 to 0.57 mm. Chlorophyll A and B decreased by the stress effect in all treatments. Carotenoids increased in Chavignée only, while remained constant in the Ganges plants.

Table 3. Growth parameters and nutrient content of shoots and roots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses. At the organ level, different letters indicate significant differences ($p < 0.05$)

Organ	Population	Treatment	Dry weight (g)		K (g/kg)		P (g/kg)		S (g/kg)		Ca (g/kg)		Mg (g/kg)		Na (g/kg)		Na/K (10^{-2})	
Shoot	Chavignée	CTRL	5.37	a	113.5	ab	6.09	ab	11.8	a	45.7	a	7.40	a	0.11	a	0.16	a
		PEG	3.32	a	137.1	a	7.43	a	12.6	a	38.6	ab	6.69	a	0.19	a	0.24	a
		PHE	6.24	a	94.3	b	4.34	bc	6.8	bc	36.3	b	6.00	a	0.86	a	1.52	a
		SAL	3.76	a	101.1	b	6.60	ab	10.2	ab	39.4	ab	6.83	a	41.0	b	71.5	b
	Ganges	CTRL	5.69	a	36.9	c	2.23	c	6.21	c	9.57	c	2.27	b	0.001	a	0.004	a
		PEG	3.59	a	50.2	c	2.67	c	5.42	c	13.7	c	3.56	b	0.02	a	0.07	a
		PHE	3.44	a	34.8	c	2.28	c	4.73	c	13.7	c	3.39	b	0.02	a	0.11	a
		SAL	4.17	a	43.5	c	2.69	c	4.81	c	8.9	c	2.38	b	19.1	c	75.8	b
	Chavignée		4.63	a	113.0	a	6.19	a	10.59	a	40.3	a	6.76	a	9.40	a	16.3	a
	Ganges		4.22	a	41.4	b	2.47	b	5.29	b	11.5	b	2.90	b	4.80	a	19.0	a
		CTRL	5.53	ab	75.2	a	4.16	ab	9.04	a	27.7	a	4.84	a	0.05	a	0.08	a
		PEG	3.45	a	93.6	b	5.05	b	9.01	a	26.2	ab	5.12	a	0.10	a	0.15	a
		PHE	4.69	b	61.3	a	3.19	a	5.65	b	23.8	b	4.55	a	0.39	a	0.73	a
		SAL	3.99	ab	69.1	a	4.43	b	7.24	a	22.5	ab	4.36	a	28.9	b	73.9	b
Root	Chavignée	CTRL	0.66	a	12.1	ab	2.57	a	7.13	a	13.4	a	4.27	a	0.43	a	6.51	a
		PEG	0.48	a	11.1	ab	3.92	a	7.83	a	12.8	ab	2.87	b	0.40	a	6.85	a
		PHE	0.90	a	16.9	ab	2.80	a	9.13	a	12.6	ac	5.10	a	0.47	a	6.18	a
		SAL	0.89	a	4.24	b	3.16	a	8.15	a	8.73	d	1.76	c	9.87	b	398.1	b
	Ganges	CTRL	0.85	a	5.54	b	2.90	a	8.18	a	12.2	ac	2.41	bc	0.27	a	12.1	a
		PEG	0.30	a	27.4	a	3.32	a	9.27	a	10.5	bcd	2.58	bc	0.43	a	5.93	a
		PHE	0.71	a	10.1	ab	4.12	a	10.0	a	11.7	ac	2.35	bc	0.32	a	7.37	a
		SAL	0.93	a	5.32	b	4.27	a	8.75	a	9.96	cd	1.76	c	18.5	c	559.6	c
	Chavignée		0.71	a	11.1	a	3.13	a	8.00	a	12.0	a	3.51	a	2.53	a	93.6	a
	Ganges		0.70	a	12.1	a	3.65	a	9.05	a	11.1	a	2.28	b	4.89	a	146.3	a
		CTRL	0.76	a	8.82	a	2.74	a	7.66	a	12.7	a	3.34	a	0.35	a	9.29	a
		PEG	0.39	a	19.2	a	3.62	a	8.55	a	11.6	a	2.72	b	0.42	a	6.39	a
		PHE	0.79	a	13.1	a	3.53	a	9.62	a	12.1	a	3.57	a	0.38	a	6.84	a
		SAL	0.91	a	4.84	a	3.78	a	8.48	a	9.4	b	1.76	c	14.6	b	487.8	b

Table 4. Root architecture parameters and leaf pigment concentrations of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses. TRL: total root length (m), TRS: total root surface area (m2), MRD: mean root diameter (mm), ChLA: chlrophyll A concentration (µg/mg FW), ChLB: chlorophyll B concentration (µg/mg FW), Carotenoids concentration (µg/mg FW). Different letters indicate significant differences ($p < 0.05$).

Population	Treatment	Root Architecture						Leaf Pigments					
		TRL		TRS		MRD		ChLA		ChLB		Carotenoids	
Chavignée	CTRL	10.00	ab	1.39	ab	0.45	a	0.86	a	0.34	a	0.13	ab
	PEG	6.63	ac	0.95	bc	0.46	a	0.67	bc	0.32	abc	0.18	cd
	PHE	6.97	ade	1.06	bc	0.49	ab	0.78	ab	0.28	cd	0.17	ad
	SAL	9.01	ab	1.37	ab	0.47	ab	0.60	c	0.26	bd	0.19	d
Ganges	CTRL	12.38	b	1.45	b	0.43	a	0.79	ab	0.33	ac	0.13	ab
	PEG	3.22	c	0.43	c	0.41	a	0.55	c	0.23	d	0.12	b
	PHE	10.21	be	1.24	ab	0.40	a	0.60	c	0.24	d	0.14	abc
	SAL	4.61	cd	0.83	ac	0.57	b	0.58	c	0.25	d	0.12	b
Chavignée		8.17	a	1.19	a	0.47	a	0.73	a	0.30	a	0.16	a
Ganges		7.61	b	0.99	a	0.45	a	0.63	a	0.26	a	0.13	a
	CTRL	11.19	a	1.42	a	0.44	a	0.82	a	0.33	a	0.13	a
	PEG	4.93	b	0.69	a	0.43	a	0.61	bc	0.27	ab	0.15	b
	PHE	8.77	ab	1.16	a	0.44	a	0.68	ab	0.26	bc	0.15	b
	SAL	6.56	ab	1.07	a	0.53	a	0.59	c	0.25	c	0.15	b

3.3 Trace elements uptake

Figure 20 shows the concentrations of trace metals inside the shoots and roots of both populations of *N. caerulescens*. Globally, drought led to an increase in Cd and Zn concentrations in the shoots of both populations while PHE and salinity decreased them, with a stronger reduction effect under PHE exposure. However, an exception was observed for Zn concentration in the shoots of Ganges population where an increase in its concentration was observed under all forms of stress. In the roots, there was a difference in the response between Cd and Zn uptake. For Cd, all stresses led to an increase in concentration, except for the reduction caused by salinity in Chavignée. Conversely, for Zn, all stresses caused an increase in concentration, particularly for the salinity treatment in Chavignée.

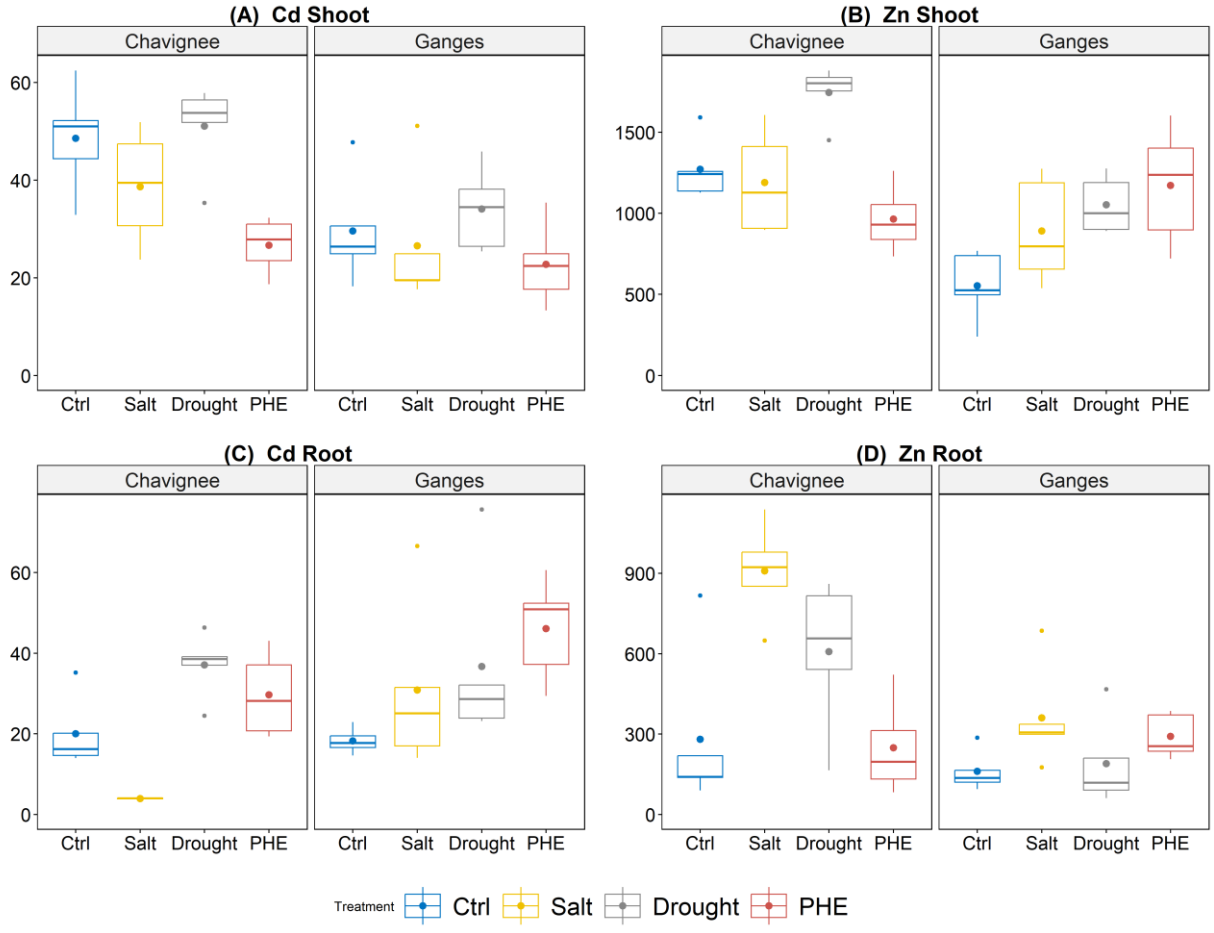

Figure 20. Concentrations (mg kg^{-1}) of Cd and Zn in shoots and roots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses.

Overall, due to the decrease of the produced biomass, the quantity of the metals accumulated per plant decreased when exposed to all stresses (Figure 21). Additionally, the translocation factor (TF) of Cd decreased in both populations, except for the increase observed under salinity stress in Chavignée. Similarly, the TF of Zn decreased in both populations, except for an increase observed under drought stress in the Ganges plants.

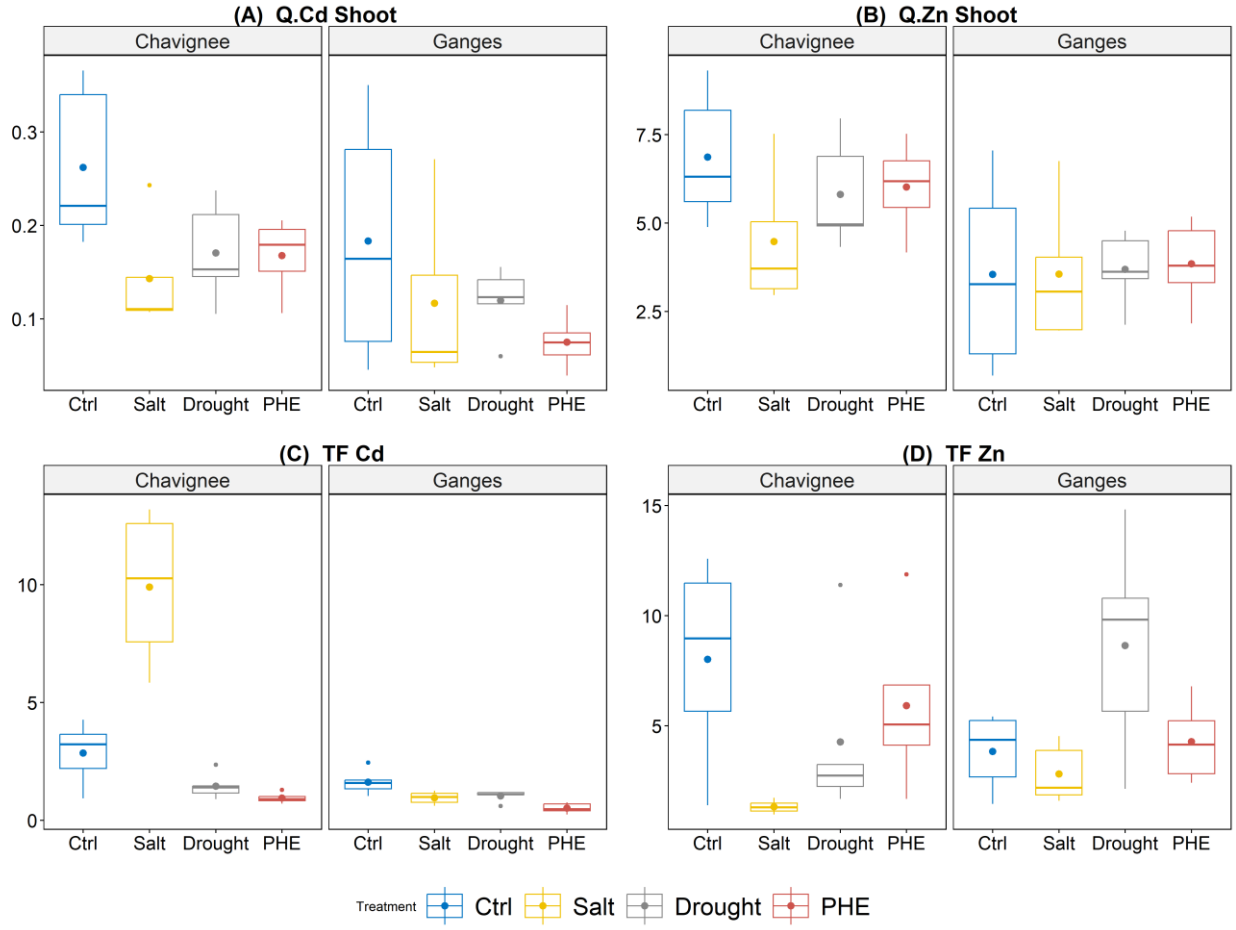

Figure 21. Quantity (µg plant^{-1}) and shoot/root translocation factors (TF) of Cd and Zn extracted by shoots and roots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses.

3.4 Stress indicators

All stress treatments exerted a significant effect on both populations by increasing the MDA content by a factor varying from 1.7 to 2.4, with the MDA content being higher in Ganges than

in Chavignée under the drought and PHE stresses (Figure 22). At the control level, Ganges plant contained significant higher levels of proline than the Chavignée by 2.6 folds. The stresses increased the proline in both populations. Consistently, both total flavonoids and total phenolic compounds increased by the effect of all stress when compared to the controls of both populations, and the phenolic compounds in the control of Ganges was significantly higher than that in Chavignée by 1.6 folds.

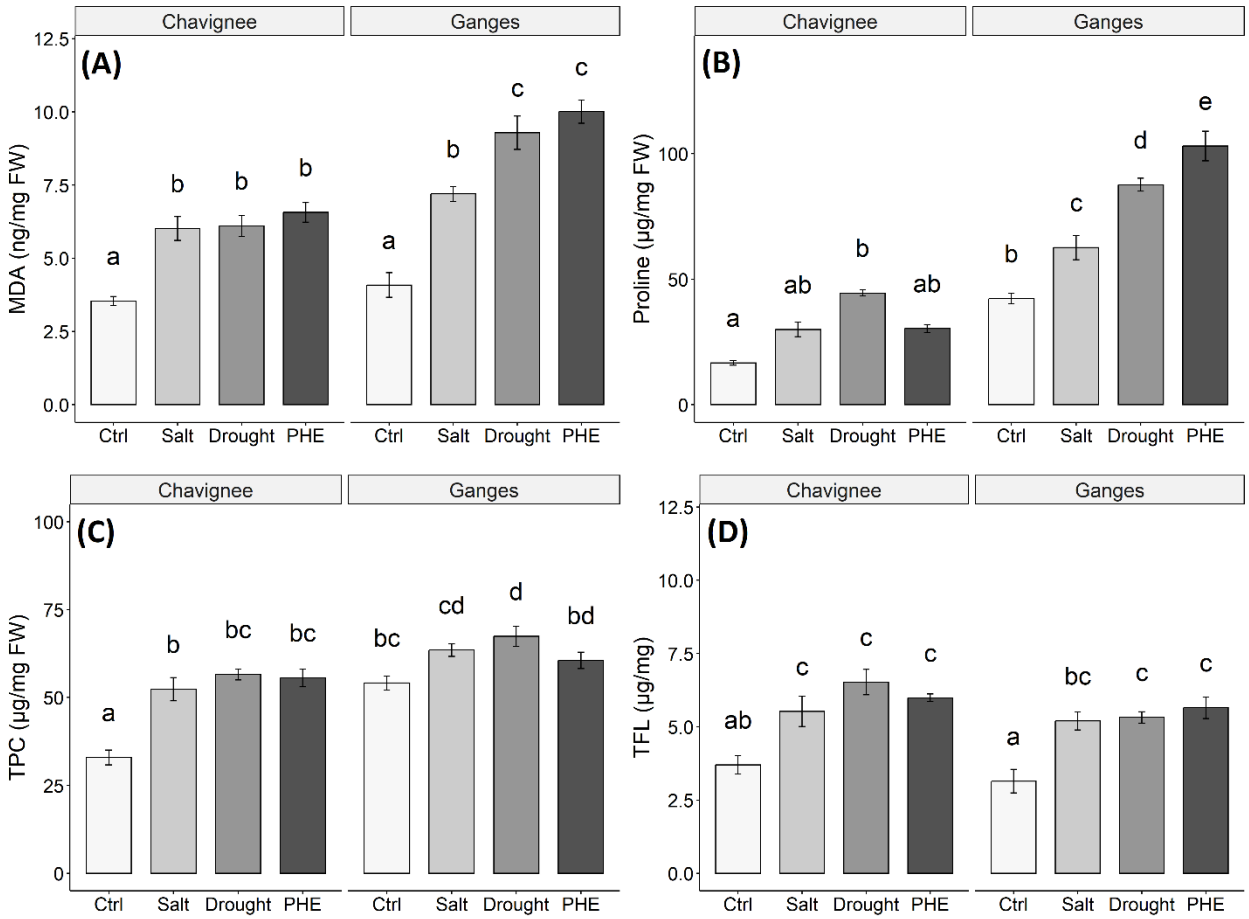

Figure 22. Stress biomarkers and metabolites content in shoots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses. MDA: malondialdehyde, TPC: total phenolic compounds, TFL: total flavonoids. Different letters indicate significant differences ($p < 0.05$).

In Figure 23, CAT, APX and DHAR were significantly higher in the controls of Ganges than Chavignée. Inversely, SOD was significantly higher in the control Chavignée than in that of Ganges. In Chavignée, SOD and CAT activities significantly increased upon exposure to

drought and salinity stressors. In Ganges SOD significantly increased due to drought and PHE while CAT, APX and DHAR increased due to the three stressors. As for GPOD activity, there was an increase in both pop under all stresses.

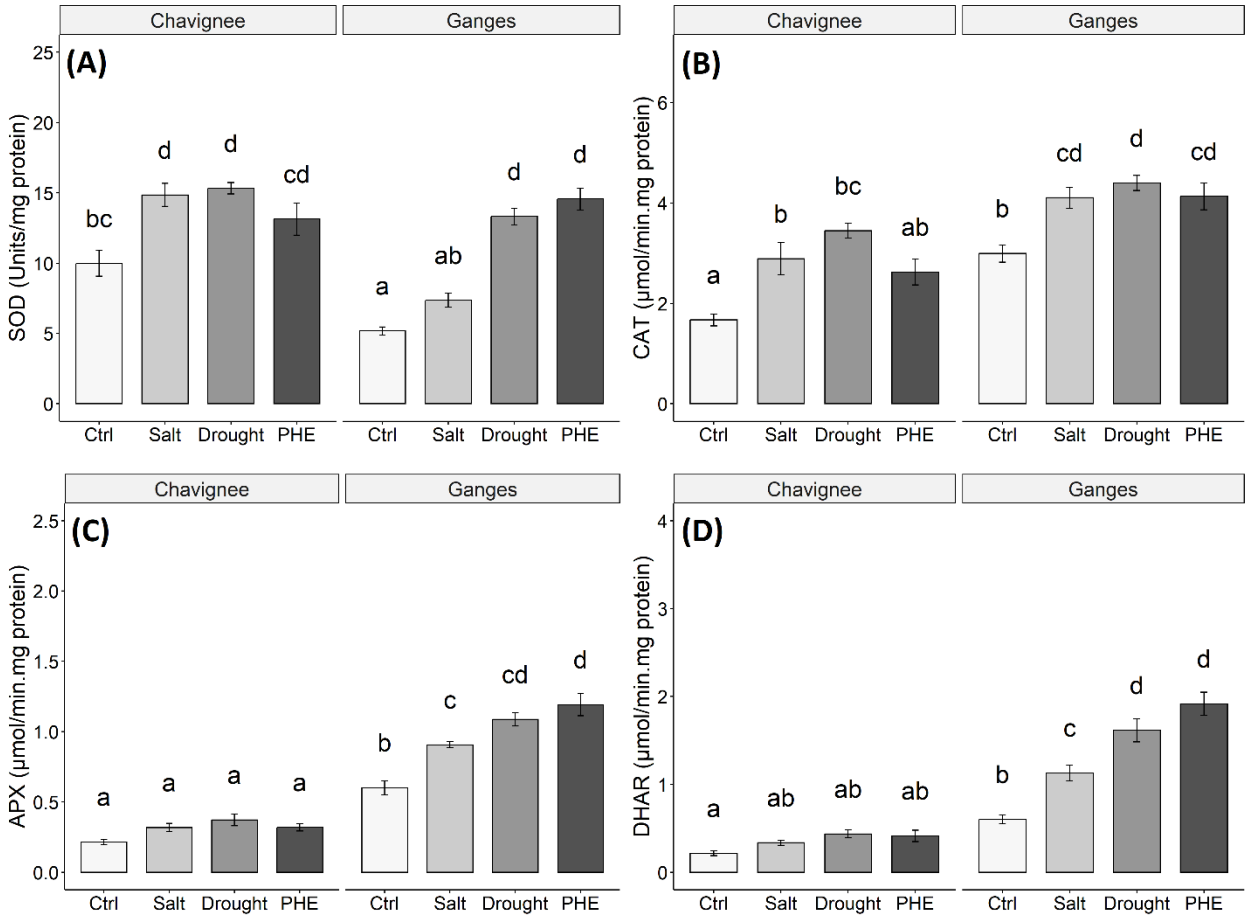

Figure 23. Activities of antioxidant enzymes in shoots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses. SOD: superoxide dismutase, CAT: catalase, APX: ascorbate peroxidase, DHAR: dehydroascorbate reductase. Different letters indicate significant differences ($p < 0.05$)

3.5 Multivariate analysis

A multi-factor analysis was conducted to evaluate the relationships and correlations between four groups of variables describing plant growth, nutrient uptake, metal uptake and stress response (Figure 24).

The first 2 axes of the analysis account for 47.2% of the total inertia present in the dataset. This indicates that almost half of the variability within the cloud of variables is captured by these two axes. This is a considerable percentage which is significantly higher than the base value, underscoring the statistical significance of the variability explained by this analysis.

The graph of individuals on the factorial design shows that dimension 1 opposes the Ganges population (on the left side of the graph, characterized by a strongly negative coordinate on the axis) against the Chavignée treatment (in the center-right of the graph, characterized by a slightly positive coordinate on the axis). Dimension 2 contrasts the drought stress treatment (at the top of the graph, characterized by a positive coordinate on the axis) and salt stress treatment (in the center-left of the graph, characterized by a slightly positive coordinate on the axis), with the control treatment (at the bottom of the graph, characterized by a slightly negative coordinate on the axis).

Regarding the representation of the variables on the factorial plane (Figure 24, C), dimension 1 faces two groups of strongly contributing variables. The variables MDA, proline, TPC, CAT, APX, DHAR and GR are characterized by a strongly negative coordinate on the axis. These variables all belong to the group of stress indicators. The variables P.S, Mg.S, S.S, Ca.S, K.S belonging to the nutrient uptake group are characterized by a strongly positive coordinate on the axis. Dimension 2 contrasts two groups of strongly contributing variables. The variables Zn.S, Ni.S, Zn.R, Cd.S and carotenoids are characterized by a strongly positive coordinate on the axis and, on the other hand, the variables Area.R, Length.R, ChlA and ChlB are characterized by a strongly negative coordinate on the axis. With the exception of carotenoids, which belongs to the stress indicators group, all variables belong to the growth indicators and metal uptake groups.

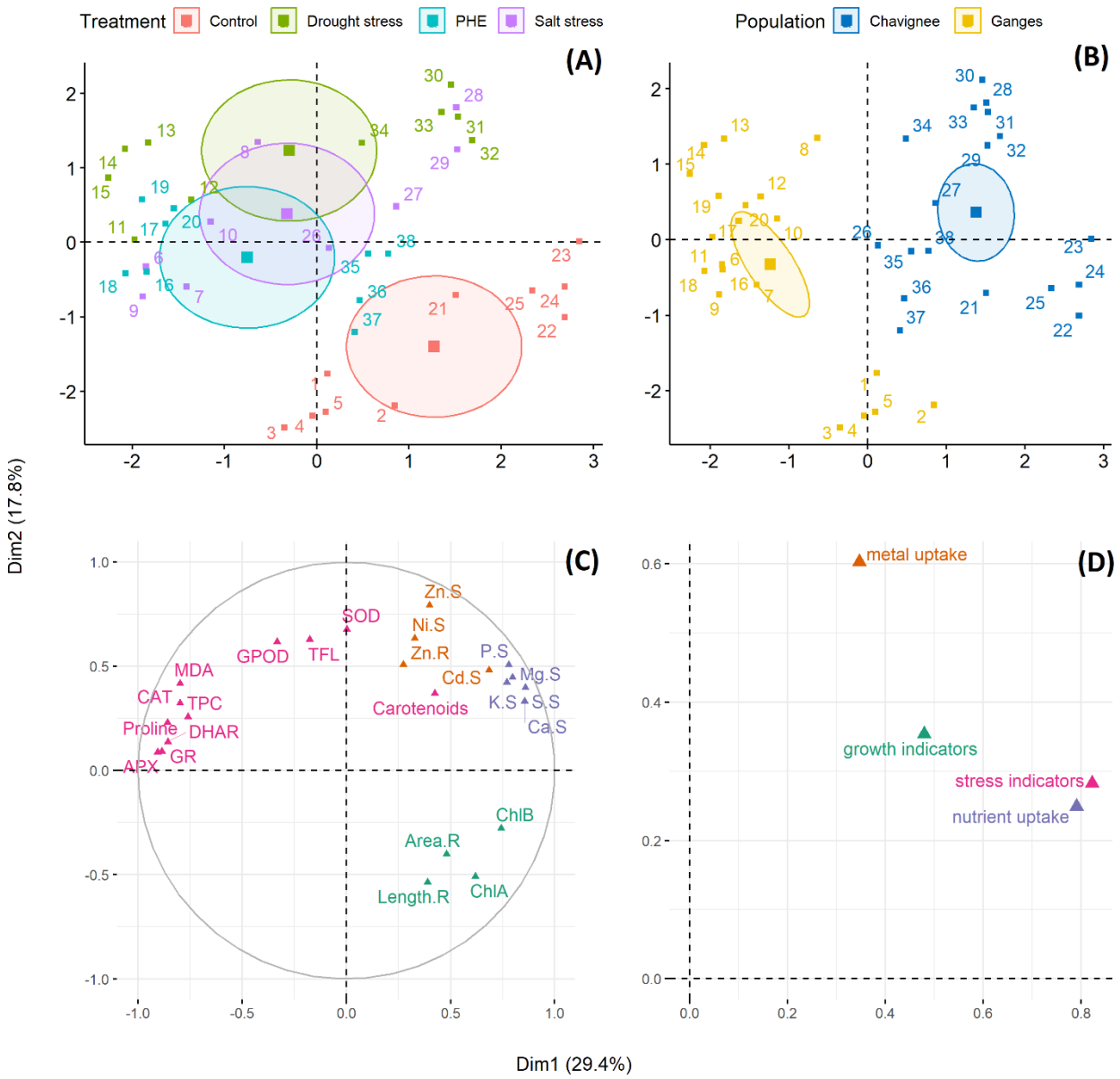

Figure 24. Multiple factor analysis (MFA) of individuals and variables. Labeled individuals are those with the greater contribution to the construction of the plane. Individuals are colored according to their membership in modalities of the variables "Treatment" (A) and "Population" (B). The labeled variables are those that are best represented on the map. The variables are colored according to their membership in the variable groups. The labeled individuals are those best represented on the map (C). The numbers (in %) next to each axis represent the variance explained by the respective principal components.

4 Discussion

This research delves into the investigation of the antioxidative response of two populations of *N. caerulescens* (Ganges and Chavignée) when exposed to various abiotic stresses. The main focus of the study is to analyze aspects such as growth parameters, stress biomarkers, antioxidant enzymes activities in addition to the phytoextraction process.

Drought and salinity had the ability to reduce plants growth belonging to both tested population (However, the Na/K ratio was lower in the roots of the Chavignée when compared to that of Ganges's.

4.1 Root architecture and leaf pigments

Overall, in

Table 4, all stresses affected root architecture parameters in the studied populations, with a decrease in the TRL and TRS. The effect was more pronounced in Ganges plants mainly under drought and salt stress. Nevertheless, MRD increased significantly in the Ganges roots under salinity from 0.43 to 0.57 mm. Chlorophyll A and B decreased by the stress effect in all treatments. Carotenoids increased in Chavignée only, while remained constant in the Ganges plants.

Table 3). These findings align with earlier studies on the phytotoxic effects of drought and salinity (Ibrahim et al., 2019b; Yaojun Zhang et al., 2020). Concentrations of K, P and S nutrients decreased under all stresses except under drought conditions, where the concentrations increased in both plants However, the Na/K ratio was lower in the roots of the Chavignée when compared to that of Ganges's.

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Table 3). Likewise, other studies have reported extensively the reducing effect of drought, salt and PAH stresses on nutrients uptake (Molina Lázaro and Segura Ana, 2021; Ors et al., 2021; Sahin et al., 2018).

In plants dealing with drought, it seems to be more important to accumulate K than producing organic solutes during the initial phases. This is because the osmotic adjustment achieved through the ions uptake, especially potassium, seems to be more energy efficient process (Hu and Schmidhalter, 2005). Potassium plays also a crucial role in enhancing the plant's ability to resist drought conditions by several functions including the regulation of stomatal conductivity, osmoregulation, homeostasis, and sustaining turgor pressure (Hasanuzzaman et al., 2018). However, salt stress caused the overaccumulation of Na in both populations while decreasing the concentration of K (However, the Na/K ratio was lower in the roots of the Chavignée when compared to that of Ganges's.

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Table 3), leading to the imbalance of Na/K ratio in plant cells (Angon et al., 2022). Both populations accumulated more Na in their aerial parts under salts stress, with more pronounced effect in Chavignée plants. Regarding K, Chavignée plants translocated more K from their roots to the aerial parts to mitigate the impact of high Na concentration, in contrast to Ganges plants. This suggests that the two tested populations were coping with salt stress in different ways. The Ganges seemed to exclude Na from its shoot, whereas the Chavignée maintained the Na/K ratio through higher K. In some salt tolerant plant species, Na is re-translocated to the root system after reaching the leaves through the shoot-exclusion mechanism by a specific Na transporter found on the plasma membrane of the root cells around xylem vessels. This serves in the protection of the leaves blades from Na, at which most of the metabolic processes occur (Chen et al., 2018; Malakar and Chattopadhyay, 2021; Munns et al., 2012).

Photosynthetic leaf pigments play a crucial role in capturing light, and thus in plant growth and photosynthesis. Under environmental stresses, chlorophyll degradation and pigments photooxidation are considered as symptoms of oxidative stress (Ma et al., 2020). In this study, chlorophyll A and B concentrations were reduced in both populations when exposed to drought, salinity, and PHE stresses (

Table 4). (Sarker and Oba, 2018)reported decline in the chlorophyll content in *A. tricolor* under drought. (Ranjbar, 2016) reported that salinity stress decreased leaf pigments especially chlorophyll in *Brassica napus L.* Drought and salinity causes the reduction of chlorophyll through various mechanisms, including excessive swelling of chloroplasts, loss of chloroplast membranes, formation of intracellular lipid droplets, and distortion of lamellae vesiculation (Ma et al., 2020). Also, phenanthrene was proved to trigger the degradation of chlorophyll in leaves through enhancing the production of enzymes that catalyze the phytol group removal from chlorophyll A, such as chlorophyllases (Shen et al., 2017; Willows et al., 2023).

In the current study, all stresses had a negative impact on TRL and TRS of the Ganges and Chavignée populations. The reduction was more marked in the Ganges plants especially in the cases of drought and salt stresses under which the MRD increased significantly (

Table 4). It is well documented that many plants develop deeper and longer root systems to cope drought stress and try to search for water (Brunner et al., 2015). The variation of root growth reduction under drought in both populations suggests that the effect of drought can depend on the species in the case of *N. caerulescens*. Regarding salinity, higher levels of stress generally lead to a decline in root system development (Robin et al., 2016). Furthermore, the increase of root diameter in the Ganges population under salt stress in this study may be attributed to the adaptation mechanism undergone by this population. The observed modification of root architecture in response to salt stress may contribute to salinity adaptation mechanisms (Rewald et al., 2012), involving the preference of thicker roots, which can serve as an effective physical barrier to reduce salt uptake and water loss in the stele (Tan et al., 2020). This may be explained also by a less Na accumulation in Ganges plants in comparison to Chavignée under salt stress. In the other hand, PHE have been shown to inhibit the root elongation and development of *N. caerulescens* and prevent the root hair elongation of the rhizodermal cells (Zelko et al., 2017).

Metal concentrations measured in shoots and roots of both populations of *N. caerulescens* showed that drought increased metals concentrations in the shoots while PHE and salinity decreased them (Figure 20). Metallophytes can cope with water deficit environments by their capacity to accumulate high quantities of toxic ions (Labudda et al., 2022). The preference of accumulating metallic ions within the epidermal leaf cells enables the reduction in cuticular transpiration, thereby achieving a lower water loss in the plant (Bhatia et al., 2005). Another postulated explanation is that the accumulation of metals, especially in the context of hyperaccumulation, serves as an osmolyte to overcome the limitations imposed by water

scarcity (Rascio and Navari-Izzo, 2011). Supporting evidence for the osmoregulatory role of metals can be found in a study conducted by (Bhatia et al., 2005), where they proved that declined soil moisture levels led to a significant increase in the content of Ni in the aerial parts of the Ni hyperaccumulator *Stackhousia tryonii*. In the Ganges plants, root to shoot translocation factors of Cd and Zn decreased under saline conditions (Figure 21). (Cheng et al., 2019)) reported that in some halophytes, the addition of NaCl resulted in a decrease in shoot Cd accumulation through reducing the root to shoot translocation of Cd. This was attributed to the rapid formation of Cd–S complexes, which have low mobility, within the root. However, Chavignée plants under salt stress were more likely to transfer greater amounts of Cd to the shoot (Figure 21). This may be due to the modification of NaCl to the subcellular distribution of Cd in leaves, which might be attributed to an increased concentration of polygalacturonic acid groups in the cell wall, and consequently enhancing the Cd binding capacity of the cell wall (S. Zhang et al., 2020). Beside that, the application of PHE enhanced the absorption of Cd through the roots while reducing the efficiency of phytoextraction in both populations of *N. caerulescens* (Figure 21). (Lin et al., 2008) found similar results, the presence of PYR in copper-contaminated soil reduced the ability of *Zea mays* (maize) to extract Cu through phytoextraction. Likewise, (Zhang et al., 2009) observed that Cd phytoextraction was hindered in soil contaminated with multiple pollutants. Conversely, (Sun et al., 2013) showed that *Juncus subsecundus* exhibited greater accumulation of Cd in its shoots compared to its roots; phytoextraction potential is enhanced in plants by aboveground accumulation. These findings imply that the interactions between metal and organic pollutants can cause either antagonistic or synergistic effects on the bioaccumulation patterns, influenced by various external and internal factors such as type of plant, pollutant concentration and characteristics, and soil conditions (Jeelani et al., 2018, 2017).

Membrane lipid peroxidation is a biomarker of cellular damage commonly expressed as MDA content. Our findings revealed that the exposure of plants to the stresses significantly increased the MDA content in both populations. However, the increase was more intense in the Ganges plants under drought and PHE (Figure 22). It has been widely reported that the MDA contents increased under individual drought and saline conditions (Angon et al., 2022). Additionally, MDA accumulation in plants treated with PHE has been previously reported in *Arabidopsis thaliana* (Liu et al., 2009b), *Triticum aestivum L*. and *Medicago sativa* L. (Salehi-Lisar and Deljoo, 2015). In this study, the production of MDA was lower in Chavignée compared to the Ganges for drought and PHE, which indicated that the Chavignée demonstrated more efficient ROS scavenging system. Indeed, previous research has demonstrated that the MDA content can increase in *N. caerulescens* when exposed to Cd, despite Cd not being typically considered a stressor for this particular hyperaccumulating plant (Wang et al., 2008).

Plants have developed various biochemical mechanisms to defend the detrimental effects of environmental stresses. Among these mechanisms, phenolic compounds play a vital role as they are the most abundant secondary metabolites in plants and serve as important antioxidants that help neutralize the excessive amounts of ROS caused by the oxidative stress. Flavonoids, which are a subgroup of phenolic compounds, are also well-known for their antioxidant properties (Tohidi et al., 2017). In this study, both *N. caerulescens* populations showed a notable increase in the levels of total phenolic and flavonoid compounds in response to all stresses. This agrees with previous observations who reported that exposure of plants to salt (Kiani et al., 2021), drought (Sarker and Oba, 2018) or PHE (Tarigholizadeh et al., 2021), remarkably increases the total phenolic and flavonoids compounds.

This experiment showed that all stresses caused proline to increase in both tested populations of *N. caerulescens* (Figure 22). Proline is an osmolyte whose accumulation has been witnessed as an indicator of stress tolerance in plants exposed to abiotic stressors such as drought, salinity

(Giordano et al., 2021) and PAHs (Song et al., 2022). It possesses an antioxidant activity and plays a crucial role in activating detoxification systems. Additionally, it protects the redox balance, acts as a protein precursor and energy source (Mansour and Ali, 2017). The high amounts of produced proline in the Ganges plants compared to the Chavignée ones (Figure 22), suggests that the former is more affected by the stresses. Nonetheless, some authors argued that the accumulation of proline in rice under saline conditions was a salt injury symptom ensuing from enhanced activity of the enzyme ornithine δ-aminotransferase and glutamate. According to this opinion, proline accumulation is seen as a consequence of stress induced metabolic disturbances rather than a beneficial adaptive response (Cai and Gao, 2020).

Plants contain antioxidant enzymes that protect them from the harmful effects of oxidative stress caused by abiotic and biotic stresses (Rajput et al., 2021). The enzymatic activities of SOD, CAT and GPOD increased significantly in both the Ganges and Chavignée under salinity, drought, and PHE stresses (Figure 23). SOD catalyzes the conversion of O_2^- into H_2O_2, which are subsequently eliminated by CAT, APX and GPOD (Elsayed et al., 2019). In the current study, there appears to be a linkage between SOD activity and the activities of CAT, and GPOD, as evidenced by the simultaneous increase in SOD activity associated with increases in CAT, and GPOD activities. Furthermore, under all stress conditions, the activities of antioxidant enzymes such as APX, DHAR and GR were significantly higher than control in the Ganges, although having unchanged activities in Chavignée plants (Fig. 19). (Devi et al., 2012) noted that some drought tolerance characteristics in *Triticum aestivum L.* can be linked to low APX and GR in the shoot under stress. By combining antioxidant enzymes observations, it can be suggested that the Chavignée plants exhibited more stress tolerance characteristics than the Ganges ones. Moreover, both the treatment and population effects were significant in this study. An obvious effect was obtained for the stresses treatments over the control one, with a similar stress effect, in addition to the effect of the Ganges population over the Chavignée one (Figure

24). The observed variations in response between the two populations may be attributed to the adaptations acquired in the respective soils from which each population originated. These adaptations may include specific detoxification strategies for pollutants and regulations of the antioxidative system. A similar pattern was observed with (Visioli et al., 2010) where an increase in the production of antioxidant compounds such as Glutathione S-transferase (GST), thioredoxin, and cytochrome P450 was observed at 10 µM Ni in *Thlaspi caerulescens* plants originating from a metalliferous soil. In contrast, the levels of these compounds in the non-metalliferous population did not exhibit significant changes across the different treatments.

5 Conclusions

In summary, when subjected to drought, salinity, and PHE stresses, it was observed that harmful reactive oxygen species (ROS) were produced, leading to oxidative stress in *N. caerulescens*. This oxidative stress had a negative impact on phytoextraction efficiency and ultimately resulted in reduced biomass. The Ganges and Chavignée populations responded to stresses in distinct mechanisms. However, the differences in response and growth between the two populations may be inherent and influenced by factors such as the soil type (metalliferous or non-metalliferous) and the habitat from which each population originated.

Chapter IV

Application of indole-3-acetic acid and 24-epibrassinolide may enhance the growth and phytoextraction efficiency of *Noccaea caerulescens*

This chapter examines the antioxidant activities and phytoextraction potential of the Ganges and Chavignée populations of *N. caerulescens* in environments contaminated with heavy metals and treated with IAA, EBR, or a mixture of phytohormones. It delves into the concentrations and translocation trends of heavy metals within these populations. The chapter also underscores the stress markers and the roles of antioxidative enzymes in the plants under these stress conditions.

Abstract

The study explored the use of external plant growth regulators to enhance the process of extracting metals from polluted soils by plants. The research specifically focused on understanding how the Ganges and Chavignée populations of *Noccaea caerulescens*, a known metal hyperaccumulator, vary in their ability to extract Cd and Zn. The study examined metal accumulation, plant growth, and antioxidant response. The *N. caerulescens* plants, cultivated in a hydroponic solution with moderate levels of Cd and Zn, received a foliar application of 10^{-8} M indole-3-acetic acid, 10^{-9} M 24-epibrassinolide, or a combination of both hormones. The findings indicated that every hormonal intervention led to a more pronounced accumulation of Cd and Zn in the Ganges population compared to Chavignée. Furthermore, while all treatments amplified antioxidative activities in the Ganges plants, only EBR and the hormone mixture treatment showed similar effects in Chavignée. The data points towards the potential efficacy of hormonal treatments in boosting the metal extraction capabilities of the Ganges variety, with the combined hormone treatment appearing to be the most potent.

1 Introduction

The rapid development of industry has accelerated the contamination of soil with heavy metals. Due to the non-biodegradable nature of their ions and long biological half-lives, heavy metals in polluted soils have emerged as a critical environmental issue on a global scale (Ali et al., 2013b; Rajewska et al., 2016). The accumulation of heavy metals in soils is closely attributed to many factors including treatment of sewage sludge, waste disposal activities and excessive use of agricultural fertilizers and pesticides (Alengebawy et al., 2021; Alsafran et al., 2022). Among heavy metals, cadmium (Cd) is commonly a non-essential toxic heavy metal that has the ability to accumulate in plants resulting in the deterioration of soil function and inhibition of plant growth (Chen et al., 2019; Sharma and Archana, 2016). Zinc (Zn) and nickel (Ni), on the other hand, are essential nutrients for plants, however their excessive accumulation in plants can be toxic to them (Kumar et al., 2022; Okereafor et al., 2020).

Hence, physical, chemical, and biological strategies have been implemented to remediate contaminated soils (Kabata-Pendias, 2010). Among these approaches, phytoextraction of heavy metals has gained popularity as a cost-effective and environmentally friendly remediation method (Suman et al., 2018). This technique employs hyperaccumulator plants that are able to extract high levels of heavy metals from the soil and accumulate them in various harvestable components, consequently decreasing their concentrations in the soil (Siyar et al., 2022). Nevertheless, the limited removal efficiency and biomass production of those plants are basic obstacles for phytoextraction. Actually, biomass production and bioaccumulation ability of the hyperaccumulator plants being employed, soil characteristics, time needed for maximal biomass development and other factors completely determine how effective phytoextraction is in field settings (Yu et al., 2020).

The application of phytohormones or plant growth regulators (PGRs) is an alternative approach that has been proposed to improve the effectiveness of plants' ability to phytoextract metals. Exogenous PGRs have a range of mechanisms of action and have various effects on plants. There have been reports of PGRs' involvement in phytoextraction in a variety of plants, including indole-3-acetic acid (IAA) and 24-epibrassinolide (EBR) (Bulak et al., 2014; Chen et al., 2022; Siddiqui et al., 2023). The interaction among various hormones plays a crucial role in their functions within plants. Brassinosteroids have the ability to regulate the growth and development of plants; they influence the movement of auxins and modify their functions (Bajguz and Hayat, 2009). Several physiological investigations have demonstrated that their combined usage with auxins produces more favorable outcomes compared to their individual use. Significantly positive synergism between brassinosteroids and auxins were reported in experiments involving *Berberis thunbergii L.* (Pacholczak et al., 2021) and *Arabidopsis* (Ahammed et al., 2015).

Several PGRs have also been shown to serve in scavenging free radicals, controlling the antioxidant defense system, and thus protecting the plant from oxidative stress (He et al., 2017; Verma et al., 2012). Enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and dehydroascorbate reductase (DHAR) in addition to compounds as proline and flavonoids are antioxidants linked to reactive oxygen species (ROS) detoxification in plants. These are produced to defend against oxidative stress (Moursi et al., 2021; Shahzad et al., 2018). For instance, in the case of zinc-stressed radish, (Ramakrishna and Rao, 2012) showed that exogenous administration of EBR decreased ROS production, lipid peroxidation, and carbonyl levels to alleviate oxidative stress.

Noccaea caerulescens (Brassicaceae) stands out as a prominent species used to explore the physiological and molecular dimensions of hyperaccumulator plants. While it is able to accumulate Cd, Zn, and Ni, the capacity to accumulate each metal varies among different

populations (Gonneau et al., 2017b). The aim of this study was to assess how the foliar application of EBR and IAA, impact the phytoextraction efficiency and antioxidative response of two populations of the hyperaccumulator *N. caerulescens*. These are two edaphic types: non-metalliferous (Chavignée population) and metalliferous (Ganges population) habitat. The obtained results should provide a comparative investigation of how phytohormones affect the ability of hyperaccumulator plants to extract metals and defend themselves against them.

2 Materials and Methods

2.1 Plant cultivation

Seeds of *N. caerulescens* (J. & C. Presl) belonging to two different populations were collected, the metallicolous population originated from a mining site in Ganges (Occitanie, France) and the non-metallicolous found in Chavignée (Bourgogne-Franche-Comté, France) (Gonneau et al., 2014). The germination of seeds was done for a period of 14 days in a climatic growth chamber with a photoperiod of 16 h and a temperature of 23 °C/18 °C day/night, 70% humidity and a photon flux density of 196 µmol photons m^{-2} s^{-1}.

The pre-cultivation phase, lasting for 56 days, was divided into two equal successive periods. During the first period, seedlings from both populations were cultivated hydroponically and were randomly allocated to two polyethylene trays measuring 36 cm in length, 26 cm in width, and 10 cm in depth. These trays were filled with a nutritive solution of 10 L. In the second period, the seedlings were transferred into another two trays of 21 L volume. This change was implemented to prevent mechanical stress on the roots. Each tray's cover plate accommodated 24 plants, each placed in holes that contained a growing basket with a sponge serving as support for the seedling. The nutrient solution composition, following (Küpper et al., 2007), included the following elements dissolved in deionized water: 1 mM $Ca(NO_3)_2$, 1 mM KNO_3, 0.1 mM KH_2PO_4, 50 µM KCl, 0.5 mM $MgSO_4$, 20 µM NaFe (III) EDTA, 10 µM H_3BO_3, 1 µM $MnCl_2$,

10 µM $ZnSO_4$, 0.7 µM $NiSO_4$, and 0.2 µM $CuSO_4$, along with 0.2 µM Na_2MoO_4. The pH of the solution was adjusted to 5.5 using 2 mM 2-(Nmorpholino) ethanesulfonic acid (MES) buffer and 0.2 M KOH. Starting from day 21, cadmium was introduced to the nutrient solution as $Cd(NO_3)_2$ at a moderate concentration of 0.25 mM. To facilitate aeration of the roots, capillary tubes that bubble filtered air were submerged in the nutrient solution. The used nutrient solution was renewed on a weekly basis.

Hormonal solutions were prepared based on previous results, by dissolving high purity IAA and EBR solutes (Sigma Aldrich®, USA) in ethanol followed by the dilution with deionized water until reaching the desired concentrations of 10^{-9} M for EBR and 10^{-8} M for IAA. The final ethanol concentrations in the EBR and IAA solutions were 1:2,000,000 and 1:200,000 respectively. During the 21 days cultivation phase, plants were grown under same controlled conditions while being subjected to foliar hormonal applications. The plants of both tested populations, which were 56 days old, were evenly placed on 8 stainless steel trays. Each tray was filled with 8.3 L of nutrient solution having the same composition as the ones used in the initial pre-cultivation phase. The experiment encompassed 4 hormonal treatments: Ctrl: control, EBR, IAA and EBR+IAA (Mix). Each plant was foliary sprayed with 2 mL hormonal solutions once per week. Control plants were sprayed with ethanol-water solution with ethanol concentration equal to that in IAA solution (highest ethanol concentration).

For both the Ganges (Ga) and Chavignée (Ch) plant populations, each treatment was replicated 5 times, resulting in a total of 40 plants in the experiment.

2.2 Plant harvest

After subjecting the plants to hormonal applications for a period of 21 days, the roots and shoots were separated at the base of the shoot. Root architecture parameters such as total root length (TRL), total root surface (TRS) and mean root diameter (MRD) were measured using

WinRhizo® software (Regent Instruments Inc., Canada). Root dry weight was measured after oven drying at 70 °C for 48 hours. After measuring the weights of fresh shoots, they were frozen in liquid nitrogen and crushed. They were then divided into two portions: the first one was freeze-dried for dry weight measurements in addition to elemental and biochemical analysis. The second portion was stored at -80 °C for further enzymatic analysis.

2.3 Assessment of plant element content

Pre-cooled agate mortar was used to grind the oven dried roots and freeze-dried shoots into fine particles. Next, 50 mg of the plant material was digested in 8 mL of 65% nitric acid for 12 hours and 4 mL of 30% hydrogen peroxide for 3.5 hours at 95 °C using the DigiPREP® system (SCP Science, Baie-d'Urfé, QC, Canada). The extracts were then filtered at 0.45 µm and diluted to 25 mL with ultrapure water (18 MΩ). The validity of the results was checked by periodically adding a blank, a control material of *N. caerulescens* with a known composition (analyses made by INRAE-USRAVE, Villenave d'Ornon, France), and a certified solution (EU-H3, SCP Science, Courtaboeuf, France) to the batches to be analyzed.

2.4 Assessment of leaf pigments, metabolites, and stress biomarkers

Freeze dried samples (10 mg) were extracted with 80% (v/v) cold ethanol, and the resulting crude extract was centrifuged. The supernatant was collected and utilized to quantify photosynthetic pigments (chlorophyll A and B, and carotenoids), metabolites (total phenolic compounds, and total flavonoids) and stress biomarkers (proline, malondialdehyde (MDA)) using a microplate reader (Safas Xenius, Monaco) following the protocol of (López-Hidalgo et al., 2021). (mentioned in detail in chapter III)

2.5 Antioxidant enzymes assays

Crude protein extracts were prepared using the technique described by (Murshed et al., 2008). Aerial part samples, which were stored at –80 °C and weighed between 0.20 to 0.40 g, were mixed with 1 ml of a buffer solution consisting of 50 mM MES/KOH (pH 6.0), 40 mM KCl, 2 mM CaCl2, and 1 mM AsA. This mixture was homogenized, and the resulting extracts were then subjected to centrifugation at 4 °C for 15 min at 16000g. The supernatants obtained were directly used for the analysis of enzymatic activities. Protein content in the extracts was determined according to Bradford's method (Bradford, 1976b).

All enzyme activities were measured by kinetic reactions done in volume of 200 µL at 25 °C using a microplate reader (Safas Xenius, Monaco). APX, DHAR, and glutathione reductase (GR) activities were determined following the method described by (Murshed et al., 2008). SOD activity was measured using the method (Dhindsa et al., 1981). CAT activity was measured according to the method of (Aebi, 1984b). The activity was calculated by monitoring the decrease in the reaction rate at 240 nm. Finally, guaiacol peroxidase (GPOD) activity was measured following the method downscaled for the microplate and modified from that of (Castillo et al., 1984b). (mentioned in detail in chapter III)

2.6 Statistical analysis

Statistical analysis was conducted using R software version 4.3.1. To assess the impact of treatment, population, and their interaction, a two-way ANOVA test was done. Subsequently, Tukey's HSD test was employed through the "multcomp" package under conditions that assume data parametricity. To explore the connections among variables concerning: i) plant growth, ii) nutrient uptake, iii) metal uptake, and iv) stress response, a multiple-factor analysis (MFA) was performed utilizing the "FactomineR" package.

3 Results

3.1 Growth indicators: nutritive status and plant development

Overall, the two populations showed notable differences in plant growth markers and mineral levels in both shoot and root (Table 5). Specifically, mineral levels in the shoots of Chavignée plants were significantly higher by 2 to 4 times than in the Ganges. Indeed, there was a trend of increased root and shoot biomass in both the Ganges and Chavignée groups after hormonal mixture treatment, even not significant. Regarding the minerals contents, there was a rise in potassium (K) levels in the shoots and roots of the Ganges population in all hormonal treatments. However, this increase was only observed after the hormonal mixture application in the shoots and roots of the Chavignée plants. In addition, the influence of IAA led to an increase in sulfur (S) and phosphorous (P) levels in the roots for both populations. After hormonal mixture application, sodium (Na) levels went down in the shoots and roots of the two groups.

Table 5. Growth parameters and nutrient content of shoots and roots of two *N. caerulescens* populations in response to IAA, EBR and hormones mixture foliar application. At the organ level, different letters indicate significant differences ($p < 0.05$)

Organ	Population	Treatment	Dry weight (g)		K (g/kg)		P (g/kg)		S (g/kg)		Ca (g/kg)		Na (mg/kg)	
Shoot	Chavignée	H2O	5.37	a	113.6	a	6.09	a	11.9	a	45.7	a	106.7	ab
		IAA	5.63	a	105.5	a	5.47	a	12.0	a	40.3	ab	182.8	a
		EBR	5.87	a	106.1	a	5.61	a	10.6	a	35.5	b	19.4	bc
		MIX	6.23	a	116.4	a	6.38	a	12.2	a	38.8	ab	42.3	bc
	Ganges	H2O	5.69	a	36.9	b	2.23	b	6.21	b	9.57	c	0.82	c
		IAA	5.58	a	42.8	b	2.23	b	5.19	b	13.8	c	4.9	c
		EBR	4.55	a	44.9	b	2.60	b	6.46	b	15.7	c	65.9	bc
		MIX	8.23	a	43.8	b	1.81	b	4.84	b	8.51	c	0.69	c
	Chavignée		5.77	a	110.4	a	5.89	a	11.7	a	40.1	a	87.8	a
	Ganges		5.97	a	41.9	b	2.22	b	5.68	b	11.9	b	16.4	b
		H2O	5.53	a	75.2	a	4.16	a	9.04	a	27.7	a	53.8	a
		IAA	5.60	a	74.1	a	3.85	a	8.61	a	27.1	ab	93.8	b
		EBR	5.28	a	78.9	a	4.27	a	8.76	a	26.7	b	40.0	c
		MIX	7.12	a	84.2	a	4.35	a	8.90	a	25.3	b	23.8	ac
Root	Chavignée	H2O	0.66	a	12.1	a	2.57	ab	7.13	a	13.4	a	428.6	ab
		IAA	0.97	a	14.5	a	2.84	ab	8.56	ab	13.1	a	176.7	ab
		EBR	0.75	a	14.1	a	3.13	ab	11.5	b	12.7	ab	354.7	ab
		MIX	1.05	a	20.7	ab	2.44	a	8.59	ab	11.9	ab	121.4	a
	Ganges	H2O	0.85	a	5.54	a	2.90	ab	8.18	ab	12.1	ab	267.5	ab
		IAA	1.01	a	37.3	b	3.86	ab	11.5	b	10.3	b	512.0	b
		EBR	0.67	a	6.90	a	4.83	b	8.27	ab	11.1	ab	430.2	ab
		MIX	1.47	a	24.5	ab	2.60	ab	9.06	ab	10.0	b	242.6	ab
	Chavignée		0.86	a	15.3	a	2.75	a	8.96	a	12.8	a	270.3	a
	Ganges		0.99	a	18.9	a	3.53	b	9.32	b	10.9	a	366.0	a
		H2O	0.76	a	8.82	a	2.74	a	7.66	a	12.7	a	348.1	a
		IAA	0.99	a	25.9	a	3.35	a	10.0	a	11.7	a	344.4	a
		EBR	0.72	a	10.9	a	3.89	a	10.1	b	12.0	a	388.2	a
		MIX	1.24	a	22.4	a	2.51	a	8.80	a	11.0	a	175.2	a

3.2 Root architecture and leaf pigments

Table 6 shows that the hormone mixture influenced the architectural characteristics of plant roots, leading to an increase in TRL and TRS for both populations. This impact was significantly greater in the Ganges plants. On the other hand, MRD increased substantially in Chavignée roots across all hormone treatments. However, only IAA increased MRD in Ganges

plants. Hormones also increased Chlorophyll A and B levels in each treatment. However, only Chavignée showed a significant increase in carotenoids.

Table 6. Root architecture parameters and leaf pigment concentrations of two *N. caerulescens* populations in response to IAA, EBR and hormone mixture foliar application. TRL: total root length (m), TRS: total root surface area (m2), MRD: mean root diameter (mm), ChLA: chlrophyll A concentration (µg/mg FW), ChLB: chlorophyll B concentration (µg/mg FW), Carotenoids concentration (µg/mg FW). Different letters indicate significant differences ($p < 0.05$).

Population	Treatment	Root Architecture						Leaf Pigments					
		TRL		TRS		MRD		ChLA		ChLB		Carotenoids	
Chavignée	H2O	10.0	a	1.39	ab	0.45	ab	0.86	ab	0.34	a	0.13	a
	IAA	9.30	a	1.54	ab	0.53	cd	0.98	bc	0.38	ab	0.20	bc
	EBR	7.92	a	1.36	ab	0.54	c	1.09	c	0.45	b	0.24	b
	MIX	11.9	ab	1.94	ab	0.51	acd	0.98	bc	0.45	b	0.21	bd
Ganges	H2O	12.4	ab	1.45	ab	0.43	b	0.79	a	0.33	a	0.13	a
	IAA	8.99	a	1.42	ab	0.51	acd	0.84	ab	0.38	ab	0.16	ac
	EBR	7.92	a	1.11	a	0.44	b	0.85	ab	0.38	ab	0.15	ac
	MIX	16.1	b	2.17	b	0.47	bd	0.83	ab	0.41	ab	0.18	acd
Chavignée		9.78	a	1.56	a	0.51	a	0.98	a	0.41	a	0.19	a
Ganges		11.3	b	1.53	a	0.46	a	0.83	b	0.37	b	0.15	b
	H2O	11.2	a	1.42	a	0.44	a	0.82	a	0.33	a	0.13	a
	IAA	9.14	a	1.48	a	0.52	b	0.91	ab	0.38	ab	0.18	b
	EBR	7.92	a	1.25	a	0.49	b	0.98	b	0.42	c	0.20	c
	MIX	13.8	a	2.04	a	0.49	b	0.92	ab	0.43	bc	0.19	bc

3.3 Trace element uptake

Figure 25 shows the concentrations of trace metals inside the shoots and roots of both populations of *N. caerulescens*. In shoots, IAA, EBR, and the mixture decreased the concentrations of Cd and Zn in Chavignée while increasing them in the Ganges population. Specifically, IAA and EBR increased the Zn concentration significantly inside the Ganges' s shoots by 2 folds. Additionally, in roots, application of IAA slightly increased Cd concentration in both populations. Besides, EBR increased the root's Zn concentration especially in Chavignée by 2 folds.

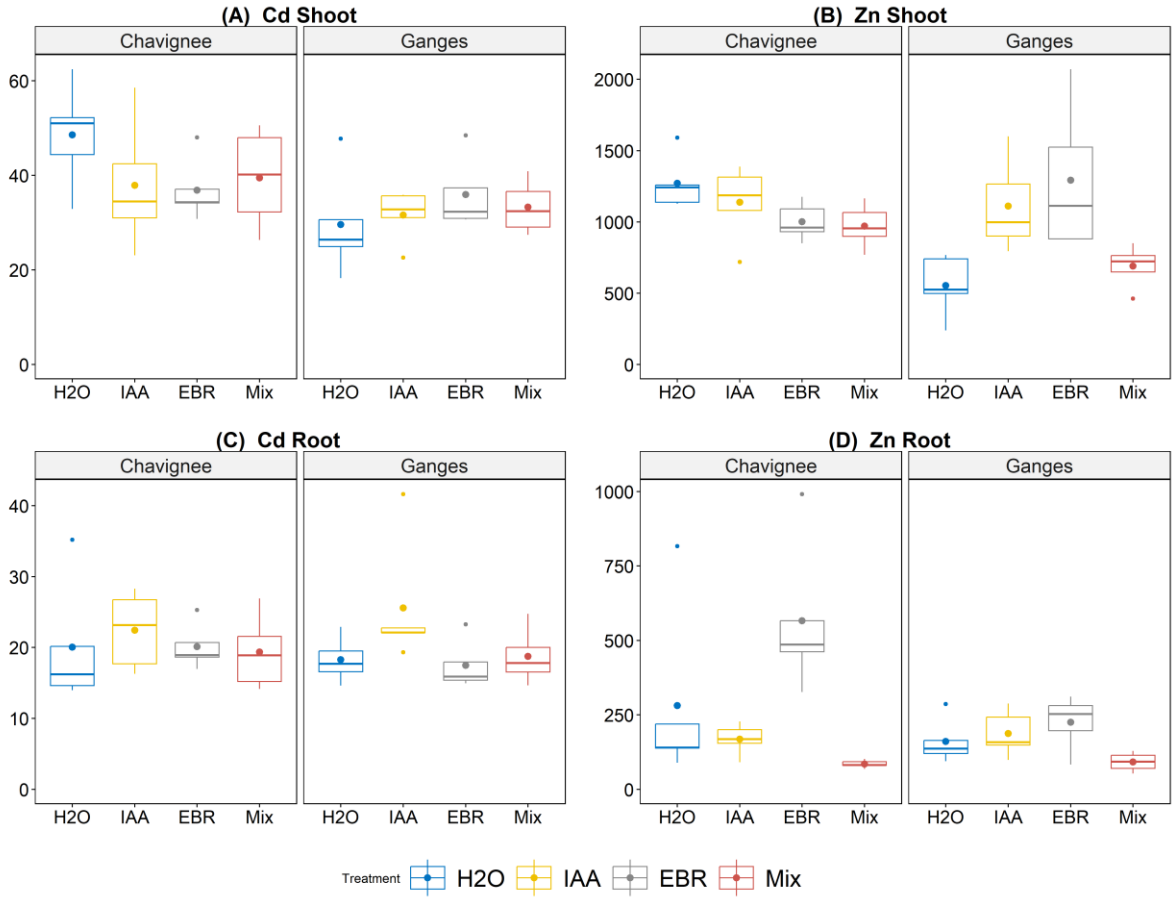

Figure 25. Concentrations (mg kg^{-1}) of Cd and Zn in shoots and roots of two *N. caerulescens* populations in response to IAA, EBR, and hormone mixture foliar application.

3.4 Stress indicators

In the control group, the Ganges plants displayed proline levels that were significantly two times higher than those in the Chavignée, as illustrated in Different letters indicate significant differences ($p < 0.05$) (Figure 26). The content of MDA in both populations was unaffected by hormonal treatments. However, EBR treatment notably raised the proline levels in both groups. Consistently, all hormone treatments significantly elevated the TPC content in both populations, with an increase ranging from 1.7 to 2.1 times, where the Ganges plants showed remarkably higher TPC content compared to Chavignée. As for the TFL content, Chavignée witnessed a significant rise when treated with IAA and EBR, whereas, in Ganges, such an increase was seen with all hormone treatments compared to the controls.

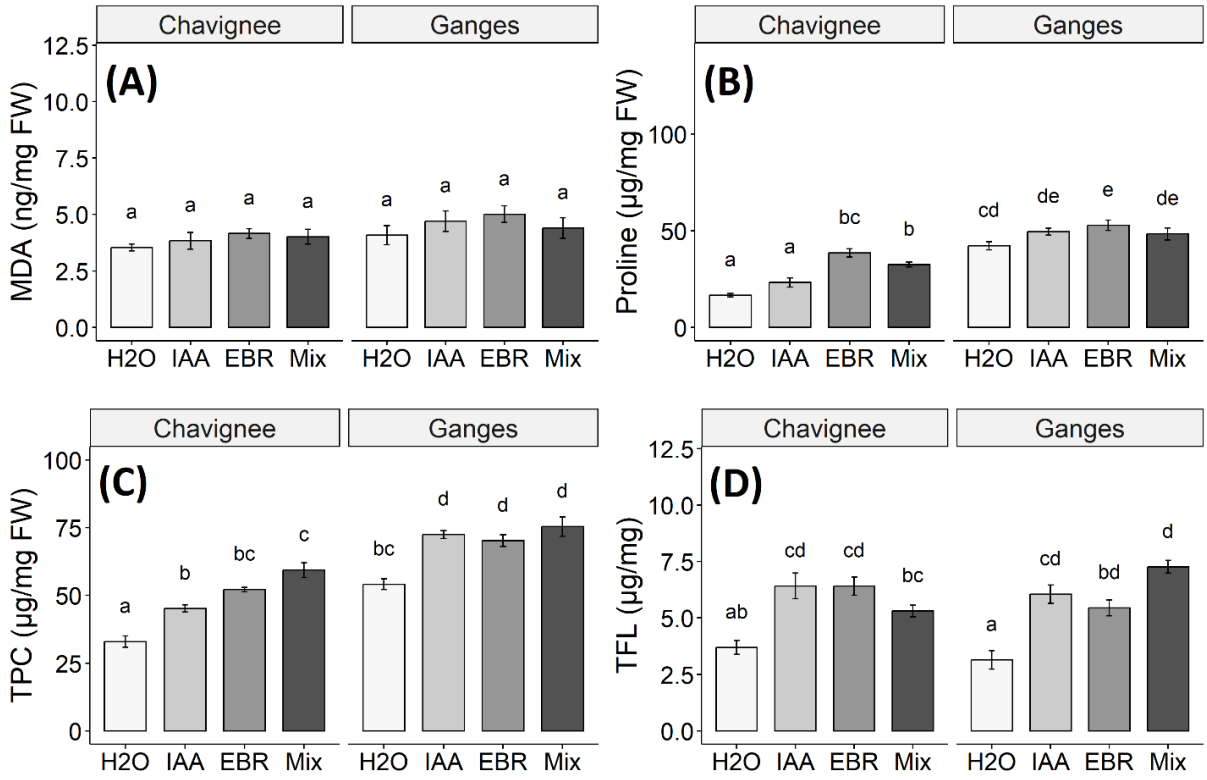

Figure 26. Stress biomarkers and metabolite content in shoots of two *N. caerulescens* populations in response to IAA, EBR, and hormone mixture foliar application. MDA: malondialdehyde, TPC: total phenolic compounds, TFL: total flavonoids. Different letters indicate significant differences ($p < 0.05$).

As shown in Figure 27, the levels of CAT, APX, and GR were significantly higher in the Ganges controls compared to Chavignée. When exposed to hormonal treatments in Ganges, there was an increase in the activities of CAT, APX, and GR, especially with EBR and the combined hormone treatments, with the latter notably increasing the CAT activity. On the other hand, in Chavignée, the activities of CAT, APX, and GPOD had a significant rise when treated with both EBR and the combined hormones. Specifically, both EBR and the hormone combination doubled the GPOD levels, and EBR alone elevated the CAT and APX activities by a factor of 1.5.

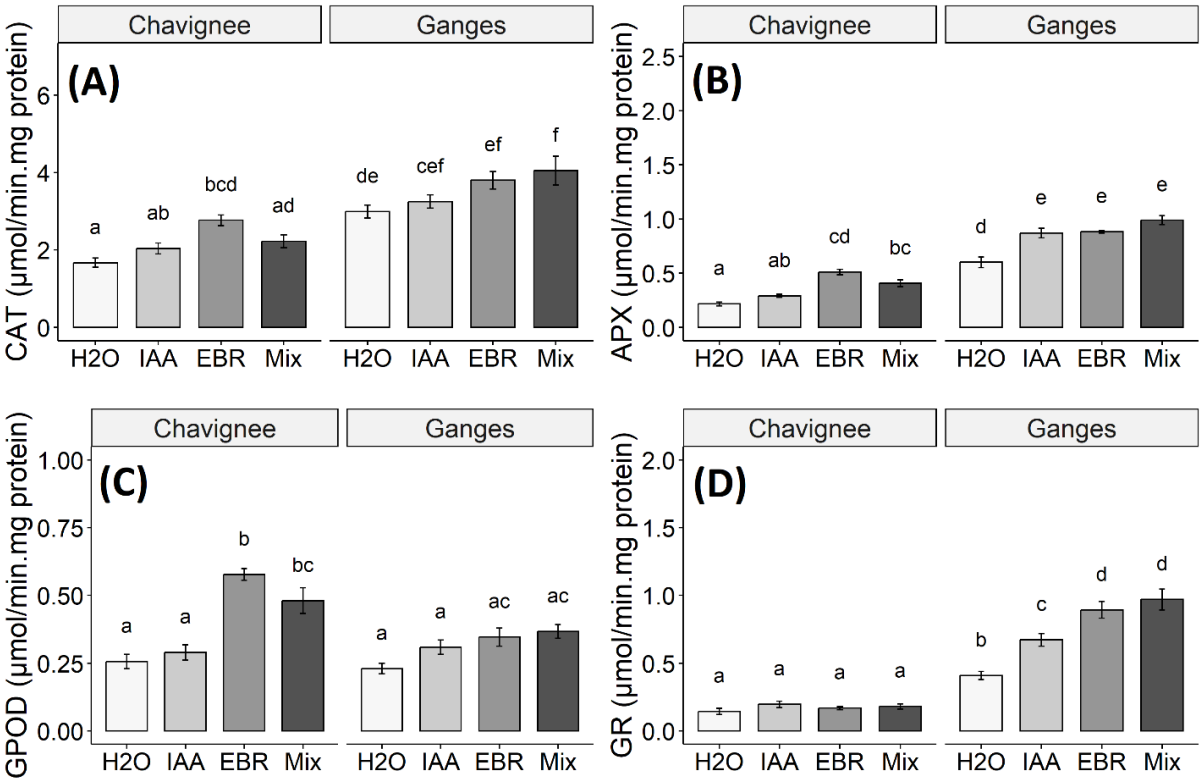

Figure 27. Activities of antioxidant enzymes in shoots of two *N. caerulescens* populations in response to IAA, EBR, and hormone mixture foliar application. CAT: catalase, APX: ascorbate peroxidase, GPOD: guaiacol peroxidase, GR: glutathione reductase. Different letters indicate significant differences ($p < 0.05$).

3.5 Multivariate analysis

A multivariate analysis was performed to assess the interconnections and associations among four categories of variables related to plant growth, nutrient absorption, metal assimilation, and response to stress (Figure 28). The initial two dimensions of this assessment represent 44.1% of the overall inertia observed in the dataset. This means that nearly half of the data spread is encompassed by these two dimensions. This is a noteworthy portion that surpasses the baseline value, emphasizing the statistical relevance of the variance elucidated by this study.

The plot of individual entities within the factorial layout reveals that dimension 1 contrasts the Ganges population (located on the graph's left side with a prominent negative value on the axis) with the Chavignée population (on the graph's right with a positive value on the axis) (Figure 28B). Dimension 2 differentiates between the EBR+IAA mixture treatment (positioned atop

the graph with a positive value on the axis) and the IAA and EBR treatments (located centrally on the graph with a marginally positive value on the axis) from the control treatment (found at the graph's base with a marginally negative value on the axis) (Figure 28A).

In terms of the placement of variables on the factorial plane (shown in Figure 28C), dimension 1 juxtaposes three sets of highly influential variables. The variables such as MDA, proline, TPC, CAT, APX, DHAR, and GR, all of which fall under stress markers, are defined by a notably negative value on the axis. Meanwhile, the variables P.S, Mg.S, S.S, Ca.S, K.S from the nutrient absorption category, and Cd.S from metal assimilation have a pronounced positive value on the axis. Dimension 2 highlights the plant growth indicators that have a significant contribution, namely DW.S, DW.R, Area.R, Length.R, ChlA, and ChlB, all possessing a pronounced positive value on the axis.

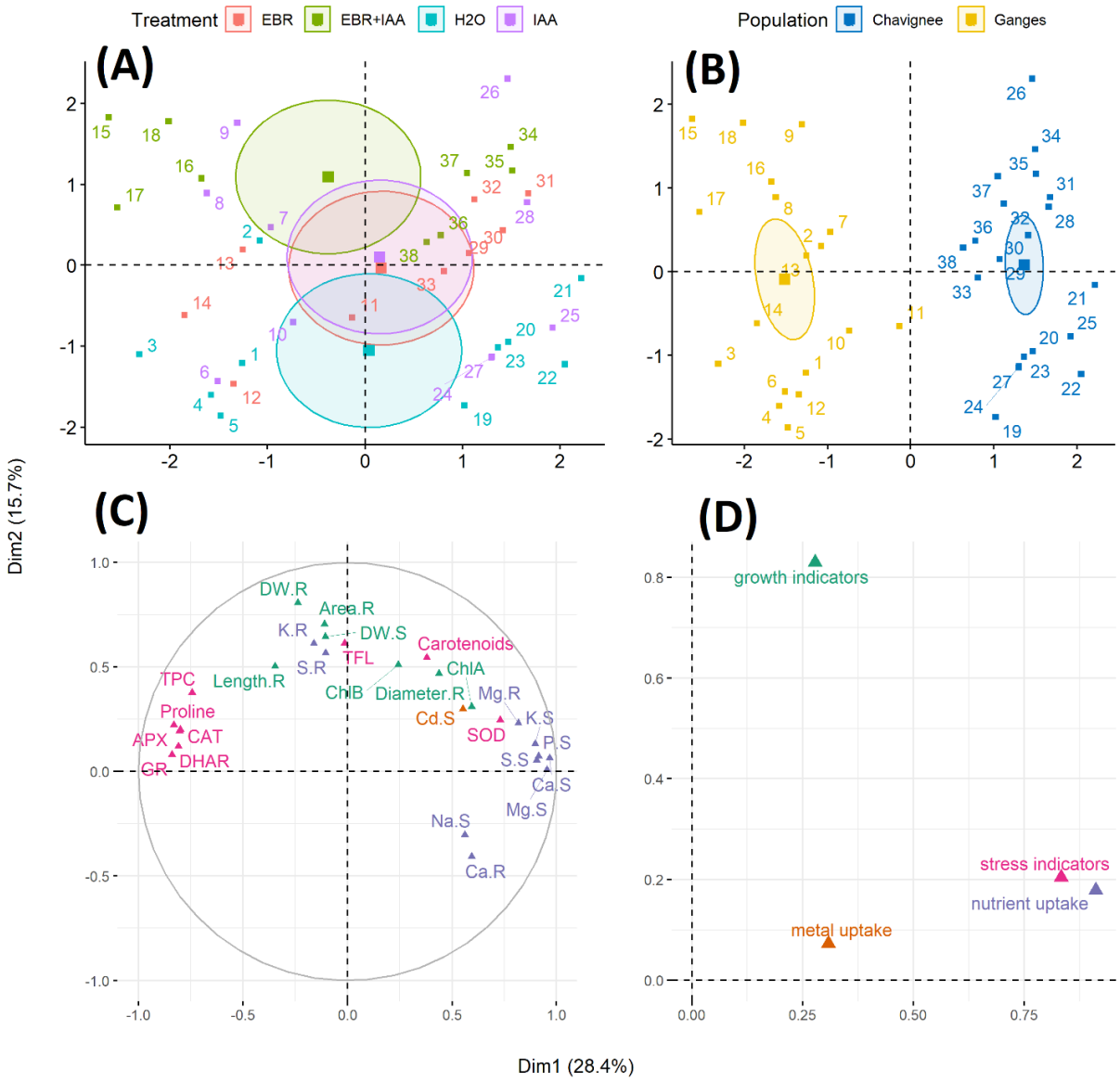

Figure 28 Multiple factor analysis (MFA) of individuals and variables. Labeled individuals are those with the greater contribution to the construction of the plane. Individuals are colored according to their membership in modalities of the variables "Treatment" (A) and "Population" (B). The labeled variables are those that are best represented on the map. The variables are colored according to their membership in the variable groups. The labeled individuals are those best represented on the map (C). The numbers (in %) next to each axis represent the variance explained by the respective principal components.

4 Discussion

This study explores the antioxidative response of two *N. caerulescens* populations (Ganges and Chavignée) exposed to foliar applications of IAA and EBR hormones. The primary aim is to examine factors like growth metrics, stress indicators, activities of antioxidant enzymes, and the phytoextraction mechanism.

All hormonal treatments could put up the root dry weights belonging to both tested populations (Table 5). However, shoot dry weight was enhanced by the hormonal mixture only in the two populations. These findings align with earlier studies on the effects of EBR and IAA on the plants' growth (Mir et al., 2020; K. Niu et al., 2023; Baolan Wang et al., 2015).

In recent research, a combination of hormones positively influenced the TRL and TRS in the Ganges and Chavignée groups (Table 6). Earlier research has indicated that brassinosteroids (BRs) and auxin work together in the development of lateral roots. BRs primarily operate at the start of the lateral root primordia (LRP) while auxin is essential for both the beginning and the emergence phases of forming lateral roots (Bao et al., 2004; Wei and Li, 2016). In these stages, BRs enhance LRP start by boosting the upward movement of auxin in the root, rather than by changing the internal IAA (Tian et al., 2018).

Metal concentrations indicated a decrease in shoots of the Chavignée population due to all hormonal treatments while increasing in shoots of Ganges plants (Figure 25). In *Picris divaricata*, 10 - 100 µM IAA increased the Pb content in leaves by about 28.4 to 37.3 %, but not in roots (Du et al., 2011). However, other previous studies have shown that plants exposed to IAA or EBR tend to have reduced accumulation and translocation of heavy metals (like Cd, Cu, Pb, and Zn) from shoots to roots. (Zhu et al., 2013) highlighted that in *Arabidopsis thaliana*, auxin helped counteract the effects of Cd^{2+} in shoots by promoting its retention in the roots, thereby limiting its movement to the shoots. Additionally, when Oryza sativa L. seedlings

were treated with EBR, there was a noticeable reduction in Cr^{2+} uptake (Sharma et al., 2016). While such effects might not be ideal for enhancing phytoextraction techniques, they could be valuable for the reestablishment or stabilization of polluted soils. In such contexts, the primary goal is to prevent metal transfer to plants, ensuring metals don't find their way into the food chain and broader environment (Barbafieri and Tassi, 2011). Our results suggest that in a metallic environment without any other stress, the hormonal application is beneficial in increasing the phytoextraction of metals specifically in the Ganges population.

Indeed, research has indicated that while Cd isn't typically a stress factor for the hyperaccumulating plant *N. caerulescens*, it does exhibit oxidative stress symptoms when exposed to it (Wang et al., 2008). Plants maintain ROS levels in their cells through an antioxidant system, which includes low molecular mass antioxidants like proline, flavonoids, phenols, carotenoids, and antioxidant enzymes (Kasote et al., 2015; Mehla et al., 2017). Regarding stress indicators, all hormonal treatments increased the TPC and TFL levels in both studied populations. Yet, only when EBR was applied did the proline level rise in Chavignée plants (In the control group, the Ganges plants displayed proline levels that were significantly two times higher than those in the Chavignée, as illustrated in Different letters indicate significant differences ($p < 0.05$) (Figure 26). The content of MDA in both populations was unaffected by hormonal treatments. However, EBR treatment notably raised the proline levels in both groups. Consistently, all hormone treatments significantly elevated the TPC content in both populations, with an increase ranging from 1.7 to 2.1 times, where the Ganges plants showed remarkably higher TPC content compared to Chavignée. As for the TFL content, Chavignée witnessed a significant rise when treated with IAA and EBR, whereas, in Ganges, such an increase was seen with all hormone treatments compared to the controls.

Phenolic substances, which act as non-enzymatic antioxidant agents, help counteract the adverse impacts of ROS and are adept at binding metals (Kruk et al., 2022; Mierziak et al.,

2014). Hence, given the application of EBR and the observed increase in TPC and TFL levels, it's likely to infer that this rise in antioxidant levels played a defensive role against heavy metal stress in *N. caerulescens*.

The key antioxidant enzymes in plants that help maintain redox balance include GPOD, CAT, APX, and GR (Rajput et al., 2021). Notably, APX and GR play a crucial role in the ascorbate-glutathione cycle, functioning within the photosynthetic machinery to neutralize H2O2 and safeguard chloroplasts during stress conditions, as indicated by (Singh et al., 2018). In this experiment, the application of IAA, EBR, and the mixture of both boosted the activities of CAT, APX, and GR enzymes in Ganges plants. On the other hand, only EBR and the combined treatment enhanced the activities of CAT, APX, and GPOD in Chavignée plants (Figure 27). Previous research by Agami and Mohamed (2013) highlighted IAA's potential to strengthen antioxidant defenses in wheat plants under Cd stress. Likewise, EBR is known to mitigate oxidative stress from metals, a trait observed in various plants like *Brassica juncea* (Siddiqui et al., 2018) and *Raphanus sativa* (Choudhary et al., 2012). This antioxidative property may be linked to the function of BR signaling kinase (BSK 1) in amplifying salicylic acid levels to combat oxidative harm (Deng et al., 2016). The data indicates that Chavignée plants might be less responsive to IAA concerning antioxidant enzyme generation. Moreover, in comparison with Chavignée, the Ganges plants exhibited higher GR activity, particularly when paired with APX. This points to a more efficient ascorbate-glutathione cycle in Ganges (Hasanuzzaman et al., 2019).

The distinct performance between Ganges and Chavignée plants (Figure 28, B), combined with Ganges plants showcasing more stress markers and decreased nutrient absorption (Figure 28, C), likely stems from their adaptation to their metalliferous native environment. Furthermore, the differences between the hormonal combination and control treatments, as shown in (Figure

28, A), imply that the combined hormonal treatment was the most influential on both *N. caerulescens* populations.

5 Conclusion

In this study involving *N. caerulescens* populations of Ganges and Chavignée grown under hormonal treatments, foliar application of IAA, EBR and the combination of both hormones proved more effective in promoting phytoextraction in the Ganges plants compared to the Chavignée plants. The Ganges plants displayed a stronger antioxidative reaction to the hormonal treatments. Every hormonal intervention amplified the activities of antioxidant enzymes in the Ganges plants, whereas the Chavignée plants showed a heightened antioxidative reaction primarily to EBR and the combined hormone treatment. Overall, the combined hormone treatment showcased a synergistic effect that was prominently observed in both plant populations.

Chapter V

Application of phytohormones may alleviate abiotic stresses and improve phytoextraction with *Noccaea caerulescens*

This chapter investigates the antioxidant mechanisms and phytoextraction capabilities of the Ganges and Chavignée populations of *N. caerulescens* in environments tainted with heavy metals and exposed to abiotic stresses such as drought, salinity, or PAH, while also being treated with IAA, EBR, or a combination of phytohormones. It offers a deep look into the levels and movement dynamics of heavy metals among these populations. Moreover, the chapter emphasizes the stress indicators and the functions of antioxidative enzymes in the plants in these challenging conditions.

This work was presented at the ICOBTE international conference in 2023.

Mohammad Chafik Sherri, Catherine Sirguey, Kassem Hamze, Ali Kanso, Stéphanie Ouvrard. Application of phytohormones may alleviate abiotic stresses and improve phytoextraction with *Noccaea caerulescens.* 16th International Conference of biochemistry of Trace Elements, Sep 2023, Wuppertal, Germany.

Abstract

Noccaea caerulescens represents one of the hyperaccumulator plants used in phytoextraction of trace metals from contaminated soils. However, the implementation of treatment processes based on their extraction capacities is challenged by different abiotic stresses present in multi-contaminated environments. This study aimed to explore the phytoextraction efficiency and antioxidant response of *N. caerulescens* when subjected to different abiotic stresses as well as the role of phytohormone application on the plant response. For that, plants from two populations of *N. caerulescens* originating from metallicolous (Ganges) and non-metallicolous (Chavignée) sites were cultivated in hydroponics. They were first exposed to moderate trace metal concentration for 8 weeks and then various stresses and hormone treatments were applied for 3 weeks: salinity (NaCl at 45 mM), drought (polyethylene glycol at 5%), and organic contaminants (phenanthrene at 200 µM), along with the exogenous application of the phytohormones 24-epibrassinolide (10^{-9} M) and indole-3-acetic acid (10^{-8} M). The different combinations of stresses and hormone application led to 12 treatments per population conducted in five replicates. Differences were observed in the activity levels of enzymes and the concentrations of extracted metals between the two populations. The response of both populations was both stress and hormone-dependent. Chavignée plants exhibited superior growth characteristics, whereas Ganges demonstrated enhanced antioxidative responses. For both populations, the combination of hormones was the most effective treatment across all stress conditions. There were variations in growth, metal extraction, and antioxidant defense responses between the two populations, indicating that their differing defense capacities might contribute to their tolerance levels. These variations could be attributed to adaptations acquired by each population based on the type of soil they originated from.

1 Introduction

In nature, plants continually face a myriad of environmental challenges that influence their growth, reproduction, overall yield, and longevity (Parmesan and Hanley, 2015). The intensification of industrial activities and shifts in climate patterns have heightened the severity of the abiotic stresses. Notably, stresses such as heavy metal contamination, soil salinity, drought, and persistent organic pollutants (*e.g.* polycyclic aromatic hydrocarbons (PAHs)) have been identified as significant factors reducing plants' productivity. Consequently, understanding how plants react to coexisting stresses like heavy metals in combination with other abiotic factors has become a matter of urgency (Mora et al., 2015; Mankin et al., 2019). These environmental stresses induce metabolic disruptions in plants, necessitating metabolic adjustments to maintain essential life processes.

Particularly, salt and drought stresses have been pinpointed as severe threats, known to curtail the commercial yield of many plants by causing biochemical and physiological disturbances (Morton et al., 2019; Ziogas et al., 2021). Such stresses restrict plant growth and yield due to factors like osmotic imbalance, nutrient disruptions, and heightened oxidative stress (Abrar et al., 2022). Specifically, salt stress leads to a surge in sodium (Na^+) and chloride (Cl^-) ion concentrations in the cellular environment, causing potential cellular harm (Balasubramaniam et al., 2023). On the other hand, drought leads to effects like stomatal closure, photosynthesis inhibition, diminished leaf span, stunted growth, reduced water retention, heightened osmolyte production, and oxidative stress characterized by elevated reactive oxygen species (ROS) generation (Seleiman et al., 2021). Moreover, PAHs, the toxic, long-lasting, and carcinogenic by-products of burning carbon-rich fuels, present another concern (Patel et al., 2020). Owing to their chemical composition, which includes non-functionalized benzene rings and a medium to high molecular weight, these compounds exhibit limited solubility (Abdel-Shafy and Mansour, 2016).

Plants primarily engage with PAHs through adsorption, either on the root system or within the initial cellular layers (Molina and Segura, 2021a). Past research indicated that PAHs can trigger oxidative stress in plants, hamper growth, and lead to issues like leaf malformation and tissue decay (Alegbeleye et al., 2017; Pašková et al., 2006b).

Soil contaminants, with a particular focus on heavy metals (HMs) and metalloids like Cr, Cd, Ni, Zn, As, and Hg, are among the most frequently observed pollutants (Rashid et al., 2023). The rising introduction of HMs into the land environment poses a significant threat to the output of farmlands (Simon, 2016). Over time, a variety of methods have been employed to remediate these polluted environments. Such methods span across biological, physical, and chemical domains (Kim et al., 2022). Among all, phytoremediation involves utilizing plants to cleanse polluted surroundings (Yan et al., 2020b). For addressing heavy metal contamination specifically, one of the subsets of phytoremediation is phytoextraction. This approach employs plants known as hyperaccumulators, which inherently possess the capability to absorb high quantities of metals from the soil and transport them from roots to shoots (Trakal et al., 2015). These plants are also uniquely equipped to endure and accumulate elevated metal concentrations by neutralizing the metals within their structures (Sheoran et al., 2011).

Amongst hyperaccumulating plants, *Noccaea caerulescens* (Brassicaceae) stands out as an exemplary species for probing the physical and molecular dynamics of such plants. This plant is known for its ability to hyperaccumulate metals like Cd, Zn, and Ni (Mandáková et al., 2015b). However, the efficiency with which it accumulates each metal varies depending on the plant population (Gonneau et al., 2017c). Nonetheless, the growth and proliferation of *N. caerulescens* can occasionally face obstacles, especially in soils that are heavily burdened by simultaneous contamination with multiple contaminants.

Abiotic stresses lead to oxidative stress in plants, marked by excessive production of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and others

(Sachdev et al., 2021). Excessive ROS can harm various plant components, including DNA, proteins, and lipids, leading to potential plant death (Choudhury et al., 2017). To counteract this damage, plants employ a combination of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and others, while non-enzymatic ones include proline and phenolic compounds. Together, they neutralize excess ROS, acting as a defense mechanism against oxidative stress (Dvořák et al., 2021; You and Chan, 2015).

To help plants cope with abiotic stresses, exogenous application of plant growth regulators (PGRs) or phytohormones seems beneficial (Bueno and Cordovilla, 2021). These external PGRs function in multiple ways, eliciting a variety of responses in plants (Sabagh et al., 2021). Some reports suggest that phytohormones like indole-3-acetic acid (IAA) and 24-epibrassinolide (EBR) have played a role in the phytoextraction process and enhanced the antioxidative defense in diverse plants when facing abiotic stresses (Alam et al., 2020; Vaz et al., 2023; Wani et al., 2016).

The interplay between different hormones is essential for their efficacy in plants (McAtee et al., 2013). Brassinosteroids, for instance, manage plant growth and development, impacting the distribution and functionality of auxins. Experimental research has shown that combining them with auxins often yields better results than using them separately (De Grauwe et al., 2005; Tian et al., 2018). Positive interactions between brassinosteroids and auxins were observed in studies on *Berberis thunbergii L.* (Pacholczak et al., 2021) and *Arabidopsis* (Ahammed et al., 2015).

Our research seeks to investigate the impact of the exogenous application of IAA and EBR phytohormones on the phytoextraction capability and antioxidative response of two populations of the hyperaccumulator *N. caerulescens* facing prevalent abiotic stresses found in polluted soils, specifically PAHs, salt, and drought stresses. These populations originate from two soil types: the non-metalliferous (Chavignée) and the metalliferous (Ganges)

environments. Through this study, we aim to provide a detailed and comparative insight into plant reactions to stress, highlighting both their capacity to remove environmental contaminants and their resilience strategies against abiotic pressures.

2 Materials and Methods

2.1 Growth conditions

Noccaea caerulescens (J. & C. Presl) seeds from two distinct sources were grown: one from a mining site in Ganges (Occitanie, France) known as the metallicolous population, and the other from Chavignée (Bourgogne-Franche-Comté, France) recognized as the non-metallicolous population (Gonneau et al., 2014). The seeds underwent a 14-day germination process in a controlled environment chamber set at a 16-hour photoperiod, temperatures of 23 °C/18 °C for day/night, 70% humidity, and a light intensity of 196 µmol photons m−2 s−1.

The subsequent pre-growth stage lasted 56 days and consisted of two phases. Initially, the sprouted seeds from both groups were grown hydroponically in two 36x26x10 cm polyethylene trays, each containing 10 liters of a nutrient solution. Later, they were transferred to two larger 21-liter trays. The cover of each tray had 24 openings, each housing a growing basket with a sponge for seedling support. The nutrient mixture, based on (Küpper et al., 2007), comprised various essential elements diluted in distilled water, including 1 mM $Ca(NO_3)_2$, and 1 mM KNO_3, among others. The mixture's pH was maintained at 5.5 using a 2 mM MES buffer and 0.2 M KOH. From the 21st day onwards, cadmium in the form of $Cd(NO_3)_2$ was added to the mixture at a concentration of 0.25 mM. For root aeration, tubes delivering filtered air bubbles were immersed in the solution. The nutrient mixture was refreshed weekly.

2.2 Stress culture and hormonal application

During the culture phase, plants were cultivated under controlled conditions, even when exposed to various stresses. Over 21 days, plants 56 days old plants were cultivated under stress conditions and received foliar hormonal treatments.

Plants were uniformly arranged across metal trays, with each tray holding 8.3 L of a nutrient mixture. The mixture in every tray was consistent with that used during the pre-growth phase, but each had an added stress-inducing agent based on the specific treatment. The study incorporated three distinct stress treatments:

i) Drought (PEG) - incremental addition of 5% polyethylene glycol (PEG 6000)
ii) Salinity (Sal) - incremental addition of 40 mM sodium chloride (NaCl).
iii) Phenanthrene (PHE) - addition of PHE (a representative PAH) at a concentration of 200 μM.

A day before the stress induction into the nutritive solutions, plants were sprayed with phytohormones which were formulated based on prior findings. High-grade IAA and EBR compounds (Sigma Aldrich®, USA) were initially dissolved in ethanol and subsequently diluted with distilled water to achieve the intended concentrations of 10^{-9} M for EBR and 10^{-8} M for IAA. The resultant ethanol ratios in the EBR and IAA solutions were 1:2,000,000 and 1:200,000, respectively. The study involved four hormonal treatments for each stress treatment: Ctrl (H2O), EBR, IAA, and a combination of EBR+IAA (Mix). Each plant received a 2 mL foliar spray of the hormonal mixtures weekly. For the control group, an ethanol-water mix was used, with the ethanol level matching that of the IAA solution (the solution with the highest ethanol concentration).

For both the Ganges (Ga) and Chavignée (Ch) plant groups, each treatment was replicated five times, leading to a total of 120 plants used in the experiment.

2.3 Physiological, morphological, and biochemical analysis

The protocols utilized for the analysis of plant material are the same as the ones used in the second and third articles.

3 Results

3.1 Growth and nutritive parameters

In Table 7, during drought stress, both Ganges and Chavignée populations exhibited increased biomass after being treated with EBR and the hormone mixture. However, in contrast to the Ganges population, under the same drought conditions, only Chavignée demonstrated a rise in the dry biomass of shoots upon exposure to IAA. Exposure to the hormonal mixture led to a rise in the shoot biomass for both populations during salt and PHE stresses.

Generally, mineral concentrations in Chavignée shoots were remarkably 2 to 4 times greater than in Ganges shoots. Notably, under drought conditions, the Ganges plants displayed an increase in potassium (K) concentrations when treated with EBR and the combined hormones. Furthermore, all hormonal treatments boosted the K concentration in Chavignée during PHE stress, yet only the combined hormones achieved this in the Ganges plants under the latter stress. In a similar line, every hormonal treatment decreased the sodium (Na) levels in Chavignée during PHE stress, but only the combined treatment accomplished this reduction in Ganges plants under the same stress.

Table 7. Growth parameters and nutrient content of shoots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses, with hormonal treatments including control (H_2O), IAA, EBR, and EBR+IAA (Mix). At the organ level, different letters indicate significant differences ($p < 0.05$)

Population	Stress	Hormone	Dry Weight (g)		K (g/kg)		Na (mg/kg)		Na/K (10^{-4})	
Ganges	Drought	H2O	3.59	ab	50.3	ab	18.8	a	3.84	a
		EBR	4.3	ab	91.6	bc	51.5	a	5.86	a
		IAA	3.11	ab	63.3	ab	173.2	a	16.6	a
		Mix	4.02	ab	93.5	b	129.7	a	13.9	a
	PHE	H2O	3.44	ab	34.8	a	21.7	a	6.28	a
		EBR	2.97	b	33.8	a	96.2	a	22.1	a
		IAA	2.8	b	32.7	a	49.0	a	15.2	a
		Mix	4.99	ab	41.3	a	81.5	a	21.1	a
	Salt	H2O	4.17	ab	43.5	a	19176	b	4456	b
		EBR	2.93	b	38.9	a	18396	b	4912	b
		IAA	3.75	ab	43.7	a	17627	b	4174	b
		Mix	5.59	ab	44.5	a	8781	c	1999	c
Chavignée	Drought	H2O	3.32	a	137	ab	185.6	a	14.3	a
		EBR	4.5	a	114.1	b	92.4	a	8.69	a
		IAA	4.6	a	123.9	ab	1102	a	90.4	a
		Mix	3.94	a	128.2	bc	121.9	a	9.95	a
	PHE	H2O	6.24	a	94.3	a	858.3	ab	89.2	a
		EBR	4.65	a	116	a	15.8	a	1.37	a
		IAA	5.47	a	109.3	a	1009	a	99.7	a
		Mix	5.18	a	133.6	a	34.4	a	2.5	a
	Salt	H2O	3.76	a	101.1	ab	41093	c	4205	cd
		EBR	3.57	a	87	a	16535	c	1827	cd
		IAA	4.51	a	106.2	ab	22132	cd	2174	bcd
		Mix	4.28	a	88	ac	30400	bd	3398	ad

3.2 Leaf pigments and root architecture

As illustrated in Table 8, when subjected to drought stress, the application of EBR and the combined hormones notably enhanced the TRL and TRS in the Ganges population. In contrast, for Chavignée, only the combined hormones augmented the TRL and TRS. During PHE stress, every hormonal treatment amplified both TRL and TRS in Chavignée, whereas only the hormone mixture yielded a similar enhancement in the Ganges population. When exposed to salinity, Chavignée plants experienced an increase in TRL and TRS across all hormonal

treatments, but in Ganges, only IAA elicited this rise. Notably, in the Ganges population, the application of IAA reduced the MRD from 0.57 mm to 0.44 mm. Regarding leaf pigments, all hormonal treatments augmented ChlA and carotenoid concentrations in Chavignée, while EBR hormone only had an increasing effect on ChlA in the Ganges plants specifically under PHE stress.

Table 8. Root architecture parameters and leaf pigment concentrations of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses, with hormonal treatments including control (H2O), IAA, EBR, and EBR+IAA (Mix). TRL: total root length (m), TRS: total root surface area (m^2), MRD: mean root diameter (mm), ChLA: chlorophyll A concentration (µg/mg FW), ChLB: chlorophyll B concentration (µg/mg FW), Carotenoids concentration (µg/mg FW). Different letters indicate significant differences ($p < 0.05$)

Population	Treatment	Hormone	Leaf Pigments						Root Architecture					
			ChlA		ChlB		Carotenoids		Length (m)		Area (m^2)		Diameter (mm)	
Ganges	Drought	H2O	0.69	c	0.27	e	0.14	b	3.2	c	0.43	g	0.41	bc
		EBR	0.71	ac	0.28	aef	0.15	be	6.09	cdef	0.79	dg	0.42	abc
		IAA	0.55	bc	0.23	ef	0.12	be	4.96	cd	0.56	cg	0.46	abc
		Mix	0.69	abd	0.26	cef	0.14	adef	8.35	bdg	1.31	bdf	0.50	bd
	PHE	H2O	0.77	cd	0.30	eg	0.17	bd	10.2	be	1.24	bdf	0.40	c
		EBR	0.81	ab	0.37	cfgh	0.18	ce	10.0	bf	1.45	bef	0.47	abc
		IAA	0.60	abd	0.24	cef	0.14	cde	8.91	bd	1.09	bcd	0.39	c
		Mix	0.70	ab	0.28	bdi	0.16	c	12.4	ab	1.81	af	0.46	abc
	Salt	H2O	0.75	cd	0.31	eh	0.14	b	4.61	cd	0.83	deg	0.57	d
		EBR	0.77	ab	0.33	cfh	0.15	be	4.00	cg	0.58	cg	0.47	abc
		IAA	0.58	ab	0.25	cfi	0.12	be	8.73	bd	1.20	bcdf	0.44	abc
		Mix	0.76	ab	0.32	bc	0.14	adef	5.48	cdf	0.76	dg	0.44	abc
Chavignée	Drought	H2O	0.67	e	0.32	f	0.18	a	6.63	a	0.95	bc	0.46	ad
		EBR	1.08	ace	0.44	cf	0.24	ac	6.28	a	0.90	b	0.47	acd
		IAA	0.73	bce	0.36	cf	0.21	abd	6.93	a	0.97	bc	0.45	ad
		MIX	0.92	bce	0.36	cf	0.19	abd	8.05	a	1.12	ab	0.44	a
	PHE	H2O	0.78	de	0.28	af	0.17	abd	6.97	a	1.06	ab	0.49	ab
		EBR	0.97	abcd	0.36	cdf	0.22	cd	11.0	a	1.52	ab	0.44	a
		IAA	0.82	ace	0.30	cef	0.21	bc	9.21	a	1.35	ab	0.47	acd
		MIX	1.10	ac	0.38	bd	0.24	c	10.1	a	1.55	ab	0.50	ab
	Salt	H2O	0.60	be	0.26	cef	0.19	ab	9.01	a	1.37	ab	0.47	ab
		EBR	0.91	abcd	0.42	bcf	0.26	abd	11.8	a	1.70	ab	0.46	ad
		IAA	0.87	abcd	0.35	bcf	0.21	abd	12.7	a	1.79	ab	0.45	ad
		MIX	0.77	abcd	0.30	bc	0.19	ac	12.7	a	1.85	ac	0.44	a

3.3 Metal accumulation

Under drought conditions, IAA reduced the concentrations of both Cd and Zn in Chavignée plant shoots but elevated these levels in the Ganges population (Figure 29). Furthermore, under

PHE stress, the application of EBR and the combined hormones doubled the concentrations of Cd and Zn in Chavignée shoots, and in the Ganges population, there was a 1.5-fold increase.

16

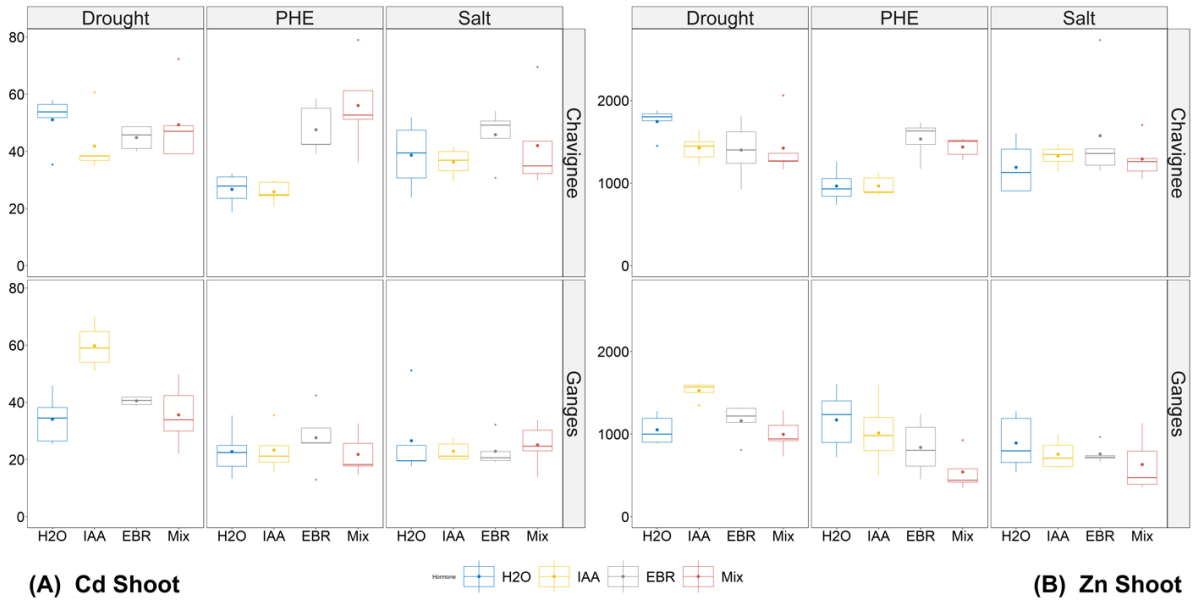

Figure 29. Concentrations (mg/kg) of Cd and Zn in shoots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses, with hormonal treatments including control (H_2O), IAA, EBR, and EBR+IAA (Mix).

3.4 Stress indicators and antioxidative parameters

In Figure 30, All the hormonal treatments reduced the MDA levels in both populations under drought, salinity, and PHE stresses. For proline, its levels rose in the Ganges plants due to the influence of both EBR and the combined hormones under all stress conditions. Moreover, every hormonal application contributed to elevating TPC and TFL levels in both groups across all examined stress conditions.

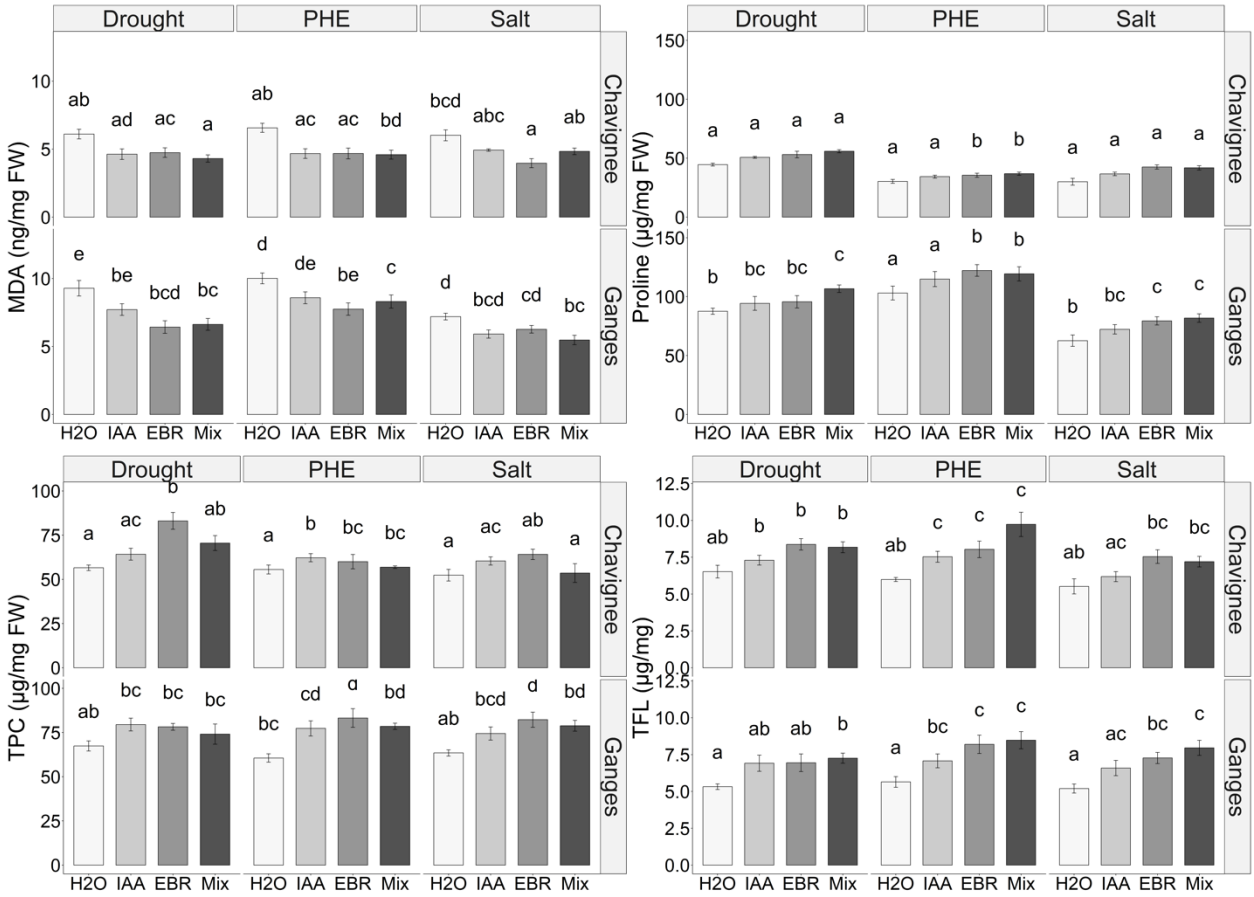

Figure 30. Stress biomarkers and metabolite content in shoots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses, with hormonal treatments including control (H_2O), IAA, EBR, and EBR+IAA (Mix). MDA: malondialdehyde, TPC: total phenolic compounds, TFL: total flavonoids. Different letters indicate significant differences ($p < 0.05$)

In Figure 31, during drought and PHE stresses, the activities of SOD and CAT enzymes were enhanced by all hormones, especially EBR, and the combined hormones, in both populations. Yet, under drought conditions, hormones specifically boosted APX activity only in Ganges plants, while EBR and the combined treatment heightened DHAR activity. In a similar line, under salinity stress, all hormonal applications elevated SOD and CAT activities across both populations. However, the combined hormone treatment was particularly potent in amplifying APX and DHAR activities under saline conditions.

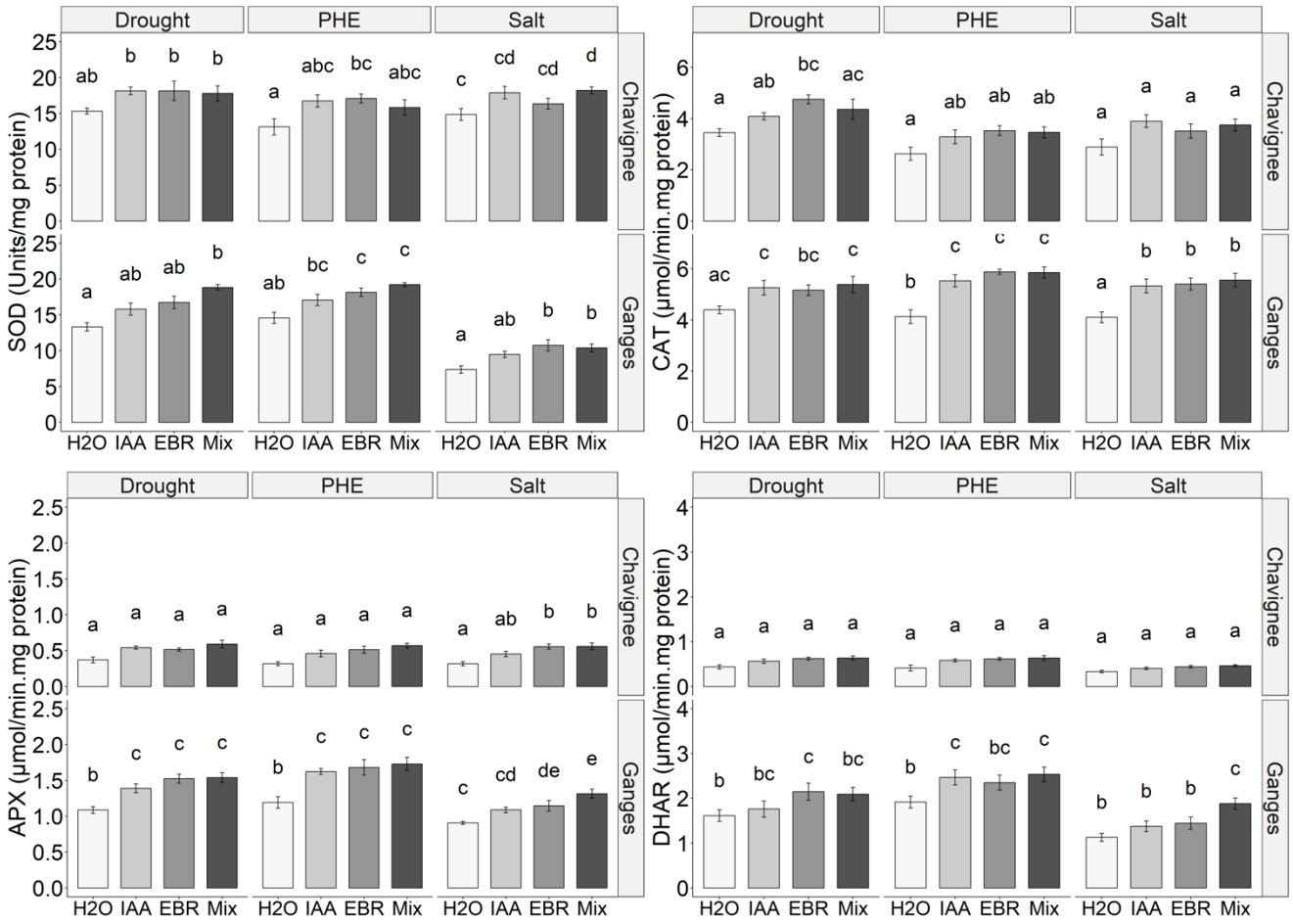

Figure 31. Activities of antioxidant enzymes in shoots of two N. caerulescens populations in response to drought, salinity, and PHE stresses, with hormonal treatments including control (H2O), IAA, EBR, and EBR+IAA (Mix). SOD: superoxide dismutase, CAT: catalase, APX: ascorbate peroxidase, DHAR: dehydroascorbate reductase. Different letters indicate significant differences ($p < 0.05$)

4 Multivariate analysis

A multivariate analysis was conducted to explore the interrelationships among four sets of variables: plant growth, nutrient uptake, metal uptake, and stress response, as shown in Figure 32. The first two dimensions accounted for 34% of the total variation observed in the data.

The visual representation of individual points within the factor plot indicates that the first dimension distinguishes between the Ganges population (appearing on the left of the plot with a notably negative value) and the Chavignée treatment (positioned on the right with a positive value) as illustrated in Figure 32, B. Also, the first dimension separates the control (positioned on the right with a slightly positive value), drought, salinity, and PHE treatments. The second dimension separates the combined EBR+IAA treatment (located at the top with a positive value) from the singular IAA and EBR treatments (centered with a slight positive value) and the control (at the bottom with a remarkable negative value) as depicted in Figure 32, A.

Regarding variable positioning in the factorial space (Figure 32, A), the first dimension contrasts three groups of key variables. The variables like MDA, proline, TPC, CAT, APX, DHAR, and GR, which are stress indicators, are characterized by a distinctively negative axis value. In contrast, variables such as P.S, Mg.S, S.S, Ca.S, K.S related to nutrient uptake, and Cd.S related to metal uptake stand out with a distinctly positive axis value. The second dimension emphasizes vital plant growth markers like DW.S, DW.R, Area.R, Length.R, ChlA, and ChlB, each having a marked positive value on the axis.

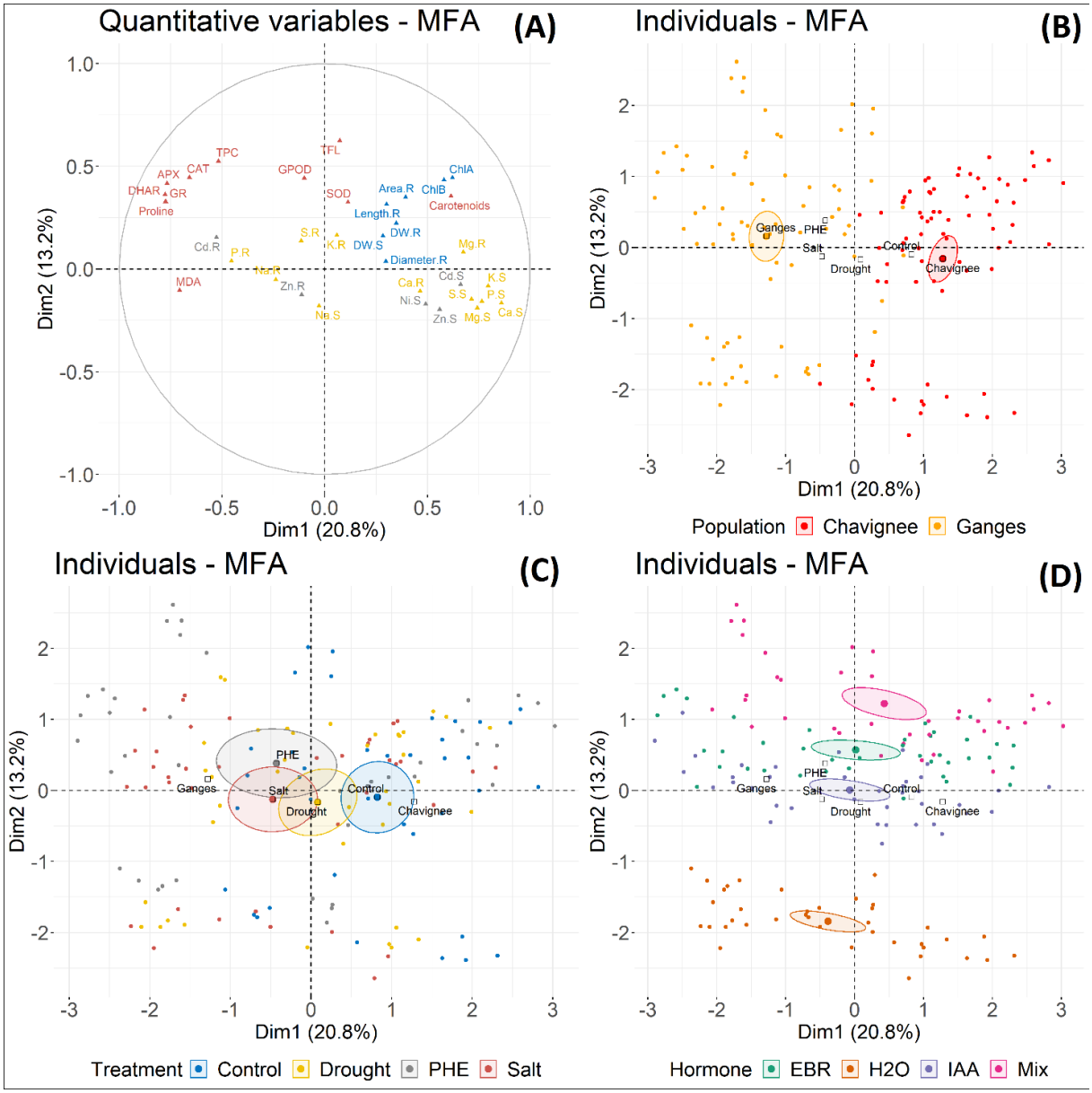

Figure 32. Multiple factor analysis (MFA) of individuals and variables. Labeled individuals are those with the greater contribution to the construction of the plane. Individuals are colored according to their membership in modalities of the variables "Population" (B), "Treatment" (C), and "Hormone" (D). The labeled variables are those that are best represented on the map. The variables are colored according to their membership in the variable groups. The labeled individuals are those best represented on the map (A). The numbers (in %) next to each axis represent the variance explained by the respective principal components.

5 Discussion

This research delves into the antioxidative reactions of two *N. caerulescens* groups (Ganges and Chavignée) under drought, salinity, and PHE stresses when subjected to foliar treatments of IAA and EBR hormones. The central objective is to examine features such as growth parameters, stress markers, the performance of antioxidant enzymes, and the process of phytoextraction.

In drought conditions, both EBR and a hormone mixture enhanced plant growth across the two populations, with IAA specifically benefiting Chavignée. The mixture further facilitated growth for both tested groups under salt and PHE stresses (Table 7). These outcomes resonate with prior research highlighting the growth-promoting effects of EBR and IAA under drought (Khan et al., 2021; Youzhi Zhang et al., 2020), saline (Kou, 2019; Latef et al., 2021), and PAH pressures (Li et al., 2015; Molina and Segura, 2021a). The augmented K levels in the Ganges plants due to EBR and the hormone mixture during drought (Table 7), could be linked to potassium adjustments in guard cells to manage stomatal movement (Ha et al., 2016). Moreover, while all hormones reduced the elevated Na concentration in salt-stressed Chavignée plants, only the mixture achieved this for the Ganges (Table 7). EBR is known to mitigate salt stress impacts in various plants, partly by regulating sodium levels via sodium transporter modulation and promoting potassium uptake (Yu et al., 2023). In contrast, IAA plays a role in strengthening sodium rejection processes (Ma et al., 2022).

In terms of root development, the hormone blend expanded both the TRL and TRS in the studied populations under drought, salt, and PHE pressures (Table 8). A notable synergy between IAA and EBR in augmenting root growth is evident (Zhengyang et al., 2023). The simultaneous use of these hormones has been recognized to steer lateral root growth and has shown that EBR boosts upward auxin movement in Arabidopsis (Bao et al., 2004). Under saline

conditions, all hormones reduced MRD in Chavignée plants, but only IAA achieved this for the Ganges (Table 8). Salinity often prompts plants to develop thicker roots, serving as barriers against salt absorption and moisture loss (Tan et al., 2020). The decline in MRD indicates a hormonal-driven alteration in root growth strategy, aiming to restore typical root conditions.

The impact of hormone treatments on the efficiency of plants to extract heavy metals is influenced by the specific plant species, type of heavy metal, and dosage applied. In our research, EBR and the combined hormones raised the concentrations of Cd and Zn across both populations studied. However, IAA increased Cd and Zn levels in the Chavignée plant shoots but decreased them in the Ganges (Figure 29). Past research found that maize shoots exhibited a notable reduction in Pb concentrations when treated with IAA, stemming from the intensified competition between Ca and Pb at the binding sites of transport proteins (Wang et al., 2007). Conversely, (Jan et al., 2021) noted a significant 178% rise in Cd concentration in *Dysphania ambrosioides* shoots with IAA treatment. The influence of external growth regulators on plants' ability to remove heavy metals is intricate, necessitating more extensive research to fully understand and standardize the effects.

Malondialdehyde (MDA), a lipid peroxidation product, is a marker for oxidative stress in plants (Morales and Munné-Bosch, 2019). In our study, every hormone treatment lowered MDA levels in both populations (Figure 30). Previous studies have confirmed that EBR and IAA can reduce MDA concentrations in plants under stress, largely by boosting antioxidant enzyme activities and fortifying cell membranes (Talaat et al., 2015; Wu et al., 2017). As for proline, the combined hormone treatment and EBR only managed to elevate its levels in the Ganges plants under all stress conditions (Figure 30). Proline, an amino acid accumulating in plants, acts as an osmoprotectant, assisting in stabilizing cellular structures and addressing osmotic imbalances (Chen and Zhang, 2016). This suggests that the Ganges population might exhibit stronger antioxidative responses in metal-rich environments.

During drought and PHE stresses, EBR and the hormone mixture boosted the functionality of the CAT and SOD enzymes (Figure 31). CAT primarily functions within peroxisomes, playing a pivotal role in neutralizing H_2O_2, which arises from the β-oxidation of fatty acids or glyoxysomes' photorespiration (Foyer and Noctor, 2005). SOD, on the other hand, is a metal-containing enzyme in plants responsible for transforming superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2). This process is essential for countering cell damage caused by reactive oxygen species (Wang et al., 2018). A study by (Ahammed et al., 2012a) observed heightened CAT and SOD activities in tomato plants stressed by PHE after EBR treatment. Moreover, both EBR and the hormone combination amplified the actions of APX and DHAR exclusively in the Ganges plants under all stress conditions, as illustrated in Figure 31.

The ascorbate-glutathione cycle, pivotal for detoxifying H_2O_2 in plant cells, revolves around the coordinated action of several enzymes, APX and DHAR being integral components (Hasanuzzaman et al., 2019). APX catalyzes the reduction of H_2O_2 to water using ascorbate as the electron donor, converting it to monodehydroascorbate (MDHA) which can spontaneously dismutate or be enzymatically reduced to regenerate ascorbate through the action of MDHA reductase (Li, 2023). However, a fraction of MDHA may non-enzymatically convert to dehydroascorbate (DHA). Here, DHAR plays its crucial role by reducing DHA back to ascorbate using reduced glutathione (GSH) as the electron donor, which is subsequently regenerated by glutathione reductase (GR) (Terai et al., 2020). An enhancement in the activities of APX and DHAR ensures efficient reduction of H_2O_2 while simultaneously maintaining the pool of reduced ascorbate and glutathione, thus reinforcing the strength of the ascorbate-glutathione cycle against oxidative stress (C. Niu et al., 2023; Rajput et al., 2021).

6 Conclusion

The findings from this study revealed that the two tested *N. caerulescens* populations exhibited a response that was both stress and hormone-dependent. Chavignée plants showed better growth parameters, while Ganges displayed higher antioxidative activities. For both populations, the combination of hormones was the most effective treatment across all stress conditions. Specifically, for Chavignée plants under drought conditions, EBR also played a role in alleviating stress. When subjected to PHE stress, Chavignée reacted positively to all hormonal treatments, unlike Ganges, which only responded to the hormone mixture. Under drought, IAA boosted phytoextraction efficiency in Chavignée, while both EBR and the hormone combination improved phytoextraction efficiency for both populations during PHE stress. The variation in growth and reaction could be inherent and linked to the kind of soil (whether it contains metals or not) and the environment where each of the two populations came from.

General Discussion

The trait of metal hyperaccumulation in plants is complicated, requiring numerous biochemical adjustments (van der Ent et al., 2015). Moreover, the prior assumption that these adaptations may come at a cost in terms of resource allocation is supported by various experimental results (Shrivastava et al., 2019). When considering the evolution of metal hyperaccumulation and possible trade-offs between defense mechanisms in these plants, it is necessary to consider the environments in which they are found and the often unique challenges these provide (Fones and Preston, 2013c).

1 Ecotype has a significant effect on the stress response of *N. caerulescens*

The Ganges population, collected from a metalliferous site, showed higher antioxidant activity when subjected to stresses such as drought, salinity, and phenanthrene. However, the Chavignée population, originating from a non-metalliferous site, showed more favorable growth parameters and appeared to be more tolerant to the imposed stresses. This could be attributed to the fact that plants growing in metalliferous soils are often exposed to elevated levels of heavy metals (Singh et al., 2016). In response to metal toxicity, these plants may have developed enhanced antioxidant defenses to combat oxidative stress. This pre-existing high antioxidant activity, acquired due to their natural habitat, may give them an advantage when exposed to other abiotic stresses. The increase in antioxidant activity can counteract the ROS generated due to abiotic stresses, thereby providing better protection against oxidative damage (Nadgórska-Socha et al., 2013; Nouman et al., 2020).

The Ganges population has evolved to actively take up cations, including trace metal elements, to concentrate them in its tissues which requires additional energy expense due to metabolic

(such as transport, sequestration, relocation, and concentration) and genetic modifications (Zemanová et al., 2016; Zhang et al., 2023). Consequently, these plants require a significant amount of ATP to mitigate the harmful effects of the metals they accumulate (Maestri et al., 2010b). However, these plants can counterbalance this energy demand by decreasing the energy spent on other functions (Farinati et al., 2009b) or by moderating the ROS signals triggered by different stresses (Fones and Preston, 2013b). This suggests that while the Ganges plants invest more in defensive mechanisms, the Chavignée population might prioritize growth and resource allocation. The absence of heavy metals in the Chavignée environment may allow these plants to invest more energy into growth and reproduction, making them more resilient to non-metal stresses (Morkunas et al., 2018). Their overall higher vigor may enable them to cope better with abiotic stresses than their Ganges counterparts, despite the latter exhibiting higher antioxidative activity. In addition, metal-rich natural soils and soils contaminated by past anthropogenic activities, such as those affected by mining, frequently exhibit nutrient deficiencies (Rashid et al., 2023). This could also shed light on why the Ganges plants from metal-rich areas accumulate less nutrients than those from Chavignée.

When metals accumulate to high levels, they can be toxic to hyperaccumulators in several ways, such as by generating oxidative stress, binding to thiol groups in proteins, or competing with other metals for binding sites in proteins (Dutta et al., 2018). An increase in superoxide production with zinc treatment was observed in *N. caerulescens* grown hydroponically, while these plants showed no stress signs (Fones et al., 2013). This suggests that excessive ROS may be produced during metal transport or sequestration, highlighting the idea that metal hyperaccumulation requires increased ROS tolerance in plants (Pasricha et al., 2021). Enzymatic activities for catalase and peroxidase, as well as those related to glutathione metabolism, are more abundant in hyperaccumulators than in non-accumulator species (Hammond et al., 2006). This observation is supported by increased antioxidant enzyme

activities and glutathione levels (Fones and Preston, 2013b). Furthermore, *N. caerulescens* growing in metal-rich soils show higher β-1,3-glucanase expression compared to those growing in non-metalliferous soils (Tuomainen et al., 2010). As part of the cellulose synthesis pathway, this enzyme might help modify the cell wall, protecting plants against both pathogens and excessive metals (Liu et al., 2023). Proteomic analysis of *N. caerulescens* in Ni-polluted soil revealed increased levels of an antifungal protein and several defensin-like proteins typically triggered by pathogens (Visioli et al., 2012). Defensins are known to be produced by plants in response to various stresses, including drought, cold, and specific signaling pathways (Kovaleva et al., 2020). This modulation of proteins associated with different stresses in hyperaccumulators supports their evolutionary adaptability to different environmental stresses, especially in ecotypes originating from metalliferous soils.

2 Hormonal treatments enhance metal accumulation in Ganges *N. caerulescens* while limiting it in Chavignée: Divergent ecotypic responses

In the Ganges plants, the observed increase in Cd and Zn concentrations in shoots, in the absence of other external stresses and in response to the foliar hormonal treatments, contrasts with the Chavignée population. Coupled with an enhanced antioxidant response in the Ganges population due to the hormonal application, it's possible that IAA and EBR could enhance the activity or expression of metal transporters or chelators (Gulcin and Alwasel, 2022) in Ganges plants. Since hormones regulate a variety of physiological functions in Ganges plants, including ion transport (Vaishnav and Chowdhury, 2023), these particular hormones could enhance their native metal uptake capacity.

Conversely, in the Chavignée population, the hormones may affect the plant's signaling in a distinct manner, potentially enhancing the activity or expression of proteins that negatively regulate metal uptake, thus impacting the plant's ability to compartmentalize metals and

reducing their movement to aboveground parts (Ur Rahman et al., 2023). This may indicate that the metalliferous ecotype, through the effect of hormones, adopts an accumulator strategy for tolerance, rather than an excluder one. For Chavignée plants, metals might be perceived as toxic beyond certain accumulation limits. In such situations, hormones may act to reduce metal accumulation below these thresholds in aboveground parts. Shen et al. (2022) showed, in hydroponic cultures, that the introduction of abscisic acid decreased the concentration of Cd in xylem sap and its accumulation in shoots. Similarly, the use of EBR led to reduced Ni uptake in *Brassica juncea*, as observed Kanwar et al. (2012). Heavy metal xylem loading, notably Cd, is primarily influenced by plant transpiration. Therefore, the observed decrease in Cd and Zn uptake in the shoots of Chavignée plants due to the hormonal effect could also be due to stomatal closure leading to reduced transpiration.

3 Synergistic effects of IAA and EBR enhance stress tolerance and antioxidant activation in *N. caerulescens*: a pathway to improved phytoremediation

In this research, the combined use of phytohormones was most effective in mitigating symptoms of drought, salinity, and PHE stress, and in enhancing antioxidant compounds in both *N. caerulescens* populations studied. The interaction between auxin and BRs (Brassinosteroids) are known to regulate multiple facets of plant growth and development, encompassing individual and interconnected levels (Tian et al., 2018). Even though this interaction has been a topic of research for many years, its details, especially in metal hyperaccumulating plants, remain not fully interpreted. BRs significantly influence auxin transport by altering the cellular positioning of auxin transporters responsible for both influx and efflux (Ibañes et al., 2009). The convergence of BR and auxin signaling pathways play a major role during root formation (Kim et al., 2006). Moreover, the synergy between BR and auxin plays a role in modulating plant responses to stress (Djemal et al., 2023). In cucumber

plants with auxin mutations exposed to varied stressors like salt, cold, and PEG, a noticeable expression of numerous BR-associated genes was observed (Yan et al., 2012).

Even though BRs and auxin play crucial roles in managing responses to salt stress and promoting root growth, the exact molecular dynamics of this interaction remain unclear. When exposed to salt stress, transcription factors associated with the BR pathway have been found to influence auxin balance by adjusting the expression of genes involved in auxin formation, binding, and breakdown (Djemal et al., 2023). Moreover, when these hormones are combined under salinity stress conditions, they promote hypocotyl growth, aid in root maturation, and influence halotropism and gravitropism (Devi et al., 2022). Recent evidence suggests that the combination of BRs and auxin aids in the production of GRAS proteins, which are thought to enhance plant resistance to abiotic stresses, especially drought (Khan et al., 2022). Therefore, to fully understand the symbiotic relationship between IAA and EBR in augmenting abiotic stress resilience in areas with multiple contaminants, in depth future research is essential.

Conclusion

Multi-contamination of soil by multiple organic and inorganic contaminants is considered an obstacle for the hyperaccumulator plants to develop and phytoextract metals. The aim of this thesis was to explore the impact of drought, salinity and PAHs in combination with heavy metals on the phytoextraction efficiency and antioxidative response of the Ganges and Chavignée populations of the metal hyperaccumulator *N. caerulescens*. In addition, it examined the impact of phytohormones application on the functioning of *N. caerulescens*. In essence, basically PHE stress led to the generation of detrimental reactive oxygen species (ROS), which in turn caused oxidative stress in *N. caerulescens*. This stress diminished the plant's phytoextraction capabilities and led to a decrease in biomass. Both the Ganges and Chavignée populations exhibited fairly comparable antioxidant defensive reactions under PHE stress. Nevertheless, Chavignée plants seemed to be slightly more tolerant than the Ganges for this stress. In addition, exposure to conditions of drought, salinity, and PHE also induced the production of harmful ROS, causing oxidative stress in *N. caerulescens*. This oxidative challenge adversely impacted phytoextraction capacity and led to a biomass decrease. Each population, Ganges and Chavignée, had unique stress coping mechanisms. The disparities in their growth and responses can be attributed to factors like the nature of their originating soil (metal-bearing or otherwise) and their native environments.

In the research centered on *N. caerulescens* populations of Ganges and Chavignée under hormonal treatments, the foliar use of IAA, EBR, and their combined form was more beneficial in enhancing phytoextraction in Ganges than in Chavignée. Ganges manifested a more robust antioxidative response to these hormonal treatments. Also, it experienced heightened antioxidant enzyme activities with each hormonal treatment, whereas Chavignée's enhanced

antioxidative response was more evident with EBR and the combined hormone treatment. Collectively, the mixture of hormones displayed a combined effect notable in both plant groups.

This study's results indicated that the two examined N. caerulescens populations had varied responses based on the type of stress and hormone exposure. Chavignée had superior growth attributes, whereas Ganges demonstrated elevated antioxidative functions. Among the treatments, the combined hormone treatment was the most beneficial across all stress scenarios. In drought, EBR also aided Chavignée in stress mitigation. Facing PHE stress, Chavignée positively reacted to all hormonal therapies, contrasting with Ganges, which responded chiefly to the combined hormones. Under drought-induced stress, IAA enhanced phytoextraction efficacy in Chavignée. Simultaneously, both EBR and the joint hormone therapy augmented phytoextraction performance in both groups during PHE exposure. The differential growth and responses might be intrinsic, tied to their soil type (metal-rich or not), and their respective originating environments.

Hence, future research could prioritize the examination of metabolites to gain a comprehensive understanding of how *N. caerulescens* reacts to combined stresses, alongside the phytohormones. Moreover, experimenting with a wider range of phytohormones and their combinations on *N. caerulescens* might offer deeper insights into the best approaches for enhancing phytoextraction.

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Appendices

Appendix 1. Shoot and root growth parameters of two *Noccaea caerulescens* populations in response to PHE stress. At the organ level, different letters indicate significant differences ($p<0.05$) after a 2-way anova. The results are presented for each individual factor (population and treatment) and their interaction (population * treatment). CHA: non-metallicolous population (Chavignée), GAN: metallicolous population (Ganges).

Organ	Population	Treatment	Fresh weight (g)		Dry weight (mg)		Water content (%)		C ($g\ kg^{-1}$)		N ($g\ kg^{-1}$)		C/N		P ($g\ kg^{-1}$)		S ($g\ kg^{-1}$)		K ($g\ kg^{-1}$)		Na ($g\ kg^{-1}$)		Na/K molar ratio	
Shoot	CHA	CTRL	1.01	a	169	a	82.4	b	363	a	41,9	a	8.66	a	2.61	a	7.78	a	18.2	a	17.2	b	1.69	b
		PHE	0.84	a	249	a	70.1	a	374	a	31,1	a	12.0	b	1.76	a	7.26	a	19.8	a	16.2	ab	1.39	ab
	GAN	CTRL	1.20	a	230	a	78.3	ab	390	a	33,1	a	11.8	ab	2.46	a	7.53	a	20.7	a	9.48	a	0.81	a
		PHE	0.63	a	175	a	71.6	ab	404	*	25,6	*	15.8	*	1.71	*	5.47	*	11.6	*	9.90	*	1.45	*
	CHA	CTRL+PHE	0.96	a	193	a	78.7	a	367	a	38,6	b	9.5	b	2.36	a	7.62	a	18.7	a	16.9	b	1,60	b
	GAN	CTRL+PHE	0.99	a	209	a	75.8	a	392	b	31,9	a	12.3	a	2.34	a	7.19	a	19.2	a	9.54	a	0,92	a
	CHA+GAN	CTRL	1.90	a	195	a	80.7	b	375	a	38,2	b	9.8	b	2.55	b	7.68	a	19.2	a	14.1	a	1,33	a
	CHA+GAN	PHE	0.73	a	212	a	70.8	a	382	a	29,7	a	12.9	a	1.74	a	6.82	a	17.8	a	16.3	a	1,40	a
Root	CHA	CTRL	0.16	ab	42,1	a	72.9	b	448	a	28,7	a	15.6	a	2.54	a	6.58	a	8.66	ab	3.58	a	0.71	ab
		PHE	0.06	a	36,6	a	37.3	a	455	a	23,9	a	19.0	a	1.91	a	5.69	a	4.75	a	3.20	a	1.14	b
	GAN	CTRL	0.26	b	48,9	a	80.5	b	430	a	29,6	a	14.5	a	3.12	a	8.56	a	10.3	b	3.35	a	0.59	a
		PHE	0.09	ab	23,7	a	72.7	b	448	a	30,8	a	14.5	a	1.87	a	8.01	a	7.80	ab	3.65	a	0.81	ab
	CHA		0.12	a	40,3	a	61.0	a	450	a	27,1	a	16.6	a	2.33	a	6.28	a	7.35	a	3.45	a	0,85	a
	GAN		0.20	b	39,4	a	77.6	a	437	a	30,1	a	14.5	a	2.65	a	8.36	a	9.35	a	3.46	a	0,67	a
	CHA+GAN	CTRL	0.20	a	45,2	a	76.3	b	440	a	29,1	a	15.1	a	2.81	a	7.48	a	9.40	b	3.47	a	0,66	a
	CHA+GAN	PHE	0.07	a	30,2	a	55.0	a	451	a	27,4	a	16.5	a	1.89	a	6.85	a	6.27	a	3.21	a	0,98	a

Appendix 2. Concentrations and quantities of Cd, Ni and Zn in shoots and roots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses. Different letters indicate significant differences ($p < 0.05$).

Organ	Population	Treatment	Cd (mg/kg)		Q.Cd (mg)		Ni (mg/kg)		Q.Ni (mg)		Zn (mg/kg)		Q.Zn (mg)	
Shoot	Chavignée	CTRL	48.6	ab	0.262	a	56.9	ab	0.307	a	1271.0	ab	6.86	a
		PEG	51.1	b	0.171	ab	60.9	a	0.204	a	1745.0	a	5.81	a
		PHE	26.7	ac	0.168	ab	26.6	b	0.167	a	963.9	bc	6.02	a
		SAL	38.6	bc	0.143	ab	45.8	ab	0.164	a	1189.6	ab	4.48	a
	Ganges	CTRL	29.6	ac	0.183	ab	30.6	ab	0.205	a	553.6	c	3.55	a
		PEG	34.1	bc	0.119	ab	54.4	ab	0.189	a	1051.5	bc	3.69	a
		PHE	22.7	c	0.075	b	34.6	ab	0.112	a	1171.9	b	3.85	a
		SAL	26.6	c	0.117	ab	47.1	ab	0.207	a	890.7	bc	3.56	a
	Chavignée		42.2	a	0.19	a	48.8	a	0.22	a	1316.3	a	5.85	a
	Ganges		28.2	b	0.12	a	41.7	b	0.18	a	916.9	b	3.66	b
		CTRL	39.1	a	0.22	a	43.7	a	0.26	a	912.3	a	5.21	a
		PEG	42.6	a	0.14	a	57.7	a	0.20	a	1398.2	b	4.75	a
		PHE	24.5	b	0.12	a	31.0	b	0.14	a	1079.5	a	4.81	a
		SAL	31.9	ab	0.13	a	46.5	ab	0.19	a	1023.5	a	3.97	a
Root	Chavignée	CTRL	20.0	ab	0.013	a	12.1	a	0.001	a	280.9	ab	0.150	a
		PEG	37.1	bc	0.017	a	11.1	a	0.001	a	608.0	ac	0.271	a
		PHE	29.7	ab	0.024	a	16.9	a	0.001	a	249.2	ab	0.179	a
		SAL	30.9	a	0.004	a	4.24	a	0.001	a	908.6	c	0.767	b
	Ganges	CTRL	18.3	ac	0.015	a	5.54	a	0.001	a	160.4	b	0.128	a
		PEG	36.7	bc	0.011	a	27.4	a	0.001	a	189.4	b	0.059	a
		PHE	46.1	b	0.033	a	10.1	a	0.001	a	291.1	ab	0.207	a
		SAL	30.8	ab	0.037	a	5.32	a	0.001	a	360.8	ab	0.407	ab
	Chavignée		23.3	a	0.014	a	1.2	a	0.001	a	504.2	a	0.33	a
	Ganges		33.0	a	0.024	a	1.4	a	0.001	a	250.4	a	0.20	a
		CTRL	19.1	ab	0.014	a	1.31	a	0.001	a	220.6	a	0.14	a
		PEG	36.9	b	0.014	a	1.42	a	0.000	a	398.7	ab	0.17	a
		PHE	38.8	b	0.029	a	1.22	a	0.001	a	272.5	a	0.19	a
		SAL	18.9	a	0.022	a	1.37	a	0.001	a	604.3	b	0.57	b

Appendix 3. Stress biomarkers and metabolite content in shoots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses. MDA: malondialdehyde (ng mg^{-1} FW), proline (µg mg^{-1} FW), TPC: total phenolic compounds (µg mg^{-1} FW), TFL: total flavonoids (µg mg^{-1} FW). Different letters indicate significant differences ($p < 0.05$)

Population	Treatment	MDA		Proline		TFL		TPC	
Chavignée	CTRL	3.54	a	16.6	a	3.70	ab	33.0	a
	PEG	6.10	b	44.5	b	6.52	c	56.5	bc
	PHE	6.57	b	30.3	ab	5.99	c	55.6	bc
	SAL	6.02	b	30.0	ab	5.52	c	52.3	b
Ganges	CTRL	4.09	a	42.2	b	3.14	a	54.1	bc
	PEG	9.29	c	87.6	d	5.32	c	67.4	d
	PHE	10.0	c	103.0	e	5.65	c	60.6	bd
	SAL	7.20	b	62.6	c	5.20	bc	63.5	cd

Appendix 4. Activities of antioxidant enzymes in shoots of two *N. caerulescens* populations in response to drought, salinity, and PHE stresses. SOD: superoxide dismutase, CAT: catalase (µmol min^{-1} mg^{-1} protein), GR: glutathione reductase (µmol min^{-1} mg^{-1} protein), GPOD: guaiacol peroxidase (µmol min^{-1} mg^{-1} protein), APX: ascorbate peroxidase (µmol min^{-1} mg^{-1} protein), DHAR: dehydroascorbate reductase (µmol min^{-1} mg^{-1} protein). Different letters indicate significant differences ($p < 0.05$).

Population	Treatment	CAT		SOD		GR		GPOD		APX		DHAR	
Chavignée	CTRL	1.67	a	9.98	ab	0.14	a	0.26	a	0.22	a	0.22	a
	PEG	3.45	bc	15.3	c	0.25	ab	0.49	b	0.37	a	0.44	ab
	PHE	2.62	ab	13.1	ac	0.26	ab	0.48	bc	0.32	a	0.41	ab
	SAL	2.89	b	14.9	c	0.18	ab	0.54	b	0.32	a	0.34	ab
Ganges	CTRL	2.99	b	5.18	d	0.41	b	0.23	a	0.60	b	0.60	b
	PEG	4.40	d	13.3	c	1.10	d	0.49	b	1.09	cd	1.61	d
	PHE	4.13	cd	14.6	c	1.18	d	0.61	b	1.19	d	1.92	d
	SAL	4.10	cd	7.37	bd	0.69	c	0.34	ac	0.91	c	1.13	c

Appendix 5. Growth parameters and nutrient content of shoots and roots of two *N. caerulescens* populations in response to IAA, EBR and hormone mixture foliar application. Different letters indicate significant differences ($p < 0.05$).

Organ	Population	Treatment	Dry weight (g)		K (g/kg)		P (g/kg)		S (g/kg)		Ca (g/kg)		Mg (g/kg)		Na (mg/kg)	
Shoot	Chavignée	H2O	5.37	a	113.6	a	6.09	a	11.9	a	45.7	a	7.40	a	106.7	ab
		IAA	5.63	a	105.5	a	5.47	a	12.0	a	40.3	ab	7.01	ab	182.8	a
		EBR	5.87	a	106.1	a	5.61	a	10.6	a	35.5	b	5.50	c	19.4	bc
		EBR+IAA	6.23	a	116.4	a	6.38	a	12.2	a	38.8	ab	6.04	bc	42.3	bc
	Ganges	H2O	5.69	a	36.9	b	2.23	b	6.21	b	9.57	c	2.27	de	0.82	c
		IAA	5.58	a	42.8	b	2.23	b	5.19	b	13.8	c	3.27	de	4.9	c
		EBR	4.55	a	44.9	b	2.60	b	6.46	b	15.7	c	3.58	d	65.9	bc
		EBR+IAA	8.23	a	43.8	b	1.81	b	4.84	b	8.51	c	2.06	e	0.69	c
	Chavignée		5.77	a	110.4	a	5.89	a	11.7	a	40.1	a	6.49	a	87.8	a
	Ganges		5.97	a	41.9	b	2.22	b	5.68	b	11.9	b	2.79	b	16.4	b
		H2O	5.53	a	75.2	a	4.16	a	9.04	a	27.7	a	4.84	a	53.8	a
		IAA	5.60	a	74.1	a	3.85	a	8.61	a	27.1	ab	5.14	ab	93.8	b
		EBR	5.28	a	78.9	a	4.27	a	8.76	a	26.7	b	4.65	c	40.0	c
		EBR+IAA	7.12	a	84.2	a	4.35	a	8.90	a	25.3	b	4.27	bc	23.8	ac
Root	Chavignée	H2O	0.66	a	12.1	a	2.57	a b	7.13	a	13.4	a	4.27	ab	428.6	ab
		IAA	0.97	a	14.5	a	2.84	a b	8.56	a b	13.1	a	4.99	ac	176.7	ab
		EBR	0.75	a	14.1	a	3.13	a b	11.5	b	12.7	ab	3.65	bd	354.7	ab
		EBR+IAA	1.05	a	20.7	a b	2.44	a	8.59	a b	11.9	ab	5.11	c	121.4	a
	Ganges	H2O	0.85	a	5.54	a	2.90	a b	8.18	a b	12.1	ab	2.41	e	267.5	ab
		IAA	1.01	a	37.3	b	3.86	a b	11.5	b	10.3	b	3.14	df	512.0	b
		EBR	0.67	a	6.90	a	4.83	b	8.27	a b	11.1	ab	2.40	ef	430.2	ab
		EBR+IAA	1.47	a	24.5	a b	2.60	a b	9.06	a b	10.0	b	2.80	ef	242.6	ab
	Chavignée		0.86	a	15.3	a	2.75	a	8.96	a	12.8	a	4.50	a	270.3	a
	Ganges		0.99	a	18.9	a	3.53	b	9.32	b	10.9	a	2.70	b	366.0	a
		H2O	0.76	a	8.82	a	2.74	a	7.66	a	12.7	a	3.34	a	348.1	a
		IAA	0.99	a	25.9	a	3.35	a	10.0	a	11.7	a	4.07	b	344.4	a
		EBR	0.72	a	10.9	a	3.89	a	10.1	b	12.0	a	3.10	c	388.2	a
		EBR+IAA	1.24	a	22.4	a	2.51	a	8.80	a	11.0	a	4.09	b	175.2	a

Appendix 6. Concentrations and quantities of Cd, Ni and Zn in shoots and roots of two *N. caerulescens* populations in response to IAA, EBR and hormone mixture foliar application. Different letters indicate significant differences ($p < 0.05$).

Organ	Population	Treatment	Cd (mg/kg)		Q.Cd (mg)		Ni (mg/kg)		Q.Ni (mg)		Zn (mg/kg)		Q.Zn (mg)	
Shoot	Chavignée	H2O	48.6	ab	0.26	a	56.9	a	0.307	a	1271.0	a	6.86	a
		IAA	37.9	b	0.23	a	46.2	a	0.278	a	1137.5	a	6.55	a
		EBR	36.9	ac	0.22	a	42.0	a	0.246	a	1001.4	ab	5.90	a
		EBR+IAA	39.5	bc	0.26	a	43.0	a	0.283	a	970.5	ab	6.02	a
	Ganges	H2O	29.6	ac	0.18	a	30.6	a	0.205	a	553.6	b	3.55	a
		IAA	31.6	bc	0.17	a	47.6	a	0.277	a	1111.2	ab	6.28	a
		EBR	35.9	c	0.17	a	55.3	a	0.275	a	1293.0	a	6.37	a
		EBR+IAA	33.3	c	0.27	a	32.4	a	0.259	a	689.2	ab	5.42	a
	Chavignée		40.7	a	0.24	a	47.0	a	0.28	a	1095.1	a	6.33	a
	Ganges		32.4	b	0.20	a	41.2	b	0.25	a	902.9	b	5.35	b
		H2O	39.1	a	0.22	a	43.7	a	0.26	a	912.3	a	5.21	a
		IAA	34.8	a	0.20	a	46.9	a	0.28	a	1124.3	a	6.41	a
		EBR	36.5	a	0.20	a	47.9	a	0.26	a	1131.0	a	6.11	a
		EBR+IAA	36.7	a	0.27	a	38.3	a	0.27	a	845.5	a	5.75	a
Root	Chavignée	H2O	20.0	a	0.01	a	1.4	a	0.001	a	280.9	ab	0.15	ab
		IAA	22.4	a	0.02	a	1.4	a	0.001	a	168.6	ac	0.18	ab
		EBR	20.1	a	0.02	a	1.4	a	0.001	a	566.4	ab	0.48	a
		EBR+IAA	19.3	a	0.02	a	1.4	a	0.001	a	85.2	c	0.09	b
	Ganges	H2O	18.3	a	0.02	a	1.2	a	0.001	a	160.4	b	0.13	ab
		IAA	25.6	a	0.02	a	1.2	a	0.001	a	187.2	b	0.16	ab
		EBR	17.5	a	0.01	a	1.2	a	0.001	a	225.3	ab	0.16	ab
		EBR+IAA	18.8	a	0.03	a	1.2	a	0.002	a	91.8	ab	0.13	ab
	Chavignée		20.5	a	0.02	a	1.4	a	0.001	a	275.3	a	0.23	a
	Ganges		20.2	a	0.02	a	1.2	a	0.001	a	167.0	a	0.15	a
		H2O	19.1	a	0.01	a	1.30	a	0.001	a	220.6	a	0.14	a
		IAA	24.0	a	0.02	a	1.30	a	0.001	a	177.9	a	0.17	ab
		EBR	18.9	a	0.01	a	1.31	a	0.001	a	414.8	b	0.34	b
		EBR+IAA	19.1	a	0.02	a	1.31	a	0.002	a	88.1	a	0.11	a

Appendix 7. Stress biomarker and metabolite contents in shoots of two *N. caerulescens* populations in response to IAA, EBR and hormone mixture foliar application. MDA: malondialdehyde (ng mg^{-1} FW), proline (µg mg^{-1} FW), TPC: total phenolic compounds (µg mg^{-1} FW), TFL: total flavonoids (µg mg^{-1} FW). Different letters indicate significant differences ($p < 0.05$).

Population	Treatment	MDA		Proline		TFL		TPC	
Chavignée	H2O	3.54	a	16.6	a	3.70	ab	33.0	a
	IAA	3.83	a	23.2	a	6.42	cd	45.2	b
	EBR	4.16	a	38.4	bc	6.41	cd	52.2	bc
	EBR+IAA	4.02	a	32.5	b	5.31	bc	59.4	c
Ganges	H2O	4.09	a	42.2	cd	3.14	a	54.1	bc
	IAA	4.71	a	49.4	de	6.05	cd	72.5	d
	EBR	5.01	a	52.8	e	5.45	bd	70.3	d
	EBR+IAA	4.40	a	48.3	de	7.27	d	75.4	d

Appendix 8. Activities of antioxidant enzymes in shoots of two *N. caerulescens* populations in response to IAA, EBR and hormone mixture foliar application. SOD: superoxide dismutase, CAT: catalase (µmol/min.mg protein), GR: glutathione reductase (µmol min^{-1} mg^{-1} protein), GPOD: guaiacol peroxidase (µmol min^{-1} mg^{-1} protein), APX: ascorbate peroxidase (µmol min^{-1} mg^{-1} protein), DHAR: dehydroascorbate reductase (µmol min^{-1} mg^{-1} protein). Different letters indicate significant differences ($p < 0.05$).

Population	Hormone	CAT		SOD		APX		GPOD		GR		DHAR	
Chavignée	H2O	1.67	a	9.98	abc	0.22	a	0.26	a	0.14	a	0.22	a
	IAA	2.04	ab	11.5	bc	0.29	ab	0.29	a	0.20	a	0.27	ab
	EBR	2.77	bcd	11.9	b	0.51	cd	0.58	b	0.17	a	0.22	a
	EBR+IAA	2.22	ad	11.6	bc	0.41	bc	0.48	bc	0.18	a	0.27	ab
Ganges	H2O	2.99	de	5.18	d	0.60	d	0.23	a	0.41	b	0.60	bc
	IAA	3.25	cef	6.94	de	0.87	e	0.31	a	0.67	c	0.82	c
	EBR	3.80	ef	8.07	ade	0.88	e	0.35	ac	0.89	d	1.27	d
	EBR+IAA	4.05	f	8.58	ce	0.99	e	0.37	ac	0.97	d	1.64	d

Title: Management of the antioxidant response of the hyperaccumulator plant *Noccaea caerulescens* to abiotic stress

Abstract: Polluted soils often contain abiotic stressors for hyperaccumulator species, not because of the presence of metals, but because of the presence of organic co-pollutants, high salinity, or drought conditions. These conditions can in extreme cases prevent the establishment of the species and therefore the implementation of a phytoremediation strategy, or at least modify its functioning, in particular its capacity to hyperaccumulate metals. It is therefore necessary to evaluate the response of hyperaccumulators subjected to a variety of abiotic stresses in order to predict and control the efficiency of the phytoextraction process. This thesis aims to characterize the antioxidant response and the phytoextraction efficiency of two populations of the Cd, Ni and Zn hyperaccumulator *N. caerulescens* to a variety of abiotic stresses and the consequences on the production of biomolecules of interest. Additionally, the study aims to examine the impact of the foliar application of indole acetic acid and 24-epibrassinolide phytohormones on the antioxidant response and phytoextraction of *N. caerulescens* in multi-stress environment, aiming to develop strategies for protecting plants during phytoextraction.

Key words: phytoremediation, hyperaccumulator, abiotic stress, antioxidant response, phytohormones

Titre : Gestion de la réponse antioxydante chez la plante hyperaccumulatrice *Noccaea caerulescens* face aux stress abiotiques

Résumé : Les sols pollués contiennent souvent des facteurs de stress abiotiques pour les espèces hyperaccumulatrices, non pas à cause de la présence de métaux, mais en raison de la présence de co-polluants organiques, d'une salinité élevée ou de conditions de sécheresse. Dans des cas extrêmes, ces conditions peuvent empêcher l'établissement de l'espèce et donc la mise en œuvre d'une stratégie de phytoremédiation, ou du moins en modifier le fonctionnement, notamment sa capacité à hyperaccumuler les métaux. Il est donc nécessaire d'évaluer la réponse des hyperaccumulatrices soumises à divers stress abiotiques afin de prévoir et contrôler l'efficacité du processus de phytoextraction. Cette thèse vise à caractériser la réponse antioxydante et l'efficacité de la phytoextraction de deux populations de *Noccaea caerulescens*, une hyperaccumulatrice de Cd, Ni et Zn, face à divers stress abiotiques et les conséquences sur la production de biomolécules d'intérêt. De plus, l'étude vise à examiner l'impact de l'application foliaire d'acide indole acétique et de phytohormones 24-epibrassinolide sur la réponse antioxydante et la phytoextraction de *N. caerulescens* dans un environnement multi-stress, dans le but de développer des stratégies pour protéger les plantes pendant la phytoextraction.

Mots clés : phytoremédiation, hyperaccumulateur, stress abiotique, réponse antioxydante, phytohormones