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Dissertation

NICKEL UPTAKE AND TRANSPORT IN THE HYPERACCUMULATOR *NOCCAEA CAERULESCENS*

Submitted by

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for the

Degree of Doctor of Philosophy of Sun Yat-sen University

and

Doctorate of Université de Lorraine

in Agronomy

-oOo-

Dissertation prepared under joint supervision:

School of Environmental Science and Engineering, Sun Yat-sen University
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-oOo-

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Abstract

Hyperaccumulating plants are capable of accumulating extraordinary concentrations of heavy metals, *e.g.* Ni, Zn and Cd, in their shoots. Metal hyperaccumulation is a rare phenomenon in higher plants. To date, more than 500 hyperaccumulating species have been identified globally, of which the majority are Ni hyperaccumulators. During the past decades, hyperaccumulation mechanisms have been extensively studied, but most attention has been paid to Zn hyperaccumulators. Much less is known about the Ni homeostasis mechanisms in Ni hyperaccumulators, in particular root uptake and long distance transport processes. Therefore, the objectives of this thesis were to assess i) the properties of Ni uptake systems in roots, and ii) how Ni moves in phloem and the significance of phloem translocation on Ni hyperaccumulation. Here are the main approaches and findings:

(1) A hydroponic cultivation was conducted to investigate the isotope fractionation of Ni and Zn during plant uptake and translocation processes. The non-accumulator *Thlaspi arvense*, the Ni hyperaccumulator *Alyssum murale* and the Ni and Zn hyperaccumulator *Noccaea caerulescens* were grown in low (2 μM) and high (50 μM) Ni and Zn solutions. Results showed that plants were inclined to absorb light Ni isotopes, presumably due to the functioning of low-affinity transport systems across root cell membrane. The Ni isotope fractionation between plant and solution was greater in the hyperaccumulators grown in low Zn treatments ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.63 to -0.90‰) than that in the non-accumulator *T. arvense* ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.21‰), thus indicating a greater permeability of the low-affinity transport system in hyperaccumulators. Moreover, Zn could compete with Ni during the uptake process, which reduced Ni concentration in plants and decreased the extent of Ni isotope fractionation ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.07 to -0.11‰), indicating that plants might take up Ni via Zn transport systems.

(2) *Noccaea caerulescens* was grown in hydroponic solutions containing different concentrations of Ni, Zn, Fe and Co, in order to investigate the competition effect of these

elements during root uptake process. Furthermore, the gene expression levels of the Zn transporter *ZIP10* and the Fe transporter *IRT1* were determined by quantitative real-time reverse transcriptase PCR. Results showed that Zn could severely suppress Ni uptake. Also, Ni absorption was stimulated by Fe deficiency. In addition, the expression levels of *ZIP10* and *IRT1* were increased after Ni exposure. These results suggested that Ni is mainly taken up via Zn transporters, while Fe transport systems also play a role for Ni uptake. The great competition between Co and Ni further indicated that Ni transport systems seem to be non-specific.

(3) Rb and Sr were used in the experiment to assess the effect of xylem transport and phloem translocation on Ni accumulation in both young and old leaves. Results showed that old leaves of *N. caerulescens* grown in Ni-, Rb- and Sr-rich solutions contained more than 60% of the total Ni, Rb and Sr content in shoots, indicating old leaves are the main sink for xylem transport. The high metal concentrations in young leaves also suggested that these tissues are also an active sink for xylem transport. In another experiment, $^{61}Ni^{2+}$ was applied to old leaves as a foliar spray to assess Ni partitioning via phloem translocation. We found that 89% of ^{61}Ni exported from old leaves was translocated upward to young leaves, whereas only 11% moved downward to roots, indicating that young leaves are the main sink for phloem translocation.

(4) Phloem exudates from leaves of *N. caerulescens* were extracted by the EDTA-stimulated phloem exudation method, and the metal and organic compound concentrations in the exudates were determined. Results showed that Ni was enriched in the phloem exudates, indicating high Ni phloem loading and translocation capacity in *N. caerulescens*. Amino acids, *e.g.* nicotianamine and histidine, were present in exudates at concentrations insufficient for Ni chelation. On the contrary, organic acids, especially malate, were present at high concentrations in all treatments, and were probably involved in Ni complexation in phloem.

In conclusion, the hyperaccumulator *N. caerulescens* takes up Ni mainly via low-affinity transport system, which seems to be Zn and Fe transporters; xylem transport is the main source for Ni accumulation in both young and old leaves, while phloem translocation also acts

as an important source for young leaves; Ni is enriched in phloem sap and mainly chelated by organic acids during phloem translocation.

KEY WORDS: Nickel; hyperaccumulator; *Noccaea caerulescens*; root uptake; phloem translocation; isotope fractionation

ABSORPTION ET TRANSPORT DU NICKEL CHEZ LES HYPERACCUMULATEURS

Résumé étendu en français

Contexteet enjeux

Les plantes hyperaccumulatrices sont capables d'accumuler dans leurs parties aériennes des concentrations extraordinaires de métaux lourds, comme par exemple le Ni, le Zn et le Cd. L'hyperaccumulation métallique est un phénomène rare chez les plantes supérieures et, à ce jour, plus de 500 espèces hyperaccumulatrices ont été identifiées dans le monde, dont la majorité sont des hyperaccumulateurs Ni. Au cours des dernières décennies, les mécanismes de l'hyperaccumulation ont été largement étudiés, en particulier pour le Zn. Les processus considérés comprennent la stimulation de l'absorption des métaux par les racines, la réduction de la séquestration du métal dans les vacuoles des racines, l'efficacité du chargement et du transport dans le xylème et la forte séquestration du métal et sa compartimentation dans les feuilles (Figure 1.1, Chapitre 1). En revanche, peu ou pas d'études ont été menées pour comprendre et décrire l'homéostasie du Ni dans les hyperaccumulateurs.

Etat de l'art et questionnement sur les processus d'absorption et de translocation du Ni chez les hyperaccumulateurs

La littératurerapporte que l'absorption du Nipar les racines peut être inhibée par une faible température, par les inhibiteurs du métabolisme et des conditions anaérobies(Puschenreiter et al., 2005; Aschmann et Zasoski, 1987), ce qui suggère que ce processus est dépendantdu métabolisme. L'absorption du Ni par des plants de soja suit effectivement un processus cinétique de type Michaelis-Menten (Cataldo et al., 1978). La constante K_m de Michaelis-Menten de l'absorption du Ni par des plants d'avoinevarie de 12 µM (Aschmann et Zasoski, 1987) à 80 µM pour le protoplaste de racines *Vigna radiata* (Zhang et al., 2001).

Pour l'hyperaccumulateur de Ni *Thlaspi goesingense*, K_m est de 36,1 µM (Puschenreiter et al., 2005). Les valeurs relativement importantes Km indiquent que le Ni pourrait être absorbé par les systèmes de transport à faible affinité chez les plantes supérieures. Mais, à ce jour, aucun transporteur du Ni à haute affinité n'a été identifié chez les plantes supérieures.

Des interactions entre le Ni et d'autres cations existent au cours du processus d'absorption racinaire. Pour hyperaccumulateur de Zn/Ni *T. pindicum*, l'absorption du Ni est inhibée par l'addition de Zn^{2+}, tandis qu'à l'inverse, Ni a peu d'effet sur l'absorption du Zn (Taylor et Macnair, 2006). De même, *Noccaea caerulescens*, hyperaccumulateurde Ni, préfère le Zn au Ni (Assunção et al., 2001). En outre, l'accumulation du Fe dans les racines de l'hyperaccumulateur de Ni *Alyssum inflatum*est stimulée lorsqu'il est exposé à des concentrations élevées de Ni (Ghasemi et al., 2009). Le transporteur de Fe^{2+} IRT1 et le transporteur de Zn ZIP10 pourraient être impliqués dans l'accumulation Ni par *N. Caerulescens* (Halimaa et al., 2014). En plus de Zn et de Fe, Mn semble interagir négativement avec le Ni au cours de l'absorption (Broadhurst et al., 2009). Fait intéressant, Reeves et Baker (1984) ont constaté que *T. goesingense* cultivé sur des substrats enrichis en métaux peut accumuler des concentrations similaire et très élevées de Ni, Zn, Mn et Co dans leurs parties aériennes. Ces résultats suggèrent que l'absorption du Ni par les racines impliquerait des transporteurs spécifiques à d'autres élémentsdivalents, comme les oligoéléments. Cependant, l'effet d'antagonismede ces éléments sur l'absorption du Ni n'est pas clairement établi.

En atteignant le cytoplasme des cellules des racines, les ions métalliques seraient rapidement complexés par des ligands organiques comme les acides aminés pour limiter leur précipitation (Haydon et Cobbet, 2007; Harris et al., 2012). Les concentrations de l'histidine (HIS) dans les racines de l'hyperaccumulateur *A. lesbiacum* sont constitutivement élevées par rapport à celles mesurées dans le non-hyperaccumulateur *Brassica juncea* (Kerkeb et Kramer, 2003), un phénomène qui serait dû à la surexpression de la voie biosynthétique de l'HISdans l'hyperaccumulateur (Ingle et al., 2005). La chélation du Ni par l'HIS pourrait alors

supprimer son transport vers les vacuoles des cellules racinaires, et améliorer sa mobilité et son transport radial dans le symplasme, de façon analogue auZn (Richau et al., 2009; Kozhevnikova et al., 2014). La fourniture de l'HIS à des hyperaccumulateurs et à des non-hyperaccumulateurs pourraient augmenter leur tolérance au Ni et le transfert de la racine vers les parties aériennes (Kramer et al., 1996 ; Kerkeb et Krämer 2003). En plus de l'HIS, la nicotianamine (NA) pourrait également augmenter le transport du Ni (Cornu et al., 2014).

En atteignant le parenchyme du xylème, le Ni serait exporté vers les vaisseaux du xylème. La part du Ni transloqué des racines vers les parties aériennes serait similaire pour l'hyperaccumulateur *T. goesingense* et le non-hyperaccumulateur *T. arvense* (Kramer et al., 1997). Cependant, comme aucun transporteur d'efflux spécifique au Ni n'a identifié, il n'est pas encore prouvé que le Ni est transporté efficacement vers le xylème. Après la pénétration dans les vaisseaux du xylème, le transport du Ni est principalement assuré par la transpiration foliaire (Robinson et al., 2003 ; Centofanti et al., 2012).

La spéciation du Ni dans le xylème des hyperaccumulateurs a été largement étudiée. Les mesures montrent que 48% du Ni de la sève brute de *A. lesbiacum* est sous forme d'ion hydraté libre, tandis que les autres fractionssont chélatées par l'HIS (19%), la glutamine (15%), le citrate (9%) et le malate (3%) (Kramer et al., 1996). Le Nide la sève brute d'*Alyssum* ssp. *Lusitanicum* estaussi principalement sous forme de cation hydraté libre (environ 70%) (Alves et al., 2011). Chez *N. caerulescens* la part du Ni de la sève brute complexée par NA est très faible (8,5%) (Mari et al., 2006). Chez les espèces du genre *Alyssum*, les concentrations en chélateurs organiques sont trop faibles pour que tout le Ni présent dans la sève brute soit sous forme de complexe, confirmant ainsi que le Ni est essentiellement présent sous la forme de cation hydraté (Centofanti et al., 2013).

Les feuilles sont les principaux organes de stockage de la grande majorité du Ni issu des racines.Lamicroanalyse de feuilles de *A. lesbiacum*, *A. bertolonii* et *T. goesingense*a montré que le Ni est distribué de manière préférentielle dans les cellules de l'épiderme, très probablement dans les vacuoles (Küpper et al., 2001 ; Broadhurst et al.,2004). Les

transporteurs d'efflux de cations, par exemple TgMTP1 et PgIREG1,sont fortement exprimés dans le tonoplaste, et transfèrent efficacement le Ni dans les vacuoles des cellules des feuilles (Persans et al., 2001 ; Merlot et al., 2014). L'étude par Micro-PIXE de la localisation du Ni dans *A. lesbiacum* a montré que le Ni est trèsfortement séquestré dans les trichomes épidermiques sur la surface des feuilles (Krämer et al., 1997). L'enrichissement de Ni a également été observé dans les pointes des feuilles (McNear et al., 2005). En dépit du stockage préférentiel du Ni dans les sites non actifs, comme les vacuoles des cellules épidermiques, la basedes trichomes et l'apoplasme, il convient de noter que le mésophylle est aussi un compartiment important de stockage du Ni dans les feuilles (Broadhurst et al., 2004).

Pour atténuer l'effet toxique, le Ni dans les feuilles de hyperaccumulateurs est principalement complexé par des acides carboxyliques. Le chélateur principal du Ni dans plusieurs hyperaccumulateurs ligneux est l'acide citrique (Lee et al., 1977; 1978). Alors que chez les espèces du genre *Alyssum*, le Ni est associé principalement avec les acides malique et malonique (Brooks et al., 1981 ; Montarges-Pelletier et al., 2008).

Le transport du Ni dans le xylème et la compartimentation du métal dans les feuilles sont maintenant bien élucidés. Par contre, peu d'attention n'a été accordée aux processus de translocation du Ni via le phloème dans les hyperaccumulateurs, malgré des observations suggérant l'importance de cette voie de transport. En particulier, un enrichissement enNi dans le phloème a été enregistrépour unegamme d'hyperaccumulateurs ligneuxtropicaux. Par exemple, la sève élaborée contient 16,9% de Ni chez l'hyperaccumulateur *Phyllanthus balgooyi* de Sabah (van der Ent et Mulligan, 2015). De même, la sève du phloème de *Euphorbia helenae* subsp. *grandifolia*, de Cuba, contient 3,1% Ni (Reeves et al., 1996). Cependant, la fonction de translocation du phloème et la spéciation de cette grande concentration de Ni dans la sève élaboréedes hyperaccumulateurs restent encore inconnue.

Objectifs de la thèse

En résumé, l'absorption par les racines et les processus de translocation, notamment via le phloème ont suscité encore trop peu d'intérêt et sont encore mal connus, alors que ces processus sont essentiels pour expliquer le phénomène d'hyperaccumulation. Ainsi, cette thèse a été entreprise pour acquérir une meilleure compréhension de l'absorption du Ni et de son transport chez les hyperaccumulateurs, notamment en ce qui concerne le rôle du phloème. L'hyperaccumulateur de Ni et de Zn, *Noccaea caerulescens* (J. & C. Presl) FK Mey (anciennement *Thlaspi caerulescens),* considéré comme une espèce modèle dans les études des mécanismes de hyperaccumulation (Milner et Kochian 2008, Krämer 2010) a été principalement étudié. L'espèce a été comparée à un autre hyperaccumulateur de Ni, *A. murale*, déjà bien connu, dont les perspectives d'application en agromine sont démontrées (Bani et al., 2007) et le non-hyperaccumulateur *Thlaspi arvense*.

Fractionnement isotopique du Ni chez les hyperaccumulateurs et ses implications

Des études récentes ont suggéré que le fractionnement isotopique dans les plantes supérieures pourrait être utilisé pour tracer les processus physiologiques impliqués dans l'homéostasie des métaux. Ainsi, le transport à haute affinité favorise les isotopes lourds, tandis que le transport à faible affinité, par exemple par canal ionique et pompe électrogénique, favorise les isotopes légers (Weiss et al., 2005). Le passage du transport d'une haute àune faible affinité se traduirait alors par un déplacement isotopique de -0,2 à -0,8 ‰ pour l'absorption du Zn par des diatomées marines (John et al., 2007). Par conséquent, l'analyse de la composition isotopique pourrait être un outil utile pour étudier les systèmes de transport en métal fonctionnant dans les plantes supérieures.

Trois espèces avec différents modalités d'accumulation du métal, à savoir le non-hyperaccumulateur *T. arvense* L., l'hyperaccumulateur Ni *A. murale* Waldst. & Kit., et l'hyperaccumulateur de Ni/Zn *N. caerulescens* (J. & C. Presl) F. K. Mey ont été cultivées en hydroponie avec des concentrations basse (2 µM) et haute (50 µM) de Ni et Zn. Le

fractionnement isotopique entre la plante et les substrats, ainsi que les parties aériennes et les racines ont été ensuite mesurés.

Les résultats ont montré que toutes les plantes avaient tendance à absorber les isotopes légers du Ni. Les valeurs de $\delta^{60}Ni_{plante\text{-}solution}$ variaient de -0,90 à -0,21‰, vraisemblablement en raison de l'implication de systèmes de transport à faible affinité à travers la membrane cellulaire de la racine. Le fractionnement isotopique du Ni entre la plante et la solution a été plus important dans les hyperaccumulateurs cultivés en présence d'une faible concentration en Zn ($\delta^{60}Ni_{plante\text{-}solution}$ = -0,63 à -0,90 ‰) que dans le non-accumulateur *T. arvense* ($\delta^{60}Ni_{plante\text{-}solution}$ = -0.21‰), ce qui indique une plus grande perméabilité du système de transport à faible affinité chez les hyperaccumulateurs. En outre, le Zn pourrait rivaliser avec le Ni pendant le processus d'absorption, ce qui réduirait la concentration du Ni dans les plantes et entraînerait la diminution de l'amplitude du fractionnement isotopique du Ni ($\delta^{60}Ni_{plante\text{-}solution}$ = -0,07 à -0,11‰). Ainsi, nous démontrons que les plantes pourraientprélever le Ni par l'intermédiaire des systèmes de transport du Zn .

Interactions du Ni avec d'autres éléments chezNoccaea caerulescens

Trois expériences ont été menées pour étudier l'effet d'éléments minéraux (Zn, Fe, Mn, Cu et Co) sur l'absorption du Ni et les transporteurs potentiels de Ni. Dans l'expérience 2.1, *N. caerulescens* a été cultivé dans des solutions nutritives contenant 0, 10, 50 et 100 µM de Ni; dans l'expérience 2.2, les plants ont été traités avec différentes concentrations de Zn, Fe et Co (tableau 2.1). Les concentrations en éléments nutritifs dans les racines et les parties aériennes ont été analysées. Dans l'expérience 2.3, les niveaux d'expression génique du transporteur deZn ZIP10 et du transporteur de Fe IRT1 ont été déterminés par Q-PCR.

Les résultats ont montré que *N. caerulescens* pourrait accumuler des concentrations exceptionnelles de Zn et Ni dans ses feuilles. Une forte corrélation négative a été observée entre les concentrations de ces deux éléments dans les plantes. Les résultats de la Q-PCR ont

également montré que l'exposition du Ni pourrait stimuler l'expression de NcZIP10 dans les racines, ce qui indique que le Zn et le Ni sont en concurrence pour les sites de liaison dans les membranes des cellules de la racine.

Les interactions entre Ni et Fe sont cependant différentes. Plus la concentration du Ni en solution est élevée plus l'absorption du Fe est stimulée. Egalement, la carence Fe augmente de manière significative l'absorption du Ni. De plus, la présence du Ni pourrait augmenter le niveau d'expression de l'IRT1 dans les racines de *N. caerulescens*. Ces résultats suggèrent fortement que le Ni pourrait être absorbé en partie des systèmes de transport du Fe.

Dans l'expérience 2.2, nous avons également testé l'interaction entre le Ni et le Co. *N. caerulescens* semble préférer le Coau Ni. Une grande concurrence entre les deux métaux existe au cours du processus d'absorption. Le Co est pas considéré comme un élément essentiel et il est habituellement à l'état de traces dans les plantes supérieures (Epstein et Bloom, 2005). Il ne devrait pas exister de système de transport spécifique du Co chez les plantes supérieures. Par conséquent, la grande interférence entre Co et Ni indique que le Ni est absorbé par les transporteurs non-spécifiques dans les hyperaccumulateurs.

Distribution du Ni dans des feuilles de N. caerulescens pendant le transport dans le xylème et la translocation dans le phloème

Les feuilles matures sont un puits important pour le xylème et une source de Nipour le phloème, tandis que les jeunes feuillesaccumulent peu à partir du xylème mais beaucoup à partir du phloème. Par conséquent, il était nécessaire de préciser les contributions respectives du transport par le xylème et lephloème dans la translocation et l'accumulation du Ni dans les feuilles de différents âges.

Dans l'expérience 4.1, *N. caerulescens* a été cultivé dans une solution nutritive contenant du Ni, du Sr et du Rb. Le Sr est un élément immobiledans le phloème, tandis que le Rb est

facilement transportable dans le phloème (Kuppelwieser et Feller, 1991). Par conséquent, le Sr peut être unindicateur pour le transport dans le xylème alors que le Rb un indicateur pour la translocation dans le phloème. Les concentrations de Ni, Sr et Rb dans les feuilles jeunes et les feuilles âgées de *N. caerulescens*ont été déterminées. Les résultats ont montré que les feuilles âgées de *N. caerulescens* contiennent plus de 60% de Ni, Rb et Sr totaux contenus dans les parties aériennes. Cela démontre que les feuilles âgées sont le principal puits du Ni transporté par le xylème. Les fortes concentrations de métaux dans les jeunes feuilles suggèrent également que ces tissus sont un puits actif pour le transport de xylème. L'augmentation du rapport Ni/Sr de 0,74 (traitement à 6 jours) à 1,09 (traitement à 42 jours) indique qu'une quantité additionnelle de Ni a atteint les jeunes feuilles, probablement via le phloème. Cette translocation à partir du phloème est sans doute très importante pour l'accumulation de Ni dans les jeunes feuilles, quand le Ni est présent dans les feuilles âgées à forte concentration.

Dans l'expérience 4.2, une application foliaire de Ni a été effectuée pour étudier plus précisément le mouvement vers le haut et vers le bas du Ni pendant la translocation dans le phloème. Du$^{61}Ni^{2+}$ a été appliqué aux vieilles feuilles en pulvérisation foliaire et le contenu de ^{61}Ni dans les jeunes feuilles et les racines ont ensuite été déterminées. Nous avons mesuré que 89% du^{61}Ni sont exportés depuisles feuilles âgées vers les jeunes feuilles, tandis que seulement 11% a été déplacé vers les racines, ce qui soutient que les jeunes feuilles sont le puits principal pour la translocation du phloème.

Spéciation du Ni dans la sève élaborée de N. caerulescens

Dans le chapitre précédent, nous avons constaté que la translocation via le phloème peut transférer quantité importante de Ni pour les jeunes feuilles de *N. caerulescens*. Des observations de terrain ont également montré que les hyperaccumulateurs de Ni qui poussent sur les sols ultramafiques peuvent accumuler des concentrations élevées de Ni dans les fleurs et dans les graines (Robinson et al., 1997, Zhang et al., 2014 ; Groeber et al., 2015). Comme

les organes reproducteurs sont généralement considérés comme étant peu alimentés par lexylème mais essentiellement par lephloème (Taiz et Zeiger 2010), ces résultats confirment que le Ni peut être efficacement transloqué via le phloème dans ces hyperaccumulateurs. En outre, l'enrichissement duNi dans le phloème a été rapporté pour un ensemble d'hyperaccumulateurs ligneux tropicaux (van der Ent et Mulligan, 2015).

Ainsi, dans cette dernière partie, nous avons porté l'attention sur les propriétés de la sève du phloème de *N. caerulescens*. Les objectifs étaient les suivants: i) mesurer les concentrations de Ni dans la sève élaborée ; ii) rechercher les ligands complexants potentiels pour le Ni dans la sève du phloème.Des exsudats des feuilles de *N. caerulescens* ont été extraits par la méthode de l'exsudation stimulée par l'EDTA. Les concentrations des métaux et des composés organiques dans les exsudats ont été mesurées.Les résultats ont montré que les exsudats du phloème sont enrichis en Ni. Les concentrations sont beaucoup plus grandes que celles observées dans la sève élaborée des plantes non-hyperaccumulatrices ainsi que dans la sève brute des hyperaccumulateurs (Álvarez-Fernández et al., 2014). Ceci confirme la très haute capacité du phloème à se charger en Ni et à le transloquer au sein de *N. caerulescens*. Si des acides aminéscomme, par exemple, la NA et l'HIS, étaient présents dans les exsudats, leurs concentrations étaient largement insuffisantes pour assurer la chélation du Ni. Au contraire, les acides organiques, en particulier le malate, étaient présents à des concentrations élevées dans tous les traitements. Nous démontrons ainsi qu'ils sont probablement très impliqués dans la complexation du Ni dans le phloème.

Conclusions

Dans cette thèse, nous avons mis l'accent sur les processus d'absorption et de transport du Ni et mené une série d'expériences afin de clarifier les modalités d'absorption du Ni par les racines et la translocation du métal par le xylème et le phloème. Les principales conclusions du travail permettent de mieux comprendre les processus d'hyperaccumulation. Tout d'abord, les hyperaccumulateurs possèdent un système de transport du Ni à faible affinité et à haute

efficacité. L'absorption du Ni semble impliquerdes transporteurs de Zn et Fe. Ensuite, le transport au sein du xylème est de première importance pour l'accumulation du Ni dans les feuilles, en particulier dans les feuilles matures. Mais le nickel peut aussi être efficacement transporté dans le phloème, puis transféré à partir des feuilles âgées vers les jeunes feuilles. Le phloème joue donc un rôle essentiel dans l'accumulation du Ni dans les jeunes feuilles. Enfin, dans le phloème, le Ni est principalement chélaté par des acides organiques, de type malate. Ces avancées contribuent à une connaissance plus complète de l'ensemble des processus qui conduisent à l'hyperaccumulation du Ni, notamment chez l'hyperaccumulateur *N. caerulescens* (Figure 6.1).Au plan pratique, les procédés de dépollution des sols par phytoextraction ou de valorisation des métaux stratégiques contenus dans les sols par la voie de l'agromine, qui utilisent des plantes hyperaccumulatrices,peuvent ainsi bénéficier, pour leur optimisation, de ces connaissances nouvelles sur l'homéostasie du Ni chez les hyperaccumulateurs.

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1. Literature review and objectives of the thesis

1.1 Discovery of metal hyperaccumulators and their application

Nickel is the latest element to be enlisted as one of the essential mineral elements in higher plants (Marschner 1995; Gerendas et al. 1999; Epstein and Bloom 2005). Although Ni concentrations in plants are comparatively low, normally ranging from 0.01-5 mg kg^{-1} (Welch 1981), this element was found to play an important role in plant physiology. Nickel was discovered as an active center of urease (Dixon et al. 1975), an enzyme that is widely distributed in higher plants (Hogan et al. 1983) and with a function of catalyzing the hydrolysis of urea, Therefore, the existence of urease could prevent the accumulation of urea, which is generated during the metabolic process and would be toxic to plants when presents in high concentrations. As an irreplaceable metallic center in urease, Ni is essential for higher plants though it is usually presented in ultramicro concentrations. In spite of the effect of urease activation, other physiological functions of Ni still remain obscured in higher plants (Gerendas et al. 1999).

There has been much more concern on the soil contamination of Ni than its deficiency due to the increasing Ni emission and contamination around the world (Nieminen et al. 2007). Serpentine regions are one of the most widely distributed Ni contaminated areas due to the weathering of Ni-rich ultramafic rocks. The Ni contents in serpentine soil usually vary from 1000 to 7000 mg kg^{-1} (Li et al. 2003). To cope with these high levels of metal, native plant species have evolved several mechanisms. Exclusion and accumulation are the two basic strategies for plants in response to the metalliferous environment (Baker 1981). Most plants choose the strategy of exclusion to alleviate metal stress. This strategy has been grouped into two classes, internal mechanisms where metals enter the symplast but are subsequently rendered harmless, and exclusion mechanisms where tolerance is based on the plant's ability to prevent entry of metals into the symplast (Taylor 1987). Whichever mechanism takes effect, it would result in metal retention in roots.

In comparison to metal exclusion, hyperaccumulation of Ni is a relatively rare response of endemic species to serpentine soils (Baker 1987). The plants which are able to accumulate Ni

to 1000 mg kg^{-1} (0.1%) dry weight in their shoots are defined as hyperaccumulators (Jaffre et al. 1976; Brooks et al. 1977). Approximately 400 species of Ni hyperaccumulator have been identified so far (van der Ent et al. 2013). Instead of using root sequestration for metal detoxification, hyperaccumulators choose the strategy to transfer most Ni from roots to shoots, and finally store and detoxify the metal in leaves. Therefore, the Ni concentration in shoots is usually (much) higher than that in roots. Some reported that these plants were capable of accumulating large amounts of Ni (up to 3%) in their leaves (Reeves et al. 1983). Chaney (1983) first recognized this outstanding characteristic and put forward the idea of phytoextraction by means of hyperaccumulators, which has become a research hotspot since the 90s (Salt et al. 1998; Pilon-Smits 2005). Some researchers also conducted phytomining tests, which grew hyperaccumulators on contaminated soil and then recover the metal from harvested plant biomass, which can mitigate soil pollution and obtain economic benefits simultaneously (Nicks and Chambers 1995; Robinson et al. 1997a; Robinson et al. 1997b; Li et al. 2003; Bani et al. 2007).

Nickel seems to be the best candidate for agromining (the whole agronomic process of phytoming; van der Ent et al. 2015) due to its high market price and vast area of suitable mining area, e.g. serpentine regions. The optimization of agromining technologies requires a thorough knowledge of the metal extraction processes and mechanisms. Hence, it is extremely important to understand the physiology, particularly the metal uptake and transport mechanisms, of Ni hyperaccumulators to improve the phytoextraction efficiency.

1.2 Hyperaccumulation mechanisms: general concept

The past decades have witnessed a great progress on the understanding on the physiology of hyperaccumulators. Much knowledge has been gained, in particular for Zn hyperaccumulation processes (Milner and Kochian 2008). The general hyperaccumulation mechanisms described in this section come mainly from the results of these researches.

(1) *Stimulated metal absorption in roots.* Metal influx transporters are generally highly expressed in root cell membranes of hyperaccumulators, regardless of plant metal status (deficient or sufficient). For instance, *ZNT1*, a high-affinity Zn transporter was expressed at

very high levels in roots of *Noccaea caerulescens*, which resulted in increased Zn influx into root symplast (Pence et al. 2000).

(2) *Reduced metal sequestration in root vacuoles.* Metal ions in root symplast are readily chelated by organic ligands (Haydon and Cobbett 2007). It was demonstrated that Zn was mainly bound to histidine (His) in roots of *N. caerulescens* (Salt et al. 1999), and the complexation by His could inhibit Zn sequestration into cell vacuoles (Kozhevnikova et al. 2014a). Hyperaccumulators usually stored much less metal in its root vacuoles compared to non-hyperaccumulators (Lasat et al. 1998).

(3) *Efficient xylem loading and transport.* In hyperaccumulators, metals in roots could be efficiently transported out from the symplast to xylem vessels. This exceptional capability is suggested to play a key role for metal hyperaccumulation (Krämer 2010; Sterckeman et al. 2015). Hanikenne et al. (2008) demonstrated that *HMA4*, a Zn efflux transporter gene, was highly expressed in the Zn hyperaccumulator *Arabidopsis halleri*, which resulted in highly Zn efflux into xylem. Similar gene was identified in *N. caerulescens* (Papoyan and Kochian 2004). Metals in xylem sap are readily transferred upward to shoots, following the transpiration flow. Results showed that Zn was mainly present as hydrated cation during the xylem transport (Lu et al. 2013; Salt et al. 1999).

(4) *Strong metal sequestration and compartmentation in leaves*. Leaves are the major sink for heavy metals in hyperaccumulators. Most metal ions would be absorbed from xylem sap and transferred into leaf symplast. Küpper et al. (1999) found that Zn was preferentially stored in epidermal cell vacuoles, the non-active sites of leaves, in which Zn was mainly bound to organic acids for detoxification (Salt et al. 1999).

Figure 1.1 briefly depicts the above four processes.

Moreover, a recent study revealed that a Zn hyperaccumulator had enhanced metal remobilization ability by phloem translocation (Lu et al. 2013), which may be also an important process involving in metal hyperaccumulation.

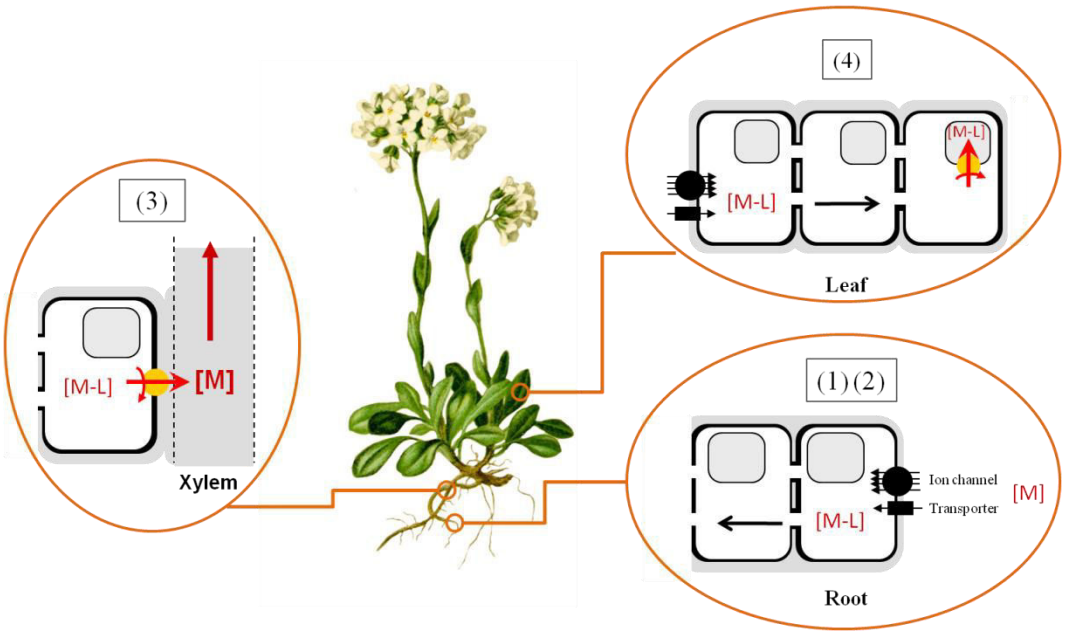

Figure 1.1 General mechanisms involving in metal hyperaccumulation, which includes (1) stimulated metal absorption in roots, (2) reduced metal sequestration in root vacuoles, (3) efficient xylem loading and xylem transport and (4) strong metal sequestration and compartmentation in leaves. [M]: Metal ion; [M-L]: Metal-ligand complex.

1.3 Ni homeostasis in higher plants: current understanding

1.3.1 Ni uptake process

Indirect results indicated that Ni is absorbed by plants as Ni ion (Ni^{2+}). Addition of chelating agents (citric acid, EDTA, NTA, DTPA, etc.) would decrease the uptake of Ni by *Berkheya coddii* in spite of an increase in the available Ni content in soil (Robinson et al. 1999; Robinson et al. 1997a), presumably due to a reduction of Ni ion concentration after chelation. Likewise, it was demonstrated that the Zn/Ni hyperaccumulator *Thlaspi goesingense* took up Ni^{2+} but excludes metal–organic complexes (Puschenreiter et al. 2005). Kerkeb and Kramer (2003) also proved that Ni was taken up by the non-hyperaccumulator *Brassica juncea* predominately as free aqueous cation.

Few studies had been conducted to investigate the uptake pattern of Ni in higher plants. Nickel absorption in roots was found to be inhibited by low temperature, metabolic inhibitors and anaerobic root conditions (Aschmann and Zasoski 1987; Puschenreiter et al. 2005), suggesting that the uptake process is a metabolic-dependent process. Cataldo et al. (1978)

showed that Ni absorption by soybean seedlings was a kinetic process, which fits Michaelis-Menten kinetics. Aschmann and Zasoski (1987) had measured the Michaelis-Menten constant (K_m) of Ni uptake in oat plants, the value of which was 12 μM. Larger K_m value (80 μM) was found in the root protoplast of mung bean (*Vigna radiata*) (Zhang et al. 2001). In the Ni hyperaccumulator *T. goesingense*, the K_m value was measured to be 36.1 μM (Puschenreiter et al. 2005). The relatively large K_m values indicate that Ni might be absorbed via low-affinity transport systems in higher plants. To date, no high-affinity Ni transporter has ever been identified in higher plants.

Interaction between Ni and other cations were also observed during root uptake process. Competition kinetic studies showed that in soybean and barley, Cu^{2+} and Zn^{2+} inhibited Ni^{2+} influx strongly and competitively, while Ca^{2+} and Mg^{2+} were non-competitive inhibitors of Ni^{2+} influx (Cataldo et al. 1978; Körner et al. 1987). In *Arabidopsis thaliana*, Fe deficiency increased Ni accumulation in roots, while Ni exposure stimulated Fe uptake (Nishida et al. 2011). A molecular study had shown that Ni could be absorbed via the Fe^{2+} transporter, *IRT1* in *A. thaliana* (Nishida et al. 2011; 2012), due to the lack of substrate specificity of *AtIRT1* (Schaaf et al. 2006).

Similarly, for the Zn/Ni hyperaccumulator *Thlaspi pindicum*, Ni absorption was inhibited by the addition of Zn^{2+} in hydroponic culture solution, whereas Ni had little effect on Zn uptake (Taylor and Macnair 2006). Likewise, several Ni-hyperaccumulating populations of *N. caerulescens* were shown to prefer Zn over Ni in experimental conditions (Assunção et al. 2001). Moreover, Fe accumulation in roots of the Ni hyperaccumulator *Alyssum inflatum* was stimulated when exposed to elevated Ni concentrations (Ghasemi et al. 2009). Halimaa et al. (2014) suggested that the Fe^{2+} transporter *IRT1* and Zn transporter *ZIP10* might be involved in Ni accumulation in *N. caerulescens*, according to SOLiD high-throughput sequencing of the root transcriptomes study. In addition to Zn and Fe, Ni seems to interact with Mn during the uptake process, as negative correlation was found between the Mn and Ni concentrations in leaves of the Ni hyperaccumulator *Alyssum murale* (Leigh Broadhurst et al. 2009). Interestingly, Reeves and Baker (1984) found that *T. goesingense* grown on metal-enriched substrates accumulated similar, extremely high concentrations of Ni, Zn, Mn and Co in their shoots. These results suggest that Ni absorption in roots may involve the transporters of other

divalent micronutrient elements, e.g. Zn, Fe and Mn. However, the competition effect of these elements on Ni is yet to be clarified.

1.3.2 Xylem loading and transport process

When reaching cytoplasm of root cells, metal ions would rapidly be complexed by organic ligands to prevent precipitation (Haydon and Cobbett 2007), and amino acids are favorable for metal complexation in such alkaline condition (Harris et al. 2012).

His concentrations were found to be constitutively high in roots of the Ni hyperaccumulator *Alyssum lesbiacum* in comparison to the non-hyperaccumulator *B. juncea* (Kerkeb and Krämer 2003), which is due to the overexpression of the His biosynthetic pathway in the hyperaccumulator (Ingle et al. 2005). Interestingly, the Ni chelation with His could suppress Ni transport into root vacuoles, and thus enhance the Ni mobility and facilitate radial transport in root symplast, which is quite similar to that of Zn (Richau et al. 2009; Kozhevnikova et al. 2014b). Supplying His to both hyperaccumulators and non-hyperaccumulators could increase both their Ni tolerance and root to shoot transport (Kramer et al. 1996; Kerkeb and Krämer 2003). In addition to His, nicotianamine (NA) could also increase root – shoot transport of Ni (Cornu et al. 2014).

When reaching xylem parenchyma, Ni would be exported to the xylem vessels. Kramer et al. (1997) showed that Ni translocation rates from roots to shoots were the same in the hyperaccumulator *T. goesingense* and the non-hyperaccumulator *T. arvense*, as long as both species were unaffected by Ni toxicity. It is still arguable if Ni could be efficiently transported out to the xylem, as specific Ni efflux transporter has not been identified yet. When entering xylem vessels, Ni transport is mainly driven by leaf transpiration (Robinson et al. 2003; Centofanti et al. 2012).

The Ni speciation in xylem sap of hyperaccumulators has been extensively studied. Kramer et al. (1996) first reported that 48% Ni in xylem sap of *A. lesbiacum* remained as free hydrated ion, while other fraction was chelated by histidine (19%) , glutamine (15%) , citrate(9%) and malate (3%). A field study conducted by Alves et al. (2011) showed that Ni existed in the xylem sap of *Alyssum serpyllifolium* ssp. lusitanicum occurs mainly as a free hydrated cation (about 70%) and complexed with carboxylic acids, e.g. citric acid (18%). Mari et al. (2006)

demonstrated that a fraction of Ni (8.5%) was chelated by NA in xylem sap of *N. caerulescens*. A recent study showed that in several *Alyssum* species, the concentrations of organic chelators were too low to account for the complexation of all the Ni present in the xylem sap, and suggested that most of the Ni in xylem sap is present as the hydrated cation (Centofanti et al. 2013).

1.3.3 Xylem unloading and leaf comparmentation process

When reaching leaves, Ni would be unloaded from xylem and transferred into leaf symplast. Leaves are the major storage organs for the large amount of Ni translocated from roots. To clarify the Ni distribution in leaves, Küpper et al. (2001) conducted a pot experiment, using three Ni hyperaccumulators, i.e. *A. lesbiacum*, *Alyssum bertolonii* and *T. goesingense*. The energy dispersive X-ray microanalysis showed that Ni was distributed preferentially in the epidermal cells, most likely in the vacuoles. This finding was confirmed by Broadhurst et al. (2004) and Tappero et al. (2007), who found that Ni was highly concentrated in epidermal cell vacuoles in *A. murale*. Molecular studies have showed that cation efflux transporters, e.g. *TgMTP1* and *PgIREG1* were highly expressed in tonoplast, which efficiently transfer Ni into leaf cell vacuoles (Persans et al. 2001; Merlot et al. 2014). Micro-PIXE was also used to investigate the Ni localization in *A. lesbiacum*, and results showed that Ni was sequestered to a considerable degree within the epidermal trichomes on the leaf surface (Krämer et al. 1997). In the asteraceous Ni hyperaccumulator *B. coddii*, significantly higher Ni concentrations were found in the apoplast of the upper epidermis cells, especially within the cuticle (Robinson et al. 2003). Nickel enrichment was also observed in the leaf tips, possibly resulting from release of excess Ni with guttation fluids (McNear et al. 2005). In spite of the preferential storage of Ni in non-active sites of leaf, such as epidermal cell vacuoles, trichome bases and apoplast, it was noteworthy that palisade mesophyll was an increasingly important compartment as Ni concentrations in leaves increased (Broadhurst et al. 2004).

To alleviate the toxic effect, Ni in leaves of hyperaccumulators is mainly complexed by carboxylic acids. Field investigations had showed that the main chelator for Ni in several woody hyperaccumulators is citric acid (Lee et al. 1977; 1978; Callahan et al. 2012). While in

Alyssum species, Ni was associated principally with malic and malonic acids (Brooks et al. 1981; Montargès-Pelletier et al. 2008).

1.3.4 Phloem translocation process

Compared to the extensive studies on xylem transport and leaf compartmentation processes, little attention has been paid to Ni phloem translocation process in hyperaccumulators. Nickel enrichment in phloem has been documented in a number of woody hyperaccumulators growing on tropical ultramafic soils. For instance, 16.9% Ni was found in the freeze-dried phloem sap of the Ni hyperaccumulator *Phyllanthus balgooyi* from Sabah, Malaysia (van der Ent and Mulligan 2015). The phloem sap of *Euphorbia helenae* subsp. *grandifolia*, which grows in Cuba, also contained 3.1% Ni (Reeves et al. 1996). However, the speciation of such highly concentration of Ni in phloem sap and the function of phloem translocation in hyperaccumulators still remain obscured.

In non-hyperaccumulators, Ni is found to be rather mobile in phloem and can be readily transferred from sources to sinks (Neumann and Chamel 1986; Page and Feller 2005; Page et al. 2006). Both downward and upward movements exist during phloem translocation. Riesen and Feller (2005) found that radioactive ^{63}Ni fed into the leaf lamina in wheat seedlings was rapidly transferred up to younger leaves and down to roots. Fismes et al. (2005) also found that in three vegetables (lettuce, radish and bean), ^{63}Ni migrated throughout the whole plants following foliar application, and mainly toward young leaves, seeds and roots. Wiersma and van Goor (1979) found that Ni was bound to organic compounds with a molecular weight in the range of 1000 – 5000 in phloem sap of *Ricinus communis*.

In summary, the xylem transport and leaf compartmentation processes involving Ni hyperaccumulation have been extensive studies during the past decades, and much knowledge has been obtained regarding these topics. In comparison, the root uptake and phloem translocation processes are still poorly understood.

1.4 Objectives of the thesis

This thesis was undertaken to gain a better understanding on the Ni uptake and transport in

hyperaccumulators. *Noccaea caerulescens* (J. & C. Presl) F. K. Mey (formerly *Thlaspi caerulescens*), which is a Ni/Zn hyperaccumulator and regarded as a model species in the studies of hyperaccumulation mechanisms (Milner and Kochian 2008; Krämer 2010), was used as the main plant candidate. Also, the Ni hyperaccumulator *A. murale* and non-hyperaccumulator *Thlaspi arvense* were used in some experiments. The objectives of the thesis are to explore (1) the properties of Ni uptake systems in roots, and (2) how Ni moves in phloem and the significance of phloem translocation on Ni hyperaccumulation.

And here are the aims and approaches of each chapter:

In Chapter 2, the function of high- and low-affinity transport systems in roots of hyperaccumulators was explored with the use of isotope fractionation analysis.

In Chapter 3, the interaction between Ni and other mineral nutrient elements (Zn, Fe, Co, etc.) in *N. caerulescens* during the root uptake process was examined by means of absorption kinetics and gene expression studies.

In Chapter 4, the contribution of xylem transport and phloem translocation on Ni accumulation in young and old leaves of *N. caerulescens* was explored by root and leaf Ni application.

In Chapter 5, the compositions of phloem sap, including metal, organic and amino concentrations, were analyzed to clarify the Ni enrichment and speciation in phloem. Phloem sap was extracted by the EDTA-stimulated phloem exudation method.

Figure 1.2 sums up the challenges of the thesis.

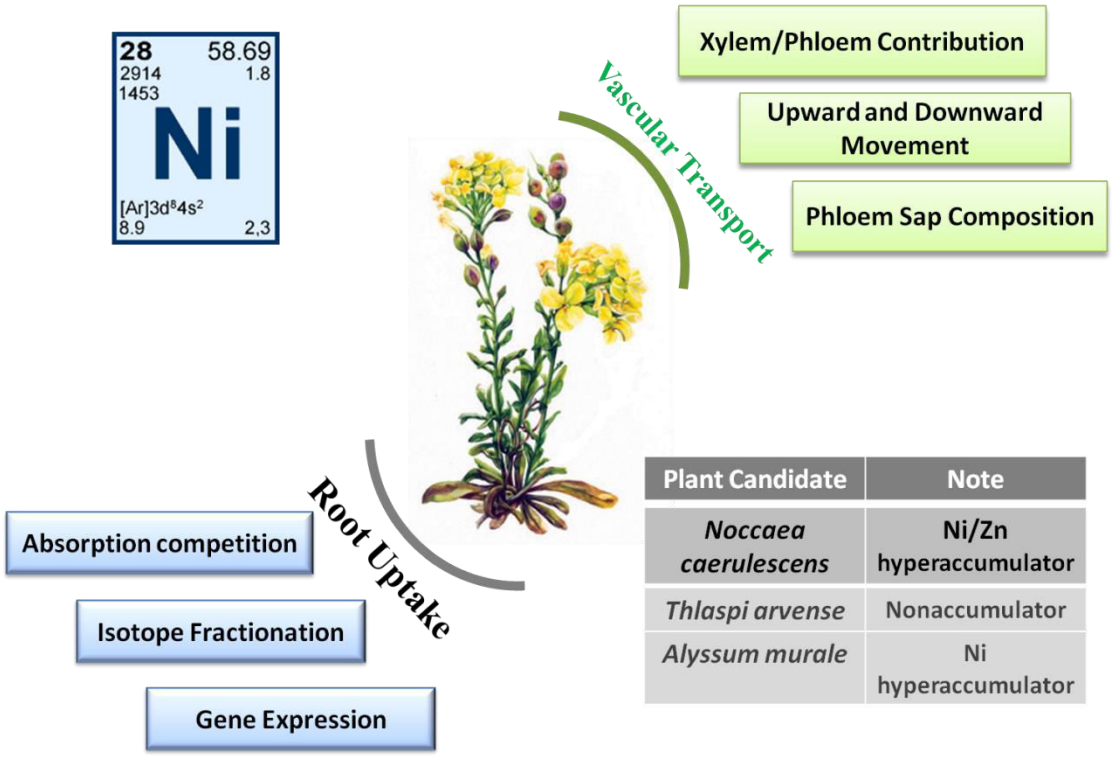

Figure 1.2 Main structure of the thesis. This study mainly focused on the Ni root uptake and vascular transport processes in the hyperaccumulator *N. caerulescens*. In the first section of thesis (Chapter 2 and 3), we conducted isotope fractionation, absorption kinetics and molecular studies to clarify the properties of Ni transport systems in roots; while the function of phloem translocation on Ni accumulation in shoots and the characteristics of phloem translocation (bidirectional movement, phloem sap compositions) were explored in the second part of the thesis (Chapter 4 and 5).

2. Isotope fractionation of Ni in hyperaccumulators*

2.1 Introduction

The Ni absorption mechanisms in hyperaccumulators as well as normal plants (non-hyperaccumulators) are far from being fully understood. In non-hyperaccumulators, Ni seems to be taken up via low-affinity transport systems. Ni absorption by soybean plants grown in various Ni concentrations fits Michaelis-Menten kinetics (1978), and measurement of the Michaelis-Menten constant (K_m) for Ni uptake in oat plant showed that the K_m value was 0.012 mM (Aschmann and Zasoski 1987). Similar K_m values (0.08 – 0.10 mM) for the symplastic influx were recorded for the Ni hyperaccumulator *Leptoplax emarginata* and non-hyperaccumulator maize (*Zea mays*) (Redjala et al. 2010). However, until now, no high-affinity Ni transporter has been identified in higher plants, and little is known about how hyperaccumulators are able to take up Ni so efficiently.

Recent studies have suggested that isotope fractionation in higher plants could be used to trace the physiological process involved in metal homeostasis. Weiss et al. (2005) have proposed that carrier-mediated transport, or high-affinity transport, favors heavy isotopes, while low-affinity transport, e.g. ion channel and electrogenic pump, favors light isotopes. This is consistent with the fact that light isotopes can move faster, while heavy isotope can form stronger covalent bonds. Low affinity transporters generally can move ions at rates of several millions per second, which facilitate the assimilation of light isotopes, while carrier uptake involves covalent binding which facilitate heavy isotopes (Weiss et al. 2005; Maathuis 2007). John et al. (2007) demonstrated that a switch from high- to low-affinity transport would result in an isotopic shift from -0.2 to -0.8‰ for Zn uptake in marine diatoms. Therefore, isotopic composition analysis could be a useful tool to study the functioning metal transport systems in higher plants.

*Deng THB, Cloquet C, Tang YT, Sterckeman T, Echevarria G, Estrade N, Morel JL, Qiu RL, 2014. Nickel and zinc isotope fractionation in hyperaccumulating and nonaccumulating plants. *Environmental Science & Technology* 48(20):11926-33.

To date, most of the studies regarding isotope fractionation of micro-nutrients in higher plants have focused on Zn, Fe and Cu. Little is known about Ni isotope fractionation in plants. Therefore, the objectives of this work were therefore: 1) to study the Ni isotope fractionation between plant organs and the growing media; 2) to identify the Ni transport systems in both hyperaccumulators and non-hyperaccumulators. Three plant species with different metal accumulation patterns, i.e. the non-hyperaccumulator *Thlaspi arvense* L., the Ni hyperaccumulator *Alyssum murale* Waldst. & Kit., and the Ni/Zn hyperaccumulator *N. caerulescens* (J. & C. Presl) F. K. Mey were used in this study.

2.2 Materials and Methods

2.2.1 Plant cultivation and harvest

Seeds of *T. arvense* (collected in Nancy, France), *A. murale* (collected in Pojska, Albania) and *N. caerulescens* (collected in Puy de Wolf, France) were sown on agar and germinated in the dark at 25 °C for 5 days. Then 24 seedlings of each species were transferred to 5 L nutrient solutions in a growth chamber for pretreatment. The solution for *T. arvense* contained the following nutrients (in µM): 1000 $Ca(NO_3)_2$, 1000 KNO_3, 500 $MgSO_4$, 100 KH_2PO_4, 50 KCl, 10 H_3BO_3,1 $MnCl_2$, 0.2 $CuSO_4$, 0.2 Na_2MoO_4, 5 Fe(III)-EDTA, 2 $NiSO_4$ and 2 $ZnSO_4$. Two mM 2-morpholinoethanesulphonic acid (MES) was used to buffer the pH, which was adjusted to 5.8 by the addition of 1 M KOH. The nutrient solution used to cultivate *A. murale* and *N. caerulescens* was based on the previous one, with a lower Ca/Mg ratio, to mimic the soil conditions of serpentine areas where the plant seeds were collected; the Ca and Mg concentrations were 500 and 1000 µM, respectively. The growth conditions were 22/18 °C day/night temperatures, 70% relative humidity, 16 h photoperiod and 150 μmol s^{-1} m^{-2} light intensity.

After 14 days of pretreatment, the seedlings were transferred to 2 L containers and treated with low and high levels (2 and 50 µM) of Ni and Zn nutrient solutions. The treatments were (Ni/Zn sulfate in µM/µM): T.a. 2/2 (*T. arvense*), A.m. 50/2 (*A. murale*), A.m. 50/50 (*A. murale*), N.c. 2/2 (*N. caerulescens*), N.c. 50/2 (*N. caerulescens*) and N.c. 50/50 (*N.*

caerulescens). To avoid iron deficiency, 20 µM instead of 5 µM of Fe(III)-EDTA was used in 50/2 and 50/50 treatments. Each treatment replicated three times and each contained one plant. The solutions were renewed weekly during the first two weeks and then twice a week.

Plants were harvested after 12 d (for *T. arvense*) or 28 d (for *A. murale* and *N. caerulescens*) of treatment. Roots were soaked in 1 mM $LaCl_3$ and 0.05 M $CaCl_2$ solution for 15 min at 0°C to remove the Ni and Zn adsorbed on the root surface (Weiss et al. 2005). The plants were washed by ultrapure water (Millipore, 18.2 MΩ cm^{-1}), then separated into root, stem and leaf (for *T. arvense* and *A. murale*), or root and shoot (for *N. caerulescens*, which was at the rosette stage), and later dried at 70 °C for 3 days. The dry samples were ground to fine powders (0.5 mm sieve) for analysis.

2.2.2 Analytical methods

All the harvested plant samples (between 3.4 to 95.8 mg) were placed in Teflon beakers and digested by 5 ml of concentrated HNO_3 on a hot plate. After digestion, the solutions were evaporated to dryness and the residues were dissolved by 1 mL of 0.1 M HNO_3. The Ni and Zn concentrations were determined by ICP-MS (Perkin-Elmer ICP-MS SCIEX Elan 6000 or Thermo X7). To evaluate blank contribution, a procedural blank was introduced into each sample series. The average blank measured throughout the study was 35 ± 5 ng of Zn (n=3), which is negligible compared to the Zn contents in samples (7 – 230 µg). Ni in the blanks was under the determination limit (< 0.9 ng).

Ni and Zn purified fractions for isotope analyses were recovered simultaneously from the same aliquot of sample. The Zn purification method for the column chemistry was adapted from Cloquet et al. (2006), while Ni method from Quitte and Oberli (2006) and Gueguen et al.(2013). Initially, a sample which contained 2 µg Ni was equilibrated overnight with 2.3 µg of double-spike (mixture of equivalent amount of ^{61}Ni and ^{62}Ni, bought from Oak Ridge National Laboratory). After evaporation, the residue was dissolved in 1 ml of 6 M HCl. The sample was loaded onto 2 ml AG1-X8 (Bio-Rad) resin bed. At this step of the chemistry, Zn was fixed in the resin bed and could be eluted. Fe was removed using 10 ml of 0.5 M HCl,

while Zn fraction was recovered by elution of 10 ml of 0.5 M HNO_3. The latter purification step was repeated twice.The loading and rinsing solutions from the first column (15 ml of 6 M HCl) were collected and dried for the second step of purification for Ni. The dried residue was dissolved in 1 ml of 1 M HCl, mixed with 0.3 ml of 1 M ammonium citrate and the pH of the mixture was adjusted to 8 – 9 by adding NH_4OH. Then, sample was loaded onto 0.5 ml resin bed of a Ni-specific resin containing dimethylglyoxime (DMG) as Ni-chelating agent. Beforehand, the resin was conditioned in ultrapure water and 2 ml of 0.2 M ammonium citrate (pH 8 – 9), which was used for matrix elution as well (4 ml). After rinsing, Ni was eluted by 4 ml of 3 M HNO_3. The eluting solution was dried and 5 ml of concentrated HNO_3 was added to break down DMG complex. This process was repeated 3 – 4 times to digest the DMG completely, so that purified Ni could be obtained. A final step for Ni purification was added, by performing another AG1-X8 column to ensure complete Fe removal.

Ni and Zn isotope measurements were carried out by MC-ICP-MS (Neptune Plus, Thermo Scientific) at CRPG-CNRS, University of Lorraine, France. Details on Zn isotope measurements are given in Tang et al. (2012). Briefly, Zn samples were diluted to obtain the same signal as measured in 100 ng g^{-1} $Zn_{IRMM\ 3702}$ solution, and $Cu_{NIST\ 976}$ was added to both standard and samples for mass bias correction. In addition to Cu doping, standard-sample-standard correction was carried out to account for the difference between Cu and Zn behavior. The masses measured were ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{63}Cu, ^{65}Cu. Mass dependent fractionation was verified for all samples. Throughout the study, $Zn_{JMC\ lyon}$ was regularly measured providing a $\delta^{66}Zn$ = -0.28 ± 0.05‰ (n=27). Such a value is in agreement with the published values and can be used to recalculate all data against $Zn_{JMC\ lyon}$. Meanwhile, reference material BCR-482 (lichen) was digested and analysed, having a $\delta^{66}Zn$ = -0.26‰ ± 0.07 (n=3) (Aebischer, pers. comm.), which is in agreement with previous published data (Cloquet et al. 2006).

For Ni isotope measurement, purified Ni was re-dissolved in 1 ml of 0.1 M HNO_3. Then the solution was diluted to 150 ng g^{-1} of Ni before being loaded via the Aridus II (Cetac) into the MC-ICP-MS in medium resolution mode. Spiked-standards NIST 986 and spiked-samples were run at similar concentrations sequentially as in the classic sample-standard bracketing

method. The calibration of the double-spike was conducted following the calibration method provided by Rudge et al. (2009). The whole analytical protocol was applied to two reference materials previously characterized, namely BHVO-2 (basalt) and SDO-1(sedimentary rock). Several preparations and measurements of these reference materials showed the values are in agreement with those published (Gall et al. 2012; Gueguen et al. 2013) (^{60}Ni = -0.01 ± 0.05‰ (2SD, n=11) for BHVO-2 and ^{60}Ni = 0.54 ± 0.05‰ (2SD, n=11) for SDO-1).The external reproducibility (2SD) of the method is thus 0.05‰.

Ni isotopic compositions are expressed in delta per mill (‰) relative to NIST SRM 986, while Zn composition is relative to IRMM 3702: δ^{60}Ni (‰) = [(^{60}Ni/^{58}Ni)$_{sample}$ / (^{60}Ni/^{58}Ni)$_{NIST\ 986}$ - 1] × 1000, δ^{66}Zn (‰) = [(^{66}Zn/^{64}Zn)$_{sample}$ / (^{66}Zn/^{64}Zn)$_{IRMM\ 3702}$ - 1] × 1000.

The average isotope compositions of the shoots of *T. arvense* and *A. murale*, and of the whole plants were calculated according to the following equations:

$$\delta^{60}Ni_{shoot} = \frac{\sum_i m_i c_i^{Ni} \delta^{60}Ni_i}{\sum m_i c_i^{Ni}} \quad \text{and} \quad \delta^{66}Zn_{shoot} = \frac{\sum_i m_i c_i^{Zn} \delta^{66}Zn_i}{\sum m_i c_i^{Zn}}$$

where m_i, c_i^{Ni} and c_i^{Zn} are the mass of plant part *i* (stem or leaf) and its concentrations of Ni and Zn, respectively;

$$\delta^{60}Ni_{plant} = \frac{\sum_j m_j c_j^{Ni} \delta^{60}Ni_j}{\sum m_j c_j^{Ni}} \quad \text{and} \quad \delta^{66}Zn_{plant} = \frac{\sum_j m_j c_j^{Zn} \delta^{66}Zn_j}{\sum m_j c_j^{Zn}}$$

where m_j, c_j^{Ni} and c_j^{Zn} are the mass of plant part *j* (root or shoot) and its concentrations of Ni and Zn, respectively.

Isotope fractionation between the two components A and B is expressed as $\Delta^{60}Ni_{A-B}$ and $\Delta^{66}Zn_{A-B}$, with $\Delta^{60}Ni_{A-B} = \delta^{60}Ni_A - \delta^{60}Ni_B$, $\Delta^{66}Zn_{A-B} = \delta^{66}Zn_A - \delta^{66}Zn_B$.

2.3 Results

2.3.1 Plant biomass and metal concentrations

All the plants grew healthily with the exception of A.m. 50/50, which presented retarded growth symptoms, probably due to Zn toxicity. This is clearly reflected in the plant biomass data (Figure 2.1a). It is noticeable that *N. caerulescens* achieved similar biomasses in all the three treatments, indicating that the solution Ni and Zn concentrations used in this experiment had no significant effect on its growth.

The Ni and Zn concentrations in plant organs showed clear species-specific patterns (Figure 2.1b, c). *T. arvense*, the non-hyperaccumulator, took up relatively small amounts of Ni and Zn, with a root-shoot translocation factor (shoot concentration / root concentration) of around 0.1 for both elements. *A. murale*, the Ni hyperaccumulator, presented high Ni concentrations in shoots (3570 µg/g in A.m. 50/2 treatment), while most of the Zn was sequestrated in roots. *N. caerulescens* could hyperaccumulate both Zn and Ni in its shoots.

A competition effect between Ni and Zn in the uptake process was also observed. When Zn in solution increased from 2 to 50 µM, the Ni concentrations in shoots of *N. caerulescens* and *A. murale* dropped by 38% and 62%, respectively (Figure 2.1b). When Ni in solution increased from 2 to 50 µM, the Zn concentration in *N. caerulescens* shoots decreased on average by 39%.

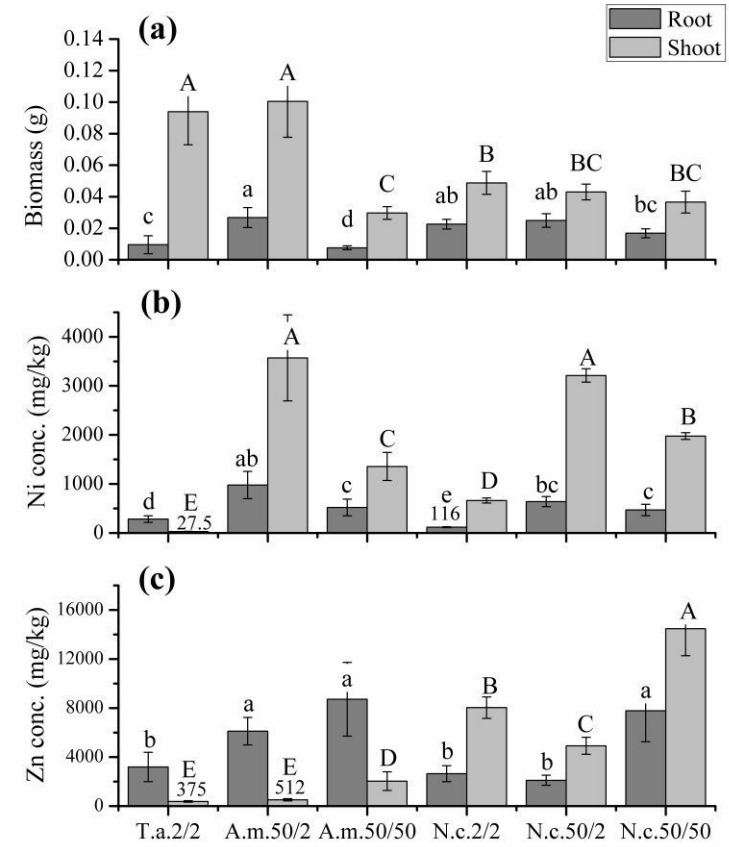

Figure 2.1 Biomass (a), Ni and Zn concentrations (b,c) of the non-hyperaccumulator *T. arvense* (T.a.), the Ni hyperaccumulator *A. murale*(A.m.) and the Ni and Zn hyperaccumulator *N. caerulescens* (N.c.). The numbers in the treatment names (2/2, 50/2 and 50/50) represent Ni and Zn concentrations in nutrient solutions (in µM). Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

2.3.2 Nickel and Zn isotopic compositions.

Figure 2.2 presents the Ni and Zn isotopic compositions (^{60}Ni and ^{66}Zn in ‰) in plants and Figure 2.3a presents the extent of fractionation between plant and solution. All the plants were inclined to absorb light Ni isotopes, with $\Delta^{60}Ni_{plant\text{-}solution}$ values ranging from -0.90 to -0.21‰. It is noticeable that the hyperaccumulators had larger isotopic shift ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.90 to -0.63‰), in particular in low Ni treatment ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.90‰ in N.c. 2/2).

Compared to Ni however, Zn isotopes had a smaller shift with $\Delta^{66}Z\text{-}n_{plant\text{-}solution}$ values of -0.23 to +0.20‰ (Figure 2.3a).

The competition between Ni and Zn also had an influence on the isotopic compositions of both *A. murale* and *N. caerulescens*. The Ni isotope fractionation in high Zn treatments ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.11 to -0.07‰) became less pronounced, in comparison with their corresponding low Zn treatments ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.73 to -0.63‰) (Figure 2.3a). This indicated that Zn had a great impact on Ni isotope fractionation during root absorption.

For Zn, shoots were enriched in light isotopes and the isotope fractionation between shoots and roots was relatively large ($\Delta^{66}Zn_{shoot\text{-}root}$ = -0.80 to -0.44‰). By contrast, heavy Ni isotopes were enriched in the shoots of *T. arvense* ($\Delta^{60}Ni_{shoot\text{-}root}$ = +0.25‰), while the hyperaccumulators *A. murale* and *N. caerulescens* still favored light Ni isotopes (-0.47 to -0.14‰), but to a lesser extent relative to Zn (Figure 2.2 and 2.3b).

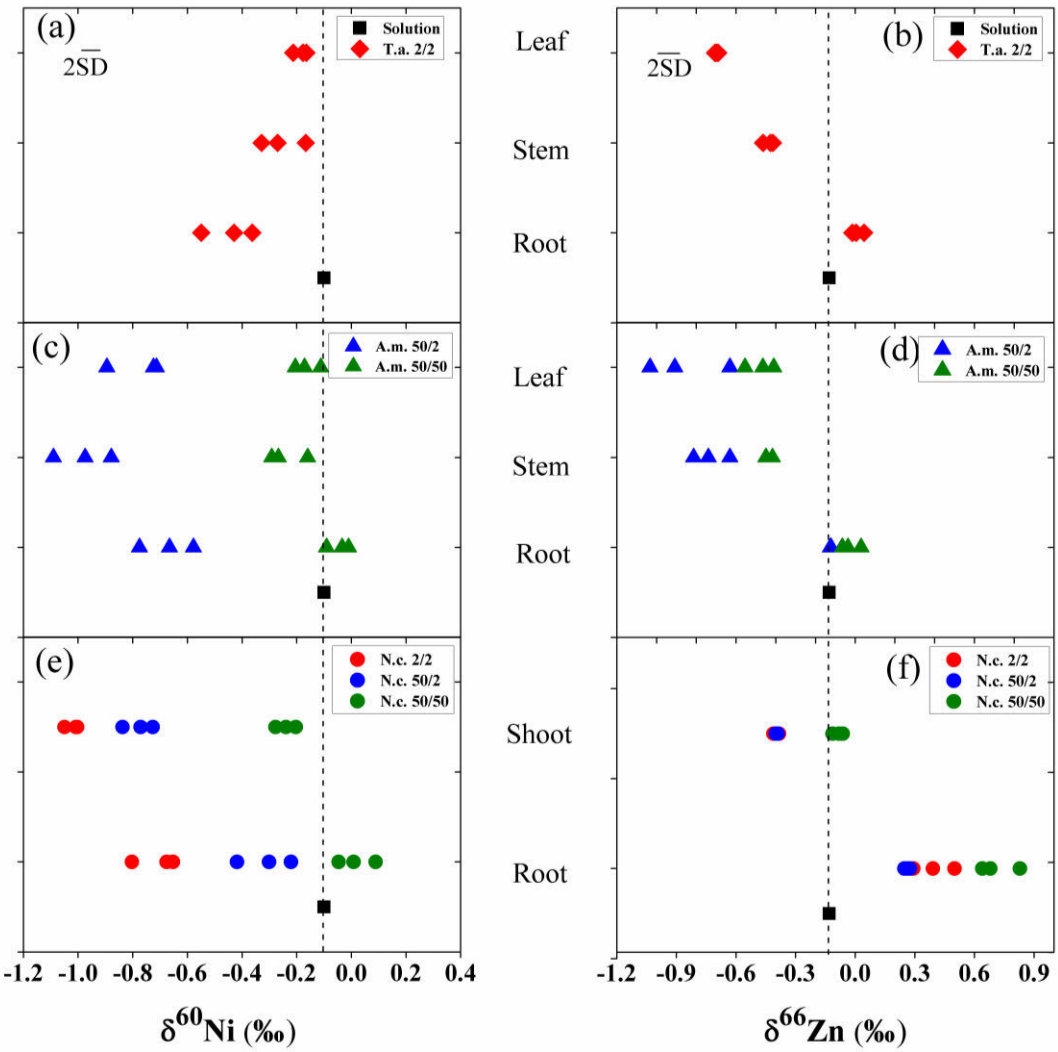

Figure 2.2 Ni (a,c,e) and Zn (b,d,f) isotope compositions (□^{60}Ni and □^{66}Zn in ‰) of plant organs and nutrient solutions (square). *T. arvense* (T.a.) (diamond) and *A. murale* (A.m.) (triangle) are separated into root, stem and leaf, while *N. caerulescens* (N.c.) (circle) is separated into root and shoot. The numbers in the treatment names (2/2, 50/2 and 50/50) represent Ni and Zn concentrations in nutrient solutions (in µM). Error bars show 2SD of the measurements (0.05‰ for Ni and 0.07‰ for Zn).

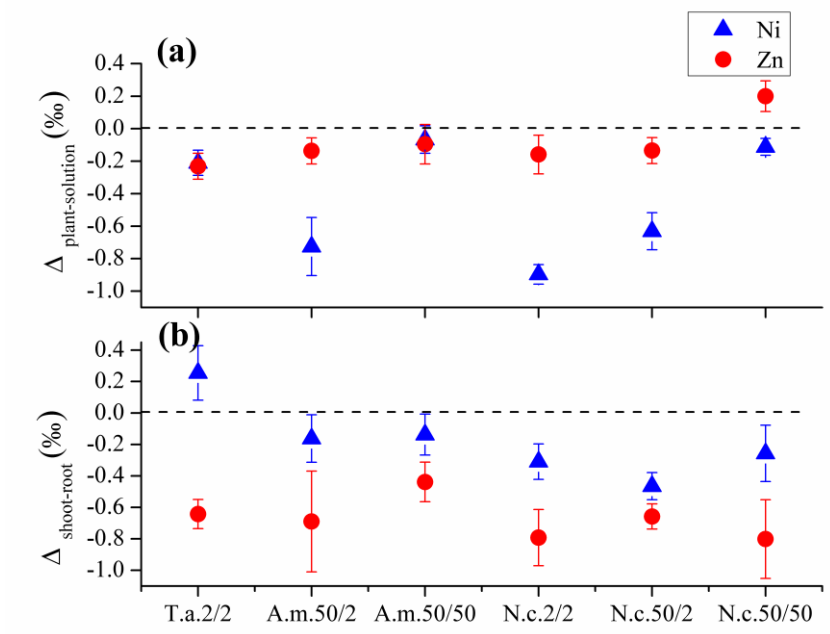

Figure 2.3 Ni (triangle) and Zn (circle) isotope fractionation between plant and solution (a) and between shoot and root (b). Error bars show 2SD of the three replicates. The numbers in the treatment names (2/2, 50/2 and 50/50) represent Ni and Zn concentrations in nutrient solutions (in μM).

2.4 Discussion

Both kinetic and equilibrium fractionations might be occurred during the Ni and Zn uptake processes. For instance, ion speciation could cause equilibrium fractionation, as heavy isotopes are preferentially chelated by ligands, which results in light isotope enrichment in free ion pools (Bigeleisen and Mayer 1947; Weiss et al. 2005; Jouvin et al. 2012). And the transmembrane absorption process could result in kinetic fractionation. Two major processes, which could influence the isotopic signature of plants would be discussed in this section, i.e. ion chelation in media and ion transport across root cell membrane.

2.4.1 Ion chelation in media

Plants usually take up trace elements in the form of free hydrated ions, e.g. Zn^{2+} and Ni^{2+}. However, many organic compounds existing in the growing media could chelate large

fractions of Zn and Ni, which could change the isotopic signatures of free ions. Results showed that Zn bound to purified humic acid is heavier than free Zn^{2+} ($\Delta^{66}Zn$ = +0.24‰ ± 0.06), when pH > 6 (Jouvin et al. 2009). Therefore, increasing the concentration of organic ligands would result in a depletion of heavy metal isotopes in free ion pools, which would potentially affect the isotopic composition in plants. For instance, rice, lettuce and tomato grown in the solution where more Zn was chelated (free Zn fraction = 0.03%), presented 0.09 – 0.21‰ greater negative isotopic shift than another treatment with higher Zn ion activity (free Zn fraction = 35%) (Weiss et al. 2005).

In this study, organic ligands exuded by roots would have little interference on Ni and Zn speciation because plant seedlings were grown in large quantities of solutions (2 L per plant), which were renewed once to twice a week. Thus, the concentrations of organic ligands potentially exuded by roots, were assumed to be low. Therefore, EDTA, which was introduced with the Fe salt, was the major organic ligand in the nutrient solutions. According to the GEOCHEM-EZ calculation, 80 – 89% of Zn remained as free hydrated ion in all the treatment solutions (Table 2.1), which should theoretically represent the isotopic signature of the whole pool and had no significant impact on plant isotopic compositions. For Ni, around 55% was present as Ni^{2+} in high Ni treatments (Ni/Zn 50/2 and 50/50), whereas only 6.5% of Ni remained as Ni^{2+} and 93% was chelated by EDTA in low Ni treatment (Ni/Zn 2/2) (Table 2.1). In this study, it is not possible to provide the precise isotopic compositions of free Ni ion in low and high Ni treatments. However, it could be postulated that the small free ion pool in low Ni treatment should have larger negative isotopic shift than that in high Ni treatment, which might cause lighter isotope enrichment in plants. Indeed, our results conformed to this hypothesis. It came out that *N. caerulescens* grown in low Ni treatment had the greatest negative isotopic shift ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.90‰ in Ni/Zn 2/2 treatment), whereas the isotopic shift became less pronounced in high Ni treatment ($\Delta^{60}Ni_{plant\text{-}solution}$ = -0.63‰ in Ni/Zn 50/2 treatment).

Table 2.1 Distribution of Ni and Zn between free and EDTA-chelated pools in different treatments according to Geochem-EZ calculation.

Treatment	Ni(II)		Ni-EDTA		Zn(II)		Zn-EDTA	
	μM	%	μM	%	μM	%	μM	%
Ni/Zn 2/2	0.13	6.47	1.86	93.2	1.60	80.0	0.29	14.5
Ni/Zn 50/2	27.5	55.0	19.7	39.4	1.78	89.1	0.02	0.80
Ni/Zn 50/50	27.7	55.5	19.3	38.7	44.4	88.8	0.39	0.78

2.4.2 Ion transport across root cell membrane

Plants generally assimilate metallic nutrients via two pathways, i.e. an apoplastic and a symplastic route. It is presumable that Ni and Zn are taken up mainly through the symplastic pathway, and a purely apoplastic route for the entry into the xylem is of minor significance (Ernst et al. 2002; Kerkeb and Krämer 2003). Thus, uptake of Ni and Zn by plants is mainly controlled by the absorption of root cells and the isotopic signatures of Ni and Zn of the whole plant should represent the uptake mechanisms of root cell membrane.

The effect of high- and low-affinity transport on isotope fractionation could explain what is observed in our experiment. *Thlaspi arvense* and *A. murale*, the non-hyperaccumulators of Zn, were isotopically light relative to the solution (-0.23 to -0.10‰) in all the treatments. In contrast, *N. caerulescens*, the Zn hyperaccumulator, was enriched in heavy isotopes in high Zn treatment ($\Delta^{66}Zn_{plant\text{-}solution}$ = +0.20‰). These divergent results suggest that different Zn transport systems are functioning in hyperaccumulators and non-hyperaccumulators. Plants could switch from high- to low-affinity transport systems as the metal concentrations change from deficient to sufficient levels (Epstein and Bloom 2005). Usually, a high-affinity transport system only plays an important role at extremely low concentrations. In bread wheat, 10 nM of Zn^{2+} is assumed to be the critical concentration between high- and low-affinity transport (Hacisalihoglu et al. 2001). However, high-affinity transport could function in a wider range of concentrations in hyperaccumulators. The first high-affinity

transporter gene, ZNT1, has been cloned from *N. caerulescens* (Pence et al. 2000). This Zn transporter is expressed to very high levels in this hyperaccumulating plant, in both Zn-deficient and Zn-sufficient status. Whereas in the non-hyperaccumulator *T. arvense*, the transporter is expressed to very low levels in plants grown in Zn-sufficient solution (1 μM). In our case, 2 and 50 μM of Zn were used in the solution culture. Thus for Zn, low-affinity uptake should take effect predominantly in the non-hyperaccumulators *T. arvense* and *A. murale*, which resulted in light isotope enrichment. While both high- and low-affinity transport systems were functioning effectively in the hyperaccumulator *N. caerulescens*, which resulted in a final isotopic shift of +0.20‰ in high Zn treatment.

The Ni isotope fractionation pattern is different from that of Zn. All species presented light Ni isotope enrichment (Figure 2.2, 2.3a), which may reflect the functioning of low-affinity transport systems. This is corroborated with Aschmann et al. (1987) and Redjala et al. (2010), who inferred that Ni is transported through a low-affinity transport system from uptake kinetic studies. Likewise, Assunção et al. (2008) proposed that *N. caerulescens* seems to express low-affinity systems for Ni accumulation. The hyperaccumulators *A. murale* and *N. caerulescens* presented greater isotopic shifts than the non-hyperaccumulator *T. arvense* in low Zn treatments (-0.90 to -0.63‰ vs. -0.21‰), indicative of a greater permeability for the low-affinity transport systems in hyperaccumulators.

It is quite notable that Zn was able to reduce Ni absorption by *A. murale* and *N. caerulescens*. Meanwhile, the extent of Ni isotope fractionation in the hyperaccumulators was also decreased in high Zn treatments (A.m. 50/50, N.c. 50/50). This could be ascribed to Ni and Zn competition during root uptake process, which indicates that Ni may share the transport systems with Zn. Thus, it could be speculated that high levels of Zn could compete with Ni, block the Ni transport pathway, and decrease the Ni internalization flow, resulting in not only a reduction of Ni uptake but also less isotope fractionation. Interestingly, *A. murale* and *N. caerulescens* had similar Ni isotope fractionation behaviors in both low Zn and high Zn treatments, thereby suggesting similar Ni transport systems may exist in these species.

Little is known about the Ni uptake strategy in higher plants. Since Ni requirement in plant physiology is relatively low, and plants growing in nature usually do not suffer from Ni

deficiency, high-affinity transporter is not expected to be evolved in higher plants, even in hyperaccumulators. From our observations along with previous studies, we propose that all plants take up Ni via low-affinity transport systems. In Ni hyperaccumulators, this transport system may be expressed in higher levels with greater permeability for Ni.

2.5 Conclusion

In this chapter, we have analyzed the Ni and Zn isotope fractionation patterns in both hyperaccumulators (*A. murale* and *N. caerulescens*) and a non-hyperaccumulator (*T. arvense*) to study the Ni transport systems during the root uptake process. Results showed that all plants were inclined to absorb light Ni isotopes, presumably due to the functioning of low-affinity transport systems across root cell membrane. The Ni isotope fractionation between plant and solution was greater in the hyperaccumulators than that in the non-hyperaccumulator, thus indicating a greater permeability of the low-affinity transport system in hyperaccumulators. Moreover, the great Zn interference on the extent of Ni isotope fractionation indicates that hyperaccumulators might take up Ni via a low-affinity transport system of Zn. The main findings of this study are illustrated in Figure 2.4.

In next chapter, we would focus on the interaction between Ni, Zn and other metallic elements to further clarify the properties of Ni transport systems.

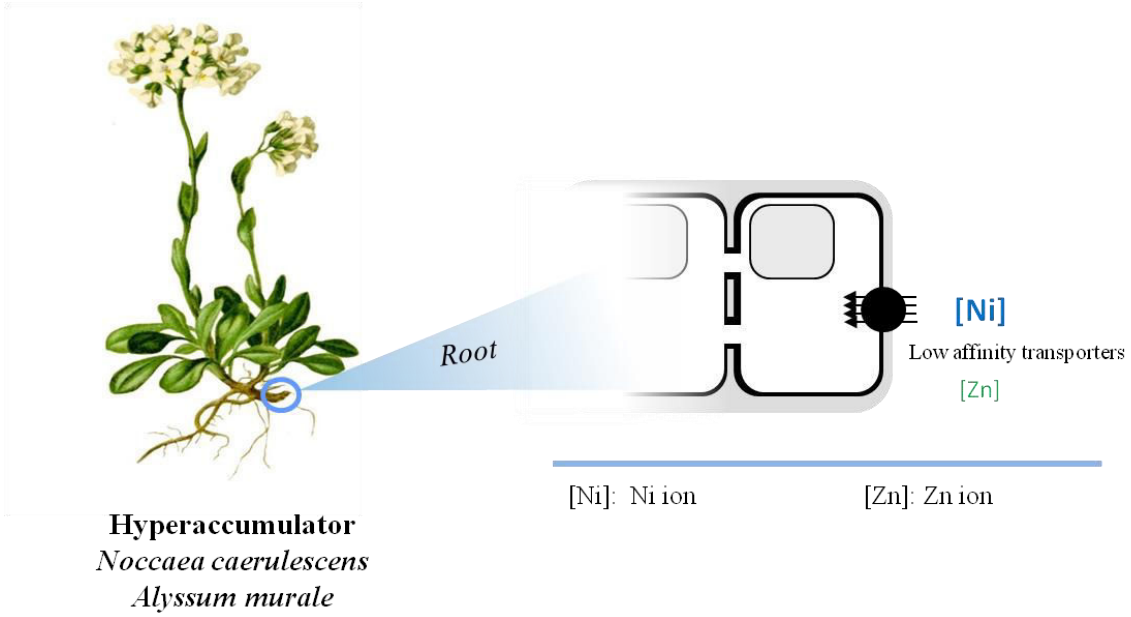

Figure 2.4 Illustration of the main findings in Chapter 2. Root uptake process of Ni in the hyperaccumulators *N. caerulescens* and *A. murale* were mainly controlled by low-affinity transport systems, which may be Zn transporters.

3. Interaction of Ni with other elements in *Noccaea caerulescens* during the root uptake process

3.1 Introduction

In Chapter 2, we have found that Ni is absorbed mainly by low-affinity transport systems in hyperaccumulators, which may involve in Zn transporter(s).

Previous research have found that Zn, Cu and Fe could competitively inhibit Ni uptake in several non-hyperaccumulating plants (Cataldo et al. 1978; Körner et al. 1987; Nishida et al. 2011; Nishida et al. 2012), which suggested that Ni may be taken up via the carrier sites of other micronutrient elements. Unlike non-hyperaccumulating plants, which assimilate trace amount of Ni, hyperaccumulators are capable of accumulating more than 1000 mg kg^{-1} Ni in their shoots, some could even reach 30,000 mg kg^{-1} (Brooks et al. 1977; Reeves et al. 1983). Therefore, these special plants may possess highly efficient Ni uptake systems in roots, and great competition between Ni and other elements are existed during the absorption process. In the Zn/Ni hyperaccumulator *Thlaspi pindicum*, Ni absorption and translocation in plants were strongly inhibited by the addition of Zn^{2+} into nutrient solution (Taylor and Macnair 2006). The competition between Ni and Zn was also found for the Zn/Cd/Ni hyperaccumulator *Noccaea caerulescens* and the Ni hyperaccumulator *Alyssum murale* (Chapter 2; Assunção et al. 2001). In addition to Zn, Ni seems to interact with Fe and Mn during the uptake process; increased Fe uptake was found in the Ni hyperaccumulator *Alyssum inflatum* when exposed to elevated Ni concentrations (Ghasemi et al. 2009), while negative correlation was observed between the Mn and Ni concentrations in leaves of *A. murale* (Leigh Broadhurst et al. 2009). Interestingly, Ni hyperaccumulator *Berkheya coddii* and *A. murale* could also hyperaccumulate Co, the non-essential mineral element in higher plants (Keeling et al. 2003; Tappero et al. 2007). Furthermore, Reeves and Baker (1984) showed that *Thlaspi goesingense*, a multi-metal hyperaccumulator, grown on metal-enriched substrates accumulated similar, extremely high concentrations of Ni, Zn, Co and Mn in their shoots. These results imply that Ni absorption in hyperaccumulators may also involve several transport systems of

micronutrient elements in roots.

To understand the interactions between metal ions during root uptake process, this chapter was aimed at studying 1) the effect of mineral elements (Zn, Fe, Mn, Cu and Co) on Ni absorption and transport, and 2) the impact of Ni on gene expression of transporters in ZIP (Zinc-regulated transporter, Iron-regulated transporter-related Protein) family.

Since our current knowledge on the interaction between Ni and other mineral ions is based on the research from several hyperaccumulators, which may possess different Ni uptake mechanisms, we chose the Zn/Cd/Ni hyperaccumulator model species *N. caerulescens* (J. & C. Presl) F. K. Mey (formerly *Thlaspi caerulescens*) in this work.

3.2 Materials and methods

3.2.1 Nickel uptake of N. caerulescens *under different concentrations of Ni treatments (Expt. 3.1)*

Seeds of *N. caerulescens* (originating from Puy de Wolf in France, a population that grows on ultramafic soils) were sown on peat matrix (Fafard Custom Growing Mix, Canada) and germinated in the dark at 22 °C for 5 days. The seedlings were then transferred to a growth chamber. The growth conditions were 22/18 °C day/night temperatures, 60% relative humidity, 16 h photoperiod and 120 μmol s^{-1} m^{-2} light intensity. After 39 days, the plants of uniform size were selected for treatment. Sets of two seedlings were transplanted to a container filled with 0.8 L nutrient solution which contained the following nutrients (in µM): 1000 $Ca(NO_3)_2$, 1000 KNO_3, 500 $MgSO_4$, 100 KH_2PO_4, 50 KCl, 10 H_3BO_3,1 $MnCl_2$, 0.2 $CuSO_4$, 0.5 $ZnSO_4$, 0.2 Na_2MoO_4, 20 Fe(III)-EDDHA. Two mM 2-morpholinoethanesulphonic acid (MES) was used to buffer the pH, which was adjusted to 5.8 by the addition of 5% KOH. Four treatments were set up, i.e. CK, Ni10, Ni50 and Ni100, which contained 0, 10, 50 and 100 µM of Ni in nutrient solutions. All the treatments were replicated three times. The solutions were renewed every 3 days.

After 27 days of treatment, plants were harvested. All the plant roots were submerged in 20 mM Na_2-EDTA solution for 15 min to desorb the metal ions attached on the root surface.

Then plants were separated into roots and shoots, washed by deionized water and blotted. The plant parts were put into paper bags and dried in 60 °C. Dry matters were digested by concentrated HNO_3 and $HClO_4$, and metal concentrations (Ni, Zn, Fe, Mn and Cu) were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (iCAP 6500 Duo, Thermo Scientific, USA).

3.2.2 Interaction between Ni, Zn, Fe and Co in N. caerulescens (Expt. 3.2)

Noccaea caerulescens seeds were germinated and grew on peat matrix for 3.5 months (Details were described in Expt. 3.1). Then 15 plants in uniform size were transferred to 0.8 L containers. Five treatments were set up, i.e. CK, -Zn, +Zn, -Fe and +Co (see Table 3.1). Every treatment replicated three times and each replicate contain one plant.

After 27 days of treatment, plants were harvested, dried and digested. Nickel, Zn, Fe and Co concentrations were determined by ICP-AES (iCAP 6500 Duo, Thermo Scientific, USA).

Table 3.1 Nickel, Fe, Zn and Co concentrations in the nutrient solutions of Expt. 3.2

Treatment	Metal conc.(μM)			
	Ni	Zn	Fe	Co
CK	50	5	20	0
-Zn	50	0	20	0
+Zn	50	50	20	0
-Fe	50	5	0	0
+Co	50	5	20	50

3.2.3 Gene expression regarding Ni uptake in N. caerulescens (Expt. 3.3)

Seeds of *N. caerulescens* (originating from Puy de Wolf in France, a population that grows on

ultramafic soils) were sown on peat matrix (Fafard Custom Growing Mix, Canada) and germinated in the dark at 22 °C for 7 days. Seedlings were then transferred to a growth chamber. Growth conditions were 22/18 °C day/night temperatures, 60% relative humidity, 16 h photoperiod and 120 μmol s^{-1} m^{-2} light intensity. After 1 month, 40 seedlings were transferred to 4 L nutrient solution for hydroponic pre-culture. And the solution composition was described in Expt. 3.1.

After 1 month of pre-cultivation in standard solution, plants of uniform size were selected for treatment. Sets of two plants were transplanted to a container filled with 2 L nutrient solution. Three treatments were set up, i.e. CK (control; Ni 0, Zn 5 μM), Zn100 (Ni 0, Zn 100 μM) and Ni100 (Ni 100, Zn 5 μM). All treatments were replicated three times. Solutions were renewed every 6 days.

After 5 months of treatment, roots were abscised from plants, frozen in liquid N_2, and then stored in -80°C refrigerator. The expression levels of two genes, i.e. Zn transporter *ZIP10* and Fe transporter *IRT1*, which were speculated to involve in Ni uptake (Halimaa et al. 2014), were analyzed by quantitative real-time reverse transcriptase PCR, according to the method from Halimaa et al. (2014). The *ZIP10* primers were ZIP10-F CGCCTCCGGAATCATCCTTT, and ZIP10-R CTTGTGCCAGGGGTTATCGT. The *IRT1* primers were IRT1-F GGCCACGAGCCTATACACC, and IRT1-R TATATGTGCGTACGAACCATGGCAA. *AtActin2* was used as the reference to normalize the expression level of the candidate genes.

3.3 Results

3.3.1 Biomass, metal uptake and translocation factor of N. caerulescens *in Expt. 3.1*

Plants grew healthily, and without any significant difference in biomass yielding in all the treatments (Figure 3.1). However, a biomass increment could still be observed in Ni10 treatment.

To investigate the metal interaction in *N. caerulescens*, Ni, Zn, Fe, Mn and Cu concentrations

in both shoots and roots were determined and the average metal concentrations in the whole plant level were present in Figure 3.2. Plants contain high concentrations of Ni, ranging from 2300 to 6000 mg kg^{-1}. Iron and Mn accumulation in plants were significantly affected by Ni addition, though the two elements had different behaviors. Iron concentration increased from 500 mg kg^{-1} in CK to 1300 mg kg^{-1} in Ni100 treatment, while Mn concentration decreased from 300 mg kg^{-1} to 80 mg kg^{-1}. Zinc concentrations were relatively low (320 – 420 mg kg^{-1}) in comparison to Ni, due to the low concentration of Zn (0.5 μM) applied in nutrient solutions. As plants might deplete all Zn in solutions, the impact from Ni addition was insignificant for Zn accumulation. Copper concentrations were relatively low (12 – 15 mg kg^{-1}) in all the treatments, which were not influenced by Ni.

The metal root – shoot translocation in plants could be indicated by translocation factor (TF = shoot / root concentrations). Table 3.2 presented the TF from Expt. 3.1. Obvious, root to shoot transport of Zn, Mn and Cu was affected Ni addition.

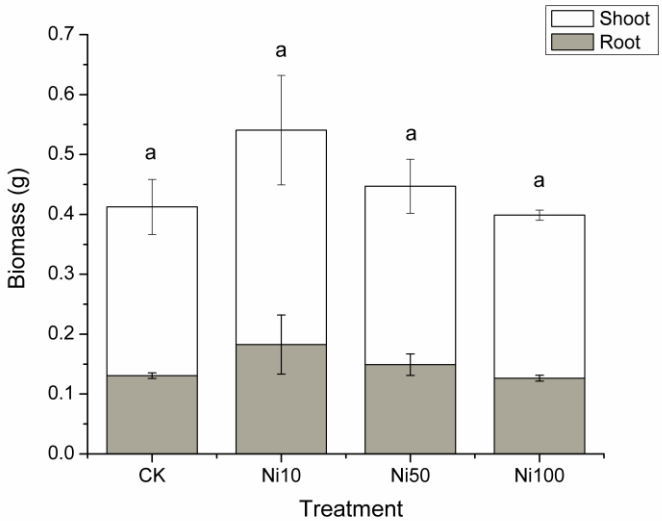

Figure 3.1 Biomass of *N. caerulescens* exposed to different concentrations of Ni in Expt. 3.1. Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

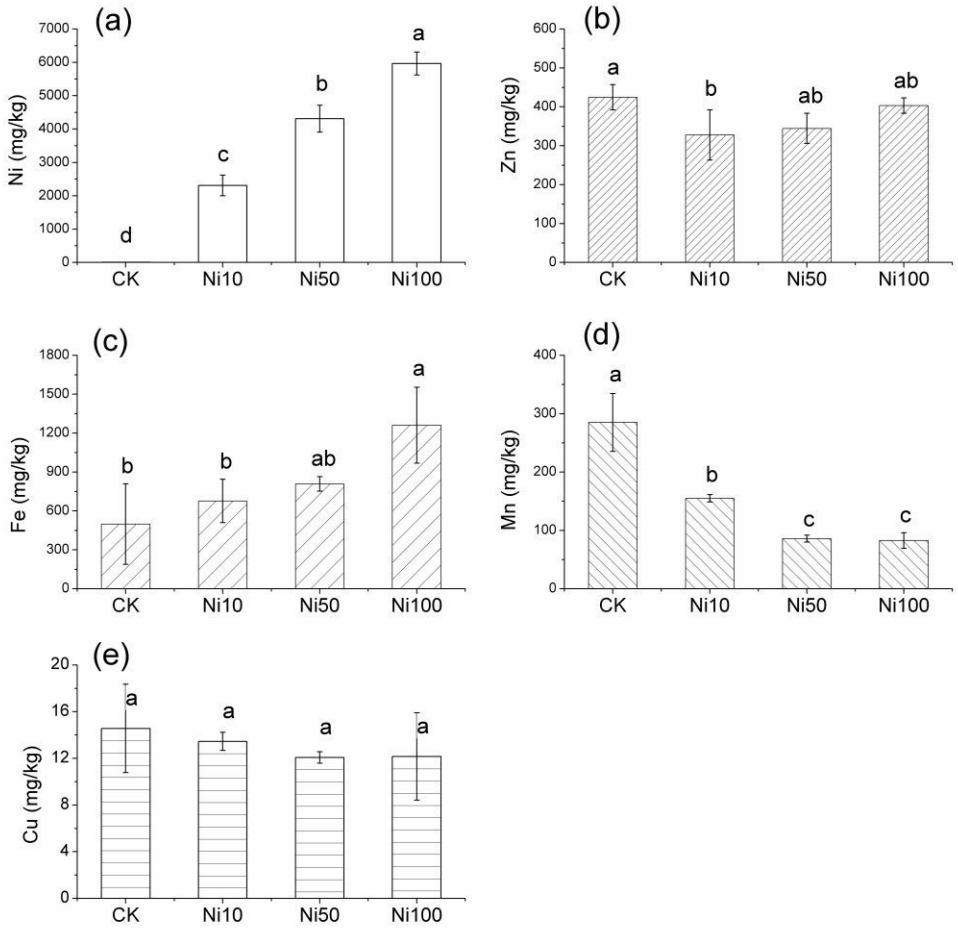

Figure 3.2 Nickel, Zn, Fe, Mn and Cu concentrations in whole plant level of *N. caerulescens* in Expt. 3.1. (a) Ni; (b) Zn; (c) Fe; (d) Mn; (e) Cu. Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

Table 3.2 Translocation factor (TF) of Ni, Zn, Fe, Mn and Cu in *N. caerulescens* in Expt. 3.1. TF is the ratio between shoot and root metal concentration. Data are means of three replicates. Different letters indicate significantly different at $p < 0.05$ (Duncan's Test).

Treatment	Ni		Zn		Fe		Mn		Cu	
CK	-	-	1.43	a	0.12	a	4.64	c	0.23	a
Ni10	1.35	a	1.33	b	0.07	a	1.20	bc	0.04	b
Ni50	1.40	b	0.91	b	0.07	a	1.96	a	0.05	b
Ni100	1.43	b	0.85	b	0.06	a	2.50	b	0.04	b

3.3.2 Biomass, metal uptake and translocation factor of N. caerulescens *in Expt. 3.2*

In Expt. 3.2, *N. caerulescens* was exposed to -Zn, +Zn, -Fe or +Co nutrient solutions. Zn addition had significant positive impact on the biomass increment of the plants (Figure 3.3). Iron or Zn deficiency was quite obvious in –Zn / –Fe treatments: yellowish leaves could be observed, in particular for the young leaves. The leaf tips turn brown in the plants growing in +Co solutions. However, -Zn, -Fe and +Co treatments had no significant difference from CK in biomass yielding, which might be due to the relatively short treatment period and the old age of the plants.

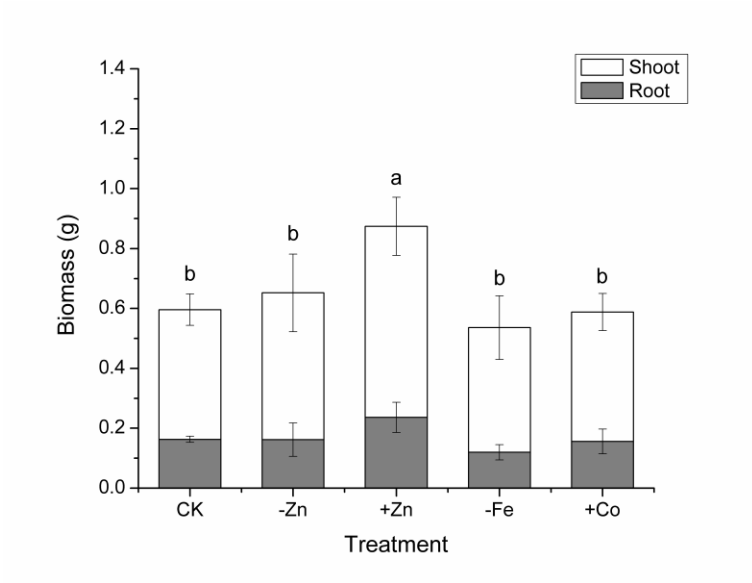

Figure 3.3 Biomass of *N. caerulescens* in five treatments of Expt. 3.2. Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

Figure 3.4 showed the Ni, Zn, Fe and Co concentrations in whole plant level in all the treatments. Nickel accumulation was clearly strengthened by Zn/Fe deficiency and suppressed by Zn/Co addition (Figure 3.4a). It is noticeable that Zn concentration was quite similar to that of Ni in CK treatment (about 2000 mg kg^{-1}), thought Ni was added 10 times higher than Zn in nutrient solution. When Zn and Ni were present in the same level in solution (50 μM), *N. caerulescens* accumulated Zn that was 9 times higher than Ni (Figure 3.4a, b). Zinc and Fe seemed to have little interaction. –Zn and +Zn had no impact on Fe assimilation, while Fe deficiency had little influence on Zn uptake as well. Cobalt was also favored by *N. caerulescens*. The Co concentration in +Co treatment reached 2200 mg kg^{-1}, which was higher than that of Ni (1000 mg kg^{-1}). In addition to its significant suppression on Ni accumulation, the addition of Co could promote Fe concentration (Figure 3.4c), and suppress Zn concentration (Figure 3.4b).

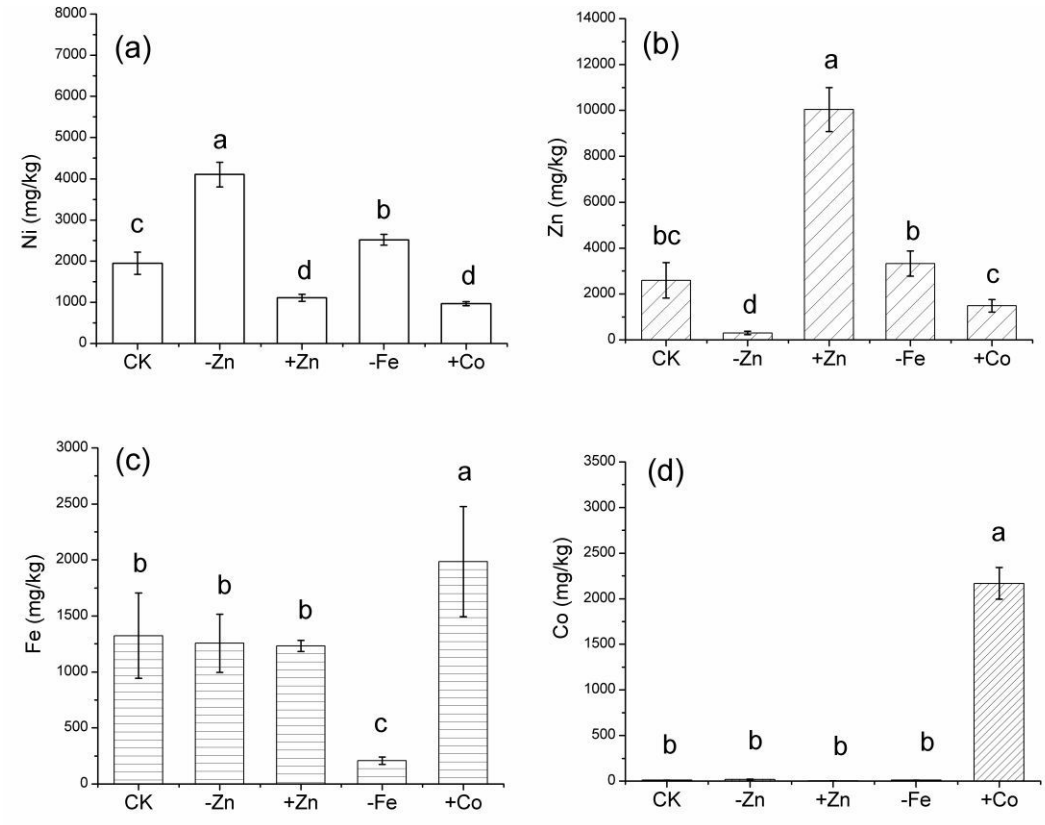

Figure 3.4 Nickel, Zn, Fe and Co concentrations in whole plant level of *N. caerulescens* in Expt. 3.2. (a) Ni; (b) Zn; (c) Fe; (d) Co. Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

Table 3.3 presented the TF of Ni, Zn, Fe and Co in different treatments. For Ni TF, Significant variation could be seen between –Zn and +Zn treatments, while –Fe and +Co seemed to have little influence on the root-shoot translocation of Ni.

Table 3.3 Translocation factor of Ni, Zn, Fe and Co in *N. caerulescens* in Expt.3.2. Data are means of three replicates. Different letters indicate significantly different at $p < 0.05$ (Duncan's Test).

Treatment	Ni	Zn	Fe	Co

CK	2.41	ab	0.59	b	0.03	b	-	-
-Zn	3.73	a	2.61	a	0.02	b	-	-
+Zn	1.98	b	0.49	b	0.03	b	-	-
-Fe	2.96	ab	0.86	b	0.22	a	-	-
+Co	2.30	ab	0.77	b	0.01	b	2.14	-

3.3.3 Gene expression of ZIP10 and IRT1 in roots of N. caerulescens *in Expt. 3.2*

In Expt. 3.3, we analyzed the root expression of Zn and Fe transporters under Ni and Zn exposure. Results showed that Ni and Zn treatments could both increase the expression level of *NcZIP10*, the Zn transporter, while the Fe transporter *NcIRT1* was stimulated only by Ni addition (Figure 3.5).

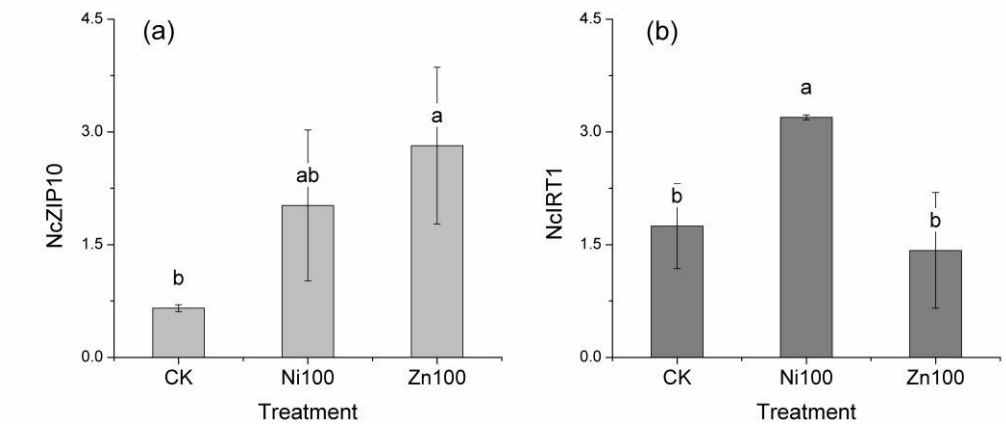

Figure 3.5 Expression levels of *ZIP10* (a) and *IRT1* (b) in roots of *N. caerulescens* from Expt. 2.3. Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

3.4 Discussion

3.4.1 Interaction between Ni and Zn

Noccaea caerulescens is a multi-metal hyperaccumulator, which is able to hyperaccumulate high levels of Zn (>10,000 mg kg^{-1}) and Ni (1000 mg kg^{-1}) (Milner and Kochian 2008). The exceptional Zn and Ni accumulation in this species is clearly shown in our results. Great competition was found between Zn and Ni during the uptake process (Assunção et al. 2001), which is confirmed in this work, indicating Zn and Ni are competing for binding sites in root cell membrane. It is noticeable that *N. caerulescens* is originally a Zn hyperaccumulator, which has strong preference on Zn absorption over Ni. Several Zn transporters in this species, including a high-affinity Zn transporter, *NcZNT1*, were identified to be constitutively overexpressed in roots, which leads to large Zn influx into the plants (Assunção et al. 2001; Pence et al. 2000). Because Ni and Zn share similar chemical properties, Ni may be absorbed via the existing transport systems of Zn in *N. caerulescens*. In fact, Halimaa et al. (2014) showed that the Zn transporter *NcZIP10* might be a putative Ni transporter. Our results also indicate that Ni exposure could stimulate the expression of *NcZIP10* in roots of *N. caerulescens*. However, Ni uptake is less efficient compared to Zn uptake, due to the lower binding affinity between Ni^{2+} and the Zn transporters. In addition, the Ni/Zn mutual inhibition on root-shoot translocation suggests that the elements further compete with each other during the xylem loading and transport process *in vivo*. Therefore, Ni seems to be deeply involved in the Zn homeostasis systems in *N. caerulescens*.

3.4.2 Interaction between Ni and Fe

The interaction between Ni and Fe is quite similar to that in the non-accumulator *A. thaliana*. Nickel exposure could enhance the relative transcription level of *IRT1* and *FRO2*, a ferric reductase in the root epidermis, resulting in Fe accumulation in plants; and it was postulated that Ni accumulation could induce an Fe-deficient response in *A. thaliana* (Nishida et al. 2012). *IRT1*, the primary Fe^{2+} transporter, could mediate transport of other divalent cations due to its lack of substrate specificity (Schaaf et al. 2006), including Ni^{2+} (Halimaa et al.

2014). Therefore, it seems that Ni could be absorbed via part of the Fe transport systems. However, it should be noted that although both Fe and Zn could interact with Ni during the uptake process, the impact from Zn is apparently greater than that of Fe, as much more Ni was accumulated in Zn-deficient treatment than Fe-deficient one (Figure 2.4). In addition, Fe seems to have no influence on the root to shoot translocation of Ni (Table 2.2 and 2.3).

3.4.3 Interaction between Ni and Mn, Cu

Leigh Broadhurst et al. (2009) found that leaf Ni concentrations decreased as Mn increased in the Ni hyperaccumulator *A. murale* and *Alyssum corsicum*, and they suggested that Ni hyperaccumulation in *Alyssum* species might have developed from a Mn handling system. In our work, elevated Ni (Figure 3.2) and Zn (Data not shown) could significantly suppress Mn uptake in *N. caerulescens*. It is not surprising to observe this phenomenon, as many metal transporters in *ZIP* family can simultaneously mediate the uptake of several divalent cations, including Zn^{2+}, Fe^{2+}, Ni^{2+} and Mn^{2+} (Milner et al. 2013; Mizuno et al. 2005). The competition between Ni and Mn is clear, however, cannot be exaggerated. Mn absorption ability is not distinctive in *N. caerulescens* (Robinson et al. 1998). Even in the study of Leigh Broadhurst et al. (2009), Mn accumulation was generally one order of magnitude lower than Ni. These observations suggest that Ni hyperaccumulators do not possess highly-efficient Mn transport systems. Therefore, the Ni absorbed via Mn transporters should be of minor importance in *N. caerulescens*. This is also true for Cu. The average Cu concentrations in *N. caerulescens* are less than 20 mg kg^{-1}, which is negligible to affect Ni uptake and transport in plants.

3.4.4 Interaction between Ni and Co

Cobalt is not considered to be an essential nutrient element and usually present in trace levels in higher plants (Epstein and Bloom 2005). However, strong Co absorption ability and great competitions between Co and Ni were found in Ni hyperaccumulators, e.g. *B. coddii* and *A. murale* (Keeling et al. 2003; Tappero et al. 2007). In this work, we also demonstrated that Co could severely suppress Ni and Zn uptake, and *N. caerulescens* seems to prefer Co to Ni,

suggesting Co and Ni may share similar transport systems in roots. Since no Co-specific transport system is expected to exist in higher plants, the great interference between Co and Ni indicates that Ni is also absorbed via non-specific transporters in the hyperaccumulators.

3.5 Conclusion

In this chapter, we assessed the interaction between Ni and other elements during the uptake and transport process of *N. caerulescens*. Results showed that great competition was existed between Ni and Co, the non-essential element, indicating Ni transport systems are non-specific. Zinc could severely suppress Ni uptake, while Ni addition could stimulate Fe uptake. In addition, the expression levels of the Zn transporter *ZIP10* and the Fe transporter *IRT1* were increased after Ni exposure. These results suggest that Ni is mainly taken up via Zn transporters, while Fe transport systems also play a role for Ni uptake. The main findings of this study are illustrated in Figure 3.6.

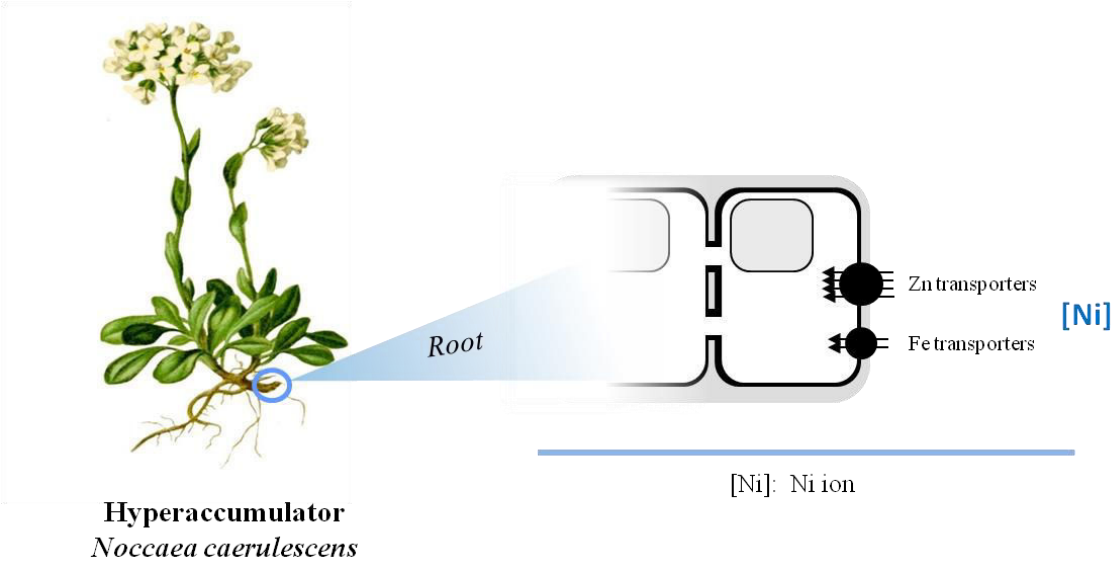

Figure 3.6 Illustration of the main findings in Chapter 3. Specific (high-affinity) Ni transporters may not exist in hyperaccumulators. Nickel is mainly absorbed via Zn and Fe transporters in roots of *N. caerulescens*.

4. Nickel partitioning in leaves of *Noccaea caerulescens* during xylem and phloem transport

4.1 Introduction

As all metals in shoots of hyperaccumulators are transported from roots, most credits of hyperaccumulation ability in shoots have been ascribed to efficient xylem loading process (Papoyan and Kochian 2004; Hanikenne et al. 2008; Krämer 2010). For example, *HMA4*, which mediates Zn and Cd efflux from root symplast to xylem vessels, are highly expressed in roots of *Noccaea caerulescens*, the Zn/Cd/Ni hyperaccumulator (Bernard et al. 2004; Papoyan and Kochian 2004), resulting in high efflux of metals into xylem. When grafting rootstock of *N. caerulescens* with shoots of *Thlaspi perfoliatum*, a non-hyperaccumulator, hyperaccumulation of Zn in shoots did happen as well (Guimarães et al. 2009). Furthermore, Sterckeman et al. (2015) showed that shoot pruning had no effect on Cd accumulation in shoots of *N. caerulescens*. These results indicate that xylem loading and transport is of pivotal importance for hyperaccumulation of Zn, Cd as well as Ni.

Xylem flow is mainly driven by transpiration, and leaves of different ages generally have different transpiration rates and thus receive different volumes of xylem fluid (Taiz and Zeiger 2010). Therefore, mature and young leaves may have different metal accumulation patterns. The pioneering work conducted by Perronnet et al. (2003) showed that 8-month-old *N. caerulescens* accumulated much higher concentrations of Zn in mature leaves, which contained 93% of the total Zn content in shoots, while lower Zn concentrations were found in young leaves. The Ni distribution pattern in leaves of hyperaccumulator *Berkheya coddii* was quite similar to that of *N. caerulescens* (Robinson et al. 2003).

In addition to xylem transport, phloem translocation also plays an important role for nutrient transport in plants. But unlike xylem transport, the main sources for phloem translocation are generally mature leaves (Taiz and Zeiger 2010). Nickel is rather mobile in phloem and can be readily transferred from sources to sinks, which could be young leaves, seeds and roots (Neumann and Chamel 1986; Page and Feller 2005; Page et al. 2006). Hyperaccumulators usually accumulate high concentrations of Ni in mature leaves, which may act as a large Ni

source for phloem translocation. Therefore, Ni output in mature leaves and input into sink organs, e.g. young leaves, via phloem might also have some impact on Ni accumulation in hyperaccumulators.

It could be recognized that mature leaves are a strong sink for xylem and a strong source for phloem, while young leaves a weak sink for xylem and a strong sink for phloem. Therefore, Ni accumulation in mature and young leaves of hyperaccumulators is affected by both xylem transport and phloem translocation. And it would be of interest to clarify the Ni partitioning between mature and young leaves during xylem and phloem transport.

In this work, *N. caerulescens* was grown in a nutrient solution containing Ni, Sr and Rb. Strontium is a phloem-immobile element, while Rb is easily transported in phloem (Kuppelwieser and Feller 1991). Hence, Sr is considered as an indicator for xylem transport while Rb as an indicator for phloem translocation. Moreover, a foliar Ni application experiment was conducted to further investigate the Ni partitioning between young leaves and roots during its bidirectional movement in phloem.

4.2 Materials and methods

4.2.1 N. caerulescens *grown in Ni, Sr and Rb solutions (Expt. 4.1)*

Seeds of *N. caerulescens* (Chavignée) were sown on peat (Fafard Custom Growing Mix, Canada) and germinated in the dark at 22 °C for 7 days. Then the seedlings were transferred to a growth chamber. The growth conditions were 22/18 °C day/night temperatures, 60% relative humidity, 16 h photoperiod and 120 μmol s^{-1} m^{-2} light intensity. After 1 month of growing, 40 seedlings were transferred to 4 L nutrient solution for hydroponic culture. The standard solution contained the following nutrients (in µM): 1000 $Ca(NO_3)_2$, 1000 KNO_3, 500 $MgSO_4$, 100 KH_2PO_4, 50 KCl, 10 H_3BO_3,1 $MnCl_2$, 0.2 $CuSO_4$, 5 $ZnSO_4$, 0.2 Na_2MoO_4, 20 Fe-EDDHA. Two mM 2-morpholinoethanesulphonic acid (Fismes et al.) was used to buffer the pH, which was adjusted to 5.8 by the addition of 5% KOH. The growth conditions were 22/18 °C day/night temperatures, 60% relative humidity, 12 h photoperiod and 120 μmol s^{-1} m^{-2} light intensity. The solution was renewed every 6 d.

After 2 months of pretreatment, plants were treated with standard solution added with 100 µM

$NiSO_4$, RbCl and $SrCl_2$, respectively. And the solutions were renewed every 3 d.

In the 6th, 18th and 42th d of treatment, two or three pieces of young leaves (YL, < 30 d of age) and old leaves (OL, > 30 d of age) were removed from the rosette of three or four plants, then washed and dried. The samples were digested and the Ni, Rb and Sr concentrations in leaves were determined by ICP-OES. In addition, during the final harvest (42 d), all plant shoots were separated into YL and OL, and then dried and weighed.

4.2.2 Foliar application of ^{61}Ni in N. caerulescens *(Expt. 4.2)*

To avoid contamination from other sources, we chose a rare Ni stable isotope - ^{61}Ni (natural abundance: 0.0114) as the Ni^{2+} source. A certain volume of 10 μg mL^{-1} $^{61}Ni(NO_3)_2$ (^{61}Ni abundance: 0.9944; Inorganic Ventures, USA) solution was dried at 90 °C and concentrated to 1 mM. The pH was adjusted to 5.8 by 5% KOH and 0.01 (v/v) Silwet L-77 spray adjuvant was added in the solution to increase leaf tissue permeability.

The method of foliar application was adapted from Lu et al. (2013). Plant seedlings were cultivated in nutrient solution for 3 months and then transferred to 1 L containers. All the fully expanded leaves, except one, were cut to ensure the remaining leaf was the oldest and located in the lowest position of the rosette. This remaining leaf was then soaked in 5.0 mL of the prepared ^{61}Ni solution for 10 s. Each plant was treated as one replicate, and there were 3 replicates. The foliar application was conducted once every day for 3 d, and plants were cultivated for another 3 d before harvesting. All plants were constantly exposed to nutrient solution without added Ni during the whole cultivation period.

At harvest, the ^{61}Ni-spiked leaf in each plant was cut and discarded. Then the plants were divided into roots and shoots. Samples were dried and then digested by concentrated HNO_3. ^{61}Nickel concentration was determined by ICP-MS (iCAP Q, Thermo Scientific), whilst ^{60}Ni (natural abundance: 0.2622) was also analyzed to monitor Ni contamination from other sources.

4.3 Results

4.3.1 Nickel, Rb and Sr accumulation in young and old leaves

In Expt. 4.1, we determined the Ni, Rb and Sr concentrations of both young (YL) and old leaves (OL) after 6, 18 and 42 d of treatment. Results showed that the concentrations of Sr, the indicator of xylem transport, increased from 550 (6 d) to 3330 mg kg^{-1} (42 d) in OL (Figure 4.1c), suggesting the continuously import from xylem. However, Sr concentrations in YL did not have significant difference in all treatments, indicating the transpiration in YL remained stable during the whole cultivation. The time-course variation for Ni (Figure 4.1a) was quite similar to that of Sr, which might suggest that Ni accumulation in both young and old leaves is mainly controlled by xylem transport. Interestingly, the phloem mobile element, Rb, presented different accumulation pattern in YL (Figure 4.1b), whose concentrations were positively correlated with treatment time.

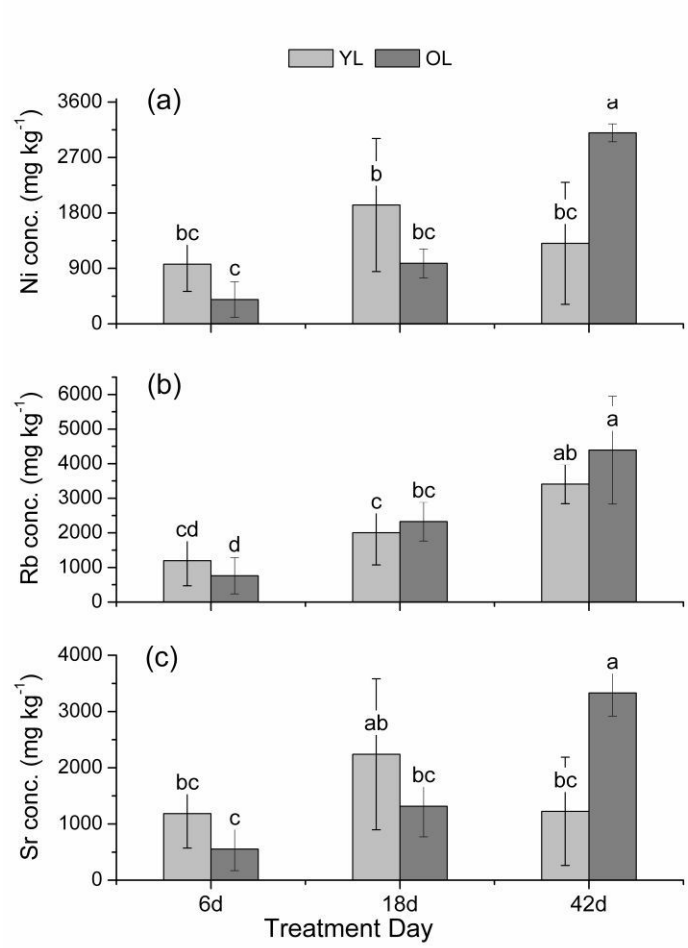

Figure 4.1 Concentrations of Ni (a), Rb (b) and Sr (c) in young (YL) and old leaves (OL). Error bars show standard deviation (SD) of the three to four replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

To assess the phloem contribution, we measured Ni/Sr and Rb/Sr ratio in YL and OL. The Ni/Sr ratios of YL increased with time, from 0.74 (6 d) to 1.09 (42 d) (Figure 4.2a), and all were greater than those of OL, indicating YL had additional Ni source after long-term treatment. For Rb/Sr ratio, it is quite obvious the ratio become much larger in YL of 42 d treatment.

The effect of xylem and phloem transport on metal accumulation was shown in Figure 4.3, which presented the Ni, Rb and Sr allocation in YL and OL. Apparently, OL was the main sink for the three elements, which comprised more than 60% of the total metal content in shoots. The phloem mobile elements, Rb and Ni, were a bit more enriched in YL than Sr.

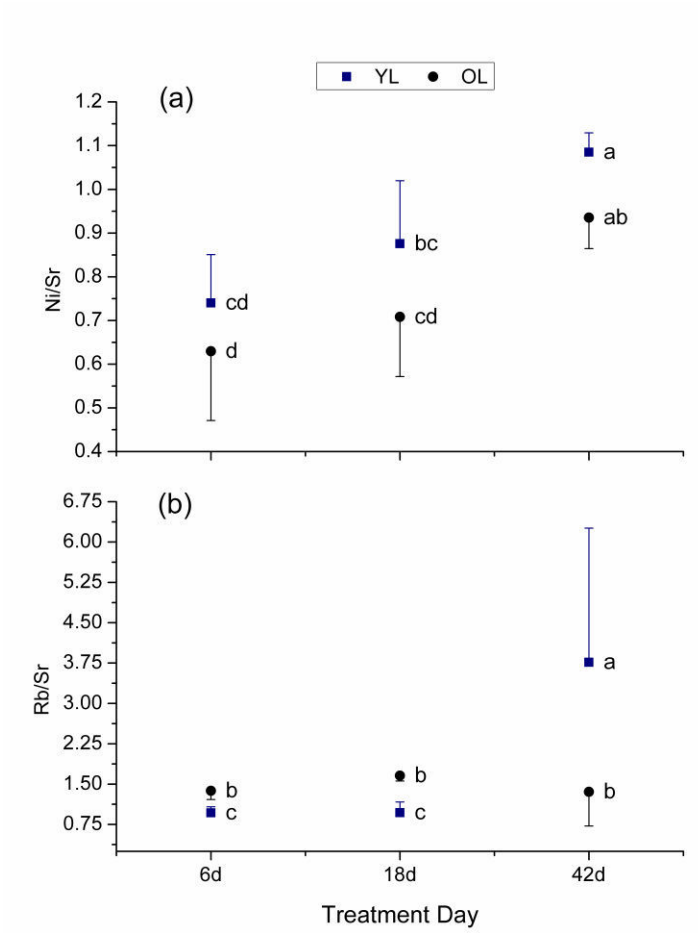

Figure 4.2 Values of Ni/Sr (a) and Rb/Sr ratios (b) in young (YL) and old leaves (OL). Error bars show standard deviation (SD) of the three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's Test).

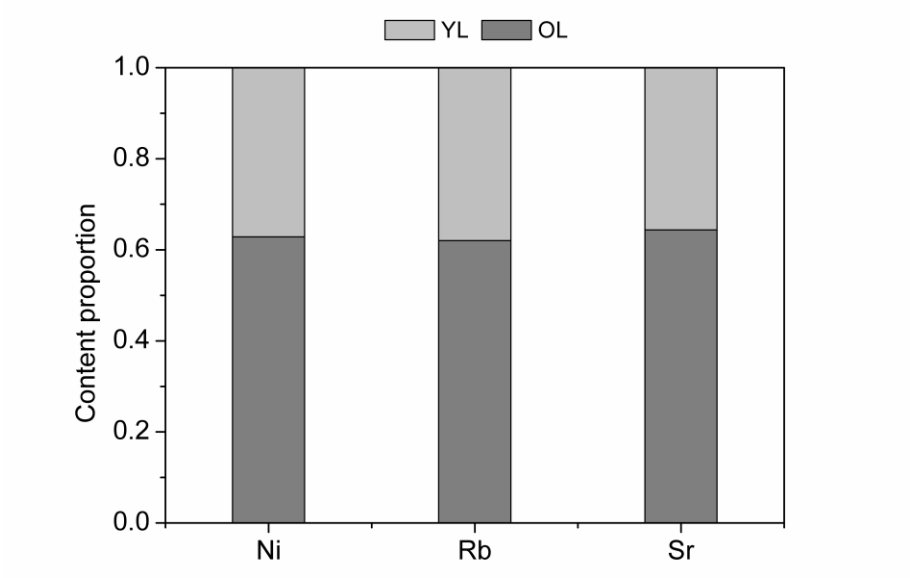

Figure 4.3 Nickel, Rb and Sr content proportion of young (YL) and old leaves (OL).

4.3.2 Upward and downward movement of Ni in phloem

Table 4.1 showed the results from ^{61}Ni foliar application experiment. According to ^{60}Ni concentration in plants, and the natural abundances of ^{60}Ni and ^{61}Ni, we calculated that less than 1% of the total ^{61}Ni content was brought by contamination, which could be neglected. Thus, it can be considered all of the ^{61}Ni in plants was exported from the spiked leaf via phloem translocation. And the results show that 89% of the ^{61}Ni moved upward to shoots, and 11% was transported downward to the roots, suggesting that young growing leaves are the main sink for phloem flow in *N. caerulescens*.

Table 4.1 Biomass and ^{61}Ni concentration in different parts of *N. caerulescens* from foliar ^{61}Ni application experiment (Expt. 4.2). Data are means ± standard deviation of three replicates.

Plant parts	Biomass	Concentration (mg kg^{-1})		^{61}Ni content	Ni content
		^{60}Ni	^{61}Ni	(µg)	ratio
Root	43.8±11.2	1.01±0.26	5.34±1.79	0.226 ± 0.071	11%

Shoot	56.7± 8.3	0.484±0.048	33.5±13.8	1.86 ± 0.68	89%

4.4 Discussion

4.4.1 Nickel partitioning during xylem transport

In this work, we found that old leaves are the main sink for xylem transport, in particular after long term treatment. This is quite consistent with the findings from Robinson et al. (2003) and Perronnet et al. (2003). Xylem transport is mainly driven by transpiration (Taiz and Zeiger 2010). Old leaves generally have larger leaf surface and greater biomass in comparison to young leaves, and receive most of the xylem fluid. Thus, most Ni transported by xylem is deposited and stored in old leaves.

4.4.2 Nickel partitioning during phloem translocation

Phloem translocation is a bidirectional transport. Both downward and upward movements exist in phloem. Riesen and Feller (2005) found that radioactive ^{63}Ni fed into the leaf lamina in wheat seedlings was rapidly transferred up to the younger leaves and down to the roots. Fismes et al. (2005) also found that in three vegetables (lettuce, radish and bean), ^{63}Ni migrated throughout the whole plants following foliar application, and mainly towards young leaves, seeds and roots.

Here, bidirectional translocation of Ni in phloem was investigated. We calculated the Ni partitioning between downward (roots) and upward (shoots) movements, and found that young leaves are the main sink for phloem translocation, which receive most of the phloem-origin Ni, while roots are a weak sink for phloem sap. This is corroborated with sugar partitioning in plants (Fondy and Geiger 1980). Young leaves are stronger sinks than roots, which can deplete the sugar content in the sieve elements more readily and thus increase the pressure gradient and the rate of phloem translocation towards themselves (Taiz and Zeiger 2010). Therefore, it could be speculated that Ni moves passively in the sieve elements,

following the sugar flow. And Ni partitioning in *N. caerulescens* might be a reflection of sugar partitioning in plants.

4.4.3 Contribution of xylem transport and phloem translocation on Ni accumulation in leaves

In the previous sections, we have discussed metal distribution in YL and OL via xylem and phloem transport, and found OL is the main sink for xylem fluid and main source for phloem translocation, while YL is an active sink for xylem fluid and the main sink for phloem flow. Therefore, it would be of interest to clarify the effect of these processes on the metal accumulation in YL and OL.

Firstly, it should be noted that in the short-term treatment (6 d), low concentrations of Ni were accumulated in old leaves (Figure 4.1a). Due to the strong sequestration ability in leaves of *N. caerulescens* (Milner and Kochian 2008), little Ni was expected to be exported out from OL via phloem in such low Ni concentrations. Therefore, it could be concluded that Ni accumulated in young leaves after short-term Ni exposure was mainly imported by xylem, and phloem-origin Ni was negligible. While in the long-term treatment (42 d), Ni accumulation in OL became evident and may act as a strong phloem source. This is reflected in the Ni/Sr ratio of YL (Figure 4.2a). The increment of Ni/Sr ratio from 0.74 (6d) to 1.09 (42d) suggests that additional Ni has reached young leaves, which shall be via phloem. This trend is more clearly presented by Rb/Sr ratio, as we can observe much higher Rb/Sr values in YL after long-term treatment, indicating the extensive supply from phloem. The difference between Ni and Rb could be ascribed to the variation of phloem mobility, as Rb is generally more mobile than Ni in phloem (Neumann and Chamel 1986). Therefore, the effect of phloem translocation is more obvious on Rb accumulation in YL.

4.5 Conclusion

In this study, we have investigated the Ni partitioning in leaves during xylem transport and phloem translocation, and their contribution on Ni accumulation. Results showed that old

leaves are the main sink for xylem transport and the main source for phloem translocation, while young leaves are also an active sink for xylem transport and the main sink for phloem translocation. Phloem translocation becomes increasingly important for Ni accumulation in young leaves, when high concentration of Ni is present in old leaves. The main findings of this study are illustrated in Figure 4.4.

The results of this work also raise new questions concerning phloem translocation, e.g. how Ni is loaded into phloem tissues, how Ni is transported in phloem sieve elements, etc, which will be the work of next chapter.

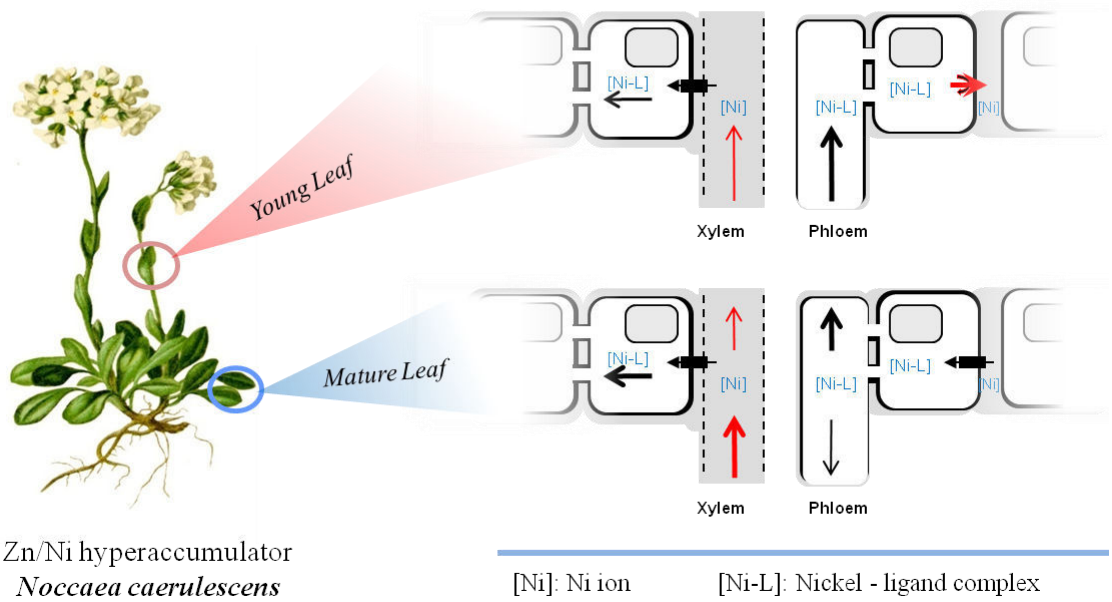

Figure 4.4 Illustration of Ni partitioning in leaves of *Noccaea caerulescens* during xylem transport and phloem translocation. Xyelm transport is the primary Ni source for above ground tissues. Mature leaves receive most of the xylem fluid and become the main source for xylem transport. They also act as the main source for phloem translocation, which could export significant amount of Ni. Young leaves are the main sink for phloem sap and thus receive most Ni in phloem, which is important for Ni accumulation in these tissues.

5. Nickel speciation in phloem sap of *Noccaea caerulescens*[*]

5.1 Introduction

The physiological processes associated with hyperaccumulation include: i) stimulated metal influx across root cell plasma membranes (Pence et al. 2000); ii) reduced metal sequestration in root vacuoles (Lasat et al. 1998); iii) increased loading into xylem for transport to shoots (Papoyan and Kochian 2004; Hanikenne et al. 2008) and iv) stimulated metal influx across leaf cell plasma membranes and sequestration in leaf vacuoles (Küpper et al. 2001; Küpper et al. 2004).

In the previous chapter, we also found that phloem translocation may transfer significant amount of Ni for young leaves of *Noccaea caerulescens*. Field investigations reported that Ni hyperaccumulators growing on ultramafic soils can accumulate high concentrations of Ni in flowers and seeds (Robinson et al. 1997b; Zhang et al. 2014; Groeber et al. 2015). Reproductive organs are usually considered to have weak xylem transport but strong phloem translocation activities (Taiz and Zeiger 2010). Therefore, these results suggest that Ni may be efficiently translocated in the phloem of hyperaccumulators. Furthermore, Ni enrichment in phloem has been documented in a number of woody hyperaccumulators growing on tropical ultramafic soils. For instance, 16.9% of Ni was found in the freeze-dried phloem sap of the Ni hyperaccumulator *Phyllanthus balgooyi* from Sabah, Malaysia (van der Ent and Mulligan 2015); and up to 25.7% Ni was recorded in the latex of *Pycnandra acuminata* from New Caledonia (Jaffre et al. 1976), amounting to the highest Ni concentration ever found in living material.

Phloem sap is slightly alkaline (pH = 7 – 8) and enriched with organic compounds, *e.g.* sugars, amino and organic acids (Taiz and Zeiger 2010). Metal ions, *e.g.* Ni^{2+} and Zn^{2+}, are usually chelated with organic compounds, and amino acids are favorable for metal complexation in such alkaline condition (Harris et al. 2012). Zinc-nicotianamine (NA) complex has been

[*] Deng THB, Tang YT, van der Ent A, Sterckeman T, Echevarria G, Morel JL, Qiu RL. 2015. Nickel translocation via the phloem in the hyperaccumulator *Noccaea caerulescens* (Brassicaceae). *Plant and Soil*. DOI: 10.1007/s11104-016-2825-1.

found in the phloem sap of castor bean and rice (Hazama et al. 2014; Nishiyama et al. 2012). In addition to NA, Harris et al. (2012) predicted that cysteine (Cys) also plays a role for Zn chelation in phloem sap, according to a speciation model. The only study regarding Ni speciation in phloem sap was carried out by Wiersma and van Goor (1979), who found that Ni was bound to organic compounds with a molecular weight in the range of 1000 – 5000 in phloem sap of *Ricinus communis*. However, no information is available for Ni complexation in phloem sap of hyperaccumulators.

The high concentration of Ni in phloem of hyperaccumulators justifies the investigation on the Ni chemical speciation in this compartment. Therefore, this work was designed to bring new information about 1) the Ni concentrations in the phloem sap, and 2) the potential Ni chelating ligands during phloem translocation, by studying the hyperaccumulator model species *N. caerulescens*.

5.2 Materials and methods

5.2.1 Confirmation of the feasibility of the EDTA-stimulated phloem exudation method (Expt. 5.1)

Seeds of *N. caerulescens* (Puy de Wolf) were sown on peat matrix (Fafard Custom Growing Mix, Canada) and germinated in the dark at 22 °C for 7 d. Seedlings were then transferred to a growth chamber. Growth conditions were 22/18 °C day/night temperatures, 60% relative humidity, 16 h photoperiod and 120 μmol s^{-1} m^{-2} light intensity. After 1 month, 40 seedlings were transferred to 4 L nutrient solution for hydroponic pre-culture. The standard solution contained the following nutrients (in µM): 1000 $Ca(NO_3)_2$, 1000 KNO_3, 500 $MgSO_4$, 100 KH_2PO_4, 50 KCl, 10 H_3BO_3,1 $MnCl_2$, 0.2 $CuSO_4$, 5 $ZnSO_4$, 0.2 Na_2MoO_4, 20 Fe-EDDHA. Two mM 2-morpholinoethanesulphonic acid (MES) was used to buffer the pH, which was adjusted to 5.8 by the addition of 5% KOH.

After 4 months of pretreatment, 4 plants with uniform size were selected and each was placed in a 1 L pot. Plants were grown in 0.75 L nutrient solution, with the addition of 100 µM $NiSO_4$, RbCl (rubidium chloride) and $SrCl_2$ (strontium chloride), respectively, and solutions

were renewed every 3 d. After 20 d of treatment, old leaves were cut from rosette and used for phloem exudate extraction, according to the method described earlier. Three treatments were set up: 1) 1 h of Milli-Q water (MQW) + 5 h of MQW extraction; 2) 1 h of EDTA-K_2 + 5 h of MQW extraction; 3) 1 h of EDTA-K_2 + 1 h of MQW extraction. Phloem exudates were then determined for their Ni, Rb and Sr concentrations by ICP-MS. In addition, metal concentrations in leaves were also analyzed.

5.2.2 Extraction of phloem exudate from expanding and old leaves of N. caerulescens *(Expt. 5.2)*

After 1 month of pre-cultivation in standard solution, plants of uniform size were selected for treatment. Sets of two plants were transplanted to a container filled with 2 L nutrient solution. Three treatments were set up, i.e. CK (control; Ni 0, Zn 5 μM), Zn100 (Ni 0, Zn 100 μM) and Ni100 (Ni 100, Zn 5 μM). All treatments were replicated three times. Solutions were renewed every 6 d.

After 5 months of treatment, leaves were cut from the rosette and used for phloem exudate extraction, the method of which was adapted from Tetyuk et al.(2013). Briefly, 30 old leaves (>2 months of age) from each pot were cut at the base of the petioles and then placed in Petri dishes containing 20 mM K_2-EDTA solution. Leaves were recut at the base of the petioles and then transferred to a centrifuge tube (5 ml, Eppendorf) containing 4 mL of 20 mM K_2-EDTA, with the cut end of leaves submerged in the solution. Potassium-EDTA was used to prevent sealing of phloem sieve elements. After 1 h, leaves were washed by Milli-Q water and then transferred to a new tube, which contained another extraction solution (4 mL Milli-Q water). The final extraction period was 5 h with the tubes placed in a moist and illuminated environment to minimize leaf transpiration. Phloem exudate solution was frozen in liquid N_2, followed by lyophilization, and finally re-dissolved in 1 mL of Milli-Q water. In addition, 30 expanding leaves (ca. 1 month of age) from each pot were also cut from the rosette and used for phloem exudate collection. Phloem exudates were then divided into several aliquots for analysis of mineral nutrient elements, amino acids and organic acids.

Mineral nutrient elements (Ni, Zn and Fe) were determined by ICP-MS (iCAP Q, Thermo Scientific, USA). Iron, a micronutrient element in plants, was analyzed in this experiment because its concentrations in plant tissues (ca. 1.8 µmol g^{-1}; Epstein and Bloom 2005) and phloem saps (40 – 80 µM; Hocking 1983; Schmidke and Stephan 1995; Yoneyama et al. 2010; Ando et al. 2012) generally remain stable. Therefore, Fe can be regarded as a reference element, with which the concentrations of other elements in phloem sap can be compared.

Malate and citrate, which are the most abundant organic acids and play important roles for metal chelation in shoots of *N. caerulescens* (Montargès-Pelletier et al. 2008; Tolrà et al. 1996), were determined by ion spectrometry (IC) (DX 600, Dionex, USA) according to AS11 and AS11-HC Anion-Exchange Column Instruction Manual (Dionex, USA). In brief, the analysis was conducted using a 4-mm diameter AS11 model pre-column and column. A gradient program, ranging from 3 to 20 mM of eluent, was used to separate the acids in 30 min, with a flow rate of 1.0 mL min^{-1}.

For amino acid compounds, special attention was paid to nicotianamine (NA) and histidine, which have high chelating affinities with Ni and Zn and were previously reported to play a role in metal chelation and hyperaccumulation (Kozhevnikova et al. 2014a; Kramer et al. 1996; Mari et al. 2006; Callahan et al. 2007; Richau et al. 2009). Nicotianamine was determined by liquid chromatography - tandem mass spectrometry (LC-MS/MS) (TSQ Quantum Ultra, Thermo Scientific, USA) with C18 ion exchange analytical column (1.9 µm , 100 mm × 2.1 mm; Hypeisil GOLD, Thermo Scientific, USA) applying the following gradient system: 0 min, 10% eluent A (acetonitrile) and 90% eluent B (0.1% HCOOH / water); 3 min, 90% eluent A; 4.1 min, 10% eluent A (flow rate 0.3 mL/min; sample injection volume 5 µL). The MS profiles were carried out in the following conditions: source heater temperature, 300 °C; sheath gas (N_2), 45 arbitrary units; auxiliary gas (N_2), 5 arbitrary units; spray voltage, +3.0 kV; capillary temperature, 300 °C. The quantitative analysis of NA was detected in SRM mode. Three product ions $(M+H)^+$ were acquired, i.e. *m/z* 286.2, 185.2 and 114.1, with source collision induced dissociation (CID) energy of 10 , 18 and 27 V, respectively, while *m/z* 304.2 → *m/z* 185.2 was used for quantitative ion pairs. Nicotianamine standard was bought from Toronto Research Chemicals Inc., Canada. Twenty

common amino acids were determined by amino acid analyzer (Syknm-S7130, Germany), according to National Standard Guideline of China for Analysis of Amino Acids (JY/T019-1996).

After phloem extraction, the expanding and old leaves, along with roots and young leaves (10 – 15 d of age), were put in paper bags and dried in 60 °C, and then digested by concentrated HNO_3. Heavy metal concentrations in digested solutions were determined by ICP-AES (iCAP 6500 Duo, Thermo Scientific, USA).

5.3 Results

5.3.1 Nickel, Rb and Sr concentrations in Expt. 5.1

Table 5.1 presents the Ni, Rb and Sr concentrations in exudates and leaves of *N. caerulescens* in Expt. 5.1. Great differences were existed between the MQW and the EDTA treatments: relatively high concentration of Sr was found in MQW treatment, while much less Sr in EDTA treatments. Moreover, the Rb/Sr mole ratio of phloem exudates was around 7 in MQW treatment, which is quite similar to that in leaves; whereas Rb/Sr mole ratio could reach 40-60 in EDTA treatments (Figure 5.1). These results suggested that the exudates extracted by MQW and EDTA have different properties and origins.

Table 5.1 Ni, Rb and Sr concentrations in exudates and leaves of *N. caerulescens* after 20 d of treatment in Expt. 5.1

Treatment	Conc. in phloem exudate (μM)			Conc. in leaves (μmol g^{-1})		
	Ni	Rb	Sr	Ni	Rb	Sr
1h MQW + 5h MQW	1.50	5.06	0.73			
1h EDTA + 5h MQW	11.3	9.57	0.15	15.1	40.1	6.32
1h EDTA + 1h MQW	7.23	2.59	0.06			

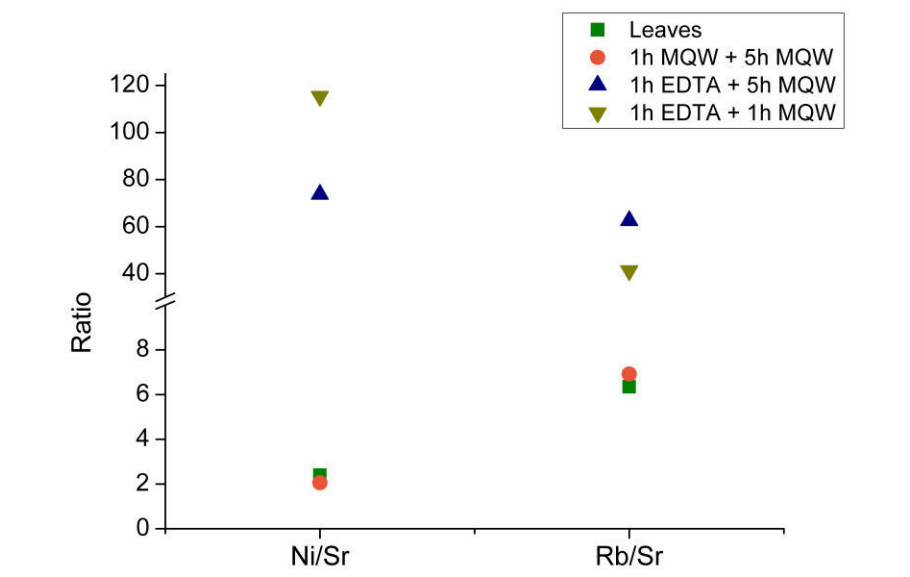

Figure 5.1 Ni/Sr and Rb/Sr mole ratios in exudates and leaves of *N. caerulescens* after 20 d of treatment in Expt. 5.1

5.3.2 Nickel and Zn concentrations in leaves and phloem exudates of expanding and old leaves in Expt.5.2

The Ni and Zn concentrations of different plant parts in Ni100 and Zn100 treatments are presented in Table 5.2. After the long-term treatment (5 months), all leaves accumulated high levels of Ni or Zn, and concentrations increased with aging. Nickel and Zn concentrations could reach 130 and 161 µmol g^{-1} dry weight, respectively, in fully-expanded old leaves (>2 months of age), in relation with the exceptional Ni and Zn accumulation ability in *N. caerulescens*. It is noticeable that young leaves (10 – 15 days of age) could also accumulate up to 72.5 µmol g^{-1} of Ni or 82.9 µmol g^{-1} of Zn.

Table 5.2 Ni and Zn concentrations in different plant parts of *N. caerulescens* in Expt. 5.2. Data are means ± standard deviation of three replicates.

Treatment	Element		Roots	Young leaves (10-15 d)	Expanding leaves (30 d)	Old leaves (>60 d)
Ni100	Ni	μM	33.6 ± 4.5	72.5 ± 10.4	107 ± 33	130 ± 29
Zn100	Zn		46.4 ± 11.7	82.9 ± 3.1	129 ± 12	161 ± 13

For phloem exudates, significant variation could be observed between expanding and old leaves. The Ni and Zn concentrations in exudates were 56 and 101 μM, respectively in old leaves, while a 27 and 26% reduction was recorded in expanding leaves (Figure 5.2b, d). These results suggest that old leaves are more efficient in loading metals into phloem tissues, and may act as the main metal source for phloem translocation.

Iron concentrations in leaves and phloem exudates are shown in Figure 5.2e, f. Stable concentrations of Fe were found in leaves (~ 2.5 μmol g^{-1}) of *N. caerulescens* grown in all treatments, which is similar to non-hyperaccumulating plants (ca. 1.8 μmol g^{-1}; Epstein and Bloom 2005). Iron concentrations in phloem exudates also remained quite stable (1.0 – 2.9 μM). Therefore, it could be speculated that Fe concentrations in phloem sap of *N. caerulescens* could be comparable to that of non-hyperaccumulating plants. It is noticeable that concentrations of Ni and Zn in phloem exudates were far greater than that of Fe (ca. 20 and 70 times, respectively).

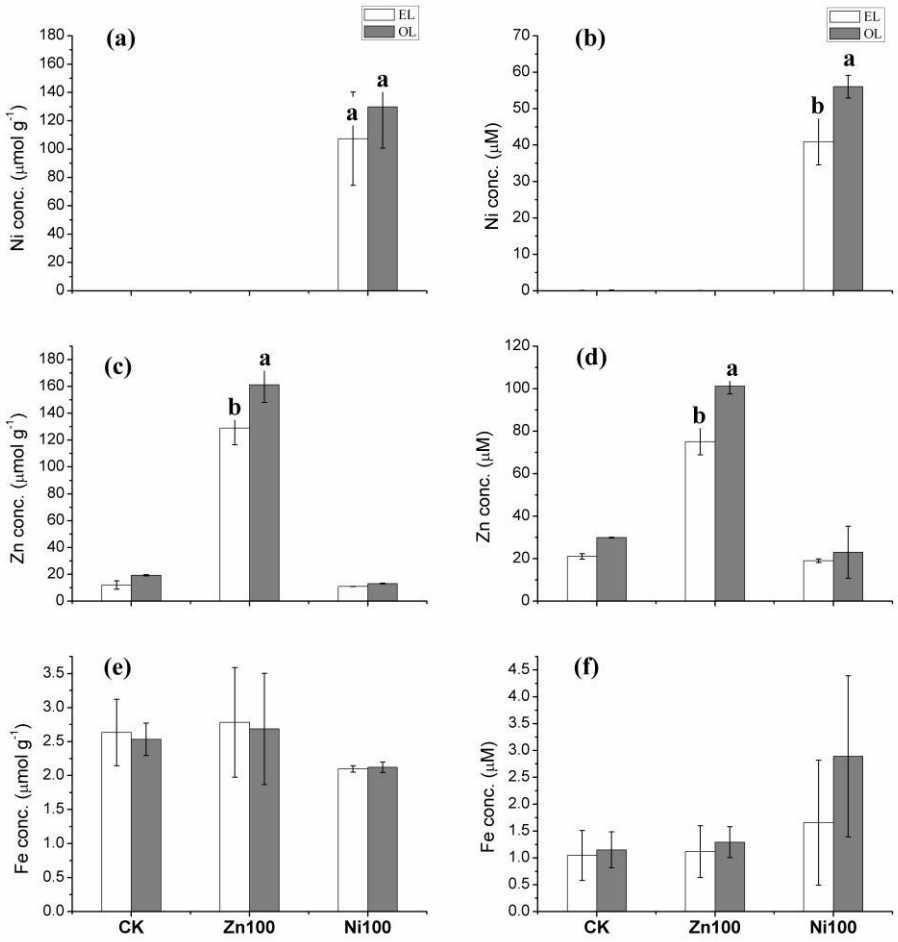

Figure 5.2 Concentrations of Ni, Zn and Fe in leaves (a, c, e) and phloem exudates (b, d, f) of expanding (EL) and old leaves (OL) of *N. caerulescens*. Data are means ± standard deviation of three replicates. Different letters in the column indicate values significantly different at $P < 0.05$, Duncan's Test.

5.3.3 Organic compounds in phloem exudate of old leaves

To investigate the potential chelating ligands for Ni and Zn during phloem translocation, relevant amino acids and organic acids in exudates were given in Figure 5.3. Concentrations of malate, the prevailing organic acid in the phloem exudates, were constitutionally high (149 – 169 μM) in all treatments, which were themselves sufficient to chelate all Ni or Zn (1:1) in the exudates, implying that organic acids should play an important role for Ni and Zn speciation in phloem sap of *N. caerulescens*.

In contrast, concentrations of amino acids were relatively low compared to those of Ni and Zn in phloem exudates. In particular, only trace concentrations (< 1 μM) of NA and His were detected in all treatments, which are negligible to chelate Ni and Zn in exudates. Thus, amino acids seem to play a minor role in Ni and Zn chelation during phloem translocation in *N. caerulescens*.

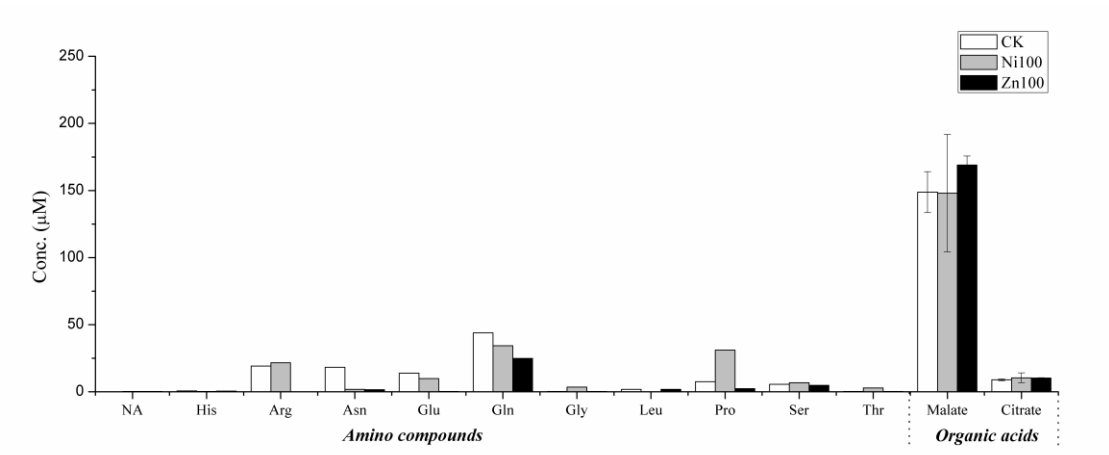

Figure 5.3 Concentrations of amino acids and organic acids in phloem exudate of *N. caerulescens*. Data of amino acids have one replicate; data of organic acids are means of two replicates.

5.4 Discussion

5.4.1 Confirmation of the exudate properties

The EDTA-stimulated phloem exudation method has been used in many studies and is proved to be efficient for phloem sap collection (King and Zeevaart 1974; Chen et al. 2001; Deeken et al. 2008; Guelette et al. 2012). The phloem exudate collected by this method could be used to analyze proteins, small molecules, lipids, and RNAs (Tetyuk et al. 2013). However, there was an uncertainty regarding the relevance of this method for metal extraction in phloem sap. Thus, we conducted a preliminary experiment. Strontium is a phloem-immobile element, while Rb is easily transported in phloem (Kuppelwieser and Feller 1991). Therefore, Sr

concentration is expected to be low, while Rb should be enriched in phloem. The results of this experiment were in good agreement with our expectation. The leaf exudation extracted by EDTA contained extremely low amount of Sr and had high Rb/Sr ratio (Table 5.1 and Figure 5.1), indicating its phloem origin. On the contrary, the water-extracted exudation had a high Sr concentration and low Rb/Sr ratio. In particular, the Rb/Sr ratio in the exudation was quite similar to that in leaves, indicating that this exudation could originate from leaf mesophyll cells. The Ni behavior was quite similar to that of Rb. Therefore, it can be concluded that the EDTA-facilitated extraction method is efficient for phloem exudate collection and subsequent metal determination.

5.4.2 Nickel enrichment in phloem sap

Because the EDTA-stimulated exudation method can only produce dilute phloem exudates, we cannot determine the true Ni and Zn concentrations in the original phloem saps. However, according to the ratio between Fe concentration in diluted exudates from this study (1.0 – 2.9 μM), and that in phloem saps from publications (40 – 80 μM; Hocking 1983; Schmidke and Stephan 1995; Yoneyama et al. 2010; Ando et al. 2012), we estimated that Ni and Zn concentrations in original phloem sap of *N. caerulescens* could reach 800 to 1600 μM and 2800 to 5600 μM, respectively. These concentrations are far greater than those in phloem sap of non-hyperaccumulating plants as well as in xylem fluids of hyperaccumulators (Álvarez-Fernández et al. 2014 and the references therein). Nickel enrichment in phloem has been documented in a number of woody hyperaccumulators growing on tropical ultramafic soils, such as the earlier noted *P. balgooyi*, *P. acuminata*, and also in *Euphorbia helenae* subsp. *grandifolia* which grows in Cuba (Reeves et al. 1996). Moreover, enhanced ability for Zn remobilization via phloem was found in the Zn hyperaccumulator *Sedum alfredii* (Lu et al. 2013). These results, along with our study, suggest that Ni or Zn enrichment in phloem may be a common phenomenon in hyperaccumulating species.

In addition, high Ni and Zn concentration in phloem saps also suggests that *N. caerulescens* has an extraordinary phloem loading capability for heavy metals. However, little is known

about how mineral nutrients are loaded into phloem tissues. According to our knowledge on the sucrose phloem loading process, both apoplastic and symplastic pathways exist in higher plants. In *Arabidopsis thaliana*, apoplastic loading is the most likely sucrose transport pathway due to the lack of plasmodesmata connections between the phloem parenchyma and the sieve element-companion cell complex (Haritatos et al. 2000). *Noccaea caerulescens*, which shares approximately 88% nucleotide sequence identity within coding regions with *A. thaliana* (Peer et al. 2006), may have similar phloem structure. If this is indeed the case, then phloem tissues in *N. caerulescens* should have great Ni and Zn absorption ability by taking up large quantities of Ni and Zn from ambient apoplastic fluid.

5.4.3 Nickel speciation in phloem sap

For non-hyperaccumulating plants, Ni and Zn in phloem sap are present at low concentrations, which can be easily chelated by the redundant amino acids, and even high molecular weight organic molecules (Wiersma and van Goor 1979; Nishiyama et al. 2012; Hazama et al. 2014). However, it should be noted that Ni and Zn are not micronutrients in the hyperaccumulator *N. caerulescens*. Their concentrations in phloem sap are several tens of times higher than those in non-hyperaccumulating plants. Therefore, Ni and Zn chelation with high molecular weight organic compound (i.e. S- and N-donors) are unlikely to happen, due to the substantial amounts of ligands that would be required. The relatively low concentrations of amino acids in phloem sap are also insufficient for chelating all Ni or Zn.

To further clarify the Ni/Zn chelation in phloem sap, we have used Geochem EZ speciation model to estimate the Ni and Zn speciation in Ni100 and Zn100 treatment, respectively. The phloem sap pH was fixed at 7.5, and the input concentrations of Ni, Zn and organic compounds were calculated according to the method from the previous section (5.4.2). The results showed that Ni was mainly bound to malate (50%), while Alanine (20%) and Glutamic acids (10%) also play roles on the Ni chelation (Figure 5.4a). Similar results were obtained for Zn, which was complexed by malate (51%), Glutamic acid (16%), citrate (9%), etc (Figure 5.4b).

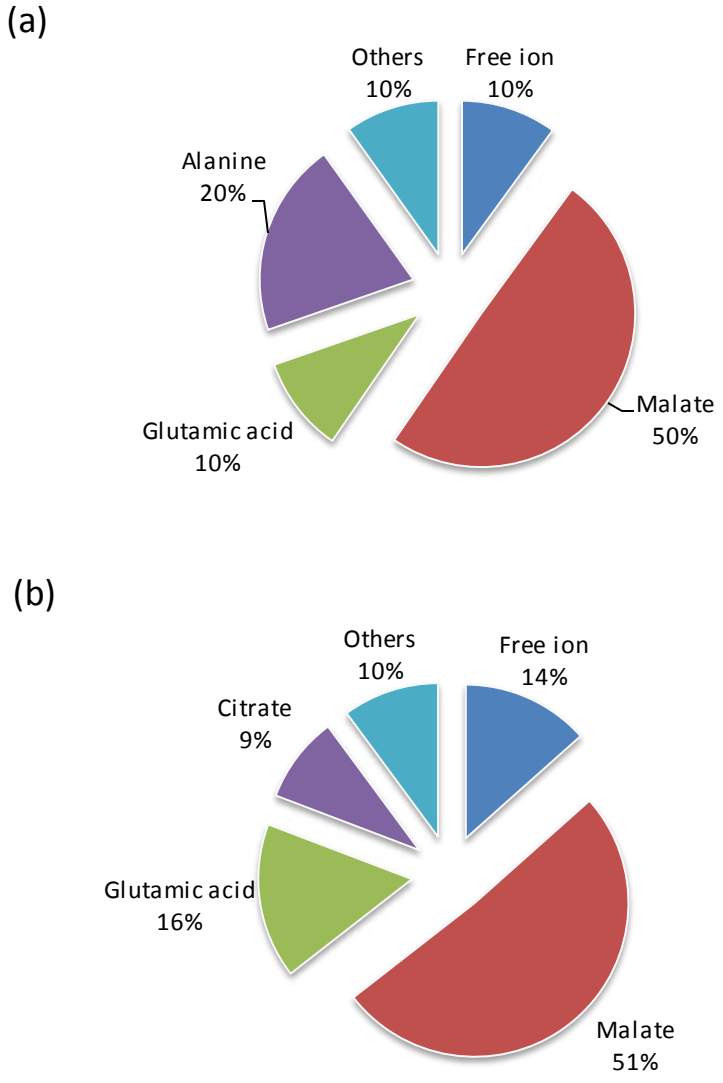

Figure 5.4 Nickel (a) and Zn (b) speciation in phloem sap of *N. caerulescens*, which is calculated by Geochem-EZ.

Therefore, organic acids, which present in high levels in phloem sap of *N. caerulescens*, seem to be the main ligands for Ni and Zn chelation, as complexation with organic acids can also increase the metal mobilities in phloem (Miranda et al. 2001; Epstein and Bloom 2005). In fact, citrate has already been discovered to be the main Ni chelator in the latex of the Ni hyperaccumulator *P. acuminata* (Schaumloffel et al. 2003).

5.4.4 Significance of phloem translocation on Ni accumulation in leaves

The observation of high Ni concentrations in phloem saps and large Ni influx into young leaves (Chapter 4) suggests that phloem translocation may play an important role for Ni accumulation in these tissues. A rough calculation can be made to estimate the contribution of phloem translocation for Ni accumulation in young leaves, when diurnal change and competition between sinks are left out. In Ni100 treatment, 30 old leaves exported 0.057 µmol of Ni in 5 h, thus the Ni exporting rate was 0.0114 µmol h^{-1}. If 89% of the Ni had moved up to a young leaf (0.01 g dry weight), then the leaf would gain 24.7 µmol g^{-1} Ni in 24 h. Similarly, phloem-based Zn was estimated to be 48.3 µmol g^{-1}. This is a non-negligible sum of Ni or Zn compared to the Ni or Zn concentrations in young leaves (72.5 and 82.9 µmol g^{-1} for Ni and Zn, respectively; Table 5.2), which may partly explain the fast accumulation of Ni or Zn in young leaves in comparison to expanding and old leaves, in particular when young leaves are considered to be strong sinks for phloem sap.

5.5 Conclusion

In this chapter, we have investigated the concentrations of metals (Ni, Zn and Fe), organic and amino acids in phloem sap of *N. caerulescens* in order to clarify how Ni is transported in phloem tissues. Results showed that high concentrations of Ni and malate are present in phloem sap, which may be transported as Ni-malate complexes. In contrast, amino acids are present in low concentrations, which may play a minor role for Ni complexation in phloem. Significant amounts of Ni can be retranslocated from mature leaves to young leaves via phloem and thus makes a great contribution for Ni accumulation in these tissues. The main findings of this study are illustrated in Figure 5.5.

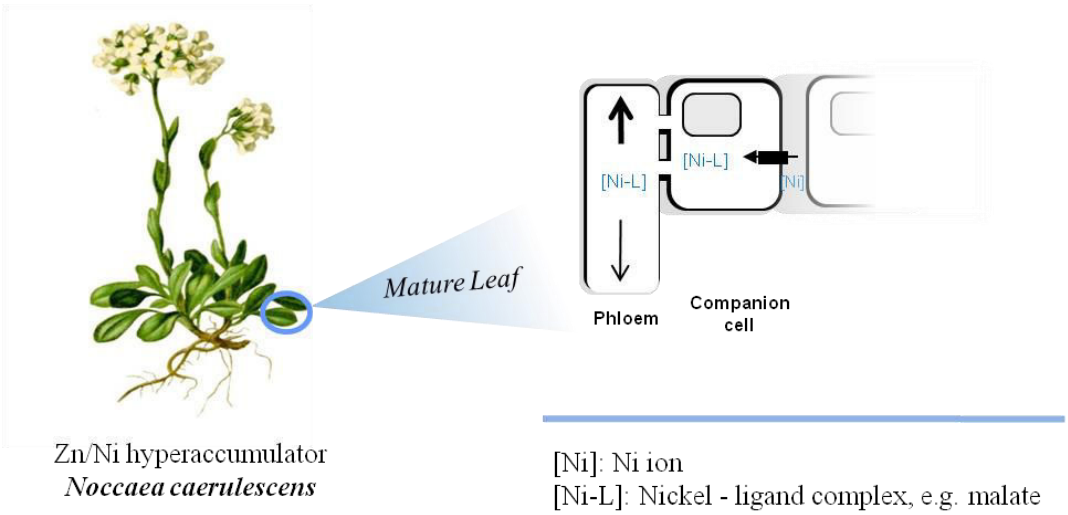

Figure 5.5 Illustration of the main findings in Chapter 5. Nickel is enriched in phloem sap of *N. caerulescens*, which probably chelated by organic acids, e.g. malate. The Ni carried by phloem flow can have significant impact on Ni accumulation in young leaves.

6. Main conclusions and future scope

6.1 Main findings of the thesis

Our current understanding on hyperaccumulation mechanisms are mainly derived from research on Zn hyperaccumulators. Much less is known about Ni homeostasis in hyperaccumulators, in particular the Ni absorption and phloem translocation processes.

In this study, we have conducted isotope fractionation, absorption kinetics and molecular studies to clarify how Ni is taken up by roots of hyperaccumulators. Meanwhile, the characteristics of phloem translocation, including its bidirectional movement and phloem sap compositions, as well as the contribution of phloem on Ni accumulation in shoots were explored to clarify how Ni is transported and retranslocated *in vivo*. Here are the main findings of this study:

For Ni uptake in roots, the Zn/Ni hyperaccumulator *Noccaea caerulescens* and the Ni hyperaccumulator *Alyssum murale* both possess high-efficient low-affinity Ni transport systems, which seem to have greater permeability than that of the non-hyperaccumulator *Thlaspi arvense*. Interaction between Ni and Zn, Fe, Co, and Mn was found during the root uptake process of *N. caerulescens*. Nickel absorption seems to be involved in non-specific transporters from ZIP (Zinc-regulated transporter, Iron-regulated transporter-related Protein) family, in particular Zn and Fe transporters.

For Ni circulation in plants, xylem transport is of primary importance for the Ni accumulation in leaves, particularly for mature leaves. Nickel can be efficiently absorbed by phloem tissues and then retranslocated from mature leaves to young leaves, which is important for the Ni accumulation in young leaves. Nickel is enriched in phloem saps of *N. caerulescens*, which may be mainly chelated by organic acids, e.g. malate.

6.2 Conceptual model for Ni homeostasis in hyperaccumulators

On the basis of the results from this study, along with knowledge from the literature, we put

forward a conceptual model for Ni homeostasis in *N. caerulescens* (Figure 6.1). Nine physiological processes are included:

(1) Rhizospheric Ni ions are efficiently absorbed into root symplast via low-affinity transport systems, which mainly belong to Zn and Fe transporters (Chapter 2 and 3).

(2) In root cytoplasm, assimilated Ni ions are readily chelated by organic compound, e.g. histidine (Kerkeb and Krämer 2003). Most Ni will then be transferred radially from epidermis to pericycle, due to the weak sequestration ability by root vacuoles (Richau et al. 2009).

(3) Nickel is then efficiently exported from xylem parenchyma and loaded into xylem vessels, which may be due to the high expression of specific efflux transporter(s), *e.g. NcHMA4* (Papoyan and Kochian 2004).

(4) Nickel moves up to shoots following the xylem flow, most of which find their final destination in mature leaves due to the strong transpiration in these tissues, while young leaves receive relatively small amount of metals (Chapter 4). Nickel is presented mainly as free hydrated cations during xylem transport (Centofanti et al. 2013).

(5) As xylem flow reaches minor veins and fills in the apoplastic space in leaves, Ni ions are then unloaded into the leaf symplast, or stored in apoplast (Robinson et al. 2003).

(6) Nickel in leaf cytoplasm will be readily transferred into vacuoles, in particular in epidermis cells (Küpper et al. 2001). Nickel in vacuoles is mainly chelated by organic acids for detoxification (Montargès-Pelletier et al. 2008).

(7) Phloem companion cells, which are soaked in Ni-rich apoplastic fluid, may contain large amount of Ni influx transporters in cell membranes, which are able to absorb large quantities of metal ions into the cytosols. The imported Ni ions are chelated by a variety of organic molecules, e.g. malate (Chapter 5).

(8) Metal-ligand complexes are then transferred from companion cells to phloem sieve elements via plasmodesmata. Nickel moves passively in sieve elements, following the phloem flow, which is mainly driven by osmotic pressure generated by photosynthate (mainly sucrose) concentration gradients between sources and sinks (Taiz and Zeiger 2010). Upward

movement appears to be the main direction for phloem translocation (Chapter 4).

(9) When reaching sink organs, Ni may be exported out from phloem tissues to the apoplast again, which are then taken up by the surrounding cells (Lu et al. 2013).

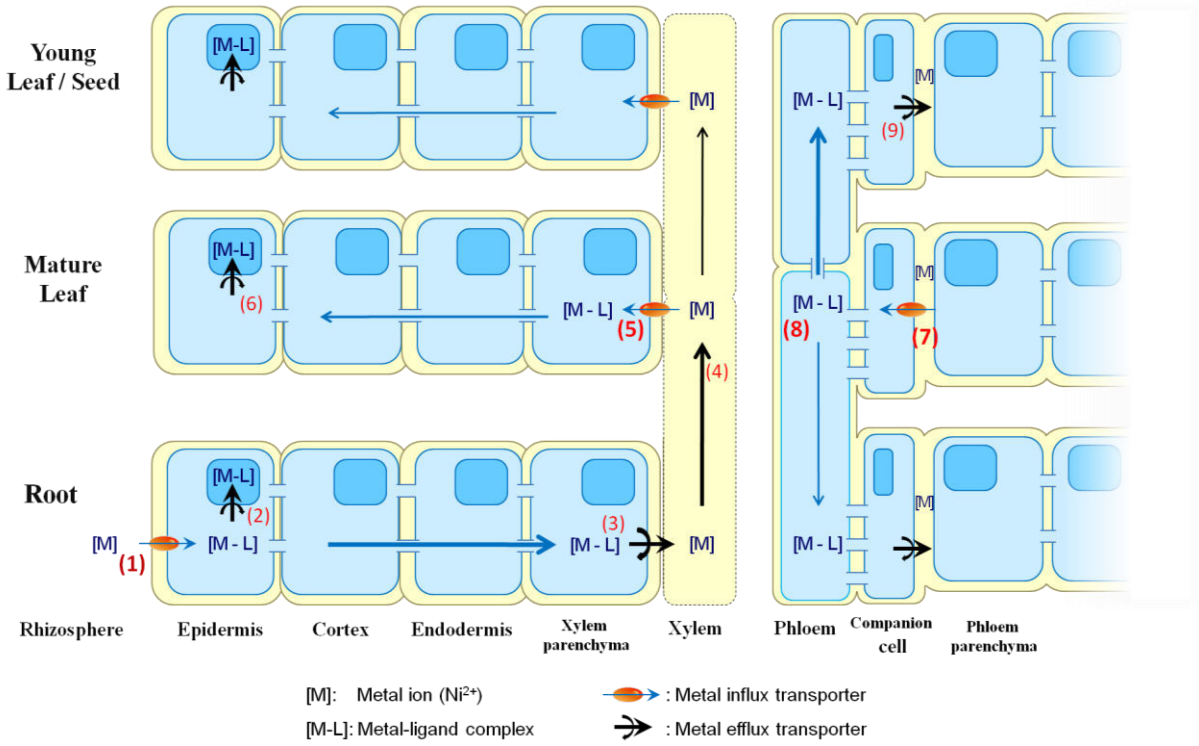

Figure 6.1 Conceptual model for Ni homeostasis processes in *N. caerulescens*. (1) root uptake; (2) root sequestration and radial transport; (3) xylem loading; (4) xylem transport; (5) xylem unloading; (6) leaf sequestration; (7) phloem loading; (8) phloem translocation; (9) phloem unloading. The findings of this thesis have expanded our knowledge on Process 1, 5, 7 and 8.

6.3 Future scope

(1) In this study, we have found that Ni uptake in roots of *N. caerulescens* could be associated with ZIP protein family, e.g. *ZIP10* and *IRT1*. It should be noted that ZIP family includes plenty of transporter genes, many of which may be related to Ni absorption. Therefore, molecular studies are needed to further explore the candidate genes involving Ni uptake in both roots and shoots (Process 1, 5 and 7, Figure 6.1) of hyperaccumulators, in particular for

the Ni hyperaccumulator *Alyssum murale*, which does not possess Zn hyperaccumulation ability.

(2) In this study, we have determined the compositions of diluted phloem exudates in *N. caerulescens* and found high levels of Ni and malate. In future research, it would be better to extract original phloem sap from the plants, by means of delicate techniques, e.g. aphid stylectomy. The properties of phloem sap, including the pH and the speciation of Ni would be of interest to analyze. In addition, the physiology of phloem loading and unloading still remains elusive, which could also be a good research point for future studies.

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