Rôles des endophytes fongiques racinaires dans la tolérance de Fallopia aux éléments Traces Métalliques

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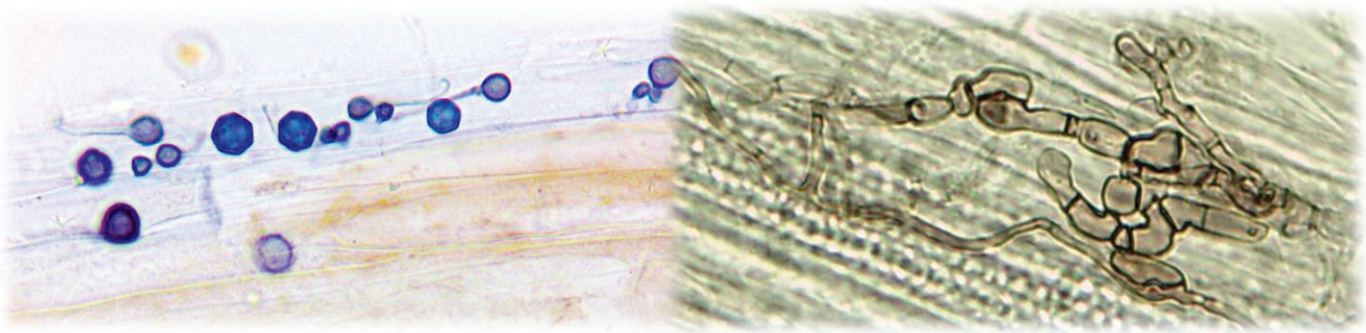

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Louise

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Résumé

En deux phrases et deux questions

Les Renouées asiatiques se développent très bien sur les milieux contaminés aux métaux. Quelle est donc leur stratégie ? Dans leurs racines se trouvent des champignons : sont-ils impliqués dans la tolérance des Renouées aux métaux ?

Français

Depuis la révolution industrielle et les émissions de contaminants qui en découlent, les sols sont soumis à des concentrations de plus en plus fortes en éléments traces métalliques (ETM). Ceux-ci, non-biodégradables et toxiques à faible dose pour la majorité des organismes, perturbent les écosystèmes et posent des problèmes environnementaux et sanitaires majeurs. Pourtant, de nombreux organismes, notamment végétaux, ont pu s'adapter à la présence d'ETM dans leurs milieux : c'est le cas des Renouées asiatiques qui s'étendent en Europe, y compris dans les milieux contaminés aux ETM. Les Renouées sont, comme la majorité des plantes, associées avec des microorganismes, dont certains, notamment des champignons présents dans leurs racines, pourraient être impliqués dans la tolérance de cette plante aux ETM. **L'objectif général de cette thèse est de tester cette hypothèse, en étudiant les espèces fongiques associées aux racines des Renouées asiatiques et leurs rôles dans la tolérance de ce taxon à la pollution métallique des sols, en conditions contrôlées.**

Pour cela, des rhizomes de Renouées (Renouée du Japon, Renouée de Sakhaline et leur hybride Renouée de Bohême) ont été mis en culture sous serre, en présence ou non d'ETM (Cd, Cr, Zn et leur mélange). La colonisation fongique des racines a été quantifiée par microscopie et des souches ont été isolées afin de les caractériser. Les métabolites secondaires présents dans les racines et dans certaines souches isolées ont été identifiés et quantifiés. L'ensemble de ces données a été mis en relation avec les traits de performance de la plante, en fonction des conditions métalliques.

Une étude bibliographique (Axe 1) inventoriant les endophytes fongiques présents dans les racines des plantes et leurs effets sur la tolérance de leur hôte aux ETM a permis de mettre en évidence l'ubiquité (diversité d'hôtes et d'environnements) des endophytes fongiques racinaires, leur diversité taxonomique et leurs propriétés stimulatrices de la croissance de

leur hôte végétal. En milieu contaminé aux ETM, ces endophytes inoculés aux plantes sont également bénéfiques à leur croissance, mais avec des effets divers sur le prélèvement et le transfert des ETM dans les tissus végétaux.

Deux expérimentations en conditions contrôlées ont été menées pour étudier l'effet des métaux sur les Renouées d'une part, et identifier les endophytes qui lui sont associés d'autre part (Axe 2). Ces expérimentations ont confirmé la tolérance des Renouées aux ETM, leurs traits étant peu voire pas affectés par les contaminations. 27 endophytes appartenant aux Dothideomycetes, Sordariomycetes et Eurotiomycetes en majorité, ont été isolés de leurs racines. Certains d'entre eux ont été testés pour leur tolérance aux ETM et d'autres pour la production de métabolites secondaires. Parmi ces souches, *Fusarium oxysporum f. sp. dianthi*, *Trichoderma* sp., *Diaporthe* sp. et l'isolat correspondant probablement à *Lachnum* sp. semblent particulièrement intéressantes pour leur tolérance aux ETM et la production de composés bénéfiques aux plantes (phytohormones, antioxydants, chélateurs et antimicrobiens).

Enfin, l'effet des endophytes sur la tolérance de la Renouée aux ETM a été exploré, en modifiant le contenu endophytique des plantes et par quantification microscopique de certains endophytes fongiques racinaires (Axe 3). Certains d'entre eux, tels que les *Olpidium*, sont bénéfiques aux Renouées en absence de contamination, mais d'autres tels que les endophytes septés (DSE, Dark Septate Endophytes) stimulent la croissance en présence de Zn. Les DSE sont plus abondants lors du stress métallique. En parallèle, l'ajout de Zn dans le sol provoque une augmentation de la concentration en torosachrysone dans les racines, elle-même corrélée à la fréquence de colonisation des racines par les DSE. Ces résultats peuvent s'inscrire dans la théorie de « l'appel à l'aide », la plante soumise à un stress sécrétant des molécules messagères chargées de recruter des microorganismes pour l'aider à y faire face.

L'ensemble de ces études conforte l'importance des endophytes fongiques racinaires dans la tolérance de leur plante hôte aux contaminants métalliques, et a permis d'identifier des champignons spécifiques et des molécules particulières qui interviendraient plus particulièrement dans ce mutualisme chez *Fallopia*. De futures expérimentations envisagent d'approfondir le rôle de ces endophytes et molécules identifiés dans la tolérance de l'hôte aux ETM. Ces résultats s'intègrent dans la compréhension des invasions végétales et pourront être utiles en phytorémédiation afin d'améliorer la croissance de plantes extractrices de contaminants métalliques.

English

Since the industrial revolution and the resulting contaminant emissions, soils have been subjected to increasingly high concentrations of trace metal elements (MTEs). These, which are non-biodegradable and toxic at low doses for most organisms, disrupt ecosystems and pose major environmental and health problems. However, many organisms, especially plants, have been able to adapt to the presence of MTEs in their environment: this is the case of Asian knotweeds, which are spreading in Europe, including in environments contaminated with MTEs. Like the majority of plants, knotweeds are associated with microorganisms, some of which, notably fungi present in their roots, could be involved in the tolerance of this plant to MTEs. The general objective of this thesis is to test this hypothesis, by studying the fungal species associated with the roots of Asian knotweeds and their roles in the tolerance of this taxon to metallic pollution of soils, under controlled conditions.

For this purpose, rhizomes of knotweed (Japanese knotweed, Sakhalin knotweed and their hybrid Bohemian knotweed) were grown in greenhouses, in the presence or absence of MTEs (Cd, Cr, Zn and their mixture). Fungal colonization of the roots was quantified by microscopy and strains were isolated to characterize them. Secondary metabolites present in the roots and in some isolated strains were identified and quantified. All these data were related to plant performance traits, depending on the metal conditions.

A bibliographical study (Axis 1) inventorying the fungal endophytes present in plant roots and their effects on the tolerance of their host to MTEs has highlighted the ubiquity (host and environmental diversity) of root fungal endophytes, their taxonomic diversity and their growth stimulating properties of their plant host. In environments contaminated with MTEs, these endophytes inoculated to plants are also beneficial to their growth, but with various effects on the uptake and transfer of MTEs in plant tissues.

Two experiments under controlled conditions were carried out to study the effect of metals on Knotweed on the one hand, and to identify the endophytes associated with it on the other (Axis 2). These experiments confirmed the tolerance of Knotweeds to MTEs, as its traits were little or not affected by contamination. 27 endophytes belonging to

Dothideomycetes, mainly Sordariomycetes and Eurotiomycetes, were isolated from their roots. Some of them were tested for tolerance to MTEs and others for the production of secondary metabolites. Among these strains, *Fusarium oxysporum f. sp. dianthi*, *Trichoderma* sp., *Diaporthe* sp. and the isolate probably corresponding to *Lachnum* sp. seem particularly interesting for their tolerance to MTEs and the production of compounds beneficial to plants (phytohormones, antioxidants, chelating agents and antimicrobials).

Finally, the effect of endophytes on knotweed tolerance to MTEs was explored by modifying the endophytic content of plants and by microscopic quantification of certain root fungal endophytes (Axis 3). Some of them, such as *Olpidium*, are beneficial to knotweeds in the absence of contamination, but others, such as Dark Septate Endophytes (DSEs), stimulate growth in the presence of Zn. DSEs are more abundant during metal stress. At the same time, the addition of Zn to the soil causes an increase in the concentration of torosachrysone in the roots, which in turn correlates with the frequency of root colonization by the DSEs. These results may be consistent with the "plant call for support" theory, where the stressed plant secretes messenger molecules that recruit microorganisms to help it cope.

All these studies confirm the importance of root fungal endophytes in the tolerance of their host plant to metallic contaminants. This thesis has made it possible to identify specific fungi and singular molecules that would intervene more particularly in this mutualism with *Fallopia*. Future experiments plan to further investigate the role of these identified endophytes and molecules in host tolerance to MTEs. These results are part of the understanding of plant invasions and could be useful in phytoremediation in order to improve the growth of plants that extract metallic contaminants.

Introduction

L'intensification des activités humaines depuis la révolution industrielle est à l'origine de l'émission d'un grand nombre de contaminants dans l'environnement. Dans les zones urbaines, les sols sont souvent caractérisés par des teneurs élevées en éléments traces métalliques (ETM) tels que le plomb (Pb), le zinc (Zn), le cadmium (Cd) ou encore le chrome (Cr), résultant d'un mélange d'émissions industrielles, domestiques et automobiles. Non-biodégradables, ces contaminants présentent une toxicité à faible concentration pour une très grande majorité d'êtres vivants, conduisant à une modification de la structure et du fonctionnement des écosystèmes. Par exemple, Cd, Cr et Zn, réduisent la germination et la croissance des plantes, tout en modifiant l'accès aux nutriments et en provoquant une chlorose (blanchissement des feuilles par perte de chlorophylle) (Fattahi et al., 2019; Rasafi et al., 2016; Shaikh et al., 2013; Yadav, 2010).

Pourtant, certains organismes se sont adaptés : ils ne développent pas de signes de toxicité malgré la présence d'ETM, comme par exemple les plantes poussant sur la prairie métallicole de Mortagne-du-Nord (France), site *Natura 2000* (Lemoine and Pauwels, 2014). On retrouve parmi ces organismes, des espèces végétales envahissantes qui permettent d'étudier les performances végétales dans les milieux contaminés aux ETM. C'est le cas des Renouées asiatiques, formant le complexe d'espèces *Fallopia* (Polygonaceae) : capables de coloniser les sols urbains contaminés (Sołtysiak et Brej, 2014), les Renouées asiatiques ont ainsi rapidement colonisé l'Europe et l'Amérique du Nord par une multiplication végétative très performante additionnée d'une importante reproduction sexuée issue de l'hybridation interspécifique entre les espèces parentales *F. japonica* et *F. sacchalinensis*, à l'origine de l'espèce hybride *F.* x *bohemica* (Bailey et al., 2009). Bien qu'il existe un certain nombre d'études concernant les teneurs en ETM dans les sols où se développent ces taxons (dans l'aire naturelle ou sur l'aire d'introduction) et/ou dans les différentes parties de la plante (Hlavati Širka et al., 2016; Nishizono et al., 1989; Rahmonov et al., 2019; Sołtysiak et al., 2011), le mécanisme de tolérance des Renouées aux ETM reste peu compris.

Décrites comme non-mycorhiziennes, les racines des Renouées asiatiques sont pourtant colonisées par des champignons endophytes (Gucwa-Przepióra et al., 2016). Au microscope, les endophytes septés, ou « Dark Septate Endophytes » (DSE) et les *Olpidium* (Olpidiomycota) sont les plus nombreux et aisément identifiables. Les DSE sont

non seulement résistants aux métaux mais auraient également un rôle bénéfique pour leur plante hôte en présence de ces contaminants (Berthelot et al., 2017; He et al., 2017; Jin et al., 2018; Yamaji et al., 2016).

Les champignons endophytes racinaires, à l'interface entre le sol et l'hôte, pourraient accroître la performance des plantes en milieu contaminé via la sécrétion d'hormones de croissance végétale (Khan and Lee, 2013) ou par la synthèse de molécules capables de chélater les ETM ou de limiter leur toxicité, tels que le glutathion, des phytochélatines ou des composés phénoliques. Les métabolites fongiques peuvent également renforcer le système de défense de l'hôte ou modifier la biodisponibilité des contaminants métalliques, leur transport et leur stockage par la plante (Domka et al., 2019b).

Ces éléments suggèrent que **la colonisation des racines par des champignons endophytes aurait un rôle prépondérant dans la tolérance des Renouées asiatiques aux ETM.**

L'objectif général de cette thèse est de tester cette hypothèse, en étudiant les espèces fongiques associées aux racines des Renouées asiatiques et leurs rôles dans la tolérance de ce taxon à la pollution métallique des sols, en conditions contrôlées.

Après une présentation du contexte, la thèse comportera trois axes complémentaires suivies d'une discussion générale :

Axe 1 : Une étude bibliographique des endophytes fongiques présents dans les racines des plantes et leurs effets sur la tolérance de leur hôte aux ETM ;
Axe 2 : L'étude de l'effet des métaux sur les Renouées et les endophytes associés ;
Axe 3 : L'étude de l'effet des endophytes sur la tolérance de la Renouée aux ETM.

Chapitre 1 : Cadre général

Origines, devenir et impacts des ETM sur les écosystèmes

Des ETM d'origine principalement anthropiques

Les ETM sont présents de manière naturelle dans les roches, à des concentrations pouvant varier selon le type de roche et l'histoire de la région. Ces concentrations locales naturelles constituent le fonds géochimique local, pouvant être élevé dans des régions volcaniques notamment. Cependant, la révolution industrielle a entraîné une explosion de la production anthropique d'ETM, qui continue d'augmenter. Ne serait-ce qu'entre les années 1930 et 1985, la production primaire de Zn a été multipliée par 4, celle de Cd par 15 et celle de Cr par 18 (Senesil et al., 1999). De nombreuses activités anthropiques sont sources d'ETM : l'industrie minière (Ag, Cd, Cu, Hg, Ni, Pb, Zn), les industries plastiques, métallurgiques ou électroniques (As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Tl, Zn), l'utilisation d'énergie fossile (As, Cr, Pb, Se), les fertilisants, pesticides et fongicides (As, Cd, Cu, Hg, Pb, Zn), le trafic routier (Cu, Pb, Zn) et bien d'autres encore (Adriano, 2001; Nikolaeva et al., 2019).

Cette production s'accompagne d'émissions et de dispersion dans les écosystèmes, notamment via l'atmosphère. Les émissions anthropiques sont nettement supérieures aux émissions naturelles : 88 % du Cd , 96 % du Pb et 99 % du Zn émis dans l'atmosphère sont d'origine anthropique **(Tableau 1,** Valavanidis et Vlachogianni (2010)**)** !

Les ETM de l'atmosphère se déposent sur les sols où, non-biodégradables, ils ont tendance à s'accumuler. Ainsi, les sols s'enrichissent en ETM - par rapport au fonds géochimique - dans les zones sous influence d'activités anthropiques **(Figure 1.1)**. Les zones urbaines et industrielles sont les plus grandes sources de contamination, ainsi que les zones agricoles subissant l'épandage de boues d'épuration et l'utilisation de pesticides, fongicides et fertilisants (Bourennane et al., 2010).

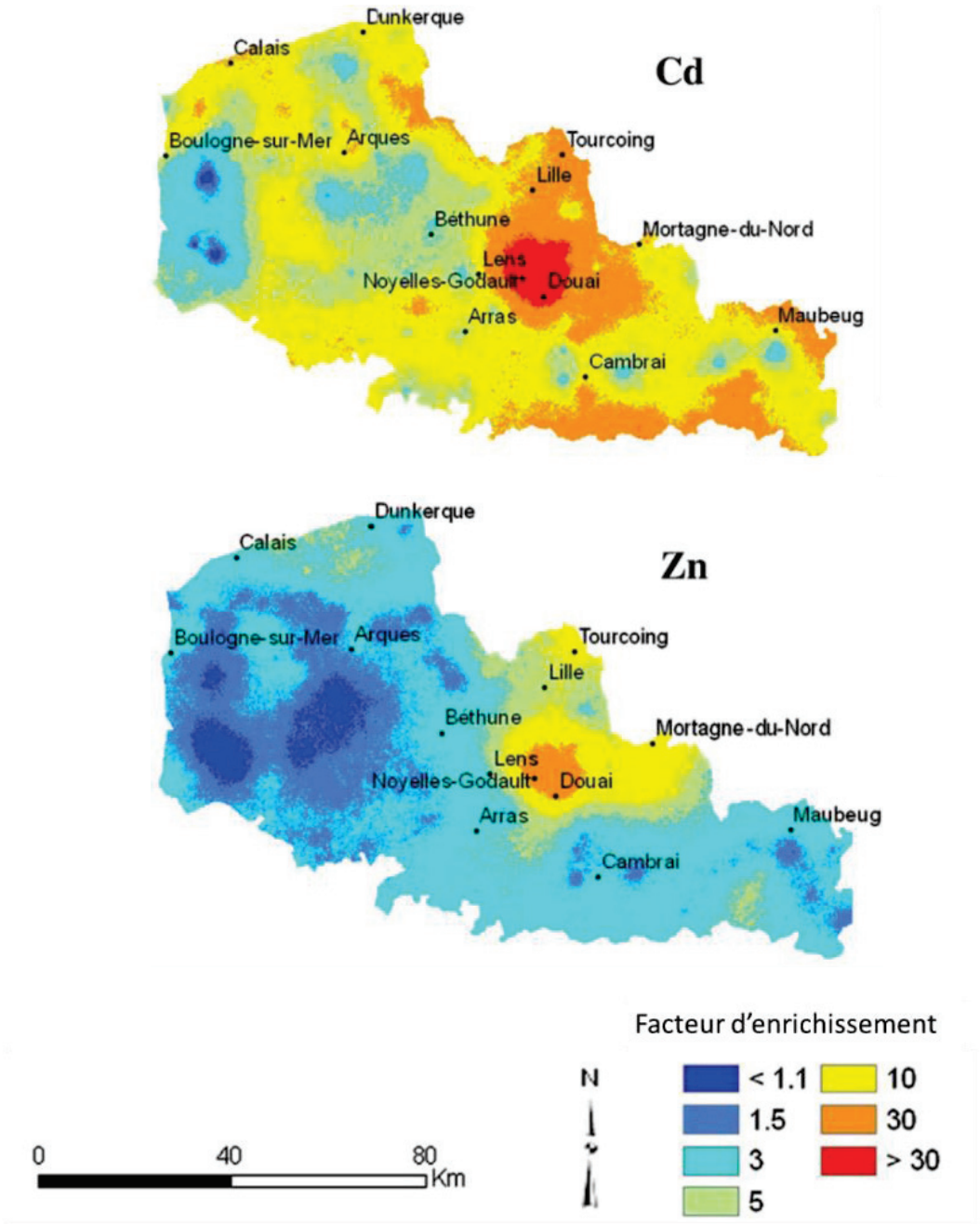


Figure 1.1 : Enrichissement des sols en Cd et Zn dans le département du Nord (France). *Ces cartes combinent mesures des concentrations en ETM (Cd et Zn) dans le sol et modélisation. Le facteur d'enrichissement correspond à une concentration d'ETM supérieure à celle attendue d'après les fonds géochimiques, et montre ainsi l'impact des activités anthropiques. Par exemple, autour de Douai, le cadmium est plus de 30 fois plus concentré dans les sols que dans des sites proches considérés comme non-contaminés. Extrait de Bourennane et al. (2010).*

Tableau 1.1 : Émissions d'ETM et concentrations observées et réglementaires dans les sols. FG : fonds géochimique. Les chiffres sont donnés pour quelques ETM parmi les plus étudiés, avec des exemples de concentrations auxquelles ils ont été retrouvés dans des sols contaminés ou non.

MTE	Émissions atmosphériques annuelles (1000 t/an)		Émissions annuelles (1000 t/an)	Concentration dans les sols (mg/kg)			Réglementation	
	Naturelles	Anthropiques	Anthropiques (1985)	Médiane française	Non-contaminé (Fonds géochimique local)	Contaminé	Seuil dans les sols (mg/kg) de toxicité écologique ou de risque pour la santé (Finlande)	Concentrations maximales autorisées (mg/kg) dans les boues d'épandage (France)
As	7,8	24	45		12,7 (4)	16 ± 13,5 % (4)	50	
Cd	1	7,5	19	0,30	0,118 (4) 0.02 - 0.14 (6) 0,30 (7)	2,45 ± 83,4 % (4) 2.2 - 4.0 (6) 2,08 (7)	10	2
Cr			9940	37,6	14 – 21 (6) 5,1 (7)	43 – 89 (6) 59,8 (7)	200	150
Cu	19	56	8114	13,8	23,5 (4) 2 – 6 (6)	46,4 ± 60,3 % (4) 99 – 230 (6)	150	100
Hg			6,8	0,05	0,086 (4)	2,91 ± 110,6 % (4)	2	1
Ni	26	47	778	20,4	4 – 8 (6)	19 – 42 (6)	100	50
Pb	19	450	3077	25,6	16,3 (4) 4 – 8 (6) 9,6 (7)	252 ± 78,4 % (4) 184 – 254 (6) 110,8 (7)	200	100
Zn	4	320	6042	59,0	65,8 (4) 9 – 19 (6) 60,5 (7)	286 ± 44,6 % (4) 394 – 712 (6) 2623,0 (7)	250	300
Référence	*1*	*1*	*5*	*2*	*4, 6, 7*	*4, 6, 7*	*3*	*2*

1 : Valavanidis and Vlachogianni (2010)
2 : Baize et al. (2006)*, médiane française*
3 : Tóth et al. (2016)
4 : Xiao et al. (2017) *en Chine : mine d'or artisanale*
5 : Senesil et al. (1999)
6 : Lacercat-Didier et al. (2016) *en France : zone irriguée par des eaux usées*
7 : Sołtysiak et al. (2011) *en Pologne : zone non-développée (zone 11) vs parking en zone urbaine (zone 19)*

Dans le sol, des ETM plus ou moins disponibles pour la plante

Les ETM interagissent avec les propriétés physico-chimiques du sol au niveau des trois phases qui le composent : solide, liquide et gaz. La phase solide comprend des minéraux (silicates et argiles principalement), une phase organique (humus et autres composés organiques à l'exclusion des êtres vivants ou peu dégradés), des précipités et de la biomasse, morte ou vivante **(Figure 1.2)**. La phase liquide, appelée « solution de sol » ou encore phase aqueuse, est constituée d'eau, de minéraux et autres solutés. La phase gazeuse est quant à elle en relation directe avec l'atmosphère (Adriano, 2001). Ces trois phases étant en équilibre thermodynamique, les ETM déposés par l'atmosphère sont susceptibles de passer d'un compartiment à un autre lors de modifications physico-chimiques. Par exemple, Cd(II) dans le sol peut être trouvé sous les formes de Cd^{2+}, $CdSO_4$, $CdCl^+$, et $CdHCO_3^+$ sur sol basique. Zn(II) peut être ionique (Zn^{2+}) ou organique (Zn-org) (Prasad, 2004). Les ETM sont biodisponibles, c'est-à-dire accessibles à la plante, lorsqu'ils sont présents dans la solution du sol. La minéralisation de la biomasse, la désorption d'ETM adsorbés sur l'humus, la présence d'argiles et d'oxydes (formant le complexe argilo-humique), ainsi que la dissolution de précipités conduisent à l'augmentation de la biodisponibilité des ETM dans le sol. Ces processus sont contrôlés par des facteurs environnementaux tels que l'acidité du sol (un pH bas favorise la dissolution) et la capacité d'échange de cations (c'est-à-dire la capacité d'un volume de sol de fixer réversiblement des ions, dont les ETM, sur son complexe argilo humique).

Il est intéressant de constater que la plante, via l'exsudation de métabolites secondaires dans la solution de sol, peut modifier ces facteurs et donc la solubilité des ETM. En effet, ces composés de faible poids moléculaire sont riches en groupements chimiques (fonctions carboxyles, cétones ou phénols notamment) ayant de fortes capacités de chélation des ETM, des propriétés antioxydantes et de piégeage des radicaux libres, et pouvant également servir de ressource ou de moyen de communication avec les organismes du sol. La modification de la biodisponibilité des ETM par les exsudats racinaires peut avoir lieu de manière directe, via l'acidification du sol, la chélation d'ETM, ou des réactions de précipitation ou d'oxydo-réduction. Par exemple, des composés organiques excrétés par les racines peuvent augmenter la solubilité du Cd en formant un complexe soluble Cd-molécule organique. De manière indirecte, les exsudats interagissent avec les microorganismes du sol

et modifient la croissance des racines, modulant les capacités de prélèvement d'ETM par la plante (Adriano, 2001).

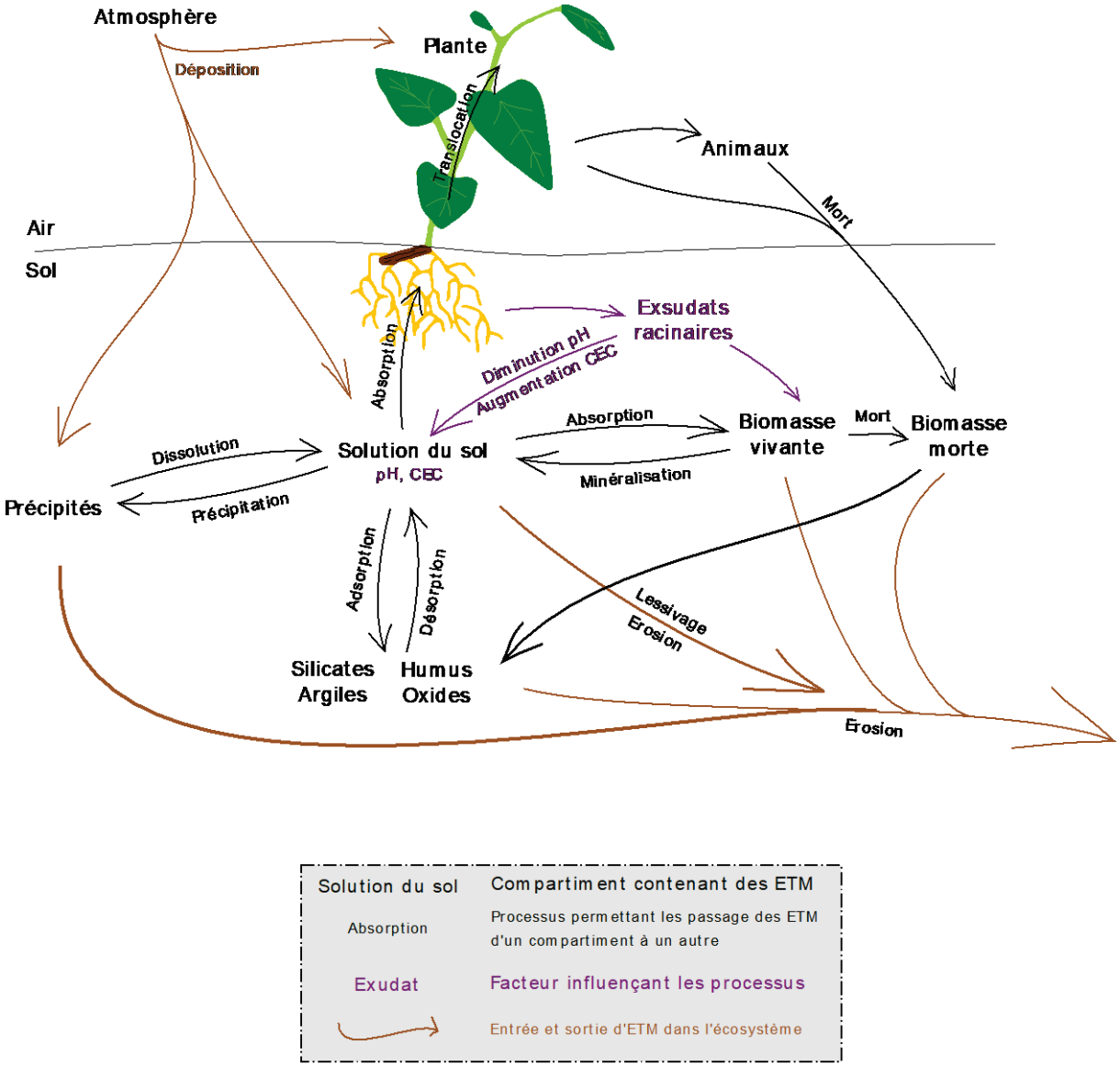


Figure 1.2 : Cycle géochimique des ETM. Modifié d'après Adriano, 2001.

Ainsi, les ETM émis par les activités anthropiques et déposés par l'atmosphère deviennent partie intégrante des écosystèmes, modifiant les équilibres biologiques qui les caractérisent.

Des ETM ayant un fort impact sur les écosystèmes

Si les ETM semblent être toxiques pour les plantes, c'est en réalité l'écosystème dans sa globalité qui est impacté, des microorganismes aux forêts entières. En effet, il a été démontré que les ETM étaient (au moins en partie) responsables du « nouveau déclin des

forêts », réduisant le nombre d'arbres (Prasad, 2004). La croissance des végétaux est inhibée, l'activité des microorganismes également (Plekhanova et al., 2019). Mais, outre le nombre d'organismes, ce sont les fonctions de l'écosystème qui sont dégradées : ainsi, la présence d'ETM dans le sol inhibe la productivité primaire, la fixation d'azote, la minéralisation de C, N, S et P, la décomposition de la litière ainsi que la synthèse et l'activité d'enzymes des sols (Babich and Stotzky, 1985). Il en va de même dans les milieux aquatiques, où respiration, productivité primaire et décomposition de la litière végétale sont réduites par la présence d'ETM, y compris à de très faibles concentrations (Peters et al., 2013). Les ETM y sont transmis le long de la chaîne alimentaire, avec une accumulation importante chez les poissons, provoquant des troubles histopathologiques et hormonaux (Mahino et al., 2014). Les ETM sont également transmis le long de la chaîne alimentaire en milieu terrestre, via leur accumulation dans les plantes, herbivores et carnivores (Prasad, 2008). Un intérêt anthropocentrique nous pousse à ajouter que, bien évidemment, l'espèce humaine n'est pas exclue de ces impacts et subit également les effets des ETM (Clemens and Ma, 2016; Valavanidis and Vlachogianni, 2010) : la maladie potentiellement mortelle d'Itai-Itai (littéralement « Aïe – Aïe ») est un exemple d'empoisonnement de la population au Cd, à proximité d'une mine de Zn au Japon, qui a pour effet de fragiliser les os et abîmer les reins (Kasuya et al., 1992).

En tant que productrices primaires et donc source d'énergie pour l'ensemble de la chaîne alimentaire, les plantes sont au cœur des relations écosystémiques. C'est pourquoi nous allons nous intéresser plus particulièrement à l'effet des métaux sur les plantes.

Effet des métaux sur les plantes et réponse de la plante

Symptômes métallifères

Bon nombre de plantes ont des symptômes similaires face à l'excès d'ETM : une croissance des racines et des parties aériennes altérée, une chlorose liée à une diminution de la photosynthèse, une réduction de la germination des graines, ainsi que la présence de tissus abîmés voire morts (Påhlsson, 1989; Rasafi et al., 2016; Shahid et al., 2019; Shaikh et al., 2013; Yadav, 2010). Le Cd provoque également une réduction de la surface des feuilles (comme le Pb) et de la floraison (Fattahi et al., 2019; Påhlsson, 1989; Rasafi et al., 2016). Ces symptômes extérieurs sont le reflet de modifications internes. La balance nutritive est

altérée : par exemple, les couleurs rouges des feuilles face à un excès de Zn reflètent un déficit en phosphore (Yadav, 2010), la concentration en fer dans les tissus peut diminuer suite à un excès de Cd (Påhlsson, 1989), etc. Le métabolisme azoté est également perturbé (Påhlsson, 1989). Par ailleurs, les ETM altèrent la structure des enzymes et la perméabilité membranaire (Miransari, 2011). Ainsi, le métabolisme énergétique (photosynthèse) et le métabolisme nutritif (nutriments) sont affectés par les ETM.

Les ETM, du fait de leur fort pouvoir oxydant, génèrent des espèces réactives de l'oxygène (ROS) qui causent un stress oxydatif important (Yadav, 2010). Ce stress est caractéristique de nombreux stress abiotiques tels que la salinité, des températures basses ou encore des déficits en nutriments (Landi et al., 2020) et les réponses peuvent donc être similaires (Sharma et al., 2019). Un équilibre peut être rétabli en ajustant la quantité d'antioxydants (Hasanuzzaman et al., 2018).

Stratégies de tolérance

Malgré ces effets généralement délétères pour les végétaux, certaines plantes subsistent sur les milieux contaminés aux ETM, sans présenter de symptôme particulier : elles sont appelées « métallophytes ». Face aux ETM, les plantes subsistant sur les milieux contaminés peuvent présenter différentes stratégies : celle de maintenir une concentration métallique interne faible en limitant l'entrée d'ETM dans ses tissus, stratégie dite de l' « exclusion » ; celle de laisser pénétrer les ETM dans leurs tissus (bio-indication), voire de les prélever activement dans le sol (accumulation ou hyperaccumulation), tout en limitant leur toxicité dans les tissus par détoxification ou stockage (Baker, 1981; van der Ent et al., 2013; Ghosh and Singh, 2005; Goolsby and Mason, 2015; Lasat, 1999). Ces différentes stratégies nécessitent de contrôler la biodisponibilité des métaux dans le sol et leur entrée dans les racines (exclusion ou internalisation), ainsi que leur transport au sein de la plante et leur stockage pour les plantes accumulatrices (Valavanidis and Vlachogianni, 2010).

Les ETM dans la plante : prélèvement, transport et stockage

Regardons ce que deviennent les métaux aux environs d'une plante et en son sein **(Figure 1.3)**. Les ETM traversent différents compartiments : le sol, la racine, le xylème, la feuille. Les déplacements peuvent être symplasmiques (les cytosols étant en continuité entre cellules au sein d'un compartiment) ou apoplasmiques (via les parois cellulaires entre

compartiments). Pour passer d'un compartiment à un autre, des transporteurs sont nécessaires. Or, la nature de ces transporteurs membranaires dépend de leur localisation : de manière simplifiée, certains transporteurs de la famille ZIP (« ZRT, IRT-like Protein » avec ZRT : Zinc-Regulated Transporter et IRT : Iron-Regulated Transporter) sont localisés à la surface de la racine et permettent l'internalisation des ETM ; par exemple HMA4 (Heavy Metal ATP-ase) et les protéines YSL (Yellow Stripe-Like) permettent leur chargement dans le xylème et leur déchargement dans les feuilles, et MTP1 (Metal Transporter Protein 1) leur transfert vers les vacuoles (Verbruggen et al., 2009). Il a été montré, chez deux cultivars de *Brassica napus*, l'un hyperaccumulateur de Cd et l'autre non, que les gènes des transporteurs *Nramp* qui participent à la remobilisation des ETM depuis les vacuoles racinaires étaient positivement régulés chez l'hyperaccumulateur (Wang et al., 2019). Chez le peuplier (*Populus trichocarpa*), une forte contamination au Zn et Cd entraîne une diminution de la quantité de transporteurs racinaires HMA4 mais une augmentation de transporteurs foliaires ZIP1, ceci limitant logiquement le transfert des ETM des racines vers les feuilles. Des champignons mycorhiziens augmentant le transporteur MT2b réduisent parallèlement le transfert du Cd vers les parties aériennes (De Oliveira et al., 2020). Ainsi, un fin contrôle est possible par ajustement génétique (transcription et traduction) de la quantité de chaque transporteur. Cependant, les plantes n'ont pas développé de mécanisme spécifique de transport pour les éléments non-essentiels tels que le Cd. De fait, le Cd partage les mécanismes de tolérance et d'accumulation du Zn (Verbruggen et al., 2009, 2013). Il a ainsi été montré que le transporteur du Zn ZnT1 (Zinc Transporter) présente également une faible affinité avec le Cd (Pence et al., 2000), et que 97 % du transport du Cd dans *Arabidopsis thaliana* se fait via les transporteurs du Zn HMA2 et HMA4 (Wong and Cobbett, 2009). Ainsi, l'accumulation des différents ETM est souvent positivement corrélée en milieu poly-contaminé (He et al., 2015). Le stockage des métaux peut se faire non-seulement dans les vacuoles, mais également dans les parois cellulaires (Nishizono et al., 1989).

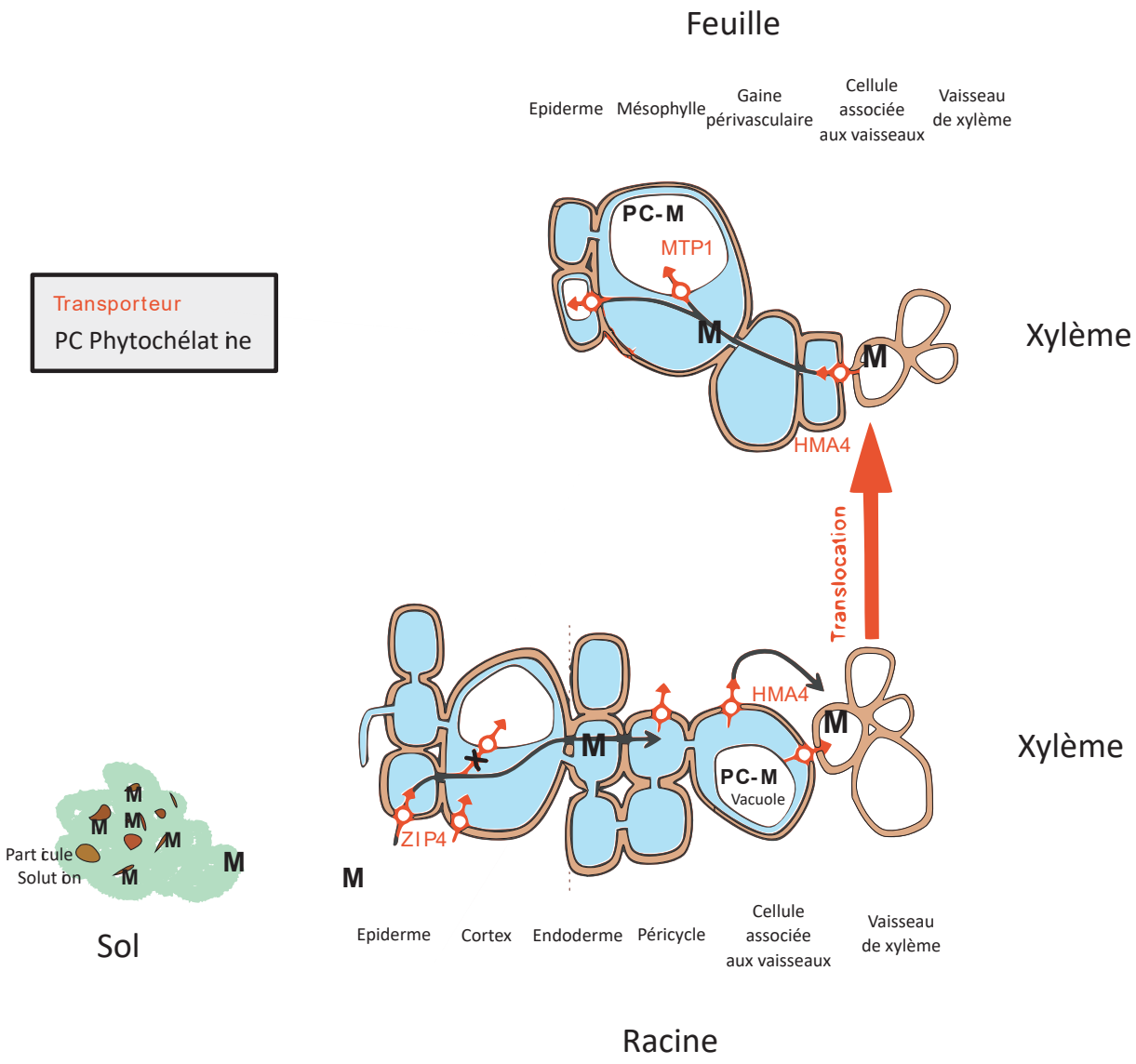


Figure 1.3 : Les ETM dans la plante : de la racine aux feuilles, à partir du sol et en passant par le xylème. *On note la possibilité de transport symplasmique (passage de l'endoderme) et apoplasmique (chargement et déchargement du xylème). ZIP : Zinc Protein, HMA : Heavy Metal ATPase, MTP : Metal Transporter Protein. M : ETM. Modifié d'après Verbruggen et al. (2009).*

Points de régulation

Les ETM interagissent également avec des ions, des peptides et de petites molécules issues du métabolisme secondaire de la plante (Singh et al., 2016; Yadav, 2010). Le glutathion (ou GSH) est un tripeptide qui, une fois condensé, forme les phytochélatines. Glutathion et phytochélatines possèdent une forte affinité pour les ETM, et participent à leur séquestration et à la défense contre le stress oxydatif (Delalande, 2010; Yadav, 2010). Les phytochélatines forment un complexe avec les ions métalliques dans le cytosol et sont ensuite transportées sous cette forme dans la vacuole. Ces molécules sont présentes non seulement chez les plantes, mais aussi chez les champignons et autres organismes, suggérant leur rôle important (Yadav, 2010). Les métallothionéines, particulièrement riches en cystéines dont les groupes sulfhydryles forment des liaisons avec des cations divalents, jouent un rôle similaire aux phytochélatines (Singh et al., 2016). Outre ces peptides, des acides organiques tels que le malate, le citrate et l'oxalate, des acides aminés (en particulier la proline et l'histidine), l'α-tocophérol et des polyphénols (via leurs groupes hydroxyle et carboxyle) chélatent les ETM. La proline, l'histidine et les phénols s'accumulent lors de contaminations métalliques et réduisent la formation de ROS ; les acides organiques participent au transport des ETM le long du xylème et à leur séquestration dans les vacuoles (Singh et al., 2016).

Le transport se fait principalement de manière active via le catabolisme d'ATP (ex : le chargeur du xylème à ATP HMA) ou l'utilisation du gradient de protons (Rascio and Navari-Izzo, 2011; Zimmermann et al., 2009). La diffusion de cations peut cependant être facilitée par des canaux passifs transmembranaires. Cependant, le transport est dépendant de la forme de l'ETM. En effet, le prélèvement au niveau des racines peut se faire sous forme ionique tandis que le transport dans les vacuoles est spécifique du complexe phytochélatine-ETM (Prasad, 2004; Rascio and Navari-Izzo, 2011; Zenk, 1996).

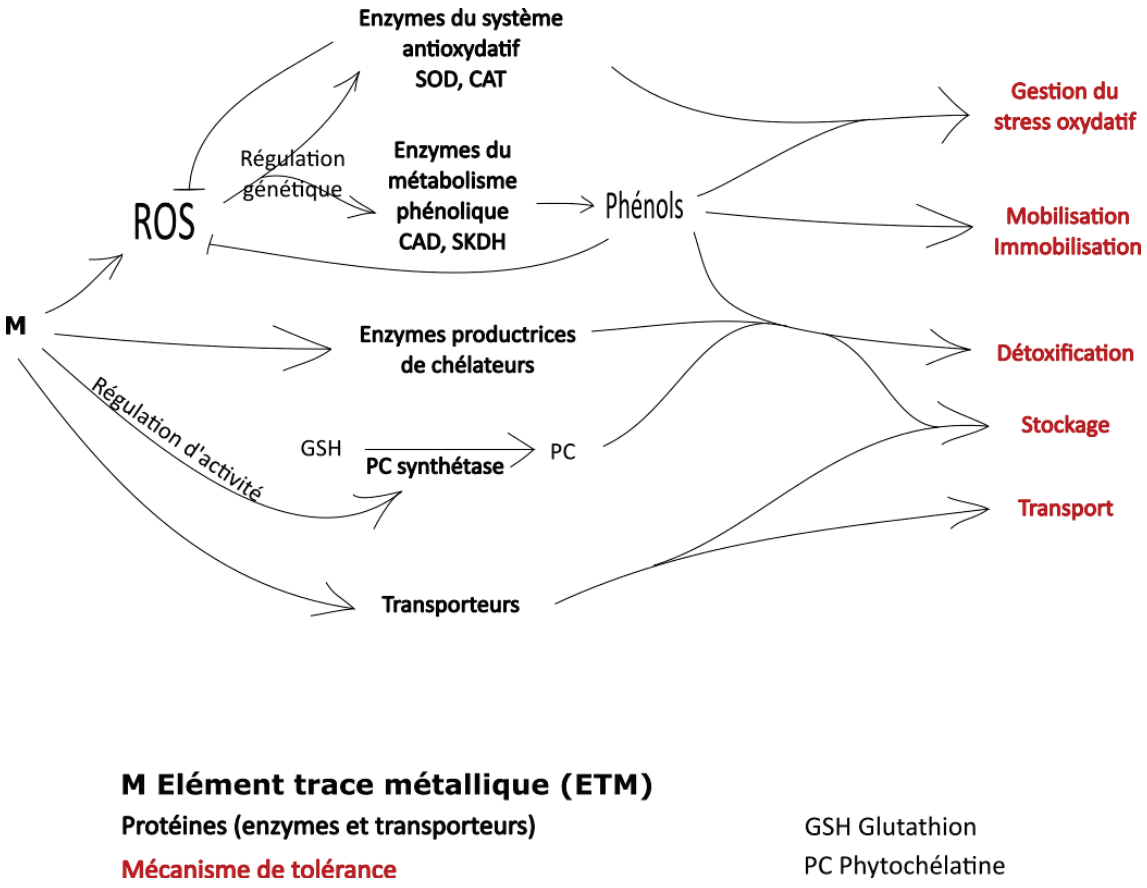


Figure 1.4 : Mécanismes de tolérance d'une plante aux ETM. *ROS : Reactive Oxygen Species.*

Ainsi, l'accumulation d'ETM par les plantes peut être finement régulée, au niveau génétique, au niveau de l'activité enzymatique, ainsi qu'au niveau du métabolisme **(Figure 1.4)** (Sharma et al., 2019). Par exemple, le Cd active la phytochélatine-synthase qui va elle-même catalyser la production de phytochélatines (Zenk, 1996). Enfin, si des molécules chimiques sont produites à l'intérieur de la plante, certaines sont également excrétées, principalement au niveau des racines, conduisant à une mobilisation ou au contraire à une phytostabilisation des ETM dans le sol (Kabata-Pendias, 2004).

Les métabolites secondaires

Les métabolites secondaires sont de petites molécules organiques, produites par les êtres vivants (principalement les plantes), et étant fortement impliqués dans les interactions de la plante avec son environnement (biotique principalement mais également abiotique). Ils sont classés en trois grandes familles principales selon leur voie de biogénèse : composés azotés (alcaloïdes), polyphénols, et terpénoïdes. Les composés appartenant à ces deux dernières familles sont souvent présents sous forme d'hétérosides dans la plante.

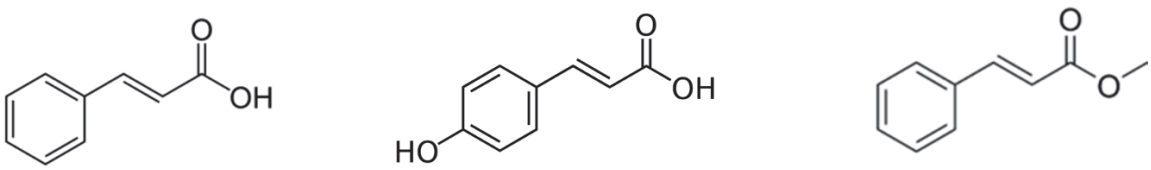
Chez les Renouées asiatiques, les polyphénols sont très abondants et diversifiés. On y trouve en particulier des procyanidines, des cinnamates, des anthraquinones, et des stilbènes (Piola et al., 2013). Afin de mieux comprendre leurs rôles potentiels dans la gestion des ETM par les Renouées, nous allons détailler ces quelques classes et leurs propriétés connues.

Les grandes familles de métabolites secondaires chez Fallopia

Les dérivés cinnamiques

L'acide cinnamique est un précurseur d'une grande partie des composés phénoliques, les phénylpropanoïdes (Landi et al., 2020; p.21-bal.com/biolog). Ceux-ci comprennent les dérivés directs de l'acide cinnamique (esters et alcools) **(Figure 1.5)**, les coumarines, les flavonoïdes et les stilbènes (p.21-bal.com/biolog; Umezawa, 2010).

Les dérivés cinnamiques sont susceptibles d'intervenir dans la réponse au stress. En effet, certains dérivés cinnamiques comme le méthylcinnamate voient leur concentration augmenter dans les plantes en réponse à un stress métallique, réponse qui reste dépendante de l'identité de la plante et du métal. En effet, le Cu entraîne une augmentation du méthylcinnamate dans les tissus du basilic, mais pas le Cd ni le Pb ; et aucun effet de ces trois ETM n'est observé chez la menthe aquatique (Kunwar et al., 2015). L'acide coumarique appliqué sur les feuilles diminue les concentrations en Cd et Cu dans les feuilles mais aussi dans les racines de la grande camomille, ainsi que la concentration totale en métabolites secondaires (Hojati et al., 2016). L'inoculation d'un facteur de virulence de *Fusarium*, mimant une infection par ce pathogène, entraîne la production d'acides hydroxycinnamiques chez le blé (Doppler et al., 2019). Ces acides sont à l'origine de conjugués monolignols qui renforcent les parois cellulaires et inhibent le pathogène ; une production accrue de flavonoïdes à partir de ces acides est également observée.

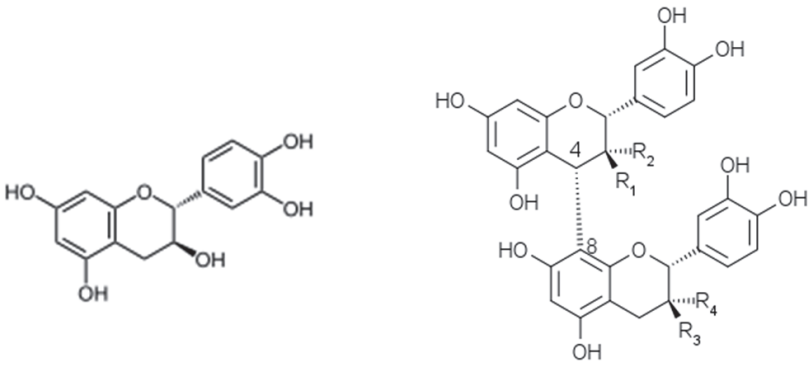


Acide cinnamique | Acide p-coumarique | Methylcinnamate

Figure 1.5 : Structure chimique de l'acide cinnamique et de quelques dérivés.

Les procyanidines et leurs dérivés

Les procyanidines dérivent de la polymérisation de monomères de catéchine ou d'épicatéchine (Ricardo da Silva et al., 1991) **(Figure 1.6)**. Les procyanidines sont présentes dans les feuilles des plantes (Niederleitner et al., 1994), leurs graines (Ricardo da Silva et al., 1991), fruits (Hammerstone et al., 2000) et racines (Bardon et al., 2016). Elles ne sont pas, à notre connaissance, produites par des champignons. Une forte corrélation positive entre le contenu en procyanidines et les capacités antioxydantes du chocolat a été montrée (Gu et al., 2006). Ces propriétés antioxydantes augmentent avec le degré de polymérisation des procyanidines (Zhao et al., 1999). La galloylation des procyanidines a un effet controversé sur les propriétés antioxydantes, inhibant la peroxydation (Zhao et al., 1999) ou au contraire augmentant les propriétés antioxydantes (Saito et al., 2004).



(+)-Catéchine | Procyanidine dimérique
R1-4 : groupes H ou OH

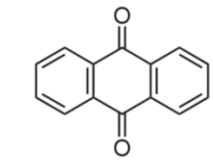
Figure 1.6 : Procyanidine dimère et l'un de ses monomères, la catéchine.

Les procyanidines semblent augmenter lorsque les plantes sont soumises à un stress : c'est le cas dans les feuilles de cerisier après infection par un champignon pathogène (Niederleitner et al., 1994), alors qu'à l'inverse la mycorhization réduit les concentrations

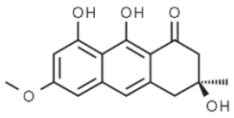
en procyanidines dans les racines de hêtre (Beyeler and Heyser, 1997). Ainsi, ces composés pourraient avoir un rôle dans la protection de la plante face à un stress biotique ou abiotique. Elles interagissent également avec les microorganismes, inhibant par exemple la bactérie promotrice de croissance *Azospirillum* (Vallejo-Ochoa et al., 2018). Dans le sol, les procyanidines sont connues pour inhiber la dénitrification bactérienne, ce qui augmenterait l'azote disponible pour la plante (Bardon et al., 2014; Galland et al., 2019). Ainsi, il pourrait y avoir un « trade-off » entre l'inhibition de la dénitrification par les procyanidines et le recrutement d'endophytes (réduit par ces mêmes molécules).

Les anthraquinones

Les anthraquinones sont très variées (Dave and Ledwani, 2012), pouvant également être dimérisées **(Figure 1.7)**. Certaines anthraquinones comme l'émodine génèrent des espèces réactives de l'oxygène (acronyme anglais ROS) (Dave and Ledwani, 2012; Yen et al., 2000), mais d'autres ont au contraire des effets fixateurs de ces molécules réactives et délétères (Yen et al., 2000). Les activités antioxydantes et de fixation des ROS sont globalement moins marquées que celles des flavonols (Zhang et al., 2005).



Squelette anthraquinone



Torosachrysone

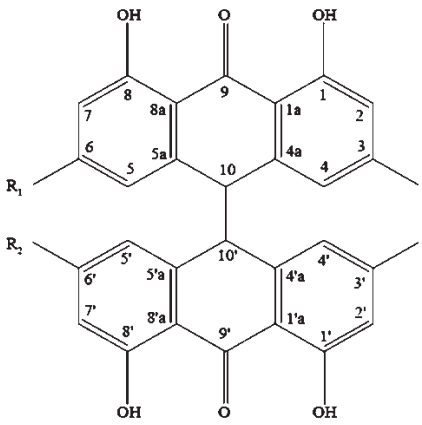


Figure 1.7 : Structure chimique des anthraquinones et dérivés.

Chez les champignons, la torosachrysone est un précurseur de pigments anthraquinoniques (Gill and Steglich, 1987). Ce composé est induit dans les racines de *F. japonica* et *F.* x *bohemica* par la présence d'une multi-contamination métallique (Michalet et al., 2017). Or, la torosachrysone a été précédemment isolée de plantes (Kitanaka and Takido, 1984) mais aussi de champignons (Gill and Steglich, 1987; Gill et al., 2000).

Les stilbènes

Les stilbènes **(Figure 1.8)** sont une famille de composés que seules certaines plantes produisent, à partir de l'acide cinnamique (Chong et al., 2009). Sous forme glycosylée, les stilbènes peuvent être stockés, transportés et/ou protégés contre la peroxidation. *F. japonica* est répertoriée comme accumulatrice de stilbènes, particulièrement de picéide (16 mg/g de poids sec) et de resvératrol (1.8 mg/g de poids sec) dans ses tissus. La synthèse de stilbènes est induite lors de stress biotiques ou abiotiques, tels qu'une infection par des pathogènes fongiques ou une contamination à l'aluminium. Globalement, les stilbènes interviennent dans trois domaines physiologiques : la signalisation chimique inter-organismes, la réponse au stress oxydatif et la défense constitutive ou inductible contre des pathogènes (Chong et al., 2009).

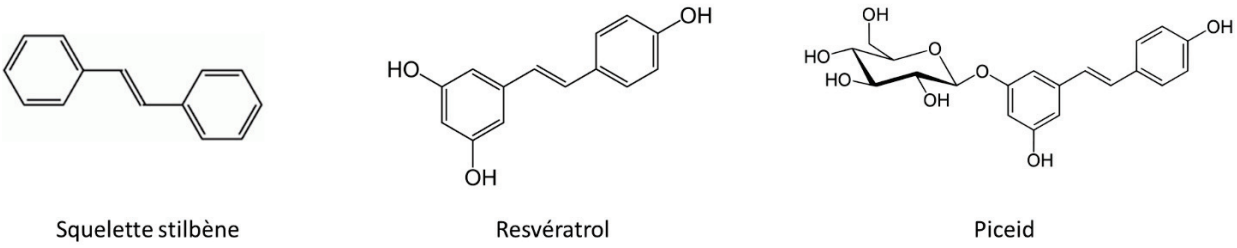


Figure 1.8 : Structure chimique de stilbènes et dérivés.

Ainsi, les métabolites secondaires retrouvés chez *Fallopia*, principalement des polyphénols, pourraient jouer un rôle essentiel dans les stratégies de tolérance de cette plante face aux ETM, par action directe (chélation des métaux, activité antioxydante, antiradicalaire, etc.) ou indirecte (communication entre organismes, stimulation de réactions de défense, etc.).

Les grandes fonctions des polyphénols dans la tolérance face aux ETM

Chélation des ETM

La chélation des ETM par les polyphénols a lieu à pH physiologique (Hider et al., 2001). Un cation métallique peut se lier via des liaisons hydrogènes à un groupe OH lié à un noyau aromatique : ce noyau stabilise cette nouvelle liaison. Les polyphénols présentent plusieurs noyaux aromatiques, et souvent plusieurs sites de liaison : ainsi, la quercetine peut se lier à 3 ions métalliques simultanément. Ces liaisons multiples conduisent à la formation de complexes, pouvant aller jusqu'à la précipitation de l'ensemble (McDonald et al., 1996; Zhang et al., 2016). Selon leur degré d'oxydation, les polyphénols sont susceptibles de chélater différentes formes

d'ETM. Ainsi, le catéchol se liera avec Fe(III), tandis que sa forme oxydée, l'orthoquinone, se liera avec Fe(II) (Hider et al., 2001). La chélation des ETM par les polyphénols limitent leur toxicité.

Lutte contre le stress oxydatif

La formation d'espèces réactives de l'oxygène est habituelle dans les cellules, du fait notamment de l'activité mitochondriale. Les ETM favorisent le stress oxydatif lié aux ROS : ils permettent l'initiation de la réaction conduisant de ROS peu réactifs à des ROS très réactifs, qui provoquent ensuite une chaîne d'oxydation de molécules biologiques (Alov et al., 2015). Outre l'action directe de chélation des ETM, les polyphénols de faible poids moléculaire participent à la protection contre ce stress oxydatif, pouvant fixer les ROS, piéger les radicaux libres et de par leur action antioxydante réduire les molécules biologiques qui ont été oxydées (Alov et al., 2015; Lu and Yeap Foo, 2000).

Messagers : un appel au secours

Les polyphénols sont au cœur des interactions plantes-microorganismes. Ils peuvent en effet jouer le rôle de « recruteurs » de microorganismes bénéfiques pour la plante, en particulier lorsqu'elle est confrontée à un stress (Thijs et al., 2016). Ainsi, une sélection spécifique de bactéries endophytes mutualistes s'opère *via* les exsudats racinaires (Chagas et al., 2018; Gaiero et al., 2013; Jha et al., 2018).

Finalement, ces métabolites secondaires peuvent intervenir de trois manières non-exclusives dans la tolérance des plantes aux métaux : via la chélation directe des ETM, favorisant leur accumulation, transport et stockage ; via leur activité antioxydante et de piégeage de radicaux libres, luttant ainsi contre le stress oxydatif induit par les ETM ; via leur rôle de messager, favorisant le recrutement d'endophytes particulièrement bénéfiques à la plante lors d'un stress métallique.

Un modèle de plantes métallophytes : les Renouées asiatiques, un complexe d'espèces envahissant

Biogéographie des Renouées asiatiques

Figure 1.9 : Berge d'une rivière envahie par des Renouées asiatiques (Savoie, France). *©Louise BARBERIS*

« Renouées asiatiques » (ou Asian knotweeds en anglais) est un terme général comprenant deux espèces dites parentales, la Renouée du Japon (*Fallopia japonica*) et la Renouée de Sakhaline (*Fallopia sachalinensis*), ainsi que leur hybride *Fallopia* x *bohemica* (voir **Cadre 1.1** pour les autres noms). Un flux génétique étant possible par hybridation, on parle donc de « complexe d'espèces *Fallopia* ».

Cadre 1.1 : Les différents noms scientifiques des Renouées asiatiques (Darbyshire 2003)

Renouée du Japon :	*Fallopia japonica* (Houttuyn) Ronse-Decraene *Reynoutria japonica* Houttuyn *Polygonum cuspidatum* (Sieb. & Zucc.) Japanese knotweed *Japanese bamboo, Mexican bamboo, fleeceflower, horse-buckwheat, Japanese Fleeceflower, bamboo, canne, Jérusalem, jonc canadien, jonc de Saint-Joseph, renouée japonaise, Sainte-Anne, sarrasin des Indes, renouée de Siebold, and persicaire cuspidée*
Renouée de Sakhaline :	*F. sachalinensis* (F. Schmidt) Ronse-Decraene *R. sachalinensis* (F. Schmidt) Nakai *F. sachalinensis* (F. Schmidt ex Maxim.) Ronse Decraene Giant knotweed *Elephant ear, sachaline, Sachaline knotweed, persicaire de Sachaline, renouée d'île Sachalin*
Renouée de Bohême :	*F.* x *bohemica* (Chrtek and Chrtková) J.P. Bailey *R. bohemica* Chrtek and Chrtkova Bohemian knotweed

Les Renouées asiatiques sont des plantes herbacées géophytes rhizomateuses : elles subsistent l'hiver uniquement dans le sol sous forme de rhizomes pouvant atteindre 8 cm de diamètre, 20 m de long et 2-3 m de profondeur ; les tiges se développent du printemps à l'automne, atteignant jusqu'à 3 m de hauteur (Barney et al., 2006). Le système rhizomal représente environ les deux-tiers de biomasse totale de la plante en été. Son développement végétatif via le système rhizomateux conduit à la formation de vastes « patchs » denses, pouvant couvrir plusieurs centaines de mètres carrés (**Figure 1.9**, Barney et al., 2006).

Les trois « espèces » au sein du complexe sont distinguables morphologiquement **(Figure 1.10)** : la base des feuilles *Fallopia japonica* est tronquée, les feuilles matures de *Fallopia sachalinensis* sont de taille très importante (jusqu'à plus de 30 cm de long), à base échancrée et apex acéré (feuilles cordées), tandis que les feuilles de *Fallopia* x *bohemica* ont une taille et une morphologie intermédiaire entre les deux espèces parentales (Barney et al., 2006). Les panicules florales sont plus grandes que la feuille sous-tendante chez *F. japonica*, mais plus petites chez *F. sachalinensis*. D'autres critères, tels que la striation de la cuticule foliaire, les stomates et les poils, permettent de différencier les trois espèces (Bailey et al., 2009). La morphologie est par ailleurs un excellent critère de différentiation génétique (Bailey et al., 2009).

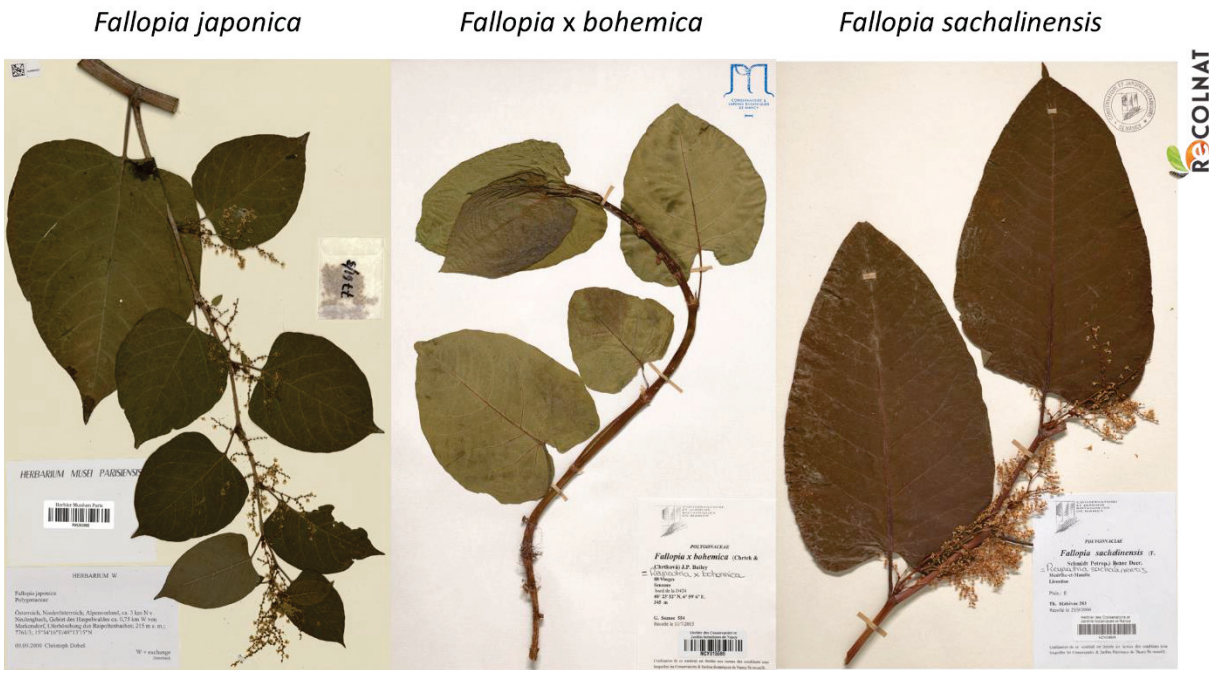


Figure 1.10 : Morphologie des Renouées asiatiques. *Les trois planches ont la même échelle. On note la base des feuilles rectiligne de* Fallopia japonica*, les feuilles matures de taille très importante (jusqu'à plus de 30 cm de long) et à base échancrée de* Fallopia sachalinensis*, des feuilles intermédiaires pour* Fallopia *x* bohemica*. Planches issues des herbiers du Museum National d'Histoire Naturelle (Paris) et du Conservatoire et Jardins Botaniques de Nancy, numérisées par Recolnat (RECOLNAT-ANR-11-INBS-0004).*

Originaires d'Asie de l'Est (Japon, Chine, Corée) où elles colonisent les sols volcaniques – riches en ETM - (Adachi et al., 1996), les Renouées asiatiques ont été introduites en Europe et en Amérique du Nord au cours du XIXème siècle pour un usage ornemental et fourrager (Barney et al., 2006; Lavoie, 2017). Seul un génotype de *F. japonica* est présent en Europe, malgré une polyploïdie variable, suggérant l'expansion d'un seul et même clone de manière végétative à partir de son introduction au début des années 1850 (Thiébaut et al., 2020). Ce clone est mâle-stérile, c'est-à-dire qu'il ne contient pas d'étamines et ne peut donc pas produire de pollen. *F. sachalinensis* est importée à plusieurs reprises à partir de 1853 : elle est bien plus variable génétiquement que *F. japonica*, et peut se reproduire via fleurs et graines. Ces deux espèces se côtoyant dans les jardins botaniques, elles ont formé par croisement (on parle d'espèces parentales) l'hybride *F.* x *bohemica*, présent en Angleterre dès 1872 et vingt ans plus tard en France (Thiébaut et al., 2020). *F.* x *bohemica* se reproduit lui-aussi de manière sexuée, y compris avec les espèces parentales. On observe en effet des graines fertiles de *F.* x *bohemica* sur des pieds de *F. japonica*, issues du dépôt de pollen d'un

hybride sur la fleur femelle de *F. japonica* (Barney et al., 2006; Lavoie, 2017; observations personnelles). L'hybride *F.* x *bohemica* présente donc une très grande diversité génétique (Pyšek et al., 2003).

De parfaites « mauvaises herbes »

Si les Renouées asiatiques furent appréciées pour leurs qualités esthétiques et utilisées comme fourrage (Thiébaut et al., 2020), elles s'étendirent le long des cours d'eau, des routes, des voies ferrées et des zones perturbées, y compris en zones urbaines et contaminées au ETM (Sołtysiak and Brej, 2014, 2019) qu'elles accumulent dans leurs tissus (Qian et al., 2012; Rahmonov et al., 2019). Elles devinrent rapidement incontrôlables jusqu'à être inscrites sur la liste de l'UICN (Union for the Conservation of Nature) des 100 espèces exotiques envahissantes parmi les plus néfastes au monde (Invasive Species Specialist Group 2016) (Lavoie, 2017).

« Néfastes », car elles ont des effets environnementaux, économiques et sociaux délétères (Williams et al., 2010). En effet, les patchs denses sur les berges des rivières en rendent l'accès difficile **(Figure 1.9)**, empêchent le développement d'espèce natives, notamment ligneuses, qui ne peuvent plus remplir leur rôle de maintien des berges en hiver ; le niveau de petits cours d'eau adjacents se trouve abaissé et la visibilité le long des routes se trouve réduite en été, etc. (Lavoie, 2017). Les dégâts environnementaux sont importants également : si la productivité primaire du lieu est augmentée (la biomasse des Renouées étant nettement supérieure à celle des plantes natives environnantes), c'est au détriment des espèces natives, des macroinvertébrés (acariens, mollusques, collemboles), des bactéries et des champignons mycorhiziens. Seuls quelques espèces d'oiseaux, des invertébrés détritivores et les champignons (souvent saprobes) profitent de la présence des Renouées et de leur litière importante (Lavoie, 2017), de même que les insectes pollinisateurs apprécient la floraison tardive et des fourmis le nectar extrafloral (Johnson et al., 2019). La phytotoxicité des plants vivants de *Fallopia*, de sa litière, de ses feuilles, d'extraits de rhizomes et de sol précédemment en contact avec des feuilles et des rhizomes a été démontrée sur les plantes natives, bien que l'inhibition des plantes natives semble majoritairement liée à de la compétition directe pour les ressources (Mincheva et al., 2016).

« Envahissantes », car il est extrêmement difficile de prévenir leur installation, et qu'elles peuvent survivre malgré de fortes perturbations. Les Renouées asiatiques sont capables de

se régénérer à partir de fragments de rhizomes de moins de 0,8 g contenant seulement un nœud **(Figure 1.11)** (Barney et al., 2006, observation personnelle). Leur production d'akènes est importante (jusqu'à 127 000 sur un même pied), pour des graines d'environ 1-2 g (Barney et al., 2006). La présence d'ailettes sur les akènes favorise l'anémochorie (transport par le vent) et l'hydrochorie (transport par l'eau) (Barney et al., 2006; Lamberti-Raverot et al., 2019). Le déplacement de fragments de rhizomes par l'espèce humaine lors de chantiers est le premier moyen d'expansion des Renouées, tandis que la dispersion des graines par les cours d'eau facilite la colonisation des berges en aval (Barney et al., 2006; Lamberti-Raverot et al., 2019; Rouifed et al., 2014). Une fois implantée, la couper une fois ou l'arracher n'affecte pas sa survie : cela peut même augmenter localement son expansion. De fait, les gestionnaires souhaitant réduire significativement sa présence ont recours à des coupes régulières ou l'emploi de pesticides (Barney et al., 2006; Rouifed et al., 2020). Cela représente un coût écologique et financier très élevé (Williams et al., 2010).

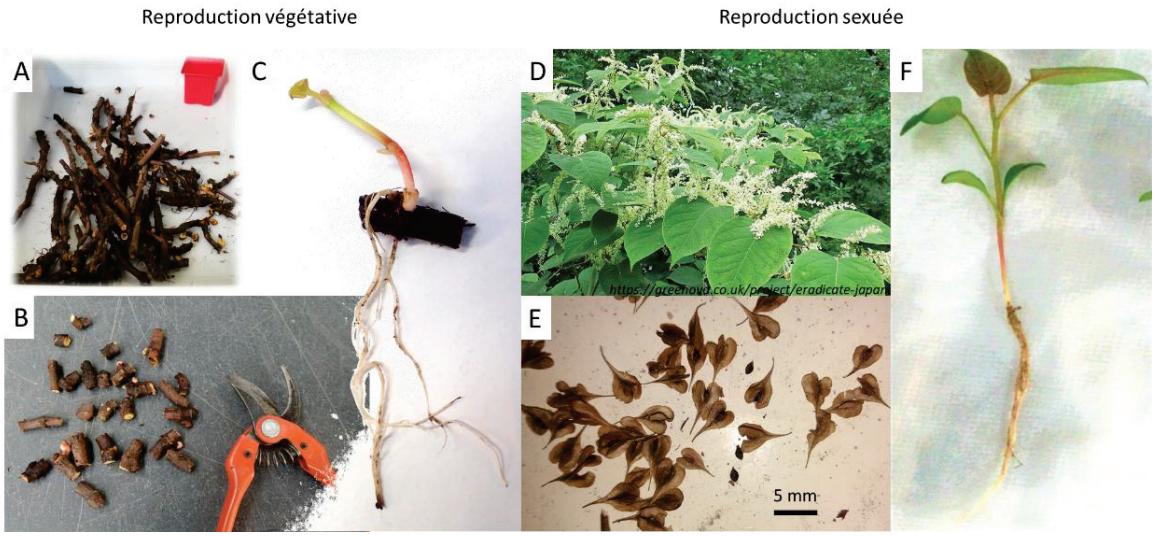


Figure 1.11 : Reproduction des Renouées. *A : rhizomes de* F. *x* bohemica *(Feyzin, France), B : fragments de ces rhizomes contenant un nœud et capables de régénérer dans le sol, C : plantule régénérée à partir d'un fragment de rhizome, avec racines adventives, D : panicules florales de* F. japonica*, E : akènes et graines de* F. *x* bohemica*. F : plantule régénérée à partir d'une graine, avec racine pivotante. ©Louise BARBERIS sauf D*

Les Renouées, et en particulier la Renouée du Japon, sont donc considérées comme un nœud gordien (Kurose et al., 2006), que les humains essaient de trancher avec du glyphosate ou du dicamba (Barney et al., 2006). D'autres tentent des méthodes alternatives,

testant la fauche ou l'addition de pathogènes (Kurose et al., 2006; Rouifed et al., 2020). Il n'en reste pas moins que les Renouées asiatiques sont un exemple de plantes très performantes, qui présentent également des effets positifs : elles sont mellifères, fourragères, ornementales, et parviennent à pousser dans des zones ou d'autres plantes n'y parviendraient pas.

Comprendre l'invasion

Les Renouées sont donc considérées comme envahissantes dans leur nouvelle aire de répartition : plusieurs hypothèses non-exclusives tentent d'expliquer les invasions :

- L'hypothèse de la « **libération de ses ennemis** » (« *Enemy release hypothesis* »)

Cette hypothèse repose sur l'idée que toute plante est régulée par des herbivores et autres pathogènes (Keane and Crawley, 2002). Certains sont spécialistes d'une plante, d'autres, généralistes, s'accommodent de plusieurs hôtes. Or, une espèce qui arrive dans un nouveau milieu ne transporte pas (en général), ses pathogènes. Ainsi, dans sa nouvelle aire géographique, elle se trouve exempte d' « ennemis » spécialistes, tandis que les plantes natives restent soumises aux leurs. La plante exotique présente donc une capacité compétitive accrue par rapport aux plantes qui l'entourent. Cette hypothèse est soutenue dans certains cas, mais n'est pas confirmée de manière généralisée (Colautti et al., 2004; Heger and Jeschke, 2014; Liu and Stiling, 2006).

Les Renouées, dans leur aire d'expansion, montrent peu de prédateurs, bien que les otiorhynques (Coléoptères), les moineaux domestiques, quelques acariens et herbivores domestiques les consomment en Europe (Schnitzler and Müller, 1998). À notre connaissance, aucune maladie fongique ou bactérienne des Renouées n'a été répertoriée jusqu'à présent.

- L'hypothèse des « **nouvelles armes** » (« *Novel weapon hypothesis* »)

Les plantes peuvent interagir entre elles ou avec les microorganismes via la sécrétion dans le sol de composés issus du métabolisme secondaire. Ces interactions chimiques peuvent être positives ou négatives : on parle d'allélopathie (Rice, 1984, 2013). Dans une communauté native où les plantes ont co-évolué, des défenses sont mises en place contre les effets des composés allélopathiques. Mais, lorsqu'une plante exotique arrive dans un nouvel environnement, les plantes locales font face à de nouvelles armes auxquelles elles n'ont pas pu s'adapter (Callaway and Ridenour, 2004; Hierro and Callaway, 2003). Les armes

en question, initialement considérées comme des armes chimiques et restreintes aux composés allélopathiques, peuvent être étendues aux armes biologiques représentées par les pathogènes portés par les espèces invasives (Vilcinskas, 2015). Ce scénario des « nouvelles armes » a été démontré dans de nombreux cas (He et al., 2009; Kim and Lee, 2011; Ni et al.; Thorpe et al., 2009) bien qu'il ne puisse être généralisé (Oduor et al., 2020; Yannelli et al., 2020). Ainsi, certaines espèces invasives telles qu'*Eupatorium rugosum*, *Ambrosia artemisiifolia* var. *elatior* et *Rudbeckia bicolor* présentent en moyenne plus de composés phénoliques *in situ* que les natives, ainsi qu'un composé commun à toutes les invasives (le scopolétol), et non-présent chez les natives (Kim and Lee, 2011). Les extraits de ces invasives ont des effets inhibiteurs de germination et de croissance sur trois plantes testées (*Rumex* acetocella, *Oenothera odorata* et *Plantago asiatica*) presque deux fois supérieurs à ceux des natives.

Les Renouées ont des teneurs et une diversité en polyphénols élevée (Michalet et al., 2017; Nawrot-Hadzik et al., 2018, 2019; Piola et al., 2013), et des effets allélopathiques avérés (Dommanget et al., 2014; Murrell et al., 2011; Serniak, 2016). L'effet inhibiteur de *Fallopia* x *bohemica* sur des plantes natives est supprimé lorsque les polyphénols sont neutralisés par du charbon actif (Murrell et al., 2011); des lessivats de *F.* x *japonica* inhibent la croissance de boutures de plantes natives (Dommanget et al., 2014); et le resvératrol, l'émodine et la (-)-épicatéchine (trois composés majeurs des racines et rhizomes de *Fallopia*) limitent la croissance racinaire (mais pas la germination ni la croissance aérienne) de plantes natives (Serniak, 2016). Outre ces trois composés, plus de 150 autres ont été détectés dans les rhizomes du complexe d'espèces *Fallopia*. Ces composés appartiennent aux stilbènes, procyanidines, flavanols, anthraquinones, carbohydrates, dérivés cinnamiques, phénylpropanoides, lignanes et naphtoquinones (Nawrot-Hadzik et al., 2019). L'hybride *F.* x *bohemica* est le mieux « armé », possédant des métabolites non retrouvés dans les deux espèces parentales (Piola et al., 2013). Les métabolites secondaires de la Renouée du Japon ont des effets marqués sur les plantes, mais également sur la faune du sol, modifiant les équilibres de la chaîne alimentaire (Abgrall et al., 2018). Quelques études montrent cependant un effet négatif des Renouées indépendant de leur métabolisme secondaire, suggérant un effet compétitif direct (Mincheva et al., 2016; Parepa et al., 2012).

- L'hypothèse des « **mutualismes augmentés** » (« *Enhanced mutualism hypothesis* »)

Les plantes sont, la plupart du temps, associées à des microorganismes qui leurs sont bénéfiques. Ainsi, au moins 85 % des espèces sont mycorhizées ; les champignons racinaires

avec lesquels elles vivent en symbiose leur facilitant entre autres l'accès aux nutriments (Brundrett and Tedersoo, 2018). D'autres bactéries et champignons endophytes peuvent être bénéfiques à leur hôte. L'hypothèse des « mutualismes augmentés » stipule qu'une plante exotique envahissante pourra s'associer avec de nouveaux mutualistes dans son aire d'expansion, ayant un effet bénéfique fort (Sun and He, 2010). Quelques études soutiennent cette hypothèse (Callaway et al., 2011; Sun and He, 2010).

Les Renouées asiatiques, comme les autres espèces de la famille des Polygonaceae, sont reconnues comme étant non-mycorhiziennes ; pourtant des champignons, appelés endophytes fongiques, colonisent les racines de Renouées sans symptôme apparent de maladie (Gucwa-Przepióra et al., 2016) (**Figure 1.12**, observations personnelles). Certains d'entre eux, notamment les Dark Septates Endophytes (DSE) présentent des fonctions mutualistes chez d'autres plantes (Jumpponen and Trappe, 1998), particulièrement en milieu contaminé aux ETM (Domka et al., 2019b). Mieux connaître leurs rôles dans les racines des Renouées est donc nécessaire : y sont-ils mutualistes ? Si oui, l'hypothèse des mutualismes augmentés est pertinente pour les Renouées asiatiques.

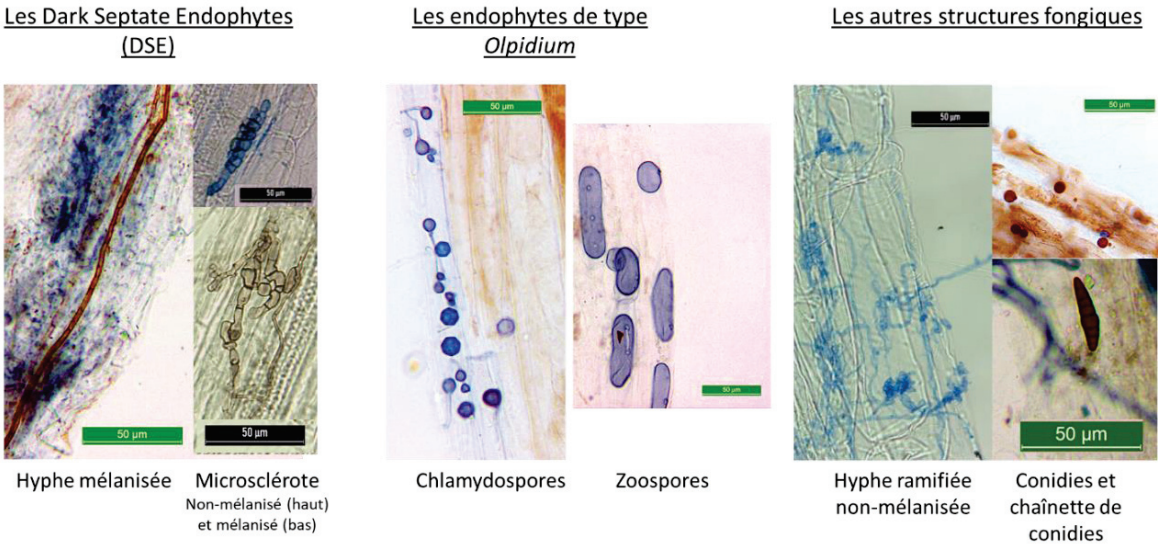


Figure 1.12 : Endophytes fongiques présents dans les racines des Renouées asiatiques. *Observation au microscope optique après coloration au bleu de méthyle. ©Louise BARBERIS & Sophie POUSSINEAU.*

Ces trois hypothèses font intervenir des microorganismes ou peuvent se décliner au niveau des microorganismes du sol (Reinhart and Callaway, 2006). En effet, outre les herbivores, une plante exotique peut être libérée de pathogènes bactériens ou fongiques (« libération de ses ennemis »). De même, les nouvelles armes peuvent inhiber les mutualistes microbiens plutôt que les plantes (Callaway et al., 2008) : cette hypothèse est alors connue sous le nom des « mutualismes dégradés » (Pinzone et al., 2018).

Les Renouées asiatiques sont donc les “mauvaises herbes idéales”, possédant une forte compétitivité, des capacités d’hybridation interspécifique, une dispersion (par voie sexuée et végétative) importante, des armes chimiques et une capacité d’adaptation élevée (Gillies et al., 2016). Ainsi que de potentiels mutualistes.

Les caractéristiques des Renouées – performance, tolérance aux ETM (détaillée dans l’axe 2), allélopathie, champignons endophytes – en font un excellent modèle pour tester le rôle des endophytes fongiques dans la tolérance de la plante aux ETM.

Problématique - Rappel

Ces éléments suggèrent que **la colonisation des racines par des champignons endophytes aurait un rôle prépondérant dans la tolérance des Renouées asiatiques aux ETM.**

L'objectif général de cette thèse est de tester cette hypothèse, en étudiant et en identifiant les espèces fongiques associées aux racines des Renouées asiatiques (*F. japonica*, *F. sacchalinensis* et *F.* x *bohemica)* et leurs rôles dans la tolérance de ce taxon à la pollution métallique des sols, en conditions contrôlées.

La thèse comporte trois axes complémentaires suivies d'une discussion générale :

Axe 1 : Une étude bibliographique des endophytes fongiques présents dans les racines des plantes et leurs effets sur la tolérance de leur hôte aux ETM ;
Axe 2 : L'étude de l'effet des métaux sur les Renouées et les endophytes associés ;
Axe 3 : L'étude de l'effet des endophytes sur la tolérance de la Renouée de Bohème aux ETM.

Une attention particulière a été donnée à la croissance de la plante hôte, aux concentrations métalliques dans les tissus végétaux, à la colonisation fongique (en particulier par les *Olpidium* et DSE), ainsi qu'au métabolisme secondaire racinaire des plantes et de certaines souches de champignons isolées. L'identité et la tolérance aux ETM de ces dernières ont également été déterminées.

Les hypothèses principales sont les suivantes :

(1) Les Renouées ont une croissance peu altérée par la contamination métallique ;
(2) La présence de métaux induit une modification de la colonisation racinaire par des endophytes fongiques, avec en particulier une augmentation des DSE (théorie du « Plant call for support ») ;
(3) Les plantes présentent de meilleurs traits de croissance et/ou tolèrent mieux le stress métallique lorsqu'elles sont intensément colonisées par certains champignons ;
(4) La présence de métaux induit un changement métabolique au niveau des racines, en lien avec la performance de la plante et/ou la colonisation fongique.

Chapitre 2 : Matériels & méthodes

Sites de prélèvements

Afin de tester les effets des ETM et des champignons endophytes sur la Renouée, nous avons mis en culture de petits fragments de rhizomes (autour d'un gramme) possédant un nœud leur permettant de régénérer, et issus de plantes prélevés sur le terrain dans la région lyonnaise **(Figure 2.1)**.

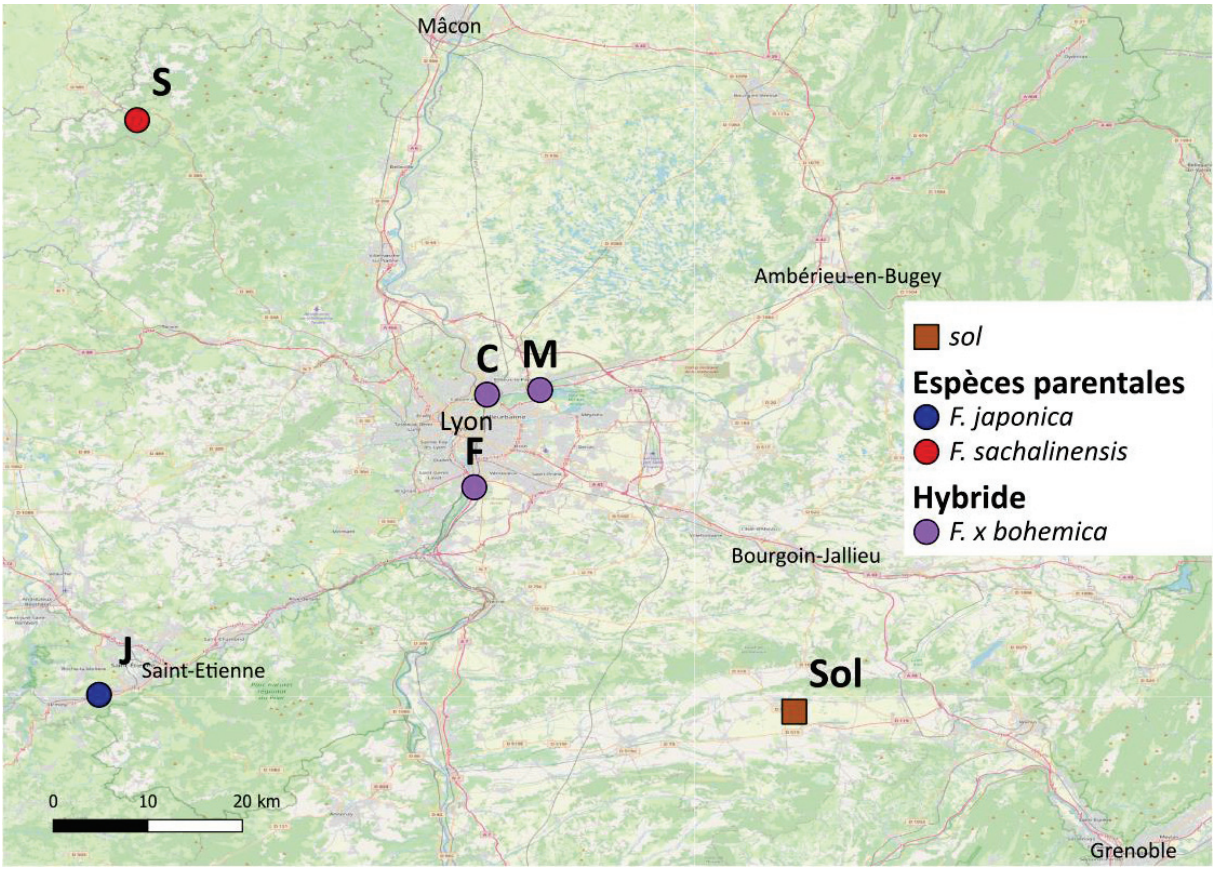


Figure 2.1 : Localisation des prélèvements de rhizomes et de sol prairial. *Le sol prélevé (15 centimètres supérieurs), exempt de contamination aux ETM, est le support de culture des futures expérimentations.*

Pour prendre en compte la diversité du complexe d'espèces *Fallopia*, nous avons récolté des rhizomes à partir de patchs correspondant à chaque espèce parentale (*F. japonica* et *F. sacchalinensis*) et de trois patchs de *F.* x *bohemica* identifiés morphologiquement et poussant sur trois sites distincts de la métropole du Grand Lyon, distants de plus de 6 km **(Figure 2.1)** :

- Miribel-Jonage (**M**, 45.8094 N; 4.9352 E), à proximité d'une forêt. Le sol n'y présente pas de contamination métallique particulière ;
- Caluire (**C**, 45.8032 N; 4.8638 S), proche d'une déchèterie, qui présente une contamination au Cu (150 mg/kg) et au Pb (180 mg/kg) ;
- Feyzin (**F**, 45.6787 N; 4.8466 S), à proximité d'une raffinerie et d'une autoroute, présentant un sol contaminé au Zn (450 mg/kg).

Voir Tableau 4A.S2 page 99 pour plus d'informations.

Les plantes de Caluire présentent des feuilles larges et plutôt cordiformes similaires à celles de *F. sachalinensis*, tandis que celles de Feyzin et Miribel possèdent des feuilles plus proches de la forme triangulaire de celles de *F. japonica*. De ce fait, nous parlerons par la suite de « morphotypes ».
Les rhizomes de *F. japonica* ont été collectés au Chambon-Feugerolle (**J**, 45.3997 N; 4.3417 S) et ceux de *F. sachalinensis* à Belleroche (**S**, 46.1723 N; 4.3930 E).

Le sol prairial, que nous souhaitions riche en microorganismes et exempt de contamination, a été collecté sur les 15 premiers centimètres d'un terrain utilisé pour du pâturage extensif à La Côte Saint-André (45.3769 N; 5.2772 E) **(Figure 2.2)**.

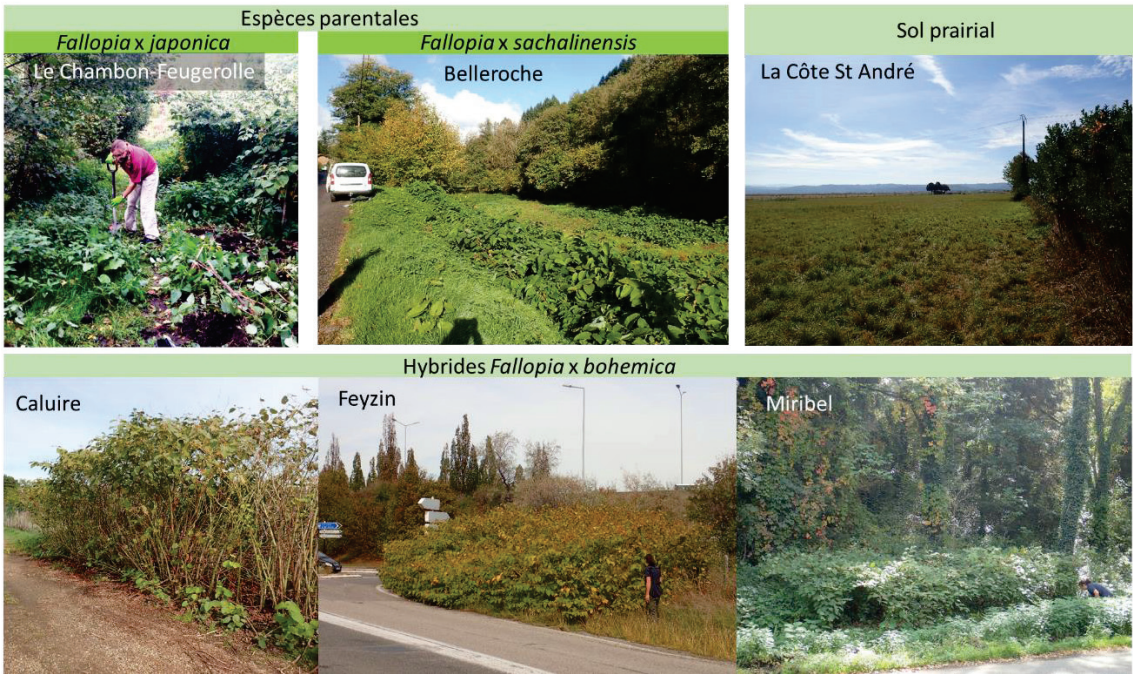


Figure 2.2 : Aspects des lieux de prélèvement du sol prairial et des Renouées. *©Louise BARBERIS et Florence PIOLA.*

Culture fongique

Afin d'identifier les champignons endophytes présents dans les racines des Renouées asiatiques et de créer une banque de souches fongiques permettant ainsi leur utilisation dans de futures expériences d'inoculation, des cultures ont été réalisées en milieu malt-agar à partir de fragments de racines désinfectés en surface selon le protocole mis au point par Berthelot et al. (2016).

Désinfection :

- 3 min dans de la Javel (5 %) avec une goutte de produit vaisselle
- 30 sec dans de l'eau oxygénée à 30 %
- rinçage à l'eau distillée

Les racines ainsi dépourvues d'organismes fongiques ou bactériens en surface et ne contenant donc plus que les endophytes, sont déposées en en boîte de Pétri sur un milieu stérile de composition suivante :

- 1,2 % malt
- 1,5 % agar
- 1 mL/L chloramphénicol (antibiotique)
- ajustement du pH à 5,5 ± 0.1 avec HCl ou NaOH
- Selon les expériences, ajout de métal sous forme de sel pour une concentration finale de Cd: 1.5 mg/L, Cr: 200 mg/L, Zn: 400 mg/L ou mix: Cd+Cr+Zn.

Figure 2.3 : Isolement d'endophytes fongiques racinaires de* Fallopia *x* bohemica *sur milieu malt-agar. *©Elisabeth MERTZ*

Les endophytes fongiques racinaires sont ensuite cultivés à l'obscurité entre 24 et 27 °C. Après quelques semaines **(Figure 2.3)**, les mycelia sont ré-inoculés sur des milieux malt-agar frais.

Analyse métabolomique

Afin d'étudier le métabolisme secondaire des Renouées asiatiques, nous avons soumis des extraits racinaires à une analyse chromatographique en phase liquide à ultra-haute performance (acronyme anglais : UHPLC pour *Ultra-High Performance Liquid Chromatography*) permettant de séparer les composés **(Figure 2.4)**. Les composés une fois séparés sont identifiés grâce à leur spectre UV (obtenu par un détecteur de type DAD - *Diode Array Detector*) et leur spectre de masse haute résolution obtenu via un spectromètre de masse de type QTOF (*Quadrupole Time Of Flight*). Une fois ionisés dans la source les ions majeurs sont sélectionnés au niveau de la chambre de collision afin de subir une fragmentation spécifique dont les résidus sont également analysés par spectrométrie de masse, permettant d'obtenir le spectre MS/MS et donnant ainsi des indications sur les groupements présents dans les composés **(Figure 2.4)**.

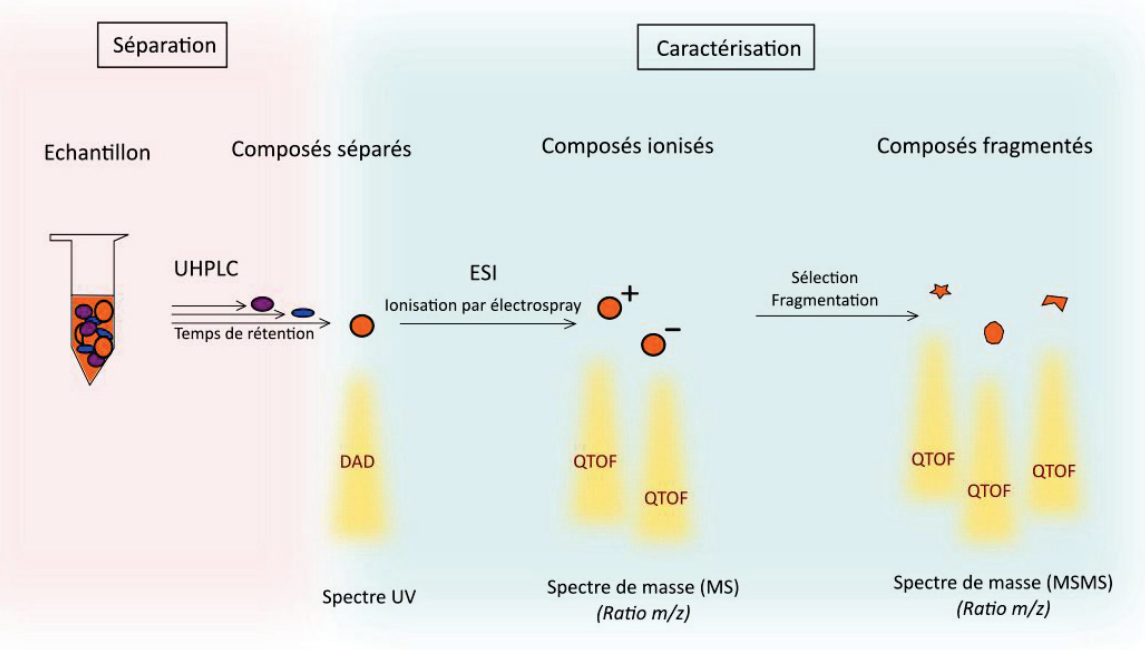


Figure 2.4 : Principe de la séparation et de la caractérisation des métabolites secondaires par UHPLC-DAD ESI/QTOF. *UHPLC : Ultra-High Performance Liquid Chromatography. DAD : Diod Array Detector. ESI : ElectroSpray Ionization. QTOF : Quadrupole Time-of-Flight.*

Pour chaque plante, environ 100 mg de racines sont collectés lors du dépotage et immédiatement plongés dans l'azote liquide afin de stopper le métabolisme, avant d'être conservés à -80 °C puis lyophilisés. Chaque échantillon est ensuite broyé finement (TissueLyserII® - Qiagen; F= 300/sec., 3 min.), puis extrait par 1 mL de MeOH-H_2O (1:1) durant 15 minutes aux ultra-sons, de manière à maximiser le contact entre tissus racinaires et solvant d'extraction. La phase liquide (contenant les métabolites extraits) est conservée (après centrifugation à 5000 tours/min pendant 10 minutes), puis l'extraction est répétée avec 1 mL de MeOH pur. Les phases liquides sont ensuite réunies, et l'ensemble est séché sous vide (Speedvac® – Labenco) et stocké à -20°C jusqu'à analyse. Pour l'analyse, les extraits sont dissouts dans du MeOH 80 % à une concentration de 10 mg/mL.

Les échantillons sont ensuite analysés par UHPLC-DAD ESI/QTOF (Agilent 1290 infinity couplée à un Agilent ESI/QTOF 6530, Agilent Technologies), dont les paramètres sont précisés pour chaque expérience. Pour chaque échantillon un chromatogramme (abondance de composés en fonction du temps de rétention) UV à 280 nm est obtenu (permettant ainsi de cibler plus spécifiquement les composés aromatiques absorbant à cette longueur d'onde), chaque pic représentant un composé (ou plusieurs composés co-élués lorsque la séparation n'est pas optimale) dont l'aire représente l'abondance du composé. À chaque pic (et donc composé) correspond un spectre UV, et lorsque détectables un ion pseudomoléculaire en mode positif ($[M+H]^+$), et un en mode négatif ($[M-H]^-$) ainsi que leurs spectres MSMS respectifs correspondants.

À l'aide du logiciel MassHunter Qualitative Analysis (Agilent Technologies), les pics des chromatogrammes UV **(Figure 2.5)** sont intégrés puis sont alignés selon leur temps de rétention dans une matrice à l'aide d'un échantillon QC (Quality Control) : le QC est un échantillon composite représentatif de l'ensemble des échantillons d'une même expérimentation. L'intégration et l'alignement permettent une quantification relative des différents composés entre échantillons. Chaque composé est également identifié par analyse de ses spectres UV, MS et MSMS et par comparaison avec la littérature, en utilisant les bases de données SciFinder pour les composés végétaux et NPAtlas pour les composés connus chez les microorganismes.

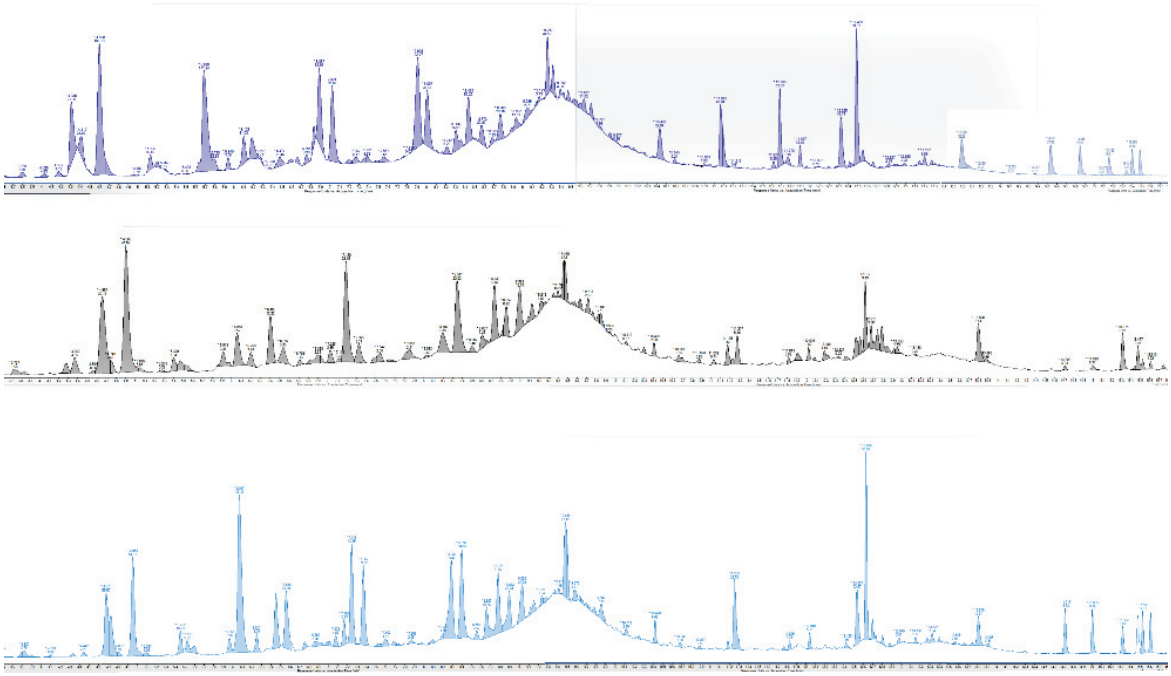


Figure 2.5 : Extraits des chromatogrammes UV à 280 nm des QC des extraits racinaires issus de trois expériences. *Chaque pic représente un métabolite, la surface intégrée correspondant à son abondance relative. Temps de rétention en abscisse, indice d'abondance en ordonnée (en unité arbitraire).*

Quantification fongique

La quantification fongique des endophytes racinaires a été réalisée au microscope optique (grossissement x 40 et x 100). Pour cela, les racines ont été préalablement décolorées dans du KOH 10 % à 90 °C (deux bains d'1 h environ). Ensuite, les racines ont été rincées à l'eau distillée et trempées pendant 1 h dans de l'HCl 1 %, avant d'être à nouveau rincées puis colorées au bleu de méthyle pendant une nuit au minimum. Elles ont été conservées dans du glycérol acide (glycérol 50 %, HCl 0,05 % dans de l'eau distillée) à 4°C avant les observations microscopiques.

Trois catégories d'endophytes ont été identifiées : les Dark Septate Endophytes (ou DSE) avec des hyphes septées et peu ramifiées souvent mélanisées et des microsclérotes ; les *Olpidium*, présentant des conidies et chlamydospores non mélanisées ; et les autres endophytes qui ne correspondent à aucune de ces deux catégories **(Figure 1.12)**.

La méthode de quantification décrite par Trouvelot et al. (1986) qui a été développée pour la quantification des champignons mycorhiziens arbusculaires a été adaptée pour l'étude des champignons endophytes racinaires des Renouées. Cette méthode repose sur une estimation visuelle du pourcentage d'infection fongique d'une racine, et ce dans 30 fragments de racines de l'ordre de 1 cm et issus d'un même individu.

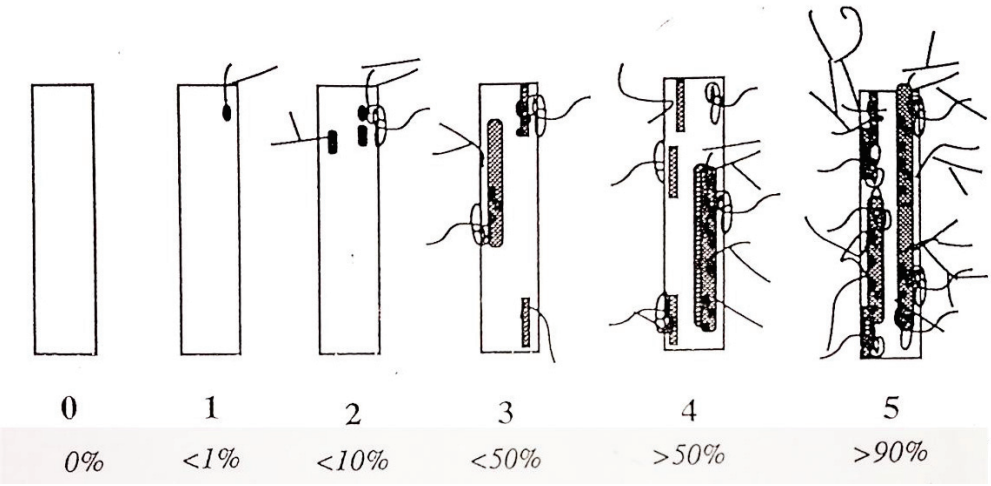


Figure 2.6 : Méthode de Trouvelot et al. (1986) pour la quantification des mycorhizes. *Chaque fragment de racine est classé dans une des 6 catégories proposées ci-dessus, selon la surface totale colonisée par les endophytes.*

Les endophytes ont été quantifiés sur 20 fragments par plantule représentant l'ensemble du système racinaire : la surface colonisée par chaque type d'endophyte a été estimée visuellement et attribuée à l'une des 6 catégories suivantes : pas de champignon (catégorie 0), trace (catégorie 1), < 10 % de la surface couverte par le champignon (catégorie 2), 10 – 50 % (catégorie 3), 50 – 90 % (catégorie 4), > 90 % (catégorie 5). Trois indices ont ensuite été calculés :

Fréquence de colonisation **F** = nombre de fragments colonisés / N

Intensité de colonisation **I** = (95n5 + 70n4 + 30n3 + 5n2 + n1) / N

Intensité de colonisation des fragments colonisés **i** = I * 100 / F

Avec N = nombre de fragments observés; n1, n2, n3, n4 et n5 correspondant au nombre de fragments dans les catégories 1, 2, 3, 4 et 5 respectivement.

Pour s'assurer de la fiabilité de la quantification des endophytes, toutes les observations microscopiques ont été faites par une seule personne, dans un ordre aléatoire et en aveugle (sans information visible des conditions de culture de l'échantillon).

Obtention de rhizomes et akènes dépourvus d'endophytes

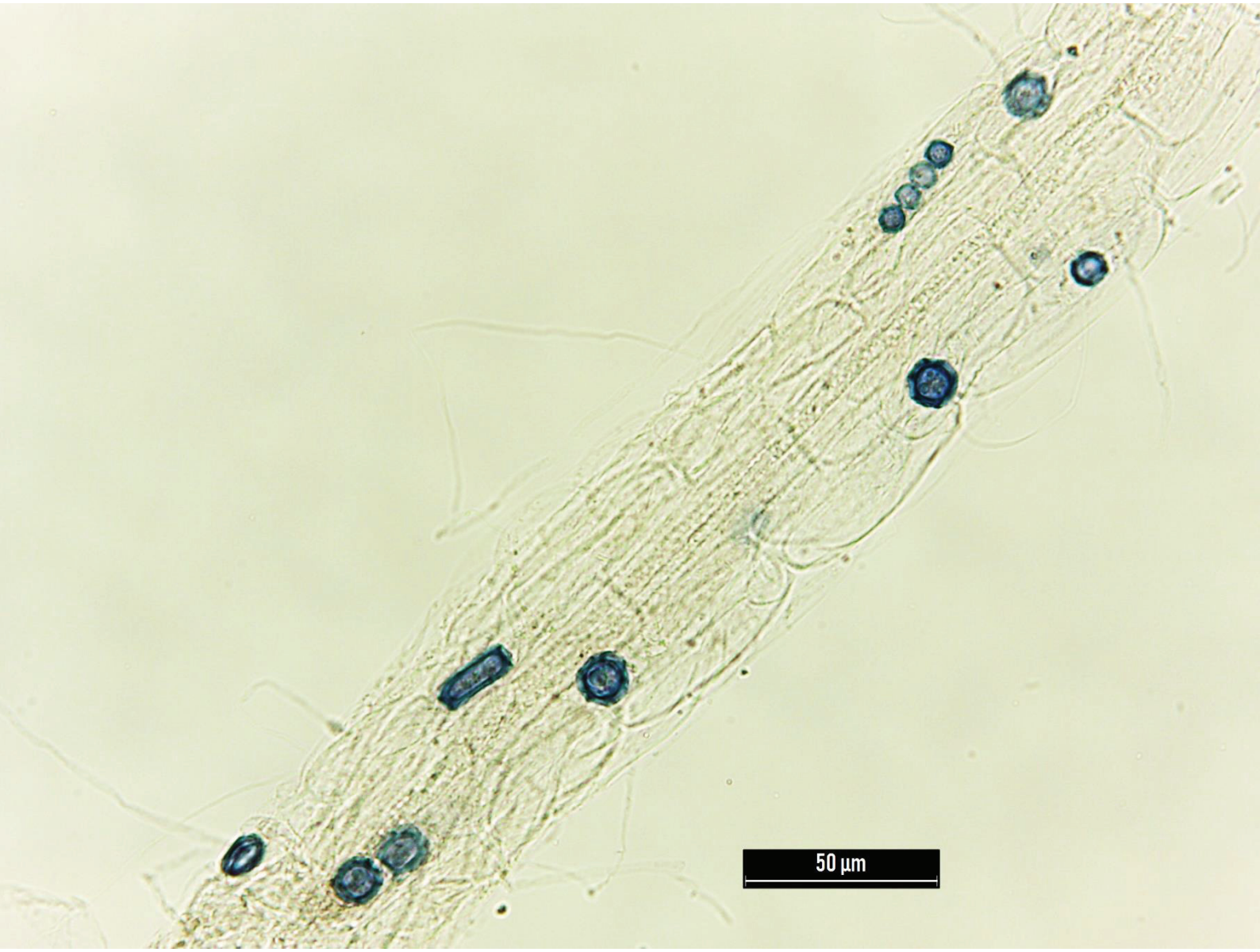
L'une des difficultés que nous avons rencontrées lors de l'élaboration de l'expérience d'inoculation des endophytes sur des plantules dépourvues d'endophytes est l'obtention de rhizomes dépourvus d'endophytes. Des expériences de désinfection en profondeur ont été réalisées avec des protocoles variés : mise en suspension des rhizomes dans différentes associations d'huiles essentielles à propriétés antifongiques, ou trempage dans de l'eau de javel, ce à des concentrations variées et pendant des durées allant d'une heure à vingt-quatre heures. La présence d'endophytes dans les rhizomes après le traitement est testée par dépôt en milieu gélosé **(Figure 2.7 gauche)** –on remarque la croissance fongique dans toutes les conditions-, et la capacité de régénération est vérifiée en perlite **(Figure 2.7 droite)** – régénération limitée dans les conditions les plus concentrées.

Figure 2.7 : Expérience de stérilisation des rhizomes aux huiles essentielles. *À gauche : test de la présence d'endophytes après traitement de stérilisation. À droite : test de la capacité de régénération des rhizomes après traitement. ©Louise BARBERIS*

Ces expériences confirment la présence d'endophytes dans les rhizomes. Aucun des traitements testés n'a permis d'obtenir des rhizomes dépourvus d'endophytes et encore capables de régénérer.

Pourrait-on utiliser les akènes pour obtenir des plants dépourvus d'endophytes ?

J'ai donc testé la présence de souches endophytiques aptes à coloniser les racines dans les akènes. Pour cela, j'ai stérilisé des akènes en surface (traitement rapide éthanol et eau de javel), puis je les ai mis à germer sur gélose (milieu axénique). L'observation microscopique des radicules après coloration au bleu de méthyle n'a pas permis d'observer d'endophytes sur plus de 15 akènes. Ces observations sont toutefois à confirmer en utilisant par exemple les outils moléculaires (méta-barcoding sur les akènes et sur les radicules).


50 µm

Chapitre 3 : Endophytes fongiques racinaires et leurs effets sur la tolérance de leur plante hôte aux métaux (Axe 1)

Préalable

L'hypothèse principale de la thèse est que les endophytes fongiques racinaires pourraient jouer un rôle importance dans la tolérance des Renouées asiatiques aux métaux.

Nous savons que les symbioses mycorhiziennes sont des lieux d'échange de carbone vers le champignon et de nutriments vers la plante et qu'elles ont un rôle clairement identifié dans la résistance des plantes aux ETM

Si la Renouée est non mycorhizée, quelques résultats présentés précédemment, notamment la présence de métabolites secondaires communs aux plantes et champignons et la présence de champignons dans les racines des Renouées, ont conduit à la recherche de données sur les endophytes fongiques racinaires non mycorhiziens, afin de répondre aux questions suivantes : quelle est leur nature ? Avec quelles familles de plantes sont-ils associés ? Quels sont leurs rôles vis-à-vis des plantes qu'ils colonisent et en particulier, jouent-ils un rôle dans la tolérance des plantes aux ETM ?

Pour répondre à ces questions, une synthèse des données bibliographiques a été réalisée : d'abord, un inventaire de tous les endophytes fongiques qui ont été recensés dans des racines a été dressé afin d'établir l'identité et la diversité de ces endophytes et de leurs associations avec des plantes. Ensuite, tous les endophytes racinaires qui ont été testés pour leur rôle dans la tolérance aux métaux de leur plante hôte ont été recensés, un des objectifs étant de déceler des constantes taxonomiques dans les mécanismes d'actions des endophytes sur la gestion des ETM.

Article sous presse

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Root fungal endophytes: identity, phylogeny and roles in plant tolerance to metal stress

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Abstract

Metal trace elements accumulate in soils mainly because of anthropic activities, leading living organisms to develop strategies to handle metal toxicity. Plants often associate with root endophytic fungi, including nonmycorrhizal fungi, and some of these organisms are associated with metal tolerance. The lack of synthetic analyses of plant-endophyte-metal tripartite systems and the scant taxonomy of these organisms led to this review aiming (1) to inventory non-mycorrhizal root fungal endophytes described with respect to their taxonomic diversity and (2) to determine the mutualistic roles of these plant-fungus associations under metal stress. More than 1500 species in 100 orders (mainly Hypocreales and Pleosporales) were reported from a wide variety of environments and hosts. Most reported endophytes had a positive effect on their host under metal stress, but with various effects on metal uptake or translocation and no clear taxonomic consistency. Future research considering the functional patterns and dynamics of these associations is thus encouraged.

Keywords: metallic trace element, fungal endophytes, taxonomy, accumulation, mutualism, plant-fungi interactions

Introduction

Metal accumulation in soils is a growing concern in developed and developing countries. Fertilizers and pesticides rich in As, Pb, Cr, Cu and Zn, among others (Senesil et al., 1999), contribute directly to the deposition of metal trace elements (MTEs) in soils. MTEs may also be emitted to the atmosphere by road traffic (Cu, Zn, Pb) (Nikolaeva et al., 2019), industries (Bourennane et al., 2010), or coal combustion (Cd, Cu, Ni, Pb, Zn…) and may be deposited on soil secondarily (Senesil et al., 1999). As metal ions are not biodegradable, they tend to accumulate and persist in soil over more than two years (Senesil et al., 1999).

As primary producers, vascular plants are essential components of the terrestrial food chain. Thus, MTE transfer and its biological effects on plants constitute crucial information for understanding the environmental fate of these pollutants. Contaminated sites present toxicity for plants, leading to scarce vegetation and negatively affecting agriculture and human health. For example, Zn and Cd are easily taken up by plants, causing chlorosis and stunted growth and disturbing N metabolism (Påhlsson, 1989); Cr reduces germination, yield and plant height and leads to the formation of thick roots (Shanker et al., 2005). MTEs in general induce oxidative stress and ionic homeostasis disturbance in plants (Yadav, 2010). However, several plant species developed metal tolerance, either to one metal (Zn for instance (Påhlsson, 1989)) or to several metals with a common mechanism (recapitulative figures in Domka et al. (2019); Singh et al., 2016). Metabolomics, ionomics and proteomics have shown numerous modifications in plant metabolism in the presence of heavy metals (Singh et al., 2016), such as phytochelatins and glutathione, which increase metal contamination (Seth et al., 2012). Amino acids, organic acids and phenols participate in the chelation and transport of metals, whereas glutathione and alpha-tocopherol are involved in the scavenging of ROS and lipid peroxides. Peptides such as phytochelatins and metallothioneins bind metal ions, and hormones such as salicylic acid or abscisic acid participate in plant systemic responses to abiotic stress (Hu et al., 2020; Raza et al., 2020; Saeed-Ur-Rahman et al., 2020; Singh et al., 2016). Metal ions bound to phytochelatins are transported from the cytosol to the vacuolar compartment, thus detoxifying the cytosol and limiting oxidative stress (Yadav, 2010; Zenk, 1996).

The plant and fungal kingdoms are strongly linked, and more than 85% of vascular plants form symbiotic associations with mycorrhizal fungi (Brundrett and Tedersoo, 2018). Although seven categories of mycorrhizal symbioses have been reported (Finlay, 2008), two

types, namely, arbuscular mycorrhizal and ectomycorrhizal symbioses, have been extensively studied for their role in plant-MTE interactions. Although the literature concerning the role of ericoid mycorrhizal (ErM) fungi is not as extensive as that for arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF), some data are also available for this group.

At metal-contaminated sites, AMF are found in more than 80% of plants across a wide diversity and have been shown to promote plant growth (Wang, 2017), demonstrating that not only are fungi tolerant to metals, but they may also help plants tolerate metals. Therefore, the role of AMFs and EMFs on plant tolerance to metal stress has been widely studied (*e.g.,* Luo et al., 2014; Miransari, 2011; Shi et al., 2019; Zhan et al., 2019). AMFs increase plant growth and alleviate metal stress, especially under high metal concentrations. Stress alleviation may come from increased plant growth (metal dilution effect) and/or decreased concentrations of available metals in soil (Khan, 2005; Miransari, 2011) but also from participation and improvement of plant defences (Ferrol et al., 2016). Ectomycorrhizae are present in approximately 2% of plant species, mainly trees (Brundrett, 2009; Brundrett and Tedersoo, 2018). Some EMFs immobilize metals in soil, whereas other EMFs promote metal uptake by the host plant. Metal transporters produced by EMFs are important in mediating tolerance (Luo et al., 2014). Thus, in mycorrhizal plants, both AMFs and EMFs can alleviate plant metal stress by sequestrating metal ions, improving the nutritional and antioxidative status of the plant and stimulating the expression of genes involved in metal accumulation in both partners (Shi et al., 2019; Yu et al., 2020). Similarly, ErM favour plant growth under Cu and Zn contamination with lower MTE concentrations in shoots but higher concentrations in roots, suggesting an adsorption mechanism (Bradley et al., 1982), or with lower concentrations in roots, suggesting a filtering effect (Casarrubia et al., 2020). The metal tolerance of fungal partners may explain in some way their host plant metal tolerance (Bradley et al., 1982): in particular, the involvement of several fungal genes coding for antioxidant enzymes, metal transporters, and DNA damage repair proteins are under investigation (Daghino et al., 2016). Transporters and metallothioneins, which could be involved in MTE sequestration in roots or MTE transport into plant tissues, have been identified in all three types of mycorrhizae (Ruytinx et al., 2020) and seem to mitigate oxidative stress (Zou et al., 2020), but the precise mechanism at the plant root-fungi interface remains unknown (Becquer et al., 2019).

In addition to mycorrhizae, other fungi associate with plant roots without forming an exchange structure. Dark-septate endophytes (DSEs) belong to this category. Rather well studied, they are often named “pseudomycorrhizae” (Jumpponen and Trappe, 1998) because of their specific association with plant roots and their mutualistic effects on plants. A meta-analysis of inoculation experiments showed that DSEs have positive or neutral effects on plant growth (Newsham, 2011). Keeping these four groups –AMF, EMF, ErM and DSE– aside, a high diversity of fungi is still found in plants. Some are classified as parasitic despite harbouring non-pathogenic interactions with some plants, while others are truly unstudied. *Olpidium* or *Mucor* are examples of less studied fungal genera defined as “endophytes” (Zahoor et al., 2017; Zubek et al., 2016).

Some authors defined the term “endophytes” as either bacterial or fungal symbionts within plant tissues that, during at least part of their life cycle, do not cause any visible signs of tissue damage or adverse effects on the host (Kageyama et al., 2008; Schulz and Boyle, 2005; Wilson, 1995).

Numerous reviews on plant-fungus relationships, plant-MTE interactions and fungus-MTE interactions exist **(Figure 3.1)**. Some of them combine plant, fungi and heavy metal relationships, but most of them are restricted to some plants (trees for example) or some microorganisms (AMFs mainly, DSEs, bacteria, etc.). Only a few studies considered the tripartite plant-endophyte-metal system at the whole ecosystem level, and even fewer used *in situ* analyses. Despite the fact that the roles of fungal endophytes on plant metal tolerance, including DSEs, were well and recently reviewed by Domka et al. (2019), the contemporaneous bibliography did not explore the phylogeny of this large taxonomical group that is formed by endophytic fungi. Fungal endophyte biology may be highly diverse, and as a result, the mechanisms of metal stress alleviation may vary between these groups, although some might be conserved between closely related species.

Consequently, the objectives of this review are (1) to report phylogenetic relationships of fungal endophytes described to date and (2) to explore their mutualistic function in the context of metal contamination and by way of taxonomy. As roots are directly in contact with contaminated soil where MTE transfer occurs, we chose to inventory root fungal endophytes exclusively. Thus, we answered the following questions: which are the fungal endophytes present in plant roots? Do they participate in plant tolerance to metal contamination? Is there any evident relationship between their mutualistic function and their taxonomy?

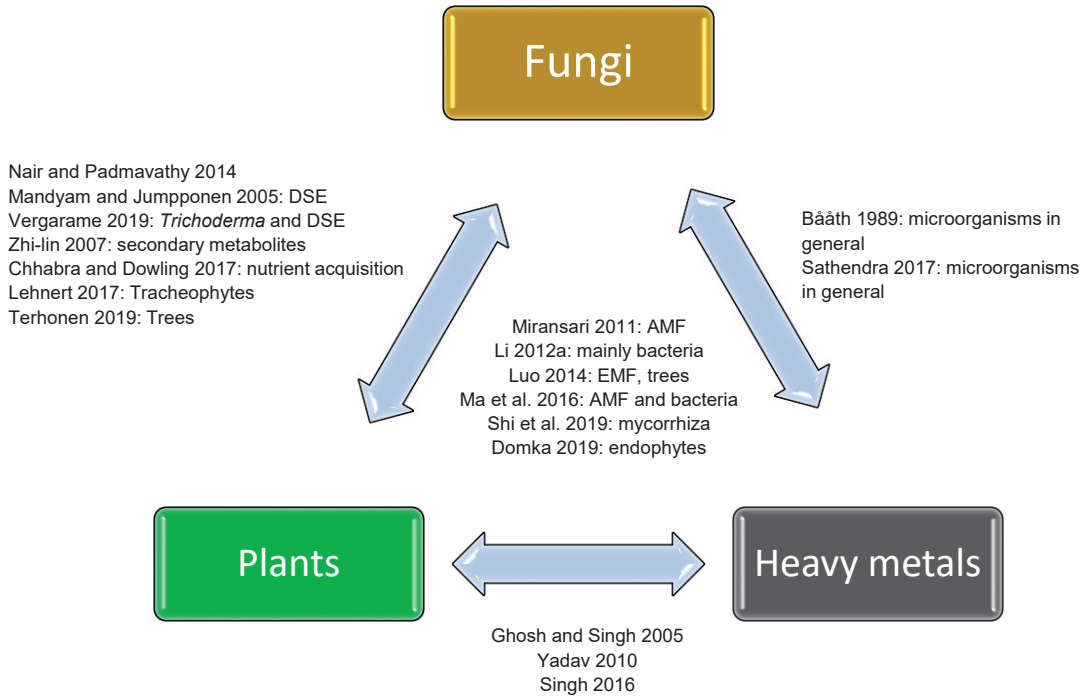

Figure 3.1: Existing reviews on plant-fungi-MTE interactions. *EM: Ectomycorrhiza Fungi, AMF: Arbuscular Mycorrhizal Fungi.*

This review identifies fungal endophytes described in various plant roots and environments and places them in a global phylogeny of fungi. Then, different aspects of metal stress alleviation by endophytes are reported in light of taxonomy. We propose future investigations to further elucidate the roles of endophytic fungi in plant metal tolerance.

Identification and phylogenetic analysis

This first chapter presents an inventory of fungal endophytes that were identified in plant roots **(Table 3.1)**: this review does not include mycorrhizal fungi. A total of 144 articles studied fungal root communities and identified them by sequencing approaches. The complete inventory is available in the supplementary data **(Table S1)**, with more than 1500 different species referenced. Hereafter, all orders of identified species are presented.

Table 3.1: Fungal root plant endophytes: Ascomycota. *ND: not determined, NS: not specified.*

Class (-mycetes)	Order	Type of plant	References
Archaeorhizo-	Archaeorhizomycetales	Forest plants	102
Asco-	Incertae sedis	Forb, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	12, 17, 22, 48, 61, 68, 95, 120, 142
Asco-	ND	Forb, Orchidaceae, Poaceae, Shrub, Tree	3, 13, 33, 47, 82, 83, 91, 99, 100, 103, 110, 113, 120, 122
Chaetothyrio-	ND	Shrub	17
Coelo-	ND	Forb	13
Dothideo-	Botryosphaeriales	Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	2, 6, 12, 20, 21, 28, 33, 48, 49, 62, 67, 81, 82, 85, 89, 102, 107, 109, 115, 116
Dothideo-	Capnodiales	Aquatic plant, Arborescent Poaceae, Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	2, 8, 12, 17, 19, 20, 21, 28, 29, 31, 38, 43, 49, 51, 54, 55, 57, 58, 62, 66, 68, 70, 74, 81, 82, 85, 90, 91, 92, 98, 102, 107, 109, 110, 112, 113, 116, 117, 122, 129
Dothideo-	Dothideales	Forb, Forest plants, Orchidaceae, Poaceae, Subshrub, Tree	9, 12, 16, 21, 28, 30, 32, 33, 43, 51, 57, 61, 87, 90, 91, 92, 102, 113, 131
Dothideo-	Incertae sedis	Forb, Forest plants, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	4, 12, 33, 53, 70, 76, 81, 82, 87, 101, 102, 116, 120
Dothideo-	Kirschsteiniotheliales	Forest plants	102
Dothideo-	Minutisphaerales	Forest plants, Orchidaceae, Poaceae	12, 63, 102
Dothideo-	Myriangiales	Forest plants	102
Dothideo-	ND	Forest plants, Poaceae, Shrub, Tree	33, 64, 72, 76, 81, 90, 102
Dothideo-	Neocelosporiales	Shrub	81
Dothideo-	Pleosporales	Aquatic plant, Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree, NS	2, 3, 4, 8, 9, 10, 12, 13, 16, 20, 21, 23, 27, 28, 29, 31, 32, 33, 34, 35, 36, 37, 41, 42, 43, 44, 45, 49, 50, 51, 53, 54, 57, 58, 62, 65, 66, 67, 68, 69, 70, 73, 76, 77, 78, 79, 80, 81, 82, 83, 85, 86, 87, 89, 90, 91, 92, 93, 97, 98, 101, 102, 104, 105, 107, 108, 109, 110, 113, 115, 116, 117, 120, 123, 128, 129, 131, 140, 141, 142, 143, 145
Dothideo-	Tubeufiales	Forest plants, Tree	65, 102
Dothideo-	Venturiales	Forb, Forest plants	102, 139
Eurotio-	Chaetothyriales	Forb, Forest plants, Halophytes, Orchidaceae, Poaceae, Subshrub, Tree, NS	4, 8, 12, 14, 20, 21, 22, 36, 43, 49, 51, 52, 55, 57, 62, 63, 66, 70, 72, 76, 83, 85, 91, 99, 101, 102, 118, 120, 121
Eurotio-	Eurotiales	Aquatic plant, Arborescent Poaceae, Carnivorous, Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree, NS	1, 2, 9, 12, 13, 15, 16, 17, 19, 25, 29, 33, 34, 36, 39, 43, 44, 46, 48, 49, 51, 53, 54, 57, 61, 62, 66, 67, 68, 70, 73, 74, 81, 82, 85, 87, 88, 89, 90, 91, 92, 96, 101, 102, 104, 106, 107, 109, 110, 113, 115, 116, 117, 119, 120, 123, 125, 129, 131, 134, 135, 136, 137, 140
Eurotio-	ND	Forest plants, Tree	76, 102
Eurotio-	Onygenales	Hyperaccumulator, Poaceae	62, 86, 92
Eurotio-	Phaeomoniellales	Forest plants, Poaceae	102, 120
Geoglosso-	Geoglossales	Forest plants	102
Incertae sedis	Incertae sedis	Arborescent Poaceae, Fern, Forb, Forest plants, Orchidaceae, Poaceae, Shrub, Tree	2, 9, 10, 12, 13, 19, 21, 29, 45, 56, 57, 63, 82, 83, 93, 97, 102, 103
Incertae sedis	ND	Forb, Forb or Poaceae, Subshrub	44, 66, 87
Lecanoro-	Lecanorales	Poaceae	63
Lecanoro-	ND	Forest plants	102
Lecanoro-	Ostropales	Forest plants, NS	101, 102
Leotio-	Chaetomellales	Forb or Poaceae, Hyperaccumulator, Orchidaceae	20, 21, 62, 66

Leotio-	Erysiphales	Poaceae	120
Leotio-	Helotiales	Aquatic plant, Arborescent Poaceae, Carnivorous, Fern, Forb, Forest plants, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree, NS	2, 3, 4, 10, 12, 17, 19, 21, 29, 30, 33, 34, 36, 40, 44, 47, 48, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 63, 67, 68, 70, 72, 76, 79, 81, 82, 83, 87, 88, 92, 93, 95, 97, 98, 99, 100, 101, 102, 103, 107, 109, 114, 115, 118, 120, 122, 124, 128, 131, 145
Leotio-	Incertae sedis	Forb, Forest plants, Orchidaceae, Shrub, Tree, NS	10, 44, 51, 55, 61, 76, 81, 101, 102, 141
Leotio-	ND	Tree, Orchidaceae, Forest plants	20, 48, 102
Leotio-	Rhytismatales	Forest plants, Poaceae, Shrub, NS	81, 91, 92, 101, 102
ND	ND	Aquatic plant, Carnivorous, Forb, Forest plants, Halophytes, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	17, 48, 51, 54, 55, 67, 72, 80, 81, 82, 88, 102, 106, 110, 129
Neolecto-	Neolectales	Forest plants	102
Orbilio-	ND	Orchidaceae	55
Orbilio-	Orbiliales	Forest plants, Orchidaceae, Shrub, Subshrub, Tree	12, 29, 78, 95, 102
Pezizo-	Pezizales	Forb, Forest plants, Poaceae, Shrub, Tree	12, 58, 81, 82, 86, 102, 103
Saccharo-	Saccharomycetales	Aquatic plant, Forb, Forest plants, Orchidaceae, Poaceae, Tree, NS	21, 51, 54, 55, 77, 90, 91, 92, 101, 102, 115, 144
Sareo-	Sareales	Shrub	81
Sordario-	Amphisphaeriales	Fern, Forb, Forest plants, Halophytes, Orchidaceae, Poaceae, Tree, NS	12, 21, 23, 28, 29, 49, 57,68, 93, 101, 102, 107, 112, 113, 130, 138
Sordario-	Atractosporales	Forest plants, Tree	76, 102
Sordario-	Cephalothecales	Forest plants, Poaceae	91, 102
Sordario-	Chaetosphaeriales	Forest plants, Orchidaceae, Poaceae, Shrub, Tree, NS	4, 12, 33, 48, 55, 57, 58, 76, 92, 94, 101, 102, 107, 109, 145
Sordario-	Coniochaetales	Forb, Forest plants, Poaceae, Shrub, Tree	36, 48, 63, 66, 81, 87, 102, 126, 131
Sordario-	Coronophorales	Forest plants	102
Sordario-	Diaporthales	Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Tree, NS	2, 12, 22, 23, 28, 29, 33, 34, 45, 51, 62, 65, 66, 67, 68, 73, 78, 81, 87, 90, 91, 92, 98, 101, 102, 109, 112, 115, 117, 119, 120, 144, 145
Sordario-	Glomerellales	Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Tree	3, 4, 9, 12, 13, 20, 21, 22, 28, 34, 37, 43, 45, 49, 53, 62, 66, 73, 78, 79, 81, 91, 96, 98, 102, 104, 107, 110, 112, 115, 144
Sordario-	Halosphaeriales	Poaceae	86
Sordario-	Hypocreales	Aquatic plant, Arborescent Poaceae, Carnivorous, Fern, Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree, NS	2, 3, 4, 5, 6, 7, 10, 12, 13, 15, 16, 17, 18, 19, 21, 22, 24, 28, 29, 31, 32, 33, 35, 36, 37, 41, 42, 44, 48, 49, 50, 51, 52, 57, 58, 60, 61, 62, 63, 66, 67, 68, 69, 70, 71, 72, 73, 74, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 96, 97, 98, 101, 102, 104, 105, 106, 107, 109, 110, 111, 113, 115, 117, 118, 119, 120, 123, 125, 126, 129, 130, 133, 136, 140, 141, 144, 145
Sordario-	Incertae sedis	Orchidaceae, Poaceae, Subshrub	12, 21, 33, 116
Sordario-	Lulworthiales	Forb, Poaceae, Shrub, Subshrub, Tree	2, 13, 51, 76, 82, 108, 116
Sordario-	Magnaporthales	Forb, Poaceae, Shrub	8, 33, 41, 62, 70, 145
Sordario-	Melanosporales	Forb, Forb or Poaceae, Orchidaceae, Shrub, Subshrub, Tree	12, 20, 21, 57, 66, 68, 81, 116, 140
Sordario-	Microascales	Forb, Forest plants, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Tree	2, 4, 5, 12, 13, 45, 57, 62, 67, 82, 91, 92, 102, 106
Sordario-	Myrmecridiales	Forest plants	102
Sordario-	ND	Forest plants, Orchidaceae, Poaceae, Shrub, Tree	33, 55, 76, 102, 145
Sordario-	Ophiostomatales	Forest plants, Poaceae, Tree	43, 50, 57, 102
Sordario-	Phomatosporales	Orchidaceae	12
Sordario-	Phyllachorales	Poaceae	92
Sordario-	Pleurotheciales	Forest plants, Poaceae	63, 102

Sordario-	Sordariales	Climber, Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	2, 5, 12, 13, 16, 20, 21, 23, 28, 29, 30, 41, 42, 49, 51, 53, 57, 61, 62, 63, 66, 67, 68, 69, 70, 72, 73, 81, 85, 86, 87, 91, 92, 102, 106, 107, 116, 123, 127, 129, 145
Sordario-	Togniniales	Forb, Forest plants, Tree	90, 102, 140
Sordario-	Trichosphaeriales	Poaceae	92
Sordario-	Xenospadicoidales	Forest plants	102
Sordario-	Xylariales	Forb, Forest plants, Halophytes, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	2, 4, 5, 8, 11, 12, 20, 21, 22, 23, 28, 30, 32, 33, 34, 36, 37, 41, 45, 57, 62, 63, 66, 70, 72, 81, 85, 91, 92, 102, 103, 107, 112, 116, 119, 120, 123, 129, 140
Taphrino-	Taphrinales	Forest plants	102

Table 3.2: Fungal root plant endophytes except Ascomycota. *ND: not determined, NS: not specified.*

Division (-mycota)	Class (-mycetes)	Order	Type of plant	References
Basidio-	Agarico-	Agaricales	Aquatic plant, Forb, Forest plants, Orchidaceae, Poaceae, Shrub, Subshrub, Tree, NS	12, 17, 18, 21, 24, 28, 32, 33, 41, 42, 48, 50, 52, 54, 57, 58, 63, 64, 76, 85, 86, 91, 92, 94, 100, 101, 102, 108, 109, 120, 122, 140, 145
Basidio-	Agarico-	Atheliales	Forb, Forest plants, Shrub, Subshrub, Tree, NS	47, 52, 102, 131
Basidio-	Agarico-	Auriculariales	Forest plants, Orchidaceae, Tree	12, 76, 102
Basidio-	Agarico-	Boletales	Forest plants, Tree	76, 94, 102
Basidio-	Agarico-	Cantharellales	Aquatic plant, Forb, Forest plants, Orchidaceae, Poaceae, Shrub, Tree	2, 12, 16, 22, 33, 35, 54, 55, 58, 64, 75, 81, 83, 85, 94, 98,102, 115
Basidio-	Agarico-	Corticiales	Forest plants, Poaceae, Subshrub, Shrub	52, 92, 102
Basidio-	Agarico-	Geastrales	Forest plants, NS	101, 102
Basidio-	Agarico-	Hymenochaetales	Forest plants, Orchidaceae, Poaceae, Shrub	12, 24, 42, 81, 102
Basidio-	Agarico-	Incertae sedis	Orchidaceae	12
Basidio-	Agarico-	ND	Forest plants, Shrub	102, 106
Basidio-	Agarico-	Phallales	Forest plants, Poaceae	86, 102
Basidio-	Agarico-	Polyporales	Aquatic plant, Forb, Forest plants, Orchidaceae, Poaceae, Shrub, Tree	8, 12, 61,64, 68, 81,92, 102, 106, 108, 132, 134
Basidio-	Agarico-	Russulales	Forest plants, Orchidaceae, Shrub, Tree	12, 43, 68, 76, 81, 86, 94, 102, 109
Basidio-	Agarico-	Sebacinales	Forest plants, Orchidaceae, Subshrub, Shrub	22, 52, 102
Basidio-	Agarico-	Thelephorales	Forest plants, Orchidaceae, Tree, NS	12, 47, 59, 94, 101, 102
Basidio-	Agarico-	Trechisporales	Forest plants	101, 102
Basidio-	Agaricostilbo-	Agaricostilbales	Forest plants, Poaceae	92, 102
Basidio-	Agaricostilbo-	ND	Forest plants	102
Basidio-	Atractiello-	Atractiellales	Tree	76
Basidio-	Basidio-	ND	Forb, Orchidaceae, Poaceae, Shrub	12, 23, 34, 52, 120, 122
Basidio-	Botryo-	Heterogastridiales	Forest plants	102
Basidio-	Cystobasidio-	Cystobasidiales	Forest plants	102
Basidio-	Cystobasidio-	Erythrobasidiales	Forest plants	102
Basidio-	Dacry-	Dacrymycetales	Forest plants	102
Basidio-	Exobasidio-	Exobasidiales	Forest plants, Halophytes	49, 102
Basidio-	Exobasidio-	Malasseziales	Forest plants, Poaceae	63, 102, 108
Basidio-	Incertae sedis	Incertae sedis	Forest plants	102
Basidio-	Microbotryo-	Incertae sedis	Forest plants	102
Basidio-	Microbotryo-	Leucosporidiales	Forest plants	102
Basidio-	Microbotryo-	Microbotryales	Forest plants	102
Basidio-	Microbotryo-	ND	Forest plants	102
Basidio-	Microbotryo-	Sporidiobolales	Forest plants, Orchidaceae, Poaceae, Subshrub, Tree	57, 91, 92, 102, 105, 116
Basidio-	ND	ND	Forest plants, Shrub, Tree	17, 57, 102

Basidio-	Puccinio-	ND	Forest plants	102
Basidio-	Tremello-	Cystofilobasidiales	Forest plants, Poaceae	92, 102
Basidio-	Tremello-	Filobasidiales	Forest plants	102
Basidio-	Tremello-	ND	Forest plants	102
Basidio-	Tremello-	Tremellales	Aquatic plant, Forb, Forest plants, Poaceae, Tree	43, 54, 92, 102, 142
Basidio-	Tremello-	Trichosporonales	Forb, Forest plants, Poaceae	37, 102, 120
Basidio-	Ustilagino-	Ustilaginales	Forest plants, Poaceae	92, 102
Basidio-	Wallemio-	Geminibasidiales	Forest plants	102
Basidio-	Wallemio-	Wallemiales	Forest plants, Poaceae	102, 120
Basidiobolo-	Basidiobolo-	Basidiobolales	Forest plants	102
Chytridio-	Chytridio-	Chytridiales	Aquatic plant	54
Chytridio-	Lobulo-	Lobulomycetales	Forest plants, Poaceae	102, 108
Chytridio-	Monoblepharido-	Monoblepharidales	Aquatic plant	54
Chytridio-	ND	ND	Aquatic plant, Poaceae	54, 108
Chytridio-	Rhizophlyctido-	Rhizophlyctidales	Aquatic plant, Forest plants	54, 102
Chytridio-	Spizello-	Spizellomycetales	Poaceae	64
Entomophthoro-	Entomophthoro-	Entomophthorales	Forest plants	102
Incertae sedis	Deutero-	ND	Forb	13
Incertae sedis	Incertae sedis	Incertae sedis	Tree	65
Kickxello-	Kickxellomycetes	Kickxellales	Forest plants	102
Kickxello-	Kickxellomycetes	Kriegeriales	Forest plants	102
Mortierello-	Mortierello-	Mortierellales	Forest plants, Halophytes, Poaceae, Orchidaceae, Shrub, Tree, NS	49, 52, 55, 58, 63, 68, 81, 92, 102
Mucoro-	Incertae sedis	Endogonales	Aquatic plant, Forest plants	54, 63
Mucoro-	Mucoro-	Mucorales	Forb, Forest plants, Hyperaccumulator, Non-hyperaccumulator, Orchidaceae, Poaceae, Shrub, Tree	12, 15, 26, 29, 43, 44, 57, 62, 66, 67, 68, 81, 85, 89, 96, 102, 107, 115, 135
Mucoro-	ND	ND	Tree	72, 76
Mucoro-	Umbelopsido-	Umbelopsidales	Forest plants, Orchidaceae, Poaceae, Shrub, Subshrub, Tree, NS	43, 48, 57, 61, 68, 72, 81, 100, 101, 102, 116, 118, 145
ND	ND	ND	Aquatic plant, Arborescent Poaceae, Carnivorous, Forb, Forest plants, Hyperaccumulator, Orchidaceae, Poaceae, Shrub, Subshrub, Tree	2, 9, 13, 16, 17, 19, 21, 22, 23, 24, 29, 30, 33, 35, 37, 40, 41, 43, 45, 48, 54, 58, 62, 65, 66, 67, 72, 79, 81, 83, 86, 88, 92, 95, 98, 99, 102, 103, 105, 108, 110, 120, 122, 133
Oo-	Oo-	ND	Forb	131
Oo-	Oo-	Peronosporales	Forb	131
Oo-	Oo-	Pythiales	Forb, Poaceae, Shrub, Tree	58, 78, 115, 131
Zoopago-	Zoopago-	Zoopagales	Tree	93
Zygo-	Zygo-	ND	Poaceae, Shrub	81, 96

Root endophytes were found wherever scientists searched for them by using molecular sequencing. Indeed, from grasslands to aquatic systems, deserts, forests, bogs, dunes, mountains and metal-polluted environments, fungi belonging to 101 orders, 40 classes and 12 divisions were retrieved and included in this review. However, many fungi are still of undetermined order.

Fungal communities may vary according to the season, site, soil characteristics and host plant. Root fungal communities were extremely different between the early and late seasons in *Bouteloua gracilis* and *Gutierrezia sarothrae* (Kageyama et al., 2008), and root culturable endophytes were strictly different between spring and summer in the carnivorous plant *Drosera rotundifolia* (Quilliam and Jones, 2010). The colonization percentage by DSEs was 6-fold higher in May (12%) than in April (2%) in *Salix humboldtiana* (Becerra et al., 2009). Thus, communities of fungal endophytes are highly variable in quality and quantity during the growing season, indicating temporal variability, which may be related to different fungal growth velocities, different fungal phenologies or variable fungal recruitment by the plant, depending on its growth state and environmental changes.

Endophyte communities also vary in terms of diversity and colonization levels between geographical sites. For example, the DSE colonization percentage in *Solanum nigrum* varied from 1 to 10% between four sites at three different elevations (Muthukumar and Sathya, 2017), and endophyte richness was greater in *Festuca paniculata* roots in unmown grasslands than in mown grasslands, with Eurotiomycetes being specific to mown grasslands (Mouhamadou et al., 2011). Local pedoclimatic conditions may represent abiotic filters limiting fungal colonization and include the variation of physico-chemical parameters such as elevation, slope orientation, climate, and soil characteristics (pH, N and K concentrations, granulometry, etc.). For instance, in the halophyte plant *Inula crithmoides*, one undetermined DSE belonging to the Pleosporales was positively correlated with the salt gradient (Maciá-Vicente et al., 2012). Nevertheless, these differences between sites may also be explained by limited fungal dispersion or biotic filters or even by the abundance of DSEs in soil that were not systemically recorded.

Each order of fungi is found in several types of plants (e.g., Pleosporales and Chaetosphaeriales in Orchidaceae, trees, subshrubs and shrubs, Poaceae, and forest plants, while Pleosporales is also reported in aquatic plants, forbs, halophytes, and hyperaccumulators) **(Table 3.1, Table 3.2)**, indicating rather generalist plant-fungus associations. However, fungal endophyte communities depend on the host plant. Indeed, endophytic assemblages differ between two plant species belonging to the same genera, for example, between the halophyte *Inula crithmoides* and the non-halophyte *Inula viscosa* (Maciá-Vicente et al., 2012). The frequencies of association between endophytes and different trees (*Betula papyrifera*, *Abies balsamea*, and *Picea glauca*) revealed the preferences of some fungi for a specific tree; for example, *Phialocephala fortinii* associates preferentially with *P. glauca* and *Oidodendron* sp. with *B. papyrifera* (Kernaghan and Patriquin, 2011). Similarly, in two grasses, *Phoma herbarum* and *Microdochium* sp. were found only in *Bouteloua gracilis*, whereas *Lophiostoma* sp. was found only in *Gutierrezia sarothrae* (Kageyama et al., 2008).

Root fungal endophytes were found among the great majority of Ascomycota (52 identified orders, **Table 3.1**)**,** followed by Basidiomycota, Chytridiomycota, Mucoromycota, and Oomycota (32, 5, 3 and 2 orders, respectively, **Table 3.2**). Orders that are found in a large number of studies (more than a quarter of studies) are Hypocreales (Sordariomycetes), Pleosporales (Dothideomycetes), Helotiales (Leotiomycetes), Eurotiales (Eurotiomycetes) and Xylariales (Sordariomycetes) **(Figure 3.2)**.

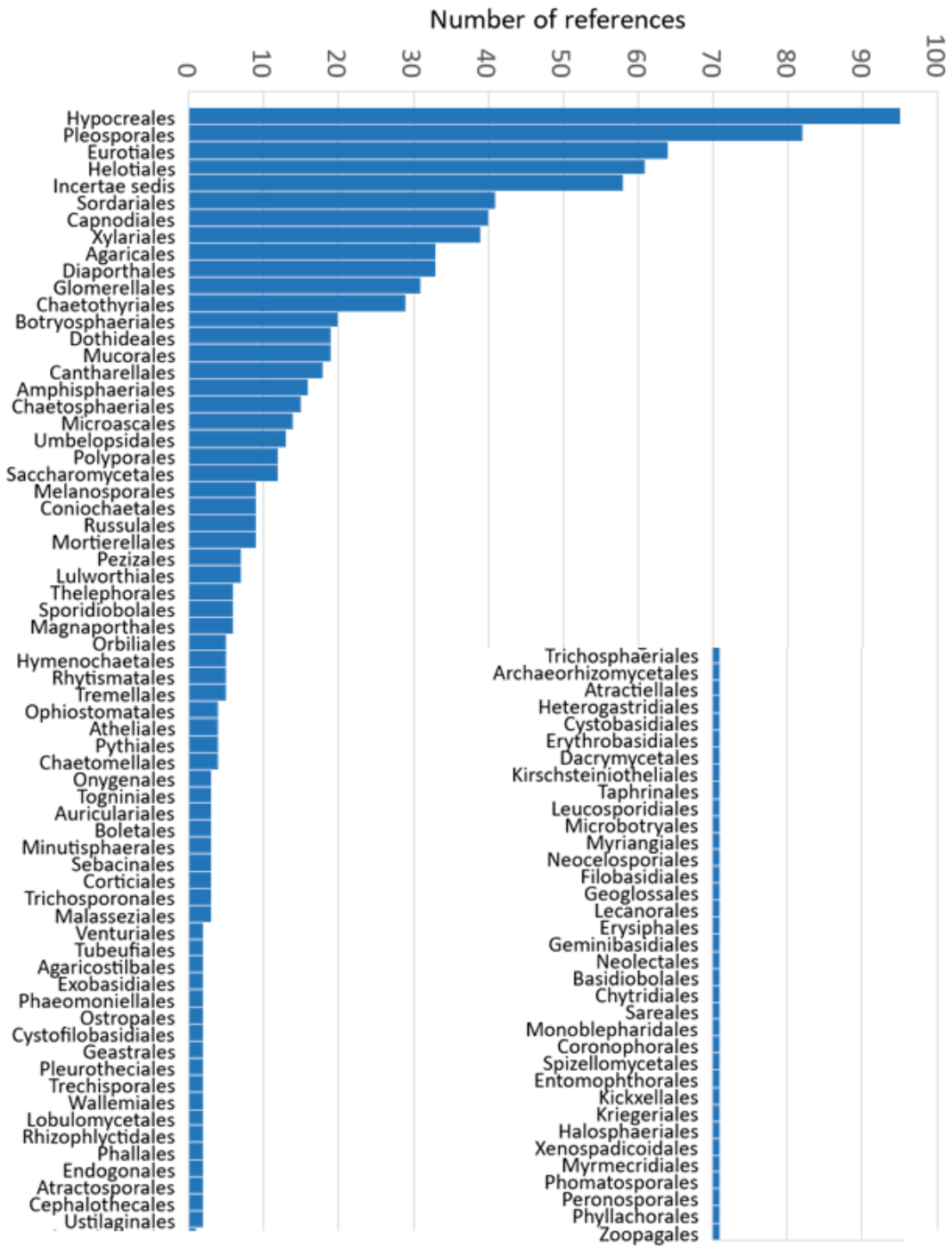

Figure 3.1: Number of references per order of fungal endophytes.

Thus, fungal root endophytes are highly diverse. We placed them on a phylogenetic tree of Eumycetes **(Figure 3.3)**. Similar to foliar endophytes (Higgins et al., 2007), root endophytes are found throughout the phylogeny of Eumycetes and do not form a monophyletic group. This suggests, similar to mycorrhizae (Fitter and Moyersoen, 1996), that the ability to live within plant roots without harming them appeared several times in evolution.

Figure 3.3: Endophytes in the phylogeny of Eumycetes. *Root fungal occurrences are defined as one fungus/one plant host/one environment/one reference article. Modified from Nagy and Szöllősi (2017).*

Among all fungal endophytes, the dark septate endophyte (DSE) morphological group is commonly – and more than other endophytes - studied for its potential beneficial association with plants. Several orders were reported to contain DSEs: Capnodiales, Chaetosphaeriales, Chaetothyriales, Dothideales, Elaphomycetales, Eurotiales, Helotiales,

Hypocreales, Leotiales, Microascales, Onygenales, Pleosporales, Pezizales, Saccharomycetales, Sordariales, Taphrinales, and Xylariales (Grünig et al., 2011; Jumpponen and Trappe, 1998; Knapp and Kovács, 2016; Newsham, 2011). DSEs therefore constitute a paraphyletic group (Yuan et al., 2011), defined by their similar morphology *(i.e.*, intercellular melanised and septate hyphae and intracellular microsclerotia). We note that the seven orders that were the most often found from our results (Hypocreales, Pleosporales, Helotiales, Eurotiales, Xylariales, Capnodiales and Sordariales) are known to contain DSEs (Jumpponen and Trappe, 1998; Knapp and Kovács, 2016; Newsham, 2011).

Root fungal endophytes are commonly found in metal-contaminated soils (Domka et al., 2019b; Lacercat-Didier et al., 2016) and increase plant metal tolerance (Domka et al., 2019b). The high diversity of root endophytes observed in this study suggests that their roles in plant metal tolerance may strongly differ as well as the mechanisms of plant-fungus tolerance to metal. Thus, in this work, the role of these organisms in plant metal tolerance and accumulation is analysed and compared with respect to their taxonomic diversity.

Fungal endophytes and plant metal tolerance

We made an inventory of root endophytes that were experimentally tested on plants in the context of metal contamination **(Table 3.3)**. When described, we reported the effect of those endophytes on plant growth, metal uptake and metal translocation from roots to aerial parts. A complete inventory of the effects of endophytes on other plant traits in the context of metal contamination is available in the supplementary data.

Of the 118 plant-fungus associations for which the MTE was specified, Cd was the most commonly studied (49), followed by Pb (24) and Zn (15) **(Figure 3.4A)**. These contaminants are particularly abundant in anthropized soils, especially originating from coal combustion (Bourennane et al., 2010; Senesil et al., 1999) and agriculture for Cd (Bourennane et al., 2010). These three elements presented enrichment factors from 10 to 30 in soils of industrial regions, much higher than those of other trace elements (Bourennane et al., 2010). A relatively equal number of studies examining monocontamination (35 associations) or polycontamination (27 associations) was observed **(Figure 3.4B)**. Nine pot experiments directly tested field soil or wastewater contamination, in contrast to the rest of the studies that used artificially mono- or polycontaminated soils. Metal availability for plants is related to its speciation in soil (Kabata-Pendias, 2004), which cannot be controlled in greenhouses. Using field soil as a substrate for experiments limits this bias and should be encouraged, although it makes the interactions between different metals more complex.

Table 3.3: Fungal endophytes and their effects on plant growth, metal uptake and translocation on metal-contaminated soil. *Oth: other endophyte. Root metal concentration was included in metal uptake, and shoot metal concentration was included in metal translocation. A line corresponds to one fungus * one plant host genera * one response dynamic * one reference. Green: positive effect, red: negative effect, ocher: neutral effect. Rs: research, Rv: review.*

Division -mycota	Class - mycetes	Order	Fungal endophytes	Type of endophyte	Metal	Contamination	Plant	Type of article	Plant growth / nutrition	Metal uptake	Metal translo-cation	References
-	-	-	Endophyte community	Oth	Pb, Zn, Cd	poly (field soil)	*Arabis alpina*	Rs	+		-	Sharma 2019
-	-	-	Endophyte fungus	Oth	Cd	mono	*Lolium arundinaceum*	Rs	+	+	+	Ren 2011
Asco-	-	-	-	DSE	-	-	*Salix caprea*	Rv		-		Terhonen 2019
Asco-	-	-	-	DSE	-	-	-	Rv				Veragarame 2019
Asco-	Dothideo-	Botryosphaeriales	*Lasiodiplodia*	Oth	Cd, Pb	poly	*Brassica napus*	Rs	+	+	+	Deng 2014
Asco-	Dothideo-	Pleosporales	*Acrocalymma vagum*	DSE			*Tobacco*	Rs	+		-	Jin 2017
Asco-	Dothideo-	Pleosporales	*Acrocalymma vagum*	DSE	Cd	mono	*Medicago sativa*	Rs	+		+	Hou 2020
Asco-	Dothideo-	Pleosporales	*Acrocalymma vagum*	DSE	Cd	mono	*Ammopiptanthus mongolicus*	Rs	+		+	Hou 2020
Asco-	Dothideo-	Pleosporales	*Alternaria alternata*	Oth	Cd	mono	*Solanum nigrum*	Rs	+	-		Khan 2017a
Asco-	Dothideo-	Pleosporales	*Lewia sp.*	Oth	Pb	mono	*Festuca arundinacea*	Rs	+	+	**no effect**	Ortega-Aguilar 2020
Asco-	Dothideo-	Pleosporales	*Peyronellaea sp.*	Oth	Pb	mono	*Zea mays*	Rs	**+ with some strains**	**+ with some strains**	**+ with some strains**	Shen 2013
Asco-	Dothideo-	Pleosporales	*Peyronellaea sp.*	Oth	Zn	mono	*Zea mays*	Rs	+	**+ with some strains**	+	Shen 2013
Asco-	Dothideo-	Pleosporales	*Peyronellaea sp.*	Oth	Cd	mono	*Zea mays*	Rs	+	**+ with some strains**	**+ with some strains**	Shen 2013
Asco-	Eurotio-	Chaetothyriales	*Exophiala pisciphila*	DSE	Cd, Pb, Zn	mono	*Zea mays*	Rs			-	Li 2011
Asco-	Eurotio-	Chaetothyriales	*Exophiala pisciphila*	DSE	Cd	mono	*Zea mays*	Rs	+		-	He 2017

Asco-	Eurotio-	Chaetothyriales	*Phialophora mustea*	DSE	Cd	poly (field soil)	*Betula pendula*	Rs	+		no effect	Berthelot 2017
Asco-	Eurotio-	Chaetothyriales	*Phialophora mustea*	DSE	Cd	poly (field soil)	*Populus tremula x alba*	Rs	no effect		no effect	Berthelot 2017
Asco-	Eurotio-	Eurotiales	*Aspergillus flavus*	Oth	Cd, Ni	mono	-					Oyewole 2019
Asco-	Eurotio-	Eurotiales	*Aspergillus niger*	Oth	Cd, Ni	mono	-					Oyewole 2019
Asco-	Eurotio-	Eurotiales	*Paecilomyces formosus*	Oth	Al, Cd, Ni	poly (+ drought & heat)	*Glycine max*	Rs	+	-	-	Bilal 2020
Asco-	Eurotio-	Eurotiales	*Penicillium funiculosum*	Oth	Cu	mono	*Glycine max*	Rs	+	-	-	Khan&Lee 2013
Asco-	Eurotio-	Eurotiales	*Penicillium funiculosum*	Oth	Al, Cd, Ni	poly (+ drought & heat)	*Glycine max*	Rs	+	-	-	Bilal 2020
Asco-	Eurotio-	Eurotiales	*Penicillium janthinellum*	Oth	Cd	mono	*Solanum lycopersicum*	Rs	+	-	-	Khan 2014
Asco-	Eurotio-	Eurotiales	*Penicillium janthinellum*	Oth	Al	mono	*Solanum lycopersicum*	Rs	+	+	+	Khan 2015
Asco-	Eurotio-	Eurotiales	*Penicillium notatum*	Oth	Cd, Ni	mono	-					Oyewole 2019
Asco-	Eurotio-	Eurotiales	*Penicillium roqueforti*	Oth	Ni, Cd, Zn, Pb	poly (waste water contaminated soil)	*Solanum surattense or wheat? (contradiction in the article)*	Rs	+	-	-	Ikram 2018
Asco-	Eurotio-	Eurotiales	*Penicillium sp.*	Oth	Cd	mono	*Brassica napus*	Rs	+	-	+	Shi 2017
Asco-	Eurotio-	Eurotiales	*Penicillium sp.*	Oth	Pb	mono	*Brassica napus*	Rs	+	no effect	+	Shi 2017
Asco-	Eurotio-	Eurotiales	*Penicillium sp.*	Oth	Cd	poly	*Brassica napus*	Rs	+	no effect	+	Shi 2017
Asco-	Eurotio-	Eurotiales	*Penicillium sp.*	Oth	Pb	poly	*Brassica napus*	Rs	+	no effect	no effect	Shi 2017
Asco-	Leotio-	-	*Leptodontidium sp.*	DSE	Cd	poly (field soil)	*Betula pendula*	Rs	+		no effect	Berthelot 2017
Asco-	Leotio-	-	*Leptodontidium sp.*	DSE	Cd	poly (field soil)	*Populus tremula x alba*	Rs	no effect		no effect	Berthelot 2017
Asco-	Leotio-	Helotiales	*Rhizodermea veluwensis*	Oth	Cu, Ni, Zn, Cd, Pb	poly	*Clethra barbinervis*	Rs	+	-	-	Yamaji 2016
Asco-	Leotio-	Helotiales	*Cadophora sp.*	DSE	Cd	poly (field soil)	*Betula pendula*	Rs	no effect		no effect	Berthelot 2017
Asco-	Leotio-	Helotiales	*Cadophora sp.*	DSE	Cd	poly (field soil)	*Populus tremula x alba*	Rs	no effect		no effect	Berthelot 2017
Asco-	Leotio-	Helotiales	*Phialocephala fortinii*	DSE	-	-	*Clethra barbinervis*	Rv				Terhonen 2019
Asco-	Leotio-	Helotiales	*Phialocephala fortinii*	DSE	Cu, Ni, Zn, Cd, Pb	poly	*Clethra barbinervis*	Rs	+	-	-	Yamaji 2016

Asco-	Leotio-	Helotiales	*Phialophora / Cadophora complex*	DSE	Cd	mono	*Salix caprea*	Rs		-	Likar & Regvar 2013
Asco-	Leotio-	Helotiales	*Rhizodermea veluwensis*	AMF	-	-	*Clethra barbinervis*	Rv			Terhonen 2019
Asco-	Leotio-	Helotiales	*Rhizoscyphus sp. = Hyaloscypha = Meliniomyces*	Ericoid	Cu, Ni, Zn, Cd, Pb	poly	*Clethra barbinervis*	Rs	+	-	-	Yamaji 2016
Asco-	Leotio-	Helotiales	*Scytalidium lignicola*	DSE	Cd	mono	*Medicago sativa*	Rs	+		+	Hou 2020
Asco-	Leotio-	Helotiales	*Scytalidium lignicola*	DSE	Cd	mono	*Ammopiptanthus mongolicus*	Rs	+		+	Hou 2020
Asco-	Sordario	Diaporthales	*Phomopsis fukushii*	DSE	Cd	-	*Solanum nigrum*	Rv		+	+	Domka 2019
Asco-	Sordario-	Diaporthales	*Phomopsis fukushii*	Oth	Cd	mono	*Solanum nigrum*	Rs	+	+	+	Khan 2017b
Asco-	Sordario-	Glomerellales	*Glomerella truncata*	Oth	Cd	mono	*Solanum nigrum*	Rs	+	**+ (Cd 15 mg/kg) or - (Cd 5 mg/kg)**	+	Khan 2017b
Asco-	Sordario-	Hypocreales	*Fusarium oxysporum*	Oth	Cd	mono	*Wheat*	Rs		-		Rahimi Tamandegani & Zafari 2019
Asco-	Sordario-	Hypocreales	*Fusarium oxysporum*	Oth	Cd	mono	*Barley*	Rs	+			Mostafa 2019
Asco-	Sordario-	Hypocreales	*Fusarium sp.*	Oth	Cd	mono	*Brassica napus*	Rs	+	**no effect**	**no effect**	Shi 2017
Asco-	Sordario-	Hypocreales	*Fusarium sp.*	Oth	Pb	mono	*Brassica napus*	Rs	+	-	**no effect**	Shi 2017
Asco-	Sordario-	Hypocreales	*Fusarium sp.*	Oth	Pb, Cd	poly	*Brassica napus*	Rs	+	**no effect**	**no effect**	Shi 2017
Asco-	Sordario-	Hypocreales	*Neotyphodium*	Oth	Cd	mono	*Festuca arundinacea, Festuca pratensis*	Rs	+	+	+	Soleimani 2010
Asco-	Sordario-	Hypocreales	*Purpureocillium sp.*	Oth	Cu	mono	*Kandelia candel*	Rs	+	-	-	Gong 2017
Asco-	Sordario-	Hypocreales	*Trichoderma asperellum*	Oth	Zn, Cd, Pb, Fe	Poly	*Lactuca serriola*	Rs	**no effect**	**no effect**	**no effect**	Wazny 2018
Asco-	Sordario-	Hypocreales	*Trichoderma asperellum*	Oth	Cu	mono	*Onion*	Rs	+	-	-	Téllez-Vargas 2017
Asco-	Sordario-	Hypocreales	*Trichoderma asperellum*	Oth	Pb	poly	*Suaeda salsa*	Rs	+	-	-	Li 2019
Asco-	Sordario-	Hypocreales	*Trichoderma asperellum*	Oth	Pb, Cd	mono	*Arabidopsis thaliana*	Rs	+			Zhang 2018
Asco-	Sordario-	Hypocreales	*Trichoderma harzianum*	Oth	-	-	-	Book				Zaidi 2014
Asco-	Sordario-	Hypocreales	*Trichoderma harzianum*	Oth	Cd, Mn,	poly	*Salix fragilis*	Rs	+	**no effect**	+	Adams 2007

					Ni, Pb, Zn							
Asco-	Sordario-	Hypocreales	*Trichoderma logibrachiatum*	Oth	Pb	mono	*Helianthus annuus*	Rs				Devi 2017
Asco-	Sordario-	Hypocreales	*Trichoderma pseudokoningii*	Oth	Cd, Cr, Cu, Fe, Zn	poly	*Pennisetum glaucum*	Rs	+	+		Firdaus-e-Bareen 2012
Asco-	Sordario-	Sordariales	*Chaetomium cupreum*	Oth	Cu	mono	*Eucalyptus globosus*	Prod of IAA and siderophores	+	-		Ortiz 2019
Basidio-	Agarico-	Polyporales	*Trametes hirsuta*	Oth	Pb	mono	*Triticum aestivum*	Rs	+	+	+	Malik 2020
Basidio-	Microbotryo-	Sporidiobolales	*Rhodotorula sp.*	Yeast	Cd, Cu, Pb	poly	*Brassica napus, B. alboglabra, B. campestris ssp. Cinensis var. utilis*	Rs	**no effect**	+		Wang 2013
Basidio-	Microbotryo-	Sporidiobolales	*Rhodotorula sp.*	Yeast	Cd, Cu, Pb	poly	*Brassica campestris ssp. Cinensis var. communis*	Rs		+		Wang 2013
Mucoro-	-	-	*Mucor circinelloides*	Oth	Pb, Cd	mono	*Arabidopsis thaliana*	Rs	+			Zhang 2018
Mucoro-	-	-	*Mucor circinelloides, Mucor racemosus*	Oth	Pb, Cd	poly	*Brassica napus*	Rs	+	**no effect**	**no effect**	Zhu 2015
Mucoro-	-	-	*Mucor sp.*	Oth	Zn, Cd, Pb, Fe	poly	*Lactuca serriola*	Rs	+	+	+	Wazny 2018
Mucoro-	-	-	*Mucor sp.*	Oth	Zn, Fe	-	*Arabidopsis arenosa*	Rv		-	+	Rozpądek 2018
Mucoro-	-	-	*Mucor sp.*	Oth	Cd	-	*Arabidopsis arenosa*	Rs	+	**no effect**	+	Rozpądek 2018
Mucoro-	-	-	*Mucor sp.*	Oth	Zn, Cr, Co, Mn, Cu	poly	*Brassica campestris*	Rs	+	-		Zahoor 2017
Mucoro-	-	-	*Mucor sp.*	Oth	Zn, Fe	poly (field soil)	*Arabidopsis arenosa*	Rs	+	-	+	Rozpądek 2018
Mucoro-	-	-	*Mucor sp.*	Oth	Zn, Fe, Cd	poly (field soil)	*Arabidopsis arenosa*	Rs	+			Domka 2019

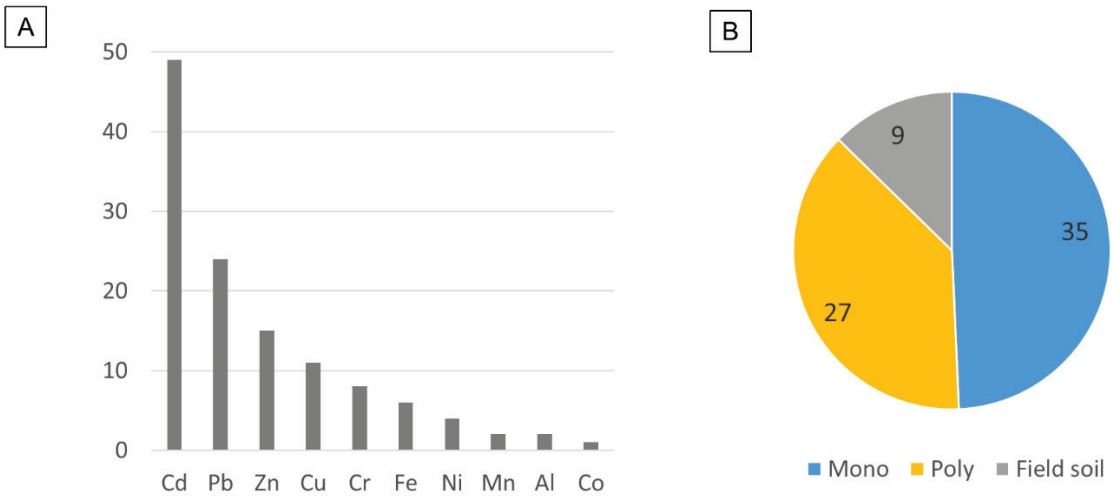

Figure 3.4: Research on fungal endophytes in relation to plant tolerance to MTE: which MTE and which protocol? *A: number of counts for each metal; B: Number of counts for each protocole of contamination (mono-, poly- contamination or field soil). Each count corresponds to a line of the* ***Table 3****, i.e. one fungus * one plant host genera * one response dynamic * one reference.*

Almost all tested endophytes led to better welfare of plants in the presence of metal trace elements **(Table 3.3, Table 3.4, Figure 3.5A)**. Plant metal tolerance may be associated with both metal accumulation in roots - metal uptake **(Figure 3.5B)** or shoots - metal translocation **(Figure 3.5C)** or with a reduction in the metal concentrations of plant parts (i.e., exclusion). This shows two strategies of metal tolerance: MTE avoidance and MTE storage, confirming the previously reported smaller inventory of Domka et al. (2019).

Table 3.4: Correlation between the effects of fungal endophytes metal uptake and translocation. *Green and bold writing: positive effect on plant growth, *: no effect on plant growth. Lines and columns: effects on metal translocation (lines) and uptake (columns). Green: positive-, red: negative-, grey: neutral effect.*

		Metal uptake			No information on metal uptake
		-	=	+	
Metal translocation	-	***Trichoderma asperellum*** ***Rhizodermea veluwensis*** ***Phialocephala fortinii*** ***Penicillium roqueforti*** ***Penicillium funiculosum*** ***Penicillium janthinellum*** ***Purpureocillium* sp.** ***Paecilomyces formosus***		***Piriformospora indica***	***Endophyte community*** ***Exophiala pisciphila*** *Phialocephala fortinii** *Phialophora/Cadophora complex** *Acrocalymma vagum**
	=	***Fusarium* sp.**	*Trichoderma asperellum** *Fusarium* sp.* ***Penicillium* sp.** *Peyronella* sp.*	***Lewia sp.***	***Phialophora mustea*** ***Leptodontidium* sp.** *Phialophora mustea* *Leptodontidium* sp. *Cadophora* sp.
	+	***Mucor* sp.** ***Penicillium* sp.** *Glomerella truncata**	*Mucor* sp.* ***Penicillium* sp.** *Peyronella* sp.*	***Lasodiplodia* sp.** *Phomopsis fukushi** *Endophyte fungus** ***Neotyphodium*** *sp.* ***Mucor* sp.** ***Peyronella* sp.** ***Penicillium janthinellum*** ***Glomerella truncata*** ***Trametes hirsuta***	***Acrocalymma vagum*** ***Scytalidium lignicola*** ***Trichoderma harzianum***
No information on metal translocation		***Alternaria alternata*** *Fusarium oxysporum** ***Chaetomium cupreum*** *Piriformospora indica** ***Mucor sp.***		*Aspergillus flavus** *Aspergillus niger** *Penicillium terreus** ***Aspergillus flavus*** ***Penicillium chrysogenum*** ***Trichoderma pseudokoningii*** *Rhodotorula* sp.*	*Aspergillus flavus** *Aspergillus niger** *Penicillium notatum** ***Exophiala pisciphila*** DSE* *Trichoderma harzianum** *Trichoderma logibrachiatum** ***Trichoderma asperellum*** ***Fusarium oxysporum*** ***Mucor circinelloides*** ***Mucor racemosus*** ***Mucor* sp.**

Metal uptake and metal translocation are not correlated **(Table 3.4)**: fungi may increase metal concentrations in roots but decrease metal translocation to shoots (like AMF, Miransari, 2011). Fungi may also decrease metal uptake but increase its translocation. Finally, metal uptake and translocation may both vary together, either increasing or decreasing in the presence of fungi. These effects depend on fungus/plant/metal identities, with the same fungal species having various effects according to its host plant and the contaminant. For example, *Trichoderma asperellum* does not have any effect on *Lactuca serriola* growth (Ważny et al., 2018), whereas it favours onion and *Suaeda salsa* growth in association with decreased metal uptake and translocation (Li et al., 2019; Téllez Vargas et al., 2017). Similarly, *Penicillium janthinellum* facilitates metal exclusion in *Solanum lycopersicum* in the presence of Cd (Khan et al., 2014) but increases metal accumulation in the presence of Al (Khan et al., 2015). Unfortunately, data are lacking concerning metal uptake and translocation induced by many fungal endophytes.

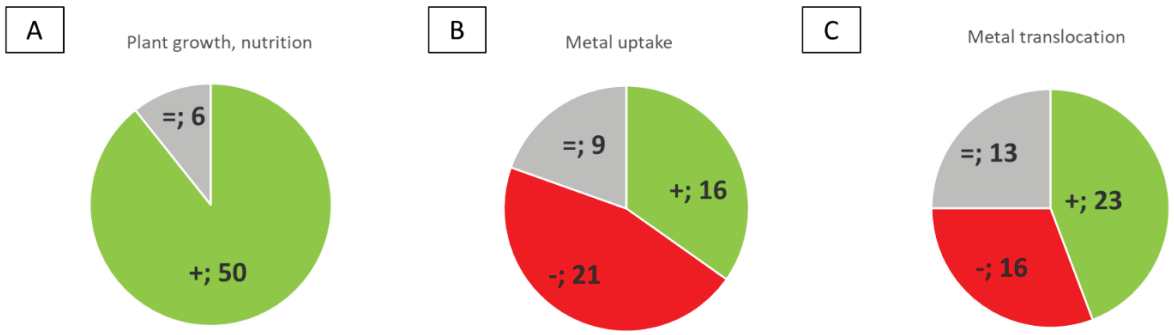

Figure 3.5: Fungal endophyte effects in the presence of metal on plant growth (A), metal uptake (B) and MTE translocation (C). **Green: positive effect, red: negative effect, grey: neutral effect.**

These strategies are not linked with taxonomy, and much variability in strategies is observed, including within a single species. We observed that some fungi, such as *Fusarium oxysporum*, commonly considered pathogens (Michielse and Rep, 2009; Poletto et al., 2020), may have positive effects on plant growth in the context of metal contamination (Mostafa et al., 2019). This fungus has also been shown to decrease some biomarkers of oxidative stress in the legume *Cicer arietinum* under Cd contamination, probably acting like a filter (Laib et al., 2020).

Considering plant taxonomy, we did not observe any common strategies for trees or members of the Poaceae. Even for a given plant species, different fungi may have different effects. Indeed,

under Cd/Pb polycontamination, *Brassica napus* is always stimulated by endophytes, but the metal uptake and translocation strategies differ; the fungal endophyte *Lasiodiplodia* sp. increases metal uptake and translocation (Deng et al., 2014), whereas *Fusarium* sp., *Mucor circinelloides* and *Mucor racemosus* have no effect on these parameters (Shi et al., 2017; Zhu et al., 2015). *Penicillium* sp. have no effect on metal uptake but increase Cd (but not Pb) translocation to shoots (Shi et al., 2017). For *Zea mays*, metal uptake and translocation may be increased by some strains of the endophyte *Peyronellaea* sp. (Shen et al., 2013), but metal translocation is decreased by *Exophiala pisciphila* (He et al., 2017; Li et al., 2011). Thus, in the presence of metal, the different fungi present in a given plant could have antagonistic effects.

Therefore, fungal endophytes have more than one way to improve metal tolerance in plants, and the different strategies are not linked to taxonomy. Different mechanisms of plant protection against MTE by fungal endophytes were reviewed in Domka et al. (2019). Here some of the highlights: endophytic fungi can accumulate high quantities of MTE in their mycelia (48.6 mg Cr/g dry fungal biomass, corresponding to 81% of the total Cr in media concentrated at 600 µg/mL) (Zahoor et al., 2017). The accumulation occurs through the production of metal chelating molecules such as glutathione (GSH), phytochelatins and metallothioneins (Domka et al., 2019b). These small molecules bind toxic metals and lead to their detoxification and storage in the vacuole. Endophytic fungi may also secrete chelating molecules in the rhizosphere, preventing metals from entering the root. These molecules include citrates, organic acids, siderophores, exopolysaccharides (EPSs), and phenolic compounds. Melanin, present in the fungal cell wall, is reported for its ability to bind metal ions. In contrast, fungi may improve plant metal accumulation by stimulating plant detoxification systems. Endophytes may indirectly improve plant growth in contaminated soil in other ways, such as the production of phytohormones or the mobilization of nutrients (Domka et al., 2019b). If the global metal content in roots or shoots is not an indicator of plant tolerance, it is possible that subcellular locations of metal ions would be more important for plant welfare. The DSE *Exophiala pisciphila* was shown to increase the subcellular compartmentalization of *Zea maize* in response to Cd and to engage in the remodelling of plant cell walls, correlating with an increase in Cd content (Shen et al., 2020).
Thus, endophytic fungi may immobilize MTE in the rhizosphere or within their mycelia. They may also favour plant (hyper)accumulation and storage and/or favour plant health independently of MTEs. All three strategies lead to better tolerance of host plants to MTEs.

Perspectives on the roles of endophytic fungi in plant metal tolerance

Ecology and evolution of plant-endophyte associations

This review, including major inventories of root fungal endophytes and their effect on plant metal tolerance, highlights the high taxonomic diversity of endophytes and their different effects on metal accumulation (uptake and translocation) in plants.

We referenced endophytes identified in plants growing in all types of environments and representing a large taxonomic diversity of Fungi. This suggests the convergent and redundant appearance of endophytism in different times and spaces during the co-evolution of plants and fungi. Present in plants without generating any symptom of disease, endophytes can shift their lifestyle, being latent saprotrophs or pathogens, temporary residents, mutualists or commensal (Suryanarayanan, 2013). Some endophytes can survive as decomposers on leaves after the death of plant tissues, suggesting that mutualism could derive from saprophytism (Suryanarayanan, 2013). This theory relates directly to the saprotrophism, symbiosis and pathogenesis continuum described by Veneault-Fourrey and Martin (2011) and the potential transition of some fungi from saprotrophism to the ectomycorrhizal lifestyle (e.g., brown-rot fungi). Using a phylogenetic approach, Delaye et al. (2013) showed that at least four changes occurred in fungi when shifting from endophytism to necrotrophism (fungi living in dead tissues) and at least four different shifts occur in the opposite direction. However, when shifts occurred towards biotrophic pathogenicity, no return towards endophytism occurred. Thus, pathogenicity is an evolutionarily stable trait, but endophytism is not (Delaye et al., 2013). According to paleobotany, endophytism (including all living fungi in unharmed plant tissues) dates as far back as 400 MYA in terrestrial plants with differentiated organs as well as in plants with prostrates (Krings et al., 2012). The major groups of Fungi were already diversified, and the structures involved in plant-fungus interactions were similar to those of today. The association of plants with fungi may have been a prerequisite for land colonization by plants.

This wide association between plants and fungi and the evolutionary convergence and redundancy of this association raises the question of the costs and benefits of the association for both partners. Leaf endophytes may protect plants against fungal pathogens, herbivory and abiotic stress but probably also interfere with photosynthesis (change the photosynthetic spectrum, consume photosynthetates, and use CO_2 for respiration) (Suryanarayanan, 2013). Under extreme resource limitation, because they utilize host photosynthates, endophytes are thought to be a cost for plants. Indeed, the leaf endophyte *Neotyphodium lolii* was shown in *Lolium perenne* to reduce

photosynthetic activity and the proportion of living shoots (Cheplick, 2007). However, the costs and benefits of root endophyte associations in plants as well as the costs and benefits from the point of view of the fungus are rarely discussed (but see Kusari et al. (2012)).

MTE avoidance or accumulation

In this review, we particularly explored the benefits of root endophytes in plant metal tolerance. Two main strategies of plant tolerance via fungi are observed: MTE avoidance and MTE accumulation.

We were not able to retrieve any fungal taxonomic patterns related to a given effect on plant metal tolerance: taxonomy does not seem to be a good predictor of the diversity of mechanisms of plant metal stress alleviation by endophytes. Furthermore, the same fungal species showed antagonistic effects on different plants, decreasing or increasing metal accumulation. This is the case for *Piriformospora indica*, which increases wheat but decreases maize metal uptake (Asilian et al., 2019; Shahabivand et al., 2012), or *Trichoderma asperellum*, which has no effect on *Lactuca serriola* metal uptake and translocation but shows a negative effect on onion and *Suaeda salsa* metal uptake and translocation (Li et al., 2019; Téllez Vargas et al., 2017; Ważny et al., 2018). It has been shown for AMF that fungal tolerance may vary individually, with local adaptation to metal contamination at the intraspecific level (Colpaert et al., 2004; Jourand et al., 2010; Vallino et al., 2011). Similarly, the endophyte *Peyronellaea* sp. displays various effects on metal uptake and translocation, depending on the tested strain (Shen et al., 2013). Thus, further studies should be developed to analyse the effects of each fungal species at the infraspecific level before integrating them at the fungal community level to better assess their effect on plant metal tolerance. Fundamental studies should further identify functional similarities between fungi that share the same strategies or the conditions that determine the balance between different strategies.

Mechanisms and evolution of metal tolerance in the plant-endophyte association

Cellular mechanisms of metal tolerance pre-exist (i.e., are present but not necessarily expressed), including in some plants growing in uncontaminated sites. These plants are able to grow when transferred to contaminated soil (Meyer et al., 2016). In *Arabidopsis halleri*, the plasma membrane pump HMA4 (HEAVY METAL ATPASE 4) involved in metal translocation and detoxification pre-existed before metal adaptation (Meyer et al., 2016). Metal tolerance may also be the exaptation of another trait: “the current function of a trait may not be that for which the trait originally

evolved (the latter being adaptation)" (Boyd, 2004). Indeed, uptake and translocation of toxic elements use the same mechanisms as those dedicated to the acquisition and transport of micronutrients (Tangahu et al., 2011). Metal tolerance implies that metal chelators (phytochelatins, metallothioneins, phenols, organic acids, etc.), as well as molecules limiting oxidative stress, such as alpha-tocopherol, scavenges ROS and lipid peroxides (Singh et al., 2016). Glutathione is involved in the production of phytochelatins and the reduction of oxidative stress (Zenk, 1996). Glutathione may be induced by the growth hormone salicylic acid, which is known for regulating many physiological processes, such as local and systemic plant-pathogen resistance and tolerance against abiotic stress (Singh et al., 2016). For both mycorrhizal and nonmycorrhizal fungi, similar mechanisms have been described. Fungi may immobilize metal ions in soil through the excretion of chelators, such as the glycoprotein glomalin from *Glomus* spp., and those on chitin-containing cell walls, which offer many binding sites to metals (Bellion et al., 2006; González-Guerrero et al., 2009). DSEs constitutively produce melanin in their cell walls, and melanin is an important antioxidant (Zhan et al., 2011). This pigment is shown to increase in the presence of Cd (Zhan et al., 2011) and Pb (Ban et al., 2012), suggesting exaptation. In the cytosol, glutathione and metallothioneins chelate metal ions, and efflux pumps are activated to transport metal ions out of the cell or into vacuoles. Finally, oxidative stress induced by metals is neutralized by the induction of superoxide dismutase and the production of antioxidant molecules (Bellion et al., 2006; González-Guerrero et al., 2009). Common molecules to both plant and fungal partners, such as polyphenols (Michalet et al., 2017; Pham et al., 2017), glutathiones, metallothioneins, and metal transporters (González-Guerrero et al., 2009; Zenk, 1996), suggest complex interactions between the two partners. In particular, plant defences against MTEs (glutathione, phytochelatins and metallothioneins) were reported to be either lowered when associated with an arbuscular fungus (Ferrol et al., 2016; González-Guerrero et al., 2009) or increased (Ferrol et al., 2016). These correlations between symbiotic fungi and plant defences may be either related to a direct production of defence molecules by fungi or an induction of plant gene expression and protein synthesis by fungi (Ferrol et al., 2016).

Basal (metabolic) oxidative stress may be amplified by environmental stresses such as salt, cold or drought stresses (Xiong et al., 2002). Thus, metal tolerance mechanisms could derive from plant adaptation to other abiotic stresses. In the same way, plant metal tolerance inherited from the endophyte association may also be an exaptation of the plant-endophyte association that could be seen as an extension of plant functional traits.

Conclusion

A wide variety of fungal endophytes are present in plant roots worldwide and in all ecosystems where they were investigated. They belong predominantly to Ascomycota, with some Mucoromycotina and Basidiomycota species. These fungi participate in plant tolerance to metal stress, improving plant growth and physiology. However, root fungal endophytes influence root metal uptake and root-to-shoot translocation inconsistently. Plant MTE tolerance via root endophytes may result from the beneficial interactions of this association compared to plant investment in their own defence systems and common defensive molecules. The mutualistic function of root fungal endophytes does not seem to be related to their taxonomy, since different association types are observed intraspecifically.

Research perspectives

Endophyte research is often oriented to applications in phytoremediation, agricultural yield improvement or metabolite production. Isolation of endophytes is therefore a purely technical step for many researchers, who thus do not provide information such as plant tissue provenance (e.g., Biswas et al., 2020). It would be of great value to share this information, which can be useful for more fundamental research (the previously cited article and others could not have been included in this inventory).

Current research mainly focuses on Cd, letting the effects of other anthropically emitted MTEs, such as Cr and Ni, go largely unexplored. Although approximately half of the studies combine several MTEs, we still do not have a clear understanding of the interactions between MTEs *in situ* (Påhlsson, 1989). Studies using contaminated field soil are encouraged. In amended soils, added metals will not be complexed as they would be *in situ*, leading to different availabilities for plants. Using field soil as a substrate would help to control the bias of metal availability and gain insights into interactions between MTEs. In addition, using field soil could help to determine the processes of microflora recruitment and thus the stability of plant-fungi associations.

One could find here potential applications for phytoremediation: as endophytes stimulate plant growth and, in some cases, phytoaccumulation of MTEs in aerial parts, we could argue that carefully chosen endophytes may be inoculated into polluted soils with particular plants to increase the degree of phytoextraction of metals (Berthelot et al., 2017; Deng and Cao, 2017). However, endophytes will be amended to existing communities, and though some studies have

been conducted on the competition between various fungi *in vitro* (Berthelot et al., 2019), we have little idea of their competitive abilities against endogenous communities *in situ*. Further studies should thus include soil collected from the field to disentangle those processes.

The competitive abilities of endophytes are determined not only by direct interactions between fungi but also by their interactions with the host plant and other microbial and biotic communities. Indeed, plants have evolved some mechanisms to distinguish pathogens from beneficial microbes through specific receptors, nutrient monitoring, damage sensing, and probably other ways that remain to be explored (Plett and Martin, 2018). Some authors hypothesized that under abiotic stresses, plants may recruit beneficial microbes through the modulation of root secondary metabolism, leading them to better cope with these stresses, i.e., the “plant call for support hypothesis” (Thijs et al. 2016). Future research should examine the mechanisms of the association between plants and fungi and their dynamics: is this association occurring randomly? To what extent do plants recruit fungi that are the most beneficial and how?

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75 Nontachaiyapoom 2010
76 Obase&Matsuda 2014
77 Orole&Adejumo 2011
78 Park 2012
79 Park 2012 - age
80 Park 2012 - varieties
81 Park 2017
82 Paul 2007
83 Pecoraro 2011
84 Pereira 2019
85 Pili 2016
86 Porras-Alfaro 2008
87 Porras-Alfaro 2014
88 Quilliam&Jones 2010
89 Russo 2016
90 Sadeghi 2019
91 Sánchez Márquez 2010
92 Sánchez Márquez 2012
93 Sati&Belwal 2005
94 Schoen 2018
95 Sharples 2000
96 Sikora 2008
97 Sridhar&Bärlocher 1992
98 Tan 2012
99 Tejesvi 2010
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101 Toju 2013
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103 Upson 2009
104 Vallino 2009
105 Vaz 2009
106 Vega 2010
107 Verma 2011
108 Vohník 2019
109 Wu 2012
110 Wu 2013
111 Xia 2011
112 Xing 2011
113 Xing&Guo 2011
114 Yamaji 2016
115 Yang 2018
116 Yeh&Kirschner 2019
117 You 2012
118 Yu 2015
119 Yuan 2009
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121 Zhang 2008
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123 Zhang 2014
124 Zijlstra 2005
125 Aban 2019
126 Chen 2020
127 Hosseyni Moghaddam 2020
128 Hou 2020
129 Li 2020
130 Lutfia 2020
131 Macía-Vicente 2020
132 Malik 2020
133 Parthibhan 2020
134 Rajagopal 2020
135 Ravuri&Shivakumar 2020
136 Salazar-Ramírez 2020
137 Singh 2020
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139 Tazik 2020
140 Teimoori-Boghsani 2020
141 Turbat 2020
142 Ulloa-Muñoz 2020
143 Wu 2020
144 Zhou 2020
145 Yang 2018a

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Synthèse

Cette revue bibliographique montre que les endophytes fongiques racinaires sont extrêmement variés : 1500 espèces dans 100 ordres (principalement chez les Hypocreales et les Pleosporales) ont été référencées, se répartissant sur l'ensemble de la phylogénie des Eumycètes. Ils sont ubiquistes, colonisant les milieux terrestres aussi bien qu'aquatiques, les forêts, prairies, déserts et montagnes, ainsi que les milieux contaminés aux ETM. Presque tous les endophytes testés se sont montrés bénéfiques à leur hôte en présence d'ETM, bien qu'aucun mécanisme universel de résistance aux ETM ne puisse être mis en évidence. Dans certaines associations, les endophytes favorisent le prélèvement des ETM et leur translocation vers les parties aériennes (ex : *Phomopsis fukushi* associé à la tomate), dans d'autres cas au contraire ils jouent plutôt le rôle de filtre en inhibant ces deux paramètres (ex : *Trichoderma asperellum* associé à l'oignon). Ces deux mécanismes ne sont cependant pas automatiquement couplés et certaines associations favorisent l'un en inhibant l'autre (*Piriformospora indica* avec le blé par exemple). Aucun lien n'a pu être montré entre le rôle des champignons dans l'une ou l'autre de ces stratégies et leur origine taxonomique, un même champignon pouvant avoir des effets différents sur le prélèvement et la translocation selon leur hôte ou les conditions environnementales (*Penicillium janthinellum* filtre le Cd mais permet l'accumulation d'Al chez *Solanum lycopersicum*).

En conclusion, cette première étude a permis de démontrer que les endophytes fongiques peuvent expliquer, au moins en partie, la tolérance de certaines plantes aux ETM. Est-ce le cas chez les Renouées asiatiques ?

Chapitre 4 : Effet des métaux sur les Renouées et les endophytes associés (Axe 2)

Avant de tester l'effet des endophytes sur la relation entre ETM et Renouées, il est nécessaire de bien comprendre quelle est cette relation, et comment sont affectés les plantes et les endophytes eux-mêmes par les ETM. Ainsi, nous commencerons par caractériser les effets des ETM sur les Renouées, puis caractériserons les endophytes fongiques présents dans les racines des Renouées et leur tolérance aux ETM.

Chapitre 4A : Tolérance des Renouées aux métaux

L'objectif de cet article est de tester l'effet de mono-contaminations aux ETM (Cd, Cr et Zn) sur les performances des Renouées asiatiques. Les performances sont mesurées par des traits de croissance (masses des différentes parties, hauteur) et des traits physiologiques (teneur en pigments foliaires). Ces mesures sont complétées par l'étude des variations de profils des composés phénoliques dans les parties racinaires.

L'expérimentation sous serre, la mesure des traits de croissance et l'obtention des extraits a été réalisée par Wilfried Chevallier lors de son stage de Master 2 – Biologie Végétale (UCBL). J'ai réalisé pour ma part toute l'analyse des données et rédigé l'article présenté ci-après.

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Responses of the species complex *Fallopia* × *bohemica* to single-metal contaminations to Cd, Cr or Zn: growth traits, metal accumulation and secondary metabolism

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Abstract

Plant responses to heavy metals and their storage constitutes a crucial step to understand the environmental impacts of Metallic Trace Elements (MTEs).

In controlled experiments, we previously demonstrated the tolerance and resilience of Japanese knotweed to soil artificial polymetallic contamination. Using the same experimental design, we tested here the effect of three individual MTEs on *Fallopia* × *bohemica* performance traits. Rhizome fragments from three different sites (considered as distinct morphotypes) were grown in a greenhouse for one month on a prairial soil artificially contaminated with either Cd, Cr (VI) or Zn at concentrations corresponding to relatively highly polluted soils.

Our results confirmed the high tolerance of Bohemian knotweed to metal stress, though, plant response to MTE pollution was dependant on MTE identity. Bohemian knotweed was stimulated by Cr (VI) (increased root and aerial masses), did not display any measurable change in performance traits under Cd at the high dose of 10 $mg.kg^{-1}$, and uptook all MTEs in its rhizome, but only Zn was transferred to its aerial parts. We also highlighted changes in root secondary metabolism that were more accentuated with Zn, including the increase of anthraquinone, stilbene and biphenyl derivatives. These results compared to multi-contamination experiments previously published suggest complex interactions between metals and plant, depending principally on metal identity and also suggest a potential role of soil microbes in the interactions.

Introduction

Because of the intensification of anthropogenic activities such as road traffic, mining, fertilisation or industrialisation, soils receive constantly higher concentrations of contaminants (through their atmospheric redeposition), leading to their accumulation over time (Bourennane et al., 2010; Senesil et al., 1999). A large part of these contaminants is represented by Metallic Trace Elements (MTEs), also referred to as Heavy Metals (HMs), which may reach sometimes very high concentrations in severely disturbed sites. Some of these MTEs do not have any known biological function and present toxicity for most organisms (*e.g.* cadmium-Cd, chromium (VI)-Cr (VI)), whereas some others are essential but may also become toxic when present in excess (*e.g*. zinc-Zn). In addition, MTEs are accumulated through the food chain and become dangerous for human health (Clemens and Ma, 2016; Clemens et al., 2013). Thus, high concentrations of MTEs in soils lead to major changes at the whole ecosystem level (Babich and Stotzky, 1985; Mahino et al., 2014; Peters et al., 2013).

In plants, as in most organisms, MTE toxicity is expressed by the alteration of enzyme structures and plasma membrane permeability, the disturbance of ionic homeostasis and an induction of oxidative stress (Miransari, 2011; Saleem et al., 2020; Yadav, 2010). For example, Cd, Cr and Zn were reported to inhibit plant growth, alter nutrient balance, induce chlorosis, and affects the germination process (Fattahi et al., 2019; Rasafi et al., 2016; Shahid et al., 2019; Shaikh et al., 2013; Yadav, 2010). Although these toxic effects concern most plant species, some of them, referred to as metallophytes, can grow on highly contaminated soils without showing any toxicity symptoms. Plant tolerance to these metal contaminated soils consists in two main strategies: excluders, which maintain a constant low concentration of MTEs in aerial parts; and accumulators, for which MTEs are transferred to aerial parts. In the latter ones, we can distinguish indicators, for which the concentration of MTEs in leaves is similar to that in soil; and hyperaccumulators, for which the concentration of metals in shoots is several order of magnitude higher than in soil (Baker, 1981; van der Ent et al., 2013; Ghosh and Singh, 2005; Goolsby and Mason, 2015; Lasat, 1999). This latter strategy implies the use of mechanisms involved in MTE transfer from soil to belowground parts and then to aerial parts, from uptake to sequestration (Yadav, 2010).

In this context, the Japanese knotweed species complex (*Fallopia* spp.) has been described to grow occasionally on metal contaminated soils and to accumulate MTEs, though variability in the type of MTE accumulated exists in the various reports from literature **(Table S1)**. *Fallopia japonica* (Houtt.) Ronse Decraene var. *japonica* (syn. *Reynoutria japonica*) and *F. sacchalinensis* (F. Schmidt ex Maxim.) Ronse Decraene (syn. *R. sacchalinensis*) of the Polygonaceae family originate both from Japan and extended all over Europe and in North America. Their interspecific hybrid *F.* × *bohemica* possesses higher invading capabilities than its parents (Bailey et al., 2009; Pyšek et al., 2003). Because of its ability to reproduce with its parents, it presents a high genotypic variability with different levels of ploidy (Bailey et al., 2009). Japanese knotweeds also expand through vegetative multiplication, rhizomes being able to develop to highly regenerative propagules growing into patches more or less developed. Japanese knotweeds and more specifically the hybrid *F.* × *bohemica* have been found to expand well in severely disturbed urban and industrial settings along roads and railways for example (Sołtysiak and Brej, 2012, 2014), where high concentrations of MTEs are present, leading to the hypothesis that these metal-rich soils could specifically promote the expansion of this plant.

Table 4A.S1: MTE concentration in soil and in* Fallopia *spp. organs from published field studies. CF: concentration factor = [Leaves]/[Soil]. *Table S1 includes all published field studies where MTE content were measured in* Fallopia *spp. and in the surrounding soil. In the studies describing several sites, only 3-4 were kept. J: Fallopia japonica, B: Fallopia x bohemica, S: Fallopia sachalinensis.*

Country	Study site	*Fallopia* species	Metal concentrations (mg/kg)												References
			Cd			Cr			Pb			Zn			
			Soil	*Leaves*	**CF**	*Soil*	*Leaves*	**CF**	*Soil*	*Leaves*	**CF**	*Soil*	*Leaves*	**CF**	
Japan	Ashio	*J*	6.1	6.6	**1.1**	-	-	**-**	-	-	**-**	466	247	**0.5**	Nishizono et al. 1989
	Bandai	*J*	96	12.2	**0.1**	-	-	**-**	-	-	**-**	9195	1614	**0.2**	
	Tama	*J*	2.4	2.1	**0.9**	-	-	**-**	-	-	**-**	112	57	**0.5**	
Poland/Czech Republic	Tramwajowa street (area 3)	*J*	0.28	6.69	**23.9**	8.6	1.2	**0.1**	15.1	5	**0.3**	54.5	60	**1.1**	Sołtysiak et al. 2011
	Dejvická street (area 4)	*J*	0.69	0.04	**0.1**	32.7	0.62	**0.0**	54.6	0.7	**0.0**	372.9	44.3	**0.1**	
	Paczkowska street (area 19)	*B*	2.08	2.22	**1.1**	59.8	1.4	**0.0**	110.8	3.84	**0.0**	2623	53.9	**0.0**	
	Holešovičkách street (area 25)	*B*	2.34	0.1	**0.0**	42.4	1	**0.0**	68.4	0.6	**0.0**	339.1	48.8	**0.1**	
USA	Liberty State Park (New Jersey)	*J*	-	-	**-**	43.4	0.4	**0.0**	-	-	**-**	96.3	98.3	**1.0**	Qian et al. 2012
Poland	Sosnowiek-Zagórze	*J*	6.7	5.3	**0.8**	9.7	1.3	**0.1**	158.1	9.7	**0.1**	501.2	541.7	**1.1**	Rahmonov et al. 2014
	Sosnowiek-Debowa Góra	*J*	4.2	1.1	**0.3**	15.1	1.8	**0.1**	120.0	4.5	**0.0**	401.2	64.7	**0.2**	
	Chorzóv	*J*	1.4	0.3	**0.2**	27.4	1.1	**0.0**	43.1	4.9	**0.1**	274.8	55.3	**0.2**	
Czech Republic	Prague and vicinities	B, J, S	0.9	5	**5.6**	-	-	**-**	57.2	4.3	**0.1**	-	-	**-**	Berchová-Bímová et al. 2014
Serbia	Borča	*B*	0.8	2.5	**3.1**	29.6	2.7	**0.1**	32.4	10.8	**0.3**	84.6	49.7	**0.6**	Hlavati Širka et al. 2016
	Refinery	*B*	0.8	1.3	**1.6**	22.8	1.4	**0.1**	35.3	7.4	**0.2**	107.7	35.7	**0.3**	
	Topčider	*B*	0.5	0.1	**0.2**	47.7	1.6	**0.0**	55.3	4	**0.1**	85.1	45.7	**0.5**	
Poland	Park Grabek (PGrab-1)	*J*	1.44	0.3	**0.2**	25.3	1.1	**0.0**	43.1	4.9	**0.1**	277.1	55.2	**0.2**	Rahmonov et al. 2019
	Parc Sielcki (PSiel-3)	*J*	0.73	0.2	**0.3**	24.7	1	**0.0**	20.7	1.1	**0.1**	92.1	40.5	**0.4**	
	Zielona Parc (PZiel-4)	J	0.21	2.2	**10.5**	25.6	1.5	**0.1**	12.3	2.1	**0.2**	51.4	75.8	**1.5**	

Similarly, in a previous study, we assessed the effect of soil multi-metal contamination on Japanese knotweed performance traits in controlled experiments in a greenhouse (Michalet et al., 2017). The results showed that after three months of culture in a soil artificially polluted with Cd, Cr, Pb and Zn at doses close to the authorized limits in sewage sludges, a delay in rhizome regeneration was observed, but only a slight decrease in aboveground and belowground organ masses was noted, whereas plant height was not affected compared to control plants growing in the non-contaminated soil. We thus concluded that this plant possesses high tolerance and resilience capacities toward polymetallic stress. Metal concentrations were also measured in plant parts after 3 months and showed that Japanese knotweed accumulated all tested MTEs. On the other hand, the concentration of Zn and Cr in shoots were close to that of soil level in contaminated soils, but for Cd the bioconcentration factor measured between shoots and soil levels reached values up to nearly 10 for some individuals. Japanese knotweed seems thus able to discriminate MTEs and to select which ones to accumulate.

Metal accumulation in plants is dependent on metal transporters like the root xylem loader HMA4, responsible of Zn hyperaccumulation, and Zn and Cd tolerance in *Arabidopsis halleri* (Krämer, 2010). Plant metal tolerance is ensured by other very diverse molecules, including peptides that directly bind metal ions (e.g. metallothioneins), hormones that stimulate plant growth and engender systemic responses to abiotic stresses, and low-molecular weight metabolites, which may chelate metal ions and scavenge free radicals generated by reactive oxygen species (ROS) (Singh et al., 2016). Those compounds are implicated in metal uptake, translocation and sequestration, as well as in the limitation of oxidative stress. *In vitro*, phenols have a high tendency to chelate MTEs thanks to their high number of hydroxyl and carboxyl groups (Kasprzak et al., 2015). For example, coumarin deficient plant mutants present Fe deficiency (Clemens, 2019), whereas flavonoid deficient mutants show higher sensitivity to Cd (Corso and Torre, 2020). Furthermore, MTEs were reported to induce phenols in plants (Corso and Torre, 2020; Singh et al., 2016). In *Fallopia* spp., a high quantity and diversity of secondary metabolites (procyanidins, flavonols, anthraquinones and stilbenes principally) are characterized in roots, particularly in the hybrid (Nawrot-Hadzik et al., 2018, 2019; Piola et al., 2013). After one month of growth under metal stress, the profiles of UV-absorbing compounds present in *Fallopia* root extracts are modified (Michalet et al., 2017). Some compounds like the anthraquinone derivative torosachrysone seemed to be overaccumulated over time.

Secondary metabolites are also strongly involved in plant biotic interactions, from negative ones - as inhibitors of neighbouring plant growth by allelopathic effects (Weir et al., 2004), or as antimicrobial protecting plants from pathogenicity (Chomel et al., 2016) for example-, to positive ones - as chemical signals involved in the establishment of mutualistic associations (Kawasaki et al., 2012). In the "plant call for support hypothesis", Thijs et al. assume that plants may recruit beneficial microbes under abiotic stress through the modulation of root secondary metabolism, which leads at the end to a better adaptation of plants toward abiotic stresses (Thijs et al., 2016). In addition, root secondary metabolites drive rhizosphere microbial communities and regulate also nutrient cycle (Bardon, 2014; Bardon et al., 2014; Michalet et al., 2013).

The aim of this study is thus to characterize the effects of individual MTEs (Cd, Cr, Zn) on *F.* × *bohemica* performance traits including rhizome regeneration, growth traits, changes in root secondary metabolism and MTE accumulation. We hypothesize that plant response to metal stress is dependent on MTE identity, and that root secondary metabolites will be affected by metals. In particular, we expect an increase of torosachrysone under metal stress, and more generally an increase in antioxidant metabolites.

For this purpose, we grew rhizome fragments of *F.* × *bohemica* originating from three different area located in the Grand Lyon metropolis during four weeks in a greenhouse on a prairial non-polluted soil that was artificially polluted or not with Cd, Cr or Zn at concentrations above the authorized limits in sewage sludge (Baize et al., 2007). The sites were chosen distant from at least 6 km between each other with contrasted environmental context, one being non-polluted, and two being localised on disturbed areas considered as polluted with one or more MTEs.

Material and methods

Plant collection

Rhizome fragments were obtained from individuals morphologically identified as *F.* × *bohemica* by F. Piola using leaf shape and size (Bailey et al., 2009) and collected from the field at three distinct sites localized in Grand Lyon metropolis: a non-disturbed zone close to woods in Miribel-Jonage (M, 45.8094°N; 4.9352°E, further referred to as "Miribel"), for which soil did not present any metallic contamination; close to a waste recycling centre with a soil contaminated with Cu (111 mg/kg) and Pb (167 mg/kg) in Caluire (C, 45.8032°N; 4.8638°E); and close to a petrol station and motorways in a soil contaminated with Zn (455 mg/kg) in Feyzin (F, 45.6787°N; 4.8466°E). Each individual coming from each of the three populations possesses distinct morphological traits **(Figure 4A.S1)**, so we will refer to them as "morphotypes". As morphology represents the first criteria for genotype distinction (Bailey et al., 2009), this suggests that genotypic diversity within *F.* × *bohemica* species complex is taken into account. Soil physico-chemical characteristics of each collecting site are presented on **Table 4A.S2**. Plant collection took place just before greenhouse experiments in February 2017.

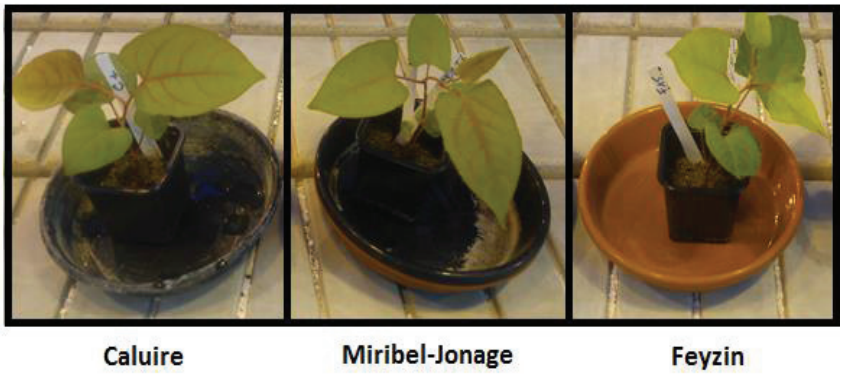


Figure 4A.S1: Pictures of young plants of each morphotype after 30 days of growth in control soil. *Caluire produces rather small plants with large and round leaves; in contrast Feyzin and Miribel produce smaller and more triangular leaves, but Miribel produces higher plants with higher aerial and belowground biomasses.*

Table 4A.S2: Physico-chemical characteristics of the soil used in this study and soils corresponding to each collection site. In bold: high MTE content.

Parameter	Prairial soil (used in greenhouse experiment)	Caluire	Feyzin	Miribel
Sand (%)	43.0	69.6	56.0	38.4
Silt (%)	43.3	26.4	19.6	43.6
Clay (%)	13.7	17.6	10.8	18.0
pH $_{H2O}$	5.76	7.73	8.02	8.00
C_{org} (g/kg)	27.5	59.9	22.5	18.1
OM (g/kg)	47.3	104.0	39.0	31.2
C:N ratio	10	ND	ND	ND
CEC ($cmol^{(+)}.kg^{-1}$)	10.50	13.00	7.93	8.95
Cd (mg/kg)	< 0.1	0.4	0.9	0.2
Cr (mg/kg)	23.4	49.0	34.7	59.9
Cu (mg/kg)	8.2	**111.0**	20.3	17.6
Ni (mg/kg)	13.7	25.7	19.3	31.7
Pb (mg/kg)	32.9	**167.0**	41.9	21.5
Zn (mg/kg)	42.9	188.0	**455.0**	64.3

Greenhouse experiment

A greenhouse experiment was conducted and occurred between February and March 2017. It followed the protocol published by Michalet et al. (2017), with modifications: a prairial soil (< 15 - 20 cm depth) collected at La Côte Saint-André (45.3769°N; 5.2772°E) and exempt of heavy metal pollution (soil physico-chemical characteristics are presented on **Table 4A.S2**), was sieved (2 mm mesh size). It was then rehydrated to its field capacity (40% m/v) by adding either Ultra-Pure water (UP) (control) or a solution containing either $CdCl_2$, CrO_2Cl_2 or $ZnCl_2$. The concentrations were chosen to theoretically achieve contamination in soil that were slightly over common recognized soil pollution threshold in sewage sludge (Baize et al., 2006) and the critical toxicity level (Krämer, 2010): 10, 200 and 400 mg/kg for Cd, Cr and Zn respectively. Then 100 ± 5 g of moistened soil were placed into 0.15 L square plastic pots and washed rhizome fragments containing one node (average fresh weight of 0.9 ± 0.05 g) of each of the 3 morphotypes of *F.* × *bohemica* collected were planted and grown in greenhouse (13 h day 22°C/11 h night 18°C) with light intensity of about 8-10 klux. Soil moisture was manually controlled by adding water every two-three days.

Water was added from the bottom of the pot (in the plate), so that field capacity was relatively maintained by capillarity throughout the whole experiment.

The experimental design was as follow: 3 morphotypes x 4 metal conditions with 7-9 plants (biological replicates) per condition except for the morphotype Miribel. For Miribel, we were not able to get enough budding rhizome (the patch was rather small compared to the 2 other morphotypes), this is the reason why only 4-7 plants (replicates) were used in each condition for this morphotype. Further a mislabelling of pots lead to an inversion between Cd and control treatment, which explains why replicates are not strictly homogeneously distributed between conditions for this morphotype.

Growth trait measures

After 40 days of growth, the photosynthetic height was measured with a calliper from soil surface to the maximal height of the last leaf. Foliar pigments (chlorophyll, flavonols and their ratio) were measured with a DUALEX SCIENTIFIC +TM (Force A) polyphenol and chlorophyllmeter, on the third leaf of each plant.

Then, plants were delicately uprooted, and the subterranean parts were washed in tap water. Roots were then cut from rhizome and a subpart of roots was immediately dropped into liquid nitrogen to fix secondary metabolism before lyophilisation. Root, rhizome and shoot fresh biomasses were measured, and the resting part of roots was dried at 65°C during 24h before weighing.

Extraction and analysis of compounds in root extracts

About 100 mg of freeze-dried fine roots were grounded (TissueLyserII® - Qiagen; F= 300/sec., 3 min.) and sonicated with a 1 mL mixture of MeOH-H_2O (1:1) for 15 minutes. After the supernatant was removed, the remaining powder was sonicated with 1 mL MeOH and both supernatants were pooled and concentrated under vacuum (Speedvac® – Labenco) before being stored at -20°C and dissolved at 10 mg/mL in MeOH 80% before analysis.

Samples were analysed by UHPLC-DAD ESI/QTOF (Agilent 1290 infinity linked with Agilent ESI/QTOF 6530, Agilent Technologies, USA), using a Poroshell® 120 EC-18 column (2.7 µm, 3.0 x 100 mm; Agilent Technologies, USA). The gradient was of 0.1% formic acid in water (A) and acetonitrile (B) as follows: 1% of B from 0 to 1.5 min, and growing with a linear gradient to 15% of B at 8 min; 60% of B at 14 minutes and 100% of B at 16 min for 1 min. All solvents were LC-MS

grade (Optima). The flow rate was adjusted at 1.0 mL/min and the injection volume was 2 µL. UV spectra were recorded between 190 and 600 nm. The ESI source was optimized as follows: positive and negative ionization modes, scan spectra from *m/z* 80 to 2000, capillary voltage 3.5 kV, fragmentor 100 V, fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 12 L/min and a temperature of 310°C at 40 psi.

Compounds were identified by analysis of their UV, HRMS and HRMS/MS spectra using MassHunter Qualitative Analysis (Agilent Technologies, USA) and by comparison with literature.

MTE in plants and soil

Sub-samples of fine roots and shoots were lyophilized and powdered, then 100 mg were digested at 125°C for 180 minutes in 4 mL of nitric acid (68%) and 1 mL H_2O_2 (35%) using a Heating digester (DK6, Velp Scientifica). Twenty-five mL of deionized water were then added before analysis by atomic absorption spectrometry with an acetylene flame atomizer (SpectrAA 220 Z; Varian, France). Cd, Cr and Zn contents were measured at a specific wavelength (228.8, 357.9 and 213.9 nm respectively). The HNO_3/H_2O_2 reagent was used as blank. Soil metal content was analysed at the CRPG of Vandoeuvre-les-Nancy. Briefly, after grinding and homogenization, soil samples were mineralized by HF, then Cr and Zn concentrations were measured by ICP-AES (NF ISO 220-36) and Cd concentrations by ICP-MS (NF EN ISO 17294-2).

Statistics

All statistical analyses were done with R x64 3.4.1 (R Core Team, 2017).

Vegetative traits and foliar metabolites

Plant height and aerial, rhizome and root biomasses were measured as vegetative traits. Foliar metabolites included chlorophyll, flavonols and NBI (Nitrogen Balance Index), was calculated as the ratio chlorophyll/flavonols. We computed ANOVAs type II (package car (Fox et al., 2019)), which are adapted to unbalanced data (2 to 7 repetitions/condition in our study). Morphotype, MTE and their interaction were used as explicative variables and trait measures as dependent variable. ANOVAs type II are valid only if they do not find interaction, which is the case in our study. Then ANOVA residuals were tested for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test).

To discriminate MTE effect, pairwise tests were computed within each morphotype for each trait. Pairwise tests were chosen according to the Shapiro-Wilk normality test and Bartlett test of homogeneity of variances on ANOVA residuals. When both normality and homoscedasticity were respected, then a Student pairwise test pooling variances was performed; if only homoscedasticity was not true, then a Student pairwise test without considering variances as equal was achieved; otherwise, pairwise comparisons using non-parametric Wilcoxon rank sum test were done. All pairwise tests were performed with Holm corrections of p-values. Foliar metabolites pairwise comparisons were done by using non-parametric tests.

Root secondary metabolites profiling

To evaluate differences in metabolite profiles between treatments, peaks in UV chromatograms recorded at λ 280 nm were integrated and aligned into a matrix to perform multivariate analyses. To eliminate contaminant incorporation in the analysis, we selected all the integrated peaks with peak area above 2 mAU in at least one condition, giving a total number of 121 peaks. In order to limit the variations due to differences in concentration between extracts, peak areas were expressed relatively to the sum of all integrated peaks for each sample.

A Principal Component Analysis (PCA) (package ade4 (Dray and Dufour, 2007)) was achieved using the scaled relative area of peaks detected in each sample as dependent variable, peak number as active element, and morphotypes and MTEs as illustrative elements. Three axes were kept. As the grouping of metabolites was better according to morphotypes than to MTEs, we performed a Partial Least Squares-Discriminant Analysis (PLS-DA) (package MixOmics (Lê Cao et al., 2017)) with MTEs as grouping factor.

We identified in priority compounds that explain the best the separation of samples according to their MTE treatment. Chosen metabolite are coloured on the PLS-DA and correspond to metabolites for which corrected variance is above a threshold of 0.8 (represented as a grey circle). For each morphotype and each compound, significant differences between the concentrations with and without MTE were tested using the same tests as for plant traits.

Constitutive production of root secondary metabolites in each morphotype was studied with a PCA inside each control treatment. PCAs were also performed within each morphotype with MTEs as illustrative data.

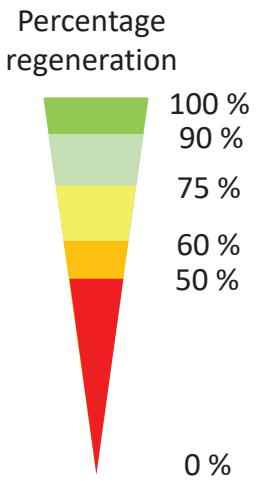
Results

Regeneration percentage

In the control treatment, where no metal contamination was achieved, all rhizomes of the three morphotypes regenerated **(Table 4A.1)**. However, in the presence of MTE, both Feyzin and Miribel morphotypes were affected (between 71% and 86% of regeneration with Cd, and between 88% and 100% with Cr, respectively), but this was especially true for Zn where, in average, only half of the rhizomes of these two morphotypes regenerated. In contrast, the morphotype Caluire was not affected by any MTE treatment, since all rhizomes regenerated in all conditions, as in the control treatment.

Table 4A.1: Percentage regeneration of* F. × bohemica *rhizomes exposed or not to MTE. *T: control, Cd: Cadmium, Cr: Chrome, Zn: Zinc. In brackets, number of regenerated rhizomes on the total number of rhizomes in the condition.*

		MTE			
		T	Cd	Cr	Zn
Morphotype	Caluire N=30	100 (7/7)	100 (7/7)	100 (7/7)	100 (9/9)
	Feyzin N=33	100 (7/7)	71 (7/9)	88 (7/8)	56 (5/9)
	Miribel N=22	100 (4/4)	86 (6/7)	100 (6/6)	40 (2/5)



Growth traits

ANOVA showed significant effects of morphotypes and MTEs on all tested traits (p-value < 0.05) including plant height and plant organ biomasses **(Figure 4A.1**, **Table 4A.S3)**. The effect of plant morphotype was higher than the one of metal pollution, thus pairwise tests were performed within morphotypes.

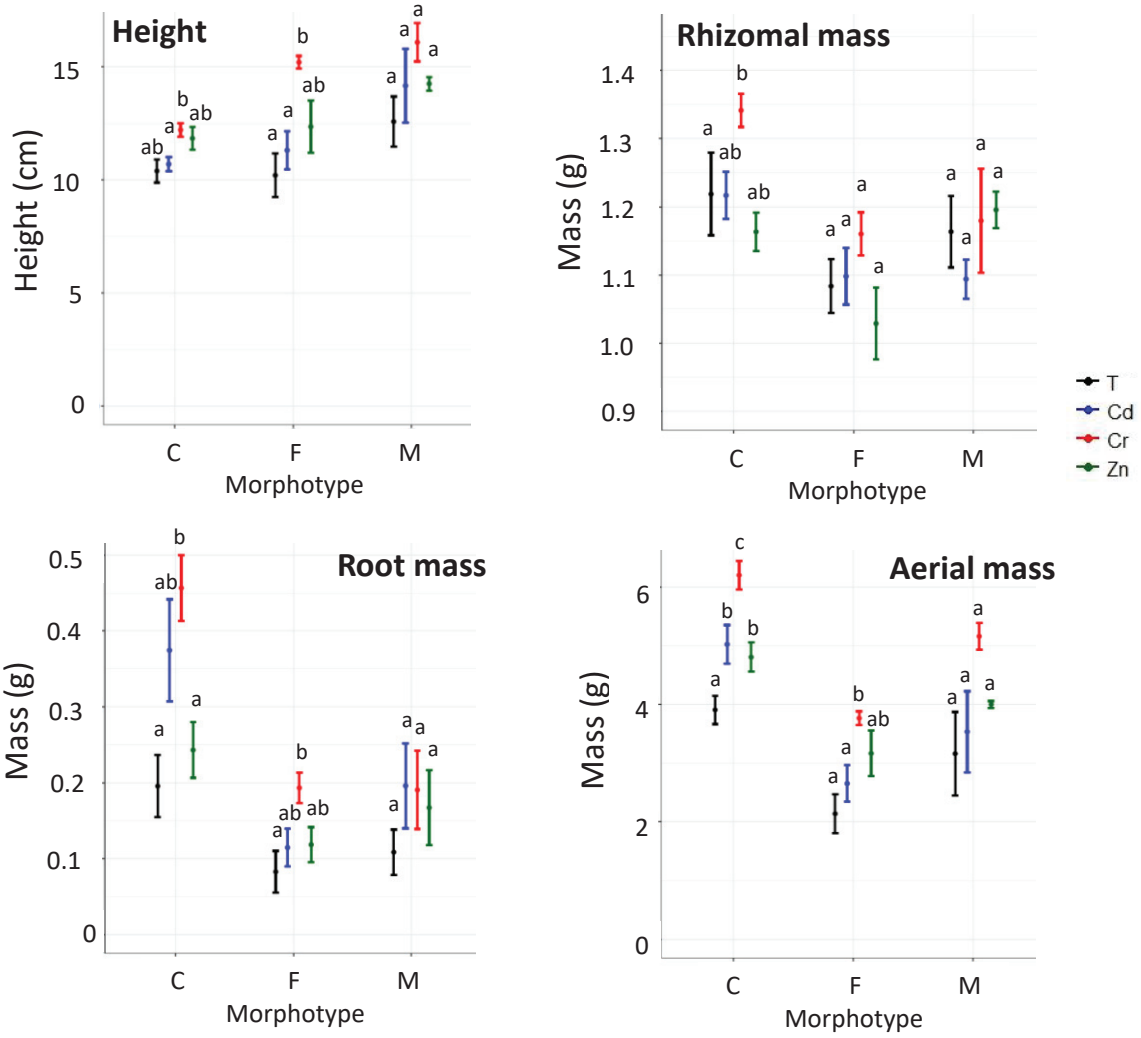


Figure 4A.1: Functional traits of Fallopia plantlets according to their MTE exposure. *Rhizomes from three morphotypes were grown 40 days in a greenhouse experiment, either without any MTE, or with one of the three chosen MTE. Mean ± SE. T: control, Cd: Cadmium, Cr: Chrome, Zn: Zinc. Morphotypes: C: Caluire, F: Feyzin, M: Miribel. N=71. Significant results are indicated with letters: within a morphotype, sharing a same letter indicates no significant difference.*

Table 4A.S3: ANOVA on functional traits. *ANalyses Of VAriances type II were performed for each vegetative trait, then validated or not by test of residual normality (Shapiro-Wilk) and homoscedasticity (Bartlett). Significant ANOVA in bold, statistically validated ANOVA in italic.*

P-values		**Functional trait**			
		Height	Root mass	Aerial mass	Rhizomal mass
ANOVA	**Morphotype**	**$1.5e^{-5}$**	**$1.3e^{-8}$**	**$9.1e^{-12}$**	**$5.9e^{-5}$**
	MTE	**$6.5e^{-6}$**	**$1.5e^{-4}$**	**$2.5e^{-8}$**	**$8.3e^{-3}$**
	Morphotype:MTE	0.43	0.20	0.80	0.6
Validity of ANOVA	**Residual normality**	$3.8e^{-3}$	*0.74*	$3.8e^{-3}$	*0.38*
	Residual homoscedasticity	$1.9e^{-3}$	$2.0e^{-3}$	*0.10*	*0.48*

Cd and Zn did not present significant effects on plant height, but Cr increased it significantly in Caluire and Feyzin morphotypes (+ 17 % and + 49 % respectively) **(Figure 4A.1)**. The effect of MTE was similar on root, rhizome and aerial biomasses: no significant effects of Cd and Zn, but with Cr, root biomasses of Caluire and Feyzin morphotypes were doubled, aerial biomasses were significantly increased (+59 % and + 76 % respectively) and rhizome biomasses also (+10 % and + 7 % respectively), but in the latter case this was only significant for Caluire morphotype. In all tested traits and for each MTE treatment, Miribel followed the same tendency as the two other morphotypes, though it did not show any significant effect compared to control treatment.

Foliar metabolites

Under Cr the foliar chlorophyll content of all morphotypes increased (+ 19 % in average), but this was significant only for Caluire and Feyzin morphotypes, whereas it decreased flavonol content especially for Caluire morphotype where it is significant (- 26 %) **(Figure 4A.2)**. No other MTE influenced foliar metabolites, except for Cd that showed a similar effect as Cr but less amplified for Caluire morphotype (+ 10 % of chlorophyll and - 18 % of flavonol) and to a lesser extent Zn, that only showed a significant decrease in flavonol for Caluire morphotype (- 18 %). The Nitrogen Balance Index (NBI), being calculated as the chlorophyll/flavonol ratio is thus increased under Cr for all morphotypes, but again this is only significant for Caluire morphotype (+ 61 %) and the effect of Cd follows the same tendency for this morphotype (+ 33 %), though, it is not significant.

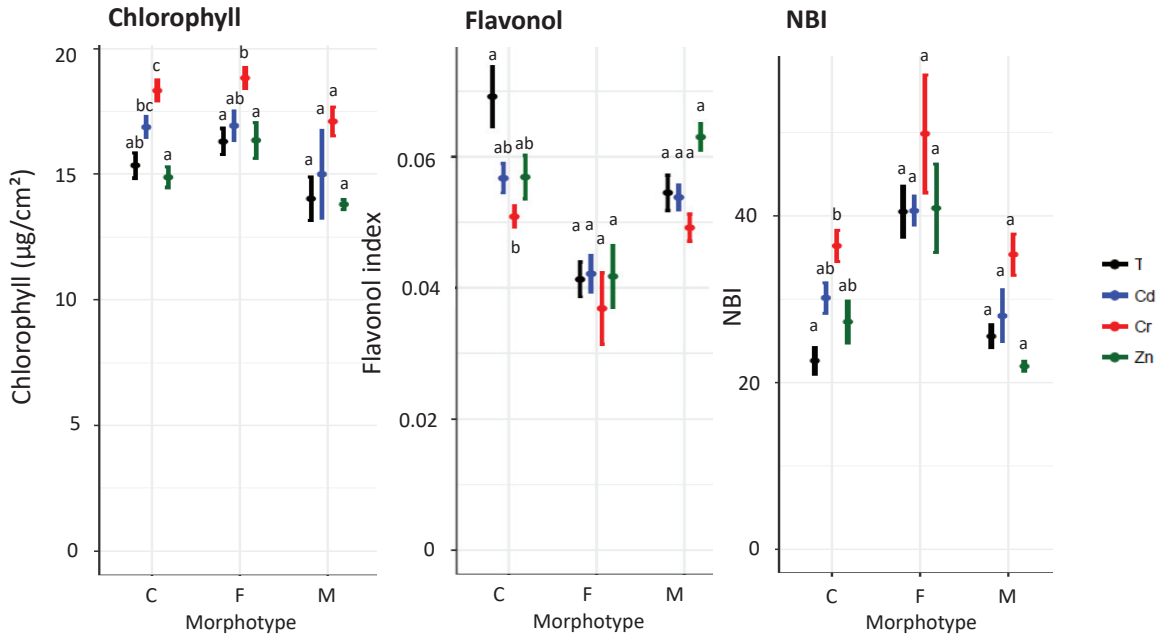


Figure 4A.2: Foliar metabolite concentration and nitrogen balance index (NBI) in response to MTE. *Rhizomes from three morphotypes were grown 40 days in a greenhouse experiment, either without any MTE, or with one of the three chosen MTEs. Foliar concentrations were measured on the third leaf of each plant. Mean ± SE. T: control, Cd: Cadmium, Cr: Chromium, Zn: Zinc. Morphotypes: C: Caluire, F: Feyzin, M: Miribel. N=71. *: 95 % confidence significant difference (pairwise t or Wilcoxon tests). Shared letters: no significant effect within the morphotype.*

MTE contents in plant parts

Very little MTEs were found in plant parts of the control treatment where no artificial contamination was achieved, and the majority of metal was concentrated in rhizome at levels close to that of soil **(Figure 4A.3 left)**. In plants exposed to metal contamination, all three MTEs were measured in rhizomes, with a concentration factor of 1 to 2 compared to soil level for Zn, 3 to 6 for Cr and 10 to 25 for Cd **(Figure 4A.3 right)**. Only Zn was accumulated in shoots, with a transfer factor of around 1 from rhizome to shoots. We observed a tendency of rhizomes from Feyzin to accumulate more Zn than other morphotypes and Miribel was the morphotype accumulating the lowest concentration of metals.

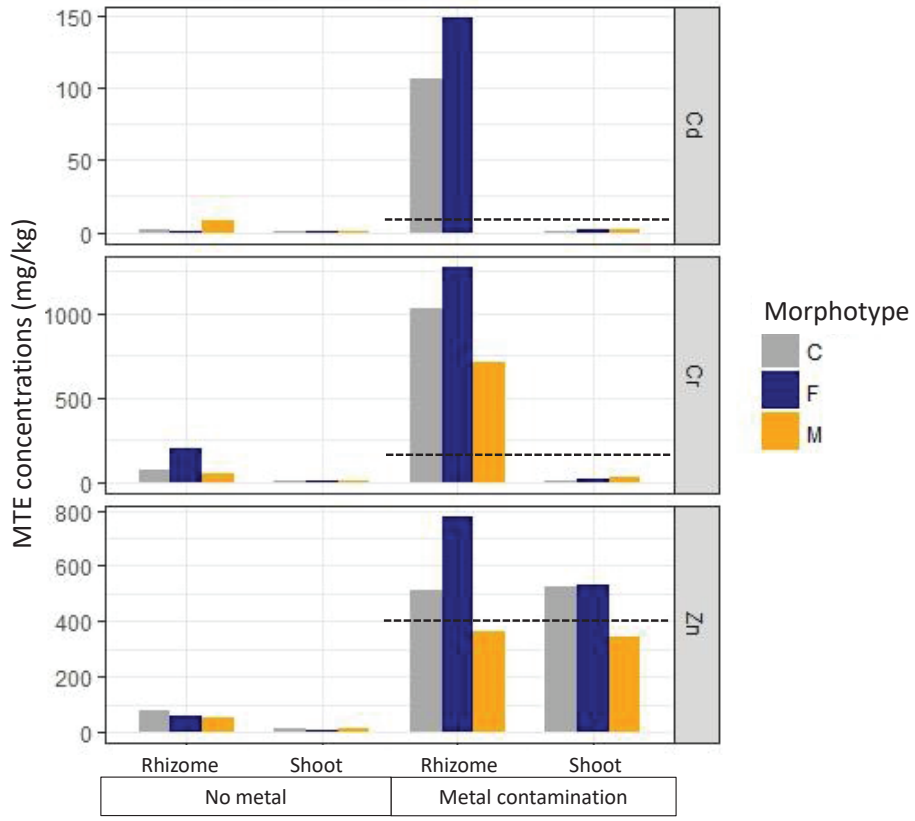


Figure 4A.3: MTE accumulation in plant parts. *Rhizomes from three morphotypes were grown 40 days in a greenhouse experiment, either without any MTE, or with one of the three chosen MTE. Cd: Cadmium, Cr: Chrome, Zn: Zinc. Morphotypes: C: Caluire, F: Feyzin, M: Miribel. MTE concentrations were measured on measured on pooled samples. Mean ± SE. Broken lines indicate soil artificial contamination. No data were available for Cd concentrations in rhizomes from Miribel.*

Root secondary metabolites

We detected 122 chromatographic peaks in *Fallopia* × *bohemica* root extracts. Root secondary metabolite profiles were highly dependent on morphotype identity **(Figure 4A.4A)**, and PCA did not allow to evidence any effect of MTEs **(Figure 4A.4B)**. As metabolite structure is stronger for morphotypes than for MTEs, it is essential to characterize the constitutive differences of each morphotype metabolism, before exploring MTE effects. A PCA on plants grown without MTEs (i.e. control treatment) was thus conducted **(Figure 4A.4C, D)**.

Feyzin morphotype possesses a distinct metabolism from Caluire and Miribel, visible on the second axis of the PCA; Miribel and Caluire separate along the third axis (**Figure 4A.4C**). Indeed, Feyzin roots are enriched in the compounds 1, 86, 89, 106 and 129 compared to Caluire and Miribel, but contain lower concentrations of the compounds 41, 44, 58, 62, 88, 97, and 108 **(Figure 4A.4D)**. Caluire is richer in compounds 13, 35, 42, and 45 compared to Miribel.

As morphotype structure may hide MTE effects on metabolites, we conducted a discriminant analysis intending to discriminate MTE treatments with the aim to identify metabolites that are the most affected in each case **(Figure 4A.5, Table 4A.2)**. The first axis separated Zn from Cd or control treatments, whereas the second axis discriminated Cr treatment. Cd and control treatments could not be distinguished **(Figure4A. 5A)**. The correlation circle **(Figure 4A.5B)** shows that MTE effect on root secondary metabolites is consistent according to their chemical family: Zn, and to a lesser extent Cr, favours anthraquinones and stilbenes (on the left) but decreases cinnamate derivatives (on the right). This could be further verified by looking at the individual variations of concentrations of discriminating compounds in MTE treatments compared to control **(Figure 4A.6)**: overall an increase in anthraquinone and stilbene concentrations were observed with Zn and Cr, whereas cinnamate and procyanidin derivatives relative concentrations decreased in these conditions. These effects were significant only for Caluire morphotype for most of the discriminating compounds.

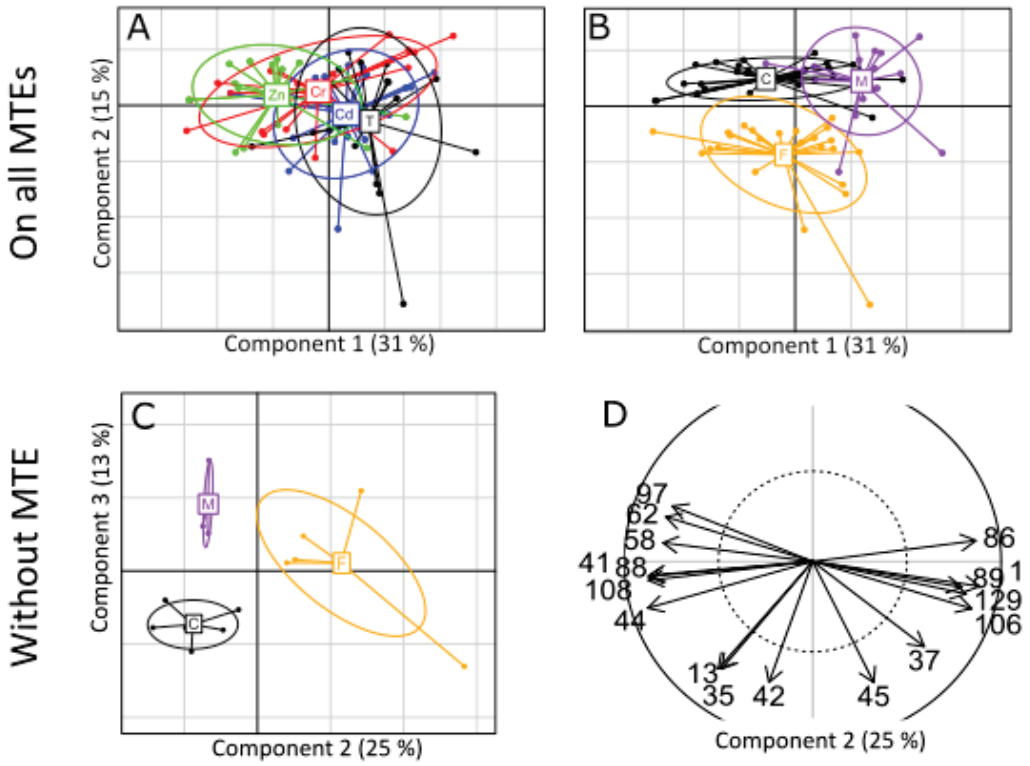


Figure 4A.4: Morphotypes and MTE separation according to secondary root metabolites: a non-discriminant analysis. *Principal Component Analysis (PCA) was performed with integrated UV peaks at 280 nm as explaining variable (121 chromatographic peaks) and their relative area (i.e. in percent of the sum of all integrated peaks detected in a given sample) in each sample as dependent variable; MTE (A) and morphotypes (B) are illustrative data. A, B: all MTE condition confounded; C, D: on T only. D: correlation circle corresponding to C. T: control without MTE; Cd: Cadmium, Cr: Chrome, Zn: Zinc. Morphotypes: C: Caluire, F: Feyzin, M: Miribel. Axes 2 and 3 of the PCA without MTE are displayed because the 1st axis do not separate morphotypes.*

In the presence of Cr, Miribel showed a distinct response compared to Caluire (or to a lesser extent Feyzin): some metabolites belonging to the procyanidin and cinnamate families were increased, sometimes significantly (compound 107 and 61, respectively a procyanidin trimer and a unknown cinnamate derivative), whereas these compounds were rather decreased under Cr in Caluire.

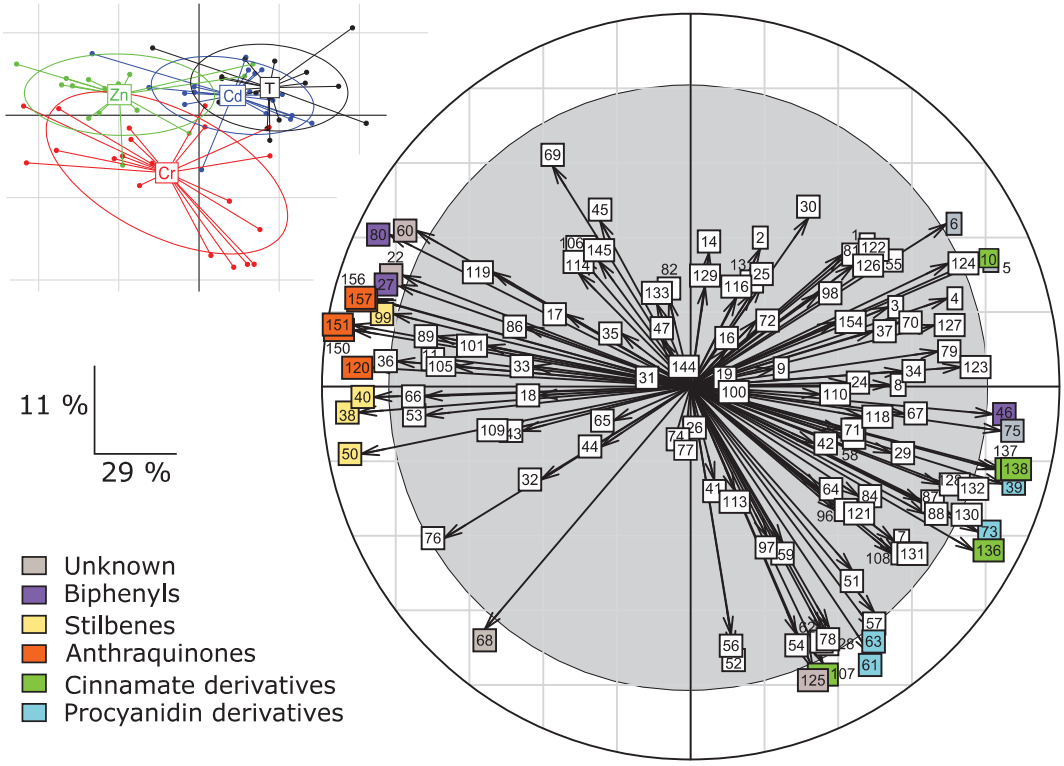


Figure 4A.5: Metabolite contribution to the separation of MTE groups: a discriminant analysis. *PLS-DA was performed on 121 integrated peaks, with MTE as grouping factor (Left). T: control, Cd: Cadmium, Cr: Chrome, Zn: Zinc. Morphotypes: C: Caluire, F: Feyzin, M: Miribel. N=71. Axis 1: 29 %, Axis 2: 11 %. Correlation circle (Right): inside the grey circle, compounds are considered as not significant. White colored peaks were not identified, whereas the identified peaks were colored according to their chemical families.*

Conversely some metabolites belonging to the stilbene and biphenyl families that were increased in Caluire under Cr treatment were decreased in Miribel (compounds 80 and 99, putatively identified as sulphated 6-methylbiphenyl-3,3',4,5'-tetraol and dihydropiceatannol hexose malonic acid respectively). These two latter metabolites are described for the first time in this plant and even as natural products. Anthraquinone derivatives bearing a hexose malonic acid moiety have already been described in this plant (Nawrot-Hadzik et al., 2019) though it is the first time to our knowledge that stilbene derivatives are described to be composed with this moiety. For biphenyl derivatives (compounds 27, 46 and 80), we were able to confirm their annotation since their MSMS spectra produced common MS^2 ions, corresponding to the loss of CO_2 (at *m/z* 231) and to a retro Diels-Adler seizure of ring A (at *m/z* 189) respectively, which are specific of this class of compounds (Nielsen and Smedsgaard, 2003). It is interesting to notice that desmethyl altenusin has already been identified to be produced both *in vitro* and *in planta* by an *Alternaria* sp. strain isolated from the roots of *Polygonum senegalense*, a plant species also belonging to the Polygonaceae family (Aly et al., 2008).

Table 4A.2: Identification of root metabolites. Metabolites to identify were chosen according to the PLS-DA (Figure 4A.6). *Nb: compound number; Ab: abundance; RT: retention time; Ref: reference; M: major compound; m: minor compound; Mm: medium compound.*

Nb	Ab	RT	UV (λ max)	m/z [M-H]-	MS/MS m/z (intensity %)	m/z [M+H]+	MS/MS m/z (intensity %)	Formula	Annotation	Chemical family	Ref
5	m	3.52	222, 266, 330sh	ND	ND	ND	ND	ND	ND	unknown	-
6	m	3.63	258, 298sh	ND	ND	ND	ND	ND	ND	unknown	-
10	M	4.31	228, 290sh,308	258.9932	135 (100), 179 (53), 136 (10)	261.004	163 (100), 145 (33), 135 (17), 117 (13)	$C_9H_8O_7S$	Sulfated hydroxy-cinnamate	Cinnamate derivative	1
22	m	5.49	232, 278	ND	ND	ND	ND	ND	ND	unknown	-
27	m	5.94	226, 278	437.11	231 (100), 189 (67), 275 (20)	439.1591	259 (23)	$C_{20}H_{22}O_{11}$	Desmethyl altenusin hexoside	Biphenyl	new
38	m	6.83	302sh, 318	405.1209	243 (100), 244 (18)	407.1312	245 (100), 135 (26), 246 (21)	$C_{20}H_{22}O_9$	Oxyresveratrol hexose = Piceatannol hexose	Stilbene	2-4
39	M	6.88	226, 276	729.1489	407 (100), 289 (44), 577 (39), 441 (38), 559(27), 451 (22), 125 (17), 169 (12)	731.1556	409 (100), 123 (84), 127 (76), 289 (68)…579 (23)	$C_{37}H_{30}O_{16}$	Procyanidin dimer monogallate	Procyanidin derivative	3-4
40	M	7.02	228, 288sh, 304, 314	ND	ND	391.1376	229 (100), 135 (22), 230 (19), 85 (16)	$C_{20}H_{22}O_8$	Resveratrol hexose = resveratroloside	Stilbene	2-4
46	m	7.55	224, 278	275.0558	189 (100), 121 (14), 165 (14), 145 (14), 231 (12)	277.0695	190 (100), 123 (85), 259(82), 235 (43), 84 (29) 217 (28), 147 (26)	$C_{14}H_{12}O_6$	Desmethyl altenusin	Biphenyl	5
50	M	7.91	226, 306, 316	389.1256	227 (100), 228 (18)	391.1366	229 (100), 85 (33), 230 (24), 135 (21)	$C_{20}H_{22}O_8$	Piceid	Stilbene	2-4
60	m	8.69	320, 356sh	317.0679	125 (100), 191 (64), 147 (16), 81 (14), 57 (12), 126 (10)	319.0794	259 (100), 151 (46), 191 (25), 260 (18), 127 (13)	$C_{16}H_{14}O_7$	ND	unknown	-
61	Mm	8.77	196, 220, 278	ND	ND	865.1881	289 (100), 577 (41), 247 (28), 427 (26), 127 (18), 409 (18), 139 (15)	$C_{45}H_{36}O_{18}$	Procyanidin trimer	Procyanidin derivative	4
63	m	8.93	226, 278	ND	ND	ND	ND	ND	Procyanidin derivative	Procyanidin derivative	-
68	m	9.21	ND	ND	ND	ND	ND	ND	ND	unknown	-
73	m	9.473	210, 226, 274	729.1489	407 (100), 729 (82), 577 (38), 441 (29), 559 (28), 169 (27), 289 (22), 125 (17)	731.1582	123 (100), 289 (82), 127 (56), 409 (40), 439 (38), 247 (37), 421 (33), 163 (27), 139 (19)	$C_{37}H_{30}O_{16}$	Procyanidin dimer monogallate	Procyanidin derivative	3-4
75	m	9.56	212, 224, 276	ND	ND	ND	ND	ND	ND	unknown	-
80	m	9.908	236, 266, 308, 340sh	311.0231	231 (100)	313.0396	315 (100), 84 (17), 215 (15), 164 (14), 136 (13), 271 (12), 156 (12), 186 (11)	$C_{13}H_{12}O_7S$	sulfated 6-methylbiphenyl-3,3',4,5'-tetraol	Biphenyl	new

99	M	11.07	236, 266, 312, 340sh	ND	ND	495.1476	247 (100), 229 (25), 248 (19), 271 (11), 127 (10)	$C_{23}H_{26}O_{12}$	Dihydro piceatannol hexose malonic acid	Stilbene	new
107	m	11.62	242, 300sh, 312	ND	ND	ND	ND	ND	Cinnamate derivative	Cinnamate derivative	-
120	M	12.33	230, 272, 318, 328, 392	287.0925	287 (100), 272 (85), 254 (62), 245 (35), 204 (33), 269 (29), 186 (23), 248 (18)	289.1074	247 (100), 271 (52), 243 (28), 243 (17), 205 (16)	$C_{16}H_{16}O_5$	Torosachrysone	Anthraquinone	6
125	m	12.39	ND	ND	ND	ND	ND	ND	ND	ND	-
136	m	13.14	252, 298sh, 316	ND	ND	ND	ND	ND	Cinnamate derivative	Cinnamate derivative	-
137	m	13.2	302sh, 314	ND	ND	ND	ND	ND	Cinnamate derivative	Cinnamate derivative	-
138	m	13.27	198, 208, 214, 298sh, 316	ND	ND	ND	ND	ND	Cinnamate derivative	Cinnamate derivative	-
150	Mm	14.53	195, 278, 330	509.1250	254 (100)	511.136	256 (100)	$C_{30}H_{22}O_8$	Emodin dianthrone	Anthraquinone	7
151	Mm	14.85	195, 278, 330	509.1250	254 (100)	511.135	256 (100)	$C_{30}H_{22}O_8$	Emodin dianthrone isomer	Anthraquinone	7
156	Mm	15.4	254, 278, 360	523.1412	254 (100)	525.1517	270 (100), 133 (41), 89 (36), 327 (27), 271 (18), 177 (11), 256 (11)	$C_{31}H_{24}O_8$	Fallopion	Anthraquinone	7
157	Mm	15.48	208, 278, 358	523.1412	254 (100)	525.1517	270 (100), 133 (41), 89 (36), 327 (27), 271 (18), 177 (11), 256 (11)	$C_{31}H_{24}O_8$	Fallopion isomer	Anthraquinone	7

1 Bugni et al. (2002)
2 Nawrot-Hadzik et al. (2018)
3 Fu et al. (2015)
4 Nawrot-Hadzik et al. (2019)
5 Aly et al. (2008)
6 Michalet et al. (2017)
7 Piola et al. (2013)

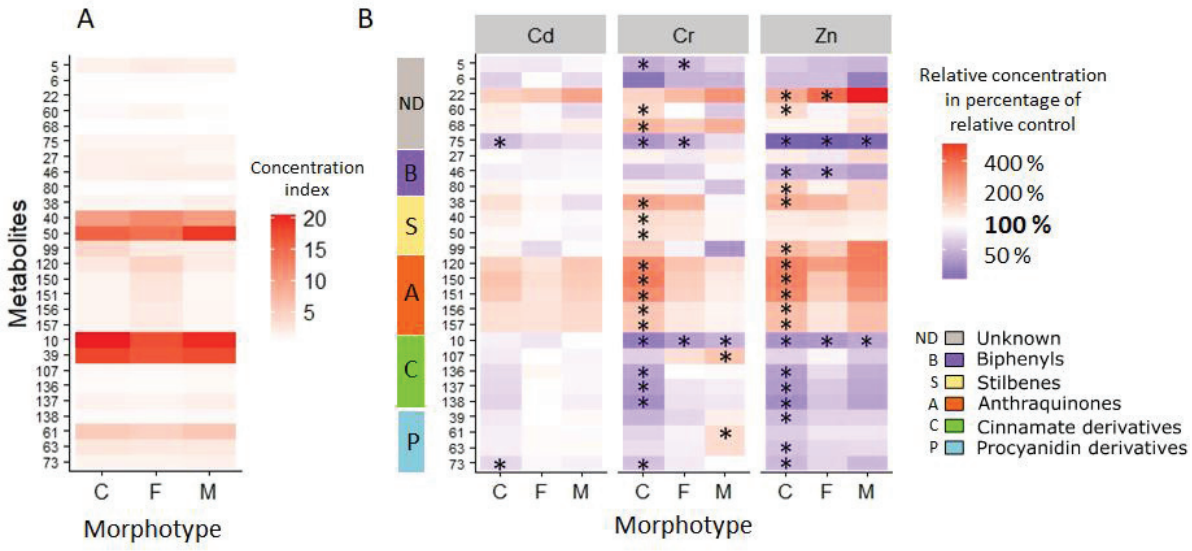


Figure 4A.6: Relative concentrations of secondary root metabolites. *Concentrations in the control of each morphotype (A). Concentrations in the presence of MTE, relative to the control of the concerned morphotype (B). T: control; Cd: Cadmium, Cr: Chrome, Zn: Zinc. Morphotypes: C: Caluire, F: Feyzin, M: Miribel. N=71. The metabolite 125 is not represented because of its very low concentration leading to disproportioned variations of concentration. *: significant difference with the control of the morphotype.*

In order to better assess the changes in metabolic patterns associated with MTE treatment between morphotypes, we conducted PCAs inside each morphotype with MTE as illustrative data **(Figure 4A.S2)**. Feyzin secondary metabolism is the less affected by MTE contamination: indeed, MTE treatments could not be separated by PCA. Conversely, Caluire and Miribel root metabolites are distinct among MTE treatments: T is separated from Zn and Cr along the first axis, and Zn is distinct from Cr along the second axis. The third axis distinguishes Cd from Cr in Miribel root metabolism. Some groups of metabolites are identifiable: the group 80, 120, 150, 151, 156, 157 comprises metabolites (a biphenyl and all identified dianthrones) previously identified as favoured by MTEs **(Table 4A.2, Figure 4A.6)**. In details, this group is increased by both Cr and Zn in Caluire morphotype, but only by Zn in Miribel morphotype **(Figure 4A.6** and **Figure 4A.S2)**.

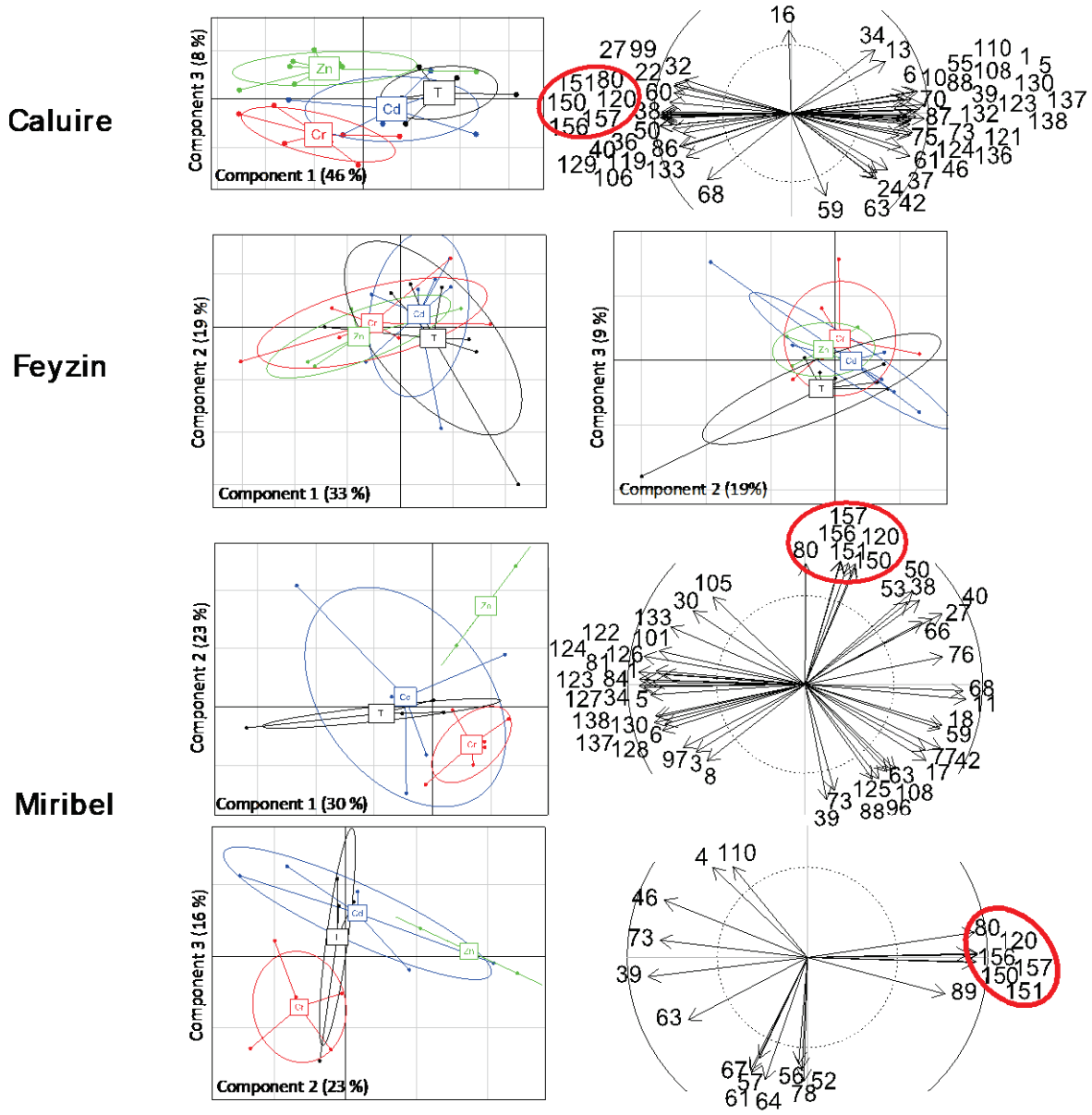


Figure 4A.S2: MTE separation according to secondary root metabolites within each morphotype: a non-discriminant analysis. *Principal Component Analysis (PCA) was performed with integrated UV peaks at 280 nm as explaining variable (121 chromatographic peaks) and their relative area (i.e. in percent of the sum of all integrated peaks detected in a given sample) in each sample as dependent variable; MTE (B) are illustrative data. T: control; Cd: Cadmium, Cr: Chrome, Zn: Zinc. Genotypes: C: Caluire, F: Feyzin, M: Miribel. Three components are displayed for Feyzin and Miribel; only the 1st and 3rd components are displayed for Caluire, as the 2nd component was supported by only one sample. Compound graphs are displayed for Caluire and Miribel, but not for Feyzin, MTE groups were not distinct on the PCA. A threshold of 0.8 was used to display compounds for Miribel axes 2 and 3 (because no compound was above a 0.9 threshold), 0.9 otherwise. Some groups of compounds are spotted by red circles, red and purple numbers. For readability, compound numbers have been moved (but not arrows).*

Discussion

In this study, we aimed at analysing the response of *F.* × *bohemica* exposed to single metal contaminations in terms of performance traits involved in early vegetative propagation including the measures of growth traits and leaf pigments, root metabolic profiles and accumulation of MTE in plants. For that, we chose three different sites located in the Grand Lyon metropolis where Bohemian knotweed naturally grows, and geographically distant from at least 6 km from each other with contrasted environmental contexts and metal pollution levels. These three different populations of individuals probably account for genetic diversity, though we were not able to discriminate two of them by using three chloroplastic markers (rpoC1, rbcL-accD and trnH-psbA) and one nuclear marker (ITS) (data not shown). Indeed, morphology is the first criteria of genotyping in this species (Bailey et al., 2009).

By the different analyses achieved in this greenhouse experiment, we confirmed the high tolerance of this plant to MTE contamination: all the three tested morphotypes were able to grow rather well on these toxic polluted soils, though the response was contrasted according to MTE treatments.

Under Cr exposure, the plants that grew were taller with higher root and aerial biomasses, illustrating a growth stimulating effect of Cr on this plant. This is in contradiction with what was described for most plant species since Cr (VI) is known to be very toxic and to cause inhibition of plant growth, inhibition of photosynthesis and chlorophyll biosynthesis, root injury and alteration of the germination process leading to lower biomasses yields as reviewed in Shanker et al. (2005), Sinha et al. (2018) or in Yadav (2010). On the other hand, preliminary experiments achieved in the same conditions and using Cr (III) (as chloride salt, $CrCl_3$) instead of Cr (VI), did not allow to reproduce the stimulating effects observed in this experiment (data not shown). From these observations, we might hypothesize that the oxidant effect of Cr (VI) might be the primary cause of the plant growth stimulation observed, rather than the effect of Cr itself. However, contrary to Cr (III), Cr (VI) uptake is active through carriers of essential anions (Cervantes et al., 2001; Sinha et al., 2018), which leads to the hypothesis that some specific mechanisms may occur in this plant in the presence of Cr (VI) and not with that of Cr (III). This needs other experiments to be further verified. Our experiment shows a short-term induction of plant traits by Cr (VI), and needs to be

confirmed over time for generalization. The influence of rhizosphere microbes in this process of growth stimulation is also to be determined since for example some soil pseudomonads were shown to alleviate Cr-stress in common wheat and even to stimulate growth in some cases (Hasnain and Sabri, 1997).

In our study, we showed that chlorophyll increases under Cr exposure whereas leaf flavonol decrease. Thus NBI, which is a proxy of nitrogen relief (Peteinatos et al., 2016), increases in this condition, which corresponds to an apparent alleviation of plant nitrogen-stress. This is quite surprising since small concentrations of Cr (VI) inhibit plant enzymes involved in nitrogen metabolism chlorophyll biosynthesis (Shanker et al., 2005; Sinha et al., 2018). The release of nitrogen-stress may be due to the facilitation of N uptake by roots, through the release of secondary metabolites in the soil. Indeed, some allelopathic molecules produced by this plant, like procyanidins, were shown to specifically inhibit bacterial denitrification enzymes (Bardon et al., 2014, 2017), thus leading to an enhanced pool of nitrate available for the plant. This is consistent with the fact that procyanidins were found to decrease in roots exposed to Cr (but also to Zn, **Figure 4A.6**), especially in the Caluire morphotype. However, because of a better nitrogen nutrition, we would have expected an increase in N-rich chlorophyll (indeed observed) but not necessarily a decrease in leaf flavonols (only significant in Caluire). Further investigations are under way to clarify this phenomenon.

In the case of Cd treatment, we were not able to evidence any difference between plant that grew with 10 $mg.kg^{-1}$ of Cd from that growing in the control non-polluted soil, and this was true for nearly every parameter measured in this study. This very high concentration, corresponding to 5 times the authorised limit for sewage sludges, which is only retrieved in very disturbed industrial settings such as mining sites, was chosen because preliminary experiments achieved with 5 $mg.kg^{-1}$ did not allow to produce any significant effect compared to control (data not shown). This is the reason why we doubled the concentration, though the results did not change. We thus demonstrate here the very high tolerance of this plant to Cd toxicity and further experiments should be needed to evaluate the highest concentration of Cd this plant could tolerate. The fact that Bohemian knotweed was able to accumulate Cd in roots at levels 10 to 25 times that of soil is a good indicator of its very high tolerance potential. Feyzin morphotype, originating from the most Zn-contaminated site, is the morphotype accumulating the highest concentration of Zn in its rhizomes, suggesting that this accumulation could be linked with local adaptation and/or maternal effects.

The exposure to Zn gave overall a similar response to that observed with Cr, though the amplitude was less pronounced, leading to non-significant changes in most cases. One major changing parameter that was only observed with Zn, is that only Zn was transferred to plant aerial parts and not the other metals which were detected in belowground parts only. This is quite surprising since our previous study using a polymetallic stress with similar metal concentrations showed that all these three MTEs were transferred to aerial parts at levels close to that of soil (Michalet et al., 2017). Thus, in the presence of relatively high levels of Zn, close to the authorised limit in soil, Bohemian knotweed seems to be able to translocate Cd and Cr in its aerial parts but not when these two metals are present individually. This suggests that Zn would activate metal transporters, which would then unspecifically transport other cations. Plants did not evolve specific mechanisms to transport non-essential elements. It is known that Zn and Cd tolerance and accumulation in plants share common genetic mechanisms (Verbruggen et al., 2009, 2013) and this could be easily explained since both are divalent metallic cations of the same group. For example, 97 % of the Cd root-to-shoot translocation of *Arabidopsis thaliana* occurs through Zn-transporters HMA2 and HMA4 (Wong and Cobbett, 2009), and transporter ZNT1 displays a high-affinity for Zn with a low affinity for Cd (Pence et al., 2000). The co-accumulation of Zn, Cd and Cr with Cd and Cr translocation being activated by Zn is also consistent with previous positive correlations between Zn and Cd accumulations (and to a lesser extent Cr and other MTEs) in water spinach (He et al., 2015).

Root metabolites, and especially phenolics that were more particularly targeted in our study, may have direct effects on metal contamination through their antioxidant and/or chelating properties, but they may also have an indirect effect by favouring the recruitment of beneficial microorganisms as stated in the “plant call for support” theory (Thijs et al., 2016). In our previous multi-contamination experiment we found torosachrysone, an anthraquinone derivative, to be the most overproduced metabolite in roots of *Fallopia* spp. exposed to Cd, Cr, Pb and Zn (Michalet et al., 2017). In this study, torosachrysone (compound 120) was also one of the most upregulated compounds in roots when exposed to Cr or to Zn. Torosachrysone was isolated from plants (Kitanaka and Takido, 1984) and fungi (Gill and Steglich, 1987; Gill et al., 2000), and is a rather original metabolite found in the roots of *Fallopia*. In addition, in this study we tentatively identified some biphenyl derivatives that were influenced by metal contamination and that are described for the first time in this plant, namely desmethyl altenusin (compound 46), its hexoside derivative (compound 27), and its decarboxylated and sulphated derivative (compound 80). Although

belonging to the same chemical group, their variations in roots according to metal stress is contrasted: whereas desmethyl altenusin concentration decreased under Cr or Zn exposure, the concentration of its decarboxylated and sulphated derivative increased, while the relative concentration of the hexoside remained stable in all treatments. Biphenyl derivatives are metabolites that are described in fungi, and the metabolites we putatively identified were shown to be produced by endophytic fungi in pure culture (Aly et al., 2008; Yuan et al., 2018a). Since this plant have been described to be colonised by non-mycorrhizal fungal endophytes such as Dark Septate Endophytes (DSE) (Gucwa-Przepióra et al., 2016) which are known to possess metal tolerance capacities (Berthelot et al., 2016), their influence on plant response to metal stress should then be further examined.

Although the effects of MTE were coherent between morphotypes with similar tendencies observed for most of the parameters studied, the amplitude of response clearly differed between them. Indeed, Caluire morphotype showed the best abilities to cope with metal stress (no negative effects on regeneration) and its growth was stimulated in some cases, especially with Cr, whereas Miribel morphotype gave the lowest amplitude of response leading to non-significant changes in most conditions and regeneration was affected, Feyzin ranging between these two morphotypes. None of the metabolites that were identified as constitutively high in each morphotype were also retrieved as implicated in plant response to MTE. Thus, the constitutive metabolism specific to each morphotype does not seem to be responsible for the differences in the observed plant metal tolerance.

Root metabolic profiles are different and respond differently to MTEs according to morphotypes. From this, it is difficult to state whether genetic or native environment is the most responsible for this apparent difference in the amplitude of response between morphotypes. One clue brought by our study is that the three chosen sites differ strongly in terms of soil type and metal pollution nature and levels: Caluire being polluted with Cu and Pb, Feyzin with Zn, and Miribel non-polluted or moderately with Cr. Plants collected in the most polluted sites were then those that show the highest amplitude of response with no toxic effects whereas in the case of plants collected from a non-polluted area the lowest intensity of response was observed, a lesser amount of metal was accumulated and regeneration was affected. We thus conclude that *F.* × *bohemica* metal tolerance is not a totally constitutive trait (as plants native from contaminated sites grow better than other

plants when re-exposed to MTE), and that native environment may influence plant response to metal stress. We consider three ways by which the environment could influence plant metal tolerance: (1) microadaptation, (2) phenotypic state of the native rhizome, and (3) native symbionts. Microadaptation is proposed to explain the high intraspecific variation in metal tolerance or hyperaccumulation in *Thlaspi caerulens* and *Arabidopsis halleri* (Corso et al., 2018; Verbruggen et al., 2009): this is an independent adaptation to local conditions through modifications of transcription and/or traduction, enabling to maintain stable MTE concentrations in the cytosol and sensible organites (Krämer, 2018). In this case, the environment would have an effect on plant metal tolerance through epi-genetic changes. The two other considerations assume that taking a rhizome is not only taking its genome, but also its chemical content (2) and/or its biotical content (3). Though we could discuss the term of "offspring" for a clonal plant, those considerations belong to the "maternal effect", that is "nongenetic influences of maternal phenotype or environment on offspring phenotypes" (Fox et al., 1997). Chemical content might include growth phytohormones, metal transporters or chelators, whereas the biotical content could encompass bacteria (Hasnain and Sabri, 1997), mycorrhizal (Hashem et al., 2019; Rasouli-Sadaghiani et al., 2019) and non-mycorrhizal fungi (Domka et al., 2019b). In particular, the rhizome from metal-contaminated soil could contain beneficial endophytes recruited by the maternal plant to cope with metal stress (Thijs et al., 2016), and then be transmitted vertically to the "offspring". Further studies are under way to disentangle some of those processes.

Conclusion

This study brings some new elements about the response of *F.* × *bohemica* species complex to the exposure to single MTE contamination, including the identification of root metabolites that might play a role in the mediation of the response. In the context of *Fallopia* development in urban and contaminated areas, we confirmed the high tolerance of this plant to MTE exposure: although its regeneration from rhizome was affected for some morphotypes, it was stimulated by the presence of Cr (VI) and that of Zn to a lesser extent, whereas high Cd pollution levels did not produce any measurable effects. The plant uptook Cd, Cr and Zn in its belowground parts, but only Zn was found in shoots. We identified root metabolites specifically increased in the presence of Zn and Cr, particularly from the stilbene and the anthraquinone families, whereas cinnamate derivatives tended to decrease. These molecules could reflect plant root interactions with rhizosphere microbes, and their production and role should be explored in future research.

We also observed clear differences in the amplitude of response between morphotypes, the one originating from the most polluted area showing the highest amplitude of response, whereas the one originating from a non-polluted area showed the lowest. Native environment may thus also influence plant response, and this might be mediated through soil microbes, highlighting here again the interest of analysing plant-microbes' interactions under metal exposure.

Declarations

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Author Contributions

FP and SM designed the project, WC conducted the experiments, PB and MLT conducted MTE analysis. LB analysed the data and wrote the article under the supervision of FP and SM.

Synthèse

Cette étude a confirmé la tolérance des Renouées aux ETM : les biomasses aériennes et racinaires n'ont été affectées ni par Cd à une dose 5 fois supérieure (10 mg/kg) à son seuil de toxicité admis, ni par Zn (400 mg/kg). En revanche, les biomasses des Renouées ont été augmentées par l'ajout de Cr(VI) (200 mg/kg). En mono-contamination, seul Zn est transféré vers les parties aériennes, à des concentrations de l'ordre de celle du sol.

Parallèlement, certains métabolites secondaires, notamment des anthraquinones et des stilbènes, sont présents en plus forte quantité dans les racines en présence d'ETM : ces molécules paraissent donc intéressantes à étudier pour leur rôle dans la tolérance des Renouées aux ETM, notamment la torosachrysone qui avait précédemment été montrée comme la molécule la plus fortement augmentée en présence de contaminants métalliques (Michalet et al., 2017). Au cours de cette étude des composés identifiés comme appartenant à la classe des biphényls **(Figure 4A.7)**, plus précisément des dérivés de l'alténusine, ont pu être détectés pour la première fois dans cette plante. Le fait que ces composés soient synthétisés par des champignons exclusivement (Cota et al., 2008; Johann et al., 2012; Liu et al., 2016; Xiao et al., 2014; Yuan et al., 2018a), et que la présence d'ETM (Zn surtout et à moindre niveau Cr) semble fortement affecter leurs concentrations dans les racines, laisse envisager un rôle potentiel de champignons endophytes dans la réponse des Renouées aux ETM.

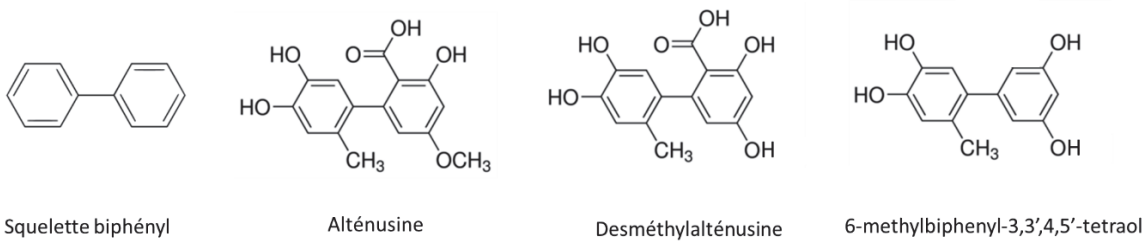
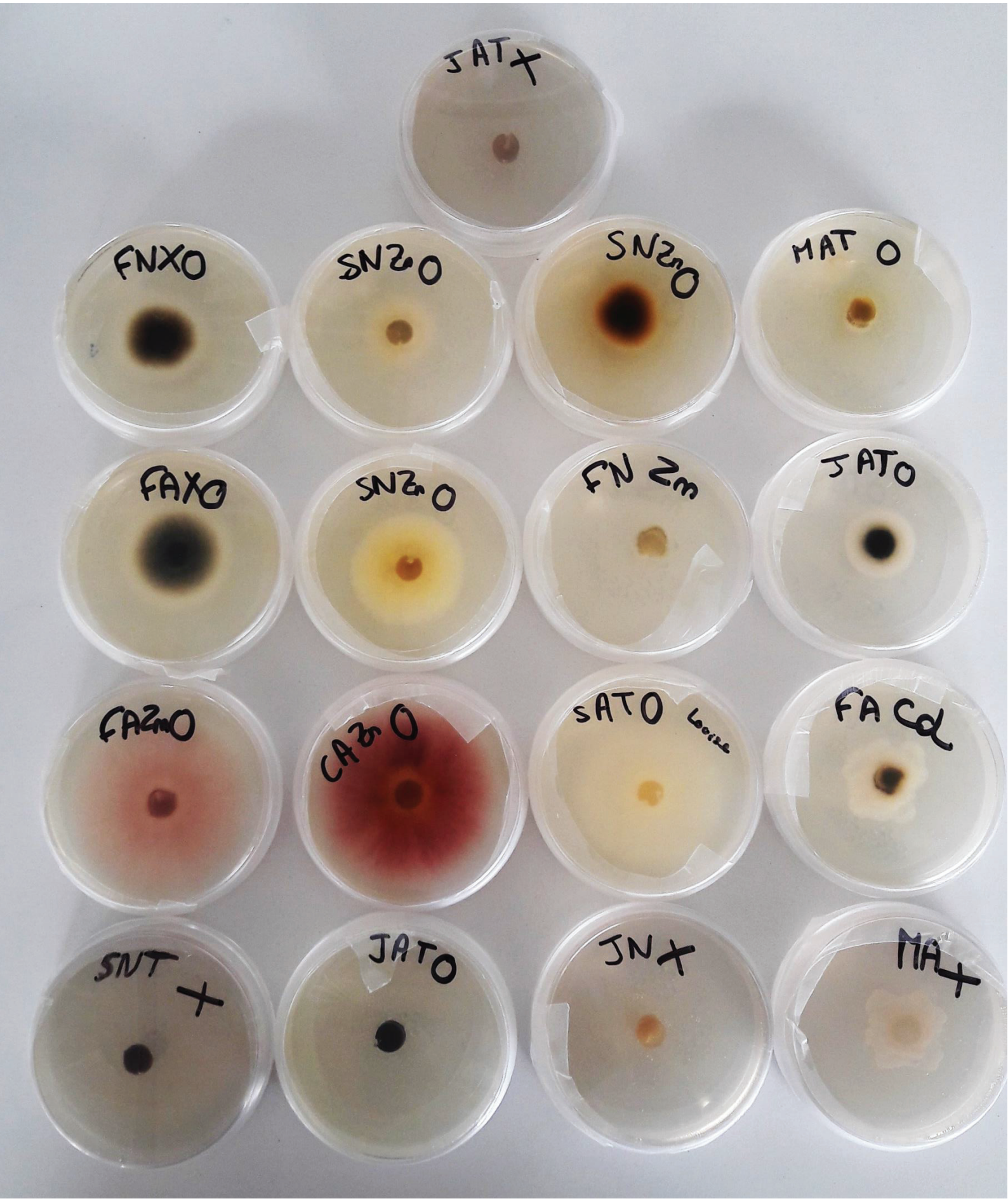


Figure 4A.7 : Structure chimique de dérivés biphényls.

Par ailleurs, cette étude a également montré le rôle important de l'environnement sur l'amplitude de la réponse au stress métallique : en effet le morphotype ayant démontré les meilleurs traits performance en présence de métaux était celui qui poussait dans un sol fortement pollué au Cu et au Pb (Caluire), alors que celui qui a démontré les moins bonnes performances était celui de Miribel poussant dans un sol non pollué et moins exposé aux activités anthropiques, celui de Feyzin poussant dans un sol contaminé au Zn ayant montré des réponses intermédiaires. Ceci démontre donc l'effet important de l'environnement natif dans lequel les plantes poussent dans la réponse au stress ; la part de l'influence de la génétique reste cependant à évaluer.

Maintenant que nous avons caractérisé les effets des ETM sur les Renouées, nous allons aborder la question des endophytes fongiques présents dans les racines des Renouées et leur tolérance aux ETM.


JAT+
FNXO
SN2O
SN2O
MAT O
FAXO
SN2O
FN 2m
JATO
FA2mO
CA2O
SATO
FA Cd
SNT +
JATO
JNX
MA+

Chapitre 4B : Isolement, identification, métabolisme secondaire et évaluation de la tolérance aux ETM des endophytes fongiques racinaires des Renouées asiatiques

Cette expérimentation a pour objectifs de :

- Connaître les endophytes fongiques racinaires associés aux Renouées
- Etudier le métabolisme secondaire de quelques-unes des souches isolées
- Tester la capacité des endophytes à pousser sur milieu contaminé aux ETM
- Constituer une banque de souches d'endophytes fongiques, qui seront utilisables pour de prochaines expérimentations

La stratégie mise en place est de maximiser les conditions de culture afin d'obtenir un maximum d'endophytes racinaires différents : les trois espèces formant le complexe *Fallopia* sont mises en culture sous serre dans un sol prairial, en présence de différents ETM (Cd, Cr, Zn ou leur mélange) ou en leur absence. Une condition supplémentaire, destinée à modifier les communautés endophytes natives et à limiter l'effet maternel lié aux endophytes déjà présents dans les rhizomes, est ajoutée : c'est l'acclimatation. Elle consiste à laisser les rhizomes dans du sol prairial pendant plusieurs mois, permettant ainsi le recrutement d'endophytes en provenance de ce sol riche, et la perte d'endophytes qui ne seraient plus « utiles » à la plante sortie de son contexte environnemental.

J'ai réalisé la culture de plantes à partir de laquelle toutes les autres expérimentations ont été effectuées. Les expérimentations suivantes (tests de tolérance des endophytes aux ETM, identification moléculaire et métabolomique) et une grande partie de la bibliographie sur les souches isolées ont été réalisées par quatre stagiaires co-auteurs de l'article (Thomas NICOLE, Guillaume HAMION, Thibaut GODOT et Marylène SIMON), que je remercie vivement.

Article en préparation

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Root fungal endophytes in *Fallopia*: identification, metal tolerance and secondary metabolites

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Keywords: metal trace elements, *Fallopia*, root fungal endophytes, secondary metabolites, phylogeny

Abstract

The *Fallopia* species complex (Polygonaceae) is expanding in Europe and North America, including on metal contaminated soils. From previous experiments and literature data, we hypothesized that root fungal endophytes participates in *Fallopia* tolerance to metal trace elements (MTEs), the aim of this study was then 1) to isolate and identify root fungal endophytes, 2) to test their tolerance to metals, 3) to analyse their secondary metabolism and 4) to discuss their potential role in plant metal tolerance.

We collected and grew five morphotypes of *Fallopia* (one morphotype of each parental species and three morphotypes of their hybrid) on either Cd, Cr, Zn, their mix or no MTE in a greenhouse experiment. Root fungal endophytes were isolated either on malt-agar medium with the metal to which their host plant was exposed to, or without metal. Molecular sequencing with ITS was used to identify fungi, their tolerance to metals was tested on Petri dishes with different MTE and their metabolite profiles were analysed by UHPLC-DAD-ESI/QTOF.

We isolated 27 strains from *Fallopia* roots, from which 22 were identified, and belong to Dothideales, Sordariales, and Eurotiales (Ascomycota). The endophyte *Diaporthe* sp. (FACd), supposed to be *Diaporthe neotheicola*, presents interesting properties: tolerant to Cd *in vitro* (but not to Cr or Zn), this isolate produces melanin, auxin and biphenyls including desmethylaltenusin, a compound which was previously shown to be increased in plant roots when exposed to Zn or Cr. This isolate seems then to possess an ecological relevance in *Fallopia* response to metal stress, and this needs to be further explored.

Introduction

Metal trace elements (MTEs), also referred to as heavy metals, are accumulating in soil since the industrial revolution characterized by the increase of anthropogenic activities. Indeed, road traffic (Nikolaeva et al., 2019), agriculture, industries and mining (Bourennane et al., 2010; Senesil et al., 1999) emit metal particles, such as cadmium (Cd), chromium (Cr), zinc (Zn) and lead (Pb), directly in soil or indirectly through redeposition from the atmosphere. As they are not biodegradable, they may reach very high concentrations in soils. Some metal trace elements (MTEs) such as Zn or Fe are essential for living organisms whereas other do not enter in the composition of any biological element (Cd and Pb for example)(Yadav, 2010). However, in excess, they all present toxicity for most organisms from plants (Prasad, 2004) to animals (Mahino et al., 2014) and microorganisms, leading to modifications at the ecosystem level (Babich and Stotzky, 1985; Gall et al., 2015). However, some plants maintain high performance traits and extend in these disturbed environments.

The *Fallopia* species complex, also known as Asian knotweeds, which comprises the two parental species (*F. japonica* and *F. sachalinensis*) and their hybrid *F.* x *bohemica* extended in Europe (Thiébaut et al., 2020), including in metal-contaminated urban areas (Sołtysiak and Brej, 2014). At the time when no consensual and efficient solution has been developed to limit their expansion, it is thus of major importance to understand how these plants could tolerate such important concentrations of MTEs and further expand in highly contaminated environments. The tolerance of knotweeds to MTEs was previously assessed in controlled conditions, either under multicontamination (Michalet et al., 2017) or monocontamination (Barberis et al., 2020). In multicontamination, all tested MTEs (Cd, Cr and Zn) except Pb were accumulated and transferred to leaves, whereas in monocontaminations, Cd and Cr were not transferred and only Zn was accumulated in leaves. In addition, Bohemian knotweed (*F.* x *bohemica*) demonstrated a high tolerance to Cd, even at high doses (10 mg/kg). We also observed a surprising stimulating effect of Cr (VI) on growth traits, whereas for Zn, this effect was less pronounced (Barberis et al., 2020). Cr (VI) and Zn also induce changes in root phenolic compounds, which is not the case of Cd: especially, torosachrysone - an anthraquinone derivative - is more abundant in roots of plants exposed to Zn or Cr, whereas biphenyl derivatives are also significantly modulated (Barberis et al., 2020). Interestingly, these biphenyl derivatives, which are derived from altenusin, a toxin

described in fungi of the *Alternaria* genus, are exclusively known to be produced in the fungal reign (Aly et al., 2008; Yuan et al., 2018a).

Despite the fact that *Fallopia* species complex is recognized as non-mycorrhizal, other fungi colonize its roots including dark septate endophytes (DSEs) (Gucwa-Przepióra et al., 2016). These fungi together with other non-mycorrhizal root endophytes are taxonomically (Barberis et al., 2020b) and functionally (Rodriguez et al., 2009) diverse but far less studied than mycorrhiza, although they are ubiquitous, abundant and seem to take part in plant-soil interactions (Jumpponen and Trappe, 1998; Zhang et al., 2006). From field study, a positive correlation was found between the concentration of MTEs in soil and endophyte root colonization of rosemary (Affholder et al., 2014). The colonization of *Deschampsia flexuosa* by AMFs was reduced on contaminated sites whereas DSEs were favoured in these soils (Ruotsalainen et al., 2007). Further studies showed the high tolerance of fungal endophytes to MTEs (Domka et al., 2019b; Li et al., 2012a, 2012b). Thus, fungal endophytes may modify plant response to MTEs (Barberis et al., 2020b), either favouring the exclusion of metal, or their (hyper)accumulation. For example, DSEs reduce the intake of Cd and Zn by *Salix caprea,* and this is correlated with a better chlorophyll status (Likar and Regvar, 2013).

Endophytes produce secondary metabolites that may enhance plant growth directly: this is probably the case of the root fungus *Penicillium funiculosum*, through its production of plant growth promoting hormones, namely gibberellins (Khan and Lee, 2013). Glutathione, phytochelatins, metallothionins, organic acids, siderophores, exopolysaccharides (EPSs), and phenolic compounds are other compounds produced by fungal endophytes, which directly chelate metal particles inside the root or outside, and may participate in the immobilization of MTEs in the rhizosphere. Therefore, plant tolerance to MTEs may rely on fungal metabolites produced by root endophytic fungi (Domka et al., 2019b).

In this context, we hypothesize that root fungal endophytes of the *Fallopia* species complex participates in plant tolerance to MTEs, the first step to test this hypothesis being the exploration of the capacity of *Fallopia* to host fungal endophytes adapted to MTEs in its roots. The aim of this study is then 1) to isolate and identify root fungal endophytes, 2) to test their tolerance to metals, 3) to analyse their secondary metabolism and 4) to discuss their potential role in plant metal tolerance.

To take into account the genetic diversity of *Fallopia* species complex and the diversity of soil contaminant levels on which it grows, we collected and grew five morphotypes of *Fallopia* (one morphotype of each parental species and three morphotypes of their hybrid) on either Cd, Cr, Zn, their mixture or no MTE in a greenhouse experiment. Rhizomes were either directly collected from the field or previously acclimated for three months in a prairial, non-contaminated soil, to allow the modification of native fungal communities, more precisely those inherited from-/adapted to-native plant environment (i.e. related to "maternal effect"), and that are not associated with plant vital processes. Root fungal endophytes were isolated either on malt-agar medium with the metal to which their host plant were exposed to, or without metal. Molecular sequencing with ITS was used to identify fungi, their tolerance to metals was tested on Petri dishes with different MTE and their metabolite profiles were analysed by UHPLC-DAD-ESI/QTOF.

Material and methods

Plant and soil collection

To cover *Fallopia* complex species diversity, rhizome fragments were obtained from three individuals morphologically identified as *F.* x *bohemica* (F. Piola, personal communication) using leaf shape and size (Bailey et al., 2009), one individual of *F. japonica* and one of *F. sacchalinensis*. *F.* x *bohemica* were collected at three distinct sites localized in Grand Lyon area (France) (Barberis et al., 2020): a non-disturbed site close to woods in Miribel-Jonage (M, 45.8094°N; 4.9352°E), for which soil did not present any metallic contamination; close to a waste recycling centre with a soil contaminated with Cu (150 mg/kg) and Pb (180 mg/kg) in Caluire (C, 45.8032°N; 4.8638°E); and close to a petrol station and motorways in a soil contaminated with Zn (450 mg/kg) in Feyzin (F, 45.6787°N; 4.8466°E). Distant from at least six kilometres between each other and presenting variability in their leaf morphology, those three sites are considered as different morphotypes, reflecting part of the hybrid variability. *F. japonica* was collected in Le Chambon-Feugerolle (J, 45,3997°N; 4,3417°E) and *F. sachalinensis* in Belleroche (S, 46,1723°N; 4,3930). To allow the modification of fungal communities, notably through the release of environmental MTE pressure, rhizomes collected in October 2017 were acclimated three months in a prairial non-contaminated soil (acclimation condition, A): for that, they were washed with tap water and conserved outside in 20 L-pots filled with sieved soil and covered. Other rhizomes were collected in January 2018, just before greenhouse experiments (no acclimation, N).

Prairial soil (< 15 - 20 cm depth) was collected at La Côte Saint-André (45.3769°N; 5.2772°E) in September 2017 for the acclimation. This soil exempt of heavy metal contamination and the physicochemical characteristics of this soil are presented in Barberis et al. (2020).

Greenhouse culture

A greenhouse experiment was conducted between January and February 2018. The experimental design follows Barberis et al. (2020), with some modifications. Soil was sieved (2 mm mesh size) and rehydrated to its field capacity (40% m/v) either with UP water or with a solution containing either $CdCl_2$, $CrCl_3$, $ZnCl_2$ or a mixture of these metal salts. The concentrations were chosen to theoretically achieve contamination in soil that were slightly over common recognized soil pollution threshold (Baize et al., 2006) for Cd, Cr and Zn: 1.5, 200 and 400 mg/kg respectively. Then 100 ± 5 g of moistened soil were placed into 0.15 L plastic pots and washed rhizome fragments containing one node (average weight of 1.2 ± 0.1 g) of each of the 5 genotypes of *Fallopia* collected were planted and grown in greenhouse (13 h day 22°C/11 h night 18°C) with light intensity of about 8-10 klux. Soil moisture was manually controlled by adding tap water every two-three days, from the bottom to limit metal leaching. Plantlets were grown for one month and their position was randomly changed 3 times/week.

The experimental design was as follow: 5 morphotypes x 5 metal conditions x 2 acclimations x 7 replicates.

To maximize the isolation of endophytes, a second experiment was achieved with less conditions but more samples/condition: rhizomes of *F.* x *bohemica* from Caluire, not-acclimated, were grown on loam, with $CrCl_3$ (400 mg/kg) or without. The design was thus as follow: 1 morphotype x 2 metal conditions x 12 replicates.

Isolation of root fungal endophytes

After one month of growth, plants were carefully uprooted and two root fragments of 1 cm-/sample were cut and conserved at 4°C in their soil.

The protocol of isolation is adapted from Berthelot et al. (2016): roots were pooled in two groups/condition (repetitions): the first condition is isolation on agar with metal and the second condition is isolation on agar without metal. Root fragments were then disinfected in surface: 3 min in bleach 5 % added with a drop of dishwashing liquid; 30 s in H_2O_2 30 %, followed by rinsing

in autoclaved distilled water. They were then deposited on malt-agar media (1.2 % malt, 1.5 % agar, 1 mL/L chloramphenicol, pH adjusted at 5.5 ± 0.1 with HCl or NaOH) in Petri dishes ± metal (Cd: 1.5 mg/L, Cr: 200 mg/L, Zn: 400 mg/L or mix: Cd+Cr+Zn), roots being put on the same metal condition of growth, in dishes. Fungal root endophytes were cultured at 24 °C in the dark. For the second experiment, the same protocol was used, except that plants were harvested after two months of growth and that 6 root fragments of each sample were disinfected, 3 for isolation with Cr (400 mg/L) and 3 for isolation with non-contaminated malt-agar gel. After a few weeks, fungi were sub-cultured on new malt-agar dishes, keeping the same metal condition.

As we hypothesize that *Fallopia* tolerance to MTE may be supported by DSEs, we added in the phylogenetic analyses four DSEs from chrono-environnement library (coded FO6, M10, KO3 and KO4) isolated from poplar roots (data not shown), for confrontation with the other isolates. Isolates and their plants and media of origin are presented in **Table S1**.

Molecular identification of fungal endophytes

Genomic DNA of the isolated *Fallopia* fungi and of the 4 strains from the fungal library of the laboratory was isolated from peripheral parts of the mycelium following the fungal genomic DNA extraction protocol described by in **Annexe 4B.1**.

Annexe 4B.1: DNA extraction from fungal culture.

Extraction Solution

Stocks: 1 M Tris solution (pH=8.0)

1) add 10 ml of 1 M Tris stock into clean (autoclaved) 100 ml vessel
2) add 1.86 g KCl
3) add 0.37 g EDTA
4) add 80 ml DI H2O and shake until solutes dissolve
5) titrate with 1 M NaOH to pH = ~ 9.5-10.0
6) top up to 100 mL with DI H2O
7) filter sterilize into sterilized 2 mL tubes for storage

Dilution Solution

1) add 3 g of BSA (e.g. Sigma or Omni – 98-99% purity, heat shock fractionated) into clean (autoclaved) vessel
2) top up to 100 mL with DI H2O
3) shake BSA into solution
4) filter sterilize into sterilized 2 mL tubes for storage

Procedure

1. Pipette Extraction Solution into 8-strip tubes (or 96-well plates). For cultured mycelium 20 µL is sufficient. For larger samples use up to 100 µL.
2. Add tissue sample to tube/well with Extraction Solution. Submerge sample and smash sample if possible (we use pipette tips or sterilized dissecting needles). Try not to add it any agar.
3. Incubate at room temp for 10+ minutes then incubate for 10 minutes at 95 C.
4. Add an equal volume of Dilution Solution to the tubes so that the final Extraction:Dilution solution ratio is 1:1.
5. Samples are now ready for PCR. We typically make 1/10 and 1/100 dilutions of the raw extract.
6. Store DNA extractions in freezer.

Approximately 1 kb of the rDNA ITS1/5.8/ITS2 region was amplified via PCR using primers NS7 (5'-GAGGCAATAACAGGTCTGTGATGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described by White et al. (1990). Reactions mixes contained 10-60 ng of fungal DNA, 0.5 µM of both primers, 100 µM dNTPs, 1.5 mM $MgCl_2$, 1 unit of the Phire Green Hot Start Polymerase (Thermofisher Scientific), in a final volume of 50 µL enzyme buffer. The conditions for thermocycling were as follow: an initial denaturation step at 98°C for 30 s followed by 30 cycles of 98°C 5 s, 53°C 5 s, 72°C 10 s, and a final extension at 72 °C for 1 min. PCR products were purified using the GenElute PCR Clean-Up kit (Sigma-Aldrich) or by a gel band purification kit (Macherey-Nagel NucleoSpin) and sequencing was performed by Genewiz Europe (Germany). Comparisons with sequences of the GenBank and UNITE databases were performed with the search algorithm BLAST (Altschul et al., 1990). The identifications of the endophytes were determined based on parameters such as the percentage of query cover and similarity with 0.0 expected values (Evalues). The cut-offs used to assign a particular precision to the identification at the species and genus rank, based on obtained BLAST results with total or near total coverage, were as follows: (1) sequence similarity of 100%: positive species, (2) sequence similarity of 99%: possibly this species, (3) genus rank only was adopted when equal BLAST top score similarity values have been obtained for several species, all from the same genus (ranging from 96 to 100%) (Hofstetter et al., 2019). The consensus sequence for each endophyte will be submitted to GenBank to obtain their respective accession numbers (http://www.ncbi.nlm.nih.gov/Genbank). Sequences were edited in Bioedit v7.0.5. (Hall, 1999) and aligned using Clustal W (Thompson et al., 1994). The phylogenetic relationships among the endophytic fungi isolated in *Fallopia* and in poplar root (fungi library of the laboratory) were studied by using a maximum-likelihood (ML) approach (1000 bootstrap replicates, K2P distances) using MEGA6 (Tamura et al., 2013). Gaps and missing data were removed from the analysis using the "complete deletion" option. The sequence of *Morchella virginiana* (JQ723113.1) was used as out-group.

Assessment of fungal sensitivity to metals

Nine isolated strains were tested for their sensitivity to MTEs. The sensitivity of the strains to different metals was evaluated by investigating the fungal growth rate. Plugs (4 mm) of each strain were cut from the edge of actively growing 2-weekold colonies and placed on malt-agar media (as described above) and previously amended with $CdCl_2$ (Cd at 10 mg/L), $CrCl_3$ (Cr at 200 mg/L), $ZnCl_2$ (Zn at 400 mg/L), those three MTEs together (condition named X), or no metal (control). Fungal strains were incubated in the dark at 24°C during 11 days. Fungal growth diameter was measured with a stereomicroscope (× 10 magnification) every day during 11 days.
Fungal growth rate in metal amended media was thus calculated (in mm/day) and reported to the growth rate in control medium.

Extraction and analysis of fungal metabolites

To cover the maximum of metabolite diversity produced by endophytes, the ten isolates were cultured in solid medium (1.2 % malt, 1.5 % agar, 1 mL/L chloramphenicol, pH adjusted at 5.5 ± 0.1 with HCl or NaOH) in Petri dishes and in liquid medium (same composition as solid medium but without agar). A plug of agar - mycelium (5 x 5 mm) served as inoculum in both types of culture. Culture lasted about one month in both media at 23°C in the obscurity under agitation at 150 rpm. Secondary metabolites were extracted by dichloromethane (DCM), ethylacetate (AcOEt) and methanol (MeOH) in a 80:20 ratio with UP water, successively. For liquid cultures, 1 mL of liquid supernatant and 100 mg of fresh mycelium were used. For solid cultures, half of the colonized agar medium (between 1 to 4 g fresh mass) were used. For each fungal isolate, we thus have three sources of metabolites (liquid supernatant, mycelia from liquid and solid cultures).
Samples were extracted with 1 mL of DCM (vortexed 1 min, sonicated 15 min, and centrifuged 5 min at 5000 rpm). Then, 800 µL of the organic phase was transferred and dried in a speed-vacuum (Speedvac® – Labenco). DCM extraction was repeated thrice, and extracts were pooled.
Then, extractions with AcOEt and MeOH: H_2O were achieved following the same protocol, except for liquid supernatants that were dried in a speed-vacuum before MeOH-H_2O extraction. In order to remove the remaining water in MeOH – H_2O extracts, a step of lyophilisation was added. 90 fungal extracts were thus obtained (10 isolates * 3 media (liquid phase, mycela in liquid culture and in solid culture * 3 extraction solvents). Controls of each extraction were made using culture media without fungus, leading to 6 control extracts (2 media - liquid and solid * 3 extraction solvents).

Dry extracts were resuspended in MeOH:H_2O (80:20) at 10 mg/mL and analysed by UHPLC-DAD ESI/QTOF (Agilent 1290 infinity linked with Agilent ESI/QTOF 6530, Agilent Technologies, USA), using a Poroshell® 120 EC-18 column (2.7 µm, 3.0 x 100 mm; Agilent Technologies, USA), and following the protocol of Barberis et al. (2020). The gradient ranges from 0.1% formic acid in water (A) to acetonitrile (B) as follows: 1% of B from 0 to 1.5 min, and growing with a linear gradient to 15% of B at 8 min; 60% of B at 14 minutes and 100% of B at 16 min for 1 min. All solvents were LC-MS grade (Optima). The flow rate was adjusted at 1.0 mL/min and the injection volume was 2 µL. UV spectra were recorded between 190 and 600 nm. The ESI source was optimized as follows: positive ionization in autoMS/MS mode, scan spectra from *m/z* 80 to 2000, capillary voltage 3.5 kV, fragmentor 100 V, fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 12 L/min and a temperature of 310°C at 40 psi. Compounds were identified by analysis of their UV, HRMS and HRMS/MS spectra using MassHunter Qualitative Analysis version B.07.00 (Agilent Technologies, USA) and by comparison with literature using online databases: SciFinder-n (CAS; https://www.cas.org/products/scifinder), PubChem (NCBI; https://pubchem.ncbi.nlm.nih.gov) and NP Atlas (https://www.npatlas.org/joomla).

Results and Discussion

Isolation, molecular identification and phylogenetic analysis of root fungal endophytes from Fallopia spp.

In total, 27 root fungal endophytes from *Fallopia* spp. were successfully isolated from their roots **(Table 4B.S1)**. This is a similar number compared to other herbaceous plants (Beena et al., 2000; González-Teuber et al., 2017; Herrera et al., 2010; Knapp et al., 2012; Teimoori-Boghsani et al., 2020; Wu et al., 2012).

Table 4B.S1: Sample names, culture provenance and isolation medium. *A: acclimated, N: not acclimated; T: control without MTE, Cd: cadmium, Cr: chromium, Zn: zinc, X: mixture of Cd, Cr and Zn. J:* F. japonica*, S:* F. sachalinensis*, B:* F. *x* bohemica*.*

Experiment	Isolates	*Fallopia* species	Morphotype	Plant culture MTE	Acclimation	Fungal culture MTE
1	JACd	*J*		Cd	A	?
	JAT	*J*		T	A	X
	JNX	*J*		X	N	X
	SAT	*S*		T	A	ø
	SNT	*S*		T	N	X
	SNZn	*S*		Zn	N	ø
	MACd	*B*	Miribel	Cd	A	?
	FACd	*B*	Feyzin	Cd	A	Cd
	FNCd	*B*	Feyzin	Cd	N	ø
	MAT	*B*	Miribel	T	A	ø
	FNX	*B*	Feyzin	X	N	ø
	MAX	*B*	Miribel	X	A	X
	MAX0	*B*	Miribel	X	A	ø
	FAX	*B*	Feyzin	X	A	ø
	CAZn	*B*	Caluire	Zn	A	ø
	FAZn	*B*	Feyzin	Zn	A	ø
	FNZn	*B*	Feyzin	Zn	N	Zn
2	CCr1	*B*	Caluire	Cr	N	Cr
	CCr3	*B*	Caluire	Cr	N	Cr
	CCr4	*B*	Caluire	Cr	N	Cr
	CCr6	*B*	Caluire	Cr	N	Cr
	CCr7	*B*	Caluire	Cr	N	Cr
	CCr2	*B*	Caluire	Cr	N	ø
	CCr5	*B*	Caluire	Cr	N	ø
	CT3	*B*	Caluire	T	N	Cr
	CT1	*B*	Caluire	T	N	ø
	CT2	*B*	Caluire	T	N	ø

An ITS sequence (~ 1kb), comprising most of the region between the position of primers NS7 and ITS4, was obtained for 26 and 4 root fungal endophytes from *Fallopia* spp. and *Populus* spp. respectively. Only one isolate exhibited a poor chromatogram quality (*i.e.,* isolate coded FNX). The BLAST research has enabled the identification of 22 isolates (18 from *Fallopia* spp. and 4 from *Populus* spp.) with total or near total coverage and a sequence similarity ranging from 95 to 100%.

Eight isolates were problematic, one did not match with any database sequence (*i.e.,* isolate coded SNT), 2 were fungal contaminants (Basidiomycota, *i.e.,* isolates coded FNZn and CCr6) and removed from the dataset, as well as the 5 others (*i.e.,* isolates coded MAT, JNX, CCr5, CT2 and CT3) for which the assignment at the genus rank were not significant (sequence similarity < 94%). The BLAST results allowed us to name 10 isolates at species rank (including those with sequence similarity of 99) and 12 were identified to genus. The 4 isolates from poplar roots were identified as members of Helotiales (Leotiomycetes) with 1 *Cadophora orchidicola* and 2 *Cadophora sp.*, recognized as formal DSEs genus (Addy et al., 2005), and Eurotiales (1 *Penicillium glandicola*) **(Table 4B.1, Figure 4B.1)**. The 18 endophytes from *Fallopia* roots belong also to the phylum Ascomycota and are distributed in 3 classes and 8 orders **(Table 4B.1, Figure 4B.1)**. The phylogenetic analysis revealed that most of the isolates were in the class of the Sordariomycetes, which were further classified into 5 orders Chaetosphaeriales, Diaporthales, Hypocreales, Ophiostomatales and Xylariales. The second class the most represented is Dothideomycetes with 2 orders, Capnodiales and Pleosporales. Here, the Dothideomycetes do not form a phylogenetic group with Capnodiales and Pleosporales more related to the Sordariomycetes and the Eurotiomycetes respectively, and this could be due to the low support of the groups at the class level. Finally, in the Eurotiomycetes, only representatives of the genus *Penicillium* classified into the order of the Eurotiales were isolated.

Except Ophiostomatales, the fungal orders represented in *Fallopia* roots are widely found in other plant roots (Barberis et al., 2020b). Almost all isolates belong to orders (Capnodiales, Pleosporales, Eurotiales, Hypocreales and Xylariales), which are described to contain DSEs (Knapp et al., 2015).

Table 4B.1: Molecular Identification of root endophytes isolated from* Fallopia *spp. using the universal primers NS7 and ITS4. *In light red: DSEs from poplar. For precise protocol of obtention of isolates, see Table 1. *Identification of SNT is based on its clustering in the tree reconstruction* ***(Figure 4B.1)****. Sequences accession numbers from this study will be indicated upon publication.*

Isolates	Division	Class	Order	Putative species	Query cover (%)	Similarity (%)	Genbank Accession number
JACd	Ascomycota	Dothideomycetes	Capnodiales	*Cladosporium sp.*	98	99	...
CCr2	Ascomycota	Dothideomycetes	Capnodiales	*Cladosporium sp.*	98	99	...
CT1	Ascomycota	Dothideomycetes	Capnodiales	*Cladosporium sp.*	100	100	...
SNZn	Ascomycota	Dothideomycetes	Pleosporales	*Ascochyta sp.*	100	98	...
SAT	Ascomycota	Dothideomycetes	Pleosporales	*Epicoccum nigrum*	100	99	...
FAX	Ascomycota	Dothideomycetes	Pleosporales	*Periconia macrospinosa*	100	100	...
FNCd	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium commune*	100	99	...
KO4	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium glandicola*	100	99	...
MACd	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium glandicola*	100	99	...
CCr3	Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium simplicissimum*	77	100	...
KO3	Ascomycota	Leotiomycetes	Helotiales	*Cadophora sp.*	73	95	...
FO6	Ascomycota	Leotiomycetes	Helotiales	*Cadophora sp.*	100	96	...
M10	Ascomycota	Leotiomycetes	Helotiales	*Cadophora orchidicola*	100	99	...
JAT	Ascomycota	Sordariomycetes	Chaetosphaeriales	*Codinaea sp.*	90	99	...
FACd	Ascomycota	Sordariomycetes	Diaporthales	*Diaporthe sp*	98	97	...
CCr4	Ascomycota	Sordariomycetes	Ophiostomatales	*Sporothrix sp.*	69	97	...
CAZn	Ascomycota	Sordariomycetes	Hypocreales	*Fusarium oxysporum*	100	100	...
FAZn	Ascomycota	Sordariomycetes	Hypocreales	*Fusarium oxysporum*	100	100	...
MAX0	Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma koningii*	100	100	...
MAX	Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma sp.*	96	99	...
CCr1	Ascomycota	Sordariomycetes	Xylariales	*Arthrinium sp.*	80	99	...
CCr7	Ascomycota	Sordariomycetes	Xylariales	*Arthrinium sp.*	78	100	...
SNT*	Ascomycota?	Dothideomycetes?	Pleosporales?		NA	NA	...

Five different species of **Pleosporales** (Dothideomycetes) were isolated in *Fallopia* roots. Pleosporales is a very large order comprising fungal strains isolated from a wide variety of ecological niches, from saprobes to hyperparasites, including pathogens, epiphytes and endophytes (Zhang et al., 2012). Despite the fact that several species of the ***Ascochyta*** genus cause major damages on Fabaceae cultures (Kaiser, 1997; Nene, 1982), *Ascochyta* species have been found several times in asymptomatic roots (Bayman and Otero, 2006; Kernaghan and Patriquin, 2011; Sadeghi et al., 2019), stems (Suryanarayanan et al., 2005) or leaves (Hashizume et al., 2010). Literature is nevertheless focused on the pathogenic effects of *Ascochyta* species, and as far as we know, no study have evaluated the beneficial effects of *Ascochyta* on plants. ***Epicoccum nigrum*** is a generalist, epiphytic or endophytic (Fávaro et al., 2012). It was described in plant roots in numerous environments, including sea, mountain, and metal-contaminated areas (Bayman and Otero, 2006; Li et al., 2016; Vaz et al., 2009), as well as in a variety of plant hosts (Furtado et al., 2019; Li et al., 2016; Martins et al., 2016; Paul et al., 2007). *E. nigrum* is used in biological control of pathogenic fungi (Cal et al., 2009; Fávaro et al., 2012; Hashem and Ali, 2004; Larena and Melgarejo, 2009) and bacteria (Madrigal et al., 1991). *E. nigrum* may induce root system development (Fávaro et al., 2012). ***Periconia macrospinosa*** is a common root endophyte (Ghimire et al., 2011; Knapp et al., 2012; Maciá-Vicente et al., 2008; Pili et al., 2016), forming typical structures of DSEs (Mandyam et al., 2010). *P. macrospinosa* improves plant growth (Kageyama et al., 2008) through the mobilisation of soil nutrients (Yakti et al., 2018), and possesses antifungal and antibacterial properties (Kim et al., 2004). However, it has sometimes been reported as root and leaf pathogen (Sarkar et al., 2019; Van Dyk, 2004).

***Cladosporium* spp.** (Capnodiales, Dothideomycetes) were isolated three times in this study. The *Cladosporium* genus is ubiquitous, and include fungi from diverse ecological niches, reported as plant pathogens (Oh et al., 2018; Rivas and Thomas, 2005), fungal pathogens (Nasini et al., 2004), or root endophytes (Barberis et al., 2020b; Bayman and Otero, 2006; Sadeghi et al., 2019; Verma et al., 2011; Yeh and Kirschner, 2019). In particular, some are mutualists, such as *Cladosporium sphaerospermum*, described as a plant growth promoting endophyte (Hamayun et al., 2009). Some *Cladosporium* isolates adsorb MTEs on their surface (Pethkar et al., 2001), particularly in their melanin (Buszman et al., 2006).

Numerous isolates identified in this study belong to the ***Sordariomycetes*** clade, which contains phytopathogens, as well as saprobes, epiphytes, lichenised fungi, and endophytes (Maharachchikumbura et al., 2016). Within the same genera ***Arthrinium***, both pathogenicity and

endophytism have been reported. Indeed, the ***Arthrinium*** genus contains two major plant pathogens among other, namely *A. sacchari* and *A. marii*, causing important damage on wheat (Mavragani et al., 2007), sugar cane (Ming, 1995), and olive tree cultures (Gerin et al., 2019). But this genus also contains fungal endophytes, in particular localized in roots (Bayman and Otero, 2006; Chen et al., 2011; Furtado et al., 2019; Maciá-Vicente et al., 2008; Ramos et al., 2010; Sánchez Márquez et al., 2010). Little is known about the genus ***Codinaea***, but at least one plant pathogenic species has been characterized (Dean et al., 2012; Menzies, 1973). Two isolates of ***Fusarium oxysporum f. sp. dianthi*** have been isolated in this study. *Fusarium oxysporum* is often considered as a plant pathogen (Michielse and Rep, 2009), in particular the well-known *F. oxysporum f. sp. dianthi* (Lemanceau et al., 1993); nevertheless, *F. oxysporum* has been isolated from numerous plant roots without causing any symptoms (Barberis et al., 2020b). Despite its pathogen lifestyle (Manicom et al., 1990), *Fusarium oxysporum f. sp. dianthi* may induce resistance against fungal phytopathogens (Kroon et al., 1991).

No less than 23 species of ***Trichoderma*** have already been reported as root endophytes (Barberis et al., 2020b). This genus includes saprobes as well (Naar and Kecskés, 1998) and is common in soils and plant roots (Harman et al., 2004). Associated to roots, *Trichoderma* spp. present a morphology of DSE (Chadha et al., 2014). Some *Trichoderma* isolates enhance plant growth (Contreras-Cornejo et al., 2009) and reduce nematode damage (Meyer et al., 2001). *Trichoderma* fungi produces a wide range of secondary metabolites, plant growth regulators including auxin, antibiotics, and antifungal compounds (Chadha et al., 2014). The colonization of plant roots by *Trichoderma* endophytes leads to the alleviation of abiotic stress, through the increase of plant enzymes involved in the regulation of oxidative stress (Zaidi et al., 2014). The species ***Trichoderma koningii*** improves plant growth while reducing the severity of plant fungal disease (Duffy et al., 1997) or bacterial disease (Hadar, 1984). This species is resistant to Cu (Nykiel-Szymańska et al., 2018).

The ***Penicillium*** genus, from which three representatives were isolated in this study, is often reported as root endophytes (Barberis et al., 2020b). Those endophytes are ubiquitous, in numerous environments, and in non-phylogenetically related plant hosts (Barberis et al., 2020b). *Penicillium* fungi produce a large variety of chemical compounds (Cole and Cox, 1981; Pitt, 2002). Those compounds often possess antibacterial properties, for example in *Penicillium commune* (Gao et al., 2011; Shang et al., 2012). The reduction of bacterial disease through plant growth promotion by a *Penicillium* species, , has been demonstrated in *Arabidopsis thaliana* (Hossain et

al., 2008). Phytohormones, such as gibberellins (Choi et al., 2005; Khan et al., 2008), and auxin (Ikram et al., 2018) are produced by these strains, suggesting a potential direct effect on plant growth. Indeed, the endophyte and gibberellin-producing *Penicillium citrinum* promotes plant growth (Khan et al., 2008). Some *Penicillium* species (*P. simplicissum*, *P. commune*) tolerate and bioaccumulate MTEs (Harimisa Andriamafana, 2018; Iskandar et al., 2011; Sharma and Malaviya, 2016). *Penicillium glandicola* (Bezerra et al., 2012) and *Penicillium commune* (Jin et al., 2013) were previously reported as endophytes. Anyway, if some plant-pathogens are reported among the *Penicillium* genus (Li et al., 2015; Valdez et al., 2006), *Penicillium* species are mainly described as plant growth-promoting fungi (Radhakrishnan et al., 2014).

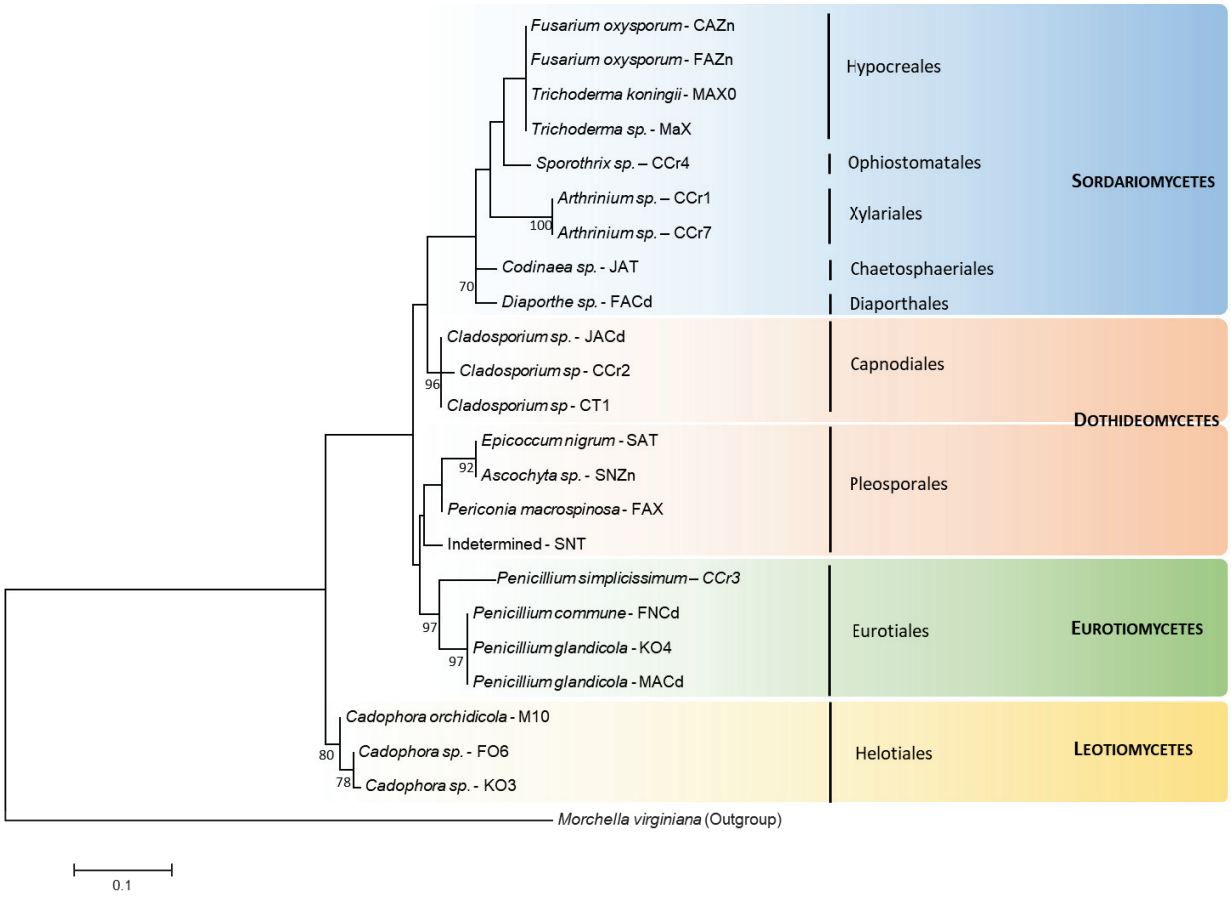

Figure 4B.1: Phylogenetic relationships among the endophyte fungi isolated in* Fallopia *roots using a maximum-likelihood approach (1000 bootstrap replicates, Kimura two-parameters). *Root endophytes from poplar roots were added (fungi library of the laboratory). Bootstrap value > 70 %.*

Metal tolerance profil of endophytes fungi from Fallopia spp.

In vitro, all isolates were tolerant to Cd, *i.e.* their growth rate was not affected by Cd **(Figure 4B.2)**. However, fungal isolates display variable pattern of tolerance for other MTEs. Only the three Hypocreales isolates could grow on the mixture of Cd, Cr and Zn, with a 5-fold reduced speed compared to their respective control. They could also grow on Cr and Zn too. On the opposite, SAT (*Epicoccum nigrum*, Pleosporales) and FACd (*Diaporthe* sp., Diaporthales) did not grow at all on any of the MTEs except Cd. SNZn (*Ascochyta* sp.*,* Pleosporales) and FAX (*Periconia macrospinosa*, Pleosporales) had a growth divided by three on Zn and Cr compared to the control. FNX (undetermined) was little affected by Zn but no growth could be observed on Cr contaminated medium.

Eight isolates (all except the unidentified FNX) on the nine tested demonstrated a similar growth on Zn compared to Cr.

Code	Fallopia species	Plant culture MTE	Acclimation	Fungal culture MTE	Class	Order	Species
SNZn	*S*	Zn	N	ø	Dothideomycetes	Pleosporales	*Ascochyta sp.*
FAX	*B*	X	A	ø	Dothideomycetes	Pleosporales	*Epicoccum nigrum*
SAT	*S*	T	A	ø	Dothideomycetes	Pleosporales	*Periconia macrospinosa*
FACd	*B*	Cd	A	Cd	Sordariomycetes	Diaporthales	*Diaporthe sp*
CAZn	*B*	Zn	A	ø	Sordariomycetes	Hypocreales	*Fusarium oxysporum*
FAZn	*B*	Zn	A	ø	Sordariomycetes	Hypocreales	*Fusarium oxysporum*
MAX	*B*	X	A	X	Sordariomycetes	Hypocreales	*Trichoderma sp.*
FNX	*B*	X	N	ø	NA	NA	NA

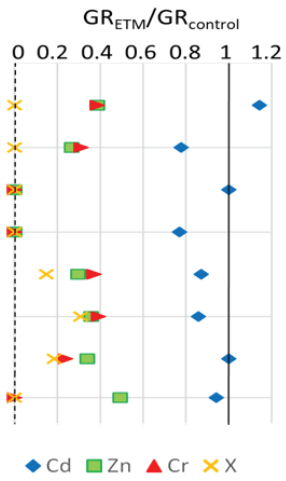

Figure 4B.2: MTE tolerance of fungal strains. *GR: fungal growth rate. The solid line indicates the same growth rate between the fungus grown on metal and the same fungus grown on a control medium without metal; the broken lines indicates no growth of fungus on metal-contaminated medium. Cd (10 mg/L), Zn (400 mg/L), Cr (200 mg/L), X (Cd + Zn + Cr at same concentrations).*

Cd at the applied concentration (10 mg/L) does not seem to present a significant toxicity for endophyte growth. Tough, this concentration corresponds to highly contaminated field soils such as mining soils (Baize et al., 2006; Xiao et al., 2017). This is consistent with field observations that endophytes are present in MTE highly contaminated sites (Barberis et al., 2020b; Weissenhorn et

al., 1993), although some fungi are strongly inhibited beyond 5 mg/L Cd *in vitro* (Weissenhorn et al., 1993). Plant-fungi associations are thus maintained on metal contaminated soils. However, on poly-contaminated media, only Hypocreales were able to grow, and at a speed severely reduced (from 3 to 6 folds). Thus, on polymetal-contaminated soils, we could expect a global reduction of root colonization by fungi, with a limited effect on Hypocreales that are more tolerant. However, this is not was is reported from field studies: a positive correlation was found between the concentration of MTEs in soil and endophyte root colonization of rosemary (Affholder et al., 2014). In addition, if the colonization of *Deschampsia flexuosa* by AMFs was reduced on contaminated sites, DSEs were favoured on those soils (Ruotsalainen et al., 2007). It is important to keep in mind that in our study, fungi were cultured *in vitro* and then directly in contact with MTE in agar media, whereas in the field, MTEs may be adsorbed on soil particles that reduce their bioavailability, and fungi are better protected inside plant roots.

Metabolites produced by endophytes fungi isolated from Fallopia spp.

33 compounds were identified from 10 fungal cultures extracts, cultivated either in solid or in liquid medium **(Table 4B.2)**. Detected metabolites belong to different families. Some compounds, such as biphenyl derivatives and cyclodipeptides, are specific to an isolate **(Table 4B.3)**. Other compounds, such as tryptophane derivatives (phytohormones) and agaridoxin, are shared by numerous fungi. Sphingolipids are produced by all tested endophyte isolates.

To understand the relationships between plant and endophytic fungi, it is essential to look at secondary metabolism in all partners. These metabolites may serve as communication between partnerss, or modify environmental conditions, including the modulation of MTE bioavailability in soil or their transfer into plant parts. Sphingolipids, such as phytosphingosine and its derivative, were detected in all fungal strain extracts analysed. These compounds, although being structural components of membrane systems, are also involved in plant-microorganism communications (Ali et al., 2018).

The production of Lachnumfuran A, a compound that has only been isolated from *Lachnum papyraceum* (Shan et al., 1996), suggests that the isolate JNX belong to the *Lachnum* genus (Helotiales). This is consistent with the presence of lachnumlactone A (or papyracon A/D, the identity is not well resolved), also identified in *Lachnum* fungi (Nielsen and Smedsgaard, 2003). The isolate JNX is thus further referred to as “Lachnum sp.”.

All studied Dothideomycetes isolates (*Periconia macrospinosa, Epicoccum nigrum*, *Pleosporales* sp. and *Ascochyta* sp.), the *Diaporthe* sp. and the *Lachnum* sp. isolates produce melanin precursors. Melanin is a dark pigment, present in fungal cell walls of dark septate endophytes (DSEs), suggesting that these isolates could be classified as DSEs. In addition to GDHB, HGA and THN produced by all previously cited isolates, *Epicoccum nigrum* also produces L-Tyrosine, another melanin precursor (Toledo et al., 2017), and pantothenic acid, which stimulates melanogenesis (Albuquerque et al., 2014). Melanin possesses antioxidant, radical scavenging and chelating properties that may decrease the effects of abiotic stresses, especially metal stress (Pombeiro-Sponchiado et al., 2017).

Indole-3-acetic acid (IAA), and its derivative Indole-3-acetamide (IAM), detected in six isolates, namely *Diaporthe* sp. (FACd), *Fusarium oxysporum* (CAZn)*, Epicoccum nigrum* (SAT), *Codinaea* sp. (JAT), *Ascochyta* sp. (SNZn), and *Lachnum* sp. (JNX), are phytohormones that promote plant growth, and also favour the establishment of fungal endophyte in roots (Luo et al., 2016).

Penicillium commune is known for its high diversity of secondary metabolites (Nicoletti and Trincone, 2016). Indeed, we could identify in the isolate of this genus (strain FNCd) six metabolites not detected in the other tested endophytes. Those six compounds, penipacid, meleagrin, roquefortine, chrysogine, glandicolin, and penilloid, are reported in multiple species within the *Penicillium* genus (Nicoletti and Trincone, 2016) and are well-known for their antimicrobial activities, though these were principally determined in animal-pathogenic strains and not in plant-pathogenic strains.

Diaporthe sp. (strain FACd) presents a very specific metabolome: it is the only analysed strain to produce biphenyls, and more precisely, altenusin and six other biosynthetic derivatives including desmethylaltenusin **(Table 4)**. Altenusin and its derivatives have been identified in *Alternaria* (Aly et al., 2008) or *Talaromyces* (Yuan et al., 2018a) endophytic fungi and are exclusively described in the fungal reign; incidentally altenusin and altenuene are considered as mycotoxins contaminating food (Escrivá et al., 2017). Altenusin and its derivatives were also reported to be chemotaxonomic markers enabling to distinguish *Diaporthe*/*Phomopsis* and *Valsa*/*Cytospora* clades (Abreu et al., 2012), which leads us to hypothesize that this strain could be *Diaporthe neotheicola*. However, desmethylatenusin, its hexoside derivative, and its decarboxylated and sulfated derivative were detected in *Fallopia* root extracts where their concentrations were strongly affected when plants were exposed to Zn or Cr, indicating that they may play an important role in the regulation of metal stress in this plant (Barberis et al., 2020). All this suggests that desmethylaltenusin detected

in plant roots is probably produced by *Diaporthe* sp. fungal strains, and might be transformed *in planta* (either by a shift of fungal metabolism when living inside plant roots, or by biochemical modifications performed by plant or other associated microorganisms)... It is interesting to note that *Polygonum senegalense* (belonging to same family of the Polygonaceae as *Fallopia*) has been shown to be associated with a leaf fungal endophyte of the genus *Alternaria* able to produce the same type of altenusin derivatives as *Diaporthe* sp. (namely altenusin, desmethylaltenusin, altenuen and alternaric acid), which were also detected in *P. senegalense* healthy leaf extracts (Aly et al., 2008). These compounds were shown to possess cytotoxic activities, but their ecological role is still to be determined.

Fusarium oxysporum produces bikaverin, norbikaverin, fusaric acid, deshydrofusaric acid, four specific compounds of the *Fusarium* genus. The two first demonstrated antifungal and antiprotozoal activities (Limón et al., 2010) whereas the two other showed anti quorum-sensing properties (Tung et al., 2017).

We were not able to detect any secondary metabolite in *Trichoderma koningii* extracts because of the too low quantities of biological material obtained for this strain.

Table 4B.2: Spectral information of fungal secondary metabolites identified and their provenance. *S: solid culture, L: liquid culture.*

Code	RT (min)	$[M+H]^+$ (*m/z*)	MS/MS (*m/z*)	Formula	Identification	Culture type (S/L-isolate)	References
C1	1	182,079	165; 136; 119	$C_9H_{11}NO_3$	L-tyrosine	S-SAT	Spectrum MoNA0104844
C2	1,78	161,057	143; 133; 115	$C_{10}H_8O_2$	1,8-dihydroxynaphtalene (DHN)	S-FACd	Pombeiro-Sponchiado *et al*., 2017
C3	3,42	220,118	202; 184; 142; 124	$C_9H_{17}NO_5$	Pantothenic acid	S/L-SAT	Spectrum FiehnHILIC0006555 ; Sugie *et al*., 2001
C4	3,96	169,047	151; 133; 123; 109	C_8H_8O4	Homogentisique acid (HGA)	S-FAX	Spectrum CCMSLIB000057208516
C5	4,34	196,096	178; 150; 122; 107	$C_{10}H_{13}NO_3$	*O*-Benzyl serine	L-JAT	Du *et al*., 2005 ; QG. Sathyaprabha *et al*., 2011
C6	4,6	322,126	193	$C_{17}H_{15}N_5O_2$	Penilloïd A	S/L-FNCd	He *et al*., 2013
C7	4,8	255,096	219; 195; 165; 131	$C_{11}H_{14}N_2O_5$	Agaridoxin (GDHB)	S-MAT/JNX/SAT/SNZn/FAX/FACd	Pombeiro-Sponchiado *et al*., 2017
C8	5,59	178,085	160; 132; 117; 105	$C_{10}H_{11}NO_2$	Deshydrofusaric acid	S-CAZn	Idris *et al*., 2003 ; Capasso *et al*., 1996
C9	6,15	175,085	158; 130; 103	$C_{10}H_{10}N_2O$	Indole-3-acetamide (IAM)	S/L-SAT/CAZn/ JNX	Luo *et al*., 2016
C10	6,28	251,126	215; 200; 173; 157; 145; 123	$C_{14}H_{18}O_4$	Lachnumfuran A	S/L-JNX	Shan *et al*., 1996
C11	6,31	191,079	173; 155; 145; 130	$C_{10}H_{10}N_2O_2$	Chrysogine	S/L-FNCd	Ali *et al*., 2013 Garcia-Estrada *et al*., 2011 ; Scott et Kennedy, 1976
C12	6,76	193,047	165; 135; 119	$C_{10}H_8O_4$	1,3,6,8-tetrahydroxynaphthalene (THN)	S-JNX/FAX	Pombeiro-Sponchiado *et al*., 2017
C13	7,42	180,099	162; 134; 117	$C_{10}H_{13}NO_2$	Fusaric acide	S-CAZn	Idris *et al*., 2003 ; Capasso *et al*., 1996
C14	8,1	309,09	291; 273; 255; 245; 227; 203	$C_{15}H_{16}O_7$	Hydroaltenusine	S-FACd	
C15	8,2	277,068	261; 219; 207; 191; 177; 163	$C_{14}H_{12}O_6$	Desmethylaltenusin	S-FACd	Aly *et al*., 2008
C16	8,8	176,068	130; 103	$C_{10}H_9NO_2$	Indole-3-acetic acid (IAA)	S-SAT/CAZn/JAT/FACd/SNZn	Spectrum MoNA0100177 ; Luo *et al*., 2016
C17	9,2	267,120	231; 203; 185; 157; 142; 131	$C_{14}H_{18}O_5$	Lachnumlactone A/ Papyracon A/ Papyracon D	S/L-JNX	Nielsen et Smedsgaard, 2003
C18	9,37	265,148	219; 144; 120; 103	$C_{14}H_{20}N_2O_3$	Penipacid B	L-FNCd	Li *et al*., 2013
C19	9,5	279,084	219; 191; 145; 121	$C_{14}H_{14}O_6$	Alternaric acid	S-FACd	Aly *et al*., 2008
C20	10,05	293,099	275; 257 ;229 ; 151	$C_{15}H_{16}O_6$	Altenuene	S-FACd	Ostry, 2008
C21	10,31	289,067	243; 207; 177	$C_{15}H_{12}O_6$	Deshydroaltenusin	S-FACd	Nakanashi *et al*., 1995 Ostry, 2008
C22	10,31	389,145	351; 323; 267; 179	$C_{17}H_{24}O_{10}$	Géniposide	L-MAT	Lelono *et al*., 2009
C23	10,43	420,162	351; 335; 318; 289; 261; 234	$C_{22}H_{21}N_5O_4$	Glandicoline B	S-FNCd	Ali *et al*., 2013 Garcia-Estrada *et al*., 2011 ; Scott et Kennedy, 1976
C24	10,75	291,085	273; 255; 227; 199	$C_{15}H_{14}0_6$	Alténusine	S-FACd	Liu *et al*., 2016 ; Johann *et al*., 2012 ; Aly *et al*., 2008 ; Ayer et Racok, 1990 ;
C25	10,8	434,180	403; 334; 318; 289	$C_{23}H_{23}N_5O_4$	Meleagrine	S/L-FNCd	Ali *et al*., 2013 Garcia-Estrada *et al*., 2011 ; Scott et Kennedy, 1976 ; Kristian
C26	11,4	390,192	322 ; 193	$C_{22}H_{23}N_5O_2$	Roquefortine	S/L-FNCd	Ali *et al*., 2013 ; Garcia-Estrada *et al*., 2011 ; Scott et Kennedy, 1976
C27	11,95	195,099	177; 163; 147; 135; 117; 107	$C_{11}H_{14}O_3$	Butylparabène	S-FACd/SNZn	Andersen *et al*., 2009
C28	12,09	273,073	255; 240; 227; 212; 199; 185; 171; 129	$C_{15}H_{12}O_5$	Djalonensone	S-FACd	Ostry, 2008
C29	12,7	369,06	352; 341; 297; 125; 107	$C_{19}H_{12}O_8$	Norbikaverin	S/L-CAZn	Nielsen et Smedsgaard, 2002 ; Deshmukh *et al*., 2014
C30	13,6	383,073	355; 340; 323; 270; 227	$C_{20}H_{14}O_8$	Bikaverin	S/L-CAZn	Nielsen et Smedsgaard, 2002 ; Deshmukh *et al*., 2014
C31	14,1	316,28	282; 270; 252; 240; 298; 170; 135; 109	$C_{18}H_{37}NO_3$	Deshydrophytosphingosine	S/L-FAX	Lee et Kim, 2002
C32	14,4	298,27	280; 250; 133; 121; 109	$C_{18}H_{35}NO_2$	*trans*-4-*trans*-8-Sphingadienine	S/L-FAX	Ohnishi *et al*., 1996 ; Lee et Kim, 2002
C33	14,46	318,29	300; 282; 270; 264; 252; 109	$C_{18}H_{39}NO_3$	Phytoshingosine	S/L-FAX	Ali *et al*., 2018 ; Bielawski *et al*., 2006

Table 4B.3: Production of secondary metabolites by fungal root endophytes analysed and their reported biological activities. *The "/" symbol indicates different identification propositions of a same compound.*

Compound	Isolate	Compound family	Biological activities
Altenusin	*FACd - Diaporthe* sp.	Biphenyl derivatives	- Antifungal (Johann et al., 2012) - Antioxidant (Chen et al., 2018; Xiao et al., 2014)
Deshydroaltenusin			
Alternarienonic acid			
Hydrated altenusin			
Altenuene			
Djalonensone			
Desmethylaltenusin			
Butylparaben	*FACd - Diaporthe* sp.	Paraben	- Antibiotic (Flasiński et al., 2018) - Antifungal (Liu et al., 2012)
Indole-3-acetic acid (IAA)	*SAT - Epicoccum nigrum* *CAZn - Fusarium oxysporum* *JAT - Codinaea sp.* *FACd - Diaporthe* sp. *SNZn - Ascochyta* sp.	Tryptophane derivatives	- Phytohormone (plant growth promotion) (Luo et al., 2018)
Indole-3-acetamide (IAM)	*SAT - Epicoccum nigrum* *CAZn - Fusarium oxysporum*		
Agaridoxin (GDHB)	*FACd - Diaporthe* sp. *JNX – Lachnum* sp. *SAT - Epicoccum nigrum* *MAT* - unidentified *SNZn - Ascochyta* sp.	Phenolic acids	Melanin precursors (Pombeiro-Sponchiado et al., 2017): - Black pigment - Antioxidant - Protection against abiotic stresses (metal trace elements, temperature extremes) - antimicrobial, antiviral - radio/photo-protection
Homogentisic acid (HGA)	*FAX - Periconia macrospinosa*		
1,3,6,8-Tetrahydroxynaphthalene (THN)	*JNX – Lachnum* sp.	Naphtalene	
1,8-Dihydroxynaphtalene (DHN)	*FAX - Periconia macrospinosa*		
Phytosphingosine	*All isolates*	Sphingolipids	- Plasma membrane structure (Ali et al., 2018) - Cell proliferation and apoptosis (Peer et al., 2010) - Plant signal transduction during stress - Plant – microorganism communication (Ali et al., 2018)
Deshydrophytosphingosine			
trans-4-trans-8-Sphingadienine			
Bikaverin	*CAZn - Fusarium oxysporum*	Naphthoquinones	- Reddish pigment (Limón et al., 2010) - Antifungal; antiprotozoar (Limón et al., 2010)
Norbikaverin			
Chrysogine	*FNCd - Penicillium commune*		- Yellow pigment (Viggiano et al., 2018)
Roquefortine C	*FNCd - Penicillium commune*	Cyclodipeptides	- Antibiotics (Koolen et al., 2012; Kopp and Rehm, 1979; Zheng et al., 2013)
Meleagrin			
Glandicoline B			
Penilloid A			
Pantothenic acid (Vitamin B5)	*SAT - Epicoccum nigrum*	Substituted amino acids	- Melanogenesis stimulation (Albuquerque et al., 2014) - Fungal growth stimulation (Albuquerque et al., 2014) - Precursor of coenzyme A (Raman and Rathinasabapathi, 2004)
O-Benzylserine	*JAT - Codinaea* sp.		- Growth hormone secretagogue in humans (Li et al., 2008)
Lachnumfuran A	*JNX – Lachnum* sp.	-	- Antibiotic (Shan et al., 1996) - Nematicid (Shan et al., 1996)
Geniposide	*MAT* - unidentified	Iridoids	
Fusaric acid	*CAZn - Fusarium oxysporum*	-	- Anti quorum sensing (Tung et al., 2017)
Deshydrofusaric acid			
L-Tyrosine	*SAT - Epicoccum nigrum*	Amino acid	- Melanin precursor and melanogenesis promotor (Slominski et al., 2012) - Antioxidant (Gülçin, 2007) - Metal chelator (Gülçin, 2007)
Penipacid B	*FNCd - Penicillium commune*	Aromatic compound/ Phenolic acids	- Cytotoxic (Dyshlovoy and Honecker, 2015; Li et al., 2013)
Lachnumlactone A / Papyracon A / Papyracon D	*JNX – Lachnum* sp.	-	- Nematicid (Shan et al., 1996)

From this study, a high taxonomic diversity of root fungal endophytes has been identified in *Fallopia* roots. Some of them are more tolerant to MTEs than other strains, and their metabolite production is diverse: we will further explore these properties regarding their potential role in *Fallopia* metal tolerance.

Do root fungal endophytes account for Fallopia tolerance to MTEs?

Fungal endophytes are numerous and diverse in *Fallopia* roots. They may be of importance for *Fallopia* tolerance to metals if: (1) they display poor sensitivity to MTEs (at least when associated with plant roots), (2) they promote plant growth on metal conditions, by (i) directly modulating metal stress, (ii) improving plant resistance against pathogens, (iii) directly increasing growth through hormonal communication, or (iv) improving plant nutrition. These properties are synthesized for each isolate in **Table 4B.4**.

The endophyte *Diaporthe* sp. (FACd) presents almost all properties cited above: tolerant to Cd *in vitro*, this isolate produces melanin, a strong antioxidant and a metal-chelator. In addition, *Diaporthe* sp. isolate produces auxin and biphenyls including desmethylaltenusin. The fact that this compound was detected at significant levels in plant root extracts and that its concentration is strongly affected by metal contamination (Barberis et al., 2020), confirms that this isolate may possess an ecological relevance in *Fallopia* response to metal stress. However, this isolate was not tolerant to Cr or Zn *in vitro*. It remains possible that its association with plant roots would protect it against these metal stresses. The isolate JNX, hypothesized to belong to the *Lachnum* genus, presents similar properties. Unfortunately, its tolerance to metals has not been assessed in this study. This isolate is also likely to help *Fallopia* in metal-contaminated environments. We reviewed the effects of fungal root endophytes on plant metal tolerance (Barberis et al., 2020b) and as far as we know, no study was conducted with these fungi to test their effects on plant metal tolerance.

***Table 4B.4: Synthesis on root endophytes of* Fallopia *x* bohemica.** *The identification of samples, their tolerance to MTEs, their production of metabolites, and their biology according to bibliography are reported. The "-" symbol indicates that this type of metabolite has not been found in this study. An empty case indicates that no data is available. ERM: Ericoid mycorrhiza. ": idem as above. *Biocids: antifungal, antibacterial, antiprotozal or nematicide. °: details and bibliography in* **Table 4B.4**. *In light grey: endophytes that have been isolated on MTE-contaminated media. [1]Identification according to secondary metabolism; [2]Identification according to phylogeny.*

	Taxonomy					Metabolites			
Sample	**Division -mycota**	**Class -mycetes**	**Order**	**Proposed identification**	**MTE tolerance**	**Melanin precursors**	**Phytohormone (IAA or IAM)**	**Biocids***	**Biology relatively to their plant host°**
JACd	Asco-	Dothideo-	Capnodiales	*Cladosporium* sp.					*Plant pathogen or endophyte, may promote plant growth*
CCr2	Asco-	Dothideo-	Capnodiales	*Cladosporium* sp.					"
CT1	Asco-	Dothideo-	Capnodiales	*Cladosporium* sp.					"
SNZn	Asco-	Dothideo-	Pleosporales	*Ascochyta* sp.	*Cd, (Cr, Zn)*	*Yes*	*Yes*	-	*Pathogen or neutral*
MAT	Asco-	Dothideo-	Pleosporales	*Pleosporales* sp.		*Yes*	-	-	*Saprobe, pathogen, endophyte, or hyperparasite*
SAT	Asco-	Dothideo-	Pleosporales	*Epicoccum nigrum*	*Cd*	*Yes*	*Yes*	-	*Endophyte used in disease control*
FAX	Asco-	Dothideo-	Pleosporales	*Periconia macrospinosa*	*Cd, (Cr, Zn)*	*Yes*	-	-	*Improves plant growth, sometimes leaf pathogen, antifungal/antibacterial properties*
CCr5	Asco-	Dothideo-	Pleosporales	*Pleosporales* sp.					*Saprobe, pathogen, endophyte, or hyperparasite*
FNCd	Asco-	Eurotio-	Eurotiales	*Penicillium commune*		-	-	*Yes*	*Promotes plant growth, biocontrol of diseases*
MACd	Asco-	Eurotio-	Eurotiales	*Penicillium glandicola*					"
CCr3	Asco-	Eurotio-	Eurotiales	*Penicillium simplicissimum*					"
JNX[1]	Asco-?	Leotio-?	Helotiales?	*Lachnum* sp.		*Yes*	*Yes*	*Yes*	*DSE or ERM, promotes plant growth*
CT2	Asco-	Leotio-	*Incertae sedis*	*Leotiomycetes* sp.					*May be DSE or ERM*
JAT	Asco-	Sordario-	Chaetosphaeriales	*Codinaea* sp.		-	*Yes*	-	*Pathogen*
FACd	Asco-	Dothideo-	Diaporthales	*Diaporthe* sp.	*Cd*	*Yes*	*Yes*	*Yes*	
CAZn	Asco-	Sordario-	Hypocreales	*Fusarium oxysporum f. sp. dianthi*	*Cd, (Cr, Zn, X)*	-	*Yes*	*Yes*	*Pathogen or endophyte, promotes resistance against pathogens*
FAZn	Asco-	Sordario-	Hypocreales	*Fusarium oxysporum f. sp. dianthi*	*Cd, (Cr, Zn, X)*				"
MAX0	Asco-	Sordario-	Hypocreales	*Trichoderma koningii*					*DSE, promotes plant growth and reduces diseases*
MAX	Asco-	Sordario-	Hypocreales	*Trichoderma* sp.	*Cd, (Cr, Zn, X)*				*Endophyte or saprobe, DSE on roots, may promote plant growth and reduce abiotic stress*
CT3	Asco-	Sordario-	*Incertae sedis*	*Sordariomycetes* sp.					
CCr4	Asco-	Sordario-	Ophiostomatales	*Sporothrix* sp.					
CCr1	Asco-	Sordario-	Xylariales	*Arthrinium* sp.					*Pathogen or endophyte*
CCr7	Asco-	Sordario-	Xylariales	*Arthrinium* sp.					
FNZn	Basidio-	Agarico-	Agaricales	*Coprinopsis urticicola*	*Cd, Zn*				*Increases plant growth, decreases fungal disease*
CCr6	Basidio-	Tremello-	Filobasidiales	*Filobasidiales* sp.					
FNX	ND	ND	ND	ND	Cd, (Zn)				
SNT[2]	Asco?	Dothideo?	Pleosporales?	*Pleosporales* sp.?					

Fusarium oxysporum f. sp. dianthi isolate is more tolerant to MTEs than *Diaporthe* and *Lachnum* isolates, including on multicontamination, and produces phytohormones and biocids. Thus, *F. oxysporum* is a good candidate for improvement of plant metal tolerance. Indeed, *Fusarium oxysporum* has been shown to produce a positive effect on barley growth in Cd-contaminated soil (Mostafa et al., 2019). With *F. oxysporum f. sp. dianthi*, *Trichoderma* sp. is the other isolate to tolerate all tested MTEs, including in poly-contamination. Unfortunately, its metabolite content has not been studied here. Its white color suggests that it does not contain melanin, excluding a DSE nature. Fungi from the *Trichoderma* genus (*T. harzianum*, *T. asperellum*, *T. pseudokoningii*) have positive effects on plant growth (Adams et al., 2007; Firdaus-e-Bareen et al., 2012; Li et al., 2019; Téllez Vargas et al., 2017; Zhang et al., 2018). *T. koningii* is a DSE, known to promote plant growth and to reduce abiotic stress. Nevertheless, its very slow growth did not allow to test its MTE tolerance and its metabolite production. The specific effect of *T. koningii* on metal contaminated environment has also not been tested (Barberis et al., 2020b). Further studies should be conducted on this strain to precise its identification, its metabolome, its biology and thus its potential role in plant metal tolerance.

In metal-contaminated environment, endophytes from the *Penicillium* genus displayed positive effects on plant growth and nutrition, with contrasting effects on plant metal accumulation (Barberis et al., 2020b). However, *P. commune* and *P. glandicola* were not tested previously for their role in plant metal tolerance. *P. commune* is reported to promote plant growth and inhibit plant diseases through the production of toxins; these positive effects should be confirmed in metal-contaminated soil.

Despite being described in literature as pathogen, or neutral to plants, *Ascochyta* sp. isolate is tolerant to Cd, and Cr and Zn to some extent, contains melanin and produces phytohormones. This strain possesses characteristics that may be beneficial for plants in metal contaminated soils, but this has still never been tested (Barberis et al., 2020b). Like *Ascochyta* sp., *Periconia macrospinosa* is tolerant to Cd, and to some extent to Cr and Zn, and this could be related to its production of melanin of different types (HGA and DHN) and its DSE nature (Hall, 1986; Mandyam et al., 2010). Beneficial effects of *P. macrospinosa* isolate on plant growth and disease resistance have been reported (Ginting et al., 2013; Yakti et al., 2018), it seems then legitimate to believe that this isolate could improve *Fallopia* tolerance to MTEs. However, it is also described as a plant pathogen (Sarkar et al., 2019).

Epicoccum nigrum is an endophyte commonly used in disease biocontrol (Hashem and Ali, 2004; Larena and Melgarejo, 2009; Mari et al., 2007). *E. nigrum* is tolerant to Cd only, but possesses melanin and produces phytohormones. Like *Ascochyta* sp., *P. macrospinosa*, *T. koningii*, *Cladosporium* sp. and *Lachnum* sp., the effect of *E. nigrum* on plant metal tolerance has not been tested (Barberis et al., 2020b).

Although *Codinea* genus is described to include plant-pathogens (Dean et al., 2012; Menzies, 1973), we showed that *Codinea* sp. isolate is able to produce phytohormones, it could thus also be a potent beneficial fungal strain.

We lack information on other strains isolated in this study. *Arthrinium* sp. may be an endophyte (Chen et al., 2011; Furtado et al., 2019; Ramos et al., 2010) as well as a plant-pathogen (Gerin et al., 2019; Mavragani et al., 2007); fungi of the *Pleosporales* order possess very various lifestyles, which is also true for *Sordariomycetes* (Maharachchikumbura et al., 2016). *Sporothrix* sp. is rather known as plant and animal pathogen (Rodrigues et al., 2013; Wingfield et al., 1993) but is also reported as endophyte (Khidir et al., 2010; Kwaśna et al., 2016; Toju et al., 2018). Efforts to access to more precise identification of these isolates are necessary and further experiments should be conducted with these isolates to better assess their potent role in plant tolerance to metal stress.

We used here culture-dependent methods to identify plant root endophytes. In order to better assess the ecological role of root fungal endophyte communities in *Fallopia* in the context of metal stress, further field- and controlled- studies shall be conducted by including the quantification of the identified isolates in different metal contexts, and by using meta-barcoding in order to also include unculturable fungi in the analysis. Using meta-barcoding directly on plant roots would improve our knowledge of fungal endophyte communities in *Fallopia* roots and precise their role in plant tolerance to metal stress.

Conclusion

To explore the hypothesis that root fungal endophytes of the *Fallopia* complex species participate in plant tolerance to MTEs, we isolated, identified and characterized 27 isolates from *Fallopia* roots, 8 of them were tested for their metal sensitivity *in vitro*, and 10 isolates were analysed for their secondary metabolite production. Among the identified isolates, few have ever been tested for their role in plant metal tolerance. Here, we could enlighten interesting properties associated with these isolates relative to antioxidant, radical scavenging or metal chelating properties (due to melanin presence), to their *in vitro* tolerance to MTEs, especially to Cd, and to their production of melanin, phytohormone or toxins. *Fusarium oxysporum f. sp. dianthi*, *Diaporthe* sp., *Epicoccum nigrum* and *Ascochyta* sp. isolates displayed all these features and are thus likely to help *Fallopia* to deal with metal stress. In particular, the strain *Diaporthe* sp., which produces desmethylaltenusin, a biphenyl shown to increase in plant roots exposed to Zn or Cr (Barberis et al., 2020), seems then to possess an ecological relevance in *Fallopia* response to metal stress, and this needs to be further explored.

Further experiments shall be conducted for other isolates such as *Trichoderma* sp. and *Lachnum* sp., or the strains *Codinaea* sp. and *Arthrinium* sp. that are rather described as plant pathogens.

In addition, to complete our characterization of *Fallopia* root endophytes, the role of these endophytes on plant metal tolerance has to be tested by direct inoculation to plants, either in sterilised soil, or through bioaugmentation in living soil.

This research may find applications both in phytoremediation, by identifying beneficial fungi that could be used in association with adapted plants to improve the decontamination of metal-polluted soils, or in the control of *Fallopia* expansion, by inhibiting specifically these beneficial fungi that improve plant growth and/or its tolerance to metal stress.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

LB, PB, CBR, FP and SM designed the project; LB conducted the plant experiments; LB and MS isolated root endophytes; PB supervised the fungal collection, MS tested endophyte tolerance to metals; CBR and TN did the molecular identification and analysed the data; TG realised a bibliographic synthesis on the isolated species; GH realised the metabolic analysis. LB synthesized the data and wrote the article, with the constructed commentaries of CBR, PB, FP and SM.

Acknowledgments

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Synthèse

Au total, 27 souches ont été isolées des racines des Renouées asiatiques. Ces souches présentent des propriétés intéressantes vis-à-vis de l'exposition aux ETM, telles que leur tolérance aux ETM (notamment au Cd), la production de composés chélateurs/antioxydants et de phytohormones. Les souches *Diaporthe* sp., *Fusarium oxysporum f. sp. dianthi*, *Cladosporium* sp., *Epicoccum nigrum* et *Ascochyta* sp., du fait de leur tolérance aux ETM et de leur production de composés bénéfiques pour leur hôte (phytohormone) ou protecteur face au stress (mélanine), sont les plus susceptibles d'être bénéfiques à leur hôte en présence d'ETM. Par ailleurs, la souche *Diaporthe* sp. a été identifiée comme productrice de dérivés de l'alténusine (dont la desméthylalténusine, composé commun identifié dans les deux types d'extraits, racinaires et fongiques), ce qui suggère (i) que cette souche pourrait jouer un rôle important dans la tolérance de la plante aux ETM et (ii) qu'une transformation *in planta* de ce composé semble se produire étant donné que dans les racines sont également retrouvés le dérivé glysosylé de la desméthylalténusine, et son dérivé décarboxylé et sulfaté (Article Chapitre 4A). Le rôle de ces métabolites dans l'éventuelle prise en charge des ETM ou de leurs effets reste à démontrer.

Par ailleurs, les traits de croissance ont été mesurés dans les différentes conditions **(Figure 4B.1)**. Malheureusement, la multiplication des conditions (50) a limité le nombre de réplicats par condition et donc la puissance statistique et l'analyse des données. De plus des problèmes liés à la régulation de la température et de l'hygrométrie dans la serre n'ont pas permis d'avoir une croissance des plantes optimale et satisfaisante, ces données n'ont donc pas été valorisées par la suite. Nous nous contenterons d'observer que sans acclimatation, la régénération est meilleure sur Cd (bleu) que sur Zn ($p < 0.05$). Qualitativement nous observons que le morphotype Feyzin en présence de Zn régénère mieux après acclimatation que sans acclimatation, ses masses aérienne et racinaire étant également augmentées par l'acclimatation. Cela nous laisse penser à un effet des endophytes et de l'environnement natif que nous souhaitons explorer : c'est pourquoi nous choisissons pour la suite des expérimentations de nous concentrer sur ce morphotype Feyzin, et de se focaliser sur le zinc comme contaminant, étant donné que ce morphotype pousse sur un sol contaminé à ce métal.

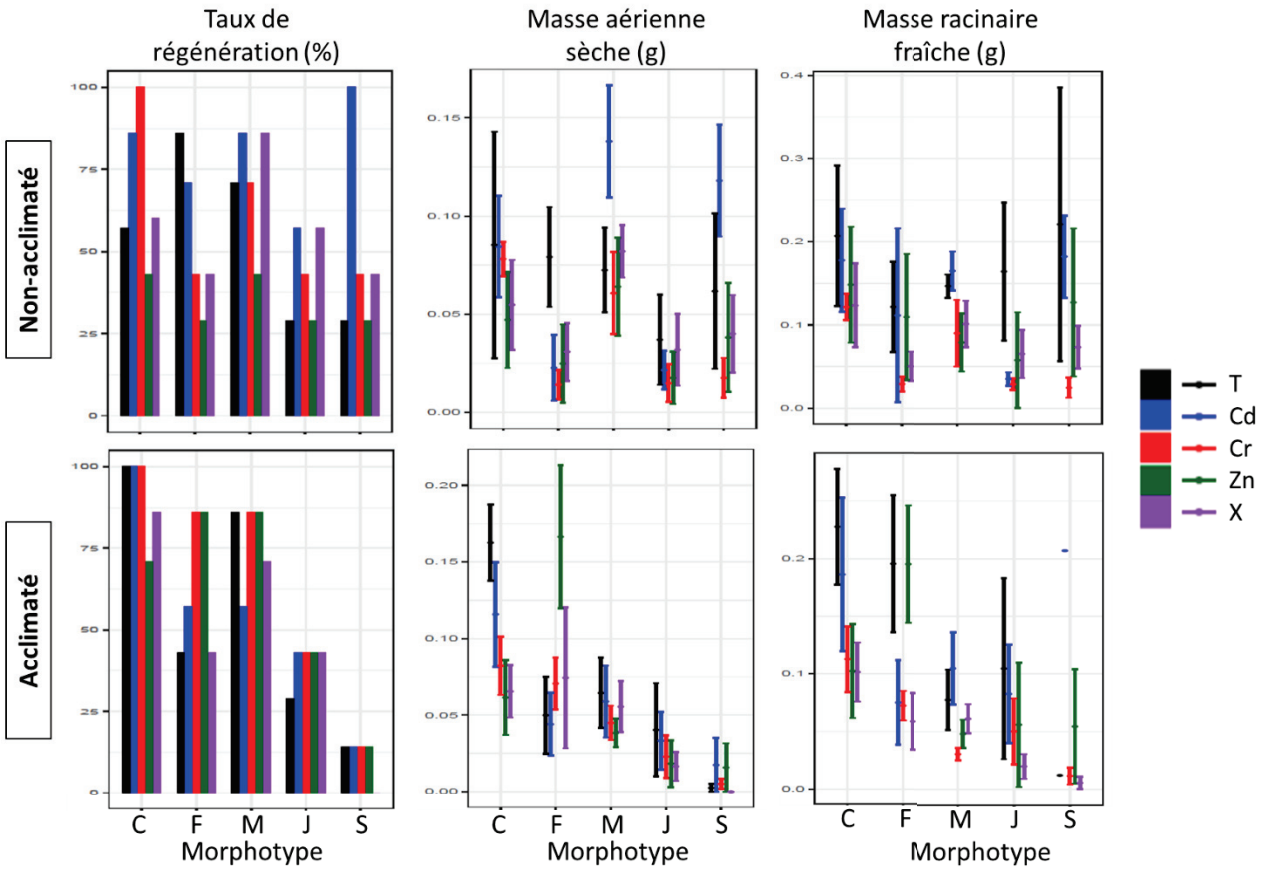
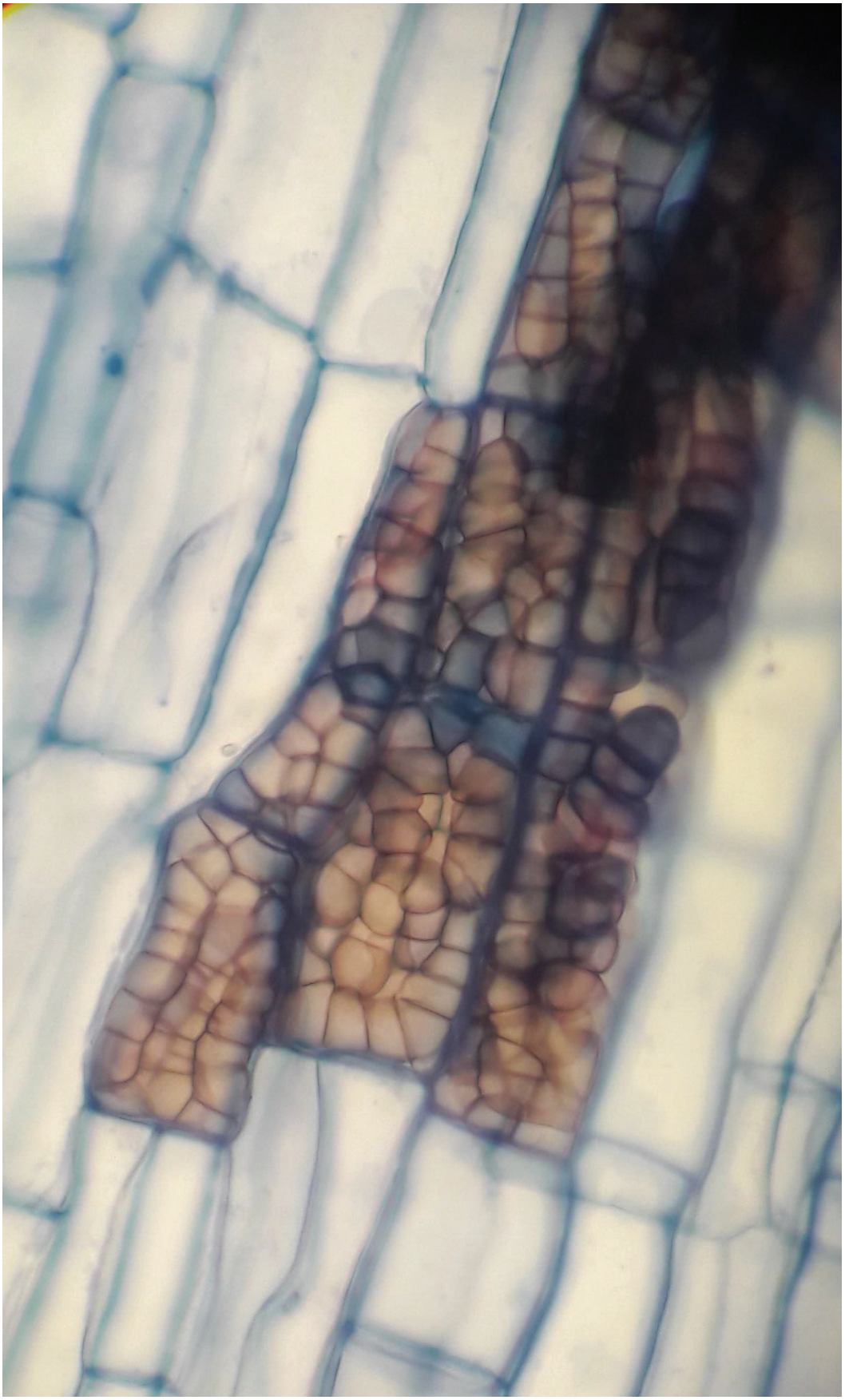


Figure 4B.1 : Traits de croissance des plantes. *C : Caluire, F : Feyzin, M : Miribel (*F. *x* bohemica*), J :* F. japonica*, S :* F. sachalinensis*.*

Nous avons confirmé ici d'un côté, la tolérance des Renouées aux ETM, et de l'autre identifié les endophytes associés à leurs racines et caractérisé la réponse *in vitro* de certains d'entre eux aux ETM, ainsi que leur production de métabolites secondaires pouvant améliorer la réponse de la plante au stress. Dans quelle mesure ces endophytes tolérants aux ETM ont-ils une importance écologique dans la tolérance des Renouées aux ETM ?



Chapitre 5 : Relation entre endophytes fongiques racinaires et tolérance de la Renouée aux ETM (Axe 3)

Préalable

Avec le morphotype de Feyzin soumis à une contamination au Zn, nous cherchons à estimer le taux d'infection par des champignons endophytes dans les racines et à mettre ceci en relation avec les traits de croissance (masses aérienne et racinaire), les modifications de profil des polyphénols racinaires et les concentrations métalliques dans les différentes parties de la plante. Afin de faire varier la diversité et l'abondance en endophytes initialement présents dans les fragments de rhizome indépendamment du traitement métallique et de s'affranchir et de la pression exercée par le zinc dans l'environnement natif, nous réitérons l'usage de l'acclimatation.

J'ai réalisé l'intégralité des expérimentations de cet article ainsi que le recueil et traitement des données, avec l'aide de Dylan ANDEOL, excepté la quantification des endophytes qui, après mise au point du protocole, a été confiée à Sophie Poussineau, technicienne au laboratoire LEHNA et que je remercie pour le long travail qu'elle a réalisé (environ 56 heures d'observation). L'identification des métabolites lors de cette expérimentation comme des précédentes, a été réalisée par Serge Michalet.

Article en préparation

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The small to the rescue of the tall: the growth of *Fallopia* × *bohemica* on Zn contamination favoured by root fungal endophytes

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Abstract

With the intensification of human activities and atmospheric emissions, Zinc (Zn) tends to accumulate in soils impacted by anthropogenic activities. To grow on these contaminated soils, plants evolved different metal tolerance strategies, including the production of various metabolites displaying antioxidant or metal-chelating properties reducing metal toxicity, and the association with beneficial root endophytic fungi that may help it to cope with the stress. **In this study, we investigated the plant-fungi-metal tripartite system in bohemian knotweed with a specific attention to root fungal colonisation, plant metal accumulation, plant growth traits, and root secondary metabolism, hypothesizing that root fungal endophytes participate in the tolerance of their host to Zn contamination.** For that, *Fallopia* x *bohemica* rhizomes collected from a Zn-contaminated site were grown for 30 days in a greenhouse in a meadow soil with or without Zn. To modify native endophyte communities, we used a protocol of acclimation where rhizomes were kept two months before the experiment in a non-polluted and sterilized meadow soil, either reinoculated with fungi (F+) or without (F-).

Through microscopic quantifications of fungal colonisation, we showed that endophytes are correlated with root and shoot concentrations of Zn, but this process is not involved in plant metal

tolerance. Some endophytes, such as *Olpidium*, seem to be beneficial for plant growth in the absence of Zn, whereas others, such as DSEs, are beneficial only in the presence of Zn-contamination. DSEs are more abundant in roots exposed to Zn, leading them to be good candidates for plant metal tolerance improvement. In parallel, we were able to evidence strong correlations between DSE frequency of colonisation in roots and torosachrysone, a compound being increased in roots exposed to metal stress. The precise role of this compound is still to be elucidated, but it could be involved in fungal (including DSEs) recruitment, which could be an illustration of the "Plant call for support" theory.

Introduction

Human activities (housing, industry, road traffic and agriculture) produce atmospheric emissions of trace elements, such as Zinc (Zn). These emissions can be transported from short to long distance, and Zn tends to accumulate in soils impacted by anthropogenic activities (Bourennane et al., 2010; Valavanidis and Vlachogianni, 2010) and crosses the food chain in ecosystems (Meena et al., 2017). Zn originates from lithogenic sources, but also from the use of fertilisers and pesticides (Senesil et al., 1999), road traffic (Meena et al., 2017; Nikolaeva et al., 2019) and coal combustion (Bourennane et al., 2010). Zn is an essential element for plants, but it turns to be toxic at high concentrations (Rizwan et al., 2019). Indeed, Zn in excess (> 1 mg/L) provokes oxidative stress, affects root and shoot growth, induces chlorosis and decreases photosynthesis rate (Påhlsson, 1989; Yadav, 2010). Zn also disturbs nutrient equilibrium in plant and nitrogen metabolism (Påhlsson, 1989).

Some plants developed metal tolerance strategies, either specific to Zn, or to a larger panel of metal contaminants (Påhlsson, 1989). Two main strategies are used to adapt to metal stress: either metal exclusion, or metal accumulation (Baker, 1981). The first consists in maintaining a constant low concentration of metal in plant tissues despite high concentrations in soil; this results from the phytostabilization of metals in soil or their active efflux outside roots. Metal accumulation is defined by higher concentrations of MTEs in plant tissues than in soil, and this results from their active uptake in roots and their transfer and sequestration into plant tissues.

Whether it is for exclusion or accumulation, a chemical adaptation of plants is required, particularly to deal with the oxidative stress generated by metals; this is especially true in the accumulating plants were homeostasis has to be maintained (Singh et al., 2016; Tangahu et al.,

2011). For example, metal tolerance includes the production of reduced glutathione (GSH), which reduces oxidative stress. GSH is also the precursor of phytochelatins involved in metal transport and sequestration (Yadav, 2010). Singh et al. (2016) underlined the importance of omics studies to understand plant metal tolerance: from ionomics to proteomics and metabolomics, a wide variety of ions, peptides and small molecules such as polyphenols take part in limiting metal toxicity. Polyphenol synthesis in particular is reported to increase with the presence of metals (Singh et al., 2016). Polyphenols are strong antioxidant, their numerous hydroxyl and carboxyl groups make them good metal chelators, and their highly oxygenated and conjugated structures allow them to scavenge free radicals. Individual phenols could have also specific roles in plant metal tolerance. For example, in *Pteris vittata*, increased concentrations of 3-caffeoylquinic acid and A-type procyanidin dimer are observed in roots of plants that grew on a metal-contaminated soil in a mining site, whereas concentrations of acetylated caffeoylquinic acid are decreased (Pham et al., 2017).

Chemical responses to Zn, and more widely to other metal trace elements (MTEs) may admittedly come from plants, but also from fungi via mutualistic interactions. It is suggested that the root fungus *Penicillium funiculosum* enhances plant tolerance to metal through the fungal production of gibberellins, which are plant growth promoting hormones (Khan and Lee, 2013). Other metabolites produced by endophytes, such as glutathione, phytochelatins, metallothioneins, directly chelate metal ions inside the root. Outside the root, metal-binding metabolites, such as citrates, organic acids, siderophores, exopolysaccharides (EPSs), and phenolic compounds may also be exuded by endophytes, hence leading to the immobilization of metals in the rhizosphere and to the modulation of their bio-availability (Domka et al., 2019b). Indirectly, secondary metabolites produced by endophytes may also improve plant tolerance through the activation of plant defence systems. In the other way, plants may also modulate their root secondary metabolism in order to attract and recruit beneficial microorganisms that could help it to cope with the stress; this is the “plant call for support” hypothesis (Thijs et al., 2016).

Plants often associate with fungi leading to beneficial interactions through mycorrhizal or non-mycorrhizal associations. Non-mycorrhizal fungal endophytes are facultative symbionts, which lack specialized exchange interface (Brundrett, 2009) but promote plant growth (Hiruma et al., 2018). Fungal endophytes are encountered all over the world in a wide variety of plant hosts and environments (Barberis et al., 2020b). On metal-enriched soil, the presence of both mycorrhizal (Pawlowska et al., 1997; Weissenhorn et al., 1993) and non-mycorrhizal (Barberis et al., 2020b;

Domka et al., 2019b) endophytic fungi is reported in plant roots. Most of the time in the context of metal stress, including Zn stress, fungal root endophytes are beneficial for plants by stimulating their growth and/or by limiting metal toxicity, however, the mechanisms of plant fungal-associations in the context of metal contamination remains unclear, as fungal endophytes either promote plant metal accumulation and translocation, or on the contrary are involved in their exclusion (Barberis et al., 2020b).

Root fungal associations depend on five factors: fungal species pool, dispersal, abiotic habitat filters, host-imposed habitat filters, and microbial species interactions (Saunders et al., 2010). Abiotic habitat filters include MTE concentrations in soil. For example, Dark Septate Endophytes (DSEs) are more abundant in metal-rich soils than in non-contaminated ones (Liu et al., 2017). Host-imposed habitat filters are also related to abiotic habitat filters since plant physiology and biochemistry depend on physicochemical changes in the environment. When conditions change, then plant-endophyte associations must adapt or acclimate, either by plant-driven recruitment of adapted endophytes, or by the adaptation of already present endophytes. Changing soil metal concentrations, for example by exposing plants grown on a metal-contaminated soil to a non-contaminated soil might then modulate plant-endophyte associations and this can be used as an experimental mean of modification of plant native endophytic associations; this experimental procedure being further called “acclimation”.

In this context, we used the invasive bohemian knotweed *Fallopia* x *bohemica* as model plant to investigate the plant-fungi-metal tripartite system. Indeed, *F.* x *bohemica* is able to grow on highly metal-contaminated soils (Sołtysiak and Brej, 2014), accumulates MTEs in leaves (Rahmonov et al., 2014) and regenerates easily with rhizomes (Bailey et al., 2009; Pyšek et al., 2003). *F.* x *bohemica* roots are colonised by fungal endophytes including DSEs but they are not mycorrhized (Gucwa-Przepióra et al., 2016). Among root fungal endophytes, DSE and *Olpidium* are easily identifiable in *Fallopia* roots (personal observations). *F.* x *bohemica* biomass was neither affected by Zn mono-contamination (400 mg/kg of soil), nor by other metals such as chromium - Cr and cadmium - Cd (Barberis et al., 2020). Bohemian knotweeds are known to contain a high diversity of polyphenols in their roots, including procyanidins, anthraquinones, stilbenes, cinnamic acids (Piola et al., 2013), or biphenyls (Barberis et al., 2020). Stilbene, anthraquinone and some biphenyl concentrations were reported to increase in roots of knotweeds exposed to Zn (Barberis et al., 2020). Torosachrysone, an anthraquinone derivative and one of the most increased metabolite in roots exposed to metals (Barberis et al., 2020; Michalet et al., 2017), is encountered

in both plants and fungi (Gill et al., 2000; Kitanaka and Takido, 1984), whereas biphenyl derivatives, desmethylaltenusin and derivatives, are exclusively described in the fungal reign (Aly et al., 2008; Yuan et al., 2018b).

We hypothesize that fungal root endophytes, especially DSEs, play a major role in the tolerance of their host to Zn contamination. To test this hypothesis, knotweed rhizomes collected from a Zn-contaminated site were grown for 30 days in a greenhouse in a meadow soil with or without Zn. To modify native endophyte communities, we used a protocol of acclimation, i.e. a change of soil conditions (biotic and abiotic), where rhizomes were conserved for two months before the experiment in a non-polluted and sterilized meadow soil, either reinoculated with fungi (F+) or without (F-). This acclimation phase releases Zn environmental pressure and offers a new pool of endophytes (or not). We then expect: *(1) a modification of native fungal communities during acclimation, (2) an increase in endophytes, and particularly DSEs, in roots in the presence of Zn, (3) a better regeneration and growth of plants with increased colonisation by fungal endophytes, (4) a change in root metabolism under Zn contamination.*

Material and methods

Plant collection and acclimation

Rhizome fragments of *F.* x *bohemica* were collected in December 2018 (for the acclimation experiment) and in February 2019 (corresponding to non-acclimated rhizomes) in Feyzin (France) next to a petrol station and motorways (45.6787° N; 4.8466° E), where the soil is contaminated with Zn (455 mg/kg). At both time of collection, plants were in dormant phase with no photosynthetic activities. For the acclimation experiment, 80 kg of meadow soil (< 15 - 20 cm depth) exempt of heavy metal pollution were collected at La Côte Saint-André (45.3769° N; 5.2772° E), sieved (4.5 mm mesh size) and sterilised with gamma-rays (25 min., 50 kGy max). The same soil was used for greenhouse experiment but without sterilisation. Soil physicochemical characteristics of the collecting sites are presented on **Table 5.S1**.

Table 5.S1: Physicochemical characteristics of the soils corresponding to each collection site. *In bold: high MTE content.*

Parameter	Meadow soil (used for acclimation and plant culture)	Feyzin
Sand (%)	43.0	56.0
Silt (%)	43.3	19.6
Clay (%)	13.7	10.8
pH $_{H2O}$	5.76	8.02
C_{org} (g/kg)	27.5	22.5
OM (g/kg)	47.3	39.0
C:N ratio	10	ND
CEC ($cmol^{(+)}.kg^{-1}$)	10.50	7.93
Cd (mg/kg)	< 0.1	0.9
Cr (mg/kg)	23.4	34.7
Cu (mg/kg)	8.2	20.3
Ni (mg/kg)	13.7	19.3
Pb (mg/kg)	32.9	41.9
Zn (mg/kg)	42.9	**455.0**

For the acclimation experiment, washed rhizomes were acclimated for two months in two different soil conditions: either in the presence of soil fungi (F+), or without (F-). For that, after sterilisation, half of the soil was reinoculated at 10 mL / kg with total “soil juice” (prepared with 100 g of fresh soil shaken in 1 L of distilled water), the other half was reinoculated with the same “soil juice” but filtered at 10 µm, in order to reintroduce soil microflora communities deprived of fungi (Bobbitt and Betts, 1992). Two plastic pots of 100 L were filled with fine layers, alternating soil, juice, and rhizomes. Both pots were covered and conserved outside during 6 weeks in winter: rhizomes remained in dormant phase, without regenerating, as in the case of field plants.

Plant culture

A greenhouse experiment was conducted between February and March 2019. It followed the protocol published by Barberis et al. (2020), with modifications. Soil was rehydrated to its field capacity (40% m/v) by adding either Ultra-Pure water (UP) (control: T) or a solution containing $ZnCl_2$ to achieve 400 mg Zn / kg of soil (Z), concentration slightly higher than the recognized pollution threshold (300 mg / kg; Baize et al. 2007) and similar to *in situ* rhizosphere soil level. Rhizomes acclimated with fungi (F+) or without (F-) and not-acclimated rhizomes (N) were washed, and fine roots removed. Then 100 ± 1 g of moistened soil were placed into 0.15 L plastic pots and fresh rhizome fragments containing one node (average weight of 0.8 ± 0.1 g) of *F.* x *bohemica* were planted and grown in greenhouse (10 h day/14 h night, temperatures from 16°C to 29°C). Soil moisture was manually controlled by adding tap water every two-three days. Water was added from the bottom of the pot (in the plate), so that field capacity was relatively maintained by capillarity throughout the whole experiment.

The experimental design was as follow: 3 acclimation conditions (N, F+ and F-) * 2 metal conditions (T and Z) * 15 biological replicates per condition.

Plant growth traits

After 30 days of growth, plants were delicately uprooted, and the subterranean parts washed with tap water. Root, rhizome and shoot fresh biomasses were measured, and 20 root fragments of 1 cm length, randomly distributed across the whole plant root system, were dropped in ethanol 70 % for fungal colonisation quantification. The resting part of roots was immediately dropped into liquid nitrogen for secondary metabolism analysis. Rhizome and shoots were dried at 65°C during 24h before weighing and were further used to analyse their metal content.

Extraction and analysis of compounds in root extracts

Fine roots were freeze-fried. To get enough root material for metabolite analysis, we randomly kept 5 samples/condition that contain more than 10 mg dry root. A pool of 12 samples (2 per conditions) constituted the quality control (QC).

The extraction protocol follows Barberis et al. (2020): dry roots were grounded (TissueLyserII® - Qiagen; F= 300/sec., 3 min.) and sonicated with a 1 mL mixture of MeOH-H_2O (1:1) for 15 minutes. After the supernatant was removed, the remaining powder was sonicated with 1 mL MeOH and

both supernatants were pooled and concentrated under vacuum (Speedvac® - Labenco) before being stored at -20°C, and dissolved at 10 mg/mL in MeOH 80% before analysis. Samples were analysed by UHPLC-DAD-ESI/QTOF (Agilent 1290 infinity linked with Agilent ESI/QTOF 6530, Agilent Technologies, USA), using a Poroshell® 120 EC-18 column (2.7 µm, 3.0 x 100 mm; Agilent Technologies, USA). The gradient between 0.1% formic acid in water (A) and acetonitrile (B) was as follows: 1% of B from 0 to 1.5 min, and growing with a linear gradient to 15% of B at 8 min; 60% of B at 14 minutes and 100% of B at 16 min for 1 min. All solvents were LC-MS grade (Optima). The flow rate was adjusted at 1.0 mL/min and the injection volume was 2 µL. UV spectra were recorded between 190 and 600 nm. The ESI source was optimized as follows: positive and negative ionization in AutoMS/MS mode, scan spectra from *m/z* 80 to 2000, capillary voltage 3.5 kV, fragmentor 100 V, fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 12 L/min and a temperature of 310°C at 40 psi.

Compounds were identified by analysis of their UV, HRMS and HRMS/MS spectra using MassHunter Qualitative Analysis (Agilent Technologies, USA) and by comparison with literature.

Quantification of endophytes

Roots were decolored with two successive water-baths in KOH 10 % at 90°C during 1 h and 30 min respectively. Then, roots were rinsed with distilled water, dropped in HCl 1 % during 1 h at ambient temperature, rinsed and stained in methyl blue (Sigma-Aldrich) at 0.05 % and let overnight at 4°C. Stained roots were then stored at 4°C in acid glycerol (50 % glycerol, HCl 0.05 % in distilled water) before microscopic observation. For endophyte quantification, we adapted the method of Trouvelot et al. (1986) previously designed for mycorrhizal quantification. We distinguished three types of endophytes: DSEs, with septate microsclerota and melanised hyphae with few ramifications (Jumpponen and Trappe, 1998); *Olpidium*, under the form of chlamydospores, either hexagonal or round (Tomlinson and Garrett, 1964); and other endophytes, group containing all other fungal forms that could not be clearly attributed to DSEs or *Olpidium*. Endophytes were quantified on 20 root fragments of 1 cm for each plant: the area covered by each type of fungi was visually estimated for each root fragment and attributed to one of 6 possibilities : no fungi, trace (class 1), < 10 % (class 2), 10 – 50 % (class 3), 50 – 90 % (class 4), > 90 % (class 5). Three indices were then calculated:

Frequency of colonisation **F** = number of colonized fragments / N

Intensity of colonisation **I** = (95n5 + 70n4 + 30n3 + 5n2 + n1) / N

Intensity of colonisation in colonized fragments **i** = I * 100 / F

With N = number of observed fragments; n1, n2, n3, n4 and n5 corresponding to the number of fragments in class 1, 2, 3, 4 and 5 respectively.

To ensure the reliability of endophyte quantification, all microscopic observations were done by a single person, in a random order and blinded from culture conditions. The use of 20 fragments/plant, representative on the whole root system, in 15 plants/conditions, gives a confident basis for comparison of the endophytic colonisation between conditions. This microscopic quantification of fungal colonisation in all root fragments collected in this study took around of 56 hours of observations in total.

Zn content in plant and soil

Sub-samples of dried rhizomes and shoots were powdered, then 100 mg were digested at 125°C for 180 minutes in 4 mL of nitric acid (68%) and 1 mL H_2O_2 (35%) using a Heating digester (DK6, Velp Scientifica). 25 mL of deionized water were then added before analysis by atomic absorption spectrometry with an acetylene flame atomizer (SpectrAA 220 Z; Varian, France). Zn contents were measured at the specific wavelength of 213.9 nm. The HNO_3/H_2O_2 reagent was used as blank. Soil metal content was analyzed at the CRPG of Vandoeuvre-les-Nancy. Briefly, after grinding and homogenization, soil samples were mineralized by HF, then Cr and Zn concentrations were measured by ICP-AES (NF ISO 220-36) and Cd concentrations by ICP-MS (NF EN ISO 17294-2).

Statistics

All statistical analyses were achieved by using R x64 3.4.1 (R Core Team, 2017).

Vegetative traits and foliar metabolites

The same statistical protocol was used as in Barberis et al. (2020). For regeneration, we used pairwised comparison of proportions, considering that a rhizome regenerated when we could measure a height of at least 0.5 cm. We computed ANOVAs type II (package car (Fox et al., 2019)), which are adapted to unbalanced data (7 to 15, because of the absence of regeneration of certain plants). Acclimation, Zn and their interaction were used as explicative variables and trait measures, endophyte indexes or metal concentrations as dependent variables. ANOVAs type II are valid only if they do not find interaction, which is the case in our study. Then ANOVA residuals were tested for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test).

To discriminate Zn effect, pairwise tests were computed within each genotype for each trait. Pairwise tests were chosen according to Shapiro-Wilk normality test and Bartlett test of homogeneity of variances on ANOVA residuals. When both normality and homoscedasticity were respected, then a Student pairwise test pooling variances was performed; if only homoscedasticity was not true, then a Student pairwise test without considering variances as equal was achieved; otherwise, pairwise comparisons using non-parametric Wilcoxon rank sum test were done. All pairwise tests were performed with Holm corrections of p-values. To precise the effects on endophytes and on metal concentrations, we repeated those tests within each acclimation condition, and within each Zn condition.

Correlations were searched with linear models between vegetative traits (aerial and root masses) and colonisation by endophytes, as well as between Zn concentration in plant (aerial and root parts) and colonisation by endophytes. We used $p \leq 0.01$ and $r^2 > 0.2$ as criterions for strong significance and relevance of correlations.

Root secondary metabolites profiling

To evaluate differences in metabolite profiles between treatments, peaks in UV chromatograms recorded at λ 280 nm were integrated and aligned into a matrix (by comparison with QC) to perform multivariate analyses. To eliminate contaminant incorporation in the analysis, we selected all the integrated peaks with peak area above 2 mAU in at least one condition, giving a total number of 108 peaks. In order to limit the variations due to differences in concentration between extracts, peak areas were expressed relatively to the sum of all integrated peaks for each sample.

Principal Component Analysis (PCA) (package ade4, Dray and Dufour, 2007) was achieved using the scaled relative area of peaks detected in each sample as dependent variable, peak number as active element, and acclimation and Zn as illustrative elements. Three axes were kept.

We identified in priority compounds that explain the best the separation of samples according to their Zn treatment and to their acclimation.

Results

Root fungal endophytes colonisation

The global intensity of root colonisation by all endophytes quantified in roots (i.e. DSEs, *Olpidium* and other forms of fungi) is significantly reduced from around one third after acclimation treatment **(Figure 5.1 top left)**. On the other hand, in rhizome acclimated in F- soil, the global intensity of root colonisation by endophytes was multiplied by two in the presence of Zn, whereas in the other conditions this augmentation is not significant, though it is significant when all conditions are included **(Figure 5.1 top left)**. In F- soil, Zn increases significantly DSE frequency of colonisation, and to a less extent that of *Olpidium*, whereas in the other conditions Zn does not affect *Olpidium* or DSEs frequencies, but it rather increases the intensity of colonisation in colonised root fragments, though this effect is not significant **(Figure 5.1 right)**.

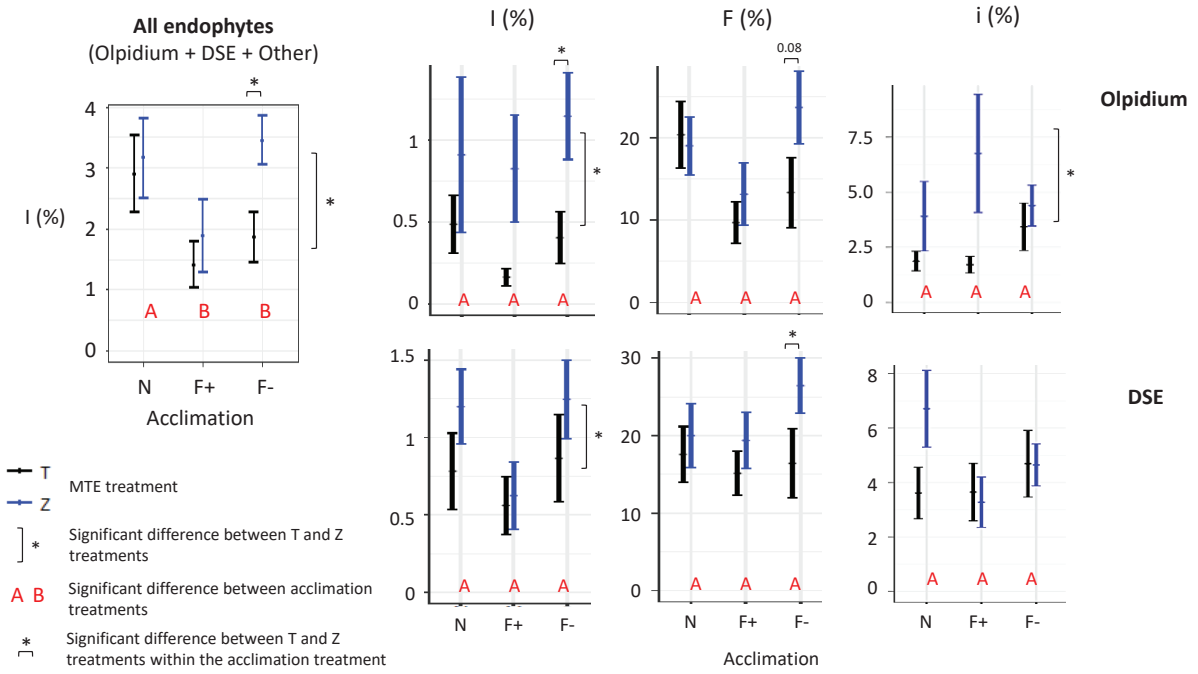

Figure 5.1 : Colonisation by fungal endophytes according to acclimation and Zn treatment. *I: global intensity of colonisation; i: intensity of colonisation in colonised fragments == clustering; F: frequency of colonisation == repartition of the colonisation. N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi; T: control without Zn, Z: zinc (400 mg/kg). Significant differences determined at p-value < 0.05.*

Finally, independently of acclimation condition, *Olpidium* and DSE global intensities of colonisation increase with Zn treatment (x 2 and x 1.5 respectively), and the intensity of colonisation by *Olpidium* in colonised fragments is also doubled with Zn.

Zn content in plant parts

Zn was not detectable in plant parts cultivated in control soils **(Figure 5.2 left)**. In the presence of Zn contamination, Zn was detected in both rhizomal and aerial parts of the plants (between 100 and 1300 mg/kg). Aerial parts accumulated more Zn than underground parts, especially in both conditions of acclimation (x 1.5 in acclimated plants).

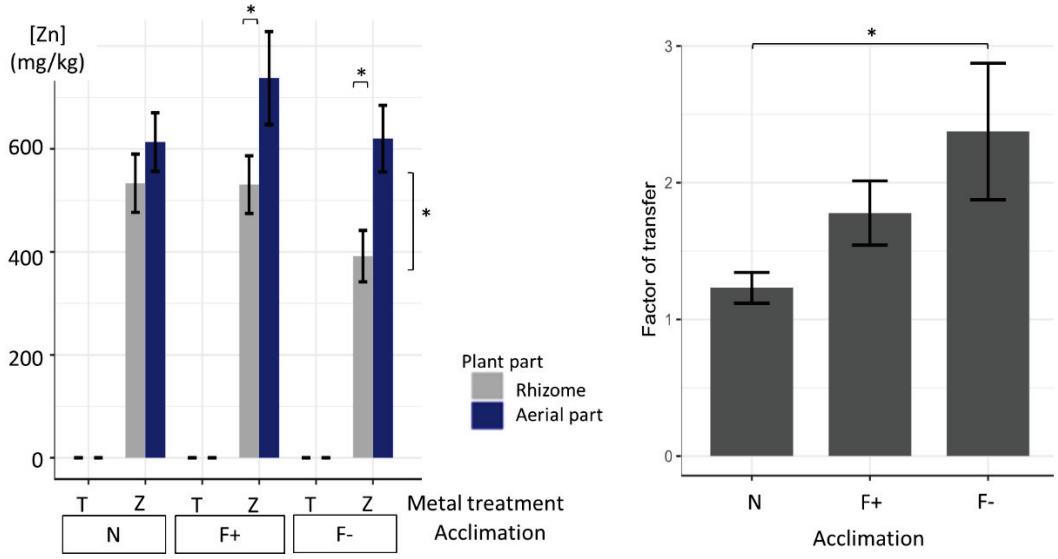

Figure 5.2: Zn concentrations in plant parts and root-to-shoot transfer factor, according to acclimation and metal treatment. *Left: Zn concentrations in plant parts, Right: Root to shoot factor of transfer on Zn contamination. N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi, T: control without Zn, Z: zinc.*

Zn root-to-shoot translocation factor is above 1 for 75 % of plants, meaning that *F.* x *bohemica* slightly concentrates Zn in its aerial parts. This factor is significantly increased after acclimation in F- soil compared to no acclimation **(Figure 5.2 right)**.

Linear models did not show any significant correlation between Zn concentration in plant tissues and root colonisation by fungal endophytes.

Regeneration percentage

All non-acclimated rhizomes (N) regenerated, both in presence and in absence of Zn **(Figure 5.3)**. We observed a negative effect of acclimation with fungi (F+): only 80 % and 60 % of rhizomes regenerated on non-contaminated and Zn-contaminated soils, respectively. This negative effect is partially lifted with the acclimation in F- soil where around 80 % of rhizomes regenerated in both control and Zn conditions, without significant differences with the two other acclimation conditions. Considering all acclimation treatments, no significant effect of Zn was observed on rhizome regeneration.

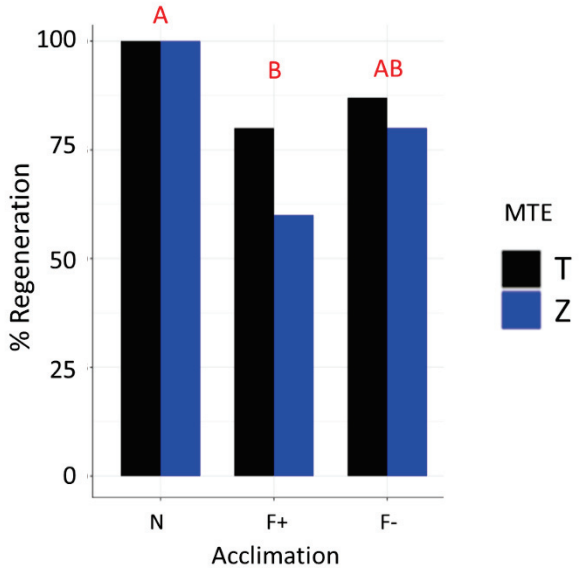

Figure 5.3: Regeneration percentage of rhizomes. *N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi, T: control without Zn, Z: zinc (400 mg/kg). Non-sharing same letters mark significant differences (p-value < 0.05) between acclimation treatments.*

Plant growth traits

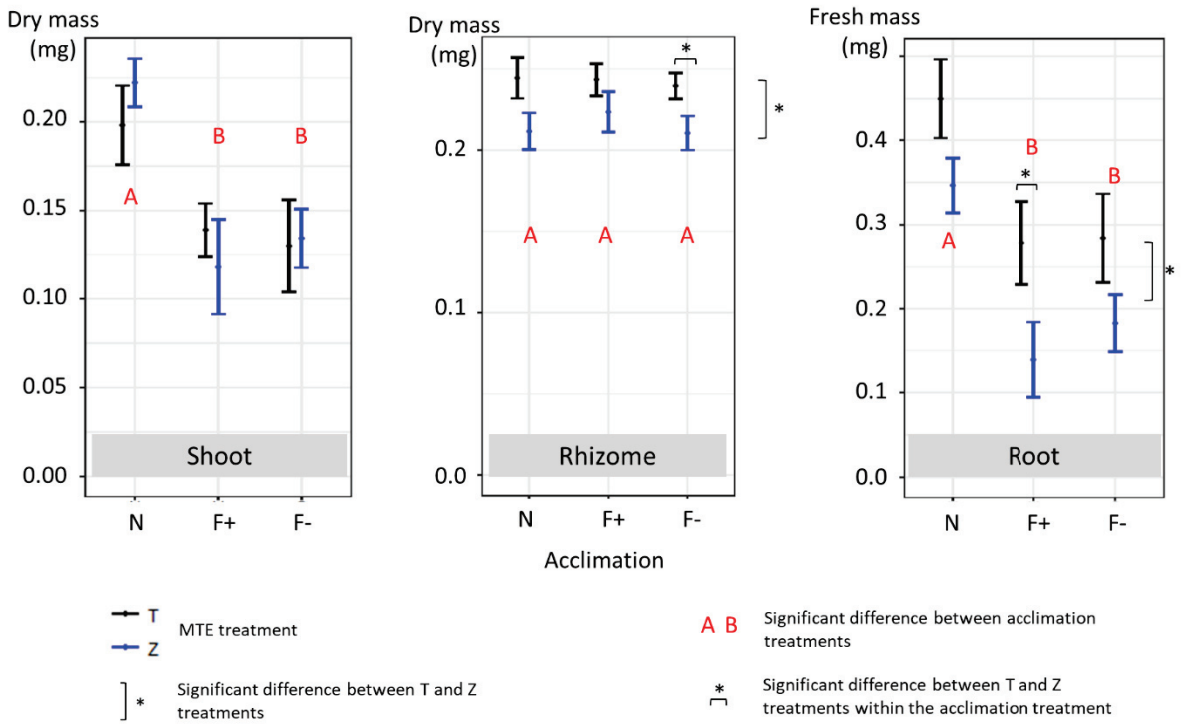

Figure 5.4: Plant growth traits. *N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi, T: control without Zn, Z: zinc. Significant differences are indicated at p-value < 0.05.*

Both F+ and F- acclimated plants have reduced shoot (-38 %) and root (-44 %) masses **(Figure 5.4)**. Independently of acclimation, Zn-contamination decreases rhizome **(Figure 5.4B)** and root **(Figure 5.4C)** masses (respectively -11 % and -33 % compared to controls); in contrast, shoot mass is not affected **(Figure 5.4A)**.

Moreover, a negative correlation between Zn concentration in rhizomes and root mass is observed, but only in F- soil **(Table 5.1)**.

Table 5.1: Correlation between Zn concentration in plants and plant biomass. *Red: negative slope. N: not acclimated, V: acclimated in living soil, S: acclimated in sterilised soil reinoculated with bacteria; T: control without Zn, Zn: zinc.*

Part	Plant mass	Acclimation	Zn	p.value	r^2	Slope	Equation
[Zn]rhizome	Root mass	F-	All	0.0049	0.25	-1.4	y= -1.4 x + 0.63

All strong correlations ($p \leq 0.01$ and $r^2 > 0.2$) between *Olpidium* or DSE colonisation and plant biomasses are shown on **Table 5.2**. In uncontaminated soil, shoot mass is correlated with *Olpidium* frequency of colonisation, independently of the acclimation treatment ($p= 0.004$; $r^2= 0.211$). No correlation could be observed between *Olpidium* colonisation and plant biomasses in Zn-contaminated soil.

DSE colonisation does not correlate with shoot mass. However, a positive correlation between DSE intensity of colonisation and root mass is observed in Zn-contaminated soil ($p= 0.003$; $r^2= 0.25$). This correlation explains even more data variability in non-acclimated rhizomes ($p= 0.006$; $r^2= 0.45$) where root mass is also strongly linked with DSE frequency of colonisation ($p= 0.003$; $r^2= 0.49$).

Table 5.2: Significant correlations between root endophyte colonisation and plant mass. *Green, positive slope. N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi, T: control without Zn, Zn: zinc. I: global intensity of colonisation; i: intensity of colonisation in colonised fragments == clustering; F: frequency of colonisation == repartition of the colonisation.*

Endophyte	Index	Plant part	Acclimation	Zn	p.value	r^2	Slope	Equation
Olpidium	F	Shoot	All	T	0.00369	0.211	0.0027	y= 0.0027 x + 0.12
DSE	I	Root	All	Zn	0.00308	0.25	0.084	y= 0.084 x + 0.18
DSE	F	Root	N	Zn	0.00349	0.494	0.0055	y= 0.0055 x + 0.24
DSE	I	Root	N	Zn	0.00587	0.454	0.091	y= 0.091 x + 0.24

Root secondary metabolites

We detected 108 UV-absorbing compounds in knotweed root extracts. We selected all metabolites that presented significant variations either with acclimation or with Zn (according to ANOVA when normality and homoscedasticity were respected, or according to pairwise Wilcox test when ANOVA was invalid). The 27 obtained compounds, whose concentrations are highly modified due to acclimation or Zn, are presented in **Table 2**, and were used to construct a principal component analysis (PCA) presented in **Figure 5.5**.

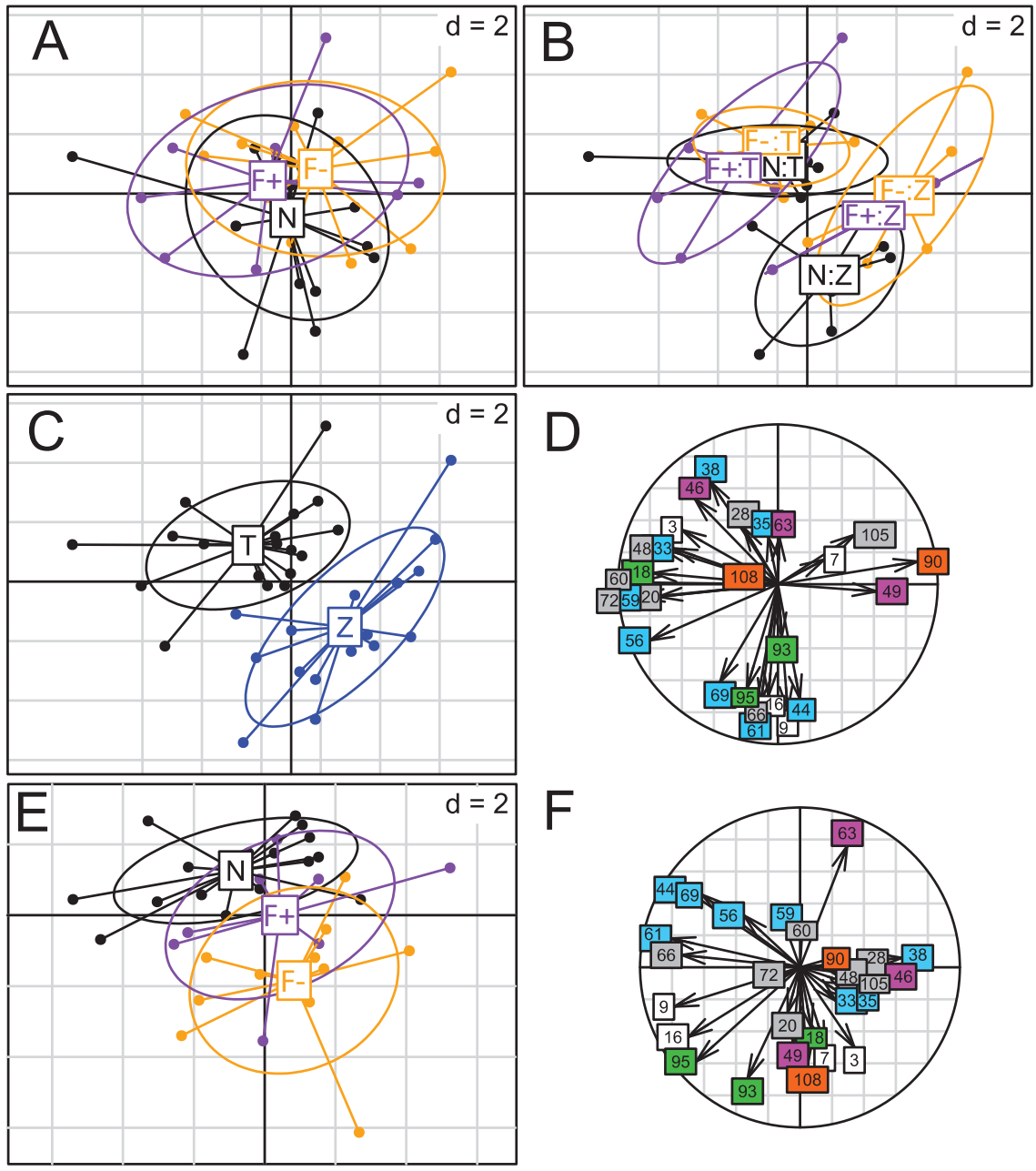

Figure 5.5: Effect of acclimation and Zn treatments on metabolite profiles. *PCA on 27 discriminant metabolites. A-D: axis 1 (29 %) and axis 2 (22 %); E-F: axis 2 and axis 3 (13 %). A, B, C, E: PCA plots; D, F: correlations circles. N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi; T: control without Zn, Z: zinc.*

Zn condition is well separated on the first two axes **(Figure 5.5C)** and acclimation conditions on the third axis with a less good separation **(Figure 5.5E)**; those three axes accounting for 64 % of the variability.

We can distinguish two main groups of compounds impacted by Zn treatment **(Figure 5.5D)**: compounds that tend to decrease with Zn (compounds 3, 18, 20, 46, 48, 56, 59, 60, 72), and compounds that increase with Zn which are different according to acclimation. Indeed, metabolites from non-acclimated plants variate along the second axis (compounds 9, 16, 44, 61, 66, 69 and 95), whereas metabolites from plants acclimated in F- soil variate along the first axis (compounds 49, 90, 105) **(Figure 5.5B, D)**.

Compound 63 is more present in the non-acclimated condition, whereas compounds 93, 95 and 108 seem to be more characteristic of plants acclimated in F- soil **(Figure 5E, F)**. Acclimation in F+ soil leads to a profile in-between the two other acclimation conditions.

Compound families do not account for regularity in the variation of metabolite profiles according to treatments.

Table 5.3: Identification of root metabolites. *N°: compound number; RT: retention time; M: major compound; m: minor compound; Mm: medium compound.*

N°	Abondance	RT	UV (λ max)	*m/z* [M- H]-	MS/MS *m/z* (intensity %)	*m/z* [M+H]+	MS/MS *m/z* (intensity %)	Formula	Annotation	Chemical family
3	m	1.732	ND	ND	ND	ND	ND	ND	ND	ND
7	m	2.08	ND	ND	ND	ND	ND	ND	ND	ND
9	m	2.76	ND	ND	ND	ND	ND	ND	ND	ND
16	m	3.86	ND	ND	ND	ND	ND	ND	ND	ND
18	M	4.31	228, 290(sh),308	258.9932	135 (100), 179 (53), 136 (10)	261,004	163 (100), 145 (33), 135 (17), 117 (13)	$C_9H_8O_7S$	**Sulfated hydroxy-cinnamate**	Cinnamate derivative
20	m	4.725	ND	ND	ND	ND	ND	ND	ND	ND
33	m	6.452	222, 278	ND	ND	ND	ND	ND	Procyanidin derivative	Procyanidin derivative
35	m	6.88	226, 276	729.1489	407 (100), 289 (44), 577 (39), 441 (38), 559(27), 451 (22), 125 (17), 169 (12)	731,1556	409 (100), 123 (84), 127 (76), 289 (68)…579 (23)	$C_{37}H_{30}O_{16}$	**Procyanidin trimer**	Procyanidin derivative
38	m	6.95	ND	ND	ND	ND	ND	ND	ND	ND
44	m	7.12	226, 278	ND	ND	ND	ND	ND	Procyanidin derivative	Procyanidin derivative
46	m	7.55	224, 278	275.0558	189 (100), 121 (14), 165 (14), 145 (14), 231 (12)	277,0695	190 (100), 123 (85), 259(82), 235 (43), 84 (29) 217 (28), 147 (26)	$C_{14}H_{12}O_6$	Desmethylaltenusin	Biphenyl
48	m	7.72	ND	ND	ND	ND	ND	ND	ND	ND
49	m	7.84	208, 220, 250, 272, 294	379,0121	299 (10), 213 (46), 255 (21), 174 (11)	381,0255	217 (100), 297 (42), 301 (30), 189 (16), 243 (10)	$C_{16}H_{12}O_{11}S$	Unknown sulfated biphenyl	Biphenyl
56	Mm	8.432	196, 210, 226, 278	577,137	407 (100), 304 (59), 289 (44), 125 (28), 244 (21), 151 (21)	579,1514	127 (100), 287 (95), 123 (87), 301 (64), 271 (63), 291 (51)	$C_{30}H_{26}O_{12}$	Procyanidin dimer (not B)	Procyanidin derivative
59	m	8.93	226, 278	ND	ND	ND	ND	ND	Procyanidin derivative	Procyanidin derivative
60	m	9.048	ND (coelution)	ND	ND	ND	ND	ND	ND	ND
61	m	9.048	ND (coelution)	535.0559	535 (100), 289 (94), 329 (84), 183 (69), 455 (26)	537,0694	139 (100), 123 (63), 167 (28), 246 (20), 140 (16), 151 (14)	$C_{26}H_{16}O_{13}$	Procyanidin derivative	Procyanidin derivative
63	m	9.207	236, 282, 302 (sh), 318 (sh)	231.0652	188 (100), 231 (57), 189 (29), 159 (23), 182 (15), 116 (14)	233,0789	191 (100)	$C_{13}H_{12}O_4$	6-methylbiphenyl-3,3',4,5'-tetraol	Biphenyl
66	m	9.301	ND	ND	ND	ND	ND	ND	ND	ND

69	m	9.473	210, 226, 274	729.1489	407 (100), 729 (82), 577 (38), 441 (29), 559 (28), 169 (27), 289 (22), 125 (17)	731,1582	123 (100), 289 (82), 127 (56), 409 (40), 439 (38), 247 (37), 421 (33), 163 (27), 139 (19)	$C_{37}H_{30}O_{16}$	**Procyanidin dimer monogallate**	Procyanidin derivative
72	m	10.75	ND	ND	ND	ND	ND	ND	ND	ND
90	M	12.33	230, 272, 318, 328, 392	287.0925	287 (100), 272 (85), 254 (62), 245 (35), 204 (33), 269 (29), 186 (23), 248 (18)	289,1074	247 (100), 271 (52), 243 (28), 243 (17), 205 (16)	$C_{16}H_{16}O_5$	**Torosachrysone**	Anthraquinone
93	M	12.44	230, 298(sh), 318	ND	ND	ND	ND	ND	ND	Cinnamate derivative
95	M	12.49	232, 298(sh), 320	ND	ND	ND	ND	ND	ND	Cinnamate derivative
105	m	13.93	ND	ND	ND	ND	ND	ND	ND	ND
108	m	15.32	208, 278, 358	ND	ND	ND	ND	ND	ND	Anthraquinone

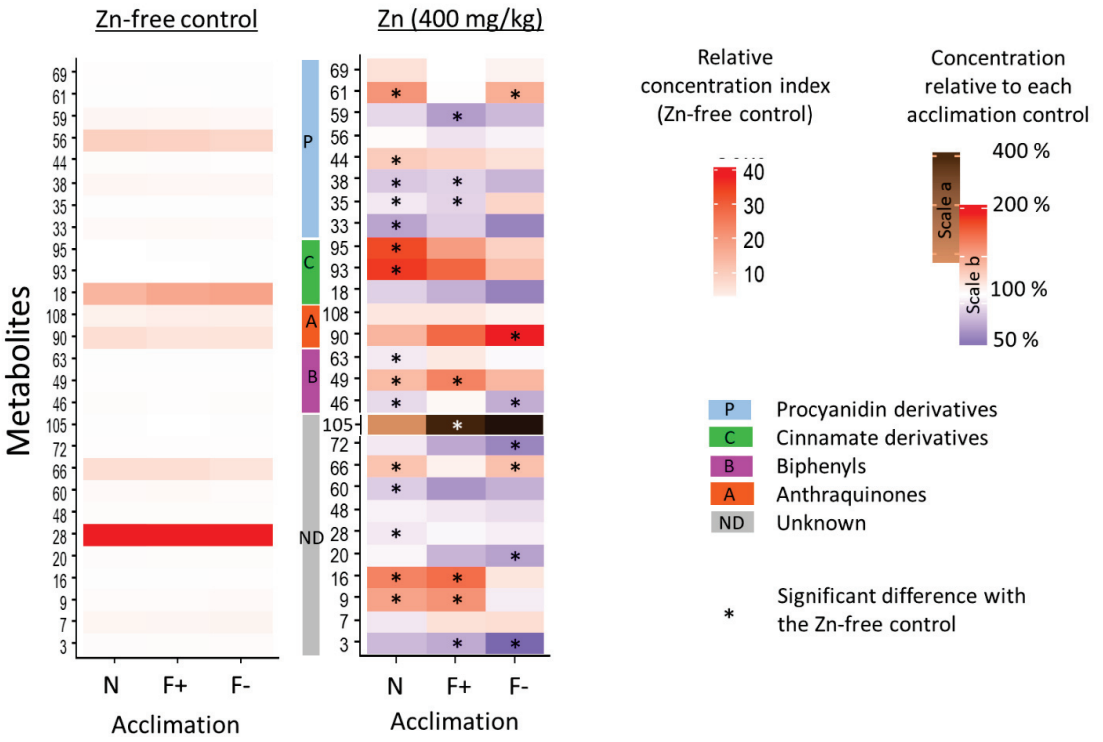

Figure 5.6: Variations in metabolites concentrations. *Heatmap representing the 27 discriminant metabolites selected and their variations of concentrations between the different treatments. Zn-free control: relative concentrations within the control in each acclimation treatment. Zn (400 mg/kg): concentrations in Zn treatment relative to their control in the same acclimation treatment. N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi, T: control without Zn, Z: zinc. Significant differences indicated with p < 0.05 (pairwise test). For readability, two scales were used: scale "a" used for compound 105, scale "b" for other compounds.*

Figure 5.6 shows the concentrations of the 27 discriminant metabolites in non-contaminated plants (left), and their changes in concentration when Zn is added (right). The identified metabolites are either major compounds (18, 28, 56, 66, 90) or minor (3, 16, 20, 35, 44, 46, 48, 49, 61, 63, 72, 93, 95, 105).

In the control treatment, little differences in metabolite concentrations are observed between acclimation treatments. On the other hand, Zn treatment has a marked effect on multiple metabolites, but as noted previously, metabolites changes do not follow the same pattern between acclimation conditions. For example, 15 metabolites are significantly affected by Zn treatment in non-acclimated plants, whereas only 8 were significantly

affected in both F- and F+ acclimated plants, with only two in common to both conditions (compounds 3 and 105).

Some compounds are particularly increased with Zn contamination, such as the biphenyl 49, the anthraquinone 90, the cinnamate derivatives 93 and 95, the procyanidin derivative 61, and the unknown compounds 9, 16 and 105. On the other hand, compounds such as the biphenyls 46 and 63, and the procyanidin derivatives 33, 35, 38 and 59, tend to decrease with Zn stress.

Once more, we do not see any constancy in compound concentration changes, related to their chemical family.

Torosachrysone (compound 90), which has already been shown to be the most increased compound in *Fallopia* roots in the presence of Zn (Barberis et al., 2020), or in the presence of multiple metals (Michalet et al. 2017), has been further studied for its potent role in plant/endophyte response to Zn by looking at specific correlations between the different parameters measured in this study and its variation of concentration. Torosachrysone was neither significantly correlated with plant biomass, nor with Zn content in plant tissues ($p > 0.01$ or $r^2 > 0.2$).

Though, in non-acclimated rhizomes, and independently of Zn treatment, a positive correlation is observed between root concentration of torosachrysone and DSE colonisation frequency, and this explains more than half of the variability of DSE colonisation in this condition **(Table 5.4)**. This positive correlation is also observed when taking all acclimation treatments into account, but it is less significant ($p= 0.0265$, $r^2= 0.169$).

Table 5.4: Correlations ($p \leq 0.01$ & $r^2 > 0.2$) between torosachrysone (compound 90) concentrations and endophyte colonization. *Green, positive slope. Z: Zn treatment. N: not acclimated, F: frequency of colonisation.*

Endophyte	Metabolite	Index	Acclimation	MTE	p.value	r^2	Slope	Equation
DSE	90	F	N	All	0.00513	0.56	15	y= 15 x + 3

Discussion

The objective of this study was to assess the plant-fungus-metal tripartite system with a specific view on plant growth, root secondary metabolism, plant metal concentrations and root endophytic colonisation. We hypothesized that fungal endophytes play a role in the tolerance of their host to Zn contamination. In details, we expected:

(1) a modification of root fungal colonization by acclimation treatment

(2) an increased colonization of root endophytes, and particularly DSEs, in the presence of Zn (Plant call for support theory)

(3) a better regeneration and growth of plants with increased colonisation by fungal endophytes

(4) a change in root phenolics under Zn contamination and correlations between metabolites, plant growth traits and endophytes.

Root fungal colonisation: modification by acclimation and Zn

We used an acclimation protocol to modify endophyte colonisation and thus better assess their role in plant metal tolerance. In details, we expected a decrease in endophytes tolerant to Zn (including DSEs) during acclimation because of the release of Zn environmental pressure during this step. Our microscopic observation of root fungal colonisation confirms the global deficit in root endophytes after acclimation, although this is not significant for DSEs or *Olpidium*. It must be noted though, that the global intensity of colonisation remains low, with a global level of infection of around 4 % in the most colonised plants of our experiment (corresponding to non-acclimated plants).

This decrease of the global level of root fungal infection in acclimated plants, independently of Zn contamination, could be associated with their poorer performance traits compared to non-acclimated plants, i.e. reduced regeneration and lower shoot and root growth. When (re)exposed to Zn, the global level of root fungal infection is restored in F- acclimated plants, but this is not associated with better performance traits, only with an elevated transfer factor of Zinc from roots to shoots.

As expected, in the presence of Zn contamination, and independently of acclimation, we were able to confirm the increase level of root fungal infection, particularly for DSEs and *Olpidium*. This is consistent with field observations, where DSEs were shown to be abundant in metal-degraded environments (Zhang et al., 2013), and even more than in undisturbed

soils (Liu et al., 2017). Moreover, a positive correlation was found between the concentration of MTEs in soil and DSE root colonisation of rosemary (Affholder et al., 2014). Root endophytes are diverse and their colonization patterns may be modified under metal stress: for example, the colonisation of *Deschampsia flexuosa* by AMFs was reduced in contaminated sites whereas DSEs were favoured in these soils (Ruotsalainen et al., 2007).

The increase of *Olpidium* and DSEs in roots of *Fallopia* grown in Zn contaminated soils can be interpreted in two ways: (1) Zn stimulates root colonisation of already present plant-associated endophytes, or (2) under Zn stress plant recruits new endophytes, in order to cope with this stress (plant call for support hypothesis). Frequency is a proxy for the repartition of fungal colonisation; an elevation of frequency can be interpreted as new points of infection (i.e. the recruitment of new endophytes), whereas an elevation of the intensity of colonisation in colonized fragments suggests the extension of already present endophytes (i.e. growth stimulation of native endophytes). In our study, Zn increases the frequency of colonisation of all endophytes in roots of plants acclimated in F- soil (including DSE and *Olpidium* frequencies), while their intensity of colonisation in colonized fragments remained constant. Thus, to cope with Zn after acclimation in F- soil, plants would need to recruit more fungal endophytes than in other conditions, which could only be explained by the absence of fungi during the acclimation period, since this effect is not observed in plants acclimated in F+ soil. We suggest that acclimation in F- soil led to a loss of native fungal endophytes, especially those associated with plant metal tolerance, which would then be compensated by the recruitment of new endophytes (more particularly DSEs and *Olpidium* in this case), when plants are (re)exposed to Zn contamination. These endophytes are supposed to be beneficial for the plant and reduce Zn toxicity, though their effect on plant growth traits does not seem to be as good as the effect of native endophytes, which intensity of infection is increased by Zn in non-acclimated plants, and this could be associated to better plant growth traits in this condition. In uncontaminated soils, this recruitment would not be necessary. Zn tended to increase the global intensity of colonisation by *Olpidium* in F+ acclimated plants through the increase of the intensity of colonisation in colonized fragments, but not with an increased frequency of colonisation as in the case of F- acclimated-plants. This suggests that during growth in greenhouse, *Olpidium* endophytes developed either from native root endophytic community without new recruitment in the case of F+ acclimated plants, but with recruitment of new *Olpidium*

in the case of F- acclimated plants. However, we still lack information about the dynamics of endophytes (Suryanarayanan, 2013) and especially *Olpidium*.

Healthy plants under Zn contamination

We confirmed the high regeneration potential of *Fallopia* × *bohemica* in metal polluted soils (Bailey et al., 2009; Barberis et al., 2020; Pyšek et al., 2003) since Zn did not have any significant effect on regeneration. This is quite surprising because this precise morphotype was the most sensitive to Zn in term of regeneration in a previous study where almost half of the rhizome did not regenerate with Zn (same morphotype, same soil, same Zn concentration) (Barberis et al., 2020). This may rely to the observed global extension and vigour of this patch during the last 2-3 years, suggesting a better metal tolerance over time of this morphotype. This good regeneration ability of rhizome under Zn contamination is partially lost with acclimation, especially in F+ soil, where rhizome exposed to Zn regenerated less than in control. This suggests that native communities in rhizomes are the most beneficial for their plant hosts, interrogating co-evolution abilities of plants and endophyte communities. This co-evolution has already been shown for bacterial endophytes and plant hosts, phytobiomes being dynamic and shaped by plant recruitment through root exudates (Baltrus, 2017).

We also partially confirmed the tolerance of *Fallopia* × *bohemica* to Zn (Barberis et al., 2020), as shoot biomasses are not impacted by the contaminant. However, the height gain observed under Zn contamination (data not shown) being not associated with increased shoot biomass can be interpreted as etiolation. Furthermore, and contrary to previous study (Barberis et al., 2020), Zn decreases plant belowground biomasses, which are the only plant parts directly in contact with the contaminant.

Acclimation reduces shoot and root biomasses at the same extent in both F+ and F- soil. Thus, this negative effect does not seem to be only related to fungi but could also be due to abiotic factors or to bacteria. When acclimation occurred in F+ soil, no etiolation was observed (data not shown), indicating that fungi participate in the shape of their host.

Zn was not detected in plants grown in non-contaminated soil, whereas Zn-concentrations measured in plant parts (aerial and rhizomal) were slightly above soil concentration in Zn-contaminated soil, confirming that *F.* x *bohemica* could be defined as a bio-indicator species for Zn contamination. Without acclimation, Zn-levels are similar in

rhizomes and in aerial parts, which is rather consistent with previous findings where rhizomes seemed to accumulate slightly more Zn than shoots (Barberis et al., 2020). Aerial parts accumulated more Zn than underground parts in both conditions of acclimation, suggesting that native fungal communities lost during acclimation may influence metal accumulation and transfer in their host. This effect of acclimation may also be related to bacterial communities (Ma et al., 2016a; Singh et al., 2018).

DSEs are suspected to be involved in Fallopia *metal tolerance*

We expected a better regeneration and growth of plants displaying increased root colonisation by fungal endophytes. Indeed, the frequency of colonized fragments by *Olpidium* is positively correlated to shoot mass in uncontaminated soil. This suggests that the recruitment of *Olpidium* in *Fallopia* roots would be beneficial for its host, a result quite surprising because *Olpidium* is often considered as a plant pathogen (Lay et al., 2018; Urcelay et al., 2011). Nevertheless, this pathogenicity may be related to their ability to be viruses vector, such as the blueberry-associated mosaic virus (Shands et al., 2017) or the lettuce big-vein virus (Tomlinson and Garrett, 1964) and lots of *Olpidium* are reported in asymptomatic plants (Piszczek et al., 2019; Rożek et al., 2019; Tomlinson and Garrett, 1964; Urcelay et al., 2011; Zubek et al., 2011). Some *Olpidium* may thus be commensal or beneficial for their hosts. However, *Olpidium* does not seem to be beneficial for *Fallopia* during Zn stress, as no correlation was found between *Olpidium* and plant biomasses in the presence of Zn contamination. On the contrary, DSEs are positively correlated with plant root biomass in Zn-contaminated soil, especially in non-acclimated plants. Thus, we hypothesize that DSEs help *F.* x *bohemica* to tolerate Zn, protecting roots, which are directly in contact with contamination. These hypotheses are only supported by correlations, and must be verified through causal experiments, by plant inoculation of DSEs and *Olpidium* individually for example.

We hypothesized that small chemicals (secondary metabolites) should be the intermediary components behind those observed effects of Zn on plant growth and on endophyte colonisation and the effects of endophyte colonisation on plant metal tolerance.

Secondary metabolites: Torosachrysone, a potential recruiter of DSEs?

Root secondary metabolites profiles were analysed to explore their potent role in plant metal tolerance. Three main hypotheses can be formulated: (1) metabolites are not implied in Zn stress, (2) metabolites are secreted for their direct effect on metal tolerance, either by plants or by fungi and (3) under metal stress, metabolites are secreted by the plant to recruit beneficial endophytes to cope with Zn (Plant call for support theory, Thijs et al., 2016). The first hypothesis is rejected out hand because we could easily distinguish metabolites that are increased with Zn-contamination, and, on the contrary metabolites that are decreased. The second hypothesis implies that some metabolites are specific to Zn contamination, in relation to fungal colonisation if those metabolites are mainly produced by fungi, but potentially independent of fungal colonisation if they are produced by the plant. Torosachrysone (compound 90), an anthraquinone derivative, increased in the presence of Zn, consistently with previous studies on mono- (Barberis et al., 2020a) and multi-contamination (Michalet et al., 2017). This is the case in particular after acclimation in F-soil, whereas the same tendency is observed without significance on both other conditions of acclimation (whereas it was found significantly increased in the same morphotype, with the same soil, at the same level of Zn contamination, and in non-acclimated conditions in Barberis et al. (2020a)). On the other hand we were able to demonstrate that torosachrysone concentration in roots was correlated with DSE frequency of colonization, which means that torosachrysone could be seen as an agent of fungal recruitment: when its concentration is increased in roots, then more DSEs infect roots.

Torosachrysone does not show any correlation with plant biomass. However, its presence in roots is positively correlated with DSE colonisation, which is positively correlated with plant biomass. Thus, the role of torosachrysone in the plant-fungi-MTE tripartite system merits further exploration.

A summary of the correlations found in this study between endophytes and their plant hosts on Zn contamination is presented in **Figure 5.7**.

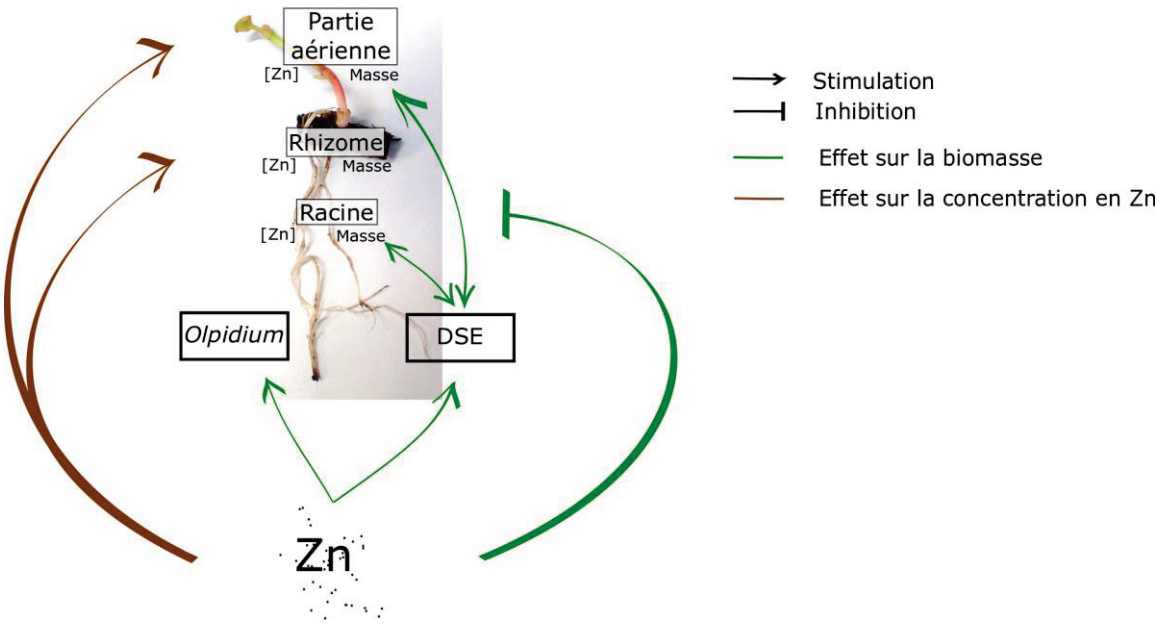

Figure 5.7: Synthesis of the potential roles of endophytes on plant traits in Zn-contaminated soil. *N: not acclimated, F+: acclimation with fungi, F-: acclimation without fungi.*

Conclusion

This study shows interrelations in the plant-fungi-metal tripartite system illustrated by the study of *Fallopia* x *bohemica* submitted to Zn stress. Coupled with microscopic quantifications of fungal colonisation, we showed that some endophytes, such as *Olpidium*, are beneficial for *Fallopia* growth in the absence of Zn, whereas other, such as DSEs, are beneficial only in the presence of Zn-contamination. DSEs are more abundant with metal stress and their root colonization intensity correlate with root mass, making them a good candidates for *Fallopia* metal tolerance improvement. In parallel, Zn contamination induces the increase of some root metabolites, torosachrysone in particular, for which a correlation with DSE frequency of colonization in roots was observed. This is consistent with the "Plant call for support theory" (Thijs et al., 2016), the plant producing metabolites to attract beneficial fungi to its rescue. Torosachrysone, could then be an agent of fungal recruitment. Further studies are needed to evaluate the reality of this hypothesis.

Author Contributions

FP, SM, PB and LB designed the project, LB conducted the experiments (with the help of Marylène SIMON and Dylan ANDEOL), analysed data and wrote the article under the supervision and constructive discussions of FP, PB and SM. SP quantified the endophytes, PB and Marie-Laure TOUSSAINT conducted MTE analysis, SM and LB conducted metabolite analysis with the participation of Dylan ANDEOL.

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Synthèse

Nous avons montré dans cet article que certains endophytes tels que les *Olpidium* sont bénéfiques à leur hôte en l'absence de Zn, alors qu'au contraire d'autres comme les DSE sont bénéfiques en présence de Zn. La colonisation des racines par les DSE est plus importante en milieu contaminé ce qui en fait de bons candidats pour l'amélioration de la tolérance végétale au Zn. Par ailleurs nous avons pu confirmer l'hypothèse selon laquelle l'acclimatation était associée à un remaniement des endophytes fongiques dans les rhizomes : en pratique, une diminution importante de la colonisation par des endophytes fongiques a pu être constatée pour les plantes acclimatées, et les traits de performance de ces plantes étaient nettement inférieures aux plantes acclimatées, particulièrement en présence de zinc. En parallèle, nous avons constaté une modification du profil métabolique racinaire avec le traitement Zn. La concentration en torosachrysone, un dérivé anthraquinonique, est accrue suite à la contamination, et plus particulièrement dans les racines des plantes acclimatées (surtout dans le sol dépourvu de champignons). Or, la concentration de ce composé dans les racines est positivement corrélée à la fréquence de colonisation par les DSE, paramètre permettant de mettre en évidence l'apparition de nouvelles infections fongiques (fréquence plus élevée = plus de points d'infections).

Tous ces résultats sont compatibles avec la théorie de « l'appel à l'aide » (Thijs et al., 2016), à savoir que la plante, pour faire face au stress métallique, modifierait son métabolisme racinaire et ainsi exsuder des composés messagers, telle la torosachrysone dans le cas de la Renouée, afin de recruter des endophytes qui lui seraient bénéfiques, en l'occurrence des DSE.

Les DSE ne favorisent pas la tolérance de leur hôte aux ETM via une « filtration » ou au contraire une accumulation des ions métalliques dans les tissus végétaux, puisqu'aucune correlation n'a été trouvée entre le degré de colonisation des racines et les concentrations tissulaires en ETM.

Cet article montre les étroites relations entre ETM, plante et endophytes, bien que les relations de causalité restent à établir.

Chapitre 6 : Discussion générale

Aspects mécanistiques

Au cours de cette thèse, nous avons testé l'hypothèse que **la tolérance des Renouées asiatiques aux ETM pourrait en partie reposer sur une colonisation des racines par des champignons endophytes.**

Plantes et ETM

Une étude bibliographique introductive traite du devenir des ETM dans l'environnement et de leurs effets sur les écosystèmes et plus particulièrement sur les plantes **(Chapitre 1)** : les ETM sont déposés depuis l'atmosphère dans le sol, où ils seront plus ou moins mobiles et assimilés (**Figure 6.1**, en noir). Sous forme soluble, ils sont biodisponibles et peuvent être prélevés par les plantes et les champignons, puis éventuellement transloqués vers les parties aériennes. Le stockage des ions métalliques est possible notamment dans les vacuoles. Les ETM sont en général dommageables aux plantes, notamment par l'induction d'un stress oxydatif (Miransari, 2011; Saleem et al., 2020; Yadav, 2010).

Pourtant, chez les Renouées asiatiques, les ETM ont peu d'effet sur la croissance (**Chapitres 4A et 5**, **Figure 6.1** en vert et rose). Les biomasses des parties souterraines ont été diminuées par le Zn lors d'une seule des expériences, et le Cr(VI) a même montré un effet stimulant sur la croissance. Le Cd, même à des concentrations au niveau du seuil de toxicité pour lesquelles la plupart des organismes vivants sont fortement affectés lorsqu'ils y survivent (Tóth et al., 2016), n'a aucun effet discernable par rapport aux plantes non polluées sur la base des différents paramètres mesurés. Cela confirme la bonne tolérance de ce taxon aux ETM, qui avait déjà été suggérée à plusieurs reprises (Michalet et al., 2017; Sołtysiak and Brej, 2014).

Les endophytes fongiques racinaires des plantes en général, des Renouées asiatiques en particulier

Les endophytes fongiques racinaires sont variés, ubiquistes et nombreux, y compris dans des sols contaminés aux ETM (**Chapitre 3**, **Figure 6.1**, en bleu). La majorité des endophytes isolés et inoculés dans des conditions contrôlées à des plantes soumises à un stress métallique ont favorisé la croissance de leur hôte, tout en ayant des effets positifs ou négatifs sur le prélèvement et l'accumulation d'ETM par la plante.

Dans les racines des Renouées asiatiques, les endophytes fongiques sont divers **(Chapitre 4B)** : 27 souches ont été isolées et identifiées. Cette diversité fongique n'est pas très élevée comparativement à ce qui est trouvé chez d'autres espèces végétales avec des protocoles d'isolement similaires aux nôtres **(Chapitre 3)**. En effet, les Poacées *Holcus lanatus*, *Panicum virgatum*, et *Hordeum murinum* ont révélé respectivement 59, 50 et 30 espèces d'endophytes racinaires (Ghimire et al., 2011; Murphy et al., 2015; Sánchez Márquez et al., 2010). De nombreuses autres études ont mis en évidence entre 10 et 30 espèces d'endophytes dans une espèce de plante hôte herbacée et ligneuse (Beena et al., 2000; González-Teuber et al., 2017; Herrera et al., 2010; Knapp et al., 2012; Teimoori-Boghsani et al., 2020; Wu et al., 2012). Il est évident que le nombre d'endophytes isolés dépend non-seulement du nombre réel d'endophytes présents et de la proportion d'entre eux qui sont cultivables, mais également de l'effort d'échantillonnage. En ce qui concerne les Renouées asiatiques, les isolats identifiés appartiennent presque tous à des espèces différentes **(Chapitre 4B)**, ce qui laisse à penser que le nombre d'espèces endophytiques réel est bien supérieur à 27. Il serait donc intéressant d'augmenter l'effort d'échantillonnage, et d'utiliser des méthodes moléculaires (méta-barcoding) directement sur les racines afin de mieux estimer l'ensemble de la diversité fongique en intégrant notamment les espèces non-cultivables. Quelques études moléculaires indépendantes des méthodes d'isolement réalisées sur le soja (*Glycine max*) ont permis d'identifier entre 40 et 50 espèces d'endophytes fongiques racinaires (Fernandes et al., 2015; Yang et al., 2018a). Toutefois, les méthodes moléculaires doivent être couplées à des méthodes d'isolement afin de caractériser fonctionnellement les endophytes présents.

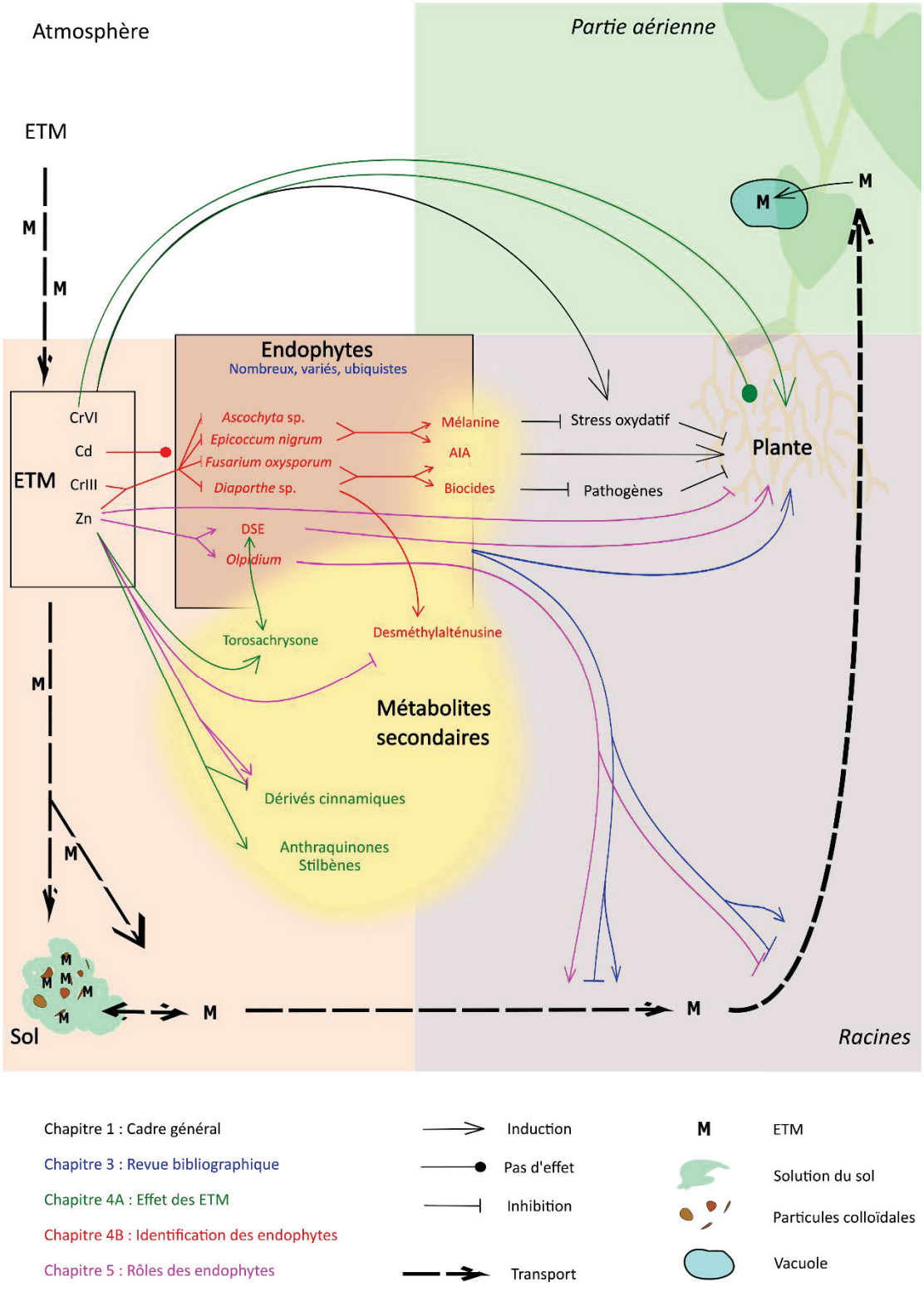

Figure 6.1 : Schéma synthétisant l'effet des endophytes fongiques racinaires sur la tolérance des Renouées aux ETM et intégrant les principaux résultats présentés dans ce manuscrit.

Parmi les endophytes de *Fallopia*, certains produisent de l'auxine (AIA), hormone stimulant la croissance des plantes. Une telle production est connue chez *Acremonium* sp., chez le champignon ectomycorhizien *Laccaria bicolor* ou encore chez la levure endophytique *Williopsis saturnus*, tous trois promouvant la croissance de leur hôte (Felten et al., 2009; Han et al., 2020; Nassar et al., 2005). L'endophyte *Phomopsis liquidambari*, s'il n'a pas été montré comme producteur d'auxine, régule sa concentration *in planta* (Li et al., 2018a). L'inoculation de ce champignon provoque le même effet bénéfique sur la croissance que l'apport d'auxine dans le sol. Des endophytes de *Fallopia* produisent également de la mélanine, molécule de protection contre le stress oxydant ; ou des antimicrobiens, molécules bénéfiques aux plantes grâce à leur pouvoir inhibiteur de pathogènes (**Chapitre 4B**, **Figure 6.1**, en rouge). La mélanine, dont la concentration augmente en présence d'ETM (Ban et al., 2012), est en outre une des composantes caractéristiques des Dark Septate Endophytes, endophytes particulièrement présents et tolérants aux milieux contaminés aux ETM (Grünig et al., 2011; Zhan et al., 2011; Zhang et al., 2013). Aucun des endophytes isolés des racines des Renouées n'est inhibé par une monocontamination au Cd, bien qu'ils le soient partiellement ou totalement par Cr(III) et Zn. Cette sensibilité variable selon la nature des ETM est cohérente avec de précédentes études (Zhang et al., 2008). Elle pose cependant la question de la tolérance aux polycontaminations qui correspondent aux conditions observées sur le terrain. Les endophytes fongiques sont malgré tout très présents en milieu contaminé (**Chapitre 3**, Zhang et al., 2013). Cependant, la sensibilité aux métaux peut être différente *in vitro* et *in situ* : ainsi, les souches FNX (non-déterminée) et FAX (*Periconia macrospinosa)*, isolées de racines de *Fallopia* x *bohemica* soumises à une polycontamination, se sont révélées incapables de croître *in vitro* dans un milieu aux mêmes concentrations métalliques **(Chapitre 4B)**.

L'observation au microscope des racines a permis d'identifier morphologiquement la présence de Dark Septate Endophytes (DSE) et d'*Olpidium* **(Chapitres 4B et 5)**. Ces deux types d'endophytes sont plus abondants en présence de Zn **(Chapitre 5)**. Les *Olpidium*, pourtant habituellement considérés comme pathogènes car vecteurs de maladies virales (Shands et al., 2017), sont associés à une accumulation de Zn dans les racines de *F.* x *bohemica* sans impact sur la croissance des plantes **(Chapitre 5)**. Il est possible que les *Olpidium* puissent, comme d'autres champignons endophytes, jouer le rôle de filtre en stockant les ETM dans leur mycélium (Domka et al., 2019b). Les DSE sont quant à eux corrélés à une croissance plus importante des racines en présence de Zn **(Chapitre 5)**,

conformément à de précédentes études où deux DSE, *Acrocalymma vagum* et *Scytalidium lignicola*, augmentaient la hauteur aérienne (x 2), le nombre de feuilles (x 1,5) et les longueurs et surfaces racinaires (+ 20 %) de *Medicago sativa* en présence de Cd (Hou et al., 2020). Leur augmentation lors d'un stress métallique combinée à leur effet positif sur la croissance en présence d'ETM en font de bons candidats mutualistes pour la tolérance de la plante aux ETM. En cela la souche *Diaporthe* sp. qui pourrait appartenir aux DSE fonctionnellement et qui produit de la mélanine, de l'auxine et des dérivés biphényls pourrait être une bonne candidate impliquée dans cette association mutualiste.

Une prochaine expérimentation, reportée du fait de la situation sanitaire particulière de 2020, doit permettre de déterminer comment chaque souche d'endophyte précédemment isolée affecte les performances des Renouées asiatiques en présence de contamination métallique. L'objectif serait donc d'inoculer des champignons sur des graines préalablement stérilisées en surface et mises à cultiver en conditions axéniques et de comparer la croissance, l'accumulation d'ETM et le métabolisme secondaire de ces plants à celle de plants non-inoculés **(Figure 6.2)**.

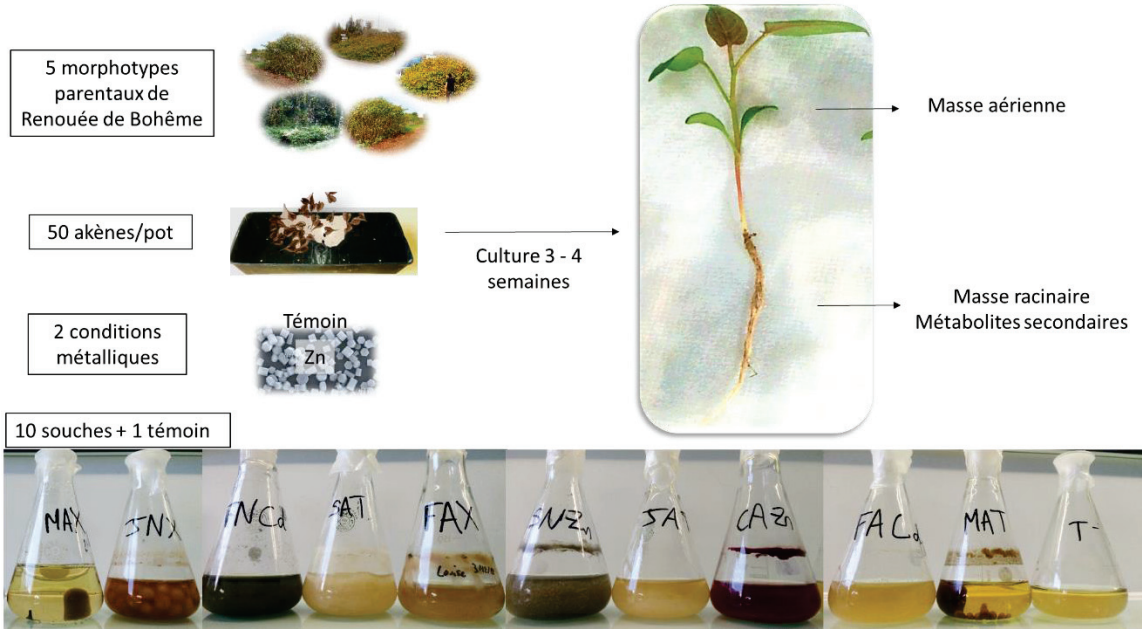

Figure 6.2 : Protocole d'expérience d'inoculation des souches fongiques sur des akènes de Renouée de Bohême. *©Louise BARBERIS*

Cette expérience d'inoculation nous permettra de déterminer si une ou plusieurs souches particulières interviennent dans la tolérance de la Renouée de Bohême aux ETM. Si c'est le cas, elles pourraient être à la base de futures expérimentations permettant la compréhension de leurs mécanismes d'action.

Les productions de métabolites secondaires de ces endophytes

Les champignons endophytes, comme les plantes, produisent et sécrètent des composés phénoliques et autres molécules organiques, qui peuvent participer à la chélation d'ETM dans le sol ou les tissus et donc favoriser la tolérance aux ETM (Domka et al., 2019b).

Certains métabolites secondaires, dont la concentration racinaire est augmentée en présence de Zn (desméthylaténusine glycosylée, et son analogue génine sulfaté et décarboxylé), ou diminuée (desméthylalténusine) (**Chapitres 4A et 5**, **Figure 6.1** en rose et vert) sont également décrits chez les champignons. Chacune des souches isolées des racines des Renouées possède un patron de production de métabolites différent **(Chapitre 4B)**. Il serait intéressant d'approfondir cette étude métabolomique des souches, notamment en étudiant les modifications du métabolisme secondaire fongique induites par le stress métallique et en comparant des extraits fongiques avec les extraits racinaires par une approche de type réseau moléculaire : cela nous permettrait d'identifier des molécules structurellement proches entre les deux types d'organismes. Une meilleure connaissance des métabolites fongiques retrouvés dans les extraits racinaires, sous forme intacte ou sous forme modifiée, permettra de mettre en évidence les souches fongiques en interaction avec la plante et ayant un rôle écologique pour elle, notamment dans la tolérance aux métaux.

Une médiation par certains métabolites secondaires ?

Nous avons deux hypothèses principales sur l'implication des métabolites secondaires dans la tolérance des plantes en général aux ETM, des Renouées asiatiques en particulier.

D'une part, les métabolites secondaires peuvent jouer le rôle de « recruteurs » de microorganismes bénéfiques pour la plante. Les métabolites impliqués seraient donc d'origine végétale. C'est le cas par exemple de *Panax ginseng*, qui recrute l'endophyte *Acremonium* sp. *via* la production d'acide jasmonique (Han et al., 2020). De la même manière, une sélection spécifique de bactéries endophytes mutualistes se déroule *via* les exsudats racinaires (Chagas et al., 2018; Gaiero et al., 2013; Jha et al., 2018). Dans ce cas, on

s'attend à une augmentation de la production de composés messagers en présence du stress ; une augmentation artificielle de leur concentration au niveau des racines devrait provoquer une augmentation de l'abondance de certains endophytes. L'effet positif attendu en présence d'endophytes devrait être annulé dans un sol stérilisé (absence d'endophytes).

D'autre part, les métabolites secondaires peuvent avoir un rôle direct sur la gestion du stress métallique et du stress oxydatif induit par les ETM, *via* les processus de mobilisation/immobilisation, de chélation, de transport et de détoxification. Dans ce cas, les métabolites peuvent être d'origine végétale ou fongique. L'ajout de ces métabolites doit, y compris en l'absence d'endophytes, améliorer la croissance de la plante.

Ainsi, afin de tester le rôle des métabolites secondaires dans la tolérance des plantes aux ETM, des expériences de croissance d'endophytes en présence de chaque composé pourront être menées *in vitro* dans un premier temps, en mésocosme dans un second temps **(Figure 6.3)**. Nous avons déjà identifié quelques candidats, dont la concentration augmente avec les ETM (notamment la torosachrysone). Pour distinguer les deux modes d'action possibles des métabolites, il est nécessaire d'intégrer deux conditions pour les endophytes (présence ou absence).

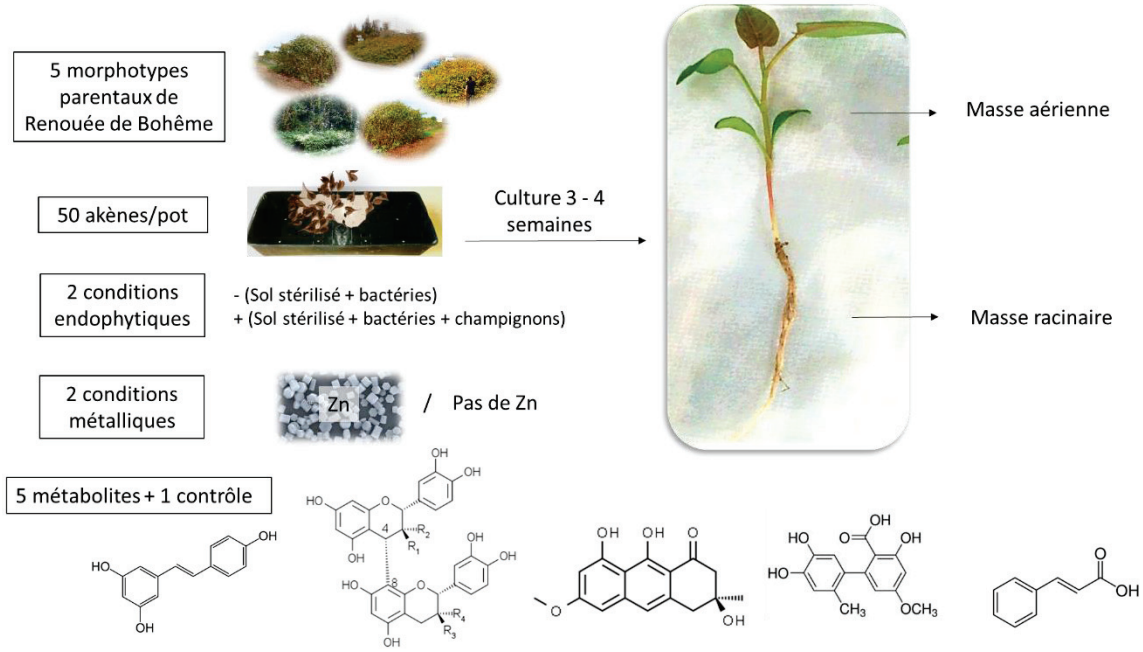

Figure 6.3 : Protocole d'expérience d'inoculation de métabolites secondaires sur des akènes de Renouée de Bohême. *©Louise BARBERIS*

Les travaux effectués au cours de cette thèse démontrent donc l'importance écologique des endophytes fongiques racinaires dans la tolérance des Renouées asiatiques au stress métallique. Au-delà de l'aspect purement mécanistique de la tolérance aux ETM de l'association plante-endophytes, il est utile de s'intéresser aux conditions permettant cette association et à son impact sur les écosystèmes.

Écologie des associations endophytiques

La transmission des endophytes

Tout comme les pathogènes (Lipsitch et al., 1996), les endophytes peuvent théoriquement être transmis verticalement (de la plante « mère » à la plante « fille ») ou bien horizontalement (d'une plante voisine à une autre, ou bien par recrutement à partir de l'environnement). Les deux modes de transmission peuvent être utilisés par une même espèce endophytique, comme *Epichloë sylvatica* sur *Brachypodium sylvaticum* (Poaceae) (Brem and Leuchtmann, 1999). Chez les herbacées, une transmission des endophytes via le pollen et les graines a été clairement mise en évidence et semble répandue (Hodgson et al., 2014). Les graines en général contiennent de nombreux endophytes variés (bactéries, champignons, protozoaires) dont certains au moins favorisent la croissance de leur hôte et l'allègent d'un stress (Shahzad et al., 2018). Dans le cas des Renouées asiatiques, la transmission verticale pourrait avoir lieu lors de la reproduction sexuée et/ou végétative : les différentes expérimentations effectuées au cours de cette thèse permettent d'explorer partiellement cette transmission.

Les Renouées colonisent de nouveaux milieux entre autres par dispersion de fragments de rhizomes qui peuvent eux-mêmes régénérer et produire de nouvelles plantes. Or, comme pour d'autres espèces (Shoemaker, 2010), les rhizomes sont colonisés par des endophytes. En effet, nous avons été dans l'incapacité d'obtenir des rhizomes dépourvus d'endophytes et toujours capables de régénérer : il existe donc une forte transmission verticale des endophytes par voie végétative.

En revanche, l'observation microscopique des radicules issues de la germination d'akènes désinfectés en surface n'a pas permis d'observer d'endophytes : il n'y aurait donc pas de transmission verticale par la voie sexuée d'endophytes capables de coloniser des racines.

Outre les transmissions verticales, nous avons exploré la transmission horizontale par l'utilisation de l'acclimatation. En effet, les rhizomes provenant du terrain ont été laissés pendant plusieurs mois dans du sol non-appauvri en champignons ou au contraire déficient en champignons (sol stérilisé aux rayons gamma et réinoculé uniquement avec les bactéries de la solution de sol) : le pool d'endophytes disponibles pour un recrutement par les plantes est donc différent. Lors de cette acclimatation, il est supposé qu'un remaniement de la colonisation racinaire endophytique est possible suite à une perte de la pression environnementale métallique. En effet, nous avons observé que des plantules provenant du même morphotype, après régénération du rhizome dans un même sol en présence de Zn, montrent une colonisation des racines plus intense après acclimatation sans champignons comparativement à l'acclimatation en sol non-appauvri en champignons (Chapitre 5). Cela laisse donc supposer qu'un recrutement d'endophytes à partir du sol est possible. Les modifications des métabolites secondaires selon que l'acclimatation a eu lieu dans un sol appauvri en champignons ou non suggèrent que des processus fongiques depuis le sol sont à l'œuvre. Il existerait donc une transmission horizontale des endophytes. Ces hypothèses de perte d'endophytes inutiles en l'absence de contraintes et de recrutement d'endophytes bénéfiques, que certaines études confirment pour les champignons mycorhiziens (Martín-Robles et al., 2018), restent à confirmer avec les champignons endophytes.

Les Renouées asiatiques peuvent donc se transmettre verticalement des endophytes par voie végétative, ceci pouvant favoriser la régénération de plantules à partir de rhizomes. Mais elles peuvent également recruter des endophytes en provenance du sol où elles se trouvent. Les mécanismes par lesquels les plantes recrutent sont principalement chimiques, *via* la sécrétion de métabolites secondaires : les phytohormones sont proposées comme actrices de ce recrutement (Manzotti, 2017) et d'autres composés phénoliques sont probablement impliqués. Ainsi, à partir d'un même sol, les communautés endophytiques effectivement recrutées dépendent de la plante hôte (Pacifico et al., 2019). Un tel recrutement spécifique, particulièrement en situation de stress, pourrait favoriser les associations avec des endophytes d'ores et déjà adaptés au milieu et pouvant se révéler plus favorables aux Renouées : c'est la théorie de « l'appel à l'aide » (Thijs et al., 2016).

L'association Renouées – endophytes : quel impact écologique ?

Toutes ces études mènent à la compréhension de la tolérance des Renouées asiatiques aux ETM. Un des intérêts fondamentaux de cette compréhension est de mieux appréhender leur potentiel envahissant et leur impact sur les écosystèmes. Deux questions se posent :

- les Renouées sont-elles plus compétitives en présence d'ETM ? C'est-à-dire, les ETM favorisent-ils la dominance des Renouées dans un écosystème et donc la disparition des espèces natives ? Actuellement, des études existent sur des plantes invasives qui, comme les Renouées, se développent bien sur les milieu contaminés (Bajwa et al., 2016; Sołtysiak and Brej, 2014), mais il n'y a pas à notre connaissance d'articles explorant l'effet d'une contamination du milieu sur le potentiel invasif d'une plante.

- les endophytes fongiques améliorent-ils la compétitivité des Renouées, particulièrement en milieu contaminé ? C'est l'hypothèse que font les auteurs d'une étude en milieu urbain (Sołtysiak and Brej, 2014). Cependant, cette augmentation de compétitivité n'est pas évidente : il a été montré à plusieurs reprises que les AMF ne sont pas forcément favorables aux plantes les plus compétitives dans un contexte de co-existence avec d'autres espèces (Calvache et al., 2017; Moora and Zobel, 1996; Veresoglou et al., 2018). Comme pour les ETM, la démonstration d'une compétitivité accrue grâce aux endophytes fongiques est à ma connaissance inexistante.

Des études de compétition entre Renouées et espèces natives pourront être menées, en présence et en l'absence d'ETM et/ou d'endophytes, afin de répondre à ces questions.

Les Renouées asiatiques, et particulièrement la Renouée du Japon et la Renouée de Bohême, sont considérées comme envahissantes en Europe et en Amérique du Nord. Connaître les conditions d'une croissance et/ou d'une compétitivité optimale de ces plantes nous permet de mieux appréhender le potentiel de colonisation de ces espèces. Nous avons vu dans cette étude qu'il n'est pas seulement question de climat ou de type de sol, mais également des concentrations en ETM et des contenus qualitatifs et quantitatifs en champignons présents dans le sol et dans les rhizomes.

Phytoremédiation

Toutes précautions gardées du fait de la nature envahissante des Renouées asiatiques, ces nouvelles connaissances pourraient être appliquées en phytoremédiation : utiliser des plantes pour dépolluer un site (phytoextraction) ou *a minima* stabiliser les contaminants (phytostabilisation) pour protéger les sites alentours. Cette technique peu onéreuse (Lewandowski et al., 2006; Wan et al., 2016) nécessite des espèces végétales peu sensibles aux contaminants impliqués.

Ici, nous avons exploré un mécanisme de tolérance des plantes aux ETM, qu'il pourrait être intéressant d'appliquer sur le terrain : l'inoculation d'endophytes bénéfiques pourrait être envisageable pour améliorer la croissance des plantes sur ces milieux. Cette stratégie d'inoculation est déjà proposée avec des champignons mycorhiziens et de bactéries promotrices de croissance (bactéries PGPR ou *Plant Growth Promoting Rhizobacteria*), dans le but d'améliorer la croissance des plantes en milieu contaminé par des molécules organiques ou inorganiques (Coninx et al., 2017; Gaur and Adholeya, 2004; Xun et al., 2015). Différentes méthodes d'inoculation sont proposées, à partir d'inoculum issu d'un écosystème similaire ou bien un inoculum commercial à la composition connue (White et al., 2008) : il est possible d'inoculer les graines, ou d'introduire dans le milieu des plants « sources » inoculés qui permettront la transmission horizontale de souches fongiques aux plantes environnantes (Koziol and Bever, 2017; White et al., 2008). Ces méthodes, déjà commercialisées pour les champignons mycorrhiziens, sont transférables à l'inoculation d'endophytes, et même potentiellement facilitées car les endophytes fongiques peuvent croître indépendamment des plantes : l'épandage d'un mulch contenant des mycelia pourrait être également envisageable.

Pour la phytoextraction, le développement rapide d'une biomasse importante est intéressant afin de maximiser les quantités de contaminants extraits. Les Renouées asiatiques présentent ces deux caractéristiques – tolérance et biomasse importante – nécessaires à leur emploi dans la phytoextraction. Elles ne sont certes pas hyperaccumulatrices, mais les concentrations métalliques dans leurs tissus aériens lors d'une poly-contamination correspondent environ aux concentrations dans le sol (**Chapitre 4A, Chapitre 5**) : grâce à leur biomasse importante, elles pourraient extraire des quantités significatives d'ETM d'un sol pollué au-delà des normes fixées : pour une biomasse de 13t/ha et une accumulation de 400 mg/kg de biomasse sur un sol à 400 mg/kg, 5,2 kg de Zn seraient

extraits chaque année, ce qui est inférieur à l'extraction par l'hyperaccumulatrice *Cardaminopsis halleri* (33 kg Zn/ha) (Terry and Banuelos, 2000) mais supérieur à ce qui est réalisé avec *Salix viminalis* (13,4 kg Zn/ha en 5 ans) (Hammer et al., 2003). Les extraits pourraient être valorisés par « phytomining » avec une extraction complète pour ETM en concentration suffisante (Chaney et al., 2018), ou en écocatalyse, c'est-à-dire l'utilisation directe de produit de fermentation riche en ETM pour catalyser des réactions chimiques (Grison, 2015). Le principal verrou actuellement à l'utilisation de ces plantes en phytoremédiation, outre la profondeur de sol explorée par les racines, est leur caractère envahissant : laisser se développer une plante non-maîtrisée est un cap difficile à accepter. Pourtant, ce caractère envahissant pourrait avoir un intérêt pour la réhabilitation de milieux contaminés : d'autres espèces envahissantes telles que *Parthenium hysterophorus* ou la plante aquatique *Eichhornia crassipes* sont proposées pour la phytoremédiation, justement du fait de leur capacité à générer beaucoup de biomasse y compris en milieu contaminé (Bajwa et al., 2016; Rai, 2015).

Notons que les Renouées asiatiques, dont le développement est certes difficile à maîtriser, présentent de nombreux atouts : ce sont des plantes fourragères (pour les milieux non-contaminés), nectarifères qui nourrissent les pollinisateurs en fin de saison, et qui ne sont pas perçues négativement dans le paysage par la population naïve (Rouifed et al., 2018; Thiébaut et al., 2020). Par ailleurs, la nature entame elle-même son travail de régulation : jusqu'ici peu sujettes aux maladies en Europe, il devient de moins en moins rare d'observer de l'herbivorie et des symptômes de maladie : les Renouées asiatiques deviennent progressivement partie intégrante des écosystèmes, permettant également la revégétalisation rapide de sites dégradés. Utiliser ces plantes en phytoremédiation serait à réfléchir d'abord dans leur milieu natif (Asie du Sud-Est) avant toute considération d'application en Europe où leur développement est actuellement non-maîtrisé.

Conclusion

Au cours de cette thèse, nous avons testé l'hypothèse que **la tolérance des Renouées asiatiques aux ETM pourrait reposer sur une colonisation des racines par des champignons endophytes.**

Une étude bibliographique (Axe 1) approfondie des endophytes fongiques racinaires recensés dans le monde montre l'ubiquité et la très grande diversité de ces microorganismes, qui colonisent des plantes mycorhiziennes ou non, y compris dans des milieux contaminés aux ETM. En présence d'ETM, les endophytes racinaires régulent l'accumulation et la translocation des métaux dans leur plante hôte, parfois en augmentant les transferts, parfois en les diminuant, sans qu'aucune logique taxonomique n'ait pu être identifiée. Pourtant, et indépendamment des effets sur l'accumulation, l'association des plantes avec des endophytes fongiques racinaires améliore leur croissance et leur tolérance aux ETM.

Les Renouées asiatiques sont très tolérantes aux ETM (Axe 2) : non seulement elles prospèrent en milieu urbain et industriel contaminés, mais elles sont même stimulées par certains ETM tels que le chrome, et n'ont aucun problème à pousser sur un milieu contaminé au cadmium à des concentrations toxiques pour la plupart des organismes. Or, nous avons détecté des endophytes fongiques dans les racines des Renouées asiatiques : deux morphologies principales sont identifiables au microscope (les Dark Septate Endophytes et les *Olpidium*) ; et 27 souches ont été isolées et cultivées à partir des trois espèces formant le complexe *Fallopia* et ayant poussé sur des milieux variés en contamination métallique. Les souches isolées sont toutes tolérantes au cadmium *in vitro*, mais sensibles à la poly-contamination (certaines beaucoup moins que d'autres), la réaction face au chrome et au zinc étant plus variée.

En modifiant les communautés endophytiques par une stratégie d'acclimatation en sol riche ou pauvre en champignon (Axe 3), nous avons montré que la tolérance de la Renouée de Bohême au zinc repose sur des processus fongiques. En particulier, les DSE sont plus abondants en présence de zinc et sont, sur ce milieu contaminé, associés à une biomasse plus élevée du jeune plant. Cette corrélation est particulièrement intéressante : le lien de causalité reste cependant à être démontré, *via* l'inoculation de souches fongiques sur des graines en milieu axénique par exemple.

Au cours de toutes ces expérimentations (Axes 2 et 3), les métabolites secondaires présents dans les racines et dans les différentes souches fongiques ont été explorés. Deux familles de métabolites racinaires sont favorisées par les ETM : les anthraquinones et les stilbènes. Au contraire, les procyanidines et dérivés cinnamiques sont moins présents. Certains biphényls augmentent en présence de métaux tandis que d'autres diminuent. La torosachrysone, une anthraquinone, est positivement corrélée à la fréquence de colonisation d'endophytes : elle pourrait être impliquée dans le recrutement de ces endophytes qui favoriseraient par la suite la tolérance aux ETM de la plante hôte.

Finalement, ces résultats vont dans le sens de nos hypothèses de départ, bien que certains liens de causalités restent à démontrer :

(1) Les Renouées asiatiques ont une croissance peu altérée par la contamination métallique ;
(2) Certains endophytes sont augmentés par les ETM
(3) La croissance des plantes est favorisée par certains endophytes fongiques ;
(4) La présence de métaux induit un changement métabolique, en lien avec la colonisation fongique.

Ces recherches participent à développer les connaissances autour des microorganismes et de leur importance dans les processus écologiques. Les connaissances apportées sur la relation entre les Renouées et les endophytes fongiques, son importance écologique dans un contexte de contamination et les mécanismes moléculaires qui se cachent derrière demandent à être complétées et généralisées (ou non) à d'autres plantes et d'autres contextes environnementaux.

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