Université de Lille
Ecole doctorale « Sciences de la Matière, du rayonnement et de l'environnement »
Unité Evolution, Ecologie et Paléontologie, Unité Mixte de Recherche UMR 8198

THÈSE

Présentée en vue de l'obtention du grade de

Docteur de l'université de Lille

Spécialité : Biologie Evolutive et Ecologie

Par Julien NOWAK

Evolution expérimentale de *Noccaea caerulescens* en condition de stress métallique : impact sur l'évolution de traits fonctionnels potentiellement impliqués dans la tolérance aux métaux

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Directrice de thèse : Hélène FREROT-PAUWELS
Co-encadrant : Maxime PAUWELS

Soutenance publique le 01 février 2019

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Noccaea caerulescens

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Liste des abréviations et symboles

Abréviation	**Signification**
DP	Descendants population
EP	Experimental population
ETM	Elément trace métallique
LC	La Calamine (Population métallicole de *Noccaea caerulescens*)
LE	Lellingen (Population non-métallicole de *Noccaea caerulescens*)
NS	Non significatif
PP	Parents population
PR	Prayon (Population métallicole de *Noccaea caerulescens*)
Zn	Zinc
WIL	Wilwerwiltz (Population non-métallicole de *Noccaea caerulescens*)
WIN	Winseler (Population non-métallicole de *Noccaea caerulescens*)

Symbole	**Signification**
F_{ST}	Indice de différenciation génétique neutre
Q_{ST}	Indice de différenciation de traits quantitatifs
S	Différentiel de sélection
ß	Gradient de sélection linéaire
γ	Gradient de sélection quadratique

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I. Introduction générale

1.1. L'adaptation locale : concepts et processus mis en œuvre

1.1.1. Émergence et définition du concept

Une certaine harmonie semble se dégager lorsqu'on observe un individu dans son environnement. Si bien que cette adéquation fut longtemps mise en avant pour justifier une vision finaliste de la nature et reste encore un argument en faveur du créationnisme [Hodge & Radick, 2006; Sober, 2008]. C'est avec sa théorie de la sélection naturelle, que Darwin proposa l'idée que cette adéquation apparente n'était le résultat que d'un processus sélectif imposé par les contraintes environnementales. Cette théorie repose sur trois principes : la variation, la sélection et l'hérédité des traits phénotypiques, l'idée étant que dans une population soumise à un environnement précis, il existe une variation, pour un ou plusieurs traits, qui permet à certains individus de se reproduire plus que les autres. Ces individus, disposant d'un avantage sélectif, auront alors plus de descendants que le reste de la population. Dès lors, si ce trait est transmissible à la descendance, les porteurs de cet avantage sélectif augmenteront en fréquence dans la population au fil des générations.

Après la découverte des gènes, structures permettant l'hérédité des traits phénotypiques, la théorie darwinienne fut mise à jour et de nouvelles forces évolutives ont été intégrées : (1) La mutation, représentant la modification accidentelle de l'information génétique, (2) la migration, représentant la transmission d'allèles d'une population à l'autre et (3) la dérive génétique, représentant l'évolution aléatoire d'une fréquence allélique. Ces trois forces, en impactant aléatoirement l'information génétique, représentent une source importante de la variation phénotypique observée dans une population et c'est cette variation qui permettra à la population de faire face aux changements environnementaux par effet de la sélection naturelle.

Ainsi, lorsque des populations naturelles sont confrontées à un changement environnemental, biotique ou abiotique, le phénotype moyen de ces populations se trouve éloigné du phénotype optimal qui permettrait de maximiser les capacités de survie et de reproduction des individus dans ces nouvelles conditions. Dès lors, grâce à l'effet de la sélection naturelle, le phénotype moyen de la population sera progressivement modifié pour tendre vers un nouvel optimum phénotypique. Ce processus par lequel une population tend à maximiser les capacités de survie et de reproduction des individus qui la compose est appelé adaptation, et conduit à un nouveau phénotype qui sera lui-même qualifié d'adaptation.

Les espèces occupent rarement des aires de répartitions dont les conditions écologiques sont homogènes et les contraintes environnementales différent selon la population étudiée, ce qui peut conduire, par sélection divergente, à des phénotypes locaux optimaux [Haldane, 1948; Kawecki & Ebert, 2004]. Cette adaptation locale créée donc des situations évolutives, où la sélection naturelle agit sur plusieurs populations soumises à des milieux différents pour aboutir à des phénotypes divergents. Dès lors, il est possible d'obtenir des informations, soit sur la nature ou les bases génétiques des traits adaptatifs, soit sur la nature des pressions de sélection entrainant la divergence phénotypique entre les populations, en étudiant, respectivement, le résultat ou les processus évolutifs impliqués dans cette adaptation.

1.1.2. Détecter l'adaptation locale par l'étude de son résultat

Selon la théorie, l'adaptation locale se manifeste par une meilleure valeur sélective des individus d'une population dans son milieu d'origine [Kawecki & Ebert, 2004]. La valeur sélective étant, ici, utilisée pour décrire la capacité des individus à survivre, se développer et se reproduire [Krimbas, 2004] (cette notion sera discutée plus tard dans le manuscrit).

Ainsi, la détection de l'adaptation locale peut se faire par transplantations réciproques. Cela consiste, d'une part, à comparer la valeur sélective des génotypes locaux à celle de génotypes étrangers dans leur milieu (critère *local vs foreign*) et, d'autre part, à comparer la valeur sélective d'un génotype dans plusieurs milieux, la valeur sélective étant, en théorie, plus élevée dans son milieu d'origine que dans d'autres habitats (critère *home vs away*). Bien que ces transplantations représentent une démarche adaptée pour détecter l'adaptation locale, notamment parce qu'elles permettent de prendre en considération l'ensemble des facteurs environnementaux, il n'est pas toujours possible de réaliser ce genre de mesures. C'est pourquoi il existe une alternative visant à recréer les conditions écologiques rencontrées *in situ* en serre ou en terrain expérimental pour comparer les phénotypes des différentes populations [Kawecki & Ebert, 2004].

Néanmoins, la seule analyse de la divergence phénotypique mesurée entre les populations ne permet pas de démontrer que les contraintes environnementales subies par les populations soient à l'origine de la variation observée et donc d'une adaptation. La dérive, par exemple, peut entrainer une évolution de la population par des phénomènes aléatoires. Les effets de la dérive génétique sont d'autant plus forts que la population est petite, car un allèle

sera plus fréquent dans la population et aura plus de chance de se fixer dans une petite population [Lande, 1976].

Une façon de montrer qu'il y a eu une sélection pour un caractère donné est de calculer l'indice de différenciation de traits quantitatifs (Q_{ST}), représenté par la variation des traits mesurés, [Spitze, 1993] entre les populations et de le comparer avec l'indice de différentiation génétique neutre (F_{ST}) [Wright, 1951]. Ainsi, si les traits évoluent uniquement sous l'effet de la dérive génétique, les indices de différenciation seront égaux ($F_{ST} = Q_{ST}$). A l'inverse une différence entre les deux indices sera interprétée comme une preuve de sélection [Hendry, 2002; Whitlock, 2008], et donc d'adaptation. Dès lors on considérera une sélection diversifiante ou stabilisante si la valeur de F_{ST} est, respectivement, inférieure ou supérieure à celle de Q_{ST}. Plusieurs cas de sélections ont été détectés grâce à cette méthode. Ainsi, en comparant les Q_{ST} obtenus sur plusieurs traits phénotypiques, et les F_{ST} entre plusieurs populations de *Noccaea caerulescens,* Jimenez *et al.* (2007) ont montré une sélection divergente forte entre des populations provenant de sites contaminés aux métaux et celles provenant de sites non contaminés. Une expérience similaire conduite sur *Arabiopsis halleri* suggère également que l'adaptation de certaines populations aux sites pollués proviendrait d'une sélection directionnelle [Meyer *et al.*, 2010].

Néanmoins, ces méthodes ne permettent que d'étudier l'adaptation locale *a posteriori*. Aussi, le phénotype de la population ancestrale fait généralement défaut et ne permet pas de discuter la dynamique de l'évolution [Franks, 2011]. Enfin, en étudiant les phénotypes actuels les effets de toutes les conditions environnementales sont confondus, et il est difficile de faire des interprétations sur l'effet d'une seule pression de sélection. Ainsi, sans preuve d'un changement phénotypique temporel entre la population ancestrale et les populations sélectionnées, ou une observation directe de l'action d'une pression de sélection, toutes les interprétations adaptatives des modèles observées peuvent être considérées comme des *just-so stories* qui ne peuvent exclure d'autres scénarios [Olson & Arroyo-Santos, 2015]. Une méthode pour étudier ces processus évolutifs consiste à faire évoluer expérimentalement les populations.

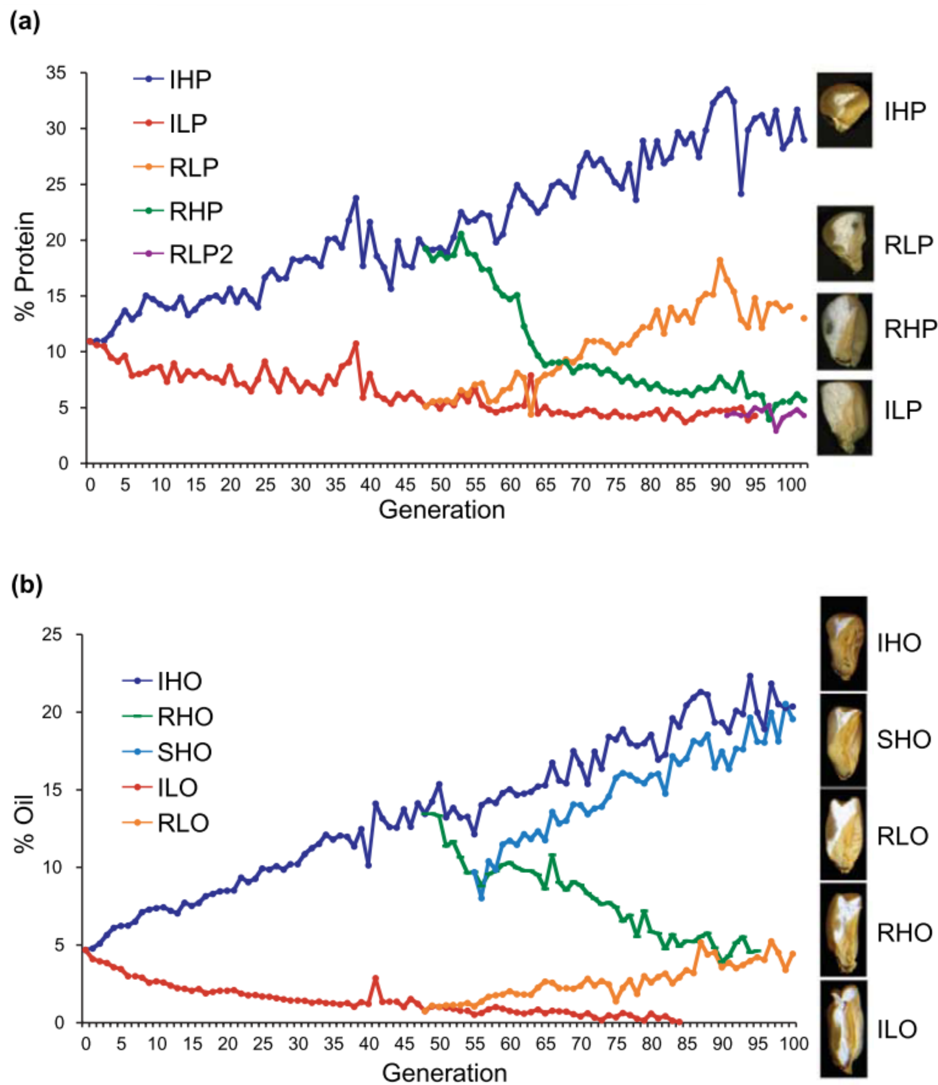


Figure 1 : Réponse à la sélection des lignées de maïs de l'Illinois pour le taux de protéines (a) ou d'huile (b) contenu dans les grains. La sélection a été réalisée sur 103 générations pour un taux important d'huile (IHP), un taux élevé de protéines (IHP), un taux faible d'huile (ILO) ou un taux faible de protéines (ILP). Au bout de 48 générations, la sélection a été inversée pour produire à partir des lignées IHP et IHO des lignées à faibles taux d'huile (RHO) ou de protéines (RHP), à l'inverse, des lignées à fort taux d'huile (RLO) ou de protéines (RLP) ont été sélectionnées, respectivement, à partir des lignées ILO et ILP. D'autres réversions ont eu lieu à la 55ème et à la 90ème génération, la première est une sélection pour des grains à haute teneur en huile (SHO) dans la lignée RHO et la seconde une sélection pour une forte teneur en protéine (RLP2) à partir de la lignée ILP. À chaque génération, les grains de 60 à 120 plantes ont été analysés, seul les 20% des plantes qui possédaient les plus hautes (ou faibles) teneurs en huile ou en protéines ont été sélectionnées pour constituer la génération suivante [Moose *et al.*, 2004].

1.1.3. Utilisation de l'évolution expérimentale pour étudier les processus évolutifs impliqués dans l'adaptation locale.

1.1.3.1. Historiques et émergence des modèles biologiques

L'évolution expérimentale est une approche qui consiste à utiliser des expériences menées en conditions contrôlées pour étudier les processus évolutifs [Reboud & Sicard, 2010] et donc tester la validité d'une interprétation adaptative [Kawecki *et al.*, 2012]. Cette idée d'observer l'évolution en action n'est pas récente. Darwin, lui-même, proposa l'idée d'utiliser des organismes à cycle de vie court, et donc à temps de génération rapide, pour observer l'effet de la sélection naturelle au fil des générations [Reboud & Sicard, 2010]. C'est en 1878 que fut réalisée l'une des premières expériences d'évolution. Ainsi, en utilisant des populations de *Cercomonas sp.*, des protozoaires à temps de génération rapide, qu'il cultiva pendant sept ans dans des températures de plus en plus importantes, William Dallinger montra qu'il était possible de sélectionner une population capable de se maintenir à 70°C, mais qui était incapable de survivre dans les conditions initiales de l'expérience [Dallinger, 1878]. Un peu plus tard, en 1896, une expérience de sélection artificielle fut réalisée sur des populations de maïs. Dans cette expérience, une sélection directionnelle fut appliquée pour favoriser des concentrations extrêmes en huile et en protéines et neuf populations, exprimant des phénotypes très différents, ont été sélectionnées durant plus de cent générations [Figure 1 - Moose *et al.*, 2004].

Durant plusieurs décennies, les projets d'évolution expérimentale se multiplient et, très vite, des modèles biologiques adaptés à ce type d'études émergent. Aussi, le terme d'évolution expérimentale s'est étendu aux expériences réalisés en laboratoire et en milieu naturel du moment où l'étude envisage plusieurs générations et a pour but de tester le processus d'évolution ou son résultat [Reboud & Sicard, 2010].

Ainsi, bien que l'évolution expérimentale soit théoriquement applicable à de nombreuses espèces, la majorité des études sont restreintes à une poignée d'organismes à cycle de vie court. En témoigne la bibliographie associée, par exemple, à *Drosophila melanogaster* ou *Escherichia coli*. Ces deux organismes étant impliqués dans deux des plus longues expériences d'évolution, en terme de générations [Lenski, 2004; Rose et al., 2004; Reboud & Sicard, 2010]. L'utilisation des plantes, hormis des espèces utiles en agronomie, est quant à elle, plutôt rare et se cantonne, généralement, à l'utilisation d'une espèce modèle qui est *Arabidopsis thaliana* [Kawecki *et al.*, 2012].

1.1.3.2. Hypothèses testables

L'évolution expérimentale donne la possibilité d'analyser l'effet de chaque force évolutive : la mutation, la sélection naturelle, la dérive génétique et la migration, lorsqu'elles agissent indépendamment les unes des autres ou lorsqu'elles sont en interaction et permet donc de tester de nombreuses hypothèses en rapport avec les processus évolutifs.

- Certains protocoles permettent de décrire la dynamique des mutations, en se centrant sur leur fréquence d'apparition, leur accumulation, leur nature ou leurs effets. [Sniegowski *et al.*, 1997; Bataillon, 2000; Chu *et al.*, 2018]. Par exemple, chez *E. Coli*, une étude menée sur 12 populations clonales, soumises à des conditions environnementales similaires pendant 10 000 générations, a montré l'apparition d'allèles mutateurs qui se sont fixés dans 3 populations [Sniegowski *et al.*, 1997].

- En testant l'influence de la sélection naturelle, l'évolution expérimentale permet d'observer l'impact d'une ou plusieurs pressions de sélection sur l'évolution des traits. Ainsi, chez *Drosophila melanogaster,* des populations tolérantes à des niveaux d'hypoxie élevée ont été sélectionnées après 200 générations dans des conditions de plus en plus pauvres en oxygène [Zhou *et al.*, 2011]. De même chez *A. thaliana*, il a été montré une évolution pour une précocité à la floraison après 2 générations de sélection dans des conditions de stress hydrique élevé, ce stress étant simulé par un arrêt de l'arrosage après 25 jours suivant le semis [Brachi *et al.*, 2012].

- D'autres expériences rendent possible l'analyse des effets de la dérive, en faisant varier la taille efficace de la population [Weber, 1990; Porcher *et al.*, 2004; Samani & Bell, 2010]. Chez *D. melanogaster*, par exemple, une sélection pour une augmentation de la taille des ailes a été opérée sur plusieurs populations de tailles différentes (n=40, 200 ou 1000) et a montré que la réponse à la sélection était proportionnelle à la taille des populations [Weber, 1990]. Aussi, chez *A. thaliana*, une étude a été menée pour tester l'impact de différents modes de sélection (sélection stabilisante *vs* sélection diversifiante) et de la taille des populations (n=100, 25 et 10) sur la réponse de plusieurs traits quantitatifs (taille de la plante, nombre de hampes florales, nombre de fruits, etc.). Pour cela, 4 réplicas de 3 populations de tailles différentes (n=100, 25 et 10) ont été utilisés, 2 réplicas ont été soumis à une sélection stabilisante où les individus à cycle de vie court ont été favorisés et 2 réplicas ont été soumis à une sélection diversifiante où seule une partie des individus a été sélectionnée pour un cycle de vie plus court. A

chaque génération les Q_{ST} de chaque trait ont été calculés entre les réplicas d'une même condition et il a été montré que dans des tailles de population limitées (n=25 et n=10) le mode de sélection n'avait pas d'impact sur le Q_{ST}, signifiant un impact très fort de la dérive sur la divergence phénotypique entre les populations [Porcher *et al.*, 2004].

- Enfin, en introduisant des individus provenant d'environnements différents il est possible de simuler de la migration et d'en étudier ses effets. Ainsi, chez *Daphnia sp*., l'effet de vigueur hybride, c'est-à-dire une augmentation de la valeur sélective des descendants provenant d'un croisement entre des individus de lignées différentes, a été montré expérimentalement en simulant de la migration entre plusieurs populations [Ebert, 2002].

En dehors de la biologie évolutive, l'évolution expérimentale est également utilisée dans d'autres domaines comme l'agriculture, où dans le cadre de sélections artificielles, des variétés végétales et animales sont généralement sélectionnées pour leur productivité ou leurs caractéristiques ornementales [Gjedrem, 1983; Moose *et al.*, 2004; Zerai *et al.*, 2010]. Aussi, l'évolution expérimentale permet de nouveaux modes de conservation des ressources génétiques, notamment pour les espèces cultivées. En effet, la conservation de la diversité génétique des espèces cultivées passe par la conservation de graines (ou d'autres propagules) provenant de plusieurs milliers d'accessions [Frankel *et al.*, 1995]. Néanmoins, cette conservation « statique » peut être limitée par la nécessité de s'adapter rapidement à une nouvelle pression de sélection, comme celle imposée par le changement climatique, la présence d'un pathogène ou des pressions anthropiques. Dans ce contexte, l'évolution expérimentale peut être utilisée pour soumettre des populations d'espèces cultivées à de nouvelles pressions de sélection, permettant une gestion dynamique des ressources génétiques [Henry *et al.*, 1991] qui peut être utilisée pour répondre aux besoins de futures sélections [Paillard *et al.*, 2000a,b; Goldringer *et al.*, 2006].

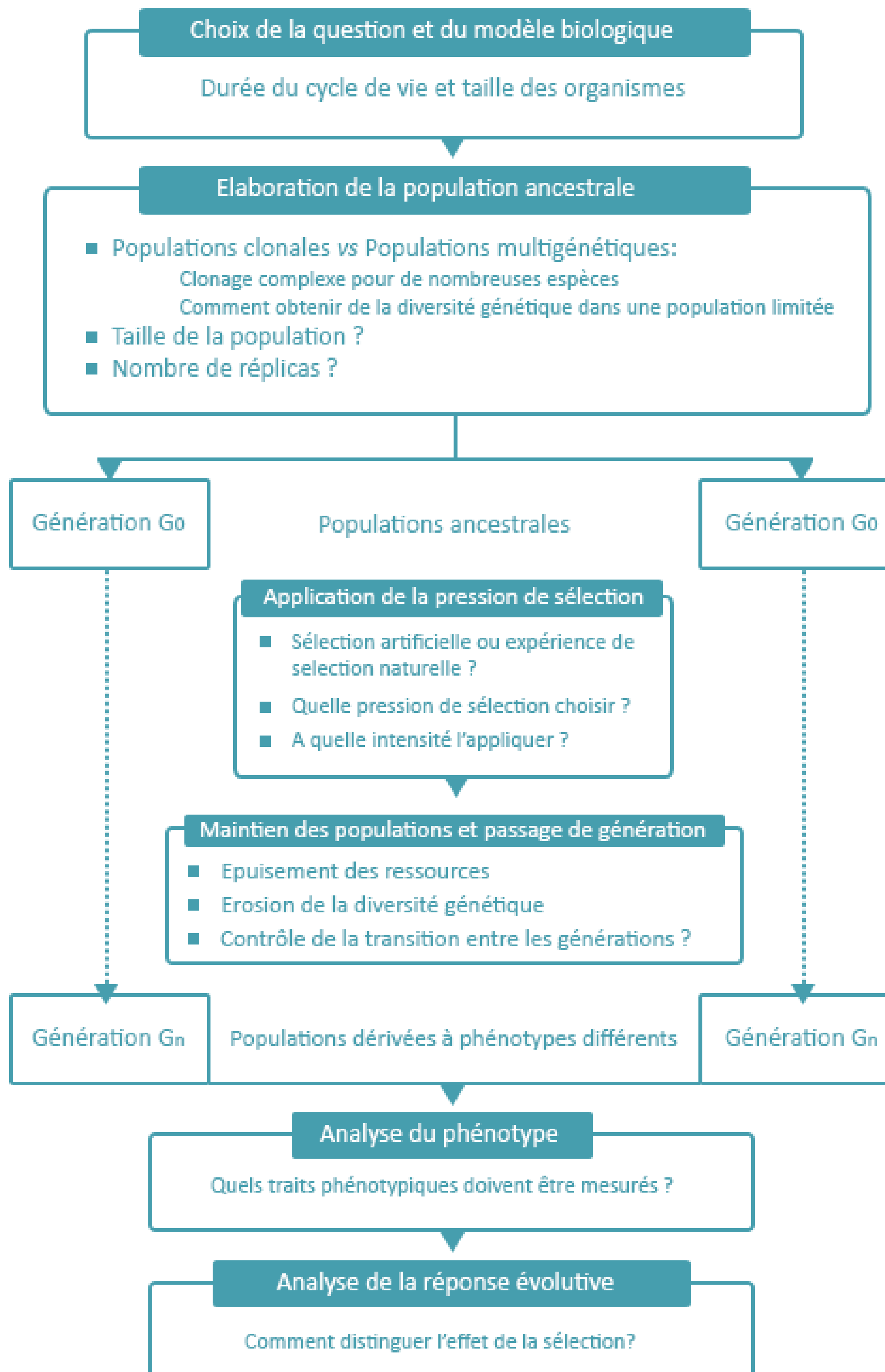


Figure 2 : Schéma récapitulatif de la mise en place d'une approche d'évolution expérimentale et des contraintes qui s'appliquent à chaque étape de sa mise en œuvre.

1.1.3.3. Principe de l'évolution expérimentale et contraintes à l'application

Bien que l'évolution expérimentale soit un outil puissant qui regroupe la mise en œuvre de protocoles diversifiés, une structure générale, faisant intervenir plusieurs étapes clés, peut être dégagée. Nous aborderons ici, ces différentes étapes ainsi que les contraintes qui peuvent s'appliquer [Figure 2].

1.1.3.3.1. Choix de la question et du modèle biologique

Dans un premier temps, il faut choisir une ou plusieurs espèces cibles qui permettront de répondre à une question posée. Ces choix conditionneront le reste du protocole.

Les hypothèses sur l'effet des différentes forces évolutives, comme l'influence du milieu (pressions de sélections biotiques ou abiotiques) ou des paramètres démographiques (taille de la population, connexion entre les populations, etc.) sur l'évolution des populations, s'appliquent à un large panel d'organismes voire même à tous les organismes. De ce fait, le choix du système d'étude devrait être une question de commodité. Néanmoins, pour observer un effet des forces évolutives testées, un nombre élevé de génération est, généralement, nécessaire. De ce fait, la grande majorité des études en évolution expérimentale se font sur quelques espèces (*E. coli, Pseudomonas sp, Drosophila sp.*) ou associations d'espèces (*Daphnia sp.* / pathogènes, bactériophage / bactéries) à cycle de vie court [Reboud & Sicard, 2010].

1.1.3.3.2. Elaboration de la population ancestrale

La seconde étape consiste à établir soit une soit plusieurs populations ancestrales, qui seront soit clonales soit multigénétiques. Les populations clonales permettent, par exemple, d'observer la fréquence d'apparition des mutations ou leur accumulation au fil des générations [Samani & Bell, 2010]. Les populations multigénétiques permettent, quant à elles, de favoriser les expériences dont le but est d'étudier la sélection [Zhou *et al.*, 2011; Brachi *et al.*, 2012] ou l'influence qu'ont d'autres forces évolutives sur cette dernière.

Dès lors, l'utilisation d'espèces de grande taille impose des contraintes spatiales qui se reflèteront sur la taille des populations. Ainsi, l'utilisation d'une grande population nécessitera de grandes surfaces, ce qui est difficilement applicable en milieu contrôlé, pour des raisons pratiques et/ou matérielles. A l'inverse l'utilisation d'une petite population peut avoir plusieurs conséquences sur l'évolution de celle-ci. Dans un premier temps, puisque dans une population

expérimentale, la diversité génétique présente est inférieure à celle retrouvée à l'état naturel, la sélection ne pourra agir que sur un nombre inférieur d'allèles [Kawecki *et al.*, 2012]. La réponse à la sélection sera donc proportionnelle à la taille de la population [Weber, 1990, cf. détails partie *1.3.2*]. Dans un deuxième temps, une petite taille de population peut amener à un effet fort de la dérive génétique, pouvant masquer les effets de la sélection [Porcher *et al.*, 2004, cf. détails partie *1.3.2*]. Enfin, dans le cas où l'évolution expérimentale sert à tester l'effet des mutations, il est nécessaire que la taille des populations soit importante, puisque le nombre de mutations à chaque génération dépend du nombre de génomes (nombre de mutation = $\mu \times 2N$ pour les organismes diploïdes et $\mu \times N$ pour les haploïdes, μ étant le taux de mutation et N le nombre d'individus) [Kawecki *et al.*, 2012].

De manière plus générale, ces contraintes spatiales limitent l'utilisation de réplicas et nécessitent de réaliser un nouveau compromis entre la taille de la population et le nombre de réplicas considérés. L'utilisation de réplicas étant indispensable aux contrôles de ces processus stochastiques mais aussi à l'exploration des différentes réponses évolutives. En effet, même si deux populations ancestrales sont génétiquement homogènes et sont soumises aux mêmes conditions, il est possible qu'aux termes de l'expérience, les populations dérivées soient très différentes. En mettant de côté la mutation et la dérive génétique qui, par essence, sont des processus aléatoires, l'hétérogénéité de la sélection peut, en partie, expliquer ces variations car elle permettrait, selon le réplica considéré, la sélection de phénotypes différents [Porcher *et al.*, 2004].

En fonction de la question choisie, il faut ensuite contrôler la diversité génétique de la population ancestrale. Ainsi, l'obtention d'une population clonale peut se faire facilement chez certains organismes, comme les procaryotes, les eucaryotes unicellulaires, les plantes ou certains animaux, mais reste relativement complexe pour beaucoup d'espèces.

A l'inverse, l'obtention d'une population multigénétique nécessite de rassembler une large gamme de génotypes. Pour cela, il peut être envisagé, dans un premier temps, d'utiliser la variation génétique d'une ou plusieurs populations sources pour des espèces disposant encore de variabilité locale [Kawecki *et al.*, 2012; Schlötterer *et al.*, 2014]. Cependant, lorsque cette variance est faible, il y a plusieurs moyens d'en créer artificiellement. Dans un premier temps, il est possible de réaliser des croisements entre individus phénotypiquement différents. Ces croisements peuvent se faire entre individus provenant d'accessions différentes [Thomas *et al.*, 1991; Rhoné *et al.*, 2010], ou, plus rarement, entre individus d'espèces différentes [Angert *et*

al., 2008]. Une autre technique consiste à augmenter artificiellement le nombre de mutations en soumettant les individus à un agent mutagène physique ou chimique [Nehnevajova *et al.*, 2007, 2012].

1.1.3.3.3. Application de la pression de sélection

La troisième étape n'est applicable qu'aux expériences dont le but est d'étudier l'effet de la sélection; elle consiste à appliquer et à maintenir une pression de sélection au fil des générations. Dès lors, deux cas sont possibles, la sélection artificielle et l'expérience de sélection naturelle (ou *Laboratory Natural Selection*). Dans le premier cas, la sélection est appliquée par l'expérimentateur sur un caractère précis [Gjedrem, 1983; Moose *et al.*, 2004; Zerai *et al.*, 2010]. Dans le second, on soumet une population à une ou plusieurs contraintes environnementales et on observe, après plusieurs générations, les réponses évolutives possibles [Brachi *et al.*, 2012; Kawecki *et al.*, 2012].

Dès lors, qu'il s'agisse d'une sélection artificielle ou d'une expérience de sélection naturelle, il est important de savoir avec quelle intensité sera appliquée la pression de sélection. Généralement, dans le cas d'une sélection artificielle, on sélectionne pour un trait d'intérêt et on fixe le nombre d'individus, porteurs de ce trait, qui pourront se reproduire et contribuer à la génération suivante, la pression de sélection appliquée est donc assez forte. Par exemple, lors de la sélection des grains, dans le cas de l'expérience réalisée sur le maïs depuis 1896, les taux d'huile et de protéines ont été mesurés sur un nombre de plantes variable selon la génération (60 à 120), et seuls 20% des individus ont été sélectionnés pour constituer la génération suivante [Moose *et al.*, 2004].

Dans le cas d'une expérience de sélection naturelle, quand c'est possible, il est préférable d'appliquer des pressions de sélection d'intensités variables. Ce qui permettra de trouver la pression de sélection optimale permettant un maintien d'un certain nombre d'individus sans entrainer la mort de la population. Ainsi, dans le cadre de l'expérience de sélection naturelle réalisée par Brachi *et al.*, (2012) sur *Arabidopsis thaliana*, des populations multigénétiques identiques ont été soumises à des pressions de sélection plus ou moins intenses portant soit sur une compétition interspécifique (avec *Poa annua*) soit un stress hydrique. Après deux générations, une évolution de la phénologie a pu être observée uniquement dans les populations où le stress hydrique était assez important (arrêt de l'arrosage à partir de 25 jours), là où, dans les populations non ou peu stressées, aucune différence n'a été remarquée même

après quatre générations. Cependant, une pression de sélection trop importante peut entrainer une extinction de la population à plus ou moins long terme.

1.1.3.3.4. Maintien des populations et passage de génération

La quatrième étape consiste à maintenir la population sur plusieurs générations. Dès lors, les expériences réalisées en milieux fermés, comme celles réalisées en serre ou en mésocosmes, peuvent poser un problème au niveau de l'épuisement des ressources au fil des générations [Kawecki *et al.*, 2012]. Dans le cas d'expériences réalisées sur des micro-organismes, l'utilisation d'un chémostat permet d'apporter un flux régulier de nutriment en quantité limitante, ce qui maintiendra constant le taux de croissance et la densité de population. La population pourra évoluer sur une longue période de temps dans des conditions stables [Novick & Szilard, 1950; Reboud & Sicard, 2010].

Une autre contrainte concerne l'érosion de la diversité génétique au fil des générations. En effet, tout au long de l'expérience, la variabilité génétique peut, par l'effet de dérive ou de la sélection, s'épuiser. Ce qui pourrait entrainer la fixation d'un seul génotype au sein de la population avant la fin de l'expérience. Cependant, il est possible de restaurer une part de la variabilité en permettant la migration d'individus entre réplicas. Par exemple, dans l'étude de Porcher *et al.*, (2004) réalisée sur *Arabidopsis thaliana*, les auteurs ont simulé de la migration en semant à chaque génération, 2% de graines provenant d'autres populations. A l'inverse, l'expérience peut être compromise par une migration importante entre les populations [Kawecki *et al.*, 2012], qui empêcherait la divergence génétique. Il est donc important de limiter les flux de gènes entre les populations.

Enfin, le passage de génération est une étape qui est rarement contrôlée et les seuls protocoles y faisant référence sont ceux utilisés en sélection artificielle. Le trait contrôlé et le pourcentage de la population sélectionné pour contribuer à la génération suivante étant généralement mentionnés. Néanmoins dans le cas d'expériences de sélection naturelle, laisser les populations évoluer naturellement entrainerait des conditions environnementales hétérogènes d'un réplica à l'autre et les différences observables entre les populations expérimentales ne seront pas uniquement dues à l'effet de la pression de sélection considérée [Kawecki *et al.*, 2012]. Ainsi, une densité d'individus plus ou moins importante entre les réplicas pourrait, par exemple, entrainer un niveau de compétition intraspécifique variable et, par conséquent, une réponse à la sélection différente.

De ce fait, pour passer d'une génération à l'autre en limitant ces effets stochastiques, la contribution de chaque individu à la génération suivante pourrait être réalisée proportionnellement à leur valeur sélective. De cette manière, les individus présentant une forte valeur sélective à la génération G_n auront une descendance fortement représentées à la génération G_{n+1} et à l'inverse, les individus présentant une faible valeur sélective auront une descendance moins représentée à la génération suivante. Néanmoins, cette méthode nécessite de pouvoir estimer correctement cette valeur sélective.

Le concept de valeur sélective (ou *fitness*) vise à quantifier la sélection naturelle et décrit la capacité d'un individu, ou plus largement d'un génotype, à se reproduire [Krimbas, 2004]. A ce titre, la valeur sélective ne doit pas être comprise comme résultante d'une histoire de vie (nombre de descendants viables, durée du cycle de vie, etc.) mais comme le reflet d'une tendance d'un génotype à avoir une histoire de vie donnée (espérance du nombre de descendants viables, espérance de longévité, etc.) [Lenormand *et al.*, 2016]. En effet, la résultante d'une histoire de vie, ou valeur sélective réalisée (*fitness* réalisée) est, à la fois, liée à son espérance, ou valeur sélective espérée (*fitness* espérée), mais également à des événements aléatoires qui peuvent affecter l'histoire de vie d'un organisme [Brandon, 1990; Krimbas, 2004].

Ainsi, la valeur sélective espérée peut être estimée par la moyenne des valeurs sélectives réalisées d'individus de génotypes identiques [Lenormand *et al.*, 2016] et, idéalement, la valeur sélective réalisée peut être estimée à travers le nombre de descendants viables que produira un individu tout au long de son cycle de vie [Krimbas, 2004]. Ce nombre de descendants est, selon les cas, difficile, voire impossible à mesurer. Dès lors, la valeur sélective réalisée peut, également, être estimée à travers la mesure de différents traits fonctionnels, morphologiques, physiologiques et phénologiques, plus facilement mesurables, directement liés aux différentes composantes des performances individuelles, à savoir la survie, la croissance et la reproduction [Arnold, 1983; Le Galliard *et al.*, 2004; Violle *et al.*, 2007].

Par soucis de lisibilité, j'utiliserai dans la suite de ce manuscrit, le terme de valeur sélective, pour désigner la valeur sélective espérée, et le terme de performance individuelle pour faire référence à la valeur sélective réalisée.

1.1.3.3.5. Analyse du phénotype et suivi des populations

La cinquième étape consiste à évaluer la réponse phénotypique. Dans le cas de la sélection artificielle, la réponse concerne le trait qui a été suivi pour réaliser la sélection des individus et est donc facilement mesurable en comparant la valeur de ce trait entre la population sélectionnée et la population ancestrale. Ainsi, dans le cas de l'expérience réalisée depuis 1896 sur le maïs, les plantes ont été sélectionnées directement pour les taux de protéines ou d'huiles contenues dans leurs graines. Après 103 générations, les analyses ont montré un changement important dans la constitution des grains [Moose *et al.*, 2004].

Dans le cas d'une sélection naturelle en laboratoire la sélection s'opère directement sur la valeur sélective des individus et il est difficile de connaitre à l'avance quelle sera la réponse à la sélection. En effet, nous l'avons vu, la valeur sélective peut indirectement être estimée par une performance individuelle, qui est, elle-même, liée à une multitude de traits fonctionnels qui agiront sur ses différentes composantes, à savoir la survie, la croissance et la reproduction [Violle *et al.*, 2007]. Il est alors possible d'étudier plusieurs caractères liés à la performance individuelle qui seront ensuite comparés entre les populations évoluées et ancestrales. Ces traits sont diversifiés et dépendent de l'organisme considéré. Chez les animaux ou les plantes, il est possible de mesurer des traits liés à la croissance ou à la reproduction des individus. Chez la drosophile par exemple, on peut mesurer le poids sec des individus, le temps d'éclosion ou les événements de reproductions précoces [Stearns, 2000]. Chez les plantes, on distinguera des traits morphologiques liés à la période végétative (nombre de feuilles, surface de la plante, etc. [Didiano *et al.*, 2014]) ou à la période reproductrice (nombre de fruits, taille des fruits, etc. [Brachi *et al.*, 2012]), des traits physiologiques liés aux fonctions de l'organisme (rendement photosynthétique, respiration, etc. [Paillard *et al.*, 2000a,b]) et des traits phénologiques liés aux différentes étapes du cycle de vie (germination, floraison, etc. [Brachi *et al.*, 2012]).

Chez les microorganismes comme *E. Coli*, les populations utilisées sont souvent clonales, ainsi la valeur sélective s'évalue à l'échelle de la population, en mettant en compétition une population avec une autre. Ces compétitions sont réalisées en soumettant deux populations au même milieu, et donc aux mêmes ressources, pendant plusieurs générations. Les cellules appartenant à chaque population sont alors comptées au début et à la fin de l'expérience de compétition [Lenski, 2004].

Dès lors, en fonction du trait étudié, de la nature et ou de la force des pressions de sélection appliquées, la variation phénotypique ne sera pas la même. Par exemple, en

soumettant plusieurs plants de sésames, *Sesamum indicum*, à différents stress abiotiques, Bor *et al.*, (2009), ont montré que la croissance des plantes diminuait lors d'un stress salin, d'un stress lié à de fortes quantités de sélénium, d'un stress lié à la sécheresse ou d'un stress lié à une augmentation des températures. Néanmoins, la réponse était plus forte chez les plants soumis au sélénium, notamment dès les premiers jours qui ont suivis la contamination.

Il faut également prendre en compte que la sélection pour un trait peut avoir entraîné, indirectement, celle d'un autre trait [Lande & Arnold, 1983]. En effet, certains traits, qualifiés de traits intégrés, sont partiellement liés les uns aux autres, soit (1) car ils partagent des bases génétiques communes, c'est le cas des traits phénotypiques liés à aux gènes pléiotropes, par exemple, la mutation du gène ZmCAD2 améliore la digestibilité du maïs mais réduit également son rendement [Barrière *et al.*, 2013] (2) ont des fonctions communes, ou (3) sont liés par des compromis (*trade-offs*) [Garland, 2014].

La réponse phénotypique d'un génotype varie également en fonction de la condition environnementale. Cette capacité est appelé plasticité phénotypique [Agrawal, 2001]. Plusieurs indices existent pour mesurer cette plasticité [Valladares *et al.*, 2006], mais les normes de réactions, qui sont des courbes décrivant la réponse phénotypique de chaque génotype en fonction de l'environnement restent le meilleur moyen de la visualiser. Dès lors, plus le phénotype d'un génotype varie en fonction de l'environnement, plus le trait sera qualifié de plastique, à l'inverse si le phénotype est maintenu constant entre les environnements on parlera de canalisation [Nijhout, 2003]. Cette canalisation suggère la mise en place de mécanismes permettant de limiter la réponse phénotypique en compensant les effets des variations environnementales. A l'inverse, la plasticité peut être interprétée comme une réponse passive de l'organisme à son environnement. La croissance d'un individu peut, par exemple, être limitée par la quantité de ressources disponible. Néanmoins, cette plasticité peut également être interprétée comme une réponse de l'organisme pour lutter contre un stress environnemental. De ce fait, beaucoup d'études discutent de la possibilité d'une plasticité adaptative, c'est-à-dire que cette capacité à moduler son phénotype aurait pu être sélectionnée dans des populations confrontées à des changements environnementaux réguliers [Via *et al.*, 1995; Dudley & Schmitt, 1996; Van Buskirk, 2002] allant jusqu'à la possibilité de l'étudier *via* l'évolution expérimentale [Garland & Kelly, 2006].

Enfin, bien que le phénotype puisse être modifié en fonction de l'environnement, il est possible que les relations entre traits restent inchangées, on parle alors d'intégration phénotypique [Pigliucci, 2003].

De ce fait, étudier la réponse phénotypique lors d'une évolution expérimentale nécessite un suivi très important des populations étudiées. Réaliser le phénotypage le plus intégratif possible est donc un bon moyen de détecter les traits qui répondront à la sélection et permet également d'évaluer l'impact de cette sélection sur la corrélation entre les traits. De plus, réaliser le phénotypage des mêmes génotypes dans diverses conditions expérimentales est un bon moyen d'identifier les conditions optimales pour l'expression des phénotypes, et aussi de détecter une potentielle évolution de la plasticité phénotypique.

1.1.3.3.6. Analyse de la réponse évolutive

La sixième et dernière étape consiste à analyser la réponse évolutive. En effet, plusieurs facteurs peuvent intervenir sur la réponse phénotypique et il est important d'en prendre compte avant toute interprétation. (1) Comme cité précédemment l'effet de la dérive génétique peut masquer celui de la sélection [Porcher *et al.*, 2004; Kawecki *et al.*, 2012; Baldwin-Brown *et al.*, 2014]. (2) D'autres facteurs de sélections peuvent également s'ajouter et masquer le signal de la pression de sélection appliquée. Par exemple, beaucoup d'expériences de sélection se déroulent en terrain expérimental ou en serre et les conditions de culture peuvent représenter une pression de sélection supplémentaire, simplement par le fait qu'elles sont différentes des conditions environnementales retrouvées *in situ*. L'utilisation d'une population témoin, où la pression de sélection choisie ne sera pas appliquée est donc indispensable [Brachi *et al.*, 2012]. (3) Les parents, notamment la mère, peuvent avoir une influence sur le phénotype de leur descendance par des effets autres que génétiques [Mousseau & Fox, 1998]. Une partie de cette influence non-génétique concerne l'effet du phénotype maternel sur le phénotype de ses descendants, on parlera d'effets maternels [Solemdal, 1997; Wolf & Wade, 2009]. Chez les plantes, les effets maternels se traduisent, notamment, par des différences au niveau de la taille des graines, de leur composition et de leur influence directe sur la dormance ou la germination [Roach & Wulff, 1987; Platenkamp & Shaw, 1993]. Enfin, plus généralement, l'environnement maternel peut contraindre la variation phénotypique de la descendance *via* un ajustement du phénotype maternel. Ce phénomène appelé plasticité transgénérationnelle [Galloway & Etterson, 2007; Herman & Sultan, 2011] représente une source fréquente, et souvent gênante, de variation phénotypique qui masque l'effet génétique [Falconer, 1981].

Une méthode employée pour tester la valeur adaptative d'un trait et l'effet d'un facteur environnemental en tant que pression de sélection est d'estimer le différentiel de sélection et

les gradients de sélection. Ainsi, en se basant sur des régressions linéaires multiples entre la valeur sélective des individus dans différents environnements contrôlés et la variation phénotypique observée, il est possible de prédire la nature et la force de la sélection exercée par la pression de sélection testée [Lande & Arnold, 1983; O'Neil, 1997]. En effet, l'idée de sélection se traduit par le fait que certains individus vont, grâce à leur phénotype, produire plus de descendants que d'autres après une génération. Ce processus peut être quantifié via le différentiel de sélection S. Il se mesure par la différence entre la moyenne du phénotype pondéré par la valeur sélective de chaque individu et la moyenne non pondérée. Concrètement il peut être mesuré par le calcul du coefficient d'une régression simple entre la valeur sélective relative de chaque individu, calculée par le rapport entre la valeur sélective de l'individu et la moyenne des valeurs sélectives au sein de la population, et le trait phénotypique standardisé [Lande & Arnold, 1983; O'Neil, 1997]. Lorsqu'on analyse plusieurs traits simultanément, on utilise des régressions multiples entre la fitness relative et les traits standardisés pour obtenir les gradients de sélection linéaires (ß) et quadratiques (γ) qui indiquent respectivement, la direction et la force de la sélection, et si la sélection est stabilisante ($\gamma < 0$) ou disruptive ($\gamma > 0$).

En comparant les Q_{ST} et les F_{ST}, calculés entre les populations ancestrales et dérivées il est possible de mettre en évidence que la réponse évolutive est liée à l'effet de la sélection (cf. partie *1.2*). De la même façon, il est possible de contrôler les processus stochastiques, comme la dérive ou l'hétérogénéité de la réponse à la sélection en utilisant les F_{ST} calculés entre les réplicas. Cet indice étant calculé sur des marqueurs neutres, non soumis à la sélection, la différence entre les populations ne pourra venir que des paramètres stochastiques, mettant en évidence un effet de la dérive ou une hétérogénéité de réponse à la sélection [Porcher *et al.*, 2004; Kawecki *et al.*, 2012].

Enfin, il est possible d'étudier les bases génétiques de la réponse observée. Par exemple, en 2004, après plus de 100 générations de sélection divergentes, des analyses QTL (*Quantitative traits loci*) ont été réalisés sur des croisements entre des populations de maïs à phénotypes opposés (IHP*ILP ou IHO*ILO - Figure 1). Ces analyses, basées sur des corrélations entre phénotypes et génotypes après croisement contrôlés de plantes à phénotypes divergents, ont montrées que les concentrations en huile et en protéine des grains étaient largement déterminées par des effets provenant de plusieurs gènes [Moose *et al.*, 2004]. D'autres analyses QTL ont estimé que le nombre de gènes impliqués dans la concentration de protéines varierait entre 102 et 178, tandis que de 14 à 69 gènes seraient impliqués dans la concentration d'huile [Dudley & Lambert, 2004]. Aussi, de nouvelles techniques permettent

aujourd'hui de séquencer directement les populations expérimentales et d'observer la réponse génomique [Schlötterer *et al.*, 2014].

1.2. Le cas de l'adaptation aux sols métallifères par les plantes

1.2.1. Les sols métallifères

La pollution, particulièrement celle générée par les éléments traces métalliques (ETM) représente une des composantes majeures du changement global. Néanmoins, cette pollution est particulière car tous les ETM sont présents naturellement à l'état de trace dans les sols et ont, par conséquent, trouvé un rôle dans de nombreux processus biologiques [He *et al.*, 2005; Nicholas, 2012]. On distinguera donc les métaux essentiels, ou oligo-éléments, indispensables au bon fonctionnement des processus biologiques, comme le zinc (Zn), le fer (Fe) ou le cuivre (Cu), des éléments qui n'ont aucune fonction biologique bien connue, et qui seront toxiques pour la plupart des espèces, même à de très faibles concentrations, comme le cadmium (Cd), le mercure (Hg) ou le plomb (Pb).

Le plus souvent, les ETM ne sont retrouvés qu'en faible concentration dans les sols, mais, localement, cette concentration peut fortement augmenter. On parlera alors de sites métallifères. L'origine de ces sites peut être naturelle et est souvent associée à la minéralisation de roches-mères riches en ETM. C'est le cas, par exemple, des sols serpentiniques, produit par l'érosion de roches ultramafiques, riches en fer, nickel, chrome et cobalt [Brooks, 1987; Kazakou *et al.*, 2008] ou des sols cuprifères, naturellement riches en cuivre [Brooks & Malaisse, 1990; Faucon *et al.*, 2007]. Ainsi, bien que ces milieux soient considérés comme extrêmes, ils abritent une grande diversité d'espèces qui est liée à une longue évolution des espèces [Ernst, 2006]. A l'inverse, de nombreux sites métallifères ont pour origine une contamination anthropique provenant, par exemple, de l'industrie métallurgique, de l'épandage de boues d'épurations, de l'utilisation de biocides ou de l'exploitation minière [Antonovics *et al.*, 1971]. Ces sites, plus récents, abritent une diversité réduite du fait des fortes pressions de sélection qui s'appliquent.

1.2.2. L'adaptation des plantes aux sols métallifères

Du fait de leur seuil de toxicité relativement bas et de leur persistance dans l'environnement, les ETM peuvent être considérés comme une pression de sélection importante

pour de nombreuses espèces [Sánchez, 2008] et notamment pour les plantes qui, du fait de leur immobilité, seront contraintes à s'adapter rapidement. Il est également à noter qu'au-delà de la concentration en éléments traces métalliques, d'autres pressions de sélection peuvent s'appliquer au niveau des sites métallifères [Antonovics *et al.*, 1971; Frérot *et al.*, 2018] comme le contenu en nutriments et en matière organique, avec des sols métallifères généralement déficients en éléments essentiels, comme l'azote, le calcium, le phosphore ou le potassium [Bradshaw *et al.*, 1960; Macnair, 1987], la sécheresse [Macnair, 1987], le stress lumineux [Jiménez-Ambriz *et al.*, 2007] ou la réduction d'interactions biotiques positives [Antonovics *et al.*, 1971]. Dès lors, ces contraintes ont conduit à la formation de communautés végétales particulières, composés de plantes caractérisées par leur capacité à tolérer les fortes concentrations en éléments traces des milieux dont elles sont issues : les plantes métallophytes.

La tolérance aux métaux, définie par la capacité à survivre, se développer et se reproduire dans des sols enrichis en éléments traces métalliques [Macnair, 1993] est déterminée génétiquement [Macnair, 1983; Arrivault *et al.*, 2006; Chiang *et al.*, 2006; Hanikenne & Nouet, 2011]. Il s'agit d'un caractère adaptatif permettant l'installation des espèces sur des sols enrichis en ETM, et dont l'expression variera en fonction de la quantité en ETM biodisponible [Baker & Walker, 1990]. La tolérance aux métaux peut s'évaluer à travers différentes composantes des performances individuelles. Ainsi, on trouve dans la littérature plusieurs évaluations de la tolérance, à partir de traits physiologiques comme la mesure de la chlorose des feuilles [Assunção *et al.*, 2003], le contenu en pigments photosynthétiques ou en enzymes antioxydantes [Kolbas *et al.*, 2018] ou de traits morphologiques liés à la phase végétative [Wong & Bradshaw, 1982; Macnair, 1983; Assunção *et al.*, 2003], ou à la phase reproductrice [Dechamps *et al.*, 2007, 2011; Jiménez-Ambriz *et al.*, 2007].

Les plantes tolérantes aux métaux, adoptent également plusieurs stratégies au niveau de la gestion des ETM [Baker, 1981; van der Ent *et al.*, 2013]. Dès lors, on distinguera les plantes excluantes qui limitent l'entrée des ETM dans les parties aériennes, des plantes bioindicatrices dont la concentration foliaire en ETM est proportionnelle à celle du sol, et des plantes hyperaccumulatrices qui accumulent de fortes quantité d'ETM au niveau de leurs parties aériennes [Figure 3]. Le seuil pour définir l'hyperaccumulation est variable en fonction de l'élément considéré [van der Ent *et al.*, 2013].

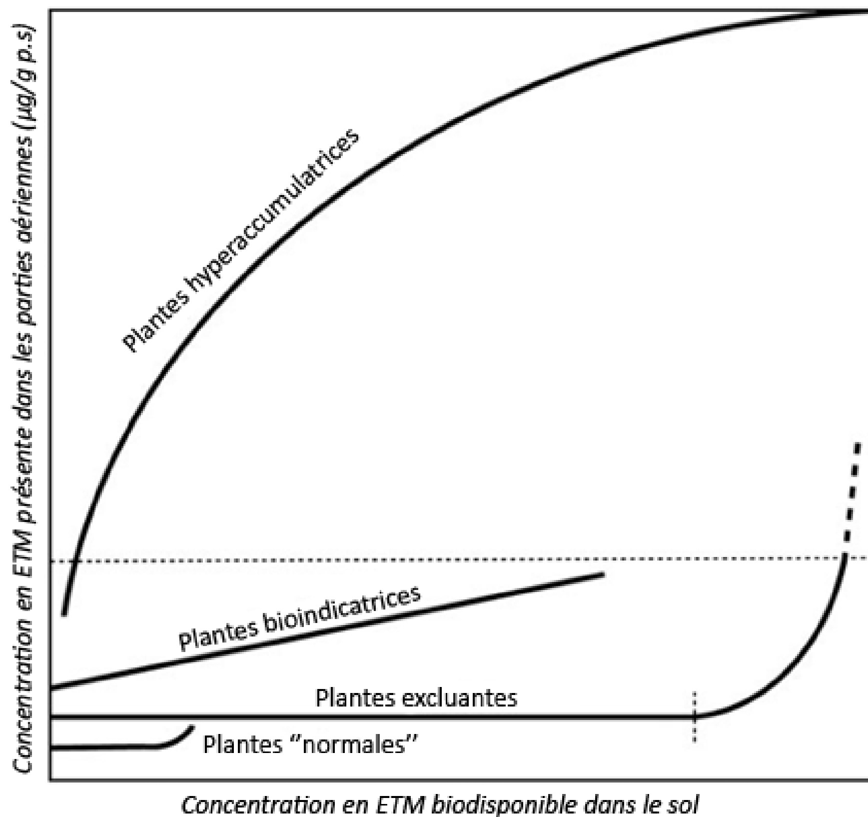


Figure 3 : relation entre la concentration en ETM présente dans les feuilles et celle présente dans le sol pour des plantes non tolérantes (« normales »), et tolérantes. On distinguera les plantes excluantes qui limitent l'entrée des ETM dans les parties aériennes, des plantes bioindicatrices dont la concentration foliaire en ETM est proportionnelle à celle du sol, et des plantes hyperaccumulatrices qui accumulent de fortes quantité d'ETM au niveau de leurs parties aérienne [d'après van der Ent *et al.*, 2013].

1.2.3. Les pseudométallophytes : des modèles pour l'étude de l'adaptation locale

La tolérance aux métaux peut s'exprimer à la fois chez tous les individus d'une espèce ou seulement chez certains individus. Ainsi, on distinguera les métallophytes absolues, qui sont des taxons uniquement présents sur les sols métallifères, des pseudométallophytes (ou métallophytes facultatives) qui occuperont à la fois les milieux contaminés et non contaminés d'une même région phytogéographique [Antonovics *et al.*, 1971]. De même, au sein des pseudométallophytes, on distinguera plusieurs groupes édaphiques, dont les populations métallicoles et non-métallicoles qui se développent, respectivement, sur les sites métallifères et non métallifères.

Parmi ces espèces, deux modèles biologiques ont émergés assez récemment : *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey et *Arabidopsis halleri* (L.) O'Kane et Al-shebaz. Ces deux espèces de la famille des Brassicaceae sont particulièrement étudiées pour leurs capacités de tolérance aux métaux et d'hyperaccumulation. Chez ces deux espèces, la tolérance et

l'hyperaccumulation du zinc et du cadmium sont des caractères constitutifs, c'est-à-dire que les deux types édaphiques expriment ces caractères. Cependant la valeur exprimée pour ces caractères diffèrera en fonction du groupe édaphique et de l'élément considéré.

Ainsi, pour le zinc, plusieurs études visant à observer les niveaux d'expression de tolérance et d'hyperaccumulation dans les deux groupes édaphiques ont montré que les individus métallicoles, retrouvés sur des sols calaminaires riches en zinc, plomb et cadmium, étaient plus tolérants mais accumulaient moins les métaux que les individus non-métallicoles [Meerts & Van Isacker, 1997; Escarré *et al.*, 2000; Bert *et al.*, 2002; Assunção *et al.*, 2003b; Sterckeman *et al.*, 2017]. En outre, des comparaisons entre Q_{ST} et F_{ST} calculés entre des populations de types édaphiques différents ont mis en avant un effet fort de la sélection sur la divergence phénotypique observée sur plusieurs traits végétatifs et reproducteurs entre les populations. Cette comparaison, réalisée sur *N. caerulescens* a montré que cette divergence pourrait venir d'une sélection divergente entre les deux types édaphiques [Jiménez-Ambriz *et al.*, 2007; cf détail de l'étude partie *1.2*] alors qu'elle viendrait d'une sélection stabilisante chez *A. halleri* [Meyer *et al.*, 2010; cf détail de l'étude partie *1.2*].

Ces résultats suggèrent donc une adaptation locale qui peut être étudiée de nombreuses manières. Ainsi, de nombreux auteurs se sont d'abord orientés vers l'analyse des bases génétiques de la tolérance et de l'hyperaccumulation à travers des croisements intraspécifiques [Karam *et al.*, Under review; Assunção *et al.*, 2003] ou interspécifiques [Willems *et al.*, 2007; Frérot *et al.*, 2010; Meyer *et al.*, 2016] quand d'autres, se sont intéressés à la structure génétique des populations [Pauwels *et al.*, 2005; Gonneau *et al.*, 2017] ou aux facteurs environnementaux pouvant expliquer leur répartition [Frérot *et al.*, 2018; Sirguey *et al.*, 2018].

Dans ce contexte, il peut être envisagé d'utiliser l'évolution expérimentale pour tester les processus évolutifs impliqués dans l'adaptation locale aux sols métallifères par des espèces pseudométallophytes.

1.2.4. Utilisation des pseudométallophytes en évolution expérimentale pour l'étude de l'adaptation aux sols métallifères ?

L'évolution expérimentale impose de nombreuses contraintes aux différentes étapes de sa mise en œuvre (cf. partie 1.3.3). Il s'agira, dans cette partie, de discuter des contraintes qui s'appliquent à l'utilisation de plantes, et particulièrement d'espèces pseudométallophytes, dans la mise en place d'une expérience de sélection naturelle.

1.2.4.1. La durée du cycle de vie

La pauvreté relative en expériences réalisées sur les plantes, reflète, en partie, leur long temps de génération par rapport aux organismes couramment utilisés en évolution expérimentale (2 mois pour *Arabidopsis thaliana* contre 2 semaines pour la drosophile ou quelques heures pour les microorganismes) [Kawecki *et al.*, 2012]. De plus, pour observer un effet de la pression de sélection, un nombre élevé de générations est généralement nécessaire.

De nombreuses espèces végétales peuvent également avoir un cycle de vie complexe impliquant plusieurs évènements de reproduction. L'utilisation de telles espèces deviendrait difficile à gérer au bout de quelques générations et imposerait des contraintes temporelles (suivi des populations sur plusieurs années), logistiques (suivi de chaque événement de reproduction, gestion de chaque descendance et de chaque population), méthodologiques (gestion de la compétition entre les parents et les descendants, contribution d'une plante sur plusieurs générations) ou matérielle (ressources limitées). Ainsi, l'utilisation de plantes sémelpares et, si possible, annuelles parait être la solution la plus envisageable.

1.2.4.2. La taille des populations, le nombre de réplicas et la diversité génétique présente dans la population ancestrale

Les plantes imposent également une contrainte au niveau de leur taille, ce qui nécessite de réaliser des compromis entre la taille des populations et le nombre de réplicas considérés (cf partie 1.3.3.2). Aussi, cette contrainte de taille limitera la diversité présente dans la population ancestrale. Il est donc important de la maximiser dès le départ.

Enfin, la taille limitée des populations impose des contraintes lors de la transition entre les générations (cf. partie *1.3.3.4*), en générant, par exemple des effets stochastiques liés à une

différence de densité entre les populations expérimentales. Ainsi, il peut être envisagé de contrôler la descendance de chaque individu en semant (ou en transplantant) un nombre de graines (ou de plantules) proportionnel à leur performance. Ce qui nécessite une bonne estimation des performances individuelles.

1.2.4.3. Choix de la pression de sélection, de son intensité et des conditions de son application

Généralement, les protocoles d'évolution expérimentale tentent de recréer une situation évolutive rencontrée dans la nature, en diminuant sa complexité [Reboud & Sicard, 2010]. De cette manière, l'étude des processus évolutifs impliqués dans l'adaptation aux sites métallifères peut être réalisée progressivement en analysant, par exemple, l'effet des différentes pressions de sélection indépendamment les unes des autres.

Dans ce contexte, il est difficilement envisageable de soumettre les populations à un sol prélevé directement des sites métallifères, car beaucoup de facteurs biotiques et abiotiques pourraient interagir, ce qui limiterait les interprétations. De plus, il ne serait pas possible de réaliser une condition contrôle, où un réplica ne sera soumis à aucun de ces paramètres. D'autres limites existent et concernent également l'utilisation de sols provenant de sites non-métallifères. D'une part, l'utilisation d'un sol naturel pose de nombreux problèmes pratiques, notamment dans les cas où de grandes quantités de sol doivent être prélevées (transport, prélévements, autorisations, etc.). D'autre part, les conditions environnementales rencontrées dans la nature peuvent changer et, dans le cas où l'expérience nécessiterait un renouvellement des ressources, le sol apporté pourrait ne pas être le même d'une génération à l'autre. Ainsi, dans un souci de standardisation et de répétabilité l'utilisation d'un substrat standardisé, comme un terreau horticole, est préférable.

Une fois la pression de sélection choisie, il faut ensuite définir l'intensité avec laquelle elle sera appliquée. En effet, cette pression de sélection doit être assez forte pour entrainer une évolution de la population sans entrainer son extinction à plus ou moins long terme. Cet ajustement nécessitera une phase de test ou un nombre de réplicas plus importants.

1.2.4.4. L'utilisation des pseudométallophytes est-elle donc pertinente ?

Les plantes imposent donc deux contraintes majeures : la durée de leur cycle de vie et leur taille.

La durée de leur cycle de vie est limitante pour suivre les populations sur un grand nombre de génération. Néanmoins, des expériences de sélection naturelle, réalisés sur des plantes ont montré qu'une réponse à la sélection était possible en peu de générations à partir du moment où la pression de sélection est importante et qu'il existe une grande diversité génétique dans la population [Brachi *et al.*, 2012, cf détail partie *1.3.2*]. En 2008, par exemple, une étude a montré une évolution de la phénologie et de la biomasse après une génération suite à un stress lié à l'altitude chez une population hybride de *Mimulus cardinalis* et *Mimulus lewisii* [Angert *et al.*, 2008]. En conséquence, dans le cas des espèces pseudométallophytes, l'utilisation de plusieurs populations de *Noccaea caerulescens* décrites comme sémelpares annuelles ou biannuelles [Dechamps *et al.*, 2007] peut donc être envisagée.

La taille des plantes est un autre facteur limitant qui nécessite de réaliser des compromis entre le nombre de réplicas et la taille des populations, ce qui joue également sur la diversité génétique présente à la création de la génération ancestrale. Néanmoins, *Arabidopsis halleri* et *Noccaea caerulescens* sont des espèces de petite taille qui permettent de manipuler des effectifs de taille raisonnable. De plus, concernant la diversité génétique, de nombreuses populations ont été recensées; il peut donc être envisagé d'utiliser la *standing genetic variation* présente dans ces populations [Molitor *et al.*, 2004; Pauwels *et al.*, 2005; Kawecki *et al.*, 2012].

Dans ce contexte, l'utilisation de *Noccaea caerulescens* ou *Arabidopsis halleri* ne semble pas limitante à l'application d'un projet d'évolution expérimentale.

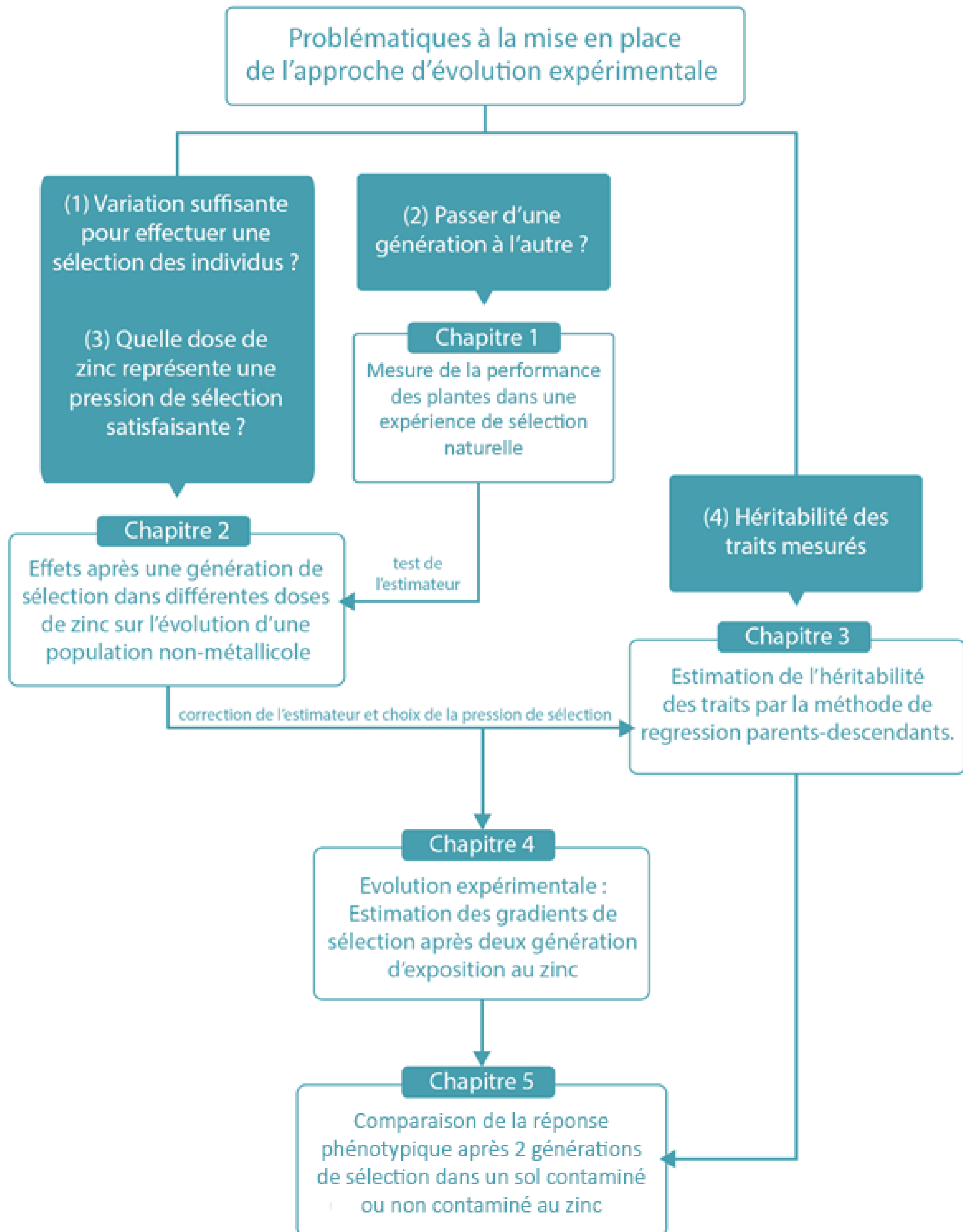


Figure 4 : Séparation des différents chapitres

1.3. Objectifs et déroulement de la thèse

Comme vu précédemment, les sites métallifères diffèrent généralement des sites non métallifères par divers paramètres environnementaux, autres que les fortes concentrations en éléments traces métalliques en présence, de sorte que l'adaptation locale aux sols métallifères peut être une réponse à différentes contraintes environnementales pouvant interagir. Par conséquent, l'effet de la toxicité des éléments traces métalliques en tant que seule pression de sélection reste à démontrer. Dans ce contexte, une approche d'évolution expérimentale a été initiée afin d'étudier l'effet d'une contamination en zinc sur l'évolution d'une population non-métallicoles de *Noccaea caerulescens*.

Les objectifs majeurs de cette thèse étant de (1) de tester si une contamination du substrat par le zinc peut représenter une pression de sélection pour des populations non métallicoles (attendues comme moins tolérantes) et (2) tester l'effet de cette sélection expérimentale sur l'évolution de plusieurs traits fonctionnels, potentiellement corrélés, associés à la tolérance aux métaux et à l'hyperaccumulation.

La mise en place de cette expérience soulève, cependant, certaines problématiques (1) la diversité génétique (et donc phénotypique) présente dans les populations naturelles est-elle suffisante pour pouvoir opérer une sélection des individus en milieu contrôlé ou semi-contrôlé? (2) Comment passer d'une génération à l'autre dans une population de taille finie, en limitant la dérive génétique ? (3) Quelle dose de zinc représente une pression de sélection satisfaisante? (4) Quelle est la part héritable de la variation phénotypique observée à cette concentration ? Pour répondre, plusieurs expériences ont été réalisées et seront intégrées aux 3 premiers chapitres. Les chapitres 4 et 5 correspondront, d'une part, à la mise en place du projet d'évolution expérimentale et à la dynamique adaptative générée par les différentes conditions de culture et, d'autre part, à la comparaison des phénotypes exprimés entre les populations ancestrales et dérivées en conditions contrôlées [Figure 4].

CHAPTER 1

2. Measuring plant performance in Laboratory Natural Selection context

2.1. Brief introduction

Experimental design in experimental evolution limits the size of investigated populations, especially when plants are involved. Therefore, to limit potential effects of genetic drift, the transition from one generation to the next can benefit from a strict control of the offspring representation. In contrast, allowing the population to evolve naturally may result in observable differences between the experimental populations that would not be only due to the effect of the considered selection pressure [Kawecki *et al.*, 2012]. To make the transition from one generation to the next by limiting stochastic effects, the contribution of each individual to the next generation can be made in proportion to their fitness. However, this method requires being able to provide a good fitness estimation. Fitness is defined as the ability of an individual to survive and reproduce [Krimbas, 2004]. Fitness represents a theoretical concept that is complex to measure. In plants, it can be estimated, indirectly, according to the measure of several morphological, physiological and phenological functional traits related to the three components of individual performance: survival, growth and reproduction [Violle *et al.*, 2007]. In semelparous plants, individual performance can be estimated through the seed set [Primack & Kang, 1989]. Nevertheless, in natural or semi-natural conditions, seeds are difficult to harvest exhaustively, because they tend to disperse easily with rain or wind. Several estimation of the seed set have been previously referenced. For example, in Brassicaceae, it may be possible to use the number of fertile fruits produced during the life cycle [Dechamps *et al.*, 2008] or the total fruit length estimated by multiplying the number of fertile fruits by the average fruit length [Brachi *et al.*, 2012].

In this context, we used several biological material from natural accessions, inbred or outbred crosses of *Noccaea caerulescens* submitted to non-polluted or zinc polluted soil in order to study the correlations between the exact number of seeds produced by plants and various vegetative and reproductive traits. Data were used to deduce the best seed set estimation in each condition.

Table 1: Characteristics of the study sites. Soil samples were taken at a depth of 0–15 cm for study 1 and 0-10 cm for study 2. Metal concentrations represented the total metal concentrations contained in soil sampling and were expressed as mg metal kg^{-1} soil.

	Lellingen[1]	**Wilwerwiltz[2]**	**Winseler[2]**	**La Calamine[1]**	**Prayon[3]**
Abbreviation	LE	WIL	WIN	LC	PR
Number of families	15	15	15	25	25
Geographic coordinates	49°59’ N. 6°00’ E	49°57′ N. 5°53′ E	49°58′ N. 5°59′ E	50°41'44 N. 05°59'39 E	50°34’52 N. 5°40’02 E
pH	5.7	5.9	5.8	6.8	6.9
Zn	126 ± 4.3	139	164 - 274	101563 ± 14329	75700 ± 13500
Cd	< 1± 0.0	<2	0.7 - 4.3	217 ± 59	667 ± 85
Pb	48 ± 3.5	54	80 – 136	8998 ± 2524	9620 ± 1460
Ni	48 ± 2.6	42	66 - 157	8998 ± 2524	211 ± 57

[1](Assunção et al., 2003a) [2](Reeves et al., 2001) [3](Roosens et al., 2003)

Table 2 : List of individuals used directly for a phenotypic survey (experiment 1) and involved in controlled crosses (parents of F1 used in experiment 2) per population. LE=Lellingen, WIL=Wilwerwiltz, WIN=Winseler, LC = La Calamine and PR=Prayon. Individuals in bold characters were used in controlled crosses.

Lellingen	**Wilwerwiltz**	**Winseler**	**La Calamine**	**Prayon**
LE.02	WIL.10	WIN.02	LC.05	PR. 02
LE.03.1	WIL.13	WIN.04	LC.06	PR.04
LE.03.2	WIL.15	WIN.05	LC.08	PR.06
LE.07	WIL.18	WIN.06	LC.09	PR.09
LE.11	WIL.19	WIN.07	LC.10	PR.10
LE.12.1	WIL.22	WIN.08	LC.13	PR.13
LE.12.2	WIL.24	WIN.13	LC.15	PR.15
LE.15	WIL.25.1	WIN.14	LC.17	PR.23
LE.16	WIL.25.2	WIN.17	LC.18	PR.28
LE.17	WIL.26	WIN.18	LC.26	PR.30
LE.19	WIL.27.1	WIN.19	LC.27	PR.31
LE.20	WIL.27.2	WIN.21	LC.28	PR.37
LE.24	WIL.30	WIN.22	LC.32	PR.39
LE.27	WIL.33	WIN.24	LC.33	PR.40
LE.28	WIL.34	WIN.26	LC.34	
LE.02.1	**WIL.27.1**	**WIN.21.1**	**LC.02**	**PR.21.1**
LE.28	**WIL.34.1**	**WIN.26.1**	**LC.08**	**PR36.1**
			LC.36	**PR.36.2**

2.2. Material and methods

2.2.1. Biological material

Noccaea caerulescens [Figure S1] seeds families were sampled in natural populations in June 2013. Sampled sites were “La Calamine” (LC – 25 families) and “Prayon” (PR – 25 families), two smelting sites from Belgium abandoned several decades ago (Assunçào et al., 2003), and “Lellingen” (LE – 15 families), “Wilwerwiltz” (WIL – 15 families) and “Winseler” (WIN – 15 families), three non-metalliferous sites from Luxembourg (Reeves et al., 2001) [Table 1].

In September 2013, 30 seeds by plant family were sown in seedling trays containing peat-based compost, placed one week in a cold room at 4°C and transferred in a greenhouse for six weeks. Then, 84 individuals from five populations (LE=17, WIL=17, WIN=17, LC=16 and PR=17) were transplanted into individual pots containing 1 kg of peat and clay-based compost (NEUHAUS® N13 - 70%)/ 2-5mm chabasite type zeolite (30%) mix and placed, after a randomization, in a tunnel greenhouse in order to induce flowering. Then, 72 of 84 individuals (LE=15, WIL=15, WIN=15, LC=13 and PR=14) [Table 2] were used directly in a phenotypic survey of local (i.e. produced through intrapopulational crosses) accessions [Experiment 1 in Figure 5], and the remaining 12 individuals (LE=2, WIL=2, WIN=2, LC=3 and PR=3) [Table 2] were involved in inter-edaphic groups (M*NM, NM*M) crosses to perform a phenotypic survey of hybrid (F1) individuals [Experiment 2 in Figure 5]. Crosses were manually performed by rubbing open anthers of male parents on the stigmas of female parents. On each parental plant, some flowers were also self-pollinated [Table 3].

In september, 30 seeds harvest form each controlled were sown and 15 representatives from inbreed and outbreed progenies were transferred into individual pots using the above-described protocol. For 10 of 15 descendants, the soil mix was contaminated with 750 mg of zinc by kg of fresh compost (20 mL of a solution at 0.57M of ZnSO4, 7 H2O) [Figure 5]. This moderate level of zinc exposure was chosen to induce toxicity without causing plant mortality that would have prevented phenotyping and seed harvesting. Pots were randomized and placed in a ventilated and unheated greenhouse in order to induce flowering.

2.2.2. Phenotypic survey

In 2014 and 2015, respectively, the 72 local plants and the 360 F1 plants were phenotyped for several vegetative and reproductive traits during their life cycle [Figure 5].

Table 3: List of controlled crosses. M=metallicolous, NM=nonmetallicolous, LE=Lellingen, WIL=Wilwerwiltz, WIN=Winseler, LC = La Calamine and PR=Prayon

<table>
<tr><th></th><th>Cross type</th><th colspan="2">F1 origin</th><th>F1 pedigree</th></tr>
<tr><td rowspan="12">Self-crosses</td><td>M self</td><td>PR*PR</td><td rowspan="3">PR</td><td>PR.36.1*PR.36.1</td></tr>
<tr><td>M self</td><td>PR*PR</td><td>PR.36.2*PR.36.2</td></tr>
<tr><td>M self</td><td>PR*PR</td><td>PR.21*PR.21</td></tr>
<tr><td>M self</td><td>LC*LC</td><td rowspan="3">LC</td><td>LC.02*LC.02</td></tr>
<tr><td>M self</td><td>LC*LC</td><td>LC.36*LC.36</td></tr>
<tr><td>M self</td><td>LC*LC</td><td>LC.08*LC.08</td></tr>
<tr><td>NM self</td><td>LE*LE</td><td rowspan="2">LE</td><td>LE.28*LE.28</td></tr>
<tr><td>NM self</td><td>LE*LE</td><td>LE.02*LE.02</td></tr>
<tr><td>NM self</td><td>WIL*WIL</td><td rowspan="2">WIL</td><td>WIL.34*WIL.34</td></tr>
<tr><td>NM self</td><td>WIL*WIL</td><td>WIL.27*WIL.27</td></tr>
<tr><td>NM self</td><td>WIN*WIN</td><td rowspan="2">WIN</td><td>WIN.26*WIN.26</td></tr>
<tr><td>NM self</td><td>WIN*WIN</td><td>WIN.21*WIN.21</td></tr>
<tr><td rowspan="12">Inter-edaphic crosses</td><td>M*NM</td><td>PR*LE</td><td rowspan="2">C1</td><td>PR.36.1*LE.28</td></tr>
<tr><td>NM*M</td><td>LE*PR</td><td>LE.28*PR.36.1</td></tr>
<tr><td>M*NM</td><td>PR*WIL</td><td rowspan="2">C2</td><td>PR.36.2*WIL.34</td></tr>
<tr><td>NM*M</td><td>WIL*PR</td><td>WIL.34*PR.36.2</td></tr>
<tr><td>M*NM</td><td>PR*WIN</td><td rowspan="2">C3</td><td>PR.21*WIN.26</td></tr>
<tr><td>NM*M</td><td>WIN*PR</td><td>WIN.26*PR.21</td></tr>
<tr><td>M*NM</td><td>LC*WIN</td><td rowspan="2">C4</td><td>LC.02*WIN.21</td></tr>
<tr><td>NM*M</td><td>WIN*LC</td><td>WIN.21*LC.02</td></tr>
<tr><td>M*NM</td><td>LC*LE</td><td rowspan="2">C5</td><td>LC.36*LE.02</td></tr>
<tr><td>NM*M</td><td>LE*LC</td><td>LE.02*LC.36</td></tr>
<tr><td>M*NM</td><td>LC*WIL</td><td rowspan="2">C6</td><td>LC.08*WIL.27</td></tr>
<tr><td>NM*M</td><td>WIL*LC</td><td>WIL.27*LC.08</td></tr>
</table>

The following vegetative traits were measured at the first flower bud emergence for each plant: the two larger diameters, to calculate the surface of the plant (*SP*) as an ellipse surface, the number of leaves (*NL*), leaf length (*LL*), leaf width (*LW*) from the mean of the 3 longest leaves, estimated leaf morphology ($LM = \frac{LL}{LW}$), and estimated leaf surface ($LS = \frac{LL}{2} * \frac{LW}{2} * \pi$). Plants were bagged at the first siliques onset in order to harvest the entire seed set. At the end of seed production, seeds were harvested and we measured the seed set (*SS*) with seed counter (elmor C3 Seed Counter, elmor Ltd.). Afterwards, plants were harvested, dried (at 20°C, during 60 days) and measured for the following reproductive traits: the number of flower stems (NFS), the length of the longest stem (*maxLFS*), the total number of siliques (*NS*), the number of aborted and non-aborted siliques (*NAS* and *NNAS*, respectively), the ratio between *NNAS* and *NAS* (*RS*) and the mean silique length (SL), measuring the length of 5 siliques per flower stem. The total length of siliques ($totalLS = NNAS * SL$) was calculated multiplying the total number of non-aborted siliques by the mean length of siliques. The total length of siliques is known to be an accurate estimator of seed set in *Arabidopsis thaliana* [Brachi *et al.*, 2012; Roux *et al.*, 2016], the number of non-aborted siliques was also used as a proxy of the seed set in *Noccaea caerulescens* [Dechamps *et al.*, 2008]. In parallel, 140 siliques randomly chosen in experiment 1 were measured and the number of seeds they contained was counted.

2.2.3. Statistical analyses

We first joined the datasets of experiments 1 and 2 in order to perform a principal component analysis and Pearson's correlations corrected for multiple tests by applying a FDR Benjamini-Hochberg adjustment. The aim was to determine which traits were the best correlated with seed set. Then, a Wilcoxon-Mann-Whitney's test was performed on the whole data set to test the effect of experimental conditions between experiment 1 and experiment 2. Afterwards, the data from each experiment were analyzed independently. For individuals from experiment 1, a hierarchical ANOVA was used to test the effect of the edaphic origin and the populations (nested in edaphic origin). For the individuals from experiment 2, we tested the effect of the cross type (M or NM selfing and inter-edaphic cross: M*NM and NM*M), the F1 origin (corresponding to the source population of parents, this factor was nested in cross type), the F1 pedigree (corresponding to the family of parents, this factor was nested in F1 origin) (see Table 3), the contamination level and the interaction between these factors with a cross-factor hierarchical ANOVA.

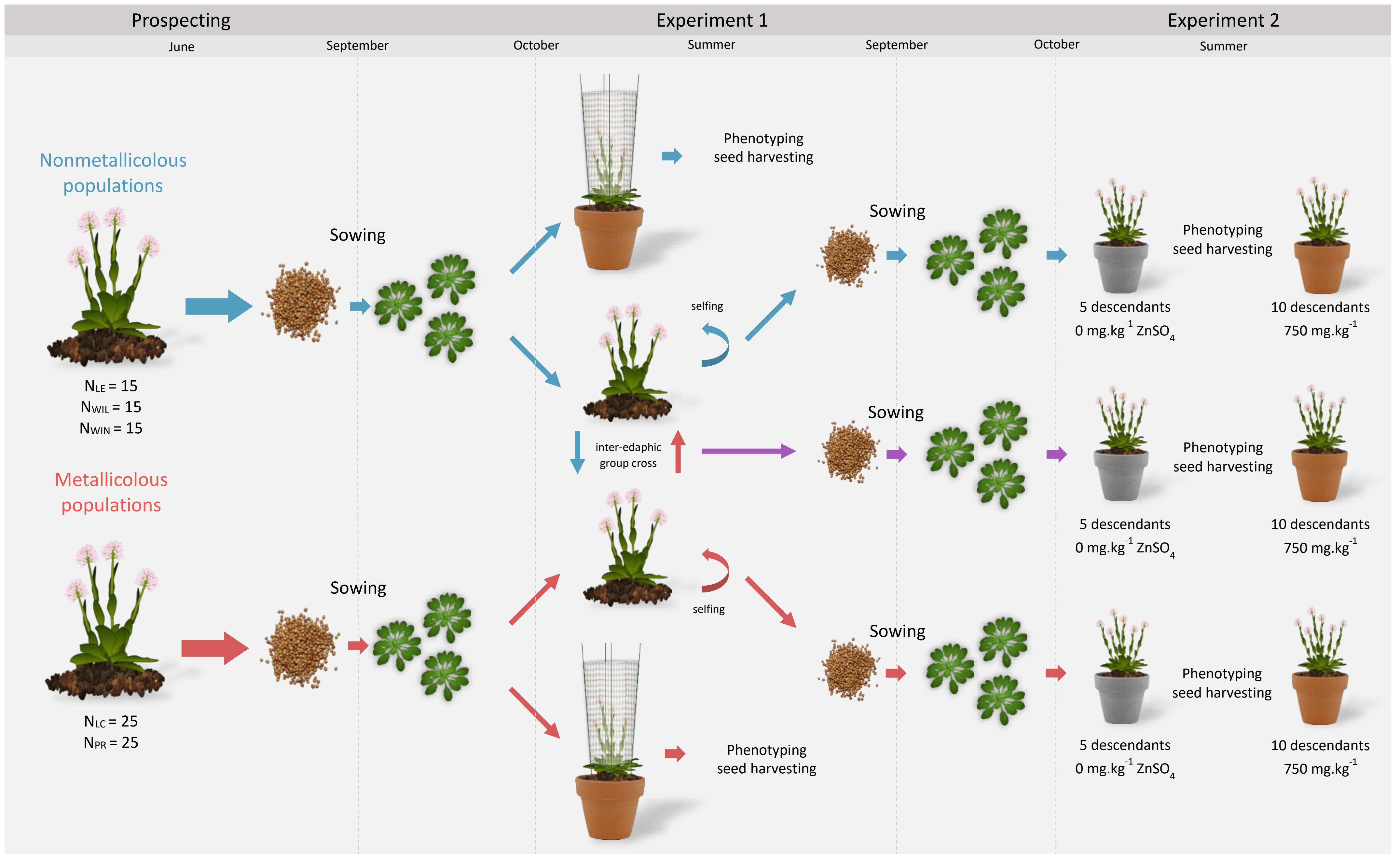


Figure 5 : summary scheme of experiment 1 and experiment 2. First, in june 2013, 95 seeds families were harvested in natural nonmetallicolous (LE=Lellingen, WIL=Wilwerwiltz, WIN=Winseler) and metallicolous (LC=La Calamine, PR= Prayon) populations. 30 seeds per families were sown and 84 individuals were transferred in individual pots containing non-polluted soil and placed in tunnel greehouse. 72 individuals were used directly in a phenotypic survey and seeds were exhaustively harbested, the remaining 12 individuals were involved in several inter-edaphic group or selfing. In september, 30 seeds per each controlled cross were sown and 15 representants from inbred and outbred progenies were transferred in individual pots containing polluted or non-polluted soil and placed in ventilated and unheated greenhouse. 360 individuals were also phenotyped and seed were exhaustively harvested.

Also, we performed a series of Pearson's correlations by progressively separating the datasets according to these factors, excepting F1 pedigree, to determine which trait was the best estimator of the number of seeds in each subgroup. F1 pedigree factor was not investigated because the number of individual in each subgroup was insufficient to perform regressions ($n \leq 10$). Finally, a Pearson's regression was performed on siliques measurement data to investigate to what extent the variation in seed number could be explained by the variation in silique length.

2.3. Results

2.3.1. Correlation among traits

PCA performed on the whole dataset showed a clear separation between vegetative and reproductive traits [Figure 6]. The first component represented 38% of the data variation and included almost all reproductive traits. The second component represented 25% of the data variation and included most of the vegetative traits. The correlations matrix based on the Pearson's correlations [Figure 7] confirmed this independence among both type of traits. Accordingly, traits showing the strongest correlation with seed set were the number of non-aborted siliques (r=0.73, p-value = $1.89.10^{-65}$) and the total length of siliques (r=0.85, p-value =$1.66.10^{-108}$). Thus, we focused on these two traits for the following analyzes.

By separating the datasets, the number of non-aborted siliques and total length of siliques seemed to show the same pattern, i.e. these traits were both well-correlated with seed set [Table 4 – Figure S2]. In most cases, the regressions were highly significant, and the correlation coefficients were very high (above r=0.7). Nevertheless, on the whole dataset, the correlation coefficient was slightly better for the total length of siliques, as it was the case in some subsets (Winseler self-cross, and C2 inter-edaphic cross). In addition, the regressions were not significant for the La Calamine self-cross cultivated in non-polluted conditions.

Finally, we observed a moderate significant correlation between the silique length and the number of seeds in the siliques (r=0.45, p.value=$1.6.10^{-8}$) [Figure 8].

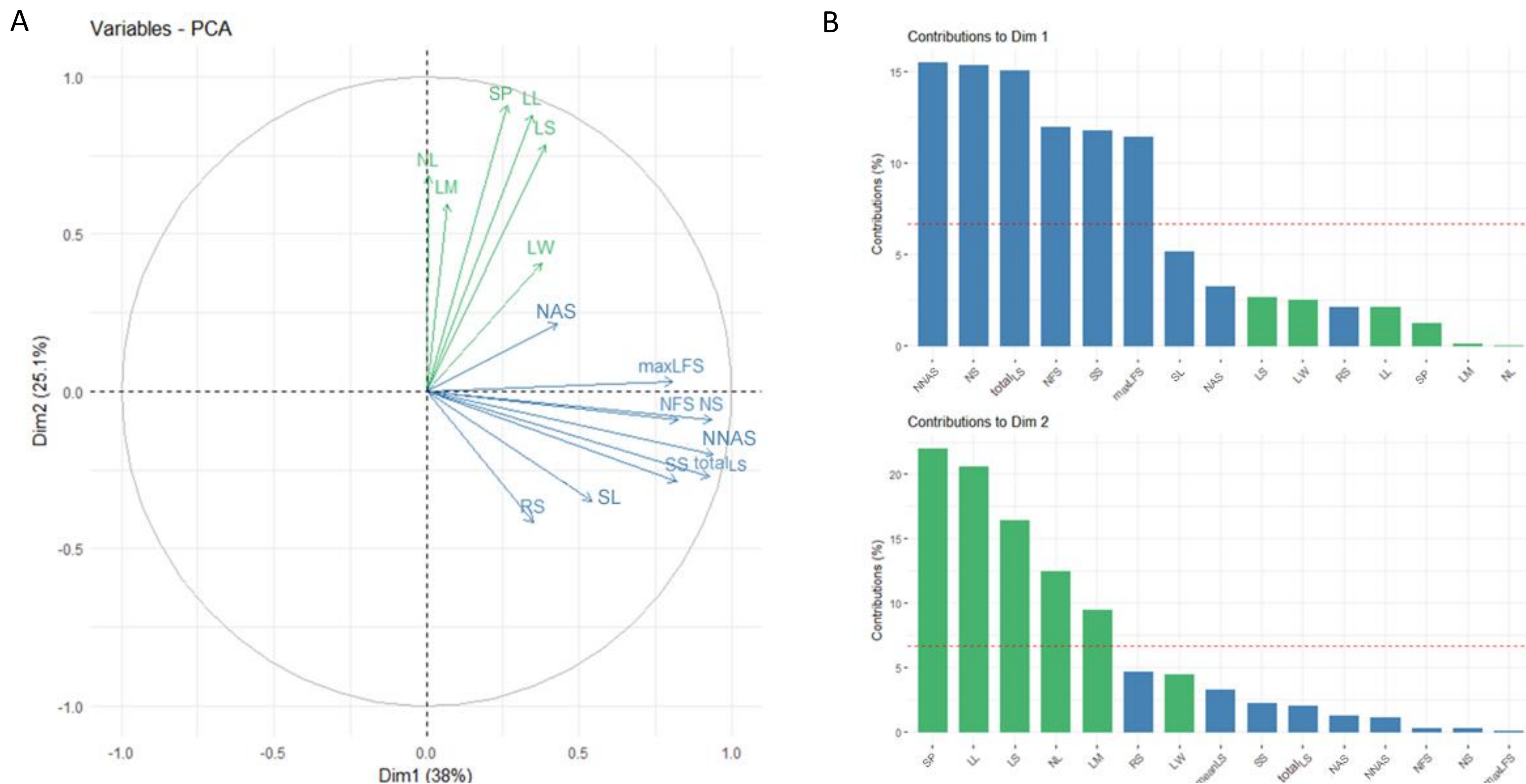


Figure 6: PCA performed on experiment 1 and 2 dataset (A) Correlation circle. (B) Variable contribution in each dimension showing vegetative (green bars) and reproductive (blue bars) traits. SP: surface of the plant; NL: number of leaves; LL: leaf length; LW: leaf width; LS: estimated leaf surface; LM: estimated leaf morphology; NFS: number of flower stems; maxLFS: the length of the longest stem; NNAS: the total number of non-aborted siliques; NAS: the total number of aborted siliques; NS: the total number of siliques; RS : ratio between the number of non-aborted siliques and aborted siliques; SL: the mean length of silique for the entire plant; SS: seed set; totalLS: total length of siliques

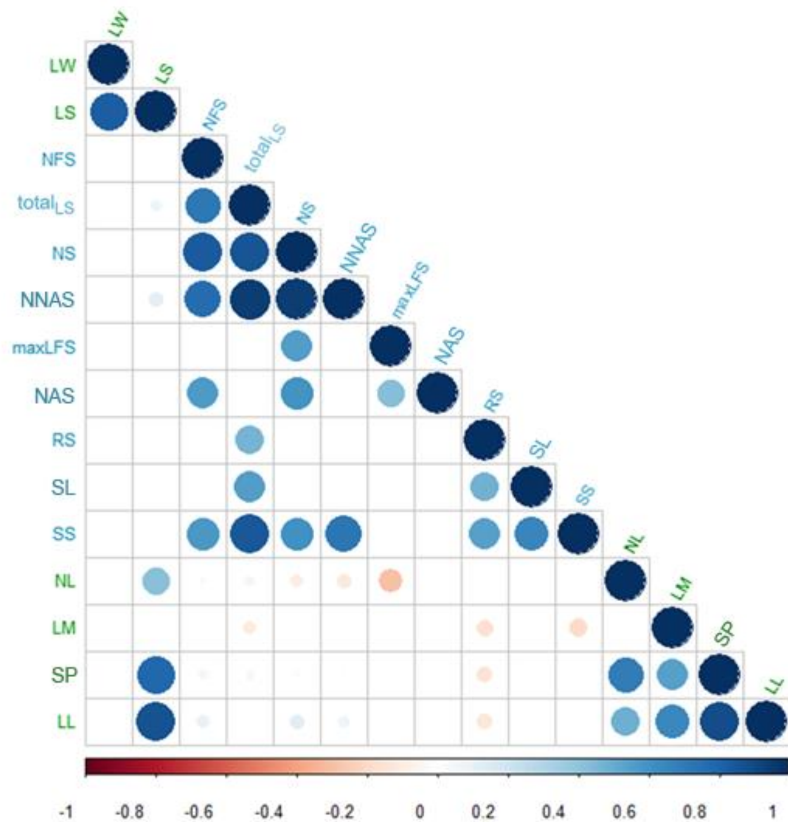


Figure 7: Correlation matrixes among vegetative (in green) and reproductive (in blue) traits in experiment 1 and 2 dataset. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). Correlation was only represented when it was significant. SP: plant surface; NL: number of leaves; LL: leaf length; LW: leaf width; LS: estimated leaf surface; LM: estimated leaf morphology NFS: number of flower stems; maxLFS: the length of the longest stem; NNAS: the total number of non-aborted siliques; NAS: the total number of aborted siliques; NS: the total number of siliques; RS : ratio between the number of non-aborted siliques and aborted siliques; SL: the mean length of silique for the entire plant; SS: seed set; totalLS: total length of siliques

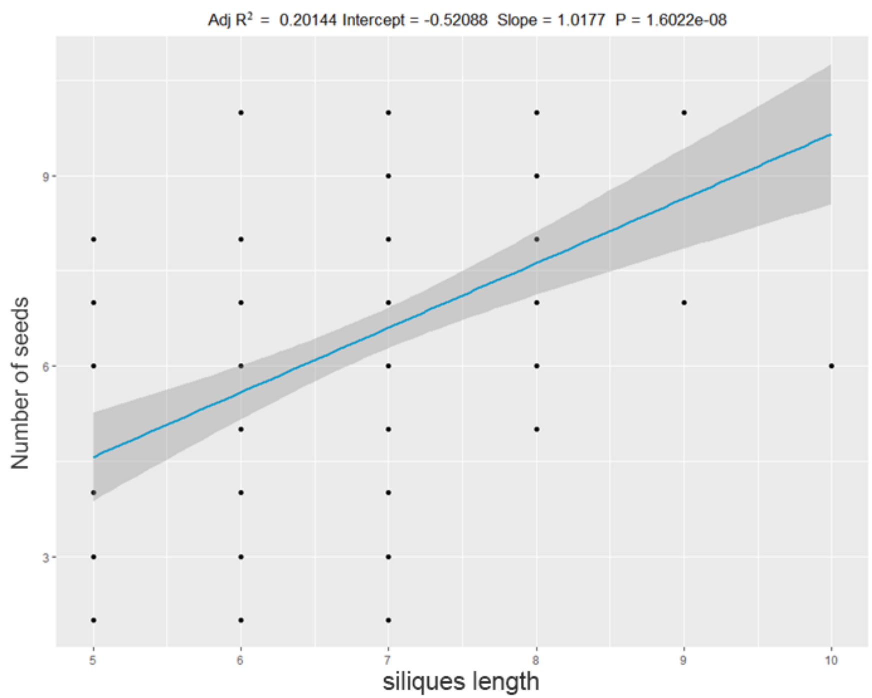


Figure 8 : Number of seeds per siliques according to siliques length Pearson's correlation between variable was represented in blue and the 95% confidence interval was represented in grey.

Table 4 : Pearson's correlations between seed set and non-aborted siliques (NNAS) or seed set estimation (totalLS) calculated on experiment 1 and 2 dataset. Data were progressively separated according to edaphic origin and population for experiment 1 and according to Condition, Cross type and F1 origin for experiment 2. r represent the Pearson's correlation coefficient. p-value represent the result of the correlation test. C represent inter-edaphic group cross C1 = PR*LE. C2= PR*WIL. C3= PR*WIN. C4= LC*WIN. C5= LC*LE and C6= LC*WIL. In bold characters, we represent non-significant correlations

Experiment	Edaphic origin		Population	NNAS (r. p-value)		totalLS (r. p-value)	
whole dataset	-			0.7299855	< 2.2e-16	0.8496925	< 2.2e-16
Experiment 1	-			0.9367964	< 2.2e-16	0.9226853	< 2.2e-16
-	Metallicolous			0.9480484	6.716e-11	0.9547774	1.846e-11
-	-		La Calamine	0.9525491	2.094e-05	0.9697148	3.548e-06
-	-		Prayon	0.9453771	1.125e-05	0.9348068	2.456e-05
-	Non-metallicolous			0.9093385	< 2.2e-16	0.8772607	2.695e-15
-	-		Lellingen	0.9207221	1.134e-06	0.9101384	2.494e-06
-	-		Wilwerwiltz	0.8758473	1.872e-05	0.8323431	0.0001182
-	-		Winseler	0.920108	1.19e-06	0.8683336	2.691e-05
	Condition	**Cross Type**	**F1 Type**	**NNAS (r. p-value)**		**totalLS (r. p-value)**	
Experiment 2	-	-	-	0.8286867	< 2.2e-16	0.8515294	< 2.2e-16
-	Both conditions	Metallicolous self- cross	-	0.8489757	< 2.2e-16	0.8226861	< 2.2e-16
-	-	-	La Calamine	0.8512369	9.418e-09	0.7799927	9.904e-07
-	-	-	Prayon	0.84039	1.18e-11	0.8300735	3.525e-11
-	-	Non-metallicolous self- cross	-	0.856889	< 2.2e-16	0.8934953	< 2.2e-16
-	-	-	Lellingen	0.894318	2.015e-08	0.8822184	5.655e-08
-	-	-	Wilwerwiltz	0.922044	8.438e-12	0.9405464	3.158e-13
-	-	-	Winseler	0.6931607	4.334e-05	0.801441	5.071e-07
-	-	inter-edaphic cross type	-	0.8150167	< 2.2e-16	0.8610564	< 2.2e-16
-	-	-	C1	0.8217362	2.598e-08	0.8346617	9.86e-09
-	-	-	C2	0.6219666	0.0002433	0.7021017	1.531e-05
-	-	-	C3	0.893734	2.963e-11	0.8951911	2.465e-11
-	-	-	C4	0.7436277	5.772e-06	0.862695	3.556e-09
-	-	-	C5	0.9299358	3.023e-13	0.9286291	3.85e-13
-	-	-	C6	0.8337194	3.605e-08	0.8726337	1.42e-09

Table 4 (continued): Pearson's correlations between seed set and non-aborted siliques (NNAS) or seed set estimation (totalLS) calculated on experiment 1 and 2 dataset. Data were progressively separated according to edaphic origin and population for experiment 1 and according to Condition, Cross type and F1 origin for experiment 2. r represent the Pearson's correlation coefficient. p-value represent the result of the correlation test. C represent inter-edaphic group cross C1 = PR*LE. C2= PR*WIL. C3= PR*WIN. C4= LC*WIN. C5= LC*LE and C6= LC*WIL. In bold characters, we represent non-significant correlations

	Condition	Cross Type	F1 Type	NNAS (r. p-value)		totalLS (r. p-value)	
Experiment 2	0 mg.kg^{-1} of Zinc	-	-	0.8704925	< 2.2e-16	0.886857	< 2.2e-16
-	-	Metallicolous self- cross	-	0.8726039	2.195e-05	0.8988689	5.227e-06
-	-	-	La Calamine	**0.9138965**	**0.0861**	**0.9424099**	**0.05759**
-	-	-	Prayon	0.8787293	0.0003691	0.8322783	< 2.2e-16
-	-	Non-metallicolous self- cross	-	0.901753	5.766e-11	0.920282	4.242e-12
-	-	-	Lellingen	0.892773	0.001195	0.8434291	0.004276
-	-	-	Wilwerwiltz	0.9457804	3.54e-05	0.9607195	9.933e-06
-	-	-	Winseler	0.9522529	7.473e-05	0.8817415	< 2.2e-16
-	-	inter-edaphic cross type	-	0.8485126	< 2.2e-16	0.8817415	< 2.2e-16
-	-	-	C1	0.9671175	4.916e-06	0.9392789	5.525e-05
-	-	-	C2	0.8219588	0.003525	0.8442427	0.002124
-	-	-	C3	0.8449239	0.002089	0.834006	0.002705
-	-	-	C4	0.9369435	0.0005975	0.9804138	1.851e-05
-	-	-	C5	0.8386136	0.00473	0.8127019	0.007755
-	-	-	C6	0.8223497	0.006509	0.8946807	0.001125
-	750 mg.kg^{-1} of Zinc	-	-	0.8091324	< 2.2e-16	0.8322783	< 2.2e-16
-	-	Metallicolous self- cross	-	0.8285386	1.901e-14	0.7966295	9.777e-13
-	-	-	La Calamine	0.8427857	2.369e-07	0.7630448	1.45e-05
-	-	-	Prayon	0.7951651	2.557e-07	0.7804443	5.939e-07
-	-	Non-metallicolous self- cross	-	0.8281708	2.107e-13	0.8801524	2.2e-16
-	-	-	Lellingen	0.8948344	3.619e-05	0.9048477	2.129e-05
-	-	-	Wilwerwiltz	0.8988834	9.375e-07	0.9260022	9.771e-08
-	-	-	Winseler	0.6202094	0.004612	0.7762989	0.000152
-	-	inter-edaphic cross type	-	0.796877	< 2.2e-16	0.8501441	< 2.2e-16
-	-	-	C1	0.7200511	0.0003436	0.7488297	0.0001454
-	-	-	C2	0.4893031	0.02855	0.5943457	0.005717
-	-	-	C3	0.8865685	1.943e-07	0.8897279	1.525e-07
-	-	-	C4	0.7033347	0.0005409	0.8324495	5.288e-06
-	-	-	C5	0.8080001	1.638e-05	0.8218273	8.825e-06
-	-	-	C6	0.800084	3.911e-05	0.8495235	4.18e-06

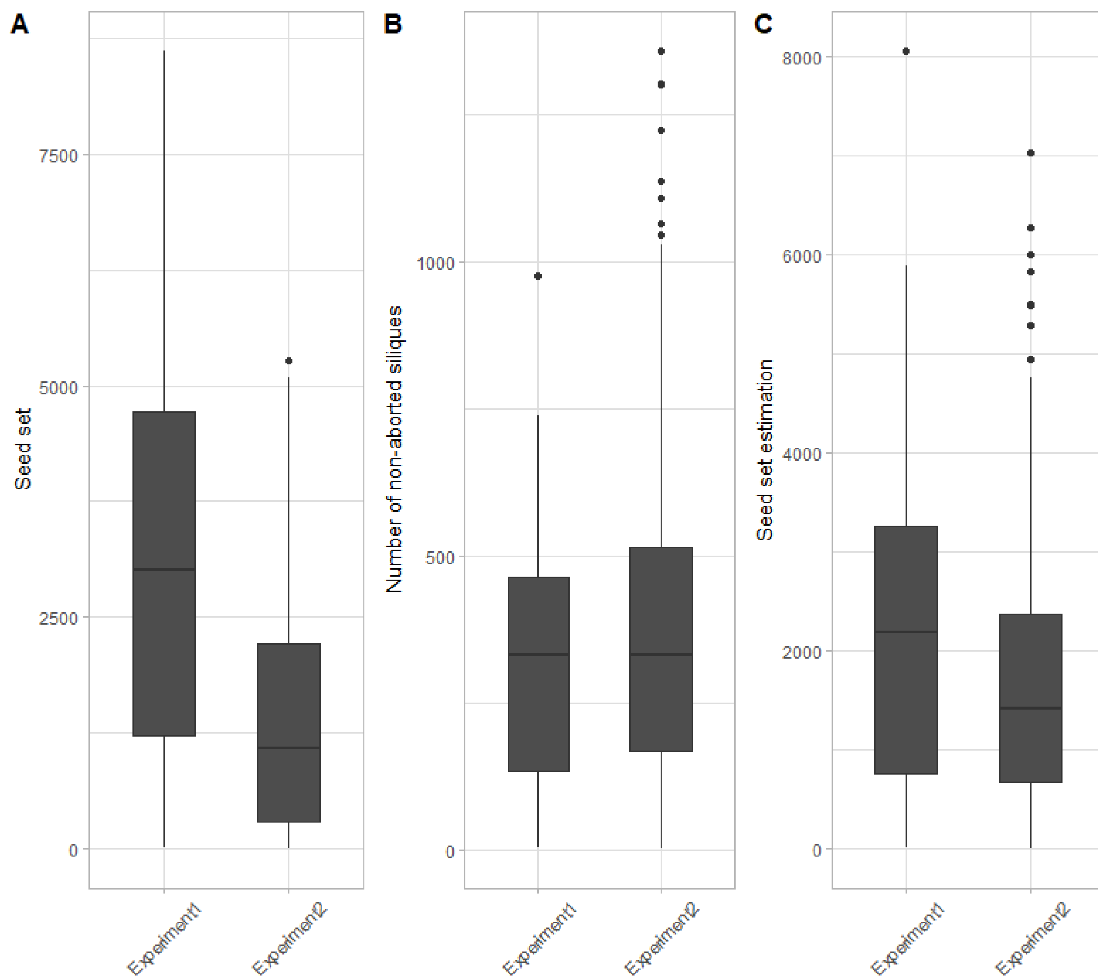


Figure 9: Comparison of (A) number of seeds, (B) number of non-aborted siliques, (C) total length of siliques according to experiment

Table 5 : Analyzes of variance of seed set, number of non-aborted siliques and total length of siliques in two edaphic groups of Noccaea caerulescens (three populations for nonmetallicolous edaphic groups and two populations for metallicolous edaphic groups). Analyze was performed on data from individuals growing in experiment 1

	df	MS	F	P value
Seed set				
Edaphic origin	1	94572570	15.65	0.0235
Population within Edaphic origin	3	19442655	2.36	0.0801
Residuals	61	167440551		
Number of non-aborted siliques				
Edaphic origin	1	780767	10.88	0.0389
Population within Edaphic origin	3	230507	2.292	0.0871
Residuals	61	2045170		
Total length of siliques				
Edaphic origin	1	53113778	11.5	0.0371
Population within Edaphic origin	3	14967891	2.76	0.0496
Residuals	61	110180504		

2.3.2. Seed set variation

The Wilcoxon-Mann-Whitney's tests, performed on the whole data set, showed a significant difference for seed set (W = 6052.5, p-value = $4.378.10^{-10}$) and total length of siliques (W = 8612.5, p-value = 0.02003) between experiment 1 and 2, with higher seed set for individuals from experiment 1 (3054 ± 2082.45) than for individuals from experiment 2 (1386.4 ± 1253.9) and higher total length of siliques for individuals from experiment 1 (2220 ± 1657.6) than for individuals from experiment 2 (1686.3 ± 1284). However, no significant difference was found on the number of non-aborted siliques (W = 11444, p-value = 0.2841) [Figure 9].

For experiment 1, we observed significant differences between edaphic origins for the seed set, the number of non-aborted siliques and the total length of siliques, with higher values for the non-metallicolous origin (*SS*= 3873.6 ± 1872.1, *NNSA*= 397.9 ± 200.6, *totalLS*= 2834 ± 1569, respectively) compared to the metallicolous origin (*SS*= 1297.8 ± 1278.2, *NNAS*= 163.3 ± 158.9, *totalLS*= 902.7 ± 917.3, respectively) [Table 5]. The effect of population was slightly significant only for the total length of siliques but post-hoc test did not confirm this difference [Figure 10].

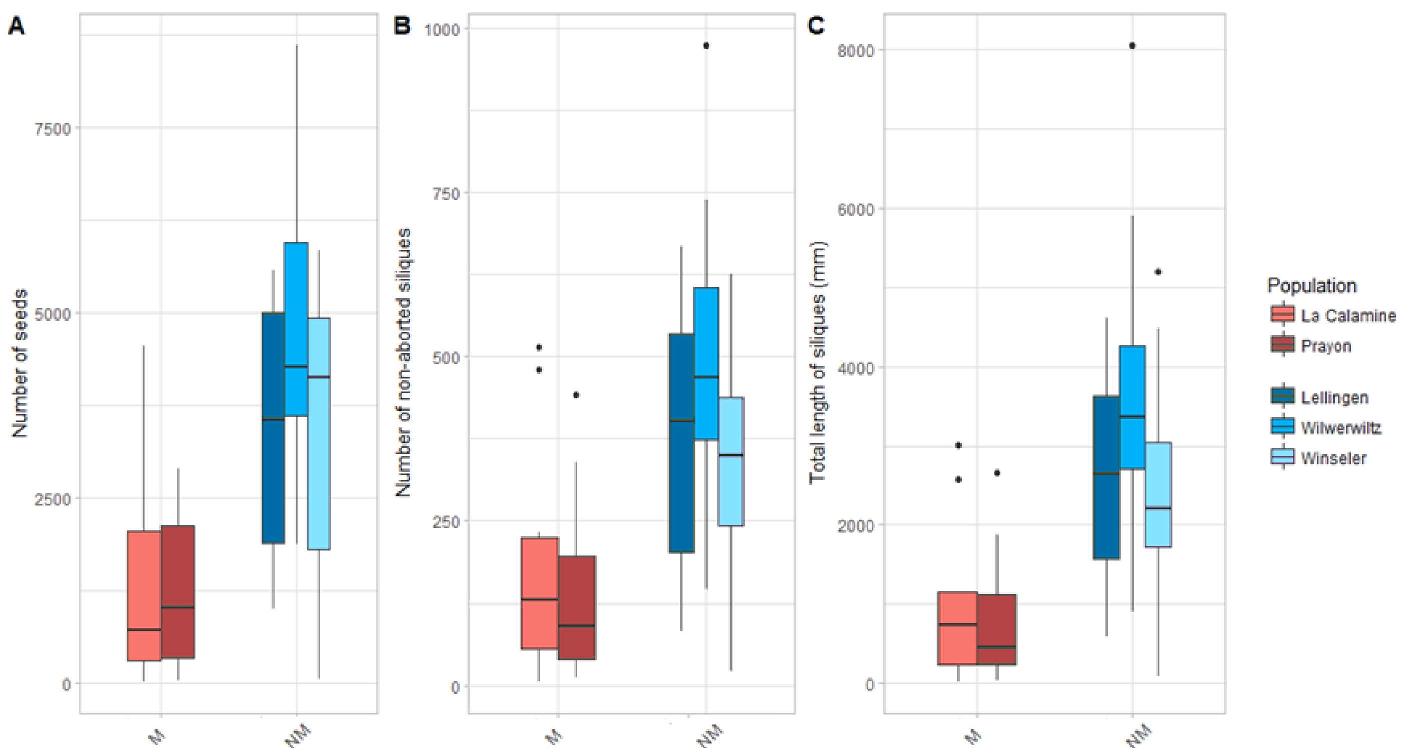


Figure 10 : Comparison of (A) number of seeds, (B) number of non-aborted siliques, (C) total length of siliques, according to edaphic origin in experiment 1. Hierarchical ANOVA found a significant difference for the seed set, the number of non-aborted siliques and the total length of siliques between edaphic origins. The effect of population was slightly significant only for the total length of siliques but post-hoc test did not confirm this difference

For experiment 2, we observed significant differences for seed set, total length of siliques and the number of non-aborted siliques according to the contamination level [Table 6]. Indeed, individuals grown on polluted soil produced higher seed set, more non-aborted siliques and have higher total length of siliques (*SS*=1604.5 ± 1290.4, *NNAS* =421.1 ± 264.9, totalLS=1932.1 ± 1308.9, respectively) than individuals grown on non-polluted soil (*SS* = 953.6 ± 1056.8, *NNAS* = 253 ± 194.5, *totalLS* = 1140.2 ± 1041.3, respectively).

A strong interaction between F1 origin and contamination level, mostly due to the response of LC*LE cross (C5) in polluted conditions was also observed [Table 6 and Figure 11]. However, the trend remained the same, with individuals grown on polluted soil producing a higher seed set, more non-aborted siliques and a higher total length of siliques. Each cross responded more or less differently to soil pollution. In the same way, we observed significant differences for the number of non-aborted siliques and seed set estimation according to the interaction between contamination level and F1 pedigree.

Table 6 : Analyzes of variance of seed set, number of non-aborted siliques and total length of siliques in several F1 of Noccaea caerulescens subjected to two contamination levels (non-polluted soil and 750mg.kg^{-1} zinc contaminated soil with zinc sulfate). We tested the effect of cross type, the effect of F1 type, the effect of F1 name, contamination level and interactions between these factors.

	Seed set				Number of non-aborted siliques				Total length of siliques			
	df	MS	F	**P value**	df	MS	F	**P value**	df	MS	F	**P value**
Cross type	2	18361847	1.28	**0.3341**	2	710885	3.15	**0.0956**	2	16908453	2.57	**0.1389**
Contamination	1	28728797	7.68	**0.0253**	1	1382836	9.11	**0.0151**	1	32145693	8.05	**0.0204**
Cross type*Contamination	2	13917302	1.83	**0.2250**	2	695561	2.25	**0.1634**	2	12824083	1.58	**0.2609**
F1 type within Cross type	8	66627471	1.33	**0.2937**	8	1112259	0.62	**0.7439**	8	33539490	0.64	**0.7353**
F1 pedigree within F1 origin	14	59019240	4.18	**0.0036**	14	1321379	2.38	**0.0632**	14	440992859	3.04	**0.0265**
Contamination*(F1 origin within Cross type)	8	28195048	3.71	**0.0179**	8	1367928	4.32	**0.0128**	8	36169031	4.35	**0.0124**
Contamination*(F1 pedigree within F1 origin)	12	12352906	1.09	**0.3701**	12	474937	0.96	**0.4841**	12	12451695	1.06	**0.3922**
Residuals	272	266771249			272	11171890			271	264656369		

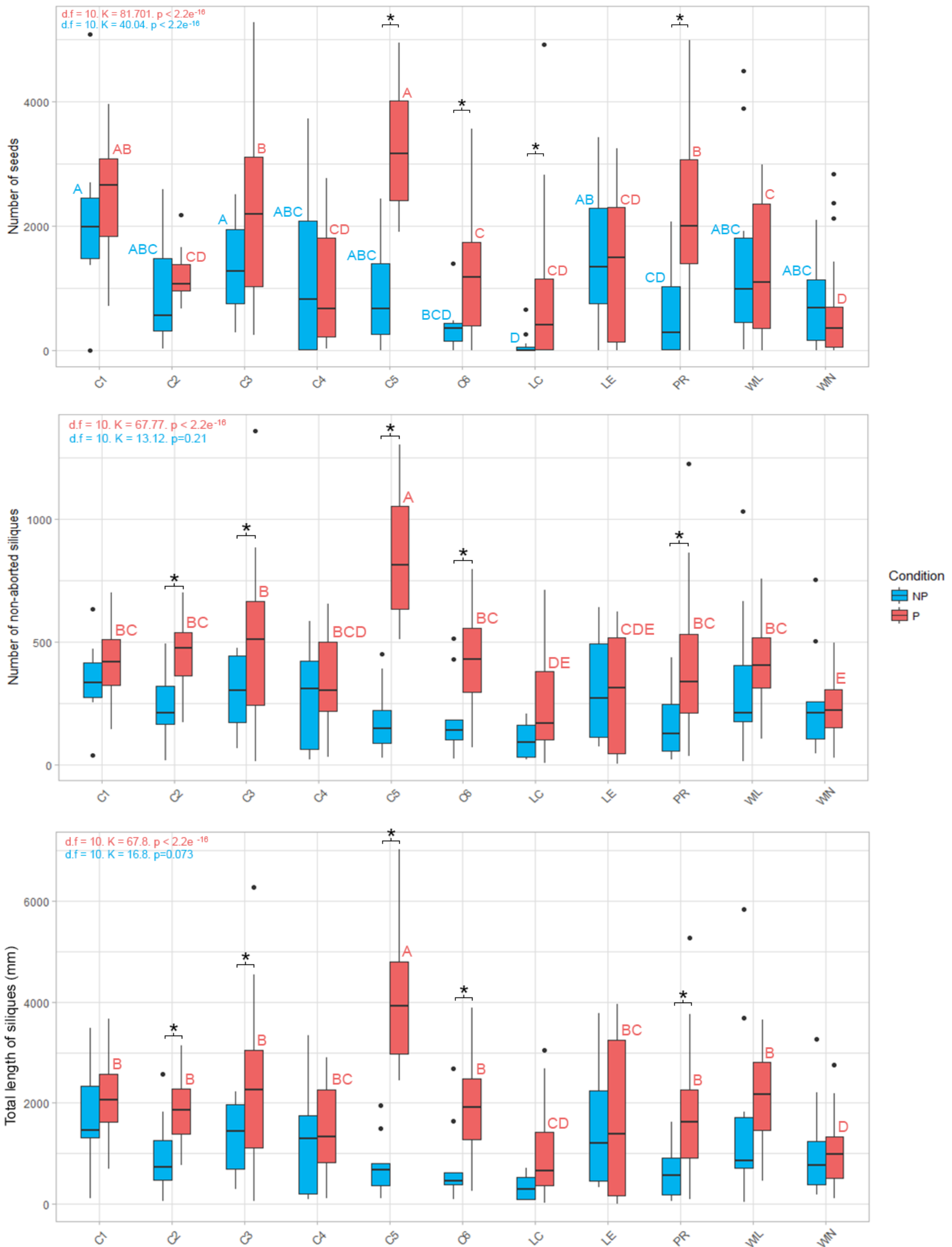


Figure 11 : Comparison of (A) number of seeds. (B) number of non-aborted siliques. (C) total length of siliques according to F1 Type : LE=Lellingen. WIL=Wilwerwiltz. WIN=Winseler. LC = La Calamine. PR=Prayon. C represent inter-edaphic cross type C1 = PR*LE. C2= PR*WIL. C3= PR*WIN. C4= LC*WIN. C5= LC*LE and C6= LC*WIL. Red plot represent 750 $mg.kg^{-1}$ of zinc polluted (P) condition. Blue plot represent non polluted (NP) condition. K= Kruskal-Wallis statistic, d.f= degree of freedom, p= p.value. Boxplots with the same letters are not significantly different at the 5% level. * represent significant difference between detected by Wilcoxon test between both condition for each cross

2.4. Key results and short discussion

2.4.1. Correlation between traits and choice of seed set estimator

Regardless of the considered data subset, Pearson's correlations showed two groups of variables, which were not correlated: vegetative variables and reproductive variables. In detail, in each group, many correlations were positives and significant, but the value of the correlations varied according to the considered traits. Thus, for correlations between vegetative variables, some traits were less correlated or not correlated, with others. This is the case of the width of the leaves, only correlated to the leaf surface, or the estimated leaf mophology only poorly correlated to the plant surface and leaf length. Same results were observed for correlations between reproductive traits, with the length of the longest stem that was only poorly correlated to the number of siliques and the number of aborted siliques, or the ratio between non-aborted siliques and aborted siliques and mean silique length that were correlated with each other and with the seed set. Conversely, some variables were strongly correlated, such as the number of flower stems, the total length of siliques, the number of siliques, the number of non-aborted siliques and the seed set.

Traits that were most correlated to the seed set were the length of siliques and the number of non-aborted siliques. In detail, we gradually separated the data set, by experience, by condition (in experiment 2), by edaphic groups, by population (F1 origin in experiment 2) or by family (F1 pedigree in experiment 2) and the two estimators were almost consistent and return similar correlation to the seed set. Nevertheless, in the case of large diversified populations (whole experiment1+experiment2 dataset), the total length of siliques (mean length of siliques x number of non-aborted siliques) seemed to be a better estimator of the seed set ($r=0.85$, p-value = 1.66×10^{-108}) than the number of non-aborted siliques ($r=0.73$, p-value = 1.89×10^{-65}). In this situation, several strategies can be assumed to increase the seed set. Seed production can be increased by either the production of additional siliques, or by the production of large siliques. Indeed the correlation between the number of seeds contained in the silique and the length of this silique was significant and positive ($r=0.45$, p-value = 1.6×10^{-8}). Thus, total length of siliques seemed to be more appropriate as a seed set estimator for the other experiments.

2.4.2. Effect of contamination on seed production of different biological materials

Moreover, our results showed a high variability in the response to zinc contamination according to the biological material studied. In metallicolous plants (individuals from Prayon and la Calamine) seed production was reduced and was significantly lower than the seed production of non-metallicolous individuals (individuals from Lellingen, Wilwerwiltz, and Winseler) in non-contaminated soils. Conversely, non-metallicolous individuals produced significantly less seed than individuals from Prayon in a zinc contaminated soil, but did not show significant differences with the seed production of individuals from la Calamine. No difference was observed between seed production in the non-metallicolous accessions according to the condition. Similar results had been shown in the same populations [Dechamps *et al.*, 2008] and in populations in southern France [Jiménez-Ambriz *et al.*, 2007] in other experiments.

These results suggest that enhanced metal tolerance would be disadvantageous in non-contaminated environments. However, this hypothesis called "cost of tolerance" [Macnair, 1993] is not yet clear. Indeed, even if some evidences had been provided to support this hypothesis in other species, with, for example, a fitness decrease observed in the tolerant edaphic group of *Agrostis tenuis* [Jain & Bradshaw, 1966] or a decrease in root growth rate in mining populations of *Agrostis cappilaris* [Wilson, 1988], this “cost” could not be demonstrated in other species (for example in *Mimulus guttatus*, [Harper *et al.*, 2003].

Finally, we observed a large variability in the seed production according to population from each edaphic group under both conditions, particularly in zinc contaminated soil. For example, progeny from Wilwerwiltz inbreed crosses in polluted environments produced more seeds than progeny from Winseler inbreed crosses. This variation suggests genetic diversity within natural populations, allowing difference in response to zinc contamination. Thus, this diversity could be used to operate a selection on some individuals in different zinc concentrations.

Supplemental material

Figure S1 : *Noccaea caerulescens*. Details of aborted (a) and non-aborted (b) siliques. Photo: Maarten Strack van Schijndel (modified)

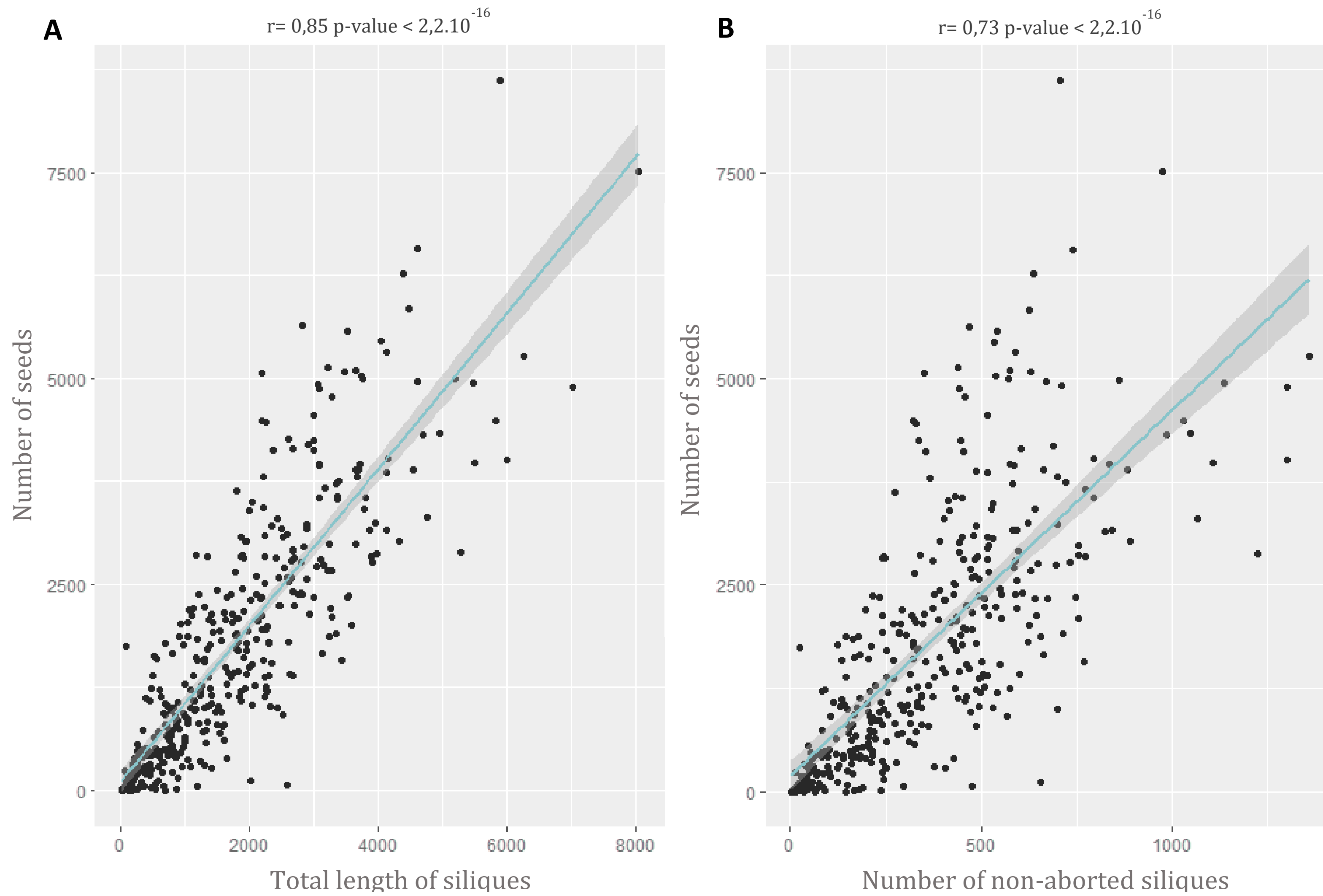


Figure S2: Number of seeds according to (A) the total length of siliques and (B) the number of non-aborted siliques

CHAPTER 2

3. Can Zinc pollution of soil promote adaptive evolution in plants? Insights from a one-generation selection experiment

3.1. Brief introduction

The first main objective of our experimental evolution approach was to test the capacity of soil zinc alone to act as a selective pressure on nonmetallicolous population of *Noccaea caerulescens*. To do so, the use of a more or less diluted calamine soil with compost [Escarré et al., 2000; Frérot et al., 2005] was not possible, in particular because, calamine soils are contaminated with several metals, such as zinc, cadmium or lead. In addition, it would not be possible using polluted soil samples to create a metal-free control soil with the same physicochemical properties. Therefore, we used a mix of compost and zeolite as control treatment and we added zinc sulphate as powder to obtain a polluted soil. The strategy also allow to repeatedly generate a particular level of soil pollution, once it has been properly defined. The chosen zinc concentration must be high to generate differences on individual performances. But zinc concentration must not cause the extinction of population, either by affecting the survival of all individuals or by limiting the number of seeds produced by the population.

In order to choose appropriate conditions of zinc exposure, we exposed several replicates of the same nonmetallicolous population to 4 different zinc concentrations and we observed the response of different vegetative and reproductive functional traits. At the end of one generation, an individual performance was calculated using the seed set estimation highlighted in Chapter 1. These performance estimates were used to create descendant populations. Then descendent populations were exposed to zinc in controlled conditions to be phenotyped in order to compare their tolerance levels. Tolerance tests were performed using a battery of morphological, physiological and biochemical traits listed from the literature.

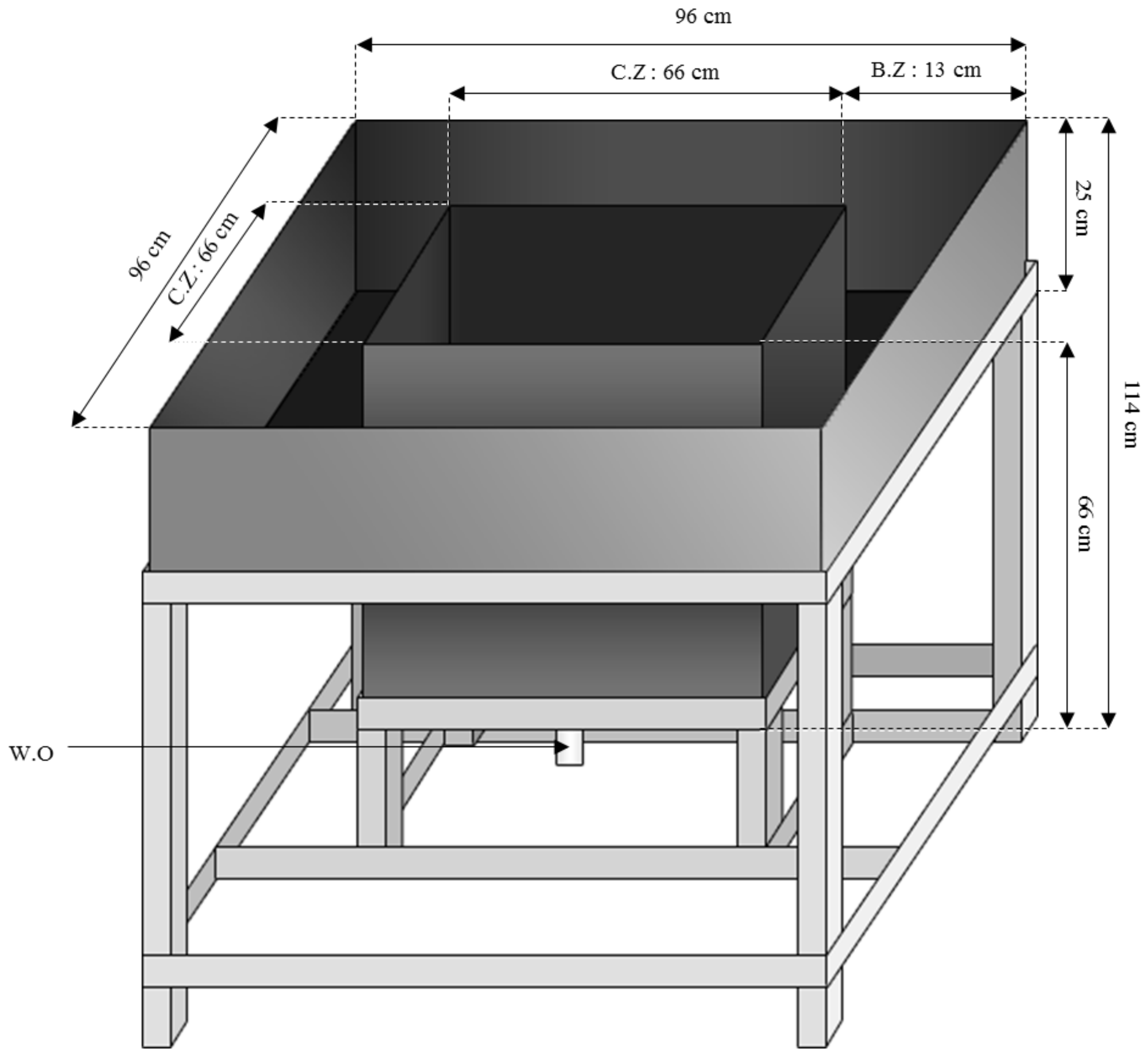


Figure 12 : Lysimeter schema. B.Z: Buffer zone. C.Z: Cultivation zone. W.O: Water outlet

3.2. Material and methods

3.2.1. Design of parent experimental populations

In June 2013, maternal families were collected in three non-metallicolous populations of *Noccaea caerulescens* from Luxembourg, at Winseler (WIN, 20 families), Wilwerwiltz (WIL, 15 families) and Lellingen (LE, 15 families) in [Table 1]. In September 2013, 15 seeds per family were sown in seedling trays containing peat-based compost, placed one week in a cold room at 4°C and transferred in a glasshouse for six weeks.

Available material was used to build four parental experimental populations (PP). To be selected, a sown family should have produced at least four seedlings to be represented once, and a multiple of four seedlings to be represented more than once, in each PP. Thus, each family was equally represented in the different PPs to homogenize the genetic composition of PP. We selected as much family fulfilling these criteria as possible in order to maximize the level of genetic diversity within each PP. Finally, PPs were made of 49 individuals from 23 families (13 from WIN, 10 from WIL and 1 from LE; Table S1).

To confirm that PPs were genetically similar, all (196) individuals were genotyped using 14 microsatellite markers from NcM1 and NcM3 multiplexes mentioned in [Mousset *et al.*, 2015]. The extraction and genotyping protocols detailed in Mousset et al. (2015) were followed. Microsatellite markers Ncpm13 and Nc7b were removed from analyses because they showed no polymorphism in the natural populations we sampled (data not shown). Pairwise F_{ST} values were calculated from microsatellite data using SPAGeDi 3 [Hardy & Vekemans, 2002].

3.2.2. Transfer and cultivation of parent populations in outdoor mesocosms

In late October 2013, PPs were transferred in distinct mesocosms. Mesocosms were made with square tubs designed from the lysimeter model proposed in [Ruttens *et al.*, 2006]. Tubs had an area of 0.436 m² enclosed by a 13 cm wide buffer zone filled with compost in order to restrain border effects and create a thermic buffer [Figure 12]. Tubs were filled with a mix of 140 kg of peat and clay-based compost and 70 kg of Zeolite that was shown to performed better that peat-based compost alone to follow plants over their entire lifecycle (unpublished data). Mesocosms were placed outdoor, to allow plants to complete their lifecycle under natural climatic conditions, in a 60 m² external area, mesocosms being separated by at least four meters (15 meters maximum), to limit pollen transfer among mesocosms.

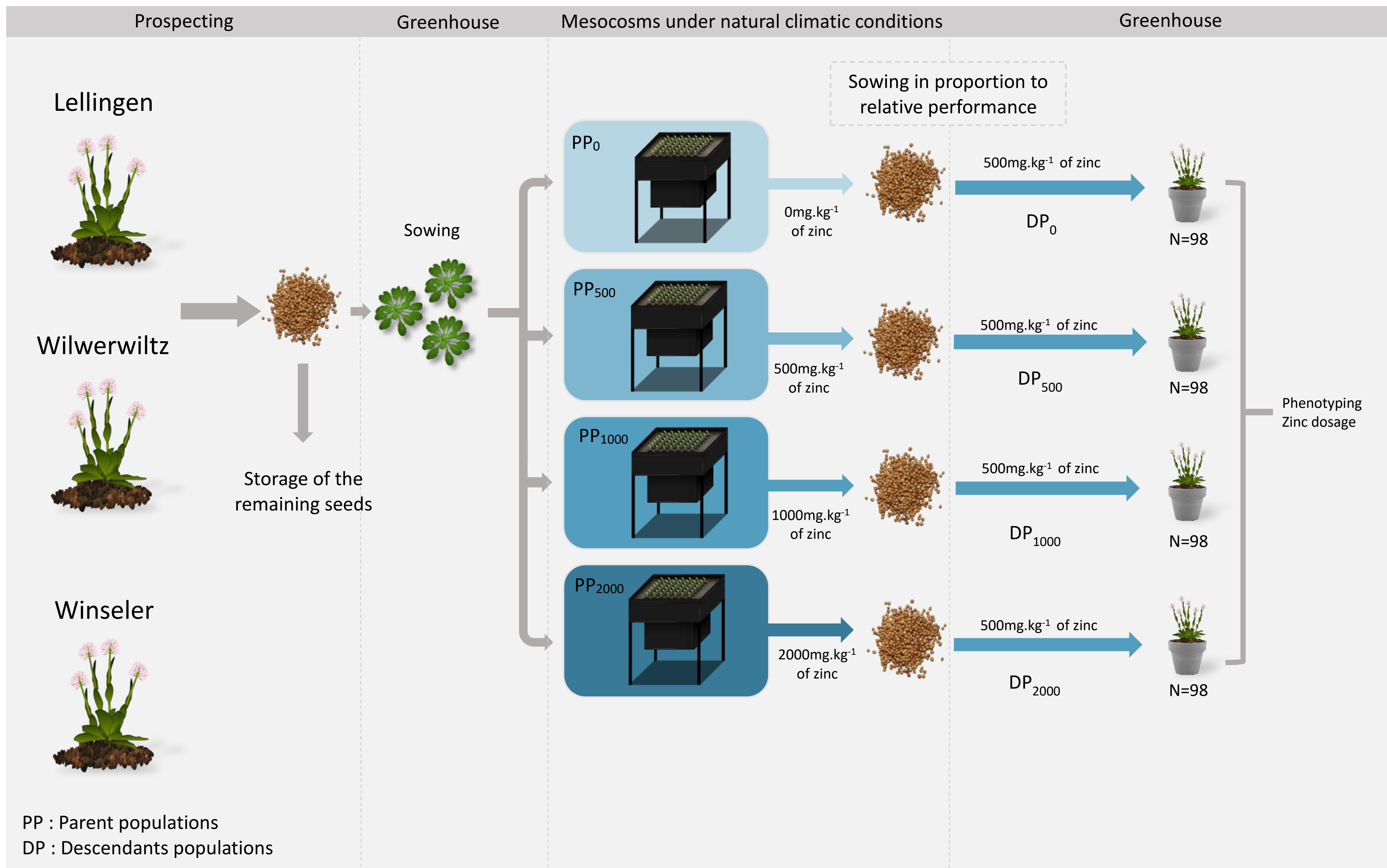


Figure 13 : Summary scheme of the experiment. 4 replicates of the same parent populations (PP) were built with seedlings from 3 natural accessions (Lellingen, Wilwerwilte and Winseler). Each PP was submitted to different zinc concentrations and was named according to the level of zinc contamination (PP_0, PP_{500}, PP_{1000} and PP_{2000}). After one generation, seeds were harvested and relative performance were estimated for each individual. These estimates performances were used to create descendant population from each PP. DP were exposed to zinc in controlled conditions to be phenotyped in order to compare their tolerance levels. Zinc dosage was also performed to compare accumulation levels among DP.

In three out of four mesocosms, different doses of zinc sulfate ($ZnSO_4$, $7H_2O$) were introduced in powder form to obtain contamination at 500 $mg.kg^{-1}$ of fresh substrate (439.73 g of $ZnSO_4$, $7H_2O$), 1000 $mg.kg^{-1}$ (879.46 g of $ZnSO_4$, $7H_2O$) and 2000 $mg.kg^{-1}$ (1758.92 g of $ZnSO_4$, $7H_2O$). The fourth mesocosm was a non-contaminated control environment. In order to obtain homogeneous substrates, mixes were made into a cement mixer.

Each PP was thus named according to the level of zinc contamination to which it was exposed: PP_0, PP_{500}, PP_{1000} and PP_{2000}. In October 2013, seedlings were transfered into mesocosms at the center of equal area cells of a 7*7 grid [Table S1]. Plants were cultivated in their respective mesocosms up to the harvest of seeds in summer 2014 [Figure 13].

3.2.3. Estimation of individual plant performance under zinc exposure in mesocosms

In order to assess the performance of plants under zinc exposure in PPs, and their ability to participate to the next generation, survival and several vegetative and reproductive traits were followed. The death date and the corresponding phenological state were recorded for each plant. Several vegetative traits were measured at the first flower bud emergence. This included plant surface (*PS*), calculated as a surface of an ellipse from the two larger diameters, the number of leaves (*NL*), leaf length (*LL*) and leaf width (*LW*) from the mean of the three longest leaves. At the end of the life cycle, mature seeds were collected from every flowering plant that produced seeds. Plants were then harvested to measure several reproductive traits. This included the number of flower stems (*NFS*), the length of the longest stem (*maxLFS*), the sum of lengths of flower stems (*sumLFS*), the total number of non-aborted siliques (*NS*) and the mean length of silique for the entire plant (*meanLS*) estimated from the length of 5 siliques per flower stem. The methodology for the measurement of *meanLS* was validated through a preliminary experiment conducted on 10 plants, on which we measured 100%, 75%, 50%, 25% of siliques by stem, or 10 or 5 scattered siliques by stem. The best compromise between measuring effort and the absence of significant difference with 100% of measured siliques corresponded to five siliques per stem (data not shown).

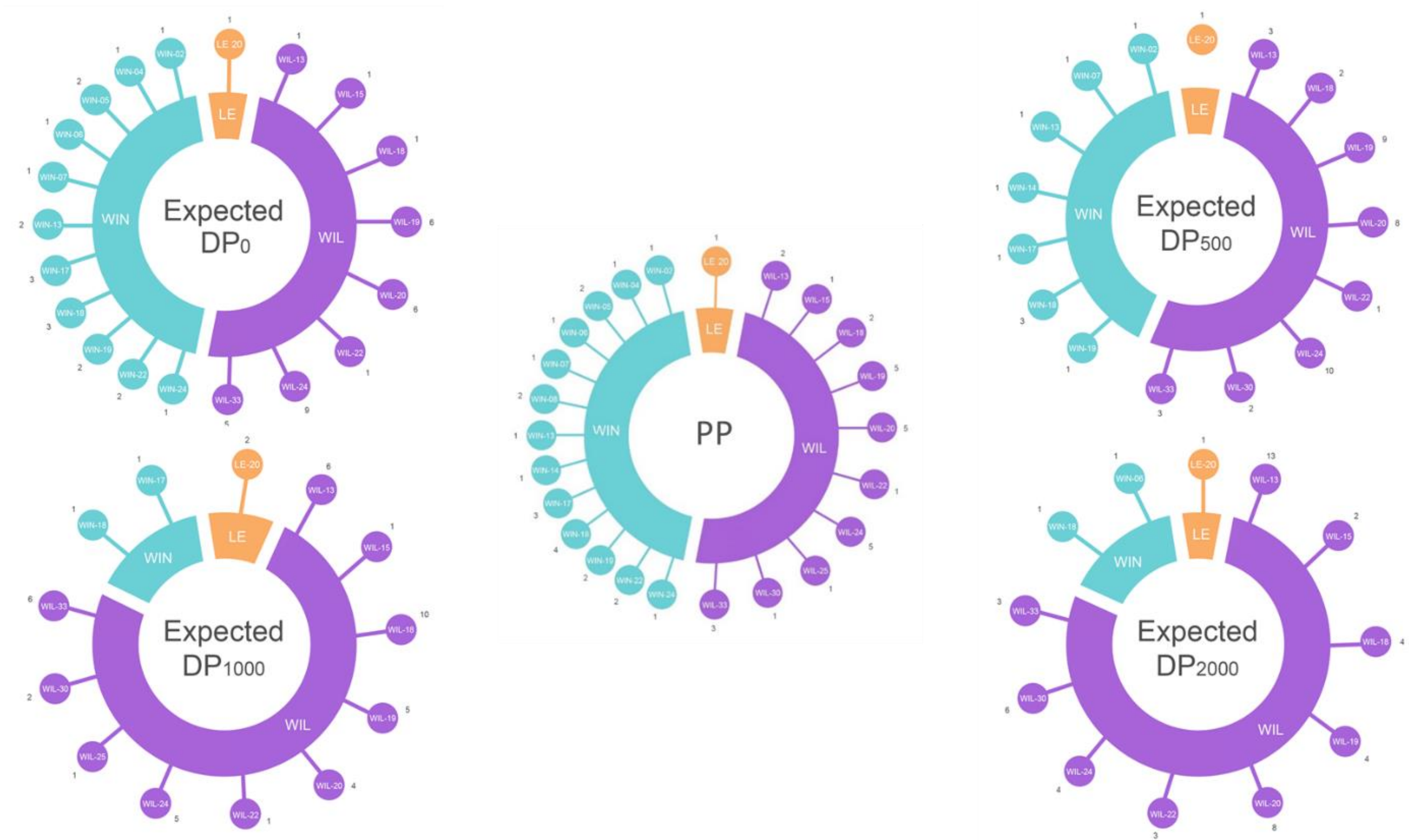


Figure 14: Expected composition of each descendant population (DP_0, DP_{500}, DP_{1000}, DP_{2000}) according to the relative performance of individual mother plants from the corresponding parent populations. PP: general composition of each parent population; WIN: Winseler population; WIL: Wilwerwiltz population; LE: Lellingen population.

3.2.4. Composition of descendant populations from performance estimates

Contributions of maternal plants to the next generation, *i.e.* expected numbers of descendants, are related to their relative performance [Violle *et al.*, 2007]. They can be appropriately assessed comparing reproductive outputs. In plants, reproductive output could be estimated through the seed set. However, due to the large number of siliques, the initiation of fruit dehiscence and seed release by environmental factors such as rain and wind, it was not possible to exhaustively collect seeds and to measure seed set without risk of bias. Therefore, we used a proxy of seed number, called W_{est}, calculated from the product of the total number of non-aborted siliques (*NS*) and the mean length of siliques (*meanLS*) [Brachi *et al.*, 2012; Roux *et al.*, 2016]. Then, plant relative performance ω_{est} was estimated by dividing the individual reproductive output W_{est} by the sum of reproductive outputs over all individuals from the same PP, $\omega_{est_i} = W_{est_i}/\sum_i^{49} W_{est_i}$, were i is the ith plant among 49 in a same PP.

After one generation of selection, four descendant populations (DP) were expected from the four PPs, and were named according to the level of zinc contamination applied to PPs: DP_0, DP_{500}, DP_{1000} and DP_{2000}. The expected composition of DPs was determined using the relative performance of individual mother plants ω_{est_i} in the corresponding PPs to calculate an expected number of descendants [Figure 14]. For each mother plant, the expected number of descendants was calculated by multiplying the relative performance estimate ω_{est_i} by the experimental population size (n=49), and rounding to the nearest integer.

To build DPs, seeds harvested from the four PPs were sown during the following December in seedling trays containing compost. Per mother plant, the number of seeds sown was equal to the expected number of descendants calculated as explained above, multiplied by six: multiplied by three to take into account the possibility of low germinating rates (*i.e.* expected germination rates were 33%) and multiplied by two to enlarge the sample size of each DP (*i.e.* to perform phenotyping on 98 individuals per DP rather than 49). Seedling trays were placed one week in a cold room at 4°C and transferred in a glasshouse for eight weeks [Figure 13].

3.2.5. Test of zinc tolerance in descendant populations

In order to compare the level of zinc tolerance among DPs derived from PPs exposed to various level zinc in soil, a tolerance test was carried out. At a called time T_0, considered as the

beginning of the tolerance test, available DP seedlings were transferred into individual pots. Pots contained 1 kg of peat and clay-based compost (NEUHAUS® N13 - 70%)/ 2-5 mm chabasite type zeolite (30%). They were contaminated with 500 mg of zinc by kg of fresh compost (20 mL of a solution at 0.38 M of $ZnSO_4$, 7 H_2O with manual homogenization). A moderate level of zinc exposure was chosen for two main reasons. First, zinc was expected to provoke toxicity but not plant mortality that would have prevented phenotyping. We took advantage of the results from the culture of PPs in mesocosms, in which mortality significantly increased from 1000 mg kg^{-1} of zinc exposure. Second, plants had to be healthy enough to actively control metal homeostasis, in particular zinc accumulation, because measuring metal accumulation in weakened plants may give spurious results and lead to erroneous interpretations [van der Ent et al., 2013].

Pots were randomized and placed in the greenhouse on a testing table. To limit micro-environmental effects, pots were rotated twice a week. Several traits were recorded at T_0 and after 2 months of experiment (T_2). At T_0 and T_2, the number of leaves and the plant surface (through calculation of an ellipse surface using the two largest orthogonal diameters) were measured, in order to calculate a growth rate based on leaves number (NL_{GR}) and plant surface (PS_{GR}). At T_2, chlorosis level was estimated by visually classifying plants in 4 categories (1: healthy plant, 2: partially chlorotic plant, 3: entirely chlorotic plant and 4: dead plant). Average chlorophyll content was also measured from three leaves for each plant with a Chlorophyll Meter (Hansatech Instruments® - CL-01 Chlorophyll Content Meter). At T_2, a series of biomarkers were analyzed to study the impact of zinc in plant tissues on different physiological processes. They were related to (1) pigment and secondary compounds dosage (Chlorophyll a/b, carotenoids, phenolic compounds, flavonoids and tannins) and (2) antioxidant enzymes activity (superoxide dismutase and ascorbate peroxidase). Each biomarker was measured according to the method detailed in [Al Souki, 2017]. zinc concentrations in aerial parts were also measured in three matures leaves following the zincon method developed for *Arabidopsis halleri* [Macnair & Smirnoff, 1999]. This method is based on UV-visible spectrophotometry using zincon as coloured zinc-chelating agent and has been previously validated for *Noccaea caerulescens* [Frérot *et al.*, 2005].

3.2.6. Statistical analyses

For data obtained on PPs in mesocosms, a chi² test was used to compare survival rates among parent populations, pairwise tests were also performed with Bonferroni adjusted p-values. The proportion of plants that produced seeds were also compared among PPs using a chi² test and pairwise tests with Bonferroni adjusted p-values.

A principal component analysis (PCA) was performed in order to test whether some traits could contribute to some point cloud structure according to zinc contamination levels in PPs. Kruskal-Wallis comparisons tests were also performed on each vegetative and reproductive variable to investigate the potential effect of zinc exposure on plants. This non-parametric test was used because the conditions for ANOVA were not fulfilled. When significant differences were found, *post-hoc* Conover tests were performed with Benjamini-Hochberg adjusted p-values.

Data obtained on DPs in controlled conditions were used to test the effect of zinc exposure of PPs in mesocosms on the evolution of metal-related traits. Again, a PCA was performed in order to test whether some traits could contribute to some point cloud structure among DPs. As an ordinal variable, chlorosis level was not included in PCA. Non-parametric tests of Kruskal-Wallis were performed for all the continuous variables and for chlorosis. If significant differences were found, *post-hoc* Conover tests were performed with Benjamini-Hochberg adjusted p-values. Finally, to test whether zinc contamination levels could have modified phenotypic correlations among traits, correlation matrixes for each DP were computed and compared using Steiger's tests.

Comparisons tests and correlations were performed with R 3.3.2 [R Core Team, 2013], PCA and graphical representations required installation of package "factoMineR" and "ggplot2".

3.3. Results

3.3.1. Genotyping of parent populations

None of the pairwise differentiation indices (F_{ST}) was significant [Table S2], indicating strong genetic homogeneity among the four parent populations on the basis of 12 microsatellites markers.

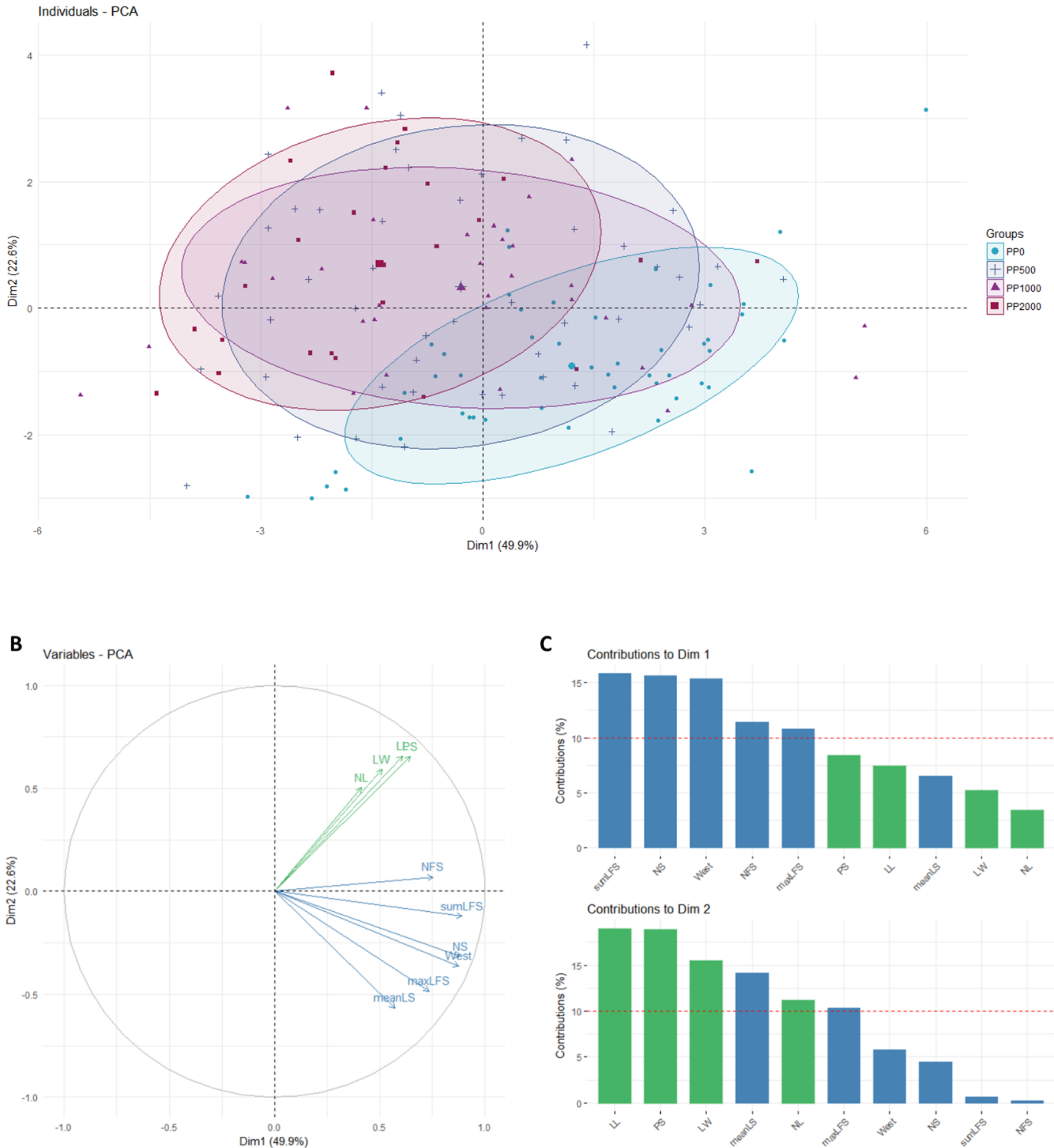


Figure 15 : Principal components analysis results on PP data. (A) Projection of individuals. (B) Correlation circle. (C) Variable contribution in each dimension showing vegetative (green bars) and reproductive (blue bars) traits. PP: parent population; PS: plant surface; NL: number of leaves; LL: leaf length; LW: leaf width; NFS: number of flower stems; maxLFS: the length of the longest stem; sumLFS: the sum of lengths of flower stems; NS: the total number of non-aborted siliques; meanLS: the mean length of silique for the entire plant; West: estimated absolute fitness.

3.3.2. Comparison of plant performance according to zinc exposure in parental populations

The level of zinc exposure significantly affected survival rates in PPs (chi² = 50.92, df = 3, p-value = 5.088×10^{-11}, Figure 16A), with a decrease of survival with the contamination level (PP_0= 100%; PP_{500}=98%; PP_{1000}=67.3%; PP_{2000}=51%). Pairwise tests distinguished two groups showing significant differences: PP_0+PP_{500} and PP_{1000}+PP_{2000}. The proportion of plants producing seeds decreased from PP_0 to PP_{2000} (PP_0: 100%, PP_{500}: 87.7%, PP_{1000}: 57.1% and PP_{2000}: 32.6%), and chi² test showed significant differences among PPs (chi² = 63.988, df = 3, p-value = 8.256×10^{-14}, Figure 16B).

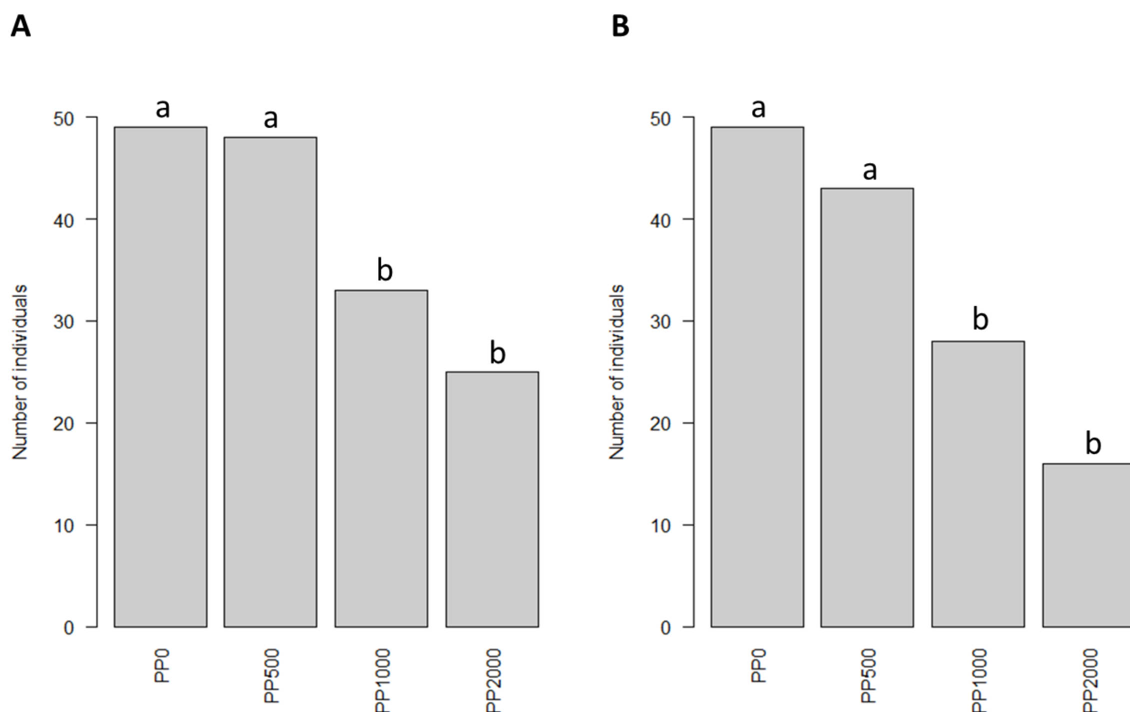


Figure 16: Number of individuals that (A) survived and (B) produced seeds in each parent population. Barplots with the same letter are not significantly different at the 5% level.

For surviving plants, the projection of individuals on the two first components of the PCA on vegetative and reproductive traits revealed a slight separation of point clouds, in particular between PP_0 and PP_{2000} [Figure 15A]. In particular, individuals from PP_0 displayed among the highest coordinates on the first component and the lowest on the second component, whereas individuals from PP_{2000} displayed among the lowest coordinates on the first component and the highest on the second component. The first component explained 49.9% of the variance and combined all reproductive traits, whereas the second component explained 22.6% of the variance and combined vegetative and reproductive traits [Figure 15B,C].

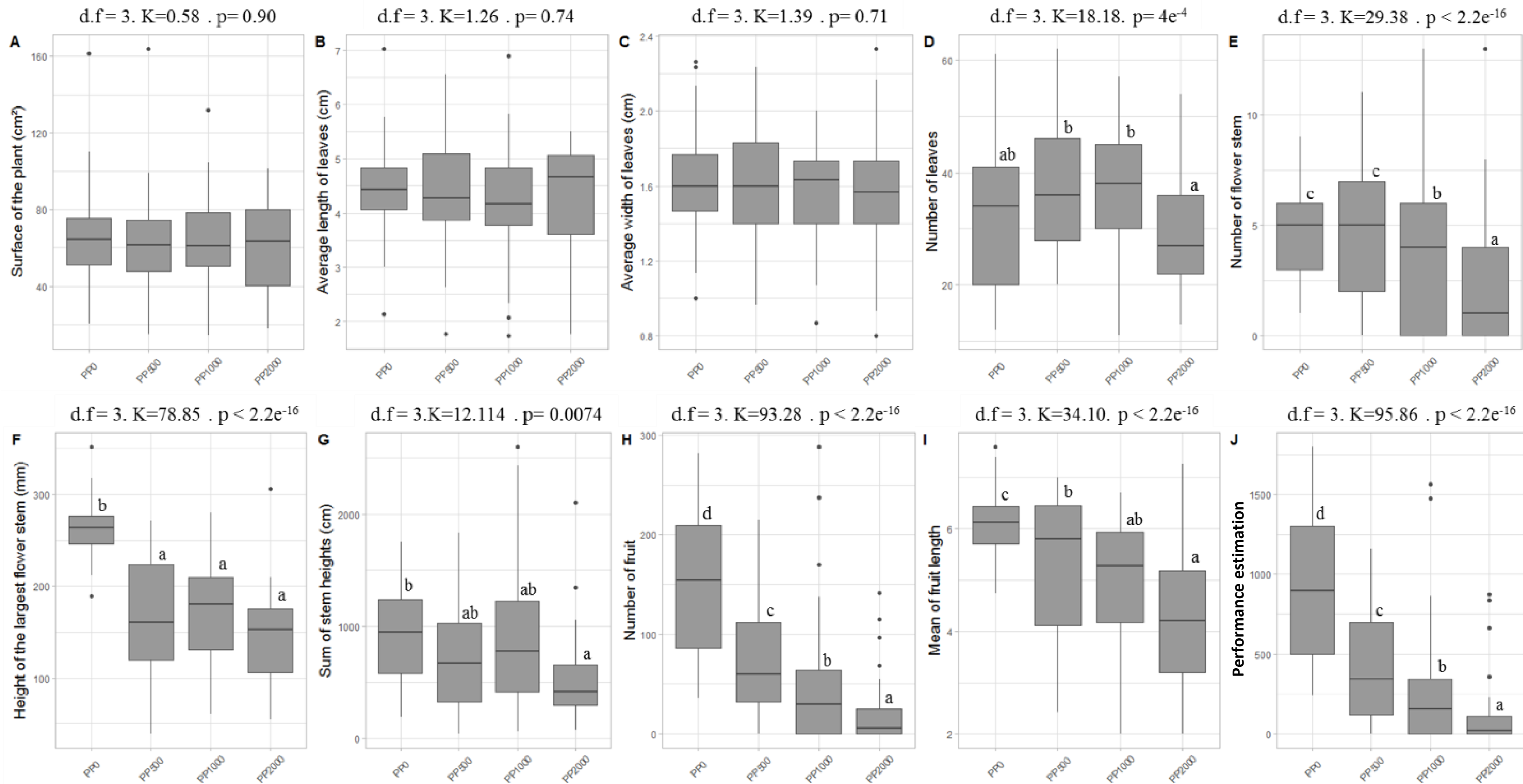


Figure 17 : Effect of zinc on vegetative traits (A, B, C and D), reproductive traits (E, F, G, H and I) and performance estimation (J) for each parental population. K= Kruskal-Wallis statistic. PP: parent population. Boxplots with the same letters are not significantly different at the 5% level.

Conversely, individuals from PP_{500} and PP_{1000} showed great phenotypic variability with confidence ellipses largely overlapping the others, either on the first component (for PP_{1000}) or on the second component (for PP_{500}).

Kruskall-Wallis and *post-hoc* tests performed on each variable showed no difference among PPs on the plant surface [Figure 17A], average length and width of the three longest leaves [Figure 17B,C]. Differences among PPs on vegetative traits only appeared for leaf number [Figure 17D], with PP_{2000} showing the lowest leaf number. On the contrary, reproductive traits always distinguished PPs, showing a reduction of traits values with increasing zinc exposure [Figures 17E-I]. Reproductive outputs were strongly reduced by zinc exposure, with more than a 4-fold reduction in PP_{2000} compared to PP_0 [Figure 17J]. Interestingly, the plants maintaining the best reproductive outputs in PP_{500}, PP_{1000} and PP_{2000} belonged to the same families, such as WIL 30, WIL 13, WIL 18 and WIL 24 [Figure 18].

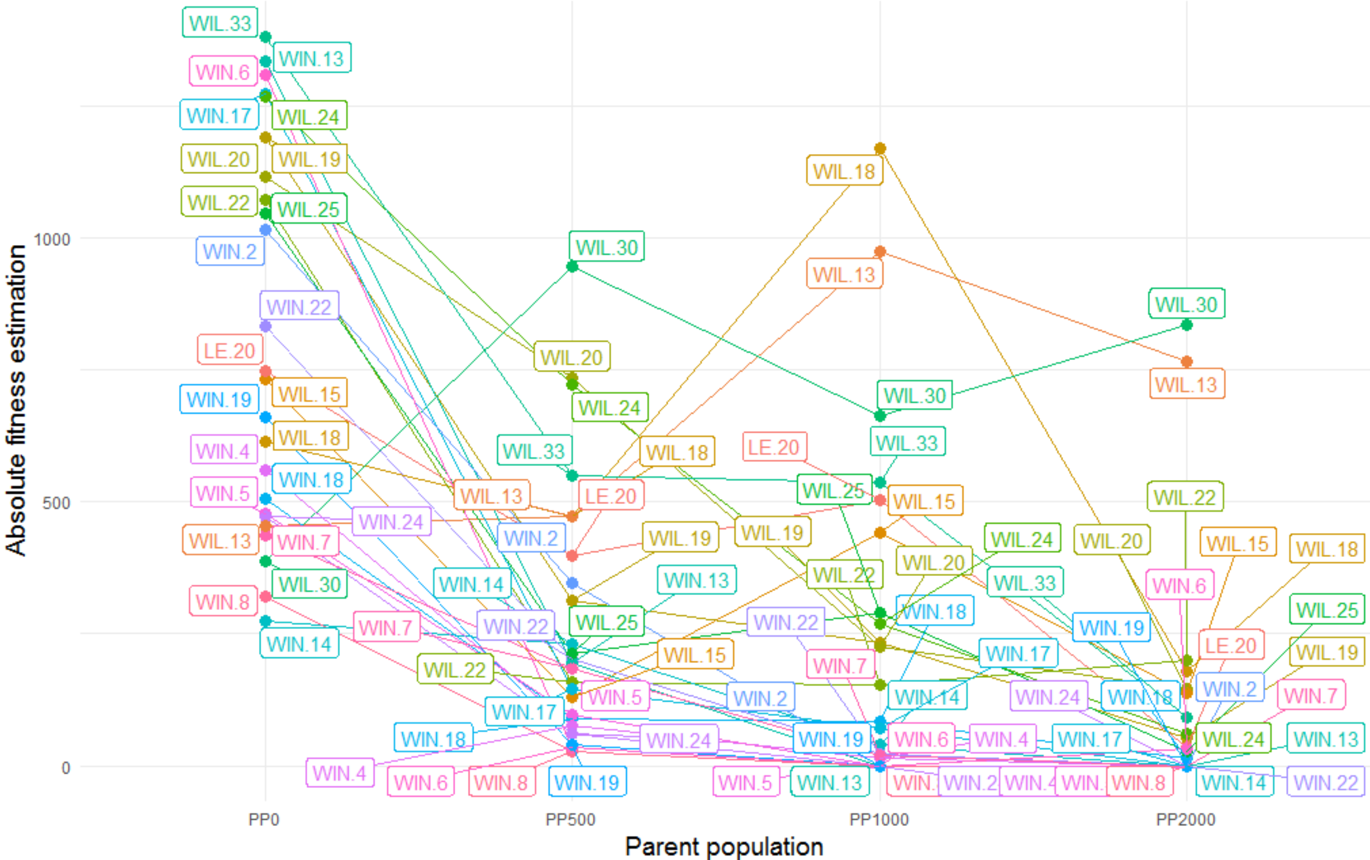


Figure 18: Average absolute performance estimation by plant family per parent population. PP: parent population; WIN: Winseler population; WIL: Wilwerwiltz population; LE: Lellingen population. Each colour represents a given plant family

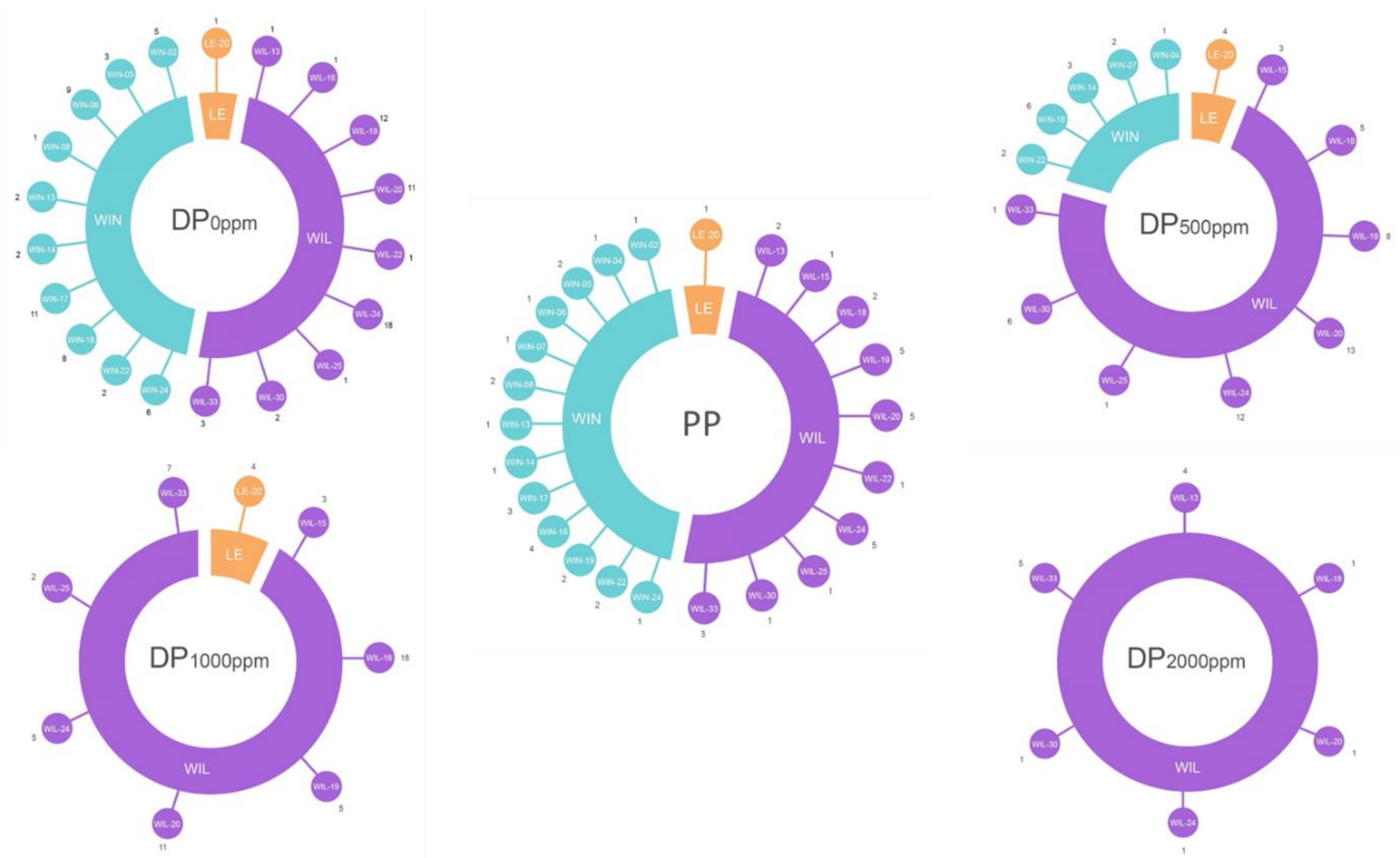


Figure 19 : Observed composition of each descendant population (DP_0, DP_{500}, DP_{1000}, DP_{2000}) according to the relative fitness of individual mother plants from the corresponding parent populations. PP: general composition of each parent population; WIN: Winseler population; WIL: Wilwerwiltz population; LE: Lellingen population

3.3.3. Comparison of zinc tolerance levels among descendant populations

Due to poor germination rates, especially for DP_{1000} and DP_{2000}, the composition of DPs could not correspond to expectations from performance estimates [Figure 14]. In particular, some progenies were not represented at all, or not as much as they were expected to be from estimated performance values. Therefore, the realized composition of DPs included all available seedlings [Figure 19].

Chlorosis level. At T_2, chlorosis at level 4 (mortality) was only observed in DP_0 and DP_{500} [Figure 20]. Chlorosis at level 3 (plant entirely chlorotic) was observed in DP_0 and DP_{500} and DP_{1000}. In DP_{2000}, chlorosis, when present, was only partial (level 2). However, no significant difference in chlorosis levels was detected among DPs (d.f = 3, χ^2= 4.047, p=0.2578).

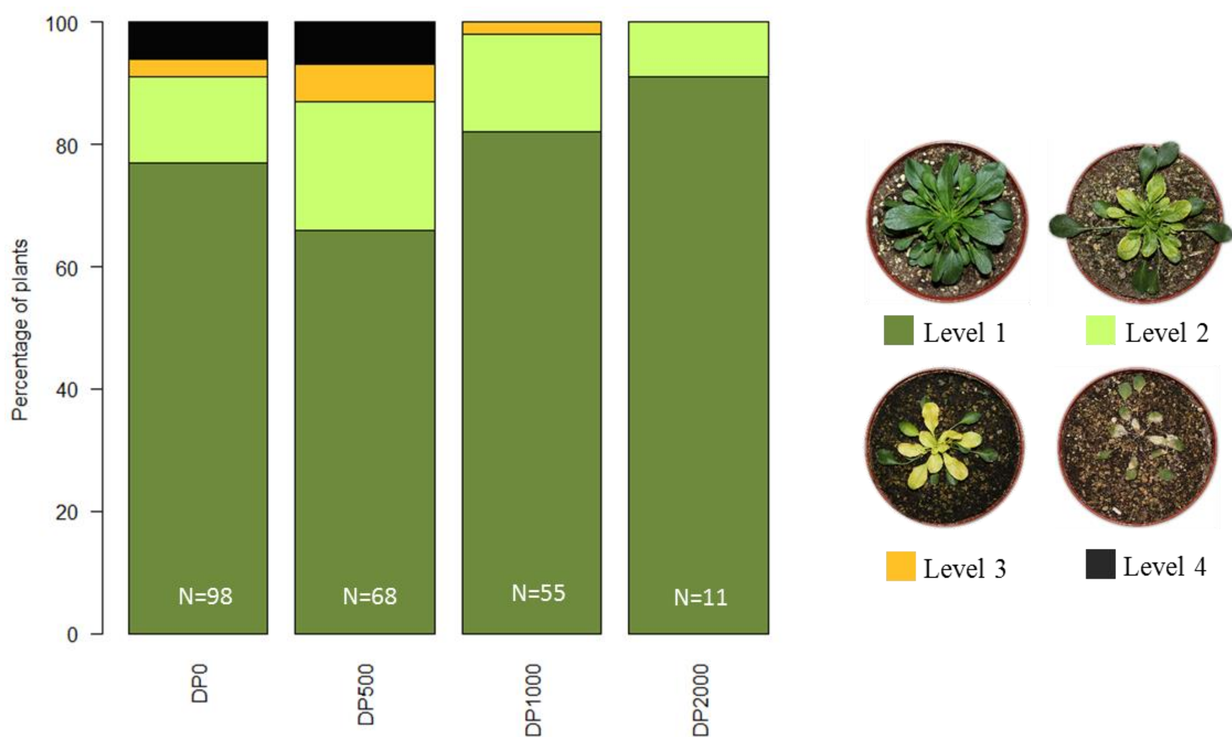
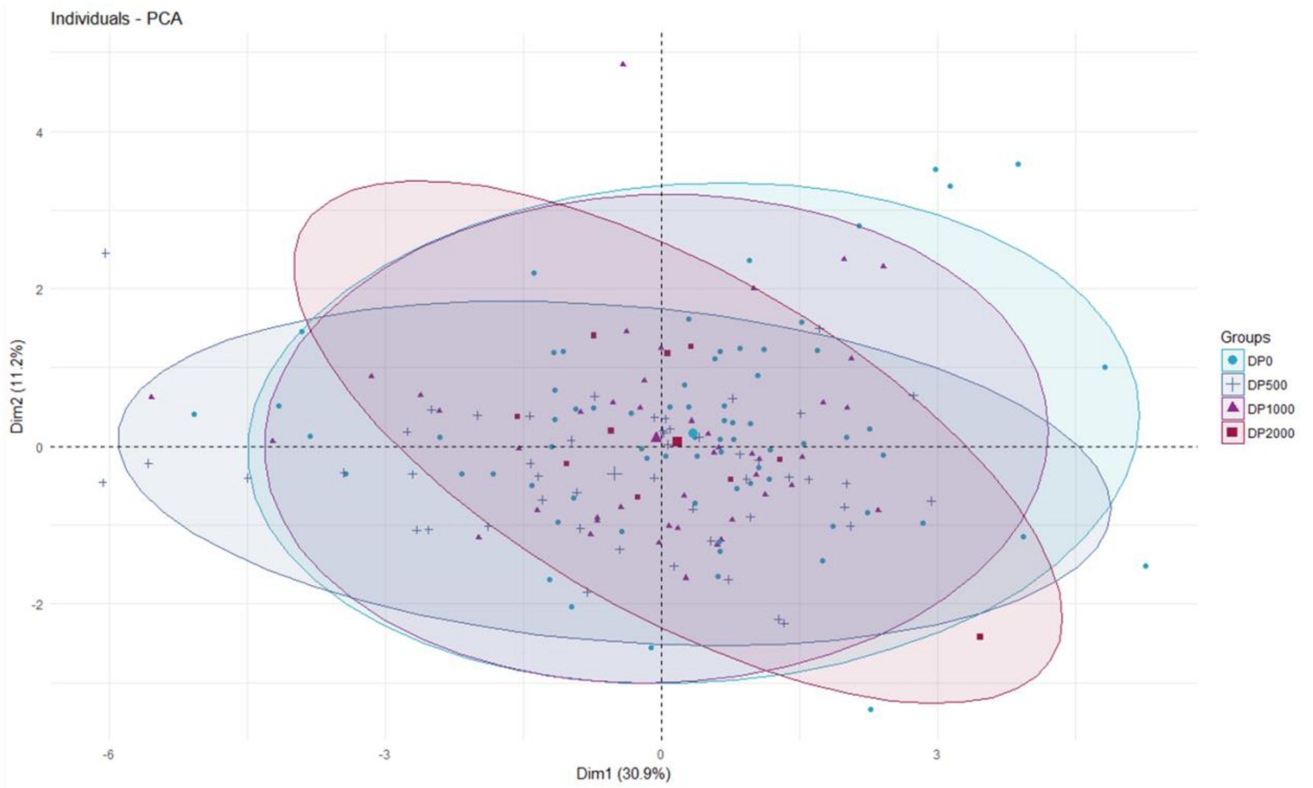
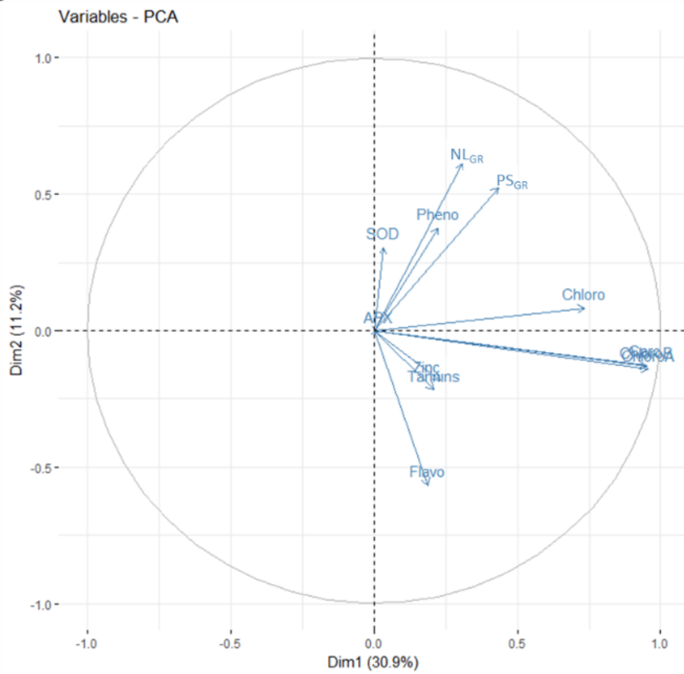


Figure 20 : Percentage of plants associated with each Chlorosis level. N=individuals number. Level 1: Healthy plant. Level 2: Partially chlorotic plant. Level 3: Entirely chlorotic plant. Level 4: Dead plant.

A



B



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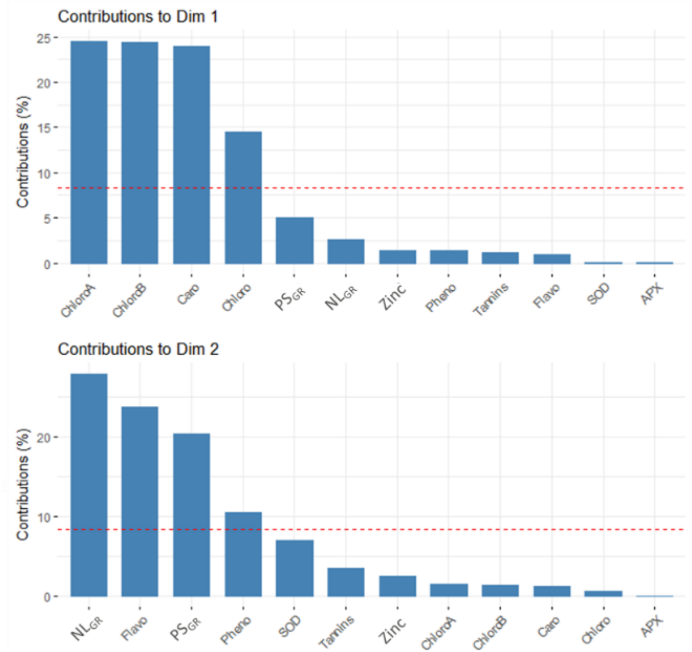


Figure 21 : Principal components analysis results on DP data at T_2. (A) Projection of individuals. (B) Correlation circle. (C) Variable contribution in each dimension. DP: parent population; ChloroA: leaf concentration in chlorophyll a; ChloroB: leaf concentration in chlorophyll b; Chloro: chlorophyll rate measured with a Chlorophyll Meter; Zinc: leaf zinc concentration; Caro: leaf concentration in carotenoids; Flavo: leaf concentration in phenolic compounds; Tannins: leaf concentration in tannins; APX: activity of the ascorbate peroxidase enzyme; SOD: activity of the superoxide dismutase enzyme; NL_{GR}: growth rate based on leaves number; PS_{GR}: growth rate based on plant surface.

The principal components analysis performed on continuous data showed that no clear point cloud structure was visible on both axes [Figure 21A]. Photosynthetic pigments were mainly represented on the first component (30.9% of variance), while secondary compounds and growth rates were mainly represented on the second component (11.2% of variance) [Figure 21B].

Antioxydant enzymes. APX activity showed no significant differences among DPs [Figure 22A]. In contrast, significant differences were found on SOD activity among DPs [Figure 22B]. This result was not confirmed by Conover's tests, even though DP_{500} and DP_{1000} tended to have significantly lower SOD levels.

Photosynthetic pigments and secondary compounds. Overall, there were no significant differences in pigments dosages among DPs, even though DP_{500} showed a tendency towards lower values (Chlorophyll A, Chlorophyll B and carotenoids, Figure 22C,D,E). In contrast, chlorophyll content measured with chlorophyll meter showed significant differences [Figure 22F]. This result was confirmed by Conover's tests, showing significantly lower chlorophyll contents in DP_{500} than in DP_0 and DP_{1000}. There were no significant differences in secondary compound dosages among DPs [Figure 22G-I].

Growth rates. For both growth rates measured on leaf number and plant surface, DP_{500} had significant lower values than DP_0 and DP_{1000} for SP_{Gr} [Figure 23A] and only than DP_{1000} for NL_{Gr} [Figure 23B].

Shoot zinc concentrations. Kruskal-wallis tests showed significant differences among DPs (d.f = 3, χ^2= 17.036, p= 5×10^{-4}). Plants from DP_{1000} and DP_{2000} presented significantly lower zinc concentrations (DP_{1000}: 9896 ± 2407 $mg.kg^{-1}$ d. wt; DP_{2000} : 9068 ± 2566 $mg.kg^{-1}$ d. wt, mean ± SD) than DP_0 and DP_{500} (DP_0 : 11433± 2182 $mg.kg^{-1}$ d. wt, DP_{500} : 10947 ± 2231 $mg.kg^{-1}$ d. wt) [Figure 23C].

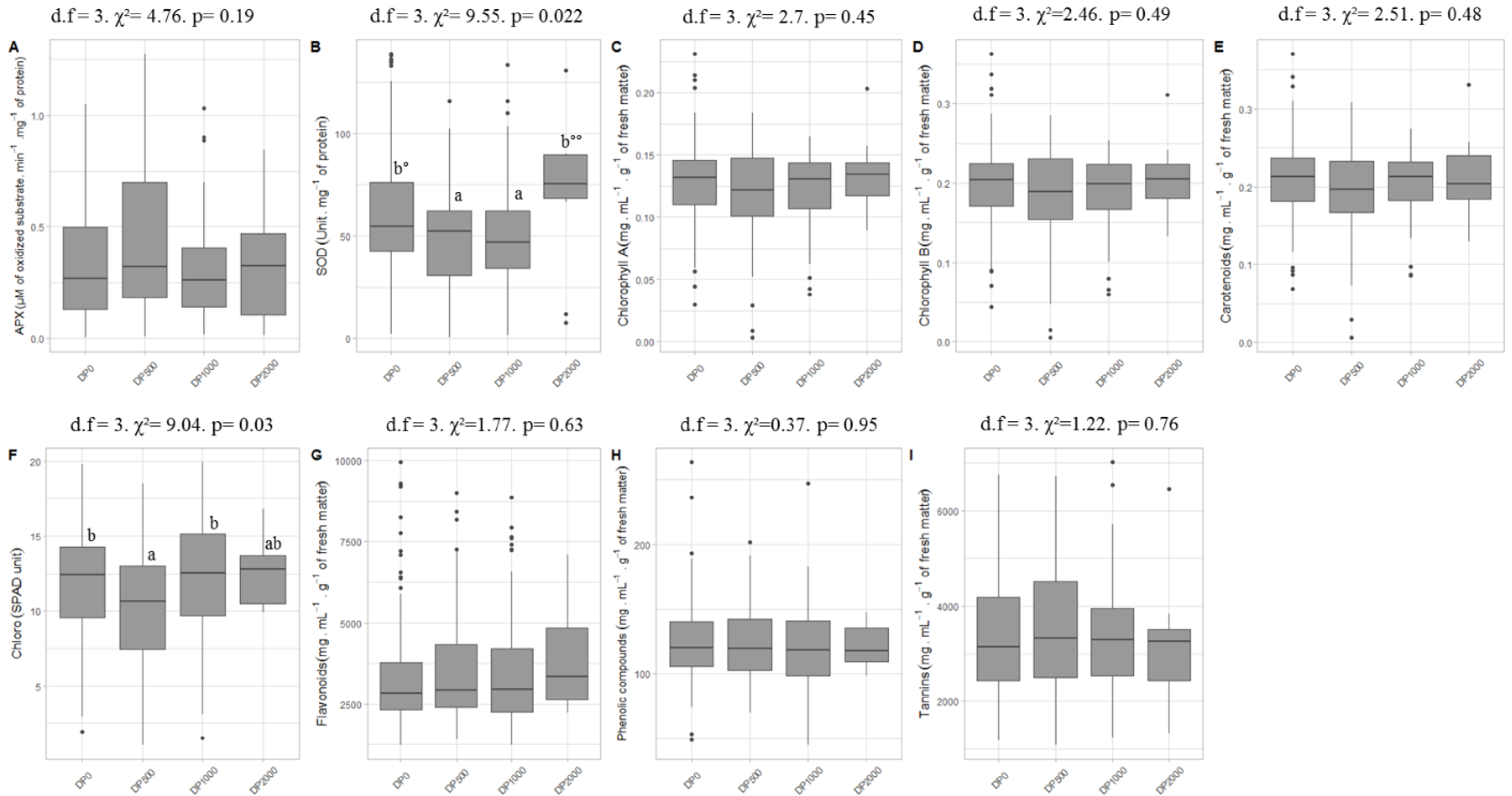


Figure 22 : Comparison of biomarker values in descendant populations. (A) Ascorbate peroxidase (APX) Activity. (B) Superoxide dismutase (SOD) Activity. (C) Carotenoids concentrations. (D) Chlorophyll a concentrations. (E) Chlorophyll b concentrations. (F) Chlorophyll rates. (G) Phenolic compounds concentrations. (H) Flavonoids concentrations. (I) Tannins concentrations. Boxplots with the same letters are not significantly different at the 5% level.

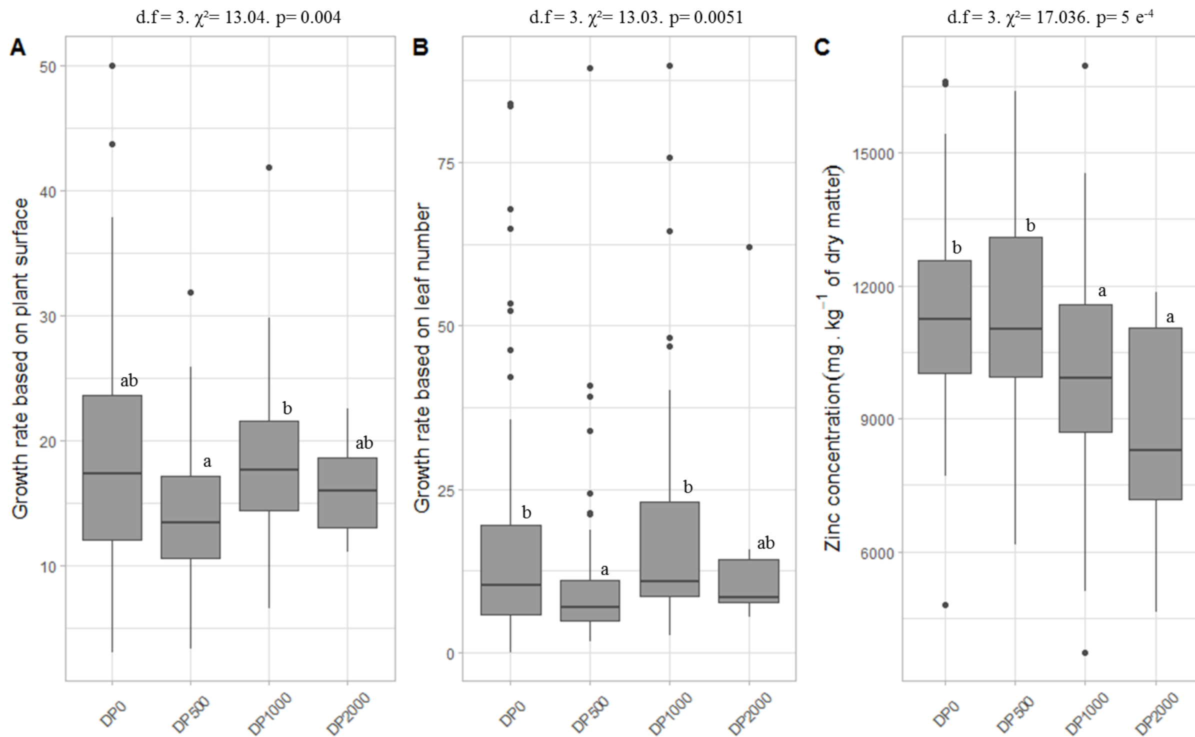


Figure 23 : Comparison of growth rates based on (A) leaf number or (B) plant surface and (C) zinc concentrations in descendant populations. Boxplots with the same letters are not significantly different at the 5% level.

DP correlation matrix comparisons. All correlation matrixes showed the same significant positive correlations among photosynthetic pigments. The main differences among matrixes came from specific significant negative correlations among other traits. In particular, DP_{500} and DP_{1000} were significantly different to DP_0 on the basis of only a few negative correlations [Figure 24]. Striking differences appeared between DP_0 and DP_{2000}, since several specific negative correlations could be observed mostly involving on one hand SOD activity with photosynthetic pigments, APX activity and tannins concentrations, and on the other hand photosynthetic pigments and flavonoids concentrations with phenolic compounds and somehow with growth rates (in particular, flavonoids concentrations with PS_{GR}).

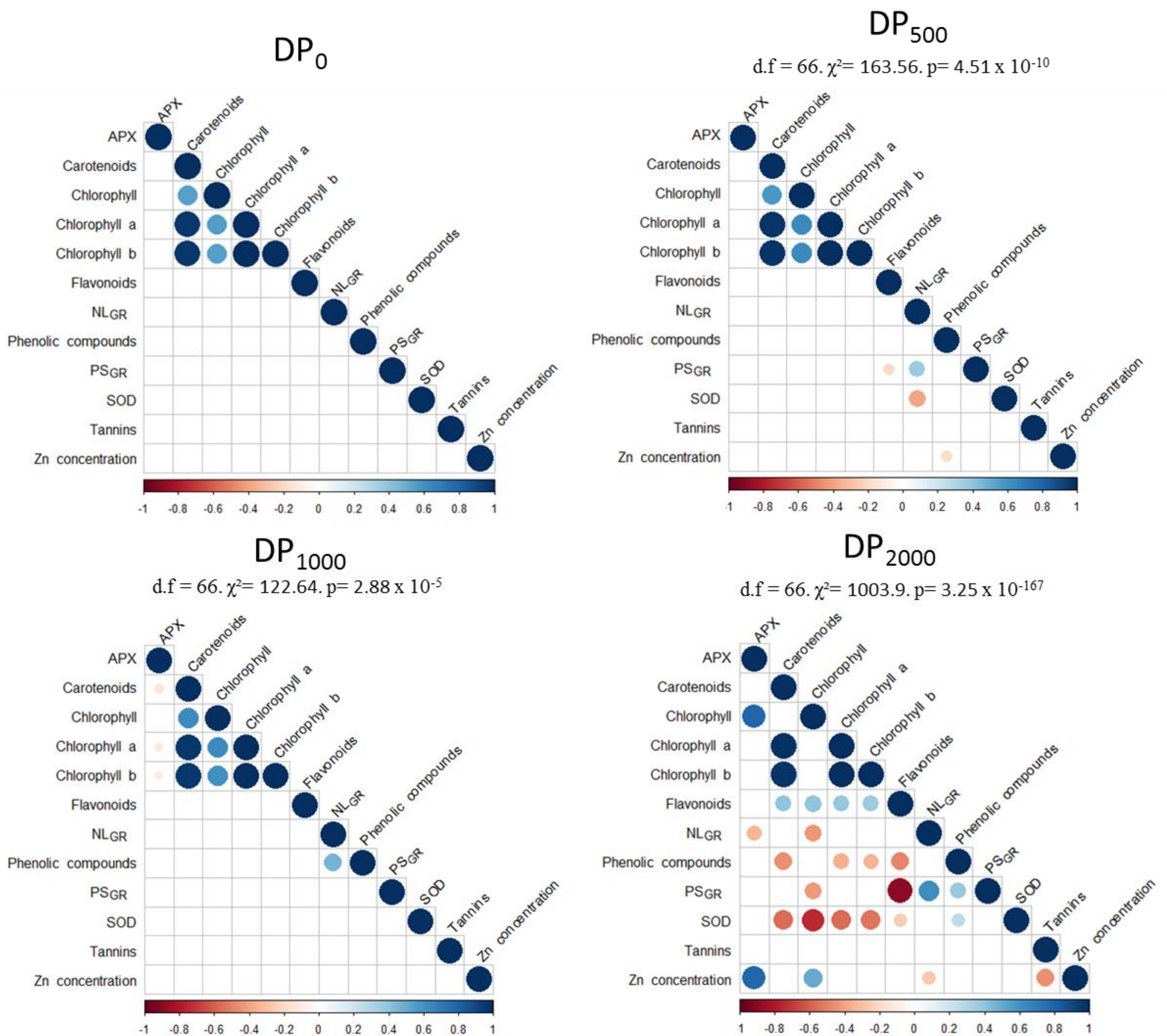


Figure 24: Correlation matrixes among traits in descendant populations. The gradient of colours represents sign and strength of correlation. Steiger's tests indicate comparisons between the DP_0 correlogram and other DPs. APX: ascorbate peroxidase activity; Carotenoids: carotenoids concentration; Chlorophyll: chlorophyll rate; Chlorophyll a: chlorophyll a concentration; Chlorophyll b: chlorophyll b concentration; Flavonoids: flavonoids concentration; NL_{GR}: growth rate based on leaf number; Phenolic compounds: phenolic compounds concentration; PS_{GR}: growth rate based on plant surface; SOD: superoxide dismutase activity; Tannins: tannins concentration; Zn concentration: shoot zinc concentration.

3.4. Key results and short discussion

3.4.1. Which zinc dose represents sufficient selection pressure?

In our experiment, increasing the level of zinc contamination of the substrate from 0 to 2000 mg kg^{-1} significantly decreased the percentage of plants surviving and producing seeds [Figure 16]. Accordingly, we observed a strong response of reproductive traits with a progressive reduction of mean values with zinc contamination. In contrast, except for the leaf number, our results showed no significant difference among PPs for vegetative traits [Figure 15, Figure 17]. Interestingly, when looking at the identity of families that have been able to survive, produce seeds and maintain a certain level of performance, the effect of increasing the level of zinc contamination in mesocosms was not random. Indeed, the loss of families was apparently gradual, so that most of the families that produced seeds in PP_{2000} also did so in other conditions. In particular, there was a gradual loss of Winseler families with the zinc contamination. Over 13 WIN families initially represented, 13, 12, 2 and 2 produced seeds in PP_0, PP_{500}, PP_{1000} and PP_{2000}, respectively. In comparison, over 10 WIL families initially represented, 10, 10, 10 and 9 produced seeds in PP_0, PP_{500}, PP_{1000} and PP_{2000}, respectively. The same pattern was also observed after ranking seed-producing families from their estimated performances [Figure 18]. The same families, mostly from Wilwerwitz (e.g. WIL 13, WIL 18, WIL 30) showed the highest performances estimates in contaminated culture conditions. As a result, the expected composition of DPs was more and more biased towards an overrepresentation of the same few WIL families [Figure 19]. Consequently, WIN families were not represented at all for the phenotyping of PP_{1000} and PP_{2000}.

The fact that same families were selected in different concentrations suggests a genetic component in the ability of plants to handle zinc exposure. Also, since zinc stress mostly affects survival and reproductive traits, it can be considered as a selective agent influencing the ability of plants to get their genes represented at the next generation

Results of this experiment allowed us to define the zinc concentration that will be applied in the final experimental evolution protocol. Although 1000 $mg.kg^{-1}$ zinc concentration appears to be an ideal concentration, the majority of individuals from Winseler did not survive or produce seeds. However, when the populations of the final experimental design were built, the Wilwerwiltz population was not accessible. At 500 $mg.kg^{-1}$, the concentration did not seem sufficient to make a selection. Therefore, we chose an intermediate concentration of 750 $mg.kg^{-1}$ of zinc

3.4.2. Improvement of the performance estimation

In this experiment, we tested the performance estimator developed in chapter 1. Thus we calculated individual performance through the estimated total size of the siliques. These performance estimates were used to build expected DP [Figure 14]. Considering a germination rate of 33%, 3 seeds per expected descendant were sown. Nevertheless, germination rates were extremely variable and very low in populations exposed to the highest concentrations (PP1000 and PP2000). Significantly changing the composition of the DP [Figure 19].

These results suggested that, in addition to affecting fecundity and reproductive output, zinc would have an effect on seed quality. Indeed, several studies have shown that, metals could reduce seed size or seed weight or cause the production of poorly or non-viable seeds [Kranner & Colville, 2011].

Consequently, the estimation of the number of seeds is not sufficient to correctly estimate the individual performance. And it is essential to take into account the viability of seeds, or seedlings, in order to correctly estimate the number of viable descendants. Thus, we estimated the germination rate and the survival rate of seedling after 5 weeks by sowing 33 seeds per individual. Then, we calculated the individual performance by multiplying the estimated total size of the siliques with survival rate of seedling.

Supplemental material

Table S1 : Population composition of parent populations represented by the plant position in each mesocosm on a 7*7 grid. Each name corresponds to the name of the mother plant. Each mesocosm received a descendant from the same mother plant.

	1	**2**	**3**	**4**	**5**	**6**	**7**
1	WIN-18	WIL-18	WIL-19	WIN-14	WIN-04	WIN-05	WIN-08
z	WIN-22	WIL-22	WIL-20	WIL-15	WIN-13	WIL-33	WIN-24
3	WIN-07	WIN-17	WIL-25	LE-20	WIL-13	WIN-02	WIN-06
4	WIL-24	WIN-19	WIL-30	WIN-18	WIL-18	WIL-19	WIN-05
5	WIN-08	WIN-22	WIL-20	WIL-33	WIN-17	WIL-13	WIL-24
6	WIN-19	WIN-18	WIL-19	WIL-20	WIL-33	WIN-17	WIL-24
7	WIN-18	WIL-19	WIL-20	WIL-24	WIL-19	WIL-20	WIL-24

Table S2 : Pairwise genetic differentiation indices (FST) calculated from the polymorphisms of 12 microsatellite markers among the four parental populations (PPs). None of the FST values was significant.

	$\mathbf{PP_{0}}$	$\mathbf{PP_{500}}$	$\mathbf{PP_{1000}}$	$\mathbf{PP_{2000}}$
$\mathbf{PP_{0}}$	-	-0.0162	-0.0169	-0.0142
$\mathbf{PP_{500}}$	-0.0162	-	-0.0177	-0.0151
$\mathbf{PP_{1000}}$	-0.0169	-0.0177	-	-0.0138
$\mathbf{PP_{2000}}$	-0.0142	-0.0151	-0.0138	-

CHAPTER 3 (Experiment in progress)

4. Heritability of functional traits in metallicolous or nonmetallicolous populations of *Noccaea caerulescens* submitted to zinc contamination.

4.1. Brief introduction

In experimental evolution, phenotypic divergence observed between ancestral and derived populations can be interpreted as genetically determined. Nevertheless, these differences may result from other transgenerational mechanisms, such as epigenetics or maternal effects [Herman & Sultan, 2011; Weinhold, 2018]. In order to to evaluate the proportion of genetic factors in phenotypic variation, we estimated the heritability of the functional traits that will be measured in our final experimental design.

Thus, the broad-sense heritability of several functional, morphological, physiological and phenological traits was estimated using parent-offspring regression in nonmetallicolous and metallicolous populations exposed or not to the zinc concentration chosen in Chapter 2.

4.2. Material and methods

4.2.1. Origin of plants, set up of parent individuals

In June 2016, 40 families of seeds were collected in 2 populations of *Noccaea caerulescens*. 20 families were sampled in Lellingen, a non-metallicolous population from Luxembourg, and 20 families were sampled in Prayon, a metallicolous population located in Belgium [Table 1]. In September 2016, 15 seeds per family were sown in seedling trays containing peat-based compost, placed one week in a cold room at 4°C, transferred in a glasshouse and watered with osmosis water every 2 days. A germination survey was performed every 2 days to record germination dates.

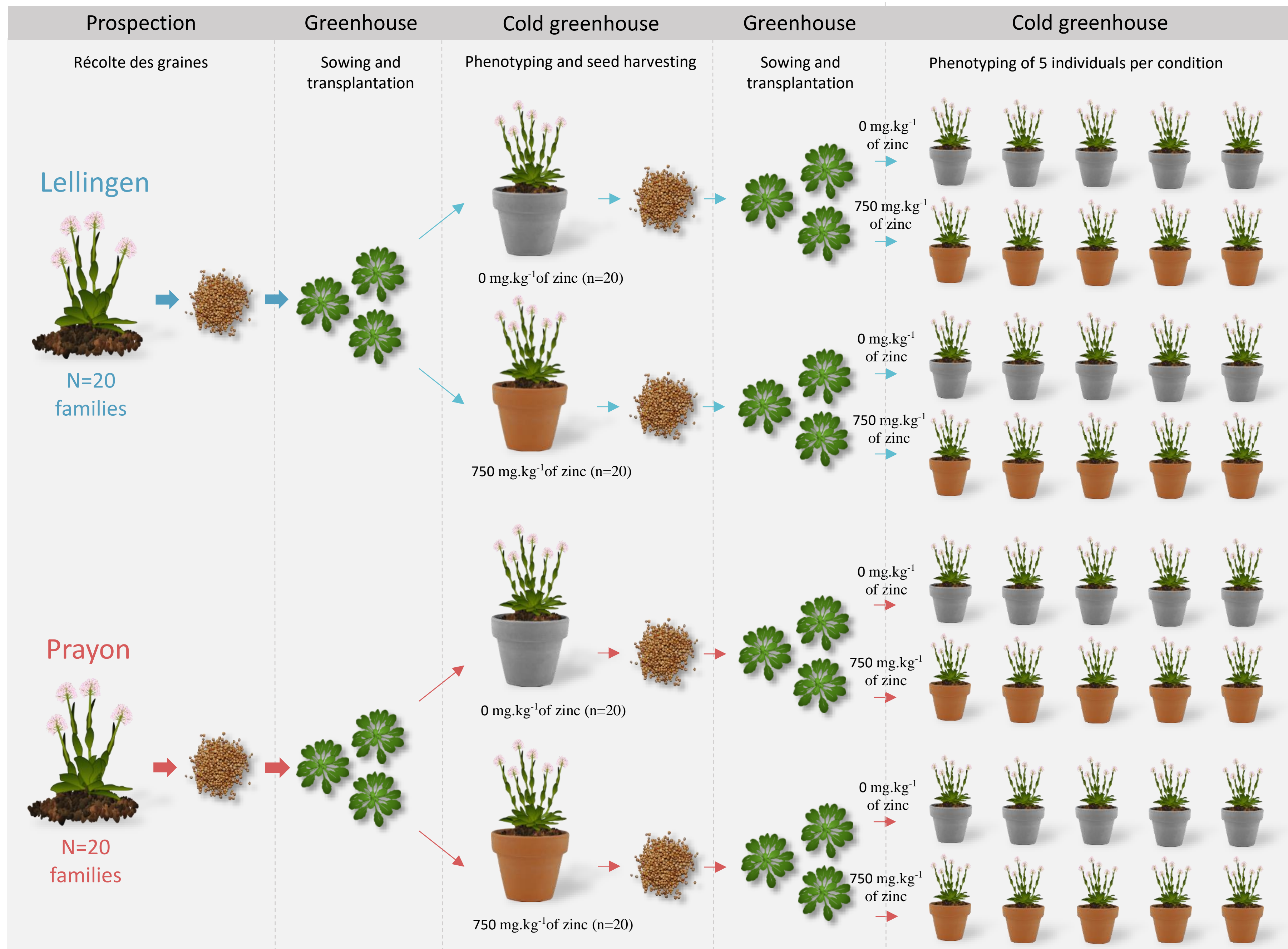


Figure 25 : Summary scheme of the experiment. 20 seeds families from one metallicolous and one nonmetallicolous natural accessions were sown. 2 seedlings from each family were cultivated in controlled conditions either in pots containing zinc contaminated or non-contaminated soil. Individuals were phenotyped on several morphological, physiological and phenological traits. Seeds were also harvested in order to obtain 10 descendants from each individual. Descendants were cultivated in same controlled conditions. 5 individuals were cultivated in zinc contaminated soil and the 5 remains were cultivated in non-contaminated soil. Individuals were followed on the same traits as those measured in parental population and broad sense heritability was calculated for each trait using parent-offspring regression.

After 8 weeks, at time t0, two individuals from each family, were transferred in two 1 L pot containing a mix of 70% of peat-based compost and 30% of zeolite, respectively contaminated or not at 750 $mg.kg^{-1}$ of zinc. Contamination of each pot was carried out using 20 mL of zinc sulfate solution at 0.57 M, and performing manual homogenization of the soil in each pot 2 days after contamination. Then, 80 pots were randomized and placed in a ventilated and unheated greenhouse, in particular to induce flowering [Figure 25].

4.2.2. Monitoring of individuals

During a complete generation, i.e. from seed sowing to the germination of offspring seeds, plants were phenotyped for several phenological, physiological, biochemical and morphological traits [Table 7].

Phenological traits: we measured the number of days from sowing to germination (*GERM*), from germination to bolting time (*BT*), between germination and anthesis (*ANT*), between germination and the first fruit emergence (*FFE*) on the main flowering stem, between germination and the senescence of the last flower on the main flowering stem, corresponding to the end of flowering (*EF*), between germination and the dehiscence of the last silique (*DEH*), between bolting and anthesis, corresponding to the bolting period (*BP*), between anthesis and the end of flowering, corresponding to the flowering period (*FP*), and between anthesis and dehiscence, corresponding to the reproductive period (*RP*). All these time intervals are converted into growth degree days.

Physiological traits: Plants were simultaneously phenotyped for photosynthetic performance of the photosystem II at t0, and t1, 25 weeks after sowing, when a first plant initiated the emergence of a first flower bud, ($Phi\text{-}PSII_0$ and $Phi\text{-}PSII_1$, respectively), using a portable fluorometer (PAM-2100, Walz). Plant were also characterized for chlorosis level of the rosette according to 4 categories (1: healthy plant, 2: partially chlorotic plant, 3: fully chlorotic plant and 4: dead plant – Figure 20); simultaneously at t1 ($STATE_1$), and separately at their specific bolting or anthesis time ($STATE_{BT}$ and $STATE_{ANT}$, respectively). Finally, 3 representative leaves were collected, scanned to digitally obtain the surface of each leaf and weighed before and after drying (3 days in a drying oven at 60°C). It was then possible to calculate the average specific leaf area [Cornelissen *et al.*, 2003] (*SLA* - formula 1), the average dry matter content [Cornelissen *et al.*, 2003] (*LDMC*-formula 2) and the average leaf thickness estimation [Vile *et al.*, 2005] (*LT* - formula 3)

Table 7: List of vegetative morphological (in green), phenological (in blue), physiological (in grey), biochemical (in yellow) and reproductive (in red) traits that have been measured. GDD: growth degree days. OD: optical density. t0: transplanting time - 8 weeks after sowing. t1: 25 weeks after sowing, when a first plant initiated the emergence of a first flower bud.

abbreviation	MPP trait (unit)	measured at
LN_{t0}	Leaf number	t0
SP_{t0}	Surface of of the plant (cm²)	t0
LN_{t1}	Leaf number	t1
SP_{t1}	Surface of of the plant (cm²)	t1
LL_{t1}	Leaf length (cm)	t1
PR_{t1}	Petiolic ratio	t1
LS_{t1}	Leaf surface (cm²)	t1
LM_{t1}	Leaf morphology	t1
LN_{BT}	Leaf number	bolting
SP_{BT}	Surface of of the plant (cm²)	bolting
LL_{BT}	Leaf length (cm)	bolting
PR_{BT}	Petiolic ratio	bolting
LS_{BT}	Leaf surface (cm²)	bolting
LM_{BT}	Leaf morphology	bolting
HR	Height of the rosette	bolting
SP_{ANT}	Surface of of the plant (cm²)	anthesis
GERM	Germination date (GDD)	germination
BT	Bolting date (GDD)	bolting
ANT	Anthesis date (GDD)	anthesis
BP	interval between bolting and anthesis (GDD)	anthesis
FFE	First fruit emergence date (GDD)	first fruit emergence
EF	End of flowering (GDD)	end of flowering
FP	Flowering period (GDD)	end of flowering
DEH	Dehiscence of the last fruit date (GDD)	dehiscence of the last fruit
RP	Reproductive period (GDD)	dehiscence of the last fruit
$PHI\text{-}PSII_0$	Photosynthetic yield	t0
$PHI\text{-}PSII_1$	Photosynthetic yield	t1
$STATE_1$	Chlorosis level of the rosette	t1
$STATE_{BT}$	Chlorosis level of the rosette	BT
$STATE_{ANT}$	Chlorosis level of the rosette	ANT
SLA	Specific leaf area ($m^2.kg^{-1}$)	bolting
LDMC	Leaf dry matter content ($mg.g^{-1}$)	bolting
LT	Leaf thickness (µm)	bolting
CHLORO	Chorophyll concentration measured at chlorophyll meter (SPAD unit)	bolting
Chla	Chlorophyll a concentration (OD.g-1)	bolting
Chlb	Chlorophyll b concentration (OD.g-1)	bolting
Car	Carotenoids concentration (OD.g-1)	bolting
Antho	Anthocyanins concentration (OD.g-1)	bolting
Flavo	flavonoids concentration (OD.g-1)	bolting
Pheno	Phenolic compounds concentration (OD.g-1)	bolting
Tan	Tannins concentration (OD.g-1)	bolting

Table 7 (continued) : List of vegetative morphological (in green), phenological (in blue), physiological (in grey), biochemical (in yellow) and reproductive (in red) traits that have been measured. GDD: growth degree days. OD: optical density. t0: transplanting time - 8 weeks after sowing. t1: 25 weeks after sowing, when a first plant initiated the emergence of a first flower bud.

abbreviation	MPP trait (unit)	measured at
NR	Number of racemes	end of life cyle
NP	Number of panicles	end of life cyle
NRAM	Number of ramifications	end of life cyle
NFS	Number of flower stems	end of life cyle
PR	Proportion of raceme	end of life cyle
PP	Proportion of panicles	end of life cyle
RPR	ratio between panicles and raceme	end of life cyle
maxLFS	length of the largest flower stem (cm)	end of life cyle
HFF	Heigth to the first fruit on the largest flower stem (cm)	end of life cyle
NS	Number of siliques	end of life cyle
NAS	Number of aborted siliques	end of life cyle
NNAS	Number of non-aborted siliques	end of life cyle
PAS	Propotion of aborted siliques	end of life cyle
PNAS	Propotion of non-aborted siliques	end of life cyle
RS	Ratio between aborted and non-aborted siliques	end of life cyle
SL	Siliques length (mm)	end of life cyle
SS	seed set estimation	end of life cyle
SR	Survival rate of descendant calculated after 5 weeks	end of life cyle
ND	Number of descendants	end of life cyle

$$(1)\ SLA = \frac{Leaf\ surface\ (m^2)}{Leaf\ dry\ mass\ (kg)}$$

$$(2)\ LDMC = \frac{Leaf\ dry\ mass\ (mg)}{Leaf\ fresh\ weight\ (g)}$$

$$(3)\ LT = \frac{1}{SLA * DMC}$$

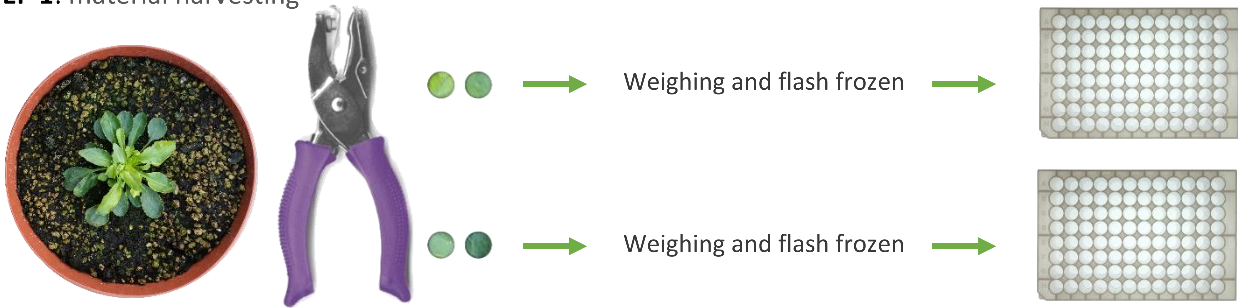
<u>Biochemical traits:</u> Biochemical traits (hereafter called "biomarkers") were assessed at BT for each individual, using leaf punches collected on 4 representative leaves. First, concentrations in photosynthetic and photoprotective pigments were analyzed: chlorophyll a (*Chla*), chlorophyll b (*Chlb*) and carotenoids (*Car*). Carotenoids protect chlorophylls and cell membranes during light stress, by avoiding ROS production and lipid peroxidation or by dissipating excess energy into heat [Gill & Tuteja, 2010; Baek *et al.*, 2012]. Then, we dosed the secondary compounds: phenolic compounds (*Pheno*), tannins (*Tan*), flavonoids (*Flavo*) and anthocyanins (*Antho*). These compounds are useful for their antioxidant role, for plant growth [Das & Roychoudhury, 2014], for metal tolerance and detoxification [Michalak, 2006] or pathogen resistance [Wink, 1988]. Photosynthetic pigments and secondary compounds were dosed by spectrophotometry after extraction in a 95% methanol solution using the method described in Al souki 2017 [Box 1].

Simultaneously, , the total chlorophyll concentration was estimated with a chlorophyll meter (Hansatech Instruments® - CL-01 Chlorophyll Content Meter) on 5 leaves representative of the plant chlorosis level (*CHLORO*).

<u>Vegetative traits</u>: Leaf number was measured at t0 (LN_{t0}), t1 (LN_{t1}), and at bolting time (LN_{BT}). Surface of plants (*SP*) was calculated as the surface of an ellipse from the two largest orthogonal diameters of the rosette, at t0, t1, bolting time and at anthesis time (SP_{t0}, SP_{t1}, SP_{BT} and SP_{ANT}, respectively). From 3 leaves, the length of the petiole (*P*), length of the blade (*L*) and width of the blade (*W*) were measured at t1 and BT in order to calculate different leaf biometric indices [Mouton, 1976], including the total leaf length ($LL=P+L$), the petiolic ratio ($PR = \frac{P}{L}$), leaf blade surface ($LS = \frac{L}{2} * \frac{W}{2}$) and leaf morphology ($LM = \frac{L}{W}$) at t1 ($LL_{t1}$, PR_{t1}, LS_{t1} and LM_{t1}) and at bolting time (LL_{BT}, PR_{BT}, LS_{BT} and LM_{BT}). Finally, the height of the rosette (*HR*) was measured at bolting time to approximates the depth of rooting [Cornelissen *et al.*, 2003].

BOX 1: Biomarker dosages

STEP 1: material harvesting



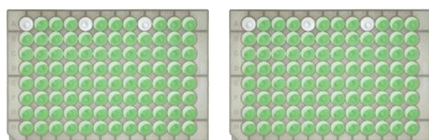
At bolting, we harvested 2*2 pieces of 6 mm on leaves that represent the chlorotic state of the plant. Each lot of punches were weighted, flash frozen in liquid nitrogen (−195.79 °C) and placed in 96 deep well plate of 2mL. In each well, a 4mm glass bead was added. The plates were stored at -80°C.

STEP 2: Pigments and secondary compounds extraction

Samples were crushed in the ball mill for 1 min 30 at 30 s^{-1}. Then, we added 1.5 mL of 95% methanol in every well. The sample was homogenized with a ball mill (2 min at 15 s^{-1}). Plates were placed in the dark at room temperature.

STEP 3: Pigment dosages

After 24 h, plates were homogenized with a ball mill (2 min at 15 s^{-1}). Then, we pooled 50µL per well of each plate in 3 microplates. Microplates were read on spectrophotometer after agitation (1 min, speed: 840)

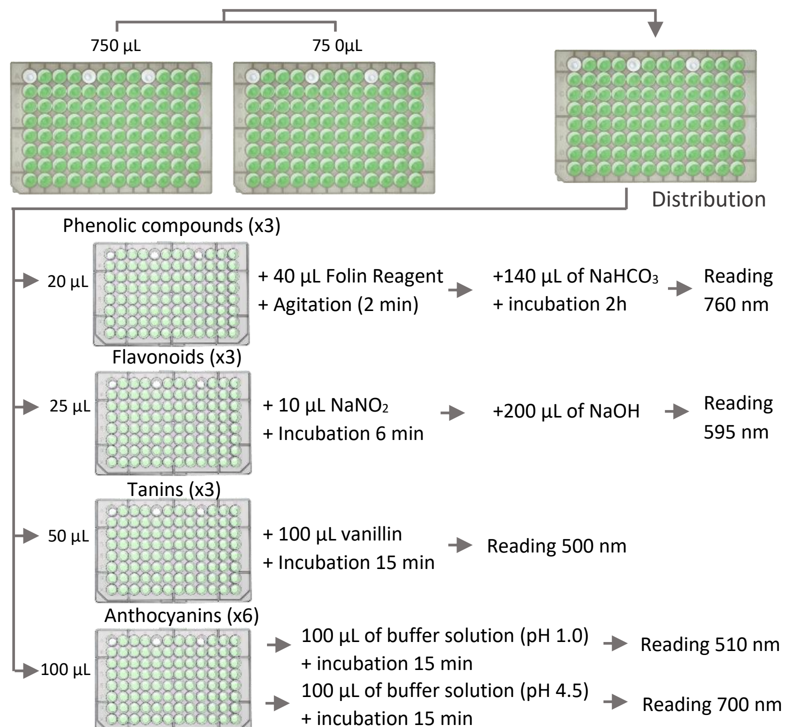


50 µL
50 µL
x3

- Reading at 470 nm : Carotenoids
- Reading at 652 nm : Chlorophyll b
- Reading at 666 nm : Chlorophyll a

STEP 4: secondary compounds dosage

After 48 h, we pooled 750 µL per well of both extraction plate in 96 deep well plate of 2 mL after centrifugation (5 min at 5000 G). Then we followed each protocol:



Box 1 : Method for biomarkers dosages

Reproductive traits: several traits related to the architecture of reproductive structures and fecundity were recorded. After maturation of the last fruit, individuals were harvested, dried (20°C during 2 months) and phenotyped. Then, we measured the number of flower stem (*NFS*), which included the number of ramified flower stems, hereafter called "panicles" (*NP*), and non-ramified flower stems, hereafter called "raceme" (*NR*), also, we measured the number of ramifications (*NRAM*). This allowed to calculate the proportion of racemes ($PR = \frac{NR}{NFS}$), the proportion of panicles ($PP = \frac{NP}{NFS}$), and the ratio between raceme and panicles ($RPR = \frac{NR}{NP}$). The length of the main flower stem (*maxLFS*) and the distance between the beginning of the flower stem and the first silique on the main flower stem, hereafter called "height to the first fruit" (*HFF*). We considered the main flower stem as the largest one.

Concerning fecundity, we counted the number of aborted and non-aborted siliques and measured 10 siliques per flower stem. This allowed to calculate the number of aborted siliques (*NAS*), the number of non-aborted siliques (*NNAS*) and the number of siliques ($NS=NAS+NNAS$). In the same way, the proportion of aborted ($PNAS = \frac{NAS}{NS}$) or non-aborted siliques ($PNNSA = \frac{NNAS}{NS}$) and the ratio between non-aborted and aborted siliques ($RS = \frac{NNAS}{NAS}$) were calculated.

Due to a significant correlation between the number of seeds and fruit size [Brachi *et al.*, 2012; Roux *et al.*, 2016; Chapter 1], seed set can be estimated by the total fruit length. Thus, we calculated the average silique length (*SL*), and multiplied it with the number of non-aborted siliques to estimate the seed set ($SS = NNAS * SL$)

At the end of plant life cycle, 100 seeds from each individual offspring were randomly harvested and weighed after drying (48 hours at 80°C - *SW*) [Cornelissen *et al.*, 2003].

Finally, 33 seeds per individual were sown in seedling trays containing peat-based compost, placed one week in a cold room at 4°C, transferred in a glasshouse for 5 weeks and watered with osmosis water every 2 days. Then, we calculated a survival rate after 5 weeks (*SR*) to estimate the number of descendants producted by each individual ($ND = SR * SS$)

4.2.3. Set up of descendant individuals

In September 2017, 33 seeds per individual were sown in seedling trays containing compost, placed 2 days in a cold room at 4°C and transferred in a glasshouse for 5 weeks. Then, we transferred 10 descendants per family in individual pots containing a mix of 70% of peat-based compost and 30% of zeolite contaminated or not with zinc sulfate. For each family, 5 individuals were transferred in pots containing a contaminated substrate at 750mg.kg^{-1} of zinc (same contamination level and method as for parents) and 5 individuals were transferred in pots containing non-contaminated substrate. Transplantations were performed 2 days after contamination and after manual homogenization of the soil in each pot. 640 pots were then randomized and placed in a ventilated and unheated greenhouse in order to induce flowering [Figure 25].

Individuals were followed during their life cycle on the same traits as those measured in parental population.

4.2.4. Estimation of phenotypic plasticity

Our experimental design was based on the cultivation of 5 similar genotypes (either full or half-sibs) in 2 environmental conditions. Thus, plasticity indexes were calculated through relative distances between phenotypes of comparable genotypes in two different conditions [Valladares *et al.*, 2006]. Relative distance allows to compare plasticity indexes independently of the character value. It was calculated according to the following formula:

$$PI_{ij->i'j'} = \frac{|x_{ij'} - x_{ij}|}{x_{ij'} + x_{ij}}$$

Where i is the individual and j is the treatment. x_{ij} represent the trait value (or the mean value of all genotypes) of an individual (or family) i in the environment j.

4.2.5. Statistical analysis

For parent individuals, we tested the effect of the edaphic group, of the contamination level and the interaction between both factors with permutational univariate analyses of variance [Anderson, 2001]. When significant differences were detected, we performed permutational pairwise t-tests with probability adjustments using the Benjamini-Hochberg (BH) procedure [Benjamini & Hochberg, 1995]. Because chlorosis state was an ordered qualitative variable, we

directly compared the four independent groups (two edaphic groups in two contamination levels) with Kruskal-Wallis tests. When significant differences were detected, we performed pairwise Conover tests with BH adjusted p-values. We assessed the effect of edaphic group and contamination level on phenotypic integration by calculating Pearson's correlations between raw variables using BH adjusted p-values. The calculated variables were not used because they were highly correlated with the raw variables. Correlation matrixes were compared with each other by Steiger's tests. Lastly, we compared plasticity indexes between both edaphic groups with Wilcoxon-Mann-Whitney tests for paired data.

To explore the data from descendants and detect potential maternal effects, we tested the effect of parental origin (four levels: non-metallicolous parents cultivated on non-polluted or polluted soil, metallicolous parents cultivated on non-polluted or polluted soil), the effect of contamination level (whatever the parental origin) and the interaction between both factors with permutational univariate analyses of variance. When significant differences were detected, we performed permutational pairwise t-tests with BH adjusted p-values. For chlorosis state, we proceeded as described above, by using Kruskal-Wallis tests and then pairwise Conover tests with BH adjusted p-values. Again, we assessed the effect of edaphic group and contamination level on phenotypic integration by calculating Pearson's correlations between variables and computing BH adjusted p-values, and by comparing correlation matrixes using Steiger's tests.

Lastly, we tested the effect of parental origin and parental condition and interaction between both factors on plasticity indexes calculated on all variables with permutational univariate analyses of variance. When significant differences were detected, we performed permutational pairwise t-tests using BH adjusted p-values. Finally, because sample sizes were balanced among families, broad-sense heritability was estimated using a parent-offspring regression analysis, using this model [Ollivier, 1971; Lynch & Walsh, 1998]:

$$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i$$

Where Y_i is the average character value for descendants (full and half-sibs) from the ith family, β_0 is the intercept, β_1 is the regression coefficient, X_i is the character value of the mother of the ith family, ε_i is random error. The regression coefficient was then used to calculate the broad-sense heritability of the traits as followed:

$$h^2{}_i = 2\beta_1$$

The broad-sense heritability of the different traits was estimated by edaphic group and contamination level.

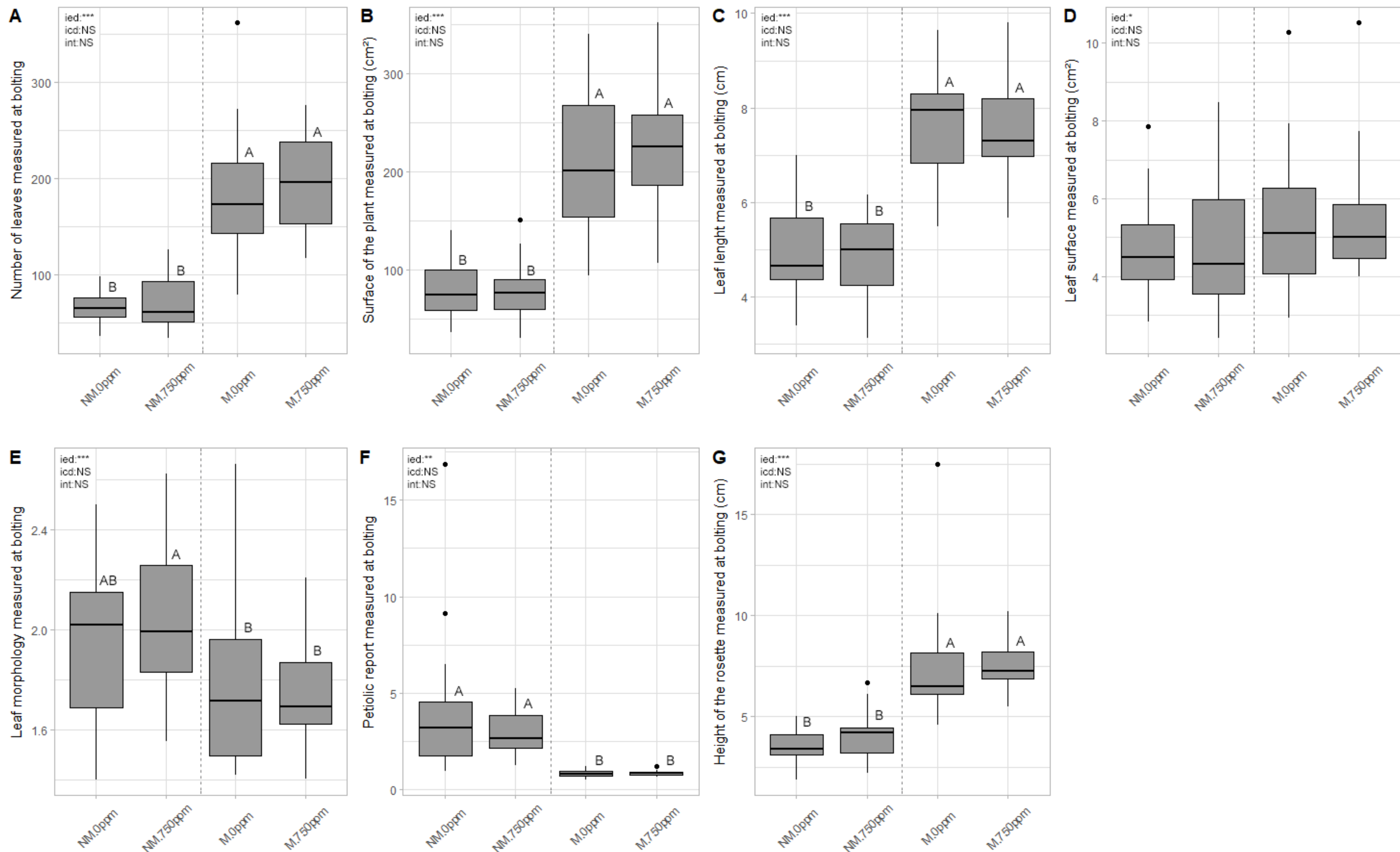


Figure 26 : results of permutational ANOVA and pairwise test on vegetative morphological traits measured at bolting. Similarly results were observed at t1 and anthesis. Dotted line represent separation between both edaphic groups. NM: nonmetallicolous populations; M: metallicolous population; 0ppm: non polluted soil; 750ppm: zinc polluted soil. "ied" (inter edaphic group difference) represent the result for the edaphic group factor, "icd" (inter contamination level difference) represent the result for the contamination factor, "int" (interaction) represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Boxplots with the same letters are not significantly different at the 5% level

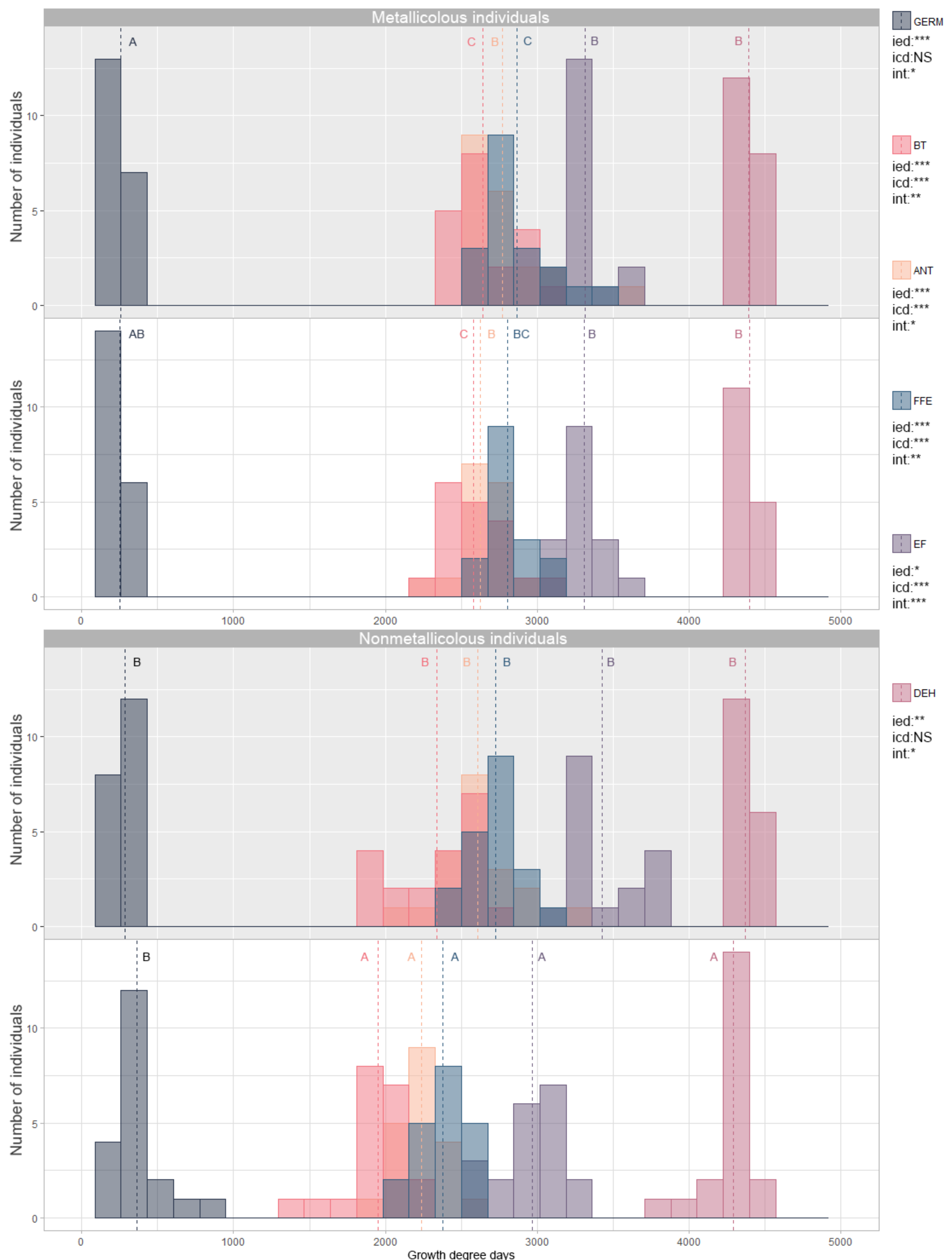


Figure 27: representation of the life cycle of metallicolous and nonmetallicolous populations in both contamination level. White background represent the non-polluted soil, grey background represent the zinc polluted soil. We represent the repartition of every phenological stage on the same time axis (converted on growth degree days) for each population - GERM: germination, BT: bolting, ANT: anthesis, FFE: first fruit emergence, EF: end of flowering, DEH: Dehiscence. For each phenological stage, we represent the results of permutational ANOVA and pairwise test: “ied” (inter edaphic group difference) represent the result for the edaphic group factor, “icd” (inter contamination level difference) represent the result for the contamination factor, “int” (interaction) represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Same letters for each trait are not significantly different at the 5% level.

4.3. Results

4.3.1. Influence of zinc contamination on parental populations

No significant difference was detected between edaphic group, contamination level and among interaction between both factors for vegetative traits measured at t0 (LN_{t0}, SP_{t0}, $PHI\text{-}PSII_{t0}$), except for the number of leaves (significant interaction: d.f=1; F=4; P value = 0.047) [Table S3]. In the same way, no difference was detected by pairwise tests [Figure S1]. For vegetative morphological traits measured at t1, at bolting and at anthesis of each individual, we only observed significant differences between edaphic groups. In comparison to non-metallicolous individuals, metallicolous individuals displayed more leaves [Figure 26A], higher plant surface [Figure 26B], leaf length [Figure 26C], leaf surface [figure 26D] and height of the rosette [Figure 26E], but different leaf blade morphology [Figure 26F] and lower petiolic ratio [Figure 26G].

For almost all phenological traits, significant differences among edaphic group, contamination level, and interaction between both factors were detected. Metallicolous individuals germinated earlier but have a later bolting, anthesis, first fruit emergence and dehiscence date than non-metallicolous ones [Figure 27]. In addition, all the flowering period seemed shorter for metallicolous individuals, as indicated by the significantly shorter interval between bolting and anthesis or flowering period [Figure S2]. An effect of contamination level was only visible on non-metallicolous individuals. Indeed, bolting, anthesis, first fruit emergence and dehiscence dates were significantly later for non-metallicolous individuals submitted to zinc contamination [Figure 27].

Similary, significant effect of edaphic group, contamination level and interaction between both factors on several physiological and biochemical traits were revealed. Metallicolous individuals had therefore significant lowest specific leaf area [Figure 28A], highest leaf thickness [Figure 28B], lowest chlorophyll concentration [Figure 28C] and highest flavonoids concentration [Figure 28D]. As indicated by the significant interaction effects for SLA, leaf thickness and chlorophyll concentration, the effect of contamination level was mainly significant for non-metallicolous individuals. Indeed, at 750 $mg.kg^{-1}$, trait values from non-metallicolous individuals tended to reach those from metallicolous individuals, except for flavonoids concentration.

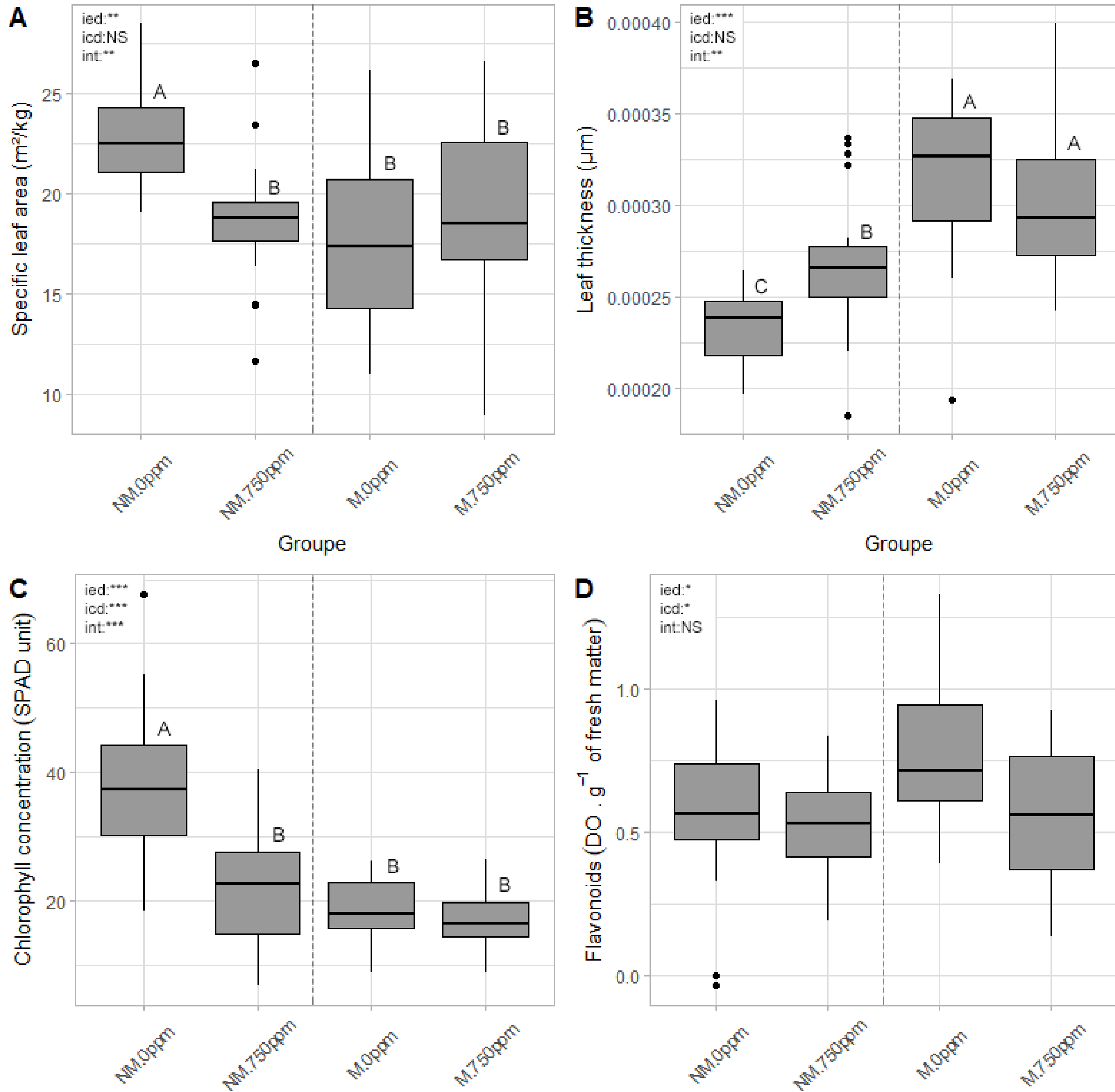


Figure 28: results of permutational ANOVA and pairwise test on physiological (A, B) and biochemical traits (C, D). Dotted line represent separation between both edaphic groups. NM: nonmetallicolous populations; M: metallicolous population; 0ppm: non polluted soil; 750ppm: zinc polluted soil. “ied” (inter edaphic group difference) represent the result for the edaphic group factor, “icd” (inter contamination level difference) represent the result for the contamination factor, “int” (interaction) represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Boxplots with the same letters are not significantly different at the 5% level.

In the same way, zinc contamination significantly increased chlorosis only for non-metallicolous individuals at t1 (df = 3, K= 15.8, P-value = 0.002) at bolting (df = 3, K=8.9, p-value = 0.03) [Figure 29] and anthesis (df = 3, K= 8.7, P-value = 0.03).

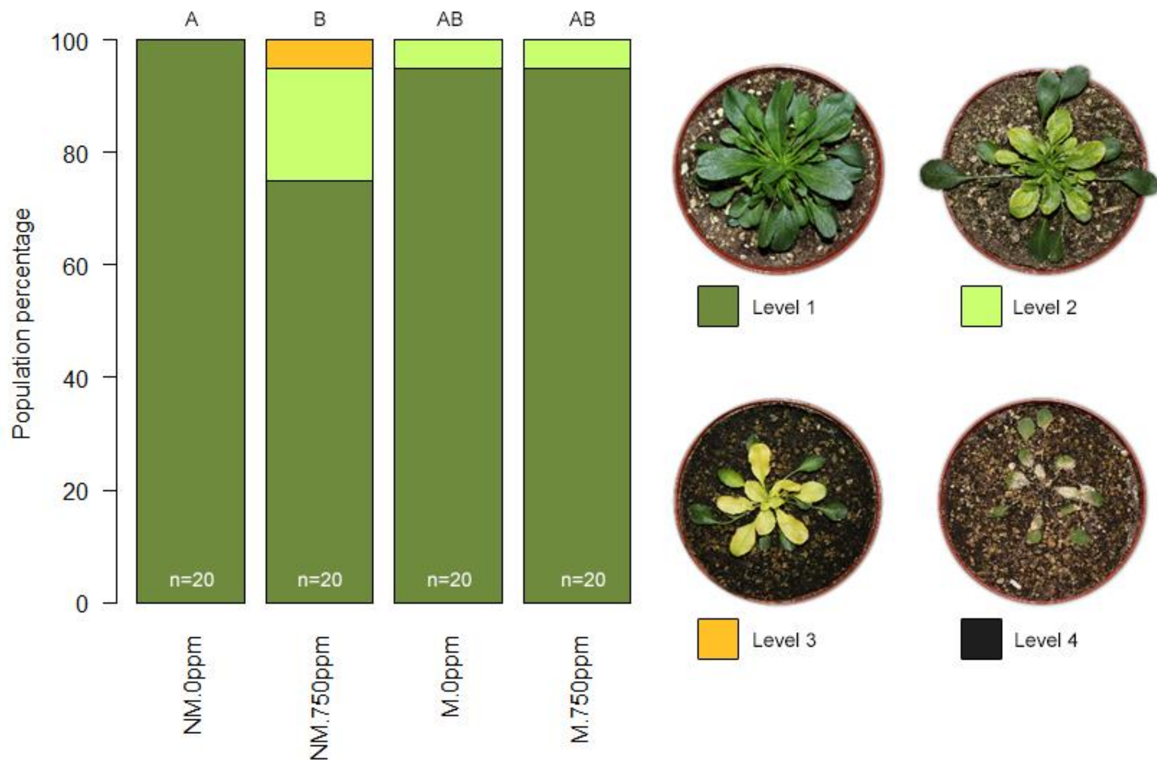


Figure 29: percentage of plants associated with each chlorosis level at bolting (similar results were observed at t1 and anthesis). n=individuals number. Level 1: Healthy plant. Level 2: Partially chlorotic plant. Level 3: Entirely chlorotic plant. Level 4: Dead plant. Barplots with the same letters are not significantly different at the 5% level with Conover's pairwise test.

Concerning traits related to the architecture of reproductive structures, we observed that metallicolous individuals produced significantly more raceme [Figure 30A] but less panicles than non-metallicolous individuals [Figure 30B]. Furthermore, for a similar length of the main flower stem [Figure 30C], non-metallicolous individuals were more affected by zinc contamination as indicated by reduction of the distance to the first fruit [Figure 30D].

Concerning traits related to fecundity, we observed a highly significant effect of contamination level in particular on non-metallicolous individuals. Thus, for an equivalent total production of siliques between edaphic groups, non-metallicolous individuals cultivated in zinc-contaminated conditions showed a higher proportion of aborted siliques [Figure 31A], a lower number of descendants [Figure 31B], and a lower average silique length although not significantly different from those of metallicolous individuals [Figure 31C]. For the seed

weight, we observed a significant difference between edaphic groups with the highest values for non-metallicolous individuals [Figure 31D].

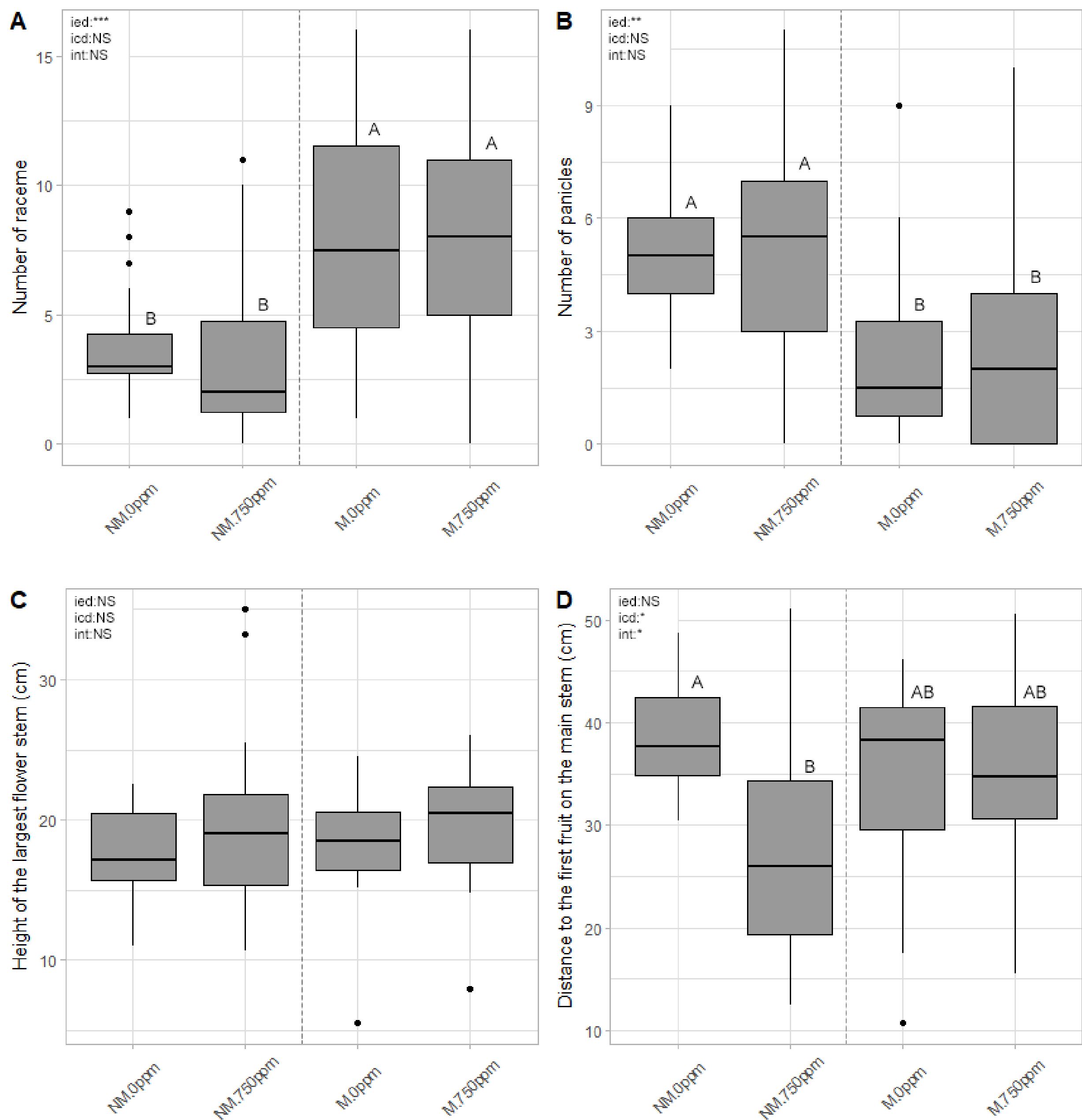


Figure 30: results of permutational ANOVA and pairwise test on traits related to reproductive structure architecture. Dotted line represent separation between both edaphic groups. NM: nonmetallicolous populations; M: metallicolous population; 0ppm: non polluted soil; 750ppm: zinc polluted soil. "ied" (inter edaphic group difference) represent the result for the edaphic group factor, "icd" (inter contamination level difference) represent the result for the contamination factor, "int" (interaction) represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Boxplots with the same letters are not significantly different at the 5% level.

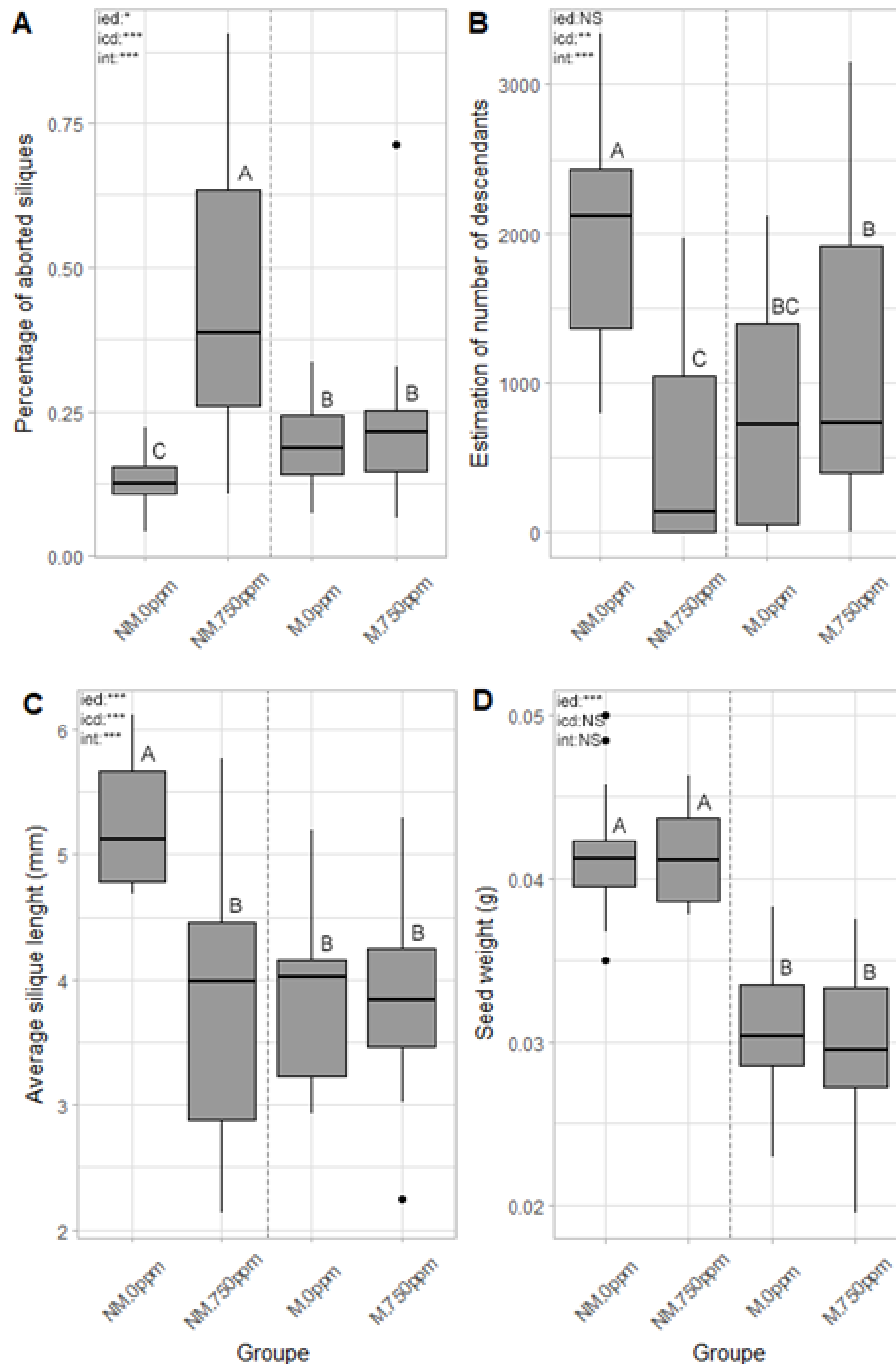


Figure 31 : results of permutational ANOVA and pairwise test on traits related to fecundity (A, B, and C) and seed weight (D). Dotted line represent separation between both edaphic groups. NM: nonmetallicolous populations; M: metallicolous population; 0ppm: non polluted soil; 750ppm: zinc polluted soil. "ied" (inter edaphic group difference) represent the result for the edaphic group factor, "icd" (inter contamination level difference) represent the result for the contamination factor, "int" (interaction) represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Boxplots with the same letters are not significantly different at the 5% level

Correlations matrixes were calculated for each combination of edaphic group with zinc level and compared [Figure 32]. All matrixes were significantly different from each other (df = 1485, P value $<1.10^{-16}$). However, some correlation groups differed between edaphic groups especially correlations between some reproductive traits (NNAS, RNS, SS, SL, ND, SR) and vegetative morphological traits. These correlations were mostly negative for metallicolous individuals [Figure 32C, 32D], when the majority of them were positive for non-metallicolous individuals [Figure 32A, 32B]. In addition, several correlations were significantly negative between some phenological traits (mainly ANT, BP, EF, FFE) and morphological traits in non-metallicolous indivduals, and became significantly positive in metallicolous ones. The effect of contamination level was more tenuous in metallicolous edaphic group [Figure 32C, 32D]. However, correlations between biomarkers and morphological traits were stronger in non-polluted condition, as well as between phenological traits and some physiological traits (LDMC, LT, SLA). On the contrary, correlations became weaker in non-polluted conditions between some reproductive traits (NSNA, RNS, SL, SS, ND, SR) and phenological traits. Interestingly, we observed contrasted responses to zinc contamination according to edaphic group. Indeed, for non-metallicolous individuals, correlations were overall stronger when individuals were cultivated in zinc contaminated soil [Figure 32B].

Finally, we analyzed the effect of edaphic group on phenotypic plasticity. Overall, we observed that non-metallicolous individuals had higher plasticity indexes than metallicolous individuals for almost all traits [Table S4]. This was especially true for all phenological traits (excepting interval between bolting and anthesis) [Figure 33B], for biochemical traits (excepting for flavonoids and tannin concentrations) [Figure 33D], and for reproductive traits [Figure 33E]. However, except for plasticity index calculated on petiolic ratio [Figure 33A], no significant difference for other vegetative morphological traits was detected. Similarly, we observed no significant difference for almost all traits related to reproductive structures excepting proportion of raceme and the ratio between panicles and racemes, and for seed weight [Figure 33F]. Conversely, for physiological traits, except for the leaf thickness, metallicolous individuals displayed higher plasticity indexes [Figure 33C].

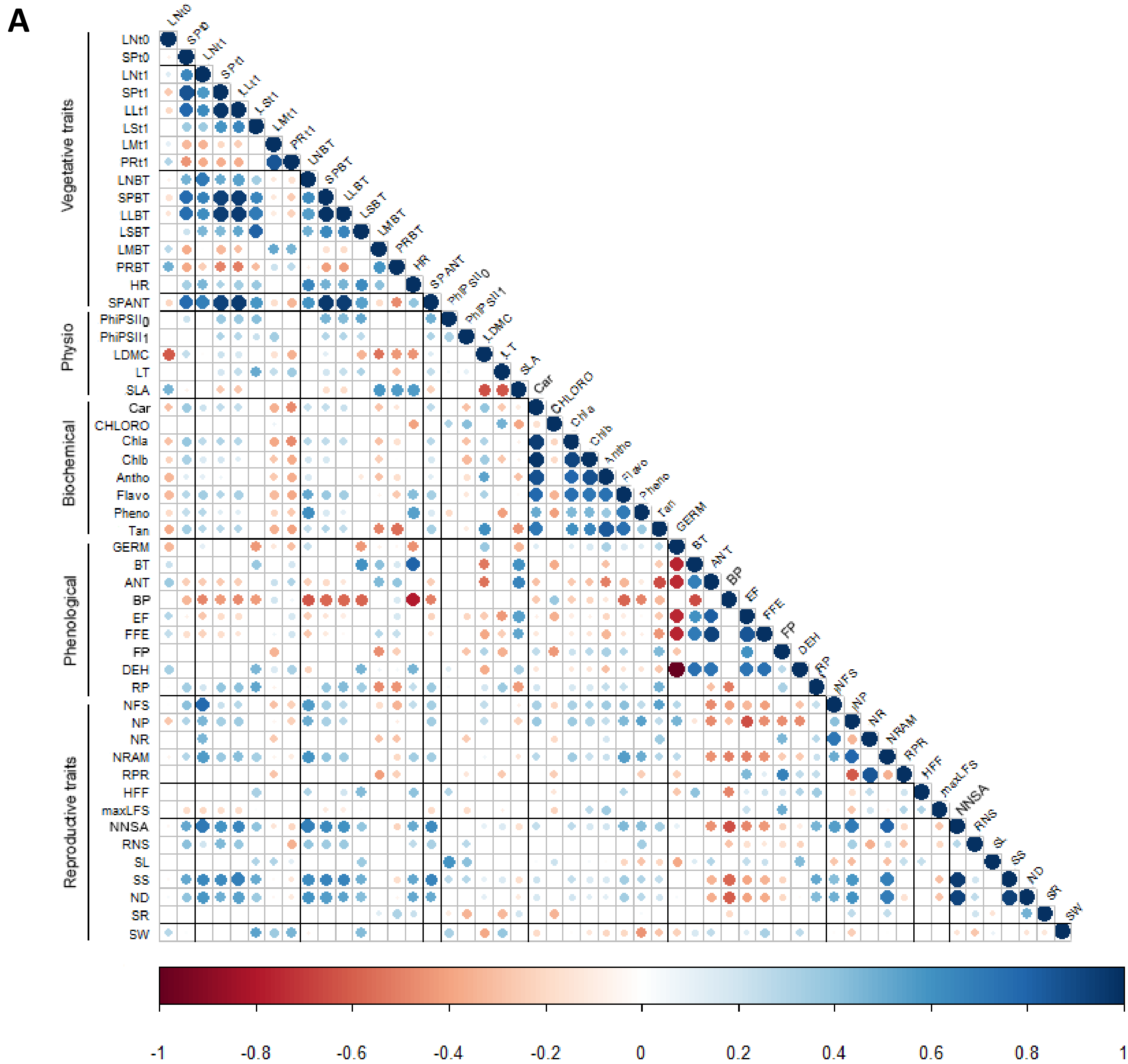


Figure 32: Correlation matrixes among traits in (A) nonmetallicolous population submitted to non-polluted soil, (B) nonmetallicolous population submitted to zinc polluted soil, (C) metallicolous population submitted to non-polluted soil, and (D) metallicolous population submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_0$, $PhiPSII_1$: average photosynthetic yield measured at t0 and t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; ChlA, ChlB, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: Reproductive period; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: distance to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RNS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after 5 weeks after sowing; SPMFS: proportion of seed set produced by main flower stem; SPP: proportion of seed set produced by panicles; SPR: proportion of seed set produced by racemes; SW: seed weight.

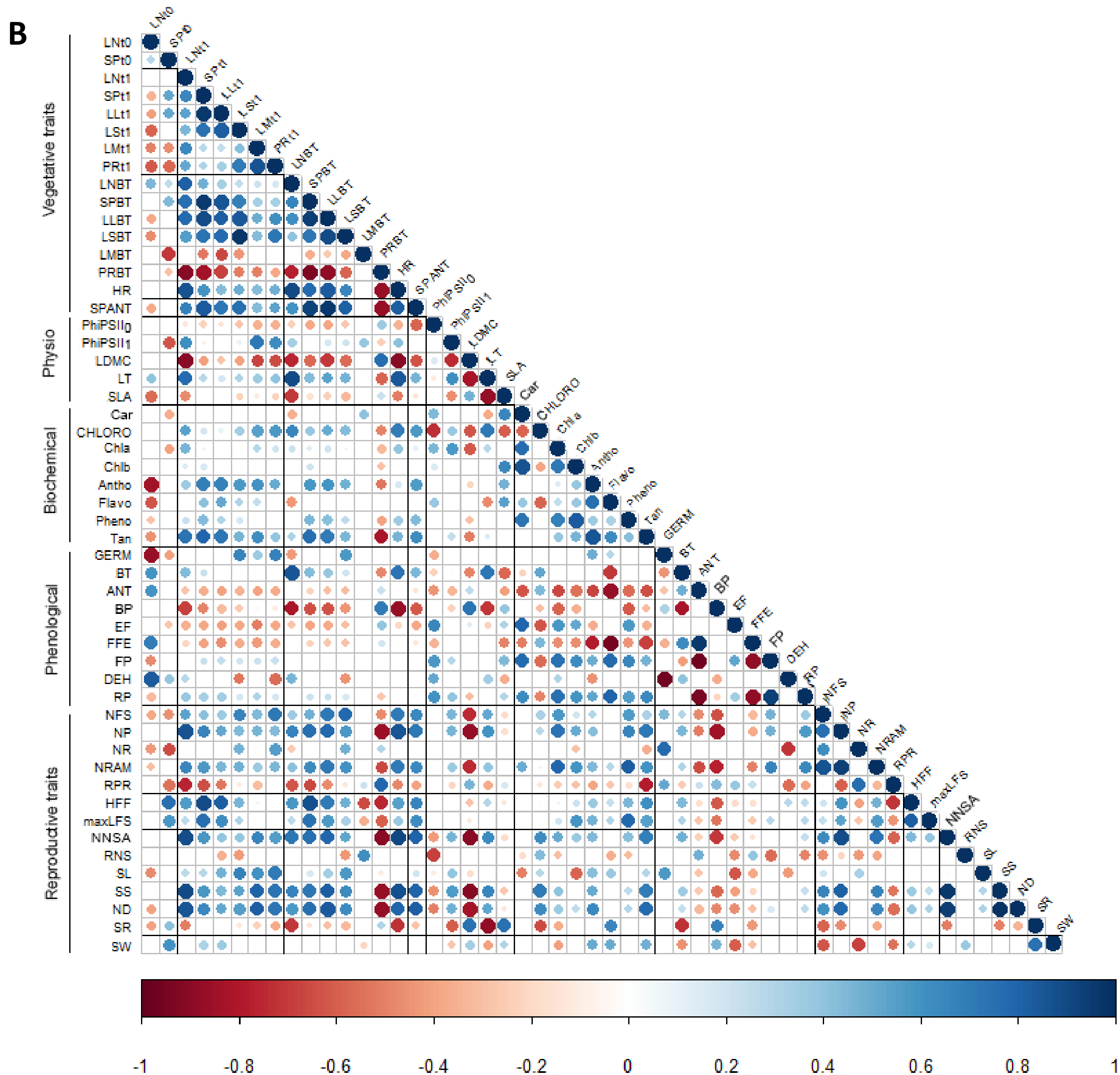


Figure 32 (continued): Correlation matrixes among traits in (A) nonmetallicolous population submitted to non-polluted soil, (B) nonmetallicolous population submitted to zinc polluted soil, (C) metallicolous population submitted to non-polluted soil, and (D) metallicolous population submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_0$, $PhiPSII_1$: average photosynthetic yield measured at t0 and t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; ChlA, ChlB, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: Reproductive period; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: distance to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RNS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after 5 weeks after sowing; SPMFS: proportion of seed set produced by main flower stem; SPP: proportion of seed set produced by panicles; SPR: proportion of seed set produced by racemes; SW: seed weight.

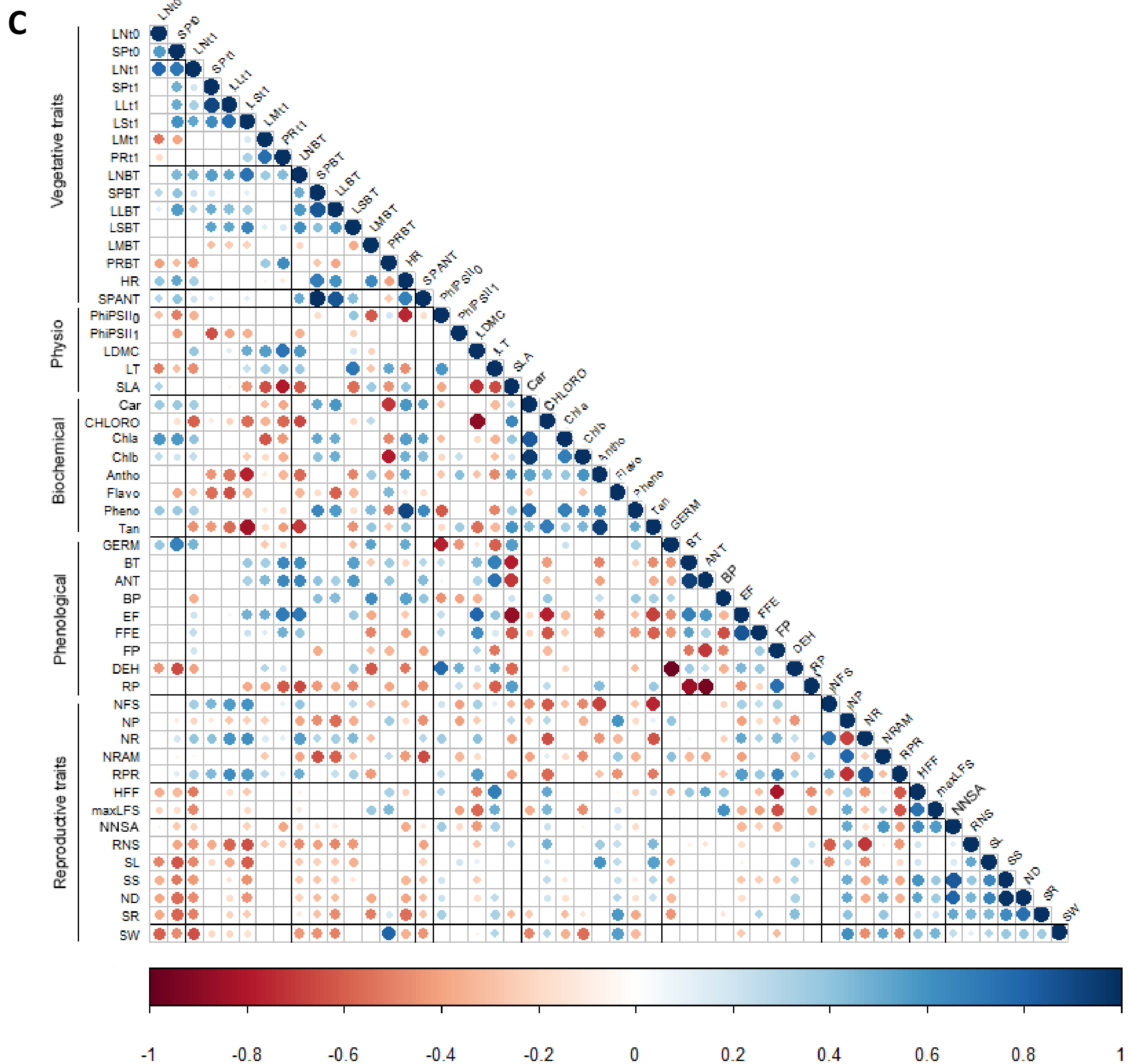


Figure 32 (continued): Correlation matrixes among traits in (A) nonmetallicolous population submitted to non-polluted soil, (B) nonmetallicolous population submitted to zinc polluted soil, (C) metallicolous population submitted to non-polluted soil, and (D) metallicolous population submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_0$, $PhiPSII_1$: average photosynthetic yield measured at t0 and t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; ChlA, ChlB, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: Reproductive period; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: distance to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RNS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after 5 weeks after sowing; SPMFS: proportion of seed set produced by main flower stem; SPP: proportion of seed set produced by panicles; SPR: proportion of seed set produced by racemes; SW: seed weight.

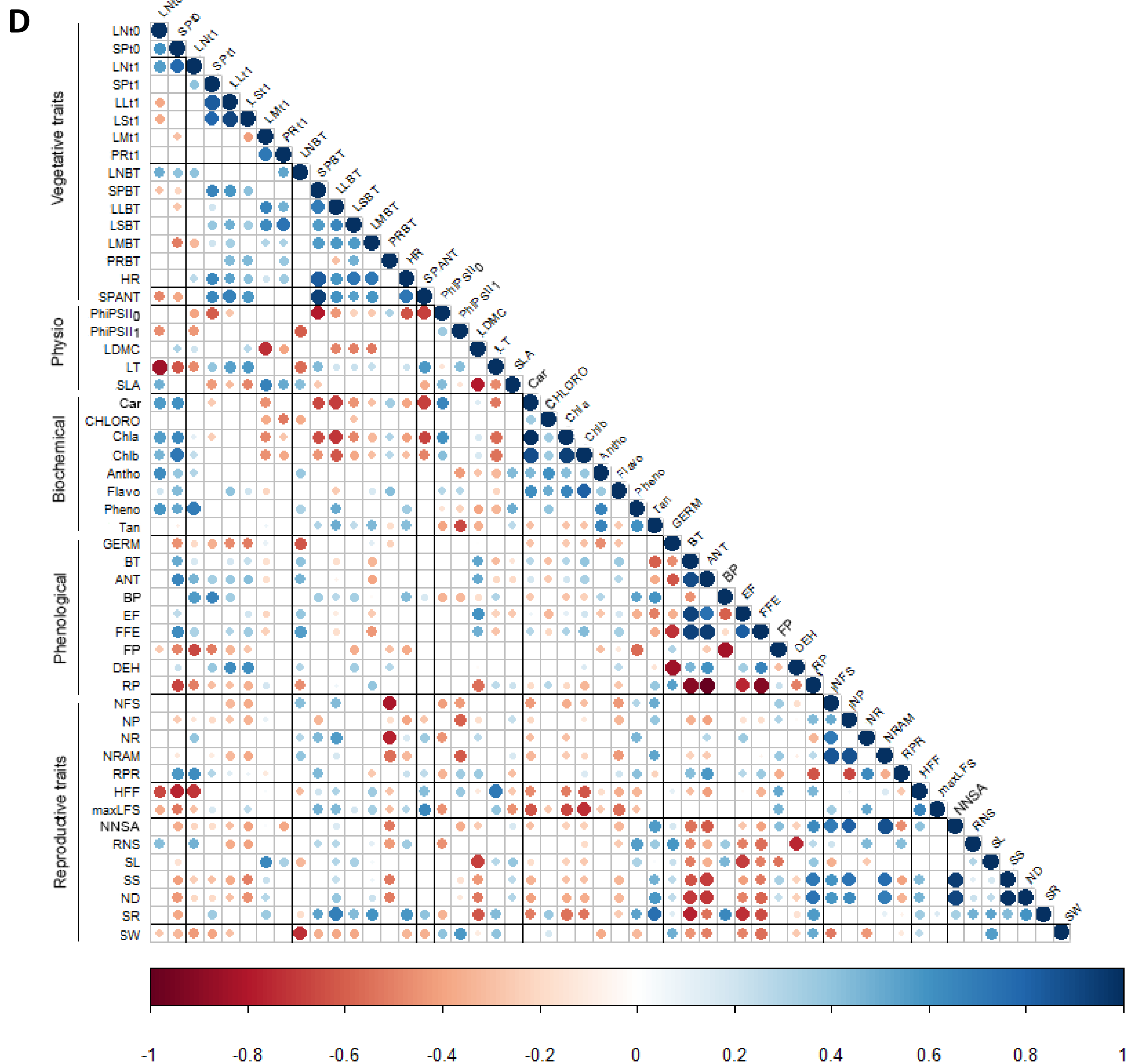


Figure 32 (continued): Correlation matrixes among traits in (A) nonmetallicolous population submitted to non-polluted soil, (B) nonmetallicolous population submitted to zinc polluted soil, (C) metallicolous population submitted to non-polluted soil, and (D) metallicolous population submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_0$, $PhiPSII_1$: average photosynthetic yield measured at t0 and t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; ChlA, ChlB, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: Reproductive period; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: distance to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RNS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after 5 weeks after sowing; SPMFS: proportion of seed set produced by main flower stem; SPP: proportion of seed set produced by panicles; SPR: proportion of seed set produced by racemes; SW: seed weight.

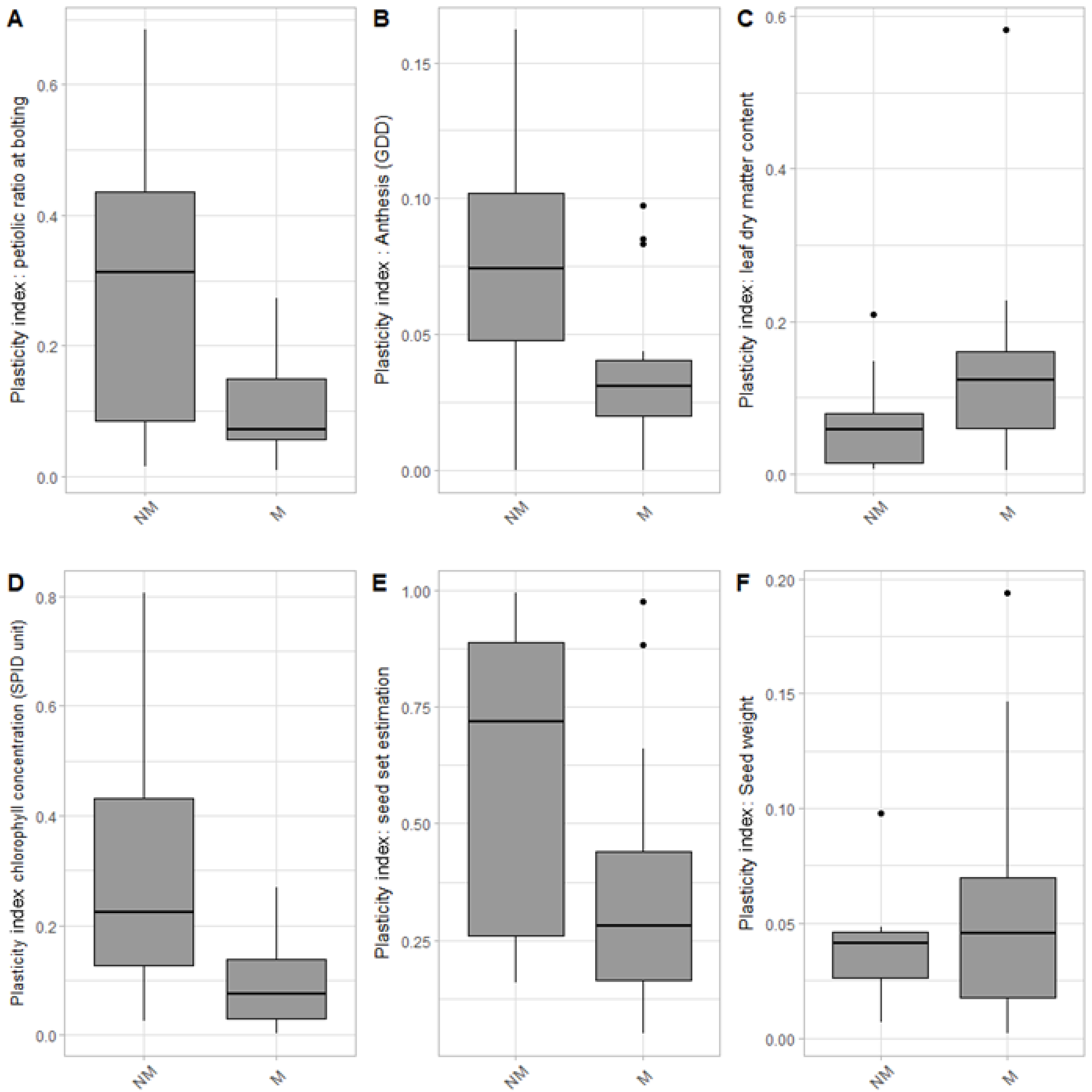


Figure 33: results of Wilcoxon-Mann-Whitney's test on plasticity index based on a vegetative morphological trait (A), a phenological trait (B), a physiological trait (C), a biochemical trait (D), a trait relative to fecundity (E), and seed weight.

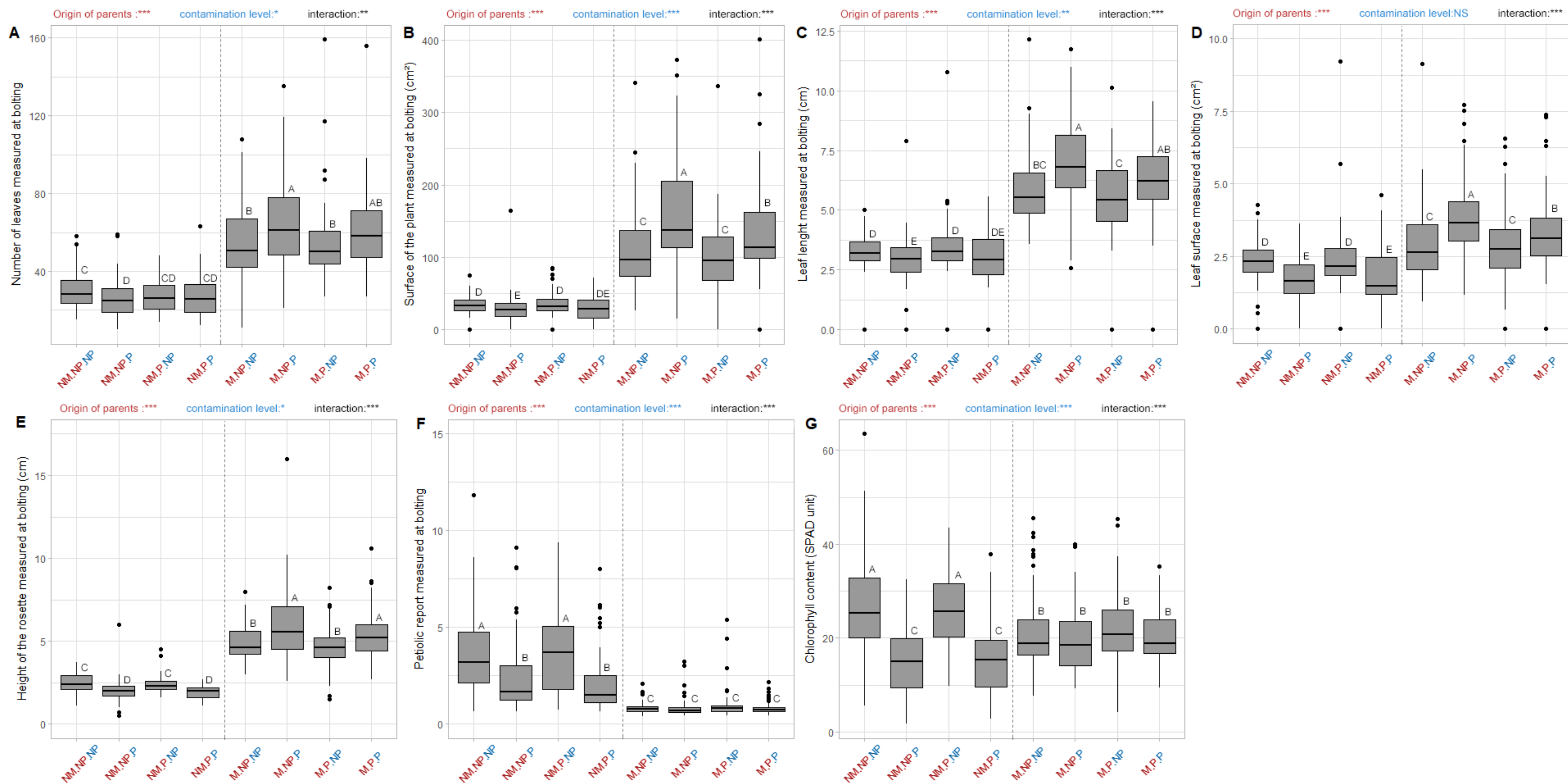


Figure 34: results of permutational ANOVA and pairwise test on vegetative morphological traits measured at bolting (A to F) a biochemical trait (G). Similarly results were observed at t1 and anthesis). Dotted line represent separation between both edaphic groups. On top of graphics we resumed the result for each factor: the origin of parents in red (nonmetallicolous populations in zinc polluted soil (NM.P) or non-polluted soil (NM.NP), and metallicolous populations in zinc polluted soil (M.P) or non-polluted soil (M.NP)), the contamination level applied on descendants in blue (zinc polluted soil (P) and non-polluted soil (NP)), and the interaction between both factor. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Boxplots with the same letters are not significantly different at the 5% level.

4.3.2. Influence of parental conditions on descendant populations

No significant difference was observed for vegetative traits measured at t0 among parental origins, contamination levels or interaction between both factors, except for the number of leaves with highest values for non-metallicolous individuals [Table S5].

Concerning vegetative morphological traits measured at t1, bolting and anthesis, significant differences were mainly assigned to edaphic origin of the parents. Metallicolous origin conferred higher number of leaves [Figure 34A], higher surface of the plant [Figure 34B], higher leaf length [Figure 34C], higher leaf surface [Figure 34D], and higher height of rosette [Figure 34E], whereas non-metallicolous origin only conferred higher petiolic ratio [Figure 34F]. For all these vegetative traits, non-metallicolous parents cultivated on non-polluted soils produced descendants that were negatively affected by zinc contamination. It was also the case for non-metallicolous parents cultivated in zinc polluted soil for leaf surface [Figure 34D], height of the rosette [Figure 34E], petiolic ratio [Figure 34F], and chlorophyll content [Figure 34G]. Descendants from metallicolous parents were also affected by zinc contamination for all traits except petiolic ratio and chlorophyll content, but always positively [Figures 34A, 34B, 34C, 34D, 34E]. Interestingly, the contamination level in which parents were cultivated influenced the response of descendants cultivated in zinc-polluted conditions in two cases and only for metallicolous individuals: for the surface of the plant [Figure 34B] and the leaf surface [Figure 34D]. In both cases, values for metallicolous descendants from non-polluted soil were higher than values for metallicolous descendants from polluted soil (M.NP.P > M.P.P).

In the same way, differences in chlorosis levels measured at t1 ($df = 7$, $K=91.25$, p-value $< 2.2x10^{-16}$), at bolting ($df = 7$, $K= 101.95$, p-value $< 2.2x10^{-16}$) [Figure 35], and anthesis ($df = 7$, $K= 85.35$, p-value $< 2.2x10^{-16}$) were detected, and were mostly related to an increased chlorosis in non-metallicolous populations cultivated in zinc-polluted soil.

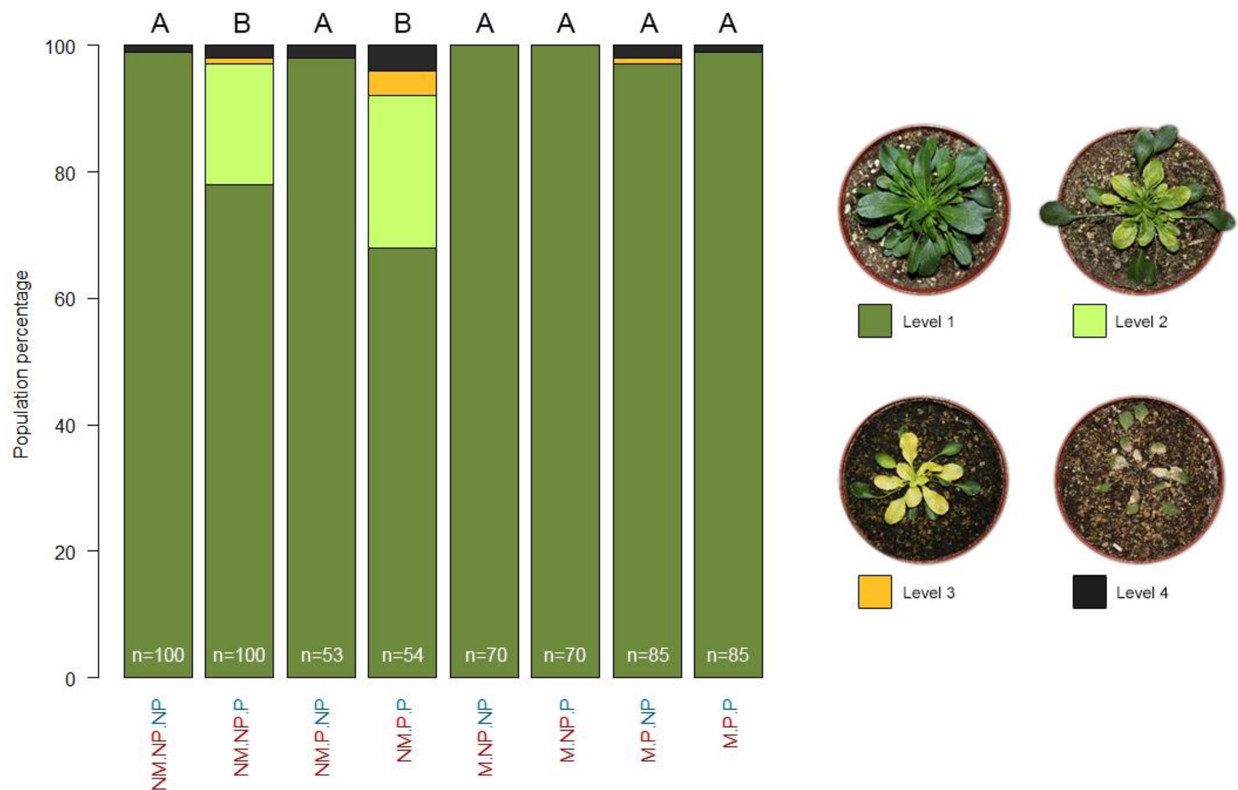


Figure 35: results of Kruskal-Wallis and Conover's pairwise test on chlorosis state measured at bolting. For each group we represent the origin of parents in red (nonmetallicolous populations in zinc polluted soil (NM.P) or non-polluted soil (NM.NP), and metallicolous populations in zinc polluted soil (M.P) or non-polluted soil (M.NP)), and the contamination level applied on descendants in blue (zinc polluted soil (P) and non-polluted soil (NP)). Barplots with the same letters are not significantly different at the 5% level.

Concerning phenological traits, significant effect of parent origin, contamination level and interaction between both factors were observed on all traits except for interval between sowing and germination (GERM), and interval between germination and dehiscence of the fruit (DEH) [Figure 36]. In particular, a longer interval between germination and bolting (BT), between germination and anthesis (ANT), between germination and first fruit emergence (FFE), and between germination and end of flowering period (EF) were specific of metallicolous individuals [Figures 36A and 36B]. As dehiscence time was the same for the two edaphic groups, the reproductive period was also shorter for metallicolous individuals. Also, there was an increase of BT, ANT, FFE and EF for non-metallicolous individuals cultivated on zinc polluted soil [Figures 36C and 36D], while no significant difference was observed between both conditions for metallicolous individuals [Figures 36A and 36B].

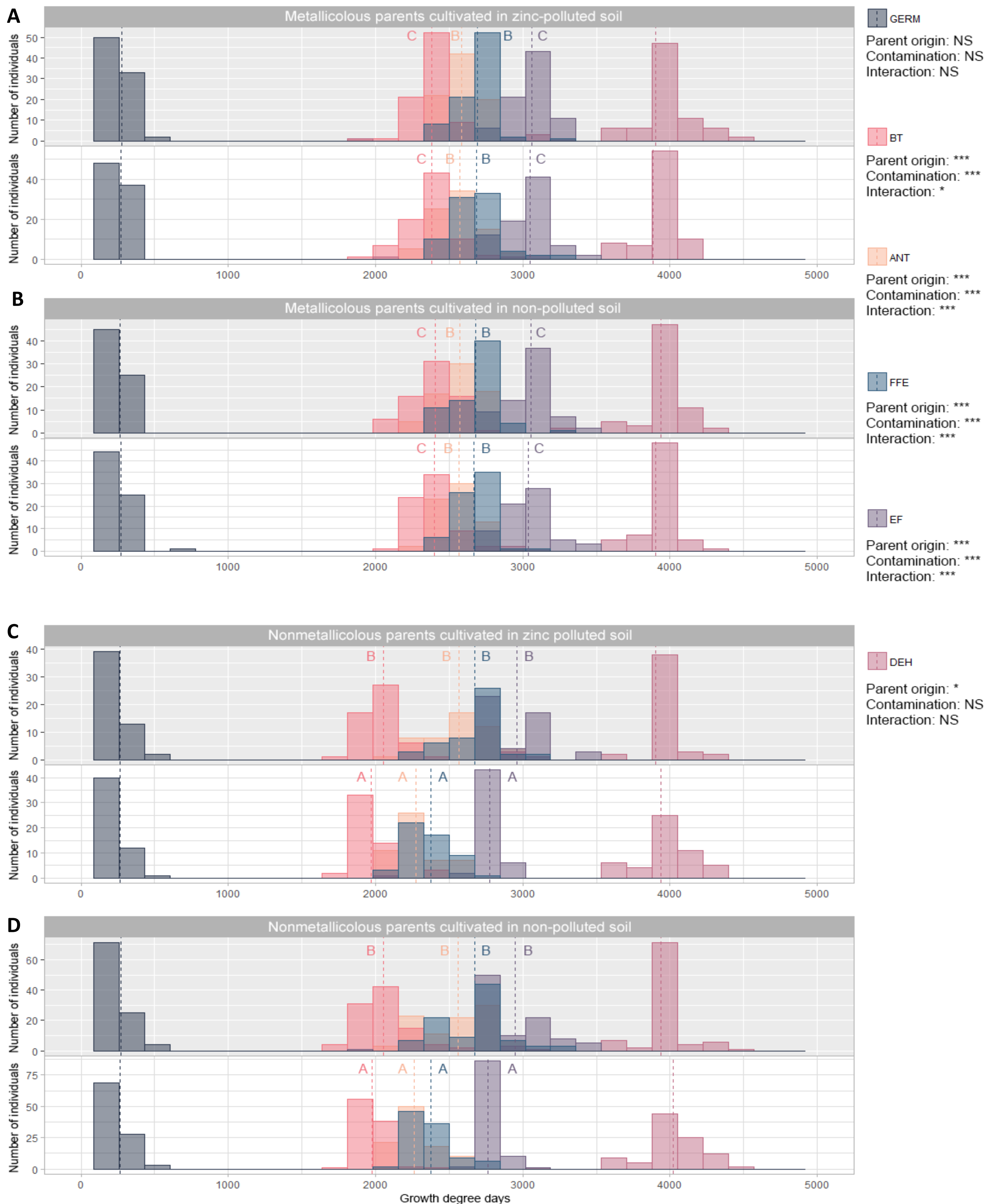


Figure 36: representation of the life cycle of offspring from (A) metallicolous individuals cultivated in zinc polluted soil, (B) metallicolous individuals cultivated in non-polluted soil, (C) nonmetallicolous individuals cultivated in zinc polluted soil, and (D) nonmetallicolous individuals cultivated in non-polluted soil. White background represent the offspring cultivated on non-polluted soil, grey background represent the offspring cultivated on zinc polluted soil. We represent the repartition of every phenological stage on the same time axis (converted on growth degree days) for each offspring - GERM: germination, BT: bolting, ANT: anthesis, FFE: first fruit emergence, EF: end of flowering, DEH: Dehiscence. For each phenological stage, we represent the results of permutational ANOVA and pairwise test: “Parent origin” represent the result for the origin of parents factor, “Contamination” represent the result for the contamination level factor, “interaction” represent the result for the interaction between both previous factors. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Same letters for each trait are not significantly different at the 5% level.

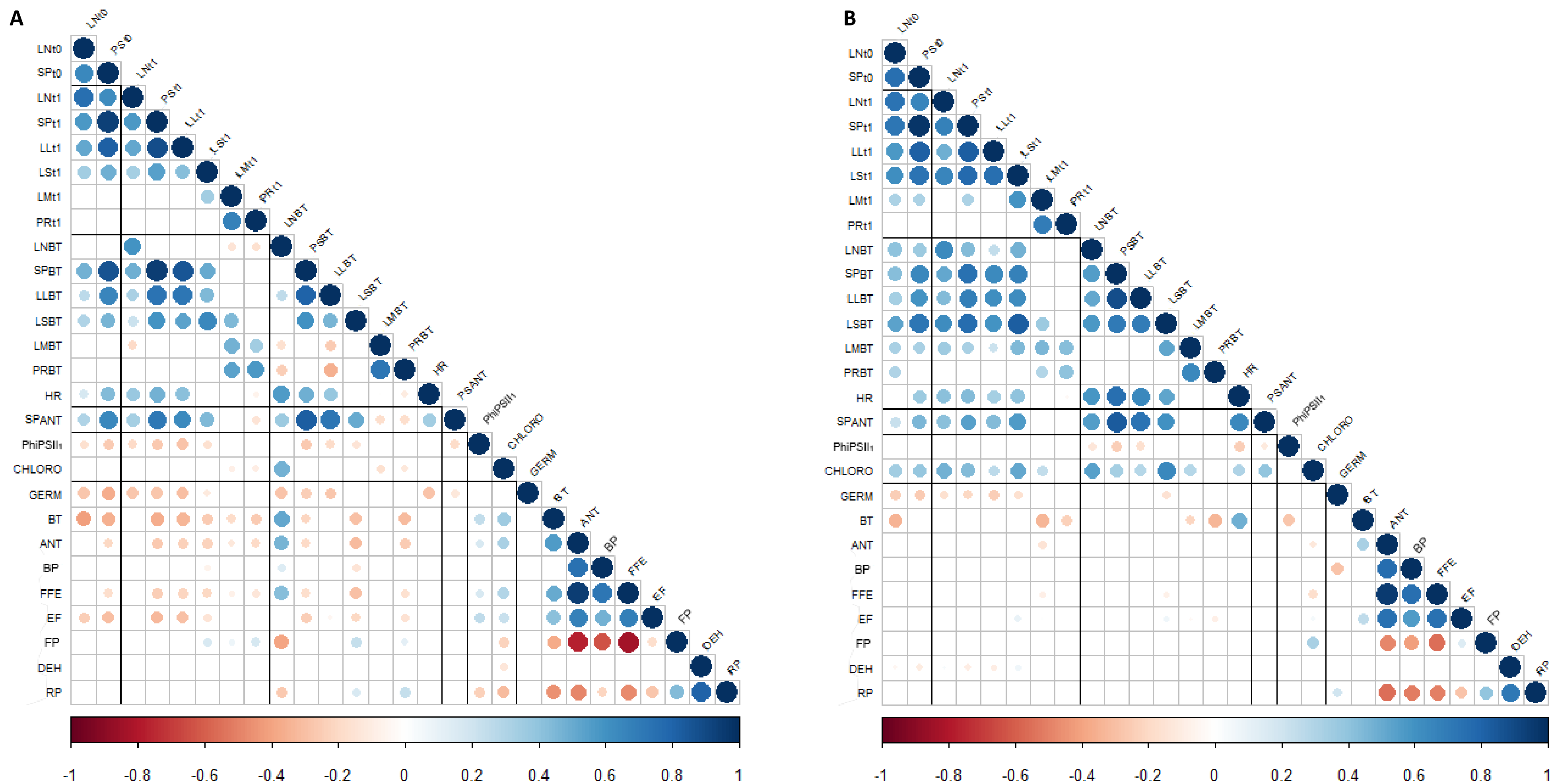


Figure 37 : Correlation matrixes among traits in offspring of nonmetallicolous population submitted to non-polluted soil: (A) the offspring was submitted to non-polluted soil; (B) the offspring was submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_1$: average photosynthetic yield measured and t1; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: time interval between anthesis and dehiscence.

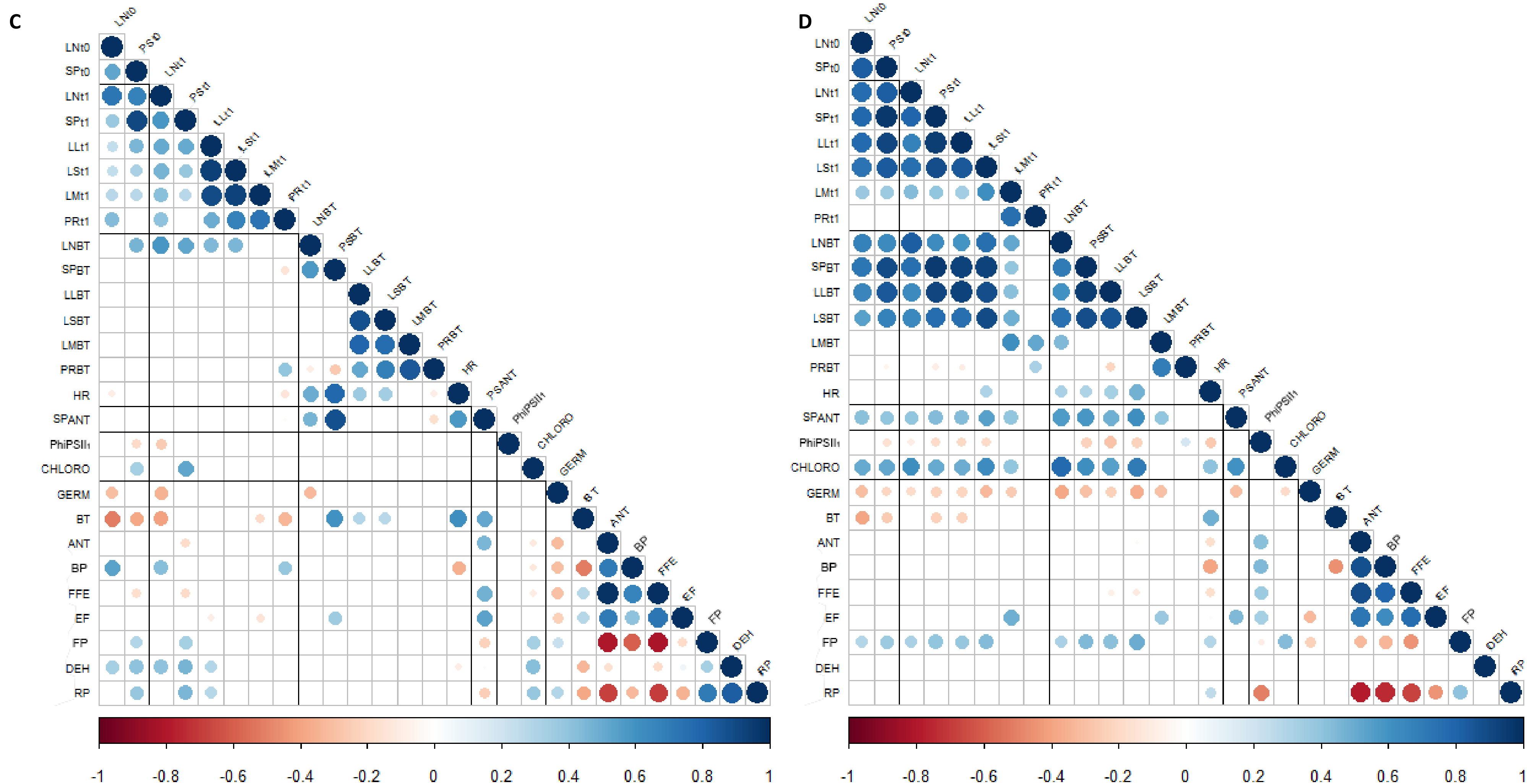


Figure 37 (continued): Correlation matrixes among traits in offspring of nonmetallicolous population submitted to zinc polluted soil: (C) the offspring was submitted to non-polluted soil; (D) the offspring was submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_1$: average photosynthetic yield measured and t1; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: time interval between anthesis and dehiscence.

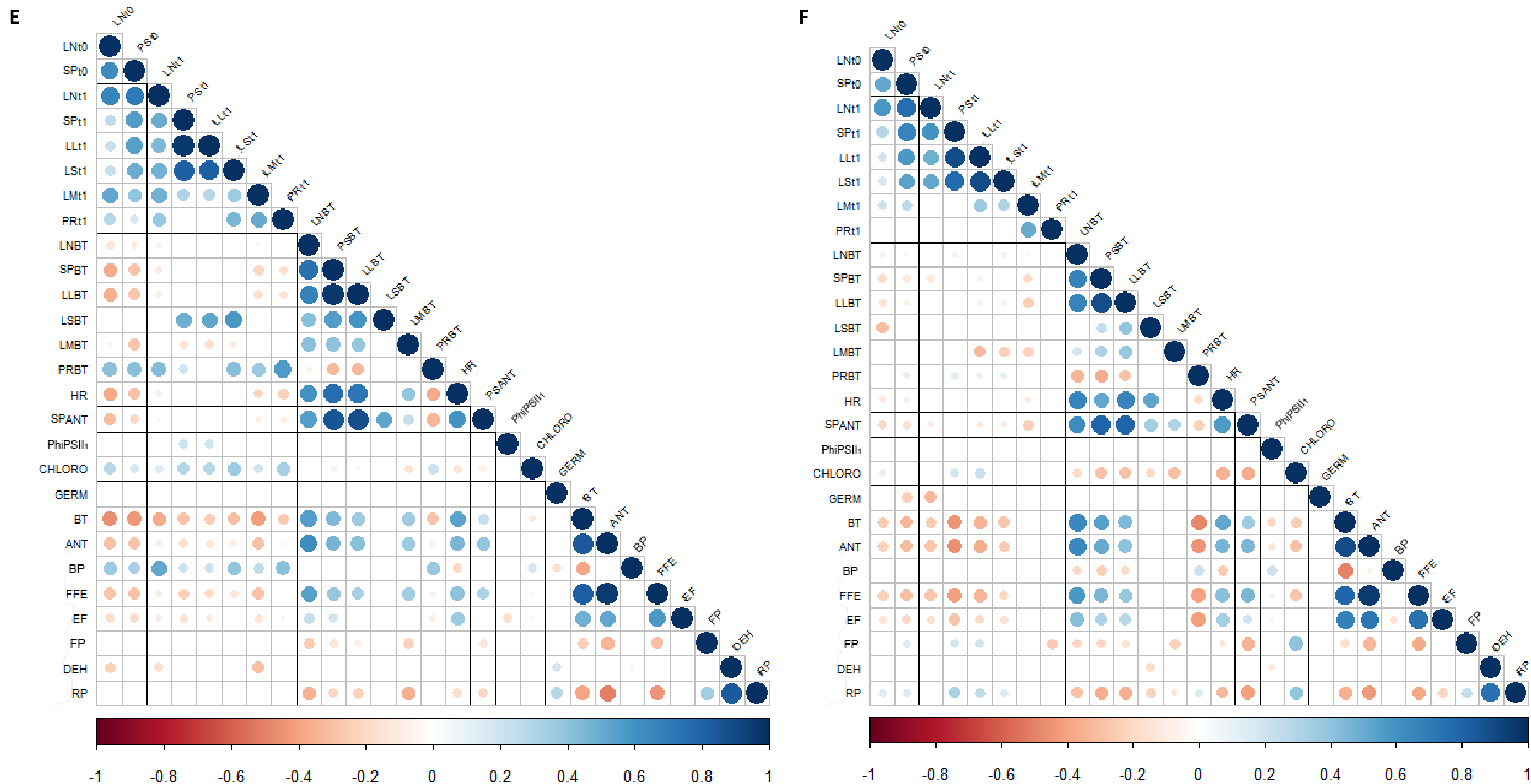


Figure 37 (continued): Correlation matrixes among traits in offspring of metallicolous population submitted to non-polluted soil: (E) the offspring was submitted to non-polluted soil; (F) the offspring was submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_1$: average photosynthetic yield measured and t1; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: time interval between anthesis and dehiscence.

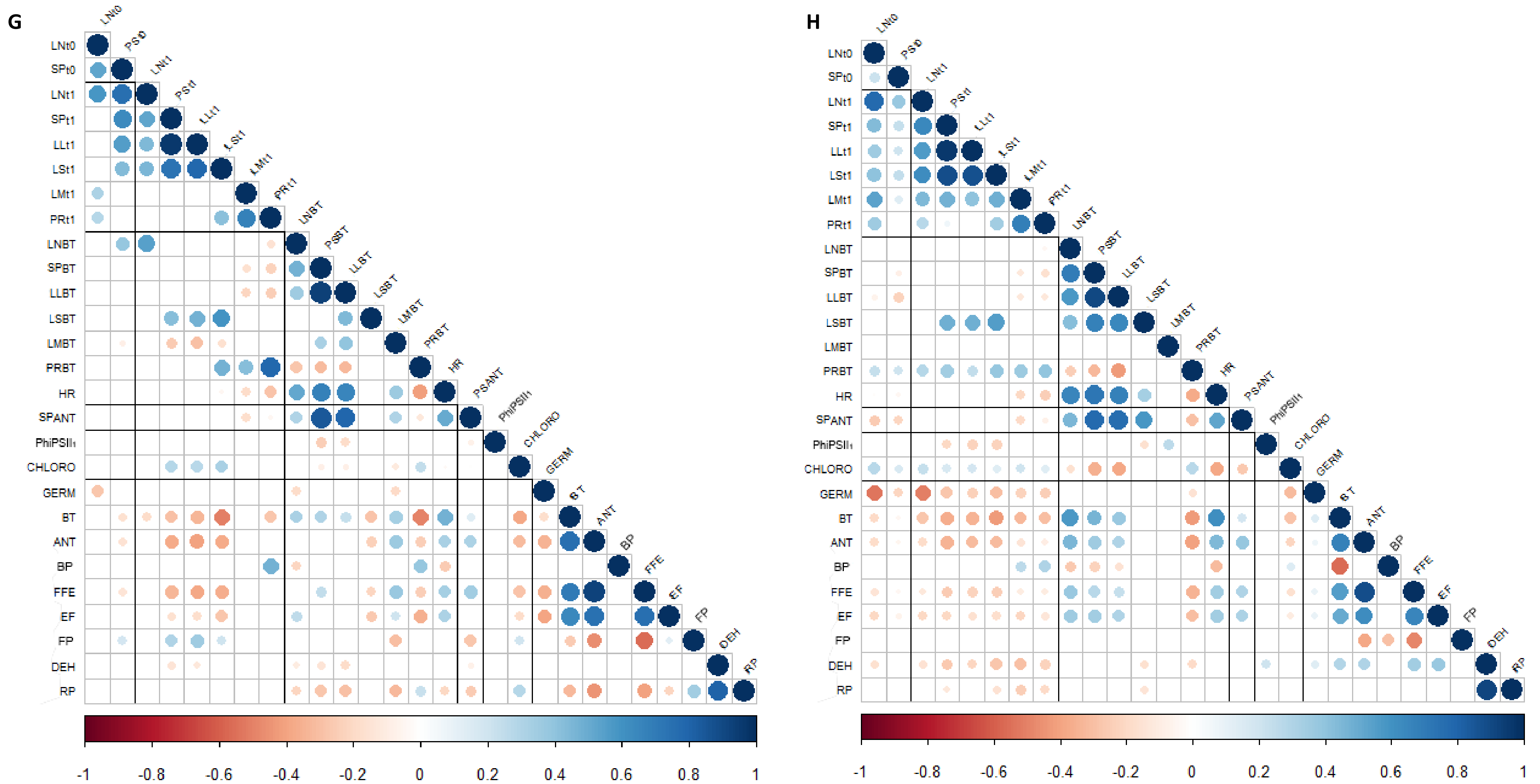


Figure 37 (continued): Correlation matrixes among traits in offspring of metallicolous population submitted to zinc polluted soil: (G) the offspring was submitted to non-polluted soil; (F) the offspring was submitted to zinc polluted soil. The gradient of colour represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PhiPSII_1$: average photosynthetic yield measured and t1; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: time interval between anthesis and dehiscence

Correlations matrixes were all different from each other (df = 729, P value $<1.10^{-16}$) [Figure 37]. As for parents, several correlation coefficients were stronger for non-metallicolous descendants cultivated in zinc polluted soil [Figures 37B and 37D]. Some correlations were specific from an edaphic group. In particular, significant positive correlations between morphological traits measured at t0 and t1 and the same traits measured at bolting and anthesis were detected only for non-metallicolous matrixes [Figures 37 A, 37B and 37D], except for the descendants cultivated in non-polluted soil with parents cultivated in zinc-polluted soil (NM-P-NP) [Figure 37C]. Significant positive correlations between chlorophyll content (CHLORO) and the vegetative traits were also detected in non-metallicolous descendants cultivated in zinc-polluted soil (NM-NP-P and NM-P-P) in comparison to non-metallicolous descendants cultivated in non-polluted soil (NM-P-NP and NM-NP-NP), but were weaker for all metallicolous descendants[Figure 37E to 37H]. Overall, we also observed more significant correlations between phenological traits and morphological traits measured at bolting in metallicolous descendants compared to non-metallicolous ones.

Finally, we analyzed the effect of parental edaphic group, parental condition and interaction between both factors on phenotypic plasticity [Table S6]. Overall, a significant effect of parental edaphic group was detected for almost traits, with non-metallicolous individuals having higher plasticity indexes than metallicolous ones (see for example Figures 38B and 38C). Only surface of the plant measured at anthesis showed a significant effect of interaction between edaphic group and parental condition [Figure 38A].

4.3.3. Heritability estimation

Most of the vegetative traits seemed to be heritable in at least one condition, but none phenological trait appeared heritable [Table 8]. The strongest regression coefficients were found for parents cultivated in their favorable conditions (non-metallicolous individuals cultivated in non-polluted soil and metallicolous individuals cultivated in polluted soil), in particular for vegetative traits measured at bolting time and for the regression between non-metallicolous parents cultivated in non-polluted soil and all their descendants.

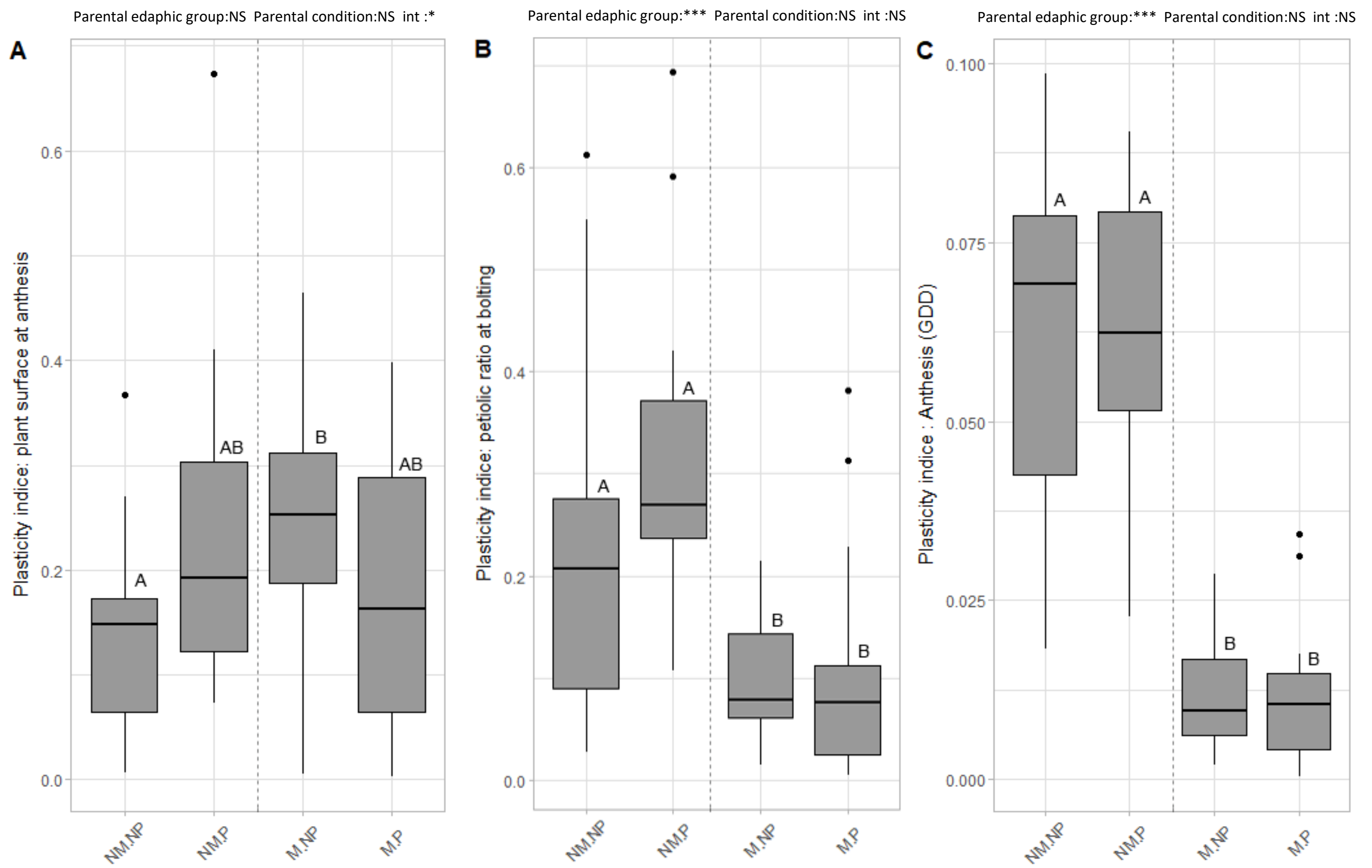


Figure 38: Results of permutational analysis of variance on plasticity indexes calculated on vegetative morphological trait (A and B) and a phenological trait (C): Effect of parental edaphic group (nonmetallicolous or metallicolous populations), parental condition (zinc polluted or non-polluted soil) and interaction between both factors. NM: nonmetallicolous parents; M: metallicolous parents; NP: non-polluted soil; P: polluted soil. * Character indicate significant difference between edaphic groups with p-value ≤ 0.05. Boxplots with the same letters are not significantly different at the 5% level.

Table 8: estimation of broad sense heritability (H²) by parent-offspring regression; NM: nonmetallicolous individuals; M: metallicolous individuals; NP: non-polluted soil; P: zinc polluted soil. LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured on 3 largest leaves at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured on 3 largest leaves at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured on 3 largest leaves at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured on 3 largest leaves at t1 and bolting. $PHI\text{-}PSII_1$: average photosynthetic yield measured t1 on 3 leaves representative of the chlorosis state of the plant; HR: Height of the rosette measured at bolting. CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting on 5 leaves representative of the chlorosis state of the plant. °: p-value ≤ 0.1, *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference, NE: estimation error ($h^2>1$ or $h^2<0$).

Parental population	NM in NP soil		NM in P soil		M in NP soil		M in P soil	
Offspring condition	NP soil	P soil	NP soil	P soil	NP soil	P soil	NP soil	P soil
Phenotypic trait	H²	H²	H²	H²	H²	H²	H²	H²
LN_{t0}	0.58 NS	0.56 NS	0.26 NS	0.38 NS	NE	NE	NE	NE
PS_{t0}	0.36 NS	0.30 NS	0.26 NS	0.46 NS	0,35 NS	0.4 NS	0.01 NS	0.31 NS
LN_{t1}	0.02 NS	0.14 NS	0.05 NS	0.29 NS	0.06 NS	0.02 NS	0.18 NS	0.03 NS
PS_{t1}	**0.34 ***	**0.32 ***	0.12 NS	0.26 NS	0.27 NS	0.23 NS	0.13 NS	0.26 NS
LL_{t1}	**0.46 ***	0.42 NS	NE	0.16 NS	**0.45 ***	**0.98 °**	0.16 NS	0.64 NS
PR_{t1}	NE	NE	NE	NE	NE	0.8 NS	NE	0.41 NS
LS_{t1}	**0.76 *****	**0.28 °**	NE	0.14 NS	0.28 NS	0.4 NS	0.39 NS	**0.5 ***
LM_{t1}	NE	0.14 NS	NE	NE	0.18 NS	0.62 NS	**0.84 ****	**0.9 ***
$PHI\text{-}PSII_1$	NE	0.50 NS	NE	NE	**2.1 ****	0.88 NS	NE	NE
LN_{BT}	0.23 NS	0.13 NS	**0.34 ***	**0.38 ***	0.25 NS	0.34 NS	0.1 NS	**0.2 °**
PS_{BT}	**0.36 ****	**0.46 ***	**0.33 ***	0.26 NS	0.22 NS	0.66 NS	**0.68 ****	**0.74 ****
LL_{BT}	**0.6 *****	**0.72 ****	0.35 NS	0.35 NS	0.56 NS	0.56 NS	0.19 NS	0.37 NS
PR_{BT}	0.05 NS	**0.16 °**	NE	NE	NE	NE	0.58 NS	0.82 NS
LS_{BT}	**0.52 *****	**0.56 ****	0.24 NS	0,28 NS	0.62 NS	**0.71 ***	**0.4 °**	**0.7 *****
LM_{BT}	NE	**0.54 ***	1.6 NS	0.22 NS	0.09 NS	**0.94 ***	0.75 NS	0.19 NS
HR	**0.6 ****	**0.5 ***	0.22 NS	0.2 NS	0.01 NS	0.08 NS	0.37 NS	0.4 NS
CHLORO	**0.25 ***	0.1 NS	0.06 NS	0.12 NS	NE	NE	0.66 NS	NE
PS_{ANT}	**0.26 ***	0.16 NS	**0.38 °**	0.26 NS	0.26 NS	0.89 NS	**0.89 ***	**0.83 ***

Table 8 (continued): estimation of broad sense heritability (H^2) by parent-offspring regression; NM: nonmetallicolous individuals; M: metallicolous individuals; NP: non-polluted soil; P: zinc polluted soil. GERM: time interval between sowing and germination converted on GDD (Growth degree days); BT: time interval between germination and bolting converted on GDD; ANT: time interval between germination and anthesis converted on GDD; BP: bolting period converted on GDD; FFE: time interval between germination and first fruit emergence on the main flower stem converted on GDD; EF: time interval between germination and end of flowering period converted on GDD; FP: flowering period converted on GDD; DEH: time interval between germination and dehiscence converted on GDD; RP: time interval between anthesis and dehiscence converted on GDD. °: p-value ≤ 0.1, *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference, NE: estimation error ($h^2>1$ or $h^2<0$).

Parental population	NM in NP soil		NM in P soil		M in NP soil		M in P soil	
Offspring condition	NP soil	P soil	NP soil	P soil	NP soil	P soil	NP soil	P soil
Phenotypic trait	H^2	H^2	H^2	H^2	H^2	H^2	H^2	H^2
GERM	NE	0.21 NS	NE	NE	0.92 NS	**NE**	0.93 NS	NE
BT	0.07 NS	0.23 NS	0.01 NS	NE	**0.74 °**	**0.96 °**	0.07 NS	0.14 NS
ANT	NE	NE	**0.38 °**	NE	0.56 NS	**0.8 °**	0.13 NS	NE
BP	0.38 NS	0.64 NS	NE	NE	0.43 NS	NE	NE	0.24 NS
FFE	NE	NE	0,69 (0.5) NS	NE	0.74 NS	1 NS	0.18 NS	0.06 NS
EF	NE	NE	0.07 (0.26) NS	0.49 NS	0.65 NS	0.87 NS	0.28 NS	NE
FP	0.03 NS	0.06 NS	**0.48 °**	0.06 NS	NE	NE	0.31 NS	NE
DEH	NE	NE	NE	0.08 NS	2.75 NS	NE	0.01 NS	0.87 NS
RP	0.94 NS	NE	NE	1 NS	NE	**0.72 °**	0.35 NS	NE

4.4. Key results and short discussion

4.4.1. Differences between both edaphic groups

Results obtained on the parental generation showed a strong divergence of phenotypes expressed in both edaphic groups in response to different environmental conditions. These differences concerned all categories of functional traits except pigment concentrations measured after methanol extraction or some reproductive traits. Thus, we observed that metallicolous individuals expressed higher phenotypic values for vegetative traits, with more leaves, a larger plant surface or a higher rosette height. Similarly, we observed a longer vegetative period in metallicolous individuals.

The level of phenotypic expression for reproductive traits also differed depending on the condition studied. In uncontaminated soil, performance appeared to be higher in nonmetallicolous individuals with a higher expected number of descendants. In contaminated soil, the trend was reversed and performances were higher in metallicolous populations.

Furthermore, we observed that phenotypic plasticity of most traits was more important in the nonmetallicolous population. Metallicolous individuals appeared to maintain the same phenotypic levels under both conditions (canalization) while nonmetallicolous individuals modified their phenotype in the presence of zinc, with lower phenotypic levels in zinc contaminated soil.

Similar differences were already observed among other populations [Jiménez-Ambriz *et al.*, 2007; Dechamps *et al.*, 2008] and allowed generalization of the conclusions about metal tolerance differences between edaphic groups of *Noccaea caerulescens* (Meerts & Van Isacker, 1997; Escarré *et al.*, 2000). Indeed, we showed that nonmetallicolous individuals were more sensitive to zinc concentration in soil by affecting, essentially, the production of descendants.

In addition, more global conclusions cannot be made because we only observed a phenotypic variation between 2 populations from different edaphic groups. The "edaphic group" effect was confounded with other genetic (specific to the populations studied) and environmental factors that can influence the phenotype of individuals.

4.4.2. Estimation of broad sense heritability

Few significant heritabilities were observed for vegetative traits measured at t0 and t1 or for phenological traits. Conversely, many vegetative traits measured at bolting or anthesis showed variable heritability depending on conditions and edaphic group.

These heritabilities values were significant and strong for nonmetallicololous parents cultivated on uncontaminated soil, especially when the offspring was cultivated on contaminated soil. This encouraging result suggests that a genetic response of vegetative traits should be possible in our experimental evolution approach. In comparison, metallicolous populations showed fewer significant heritability values.

Supplemental material

Table S3 : Results of permutational analysis of variance: Effect of edaphic group, contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured on 3 largest leaves at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured on 3 largest leaves at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured on 3 largest leaves at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured on 3 largest leaves at t1 and bolting. PHI-$PSII_0$, PHI-$PSII_1$: average photosynthetic yield measured at t0 and t1 on 3 leaves representative of the chlorosis state of the plant; HR: Height of the rosette measured at bolting. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant.

	Edaphic group (df=1)				Contamination (df=1)				Edaphic group * Contamination interaction (df=1)			
Variable	**MS**	**F**	**P value**	**P value (Perm)**	**MS**	**F**	**P value**	**P value (Perm)**	**MS**	**F**	**P value**	**P value (Perm)**
LN_{t0}	0.45	0.12	0.72	0.69	6.05	1.67	0.2	0.2	14.45	4	**0.049**	**0.047**
SP_{t0}	967.3	3.41	0.07	0.07	780.7	2.75	0.1	0.09	193.3	0.68	0.41	0.42
PHI-$PSII_0$	33.8	0.07	0.79	0.78	1150.1	2.44	0.12	0.12	0.56	0.001	0.97	0.97
LN_{t1}	1445	8.83	**0.004**	**0.005**	20	0.12	0.73	0.74	174.05	1.06	0.31	0.31
SP_{t1}	39915	41.34	**$1.02 .10^{-08}$**	**0.001**	189.36	0.2	0.66	0.66	232.2	0.24	0.62	0.62
LL_{t1}	32	34.2	**$1.2 .10^{-07}$**	**0.001**	0.51	0.54	0.46	0.46	0.000055	0.000059	0.99	0.99
PR_{t1}	10.6	31.8	**$2.7 .10^{-07}$**	**0.001**	0.58	1.76	0.19	0.21	0.76	2.29	0.13	0.14
LS_{t1}	7.74	4.15	**0.04**	**0.05**	0.02	0.01	0.91	0.9	3.44	1.85	0.18	0.16
LM_{t1}	0.4	11.3	**0.001**	**0.002**	0.1	2.86	0.09	0.08	0.01	0.33	0.56	0.55
PHI-$PSII_1$	0.02	0.000078	0.99	0.99	399	1.41	0.24	0.22	364.1	1.29	0.26	0.28
LN_{BT}	3799.4	161.5	**0**	**0.001**	18.4	0.78	0.38	0.4	0.72	0.03	0.86	0.85
SP_{BT}	4739	147.1	**0**	**0.001**	11.9	0.37	0.55	0.54	15.8	0.49	0.49	0.49
LL_{BT}	1.85	134.9	**0**	**0.001**	0.0026	0.19	0.67	0.68	0.0012	0.08	0.77	0.77
PR_{BT}	1.83	37.4	**0**	**0.001**	0.07	1.4	0.24	0.26	0.08	1.58	0.21	0.24
LS_{BT}	0.14	4.23	**0.043**	**0.045**	0	0.0002	0.99	0.99	0.0003	0.009	0.93	0.94
LM_{BT}	0.01	13.5	**0.0005**	**0.003**	0.0004	0.38	0.54	0.53	0.0005	0.51	0.47	0.47
HR	3.6	99.5	**0**	**0.001**	0.012	0.33	0.57	0.59	0.02	0.6	0.44	0.46
SP_{ANT}	4799	154.6	**0**	**0.001**	8.2	0.26	0.61	0.62	36.9	1.19	0.28	0.27

Table S3 (continued): Results of permutational analysis of variance: Effect of edaphic group, contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. GERM: time interval between sowing and germination converted on GDD (Growth degree days); BT: time interval between germination and bolting converted on GDD; ANT: time interval between germination and anthesis converted on GDD; BP: Bolting period converted on GDD; FFE: time interval between germination and first fruit emergence on the main flower stem converted on GDD; EF: time interval between germination and end of flowering period converted on GDD; FP: flowering period converted on GDD; DEH: time interval between germination and dehiscence converted on GDD; RP: time interval between anthesis and dehiscence converted on GDD; SLA: average specific leaf area calculated at bolting on 3 leaves representative of the chlorosis state of the plant; LDMC: average leaf dry matter content calculated at bolting on 3 leaves representative of the chlorosis state of the plant; LT : average leaf thickness calculated at bolting on 3 leaves representative of the chlorosis state of the plant; ChlA, ChlB, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting on 5 leaves representative of the chlorosis state of the plant. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant.

	Edaphic group (df=1)				Contamination (df=1)				Edaphic group * Contamination interaction (df=1)			
Variable	**MS**	**F**	**P value**	**P value (Perm)**	**MS**	**F**	**P value**	**P value (Perm)**	**MS**	**F**	**P value**	**P value (Perm)**
GERM	1170.6	11.3	**0.001**	**0.001**	328.1	3.17	0.08	0.08	512.6	4.9	**0.03**	**0.03**
BT	55020.5	82.9	**0**	**0.001**	12793.1	19.28	**0**	**0.001**	6738.4	10.15	**0.002**	**0.004**
ANT	19217.6	30.3	**0**	**0.001**	16495.8	27	**0**	**0.001**	3255.6	5.13	**0.03**	**0.03**
BP	8150.16	25.6	**0**	**0.001**	118.1	0.37	0.54	0.53	395.9	1.24	0.27	0.27
FFE	20548	45.7	**0**	**0.001**	10683.6	23.74	**0**	**0.001**	5009.7	11.13	**0.001**	**0.002**
EF	3217.9	6.27	**0.015**	**0.014**	13661.8	26.6	**0**	**0.001**	12904.6	25.13	**0**	**0.001**
FP	4964.4	15.6	**0.0002**	**0.001**	117	0.37	0.55	0.56	2002.9	6.31	**0.01**	**0.015**
DEH	1099.7	8.76	**0.004**	**0.003**	338.6	2.7	0.1	0.13	483.1	3.85	**0.05**	**0.05**
RP	11492.1	19.7	**0**	**0.001**	11763.7	20.16	**0**	**0.001**	1119.7	1.92	0.17	0.17
SLA	1.56	8.12	**0.006**	**0.005**	0.7	3.67	0.06	0.06	1.7	8.9	**0.004**	**0.004**
LDMC	0.0013	0	1	1	72.9	1.36	0.25	0.29	0.007	0.0001	0.99	1
LT	0	46.5	**0**	**0.001**	0	1.8	0.18	0.18	0	8.99	**0.004**	**0.002**
CHLORO	38.1	38.86	**0**	**0.001**	17.6	17.9	**0.0001**	**0.001**	12.9	13.1	**0.0005**	**0.001**
ChlA	0.07	1.85	0.18	0.19	0.003	0.07	0.79	0.76	0	0.0002	0.99	0.98
ChlB	0.0005	0.006	0.94	0.94	0.03	0.35	0.55	0.54	0.0017	0.02	0.89	0.88
Caro	0.001	0.011	0.92	0.92	0.0001	0.001	0.97	0.97	0.005	0.06	0.81	0.82
Antho	0.13	0.35	0.55	0.54	1.77	4.61	**0.03**	**0.05**	0.05	0.12	0.73	0.72
Flavo	0.003	4.56	**0.04**	**0.04**	0.003	4.56	**0.04**	**0.04**	0.0015	2.03	0.16	0.14
Pheno	0.0005	0.02	0.88	0.91	0.02	0.84	0.36	0.37	0.006	0.25	0.62	0.66
Tan	0.0005	0.12	0.73	0.73	0.02	5.07	**0.03**	**0.02**	0.0004	0.1	0.75	0.76

Table S3 (continued): Results of permutational analysis of variance: Effect of edaphic group, contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. NR: number of racemes; NP: number of panicles; NRAM: number of ramifications; NFS: number of flower stems; PPR: proportion of racemes; PPP: proportion of panicles; RPR: ratio between number of panicles and racemes; maxLFS: height of the largest flower stem; HFF: distance between the beginning of the flower stem and the first silique on the main flower stem; NS: number of silique produced by whole plant; NAS: number of aborted siliques produced by whole plant; NNAS: number of non-aborted siliques produced by whole plant; PNAS: proportion of aborted siliques on the whole plant; PNNAS: proportion of non-aborted siliques on the whole plant; RNS: ratio between the number of non-aborted and the number of aborted siliques on the whole plant; SL: silique length calculated on the whole plant; SS: estimated seed set produced by whole plant; SR: survival rate after 5 weeks after sowing; ND: estimated number of descendants (SS*SR). SW: seed weight measured on 100 seeds after drying (48h at 80°C). df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant.

	Edaphic group (df=1)				Contamination (df=1)				Edaphic group * Contamination interaction (df=1)			
Variable	**MS**	**F**	**P value**	**P value (Perm)**	**MS**	**F**	**P value**	**P value (Perm)**	**MS**	**F**	**P value**	**P value (Perm)**
NR	4.9	26.02	**0**	**0.001**	0.0085	0.004	0.83	0.85	0.004	0.02	0.89	0.9
NP	1.44	14.3	**0.0003**	**0.002**	0.01	0.13	0.72	0.71	0.004	0.04	0.84	0.86
NRAM	0.69	0.51	0.48	0.48	0.2	0.15	0.7	0.7	0.14	0.1	0.75	0.76
NFS	0.33	1.02	0.31	0.31	0.04	0.12	0.73	0.74	0.53	1.62	0.21	0.2
PPR	0.033	37.4	**0**	**0.001**	0	0.03	0.86	0.86	0	0.004	0.95	0.95
PPP	0.03	37.4	**0**	**0.001**	0	0.03	0.86	0.84	0	0.004	0.95	0.94
RPR	2.89	14.45	**0.0004**	**0.001**	0.01	0.05	0.82	0.8	0.08	0.4	0.53	0.53
maxLFS	0.002	0.007	0.93	0.94	1	3.34	0.07	0.063	0.11	0.35	0.55	0.57
HFF	0.36	0.34	0.56	0.56	6.14	5.95	**0.02**	**0.02**	8.7	8.5	**0.005**	**0.012**
NS	13.49	0.02	0.89	0.9	2401.7	3.16	0.08	0.07	8919.3	11.7	**0.001**	**0.003**
NAS	8.68	0.23	0.63	0.63	19.9	0.54	0.47	0.45	23.6	0.63	0.43	0.41
NNAS	161.5	0.3	0.58	0.57	3805.1	7.23	**0.009**	**0.02**	6019.9	11.44	**0.001**	**0.001**
PNAS	0.001	4.52	**0.04**	**0.04**	0.007	24.98	**0**	**0.001**	0.005	17.87	**0.0001**	**0.001**
PNNAS	0.001	4.52	**0.04**	**0.03**	0.007	24.98	**0**	**0.001**	0.005	17.87	**0.0001**	**0.001**
RNS	0.03	0.18	0.67	0.7	2.16	14.3	**0.0003**	**0.001**	2.02	13.4	**0.0005**	**0.001**
SL	0.12	15.27	**0.0002**	**0.001**	0.12	16.34	**0.0001**	**0.001**	0.12	16	**0.0002**	**0.001**
SS	18934.9	1.93	0.17	0.16	139242.7	14.2	**0.0003**	**0.003**	176882.8	18	**0.0001**	**0.001**
SR	0.0001	0.214	0.64	0.67	0	0.17	0.68	0.68	0	0.03	0.86	0.87
ND	20697.6	2.6	0.11	0.11	90831.9	11.4	**0.0012**	**0.002**	175188.5	21.98	**0**	**0.001**
SW	0	94	**0**	**0.001**	0	0.41	0.53	0.51	0	0.34	0.56	0.58

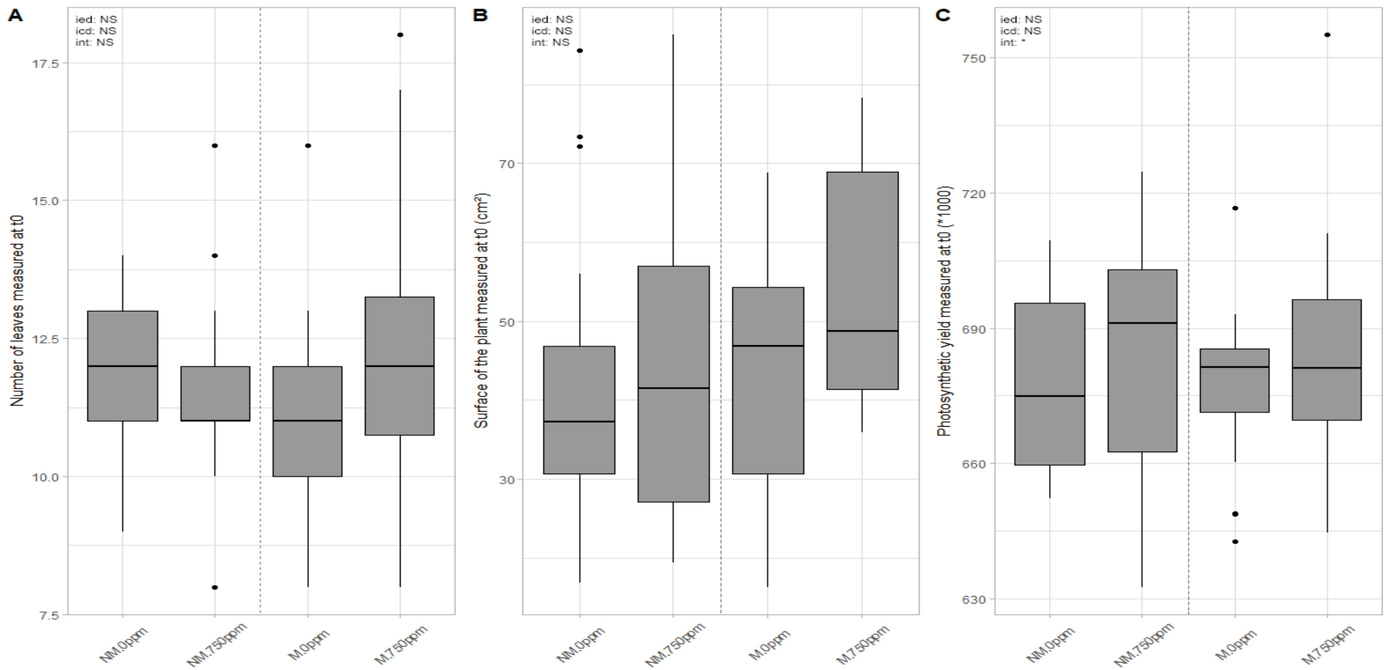


Figure S3: results of permutational ANOVA and pairwise test on vegetative morphological traits (A, and B) and photosynthetic yield (C) measured at t0. Dotted line represent separation between both edaphic group. NM: nonmetallicolous populations; M: metallicolous population; 0ppm: non polluted soil; 750ppm: zinc polluted soil. “ied” (inter edaphic group difference) represent the result for the edaphic group factor, “icd” (inter contamination level difference) represent the result for the contamination factor, “int” (interaction) represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Boxplots with the same letters are not significantly different at the 5% level.

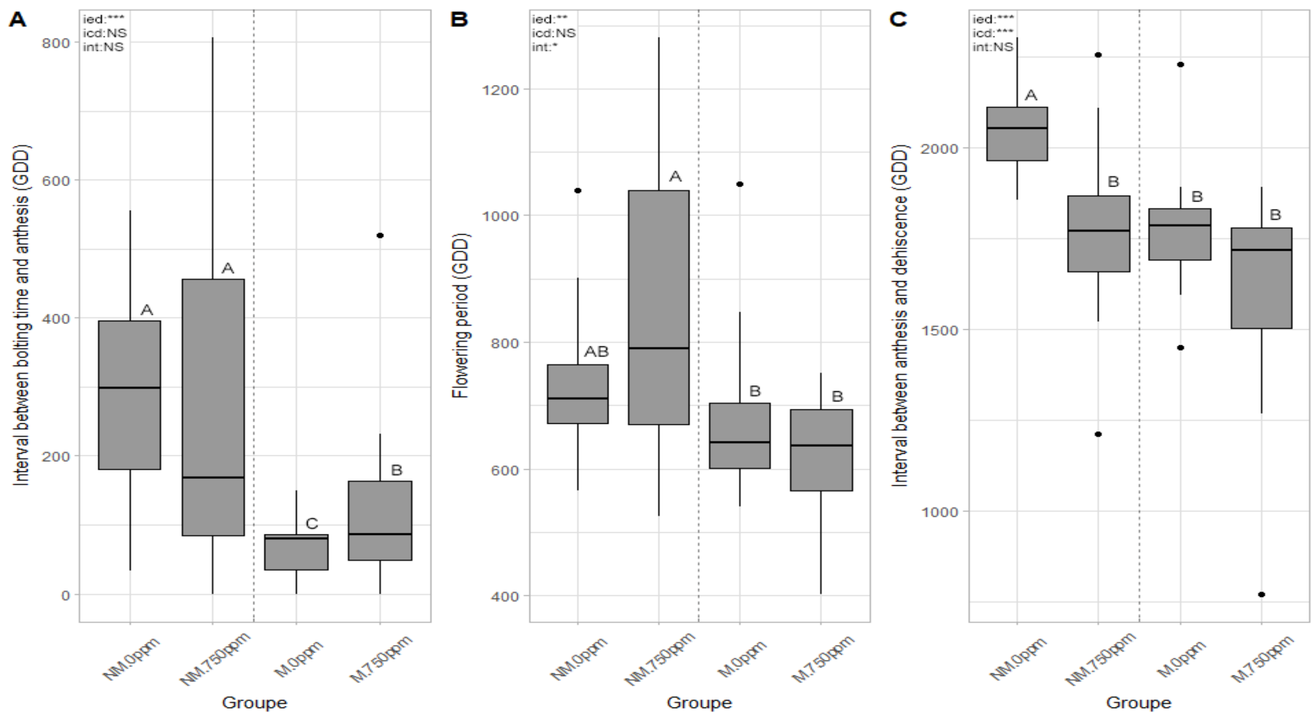


Figure S4 : results of permutational ANOVA and pairwise test on phenological traits. Dotted line represent separation between both edaphic groups. NM: nonmetallicolous populations; M: metallicolous population; 0ppm: non polluted soil; 750ppm: zinc polluted soil. “ied” (inter edaphic group difference) represent the result for the edaphic group factor, “icd” (inter contamination level difference) represent the result for the contamination factor, “int” (interaction) represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001, NS: no significant difference. Boxplots with the same letters are not significantly different at the 5% level.

Table S4: Results of Wilcoxon-Mann-Whitney's comparison tests on plasticity indexes between both nonmetallicolous and metallicolous edaphic group test. rdpi: relative distance plasticity index, N_{NM}: sample size of nonmetallicolous population; N_M: sample size of metallicolous population. U: Wilcoxon-Mann-Whitney's statistic; P: P-value. When significant differences were observed, we indicated the direction of the difference: $\overline{NM}$ was the mean in nonmetallicolous population $\overline{M}$ was the mean in nonmetallicolous population. PLN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. $PHI\text{-}PSII_0$, $PHI\text{-}PSII_1$: average photosynthetic yield measured at t0 and t1; GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: Bolting period; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: time interval between germination and end of flowering period; FP: flowering period; DEH: time interval between germination and dehiscence; RP: time interval between anthesis and dehiscence. SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness.

rdpi	N_{NM}	N_M	U	P	direction of the difference		
LN_{t0}	20	20	264	0,08			
SP_{t0}	20	20	244	0,24			
$PHI\text{-}PSII_0$	20	20	196	0,925			
LN_{t1}	20	20	216	0,62			
SP_{t1}	20	20	193	0,86			
LL_{t1}	20	20	238	0,31			
PR_{t1}	20	20	104	0,009	$\overline{NM}$	>	$\overline{M}$
LS_{t1}	20	20	188	0,76			
LM_{t1}	20	20	174	0,49			
$PHI\text{-}PSII_0$	20	20	186	0,72			
LN_{BT}	20	18	224	0,2			
SP_{BT}	20	18	188	0,83			
LL_{BT}	20	18	190	0,78			
PR_{BT}	20	18	93	0,01	$\overline{NM}$	>	$\overline{M}$
LS_{BT}	20	18	169	0,76			
LM_{BT}	20	18	185	0,9			
HR	20	18	187	0,85			
SP_{ANT}	20	16	169	0,79			
GERM	20	20	127,5	0,048	$\overline{NM}$	>	$\overline{M}$
BT	20	18	62	0,0003	$\overline{NM}$	>	$\overline{M}$
ANT	20	16	72,5	0,006	$\overline{NM}$	>	$\overline{M}$
BP	20	16	149,5	0,75			
FFE	20	16	87	0,02	$\overline{NM}$	>	$\overline{M}$
EF	17	15	35	0,0005	$\overline{NM}$	>	$\overline{M}$
FP	17	15	60	0,01	$\overline{NM}$	>	$\overline{M}$
DEH	18	16	84,5	0,04	$\overline{NM}$	>	$\overline{M}$
RP	18	16	96	0,1			
SLA	20	18	240	0,08			
LDMC	20	18	269	0,0085	$\overline{NM}$	<	$\overline{M}$
LT	20	18	202	0,534			

Table S4 (continued): Results of Wilcoxon-Mann-Whitney's comparison tests on plasticity indexes between both nonmetallicolous and metallicolous edaphic group test. rdpi: relative distance plasticity index, N_{NM}: sample size of nonmetallicolous population; N_M: sample size of metallicolous population. U: Wilcoxon-Mann-Whitney's statistic; P: P-value. When significant differences were observed, we indicated the direction of the difference: $\overline{NM}$ was the mean in nonmetallicolous population $\bar{M}$ was the mean in nonmetallicolous population. ChlA, ChlB, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting; NR: number of racemes; NP: number of panicles; NRAM: number of ramifications; NFS: number of flower stems; PPR: proportion of racemes; PPP: proportion of panicles; RPR: ratio between number of panicles and racemes; maxLFS: height of the largest flower stem; HFF: distance between the beginning of the flower stem and the first silique on the main flower stem; NS: number of silique produced by whole plant; NAS: number of aborted siliques produced by whole plant; NNAS: number of non-aborted siliques produced by whole plant; PNAS: proportion of aborted siliques on the whole plant; PNNAS: proportion of non-aborted siliques on the whole plant; RNS: ratio between the number of non-aborted and the number of aborted siliques on the whole plant; SL: silique length calculated on the whole plant; SS: estimated seed set produced by whole plant; SR: survival rate after 5 weeks after sowing; ND: estimated number of descendants (SS*SR). SW: seed weight measured on 100 seeds after drying (48h at 80°C)

rdpi	N_{NM}	N_M	U	P	direction of the difference		
CHLORO	20	18	62	0,0003	$\overline{NM}$	>	$\bar{M}$
ChlA	20	18	80	0,003	$\overline{NM}$	>	$\bar{M}$
ChlB	20	18	121	0,08	$\overline{NM}$	>	$\bar{M}$
Car	20	18	112	0,05	$\overline{NM}$	>	$\bar{M}$
Antho	20	18	113	0,05	$\overline{NM}$	>	$\bar{M}$
Flavo	20	18	168	0,74			
Pheno	20	18	120	0,08			
Tan	20	18	141	0,26			
NR	18	16	103,5	0,167			
NP	18	16	148,5	0,4			
NRAM	18	16	170,5	0,37			
NFS	18	16	205,5	0,47			
PPR	18	16	38	0,0003	$\overline{NM}$	>	$\bar{M}$
PPP	18	16	152	0,33			
RPR	18	16	36	0,06			
maxLFS	18	16	107	0,21			
HFF	18	16	98	0,12			
NS	18	16	165,5	0,67			
NSA	18	16	159	0,62			
NSNA	18	16	94	0,09			
PNSA	18	16	31	0,00003	$\overline{NM}$	>	$\bar{M}$
PNSNA	18	16	28	0,000016	$\overline{NM}$	>	$\bar{M}$
RNS	18	16	33	0,000044	$\overline{NM}$	>	$\bar{M}$
SL	18	16	94	0,09			
SS	18	16	83	0,036	$\overline{NM}$	>	$\bar{M}$
SR	11	14	58	0,31			
ND	20	19	131,5	0,09			
SW	7	12	47	0,71			

Table S5: Results of permutational analysis of variance: Effect of origin of parents (nonmetallicolous populations in zinc polluted soil or non-polluted soil, and metallicolous populations in zinc polluted soil or non-polluted soil), contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured on 3 largest leaves at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured on 3 largest leaves at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured on 3 largest leaves at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured on 3 largest leaves at t1 and bolting. $PHI\text{-}PSII_1$: average photosynthetic yield measured t1 on 3 leaves representative of the chlorosis state of the plant; HR: Height of the rosette measured at bolting. CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting on 5 leaves representative of the chlorosis state of the plant. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant at 5% level

	Origin of parents					Contamination					Origin of parents * Contamination interaction				
Variable	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**
LN_{t0}	3	0,11	7,9	**0**	**0,001**	1	0,02	3,9	0,05	0,06	3	0,014	0,96	0,41	0,4
SP_{t0}	3	0,34	0,22	0,88	0,9	1	0,06	0,11	0,74	0,77	3	1,73	1,11	0,34	0,33
LN_{t1}	3	0,12	0,78	0,51	0,48	1	0,24	4,55	**0,03**	**0,03**	3	0,84	5,3	**0,0014**	**0,003**
SP_{t1}	3	50,47	42,11	**0**	**0,001**	1	0,07	0,19	0,67	0,67	3	4,38	3,65	**0,012**	**0,014**
LL_{t1}	3	0,14	35,11	**0**	**0,001**	1	0,0002	0,17	0,68	0,68	3	0,01	3,17	**0,024**	**0,019**
PR_{t1}	3	0,39	44,5	**0**	**0,001**	1	0,15	50,49	**0**	**0,001**	3	0,12	13,9	**0**	**0,001**
LS_{t1}	3	0,005	1,11	0,34	0,31	1	0,009	6,82	**0,009**	**0,009**	3	0,04	9,67	**0**	**0,001**
LM_{t1}	3	0,004	8,84	**0**	**0,001**	1	0,005	30,44	**0**	**0,001**	3	0,0044	9,12	**0**	**0,001**
$PHI\text{-}PSII_1$	3	27,2	2,64	**0,05**	**0,043**	1	7,78	2,26	0,13	0,14	3	16,1	1,56	0,2	0,19
LN_{BT}	3	233,43	199	**0**	**0,001**	1	1,6	4,1	**0,04**	**0,04**	3	7,88	6,72	**0,0002**	**0,001**
SP_{BT}	3	2237,45	222,76	**0**	**0,001**	1	81,93	24,47	**0**	**0,001**	3	154	15,33	**0**	**0,001**
LL_{BT}	3	2,29	278,2	**0**	**0,001**	1	0,027	10	**0,0016**	**0,002**	3	0,13	15,68	**0**	**0,001**
PR_{BT}	3	1,15	98,5	**0**	**0,001**	1	0,15	38	**0**	**0,001**	3	0,13	11,1	**0**	**0,001**
LS_{BT}	3	0,35	54,67	**0**	**0,001**	1	0,0013	0,6	0,44	0,45	3	0,13	20,37	**0**	**0,001**
LM_{BT}	3	0,0046	5,36	**0,0012**	**0,002**	1	0,0055	19,37	**0**	**0,001**	3	0,011	13	**0**	**0,001**
HR	3	2,23	355,85	**0**	**0,001**	1	0,008	3,88	**0,049**	**0,042**	3	0,1	16,4	**0**	**0,001**
CHLORO	3	0,06	0,2	0,9	0,89	1	9,28	94,12	**0**	**0,001**	3	5,9	20,04	**0**	**0,001**
SP_{ANT}	3	3499,5	228,1	**0**	**0,001**	1	217,85	42,6	**0**	**0,001**	3	335,9	21,9	**0**	**0,001**

Table S5 (continued): Results of permutational analysis of variance: Effect of origin of parents (nonmetallicolous populations in zinc polluted soil or non-polluted soil, and metallicolous populations in zinc polluted soil or non-polluted soil), contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. GERM: time interval between sowing and germination converted on GDD (Growth degree days); BT: time interval between germination and bolting converted on GDD; ANT: time interval between germination and anthesis converted on GDD; BP: bolting period converted on GDD; FFE: time interval between germination and first fruit emergence on the main flower stem converted on GDD; EF: time interval between germination and end of flowering period converted on GDD; FP: flowering period converted on GDD; DEH: time interval between germination and dehiscence converted on GDD; RP: time interval between anthesis and dehiscence converted on GDD. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters significant at 5% level

	Origin of parents					**Contamination**					**Origin of parents * Contamination interaction**				
Variable	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**
GERM	3	13,8	0,48	0,7	0,72	1	1,38	0,14	0,7	0,7	3	4,04	0,14	0,93	0,94
BT	3	35487,8	374,9	**0**	**0,001**	1	450,6	14,28	**0,0002**	**0,001**	3	357,1	3,77	**0,011**	**0,014**
ANT	3	6166,3	45,6	**0**	**0,001**	1	5486,2	121,7	**0**	**0,001**	3	5195,7	38,4	**0**	**0,001**
BP	3	12728,5	112,65	**0**	**0,001**	1	2713,6	72	**0**	**0,001**	3	2923,45	25,87	**0**	**0,001**
FFE	3	6211,7	46,5	**0**	**0,001**	1	5516,8	123,9	**0**	**0,001**	3	5302,4	39,7	**0**	**0,001**
EF	3	9221,9	82,23	**0**	**0,001**	1	2337,5	62,53	**0**	**0,001**	3	1662,25	14,82	**0**	**0,001**
FP	3	254,5	3,67	**0,012**	**0,019**	1	700,8	30,3	**0**	**0,001**	3	1029,4	14,84	**0**	**0,001**
DEH	3	1214	3,02	**0,03**	**0,03**	1	61,7	0,46	0,5	0,5	3	628,3	1,56	0,2	0,2
RP	3	9596,8	24,5	**0**	**0,001**	1	7174,6	55	**0**	**0,001**	3	9596,8	24,54	**0**	**0,001**

Table S6: Results of permutational analysis of variance on plasticity indexes: Effect of parental edaphic group (nonmetallicolous or metallicolous populations), parental condition (zinc polluted or non-polluted soil) and interaction between both factors. Each test was realized with 1000 permutations. LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (8 weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured on 3 largest leaves at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured on 3 largest leaves at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured on 3 largest leaves at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured on 3 largest leaves at t1 and bolting. $PHI\text{-}PSII_1$: average photosynthetic yield measured t1 on 3 leaves representative of the chlorosis state of the plant; HR: Height of the rosette measured at bolting. CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting on 5 leaves representative of the chlorosis state of the plant. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant at 5% level.

	Parental edaphic group					Parental condition					Parental edaphic group * Parental condition interaction				
Variable	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**
LN_{t0}	1	0	0,51	0,48	0,48	1	0	0,14	0,71	0,71	1	0	0,04	0,84	0,84
SP_{t0}	1	0	0,08	0,78	0,76	1	0	0,04	0,85	0,86	1	0	0,04	0,85	0,86
LN_{t1}	1	0,0002	3,21	0,08	0,1	1	0	0,85	0,36	0,37	1	0,0001	1,49	0,23	0,21
SP_{t1}	1	0	0,24	0,62	0,64	1	0	0,25	0,62	0,6	1	0	0,2	0,66	0,67
LL_{t1}	1	0	0,45	0,5	0,51	1	0	19	0,66	0,67	1	0	0,3	0,59	0,6
PR_{t1}	1	0,01	35,4	**0**	**0,001**	1	0,0001	0,26	0,61	0,6	1	0	0,0024	0,96	0,95
LS_{t1}	1	0,0009	5,08	**0,03**	**0,03**	1	0,0003	1,52	0,22	0,23	1	0	0,03	0,87	0,85
LM_{t1}	1	0,0011	21,11	**0**	**0,001**	1	0,0001	2,53	0,12	0,12	1	0,0001	2,63	0,11	0,11
$PHI\text{-}PSII_1$	1	0	0,09	0,76	0,78	1	0	0,14	0,71	0,72	1	0	0,24	0,63	0,65
LN_{BT}	1	0	0,45	0,51	0,5	1	0	0,16	0,69	0,68	1	0,0001	0,86	0,36	0,35
SP_{BT}	1	0,0002	1	0,32	0,32	1	0,0001	0,31	0,58	0,55	1	0,0003	1,22	0,27	0,3
LL_{BT}	1	0	0,56	0,56	0,45	1	0	0,7	0,41	0,44	1	0,0002	3,46	0,07	0,07
PR_{BT}	1	0,0072	24,25	**0**	**0,001**	1	0,0006	2,18	0,14	0,14	1	0,0007	2,22	0,14	0,17
LS_{BT}	1	0,0005	2,78	0,1	0,11	1	0,0003	1,6	0,21	0,21	1	0,0003	1,34	0,25	0,24
LM_{BT}	1	0,0007	11,3	**0,0014**	**0,002**	1	0,0002	3	0,03	0,09	1	0,0005	7,12	0,008	0,003
HR	1	0,0003	3,34	0,07	0,08	1	0	0,31	0,58	0,58	1	0	0,03	0,86	0,87
CHLORO	1	0,01	36,34	**0**	**0,001**	1	0	0,04	0,85	0,84	1	0	0,0014	0,97	0,98
SP_{ANT}	1	0,0002	0,81	0,37	0,37	1	0,0001	0,37	0,54	0,56	1	0,0017	6,39	**0,01**	**0,01**

Table S6 (continued): Results of permutational analysis of variance on plasticity indexes: Effect of parental edaphic group (nonmetallicolous or metallicolous populations), parental condition (zinc polluted or non-polluted soil) and interaction between both factors. Each test was realized with 1000 permutations. GERM: time interval between sowing and germination converted on GDD (Growth degree days); BT: time interval between germination and bolting converted on GDD; ANT: time interval between germination and anthesis converted on GDD; BP: Bolting period converted on GDD; FFE: time interval between germination and first fruit emergence on the main flower stem converted on GDD; EF: time interval between germination and end of flowering period converted on GDD; FP: flowering period converted on GDD; DEH: time interval between germination and dehiscence converted on GDD; RP: time interval between anthesis and dehiscence converted on GDD. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters significant at 5% level.

	Parental edaphic group					Parental condition					Parental edaphic group * Parental condition interaction				
Variable	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**
GERM	1	0	0,26	0,61	0,59	1	0,0001	3,09	0,08	0,08	1	0	0,03	0,87	0,87
BT	1	0	6,26	**0,01**	**0,015**	1	0	0,4	0,53	0,51	1	0	0,12	0,73	0,74
ANT	1	0,0006	124,6	**0**	**0,001**	1	0	0,002	0,97	0,96	1	0	0,01	0,92	0,9
BP	1	0,0062	28,78	**0**	**0,001**	1	0	0,07	0,79	0,77	1	0	0,03	0,86	0,87
FFE	1	0,0005	100,76	**0**	**0,001**	1	0	0,38	0,54	0,54	1	0	0,02	0,89	0,88
EF	1	0,0001	40,57	**0**	**0,001**	1	0	0,16	0,69	0,68	1	0	0,16	0,69	0,7
FP	1	0,003	12,5	**0,0008**	**0,002**	1	0,0001	0,35	0,55	0,55	1	0	0,0002	0,99	0,99
DEH	1	0	1,64	0,2	0,22	1	0	0,45	0,5	0,53	1	0	0,53	0,47	0,45
RP	1	0,0015	27,3	**0**	**0,001**	1	0	0,1	0,75	0,74	1	0	0,01	0,92	0,92

CHAPTER 4

5. Influence of zinc polluted soil on the evolution of phenotypic traits: Predictions based on descendant production during a two-generation selection experiment

5.1. Brief introduction

In previous chapters we developed a set-up to perform experimental evolution in *Noccaea caerulescens*. The previous experiments showed that diversity in the response to zinc exists within natural nonmetallicolous and metallicolous populations. They also revealed that this diversity, expressed through differences in performance between individuals, could be sufficient for selection to operate. In addition, we opted for a zinc concentration of 750 $mg.kg^{-1}$, which would represent an optimal intensity of selective pressure, because it allowed us to highlight this diversity but not cause the extinction of the population.

In this chapter, we initiated the experimental evolution project and monitored the experimental populations over two generations. In addition, we estimated the selection differentials and selection gradients on several functional traits in each experimental populations in order to test the effect of zinc on the phenotype evolution.

5.2. Material and methods

5.2.1. Origin of plants, design of experimental populations and monitoring of individuals

Several *Noccaea caerulescens* seed families were sampled in June 2014, in "La Calamine" (C – 25 families) and "Prayon" (P – 23 families), two smelting sites from Belgium abandoned several decades ago [Assunção *et al.*, 2003; Roosens *et al.*, 2003]. Seeds were also collected in "Lellingen" (L – 25 families), and "Winseler" (W – 28 families), two non-metalliferous sites from Luxembourg [Dechamps *et al.*, 2008 - Table 1]. In September 2014, 15 seeds from each family were sown in sowing trays containing peat-based sowing soil. The trays were placed one week in a cold room at 4°C and placed in a greenhouse (20°C) for 6 weeks and were watered with osmosis water every 2 days.

Depending on germination rates, available seedlings were used to build 4 experimental populations (EP). EP_1, EP_2 and EP_3 were built from non-metallicolous families of the two geographical origins in order to have a maximum genetic diversity within experimental populations. As these EPs were also biological replicates, they were built with the same families in the same proportions. EP_4 was built with metallicolous individuals from the two geographical origins in order to have a maximum genetic diversity. Finally, each population was composed with 98 individuals from 36 families for EP_1, EP_2 and EP_3 (L=24 families, W=12 families) and 98 individuals from 48 families for EP4 (C=25 families, P=23 families) [Table 9].

Table 9 : Individuals used in the construction of experimental populations (EP)

EP1, EP2 & EP3				EP4			
Lellingen		Winseler		La Calamine		Prayon	
L101	2	W101	4	C101	2	P101	2
L102	2	W102	4	C102	2	P102	2
L103	2	W103	4	C103	2	P103	2
L104	2	W104	2	C104	2	P104	2
L105	4	W105	4	C105	2	P105	2
L106	2	W106	4	C106	2	P106	2
L107	2	W107	4	C107	2	P107	2
L108	4	W109	4	C108	2	P109	4
L109	2	W112	4	C109	2	P110	2
L110	4	W122	2	C110	2	P111	2
L111	2	W125	4	C111	2	P112	2
L112	2	W128	2	C112	2	P113	2
L113	2			C113	2	P114	2
L114	2			C114	2	P116	2
L115	2			C115	2	P117	2
L116	2			C116	2	P119	2
L117	4			C117	2	P120	2
L118	2			C118	2	P121	2
L119	2			C119	2	P122	2
L120	2			C120	2	P123	2
L121	2			C121	2	P124	2
L122	2			C122	2	P125	2
L124	2			C123	2	P126	2
L125	2			C124	2		
				C125	2		

In late October 2014, each EP was transferred in two mesocosms. Mesocosms were designed according to the lysimeter model proposed by Ruttens [Ruttens *et al.*, 2006]. They represent square tubs with an area of 0.436 m² enclosed by a 13 cm wide buffer zone. Buffer zones were filled with compost to create a thermal buffer [Figure 12]. Tubs were filled with a mix of 140 kg of peat and clay based compost and 70 kg of zeolite which allows a better growth of plants and therefore a better monitoring of individuals than in compost alone (unpublished data). Mesocosms were placed outside so that plants can complete their life cycle under natural climatic conditions. For EP_2, EP_3 and EP_4, zinc sulfate ($ZnSO_4$, $7H_2O$) was introduced in powder form to obtain a contamination of 750 mg of zinc per kg of fresh substrate (659.59 g of $ZnSO_4$, $7H_2O$). To ensure homogeneous substrates, mixes were made into a cement mixer. Therefore, EP_1 has made with individuals of non-metallicolous origin developing in a non-metallicolous control environment, EP_2 and EP_3 were made with individuals of non-metallicolous origin developing in a test environment on zinc contaminated soil, and EP_4 was made with individuals of metallicolous origin developing in a metallicolous control environment on contaminated soil. The seedlings were transferred using a 7*7 cell grid and the plants were placed in the center of each cell [Table 10]. Dead plants were replaced for 4 weeks (as death was probably due to transplantation). After this date the plants were not replaced. In order to limit pollen flow among EPs, they were placed on an area of 60 m² with a minimum distance between the EPs of 4 m (15 m maximum). In addition, an insect and wind proof cage system was established over 3 of 4 EP from the first anthesis. Thus, one EP out of 4 was left accessible to wind and pollinators while the 3 others remained inaccessible. A rotation of the cages was carried out every 3.5 days [Figure S5].

Each individual was monitored for several vegetative and reproductive traits throughout its life cycle. First, the death date and the corresponding phenological stage were recorded for each plant. Vegetative traits were measured at the emergence of the first floral bud, and included: the surface of the plant (*SP*), calculated as the surface of an ellipse from the two larger diameters of the rosette, and the number of leaves (*LN*). At the end of the life cycle (summer 2015), seeds were collected from each plant and plants were harvested, dried (20°C for 2 months) and phenotyped for several reproductive traits : the number of flower stems (*NFS*), the length of the largest flower stem (*maxLFS*), the number of siliques (*NS*), the number of aborted (*NAS*) and non-aborted (*NNAS*) siliques, the ratio between the number of non-aborted and aborted siliques (*RS*) and the average length of a silique estimated from the length of 10 siliques per flower stem (*SL*).

Table 10 : Experimental population composition

EP1, EP2 et EP3

Mesocosm 1

	1	2	3	4	5	6	7
1	W112	W104	L114	L121	W128	L112	L103
2	W101	L125	L105	L102	W125	L105	L115
3	W105	W109	L118	L110	L109	L101	W109
4	L108	W103	W101	L104	L106	L122	W103
5	W102	L107	L113	W122	W102	W106	L111
6	W107	L108	L119	L116	L110	L117	W112
7	W125	W107	L124	W106	L120	L117	W105

Mesocosm 2

	1	2	3	4	5	6	7
1	W103	W128	W125	W109	W106	W105	L108
2	L109	L105	W122	L117	L110	W109	W101
3	W112	L125	L113	W102	L120	L106	L110
4	L103	W105	L124	L108	L111	W103	L117
5	W101	W106	L101	W107	L104	L119	L115
6	L122	L121	W102	L116	L114	W104	L112
7	W107	L102	W125	W112	L107	L118	L105

EP4

Mesocosm 1

	1	2	3	4	5	6	7
1	C113	P101	C123	C103	P105	C107	C119
2	P122	P125	C116	P117	P112	P113	P124
3	C112	P111	P116	C105	C106	C104	C102
4	P109	P107	P114	P103	P110	P109	P104
5	P102	C124	P126	P120	P123	C120	C117
6	C114	P106	C125	C108	P119	C109	C111
7	C110	C121	C118	P121	C122	C101	C115

Mesocosm 2

	1	2	3	4	5	6	7
1	C109	C122	P109	P101	P126	P122	C113
2	C124	C103	P125	C123	C107	P105	P102
3	C114	P113	C115	C101	P103	C117	P111
4	P119	C116	C110	P107	C106	C102	C111
5	P104	P109	P106	C121	P123	C108	P124
6	C105	P117	C119	C125	C120	P112	C118
7	P116	P121	P120	C104	C112	P114	P110

5.2.2. Estimation of performances and generation transition

In order to avoid changes in intra-specific competition over generations, the density of EPs were controlled. Sowing of the G_{n+1} generation was carried out in proportion to the performance of the individuals from the G_n generation. Consequently, plants with a higher performance in the G_n generation had more offspring represented in the G_{n+1} generation and, conversely, individuals with a lower performance had less or no offspring [Westneat & Fox, 2010].

Mesocosms being placed in semi-natural conditions, entire seed sets were difficult to harvest because they dispersed easily with rain or wind. Therefore, the performance was estimated by an expected number of descendants. For that we calculate a seed set proxy: the product of the number of non-aborted siliques by the average size of siliques ($SS = NNAS * SL$ [Brachi *et al.*, 2012; Roux *et al.*, 2016; Chapter 1]. In parallel, in September 2015, 33 seeds of each individual were sown in clay and peat-based sowing compost in order to calculate a survival rate of seedling at 5 weeks (SR). This allowed to estimate an expected number of descendants multiplying the seed set estimation by the survival rate at 5 weeks ($ND = SS * SR$).

Once the expected number of descendants was estimated, we calculated a relative performance per individual ($\omega_{\sum ND_i}$) by dividing the expected number of descendants of each individual by the sum of the number of descendants over all individuals from the same EP ($\omega_{ND_i} = \frac{ND_i}{\sum_i^n ND_n}$). Then, this estimator of relative performance was used to constitute the G_{n+1} generation of each EP so as to maintain a population size of 98 individuals. To do this, the relative performance was multiplied by 98 ($98 \times \omega_{ND_i}$) and then rounded to the nearest integer ($\approx 98\omega_{ND_i}$) ($[98\omega_{ND_i}]$). If the obtained population size was less than 98, we added a descendant to the individuals whose difference $98\omega_{ND_i} - [\approx 98\omega_{ND_i}]$ was the largest positive number. Conversely, if the population size was greater than 98, we removed a descendant from individuals whose difference between $98\omega_{ND_i} - [98\omega_{ND_i}]$was the largest negative number.

Transplants of G_1 were performed at the end of October 2015. Then, individuals were followed throughout their life cycle and phenotyped on all the traits described above. The cycle is then repeated from generation to generation [Figure 39].

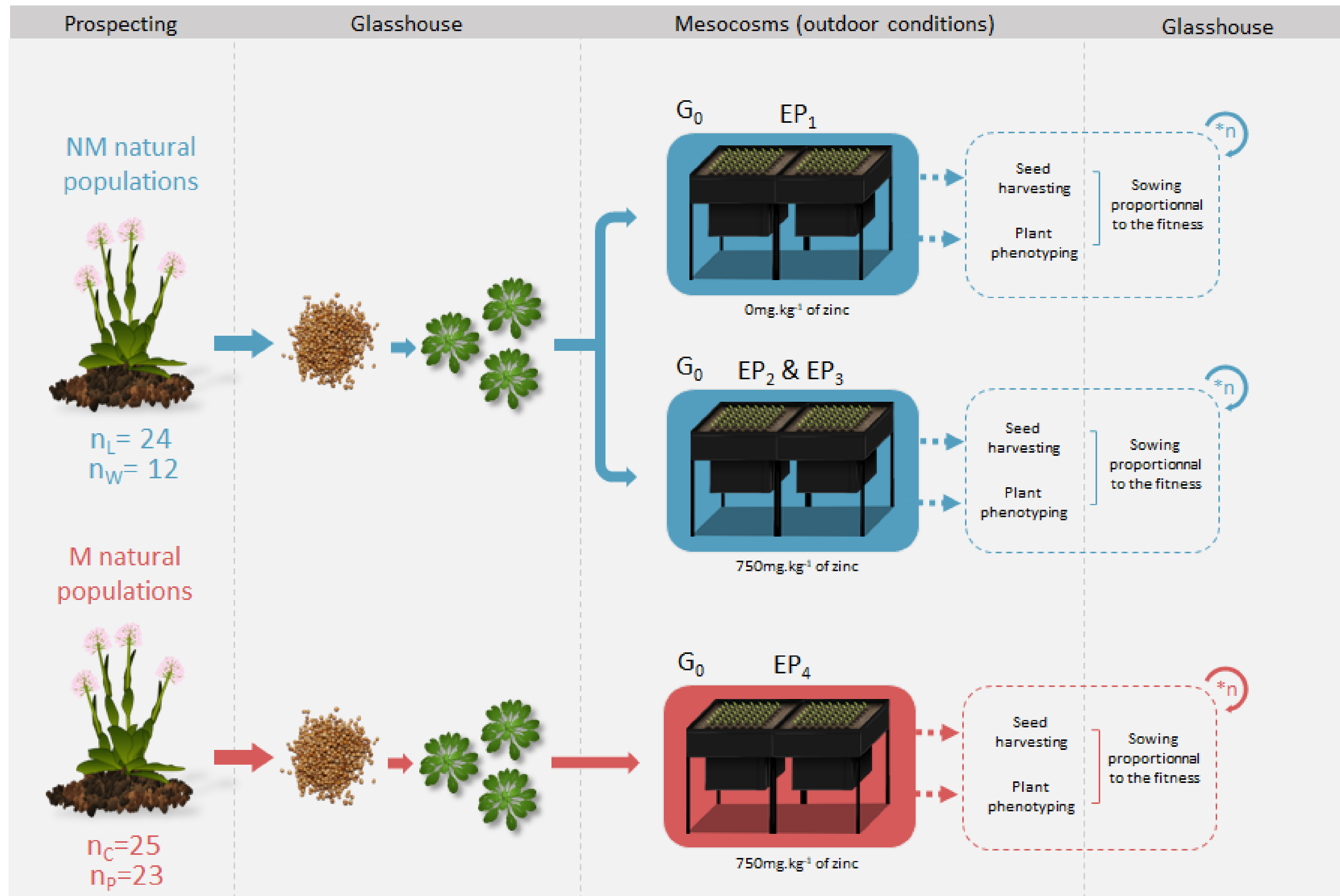


Figure 39 : Summary scheme of the experiment. 4 experimental populations (EP) were built with seedlings from 4 natural metallicolous (M – Prayon and La Calamine) and nonmetallicolous (NM - Lellingen and Winseler) accessions. EP_1, EP_2 and EP_3 represent 3 replicas of the same nonmetallicolous composite population. EP_4 represent a metallicolous composite population. Each EP was cultivated in two mesocosm filled with mix of compost and zeolite. For EP2, EP3 and EP4, soil was enriched with 750mg.kg^{-1} of zinc. Individuals from each population was phenotyped and seeds were harvested in order to estimate individual performance. These performances were used to create the generation n+1.

5.2.3. Statistical analyses

For the data obtained at the end of G_0, a Pearson's chi-squared test was used to compare survival rates among EPs, the rates of plants that produced seeds were also compared among EPs using a chi^2 test. We performed a principal component analysis using the 10 vegetative and reproductive variables in order to detect a putative point cloud structure according to EPs. Then, Kruskal-Wallis comparisons tests were used to evaluate differences among EPs on each vegetative and reproductive variable. When significant differences were found, *post-hoc* Conover tests were performed with Benjamini-Hochberg adjusted p-values. For the expected number of descendants, we also performed Fligner variance tests to evaluate the effect of EPs on the variance of the trait. When significant differences were detected, we performed a pairwise Fisher variance test with Benjamini-Hochberg adjusted p-values. Same statistical analyses were performed on the data from G_1. Then we compared traits values between G_0 and G_1 in each EP with Wilcoxon-Mann-Whitney tests.

To test the effect of zinc on phenotypic traits evolution we performed selection analyses at each generation. First we calculated standardized selection differentials in each EP for each phenotypic trait, except those used to estimate the number of descendants, *i.e.* the performance proxy itself (NS, NNAS NAS and meanLS), as regression coefficients from simple linear regressions [Lande & Arnold, 1983; Brachi *et al.*, 2012]:

$$\omega_{\overline{ND_i}} = \mu + X_i + \varepsilon$$

Where $\omega_{\overline{ND_i}}$ is an estimator of the relative performance of each individual i calculated in each EP by dividing the expected number of descendants of each individual by the average expected number of descendants over all individuals from the same EP. In this equation, μ was the intercept and X_i corresponded to the standardized value of the phenotypic trait considered for individual i, and ε is the residual term. We corrected for multiple tests by applying a Benjamini-Hochberg adjustment.

In the same way, we estimated the linear (ß) and quadratic (γ) selection gradients with polynomial regressions. ß selection gradient informs on the direction and intensity of the selection and γ selection gradient informs on the selection type. If $\gamma < 0$, selection is stabilizing and if $\gamma > 0$, selection is disruptive [Lande & Arnold, 1983; Brachi *et al.*, 2012]. First, we performed a polynomial regression of the linear and quadratic terms of each of the n phenotypic trait tested, except the i trait of interest:

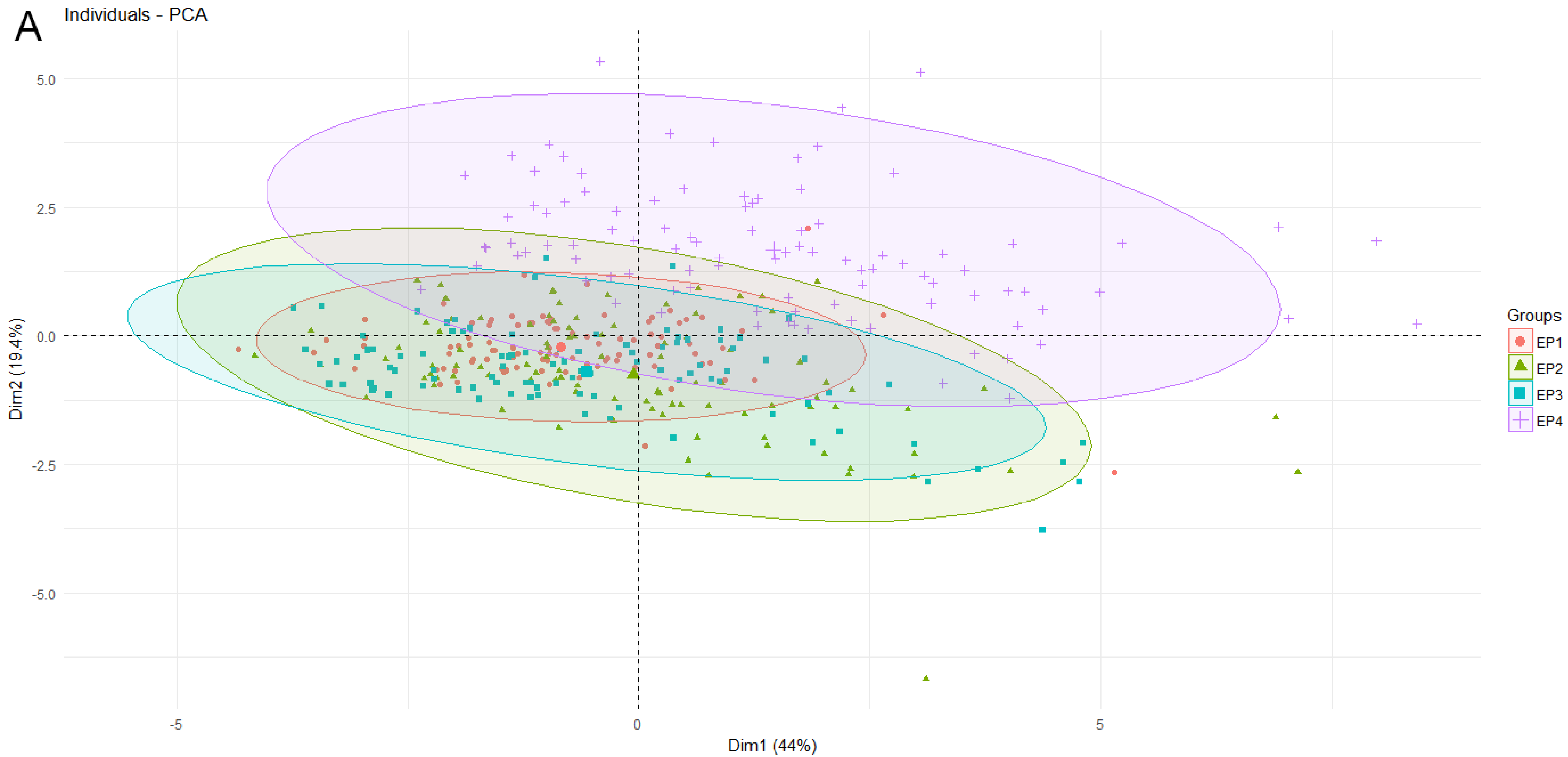
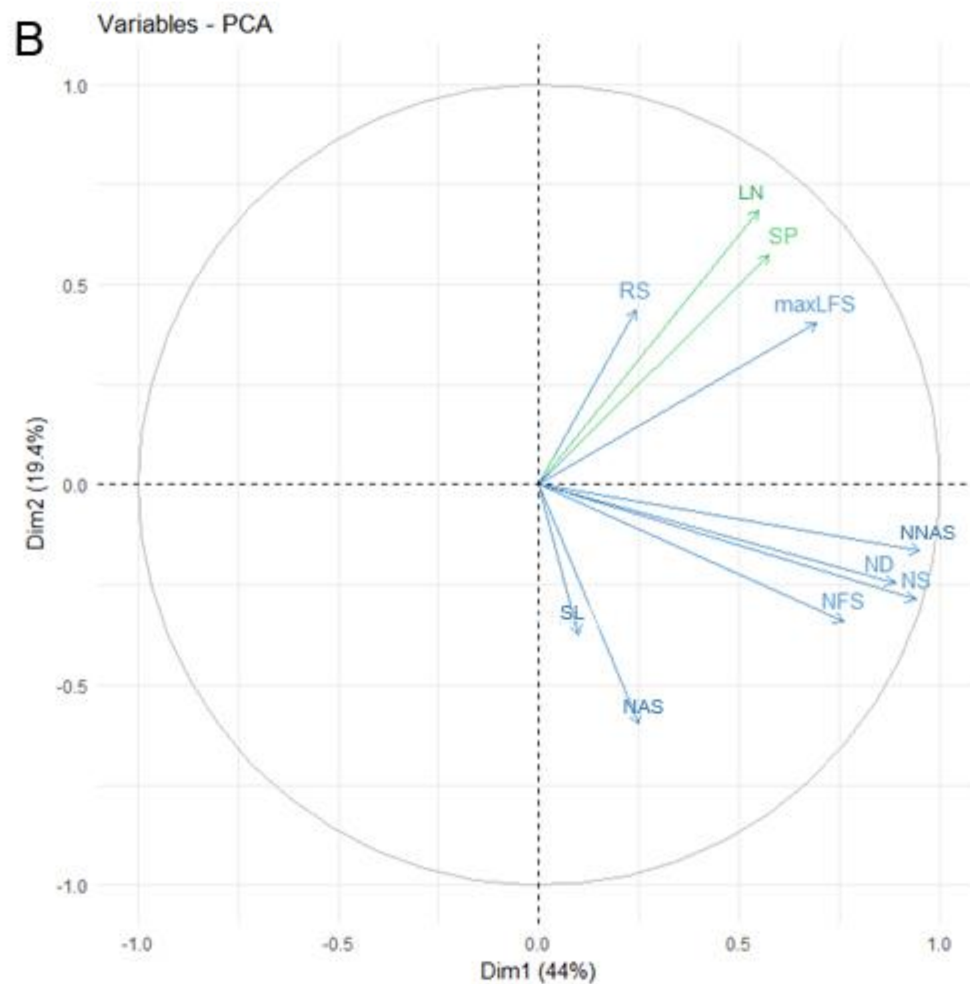


Figure 40 : Principal components analysis results on G_0 data. (A) Projection of individuals. (B) Correlation circle. (C) Variable contribution in each dimension showing vegetative (green bars) and reproductive (blue bars) traits. EP: experimental population; SP: plant surface; NL: number of leaves; NFS: number of flower stems; maxLFS: the length of the longest stem; NS: the total number of non-aborted siliques; NAS: number of aborted siliques; NNAS: number of non-aborted siliques; RS: ratio between the number of non-aborted and aborted siliques; SL: the mean length of silique for the entire plant; ND: expected number of descendants.



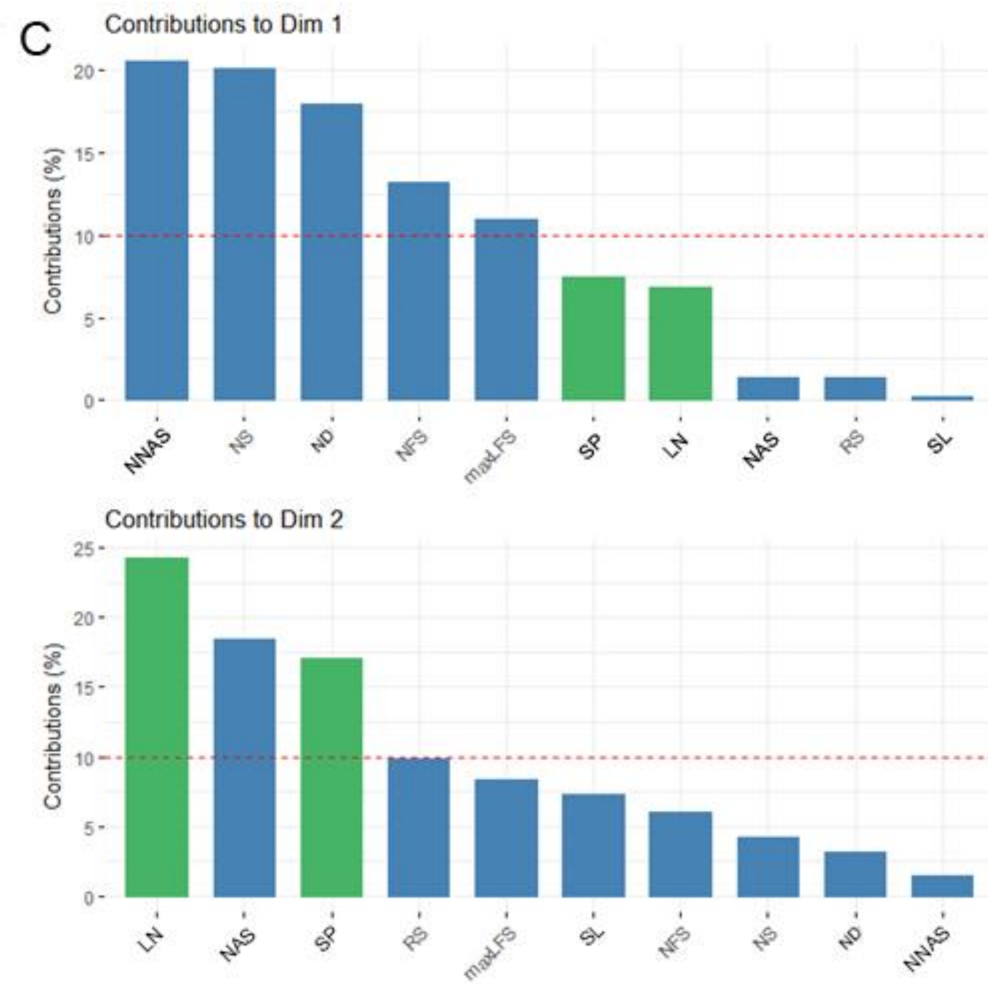


Figure 40 (continued): Principal components analysis results on G_0 data. (A) Projection of individuals. (B) Correlation circle. (C) Variable contribution in each dimension showing vegetative (green bars) and reproductive (blue bars) traits. EP: experimental population; SP: plant surface; NL: number of leaves; NFS: number of flower stems; maxLFS: the length of the longest stem; NS: the total number of non-aborted siliques; NAS: number of aborted siliques; NNAS: number of non-aborted siliques; RS: ratio between the number of non-aborted and aborted siliques; SL: the mean length of silique for the entire plant; ND: expected number of descendants.

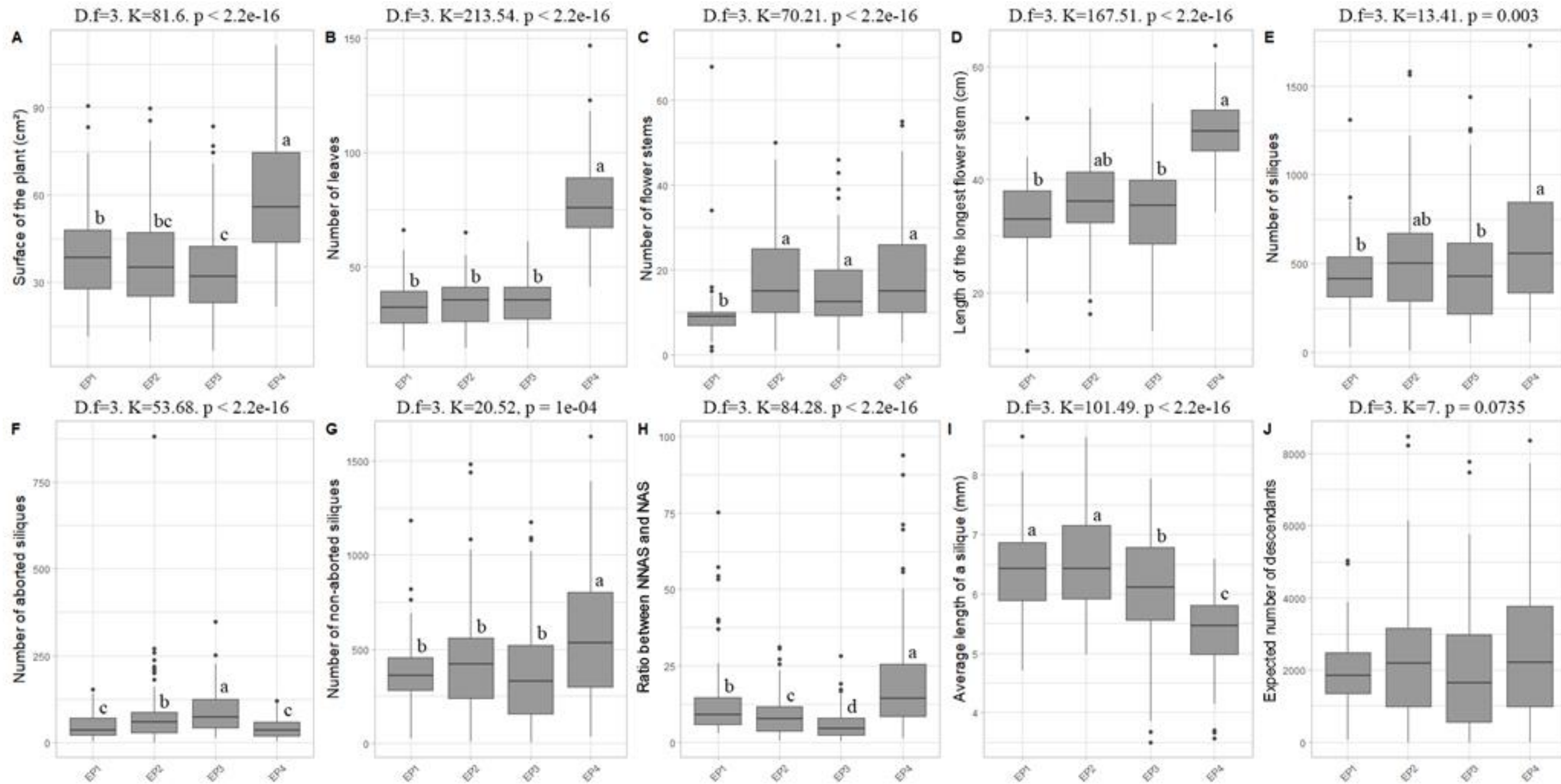


Figure 41 : Effect of zinc on vegetative traits (A and B), reproductive traits (C-I) and fitness estimation (J) for each experimental population at G0. K= Kruskal-Wallis statistic, d.f= degree of freedom, p= p.value. EP: experimental population. Boxplots with the same letters are not significantly different at the 5% level.

$$\omega_{\overline{ND_i}} = \mu + X_{n-i} + X_{n-i}^{2} + \varepsilon$$

Where $\omega_{\overline{ND_i}}$ was the relative performance of each individual calculated as described above, μ was the intercept and X_i corresponded to the standardized value of the phenotypic trait of individual i, and ε was the residual term. Then, the residual term was used to perform the polynomial regression of trait i (of interest).

$$\varepsilon = \mu + X_{i} + X_{i}^{2} + \varepsilon'$$

5.3. Results

5.3.1. Comparison of plant performance in G_0

There were no differences among EPs for survival rate. Indeed, no mortality was recorded in G_0. The proportion of plants that produced seeds were not significantly different among EPs (χ^2= 7.42, df = 3, p-value = 0.0597), although proportions were slightly lower in EP_3 (97%) and EP_4 (96%) compared to EP_1 (100%) and EP_2 (100%).

PCA analysis showed that the first component explained 44% of the variance and combined most reproductive traits, whereas the second component explained 19.4% of the variance and combined all vegetative and two reproductive traits [Figure 40]. The projection of individuals on the two first components of the PCA on vegetative and reproductive traits showed a clear separation of point clouds between EP_4 and other EPs in particular along the second axis, indicating higher vegetative values, smaller mean silique lengths and lower numbers of aborted siliques in this EP [Figure 40A]. Although the three points clouds corresponding to EP_1, EP_2 and EP_3 largely overlapped, the variance of values appeared to be lower in EP_1 compared to EP_2 and EP_3.

Kruskal-Wallis tests and post-hoc tests showed significant differences among EPs for all variables except the expected number of descendants [Figure 41], and were congruent with PCA results. Indeed, for vegetative traits, significant differences were mainly observed between EP_4 and other EPs, with highest values of plant surface [Figure 41A] and number of leaves [Figure 41B] in EP_4. Individuals in EP_4 had also smaller number of aborted siliques (although similar to the one of EP_1, Figure 41F), higher rates of non-aborted siliques to aborted siliques [Figure 4H] and smaller average silique lengths [Figure 41I]. For some variables, values in EP_2 or EP_3 were not different from those in EP_1 and EP_4: length of the longest stem in EP_2 [Figure 41D], number of siliques in EP_2 [Figure 41E], and average length of siliques in EP_3 [Figure

41I]. Interestingly, values for number of aborted siliques were significantly higher in EP_2 and EP_3 than in EP_1 and EP_4 [Figure 41F, 41H]. Moreover, individuals in EP_2 and EP_3 tended to have smallest plant surfaces [Figure 41A]. Only for the number of flower stems, there was a significant difference between EP_1 (lower values) and other EPs, with the other EPs showing non-significant differences among them [Figure 41C]. Finally, there was no significant difference in the expected number of descendants among EPs [Figure 41J].

However, Fligner test showed a significant difference in the variance of the expected number of descendants among EPs (χ^2= 37.648, df = 3, P-value = 3.35x10^{-8}) with significant higher variances in EP_2, EP_3 and EP_4 than in EP_1.

The average of the expected number of descendants was calculated for each family in each EP [Figure 42]. We observed that the families with the highest expected number of descendants were the same in EP_2 and EP_3. Thus, some families (W103, W105, W106, W107, W109, W112 and L105) were present in the quartile of the highest performing individuals in EP_2 to EP_3 but not necessarily in EP_1. Similarly, several families (L102, L103 and L116) were present in the quartile of the worst performing plants in EP_2 and EP_3 but not necessarily in EP_1.

5.3.2. Comparison of plant performance in G_1

We observed significant differences among EPs for survival rates (χ^2= 35.52, df = 3, P-value = 9.46x10^{-8}) with, significant lower survival rate in EP_2 and EP_4 compared to EP_1 and EP_3 [Figure 43A]. The number of plants producing seeds was also significantly different among EPs (χ^2= 122.49, df=3, P-value = < 2.2x10^{-16}), with the highest proportion in EP_1, the lowest rate in EP_2 and intermediate rates in EP_3 and EP_4 [Figure 43B].

PCA analysis showed that the first component explained 27.3% of the variance and combined vegetative and reproductive traits, whereas the second component explained 21.2% of the variance and combined only reproductive traits [Figure 44]. The projection of individual on the two first components of the PCA on vegetative and reproductive traits did not showed strong separation of point clouds among EPs (Figure 44A). There was only a slight separation between EP_1 and other EPs along the second axis, in particular with EP_2 and EP_3, mostly representing differences in number of aborted siliques and length of siliques [Figure 44A]. In addition, the variance of values appeared to be lower in EP_1 compared to other EPs.

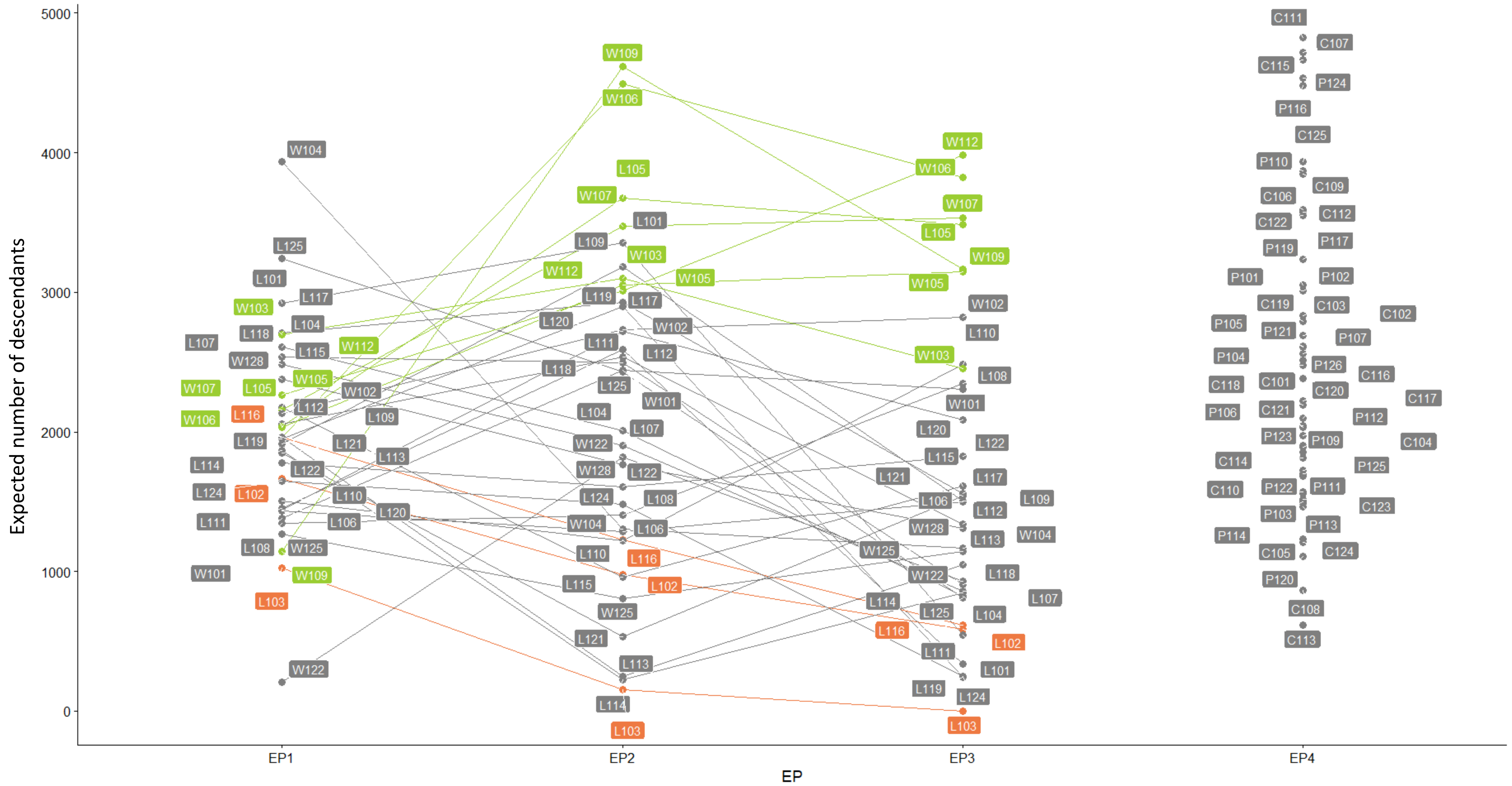


Figure 42: Mean per family of expected number of descendants calculated on G_0 data. Green points represent families of the first quartile of the most successful plants present in EP2 and EP3. Orange points represent families of the last quartile of the less successful plants present in EP2 and EP3. Grey points represent the other families. EP: experimental population, C: La Calamine, L: Lellingen, P: Prayon, W: Winseler

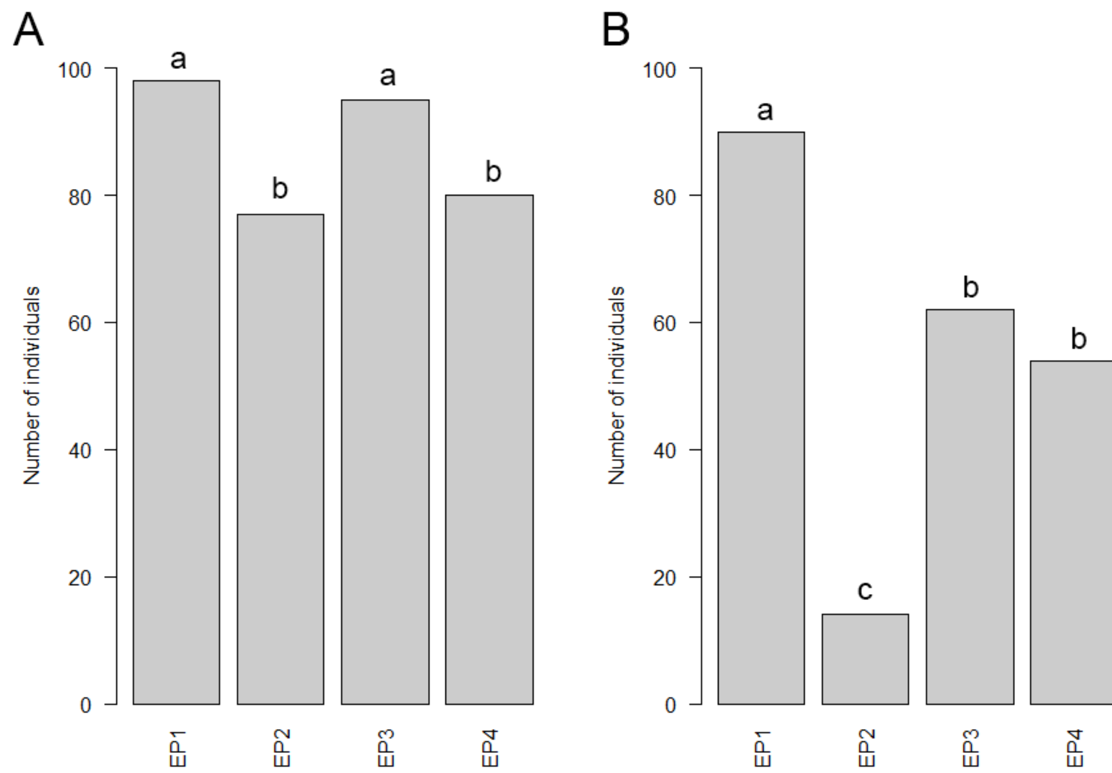


Figure 43: Number of individuals that (A) survived and (B) produced seeds in each experimental population from G_1. Barplots with the same letter are not significantly different at the 5% level.

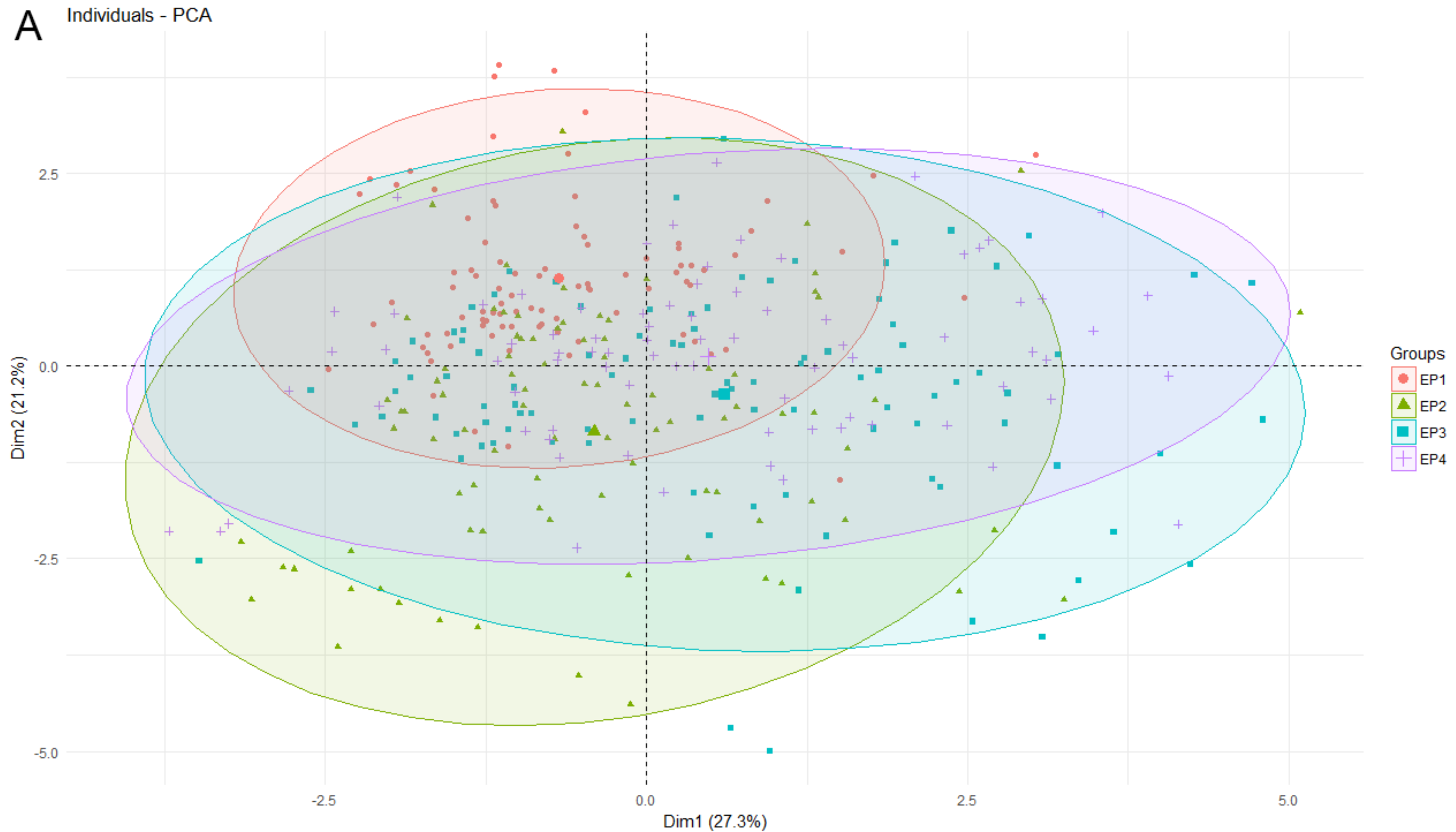


Figure 44: Principal components analysis results on G_1 data. (A) Projection of individuals. (B) Correlation circle. (C) Variable contribution in each dimension showing vegetative (green bars) and reproductive (blue bars) traits. EP: experimental population; SP: plant surface; NL: number of leaves; NFS: number of flower stems; maxLFS: the length of the longest stem; NS: the total number of non-aborted siliques; NAS: number of aborted siliques; NNAS: number of non-aborted siliques; RS: ratio between the number of non-aborted and aborted siliques; SL: the mean length of silique for the entire plant; ND: expected number of descendants.

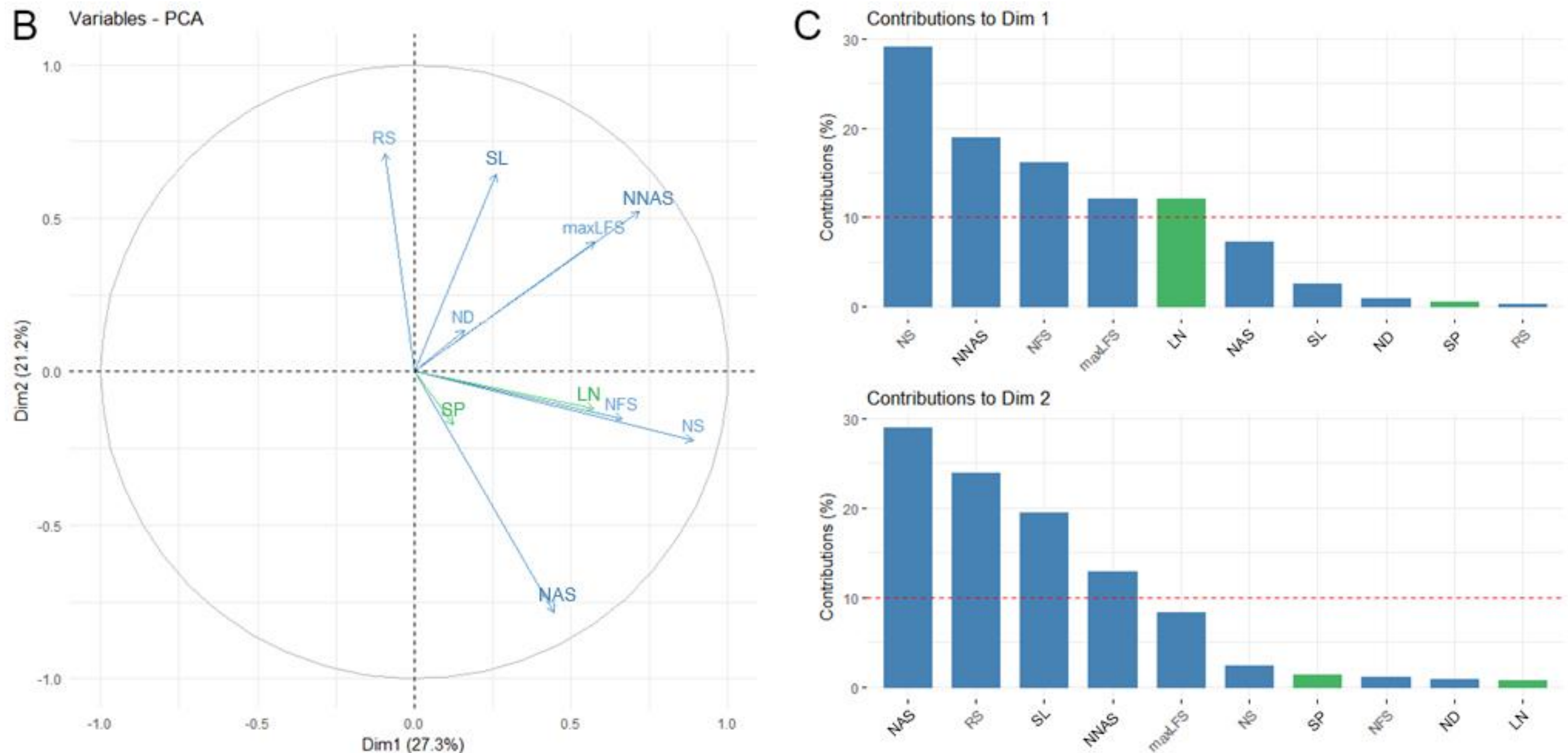


Figure 43 (continued): Principal components analysis results on G_1 data. (A) Projection of individuals. (B) Correlation circle. (C) Variable contribution in each dimension showing vegetative (green bars) and reproductive (blue bars) traits. EP: experimental population; SP: plant surface; NL: number of leaves; NFS: number of flower stems; maxLFS: the length of the longest stem; NS: the total number of non-aborted siliques; NAS: number of aborted siliques; NNAS: number of non-aborted siliques; RS: ratio between the number of non-aborted and aborted siliques; SL: the mean length of silique for the entire plant; ND: expected number of descendants.

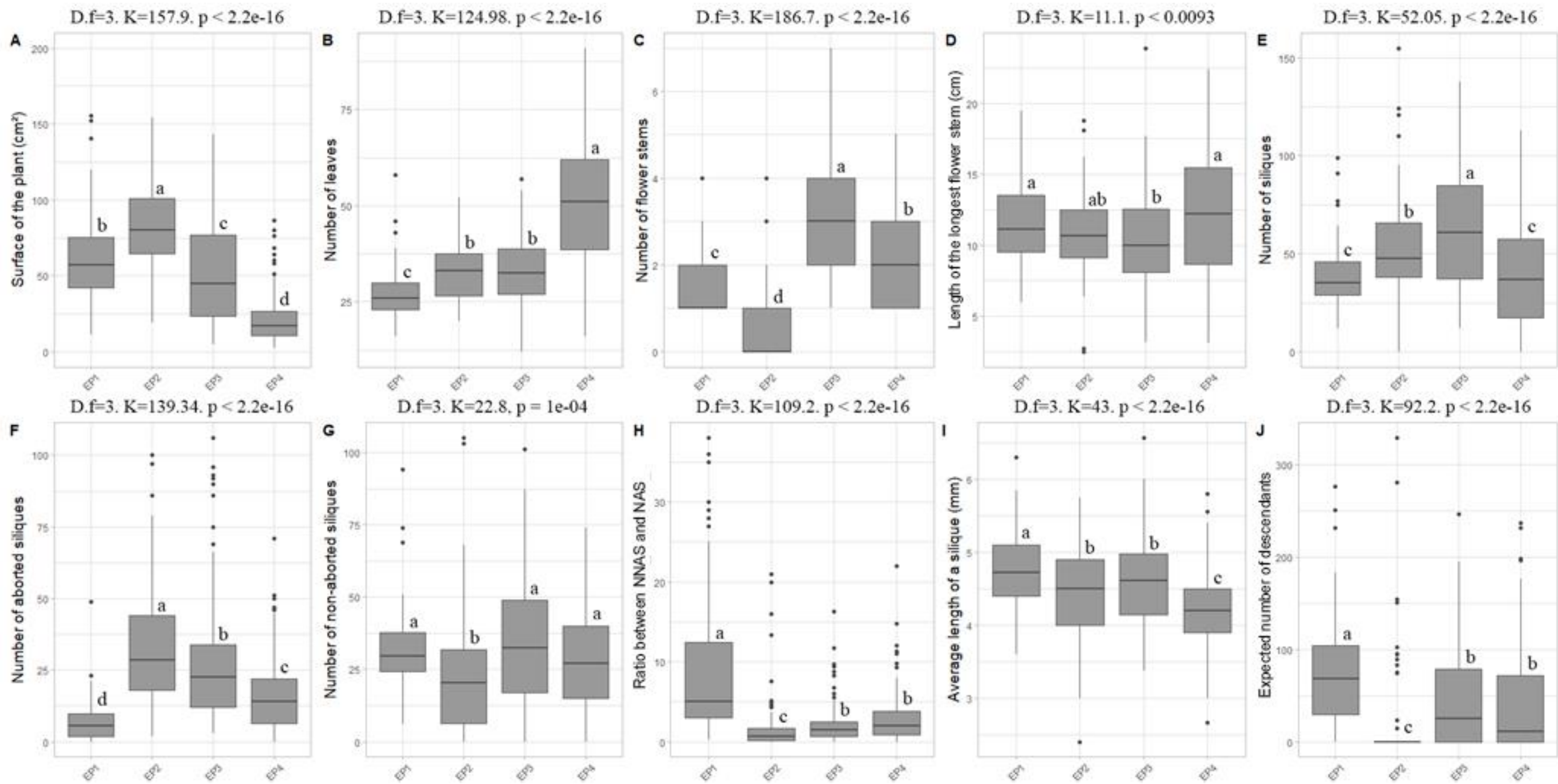


Figure 45: Effect of zinc on vegetative traits (A and B), reproductive traits (C-I) and fitness estimation (J) for each experimental population at G1. K= Kruskal-Wallis statistic, d.f= degree of freedom, p= p.value. EP: experimental population. Boxplots with the same letters are not significantly different at the 5% level.

Kruskal-Wallis tests and post-hoc tests showed significant differences among EPs for all variables, and were congruent with PCA results [Figure 45]. Indeed, EP_1 individuals significantly displayed the lowest values for number of leaves [Figure 45B] and number of aborted siliques (Figure 45F), but the highest ratio of non-aborted to aborted siliques [Figure 45H] and the highest values for average length of siliques [Figure 45I]. In addition, EP_2 and EP_3 individuals significantly showed the highest values for the number of aborted siliques, especially in EP_2 [Figure 45F]. EP_2 and EP_3 also significantly displayed the highest values for plant surface [Figure 45A], the number of siliques [Figures 45E], and the number of flower stems, in particular EP_3 [Figure 45C]. However, EP_2 and EP_3 showed contrasted results for several traits. Indeed, comparing to EP_3, EP_2 individuals had higher values for plant surface [Figure 45A], lower (actually, the lowest) values for the number of flower stems [Figure 45C], higher values for the number of siliques [Figure 45E] and the number of aborted siliques (the highest) [Figure 45F], but the lowest values for the number of non-aborted siliques [Figure 45H]. In contrast, EP_4 individuals significantly had the smallest plant surface with the highest number of leaves [Figures 45A, 45B], and the smallest average length of siliques [Figure 45I], whereas the numbers of siliques and non-aborted siliques were not different from those of EP_1 [Figures 45E, 45G].

Finally for the expected number of descendants, we observed significant differences among EPs [Figure 45J], with highest number of descendants in EP_1, the lowest number of descendants in EP_2 and intermediate values in EP_3 and EP_4.

Fligner test showed significant difference in the variance of the expected number of descendants among EPs (χ^2= 75.512, df = 3, P-value = 2.81×10^{-16}) but pairwise tests did not confirm this tendency.

The average number of descendants was calculated for each family in each EP [Figure 46]. Unlike G_0, only a few families were present in the quartile of the highest performing individuals in EP_2 to EP_3 but not necessarily in EP_1 (L108 and L112) or in the quartile of the worst performing plants in EP_2 and EP_3 but not necessarily in EP_1 (L104, L120 and L125). However, we observed that some families eliminated in EP_2 at G_0 were lost in EP_3 at G_1 (L113, L114 and L121) and, in the same way, some families eliminated in EP_3 at G_0 were lost in EP2 at G_1 (L101, L102, L111, L116, L119 and L124).

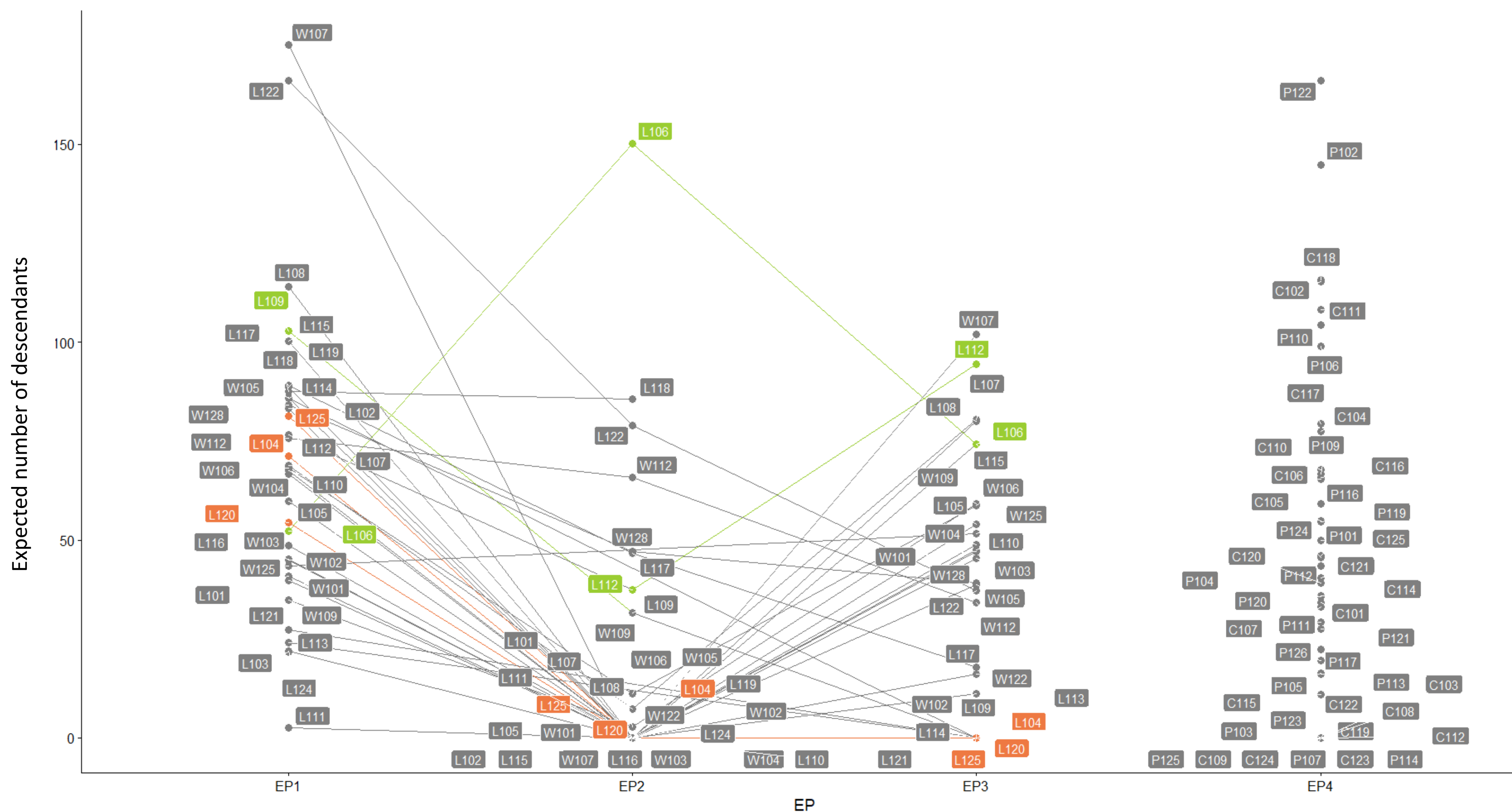


Figure 46: Mean per family of number of descendant estimation calculated on G_1 data. Green points represent families of the first quartile of the most successful plants present in EP2 and EP3. Orange points represent families of the last quartile of the most successful plants present in EP2 and EP3. Grey points represent the other families. EP: experimental population, C: La Calamine, L: Lellingen, P: Prayon, W: Winseler.

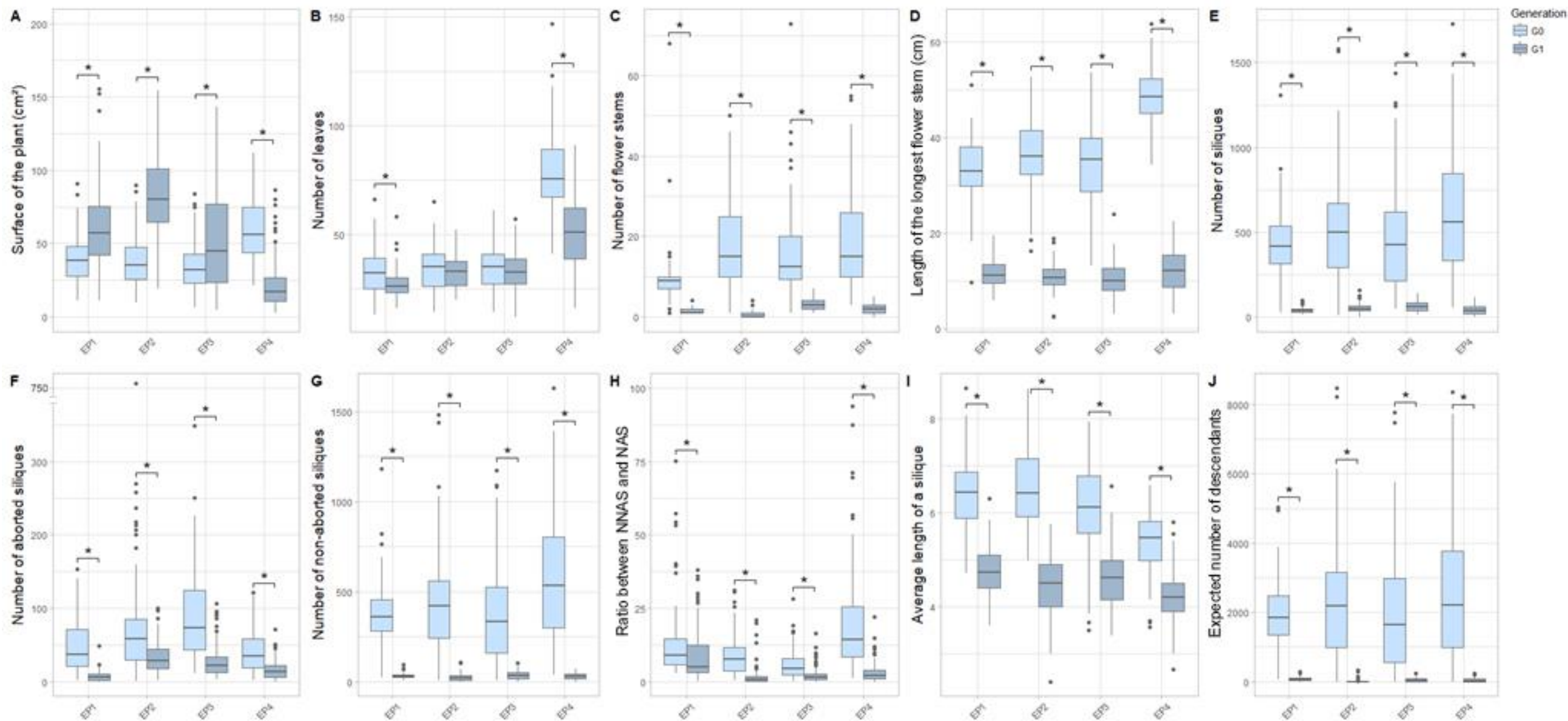


Figure 47: Effect of zinc on vegetative traits (A and B), reproductive traits (C-I) and fitness estimation (J) for each experimental population at G_0 and G_1. Light blue boxplots represent G_0 individuals, dark blue boxplots represent G_1 individuals. EP: experimental population; * symbol indicates significant difference between G_0 and G_1 individuals detected by Wilcoxon-Mann-Whitney test.

5.3.3. Inter-generational comparison of plant performance and selection analysis

Wilcoxon-Mann-Whitney test showed significant difference between G_0 and G_1 individuals for all traits in all EPs except for the number of leaves in EP_2 and EP_3 [Figure 47]. In the same way, for all traits except surface of the plant in EP_1, EP_2 and EP_3, individuals from G_0 had higher performance than G_1 individuals.

We calculated standardized selection differentials (S), linear selection gradients (ß) and quadratic selection gradients (γ) for traits that were not included in the expected number of descendants [Table 11]. At G_0, we detected significant standardized selection differentials for each trait in EP_1 EP_2 and EP_3. For EP_4 we detected significant standardized selection differentials only for the number of flower stems and the length of the largest flower stem. Also, we observed that standardized selection differentials were higher for number of flower stems, length of the longest flower stem and ratio between non-aborted and aborted siliques in EP_2 and EP_3 compared to EP_1. Concerning selection gradients, we detected slightly positive directional selection for all traits except the number of leaves in EP_1. In EP_2, we detected strong positive directional selection for the number of flower stems, the length of the largest flower stem and the ratio between non-aborted and aborted siliques. In EP_3, we detected a positive directional selection only for the number of flower stems. In EP_4, strong positive directional selection was detected for the number of flower stems the ratio between non-aborted and aborted siliques. Concerning the stabilizing/disruptive selection gradients, we observed a few significant quadratic regression coefficients in EP_2 with disruptive selection for the length of the largest flower stem and stabilizing selection for the ratio between non-aborted and aborted siliques. Also, we observed a significant quadratic regression coefficient in EP_4 for a disruptive selection for the plant surface, and a nearly significant quadratic regression coefficient for a stabilizing selection for the number of flower stems.

At G_1, only a few standardized selection differentials were significant compared to G_0 in EP_1, EP_2 and EP_3. In addition, all standardized selection differentials were significant in EP_4. Thus we detected significant standardized selection differentials for the length of the largest flower stem in all EPs, with higher values in EP_2, EP_3 and EP_4 compared to EP_1. Also, in EP_2, we detected significant standardized selection differential for the ratio between non-aborted and aborted siliques, and, in EP_3, for the surface of the plant. Concerning selection gradients, we observed significant positive regression coefficients for the length of the largest flower stem, and the ratio between non-aborted and aborted siliques in EP_1, EP_2 and EP_4. For EP_3, this selection gradient tended to be significant for a positive selection.

Table 11: Result of selection analysis with selection differentials (S), selection gradients (b), quadratic selection coefficients (c) for the surface of the plante (SP), the number of leaves (LN), the number of flower stems (NFS), the length of the largest flower stem (maxLFS) and the ratio between non-aborted and aborted siliques (RS). Selection is stabilizing when γ<0 and disruptive when γ>0. SE : standard error, ° P<0.1, * P< 0.05, ** P<0.01, *** P <0.001.

Generation	Experimental population	trait	S (SE)	ß (SE)	γ (SE)
G_0	EP1	SP	0.26 (0.04)***	0.08 (0.03)*	0.05 (0.04)
		LN	0.19 (0.05)***	0.03 (0.03)	-0.10 (0.04)
		NFS	0.25 (0.04)***	0.14 (0.06)*	$6.8.10^{-4}$ (0,02)
		maxLFS	0.25 (0.04)***	0.09 (0.03)*	-0.02 (0.04)
		RS	0.13 (0.05)**	0.12 (0.05)*	-0.04 (0.02)
	EP2	SP	0.27 (0.07)***	-0.05 (0.05)	0.04 (0.06)
		LN	0.25 (0.07)***	0.06 (0.04)	0.1 (0.06)
		NFS	0.47 (0.06)***	0.39 (0.05)***	-0.01 (0.08)
		maxLFS	0.35 (0.06)***	0.11 (0.04)**	0.12 (0.06)*
		RS	0.24 (0.07)***	0.27 (0.06)***	-0.2 (0.06)**
	EP3	SP	0.37 (0.08)***	0.03 (0.06)	-0.06 (0.08)
		LN	0.23 (0.08)**	-0.03 (0.05)	0.02 (0.08)
		NFS	0.46 (0.07)***	0.22 (0.07)*	0.04 (0.04)
		maxLFS	0.56 (0.06)***	0.11 (0.05)	0.1 (0.08)
		RS	0.45 (0.07)***	0.16 (0.07)	-0.06 (0.06)
	EP4	SP	0.12 (0.07)	-0.005 (0.04)	0.16 (0.06)*
		LN	0.14 (0.07)	-0.02 (0.04)	0.04 (0.06)
		NFS	0.59 (0.05)***	0.46 (0.07)***	-0.14 (0.08)
		maxLFS	0.36 (0.07)***	0.01 (0.04)	0.04 (0.06)
		RS	0.09 (0.07)	0.21 (0.09)*	-0.04 (0.02)
G_1	EP1	SP	0.14 (0.08)	0.07 (0.09)	0.02 (0.1)
		LN	0.08 (0.07)	-0.01 (0.09)	0.004 (0.08)
		NFS	0.02 (0.08)	0.10 (0.13)	-0.14 (0.14)
		maxLFS	0.25 (0.07)**	0.23 (0.08)*	-0.006 (0.12)
		RS	0.08 (0.08)	0.37 (0.13)*	-0,34 (0.14)
	EP2	SP	0.49 (0.33)	-0.95 (0.9)	0.42 (0.3)
		LN	-0.05 (0.35)	0.13 (0.31)	-0.06 (0.54)
		NFS	0.26 (0.33)	0.55 (0.50)	-0.14 (0.44)
		maxLFS	1.19 (0.34)**	0.6 (0.29)*	1.06 (0.36)**
		RS	1.09 (0.34)**	2.50 (0.75)**	-0.94 (0.38)*
	EP3	SP	0.24 (0.12)*	0.18 (0.13)	-0,18 (0.2)
		LN	0.08 (0.12)	-0.04 (0.1)	-0.06 (0.14)
		NFS	0.08 (0.12)	0.06 (0.11)	-0.16 (0.18)
		maxLFS	0.45 (0.11)***	0.26 (0.12)	-0.22 (0.12)
		RS	0.51 (0.11)	0.3 (0.19)	0.02 (0.12)
	EP4	SP	0.40 (0.14)**	0.09 (0.19)	-0.01 (0.18)
		LN	0.51 (0.14)***	-0.006 (0.11)	0.08 (0.18)
		NFS	0.63 (0.12)***	0.43 (0.23)	-0.16 (0.34)
		maxLFS	0.81 (0.13)***	0.29 (0.12)*	0.26 (0.2)
		RS	0.31 (0.15)*	0.56 (0.19)*	-0.28 (0.12)

Therefore, we observed that these coefficients were higher in EP_2 compared to other EPs, in particular for plant surface and ratio of non-aborted to aborted siliques. Finally, concerning stabilizing/disruptive selection gradients, we observed only significant quadratic regression coefficients for a disruptive selection on the length of the longest flower stem and a stabilizing selection on the ratio between non-aborted and aborted siliques in EP_2. Also, the quadratic regression coefficient for the ratio between non-aborted and aborted siliques tended to be significant in EP_1 for a stabilizing selection.

5.4. Key results and short discussion

5.4.1. Zinc impact on the experimental population composition

Focusing on the average performance of each family in each EP, at each generation it seems that the response to zinc exposure was not random. At the G_0, several same families (L105, W103, W105, W105, W106, W109 and W112) expressed the best performances in EP_2 and EP_3. Similarly, same families (L102, L103 and L116) express the lowest levels of performance in EP_2 and EP_3 [Figure 42].

At the G_1, few similar families expressed the best (L108 and L112) or worst (L104, L120 and L125) performances in EP_2 and EP_3. Nevertheless, several families lost in EP_2 at the end of G_0 (L113, L114 and L121) were lost at the end of G_1 in EP_3 and, conversely, families lost in EP_3 at the end of G_0 (L101, L102, L111, L116, L119 and L124) were lost at the end of G_1 in EP_2 [Figure 46].

The fact that the same families were selected suggests a genetic component in the ability of plant to handle Zn exposure. Since Zn stress mostly affect survival and reproductive traits, it can be considered as a selective agent influencing the ability of plants to get their genes represented at the next generation (individual fitness - Westneat & Fox, 2010).

5.4.2. Impact of zinc on the evolution of traits: Prediction based on descendant production

Calculating the selection differentials at the end of G_0 shows that all traits responded positively and significantly to selection in EP_1, EP_2 and EP_3. Nevertheless, the calculated differentials were higher for reproductive traits in EP_2 and EP_3. In EP_4, only the number of floral stems and the size of the largest stems seem to respond positively and significantly to the

selection. At G_1, the selection differentials, calculated for each population, showed very different results compared to the G_0. Indeed, only selection differential calculated over the length of the largest floral stem is positive and significant in all populations. Also, the selection differentials calculated on all traits were significant and positive in EP_4.

Nevertheless, selection differential values include both direct and indirect selection on related traits [Lande & Arnold, 1983]. Indeed, linear and quadratic selection gradients were also calculated for each trait in each population for each generation. Generally, different results were observed between both generations. Thus, at G_0, we observed a positive selection for a higher number of flower stems and a higher RS ratio. At G_1, we observed a positive selection for a higher floral stem length and a higher RS ratio.

Differences observed between G_0 and G_1 could be explained by different factors. (1) The differences observable in G_1 could be related to the result of the selection in G_0. For example, by strong selection for the number of flower stems in G_0, it was possible to lose a part of the available polymorphism. Thus, the selection could not operate on the same trait in G_1. (2) Differences could also be explained by different culture conditions between both generations. This hypothesis was supported by the fact that all populations were infected with a fungal pathogen, identified as *Sclerotinia sclerotirum* (personal communication: Chloé Ponitzki). This pathogen appeared during the reproduction of individuals and caused significant damage to reproductive structures. (3) The substrate was not changed between both generations and this difference in response could be related to resource depletion.

Figure S5 : Insect and wind proof cage system

CHAPTER 5

6. Effects of two-generation selection under zinc exposure on the evolution of zinc tolerance-related traits in experimental populations of *Noccaea caerulescens*.

6.1. Brief introduction

As described in the previous chapter, selection was applied measuring performance in 4 experimental populations (EP_1 to EP_4) of *N. caerulescens* during two generations (G_0 and G_1). At the end of each generation, selection analyses were carried out, through the calculation of standardized selection differentials and selection gradients (chapter 5). However, interpretation of these results were limited because few traits were analyzed and it was possible that other traits were selected, but were not measured.

In order to observe the phenotypic evolution of our experimental populations, representants of generations G_0 and G_2 from each EP were cultivated under controlled conditions in different zinc concentrations. The most possible integrative phenotyping, including morphological, physiological and phenological traits, was performed on all individuals. Phenotypic levels were also compared among populations and generations.

6.2. Material and methods

6.2.1. Phenotypic survey of EPs after 2 years of experimental evolution

In september 2016, after two generations of experimental evolution, 33 seeds of each family used to create G_0 [Table 9-10] and G_2 were sown in seedling trays containing peat-based compost. They were placed one week in a cold room at 4°C, transferred in a glasshouse and watered with osmosis water every two days. A germination survey was performed every two days. After eight weeks, we transferred two replicates of each derived population after two generations (G_2), two replicates of metallicolous ancestral population ($G_{0\text{-}M}$) and three replicates of nonmetallicolous ancestral population ($G_{0\text{-}NM}$) were built following the exact same design used for the composition of EPs in mesocosms. However, here, each plant was transferred in individual pots containing a mix of 70% of peat-based compost and 30% of zeolite contaminated at several levels of heptahydrate zinc sulfate.

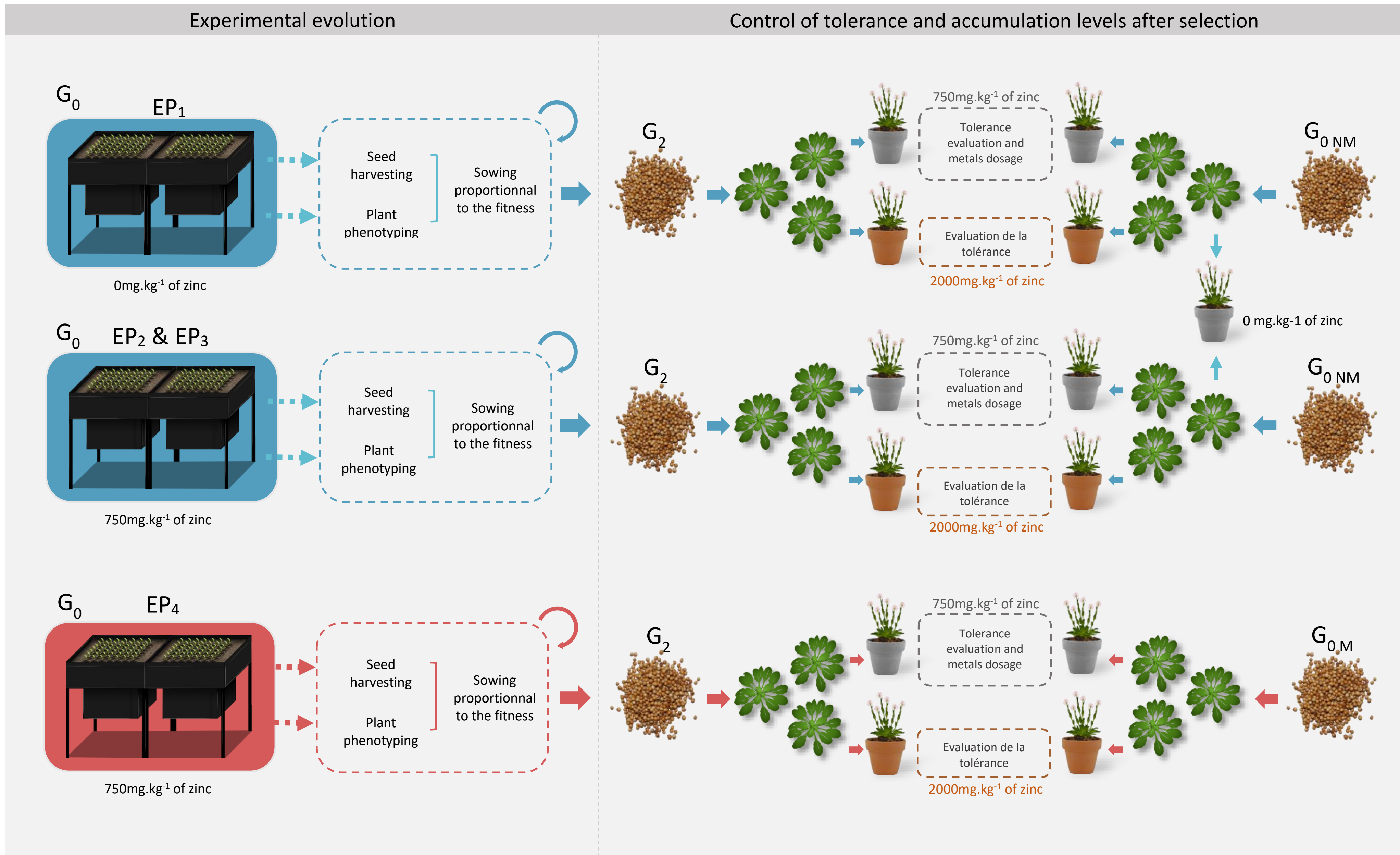


Figure 48: Control of tolerance and accumulation levels after selection. G_0: ancestral population; G_2: derived population after two generations of selection: EP: experimental population.

Thus, one replicate of $G_{0\text{-}NM}$ was transferred in pots containing non contaminated substrate, one replicate of $G_{0\text{-}NM}$, G_2 and $G_{0\text{-}M}$ were transferred in pots containing substrate at 750 mg.kg^{-1} of zinc, and one replicate of $G_{0\text{-}NM}$, G_2 and $G_{0\text{-}M}$ were transferred in pots containing substrate at 2000 mg.kg^{-1} of zinc. Contamination of each pot was carried out using 20 mL of heptahydrate zinc sulfate solution ($ZnSO_4$, $7H_2O$) at 0.57 M or 1.53 M for a contamination at 750 mg.kg^{-1} or 2000 mg.kg^{-1} of zinc, respectively. Transplantations of plants in pots were performed 2 days after contamination following manual homogenization of the soil. Then, eight weeks after sowing, pots were randomized and placed in a ventilated and unheated greenhouse in order to induce flowering [Figure 48].

During a complete generation, i.e. from seed sowing to the germination of offspring seeds, plants were phenotyped for several phenological, physiological, biochemical and morphological traits [Table 12].

Phenological traits: we measured the number of days from sowing to germination (*GERM*), from germination to bolting time (*BT*), between germination and anthesis (*ANT*), between germination and the first fruit emergence (*FFE*) on the main flowering stem, between germination and the senescence of the last flower on the main flowering stem, corresponding to the end of flowering (*EF*), between germination and the dehiscence of the last silique (*DEH*), between bolting and anthesis, corresponding to the bolting period (*BP*), between anthesis and the end of flowering, corresponding to the flowering period (*FP*), and between anthesis and dehiscence, corresponding to the reproductive period (*RP*). All these time intervals were converted into growth degree days.

Physiological traits: Plants were simultaneously phenotyped for photosynthetic performance of the photosystem II at t1, 25 weeks after sowing, when a first plant initiated the emergence of a first flower bud, ($Phi\text{-}PSII_1$), using a portable fluorometer (PAM-2100, Walz). Plant were also characterized for chlorosis level of the rosette according to four categories (1: healthy plant, 2: partially chlorotic plant, 3: fully chlorotic plant and 4: dead plant – Figure 20), at t1 ($STATE_1$), and separately at their specific bolting or anthesis time ($STATE_{BT}$ and $STATE_{ANT}$, respectively). Finally, three representative leaves were collected, scanned to digitally obtain the surface of each leaf and weighed before and after drying (three days in a drying oven at 60°C). It was then possible to calculate the average specific leaf area [Cornelissen *et al.*, 2003] (*SLA* - formula 1), the average dry matter content [Cornelissen *et al.*, 2003] (*LDMC*-formula 2) and the average leaf thickness estimation [Vile *et al.*, 2005] (*LT* - formula 3)

Table 12 : List of vegetative morphological (in green), phenological (in blue), physiological (in grey), biochemical (in yellow) and reproductive (in red) traits that have been measured. Also, CSR scores (in orange) were estimated by [Pierce *et al.*, 2016] algorithm and several elements were dosed (in purple). GDD: growth degree days. OD: optical density. t0: transplanting time - eight weeks after sowing. t1: 25 weeks after sowing, when a first plant initiated the emergence of a first flower bud.

abbreviation	MPP trait (unit)	measured at
LN_{t0}	Leaf number	t0
SP_{t0}	Surface of of the plant (cm^2)	t0
LN_{t1}	Leaf number	t1
SP_{t1}	Surface of of the plant (cm^2)	t1
LL_{t1}	Leaf length (cm)	t1
PR_{t1}	Petiolic ratio	t1
LS_{t1}	Leaf surface (cm^2)	t1
LM_{t1}	Leaf morphology	t1
LN_{BT}	Leaf number	bolting
SP_{BT}	Surface of the plant (cm^2)	bolting
LL_{BT}	Leaf lenght (cm)	bolting
PR_{BT}	Petiolic ratio	bolting
LS_{BT}	Leaf surface (cm^2)	bolting
LM_{BT}	Leaf morphology	bolting
HR	Height of the rosette	bolting
SP_{ANT}	Surface of of the plant	anthesis
GERM	Germination date (GDD)	germination
BT	Bolting date (GDD)	bolting
ANT	Anthesis date (GDD)	anthesis
INT	interval between bolting and anthesis (GDD)	anthesis
FFE	First fruit emergence date (GDD)	first fruit emergence
EF	End of flowering (GDD)	end of flowering
FLO	Flowering period (GDD)	end of flowering
DEH	Dehiscence of the last fruit date (GDD)	dehiscence of the last fruit
RP	Reproductive period (GDD)	dehiscence of the last fruit
PHI-PSII	Photosynthetic yield	t1
$STATE_1$	Chlorosis level of the rosette	t1
$STATE_{BT}$	Chlorosis level of the rosette	BT
$STATE_{ANT}$	Chlorosis level of the rosette	ANT
SLA	Specific leaf area ($m^2.kg^{-1}$)	bolting
LDMC	Leaf dry matter content ($mg.g^{-1}$)	bolting
LT	Leaf thickness (µm)	bolting
CHLORO	Chorophyll concentration measured at chlorophyll meter (SPAD unit)	bolting
Chla	Chlorophyll a concentration (OD.g-1)	bolting
Chlb	Chlorophyll b concentration (OD.g-1)	bolting
Car	Carotenoids concentration (OD.g-1)	bolting
Antho	Anthocyanins concentration (OD.g-1)	bolting
Flavo	flavonoids concentration (OD.g-1)	bolting
Pheno	Phenolic compounds concentration (OD.g-1)	bolting
Tan	Tannins concentration (OD.g-1)	bolting

Table 2 (continued): List of vegetative morphological (in green), phenological (in blue), physiological (in grey), biochemical (in yellow) and reproductive (in red) traits that have been measured. Also, CSR scores (in orange) were estimated by [Pierce *et al.*, 2016] algorithm and several elements were dosed (in purple). GDD: growth degree days. OD: optical density. t0: transplanting time - eight weeks after sowing. t1: 25 weeks after sowing, when a first plant initiated the emergence of a first flower bud.

abbreviation	MPP trait (unit)	measured at
NR	Number of racemes	end of life cyle
NP	Number of panicles	end of life cyle
NRAM	Number of ramifications	end of life cyle
NFS	Number of flower stems	end of life cyle
PR	Proportion of raceme	end of life cyle
PP	Proportion of panicles	end of life cyle
RPR	ratio between panicles and raceme	end of life cyle
maxLFS	length of the largest flower stem (cm)	end of life cyle
HFF	Height to the first fruit on the largest flower stem (cm)	end of life cyle
NS	Number of siliques	end of life cyle
NAS	Number of aborted siliques	end of life cyle
NNAS	Number of non-aborted siliques	end of life cyle
PAS	Propotion of aborted siliques	end of life cyle
PNAS	Propotion of non-aborted siliques	end of life cyle
RS	Ratio between aborted and non-aborted siliques	end of life cyle
SL	Siliques length (mm)	end of life cyle
SS	seed set estimation	end of life cyle
SR	Survival rate of descendant calculated after 5 weeks	end of life cyle
ND	Number of descendants	end of life cyle
CSR-C	C score calculated with Pierce *et al.* algorithm	bolting
CSR-S	S score calculated with Pierce et al. algorithm	bolting
CSR-R	R score calculated with Pierce et al. algorithm	bolting
Al	Aluminum concentration (mg.kg^{-1})	bolting
B	Bore concentration (mg.kg^{-1})	bolting
Ca	Calcium concentration (mg.kg^{-1})	bolting
Cu	Copper concentration (mg.kg^{-1})	bolting
Fe	Iron concentration (mg.kg^{-1})	bolting
Mg	Magnesium concentration (mg.kg^{-1})	bolting
Mn	Manganese concentration (mg.kg^{-1})	bolting
P	Phosphorus concentration (mg.kg^{-1})	bolting
K	Potassium concentration (mg.kg^{-1})	bolting
Na	Sodium concentration (mg.kg^{-1})	bolting
S	Sulfur concentration (mg.kg^{-1})	bolting
Zn	Zinc concentration (mg.kg^{-1})	bolting

$$(1)\ SLA = \frac{Leaf\ surface\ (m^2)}{Leaf\ dry\ mass\ (kg)}$$

$$(2)\ LDMC = \frac{Leaf\ dry\ mass\ (mg)}{Leaf\ fresh\ weight\ (g)}$$

$$(3)\ LT = \frac{1}{SLA * DMC}$$

Biochemical traits: Biochemical traits (hereafter called "biomarkers") were assessed at BT for each individual, using leaf punches collected on 4 representative leaves. First, concentrations in photosynthetic and photoprotective pigments were analyzed: chlorophyll a (*Chla*), chlorophyll b (*Chlb*) and carotenoids (*Car*). Carotenoids protect chlorophylls and cell membranes during light stress, by avoiding ROS production and lipid peroxidation or by dissipating excess energy into heat [Gill & Tuteja, 2010; Baek *et al.*, 2012]. Then, we dosed the secondary compounds: phenolic compounds (*Pheno*), tannins (*Tan*), flavonoids (*Flavo*) and anthocyanins (*Antho*). These compounds are useful for their antioxidant role, for plant growth [Das & Roychoudhury, 2014], for metal tolerance and detoxification [Michalak, 2006] or pathogen resistance [Wink, 1988]. Photosynthetic pigments and secondary compounds were dosed by spectrophotometry after extraction in a 95% methanol solution using the method described in Al souki 2017 [Box 1].

Simultaneously, the total chlorophyll concentration was estimated with a chlorophyll meter (Hansatech Instruments® - CL-01 Chlorophyll Content Meter) on five leaves representative of the plant chlorosis level (*CHLORO*).

Vegetative traits: Leaf number was measured at t0 (LN_{t0}), t1 (LN_{t1}), and at bolting time (LN_{BT}). Surface of plants (*SP*) was calculated as the surface of an ellipse from the two largest orthogonal diameters of the rosette, at t0, t1, bolting time and at anthesis time (SP_{t0}, SP_{t1}, SP_{BT} and SP_{ANT}, respectively). From three leaves, the length of the petiole (*P*), length of the blade (*L*) and width of the blade (*W*) were measured at t1 and BT in order to calculate different leaf biometric indices [Mouton, 1976], including the total leaf length ($LL=P+L$), the petiolic ratio ($PR = \frac{P}{L}$), leaf blade surface ($LS = \frac{L}{2} * \frac{W}{2}$) and leaf morphology ($LM = \frac{L}{W}$) at t1 ($LL_{t1}$, PR_{t1}, LS_{t1} and LM_{t1}) and at bolting time (LL_{BT}, PR_{BT}, LS_{BT} and LM_{BT}). Finally, the height of the rosette (*HR*) was measured at bolting time to approximate the depth of rooting [Cornelissen *et al.*, 2003].

Reproductive traits: several traits related to the architecture of reproductive structures and fecundity were recorded. After maturation of the last fruit, individuals were harvested, dried (20°C during two months) and phenotyped. Then, we measured the number of flower stem (*NFS*), which included the number of ramified flower stems, hereafter called "panicles" (*NP*), and non-ramified flower stems, hereafter called "raceme" (*NR*), as well as, we measured the number of ramifications (*NRAM*). This allowed to calculate the proportion of racemes ($PR = \frac{NR}{NFS}$), the proportion of panicles ($PP = \frac{NP}{NFS}$), and the ratio between raceme and panicles ($RPR = \frac{NR}{NP}$). The length of the main flower stem (*maxLFS*) and the distance between the beginning of the flower stem and the first silique on the main flower stem, hereafter called "height to the first fruit" (*HFF*). We considered the main flower stem as the largest one.

Concerning fecundity, we counted the number of aborted and non-aborted siliques and measured 10 siliques per flower stem. This allowed to calculate the number of aborted siliques (*NAS*), the number of non-aborted siliques (*NNAS*) and the number of siliques (*NS*=*NAS*+*NNAS*). In the same way, the proportion of aborted ($PNAS = \frac{NAS}{NS}$) or non-aborted siliques ($PNNSA = \frac{NNAS}{NS}$) and the ratio between non-aborted and aborted siliques ($RS = \frac{NNAS}{NAS}$) were calculated.

Due to a significant correlation between the number of seeds and fruit size [Brachi *et al.*, 2012; Roux *et al.*, 2016; Chapter 1], seed set can be estimated by the total fruit length. Thus, we calculated the average silique length (*SL*), and multiplied it with the number of non-aborted siliques to estimate the seed set ($SS = NNAS * SL$)

Finally, 33 seeds per individual were sown in seedling trays containing peat-based compost, placed one week in a cold room at 4°C, transferred in a glasshouse for five weeks and watered with osmosis water every two days. Then, we calculated a survival rate after five weeks (*SR*) to estimate the number of descendants producted by each individual ($ND = SR * SS$)

To compare metal accumulation levels among derived and ancestral populations we performed a tissue analysis. This analysis consists in dissolving plant samples in strong acids, and measuring the metals concentration by induced coupled plasma atomic emission spectrometry (ICP-AES). First, three leaves representative of the chlorotic state of the plant were collected in the peripheral rosette, at bolting stage. We harvested the leaves on the periphery of the rosette because they represent mature leaves at the maximum of their accumulation capacity [Perronnet *et al.*, 2003]. The leaves were washed with demineralized

water and dried for three days at 60°C. Then, samples were crushed with mortar and mineralized using a method adapted to samples with low biomass. Thus, 50 mg of dry matter was added to 20 mL of HNO_3 solution, the solution was left at room temperature for four hours and placed 24 hours at 80°C. Once the samples had cooled, 2 mL of H_2O_2 were added and the solution was left at room temperature until the reaction was complete. Then, mineralizates were filtered and filled to 10 mL with demineralized water. Furthermore, standard samples of *Noccaea caerulescens* were mineralized. The contents of aluminum (Al), bore (B), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), sodium (Na), sulfur (S) and zinc (Zn) were then determined by ICP-AES. ICP analysis was performed only on $G_{0\text{-}M}$, $G_{0\text{-}NM}$, $G_{2\text{-}EP1}$, $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$ populations submitted to 750 $mg.kg^{-1}$ of zinc pollution, because we wanted (1) to evaluate the role of zinc selection on nonmetallicolous derived populations, $G_{0\text{-}M}$ and $G_{0\text{-}NM}$ being used as metallicolous and non-metallicolous control, and (2) to use plants healthy enough to actively control metal homeostasis, in particular zinc accumulation. Indeed, measuring metal accumulation in weakened plants may give spurious results and lead to erroneous interpretations [van der Ent *et al.*, 2013].

6.2.2. Estimation of ecological strategies

According to Grime (1977), plants adopt different ecological strategies in response to two types of environmental factors: either stress related to a decrease in resources or stress related to disturbance. Stress was defined as resulting from environmental factors that reduce plant growth, and disturbance was defined as resulting from environmental factors that cause total or partial destruction of plant biomass [Grime, 1977]. In theory, differences in stress and disturbance intensity leads to quantitative variation in three ecological strategies: competition strategy (C) in optimal habitats with low stress and disturbance intensities, with individuals that invest resources in competitive structures (rapid growth or large organs), stress-tolerance strategy (S) in stressed habitat with low disturbance, with individuals that limit resources exploitation in favor of plant tissues protection, and ruderal strategy (R) in regularly disturbed environments with low stress intensity, with individuals that invest in traits adapted to the temporary exploitation of favorable conditions. CSR scores were calculated for each individual in all populations using method and algorithm developed by [Pierce *et al.*, 2016]. This algorithm used three leaf traits: the leaf surface (LS), the leaf dry matter content (LDMC) and specific leaf area (SLA) to calculate the CSR score. Each CSR score represent the percentage associated to C, S and R axes.

6.2.3. Statistical analysis on phenotypic data

First, we analyzed the effect of population ($G_{2\text{-}EP1}$, $G_{2\text{-}EP2}$, $G_{2\text{-}EP3}$, $G_{2\text{-}EP4}$, $G_{0\text{-}NM}$ and $G_{0\text{-}M}$), contamination level (750 $mg.kg^{-1}$ or 2000 $mg.kg^{-1}$ of zinc), and interaction between both factors on each trait independently with two-way permutational analysis of variance (permANOVA). When significant differences were detected, we performed permutational pairwise tests with probability adjustments using the Benjamini-Hochberg (BH) procedure. Because chlorosis state was an ordered qualitative variable, we directly compared the four independent groups (two edaphic groups in two contamination levels) with Kruskal-Wallis tests. When significant differences were detected, we performed pairwise Conover tests with BH adjusted p-values. We used the $G_{0\text{-}NM}$ population submitted to non-contaminated soil as the non-stressful situation for nonmetallicolous population. Thus, it was included on *post-hoc* analysis in order to determine whether populations of nonmetallicolous origin submitted or not to zinc-contaminated soil showed a differential phenotypic response.

Concerning element contents, we tested the effect of population with one way permANOVA, and permutational pairwise tests with BH p-value adjustment.

Concerning CSR scores, we tested the percentage of each ecological strategy exhibited by each population in both conditions. Thus, we used permANOVA to evaluate the effect of population, contamination level and interaction between both factors. When significant differences were detected, we performed permutational pairwise *t* tests with probability adjustments using the Benjamini-Hochberg method.

Lastly, in order to test the effect of population and contamination on phenotypic integration, we calculated Pearson's correlations among raw variables and evaluated their significance using a correlation test with BH-adjusted p-values. The calculated variables were not used because they were highly correlated with the raw variables. Correlation matrixes were compared by performing Steiger's tests.

6.3. Results

6.3.1. Phenotypic comparison of ancestral and derived populations

We analyzed the effect of population ($G_{2\text{-}EP1}$, $G_{2\text{-}EP2}$, $G_{2\text{-}EP3}$, $G_{2\text{-}EP4}$, $G_{0\text{-}NM}$ and $G_{0\text{-}M}$), contamination level (750 $mg.kg^{-1}$ or 2000 $mg.kg^{-1}$ of zinc), and interaction between both factors on each trait independently. PermANOVA detected effect of both factors and their interaction for almost all traits [Table S7].

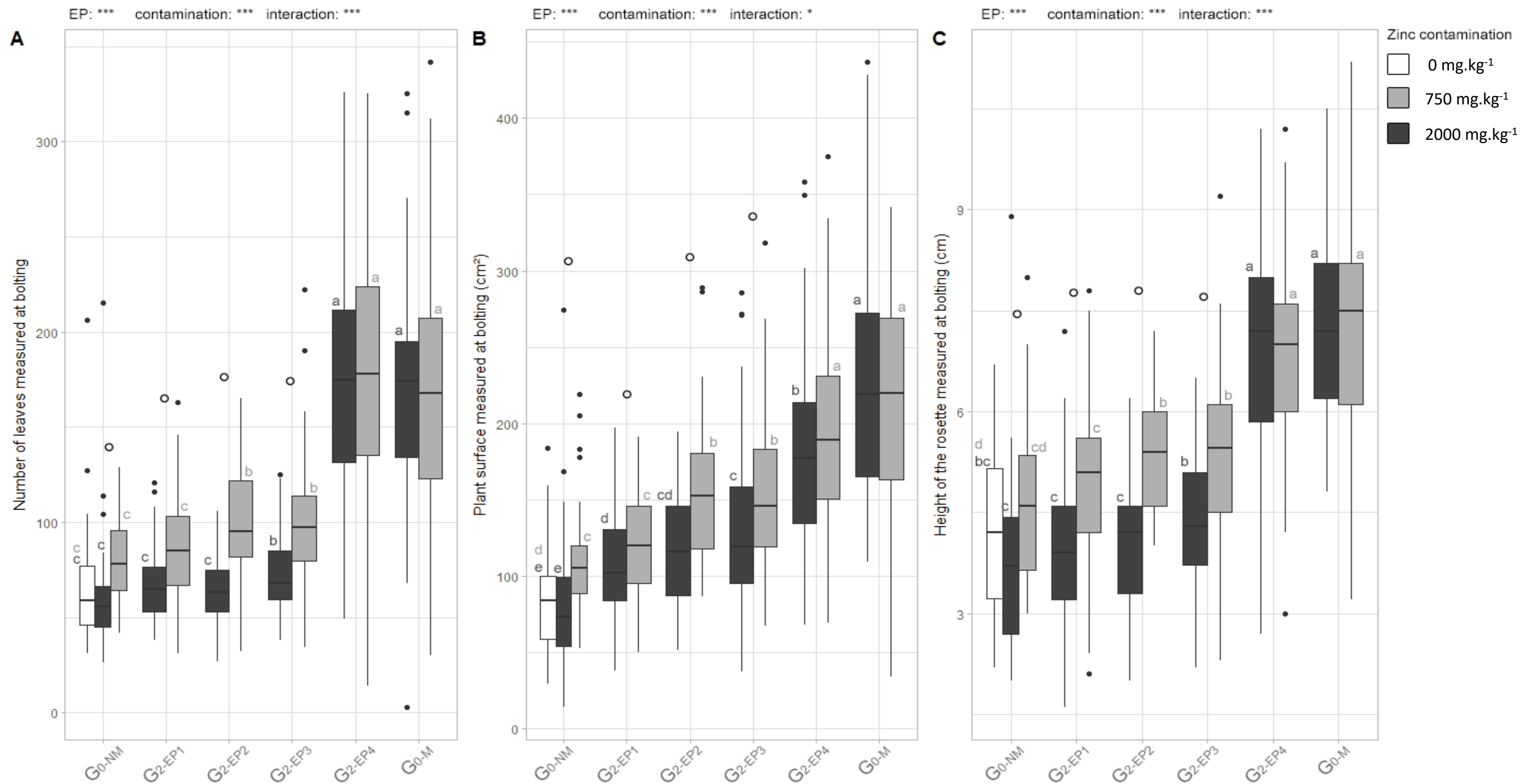


Figure 49: results of permutational ANOVA and pairwise test on vegetative traits measured at bolting, the number of leaves (A), the surface of the plant (B), and the height of rosette (C). Similarly results were observed at t1 and anthesis. White boxplot represent $G_{0\text{-}NM}$ population submitted to non-contaminated soil, light grey boxplot represent populations submitted to 750 $mg.kg^{-1}$ of zinc, and dark grey boxplot represent populations submitted to 2000 $mg.kg^{-1}$ of zinc. G_0: ancestral population; G_2: derived population after two generations; NM: nonmetallicolous populations; M: metallicolous population; "EP" (Experimental population) represent the result for the experimental population factor, "contamination" represent the result for the contamination factor, "interaction" represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001. Boxplots with the same letters are not significantly different at the 5% level, light grey and dark grey letters represent differences among experimental populations submitted to 750 mg.kg-1 and 2000 mg.kg-1 respectively, $G_{0\text{-}NM}$ submitted to non-contaminated soil was compared to other populations in both conditions. ° character represent significant difference between contamination levels in each population.

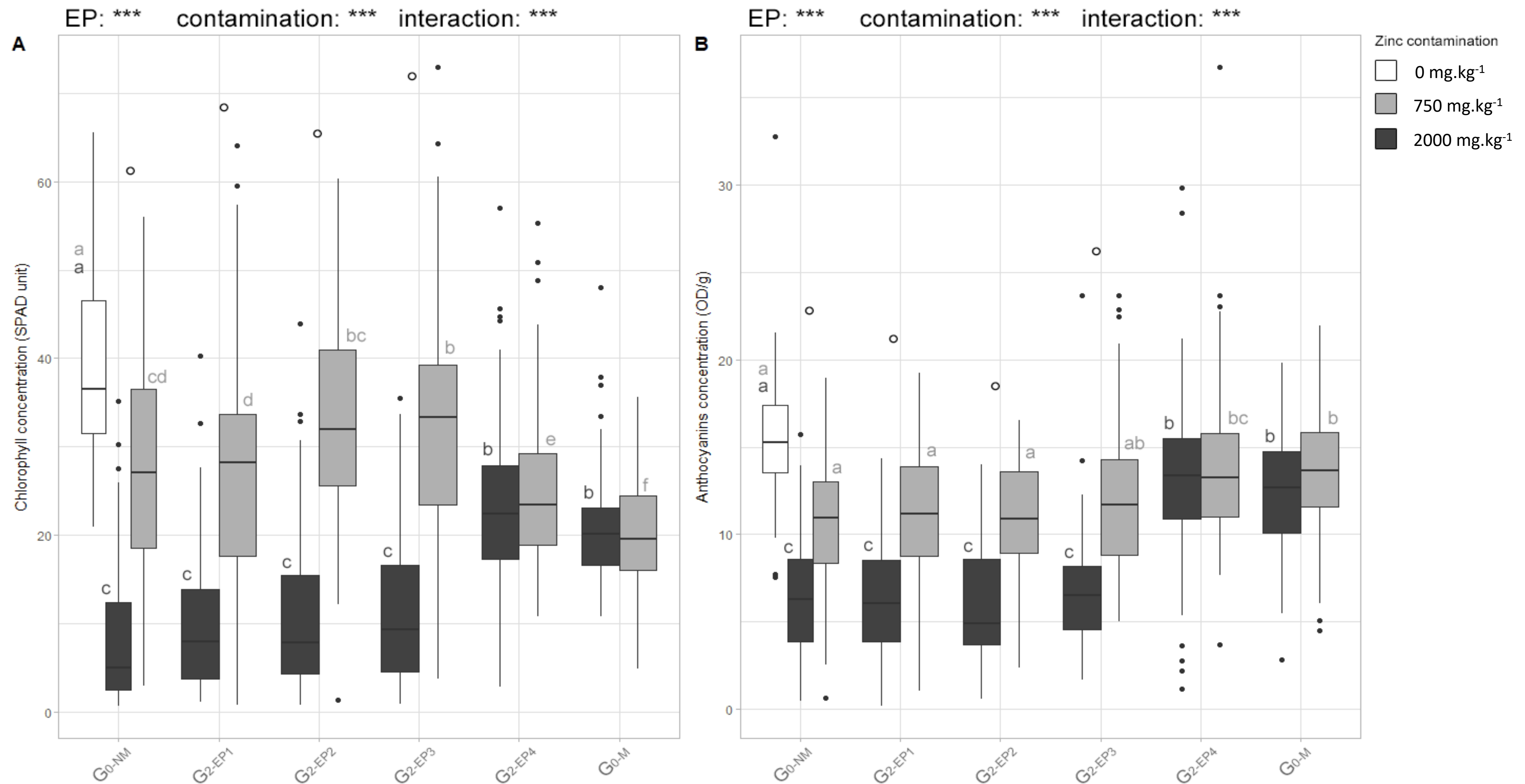


Figure 50: results of permutational ANOVA and pairwise test on biochemical traits, chlorophyll concentration measured with chlorophyll meter (A), and anthocyanin concentration (B). White boxplot represent $G_{0\text{-}NM}$ population submitted to non-contaminated soil, light grey boxplot represent populations submitted to 750 mg.kg^{-1} of zinc, and dark grey boxplot represent populations submitted to 2000 mg.kg^{-1} of zinc. G_0: ancestral population; G_2: derived population after two generations; NM: nonmetallicolous populations; M: metallicolous population; "EP" (Experimental population) represent the result for the experimental population factor, "contamination" represent the result for the contamination factor, "interaction" represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001. Boxplots with the same letters are not significantly different at the 5% level, light grey and dark grey letters represent differences among experimental populations submitted to 750 mg.kg-1 and 2000 mg.kg-1 respectively, $G_{0\text{-}NM}$ submitted to non-contaminated soil was compared to other populations in both conditions. ° Character represent significant difference between contamination levels in each population.

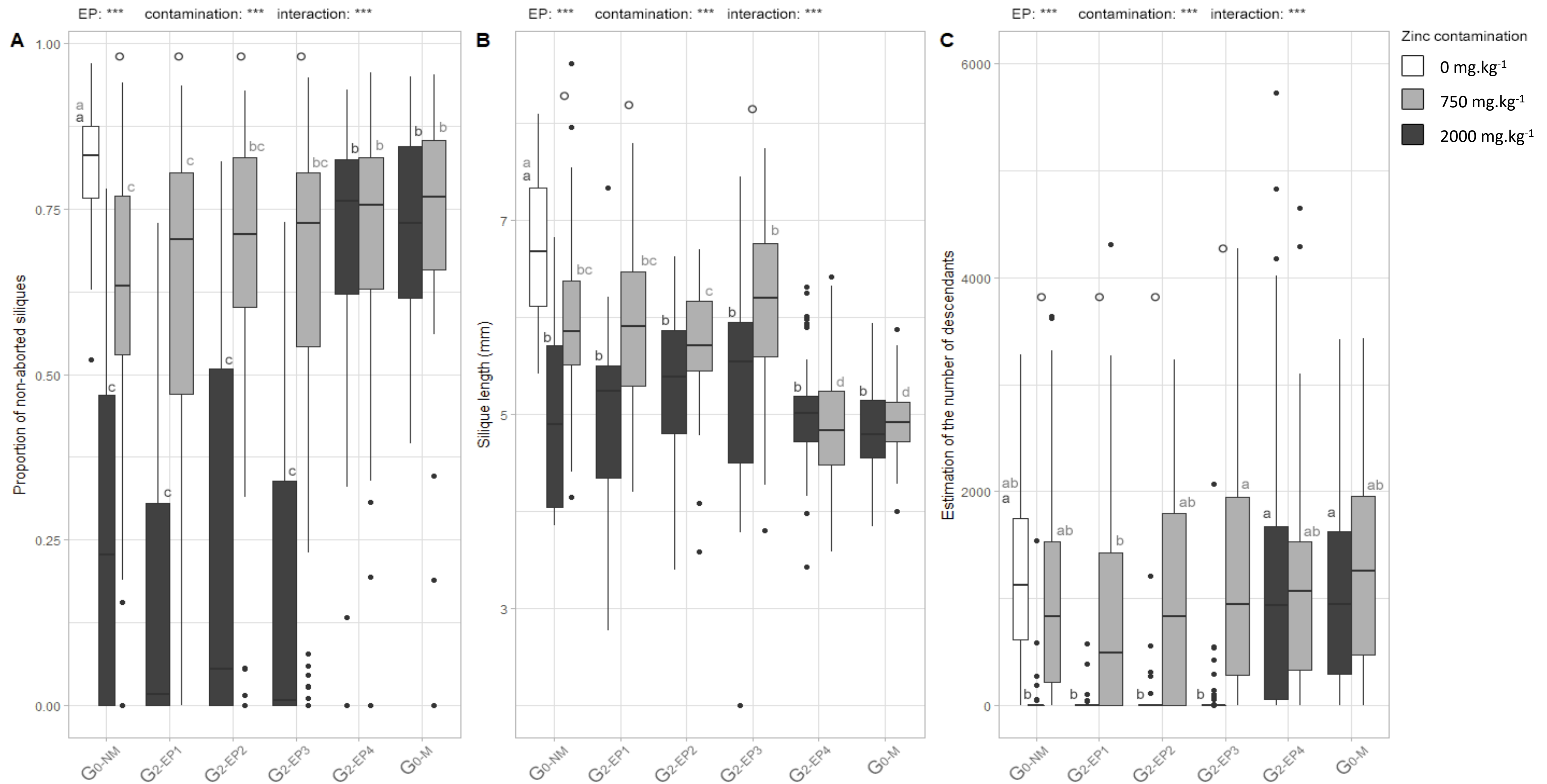


Figure 51: results of permutational ANOVA and pairwise test on reproductive traits, proportion of non-aborted siliques (A), siliques length (B), and estimation of the number of descendants (C). White boxplot represent $G_{0\text{-}NM}$ population submitted to non-contaminated soil, light grey boxplot represent populations submitted to 750 mg.kg^{-1} of zinc, and dark grey boxplot represent populations submitted to 2000 mg.kg^{-1} of zinc. G_0: ancestral population; G_2: derived population after two generations; NM: nonmetallicolous populations; M: metallicolous population; "EP" (Experimental population) represent the result for the experimental population factor, "contamination" represent the result for the contamination factor, "interaction" represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001. Boxplots with the same letters are not significantly different at the 5% level, light grey and dark grey letters represent differences among experimental populations submitted to 750 mg.kg-1 and 2000 mg.kg-1 respectively, $G_{0\text{-}NM}$ submitted to non-contaminated soil was compared to other populations in both conditions. ° Character represent significant difference between contamination levels in each population.

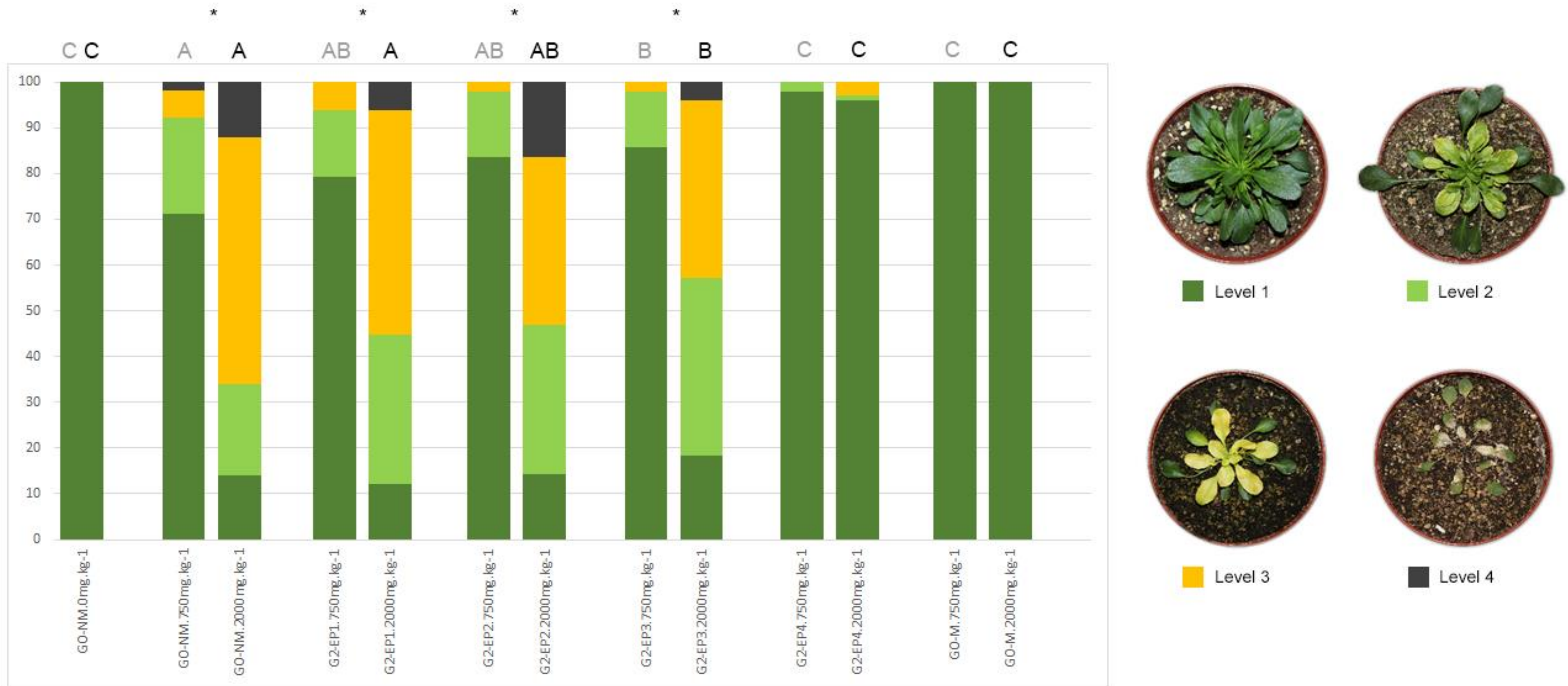


Figure 52: Percentage of plants associated with each chlorosis level measured at bolting in non-contaminated, 750 mg.kg^{-1} of zinc contaminated soil and 2000 mg.kg^{-1} of zinc contaminated soil. Same results were observed at t1 and at anthesis. Level 1: Healthy plant. Level 2: Partially chlorotic plant. Level 3: Entirely chlorotic plant. Level 4: Dead plant. Kruskal-Wallis test showed significant differences (d.f= 12, K=545.7, p-value < 2.2e-16) among populations. Barplots with same letters are not significantly different at the 5% level, light grey and dark grey letters represent differences among experimental populations submitted to 750 mg.kg-1 and 2000 mg.kg-1 respectively, $G_{0\text{-}NM}$ submitted to non-contaminated soil was compared to other populations in both conditions. * Character represent significant difference between contamination levels in each population.

At first, either in the metallicolous or nonmetallicolous control populations, we observed only few significant differences between ancestral and derived populations ($G_{0\text{-}M}$ vs $G_{2\text{-}EP4}$ and $G_{0\text{-}NM}$ vs $G_{2\text{-}EP1}$). This mostly concerned some vegetative traits for both control populations (e.g. LN_{t0}, SP_{t0}, SPBT [Figure 49A]), and, only for the nonmetallicolous control population, some traits related to the architecture of reproductive structures (e.g. NP, NRAM or PR). These results were observed in either zinc contamination conditions.

Metallicolous and nonmetallicolous control populations ($G_{0\text{-}M}$ vs $G_{0\text{-}NM}$ or $G_{2\text{-}EP4}$ vs $G_{2\text{-}EP1}$) showed many differences for both vegetative and reproductive traits. Thus, at 750 $mg.kg^{-1}$ of zinc exposure, we observed higher number of leaves [Figure 49B], higher plant surface [Figure 49A] or higher height of rosette [Figure 49C] higher specific leaf area and lower leaf dry matter content in metallicolous populations. Metallicolous populations also exhibited more photosynthetic pigments [Figure 50A] and more secondary compounds [Figure 50B]. They also showed a tendency to make more racemes, a significant highest proportion of non-aborted siliques [Figure 51A], but significant lower silique length [Figure 51B]. However, no significant differences were observed for the seed set estimation or the number of descendant estimation [Figure 51C]. Differences between control populations were also visible in duration of some phenological stages. Thus, we observed a longer vegetative period, with a later bolting time but a shorter bolting period in metallicolous populations. At last, we observed strong differences in chlorosis levels between both control populations, with higher chlorosis levels in nonmetallicolous populations [Figure 52]. In 2000 $mg.kg^{-1}$ of zinc exposure, differences among control ancestral and derived populations became more pronounced mostly because the values for nonmetallicolous populations strongly changed, but the same tendencies remained [Figures 49, 50 and 51].

Comparisons between test derived populations and nonmetallicolous control derived ($G_{2\text{-}EP1}$ vs $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$) or ancestral population ($G_{0\text{-}NM}$ vs $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$) showed that in 750 $mg.kg^{-1}$ of zinc exposure almost all vegetative morphological traits measured at t1, bolting or anthesis significantly changed in exposed derived populations $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$, with higher number of leaves [Figure 49A], higher surface of plant [Figure 49B], higher leaf length, higher leaf surface and higher height of rosette [Figure 49C]. In addition, significantly shorter germination time, lower specific leaf area, and higher leaf thickness were observed in $G_{2\text{-}EP2}$ compared to $G_{0\text{-}NM}$, as well as significantly higher photosynthetic yield, higher number of ramification, higher number of siliques, higher seed set estimation and higher number of descendant [Figure 51C] in $G_{2\text{-}EP3}$ compared to $G_{2\text{-}EP1}$. Chlorosis level was also significantly

lower in $G_{2\text{-}EP3}$ compared to $G_{2\text{-}EP1}$ [Figure 52]. In 2000 $mg.kg^{-1}$ of zinc exposure, we observed differences only on vegetative traits, with significant higher number of leaves, higher plant surface [Figure 49B] and higher leaf length in $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$ compared to $G_{0\text{-}NM}$ and only for $G_{2\text{-}EP3}$ compared to $G_{2\text{-}EP1}$. We also observed higher height of rosette in $G_{2\text{-}EP3}$ compared to $G_{0\text{-}NM}$ and $G_{2\text{-}EP1}$ [Figure 49B].In addition, $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$ differed for only three traits, in 750 $mg.kg^{-1}$ of zinc exposure only: leaf number measured at t0, leaf morphology measured at bolting, and silique length [Figure 51B].

Finally, test derived populations and metallicolous control derived populations ($G_{2\text{-}EP4}$ vs $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$) were less differentiated than metallicolous and nonmetallicolous control populations ($G_{0\text{-}M}$ vs $G_{0\text{-}NM}$ or $G_{2\text{-}EP4}$ vs $G_{2\text{-}EP1}$). Thus, in 750 $mg.kg^{-1}$ of zinc exposure, no significant differences were observed for most of the biochemical and reproductive traits. Significant differences appeared only for the chlorophyll content [Figure 50A], anthocyanin concentration [Figure 50B], and siliques length [Figure 51B], with more chlorophyll concentration in $G_{2\text{-}EP2}$/ $G_{2\text{-}EP3}$ than $G_{2\text{-}EP4}$, less anthocyanin concentration in $G_{2\text{-}EP2}$ than $G_{2\text{-}EP4}$ and higher siliques length in $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$. In 2000 $mg.kg^{-1}$ of zinc exposure, $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$ behave like nonmetallicolous ancestral and control populations, thus showing significant differences with $G_{2\text{-}EP4}$.

Compared to other nonmetallicolous populations ($G_{0\text{-}NM}$, $G_{2\text{-}EP1}$, $G_{2\text{-}EP2}$, $G_{2\text{-}EP3}$) in contaminated conditions (750 and 2000 $mg.kg^{-1}$ of zinc), unexposed $G_{0\text{-}NM}$ exhibited lower values of vegetative morphological traits [Figure 49A, Figure 49B, Figure 49C], higher pigments and secondary compounds concentrations [Figure 50A, Figure 50B] and higher fecundity [Figure 51A, Figure 51B, Figure 51C].

6.3.2. Comparison of metal contents among ancestral and derived populations

PermANOVA detected population effect on aluminum, bore, potassium, magnesium, manganese, phosphorus, sulfur and zinc concentrations [Table S8]. Except for aluminum concentration, significant differences were observed between $G_{0\text{-}M}$ and other populations, with higher concentrations of bore [Figure 53A], potassium [Figure 53B], manganese, and phosphorus, and lower concentrations of sulfur and zinc [Figure 53C]. We also observed differences among populations from nonmetallicolous origin for bore [Figure 53A], potassium [Figure 53B], phosphorus, and zinc concentrations [Figure 53C], with higher concentrations of bore and potassium in $G_{2\text{-}EP3}$ than $G_{2\text{-}EP1}$ and lower concentrations of zinc in $G_{2\text{-}EP3}$ than in $G_{2\text{-}EP2}$.

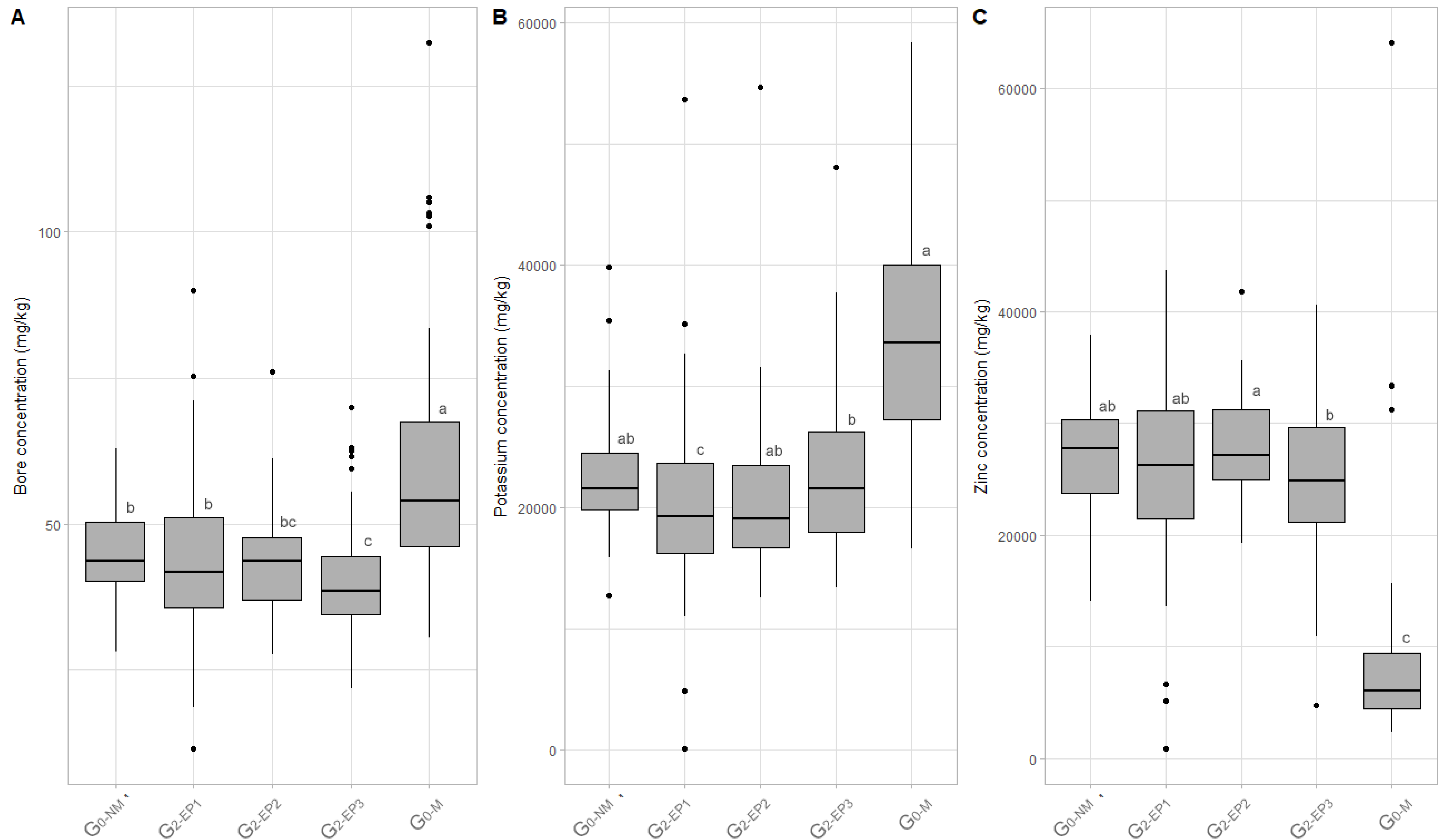


Figure 53: results of permutational ANOVA and pairwise test on element contents, bore concentration (A), potassium concentration (B), and zinc concentration (C). G_0: ancestral population; G_2: derived population after 2 generations; NM: nonmetallicolous populations; M: metallicolous population; EP: Experimental population. Boxplots with the same letters are not significantly different at the 5% level.

6.3.3. CSR classification of ancestral and derived populations

Most of variation within and among populations occurred along the S-R axis and differed according to zinc condition. Indeed, in 750 $mg.kg^{-1}$ of zinc exposure, most of the individuals were classified as CR or CRS [Figure 54A], while in 2000 $mg.kg^{-1}$ of zinc exposure, most of the individuals were classified as CR, CRS, and SR [Figure 54B]. For unexposed G_{0-NM} population, most of the individuals were classified as CR. Also, in 750 $mg.kg^{-1}$ and 2000 $mg.kg^{-1}$ of zinc exposure, there were more individuals classified as CR in populations of metallicolous origin.

PermANOVA showed an effect of population, contamination and their interaction for C and S scores, and only an effect of population for R score. Thus, for 750 $mg.kg^{-1}$ of zinc exposure, we observed higher C scores in G_{2-EP2} and G_{2-EP3} but lower C scores in G_{0-NM} and G_{2-EP1}, while intermediate scores were observed in G_{2-EP4} and G_{0-M} [Figure 54C]. We observed higher S scores in G_{2-EP1} and lower S scores in G_{2-EP4} and G_{0-M}, while intermediate scores were observed in G_{0-NM}, G_{2-EP2} and G_{2-EP3} [Figure 54D]. Finally, we observed higher R scores in G_{2-EP4} and G_{0-M}, lower R scores in G_{2-EP1} and G_{2-EP2}, and intermediate values in G_{2-EP3} and G_{0-NM} [Figure 54E]. In the 2000 $mg.kg^{-1}$ zinc exposure, significant differences between populations of metallicolous and nonmetallicolous origin were mostly detected for S and C scores.

Finally, we observed that unexposed G_{0-NM} population significantly exhibited the lowest S scores, the highest R scores and intermediate C scores.

6.3.4. Comparison of correlation matrixes

All correlation matrixes were different from each other ($p<1.10^{-16}$) [Figure S3]. We particularly observed several differences between populations from metallicolous and non-metallicolous origins. There were fewer differences between the correlation matrices of the metallicolous populations in the two contaminated conditions, where as in nonmetallicolous populations, we observed a general increase of significant correlations in high zinc concentration (2000 $mg.kg^{-1}$). We also observed significant negative correlations between flowering period and fecundity (NNAS, SS or ND) for nonmetallicolous populations, especially in high zinc concentration. In the same way, for nonmetallicolous populations, we observed significant but weak correlations between vegetative traits and element contents. Moreover, we also observed negative correlations between phenological and reproductive traits in all matrixes.

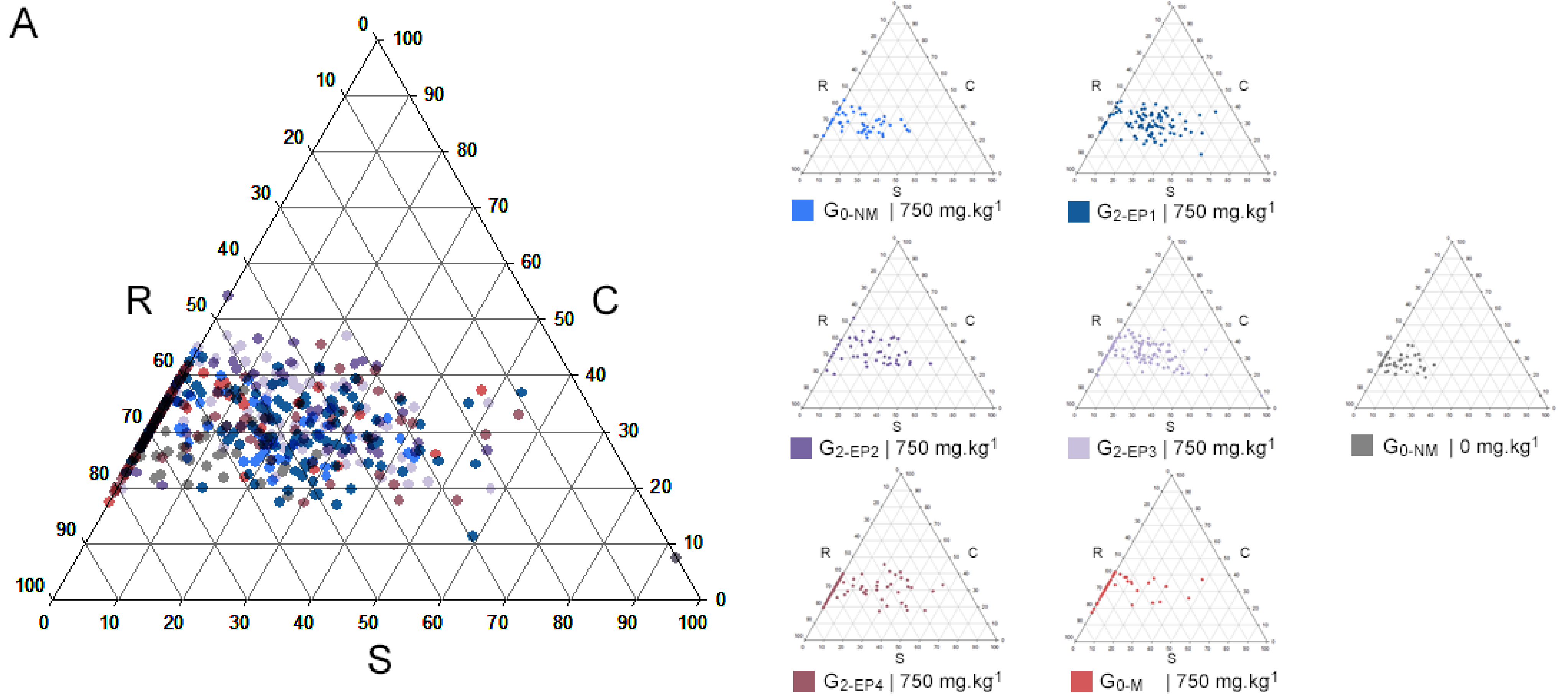
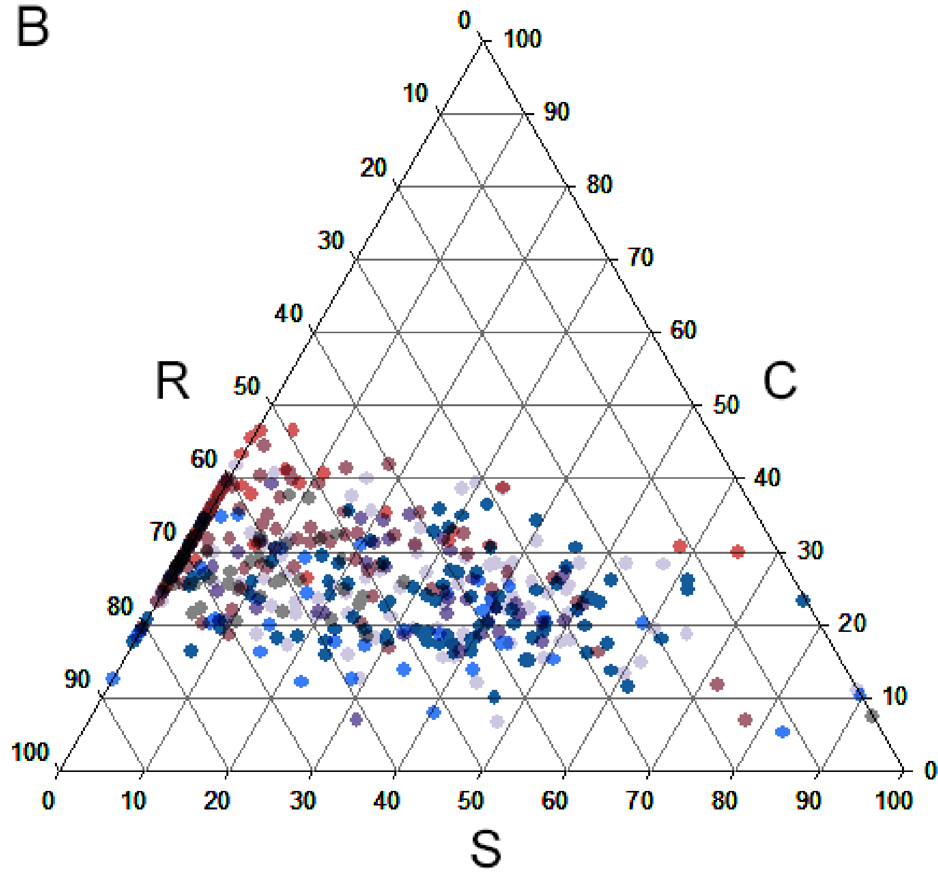


Figure 54: (A) CSR representation of individuals from ancestral (G_0) and derived populations (G_2) submitted to 750 mg.kg-1 of zinc contaminated soil. Dots were colored according to populations. For more visibility, we represented each population independently. As reference of non-stressful situation of nonmetallicolous population, we represented the $G_{0\text{-NM}}$ population submitted to non-contaminated soil.



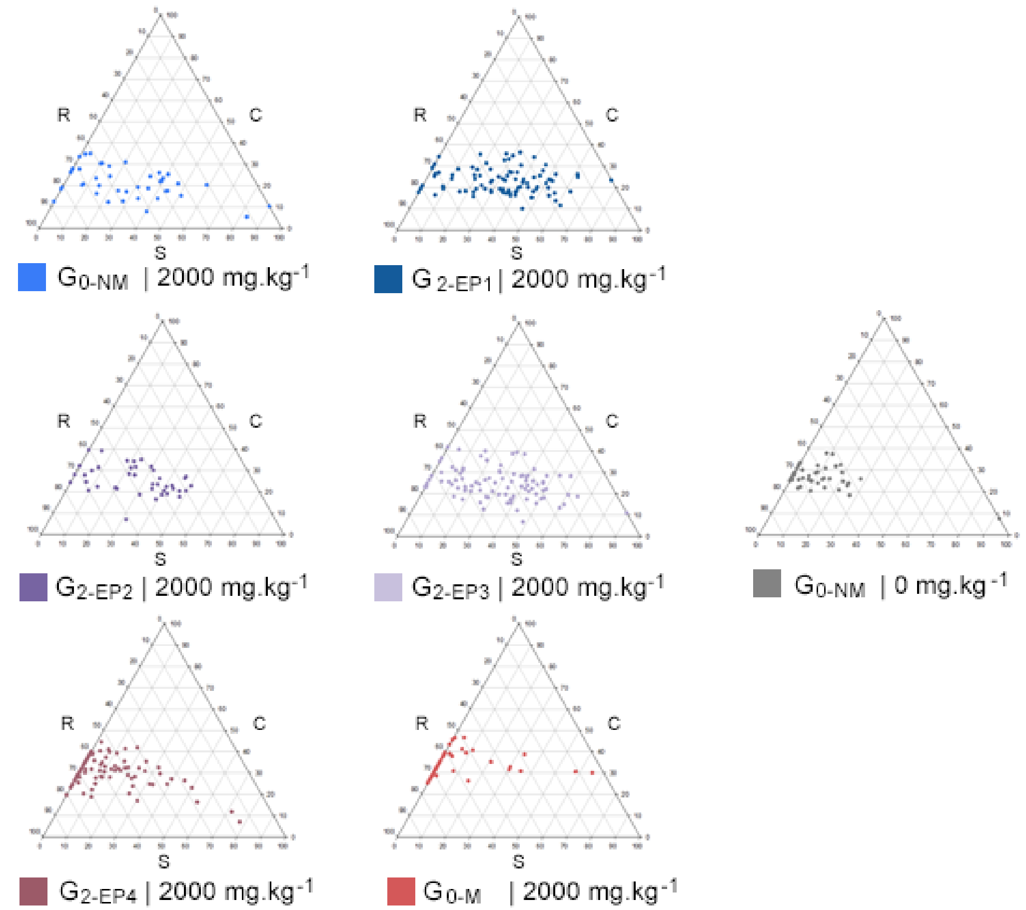


Figure 54 (continued): (B) CSR representation of individuals from ancestral (G_0) and derived populations (G_2) submitted to 2000 mg.kg-1 of zinc contaminated soil. Dots were colored according to populations. For more visibility, we represented each population independently. As reference of non-stressful situation of nonmetallicolous population, we represented the $G_{0\text{-}NM}$ population submitted to non-contaminated soil.

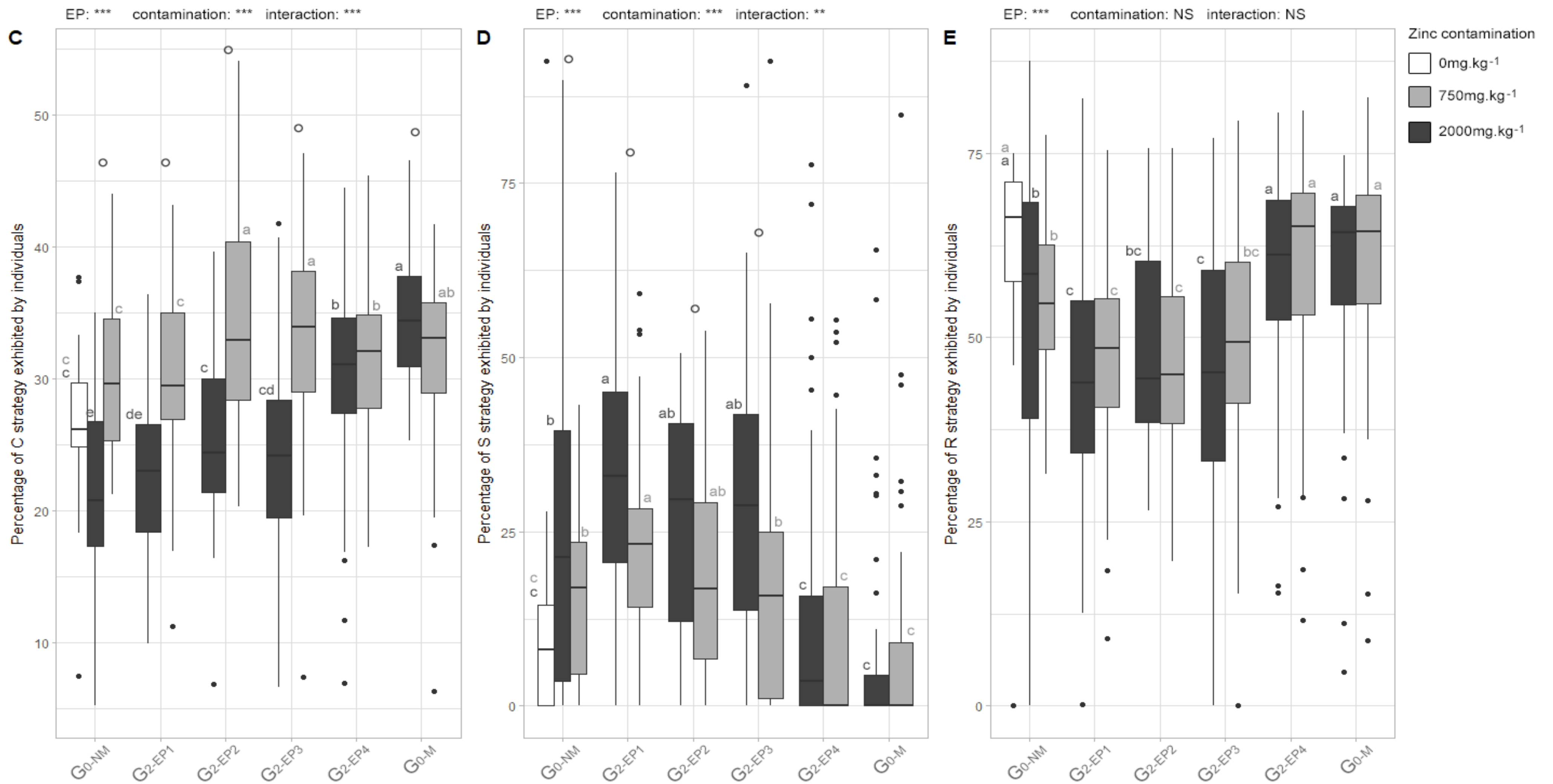


Figure 54 (continued): results of permutational ANOVA and pairwise test on CSR scores, percentage of C strategy (A), percentage of S strategy (B), and percentage of R strategy (C). White boxplot represent $G_{0\text{-NM}}$ population submitted to non-contaminated soil, light grey boxplot represent populations submitted to 750mg.kg^{-1} of zinc, and dark grey boxplot represent populations submitted to 2000mg.kg^{-1} of zinc. G_0: ancestral population; G_2: derived population after 2 generations; NM: nonmetallicolous populations; M: metallicolous population; "EP" (Experimental population) represent the result for the experimental population factor, "contamination" represent the result for the contamination factor, "interaction" represent the result for the interaction between edaphic group and contamination level. *: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001. Boxplots with the same letters are not significantly different at the 5% level, light grey and dark grey letters represent differences among experimental populations submitted to 750mg.kg-1 and 2000mg.kg-1 respectively, $G_{0\text{-NM}}$ submitted to non-contaminated soil was compared to other populations in both conditions. ° Character represent significant difference between contamination levels in each population.

6.4. Key results and short discussion

6.4.1. Response of control populations

First, results of the comparisons of phenotypic levels, at both concentrations, showed few differences between $G_{2\text{-}EP4}$ and $G_{0\text{-}M}$, suggesting that our experimental design did not induce a counterselection of tolerance in metallicolous plants. On the other hand, some differences could be detected between $G_{2\text{-}EP1}$ and $G_{0\text{-}NM}$, the direction of the difference depending on the studied trait. These differences could be explained by several reasons. (1) Culture conditions may have induced direct or indirect selection for more biomass. (2) It was possible that transgenerational effects may be expressed in both populations. The parental environment (natural soil for parents of $G_{0\text{-}NM}$ population or artificial soil for $G_{1\text{-}EP1}$) would induce the expression of different phenotypes in each progeny. (3) An effect of genetic drift, related to population size, which would induce a random evolution of the phenotype.

6.4.2. Evolution of the nonmetallicolous population phenotype after two generations of selection in zinc contaminated soil

The most interesting results concerned the phenotypic response observed in $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$. Globally, we observed a strong response for vegetative traits in these two populations compared to $G_{2\text{-}EP1}$. Plants of $G_{2\text{-}EP2}$ and $G_{2\text{-}EP3}$ produced significantly more leaves, larger plant surface, higher rosette and more chlorophyll (measured with a chlorophyll meter). Also, the response appears to be stronger in $G_{2\text{-}EP3}$ with significantly different phenotypes compared to $G_{2\text{-}EP1}$ for some physiological and reproductive traits, with less chlorosis, more flower stems, better progeny survival rate and a higher expected number of descendants.

It is also important to note that the responses of the $G_{2\text{-}EP3}$ and $G_{2\text{-}EP2}$ populations converge. Indeed, very few significant differences between the two populations were observed.

The decrease of chlorosis [Chaney, 1993; Assunção *et al.*, 2006; Rout & Das, 2009], the increase of biomass [Ingrouille & Smirnoff, 1986; Escarré *et al.*, 2000], or the number of seeds (and therefore descendants) [Jiménez-Ambriz *et al.*, 2007; Dechamps *et al.*, 2008] were used in the literature to show higher tolerance levels. Thus, it appears that the EP_2 and EP_3 evolved to higher tolerance levels.

Supplemental material

Table S7: Results of permutational analysis of variance: effect of experimental population, contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured on three largest leaves at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured on three largest leaves at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured on three largest leaves at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured on three largest leaves at t1 and bolting. Phi-PSII: average photosynthetic yield measured at t1 on three representative leaves; HR: Height of the rosette measured at bolting. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant.

	Experimental population (EP)					Condition					EP * Condition interaction				
Variable	df	MS	F	P value	P value (Perm)	df	MS	F	P value	P value (Perm)	df	MS	F	P value	P value (Perm)
LN_{t0}	5	5,36	53,79	**0**	**0,001**	1	0,09	4,53	**0,03**	**0,04**	5	0,085	0,85	0,51	0,54
SP_{t0}	5	288,51	49,65	**0**	**0,001**	1	4,67	4,02	0,045	0,056	5	6	1,03	0,4	0,39
LN_{t1}	5	153	92,88	**0**	**0,001**	1	36,97	112,24	**0**	**0,001**	5	6,83	4,15	**0,001**	**0,001**
SP_{t1}	5	257,5	29,37	**0**	**0,001**	1	40	22,8	**0**	**0,001**	5	8,46	0,96	0,44	0,46
LL_{t1}	5	0,16	28,86	**0**	**0,001**	1	0,02	17,6	**0**	**0,001**	5	0,009	1,67	0,14	0,136
PR_{t1}	5	0,02	56,02	**0**	**0,001**	1	0,0007	9,69	**0,002**	**0,003**	5	0,0007	1,82	0,1	0,09
LS_{t1}	5	0,46	45,6	**0**	**0,001**	1	0,06	30	**0**	**0,001**	5	0,04	3,9	**0,002**	**0,002**
LM_{t1}	5	0,002	8,54	**0**	**0,001**	1	0,001	29,2	**0**	**0,001**	5	0,009	3,55	**0,003**	**0,006**
Phi-PSII	5	0,0004	15,95	**0**	**0,001**	1	0,0005	105,64	**0**	**0,001**	5	0,0002	10,35	**0**	**0,001**
LN_{BT}	5	2138,2	202,7	**0**	**0,001**	1	77,05	36,52	**0**	**0,001**	5	33,46	3,17	**0,008**	**0,012**
SP_{BT}	5	1481,3	94,88	**0**	**0,001**	1	67,98	21,77	**0**	**0,001**	5	40,97	2,62	**0,023**	**0,04**
LL_{BT}	5	0,35	55,16	**0**	**0,001**	1	0,03	20,7	**0**	**0,001**	5	0,02	2,71	**0,02**	**0,025**
PR_{BT}	5	0,06	143	**0**	**0,001**	1	0,002	2,73	0,1	0,09	5	0,0007	1,75	0,12	0,11
LS_{BT}	5	0,46	20,69	**0**	**0,001**	1	0,1	22,3	**0**	**0,001**	5	0,08	3,73	**0,0024**	**0,005**
LM_{BT}	5	0,003	4,9	**0,0002**	**0,001**	1	0,001	7,61	**0,006**	**0,006**	5	0,003	4,47	**0,0005**	**0,001**
HR	5	1,33	162,9	**0**	**0,001**	1	0,1	60,46	**0**	**0,001**	5	0,08	9,47	**0**	**0,001**
SP_{ANT}	5	1433,94	81,53	**0**	**0,001**	1	84,55	24,04	**0**	**0,001**	5	41,3	2,35	**0,04**	**0,035**

Table S7 (continued): Results of permutational analysis of variance: effect of experimental population, contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. GERM: time interval between sowing and germination converted on GDD (Growth degree days); BT: time interval between germination and bolting time converted on GDD; ANT: time interval between germination and anthesis converted on GDD; BP: bolting period - time interval between bolting and anthesis converted on GDD; FFE: time interval between germination and first fruit emergence on the main flower stem converted on GDD; EF: end of flowering - time interval between germination and the dehiscence of the last flower on the main flower stem converted on GDD; FP: flowering period - time interval between anthesis and the dehiscence of the last flower on the main flower stem converted on GDD; DEH: time interval between germination and dehiscence of the last silique converted on GDD; RP: time interval between anthesis and dehiscence of the last silique converted on GDD; SLA: average specific leaf area calculated at bolting on three leaves representative of the chlorosis state of the plant; LDMC: average leaf dry matter content calculated at bolting on three leaves representative of the chlorosis state of the plant; LT : average leaf thickness calculated at bolting on three leaves representative of the chlorosis state of the plant; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting on five leaves representative of the chlorosis state of the plant. D.f: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant.

	Experimental population (EP)					Condition					EP * Condition interaction				
Variable	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**
GERM	5	321,62	6,07	**0**	**0,001**	1	48,65	4,59	**0,03**	**0,032**	5	28,71	0,54	0,74	0,74
BT	5	1107,1	3,14	**0,008**	**0,007**	1	4567,67	64,87	**0**	**0,001**	5	624,84	1,77	0,11	0,13
ANT	5	790,77	3,23	**0,007**	**0,004**	1	3708,8	75,8	**0**	**0,001**	5	905,2	3,7	**0,003**	**0,003**
BP	5	3185,14	18,08	**0**	**0,001**	1	180,69	5,13	**0,02**	**0,03**	5	452,36	2,57	**0,026**	**0,019**
FFE	5	843,24	3,38	**0,005**	**0,003**	1	3940,8	78,91	**0**	**0,001**	5	1160,7	4,64	**0,0003**	**0,001**
EF	5	3255,2	8,81	**0**	**0,001**	1	5160,75	69,82	**0**	**0,001**	5	2876,4	7,78	**0**	**0,001**
FP	5	2006,583	8,38	**0**	**0,001**	1	1382,06	28,86	**0**	**0,001**	5	1591,7	6,65	**0**	**0,001**
DEH	5	5675	3,98	**0,0014**	**0,003**	1	1675,22	5,88	**0,016**	**0,013**	5	2589,96	1,82	0,11	0,1
RP	5	4913,335	3,49	**0,004**	**0,005**	1	5,19	0,018	0,89	0,89	5	1173,9	0,83	0,53	0,49
SLA	5	2,44	23,17	**0**	**0,001**	1	0,013	0,64	0,42	0,43	5	0,11	1,07	0,38	0,4
LDMC	5	263,64	18,02	**0**	**0,001**	1	23,05	7,88	**0,005**	**0,004**	5	5,08	0,35	0,88	0,88
LT	5	0	2,85	**0,015**	**0,016**	1	0	1,89	0,17	0,18	5	0	1,86	0,1	0,09
CHLORO	5	4,9	7,9	**0**	**0,001**	1	38,58	311,57	**0**	**0,001**	5	20,35	35,87	**0**	**0,001**
Chla	5	0,39	6,98	**0**	**0,001**	1	0,14	12,44	**0,0004**	**0,001**	5	0,17	3,04	**0,01**	**0,01**
Chlb	5	0,09	1,4	0,22	0,2	1	0,013	0,96	0,33	0,34	5	0,05	0,83	0,53	0,53
Car	5	0,069	0,46	0,8	0,8	1	0,05	1,63	0,2	0,21	5	0,12	0,8	0,55	0,54
Antho	5	4,18	48,9	**0**	**0,001**	1	2,43	142,24	**0**	**0,001**	5	0,98	11,5	**0**	**0,001**
Flavo	5	0,017	4,07	**0,001**	**0,001**	1	0,0004	0,49	0,48	0,49	5	0,0028	0,66	0,65	0,65
Pheno	5	0,18	20,5	**0**	**0,001**	1	0,05	30,2	**0**	**0,001**	5	0,01	1,19	0,31	0,34
Tan	5	0,052	29,09	**0**	**0,001**	1	0,036	100,41	**0**	**0,001**	5	0,02	6,93	**0**	**0,001**

Table S7 (continued): Results of permutational analysis of variance: effect of experimental population, contamination level and interaction between both factors on phenotypic traits. Each test was realized with 1000 permutations. NR: number of racemes; NP: number of panicles; NRAM: number of ramifications; NFS: number of flower stems; PR: proportion of racemes; PP: proportion of panicles; RPR: ratio between number of panicles and racemes; maxLFS: height of the largest flower stem; HFF: height to the first fruit; NS: number of silique produced by whole plant; NAS: number of aborted siliques produced by whole plant; NNAS: number of non-aborted siliques produced by whole plant; PAS: proportion of aborted siliques on the whole plant; PNAS: proportion of non-aborted siliques on the whole plant; RS: ratio between the number of non-aborted and the number of aborted siliques on the whole plant; SL: silique length calculated on the whole plant; SS: estimated seed set produced by whole plant; SR: survival rate of descendant after five weeks after sowing; ND: estimated number of descendants (SS*SR). CSR-C, CRS-S, CSR-R: C, S and R score calculated with Pierce *et al.* (2007) algorithm. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant.

	Experimental population (EP)					**Condition**					**EP * Condition interaction**				
Variable	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**	**df**	**MS**	**F**	**P value**	**P value (Perm)**
NR	5	0,25	2,36	**0,038**	**0,034**	1	0,019	0,93	0,34	0,35	5	0,13	1,23	0,29	0,29
NP	5	1,53	15,15	**0**	**0,001**	1	0,8	39,67	**0**	**0,001**	5	1,04	10,3	**0**	**0,001**
NRAM	5	20,13	17,62	**0**	**0,001**	1	9,85	43,12	**0**	**0,001**	5	12,21	10,69	**0**	**0,001**
NFS	5	3,5	38,42	**0**	**0,001**	1	4,37	239,75	**0**	**0,001**	5	2,24	24,55	**0**	**0,001**
PR	5	0,008	9,2	**0**	**0,001**	1	0,0054	30,77	**0**	**0,001**	5	0,008	9,35	**0**	**0,001**
PP	5	0,008	9,2	**0**	**0,001**	1	0,0054	30,77	**0**	**0,001**	5	0,008	9,35	**0**	**0,001**
RPR	5	0,21	3,34	**0,005**	**0,009**	1	0,0087	0,69	0,41	0,44	5	0,035	0,56	0,73	0,72
maxLFS	5	24,8	48,39	**0**	**0,001**	1	19,08	186,11	**0**	**0,001**	5	14,06	27,44	**0**	**0,001**
HFF	5	0,91	7,37	**0**	**0,001**	1	1,51	61,22	**0**	**0,001**	5	1,53	12,42	**0**	**0,001**
NS	5	24443,6	50,16	**0**	**0,001**	1	25251	259,11	**0**	**0,001**	5	14661,52	30,1	**0**	**0,001**
NAS	5	391,4	5,02	**0,0004**	**0,001**	1	462,91	29,68	**0**	**0,001**	5	460,84	5,91	**0**	**0,001**
NNAS	5	18631,1	37,12	**0**	**0,001**	1	10872,2	108,32	**0**	**0,001**	5	7941,97	15,82	**0**	**0,001**
PAS	5	0,02	55,7	**0**	**0,001**	1	0,015	212,04	**0**	**0,001**	5	0,01	28,23	**0**	**0,001**
PNAS	5	0,02	55,62	**0**	**0,001**	1	0,015	211,32	**0**	**0,001**	5	0,01	28,17	**0**	**0,001**
RS	5	1,05	18,42	**0**	**0,001**	1	0,62	54,37	**0**	**0,001**	5	0,31	5,49	**0,0001**	**0,001**
SL	5	0,079	18,02	**0**	**0,001**	1	0,049	55,8	**0**	**0,001**	5	0,043	9,85	**0**	**0,001**
SS	5	298512,9	27,3546	**0**	**0,001**	1	521749,5	239,06	**0**	**0,001**	5	312252,9	28,61	**0**	**0,001**
SR	5	0,013	31,67	**0**	**0,001**	1	0,0063	76,43	**0**	**0,001**	5	0,004	9,78	**0**	**0,001**
ND	5	81632,1	19,44	**0**	**0,001**	1	100404,7	119,53	**0**	**0,001**	5	61899,8	14,74	**0**	**0,001**
CSR-C	5	5,18	21,5	**0**	**0,001**	1	5,73	118,97	**0**	**0,001**	5	4,5	18,7	**0**	**0,001**
CSR-S	5	51,2	33,5	**0**	**0,001**	1	9,3	30,3	**0**	**0,001**	5	5,66	3,7	**0,0025**	**0,002**
CSR-R	5	29,68	23,77	**0**	**0,001**	1	0,42	1,7	0,19	0,21	5	0,49	0,39	0,85	0,87

Table S8: Results of permutational analysis of variance: effect of experimental population on aluminium (Al), bore (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), sulfur (S) and zinc (Zn) concentration. Element contents were determinate by ICP-AES emission spectrophotometry from three matures leaves harvested at bolting time. df: degree of freedom, MS: mean square, F: F statistic of ANOVA, P value: parametric p-value calculated with ANOVA, P value (Perm): The p-value of the permutation test. P-value in bold characters was significant.

	df	MS	F	P value	P value (Perm)
Al	4	177,54	2,86	**0,024**	**0,015**
B	4	3424,8	21,88	**4,80E-16**	**0,001**
Ca	4	6349248	0,18	0,95	0,95
Cu	4	0,44	0,38	0,38	0,8
Fe	4	290,75	0,41	0,8	0,84
K	4	1658591835	35,96	**5,56E-16**	**0,001**
Mg	4	140286110	24,88	**4,88E-18**	**0,001**
Mn	4	32298,38	4,42	**0,002**	**0,002**
Na	4	973724,5	2,31	0,06	0,07
P	4	2187701,5	3,98	**0,004**	**0,005**
S	4	89965619	8,92	**7,50E-07**	**0,001**
Zn	4	2975862719	55,86	**5,10E-36**	**0,001**

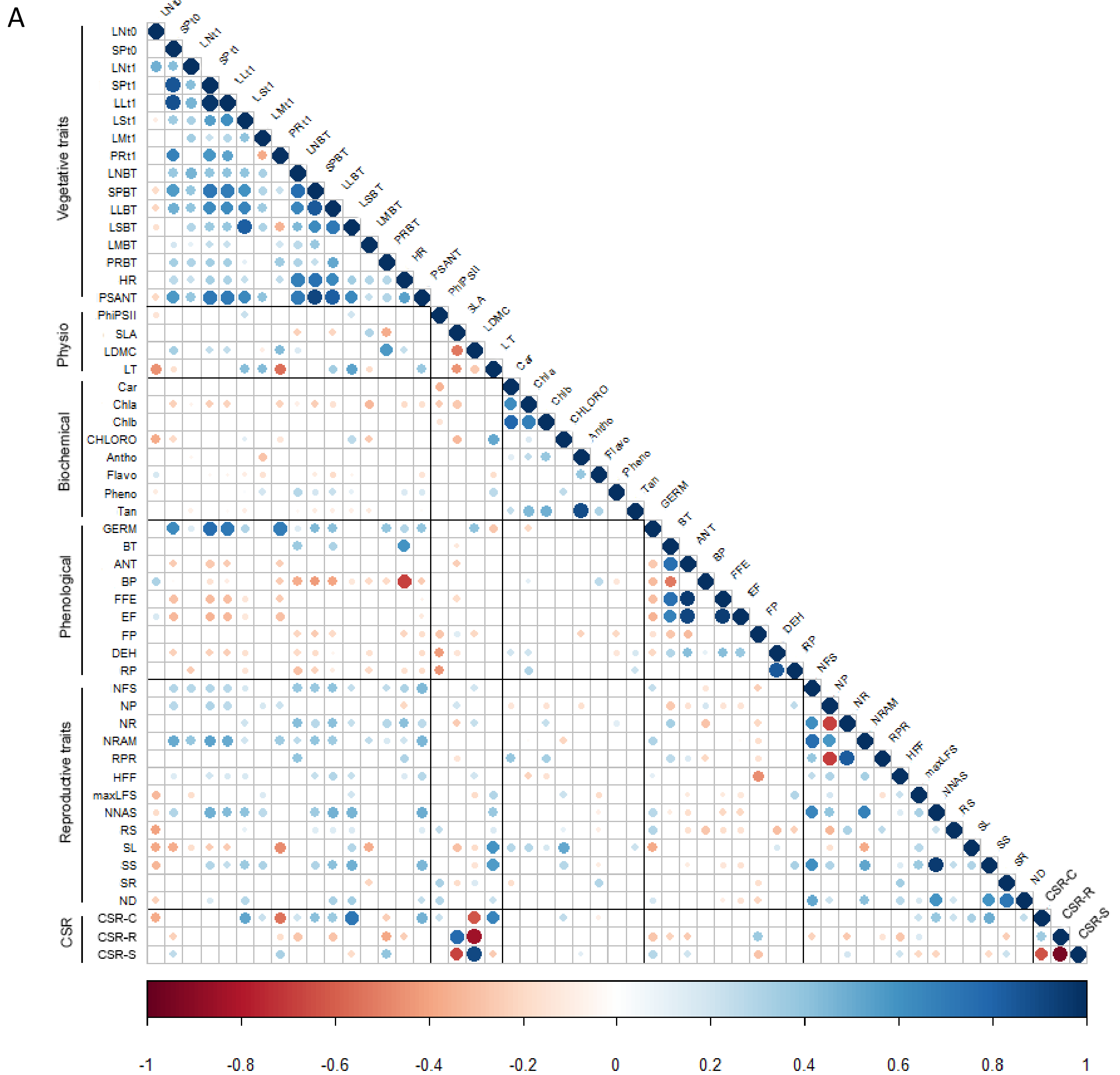


Figure S6: Correlation matrixes among traits in (A) $G_{0\text{-}NM}$ population submitted to non-polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

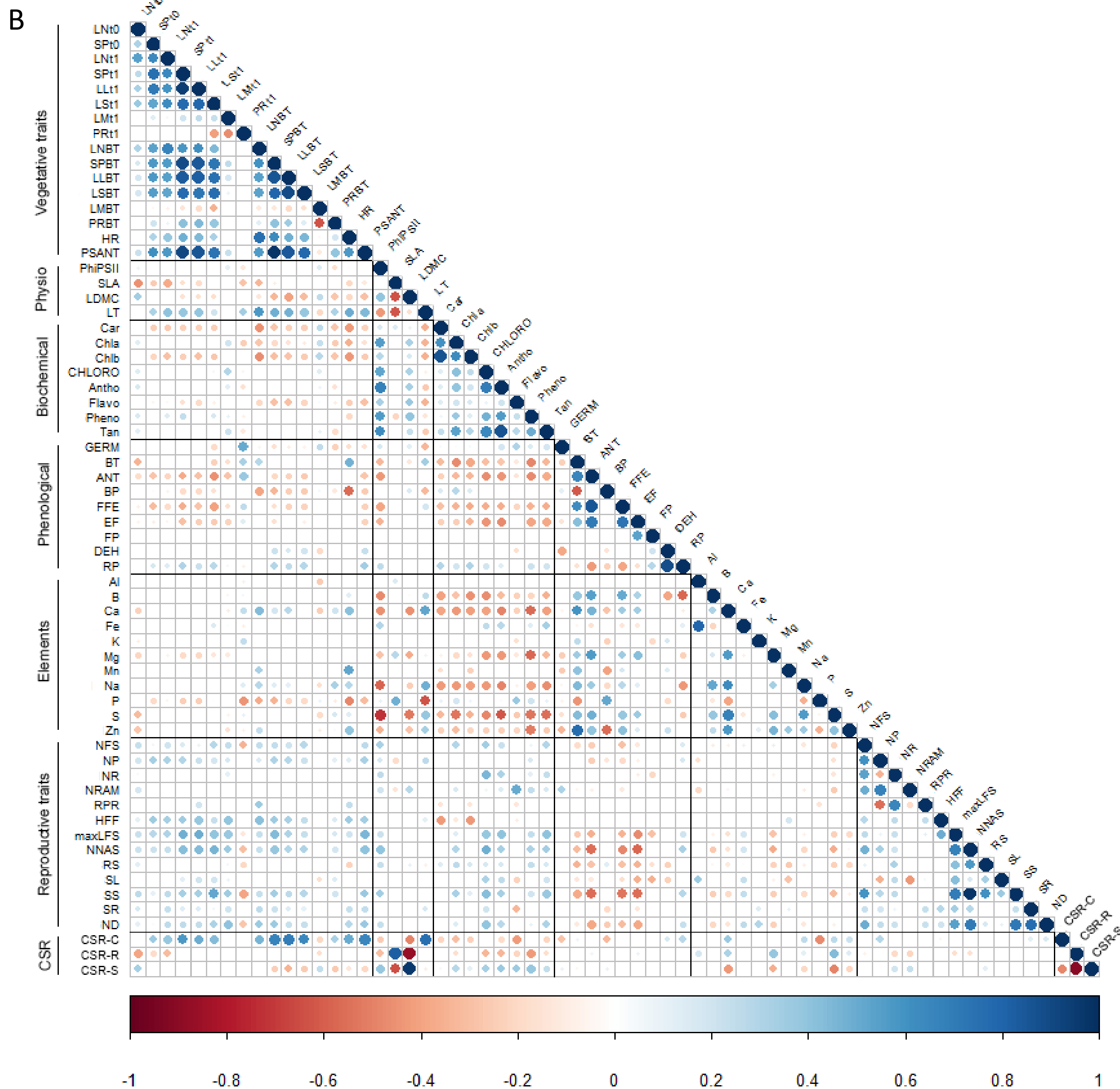


Figure S6 (continued): Correlation matrixes among traits in (B) $G_{0\text{-}NM}$ population submitted to 750 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

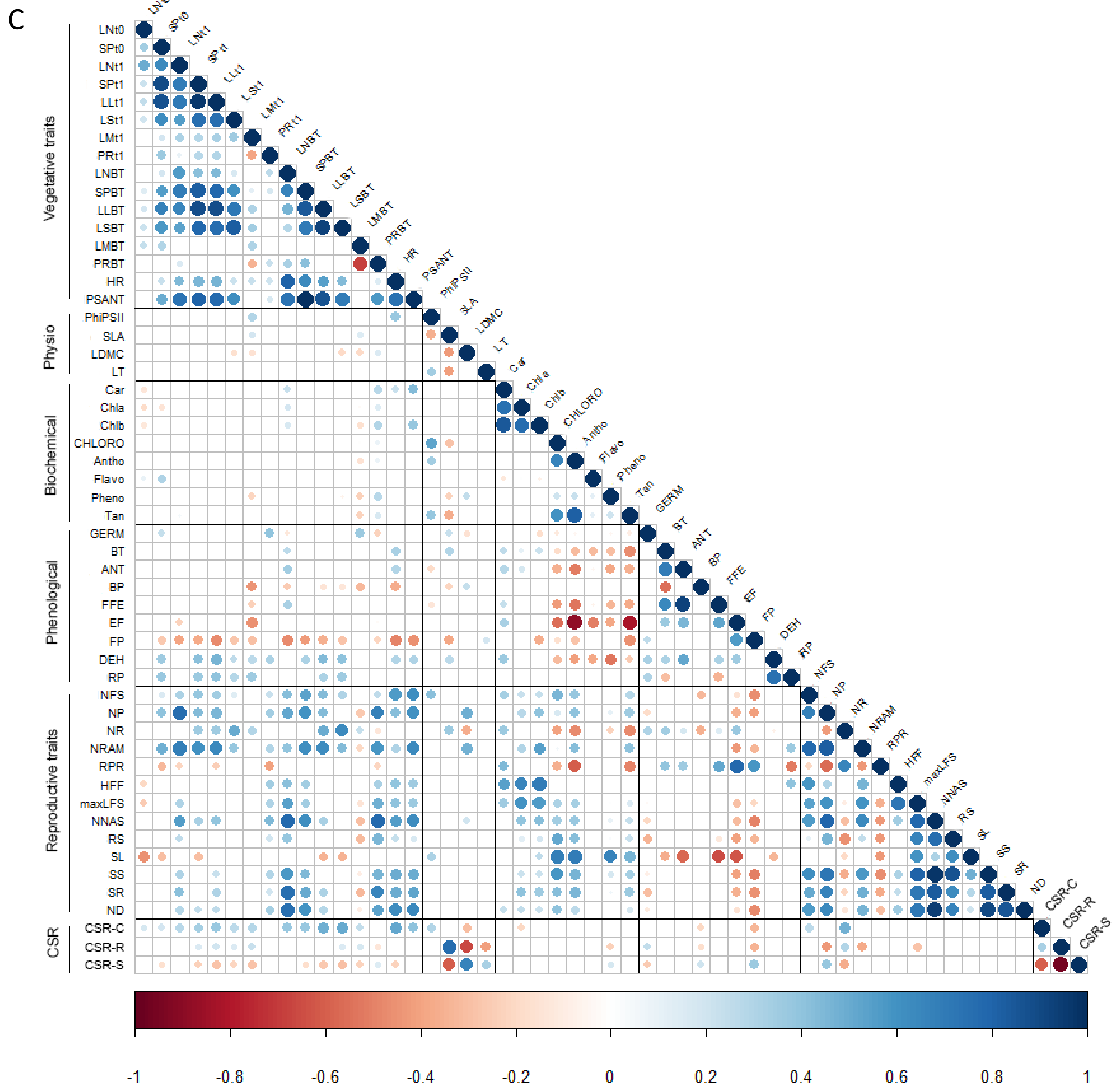


Figure S6 (continued): Correlation matrixes among traits in (C) $G_{0\text{-}NM}$ population submitted to 2000 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

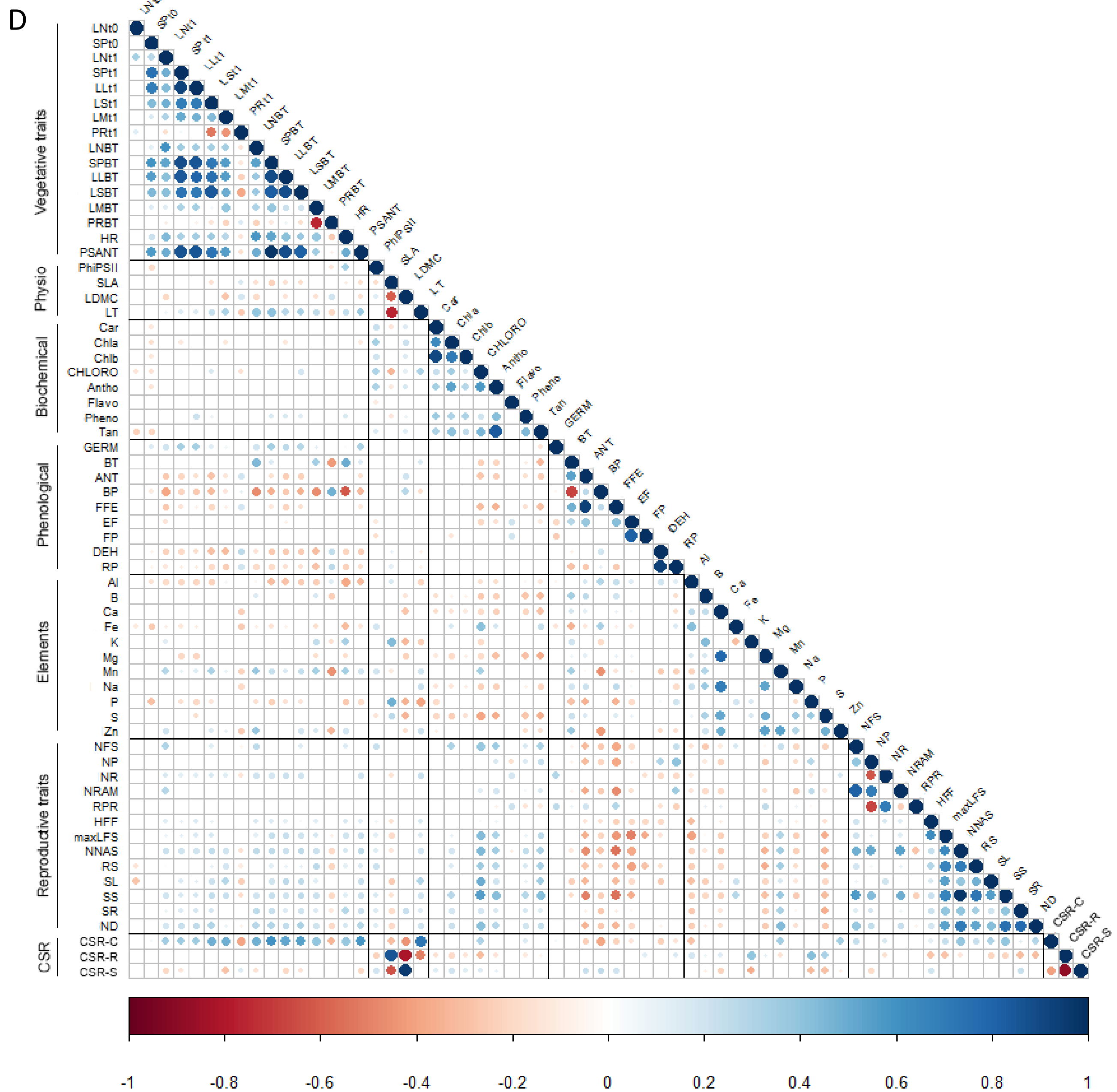


Figure S6 (continued): Correlation matrixes among traits in (D) $G_{2\text{-}EP1}$ population submitted to 750 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

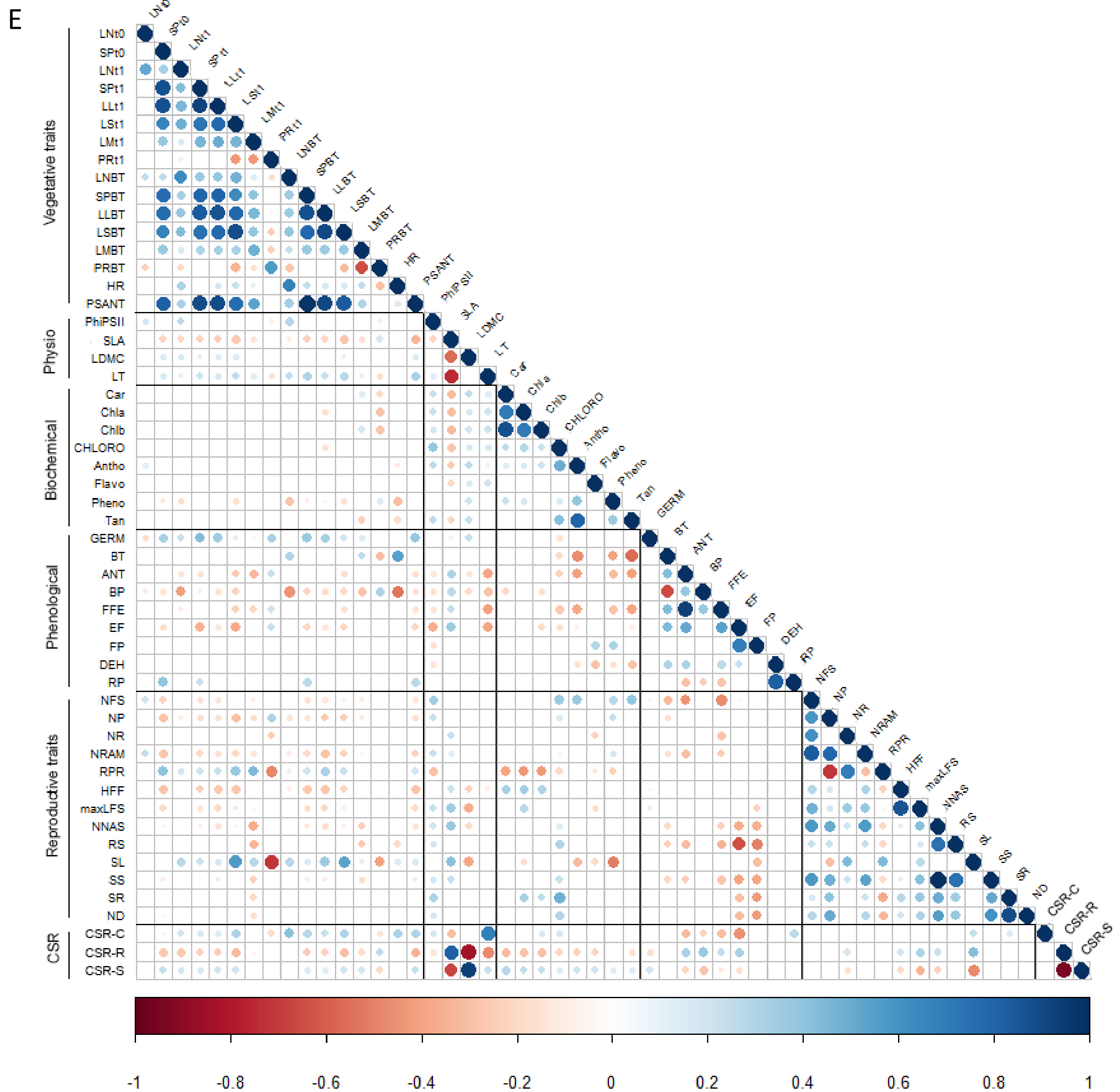


Figure S6 (continued): Correlation matrixes among traits in (E) $G_{2\text{-EP1}}$ population submitted to 2000 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

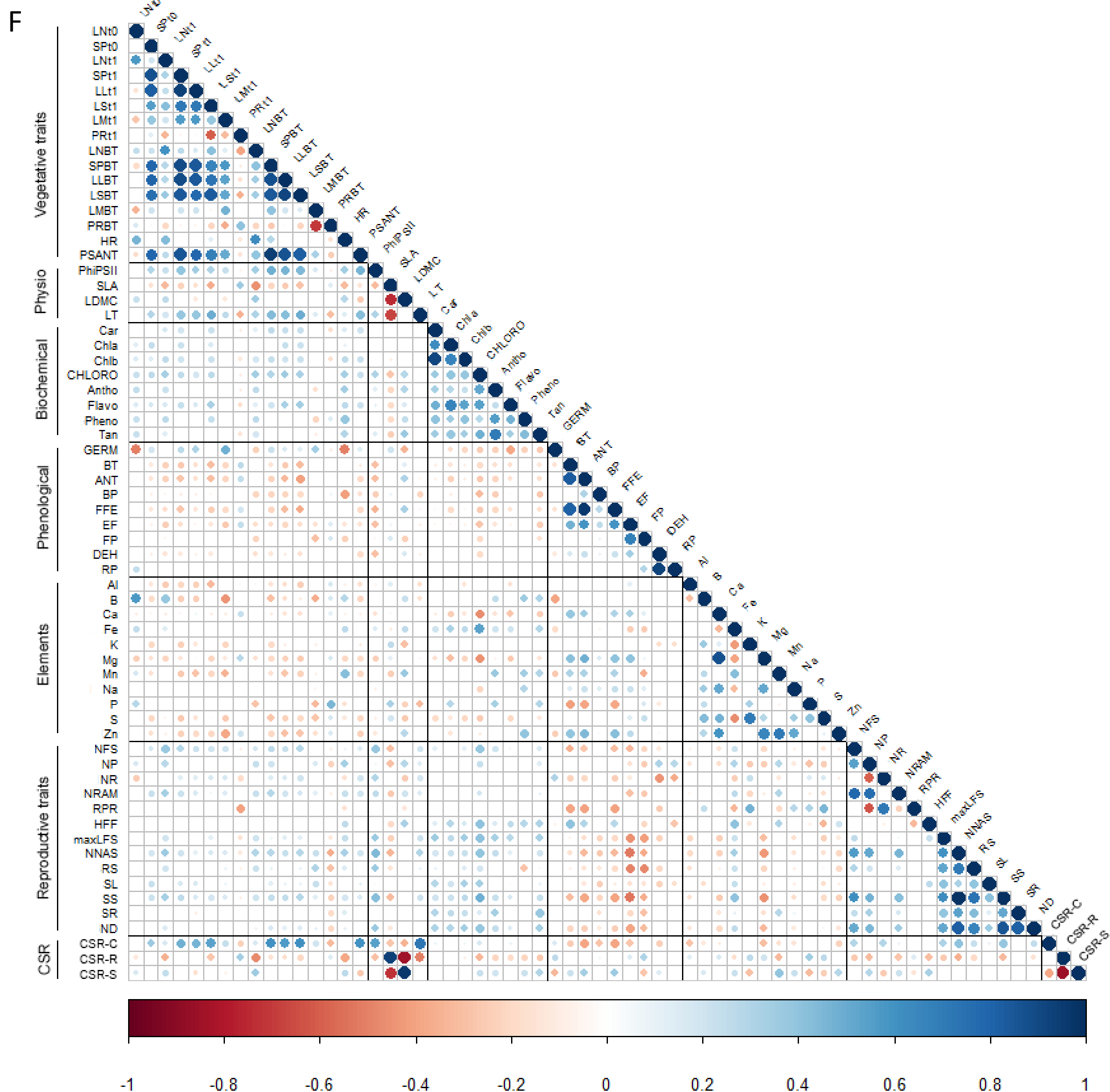


Figure S6 (continued): Correlation matrixes among traits in (F) $G_{2\text{-}EP2}$ population submitted to 750 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

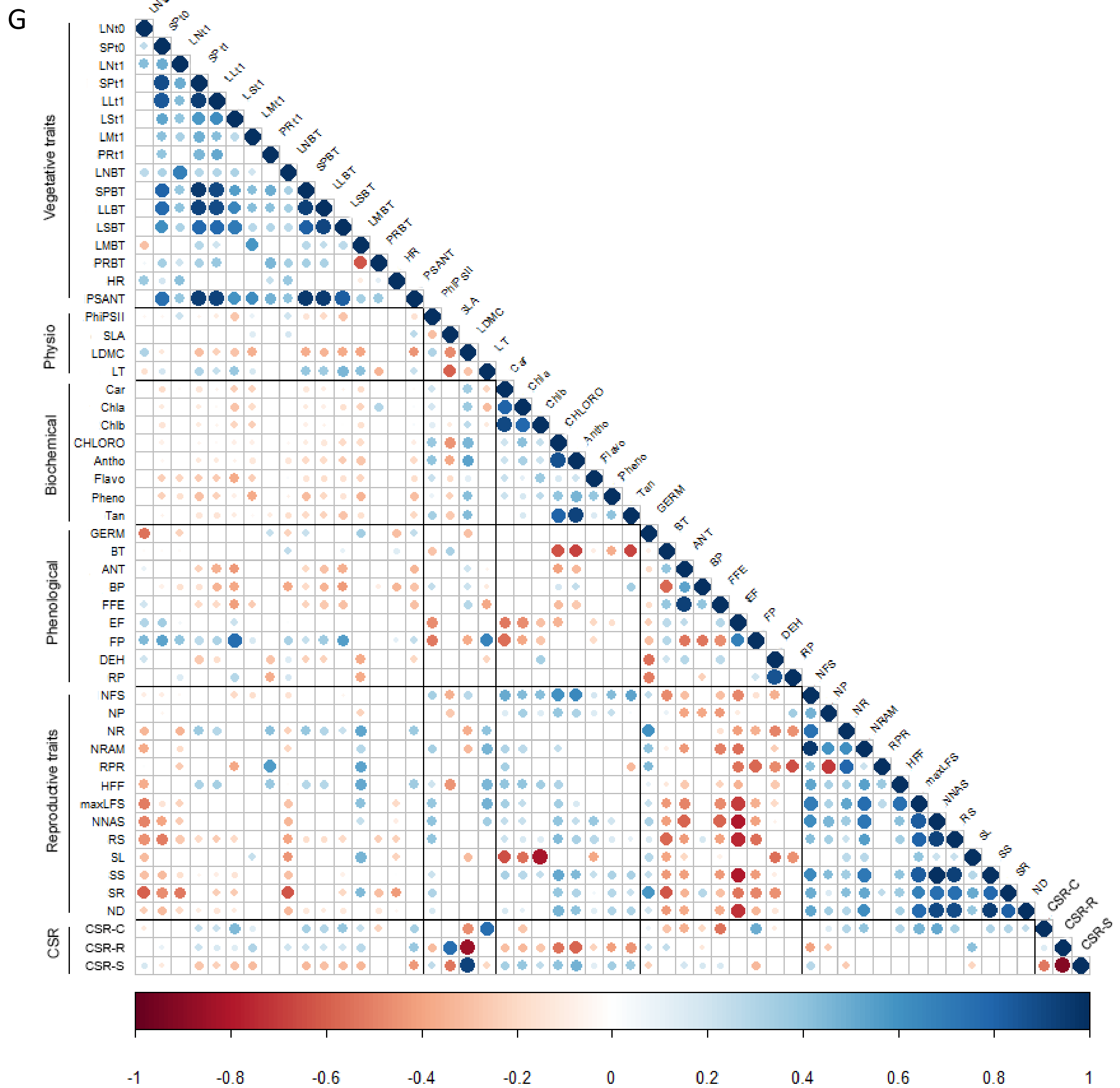


Figure S6 (continued): Correlation matrixes among traits in (G) $G_{2\text{-}EP2}$ population submitted to 2000 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

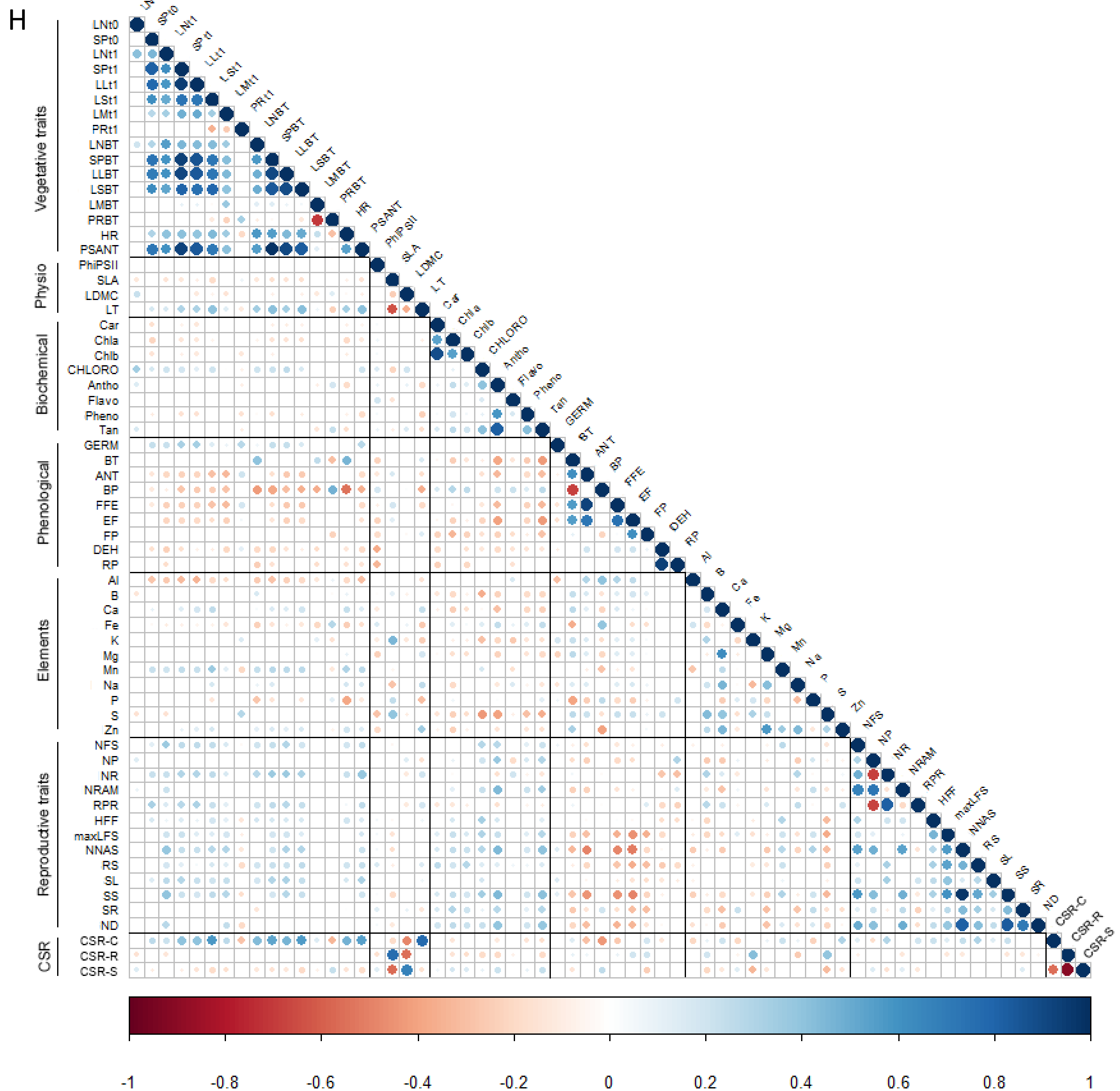


Figure S6 (continued): Correlation matrixes among traits in (H) $G_{2\text{-}EP3}$ population submitted to 750 $mg.kg^{-1}$ of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

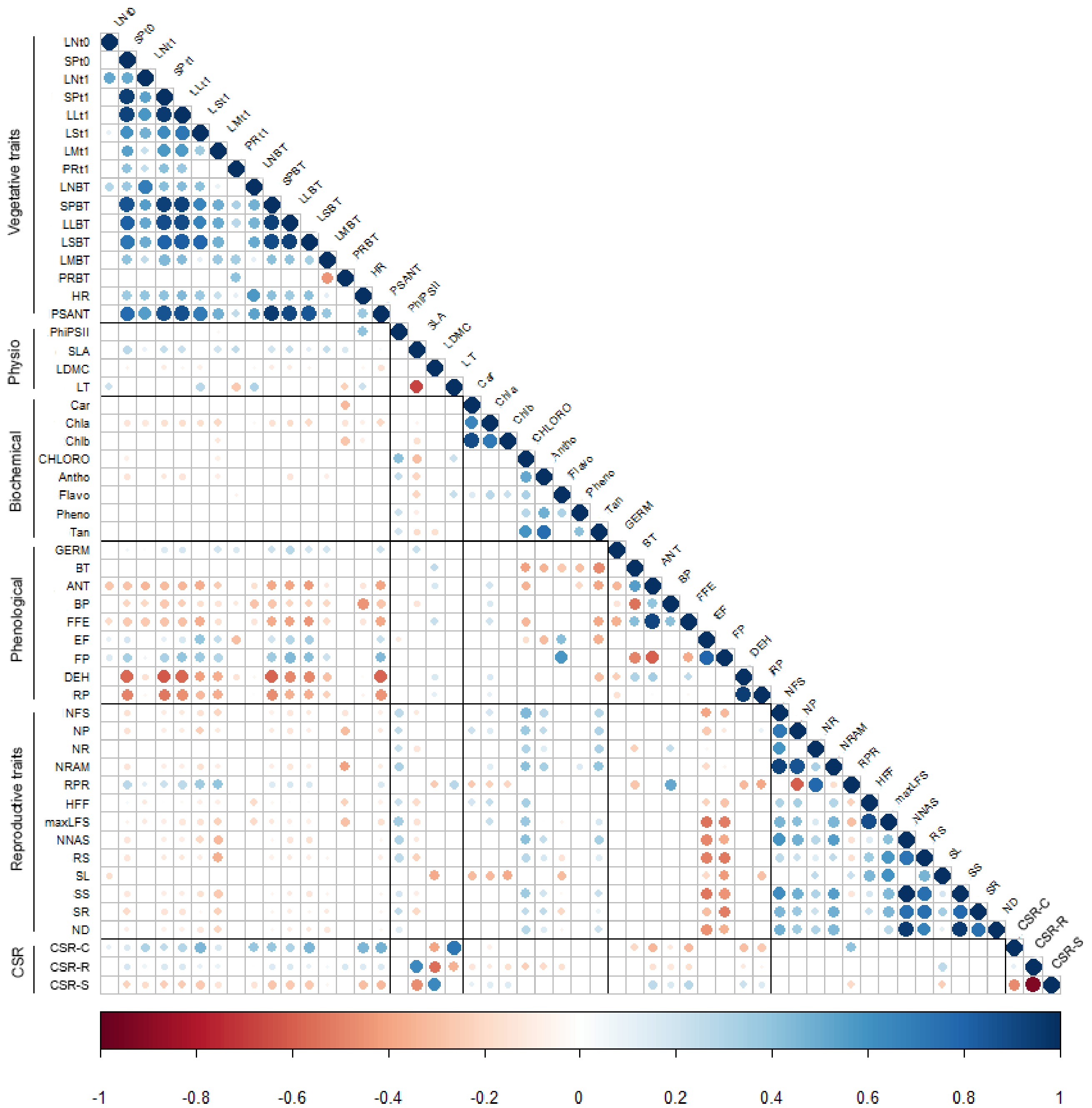


Figure S6 (continued): Correlation matrixes among traits in (I) G$_{2\text{-EP3}}$ population submitted to 2000 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN$_{t0}$, LN$_{t1}$, LN$_{BT}$: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP$_{t0}$, SP$_{t1}$, SP$_{BT}$, SP$_{ANT}$: surface of the plant measured at t0, t1, bolting and anthesis; LL$_{t1}$, LL$_{BT}$: average leaf length measured at t1 and bolting; PR$_{t1}$, PR$_{BT}$: average petiolic ratio measured at t1 and bolting; LS$_{t1}$, LS$_{BT}$: average leaf surface measured at t1 and bolting; LM$_{t1}$, LM$_{BT}$: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

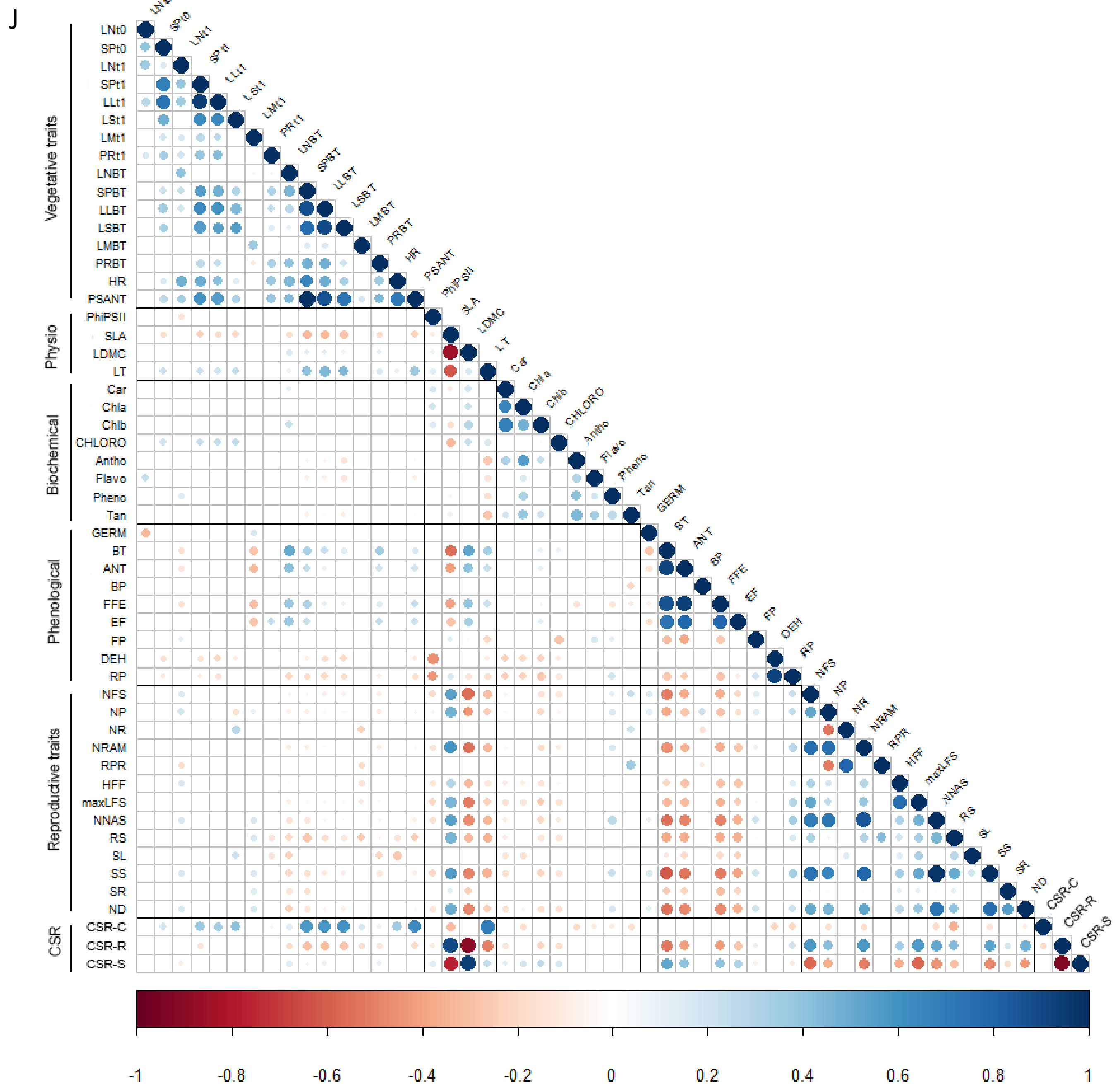


Figure S6 (continued): Correlation matrixes among traits in (J) $G_{2\text{-}EP4}$ population submitted to 750 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

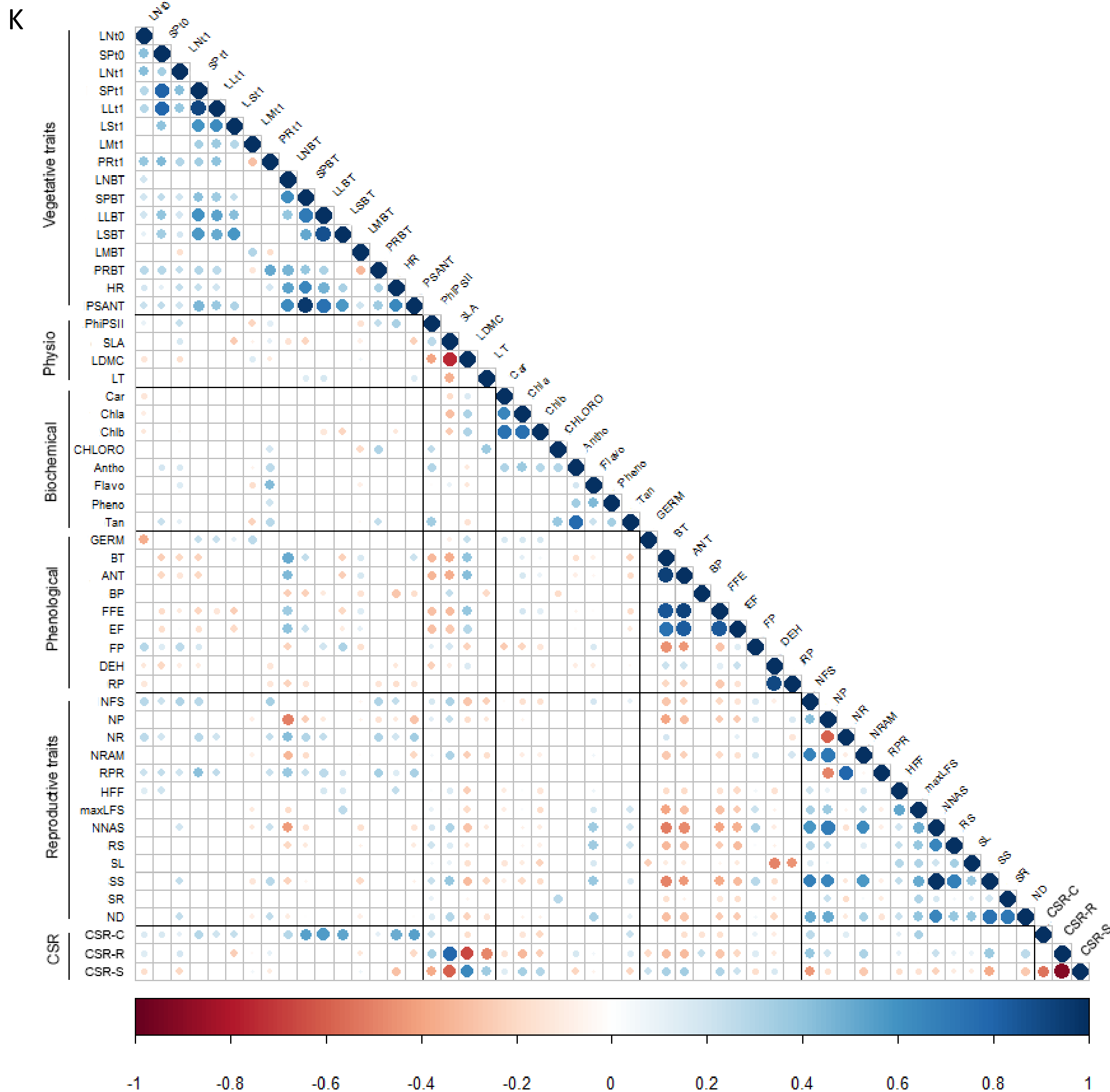


Figure S6 (continued): Correlation matrixes among traits in (K) $G_{2\text{-}EP4}$ population submitted to 2000 $mg.kg^{-1}$ of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

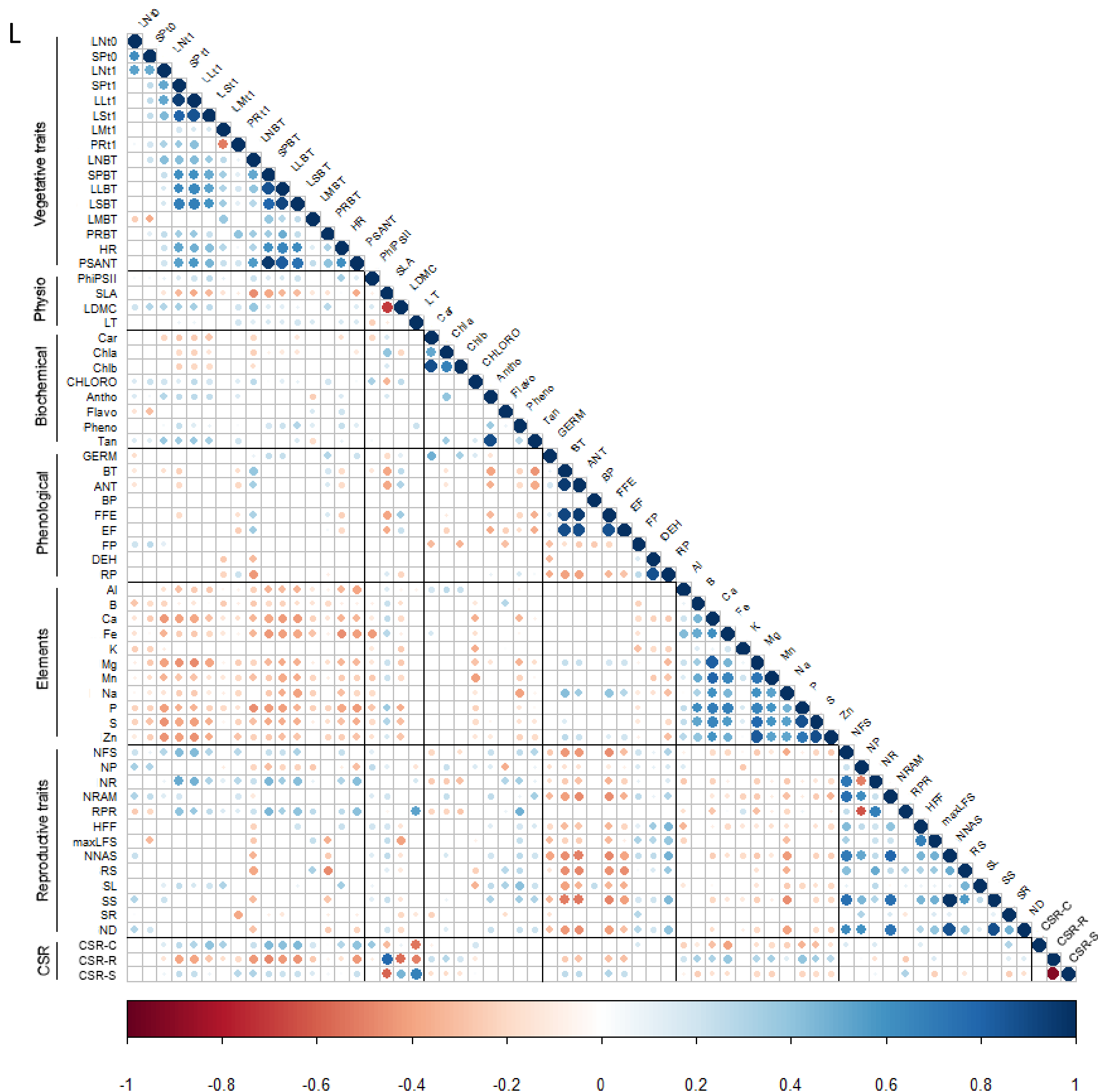


Figure S6 (continued): Correlation matrixes among traits in (L) $G_{0\text{-}M}$ population submitted to 750 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

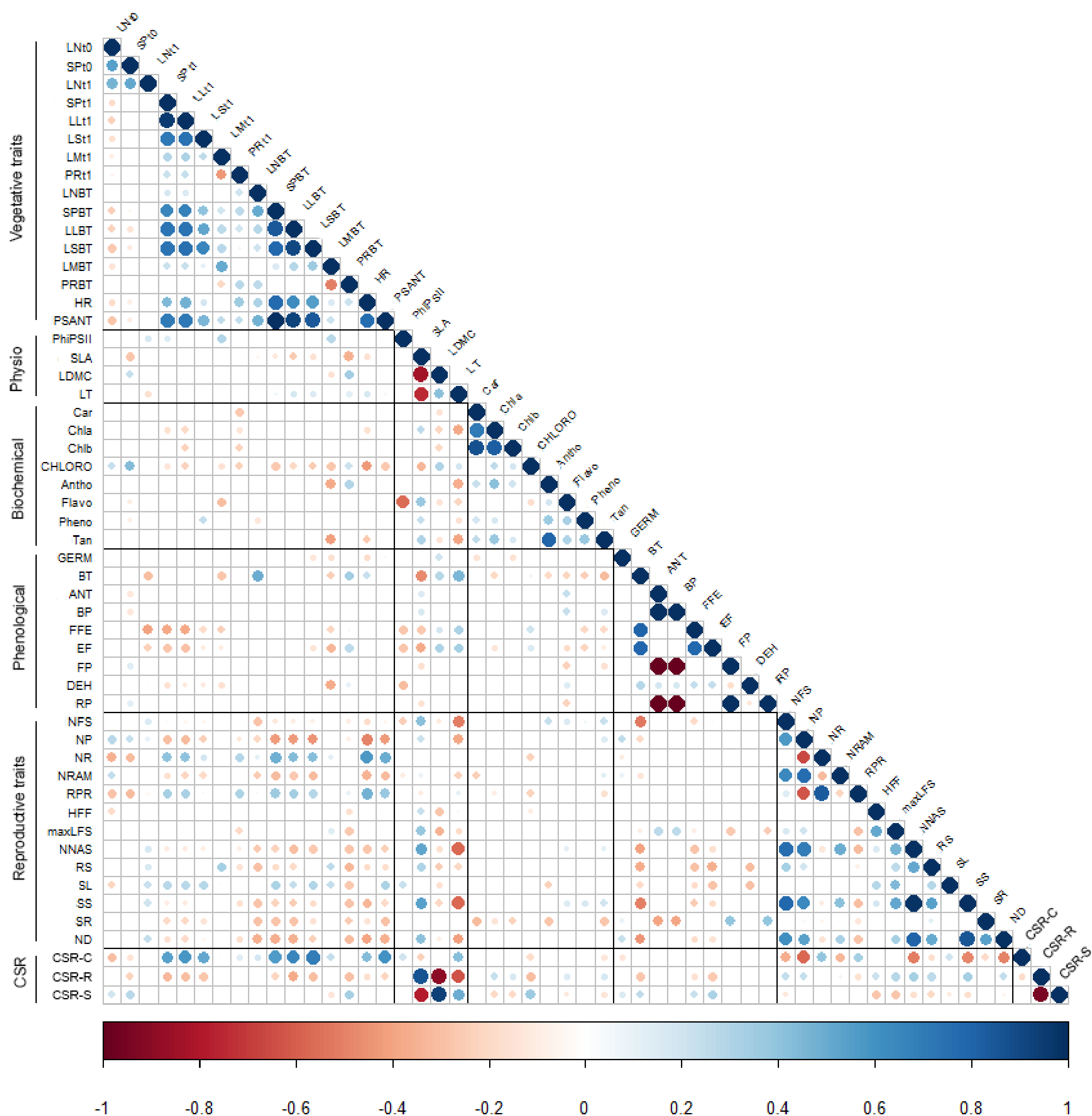


Figure S6 (continued): Correlation matrixes among traits in (M) $G_{0\text{-}M}$ population submitted to 2000 mg.kg^{-1} of zinc polluted soil. The gradient of colors represents sign and strength of correlation (red: r=-1, white: r=0 and blue: r=1). LN_{t0}, LN_{t1}, LN_{BT}: leaf number measured at t0 (eight weeks after sowing), t1 (25 weeks after sowing) and bolting time; SP_{t0}, SP_{t1}, SP_{BT}, SP_{ANT}: surface of the plant measured at t0, t1, bolting and anthesis; LL_{t1}, LL_{BT}: average leaf length measured at t1 and bolting; PR_{t1}, PR_{BT}: average petiolic ratio measured at t1 and bolting; LS_{t1}, LS_{BT}: average leaf surface measured at t1 and bolting; LM_{t1}, LM_{BT}: average leaf morphology measured at t1 and bolting. HR: Height of the rosette measured at bolting. PhiPSII: average photosynthetic yield measured at t1; SLA: average specific leaf area; LDMC: average leaf dry matter content; LT: average leaf thickness; Chla, Chlb, Car, Antho, Flavo, Pheno, Tan: Chlorophyll a, Chlorophyll b, carotenoids, anthocyanins, flavonoids, phenolic compounds and tannin concentration measured at bolting after methanol extraction; CHLORO: average chlorophyll concentration measured with chlorophyll meter at bolting. GERM: time interval between sowing and germination; BT: time interval between germination and bolting; ANT: time interval between germination and anthesis; BP: bolting period, time interval between bolting and anthesis; FFE: time interval between germination and first fruit emergence on the main flower stem; EF: End of flowering, time interval between germination and dehiscence of the last flower on the main flower stem; FP: flowering period, time interval between anthesis and end of flowering; DEH: time interval between germination and dehiscence of the last silique; RP: reproductive period, time interval between anthesis and dehiscence; NFS: number of flower stem; NP: number of panicles; NR: number of racemes; NRAM: number of ramifications; RPR: ratio between panicles and racemes; HFF: height to the first fruit on the main flower stem; maxLFS: height of the largest flower stem; NNAS: number of non-aborted siliques; RS: ratio between non-aborted and aborted siliques; SL: average silique length; SS: estimation of seed set; ND: estimation of number of descendants; SR: survival rate after five weeks after sowing; CSR-C, CSR-S, CSR-C: C, S and R score calculated with Pierce *et al.* algorithm (2017).

7. Discussion générale

Les objectifs majeurs de cette thèse étaient de tester si une contamination au zinc, seule, pouvait représenter une pression de sélection pour des populations non-métallicoles, considérées comme moins tolérantes que les populations métallicoles, et de tester l'effet de cette pression de sélection potentielle sur l'évolution de plusieurs traits fonctionnels possiblement associés à la tolérance aux métaux et à l'hyperaccumulation. Pour cela, nous avons mis en place un projet d'évolution expérimentale visant à simuler la colonisation d'un substrat contaminé par le zinc par des populations non-métallicoles de *Noccaea caerulescens*. La mise en place de cette approche a soulevé plusieurs questions:

(1) la diversité génétique, et donc phénotypique, présente dans les populations naturelles est-elle suffisante pour pouvoir opérer une sélection des individus en milieu contrôlé ou semi-contrôlé?

(2) Comment passer d'une génération à l'autre dans une population de taille finie, en favorisant les effets de la sélections sur ceux de la dérive génétique ?

(3) Quelle dose de zinc représente une pression de sélection satisfaisante?

(4) Quelle est la part héritable de la variation phénotypique observée à cette concentration ?

Les réponses pouvant être apportées ont été testées expérimentalement dans les trois premiers chapitres.

A la suite de la mise en place de ce dispositif, il a également été question de connaitre la nature des traits qui ont possiblement évolués. Ainsi, deux expériences, exposées dans les chapitres 4 et 5, ont été réalisés pour répondre à cette question.

7.1. Les étapes préliminaires à la mise en œuvre du projet d'évolution expérimentale

7.1.1. Une diversité génétique initiale suffisante dans les populations naturelles ?

La construction des populations expérimentales a été réfléchie de façon à introduire une diversité génétique initiale suffisante. Pour cela, les graines de plusieurs individus ont été

récoltées dans plusieurs populations métallicoles de Belgique (La Calamine et Prayon) et non-métallicoles du Luxembourg (Lellingen, Wilwerwiltz et Winseler), ces populations étant largement référencées [Assunção *et al.*, 2003; Molitor *et al.*, 2004; Dechamps *et al.*, 2007, 2008, 2011]. Plusieurs individus provenant de ces familles de graines ont été semés puis cultivés dans différentes conditions environnementales afin d'évaluer leur survie et de contrôler leurs niveaux d'expressions phénotypiques. Ainsi, dès leur mise en culture dans les expériences de préparation à l'évolution expérimentale (chapitres 1 et 2), les différences de réponses observées entre les populations et entre les familles issues d'une même population ont montré qu'il existait une variation phénotypique forte dans la capacité des plantes à gérer l'exposition au zinc. Dans le chapitre 1, les résultats des deux expériences ont montré une grande variance dans la production de graines au sein même des populations. Par exemple, dans la population Lellingen, la production de graines variait du simple (chez LE.07) au quintuple (chez LE.15) dans l'experience 1, alors que dans l'expérience 2, la production de graine pouvait varier du simple au double pour les descendants d'une même plante mère dans les deux conditions (chez les descendants produits par autofécondation de LE.02).

Dans le chapitre 2, il est intéressant de noter que lorsqu'on examine l'identité des familles qui ont pu survivre, produire des graines et maintenir un certain niveau de performance, l'effet de l'augmentation des niveaux de zinc dans les mésocosmes n'était pas aléatoire. En effet, la perte de famille a été graduelle, de sorte que les familles ayant produits des graines dans la population exposée aux plus fortes doses ont également produits des graines dans les autres conditions. Dans le détail, nous avons observé une perte progressive des familles provenant de Winseler avec l'augmentation des concentrations en zinc [Table 13]. En effet, sur les 13 familles provenant de Winseler initialement présentes, 13, 12, 2 et 2 familles ont respectivement produits des graines dans les populations PP_0, PP_{500}, PP_{1000} et PP_{2000}. En comparaison, sur les 10 familles provenant de Wilwerwiltz présentes, 10, 10, 10 et 9 familles ont respectivement produits des graines dans les populations PP_0, PP_{500}, PP_{1000} et PP_{2000}.

Table 13 : taux de survie calculés sur l'effectif global (n=49), sur les descendants provenant de Lellingen (LE), Wilwerwiltz (WIL) et Winseler (WIL)

taux de survie	n	0mg.kg^{-1}	500mg.kg^{-1}	1000mg.kg^{-1}	2000mg.kg^{-1}
Global	49	100%	98%	67,3%	51%
WIL	26	100%	100%	92,3%	80,7%
WIN	22	100%	95,4%	36,4%	13,6%
LE	1	100%	100%	100%	100%

La même tendance a également été observée après avoir classé les familles ayant produits des graines en fonction de leur performance moyenne [Figure 18]. Les mêmes familles, provenant pour la plupart de Wilwerwiltz (par exemple. WIL13, WIL18 ou WIL30) ont montré les performances les plus élevées dans les trois conditions de cultures en présence de zinc.

Le fait que les mêmes familles expriment les performances les plus élevées dans toutes les conditions en présence de zinc, suggère une composante génétique responsable de cette capacité à gérer l'exposition au zinc. De plus, au sein des populations étudiées, il devrait exister une diversité génétique assez forte qui pourrait être utilisée comme matériel de départ afin d'opérer une sélection [Pauwels *et al.*, 2006].

Cette idée selon laquelle une sélection opérée sur la diversité génétique initiale serait à la base d'une divergence entre les populations métallicoles et non-métallicoles a été abordée plusieurs fois dans la littérature. En 1975, une étude conduite sur *Agrostis stolonifera* montrait que les populations tolérantes au cuivre étaient composés de nombreux génotypes et supposait que ces populations avaient évolué par sélection de génotypes tolérants, ou partiellement tolérants, pouvant exister dans les populations non tolérantes à de faibles fréquences [Wu *et al.*, 1975].

De même, des études plus récentes ont suggéré que la variation pour la tolérance aux métaux pourrait préexister dans les populations non-métallicoles et être sélectionnée lors de la colonisation d'un site métallifère [Meyer *et al.*, 2016]. Dans cette dernière étude, une analyse QTL réalisée à partir de croisements interspécifiques entre des individus non-métallicoles d'*Arabidopsis halleri* et des individus d'une espèce non-métallophyte, *Arabidopsis lyrata petraea*, a montré l'existence d'un QTL expliquant 22.6% et 31.2% de la variance phénotypique pour, respectivement, la tolérance au zinc et au cadmium. Or, ce QTL co-localisait avec le gène *AhHMA4* (Arabidopsis halleri Heavy Metal ATPase 4), ayant subi une triplication chez *A. halleri* (responsable en partie de l'augmentation du niveau d'expression chez cette espèce), qui daterait de la séparation entre *A. halleri* et *A. lyrata* et qui serait, par conséquent, antérieure à l'anthropisation des milieux [Hanikenne *et al.*, 2008].

Enfin, en 2017, une étude de génétique des populations suggérait également une sélection au niveau local à partir de la standing variation chez *N. caerulescens* [Gonneau *et al.*, 2017]. Dans cette étude, la structure génétique neutre de 62 populations de *N. caerulescens* a été analysée et a montré l'existence de trois sous-unités génétiques (SU1, SU2 et SU3) géographiquement cohérentes. Ces sous unités impliquent que la structure génétique neutre n'a pas fortement été impactée par la présence de l'espèce dans les différents types édaphiques

(Calaminaire, serpentinique et non-métallifère) et serait mieux expliquée par des événements historiques. La divergence entre SU1 et SU2/SU3 pourrait alors être expliquée par la dernière glaciation alors que la divergence entre SU2 et SU3 impliquerait une recolonisation postglaciaire. La présence de populations métallicoles (calaminaires et serpentiniques) et non-métallicoles dans SU1, SU2 et SU3 suggère la colonisation indépendante des sites métallifères après la formation de chaque sous-unité, suivie d'une adaptation locale par sélection de la standing variation [Gonneau *et al.*, 2017].

7.1.2. Comment effectuer le passage de génération ?

Pour les raisons évoquées dans le chapitre 1, nous avons choisi de contrôler le passage des générations par l'estimation de la contribution relative de chaque individu dans la descendance. Dans ce contexte, l'expérience du chapitre 1 visait à développer un estimateur du nombre de graines. Nous avons surtout testé la validité de deux estimateurs du nombre de graines couramment utilisés chez les Brassicaceae, le nombre de fruit fertiles [Dechamps *et al.*, 2008] et la taille totale des silicules [Brachi *et al.*, 2012; Roux *et al.*, 2016].

Les résultats présentés dans le chapitre 1 montraient que les traits qui semblaient les plus corrélés au nombre de graines étaient le nombre de fruits non-avortées et la taille totale des fruits, ces corrélations étant généralement comprises entre r = 0.8 et r = 0.9 dans l'ensemble des jeux de données considérés. Dans le détail, en séparant progressivement le jeu de données, par expérience, par condition (seulement pour l'expérience 2), par type édaphique, par population (origine de la F1 dans l'expérience 2) ou par famille (pedigree de la F1 dans l'expérience 2), ces deux traits montraient quasiment les mêmes corrélations au nombre de graines.

Néanmoins dans le cas d'un grand effectif diversifié (groupement des données des expériences 1 et 2, avec des individus de types édaphiques différents et exposés à des conditions de cultures différentes), la taille totale des fruits semblait être un meilleur estimateur (r = 0.85, p-value = 1.66×10^{-108}) que le nombre de siliques non avortées (r = 0.73, p-value = 1.89×10^{-65}).

Plusieurs stratégies peuvent être rencontrées pour augmenter le nombre de graines: soit la plante produit plus de silicules, soit elle produit des silicules de plus grande taille. Ceci n'est possible qu'à condition que la taille d'une silicule soit directement corrélée au nombre de graines qu'elle contient.

Cette relation entre la taille d'une silicule fruits et le nombre de graines qu'elle contient a également été testée et, bien que la corrélation soit assez faible (r = 0.45), elle est significative

et positive. La valeur assez faible de cette corrélation pourrait être, en partie, liée à la précision de la mesure des silicules qui est effectuée au millimètre près. Cette mesure n'est certainement pas assez précise au vue de la taille des graines. Ainsi, une méthode de mesure numérique de la taille des silicules a été explorée récemment (résultats non publiés). Les résultats de cette méthode montrent une corrélation nettement plus importante entre le nombre de graines d'une silique et la surface du placenta (organe qui porte les graines) (r=0.73 figure 55). Néanmoins cette méthode nécessite une optimisation pour être utilisée sur de grands échantillons. Il se pourrait également que les silicules de grande taille contiennent parfois des graines avortées, mais nous ignorons ce qui influencerait la proportion de graines avortées. Il ne s'agit pas nécessairement d'un effet du métal puisque nous avons vu qu'une variation autour du contenu en graines des silicules existait dans une expérience en condition non polluée.

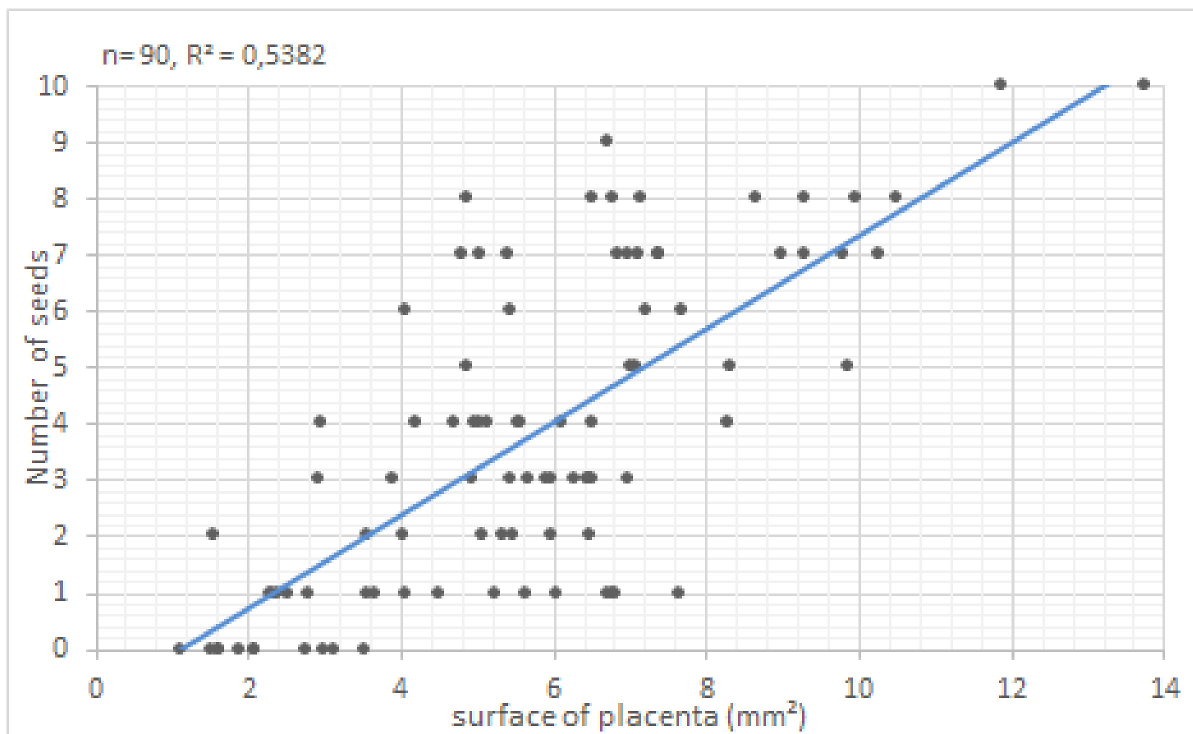


Figure 55 : corrélation entre le nombre de graines contenues dans une silicule et la surface du placenta mesurée numériquement

Ainsi, l'estimation de la taille totale des silicules semble être une bonne alternative au nombre de graines.

Dans le chapitre 2, nous avons mis en pratique cet estimateur pour constituer une descendance pour chaque PP. Ainsi, la taille totale des silicules a été calculée et la performance

relative de chaque individu, dans chaque population, a été estimée selon cette valeur. Cette performance relative a ensuite été utilisée pour constituer une descendance (DP pour *descendant populations*) « attendue » de 49 individus dans chaque PP [Figure 14]. En considérant un taux de germination de 33%, trois graines par descendant attendu ont été semées. Néanmoins, les taux de germinations étaient extrêmement variables et très bas dans les populations exposées aux plus fortes concentrations (PP_{1000} et PP_{2000}). Modifiant considérablement la composition des DP [Figure 19].

Ces résultats suggèrent qu'au-delà d'affecter la production d'organes reproducteurs en réduisant le nombre de hampes florales, le nombre de fruits ou la taille totale des silicules, le zinc aurait un effet sur la qualité des graines. En effet, plusieurs études ont montré que les métaux pouvaient provoquer une réduction de la taille ou du poids des graines ou causer la production de graines peu ou non-viables [synthèse Kranner & Colville, 2011]. Néanmoins l'effet du métal sur la qualité des graines serait dépendant de l'espèce et de l'élément considérés. Ainsi, l'exposition du soja (*Glycine max*) à 0,05 $mg.L^{-1}$ de cadmium réduit le poids des graines mais n'a pas d'effet sur le nombre de graines moyen contenu dans une gousse. A l'inverse une exposition à 0,1 $mg.L^{-1}$ de nickel réduit le nombre de graines moyen contenu dans une gousse mais n'a pas d'effet sur le poids des graines [Malan & Farrant, 1998]. Dans cette étude, le cadmium réduisait significativement l'accumulation de réserve dans la graine alors que le nickel menait à la production de gousses déformées contenant des graines non-viables.

La plupart des effets toxiques engendrés par l'exposition aux métaux est lié à un stress oxydatif engendré par la production d'espèces réactives de l'oxygènes (ou ROS pour *reactive oxygen species*) [Schützendübel & Polle, 2002; Sharma & Dietz, 2009; Sytar et al., 2013]. Ainsi, les métaux accumulés dans les graines peuvent entrainer la production de ROS qui vont entrer en réaction avec les protéines ou les lipides accumulés dans la graine, causant ainsi leur dégradation [synthèse Kranner & Colville, 2011].

De ce fait, la seule estimation du nombre de graines ne suffit pas et il est important de prendre en compte la viabilité des graines, voire des plantules, pour estimer correctement un nombre de descendants viables. Ainsi, nous avons estimé le taux de germination en semant 33 graines par individus, puis le taux de survie des plantules après cinq semaines, de façon à estimer un nombre de descendants viables.

7.1.3. Quelle pression de sélection appliquer ? Avec quelle intensité ?

Le but de l'expérience est de tester l'effet du zinc en tant que seule pression de sélection sur l'évolution d'une population non-métallicole. Ainsi l'utilisation d'un sol calaminaire plus ou moins dilué avec du terreau [Escarré *et al.*, 2000; Frérot *et al.*, 2005] n'était pas envisageable. Les sols calaminaires étant contaminés par plusieurs métaux, comme le zinc, le cadmium ou le plomb, sous une multitude de formes chimiques plus ou moins assimilable par les plantes. De plus il ne serait pas possible de créer un sol témoin, sans métaux, ayant les mêmes propriétés physico-chimiques. Nous avons donc utilisé un mélange de terreau, à base de tourbe, et de zéolithe auquel a été ajouté du sulfate de zinc hepta-hydraté ($ZnSO_4$, $7H_2O$) sous forme de poudre.

Ensuite, la concentration en zinc choisie se doit d'être assez forte pour générer des différences de performances chez les individus mais ne pas être trop forte, ce qui entrainerait l'extinction de la population à plus ou moins long terme, soit en affectant la survie de tous les individus, soit en limitant le nombre de graines produites par la population, ce qui ne permettrait pas de constituer les générations suivantes.

Dans le chapitre 2, nous avons mis en place une expérience visant à choisir cette concentration optimale.

Les résultats des comparaisons entre PPs cultivées à différentes concentrations en zinc ont montré que le zinc agissait principalement sur la survie des individus et la production d'organes reproducteurs. Une dose croissante de zinc réduisant progressivement le taux de survie [Figure 16A], le pourcentage de plantes ayant produit des graines [Figure 16B] et la valeur moyenne des différents traits reproducteurs [Figure 17].

Dans cette expérience, la sélection provoquée par l'exposition au zinc a en partie favorisé l'évolution vers des niveaux de tolérance plus élevés dans les DPs. Notamment, nous avons observé une tendance à une diminution de la chlorose chez la progéniture des PP les plus exposés, sans plantes entièrement chlorotiques dans DP_{2000}. L'activité de la superoxyde dismutase (SOD) tend également à être plus élevée dans DP_{2000} comparée aux autres DP [Figure 22B].

Les résultats de cette expérience semblent suggérer qu'une sélection par l'exposition au zinc est possible et qu'elle permettrait une évolution de traits fonctionnels liés à la tolérance ou l'hyperaccumulation du zinc. Cela nous a permis de définir la concentration en zinc qui sera

appliquée dans le dispositif d'évolution expérimentale final. Ainsi, 1000 $mg.kg^{-1}$ pouvait apparaître comme une concentration idéale car elle semblait provoquer une sélection, tout en préservant une survie suffisante à la création des générations successives. Cependant, la majorité des individus provenant de Winseler n'ont pas survécu ou n'ont pas produit de graines à cette concentration. Or, au moment de créer les populations du dispositif final, la population Wilwerwiltz n'était pas accessible. De plus, à 500 $mg.kg^{-1}$, la concentration ne semblait pas suffisante pour opérer une sélection. Par conséquent, nous avons choisi une concentration intermédiaire de 750 $mg.kg^{-1}$ de zinc.

7.1.4. Quelle est la part héritable de la variation phénotypique observée?

En évolution expérimentale, la divergence phénotypique observée entre les populations ancestrales et dérivées peut être interprétée comme déterminée génétiquement. Néanmoins, ces différences peuvent résulter d'autres mécanismes transgénérationnels, comme l'épigénétique ou les effets maternels [Herman & Sultan, 2011; Weinhold, 2018]. Aussi pour évaluer la part des facteurs génétiques dans la variation phénotypique il a été envisagé d'estimer l'héritabilité des traits fonctionnels qui ont été mesurés dans le dispositif final.

Dans le chapitre 3, l'héritabilité au sens large de plusieurs traits fonctionnels, morphologiques, physiologiques et phénologiques [Table 7] a été estimée selon une régression parents-descendants dans plusieurs populations exposées, ou non, au zinc.

L'héritabilité au sens large a été estimée pour chaque trait dans chaque situation possible :

- Parent NM cultivé sur substrat non-contaminé / Descendance cultivée sur substrat non-contaminé
- Parent NM cultivé sur substrat non-contaminé / Descendance cultivée sur substrat contaminé
- Parent NM cultivé sur substrat contaminé / Descendance cultivée sur substrat non-contaminé
- Parent NM cultivé sur substrat contaminé / Descendance cultivée sur substrat contaminé
- Parent M cultivé sur substrat non-contaminé / Descendance cultivée sur substrat non-contaminé

- Parent M cultivé sur substrat non-contaminé / Descendance cultivée sur substrat contaminé
  - Parent M cultivé sur substrat contaminé / Descendance cultivée sur substrat non-contaminé
  - Parent M cultivé sur substrat contaminé / Descendance cultivée sur substrat contaminé

Ainsi, la régression de la valeur moyenne des descendants sur la valeur de son parent est égale à la moitié de l'héritabilité au sens large (H^2) [Ollivier, 1971; Lynch & Walsh, 1998].

Très peu d'héritabilités significatives ont été observées pour les traits végétatifs mesurés au moment de la transplantation des individus (huit semaines après le semis) ou 25 semaines après le semis ou pour les traits phénologiques. En revanche, beaucoup de traits végétatifs mesurés à la montaison ou à l'anthèse ont montré une héritabilité plus ou moins forte selon les conditions de culture et le type édaphique.

Les héritabilités calculées étaient significatives et assez fortes pour les parents non métallicoles cultivées sur substrat non contaminé, et en particulier lorsque les descendants se développaient sur substrat contaminé. Ce résultat suggère qu'une réponse génétique des traits végétatifs devrait effectivement être possible dans notre dispositif d'évolution expérimentale. En comparaison, les populations métallicoles montraient moins de valeurs d'héritabilité significatives.

Cette résultats contredisent, en partie, les résultats décrits dans la thèse de G. Jiménez-Ambriz (2006) Où l'héritabilité au sens strict a été calculée sur plusieurs traits phénologiques et morphologiques, en lien avec la croissance et la reproduction.

Les résultats ont montré des valeurs d'héritabilité de la taille des plantes mesurée à la montaison nettement plus faibles que dans notre expérience chez les deux groupes édaphiques et quel que soit le niveau de contamination. En effet, il a été montré une différence assez forte des valeurs d'héritabilité entre les deux types édaphiques ($h^2_M > h^2_{NM}$) mais aucun effet significatif de la condition de culture a été observé ce qui n'est pas le cas dans notre expérience.

A l'inverse, les résultats de nos expériences convergent sur la mesure de l'héritabilité des traits phénologiques. Dans les deux cas, les valeurs sont, généralement, non significatives. Ces observations convergent également avec les travaux de Stirling *et al.*, (2002) où il a été montré que, globalement, les traits morphologiques étaient plus héritables que les traits liés au cycle biologique. Ce schéma pouvant s'expliquer par une plasticité plus importante des traits phénologiques.

Les différences observées entre les résultats de notre expérience et ceux exposés par G. Jiménez-Ambriz, (2006) peuvent être expliquées par différents facteurs. Le plus évident est que l'héritabilité au sens strict ne prend pas en compte les effets de dominance et les effets d'épistasie. Aussi, l'héritabilité est variable à l'intérieur de l'espèce [Egli, 2004] et dépend de la population (ou de l'échantillon de la population) et des conditions considérées. Enfin, l'utilisation d'une régression dans notre expérience a été limitée par la perte de plusieurs familles, réduisant ainsi le nombre de points utilisables.

7.2. Etudier les effets du zinc sur l'évolution d'une population non-métallicole de Noccaea caerulescens par une approche d'évolution expérimentale

7.2.1. Suivi des populations expérimentales et prédictions de l'évolution des traits phénotypiques par le calcul des gradients de sélection

Les trois premiers chapitres nous ont permis de réfléchir à la mise en place d'un dispositif expérimental répondant aux différentes problématiques. Ainsi, pour générer une diversité suffisante, plusieurs familles de graines ont été échantillonnées à Prayon et La Calamine, deux sites métallifères belges, et à Winseler et Lellingen, deux sites non métallifères du Luxembourg.

Les graines ont été semées et, en fonction des taux de germination, quatre populations expérimentales (EP) de 98 individus. EP_1, EP_2 et EP_3 ont été constituées à partir d'individus non métallicoles de façon à (1) avoir un maximum de diversité génétique au sein de chaque EP mais (2) d'avoir les mêmes familles dans les mêmes proportions entre les EP. EP_4 a été constitué avec des individus métallicoles de façon à avoir un maximum de diversité génétique. Chaque population a été cultivée en extérieur dans deux mésocosmes. EP1 a été cultivée sur un substrat non-contaminé, EP_2 et EP_3 sur un substrat contaminé à 750 $mg.kg^{-1}$ de zinc et EP_4 dans un substrat contaminé à 750 $mg.kg^{-1}$ de zinc. De façon à avoir un témoin non-métallicole (EP_1) avec des individus non-métallicoles sur du substrat non contaminé, un témoin métallicole (EP_4) avec des individus métallicoles sur du substrat contaminé et deux populations tests (EP_2 et EP_3) avec des individus non-métallicoles sur un substrat contaminé [Figure 39].

Les flux de gènes entre les populations expérimentales ont été limités en utilisant un système de cage qui recouvre l'intégralité de la population. Les cages sont alors déposées sur trois populations sur quatre et une rotation est effectuée tous les 3,5 jours.

Chaque individu est suivi sur un ensemble de traits fonctionnels durant son cycle de vie et ses graines sont récoltées, permettant ainsi d'estimer ses performances via un nombre de

descendants espéré (produit de la taille totale des silicules et du taux de survie des plantules après cinq semaines suivant la germination).

A la fin de chaque génération G_n, la performance relative de chaque individu est estimée dans chaque population pour constituer la génération G_{n+1}. Dans le cadre de cette thèse, les populations ont été suivies jusqu'à l'obtention d'une G_2 sous forme de graines.

Dans le chapitre 4, nous avons analysé les phénotypes exprimés dans chaque EP à la génération G_0 et à la génération G_1. Afin de tester l'effet du zinc sur l'évolution des traits, nous avons calculé les différentiels de sélections standardisés (S) et les gradients de sélections linéaires (ß) et quadratiques (γ), renseignant respectivement sur l'écart entre le phénotype des individus sélectionnés et la moyenne du phénotype dans la population dont ils sont issus (S), la direction et la force de la sélection (ß) et le mode de sélection ($\gamma<0$: sélection stabilisante ; $\gamma>0$: sélection disruptive)

Dans un premier temps, les résultats du phénotypage de la G_0 montrent des différences significatives entre les deux témoins, EP_1 et EP_4, pour un grand nombre de traits [Figure 41]. Les individus d'EP_4 ont un nombre de feuilles plus élevé, une surface de rosette plus grande, un nombre de hampes florales plus élevé, des hampes florales plus grandes, un plus grand nombre de silicules non avortées mais une taille moyenne de silicule plus faible. Ces différences étant liées à la fois aux types édaphiques utilisés dans la confection des EP mais aussi aux conditions de cultures. Ces résultats sont cohérents avec ceux des expériences que nous avons déjà réalisées [Chapitre 1, Chapitre 3]. Aussi, des observations similaires ont pu être faîtes dans des populations du Sud de la France [Jiménez-Ambriz *et al.*, 2007], avec les individus métallicoles exposés à un substrat contaminé (provenant de Malines, Moyen Age et Saint Bresson) ayant une surface de rosette plus importante et un nombre de hampes florales plus important que les individus non-métallicoles (provenant de Baraquette, Serrane et Tude) cultivés sur un substrat peu contaminé. Ces résultats contrastent, cependant, avec des observations réalisées *in situ* par Dechamps *et al.*, (2008) où des phénotypes similaires entre des individus provenant de Prayon et des individus provenant de Winseler ont pu être observé pour le nombre de feuilles ou le nombre de fruits fertiles.

L'exposition au zinc entraine également des différences significatives entre les populations d'origine non-métallicoles. Ainsi, comparé à EP_1 on observe une diminution de la surface des plantes dans EP_3 et une tendance dans EP_2, une augmentation du nombre de hampes florales et du nombre de silicules avortées dans EP_2 et EP_3, et une diminution de la taille moyenne des silicules dans EP_3. Néanmoins, le zinc ne semble pas affecter la survie des populations.

Enfin, le nombre de descendants attendu ne semble pas significativement différent entre les EP. Néanmoins la variance de ce trait est significativement plus grande dans EP_2, EP_3 et EP_4 comparé à EP_1, suggérant ainsi des niveaux de performances variables à l'intérieur de chaque population exposé au zinc.

En outre, en s'intéressant à la performance moyenne de chaque famille dans chaque EP, il semble que la réponse à l'exposition au zinc ne soit pas aléatoire. Plusieurs familles identiques (L105, W103, W105, W106, W109 et W112) expriment, en effet, les meilleures performances dans EP_2 et EP_3. De même, des familles similaires (L102, L103 et L116) expriment les niveaux de performance les plus bas dans EP_2 et EP_3 [Figure 42]. Ces résultats confirment et généralisent ceux observées dans le chapitre 2.

Les calculs des différentiels de sélection (*S*) réalisés à la fin de la G_0 montrent que l'ensemble des traits ont répondu positivement et significativement à la sélection dans EP_1, EP_2 et EP_3. Les différentiels étant plus élevés pour les traits reproducteurs dans EP_2 et EP_3. Dans EP_4 seuls le nombre de hampes florales et la taille de la plus grande hampe semblent répondre positivement et significativement à la sélection. Néanmoins, les valeurs des différentiels incluent à la fois la sélection directe du trait et la sélection indirecte liée à la sélection de traits corrélés. C'est pourquoi, les gradients de sélection linéaire (ß) et quadratique (γ) ont également été calculés pour chaque trait dans chaque population.

Ainsi, sur l'ensemble des populations, une sélection positive semble agir sur plusieurs traits reproducteurs comme le nombre de hampes florales ou le rapport entre le nombre de silicules non-avortées et le nombre de silicules avortées (rapport RS). Les gradients quadratiques ont montré, uniquement, une sélection stabilisante ($\gamma < 0$) pour le rapport RS et une sélection disruptive ($\gamma > 0$) pour la longueur de la plus grande hampe florale dans EP_2 et une sélection disruptive pour la surface de la plante dans EP_4.

Un nombre de hampe florale et un rapport RS plus élevés semblent donc être sélectionnés dans l'ensemble des EP, néanmoins l'intensité de la sélection semble être plus importante dans les EP en présence de zinc (EP_2, EP_3 et EP_4). Un nombre de hampe florale plus élevé peut être favorisé car il augmenterait, logiquement, le nombre de silicules (et donc de graines) produits. Un rapport RS plus élevés peut traduire une meilleure pollinisation des fleurs ou une meilleure allocation des ressources dans la production de fruits, et donc une augmentation du nombre de graines [Stephenson, 1981]. On peut également supposer qu'un rapport RS plus élevé pourrait traduire un meilleur « état de santé » de la plante.

En effet, le rapport RS est assez étroitement et positivement corrélé aux variables végétatives et permettrait d'expliquer, en partie, le différentiel de sélection positif observé pour le nombre de feuilles et la surface de la plante dans EP_1, EP_2 et EP_3. Une autre explication à l'observation de différentiels de sélection positifs pour les traits végétatifs serait une sélection indirecte liée à la sélection d'autres traits non mesurés [Lande & Arnold, 1983; O'Neil, 1997].

Après avoir constitué la G_1 de chaque EP et suivi les phénotypes des différents individus, nous avons remarqué des différences nettes entre les réponses exprimées par les populations entre la G_0 et la G_1. Ainsi, comparé à la G_0, le taux de survie des individus calculée à la G_1 dans chaque EP semble nettement plus bas dans EP_2 et EP_4 [Figure 43A]. Le pourcentage de plantes ayant produits des graines était également plus faible dans EP_2, EP_3 et EP_4 [Figure 43B]. De même les niveaux exprimés des traits phénotypiques étaient, globalement, plus bas en G_1 comparé à la G_0, excepté pour la surface des feuilles et le nombre de feuilles [Figure 47].

Le détail des comparaisons entre les EP à la génération G_1 montre également une structure très différente de celle observée à la G_0. Par exemple, les phénotypes exprimés par les plantes des populations témoins EP_1 et EP_4 divergent moins qu'en G_0 et les tendances observées en G_0 se sont parfois inversées. Ainsi, les différences significatives entre EP_1 et EP_4 concernant la taille des hampes florales ou le nombre de silicules observables en G_0 ne l'étaient plus en G_1.

Par ailleurs, les différences observées entre EP_1 EP_2 et EP_3 dépendent du trait considéré [Figure 45]. Généralement, les réponses phénotypiques dans EP_2 et EP_3 ont convergé et étaient significativement différentes de celles exprimées dans EP_1. Néanmoins, pour la surface de la plante, le nombre de hampes florales et le nombre de silicules non avortées, la réponse phénotypique diverge entre EP_2 et EP_3.

En s'intéressant à la performance de chaque famille dans chaque EP à la génération G_1, peu de familles communes ont exprimé les meilleures (L108 et L112) ou les pires (L104, L120 et L125) performances dans EP_2 et EP_3 [Figure 46]. Néanmoins, plusieurs familles perdues dans EP_2 à la fin de la G_0 (L113, L114 et L121) ont été perdues à la fin de G_1 dans EP_3 et, réciproquement, des familles perdues dans EP_3 à la fin de la G_0 (L101, L102, L111, L116, L119 et L124) ont été perdues à la fin de la G_1 dans EP_2.

Les différentiels de sélection ont été calculés en G_1 dans chaque population et montrent des résultats très différents de ceux observées à la G_0. En effet, seul le différentiel de sélection calculé sur la longueur de la plus grande hampe florale est positif et significatif dans l'ensemble

des populations. De plus, les différentiels de sélection calculés sur l'ensemble des traits sont significatifs et positifs dans EP_4.

Les gradients de sélection linéaires ont montré une sélection positive de la longueur de la hampe principale et du rapport entre le nombre de silicules non-avortées et le nombre de silicules avortées sur l'ensemble des populations, excepté EP_3. Enfin les gradients de sélection quadratiques ont montré une sélection stabilisante pour le rapport entre le nombre de silicules non-avortées et le nombre de silicules avortées uniquement dans EP_2 et une sélection disruptive pour la longueur de la plus grande hampe.

Ces résultats suggèrent, une nouvelle fois, une sélection positive agissant sur les traits reproducteurs. Néanmoins, la sélection ne semble pas agir sur les mêmes traits. Ici, en G_1, un nombre de hampes florales élevé ne semble pas sélectionné. Néanmoins, le nombre de structures florales était beaucoup moins variable qu'à la génération G_0, limitant ainsi la sélection. Les plantes observées avaient, généralement, une hampe florale principale développée, portant des silicules non-avortées, et une ou plusieurs hampes florales non développées, portant des silicules, pour la plupart, avortées. La sélection serait donc visible uniquement sur la longueur de la hampe florale principale car plus celle-ci est longue, plus elle porte de fruits et donc plus la plante produit de graines.

Les différences observées entre la G_0 et la G_1 peuvent être expliquées par plusieurs facteurs. Premièrement, les différences observables en G_1 pourraient être liées au résultat de l'effet de la sélection appliquée en G_0. Par exemple, une forte sélection appliquée à la G_0 sur le nombre de hampes florales pourrait conduire à une perte de polymorphisme pour ce trait, ce qui limiterait l'effet de la sélection sur ce trait à la génération G_1. Deuxièmement, le substrat n'a pas été remplacé entre les deux générations et il est possible que les différences de réponses soient liées à une diminution des ressources disponibles en G_1. Enfin, le fait de conserver le même substrat de génération en génération entraine un changement des conditions de culture et permet, par exemple, à des pathogènes de s'installer. En effet, durant la G_1, l'ensemble des populations a été infecté par un champignon, identifié comme *Sclerotinia sclerotiorum* (Communication personnelle Chloé Ponitzki). L'apparition de ce champignon coïncidait avec la phase reproductrice des plantes où il s'est attaqué essentiellement au collet de la plante, entrainant ainsi, l'avortement des différentes structures florales [Figure 56].

Figure 56 : A. Champignon identifié comme *Sclerotinia sclerotiorum* (Communication personnelle Chloé Ponitzki). B. détail de dégâts provoqués sur le collet

L'installation de ce champignon a donc nettement affecté l'ensemble des populations en réduisant la performance des individus. Néanmoins, les dégâts les plus importants ont été observés dans les EP qui ont été exposés au zinc. Particulièrement EP_2 et EP_4.

Cette observation peut s'expliquer par le fait que les populations EP_2 et EP_3 étaient déjà affectées par la présence de zinc et n'ont pas pu lutter efficacement contre la présence du pathogène. La diminution de la production de glucosinolate, un composé organique impliqué dans la défense contre l'herbivorie et/ou des pathogènes, dans les feuilles en présence de fortes concentrations en zinc dans l'environnement de culture a déjà été démontré [Tolrà *et al.*, 2001]. Néanmoins, cette étude suggérait qu'un compromis entre l'hyperaccumulation du zinc et la production de glucosinolate pouvant conférer un avantage adaptatif puisque le zinc accumulé pouvait également protéger les plantes des herbivores et/ou des pathogènes [Boyd & Martens, 1992; Boyd, 1998, 2007]. Nos observations vont à l'encontre de cette hypothèse puisque dans EP_2 et EP_3, nous devrions retrouver les plus fortes concentrations en métaux dans les feuilles, ce qui devrait limiter l'installation des pathogènes.

De même, il a été montré que les concentrations foliaires en glucosinolates étaient plus faibles chez les plantes métallicoles [Noret *et al.*, 2004, 2007], ce qui pourrait également expliquer les dégâts causés par le pathogène dans EP_4.

A la suite de cette expérience, il a été décidé de renouveler les conditions à chaque génération pour éviter l'installation d'autres espèces de pathogènes.

7.2.2. Comparaison de la réponse phénotypique dans des conditions contrôlées

Que ce soit dans l'expérience du chapitre 2, visant à choisir une concentration en zinc optimale, ou dans l'évolution expérimentale décrite dans le chapitre 4, le zinc affecte la survie, les capacités de croissance et de reproduction, il semble donc agir comme une pression de sélection.

Les gradients de sélection calculés dans le chapitre 4 prédisaient que la sélection semblait favoriser l'évolution des traits reproducteurs par opposition aux traits végétatifs dans l'ensemble des populations.

Aussi dans ces deux expériences, la sélection par l'exposition au zinc pourrait favoriser l'évolution du phénotype de la population. Pour observer cela, les descendances des populations utilisées dans les chapitres 2 et 4 ont été cultivées en serre, en condition contrôlées pour pouvoir

comparer, dans des conditions identiques, l'expression de plusieurs traits phénotypiques entre les populations dérivées et/ou ancestrales.

Dans le chapitre 2, la descendance (DP) de chaque PP a été cultivée en serre chaude dans un substrat contaminé à 500 $mg.kg^{-1}$ de zinc et plusieurs traits fonctionnels morphologiques et physiologiques ont été analysés, et non les traits reproducteurs pour différentes raisons exposées plus bas. Les résultats étaient assez contrastés. Pour certains caractères, y compris les concentrations en pigments et en composés secondaires ainsi que l'activité de l'ascorbate peroxydase, aucune différence n'a été observée entre les DP. Pour les autres caractères, nous avons observé une nette tendance à la divergence phénotypique entre les DP. Par exemple, nous avons observé une tendance à une diminution de la chlorose chez la progéniture des PP les plus exposées, sans plantes entièrement chlorotiques dans DP_{2000}. L'activité de la superoxyde dismutase (SOD) tend également à être plus élevée dans DP_{2000} comparée aux autres DP [Figure 22B]. Aussi, l'augmentation de l'activité de la SOD semble être associée à de nouvelles corrélations entre les traits dans DP_{2000}, ces corrélations étant absentes dans d'autres DP [Figure 24], suggérant ainsi que la sélection cible probablement plusieurs traits corrélés entre eux, ou avec d'autres traits non sélectionnés.

L'absence de réponse forte pose la question de savoir si la tolérance au zinc a réellement évolué entre les populations parentales et leur descendance ou si elle a été correctement évaluée. Deux choix méthodologiques peuvent expliquer l'absence de signal fort.

- Tout d'abord, parce qu'il fallait des parties végétatives non sénescentes pour effectuer différents dosages de biomarqueurs et de zinc, et parce que l'échantillonnage de ces parties végétatives aurait pu entrainer un biais dans l'expression des traits reproducteurs, nous nous sommes concentrés sur les caractères végétatifs. Cependant, les traits reproducteurs auraient pu révéler des différences plus importantes entre les DP car ils peuvent être plus proches de la valeur sélective [Violle *et al.*, 2007] et donc de la tolérance. En effet, des différences significatives dans la tolérance aux métaux ont déjà été montrées entre les accessions métallicoles et non-métallicoles avec l'utilisation de traits reproducteurs [Dechamps *et al.*, 2007, 2008; Jiménez-Ambriz *et al.*, 2007].
- Deuxièmement, l'exposition des DP à une seule concentration de zinc n'a peut-être pas permis de générer des réponses contrastées entre les DP. Le niveau d'exposition (500 $mg.kg^{-1}$) peut ne pas être assez élevé, ou l'absence de conditions contrôle (0 $mg.kg^{-1}$), permettant le calcul des indices de tolérance, peut avoir limité notre capacité à détecter les

différences de tolérance. De plus, la statistique de l'indice de tolérance a d'abord été conçue à partir de la croissance racinaire et on peut se poser la question de sa pertinence dans la mesure d'autres traits. De plus, l'utilisation d'indices de tolérance présente d'autres inconvénients qui n'ont pu être résolu, notamment la nécessité de cloner des génotypes chez une espèce non clonale et la nécessité de disposer d'un grand échantillon pour limiter la variance d'erreur [Macnair, 1993].

Enfin, en raison des tailles d'échantillons déséquilibrés (N_{DP0}=98, N_{DP500}= 68, N_{DP1000}= 55, N_{DP2000}=11), les analyses statistiques n'ont peut-être pas permis de déceler les différences entre les DP.

Nous avons aussi observé des résultats significatifs qui peuvent être comparé à des études antérieures. Ainsi, on s'attend généralement à ce que la chlorose révèle la phytotoxicité du zinc [Chaney, 1993; Rout & Das, 2009]. En effet, l'absence de chlorose à la plupart des niveaux d'exposition au zinc dans les tests hydroponiques a déjà été utilisée pour montrer des niveaux de tolérance plus élevés des populations métallicoles de *N. caerulescens* par rapport aux populations non-métallicoles [Assunção *et al.*, 2006]. On peut aussi supposer qu'une activité plus élevée de la SOD renforce le système de défense antioxydant [Sharma & Dietz, 2009], en prévenant, par exemple, les dommages causés par les ROS aux acides nucléiques ou aux pigments photosynthétiques [Sytar *et al.*, 2013]. Ainsi, l'augmentation des capacités antioxydantes pourrait être l'un des mécanismes responsables de l'augmentation de la tolérance des métaux chez les hyperaccumulateurs [Lin & Aarts, 2012]. Chez *N. caerulescens*, par exemples, il a été suggéré qu'une augmentation de l'activité des enzymes antioxydantes, pourrait entraîner une diminution de l'accumulation de ROS lors d'un stress lié à l'exposition au cadmium, entraînant indirectement une tolérance accrue au cadmium [Wang *et al.*, 2008].

Dans le chapitre 5, des représentants de la G_2 et de la G_0 de chaque EP ont été cultivés en serre froide dans un substrat contaminé soit à 750 mg.kg^{-1} soit à 2000 mg.kg^{-1} de zinc. Des représentants de la G_0 non-métallicole ont été également cultivés sur substrat non contaminé pour observer la réponse phénotypique d'une population non-métallicole supposée non stressée. Les individus sont suivis durant tout leur cycle de vie sur des traits fonctionnels morphologiques, physiologiques et phénologiques. Dans un premier temps, toutes les populations d'origine non-métallicoles ($G_{2\text{-}EP1}$, $G_{2\text{-}EP2}$, $G_{2\text{-}EP3}$ et $G_{0\text{-}NM}$) ont exprimé des niveaux phénotypiques plus bas lorsqu'elles étaient exposées à 2000 mg.kg^{-1} de zinc par rapport à une exposition à 750 mg.kg^{-1} (croissance limitée, plus de chlorose, performances réduites, etc.), ce

qui n'a pas été le cas pour les populations d'origine métallicole ($G_{0\text{-}M}$, $G_{2\text{-}EP4}$). Ce résultat concerne l'ensemble des traits excepté la concentration en certains biomarqueurs (Chlorophylle B, caroténoïdes et flavonoïdes) et certains traits phénologiques (Intervalle entre la montaison et la floraison ou la période de reproduction), mais reste le plus visible sur les traits reproducteurs. Cette observation est en accord avec d'autres études montrant une sensibilité au métaux plus importante des traits reproducteurs par rapport aux traits végétatifs [Jiménez-Ambriz *et al.*, 2007; Dechamps *et al.*, 2008].

Les résultats des comparaisons, aux deux concentrations, ont montré, également, peu de différences entre $G_{2\text{-}EP4}$ et $G_{0\text{-}M}$ suggérant que notre dispositif expérimental n'entrainait pas l'évolution du phénotype des plantes métallicoles. A l'inverse quelques différences ont pu être mises en avant entre $G_{2\text{-}EP1}$ et $G_{0\text{-}NM}$, le sens de la différence dépendant du trait étudié. De façon générale, les différences observées pour les traits végétatifs vont dans le sens $G_{2\text{-}EP1} > G_{0\text{-}NM}$, alors que les différences observées pour les traits reproducteurs vont dans le sens opposé. Ces différences peuvent s'expliquer de plusieurs façons :

- Les conditions de cultures ont pu entrainer une sélection directe, ou indirecte, pour plus de biomasse. Les résultats des gradients de sélection n'allaient pas dans ce sens mais les différentiels de sélection calculés en G_0 suggéraient une sélection indirecte pour plus de feuilles et une surface de rosette plus grande.
- Il est possible que des effets transgénérationnels s'expriment dans les deux populations. Les conditions de culture des parents (sol naturel pour les parents de la population $G_{0\text{-}NM}$ et substrat artificiel pour la $G_{1\text{-}EP1}$) entraineraient alors l'expression de phénotypes différents dans chaque descendance.
- Il est possible que le phénotype de la population EP_1 ait évolué par effet de la dérive génétique.

Les résultats les plus intéressants concernent cependant la réponse phénotypique observée dans $G_{2\text{-}EP2}$ et $G_{2\text{-}EP3}$. Globalement, nous avons observé une réponse forte pour les traits végétatifs dans ces deux populations comparées à $G_{2\text{-}EP1}$, les plantes de $G_{2\text{-}EP2}$ et $G_{2\text{-}EP3}$ présentant, de façon significative, plus de feuilles, une surface plus grande, une rosette plus haute et plus de chlorophylle. La réponse semble plus forte dans $G_{2\text{-}EP3}$ avec des phénotypes significativement différents de $G_{2\text{-}EP1}$ pour certains traits physiologiques et reproducteurs, notamment moins de chlorose, plus de hampes florales produites, un meilleur taux de survie des descendants et un nombre de descendants espéré plus important.

Il est également important de souligner que les réponses des populations G_{2-EP2} et G_{2-EP3} convergent. En effet très peu de différences significatives entre les deux populations ont été observées.

La diminution de la chlorose [Chaney, 1993; Assunção *et al.*, 2006; Rout & Das, 2009], l'augmentation de la biomasse [Ingrouille & Smirnoff, 1986; Escarré *et al.*, 2000], ou du nombre de graines (et donc de descendants) [Jiménez-Ambriz *et al.*, 2007; Dechamps *et al.*, 2008] étant utilisés dans la littérature pour montrer des niveaux de tolérances plus élevés, il semble raisonnable de considérer que les populations EP_2 et EP_3 aient évolué pour des niveaux de tolérance plus élevés. Ces résultats appuient et généralisent également les tendances observées dans les DP des populations exposées à 1000 $mg.kg^{-1}$ et 2000 $mg.kg^{-1}$ de zinc dans le chapitre 2.

Par ailleurs, les scores CSR exprimés dans chaque population ont été analysés et allaient, en partie, dans le sens des observations précédentes. Ainsi, Le score C était significativement plus élevé dans G_{2-EP2} et G_{2-EP3} alors que le score S diminuait significativement dans G_{2-EP3}, et tendait à diminuer dans G_{2-EP2}, ces résultats étant interprétés comme une sélection pour un développement important de la biomasse [Grime, 1977]. A l'inverse, dans G_{2-EP1}, G_{2-EP2} et G_{2-EP3}, une diminution significative du score R a été observée par rapport à G_{0-NM}, suggérant une contre sélection de cette stratégie, interprétée dans la littérature comme favorisant un cycle de vie court avec une production importante du nombre de graines, probablement liée aux conditions de cultures sur les populations non-métallicoles. Ces observations contrastent avec les résultats du chapitre 4. En effet, les gradients de sélection calculés à la G_0 prédisaient une évolution des traits reproducteurs. Néanmoins, nous observons surtout une évolution des traits végétatifs.

Enfin, le zinc a également été dosé dans les deux expériences. La méthode zincon a ainsi été utilisée, dans le chapitre 2, pour analyser le contenu en zinc de l'ensemble des DP. Dans le chapitre 5, un dosage ICP a été réalisé pour doser plusieurs éléments dans les populations G_{0-NM}, G_{2-EP1}, G_{2-EP2}, G_{2-EP3} et G_{0-M} exposées à 750$mg.kg^{-1}$ de zinc.

Dans le chapitre 5, la concentration foliaire en zinc était significativement plus basse dans la population G_{0-M} comparé aux autres populations. Cette différence étant due à l'origine édaphique de chaque population. G_{0-M} est constituée d'individus métallicoles, connus dans la bibliographie pour accumuler moins de zinc que les individus non-métallicoles [Meerts & Van Isacker, 1997; Escarré *et al.*, 2000]. Une différence significative a aussi été observée entre G_{2-EP2} et G_{2-EP3} allant dans le sens d'une accumulation plus faible de zinc dans G_{2-EP3}. Cette

différence tendant à être significative également entre $G_{0\text{-NM}}$/$G_{2\text{-EP1}}$ et $G_{2\text{-EP3}}$. Ces résultats suggèrent qu'une sélection pour une meilleure tolérance au zinc n'entraîne pas, ou pas rapidement, de différences significatives sur l'évolution de l'hyperaccumulation dans une population non-métallicole, dans nos conditions de sélection.

Néanmoins, dans le chapitre 2, les résultats ont montré des niveaux d'accumulation de zinc plus bas dans la descendance des populations les plus exposées (1000 et 2000 mg.kg^{-1}) que dans autres populations (0 et 500 mg.kg^{-1}). Ces résultats pouvant expliquer la baisse des niveaux de chloroses observée dans DP_{1000} et DP_{2000}. En effet, la réduction de la charge métallique peut correspondre à une réduction de la toxicité des métaux [Schat *et al.*, 2000].

Les niveaux d'accumulations plus bas dans DP_{1000} et DP_{2000} suggèrent que dans des conditions de sélection plus intenses que celles appliquées dans le chapitre 5, une sélection pour une augmentation de la tolérance pourrait provoquer une contre-sélection des capacités d'accumulation. Des études de coségrégation ont également mis en évidence un certain degré de corrélation génétique entre ces caractères [Assunção *et al.*, 2003a; Frérot *et al.*, 2005]. Néanmoins, les relations évolutives entre la tolérance et l'hyperaccumulation des métaux ne sont pas encore claires.

A.J.M. Baker [Baker, 1981] a suggéré que l'hyperaccumulation des métaux était l'une des stratégies physiologique de la tolérance aux métaux, car elle permettrait de concentrer les métaux dans des feuilles caduques, ce qui diminuerait la charge métallique globale. Des hypothèses alternatives, non exclusives, ont également été formulées [Boyd & Martens, 1992]. L'hypothèse de résistance à la sécheresse suppose que l'hyperaccumulation des métaux aurait pu être sélectionnée car elle augmenterait l'osmolarité cellulaire en milieu sec. L'hypothèse de l'allélopathie, ou d'interférence, suggère que les plantes hyperaccumulatrices enrichissent localement la surface du sol empêchant la colonisation par d'autres espèces non (ou peu) tolérantes [Boyd & Martens, 1992; Boyd, 1998]. L'hypothèse de défense, qui est l'hypothèse évolutive principale de l'hyperaccumulation [Vesk & Reichman, 2009], suppose que les métaux accumulés pourraient être avantageux car ils rendent les plantes toxiques pour les herbivores ou les pathogènes [Boyd & Martens, 1992; Boyd, 1998, 2007]. Un développement récent de cette hypothèse suggère même que l'hyperaccumulation pourrait être stimulée par une blessure [Plaza *et al.*, 2015]. Enfin, l'hypothèse d'absorption par inadvertance suppose que l'hyperaccumulation des métaux pourrait avoir évolué accidentellement, comme sous-produit d'un autre mécanisme ayant une fonction adaptative. Dans ce cas, l'hyperaccumulation serait

donc non fonctionnelle, non avantageuse et n'aurait aucun impact sur la valeur sélective des plantes.

Les résultats de cette expérience semblent donc suggérer qu’une sélection par l’exposition au zinc est possible et qu’elle permettrait une évolution de traits fonctionnels liés à la tolérance ou l’hyperaccumulation du zinc. Néanmoins, le résultat de cette sélection semble dépendant des concentrations appliquées et du nombre de générations.

Les différences observées entre les populations dérivées (DP ou G_2) peuvent également être le résultat d’autres mécanismes transgénérationnels [Herman & Sultan, 2011; Weinhold, 2018].

Conclusions et perspectives

La mise en place d'une évolution expérimentale pour simuler la colonisation d'un substrat enrichi au zinc par une population non-métallicole de *Noccaea caerulescens* a permis de montrer, d'une part, que le zinc pouvait représenter une pression de sélection importante car il affectait la survie et la reproduction des individus en entrainant une variation des performances individuelles. D'autre part, cette variation a permis la sélection de plusieurs familles identiques au fil des générations, suggérant que des mécanismes génétiques étaient impliqués dans la gestion du stress entrainé par l'exposition au zinc. Nous avons également montré que cette sélection pour de meilleures performances pouvait entrainer une évolution rapide des traits liés à la biomasse (nombre de feuilles, surface de la rosette, hauteur de la rosette, etc.), ces traits étant directement impliqués dans la tolérance aux métaux. De plus, les phénotypes des plantes sélectionnées tendent à se rapprocher des phénotypes exprimés dans les populations métallicoles.

Néanmoins, cette seule analyse phénotypique ne suffit pas à montrer le potentiel adaptatif des traits végétatifs. La différence observée pouvant s'expliquer par d'autres processus comme la dérive ou la plasticité transgénérationnelle. Ainsi, les comparaisons d'indices de différenciations de traits quantitatifs (Q_{ST}) avec les indices de différenciation génétiques neutres (F_{ST}) permettraient de mettre en évidence l'effet d'une sélection. Dans cette optique, une approche GBS (Genotyping by sequencing) a été initiée pour réaliser un génotypage des représentants de la G_0 et de la G_2, à partir de polymorphismes mononucléotidiques (SNP). Ces données pourront être utilisées pour réaliser des comparaisons Q_{ST}, F_{ST} entre populations ancestrales et dérivées de façon à mettre en évidence un effet de la sélection.

Une étude de la réponse génomique, utilisant ces données de séquençage, a également été initiée par Lucas Prost, dans le cadre d'un projet de Master. Les premiers résultats suggèrent que certaines régions génomiques ont répondu à la sélection engendrée par le zinc. Néanmoins les régions génomiques ayant répondus dans EP_2 et EP_3 étaient différentes. Ces résultats combinés à la convergence phénotypique observée entre les deux EP supposeraient qu'un grand nombre de gènes sont impliqués dans la tolérance au zinc.

L'utilisation des données phénotypiques et des données de séquençage permettrait de mieux identifier les gènes impliqués dans la tolérance aux métaux.

Aussi, compte tenu de l'intensité de notre pression de sélection, plusieurs générations sont attendues pour observer une éventuelle évolution des traits reproducteurs. Actuellement, l'expérience en est à la G_3 et il est envisagé d'aller, au minimum jusqu'à la G_5 pour effectuer une comparaison des phénotypes en milieu contrôlé.

Il peut également être envisagé de réaliser l'expérience inverse, c'est-à-dire soumettre une population d'origine métallicole à un substrat non-contaminé pour observer l'évolution du phénotype.

Enfin, les pressions de sélection qui s'opèrent en milieu naturel lors d'une colonisation d'un substrat métallifère sont nombreuses et il peut aisément être envisagé de tester l'effet de plusieurs métaux ou d'autres pressions de sélection biotiques ou abiotiques.

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Annexe

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RESEARCH PAPER

Can zinc pollution promote adaptive evolution in plants? Insights from a one-generation selection experiment

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Abstract

Human activities generate environmental stresses that can lead plant populations to become extinct. Population survival would require the evolution of adaptive responses that increase tolerance to these stresses. Thus, in pseudometallophyte species that have colonized anthropogenic metalliferous habitats, the evolution of increased metal tolerance is expected in metallicolous populations. However, the mechanisms by which metal tolerance evolves remain unclear. In this study, parent populations were created from non-metallicolous families of *Noccaea caerulescens*. They were cultivated for one generation in mesocosms and under various levels of zinc (Zn) contamination to assess whether Zn in soil represents a selective pressure. Individual plant fitness estimates were used to create descendant populations, which were cultivated in controlled conditions with moderate Zn contamination to test for adaptive evolution in functional traits. The number of families showing high fitness estimates in mesocosms was progressively reduced with increasing Zn levels in soil, suggesting increasing selection for metal tolerance. In the next generation, adaptive evolution was suggested for some physiological and ecological traits in descendants of the most exposed populations, together with a significant decrease of Zn hyperaccumulation. Our results confirm experimentally that Zn alone can be a significant evolutionary pressure promoting adaptive divergence among populations.

Keywords: Abiotic stress, anthropogenic habitats, experimental evolution, hyperaccumulation, fitness estimation, local adaptation, tolerance.

Introduction

Local adaptation is a major topic in evolutionary biology, especially in the current context of global change, because anthropogenic activities have generated environmental modifications in time and/or space (Grimm *et al.*, 2008). Modified environmental conditions might represent significant stresses for pre-existing populations of plants and other organisms, whose survival may rely on the capacity of individuals to either migrate or rapidly adapt their phenotype (Alberti *et al.*, 2017). There are several known examples of local adaptation after anthropogenic modifications. The first of these is the textbook example of industrial melanism in the British peppered moth (Majerus, 2009; van't Hof *et al.*, 2016), and there is now an increasing number of examples from species evolving in urban areas (Johnson and Munshi-South, 2017). These examples suggest that local adaptation can occur rapidly. Most of the time, however, available data are not sufficient to support local adaptation, for a number of reasons. First, local adaptation is generally tested only indirectly, using correlations between environmental and

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phenotypic variations (Thompson *et al.*, 2016). Second, temporal data on phenotypic changes from ancestral to selected populations (Franks, 2011) are usually lacking, so that it is not possible to discuss the dynamics of evolution. Third, the absence of historical information does not allow us to exclude alternative scenarios for phenotypic evolution, so that any adaptive interpretation of currently observed patterns may be considered as a 'just-so story' (Olson and Arroyo-Santos, 2015).

In this context, experimental approaches allowing the testing of evolutionary hypotheses can be particularly helpful. Experimental evolution involves the development of experimental designs to test for the effects of evolutionary forces on the evolution of populations surveyed over time in either controlled or natural environments (Garland and Rose, 2009; Kawecki *et al.*, 2012). A particular type of experimental evolution involves selection experiments focusing on the role of selection in evolution (Fuller *et al.* 2005). A selection experiment combines experimental populations and environmental conditions to test whether an environmental parameter acts as a selective agent. Selection experiments are generally long-term experiments that follow hundreds to thousands of generations. This is, however, limited to organisms with (very) short generation times, such as *Escherichia coli* (Lenski, 2017) or *Drosophila melanogaster* (Graves *et al.*, 2017). In addition, some studies have revealed that adaptive changes can be observed over a smaller number of generations. These studies concerned the evolution of resistance to starvation in *D. melanogaster* (Kubrak *et al.*, 2017) or the reduction in the length of the life cycle in response to water stress in *Arabidopsis thaliana* (Brachi *et al.*, 2012). In some cases the effects of experimental selection can even be observed after one generation, for example, for the evolution of above-ground biomass or phenology in response to elevation-related stresses in hybrid populations of *Mimulus cardinalis* and *Mimulus lewisii* (Angert *et al.*, 2008).

Local adaptation to metalliferous environments represents a unique model to understand adaptive evolution in anthropogenic habitats. Anthropogenic metalliferous soils have elevated soil concentrations of trace metal elements (TMEs) such as zinc (Zn), cadmium (Cd), and lead (Pb). TMEs have a relatively low toxicity threshold and persist in the environment, and they can be considered important selective pressures for biological species (Sánchez, 2013). Thus, in pseudometallophyte plant species that colonize both metalliferous soils (metallicolous populations) and non-polluted soils (non-metallicolous populations), adaptive divergence between populations is expected. Accordingly, ecogeographic patterns showing phenotypic differentiation between metallicolous and non-metallicolous populations, with higher levels of tolerance to the TMEs to which they are exposed among metallicolous populations, is usually interpreted in adaptive terms, assuming that soil metals are the major selective pressures (Jiménez-Ambriz *et al.*, 2007; Dechamps *et al.*, 2008; Meyer *et al.*, 2009; Babst-Kostecka *et al.*, 2016).

Such an *a posteriori* interpretation of ecogeographic patterns can be challenged in various ways. There is no experimental evidence of the role of soil metal toxicity in adaptive divergence between metallicolous and non-metallicolous populations. On the contrary, recent data suggest that, besides metal pollution, other environmental factors that distinguish the ecology of metalliferous and non-metalliferous sites in anthropogenic landscapes may promote adaptive divergence among populations (Frérot *et al.*, 2018). Considering that metal tolerance can be defined as the ability to survive and reproduce on soils containing high concentrations of metals that are toxic for most plant species (Macnair, 1993), with no reference to any selective agents, any adaptation to a particular feature of metalliferous soils—even if it is not directly related to metal toxicity—could promote divergence in metal tolerance levels between metallicolous and non-metallicolous populations. This nuance may be even more relevant for pseudometallophytes showing species-wide quantitative variations in metal homeostasis mechanisms. In such species, non-metallicolous individuals showing basic physiological tolerance may be considered pre-adapted to internal metal toxicity (Meyer *et al.*, 2016), so that the evolution of increased tolerance to metalliferous soils could primarily require adaptation to other selective pressures. Finally, population genetic data suggest that demographic history may also play a role in between-population phenotypic divergence (Gonneau *et al.*, 2017). In some cases, trait values observed in metallicolous populations may not even be derived but rather may be ancestral (Babst-Kostecka *et al.*, 2018).

In this study, we developed a selection experiment on *Noccaea caerulescens*, a small Brassicaceae pseudometallophyte occurring on non-metalliferous, calamine (Zn- and Cd-enriched), and serpentine (Ni-enriched) soils. *N. caerulescens* is also known to hyperaccumulate Zn, Cd, and Ni. The species is a model system for the study of plant–soil interactions, particularly in metal-contaminated soils (Assunção *et al.*, 2003*b*; Milner and Kochian, 2008). Species-wide quantitative variation in metal tolerance and hyperaccumulation has been reported, and local adaptation to metal contamination has been assumed to account for phenotypic divergence between metallicolous and non-metallicolous populations (Meerts and van Isacker, 1997; Dechamps *et al.*, 2007; Gonneau *et al.*, 2014). Our first objective in this study was thus 2-fold. First, we wanted to investigate whether soil Zn pollution could represent a selective pressure promoting adaptive divergence among natural populations from standing genetic variation. To do so, we constructed several genetically homogeneous experimental populations from non-metallicolous progenies and subjected them to contaminated soil with different Zn doses in mesocosms for one generation. Plant performance was then estimated and compared, to assess whether selection could be assumed to be occurring. Second, we wanted to test whether one generation of selection from standing genetic variation could have modified the tolerance level of the descendant generation. To do this, performance estimates were used to create descendant populations. Descendant populations were exposed to Zn in controlled conditions and phenotyped in order to compare their tolerance levels. Tolerance tests were performed using a battery of morphological, physiological, and biochemical traits described in the literature.

Materials and methods

Design of parent experimental populations

In June 2013, maternal families were collected from three non-metallicolous populations of *N. caerulescens* in Luxembourg, at Winseler (WIN; 20 families), Wilwerwiltz (WIL; 15 families), and Lellingen (LE; 15 families) (Table 1). In September 2013, 15 seeds per family were sown in

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Table 1. *Characteristics of the study sites*

		Lellingen[a]	Wilwerwiltz[b]	Winseler[b]
Geographic coordinates		49°59′N, 6°00′E	49°57′N, 5°53′E	49°58′N, 5°59′E
Soil pH		5.7	5.9	5.8
Total soil metal concentration (mg kg^{-1})	Zn	126 ± 4.3	139	164–274
	Cd	<1 ± 0.0	<2	0.7–4.3
				2.5–2.6
	Pb	48 ± 3.5	54	80–136
				122–168
	Ni	48 ± 2.6	42	66–157
				59–65

Soil samples were taken at a depth of 0–15 cm for study 1 and 0–10 cm for study 2. The concentration of metals is expressed as mg metal kg^{-1} soil.

[a] Assunção *et al.*, 2003*a*.

[b] Reeves *et al.*, 2001.

seedling trays containing peat-based compost, placed in a cold room at 4 °C for 1 week, and then transferred to a glasshouse for 6 weeks.

Available material was used to build four parental experimental populations (PPs). To be selected, a sown family had to have produced at least four seedlings to be represented once, and a multiple of four seedlings to be represented more than once, in each PP. Thus, each family was equally represented in the different PPs to homogenize the genetic composition of PPs. We selected as many family fulfilling these criteria as possible to maximize the genetic diversity within each PP. The final PPs were made up of 49 individuals from 23 families (13 from WIN, 10 from WIL, and 1 from LE; see Supplementary Table S1 at *JXB* online).

To confirm that PPs were genetically similar, all 196 individuals were genotyped using 14 microsatellite markers from the NcM1 and NcM3 multiplexes mentioned in Mousset *et al.* (2015). The extraction and genotyping protocols detailed in Mousset *et al.* (2015) were followed. Microsatellite markers Ncpm13 and Nc7b were removed from analyses because they showed no polymorphism in the natural populations we sampled (data not shown). Pairwise fixation index (F_{ST}) values were calculated from microsatellite data using SPAGeDi 3 (Hardy and Vekemans, 2002).

Transfer and cultivation of parent populations in outdoor mesocosms

In October 2013, PPs were transferred to distinct mesocosms. Mesocosms were made using square tubs designed from the lysimeter model proposed by Ruttens *et al.* (2006). Tubs had an area of 0.436 m^2 enclosed by a 13 cm-wide buffer zone filled with compost to restrain border effects and create a thermic buffer (Supplementary Fig. S1). Tubs were filled with a mixture of 140 kg of peat- and clay-based compost and 70 kg of zeolite; this mixture was found to perform better than peat-based compost alone when following plants over their entire life cycle (unpublished data). Mesocosms were placed outdoors to allow plants to complete their life cycle under natural climatic conditions, in a 60 m^2 external area; mesocosms were separated by at least 4 m (15 m maximum) to limit pollen transfer between them.

In three out of four mesocosms, different doses of zinc sulfate ($ZnSO_4{\cdot}7H_2O$) were introduced in powder form to obtain soil contamination at concentrations of 500 mg kg^{-1} (439.73 g of $ZnSO_4{\cdot}7H_2O$), 1000 mg kg^{-1} (879.46 g of $ZnSO_4$, $7H_2O$), and 2000 mg kg^{-1} (1758.92 g of $ZnSO_4{\cdot}7H_2O$). The fourth mesocosm was used as a non-contaminated control environment. In order to obtain homogeneous substrates, the mixtures were made in a cement mixer.

Each PP was named according to the level of Zn contamination to which it was exposed: PP_0, PP_{500}, PP_{1000}, and PP_{2000}. In late October 2013, seedlings were transferred into mesocosms at the center of cells of equal area arranged in a 7 × 7 grid (Supplementary Table S1). Plants were cultivated in their respective mesocosms until seeds were harvested in summer 2014.

Estimation of individual plant fitness under Zn exposure in mesocosms

To assess the performance of plants in PPs under Zn exposure, and their ability to participate in forming the next generation, survival and several vegetative and reproductive traits were followed. The date of death and the corresponding phenological state were recorded for each plant. Several vegetative traits were measured at the emergence of the first flower bud. These comprised the plant surface (PS), calculated as the surface area of an ellipse from two orthogonal diameters of the rosette, the number of leaves (NL), and the mean leaf length (LL) and leaf width (LW), calculated from the three longest leaves. At the end of the life cycle, mature seeds were collected from every flowering plant that produced seeds. Plants were then harvested and several reproductive traits were measured. These included the number of flower stems (NFS), the length of the longest stem (maxLFS), the sum of the lengths of flower stems (sumLFS), the total number of non-aborted siliques (NS), and the mean length of siliques for the entire plant (meanLS), estimated from the length of five siliques per flower stem. The methodology for the measurement of meanLS was validated in a preliminary experiment conducted on 10 plants, on which we measured 100%, 75%, 50%, or 25% of siliques per stem, or 10 or five scattered siliques per stem. The best compromise between the effort of measurement and the absence of a significant difference from measuring 100% of siliques corresponded to measuring five siliques per stem (data not shown).

Composition of descendant populations from fitness estimates

The contributions of maternal plants to the next generation, that is, the expected numbers of descendants, are related to their relative fitness (Violle *et al.*, 2007). They can be appropriately assessed by comparing reproductive outputs. In plants, reproductive output can be estimated from the seed set. However, owing to the large number of siliques and the initiation of fruit dehiscence and seed release by environmental factors such as rain and wind, it was not possible for us to exhaustively collect seeds and to measure seed set without risk of bias. Therefore, we used a proxy of seed number, called W_{est}, calculated as the product of NS and meanLS (Brachi *et al.*, 2012; Roux *et al.*, 2016). Plant relative fitness (ω_{est}) was then estimated by dividing the individual reproductive output W_{est} by the sum of reproductive outputs over all individuals from the same PP:

$$\omega_{est_i} = W_{est_i} \Big/ \sum_i^{49} W_{est_i}$$

where *i* is the *i*th plant among the 49 plants in the same PP.

After one generation of selection, four descendant populations (DPs) were expected from the four PPs, and were named according to the level of Zn contamination applied to the PPs: DP_0, DP_{500}, DP_{1000}, and DP_{2000}. The expected composition of the DPs was determined using the relative fitness of individual mother plants, ω_{est_i}, in the corresponding PPs to calculate an expected number of descendants (Fig. S2). For each mother plant, the expected number of descendants was calculated by multiplying

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the relative fitness estimate ω_{est_i} by the experimental population size (n=49) and rounding to the nearest integer.

To construct the DPs, seeds harvested from the four PPs were sown in December 2014 in seedling trays containing compost. The number of seeds sown from each mother plant was equal to the expected number of descendants calculated as explained above, multiplied by 6: that is, the number of descendants was multiplied by 3 to take into account the possibility of low germination rates (i.e. the expected germination rate was 33%) and multiplied by 2 to enlarge the sample size of each DP (so that phenotyping could be performed on 98 individuals per DP rather than 49). Seedling trays were placed in a cold room at 4 °C for 1 week and then transferred to a glasshouse for 8 weeks.

Test of Zn tolerance in descendant populations

To compare the levels of Zn tolerance among DPs derived from PPs exposed to various levels of Zn in soil, a tolerance test was carried out. At a time T_0, considered to be the beginning of the tolerance test, the available DP seedlings were transferred into individual pots containing 1 kg of a peat- and clay-based compost (70%)/zeolite (30%) mix, contaminated with 500 Zn kg^{-1} by adding 20 ml of a solution of 0.38 M $ZnSO_4 \cdot 7 H_2O$). This moderate level of Zn exposure was chosen for two main reasons. First, Zn was expected to provoke toxicity but not plant mortality, which would have prevented phenotyping. We made use of the results from the culture of PPs in mesocosms, in which mortality significantly increased from 1000 mg kg^{-1} of Zn exposure. Second, plants had to be healthy enough to actively control metal homeostasis, in particular Zn accumulation, because measuring metal accumulation in weakened plants may give spurious results and lead to erroneous interpretations (Van der Ent *et al.*, 2013).

Pots were randomized and placed in a greenhouse on a testing table. To limit microenvironmental effects, the pots were rotated twice a week. Several traits were recorded at T_0 and after 2 months (T_2). At T_0 and T_2, the number of leaves and the plant surface area (calculated as the surface area of an ellipse from the two larger diameters) were measured in order to calculate a growth rate based on the number of leaves (NL_{GR}) and the plant surface (PS_{GR}). At T_2, the level of chlorosis was estimated by visually classifying plants into four categories (1, healthy; 2, partially chlorotic; 3, entirely chlorotic; 4, dead plant). Average chlorophyll contents were also measured from three leaves per plant by using a CL-01 Chlorophyll Content Meter (Hansatech Instruments, King's Lynn, UK). At T_2, a number of biomarkers were analyzed to study the impact of Zn in plant tissues on different physiological processes. These biomarkers were related to (i) levels of pigments and secondary compounds (chlorophyll *a*/*b*, carotenoids, phenolic compounds, flavonoids, and tannins) and (ii) antioxidant enzyme activity [superoxide dismutase (SOD) and ascorbate peroxidase (APX)]. Each biomarker was measured according to the method detailed by Al Souki (2017). Zn concentrations in aerial parts were also measured in three mature leaves following the zincon method developed for *Arabidopsis halleri* (Macnair and Smirnoff, 1999). This method is based on UV-visible spectrophotometry using zincon as a colored Zn-chelating agent and has been previously validated for *N. caerulescens* (Frérot *et al.*, 2005)

Statistical analyses

For data obtained on PPs in mesocosms, a χ^2 test was used to compare survival rates among parent populations; pairwise tests were also performed with Bonferroni-adjusted P-values. The rates of plants that produced seeds were also compared among PPs using a χ^2 test and pairwise tests with Bonferroni-adjusted P-values.

A principal component analysis (PCA) was performed in order to test whether some traits could contribute to some of the point cloud structure according to zinc contamination levels in PPs. Kruskal–Wallis comparison tests were also performed on each vegetative and reproductive variable to investigate the potential effect of Zn exposure on the plants. This non-parametric test was used because the conditions for ANOVA were not fulfilled. When significant differences were found, *post hoc* Conover tests were performed with Benjamini–Hochberg adjusted P-values.

Data obtained on DPs in controlled conditions were used to test the effect of Zn exposure of the PPs in mesocosms on the evolution of metal-related traits. As above, a PCA was performed to test whether some traits could contribute to some of the point cloud structure among DPs. As an ordinal variable, the level of chlorosis was not included in the PCA. Non-parametric Kruskal–Wallis tests were performed for all the continuous variables and for chlorosis. If significant differences were found, *post hoc* Conover tests were performed with Benjamini–Hochberg adjusted P-values. Finally, to test whether Zn contamination levels could have modified phenotypic correlations among traits, correlation matrices for each DP were computed and compared using Steiger's tests.

Comparison tests and correlations were performed with R 3.3.2 (R Core Team, 2016); PCA and graphical representations required the installation of the packages factoMineR and ggplot2.

Results

Genotyping of parent populations

None of the pairwise differentiation indices (F_{ST}) was significant (Supplementary Table S2), indicating strong genetic homogeneity among the four parent populations on the basis of 12 microsatellite markers.

Comparison of plant performance according to Zn exposure in parent populations

The level of Zn exposure significantly affected survival rates in PPs (χ^2=50.92, df=3, P=5.088 × 10^{-11}; Fig. 1A): as the contamination level increased, survival rates decreased (PP_0=100%, PP_{500}=98%, PP_{1000}=67.3%, PP_{2000}=51%). Pairwise tests distinguished two pairs of groups showing significant differences: PP_0/PP_{500} and PP_{1000}/PP_{2000}. The number of plants producing seeds decreased as the Zn contamination level increased (PP_0=100%, PP_{500}=87.7%, PP_{1000}=57.1%, PP_{2000}=32.6%); a χ^2 test showed significant differences among PPs (χ^2=63.988, df=3, P=8.256 × 10^{-14}; Fig. 1B).

For surviving plants, the projection of individuals on the two first components of the PCA on vegetative and reproductive traits revealed a slight separation of point clouds, in particular between PP_0 and PP_{2000} (Supplementary Fig. S3A). In particular, individuals from PP_0 displayed among the highest coordinates on the first component and the lowest on the second component, whereas individuals from PP_{2000} displayed among the lowest coordinates on the first component and the highest on the second component. The first component explained 49.9% of the variance and combined all reproductive traits, whereas the second component explained 22.6% of the variance and combined vegetative and reproductive traits (Supplementary Fig. S3B, C). Conversely, individuals from PP_{500} and PP_{1000} showed great phenotypic variability. with confidence ellipses largely overlapping the others, either on the first component (for PP_{1000}) or on the second component (for PP_{500}).

Kruskall–Wallis and *post hoc* tests performed on each variable showed no difference among PPs in terms of the plant surface (Fig. 2A) or the average length and width of the three longest leaves (Fig. 2B, C). Differences among PPs on vegetative traits were apparent only for leaf number (Fig. 2D),

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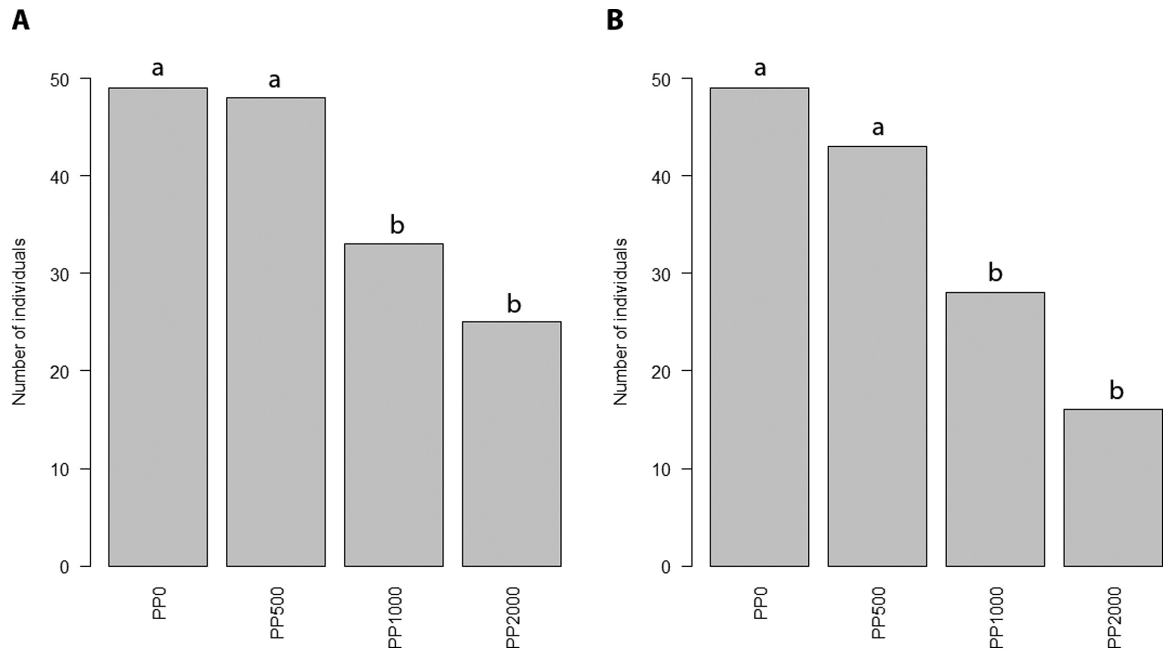

Fig. 1. Number of individual plants that (A) survived and (B) produced seeds in each parent population (PP). Bars with different letters are significantly different ($P \leq 0.05$).

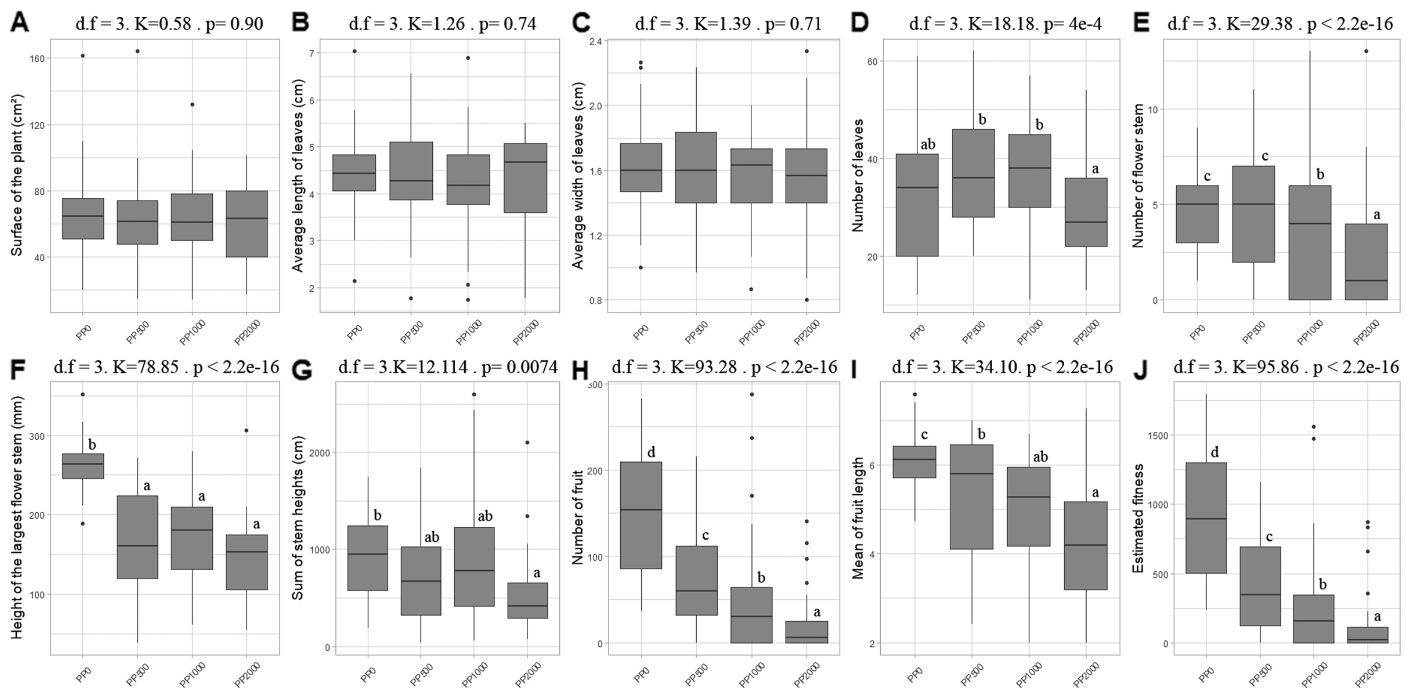

Fig. 2. Effect of zinc on vegetative traits (A–D), reproductive traits (E–I), and fitness estimation (J) for each parental population (PP). K, Kruskal–Wallis statistic. Boxplots with different letters are significantly different ($P \leq 0.05$).

with PP_{2000} showing the lowest leaf number (Supplementary Table S3). By contrast, reproductive traits always distinguished PPs, with a reduction of trait values with increasing Zn exposure (Fig. 2E–I). Reproductive outputs were strongly reduced by Zn exposure, with more than a 4-fold reduction in these outputs in PP_{2000} compared with PP_0 (Fig. 2J). Interestingly, the plants that maintained the best reproductive outputs in PP_{500}, PP_{1000}, and PP_{2000} belonged to the same families, such as WIL 30, WIL 13, WIL 18, and WIL 24 (Fig. 3).

Comparison of Zn tolerance levels among descendant populations

Because of poor germination rates, especially for DP_{1000} and DP_{2000}, the composition of DPs did not correspond to the expectations from the fitness estimates (Supplementary Fig. S2). In particular, some progenies were represented less than they were expected to be from estimated fitness values, or not at all. Therefore, the composition of the DPs included all available seedlings (Fig. 4).

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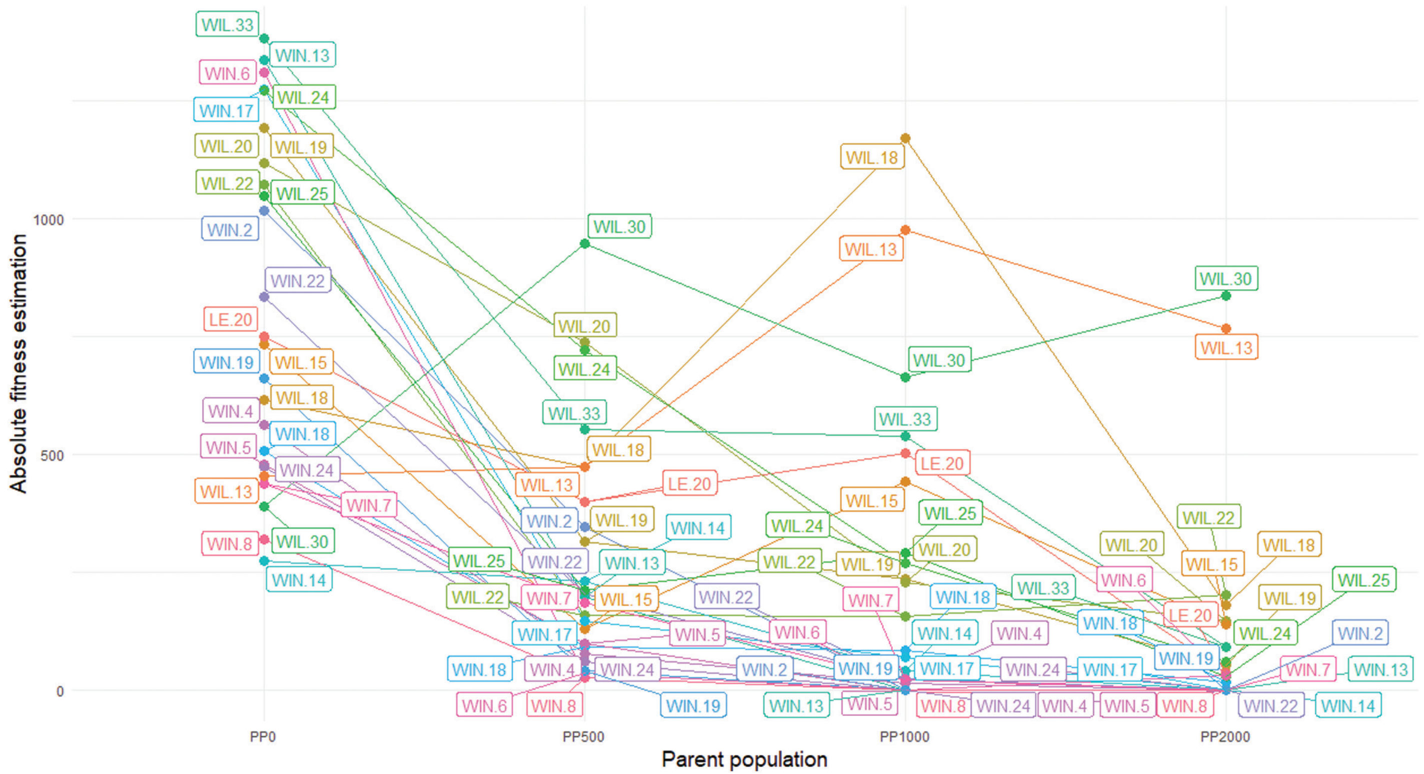

Fig. 3. Average absolute fitness estimation by plant family per parent population. LE, Lellingen population; PP, parent population; WIL, Wilwerwiltz population; WIN, Winseler population. (This figure is available in colour at *JXB* online.)

Fig. 4. Observed composition of each descendant population (DP_0, DP_{500}, DP_{1000}, and DP_{2000}) according to the relative fitness of individual mother plants from the corresponding parent populations. LE, Lellingen population; PP, general composition of each parent population; WIL, Wilwerwiltz population; WIN, Winseler population. (This figure is available in colour at *JXB* online.)

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Chlorosis

At T_2, chlorosis at level 4 (mortality) was observed only in DP_0 and DP_{500} (Fig. 5). Level 3 chlorosis (plant entirely chlorotic) was observed in DP_0, DP_{500}, and DP_{1000}. In DP_{2000}, chlorosis was only partial (level 2) when present. However, no significant difference in chlorosis levels was detected between the DPs (χ^2=4.047, *df*=3, *P*=0.2578).

The PCA performed on continuous data showed that no clear point cloud structure was visible on both axes (Supplementary Fig. S4A). Photosynthetic pigments were mainly represented on the first component (30.9% of variance), while secondary compounds and growth rates were mainly represented on the second component (11.2% of variance) (Supplementary Fig. S4B).

Antioxidant enzymes

APX activity showed no significant differences among DPs (Fig. 6A). In contrast, significant differences in SOD activity were found among DPs (Fig. 6B). However, this result was not confirmed by Conover's tests, although DP_{500} and DP_{1000} tended to have significantly lower SOD levels.

Photosynthetic pigments and secondary compounds.

Overall, there were no significant differences in concentrations of pigments (chlorophyll *a*, chlorophyll *b*, and

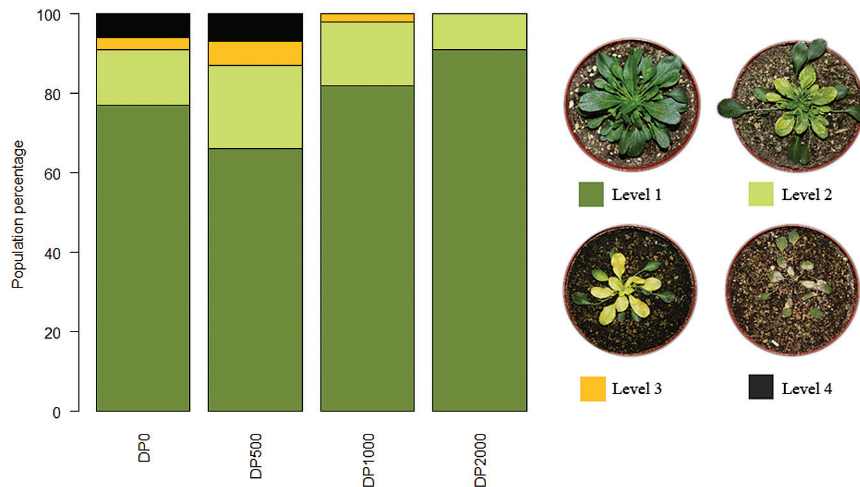

Fig. 5. Percentage of plants in each of the four descendant populations (DPs) with each level of chlorosis. Level 1, healthy; level 2, partially chlorotic; level 3, entirely chlorotic; level 4, dead. (This figure is available in colour at *JXB* online.)

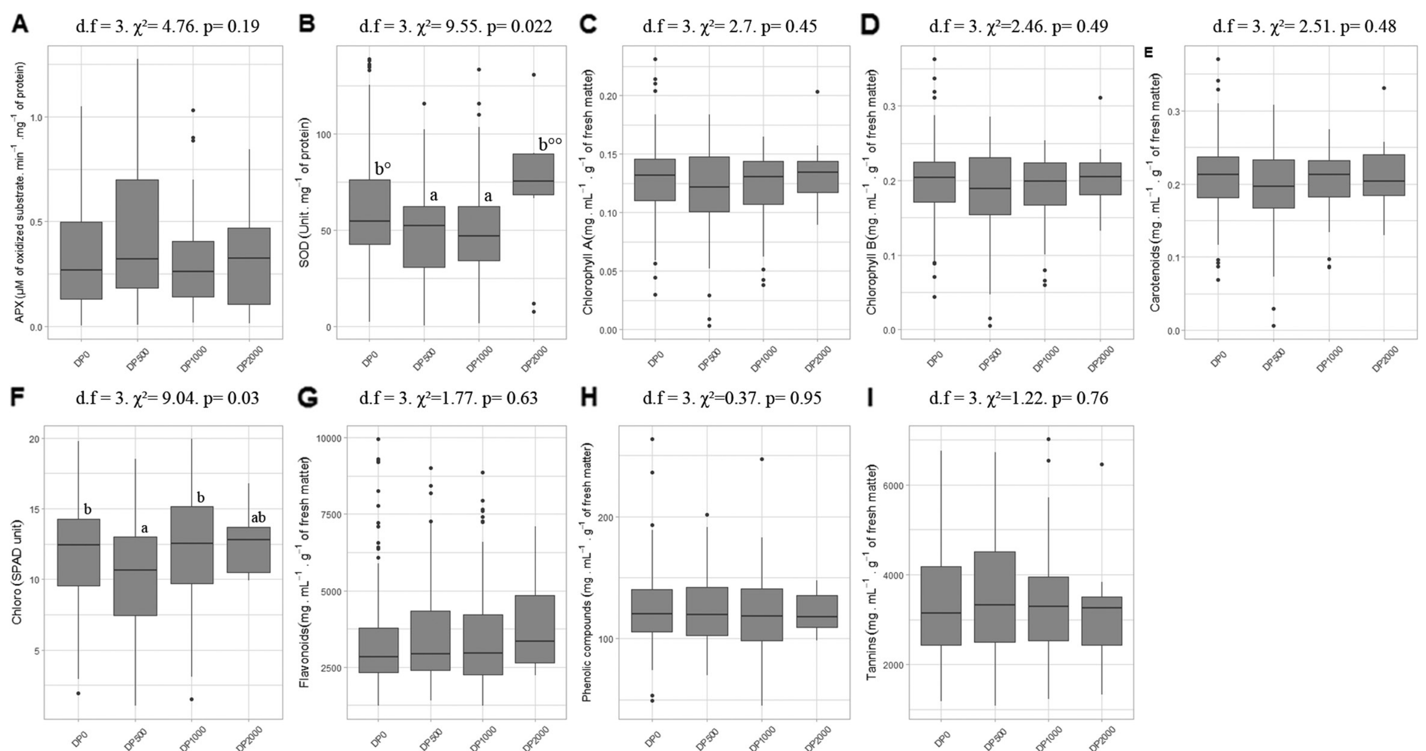

Fig. 6. Comparison of biomarker values (activity/concentration) in descendant populations (DPs). (A) Ascorbate peroxidase (APX). (B) Superoxide dismutase (SOD). (C) Chlorophyll *a*. (D) Chlorophyll *b*. (E) Carotenoids. (F) Level of chlorophyll as measured with a chlorophyll meter. (G) Flavonoids. (H) Phenolic compounds. (I) Tannins. Boxplots with different letters are significantly different (*P*≤0.05).

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carotenoids) among DPs, although DP_{500} showed a tendency towards lower values (Fig. 6C–E). By contrast, chlorophyll content measured with a chlorophyll meter showed significant differences (Fig. 6E). This result was confirmed by Conover's tests, showing significantly lower chlorophyll contents in DP_{500} than in DP_0 and DP_{1000}. There were no significant differences in the levels of secondary compounds among DPs (Fig. 6F–I).

Growth rates

For growth rates measured on leaf number and plant surface, DP_{500} had significantly lower values than DP_0 and DP_{1000} for SP_{Gr} (Fig. 7A), and a significantly lower value than DP_{1000} for NL_{Gr} (Fig. 7B).

Shoot zinc concentrations

Kruskal–Wallis tests showed significant differences among DPs (χ^2=17.036, df=3, P=5 × 10^{-04}). Plants from DP_{1000} and DP_{2000} had significantly lower mean ±SD Zn concentrations (DP_{1000}, 9896 ± 2407 mg kg^{-1} DW; DP_{2000}, 9068 ± 2566 mg kg^{-1} DW) than DP_0 and DP_{500} (DP_0, 11433 ± 2182 mg kg^{-1} DW; DP_{500}, 10947 ± 2231 mg kg^{-1} DW) (Fig. 7C).

Descendant population correlation matrix comparisons

All correlation matrices showed the same significant positive correlations among photosynthetic pigments. The main differences among matrices came from specific significant negative correlations among other traits. DP_{500} and DP_{1000} were significantly different from DP_0 on the basis of only a few negative correlations (Fig. 8). Striking differences were evident between DP_0 and DP_{2000}, since several specific correlations could be observed in DP_{2000}. Thus, SOD activity was negatively correlated with photosynthetic pigments. Photosynthetic pigments and flavonoid concentrations were also negatively correlated with phenolic compounds and growth rates. In addition, in DP_{2000} , photosynthetic pigments and flavonoid concentrations were positively correlated, as were phenolic compounds and NL_{GR} with PS_{GR}.

Discussion

Experimental selection for zinc tolerance

In our selection experiment, increasing the level of Zn contamination of the substrate from 0 to 2000 mg kg^{-1} significantly decreased the percentage of plants that survived and produced seeds (Fig. 1). We observed a corresponding strong response of plant reproductive traits, with a progressive reduction of mean values with increasing Zn contamination (Fig. 2). In contrast, except for leaf number, we found no significant difference among PPs for vegetative traits (Fig. 2, Supplementary Fig. S3, Supplementary Table S3). Interestingly, when looking at the identity of families that were able to survive, produce seeds, and maintain a certain level of performance, the effect of increasing the level of Zn contamination in mesocosms was not random. Indeed, the loss of families was apparently gradual, so that most of the families that produced seeds in PP_{2000} also did so in the other conditions. In particular, there was a gradual loss of families from the WIN site with increasing Zn contamination. Of the 13 WIN families initially represented, 13, 12, 2, and 2 produced seeds in PP_0, PP_{500}, PP_{1000}, and PP_{2000}, respectively. In comparison, of the 10 WIL families initially represented, 10, 10, 10, and 9 produced seeds in PP_0, PP_{500}, PP_{1000}, and PP_{2000}, respectively. The same pattern was also observed after ranking seed-producing families in terms of their estimated fitness (Fig. 3). The same families, mostly from WIL (e.g. WIL 13, WIL 18, and WIL 30) showed the highest fitness estimates in contaminated culture conditions. As a result, the expected composition of DPs was notably biased towards an overrepresentation of the same few WIL families

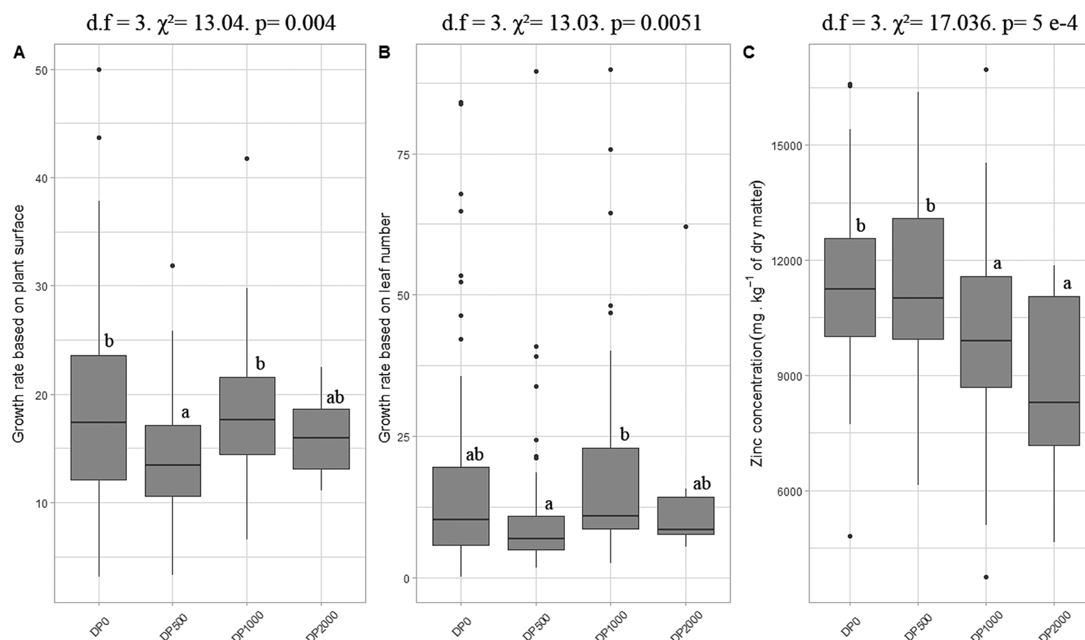

Fig. 7. Comparison of growth rates based on (A) surface of the plant or (B) leaf number and (C) zinc concentrations in descendant populations (DPs). Boxplots with different letters are significantly different (P≤0.05).

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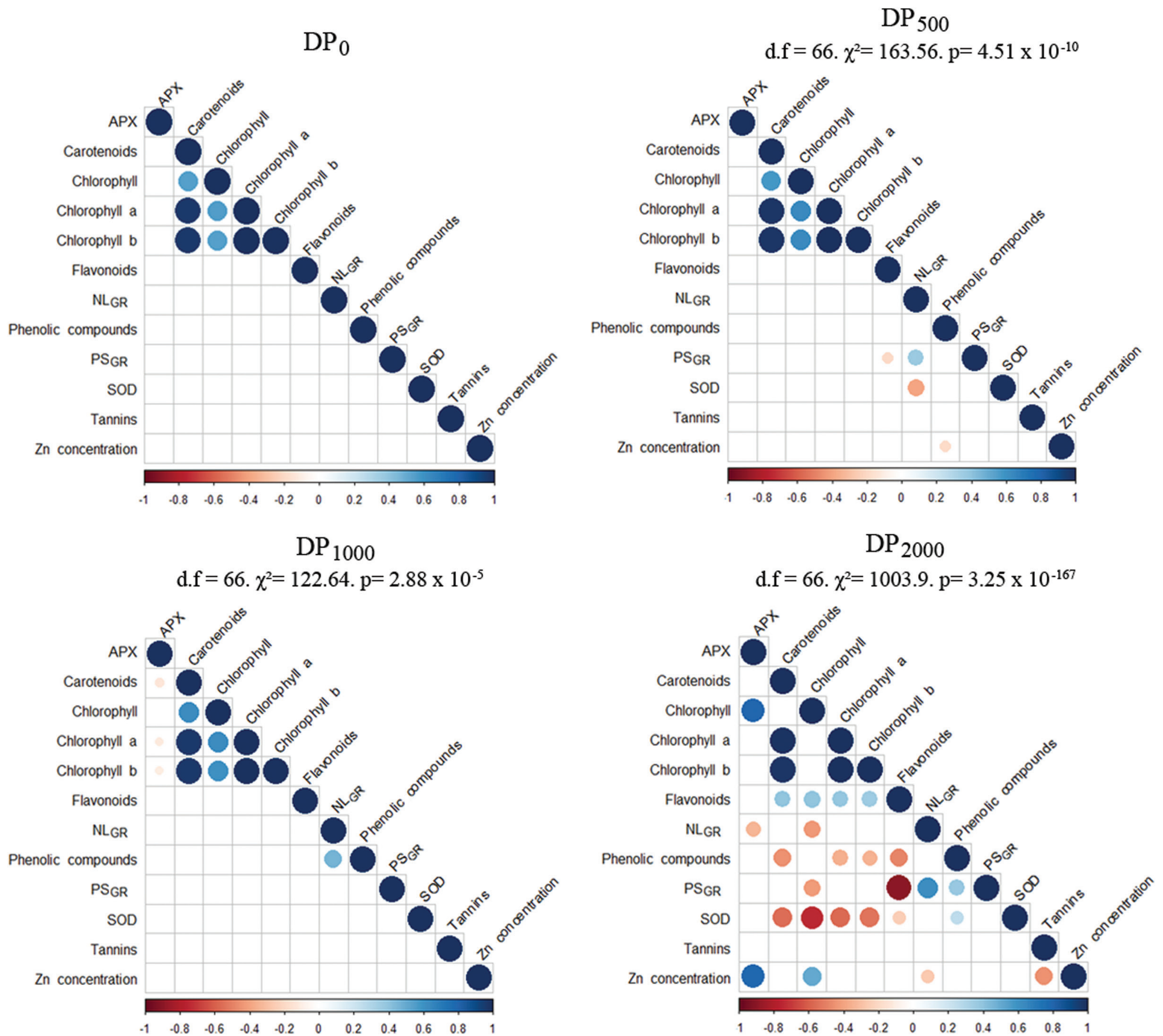

Fig. 8. Correlation matrices among traits in descendant populations (DPs). The gradient of colors represents the sign and strength of the correlation. Steiger's tests indicate comparisons between the DP_0 correlogram and those of the other DPs. APX, ascorbate peroxidase activity; Carotenoids, concentration of carotenoids; Chlorophyll, Chlorophyll level as measured with a chlorophyll meter; Chlorophyll *a*, chlorophyll *a* concentration; Chlorophyll *b*, chlorophyll *b* concentration; Flavonoids, concentration of flavonoids; NL_{GR}, growth rate based on leaf number; Phenolic compounds, concentration of phenolic compounds; PS_{GR}, growth rate based on plant surface area; SOD, superoxide dismutase activity; Tannins, concentration of tannins; Zn concentration, shoot zinc concentration. (This figure is available in colour at *JXB* online.)

(Fig. 4). By contrast, WIN families were not represented at all for the phenotyping of PP_{1000} and PP_{2000}.

The fact that the same families were selected suggests a genetic component in the ability of plants to handle Zn exposure. Since Zn stress mostly affects survival and reproductive traits, it can be considered to be a selective agent influencing the ability of plants to have their genes represented in the next generation (i.e. individual fitness). Any difference among DPs could therefore be interpreted as genetically determined. An alternative explanation would assume that altered phenotypes in DPs may result from transgenerational epigenetic mechanisms, also known as maternal effects, through a form of transgenerational stress adaption (Herman and Sultan, 2011; Weinhold, 2018). This notion remains highly controversial, however, as there is no strong evidence yet that modifications in the DNA methylome induced by environmental stresses can be inherited (Van Dooren *et al.*, 2018, Preprint).

It is clear that our results are limited to the reductionist approach we developed. In either geogenic or anthropogenic soil contamination, Zn never occurs alone, but is usually associated with a cocktail of pollutants as well as other abiotic or biotic modifications of the habitat (Frérot *et al.* 2018). At the organism level, it is known that physiological adaptations may or may not differ when plants are exposed to single or combined stresses

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(Pandey *et al.*, 2015; Zhang and Sonnewald, 2017). At the genetic level, we can expect that Zn, in combination with other stresses, or in a different chemical form, would affect a certain plant genotype in a different way, or at a different level of exposure.

Consequences of experimental selection on the evolution of metal tolerance

In our experiments, selection resulting from Zn exposure should have favored evolution towards higher Zn tolerance levels in DPs. Metal tolerance is, however, a very integrative trait that is expected to be determined by a combination of various functional (morphological, physiological, and phenological) traits that operate at lower levels of biological organization and are potentially correlated (Violle *et al.*, 2007).

The traits that we measured in this study included many traits that have been previously considered to be reliable estimates of metal tolerance in plants. Results were, however, not consistent across traits. For some traits, including levels of pigments and secondary compounds as well as APX activity, no difference was observed among DPs. For other traits, a clear trend towards phenotypic divergence among DPs was observed. A trend towards less pronounced chlorosis in the offspring of the PPs exposed to higher levels of Zn, with no entirely chlorotic plants in DP_{2000} only, was observed. DP_{2000} also showed a strong trend towards significantly higher SOD activity compared with other DPs (Fig. 6B). Increased SOD activity also appeared to be associated with new correlations among traits in DP_{2000} that were absent in the other DPs (Fig. 8). This suggests that SOD activity might not have been the only target of selection.

The absence of strong responses raises the question of whether Zn tolerance actually evolved between the PP and DP or whether evolution of Zn tolerance was properly assessed. Two methodological choices may explain the absence of a strong response. First, because we needed non-senescent vegetative plant parts to assess biomarker and metal levels, and because sampling could have biased reproductive traits, we focused on vegetative traits. However, reproductive traits could have revealed larger differences among DPs because they may be closer to fitness (Violle *et al.*, 2007) and therefore to tolerance. Significant differences in metal tolerance among *N. caerulescens* accessions have already been assumed using reproductive traits, while vegetative traits showed no significant difference (Dechamps *et al.*, 2007; Jiménez-Ambriz *et al.*, 2007). Second, exposure of DPs to a single Zn concentration may not have allowed the generation of different responses among DPs. The level of exposure of the DPs (500 ppm) may not have been high enough, or the absence of a control condition (0 ppm), which would have allowed the calculation of tolerance indices, may have limited our ability to detect differences in Zn tolerance among the DPs. The tolerance index statistic was initially designed using root growth, and its relevance to other traits may not necessarily be straightforward. Furthermore, the use of tolerance indices has some other disadvantages that could not be overcome in our study, including the necessity to clone genotypes in a non-clonal species and the need for a large sample size to limit the error variance of the ratio (Macnair, 1993). Finally, statistical analyses may have failed to detect differences between DPs because the sample sizes were unbalanced as a result of contrasting germination and survival rates (N_{DP0}=98, N_{DP500}=68, N_{DP1000}=55, N_{DP2000}=11).

In contrast, positive results can be related to previous knowledge. Chlorosis is generally expected to reflect Zn phytotoxicity (Chaney, 1993; Rout and Das, 2003). The absence of chlorosis at most levels of Zn exposure in hydroponic tests has previously been used to show higher Zn tolerance levels of metallicolous compared with non-metallicolous populations of *N. caerulescens* (Assunção *et al.*, 2006). Higher SOD activity can be assumed to reinforce the antioxidant defense system (Sharma and Dietz, 2009). It may help to prevent the damage caused to nucleic acids or photosynthetic pigments by reactive oxygen species produced because of the toxicity of excess internal Zn (Sytar *et al.*, 2013). Increased enzymatic antioxidant capacity may be one of the mechanisms responsible for increased metal tolerance in metal accumulators (Lin and Aarts, 2012). In *N. caerulescens*, it has been suggested that the enhanced activity of antioxidant enzymes such as SOD may result in less accumulation of reactive oxygen species due to Cd toxicity, indirectly resulting in increased Cd tolerance (Wang *et al.*, 2008).

Consequences of experimental selection on metal hyperaccumulation

Interestingly, our results showed a significantly lower level of Zn hyperaccumulation in the offspring of the PPs that were exposed to the highest levels of Zn (1000 and 2000 mg kg^{-1}) compared with the other conditions (0 and 500 mg kg^{-1}) (Fig. 5). This may explain the lower levels of shoot chlorosis in DP_{1000} and DP_{2000}, since a reduction of the metal burden in the shoots may correspond to reduced metal toxicity (Schat *et al.*, 2000). The lower Zn hyperaccumulation in DP_{1000} and DP_{2000} suggests that selection for increased tolerance could provoke counter-selection for accumulation capacity. A negative relationship between Zn tolerance and Zn accumulation has previously been reported at the population level in *N. caerulescens* (Meerts and van Isacker, 1997). Some degree of negative genetic correlation between these traits was also previously evidenced from cosegregation studies (Assunção *et al.*, 2003*c*; Frérot *et al.*, 2005). So far, however, the evolutionary relationships between metal tolerance and hyperaccumulation remain elusive. Baker (1981) suggested that metal hyperaccumulation was one of the two physiological strategies of metal tolerance and that it could have been selected to increase metal tolerance because the active concentration of metals in deciduous aerial parts of plants could allow metals to be released into the external environment and therefore reduce the overall metal load. Alternative, not mutually exclusive, hypotheses have also been formulated (Boyd and Martens, 1992). The hypothesis of drought resistance assumes that metal hyperaccumulation could have been selected to increase cellular osmolarity in dry environments. The hypothesis of elemental allelopathy assumes that hyperaccumulation of metal may be useful against nearby competitors (Boyd and Martens, 1992; Boyd, 1998). The elemental defense hypothesis, which Vesk and Reichman (2009) described as 'the leading hypothesis for the evolution of the metal hyperaccumulation trait in plants', assumes that the accumulation of

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metals could be advantageous because it renders plants toxic for herbivores or pathogens (Boyd and Martens, 1992; Boyd, 1998, 2007). A recent development of this hypothesis even suggests that hyperaccumulation could be stimulated by wounding (Plaza *et al.*, 2015). Another hypothesis assumes that metal hyperaccumulation could have evolved accidentally, as a by-product of other physiological modifications; if this were the case, it would therefore be non-functional, non-advantageous, and would have no impact on plant fitness. Apart from suggesting that hyperaccumulation may evolve under selection pressure, our results do not invalidate any of these other hypotheses. They suggest that, in species showing species-wide Zn hyperaccumulation, selection towards higher Zn tolerance levels may show a trade-off with evolution towards higher hyperaccumulation levels under conditions of exposure to high Zn concentrations. This may correspond to a reduction of the metal burden in the shoot when metal concentrations in soils are high.

Conclusion

Our selection experiment suggested that high Zn contamination affected several fitness components of non-metallicolous individuals of *N. caerulescens*, such as survival, reproductive traits, and seed production. After one generation of selection, a substantial response appeared at the highest Zn contamination levels. In particular, a trend towards more pronounced physiological traits associated with Zn tolerance, such as chlorophyll level and SOD activity, was observed. Our results also suggest that selection towards increased Zn tolerance could be associated with a reduction of Zn hyperaccumulation capacity. These results therefore support a role of Zn contamination as a possible selective agent shaping the evolution of both Zn tolerance and Zn hyperaccumulation. Results will have to be confirmed in further studies, in particular to check whether Zn contamination still represents a selective pressure in the field. If our results are confirmed, the molecular bases of adaptive phenotypic divergence among metallicolous and non-metallicolous populations could also be investigated.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Lysimeter schema.

Fig. S2. Expected composition of each descendant population.

Fig. S3. Principal components analysis results on PP data.

Fig. S4. Principal components analysis results on DP data at T_2.

Table S1. Population composition of parent populations.

Table S2. Pairwise genetic differentiation indices (F_{ST}).

Table S3. Results of comparison tests on traits measured in parent populations.

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Résumé

Les activités humaines génèrent des stress environnementaux pouvant entrainer l'extinction des populations végétales. Dès lors, la survie de ces populations nécessite l'évolution rapide de traits adaptatifs, qui augmenterait leur capacité de tolérance à ces stress d'origine anthropique. Par exemple, la colonisation d'un site métallifère nécessite l'évolution d'une capacité à tolérer les fortes concentrations en éléments traces métalliques (ETM) dans le sol. La tolérance aux métaux est ainsi généralement plus élevée chez les espèces se développant exclusivement sur sols métallifères (métallophytes strictes), et dans les populations métallicoles des espèces se développant occasionnellement sur sols métallifères (pseudométallophytes). Néanmoins, les mécanismes évolutifs permettant une augmentation de la tolérance aux métaux restent mal compris.

Dans ce contexte, un projet d'évolution expérimentale a été initié dans le but d'observer l'effet du zinc sur l'évolution d'une population non-métallicole de l'espèce pseudométallophyte, *Noccaea caerulescens*. Pour cela, plusieurs populations métallicoles et non-métallicoles ont été échantillonnées afin de constituer quatre populations expérimentales (EP) qui ont été cultivées en mésocosme. Une EP d'origine non-métallicole a été exposée à un sol non contaminé (EP1), deux EP d'origine non-métallicole ont été exposées à sol contaminé à 750 $mg.kg^{-1}$ de zinc (EP2 et EP3) et une EP d'origine métallicole a été exposée à un sol contaminé à 750 $mg.kg^{-1}$ de zinc (EP4). A chaque génération, la performance de chaque individu a été mesurée, et les graines récoltées, de façon à construire la génération suivante. Après deux générations, plusieurs réplicas des populations dérivées et ancestrales de chaque EP ont été cultivées en conditions contrôlées dans différentes conditions d'exposition au zinc (750 $mg.kg^{-1}$ et 2000 $mg.kg^{-1}$). Leurs capacités de tolérance ont été évaluées à travers la mesure de plusieurs traits fonctionnels.

Les résultats de cette étude montrent que le zinc représente effectivement une pression de sélection pour les populations non-métallicoles de *Noccaea caerulescens*, qui entraine une surreprésentation de certaines descendances à la génération suivante. Cette sélection semble avoir affecté de façon similaire les deux populations expérimentales d'origine non-métallicoles soumises au zinc (EP2 et EP3). En comparant différents traits fonctionnels morphologiques, physiologiques et phénologiques, potentiellement impliqués dans la tolérance aux métaux entre populations dérivées et ancestrales, nous observons une réponse phénotypique forte des populations EP2 et EP3 avec, notamment, une augmentation significatives de quasiment tous les traits morphologiques en lien avec la croissance des individus (nombre de feuilles, surface de la plante, hauteur de la plante, etc.).

Mots clés : Evolution expérimentale, adaptation locale, *Noccaea caerulescens*, hyperaccumulation, tolérance aux métaux, pression de sélection

Abstract

Human activities generate environmental stresses that can lead to the extinction of plant populations. Therefore, the survival of these populations requires the rapid evolution of adaptive traits, which would increase their ability to tolerate these anthropogenic stresses. For example, the colonization of a metalliferous site requires the evolution of an ability to tolerate high concentrations of metal trace elements (MTE) in the soil. Thus, metal tolerance is generally higher in species growing exclusively on metalliferous soils (strict metallophytes), and in metallicolous populations of species growing occasionally on metalliferous soils (pseudometallophytes). However, the evolving mechanisms for increasing metal tolerance remain unclear.

In this context, an experimental evolution project was initiated to observe the effect of zinc on the evolution of a nonmetallicolous population of the pseudometallophyte, *Noccaea caerulescens*. For this purpose, several metallicolous and nonmetallicolous populations were sampled to form four experimental populations (EP) that were cultured in mesocosm. One non-metallicolous EP was exposed to uncontaminated soil (EP1), two non-metallicolous EPs were exposed to contaminated soil at 750 $mg.kg^{-1}$ of zinc (EP2 and EP3) and one metallicolous EP was exposed to contaminated soil at 750 $mg.kg^{-1}$ of zinc (EP4). In each generation, the performance of each individual was measured, and the seeds were harvested, in order to build the next generation. After two generations, several replicas of the derived and ancestral populations of each EP were cultivated in controlled conditions under different zinc doses (750 $mg.kg^{-1}$ and 2000 $mg.kg^{-1}$). Their tolerance abilities were assessed through the measurement of several functional traits.

Results of this study showed that zinc represent a selection pressure for nonmetallicolous populations of *Noccaea caerulescens*, leading to an over-representation of some offspring in the next generation. This selection seemed to similarly affected EP2 and EP3. Comparison of different morphological, physiological and phenological functional traits, potentially involved in metal tolerance, between derived and ancestral populations showed strong phenotypic response of EP2 and EP3 with a significant increase in almost all morphological traits related to individual growth (number of leaves, plant surface, plant height, etc.).

Keywords: experimental evolution, local adaptation, *Noccaea caerulescens*, hyperaccumulation, metal tolerance, selective pressure