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# The clonal plant microbiota: assembly rules, heritability and influence on host phenotype

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présentée par

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Préparée à l'unité de recherche U.M.R. 6553 Ecobio  
Écosystèmes, Biodiversité, Evolution  
Observatoire des Sciences de l'Université de Rennes

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The clonal plant  
microbiota :  
assembly rules,  
heritability and  
influence on host  
phenotype

Thèse soutenue à Rennes  
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THE CLONAL PLANT MICROBIOTA: ASSEMBLY RULES,  
HERITABILITY AND INFLUENCE ON HOST PHENOTYPE

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LE MICROBIOTE DES PLANTES CLONALES : REGLES D'ASSEMBLAGE,  
HERITABILITE ET INFLUENCE SUR LE PHENOTYPE DE L'HÔTE





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## List of papers

This thesis is based on the following articles which are either published, in revision or in preparation.

- Article I Vannier, N., Mony, C., Bittebiere, A. K., & Vandenkoornhuyse, P. (2015). Epigenetic mechanisms and microbiota as a toolbox for plant phenotypic adjustment to environment. *Frontiers in plant science*, 6.
- Article II Vannier, N., Bittebiere, A. K., Vandenkoornhuyse, P., & Mony, C. (2016). AM fungi patchiness and the clonal growth of *Glechoma hederacea* in heterogeneous environments. *Scientific reports*, 6.
- Article III Vannier, N., Mony, C., Bittebiere, A. K., Michon-Coudouel, S., Biget, M. & Vandenkoornhuyse, P. A microorganisms journey between plant generators. In revision for *.ISME J*.
- Article IV Vannier, N., Mony, C., Bittebiere, A. K., & Vandenkoornhuyse, P. Introduction of the metaholobiont concept for clonal plants. In prep.
- Article V Vannier, N., Bittebiere, A. K., Mony, C. & Vandenkoornhuyse, P. Plant-plant interactions mediated by fungi impact plant fitness. In prep.

## Résumé Français

Depuis plusieurs années déjà, la communauté scientifique a tiré la sonnette d'alarme sur les risques accrus de crises alimentaires du fait des changements climatiques qui deviennent plus fréquents et extrêmes (Battisti et al., 2009). La productivité agricole mondiale diminue ainsi alors même qu'en parallèle la population humaine continue à croître régulièrement avec une population humaine estimée à 10 milliards à l'horizon 2100 (Lutz et al., 2001). Pour subvenir à une telle demande de nourriture la production agricole devra presque doubler (Cirera & Masset, 2010). Afin d'augmenter la production, les besoins en espaces agricoles seront en grande partie acquis par la transformation d'écosystèmes naturels (forêts) en cultures. Il est estimé ainsi la transformation de  $10^9$  hectares en agrosystèmes d'ici à 2050 (Tilman et al., 2001) induisant ainsi une perte de biodiversité et de fonctions écosystémiques et impactant considérablement la quantité de CO<sub>2</sub> émise vers l'atmosphère. Le défi pour l'agriculture durant ce siècle est donc de produire plus efficacement et durablement pour nourrir la planète tout en préservant au maximum les écosystèmes naturels. En agriculture, les contraintes locales de l'environnement ont été contournées par l'utilisation d'intrants dans les cultures (fertilisants, pesticides, etc.). Cependant, la disponibilité des ressources permettant de produire ces fertilisants est limitée et se pose donc la question de la durabilité de l'agriculture conventionnelle (Duhamel & Vandenkoornhuyse 2013). s.

Durant les dernières décennies, les communautés symbiotiques de bactéries, champignons et Archaea ont été reconnues comme des déterminants majeurs des fonctions écosystémiques à l'échelle globale (van der Heijden et al., 1998b; Leininger et al., 2006; Falkowski et al., 2008; van der Heijden et al., 2008), notamment via les fonctions de résistance et d'acquisition des ressources qu'ils fournissent. Ainsi, de nombreux chercheurs ont commencé à reconnaître le potentiel des micro-organismes associés aux plantes pour résoudre le dilemme productivité agricole-maintien de la biodiversité. La prochaine étape pour l'agriculture de demain serait donc de prendre en compte les communautés microbiennes des cultures pour optimiser les fonctions qu'elles assurent.

Afin de parvenir à cet objectif d'utilisation des communautés microbiennes à des fins d'agriculture durable, il est nécessaire d'appréhender les règles qui régissent le fonctionnement de ces communautés. Cette thèse a pour objectif de déterminer les règles d'assemblage des communautés microbiennes associées aux plantes et leur influence sur le phénotype de la plante

hôte. Elle est articulée autour de trois chapitres qui sont chacun composés d'articles en préparation, soumis ou publiés.

Chapitre 1 : Conséquences écologique et évolutive de la plasticité induite par les mutualistes.

In natura, les plantes sont colonisées par une grande diversité de micro-organismes à l'intérieur et à l'extérieur de leurs tissus. Ces microorganismes constituent leur microbiote. Ce microbiote fournit à son hôte des fonctions clés telles que l'acquisition des ressources ou la résistance aux stress biotiques et abiotiques (Friesen et al., 2011; Møller et al., 2016), influençant ainsi tous les aspects de la vie d'une plante, de l'établissement à la croissance jusqu'à la reproduction (Møller et al., 2016). La composition du microbiote des plantes est déterminée par une large gamme de facteurs environnementaux tels que les propriétés du sol comprenant le pH ou la disponibilité en nutriments et en eau (Berg & Smalla, 2009; Bulgarelli et al. 2012; Lundberg et al., 2012; Shakya et al., 2013; Schreiter et al., 2014). En effet, les micro-organismes associés aux plantes sont majoritairement recrutés depuis le sol. Du fait de leur style de vie sessile (i.e. immobile), les plantes doivent donc faire face à l'hétérogénéité de l'abondance des micro-organismes dans le sol. Les plantes ont ainsi développé différents mécanismes permettant de tamponner les effets des contraintes environnementales tels que des modifications plastiques (i.e. production de plusieurs phénotypes à partir d'un seul génotype) (Bradshaw, 1965). Ces modifications sont classiquement considérées comme étant déterminées par le génome de l'organisme. Or les mécanismes épigénétiques (régulant l'expression du génome) et les micro-organismes symbiotiques ont été identifiés à de multiples reprises comme des sources de variations phénotypiques (Richards, 2006; Friesen et al., 2011; Holeski et al., 2012; Vandenkoornhuyse et al., 2015). Dans le premier chapitre de cette thèse nous avons étudié l'influence de ces deux sources de variations phénotypiques sur le phénotype et l'adaptation des plantes.

L'article de synthèse bibliographique présenté dans cette thèse (Article I) a permis de mettre en évidence l'importance du microbiote et de l'épigénétique comme sources de plasticité phénotypique. Les avancées récentes en épigénétique et sur les symbiotes des plantes ont démontré leur impact majeur sur la survie, le développement et la reproduction des plantes. Ces sources de plasticité permettent aux plantes de s'adapter aux contraintes environnementales sur des pas de temps courts (au cours la vie de la plante) et longs (i.e. temps évolutifs). De plus, les variations phénotypiques ainsi produites peuvent être intégrées dans le génome par accommodation génétique et ainsi influencer les trajectoires évolutives. De manière générale, cette synthèse bibliographique indique également que des interactions entre microbiote et épigénétique pourraient exister et

devraient donc ,tre étudiés en parallèle des effets respectifs de ces mécanismes sur la survie et l'évolution des plantes.

Dans le second article présent dans ce chapitre (Article II), nous nous sommes intéressés au rôle des micro-organismes dans la réponse des plantes clonales à l'hétérogénéité environnementale. Les plantes clonales sont des organismes particulièrement plastiques. En effet, leur structure organisationnelle en réseau (i.e. individus clonaux connectés) permet la propagation dans l'espace ainsi que le partage des ressources et de l'information (i.e. intégration physiologique; Oborny et al., 2001). De nombreuses études ont mis en évidence que ce réseau permet le développement de deux principaux types de réponses plastiques à l'hétérogénéité des ressources: le foraging (i.e. exploration de l'espace pour les ressources) et la spécialisation (i.e. division du travail pour l'exploitation des ressources) (Slade & Hutchings, 1987; Dong, 1993; Hutchings & de Kroon, 1994; Birch & Hutchings, 1994). Par transposition, la présence de microorganismes symbiotiques dans le sol, pourrait induire le même type de réponses plastiques, en raison de leur rôle clé pour la plante (lire les synthèses Friesen et al., 2011; Møller et al., 2016).. Nous avons testé expérimentalement cette hypothèse en manipulant l'hétérogénéité de présence de champignons mycorhiziens à arbuscules (MA) et en mesurant les traits de réponses plastiques de foraging et de spécialisation de la plante clonale *Glechoma hederacea*. Dans nos expérimentations, nous avons démontré 1.) que *Glechoma hederacea* ne produit pas de réponse plastique de spécialisation et une réponse de foraging limitée à l'hétérogénéité des champignons MA et que 2.) les traits architecturaux impliqués dans les réponses de foraging des plantes ne sont pas affectés par les espèces de champignons MA testées, contrairement aux traits d'allocation des ressources liés aux réponses de spécialisation. Le fait que *G. hederacea* ne réponde que peu ou pas à l'hétérogénéité de distribution des champignons MA suggère que les plantes pourraient homogénéiser cette distribution en les transmettant d'un ramet à un autre dans le réseau. Cette hypothèse est corroborée par des observations en microscopie électronique à balayage révélant la présence d'hyphes à la surface d'échantillons de stolons et des observations en coupe montrant la présence de structures apparentes à des champignons colonisant les cellules de stolons.

## Chapitre 2 : L'héritabilité du microbiote des plantes, vers le concept de méta-holobionte

Dans ce chapitre nous avons testé expérimentalement l'hypothèse de transmission des micro-organismes entre ramets clonaux. Ce chapitre vise également à évaluer les conséquences d'un tel mécanisme de transmission pour les performances des plantes et pour la compréhension du fonctionnement et de l'évolution des plantes.

Des études précédentes ont mis en évidence l'existence de transmission de symbiotes endophytiques via la colonisation des graines de la plante hôte. Ce type de transmission a majoritairement été décrit pour le champignon *Neotyphodium coenoccephalum* qui colonise les graines et permet la résistance à certains stress environnementaux (Clay & Schardl, 2002; Selosse et al., 2004). Cet exemple est le seul décrit dans la littérature montrant une transmission verticale de micro-organismes entre générations de plantes et n'implique le transfert que d'une seule espèce de champignon à un moment spécifique de la vie de l'hôte. Comme mis en évidence dans le premier chapitre les plantes clonales pourraient potentiellement transmettre des micro-organismes, en plus des ressources et de l'information, à leurs descendants clonaux, constituant un niveau supplémentaire d'intégration physiologique.

Dans le premier article présenté dans ce chapitre (Article III), nous avons cherché à démontrer expérimentalement l'existence de transmission de microorganismes entre générations de plantes clonales. Dans nos expérimentations, nous avons détecté la présence d'archées, de bactéries et de champignons dans l'endosphère des ramets mères de *G. hederacea*. Certains de ces micro-organismes (des champignons et des bactéries) ont été détectés dans les racines et stolons des ramets filles démontrant ainsi l'héritabilité d'une partie du microbiote de la plante à ses descendants. De plus, les communautés transmises étaient similaires entre ramets filles bien que plus pauvres en nombre d'OTUs que l'assemblage présent au niveau des racines du ramet mère initial. Ce résultat démontre l'existence d'une filtration des micro-organismes durant la transmission par une diminution de la richesse et une homogénéisation des communautés transmises constituant un "core microbiote".

D'un point de vue théorique la transmission du microbiote aux descendants est avantageuse car elle permet d'assurer la présence de symbiotes bénéfiques et permet donc d'assurer la qualité de l'environnement des descendants (Wilkinson & Sherratt, 2001). Nos résultats ouvrent des perspectives sur la capacité des plantes clonales à sélectionner et transmettre préférentiellement certains micro-organismes spécifiques. Cependant, les modalités de cette filtration ne sont pour l'instant pas connues et représentent donc une perspective de recherche importante de cette thèse. Les micro-organismes transmis pourraient ainsi être filtrés en fonction de leurs capacités de dispersion (i.e. de colonisation des entre-nœuds ou stolons) ou en fonction des fonctions qu'ils remplissent pour le ramet mère (par exemple l'acquisition de ressource ou la résistance à un stress).

Cette transmission verticale de micro-organismes constitue une continuité du partenariat entre la plante et ses symbiotes (Zilber-Rosenberg & Rosenberg, 2008). Récemment, la compréhension des interactions hôte-symbiotes a évolué vers une approche holistique de l'hôte et de ses symbiotes. La théorie de l'holobionte fournit un cadre théorique pour l'étude des interactions hôte-symbiotes (Zilber-Rosenberg & Rosenberg, 2008; Theis et al., 2016). Dans l'hologenome (i.e. l'ensemble formé par le génome de l'hôte et celui de ses symbiotes) un micro-organisme peut être assimilé à un gène dans le génome. De la même manière que les gènes sont hérités lors de la reproduction sexuelle, l'héritabilité des micro-organismes entre les générations d'hôtes est ainsi un paramètre clé de l'évolution de l'hologenome. Dans ce contexte, le mécanisme démontré ici intègre les plantes dans la gamme des organismes qui peuvent être considérés et appréhendés comme des holobiontes. De plus, la structure en réseau des plantes clonales suggère un niveau supplémentaire de complexité dans l'assemblage de l'holobionte puisque dans ce réseau les holobiontes peuvent échanger ou partager une partie de leur microbiote.

Dans le second article de ce chapitre deux (Article IV) nous proposons une extension du concept de l'holobionte aux organismes clonaux organisés en réseaux : le méta-holobionte. En plus des bases théoriques de la compréhension des réseaux clonaux comme des méta-holobiontes nous proposons dans cet article de transposer des corpus de connaissances issus des théories écologiques des méta-communautés et des réseaux écologiques. Le cadre théorique des méta-communautés devrait permettre d'appréhender l'impact de la transmission des micro-organismes sur l'assemblage et la survie des communautés de micro-organismes et sur la gestion par les plantes des micro-organismes d'intérêt. Le cadre théorique de l'écologie des réseaux devrait fournir des perspectives sur l'optimisation de la résilience et des performances du réseau clonal en fonction de sa structure et de la transmission des micro-organismes qui dépend de cette structure.

### Chapitre 3 : Importance du contexte de la communauté de plantes pour l'assemblage du microbiote d'une plante hôte

La composition des communautés de micro-organismes du sol dépend de différents facteurs environnementaux dont par exemple le type de sol et ses propriétés (e.g. pH, humidité, concentration en nutriments) (Berg & Smalla, 2009; Lundberg et al., 2012; Bulgarelli et al. 2012; Shakya et al., 2013; Schreiter et al., 2014). Du fait de l'immobilité des plantes, ce pool de micro-organismes disponibles pour le recrutement détermine en grande partie leur microbiote (Lundberg

et al., 2012; Bulgarelli et al., 2012). En plus du type de sol et de ses propriétés, les plantes peuvent également modifier ce pool de micro-organismes via différents mécanismes. Les plantes sont capables de recruter préférentiellement certains micro-organismes – partir du sol (Vandenkoornhuyse et al., 2015) et de promouvoir les symbiotes les plus bénéfiques (Bever et al., 2009; Kiers et al., 2011). De plus les exsudats racinaires des plantes peuvent également augmenter ou diminuer l'abondance de micro-organismes spécifiques en fonction de l'espèce de plante considérée (pour une synthèse lire Berendsen et al., 2012). On s'attend donc – ce que les plantes modifient localement la composition des communautés microbiennes du sol. Suivant cette hypothèse, le voisinage d'une plante donnée (c'est-à-dire l'identité et l'abondance des plantes voisines) devrait influencer localement les communautés de micro-organismes du sol et donc les micro-organismes disponibles pour le recrutement par d'autres plantes de la communauté.

Ainsi, le voisinage d'une plante donnée devrait influencer l'assemblage du microbiote de la plante focale. Cependant, ce rôle potentiel du contexte de la communauté de plantes sur l'assemblage du microbiote n'a pas été étudié expérimentalement. En outre, des espèces (Oh et al., 2012; Bonito et al., 2014) et des génotypes (Bulgarelli et al., 2012; Lundberg et al., 2012) différents de plantes ont des microbiotes spécifiques ce qui suggère que les plantes peuvent promouvoir différents micro-organismes et donc avoir des effets contrastés sur les communautés du sol. Considérant la grande diversité de fonctions fournies par les symbiotes des plantes (Friesen et al., 2011, Møller et al., 2016) et leurs effets sur le phénotype et les performances des plantes mis en évidence dans cette thèse (Articles I et II), des changements de composition du microbiote devraient affecter les performances de la plante focale.

Dans ce chapitre qui est composé d'un article en préparation nous avons testé expérimentalement l'hypothèse de l'effet du contexte de la communauté de plantes sur l'assemblage du microbiote d'une plante hôte. Nous avons utilisé un dispositif de mésocosmes manipulant des communautés prairiales de plantes. Nous avons échantillonné les assemblages fongiques du sol de ces mésocosmes en utilisant *Medicago truncatula* comme plante piége et en cartographiant le recouvrement en plante sur plusieurs années.

Dans notre expérience (Article V), nous avons détecté un effet significatif de l'abondance des espèces de plantes du voisinage sur la composition du microbiote colonisant les racines de *M. truncatula*. Cette effet dépend de l'espèce de plante du voisinage avec certaines plantes ayant un effet positif et d'autres un effet négatif sur la richesse et l'équitabilité des assemblages fongiques colonisant *M. truncatula*. Nous avons démontré ainsi qu'une plante spécifique peut filtrer et déterminer les assemblages fongiques locales présentes et disponibles pour le recrutement dans le sol

comme proposé par Valyi et al., (2016). Nos résultats indiquent également que cet effet du voisinage de plantes ne se limite pas aux champignons MA mais influence tout l'assemblage fongique. L'influence du voisinage en plantes sur le microbiote racinaire se produit à l'échelle fine (i.e. de 5 à 25 cm). De plus, il s'avère que la fois le recouvrement passif et le recouvrement présent en plantes impactent les assemblages fongiques des racines suggérant que les plantes peuvent laisser une empreinte durable sur la composition des assemblages fongiques du sol. En outre, nous avons également démontré que les changements de composition des assemblages fongiques colonisant les racines impactent in fine les performances de la plante hôte *M. truncatula*. Ainsi, des assemblages fongiques plus riches ou plus équitables ont induit de meilleures performances chez les plantes hôtes. Dans leur ensemble ces résultats démontrent l'existence d'interactions plante-plante via les champignons et impactant in fine la fitness de la plante hôte et ouvrent des perspectives sur la manipulation du microbiote racinaire des plantes via leur voisinage dans l'objectif d'améliorer la productivité.

Ce travail a donc permis de mieux comprendre les modalités d'assemblage du microbiote et démontre l'existence d'une empreinte du voisinage des plantes sur la composition des communautés de micro-organismes. Un second résultat majeur est la démonstration de l'héritabilité d'une fraction de ce microbiote, sans doute un microbiote transmis essentiel au développement de son hôte. Ces travaux offrent de nouvelles opportunités de recherche qui devraient permettre de mieux comprendre le microbiote des plantes, son assemblage, ses fonctions et son influence sur le phénotype de l'hôte. Au-delà des réflexions conceptuelles induites par cette thèse, ces travaux interrogent d'un point de vue appliqué la place du voisinage des plantes en agriculture pour contribuer à la diversité du réservoir des micro-organismes symbiotiques. Une perspective majeure de ce travail est donc le développement de polycultures dans lesquelles les espèces de plantes induisent des effets de facilitation via la promotion de la diversité du microbiote.



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## GENERAL INTRODUCTION

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## General context of the thesis

### I. Scientific context

In the context of global changes (i.e. global warming), local conditions are changing and scientists have raised the alarm on the risks of food crises becoming more and more frequent (Battisti et al., 2009) due to extreme climatic events or pests spreading. Agricultural productivity is thus expected to decline worldwide and in parallel human population is continuously increasing and is expected to reach 10 billion people before 2100 (Lutz et al., 2001). By this time, it is estimated that agricultural production would need to almost double to fulfill world demands (Cirera & Masset, 2010). This need for space will be fulfilled by the transformation of natural ecosystems in cropland and following estimations about  $10^9$  ha are likely to be lost by 2050 (Tilman et al., 2001) constituting a massive loss of biodiversity and ecosystem functions. The future challenges for agriculture during this century is thus to produce enough food to support world population expansion and in parallel to limit collateral damage to the environment.

In agriculture, local environment constraints have been overstepped by the use of additives in the cultures (fertilizers, insecticides etc). However, the phosphorus that is used in fertilizers relies on high quality rock phosphate, which is a finite resource and major agricultural regions such as India, America, and Europe are already dependent on P imports (Duhamel & Vandenkoornhuysen 2013). The use of inputs have thus failed to solution the current challenges and have even impoverished soils. The last decades the symbiotic communities of bacteria, archaea and fungi have been recognized as major drivers of global ecosystem functions (van der Heijden et al., 1998b; Leininger et al., 2006; Falkowski et al., 2008; van der Heijden et al., 2008). Researchers have thus started to recognize the potential of plant-associated microorganisms to solve the agricultural productivity-biodiversity loss conundrum. Together with the root nodules, AM fungi are now recognized as one of the important symbiosis that help feed the world (Marx et al., 2004, in The roots of plant-microbe collaborations) and are promising for the development of organic farming (Gosling et al., 2006). Industrials and farmers have even started to conduct experiments using microorganisms inoculation to enhance crop resistance to pathogens or survival on deprived soils (de Vrieze, 2015 Science 'The littlest farmhands\_'). The next step will be to engineer crops microbiota in order to optimize functional characteristics provided. For example, flowering time

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and biomass are suitable candidate plant traits for the research of microorganism-services. In order to fulfill this goal, scientists will have to describe in precision the links between the microbiota assembly and functioning and the resulting plant phenotype.

In natura, plants are colonized by a high diversity of microorganisms both inside and outside of their tissues, constituting the microbiota. These microorganisms affect plant health and growth in a beneficial, harmful, or neutral way. Because of their sessile lifestyle (i.e. immobile), plants rely on these micro-organisms for many ecological needs. Indeed, plant-associated microorganisms provide important ecological functions to the plant such as the acquisition of resources or the resistance against biotic and abiotic stresses (Friesen et al., 2011; Møller et al., 2016), all along the plant life (development, survival and reproduction) (Møller et al., 2016). Considering the importance of microbiota, the last decade have seen significant advances in the description of the factors shaping the assembly of the plant microbiota. The improvements of sequencing technologies allow to describe more and more finely the composition of the microbiota. Composition and dynamics of this microbiota are driven by a large range of environmental factors such as soil properties comprising pH, nutrient and water availability (Berg & Smalla, 2009; Bulgarelli et al 2012; Lundberg et al., 2012; Shakya et al., 2013; Schreiter et al., 2014). In addition, the complexity and versatility of plant-microorganisms associations makes difficult to disentangle all the rules leading to a given microbiota composition. Thus, determining the assembly rules of the plant microbiota is a current conundrum in ecology.

Because they are immobile, plants have to cope with environmental heterogeneity such as the heterogeneity of microorganisms presence in soil. Plants classically respond to environmental heterogeneity through the production of phenotypic plasticity (i.e. production of different phenotypes from a single genotype; Bradshaw, 1965). In nature, clonal plants represent up to 70% of plant species in temperous ecosystems (van Groenendael & de Kroon, 1990). Clonal plants are organized as a network of ramets (i.e. clonal individuals constituted of leaves and roots) connected by above or below-ground modified stem (i.e. stolons and rhizomes respectively) (see Harper, 1977 for a description of clonal structure). This network allow the sharing of resources and information (i.e. physiological integration, Oborny et al., 2001) and thus allows to produce plastic responses at the scale of the network. These plants are thus able to modify the structure of the network allowing to explore the environment (i.e. foraging) ensuring resources acquisition (e.g. light or nutrients,

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Slade & Hutchings, 1987; Dong, 1993; Hutchings & de Kroon, 1994; Birch & Hutchings, 1994). Considering that microorganisms can have large effects on plant morphology (see for review Friesen et al., 2011; Møller et al., 2016), they may alter plant ability to produce such plastic responses despite it has not been demonstrated. In addition, because microorganisms provide key functions to the plant, the expectation is thus that plants should develop plastic responses to ensure the availability of beneficial symbionts like they do for resources. Despite the ubiquity of clonal plants, the possibility that they forage for microorganisms to construct their microbiota has never been investigated.

A major mechanism ensuring the availability of beneficial microorganisms for macroorganisms is the transmission of microorganisms between individuals (investigated in the Chapter 2). Two kinds of transmission have been theorized, vertical (i.e. direct transmission from parents to offsprings) and pseudo-vertical (i.e. transmission by sharing the same environment) (Wilkinson, 1997). In plants, vertical transmission has only been evidenced through seeds colonization and the most well-known example is stress-protective endophyte *Neotyphodium coenoccephalum* to the descendants in several grass plant species (Clay & Schardl, 2002; Selosse et al., 2004). In clonal plants, the progeny is connected to the parent plant in the clonal network. Stuefer et al., (2004) suggested that in these plants, microorganisms could be transmitted in addition to resources and information. Despite it has been suggested and could represent a major factor structuring microbiota assembly, such transmission of microorganisms within the clonal network has never been investigated so far.

In prairial ecosystems, many plants species are coexisting and interact in competitive or facilitative ways. A large number of studies, mainly focusing on Arbuscular Mycorrhizal Fungi (AM fungi hereafter), have investigated how the plant community can be influenced by the diversity of soil fungi (Grime et al., 1987; van der Heijden et al., 1998a; Klironomos et al., 2000; Bever et al., 2001). Reciprocally, microcosm studies based on a focal plant design have demonstrated that the surrounding plant composition influence the fungal microbiota of the focal plant-associated (Johnson et al., 2004). Such microcosms-based studies have provided insights into the possible effect of the community context on microbiota assembly. However, microcosms are oversimplifications of the plant community real context where many species co-exist in a spatio-temporal dynamic system. In multispecific plant communities, Hausmann & Hawkes (2009)



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showed that the plant community composition influenced the composition of a focal plant microbiota. As a plant harbor a specific microbiota (Hardoim et al 2008; Redford et al., 2010; Bulgarelli et al., 2012; Lundberg et al., 2012) then we can expect a plant to leave a specific fingerprint on the soil pool of microorganisms. A plant neighborhood could thus determine its microbiota assembly (investigated in Chapter 3). Nevertheless, the spatial and temporal scales of this influence are still unclear whereas it could determine the fingerprint of the plant community on the soil pool of fungi. In addition, the above-described studies mainly focused on particular type of plant symbionts, the AM fungi. Despite many other plant endophytes are known to provide important functions (see Introduction section), the influence of the plant community on bacteria, fungi, and archaea has not yet been described.

My PhD thesis aimed to address hypotheses related to these 3 current important frontiers of research in Ecology and Plant Science, (1) the importance of plant-microorganisms interactions for plant phenotype and plasticity (2) the understanding of the vertical heritability of the plant microbiota and (3) the ability of plants to manipulate the local symbiotic compartment thus to leave a fingerprint on the pool of microorganisms. The work I did also aimed to question different theoretical concepts of ecology and evolution and to provide new perspectives regarding these concepts.

## II. Structure of the PhD thesis

This thesis begins with a literature review of the existing knowledge on the plant microbiota composition, assembly rules and dynamics and the links between this microbiota and plant phenotypes. This state of the art aims at delimiting the current knowledges and gaps in our understanding of the plant microbiota and at identifying the objectives of this thesis work. The second part of this work consists in articles presented in three chapters addressing the questions raised by the literature review. Each of these chapters begins with a short presentation of the scientific context and the experimental procedure, as well as a brief summary of the main results. The results are then presented and discussed in the form of articles published, submitted or in preparation for scientific journals with a proofread committee.

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## Literature review

### 1. Microbiota composition

#### 1.1 Plants and microorganisms, an ubiquitous alliance

Plant and microorganisms have long been considered as separate but interacting organisms. This conception dividing two fields of research on plants and on microorganisms has progressively been replaced by an integrated approach to plant-associated microorganisms. This evolution in understanding of the plant has slowly evolved through the numerous studies describing how plants are systematically associated with microorganisms. For example, all plant species that were investigated were colonized by leaf endophytes (Arnold et al., 2003; Schulz & Boyle, 2005; Albrechtsen et al., 2010; Gilbert et al., 2010) and parallel studies also highlighted the difficulty of growing transplants of different species without bacteria and fungi (Hardoim et al., 2008). Indeed, microorganisms colonize every plant species known and are found in various parts of these plants: roots, stems, xylem vessels, apoplast, seeds, and nodules (Rosenblueth & Martinez-Romero 2006; Figure 1). Some of these microorganisms live within or on the surface of plant organs, live within the endosphere or episphere and are called endophytes and epiphytes, respectively. The endosphere and episphere constitute the plant microbiota. On the surface of leaves, prokaryotes are found at densities of  $10^6$  to  $10^7$  per  $\text{cm}^2$  (Lindow & Brandl 2003) and lab-based estimates of endophytic bacterial populations range from  $10^7$  to  $10^{10}$  cells per gram of tissue (Hardoim et al., 2008). Among the endophytic microorganisms colonizing plants, Arbuscular Mycorrhizal Fungi (AM fungi hereafter) have been found in 80% of all terrestrial plants (Bonfante & A nca, 2009).

Evidence of colonization of plant roots by AM fungi has been found in fossils dating from the early Devonian and Ordovician, suggesting that these fungi were already associated with plants over 400 to 460 million years ago (Remy et al., 1994; Redecker et al., 2000). AM fungi are suggested to be at the origin of major evolutionary events such as the colonization of land, and the evolution and diversification of plant phototrophs (Selosse & Le Tacon, 1998; Heckman et al., 2001; Brundrett, 2002; Bonfante & Genre, 2010). All these elements converge towards a consideration of the plant as an obligate host of numerous microorganisms. In the light of these

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observations [ ̃ ] a plant that is completely free of microorganisms represents an exotic exception, rather than the "biologically relevant" rule [ ̃ ] (Partida-Martinez & Heil, 2011).

## 1.2 Plant-microorganisms symbiosis

Symbiotic interactions between plants and the micro-organisms can be cooperative, neutral or antagonistic, thus a continuum of interactions ranging from mutualism to parasitism (i.e. an interaction being parasitic if it is disadvantageous for one of the organisms or a mutualistic when beneficial for both partners; e.g. Rico-Gray, 2001). The level of intimacy (physical association) is likely to be variable among symbionts and interactions are considered as symbioses when the physical association is strong. This is the case for many known associations in nature such as corals with the algae *Symbiodinium* (along with other symbionts, Rosenberg et al., 2007), lichens (an association between fungi and photobiontes; Spribille et al., 2016), legumes with *Rhizobia*, and more generally plants with Arbuscular Mycorrhizal Fungi. Such symbiosis has long been considered as beneficial to both organisms. Nevertheless, symbionts behavior cannot be considered as binary (i.e. either parasitic or mutualistic) but rather as more or less beneficial. This continuum in symbionts behavior has been demonstrated in different plant symbioses. For example, different AM fungi isolates can provide quantitatively different amounts of phosphorus in exchange for a given quantity of carbohydrates (Kiers et al., 2011), with cheaters racketeering their host whereas cooperators display "fair-trade" behavior (see section 3.2.3 for more details). Furthermore, a given symbiont can behave as a parasite or a mutualist depending on the ecological context. Hiruma et al. (2016) demonstrated in an elegant experimental study that the endophyte *Colletotrichum tofieldiae* transfers phosphorus to shoots of *Arabidopsis thaliana* via root-associated hyphae only under phosphorus-deficient conditions. This shift in *C. tofieldiae* behavior was associated with a phosphate starvation response of the plant indicating that the behavior of the symbiont on was dependent on the nutrient status of the host. In addition, symbiosis generally involves more than two partners that can either be parasites or mutualists (Johnson et al., 1997; Saikkonen et al., 1998) and can colonize different parts of the plant at the same time. In the above-described experiment, *C. tofieldiae* was shown to first colonize the roots and then spread to the leaves (Hiruma et al., 2016).

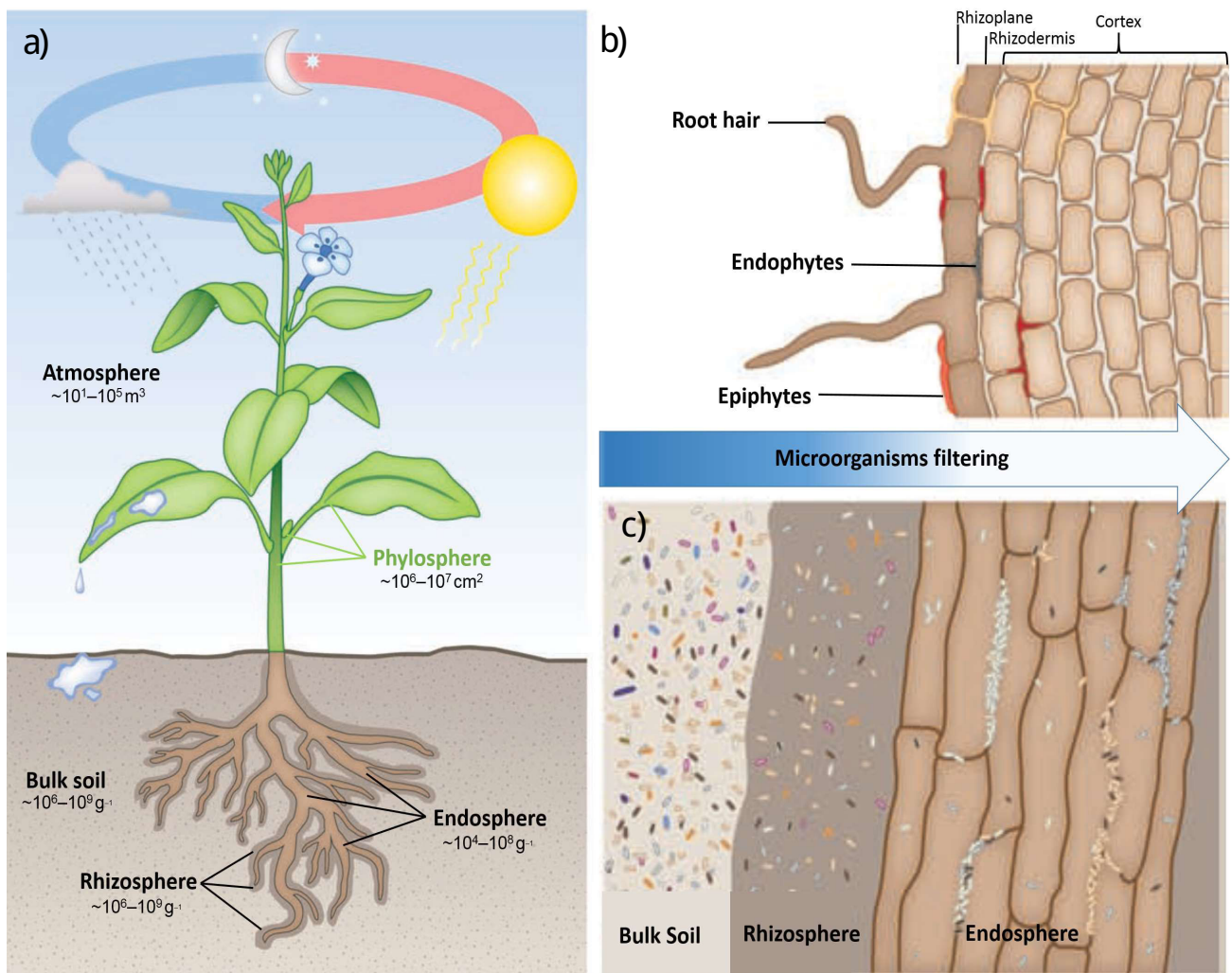


Figure 1. a) Schematic plant showing the different compartments of the plant and its environment colonized by microorganisms comprising bacteria, fungi and archaea. Numbers of bacterial cells are indicated for phyllosphere, atmosphere, rhizosphere, and root and soil bacterial communities and were taken from (Bulgarelli et al., 2013). b) Schematic root cross section of the root hair zone showing epiphytic colonization endophytes colonizing the endosphere. c) Schematic longitudinal section of a root and the rhizosphere and bulk soil around the root. The bulk soil is colonized by a wide diversity of microorganisms and they are filtered within the rhizosphere and subsequently in the endosphere. The figure was modified from Møller et al., (2016).

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### 1.3 The root microbiota: Diversity and composition

The diversity of microorganisms associated with plants roots is estimated to be in the order of tens of thousands of species (Berendsen et al., 2012). Tremendous advances in the approaches used to describe the microorganisms living in association with plants have been made in recent decades. Approaches based on the cultivation of organisms were for a long period of time the only methods available to characterize microbial communities (Vartoukian et al., 2010; Margesin & Miteva, 2011; Rosling et al., 2011). However, since most microorganisms, and especially fungi, are not cultivable (Epstein, 2013), the diversity of plant-associated microorganisms has long been underestimated. Estimations of plant-associated fungal diversity for a long time relied on spore counts and morphology (see for example Burrows & Pflieger, 2002; Landis et al., 2005).

Molecular methods developed in recent decades, such as Sanger sequencing of cloned products, PCR amplicons (polymerase chain reaction) or PCR-RFLP (restriction fragment length polymorphism) revolutionized the experimental approaches and were rapidly adopted for microorganisms identification and classification (Balint et al., 2016). This ability to rapidly detect DNA and at low cost provided information about the diversity of organisms in an environmental sample and led to numerous descriptions of the wide diversity of microorganisms associated with plants. Thanks to these studies, we now know that plants harbor an extreme diversity of archaea (Edwards et al., 2015), bacteria (Bulgarelli et al., 2012; Lundberg et al., 2012) and fungi (Vandenkoornhuysen et al., 2002b). These studies extended our knowledge of plant-associated microorganisms and led to an upward revision of their diversity. In 2002, Vandenkoornhuysen et al. demonstrated that the diversity of fungi colonizing plants roots was much greater than previously believed and included non mycorrhizal fungi of the phyla Basidiomycota and Ascomycota. The composition of this microbiota varies significantly between plant species (Hardoim et al., 2008; Redford et al., 2010; Bulgarelli et al., 2012; Lundberg et al., 2012) and also depends on the genotype of the plant (Inceoğlu et al., 2010, 2011) but to a limited extent (Bulgarelli et al., 2012, Lundberg et al., 2012; see section 3.2.3 for host genetics effect).

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Although the composition of the plant microbiota has been extensively described for different species of bacteria with estimations of the relative abundances and species richness for different organs (Figure 2) such knowledge is lacking for fungal and archaeal communities and only a few seminal papers have been published (Vandenkoornhuyse et al., 2002a; Edwards et al 2015; Colemann-derr et al., 2016). Within the fungal compartment, the most studied organisms are root-associated mycorrhizae that are either endomycorrhizal (within the plant cells) or ectomycorrhizal (outside the cell walls) forming hyphae around the roots. All AM fungi belong to the phylum Glomeromycota whereas the ectomycorrhizal fungi are spread among the Ascomycota and Basidiomycota phyla (Arnold, 2007; Rodriguez et al. 2009; Rudgers et al. 2009). In roots and shoots, endophytic Basidiomycota and Ascomycota also exist in a non-mycorrhizal form. Recent studies enlarging the fungal compartment to groups other than mycorrhizae detected over 3000 OTUs in total in the soil, episphere and endosphere of agave species with a third of these OTUs (1007 OTUs belonging to nine orders) being found in the endosphere (Colemann-derr et al., 2016).

- Phylum annotation**
- Actinobacteria
  - Bacteroidetes
  - Chloroflexi
  - Deinococcus-Thermus
  - Firmicutes
  - Nitrospirae
  - Proteobacteria
  - Spirochaetes

- Relative abundance**
- n.d.
  - < 0.1%
  - 0.1–0.5%
  - 0.5–1%
  - 1–5%
  - 5–10%
  - 10–20%
  - 20–30%
  - 30–40%
  - > 40%

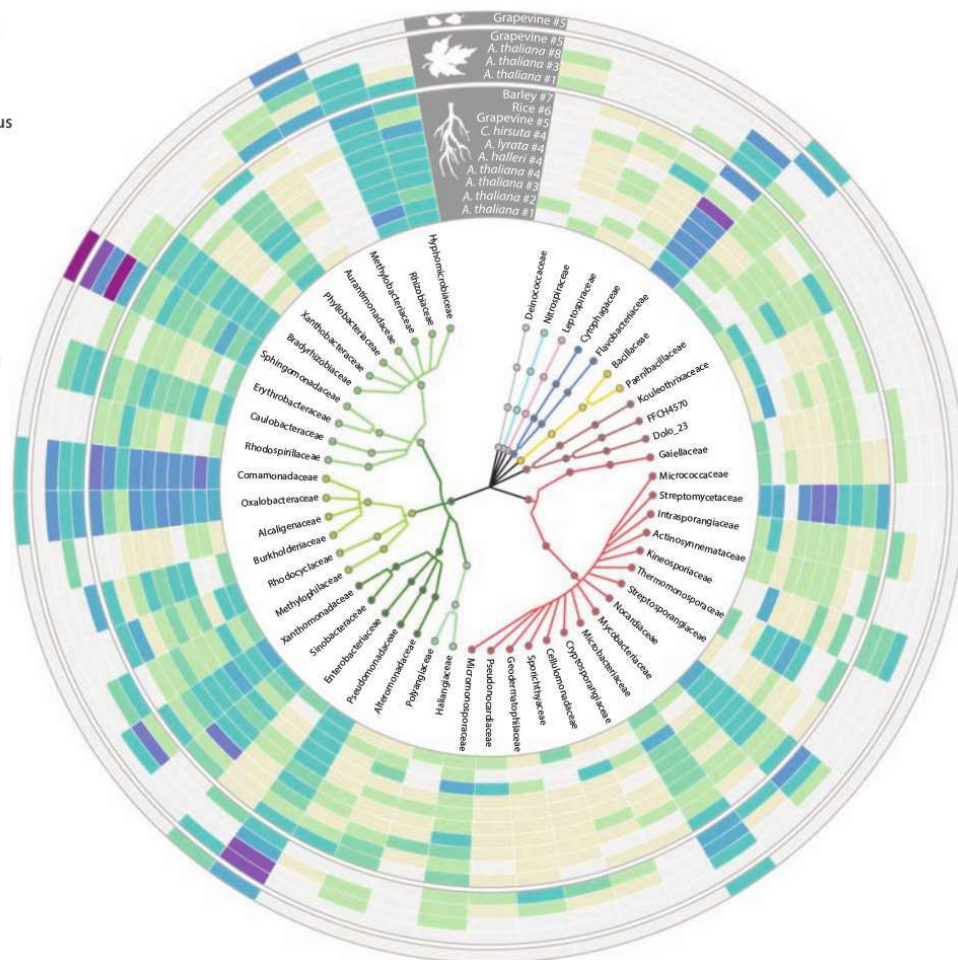


Figure 2. Phylogenetic structure of bacteria associated to the roots and leaves of different plant species. This figure was realised from sequencing data of different papers and analyzed using a reference-based operational taxonomic unit (OTU) picking method and were subsequently combined at the family level. Abbreviations: n.d., not detected. From Møller et al., (2016).

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## 2. Endophytes induced functions and phenotypic modifications

Endophytic microorganisms are involved in a variety of phenotypic changes in plants. These changes have been widely studied especially in plants of agricultural importance. Because of their sessile lifestyle plants have to cope with the environmental conditions. Abiotic conditions especially, such as light, water and others are spatially variable, so plants must cope with this heterogeneity (Hodge, 2004). There are numerous reports of fungal and bacterial symbionts conferring tolerance to a variety of stresses to host plants as well as other benefits (e.g. Friesen et al., 2011; Møller et al., 2016). The range of functions provided by roots microorganisms are reviewed in the following section.

### 2.1 Resources acquisition

Microorganisms living in association with plants improve the acquisition of different resources and, more especially, allow the acquisition of otherwise inaccessible ones. Leaf epiphytic cyanobacteria for example transfer atmospherically fixed nitrogen to plants that can represent 10 to 20% of the leaf nitrogen (Bentley & Carpenter, 1984). This ability to fix atmospheric nitrogen is displayed by at least six different bacterial phyla, the most common being Proteobacteria, and by several archaea lineages (Martinez-Romero, 2006; Friesen et al., 2011). Nitrogen acquisition also occurs within the tree roots in boreal forests thanks to Ectomycorrhizal fungi (Lindahl et al., 2007). In parallel, other nutrients can be more easily obtained through the help of endophytic organisms and a given organism can provide multiple resources. For instance, the arbuscular mycorrhizal fungi living in association with plants supply these plants with water, phosphorous, nitrogen, and trace elements (Smith & Read, 2008; Smith et al., 2009). In the arbuscular mycorrhizal fungi symbiosis, mineral nutrients uptake can be 5 and 25 times higher for nitrogen and phosphorus respectively, in mycorrhized as compared to non-mycorrhized roots (Van Der Heijden et al., 2003; Vogelsang et al., 2006). AM fungi acquire these resources more efficiently because hyphae can access narrower soil pores and increase the uptake of immobile resources (especially inorganic phosphate), by acquiring nutrients beyond the depletion zone of the root and stimulating the production of exudates that release immobile soil nutrients (Maiquet et al., 2009; Courty et al., 2010; Cairney, 2011). In



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addition, AM fungi are able to acquire both organic and inorganic nitrogen, which is not the case of plants (see Hodge & Storer, 2015 for a review on AM fungi nitrogen uptakes).

One example is the nitrogen in soil organic matter released by hydrolytic and oxidative enzymes produced by Ericoid mycorrhizae. This enhanced acquisition of resources not only directly affects the individual plant's fitness but also its phenotype and competitive interactions with other plants (see section II 2.2.1 for examples; for a review see Hodge & Storer, 2015). Indeed, a phenotypic consequence of the more efficient nutrient uptake of mycorrhizae for the plant is a reduced number of fine roots together with a lower root:shoot ratio and specific root length (Smith et al. 2009). Improved resource acquisition also allows the plant to cope more efficiently with environmental constraints.

## 2.2 Resistance to environmental constraints

### 2.2.1 Abiotic stresses

Numerous studies indicate that plant adaptation to stressful conditions may be explained by the fitness benefits conferred by mutualistic fungi (for example resources acquisition described in the section 2.1) (Stone et al., 2000; Rodriguez et al., 2004). In addition to these indirect effects of resources acquisition, fungi and bacteria also provide direct resistance to a large range of stresses through the production of certain compounds. Among the stresses alleviated by plant endophytes the main ones are salinity, extreme heat, drought and heavy metal pollutants. Salinity tolerance for example can be increased by different metabolites such as trehalose produced by bacteria like Rhizobia in nodules (Lopez et al., 2008). Such improved tolerances are not only provided by bacteria. The fungal endophyte *Curvularia* found on thermal soils in Yellowstone Park has been shown to increase tolerance to extreme heat (Redman et al., 2002). Fungal endophytes such as *Fusarium culmorum* colonizing the above- and below-ground tissues and seed coats of *Leymus mollis*, also confers salinity tolerance (Rodriguez et al., 2008). In addition a given microorganism can at the same increase resource acquisition and provide resistance to an abiotic stress (independently of its effect on resources acquisition). AM fungi increase stomatal conductance when they are inoculated to plants either under normal or drought conditions. This increase of stomatal conductance has been linked to greater drought tolerance of rice and tomato plants inoculated with such fungi (Lambers et al., 2008; Rodriguez et al., 2008). Following observation of

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the described tolerance, several microorganisms have been considered as suitable candidates for bioremediation of polluted soils. This is the case of plant growth-promoting rhizobacteria that elicit tolerance to heavy metals (Glick, 2003; Zhuang et al., 2007).

### 2.2.2 Biotic stresses

In addition to their role in the resistance against abiotic stresses plant-associated microorganisms also mediate plant resistance against biotic constraints among which the most studied are aggressions by pathogens. Indeed, endophytes are able to secrete defensive chemicals in plant tissues (Arnold et al. 2003; Clay & Schardl 2002). Different studies have identified chemicals providing defense against pathogens and produced by a myriad of microorganisms associated with plants. The range of compounds produced by symbionts consists of antimicrobials with direct effects on the pathogen or indirect effects such as a diminution of its pathogenicity. Compounds with a direct and immediate antibacterial effect include antimicrobial auxin and other phytohormones (Morshed et al., 2005). Compounds with indirect effects are for example nonanoic acid produced by the fungus *Trichoderma harzianum* that inhibits mycelial growth and spore germination of two pathogens in the tissues of *Theobroma cacao* (Aneja et al. 2005). Alternatively, microorganisms can also produce compounds such as AHL-degrading lactonases that are able to alter the communication between pathogens and thus prevent the expression of virulence genes (Reading & Sperandio, 2006). In addition, different strains and species can produce different compounds and each endophyte can itself produce several compounds. Even within a species, each strain can produce multiple antibiotics as in *Bacillus* where these antibiotics have synergistic interactions against pathogens (Haas et al., 2000). Such protection against biotic aggressors can also be mediated by stimulation of the plant immune system. Boller & Felix (2009) highlighted mutualist-induced signaling pathways initiated by flagellin/FLS2 and EF-Tu/EFR recognition receptors, allowing plants to respond to virus, pests and pathogens. The defensive responses induced by mutualists are not always localized and given microorganisms like the fungus *Trichoderma* may induce both systemic and localized resistances to a variety of plant pathogens (Harman et al., 2004). Such resistances often protect against crop damage and many plant-associated microorganisms like the latter can be used for biocontrol (Harman et al., 2004).

Biotic constraints are not limited to pathogens and many other aggressors or competitors can affect plants. One of the most studied biotic stresses alleviated by plant-associated microorganisms

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is herbivory. Mutualist-induced resistance to herbivory has been identified and described for various plant feeding herbivores. Such resistance often involves the production by the endophyte of compounds that are toxic for herbivores or that diminish plant palatability. For example, Tanaka et al., (2005) showed that the fungal endophyte *Neotyphodium* produces the secondary metabolite peramine that protects *Echinochloa festucae* from insect herbivory. Like for abiotic stress tolerance (see above section 2.2.1) a single microorganism can produce various compounds. For instance, clavicipitaceous endophytes such as *Neotyphodium* induce the production of alkaloids, lolitremes, lolines, and peramines allowing grasses to defend against herbivores (Rowan, 1993; Siegel et al., 1989; Clay, 1990; Clay & Schardl 2002). Such patterns of defense against herbivores have been mostly evidenced in grasses and also comprise other endophytes limiting mammalian herbivory through the production of lysergic acid amide (White, 1987; Gentile et al., 1999; Zhang et al., 2012). Non-toxic compounds conferring antifeeding properties include for example alkaloids that reduce rabbit herbivory on plants (Panaccione et al., 2006).

### 2.3 Growth and reproductive strategy

As has been shown in other hosts such as insects with *Wolbachia*, endophytes can also alter plant growth and reproductive strategy. In clonal plants, Streitwolf-Engel et al., (1997, 2001) showed that colonization of the roots by Arbuscular Mycorrhizal Fungi could alter the growth and reproduction of clonal plants. In this experiment, the authors found that ramets (i.e. clonal units composed of roots and shoots) production by *Prunella vulgaris* was differentially affected by the inoculation of three AM fungi isolates (see figure 3 for clonal growth plant description). The number of ramets produced changed by a factor of up to 1.8 independently of the isolates' effects on plant biomass. AM fungi can thus alter the trade-off between growth and reproduction. In addition, microorganisms can also alter the trade-off between different compartments of plant growth. In the above-described experiment the authors also found that branching (lateral ramification) was affected by the inoculation, suggesting that foraging by the plant (i.e., the strategy for resources acquisition) was modified by Arbuscular Mycorrhizal Fungi inoculation. In another experiment conducted, Sudova (2009) showed that growth and reproductive strategy modifications induced by the AM fungi vary both with the fungi identity and the plant species. Using five co-occurring plant species with 3 AM fungal isolates the authors showed that plant growth response to inoculation varied widely from negative to positive depending on the inoculum. AM inoculation led to changes in clonal growth traits such as an increase in stolon number and length only in some plant species.

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The effects of microorganisms on growth and reproductive strategy of plants appear thus to depend on the matching between plant and microorganisms identities although this idea has not been extensively tested.

As shown in this section the vast diversity of plant-associated microorganisms ensures essential functions impacting plant growth, development, survival and resistance to environmental constraints in general. However, the described studies tended to focus on describing the effects of the microbiota but not on the use of this microbiota by the plant. Considering the benefits of symbiotic associations, evolution should favorize a plant that optimizes its interactions with microbes. However, the extent to which plants might forage for microorganisms has not been investigated to date.

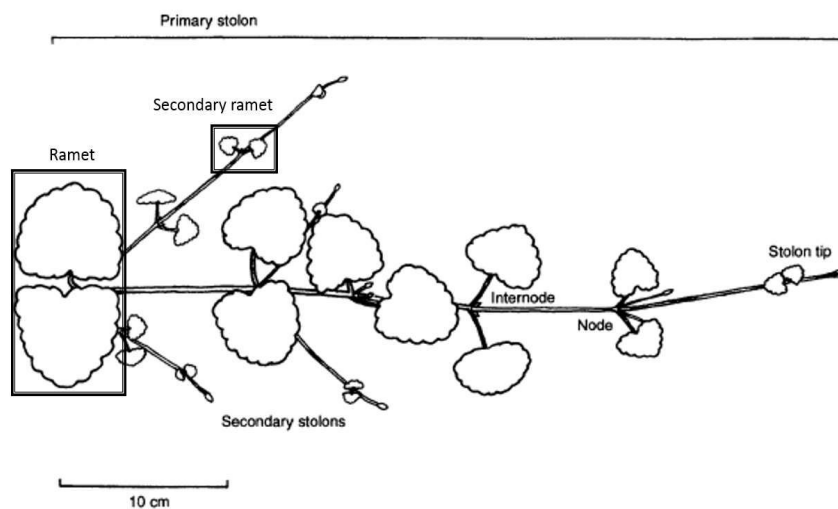


Figure 3. Growth form of the clonal plant *Glechoma hederacea*. The plant is organized as a network of ramets that are potentially independant individual units composed of roots, shoots and a node. These ramets are connected by stolons and the section of the stolon separating two nodes (i.e. two ramets) is called an internode. Modified from Birch & Hutchings, 1994.

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### 3. Plant microbiota assembly

The additive ecological functions supplied by the plant mutualists described in the previous section extend the plant's adaptation ability (e.g., Vandenkoornhuyse et al., 2015), leading to fitness benefits for the host in highly variable environments (Conrath et al., 2006) and therefore can affect evolutionary trajectories (e.g., Brundrett, 2002). In addition, because microbial communities may produce a mixture of antipathogen molecules that are potentially synergistic (see section 2.2.2), we can predict that plant hosts will be better protected against biotic stresses in the presence of more diverse microbial communities (Friesen et al., 2011). The same idea has been proposed for resources acquisition following results showing complementarity between symbionts in the acquisition of resources (Van der Heijden et al., 1998b). Thus the composition of the plant microbiota is of major importance in determining the ecological success (the fitness) of plants. In this context, the assembly rules shaping microbiota diversity and composition have only started to be described in recent years, and the current knowledge is reviewed in the following section (see figure 4 for an overview of the factors shaping microbiota assembly).

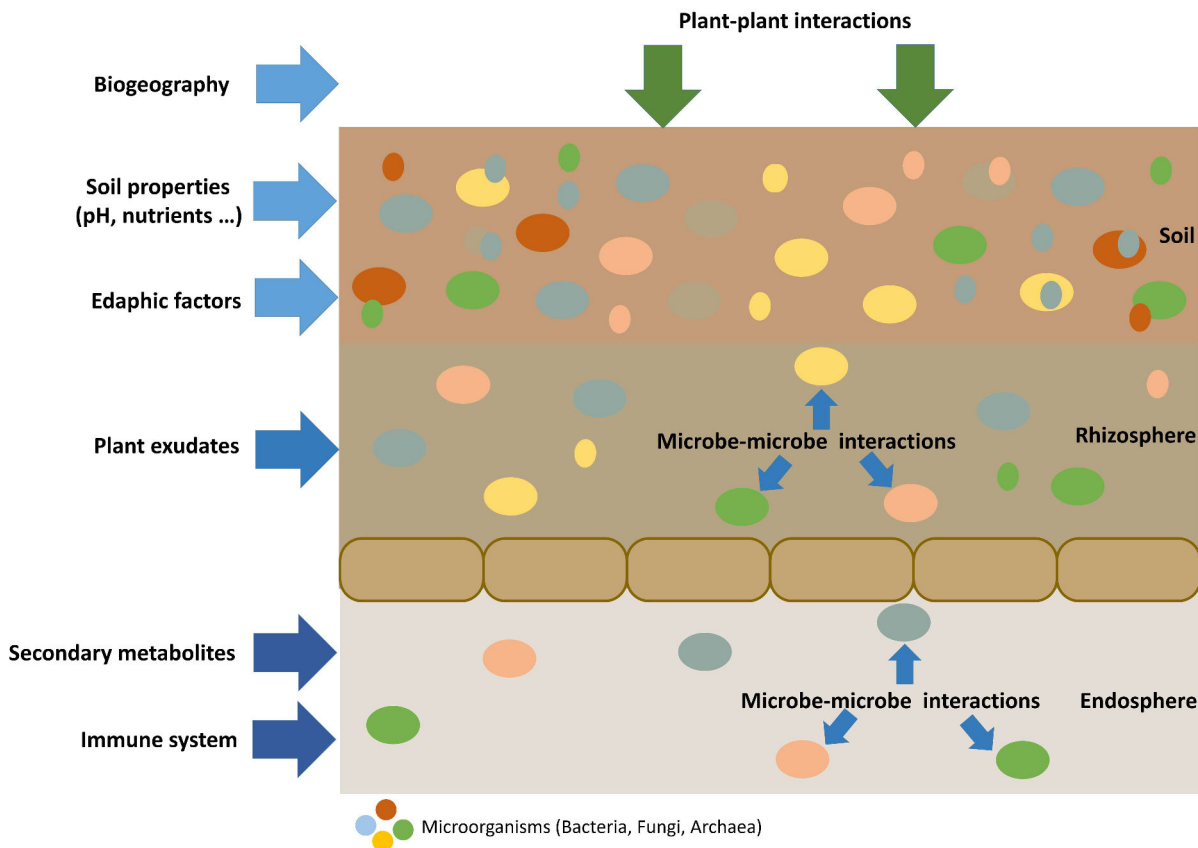


Figure 4. Schematic root surface and root endosphere as well as surrounding rhizosphere (zone of the soil under plant influence) and bulk soil. The figure shows the different abiotic and biotic factors structuring the assembly of the microorganisms community within the different compartments. These factors inducing a filter of the microorganism communities from the soil to the rhizosphere and subsequently within the endosphere are presented in this section.

### 3.1 Microbiota recruitment

#### 3.1.1 The soil as a microbial reservoir

The soil is a highly rich and diversified reservoir of microorganisms (Curtis et al., 2002; Torsvik et al., 2002; Gams, 2007; Buee et al., 2009). For example, forest soil samples of 4 g were found to contain approximately 1000 molecular operational taxonomic units assigned to fungal species (Buee et al., 2009). Recent studies investigating the composition of microbiota in different compartments (soil, rhizosphere, root and leaf endosphere) of *Agave* species showed that the majority of bacterial and fungal OTUs found in the endosphere were present in the soil (Coleman-

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derr et al., 2016). This observation was independently evidenced in the common model plant *Arabidopsis thaliana* where most of the root-associated bacteria were also found in the soil (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppli et al., 2014). Root-associated microorganisms are thus mainly recruited from the surrounding soil, and the pool of microorganisms available for recruitment in the soil determines the diversity and composition of the plant root microbiota. The above-described recent studies, in line with others, have clearly demonstrated from experimental approaches that the main environmental factors determining the soil pool (and thus the plant roots endophytic microbiota) are soil type and properties such as pH, temperature or water availability (Berg & Smalla, 2009; Bulgarelli et al 2012; Lundberg et al., 2012; Shakya et al., 2013; Schreiter et al., 2014).

### 3.1.2 Rhizosphere

While the composition of the soil microbial communities seems to be mostly determined by soil parameters (see above), the rhizosphere is a thin layer of soil at the periphery of the roots. This specific habitat of soil microorganisms contains a great abundance of microbes up to  $10^{11}$  microbial cells per g of soil and is also highly diversified with more than 30,000 prokaryotic species (e.g. Egamberdieva et al., 2008; Mendes et al., 2011). This rhizosphere corresponds to the influence zone of plant root exudates and oxygen releases (Vandenkoornhuysen et al., 2015). Exudates are highly complex mixtures comprising carbon-rich molecules and other compounds secreted by the plant as products of its metabolism. These exudates have a strong effect on the fungal and bacterial communities because they can be resources available for microorganisms, signal molecules (e.g. chemotaxy), and at the same time anti-microbial compounds (Vandenkoornhuysen et al., 2015). The large quantity of organic molecules secreted allows plants to manipulate the rhizosphere from the surrounding bulk soil reservoir (Bais et al., 2006). Differences in microbial communities in the rhizosphere of the same soil, depending on the plant species, have been highlighted (Berg et al., 2006; Garbeva et al., 2008; Berg & Smalla, 2009; Micallef et al., 2009) demonstrating the strong influence of plants and the induced 'rhizosphere effect'. Differences have also been detected within species as genotypes were shown to harbor distinct rhizosphere communities (Micallef et al., 2009). In addition some species have also been shown to create similar communities in different soils (Miethling et al., 2000). If specific richness seems to be lower in the rhizosphere than in the surrounding soil, suggesting that organisms are filtered from the original soil pool (Bulgarelli et al., 2012), the intensity of the rhizosphere effect seems however to vary (Uroz et al., 2010; Bulgarelli et

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al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014). The importance and intensity of the rhizosphere effect still need to be clarified but it has been hypothesized that plant roots select for specific microorganisms to prosper in the rhizosphere through the composition of the exudates that they release into the rhizosphere (discussed in section 3.2).

### 3.1.3 Endosphere

The endosphere constitutes the area within the plant roots and is colonized by a large variety of organisms comprising mycorrhizal and non-mycorrhizal fungi (Vandenkoornhuyse et al., 2002b; Smith and Read, 2008), bacteria (Reinhold-Hurek & Hurek, 2011) and Archaea (Sun et al., 2008). The plant's influence on microbial assemblages is stronger in this area than in the rhizosphere because of the influence of the plant immune system (Vandenkoornhuyse et al., 2015). In this area the diversity and abundance of organisms are lower than in the surrounding soil (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014), with specific assemblages differing from the soil communities (Vandenkoornhuyse et al., 2015). Several key microorganisms are indeed enriched (i.e. with higher relative abundance) in the endosphere, as compared to the soil and rhizosphere compartments, whereas others are depleted (i.e. with a lower relative abundance). Lundberg et al. (2012) detected 96 OTUs that were enriched in the endosphere of *A.thaliana* and 159 OTUs that were depleted. These results have been confirmed in a more recent study using a larger set of *Arabidopsis* and *Cardamina* hosts where Actinobacteria, Betaproteobacteria and Bacteroidetes dominated the datasets obtained from the rhizosphere and endosphere samples (Schlaeppi et al., 2014). These patterns of organisms enrichment and depletion can be attributed to the plant's ability to control its microbiota through its immune system.

## 3.2 Controls of the plant over its microbiota

### 3.2.1 Microorganisms recruitment through compounds secretion

The microorganisms communities colonizing the rhizosphere, and those that penetrate the endosphere and form the plant microbiota, have been described with regard to their role in plant health. Studies have highlighted a large panel of mechanisms that allow the plant to control this microbiota by filtering the pathogens and promoting beneficial microbes (Doornbos et al., 2012).



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These control mechanisms have been identified for both the rhizosphere and endosphere and involve exudates secretion and the plant's immune system.

Berendsen et al., (2012) reviewed the mechanisms that enabled the plant to control its rhizosphere composition and showed that plant could exude secondary metabolites such as benzoxazinoids that inhibited the growth of specific microbes in the rhizosphere (Bais et al., 2002; Zhang et al., 2011). Some studies have also evidenced plant-secreted compounds that suppress microbes cell-to-cell communication (i.e. 'quorum sensing') and thus alter the expression of microbial virulence genes. Such compounds have been identified in a variety of species such as pea (*Pisum sativum*), rice (*Oryza sativum*) and green algae (*Chlamydomonas reinhardtii*) (Teplitski et al., 2000, 2004; Ferluga & Venturi, 2009). A study on *Medicago truncatula* (Gao et al., 2003) found that ~20 compounds were produced and released in seedlings exudates that altered quorum sensing. These exudates also exhibited anti-microbial properties against pathogens and at the same time attracted beneficial organisms. An example of this attractive effect is the beneficial rhizobacterium *Pseudomonas putida* KT2440 that is tolerant to and chemotactically attracted by exudate compounds (Neal et al., 2012). Such recruitment of microorganisms, mediated by the root exudates, has been described in fungal antagonists as a response to colonization of the plant by the pathogen *Verticillium dahliae*. Other studies have indicated that upon attack by pathogens, plants respond by recruiting specific beneficial microorganisms (Rudrappa et al., 2008; Lee et al., 2012). For example, when the leaves of *Arabidopsis* were infected by *Pseudomonas syringae* pv., the roots were more colonized by *Bacillus subtilis* FB17 which is a beneficial endophyte. These observations led to the suggestion that the recruitment of specific microorganisms within the endosphere by the plant constituted a means for plants to repress pathogens (Berendsen et al., 2012). The plant is thus able to preferentially recruit microorganisms depending on its needs by repressing or facilitating the microorganisms within the rhizosphere. The control of the plant over the microbial communities is not limited to the area around the root and is even greater within the roots.

### 3.2.2 The immune system

Another way for the plant to control the composition of its microbiota is through the innate immune system. The forms of innate immunity and their role in controlling microbiota composition have been reviewed (see for example Jones & Dangl, 2006). Two forms of innate immunity exist depending on the pattern of the molecules triggering the immunity that can be pathogen-associated molecular pattern (PTI) or gene-based effectors (ETI). Boller & Felix, (2009) described how host

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plant receptors detect molecular patterns of damage and microbes and initiate the immune response by producing reactive oxygen, thickening their cell walls and activating defense genes in response to bacterial flagellin. In the `arms race\_ between plants and their pathogens, pathogens have evolved the use of effectors to suppress the above-described PTI mechanisms (Kamoun, 2006) whereas the plant has developed a perception of these effectors through ETI that induces a strong response of cell apoptosis and local necrosis (Boller & Felix, 2009). In a recent paper, Hiruma et al., (2016) suggested that the immune system has wider physiological roles than simply restricting pathogen growth and may be involved in symbiont behavior. In an experiment described previously in section 1.2 they used metabolic profiling and experimental inoculations of endophytic fungi to demonstrate that the plant's innate immune system favored the colonization of *Colletotrichum tofieldiae* during phosphate starvation and defense responses under phosphate-sufficient conditions (Hiruma et al., 2016). At the same time the immune system stimulated the indole glucosinolate metabolism (Pant et al., 2015) that induced a shift in symbiont behavior, triggering the transfer of phosphorus by the fungi from root-associated hyphae to the plant shoots. The immune system could thus be a tool allowing the plant to manipulate symbionts for its own good.

### 3.2.3 Regulation of symbiotic interactions

As explained in section 1.2, symbiosis consists of a continuum of partnerships ranging from parasitism to mutualism. The level of symbiosis efficiency is a consequence of the evolutionary trajectory of the symbiont under consideration. This means that in any kind of symbiosis cheaters and cooperators can co-exist. But why cheating? A large proportion of the plant microbiota provides beneficial functions to the plant and is thus believed to consist of mutualists. Mutualism implies reciprocal benefits with an associated cost for both partners (Cameron et al., 2008; Davitt et al., 2010; Fredericksson et al., 2012). This type of interaction favors `the tragedy of the commons\_ since it creates a conflict of interest between organisms. From an evolutionary point of view, mutualism is thus expected to be unstable and to evolve toward an asymmetric relationship with one partner benefiting from the interaction at the expense of the other. By doing so, a cheater symbiont or a host providing few benefits (at low cost) to the other in exchange for high rewards would selfishly improve its own fitness and thus invade the community. The stability of mutualism has been proposed to rely on control mechanisms on both sides of the symbiosis (Bever et al., 2009; Kiers et al., 2011). It has been clearly demonstrated that plants can exert a carbon sanction on the less beneficial Arbuscular Mycorrhizal fungi cooperators (Kiers et al., 2011) thus reducing the

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fitness of any cheaters. This mechanism of carbon sanction by the host plant has been demonstrated for both phosphorus (Bever et al., 2009; Kiers et al., 2011) and nitrogen transfer (Fellbaum et al., 2012) by AM fungi. Reciprocally on the fungal side, phosphorus is only delivered to the host in exchange for photosynthates. In this symbiosis, being a cheater thus means increasing the carbon cost to deliver the phosphorus (Kiers et al., 2011). Considering that a given host plant is colonized by multiple symbionts at the same time, cheaters can only proliferate if the plant is not able to favorise other symbionts. The level of cooperation is thus likely correlated with the level of diversity of symbionts.

#### 3.2.4 The host plant effect: genetics and biogeography

The colonization of plants by microorganisms occurs in the light of a finely tuned innate plant immune system allowing a selective recognition and filtration of microorganisms. A plant is thus capable of constructing its own microbiota. The inherent expectation is thus a strong effect of plant identity on the assembly of the microbiota. Although the initial microorganisms communities depend on the original soil they become more and more plant-specific and less diverse during colonization of the rhizosphere and endosphere (see above section 3.1). Even within these compartments, communities become increasingly plant-specific during plant growth and development (Chaparro et al., 2014; Sugiyama et al., 2014; Edwards et al., 2015; Shi et al., 2015). Plants represent microorganisms habitats and thus the root architecture or nutrient quality and quantity can determine the plant microbiota depending on the plant species (Oh et al., 2012; Bonito et al., 2014). Differences between microbiota compositions can even be detected at a finer scale such as the ecotype. *A. thaliana* ecotypes establish different rhizosphere communities from each other at a statistically significant level (Bulgarelli et al., 2012; Lundberg et al., 2012). However the same studies along with others also highlighted that environmental factors seemed to exert greater control on root-associated bacteria than the plant genotype or even plant species (Bulgarelli et al., 2012; Lundberg et al., 2012; Shakya et al., 2013; Schlaeppi et al., 2014). This suggests that plant identity might not be the primary or at least the strongest factor determining differences in plant microbiota. Recent advances indicate that the importance of the host species might be microorganism-specific. Coleman-derr et al. (2016) found that while prokaryotic communities of *Agave* species were primarily shaped by plant compartment and soil properties, fungal communities were structured by the biogeography of the plant host species. Such differences in biogeographic effect have been independently found in other studies where the root fungal communities

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(ectomycorrhiza) were differentiated more by geographic distance than bacterial communities (Peay et al., 2007; Shakya et al., 2013; Meiser et al., 2014).

### 3.3 Biotic interactions

Microorganisms living within the plant interact with each other in different ways and these interactions may determine their co-existence. The plant itself is also interacting with other plants in a community context. In this section we review the importance of the biotic interactions in assembly of the microbiota, focusing primarily on recent discoveries regarding microbe-microbe interactions and then emphasizing the under-appreciated role of the plant community context.

#### 3.3.1 Microbe-microbe interactions

Considering the fact that plant roots constitute a habitat for microorganisms and that the roots represent a finite volume, co-existing microorganisms within the plant are expected to be in competition for space and resources. Competition between bacterial and fungal species has been demonstrated at the scale of the species. Werner & Kiers (2015) demonstrated that the order of arrival of AM fungi structured the colonization of the plant roots suggesting that mycorrhizal fungi compete for root space. Specifically they evidenced that the AM fungal that first colonized plant roots (through an earlier inoculation) benefitted from a priority effect and were thus found in greater abundance. At higher taxonomic levels, a recent study focusing on the whole endospheric root fungal microbiota (L, Van et al., 2017) highlighted the coexistence of Glomeromycota, Ascomycota and Basidiomycota in *Agrostis stolonifera*. An important result of this work was the demonstration that the phylogenetic structure and phylogenetic signal measured differed among phyla. A phylogenetic overdispersion was observed for Glomeromycota and Ascomycota indicating facilitative interactions between fungi or competitive interactions resulting in the coexistence of dissimilar fungi. Conversely, the fungi within Basidiomycota displayed a clustered phylogenetic pattern suggesting an assemblage governed by environmental filtering (i.e. plant host) favoring the coexistence of closely related species (L, Van et al., 2017). These mechanisms of facilitation, competition or environmental filtering acting on the fungal microbiota composition are observed at the level of an individual plant.

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However, competitive or facilitative interactions between microorganisms in the plant microbiota are not stable over time and colonization of the root by a new microorganism can bring major changes. For example, colonization by the fungal pathogen *Rhizoctonia solani*, caused a significant shift in composition of the sugar beet and lettuce rhizosphere community often with an increase in specific family abundances (Chowdhury et al., 2013; Chapelle et al., 2016). Moreover, plant-microorganisms may themselves be colonized by bacteria and viruses (i.e. three way symbiosis) affecting symbiotic communication. For example, the endophyte *Curvularia protuberata* contains a virus that is required to induce heat tolerance (Mendez et al., 2007), the fungus being asymptomatic without the virus. Current knowledge thus indicates that microbe-microbe interactions can, like host-microbe interactions, drive plant microbiota assembly and its effect on phenotype. However, information acquired about these microbe-microbe interactions is still very limited. An example is the above-described three way symbioses that can mediate the effect of a given symbiont but whose occurrence in natural ecosystems is unknown.

### 3.3.2 The plant community context

The pool of microorganisms available in the soil conditions plant recruitment and thus determines the plant microbiota (see section 3.1.1). This soil pool depends on local environmental filtering and microorganisms dispersal. Another factor structuring this pool is plant host filtering. Indeed, as a plant is able to selectively recruit and promote microorganisms depending on its needs (see section 3.2) a given plant will influence the local soil pool. Thus at the scale of the plant community, the effect of a plant on the local soil pool of microorganisms indirectly affects the microbiota assembly of the other plants in the community. More importantly, as the filtering effect is dependent on plant identity, studies based on matrix-focal plant designs have demonstrated that this identity determines the composition of the focal plant fungal community (Johnson et al., 2004; Hausmann & Hawkes, 2009). Such host filtering effects have long been considered negligible for plant microbiota assembly in comparison to abiotic environmental factors. However, a recent review by Valyi et al. (2016) proposed that the degree of host filtering on the AM fungal community would depend on the spatial scale considered and could be stronger than environmental factors at a local scale. Taking into account that a given plant recruits its microbiota within its local environment, there is a need to evaluate the intensity of this plant community context in determining both the AM fungal and whole microbiota assembly.

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### 3.4 Microbiota transmission

Diverse modes of transmission of microorganisms between generations, also called heritability, have been described and reviewed, and organized into different classifications (McFall-Ngai, 2002; Zilber-Rosenberg & Rosenberg, 2008). Herein, we present these modes of heritability in two categories: pseudo-vertical (or horizontal) and vertical transmissions.

#### 3.4.1 Horizontal or pseudo-vertical transmission

Pseudo-vertical transmissions are indirect transmissions mediated by the environment. Two individuals sharing the same environment are able to recruit similar microorganisms from the soil pool and thus plants producing microorganisms at low dispersal distance may share a part of their microbiota to this environmental transmission. Such transmission has been largely described for a wide range of organisms with significant impact on plant phenotype such as growth-promoting rhizobacteria (Smith et al., 1999; Singh et al., 2004; Somers et al., 2004; Egamberdieva et al., 2008), rhizobium (Stougaard, 2000; Jones et al., 2007) and even Arbuscular mycorrhizal fungi and non-photosynthetic fungi when seeds fall close to the mother plant (Wilkinson, 2001; Wang & Qui, 2006).

#### 3.4.2 Vertical transmission or heritability

For plants, vertical transmission has primarily been reported for the cytoplasmic inheritance of chloroplasts (Margulis, 1993). Other true vertical transmission in plants does exist even if it has not been extensively studied. A limited diversity of endophytic fungi and bacteria are contained in the germplasm and are thus vertically transmitted in grasses (Clay & Schardl, 2002), conifers (Ganley & Newcombe, 2006), and tropical trees (U'Ren et al., 2009). Another kind of vertical transmission has been proposed for plants through vegetative propagules (Wilkinson, 2001; Zilber-Rosenberg & Rosenberg, 2008). Plants are modular organisms and thus a fragment of plant (i.e. propagule) falling on the ground is able to grow and produce a new plant. In this context, it is probable that microorganisms developing within the plant fragment are transmitted to the progeny even if this has not been experimentally demonstrated. Another form of vertical microbiota transmission was suggested for clonal plants by Stuefer et al., (2004). Clonal plants are organized as a network of ramets (i.e. clonal, potentially autonomous individuals) connected by above or below-ground

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modified stems (i.e. stolons) (Figure 3). This network structure promotes plant propagation in space (i.e. physical integration) and in some species the sharing of resources within the network to optimize fitness of the network as a whole (i.e. physiologic integration, Oborny et al., 2001). Another layer of integration could occur in such a network because the stolons could permit the transfer of microorganisms between ramets through the vascular system or via the surface of the stolons. This hypothesis has never been addressed despite its importance regarding microorganisms dispersal and plant adaptation to environmental constraints.

Considering all the benefits that can be attributed to symbiotic microorganisms (described in section 2) vertical transmission of part of or the whole microbiota would be advantageous because it would limit the costs of searching for suitable symbionts (Wilkinson & Sherratt, 2001) and thus could ensure habitat quality for the progeny. In terms of evolution, the transmission of symbiotic microorganisms between plant generations constitutes a `continuity of partnership\_ between the plant and the transmitted symbionts (Zilber-Rosenberg and Rosenberg, 2008). There is thus a need in plant science to search for vertical transmission of a significant part of the microbiota influencing the plant's fitness.

#### 4. The plant is a `holobiont\_

Following the observations described in the previous sections, a new understanding of plant-microorganisms interactions has been proposed. Experiments and observations have clearly demonstrated the fundamental role of microorganisms in all aspects of host life with important consequences on host phenotype and fitness. Thus the plant should not be considered as a standalone entity but needs to be viewed as a host together with its microbiota. In this context, the holobiont idea, originally introduced in 1994 during a symposium lecture by Richard Jefferson and the holobiont hypothesis later developed by Zilber-Rosenberg and Rosenberg (2008) allow a collective view of the functions and interactions of a host and its symbionts. Holobiont is a term derived from the Greek word *holos* (whole or entire) and is used to describe an individual host and its microbial community. This holobiont theory proposes that an entity comprising a host and its associated microbiota, can be considered as a unit of selection (i.e. not the only unit) in evolution (Zilber-Rosenberg & Rosenberg, 2008; Theis et al., 2016). The host and microbial genomes of a holobiont are collectively defined as its hologenome (Rosenberg & Zilber-Rosenberg, 2013;

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Bordenstein & Theis, 2015). This concept relies on different tenets among which the idea that microorganisms can be vertically or horizontally transmitted between generations, constituting a continuity of partnership, which shapes the holobiont phenotype. Genomic novelties can thus occur within any components of the hologenome (host or microorganisms) and holobiont evolution could lead to variations in either the host or the microbiotic genomes (Zilber-Rosenberg & Rosenberg, 2008; Rosenberg & Zilber-Rosenberg, 2013). Furthermore, variations in the hologenome can also arise by recombination in the host and/or microbiome and by acquisition of new microbial strains from the environment (Rosenberg & Zilber-Rosenberg, 2013). In this context, selection acts on the result of the interactions both between the host and its symbionts and between symbionts: the holobiont phenotype.

The recent development of the holobiont concept and hologenome theory (Bordenstein & Theis, 2015) has triggered a debate on its validity and its usefulness as a scientific framework (Moran & Sloan, 2015; Douglas & Werren, 2016; Theis et al., 2016). The primary role of this holobiont concept and related hologenome theory is to provide adequate vocabulary to describe and study host microbiota symbiosis (Theis et al., 2016) while blending the effects of complex microbiota on host phenotypes that may be cooperative or competitive (Bosch & McFall-Ngai, 2011; Vandenkoornhuyse et al., 2015). Holobionts and their hologenomes are theoretical entities that require understanding. Metaphorically, skepticism and critics are the selection pressures shaping the evolution of science and `...the hologenome concept requires evaluation as does any new idea\_ (Theis et al., 2016). One of the main critics and prospects regarding the validity and significance of the holobiont and hologenome theory is the lack of studies showing vertical transmission of microorganisms between holobiont generations (i.e. vertical transmission, or heritability).

Observational and experimental studies addressing the ubiquity (i.e. frequency of occurrence in natural populations), the fidelity (i.e. homogeneity in the organisms transmitted) and the significance (i.e. amount of microorganisms transmitted and impact on the plant phenotype) of the heritability are thus needed to build a comprehensive holobiont theory. Another aspect of interest is the role of heritability in holobiont assembly and especially in the covariance of genomes between hosts and microbiota components that can induce variation in the phenotypes on which selection acts.



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In order for the holobiont and hologenome theory to be comprehensive, it should encompass many symbiosis models. The current understanding of the plant as a holobiont suggests another layer of complexity that has not been theorized yet. Many organisms are modular but plants harbor an additional level of modularity through clonal reproduction. The network structure of clonal plants potentially allows exchanges between holobionts, and thus provides a particular model for comprehension of holobiont assembly and evolution. An extension of the holobiont and hologenome theory for clonal organisms is thus required.

## 5. Objectives of the thesis

This thesis is organised around two major objectives that are the consequence of each another. This work aims first at identifying the assembly rules governing the structure of the plant microbiota and the consequences of this assembly regarding plant phenotypes. More precisely, we aimed at determining :

- (i) The importance of the microbiota regarding plant phenotypic plasticity and adaptation (Chapter 1) with the hypothesis that fungal endophytes and specifically AM fungi influence the spatial spreading and performance of plants
  
- (ii) The occurrence of microbiota heritability between clonal generations in plants and its impact on the assembly of the microbiota (Chapter 2). Specifically, we tested the hypothesis that clonal plants are able to transmit to their clonal progeny a part of their microbiota, including important symbionts for plant fitness, through the connective structures forming the clonal network.

The second objective of the thesis is to determine the importance of the plant community context for the plant-microorganisms interactions and the outcome for plant performance (chapter 3). The hypothesis is that the abundance of a given plant in a community can modify the composition and structure of the fungal community colonizing a focal host-plant, ultimately determining its performance.



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## Chapter I: Consequences of mutualist-induced plasticity

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### I.1. Introduction

#### Scientific context

Plants are sessile organisms and have thus to cope with environmental constraints. In nature, the spatial distribution of resources and environmental constraints in general, are heterogeneous. Plants have thus evolved different mechanisms to buffer these constraints such as plastic modifications (i.e. production of different phenotypes from a single genotype) to adapt to local conditions (Bradshaw, 1965). However, the tools available for plantplastic responses are not limited to its genome. Indeed, epigenetic mechanisms (regulating the expression of the genome) and microorganisms has been repeatedly shown as sources of variations (Richards, 2006; Friesen et al., 2011; Holeski et al., 2012; Vandenkoornhuyse et al., 2015). A large number of studies have separately identified the effect of specific plant-associated microorganisms and epigenetic marking on plant phenotype. There is however a need to bring together these findings, to evaluate the importance of these sources of phenotypic variation on the plant ability to produce plastic responses and adapt to environmental constraints.

Clonal plants are particularly plastic organisms. In clonal plants, the network structure (i.e. clonal individual connected) allows the propagation in space, together with resource and information sharing (i.e. physiological integration; Oborny et al., 2001). The network thus allow to develop plastic responses to the heterogeneity at low modular level such as a leaf or a root to higher modular level such as the ramet. Many studies have evidenced two plastic responses to the heterogeneity of resources that are specific to this network structure: foraging and specialization (Slade & Hutchings, 1987; Dong, 1993; Hutchings & de Kroon, 1994; Birch & Hutchings, 1994). Plant-associated symbionts provide a large range of key functions that can be useful for the plant

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(Friesen et al., 2011; Møller et al., 2016). From a theoretical point of view, plants should thus consider microorganisms as a resource and develop foraging and specialization behaviors in response to their heterogeneous distribution. . Based on the optimal foraging theory (de Kroon & Hutchings, 1995), clonal plants are expected to aggregate ramets (i.e. clonal unit comprising shoot and roots) in favorable patches and to avoid unfavorable patches through an elongation of stolons (i.e. connective structure). In agreement with the division of labor theory (Stuefer & de Kroon, 1996), plants are also expected to develop specialization response by developing preferentially roots in a nutrient rich soil patch. Such plant response to microorganisms heterogeneous distribution has never been investigated to date. In addition, as microorganism affect plant phenotype, they could alter its ability to produce plastic response.

### Objectives of the chapter

This chapter aims at determining the role of microorganisms in the production of phenotypic plasticity to environmental heterogeneity. More precisely we address the following questions:

- 1) Is the genome the only source of phenotypic plasticity for plants ? Are microbiota and epigenetics significant sources of phenotypic plasticity allowing plant to adapt to environmental constraints ? (Article I)
- 2) Does plant develop foraging and specialization plastic responses to the spatial heterogeneity of Arbuscular Mycorrhizal Fungi presence ? Arbuscular Mycorrhizal Fungi identity is susceptible to alter plant traits, thus impacting its ability to produce foraging and plastic responses ? (Article II)

### Methods

The work presented in this chapter is based on two different approaches. The first article presented is a review investigating the current knowledges and limits on plant phenotypic plasticity induced by epigenetic and microbiota. This paper aims at identifying perspectives in the roles of microorganisms for plant response to environmental constraints.

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The second article presented herein is based on experimental cultivations of the clonal plant *Glechoma hederacea* in controlled conditions (light, temperature, water and nutrient availability, no exterior contaminations). Two different experiments are presented in this article. We tested the *G. hederacea* foraging and specialization responses to the heterogeneous distribution of AM fungi. We manipulated the heterogeneity of AM fungi distribution by setting artificial patches (separate cultivation pots) that were inoculated (presence) or not (absence) with a mixture of three AM fungi species. To test the effect of the fungal species inocula on the plant traits, plants were cultivated into larger pots and the treatments consisted of the inoculation of individual fungal species (three treatments) or no inoculation at all.

### Main results

The review presented herein (Article I) allowed to evidence the importance of microbiota and epigenetics as sources of phenotypic plasticity for plants adaptation to environmental constraints. Recent discoveries on epigenetics and plant-associated microorganisms demonstrated their impact on plant survival, development and reproduction. Microbiota and epigenetics represent non genome based sources of phenotypic variations that can be used by the plant to cope with environmental conditions from very short (within lifetime) to longer time scales (i.e. evolutive). These phenotypic variations can furthermore be integrated in the genome through genetic accommodation and thus impacts plants evolutionary trajectories. Our review of the existing knowledge overall evidenced that an interplay between microbiota and epigenetic mechanisms could occur and deserves to be investigated together with the respective impacts of these mechanisms on plant survival and evolution. In our experiments, we demonstrated 1.) in *Glechoma hederacea* no specialization and a limited foraging response to the heterogeneous distribution of AM fungi and 2.) that the architectural traits involved in the plant's foraging response were not affected by the AM fungi species tested, contrary to resource allocation traits (linked to the specialization response).

Two possible explanations were proposed: (i) plant responses are buffered by the differences observed in fungal species individual effects. Indeed, as AM fungal species have contrasted effects on plant traits and resources acquisition, in mixture their cumulative effects could be neutral; (ii) the initial heterogeneous distribution of AM fungi is perceived as homogeneous by the plant either by reduced physiological integration or due to the transfer of AM fungi propagules through the stolons.

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Indeed the fact that *G. hederacea* does not forage for arbuscular mycorrhizal fungi suggests that plant could homogenize the distribution of its symbiotic microorganisms through their transmission between ramets i.e. generations. Additional observation of Scanning electron microscopy revealed the presence of hyphae on the stolon surface and several cells close to the external surface of the stolon cross-section were invaded by structures which could be interpreted as fungi. DNA sequencing of stolon samples confirmed these results and demonstrated the presence of AM fungi in the stolons. This suggests that fungi can be transferred from one ramet to another, at least by colonization of the stolon surface and/or within the stolon tissues. The possible transfer of AM fungi between ramets suggests that plants could have evolved a mechanism of vertical transmission of a part of their microbiota. This mechanism would ensure the habitat quality for their progeny and constitutes a continuity of partnership (Wilkinson, 2001). To date, vertical transmission examples only concerns a few microorganisms colonizing plant seeds (Clay & Schardl., 2002; Selosse et al., 2004). Such mechanisms would be of importance since induced phenotypic modifications inherited between generations allow short, medium and long-time scale adaptation of plants to environmental constraints (Article I). The hypothesis of microbiota transmission between ramets is addressed in the following chapter.

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I.2 Article I: Epigenetic mechanisms and microbiota as a toolbox for  
plant phenotypic adjustment to environment







# Epigenetic Mechanisms and Microbiota as a Toolbox for Plant Phenotypic Adjustment to Environment

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## Summary

The classic understanding of organisms focuses on genes as the main source of species evolution and diversification. The recent concept of genetic accommodation questions this gene centric view by emphasizing the importance of phenotypic plasticity on evolutionary trajectories. Recent discoveries on epigenetics and symbiotic microbiota demonstrated their deep impact on plant survival, adaptation and evolution thus suggesting a novel comprehension of the plant phenotype. In addition, interplays between these two phenomena controlling plant plasticity can be suggested. Because epigenetic and plant-associated (micro-) organisms are both key sources of phenotypic variation allowing environmental adjustments, we argue that they must be considered in terms of evolution. This 'non-conventional' set of mediators of phenotypic variation can be seen as a toolbox for plant adaptation to environment over short, medium and long time-scales.

Key-words : Plant, Symbiosis, Phenotypic Plasticity, Epigenetic, Evolution

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Evolution is driven by selection forces acting on variation among individuals. Understanding the sources of such variation that has led to the diversification of living organisms, is therefore of major importance in evolutionary biology. Diversification is largely thought to be controlled by genetically-based changes induced by ecological factors (Schluter, 1994; 2000). Phenotypic plasticity, i.e., the ability of a genotype to produce different phenotypes (Bradshaw, 1965; Schlichting, 1986; Pigliucci, 2005), is a key developmental parameter for many organisms and is now considered as a source of adjustment and adaptation to biotic and abiotic constraints (e.g., West-Eberhard, 2005; Anderson et al., 2011). However, many current studies still focus on genetically generated plasticity to predict and model biodiversity response to a changing climate (Peck et al., 2015), omitting considerable individual variability. In addition, it is striking how poorly the variability is integrated and that both experiments and models most often measure population averages (Peck et al., 2015).

Because of their sessile lifestyle, plants are forced to cope with local environmental conditions and their survival subsequently relies greatly on plasticity (Sultan, 2000). Plastic responses may include modifications in morphology, physiology, behavior, growth or life history traits (Sultan, 2000). In this context, the developmental genetic pathways supporting plasticity allow a rapid response to environmental conditions (Martin and Pfennig, 2010) and the genes underlying these induced phenotypes are subjected to selection (Pfennig, 2010). If selection acts primarily on phenotype, the environmental constraints an organism has to face can lead either to directional selection or disruptive selection of new phenotypes (Pfennig, 2010). Thus, novel traits can result from environmental induction followed by genetic accommodation of the changes (West-Eberhard, 2005). These accommodated novelties, because they are acting in response to the environment, are proposed to have greater evolutionary impact than mutation-induced novelties (West-Eberhard, 2005).

The links between genotype and phenotypes are often blurred by factors including (i) epigenetic effects inducing modifications of gene expression, post-transcriptional and post-translational modifications, which allow a quick response to an environmental stress (Shaw and Etterson, 2012) and (ii) the plant symbiotic microbiota recruited to dynamically adjust to environmental constraints (Vandenkoornhuysen et al., 2015). We investigate current knowledge

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regarding the evolutionary impact of epigenetic mechanisms and symbiotic microbiota and call into question the suitability of the current gene-centric view in the description of plant evolution. We also address the possible interactions between the responsive epigenetic mechanisms and symbiotic interactions shaping the biotic environment and phenotypic variations.

Genotype-phenotype link: still appropriate?

In the neo-Darwinian synthesis of evolution (Mayr and Provine, 1997), phenotypes are determined by genes. The underlying paradigm is that phenotype is a consequence of genotype (Alberch, 1991) in a nonlinear interaction due to overdominance, epistasis, pleiotropy, and covariance of genes (see Alberch, 1991; Pigliucci, 2005). Both genotypic variations and the induction of phenotypic variation through environmental changes have been empirically demonstrated, thus highlighting the part played by the environment in explaining phenotypes. These phenotypes are consequences of the perception, transduction and integration of environmental signals. The latter is dependent on environmental parameters, including (i) the reliability or relevance of the environmental signals (Huber and Hutchings 1997), (ii) the intensity of the environmental signal which determines the response strength (Hodge, 2004), (iii) the habitat patchiness (Alpert and Simms, 2002) and (iv) the predictability of future environmental conditions with current environmental signals information (Reed et al., 2010).

The integration of all these characteristics of the environmental stimulus regulates the triggering and outcomes of the plastic response (e.g. Alpert and Simms, 2002). In this line, recent works have shown that plant phenotypic plasticity is in fact determined by the interaction between plant genotype and the environment rather than by genotype alone (El-Soda et al., 2014). Substantial variations in molecular content and phenotypic characteristics have been repeatedly observed in isogenic cells (Kaern et al., 2005). Moreover, recent analyses of massive datasets on genotypic polymorphism and phenotype often struggle to identify single genetic loci that control phenotypic trait variation (Anderson et al., 2011). The production of multiple phenotypes is not limited to the genomic information and the idea of a genotype-phenotype link no longer seems fully appropriate in the light of these findings. Besides, evidence has demonstrated that phenotypic variations are related to genes-transcription and RNAs-translation, which are often linked to epigenetic mechanisms, as discussed in the following paragraph (Rapp and Wendel, 2005).

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## Epigenetics as a fundamental mechanism for plant phenotypic plasticity

Epigenetics often refers to a suite of interacting molecular mechanisms that alter gene expression and function without changing the DNA sequence (Richards, 2006; Holeski 2012). The best-known epigenetic mechanisms involve DNA methylation, histone modifications and histone variants, and small RNAs. These epigenetic mechanisms lead to enhanced or reduced gene transcription and RNA-translation (e.g. Richards, 2006; Holeski, 2012). A more restricted definition applied in this paper considers as epigenetic the states of the epigenome regarding epigenetic marks that affect gene expression: DNA methylation, histone modifications (i.e. histone amino-terminal modifications that act on affinities for chromatin-associated proteins) and histone variants (i.e. structure and functioning), and small RNAs. These epigenetic marks may act separately or concomitantly, and can be heritable and reversible (e.g. Molinier et al., 2006; Richards, 2011; Bilichak, 2012). The induction of defense pathways and metabolite synthesis against biotic and abiotic constraints by epigenetic marks has been demonstrated during the last decade mainly in the model plant species *Arabidopsis* and tomato (e.g. Rasmann et al., 2012; Slaughter et al., 2012; Sahu et al., 2013).

Epigenetics is now regarded as a substantial source of phenotypic variations (Manning et al., 2006; Crews et al., 2007; Kucharski et al., 2008; Bilichak, 2012; Zhang et al., 2013) in response to environmental conditions. More importantly, studies have suggested the existence of epigenetic variation that does not rely on genetic variation for its formation and maintenance (Richards, 2006; Vaughn et al., 2007). However, to date, only a few studies have demonstrated the existence of pure natural epi-alleles (Cubas et al., 1999) although they are assumed to play an important role in relevant trait variation of cultivated plants (Quadrona et al., 2014). Similarly to the results observed in mangrove plants (Lira-Medeiros et al., 2010), a recent work on *Pinus pinea* which exhibits high phenotypic plasticity associated with low genetic diversity, discriminated both population and individuals based on cytosine methylation, while the genetic profiles failed to explain the observed phenotype variations (Sanz-Laguna et al., 2014). Epigenetics can provide phenotypic variation in response to environmental conditions without individual genetic diversity. It could hence provide an alternative way or an accelerated pathway for adaptive 'evolutionary' changes (Bossdorf et al., 2008). Epigenetic marks could also 'tag' a site for mutation: it is known that methylated cytosine is

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more mutable increasing the opportunity for random mutation to act at epigenetically modified sites.

### Epi-alleles, genetic accommodation and adaptation

Even if totally independent epigenetic variations (i.e., pure epi-alleles) are scarce and still need to be investigated, the evolutionary significance of the resulting epigenetically-induced phenotypic variations is being increasingly debated (Schlichting and Wund, 2014). Assuming that selection acts on phenotypes and that these phenotypes are not always genetically controlled, it can be argued that new phenotypes arising from adaptive plasticity are not random variants (West-Eberhard, 2005). Changes in the trait frequency then correspond to a 'genetic accommodation' process (West-Eberhard, 2005; Schlichting and Wund, 2014) through which an environmentally-induced trait variation becomes genetically determined by a change in genes frequency that affects the trait 'reaction norm' (West-Eberhard, 2005; Crispo 2007). It may also be suggested that genetic accommodation can result from the selection of genetic changes optimizing the novel variant's adaptive value through modifications in the form, regulation or phenotypic integration of the trait. In the "adaptation loop", the effect of environment on plant performance induces the selection of the most efficient phenotype. The epigenetic processes are not the only engines of plant phenotypic plasticity adjustment. Indeed, plants also maintain symbiotic interactions with microorganisms to produce phenotypic variations.

### Plant phenotypic plasticity and symbiotic microbiot

Plants harbor an extreme diversity of symbionts including fungi (Vandenkoornhuysen et al., 2002) and bacteria (Bulgarelli et al., 2012; Lundberg et al., 2012). During the last decade, substantial research efforts have documented the range of phenotypic variations allowed by symbionts. Examples of mutualist-induced changes in plant functional traits have been reported (Streitwolf-Engel et al., 1997; 2001; Wagner et al., 2014), which modify the plant's ability to acquire resources, reproduce, and resist biotic and abiotic constraints. The detailed pathways linking environmental signals to this mutualist-induced plasticity have been identified in some cases. For instance, Boller and Felix (2009) highlighted several mutualist-induced signaling pathways allowing a plastic response of plants to virus, pests and pathogens initiated by flagellin/FLS2 and EF-Tu/EFR recognition receptors. Mutualist-induced plastic changes may affect plant fitness by modifying plant response to its environment including (i) plant-resistance to salinity (Lopez et al.,

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2008), drought (Rodriguez et al., 2008), heat (Redman et al., 2002) and (ii) plant nutrition (e.g., Smith et al., 2009). These additive ecological functions supplied by plant mutualists extend the plant's adaptation ability (Bulgarelli et al., 2013; Vandenkoornhuyse et al., 2015), leading to fitness benefits for the host in highly variable environments (Conrath et al., 2006) and therefore can affect evolutionary trajectories (e.g. Brundrett, 2002).

In fact, mutualism is a particular case of symbiosis (i.e. long lasting interaction) and is supposed to be unstable in terms of evolution because a mutualist symbiont is expected to improve its fitness by investing less in the interaction. Reciprocally, to improve its fitness a host would provide fewer nutrients to its symbiont. Thus, from a theoretical point of view, a continuum from parasite to mutualists is expected in symbioses. However, the ability of plants to promote the best cooperators by a preferential C flux has been demonstrated both in Rhizobium/ and Arbuscular Mycorrhiza/Medicago truncatula interactions (Kiers et al., 2007; 2011). Thus, the plant may play an active role in the process of mutualist-induced environment adaptation as it may be able to recruit microorganisms from soil (for review Vandenkoornhuyse et al., 2015) and preferentially promote the best cooperators through a nutrient embargo toward less beneficial microbes (Kiers et al., 2011).

In parallel, vertical transmission or environmental inheritance of a core microbiota is suggested (Wilkinson and Sherratt, 2001) constituting a "continuity of partnership" (Zilber-Rosenberg and Rosenberg, 2008). Thus the impact on phenotype is not limited to the individual's lifetime but is also extended to reproductive strategies and to the next generation. Indeed, multiple cases of alteration in reproductive strategies mediated by mutualists such as arbuscular mycorrhizal fungi (Sudov@ 2009) or endophytic fungi (Afkhami and Rudgers, 2008) have been reported. Such microbiota, being selected by the plant and persisting through generations, may therefore influence the plant phenotype and be considered as a powerhouse allowing rapid buffering of environmental changes (Vandenkoornhuyse et al., 2015). The idea of a plant as an independent entity on the one hand and its associated microorganisms on the other hand has therefore recently matured towards understanding the plant as a holobiont or integrated "super-organism" (e.g., Vandenkoornhuyse et al., 2015).

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## Holobiont plasticity and evolution

If the holobiont can be considered as the unit of selection (Zilber-Rosenberg and Rosenberg, 2008), even though this idea is still debated (e.g. Leggat et al., 2007; Rosenberg et al., 2007), then the occurrence of phenotypic variation is enhanced by the versatility of the holobiont composition, both in terms of genetic diversity (i.e. through microbiota genes mainly) and phenotypic changes (induced by mutualists). Different mechanisms allowing a rapid response of the holobiont to these changes have been identified (1) horizontal gene transfer between members of the holobiont (i.e., transfer of genetic material between bacteria; Dinsdale et al., 2008) (2) microbial amplification (i.e., variation of microbes abundance in relation to environment variation) and (3) recruitment of new mutualists within the holobiont (Vandenkoornhuysen et al., 2015). In this model, genetic novelties in the hologenome (i.e. the combined genomes of the plant and its microbiota, the latter supporting more genes than the host) are a consequence of interactions between the plant and its microbiota.

The process of genetic accommodation described in section 3, impacts not only the plant genome but can also be expanded to all components of the holobiome and may thus be enhanced by the genetic variability of microbiota. In the holobiont, phenotypic plasticity is produced at different integration levels (i.e., organism, super-organism) and is also genetically accommodated or assimilated at those scales (i.e., within the plant and mutualist genomes and therefore the hologenome). The holobiont thus displays greater potential phenotypic plasticity and a higher genetic potential for mutation than the plant alone, thereby supporting selection and the accommodation process in the hologenome. In this context, the variability of both mutualist-induced and epigenetically-induced plasticity in the holobiont could function as a "toolbox" for plant adaptation through genetic accommodation. Consequently, mechanisms such as epigenetics allowing a production of phenotypic variants in response to the environment should be of importance in the holobiont context.

Do microbiota and epigenetic mechanisms act separately or can they interact ?

Both epigenetic and microbiota interactions allow plants to rapidly adjust to environmental conditions and subsequently support their fitness (Figure 1). Phenotypic changes ascribable to mutualists and mutualists transmission to progeny are often viewed as epigenetic variation (e.g.,



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Gilbert et al., 2010). However, this kind of plasticity is closer to an "interspecies induction of changes" mediated by epigenetics rather than "epigenetics-induced changes" based solely on epigenetic heritable mechanisms (see section on epigenetics for a restricted definition). Apart from the difficulty of drawing a clear line between epigenesis and epigenetics (Jablonka and Lamb, 2002), evidence is emerging of the involvement of epigenetic mechanisms in mutualistic interactions. An experiment revealed changes in DNA adenine methylation patterns during the establishment of symbiosis (Ichida et al., 2007), suggesting an effect of this interaction on the bacterial epigenome or at least, a role of epigenetic mechanisms in symbiosis development. Correct methylation status seems also to be required for efficient nodulation in the *Lotus japonicus* - *Mesorhizobium loti* symbiosis (Ichida et al., 2009) and miRNA "miR-397" was only induced in mature nitrogen-fixing nodules (De Luis et al., 2012). As epigenetic mechanisms are involved in the development of symbiosis, we assume that epigenetic phenomena may have significant effects on mutualist associations. As yet, little is known about the epigenetic effects and responses underlying host-symbiont interactions.

These epigenetic mechanisms and microbiota sources of plant phenotypic plasticity may act synergistically although this idea has never convincingly been addressed. As far as we know, different important issues bridging epigenetic mechanisms and microbiota remain to be elucidated such as (1) the frequency of epigenetic marking in organisms involved in mutualistic interactions, (2) the range of phenotypic plasticity associated with these marks either in the plant or in microorganisms, (3) the consequences of these marks for holobiont phenotypic integration, (4) the functional interplay between epigenetic mechanisms and microbiota in plant phenotype expression, (5) the inheritance of epigenetic mechanisms and thus their impact on symbiosis development, maintenance and co-evolution. To answer these questions, future studies will need to involve surveys of plant genome epigenetic states (e.g., methylome) in response to the presence/absence of symbiotic microorganisms. Recent progress made on bacteria methylome survey methods should represent useful tools to design future experiment on this topic (Sánchez-Romero et al., 2015).

Although research on the interaction between microbiota and epigenetics is in its infancy in plants, recent works mostly on humans support existing linkages. Indeed, a clear link has been evidenced between microbiota and human behavior (Dinan et al., 2015). Other examples of microbiota effects are their (i) deep physiological impact on the host through serotonin modulation

(Yano et al., 2015) and (ii) incidence on adaptation and evolution of the immune system (Lee and Mazmanian, 2010). Such findings should echo in plant-symbionts research and encourage further investigations on this topic. More broadly, and despite the above-mentioned knowledge gaps, our current understanding of both epigenetic mechanisms and the impact of microbiota on the expression of plant phenotype, invite us to take those phenomena into consideration in species evolution and diversification.

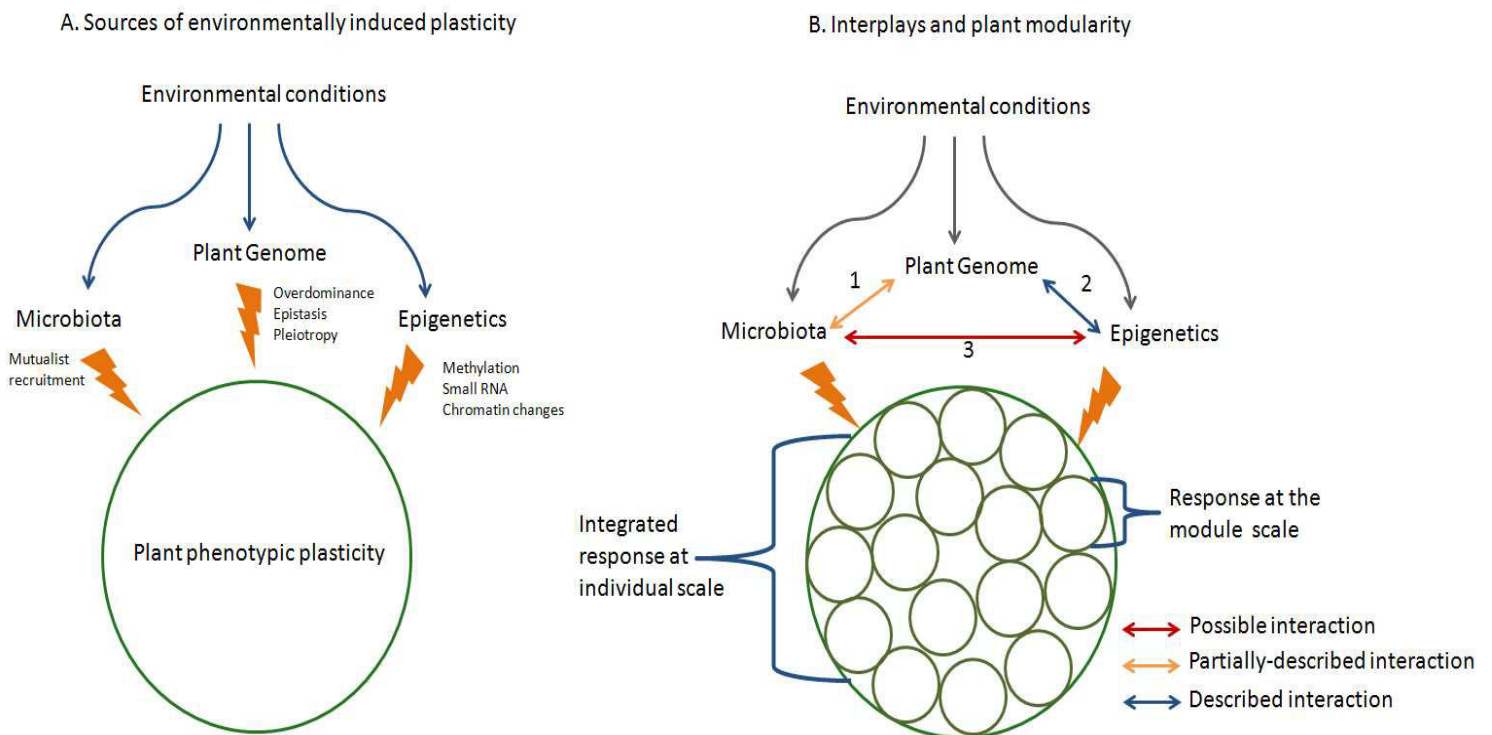


Figure 1: (A) Plant phenotypic plasticity is triggered by environmental constraints. Phenotypic changes induced are not solely genetically controlled but are also based on either epigenetic marks (box 2) or plant microbiota by recruitment of mutualists. This plant 'toolbox' allows a rapid response to environmental constraints. (B) The control over plant phenotypic plasticity may cross-talk or synergistically interplay with different possible interactions. 1) Co-evolution plant-symbiont 2) Interplay genetic/epigenetics 3) Cross-talk epigenetic/microbiota. These mechanisms also act at the modular scale of plant structure.

'Extended phenotype' and 'hologenome theory'

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Microbiota and epigenetic mechanisms play different but complementary roles in producing phenotypic variations which are then subjected to selective pressure. Diversification of traits is suggested to depend on evolutionary time (necessary for the accumulation of genetic changes, i.e., Martin and Pfennig, 2010) but rapid shifts in plant traits, as allowed by both microbiota and epigenetics, would provide accelerated pathways for their evolutionary divergence. In addition, such rapid trait shifts also permit rapid character displacement. Induction of DNA methylation may occur more rapidly than genetic modifications and could therefore represent a way to cope with environmental constraints on very short time scales (during the individual's lifetime; Rando and Verstrepen, 2007). In parallel, microbiota-induced plasticity is achieved both at a short time scale (i.e. through recruitment) and at larger time scales (i.e. through symbiosis evolution; Figure 2).

Because of the observation of transgenerational epigenetic inheritance, the relevance of epigenetically-induced variations is a current hot topic in the contexts of evolutionary ecology and environmental changes (Bossdorf et al., 2008; Slatkin, 2009; Zhang et al., 2013; Schiliching and Wund, 2014). This has stimulated renewed interest in the 'extended phenotype' (Dawkins, 1982). The central idea of Dawkins 'extended phenotype' (Dawkins, 1982) is that phenotype cannot be limited to biological processes related to gene/genome functioning but should be 'extended' to consider all effects that a gene/genome (including organisms behavior) has on its environment. For example, the extended phenotype invites us to consider not only the effect of the plant genome on its resources acquisition but also the effect of the genome on the plant symbionts as well as on nutrient availability for competing organisms.

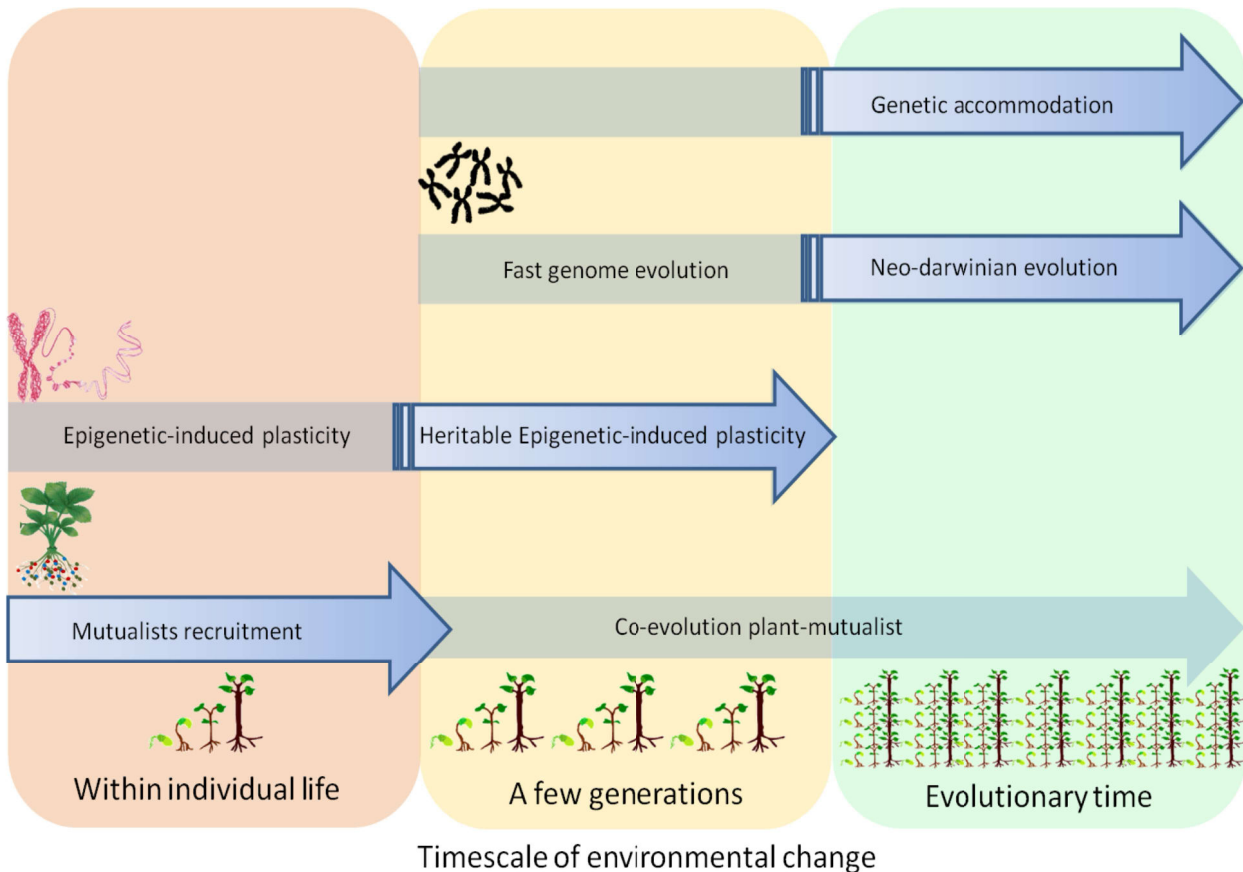


Figure 2: A plant's phenotypic variations can be inherited even in the case of a phenotypic trait not controlled by a gene/genome variation. This rapid response to environmental change involves epigenetic mechanisms and/or microorganisms recruitment within the plant microbiota. Heritable transgenerational plasticity mediated by epigenetic mechanisms and/or mutualists could be followed by genetic accommodation and long term adaptation.

More recently the development of the 'hologenome theory' (Zilber-Rosenberg and Rosenberg, 2008) posits that evolution acts on composite organisms (i.e., host and its microbiome) with the microbiota being fundamental for their host fitness by buffering environmental constraints. Both the 'extended phenotype' concept and 'hologenome theory' admit that the environment can leave a "footprint" on the transmission of induced characters. Thus, opportunities exist to revisit our understanding of plant evolution to embrace both environmentally-induced changes and related 'genetic accommodation' processes.

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I.3 Article II: AM fungi patchiness and the clonal growth of *Glechoma hederacea* in heterogeneous environments



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# SCIENTIFIC REPORTS



**OPEN** **AM fungi patchiness and the clonal growth of *Glechoma hederacea* in heterogeneous environments**

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Abstract:

The effect of AM fungi spatial distribution on individual plant development may determine the dynamics of the whole plant community. We investigated whether clonal plants display a foraging or a specialization response, as for other resources, to adapt to the heterogeneous distribution of AM fungi. Two separate experiments were done to investigate *Glechoma hederacea* response to a heterogeneous distribution of a mixture of 3 AM fungi species, and the single effects of each species on colonization and allocation traits. No specialization and only a limited foraging response to AM fungi heterogeneous distribution was found. An effect of the AM fungal species on plant mass allocation and ramet production, but not on spacer length, was detected. Two possible explanations are proposed: (i) the plant's responses are buffered by differences in individual effects of the fungal species or their root colonization intensity. (ii) the initial AM fungi heterogeneity is sensed as homogeneous by the plant either by reduced physiological integration or due to the transfer of AM fungi propagules through the stolons. Microscopic and DNA sequencing analyses provided evidences of this transfer, thus demonstrating the role of stolons as dispersal vectors of AM fungi within the plant clonal network.

Key words: *Glechoma hederacea*, Arbuscular Mycorrhizal Fungi, Phenotypic Plasticity, Clonality, Heterogeneity, Scanning Electron Microscopy, Patches



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## INTRODUCTION

In nature, environmental conditions, especially resources, vary spatially and temporally even at a fine scale. The spatial variations in resources abundance are perceived by organisms as environmental heterogeneity as long as the patches of resources are smaller than the organism and larger than the response unit<sup>1,2</sup>. Plants, because of their sessile lifestyle, have to cope with this heterogeneity and have evolved complex and diverse buffering mechanisms, such as phenotypic plasticity (i.e. production of different phenotypes from a single genotype<sup>3</sup>). Phenotypic plasticity improves the plant's ability to respond to resource heterogeneity during its lifetime by allowing trait adjustment to current environmental conditions<sup>4,5,6,7</sup>. Plasticity is expressed at different modular levels in plants<sup>8</sup> ranging from first order modules such as leaf or root to a superior modular level such as the ramet (see Harper, 1977 for modular structure description<sup>9</sup>). This plastic response results from a trade-off between environment exploration for a resource (e.g. foraging for nutrient-rich patches) and resources exploitation (e.g. uptake of the resource and establishment in the patches).

In clonal plants, each individual consists of a set of ramets connected through belowground (i.e. rhizomes) or aboveground horizontal modified stems (i.e. stolons). These connections result in a network structure and promote plant propagation in space (i.e. physical integration). In some species they also allow sharing of information and resources within the physical clone (i.e. physiological integration<sup>10</sup>). As a result of this network architecture, clonal individuals experience spatial heterogeneity at centimetric scales. They also share information about this environmental signaling between ramets. This leads to plastic responses at the local scale to optimize performance, through resource-sharing, at the clone level<sup>11</sup>. The response of clonal individuals to this small-scale heterogeneity results from a resource exploitation-exploration trade-off. Exploration responses are mostly linked to ramet positioning and induce modifications in clonal network architecture to allow foraging for available resources<sup>12,13</sup>. The optimal foraging theory predicts that ramets should maximize resource acquisition by aggregating in rich patches and avoiding poor patches<sup>12,14,15,16</sup>. Such aggregation may be achieved through modifications of the horizontal architecture of clonal plants, such as internode shortening or increased branching<sup>12,17,18</sup>. Exploitative responses involve (changes in resource acquisition traits. As a result of physiological integration, each ramet may specialize in acquiring the most abundant resource (division of labor theory<sup>19</sup>) and share it throughout the network. This specialization can involve modifications in ramet resource allocation patterns<sup>20,21</sup> whereby a higher root/shoot ratio is observed in ramets developing in nutrient-rich patches, and a lower ratio in light-rich patches<sup>20,22</sup>.

Clonal foraging and ramet specialization have been demonstrated in response to soil nutrient heterogeneity<sup>22,23,24,25</sup>. However, under natural conditions, plant-nutrients uptake is mostly mediated by symbiotic micro-organisms such as Arbuscular Mycorrhizal (AM) fungi which colonize ~80% of terrestrial

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plants<sup>26</sup>. AM fungi symbionts (i.e. Glomeromycota) colonize roots and develop a dense hyphal network exploring soil to 'harvest' mineral nutrients for the plant's benefit<sup>26</sup>. Plants with mycorrhizal roots can thus attain higher rates of phosphorus and nitrogen absorption than plants with non-mycorrhizal roots<sup>27,28</sup>. In turn, AM fungi obtain from plants, the carbohydrates required for their survival and growth<sup>29,30</sup>. In natural conditions, plant roots are colonized by a complex community of AM fungi<sup>31</sup>. These fungi display different levels of cooperation ranging from good mutualists to more selfish ones (i.e. cheaters<sup>32</sup>). Within the root-colonizing fungal assemblage, plants have been shown to preferentially allocate carbon toward the best cooperators, thereby favoring their maintenance over cheaters<sup>33</sup>. The additive nutrient supply provided by AM fungi can be assimilated as a resource for the plant. An important prediction is that plants may respond to the heterogeneous presence of AM fungi as for a nutritive resource. The plant could thus forage (optimal foraging theory) or specialize (division of labor theory) in response to AM fungi presence. The opposite hypothesis is that AM fungi and foraging or specialization are alternatives to cope with resource heterogeneity implying that plants with clonal mobility do not rely on AM fungi to respond to the heterogeneity.

Our aim in this study was to analyze a plant's plastic response to AM fungal heterogeneity by performing two experiments under controlled conditions with the clonal herb *Glechoma hederacea*. In the first experiment, we tested the plant's foraging and specialization response to the heterogeneous distribution of AM fungi. The treatments consisted of a mixture of three species of AM fungi that has been shown to display various cooperativeness in precedent studies. Two assumptions were tested: (i) according to the optimal foraging theory, clones should aggregate ramets in the patches containing AM fungi by reducing their internodes lengths and (ii) according to the division of labour theory, clones should specialize ramets with a higher allocation to roots in the presence of AM fungi than in their absence. To better understand the results obtained in experiment 1 and because of the potential impact of different cooperation levels in fungi involved in this symbiosis, we carried out a second experiment to test the effect of AM fungal identity on the foraging and specialization response of *G. hederacea*. We tested i) the effect on plant traits of the individual presence of the three different species of AM fungi used in the assemblage treatment and ii) the assumption that AM fungal species differ in their effects on the traits involved in foraging and specialization responses. In both experiments, the performance of clonal individuals was expected to be reduced in the absence of AM fungi.

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## METHODS

### Biological material

We used the clonal, perennial herb *Glechoma hederacea* which is a common Lamiaceae in woods and grasslands. *G. hederacea* clones produce new erect shoots at the nodes at regular intervals of 5 to 10 cm on plagiotropic monopodial stolons (i.e. aboveground connections). Each ramet consists of a node with two leaves, a root system and two axillary buds. In climatic chambers with constant conditions, *G. hederacea* does not flower and displays only vegetative growth<sup>12</sup>. This species is known to exhibit foraging behavior<sup>12,22,45</sup> and organ specialization<sup>22</sup> in response to nutrients or light heterogeneity. The ramets used in our experiments were obtained from the vegetative multiplication of 10 clonal fragments taken in 10 different locations sufficiently spaced to obtain different genotypes. Plants were cultivated for three months under controlled conditions to avoid parental effects linked with their original habitats<sup>51</sup>. Vegetative multiplication was carried out on a sterilized substrate (50% sand and 50% vermiculite, autoclaved at 120°C for 20 minutes) to ensure the absence of AM fungi propagules. For each experiment, the transplanted clonal unit consisted of a mature ramet (leaves and axillary buds) with one connective internode (to provide resources to support ramet survival)<sup>52</sup>, and without roots (to avoid prior mycorrhization). The AM fungi inocula used in both experiments were *Glomus* species: *Glomus intraradices* (see Stockinger et al., 2009 for discussion on *G. intraradices* reclassification<sup>53</sup>), *Glomus custos*, and *Glomus clarum*. These AM species were chosen to limit phylogenetic differences between fungi life-history traits<sup>54</sup>. *G. intraradices* has been shown to display beneficial P uptake in *Medicago truncatula*<sup>33</sup>. The use of three different AM species also ensure a range of cooperativeness in the symbionts. The inocula used in the two experiments consisted of a single-species inoculum produced in in vitro root cultures (provided by S.L. Biotechnologia Ecologica, Granada, Spain) or a mixture of equal proportions of all three inocula. The inoculations consisted of an injection of 1mL of inoculum directly above the roots, and were administered when the plants had roots of 0.5 to 1 cm in length.

### Experimental conditions

Experiment 1 was designed to test the foraging and specialization responses of *G. hederacea* to the heterogeneous distribution of AM fungi. Experiment 2 tested the effect of the species of AM fungus on the plant traits involved in these responses.

Both experiments were carried out with cultures grown on the same sterile substrate (50% sand, 50% vermiculite) in a climate-controlled chamber with a diurnal cycle of day /12h night at 20°C. Plants were watered with deionized water every two days to check for nutrient availability. Necessary nutrients were supplied by watering the plants every 10 days using a fertilizing Hoagland's solution with strongly reduced

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phosphorus content to ensure ideal conditions for mycorrhization (i.e. phosphorus stress)<sup>55,56,57</sup>. At each watering, the volumes of deionized water and fertilizing solution per pot were 25 mL and 250 mL respectively for the first and second experiments. We also controlled nutrient accumulation during the experimental period by using pierced pots that allowed evacuation of the excess watering solution. To prevent nutrient enrichment due to the inoculum, AM fungi-free pots were also inoculated with a sterilized inoculum (autoclaved at 100°C for five minutes).

Experiment 1: Effect of heterogeneous AM fungal distribution on *G. hederacea* foraging and specialization responses.

The responses of *G. hederacea* to four different spatial distributions of AM fungi were tested. *G. hederacea* was grown in series of 11 consecutive pots: two homogeneous treatments with the presence (P) or absence (A) of AM fungi in all pots; and two heterogeneous treatments with two patches of 5 pots either in presence then absence (PA) or absence then presence (AP) (Fig. 1). The two latter treatments were done to take into account a potential effect of ramet age in the plant's response to heterogeneity. These treatments were replicated for 10 clones of *Glechoma hederacea* (see Methods section 'Biological material' for precision on plants used). Each clone was grown in plastic pots (8 B 8 B 7 cm<sup>3</sup>) filled with sterile substrate. Only one ramet was allowed to root in each pot and plant growth was oriented in a line by removing lateral ramifications. The initial ramet, in all treatments, was planted in a pot without AM fungi. For each treatment, the inoculum consisted of a mixture of the three AM fungal species (*G. clarum*, *G. custos* and *G. intraradices*). Inoculations were started on the second pot of each line which actually contained the fourth ramet of the clone (exceptionally, the first three ramets rooted in the same first pot due to internode shortness, see Fig. 1). Inoculations were administered for each ramet separately when the ramet had roots of 0.5 to 1 cm in length to avoid a ramet age effect on the AM fungi colonization process.

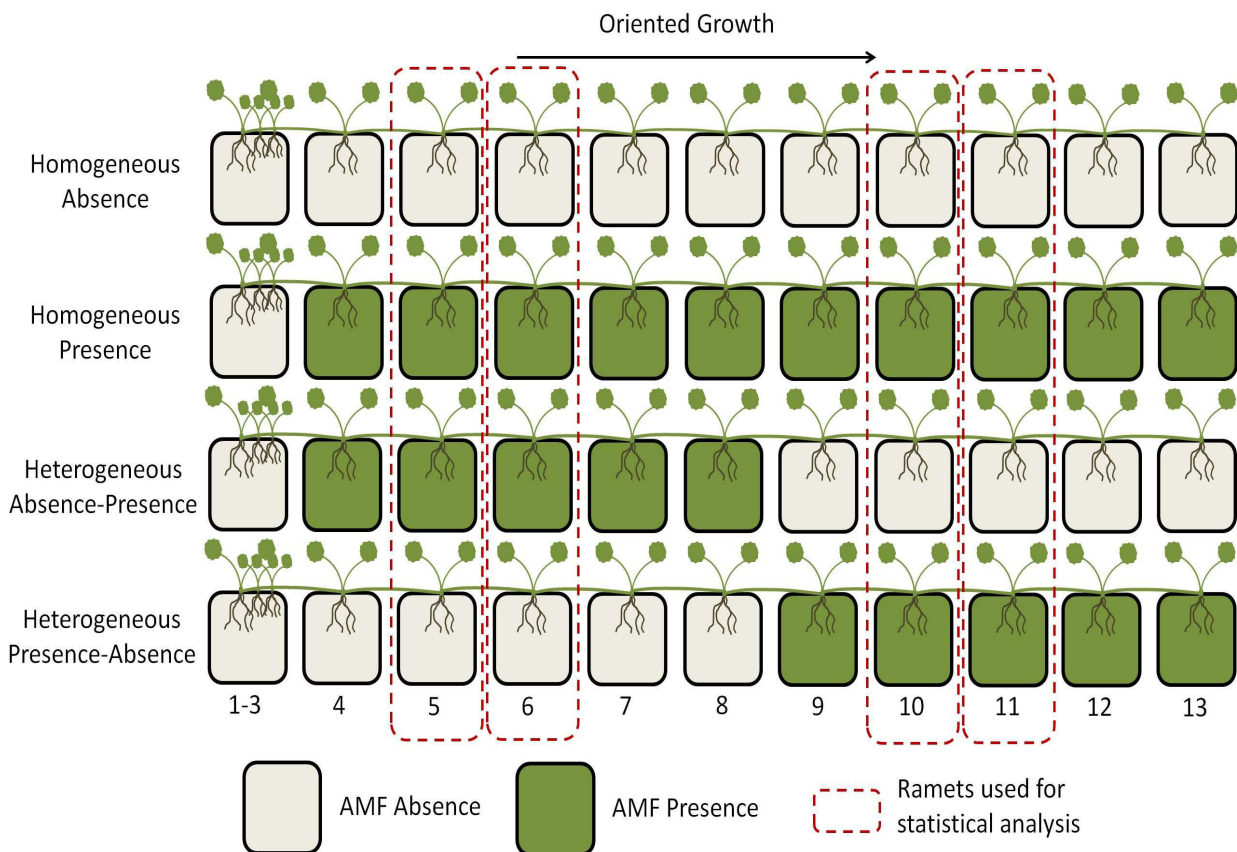


Fig.-1: Schematic drawing of the experimental design composed of pots arranged in lines. Ramets were forced to root in different pots and lateral ramifications were removed to orient growth in a line. Four treatments of AM fungal distribution were applied based on the presence or absence of AM fungi in the pots: Absence (A) (10 pots without AM fungi); Presence (P) (10 pots with AM fungi); Presence-Absence (PA) (five pots with AM fungi followed by five pots without AM fungi); Absence-Presence (AP) (five pots without AM fungi followed by five pots with AM fungi).

The clones were harvested when the final ramet (number 13) had rooted in the 11th pot. This ensured that each clone had 10 points for sampling environmental quality. The 5th, 6th, 10th and 11th ramets of each clone in the pot line (Fig. 1) were used for statistical analyses. These ramets corresponded to the second and third ramets experiencing the current patch quality. Indeed, Loupre et al. (2012) emphasized the role of the 'past experience' of the clone in developing a plastic response. The choice of these four ramets thus ensured that the clone had enough sampling points to assess the quality of its habitat i.e. in the patches where AM fungi were present or absent, in the heterogeneous treatments, and to adjust accordingly when initiating new ramets<sup>35</sup>. Each ramet was carefully washed after harvesting. The foraging response was assessed by measuring the length of the internode just after the ramet. An aggregation of ramets, with shortened internodes was expected in patches where AM fungi were present and an avoidance of patches, i.e. production of longer internodes, was expected where AM fungi were absent. Modifications in ramification

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production linked to the effect of the treatment were checked by recording the number of ramifications produced by the ramets throughout the experiment. The specialization response was examined by measuring the root/shoot ratio (R/S) i.e. the biomass allocated to the below- and above-ground resource acquisition systems, after separating the roots and shoots and after oven-drying for 72h at 65°C. We expected a higher R/S ratio in patches where AM fungi were present than in patches where AM fungi were absent. Clone performance was assessed from (i) the total biomass of the clone, calculated as the sum of ramet roots, shoots and stolons after oven-drying for 72h at 65°C and (ii) the growth rate calculated as the number of days needed for the clone to develop the 10 sampling ramets i.e. the number of days between rooting of the 4th ramet and final harvesting.

Experiment 2: Effect of AM fungal identity on *G. hederacea* performance and traits.

The effects of individual AM fungal species on *G. hederacea* foraging and specialization traits were tested using four culture treatments: 1) no AM fungi, 2) with *Glomus custos*, 3) with *Glomus intraradices*, and 4) with *Glomus clarum*. Each treatment was replicated eight times with four related ramets assigned to each treatment replicate (32 clones in total), to control for plant-genotype effects. The initial ramet of each clone had previously been cultivated on sterile substrate to ensure root system development and facilitate survival after transplanting. The initial ramets were then transplanted in pots (27.5 B 12 B 35 cm<sup>3</sup>) filled with substrate. The AM fungi inoculations consisted of three injections of 1 mL of inoculum directly on the roots of the first three rooted ramets to ensure colonization of the whole pot. The plants were harvested after six weeks. The following traits involved in foraging were measured: (i) the longest primary stolon length (of order 1) as an indicator of the maximum spreading distance of space colonization (ii) the number of ramifications as an indicator of lateral spreading and clone densification. We also measured biomass allocation to the roots, shoots and stolons at the clone level, i.e. traits involved in the specialization response, after oven-drying for 72h at 65°C. Plant performance for the entire clone was determined from: (i) the total biomass calculated as the sum of the dry weights of the shoots, roots and stolons after oven-drying for 72h at 65°C. and (ii) the number of ramets i.e. the number of potential descendants. Performance was expected to be higher in pots inoculated with fungi and to differ depending on the fungal species.

#### Statistical analysis

For experiment 1, to test whether *G. hederacea* develops a plastic foraging (internode length) or specialization (R/S ratio) response to the heterogeneous distribution of AM fungi, ANOVA analyses were performed using the linear mixed-effects model procedure in R 3.1.3<sup>58</sup> with packages "nlme"<sup>59</sup> and "car"<sup>60</sup>. Ramets of the same age were compared between genotypes to control for a possible effect of ramet age.

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For experiment 2, to determine whether the species of AM fungi induced changes in plant traits and performance, ANOVA analyses were performed using linear mixed models with the same R packages and version described above. *W* Resource allocation was tested by using the clone total biomass as covariate to take into account the trait variance associated with clone growth.

For both experiments genotype-induced variance and data dependency was controlled by considering the treatment (four modalities) as a fixed factor and the plant-clone genotype as a random factor. The effect of genotype was assessed by comparing the intra- and inter-genotype variance and was considered significant when the inter-genotype variance was strictly superior to the intra-genotype variance. When a significant effect of treatment was detected by ANOVA, post hoc contrast tests were performed using the "doBy" package<sup>61</sup> to test for significant differences between modalities. When necessary, the normality of the residuals was ensured by subjecting the data to log transformation. The total clone biomass (summed dry weights of shoots, roots, and stolons) was used as covariate to account for variance due to differences in clone performance.

## RESULTS

In both experiments *G. hederacea* traits variation was not significantly influenced by plant genotype (i.e. the inter-genotypic variance was not greater than the intra-genotypic variance).

Experiment 1: Effect of heterogeneous AM fungi distribution on *G. hederacea* foraging and specialization responses.

The hypothesis of modified foraging and specialization responses of *Glechoma hederacea* to the patchiness of AM fungal presence was tested by comparing the internode lengths and the R/S ratio between the treatments for the 5<sup>th</sup>, 6<sup>th</sup>, 10<sup>th</sup> and 11<sup>th</sup> ramets (see Methods for details on ramet selection and experimental design).

We found a significant effect of the AM fungal treatment on the 10<sup>th</sup> internode length ( $P=0.005$ ;  $F=5.74$ ) (Tab. 1, Fig. 2) with a longer internode in the PA treatment (AM fungi present then absent) than in the absence (A) and presence (P) treatments. Conversely, no significant effect was found for the 5<sup>th</sup> ramets ( $P=0.71$ ;  $F=0.45$ ) and 6<sup>th</sup> ramets ( $P=0.15$ ;  $F=1.92$ ) (Fig. 2). The 11<sup>th</sup> ramets seem to display the same response patterns as the 10<sup>th</sup> ramets, but no significant differences were detected between the treatments ( $P=0.93$ ;  $F=0.15$ ), due to a partial bimodal distribution of data in the "P" treatment with a few individuals exhibiting longer stolon. In addition, the number of ramifications for the 5<sup>th</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 11<sup>th</sup> ramets was not

significantly affected by treatment (Tab. 1). No changes in the R/S ratio in response to AM fungal treatment were detected in any of the four tested ramets (Tab.1).

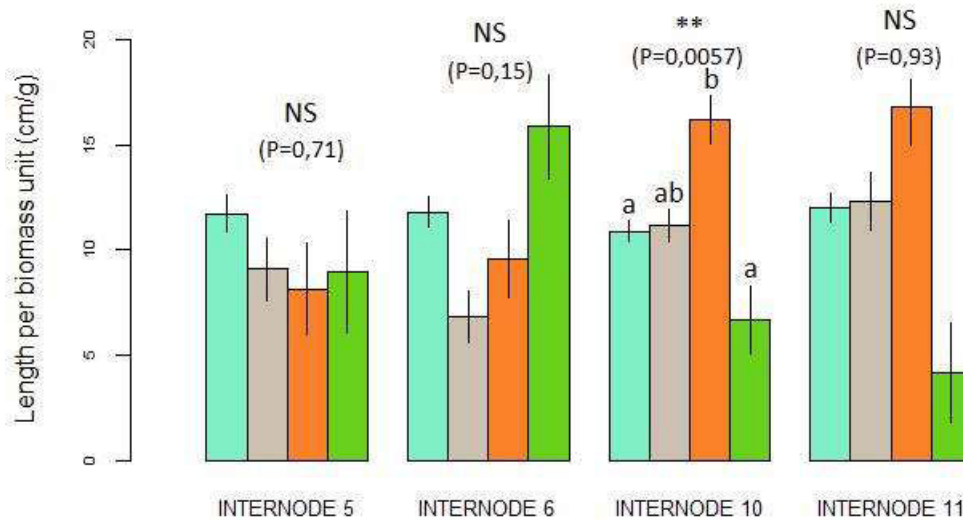


Fig.-2: Foraging response: internode length under the four treatments applied (cm per gram of ramet total biomass) (A). Specialization response: root:shoot ratio (R/S) of 5<sup>th</sup>, 6<sup>th</sup>, 10<sup>th</sup> and 11<sup>th</sup> ramets under the four applied treatments (g of roots per g of shoots after drying) (B). A absence (blue bars), Presence (grey bars), Presence-Absence (orange bars), Absence-Presence (green bars). Statistical significance of the internode length or R/S variations between treatments: NS, not significant; \*\*, P<0.01.

As regards performance, *G. hederacea* growth rate tended to vary with the AM fungal treatment (P=0.067; F=2.7) (Tab. 1), with a tendency of slower growth in the "A" treatment. No differences between treatments were detected for clone total biomass (P=0.75; F=0.39; Tab.1) indicating no difference in biomass production or performance, for the clone as a whole.



Table.-1: Results of linear models for each traits linked to the plants foraging, specialization and performance.

Trait	Treatment		Total biomass		Random factor (Genotype)	
	F-value	P-value ( =0.05)	F-value	P-value ( =0.05)	Intra : lower/estimate/upper	Inter : lower/estimate/upper
Total Biomass	0.39	0.75	-	-	0.72 / 0.95 / 1.25	0.43 / 0.81 / 1.53
Growth time	2.7	0.067	-	-	3.98 / 5.24 / 6.89	0.37 / 1.79 / 8.53
Σ <sup>Dp</sup> III↑ III×T ½ IIε ↑D <sub>p</sub>	0.45	0.71	1.58	0.22	1.19 / 1.61 / 2.18	0.75 / 1.45 / 2.8
Σ <sup>Dp</sup> III↑ III×T ½ IIε ↑D <sub>p</sub>	1.92	0.15	8.32	<0.01	1.03 / 1.4 / 1.9	0.34 / 0.83 / 2.05
Σ <sup>Dp</sup> III↑ III×T ½ IIε ↑D <sub>p</sub>	5.74	<0.01	4.38	<0.05	0.59 / 0.81 / 1.12	0.54 / 0.97 / 1.74
11 <sup>th</sup> internode length	0.15	0.93	0.02	0.87	0.96 / 1.34 / 1.86	0.41 / 0.94 / 2.17
5 <sup>th</sup> ramet root/s hoot	0.48	0.69	-	-	n/a	n/a
6 <sup>th</sup> ramet root/s hoot	0.18	0.9	-	-	n/a	n/a
10 <sup>th</sup> ramet root/s hoot	1.09	0.37	-	-	n/a	n/a
11 <sup>th</sup> ramet root/s hoot	0.46	0.7	-	-	n/a	n/a
5 <sup>th</sup> ramet number of ramifications	1.1	0.36	14.49	<0.01	0.99 / 1.31 / 1.7	0.38 / 0.83 / 1.8
6 <sup>th</sup> ramet number of ramifications	0.46	0.7	5.2	<0.01	1.06 / 1.40 / 1.85	0.81 / 1.46 / 2.64
10 <sup>th</sup> ramet number of ramifications	0.26	0.84	1.91	0.18	0.89 / 1.18 / 1.55	0.36 / 0.77 / 1.66
11 <sup>th</sup> ramet number of ramifications	0.88	0.46	1.08	0.3	0.89 / 1.18 / 1.56	0.22 / 0.59 / 1.63

F-values and P-values of the treatment and total biomass (when used as covariable) are presented, as well as lower, estimate and upper values of intra and inter genotype variance (random factor).

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Experiment 2: Effect of AM fungi identity on *G. hederacea* traits.

The hypothesis that modifications in *G. hederacea* foraging and specialization traits were affected by the AM fungal species was tested by comparing the allocation, architectural and growth traits of four treatments inoculated with different AM fungal species (see Methods for details on experimental design). Primary stolon length (an architectural trait) tended to vary ( $P=0.07$ ;  $F=2.83$ ) in response to the presence and species of AM fungi whereas the number of ramifications ( $P=0.25$ ;  $F=1.49$ ) did not (Tab. 2). Allocation to stolons was significantly affected by the presence and species of AM fungi ( $P=0.017$ ;  $F=4.51$ ) with plants inoculated with *Glomus intraradices* allocating significantly fewer resources to stolons (Fig. 3) and more to shoots ( $P=0.019$ ,  $F=4.24$ ) than plants without AM fungi. The allocation to roots, however, was not dependent on the treatment ( $P=0.68$ ;  $F=0.50$ ).

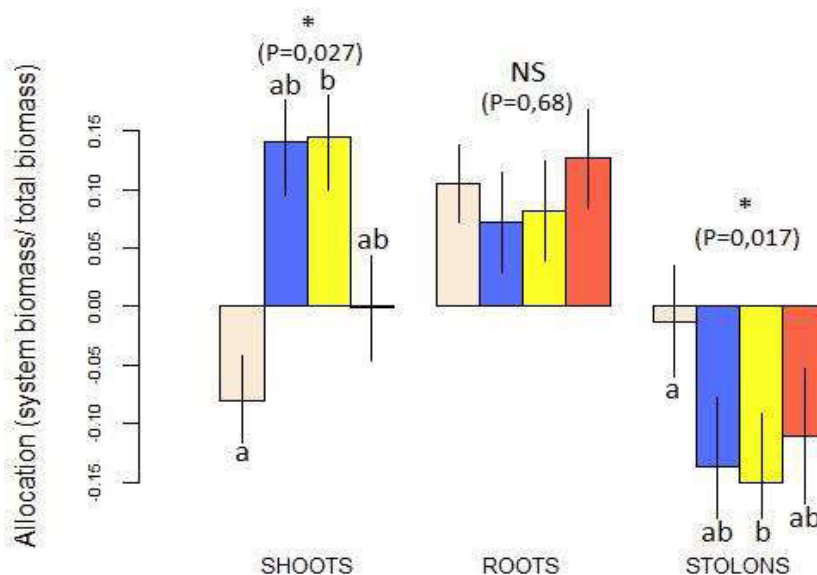


Fig.-3: Allocation traits of the whole clone for the four treatment of AM fungi inoculation: T1= no AM fungi (white bars), T2= *Glomus custos* (blue bars), T3= *Glomus intraradices* (yellow bars), T4= *Glomus clarum* (red bars). Means of each organ (shoots, roots and stolons) biomass in grams per gram of total clone biomass. Statistical significance of the organ biomass variations between treatments: NS, not significant; \*,  $P<0.05$ .

As regards performance, changes in ramet production per biomass unit ( $P=0.038$ ;  $F=3.55$ ) were detected with *G. intraradices* inducing less ramet production than *G. custos* whereas the treatments without AM fungi and with *G. clarum* did not differ significantly from the other two treatments (Fig. 4). No treatment-dependent change in total biomass was observed ( $P=0.57$ ;  $F=0.67$ ).

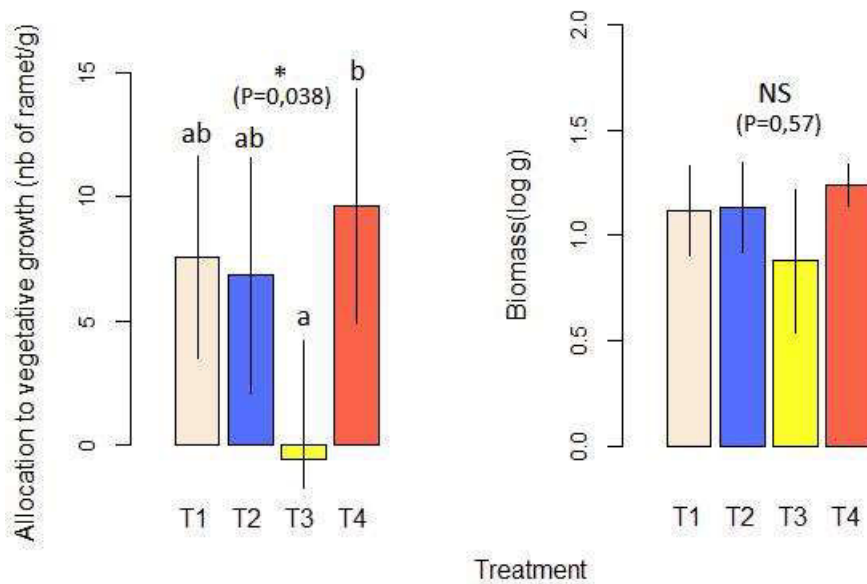


Fig.-4: Performance traits of the clone for the four treatments of AM fungi inoculation: T1= no AM fungi (white bars), T2= *Glomus custos* (blue bars), T3= *Glomus intraradices* (yellow bars), T4= *Glomus clarum* (red bars). Total clone biomass in grams after drying (A). Number of ramets per gram of total clone biomass (B). Statistical significance of the total biomass and number of ramets variations between treatments: NS, not significant; \*,  $P < 0.05$ .

Table.-2: Results of linear models for each traits linked to the plants resources allocation and performance.

Trait	Treatment		Total biomass		Random factor (Genotype)	
	F-value	P-value ( =0.05)	F-value	P-value ( =0.05)	Intra : lower/estimate/upper	Inter : lower/estimate/upper
Total Biomass	0.67	0.57	-	-	0.27 / 0.38 / 0.52	0.03 / 0.14 / 0.67
Number of ramets (allocation)	3.55	<0.05	46.6	<0.001	5.97 / 8.45 / 11.96	7.58 / 13.7 / 24.8
Primary stolon length	2.84	0.07	1.99	0.17	10.99 / 15.45 / 21.75	4.53 / 10.69 / 25.23
Number of ramifications	1.49	0.25	5.8	<0.05	0.46 / 0.66 / 0.93	0.24 / 0.53 / 1.18
Stolons weight (allocation)	4.51	<0.05	91.37	<0.001	0.08 / 0.11 / 0.17	0.03 / 0.09 / 0.22
Shoots weight (allocation)	3.96	<0.05	1528	<0.001	0.06 / 0.09 / 0.13	0.04 / 0.08 / 0.18
Roots weight (allocation)	0.5	0.68	30.72	<0.001	0.06 / 0.09 / 0.12	0.006 / 0.03 / 0.19

F-values and P-values of the treatment and total biomass (when used as covariable) are presented, as well as lower, estimate and upper values of intra and inter genotype variance (random factor).

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## DISCUSSION

The plants did display some foraging behavior in response to AM fungi heterogeneity, as elongation of the internodes was observed in patches without AM fungi after the plant had experienced patches with AM fungi. This behavior would correspond to an avoidance of resource-poor patches, as expected from the optimal foraging theory. However, this behavior was only detected at a particular ramet age (10<sup>th</sup> ramets), indicating a possible role of the ontogenic state in development of the plastic response<sup>34</sup>. This may be due to a 'lag time' in the plant's response based on the need for environmental sampling. Indeed, Loupre et al., (2012) demonstrated that clonal plants may need a minimum number of sampling points as benchmarks in order to perceive and respond to resource availability<sup>35</sup>. In their study, *Potentilla reptans* and *P. anserina* started to respond to the treatment after the 5<sup>th</sup> internode, suggesting a strong effect of patch size. A similar patch size effect had already been demonstrated in modeling studies<sup>10,36</sup>. No plastic modifications corresponding to a ramet specialization of *G. hederacea*, in response to AM fungal spatial heterogeneity, were found either. Contrary to the results expected with the specialization theory, biomass was not preferentially allocated to the roots in patches with AM fungi or to the shoots in patches without AM fungi. This absence of response was recorded for all the ramet ages tested.

These results – a mild foraging response and no specialization – give credit to the theory supported by Orntchenko & Zobel (2000) that the species with high mobility do not rely on AM fungi to cope with resource heterogeneity<sup>37</sup>. *Glechoma* with its high clonal mobility should thus show no response to AM fungi response. However, our results do not fit with the literature predictions for specialization and foraging response<sup>38</sup>. This divergence may be explained by two alternative hypotheses that are developed in the following sections. The first explanation is linked with the occurrence of an individual effect of the species of AM fungus on plant traits, which may predominate or modify the response to the presence/absence of AM fungi when all three species exist together (experiment 2); the second is linked with reduced physiological integration either due to a direct effect of AM fungi on this plant trait, or to the absence of a clear contrast between the different patches sensed by the plant.

In our second experiment, we demonstrated that the architectural traits involved in the plant's foraging response were not affected by the species of AM fungi tested, consistently with the weak response detected in the first experiment. On the contrary, significant changes in resource allocation traits (linked to the specialization response) were detected, depending on the species of AM fungus. Only one species, *G. intraradices* induced a change in allocation by the plant, in comparison to the absence of AM fungi treatment, which led to an increased allocation to shoots at the expense of stolons. Modifications of plant phenotype, depending on the AM fungal species, have already been observed in such traits<sup>39,40</sup>. These authors identified a significant effect of *Glomus* species isolates on branching, stolon length and ramet production in

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*Prunella vulgaris* and *Prunella grandiflora*. In the first analysis of the AM fungal genome, Tisserant et al. (2013) revealed existing pathways attributed to the synthesis of phytohormones or analogues<sup>41</sup>. Such molecules would have a direct effect on host phenotype. In the individual effect observed, plant response in the presence of *G. intraradices* symbiosis was coupled with decreased plant performance due to a diminution of ramet production relative to biomass in this treatment. In contrast, the *G. custos* treatment led to a decrease in the potential number of descendants of the clone. According to experiment 1, root colonization by an inoculum containing three species had no effect on plant traits associated with specialization and foraging. This suggests two alternative hypotheses: i) *G. intraradices* may be less cooperative than *G. custos* with *Glechoma hederacea* and the result is a consequence of the plant's rewarding process to the more cooperative fungus<sup>33</sup> and/or ii) root colonization by *G. custos* or *G. clarum* buffers the effect of *G. intraradices* due to a 'priority effect' (i.e. order of arrival in the colonization as a key to fungal community structure in roots)<sup>41</sup>.

To test this, the mycorrhization intensity of the three AM fungal species inoculated in the first experiment would need to be assessed by qPCR. Alternatively, the combined effects of the three AM fungal species on plant phenotype might result in the environment not being perceived as heterogeneous by the plant. This hypothesis is developed in the following section.

The intracolonial plasticity predicted by the foraging and division of labor theories is based on the ability of ramets to sense environmental heterogeneity, share information and resources within the clonal network, to locally adapt and optimize the performance of the whole clone. The weak response of *G. hederacea* to AM fungal heterogeneity could thus be explained by a decrease in physiological integration that reduces the level of resource-sharing within the clone and prevents the plant from developing an optimized foraging or specialization response. This diminution could initially be due to the presence of AM fungi. Only a few studies have been carried out on the effect of AM fungi on the degree of integration<sup>43</sup>. These authors demonstrated that AM fungi induced a decrease of physiological integration in the clonal plant *Trifolium repens* when grown in a heterogeneous environment. This effect was dependent on the presence and richness of AM fungal species. Whether this observed diminution of physiological integration is due to a direct manipulation of the host plant phenotype by the fungi remains, as far as we know, unknown. Secondly, this diminution may depend on the individual plant's perception of environmental conditions that might be sensed as homogeneous because the patch contrast is less important than expected. A reduction of plant integration is expected when the maintenance of high physiological integration is more costly than beneficial<sup>44,45</sup>, such as when the environment is resource-rich, not spatially variable<sup>46</sup> or not sufficiently contrasted<sup>10,47</sup>. Such a reduced contrast might result from the effect of the three AM fungal species on the plant phenotype (when used as a mixed inoculum), which is unlikely. A more probable mechanism of

environment homogenization could result from AM fungal transfer through the stolons. Scanning electron microscopy of the clone cultures (see protocol in supplementary material) revealed the presence of hyphae on the stolon surface (fig. 5). In addition, several cells close to the external surface of the stolon cross-section were invaded by structures which could be interpreted as fungi. DNA sequencing of stolon samples (fig. 6) confirmed these results and demonstrated the presence of AM fungi in stolons. This suggest that fungi can be transferred from one ramet to another, at least by colonization of the stolon surface (as shown in Fig. 5a) and/or within the stolon (Fig. 5b). Whether fungi are passively or actively transferred through the plant's stolon tissues, and hence to all related ramets, remains an open question. Further studies are therefore needed to confirm these fungal transfers to plant clones and to measure their intensities in contrasted environments.

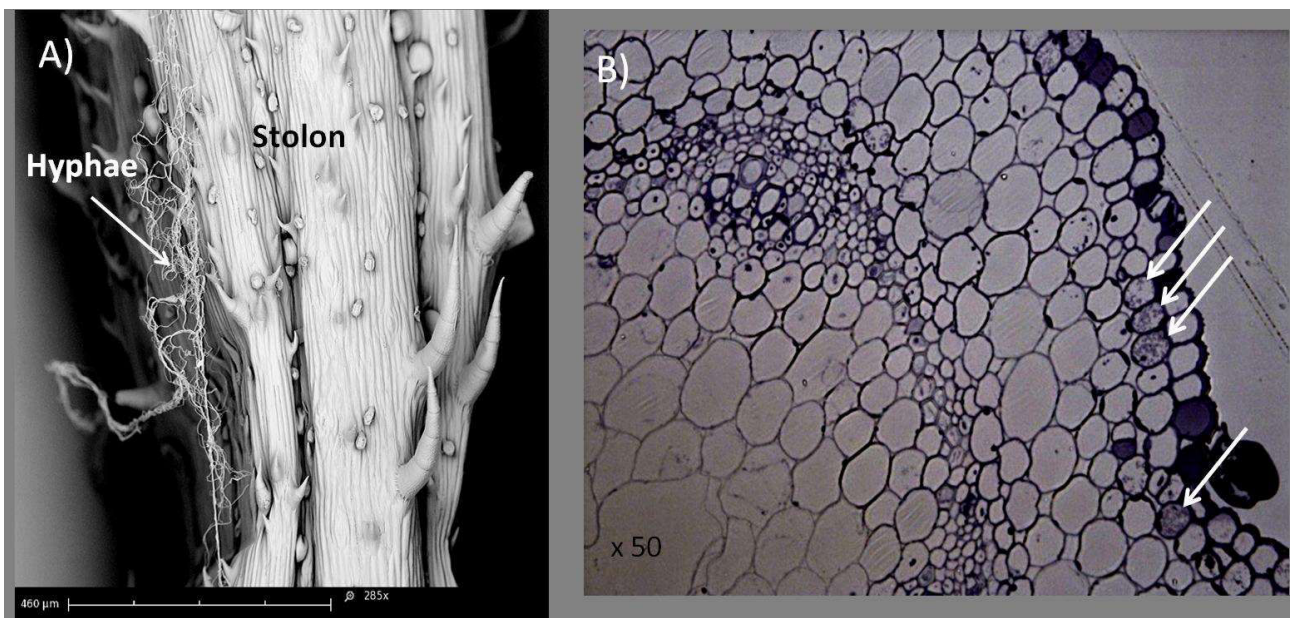


Fig.-5: Maximum likelihood tree of the GTR +I+G model using PhyML. Multiple alignment has been produced with MUSCLE<sup>62</sup>. Bootstrap values at the nodes were produced from 200 replicates. Only values above 50 are shown. Multiple alignment and tree reconstruction were performed using SEAVIEW<sup>63</sup>. OTUs have been obtained from a *Glechoma hederacea* stolon after DNA extraction using DNEasy plant mini kit (Qiagen), PCR amplification using fungal primers NS22b and SSU817, and Illumina MiSeq sequencing. In addition to reference sequences within the Glomeromycota phylum, we sampled 13 sequences among the best BLAST hits (尾).

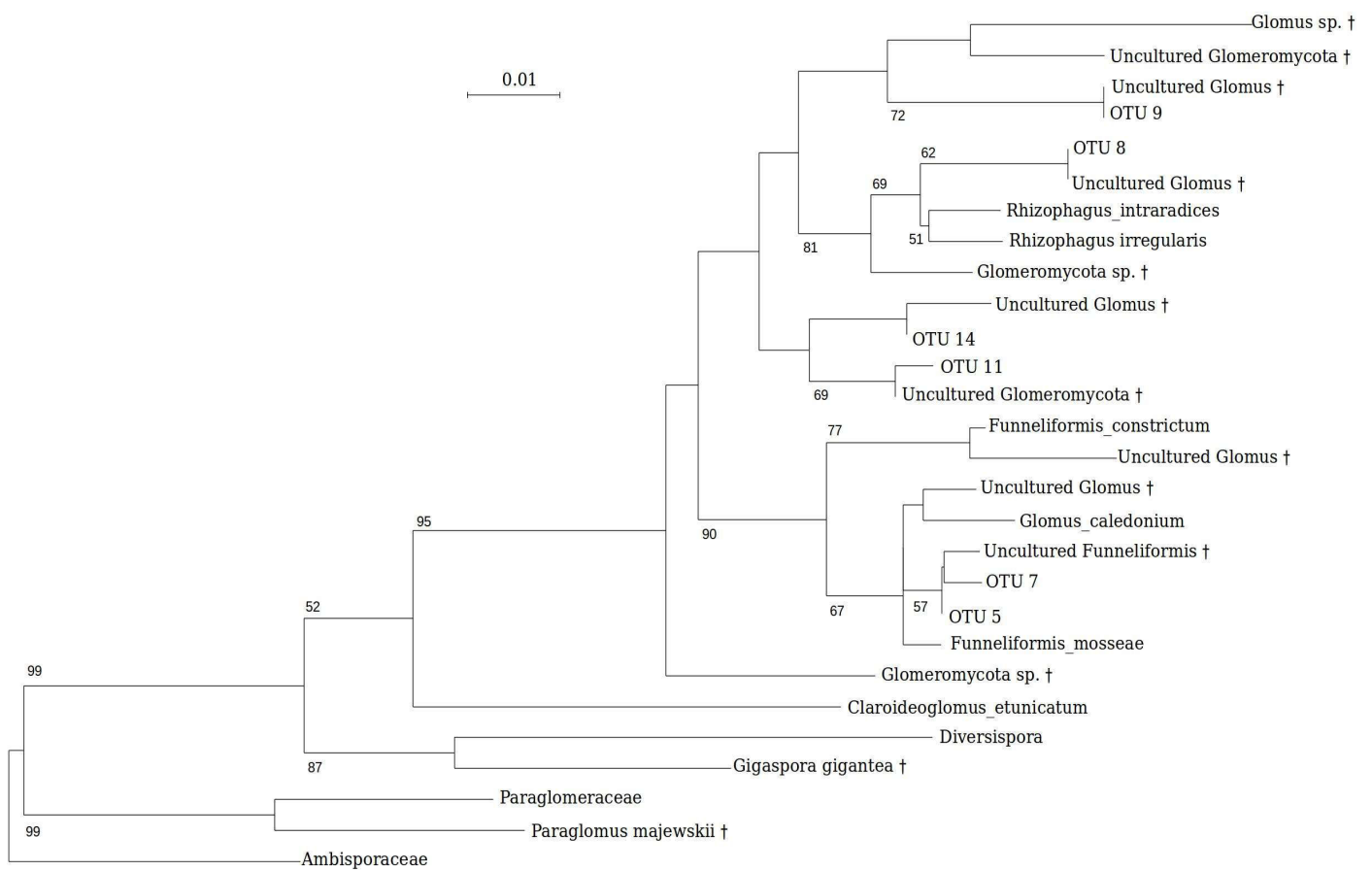


Fig.-6: Results for the microscopy analysis of stolons harvested from *G. hederacea* pre-cultures. Scanning electron microscopy of the stolon surface showing hyphae attached to the stolon hairs (A). Stolon microscopy cross-section observed with an optical microscope. Arrows indicate cortical cells invaded by structures which may be interpreted as fungi (B).



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Studies of the response of clonal plants to environmental heterogeneity have classically focused on abiotic heterogeneity<sup>48,49</sup>. Our study is the first to investigate clonal response to a heterogeneous distribution of AM fungi, based on the assumption that AM fungi can be regarded as a resource for the plant. However, in response to the heterogeneous distribution of AM fungi, *G. hederacea* clones displayed only a weak foraging response and no specialization, which suggests respectively that clones do not aggregate more especially in patches with AM fungi or maximize their proportion of roots in contact with AM fungi. We provide a first explanation by highlighting the impact of AM fungal identity on the plant phenotypes and more particularly on the allocation traits involved in specialization. More importantly, we provide evidence that stolons might be vectors for the transfer of micro-organisms between ramets, thereby buffering (through this dispersion of fungi) the initial heterogeneous distribution. If this is true, stolons will have to be regarded in a different way, and be seen as ecological corridors for the dispersion of micro-organisms allowing a continuity of partnership along the clone. Considering the plant as a holobiont<sup>31,50</sup>, this novel view of stolon function is expected to stimulate new ideas and understanding about the heritability of microbiota in clonal plants.

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## Author contribution

NV, AKB, PV and CM conceived the ideas and experimental design. NV, AKB, CM did the experiments. NV did the data analyses. NV, AKB, PV and CM did the interpretations and writing of the publication.

## Competing Financial Interests statement

No competing financial interests.

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## Chapter II: The heritability of the plant microbiota, toward the meta-holobiont concept

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### II.1 Introduction

#### Scientific context

The first chapter findings suggested that the elongation of clonal plants stolons is accompanied by the transmission of a "cohort" of microorganisms, that includes arbuscular mycorrhizal fungi, from the mother ramet to spatially distant descendants (i.e. other developing ramets). This chapter aims at experimentally testing this hypothesis and at reviewing its consequences for both plant performance and the conceptual understanding of plants functioning and evolution.

Previous studies have evidenced the existence of vertical inheritance of endophytic symbionts, colonizing host-plants through seeds. The most described example of such transmission is the stress-protective endophyte *Neotyphodium coenoccephalum* that colonizes plant seeds and is transmitted to descendants in several grass species (Clay & Schardl, 2002; Selosse et al., 2004). However, this process is the only known example of true vertical transmission in plants. It represents the transfer of only a few plant-associated microorganism and at a single moment of the plant life. In clonal plant networks, information and resources can be shared within the physical clone (i.e. physiological integration; Oborny et al., 2001). An additional level of integration may then occur through the sharing of microorganisms within the clonal network, as previously proposed by Stuefer et al. (2004) and suggested by our previous results (Article II).

From a theoretical point of view, vertical and pseudo-vertical transmissions (i.e. inheritance of conspecific symbionts from parents to offsprings sharing the same environment; Wilkinson, 1997) are advantageous because they limit the costs of foraging for suitable symbionts (Wilkinson & Sherratt, 2001). In this context, microbiota heritability allows the plant to ensure environmental

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quality for its progeny. Vertical transmission would thus permit a `continuity of partnership\_ between the plant and its symbionts (Zilber-Rosenberg & Rosenberg, 2008). Recently, the understanding of host-symbionts interactions has evolved toward an holistic perception of the host and its associated microorganisms. The holobiont theory provides a theoretical framework for the study of host-symbiont interactions (Zilber-Rosenberg & Rosenberg, 2008; Theis et al., 2016). In the hologenome, a microorganism can be equated to a gene in the genome. Like genes are inherited during sexual reproduction, a key parameter for the evolution of the hologenome is the heritability of microorganisms between host generations. In this context, the mechanism suggested in the previous chapter would integrate clonal plants into the range of organisms that can be considered as holobionts. Furthermore, the network structure of clonal plants suggests another layer of complexity in the holobiont assembly since holobionts would be susceptible to share a part of their microbiota within the clonal network. There is thus a need to develop an extension of the holobiont concept for clonal organisms organized as networks, `the meta-holobiont concept\_.

### Objectives of the chapter

This chapter aims at testing the hypothesis of the heritability of a core microbiota in clonal plants and intends to extend the current theories regarding the holobiont assembly and evolution to clonal plant networks. More precisely we address the following questions :

- 1) Is there a transmission of a cohort of microorganisms in the clonal plant *G. hederacea* through stolon elongation ? Is this cohort composed of specific microorganisms, thus constituting an inherited core microbiota ? (Article III)
- 2) To which extent this mechanisms redefine the holobiont theory for clonal organisms organized in network ? What are the perspectives of clonal plants as model organisms for the study of holobionts assembly ? (Article IV)

### Methods

This chapter is composed of two articles (III and IV). The first paper is based on an experimental approach to demonstrate the existence of a microbiota heritability mechanism in clonal plant. The



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second paper is an opinion paper addressing the consequences of this mechanism on the understanding of holobionts assembly in clonal network.

We tested the hypothesis of microorganisms' transmission to progeny through clonal integration with an experimental approach using the clonal herbaceous species *Glechoma hederacea*. Plants from 10 ecotypes were grown under controlled conditions in individual pots. The mother pot was filled with field soil in to provide an initial microbial inocula and newly emitted ramets were forced to root in separate pots containing sterilized substrate. To detect endophytic microorganisms transferred from mother to daughter ramets, we sampled roots of both mother (growing in pots containing microorganisms) and daughter ramets (growing in sterile substrate without microorganisms) as well as internodes connecting them. High-throughput amplicon sequencing of 16S and 18S rRNA genes was used to detect and identify Bacteria, Archaea and Fungi within the roots endosphere and internodes.

We constructed an opinion paper to address the significance of the above heritability mechanism on our understanding of plants fitness and evolution. In this review we mobilise knowledges on clonal plants network to propose hypotheses on whether they may use microorganisms transfer as a tool for adaptation. We also mobilise knowledges from network theory and meta-community ecology to provide directions for future research on clonal network linked to microbiota assembly and plant fitness.

## Main results

In our experiment (Article III), we detected the presence of archaea, bacteria and fungi within the root endosphere of the mother ramets. Some of these microorganisms were also found within the stolons and the roots of the daughter ramets, comprising fungi and bacteria but not Archaea. We thus demonstrated the heritability of a part of the plant microbiota to its progeny.

In addition, endophytic communities of daughter ramets roots were found to be similar between each other, while they were different from the original mother communities. We thus demonstrated a filtration process during the transmission of the microbiota (decrease in richness and homogenization of the transmitted communities). Our results confirm the hypothesis of microorganism transmission between ramets constituting thus an heritable core microbiota (Article III). Whether the transmission could occur in the reverse sense, i.e. from the daughter to the mother ramets, remains an open question.

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Microbiota transmission to the progeny is advantageous because it provides suitable symbionts and thus ensures habitat quality for the progeny (Wilkinson & Sherratt, 2001). Our results open new questions on the ability of clonal plants to preferentially select and transmit particular sets of microorganisms. Indeed, we observed that microorganisms were filtered during the transmission process but the modalities of this filtration remain unknown. Microorganisms could be filtered based on their ability to colonise the internodes (i.e. dispersal abilities) or alternatively they could be filtered depending on the functions they provide to the mother ramet. In the latter case, it would suggest an active filtering by the plant. The expectation is thus that beneficial organisms such as cooperative AM fungi would be preferentially transmitted to the progeny.

Our results invite to revise our understanding of clonal plants. Especially an extension of the holobiont concept toward the meta-holobiont for clonal plant network has been introduced (Article IV). In addition, we propose that clonal network sharing of microorganisms should be apprehended through the network theory and meta-community frameworks. The network theory framework should provide insights on how the network structure and associated microorganisms sharing could enhance the network resilience and performance as a whole. The meta-community framework should help to understanding the impact of microorganisms transmission on microorganisms communities assembly and survival and on plant management of useful microorganisms.

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## II.2 Article III: A microorganisms journey between plant generations



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## A microorganisms journey between plant generations

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## Abstract

Plants are colonized by a great diversity of symbiotic microorganisms which form a microbiota and perform additional functions for their host. This microbiota can thus be considered a toolbox enabling plants to buffer local environmental changes, with a major influence on plant fitness. In this context, the transmission of the microbiota to the progeny represent a way to ensure habitat quality. However, examples of such transmission are scarce and their importance unclear. We investigated the transmission of symbiotic partners to plant progeny within the clonal network using *Glechoma hederacea* as plant model. We demonstrated the vertical transmission of a significant proportion of the mother's symbiotic Bacteria and Fungi to the daughters and the heritability of a specific core microbiota. In this clonal plant, microorganisms are transmitted between individuals through connections, thereby ensuring the availability of symbiotic partners for the newborn plants as well as the dispersion between hosts for the microorganisms. This previously unknown ecological process allows the dispersal of microorganisms in space and across plant generations. The vast majority of plants are clonal, this process might be therefore a strong driver of ecosystem functioning and assembly of plant and microorganism communities in a wide range of ecosystems.

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## Introduction

All living plants experience interactions with ectospheric and endospheric microorganisms and are known to harbor a great diversity of symbionts including fungi (Vandenkoornhuysen et al., 2002; L, Van et al., 2017), bacteria (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014) and Archaea (Edwards et al., 2015) which collectively form the plant microbiota. This microbiota performs ecological functions that extend the plant's ability to adapt to environmental conditions (Bulgarelli et al., 2013; Vandenkoornhuysen et al., 2015). Studies using maize cultivars demonstrated that genetic control of the composition of the microbial rhizosphere by the host-plant was detectable, even if limited (Peiffer et al., 2013). Plant microbiota composition is thus, at least in part, not only a consequence of the pool of microorganisms available for recruitment in the surrounding soil but also of plant selective recruitment within the endosphere. This filtering system includes plant defense mechanisms (Berendsen et al., 2012; Yamada et al., 2016) and promotion of the best cooperators through a nutrient embargo toward less beneficial fungi (Vandenkoornhuysen et al., 2015; Kiers et al., 2011).

From a theoretical point of view, vertical and pseudo-vertical transmissions (i.e. inheritance of conspecific symbionts from parents to offspring sharing the same environment; Wilkinson et al., 1997) are advantageous because they limit the costs of foraging for suitable symbionts (Wilkinson and Sherratt, 2001). Vertical transmission would thus permit a 'continuity of partnership' between the plant and its symbionts (Zilber-Rosenberg and Rosenberg, 2008). In this context, microbiota heritability is also a way for the plant to ensure environmental quality for its progeny. In natura, plants can reproduce either by seed production or by clonal multiplication (van Groenendael and de Kroon, 1990; Klimek et al., 1997). Some studies have evidenced a vertical inheritance of endophytic symbionts colonizing host-plants through the seeds: the most well-known example is perhaps the transmission of the stress-protective endophyte *Neotyphodium coenoccephalum* to the descendants in several grass plant species (Clay and Schardl, 2002; Selosse et al., 2004).

Recent findings suggest that vegetative elongation of the horizontal stems forming the clonal plants network is accompanied by the transmission of a 'cohort' of microorganisms, that includes arbuscular mycorrhizal fungi, to spatially distant descendants (Vannier et al., 2016). This form of

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heritability of microorganisms to plant-progeny is not mediated environmentally (i.e. through environment sharing) or sexually. Such a process would support the niche construction of plant progeny while microorganisms could benefit from a selective dispersal vector allowing them to reach a similar and hence suitable host. Transmission in clonal plants has been demonstrated to involve information- and resource-sharing within the physical clone (i.e. physiological integration; Oborny et al., 2001). An additional level of integration might occur through the sharing of microorganisms within the clonal network, as previously proposed by Stuefer et al. (2004).

We tested this hypothesis of microorganisms transmission to progeny through clonal integration and addressed the new concept of a core microbiota heritability in clonal plants, using the clonal herbaceous species *Glechoma hederacea* as model. The growth form of this plant consists of a network of ramets connected through horizontal stems (i.e. aerial stolons), one of the most widespread forms of clonality (Zilber-Rosenberg and Rosenberg, 2008). Plants from 10 ecotypes were grown under controlled conditions. First, a juvenile ramet without roots (mother ramet) was transplanted into a pot containing field soil. Plant growth was oriented by forcing the newly emitted ramets (daughter ramets) of the two ramifications to root into separate pots containing sterilized substrate (Figure 1). Our aim was to detect the endophytic microorganisms present in the mother ramet roots and transferred to the daughter ramets through the clone stolons. High-throughput amplicon sequencing of 16S and 18S rRNA genes was used to detect and identify Bacteria, Archaea and Fungi within the roots endosphere and the stolon internodes. Control pots randomly distributed in the experiment were also analyzed to remove from the dataset all operational taxonomic units (OTU) which could not be attributed to a plant-mediated transfer of microorganisms (see methods in supplementary information).

## Material and methods

### Biological material

We used the clonal, perennial herb *Glechoma hederacea*, which is a common model for studying clonal plant response to environmental constraints (Slade and Hutchings 1987; Birch and Hutchings, 1994; Stuefer et al., 1996). *G. hederacea* clones produce new erect shoots at the nodes at regular intervals of 5 to 10 cm (the internodes) on plagiotropic monopodial stolons (i.e. aboveground connections). Each ramet consists of a node with two leaves, a root system and two



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axillary buds. In climatic chambers with controlled conditions and in the absence of enriched substrate, *G. hederacea* does not invest in flowering but displays only vegetative growth (Birch and Hutchings, 1994). The ramets used in our experiments were obtained from the vegetative multiplication of 10 clonal fragments taken at 10 different locations separated by at least 1 km to sample different ecotypes. Plants were grown for three months under controlled conditions to limit parental effects related to their geographic location and habitats (Dyer et al. 2010). Vegetative multiplication was carried out on a sterilized substrate (50% sand and 50% vermiculite, autoclaved twice at 120°C for 1h).

### Experimental conditions

Experiments were carried out with cultures grown on the same sterile substrate (50% sand, 50% vermiculite) in a climate-controlled chamber with a diurnal cycle of 12h day /12h night at 20°C. Plants were watered with deionized water every two days to avoid a bias in nutrient availability. Necessary nutrients were supplied by watering the plants every 10 days with a low-phosphorus watering solution to favor mycorrhization (Oborny et al., 2001). At each watering, the volumes of deionized water and fertilizing solution per pot were 25 mL. To test for the transmission of microorganisms within the clonal network we transplanted an initial ramet (mother ramet) into a pot with field soil and oriented its growth to force the newly emitted ramets to root in different individual pots containing sterilized substrate (Figure 1). During the experiment, secondary ramifications of daughter ramets were removed to limit spread and confine the growth of the plant to a simple network of five ramets comprising the mother ramet and four daughter ramets equally distributed between two stolons (two on each primary stolon). By using two stolons we could test whether the potential transmission was systematic within the clone or whether this transmission varied between stolons (i.e. transfer of random organisms from the mother pool). The transplanted clonal unit (i.e. the mother ramet) consisted of a mature ramet (leaves and axillary buds) with one connective stolon internode (to provide resources to support ramet survival; Huber and Stuefer, 1997), and without roots (to avoid prior colonization of the roots by micro-organisms). Field soil was collected from grassland harboring native *Glechoma hederacea* and located in the experimental garden of the University of Rennes. Soil was then sieved through 0.5 cm mesh to remove stones and roots. The experiment was stopped and the ramets harvested when the clone had reached the stage with a mother ramet and four rooted daughter ramets. The composition of endospheric microorganisms in the root and internode samples was analyzed by separating the clonal network

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into stolon internodes, roots and shoots for both the mother and the daughter ramets. Each internode and root sample was meticulously washed first with water, secondly with a 1% Triton X 100 (Sigma) solution (three times) and lastly with sterile water (five times). This procedure ensured removal of the ectospheric microorganisms (Vandenkoornhuysen et al., 2007). In order to control for potential contaminations, three control pots were also randomized into the experimental design. These pots were filled with the same sterile substrate and watered similarly to the other pots. Substrate from these control pots was sampled at the end of the experiment so that all contaminant microorganisms that were not plant-transmitted could be removed from the sequence analyses and from all subsequent statistical analyses. All root, internode and substrate samples were frozen at -20°C before DNA extraction and subsequent molecular work.

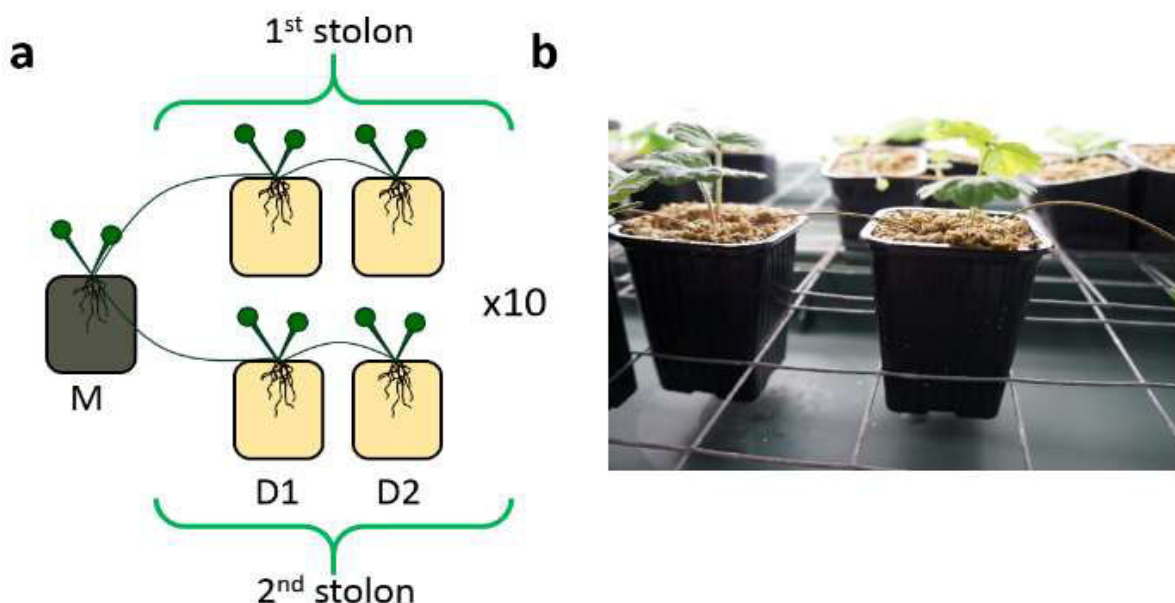


Figure 1 | Experimental design. (a), clonal ramets of 10 ecotypes were forced to root in separate individual pots and connected by stolons. At the end of the experiment, the clonal network consisted of the mother ramet and 4 daughter ramets. The daughter ramets (1<sup>st</sup> and 2<sup>nd</sup> mother ramets) were positioned along the two primary stolons produced by the mother ramet. Pots with mother ramets were filled with homogenized field soil, those with daughter ramets contained sterilized substrate, and contact was only by the internode that separated two consecutive ramets. M = mother, D1 = 1<sup>st</sup>

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daughter, D2 = 2nd daughter. (b), picture of the experimental design: the pots are only connected by the internodes.

### DNA extraction and amplification

DNA was extracted from cleaned roots and internodes, as well as from the substrate from control pots, using the DNeasy plant mini kit (Qiagen). The 18S rRNA gene was PCR amplified using fungal primers NS22b (5'-AATTAAGCAGACAAATCACT-3') and SSU817 (5'-TTAGCATGGAATAATRRAATAGGA-3') (L, Van et al., 2017). The conditions for this PCR comprised an initial denaturation step at 95°C for 4 min followed by 35 cycles of 95°C for 30 s, 54°C for 30 s and 72°C for 1 min with a final extension step at 72°C for 7 min. The 16S rRNA gene was amplified using Bacterial primers 799F (5'-AACMGGATTAGATACCKG-3') and 1223R. (5'-CCATTGTAGTACGTGTGTA-3'). The conditions for this PCR consisted of an initial denaturation step at 94°C for 4 min followed by 32 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min with a final extension step at 72°C for 10 min. The 16S rRNA gene was also amplified using a nested PCR with Archaea primer. The first PCR primers were Wo\_17F (5'-ATTCYGGTTGATCCYGSCGRG-3') and Ar\_958R (5'-YCCGGCGTTGAMTCCAATT-3') and PCR conditions comprised an initial denaturation step at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 57.5°C for 50 s and 72°C for 50 s with a final extension step at 72°C for 10 min. The second PCR primers were Ar\_109F (5'-ACKGCTCAGTAACACGT-3') and Ar\_915R (5'-GTGCTCCCCGCCAATTCCT-3') and PCR conditions comprised an initial denaturation step at 94°C for 4 min followed by 32 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min with a final extension step at 72°C for 10 min. All amplification reactions were prepared using Illumina RTG PCR beads with 21 L of extracted DNA and target PCR products were visualized by agarose gel electrophoresis.

### Sequencing and data trimming

All PCR amplifications products were purified using Agencourt AMPure XP kit. After purification, the amplifications products were quantified and their qualities checked using Agilent high sensitivity DNA chip for bioanalyzer and Invitrogen fluorimetric quantification.

All PCR amplifications products were then subjected to an end repair step and adaptor ligation using the Neb library preparation kit. Multiplexing was done with a PCR step using

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NEBnext Ultra 2 multiplex oligo (dual index). Multiplexed products were then quantified and quality checked using Agilent high sensitivity DNA chip for bioanalyzer and quantitative PCR with SmartChip Wafergen. Amplicons libraries were pooled to equimolar concentration and paired-end sequenced (2x250 bp) with an Illumina MiSeq instrument. Data trimming consisted of different steps: primer removal (Cutadapt), and classical sequence quality. An additional step consisted of checking the sequence orientation using a homemade script. This stringent data trimming resulted in 9,592,312 reads. Trimmed sequences were then analyzed using the FROGS pipeline (Escudie et al., 2015) (X.SIGENAE [<http://www.sigenae.org/>]). FROGS pre-process was performed with a custom protocol (Kozich et al., 2013) for Archaea and Fungi and with the FROGS standard protocol for bacteria reads. In this pre-process, bacteria reads were assembled using Flash (MagoT and Salzberg, 2011). The clustering step was performed with SWARM to avoid, in an innovative manner, the use of identity thresholds to group sequences in OTUs (Mahŕ et al., 2014). Following the pipeline designer's recommendations, a de-noising step was performed with a maximum distance of aggregation of 1 followed by a second step with a maximum distance of aggregation of 3. Chimera were filtered with the FROGS remove chimera tool. A filter was also applied to keep those OTUs with sequences in at least three samples to avoid the presence of artificial OTUs. All statistical analyses were also done with a five samples filter and results were similar. We herein present only the R2 Fungi and R1 Archaea results based on affiliation statistics that indicated a better quality of affiliation. OTUs affiliation was performed using Silva 123 16S for Bacteria and Archaea and Silva 123 18S for Fungi. OTUs were then filtered based on the quality of the affiliations with a threshold of at least 95% coverage and 95% BLAST identity. The stringent parameters used in FROGS enabled us to finally obtain 4,068,634 bacterial reads, 2,222,950 fungal reads and 113,008 Archaeal reads. Rarefaction curves were generated using R (package vegan 2.2-1; Oksanen et al., 2015) to determine whether the sequencing depth was sufficient to cover the expected number of operational taxonomic units (OTUs). The sequencing depth was high enough to describe the microbial communities in detail (Supplementary Figure S1). To homogenize the number of reads by sample for subsequent statistical analyses, samples were normalized to the same number of reads based on graphical observation of the rarefaction curves using the same R package. During this step, samples with less reads than the normalization value were removed from the dataset. All OTUs found in the soil of the control pots were then removed from the data set.

Sequences data are available through the accession number PRJEB20603 at European Nucleotide Archive.

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## Statistical analyses

The positions and stolon of each ramet within the network were recorded as two factors for the statistical analyses. We considered three positions in the network: the mother ramet, the 1st daughter ramet and the 2nd daughter ramet. The stolon was considered as a factor with two levels: the 1st and the 2nd stolon emitted during growth. We analyzed heritability, richness and composition of microorganisms assemblages in *G. hederacea* ecotypes. We analyzed fungi and bacteria assemblages separately. No statistical analyses were performed on Archaea data as they were found in the mother ramet roots and in the stolon internodes following the mother ramets but not in the daughter roots. All statistical analyses were performed using the R software (R Development Core Team, 2011).

## Heritability calculation and null model construction

Heritability was measured for each taxonomic group in each ecotype as the number of OTUs present in the mother ramet and shared by at least two daughter ramets (we also tested the heritability calculation for three and four daughter ramets). To determine whether the observed heritability could be expected stochastically, we compared the observed heritability against a null model. This procedure is designed to test the null hypothesis that species from the mother ramets are randomly distributed within each daughter ramet and do not reflect the selection or the dispersal of a particular set of species from the mother pool. It allows assessment of the probability that the observed heritability indexes are greater than would be expected under a null distribution (Mason et al., 2008). We built a null model for each of the 10 ecotypes by generating daughter ramets communities with a random sampling of microorganism species within the mother's pool. The probability of species sampling was the same for all species in the mother's pool (i.e. independent of their initial abundance in the mother roots). Only species identity was changed from one model to another while species richness within the daughter communities remained unmodified. For each daughter ramet community within the 10 ecotypes, 9999 virtual communities were randomly sampled from the mother's pool and the heritability indexes calculated for each of these models. Results were similar when a less stringent heritability was used (e.g. OTU present in at least one daughter ramet) but the heritability could not be more stringent because it would create null

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communities with zero inherited OTUs for most of the null communities and thus overestimate the difference between the observed and the random heritability values.

For each ecotype, we computed the Standard Effect Size (SES), calculated as described by Gotelli

and McCabe, 2002: 
$$SES = \frac{I_{obs} - \bar{I}_{null}}{\sigma_{null}}$$

where  $I_{obs}$  is the observed heritability index value,  $\bar{I}_{null}$  is the mean of the null distribution and  $\sigma_{null}$  is its standard deviation. SES aims to quantify the direction and magnitude of each ecotype heritability index compared to the null distribution. Negative SES values indicate lower heritability than in the random model (heritability of microorganisms species not present in the mother ramet), whereas positive SES values reveal higher heritability than expected by random (heritability of microorganisms from the mother ramet). A one-sample t-test with the alternative hypothesis ``greater_`` was then applied to the SES values to determine whether they were significantly greater than zero after checking for the data normality.

#### Analyses of richness through linear mixed models

Richness was calculated as the number of OTUs present in the sample. Richness was calculated separately for bacteria and fungi at the scale of the whole community and at the scale of the phyla (OTU richness in each phylum). We chose these two scales to detect general patterns in microorganisms richness and also to detect potential variation in these patterns between taxonomic groups (phyla). We conducted our analyses at the phylum scale rather than at a more precise taxonomic level because we were constrained by the sequence affiliation that produced multi-affiliation of OTUs at lower taxonomic levels. To test whether the richness was affected by the sample position in the clonal network, we performed linear mixed-effects models using R packages ``nlme_`` (Pinheiro et al., 2015) and ``car_`` (Fox and Weisberg, 2011). We initially tested for differences in richness between mother and daughter ramets. We then tested for differences in richness between 1st and 2nd daughter ramets by considering the position in the clone (1st daughter or 2nd daughter) and the stolon (1st stolon, 2nd stolon) within the plant ecotype as explanatory variables. Ecotype-induced variance and data dependency were controlled by considering the position in the clone (mother or daughter) and the stolon as fixed factor and the plant ecotype as a random factor in the mixed models. Normality of the models residuals was verified using a graphical representation of the residuals and the data were log or square-root transformed when

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necessary. For several fungal and bacterial groups exhibiting low abundances in the samples, the models testing differences in richness did not respect the normality of the residuals and thus these results are not presented.

#### Analysis of microorganisms community composition

A PLS-DA analysis was used to test whether the microbiota composition varied significantly between mother and daughter ramets and between daughter ramets. The PLS-DA consists of a Partial Least Squares (PLS) regression analysis where the response variable is categorical (y-block; describing the position in the ecotype), expressing the class membership of the statistical units (Sjöström et al., 1986; Sabatier et al., 2003; Mancuso et al., 2015). This procedure makes it possible to determine whether the variance of the x-blocks can be significantly explained by the y-block. The x-blocks (OTUs abundance) are pre-processed in the PLS-DA analysis using an autoscale algorithm (i.e., centers columns to zero mean and scales to unit variance). The PLS-DA procedure includes a cross-validation step producing a p-value that expresses the validity of the PLS-DA method regarding the data set. The PLS-DA procedure also expresses the statistical sensitivity indicating the modeling efficiency in the form of the percentage of misclassification of samples in categories accepted by the class model. Our aim in using this model was to test the variance of community composition that could be explained by the position of the ramet in the clone. The entire data set was subdivided into two or three groups depending on the groups tested (i.e. mother ramets vs 1st daughter ramets vs 2nd daughter ramets, mother ramets vs all daughter ramets and 1st daughter ramets vs 2nd daughter ramets).

#### Results

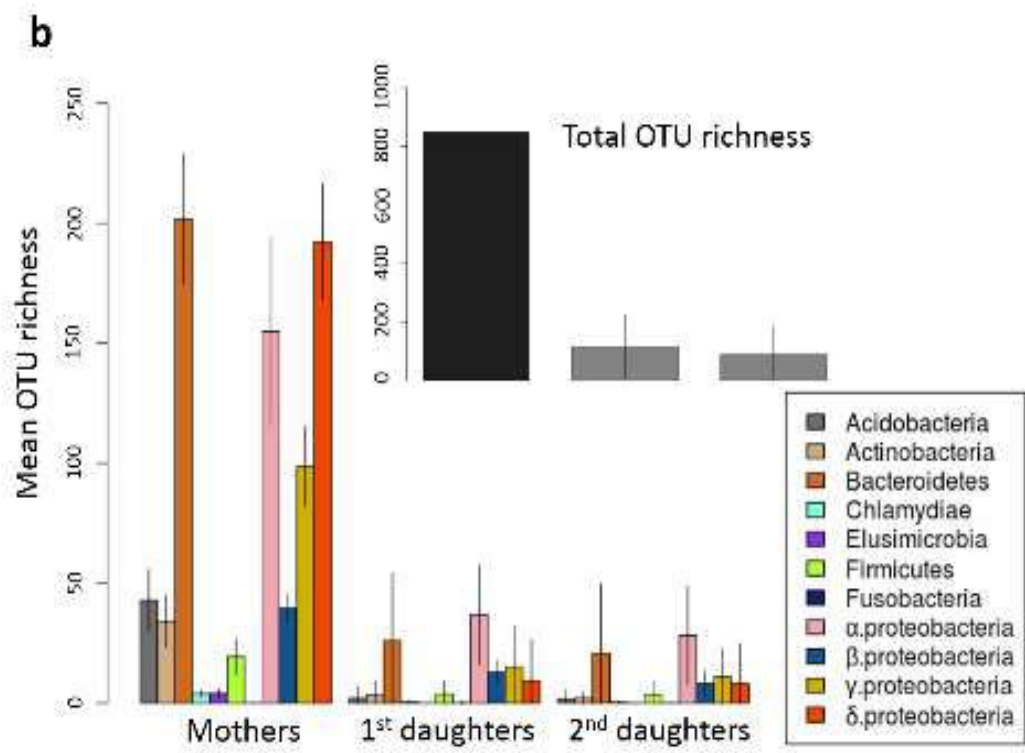
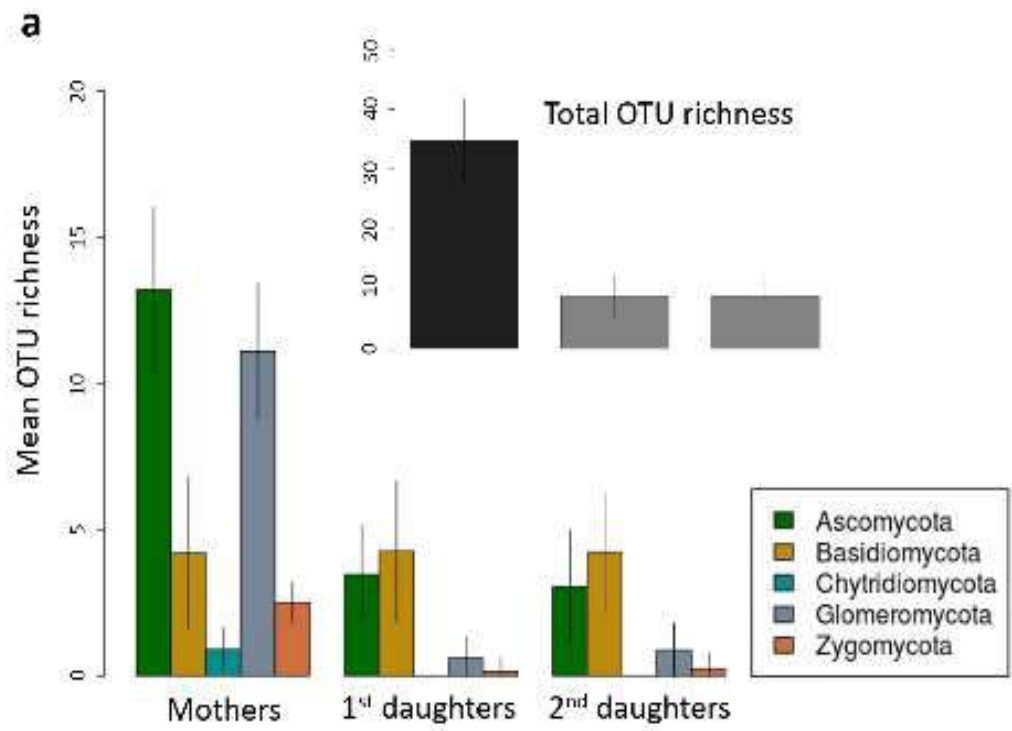
##### Archaeal, Bacterial and fungal communities in the roots of *Glochoma hederacea*

Archaea (Thaumarcheota), fungi and bacteria were found in mother ramets. Archaea were not detected in the daughter ramets, but fungi and bacteria were found in daughter roots (Figure 2). Comparison of the sequences obtained from the roots of mother and daughter ramets revealed a subset of 100% identical reads in both mother and daughter ramets, representing 34% and 15% of the daughter fungal and bacterial reads respectively. Heritability, calculated as the number of OTUs found in the mother and in the roots of at least two daughters, varied from 15 to 374 OTUs ( $\bar{I} = 100.2 \pm 118.6$ ) for bacteria and from 0 to 12 OTUs ( $\bar{I} = 6.1 \pm 3.63$ ) for fungi, depending on the ecotypes. To test whether this observed heritability was higher than would be expected

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stochastically (i.e. random dispersal of OTUs), we used a null model approach in which the identity of the fungi or bacteria species in the experimental samples was randomized while keeping the OTU richness identical. For each ecotype, we thus generated bacterial and fungal random daughter communities by sampling species from all the mother roots communities (regional pool) and compared the observed heritability in our dataset to this distribution of random heritability values. The null model approach indicated that the observed communities displayed significantly higher OTUs heritability between the roots of mothers and daughters than expected stochastically (one sample t-test with alternative hypothesis `higher\_',  $P < 0.01$   $t = 3.03$ ,  $df = 8$ , and  $P < 0.001$   $t = 6.11$ ,  $df = 9$  for fungi and bacteria respectively) (Supplementary Figure S2). In addition to the non-random presence of OTUs in daughter roots we also found communities of fungi and bacteria in the stolon internodes connecting the ramets in the network (Supplementary Figure S3). These internodes exhibited similar phyla richness to that observed in the daughter roots. The transmission of bacteria and fungi within the *G. hederacea* clonal network was thus clearly demonstrated.





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Figure 2 | Composition of the bacterial and fungal communities within the root endosphere at the different positions in the clonal network. (a), mean number of OTUs of each fungal phylum and mean total number of OTUs for all phyla together found in the root samples at the different positions in the clonal network (mother, 1st daughter or 2nd daughter). Vertical bars represent the standard error of the mean for each phylum. Significance of the linear mixed models testing the differences in OTUs richness between mothers and daughters in the clonal network is indicated: \*\*\*  $P < 0.001$ . (b), Mean number of OTUs of each bacterial phylum and mean total number of OTUs for all phyla together found in the root samples at the different positions in the clonal network (mother, 1st daughter or 2nd daughter). Vertical bars represent the standard error of the mean for each phylum. Significance of the linear mixed models testing the differences in OTUs richness between mothers and daughters in the clonal network is indicated: \*\*\*  $P < 0.001$ .

#### Microbial communities filtration during transmission

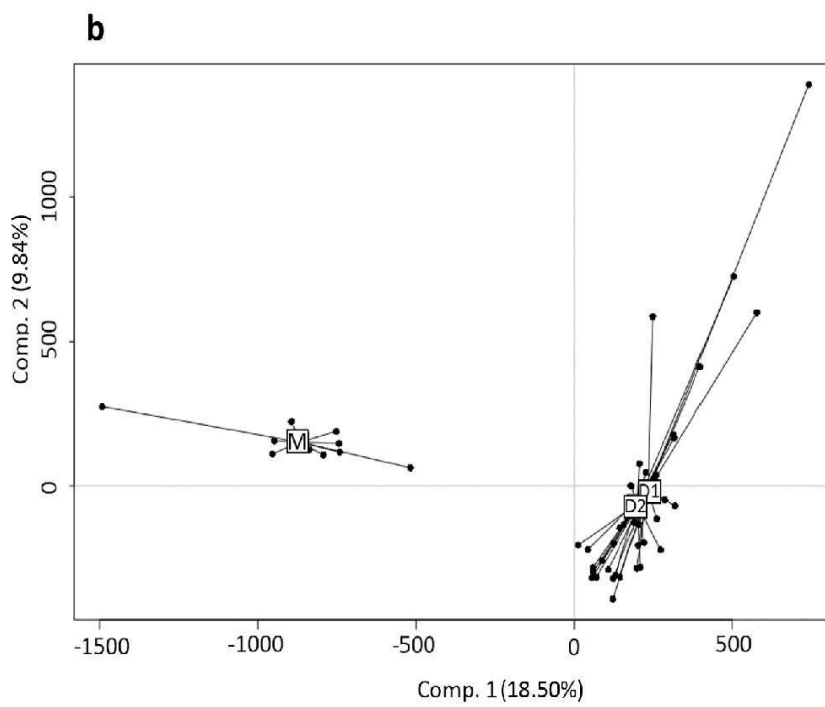
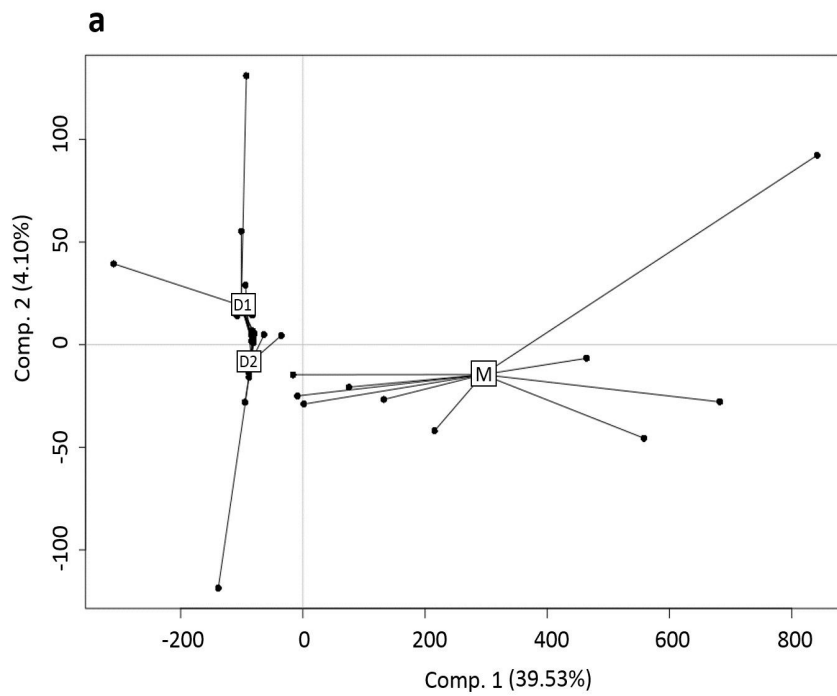
Endophytic microorganisms were strongly filtered during the transmission process. Daughter roots displayed significantly lower fungal OTUs richness than mother ramet roots with mother communities averaging 40 OTUs compared to an average of 10 OTUs in the daughter ramets (linear mixed model,  $F_{1,31}=280$ ,  $P < 0.001$ ; mother ramet:  $40 \pm 7$ ; daughter ramet:  $10 \pm 3$ ) (Figure 2; supplementary table S1). The same significant pattern was observed for Bacteria with mother communities averaging 800 OTUs compared to an average of 100 OTUs in the daughter ramets (linear mixed model,  $F_{1,39}=410$ ,  $P < 0.001$ ; mother ramet:  $800 \pm 131$ ; daughter ramet:  $100 \pm 100$ ) (Figure 2, supplementary table S1). The observed low richness of the transmitted communities indicates that the transmitted microbiota is filtered from the original pool (i.e. the mother microbiota). A significant effect of ecotype, on the richness of the transmitted microbiota, was also found (Methods for details on the statistics and random factor used). Comparison of the microorganisms in the roots of mothers and daughters revealed a general decrease in richness of most phyla during the transmission process. The fungal communities colonizing the roots were mostly from the phyla Ascomycota (106 OTUs) and Basidiomycota (39 OTUs) and to a lesser extent from Glomeromycota (24 OTUs), Zygomycota (7 OTU) and Chytridiomycota (4 OTUs) (Figure 2a). The mean OTU richness of Ascomycota and Glomeromycota was significantly lower in daughter roots than in mother roots (supplementary table S1) whereas no significant variation was observed in the OTU richness of Basidiomycota. (supplementary table S1). This striking observation clearly advocates for the presence of a fungus-dependent filtering mechanism. The bacterial communities

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colonizing the roots were distributed in 3384 OTUs mostly belonging to Proteobacteria (2009 OTUs) and Bacteroidetes (715 OTUs) which together represented about 80% of all the sequences, the remaining 20 % belonging to 6 additional phyla (Figure 2b). Consistently with fungi, the bacterial OTU richness was significantly lower in daughter roots than in mother roots for the Proteobacteria, Bacteroidetes, Acidobacteria, Actinobacteria and Firmicutes (supplementary table S1). This observation suggests that bacterial phyla are indifferently affected by the filtering mechanism.

#### The heritability of a core microbiota

The differences in microorganisms community composition between mother and daughter roots were assessed using a multi-regression approach with a Partial Least Squares Discriminant Analysis procedure (PLS-DA) (see Material and Methods, supplementary information). The advantage of this analysis is its ability to test an hypothesis based on a grouping factor of the samples in the data set (i.e. an explicative factor) and to obtain the significance of the factor as well as the part of the variance explained by the factor. With this analysis the entire dataset can be used and most of the variance conserved in contrast to NMDS approaches in which the distances between samples such as Bray-Curtis or Jaccard summary the variance between samples. Significant differences in the composition of daughters communities compared to mothers were detected for both fungi (PPLS-DA = 0.001, PMothers vs Daughters < 0.01, explained variance = 87.3%, Figure 3a) and bacteria (PPLS-DA = 0.001, PMothers vs Daughters < 0.01, explained variance = 72.4%, Figure 3b; supplementary table S2). These differences in composition between mothers and daughters can be explained by the observed diminution in richness during the transmission process. These result indicates that only a portion of the original pool of microorganisms is transmitted from the mother to the daughters (i.e. a specific set of organisms). To test the validity of the hypothesis that a plant filtering mechanism allows the transmission of a core microbiota we analyzed the filtering consistency between daughters by comparing the microbiota composition within the daughter roots using a PLS-DA procedure. The composition of the roots communities was not significantly different between the 1st and 2nd daughter ramets (PPLS-DA = 0.09 and PPLS-DA = 0.33 for fungi and bacteria respectively, supplementary table S2), thus confirming that a specific set of organisms was similarly transmitted to daughter-plants of all ecotypes.



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Figure 3 | Partial Least Square Discriminant Analysis (PLS-DA). (a), PLS-DA testing the significance of the position (mothers, 1st daughters and 2nd daughters) on the composition of the root bacterial communities. (b), PLS-DA testing the significance of the position (mothers, 1st daughters and 2nd daughters) on the composition of the root fungal communities. The groups used as grouping factor in the model are represented on the graphs. They correspond to mother, 1st and 2nd daughter ramets. 1st and 2nd ramets were grouped independently of the stolon to which they belonged. This analysis was used to test the hypothesis that roots at different ramet positions in the clonal network exhibit similar compositions of fungal and bacterial communities. The percentage of variance indicated on each axis represent the variance of the communities composition explained by the grouping factor.

#### Effect of dispersal distance and dispersal time

We found patterns of richness dilution in bacterial communities along the stolons (linear mixed model,  $F_{1,18} = 6.13$ ,  $P < 0.05$ , supplementary table S3) showing that those ramets most distant from the mother were less rich in bacteria. This finding suggests that colonization of the daughters by bacteria is limited by dispersal distance. This pattern of richness dilution also followed the course of plant development as stolons produced earlier in the experiment (i.e 1st stolon emitted by the plant) were found to be richer (linear mixed model,  $F_{1,9} = 4.92$ ,  $P < 0.05$ , supplementary table S3), which suggests that richness of the bacterial community also depends on dispersal time. Alternatively, these patterns may be linked to a cumulative filtering effect at each node of the clonal network, reducing the pool of transmitted bacteria. Conversely these richness dilution patterns were not detected for fungal communities (supplementary table S1), suggesting either that dispersion of the transmitted species was not limited or that the fungal community was already strongly filtered during the initial transmission. These two non-exclusive hypotheses are supported by our observation of a variation in the diminution of fungal community richness between mothers and daughters, probably dependent on the life history and dispersal traits of the different fungal taxonomic groups.

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## Discussion

This work provides the first demonstration of vertical transmission and heritability of a specific endospheric microbiota (fungi and bacteria) in plants. Along with other studies, it supports an understanding of the plant as a complex- rather than a standalone- entity and is aligned with the idea that the plant and its microbiota have to be considered as holobionts (Zilber-Rosenberg and Rosenberg, 2008; Vandenkoornhuyse et al., 2015; Theis et al., 2016). Our demonstration of core-microbiota transmission supports the idea that microbial consortia and their host constitute a combined unit of selection. This finding does not conflict with the idea that this heritability of microbiota (microbial components metaphorically called ‘singers’ in Doolittle and Booth, 2017), within clonal-plants, in fact consists of the heritability of a selected set of functions (the ‘song’ in Doolittle and Booth, 2017). Thus, our work reconciles aspects of the on-going debate regarding the evolutionary process at work within the holobiont entity (Bordenstein et al., 2015; Moran and Sloan, 2015; Theis et al., 2016).

For the plant, the transmission of a microbiota along plant clonal networks extends to microorganisms the concept of physiological integration previously demonstrated for information and resources. This integrated network-architecture questions the idea of a meta-holobiont organization where ramets (i.e. holobionts) can act as sinks or sources of micro-organisms. Such a structure may ensure exchanges between the holobionts, and especially between the mother source and the daughter ‘sinks’, thereby increasing the fitness of the clone as a whole. Indeed, the inheritance of a cohort of micro-organisms that has already gone through the plant filtering system provides a pool of microorganisms available for recruitment in the newly colonized environments. This ‘toolbox’ of microorganisms could allow the plant to rapidly adjust to environmental conditions and therefore provide fitness benefits in a heterogeneous environment (Clay and Schardl, 2002). This may be assimilated to plant niche construction and provide a competitive advantage when colonizing new habitats.

From the perspective of microorganisms, the stolons can be seen as ecological corridors facilitating the dispersal at a fine scale. In addition to propagules transport in the environment, this process ensures a spread of the transmitted organisms from one suitable host to another. As a consequence, transmitted symbiotic partners may benefit from a priority effect when colonizing the

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rooting system within the new environment (Werner and Kiers, 2015). Future work will thus need to address (i) the direction (uni vs. bidirectional) of microorganisms transmission within the clonal network as well as the modalities of (ii) the transmission mechanism (active or passive), and of (iii) microorganisms filtering during this transmission to determine (iv) the significance of the process in ecosystems. As regards this last aspect, plant communities are dominated by clonal plants and our findings demonstrate their fundamental role in the spreading of microorganisms between trophic levels and reveal a new ecological function of plant clonality. Considering that the heritability process demonstrated herein affects different compartments within the ecosystem, this novel ecosystem process consisting of microbiota filtering and transfer by clonal plants is of paramount importance.

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The authors of the paper declare that they do not have conflict of interest.

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SUPPLEMENTARY TABLES & FIGURES

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## A microorganisms journey between plant generations

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Supplementary table 1 | Results of linear mixed models comparing OTU richness of mother roots to daughter roots for all fungi and all bacteria as well as for specific phyla. P-values and F-values are presented as well as degrees of freedom.

Mothers vs Daughters

Fungi	num DF/ den DF	F-value	P-value ( =0.05)
All fungi	1/31	280	<0.001
Ascomycota	1/31	177	<0.001
Basidiomycota	1/31	0.07	0.79
Glomeromycota	1/31	460	<0.001
Bacteria	num DF/ den DF	F-value	P-value ( =0.05)
All bacteria	1/39	410	<0.001
Acidobacteria	1/39	509	<0.001
Actinobacteria	1/39	241	<0.001
Bacteroidetes	1/39	293	<0.001
Firmicutes	1/39	73	<0.001
Proteobacteria	1/39	402	<0.001

Supplementary table 2 | PLS-DA results of the different models tested for different grouping of ramets position in the clonal network. The significance of the model is indicated by a P-value. When the model is statistically significant, the statistical sensitivity is indicated by the number and percentage (in brackets) of misclassifications of samples in categories accepted by the class model. The modelling efficiency is also presented in the form of the percentage of variance explained by the model. When the model is significant, the P-values of the pairwise test comparison of the different groups are presented. M = mother, D1 = 1<sup>st</sup> daughter, D2 = 2<sup>nd</sup> daughter, D1-1 = 1<sup>st</sup> daughter on the first stolon, D1-2 = 1<sup>st</sup> daughter on the second stolon, D2-1 = 2<sup>nd</sup> daughter on the second stolon, D2-2 = 2<sup>nd</sup> daughter on the second stolon.

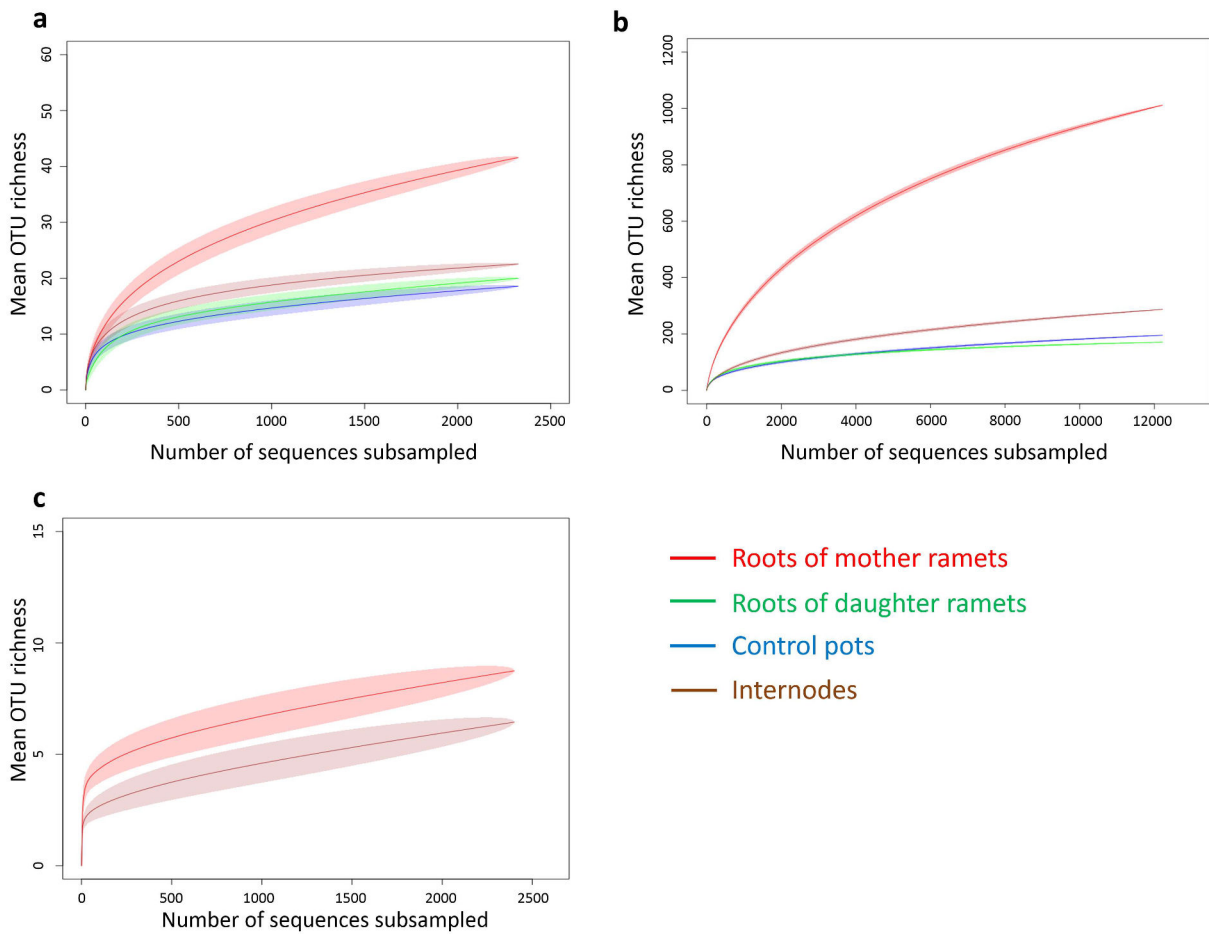
M vs D1 vs D2	P-value	Misclassifications (%)	Explained Variance %	P-values pairwise tests
Fungi	0.002	37.9 (1.3)	43.62	A-B=0.0015, A-C=0.0015, B-C=0.084
Bacteria	0.001	43.8 (1.7)	28.34	A-B=0.0015, A-C=0.0015, B-C=0.46
<b>M vs D1D2</b>				
Fungi	0.001	10.7 (1.1)	87.31	-----
Bacteria	0.001	0.2 (0.2)	72.4	-----
<b>D1 vs D2</b>				
Fungi	0.091	-----	-----	-----
Bacteria	0.333	-----	-----	-----
<b>D1-1 vs D1-2 vs D2-1 vs D2-2</b>				
Fungi	0.568	-----	-----	-----
Bacteria	0.162	-----	-----	-----

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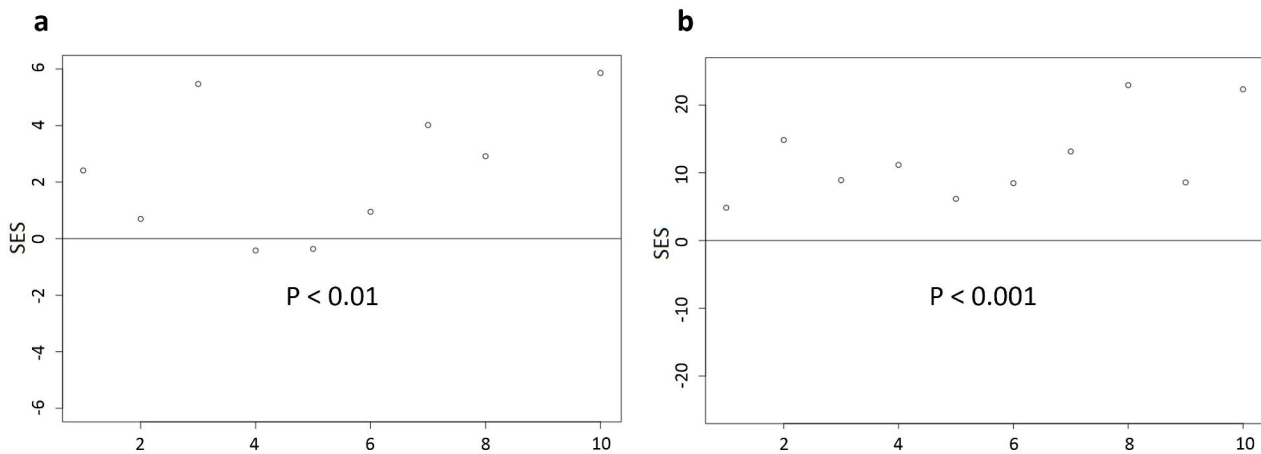
Supplementary table 3 | Results of linear mixed models comparing OTU richness between daughter roots depending on their position in the network (1<sup>st</sup> or 2<sup>nd</sup> daughter, 1<sup>st</sup> or 2<sup>nd</sup> stolon) for all fungi and all bacteria. P-values and F-values are presented as well as degrees of freedom.

1 <sup>st</sup> Daughters vs 2 <sup>nd</sup> Daughters	num DF/den DF	F-value	P-value ( =0.05)
All fungi	1/11	0.001	0.97
All bacteria	1/18	6.13	0.03
Stolon (1 <sup>st</sup> , 2 <sup>nd</sup> )	num DF/den DF	F-value	P-value ( =0.05)
All fungi	1/8	0.02	0.88
All bacteria	1/9	4.92	0.04

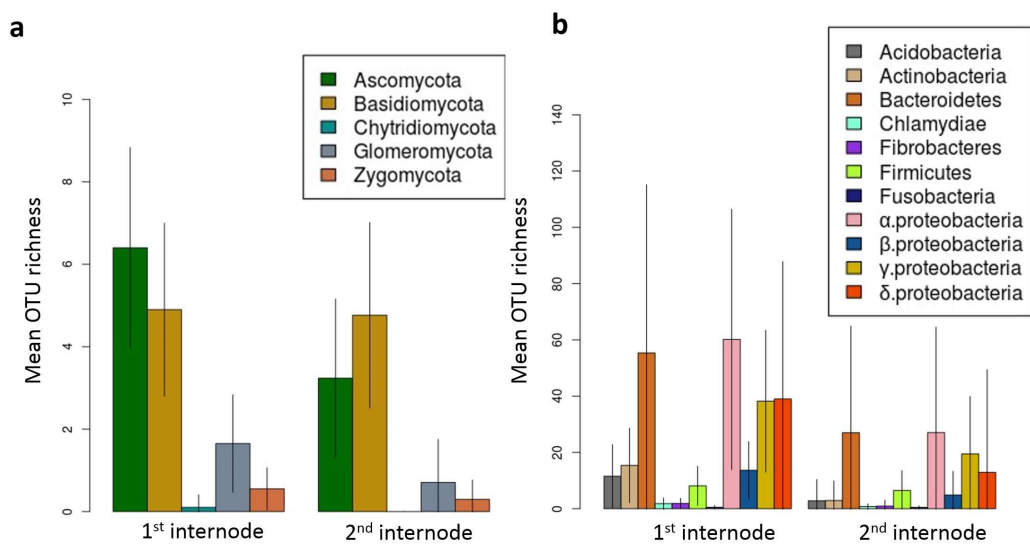




Supplementary Figure 1 | (a), mean rarefaction curves for the Bacterial communities in the mother roots samples (red), the daughter roots samples (green), the internode samples (brown) and the control pots (blue). (b), mean rarefaction curves for the fungal communities in the mother roots samples (red), the daughter roots samples (green), the internode samples (brown) and the control pots (blue). (c), mean rarefaction curves for the fungal communities in the mother roots samples (red), the daughter roots samples (green), the internode samples (brown) and the control pots (blue). Coloured areas indicate  $\pm$  SE.



Supplementary Figure 2 | Null model results. (a), standard effect size (SES) values for fungal root communities of the different ecotypes. (b), standard effect size (SES) values for bacterial root communities of the different ecotypes. One value in the graph corresponds to one ecotype. To determine whether the observed heritability could be expected stochastically, we compared the observed heritability against a null model. We built a null model for each daughter ramet of the 10 ecotypes by generating daughter communities with random samples of the microorganisms species occurring within the species pool (regional pool) of the mother ramet. Only the species identity was changed while species richness within the null daughter communities remained unmodified. We created 9999 null datasets for each daughter ramet and measured the OTUs heritability for each ecotype created in this way as the number of OTUs shared between the mother and at least 2 daughters. We then calculated the Standard effect size (SES) values of each ecotype. Negative SES values indicated that the observed heritability was lower than would be expected in the null-model (heritability of OTUs not specific to the ecotype mother ramet), whereas positive SES values revealed a higher heritability than expected (heritability of microorganisms from the mother). The horizontal line represents an SES value of 0 (no difference between the observed heritability and the null heritability). P-values indicate the significance of the one sample t-test to determine whether SES values are significantly higher than 0 (alternative hypothesis `greater\_`).



Supplementary Figure 3 | Composition of the bacterial and fungal communities within the internode samples. (a), mean number of OTUs of each fungal phylum found in the internode samples at the different positions in the clonal network (1<sup>st</sup> internodes following the mother ramets and 2<sup>nd</sup> internodes following the 1<sup>st</sup> daughter ramets). Vertical bars represent the standard error of the mean for each phylum. (b), mean number of OTUs of each bacterial group (phylum or class) found in the internode samples at the different positions in the clonal network (1<sup>st</sup> internodes following the mother ramets and 2<sup>nd</sup> internodes following the 1<sup>st</sup> daughter ramets). Vertical bars represent the standard error of the mean for each phylum.



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II.3 Article IV: Introduction of the metaholobiont concept for clonal plants.



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## Introduction of the meta-holobiont concept for clonal plants

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~In preparation~

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The genome is not the only support of phenotypic variations

The theoretical basis of neo-Darwinian evolution (i.e. the modern synthesis) is linked to the idea that a genetic variation, as a mutation, is an accidental random event having neutral consequences or inducing advantageous or disadvantageous effects on fitness with the natural selection increasing the advantageous variants in large populations (e.g. Charlesworth et al., 2017). This sorting of genetic variations by natural selection acts on the individual phenotypic value. It is thus generally believed that an organism, its functions and its ability to adapt to environmental constraints can be addressed by the analysis of its genome (i.e. the information repository of the organism). Behind this idea is the assumption that organisms develop through programmed genes. This view of genomes is an oversimplification (Goldman & Landweber, 2016). There are existing examples of physical transience in genomes (i.e. genome composition and stability are not fixed at all times in every organism) as in ciliates (e.g. *Oxytricha*) (Bracht et al., 2013). Elsewhere and less anecdotal is the existence of additional information beside the genome supporting phenotype expression. These are of two main types. Firstly, the epigenetic marks (i.e. DNA methylation, histone modification, histone variants, and small RNAs) which can be heritable and reversible. They induce a suite of interacting molecular mechanisms which impact gene expression and function and thus phenotypes without changing the DNA sequence (e.g. Richards, 2006; Holeski et al., 2012; Kawakatsu et al., 2016). Secondly, all macro-organisms, animals and plants, are interacting as hosts with symbiotic partners forming the microbiota. Such associations deeply impact the phenotypic variations, and determine host fitness (e.g. Hooper et al., 2012; Tremaroli & Bäckhed, 2012; Mc Fall-Ngai et al., 2013; Blaser, 2014; Vandenkoornhuyse et al., 2015; Vannier et al., 2015). A large proportion of population genetics studies considers the host genome solely whereas microbe-free plants are not facts but artifacts (e.g. Partida-Martinez & Heil, 2011). Thus phenotypic variations are often mistakenly attributed to genome variants and the neo-darwinian approach of species evolution fails to explain these variations. A holistic understanding of plant-microbes associations is thus needed.





Fig 1: *Glechoma hederacea* phenotypic plasticity induced by its microbiota composition. All the plants have the same genotype (i.e. same clone) grown under controlled conditions (light, temperature, hygrometry, duration, water supply). Only one of their microbiota component differed (i.e. mycorrhizal colonizer).

### The holobiont and hologenome concepts

A given macro-organism can no longer be considered as an autonomous entity but rather as the result of the host and its associated micro-organisms forming a holobiont (e.g. Bordenstein & Theis, 2015) with their collective genome forming the hologenome. The holobiont is a unit of biological organization and encompasses not solely the host and obligate symbionts but also includes [ ] the facultative symbionts and the dynamic associations with the host [ ] (Theis et al., 2016). This new understanding of what a macro-organism is, deeply modifies our perception of evolution processes in complex organisms. From this, an important idea is that genetic variations occurring in any

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genomic subunits have to be considered as hologenomic variations which may be neutral, deleterious or beneficial for the holobiont (Zilber-Rosenberg & Rosenberg, 2008; Bordenstein & Theis, 2015). This parsimonious idea is the keystone of the hologenome theory of evolution (Zilber-Rosenberg & Rosenberg, 2008). Beside these genetic changes in the hologenome, a plant, for example, can recruit micro-organism(s) within the phyllosphere and the rhizosphere to buffer environmental constraints (e.g. Vandenkoornhuyse et al., 2015). The microbiota modification in a plant holobiont, thus hologenome modifications, can be seen as a rapid acquisition of new functions to adjust to biotic and abiotic environmental fluctuations. Because the micro-organisms recruited can be vertically or pseudo-vertically transmitted (Rosenberg et al., 2009) this adjustment would be a reboot of the Lamarckian deterministic evolution (i.e. inheritance of acquired characteristics). In this context, the microbiota assembly is the repository of the information transmitted between generations.

The microbiota assembly of the holobiont

Natural selection acts on holobiont phenotype, thus on hologenome. The case of neutrality (nor advantage neither disadvantage) or circum-neutral effect on individual fitness, could explain the heterogeneity of microbiota community structure observed among hosts.

From ecological theories, both niche partitioning (through environmental filtering or through species interactions sorting) and neutral processes (Hubbell, 2001; 2005) are classically admitted to drive community assemblies and to explain diversity patterns. Coexistence is mainly assumed to result from complementarity in resource use (Webb et al., 2002). The neutral and stochastic vs deterministic host-microbiota assembly has been debated (Nemergut et al., 2013, Bordenstein & Theis, 2015). From a random community assembly model (Nemergut et al., 2013), if a large proportion of the microbiota is recruited from the environment (i.e. horizontal transmission), the microbiota community composition is not expected to differ from a random assembly. However, because the functions assumed by the microbiota are important for holobiont fitness, a specialization at the metabolic level of the microbiota is expected from a strong selection process on hologenomes. In this case, the resulting observation would be a deterministic host-microbiota assembly (i.e. non-random assembly). This microbiota specialization could act at two different levels. The first level is the micro-organisms recruitment and filtering by host, a process that should culminate in the vertical or pseudo-vertical transmission of microbiota components. The second level of specialization is, for particular micro-organisms within the microbiota, the relaxation of

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selection pressure on “useless” genes and accumulation of mutations in these particular genes toward a loss of functions evolution. In addition, microorganisms within the holobiont experience selection pressures both at the individual level within the holobiont and at the whole holobiont level.

#### Holobiont and hologenome: the special case of plants

Plants are sessile macro-organisms. This conditions their interactions with their environment. First, they are not able to escape environmental constraints and need to adapt to either abiotic (e.g. resource limitation) or biotic (e.g. competition, predation) stresses. Second, plants act on their own environmental conditions. For instance, they deplete through their nutrition mineral and water resources of their habitat, or modify microclimate and local soil characteristics through the development of above and belowground organs (Marschner et al., 1986; Orwin et al., 2010; Kardol et al., 2015). This retroaction of plants on their environment drives environmental fluctuations occurring at different time scales. In these two above-described situations, the recruitment of microorganisms within the microbiota enables to acquire new ecological functions increasing resource acquisition or buffering stressful conditions (see for review Friesen et al., 2011; Bulgarelli et al., 2013; Møller et al., 2016). Changes in the microbiota composition along the seasons or years may thus reflect the temporal needs of the plants. Considering that the environmental changes can be either continuous or disruptive and occur over short or long time-scales, the recruitment of microorganisms represents a quick and long-term adaptation to environmental constraints that is less costly for the plant than genomic plastic responses (see Alpert & Simms, 2002 for a review of plasticity advantages). Plant-associated micro-organisms then condition plant survival and fitness and provide quick phenotypic adjustments allowing adaptation to rapid and long-term environmental changes (Vannier et al., 2015).

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Plants are modular organisms and can be seen as a discrete organization of units (modules) forming a system (Harper, 1977). Their growth is iterative through undifferentiated and totipotent tissues (meristems). Such modularity is expressed at different levels of integration: from simple subunits such as a leaf, a root, or a flower (order 1 of modularity) to more complex units such as ramets (i.e. potentially autonomous clonal units composed of roots and shoots modules) produced by clonal multiplication (e.g. order 2 of modularity) (Fig. 2a). Most plants are then constituted as reticulated networks that sample the environment and can adjust their structure to the environment (van Groenendael & de Kroon, 1990). A physiological integration involving information- and resource-sharing within the clonal network has been demonstrated (e.g. Oborny et al., 2001). Physiological integration occurs for all first order modules within each second order module, at least partially during its life-cycle. Some species display physiological integration between all or part of the second order modules (Price & Hutchings, 1992), for a short period or the whole clonal plant life (e.g. splitter vs. integrator strategies). In response to small-scale environmental heterogeneity, such modularity and integration enable plastic adjustments of order 2 modules with an impact on the fitness of either or both 1<sup>st</sup> and 2<sup>nd</sup> order modules depending on integration level. (Stuefer et al., 2004; Hutchings & Wijesinghe 2008). More specifically, plastic changes have been reported in the network architecture in response to patchy distribution of favorable habitats (i.e. foraging, Fig 2b). Such changes occur along a morphological gradient from a 'phalanx' (the clonal network is dense with high branching and low internode length) to a 'guerilla' form (loose network with low branching and high internode length) (Doust, 1981). Phalanx forms promotes the exploitation of nutrient rich habitats through ramet aggregation whereas guerilla forms enable space exploration and the colonization of habitats at distance from the mother plant.

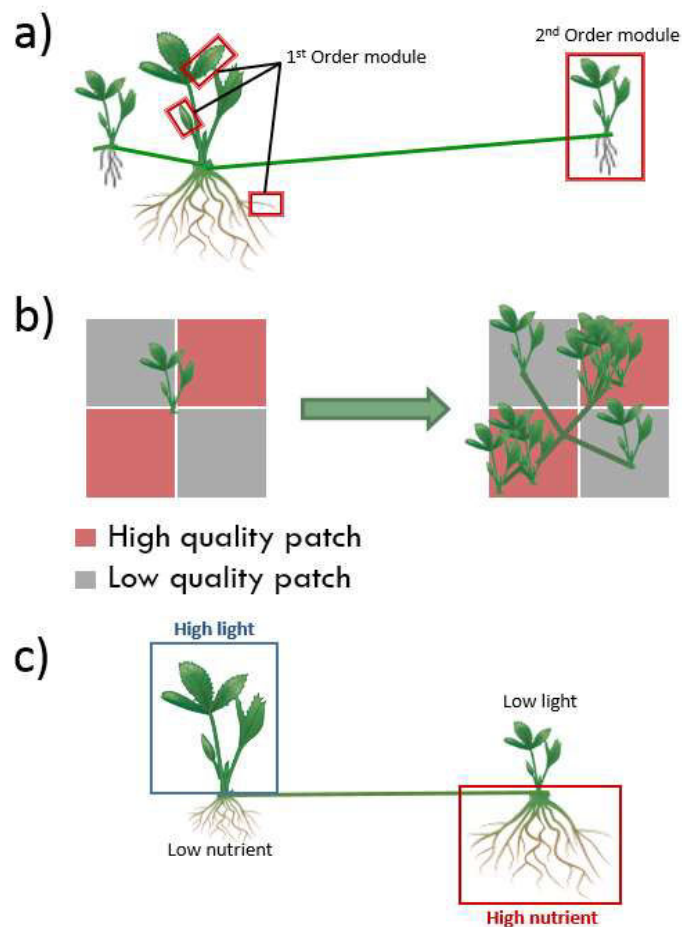


Figure 2. a) Schematic clonal plant showing the two levels of modularity within clonal plants: 1<sup>st</sup> order modules and 2<sup>nd</sup> order modules. b) Schematic clonal plant showing a plastic response of foraging in resource heterogeneous environment. The foraging response consists on ramet aggregation in good patches through the modification of internodes length while avoiding poor patches. c) Schematic of a clonal plant showing a plastic response of specialization to combine heterogeneity of light and nutrient. The response consists on preferentially develop roots in high nutrient and shoots in high light and redistribute resources within the network thanks to the physiological integration.

In addition to the sampling of resources in the environment, this plastic network also allows to sample potentially symbiotic microorganisms within the soil pool. In clonal plants, this process has never been taken into account for microbiota assembly despite the microbiota importance for plant fitness. Pseudo-vertical (i.e. acquisition by environment sharing) and vertical transmissions of microbiota ensure the presence of suitable symbionts and thus limit the associated foraging costs

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(Wilkinson & Sherratt, 2001). The question of the vertical or pseudo-vertical transmission of the microbiota is then of fundamental importance. In aggregated networks (i.e. phalanx), the clonal progeny is expected to be in contact with a similar pool of microorganisms than the mother plant (i.e. strong pseudo-vertical transmission). Conversely, in scattered networks (i.e. guerilla), a weak pseudo-vertical transmission is expected (i.e. progeny encounter different microorganisms than parents). However, it has recently been demonstrated that clonal plants are able to vertically transmit a core microbiota (i.e. a fraction of the mother microbiota) containing bacteria and fungi through their stolons (Vannier et al., 2016; Vannier et al., submitted). This vertical transmission of a subset of the mother microbiota could be seen as an insurance of habitat quality for progeny.

#### From holobiont to meta-holobiont concept

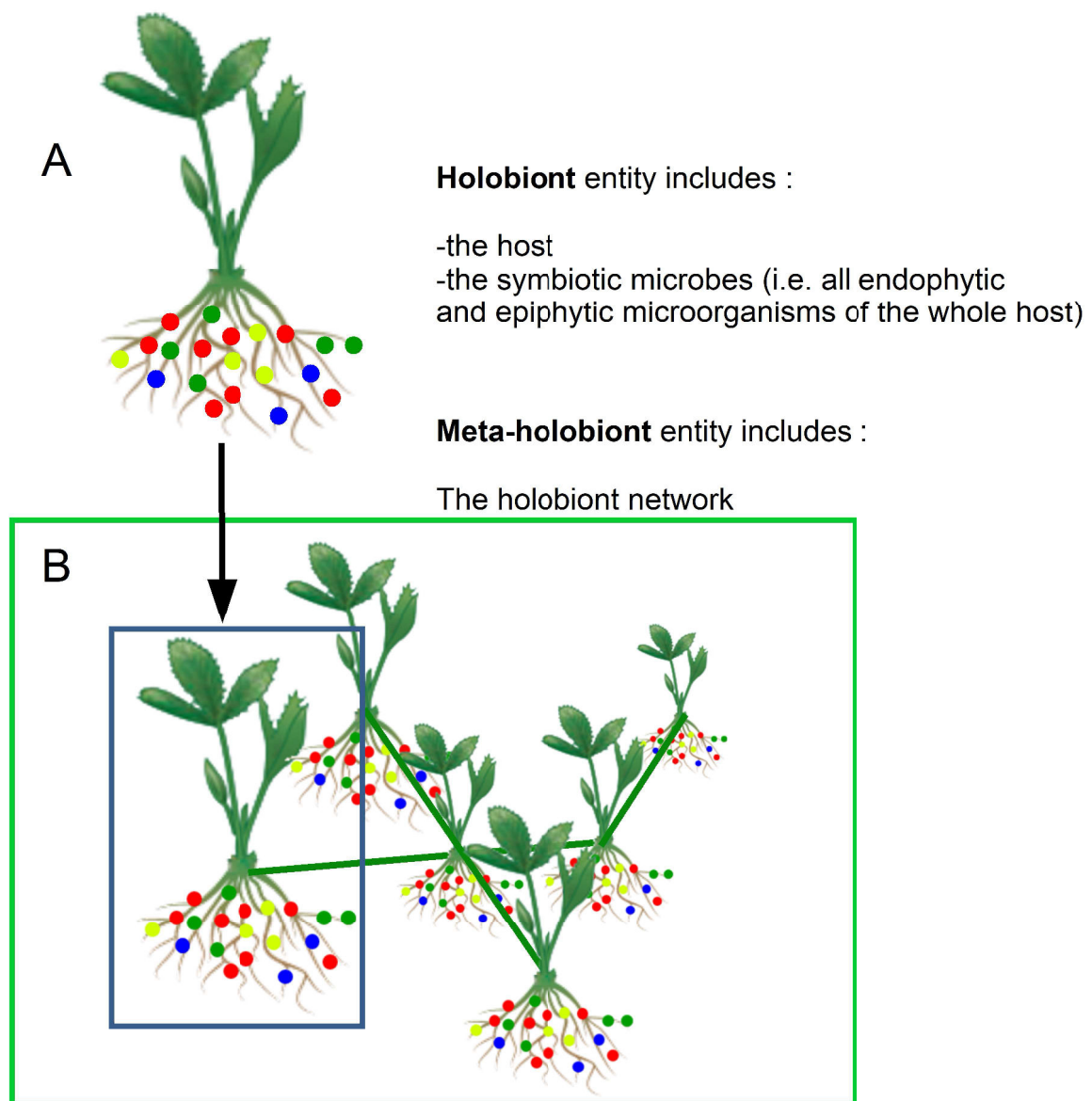
Considering that a particular clone is colonized by a complex microbiota, that at least part of this microbiota is transmitted between clonal generations and that these transmitted microorganisms alter the clone fitness (e.g. among others, arbuscular mycorrhizal fungi, AM fungi hereafter) (Vannier et al., Submitted), a clonal individual thus satisfies the holobiont and hologenome theory (Zilber-Rosenberg & Rosenberg, 2008; Theis et al., 2016). The plant clonal network represents however an additional level of organization in which holobionts are inter-connected and integrated in a higher modularity model (the clonal network). To explain this level of organization, we introduce the concept of meta-holobiont.

#### The meta-holobiont concept

Bordenstein & Theis, (2015) have proposed a framework of ten principles for the holobiont and hologenome theory which do not change the rules of evolutionary biology but redefine what a macro-organism really is. The meta-holobiont concept is primarily dedicated to better understand and define clonal organisms forming a network (i.e. like clonal plants) through which holobionts can share molecules and micro-organisms (Fig 3). The meta-holobiont concept thus posits that through a network passive or active transfers of microorganisms, resources and information between holobionts can impact an individual holobiont. The meta-holobiont also posits the existence of specific physical host structures to build the network used for these exchanges. The physical structures linking clonal plant holobionts can be stolons or rhizomes. This physical link is of crucial importance as it is the support of the plastic responses developed at both i.e. the holobiont and meta-holobiont scales.

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In the case of mycorrhizal hyphae for example, the physical link (hyphae) linking two plants is not produced by the host plant and thus does not allow integrated plastic responses at the meta-holobiont scale (in this case two plants linked by a mycorrhizal hyphae). In this case, the selection pressures occurring on one plant of the network does not necessarily impact the other plants linked by a mycelial network. Another case to consider is animal in colonies of social insects as termites which have developed active behavior of microbiota exchanges. In this particular case, the support of microbiota, information and resources exchanges is the social organization of the colonies. The envelop of applicability of the meta-holobiont concept in such example is not developed herein but we warmly encourage further discussions on this specific case.



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Fig 3: The meta-holobiont. (A) the holobiont entity and meta-holobiont entity and their respective composition are explained in (A) and (B) respectively. Colored dots correspond to epiphytic or endophytic microorganisms forming the root microbiota. Notice that roots and shoots are colonized by microorganisms.

The inherent physiological integration of the meta-holobiont network (Oborny et al., 2001) and the microorganisms transmission from mother to clonal progeny (demonstrated in Vannier et al., Submitted) and possibly reciprocally (i.e. from clonal offsprings to ascendent ramets), although this has not been demonstrated yet, clearly represent an important level to consider in the understanding of the clonal holobiont. If holobionts and hologenomes are units of biological organization for the observation and understanding of a given macro-organism (principle 1, 2, 3 in Bordenstein & Theis, 2015) then the meta-holobiont could be considered alike a supra-organism.

#### Holobiont impacts on meta-holobiont structure and reciprocal effects

In patchy resource distribution, many authors demonstrated that clonal individuals aggregate ramets through internodes shortening and increased branching in resource-rich patches, and avoid resource poor patches through spacers elongation (i.e. foraging, Fig 2b; de Kroon & Hutchings, 1995; Wijesinghe & Hutchings, 1999; Roiloa & Retuerto, 2006). Another important response of clonal plants to heterogeneity is their ability to specialize individual modules in the acquisition of the most abundant resource to the network benefit (Fig 2c; Alpert & Stuefer, 1997; Stuefer, 1998). This specialization process has been extended to the concept division of labour concept (*sensu* Stuefer et al., 1996), occurring when the spatial distributions of two resources are negatively correlated (Alpert & Stuefer, 1997; van Kleunen & Stuefer, 1999).

On the one hand, microbiota transfer within the clonal network may be an alternative to these plastic mechanisms by providing ramets the ability to compensate for nutrient-limitation. For instance the transfer of arbuscular mycorrhizal fungi (AM fungi) with high resource uptake ability at the ramet scale is probably less costly for the plants than developing an increased rooting system or than increasing spacer length to forage for better patches. Specialization may then be seen in a wider context at the holobiont level (i.e. ramets that do specialize because of the recruitment of particular microbiota). Foraging through plant trait modifications could likely not be as beneficial as



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using microbiota functions. Note that such trade-off between foraging plant traits versus the use of microbial activity for resource uptake has already been well described at the individual level with root development (see for instance Eissenstat et al., 2015; Liu et al., 2015). What we suggest herein at the meta-holobiont scale, thus only consists in an extension of plant foraging tradeoffs.

On the other hand, foraging and specialization mechanisms, may be indirectly mediated by the holobiont microbiota. Studies have demonstrated that microorganisms often manipulate plant traits (Cheplick, 2004) inducing changes in plant architecture (e.g. connection branching or elongation; Streitwolf-Engel et al., 1997; Sudova, 2009; Streitwolf-Engel, 2001; Vannier et al., 2015), biomass allocation (Du et al., 2009; Vannier et al., 2015). In parallel, other works demonstrated that high diversity of AM fungi can reduce plant physiological integration in heterogeneous environments (Du et al., 2009). These results thus suggest a retroaction between plants and their microbiota in plastic adjustments to environmental heterogeneity developed at the clone level.

In these two cases, microorganisms sharing between holobionts in the meta-holobiont impacts both the holobiont and the meta-holobiont phenotypes. The meta-holobiont concept may shed light on a new understanding of these integration and plastic responses mechanisms, and explain the large range of strategies observed between plants.

Holobionts coexistence and dynamics within the meta-holobiont network: transposition of metacommunity-based theories

Microbiota transmission within the meta-holobiont induces consequences at the holobiont scale. As previously exposed, the meta-holobiont could specialize if the environment is heterogeneous and thus alter individual holobionts microbiota. Reversely an homogenization of holobionts microbiota within the meta-holobiont can be expected in homogeneous environments. This may condition the dynamics of microbiota assembly at the meta-holobiont level because in the first case, several holobionts may represent poorer pools of genomes than others to be transferred within the meta-holobiont while in the second case, all holobionts represent the same potentialities. Understanding microbiota assembly within meta-holobiont networks and its consequence on microorganisms species dynamics is likely to be achieved based on metacommunity theories. Different models of metacommunities have been described (see Leibold et al., 2004 for a review): specialization of

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microbiota within the meta-holobiont is close to the source-sink metacommunity model whereas homogenization corresponds to the neutral or patch dynamic models. Such theories transposition to the frame microorganisms transfer through plant network should provide an interesting framework for the understanding of the meta-holobiont impacts on microorganisms dispersal in natural ecosystems.

#### Network theory and meta-holobiont properties

Because microorganisms alter holobiont phenotypes, their recruitment in each holobiont and transmission dynamics within the meta-holobiont may condition the properties of the meta-holobiont. Such properties can for instance include productivity (e.g. fitness of the meta-holobiont, plant mass productivity), adaptation to heterogeneous conditions or resilience to disturbance. There is a wide theoretical corpus of knowledge based on graph theory about the relationships between network topology and its properties. For instance the number of nodes, their connectance or shape (i.e. modularity, nestedness) within a network, condition its stability to perturbations since it determines individual fluxes at population and community levels (see review of Proulx et al., 2005). In plants in general and in clonal plants in particular, the modules topology has been demonstrated to be shaped by structural blue-print, ontogeny, and plastic response to the environmental conditions (Huber et al., 1999; Bittebiere et al., 2014). Many researches have been specifically done on clonal plants to investigate how the network topology provides emergent functions to the clonal plant such as response to heterogeneous conditions, fluctuating environments, or disturbance (see examples given in the review of Oborny et al., 2012). Transposing graph theories to the meta-holobiont concept may allow for instance to determine keystone holobionts or specific network structures that maximize the whole network performance. This could be achieved through the maximization of resilience based on holobionts' positions within the network or the degree of redundancy of microbiota compositions in the network. The meta-holobiont concept may provide a new way to consider these questions while taking into account the plant-microorganisms associations.

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## Chapter III: Importance of the plant community context for the individual plant microbiota assembly

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### III.1 Introduction

#### Scientific Context

The composition of the soil pool of microorganisms depends on different environmental factors comprising soil type and properties (e.g. pH, water content, nutrient concentration) ( Berg & Smalla, 2009; Lundberg et al., 2012; Bulgarelli et al 2012; Shakya et al., 2013; Schreiter et al., 2014). Because plants are sessile this pool of microorganisms available for recruitment determine the plant microbiota (Lundberg et al., 2012; Bulgarelli et al., 2012). In addition to soil type and properties, plants can also modify this soil pool of microorganisms through different mechanisms. Plants are able to selectively recruit microorganisms from the soil (Vandenkoornhuyse et al., 2015) and promote the most beneficial symbionts (Bever et al., 2009; Kiers et al., 2011). In addition, plant exudates have been shown to either enhance or reduce particular microorganisms abundances depending on the plant species (for a review see Berendsen et al., 2012). The expectation is thus that plants can locally alter the composition of the soil microbial pool. Following this idea, the neighborhood of a given plant (i.e. the identity and abundance of neighboring plants) should influence the local microorganisms soil pool and thus the microorganisms available for recruitment by other plants in the community. Thus the neighborhood of a given plant should influence the focal plant microbiota assembly. However, this potential role of the plant community context in the plant microbiota assembly has not been extensively described yet. In addition, plants have been shown to harbor contrasted microbiota between species (Oh et al., 2012; Bonito et al., 2014) and ecotypes (Bulgarelli et al., 2012; Lundberg et al., 2012). An expectation is thus that plant species select and promote different microorganisms and have thus contrasted effects on the soil pool and on the different microbial groups within this soil pool.

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Considering the diversity of functions provided by plant-associated endophytes (Friesen et al., 2011, Møller et al., 2016) and their effects on plant phenotypes and performance evidenced herein (Articles I and II), changes in endophyte community composition is likely to affect individual plant performance.

### Objectives of the chapter

This chapter aims at testing the hypothesis that the plant community context and especially the close neighborhood of a plant determine its fungal microbiota assembly, ultimately impacting its performance. More precisely we will address the following questions:

- 1) Does the neighborhood plant species abundances influence the richness and equitability of the focal plant fungal microbiota? Does neighborhood plant species have contrasted effect? (Article V)
- 2) At which temporal and spatial scales act this neighborhood effect? (Article V)
- 3) Are changes in fungal groups richness and equitability mediated by the neighborhood plants determining the focal plant performance? (Article V)

### Methods

This chapter is composed of a single article (V). In this study, we tested the hypothesis of a neighborhood effect on plant microbiota assembly impacting plant performance using an outdoor experimental mesocosm design with grassland plant communities. This mesocosm comprises a range of grassland plant communities varying in composition and richness. Soil cores were sampled within each mesocosm and *Medicago truncatula* was used as a trap plant to capture soil fungal species. High-throughput amplicon sequencing of 18S rRNA genes was used to detect and identify fungi within the root endosphere of *M. truncatula* individuals. To determine plant neighborhoods, plant species abundances were mapped two consecutive years (one year before and the same year than soil sampling). We thus explained the richness and equitability (equality of species abundances) of the fungal community trapped by *M. truncatula* by the abundances of plant species

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within the neighborhood of the sampling point. We considered two temporal scales of the neighborhood, the past covering (one year before sampling) and the present covering (the same year than sampling). We additionally assess the effect of the microbiota composition on *M. truncatula* biomass as a measure of its performance.

### Main results

In our experiment (Article V), we detected a significant effect of the plant neighborhood species abundances on the composition of the microbiota colonizing *M. truncatula*'s roots. This effect was plant species dependant with several plants affecting positively and others negatively the richness and the equitability of the *M. truncatula* fungal endophytes. We thus demonstrated that a particular plant can filter and determine the local fungal pool available for recruitment in the soil, as proposed by Valyi et al., (2016). This was true not solely at the AM fungal diversity level but was also the case for the other fungal groups and more widely for the whole fungal community.

Plant neighborhood influence on soil microbiota occurred at small scale (i.e. from 5 to 25 cm), stressing the importance of the local plant community context for endophytes assembly. In addition, both the present and the past plant neighborhood impacted the root fungal community thus demonstrating that plants can leave a durable fingerprint on the composition of the soil fungal pool. Furthermore, we demonstrated that such changes in the fungal composition of the host plant roots impacted *M. truncatula* performance. Higher species richness and more equitable endophyte assemblages increased the fitness of the plant.

Both results demonstrated the existence of plant-plant interactions mediated by fungi ultimately impacting the plant fitness. This offers a new understanding of the plant microbiota assembly through the underestimated role of the local plant community. The role of leaf endophytes diversity in the diversity-productivity relationship has been recently proposed (Laforest-Lapointe et al., 2017) and our result suggest that it could be extended to the root microbiota and depend on the plant community context.



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III.2 Article V: Plant-plant interactions mediated by fungi impact plant  
fitness



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## Plant-plant interactions mediated by fungi impact plant fitness

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~In preparation~

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## Summary

- ø The plant microbiota is now recognized as a major driver of plant health. The rules governing root microbiota assembly have been recently investigated and the importance of abiotic determinants has been highlighted. The role of the biotic context of the plant community however remains unclear.
- ø We tested whether the plant neighborhood may leave a fingerprint on the fungal soil pool. We used outdoor experimental mesocosms comprising various floristic composition and mapped plant distribution over two years. *Medicago truncatula* was used as a trap plant on soil samples and root DNA was sequenced to describe fungal communities. The trap plant performance was estimated through biomass measures.
- ø Fungal communities richness and equitability were influenced by the abundance of key plant species in the neighborhood. A given plant had contrasted effects on fungal phyla and classes. Additionally, we demonstrated that changes in fungal groups richness and equitability influenced the biomass of the trap plant.
- ø Our results suggest the existence of plant-plant interactions mediated by fungi and impacting plant fitness. The shift between facilitation and competition may be mediated by the fungal community. The ecosystem diversity-productivity relationship could be extended to the root microbiota and depend on the plant community context.



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## Introduction

The great diversity of soil microorganisms, micro- and meso-fauna is a key-driver of aboveground ecosystem functioning which determines vegetation composition and dynamics (e.g. van der Heijden et al., 1998; 2008; Wagg et al., 2014, Fester et al., 2014) through nutrients cycling and symbioses (e.g. Bardgett & van der Putten, 2014). These symbioses between plants and soil microorganisms are widespread in natura and are compulsory for plant development and growth (Hacquard et al, 2016; Bulgarelli et al., 2013; Vandenkoornhuysen et al., 2015). Among symbiotic microorganisms, the Arbuscular Mycorrhizal (AM) fungi have received much attention since they are arguably the world's most abundant mutualists and among the most important terrestrial symbionts :that help feed the world~ (Marx, 2004).

In exchange for carbohydrates, AM association provides many ecological functions to plants such as nutrients acquisition and protection under stressful conditions. Despite their ubiquity, the existence of such mutualisms is unexpected in terms of evolutionary trajectories (Hardin, 1968) because cheaters (i.e. providing little phosphorus in exchange for carbohydrates) are predicted to spread at the expense of cooperative partners and thus indirectly favor competing strains (West et al., 2002). The ability of plants to preferentially reward AM fungal symbionts according to their level of "cooperativeness" was demonstrated using *Medicago truncatula* (Kiers et al., 2011). This selective rewarding mechanism mitigates the fitness of less cooperative fungi, stabilizes the AM fungal symbiosis (Kiers et al., 2011) and is supposed to allow plants to filter and exclude part of the colonizers. A consequence of this filtering phenomenon is the observation of a :host-plant preference~ (Vandenkoornhuysen et al., 2002; Duhamel & Vandenkoornhuysen, 2013). This filtering effect occurring at the individual plant level is then likely to influence the local diversity and abundance of fungal propagules.

At the plant community scale, the very first experimental studies based on a matrix-focal plant design showed that the identity of the plant species in the neighborhood determines the composition of the focal plant fungal community (Johnson et al., 2004; Hausmann & Hawkes, 2009). This observation remains consistent for AM fungi in more complex mixtures of plants and can be extended to their temporal dynamics (Hausman & Hawes, 2010). The first established plant

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species has a filtering effect on the pool of AM fungi colonizing the second established plant species (Hausman & Hawes, 2010). Following these findings, the temporal scale effect has been suggested as a key parameter in understanding of the whole fungal community assembly (Bennett et al., 2013; Cotton et al., 2015). However in the above-described experiment, the temporal scale tested was very short (several weeks) and the fingerprint of a plant on later fungal communities has never been tested over a long period of time (Hausmann & Hawkes, 2010). Although the influence of plant community composition on fungal assemblages has been described (e.g. Johnson et al., 2004; Hausmann & Hawkes, 2010), the spatial scale of this influence is still unclear. In a recent review, Valyi et al. (2016) proposed that the relative influence of environmental conditions, dispersal and host filtering on the AM fungal community was dependent on on the spatial scale considered. The effect of the host plant (i.e. host filter) would then be stronger at a local scale (Valyi et al., 2016). As far as we know, there are very few empirical or experimental supports for this hypothesis. A single seminal paper demonstrated that the relationship between plant and AM fungi compositions was detectable at the 25 cm<sup>2</sup> point scale but not at the 1m<sup>2</sup> plot scale (Landis et al., 2005). However, the fact that the influence of a given plant on the composition of fungal endophytes can be detectable at a fine scale suggests that the plant communities and their dynamics may condition the AM fungal community assembly. At a given location, we can make the parsimonious assumption that the fungal propagule reservoir available is necessarily a consequence of the fungal community structure and composition within the previous hosts. If the fungal diversity pool results from the cumulative influences of plants, the consequence is that we can explain AM fungal community assembly and quantify the respective influences of the surrounding plant species.

This assumption drives the idea that the plant neighborhood determines the assembly of the fungal pool available for recruitment by the focal plant. The plant-associated fungal endophytes are known to provide benefits for plant nutrition and resistance to abiotic and biotic constraints (e.g. Friesen et al., 2011). Changes in fungal groups richness should therefore affect the ecological functions performed by the fungi to the focal plant and ultimately impact its fitness.

We herein address the hypothesis that a plant neighborhood fingerprint on the root fungal community impacts host-plant fitness. We used a mesocosm design comprising experimental assemblages of different grassland species, with varying levels of plant diversity. We spatially sampled soil cores from plots on which *Medicago truncatula* was then grown as a trap plant. Root

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fungal assemblages were characterized by amplicon sequencing. Plant neighborhoods around the soil sampling positions were characterized using centimetric maps of plant species occurrences recorded over two years. We analyzed the effect of past and current plant neighborhoods on endophyte assemblages of *M. trunculata* and ultimately on its biomass. In this study we considered two diversity scales, the sample scale (alpha diversity) and the plot scale (gamma diversity). More specifically, we tested the following hypotheses: (i) the root fungal community structure is determined by the local plant neighborhood and the relationships between plant composition and endophyte diversity and richness should be weaker at the plot than at the sample scale. The scale of influence is likely to be a few centimeters (ii) this relationship occurs across different fungal taxonomic groups (iii) the changes in the fungal communities colonizing the plant should ultimately impact the focal plant performance.

## Materials and Methods

Analyses were performed in two stages. We first analyzed the influence of past and present plant neighborhoods (i.e. plant species around the sampling point) on the fungal assemblages at the plot scale and at the sample scale. We based our analyses on indexes describing the fungal communities calculated for different taxonomic groups following a down scale approach (from the whole assemblage to the phyla and classes).

### Experimental design

To determine whether fungal communities respond to the overlying plant communities depending on their taxonomic groups, and at which spatio-temporal scales, we used 112 experimental plant communities settled in 1.30 B 1.30 B 0.25 m mesocosms in 2009 (see Benot et al., 2013, and Bittebiere et al., 2013 for additional information on the experimental design). This study was conducted in the experimental garden of the University of Rennes1. Communities were constituted from a set of plant species widely distributed in temperate grasslands of Western France (des Abbayes et al., 1971), with one to 12 plant species in each mixture. Plants were grown on a homogeneous substrate composed of sand (20%) and ground soil (80%). Weeds were regularly removed, and the mesocosms were watered every two days during the dry season. A above-ground vegetation was mown once a year in late September and plant flowers were cut off to suppress

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sexual reproduction. Plant community dynamics was therefore dependent only on the plant's clonal growth. The present plant neighborhood therefore resulted from the previous one.

#### Plant neighborhood characterization

The plant species spatial distributions in the plots changed over time due to the ongoing community dynamics. To take these dynamics into account, species occurrences were therefore mapped in all mesocosms after two and three years of experimental communities cultivation (i.e. early spring 2011 and 2012), using an 80 B 80 cm squared lattice centered on the mesocosm (Fig. 1). We recorded presence/absence data in 5 B 5 cm cells of the lattice (i.e. 256 cells in total per mesocosm). A plant species was considered as present when at least one individual rooted within the cell, with each individual belonging to a single cell. GIS (ArcGIS ver. 9.3., ESRI) was used to calculate the number of cells colonized by each plant species (i.e. their abundances) at each spatial scale tested. Plant species abundances were calculated at the plot scale as the total number of cells occupied over the square-lattice. Within the plot, we analyzed plant species abundance at five spatial scales around the central sampling point, ranging from 5 to 25 cm (Fig. 1) (see Bittebiere & Mony, 2015 for details on the method). We considered two temporal scales by performing these calculations in 2011 (past plant neighborhood) and 2012 (present plant neighborhood). Per species correlations between 2011 and 2012 for each spatial scale exceeding 90% were not detected.

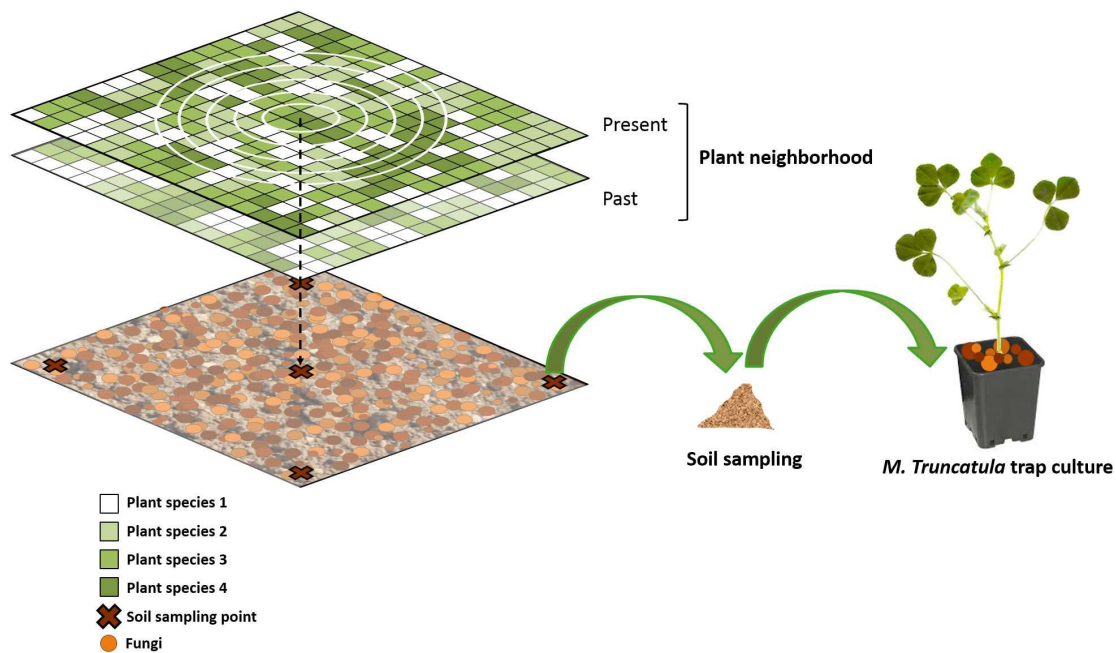


Figure 1. Sampling protocol. Plant neighborhoods were determined by mapping the abundances of the different plant species in the past (one year before sampling) and present (the same year than sampling). Five soil cores were sampled within each plot and an individual of *M. truncatula* was grown on each soil sample as a trap plant.

#### Endophyte assemblages analyses through a trap plant bioassay

To determine the fungal pool of species available for plant recruitment, we sampled five soil cores per mesocosm within the 80x80cm lattice (the four corners and the center) (i.e. 560 soil samples in total). This design enabled the fungal community to be captured both at the sample (sample in the center of the mesocosm) and the plot scales (the five sampling points of the plot). These soil samples were used as substrates for the cultivation of *Medicago truncatula* individuals, used as trap plants. *M. truncatula* was transplanted as seedlings after being germinated in sterile conditions for 3 weeks. This species is known to display a very low host-preference, trapping most of the fungal species present in the soil (Cook, 1999). *M. truncatula* individuals were cultivated for seven weeks under controlled conditions (constant temperature and water availability) with a 12h day/light cycle and nutrients were provided with a watering solution before harvesting. To evaluate *M. truncatula*

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performance, root and shoot samples from each individual were weighed at the end of experiment. Plant total fresh mass was used as a proxy of performance.

#### DNA extraction and amplicon preparation

*Medicago truncatula* root samples were carefully washed with detergent (Triton 100X, 1% V/V), thoroughly rinsed in sterile distilled water and ground to powder using a pestle and mortar under liquid nitrogen. Then, total DNA was extracted using the DNeasy plant kit (Qiagen) according to the manufacturer's recommendations. A 480 bp fragment of the fungi SSU rRNA was specifically amplified by PCR using NS22/0817 primers (L, Van et al., 2017) with PuReTaq Ready-to-go PCR beads (GE Healthcare). All the PCRs were done using fusion primers containing sequencing adapters and multiplex identifiers in addition to PCR primer (more details about amplifications in L, Van et al., 2017). For each of the 560 samples, true technical amplicon replicates were performed (i.e. two independent PCRs for each extracted DNA sample). Amplicons were purified using AMPure XP<sup>®</sup> PCR kit (Agencourt/Beckman-Coulter). Purified amplicons were then quantified (Quant-iT PicoGreen ds DNA assay, Invitrogen). An equimolecular amount of each amplicon was pooled to prepare the sequencing library. Traces of concatemerized primers were removed (LabchipXT, Caliper) before emPCR and sequencing on a GS FLX+ instrument (Roche), following the manufacturer's instructions.

#### Data trimming and contingency matrix preparation

Trimming, filtering, clustering, OTU identification and taxonomic assignments were performed as described elsewhere (e.g. Ben Maamar et al., 2015, L, Van et al., 2017). To summarize the strategy, short sequences (<200 bp), sequences with homopolymers (>8 nucleotides) or ambiguous nucleotides, sequences containing errors in the multiplex identifier or primer, were deleted from the dataset. Detected chimeric sequences using chimera.uchime were deleted. After these steps, and from the two replicates, only sequences displaying 100% identity were kept. The remaining sequences were grouped into OTUs using DNAclust (Ghodsi et al., 2011) with a 97% sequence identity threshold, and a contingency matrix was built. After these steps, the sequencing depth (i.e. number of sequences per sample to describe the community) was checked from rarefaction curves computed using the VEGAN package (Oksanen et al., 2015) in R (R Core Team, 2013). We

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removed 154 samples which did not satisfy these criteria and samples were normalized to 1351 sequences.

#### OTUs affiliation and taxonomic groups selection

A total of 3471 fungal OTUs for the 406 samples were obtained. A large proportion of these OTUs were rare (i.e. >70% of the OTUs represented by less than 25 sequences). Similarly to plant community studies, OTUs occurring in at least 1% of the samples were used for the statistical analyses. To check the sensitivity of the statistical signal of this additional filtering step, we tested other thresholds and obtained similar statistical results (data not shown). The resulting dataset contained 2057 fungal OTUs. All the statistical analyses were performed at three taxonomic levels, all fungi, within phyla (i.e. Ascomycota, Basidiomycota, Glomeromycota), and within classes (i.e. Sordariomycetes, Glomeromycetes, and Agaricomycetes) (i.e. seven datasets in total). Phyla and classes were selected according to their respective dominance in the whole assemblage and within the phyla. The Ascomycota, the Glomeromycota, and the Basidiomycota contained 1587 OTUs (77.2% of the total richness), 308 OTUs (15% of the total richness) and 100 OTUs (4.86% of the total richness), respectively. In each of these three fungal phyla, Sordariomycetes (186 OTUs), Glomeromycetes (80 OTUs) and Agaricomycetes (86 OTUs) were the dominant classes in terms of OTU richness.

#### Diversity indices calculation

Indices were calculated at the plot scale (i.e. the five samples from each mesocosm pooled) and at the sample scale (i.e. based on the sample from the center of the mesocosm only) for the seven fungal datasets (see above). To characterize the fungal community at the plot scale (gamma diversity), we calculated the OTUs richness as the total number of OTUs occurring in at least one of the five samples from the plot. At the sample scale (alpha diversity), we calculated the OTU richness (S), the Pielou equitability index (J), and the Shannon and Simpson diversity indices. Because strong correlations (i.e. > 90%) between Shannon diversity, Simpson diversity, and equitability were found, the Shannon and Simpson indices were discarded before further analyses. No strong correlations (i.e. > 90%) were found between the two remaining indices regardless of the taxonomic level analyzed. Indices were calculated using the VEGAN package (Oksanen et al., 2013) in R (R Core Team, 2015).

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## Statistical analyses

To determine whether the fungal community structure was influenced by the past and present plant neighborhoods at the sample and plot scales, we used multiple regression analyses with plant-species abundances as explanatory variables in linear model (LM) procedures. These analyses were performed on the seven fungal datasets.

First, to test for the influence of past and present plant neighborhoods on the fungal pool at the plot scale, we tested the effect of the plant abundances for each date (see above section Plant neighborhood characterization) on the fungal OTUs richness. We constructed two models for each taxonomic level analyzed (i.e. 14 models), which were optimized using a backward stepwise selection procedure of the explanatory variables based on the Akaike's Information Criterion (described below).

Second, to test for the influence of past and present plant neighborhoods on the fungal pool at the sample scale (i.e. corresponding to the soil sample from the mesocosm center), we tested the effect of the plant abundances for each date (see above section Plant neighborhood characterization) on the fungal richness and equitability for the five neighborhood sizes examined. This enabled us to determine the spatio-temporal scale of response of taxonomic groups from the local fungal pool to the plant neighborhood. One model was developed for each date and neighborhood size. We therefore constructed a total of ten models per index (two indices) and per taxonomic level analyzed (i.e. 140 models in total), and each model was optimized using a backward stepwise selection procedure of the explanatory variables.

We used the information-theoretic model comparison approach based on Akaike's Information Criterion (AIC) and compared for each index, all the optimized models through second-order AIC corrected for small sample sizes (AICc) (Burnham & Anderson, 2002). In our analyses, we considered models with smaller AICc values and with a substantial level of empirical support (i.e., a difference of AICc > 2 with other models) as the most probable (Burnham & Anderson, 2002). Thus this procedure enabled us to compute multiple models and to compare these models while minimizing the risk of producing false positives.



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To determine the impact of fungal taxonomic groups, OTUs richness, and equitability on the trap plant performance we used linear models with the indices as explanatory variables and *M. truncatula* biomass as the dependent variable. This was done at the sample scale (central point of the mesocosm) and at the three taxonomic levels analyzed (all fungi, phyla, classes).

For all models, data were log or square-root transformed when necessary to satisfy the assumption of a normal distribution of the residuals. The model coefficients and the proportion of index variation that was accounted for by the regression ( $R^2$ ) were calculated. The significance of each explanatory variable was tested with an ANOVA procedure. All the statistical analyses were performed using the packages `car` (Fox & Weisberg, 2011) and `AICcmodavg` (Mazerolle, 2012) in R (R Core Team, 2013).

## Results

### Endospheric fungal microbiota in *Medicago truncatula* roots

The 2057 fungal OTUs found in the *M. truncatula* root endosphere belonged to five phyla (i.e. Zygomycota, Chytridiomycota, Glomeromycota, Ascomycota, and Basidiomycota), with Zygomycota and Chytridiomycota together accounting for less than 3% of the total number of OTUs (Fig 2). The fungal community colonizing the roots of the trap plant *Medicago truncatula* ranged from 82 to 265 OTUs (Fig 2). The mean number of OTUs observed in a sample was 165 whereas the mean number of OTUs observed in a mesocosm was 163, an OTU richness comparable to previous studies (e.g. 133 fungal OTUs per sample in L, Van et al., 2017). The fungal composition of *M. truncatula* root endosphere was dominated by the Ascomycota OTUs which represented more than 80% of the reads and ~77% of the total fungal richness. The 1587 Ascomycota OTUs were distributed in nine classes, the Sordariomycetes being the class with the most OTUs (~11%) followed by Eurotiomycetes, Dothideomycetes, Laboulbeniomycetes, Lecanoromycetes, Orbiliomycetes, Pezizomycetes and Taphrinomycetes (Fig 2). The rest of the Ascomycota OTUs were not classified at the level of the class. Among the 308 Glomeromycota OTUs, 25% belonged to the class Glomeromycetes whereas the other OTUs were not affiliated at the level of the class (Fig 2). Within the Basidiomycota, 86% of the fungi belonged to the class Agaricomycetes. The

other 14% of the Basidiomycota OTUs were distributed in two other classes, the Agaricostilbomycetes and the Exobasidiomycetes whereas a few OTUs were unclassified at the level of the class (Fig 2).

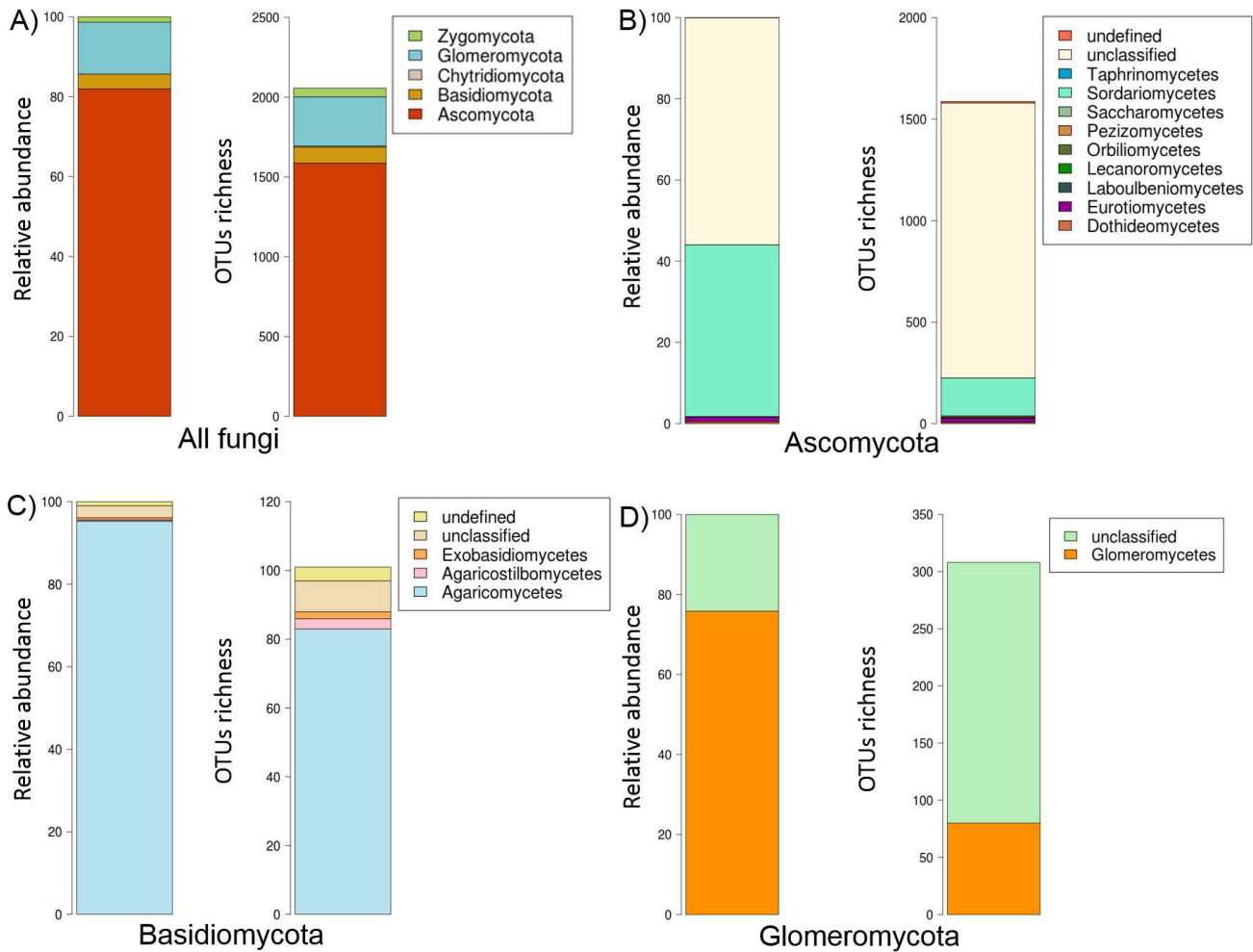


Figure 2. Relative abundance and OTUs richness of the different phyla within the whole fungal community (A). Relative abundance and OTUs richness of classes within the three major phyla Ascomycota (B), Basidiomycota (C) and Glomeromycota (D). Unclassified represent OTUs affiliated to sequences without classification and Undefined represent OTUs affiliated to unknown organisms at this taxonomic level.

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## Fungal community richness response to the plant neighborhood

### Fungal richness at the plot scale

To investigate the effect of the plant neighborhood on the richness of the fungal community at the scale of the whole mesocosm (i.e. gamma diversity), we produced linear models at the plot scale (i.e. the five sampling points of the mesocosm) with the plant species abundances as explanatory variables (Tab. 1). The plot richness of the whole fungal community was significantly determined by the present plant neighborhood. However, the proportion of the variation in fungal richness explained by the model was low ( $p=0.04$ ;  $R^2=0.04$ ). In addition, the fungal richness within the Ascomycota and Basidiomycota was not determined by the plant neighborhood and only 5% of the variance in Glomeromycota richness could be attributed to the present-plant neighborhood ( $P=0.04$ ;  $R^2=0.05$ ) (Tab. 1). When considering the past-plant neighborhood, only Basidiomycota richness was weakly determined by the plant neighborhood ( $P=0.03$ ;  $R^2=0.04$ ) whereas Ascomycota and Glomeromycota, as well as the fungal community as a whole, were not.

### Fungal richness at the sample scale

To investigate the effect of the plant neighborhood on the richness of the fungal community at fine-scale we produced linear models at the sample scale (i.e. center of the mesocosm) with plant species abundances as explanatory variables. Our multiple linear models analysis revealed that the richness of the fungal community was significantly determined by the plant neighborhood for all taxonomic groups tested (Tab 2). In comparison with the models produced at the plot scale, a larger proportion of the variations in fungal community richness could be explained at this alpha diversity scale. At the level of the whole fungal community, the richness increased significantly only with the abundance of *Agrostis tenuis* in the neighborhood whereas the abundance of the other plants had no significant effect on fungal community richness ( $P<0.05$ ;  $0.07 \leq R^2 \leq 0.1$ ). In addition, *A. tenuis* was one of the rarest species in the experiment and the effect detected could be an artifact of this rarity.

Ascomycota richness increased with the abundance of *A. tenuis* and *Festuca rubra* ( $P<0.05$ ;  $0.04 \leq R^2 \leq 0.12$ ) whereas Glomeromycota richness increased with *Brachypodium pinnatum* and *Dactylis glomerata* and decreased with *Elytrigia repens* ( $P<0.05$ ;  $0.06 \leq R^2 \leq 0.09$ ). The effects (positive or negative) of plant species at the phylum-level were not necessarily the same at the class-level. The presence of *D. glomerata* in the plant neighborhood significantly increased the

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richness of the phylum Basidiomycota ( $P < 0.05$ ,  $0.06 \leq R^2 \leq 0.09$ ) but decreased the richness of the class Agaricomycetes ( $P < 0.05$ ,  $0.07 \leq R^2 \leq 0.1$ ). The same pattern was observed for *F. rubra* increasing the richness of the phylum Ascomycota ( $P < 0.05$ ,  $0.04 \leq R^2 \leq 0.12$ ) but decreasing the richness of the class Sordariomycetes ( $P < 0.05$ ,  $0.1 \leq R^2 \leq 0.11$ ). On the contrary, several species had a consistent effect between taxonomic levels. *A. tenuis* for example significantly increased the richness of the whole fungal community and at the phylum levels for both Ascomycota and Basidiomycota, and at the class level for Sordariomycetes. The same pattern was observed with *B. pinnatum* that increased the richness of both the phylum Glomeromycota and the class Glomeromycetes.

#### Fungal equitability at the sample scale

To investigate the effect of the plant neighborhood on the equitability of the fungal community at fine-scale, we produced linear models at the sample scale (center of the mesocosm) with plant species abundances as explanatory variables. The multiple linear models analysis revealed that the equitability of the whole fungal community, of the Ascomycota, Basidiomycota, Glomeromycota, Sordariomycetes, Glomeromycetes, and Agaricomycetes were all significantly determined by the plant neighborhood (Tab 2). At the level of the whole fungal community, the equitability significantly decreased with the presence of only *Holcus mollis* in the neighborhood whereas the abundances of other plant species had no significant effect on fungal community equitability ( $P < 0.05$ ;  $0.04 \leq R^2 \leq 0.07$ ).

1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20. 21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38. 39. 40. 41. 42. 43. 44. 45. 46. 47. 48. 49. 50. 51. 52. 53. 54. 55. 56. 57. 58. 59. 60. 61. 62. 63. 64. 65. 66. 67. 68. 69. 70. 71. 72. 73. 74. 75. 76. 77. 78. 79. 80. 81. 82. 83. 84. 85. 86. 87. 88. 89. 90. 91. 92. 93. 94. 95. 96. 97. 98. 99. 100. 101. 102. 103. 104. 105. 106. 107. 108. 109. 110. 111. 112. 113. 114. 115. 116. 117. 118. 119. 120. 121. 122. 123. 124. 125. 126. 127. 128. 129. 130. 131. 132. 133. 134. 135. 136. 137. 138. 139. 140. 141. 142. 143. 144. 145. 146. 147. 148. 149. 150. 151. 152. 153. 154. 155. 156. 157. 158. 159. 160. 161. 162. 163. 164. 165. 166. 167. 168. 169. 170. 171. 172. 173. 174. 175. 176. 177. 178. 179. 180. 181. 182. 183. 184. 185. 186. 187. 188. 189. 190. 191. 192. 193. 194. 195. 196. 197. 198. 199. 200. 201. 202. 203. 204. 205. 206. 207. 208. 209. 210. 211. 212. 213. 214. 215. 216. 217. 218. 219. 220. 221. 222. 223. 224. 225. 226. 227. 228. 229. 230. 231. 232. 233. 234. 235. 236. 237. 238. 239. 240. 241. 242. 243. 244. 245. 246. 247. 248. 249. 250. 251. 252. 253. 254. 255. 256. 257. 258. 259. 260. 261. 262. 263. 264. 265. 266. 267. 268. 269. 270. 271. 272. 273. 274. 275. 276. 277. 278. 279. 280. 281. 282. 283. 284. 285. 286. 287. 288. 289. 290. 291. 292. 293. 294. 295. 296. 297. 298. 299. 300. 301. 302. 303. 304. 305. 306. 307. 308. 309. 310. 311. 312. 313. 314. 315. 316. 317. 318. 319. 320. 321. 322. 323. 324. 325. 326. 327. 328. 329. 330. 331. 332. 333. 334. 335. 336. 337. 338. 339. 340. 341. 342. 343. 344. 345. 346. 347. 348. 349. 350. 351. 352. 353. 354. 355. 356. 357. 358. 359. 360. 361. 362. 363. 364. 365. 366. 367. 368. 369. 370. 371. 372. 373. 374. 375. 376. 377. 378. 379. 380. 381. 382. 383. 384. 385. 386. 387. 388. 389. 390. 391. 392. 393. 394. 395. 396. 397. 398. 399. 400. 401. 402. 403. 404. 405. 406. 407. 408. 409. 410. 411. 412. 413. 414. 415. 416. 417. 418. 419. 420. 421. 422. 423. 424. 425. 426. 427. 428. 429. 430. 431. 432. 433. 434. 435. 436. 437. 438. 439. 440. 441. 442. 443. 444. 445. 446. 447. 448. 449. 450. 451. 452. 453. 454. 455. 456. 457. 458. 459. 460. 461. 462. 463. 464. 465. 466. 467. 468. 469. 470. 471. 472. 473. 474. 475. 476. 477. 478. 479. 480. 481. 482. 483. 484. 485. 486. 487. 488. 489. 490. 491. 492. 493. 494. 495. 496. 497. 498. 499. 500. 501. 502. 503. 504. 505. 506. 507. 508. 509. 510. 511. 512. 513. 514. 515. 516. 517. 518. 519. 520. 521. 522. 523. 524. 525. 526. 527. 528. 529. 530. 531. 532. 533. 534. 535. 536. 537. 538. 539. 540. 541. 542. 543. 544. 545. 546. 547. 548. 549. 550. 551. 552. 553. 554. 555. 556. 557. 558. 559. 560. 561. 562. 563. 564. 565. 566. 567. 568. 569. 570. 571. 572. 573. 574. 575. 576. 577. 578. 579. 580. 581. 582. 583. 584. 585. 586. 587. 588. 589. 590. 591. 592. 593. 594. 595. 596. 597. 598. 599. 600. 601. 602. 603. 604. 605. 606. 607. 608. 609. 610. 611. 612. 613. 614. 615. 616. 617. 618. 619. 620. 621. 622. 623. 624. 625. 626. 627. 628. 629. 630. 631. 632. 633. 634. 635. 636. 637. 638. 639. 640. 641. 642. 643. 644. 645. 646. 647. 648. 649. 650. 651. 652. 653. 654. 655. 656. 657. 658. 659. 660. 661. 662. 663. 664. 665. 666. 667. 668. 669. 670. 671. 672. 673. 674. 675. 676. 677. 678. 679. 680. 681. 682. 683. 684. 685. 686. 687. 688. 689. 690. 691. 692. 693. 694. 695. 696. 697. 698. 699. 700. 701. 702. 703. 704. 705. 706. 707. 708. 709. 710. 711. 712. 713. 714. 715. 716. 717. 718. 719. 720. 721. 722. 723. 724. 725. 726. 727. 728. 729. 730. 731. 732. 733. 734. 735. 736. 737. 738. 739. 740. 741. 742. 743. 744. 745. 746. 747. 748. 749. 750. 751. 752. 753. 754. 755. 756. 757. 758. 759. 760. 761. 762. 763. 764. 765. 766. 767. 768. 769. 770. 771. 772. 773. 774. 775. 776. 777. 778. 779. 780. 781. 782. 783. 784. 785. 786. 787. 788. 789. 790. 791. 792. 793. 794. 795. 796. 797. 798. 799. 800. 801. 802. 803. 804. 805. 806. 807. 808. 809. 810. 811. 812. 813. 814. 815. 816. 817. 818. 819. 820. 821. 822. 823. 824. 825. 826. 827. 828. 829. 830. 831. 832. 833. 834. 835. 836. 837. 838. 839. 840. 841. 842. 843. 844. 845. 846. 847. 848. 849. 850. 851. 852. 853. 854. 855. 856. 857. 858. 859. 860. 861. 862. 863. 864. 865. 866. 867. 868. 869. 870. 871. 872. 873. 874. 875. 876. 877. 878. 879. 880. 881. 882. 883. 884. 885. 886. 887. 888. 889. 890. 891. 892. 893. 894. 895. 896. 897. 898. 899. 900. 901. 902. 903. 904. 905. 906. 907. 908. 909. 910. 911. 912. 913. 914. 915. 916. 917. 918. 919. 920. 921. 922. 923. 924. 925. 926. 927. 928. 929. 930. 931. 932. 933. 934. 935. 936. 937. 938. 939. 940. 941. 942. 943. 944. 945. 946. 947. 948. 949. 950. 951. 952. 953. 954. 955. 956. 957. 958. 959. 960. 961. 962. 963. 964. 965. 966. 967. 968. 969. 970. 971. 972. 973. 974. 975. 976. 977. 978. 979. 980. 981. 982. 983. 984. 985. 986. 987. 988. 989. 990. 991. 992. 993. 994. 995. 996. 997. 998. 999. 1000.

|                  | Present |                |                            | Past    |                |                            |
|------------------|---------|----------------|----------------------------|---------|----------------|----------------------------|
| Taxonomic groups | P-value | R <sub>d</sub> | Significant plant species  | P-value | R <sub>d</sub> | Significant plant species  |
| All Fungi        | 0.04    | 0.04           | Asto (-)                   | 0.11    | 0.01           | -                          |
| Ascomycota       | 0.07    | 0.03           | Hmol (-)                   | 0.09    | 0.02           | -                          |
| Glomeromycota    | 0.04    | 0.05           | Cnig (-)                   | 0.08    | 0.02           | -                          |
| Basidiomycota    | 0.12    | 0.03           | Bpin (+)                   | 0.03    | 0.04           | Hlan (-)                   |
| Agaricomycetes   | 0.02    | 0.04           | Hlan (-)                   | 0.02    | 0.04           | Hlan (-)                   |
| Sordiariomycetes | 0.03    | 0.07           | Erep (+) Dglo (+) Lper (+) | 0.01    | 0.08           | Aten (+) Cnig (+) Lper (+) |
| Glomeromycetes   | 0.004   | 0.08           | Hmol (-) Cnig (+)          | 0.01    | 0.06           | Cnig (-)                   |

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Considering the fungal phyla and classes separately, plant-species effects were better revealed. For example, the equitability of the phylum Ascomycota significantly increased with *B. pinnatum* but decreased with *H. mollis* abundances ( $P < 0.01$ ;  $0.11 \leq R^2 \leq 0.14$ ), whereas Glomeromycota equitability increased with *F. rubra* abundance ( $P < 0.01$ ;  $R^2 = 0.09$ ). Several plant species had the same effect on the equitability for every fungal group whereas others had different effects between groups: the abundance of *B. pinnatum* always increased fungal equitability within the Sordariomycetes, Glomeromycetes, and Agaricomycetes whereas *A. stolonifera* increased the equitability of Glomeromycetes but decreased the equitability of Agaricomycetes. The explained variance in the models increased when lower as compared to higher fungal taxonomic levels were considered (e.g. 14% of the variance was explained for the Ascomycota phylum ( $P < 0.01$ ) while ~24% of the variance was explained for the Sordariomycetes class ( $P < 0.001$ ).

**Effect of temporal and spatial scales on the link between plant landscape and fungal community**  
We determined the spatio-temporal scale of response of the fungal community to the plant neighborhood by producing linear models with past and present plant species distributions at five neighborhood sizes (5 to 25 cm). These analyses were performed at the scale of the sample (center of the mesocosm) and the best models were selected according to the AICc criteria (see Material & Methods section). These selected models showed that fungal community richness and equitability responded equally to present and past plant neighborhoods (i.e. the AICc criteria of the models were not different; Tab. 2). Furthermore, the species explaining the variations in richness and equitability of the fungal community were the same at both temporal scales (i.e. past and present). Only the equitability of Glomeromycota, Basidiomycota, and Sordariomycetes and the richness of Glomeromycetes responded to a single temporal scale.

The whole fungal community richness and equitability responded indifferently to the plant neighborhood at the five neighborhood size scales analyzed (i.e. 5, 10, 15, 20 and 25 cm around the sampling point) (Tab. 2). Only the Glomeromycota equitability index responded to a specific neighborhood size (i.e. 25 cm), whereas the two other fungal phyla and the classes analyzed responded to at least two of the five neighborhood sizes tested for both richness and equitability. Thus a clear neighborhood effect was highlighted but no specific neighborhood size was detected in the response of the fungal community to the plant neighborhood.



Table 2. Responses of each fungal group richness and equitability at the sample scale to the past and present plant neighborhoods (results from linear models). P-values and the range of adjusted R<sup>2</sup> of best models are presented. Significant time and spatial scales of neighborhood are also indicated. Only species significantly participating to the model building are showed as well as their effect on the fungal richness and equitability (+ increasing; - decreasing). \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001.

| Taxonomic groups | Time scale of response | Spatial scale of response (radius) | R <sup>2</sup>    | Significant plant species                      |
|------------------|------------------------|------------------------------------|-------------------|--|
| All Fungi        |                        |                                    |                   |  |
| Richness         | Present/Past           | 5 to 20cm                          | 0,07- 0,1 (*)     | A ten (+)                                      |
| Equitability     | Present/Past           | 10 to 25cm                         | 0,04 - 0,07 (*)   | Hmol (-)                                       |
| Ascomycota       |                        |                                    |                   |  |
| Richness         | Present/Past           | 5 to 25cm                          | 0,04 - 0,12 (*)   | A ten (+) Frub (+)                             |
| Equitability     | Present/Past           | 5 to 20cm                          | 0,11 - 0,14 (**)  | Hmol (-) Bpin (+)                              |
| Glomeromycota    |                        |                                    |                   |  |
| Richness         | Present/Past           | 5 to 25cm                          | 0,06 - 0,09 (*)   | Erep (-) Bpin (+) Dglo (-)                     |
| Equitability     | Present                | 25cm                               | 0,09 (**)         | Frub (+)                                       |
| Basidiomycota    |                        |                                    |                   |  |
| Richness         | Present/Past           | 5 to 25cm                          | 0,06 - 0,09 (*)   | Dglo (-) Asto (-) A ten (+) Hlan (-) Cnig (-)  |
| Equitability     | Present                | 15 and 25cm                        | 0,07 (*)          | Asto (-)                                       |
| Sordariomycetes  |                        |                                    |                   |  |
| Richness         | Present/Past           | 20 and 25cm                        | 0,1 - 0,11 (*)    | Erep (-) Bpin (-) Frub (-) A ten (+)           |
| Equitability     | Past                   | 10 and 25cm                        | 0,22 - 0,24 (***) | Bpin (+) Hmol (-) Frub (+,-) A ten (+)         |
| Agaricomycetes   |                        |                                    |                   |  |
| Richness         | Present/Past           | 5 to 20 cm                         | 0,07 - 0,1 (*)    | Dglo (+) Asto (-) Hlan (-)                     |
| Equitability     | Present/Past           | 5 to 20 cm                         | 0,08 - 0,15 (**)  | Dglo (-) Asto (-) Hmol (+) Anob (-)            |
| Glomeromycetes   |                        |                                    |                   |  |
| Richness         | Past                   | 5 to 25cm                          | 0,08 - 0,1 (**)   | Bpin (+)                                       |
| Equitability     | Present/Past           | 5 to 25cm                          | 0,07 - 0,12 (*)   | Bpin (+) Asto (+) A ten (+) Hlan (+) L per (+) |





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## Effect of fungal richness and equitability on the trap plant biomass

To investigate the effect of the changes in fungal communities richness and equitability on the fitness of the trap plant we produced linear models at the sample scale (center of the mesocosm) with fungal groups richness or equitability as explanatory variables.

The biomass of the trap plant *Medicago truncatula* was not affected by the richness of its whole fungal community (Tab 3). However, the biomass increased significantly with the richness of the Basidiomycota and Glomeromycota phyla ( $P < 0.01$  and  $P < 0.05$  respectively) (Tab. 3) but not with the phylum Ascomycota (Tab 3). The combined effects of Basidiomycota and Glomeromycota richness explained ~12% of the variations in plant biomass ( $P < 0.05$ ;  $R^2 = 0.12$ ). At the class level, the same positive effect on *M. truncatula* biomass was found for Glomeromycetes and Agaricomycetes richness but not for Sordariomycetes richness ( $P < 0.01$ ;  $R^2 = 0.13$ ; Tab 3).

If a higher microbiota fungal richness positively impacted the *M. truncatula* biomass, we would expect a reciprocal effect of equitability, because the detection of rare OTUs from the same sequencing depth within a more evenly distributed community, is less likely. As expected, the data analyses indicated that the biomass of the trap plant increased with the equitability of its whole fungal microbiota community ( $P < 0.01$ ;  $R^2 = 0.07$ ; Tab 3). However, the biomass increased only with the equitability of the phylum Ascomycota but not with the Basidiomycota and Glomeromycota phyla ( $P < 0.01$ ,  $R^2 = 0.1$ ; Tab 3). At the class level, the same positive effect on *M. truncatula* biomass was found for Sordariomycetes equitability ( $P < 0.01$ ;  $R^2 = 0.1$ ; Tab 3) but not for Agaricomycetes and Glomeromycetes equitability.

Table 3. Results of linear models testing the effect of each fungal group richness and equitability on the biomass of the trap plant *M. truncatula*. ANOVA P-values, F-values and adjusted R<sup>2</sup> of the best models are presented. Only fungal groups significantly participating to the best model building are presented.

| $\frac{D_{\text{rich}}}{D_{\text{eq}}}$                   |   | $\frac{D_{\text{rich}}}{D_{\text{eq}}}$ |       |           | $\frac{D_{\text{rich}}}{D_{\text{eq}}}$ |       |           |
|---|---|---|-------|-----------|---|-------|-----------|
|   |   | $t$                                     | $C$   | $\hat{w}$ | $t$                                     | $C$   | $\hat{w}$ |
| $t$   | $! \frac{D_{\text{rich}}}{D_{\text{eq}}}$   | $***$                                   | $3.7$ | $0.18$    | $***$                                   | $1.5$ | $0.05$    |
|   | $! \frac{D_{\text{rich}}}{D_{\text{eq}}} + \frac{D_{\text{rich}}}{D_{\text{eq}}}$     | $***$                                   | $***$ | $***$     | $***$                                   | $1.5$ | $***$     |
|   | $. \frac{D_{\text{rich}}}{D_{\text{eq}}} + \frac{D_{\text{rich}}}{D_{\text{eq}}}$     | $***$                                   | $1.5$ |           | $***$                                   |       |           |
| $D_{\text{rich}} + \frac{D_{\text{rich}}}{D_{\text{eq}}}$ | $***$   | $1.5$                                   | $***$ |           |   |       |           |
| $f$   | $\{ \frac{D_{\text{rich}}}{D_{\text{eq}}} + \frac{D_{\text{rich}}}{D_{\text{eq}}} \}$ | $***$                                   | $***$ | $***$     | $***$                                   | $1.5$ | $***$     |
|   | $! \frac{D_{\text{rich}}}{D_{\text{eq}}} + \frac{D_{\text{rich}}}{D_{\text{eq}}}$     | $***$                                   | $1.5$ |           | $***$                                   |       |           |
|   | $D_{\text{rich}} + \frac{D_{\text{rich}}}{D_{\text{eq}}}$                             | $***$                                   | $1.5$ |           | $***$                                   |       |           |

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## Discussion

### Local plant neighborhood as a driver of fungal community

In agreement with our expectations (hyp.1) we demonstrated that the plant neighborhood (neighborhood plant distribution) can leave a fingerprint on the fungal community. The root fungal microbiota of *M. truncatula* was analyzed at two different scales: gamma diversity (i.e. plot scale) and alpha diversity (i.e. sample scale). The plant distribution at the plot scale (i.e. gamma diversity) poorly explained the plot richness of *M. truncatula*'s endophytic fungal microbiota in contrast to the sample scale (i.e. local diversity) (Tab 1). This result suggests that the biotic interactions structuring the fungal community (i.e., host preference and host filtering) mostly act at a centimetric scale (Hazard et al., 2013; Valyi et al., 2016).

At the sample level, our results indicated that the plant neighborhood determined, at least in part, the richness and competitive balance (i.e. equitability) of the whole fungal community colonizing the trap plant, *M. truncatula*. Such heterogeneity of microbiota composition within a given host-plant species is a recurrent observation (e.g. Davison et al., 2011; Schlaeppi et al., 2014; L, Van et al., 2017) and has been linked to plant recruitment from the soil 'reservoir' (Vandenkoornhuyse et al., 2015). Indeed, root-associated fungi in agave species have been shown to be mainly recruited from the surrounding soil (Coleman-Derr et al., 2016). The importance of abiotic factors, notably soil properties, as determinants of the soil microbial pool composition (Shakya et al., 2013; Schreiter et al., 2014; Coleman-Derr et al., 2016) has been repeatedly demonstrated and is considered as the main source of variation of the plant microbiota (Vandenkoornhuyse et al., 2015). We demonstrated here that the plant neighborhood at a centimetric scale (i.e. 5 cm to 25 cm around the sampling point) also determines in part the fungal soil pool available for plant recruitment. Our findings thus open up new avenues in the understanding of plant microbiota assembly rules by introducing the role of the local plant community context (i.e. the plant neighborhood) as a structuring factor.

### The fungal community also results from past plant neighborhoods

In agreement with our expectations (hyp. 2) we demonstrated that the past neighborhood also impacted the fungal community structure of the following year and that this was not due to correlations between past and current neighborhoods. This result was found for the whole fungal

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microbiota community level as well as for the three phyla and classes analyzed. The key interpretation of this result is that past plants do leave a `fingerprint\_ on the composition of microorganisms colonizing the present plant community. Although the fungal communities responded at both temporal scales, there was no difference in the intensity of response between the two years. The persistence of the effect of the plant over these years could be due to the short period investigated in the present study (two years) because the majority of fungal spores and propagules can survive in soil for a long time. A study involving a longer period (i.e. >2 years), for example mapping the plant communities 5 years before sampling, would allow the temporal limits of this potential soil fungal bank `memory\_ to be determined.

The structure of the root endospheric fungal community impacts *M. truncatula* biomass

In agreement with our expectations (hyp.3) we demonstrated that the performance of the *Medicago truncatula* plant was affected by the composition of the fungal endophytes community. The measures of *Medicago truncatula* performance indicated that the individual biomass production depended on the richness of Glomeromycota and Basidiomycota (but not on the total richness of fungal OTUs or on the Ascomycota richness). This relationship between Glomeromycota richness and plant performance has already been demonstrated for AM fungi (Van der Heijden et al., 1998, Klironomos et al., 2000, Hiiesalu et al., 2014), due to their beneficial effects. The increase in AM fungal diversity has experimentally been shown to result in more efficient exploitation of available resources such as soil phosphorus (Van der Heijden et al., 1998) and to decrease plant pathogens (Van der Putten et al., 2009). To our knowledge, however, the positive effect of fungal species richness on plant performance has never been demonstrated with the phylum Basidiomycota or the class Agaricomycetes. As far as we know, little is known about the functions of the endospheric Agaricomycetes in grass plants and the role of this group on plant growth has still to be clarified. Interestingly, *Medicago truncatula* performance only depended on the equitability of the whole community, the phylum Ascomycota and the class Sordariomycetes that represented the larger fungal groups in the samples. This result suggests that a community dominated by a few Ascomycota and Sordariomycetes species has a less beneficial effect on plant growth. Ascomycota is a phylum known to be composed of very diversified organisms performing various functions for host plants. In this context, an equitable community could represent a higher diversity of organisms available to recruitment in the plant `toolbox\_ for adjustment to environmental conditions (Vannier et al., 2015).

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### Plant-plant interaction mediated by fungi affects plant performance

Knowing that the fungal communities compositions were determined by the plant neighborhood and that these changes affected plant performance (biomass productivity), this study demonstrates the existence of plant-plant interactions mediated by the fungal communities. Our results indicate that these shifts in fungal community composition can have positive or negative effects on the trap plant biomass, suggesting that the neighborhood can have either a facilitative or a competitive effect on the trap plant. Studies investigating the shift between plant-plant facilitation and competition suggested that this shift is linked to environmental stress or disturbance intensity and is spatially heterogeneous (O'Brien et al., 2017). Previous studies have indicated that plant-plant facilitation (i.e. a beneficial effect of a given plant presence) may be linked to the compositions of their AM fungal communities (Montesinos-Navarro et al., 2012, Zhang et al., 2014). Montesinos-Navarro et al., (2012) argued that stronger facilitation occurs between pairs of plant species with different associated AMF. This phenomenon underlies a potential mechanism which increases AM fungal diversity in the shared rhizosphere and promotes complementarity between the beneficial effects of each AM fungus (Van der Heijden et al., 1998; Wagg et al., 2011). We herein provide the first experimental evidence of this assumption by showing that the richness of Glomeromycota increased with the abundance of specific plants in the neighborhood, which ultimately increased *M. trunculata*'s biomass. Our results also indicate that changes in plant fungal communities can be detrimental as several plant species had a negative effect on fungal richness and/or equitability. We thus propose that the shift from plant-plant facilitation to competition is mediated by changes in the fungal communities available for recruitment in the surrounding soil. More importantly, we demonstrated here that these phenomena of plant-plant facilitation and competition mediated by fungi are not restricted to AM fungi but have to be extended to other fungal groups such as Ascomycota and Basidiomycota. We thus encourage future studies to consider the whole fungal community and to take into account the feedbacks between plant and fungal communities that can affect ecosystem properties such as productivity (Cadotte et al., 2008).

### Positive and negative effects of given plants on the trap plant biomass

An increase in the plant trap biomass associated with an increase in fungal groups richness (e.g. Glomeromycetes and Agaricomycetes) and equitability (e.g. Ascomycota and Sordariomycetes) was highlighted. A positive impact of *Brachypodium pinnatum* and *Agrostis tenuis* abundance in the

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neighborhood on the productivity of the trap plant *M. truncatula*, mediated by an increase in fungal richness or equitability, was evidenced. This change in fungal soil assemblage may be due to a complementarity of the niches that plants provide to fungal symbionts. The degree of host preference differs between plants (Helgason et al., 2002), which may then constitute different niches for fungi. These differences in host preference can be due to the level of mycotrophy of the host with generalist hosts favoring a rich fungal community contrary to more specialist hosts. Such positive effects of particular plant identities have already been suggested: for instance, spore abundance in salt marsh was determined by the proximity of mycotrophic hosts (Carvalho et al., 2003), whereas the presence of the grass *Anthoxantum odoratum* increased the abundance of AM fungi in the soil regardless of the plant mixtures (De Deyn, 2010).

Conversely, we also demonstrated in our experiment the negative effects of several plant species on the richness and equitability of key fungal groups, which ultimately reduced the trap plant biomass. For example, *H. mollis*, *A. stolonifera* and *H. lanatus* indirectly decreased the productivity of the trap plant *M. truncatula*. This negative impact might be due to a host-preference effect. Indeed, several groups of fungi can harbor a limited range of host plants. A given plant can thus represent either a good or a bad choice of host. It can also be interpreted as an allelopathic-like phenomenon although it has not been evidenced before on these species. For example, a species belonging to the family *Festuca* has been shown to produce allelopathic compounds suppressing *D. glomerata* growth but depending on the competition context of the community (Viard-Crétat et al., 2012). Plant production of allelopathic compounds may suppress the germination of spores or the formation of mycorrhizal associations (Stinson et al., 2006; Callaway et al., 2008) and thus alter the reservoir of soil microorganisms.

We herein explain the competitive balance and richness of the fungal communities colonizing *Medicago truncatula* based on the close neighborhood of plants surrounding the sampling point. These findings expand the rules of plant fungal microbiota assembly to consideration of the fingerprint that a plant in the local neighborhood can leave on the soil fungal pool. These plant filtering mechanisms operated on the fungal community at a finer scale than previously thought (below 25 cm). The clear influence of plant neighborhood on the local soil microbial reservoir is an additional ecological force driving microbiota complexity and heterogeneity with strong consequences on the focal plant fitness. Recent advances demonstrated a

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significant impact of the leaf-associated microbiota diversity on ecosystem productivity (Laforest-Lapointe et al., 2017). We extend this idea to include the root-associated microbiota and support the correlation between plant-associated microbial diversity and ecosystem productivity, suggesting that manipulations of plant neighborhoods can be used to improve biodiversity-ecosystem functioning relationships.



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#### Author Contributions

A.K.B., P.V. and C.M. conceived the ideas and experimental design. A.K.B. and C.M. did the experiments. N.V. did the data analyses. N.V., A.K.B., P.V. and C.M. did the interpretations and writing of the publication.

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## GENERAL DISCUSSION AND PERSPECTIVES

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## I. Plants and microorganisms: a tight association

### Microbiota matters for plant phenotype

Considering the diversity of functions provided by the plant microbiota, it is clear that endophytes have important impacts on plants phenotype (Article I). Clonal plants that represent most of the known plants in temperate ecosystems (van Groenendael & de Kroon, 1990) are no exception to this rule (Article II). We demonstrated that the inoculation of symbionts (AM Fungal species) can alter architectural, allocative and reproductive traits (Article II). The effects of these plant-associated microorganisms on the traits involved in clonal plants foraging and specialization have been poorly described to date. Our experiments thus provided indications that AM fungi could be responsible for changes in the ability of clonal plant to produce foraging and specialization responses.

More importantly, we showed that AM fungal species used have contrasted effects on plant traits. In our experiments, the difficulty of identifying the role of a given symbiont in determining the plant phenotype resided in the fact that a given plant was colonized by different symbionts with contrasted effects at the same time (Article II). It is thus necessary to identify the range of variations due to symbiont identity. A perspective of this work would thus be to test a wider range of AM fungal partners to determine whether they specifically modify plant traits and act on performances. We performed a preliminary study (not presented in the document) testing this hypothesis. We grew individuals of *G. hederacea* in controlled conditions inoculated with a fungal isolate or not inoculated. We tested the effect of nine fungal species selected to constitute a large range of cooperative behavior (i.e. providing high benefits to low or no benefits). We found significant differences in various plant traits comprising performance traits depending on the fungal partner identity.

In addition to direct effects described herein (Article II), the microbiota can also indirectly impact plant phenotype through its interplay with epigenetic mechanisms (Article I). These two sources of phenotypic variation happening during the plant's life currently constitute two separated fields of research. The few observations of a link between epigenomic modifications and the establishment of symbiosis (Ichida et al., 2007, 2009; De Luis et al., 2012) suggest however that



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interplays does exist. These two separated fields of research will thus need to develop a common framework to determine the occurrence and significance for plant phenotype of microbiota-epigenetics interplays. Conducting a monitoring of epigenetic marking while inoculating different experimentally engineered microbiota would surely provide insightful results and could allow to disentangle complex endophytes-induced phenotypic changes.

Plants have evolved mechanisms ensuring the presence of symbionts

Plants have been interacting with microorganisms for a very long time (over 400 millions years for AM fungi; Remy et al., 1994; Redecker et al., 2000) allowing the establishment of co-evolution patterns (Brundrett, 2002). Subsequently, plants should have evolved mechanisms to optimize their association with beneficial symbionts. Many studies have described how plants have evolved, filtration, defense, promotion or regulation mechanisms regarding microorganism colonization. For example, mechanisms allowing plants to regulate the symbiosis and avoid cheating behavior with AM fungi have already been evidenced (Bever et al., 2009; Kiers et al., 2011). However, since plants are sessile organisms, their `recruitment choice\_ depends on available microorganisms in the local environment (e.g. Vandenkoornhuyse et al., 2015). In this context, the heterogeneity of beneficial microorganisms presence in the soil could be considered as a source of stress for plant (i.e. absence of beneficial microbes).

Regarding this heterogeneity, different forms of `continuity of partnership\_ could be expected to ensure beneficial interactions. Clonal plants are known to display plastic responses maximizing either exploration (foraging) or exploitation (specialization) of heterogeneous resources relying on their ability to share resources and information within the clonal network. Such responses could then be expected to buffer beneficial microorganisms heterogeneity. In our experiments (Article II), we however demonstrated that *Glechoma hederacea* only displayed a low foraging and no specialization response to AM fungi heterogeneity.

Clonal plants have however evolved another mechanism allowing to ensure the presence of microorganisms and thus their habitat quality. We evidenced the existence of vertical transmission (or heritability) of microorganisms through the connexions between ramets (Article III). This previously unknown mechanism allows the transfer of a core microbiota that ensures habitat quality of the plant progeny. This results suggest that plants physiological integration (Oborny et al., 2001)

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comprise in addition to resources and information, the sharing of microorganisms, constituting a prospect of interest.

These results open a large avenue for future research questions such as 1.) the direction of this transfer (unidirectional or bidirectional; i.e. from progeny to parent plants), 2.) the mechanisms of transfer, active through the vascular network fluxes or passive by colonization of the stolon surface; 3.) the modalities of microorganisms filtering during the process. Considering the latter hypothesis we indeed evidenced that transmitted communities are similar and an important consequence is the existence of a filtration process during the transmission that creates this similarity. This filtration process could be based either on microorganisms dispersal ability (i.e. ability to colonize stolons) or on the functions they provide to plants. Different hypotheses can be drawn from these results like the transmission of a core microbiota necessary for the clone establishment or alternatively the transfer of only the most cooperative and important fraction of the microbiota transmitted. We are currently testing the hypothesis that more beneficial symbionts could be preferentially transmitted. This experiment comprises two phases. A first phase (already completed, see above) aims at identifying the impact of the inoculation of single AM fungal species on plant traits and performance to detect beneficial and deleterious AM species. The second phase of the experiment consists in the inoculation of clonal plants with a designed mixture of AM fungal species comprising a range of symbiont qualities. This second phase aims at determining which AM fungal species are transmitted to the progeny, depending on their level of cooperation and their effects on plants.

Estimating the ecological significance of this mechanism should be the main focus of future research by determining its ubiquity, fidelity and its significance for plant phenotype expression. The next step would thus be to test if specific microbial cores can be recruited depending on plant ecological needs. For example when the plant is subjected to a stress, it should preferentially transfer organisms conferring an increased resistance to this particular stress.

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## II. Redefining the individual plant : from holobiont to meta-holobiont

Plants can no longer be considered as standalone entities

Plants are colonized by a high diversity of symbionts that individually (Article II) and collectively (Articles I and V) determine the plant phenotype and its subsequent ability to grow, reproduce and adapt to environmental conditions (Article I). This microbiota is present in every known plant and can be transmitted between generations (Article II). The plant can thus no longer be seen as an independent entity. Regarding selection and evolution processes the plant has to be considered as a complex and multiple entity comprising the plant and its microbiota forming the holobiont as well as their genomes forming the hologenome (Vandenkoornhuyse et al., 2015). Selection processes can act at different organisation levels in such complex organisms (i.e. on the plant phenotype, on the microorganisms phenotypes or on the result of their interactions).

Toward a novel understanding of clonal network organisms

The discovery of a novel microbiota heritability mechanism deeply impacts our understanding of plant phenotype and evolution. Not only plants can be considered as holobionts but the structure of clonal plants in network connecting clonal ramets extends the holobiont concept to a more complex scale that we have called the meta-holobiont (article IV). The clonal network allows indeed the dynamic sharing of microorganisms between ramets. Different holobionts are connected in clonal network allowing them to exchange of part of their hologenome. The network thus constitutes an additional level on which selection can act. Selection can act on the phenotype of the individual holobiont or on the phenotype of the network. In addition, these two levels of organization are intricated because the fitness of one member of the network could effect the fitness of the whole network.

This idea could be extended to other clonal networks as soon as organisms can be shared within the network. Future research are needed on clonal organisms in order to determine whether such structure happens to exist in other organisms or if the meta-holobiont only fits for clonal plants. Even if the meta-holobiont theory developped herein (Article V) cannot be extended to other organisms, we emphasize the suitability of the clonal plant model (and thus the meta-holobiont) for the study of microbiota assembly in the context of complex organisms.

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The role of the meta-holobiont in determining microorganisms dispersal and plant fitness in natural ecosystem is a major perspective. Especially, in the context of plant community, transmission and filtration mechanisms in the meta-holobiont could impact the dispersal of beneficial symbionts in the environment. If the meta-holobiont is able to selectively facilitate the spreading of the most useful microorganisms within the plant community it could impact the microbiota assembly of the whole plant community.

### III. From individuals to community

The community context shapes the microbiota

At the plant community scale, plants are interacting with each other in neutral, competitive, or facilitative ways. A given plant in the community is thus interacting with its neighborhood and it is likely that this neighborhood could affect the assembly of its microbiota. More importantly, at a given location, the fungal propagule reservoir (i.e. the soil pool) available for recruitment by the plant is a consequence of the fungal community within the previous host-plants. We demonstrated that the plant neighborhood determines in part this fungal soil pool available for plant recruitment (Article V). We explained the competitive balance and richness of the fungal communities colonizing the trap plant *Medicago truncatula* based on the close neighborhood of plants surrounding the soil sampling point. Valyi et al. (2016) proposed that the relative influence host filtering on the AM fungal community is stronger at local scale (Valyi et al., 2016). Our results fits perfectly with this hypothesis and introduce the role of the local plant community context (i.e. the plant neighborhood) as a factor structuring the assemblage of the plant microbiota, aside from the plant genetics and the environmental factors like soil properties. The results obtained herein (Article V) suggest that the host preference and host filtering structuring the fungal community mostly act at centimetric scale (Hazard et al., 2013; Valyi et al., 2016). The effect of the plant neighborhood occurred at finer scale than previously thought (i.e. 5 cm to 25 cm around the sampling point) on the fungal community richness and composition. Previous studies focusing on AM fungi reported that AM fungal community composition was highly heterogeneous, even at local scale (Brundrett & Abbott, 1995; Carvalho et al., 2003; Wolfe et al., 2007). Bahram et al. (2015) also reported in a meta-analysis the existence of a spatial autocorrelation of AM fungi, suggesting thus dispersal patterns of fungi at the scale of the meter.

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This corpus of knowledge, together with our results, suggest that fungi, and possibly more widely the microorganisms, are dispersal limited at the scale of the plant community. However, evidences that isolation and dispersal limitation exist for microbial assemblages are scarce (Telford et al., 2006). There is thus a need to reconsider the scale at which we study plant microbiota assembly rules. In addition, the mechanism of microbiota heritability evidenced herein (Article III) allow the dispersal of fungi in the community and calls for the development of a framework on microorganisms dispersal.

Toward the concept of micro-organisms micro-landscape

Plant host has been shown to affect the microbiota structure, in particular its composition and diversity (Berg & Smalla, 2009; Bulgarelli et al., 2012; Lundberg et al., 2012). In addition, we demonstrated that the plant neighborhood can have contrasted effects on fungal taxonomic groups (e.g. different phyla or classes) richness and equitability. Theoretically, niche partitioning and neutral processes (Hubbell, 2001, 2005) are classically admitted to drive community assemblies and explain diversity patterns. In the context of symbiotic microorganisms, plants can be considered as habitat and the assembly of the plant community is driven by the niche partitioning of microorganisms. This result thus suggest that a particular plant constitutes a host of a specific quality (favorable or unfavorable) that can be assimilated to a patch of habitat for symbiotic organisms. From here, the plant community can be assimilated to a dynamic mosaic of patches. The composition of the patches and their spatial arrangement could thus determine the microbiota assembly of plant species within the mosaic.

In this context, a major perspective is to transpose and adapt macro-landscape ecological frameworks at the scale of the microorganisms landscape. Plant community can be redefined from the fungal perspective in an attempt to characterize what habitat size, isolation and dispersal should be considered in microbial ecology. From our results, we develop the idea that for a given fungus, the plant community is a dynamic mosaic of plants of various quality that can be assimilated to patches in a landscape. An important consequence of this understanding of the fungal landscape is the transposition of macro-landscape elements to a micro-scale. For example, a perspective of this approach is to investigate the impact of the connectivity in the fungal micro-landscape. The connectivity describe the permeability of landscape elements to the dispersal of organisms. If plants can be considered as habitat of different quality for a fungus, then the abundance of a favorable host

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within the fungal landscape would increase the dispersion of the fungus. We are currently engaged in the redaction of a review (not presented herein) that aims at transposing these knowledge of macro-landscape to the micro-landscape, introducing the micro-landscape. In parallel, we conducted an experiment in collaboration (not presented herein) using experimental mesocosms to test the hypothesis that landscape parameters such as the connectivity between two hosts (defined as the presence of favorable hosts between the focal plants) could explain the similarity of their microbiota.

#### IV. Microbiota assembly and agricultural practices

The ability to engineer plant-microbiota is a key challenge to resolve the productivity-biodiversity loss conundrum. Microbiota engineering aims at optimizing specific attributes of the focal host organisms through the selection of a specific microbiota (Møller et al., 2016). In plants, several candidate traits have been identified, comprising for example flowering time and plant biomass (Panke-Buisse et al., 2015). To fulfill this goal, future research will have to describe in precision on one the hand the rules driving microbiota assembly and on the other hand the host-plant fitness resulting from a given assembly. While beneficial microorganisms have started to be commercialized (Berg, 2009; and see de Vrieze, 2015 for a chronicle) they are not always reliable on crops, especially depending on the local context. Our results provide evidences that it is not only the presence of one particular beneficial microbe that increases plant performance but also the global richness of the plant fungal microbiota (Article V). For a few years, scientists have proposed improvements for agricultural practices such as the increase in plant crop richness to promote microorganisms diversity and then enhance the diversity of ecological functions provided to the plant (Duhamel & Vandenkoornhuyse, 2013). The work presented herein demonstrated that the rules of plant microbiota assembly, comprising the richness of major fungal groups, and the resulting effects on plant phenotype depend on the plant community context (Article V). There is thus perspectives for agricultural practices to consider not only the plant richness but also the plant composition and spatial arrangement.

Studies have until now focused on either the identification of key beneficial microorganisms or the maximization of microorganisms diversity. We additionally demonstrate that opportunities exist of maximizing the equitability of the microbiota to increase plant fitness. In addition, the

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effect on plant phenotype does not necessarily rely on the plant community richness as previously thought, but can also depend on plant identities in the host neighborhood. This encourage for the development of polyculture practices. For example, combination of facilitative plants can be used in cultures to maximize key fungal groups richness thus ultimately increasing productivity.

To conclude, i can see two major perspectives arising from this PhD thesis. The first perspective is linked to the above mentioned limits of the neo-Darwinian synthesis of evolution in describing and understanding plant-microbes interactions and their evolutionary consequences. Both microbiota and epigenetics are not linked to the genome and are thus not integrated in the synthesis. The synthesis do not encompass biological units of organization like holobionts and meta-holobionts. From my perception, one of the major challenges of the next few years relies on the development of an extention for the neo-Darwinian synthesis of evolution integrating non-genome based and interactions mediated evolutionary patterns. The second pespective is linked to the concrete application of acquired knowledges for the development of a sustainable agriculture. The results obtained during this PhD highlights the complexity and the dynamics of plant microbiota assembly and of its resulting effects on plant phenotype. From my perception thus, the ability to maximize crops resistance, resilience and productivity does not reside on the study of a given beneficial microorganism but rather on the understanding of the mechanics regulating emerging properties and functioning of microbiota assemblages.





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## RESUME

Les plantes vivent en association avec une grande diversité de micro-organismes qui forment son microbiote. Ce microbiote fournit des fonctions clés qui influencent tous les aspects de la vie d'une plante, son établissement, sa croissance et jusqu'à sa reproduction. Cette thèse a pour intention de déterminer les règles d'assemblage du microbiote et ses conséquences pour le phénotype, l'adaptation et l'évolution des plantes. Pour atteindre cet objectif nous avons utilisé différentes approches expérimentales comprenant des plantes clonales comme organismes modèles ainsi que des mésocosmes prairiaux pour des analyses à l'échelle des communautés.

Nos résultats ont démontré i) que les Champignons Mycorhiziens – Arbuscules induisent d'importantes variations phénotypiques pour les traits des plantes clonales impliqués dans l'exploration de l'espace et l'exploitation des ressources. Ces changements dépendent de l'identité des symbiotes et altèrent les capacités des plantes à développer des réponses plastiques à l'hétérogénéité environnementale. ii) Les plantes ont évolué un mécanisme permettant la transmission d'une partie de leur microbiote à leur descendance, assurant ainsi la qualité de leur habitat. iii) Le contexte spécifique des communautés de plantes est un facteur majeur structurant l'assemblage du microbiote des plantes à l'échelle locale. L'abondance de certaines espèces de plante dans le voisinage d'une plante cible augmente ou diminue la diversité de son microbiote, déterminant in fine ses performances.

De manière générale, cette thèse démontre l'importance des organismes symbiotiques dans la compréhension de l'adaptation et de l'évolution des plantes. À partir des connaissances acquises nous avons développé une nouvelle compréhension des interactions symbiotiques chez les plantes clonales en introduisant la théorie du méta-holobionte comme extension de la théorie de l'holobionte.

Mots-clés : Plantes clonales ; Microbiote ; Champignons Mycorhiziens – Arbuscules ; Holobionte ; Interactions plante-plante ; Assemblage des communautés

## ABSTRACT

Plants live in association with a wide diversity of microorganisms forming the microbiota. The plant microbiota provides a variety of key functions that influence many aspects of plant's life comprising establishment, growth and reproduction. The present thesis aims at determining the assembly rules of the plant microbiota and its consequences for plant phenotype, adaptation and evolution. To fulfill this objective, we used different experimental approaches using either clonal plants as model organisms or grassland mesocosms for community-wide analyses.

Our results demonstrated i) that Arbuscular Mycorrhizal Fungi induce important phenotypic variations in clonal plants traits involved in space exploration and resources exploitation. These changes depended on the identity of the symbionts and altered the plants ability to produce plastic responses to environmental heterogeneity. ii) Plants have evolved a mechanism allowing the transmission of a part of their microbiota to their progeny, ensuring thus their habitat quality. iii) The plant community context is a major factor structuring local plant microbiota assembly. Particular plant species identity in the neighborhood increase or decrease the microbiota diversity and ultimately determine the focal plant performance.

This thesis overall demonstrates the importance of symbiotic microorganisms in the understanding of the plant adaptation and evolution. From the knowledges acquired we developed a novel understanding of symbiotic interactions in clonal plants by extending the holobiont theory to the meta-holobiont theory.

Keywords: Clonal plants; Microbiota; Arbuscular Mycorrhizal Fungi; Holobiont; Plant-plant interactions; Community assembly