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Diversité, résilience et fonctionnalité des communautés ectomycorhiziennes dans les sols urbains : du diagnostic moléculaire à l'aménagement urbain

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie des Interactions

École doctorale GAIA

Portée par le Centre d'Ecologie Fonctionnelle et Evolutive, l'agence Ilex Paysages + Urbanisme
ainsi que l'établissement public d'aménagement Euroméditerranée

Diversité, résilience et fonctionnalité des communautés ectomycorhiziennes dans les sols urbains : du diagnostic moléculaire à l'aménagement urbain

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Le 13 décembre 2023

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UNIVERSITÉ
DE MONTPELLIER

Résumé

Dans les écosystèmes urbains – tels que les parcs, les alignements d’arbres ou encore les friches industrielles – la composition des communautés ectomycorhiziennes et sa capacité à établir des interactions avec les plantes restent peu documentées. Pourtant, en Méditerranée, une vaste gamme d’arbres et d’arbustes peuplent les écosystèmes urbains, en interaction avec des champignons ectomycorhiziens. Ces organismes favorisent l’assimilation des nutriments et de l’eau par les plantes, contribuant à leur survie. Ce rôle central assuré par les champignons ectomycorhiziens pour le compte des plantes en fait des candidats privilégiés pour le développement de méthodes innovantes de conception de « sols vivants » à la base de communautés végétales plus longévives.

Cette thèse CIFRE est née d’une demande sociétale, et constitue la concrétisation d’une coopération étroite entre la recherche fondamentale en écologie des communautés, l’architecture des paysages et l’ingénierie de l’aménagement public. Elle s’appuie sur une analyse moléculaire des réservoirs fongiques des sols urbains pour concevoir des itinéraires techniques de restauration de sols dégradés d’une part, et de conception d’espaces verts résilients d’autre part. Au cours de ce travail, nous avons tout d’abord analysé le corpus scientifique disponible sur les réseaux d’interactions ectomycorhiziens, et analysé plus particulièrement l’état de l’art des interactions décrites dans les sols urbains. Nous avons ensuite questionné les limites actuelles des méthodes métabarcoding pour la caractérisation des communautés ectomycorhiziennes. Dans un troisième volet, nous avons analysé la composition de l’inoculum fongique ectomycorhizien présent au sein de sols urbains excavés après 70 ans de scellement. Nous avons développé une technique d’inoculation de la communauté fongique basée sur l’addition d’une fraction de sol issu des communautés des milieux adjacents pour la restauration écologique de tels sols. Trois résultats majeurs émergent de ce travail :

- 1) Dans les écosystèmes naturels, les connaissances sur les réseaux d’interactions ectomycorhiziens sont principalement issues d’écosystèmes forestiers, les stades successionnels arbustifs et les écosystèmes anthropisés ayant fait l’objet de peu de travaux.
- 2) Une utilisation optimale du métabarcoding pour décrire la composition des communautés fongiques ectomycorhiziennes pourrait s’appuyer sur un compromis

associant 1) un nombre élevé d'échantillons environnementaux (sols, racines) afin d'intégrer la patchiness des communautés et 2) une profondeur modeste de séquençage, afin d'optimiser la capacité de l'outil à caractériser les communautés sur un nombre réduits de lectures.

- 3) La banque de spores ectomycorhizienne résiduelle des sols scellés est pauvre en espèces, mais capable de s'établir sur les racines des végétaux après 70 ans d'isolement en sol scellés. La pratique d'inoculation d'une fraction de sol issue des milieux naturels adjacents montre des résultats prometteurs avec une mobilisation efficace par les plantules des communautés fongiques issues des garrigues.

Sur le plan appliqué, ce travail met en lumière l'importance de la composante souterraine des écosystèmes urbains, et des interactions plantes – champignons qui s'y déroulent. La réhabilitation de sols urbains par la ré-inoculation de substrats permet d'assister le développement des communautés végétales des espaces végétalisés en ville. Plus largement, cette thèse propose des pistes de recherche fondamentales et appliquées afin d'améliorer notre connaissance de l'écologie des champignons ectomycorhiziens basée sur l'expérimentation *in situ* en ville. Elle démontre aussi la nécessité d'une réflexion à plusieurs échelles du paysage, impliquant la diversité des acteurs impliqués dans le milieu urbain.

Mots-clés : Communautés ectomycorhiziennes, réseaux d'interaction ectomycorhiziens, écologie urbaine, sols scellés, métabarcoding, restauration des sols, forêts méditerranéennes, garrigues

Abstract

In urban ecosystems – such as parks, trees alignments or industrial wastelands – the composition of ectomycorrhizal communities and its capacity to establish interactions with plants remains poorly documented. However, in the Mediterranean biome, the vast majority of trees and shrubs of urban ecosystems are ectomycorrhizal host plants. Ectomycorrhizal fungi highly contribute to plant establishment, growth and survival through promoting nutrient and water assimilation of nutrients and water by plants. Consequently, ectomycorrhizal fungi are ideal candidates for the development of innovative methods for designing “living soils” at the base of longer-lived plant communities.

This PhD project emerges from a societal request and is conducted in close cooperation between fundamental research in community ecology, landscape architecture and public planning engineering. It is based on a molecular analysis of fungal reservoirs in urban soils to develop restoration methods of degraded urban soils and build resilient green spaces. During this work, we first analyzed the scientific corpus available on ectomycorrhizal interaction networks, and more particularly analyzed the state of the art of interactions described in urban soils. We questioned the current limits of metabarcoding methods for the analysis of ectomycorrhizal communities. In a third part, we analyzed the composition of the ectomycorrhizal fungal inoculum present in urban soils excavated after 70 years of sealing. Under the perspective of ecological restoration of such soils, we developed a fungal community inoculation technique based on the addition of a fraction of soil from adjacent natural ecosystems. Three major results emerge from this work:

1) In natural ecosystems, the academic corpus on common mycorrhizal networks mainly comes from forest ecosystems. The successional stage of shrubland and anthropized ecosystems having been little regarded.

2) Optimal use of metabarcoding to describe the composition of ectomycorrhizal fungal communities could rely on a compromise combining 1) a high number of environmental samples (soils, roots) in order to integrate the patchiness of the communities and 2) a depth modest sequencing, in order to optimize the tool's ability to characterize communities on a reduced number of reads.

3) The residual ectomycorrhizal spore bank in sealed soils host degraded ectomycorrhizal communities that are still capable to establish mutualistic interactions with plants after 70 years of isolation in sealed soils. The efficiency of fungal inoculation through the transfer of a fraction of soil from adjacent natural environments was assessed by the effective mobilization of shrublands ectomycorrhizal propagules by seedlings.

From an operational perspective, this work highlights the importance of the belowground biotic component of urban ecosystems –and the plant-fungus interactions established in the soil. The restoration of urban soils through the re-inoculation of ectomycorrhizal propagules in the substrate can support the development of long-lived plant communities in vegetated urban areas. This thesis proposes fundamental and applied research avenues to improve our knowledge of the ecology of ectomycorrhizal fungi based on *in situ* experimentation in cities. It demonstrates the need for reflection on several scales of the landscape, involving the diversity of actors involved in the urban environment.

Key words: Ectomycorrhizal communities, common mycorrhizal networks, urban ecology, sealed soils, metabarcoding, soil restoration, Mediterranean forests, shrublands

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Chapitre 1

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Chapitre 2

Figure 1: Study methodological pipeline

Figure 2: Effective sequencing depth of our study including a) vs theoretical calibrated depths and b) distribution of sequences of fungi and other organisms obtained in soil, on ectomycorrhizal root tips and in the rhizosphere at two sequencing depths

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Chapitre 3

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Figure 4: Composition of ectomycorrhizal fungal communities in soils (a) and on roots of 8-months old seedlings (b) grown in three types of native soils (*Quercus ilex* forest vs Mediterranean shrublands (garrigues) vs 60-yr long urban sealed soils) and in four modalities of inoculation (0.01, 0.1, 1 and 10% of final soil volume) of urban soils with each of the two natural soils (i.e. *Quercus ilex* forest vs Mediterranean shrublands). Distribution of fungal life-forms in soils (c) and on roots of 8-months old seedlings (d) in the same conditions of growth. Colors indicate fungal genera (panels a and b) or fungal life-forms (c and d). Values are the number of reads obtained from Illumina MiSeq metabarcoding of soils or root tissues

Figure 5: Proportion of ascomycete (black) and basidiomycetes (white) ectomycorrhizal fungal ASVs, in *Quercus ilex* forest vs Mediterranean shrublands (garrigues) vs 60-yr long urban sealed soils and on roots of 8-months old seedlings of plant species (*Arbutus unedo* vs *Cistus monspeliensis* vs *Pinus halepensis* vs *Quercus ilex*) grown in the three types of soils.

Table 1: Sampling design for metabarcoding of soils and ectomycorrhizal root apices, with detail of obtained dataset.

Table 2: Number of EM fungal ASVs detected by metabarcoding in three types of sampled soils (*Quercus ilex* forest vs Mediterranean shrublands (garrigues) vs 60-yr long urban sealed soils) and on roots of 8-months seedlings grown in these three types of soils.

Chapitre 4

Figure 1: Concentric view at the landscape scale of a) fundamental research issues and b) applied research issues

Figure 2: Case study of a city-based experiment in Marseille, Les Fabriques

Box 1: The case of Marseille, Les Fabriques: a pioneer district experimenting sealed soil recycling, EM fungi inoculation and alternatives of intensive watering.

Discussion générale

Figure 1 : Schéma conceptuel du lien entre diversités ectomycorhizienne résidente et transférable en milieu urbain au cours de l'évolution des paysages méditerranéens (série dynamique du chêne vert sur calcaire)

Figure 2 : Expérimentation en conditions contrôlées sur les réseaux d'interaction ectomycorhiziens avec a) design expérimental, b) photographies et c) quelques résultats préliminaires

Figure 3 : Fausse roquette (*Diplotaxis erucoides*, Brassicacées) : une rudérale non mycorhizée qui colonise les systèmes urbains, témoin du manque d'inoculum ?

Figure 4 : a), b), mise en andain des sols destinés aux fosses de plantations à Lyon, France, c) sol récemment descellé dans le quartier des Fabriques, Marseille, et d. Végétalisation du sol récemment descellé dans les fosses de plantation expérimentales du quartier des Fabriques, Marseille, France

Figure 5 : a) Mortalité d'arbres plantés (trop grand ?) sur le campus universitaire de Montpellier et b) Pépinière de production horticole pour l'aménagement urbain, Vanucci Pianta à Pistoia, Italie

Figure 6 : Vers des villes intégrées dans un continuum paysager

Encadré 1 : Quels traits fonctionnels des champignons ectomycorhiziens peuvent se révéler intéressants dans une perspective d'aménagement urbain ? (Construit à partir de Zanne *et al.*, 2019)

Encadré 2 : Faux négatif : attention aux faux amis et vrais ennemis !

Encadré 3 : Repenser les itinéraires de pépinières ?

Interstices

Planche 1 : a) Purification des amplicons automatisée, b) nettoyage de la flow-cell avant séquençage, c) séquenceur MiSeq, Illumina, d) c'est parti

Planche 2 : a) expérimentation en conditions contrôlées sur le Terrain Expérimental du CEFE, Montpellier, France, b) plantules de chêne vert, arbousier et pin d'Alep dans les rhizotrons, c) mesure des taux de colonisation des apex mycorhizés et excision des mycorhizes destinées au séquençage, d) site de prélèvement du sol scellé à Marseille, quartier des Fabriques, France

INTRODUCTION GENERALE

Glossaire de l'introduction générale

Les mots introduits par ce glossaire sont classés par ordre alphabétique. Ils sont en italique dans le corps du texte.

Amplicon : Fragment d'ADN synthétisé artificiellement par un procédé d'amplification tel que la PCR (Polymérase Chain Reaction) – notamment pour des fins de séquençage.

Banque de spores : Ensemble des propagules fongiques présentes dans le sol.

Chimère : Séquences hybrides constituées par deux ou plus séquences moléculaires. Elles peuvent être générées par des erreurs de répllication lors de la PCR. Ces séquences ne sont pas exploitables pour des analyses écologiques et sont extraites du jeu de données.

Écologie des communautés : Domaine d'étude qui porte sur les assemblages d'espèces qui coexistent dans un écosystème – sans relation trophique directe. L'écologie des communautés analyse les mécanismes environnementaux (filtrage climatique, pédologique) qui régissent l'assemblage des espèces dans un écosystème ainsi qu'aux interactions entre les individus, d'espèce différentes ou non.

Feedbacks positifs entre plantes et sol – ou *plant-soil feedbacks* : Ou boucle de rétroaction positives entre plante et sol. Ce sont des processus au cours desquels les propriétés biotiques et abiotiques de l'écosystèmes sont altérées réciproquement, sous l'action des individus peuplant l'écosystèmes – y compris les champignons ectomycorhiziens. Les feedbacks positifs entre sols et plantes ont des conséquences sur la structure physico-chimique du sol, ainsi que sur la croissance et la survie des communautés végétales.

Fructifications épigées vs hypogées : Les champignons disposent des structures reproductives dites épigées (majorité des fructifications des champignons de la lignée des Basidiomycètes) lorsqu'elles sont produites à la surface du sol. Les fructifications hypogées sont produites dans le sol. Ce sont des fructifications gastéroides de type « truffe », qui concernent majoritairement les taxons de la lignée des Ascomycètes.

Guilde trophique : L'ensemble des taxons ayant adopté une stratégie commune d'acquisition des ressources forme une guilde trophique. Chez les champignons, on retrouve plusieurs guildes trophiques dont les champignons saprotrophe, mutualistes, ou encore parasitiques.

Sclérote : Forme de vie fongique, notamment pour sa conservation dans le sol lors d'épisodes climatiques contraignants (sécheresse, hivernage). Ce sont des corps durs formés de mycélium compact.

Singleton : Séquence présente en un seul exemplaire dans un jeu de données issu de séquençage moléculaire. La suppression des singletons pour les analyses écologiques est courante. Cette suppression est toutefois problématique car le chercheur ne connaît pas la véracité de la donnée – et extrait peut-être une information biologiquement viable.

Services écosystémiques : Actions des écosystèmes sur le climat, les cycles biogéochimiques et sur la biodiversité qui bénéficient aux humains – tels que la régulation climatique, le stockage de carbone, l'absorption de l'eau par les sols, la réduction de la pollution atmosphérique, la pollinisation ou encore l'accueil de la biodiversité. Il existe également une quantification des services écosystémiques en valeur économique / marchande.

Successions végétales : Processus d'évolution de l'écosystème au cours duquel les conditions environnementales et les communautés végétales se modifient mutuellement. On distingue les phases de succession primaire – au cours desquels un espace est colonisé et transformé par des espèces dites pionnières (plantes herbacées et arborescentes) – des successions secondaires – au cours desquelles des communautés dites de fin de succession s'établissent (communautés végétales arborées).

Taq polymérase : Il s'agit d'une enzyme qui a été isolée à partir de la bactérie *Thermus aquaticus*. Elle est utilisée pour la synthèse de l'ADN lors des réactions de PCR (Polymerase Chain Reaction) en raison de sa capacité à résister dans des environnements à températures élevées (dont les 95°C appliqués à la phase de dénaturation de l'ADN).

Traits fonctionnels : Les traits fonctionnels des espèces sont des caractères morphologiques, anatomiques, physiologiques ou biotiques associés à des fonctions dans l'écosystème. Par

exemple, un trait largement employé en écologie végétale est celui de la surface foliaire spécifique, qui renseigne sur la capacité photosynthétique de la plante.

I. La guilde ectomycorhizienne : composition, fonction, méthodes d'étude

1. Le règne fongique : taxonomie, guildes et écologie

Les champignons constituent l'un des règnes du vivant les plus diversifiés et dont la majeure partie reste encore à découvrir (Hawksworth and Lücking, 2017). Seul 8 % du nombre total d'espèces estimé, c'est-à-dire entre 2.2 et 3.8 millions, ont été décrits (Hawksworth and Lücking, 2017). La diversité des champignons n'est pas que taxonomique : elle est aussi morphologique, anatomique, et fonctionnelle (Figure 1). Les champignons sont omniprésents dans les écosystèmes terrestres (Peay et al., 2010; Tedersoo, Bahram, Toots, Diédhiou, *et al.*, 2012; Větrovský *et al.*, 2019) – et également présents dans les écosystèmes marins (Nagano and Nagahama, 2012). La diversité des champignons s'organise en différentes *guildes trophiques* (voir Glossaire) – groupant les taxons en fonction de leur mode d'acquisition des ressources (Figure 1). Ces différents groupes fonctionnels jouent un rôle fondamental dans de nombreux processus écosystémiques fondamentaux. Les champignons saprotrophes – ou décomposeurs – interviennent dans les cycles biogéochimiques de l'azote et du carbone en dégradant la matière organique, dont la lignine (Figure 1, Blanchette, 1991). Les champignons pathogènes se nourrissent au détriment d'un autre organisme vivant (animal végétal, ou fongique, Doehlemann *et al.*, 2017). A l'opposé, les champignons mutualistes acquièrent leurs ressources tout en favorisant l'établissement, la croissance et la survie de leur hôte (Smith and Read, 2010; Yan *et al.*, 2019).

Les champignons mutualistes ont adopté une stratégie d'acquisition des ressources basée sur une interaction réciproque avec les végétaux (Martin, Uroz and Barker, 2017). Celle-ci concerne la vaste majorité des végétaux terrestres, et se décline de deux manières : l'une sous la forme d'endophytisme, c'est-à-dire grâce à une colonisation végétative asymptomatique dans les organes foliaires, caulinaires ou racinaires (Figure 1 ; les champignons occupent les tissus des plantes), l'autre dans le cadre d'une symbiose racinaire avec la formation d'organes chimériques dans les apex des racines fines des plantes (Figure 2). La déclinaison morpho-anatomique de cette symbiose (Figure 2) varie selon plusieurs caractères des végétaux, tels que la forme de vie de la plante (herbacée ou ligneuse, Brundrett and Tedersoo, 2018) ou sa position

au regard de l'histoire évolutive de la lignée verte taxonomique (*e.g.* Orchidacées ou Ericacées, Miura *et al.*, 2010; Vohník, 2020).

La symbiose mycorhizienne est une généralité dans le règne végétal et concerne la vaste majorité des plantes terrestres (90 %, van der Heijden *et al.*, 2015). Toutefois, cette interaction est apparue plusieurs fois indépendamment dans les lignées végétales, et s'est avérée variable au cours de l'histoire évolutive des plantes hôtes. Les interactions mycorhiziennes impliquent des champignons des lignées Asco- et Basidiomycètes dont la stratégie saprophytique a évolué vers le mutualisme (van der Heijden *et al.*, 2015). Conjointement, la capacité des plantes à établir des interactions mutualistes a notamment varié de l'absence de mycorhization, à l'endomycorhization et l'ectomycorhization (Figure 2). Par exemple, certains gènes exprimés chez les Pinacées ont témoigné de l'abandon – puis de la récupération de la capacité ectomycorhizienne au cours de l'histoire évolutive (Maherali *et al.*, 2016).

Le caractère bénéfique de la symbiose mycorhizienne semble également déterminé par les paramètres biotiques et abiotiques de l'écosystème – incluant la signature de la richesse des sols et l'accessibilité des plantes au carbone photosynthétique (van der Heijden *et al.*, 2015). La réciprocité entre les deux protagonistes de l'interaction peut s'avérer variable dans le temps – notamment dans les écosystèmes à saisonnalité variées (Figure 1, Adnan *et al.*, 2022). Dans la littérature académique, le terme de « parasitisme équilibré » pour désigner les interactions mutualistes entre plantes et champignons est parfois employé (Antonovics *et al.*, 2015, Johnson, Garham and Smith, 1997).

Figure 1 : A) Illustration des principales guildes écologiques et diversité des formes de vie chez les champignons avec **a)** Fructification de *Corpinus comatus*, **b)** *Penicillium* sur citron, *Penicillium citrinum*, **c)** pourriture blanche causée par la dégradation de la lignine par un champignon saproxylique, **d)** carpophore de *Galerina sp.* sur tronc vivant **e)** endomycorhize dans une racine fine d'un arbre tropical du genre *Inga* (Fabaceae), **f)** champignon endophyte *Neotyphodium coenophialum* dans les tissus de la fétuque élevée, **g)** racines ectomycorhizées de *Pinus halepensis*, **h)** fructification d'*Amanita caesarea*, **i)** hyphes rhizomorphes d'*Armillaria mellea*, **j)** troncs de *Platanus sp.* infectés par le chancre colorés du platane *Ceratocystis fimbriata platani*, **k)** fourmi infectée par *Ophiocordyceps unilateralis*, et **l)** gazon infecté par le genre d'oomycète *Pythium* ; et **B)** Porosité entre les guildes trophiques

A) Trois principales stratégies trophiques pour l'acquisition des ressources chez les champignons

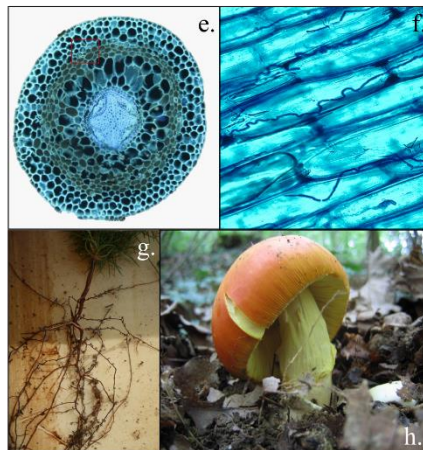
Saprophytisme

Mobilisation de la matière organique pour l'acquisition des nutriments.
Décomposition



Mutualisme

Interactions bénéfiques entre plantes et champignons. Echanges réciproques de nutriments et d'eau.
Protection sanitaire.



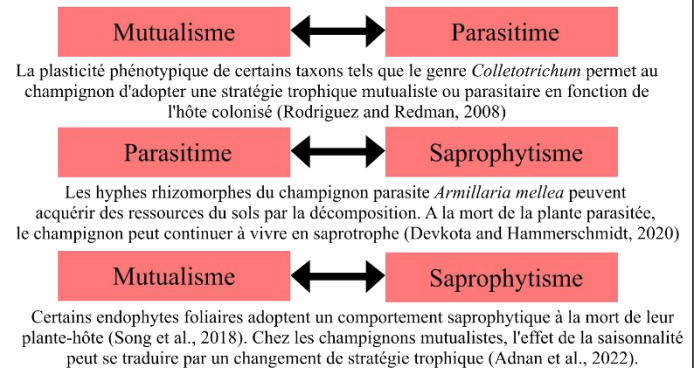
Parasitisme

Acquisition des ressources au détriment d'un autre être vivant (plante, insecte).



B) Les stratégies trophiques ne sont pas fixes

L'assignation des taxons aux guildes trophiques décrites ci-dessus peut s'avérer complexe. Les stratégies trophiques des champignons sont perméables et peuvent varier au cours de la vie du champignon. Plusieurs facteurs peuvent conduire à un changement de stratégie écologique tels que la disponibilité des ressources dans le sol ou encore la composition de la communauté végétale. Chez les champignons interagissant avec les plantes, le stade ontogénique de la plante peut structurer le mode d'acquisition des ressources du champignon. La variabilité phénotypique - intraspécifique - au sein d'un taxon peut conduire à différentes stratégies trophiques. Enfin, les stratégies d'acquisition peuvent varier au cours du temps. C'est notamment le cas dans les écosystèmes à saisonnalité marquée.



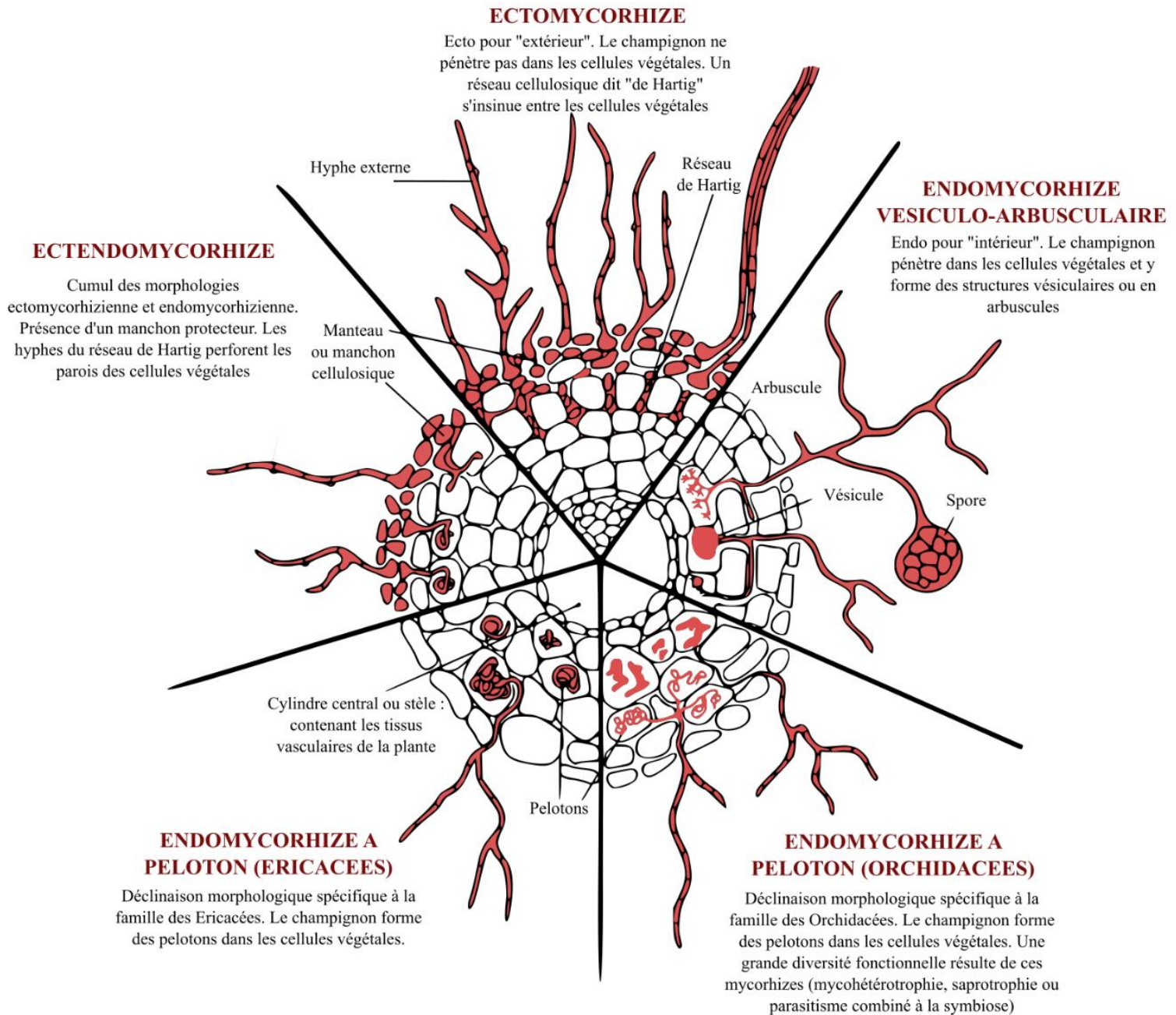
2. La symbiose ectomycorhizienne

2.1. Un mutualisme décliné en diverses morpho-anatomies et une pluralité de fonctions

Les champignons ectomycorhiziens représentent une fraction de la diversité fongique estimée à plus de 20.000 espèces (van der Heijden *et al.*, 2015). Dans la phylogénie, ces taxons sont issus de diverses lignées incluant les phylums des Basidiomycètes, Ascomycètes et Zygomycètes (Tedersoo and Smith, 2013). Quelques 6.000 espèces de végétaux ligneux et de rares plantes herbacées (Comandini, Contu and Rinaldi, 2006) ont développé ce type d'interaction particulier. A l'échelle biogéographique, cette interaction a été majoritairement documentée dans les biomes tempérés, méditerranéens et boréaux (Tedersoo, Bahram, Toots, Diedhiou, *et al.*, 2012). Moins documentée en milieu tropicale, la symbiose ectomycorhizienne y est pourtant bien représentée chez certaines familles telles que les Myrtaceae ou les Dipterocarpaceae (Alexander and Högborg, 1986; Corrales *et al.*, 2022). Dans les écosystèmes méditerranéens, l'ectomycorhize concerne essentiellement les Fagaceae (les chênes et châtaigniers, Richard *et al.*, 2005), les Cistaceae (les cistes et hélianthèmes, Comandini, Contu and Rinaldi, 2006), les Pinaceae (pins, sapins, Martin *et al.*, 2016), quelques rares Ericaceae comme les arbousiers (genre *Arbutus*, représenté par *Arbutus unedo* dans l'ouest du bassin méditerranéen, Richard *et al.*, 2005; Taschen *et al.*, 2015), ou encore les Salicaceae et les Betulaceae (Smith & Read 2010).

La manifestation macroscopique de l'interaction ectomycorhizienne se produit au niveau des apex des racines fines (à croissance primaire) des arbres (Figure 3e). Les mycorhizes (du grec « champignon-racine ») sont des structures morphologiques composites (Figure 2). Le mycélium du champignon forme un manchon mycélien protecteur – ou manteau constitué majoritairement de fibrilles de chitine – autour des apex racinaires (Figure 2, 3e). Les hyphes forment une structure en réseau dit « réseau de Hartig » qui s'imisce entre les cellules végétales sans y pénétrer (Figure 2).

Figure 2 : La symbiose mycorhizienne : différents types de morpho-anatomie. Adapté de Selse et Le Tacon, 1998. Les éléments colorés en rouge sont fongiques.



Le lien physique entre l'appareil végétatif du champignon et la plante confère des bénéfices largement documentés pour les deux organismes interagissant (Smith and Read, 2010). L'ectomycorhize est une véritable structure d'échange pour les transferts d'eau et de nutriments entre les deux protagonistes de l'interaction. Les hyphes mycéliens connectés à la mycorhize – ou mycélium extraracinaire – forme une extension du système racinaire de la plante. En explorant pour le compte de son hôte des anfractuosités du sol inaccessibles aux organes végétaux, le champignon ectomycorhizien joue un rôle fondamental dans l'absorption hydrique et minérale des plantes (Swaty *et al.*, 2004; Brunner, 2015). Il favorise l'assimilation pour la plante d'éléments nutritifs tels que l'azote, le phosphore, mais aussi le carbone à partir de formes complexes piégées dans la matière organique, ainsi que l'eau (Smith and Read, 2010; Klein, Siegwolf and Körner, 2016). La plante-hôte fournit au champignon des sucres issus de la photosynthèse (Smith and Read, 2010). Le manchon protecteur édifié par le champignon confère au végétal une protection physique contre les organismes pathogènes du sol, ainsi qu'une tolérance accrue aux polluants des sols (Van Tichelen, Colpaert and Vangronsveld, 2001; Krpata *et al.*, 2008).

2.2. Un mutualisme impliqué dans des réseaux d'interactions entre plantes

Peu de taxons ectomycorhiziens sont strictement spécialisés au regard de l'identité taxonomique de leur plante hôte. En raison de ce caractère diffus du mutualisme (Bruns, Bidartondo and Taylor, 2002), les champignons impliqués ont la capacité d'établir des interactions avec plusieurs plantes voisines, conspécifiques ou non. Un lien physique peut être établi par les mycéliums de symbiontes racinaires entre plantes voisines (Selosse *et al.*, 2006; Simard *et al.*, 2003; Taudière *et al.*, 2015). Le réseau formé par ces champignons est appelé « réseau d'interaction ectomycorhizien » (*Common Mycorrhizal Networks, CMNs*, Figure 3a, c). Les premières mentions d'échange de nutriments entre plantes voisines par la voie fongique remontent aux années 1980 avec les travaux en conditions contrôlées réalisées par David Read (1984). Dans les années 1990s, les travaux de Suzanne Simard ont démontré l'existence de transferts de carbone entre de jeunes plants ectomycorhizés d'espèces différentes impliquées dans des *successions secondaires* (voir Glossaire), en l'occurrence le bouleau à papier (comme essence pionnière donneuse de carbone) et le sapin de Douglas (comme essence de fin de succession receveuse ; Simard *et al.*, 1997). Depuis, le rôle des réseaux d'interactions ectomycorhiziens dans la nutrition des plantes a largement été mis en évidence (Selosse *et al.*,

2006). L'étendue de ceux-ci dans les écosystèmes et leur effet sur les performances des végétaux ont été explorés – et concernent non seulement l'acquisition d'éléments nutritifs pour la croissance (par exemple, le carbone, l'azote, le phosphore et l'eau) mais également la diffusion d'éléments chimiques de défenses tels que des molécules allélopathiques (Simard *et al.*, 2012). Ces réseaux sont impliqués dans de nombreux cycles biogéochimiques et sont à l'origine de boucles de rétroactions positives entre les plantes et le sol (ou *positive feedbacks loops*, voir Glossaire, (Bever, 1994; Wardle *et al.*, 2004). Ils contribuent considérablement aux échanges carbonés entre le sol et les végétaux – la voie fongique mesurant 40 % des transferts carbonés dans un écosystème de forêt tempérée (Klein, Siegwolf and Körner, 2016). En mésocosme, le rôle des réseaux d'interaction dans l'ascenseur hydraulique a montré des effets positifs sur la résistance des plantules de chênes au stress hydrique (Egerton-Warburton, Querejeta and Allen, 2007).

Figure 3 : Les réseaux d'interactions ectomycorhiziens, représentation schématique (a) photo (b), définitions admises (c), définitions des différents types d'exploration des réseaux mycorhiziens (d) et (e) illustrations de ces types d'explorations avec des mycorhizes observées dans ce travail de thèse



Les réseaux d'interactions contribuent activement à des processus à l'échelle de la communauté tels que les *successions végétales* (voir Glossaire, Selosse *et al.*, 2006; Authier, Violle and Richard, 2022). Au cours des successions primaires, ils facilitent l'établissement des plantes pionnières (Nara, 2006a). En partageant les symbiontes établis sur leurs racines, les végétaux pionniers favorisent l'installation des espèces tardives au cours des successions secondaires (Richard, Gardes and Selosse, 2009). Ainsi, les réseaux d'interactions participent à la régénération du sous-étage forestier via les transferts de carbone photosynthétique par voie fongique entre les individus de canopées et les plantes d'ombre (Selosse *et al.*, 2006; Simard *et al.*, 2003).

Pour l'heure, les connaissances acquises sont inégales entre les différentes étapes de la succession végétale. Plus particulièrement, on méconnaît l'action des réseaux d'interaction au cours d'un stade ontogénique précoce – lors de trouées. Récemment, des travaux ont mis en lumière le rôle des champignons ectomycorhiziens pouvant s'établir comme endophytes racinaires dans les racines de plantes herbacées. Ces derniers favorisent ainsi la colonisation d'écosystèmes herbacés par la strate arbustive ectomycorhizienne (Schneider-Maunoury *et al.*, 2018; Liang *et al.*, 2020). Ce phénomène pourrait s'apparenter à un effet nurse par les plantes herbacées déjà établies ; facilitant l'installation des végétaux de successions secondaires via le transfert de symbiontes racinaires (Authier, Violle and Richard, 2022).

Dans les écosystèmes, l'étendue des réseaux d'interactions a été documentée par exemple dans les forêts de sapin de Douglas, où Beiler *et al.* (2010) ont démontré la connectivité d'un arbre avec 47 autres, par plusieurs genets du champignon *Rhizopogon*. L'amélioration de la connaissance sur l'étendue des réseaux d'interaction comporte des objectifs appliqués – notamment pour la production forestière et pour celle de champignons comestibles. Des taxons ectomycorhiziens à forte valeur économique sont capables d'établir des réseaux tels que le champignon *Tricholoma matsutake* dans les forêts japonaises de *Pinus densiflora* (Lian *et al.*, 2006) ou encore *Tuber melanosporum* qui s'associe aux chênes dans les truffières (Pereira *et al.*, 2013). Toutefois, des questions persistent quant à la composition, à l'étendue et aux mécanismes qui sous-tendent l'installation des réseaux d'interactions. A ce jour, la majorité de la littérature s'est focalisée sur des forêts de sapin de Douglas (Karst, Jones and Hoeksema, 2023). Dans les milieux très anthropisés tels que les écosystèmes urbains, la littérature sur le sujet est pour l'heure quasi-inexistante (Authier, Violle and Richard, 2022). Dans ce travail,

nous analyserons les connaissances disponibles sur les réseaux d'interaction qui s'établissent le long d'un gradient d'anthropisation – jusqu'aux centres urbains.

2.3. Les interactions ectomycorhiziennes jouent un rôle majeur dans le fonctionnement des paysages

L'étude des communautés ectomycorhiziennes, des filtres gouvernants leur expression, et de leur capacité à lier les végétaux au sein de réseaux d'interactions est un sujet majeur en écologie (van der Heijden *et al.*, 2015). L'*écologie des communautés* (voir Glossaire) est une discipline qui s'intéresse aux assemblages d'espèces dans les écosystèmes et aux mécanismes qui régissent ces assemblages (Mittelbach & McGill, 2019). En particulier, cette discipline explore plus particulièrement l'effet des filtres environnementaux et des interactions biotiques sur la structure et la composition des communautés. Chez les champignons ectomycorhiziens, plusieurs filtres à la dispersion des propagules (spores, *sclérotés* – voir Glossaire, fragments mycéliens), à l'installation et à l'établissement des communautés ont été identifiés (Figure 5, Koide, Fernandez and Petprakob, 2011).

A l'échelle paysagère, la mosaïque d'écosystèmes et la connectivité entre les différents habitats influence la composition de la *banque de spores* (voir Glossaire, Glassman *et al.*, 2015). En effet, la fragmentation des habitats limite la dispersion des spores – en particulier celle des champignons dont les fructifications sont *hypogées* (par exemple les truffes, Voir glossaire, Smith, Henkel and Rollins, 2015), car elle entrave les déplacements de la faune consommatrice des fructifications (Newbound, Mccarthy and Lebel, 2010). Chez les champignons ectomycorhiziens dont la dispersion se fait par voie aérienne (majorité des fructifications *épigées*, voir Glossaire, Horton, 2017), les spores peuvent parfois coloniser les écosystèmes sur de longues distances. Par exemple, les spores du champignon *Suillus pogens* sont capables de parcourir jusqu'à 10 kilomètres (Peay *et al.*, 2012). Toutefois, la capacité de dispersion des spores aéroportées est contrainte par la fragmentation et l'isolation des habitats (Bowman, Arnold and Arnold, 2021). Dans les écosystèmes sujet à une forte pression anthropique, l'effet des barrières physiques (par exemple, des infrastructures anthropiques comme des zones urbanisées) reste à démontrer sur la capacité de colonisation des propagules ectomycorhiziennes (Golan and Pringle, 2017).

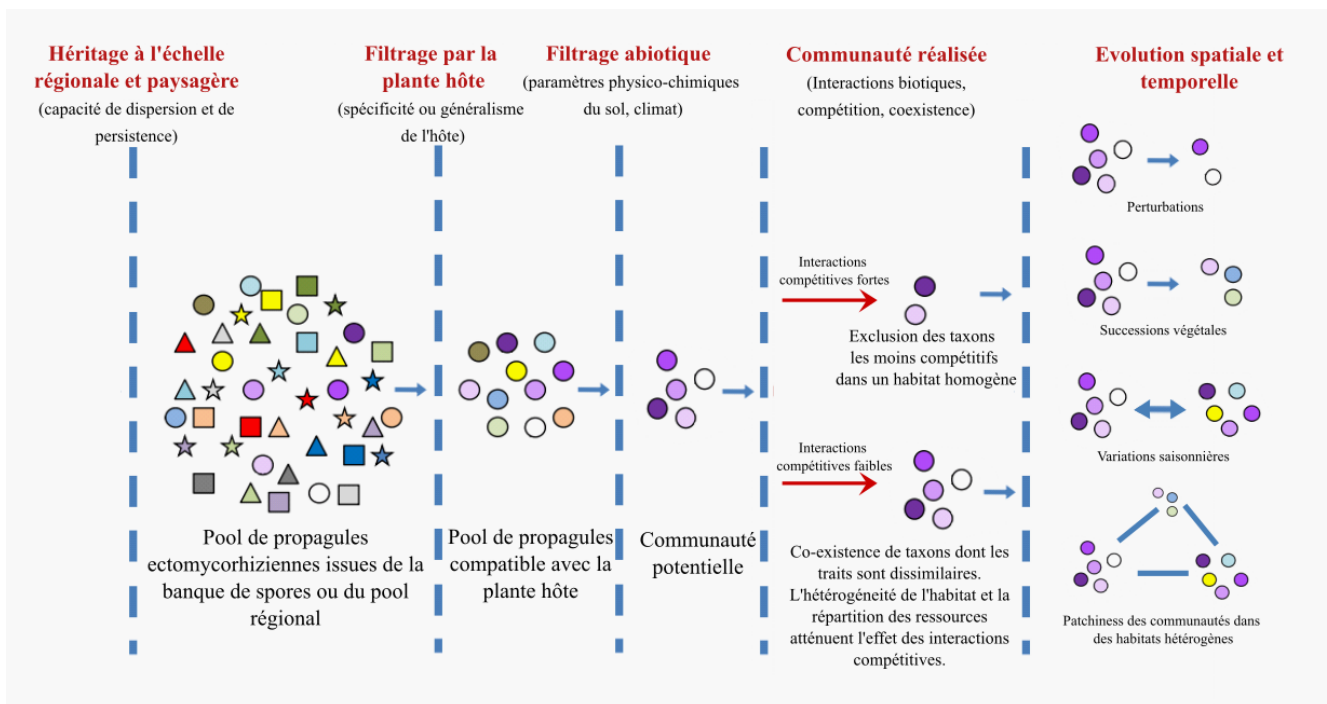
A l'échelle locale – celle du grain de paysage défini par une formation végétale donnée (par exemple bois, pelouse, lande) – les communautés ectomycorhiziennes sont majoritairement structurées par les paramètres physico-chimiques du sol (Figure 5). Elles diffèrent selon la nature de la roche mère (Branco, 2010, Johnson et al., 2017), de sa teneur en minéraux (Peay *et al.*, 2015), ainsi qu'en fonction des propriétés hydriques du sol (Patterson *et al.*, 2019). Par ailleurs, il a été largement démontré que la répartition inégale des ressources dans l'espace gouverne de fortes variations de composition des communautés ectomycorhiziennes à l'échelle locale, aussi appelée *patchiness* (Figure 4, Koide, Fernandez and Petprakob, 2011; Courty *et al.*, 2016). La disponibilité et la répartition des ressources dans le sol sont à l'origine d'interactions biologiques pouvant mener à l'exclusion compétitive de certains taxons. En particulier, dans le cas d'une répartition homogène des ressources, les taxons les moins compétitifs seront exclus (Figure 4, Koide, Fernandez and Petprakob, 2011). Si les ressources sont réparties de manière hétérogène dans l'espace, les communautés ectomycorhiziennes seront structurées en patchs de diversité dans l'espace – les taxons exploitant différentes ressources (Figure 4, Horton and Bruns, 2001; Lilleskov *et al.*, 2004; Pickles and Anderson, 2016).

Durant les successions secondaires des paysages, le processus de facilitation peut jouer un rôle majeur, et impliquer les champignons ectomycorhiziens (Taudière *et al.*, 2015) qui sont à la fois les moteurs et les passagers d'une dynamique globale entraînant l'ensemble de l'écosystème. Un premier filtre biotique à l'établissement des champignons ectomycorhiziens est celui de la plante hôte (Figure 4, Boeraeve, Honnay and Jacquemyn, 2018; Wu *et al.*, 2018). La capacité des plantes à s'associer avec certains champignons ectomycorhiziens varie fortement, lorsque l'on considère l'amplitude taxonomique et le nombre des partenaires fongiques compatibles. En d'autres termes, le « degré de spécificité » des végétaux pour leurs symbiontes varie de la spécialisation (*c.a.d.* peu de partenaires mycorhiziens) au généralisme (*c.a.d.* vaste panel de partenaires mycorhiziens, (Taudière *et al.*, 2015). Dans le cas d'une spécialisation des interactions, les cortèges ectomycorhiziens sont réduits à quelques dizaines d'espèces. C'est le cas chez certains arbres tels que les aulnes (Roy *et al.*, 2013) ainsi que chez certains taxons de champignons tels que *Suillus*, dont les différentes espèces sont strictement associées à des espèces des genres *Pinus* et *Larix* (Kretzer *et al.*, 1996; Liao, Chen and Vilgalys, 2016). A l'opposé, les champignons généralistes ont la capacité de s'associer une vaste gamme

d'espèces végétales. C'est le cas par exemple d'*Amanita pantherina*, qui peut interagir avec toutes les espèces ectomycorhiziennes présentes en Corse (Taudière et al., 2015).

A une plus grande échelle temporelle, la composition des communautés ectomycorhiziennes évolue au cours des successions végétales (Nara, 2006b; Taudière et al., 2015). En méditerranée, les communautés sont très diversifiées les écosystèmes de successions secondaires tels que les forêts de chêne vert (Richard et al., 2005) et dans une moindre mesure dans les pinèdes (Adamo et al., 2021). Le compartiment ectomycorhizien des milieux pionniers tels que les garrigues reste peu connu. Toutefois, certaines espèces arbustives telles que les Cistacées dominante dans ces formations végétales accueillent une grande diversité de champignon ectomycorhiziens sur leurs racines (Comandini, Contu and Rinaldi, 2006).

Figure 4 : Mécanismes structurant des communautés ectomycorhiziennes, de l'échelle paysagère à l'échelle de l'hôte. Adapté de Koide et al., 2004.



3. L'outil moléculaire pour l'étude des communautés EM : de la multitude de services rendus à des limites méthodologiques

Dans cette thèse, une emphase sera faite sur les outils moléculaires couramment utilisés pour l'étude des communautés ectomycorhiziennes tels que le barcoding et le métabarcoding. Ces outils représentent en eux-mêmes un vaste sujet d'étude qu'il est fondamental d'aborder afin de mieux comprendre les données qu'ils restituent et d'améliorer l'interprétation écologique de celles-ci. Étudier les outils moléculaires permettra ainsi de promouvoir la répliquabilité, la portabilité des recherches utilisant ces méthodes, ainsi que de favoriser leur transfert vers des problématiques opérationnelles.

3.1. Émergence des outils moléculaires et apport pour l'écologie des champignons

Depuis l'émergence de la mycologie moderne au début du 19^{ème} siècle et jusque dans les années 90, l'identification des champignons a reposé sur les caractères morphologiques des fructifications, ainsi que sur la culture de mycélium en laboratoire. L'obligation de se baser sur du matériel biologique visible et cultivable a longtemps limité l'identification de certains

Encadré 1 : Du barcoding au métabarcoding

Travail pionnier dans les années 1990 - Les travaux pionniers de Gardes et al. (1991) ont amorcé les débuts de l'usage de plus en plus répandu du barcoding pour l'identification des champignons ectomycorhiziens. Le barcoding consiste en l'identification d'un « code-barre » moléculaire spécifique à un taxon. En se basant sur du matériel biologique, le séquençage en Sanger permet d'identifier la séquence la plus abondante de l'échantillon.

Dans les années 2000 : vers des méthodes de séquençage plus puissantes - Les méthodes de métabarcoding (*par exemple* 454, Illumina MiSeq) permettent l'identification de l'ADN présentes appartenant à plusieurs espèces dans un même échantillon. Cela permet d'identifier une communauté de séquences à partir d'un échantillon biologique ou environnemental. Massivement utilisées notamment pour l'identification des champignons ectomycorhiziens, ces méthodes performantes et sensibles requiert toutefois un calibrage pour améliorer l'interprétabilité et la portabilité des données générées.

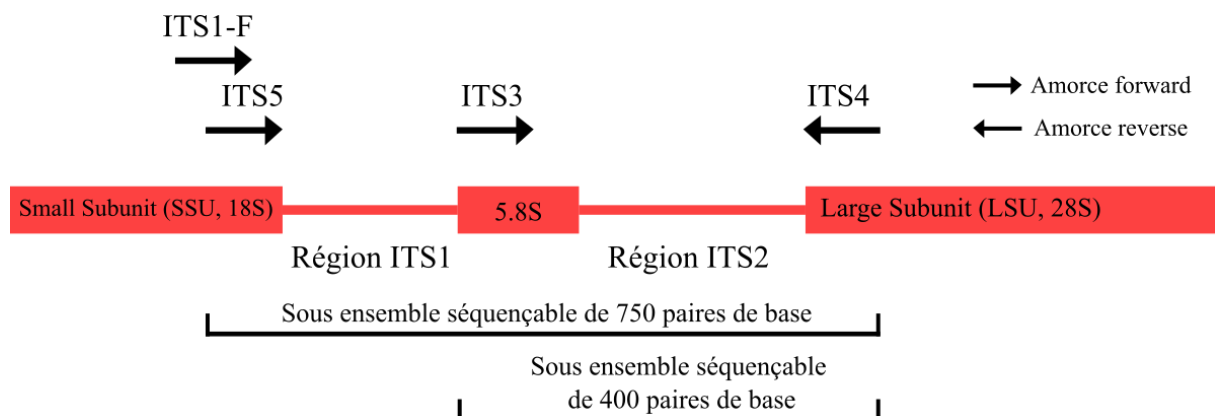
Propriétés du barcoding et métabarcoding (adapté de Janowski *et al.*, 2023)

	Barcoding (Sanger, RFLP)	Métabarcoding (454-Pyrosequencing, Illumina MiSeq)
Type d'échantillon	Echantillon biologique	Echantillon biologique ou environnemental
Longueur du gène séquencée	750 paires de bases	450 paires de bases. Perte de qualité si plus long
Nombre de séquences générées	1 séquence, la séquence dominante de l'amplicon	Des millions de séquences à partir d'un amplicon
Coût	Coût en temps / échantillon élevé.	Très faible coût par séquence
Utilisation	Taxonomie, écologie des interaction	Écologie des interactions, écologie des communautés, séquençage d'ADN dégradé
Apports	Précision taxonomique	Echantillonnage simplifié ; analyse du compartiment végétatif du champignon
Limites	Difficulté pour séquencer les espèces rares, Perte d'amplicons non analysables	Difficulté d'interprétation des données générées en raison de leur nombre

taxons. En particulier, la grande majorité des champignons ectomycorhiziens n'est pas cultivable (Hawksworth, 2001). Si aujourd'hui cette diversité est estimée à environ 20.000 taxons (Tedersoo, May and Smith, 2010), l'échantillonnage et l'analyse des critères morphologiques de ces champignons reste contrainte par le caractère diffus, erratique et fugace des épisodes de fructifications (Horton and Bruns, 2001). De plus, l'utilisation des fructifications comme proxy de l'abondance des espèces se révèle limitée par la capacité des champignons ectomycorhiziens à coloniser un sol en pratiquant la reproduction clonale – sans production de carpophores durant plusieurs années consécutives (Sanchez *et al.*, 2014). Pour cette guildes, l'identification taxonomique basée sur l'observation des traits morpho-anatomiques des apex mycorhizés, appelée morphotypage (Agerer, 2001), est limitée en raison d'un nombre élevé de caractères cryptiques, ainsi que du polymorphisme intraspécifique, notamment en fonction des hôtes végétaux associés (Ostonen *et al.*, 2013).

L'émergence des outils moléculaires adaptés aux champignons ectomycorhiziens dans les années 90 a métamorphosé l'étude des champignons ectomycorhiziens (Gardes *et al.*, 1991). Les méthodes moléculaires se basent sur l'identification d'une séquence génétique de l'ADN ribosomique ITS (Internal Transcribed Spacer, Figure 5, Gardes and Bruns, 1993). Cette région non codante est la plus couramment employée pour l'étude des communautés ectomycorhiziennes car elle est discriminante au niveau interspécifique, et peu variable au niveau intraspécifique (Gardes and Bruns, 1993).

Figure 5 : La région ITS (Internal Transcribed Spacer), marqueur universel pour l'identification des champignons ectomycorhiziens. Les amorces représentées sur ce schéma sont celles qui ont été utilisées dans ce travail.



Les premières méthodes moléculaires développées dans les années 90 sont celles de barcoding (par exemple, *Restriction Fragment Length Polymorphism* – RFLP ou Sanger, Encadré 1). Elles permettent d'identifier une séquence moléculaire à partir d'un échantillon biologique (comme par exemple un échantillon de carpophore ou de mycorhize, Encadré 1, Figure 6). Dans les années 2000, des méthodes de métabarcoding – séquençage haut débit ou NGS (Next-Generation Sequencing, par exemple 454-pyroséquençage ou Illumina, Encadré 1, Lücking *et al.*, 2021; Janowski and Leski, 2023) – permettant d'identifier un ensemble de séquences présentes dans un échantillon ont été développées. Ces méthodes sensibles et performantes permettent d'extraire de l'information taxonomique à partir d'échantillons biologiques (par exemple d'un carpophore ou d'une mycorhize) ou dans un échantillon environnemental (par exemple à partir d'une fraction de sol, d'eau ou d'air, Ruppert, Kline and Rahman, 2019; Calderon-Sanou *et al.*, 2020).

L'application des méthodes moléculaires – barcoding et métabarcoding confondus – a considérablement transformé la manière d'étudier les champignons (Horton and Bruns, 2001; Buée, Sentausa and Murat, 2016). Depuis le développement de ces méthodes, la diversité des champignons a été largement revue à la hausse (Hawksworth and Lücking, 2017; Ruppert, Kline and Rahman, 2019). Sur le plan taxonomique, l'outil moléculaire a permis une relecture et une mise à jour de la phylogénie des champignons (Tedersoo and Smith, 2013). L'identification moléculaire a permis de discriminer des taxons dont les apex mycorhizés présentent de grandes similarités (par exemple, *Tuber melanosporum* et *Tuber indicum*, Zambonelli *et al.*, 1999). Enfin, l'outil moléculaire a notablement contribué à l'identification des champignons établissant des interactions ectomycorhiziennes avec les plantes. Plus particulièrement, les analyses basées sur les données de séquençage ont permis de progresser sur notre connaissance de la distribution spatiale des interactions mutualistes, ainsi que sur notre vision du partage de partenaires dans les réseaux d'interactions bipartites (Lian *et al.*, 2006; Lofgren *et al.*, 2021). A l'échelle des communautés, les travaux basés sur le séquençage des apex ont permis de mieux décrire et comprendre les patrons de répartition des espèces et les filtres régissant la distribution des points chauds de diversité (Koide, Xu and Sharda, 2004; Schneider-Maunoury *et al.*, 2018).

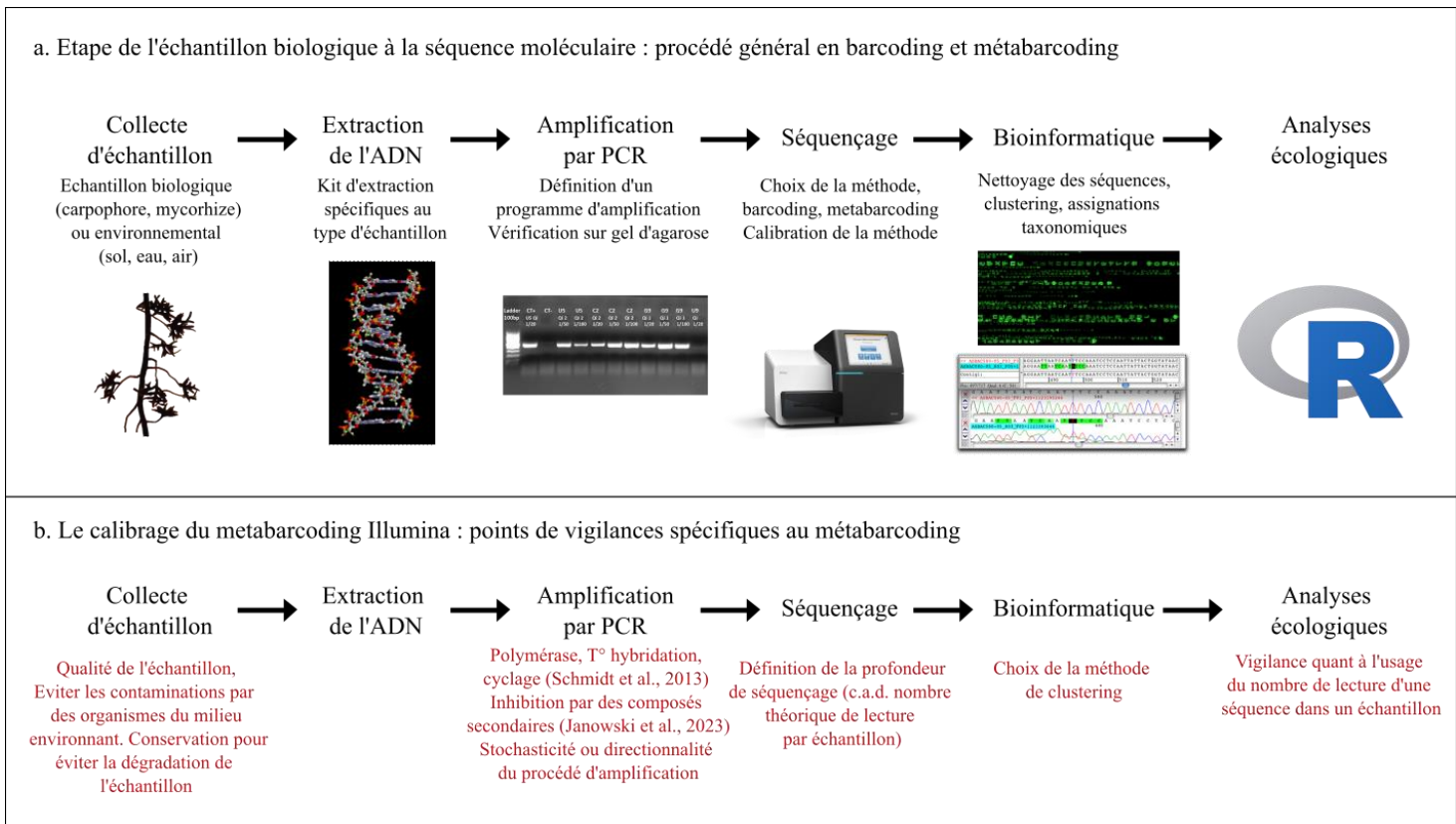
Aujourd'hui, le barcoding et le métabarcoding ADN sont des outils largement utilisés par les écologues, mais aussi par les gestionnaires des espaces naturels (voir par exemple Ji *et al.*,

2013). Parmi la pluralité de techniques développées au cours de la dernière décennie, la technologie Illumina représente un outil consensuel largement utilisé en écologie des communautés ectomycorhiziennes (Janowski and Leski, 2023). Dans ce manuscrit, nous utiliserons et nous interrogerons cette méthode. En effet, malgré son utilisation quasi exclusive, des questions persistent quant aux limites concernant l'utilisation des données issues du séquençage Illumina.

3.2. De l'échantillon à la séquence moléculaire : protocole pour l'obtention de séquences en métabarcoding Illumina

De la collecte de l'échantillon à l'analyse de la composition de la communauté ciblée, le métabarcoding ADN est constitué d'une suite d'étapes comprenant l'extraction de l'ADN, son amplification, son séquençage et l'analyse bio-informatique des bibliothèques. Toutes ces étapes représentent des sources de variabilité du résultat obtenu (Figure 6a). En effet, à chacune d'entre elles l'expérimentateur réalise des choix méthodologiques affectant l'image de la communauté qui sera produite au terme du métabarcoding (Figure 6). Sur le terrain, un protocole d'échantillonnage précautionneux (par exemple en conditionnant dans des tubes hermétiques, stériles) permet d'éviter la dégradation de l'ADN présent dans l'échantillon par des organismes décomposeurs (Janowski and Leski, 2023). Les méthodes de métabarcoding sont très sensibles et des séquences contaminantes issues d'organismes extérieurs à l'échantillon peuvent être séquencées par la machine (Figure 6b, Tedersoo *et al.*, 2022).

Figure 6 : De l'échantillon biologique à la séquence moléculaire. a) Illustration des procédés de séquençage de barcoding et metabarcoding et b) spécificité du metabarcoding



Le processus d'amplification (PCR, Polymerase Chain Reaction) du gène ciblé est une étape cruciale pour la constitution d'*amplicons* séquençables (voir Glossaire, Figure 6b). Au cours du processus d'amplification, le gène ciblé par les amorces (Figure 5) est dupliqué de manière exponentielle. Durant ce processus itératif, les deux brins d'ADN complémentaires du gène ciblé sont désappariés. La *Taq polymérase* (voir Glossaire) permet la synthèse du brin d'ADN complémentaire (Newton et al., 1997). La PCR est à la fois un procédé stochastique et déterministe. D'une part, le recrutement des molécules à amplifier dans l'ADN extrait est probabiliste : à quantité initiale égale, l'une parmi deux versions du gène extrait (correspondant à la présence de deux espèces dans l'échantillon) peut être préférentiellement amplifiée pour des raisons liées à une plus grande affinité envers les amorces universelles (Kausrud, 2023) et/ou les conditions d'amplification (température, concentration en réactifs, etc.), et apparaît dominante après amplification (Krebschull and Zador, 2015). La limitation du nombre d'itérations – ou cycles PCR – permet de limiter l'importance de cet effet (Krebschull and Zador, 2015). Cette amplification différentielle a alors pour conséquence de biaiser la composition des communautés étudiées en faveur ou défaveur de certains groupes taxonomiques (Bellemain *et*

al., 2010; Tedersoo and Lindalh, 2016), et ainsi produire une image orientée des communautés ectomycorhiziennes (Kebschull and Zador, 2015; Taylor, 2016). En fonction des types d'échantillons, l'action de la polymérase peut être perturbée par la présence de composés secondaires (par exemple tanins, mélanine, composés phénoliques) issus de la matière organique, présents en compagnie de l'ADN extrait (Janowski *et al.*, 2019).

La calibration du séquenceur en métabarcoding est une étape déterminante – notamment pour déterminer du nombre de lecture – également appelé nombre de « reads » (Figure 6). Le nombre de lecture correspond au nombre de séquences qui seront – en théorie – lues par amplicon séquencé (Smith and Peay, 2014; Kauserud, 2023). Classiquement, ce nombre de lectures par échantillon est défini en divisant la capacité de la cartouche de séquençage par le nombre d'échantillons (amplicons) à séquencer. Une cartouche de séquençage est un consommable qui contient les réactifs nécessaires à la lecture d'un certain nombre de séquence. Pour chaque « run » de séquençage – correspondant à l'utilisation d'une cartouche – l'expérimentateur est donc face à un compromis entre le nombre d'échantillons (effort d'échantillonnage, que l'on souhaite toujours le plus ambitieux possible) et le nombre de séquences qu'il pourra obtenir pour chacun d'eux (la profondeur de séquençage, ou profondeur d'exploration de cet échantillon, que l'on souhaite la plus grande possible). Les capacités des cartouches étant déterminées, l'accroissement de la profondeur de séquençage se fera au détriment du nombre d'amplicons séquencés, et réciproquement.

Lors de l'étape suivante, dite d'analyse bio-informatique, un nettoyage des données issues du séquençage est réalisé (Figure 6b). Au cours du séquençage, des biais de lecture peuvent intervenir et générer des séquences artéfactuelles et artificielles telles que des *chimères* (voir Glossaire) qui seront éliminées du jeu de données analysables. Les *singletons* (voir Glossaire) sont également extraits du jeu de données (Bjørnsgaard Aas, Davey and Kauserud, 2017). Si l'élimination des séquences chimériques ne pose pas de problème, celle des singletons est plus problématique car il est possible que des séquences assignables à un groupe taxonomique – et écologiquement plausibles soient supprimées (Kauserud *et al.*, 2012). L'ensemble des séquences utilisables sont ensuite regroupées en fonction de leur homologie, au cours d'une étape appelée *clusterisation* (Encadré 2). Ce regroupement permet la définition d'unités

taxonomiques moléculaires, encore appelées MOTUs (*Molecular Operational Taxonomic Units*), OTUs (*Operational Taxonomic Units*) ou ASVs (*Amplicon Sequence Variants*, Kõljalg *et al.*, 2013). Les séquences résultantes des clusters sont ensuite comparées à des bases de données de référence telles que GenBank (Benson *et al.*, 2016) ou UNITE (Kõljalg *et al.*, 2005) pour l'assignation taxonomique de chaque entité. D'autres bases de données regroupant les caractères écologiques des taxons (*c.a.d.* forme de vie, guildes trophiques) ont été développées à des fins d'analyses basées sur les traits d'histoire de vie des taxons (par exemple la base de données FUNGuild, Nguyen *et al.*, 2016).

Encadré 2 : Le clustering en ASV, OTU, de quoi parle-t-on ?

Qu'est-ce que le clustering ? Le clustering est une étape fondamentale lors du traitement bio-informatique des données générées par la machine de séquençage. Il consiste en le regroupement de séquences dans des « clusters » à partir d'un seuil d'homologie (souvent fixé à 97 %, Pauvert *et al.*, 2019). C'est l'identification donnée à ce groupement de séquence qui permettra l'assignation taxonomique à l'échantillon. Plusieurs méthodes de clustering existent. Chez les champignons ectomycorhiziens, de nombreuses méthodes utilisent le clustering en OTU. Plus récemment, le clustering en ASV est aujourd'hui de plus en plus répandu. C'est cette méthode que nous utiliserons dans ce manuscrit.

Le clustering en OTU. L'un est le groupement en OTUs (Operational Taxonomic Unit) qui consiste à utiliser la séquence consensus du cluster pour l'assignation taxonomique et les analyses écologiques.

Le clustering en ASVs. Le groupement en ASVs (Amplicon Sequence Variants) pour laquelle les clusters sont comparés à des séquences de références dans des bases de données.

3.3. Quelles limites connues du métabarcoding des communautés des champignons ectomycorhiziens ?

On assiste à une transférabilité du métabarcoding vers des milieux appliqués, notamment pour établir des diagnostics pour la gestion forestière (Charya and Garg, 2019; Tomao *et al.*, 2020), pour la conservation (Geml *et al.*, 2014; Suz *et al.*, 2015), ou encore pour l'urbanisme. Si aujourd'hui les outils moléculaires tels que le métabarcoding tendent à devenir des outils décisionnels, la calibration de ceux-ci est alors fondamentale.

Le métabarcoding (Encadré 1) apparaît comme une méthode puissante de caractérisation des communautés ectomycorhiziennes (Janowski and Leski, 2023) qui offre la possibilité d'identifier les composantes des communautés à partir d'échantillons environnementaux (comme par exemple des fractions de sol, d'air, d'eau). Ainsi, le scientifique n'est plus tributaire des périodes de fructification et l'identification des espèces rares en est facilitée (van der Heijden *et al.*, 2015a; Nilsson *et al.*, 2019; Ruppert, Kline and Rahman, 2019). Le métabarcoding permet ainsi d'identifier les champignons ectomycorhiziens en se basant sur

leur appareil végétatif – le mycélium – plutôt que sur leurs fructifications. Les communautés ectomycorhiziennes assimilatrices – et non plus uniquement leur part reproductrice – sont mises en lumière.

Cependant, des limites persistent quant à l'interprétation des données issues du métabarcoding pour l'estimation de la diversité des communautés ectomycorhiziennes. Comme évoqué précédemment, le nombre de lectures d'une séquence reflète imparfaitement l'abondance d'un taxon dans l'écosystème (Kausrud, 2023). Pourtant, de nombreux auteurs tendent à utiliser le nombre de lecture comme une mesure quantitative de l'abondance (Merges and Neuschulz, 2018), alors que le métabarcoding a la capacité d'extraire et d'amplifier de l'ADN de la banque de spores, du mycélium, de primordium, de sclérotes. Par ailleurs, des résultats contradictoires ont été publiés concernant la corrélation entre profondeur de séquençage et estimation de la diversité (Baldrian *et al.*, 2021). Chez les champignons endomycorhiziens, il a été observé qu'une plus grande profondeur de séquençage ne permet pas systématiquement de capturer une plus grande diversité biologique (Vasar *et al.*, 2017). Dans ce travail de thèse, nous allons tester cette corrélation dans les environnements méditerranéens, chez les communautés ectomycorhiziennes. Nous analyserons les données récoltées à l'issue de séquençage en métabarcoding le long d'une gamme de profondeur de séquençage.

II. Quelle place pour la symbiose ectomycorhizienne et ses effets dans les écosystèmes urbains ?

Dans les écosystèmes tempérés, la symbiose ectomycorhizienne et ses fonctions associées sont cruciales pour la nutrition, la croissance et la longévité des arbres (Smith and Read, 2010). La composition des communautés ectomycorhiziennes et ses bénéfices pour le fonctionnement des écosystèmes forestiers ont été largement documentés dans les milieux naturels (Richard *et al.*, 2005), notamment en contexte de forêts cultivées (Kranabetter, Durall and MacKenzie, 2009). La symbiose mycorhizienne est utilisée en sylviculture (mycorhization contrôlée ; Selosse *et al.*, 1998; Charya and Garg, 2019) et en agroforesterie (par exemple trufficulture ; Taschen *et al.*, 2015).

En favorisant l'établissement, la croissance, la coexistence et la survie des végétaux, les champignons ectomycorhiziens ont vocation à constituer un chaînon important dans le succès de leur implantation dans les écosystèmes urbains. Pourtant, la symbiose ectomycorhizienne et l'étendue des réseaux d'interactions qu'elle constitue en ville reste peu explorées. Une meilleure connaissance des communautés ectomycorhiziennes urbaines permettrait de mobiliser un levier naturel pour améliorer le fonctionnement et la durabilité des espaces verts en ville.

1. Cadre institutionnel et opérationnel autour de l'aménagement urbain

1.1. *Les écosystèmes urbains fournissent des services écosystémiques fondamentaux pour la santé environnementale des villes*

En ville, la composante biotique des sols reste peu connue, notamment dans les centres les plus urbanisés (Maltsev et al., 2017). Les villes hébergent un large panel d'habitats (Rebele, 1994), abritant une large diversité d'espèces végétales arborescentes natives et non natives (Faeth, Bang and Saari, 2011; Beninde, Veith and Hochkirch, 2015). Les écosystèmes arborés des villes se déclinent en différents fragments forestiers (Escobedo, Kroeger and Wagner, 2011), en parcs (Birge-Liberman, 2010; Viera *et al.*, 2018) et en alignements d'arbres (Koeser *et al.*, 2013 ; Roman, Battles and McBride, 2014). On y retrouve à la fois des écosystèmes anciens (par exemple des patchs forestiers préservés, Figure 7, Vogt-schilb *et al.*, 2018, Lundholm, 2006) ainsi que des écosystèmes dont l'aménagement et la végétalisation est récente (Figure 7, Daniels *et al.*, 2018). Dans ces différents écosystèmes, le degré d'anthropisation varie en fonction du niveau d'intervention humaine sur l'espace (Figure 7, Naylo *et al.*, 2019) – celui-ci pouvant varier d'un très fort degré d'intervention (par exemple, taille, arrosage, amendement, et extraction de la matière organique tombée au sol) à une évolution de l'écosystème sans intervention humaine (Vogt, Hauer and Fischer, 2015; Koeser *et al.*, 2016).

Figure 7 : Représentation de quelques écosystèmes urbains le long d'un gradient d'ancienneté et d'anthropisation



Les espaces verts participent à la qualité environnementale des villes (Brauchbach *et al.*, 2017) ainsi qu'à la qualité de vie des populations (Salmond *et al.*, 2016). Les espaces végétalisés fournissent de nombreux *services écosystémiques* (voir Glossaire, Elmquist *et al.*, 2015; Kremer *et al.*, 2016). Entre autres, ils permettent d'atténuer l'effet des îlots de chaleur (Aram *et al.*, 2019 ; Sodoudi *et al.*, 2018) et de réguler les flux hydriques (par exemple, par l'interception des précipitations et par l'infiltration de l'eau dans les sols, Elmquist *et al.*, 2013). Ils limitent la pollution atmosphérique en fixant les particules fines (Bottalico *et al.*, 2016; Selmi *et al.*, 2016; Kumar *et al.*, 2019) et jouent un rôle fondamental dans les cycles biogéochimiques en absorbant le carbone et l'azote atmosphérique (CO₂, NO₂, Pataki *et al.*, 2011; Grote *et al.*, 2016; Velasco *et al.*, 2016). De plus, ils représentent d'importants refuges pour la biodiversité au sein d'espaces fragmentés, parfois peu connectés avec les milieux naturels adjacents (Soga *et al.*, 2014; Lapoint *et al.*, 2015; Lookingbill *et al.*, 2022).

Or, les services écosystémiques rendus par les espaces naturels urbains dépendent en grande partie de la composition biologique de l'écosystème (*c.a.d.* diversité, assemblage d'espèces, Vieira *et al.*, 2018), et de sa maturité (Parsa *et al.*, 2019). Les parcs boisés anciens ont un fonctionnement écologique du milieu qui peut s'apparenter à celui des écosystèmes forestiers. En particulier, les forêts urbaines et les parcs arborés âgés abritent des communautés végétales matures qui ont tendance à mieux stocker le carbone que les écosystèmes plus récents tel que les espaces verts artificiels (Velasco *et al.*, 2016; Hui *et al.*, 2017). L'atténuation de l'effet des îlots de chaleur dépend en grande partie de la couverture végétale occupée par les plantes, laquelle s'accroît généralement avec l'ancienneté du couvert (dos Santos *et al.*, 2017). Les propriétés inhérentes aux espèces peuplant les écosystèmes urbains tels que les *traits fonctionnels* (voir Glossaire) influent sur les services écosystémiques rendus (comme par exemple le stockage de carbone ou l'accueil de la biodiversité, Goodness *et al.*, 2016).

1.2. Une mosaïque d'acteurs dans le paysage urbain : cadre institutionnel et appliqué

La dynamique actuelle d'urbanisation, l'érosion de la biodiversité et le changement climatique induisent une forte pression sur les écosystèmes urbains et sur la santé environnementale des villes (Encadré 3). Aujourd'hui, la tendance politique est à un urbanisme plus écologique (Mehdi *et al.*, 2012) centré sur les services écosystémiques rendus par les espaces végétalisés. Dans les années 2000, un cadre législatif a été mis en place et préconise des pratiques écologiques pour améliorer l'aménagement des espaces. Les lois Grenelles 1 et 2 (LOI n° 2009-967, 2009) ont établi un cadre juridique autour des plans locaux d'urbanisme (PLU) afin de limiter l'étalement urbain, et de favoriser la préservation de la biodiversité et la connectivité entre les habitats (avec notamment les trames vertes et bleues, Bergès, Roche and Avon, 2010;

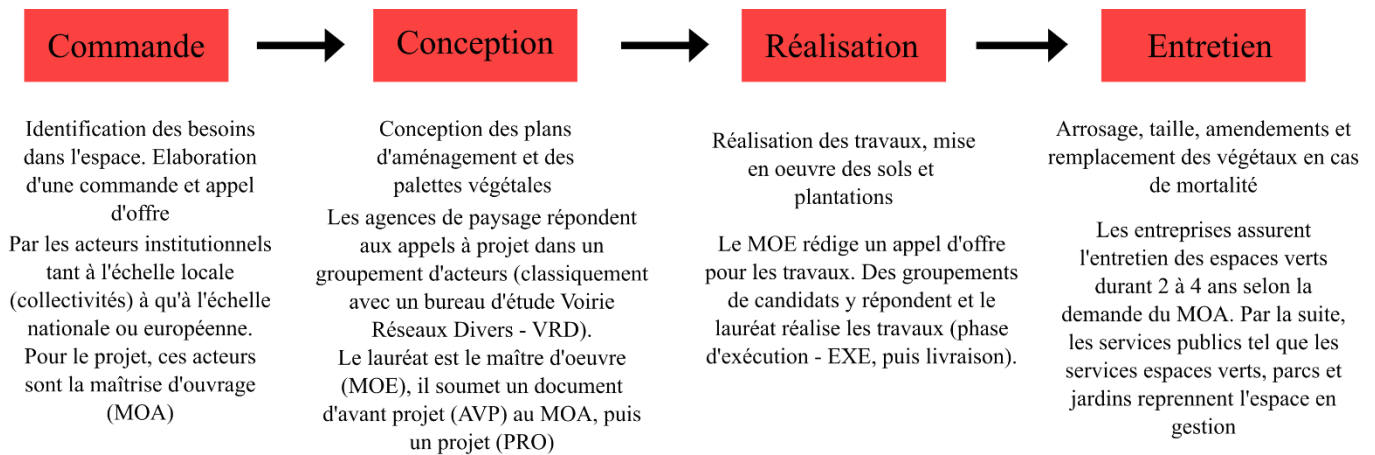
Encadré 3 : La ville en quelques chiffres

- 70 % de la population sera urbaine en 2050 (UN, 2022).
- Extension de la surface des villes avec 0.6 à 1.3 millions de km² en plus d'ici 2050 (Huang *et al.*, 2019)
- Artificialisation accrue des sols (Vidal-Beaudet and Rossignol, 2014).
- Exacerbation des effets du changement climatique : microclimats, îlots de chaleurs (Deilami *et al.*, 2018).
- 1/3 des émissions globales de CO₂ sont émises par les zones urbaines (0.5 % de la surface mondiale en 2015, Crippa *et al.*, 2021).
- Dépôts de polluants atmosphériques tels que les particules fines (Molina and Molina, 2012).
- Préjudice à la santé des populations en raison de l'altération de la qualité de l'air (pollution, températures élevées, Bernstein *et al.*, 2004)

Levray, 2015). On assiste ainsi depuis une décennie à une mutation des projets d'aménagement de l'espace public urbain. Aujourd'hui, ceux-ci impliquent davantage de végétalisation (Kowarik, 2011), la préservation des écosystèmes urbains existants (Hérivaux & Le Coent, 2021), ainsi que l'établissement de continuum écologique à la fois dans les villes, mais aussi à l'échelle paysagère (Peng, Zhao and Liu, 2017). Autrefois peu considérés dans les projets d'aménagement publics, les zones péri-urbaines sont désormais partie intégrante des problématiques de la restauration de la continuité écologique entre les villes et les écosystèmes naturels adjacents (Giaffarelli & Vagge, 2023). Ces espaces bordant les villes sont des zones de transition – dites zones tampons – entre les villes et les écosystèmes naturels qui jouent un rôle fondamental pour le maintien de la biodiversité et la connectivité écologique (Livesley, Escobedo and Morgenroth, 2016; Mortoja, Yigitcanlar and Mayere, 2020).

Les acteurs impliqués dans la conception, le maintien et la restauration des écosystèmes urbains sont particulièrement diversifiés (Figure 8). Parmi eux, les aménageurs publics ainsi que les paysagistes-concepteurs sont des acteurs centraux pour le design et l'aménagement des espaces publics dont les espaces végétalisés (Figure 8). L'objectif est aujourd'hui de créer des écosystèmes durables dans le temps, peu consommateurs en carbone et autonomes en eau (Monteiro, Cristina and Covas, 2021). Toutefois, le caractère lacunaire des connaissances concernant le développement souterrain des écosystèmes urbains ne permet pas l'émergence de pratiques intégratives favorisant la persistance des végétaux. En particulier, les propriétés biotiques des sols urbains restent peu connues et non prises en compte pour favoriser la croissance et la survie des végétaux en ville (Newbound, Mccarthy and Lebel, 2010). De plus, les mesures favorisant la biodiversité et la connectivité entre les espaces visent en premier lieu les déplacements de macroorganismes mobiles (oiseaux, amphibiens, mammifères). Les macros- et microorganismes fixes tels que les plantes et leurs associés racinaires ne font pas l'objet d'une réflexion similaire. A ce jour, très peu d'écosystèmes urbains bénéficient d'un statut de conservation (Kowarik, 2011).

Figure 8 : Cadre institutionnel, gouvernance et réalisation de l'espace vert



2. En ville, les sols sont construits sur des bases abiotiques

2.1. *Les sols urbains, les anthrosols, les technosols... Clarification sémantique*

Les sols des écosystèmes urbains sont très divers (Ossola and Livesley, 2016, Greinert, 2015) tant sur les plans physiques, chimiques et biotiques (Scharenbroch, Lloyd and Johnson-Maynard, 2005). Toutefois, les propriétés singulières des milieux urbains conditionnent la structure physique du sol – souvent plus compacte en ville (Yang and Zang, 2015). Sur le plan chimique, l'azote, limitant dans les écosystèmes naturels, est plus abondant dans les sols urbains – et ce en raison des amendements humains, des déjections animales ou encore des émissions de gaz d'origine anthropique (Pickett and Cadenasso, 2009). Les activités anthropiques – notamment industrielles – sont à l'origine d'une teneur accrue en polluants tels que les hydrocarbures et les métaux lourds dans les sols (Sager, 2020). Sur le plan fonctionnel, la capacité d'infiltration de l'eau dans le sol ainsi que le stockage de carbone sont contraints à la fois par la compaction et l'imperméabilisation des sols (scellement des sols, Pouyat *et al.*, 2007). Sur le plan biotique, la capacité d'accueil des organismes du sol semble altérée par l'activité humaine. En comparaison avec les milieux naturels adjacents, la diversité de certains groupes taxonomiques est très dégradée en ville (documenté chez les bactéries, les champignons, les vers de terres et nématodes, Huot *et al.*, 2017; Guillard *et al.*, 2018).

Dans la littérature, plusieurs termes sont couramment utilisés pour désigner les sols urbains. Le plus générique, « sol urbain », désigne tout sol se trouvant dans une zone urbanisée (Riordan *et al.*, 2021). Il désigne à la fois des sols naturels (par exemple, des sols de reliques forestières en villes, Hui *et al.*, 2017; Vogt-schilb *et al.*, 2018) comme des sols complètement artificiels (Huot *et al.*, 2017; Deeb *et al.*, 2020). Le terme d'« anthrosol » désigne tous les sols dont la composition physico-chimique a été modifiée par les activités humaines. Toutefois, dans la définition de Zikeli *et al.*, (2005), les modifications induites par l'Homme résultent simplement de l'occupation prolongée du sol et non pas des actes de conception délibérés. Les anthrosols ne sont pas spécifiques aux milieux urbains. Par exemple, les terra preta (des sols très carbonés suite à l'occupation humaine) sont des anthrosols typiques des forêts tropicales sud-américaines (Woods *et al.*, 2009).

Parce qu'il est plus précis, nous utiliserons le terme de « technosol » dans ce manuscrit. Régulièrement employé en Europe (Go and Zorpas, 2021), ce terme fait référence à tout sol dont la composition (*c.a.d.* matrice) et la structure (*c.a.d.* organisation en horizons) ont été entièrement conçues par les humains (Figure 9, Leguédois *et al.*, 2016). Dans les espaces verts, les technosols des fosses de plantations sont conçus pour répondre aux besoins physico-chimiques des végétaux. Le terme de « terres fertiles » ou de « terre végétale » est également couramment

Encadré 4 : Un dire d'acteur d'agence du paysage interrogé sur la définition des termes de terre fertile et terre végétale

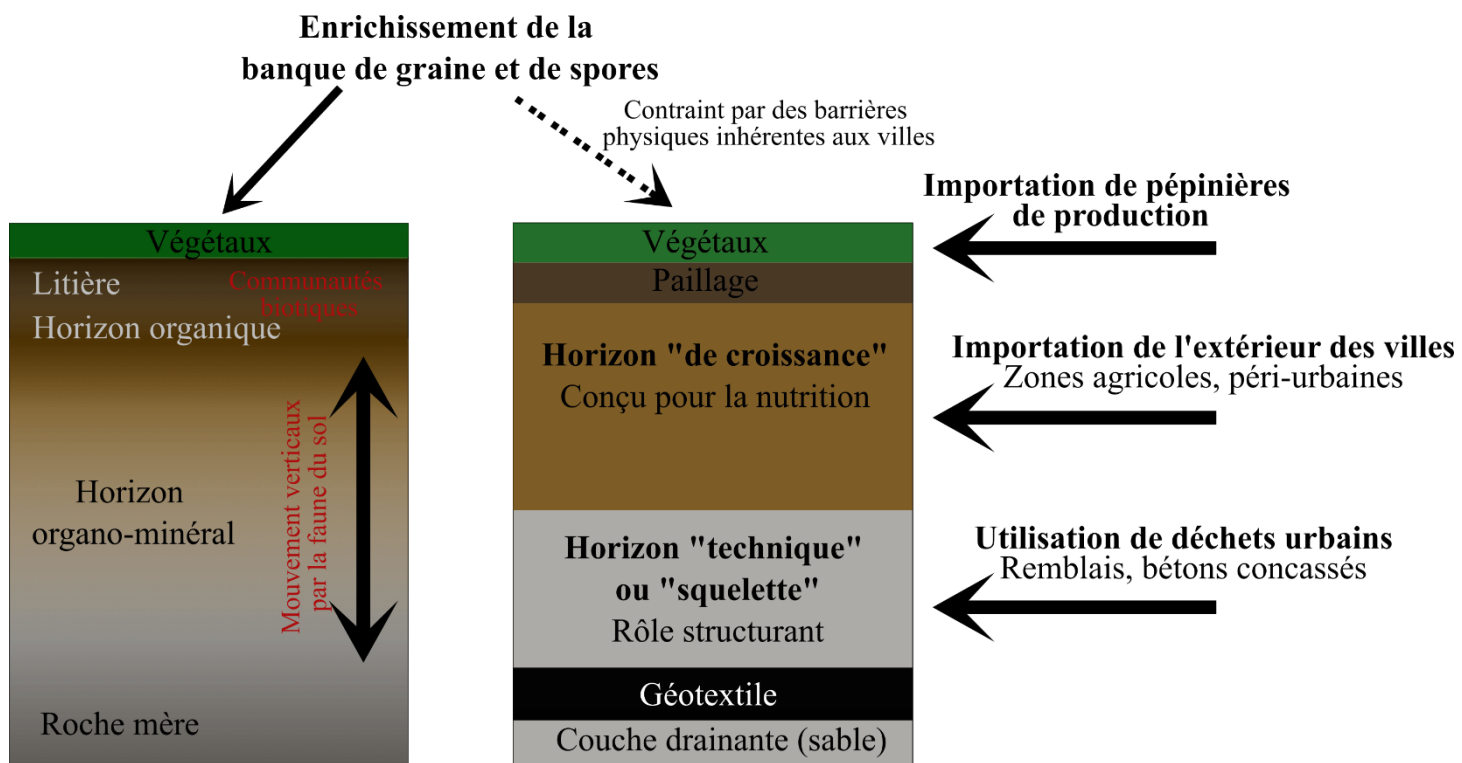
« Terre fertile fait référence à un substrat fabriqué. La méthode de fabrication peut varier [...]. Il n'y a pas vraiment de sémantique derrière ce nom. « Fertile » permet de dire que la terre sera adaptée pour faire pousser les végétaux que l'on plantera. [...] Dans l'inconscient collectif de notre métier, « terre végétale » fait référence avant tout à de la terre issue de décapage de terre agricole (terre arable). En revanche c'est également une norme qui définit un substrat avec des propriétés physico-chimiques précises. Après il faut faire attention, car nous nous posons rarement ce genre de question et tout le monde utilise des termes sans forcément qu'ils soient juste d'un point de vue scientifique ou sémantique. »

employé dans la sphère de l'aménagement public et résulte d'habitudes d'usage dans la sphère de l'aménagement public – plus que d'une définition basée sur un fondement sémantique ou scientifique (Encadré 4). Ainsi, ces deux termes font référence à des terres fabriquées, composites et dont la structure verticale se décline en différents horizons incluant une matrice drainante, une matrice de croissance racinaire, du compost et un paillage en surface (Figure 9, Deeb *et al.*, 2020). La matrice de croissance racinaire de ces technosols se compose principalement de sols importés de l'extérieur des villes, provenant des milieux agricoles ou péri-urbains (Deeb *et al.*, 2020). Ces pratiques d'importation de sols issus de l'extérieur des villes montrent des limites pouvant avoir des conséquences délétères sur les milieux naturels et péri-urbains. L'importation de volumes considérables de sols fertiles agricoles (Figure 9) génère des coûts économiques et environnementaux inhérents à leur prélèvement et leur transport (Zambon *et al.*, 2018; Deeb *et al.*, 2020). En France, par exemple, 3 millions de m³ de sols sont ainsi transférés chaque années des milieux agricoles vers les centres urbains (Rokia *et al.*, 2014). Lors de ces opérations de substitution édaphique, l'extraction de la matrice du sol présente sur le site urbain à aménager et son exportation représentent également des coûts écologiques et économiques particulièrement importants (Walsh *et al.*, 2019).

Ces pratiques de conception des sols ont été principalement étudiées sous l'angle de la reprise et de la croissance des végétaux qui s'y établissent. La composition et la quantité

d'amendement organique a montré un effet différencié sur la croissance d'arbres urbains méditerranéens (De Lucia *et al.*, 2013). Pour l'heure, le compartiment biotique de ces sols nouvellement créés n'a été que peu étudié (voir Deeb *et al.*, 2017 pour une étude sur les vers de terre) et son intégration dans les procédés de conception reste largement à explorer. Les communautés ectomycorhiziennes des technosols sont peu connues et ne font pas encore l'objet d'une réflexion pour la conception de ces sols.

Figure 9 : Schéma comparatif d'un sol-type de milieu forestier et d'un technosol



2.2. *Les sols scellés constituent-ils une ressource alternative à l'importation des sols agricoles dans les villes ?*

Comme évoqué précédemment, la conception des technosols repose en grande partie sur l'importation de matériaux extérieurs aux villes (Rokia *et al.*, 2014; Deeb *et al.*, 2020). Les alternatives telles que l'usage des sols scellés sont encore peu employées. La demande sociétale croissante pour la conception d'espaces végétalisés urbains accroît la pression sur les sols agricoles. En effet, la pédogénèse est un processus sur le temps long, de l'ordre du siècle pour construire 2 à 10 mm d'horizon organo- minéral différencié (Heimsath *et al.*, 1997). Afin de répondre aux besoins croissants des villes en sols, de nouvelles pistes de conception et de recyclage des sols sont donc à explorer. Entre particulier, le recyclage des sols scellés constitue un véritable levier, qui s'inscrit dans le développement de circuits courts visant à favoriser le ré-usage des sols présents sur les sites d'aménagement.

En ville, des volumes considérables de sols scellés sont confinés sous des infrastructures de transport, d'industrie ou de logement. Le scellement des sols sous des matériaux imperméables tels que des goudrons, des bétons ou encore des infrastructures peut durer plusieurs décennies (Scalenghe and Ajmone-Marsan, 2009). Le scellement du sol restreint l'infiltration de l'eau (Di Prima *et al.*, 2018) et l'imperméabilisation induite par le scellement limite les échanges entre les compartiments aériens et souterrains de l'écosystème – constituant une barrière physique à l'accueil et à la respiration des communautés biotiques telles que les invertébrés et les champignons (Tobias *et al.*, 2018). Le scellement affecte notamment les communautés de champignons ectomycorhiziens et en réduisant la richesse (Martinová *et al.*, 2016). Cet appauvrissement des communautés microbiennes limite certaines fonctions fondamentales telles que la minéralisation (Geisen, Wall and van der Putten, 2019; Pereira, O'Riordan and Stevens, 2021).

A l'échelle mondiale, les sols scellés couvrent environ 3% de la surface de notre planète et 2,4 % de la surface couverte par les pays membres de l'Union Européenne (Rodriguez-Espinosa *et al.*, 2021). Aujourd'hui, la désimperméabilisation des sols est motivée par la conversion des espaces industriels pour répondre aux besoins d'espaces sous la pression d'urbanisation (Tobias *et al.*, 2018; Fan *et al.*, 2020) – ou encore pour répondre à des questions de sécurité alimentaire (Rodriguez-Espinosa *et al.*, 2021). Aujourd'hui, les sols scellés

représentent une ressource abondante et disponible dans les centres urbains après un procédé de désclellement (Tobias *et al.*, 2018). Les premiers travaux portant sur les propriétés des sols scellés montrent une modification de la structure et des propriétés physico-chimique en raison du scellement : ces sols sont compactés (Layman *et al.*, 2016), pauvres en matière organiques, en nutriments (O’Riordan *et al.*, 2021) et parfois contaminés par des hydrocarbures (Charzynski *et al.*, 2017).

En raison des propriétés qui les rendent peu valorisables (structure peu organisée, pauvreté chimique et biotique, Geisen, Wall and van der Putten, 2019), les sols scellés demeurent peu utilisés pour la végétalisation, et peu étudiés sous l’angle des processus permettant leur réhabilitation (Maienza *et al.*, 2021). Pourtant, des travaux de restauration de ces sols ont produit des résultats encourageants en témoignant d’un ré-enrichissement en nutriment du sol descellé sans recourir à l’ajout d’intrants (Maienza *et al.*, 2021). En Suisse, Tobias *et al.*, (2018) ont suivi de plusieurs projets de réhabilitation de friches industrielles dont les sols étaient scellés avec des résultats prometteurs quant à la restauration des propriétés d’accueil de la biodiversité et pour la récupération des services écosystémiques perdus telles que les fonctions de rétention et d’absorption de l’eau.

3. Considérer les interactions entre sols et plantes : une piste pour la conception des sols et des communautés végétales urbaines

3.1 La réintroduction d'une fraction biotique peut permettre de concevoir des technosols fonctionnels

La banque de spores fongiques, et plus largement l'inoculum fongique présent dans les sols urbains en général et dans les sols scellés en particulier est pour l'heure encore peu connue (Newbound, Mccarthy and Lebel, 2010; Authier, Violle and Richard, 2022). Les premières analyses comparant des sols urbains et les écosystèmes naturels adjacents ont mis en évidence des différences de composition marquées. Dans les villes, les alignements d'arbres abritent des communautés ectomycorhiziennes pauvres (Bainard, Klironomos and Gordon, 2011; Martinová *et al.*, 2016) comparativement aux parcs anciens qui concentrent une diversité fongique plus élevée (Hui *et al.*, 2017; Marczylo, Macchiarulo and Gant, 2021; Figure 9). Dans les sols urbains les plus artificialisés, les communautés ectomycorhiziennes se composent majoritairement des taxons produisant des fructifications hypogées, dont de nombreux Ascomycètes parmi lesquels des Tubercacées (Cui & Mu, 2016; Karpati *et al.*, 2011; Martinová *et al.*, 2016). Cette situation contraste avec les écosystèmes forestiers, généralement dominés par les lignées de Basidiomycètes aux fructifications épigées (Figure 9), dont les Russulaceae, Cortinariaceae (Hui *et al.*, 2017; Marczylo *et al.*, 2021; Martinová *et al.*, 2016)

La capacité de dormance et de germination des espèces fongiques ectomycorhiziennes varie selon les lignées (Nara, 2009), et selon le type de forme de résistances produites (LoBuglio *et al.* 1999). Les propagules peuvent persister à moyen terme pour les taxons à fructifications hypogées (Bruns *et al.*, 2009; Taschen *et al.*, 2022) alors que la persistance des spores des fructifications épigées semblerait plus limitée dans le temps (Ishida *et al.*, 2008). Dans une perspective de restauration des sols scellés pour l'aménagement d'écosystèmes urbains, l'étude des communautés ectomycorhiziennes pourrait permettre d'estimer les capacités d'accueil biotiques de ces milieux.

La restauration biotique des sols consiste en l'ajout d'un substrat enrichi en spores, mycélium, apex ectomycorhizés, voire carpophores issus des horizons organiques de sols naturels (Repac, 2011; Taschen *et al.* 2022). Des essais d'inoculation à des fins d'enrichissement de la communauté fongique ont été développés en conditions contrôlées – par exemple en pépinière (Rincon, Alvarez and Pera, 2001) pour des applications en milieu naturel (Selosse *et al.*, 2001). Par ailleurs, la pratique de l'inoculation ectomycorhizienne dans un contexte de sol dégradé a montré des résultats prometteurs sur l'établissement et la croissance des végétaux. Par exemple, dans des sites miniers, le taux de survie de plantules de *Pinus densiflora* et *Quercus variabilis* inoculées avec *Pisolithus sp.*, *Cenococcum geophilum* et *Laccaria laccata* étaient largement supérieurs à ceux de plantules non inoculées (Zong *et al.*, 2015). Dans un projet de phytoremédiation en contexte postindustriel, l'inoculation de *Salix miyabaena* par le champignon *Sphaerosporella brunnea* a permis d'améliorer la croissance des plantules – notamment en terme de biomasse de l'appareil caulinaire (Dagher, Pitre and Hijri, 2020). Dans les villes, les pratiques d'inoculation du sol restent peu mises en œuvre. Des tentatives anciennes d'inoculation ectomycorhizienne de *Laccaria laccata*, *Cenococcum geophilum* et *Paxillus involutus* au pied d'arbres d'alignement (*Tilia spp*) ont montré un effet significatif sur la nutrition en calcium et magnésium des arbres croissance (Garbaye and Churin, 1996; Garbaye *et al.*, 1999). Plus récemment, des travaux portant sur l'inoculation par des champignons ectomycorhiziens dans des alignements de *Pinus contorta* ont montré que les arbres ayant établis des connexions avec les genres *Tuber*, *Suillus* et *Wilcoxina* avaient une résistance accrue au stress généré par les sels de déneigement (Zwiazek *et al.*, 2019). Les pratiques d'inoculation ectomycorhizienne restent pour l'heure peu pratiqués dans le cadre de la conception des technosols.

Les méthodes actuelles de conception des technosols permettent de répondre aux besoins des végétaux en nutriments et en matière organique mais ne reposent pas sur les organismes impliqués dans les interactions synergiques entre plantes. Dans les sols scellés, les communautés ectomycorhiziennes n'ont été que peu étudiées. Sous l'angle de la recherche fondamentale, ces sols avec une date de scellement connue sont des candidats adéquats pour étudier la persistance et la résilience des propagules ectomycorhiziennes. Il est alors possible d'explorer la résilience des communautés ectomycorhiziennes au cours d'un gradient temporel de scellement. De plus, l'analyse des traits des taxons ectomycorhiziens persistants peut assister la théorisation des stratégies écologiques des champignons et guider les pratiques d'inoculation

dans les sols scellés. Dans ce travail, nous explorerons les communautés ectomycorhiziennes présentes dans les sols urbains scellés et testerons leur capacité à 1) maintenir un rôle inoculant sur les végétaux, au terme de décennies de scellement, et 2) s'installer avec succès dans de tels sols à partir de transferts depuis les milieux naturels.

3.2. Les interactions entre plantes médiées par le sol constituent un levier pour la conception d'espaces verts urbains fonctionnels

Les boucles de rétroactions entre sol et plantes ont été largement documentées dans les écosystèmes naturels (Van der Putten *et al.*, 2013) et agricoles (Mariotte *et al.*, 2018) et témoignent du rôle fondamental des processus de facilitation dans les cycles des nutriments. En ville, ces *plant-soil feedback* demeurent sous-étudiés.

Dans les villes européennes, la majorité des espèces ectomycorhiziennes utilisées dans les espaces verts urbains appartiennent aux Pinaceae (*Pinus spp.*), Fagaceae (*Quercus spp.*), Malvaceae (*Tilia spp.*) ou encore Salicaceae (Ossola *et al.*, 2020; Olchowik *et al.*, 2021; Purahong *et al.*, 2022). Or, les premiers travaux de biologie moléculaire se focalisant sur les interactions plante-champignons en contexte urbain ont été essentiellement consacrés aux plantes endomycorhiziennes et montrent un déficit de mycorhization comparativement aux espaces naturels (Stabler, Martin and Stutz, 2001; Bainard, Klironomos and Gordon, 2011). Un patron contrasté a été observé chez les végétaux ectomycorhiziens. Les communautés appauvries des sols urbains montrent par exemple une colonisation des apex racinaires plus faible en ville qu'en milieu forestier chez le chêne rouge (*Quercus rubra*, Karpati *et al.*, 2011). A l'opposé, d'autres travaux utilisant des outils moléculaires ont mis en évidence une forte capacité des champignons persistants dans les sols urbains à coloniser les systèmes racinaires des végétaux – comme par exemple dans les alignements de tilleuls, majoritairement mycorhizés par le champignon *Cenococcum geophilum* (Timonen and Kauppinen, 2008). Sur le plan morphologique, les taux de colonisation des racines par les champignons ont montré des résultats contrastés. D'une part, une colonisation similaire a été observée chez le chêne rouge le long d'un gradient d'urbanisation (Tonn and Ibáñez, 2017). D'autre part, une plus faible colonisation par les champignons ectomycorhiziens dans des parcs urbains et des alignements d'arbres, en comparaison avec des écosystèmes forestiers adjacents (Bainard, Klironomos and Gordon, 2011). Les facteurs responsables de ces réponses sont peu clairs. La signature

singulière des sols urbains en nutriments, matière organique, et métaux lourds pourraient limiter la connectivité entre les plantes et leurs partenaires mycorhiziens (Jumpponen *et al.*, 2010). Toutefois, la capacité des plantes à mobiliser l'inoculum ectomycorhizien dans un contexte urbain représente un résultat encourageant pour l'utilisation des interactions plantes-champignons dans une démarche d'aménagement.

Les processus de facilitation entre plantes médiées par le mutualisme ectomycorhizien ont fait l'objet de travaux en milieu naturel (par exemple Richard *et al.* 2009). Dans les écosystèmes urbains, les communautés végétales sont élaborées par les architectes et les paysagistes-concepteurs selon des critères économiques (Hope *et al.*, 2003), autoécologiques (Jenerette *et al.*, 2016) et selon leur valeur sociale (*c.a.d.* selon l'appréciation du citoyen pour l'esthétique du végétal, Goodness, 2018). Dans ces travaux, nous proposons d'explorer la dimension fonctionnelle de ces communautés, en l'abordant par la capacité des végétaux à coopérer grâce à leurs partenaires fongiques partagés. Dans les villes, l'établissement de tels réseaux est limité par 1) la faible disponibilité en symbiotes fongiques (Bainard, Klironomos and Gordon, 2011; Karpati *et al.*, 2011; Martinová *et al.*, 2016) et 2) les limitations du développement racinaire imposées par les designs de conception des fosses de plantation. La structure compartimentée des espaces verts en fosses de plantation est limitante à la fois pour la croissance des arbres et pour l'établissement d'interactions racinaires entre végétaux voisins. Cette compartimentation résulte d'une forte occupation du sous-sol par l'Homme, les sous-sols des villes étant utilisés pour les divers réseaux anthropiques (par exemple les réseaux de transports, électricité, eau, internet, Randrup, McPherson and Costello, 2001; Levinson, 2012; Galle, Nitoslawski and Pilla, 2019). Ainsi, les végétaux doivent évoluer dans les espaces confinés, l'extension de leur système racinaire pouvant être limitée par les barrières physiques des bordures des fosses de plantation – scellées, sans continuum dans le sol entre les différentes fosses. Sur le plan fonctionnel, la compartimentation et le manque de connectivité entre les espaces de l'espace limite les échanges et l'établissement des réseaux d'interactions mutualistes avec les fosses voisines (Peng, Zhao and Liu, 2017).

III. Questions générales, hypothèses et déroulé de ce manuscrit

Aujourd'hui, les villes et les acteurs du paysage cherchent à élaborer des espaces capables de répondre aux enjeux climatiques et de contribuer au bien-être des populations urbaines. Dans cette perspective, des espaces verts durables dans le temps et pourvoyeurs de services écosystémiques sont attendus par la demande sociétale. Il est établi que la longévité et la résilience des communautés végétales s'appuient notamment sur la réalisation de connexions efficaces dans le sol, via des partenariats microbiens.

Dans ce travail, nous utilisons l'étude de cas de la métropole de Marseille pour aborder la séquence de questionnements suivante. Sur le plan fondamental, nous 1) questionnons la diversité fongique ectomycorhizienne présente dans les sols urbains et 2) nous testons la capacité de cette diversité à être spontanément mobilisée par les végétaux. A l'amont, et sur le plan méthodologique, nous 3) évaluons la capacité des outils moléculaires de métabarcoding utilisés en recherche (Illumina MiSeq) à détecter et caractériser les communautés fongiques des sols méditerranéens. A l'aval et sur le plan appliqué, nous questionnons 4) la capacité des écosystèmes naturels à fournir un inoculum fongique utilisable par les aménageurs et 5) les modalités techniques à mettre en œuvre pour réaliser de telles inoculations.

Dans ce manuscrit, nous nous intéresserons dans un premier lieu à l'étendue de la connaissance sur les réseaux d'interactions ectomycorhiziens des écosystèmes naturels aux espaces urbains (**Chapitre I**). Dans cette revue bibliographique, nous mettons en évidence et identifions l'étendue des problématiques restant à explorer dans les milieux urbains. Nous montrons que les réseaux anthropiques (*e.g.* canaux de transports et de communications, réseaux d'énergie, d'eau) sont prioritaires dans les villes, au détriment des réseaux écologiques. Nous proposerons l'idée de trame beige, un outil opérationnel qui pourrait permettre la connectivité entre les organismes fixes, tels que les plantes et les champignons ectomycorhiziens.

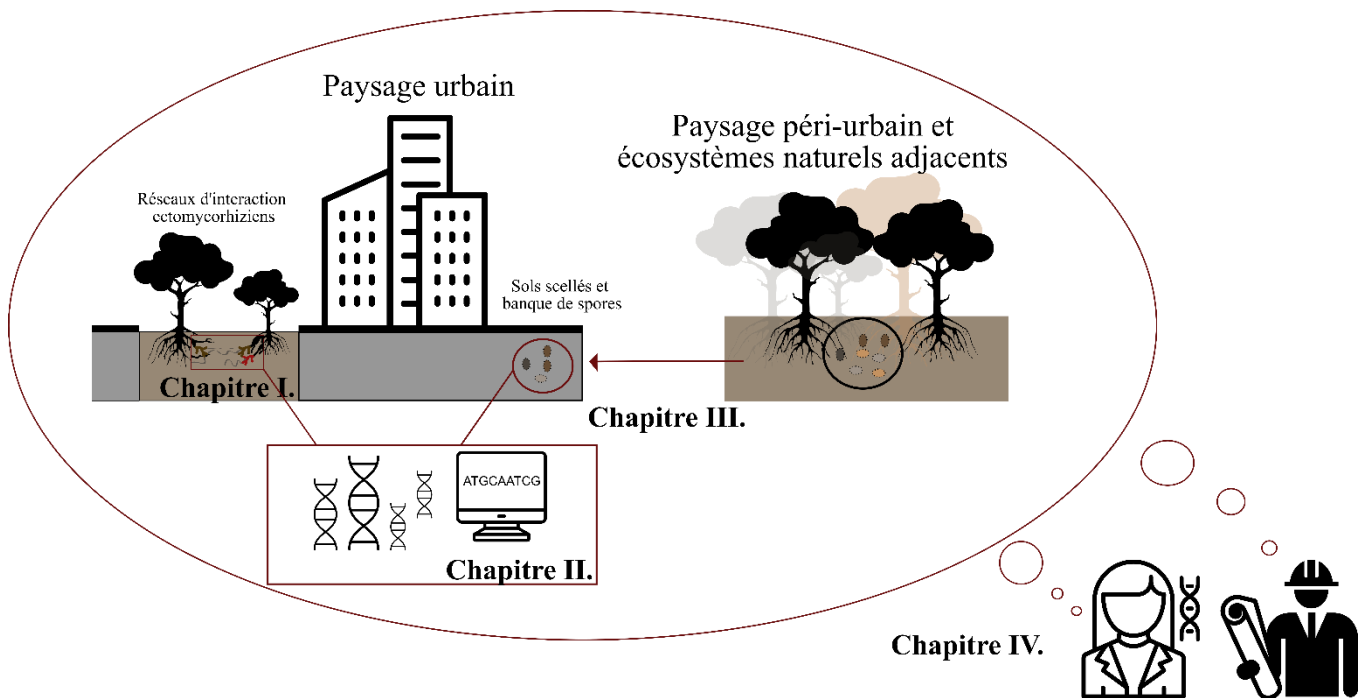
Ensuite, nous nous focalisons sur les méthodes moléculaires de barcoding et de métabarcoding pour l'identification des champignons ectomycorhiziens (**Chapitre II**). Par une

approche expérimentale, nous analyserons la diversité et la composition des communautés ectomycorhiziennes restituées par le métabarcoding selon différentes calibrations de profondeur de séquençage. Nous testerons sa répliquabilité et son calibrage afin de mieux comprendre et interpréter les données générées par la machine.

Dans le **Chapitre III**, nous questionnons la capacité des sols urbains à séquestrer un inoculum ectomycorhizien fongique viable sur le long terme. Nous utiliserons des outils de métabarcoding pour explorer les réservoirs de propagules de sols scellés et de sol d'écosystèmes péri-urbains (une forêt de chêne vert et une garrigue). Nous analyserons la capacité des spores persistantes à former des interactions avec les végétaux après des décennies de scellement sous des matériaux étanches. Sous un angle plus appliqué, nous testerons la capacité des sols des milieux péri-urbains à fournir un inoculum mobilisable par les végétaux dans le contexte limitant des sols scellés.

Dans un dernier chapitre (**Chapitre IV**), nous analyserons les particularités de villes qui permettent de questionner l'écologie des champignons ectomycorhiziens. Nous réfléchirons comment l'inclusion des composantes du paysage urbain et péri-urbain dans des questions de recherche peuvent d'une part permettre d'aborder des problématiques fondamentales d'écologie des communautés des champignons ectomycorhizien et d'écologie fonctionnelle ; et d'autre part, interroger les méthodes actuelles de conception des espaces végétalisés, notamment pour la production des ressources en sols et plantes qui les composent.

Figure 10 : Représentation schématique des différents axes de travail de ma thèse



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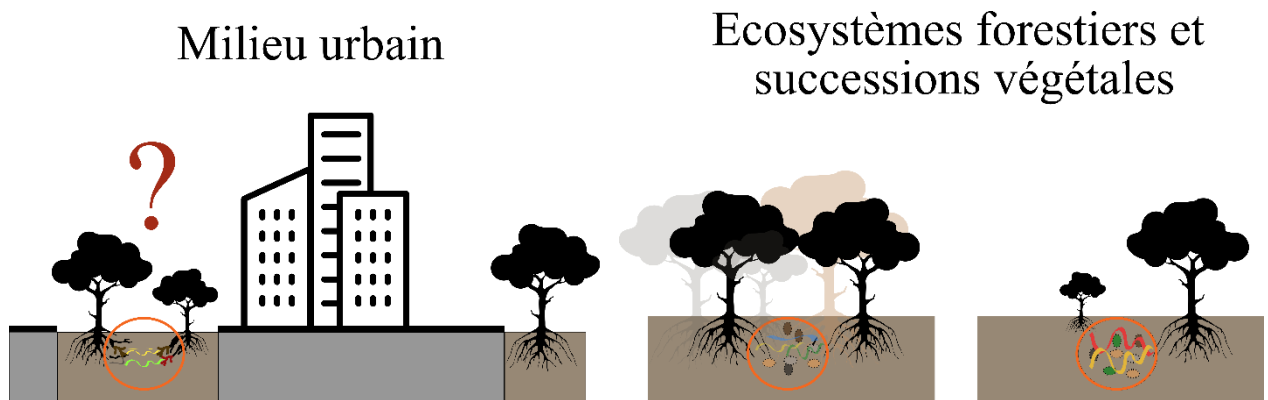
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Chapitre 1 : Les réseaux d'interaction ectomycorhiziens dans l'anthropocène : des écosystèmes naturels à l'aménagement urbain



Présentation du chapitre en français

Dans la plupart des forêts boréales, tempérées et méditerranéennes, les arbres acquièrent des ressources hydriques et minérales du sol par le biais d'associations ectomycorhiziennes. Les champignons ectomycorhiziens (ECM) sont très diversifiés et leur spécificité à l'égard des plantes hôtes varie considérablement de la spécialisation (peu de partenaires ectomycorhiziens) au généralisme (vaste panel de partenaires fongiques). En interagissant simultanément avec plusieurs plantes hôte, les champignons ectomycorhiziens peuvent être à l'origine de réseaux d'interactions qui servent de médiateurs pour les interactions nutritives entre plantes voisines. Ce phénomène intervient différenciellement au cours des successions végétales – et intervient dans la dynamique des communautés végétales (établissement, survie).

Les connaissances actuelles sur les réseaux d'interactions ectomycorhiziens reposent essentiellement sur un corpus acquis dans les écosystèmes tempérés. Les forêts intertropicales ainsi que les écosystèmes très anthropisés restent peu explorés. Dans le chapitre à venir, nous nous sommes intéressés aux réseaux d'interactions ectomycorhiziens qui peuvent s'établir entre plantes voisines, au cours de la succession végétale et le long d'un gradient d'anthropisation. D'abord, nous explorons le corpus disponible qui documente l'implication des réseaux d'interactions au cours des successions primaires et secondaires. Nous aborderons le cas des écosystèmes naturels, puis des systèmes forestiers gérés par l'Homme. Enfin, nous nous focaliserons sur les écosystèmes anthropisés et sur les caractères inhérents aux villes peuvent contraindre l'établissement des réseaux d'interactions ectomycorhiziens.

Ce travail met en avant trois conclusions principales. Tout d'abord, l'identification de taxons ectomycorhiziens dans les tissus de plantes endomycorhiziennes a révélé une interconnexion inattendue entre les plantes-hôtes ectomycorhiziennes et les plantes herbacées mycorhiziennes à arbuscules (AM). Par l'endophytisme, ces plantes herbacées peuvent jouer un rôle de réservoir de symbiontes ectomycorhiziens – avec des conséquences sur l'établissement des plantes ectomycorhiziennes dans les milieux ouverts. Ensuite, nous avons constaté le caractère inégal des connaissances sur les réseaux d'interactions ectomycorhiziens au cours de la succession végétale, en particulier dans les forêts cultivées – dont les peuplements se composent majoritairement d'hôtes ectomycorhiziens. Enfin, nous avons identifié le caractère lacunaire des connaissances sur les réseaux d'interactions ectomycorhiziens dans les villes – et ce en raison du caractère paupérisée de l'inoculum fongique résident et des conflits spatiaux entre humains et non-humains. Malgré des initiatives de gestion visant à interconnecter

les organismes mobiles par le biais de corridors écologiques (trames vertes et bleues), la conception de trames du sol (trame beige) visant à connecter les organismes fixes (champignons ectomycorhiziens) reste très complexe à difficile à développer entre les différents écosystèmes urbains et péri-urbain.

A l'issue de ce travail bibliographique, nous identifions les principales perspectives de recherche fondamentales et appliquées - qui permettront de mieux comprendre les composantes des réseaux d'interaction ectomycorhiziens dans les villes – et de favoriser leur développement dans les projets d'aménagement urbain.

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Ectomycorrhizal Networks in the Anthropocene: From Natural Ecosystems to Urban Planning

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Trees acquire hydric and mineral soil resources through root mutualistic associations. In most boreal, temperate and Mediterranean forests, these functions are realized by a chimeric structure called ectomycorrhizae. Ectomycorrhizal (ECM) fungi are highly diversified and vary widely in their specificity toward plant hosts. Reciprocally, association patterns of ECM plants range from highly specialist to generalist. As a consequence, ECM symbiosis creates interaction networks, which also mediate plant–plant nutrient interactions among different individuals and drive plant community dynamics. Our knowledge of ECM networks essentially relies on a corpus acquired in temperate ecosystems, whereas the below-ground facets of both anthropogenic ECM forests and inter-tropical forests remain poorly investigated. Here, we successively (1) review the current knowledge of ECM networks, (2) examine the content of early literature produced in ECM cultivated forests, (3) analyze the recent progress that has been made in understanding the place of ECM networks in urban soils, and (4) provide directions for future research based on the identification of knowledge gaps. From the examined corpus of knowledge, we reach three main conclusions. First, the emergence of metabarcoding tools has propelled a resurgence of interest in applying network theory to ECM symbiosis. These methods revealed an unexpected interconnection between mutualistic plants with arbuscular mycorrhizal (AM) herbaceous plants, embedding ECM mycelia through root-endophytic interactions. This affinity of ECM fungi to bind VA and ECM plants, raises questions on the nature of the associated functions. Second, despite the central place of ECM trees in cultivated forests, little attention has been paid to these man-made landscapes and in-depth research on this topic is lacking. Third, we report a lag in applying the ECM network theory to urban soils, despite management initiatives striving to interconnect motile organisms through ecological corridors, and the highly challenging task of interconnecting fixed organisms in urban greenspaces is discussed. In particular, we observe a pauperized nature of resident ECM inoculum and a spatial conflict between belowground human pipelines and ECM networks. Finally, we identify the main directions of future research to make the needed link between the current picture of plant functioning and the understanding of belowground ECM networks.

Keywords: plant-fungal interactions, ectomycorrhizal symbiosis, endophytic fungi, anthropogenic soils, forest soils, ecological succession, syngenetic cycle

Introduction

In most boreal, temperate and Mediterranean ecosystems, as in a part of tropical and subtropical forests, the canopies of few ectomycorrhizal (ECM) tree species dominate multi-layered communities of arbuscular mycorrhizal (AM) plants (Smith and Read, 2010; Tedersoo and Nara, 2010; Liang *et al.*, 2020). ECM symbiosis shapes a wide variety of landscapes worldwide, from highly-preserved old-growth forests of the northern hemisphere (Smith *et al.* 2002; Spake *et al.* 2016), to emblematic oak savannas in Mediterranean biodiversity hotspots (Kennedy *et al.* 2012; Baptista *et al.* 2015), to highly artificialized tree plantations (Giachini *et al.* 2000) and orchards (Belfiori *et al.* 2012).

Belowground, ECM assist plant mineral nutrition by hydrolyzing natural polymeric compounds contained in litter and forest organic debris (Pritsch *et al.*, 2011; Martin *et al.*, 2016), and mobilizing soil water through absorptive hyphae (Lehto and Zwiazek, 2011). Based on their ability to densely colonize tree root systems, ECM mycelia establish hundreds of thousands of connections per square meter through short roots (Dahlberg, Jonsson and Nylund, 1997; Taylor, 2002), from which thousands of kilometers of extrametrical mycelia are annually produced exploring soil for water, nutrients and new apices to colonize (Leake *et al.*, 2004; Hagenbo *et al.*, 2016), or provide physical support to plant-plant interactions in the soil.

Taxonomically, ECM mutualism is highly asymmetric, with a high diversity of some 20,000 species of Ascomycetes and Basidiomycetes linking only 6,000 species of Angio- and Gymnosperms within very few families (van der Heijden *et al.*, 2015). In the ECM symbiosis, both the host and symbiotic species highly vary in their degree of specificity with their partners, from highly specialized (*e.g.* species in the Basidiomycete genera *Alnicola* and *Alpova* are strict associates of *Alnus* spp), to broad-host range species (*e.g.* the Ascomycete *Cenococcum geophilum* associated with a wide range of Angio- and Gymnosperms; Bruns, Bidartondo and Taylor, 2002; Barham, Harend and Tedersoo, 2014). These patterns underly the ability of ECM fungal diversity to interconnect ECM hosts through compatible dispersed inoculum across landscapes (Taudiere *et al.*, 2015).

In multi-layered forests, the co-occurrence of plants from various mycorrhizal guilds constitutes the support for the establishment of common mycorrhizal networks (CMNs) linking canopies to the undergrowth through belowground mycelia. While AM networks interconnect roots from similar or different AM species of trees to shrubs and herbaceous plants (Wipf *et al.*, 2019),

fully autotrophs ECM trees exchange nutrients among each other (Klein, Siegwolf and Körner, 2016) and with mixotrophic and mycoheterotrophic orchids (e.g. Li *et al.*, 2021 for orchids) and ericaceous forest plants (Suetsugu *et al.* 2021).

Based on its ability to establish dense networks of hyphal connections among roots, ECM symbiosis strongly influences plant community composition and dynamics (Richard *et al.*, 2009; van der Heijden *et al.*, 2015; Nagati *et al.*, 2019). Broad-host range species of fungi drive interspecific plant-plant interactions through shared mycelia (Beiler *et al.*, 2010), which are then interconnected into CMNs. Reciprocally, hub species of ECM plants typically accumulate hyper-diverse communities of ECM fungi that co-occur on the local scale by their root systems (Tedersoo *et al.*, 2012; Courty *et al.*, 2016). In forest soils, these mycelial-mediated physical links among roots are involved in an underground carbon trade among co-occurring plant individuals (Simard, Jones and Durall, 2003; Klein, Siegwolf and Körner, 2016). They constitute the below-ground facet of mycohetero- and mixo-trophic plant evolutionary lineages, whose species become established and survive along with ECM trees (Bidartondo, 2005; Selosse *et al.*, 2006). From a phytocentric perspective, these interaction networks mediate positive soil feedbacks among co-occurring plants and drive ecological successions (Bever, 2002; Kennedy *et al.*, 2012).

One of the best markers of the Anthropocene lies on the dramatic degradation of physical, chemical and biological signatures of the pedosphere, and the rapid extension of human-made soil profiles in most parts of the world (e.g. *Anthrosols* and *Technosols* ; Certini and Scalenghe, 2011). As organisms are highly dependent on soil physico-chemical conditions, ECM fungal communities are critical components of soil history by responding to short-term as well as long-term human impacts (Dupouey *et al.* 2002). Inevitably, the composition and dynamics of ECM community are profoundly influenced by forest management (Tomao *et al.* 2020) and anthropic disturbance derived from agronomic practices in man-made ecosystems (Olivera *et al.* 2014; Taschen *et al.* 2015), with cascading effects on the underlying interaction networks (Correia *et al.* 2021).

Since the early 2000s and the first evidence of the pivotal role of ECM CMNs in the dynamics of temperate plant communities during both primary (Nara, 2006a) and secondary successions (Simard *et al.*, 1997), the ecological significance of ECM fungal-mediated interactions among plants has been a matter of ongoing debate (Bever *et al.*, 2010; Birch *et al.*, 2020). As a consequence, our understanding of the physical nature, functional boundaries and trophic

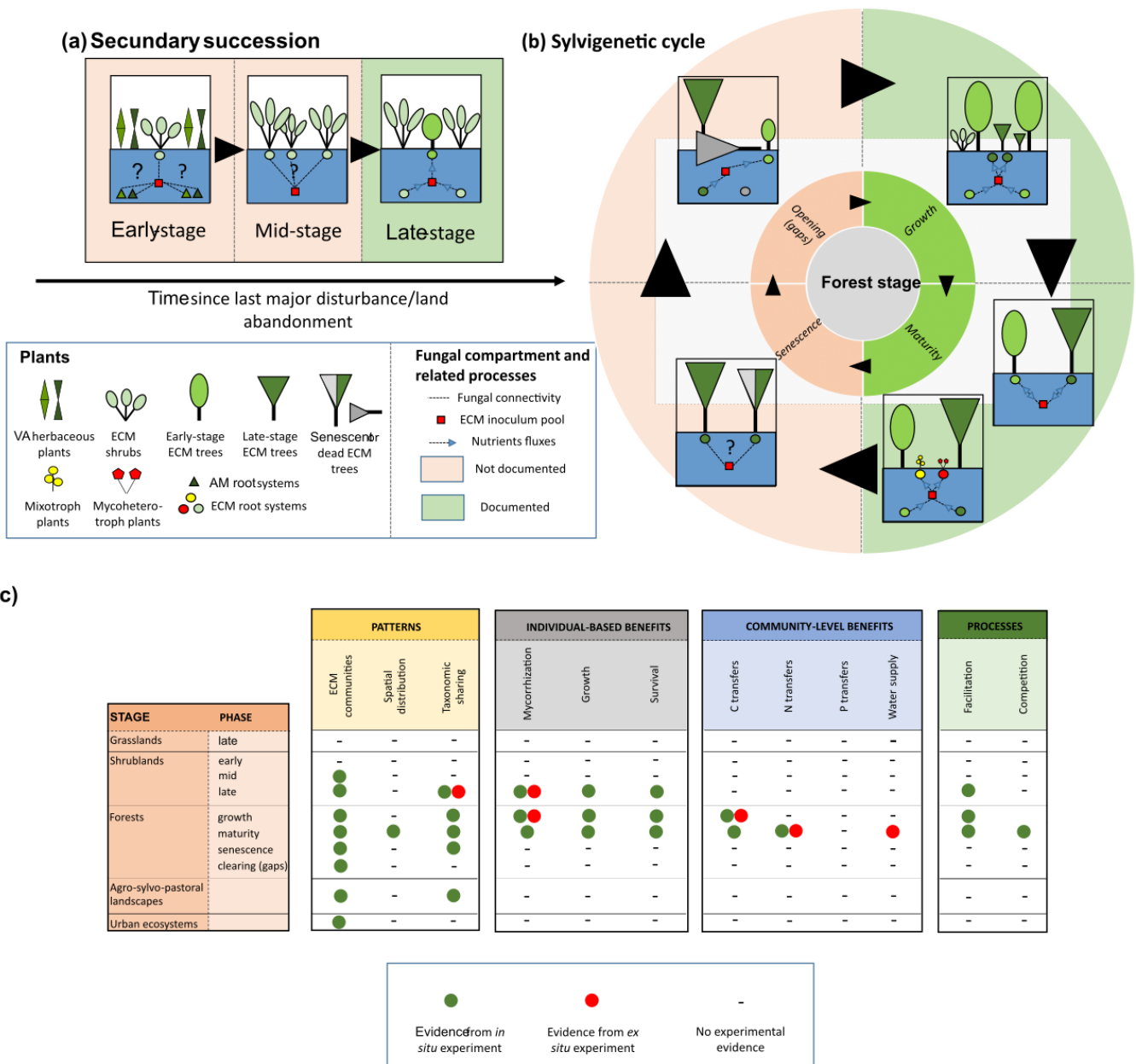
influence of ECM CMNs has considerably increased for the last decade, propelled by both the emergence of powerful metabarcoding tools and the deployment of a variety of experimental approaches in order to decipher underground ECM-based processes.

In this review, we provide an overview of major advances of our understanding of the ECM CMNs, focusing on the spatial extent, associated functions and effects of anthropic practices on mycelium sharing among plants. We evaluate the published literature and identify research gaps to determine promising research avenues along a gradient of anthropic footprint, extending from forests driven by spontaneous processes on one hand, to cultivated forests (see Glossary) and highly artificial urban ecosystems on the other. First, we provide a diachronic perspective on the role of ECM CMNs in natural ecosystems along ecological successions, with a focus on the sylvigenetic cycle (*i.e.* the ontogenetic cycle of the forest *sensu* Oldeman 1948). Second, we mobilize the state-of-the-art concerning ECM network-based research in cultivated forests, including agroforests, to discuss the unexpected diversity of ecological guilds involved in ECM networks, and the consequences of our understanding of forest functioning. Third, we assemble current knowledge concerning ECM diversity patterns in urban soils, and discuss the challenge of conciliating ECM network-based services in cities and the development of human networks. Last, we propose a framework for future research across the gradient of ecosystems explored in the first three sections of the review.

Ectomycorrhizal networks drive plant community dynamics along ecological succession

During ecological successions in temperate, Mediterranean and boreal ecosystems, two dependent processes concomitantly unfold after disturbance. While aboveground communities of pioneer herbs and shrubs are progressively enriched by tree species, a microbiological switchover occurs belowground, from primarily arbuscular mycorrhizae associations (AM) on roots of early vegetation stages to their replacement by ectomycorrhizal association patterns on tree root systems (van der Heijden *et al.*, 2015). In these ecosystems, the main tree families (*i.e.* Fagaceae, Betulaceae, Pinaceae, Salicaceae; Taudiere *et al.*, 2015) have the ability to associate with ECM fungi. Contrastingly, only two families of shrubs (*i.e.* Cistaceae and Ericaceae *pro parte* (Comandini, Contu and Rinaldi, 2006; Richard, Gardes and Selosse, 2009) are ectomycorrhizal. In this section, we successively discuss 1) the networking role of these ecologically pivotal shrub species that endure the transition between VA and ECM plant communities, and 2) our knowledge of the role of ECM CMNs during the sylvigenetic cycle, from seedling establishment to the senescence of forest trees.

Figure 1: Schematic representation of ectomycorrhizal-based networks (a) across early ecological succession and (b) during sylvigenetic cycle. Dashed lines indicate plant–plant interactions through ECM CMNs. Blue arrows indicate nutrient transfers through ECM CMNs. (c) Summary of ECM network-related effects documented along the chronosequence.



ECM networks mediate plant-plant interactions in early successional stages

Our knowledge of the ecological significance of ECM networks along succession almost entirely relies on data accumulated in boreal and temperate ecosystems of the Nearctic and palearctic regions (but see Ramanankierana *et al.* 2007 for a tropical perspective). In this documented area, and using a combination of *in situ* measurements, soil bioassays and seedling transplantation, a consistent pattern of CMN-mediated nurse effect by shrubs on ECM late successional tree species has been reported in northern America (*Arbutus menziesii* vs. *Pseudotsuga menziesii*; Molina *et al.* 1997; Horton, Bruns and Parker, 1999; Kennedy *et al.*, 2012); *Helianthemum bicknellii* vs *Quercus spp*; (Dickie *et al.*, 2004), southern Europe (*Arbutus unedo* vs. *Quercus ilex*; (Richard, Gardes and Selosse, 2009) and eastern Asia (*Salix reinii* vs *Betula ermanii* and *Larix kaempferi*; (Nara and Hogetsu, 2004; Nara, 2006b).

In the different investigated case studies, the highlighted CMNs-based facilitation process similarly lies on two complementary characteristics regarding the ecology of the nurse shrub species. First, ECM nurses have the ability to hold a prominent place in early stages of ecological succession, by either surviving after disturbance (Nara and Hogetsu, 2004) or establishing as a pioneer ECM species in a plant matrix exclusively composed of VA species (Dickie *et al.*, 2004; Richard, Gardes and Selosse, 2009; Kennedy *et al.*, 2012). Second, beyond this role of ecological hinge, ECM nurses act as inoculum relays for late successional tree species by accumulating diversified communities of ECM fungal partners on their roots, including generalist species which increase the potential for connection into common mycorrhizal networks. In previous research, the efficiency of CMNs in early vegetation stages has been evaluated by measuring the ability of these hub species to sustain the mycorrhization of the benefactor species (*e.g.* Kennedy *et al.*, 2012), and by evaluating the subsequent gain on their growth, nutrient uptake and survival (*e.g.* Nara and Hogetsu, 2004).

Understanding the drivers of tree seedling establishment and survival is a central question in forest ecology, and a major challenge for forest and land managers. As a consequence, most research has been focused on the understanding of the role of ECM CMNs at the end of a shrub-dominated stage, when ECM tree species spontaneously establish in matrices of ECM nurse shrubs. These works overlook the major part of the chronological sequence since disturbance (Figure 1a). In particular, we still know little about the early ecological bases of early ECM networks, *i.e.* upon ECM shrub establishment in VA plant communities (Figure 1a).

Interconnections among roots of VA and ECM plants through ECM-based mycelia may occur, as suggested by recent studies showing a possible dual [ECM+endophytic] niche for species of various lineages of ECM fungi in both asco- (*e.g. Tuber melanosporum* and *T. aestivum*; Schneider-Maunoury *et al.*, 2018, 2020) and basidiomycetes (*e.g. Rhizopogon spp*; Toju *et al.*, 2018). The functional role of such plant-plant interactions is not clearly understood, and may include nutrient transfer from VA to ECM plants (see Taschen *et al.*, 2020 for *Tuber melanosporum*), with possible consequences on the establishment and survival of ECM shrubs in stressful environments. Identifying both ECM fungal and VA plant hubs involved in these tripartite interaction networks may provide insights into the biotic dimension of both ECM plant and fungal fundamental niches, and offer promising perspective on restoration ecology, similarly to nurse-based processes of restoration developed on post-mining degraded ecosystems (Demenois *et al.* 2017).

Furthermore, the belowground facet of the functioning of highly resilient shrub populations is still poorly known in the later succession stages (Figure 1a). In particular, the role of mycelium sharing among conspecific ECM shrubs, as a possible mechanism underlying the drought tolerance and adaptation of these communities to disturbance remains poorly addressed. This is the case for Cistaceae, an emblematic family of evergreen shrubs dominating Mediterranean-type landscapes at low elevation (Guzmán and Vargas, 2009). Based on fruitbody surveys, there is evidence to suggest that Cistaceae host highly diversified and species-rich ECM fungal communities (Comandini, Contu and Rinaldi, 2006; Loizides, 2016; Leonardi *et al.*, 2020), yet little is known of their belowground patterns and functioning. Exploring the topology (see Glossary) of the corresponding CMNs, and their response to drought and disturbance in widely distributed monospecific stands, may contribute to a systemic understanding of the belowground adaptation processes of Mediterranean plants.

ECM networks sustain nutrient exchange among trees along the sylvigenetic cycle

As forests age, two highly dependent community dynamics concomitantly occur. Thus, the progressive replacement of short-lived early-stage ECM trees (*e.g.* Betulaceae, Salicaceae and Pinaceae *pro parte*) by long-lived shade-tolerant dryads aboveground (*e.g.* Fagaceae and Pinaceae *pro parte*) is accompanied by a marked shift in the composition of associated belowground communities (Bruns *et al.* 2002; Taudiere *et al.* 2015). On tree roots, while early-

stage trees host poor ECM fungal communities, dryads associate with multiple fungal species, and progressively accumulate species on their roots (e.g. Smith *et al.*, 2002; Richard *et al.*, 2005), with specialization degree of fungal partners increasing with tree ageing (Bruns, Bidartondo and Taylor, 2002; Barham, Harend and Tedersoo, 2014; Taudiere *et al.*, 2015). Meanwhile, the underground composition of early-stage ECM fungal communities reciprocally drives the recruitment of late-stage plants. On roots of pioneer trees, broad host range fungal hubs transfer nutrients to late-stage trees through CMNs (Teste and Simard *et al.*, 2008; Nagati *et al.*, 2019), increasing their establishment success through soil feedbacks (Liang *et al.*, 2020), most notably in arid environments (Bingham and Simard, 2012; Montesinos-Navarro, Valiente-Banuet and Verdú, 2019).

Simard's pioneer work (Simard *et al.*, 1997) propelled intensive research on the understanding of the role of ECM CMNs along forest sylvigenetic cycle. In particular, the ability of CMNs to mediate the recruitment of late-stage tree seedlings under the canopies of pioneer species has been largely explored. Interplant nutrient transferring through ECM CMNs is now considered as one of the most fascinating mechanisms involved in shade tolerance of late-stage tree species, which establish during forest ontogeny (Figure 1b; Teste and Simard, 2008; Simard *et al.*, 2012), and partially and fully heterotrophic plant lineages living in the undergrowth (Figure 1b; Li *et al.*, 2021). However, we still know little about the ecological significance of these trophic interactions among neighboring trees at the forest ecosystem level. Consistent evidence suggests that plant-plant interactions through shared ECM mycelia may play a central role in the performance of tree seedlings in mature forests in boreal (Nagati *et al.*, 2019), temperate (Teste and Simard, 2008), Mediterranean (Egerton-Warburton, Querejeta and Allen, 2007) and tropical climates (Liang *et al.*, 2020). The consequences of these interactions on the long-term ecosystem dynamics still need to be tested. For instance, community patterns and dynamics suggest that CMNs may drive soil-mediated positive feedback loops (Bever *et al.*, 2010), which in turn may shape directional succession induced by climate change in boreal ecosystems (Deslippe and Simard, 2011; Nagati *et al.*, 2019). In natural forests, the role of CMNs-based, long-term process in forest dynamics still need to be evaluated. For instance, in Mediterranean old-growth forests, shade-tolerant *Quercus ilex* seedlings survive in light-restricted conditions for decades, until the senescent phase and the advent of canopy openings (Panaiotis, Carcaillet and M'Hamedi, 1997). In gaps, these pre-established individuals avail of these conditions to emerge from the shrubby matrix and renew tree populations (Panaiotis, Loisel and Roux, 1998). In the soil of senescent *Q. ilex* forest patches, seedlings and old *Q. ilex* individuals share high

diversity of ECM symbionts on their roots (Richard *et al.*, 2005). Along this well-described sylvigenetic cycle, quantifying nutrient transfers among conspecific individuals during mature and senescent phases, may advance our knowledge of the efficiency of CMNs-based interaction in forest regeneration.

The mechanisms underlying CMNs effect on forest dynamics are not fully understood. In particular, our knowledge of the nature and the ecological importance of transferred resource among co-occurring hosts remains partial. However, CMNs undoubtedly drive carbon transfer along reversible source-sink avenues at the local scale (Teste, Simard and Durall, 2009; Song *et al.*, 2015; Cahanovitch *et al.* 2022). One of the most spectacular demonstration of this mechanism consists of carbon transfers among adult trees in temperate mixed conifer-hardwood forests (Klein, Siegwolf and Körner, 2016). This finding is the first to reveal bidirectional nutrient exchanges among co-occurring ECM mature trees, with a significant amount of the carbon accumulated on roots of adult trees being transferred from neighboring donors. Concerned four fungal genera typically dominate in old stands forests (i.e. *Russula*, *Cortinarius*, *Lactarius*, and *Tricholoma*; Courty *et al.*, 2005; Richard *et al.*, 2005),. Contrastingly, the influence of ECM CMNs on nitrogen and phosphorus sharing among co-occurring individuals remains less obvious (Figure 1c). However, ECM network-mediated nitrogen fluxes have been evidenced between co-occurring ECM tree species with contrasted nitrogen-acquisition strategies (He *et al.*, 2006; Teste *et al.*, 2015). The importance of these nutrient transfers on forest functioning is still under debate. Nevertheless, recent data suggest that they may drive feedback loops which promote tree population dynamics in monospecific tropical forests (Corrales *et al.*, 2016). More generally, nutrient transfers through ECM CMNs are highly suspected to shape soil feedbacks and drive either cyclic or directional succession in the corresponding plant communities, from the tropics to arctic ecosystems (Deslippe and Simard, 2011; Kadowaki *et al.*, 2018; Montesinos-Navarro, Valiente-Banuet and Verdú, 2019; Liang *et al.*, 2020). The knowledge accumulated during the last decade revealed an unexpectedly large biogeographical range and functional significance of ECM network-based ecosystem processes along forest ontogeny. However, research gaps still persist on both descriptive and functional facets of ECM networks in forest ecosystems. In particular, the role of ECM CMNs at the end of the sylvigenetic cycle, and particularly in natural canopy gaps, still need to be explored (Figure 1c).

The belowground architecture of root systems of ECM trees, and the spatial distribution of the associated ECM fungal symbionts are poorly predicted by the vertical projection of the corresponding canopies (Lian *et al.*, 2006; Taschen *et al.*, 2015). Likewise, documenting the spatial patterns of plant-plant physical links through CMNs provide apparently counter-intuitive connections among highly spatially-distant individuals and determine unexpected hub individuals among populations (Teste, Simard and Durall, 2009), with consequences on forest management when tree individuals sustain production of associated resources (e.g., Lian *et al.*, 2006 for Matsutake forests). At the local scale, we still know little about the distribution of links between plant and fungal individuals. However, first evidence from temperate *Pseudotsuga menziesii* uneven forests strikingly revealed the ability of ECM CMN to interconnect most co-occurring plant individuals with each-other (Beiler *et al.*, 2010). In those ecosystems, and based on the use of multi-locus, microsatellite DNA markers, Beiler, Simard and Durall (2015) revealed the nested topology of *Rhizopogon* ssp.-based ECM networks, suggesting a potential role in the resilience of tree population through the prevention of cascading effect following tree death. To the best of our knowledge, most ECM ecosystems still await for similar systemic (*i.e.* network-based) assessments of their belowground functioning, despite the promising potential of networks-derived metrics for forest management and conservation (Taudiere *et al.*, 2015).

In cultivated ecosystems, anthropic practices affect ectomycorrhizal networks

Only 2% of vascular plant species are ectomycorrhizal (van der Heijden *et al.*, 2015; Brundrett and Tedersoo, 2020). The majority of timber, softwood lumber and construction wood traded worldwide derives from this low percentage of vascular plants (Smith and Read, 2010). Most emblematic agro-sylvo-pastoral landscapes in the northern hemisphere are shaped by these plant species (*e.g.* Conedera *et al.*, 2016 for an illustration with the domesticated ECM tree *Castanea sativa*). As a consequence, the effect of silvicultural practices on ECM fungal communities, and the nature of adapted forest management practices to maintain diverse ECM communities has been intensively debated (see Tomao *et al.*, 2020 for a review). Here we successively discuss the current knowledge of the effect of human practices on ECM fungal networks in two main contexts. We first consider cultivated ecosystems as a wide variety of ECM forest types cultivated for timber production (see Glossary). Second, we review the state of the art in agroforests, *i.e.* in ECM socio-ecosystems where timber production is associated

with a wide variety of human services, including crop and fruit productions (e.g. Dehesas, Montados, planted orchards; Grove & Rackham 2001).

Anthropic disturbances shape ECM communities and networks in cultivated forests

In cultivated ECM forests, silvicultural practices generally consist of interrupting the sylvigenetic cycle at the maturity phase, truncating the end of both above- and belowground ecological successions. In most documented ecosystems dominated by either angio- or gymnosperms, both selective tree logging and clearcutting have been reported to induce marked compositional change in ECM fungal communities. The higher the intensity of practices, the higher are the deleterious effects on ECM diversity (Sterkenburg *et al.*, 2019). Specifically, it has been shown that ECM community diversity positively responds to tree diversity, basal area and canopy cover in cultivated forest systems (Tomao *et al.*, 2020; but see Craig, Woods and Hoeksema, 2016; Spake *et al.*, 2016 for case study-dependant contrasted responses). As a practical consequence, the retention of forest patches (Kranabetter, de Montigny and Ross, 2013; Varenus, 2017), green trees (Sterkenburg *et al.*, 2019), and to a lesser extent coarse woody debris (Walker *et al.*, 2012) at harvest time have been reported as efficient compensatory measures to maintain ECM fungal diversity in cultivated ecosystems. If direct effect of tree removal are widely documented, indirect effect deleterious of forest logging have been also reported, including environmental change (Varenus, 2017) soil compaction (Hartmann *et al.*, 2014) and soil amendment (Olivera, Bonet and Oliach, 2014; Almeida *et al.*, 2018).

The effect of forest management practices on ECM CMNs have been poorly investigated so far. However, the accumulated knowledge during the last decade in forest ecosystems gives a theoretical framework to analyze the response of plant-fungi bipartite networks to forest practices. From a plant-centered perspective, the reported pauperization effect of forest exploitation on ECM communities may alter the topology of ECM networks. Early evidence of age influence on network topology already exists: using high-throughput sequencing of soils, Correia *et al.*, (2021) reported contrasted ECM network topologies along a chronosequence of *Fagus sylvatica* forest establishment, plant nodes in long-established forests presenting higher numbers of connection links than in recent patches. From a fungal perspective, silvicultural practices tend to reduce the number and diversity of available plant nodes for fungal genets. As a consequence, tree logging may decrease the linkage density of ECM CMNs in cultivated

stands. When considering the highly saturated and nested nature of ECM CMNs (Beiler *et al.*, 2010; Taudiere *et al.*, 2015), one may suggest consequences of tree logging on ECM network-mediated processes in forest ecosystems, including affected tree regeneration dynamics (Pec *et al.*, 2020). On the basis of scarce preliminary works, further research is needed to illuminate the relationships between the complexity and the stability of ECM CMNs. Cultivated forests are also ideal candidate systems to study the links between the stability of ECM CMNs and the resilience of forest ecosystems

ECM fungi shape complex interaction networks in tree savannas

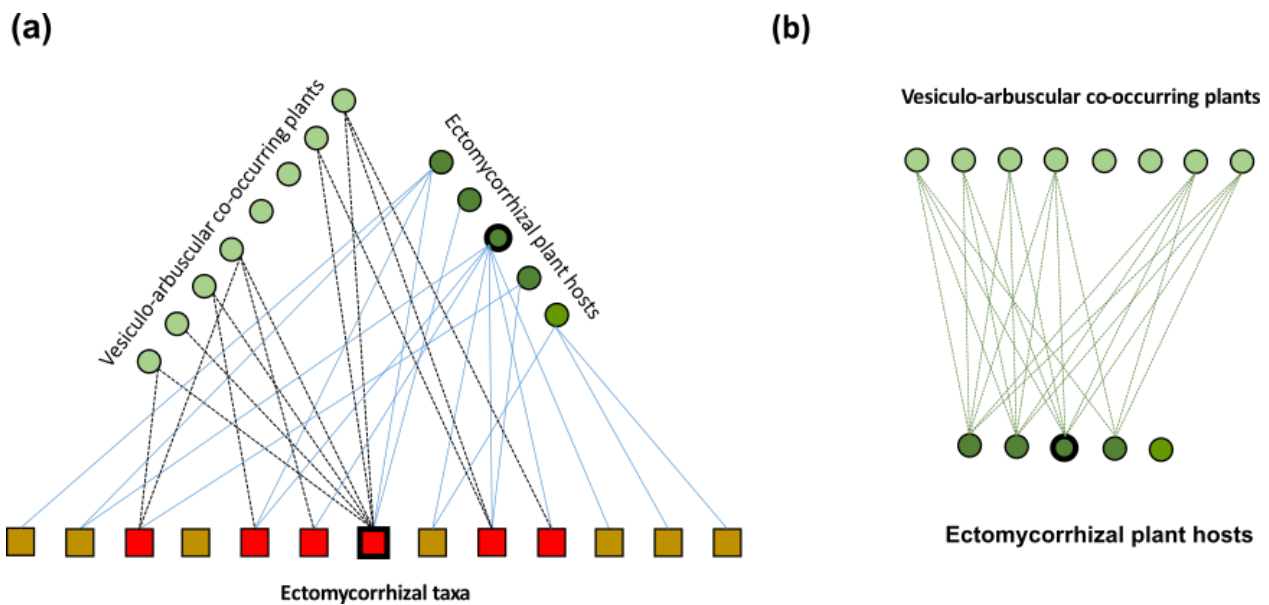
Ectomycorrhizal trees dominate millions of hectares of anthropogenic landscapes in temperate and Mediterranean ecoregions, where trees, pastures, and croplands amalgamate in complex and species-rich mosaics called tree savannas (Grove & Rackham 2001; Moreno and Pulido, 2009; see Glossary). Most ECM tree species contribute to the current highly diversified physiognomy of these systems, but the most emblematic ones are dominated by various species of oaks (*e.g.* from *Quercus velutina* dominating north America oak openings and typical *Q. suber* dehesas, which cover one-eighth of Spain), sweet chestnut (*e.g.* *Castanea sativa* montados currently covering one-sixth of the area of Portugal), or larches and pines, which dominate multifunctional transitional landscapes between subalpine forests and alpine shrublands across Europe.

Because tree savannas play important ecological, social and economic roles for societies (*e.g.* Conedera *et al.*, 2016), understanding their functioning and analyzing their biodiversity patterns has been subject of much attention (Bugalho *et al.*, 2011). Within their soils, these ecosystems concentrate some of the richest ECM communities described so far (Tedersoo *et al.*, 2006; Morris *et al.*, 2008; Baptista *et al.*, 2015; Reis *et al.*, 2018) and harbor highly specific fungal assemblies dominated by ascomycetes, in response to a unique combination of environmental and anthropic drivers (Dickie *et al.*, 2009). Despite the primary importance of tree savannas for conservation, very little is known about their belowground functioning, and ECM-mediated plant-plant interactions remain poorly explored in most of them.

Within this panorama, spontaneous oak savannas and planted orchards for the yield of the emblematic black truffle (*Tuber melanosporum*), are an exception. Indeed, during the last decade, very few ECM communities received as much attention as *T. melanosporum* truffle

grounds. In these transient ecosystems, a set of practices by truffle growers shape species-rich ECM communities, typically dominated by lineages of ascomycetes (*e.g.* Pyrenomataceae, Tuberaceae and Helvellaceae) and early-stage lineages of basidiomycetes (*e.g.* Thelephoraceae) in both spontaneous and planted truffle grounds (*e.g.* Belfiori *et al.*, 2012; Taschen *et al.*, 2015). In these communities, *T. melanosporum* and few species in the early-stage lineages of basidio- (*i.e.* Thelephoraceae, Sebacinaceae, *Inocybe* spp. and *Scleroderma* spp.) and ascomycetes (*i.e.* *Cenococcum geophilum*, Pyrenomataceae), simultaneously associate as mutualists with their host through ECM root tips, and interact as fungal endophytes on the roots of most VA plant species established under host canopies (Schneider-Maunoury *et al.*, 2018, 2020; Figure 2a). In these systems, the topology of the below-ground fungal interaction networks remains undescribed (Figure 2a). Similarly, the ecological consequences of the “beyond forest edge” spatial extent of ECM CMNs remains poorly addressed (Taschen *et al.*, 2020). In particular, the ability of ECM fungi to transcend their host range and to establish as endophytes in the tissues of AM plants (Taschen *et al.*, 2020) still need to be examined under a functional perspective. These remarkable findings pave the way for pursuing the exploration of ECM-VA plant interaction mediated by shared ECM fungal hubs, to provide systemic views of forest functioning.

Figure 2. Schematic representation of ectomycorrhizal-based multipartite networks in *Tuber ssp.* temperate agroforests, from Schneider Manaury *et al.* (2018) modified. **(a)** Species of co-occurring ectomycorrhizal and arbuscular mycorrhizal plants are represented by dark and clear green dots, respectively. The different taxa of ECM fungi are represented by squares. Interaction hubs are indicated by thickened outlines (here, *Quercus ilex* and *Tuber melanosporum*). Solid lines indicate plant-fungi ECM interaction links. Dotted lines indicate interaction links between ECM fungal taxa and VA plants. **(b)** Schematic representation of the corresponding projected VA-ECM plant network.



ECM common mycorrhizal networks in urban soils: headache in a saturated below-ground

In anthropogenic urban ecosystems, a few ECM trees and shrubs (*e.g. Tilia, Pinus, Quercus, Cistus*) hold a prominent place among a wide range of plant species adapted to the environmental conditions and physical constraints of cities (Bainard, Klironomos and Gordon, 2011; Williams, Hahs and Vesk, 2015; Jenerette *et al.*, 2016). In urban green spaces, the selection of plant species by urban land managers is in priority driven by socioeconomic constrains (Hope *et al.*, 2003). In particular, a panel of aesthetical traits, including blooming intensity, flower size and color, shape patterns of urban tree diversity in cities (Kendal, Williams and Williams, 2012; Conway and Vander Vecht, 2015; Jenerette *et al.*, 2016; Goodness, 2018). To a lesser extent, ecophysiological traits of species also matter, in particular plant tolerance to drought or freeze (Jenerette *et al.*, 2016; McPherson, Berry and van Doorn, 2018). On the other hand, plant-related extended phenotype functions (Dawkins 2016), such as fungal-mediated adaptation to drought through mycorrhizal symbionts, or nutrient uptake based on connection with neighboring individuals through ECM CMNs, still struggle to be included in selection processes among plant candidates.

Urban soils host pauperized ECM fungal communities

The composition of soil fungal communities in urban soils remains largely unexplored (Delgado-Baquerizo *et al.*, 2021). Published research consistently shows eroded patterns of ECM diversity in cities, as compared to forest ecosystems (Bainard, Klironomos and Gordon, 2011; Karpati *et al.*, 2011; Martinová *et al.*, 2016). In particular, a low ECM species richness has been observed at the local scale, on roots of planted trees in city parks (Bainard, Klironomos and Gordon, 2011; Karpati *et al.*, 2011), streetscapes (Bainard, Klironomos and Gordon, 2011), and private residential properties (Bainard, Klironomos and Gordon, 2011), with an unusual dominance of hypogeous fungi (*e.g. species in the Tuberaceae*) and an underrepresentation of ECM families which typically dominate in forest soils (*i.e. Russulaceae, Inocybaceae and Cortinariaceae*; Martinová *et al.*, 2016; Hui *et al.*, 2017; Van Geel *et al.*, 2018). Interestingly, these pauperized communities show a high ability to colonize root systems, with similar ECM colonization rates on roots in urban soils as compared to forests (Timonen and Kauppinen, 2008; Bainard, Klironomos and Gordon, 2011; Tonn and Ibáñez, 2017). This pauperized nature

of ECM communities in urban soils and subsequent homogenization of ECM fungal communities among cities on a continental scale (Schmidt *et al.*, 2017; Delgado-Baquerizo *et al.*, 2021), question about the mechanisms driving the observed compositional change and the consequences of species shift on urban ecosystem functioning. Three main mechanisms may concomitantly drive the observed patterns.

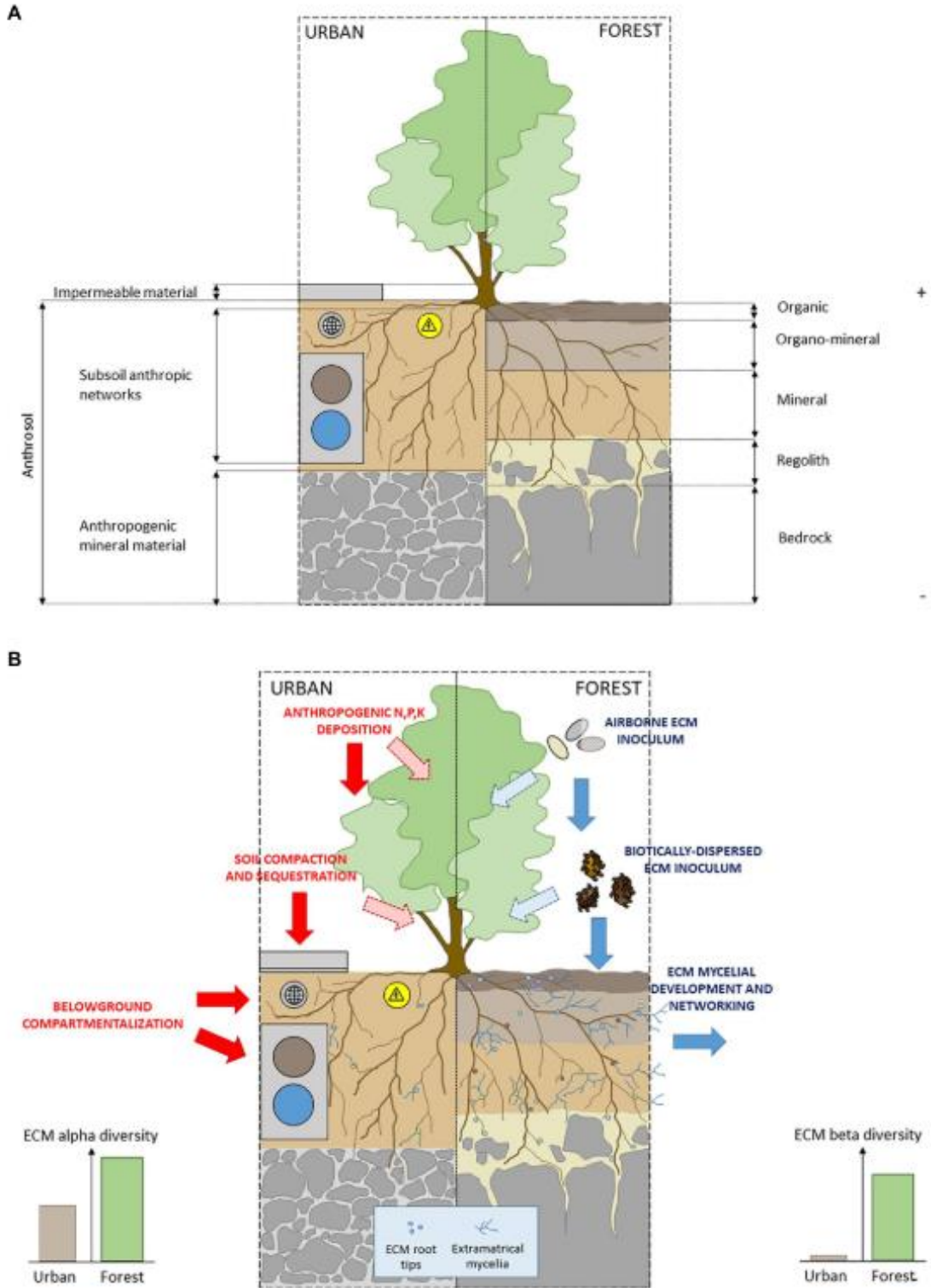
First, a deleterious effect of urban environment on both the dispersion (by physical barriers among poorly connected and highly fragmented habitats) and establishment (by unsuitable physico-chemical conditions in anthrosols; Figure 3a) are likely to act as filters on the potential communities of airborne and biotically dispersed ECM inoculum (Kasprzyk and Worek, 2006; Koide, Fernandez and Petprakob, 2011). In particular, sealing of urban soil surface may impact ECM dispersion from adjacent forests to city streets, with negative effects on ECM richness and diversity in urban soils (Martinová *et al.*, 2016; Figure 3a).

Moreover, the low alpha diversity of ECM communities in cities may reflect the specific physicochemical properties of urban soils (Newbound *et al.*, 2012; Lüttge and Buckeridge, 2020). Thus, the role of soil chemical signature as one of the main drivers of the composition, diversity, temporal dynamics and spatial patterns of ECM fungal communities has been widely documented in forest ecosystems (*e.g.* Lilleskov, Hobbie and Horton, 2011 for a review; Courty *et al.*, 2016 for a functional perspective). Unsurprisingly, in urban as in forest soils, the vegetative development (Olchowik *et al.*, 2021) and the species richness (Newbound *et al.*, 2012; Martinová *et al.*, 2016; Van Geel *et al.*, 2018) of ECM communities are negatively affected by soil alkalinity, and positively respond to organic matter and moisture content (Van Geel *et al.*, 2018). The consistent pattern of ECM richness decrease and the composition drift observed in urban soils have then to be considered in light of 1) currently widespread practices along city roads, including deicing salts contributing to the alkalization of urban soils (Czerniawska-Kusza, Kusza and Dużyński, 2004), and 2) the critically low organic matter content in city soils, in particular in sealed contexts (Scharenbroch, Lloyd and Johnson-Maynard, 2005; Alzetta *et al.*, 2012), and its deleterious consequences on the establishment of species with affinities for organic soils (*e.g.* Genney, Anderson and Alexander, 2006; Kranabetter, Durall and MacKenzie, 2009).

Last, the simplification of ECM communities in urbanized contexts may be a consequence of anthrosol assembling process in urban green spaces. This highly artificial growth matrix generally consists of a unique soil horizon intercalated between a deeper layer

of mineral anthropogenic substrate, and a cover of impermeable materials (Rodríguez-Espinosa *et al.*, 2021; Figure 3a). The specific physical soil properties of anthrosols (Lorenz and Lal, 2009) combined with surface sealing, drastically affects biological processes (Lu and Weng, 2006; Martinová *et al.*, 2016; Salvati *et al.*, 2016; Hui *et al.*, 2017) limiting the differentiation of soil profile into distinct horizons during pedogenesis (Hui *et al.* 2017; Figure 3a & 3b). As a consequence, anthrosols remain unsuitable habitats for the vertical stratification of ECM diversity from organic surface to the underlying mineral horizons (Rosling *et al.*, 2003; Genney, Anderson and Alexander, 2006), by penalizing hyper diverse ECM sub-communities with affinities for surface organic layers, (Richard *et al.* 2011).

Figure 3. (a) Schematic representation of pedogenic conditions for the development of ectomycorrhizal plants in urban and forest soil conditions and **(b)** Schematic representation of ECM network-based mechanisms and anthropic filters in urban and forest soils conditions.



Traffic jam in urban soils

During the last decade, restoring ecological networks in urban areas (Ignatieva, Stewart and Meurk, 2011) has become a priority for urban landscape planners, in order to provide suitable habitats for organisms and re-establish connectivity among fragmented meta-populations (Peng, Zhao and Liu, 2017). The development of urban green corridors connecting habitat patches has been successfully developed in cities worldwide, as an efficient strategy to maintain high levels of alpha diversity (see Glossary), in particular for motile organisms (Beninde, Veith and Hochkirch, 2015).

Contrastingly, fixed organisms such as plants and their fungal root associates still await to benefit from an adequate declination of network-based strategy for the conservation of dedicated belowground mutualistic interactions and their underlying functions. In particular, the topology of pauperized ECM CMNs in urban soils remains poorly described, the network-based signature of plant adaptation to urban environment still need to be explored, and the role of ECM CMNs for plant species coexistence in cities still need to be understood.

Yet, in urban areas as in natural ecosystems, there is strong evidence for a pivotal role of ECM colonization and diversity for promoting nutrient uptake (Newbound, Mccarthy and Lebel, 2010), acclimatation to hydric stress (Fini *et al.*, 2011), tolerance to salt exposition (Zwiazek *et al.*, 2019), and survival of ECM plants (Tonn and Ibanez, 2017). Despite this pivotal role of ECM symbiosis, planning practices fail at integrating plant-plant ECM interconnexion through CMNs in urban design. At the individual level first, urbanization imposes to belowground counterparts of trees undersized dedicated soil volumes in compartmentalized planting pits (Day *et al.*, 2010; Figure 3a). At the plant population/community level, the organisation of cities constrains root development patterns along green linearities bordering transport networks (Levinson, 2012), anthropic pipelines (*e.g.* electrical, water, internet; Galle, Nitoslawski and Pilla, 2019), and carriageway stabilizing materials (Randrup, McPherson and Costello, 2001). Moreover, from a temporal perspective, high turnover of land-use of volatile urban landscapes hampers belowground mycelial dynamics by inducing severe soil disturbance regime (Moore, 2008; Lüttge and Buckeridge, 2020).

All in all, and further considering the temptation to consider urban underground space as an opportunity to compact city development (Cui, Broere and Lin, 2021), there is clear evidence that the establishment of functional ECM CMNs in cities may collide with the current

geographical conceptualization of urban underground in the cities of the Anthropocene (Qihu, 2016; Connor and McNeill, 2022).

Perspectives and conclusions

The understanding of ectomycorrhizal interaction networks is a growing body of research. Their deterioration by human impacts is an overlooked marker of the anthropogenic footprint on terrestrial ecosystems. The pedosphere is particularly impacted by the rise to dominance of anthropic disturbances (Certini and Scalenghe, 2011). Here we aimed at providing a comprehensive overview of the recent advances on our understanding of ECM CMNs in a wide range of ecosystems differing in the intensity of anthropic influence, in order to draw up a framework of timely research avenues. From this analysis, three issues should be primarily addressed.

Knowledge gaps in natural gaps

During the last decade, the development of metabarcoding tools propelled an unprecedented level of knowledge of belowground fungal patterns in forest soils, and enlarge perspectives on fungal assembly at both local (*e.g.* Baptista *et al.*, 2015) and global (Tedersoo *et al.*, 2014; Tedersoo, Bahram and Zobel, 2020) scales. Despite significant progress been made, several grey areas persist. First, the topology of CMNs at the establishment time of pivotal ECM shrubs (Figure 1a) and the affinity of ECM fungi toward AM plant hosts, remain fascinating and poorly documented issues in ecology. Second, studies investigating network-based dynamics in soils of natural canopy gaps are surprisingly lacking. Yet, understanding the remobilization of late-stage trees inoculum heritage in soil by pioneer species to initiate a new sylvigenetic cycle (Figure 1b) is a central question for forest ecologists and managers. Last, there is urgent need to assess the effect of forest management practices on the topology of ECM networks since forest ecosystems face dramatic change through the combined effects of local impactful disturbance and global change.

Taxonomic limits and spatial patterns of ECM CMNs

One major recent advance in our knowledge of ECM symbiosis is the ability of various ECM fungal lineages to interconnect between their hosts and VA plants, in both forests (Toju *et al.*,

2018) and tree savannas (Schneider-Maunoury *et al.*, 2020). This unexpected porosity among ecological guilds of plants raises the questions of 1) the spatial limits of ECM CMNs through physical links among involved plant individuals, and 2) the taxonomic basis of these interactions in multi-layered plant communities. Moreover, the role of these mixed [ectomycorrhizal-endophytic] connections by polyvalent fungal species for forest community dynamics necessitates more effort to be fully understood (Taschen *et al.*, 2020). This finding suggests to re-examine, from a biotic and below-ground perspective, the mechanisms involved in the particularly widespread nursing effect of VA plants for ECM trees.

Toward soil corridors for underground cities

Our synthesis reveals that patterns and functions of ECM CMNs in urban soils are poorly understood. However, given the rate of soil artificialization in cities worldwide (Scalenghe and Ajmone-Marsan, 2009; Just, Frank and Dale, 2018), there is crucial need to consider urban soils and their living organisms as a valuable resource for citizens in the Anthropocene. From a landscape management perspective, cities may be ideal candidates for the establishment of ECM network-based plant synergies in artificial green spaces, by designing interconnected planted pits along soil continuums, to maintain functional and multi-scaled ECM CMNs within *beige* corridors.

In such corridors, mimicking ECM networks established in natural ecosystems may be a promising avenue for urban planners to design functionally efficient artificial green spaces. First, in the cities of the Anthropocene, the composition of plant communities may be thought to 1) favor species with high number of associated ECM fungal species (interaction hub species such as, *e.g.* Fagaceae; Taudiere *et al.* 2015) to increase the alpha diversity of ECM fungal inoculum, 2) associate nurse plants and late-stage benefactor species in multi-layered designs (Figure 1) and 3) promote synergies between AM and ECM plant guilds through endophytic interaction (Figure 2). In these artificial ecosystems, tree populations may be uneven-aged, to increase the complexity of ECM networks by promoting the emergence of interaction-hub tree individuals (*i.e.* aged individuals with high number of linked fungal individuals; Beiler *et al.* 2010), and subsequently favor the ecological stability within nested network patterns (Montoya, Pimm and Solé, 2006).

Second, from a belowground perspective, the conception of anthrosols within *beige* corridors may be rethought to reach suitable soil properties (*i.e.* biotic and abiotic conditions, including chemical and physical properties) for the establishment and the expansion of physical ECM links among plants. Accommodations may encompass a reduction of soil sealing area, the use of permeable sealing materials, the edification of stratified and organic-enriched pedological profiles, and controlled ECM inoculation by native fungi (Figure 3). Ultimately, combining above- and belowground facets of *beige* corridors may be in line with the rewilding projects that gain popularity in landscape planning.

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Glossary

Alpha-diversity: diversity of co-occurring organisms at local scale. Species richness is a widely used measurement of alpha diversity as a taxonomical facet of biodiversity. Contrastingly, the beta-diversity of metacommunities characterizes the level of composition differences between communities.

Anthropogenic: refers to processes, assemblies and ecosystem compartments driven by, or resulting from human activity. Anthropogenic landscapes typically consist of vegetation mosaics induced by long-term human activity (forestry, pastoralism, fire regime, etc.). Anthropogenic soils include pedological profiles that are physico-chemically altered by long-term anthropic pressures as well as matrices of artificial materials from urban/industrial origin (Anthrosols).

Cultivated forest: consists of highly diversified physiognomies of vegetation dominated by trees, originated from either natural process (spontaneous establishment of seedlings) of plantations, where human practices drive the structure, the composition and the dynamics of tree populations. Cultivated forests encompass forestry-based managed forests and agroforests, including planted orchards and multifunctional tree savannas.

Endophytic lifeforms: micro-organisms, including bacteria and filamentous fungi, leaving inside plant tissues (roots, seeds, leaves, bark, etc.) where they accomplish a part or their entire biological cycle. Recent research shows the ability of ectomycorrhizal fungi (e.g., *Tuber melanosporum*) to colonize root tissues of AM host as endophytes.

Network topology: set of properties characterizing an interaction network, used to infer ecological/evolutionary hypotheses, and based on the analysis of the distribution of links between the objects (nodes) constituting the network.

Sealed soils: soils that have sequestered in the mid-term by a cover of impermeable materials (e.g., tar) hindering pedogenesis.

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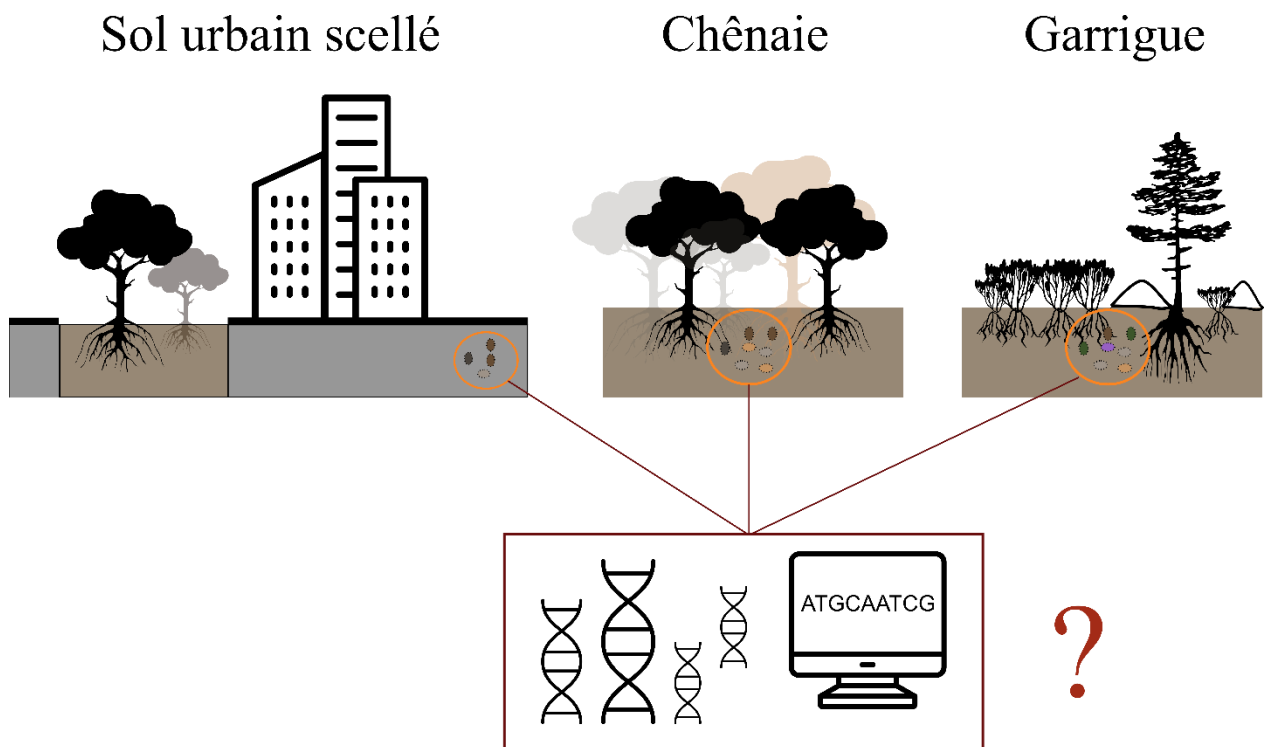
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Chapitre 2 : Esclave ou maître de la machine ? Du « bon » usage du métabarcoding en écologie des communautés ectomycorhiziennes



Présentation du chapitre en français

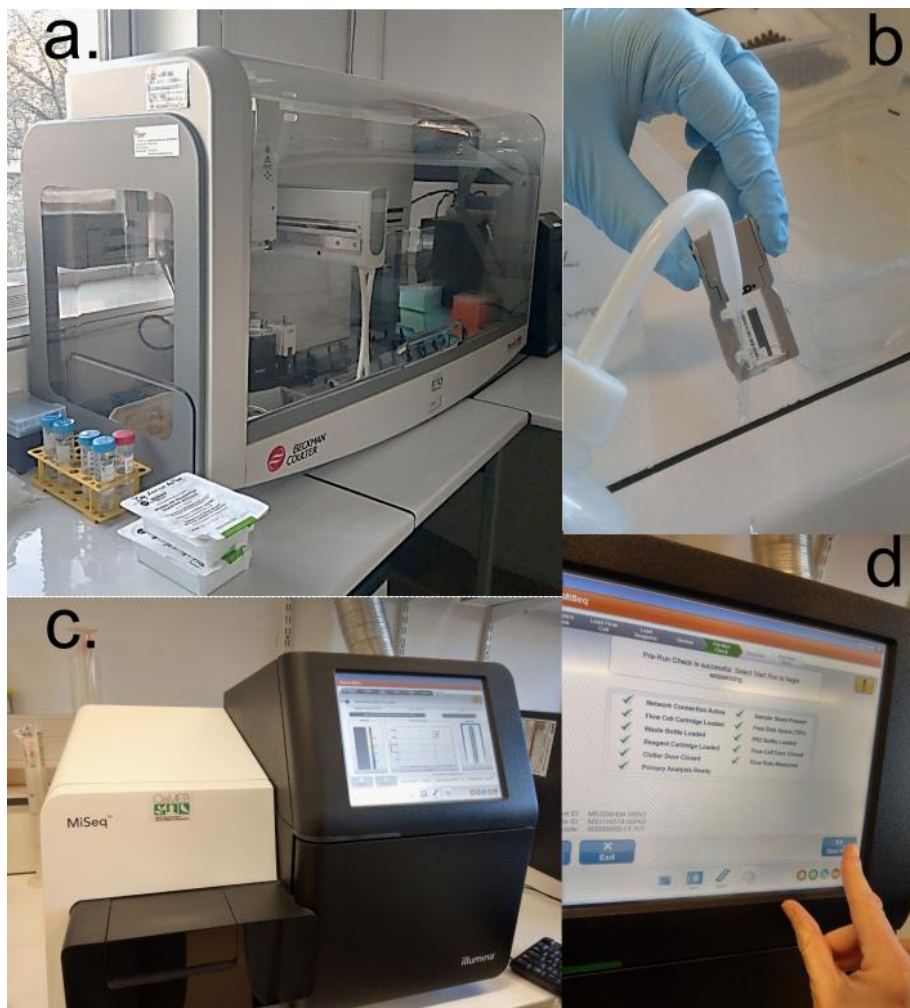
Au cours du chapitre précédent, nous avons exploré l'étendue des connaissances sur les réseaux d'interactions ectomycorhiziens qui s'établissent entre plantes voisines au cours des successions végétales. Nous avons documenté le caractère inégal de ces connaissances, avec des stades dynamiques davantage explorés que d'autres (croissance et maturation des communautés végétales vs sénescences et établissement des plantes dans les trouées). Nous avons identifié le caractère lacunaire des connaissances sur le sujet le long d'un gradient d'anthropisation : dans les systèmes les plus impactés par l'activité humaine, les réseaux d'interactions ectomycorhiziens sont peu documentés. Les propriétés inhérentes aux villes (perturbations, usage des sols, fragmentation de la trame du sol) semblent favoriser la connectivité entre les humains au détriment des organismes du sol. Par conséquent, nous avons proposé, à l'issue de ce travail, la création de trame beige du sol, afin de favoriser un continuum écologique pour les organismes du sol – incluant les champignons ectomycorhiziens. L'implantation de telles structures en ville demande néanmoins des connaissances accrues sur le compartiment fongique des sols urbains.

Chez les champignons ectomycorhiziens, les méthodes employées pour l'identification des taxons ont longtemps reposé sur l'analyse morpho-anatomique ainsi que sur la culture en laboratoire. Toutefois, la nature diffuse des fructifications des champignons ectomycorhiziens et la difficulté pour les cultiver contraint l'échantillonnage de cette guildes. Les avancées technologiques moléculaires développées dans les années 1990s telles que le barcoding ou le métabarcoding dans les années 2000s sont rapidement devenues consensuelles et très répandues dans la sphère scientifique. Ces méthodes ont permis entre autres de revoir à la hausse la diversité des champignons ectomycorhiziens, de réaliser des phylogénies moléculaires et de mieux comprendre l'écologie de ces champignons. En permettant une analyse de la communauté ectomycorhizienne à partir d'échantillon environnementaux (comme un aliquot de sol, d'eau ou d'air), le métabarcoding est désormais une méthode de référence largement employée en écologie des communautés. Or, il n'existe pas de protocole standardisé de métabarcoding pour l'étude de la guildes ectomycorhizienne – ce qui compromet la répliquabilité et la portabilité des études.

Le travail suivant utilise une approche expérimentale du séquençage en barcoding et métabarcoding pour l'identification des communautés ectomycorhiziennes à partir d'échantillons biologiques et environnementaux. Nous expérimenterons différentes calibrations

de séquençage en métabarcoding en faisant varier la profondeur de séquençage. Nous analyserons la capacité des méthodes de métabarcoding à restituer les séquences moléculaires fournies par le barcoding Sanger. Nous discuterons ces résultats à la lumière de l'écologie des communautés, tout en réfléchissant à des pistes de calibrations applicables pour le diagnostic écologique des communautés ectomycorhiziennes dans un cadre appliqué.

Planche 1 : a) Purification des amplicons automatisée, b) nettoyage de la flow-cell avant séquençage, c) séquenceur MiSeq, Illumina, d) c'est parti



Master or slave of the machine? How to handle hyper efficient metabarcoding tools in ectomycorrhizal community ecology

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Abstract

The development of metabarcoding technics allow considerable insight into the ectomycorrhizal diversity and its ecology. Nowadays, these technics are widespread and numerous community ecology studies are molecular-based. Yet, knowlegdes gaps are persisting on how the metabarcoding calibration influences the retrieved ectomycorrhizal communities. This study is based on an experimental approach of barcoding and metabarcoding. We ask the following questions:

- 1) How is ectomycorrhizal diversity influenced by metabarcoding sequencing depth?
- 2) Is the number of read an appropriate proxi of taxa abundance in the ectomycorrhizal community?
- 3) What is the capacity of metabarcoding to retrieved the same sequence as Sanger barcoding?

After sampling and sequencing the fungal ITS of three types of samples including soil, root EM apices and EM close-by environment – denominated as rhizosphere. We experiment different sequencing calibration with variable sequencing depth, our results revealed several important points. First, we notice a strong sampling effect while studying the soil ectomycorrhizal communities. EM patchiness in the soil implies the multiplication of sampling

points. Secondly, we noticed that read numbers cannot be used as proxy of abundances as it depends on sequencing depth. Next, we found that EM community's composition was differing according to sequencing depth and to sample type – suggesting that micro-scale patchiness occurs in soil. Finally, we found that Illumina metabarcoding retrieved the dominant sequences of EM root tips barcoded by Sanger – which demonstrate the efficiency of the method to identify the fungal partners of ectomycorrhizal host plants.

Our results encourage the complementarity of approaches as no technique was revealed as more efficient than another. These results have strong implications for community ecologists focusing on ectomycorrhizal mutualism – and for further applications such as conservation purposes or ecological diagnostics of urban landscapes.

Key words: ectomycorrhizal communities, metabarcoding, Illumina, barcoding, sequencing depth, diversity

Introduction

The ectomycorrhizal (EM) symbiosis is pivotal for plant growth and survival (Smith and Read, 2010). It is involved into fundamental ecosystem processes, including biogeochemical cycling (Teste *et al.*, 2017) and plant communities' dynamics (Tedersoo, Bahram and Zobel, 2020). Yet, outstanding research questions are persisting about the EM compartment in the ecosystems and its related networks (Authier, Violle and Richard, 2022; Karst, Jones and Hoeksema, 2023). The identification of ectomycorrhizal taxa has always been a challenging issue because of the biology and ecology of this guild. The observation of EM carpophores in the field is constrained by the diffuse, discrete and ephemeral nature of fructifications (Horton and Bruns, 2001). Moreover, EM fungi can rely on asexual reproduction for belowground colonization (*e.g. Sphaerosporella*, Sanchez *et al.*, 2014). On plant roots, the identification of EM fungi based on EM tip morphologies is challenged by apices polymorphism. Depending on the host plant, a single fungal taxon can exhibit several root tip morphotypes (Janowski and Leski, 2023).

To date, 20.000 identified taxa belong to the EM guild – encompassing a large range of taxonomic diversity including distant lineages from Basidiomycota, Ascomycota and Zygomycota phyla (Tedersoo and Smith, 2013; Janowski and Leski, 2023). Yet, despite years of research and empiric work, most species of the EM guild still fail at cultivation attempts (*e.g. Boletus*, *Cantharellus*, Karwa, Varma and Rai, 2011). Out of the 250 genera identified as ectomycorrhizal (Tedersoo and Smith, 2013), only a few are cultivable (*e.g. Tuber*, Reyna and Garcia-Barreda, 2014). Cultivation through the development of controlled-inoculation technics with EM spores or mycelia shows contrasted results with both successful fungal establishment (*e.g. Tuber*, Belfiori *et al.*, 2012 and *Laccaria bicolor*, Selosse *et al.*, 1998) and unsuccessful results with the rapid replacement by native fungi in the field (*e.g. Suillus* and *Inocybe* inoculation on *Pinus* species Flores-Renteria, Barradas and Alvarez-Sanchez, 2018).

The emergence of the molecular-based identification technics, considerably improved taxonomic identification of EM fungi and allowed addressing important issues about the ecology of these symbiotic organisms. In the early 90s, the barcoding methods such as RFLP and Sanger sequencing expanded rapidly after the pioneer work by T.D. Bruns and M. Gardes (Bruns and Gardes, 1991; Gardes and Bruns, 1993). This methodological development was

based on the fungal Internal Transcribe Spacer (ITS) – a repeated rDNA locus that substantially varies among taxonomic groups (Gardes *et al.*, 1991; Seifert, 2009). The most consensual method of barcoding is nowadays Sanger sequencing (Sanger and Nicklen, 1977), which consists in the sequencing of the 600-800 (rarely up to 1300) nucleotides between the small (18S) and large (28S) ribosomal subunit coding genes, from the dominant DNA molecule of a biological sample (*e.g.* mycorrhizal tip, carpophore; Nilsson *et al.*, 2012). The contribution of Sanger sequencing to the communities of fungal taxonomists and ecologists is huge. Specifically, it allows for the constitution of a reference database for taxonomic assignation, continuously implemented and stored in generalist – such as GenBank (Benson *et al.*, 2018) – or dedicated – such as UNITE (Kõljalg *et al.*, 2005) – repositories. Sanger sequencing has been and still is successfully used for taxonomic purposes and phylogenetic reconstructions (Seifert, 2009; Lücking *et al.*, 2021). Focusing on plant-fungi mutualism, barcoding assisted the identification of the fungal symbiont of EM root tips (Horton and Bruns, 2001). The consequences on community ecology are considerable, with a better insight into the ecological strategies of EM fungi (El Karkouri, Martin and Mousain, 2004) and on the fungal-mediated ecosystem processes such as ecological succession (Nara, 2006). Despite these considerable advances in the field of taxonomy, the Sanger method is intrinsically limited to explore the biodiversity of a given ecosystem (Cale *et al.*, 2021), because it yields a unique sequence, usually representing the most represented DNA prior to the PCR step. The emergence of Next-Generation Sequencing technics in 2010s (*e.g.* 454-pyrosequencing, Illumina MiSeq) alleviated these limitations and propelled community ecology studies into the current metabarcoding era. Illumina MiSeq became the most consensual technology because of its capacity to generate at low-cost and with high accuracy, millions of sequences (Schmidt *et al.*, 2013) from biological or environmental samples (*e.g.* soil, water, air, Ruppert, Kline and Rahman, 2019). The use of environmental DNA partially overcomes the sampling limitation of EM fungi – giving the access to the EM communities of the soil (*e.g.* mycelia, sclerotia, and spores, Wu *et al.*, 2019). At the community level, insights into the EM component (Tedersoo, May and Smith, 2010; Mello and Balestrini, 2018) allow the description of both soil and root-associated EM fungi.

The unprecedented quantity of generated data drastically changed our vision of the diversity of EM fungi (Hawksworth and Lücking, 2017; Ruppert, Kline and Rahman, 2019) – and assisted the spatial partitioning of diversity (Adamo *et al.*, 2020; Calderon-Sanou *et al.*, 2020). Metabarcoding-based studies improved the comprehension of ecological strategies of

EM taxa such as mixed-root-endophytic and mutualist taxa (*e.g.* *Tuber melanosporum*, Schneider-Maunoury *et al.*, 2018). These EM fungi hosted in the roots of endomycorrhizal plants highly contribute to the maintenance of local dominance of trees – because of its networking capacity (Liang *et al.*, 2020). At the community scale, metabarcoding-based studies assisted our understanding of EM fungi recruitment by host plants and revealed differential patterns of EM filtering within soil and plants (Goldmann *et al.*, 2016). Metabarcoding approaches revealed higher diversity of fungal propagules in the soil – for which patterns in the ecosystem are highly patchy (Koide, Xu and Sharda, 2004; Schneider-Maunoury *et al.*, 2018). Yet, uncertainties remain about both the EM diversity and community composition inferred from data generated by Illumina metabarcoding. Many steps separate samples from molecular sequences – including sampling collection, DNA extraction and amplification, sequencing and bioinformatic treatment – each bearing putative biases. The gathering of a reliable molecular information on a sample thus highly depends on the methodology applied during the process.

In the field, the sampling collection and storage methods were found to highly influence the retrieved fungal community after sequencing – either from a biological (Bent & Taylor, 2010) or an environmental sample (Guerrieri *et al.*, 2021). As the Illumina method is highly sensitive, direct extraction of eDNA after sampling is preconized (Guerrieri *et al.*, 2021) to avoid sample contamination by undesired propagules, *e.g.* skin fungi, contamination among samples, (Tedersoo *et al.*, 2022) or DNA degradation by decayer organisms (Clasen *et al.*, 2020).

During lab work, the steps of PCR (*i.e.* Polymerase Chain Reaction) amplification can alter the composition of EM sequences in the final dataset (Buée *et al.*, 2016; Nilsson *et al.*, 2019; Janowski and Leski, 2023). PCR biases have been consistently documented in the literature and involves at least directional amplification aspects through primers selection (Bellemain *et al.*, 2016), PCR stochasticity (Kausserud *et al.*, 2012) and amplification errors (Bjørnsgaard Aas, Davey and Kausserud, 2017). On the one hand, PCR process is directional and more efficiently amplifies short sequences of the fungal ITS (< 500 base pairs, Nilsson *et al.*, 2019) – over-representing taxa with short ITS sequences such as some Ascomycetes (Bellemain *et al.*, 2016; Kausserud, 2023). Also, a loss of base-calling quality is occurring with sequence length (Tan *et al.*, 2019). Consequently, estimates of fungal diversity are highly influenced by primer selection (Op De Beeck *et al.*, 2014) – and the presence or abundance of

some taxa may be underestimated (Bellemain *et al.*, 2016; Nilsson *et al.*, 2019). In order to solve this issue, several technics have been adopted. For EM-based studies, targeting the ITS2 domain rather than the full length ITS1-5.8S-ITS2 locus or the more length-variable ITS1 domain, is recommended to limit the unbalance of short and long sequences (Bellemain *et al.*, 2016). Moreover, a diversity of ITS primers have been designed to target the fungal ITS locus, with variable hybridization affinities for distinct fungal groups (Toju *et al.*, 2012; Op De Beeck *et al.*, 2014; Kauserud, 2023). Parallel sequencing targeting some specific groups such as the Basidiomycota lineage (Gardes and Bruns, 1993) or specific genera such as *Cortinarius* (Teasdale *et al.*, 2013) or *Cantharellus* (Ogawa *et al.*, 2017) can be advised. On the other hand, the stochasticity of the PCR process during replication (magnified by the number of cycles, Schmidt *et al.*, 2013) can alter the final abundance of a given target when compared to the initial pool of targets in the DNA extract. The dominance of a DNA molecule within a pool of molecules can become extreme after PCR amplification (Kauserud, 2023). Therefore, PCR can randomly overestimate some taxa (Buée, Sentausa and Murat, 2016), with obvious consequences on final diversity estimates. To prevent this undesired effect of PCR stochasticity, lowering the amount of PCR cycles has been consistently preconized (Castle *et al.*, 2018; Nilsson *et al.*, 2019).

The calibration of sequencing depth determines the expected number of sequence reads from a sample (Smith and Peay, 2014; Zaheer *et al.*, 2018; Kauserud, 2023). Sequencing depth mostly results from a trade-off between the sampling effort and the cost of a metabarcoding run (Shokralla *et al.*, 2012; Schmidt *et al.*, 2013). A common affirmation is to consider that increasing sequencing depth yields a higher alpha diversity within a sample – including both targeted genes and contaminant sequences (Baldrian *et al.*, 2021). Yet, to date, sequencing depth displays contrasted results on sample diversity. A high variation in the number of reads per sample has been observed, and challenges further ecological interpretations (McMurdie and Holmes, 2014; Nilsson *et al.*, 2019). Sequencing depth was found to differentially alter the diversity and composition of the targeted communities. Some studies found no significant difference in alpha diversity of soil samples sequenced at diverging depths (Mommer *et al.*, 2018). Conversely, Smith & Peay (2014) noticed a significant shift among soil fungal communities of American pine forests related to sequencing depth, with a somehow unexpected increase in both alpha and beta diversity at lower sequencing depth (Smith and Peay, 2014).

Because of its capacity to yield a large amount of sequences and despite its limitations, the Illumina method was rapidly adopted by community ecologists focusing on ectomycorrhiza (Nilsson *et al.*, 2019; Janowski and Leski, 2023). This method also reached operational actors and is now used as a diagnostic tool to guide management decisions for conservation purposes (*i.e.* fungal red-list, Geml *et al.*, 2014; Suz *et al.*, 2015), myco-forest managing (Charya and Garg, 2019; Tomao *et al.*, 2020) and ecological restoration monitoring (Yan *et al.*, 2018). However, the lack of a standardized metabarcoding protocol challenges the transferability of the molecular method (Ruppert, Kline and Rahman, 2019). Because of the multiple steps from sampling to sequence reads, a calibration specific to the EM guild is needed, including standardized PCR protocol (*i.e.* PCR cycling) and sequencing depth calibration (Smith and Peay, 2014). Today, there is no certitude that Illumina metabarcoding is a technology adapted to the estimation of relative abundances of taxa present in a sample (Fonseca, 2018). Yet, in fungal studies, read numbers are still used as a proxy of abundance (Merges and Neuschulz, 2018). Consequently, progress into the metabarcoding technics is required to ensure study repeatability and robust ecological interpretations. For both fundamental and applied purposes, a methodological calibration of molecular technics for EM fungi is thus necessary.

In this study, we 1) evaluated the effect of different sequencing methods (Sanger *vs* Illumina MiSeq) on the structure and the composition of the EM communities identified from the same samples of EM root apices (Figure 1). We then applied MiSeq metabarcoding on contrasted soil conditions, from highly mineral and species-poor EM communities from long-term sealed urban soils to highly organic and hyper-diverse substrates (*Quercus ilex* forest). In these three types of environmental samples, we 2) evaluated the effect of varying the sequencing depth on the diversity of the EM communities revealed from aliquots of the same DNA amplicon libraries (Figure 1). These results are discussed in the light of the current challenges of EM community ecologists.

Material and methods

Sampling

In March 2020, soil samples were collected from three Mediterranean habitats located within the urban area and in the landscapes surrounding the city of Marseille, France. These three study systems represent contrasted conditions of EM diversity, from the impoverished urban conditions to the highly diversified forest soils (Figure 1).

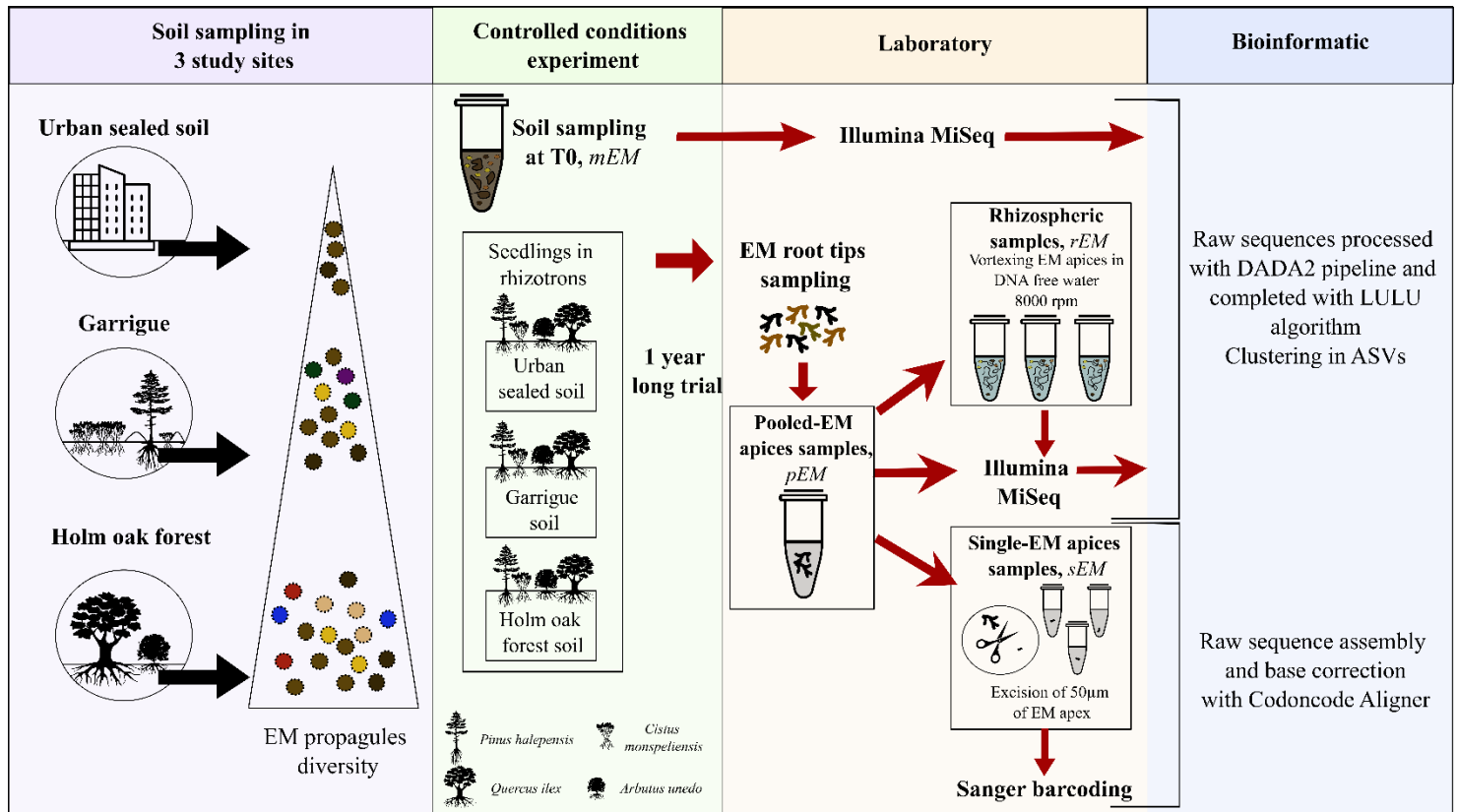
First, forest soils were sampled in a forest patch located at the Vallon des Peyrards (43°22'16.31" N, 5°22'27.09" E). The soil consists there in a thick organic litter covering a 60 cm deep organo-mineral horizon of sandy texture. The vegetation is dominated by the holm oak (*Quercus ilex*) with a dense understory of strawberry trees (*Arbutus unedo*) mixed with various endomycorrhizal shrubs of the Oleaceae and Rosaceae families. The second soil was collected in a garrigue located at Chemin de la Mûre (43°22'1.4" N, 5°23'16.51" E) on a calcareous plateau. At this site, the poorly differentiated soil consists in a superficial organic layer topping a silky-loam layer, with high volumetric rock content. The drought-tolerant vegetation is made of scattered Aleppo pine individuals (*Pinus halepensis*) dominating a shrubby layer of dense patches of kermes oak (*Quercus coccifera*) and rockroses (*Cistus monspeliensis*). The third soil was sampled at the Quartier des Fabriques (3°19' 35.86" N, 5°21'59" E), where it has been sealed by a layer of concrete in the early 1960th (*EuroMediterranée* report and *Geoportail*, 1957 aerial photograph, reference C3245-0461_1957_CDP1202_1247).

Experimental setup

The experiment was carried out at the CEFÉ laboratory, Montpellier (43°38'19" N, 3°51'43" E), from March to October 2020. We used standardized soil containers filled with 25 liters of soil, as in Taschen et al. (2020). In all, 36 containers were designed, *i.e.* 12 per soil origin. For each soil origin, the 12 replicates included two containers filled with soils previously vapor-sterilized for 24 hours at 100°C, and used as controls of the contamination through atmospheric dispersal of EM spores at the experimental field of CEFÉ laboratory. In all containers, four species of Mediterranean trees (*Quercus ilex* and *Pinus halepensis*) and shrubs

(*Arbutus unedo* and *Cistus monspeliensis*) were sown in March 2020, and used as bait plants to capture the EM inoculum present in soils during the development of their root systems.

Figure 1: Study methodological pipeline



Sample collection and characterization

At experimental setup (March 2020), 1g-soil aliquots were collected from all containers and stored at -18°C (Table 1, “matrix-EM samples”; *mEM*).

In October 2020, containers were disassembled, and all plants were gently harvested. In the laboratory, the root system of each plant individual was examined under a dissecting microscope, and a subsample of EM root tips, representing all the morphotypes (hereafter designed as “pooled-EM samples”; *pEM*), was picked using dissecting forceps, immediately placed in Eppendorf tubes and stored at -18°C until subsequent DNA analysis. For each plant individual, five EM apices representing the five dominant morphotypes were selected in each *pEM* for Sanger sequencing. For each of these five apices (hereafter designed as “single EM

apices samples”; *sEM*), the ectomycorrhizal tip was cut in half under the dissecting microscope. The sliced part of the apex was placed in a separate Eppendorf tube for Sanger sequencing, and the remaining part was reintroduced in *pEM* (Figure 1). In all, 263 pooled-EM samples and 192 single-EM samples were collected (Table 1).

From this sampling, 48 pooled-EM samples were selected to analyze the EM material immediately aggregated around the mantle and physically bound to the extramatrical mycelium of the picked EM apices (Table 1). Hereafter called “rhizospheric EM samples” (*rEM*) were obtained by adding 300µl DNA-free sterile water to each *pEM* and centrifuging the aliquot for 1 minute at 8,000 rpm (Figure 1). After shaking, *pEM* were removed from the filtrate, and transferred into new Eppendorf tubes for separate DNA analysis.

Molecular analysis of EM communities

DNA extraction

mEM and *rEM* DNA were extracted using FastDNA™ Spin Kit for Soil (MP Biomedicals, Irvine CA, USA). *pEM* and *sEM* DNA were extracted using DNeasy Plant Pro Kit (QIAGEN, Hilden, Germany). For each of these extraction kits, we followed the manufacturer’s instructions.

Illumina sequencing

The Illumina MiSeq sequencing was performed at the Sequencing and Genotyping Facility of the Cemeb Labex (GenSeq, UMR ISEM, Montpellier).

The fungal ITS2 was amplified using specific Illumina primers (ITS 3-F (5’TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG3’; ITS 4-F (5’GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG3’ plus several oligo-N) and the Phusion Flash High-Fidelity DNA polymerase (Thermo Scientific). We used the following PCR program: 98°C 2 minutes; (98°C 30 s; 50°C 45 s; 72°C 15 s) x 30; 72°C 10 minutes. The quality of PCR products was checked by electrophoresis on 2% agarose gels.

For each amplicon, 25µl aliquots were purified following the Gel-Free Size Selection Using SPRIselect For Next Generation Sequencing protocol (Verrow *et al.*, 2019) to remove unsized DNA sequences and remaining unused primers. Next, DNA libraries of ITS2 amplicons were built following the 16S Metagenomic Sequencing Library Preparation Protocol (ref. 15044223). After oligonucleotide indexing of samples, PCR products were again purified. Qualitative and quantitative analyses were performed using Fragment Analyzer (Qiagen) and Qubit fluorometer (Thermo Fisher) to help generating a balanced multiplexing to determine the adequate final concentration of amplicon library to be sequenced. The latter was set to 8 pM and the concentration of PhiX was standardized to 10 % (Table S1). Sequencing was performed on a MiSeq platform, for final paired-end reads ranging from 485 to 644pb using a nano kit of 500 cycles (MiSeq Reagent Kit v2, 500-cycles, MS-102-2003).

In a first analysis, the effect of sequencing depth on the retrieved diversity in soils was tested as follows. In a first run (Run 1, Table S1) a 10,000-reads sequencing depth was processed on 96 *mEM* amplicons, including 19 amplicons also sequenced at a depth of 50,000-reads (Run 2, Table S1), This sampling scheme allowed to maintain constant the total number of expected reads (960,000 *vs* 950,000) for allowing further comparison of the total detected diversity (Table S2). In a second analysis, the effect of sequencing depth on the retrieved diversity on roots was tested as follows. In a third run (Run 3, Table S1), a 2,600-reads sequencing depth was processed on 327 *pEM* and *rEM* amplicons, including 38 amplicons also sequenced at a depth of 25,000 reads (Run 4, Table S1). This sampling scheme allowed to maintain nearly constant the total number of expected reads (850,200 *vs* 950,000) for allowing further comparison of the total detected diversity (Table S2).

For each run of sequencing, several amplicons were triplicated and sequenced in three different wells of the plate. In all, triplicates included 6 *mEM* samples (final =18 samples), 5 *pEM* samples (final = 15 samples) and 1 *rEM* sample (final = 3 samples). In all, out of these 36 samples, 30 were retrieved with fungal sequences and 23 with EM fungal sequences (Table 1b).

Sanger sequencing

Two fragments including the fungal ITS2 of *sEM* samples (Figure 1) were selected for Sanger barcoding. A 350 base-pair long fragment encompassing part of the 5.8S rDNA and ITS2 was

amplified using ITS3 and ITS4 primers (respectively 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG3' and 5'GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG3', 192 samples). A 750 base-pair long full length ITS1-5.8S-ITS2 fragment was amplified using ITS1F or ITS5 and ITS 4 primers (ITS1F: 5'CTTGGTCATTTAGAGGAAGTAA3' and ITS5: 5'GGAAGTAAAAGTCGTAACAAGG3', 192 samples), with the polymerase Phusion Flash High-Fidelity DNA (Thermo Scientific). The PCR program was the following: 94°C 2 minutes; (94°C 30 s; 65°C 30 s (-1°C/cycle); 72°C 30 s) x 10 and then (94°C 30 s; 55°C 30 s; 72°C 30 s) x 35; 72°C 5 min. The quality of PCR products was checked by electrophoresis on 2% agarose gels and Sanger sequencing was performed at Eurofins Genomics (Ebersberg, Germany).

Table 1: a) Sequencing overview of the study and b) Sequencing overview of triplicated samples

a) Sequencing overview of the study

Sample type	Number of sequenced amplicons	Number of amplicons with sequences assigned		Number of amplicons with fungal sequences assigned		Number of amplicons with EM sequences assigned	
		Number	%	Number	%	Number	%
Soil (<i>mEM</i>)							
Total	101	97	96	97	96	78	77
10,000 sequencing depth	82	82	100	82	100	65	79
50,000 sequencing depth	19	15	79	15	79	13	68
Pooled-EM apices (<i>pEM</i>)							
Total	216	202	93	186	86	121	56
2,600 sequencing depth	178	164	92	149	84	92	52
25,000 sequencing depth	38	38	100	37	97	29	76
Rhizospheric samples (<i>rEM</i>)							
Total	86	56	65	47	54	18	21
2,600 sequencing depth	48	31	64	25	52	9	19
25,000 sequencing depth	38	25	66	22	58	9	24
Single-EM samples (<i>sEM</i>)							
ITS2 350bp	192	115	60	24	12,5	20	10
ITS2 750bp	192	62	32	62	32	21	11

b) Sequencing overview of triplicated samples

Sample type	Number of sequenced amplicons	Number of amplicons with fungal sequences assigned	Number of amplicons with EM sequences assigned
Soil (<i>mEM</i>)	18	16	16
Pooled-EM apices (<i>pEM</i>)	15	11	4
Rhizospheric samples (<i>rEM</i>)	3	3	3

Bioinformatic analyses

Illumina metabarcoding datasets

The analysis was performed on 97 successful amplicons of *mEM*, 202 successful amplicons of *sEM* and 56 of *rEM* (Table 1a). Raw sequences were processed with DADA2 pipeline v1.16 (Callahan et al., 2016; R Core Team 2022) and completed by the LULU algorithm (Frøslev *et al.*, 2017). The amplicon sequence variant (ASV) clustering method was used as in Pauvert et al. (2019). In short, we clustered 3 022 904 paired-end sequences into 6 133 ASVs, including 530 EM ASVs retrieved from 676 187 reads.

Sanger barcoding data

The taxonomic identification of DNA barcodes from Sanger sequencing was performed as following. Raw sequence data were edited and assembled using Codon Code Aligner 4.1.1 (CodonCode Corp., Centerville, Massachusetts). 177 *sEM* amplicons (Table 1a) were analyzed using either ITS3/ITS4 (115 amplicons) or ITS1/ITS5/ITS4 (62 amplicons). The sequences exhibiting an insufficient quality for appearing and correcting were discarded from the analysis. We associated the sequences in contigs based on the sample name. We removed primers and low-quality regions of the sequence. A manual correction of nucleotide base was performed. The resulting aligned sequences were blasted on UNITE database (<https://unite.ut.ee>).

Data analyses

All analysis were performed in R Studio Software (R Core Team, Version 3.6.2), using Vegan (Oksanen *et al.*, 2017), MiscMetabar (Taudière, 2023) and Phyloseq (McMurdie and Holmes, 2013) packages for ecological and community analysis.

We verify if retrieved read numbers per sample was significantly diverging according to theoretical sequencing depth using a Kruskal-Wallis test. We compared ASVs richness among sample types and different sequencing depth. We draw an accumulation curve on ASVs abundances using MiscMetabar::accu_plot() (Taudière, 2023) to estimate the number of read required to exhaustively sequence a sample.

From a compositional perspective, we analyzed the respective effect of sequencing depth, sample nature (soil *vs* plant tissues) on the retrieved EM communities. We analyzed the EM diversity and communities of the 36 triplicated samples with EM sequences retrieved after sequencing (Table 1b). We compared the number of reads and the amount of ASVs retrieved among the triplicated amplicons.

Finally, we compared the dominant EM sequences retrieved by Sanger barcoding and Illumina metabarcoding. We imported Illumina sequences into Codon Code software and performed a sequence comparison with 90 % of homology. We blasted the consensus sequences to identify the EM sequences. We calculated the percentage of corresponding Illumina sequences.

Results

Quantitative effects of sequencing depth and sampled material on dataset

The metabarcoding of soil samples at two contrasted sequencing depths provided 1) a highly variable number of reads among samples (Table S2) and 2) revealed a gap between the expected and the realized number of reads, at both depths (Figures 2). At the lowest sequencing depth (*i.e.* 10,000 reads per sample), the number of sequences per sample ranged from 179 to 42,805, and averaged 9,104 \pm 8,867 reads (Table S2 for details). When considering the data gathered on the same subsample of 15 soil cores, a mean of 20,530 \pm 11,475 reads were generated by the analysis (Table S2). At the highest sequencing depth (50,000 reads per sample), the total number of sequences per sample averaged 8,929 (*i.e.* 17.86% of the expected number of sequences), and ranged from 13 to 30,485 (Table S2).

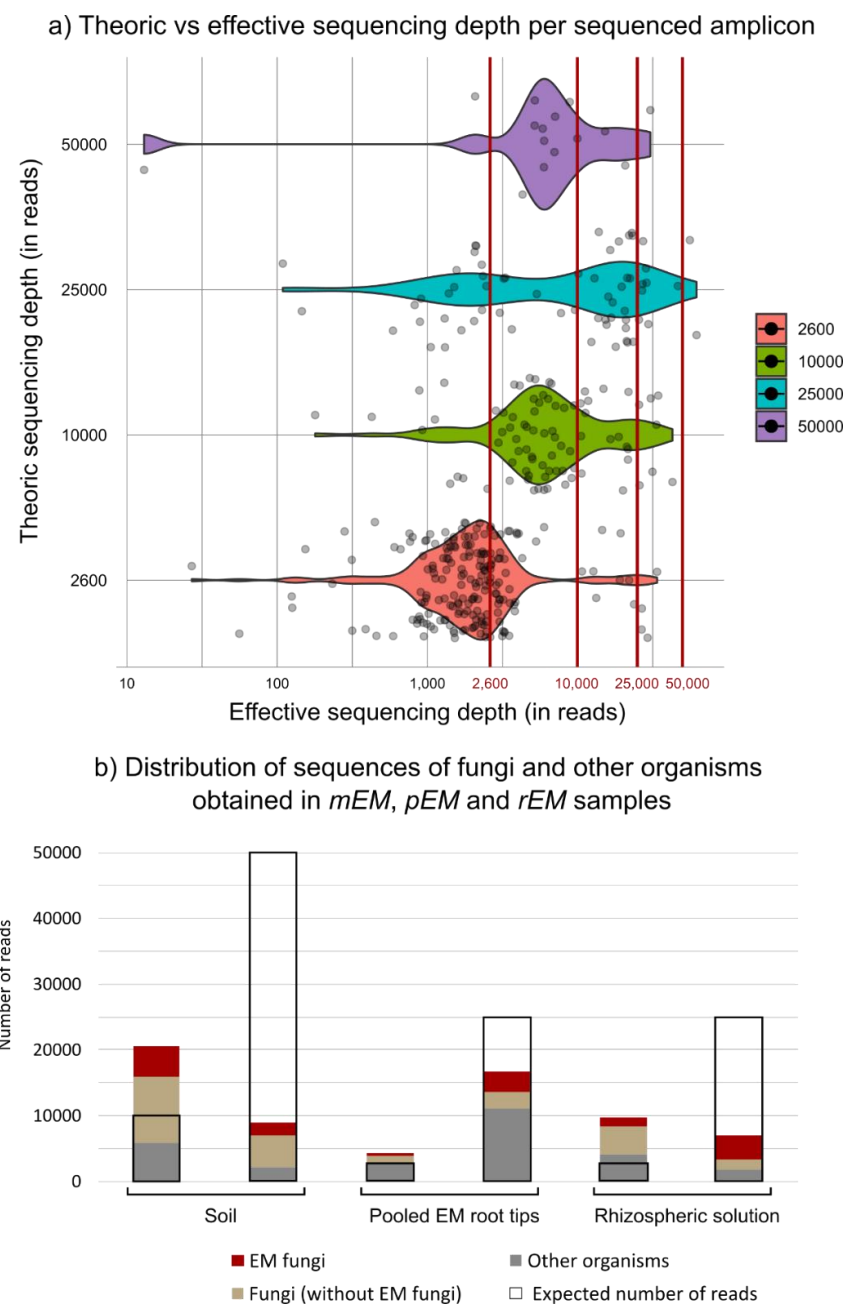
For the 33 paired EM root tips, a similar pattern was obtained, with highly variable and contrasted numbers of sequences obtained among samples, at each of the two sequencing depths (Figures 1a & 2). At low (*i.e.* 2,600 sequences) and high (*i.e.* 25,000 sequences) sequencing depths, a mean of 1,877 \pm 6,936 and 17,942 \pm 8,683 sequences per sample were respectively obtained (Table S2).

For the 14 paired rhizospheric samples, 9.642 \pm 11,183 and 6.976 \pm 13,518 sequenced were respectively generated at low (*i.e.* 2,600 sequences) and high (*i.e.* 25,000 sequences) sequencing depths (Table S2).

From metabarcoded soils, a majority of fungal sequences were identified, accounting for 71.28 and 74.7% of total number of reads retrieved at low and high sequencing depth (Table S2 and Figure S2). Contrastingly, plant sequences dominated in datasets obtained from the metabarcoding of EM root tips, with 66.16 and 65.21% of sequences assigned to the host at low and high sequencing depth, respectively (Table S2 and Figure S2). Last, the metabarcoding of the rhizosphere provided contrasted patterns at low (with a 52.29% of EM fungi sequences) and high (with an equilibrated pattern between the number of plant and fungi sequences; Table S2 and Figure 2b).

On paired samples, sequencing depth positively influenced the number of reads obtained on root tips for all organisms EM apices (KW = 27,98; p-value = 1,22e-07, Table S2), and negatively impacted the number of reads obtained in soil (KW = 9,29; p-value = 0,002, Table S2) and in the rhizosphere (KW = 3,21; p-value = 0,07, Table S2). In EM apices, sequencing depth positively influenced the number of reads obtained for fungal organisms (KW = 9,4, p-value = 0,002, Table S2). In rhizospheric sample, no significant difference was obtained when only considering fungi (KW = 0,05; p-value = 0,82, Table S2) and the EM guild (KW = 0,003; p-value = 0,97, Table S2). Similarly, in EM root tips, no significant difference was obtained on the number of read for the EM guild (KW = 2,14; p-value = 0,17; Table S2).

Figure 2: Effective sequencing depth of our study including a) vs theoretical calibrated depths and b) distribution of sequences of fungi and other organisms obtained in soil, on ectomycorrhizal root tips and in the rhizosphere at two sequencing depths



Qualitative effects of sequencing depth and sampled material on dataset

The number of ectomycorrhizal fungal ASVs detected in soils, on root tips and in the rhizosphere was positively and significantly correlated with the number of sequences (Figure 3a, b, c). In all amplicons, the detection of EM fungal diversity was 100 % complete at 1,322 reads, and in most of them (163 amplicons, *i.e.* 75%), this detection was efficient at less than 500 reads (Figure 4).

In all, 241, 78 and 24 EM ASVs were respectively detected in soil, EM apices and rhizospheric samples (Figure 5). In paired-samples of soil, the mean ASVs number retrieved per samples was higher at low sequencing depths (7 ASVs; Table S3) than at high sequencing depth (6 ASVs; Table S3). Contrastingly, in paired-samples of EM root tips and in paired-rhizospheric samples, the mean number of ASVs was lower at low sequencing depths (1,4 and 1 ASVs; Table S3) than at high sequencing depth (2,1 and 1,07, Table S3). However, the effect of the sequencing depth was not significantly influencing the number of EM ASVs (soil KW = 1,14; p-value = 0,28; paired-EM root tips KW = 1; p-value = 0,12 and paired rhizospheric samples KW = 0,0008; p-value = 0,98; Table S3).

Figure 3: Correlation analysis between the number of read and the number of EM ASVs in a) soil, b) EM root tips and c) rhizospheric samples

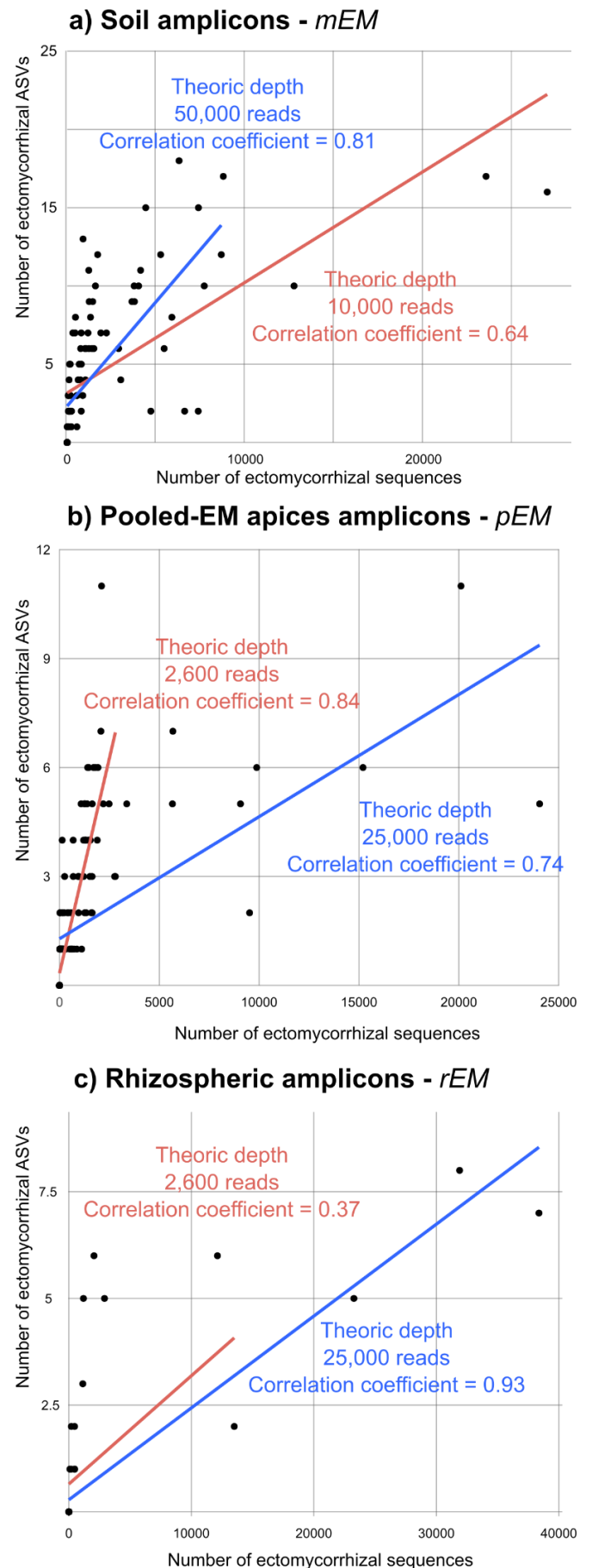
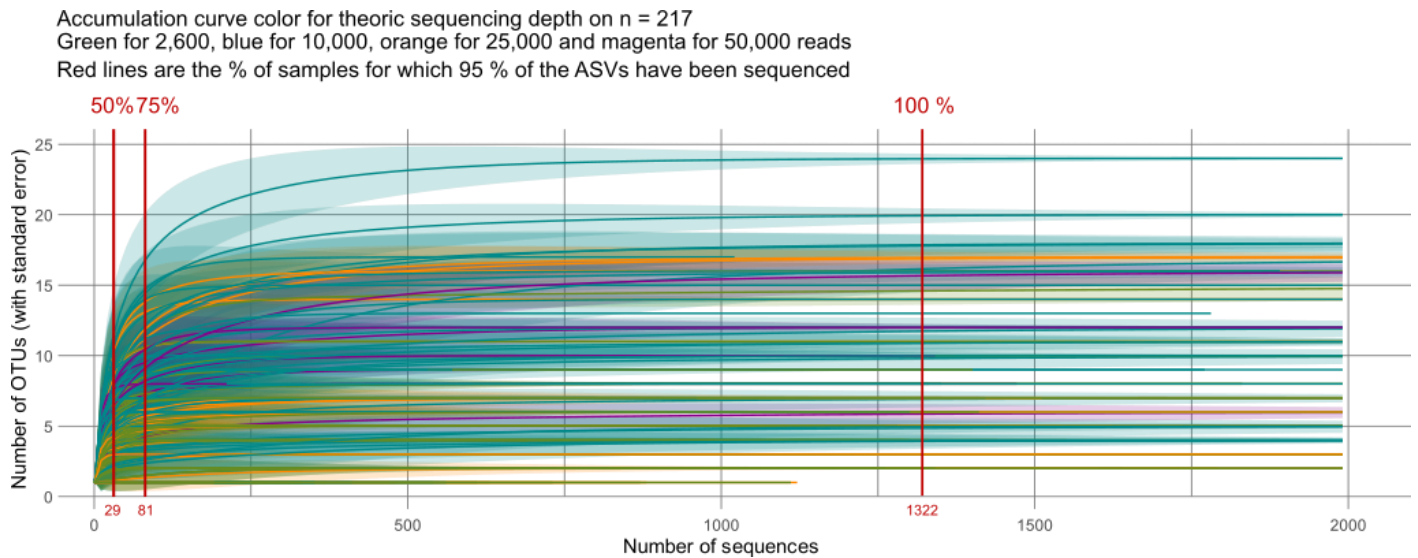


Figure 4: Accumulation curve of ectomycorrhizal fungi obtained in soil, on ectomycorrhizal root tips and in the rhizosphere at two sequencing depths. Each curve reports a sequenced sample. Shaded zone indicates standard deviation for each sequenced sample.



The composition of EM communities varies depending on sample type and sequencing depth

First, the composition EM ASVs communities significantly vary according to the sample type (*i.e.* soil, EM apices, rhizosphere; Adonis permutational F -value = 2,4 and p -value = 0,001). The difference was significantly different among the three type of samples (p -value = 0,001, 0,001 and 0,02 by Adonis pairwise post-hoc comparison test, for soil *vs* apices, soil *vs* rhizosphere and apices *vs* rhizosphere). Using a Venn diagram representation, the analysis on 355 samples with EM ASVs detected in soils, apices and rhizospheres reveals that the vast majority of EM ASVs are specific to one single sample type (Figure 5a) – short of the exception of rhizosphere samples which share 17 ASVs, including 16 ASVs shared with EM apices (Figure 5a) and 13 ASVs shared with soil samples (Figure 5a). When focusing on the effect of sequencing depth on paired samples of soils (Figure 5b), rhizosphere (Figure 5c) and root apices (Figure 5d), a pattern of high composition discrepancy was shown, and a particularly patchy distribution of them, with 182 (73.7%), 39 (50%) and 14 (58.3%) of ASVs detected in one single amplicon of soil, EM root apices and rhizosphere, respectively (Table S4).

Secondly, we found that sequencing replication of the same amplicon allow an improved coverage of EM ASVs diversity with a mean of 5.19 ASVs retrieved per triplicated sample against 3,35 ASVs retrieved in non-replicated samples ($X^2 = 13,98$ and $p = 0,0001$ by Kruskal-Wallis test). The composition of EM communities in a same amplicon is altered by sequencing replication (Figure 6). On the 12 triplicated samples (*i.e.* the same amplicon was sequenced in three different wells – resulting in 36 amplicons), eight of them exhibited various numbers of ASVs numbers, while the four remaining retrieved the same number of ASVs (Figure 6).

Figure 5: Distribution of ectomycorrhizal ASVs detected in a) all mixed samples, b) paired-soil samples, c) paired-rhizospheric samples and d) paired-EM root tips samples

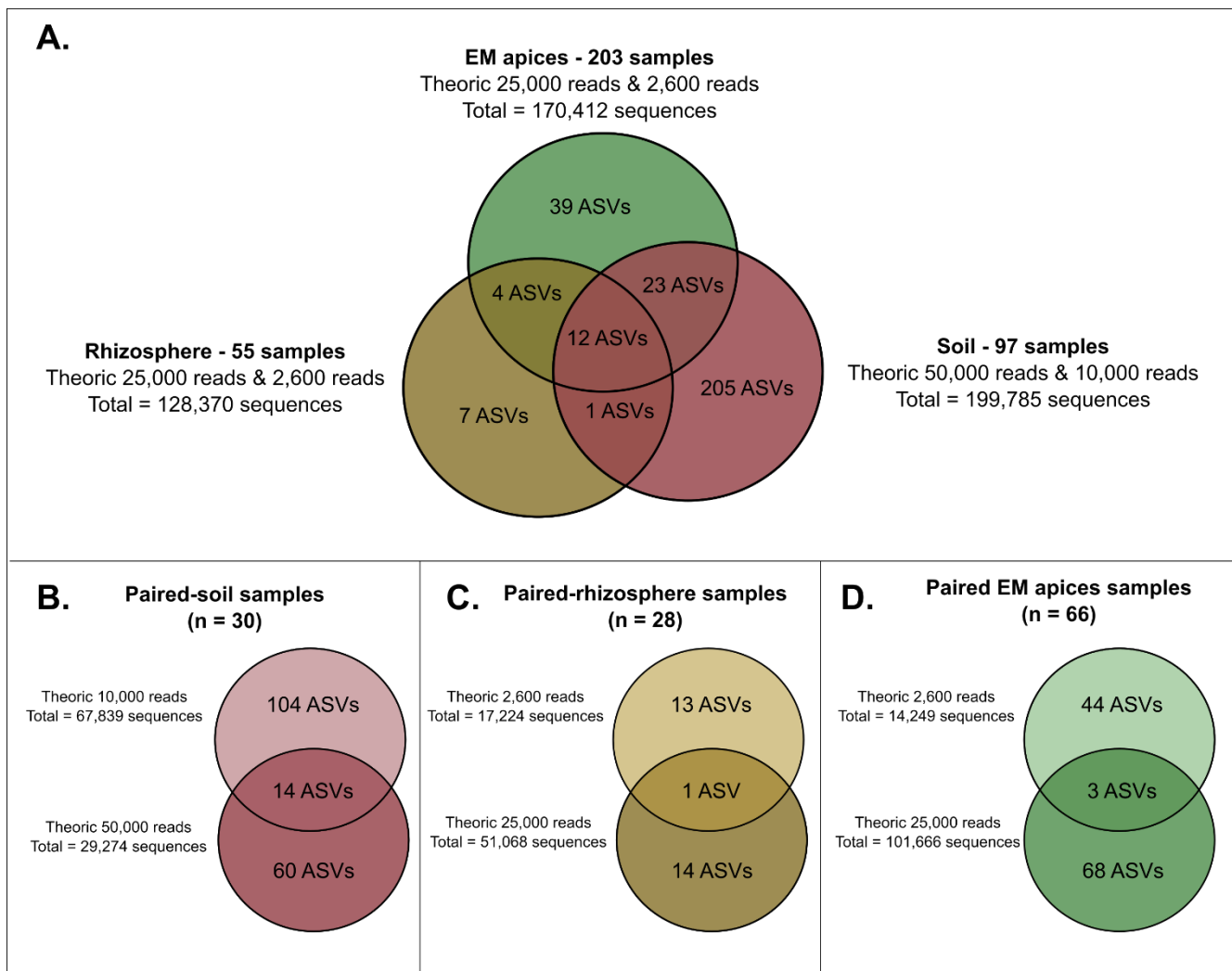
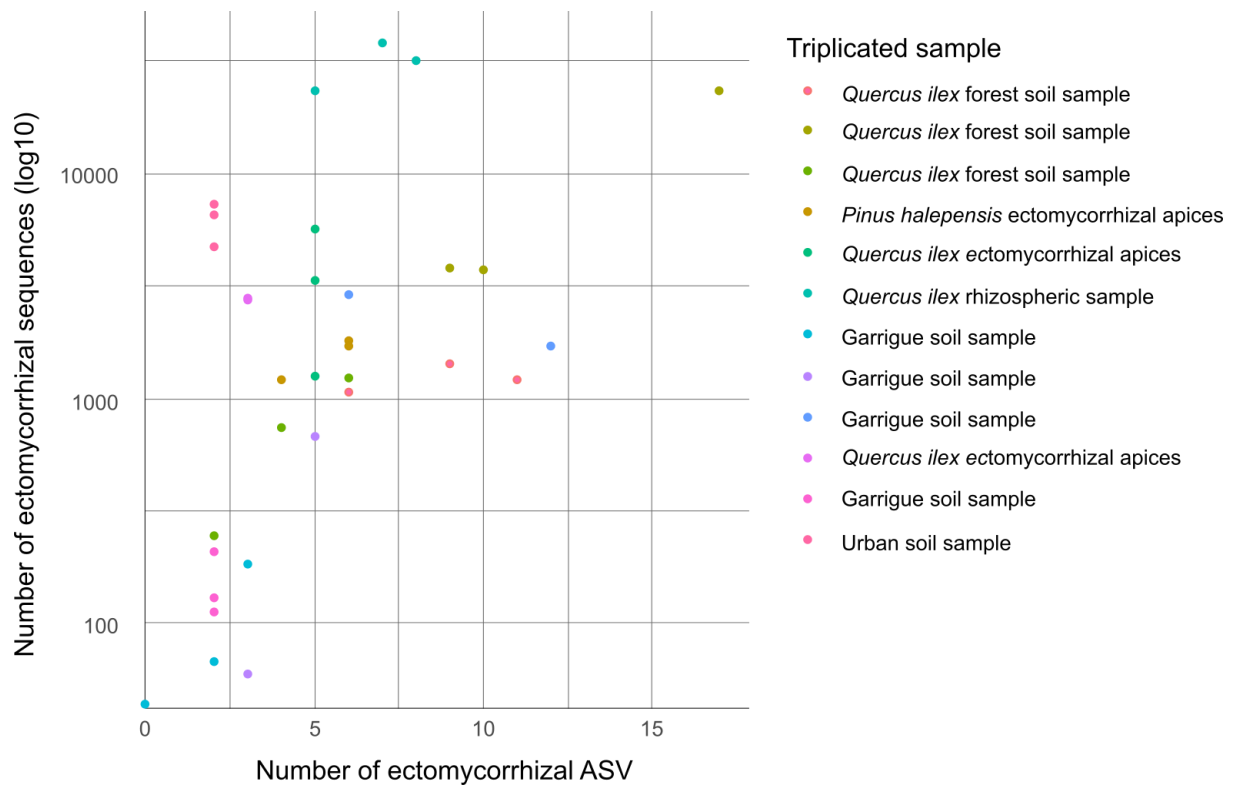
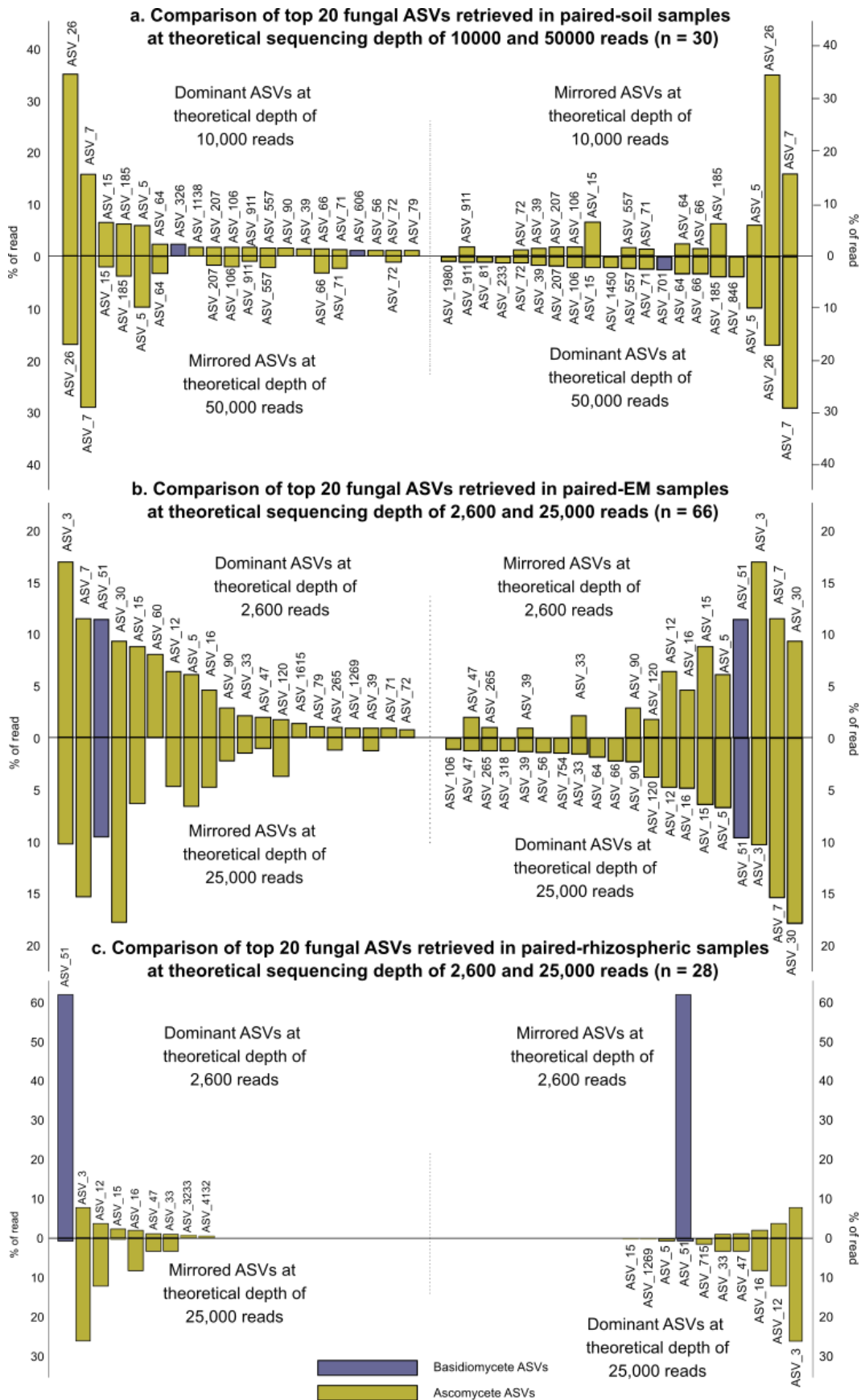


Figure 6: Sequencing replication alters the composition of ASVs among triplicated amplicons. Diversity of ectomycorrhizal fungi (ASVs) detected in triplicated amplicons.



Finally, when plotted into rank abundance diagrams (Figure 7), the EM communities revealed by low vs high sequencing depths showed highly contrasted patterns. In soils (Figure 7a), while the first two dominant ASVs ranked 1 and 2 at both sequencing depth, seven ASVs among the twenty most represented ones were not present before rank 20 at high sequencing depth (Figure 7a). On root apices, a better correspondence was obtained between pictures of the community at low and high sequencing depth (Figure 7b). The five most represented ASVs were recorded before rank five at both sequencing depths, including one Basidiomycete species which was absent from soil datasets (Figure 7b). In rhizosphere, the same five first ASVs were detected at both sequencing depths, but high highly variable numbers of reads when considering the two patterns Figure 7c). The same Basidiomycete (ASV51, *Tomentella spp*) was among the most amplified ASV in rhizosphere and on root apices (Figure 7b & 7c).

Figure 7: Correspondence between the % of reads obtained for twenty most amplified ASVs at two theoretical sequencing depths in paired-samples of (a) soil, (b) EM apices and (c) rhizospheric samples



Comparison of taxonomic assignation of dominant ASVs in Sanger barcoding and Illumina MiSeq metabarcoding

In 19 and 21 paired-samples (*i.e.* 19 and 21 pools of excised root tips sequenced as one single environmental sample by Illumina *vs* discrete parts of excised apices barcoded by Sanger; Figure 1), the comparison of the dominant ASVs shows a majority (respectively 79 % and 71 % for a short and a long fragment of ITS2) of taxa congruently dominant in both analyses (Table 2). The 21 and 29 % of remaining taxa were either not detected by metabarcoding (respectively 5 and 24 % for a short and a long fragment of ITS2; Table 2) or detected in the community as poorly represented taxa (respectively 16 and 5 % for a short and a long fragment of ITS2; Table 2). A similar pattern was obtained with using two various ITS coverage in barcoding analysis (Table 2).

Table 2: Correspondence between root tips metabarcoding and barcoding.

Number of paired-amplicons sequenced in both Illumina and Sanger	Identical dominant sequence in barcoding and metabarcoding		Only dominant in Sanger		Undetected by Illumina	
	Number	%	Number	%	Number	%
ITS2 350bp - 19 samples	15	79	1	5	3	16
ITS2 750bp - 21 samples	15	71	5	24	1	5

Discussion

Main results

Our study emphasizes the importance of evaluating the contribution of newly widespread technologies such as Illumina to our ability to increase our understanding of the composition of ectomycorrhizal communities. Through the use of two highly popular sequencing methods (Sanger barcoding *vs* Illumina), we provide several important methodological and ecological inputs for further molecular-based exploration of diversity and ecology of EM fungi. First, we noticed the highly unbalanced sampling of EM communities which results from fixed sequencing depths. Specifically, we revealed a non-systematic and erratic gap between the calibration of the sequencing depth and the obtained output and we revealed some potential consequences for subsequent community analyses. Thus, the erratic nature of among-samples quantitative Illumina sequencing outputs induces an erratic evaluation of the EM diversity, the number of reads significantly and positively correlates with the number of detected EM ASVs (Figure 3). Thirdly, we found that calibration and replication induced shifts in EM community composition. Finally, we found that the nature of the metabarcoded samples, *i.e.* soil *vs* EM apices *vs* rhizosphere interfered with sequencing depth to shape the composition of the revealed community. Hereafter, we discuss these results in the light of the methodology of the investigation of EM communities, from the field sampling, the laboratory step to the final ecological analysis.

In the field, the need of numerous sampling points to limit the consequences of EM species patchiness

EM communities have been consistently described as spatially patchy in the ecosystems (Horton and Bruns, 2001; Lilleskov *et al.*, 2004). The patchiness of EM communities results from local environmental filtering – with a spread of propagules from resource patches in forest/shrubland ecosystems (Koide, Fernandez and Petprakob, 2011). In our study, EM community patchiness was revealed through the unicity of ASVs sequenced in soil amplicons (*i.e.* processed samples), with most of the ASVs belonging to only one amplicon (Table S4). Because of the low number of shared ASVs among soil samples (Table S4), we hypothesize that our sampling scheme did not allow an exhaustive sampling of the EM community. With

accounting of ectomycorrhizal community's patchy distribution in the soil, molecular-based studies may be based on a high level of replication. An increased sampling effort can help to reduce the sampling effect observed with our data.

Bait plants are efficient tools to sample EM communities in inoculum-poor soils

Using tree seedlings as baiting procedure to capture EM inoculum is an ancient and widely used method consisting of barcoding EM apices developed on roots of plants grown in soil substrates of unknown fungal composition (Molina *et al.* 1992). In our study, this method was an efficient and complementary way to sample EM communities. Thus, metabarcoding and plant baiting procedure applied to the same soil soils revealed two different pictures of the EM communities, with X% of the total EM diversity detected by both methods (Figure 5). Several determinants may explain this difference. First, metabarcoding has the ability to detect EM fungi from various sources of biological material, including dormant spores, mycelia, sclerotia, primordia and rhizomorphs (Wu *et al.*, 2019), but without distinguishing between mobilizable and potentially inactive lifeforms. Contrastingly, bait plants only associate with the active and compatible part of this pool, with an ability to establish on seedlings of the tree species used in the experiment. Second, metabarcoding provide a picture of the EM inoculum present in a highly restricted volumes of soil. On the other hand, the barcoding of EM apices of bait plants is based on the examination on extended root systems which foraged large volumes of soil, and then get the opportunity to interact with highly patchy soil biotas. From a fundamental perspective, both methods appear as two complementary tools which may be used to characterize EM soil diversity.

The comparison between the dominant sequences retrieved by Illumina and Sanger sequencing methods applied to the same samples provided consistent results, the dominant sequences retrieved in pooled-EM samples corresponding to the dominant sequence identified using Sanger barcoding, in around 75% of the samples (Table 2). In the 25% remaining samples, Illumina and Sanger sequencing revealed different dominant species, the metabarcoding losing the signal of the fungal species identified by the barcoding of the apical part of EM root apices (Table 2). Again, this result evidences the complementarity between barcoding and metabarcoding methods, as efficient way to characterized the established (barcoding) and

surrounding (metabarcoding) fungal diversity interacting at the immediate vicinity of EM root apices.

From field sampling to sequencer settings: how to sample amplicons in metabarcoding studies?

In the laboratory, the theoretical sequencing depth was assessed by dividing the number of reads of the metabarcoding cartridge by the number of amplicons. In most samples, at all sequencing depth, this theoretical number of reads was a poor predictor of the obtained data (Figure 2a, b). This result suggests that the early steps of DNA extraction and amplification are critical steps which strongly conditions the final sequence dataset.

In our study, the number of ectomycorrhizal ASVs was highly and positively correlated with the number of reads per sample (Figure 3). With replicating the sequencing processes three times on the same amplicon (triplicated samples), we noticed that the number of ectomycorrhizal ASVs retrieved was varying among these three replications (Figure 6). Positively considered, this erratic pattern may suggest that sequencing replication of a single amplicon can be an efficient way to sample more exhaustively the ectomycorrhizal community within a sample. However, the origin of this patterns is a matter of concern, because it suggests that number of reads of an ASV should be considered as a measurement of presence but not of abundance of a given taxon. The origin of this pattern remains to be explored, but may be firstly technological. Thus, during the sequencing process, DNA molecules are randomly binding to the flow-cell (Singer *et al.*, 2019), and this stochastic process may generate random evaluation of ASVs abundance in the community.

The obtained results (*i.e.* erratic number of reads per sample and correlation between sample size and diversity; Figure 3) suggest that the analysis of metabarcoded EM fungal communities may preferentially be based on normalized measurements of the diversity, including rarefied richness, and/or the use of diversity indexes which are poorly affected by sample size (*e.g.* Fisher's alpha). In addition, the before PCR-dependent pattern of number of reads obtained in a given sample (*i.e.* the observed within-triplicates variability) is a result of primary importance, and suggests that the number of reads shouldn't be considered as a reliable measurement of abundance in community studies. Finally, under the perspective of capturing the EM fungal

diversity in soils, sequencing a high number of environmental samples at low sequencing depth, under 1000 reads, may be more efficient than over-sequencing few environmental samples, for the same total number of expected reads. This result shows that soil metabarcoding suffers the same consequences of the well-known EM fungal patchiness in soils (Horton and Bruns, 2001).

The number of read shouldn't be considered as a proxy of taxa abundance

Our study unambiguously shows the high volatility of the numbers of reads obtained by metabarcoding in from a given sample, as a consequence of both amplification step and Illumina internal process. PCR have been consistently described as influencing the proportion of DNA molecule in an amplicon (directional, stochasticity, Bellemain *et al.*, 2016; Kauserud, 2023). Other PCR-step sources of variability have been identified, including polymerase inhibition by soil complex forms of carbon. In particular, the presence of secondary metabolites molecules in DNA extract (*e.g.* melanin or polyphenol) may have altered the polymerase activity (Janowski *et al.*, 2019). In our study, *Quercus ilex* root tips and soils are known sources of complex forms of carbon (Courty *et al.*, 2016) and tannins. Several technical guidelines have been formulated to diminish the effect of polymerase inhibitors or inducers of PCR stochasticity, including the dilution of extracted DNA (Schmidt *et al.*, 2013; Janowski *et al.*, 2019; Tedersoo *et al.*, 2022). However, the choice of diluting DNA extracts results from a trade-off between inhibition intensity and research question, as dilution level may shape both alpha/beta-diversity within extracts and the capacity of metabarcoding to retrieve rare EM taxa (Castle *et al.*, 2018).

The composition of EM communities varies according to sequencing calibration and sequencing method

Based on the analysis of paired amplicons, our results show that the EM ASVs community composition was partially determined by initially chosen sequencing depth (Figure 7). From the same amplicon, two different sequencing depths detected different ASVs, which at the end were unique to one of the other datasets (Figure 7). This pattern was consistently observed in soils, on roots and in the rhizosphere, meaning that false negatives are commonly obtained in metabarcoding, whatever the sampling effort (measured as the sequencing depth). To evaluating the importance of this artefactual pattern, we doubled sequenced EM root tips,

after excision, using both metabarcoding and Sanger barcoding on the same biological material. Interestingly, our comparison revealed that ~ 75 % of the dominant ASVs detected by Illumina metabarcoding were also detected as dominant by Sanger barcoding (Table 2). Over the ~25 % of remaining ASVs, a small proportion were lost (16 and 5%; Table 2) or poorly detected (5 and 24%; Table 2) in metabarcoding. This result is of primary importance, as metabarcoding is becoming the used standard method to described EM communities, largely preferred to the barcoding of root apices.

The composition of EM community in the rhizosphere differs from those in soil and on root apices

Through studying the EM ASVs communities retrieved in three contrasted samples types (*i.e.* soil, EM apices and rhizosphere), we expected to find high similarity between root tips EM communities and rhizospheric communities. Our hypothesis was not confirmed, the composition of the community “harvested” from EM apices significantly differing from those in soil and on roots (Figure 5). Interestingly, our results revealed that rhizospheric samples contain ectomycorrhizal ASVs undetected neither in the soils or in the EM root tips (Figure 5) suggesting that, technological biased apart, ectomycorrhizal apices may host original EM communities, including taxa with high affinity for and/or establishing trophic interaction with the mycorrhizal fungi. This result may be supported by the often-observed multiple colonization of EM root apices revealed either anatomically or after Sanger sequencing (Horton & Bruns 2001). Further insights are needed to better understand the interaction pattern at behind the micro-local patchiness of EM communities.

Conclusion

During the last decade, metabarcoding provided unprecedented amount of fungal data from unexplored environments. This unique tool opened a large window on the underground counterpart of forest ecosystem by providing high resolution pictures of the composition of ectomycorrhizal communities. In this study, we show that the dataset produced by Illumina metabarcoding should be analyzed prudently when considering the number of reads, which are highly impacted by machine settings. We also show that increasing sequencing depth per sample should be considered as smoke and mirrors, and may be abandoned for the benefit of ambitious sampling efforts in the field to consider the patchiness of EM communities. Further experimental work can be improving our use of molecular tools including the effect of bioinformatic pipeline on the picture of the ectomycorrhizal communities.

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Supplementary materials

Table S1: Illumina MiSeq run details

Run	Sample type	Multiplexing	Theoretical sequencing depth	Library concentration	% PhiX	Cluster density (K/mm ²)	% Passing filter	M reads
1	Soil	95 samples	10.000 reads	8 pM	10 %	695	96	0,95
2	Soil	93 samples	10.000 reads	8 pM	10 %	701	97,3	0,95
3	Soil	19 samples	50.000 reads	8 pM	10 %	573	97,7	0,78
4	EM apices	38 samples	25.000 reads	8 pM	10 %	731	96,8	1
5	Rhizosphere samples	38 samples	25.000 reads	8 pM	10 %	637	96,3	0,87
6	EM apices and rhizosphere samples	327 samples	2.600 reads	8 pM	10 %	680	96,5	0,93

Table S2: Details of datasets according to sample type, paired-sampling, theoretical depths and assignation of the sequence to fungal group or ectomycorrhizal (EM) guild.

All raw sequences										
Sample type and theoretical depth	n	Total	Min	Max	Mean	Median	Sd	Kruskal Wallis X ²	p-value	
Soil – 50.000 reads	15	133.929	13	30.485	8.928,6	5.985	7.813,66	0,16	0,002	
Soil – 10.000 reads	82	746.554	179	42.805	9.104,32	5.991,5	8767,87			
Paired samples – 50.000 reads	15	133.929	13	30.485	8.928,6	5.985	7.813,66	9,29		
Paired-samples – 10.000 reads	15	307.952	5.222	42.805	20.530,13	21.638	11.475,29			
EM samples – 25.000 reads	38	641.057	1.053	29.571	16.869,92	18.381	8.137	70,43		1.22e-07
EM samples – 2.600 reads	165	403.137	27	26.787	2.443,25	1.879	3402,91			
Paired-EM samples – 25.000 reads	33	552.672	1.053	29.751	16.747,64	17.942	8.682,5	27,98		
Paired-EM samples – 2.600 reads	33	140.370	126	26.787	4.253,64	1.877	6.936,15			
Rhizosphere – 25.000 reads	25	241.068	109	61.901	9.642,72	2.062	18.133,45	0,99	0,07	
Rhizosphere – 2.600 reads	30	204.673	56	33.798	6.822,43	2.736,5	9.232,49			
Paired-rhizosphere – 25.000 reads	14	97.662	109	46.543	6.975,86	2.182	13.518,48	3,21		
Paired-rhizosphere – 2.600 reads	14	134.982	125	33.798	9.641,57	3.901,5	11.183,32			
All fungal sequences										
Sample type and theoretical depth	n	Total	Min	Max	Mean	Median	Sd	Kruskal Wallis X ²	P-value	
Soil – 50.000 reads	15	100.039	13	28.776	6.669,27	4.265	7.812,92	0,002	0,02	
Soil – 10.000 reads	82	545.159	53	31.888	6.648,28	4.062,5	7.359,43			
Paired samples – 50.000 reads	15	100.039	13	28.776	6.669,27	4.265	7.812,92	4,92		
Paired-samples – 10.000 reads	15	219.509	249	31.888	14.633,93	14.953	10.690,35			
Pooled-EM samples – 25.000 reads	38	213.545	0	26.135	5.619,6	2.758,5	6.415,93	20,12		0,002
Pooled-EM samples – 2.600 reads	165	169.284	0	14.844	1.025,96	733	1.717,09			
Paired-EM samples – 25.000 reads	33	187.036	0	26.135	5.667,76	2.459	6.728,81	9,4		
Paired-EM samples – 2.600 reads	33	48.822	0	14.844	1.479,45	498	3.375,56			
Rhizosphere – 25.000 reads	25	183.602	0	53.627	7.344,08	1.300	14.307,35	0,004	0,82	
Rhizosphere – 2.600 reads	30	140.381	0	29.210	4.679,37	1.509	7.488,31			
Paired-rhizosphere – 25.000 reads	14	72.277	0	38.378	5.162,64	1.595,5	10.384,78	0,05		
Paired-rhizosphere – 2.600 reads	14	76.186	0	29.210	5.441,86	1.509	8.663,77			
Ectomycorrhizal sequences										
Sample type and theoretical depth	n	Total	Min	Max	Mean	Median	Sd	Kruskal Wallis X ²	P-value	
Soil – 50,000 reads	15	29,274	0	8.651	1.951,6	1.170	2.646,44	0,15	0,46	
Soil – 10.000 reads	82	170.511	0	26.940	2.079,4	505,5	4.439,32			
Paired samples – 50.000 reads	15	29.274	0	8.651	1.951,6	1.170	2.646,44	0,53		
Paired-samples – 10.000 reads	15	67.839	0	26.940	4.522,6	1.225	7.255,11			
Pooled-EM samples – 25.000 reads	38	110.574	0	24.046	2.909,84	73,5	5.807,07	8,09		0,17
Pooled-EM samples – 2.600 reads	165	59.838	0	2.799	362,65	0	650,45			
Paired-EM samples – 25.000 reads	33	101.666	0	24.046	3.080,79	39	6.174,31	2,14		
Paired-EM samples – 2.600 reads	33	14.249	0	2.107	431,79	0	686,21			
Rhizosphere – 25.000 reads	25	106.707	0	38.378	4.268,28	0	10.654,2	0,07	0,95	
Rhizosphere – 2.600 reads	30	21.663	0	13.504	722,1	0	2.508,11			
Paired-rhizosphere – 25.000 reads	14	51.068	0	38.378	3.647,71	0	10.502,85	0,003		
Paired-rhizosphere – 2.600 reads	14	17.224	0	13.504	1.230,29	0	3.584,5			

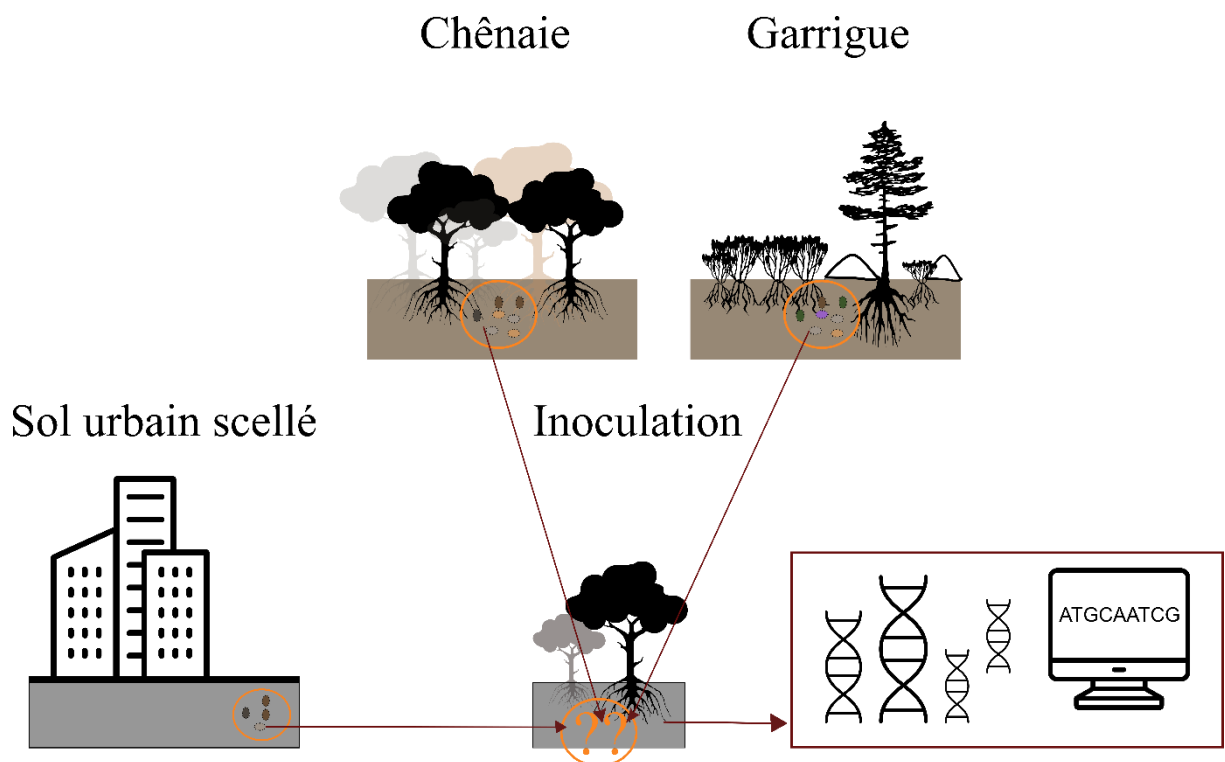
Table S3: Details of ASVs data according to sample type, paired-sampling, theoretical depths and assignation of the sequence to fungal group or EM guild.

All ASVs									
Sample type and theoretical depth	n	Total	Min	Max	Mean	Median	Sd	Kruskal Wallis X ²	p-value
Soil – 50.000 reads	15	775	2	113	51,66	50	29,07	0,12	0,07
Soil – 10.000 reads	82	4.026	1	115	40,1	47	26,45		
Paired samples – 50.000 reads	15	775	2	113	51,66	50	29,07		
Paired-samples – 10.000 reads	15	1.115	37	115	74,33	76	27,64		
EM samples – 25.000 reads	38	1.230	3	55	22,97	21,5	13,66	51,67	9,6e-06
EM samples – 2.600 reads	165	873	1	36	7,45	7	4,77		
Paired-EM samples – 25.000 reads	33	721	3	55	21,85	21	13,3		
Paired-EM samples – 2.600 reads	33	280	1	36	8,49	8	6,93		
Rhizosphere – 25.000 reads	25	156	1	27	6,24	2	7,82	0,1	0,34
Rhizosphere – 2.600 reads	30	148	1	31	4,93	3	5,97		
Paired-rhizosphere – 25.000 reads	14	96	1	26	6,86	3,5	7,32		
Paired-rhizosphere – 2.600 reads	14	79	1	31	5,64	2,5	8,04		
All fungal ASVs									
	n	Total	Min	Max	Mean	Median	Sd	Kruskal Wallis X ²	p-value
Soil – 50.000 reads	15	497	2	94	33,13	32	26,21	0,01	0,1
Soil – 10.000 reads	82	2.637	1	88	32,15	33	21,05		
Paired samples – 50.000 reads	15	497	2	94	33,13	32	26,21		
Paired-samples – 10.000 reads	15	742	5	88	49,46	53	27,13		
EM samples – 25.000 reads	38	401	0	35	10,55	7	9,27	18,33	0,002
EM samples – 2.600 reads	165	712	0	25	4,36	3	3,92		
Paired-EM samples – 25.000 reads	33	328	0	35	9,94	7	8,81		
Paired-EM samples – 2.600 reads	33	141	0	25	4,27	3	4,82		
Rhizosphere – 25.000 reads	25	102	0	19	4,08	2	5,17	0,24	0,25
Rhizosphere – 2.600 reads	30	95	0	14	3,17	1,5	3,48		
Paired-rhizosphere – 25.000 reads	14	65	0	19	4,64	3	5,08		
Paired-rhizosphere – 2.600 reads	14	45	0	14	3,21	1	4,13		
Ectomycorrhizal ASVs									
	n	Total	Min	Max	Mean	Median	Sd	Kruskal Wallis X ²	p-value
Soil – 50.000 reads	15	74	0	12	4,93	6	4,35	0,16	0,28
Soil – 10.000 reads	82	380	0	18	4,63	3	4,89		
Paired samples – 50.000 reads	15	74	0	12	4,93	6	4,35		
Paired-samples – 10.000 reads	15	118	0	18	7,87	7	6,6		
EM samples – 25.000 reads	38	86	0	11	2,26	1	2,64	7,34	0,12
EM samples – 2.600 reads	165	197	0	11	1,2	0	1,84		
Paired-EM samples – 25.000 reads	33	71	0	11	2,15	1	2,73		
Paired-EM samples – 2.600 reads	33	47	0	11	1,42	0	2,45		
Rhizosphere – 25.000 reads	25	30	0	8	1,2	0	2,45	0,06	0,98
Rhizosphere – 2.600 reads	30	25	0	6	0,83	0	1,7		
Paired-rhizosphere – 25.000 reads	14	15	0	7	1,07	0	2		
Paired-rhizosphere – 2.600 reads	14	14	0	6	1	0	2,33		

Table S4: Occurrence of ASVs in soil, root tips and rhizospheric samples

Number of retrieved ASVs	Occurrence of ASVs in soil samples	Occurrence of ASVs in EM samples	Occurrence of ASVs in rhizospheric samples
1	182	39	14
2	23	7	4
3	14	8	1
4	7	4	0
5	0	4	0
6	1	2	5
7	3	0	0
8	2	4	0
9	2	3	0
10	0	0	0
11	3	0	0
12	1	2	0
13	0	2	0
14	1	0	0
15	1	2	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	1	0
20	0	0	0
21	1	0	0

Chapitre 3 : Résilience des communautés ectomycorhiziennes des sols urbains scellés : approche expérimentale par l'inoculation



Présentation du chapitre en français

Au cours du chapitre précédent, nous avons vu que le métabarcoding fournissait une image de la communauté ectomycorhizienne influencée par l'effort d'échantillonnage et par la calibration de la profondeur de séquençage. Cette méthode est très efficace pour établir un inventaire de la diversité ectomycorhizienne de plusieurs sols – et pour autoriser la comparaison entre échantillons et entre écosystèmes. L'utilisation de plantes-pièges est une méthode efficace pour mobiliser l'inoculum ectomycorhizien du sol. Le séquençage par métabarcoding des apex ectomycorhizés permet généralement d'identifier le champignon interagissant avec la plante hôte.

Dans le chapitre à venir, nous analyserons le compartiment ectomycorhizien des sols urbains scellés. La restauration de ces sols représente un enjeu majeur pour favoriser le recyclage de cette ressource – notamment en vue d'utiliser ces substrats dans des projets de végétalisation. Par conséquent, la caractérisation du compartiment ectomycorhizien et sa capacité à tisser des interactions avec les plantes est primordiale pour développer des solutions de remobilisation de ces sols. Ainsi, au cours d'un travail expérimental mené en conditions contrôlées sur le site expérimental du Centre d'Ecologie Fonctionnelle et Evolutive à Montpellier (Planche 2), nous avons utilisé une approche de métabarcoding du sol et des apex ectomycorhizés afin d'étudier la résilience de ces communautés fongiques après 60 ans de scellement. Nous posons les questions suivantes :

- (1) Après 60 ans de scellement du sol, quelle fraction de la diversité ectomycorhizienne est encore détectable par métabarcoding dans les sols ? Quelles sont les particularités écologiques des taxons peuplant ces communautés persistantes ?
- (2) Quelle est la capacité de cet inoculum résiduel à interagir avec certains taxons de plantes typiquement utilisés pour l'espace vert ?
- (3) Quelle portion de la diversité ectomycorhizienne hébergée dans les écosystèmes adjacents des villes Méditerranéennes peut être mobilisée pour le développement de technique de restauration des sols basés sur le compartiment biotique ?

Nous avons constaté que : (i) l'imperméabilisation prolongée des sols a considérablement réduit la diversité des champignons ectomycorhiziens dans les sols urbains, (ii) les spores des champignons gastéroides à fructification hypogées ont persisté malgré le scellement et ont permis l'établissement d'interaction avec les plantules méditerranéennes et (iii) les techniques

d'inoculation par l'ajout d'une fraction de sol s'est révélée efficace, mais la transférabilité de l'inoculum dépend de l'habitat source d'inoculum. Les milieux perturbés tels que les garrigues ont fourni un inoculum préférentiellement récupéré par les plantules établies dans l'expérimentation.

Planche 2 : a) expérimentation en conditions contrôlées sur le Terrain Expérimental du CEFE, Montpellier, France, b) plantules de chêne vert, arbousier et pin d'Alep dans les rhizotrons, c) mesure des taux de colonisation des apex mycorhizés et excision des mycorhizes destinées au séquençage, d) site de prélèvement du sol scellé à Marseille, quartier des Fabriques, France



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After-sealing life in urban soils: Experimental evidence of resilience and efficiency of ectomycorrhizal inoculation

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Abstract

The restoration of urban sealed soils is a major stake for urban planners. In particular, characterizing their resilience from a biotic perspective is of primary importance for remobilizing these artificialized substrates in urban green spaces.

Using Marseille (France) as a case study, we implemented a metabarcoding next-generation sequencing approach to characterize the diversity and the composition of ectomycorrhizal (EM) fungal communities present in soils sealed for 60 years. Based on an inoculation experiment, we tested the ability of the remaining spore bank to establish EM interactions with plants. Altogether, we aimed at assessing the potential of peri-urban natural habitats to provide an efficient soil EM inoculum for the restoration of urban soils.

We found that: (i) long-term sealing drastically reduced the diversity of EM fungi in urban soils, (ii) long-leaved spores of hypogeous gasteroid fungi were efficient to establish EM symbioses with plants, and (iii) the efficiency of EM fungal inoculation depended on the natural habitat used as an inoculum source. Oak forests provided species-rich but poorly efficient inoculum. Contrastingly, soils collected in disturbed shrublands (garrigues) hosted moderately-rich EM communities containing drought-adapted species with high ability to colonize urban soils.

Our findings highlight that peri-urban landscapes are promising candidates to feed local systems of urban soil restoration. In this perspective, poorly-considered habitats such as shrublands can be considered as valuable sources of soil biota to prevent the over-exploitation of agricultural areas.

Highlights

- 1 – Urban soils host mobilizable ectomycorrhizal fungal inoculum 60 years after sealing.
- 2 – Hypogeous gastroid fungi are highly adapted to urban soil conditions.
- 3 – Inoculation-based soil rewilding promotes ectomycorrhizal diversity in urban soils
- 4 – Shrublands concentrate a high diversity of stress-adapted ectomycorrhizal fungi

Introduction

Soils are an essential component of the urban landscape and a key resource for anthropic land use. In cities, anthropic occupation mostly results in the “sealing” of soil, which consists in isolating the aboveground part of urban ecosystems from the below ground compartment (Authier et al., 2022). As a consequence of urbanization, sealed soils currently cover 2,4% of the European area (Rodriguez-Espinosa et al., 2021). Sealed soils are covered by impervious anthropic constructions such as roads, buildings, layers of concrete and asphalt (Scalenghe & Ajmone-Marsan, 2009). The deleterious effects of soil sealing include the negative influence of compaction (Layman et al., 2016), nutrient depletion (O’riordan et al., 2021) and the pauperization of biotic communities (Geisen et al., 2019; Pereira et al., 2021). In particular, this process affects the composition and dynamics of obligate root symbionts, including a high diversity of mycorrhizal fungi (Smith and Read, 2010).

In European cities, the conversion of abandoned industrial wastelands into renovated sites for new uses (*e.g.* residential, recreational, commercial) is propelled by the current densification of urban areas (Fan et al., 2020). From a landscape managing perspective, this process consists in initiatives removing impervious surfaces and making available considerable volumes of unsealed soils (Fabbri et al., 2021; Maienza et al., 2021). Yet, restoration initiatives on unsealed soils have been poorly documented so far (Maienza et al., 2021). Scarce evidence reveals the recovery of lost ecological functions of rehabilitated soils, such as nutrient and water cycling, microclimatic regulation and biodiversity support (Tobias et al., 2018).

The restoration processes of degraded soils mostly consist in a physical treatment of soil compaction (Layman et al., 2016) and a chemical re-balancing through soil nutrient enrichment (*i.e.* organic amendment; Layman et al., 2016). The restoration of soil-living fungal

communities has been poorly explored. Yet, these communities are driving plant development and survival in urban green spaces (Layman et al., 2016; Mills et al., 2020), adaptation to heavy metal pollution (Fomina et al., 2005; Jourand et al., 2014), drought tolerance (de Jalón et al., 2020; Swaty et al., 2004), and may contribute to positive feedbacks loops (Andriuzzi & Wall, 2018). As a result, developing efficient methodologies to favor biotic diversity in unsealed urban soils may contribute to restore below-ground positive feedbacks on urban plant communities.

Little is known about the composition of the fungal communities in urban soils (Authier et al., 2022; Newbound et al., 2010). The assembled corpus knowledge mostly refers to arbuscular mycorrhizal lifeforms (AM; Chaudhary et al., 2019) and to a lesser extent the ectomycorrhizal lifeform (EM; Jumpponen et al., 2010). At regional scale, the composition of urban EM communities is primarily determined by soil characteristics of cities, and secondarily by the biogeographical context (Van Geel et al., 2018). Locally, the diversity of EM communities shows contrasted patterns between pauperized assemblies hosted by streetscape alignments (Bainard et al., 2011; Martinová et al., 2016) and diversified communities in old parks (Hui et al., 2017). In urbanized conditions, these pauperized EM communities are dominated by hypogeous life-forms of fungi (Cui & Mu, 2016; Karpati et al., 2011; Martinová et al., 2016). Contrastingly species producing above-ground fruit bodies appear more sensitive to highly specific physico-chemical properties of urban soils, and preferentially interact in more forest-like functioning ecosystems such as old urban parks (Hui et al., 2017; Marczylo et al., 2021; Martinová et al., 2016).

Until the recent emergence of efficient metabarcoding tools, the qualification and the assessment of the resilience of the residual fungal inoculum present in sealed soils in a long-term perspective was unreachable. As a result, very little is known about the temporal dynamics of the EM spore bank in most fungal lineages. First evidences suggest that dormancy and germination ability of sexual spores highly vary in EM symbiosis (Nara, 2009), with many lineages of epigeous fungi showing a limited germination ability (Ishida et al., 2008). Contrastingly, the germination capacity of spores produced in truffle-like life-forms (*i.e.* gastroid fruit bodies) may be maintained over time (Bruns et al., 2009; Taschen et al., 2022). In urban conditions, the residence time of EM fungal spores sequestered under sealed soils is largely underexplored. No study addressed the ability of EM spores to germinate and colonize

plant root systems on the long-term (but see Bruns et al. 2009). From this perspective, urban soils with known sealing date make an ideal candidate to investigate the resilience of EM spore bank, and soil unsealing initiatives offer valuable opportunities to document long-term persistence of EM fungal diversity in soils.

Only few studies have considered peri-urban ecosystems as a potential source of EM inoculum for urban soils (Bainard et al., 2011; Karpati et al., 2011; Marczylo et al., 2021; Martinová et al., 2016). Highly disturbed Mediterranean landscapes offer an opportunity to investigate this question, in particular in the north-western part of the basin. In this region, mosaics of evergreen oak forests and shrublands are located adjacent to cities (Sirami et al., 2010; Vogt-Schilb et al. 2019). In this region, Mediterranean oak forests concentrate a tremendous diversity of EM fungi (Richard et al., 2005). Contrastingly, early-successional stages, including shrublands, remain poorly described, despite their pivotal role during ecological succession, as ecological hinges between open and forest habitats (Gómez et al., 2004; Richard et al., 2009). To our knowledge, the ability of urban soils to filter the pools of immigrant EM propagules dispersed from peri urban natural landscapes through abiotic filtering (Koide et al., 2011) has poorly been explored so far.

Historically, EM fungal enrichment consisted of planting tree seedlings inoculated in nursery by a single fungal species (Rincon, Alvarez and Pera, 2001; Selosse et al. 2001). The efficiency of this inoculation was mainly reported in post-mining contexts, as an adapted method to enhance seedling survival (Zong et al., 2015) and as a potential fungal-based promising decontamination process (Dagher et al., 2020). Furthermore, EM inoculation has been consistently used to promote seedling growth (Kipfer et al., 2012) and nutrition (Bâ et al., 2002). Multi-species inoculation experiments remain scarce, and suggest that different combinations of EM species differentially affect plant performance (Kipfer et al., 2012). Aside from inoculated seedlings, various practices coexist to improve EM fungal inoculum in soils. Most of them consist in the addition of EM spores and mycelia from various sources (*e.g.* litter, humus, soil, root seedlings, excised EM root tips or fruit bodies; Repac, 2011; Taschen et al. 2022). The ability of these practices to restore urban soil by enriching their EM fungal communities has not been investigated so far.

Here, we successively explore the ability of unsealed to soil to harbor long-term resilient EM fungal communities and assimilate EM diversity inherited from natural inoculum pools as part of soil-based inoculation practices. We used the case study of Marseille city (France), to (i) characterize the diversity of EM communities present in a 60-year long sealing context, based on sensitive next-generation sequencing methods and (ii) test the ability of seedlings to mobilized the EM fungal species as root symbionts in urban soils. Using a bait plant experiment, we explored the complementarity of soils from the two main components of Mediterranean landscapes (*Quercus ilex* forest vs. garrigue) to provide suitable source of EM inoculum. From an applied perspective, we tested a large inoculation gradient of soil dilution to evaluate the practicability of using natural soils as EM inoculum sources for urban green spaces.

Material and methods

Sampling

In March 2020, we collected natural (forest and garrigue) and urban sealed soils, in the urban area and the landscapes surrounding the city of Marseille, France.

Forest soils were collected in the Vallon des Peyrards (43°22'16.31" N, 5°22'27.09" E), in a secondary *Quercus ilex* L. forest. The soil at this site is composed by a thick organic litter surrounding a 60 cm deep organo-mineral horizon of sandy texture (Table S1). Garrigue soils were collected at Chemin de la Mûre (43°22'1.4" N, 5°23'16.51" E) on a calcareous plateau surrounding Marseille. The soil at this site is composed by a superficial organic layer surrounding a silky-loam layer, with high volumetric rock content (Table S1). Urban soils were collected at Quartier des Fabriques (3°19' 35.86" N, 5°21'59" E). Soil surface was continuously sealed since the early 1960th (*EuroMediterranée* report and *Geoportail*, 1957 aerial photograph, reference C3245-0461_1957_CDP1202_1247) by a layer of concrete. The soil is made of a several-meter deep silky layer (60.4 % of the mass) with 3.22 g/kg of organic matter and a C/N ratio of 9.4 (Table S1).

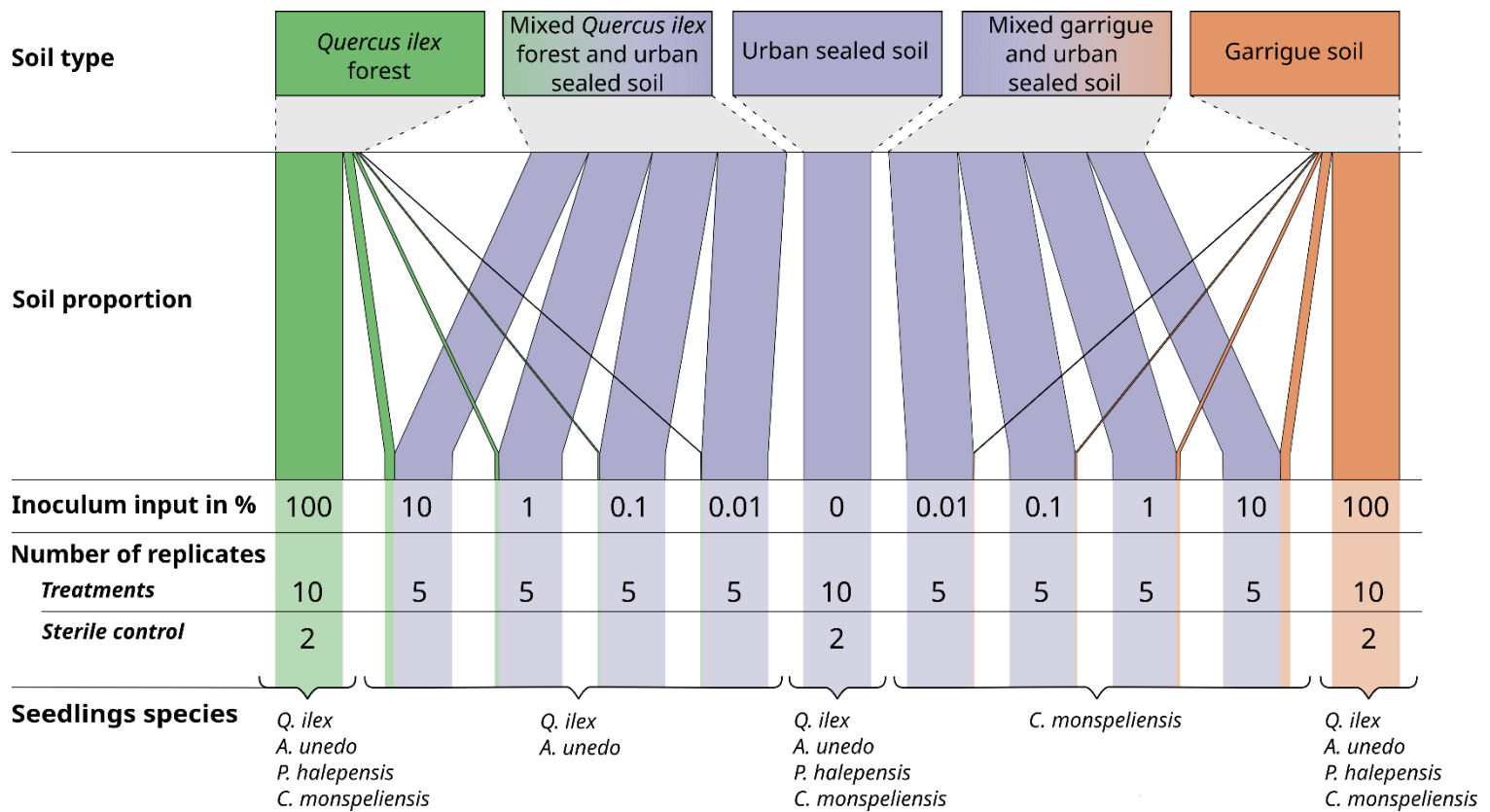
Experimental set up

The experiment was carried out at CEFE laboratory (43°38'19" N, 3°51'43" E), Montpellier, from March to October 2020. Soil containers, with standardized dimensions of 50 × 7 cm by 45 cm in depth, were built as in Taschen et al. (2020), and filled with 25 liters of soil. A total of 76 containers were assembled as follows (Fig. 1).

36 containers were filled with native soils (12 urban *vs* 12 forest *vs* 12 shrubland soils; Fig. 1). For each soil origin, two containers were vapor-sterilized for 24 hours at 100°C, and used as controls of the contamination through atmospheric-spore dispersal at CEFE laboratory. The 40 remaining containers were filled with urban soils diluted with aliquots of forest or garrigue soils, used as source of fungal inoculum (Fig. 1). For each source of inoculum, 20 containers were filled with urban soils diluted with 0.01 (five containers), 0.1 (five containers), 1 (five containers) or 10 % (five containers); Fig. 1).

Four species of Mediterranean plants (*Quercus ilex*, *Arbutus unedo*, *Cistus monspeliensis* and *Pinus halepensis*) were used as bait plants and sowed in containers (Fig. 1). These species were selected because they dominate spontaneous vegetation across Mediterranean landscapes and concentrate landscapers' interest for urban greening projects in the region.

Figure 1: Experimental design



Soil sampling

In each container, 1g-aliquots of soils were sampled in March (T_0), May (T_1) and September 2020 (T_2), conditioned and stored at -18°C . We sampled 120 samples of soils, with respectively 20 aliquots of each soils (*Quercus ilex* forest, garrigue and urban sealed soils); and 60 aliquots of inoculated soils (Table 1).

Plant sampling

Plants were integrally harvested in October 2020. The mycorrhization rate of each seedling was assessed as the proportion of colonized root apices. Mycorrhizal rates were assessed on 93, 75, 40 and 138 individuals of *Arbutus unedo*, *Quercus ilex*, *Pinus halepensis* and *Cistus monspeliensis*, respectively (Table 1). For each individual, a subsample of EM root tips, representing the visual diversity of fungi, was collected and stored at -18°C . In all, 72, 61,

33 and 97 pooled-ECM samples of individuals of *Arbutus unedo*, *Quercus ilex*, *Pinus halepensis* and *Cistus monspeliensis* were prepared for metabarcoding (Table 1).

Molecular analysis of EM communities

For each container, the analysis of EM fungal community was performed by separately metabarcoding soil aliquots and root apices of each plant individual. This analysis was performed to assess the ability of plant species to bait EM inoculum (metabarcoding of EM root apices) from a pool of EM fungi (metabarcoding of soil).

Fungal DNA was extracted using FastDNA™ Spin Kit for Soil (MP Biomedicals, Irvine CA, USA) for soil samples using DNeasy Plant Pro Kit (QIAGEN, Hilden, Germany) for root apices. The fungal ITS 2 was amplified using specific Illumina primers and the Phusion Flash High-Fidelity DNA polymerase (Thermo Scientific). The sequencing of 390 amplicon samples was performed at the Sequencing and Genotyping Platform of the Cemeb Labex (GenSeq, UMR ISEM, Montpellier).

Bioinformatic analysis of EM communities

The analysis was performed on 153 successful DNA amplicons of seedlings and 72 successful DNA amplicons of soils. Raw sequences were processed with DADA2 pipeline v1.16 (Callahan et al., 2016; R Core Team 2022) and completed by the LULU algorithm (Frøslev et al., 2017). The amplicon sequence variant (ASV) clustering method was used as in Pauvert et al. (2019). In short, we clustered 3 022 904 paired-end sequences into 6 133 ASVs, including 530 EM ASVs from 676 187 reads. ASVs were assigned to fungal guilds (ectomycorrhizal vs saprobic vs parasitic) and fungal life-forms (agaricoid vs boletoid vs clavarioid/coralloid vs corticioid vs hydroid vs gastroid vs pezizoid), using FUNGuild database (Nguyen et al., 2016).

Data analyses

Community analyses were performed using Vegan (Oksanen et al., 2017) and Phyloseq (Mcmurdie & Holmes, 2013) packages in R Studio Software (R Core Team, Version 3.6.2).

The alpha diversity of EM communities from different soil origins was compared using Hill numbers as in Taudière et. al (2015). Differences were tested by Kruskal-Wallis test as the prerequisites of normality, variance homogeneity and residual normality were not respected. Post-hoc Dunn tests were performed to determine the composition of significantly different groups of modalities. In order to compare species richness among samples of different size, a rarefaction analysis was performed on ASVs abundances using Vegan package (Oksanen et al., 2017). This analysis aims to estimate the expected amount of ASVs in a given depth of sequencing and help comparing diverging ecological situations. Finally, a beta-diversity analysis of EM communities among soil origins and among plant species was performed using *adonis2* in Vegan package (Oksanen et al., 2017).

Results

DNA metabarcoding revealed the presence of mobilizable EM fungal DNA in urban sealed soils

The metabarcoding of soil aliquots revealed the presence of EM fungal DNA in 20 (100%), 17 (85%) and 3 (15%) forest, garrigue and urban soils, respectively (Table 1). In inoculated soils, the analysis revealed fungal DNA in 20 (100%) and 7 (35%) samples inoculated with either forest or garrigue soils (Table 1).

This available EM fungal inoculum was established as ectomycorrhizas on roots of 118 out of 263 seedlings (44.87 %; Table S1), including 32 (62.74 %), 31 (73.81 %) and 18 (23.38 %) plant individuals grown in soils from forest, garrigue and sealed area, respectively (Table 1 and Table S2 for details).

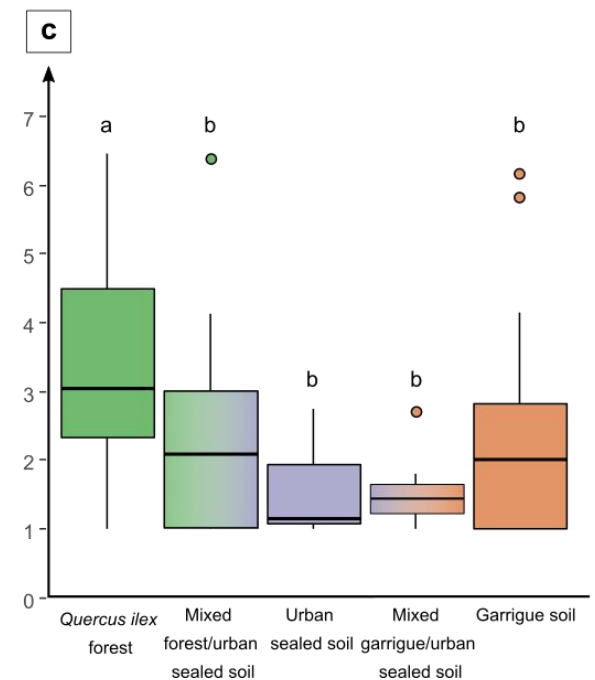
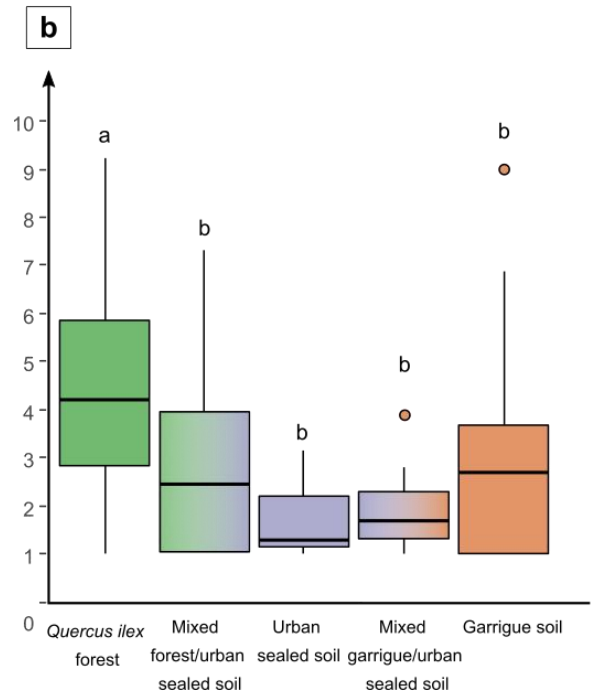
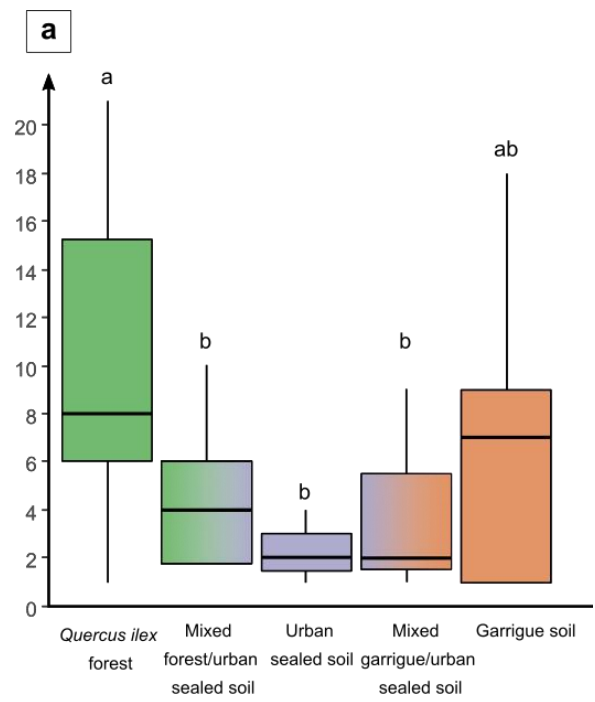
Urban sealed soils harbor low EM fungal diversity

We detected 118, 63 and 6 EM fungal ASVs in forest, garrigue and urban soils, respectively (Table 2). On average, 2.33 ASVs ($sd = 1.53$) were retrieved per urban soil sample (Fig. 2a), while forest and garrigue soil aliquots harbored a mean of 10.2 ($sd = 6.12$) and 6.4 ASVs ($sd = 5.36$) (Fig. 2a). Shannon entropy (H^1) and Shannon entropy (H^2) differed among the three soil origins (Fig. 2b and 2c; $P = 0.03$ and 0.02 by Kruskal-Wallis test for H^1 and H^2). Urban and garrigue soils showed significantly lower values of Shannon entropy ($P = 0.01$ for both soil origins, by Dunn post hoc test) and Inverse Simpson ($P = 0.02$ and 0.01 for urban and garrigue soils, by Dunn post hoc test) than forest soils. Pairwise comparisons showed no significant differences between garrigue and urban soils ($P = 0.10, 0.17$ and 0.23 for H^0, H^1 and H^2). The difference in H^0 values among soils was statistically significant ($P = 0.03$ by Kruskal-Wallis test; Fig. 2a), with lower richness in urban than in forest soils ($P = 0.01$ by Dunn post hoc test), and in garrigue than in forest soils ($P = 0.03$ by Dunn post hoc test).

Table 1: Sampling design for metabarcoding of soils and ectomycorrhizal root apices, with detail of obtained dataset.

Sample type	Number of samples			Number of reads		
	Used for estimation of colonization rate	Metabarcoded	With positive EM amplicons	Total	Mean	Sd
Native soils						
<i>Quercus ilex</i> forest soil		20	20	79962	3998.1	6206.46
Garrigue soil		20	17	42348	2491.06	3460.52
Urban sealed-soil		20	3	9467	3155.67	2989.16
Inoculated soils						
Mixed <i>Q. ilex</i> forest /urban soil		40	20	42336	1925.73	2569.33
Mixed garrigue/urban soil		20	7	12236	1223.6	2696.35
Total		120	47	186379	2781.77	4225.77
ECM apices						
<i>Arbutus unedo</i>	93	72	20	15119	755.95	1061.51
<i>Quercus ilex</i>	75	61	32	40639	1269.97	1721.03
<i>Pinus halepensis</i>	40	33	23	41126	1788.09	2994.2
<i>Cistus monspeliensis</i>	138	97	43	21803	454.23	1168.31
Total	346	263	118	118687	1005.82	1852.97

Figure 2: Alpha diversity of EM fungal communities in soils of *Quercus ilex* forests (green), Mediterranean shrublands (orange), 60-yrs long urban sealed soils (purple) and in inoculated urban soils (mixed colors). Alpha diversity was estimated using Hill numbers H^0 (a), H^1 (b) and H^2 (c).



After normalization at fixed number of reads, EM diversity was higher in forest soils (62.35 ASVs for a standardized number of 941 reads) than in garrigue soils (35.9 ASVs) and in urban sealed soils (6 ASVs; Fig. 3a). Contrastingly, normalized EM diversity on EM root apices grown in garrigue soils was higher (38.67 ASVs for a standardized number of 1581 reads) than in forest (38.67 ASVs) and urban sealed soils (14 ASVs; (Fig. 3b).

Figure 3: Rarefaction estimation of EM fungal species richness in *Quercus ilex* forests, Mediterranean shrublands (garrigues) and 60-yr urban sealed soils (a) and on roots of 8-months seedlings grown in these three types of soils (b).

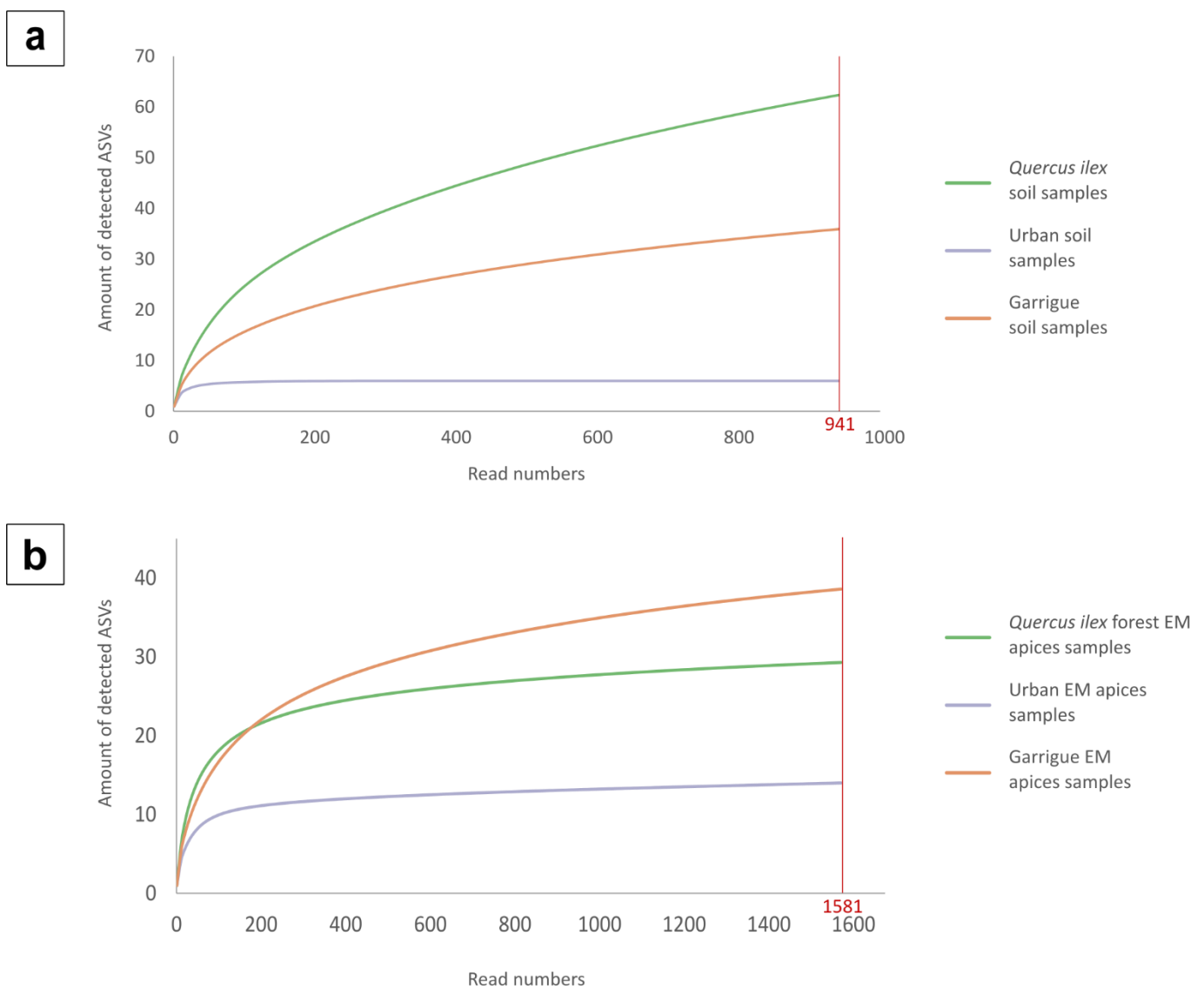
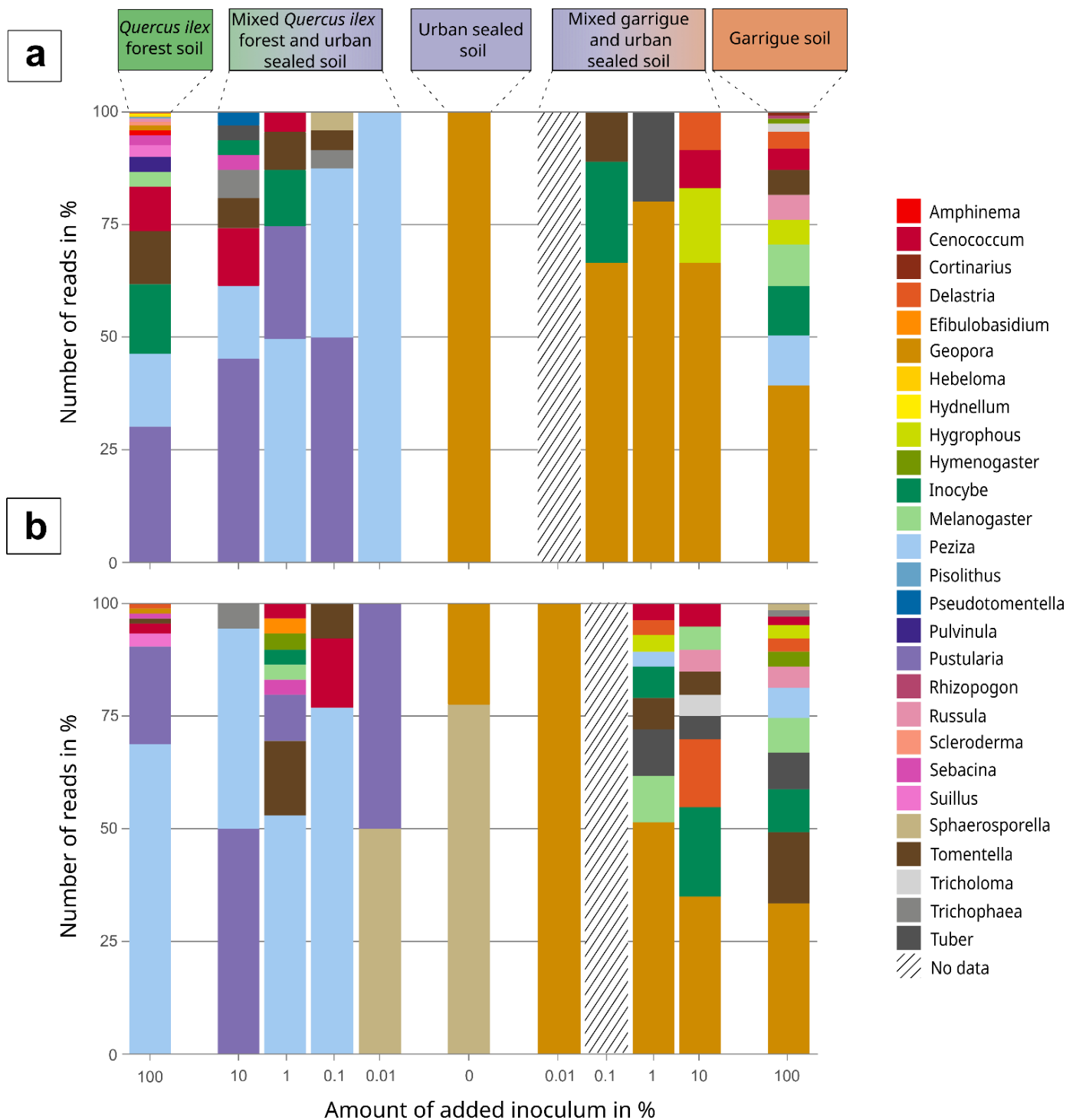
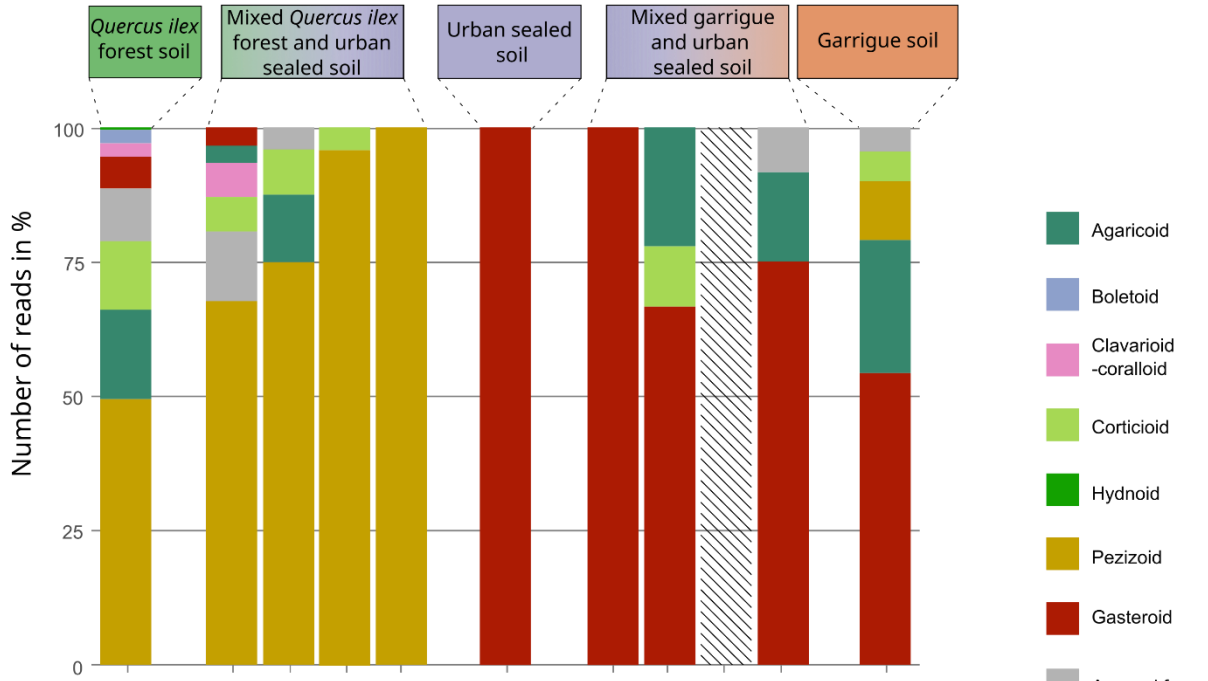


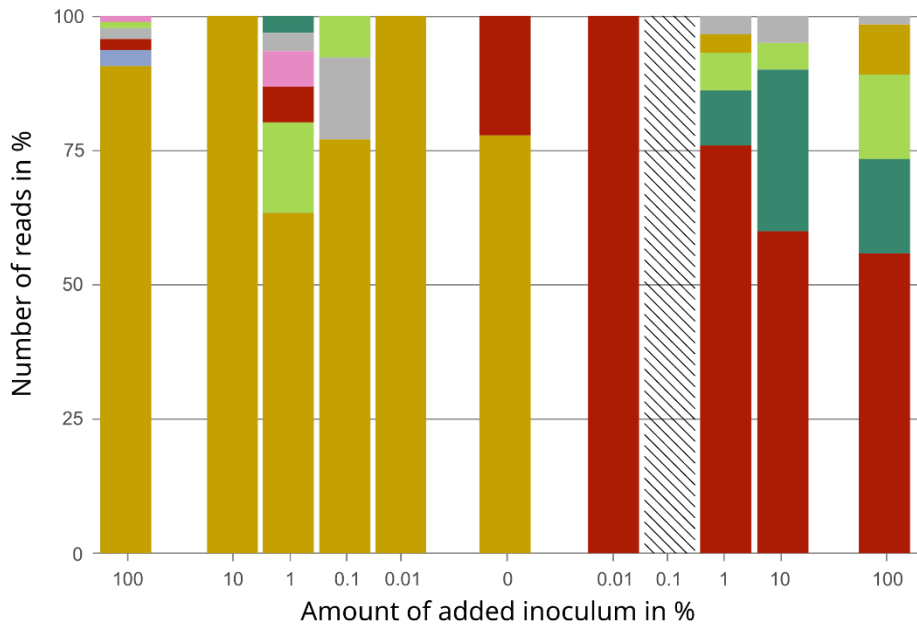
Figure 4: Composition of ectomycorrhizal fungal communities in soils (a) and on roots of 8-months old seedlings (b) grown in three types of native soils (*Quercus ilex* forest vs Mediterranean shrublands (garrigues) vs 60-yrs long urban sealed soils) and in four modalities of inoculation (0.01, 0.1, 1 and 10% of final soil volume) of urban soils with each of the two natural soils (i.e. *Quercus ilex* forest vs Mediterranean shrublands). Distribution of fungal life-forms in soils (c) and on roots of 8-months old seedlings (d) in the same conditions of growth. Colors indicate fungal genera (panels a and b) or fungal life-forms (c and d). Values are the number of reads obtained from Illumina MiSeq metabarcoding of soils or root tissues.



c



d

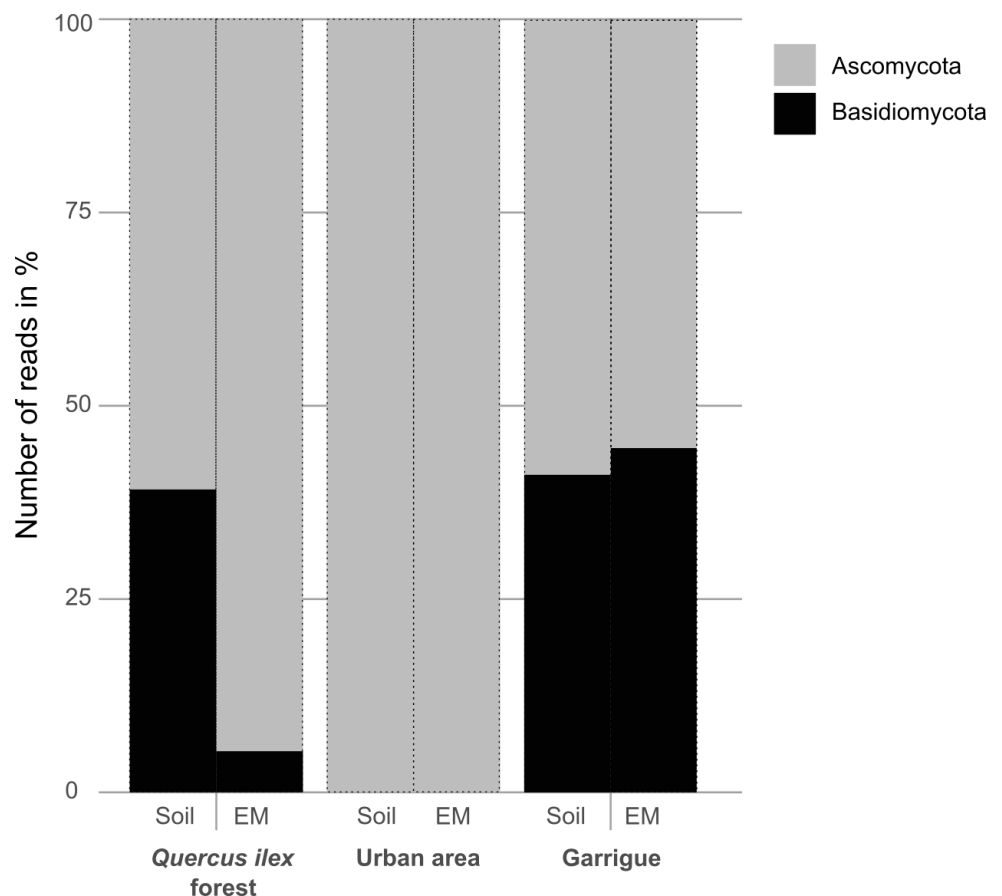


EM communities in soils of forests, garrigues and urban area show contrasted composition

The composition of EM fungal communities markedly differed among the three soil origins ($P = 0.001$ by Adonis permutational test; Table S3a). EM fungal communities were significantly different between all pairs of soils ($P = 0.003$, 0.003 and 0.001 by Adonis pairwise post-hoc comparison test, for urban vs forest, urban vs garrigue and forest vs garrigue).

Soil metabarcoding evidenced the presence of 17, 13 and 1 EM fungal genera in *Quercus ilex* forest, garrigue and urban area, respectively (Fig. 4 a). The proportion of Ascomycetes reached 39.49, 58.42 and 100% in the same three habitats (Fig 5). From this pool, only 8, 13 and 2 EM fungal genera were bait by seedlings grown in *Quercus ilex* forest, garrigue and urban area, respectively (Fig. 4 a). On roots, Ascomycetes accounted for 94.52, 44.66 and 100% of ectomycorrhizas in the same three habitats (Fig 5).

Figure 5: Proportion of ascomycete (black) and basidiomycetes (white) ectomycorrhizal fungal ASVs, in *Quercus ilex* forest vs Mediterranean shrublands (garrigues) vs 60-yrns long urban sealed soils and on roots of 8-months old seedlings of plant species (*Arbutus unedo* vs *Cistus monspeliensis* vs *Pinus halepensis* vs *Quercus ilex*) grown in the three types of soils.



Forest, garrigue and sealed soils harbor contrasted EM fungal life-forms

In forest soils, most ASVs 94.08 % produced epigeous fruit bodies (Fig. 4c), and pezizoid lifeforms (e.g. *Pustularia* and *Peziza*; Fig. 4a) accounted for half of the fungal ASVs abundance (49.43 %, Fig. 4c). A similar pattern was observed on EM root apices, with *Peziza* and *Pustularia* taxa accounting for 68.88 and 21.69 % of the EM root tips (Fig. 4b). Contrastingly, in garrigue, hypogeous gastroid fungi represented a majority of ASVs detected in soil (54.28 % of the reads; e.g. *Geopora* and *Melanogaster*; Fig. 4a & 4c) and on root apices (55.81 % of the reads; e.g. *Geopora*, *Tuber* and *Melanogaster*, 55.81 % of the reads, Fig. 4b & 4d). Similarly, gastroid fungi (*Geopora*; Fig. 4a & 4c) was the only life-form detected in urban soils. On seedling roots epigeous pezizoid-like (i.e. *Sphaerosporella*; 77.82 % of the reads, Fig. 4b & 4d) and hypogeous gastroid-like (i.e. *Geopora*; 22.18% of the reads, Fig. 4b & 4d) were the only lifeforms established as ectomycorrhizas.

Soil mixing, an efficient practice to inoculate plant seedlings

In inoculated urban soils, amplified EM fungal DNA was detected in respectively 20 (50% of the samples, 42 ASVs) and 7 (35 % of the samples, 16 ASVs) samples of mixed urban/*Quercus ilex* forest soil and mixed urban/garrigue soils (Table 1). From this pool, EM fungal inoculum established as ectomycorrhizas on roots of 36.99% (27 out 73 seedlings) of seedlings grown in mixed urban/*Quercus ilex* forest soil and 60 % (12 out 16 seedlings) in mixed urban/garrigue soils (Table S2).

Inoculated urban soils tended to exhibit (no significant difference by Dunn pairwise post-hoc test) higher specific diversity than original ones, with H^0 averaging 2.33, 4 and 3.71 ASVs per sample of urban, mixed *Quercus ilex*/urban and mixed garrigue/urban soils (Fig. 2).

Inoculation of urban soils with forest soil aliquots induced a significant compositional shift of EM community toward the composition of forest soils, in mixed substrates (F -value = 1.30, P = 0.02 by permutational Adonis, Table S3b; Fig. 4a) as on EM root apices (F -value = 1.85, P = 0.002, Table S3d; Fig. 4b). Contrastingly in mixed garrigue/urban soils, compositional shift was only detected on seedling roots (F -value = 1.35, P = 0.03, Table S3e; Fig. 4b) and not in soil (F -value = 0.99, P = 0.45, Table S3c; Fig. 4c).

Soil mixing, an efficient practice to inoculate seedlings with gastroid and pezizoid life-forms

In mixed forest/urban soils, epigeous pezizoid EM fungi was the dominant life-form whatever the dilution modality (e.g. *Peziza* and *Pustularia*; Fig. 4b & 4d). In a lesser extent, seedlings were colonized by hypogeous gastroid fungi (*Hymenogaster* and *Tuber*), while they were not detected in soils of origin (Fig. 4b).

Contrastingly, fungi producing hypogeous gastroid fruit bodies (i.e. *Geopora*, *Melanogaster*, *Tuber* and *Delastria*; Fig. 4b & 4d) dominated the EM community established on seedling roots in mixed garrigue/urban soils (Fig. 4d). ASVs in the lineages *Tuber*, *Trichophea* and *Pseudotomentella* were detected in mixed garrigue/urban soils, while they were not amplified from inoculant source (Fig. 4a).

Discussion

This study emphasizes the long-term resilience of fungal diversity in urban sealed soils, and paves promising avenues for restoration processes using the EM inoculum established in peri-urban natural habitats. We first revealed that 60-year sealing drastically reduced the diversity of EM fungi in urban soils, while maintaining active the propagules of very few gastroid species highly tolerant to severe stress. Second, we showed that evergreen oak forest fragments located within urban area concentrate species-rich communities of EM fungi with limited potential to colonize young plants grown in urban soils. We finally evidenced that degraded shrublands (*i.e.* garrigues) may constitute valuable reservoirs of fungal inoculum for urban planning, based on naturally established drought-adapted EM fungi.

We detected the presence of mobilizable EM inoculum in urban soils sealed in the early 60th. The combination of soil and root apices metabarcoding evidenced 1) a low diversity of EM species, and 2) a long-term persistence of the mobilizable EM inoculum in soil spore bank, which allowed 3) the successful establishment of species as ectomycorrhizas on roots of seedlings. Sealed soils contained a spatially fragmented pool of EM fungal inoculum. As only 15% of samples provided fungal amplicons (*vs.* 100 and 85% of forest and garrigue soils; Table 1), the spore bank in long-term sealed soils seems quantitatively and spatially limited. This result is consistent with previous research that consistently reported eroded diversity of EM communities in urban sealed soils (Martinová et al., 2016), streetscapes (Lothamer et al., 2014), private gardens and city parks (Cui & Mu, 2016), as compared to natural habitats. This poorly diversified pool of EM fungi in sealed soils may be a consequence of several processes (*e.g.* drought, pollution, chemical cycle interruption) occurring in urban ecosystems. In our study, sixty years of soils containment under impermeable materials may have induced the inactivation of most EM species present in the original soil. Furthermore, sealed soils directly suffer from the lack of ecological continuum between natural sources of inoculum around cities and the below-ground compartment of urban landscapes (Authier et al., 2022; Grilli et al., 2017). Thus sealing materials act as one of the main physical components of the urban abiotic filtering, which quantitatively and qualitatively affect the pool of immigrant EM propagules (Koide et al., 2011). Finally, soils containment under impermeable materials may have affected the survival of soil biota, by inducing drastic depletion of hydric and chemical reservoirs and deleterious long-term effects on physical soil properties (Pereira et al., 2021).

In our study, we provide evidence that spores of false-truffles (*i.e. Geopora*) retain the ability to germinate and establish as mycorrhizas after 60 years of dormancy. The ability of these species to colonize roots of seedlings was as high as those of EM communities from forest and garrigue soils (Table S4). These results confirm the highly adapted life-style of hypogeous gastroid fungi to urban environments (Martinová et al., 2016; Hui et al., 2017; Van Geel et al., 2018). Interestingly, gastroid fruit bodies, in which spores are physico-chemically protected against desiccation (Wilson et al., 2011) and biotic damages (Calhim et al., 2018) emerged as drought-adapted fungal lineages (Vogt-Schilb et al., 2022; Zambonelli et al., 2014). From an urban planning perspective, these fungal life-forms may be considered as promising candidate species for inoculation-based soil restoration initiatives in the cities of the Anthropocene.

In *Q. ilex* forest soils, the EM community was dominated by three genera of Ascomycetes (*i.e. = Peziza, Pustularia, Cenococcum*). This omnipresence of this lineage highly contrasts with previous descriptions of EM communities in Mediterranean forest. Thus, a strong dominance of Basidiomycetes has been consistently reported on *Q. ilex* seedlings growing in forests of various ages (Richard et al., 2005, de Jalon et al. 2020). Here, Basidiomycete lineages only accounted for around forty percent of DNA sequences obtained from EM root tips (Figure 5). This result is in accordance with previous studies which evidenced an underrepresentation of EM basidiomycetes lineages in urban soils (Martinová et al., 2016; Hui et al., 2017; Van Geel et al., 2018). Our research suggests that abiotic filtering in urban area may shape significant composition shift on EM communities at the expense of Basidiomycetes.

The EM fungal inoculum present in *Q. ilex* forest soils was poorly efficient to colonize the root systems of seedlings during the first year of growth in inoculated urban soils. Less than half of the soil EM diversity were successfully established on root seedlings at harvesting date. Establishment failure mostly concerned Basidiomycetes species. The mechanisms underlying this pattern remains unclear. First, nutrient-poor and highly disturbed urban soils may provide unsuitable establishment conditions for most forest Basidiomycetes. Interestingly, typical forest-associated genera of Basidiomycetes tend to dominate in mature urban ecosystems such as urban old parks (Hui et al., 2017; Marczylo et al., 2021; Martinová et al., 2016). Additionally, young seedlings might be unsuitable hosts for late-successional Basidiomycete species with high nutritional requirements (Smith & Read 2010). Considering the importance of below-ground interaction in the functioning of city greenspaces, more metabarcoding-based research

is needed to characterize EM fungal succession underground cities, and its consequences on ecosystem functioning. From a landscaping perspective, forest soils may be preserved and carefully mobilized in the urban area, to assist the establishment/survival of aged plant material, and not to design new greenspaces.

In garrigues, EM fungal communities were moderately rich and highly specific in composition (Fig. S1). In these highly anthropized habitats, a high diversity of gastroid life-forms, including species in the genera *Geopora*, *Tuber*, *Melanogaster*, *Hymenogaster* and *Delastria* accounted for half of the soil DNA sequences (Fig. 4a&c). This result suggests that xeric conditions of garrigue soils provide suitable habitats for drought-adapted lineages of EM species. To the best of our knowledge, this study is the first to report on the belowground diversity of EM communities in Mediterranean shrublands. So far, this habitat received little attention in conservation initiatives, and garrigues suffer from a lack of knowledge of its fungal diversity. As a consequence, our results may contribute to shed new light on this poorly known biota, established on roots of highly specific pioneer EM plant species belonging the Cistaceae (*i.e.* *Cistus*, *Halimium*, *Helianthemum* and *Fumana*) and Fagaceae (*Quercus coccifera*). From an operational perspective, further field surveys of EM communities in Mediterranean shrublands of various composition and succession stage may contribute to identify highly performant plant and fungal species for urban soil rewilding.

In our comparative experiment, garrigue soil was the most efficient source of EM inoculum for urban soils. After a year of growth, the EM fungal community established on roots of seedlings grown in mixed urban/garrigue soils was 32% richer than in mixed urban/forest soils. *Geopora* was the most frequently detected lineage, with more than 50% of DNA sequences in all dilution treatments. Other gastroid species isolated from garrigue soils, such as *Delastria*, *Tuber* and *Melanogaster* genera, successfully established on roots of seedlings grown in inoculated urban soils.

Across the range of tested dilutions, the addition of 1% of garrigue soil to an urban matrix allowed the establishment of the garrigue EM original community (Fig. 4b). This result opens a window on soil restoration in urban landscapes. Considering the moderated fraction of garrigue soil to successfully transfer most EM diversity in urban soils, such inoculation-based processes may contribute to reduce the current pressure of urban planning on agricultural

landscapes. Thus, Mediterranean shrublands currently cover considerably extended areas, and represent highly diversified reservoirs of drought-adapted plant and fungal diversity (Vallejo et al. 2012). Then, this habitat offers interesting perspective for both landscape management and urban planning, by providing multiple stress-adapted- plant species, as efficient alternatives of exotic and-or poorly adapted ones.

We focused our experiment on EM symbiosis, but habitats also host an underexplored diversity of arbuscular fungi, as many families of highly-adapted herbs (*e.g.* Poaceae, Fabaceae *pro parte*) and shrubs (*e.g.* Lamiaceae, Fagaceae *pro parte*) develop this specific interaction with soil fungi (Smith & Read, 2010). Under this perspective, timely research may include the evaluation of 1) short- and mid-term physio-ecological benefits of enriched mycorrhization on seedlings grown in urbanized areas, and 2) from a long-term perspective, the efficiency of inoculation-based soil restoration on the development and survival of high stem saplings planted in urban greenspaces.

More generally, to provide a comprehensive overview of the functioning of EM urban landscapes, further research should be designed. First, little is known about the fungal inoculum transfers between city centers and adjacent natural landscapes. Insight into the spatio-temporal dynamics of spore dispersal into the natural and anthropic corridors might help to understand the importance of urban filtering in the establishment of fungal diversity. Second, exploring the early-successional stages of shrublands may provide valuable information about the most adapted EM species for the restoration of urban soils in the context of global change. Third, plant-plant interaction remains poorly described in cities, based on a network perspective. Such a community level perspectives of EM plants grown in the urban area may help urban planners to design synergic plant associations, through hydric and mineral transfers among co-occurring individuals linked by of common mycelial networks. Finally, cities represent an extraordinary field-experiment area for testing the concepts and mobilizing the corpus of community ecology. Collaborative research initiatives may help landscaping actors and citizens to shape long-lived ecosystems in livable cities.

Conclusion

Our study provides promising perspective for designing greenspaces favoring efficient cooperative synergies between above- and belowground compartments of urban ecosystems. By mobilizing the most resilient root symbionts naturally present in surrounding landscapes, inoculation of urban sealed soils may contribute to lower anthropic interventionism and the related environmental and ecological costs. Thus, green spaces are soil-consuming and the current methods of soil conception exhibit high ecological and economic costs because of soil extraction, transportation and enrichment. The intense soil-consumption of city leads to the necessity of elaborating sustainable alternatives. Accordingly, the reuse of unsealed soils and their enrichment with highly-adapted ectomycorrhizal fungal communities is absolutely consistent with the increased need of soils in landscaping projects.

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Supplementary materials

Supplementary Tables

Table S1: Detail of physico-chemical parameters of the oils collected in the urban area of Marseille (France) and in two peri-urban habitats.

Table S2: Sampling details for metabarcoding of soils and ectomycorrhizal root apices, with detail of obtained dataset.

Table S3: Variance analysis of soil EM fungal communities according to soil origin **(a)**, and inoculation of urban sealed soils by either *Quercus ilex* soils **(b)** or garrigue soils **(c)**. Panels **(d)** and **(e)** show the EM fungal communities established on root apices of 8-months seedlings grown in either forest **(d)** or garrigue soil **(e)**

Table S4: Mycorrhization rate of 8-months old seedlings (i.e. *Arbutus unedo*, *Cistus monspeliensis*, *Pinus halepensis* and *Quercus ilex*) grown in soils collected in either *Quercus ilex* forest, Mediterranean shrublands (garrigues) or 60-yrs long urban sealed soils). N: number of seedlings

Supplementary Figures

Figure S1: Distribution of ectomycorrhizal fungal ASVs in soils collected in either *Quercus ilex* forest, Mediterranean shrublands (garrigues) or 60-yrs long urban sealed soils).

Extended material and methods

Table S1: Detail of physico-chemical parameters of the three soils origins

Soil type	<i>Quercus ilex</i> forest	Garrigue	Urban sealed soil
Sampling depth	10;42	7;30	NA
Sampling location	43°22'16.31",5°22'27.09"	43°22'1.4",5°23'16.51"	43°19'35.86",5°21'59"
CEC_(meq/kg)	198,24	256,6	7,1
Saturation %	100	100	100
Water pH	8,020	7,93	8,8
pH_KCl	7,560	7,37	8,2
Total limestone (g/kg)	609,440	94,44	NA
Active limestone (g/kg)	112,900	53,7	NA
Organic matter (g/kg)	53,320	45,06	3,22
Organic nitrogen N (g/Kg)	1,510	1,12	0,342
C/N (Corg/Norg)	20,500	23,47	9,4
Microbial activity intensity	8,000	10	NA
Conductivity (mS/cm)	0,14	0,18	NA
Phosphoric anhydride P205 Joret (g/kg)	0,11	0,01	NA
Potassium oxide K2O (g/kg)	0,13	0,71	0,055
Magnesium oxide MgO (g/kg)	0,23	0,26	0,33
K2O/MgO	0,57	2,73	0,2
Calcium oxide CaO (g/kg)	12,29	12,5	8,965
Iron (mg/kg)	282	101	2,9
Copper (mg/kg)	7	3,8	0,3
Zinc (mg/kg)	22,8	6,9	0,1
Coarse sand %	17	8	2,8
Fine sand %	29	16	25,7
Coarse silt %	28	21	43,1
Fine silt %	11	24	17,8
Clay %	15	31	10,6
Crusting index	0,7	1,5	3,3
RFU/LM2	13,96	18,14	NA
Soil granulometry	Sandy-loamy-clayey to sandy-loamy soil	Clayey soil	Sandy-loamy-clayey

Table S2: Sampling details for metabarcoding of soils and ectomycorrhizal root apices, with detail of obtained dataset.

Sample type	ECM apices measured for T ^{EM}	Send to NGS	Samples with ECM ASVs sequences assigned	Mean number of read per sample		
				Sum	Mean	Sd
Soil		120	47			
Holm oak forest soil		20	20	79962	3998.1	6206.46
Mediterranean shrubland soil		20	17	42348	2491.06	3460.52
Urban sealed-soil		20	3	9467	3155.67	2989.16
Mixed holm oak/urban soil		40	20			
- 0.01 %		10	1	16	16	NA
- 0.1 %		10	5	10743	2148.6	2414.6
- 1 %		10	6	17941	2563	3211.53
- 10 %		10	8	13666	1708.25	2466.95
Mixed shrubland/urban soils		20	7			
- 0.01 %		5	0	0	0	NA
- 0.1 %		5	1	8727	8727	NA
- 1 %		5	3	1532	383	325.59
- 10 %		5	3	1977	494.25	887.41
ECM apices	346	263	118			
<i>Arbutus unedo</i>	93	72	20	15119	755.95	1061.51
- In holm oak forest soil			2	105	52.5	37.48
- Mediterranean shrubland soil			5	415	83	60.27
- In urban sealed soil			3	3349	1116.33	1074.51
- Mixed urban sealed soil and holm oak forest soil						
- 0.01 %			0	0	0	NA
- 0.1 %			1	4192	4192	NA
- 1 %			4	3043	760.75	677.98
- 10 %			5	4015	803	749.02
<i>Quercus ilex</i>	75	61	32	40639	1269.97	1721.03
- In holm oak forest soil			6	4299	716.5	622.23
- Mediterranean shrubland soil			8	16901	2112.62	3153.57
- In urban sealed soil			6	7059	1176.5	811.52
Mixed urban sealed soil and holm oak forest soil						
- 0.01 %			2	3812	1906	702.86
- 0.1 %			3	2291	763.67	1268.16
- 1 %			5	3581	716.2	761.47

- 10 %			2	2696	1348	502.04
<i>Pinus halepensis</i>	40	33	23	41126	1788.09	2994.2
- In holm oak forest soil			13	19734	1518	530.02
- Mediterranean shrubland soil			7	17708	2529.71	5598.96
- In urban sealed soil			3	3684	1228	875.79
<i>Cistus monspeliensis</i>	138	97	43	21803	454.23	1168.31
- In holm oak forest soil			11	2637	239.73	238.92
- Mediterranean shrubland soil			9	2888	320.89	328.82
- In urban sealed soil			6	1714	285.67	503.36
- Mixed urban sealed soil and Mediterranean shrubland soil						
- 0.01 %			1	21	21	NA
- 0.1 %			0	NA	NA	NA
- 1 %			9	10706	1189.57	2538.07
- 10 %			7	3837	548.14	699.02

Table S3: Variance analysis of soil EM fungal communities according to soil origin (a), plant species (b) and inoculation of urban sealed soils by *Quercus ilex* soils (c) or garrigue soils (d). Panels (e) and (f) analysis the EM fungal communities retrieved in apices of 8-months seedlings grown in forest soil (e) and garrigue soil (f)

A. EM fungal communities' composition divergence analysis among three soils					
	<i>Df</i>	<i>Sum of squares</i>	<i>R</i> ²	<i>F-value</i>	<i>P-value</i>
<i>Soil type</i>	2	2	0.11	2.25	0.001
<i>Residual</i>	37	16.8	0.89		
<i>Total</i>	39	18.8	1		

B. EM fungal communities' composition divergence analysis among baiting plant species					
	<i>Df</i>	<i>Sum of squares</i>	<i>R</i> ²	<i>F-value</i>	<i>P-value</i>
<i>Plant species</i>	3	1.29	0.11	0.91	0.68
<i>Residual</i>	23	10.9	0.89		
<i>Total</i>	26	11.58	1		

Soil EM fungal communities' composition divergence analysis among an inoculation gradient ranging from urban soils to holm oak forest soil					
	<i>Df</i>	<i>Sum of squares</i>	<i>R</i> ²	<i>F-value</i>	<i>P-value</i>
<i>Dilution percentage</i>	4	2.21	0.23	1.30	0.02
<i>Residual</i>	18	7.61	0.77		
<i>Total</i>	22	9.82	1		

Soil EM fungal communities' composition divergence analysis among an inoculation gradient ranging from urban soil to garrigue soil					
	<i>Df</i>	<i>Sum of squares</i>	<i>R</i> ²	<i>F-value</i>	<i>P-value</i>
<i>Dilution percentage</i>	3	1.35	0.23	0.99	0.45
<i>Residual</i>	10	4.54	0.77		
<i>Total</i>	13	5.89	1		

Apices EM fungal communities' composition divergence analysis among an inoculation gradient of mixed urban and holm oak forest soil					
	<i>Df</i>	<i>Sum of squares</i>	<i>R</i> ²	<i>F-value</i>	<i>P-value</i>
<i>Dilution percentage</i>	5	3.58	0.32	1.85	0.002
<i>Residual</i>	20	7.72	0.68		
<i>Total</i>	25	11.3	1		

Apices EM fungal communities' composition divergence analysis among an inoculation gradient of mixed urban soil and garrigue soil					
	<i>Df</i>	<i>Sum of squares</i>	<i>R</i> ²	<i>F-value</i>	<i>P-value</i>
<i>Dilution percentage</i>	3	1.84	0.18	1.35	0.03
<i>Residual</i>	19	8.67	0.83		
<i>Total</i>	22	10.51	1		

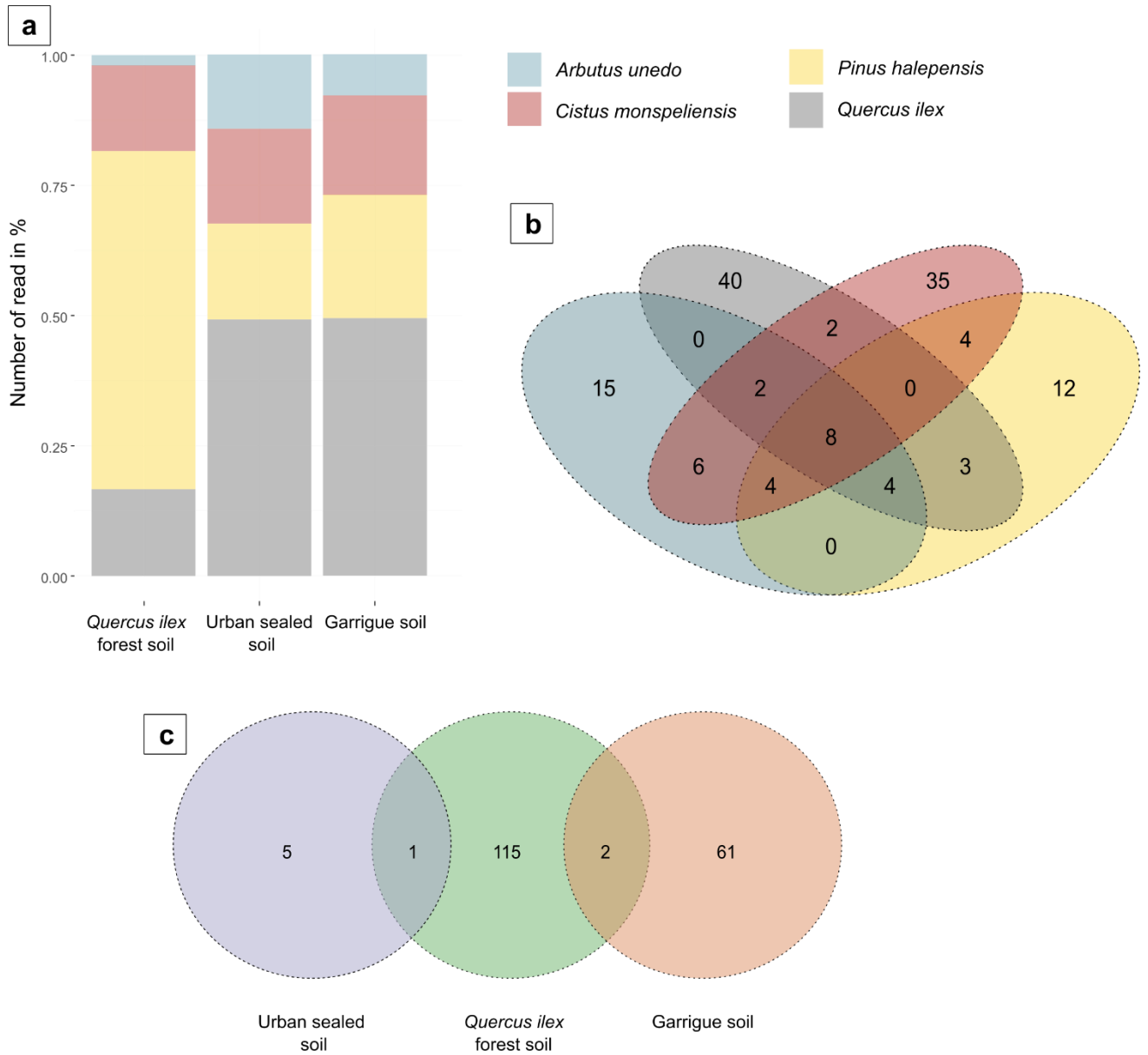
Table S4: Mycorrhization rate of 8-months old seedlings (*Arbutus unedo*, *Cistus monspeliensis*, *Pinus halepensis* and *Quercus ilex*) grown in the three types of soils (in *Quercus ilex* forest vs Mediterranean shrublands (garrigues) vs 60-yrs long urban sealed soils). N: number of seedlings

Soil origin	n	Min	Max	Median	Mean	Sd	Variation coefficient
Urban area (sealed)	99	46.03	100	96.42	93.93	8.20	0.08
<i>Quercus ilex</i> forest	98	66.66	100	93.84	93.33	5.88	0.06
Mediterranean shrubland	45	63.33	100	95.69	92.83	7.60	0.08

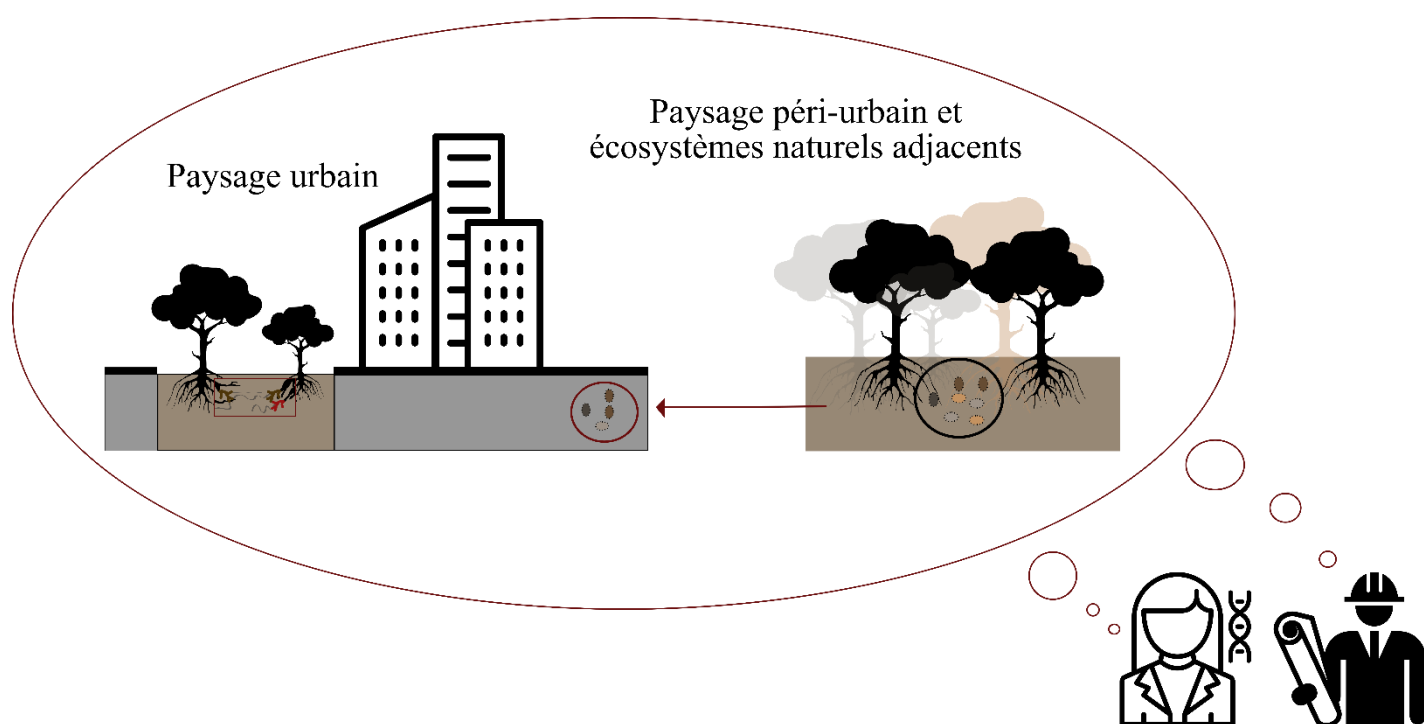
Table S5: Amount of detected ASVS per genera among sources soils

	Holm oak forest soil	Urban unsealed soil	Garrigue soil
<i>Amphinema</i>	2	0	0
<i>Cenococcum</i>	15	0	5
<i>Cortinarius</i>	0	0	1
<i>Delastria</i>	1	0	3
<i>Geopora</i>	2	6	8
<i>Hebelloma</i>	1	0	0
<i>Hydnellum</i>	1	0	0
<i>Hygrophorus</i>	0	0	5
<i>Hymenogaster</i>	0	0	1
<i>Inocybe</i>	25	0	10
<i>Melanogaster</i>	7	0	8
<i>Peziza</i>	18	0	7
<i>Pisolithus</i>	1	0	0
<i>Pulvinula</i>	5	0	0
<i>Pustularia</i>	10	0	0
<i>Rhizopogon</i>	0	0	1
<i>Russula</i>	2	0	6
<i>Scleroderma</i>	1	0	0
<i>Sebacina</i>	5	0	0
<i>Suillus</i>	5	0	0
<i>Tomentella</i>	17	0	6
<i>Tricholoma</i>	0	0	2

Figure S1: (a) distribution of ectomycorrhizal fungal ASVs on roots of 8-months old seedlings of plant species (*Arbutus unedo* vs *Cistus monspeliensis* vs *Pinus halepensis* vs *Quercus ilex*). (b) Venn diagram representing the distribution of ectomycorrhizal fungal ASVs on the four previous plant species. (c) distribution of ectomycorrhizal fungal ASVs in the three types of soils (*Quercus ilex* forest vs Mediterranean shrublands (garrigues) vs 60-yrs long urban sealed soils).



Chapitre 4 : Perspectives citadines de recherche sur les interactions ectomycorhiziennes : des centres villes à l'intégration paysagère



Présentation du chapitre en français

Au cours du chapitre précédent, nous avons exploré une technique de restauration de la communauté ectomycorhizienne des sols urbains scellés par l'inoculation d'une fraction de sol provenant des milieux naturels adjacents. Cette expérimentation conduite en conditions contrôlées a montré des résultats prometteurs pour le développement de pratiques d'économies circulaires favorisant le recyclage des sols scellés des villes. Au cours du chapitre suivant – qui est un essai de perspectives – nous analyserons comment les villes peuvent devenir de véritables laboratoires pour aborder des problématiques de recherche fondamentale et appliquée concernant les champignons ectomycorhiziens. Nous explorerons les particularités uniques des villes (usage des sols connus, fragmentation, paysage contrôlé) tout en détaillant des questions d'écologie des champignons ectomycorhiziens qui pourraient y être abordées. Sous un angle appliqué, nous détaillerons comment les villes peuvent devenir des systèmes qui s'autoalimentent en ressources – sols et plantes – en se basant sur les interactions biologiques qui s'établissent entre plantes et champignons – ainsi qu'entre plantes voisines.

Perspectives on ectomycorrhizal research: knocking on the city's door

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Abstract

Highly anthropized ecosystems have long been disregarded as useful environments to address ecological research questions, specifically questions related to ectomycorrhizal mutualism. In this paper, we argue that artificial urban ecosystems are adequate candidate spaces for experimentation on ectomycorrhizal fungi and addressing questions focused on community dynamics. We analyze several points of interest for city-based experimentation, such as land use history, land fragmentation, and strong anthropogenic footprint on soil, plants, and ecosystem dynamics. We explore fundamental and applied research questions involving ectomycorrhizal fungi and how they can be answered with in-field experimentation under urban conditions. Finally, we discuss the need of interdisciplinary approaches involving social sciences to improve landscaping methods and creating resilient plant communities within urban areas experiencing climate change.

Key words: Artificial urban ecosystems, fungal ecology, mycorrhiza, soil, plant communities, urban ecology, networks

Urban lands and fungal ecology research: where are we?

The ecological potential of cities has been highly underestimated to date. Diversity patterns in city soils remarkably contrast with those in natural ecosystems (Bach *et al.*, 2020). Uncertainties remain about the processes underlying the degradation of fungal communities in urban conditions (Cui and Mu, 2016; Martinová *et al.*, 2016; Scholier *et al.*, 2023). A comprehensive understanding of fungi-mediated interactions can help address fundamental and applied ecological issues.

Ectomycorrhizal fungi (EM) are mutualistic organisms promoting plant growth and survival (Smith and Read, 2010; Zanne *et al.*, 2019). They are involved in fundamental ecosystems processes, such as water and nutrient cycling (Read and Perez-Moreno, 2003). At the ecosystem scale, EM fungi drive positive loops between plants and soil (plant-soil feedbacks, Van der Putten *et al.*, 2013; Teste *et al.*, 2017; Mariotte *et al.*, 2018). The capacity of EM fungi to establish physical connections between neighboring plants through shared mycelia results in common mycorrhizal networks (CMNs, Selosse *et al.*, 2006; Simard *et al.*, 2012). It generates plant-plant interactions favoring plant nutrition (Simard *et al.*, 2015), drought resistance (Egerton-Warburton, Querejeta and Allen, 2007) and plant community structure (Chagnon, Bradley and Klironomos, 2020). Ectomycorrhizal taxa are ubiquitous, ranging from natural forests (Richard *et al.*, 2005) to highly anthropized urban ecosystems (Newbound, Mccarthy and Lebel, 2010; Stevenson *et al.*, 2020). Research efforts have been mostly conducted in natural ecosystems, such as temperate forests (Martin *et al.*, 2016; Policelli *et al.*, 2020) and grasslands (Vandenkoornhuyse *et al.*, 2002; Neuenkamp *et al.*, 2018). Experimental approaches in cultivated forests have led to considerable insights into ectomycorrhizal fungal patterns (Barroetaveña, Cázares and Rajchenberg, 2007; Spake *et al.*, 2016) and the dynamics of nutrient exchange between neighboring plants through common mycorrhizal networks (Wipf *et al.*, 2019; Karst, Jones and Hoeksema, 2023). From an applied perspective, research in managed forests guided forestry practices (Charya and Garg, 2019; Tomao *et al.*, 2020) and supported the creation of fungal red list (Geml *et al.*, 2014). Apart from forestry systems (Kujawska *et al.*, 2021), the fungal component of anthropized ecosystems have been poorly investigated so far (Authier, Violle and Richard, 2022).

In cities, the main ecosystems include tree dominated urban forests (Escobedo, Kroeger and Wagner, 2011), parks (Birge-Liberman, 2010; Vieira *et al.*, 2018), and artificial planted green spaces (Daniels *et al.*, 2018; Browning *et al.*, 2022). Urban tree communities are mostly composed of long-lived and pollutant resistant taxa (Van Tichelen, Colpaert and Vangronsveld, 2001). Usual taxa are ectomycorrhizal host plants from the Fagaceae, Pinaceae, Salicaceae and Tiliaceae lineages (Ossola *et al.*, 2020; Olchowik *et al.*, 2021, Purahong *et al.*, 2022). Yet, the vast majority of the research on urban fungi has been devoted to decomposers through the myco-cultivation harvest practices (Liu *et al.*, 2018; Zhang *et al.*, 2021), and their potential to decontaminate polluted soils (i.e. heavy-metal storage and degradation, Falandysz, 2016). Pathogenic fungi have also been investigated for sanitary consideration in both human health (Kasprzyk *et al.*, 2021) and plant survival (Schubert *et al.*, 2008) through monitoring the fluctuations in airborne spores (Crawford *et al.*, 2015; Leung, 2015; Buters *et al.*, 2018). In contrast, the mutualistic guild of EM fungi remains poorly studied in urban ecosystems (Bainard, Klironomos and Gordon, 2011; Hui *et al.*, 2017). In cities, numerous outstanding issues persist from fundamental and applied perspectives (Newbound, Mccarthy and Lebel, 2010). The degradation of EM communities due to increases in urban areas is unclear and needs to be investigated. The development of design methods for urban green spaces that favor the colonization and persistence of EM-propagules remains to be explored.

The compositional, organizational, and historical diversity of urban ecosystems dominated by EM plant species confer to cities unique potential for questioning the ecology of the ectomycorrhizal symbiosis. Specifically, cities are hosting a gradient of forest stand ages (Daniels *et al.*, 2018; Vogt-Schilb *et al.*, 2018; Farkas *et al.*, 2023), soil conditions (Scharenbroch, Lloyd and Johnson-Maynard, 2005; Greinert, 2015), plant communities (Goodness, 2018) and practices (Vogt, Hauer and Fischer, 2015; Aronson *et al.*, 2017; Ruíz-Pérez, Alba-Rodríguez and Marrero, 2020). This large range of ecological conditions is a great opportunity to advance research on the EM fungal communities' composition, dynamics, role in ecosystem processes, and response to anthropogenic footprint (e.g. biogeochemical cycling alteration and exposition to pollutants). At the landscape scale, cities are bordered by peri-urban areas which are hosting lower density of human population and can be dedicated to anthropized land uses such as agricultural lands. Peri-urban areas act as a buffer zone between highly anthropized urban center and natural ecosystems (Woltjer, 2014; Dadashpoor and Somayeh, 2019). Peri-urban areas are subject to high pressure of urbanization and mostly result from the

conversion of agricultural lands and forests into urbanized areas (Seto *et al.*, 2011; Dadashpoor, Azizi and Moghadasi, 2019). From the perspective of EM research, peri-urban areas are promising spaces to undercover the mechanisms of EM colonization in urban centers and to focus on the ecological connection between adjacent natural ecosystems and city centers.

In this perspective essay, we present the unique potential of urban landscapes for fundamental and applied research on ectomycorrhizal fungi, with a special attention to the plant-EM mutualistic interactions. We explore several specificities of the urban landscape, such as land use legacy, landscape connectivity, and anthropic control on abiotic and biotic features. We demonstrate how these urban specificities can fit with fundamental and applied research issues. We identify the potential challenges and present the corresponding knowledge gaps. Under an applied perspective, we explore how experimental research performed in cities can improve the creation of soil and production of plants for landscaping. Finally, we discuss the need of interdisciplinarity approaches to improve both our knowledge about urban ecosystems, as well as ways in which we can mitigate climate challenges and incorporate citizen values.

Why develop experimentation and theoretical frameworks of fungal ecology in cities?

The past land use in city archives as a precious tool to study long-term fungal legacy

In forests, soil properties (Macdonald, Bennett and Taranu, 2012) and ectomycorrhizal communities (Orgiazzi *et al.*, 2012; Correia *et al.*, 2021) are influenced by historical legacy (Diedhiou *et al.*, 2009). Discussing ecological conclusions in light of long-term history uses and practices improves the robustness of scientific results (Foster *et al.*, 2003). In cities, ethnographic resources, such as aerial photographs, cadastral maps, historical records, or citizen testimonies can inform the researcher about land occupation and past activities. In urban areas, the diversity of ecosystem ages, ranging from old and highly preserved forests to recent artificial ecosystems (Daniels *et al.*, 2018; Farkas *et al.*, 2023) located in the same area (Vogt-Schilb *et al.*, 2018) can inform research on the dynamics of EM fungal communities (Figure 1a). Currently, Mediterranean cities are hosting a mosaic of fragmented ancient forests (Vogt-Schilb *et al.*, 2018) co-occurring with recent afforestation. Planted stands of various ages result

from inner land use changes in cities, such as the rehabilitation of industrial areas into residential or recreational spaces (Figure 2, Fan *et al.*, 2020). The ability of fungi to persist in soils fully surrounded by a city as dormant propagules (*i.e.* sexual spores) provides an opportunity to understand unknown aspects of the ecology and diversity of EM fungi (Figure 1a). The fragments of ancient forests located in cities are highly valuable ecosystems for research due to soil age altering the composition of EM communities (Delgado-Baquerizo *et al.*, 2020).

At the landscape scale, including urban centers and peri-urban areas, insights into inherited and resilient fungal propagules can help understand the EM ecological strategies under highly constraining conditions (Figure 1a). In city centers, postindustrial areas previously covered by large surfaces of impermeable material are being unsealed and used for land conversion (Tobias *et al.*, 2018). Unsealed soils with a known date of confinement provide an opportunity to study the survival of EM spores after years of isolation (Figure 1a, Martinová *et al.*, 2016). Using ambitious sampling designs to explore large gradients of timescales and contexts, a significant amount of knowledge can be acquired about the community of species adapted to intense anthropogenic footprint (Figure 1a). Using a trait-based approach, this knowledge can be mobilized to theorize the fungal ecological strategies of urban-adapted fungal lifeforms (Zanne *et al.*, 2019; Aguilar-Trigueros *et al.*, 2022). Thus, fungal species retrieved after decades of sealing might be effective candidates for the development of fungal inoculation techniques in man-made anthrosols. In peri-urban areas, the EM pool of propagules is inherited from the recent conversion of agricultural lands and forests into urbanized areas. Specifically, the persistence of EM fungi and its capacity to establish mutualist interaction after land conversion remain to be explored (Figure 1a). To date, the effect of rapid land use changes on EM propagules has been poorly studied.

Cities as archipelagos: studying artificial insularity and fungal colonization processes

Most of the urban research efforts have been conducted on ecological networks and connectivity between motile organisms (*e.g.* birds, mammals) or among urban ecosystems (Ignatieva, Stewart and Meurk, 2011; Bergsten, Galafassi and Bodin, 2014, Lookingbill *et al.*, 2022). Abiotic (*i.e.* climate, soil conditions) and biotic (*i.e.* host plant, ecological interactions)

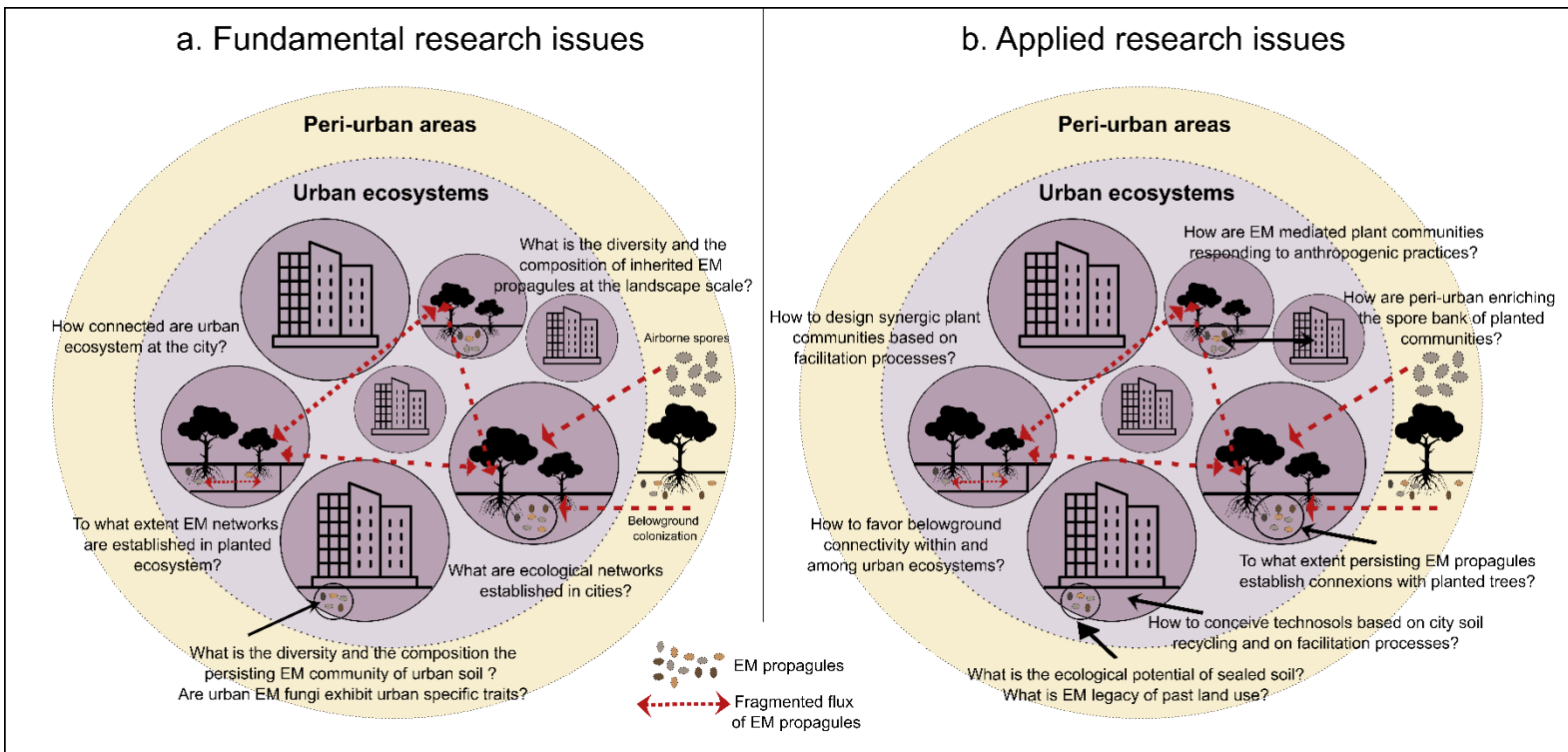
variables have a considerable influence on EM propagules and the resulting EM community structure and function (Koide, Fernandez and Petprakob, 2011). Yet, the effect of land fragmentation on the composition of ectomycorrhizal communities has been poorly investigated. In the urban landscape, there is a potential for the lack of connection across the natural ecosystems, peri-urban area, and to city centers to effect EM interactions (Figure 1a), but little is currently known (Peay *et al.*, 2007; Grilli *et al.*, 2017). At the city scale, investigations of the connections between old forest fragments and recently established stands can help to understand the ability of mature forests to provide functional EM inoculum for neighboring green spaces. We hereafter consider two geographic levels of land fragmentation, 1) the above-ground landscape fragmentation and 2) the archipelago structure of belowground habitats (Figure 1a, b).

From an aboveground perspective, land fragmentation occurs at the city center level and the landscape level with a discontinuity between natural ecosystems, peri-urban ecosystems, and urban centers. A shift between urban and rural EM airborne diversity has already been described (Abrego *et al.*, 2020), but the biotic and abiotic variables structuring EM patterns remain to be explored (Figure 1a). Specifically, understanding the ecological filtering ranging from natural, peri-urban and urban ecosystems can help develop a functional trait framework of the taxa that effectively reach urban centers. Fructification traits (*e.g.* hypogeous) and spore traits can be indicative of the anemochore or zoochore dispersal strategies (Figure 1a, Hopkins *et al.*, 2021). Such research can help understand the effective buffer zone of peri-urban ecosystem for the maintenance of EM diversity in urban centers.

In cities, subterranean land uses (*i.e.* planting pits, networks of water, electricity, internet and transportation) progressively disrupt the belowground soil (Authier, Violle and Richard, 2022). The diversity of uses generates an archipelago composition of the ground beneath the urban area (Figure 1a). Consequently, the compartmentation according to the land use generate a discontinuity in the soil matrix with the isolation of urban ecosystem from the others (Jim, 2023). From a functional perspective, the effect of belowground compartmentation among planting pits constrain the expansion of tree root systems (Jim, 2023). The effects of soil discontinuity on EM mutualism, common mycorrhizal networks, and fungi driven biogeochemical cycling have not been explored (Figure 1a). We hypothesize that compartmentation alters the connection between neighboring plants with a strong effect on

biogeochemical cycles. The physical barriers to the EM pathway in plant communities alters community level carbon trade (Klein, Siegwolf and Körner, 2016) and hydraulic pathways (Egerton-Warburton, Querejeta and Allen, 2007).

Figure 1: Concentric view at the landscape scale of a) fundamental research issues and b) applied research issues



Cities are modular spaces: experimenting from the zero-state of an artificial ecosystem

Urban greening projects gain relevance as cities are facing important structural challenges for adapting to climate change and maintaining population health (Haaland and van den Bosch, 2015; Feng & Tan, 2017; Filho *et al.*, 2019). These projects involve a deep restructuring of the urban space with soil unsealing (Tobias *et al.*, 2018), changes in soil composition (Deeb *et al.*, 2020; Rodríguez-Espinosa *et al.*, 2021), revegetation (Goodness, 2018; Mills *et al.*, 2020) and maintenance (Vogt, Hauer and Fischer, 2015; Aronson *et al.*, 2017; Ruíz-Pérez, Alba-Rodríguez and Marrero, 2020).

The design of the green space highly influences soil ecological conditions (Niemelä, 2014), its biotic capacity (Lepczyk *et al.*, 2017), and the plant-soil feedback loops (Pavao-Zuckerman and Byrne, 2009). Urban green spaces are comparable to laboratory controlled-conditions and provide valuable on-site experimentation designs for a number of reasons, 1) urban green spaces with similar abiotic and biotic properties are replicated across the city, 2) soils conditions are controlled within a man-made matrix, 3) plant community compositions are controlled (and selected), and 4) maintenance practices are planned with known quantities of water and nutrients applied.

In artificial urban green spaces, anthropic-made soils are often identified as technosols (Greinert, 2015; Fabbri *et al.*, 2021; Rodríguez-Espinosa *et al.*, 2021). The modularity of technosol production is a strength for experimental purposes due to known baseline conditions. These soils are highly diverse and the composition is based on structural features, nutrient content, and water retention capacity (Greinert, 2015). In the past, the soil organo-mineral matrix has been consistently imported from outside of cities (Vidal-Beaudet and Rossignol, 2014). Technosols can be artificially assembled using urban wastes and rehabilitated soils (Tobias *et al.*, 2018). The combination of soil material inherited from the city can mimic the soil structure of natural ecosystems with organic, organo-mineral, and mineral horizons (Deeb *et al.*, 2020). To date, the EM component of urban waste, industrial remnants, and mulch have not been investigated within technosol production. Under the hypothesis of soil biota degradation, experimenting with the restoration of biotic components in technosol assembly could be a useful tool in creating higher quality soils (Figure 1a, b).

Where should we go? Cities as reservoirs of soil and plant nurseries

Currently, there is an increased involvement of stakeholders for the establishment of in situ experimentations in cities (Evans, Karvonen & Raven, 2016). Successful experiments are made possible through close collaborations between scientists and landscapers. The concept of nature-based solutions has been widely explored in the literature (Atanasova *et al.*, 2021). In this section, we illustrate the concept of city-based solutions in early-development and mostly based on empiric initiative. Hereafter, we explore how the soil and plants used in urban green spaces can be designed to consider the success and persistence of EM mutualisms.

Soil creation: perspective of soil recycling and belowground continuum

To date, cities are highly dependent on agricultural and forest soil imports for technosol construction (Deeb *et al.*, 2020). Importing soil has a lot of negative consequences, such as the impact on adjacent agricultural ecosystems, land degradation, and the carbon cost of extraction and transport (Rokia *et al.*, 2014; Deeb *et al.*, 2020). As soil is a non-renewable resource, the development of new methods for supplementing soil is becoming an emergency for landscapers. Experimenting in cities can assist the development of city-based solution for soil assembly and for a soil continuum designed at the city scale (Figure 1b).

Experiments manipulating the recycling of recently unsealed soils can assist in the development of new methods of soil assembly (Figure 1b, 2, Tobias *et al.*, 2018; Fabbri *et al.*, 2021). Furthermore, a circular system in which cities are producing their own soil can be developed (Breure, Lijzen and Maring, 2020; Fabbri *et al.*, 2021). However, city-based soil production still requires insights to design hospitable below-ground environments for plants. Specifically, the implementation of soil biota is still lacking in restoration processes (Maltsev *et al.*, 2017). Because soil biota host mutualistic microbial communities, promoting EM communities in restored soils can enhance facilitation processes and result in positive effects on plant growth and survival. In addition, more research is needed to identify species of fungi that are adapted to urban conditions, as well as develop assembly and restoration methods based on the incorporation of mutualistic soil biota (Figure 1b).

The composition of EM communities in soil has been described in a variety of urban ecosystems, ranging from forest-like communities in parks (Hui *et al.*, 2017) to highly degraded communities in sealed soils (Martinová *et al.*, 2016). The historical legacy of past land-use, including the anthropogenic footprint, may influence the persistence and viability of EM propagules (Bruns *et al.*, 2009; Diedhiou *et al.*, 2009). In degraded soil matrices, the rehabilitation of viable EM communities can be potentially achieved with the inoculation of fungi. Fungal inoculation implies the addition of fungal spores or mycelia to the soil (Garbaye *et al.*, 1999). Mycorrhizal-based inoculation technologies have been implemented for forestry and agricultural purposes (Marx, Maul and Cordell, 1992; Marx, Marrs and Cordell, 2002) with promising results. However, before such technologies can be developed for technosols, experiments must be implemented to test the effectiveness of fungal inoculation in soil assembly for urban greening projects. (Figure 1b, 2, Box 1).

To move further into a circular technosol production system, cities can encourage natural inoculation or establishment of EM fungi. Connecting patches of land within a city and creating continuous areas of soil will promote the spread and colonization of EM fungi across the landscape (Box 1). Monitoring airborne EM propagules is a diagnostic tool to quantify the health of the ecosystem and its connectivity at the landscape scale (Figure 1b). Currently, government policies favor the connection of different ecosystems in urban landscapes. Green and blue infrastructures are a key measure of spatial connectivity (Ignatieva, Stewart and Meurk, 2011; Beaujean *et al.*, 2021) and have shown promising results, especially for motile organisms (Beninde, Veith and Hochkirch, 2015). However, the actions favoring ecological connection in urban soils do not account for soil biota continuity (Authier, Violle and Richard, 2022). In the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) global assessment (IPBES, 2019), green and blue corridors have been widely documented while the soil continuum has not been mentioned. The benefits of encouraging connectivity of soil fungi and other microbes between different urban areas within a city is still undervalued by scientific and political institutions.


Urban soil is necessary for several uses across the urban landscape. The qualities of the soil and how it will be incorporated into the ecosystem is dependent upon the intended function of the space. For example, the development of new housing developments to anticipate the demographic expansion in China leads to increased disturbance of the belowground soil (Qihu, 2016). Consequently, adopting a vision centered on soil biota would result into a beneficial of the cities soil structure and connection ranging from a continuum between ecosystems to a strict partitioning of uses between humans and non-humans (*i.e.* non-mixed land uses, Figure 2, Box 1). Such development can benefit not only to ectomycorrhizal organisms, but the entirety of soil fauna.

Figure 2: Case study of a city-based experiment in Marseille, Les Fabriques

A) Aerial view of the experimental area in highly anthropized context. Final planning to be realized by 2025



B) The experimental platform: 36 standardized planting pits



Standardized plant communities

Community composition design is based on the plant mycorrhizal status and on the community composition observed in adjacent natural ecosystems. Plants were grown in nurseries before transplantation

Two contrasting and standardized water regime

A manual watering system with low water quantities applied and an irrigation watering system with the regular watering quantities applied in urban green spaces.

Technosol matrix with a known soil EM biota


A soil matrix mostly composed by unsealed soil from the same location. 3% of soil inoculation were added to the unsealed soil. The fraction of soil inoculant have been sampled in natural adjacent ecosystems such as garrigue and oak forest.

Watermark sensors

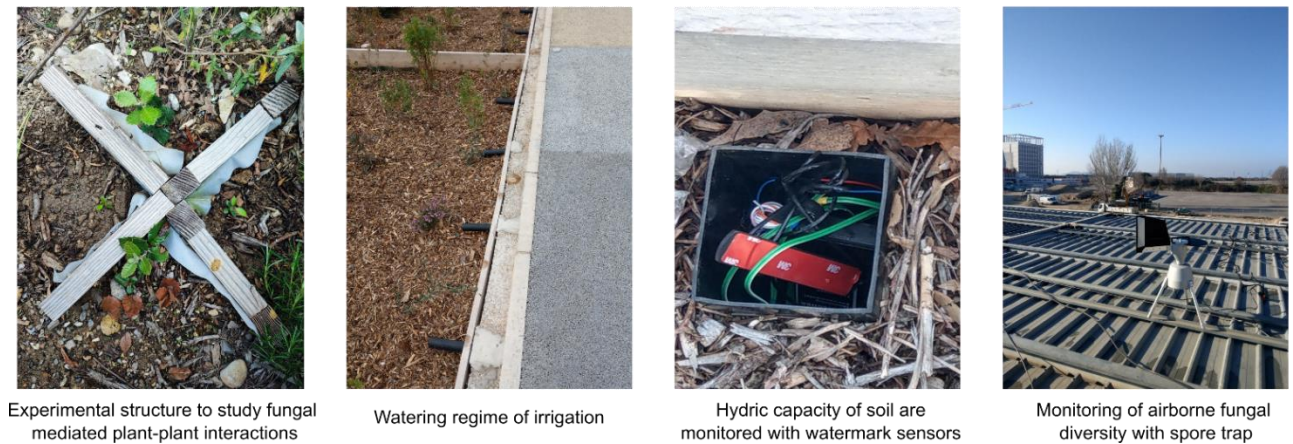
6 watermarks sensors set up per planting pits at three horizons of soil depths and following a gradient of distance from the water source. Data about soil hydration are weekly reported.

Experimental structure

2 experimental structure per planting pits hosting plantules used as bait plant for EM fungi.



C) Detail of experimental setup installed in Les Fabriques



Plant community composition: from cities as nurseries to the reduction of anthropic intervention

Planted trees in urban ecosystems experience early mortality (Roman, Battles and McBride, 2016; Hilbert et al., 2019). The potential mechanisms underlying the weakening of urban trees are 1) the shock experienced during the transplanting from nursery conditions to the outside environment, 2) the impact of competitive interactions between neighboring plants due to the high density of cooccurring plants, and 3) the difficulty of establishing without the necessary symbiotic soil biota.

Cities are highly dependent on the import of plants from external sources, such as nurseries and greenhouses (Petri *et al.*, 2016). The practices used in tree production have high economic and ecological costs. Common methods consume high amounts of soil and water while relying upon on intense chemical fertilizers (Petri *et al.*, 2016; Allen *et al.*, 2017). Planted trees experience shock during the transition between a highly controlled and nutrient rich nursery to a variable and nutrient poor urban area (Struve, 2009; Wattenhofer and Johnson, 2021). Furthermore, damage to vital plant organs have been reported after transport (Allen et al., 2017; Wattenhofer and Johnson, 2021). The loss of fine root surface during the transport, extraction, and planting of the tree is estimated to be 30%. Fine root systems are important for the uptake of water and nutrients from the surrounding soil and therefore influence the establishment success of tree (Allen *et al.*, 2017). Research and encouragement of city-based tree production can increase the success of planted trees and reduce costs of transport. Growing trees with consistent soil composition, climate conditions, and with their native mycobionts will decrease shock and early mortality rates. Localizing tree production within the target city can be beneficial for several reasons. City-based production can limit the transplanting shock as plants are adapted to local conditions. Plants have established mutualistic interactions with the native soil biota. The transporting process at the city-scale is traumatic as plants are not moved as far. Moreover, responding to the biotic requirements of the plant during the production will assist the plant recovery after transplantation. Consequently, designing plant communities focused on fungal mediated synergies can be a beneficial ecological perspective for landscapers.

Currently, the composition of plant communities in artificial urban green spaces is designed according to aesthetic, economic and social criteria, and climate requirements (Jenerette *et al.*,

2016; Goodness, 2018). The choice of plants is rarely influenced by community-level functioning. Considering the ecological niche (Violle and Jiang, 2009), resource acquisition strategies (Reich, 2014), and plant-biota feedback (Wardle *et al.*, 2004) can increase the longevity and resilience of the planted communities (Figure 2, Box 1). Ecological engineering has been used in the design of plant communities to prevent competitive interactions and favor synergic plant assemblies in highly anthropized ecosystems, such as agricultural systems (Litraco & Violle, 2015). In contrast, in urban ecosystems, ecological engineering remains under used (Faeth, Bang and Saari, 2011). To date, the ectomycorrhizal colonization of urban trees and the capacity of EM fungi to connect neighboring plants after transplantation is still unclear (Figure 1b, Last, Dighton and Mason, 1987; Bainard, Klironomos and Gordon, 2011; Authier, Violle and Richard, 2022). The current knowledge about EM pathways between neighboring plants within assembled urban communities is still incomplete and requires further exploration for future application (Figure 1b, Box 1, Klein, Siegwolf and Körner, 2016; Karst, Jones and Hoeksema, 2023). Understanding community dynamics will be integral in research about the EM networks established in plant communities. Ideally, landscapers should be able to choose certain species and types of plants to encourage synergic communities (Figure 2, Box 1) and CMNs for plant growth and survival. Advances in the design of assembled plant communities can be made through experimenting in situ. For example, city-based research of several compositions focused on understanding the interaction of the plants, as well as their functional and biotic traits can establish guidelines for plant community's conception (Box 1).

During the green space life cycle, landscapers and gardeners are involved in the physiological and architectural development of the plant communities (Roloff, 2016). The economic cost due to active management in green spaces is elevated (*e.g.* administration, planting, irrigation, fertilizing, maintenance, Roy, Byrne and Pickering, 2012). Urban green spaces are water intensive and in arid areas this raises significant challenges (Kjelgren, Rupp and Kilgren, 2000; Azeñas *et al.*, 2019; Monteiro, Cristina and Covas, 2021). For instance, the nutrients and water requirements of plants are anticipated and their excessive use disfavors the dynamics between natural allies, such as ectomycorrhizal fungi. In addition, some anthropogenic practices weaken the plants and expose them to early mortality (Hilbert *et al.*, 2019). The ecological cost of plant assistance is high: nitrogen fertilization alters the EM mycelial growth, lowers the rate of EM root colonization, and can cause a shift in EM community compositions (Nilsson and Wallander, 2003; Teste, Lieffers and Strelkov, 2012, de

Witte *et al.*, 2017). EM colonization rate of roots is lowered when there is an excessive amount of water present in the soil (Olivera, Bonet and Oliach, 2014) resulting in altered hydraulic pathways between neighboring plants (Egerton-Warburton, Querejeta and Allen, 2007). As a consequence of the loss of mutualists, plants do not have access to the otherwise unavailable nutrients via ectomycorrhizal symbiosis. City-based research about ectomycorrhizal benefits can outline natural solutions to decrease the overuse of resources and minimize the amount of labor needed to maintain urban green spaces. By using informed ecological engineering and favoring dynamic plant communities, the environmental and economic cost of plant maintenance can be greatly reduced (Figure 1b, Nesshöver *et al.*, 2017). To our knowledge, the effect of ectomycorrhizal communities on plant drought resistance has not been studied in urban ecosystems. Insights into watering and other practices are fundamental for the sustainability of both green spaces and limited resources (Figure 2, Box 1).

Box 1: The case of Marseille, Les Fabriques: a pioneer district experimenting sealed soil recycling, EM fungi inoculation and alternatives of intensive watering.

Context - A hybrid experimental setup following both fundamental and applied goals have been installed in a postindustrial district of the city of Marseille, France (**Figure 2**). This research work was initiated by the operational actors including a landscaping company (Ilex Paysage + Urbanisme Inc., Lyon, France) and a public development agency (Euroméditerranée, Marseille, France). This project is part of the restructuration of a postindustrial district into a residential area performed on a large surface of 480 acres previously dedicated to industrial and harbor activities. By the end of 2025, the district will host 18.000 new housing, 1.200.000 m² of space dedicated to commercial activities and 40 acres of public spaces – including green spaces. The area is enclosed by important transportation arteries including a rail line and a highway (**Figure 2**). The experimental platform is also an educative area where communication events are frequently organized with green space services, political institutions, landscapers, and citizens.

Followed objectives are differing according to the actors involved into this project

	Scientists	Landscapers	Urban planners
Context	Fungal mediated plant-plant interactions have not been yet explored in urban ecosystems. The response of EM communities to anthropogenic management such as water inputs remain to explore.	Plant communities are exposed to early mortality under urban conditions. The viability of anthropogenic practices such as watering is constrained by resource availability under the context of climate change.	A landscape transition is occurring in postindustrial cities. Highly populated Mediterranean cities has to respond to intense climate change.
Objectives	Analyze (i) the EM compartment of technosol based on sealed soils, (ii) the synergic interaction between neighboring plant through CMNs and (iii) the response of CMNs to contrasting management practices	Create durable and synergic plant communities. Limit reliance on water and nutrient inputs.	Create livable spaces in cities. Visibility and communicative value
Perspectives	Get insight into EM strategies under highly constraining urban context. Improve knowledge about the EM networks established at the community scale	Develop ecological and interaction-based technics of soil conception, plantation and ecosystem maintain	Develop the acquired knowledges and technics in other landscaping projects

Experimental structure - The experimental platform is a 100 meters long streetscape composed by 36 standardized planting pits (**Figure 2**). The soil was made of recently unsealed soil matrix, extracted from the same location and inoculated with 3 % of soil collected in adjacent natural ecosystems of garrigue and holm oak forest. The design of two plant palettes was made based on plant communities retrieved in adjacent natural habitats and adapted to local Mediterranean climate. The plant community is composed by ectomycorrhizal Mediterranean trees and arbustive species such as *Quercus ilex*, *Quercus pubescens*, *Arbutus unedo* and *Cistus monspeliensis*. These EM host plants were also selected because of its trait's complementarity – and its synergies. Specifically, we relied on EM facilitative interactions during early stage of ecological succession (**Gómez-Aparicio et al., 2004; Richard, Gardes and Selsosse, 2009**). The endomycorrhizal shrubby and herbaceous communities were designed as retrieved in the holm oak forest and garrigue ecosystem surrounding the cities of Marseille (*Thymus*, *Pistachia lentiscus*, *Coronilla glauca*, *Lavandula*). Two watering modalities were applied: a manual, hand driven watering and an automatic irrigation system. These two conditions are highly contrasting regarding water quantities and watering frequencies.

Perspectives – The experimentation are running until mid-year of 2024. The collection of fine roots and soil samples from the planting pits will permit the molecular identification of the soil EM community and the EM community established as symbionts. Ecological analysis will determine the effect of hydric regime on EM communities and on the related networks. The identification of shared EM symbiont on the root of neighboring plant will assist the conception of synergic plant community. From an applied perspective, the recycled soil matrix will permit to inoculate other recycled unsealed matrix for the green spaces planned at the district scale (**Figure 2**). The acquired knowledge about synergic plant association and efficient hydric regime will guide both the plant *palette* design of other green spaces of the project.

Conclusion: interdisciplinarity is a fundamental element in urban field experimentation

Under the climate emergency, cities of the future will have to be green to persist as livable environments for human societies. If not, cities' climate will make them uninhabitable as they will be less resilient and experience greater effects as compared to surrounding natural areas (*i.e.* heat island, drought, intense climatic events, Masson *et al.*, 2020). Building urban green spaces that are durable and efficient providers of ecosystem services is a priority. City-based experimentation has the potential to inform green space creation and managing practices with consequences for the organization of landscaping projects and physical structure of cities. Applied research can assist the development of circular economy systems for the soil and plants incorporated into green spaces. Specifically, cities can produce their own soil from waste, as well as grow trees within the locally sourced soil. Ectomycorrhizal fungi will become one of fundamental ecological engineers mitigating plant growth, survival, and resistance to increased drought. Resolving the uncertainties remaining about ectomycorrhizal mutualism and its capacity to connect neighboring plants cultivated in urban green spaces will assist fundamental and applied research, as well as inform the development of ecological practices at the city landscape scale.

The specifics of urban areas involve a number of contributors who influence the management of these spaces, ranging from political officials, landscapers, gardeners, land owners, and citizens as users (Hilty and Merenlender, 2003; Bush, 2020). Consequently, running experimental projects within cities requires an insight into the values and objectives of each contributor. The development of city-based research requires an interdisciplinary approach involving social, economic, and ecological sciences. By incorporating multiple perspectives, we can start to understand the complexity of issues surrounding the management of urban areas and climate change. Political decisions, such as urban planning documents (*e.g.* PLU, SCOT in France) remove administrative barriers (*e.g.* delays, decisions, Kronenberg, 2015; Bush, 2020) and allow for effective experimental designs to be implemented within urban green spaces (Ahern, 2013).

Finally, the influence of city-based experimentation can go far beyond the confines of the urban areas. The results may enlighten ecological processes occurring in other anthropized

ecosystems, such as managed forests. As cities are combining extreme micro-climate conditions (e.g. heat island, extreme climatic events), insight into the response of fungal and related plant communities guide predictions about the future of mycorrhizal association under intense perturbation in natural landscapes. Such results can help improve predictions of the evolutionary and ecological consequences of natural ecosystems experiencing climate change.

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DISCUSSION GENERALE



I. Apports théoriques et perspectives opérationnelles issues du travail de recherche

En recourant à différentes approches méthodologiques telles que la revue bibliographique et l'expérimentation en conditions contrôlées, ce travail a permis de documenter les composantes de la symbiose ectomycorhizienne dans les milieux urbains ainsi que les méthodes moléculaires qui permettent de les étudier. Plusieurs résultats majeurs émergent de ce travail :

- 1) **Chapitre 1** – Les composantes de la symbiose ectomycorhizienne ont pour l'heure été peu explorées dans les écosystèmes urbains. Une meilleure connaissance de la banque de spores, de la mycorhization des arbres urbains et des réseaux d'interactions qui s'établissent entre plantes voisines permettrait d'améliorer la manière de restaurer un continuum écologique dans les sols centré sur les organismes facilitateurs.
- 2) **Chapitre 2** – Les méthodes de métabarcoding couramment employées restituent à ce jour une image de la communauté ectomycorhizienne qui varie en fonction de la calibration de la méthode. Le nombre de lecture d'une séquence ne peut pas être utilisé pour estimer l'abondance d'un taxon dans la communauté.
- 3) **Chapitre 3** – La banque de spore des sols scellés héberge une communauté paupérisée de propagules ectomycorhiziennes, essentiellement composées d'Ascomycètes produisant des fructifications de type « truffe », hypogées. En conditions contrôlées, les plantules sont capables de mobiliser cet inoculum résiduel en tissant des interactions ectomycorhiziennes.
- 4) **Chapitre 3** – L'inoculation de la banque de spore des sols scellés – en ajoutant une fraction de sol naturel des écosystèmes adjacents – montre des résultats prometteurs. Le ré-enrichissement de la communauté requiert une faible quantité d'inoculant, de l'ordre de 1 % de la quantité de la matrice de sol. L'inoculum mobilisé par les plantules se compose majoritairement de taxon de début de succession végétale, composé de champignons hypogés tels que *Geopora*.
- 5) **Chapitre 4** – Les particularités uniques des villes (historique documenté de l'usage des sols, contrôle de certaines composantes biotiques et abiotiques des espaces végétalisés...etc.) rendent ces espaces intéressants pour les chercheurs et aménageurs –

qui peuvent mener des expérimentations pour répondre à des questions fondamentales et appliquées sur les champignons ectomycorhiziens.

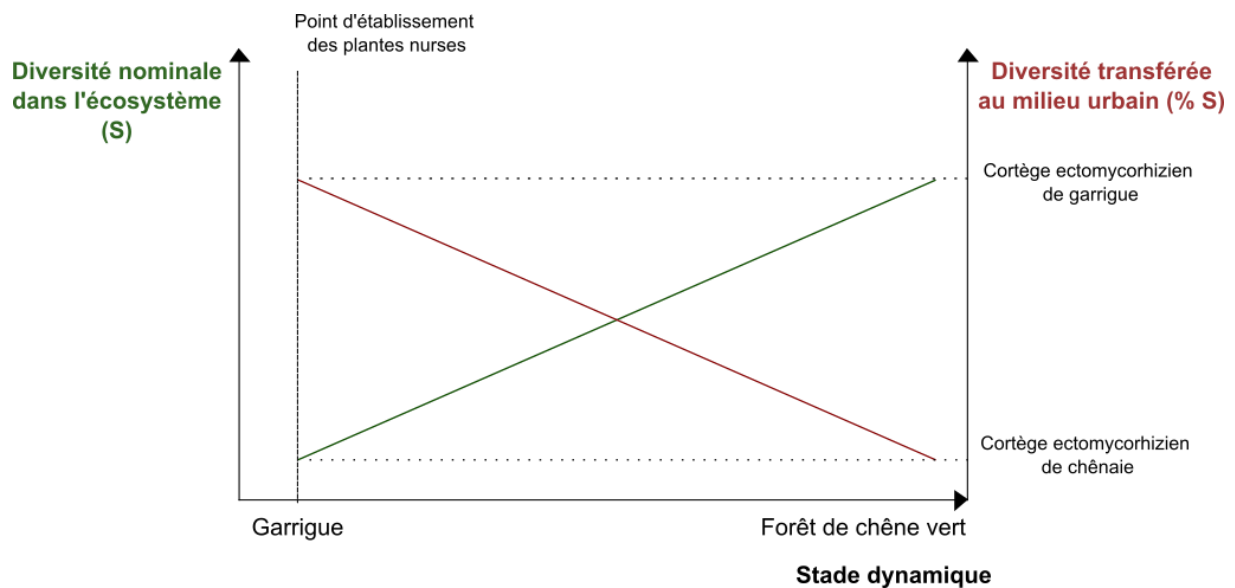
Au cours de cette discussion générale, je discuterai mes résultats en faisant appel à des outils d'écologie des communautés. Je formulerai des perspectives pour l'amélioration de l'aménagement urbain permises par les résultats de mon travail. J'explorerai en premier lieu l'absence de lien qui existe entre la diversité des propagules ectomycorhiziennes et leur transférabilité dans les sols urbains. Dans une perspective phyto-centrée, je réfléchirai comment les interactions entre plantes voisines médiées par des champignons peuvent assister la conception des palettes végétales en ville. Ensuite, je discuterai de la transférabilité des outils moléculaires pour la réalisation de diagnostics écologiques des sols urbains – destinés au design et à la gestion de ces espaces. Enfin, je réfléchirai à la place de la ville à l'échelle paysagère pour la provision des ressources nécessaires à la conception des espaces verts – notamment pour le sol et la production des végétaux.

II. Dans les sols des forêts méditerranéennes, la diversité ne garantit pas la transférabilité en ville

1. Penser la fonctionnalité myco-centrée pour la restauration des sols urbains scellés

Au cours cette thèse, nous avons exploré la capacité d'inoculation de deux formations végétales dominant les paysages de l'ouest du bassin méditerranéen, la chênaie verte, ou yeuseraie, et la garrigue. Ces milieux montrent des caractères contrastés tant sur la composition des communautés végétales que sur celle des communautés fongiques associées. Nous avons émis l'hypothèse qu'un inoculum fongique hyper-diversifié (issu de la yeuseraie) montrerait une capacité d'inoculation des sols urbains supérieure, comparativement au modeste pool fongique hébergé par la garrigue. De manière claire, nos résultats ont rejeté cette hypothèse. Ainsi, les milieux pionniers de garrigue se sont révélées être les plus performants pour coloniser les systèmes racinaires de plantules développées dans un substrat majoritairement constitué de sol urbain prélevé sous bétons. Ce résultat suggère que dans ce modèle de la symbiose ectomycorhizienne, « diversité n'est pas garantie de transférabilité », le maître mot restant « adaptabilité » permettant aux organismes ainsi importés de s'établir comme inoculum viable dans le contexte difficile des sols urbains (Figure 1). Pour l'heure, des solutions fondées sur la diversité des communautés microbiennes associées aux plantes sont développés dans le cadre de technique de biocontrôle ou de solutions fondées sur la nature (« nature-based solutions, Berg *et al.*, 2017 ; Eggermont *et al.*, 2015). Chez les champignons ectomycorhiziens exclusivement, ce cadre conceptuel n'avait pas encore été exploré – et nos résultats montrent des liens complexes entre la diversité de la communauté ectomycorhizienne et sa transférabilité dans un sol urbain.

Figure 1 : Schéma conceptuel du lien entre diversités ectomycorhizienne résidente et transférable en milieu urbain au cours de l'évolution des paysages méditerranéens (série dynamique du chêne vert sur calcaire)



Au cours du travail du Chapitre III, l'inoculum fongique le plus diversifié que nous ayons séquencé provient d'une chênaie verte mature, issue d'une recolonisation de parcelles agricoles après abandon des pratiques de cultures après la seconde guerre mondiale. Cette forêt abrite des communautés fongiques associées à cette essence de fin de succession végétale (Figure 1). On y rencontre, établies sur les systèmes racinaires des arbres, des espèces fongiques capables de persister dans des contextes de forte compétition (stratégie compétitrice au sens de Grime, 1988), principalement constituées de Basidiomycètes (Richard et al. 2010). Dans les garrigues essentiellement peuplées d'espèces pionnières (Figure 1), la communauté fongique se compose en grande partie par des champignons appartenant à la lignée des Ascomycètes dont les traits de vie semblent témoigner d'une résistance accrue à des conditions stressantes (notamment liées aux sécheresses estivales marquées dans cet écosystème) – en raison de sols peu différenciés et largement dégradés par l'action anthropique sur le long terme (Grove et Rackam 2003). Ces taxons appartiennent à des lignées produisant des fructifications hypogées, particulièrement adaptées au stress hydrique (Vogt-Schilb *et al.*, 2022)

Ainsi, ces résultats montrent que les traits écologiques de taxons ectomycorhiziens les plus adaptés à leur implantation dans les villes correspondent aux cortèges ectomycorhiziens se développant spontanément dans les milieux de début de succession secondaire (Figure 1) – dans lesquels les stress sont plus intenses qu'en système forestier. Sous l'angle de la recherche fondamentale, cela invite à utiliser les outils de l'écologie fonctionnelle pour décrire des stratégies écologiques adoptées par les champignons ectomycorhiziens dans la colonisation des milieux perturbés. Pour l'heure, ces stratégies écologiques restent peu explorées (Zanne *et al.*, 2019). A terme, cela pourrait permettre de cibler et pourquoi pas, tenter de cultiver au sein de pépinières urbaines, certaines de ces espèces adaptées pour leur capacité d'adaptation à la ville (Encadré 1).

Encadré 1 : Quels traits fonctionnels des champignons ectomycorhiziens peuvent se révéler intéressants dans une perspective d'aménagement urbain ? (Construit à partir de Zanne *et al.*, 2019)

Des traits liés à la reproduction et à la colonisation

- Capacité reproductive : Fréquence de fructification, taille de fructification
- Capacité de dispersion : taille des spores, nombre de spores
- Persistance des spores et de leur capacité de germination : ornementation, concentration en mélanine
- Stratégie généraliste ou spécialiste pour l'association avec des plantes hôtes

Des traits liés à l'acquisition ou la conservation des ressources

- Capacité d'exploration pour l'exploration des ressources : longueur des hyphes
- Capacité à extraire les ressources : activité enzymatique
- Capacité à stocker et transmettre des ressources : concentration en C, N, P dans les tissus

Des traits liés à la réponse au stress et à la perturbation :

- Forme de fructification épigées ou hypogées
- Capacité de dormance : production de sclérote, des spores longévives

Dans une perspective appliquée, nos résultats s'avèrent prometteurs pour deux raisons. La première est qu'une faible quantité d'inoculum permet d'assurer l'implantation des communautés ectomycorhiziennes dans les sols urbains scellés. Seule une fraction d'1 % du volume du sol d'une fosse de plantation suffit à établir une colonisation massive des racines courtes par les champignons ectomycorhiziens (Chapitre III). La seconde est que nos résultats amènent à réfléchir aux sources mobilisables pour renforcer l'inoculum ectomycorhizien résiduel des sols scellés. Ainsi, cela consisterait à s'appuyer sur la fonctionnalité abritée par la diversité taxonomique considérable (de l'ordre de 20,000 espèces; Tedersoo *et al.* 2017) des champignons ectomycorhiziens. En s'appuyant sur les stratégies écologiques des champignons

ectomycorhiziens issus de ces milieux stressants, le rôle de tels espaces, considérés comme dégradés et de faible valeur pour la diversité, pourrait ainsi être réenvisagé. Ainsi, une forme de réhabilitation de la représentation sociale des garrigues méditerranéennes, comme puits de diversité fongique restauratrice des sols dans les villes, pourrait accompagner de telles opérations. Par ailleurs, en limitant la source d'inoculum aux sols de garrigues, la possible demande urbaine en sols forestiers, déjà fortement prisés par les acteurs des paysages (terres de cultures, espaces de distraction/détente), serait fortement réduite pour des raisons écologiques dont les populations peuvent facilement se saisir (Figure 1).

2. Penser la fonctionnalité phyto-centrée pour la conception des écosystèmes urbains

En ville, la composition des communautés végétales reste majoritairement basée sur des critères d'adaptation aux conditions locales (Jenerette *et al.*, 2016), des critères esthétiques (Goodness, 2018) ainsi que sur une approche empirique de la cohabitation entre les plantes (Comm. Pers. Ilex Paysage + Urbanisme). Lors de la conception des palettes végétales, les interactions entre espèces végétales – compétitives ou facilitatrices - restent peu explorées. La littérature sur le sujet, et notamment le corpus relatif aux réseaux d'interactions ectomycorhiziens, est pour l'heure quasi-inexistante dans les écosystèmes anthropisés (Chapitre I). Pourtant, la réalisation de communautés végétales interconnectées entre plantes voisines via la symbiose ectomycorhizienne apparaît comme une façon prometteuse d'assister la conception de communautés végétales synergiques – et ainsi de favoriser la durabilité des plantes dans le temps (reprise après plantation, croissance). A ce jour, peu d'essais ont été réalisés en ce sens, et une expérimentation en cours, dans le cadre de cette thèse, fournira les premiers résultats de l'effet synergique entre plantes établies dans les sols urbains et choisies selon le critère de leur compatibilité ectomycorhizienne. Dans le contexte actuel de forte fluctuation des précipitations méditerranéennes (IPCC, 2023), les externalités positives produites par la facilitation pourraient soulager la pression pesant sur la ressource hydrique.

Au cours de ce travail de thèse, la question de la composition de communautés végétales synergiques s'est posée. Plus particulièrement, la conception de la structure expérimentale à Marseille, dans le quartier des Fabriques (évoquée dans le Chapitre IV) a suscité de nombreuses discussions sur la composition des palettes végétales (quels taxons prioriser et pour quelles

raisons ?). Par conséquent, nous avons expérimenté des palettes végétales composées de plantes arbustives et arborées en conditions contrôlées, en appui de ce qui se fait *in situ*, à Marseille. Ce travail consiste en une structure expérimentale d'envergure, conduite sous serre, sur le Terrain Expérimental du Centre d'Écologie Fonctionnelle et Évolutive à Montpellier (Figure 2, a, b). L'objectif appliqué de cette expérimentation est l'identification de couples de plantes méditerranéennes synergiques – pour lesquels des réseaux d'interactions ectomycorhiziens s'établissent grâce à une compatibilité maximum des plantes pour des espèces de champignons partagées. Nous utilisons des plantes ectomycorhiziennes couramment employées dans l'aménagement public d'espace végétalisés – à savoir l'arbousier, le ciste, le pin d'Alep et le chêne vert. Ce travail est aujourd'hui en cours et sera conduit à son terme au cours de l'année à venir. En couplant une approche fonctionnelle des traits aériens et souterrains des plantes (traits morphologiques et physiologiques) à une approche moléculaire des symbiontes ectomycorhiziens, nous allons dans un premier temps qualifier et quantifier les réseaux d'interactions ectomycorhiziens s'établissant entre individus d'espèces de plantes co-occurentes, d'espèce différentes ou non. Ensuite, nous évaluerons le rôle des liens mycéliens dans la réponse des plantes à la co-occurrence (croissance, nutrition, écophysologie). Nous analyserons comment varient les traits morphologiques, anatomiques et les communautés souterraines associées aux plantes lorsqu'elles se développent au voisinage de plantes d'espèces différentes ou non. Enfin, nous analyserons le rôle de la plante dite *nurse* – c'est-à-dire une plante pionnière jouant le rôle de nourrice (ici, *Arbutus unedo* et *Cistus monspeliensis*), connue pour faciliter l'établissement, la croissance et la survie des plantes de stade forestier dites « receveuse » (ici, *Pinus halepensis* et *Quercus ilex*). Nous analyserons les limites du système de nurses dans l'environnement – afin d'évaluer si cet effet est généralisable, ou s'il est dépendant du contexte au regard des conditions pédologiques et hydriques.

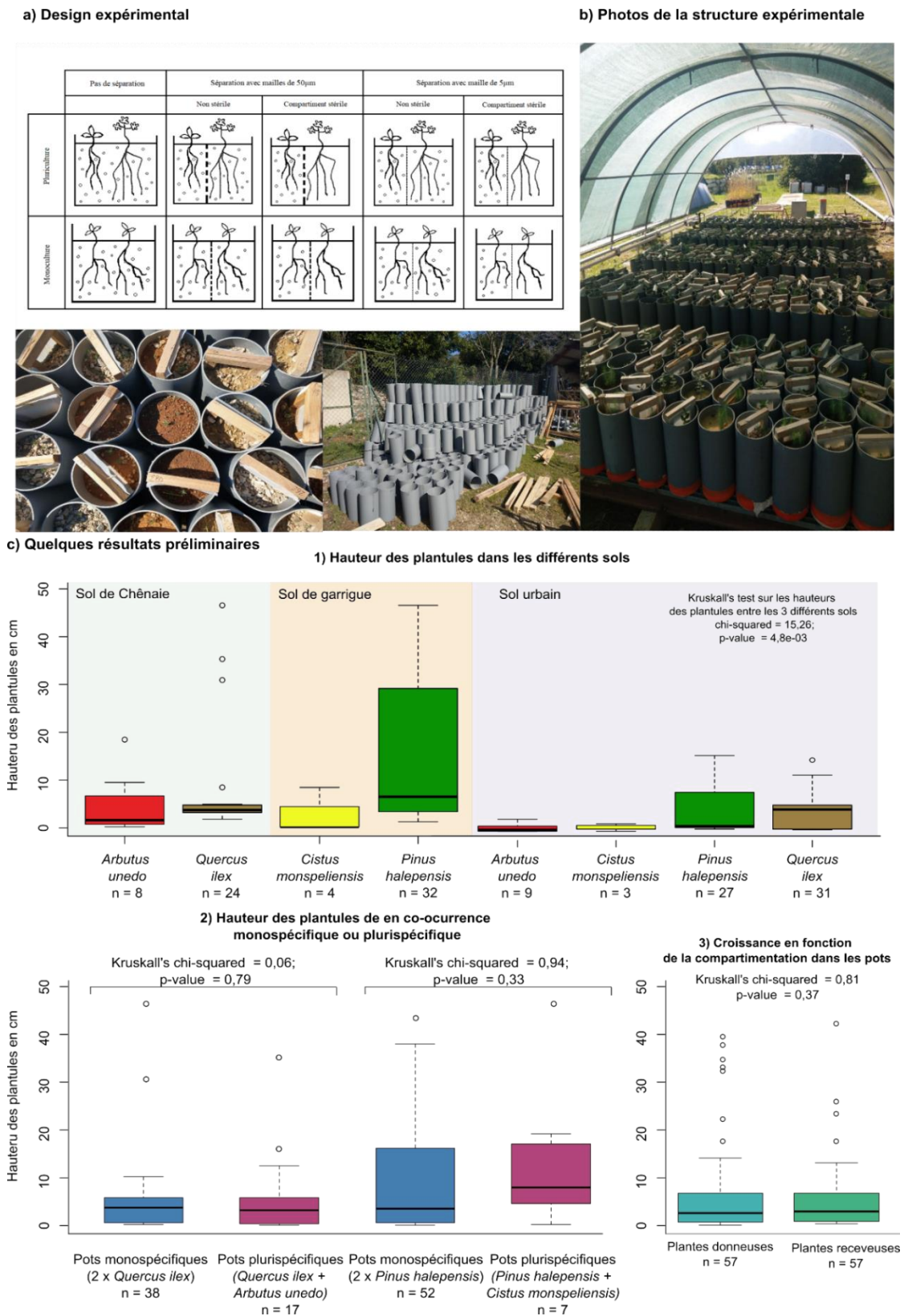
Les premiers résultats après deux ans de croissance des plantules indiquent des différences de hauteur qui pourraient être attribuées aux conditions de sol – avec notamment des hauteurs plus faibles chez les plantules établies dans les sols urbains scellés, sans doute en réponse aux faibles teneurs en matière organique de ces sols (Figure 2c1). Pour le chêne vert et le pin d'Alep, la croissance en configuration de couples plurispécifiques ou monospécifiques n'induit pas de différence marquée sur la hauteur mesurée de plantules (Figure 2c2). Conjointement, la position donneuse ou receveuse (y compris receveuse en compartiment stérile) n'induit pas non plus de différence significative de hauteur (Figure 2, c3). Aujourd'hui

seul un faible échantillon des plantes mesurées sur l'expérimentation a permis la réalisation de ces analyses préliminaires (Figure 2c). L'inclusion de l'intégralité des données récoltées permettra une analyse plus précise grâce à un échantillonnage conséquent (environ 1600 plantules). D'autres mesures biométriques et de quantification chimique dans les tissus des plantes restent à conduire sur cette expérimentation. Il faudra notamment analyser des traits des plantes qui témoignent de l'activité biotique de leurs partenaires ectomycorhiziens (empreinte des nutriments dans les tissus des plantes, concentration en N, P, K, C, développement de l'appareil aérien).

Parmi les résultats attendus de cette ambitieuse expérimentation, nous quantifierons et qualifierons l'effet de la connexion entre plantes nourrices et receveuses. Parmi les hypothèses de travail, les cortèges fongiques établis sur les plantes nurses de début de succession pourraient être mis à profit par les plantes de fin de succession, et permettra des transferts de ressources (eau, minéraux), permettant ainsi des synergies végétales dans les espaces verts urbains.

Parallèlement, le couplage de ce travail en conditions contrôlées au dispositif expérimental en conditions réelles, dans le quartier des Fabriques, à Marseille (présenté dans le Chapitre IV) permet de tester la réponse fonctionnelle des associations végétales à des pratiques d'arrosage contrastées. Dans ce travail, nous avons émis l'hypothèse que le régime d'arrosage pourrait être modulé grâce aux transferts d'eau qui peuvent être réalisés par voie fongique entre plantes voisines. Nous analyserons comment varie la composition des communautés ectomycorhiziennes qui s'établissent sur les racines des plantes dans des sols descellés en réponse à des régimes contrastés d'arrosage. L'objectif opérationnel de ce travail est d'optimiser l'usage de la ressource hydrique dans les espaces plantés de demain.

Figure 2 : Expérimentation en conditions contrôlées sur les réseaux d'interaction ectomycorhiziens avec a) design expérimental, b) photographies et c) quelques résultats préliminaires



III. Quels outils de diagnostic de la science sont transférables pour la gestion des sols urbains ?

1. Quelle transférabilité des outils de métabarcoding vers les questions opérationnelles ?

Les techniques de métabarcoding sont des outils de plus en plus appétants pour les acteurs du territoire, dont font notamment partie les établissements en charge de la gestion et de l'aménagement des paysages urbains tels que les commanditaires de ce programme de recherche. Pour l'heure, la calibration de ces outils, encore largement réservés à un usage scientifique, reste relativement empirique, car peu de travaux analysant leur performance sur des modèles biologiques ont été publiés à ce jour (mais se référer à Kauserud, 2023 pour une référence récente). Les résultats présentés dans le Chapitre II ont montré les limites l'importance de la calibration de ces outils avant tout usage. Nous allons ici apporter des éléments de réponses à trois questions que se posent le citoyen comme l'aménageur, concernant le champ d'application et la portée opérationnelle de telles méthodes.

Le métabarcoding est-il synonyme d'exhaustivité ?

Non, bien entendu, et ce pour plusieurs raisons tenant tout aussi bien des limites inhérentes à l'échantillonnage des sols, au caractère destructif de la méthode et aux aspects technologiques évoqués dans le chapitre II. Aucune méthode – moléculaire ou non – ne permet un inventaire exhaustif de la diversité des champignons ectomycorhiziens, pas plus que les inventaires les plus perfectionnés ne permettent l'évaluation de la diversité d'autres organismes beaucoup plus visibles, comme les plantes (Archaux *et al.*, 2009). Aucune méthode ne s'en revendique, donc, les mycologues étant parfaitement conscient de la capacité limitée des fructifications à rendre compte de la diversité fongique présente dans les sols. Le métabarcoding est aujourd'hui la seule méthode permettant d'accéder à la communauté fongique dans son ensemble, qu'elle soit présente sous forme de spores, sclérotés, mycélium ou encore de fructification – et permettant par conséquent d'échantillonner la fraction dormante et assimilatrice de la guildes ectomycorhiziennes – inaccessible à un mycologue. Les comparaisons effectuées entre l'échantillonnage des fructifications et les données générées par métabarcoding

témoignent d'une complémentarité entre ces deux approches – car elles fournissent toutes deux une image originale et hautement informative de la communauté ectomycorhizienne présente dans un environnement donné (Froslev *et al.*, 2019).

Dans notre travail expérimental, nous avons mis en lumière les conséquences méthodologiques de la structuration spatiale fortement irrégulière (« patchy ») des communautés ectomycorhiziennes, y compris dans l'espace contraint de rhizotrons expérimentaux de contenance limitée. Cette distribution est une caractéristique connue de ces communautés (Koide, Xu and Sharda, 2004; Pickles and Anderson, 2016), qui impose de multiplier les points d'échantillonnage pour en limiter l'impact. Ensuite, le nombre d'espèces moléculaires peut varier au sein d'un même amplicon séquencé plusieurs fois (Chapitre II, analyse d'échantillon tripliqués). Ces résultats suggèrent de multiplier le nombre d'échantillons au sein des points d'échantillonnage – et de recourir au séquençage répété du même amplicon afin de couvrir le mieux possible sa diversité.

Enfin, nos résultats ont montré que les estimations de la diversité varient en fonction de la calibration de l'appareil de séquençage, pour un même amplicon (Chapitre II, profondeur de séquençage). La diversité alpha et la composition des communautés ectomycorhiziennes diffèrent en fonction du choix de la profondeur de séquençage – sans « prime à la profondeur ». C'est en particulier le cas dans les sols forestiers, pour lesquelles les séquençages profonds se sont avérés moins performants que les séquençages superficiels, la saturation de la diversité détectée se produisant au terme de quelques centaines de reads (Chapitre II). Cette analyse a révélé la complémentarité des images fournies par les composantes physiquement établies (apex mycorhiziens) et libres (rhizosphère et sol) d'une même communauté, à différentes profondeurs de séquençage.

Le métabarcoding permet-il d'estimer l'abondance fongique?

Le métabarcoding n'est pas construit comme un outil d'évaluation des biomasses mycéliennes, contrairement à la qPCR (Parladé *et al.*, 2007). A ce titre, il ne permet pas d'estimer l'abondance des taxons ectomycorhiziens dans le sol à un instant donné, comme tous les outils disponibles (relevés de carpophore, quantification des apex ectomycorhiziens après morphotypage, etc.). Il en fournit une estimation contexte-dépendante. Cependant, un nombre

croissant de travaux tendent à utiliser, sans calibration préalable, le nombre de lecture d'une séquence (nombre de reads) comme une mesure de l'abondance des taxons (Merges *et al.*, 2018). Or, la structure des communautés ectomycorhiziennes « métabarcodées » dépend en partie de choix méthodologiques en laboratoire, dès l'amplification (réglages des thermocycleurs) et jusqu'à l'étape cruciale de la calibration du run de séquençage (Kausrud *et al.*, 2012; Janowski and Leski, 2023). Ces résultats nous rappellent si nécessaire que le nombre de lectures d'une séquence ne constitue pas un proxy de qualité de son abondance, l'estimation quantitative des champignons ectomycorhiziens restant un objectif encore peu atteignable malgré la performance sans précédent des outils de séquençage. Une analogie plausible serait qu'un mycologue devrait échantillonner durant des décennies afin de générer autant d'information qu'un run de séquençage en métabarcoding.

L'estimation qualitative des communautés ectomycorhiziennes par métabarcoding met en lumière la présence d'espèces moléculaires. Dans nos résultats issus du séquençage d'amplicons de sol et d'apex ectomycorhizés appariés (Chapitre II, Figure 7 et Encadré 2), nous avons révélé la présence probable de nombreux faux négatifs dans les résultats de métabarcoding. Ces faux négatifs sont des espèces moléculaires apparemment absentes – c'est-à-dire dont le matériel biologique a été observé avant séquençage, ou a été observé dans le même amplicon séquençé selon de modalités différentes de réglage du séquenceur. Ce résultat montre que la calibration du séquenceur influence la composition en espèces moléculaires du jeu de données. Toutefois, nos résultats ont également mis en évidence la capacité du métabarcoding et du barcoding Sanger à restituer les séquences ectomycorhiziennes dominantes issues d'un même échantillon (Chapitre II, Table 2).

Encadré 2 : Faux négatif : attention aux faux amis et vrais ennemis !

Un faux négatif est constitué d'une absence artéfactuelle d'une donnée. Ici, il s'agit de l'absence d'une entité taxonomique moléculaire dans un jeu données issu de métabarcoding, qui n'est pas une preuve suffisante de l'absence de l'espèce dans l'environnement séquençé. Au cours des analyses bio-informatiques, les singletons retirés de manière systématiques des jeux de données peuvent également constituer des faux négatifs.

Un faux positif est constitué de la présence artéfactuelle d'une donnée. Dans le cas des études utilisant le métabarcoding, il peut s'agir d'une erreur d'assignation taxonomique d'une séquence. Par conséquent, cela n'est pas un biais inhérent à la technique, mais une déformation ultérieure de biais ultérieur au séquençage – qui peut résulter de la difficulté d'assigner taxonomiquement des lignées à des fractions relativement courte du gène ITS.

Le métabarcoding permet la détection de toutes les entités biologiques fongiques présentes dans un échantillon environnemental. Cela inclut des formes de vie ralenties (sclérote), des formes de dormance (spores), des formes actives assimilatrices (mycélium) ou encore des formes reproductrices (carpophores, ascocarpes). Ainsi, les données générées par le séquençage de fraction de sol en métabarcoding ne permettent pas d'avoir une caractérisation de la fraction active de la communauté ectomycorhizienne, mais une estimation du pool potentiellement mobilisable par les végétaux, même dans les sols les plus pauvres en inoculum (Chapitre III). Cet outil permet donc de produire des diagnostics permettant par exemple de distinguer/hiérarchiser des sols dans lesquels l'inoculum ectomycorhiziens serait à renforcer en priorité.

2. Les plantes comme témoins des interactions avec les organismes du sous-sol ?

Dans nos expérimentations en conditions contrôlées (Chapitre II et III), nous avons utilisé des plantules ectomycorhiziennes afin de capturer la diversité fongique présente dans les sols. L'utilisation de plantes-piège est une technique issue de l'agronomie – aujourd'hui largement répandue pour analyser la colonisation des racines par les champignons mycorhiziens (Sýkorová *et al.*, 2007 ; Mickan *et al.*, 2016). Dans notre travail expérimental (Chapitre II et III), les plantes pièges utilisées ont été largement colonisées par les propagules ectomycorhiziennes du sol – y compris dans les sols urbains ayant subi un scellement prolongé. En vue d'analyser la composition des communautés ectomycorhiziennes établies avec les plantes hôtes, nous avons échantillonné les apex racinaires mycorhizés et nous avons séquencé l'ADN fongique extrait. Nous avons couplé l'approche agronomique d'utilisation des plantes pièges avec les outils de métabarcoding.

Dans une perspective de diagnostic écologique, l'utilisation de plante-piège représente une technique performante- aujourd'hui employée dans la plateforme expérimentale du quartier des Fabriques à Marseille (Chapitre IV). La mise en place de cette méthode peut comporter des limites – y compris logistiques et de contraintes temporelles fortes. En effet, la mycorhization de plantes pièges peut nécessiter plusieurs mois de croissance, et la capacité des plantules à se

connecter à leurs partenaires ectomycorhiziens est variable en fonction des espèces (Smith and Read 2010). Certaines plantes dont les réserves nutritives sont abondantes dans la graine ne se connectent à leurs partenaires ectomycorhiziens qu’après six mois de croissance (exemple du chêne vert, Richard *et al.*, 2009). Sur le plan technique, la collecte des plantes-piège dans leur intégralité est également une étape délicate au cours de laquelle la dégradation de l’appareil racinaire de la plante peut altérer l’échantillonnage des apex mycorhizés. Les difficultés de collecte des végétaux sont variables en raison de la grande variabilité existante dans les traits racinaires des plantes (intensité de ramification, colonisation verticale du sol, diamètre des racines fines, Iversen *et al.*, 2017 ; Guerrero-Ramirez *et al.*, 2020). Par exemple, chez le chêne vert, un pivot vertical plongeant en profondeur dès la germination rend son extraction particulièrement délicate in situ (Richard *et al.*, 2009).

Afin de répondre à la contrainte suscitée par la plantation et le suivi de plantes pièges semées de la main du scientifique ou de l’aménageur, une alternative intéressante serait d’analyser l’inoculum fongique mobilisé par les plantes s’établissant spontanément dans les villes. Pour l’heure, la flore spontanée a déjà été employée à des fins scientifiques – d’une part pour avoir un rôle de diagnostic de la diversité présente dans la ville (exemple du Projet de sciences participatives Les Sauvages de ma Rue, porté par Tela Botanica et le MNHN) et d’autre part pour analyser la réponse évolutive, morphologique, phénologique ou encore les traits de dispersion des plantes dans un contexte urbain (Cheptou *et al.*, 2008; Andrieu *et al.*, 2009). L’usage de la flore spontanée à destination d’une meilleure compréhension des interactions entre plantes et champignons est à tester, et permettrait de répondre à des questions scientifiques dont les applications sont de première importance. Parmi les espèces peuplant les communautés végétales spontanées, certaines plantes peuvent se révéler indicatrices de l’appauvrissement de la banque de spores (Figure 3, *Diplotaxis erucoïdes*, Trinchera *et al.*, 2021) ou de sa capacité biotique. D’autres, comme les espèces du genre *Helianthemum sp.* (Cistaceae), adaptées aux milieux stressants – tels que les villes (Xu *et al.*, 2023) – mobilisent des communautés ectomycorhiziennes diversifiées (Dickie *et al.*, 2004) et peuvent restituer une image particulièrement informative de la fraction mobilisable de la banque de spores présente en milieu urbain.

La plupart des plantes composant la communauté spontanée de la flore urbaine sont des plantes endomycorhiziennes. Toutefois, les travaux récents témoignant de la colonisation des

racines par des champignons ectomycorhiziens sous forme endophytiques (Schneider-Maunoury *et al.*, 2018; Liang *et al.*, 2020) permettent d'émettre l'hypothèse selon laquelle la flore spontanée pourrait jouer un rôle primordial dans l'établissement de l'inoculum ectomycorhizien en ville. Des travaux descriptifs à réaliser permettraient de repenser le rôle de la flore spontanée dans le fonctionnement des écosystèmes urbains. Par une meilleure compréhension de la fonctionnalité de cette flore spontanée (réservoir d'inoculum, plantes compagnes, paillage végétal, protection et enrichissement du sol en N), il peut ainsi être possible de repenser les pratiques de gestion de cette flore – par exemple, en intervenant moins sur son développement (arrachage) ou sur sa gestion par la chimie (désherbage, recours aux produits phytosanitaires), comme cela a déjà largement été initié dans de nombreuses collectivités.

En couplant le métabarcoding avec l'échantillonnage par la technique des plantes-pièges – incluant des plantes issues de la fraction spontanée de la flore urbaine, les aménageurs peuvent obtenir une estimation de la diversité fongique en général, et ectomycorhizienne en particulier. Les méthodes moléculaires associées au séquençage des apex racinaires peuvent se décliner soit en barcoding (qui restituera l'identité de la séquence fongique dominante), soit en métabarcoding (qui restituera la composition de la communauté fongique présente sur l'apex analysé).



Figure 3 : Fausse roquette (*Diplotaxis erucoides*, Brassicacées) : une rudérale non mycorhizée qui colonise les systèmes urbains, témoin du manque d'inoculum ?

IV. Comment intégrer ces réflexions à l'échelle paysagère ?

Dans le Chapitre IV, notre réflexion a montré que les villes pourraient emprunter des itinéraires de production de leurs propres ressources afin de subvenir à leurs besoins en sols et en plantes pour la conception de leurs espaces verts. Ici, nous détaillons ces deux aspects, tout en intégrant la ville dans un système paysager plus large – afin que dans ce cadre conceptuel, les villes soient elles-aussi des puits de ressources pour les écosystèmes naturels et péri-urbains.

1. Vers des villes capables de s'émanciper pour leurs besoins en sols

Notre travail a montré que la mobilisation des sols urbains scellés peut représenter une alternative avantageuse et écologiquement largement préférable à l'importation de sols issus des milieux agricoles et/ou forestiers péri-urbains. Toutefois, l'érosion de la diversité biotique des sols urbains scellés nécessite la mise en œuvre de méthodes de restauration adaptées de la fraction vivante de tels sols, afin de favoriser des boucles de rétroaction positives entre plantes et sols urbains. Bien que le renforcement montre des résultats prometteurs pour la remise en fonction de ces sols, sa pratique sur le long terme peut s'avérer contraignante et vectrice de pression sur les écosystèmes naturels situés à proximité des villes, par le prélèvement récurrent des horizons organiques des sols naturels. Plusieurs pistes sont à explorer pour limiter cet effet délétère d'une pratique visant la restauration biologique des sols urbains.

Une solution privilégiée pour l'accès à un inoculum fongique transférable en limitant les coûts écologiques et environnementaux pourrait reposer sur le développement d'une économie circulaire au sein des villes pour la ressource en sol. Pour l'heure, le développement de pratiques d'économie circulaire fait à la fois l'objet de développement empirique et l'objet de recherches universitaires interdisciplinaires. Les agences de paysage explorent empiriquement des méthodes de recyclage et d'inoculation des sols. Par exemple, dans la ville de Lyon, certains sols sont mis en andains durant quelques mois avant le transfert dans des fosses de plantations – afin de favoriser la colonisation de ceux-ci par la vie microbienne et la faune du sol (Figure 4a). A Marseille, dans le quartier des Fabriques, le sol inoculé des fosses de plantation de la plateforme expérimentale (Chapitre IV) a vocation à alimenter les sols des fosses de plantation des espaces végétalisés de tout le quartier (Figure 4b).

Figure 4 : a., b., mise en andain des sols destinés aux fosses de plantations à Lyon, France, c. sol récemment descellé dans le quartier des Fabriques, Marseille, et d. Végétalisation du sol récemment descellé dans les fosses de plantation expérimentales du quartier des Fabriques, Marseille, France



Des recherches complémentaires restent à mener afin que les villes accèdent à une certaine autonomie quant à leurs ressources en sol. D’abord, l’analyse des réservoirs potentiels d’inoculum ectomycorhizien au sein des écosystèmes urbains, tels que les fragments forestiers anciens, reste à compléter. Ensuite, la capacité de dispersion et de colonisation de cet inoculum reste à comprendre, à plusieurs échelles – incluant la dispersion intra-muros, et les flux avec les écosystèmes péri-urbain. Aujourd’hui, la dispersion des propagules ectomycorhiziennes est à l’étude dans le quartier des Fabriques à Marseille, avec l’implantation de pièges à spores dans plusieurs zones de collecte urbaines et péri-urbaines (Chapitre IV).

Ce travail de thèse a porté sur un cas d’étude dans une ville Méditerranéenne et dans les écosystèmes dominant les paysages proches. Les verrous de sa transférabilité vers d’autres villes

dont le climat est plus tempéré ou océanique apparaissent méthodologiquement peu contraignants, les sols scellés constituant un enjeu commun aux cités de l’anthropocène.

2. Vers des villes s’émancipant en favorisant les synergies entre plantes

Aujourd’hui, la mortalité des végétaux en ville reste particulièrement élevée durant les cinq ans qui suivent la plantation (Hilbert *et al.*, 2019; Wattenhofer and Johnson, 2021) – et ce malgré l’intervention humaine dans la gestion des arbres dans les villes (amendement, arrosage, Figure 5a). Plusieurs raisons peuvent expliquer cette mortalité accrue. Le choc de transplantation – qui survient après l’extraction des plantes en pépinière et leur transfert dans les fosses de plantation peut être expliqué par plusieurs facteurs : d’une part, les itinéraires de pépinières favorisent une croissance rapide des arbres, mais ne tiennent pas compte des besoins biotiques des végétaux (Encadré 3, Figure 5b). D’autre part, la faible diversité taxonomique (et potentiellement fonctionnelle) des communautés ectomycorhiziennes résiduelles, couplées à des conditions physico-chimiques d’accueil peu avantageuses dans les sols scellés, peuvent s’avérer limitantes pour l’établissement des végétaux.

Figure 5 : a) Chêne verts en production horticole pour l’aménagement urbain b) chênes pubescents récemment plantés en difficulté sur le campus universitaire des Sciences de Montpellier



Dans une perspective d'économie circulaire, des systèmes de production de plantes peuvent être pensés à l'échelle des villes. Plus spécifiquement, en s'appuyant sur les interactions de facilitation qui peuvent s'établir entre plantes voisines, les espaces verts urbains pourraient constituer des espaces pépinières, en ville, dans lesquels des végétaux « muscleraient » leurs communautés fongiques racinaires en co-culture avec leurs espèces nourrices. En localisant la production de végétaux dans les villes, ces derniers pourraient montrer une meilleure adaptation aux conditions climatiques et pédologiques de l'espace urbain – ce qui limiterait le choc de plantation et la mortalité constatée (Encadré 3). Toutefois, le développement de pépinières urbaines n'affranchirait pas les maîtres d'ouvrage de repenser les méthodes de gestion et de plantation en ville. Le développement de pépinières urbaines demandera certainement beaucoup de temps et de formation des services pour l'appropriation de la gestion de ces espaces.

Encadré 3 : Repenser les itinéraires de pépinières ?

Les arbres destinés à l'espace verts urbains sont produits dans des pépinières horticoles dont les plus importantes sont situées en Espagne, en Hollande et en Italie (par exemple Vanucci Piante, Comm. Pers. Ilex Paysage + Urbanisme, photo ci-dessous). Ces systèmes de production recourent à des pratiques de tuteurage et de taille qui interviennent dans le développement architectural (Toca *et al.*, 2022). La croissance des arbres est maximisée par l'usage d'engrais azotés, phosphorés et par un l'arrosage abondant (Petri *et al.*, 2016 ; Allen *et al.*, 2017). Dans ces conditions de saturation en sources de nutriments assimilables et en eau, les végétaux ainsi produits développent sur leurs racines des communautés fongiques ectomycorhiziennes sensiblement altérées, pauciflores et peu adaptées aux sols stressants (Correia *et al.*, 2021). Il a par exemple été démontré que les régimes d'arrosage compensant les pertes par évapotranspiration réduisent la mycorhization, et notamment l'installation d'espèces ectomycorhiziennes telles que les Tuberaceae (Olivera, Bonet and Oliach, 2014). Dans des systèmes de plantation forestière, la fertilisation azotée génère une baisse de la production de biomasse chez les communautés ectomycorhiziennes liées aux épicéas (Nilsson and Wallander, 2003) et aux hêtres (de Witte *et al.*, 2017) ainsi qu'une altération de la composition des communautés interagissant avec le pin tordu (*Pinus contorta*, Teste, Lieffers and Strelkov, 2012). Les itinéraires de pépinières n'incorporent que timidement la place des interactions mutualistes ectomycorhiziennes. Pourtant, l'inoculation contrôlée est réalisée avec succès depuis les années 70, notamment dans le cadre de la production de plantules à forte valeur économique pour la production de truffes (inoculation de chênes truffiers, Murat, 2015 ; Zambonelli, 2015), de lactaire sanguin (*Lactarius sanguifluus*, inoculation de pins sylvestres et maritimes, Parladé, Pera and Luque, 2004). Par ailleurs, en contexte forestier de production ligneuse, l'inoculation des essences cultivées est pratiquée depuis des décennies, notamment chez le pin Douglas, inoculé par le champignon *Laccaria bicolor* – avec un succès d'établissement et de croissance accrue des arbres (Villeneuve, Le Tacon and Bouchard, 1991 ; Selosse *et al.*, 1998). L'usage de la symbiose ectomycorhizienne représente une alternative aux engrais chimiques (Sousa *et al.*, 2012), mais aussi une aide à la croissance permettant de limiter les interventions manuelles de dégagement des jeunes plants (Selosse, 2001). Dans le contexte méditerranéen, des plantules d'*Arbutus unedo* produites en pépinière avec une inoculation ectomycorhizienne par *Pisolithus tinctorius* ont démontré de meilleures capacités d'établissement après la transplantation, une résistance accrue au stress hydrique (Navarro García *et al.*, 2011).

Les conséquences délétères du « choc de transplantation » induisent une mortalité accrue des arbres en ville (Hilbert *et al.*, 2019) – dans lesquelles la responsabilité de certaines pratiques de pépinières peut être invoquée. La collecte des arbres produits en pleine terre est souvent traumatique : environ 30 % des racines fines des individus sont excisées de l'arbre lors de l'extraction (Allen *et al.*, 2017). Un conditionnement inadéquat des végétaux (c.a.d. en motte, racines nues, container, Allen *et al.*, 2017; Wattenhofer and Johnson, 2021) ainsi que les conditions de transports peu délicates peuvent dégrader et affaiblir les végétaux (Nowak, Kuroda and Crane, 2004).

Mon travail n'a pas exploré la vie de ces végétaux avant leur arrivée en ville – dont les itinéraires de pépinières pourraient expliquer à la fois le déficit de mycorhization sur les racines des arbres transplantés et leur difficulté de reprise après la transplantation en ville. Aujourd'hui, certaines entreprises de production horticole en interaction avec des chercheurs considèrent sérieusement à repenser leurs méthodes de production pour favoriser à la fois le développement architectural de la plante et sa transplantation (Thèse Alexandre De Haldat – en préparation : « *Interactions racines/tiges chez les arbres dans un contexte de changement climatique. Applications pour la production en pépinière des arbres d'ornement* » à l'UMR AMAP, Montpellier). Dans la perspective de repenser les systèmes de production des végétaux, l'inclusion systématique des pré-requis biotiques – notamment ectomycorhizien pourrait améliorer la reprise des végétaux.

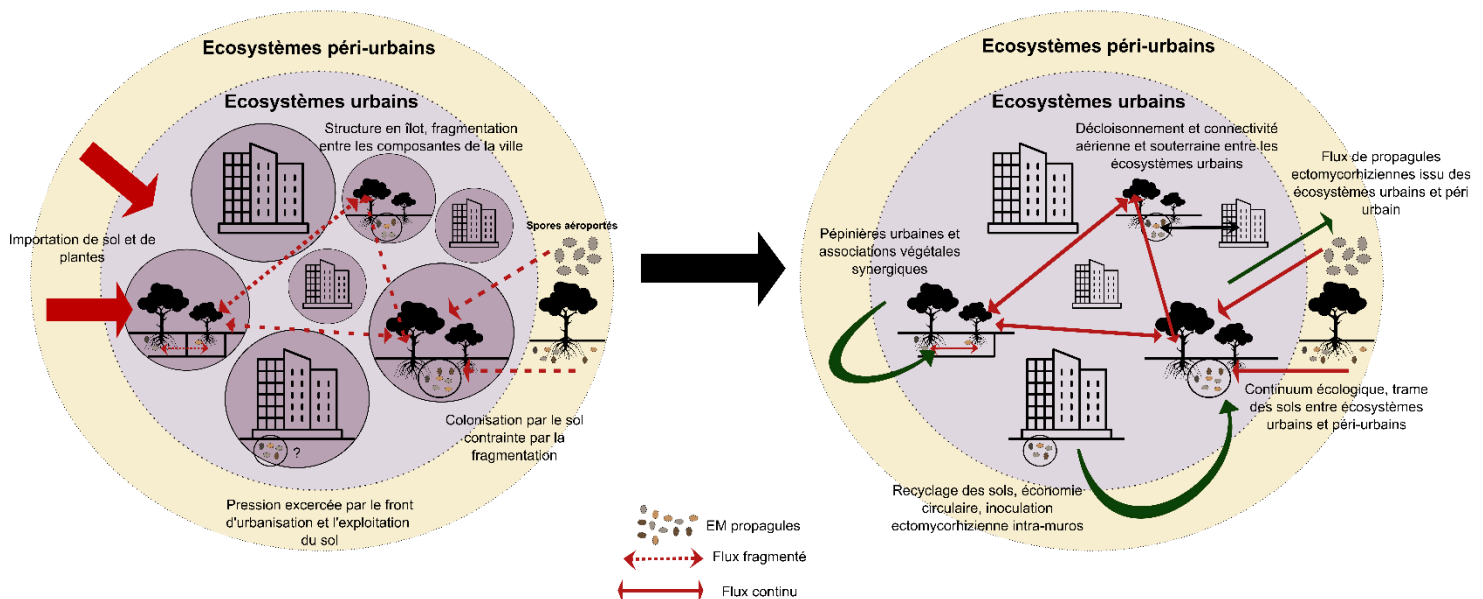
V. Conclusion générale : la connectivité comme mot clé des villes du futur

Aujourd'hui, les villes sont des systèmes qui absorbent les ressources tout en exerçant une pression intense sur les écosystèmes adjacents (Figure 6). Les enjeux actuels liés à la disponibilité des ressources en sol et au dérèglement climatique tendent à questionner les méthodes de conception des écosystèmes urbains – incitant les villes à développer des circuits d'économie circulaire pour pourvoir à leurs besoins en plantes et en sols (Figure 6). A l'échelle paysagère, une perspective à long terme serait celle de la mutation entre des villes consommatrices de ressources limitées (comme le sont les sols vivants), à des villes qui seraient productrices de ces mêmes ressources (Figure 6). Toutefois, pour favoriser les interactions biotiques, il est fondamental de promouvoir la connectivité écologique à plusieurs niveaux d'organisation – incluant la connectivité entre les écosystèmes urbains et celles avec les écosystèmes péri-urbains. Dans les Chapitres I et IV nous avons identifié le caractère lacunaire de la continuité écologique sur les trames du sol. La création de continuums écologiques – comme par exemple la trame beige du sol proposée Chapitre 1 – permettra une colonisation accrue des organismes du sols à différents niveaux d'organisation (intramuros, et avec les écosystèmes péri-urbains adjacents, Figure 6).

Figure 6 : Vers des villes intégrées dans un continuum paysager

Des villes qui absorbent les ressources...

à des villes qui s'autoalimentent et fournissent des ressources.



Une autre facette de la connectivité pour favoriser l'établissement d'écosystèmes urbains durables est celle des interactions entre les mondes de la recherche scientifique en écologie des communautés et celui de l'aménagement des espaces publics en milieu urbain. Après ces trois ans d'interactions synergiques et mutualistes, notre travail a mis en lumière la résilience de communautés ectomycorhiziennes dans les environnements postindustriels ayant subi un scellement prolongé. Ce travail offre des possibilités prometteuses tant pour la conception d'espace vert basés sur le recyclage des sols urbains et des perspectives de recherche fondamentales sur l'écologie des champignons ectomycorhiziens ambitieuses. Parmi les perspectives de fondamentales et appliquées identifiées au cours de ce travail, la plupart ne seront réalisables que par un décloisonnement entre le monde de la recherche et celui de l'aménagement urbain. Les villes sont des territoires qui concentrent des enjeux importants – démographiques, climatiques, sociaux. Elles concentrent également en leurs centres la majorité des institutions impliquées dans la construction de celles-ci. Géographiquement, tout le monde peut s'y retrouver – et la proximité peut favoriser le décloisonnement entre plusieurs domaines de compétences. Enfin, les villes étant des espaces hybrides où se confondent humains et non-humains, le concours des sciences sociales pour une meilleure compréhension s'avère nécessaire.

VI. Bibliographie de la discussion générale

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