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Par

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Impact de la fertilisation phosphatée et de la mycorhization arbusculaire sur le développement de porte-greffes de noyer fruitier micropropagés

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Impact of phosphate fertilization and arbuscular mycorrhiza on the development, nutrition and quality of micropropagated walnut rootstocks

Summary

The English walnut (*Juglans regia* L.) is the main species cultivated for the production of edible nuts. Owing to a sparse canopy and a deep rooting system, walnut is an ideal species for alley cropping, an agroforestry practice able to enhance productivity through interplant facilitative mechanisms. Walnut agroforestry requires the large scale production of seedling rootstocks selected to provide the best anchorage, vigour, and tolerance of pathogens. Due to the heterozygosis of walnut, the characteristics of agronomical interest of the chosen cultivar are not inherited *via* seed propagation. *In vitro* plant tissue culture thus plays a key role in mass propagation of high-quality walnut rootstocks. Micropropagation of walnut explants needs an *ex vitro* acclimatization phase to repair the *in vitro* induced abnormalities, and further requires a *post*-acclimatization growth in greenhouse conditions when plantlets become photoautotrophic. However, poor survival and slow growth rates are common difficulties encountered in nurseries when establishing micropropagated walnut saplings. As many other fruit and nut bearing trees, walnut exhibits a high dependency on symbiotic soil-borne arbuscular mycorrhizal (AM) fungi for development due to a coarse root architecture that limits soil inorganic phosphate (Pi) uptake. In the context of rootstock production, the aim of this thesis was to investigate at different development stages the establishment of seven walnut rootstocks of economic interest, upon inoculation or not with an AM fungus and two contrasting Pi fertilization regimes. We demonstrated that biotization with the AM fungus *Rhizophagus irregularis* decrease micropropagated walnut rootstock dependency on Pi fertilization both at the acclimatization and *post*-acclimatization stages, together with improving adequate sapling development and quality, especially in the hybrid rootstocks VLACH, VX211, and WIP3. We also showed that these benefits are rootstock-dependent, indicating that walnut mycorrhizal dependency for Pi nutrition varies between cultivars, and that the symbiotic program may be differentially recruited depending on the chosen rootstock. Detection of putative *Juglans* orthologues to the AM-essential essential proteins in the model legume *Medicago truncatula* was further performed with Orthofinder using the BLAST all-vs-all algorithm. When combined with differential gene expression analysis in mycorrhizal and non-mycorrhizal walnut RG17 rootstocks under two contrasting Pi fertilization regimes, this approach led to the identification of mycorrhiza-inducible *JrPHT1*-, *JrFATM*-, *JrRAM2*, and *JrSTR2*-like genes only under Pi starvation. To address the benefits of mycorrhizal walnuts in alley cropping, we experimentally simulated walnut agroforestry system using compartmentalized microcosm in which young walnut hybrid rootstocks RX1 (*J. regia* x *J. microcarpa*) were connected or not by a common mycelial network (CMN) to maize plants (*Zea mays* L.) grown under contrasting Pi supply levels. Within a few weeks, the inoculum reservoir formed by walnut saplings allowed the mycorrhization of maize roots, thereby giving access to the maize nutrient pool. We showed that hyphal connections under P deficient condition result for both plants in growth and nutritional benefits when compared to P sufficient condition without a CMN. We concluded that a CMN is able to alleviate Pi deficiency in co-cultivated walnut and maize plants, and may therefore contribute to limit the use of chemical fertilizers in agroforestry systems.

Impact de la mycorhization arbusculaire et de la fertilisation phosphatée sur le développement, la nutrition et la qualité de porte-greffes de noyer fruitier micropropagés

Résumé

Le noyer anglais (*Juglans regia* L.) est la principale espèce cultivée pour la production de noix comestibles. Sa canopée peu dense et son système d'enracinement profond font du noyer une espèce idéale pour la culture en allées, une pratique agroforestière pouvant améliorer la productivité en favorisant les mécanismes de facilitation inter-plantes. L'agroforesterie du noyer fruitier nécessite la production à grande échelle de porte-greffes (PG) sélectionnés pour offrir un meilleur ancrage, une meilleure vigueur et tolérance aux agents pathogènes. En raison de l'hétérozygotie du noyer qui ne reproduit pas à l'identique ces caractéristiques d'intérêt agronomique, la multiplication végétative *in vitro* joue un rôle clé dans la propagation des PG de noyer fruitier de qualité. La micropropagation des explants végétaux nécessite une phase d'acclimatation *ex vitro*, puis une post-acclimatation en serre lorsque les plantules deviennent photoautotrophes. Cependant, lors de l'établissement de vitroplants, les pépinièristes rencontrent des difficultés face à un taux de mortalité important et une croissance lente. L'appareil racinaire peu ramifié du noyer limite l'absorption du phosphate inorganique du sol (Pi), c'est pourquoi le noyer présente une forte dépendance aux champignons mycorhiziens à arbuscules (MA) symbiotiques du sol pour sa nutrition phosphatée et son développement. Dans le contexte de la production de PG de noyer fruitier, cette thèse a consisté à analyser, à différents stades de développement, l'établissement de sept PG présentant un intérêt économique, après inoculation ou non par un champignon MA dans des conditions contrastées de disponibilité en Pi. Nous avons démontré que la biotisation par le champignon MA *Rhizophagus irregularis* améliore le développement, la nutrition et la qualité des PG lors de l'acclimatation *ex vitro* et *post-vitro* en situation de carence phosphatée, en particulier chez les PG hybrides VLACH, VX211, et WIP3. Toutefois, ces avantages dépendent du PG, suggérant un recrutement différentiel du programme symbiotique selon le cultivar étudié. Ceci nous a conduit à rechercher chez le noyer les orthologues des protéines essentielles à la symbiose MA chez la légumineuse modèle *Medicago truncatula*, en utilisant Orthofinder à l'aide de l'algorithme BLAST all-vs-all. Nous avons validé les candidats *JrPHT1, JrFATM, JrRAM2* et *JrSTR2* par l'étude comparative de leur expression après inoculation ou non par *R. irregularis* dans des conditions contrastées de disponibilité en Pi. Afin d'étudier les avantages de la mycorhization du noyer en culture en allées, nous avons simulé à l'aide de microcosmes compartimentés un système agroforestier dans lequel le PG RX1 (*J. regia* x *J. microcarpa*) est relié ou non par un réseau mycélien commun (RMC) à des racines de maïs (*Zea mays* L.), cultivé dans des conditions contrastées de disponibilité en Pi. En huit semaines, le réservoir de propagules fongiques formé par les racines de noyer a permis la mycorhization du maïs, autorisant ainsi un accès aux résidus de fertilisation. Nous avons montré que le RMC développé lors d'une carence en Pi conduit chez les deux plantes en co-culture à des bénéfices en termes de croissance et de nutrition, comparables à ceux observés sans mycorhization et sans carence. Cette étude démontre qu'un RMC peut pallier une carence phosphatée chez le maïs et le noyer élevés en co-culture, et donc contribuer à limiter l'apport d'intrants chimiques en systèmes agroforestiers.

Sommaire

Introduction

1. Agroécologie et agroforesterie

Face aux enjeux agricoles actuels, la France s'est inscrite dans une démarche visant à promouvoir et assurer une transition agroécologique de son agriculture. A cet effet, la loi d'avenir pour l'agriculture, l'alimentation et la forêt (loi n° 2014-1170) a été adoptée le 13 octobre 2014. Le développement de systèmes agroécologiques est une priorité qui se traduit par la mise en œuvre de politiques publiques incitatives de promotion et de pérennisation de systèmes de production agroécologique. Elles encouragent et aident le recours à des pratiques et à des systèmes de cultures innovants dans une démarche agroécologique et se veulent de nature « à faciliter la production, le transfert et la mutualisation de connaissances, y compris sur les matériels agricoles, nécessaires à la transition vers des modèles agroécologiques ». Il est en effet considéré que les systèmes agroécologiques qui permettent de favoriser la biodiversité et les interactions biotiques au sein des agrosystèmes sont de nature à constituer des leviers pour diminuer la dépendance des productions agricoles aux intrants chimiques. Ils sont reconnus comme des systèmes performants tant sur le plan économique qu'environnemental et social. L'objectif global de ces politiques est de proposer à travers des systèmes de production agroécologique des solutions durables et efficaces qui devraient permettre à la ferme France de s'adapter aux conséquences du changement climatique et à la surexploitation des ressources naturelles. Parallèlement à la transition agroécologique promut par la loi d'avenir de l'agriculture, le code de l'environnement a été modifié pour introduire « des clauses visant au respect et la préservation de la ressource en eau, de la biodiversité, des paysages, de la qualité des produits, des sols et de l'air, la prévention des risques naturels et la lutte contre l'érosion, y compris des obligations de maintien d'un taux minimal d'infrastructures écologiques ». A ce titre, le sol est considéré comme un support nourricier vivant qu'il convient de préserver notamment par l'introduction d'infrastructures écologiques (bandes enherbées, haies, arbres) visant à renforcer et garantir la durabilité des exploitations agricoles.

L'agroforesterie est l'une des composantes principales des politiques publiques en faveur de la transition agroécologique. A cet égard, le Ministère de l'Agriculture a lancé en décembre 2015 un plan de développement de l'agroforesterie consistant à introduire des arbres et des haies dans les grandes cultures pour obtenir des bénéfices agricoles et environnementaux (https://agriculture.gouv.fr/un-plan-national-de-developpement-pour-lagroforesterie; Van Lerberghe 2015; Liagre 2012). Le terme agroforesterie apparaît en 1977 dans le cadre d'un programme canadien porté par des chercheurs du Centre de Recherche pour le Développement International visant à résoudre les problèmes de déforestation tropicale causés par l'érosion (P. K. Ramachandran Nair 2011)). Il recouvre des systèmes agroforestiers ancestraux et variés: sylvopastoralisme, pré-vergers, bocages, cultures intercalaires en vergers fruitiers, truffiers, noyeraies, et vignobles qui sont présents mondialement sous différentes formes (Coulon et al. 2000). Globalement, l'agroforesterie correspond à des dispositifs visant à potentialiser les services écosystémiques rendus par les arbres et les sols agricoles (**Fig. 1**). Ainsi, on peut donner comme exemple d'un élement de l'agroforesterie, la chute des feuilles des arbres qui constitue une source de matière organique fraîche susceptible d'être minéralisée par les microorganismes telluriques (40 % de la biomasse d'un arbre retourne au sol chaque année), ou d'être humifiée augmentant ainsi la fertilité des sols. Le développement des racines des arbres augmente la macroporosité des sols ce qui facilite l'infiltration de l'air et de l'eau en profondeur, favorisant ainsi la régulation des flux d'eau et un bon état structural du sol (https://www.agroforesterie.fr/definition). En présence d'une culture, les racines de l'arbre se développent préférentiellement en profondeur afin de limiter la compétition avec les racines de la culture intercalaire pour les ressources en surface (Allen et al. 2004). Par ailleurs, dans les systèmes agroforestiers, les racines des arbres peuvent intercepter les polluants d'origine agricole (nitrates), et limiter leur diffusion vers les eaux souterraines (Rowe et al. 1999; Cadisch, Willigen, and Suprayogo 2004). L'intérêt de l'agroforesterie réside également dans le fait que les arbres favorisent le maintien de la biodiversité en attirant les insectes rampants ou volants, en servant d'habitat aussi bien pour les oiseaux que les pollinisateurs des cultures

intercalaires et de refuge pour les auxiliaires de cultures (Liagre 2012). Les arbres en créant un microclimat du fait d'un apport d'humidité et d'ombre, limitent la prolifération de ravageurs et de diverses adventices (Altieri 1987). Les arbres jouent donc un rôle prépondérant dans la régulation des ravageurs et pourraient contribuer à limiter le recours aux produits de protection des plantes.

Les systèmes agroforestiers en permettant la séquestration du carbone dans la biomasse de l'arbre, contribuent à diminuer la teneur en CO_2 de l'atmosphère. Leur déploiement en masse pourraient contribuer à atténuer le réchauffement climatique (Van Lerberghe 2015). Compte tenu de ses nombreux intérêts, l'agroforesterie a la capacité de participer significativement au fonctionnement agroécologique du système agricole et à son efficacité économique. La filière bois étant de plus en plus sollicitée par la demande croissante en matériaux renouvelables et le développement de nouveaux marchés (e.g. utilisation du bois en chimie et dans l'industrie), une amélioration de sa production pourrait présenter à terme un intérêt financier non négligeable pour l'exploitant (Jaenicke and Beniest 2003).

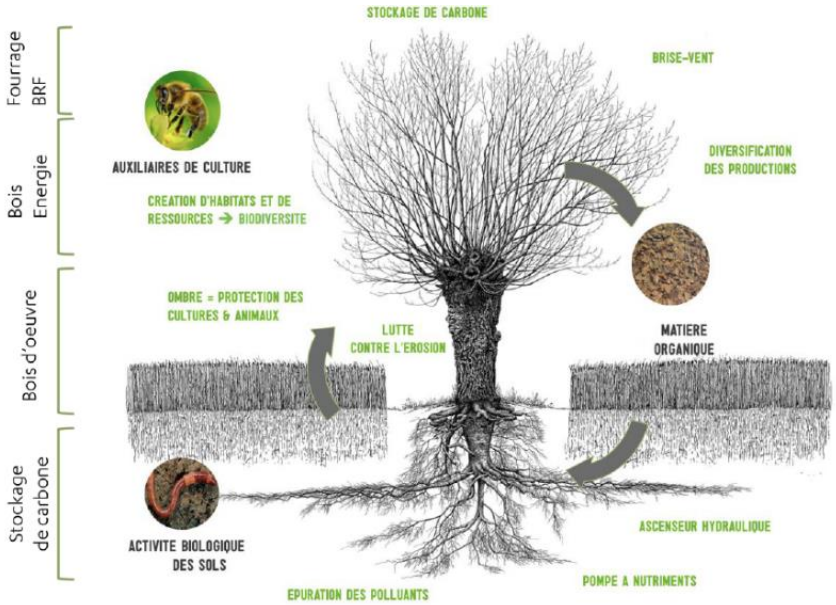


Figure 1. Représentation schématique des services écosystémiques attribués aux arbres associés à des parcelles agricoles (agroforesterie.fr).

Cependant, l'ensemble des profits issus d'un système agroforestier ne peut être effectif que lorsque la mise en place et le maintien de ce système sont correctement effectués. Peu d'agriculteurs se lancent dans la mise en oeuvre de tels systèmes notamment par manque de maitrise des itinéraires techniques : soit de production des fruits de l'arbre, soit du bois. Les réticences sont aussi souvent liées au fait que les profits tirés par la présence de l'arbre sur le système de production ne sont pas immédiats, l'ensemble des bénéfices d'un système agroforestier étant à considérer à long terme (Moorhead and David Dickens 2012). Il convient aussi de reconnaître que l'arbre peut avoir des effets négatifs sur la productivité du système, la composition et la diversité des communautés de plantes présentes sur le terrain (Dubey et al. 2016). L'agriculteur est confronté à la nécessité de considérer les conditions environnementales (e.g. type de sol, humidité, taux de phosphate, taux d'azote) et les investissements financiers nécessaires. Les études dans ce domaine restent trop peu nombreuses et seulement quelques-unes ont été conduites en conditions réelles du champ (Nerlich, Graeff-Hönninger, and Claupein 2013).

A ces contraintes pratiques et économiques s'ajoute la nécessité d'une production à grande échelle d'arbres et d'arbustes sains disponibles à la plantation. Pour faire face à cette difficulté, différentes approches ont été développées incluant la sélection de greffons choisis pour leurs propriétés agronomiques et de porte-greffes choisis pour leur rusticité. L'une des stratégies de production de greffons et de porte-greffes reposent sur des processus de multiplication végétative (Jaenicke and Beniest 2003). La multiplication végétative est un mode de reproduction asexuée naturelle qui propage des individus identiques à la différence du semis qui génère des individus à patrimoines génétiques différents. Cette méthode permet la conservation des caractères d'une variété donnée, susceptibles de se perdre ou de s'affaiblir par reproduction sexuée. La multiplication végétative permet une production en masse de plants et un racourcissement significatif du cycle de production comparativement à la reproduction sexuée des jeunes arbres notamment en s'affranchissant de l'étape de germination. Ces méthodes de production par bouturage ou marcottage sont largement utilisées pour la production de végétaux depuis les années 1970. Elles font appellent à des techniques de multiplication végétative par culture *in vitro* à grande échelle, dites de micropropagation qui ont apporté un progrès considérable par rapport aux méthodes traditionnelles de multiplication (Auge et al. 1989). Ces techniques reposent sur le concept de totipotence cellulaire énoncé par Haberlandt (Haberlandt; 1902), selon lequel toutes les informations génétiques, et en particulier le programme de l'embryogenèse, sont présentes dans chaque noyau de la cellule végétale (Norreel 1973). Ainsi, les cellules végétales prélevées sur tout organe disposent de la capacité de régénérer un individu complet identique à la plante mère (Quashie 2009). Suivants ces principes, la première régénération de plante entière à partir de cellules végétales somatiques a été obtenue chez le tabac en 1965 (Vasil and Hildebrandt 1965) ouvrant une nouvelle ère de développement de laboratoires commerciaux aux Etats-Unis et en Europe destinés à satisfaire les besoins horticoles. La micropropagation ainsi utilisée à partir d'un seul individu (plant), permet l'obtention d'un nombre considérable de plantes génétiquement identiques à la plante mère, avec un taux de multiplication moyen de l'ordre de 200 000 plants par an à partir d'une bouture (Ferry, Bouguedoura, and El Hadrami 1998; Semal 1998). Ces techniques de multiplication ont aussi le grand avantage de nécessiter peu d'espace et de s'affranchir de la saisonnalité. La micropropagation a également rendu possible la multiplication d'espèces pour lesquelles les semences sont rares, ou qui présentent des difficultés de germination et sur lesquelles les techniques de bouturage ou de greffage sont inapplicables. Ces techniques ont permis la commercialisation d'une plus grande diversité des plantes et l'amélioration des performances agronomiques ou horticoles des plantes cultivées (Skirvin et al. 2000).

2. Multiplication végétative *in vitro* des ligneux feuillus

Appliquées aux arbres, la multiplication végétative constitue un levier essentiel, voire incontournable au developpement de l'agroforesterie. De nombreuses espèces de ligneux feuillus sont multipliés *in vitro* à partir de microbouturage d'explants de méristèmes prélevés sur la tige des arbres (Sbay, Lamhamedi, and Lhafi 2015). Les méristèmes constitués de cellules indifférenciées conservent la capacité de se diviser et gardent jusqu'à leur mort leur caractère juvénile. Ils confèrent à la plante une organogenèse permanente chez les végétaux supérieurs (Margara 1982). La multiplication de méristème apicaux (situé à l'extrémité d'une tige) ou axillaires (situés au niveau d'un bourgeon axillaire ou nœud), permet l'obtention à la fois d'un grand nombre d'individus tous identiques génétiquement et indemnes de maladies (Schmid, Weber, and Keller 1984). Bien que les bourgeons apicaux aient un potentiel de croissance supérieur à ceux des bourgeons axillaires, ils ont l'inconvenient d'être souvent plus délicats à prélever. C'est pourquoi la préférence va plus généralement à l'excision des nœuds caulinaires (Sbay, Lamhamedi, and Lhafi 2015). Le processus de multiplication de feuillus *in vitro* passe par une étape de production de tiges multipliées par

bourgeonnement dont les progénitures sont plantées pour enracinement puis acclimatées aux conditions *ex vitro*. Ce microbouturage *in vitro* présenté en **Fig. 2A**, et illustré en **Fig. 2B** dans le cas spécifique du noyer choisi comme espèce modèle de cette thèse, se déroule en six étapes successives :

(1) la préparation du matériel végétal consiste en la sélection des tiges, leur désinfection, et l'excision des explants nodaux en conditions aseptiques (**Fig. 2 a-b**);

(2) l'initiation (mise en culture) vise à favoriser *in vitro* la croissance des bourgeons axillaires néoformés (**Fig. 2 c-d**);

(3) la multiplication consiste à promouvoir l'apparition de tigelles et de nombreux bourgeons actifs qui seront à leur tour fragmentés et multipliés *in vitro.* A cette étape, les cytokinines sont employées pour favoriser la multiplication cellulaire (**Fig. 2 e-f**). Il s'agit d'une étape importante puisque le coefficient de multiplication est le critère économique majeur dans le cadre d'une production commerciale ;

(4) lors la phase d'enracinement (rhizogénèse), les vitroplants sont sectionnés en tigelles individualisées qui sont repiquées sur un milieu riche en auxines, en conditions humides (**Fig.2 g-h**);

(5) l'acclimatation (sevrage) est le passage des conditions *in vitro* aux conditions *in situ* **Fig.2 i-j**)**.** Cette étape est nécessaire pour obtenir des plants suffisamment robustes pour les cultiver ultérieurement en plein champ. Les vitroplants étant élevés sur un milieu riche en sucre, dans une hygrométrie de 100% et dans des conditions aseptiques, le sevrage consiste à les adapter progressivement à l'hygrométrie naturelle, ainsi qu'à rendre progressivement les feuilles et les racines plus efficientes. À la sortie du milieu de culture, les vitroplants sont nettoyés puis repiqués en terrines dans un substrat dont la composition varie selon le coût des différents constituants (e.g. terreau, perlite, vermiculite, tourbe, sable fin). L'acclimatation s'effectue en mini-serres, à l'abri d'une luminosité trop vive, et sous une atmosphère saturée en humidité, qui est progressivement réduite;

(6) à cette phase d'acclimatation succède la phase de *post*-acclimatation au cours de laquelle les plants deviennent phoautotrophes après rempotage et élevage en serres (**Fig.2 k-l**).

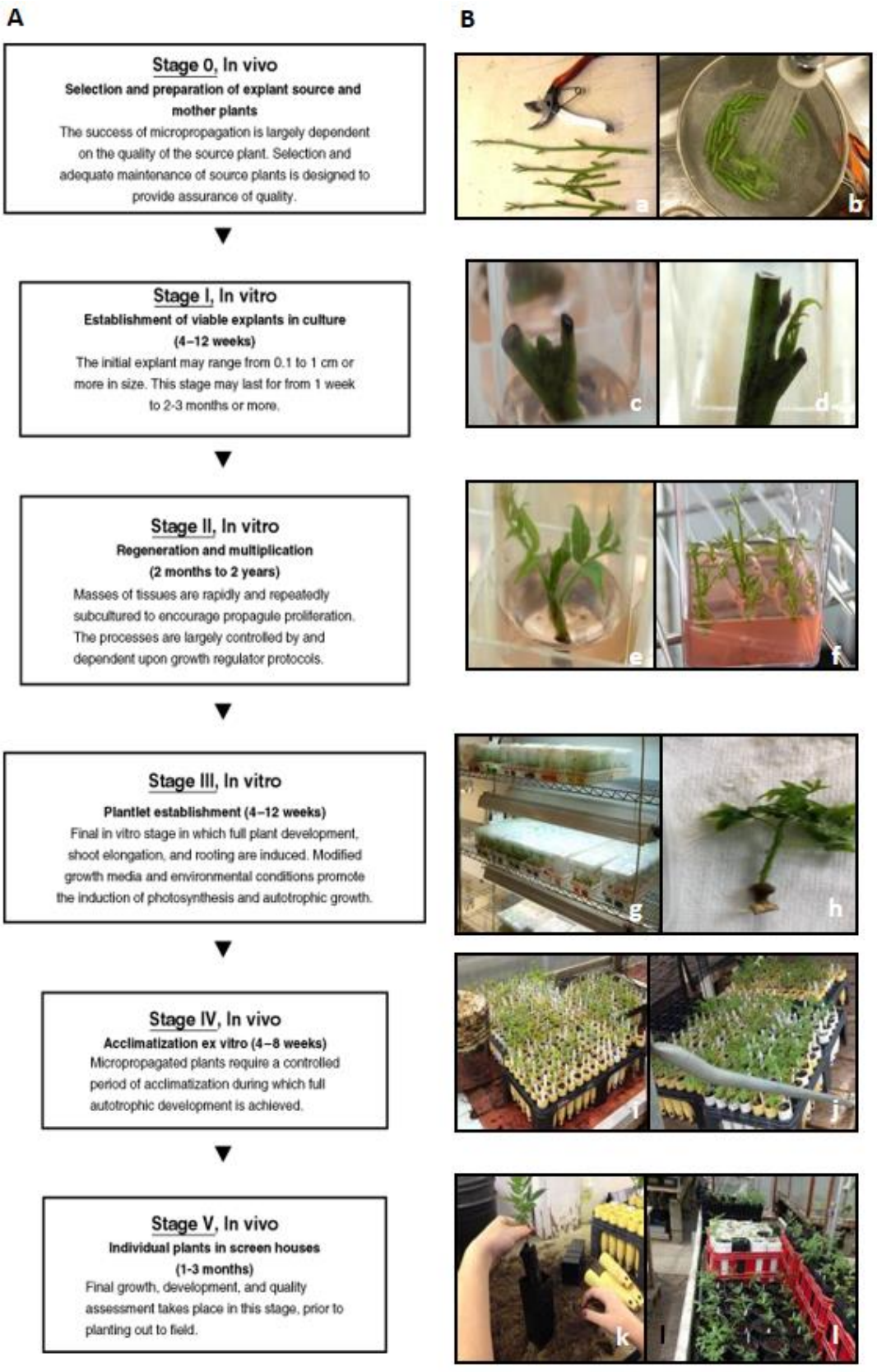


Figure 2 Les différentes étapes de la micropropagation végétale selon **A**) Altman and Loberant (2003); et **B**) illustrées dans le cas du noyer: **a**) tiges destinées à la culture in vitro; **b**) lavage avant désinfection; **c**) explant de tige cultivé in vitro: générant **d**) un bourgeon; **e**) tige excisée de l'explant; et **f**) multipliée *in vitro*; **g**) **h**) développement caulinaire et radiculaire en conditions *in vitro*; **i**) **j**) acclimatation *ex vitro*; **k**) **l**) culture en serres des plants micropropagés (http://walnutrootstock.ucanr.edu/WIP/Propagation/Micropropagation).

Bien que la micropropagation permette une multiplication très rapide de pieds-mères de haute qualité, cette production *in vitro* a toutefois ses limites. Elle est en effet onéreuse tant par la main d'œuvre que par la quantité de matériel nécessaire. La production augmente fortement au cours de la multiplication induisant un accroissement des besoins en main d'œuvre, d'espaces de culture et de fournitures (Sbay, Lamhamedi, and Lhafi 2015). La mortalité des vitroplants est non négligeable et peut atteindre 80% pour les variétés fragiles du fait des difficultés techniques rencontrées lors des étapes de sevrage et de *post*-acclimatation (Peixe et al. 2015). L'acclimatation et l'élevage en serre sont des étapes très importantes. Elles préparent les jeunes plants à résister aux difficultés auxquelles ils seront confrontés dans leurs milieux naturels. Au cours de l'acclimatation, les vitroplants passent d'un milieu axénique, saturé en eau et riche en sucre, à un milieu de plus faible hygrométrie et en conditions de ressources carbonées limitées (Borkowska 2002). Les feuilles des plantules micropropagées présentent des caractéristiques qui limitent leur autonomie lors du sevrage (Rohr et al. 2003; Chandra et al. 2010). Elles sont en effet peu épaisses avec un parenchyme supérieur constitué d'une seule couche de cellules palissadiques pauvres en chloroplastes, et les stomates non fonctionnels restent ouverts. Il en résulte un risque de déshydratation, une efficacité photosynthétique réduite, une limitation de l'adaptation autotrophe des plantules, et un épuisement des ressources carbonées disponibles. La richesse en sels minéraux des milieux de culture défavorise la rhizogénèse des vitroplants particulièrement chez les espèces ligneuses. L'absorption minérale lors de l'acclimatation des vitroplants est limitée par l'absence de poils absorbants et la faible ramification de l'appareil racinaire. Lors de la phase de *post*-acclimatation, l'absorption minérale des racines peut aussi être compromise du fait d'une superficie de contact avec le sol réduite; de la faible porosité des racines lignifiées, et des dommages mécaniques des racine suite au rempotage (Grossnickle 2012).

3. Biotisation des ligneux feuillus

Le rôle positif des microrganismes endophytes au cours des différentes étapes de la micropropagation et de l'acclimatation *ex vitro* a été montré par de nombreuses études conduites au cours des deux dernièeres décennies (**Fig. 3**). Herman a introduit en 1996 le terme biotisation pour désigner les bénéfices tirés de la co-culture de plantes micropropagées avec des bactéries ou des champignons (Kanani, Modi, and Kumar 2020). En effet, les racines des plantes sont naturellement associées à une multitude d'organismes qui jouent un rôle essentiel dans la nutrition des végétaux et influencent la productivité et la pérennité des écosystèmes forestiers (http://docs.gip-ecofor.org/public/forestier.pdf; 2018). Au niveau de la rhizosphère qui correspond à la zone de sol influencée par les racines des plantes, les microorganismes telluriques régulent les flux de nutriments par transformation des matières organiques en éléments minéraux solubles assimilables par les plantes, et la formation d'associations symbiotiques participe directement à la nutrition des arbres.

Dans ce complexe, les champignons mycorhizogènes à arbuscule (CMA) jouent un rôle essentiel. Ils font partie des endophytes les plus étudiés dans le cadre des travaux de recherche sur la biotisation des ligneux et des fruitiers (Rai 2001; Taylor and Harrier 2003; Kapoor, Sharma, and Bhatnagar 2008; Kanani, Modi, and Kumar 2020). Les racines absorbantes de plus de 80% des plantes terrestres, incluant les angiospermes, gymnospermes, pteridophytes et bryophytes, établissent des associations symbiotiques avec des CMA à l'interface sol-plante (Smith and Read 2009). Le réseau dense et étendu des hyphes des champignons mycorhiziens aide les racines de la plante à explorer un grand volume de sol et à accéder à des endroits qui leur sont inaccessibles. Le champignon contribue à améliorer la nutrition de la plante en apportant principalement de l'eau, du phosphore et de l'azote, et à l'accroissement de la biomasse végétale tout en assurant une protection contre les stress biotiques et abiotiques (Chen et al. 2018). On constate qu'en moyenne la biomasse des plantes mycorhizées est trois fois supérieure à celle des témoins non-inoculés (Hoeksema et al. 2010).

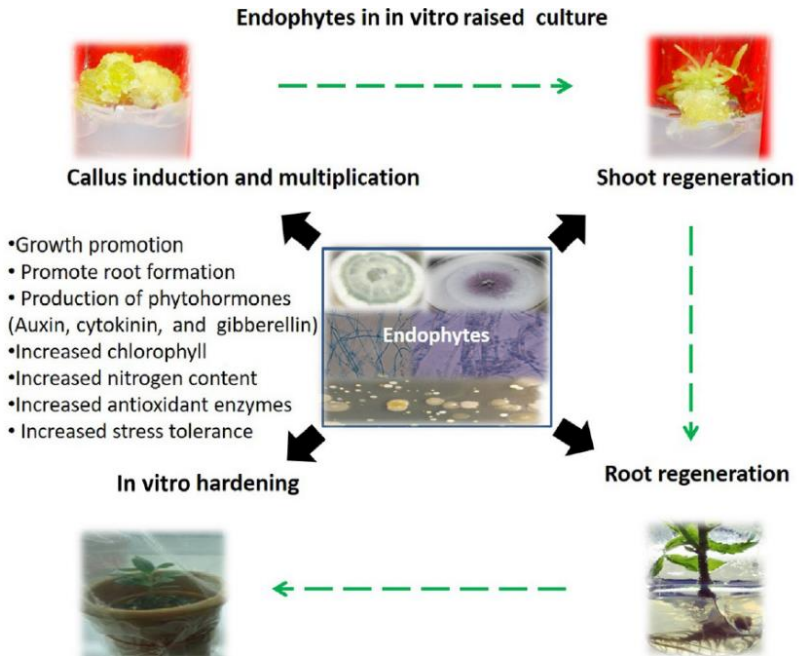


Figure 3. *Bénéfices des microorganismes endophytes à différentes étapes de la culture de plants micropropagés (Kanani et al. 2020).*

Comme l'une des fonctions majeures de la symbiose endomycorhizienne est de permettre aux arbres de s'ajuster aux conditions édaphiques (Rai 2001), l'intégration des CMA dans les différentes étapes de la micropropagation a été étudiée y compris lors de la phase d'enracinement *in vitro*, du sevrage et de la *post*-acclimation *ex vitro* de ces espèces ((Taylor and Harrier 2003), pour revue). Une amélioration de la survie et de la croissance *ex vitro* des microplants après leur mycorhization a été constatée chez de nombreuses espèces fruitières comme par exemple la vigne, le prunier, et le cerisier (Taylor and Harrier 2003). Il a ainsi été démontré que la présence de CMA au cours de l'enracinement *in vitro* du prunier-cerise (*Prunus cerasifera*) et de la vigne (*Vitis vinifera*) permet d'obtenir un système racinaire plus ramifié, améliorant l'acclimatation *ex vitro* des vitroplants (Schellenbaum et al. 1991). L'effet positif des CMA à cette étape est associé à une amélioration de la nutrition phosphatée (Locatelli and Lovato 2002; Cavallazzi et al. 2007), et à l'augmentation de la pression osmotique du système vasculaire (Nowak 2003).

Ces résultats accréditent la dépendance mycorrhizienne de la majorité des ligneux et des fruitiers pour leur croissance et leur développement. La notion de dépendance mycorhizienne exprime la mesure dans laquelle la symbiose permet de satisfaire les besoins nutritionnels pour la croissance de l'hôte végétal lorsque les ressources du sol sont limitantes ou inaccessibles (Janos 2007). Il s'agit d'une caractéristique génotypique des plantes que reflète souvent la morphologie du système racinaire Les racines des plantes présentant peu de poils absorbants sont théoriquement moins efficaces dans l'exploration du sol et l'absorption des éléments minéraux comparativement aux plantes disposant d'un système racinaire ramifié (Hetrick 1991). La dépendance mycorhizienne est essentiellement modulée par le phosphate inorganique (Pi), très peu mobile dans le sol et dont le stock dans l'environnement immédiat des racines s'épuise très vite (Balzergue 2012). Les CMA en augmentant le volume de sol exploré, permettent de dépasser cette zone d'appauvrissement grâce à leur mycélium extra-racinaire composé d'hyphes très fins et beaucoup plus longs que les poils absorbants (environ 100 fois plus) (Javot, Pumplin, and Harrison 2007). Les champignons endomycorhiziens sont capables de dégrader des molécules organiques du sol pour libérer du Pi et l'acquérir grâce à des transporteurs à haute affinité (Harrison and Buuren 1995; Maldonado-Mendoza, Dewbre, and Harrison 2001). Ce phosphate est ensuite transféré à la plante *via* des transporteurs dont l'expression est induite au niveau racinaire par la symbiose mycorhizienne à

arbuscule (Javot, Pumplin, and Harrison 2007; Parniske 2008). En retour, la plante fournit du carbone au champignon sous forme de sucres et de lipides. La génomique des CMA laisse à penser que ces champignons, bien que très riches en lipides, sont dépendants de la biosynthèse des acides gras de l'hôte (Wewer, Brands, and Dörmann 2014). Plusieurs travaux de génétique des plantes et de biochimie ont depuis démontré que les lipides de l'hôte sont une source de carbone nécessaire au partenaire fongique qui est auxotrophe pour les acides gras (Bravo et al. 2017; Keymer et al. 2017; Luginbuehl et al. 2017; Jiang et al. 2016). Bien que très bien caractérisés chez les légumineuses (e.g. *Medicago truncatula, Lotus japonicus, Glycine max*), les céréales (e.g. maïs, blé, riz, sorgho), la pomme de terre et la tomate, seuls quelques transporteurs symbiotiques de Pi ont été identifiés chez les espèces pérennes, dont le peuplier et la vigne (**Tableau 1**). De même, si la voie de biosynthèse et de transport des lipides nécessaire à la symbiose mycorhizienne à a été caractérisée chez des plantes modèles commme *M. truncatula* et *L. japonicus*, elle reste méconnue chez les ligneux feuillus.

Tableau 1. Exemples de transporteurs de Pi induits dans les racines de différentes espèces végétales en réponse à l'endomycorhization.

Espèce végétale	Transporteur	Référence
Brachypodium distachyon (Brachypode à deux épis)	BdPT7	Hong et al. 2012
Glycine max (Soja)	GmPT10	Tamura et al. 2012
Lotus japonicus (Lotier)	LjPT4	Volpe et al. 2016
Medicago truncatula (Luzerne tronquée)	MtPT4	Harrison, Dewbre, and Liu 2002
Oryza sativa (Riz)	OsPT11	Paszkowski et al. 2002; S.-Y. Yang et al. 2012
Populus trichocarpa (Peuplier baumier)	PtPT10	Loth-Pereda et al. 2011
Sorghum bicolor (Sorgho commun)	SbPT11	Walder et al. 2015
Lycopersicon esculentum (Tomate)	LePT4	Nagy et al. 2005
Lycopersicon esculentum (Tomate)	LePT5	Nagy et al. 2005
Solanum tuberosum (Pomme de terre)	StPT4	Nagy et al. 2005
Solanum tuberosum (Pomme de terre)	StPT5	Nagy et al. 2005
Vitis vinefera (Vigne cultivée)	VvPht1-2	Valat et al. 2018
Vitis vinefera (Vigne cultivée)	VvPht1-1	Valat et al. 2018
Zea mays (Maïs)	ZmPht1-6	Glassop, Smith, and Smith 2005; Willmann et al. 2013; X. Liu et al. 2016

4. Le noyer fruitier

Le noyer commun (*Juglans regia*), l'une des 25 especes du genre *Juglans*, est le principal producteur de noix comestibles. La France est le premier producteur européen de noix avec une production annuelle d'environ 35 000 tonnes, et le septième producteur mondial derrière la Chine, l'Iran, et les Etats Unis (production annuelle moyenne de 38 000 tonnes) (https://www.planetoscope.com/Epices/1819-production-mondiale-de-noix.html). En 2012, 627 921 tonnes de noix ont été récoltées en Californie sur près de 126 000 ha, ce qui représente l'équivalent d'un milliard de dollars (CA Ag. Comissioners' Rpt, 2012). Le noyer adapté à des sols profonds, calcaires, fertiles et bien drainés, préfère les climats tempérés. Il présente une tolérance limitée aux faibles températures et a des besoins en eau importants. Ces exigences pédoclimatiques expliquent la localisation des noyeraies fruitières françaises principalement en Dordogne et en Isère (Forêt-entreprise n°207; novembre 2012; http://www.pirinoble.eu/docs/fr/Noyers.pdf). On recense dans la noyeraie française essentiellement huit variétés classiques à fructification terminale (noix en bout de branches), dont Franquette, Corne, Marbot et Grandjean, supports de l'AOP Noix du Périgord. Deux autres variétés, Mayette et Parisienne, sont prépondérantes en Isère. Deux variétés, Lara et Fernor, à fructification sur brindilles latérales (noix tout le long de la branche) complètent la gamme variétale (https://www.noixduperigord.com/fr/noix-du-perigord/de-larbre-au-fruit). Outre la production de noix comestibles, les vergers de noyers produisent une grande diversité de co-produits issus des coques, des feuilles et du bois (**Fig. 4**).

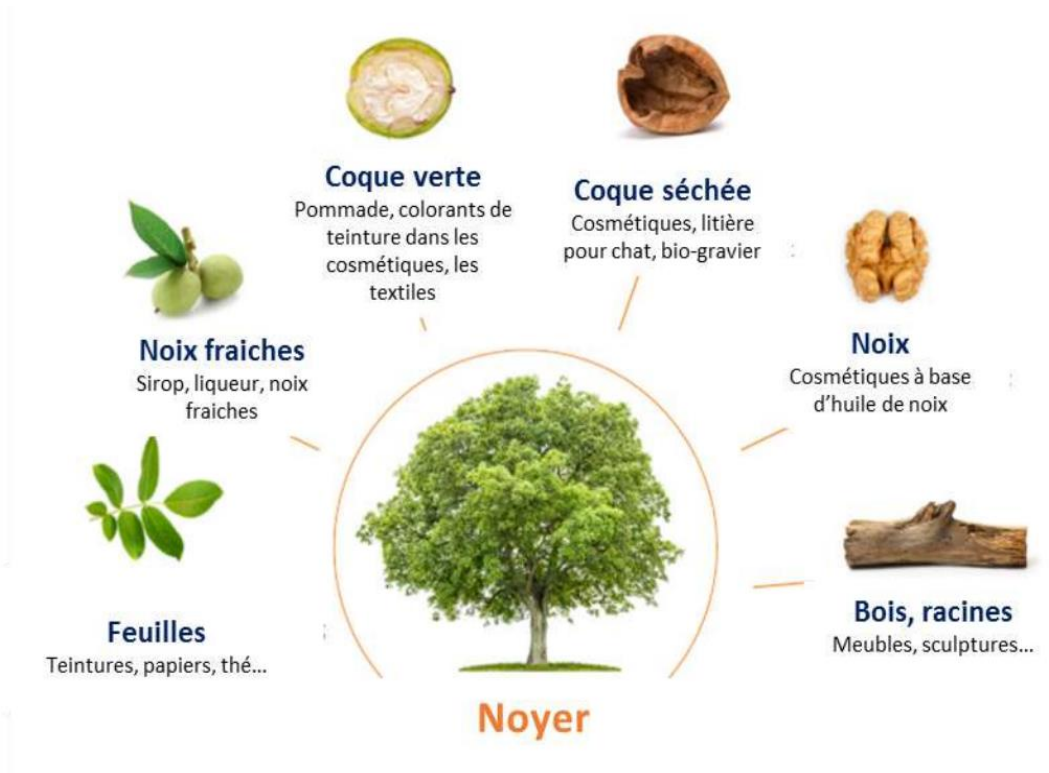


Figure 4. Différentes valorisations des co-produits générés en vergers du noyer fruitier (AlpBioEco, 2019).

4.1. Le noyer fruitier en agroforesterie

Les premières traces de la pratique de la culture sous noyers datent de l'époque romaine (Lelle and Gold 1994; leplongeon 2017). Elle a été maintenue jusqu'à nos jours malgré l'intensification et la mécanisation de l'agriculture (Dupraz et al. 2008). Avec environ 6 000 ha de cultures intercalaires dans le Dauphiné et 3 000 ha dans le Périgord (Coulon et al. 2000), les noyeraies françaises constituent le plus grand exemple de cultures intercalaires d'Europe (Dupraz et al. 2018). Les variétés traditionnelles de noyer fruitier sont productives la dixième année de plantation (https://www.noixduperigord.com/fr/noix-du-perigord/de-larbre-au-fruit). C'est pour cette raison que la pratique de cultures intercalaires sous vergers de noyers joue le rôle d'une culture relais dans l'attente de la production fruitière des noyers. La plupart des jeunes plantations de noyers sont entretenues par des cultures intercalaires constituées essentiellement de maïs (**Fig. 5**), de sorgho, de céréales d'hiver (blé, orge), ou encore d'oléoprotéagineux (soja, colza, tournesol), de tabac, de plantes fourragères (luzerne), de plantes aromatiques (lavande), de petits fruits (cassis, groseille), et d'arbres fruitiers (poiriers, pommiers, vigne) (Wolz and DeLucia 2018). Parallèlement, la vente de bois constitue un complément de revenu, les arbres de valeur sont abattus et remplacés ce qui permet d'entretenir un équilibre entre noyers jeunes et adultes (Dupraz et al. 2008).

Figure 5. Noyer et maïs en agroforesterie (USDA National Agroforestry Center, 2012).

La vocation fruitière des arbres impose des contraintes spécifiques pour la pratique des cultures intercalaires. Elle ne doit pas être un obstacle à la récolte mécanisée des noix en septembre. La filière nucicole a développé un savoir-faire de la gestion des interactions de l'arbre en co-culture pour maximiser les bénéfices de l'agroforesterie. La culture intermédiaire assure un revenu à court terme et limite les charges d'entretien de la noyeraie contrairement à un sol nu ou enherbé. Elle assure aussi l'autoconsommation des produits issus de la culture (alimentation animale), tout en stimulant le développement des jeunes noyers (Coulon et al. 2000). Les légumineuses intercalaires (pois, soja, féverole) améliorent ainsi la croissance des noyers grâce à leurs résidus azotés (Liagre, 1993). D'autres pratiques comme la mise en place de culture de maïs intercalaires sur deux années consécutives suivie d'une culture d'hiver favorisent la formation des arbres (Dupraz et al. 2008). L'interculture conduite proche des arbres favorise une croissance en hauteur des noyers (Dupraz

1994). Ainsi, en deux ans, le noyer atteint une taille suffisante pour permettre son rabattage et la formation de branches plus vigoureuses. Après la moisson, le nuciculteur peut cultiver un blé d'hiver ou de l'orge pour faciliter les interventions sur la parcelle (Dupraz et al. 2008). L'agroforesterie permet ainsi la mise en place d'une culture rentable jusqu'à la récolte des produits de l'arbre (fruits, bois, autres).

Selon certaines études, la rentabilité de ces systèmes serait avérée à long terme par la diversification des produits récoltés sur une même parcelle et du fait de l'affranchissement progressif des engrais de synthèse (Moorhead and David Dickens 2012; Nerlich, Graeff-Hönninger, and Claupein 2013). Le rôle de la symbiose mycorhizienne à arbuscule dans l'agroforesterie impliquant le noyer n'a à notre connaissance jamais été étudié sous l'angle de la nutrition minérale. Elle l'a été essentiellement en tant que vecteur d'allélopathie. En effet, les noyers adultes produisent de la juglone (5-hydroxy-1,4 naphthoquinone), qui est un inhibiteur de la respiration chez certaines plantes (Willis 2000). On trouve ce composé aromatique dans les feuilles, les fruits, l'écorce et les racines des plantes adultes au genre *Juglans* principalement. Dans les systèmes agroforestiers, la juglone peut être transportée du noyer jusqu'aux racines de la plante intercalaire via les hyphes des CMA et avoir des effets délétères. Ces effets ont été montrés par exemple sur la tomate (Achatz and Rillig 2014; Achatz et al. 2014).

4.2 La micropropagation du noyer fruitier

La production de noix nécessite d'utiliser des porte-greffes sélectionnés pour assurer un développement rapide de l'arbre, une tolérance aux organismes phytopathogènes d'origine tellurique et une croissance suffisante au-delà des premières mises à fruits. Un bon équilibre vigueur-production est indispensable au maintien dans le temps d'une production abondante, régulière et de qualité. Le noyer est encore multiplié directement par semis dans de nombreux pays. Ces semis (*J. regia* pour la majorité) sont utilisés comme porte-greffes servant à la multiplication par greffage des variétés sélectionnées (Pouquet 2010). Cette approche a l'inconvenient de créer une hétérogénéité des vergers puisque les semis ne permettent pas de produire des porte-greffes à caractéristiques génétiques identiques. Pour y remédier, des programmes de création de porte-greffes clonaux par multiplication végétative ont été initiés au début des années 1980 suite aux premiers succès de cultures aseptiques de cals de noyer (Jay-Allemand, Capelli, and Cornu 1992; Driver et al. 1984). Les recherches françaises conduites au CTIFL ont permis d'obtenir plusieurs génotypes de *J. regia* aptes à la micropropagation, dont RG2, RG6, RG12, RG15 et RG17. Après sélection sur des critères agronomiques de rendement et de calibre des noix, plusieurs clones dont RG2 ont été inscrits au catalogue des variétés de fruitiers en 1996. Ces porte-greffes restent cependant encore peu utilisés par les producteurs (Pagès and Prunet 2011). Les programmes de recherche conduits aux Etats-Unis et en particulier en Californie (UC Davis Walnut Breeding Program), se sont plus portés sur la micropropagation et la commercialisation de variétés hybrides (*J. hindsii* x *J. regia; J. hindsii x J. microcarpa*) sélectionnés pour leur tolérance aux principaux ravageurs d'origine tellurique (nématodes, agrobactéries, oomycètes) (Browne 2009). La micropropagation des tigelles de noyers hybrides est souvent limitée par une nutrition en phosphate inorganique altérée qui inhibe la croissance des vitroplants (Barbas et al. 1993). Dans le cas de ces variétés, le taux de mortalité des vitroplants de noyer enracinés peut être total après transfert en serre (Hackett et al. 2010).

5. Objectifs et organisation du mémoire de thèse

Les premières investigations sur l'impact bénéfique de la symbiose mycorhizienne à arbuscule sur l'établissement du noyer en milieu naturel ont été conduites dès les années 1980. Elles se rapportent essentiellement à la question du noyer-bois (*J. nigra*) issu de semis sur le continent américain. Il ressort de ces études que l'inoculation des racines par certains CMA permet d'améliorer la survie après la *post*-acclimataion en augmentant entre autres le nombre de racines latérales et la nutrition minérale des arbustes. Malgré le rôle de la symbiose mycorhizienne à arbuscule dans l'établissement des ligneux feuillus, peu d'études se sont intéréssées au rôle des CMA dans l'adaptation *ex vitro* de noyers micropropagés ni à leur rôle dans la nutrition phosphatée (Dolcet-Sanjuan et al. 1996; Peixe et al. 2015). Le séquençage récent de certains génomes du genre *Juglans* (Chen et al. 2019), fait que ni les transporteurs de phosphate, ni la voie de biosynthèse et de transport des lipides nécessaires à la symbiose mycorhizienne à arbuscule n'ont été caractérisés à ce jour.

Considérant par ailleurs que dans les systèmes agroforestiers, et en particulier en France, le noyer fruitier est très fréquemment associé à des cultures annuelles intercalaires comme le maïs (Wolz and DeLucia 2018) et qu'aucune étude n'a été consacrée aux échanges nutritionnels interspécifiques entre les deux cultures d'une même parcelle *via* les CMA, il a paru pertinent de conduire un projet de recherche dans le cadre de la présente thèses sur ces sujets. Cette thèse s'articule donc autour de trois objectifs principaux visant à :

1. Evaluer l'impact d'une mycorhization contrôlée sur la croissance et la nutrition de différents porte-greffes micropropagés de noyer fruitier lors de leur acclimatation et *post*-acclimatation en conditions carencées ou non en Pi ;

2. Identifier chez le noyer fruitier les orthologues des transporteurs de Pi et des protéines impliquées dans la biosynthèse et de transport des lipides décrits chez d'autres espèces et étudier leur expression en conditions carencées ou non en Pi ;

3. Analyser en système agroforestier simplifié intégrant une co-culture de maïs, l'influence de la mycorhization du noyer sur la croissance et la nutrition des deux espèces en conditions carencées ou non en Pi.

Le manuscrit se décompose en trois parties et cinq chapitres dont certains sont présentées sous la forme d'articles, soumis ou en préparation en suivant le sommaire suivant :

La partie 1 consiste en une analyse bibliographique présentant :

Chapitre I : La symbiose mycorhizienne à arbuscule

Chapitre II : L'interaction symbiose endomycorhizienne-noyer

La partie 2 présente sur trois chapitres les résultats obtenus et leurs discussions :

Chapitre III : Impact de la mycorhization lors de l'acclimatation et la *post*-acclimatation de plusieurs porte-greffes de noyer fruitier au cours d'une carence phosphatée ou non.

Chapitre IV : Identification chez le noyer fruitier des orthologues des transporteurs de Pi et des protéines impliquées dans la biosynthèse et dans le transport des lipides décrits chez d'autres espèces et étudier leur expression en conditions carencées ou non en Pi

Chapitre V : Influence en système agroforestier simplifié intégrant une co-culture de maïs, de la mycorhization du noyer sur la croissance et la nutrition des deux espèces en conditions carencées ou non en Pi

La partie 3 est consacrée aux conclusions générales et aux perspectives de ces travaux.

Partie 1 : Synthèse bibliographique

Chapitre I

La symbiose mycorhizienne à arbuscule

Le terme symbiose a été introduit en 1877 par Albert Bernhardt Frank pour décrire la coexistence mutualiste de deux organismes au sein d'un même environnement, chacun pouvant tirer des bénéfices l'un de l'autre (Frank, 1877). Anton de Bary a élargi cette définition en décrivant la symbiose comme une interaction intime et de long terme entre deux ou plusieurs espèces biologiquement distinctes, pouvant aller du mutualisme au parasitisme (de Bary, 1879). La mycorhize, etymologiquement du grec "mykitas" et "riza", signifiant respectivement "champignon" et "racine" est la symbiose la plus répandue (Smith and Read 2009). La symbiose mycorhizienne correspond à l'interaction s'établissant entre certains champignons du sol et une plante hôte. Elle se caractérise par la mise en place de structures fongiques au niveau des racines de la plante (Koide and Mosse 2004). Au cours de la symbiose mycorhizienne, le champignon apporte à la plante des éléments minéraux nécessaires à son développement, comme le phosphate (P) et l'azote (N). En retour, la plante apporte aux champignons des ressources carbonées issues de la photosynthèse. Grâce à cette symbiose, la plante a une meilleure croissance et améliore sa résistance à des stress biotiques et abiotiques (Karandashov and Bucher 2005; Parniske 2008; Smith and Read 2009). La grande diversité génétique et physiologique des champignons mycorhiziens explique leur ubiquité à tout type de sol.

Près de 6 000 espèces de Gloméromycètes, Ascomycètes et Basidiomycètes ont été recensées en tant que champignons mycorhiziens (Bonfante and Genre 2015). La classification la plus commune des différentes symbioses décrit sept types de mycorhizes qui sont classées en fonction de leurs caractéristiques structurales et des modalités de leur mise en place au sein de la plante. Deux groupes se distinguent par leur fréquence : les ectomycorhizes et les endomycorhizes. Les ectomycorhizes concernent principalement les arbres des forêts tempérées et boréales chez lesquels les hyphes fongiques ne pénètrent pas la paroi cellulaire mais entourent les différentes cellules du cortex racinaire pour former un manchon mycélien. Ce réseau d'hyphes appelé « réseau de Hartig » est une zone d'échange de nutriments entre les deux organismes. La majorité des symbioses ectomycorhiziennes implique des champignons appartenant à la classe des Basidiomycètes, mais ces symbioses peuvent aussi s'établir entre une plante et certains Ascomycètes et Zygomycètes (Read et al. 2000). A l'inverse, au cours de la symbiose endomycorhizienne, une partie des hyphes du champignon pénètre à l'intérieur des cellules du cortex racinaire de l'hôte pour établir les structures nécessaires aux échanges entre la plante et le champignon (Bonfante and Anca 2009). Il existe trois types de symbioses endomycorhiziennes : la mycorhize à arbuscule, la mycorhize éricoïde qui se forme entre les plantes de la famille des Ericacées, et la mycorhize orchidoïde strictement limitée à la famille des Orchidacées.

La symbiose mycorhizienne à arbuscule (SMA) est la symbiose endomycorhizienne la plus répandue. Elle se caractérise par la mise en place d'une structure extrêmement ramifiée d'échanges bidirectionnels : l'arbuscule (Karandashov and Bucher 2005; Parniske 2008; Bonfante and Genre 2010). Contrairement aux champignons ectomycorhiziens, les champignons mycorhiziens à arbuscule (CMA) dépendent entièrement de leur hôte pour leur nutrition carbonée, ce sont des biotrophes obligatoires (Bago et al. 2000). Ces CMA améliorent la nutrition hydrique et minérale de leur plante hôte en formant un réseau dense d'hyphes extra-radiculaires qui s'étend au-delà des racines et de la rhizosphère (Smith and Read 2009). Apparue il y a 460 millions d'années à la période de l'Ordovicien (Redecker 2000), la SMA aurait contribué à la nutrition et à l'établissement des premières plantes terrestres (Smith and Read 2009). Les CMA sont des champignons telluriques appartenant à un groupe très homogène et regroupés dans la classe des Gloméromycètes, le sous-phylum des Glomeromycotina (Spatafora et al. 2016) et le phylum des Mucoromycota (Bonfante and Venice 2020). Ces champignons sont présents dans la plupart des sols. Ils réalisent des symbioses avec plus de 80% des espèces végétales terrestres, incluant une grande diversité

d’espèces dicotylédones, monocotylédones, gymnospermes, ptéridophytes, lycopodes ou encore bryophytes (Kistner and Parniske 2002; Schüßler and Walker 2010). Certaines familles de plantes comme les Brassicaceae (choux, navet, colza, moutarde, cresson, arabette), les Chenopodiaceae (betterave, épinard), les Proteaceae (macadamier) et quelques Fabaceae dont le genre *Lupinus*, sont incapables d’établir cette symbiose et sont qualifiées de non mycotrophes (Brundrett 2009).

1 Cycle de développement des CMA

Le cycle de développement des CMA se déroule en plusieurs étapes. Lors de la première phase dite asymbiotique, les spores du champignon, dont la taille est comprise entre 40 et 150 µm (Trépanier et al. 2005), ont une capacité de germination et de croissance pendant quelques jours sans pratiquement entamer leurs réserves énergétiques (Bécard et al. 2004). Cette capacité permet au champignon de maximiser ses chances de rencontrer un partenaire symbiotique et ainsi de poursuivre son cycle de vie (Bago, Pfeffer, and Shachar-Hill 2000). En présence d'une plante hôte, le champignon entre dans la phase présymbiotique puis symbiotique (**Fig. I.1**).

1.1 Phase présymbiotique

La phase présymbiotique débute par un échange réciproque de signaux diffusibles entre la racine d'une plante hôte et le CMA (**Fig. I.1**). Des molécules contenues dans les exsudats racinaires induisent la ramification des hyphes germinatifs du CMA (Buée et al. 2000; Giovannetti et al. 1993). Les strigolactones, une classe de phytohormones dérivées du métabolisme des caroténoïdes, stimulent la croissance et la ramification des hyphes des CMA et sont également impliquées dans la germination des graines de certaines plantes parasites dont le genre *Striga* (Akiyama, Matsuzaki, and Hayashi 2005; Besserer et al. 2006). D’autres molécules sécrétées par la plante pourraient aussi être perçues par le champignon mycorhizien, mais leur rôle exact reste à preciser (Lenoir et al. 2017). Des flavonoïdes sont aussi impliqués dans la germination des spores et la ramification des hyphes mais leurs effets semblent contrastés en fonction des conditions (Steinkellner et al. 2007).

Au cours de sa croissance, le champignon secrète à son tour des molécules initiatrices du programme symbiotique de la plante hôte (Bonfante and Requena 2011). Les signaux diffusibles produits par le champignon (**Fig. I.1**), sont percus par la plante hôte et agissent sur l’expression de certains de ses gènes modifiant son métabolisme et son architecture racinaire (Weidmann et al. 2004; Oláh et al. 2005; Kuhn, Küster, and Requena 2010). Ces signaux sont appelés facteurs Myc par analogie aux facteurs Nod secrétés par les rhizobia, bactéries fixatrices d'azote. Les hyphes germinatifs secrètent en particulier un mélange de lipochitooligosaccharides (Myc LCOs) structuralement proches des facteurs Nod (Maillet et al. 2011). Ces molécules sont composées d’un squelette de N-acétylglucosamine de quatre à cinq résidus, qui peuvent être sulfatées ou non. L’application de ces molécules augmente le taux de mycorhization des plants traités et stimule la prolifération des racines latérales (Maillet et al. 2011). Les facteurs Myc et Nod par le biais des recepteurs LysM-RLK induisent chez la plante une voie de signalisation commune aux deux symbioses dénommée Common Symbiotic Pathway, impliquant en particulier les gènes *DMI* (Doesn’t Make Infection) *1*, *DMI2*, and *DMI3* (Gutjahr and Parniske 2013).

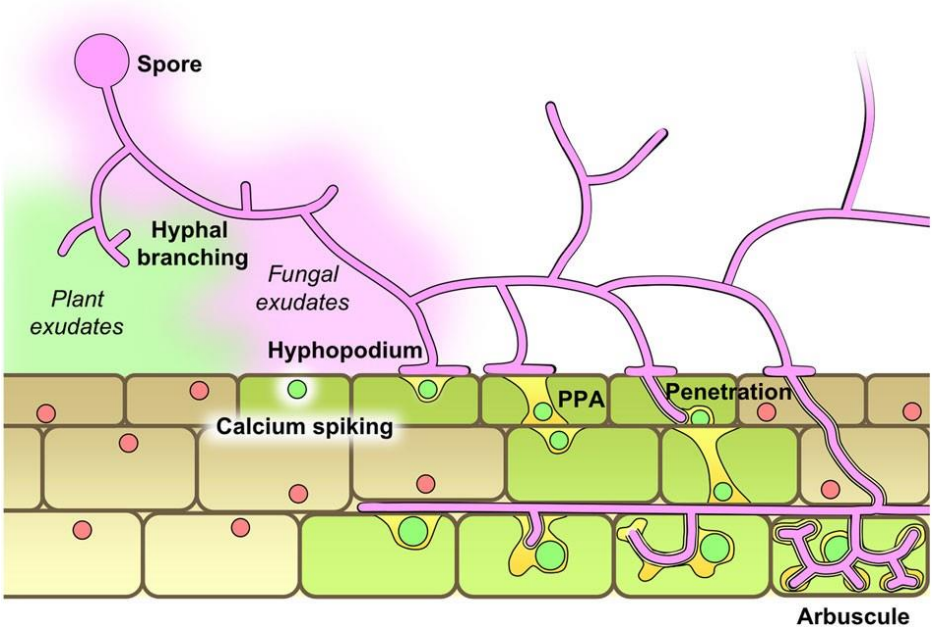


Figure I.1: Cycle de développement des champignons mycorhiziens à arbuscule. La phase asymbiotique: la spore germe mais se développe très peu sans la présence du partenaire végétal. La phase présymbiotique: échanges de signaux diffusibles sans contact direct entre les deux partenaires. La plante sécrète des exsudats perçus par le champignon, induisant sa ramification et son activité métabolique. Le champignon produit lui aussi des signaux perçus par les cellules racinaires, générant des variations de teneurs en calcium dans le cytoplasme et les noyaux, ainsi que l'activation de gènes végétaux. La phase symbiotique : le champignon forme un hyphopode à la surface de l'épiderme, la plante met en place un appareil de pré-pénétration (PPA) pour guider le développement du champignon à travers les différentes couches de cellules jusqu'aux cellules du cortex interne où sont mis en places les arbuscules où ont lieu les échanges. Le champignon achève son cycle en développant un important mycélium extra-radiculaire (ERM) qui forme une nouvelle génération de spores (Bonfante and Genre 2010).

1.2 La phase symbiotique

Une fois l'hyphe en contact avec la racine, le champignon développe des hyphopodes qui lui permettent d'adhérer aux cellules de l'épiderme de la plante hôte (**Fig. I.1**). A ce stade, le champignon induit la mise en place d'un appareil de prépénétration (PPA) dans la cellule épidermique, suite à un réarrangement polarisé du cytoplasme et du cytosquelette. Le PPA est un pont apoplasmique endocellulaire à travers lequel le champignon peut traverser les différentes couches cellulaires jusqu'aux cellules corticales (Genre et al. 2008). Une fois dans la cellule corticale, les hyphes se ramifient par dichotomie, ce qui produit des hyphes ayant un diamètre de plus en plus petit ; à partir d'un hyphe initial de 10 µm de diamètre, les dernières ramifications peuvent atteindre moins de 1 µm de diamètre (**Fig. I.2A**).

L'ensemble de ces ramifications prend une forme d'arbuste : les arbuscules, lieux des échanges symbiotiques (Parniske 2008).

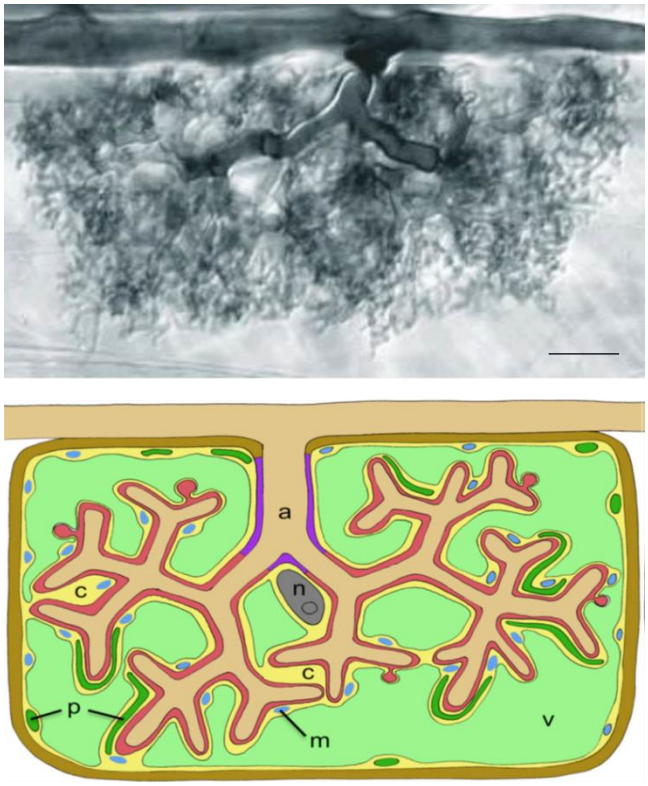


Figure I.2. Développement arbusculaire (échelle = 10 µm) selon **A)** Brundrett (2009); et **B)** représentation schématique du complexe membranaire intra-cellulaire constitué de la membrane plasmique du champignon, de la paroi fongique, d'un espace dérivé de l'apoplaste de la plante (espace périarbusculaire) et de la membrane périarbusculaire (Bapaume and Reinhardt 2012)

Simultanément, un réarrangement cellulaire conduit à la migration du noyau depuis le centre vers la périphérie de la cellule et au rassemblement de l'appareil de Golgi, des mitochondries, du réticulum endoplasmique à proximité de l'arbuscule et à la fragmentation de la vacuole (Lenoir et al. 2017; Bapaume and Reinhardt 2012). Au niveau de l'arbuscule, la mycorhize se caractérise par un complexe membranaire intra-cellulaire constitué de la membrane plasmique du champignon, de la paroi fongique, d'un espace dérivé de l'apoplaste de la plante, appelé espace périarbusculaire, et de la membrane plasmique végétale modifiée dénommée alors membrane périarbusculaire (**Fig.I.2B**). Les principaux échanges de nutriments entre le champignon et la plante interviennent au niveau de l'arbuscule par le biais de transporteurs localisés au niveau du plasmalemme fongique et de la membrane périarbusculaires de la plante hôte.

La mise en place des arbuscules se déroule en 2 à 4 jours et survivent de 7 à 10 jours suivant l'association symbiotique pour ensuite se dégrader (Parniske 2008; Pumplin and Harrison 2009). Un grand nombre d'espèces de CMA produisent des structures de réserves lipidiques, appelés vésicules, à l'intérieur ou à l'extérieur des racines. Ces vésicules peuvent devenir des propagules capables de coloniser une plante hôte (Biermann and Linderman 1983; Declerck, Strullu, and Plenchette 1998). Parallèlement à son développement intra-radiculaire, le mycélium se développe à l'extérieur de la racine pour à la fois prélever des minéraux et de l'eau dans le sol, et former une nouvelle génération de spores (**Fig. I.1**) (Olsson, Olsson, and Hammer 2014). La croissance coenocytique du mycélium externe, c'est-à-dire sans cellules cloisonnées, peut atteindre plusieurs km par gramme de sol ce qui augmente considérablement le volume prospecté (Leake et al. 2004). Le mycelium extra-radiculaire (ERM) comprend plusieurs sortes d'hyphes (**Fig. I.3**). Les plus fins (diamètre de 1 à 5 µm) sont responsables de l'acquisition des nutriments *via* la différentiation de

"branched absorbing structures" (BAS) dont la ramification augmente la surface de contact avec l'environnement (Bago et al. 2004). Les hyphes les plus épais d'un diamètre de 5 à 20 µm constituent le squelette du mycélium. Ils sont impliqués dans l'extension extra-radiculaire du champignon et dénommés "runner hyphae" (Friese and Allen 1991**;** **Fig. I.3**).

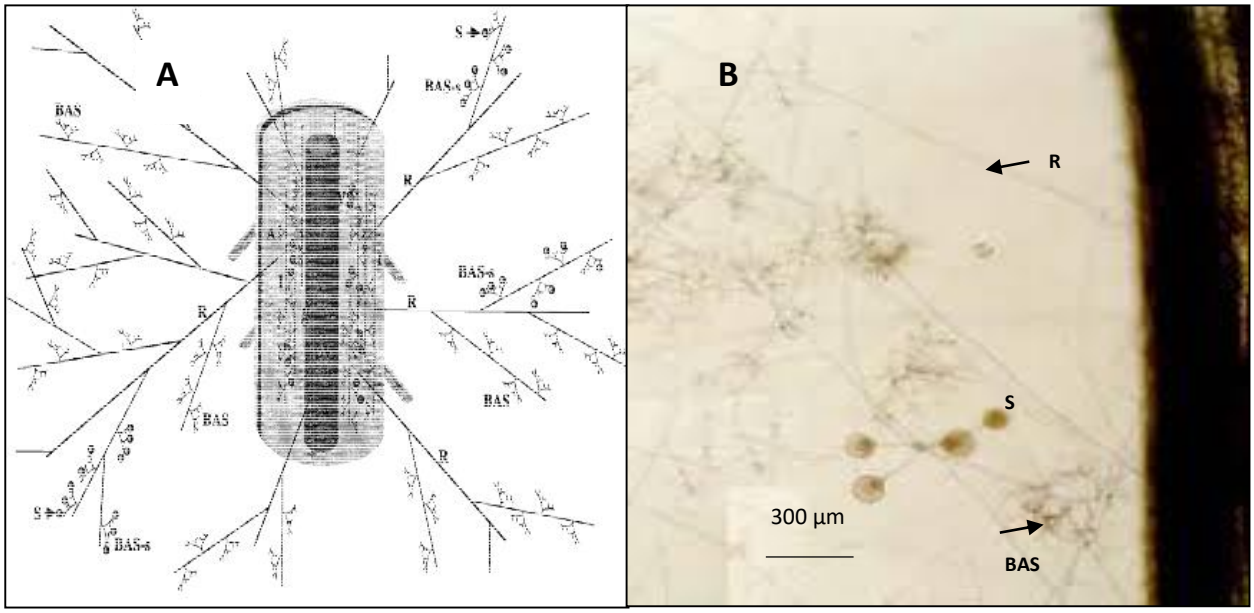


Figure I.3. Mycélium extra-radiculaire émanant d'une racine mycorhizée illusté d'après **A**) (Bago et al. 2004) et **B**) (Costa et al. 2013). Les structures du champignon détaillées sont les spores (**S**), les runner hyphae (**R**), et les branched-absorbing structures (**BAS**) (échelle = 300 µm).

L'amélioration de la nutrition minérale de la plante hôte *via* le mycélium extra-radiculaire a été attribuée à une meilleure prospection de l'environnement, à l'étroitesse des hyphes qui permet au champignon de pénétrer à l'intérieur des pores des agrégats du sol et à la ramification des hyphes qui accroît l'influx de nutriments par unité de surface fongique (Bucking, Liepold, and Ambilwade 2012).

2. Les échanges trophiques dans la SMA

2.1 Acquisition du phosphate par la voie symbiotique

Le phosphore est, avec l'azote, l'un des deux plus importants macroéléments pour le développement et la croissance des plantes. Il est un élément central des acides nucléiques, des phospholipides, des phosphoprotéines et de l'ATP (adénosine triphosphate), dont l'hydrolyse de la liaison phosphodiester en fait le plus important producteur d'énergie dans les systèmes biologiques. Le phosphore assimilable par les plantes est le phosphate inorganique (Pi) sous forme d'ions orthophosphate ($H_2PO_4^-$ et HPO_4^{2-}) qui sont absorbés au niveau des poils absorbants des racines (Marschner 1996). Le Pi présent en très faible concentration dans les sols (comprises entre 5 et 10 µM), est très peu mobile (Javot, Pumplin, and Harrison 2007). Les interactions électrostatiques du phosphate avec les ions positifs liés à l'argile et à l'humus, tels que les hydroxydes de fer et d'alumium ou le calcium, font que le phosphate est l'ion majeur le moins diffusible (Adesemoye and Kloepper 2009). Le stock de Pi dans l'environnement immédiat de la plante est donc très rapidement épuisé, ce qui crée une zone d'appauvrissement de 1 à 2 mm autour de la racine (Balzergue 2012). Différentes stratégies ont été mises en place par les plantes pour maximiser l'absorption de Pi dans le sol, comme l'augmentation de la ramification racinaire, du nombre de poils absorbants et la mise en place de mécanismes de solubilisation du phosphate par la sécrétion d'acides organiques et de phosphatases (Ferrol, Azcón-Aguilar, and Pérez-Tienda 2019).

La formation d'une association symbiotique avec les CMA constitue une stratégie alternative pour les plantes mycotrophes. Comme nous l'avons vu précedemment, cette association permet d'augmenter le volume de sol exploré et donc de dépasser la zone d'appauvrissement grâce au mycélium extra-racinaire (Javot, Pumplin, and Harrison 2007). Le mycelium sécrète des phosphatases, de l'acide citrique et des sidérophores qui augmentent la biodisponibilité du phosphate (Joner and Johansen 2000; Sato et al. 2019). La haute affinité des transporteurs fongiques de phosphate, localisés au niveau des hyphes extra-radiculaires (**Fig. I.4**), permet l'acquisition du Pi par le champignon même lorsqu'il est présent en très faible concentration dans l'environnement (Harrison and Buuren 1995; Fiorilli, Lanfranco, and Bonfante 2013; Maldonado-Mendoza, Dewbre, and Harrison 2001). De cette manière, les plantes possèdent deux chemins potentiels pour leur nutrition phosphatée (**Fig. I.4**) : l'absorption directe au niveau des poils absorbants, et l'acquisition du phosphate importé par le champignon au niveau des arbuscules (Harrison and Buuren 1995; Maldonado-Mendoza, Dewbre, and Harrison 2001; Fiorilli, Lanfranco, and Bonfante 2013). La contribution de la voie symbiotique à l'acquisition du phosphate des plantes dépend du champignon, de la plante-hôte et des ressources de l'environnement.

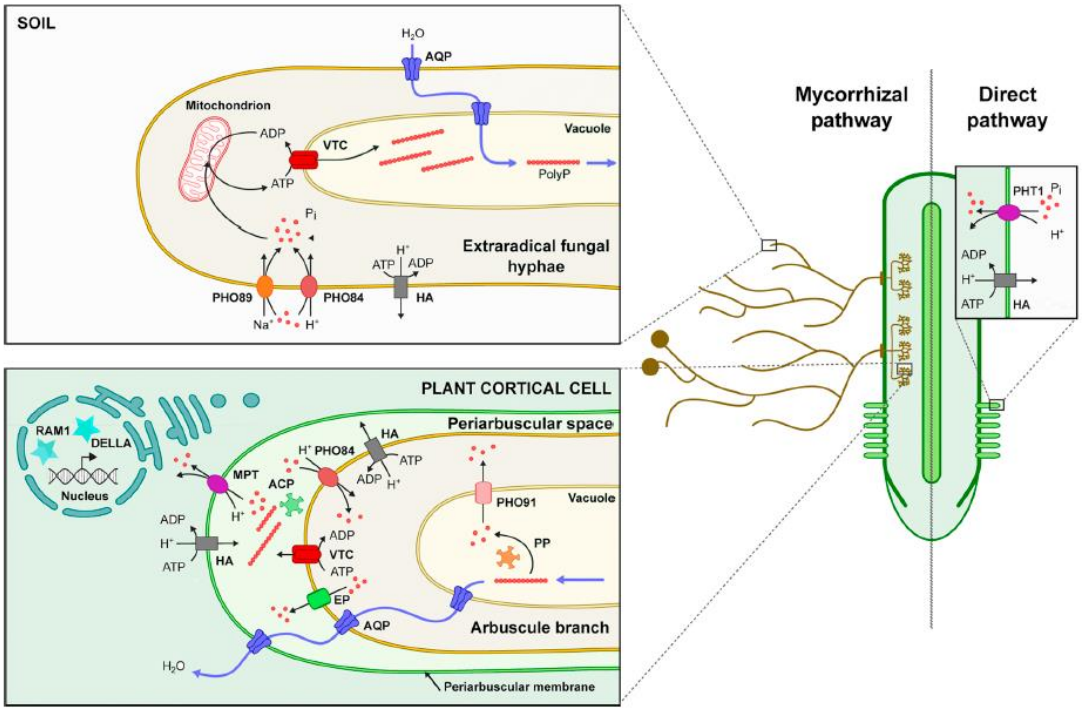


Figure I.4. Représentation schématique de l'acquisition du Pi dans une racine mycorhizée (Ferrol, Azcón-Aguilar, and Pérez-Tienda 2019)**.**

Le prélèvement du phosphate par le mycelium extra-radiculaire se fait contre un gradient de concentration. Le processus d'absorption étant énergie dépendant, le phosphore entre dans le cytoplasme *via* les transporteurs à haute affinité du type Pi:H^+ symport. Les H^+-ATPases présentent au niveau des membranes plasmiques permettent de répondre aux besoins énergétiques et génèrent les gradients électrochimiques nécessaires aux transports à travers les membranes (Rosewarne et al. 2007). Chez les CMA, les transporteurs de Pi sont homologues au transporteur PHO84 de la levure. Ils appartiennent à la famille PHT1 **(Fig. I.4)** qui regroupe des transporteurs de la membrane plasmique (Harrison and Buuren 1995; Bun-Ya et al. 1991). Les transporteurs de Pi de la famille PHT1 identifiés chez différents organismes présentent des degrés de similarité importants. Ils ont un poids moléculaire et une taille respectivement d'environ 60 kDa (550 acides aminés). Les analyses d'hydrophobicité montrent que ces transporteurs possèdent 12 segments transmembranaires (**Fig. I.5**). Tous les transporteurs fongiques de la famille PHT1 possèdent une séquence consensus GGDYPLSxxIxSE (Karandashov and Bucher 2005). GvPT, le premier transporteur à haute affinité pour le Pi a été décrit en 1995 chez *Glomus versiforme*. Par la suite, des homologues de GvPT ont été identifiés chez *Rhizophagus irregularis* (GintPT), *Glomus mosseae* (GmPT) et *Gigaspora margarita* (GigmPT) (Harrison and Buuren 1995; Maldonado-Mendoza, Dewbre, and Harrison 2001). Ces transporteurs se trouvent essentiellement au niveau de la membrane du mycélium extra-radiculaire. Une fois transporté dans le cytosol, le Pi intègre le cycle de respiration mitochondriale pour la formation d'ATP (**Fig. I.4**). Dans la vacuole, l'ATP est polymérisé en polyphosphate (polyP), polymère linéaire constitué de 3 à 1000 Pi (Ezawa et al. 2004). Le polyP est transporté à travers les hyphes non septés du champignon jusqu'aux hyphes intra-radiculaires *via* les flux cytoplasmiques principalement régulés par l'activité des aquaporines fongiques. Le polyphosphate est hydrolysé par l'action de polyphosphatases fongiques dans les hyphes intra-radiculaires (**Fig. I.4**) induisant une forte concentration en Pi dans la vacuole et par conséquence l'efflux du Pi du champignon dans le cytosol de l'arbuscule via le transporteur PHO91 (Kikuchi et al. 2014; Ferrol, Azcón-Aguilar, and Pérez-Tienda 2019). Par la suite, le Pi est transporté dans l'espace périarbusculaire par un transporteur membranaire encore non identifié (Ferrol, Azcón-

Aguilar, and Pérez-Tienda 2019). Dans cet espace, le Pi est accessible aux transporteurs végétaux impliqués dans l'acquisition symbiotique du phosphate inorganique.

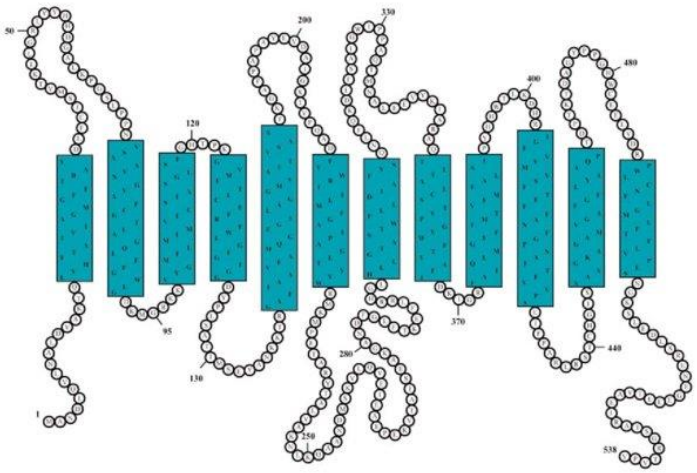


Figure I.5. Topologie du transporteur LePT1 de la tomate typique des protéines PHT1 avec 12 domaines transmembranaires, des extremités N-terminal et C-terminal cytoplasmiques et une boucle cytoplasmique entre les domaines transmembranaires 6 et 7 (Poirier and Bucher 2002).

Chez les plantes, les transporteurs de Pi du type Pi:H^+ symport sont répartis en cinq familles phylogénétiques: PHT1, PHT2, PHT3, PHT4, et PHT5 (Wang et al. 2017). La famille PHT1 qui regroupe des transporteurs présents dans la membrane plasmique est caractérisée par la séquence consensus GGDYPLSATIxSE (Karandashov and Bucher 2005). Cette famille est essentiellement impliquée dans l'acquisition du phosphate inorganique du sol. Les transporteurs localisés dans les plastes, les mitochondries, l'appareil de Golgi et la vacuole sont quant à eux regroupés respectivement dans les familles PHT2, PHT3, PHT4, et PHT5 (W. Wang et al. 2017). Seuls les transporteurs de la famille PHT1 semblent actuellement être impliqués dans l'acquisition symbiotique du Pi (Wang et al. 2017). Au sein de cette famille, quatre groupes phylogénétiques et fonctionnels se distinguent. Les groupes 1 et 3 participent au transport de Pi *via* la voie mycorhizienne (Casieri et al. 2013). Les gènes correspondants aux transporteurs du groupe 3, en particulier *StPT3* chez la pomme de terre (Rausch et al. 2001), sont fortement induits par la SMA, mais présentent une expression basale dans des racines non colonisées. Les gènes correspondant aux transporteurs du groupe 1 dont *MtPT4* et *ZmPT6* chez la luzerne tronquée et le maïs, sont uniquement exprimés chez les espèces pérennes et annuelles au cours de la mycorhization (Drain et al. 2017). Ces transporteurs se trouvent uniquement dans les cellules du cortex colonisées par le champignon et ont une faible affinité pour le Pi. Ils sont situés au niveau de la membrane périarbusculaire des plus fines ramifications des arbuscules (**Fig. I.4;** (Ferrol, Azcón-Aguilar, and Pérez-Tienda 2019). En général, l'induction de ces transporteurs spécifiques de la mycorhization s'accompagne d'une répression d'autres transporteurs PHT1 pour la plupart impliquée dans la voie directe d'absorption du Pi, comme par exemplme *MtPT1* chez la luzerne tronquée (H. Liu et al. 1998). Ces régulations opposées entre les différents groupes fonctionnels PHT1 traduisent le passage de la voie directe d'absorption du Pi à la voie mycorhizienne qui devient alors favorisée. En plus de son implication dans la nutrition de la plante, le Pi est un signal majeur nécessaire au maintien de la SMA (Garcia et al. 2016). Des apports importants en Pi inhibent le développement de la symbiose en favorisant la voie d'absorption directe moins coûteuse pour la plante (Drain et al. 2017). La répression des transporteurs de Pi qui sont strictement induits en réponse à la SMA se traduit par une accumulation de polyphosphate dans les arbuscules, une diminution significative de la concentration en P des parties aériennes de la plante et une dégénérescence prématurée des

arbuscules qui met un terme à l'interaction (Ferrol, Azcón-Aguilar, and Pérez-Tienda 2019). L'avortement de la symbiose s'explique par la non réciprocité des échanges : la plante ne reçoit plus de phosphate du champignon, elle ne lui apporte donc plus de carbone. Étant un biotrophe obligatoire, le champignon ne peut plus se développer.

En termes de régulation transcriptionelle, certains éléments *cis*-régulateurs (CRE) impliqués dans l'activation de gènes codant des transporteurs PHT1 ont été identifiés chez plusieurs plantes (Pimprikar and Gutjahr 2018). L'élément P1BS (GNATATNC), reconnu par le facteur de transcription PHR1 (PHOSPHATE STARVATION RESPONSE 1), est présent dans la plupart des promoteurs des gènes dont l'expression augmente en réponse à une carence phosphatée (Rubio 2001). Il en est de même pour l'élément *cis*-régulateur PHO (CACGTG; Oshima 1997; Hu et al. 2017), reconnu par le facteur de transcriptionn Pho4 (Ogawa et al. 2019) Harrison et al., 2004). Les motifs "root" (ATATT ou AATAT) et "W" (TTGACY, reconnu par le facteur de transcription WRKY) ont été identifiés dans les promoteurs de gènes *PHT1* chez le blé, le sorgho et le lin (Walder et al. 2015; Zhang et al. 2019). Chez Arabidopsis, WRKY est en particulier impliqué dans l'activation transcriptionnelle de gènes codant des transporteurs PHT1 en réponse à une carence phosphatée (Wang et al. 2014). 2014). Les séquences consensus NODCON2GM (CTCTT) et OSEROOTNODULE (AAAGAT) sont des motifs OSE (**o**rgan-**s**pecific **e**lements) caractéristiques des promoteurs des gènes activés dans les nodosités racinaires et les cellules corticales contenant des arbuscules, dont certains transporteurs *PHT1* inductibles par la SMA (Walder et al. 2015; Y. Zhang et al. 2019). Le facteur de transcription RAM1 (REDUCED ARBUSCULAR MYCORRHIZA 1) est également requis pour l'expression des transporteurs de phosphate spécifiques à la SMA (Park et al. 2015; Hartmann et al. 2019). L'activation transcriptionelle de *RAM1* dépend de l'élément *cis*-régulateur CGGCCG également dénommé 'AM-CYC box' identifié *in silico* dans les promoteurs des gènes induits par la SMA (Favre et al. 2014). Initialement mis en évidence dans les promoteurs de six gènes *PHT1* régulés par la mycorhization chez le tabac et l'aubergine l'élément *cis*-régulateurs MYCS (**myc**orrhiza transcription factor binding **s**équence) correspond à la séquence consensus TTTCTTGTTCT autorisant une variation nucléotidique ([T/C][T/G][T/A]CTTGTT[/C/T/G][T/C]) (A. Chen et al. 2011; Favre et al. 2014; Bravo et al. 2016; Xue et al. 2018). Chez le maïs, la séquence MYCS est toutefois absente des promoteurs de deux gènes *PHT1* inductibles par la SMA, ce qui tend à suggérer l'existence d'autres élements régulateurs impliqués dans l'activation transcriptionelle des transporteurs de Pi par la mycorhization (Liu et al. 2016). Ainsi, le motif "CTTC" (TCT(T/C)GTT), situé à la proximité de l'élément P1BS, est nécessaire et suffisant à l'expression de plusieurs gènes *PHT1* spécifiquement induits par la SMA (Chen et al. 2011; Favre et al. 2014; Lota et al. 2013). Plus récemment, le facteur de transcription CBX1 ciblant la séquence *cis*-régulatrice CTTC CRE (CTTCTTGTTC) a été mis en évidence dans le promoteur du gène *LjPT4* induit par la SMA (Xue et al. 2018). De même, Jiang et al. (2018) ont identifié chez *M. truncatula* l'élément *cis*-régulateur AW (CNTNG(N)$_7$CG) impliqué dans l'activation de *MtPT4 via* le facteur de transcription WRI5a.

2.2 Acquisition de l'azote par la voie symbiotique

L'azote (N) est, comme le phosphore, un macroélément vital. Il entre dans la formation des phospholipides, des coenzymes, des acides aminés, et contribue à 5 % du poids sec de la plante. L'azote est présent dans les sols sous forme de composés organiques (peptides et acides aminés) et inorganiques (nitrites, nitrates et ammonium). Le nitrate (NO_3^-) constitue la source d'azote inorganique majoritaire dans les sols (Marschner 1996). Chargé négativement, il n'est pas retenu par le complexe argilo-humique du sol, qui porte aussi une puissante charge négative. Le mycelium du CMA est capable de prélever l'azote sous forme inorganique avec une nette préference pour l'ammonium (NH_4^+) car la réduction du nitrate (NO_3^-) en NH_4^+ induit un cout énergétique plus important (Bücking, Mensah, and Fellbaum 2016). On connaît chez *R. irregularis*, au moins trois transporteurs de la famille des AMT/MEP (AMmonium Transporter/MEthylammonium Permeases)

impliqués dans le transport de $NH4^+$ (Casieri et al. 2013); **Fig. I.6**). *AMT1* est exprimé dans les hyphes externes et participerait à l'absorption de $NH4^+$ à partir de la solution du sol, tandis que *AMT2* et *AMT3*, exprimés préférentiellement dans les hyphes internes, participeraient aux échanges avec la plante hôte au niveau de l'interface symbiotique (Pérez-Tienda et al. 2011). Du fait de sa toxicité, dans les cellules fongiques, le $NH4^+$ est rapidement assimilé *via* le cycle GS/GOGAT en arginine (Jin et al. 2005), **Fig. I.6**).

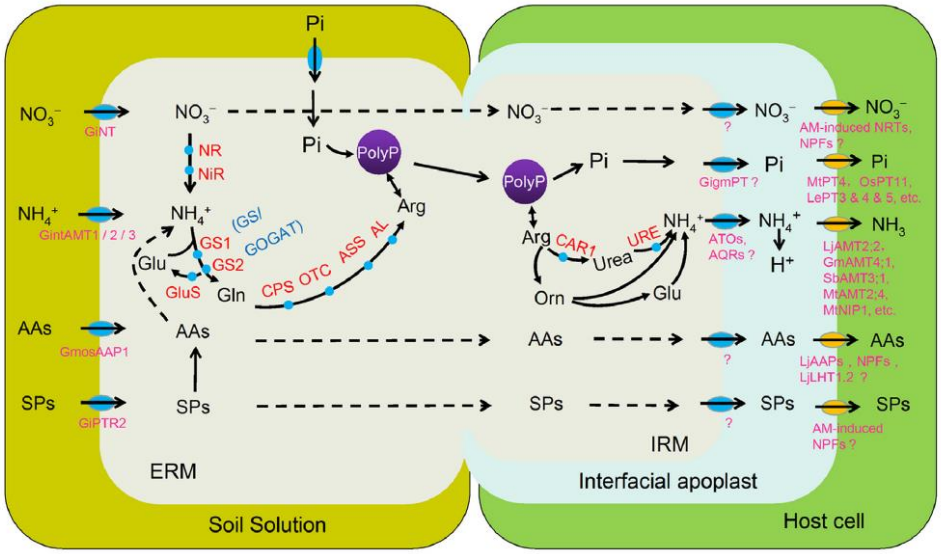


Figure I.6. Représentation schématique des mécanismes d'acquisition et d'assimilation de l'azote dans la symbiose mycorhizienne à arbuscule (A. Chen et al. 2018).

L'arginine, acide aminé basique, est co-transportée dans le mycelium intra-radiculaire (IRM) en association avec les chaînes de polyP chargées négativement puis transformée en ammonium *via* le cycle de l'urée pour enfin être transféré à la plante (Fellbaum et al. 2012; Tian et al. 2010). Deux transporteurs d'azote organique ont été caractérisés chez les CMA *Funneliformis mosseae* et *R. irregularis*, l'un d'acides aminés, AAP1 (Amino Acid Permease), impliqué dans l'absorption d'acides aminés à partir du sol (Drain et al. 2017) et l'autre de dipeptides, PTR2 (Peptide Transporter 2), dont les transcrits sont présents dans les hyphes externes et intra-radiculaires, ainsi que dans les cellules arbusculaires (Belmondo et al. 2014). Ceci suggère un rôle de PTR2 dans l'absorption de petits peptides à partir de la solution du sol, ainsi que dans leur échange au niveau de l'espace périarbusculaire.

Chez les plantes, les transporteurs d'ammonium sont répartis sur base des analyses phylogénétiques en deux sous-familles, celle des AMT1 et celle des AMT2. Les transporteurs de la sous famille AMT1 regroupent majoritairement des systèmes de transport de NH_4^+, ceux de la sous famille AMT2, plus proches des AMT/MEP fongiques ont la capacité de transporter du NH_3 à la place du NH_4^+ (Guether et al. 2009). Chez la plante hôte, plusieurs *AMTs* inductibles par la mycorhization et localisés spécifiquement dans les cellules contenant des arbuscules (Fig. I.6) ont été identifiés, en particulier chez *L. japonicus* (*LjAMT2;2*) (Guether et al. 2009), *G. max* (*GmAMT1;4*, *GmAMT3;1*, *GmAMT4;1*, *GmAMT4;4*) (Kobae et al. 2010) et *S. bicolor* (*SbAMT3;1*, *SbAMT4*) (Koegel et al. 2013). Deux rôles fonctionnels distincts ont été proposés pour deux AMT chez *M. truncatula*. AMT2;3 serait plutôt impliqué dans la détection et/ou la signalisation des conditions favorables au maintien des arbuscules, alors que AMT2;4 serait dédié à l'absorption de NH_4^+ depuis l'espace apoplastique (Breuillin-Sessoms et al. 2015). De nombreux gènes codant pour

des transporteurs putatifs d'azote organique appartenant aux familles AAP et PTR, mentionnées précédemment, sont régulés par la SMA chez les espèces modèles *M. truncatula* et *L. japonicus* (Guether et al. 2009).

2.3 La nutrition carbonée du symbiote fongique

2.3.1 Acquisition du carbone sous forme de sucre par le symbiote fongique

Les CMA peuvent acquérir jusqu'à 20% du carbone fixé *via* la photosynthèse (Bago, Pfeffer, and Shachar-Hill 2000). Chez les plantes, le saccharose (Suc), assurant le transfert longue distance de sucre du mésophile aux organes puits, est considéré comme le sucre majeur transféré à l'interface plante-champignon (Bücking and Shachar-Hill, 2005). C'est pourquoi, lors de la mise en place de la symbiose, l'activité photosynthétique augmente ainsi que l'expression dans les feuilles et les racines des plantes mycorhizées de transporteurs de saccharose (SUTs) qui dirigent les flux en direction du puits symbiotique (Boldt et al. 2011). Une fois le saccharose dans les cellules racinaires colonisées par le CMA, le phloème est déchargé par la voie symplastique de manière passive à travers les plasmodesmes cellulaires ou par la voie apoplastique au moyen des transporteurs de type SUT (Doidy et al. 2012**;** **Fig. I.7**).

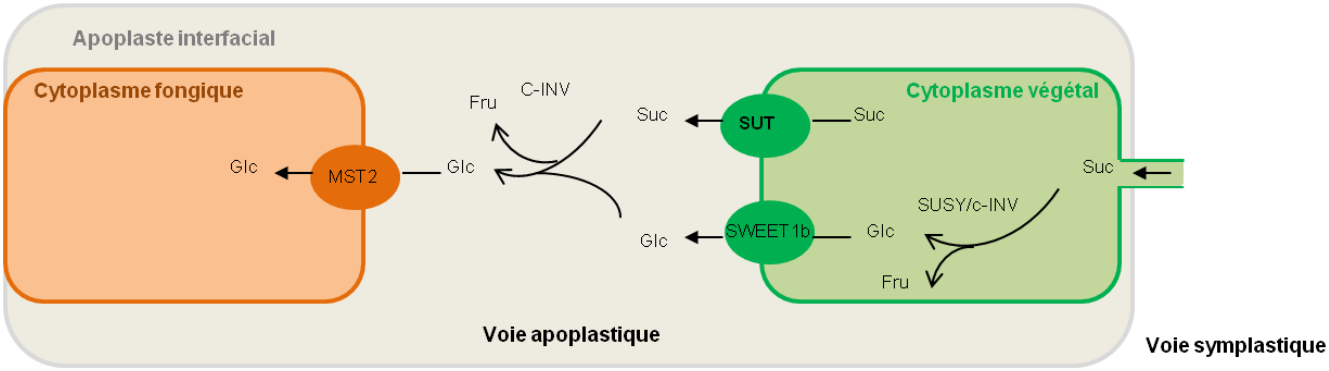


Figure I.7. Représentation schématique des voies apoplastique et symplasmique impliquées dans le transfert et l'acquisition de sucres par le champignon mycorhizien dans l'arbuscule.

Les structures intra-radiculaires fongiques sont incapables d'acquérir du saccharose (Andrea Schubert and Allara 2004). La dégradation du saccharose intervient donc au niveau du cytoplasme et de l'espace périarbusculaire et les produits d'hydrolyse sont destinés au champignon. A cet effet, le saccaharose est catabolisé par les Suc synthases cytosoliques (SUSY), pour produire de l'UDP-glucose et du fructose, et par les invertases cytosoliques (c-INV) ou apoplastiques (C-INV) pour produire du fructose et du glucose (Schaarschmidt et al. 2007; **Fig. I.7**). La détermination du rôle des transporteurs SWEET (Sugars Will Eventually be Exported Transporter) impliqués dans l'import et l'export des mono- et disaccharides dans la nutrition du symbiote est plus récente. An et al. 2019 ont démontré chez *M. truncatula*, l'implication de MtSWEET1b dans le transport du glucose dans l'espace périarbusculaire **Fig. I.7** et le maintien d'arbuscules fonctionnels.

Les CMA ont la capacité d'acquérir plusieurs types de sucres au niveau des hyphes intra- et extra-radiculaires *via* des transporteurs de monosaccharides (MST). Chez *R. irregularis*, RiMST2 est impliqué dans le transport de glucose, de fructose, de xylose, de mannose, de galactose, et des acides glucuronique et galacturonique. Ces sucres étant des constituants de l'apoplaste de l'interface plante-champignon, il est très probable que les CMA se nourrissent aux dépends des composants de la paroi végétale (Smith and Smith, 1990). Helber et al en 2011, ont détecté des transcrits de *RiMST2* au niveau des arbuscules et des hyphes intercellulaires (Helber et al. 2011; **Fig. I.7**). L'importance des sucres de la plante dans la nutrition des CMA et l'implication requise du transporteur MST2

pour une symbiose fonctionnelle ont été démontrées en utilisant une méthode d'extinction de l'expression génique dite « host-induced gene silencing ». Il a été ainsi montré que la diminution de l'expression du gène *MST2* conduit à une réduction de la colonisation des racines, couplée à une sénescence accélérée des arbuscules (Helber et al. 2011). L'expression du gène du transporteur *RiMST2* au niveau de l'ERM est spécifiquement induite par l'addition de xylose, suggérant que les CMA acquièrent également du sucre directement de la solution du sol. Des travaux plus récents ont mis en evidence l'expression des transporteurs *RiMST6* et *RiMST5 in planta* et chez les spores germées (Ait Lahmidi et al. 2016), confortant l'hypothèse de l'acquisition directe du glucose au niveau du sol.

Les hexoses absorbés par le champignon sont transformés en glycogène et en tréhalose dans les hyphes intra-radiculaires, puis transférés à l'ERM (Shachar-Hill et al. 1995; **Fig. I.8**). La beta-oxydation des acides gras et le cycle du glyoxylate sont des procédés actifs dans le mycélium extra-radiculaire (Lammers et al. 2001). Ces voies métaboliques sont impliquées dans l'utilisation des triacylglycérides (TAG), des lipides de réserve exportés du mycélium intra-radiculaire pour générer des glucides dans le mycélium extra-radiculaire. L'ERM ne peut synthétiser de TAG. D'après la faible activité des enzymes impliquées dans la glycolyse et suivant les résultats d'études par marquage ^{13}C, il a été proposé que les lipides de réserve, plutôt que les sucres, soient préférentiellement utilisés dans l'ERM, *via* la beta-oxydation des acides gras, le cycle du glyoxylate et la gluconéogenèse (**Fig. I.8**). Ainsi, le catabolisme des TAG générerait une source de carbone et d'énergie pour l'extension des hyphes dans le sol (Bago, Pfeffer, and Shachar-Hill 2000; Bago et al. 2004; Pfeffer, Bago, and Shachar-Hill 2001).

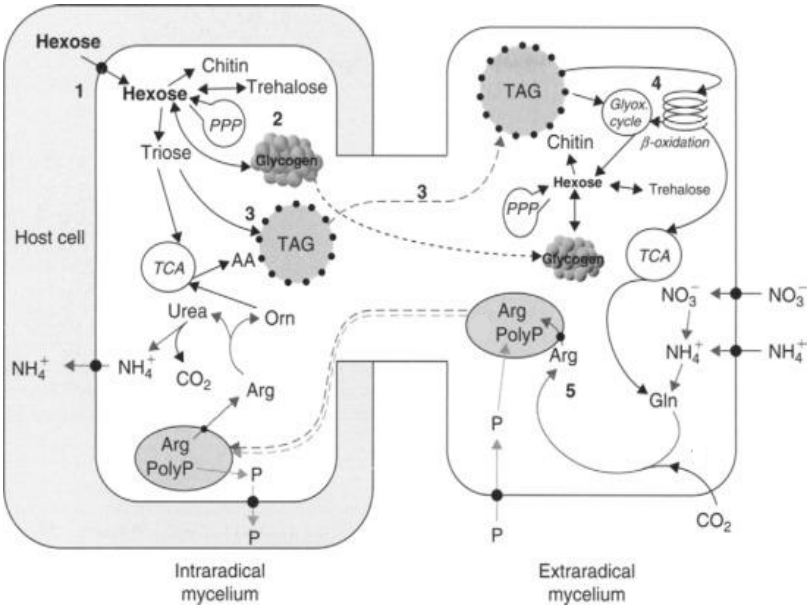


Figure I.8. Représentation schématique des principales voies métaboliques identifiées dans les hyphes intra-radiculaires (IRM) et le mycelium extra-radiculaire (ERM) des champignons mycorhiziens à arbuscule. **1**: acquisition des hexoses dans l'IRM; **2**: synthèse de glycogène et de tréhalose à partir des hexoses; **3**: synthèse de lipides de réserve (TAG) dans l'IRM et export dans l'ERM; **4**: conversion dans l'ERM des TAG *via* le cycle du glyoxylate et la néoglucogénèse en hexoses; **5**: synthèse d'arginine dans l'ERM et export dans l'IRM (Pfeffer, Bago, and Shachar-Hill 2001).

2.3.2 Acquisition du carbone sous forme de lipide par le symbiote fongique

Jusqu'à récemment, les hexoses étaient considérés comme la source essentielle de transfert de carbone de la plante hôte au CMA. Des études récentes ont mis en évidence l'acquisition par le champignon de carbone d'origine végétale sous forme lipidique. Des analyses génomiques et transcriptomiques ont révélé l'absence chez *R. irregularis* de l'enzyme FASI (fatty acid synthase I) impliquée dans la synthèse de l'acide palmitique (C16:0) et la synthèse *de novo* d'acides gras (FAs) (Tisserant et al. 2013; Trépanier et al. 2005). Toutefois, les gènes codant les enzymes responsables de l'élongation et la désaturation des FAs au-delà de la chaîne C16 sont exprimés chez le champignon (Wewer, Brands, and Dörmann 2014). Une étude phylogénomique a révélé la présence spécifique chez les plantes mycotrophes de protéines appartenant aux voies métaboliques de biosynthèse et d'export de lipides (Bravo et al. 2016). Celles-ci incluent notamment la pyruvate kinase (PK), la ketoacyl ACP (ayl carrier protein) réductase (KAR), la kétoacyl-ACP synthase, (KAS), l'enoyl-ACP réductase (ENR), la palmitoyl-acyl carrier thioestérase FATM (Fat required for Arbuscular Mycorrhizal Symbiosis), la glycerol-3-phosphate acyl transférase RAM2 (REDUCED ARBUSCULAR MYCORRHIZA 2), et les ABC transporteurs STR et STR2.

L'auxotrophie des champignons MA pour l'acide palmitique a été démontrée par l'obtention de mutants *fatm* (Bravo et al. 2017), *ram2* (Bravo et al. 2017), *str/str2* (Q. Zhang, Blaylock, and Harrison 2010), et *kas* (Keymer et al. 2017). Lorsque l'expression d'un des gènes précités est réprimée, la colonisation fongique, la ramification des arbuscules, et la teneur en lipides fongiques sont significativement réduites. L'ensemble de ces travaux a permis de construire le modèle consensuel de la nutrition lipidique des CMA qui est présenté en **Fig. I.9** (MacLean, Bravo, and Harrison 2017; Roth et al. 2018; F. Wang 2017).

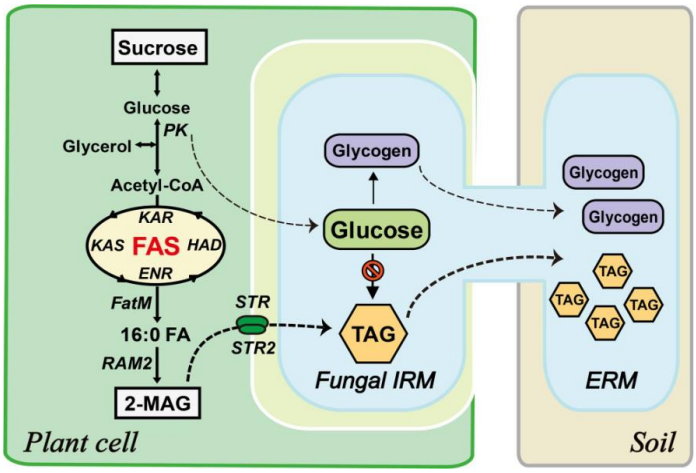


Figure I.9. Représentation schématique de l'acquisition du carbone végétal sous forme lipidique par le symbiote fongique. Rôle des enzymes FAS (fatty acid synthase), FATM (palmitoyl-acyl carrier thioestérase) dans la production de palmitate (16:0 FAs) permettant la synthèse de 16:0 β-monoacylglycérides (2-MAG) par l'action de la glycerol-3-phosphate acyl transférase RAM2. Export des 2-MAG au mycélium extra-radiculaire *via* les transporteurs ABC transporters STR and STR2 localisés au niveau de la membrane périarbusculaire. ERM, mycelium extra-radiculaire; IRM, mycelium intra-radiculaire; TAG, triacylglycérides (MacLean, Bravo, and Harrison 2017).

FATM libère du palmitate (16:0 FAs) qui sert de substrat à RAM2 pour la synthèse de 16:0 β-monoacylglycérides (2-MAG). Les 2-MAG sont ensuite exportés au mycélium extra-radiculaire par les transporters STR et STR2 localisés au niveau de la membrane périarbusculaire. Les mécanismes d'acquisition des 2-MAG au niveau de la membrane plasmique des hyphes intra-radiculaires restent encore inconus.

En termes de régulation transcriptionnelle, le facteur de transcription RAM1 (REDUCED ARBUSCULAR MYCORRHIZA 1) est non seulement requis pour l'expression des transporteurs de phosphate spécifiques à la SMA (Park et al. 2015; Hartmann et al. 2019), mais également pour l'expression des gènes végétaux impliqués dans la synthèse et l'export de lipides destinés au CMA, dont *FatM, RAM2,* et *STR* (Luginbuehl and Oldroyd, 2017). De même, deux études supplémentaires ont révélé le rôle clé des facteurs de transcription WRINKLED1-like AP2 dans le contrôle de l'échange bidirectionnel de phosphate et de lipide entre les partenaires symbiotiques**.** Chez *M. truncatula,* l'élément *cis*-régulateur AW (CNTNG(N)$_7$CG) est reconnu par le facteur de transcription WRI5a qui active l'expression des gènes *MtPT4*, *MtFatM* et *MtSTR* (Jiang et al. 2018). En recherchant chez le lotier (*L. japonicus*) le facteur de transcription ciblant la séquence *cis*-régultrice CTTC CRE (CTTCTTGTTC) présente dans le promoteur du gène *LjPT4* induit par la SMA, Xue et al. (2018) ont identifié CBX1. Ce facteur de transcription qui reconnaît également la séquence *cis*-régulatrice AW (CNTNG(N)$_7$CG), active non seulement l'expression de *LjPT4*, mais également l'expression des gènes codant les enzymes KAS1, FatM et RAM2. L'ensemble de ces données suggère donc l'existence d'une co-régulation transcriptionelle des gènes impliqués dans l'échange bidirectionnel de nutriments entre les partenaires symbiotiques (Jiang et al. 2018).

2.3.3 Régulation des échanges trophiques dans la SMA

Comme les CMA sont des biotrophes obligatoires, le carbone fourni par la plante est un bénéfice trophique indéniable pour le mycosymbiote (Konvalinková and Jansa 2016). Chez la plante mycorhizée, une relation mutualiste suppose que les bénéfices nutritionnels acquis dépassent l'investissement carboné accordé à la nutrition du champignon (Friede et al. 2016). Lorsque les plantes sont carencées, elles allouent du carbone au CMA, qui en retour leur apportent des minéraux peu mobiles dans le sol (Johnson, Graham, and Smith 1997). Si ces nutriments sont disponibles sans l'aide d'association mycorhizienne, la plante peut cesser de fournir des photosynthétats au symbiote afin de préserver ses réserves énergétiques (Blanke et al. 2005). Les CMA sont donc limités en apport de carbone, et la colonisation des racines est inhibée (Treseder and Allen 2002).

2.3.3.1 Régulation de la SMA en fonction de la biodisponibilité en phosphate

Il est reconnu depuis de nombreuses années qu'une fertilisation phosphatée de l'ordre de 100 mg ou plus de Pi/kg de sol est dévaforable à la symbiose MA (Graham 1981). Une fertilisation élevée en Pi conduit à une réduction significative du nombre d'arbuscules (Rausch et al. 2001; Bonneau et al. 2013) du fait de la répression chez la plante de l'expression des gènes des transporteurs de Pi inductibles par la SMA (Breuillin et al. 2010). Le développement des abuscules est alors interrompu avant la ramification des hyphes dans les cellules corticales (Kobae et al. 2010). Ceci s'explique principalement par le fait qu'une concentration élevée de Pi dans la racine (Sanders, 1975) induit une réduction du flux de carbone vers le champignon après plusieurs semaines d'interaction symbiotique (Olsson, Olsson, and Hammer 2014). L'acquisition par la plante de Pi provenant des arbuscules est en effet nécessaire à un flux de carbone depuis la plante vers le CMA (Kiers et al. 2011). L'apport de saccharose à l'hôte végétal stimule l'acquisition et le transsfert de Pi à la plante où l'expression du transporteur d'hexoses *MST2* chez le champignon est corrélée à celle de *MtPT4* chez *M. truncatula* (Bücking and Shachar-Hill 2005; Helber et al. 2011). Il a donc été proposé que le transfert de carbone génère un signal contrôlant l'apport de phosphate par le champignon (Javot, Pumplin, and Harrison 2007; Breuillin et al. 2010; Gutjahr and Parniske 2013; W. Wang et al. 2017). Suivant l'hypothèse la plus répandue, l'apport en saccharose réduirait l'incorporation de Pi dans les chaînes PolyP. Il en résulterait une augmentation de la quantité cytoplasmique et apoplastique de Pi dans l'IRM suivant le gradient de concentration (Bücking and Shachar-Hill 2005; Fellbaum et al. 2012). De plus, la dégradation des chaînes de PolyP dans l'IRM et l'efflux de Pi sont stimulés par l'apport en saccharose de la plante (Solaiman and Saito 2001).

Hammer et al. 2011 ont suggéré que le carbone stimulait les activités phosphatases impliquées dans le catabolisme des longues chaînes de polyP (>~20 résidus Pi, Taknishi et al. 2009) générant ainsi du Pi et des chaînes plus courtes de polyP. Toutefois, il est imporatnt de souligner que la distribution des ressources dans le sol n'est pas uniforme. Il a été montré par Whiteside et al. 2019 en utilisant un système split-root *in vitro* et en manipulant la quantité de Pi disponible de part et d'autre de chacune des deux parties racinaires, qu'un gradient trophique pouvait maintenir une interaction symbiotique *via* un flux de Pi du compartiment non carencé à celui dépourvu de phosphate.

2.3.3.2 Régulation de la SMA en fonction de la biodisponibilité en azote

La réponse de la SMA à la fertilisation azotée dépend des conditions nutritionnelles environnementales, en particulier du ratio N:P dans la solution du sol. D'après la loi du minimum selon laquelle la croissance végétale est contrôlée par le nutriment le plus limitant dans l'environnement (van der Ploeg, Böhm, and Kirkham 1999), la biodisponibilité relative de l'azote et du phosphate dans le sol détermine donc l'avantage nutritionnel conféré par la symbiose (Johnson et al. 2005). La fertilisation azotée est bénéfique à la plante uniquement si le phosphate est limitant (Johnson et al. 2005). Lorsque *M. truncatula* est limité en azote, la sénescence des arbuscules n'a pas lieu pour les mutants *mtpt4* (Javot, Pumplin, and Harrison 2007), alors que le transport symbiotique de l'azote n'est pas affecté (Breuillin-Sessoms et al. 2015). Il semble donc que les transporteurs symbiotiques de P et N (PT4; AMT2;3) fournissent des minéraux à la plante hôte, et élicitent un signal requis pour la maintenance des arbuscules. De nombreuses études indiquent qu'une fertilisation en azote inorganique de l'ordre d'au moins 100 mg de Ni/kg de sol réduit la colonisation racinaire des CMA (Blanke et al. 2005), et limite la quantité de carbone allouée au champignon (Olsson, Hansson, and Burleigh 2006; Fellbaum et al. 2012). Nouri et al. 2014 ont observé chez le pétunia inoculé par *R. irregularis* que des fertilisations élevées en potassium, calcium, magnésium, sulfate, ou fer n'ont pas d'effet significatif sur la colonisation mycorhizienne. Seul l'azote et le phosphore ont été recensés pour leur effet inhibiteur sur la SMA à l'heure actuelle. De façon analogue au Pi, le transport d'azote symbiotique est stimulé par un apport de carbone à la plante (Fellbaum et al. 2012). Ce transport d'azote s'accompagne d'une augmentation significative des activités uréase et arginase dans l'IRM du champignon MA. A l'inverse, un apport de carbone sous forme d'acétate au niveau de l'ERM n'augmente pas le transport d'azote symbiotique. Ces résultats soulignent l'importance des flux carbonés de la plante hôte dans l'acquisition de l'azote par le champignon.

2.3.3.2 Régulation des échanges trophiques en fonction des partenaires impliqués

Selon Kivlin et al. (2011), les écosystems naturels peuvent héberger jusqu'à trente espèces différentes de CMA. Certains écotypes mycorhiziens peuvent être plus ou moins efficients selon la plante hôte considérée (Sanders 2002). Le niveau de coopération des champignons mycorhiziens est estimé en coût de carbone pour l'hôte par unité de Pi transféré. Ce ratio varie en fonction des espèces et des isolats (Feddermann et al. 2010) et dépend en particulier des capacités différentes des CMA à développer un mycélium extra-radiculaire et à coloniser l'espace intra-radiculaire (Chagnon et al. 2013). Selon le modèle proposé par Kiers et al. (2011) présenté en **Fig. I.10**, les espèces les plus coopératives (e.g. *R. irregularis*) investissent le carbone fourni par la plante dans la synthèse de chitine pour la croissance du mycélium extra-radiculaire, ce qui permet l'acquisition de Pi dans le sol puis le transfert de polyP aux hyphes intra-radiculaires. Dans l'IRM, le polyP est clivé en chaînes plus courtes (PPi) en générant du Pi et son efflux à l'interface des arbuscules. Ces processus sont stimulés par l'apport carboné de la plante. A l'inverse, chez les espèces les moins coopératives, les photosynthétats sous forme de TAG sont stockés *via* le développement de vésicules (e.g. *G. aggregatum*) ou de spores (e.g. *G. custos*) et la conversion du polyP en PPi est faible dans l'IRM. En cultivant *in vitro* des racines de *M. truncatula* avec une ou plusieurs espèces de champignons (*R.*

irregularis et/ou *G. aggregatum*) et en mesurant les flux de saccharose de la plante au champignon, Kiers et al. (2011) ont montré que la plante alloue significativement plus de carbone au champignon le plus coopératif. La plante peut donc détecter et discriminer les partenaires fongiques, puis récompenser avec plus de carbone celui d'entre eux qui fournit le plus de phosphate.

De même, le partenaire fongique peut discriminer les hôtes végétaux en fonction des ressources carbonées allouées. En cultivant *in vitro* chaque champignon en présence de deux systèmes racinaires où l'apport de saccharose varie de 0 à 25 mM, il a été mis évidence que l'espèce coopérative *R. irregularis* alloue préférentiellement du Pi à la plante qui lui fournit le plus de carbone (Kiers et al. 2011). Une tendance similaire est observée dans le cas de *G. aggregatum*. Cependant la quantité de Pi transférée à la plante est significativement réduite comparativement à *R. irregularis* car le polyP, inaccessible à la plante, est faiblement dégradé dans l'IRM de *G. aggregatum* (**Fig. I.10**). Dans l'ensemble, ces résultats indiquent un contrôle bidirectionnel dans l'échange de nutriments entre partenaires mycorhiziens.

Des études en condition *ex vitro* utilisant un système split-root dans lequel chacune des deux parties du système racinaire est cultivée en présence d'un CMA plus ou moins efficient (i.e. *R. irregularis* > *F.mosseae*) ont permis d'obtenir des résultats complémentaires. Dans ce cas, *F. mosseae*, champignon le moins coopératif, alloue significativement plus de ^{33}P à la plante en présence de *R. irregularis* qu'en son absence (Argüello et al. 2016). Cette augmentation s'accompagne d'une réduction en coût ^{14}C par unité de ^{33}P pour *F. mosseae*. Ainsi, la diversité des CMA permettrait de promouvoir la coopération entre les partenaires symbiotiques.

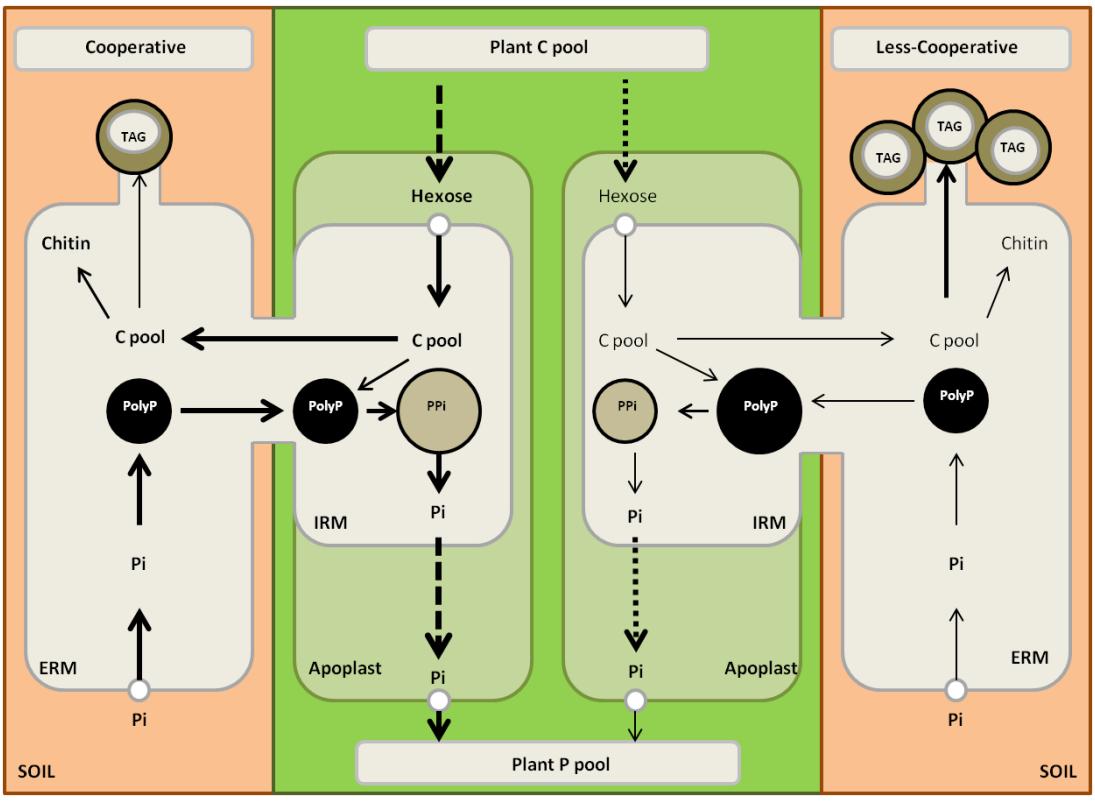


Figure I.10. Modèle illustrant les échanges de carbone et de phosphate dans des racines colonisées par un champignon mycorhizien coopératif (partie gauche du schéma) ou moins coopératif (partie droite du schéma) dans lequel l'épaisseur des flèches est proportionnelle aux flux représentés. L'hôte alloue préférentiellement du carbone au champignon coopératif qui investit ces ressources carbonées dans l'extension des hyphes (synthèse de chitine), ce qui permet un transfert de Pi sous forme de longues chaînes de polyphosphate (PolyP). La dégradation du polyP en chaînes plus coutes (PPi) et en phosphate inorganique (Pi) dans le mycélium intra-radiculaire (IRM) est facilitée par l'apport de carbone. L'accroissement du pool de Pi dans l'IRM facilite son efflux dans l'apoplaste et son transfert à l'hôte. Le champignon moins coopératif investit plus les ressources carbonées de l'hôte sous forme de triacylglycérides (TAG) dans la formation de spores et de vésicules, ce qui limite l'extension du mycélium extraradiculaire (ERM) et l'acquisition de Pi. Le phosphate est stocké dans l'IRM sous forme de polyP et la conversion en PPi et Pi est faible. Le pool de Pi dans le cytoplasme fongique est réduit et son efflux dans l'apoplaste est médié par un gradient de concentration entre le champignon et l'hôte (Kiers et al. 2011).

3. Les réseaux mycéliens communs

Les CMA présentent une faible spécificité vis-à-vis de leurs hôtes. On recense environ 270 espèces de CMA sur base de critères morphologiques pour 200 000 espèces végétales mycotrophes (Parihar et al. 2020). La faible spécificité d'hôte permet la formation de réseaux mycéliens communs (RMC) entre des individus qui coexistent même s'ils n'appartiennent pas à la même espèce (Walder et al. 2012). La mise en place de ce réseau peut résulter de la fusion par anastomose d'hyphes de champignons, génétiquement proches, en symbiose avec des plantes différentes (Giovannetti et al. 1993), qui permet la mise en commun du matériel cytoplasmique et nucléaire des deux hyphes (**Fig. I.11 A, B**). Les Glomeraceae, dont *R. irregularis*, contrairement aux Acaulosparaceae et aux Gigasporaceae, forment des anastomoses entre les hyphes du même génotype ou de génotypes voisins (Chifflot et al. 2009). La mycorhization d'une nouvelle plante par le mycélium extra-radiculaire d'un seul champignon déjà impliqué dans une symbiose permet également la formation d'un RMC (Barto et al. 2011 **Fig. I.11 C**). Divers travaux de recherche ont montré en laboratoire la possibilité pour un réseau de connecter des plantes espacées de 12 à 20 cm (Barto et al. 2011; Song et al. 2010; Babikova et al. 2013).

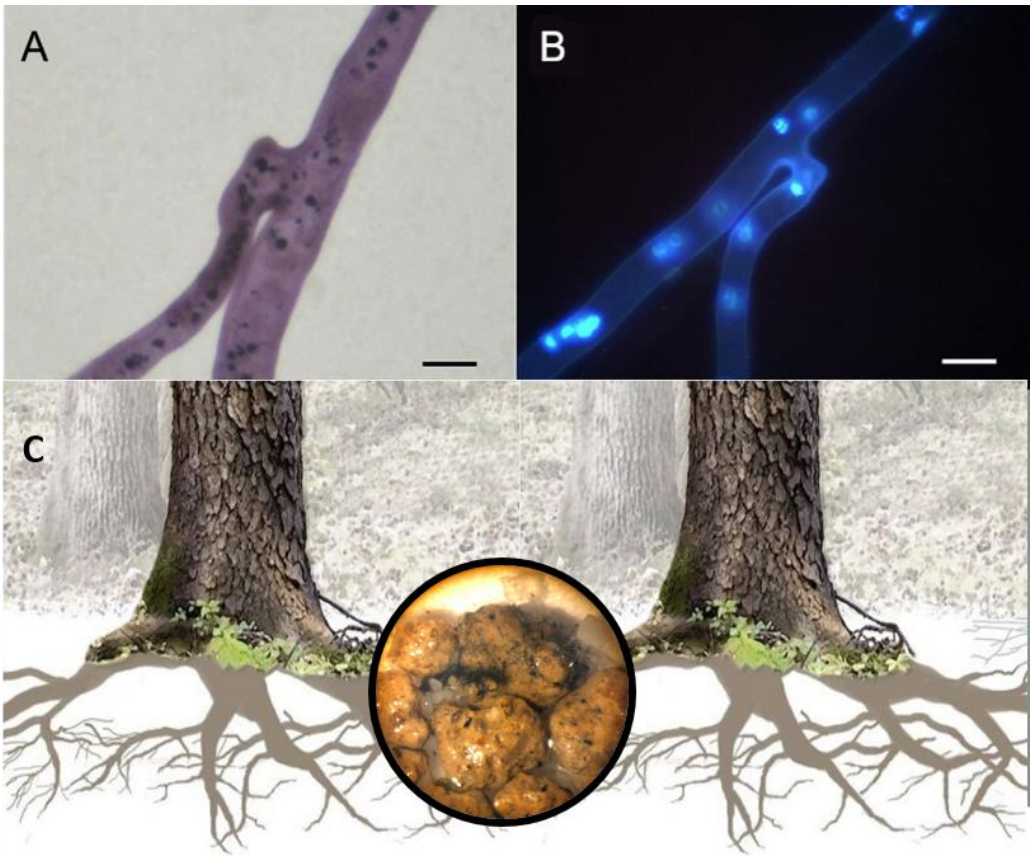
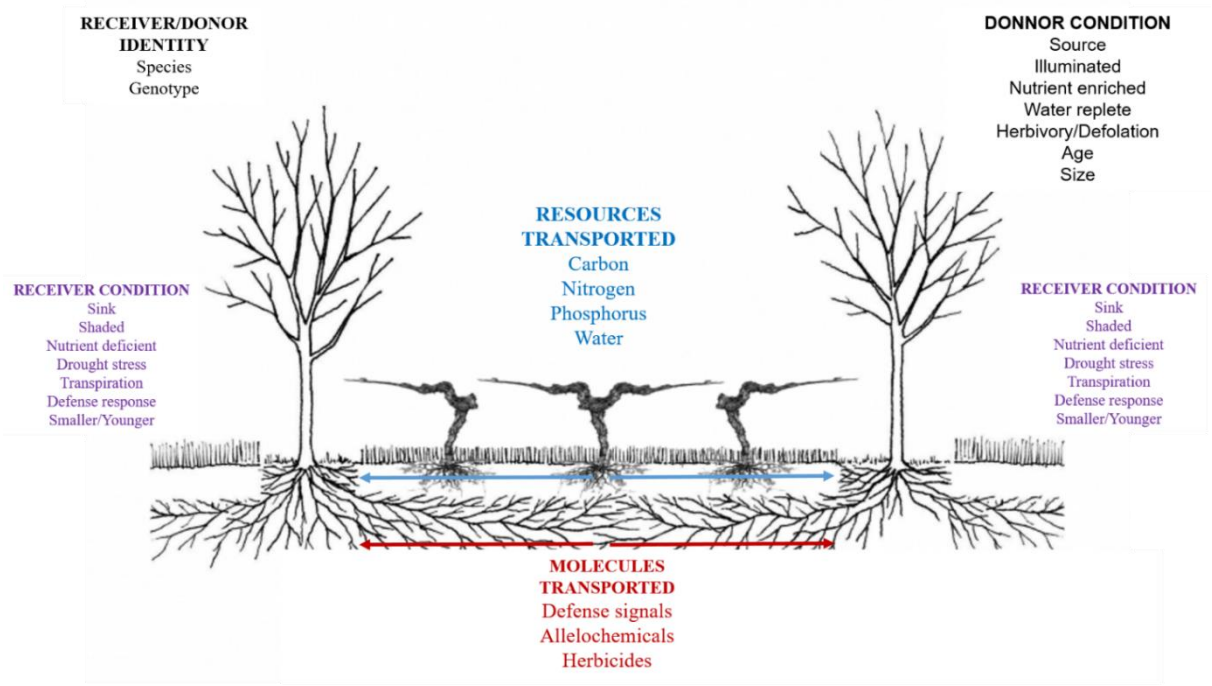


Figure I.11. Mécanismes impliqués dans la formation des réseaux mycéliens communs. Anastomose d'hyphes **A**) colorés au bleu Trypan (échelle = 5 µm), et **B**) migration de noyaux visualisée par fluorescence après coloration au 4',6-diamidino-2-phénylindole (échelle = 7 µm) (Novais et al. 2017). **C**) Mycorhization d'une autre plante par le mycélium extra-radiculaire d'un champignon déjà impliqué dans une symbiose.

En connectant physiquement les racines des plantes, les RMC permettent le transfert longue distance de nutriments et de signaux phytochimiques. Ainsi, les RMC participent à l'adaptation des végétaux aux conditions environnementales (Gorzelak et al. 2015) **Fig. I.12**). La transmission des ressources et la communication entre plantes confèrent aux RMC un rôle pilier dans le fonctionnement et la productivité des agrosystèmes malgré une répartition potentiellement asymétrique des éléments échangés entre les plantes à travers le réseau, ou entre les plantes et le champignon (Lallemand et al. 2018).



FigureI.12. Représentation schématique des ressources et des signaux transportés par réseau mycélien commun (CMN) et des conditions impliquées dans leur transfert entre plantes (modifié à partir de David Dellas, Arbre et Paysage 32)

3.1 Implication des RMC dans les flux nutritifs entre plantes

En utilisant des techniques de traçage isotopique (^{14}C, ^{15}N, ^{32}P), il a été possible d'apporter la démonstration d'un transfert de carbone et d'éléments minéraux entre deux plantes connectées par les hyphes de CMA dans les années 1980 (Chiariello, Hickman, and Mooney 1982; van Kessel, Singleton, and Hoben 1985; Heap and Newman 1980). Il a été montré depuis que les réseaux mycéliens sont impliqués dans le transport interplantes de glucides, d'acides aminés, de lipides (TAG), de vitamines, d'eau, d'arsenic, de césium et de rubidium (Oelmüller 2019) pour revue). Si des transporteurs fongiques et végétaux assurent le transfert de C, N, P à l'interface apoplastique hôte-champignon, les flux de carbone et d'éléments minéraux médiés par un RMC se font du cytoplasme de la plante donneuse au cytoplasme de la plante réceptrice, *via* le symplaste du mycélium fongique (Oelmüller 2019). Le système vacuolaire du fait de sa continuité dans les hyphes aseptés des champignons MA, serait impliqué dans le transport longue distance des nutriments entre les plantes connectées par un réseau fongique (Uetake et al. 2002). Le rapport "sources-puits" des plantes interconnectées régit le flux des ressources de la plante donneuse non carencée (source) à la plante réceptrice carencée (puits). Une demande en éléments nutritifs chez une plante donnée (e.g. croissance, ombrage, carence nutritionnelle, défioliation, pathogènes, insectes, sécheresse) peut être assurée par un transfert de nutriments provenant d'une autre plante *via* le RMC (**Fig. I.12;** (Simard et al. 2012). Le gradient trophique ainsi créé, permettrait une uniformisation de l'accès aux

ressources nutritives entre les espèces végétales (Wirsel 2004). Selon Selosse et al. (2006), le transfert d'azote *via* un réseau mycélien commun se fait sous forme d'ammonium à partir des espèces fixatrices d'azote vers les espèces non fixatrices où 40% de l'azote de la plante puits peut provenir de la plante donneuse. Moins mobile dans le sol, le transfert de Pi entre plantes connectées serait inférieur à 5% (Selosse et al. 2006) et le taux de transfert de carbone entre les plantes de l'ordre de 10% (Watkins et al. 1996).

Les flux de nutriments au sein du RMC dépendent de nombreux facteurs environnementaux. Les besoins nutritionnels des plantes interconnectées varient selon leurs stades phénologiques, la compétition interplantes, les saisons et les nutriments disponibles (Simard et al. 2012; Oelmüller 2019). En manipulant par ombrage la capacité photosynthétique de deux plantes (*M. truncatula*) connectées par un RMC, il a été montré par Fellbaum et al. (2012) que les CMA allouent une quantité supérieure de minéraux (P, N) à l'hôte non ombragé sans modification du taux de colonisation racinaire. Cette stratégie permettrait au champignon de maintenir une source de carbone alternative en cas de fluctuation de la capacité photosynthétique de la plante donneuse. En cohérence avec les données de Kiers et al. (2011), le partenaire fongique peut donc discriminer les hôtes végétaux en fonction des ressources carbonées allouées. En plus de l'influence des facteurs environnementaux, la quantité de ressources fournies et reçues dépend des partenaires impliqués. En utilisant le différentiel de signature isotopique ^{13}C entre les plantes à métabolisme photosynthétique de type C3 et C4 pour distinguer l'investissement en carbone du sorgho (*S. bicolor*, C3) de celui du lin (*Linum usitatissimum*, C4) dans un réseau mycélien commun, Walder et al. (2012) ont montré que l'allocation carbonée de chacune des plantes n'est pas proportionnelle à la quantité d'éléments minéraux fournis par le RMC (**Fig. I.13)**.

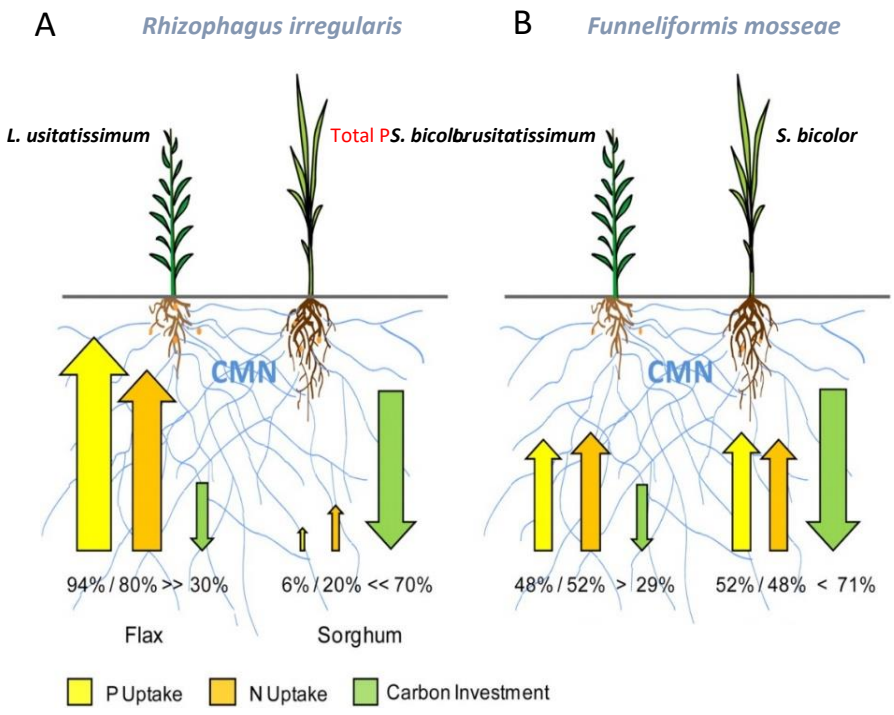


Figure I.13. Illustration des échanges médiés par un réseau mycéliem commun formé par **A**) *R. irregularis* ou **B**) *F. mosseae* connectant du lin (*L. usitatissimum*) et du sorgho (*S. bicolor*) (Walder et al. 2012).

Le sorgho d'une biomasse supérieure à celle du lin de l'ordre de 60%, investit deux fois plus de carbone que le lin dans la nutrition du champignon, qu'il s'agisse d'un réseau formé par *R. irregularis* (**Fig. I.13A**) ou par *F. mosseae* (**Fig. I.13B**). Lorsque les deux plantes sont connectées

par *R. irregularis*, le lin reçoit 80% d'azote et 94% de phosphate des ressources fournies par le réseau, alors que le sorgho, principal investisseur, n'en perçoit que 6% (N) et 20% (P) (**Fig. I.13A**). Dans le cas de *F. mosseae*, l'allocation minérale du RMC aux plantes hôtes est similaire chez le lin et le sorgho, alors que la contribution carbonée du lin est deux fois moindre que celle du sorgho (**Fig. I.13B**). Quel que soit le champignon impliqué dans le réseau, le lin bénéficie de la présence du sorgho et les biomasses végétales produites en intercultures sont supérieures à celles enregistrées en monocultures (Walder et al. 2012). En dépit de flux carbonés asymétriques entre les deux espèces annuelles, les RMC peuvent donc contribuer à la facilitation interplantes lorsque le coût carboné de la plante donneuse (ici le sorgho) est négligeable au regard de son accroissement de biomasse.

Dans leurs travaux, Calabrese et al. (2019) soulignent également une asymétrie entre le transportome du peuplier baumier (*P. trichocarpa*, C4) et celui du sorgho (C3) connectés par *R. irregularis*. Les résultats obtenus indiquent que le CMA contribue très peu à la nutrition phosphatée de l'espèce pérenne à croissance lente, contrairement à ce qui est observé chez le sorgho, espèce annuelle à croissance rapide. De plus, une activation transcriptionnelle de gènes impliqués entre-autres dans le transport de lipide et de sucre est uniquement observée chez le sorgho. La nutrition carbonée du CMA est donc principalement assurée par le sorgho au regard de son accroissement de biomasse. En accord avec les données de Kiers et al. (2011); Walder et al. (2012); et Fellbaum et al. (2012), le partenaire fongique peut discriminer les hôtes végétaux en fonction des ressources carbonées allouées.

3.1 Implication des RMC dans la transmission de signaux d'alerte

La communication plante-plante au sein d'un écosystème est également possible grâce aux réseaux mycéliens communs (Bücking, Mensah, and Fellbaum 2016; Oelmüller 2019; Gilbert and Johnson 2017; Babikova et al. 2013). Lorsqu'une plante est infectée par un organisme pathogène, elle peut transmettre des informations sur l'attaque aux plantes connectées en envoyant des signaux de défense. Les plantes saines reconnaissent ces signaux, ce qui leur permet d'activer leur système de défense et d'anticiper ainsi l'attaque. La mise en place d'un dialogue impliquant des signaux d'alerte transmis par un RMC a été démontrée par Song et al. (2010) en utilisant des plants de tomate (*L. esculentum*) connéctés par *F. mosseae*. L'infection d'une première plante par le parasite foliaire *Alternaria solani* se traduit chez la plante saine connectée par l'induction de gènes de défense. Chez les plantes infectées après connexion par le CMN, l'incidence de la maladie est réduite comparativement à des témoins non connectés. Dans le même dispositif expérimental, une attaque foliaire par la chenille du lépidoptère *Spodoptera litura* d'un premier plant se traduit chez la plante saine connectée au RMC par l'induction de gènes de défense dont l'activation transcriptionnelle dépend de composés hormonaux de type jasmonates (JAs), couplée à une résistance à l'attaque de l'insecte (Song et al. 2015). Ces résultats ont été obtenus en utilisant comme plante donneuse un mutant de tomate *spr2*, défectif pour cette voie de signalisation. Ces données accréditent la participation des hyphes fongiques dans la transmission des signaux d'alerte de type JA de la plante attaquée à la plante saine. Plus récemment, des résultats identiques ont été obtenus *in vitro* avec des plantules de pomme de terre (*S. tuberosum*) connectés par *R. irregularis*. L'infection d'une première plante par le pathogène *Phytophthora infestans* active l'expression de gènes de défense chez les plantes saines réceptrices (Alaux et al. 2020).

Suivant une approche similaire, Babikova et al. (2013) ont montré chez la fève (*Vicia faba*) qu'une plante qui n'est pas encore attaquée peut être informée de la présence de pucerons (*Acyrthosiphon pisum*) lorsque ses racines sont connectées par un réseau mycélien (*R. irregularis*) à celles d'une plante infectée. Dans ce cas, le RMC permet aux plantes saines qui vivent dans le voisinage d'une plante attaquée de réagir préventivement en produisant du salicylate de méthyle, composé organique volatil (VOC) dissuasif des pucerons mais attractif de guêpes parasitoïdes (*Aphidius ervi*) prédatrices des pucerons.

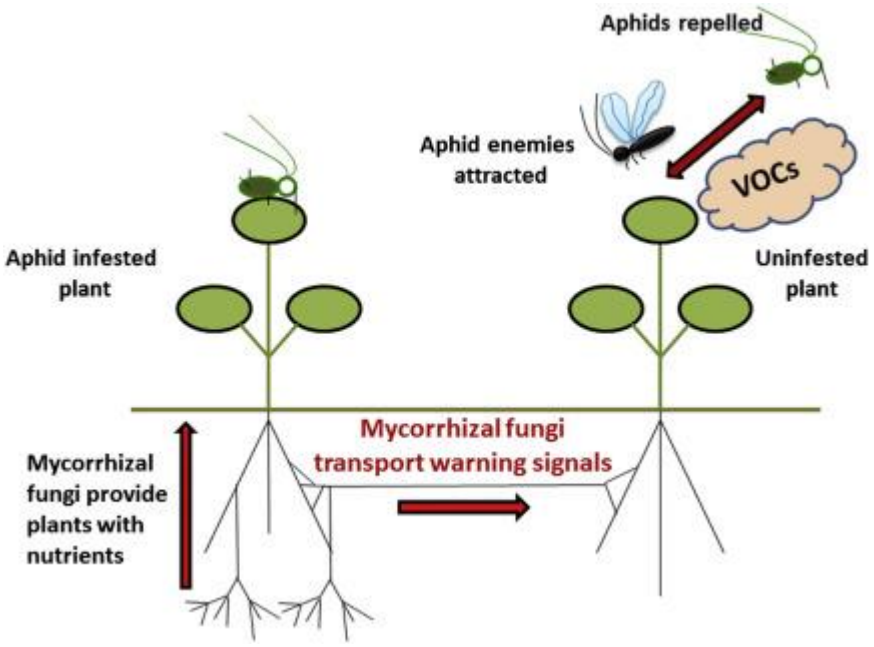


Figure I.14. Représentation schématique des interactions plante-champignon endomycorhizien-puceron-guêpe parasitoïde dans le cadre de la transmission de signaux d'alerte par un réseau mycélien commun (Gilbert and Johnson, 2017).

Le système présenté ci-dessus (**Fig. I.14**) implique donc une relation plante-herbivore entre le puceron et la fève, une symbiose mutualiste entre la fève et la guêpe, un parasitisme entre la guêpe et le puceron et une symbiose mutualiste entre la fève et le champignon. Dans un dispositif expérimental similaire mais dépourvu de guêpes parasitoïdes, Cabral et al. 2019 ont montré que la présence de pucerons dans la phyllosphère de la plante donneuse s'accompagnait d'une augmentation de la teneur en hexoses dans l'hyphosphère de la plante réceptrice. Ces hexoses pourraient subvenir au coût énergétique de la réponse immunitaire préventive observée chez la plante saine connectée par le réseau mycélien commun. La complexité des interactions multitrophiques est d'autant plus importante à souligner que les pucerons peuvent aussi réduire la quantité de carbone allouée à la mycorhize *via* une compétition trophique entre les organismes puits (i.e. puceron, CMA) pour les assimilas provenant des plantes (Charters, Sait, and Field 2020).

3.2 Implication des RMC dans la transmission de composés allélopathiques

En connectant physiquement les racines des plantes, les réseaux mycéliens communs peuvent intervenir non seulement dans la facilitation entre plantes *via* le transfert de nutriments et la transmission de signaux d'alerte (Walder et al. 2012; Fellbaum et al. 2012; Montesinos-Navarro et al. 2012), mais aussi dans la compétition par interférence chez les plantes connectées. Les RMC peuvent en effet transférer à partir d'une plante émettrice de composés allélochimiques qui inhibent la croissance des plantes réceptrices (Salahuddin et al. 2018; Barto et al. 2012). En utilisant comme plante émettrice l'œillet d'Inde (*Tagetes tenuifloia*) dont les racines produisent de l'alpha-tertiényle et des thiophènes qui nuisent à la croissance de nombreuses plantes dont la laitue cultivée (*Lactuca sativa*), Barto et al. (2011) ont montré que comparativement aux plantes non connectées par un réseau d'hyphes, la quantité d'alpha-tertiényle et de thiophènes augmente respectivement de 179 et 378 % dans la rhizosphère de la laitue en présence d'un RMC. Parallèment, la biomasse de la laitue utilisée comme plante réceptrice sensible est réduite de 50 %.

La plupart des espèces de la famille des noyers (Juglandaceae) produisent de la juglone (5 hydroxy-1, 4 napthoquinone), un composé phénolique présent naturellement dans toutes les parties de la plante mais qui est phytotoxique pour de nombreuses autres espèces comme la tomate (Jose and Gillespie 1998; Willis 2000; L. Yang, Wang, and Kong 2010). Il a été montré qu'en ajoutant de la juglone purifiée à la litière du noyer (*J. regia*) utilisé comme plante donneuse, la quantités de juglone dans la rhizosphère de la tomate augmente de 271 % et sa biomasse est reduite de 56 % lorsqu'elle est connectée au noyer *via* un RMC, comparativement à une situation où elle ne l'est pas (Achatz and Rillig, 2014).

Ces résultats montrent que le RMC transmet des quantités importantes de composés allélopathiques directement à la plante cible. En l'absence de ce RMC, la plante cible perçoit de plus faibles concentrations de ce composé par diffusion dans le sol. Ainsi, le transport de molécules assuré par les hyphes de CMA n'est pas toujours bénéfique à la plante réceptrice.

4. La SMA en systèmes agroforestiers intercalaires

4.1 Les systèmes agroforestiers intercalaires

Les systèmes agroforestiers intercalaires (SAI) sont des dispositifs où les arbres sont plantés en rangées espacées les unes des autres, de façon à aménager des espaces intercalaires (les allées) permettant la culture d'autres plantes cultivées comme le maïs, le soja, le blé ou des cultures fourragères (Carrier et al. 2019). Plusieurs études de simulations conduites à l'échelle européenne ont montré que des SAI bien conçus pourraient s'avérer plus productifs et rentables que des systèmes de monocultures agricoles et de plantations forestières classiques (Graves et al. 2009). Le fonctionnement des SAI dépend fortement des relations interspécifiques entre l'arbre et la culture. Ces interactions peuvent se décrire en termes de compétition, de différenciation de niche écologique (ou réduction de compétition), et de facilitation (**Figure I.15**). La compétition intervient lorsqu'une espèce modifie négativement l'environnement d'une autre espèce, par exemple en puissant une ressource qui devient limitante ou en génèrant un ombrage défavorable (Vandermeer 1998).

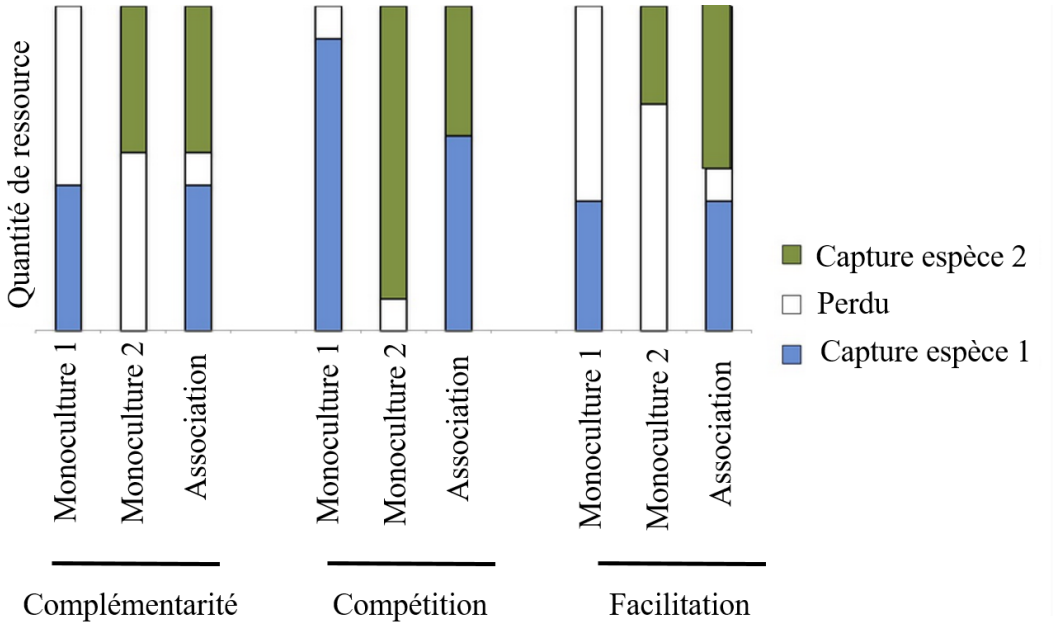


Figure I.15. Comparaison de la capture d'une ressource (e.g. eau, azote) par deux espèces (e.g. arbres et plantes) cultivées séparément, et en mélange (Dupraz 2015).

Une espèce peut avoir un avantage compétitif sur une autre en (i) acquérant une plus grande part de la ressource disponible ou en (ii) utilisant plus efficacement l'eau et/ou les nutriments pour la production de la biomasse et/ou en (iii) allouant les assimilats de façon à optimiser la survie et la croissance (Nambiar and Sands 1993). La compétition pour la lumière peut entraîner des modifications au niveau aérien du fait de surfaces foliaires supérieures pour certaines espèces et une efficience d'utilisation de l'azote pour la photosynthèse plus élevée (Benomar, DesRochers, and Larocque 2012). Au niveau souterrain, les réponses de la plante à la compétition pour les ressources sont semblables leur permettant de s'ajuster à un manque d'eau ou d'éléments nutritifs disponibles dans le sol (Nambiar and Sands 1993). Les individus peuvent par exemple augmenter leur longueur ou leur surface spécifique racinaire pour augmenter l'acquisition des ressources (Bolte and Villanueva 2006). Lorsque la biodisponibilité des ressources abiotiques diminue et que l'environnement devient plus contraignant, la compétition entre les plantes diminue pour laisser place à des interactions positives (Pretzsch, Schütze, and Uhl 2013).

La différenciation de niche écologique (réduction de compétition) induit l'utilisation d'une même ressource différée dans le temps ou dans l'espace (Loreau 1998). Il en résulte une diminution de la compétition (la compétition intraspécifique étant plus intense que la compétition interspécifique) et une plus grande quantité de ressources utilisées. Les espèces sont ainsi plus efficientes pour utiliser les ressources (Kelty and Cameron 1995). On distingue une différentiation passive, si les niches fondamentales des espèces sont différentes, et une différentiation active, résultant de la plasticité des espèces (niches réalisées). La différenciation passive résulte du décalage de phénologie entre espèces ou de l'utilisation de sources différentes. La différentiation active correspond à la ségrégation spatiale des systèmes racinaires des arbres et des cultures observée dans certains systèmes agroforestiers (Mulia and Dupraz 2006).

La facilitation correspond quant à elle à une augmentation de la croissance ou de la survie de l'espèce qui lui est associée par l'amélioration des conditions environnementales (température, ombre, disponibilité des ressources). Les exemples de facilitation les plus fréquemment cités se rapportent au cas des plantes "nurse" en milieu fortement contraint, qui protègent les stades juvéniles des plantes voisines, et les plantes fixatrices d'azote dont l'azote fixé peut bénéficier aux plantes voisines (Bruno, Stachowicz, and Bertness 2003).

Différenciation de niche et facilitation sont souvent regroupées sous le terme de complémentarité, les deux mécanismes étant étroitement imbriqués et difficiles à séparer (Loreau 1998). Les services fournis par les SAI reposent sur le principe de l'équilibre entre ces deux mécanismes majeurs, compétition et complémentarité de l'association de l'arbre et de la culture. L'intérêt de l'association n'est positif que si la complémentarité appporte des bénéfices supérieurs à la compétition (Dupraz et al. 2008).

4.2 Les interactions aériennes et souterraines en systèmes agroforestiers intercalaires

Deux grands types d'interactions entre arbres et cultures se distinguent: les interactions aériennes qui sont liées à la modification du microclimat de l'association (lumière transmise aux cultures, température et humidité de l'air, vitesse du vent), et les interactions souterraines qui sont généralement abordées en termes de modification des ressources du sol (Carrier et al. 2019).

Au niveau aérien, l'ombrage généré par les arbres a été identifié comme l'un des facteurs majeurs limitant la productivité des SAI (Reynolds et al. 2007; Bouttier et al. 2014). La biomasse d'une culture est en effet dépendante du rayonnement solaire capté pour la photosynthèse. La croissance des arbres diminue la quantité et la qualité de la lumière que la culture intercalaire intercepte (Jose et al. 2000). Le niveau de production de la culture tend donc à diminuer avec le temps au fur et à mesure que les arbres se développent (Reynolds et al. 2007). Carrier et al. (2019) ont établit une classification en fonction des espèces et de la tolérance des plantes annuelles à la concurrence des arbres au niveau de l'interface arbre-culture donnant une supériorité aux cultures

fourragères par rapport au soja et des céréales par rapport au maïs. L'impact de l'ombrage des arbres sur les rendements agricoles est aussi fonction du type de métabolisme C3 ou C4 des plantes (Reynolds et al. 2007; Udawatta et al. 2014). La photosynthèse en C4 concerne particulièrement les graminées tropicales, maïs, mil, sorgho et canne à sucre. Elle permet un rendement photosynthétique très supérieur à celui des plantes en C3 en assimilant la totalité du CO_2 atmosphérique interne à la plante. Ce mécanisme fonctionne d'autant mieux que la luminosité est vive et la température élevée. Les plantes en C4 sont donc avantagées dans les milieux ouverts à rayonnement solaire élevé (Reynolds et al. 2007). Le taux de photosynthèse nette des plantes en C3 comme le blé et le soja, sature à 50 % du rayonnement solaire incident maximal, celui des plantes en C4 comme le maïs, sature lorsque le rayonnement est maximal (100 %), soit environ à 1700 µmol $m^{-2}.s^{-1}$ (Reynolds et al. 2007). Un rayonnement lumineux en deçà de ces pourcentages induit une inhibition des processus de la photosynthèse et de la croissance de la plante. En cas de surexposition, la plante peut stopper sa photosynthèse pour préserver l'eau dans ses tissus (Carrier et al. 2019). La production maximale de matière sèche de la plante intercalaire est donc plafonnée par la quantité d'énergie lumineuse disponible pour la photosynthèse. L'élagage des arbres, leur orientation, leur densité et le choix de l'espèce (la phénologie, la structure de la canopée, la taille, la densité foliaire, la surface, l'inclinaison des feuilles et leurs caractéristiques de réflectance jouent un rôle prépondérant dans la quantité de lumière interceptée par la canopée (Guo et al. 2015; Benavides, Douglas, and Osoro 2009).

Dans la partie souterrainne, les SAI permettent une meilleure exploitation des ressources du milieu grâce à la complémentarité des arbres et des cultures pour l'utilisation de l'eau, des éléments minéraux du sol et leur impact positif sur la qualité du sol (Jose 2009). Selon Dupraz et al. (2008), trois mécanismes souterrains spécifiques, (i) filet racinaire de sécurité, (ii) pompe à nutriments, et (iii) ascenseur hydraulique, gèrent la protection des sols et l'optimisation de la production en systèmes agroforestiers intercalaires (**Figure I.16**).

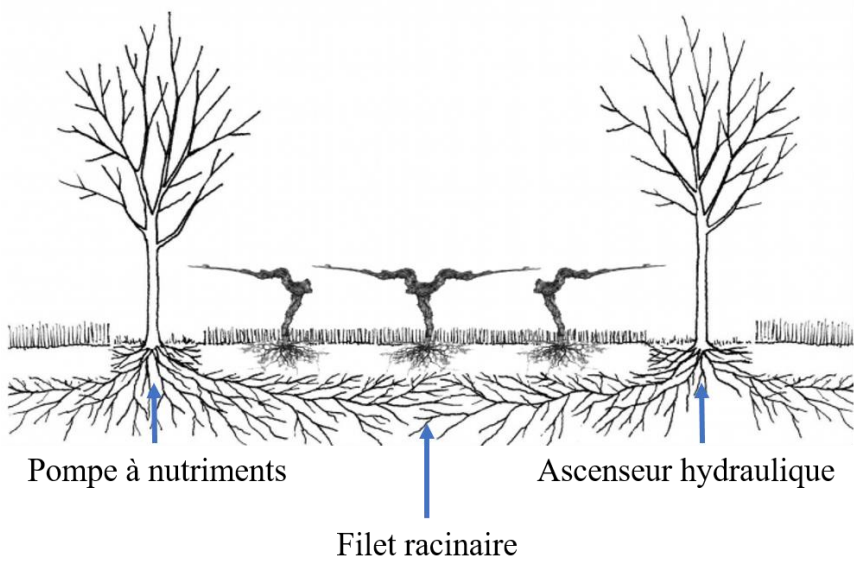


Figure I.16. Mécanismes souterrains gérant de façon bénéfique la protection des sols et l'optimisation de la production en systèmes agroforestiers intercalaires) (David Dellas, Arbre et Paysage 32).

Le filet racinaire de sécurité se rapporte à l'interception d'éléments nutritifs ou polluants drainés au-delà de la profondeur des racines des cultures, mais explorés par les racines profondes des arbres (Allen et al. 2004; Rowe et al. 1999). Les nutriments en excès lixiviés à une profondeur plus importante que la zone de racines des cultures sont récupérés par les racines des arbres et recyclés dans le système. L'azote, les cations mobiles comme le calcium, le magnésium et le potassium, sont récupérés ce qui limite la pollution des eaux de nappe (Jose 2009). La combinaison d'une minéralisation ralentie et d'une augmentation de la capacité de stockage des pluies d'automne et d'hiver diminue le risque de lixiviation d'azote.

La remontée d'éléments nutritifs par les racines profondes des arbres permet de réintroduire dans le système des éléments issus de la dégradation de la roche-mère ou de la présence passée de racines (P. K. R. Nair 1993). Cette pompe à nutriment est d'autant plus efficace que les systèmes racinaires sont profonds, ce qui est le cas en agroforesterie. Selon Thevathasan et Gordon (2004), l'efficacité du cycle de l'azote sur une parcelle agroforestière permet une réduction de la fertilisation azotée de 7 kg/ha/an (peupliers hybrides à croissance rapide âgés de 8 ans, sur une rotation de maïs, haricot, blé, avec une fertilisation annuelle moyenne de 80 kg/ha).

De plus, l'effet « pot de fleur » des cultures d'hiver (sol sec non colonisable au printemps) oblige les arbres à passer sous la zone racinée par la culture et permet une exploitation plus complète des ressources en eau (Liagre 2008). Pour piloter l'enracinement des arbres, une rotation comportant majoritairement des cultures d'hiver est donc nécessaire. Des opérations mécaniques de contrôle appelées cernages peuvent être préconisées pour délimiter la zone racinaire des arbres de celle des cultures. On utilise à cet effet un outil à dent qui passe le long des arbres pour creuser une tranchée de 50 à 100 cm incitant les appareils racinaires à s'ancrer plus en profondeur. De cette manière, les arbres peuvent remonter de l'eau puisée dans les horizons profonds et humides du sol jusqu'à des horizons de surface plus secs, par un processus physique appelé l'ascenseur hydraulique. Durant la nuit, la transpiration de l'arbre s'arrête et l'eau circule de telle sorte que toutes les parties de l'arbre se retrouvent au même potentiel hydrique. L'eau qui avait été puisée en profondeur est redistribuée et exsudée par les racines. L'eau absorbée par les racines qui se trouvaient dans les couches profondes et humides du sol est donc libérée dans les couches superficielles du sol durant la nuit (Baker and Bavel 1986; Caldwell and Richards 1989).

4.3 Gestion des systèmes agroforestiers intercalaires et SMA

Le rôle des champignons endomycorhiziens dans les systèmes agricoles est la résultante des facteurs positifs qui encouragent le développement de la SMA et des facteurs négatifs qui lui sont dommageables. Cette opposition est particulièrement exacerbée dans les systèmes alternatifs y compris les systèmes agroforestiers intercalaires en agriculture conventionnelle (Chifflot et al. 2009). Les facteurs favorables à la SMA peuvent entrer en conflit avec des pratiques culturales visant à optimiser les performances de la culture comme le labour, les pesticides ou les fertilisants (Gosling et al. 2006). A cet égard, les effets de l'agrosylviculture sur le développement de la SMA ont été énoncés en termes d'impact sur le potentiel mycorhizogène du sol et d'influence sur la dépendance mycorhizienne des espèces en co-culture (Chifflot 2008); **Tableau I.I**).

Le potentiel infectieux mycorhizogène d'un sol représente sa capacité à initier la formation d'associations mycorhiziennes à partir d'une quantité de propagules présentes dans ce sol sous forme de spores, de mycélium et de racines portant des vésicules (Declerck, Strullu, and Plenchette 1998). La dépendance mycorhizienne définit la mesure dans laquelle la plante bénéficie de la symbiose mycorhizienne pour atteindre son niveau maximal de rendement ou de croissance dans des conditions définies de fertilité du sol (Janos 2007). La dépendance mycorhizienne dépend des exigences nutritionnelles de la plante et plus précisément de la capacité de la plante à se procurer les quantités de phosphate inorganique dont elle a besoin (Plenchette, Fortin, and Furlan 1983).

Cette dépendance est grandement déterminée par le génotype de la plante qui régit sa capacité à capter les macroéléments *via* des facteurs morphologiques comme la taille et la densité des poils absorbants, la géométrie du système racinaire, et son efficacité métabolique (Plenchette, Fortin, and Furlan 1983; Declerck, Strullu, and Plenchette 1998; Janos 2007). Pour une plante donnée, il existe donc une quantité de phosphate extractible en dessous de laquelle la plante ne peut se développer sans mycorhization (Janos 2007).

Tableau I.I. Effets de l'agrisylviculture et des techniques culturales associées sur le potentiel mycorhizogène du sol et la dépendance mycorhizienne des arbres et des cultures (adapté de (Gosling et al. 2006; Rivaton 2018; Chifflot et al. 2009)

Potentiel mycorhizogène du sol	
Effets favorables	**Effet défavorables**
Cultures et arbres mycorhizotrophes **Connections arbre-culture** Augmentation du nombre de fragments d'hyphes, et pour les cultures mycorhizées futures : augmentation de la colonisation fongique et de l'absorption des nutriments **Rotation longue et variée** Amélioration du potentiel mycorhizogène des sols et de la diversité des CMA **Sources organiques à minéralisation lentes (fumier, compost, résidus de culture)** Stimulation des CMA **Effet « filet à papillon » des arbres** Interception des propagules de CMA fixées à des particules de sol transportées par le vent	**Jachère nue longue** **Monoculture** Réduction du nombre de propagules, de la colonisation par les CMA, de l'absorption des éléments **Travail du sol** **Cernage racinaire,** **Barrière en polyéthylène** **Nettoyage de la ligne d'arbres** Destruction du réseau d'hyphes des CMA, réduction et/ou retardement de la colonisation fongique, diminution du volume de sol exploré par les CMA et éventuelle réduction de l'absorption des nutriments pour la culture **Herbicides** Elimination des plantes hôtes potentielles, réduction de la colonisation et de la sporulation des CMA
Dépendance mycorhizienne des espèces en co-culture	
Effets favorables	**Effet défavorables**
Ressources du sol limitant **Compétition arbre-culture pour les ressources** **Engrais peu solubles** Induction des gènes impliqués dans le fonctionnement de la SMA	**Ombrage des arbres** **Réduction de la photosynthèse** Diminution des apports carbonés, dégénérescence des arbuscules et réduction de la sporulation **Sol riche en P** Réduction de la colonisation fongique, de la densité et de la diversité des spores **Cultures et arbres peu mycorhizotrophes** Décroissance du nombre de fragments d'hyphes, et pour les cultures mycorhizées futures : réduction et/ou retard de la colonisation fongique, réduction de l'absorption des nutriments

4.3.1 SAI et potentiel mycorhizogène du sol

La plupart des essences feuillues utilisées en agroforesterie (Aceraceae (*e.g.* érable), Ulmaceae (*e.g.* orme), Oleaceae (*e.g.* frêne), Juglandacae (*e.g.* noyer) est hautement mycorhizotrophe (Smith and Read 2009). Il est donc possible d'améliorer le statut mycorhizien de l'essence agroforestière au cours de la phase d'élevage en pépinière et d'introduire au sein du système un inoculum mycorhizien abondant et diversifié qui optimisera dans un premier temps les

performances de la plantation de l'arbre (croissance accrue, baisse de la mortalité en transplantation). L'absence de culture, par exemple dans le cas d'une jachère nue, a un impact négatif sur le nombre de propagules viables contenus dans le sol (Troeh and Loynachan 2003). De nombreuses études ont mis en évidence l'effet dépressif de la monoculture sur l'abondance et la diversité des propagules mycorhiziennes dans le sol ainsi que sur les réseaux d'hyphes mycéliens (Plenchette, Fortin, and Furlan 1983; Gosling et al. 2006). Les CMA peuvent s'associer à différentes espèces végétales mais des écotypes mycorhiziens peuvent être plus ou moins efficients selon la plante hôte considérée (Sanders 2002). La succession année après année de la même culture aboutit à la sélection d'un faible nombre d'espèces de CMA, spécifiques à cette plante (Peyret - Guzzon 2014). Johnson et al. (1992) suggèrent que la monoculture ne sélectionne que des espèces peu ou pas avantageuses pour la culture, ce qui peut en partie expliquer le déclin des niveaux de production agricole. Dans le cas de monocultures répétées d'espèces annuelles, les réseaux mycorhiziens sont altérés en profondeur ce qui induit une faible mycorhization des jeunes plants en début de culture et une faible croissance (Kuyper et al. 2004). Il a été montré que cette diminution du développement de l'espèce cultivée pouvait être attribuée à une nutrition en P limitée dans le cas du maïs (Miller and Sharitz 2000).

Grâce à la diversité des systèmes mixtes la présence de mycorhizes est plus importante dans les systèmes agroforestiers que dans des assolements séparés. L'amélioration de la structure du sol par la présence de l'arbre favorise aussi cette diversité. Les champignons mycorhiziens ne sont plus cantonnés aux racines d'une seule espèce mais peuvent se propager entre les co-cultures (Chifflot et al. 2009). Dans les noyeraies françaises, il a été montré que le sol agroforestier est plus riche en propagules mycorhizogènes que le sol agricole en monoculture. La présence des noyers agroforestiers augmente significativement la quantité de spores de CMA dans les allées cultivées (Liagre 2008). Les arbres jouent le rôle de réservoirs de propagules symbiotiques pour la culture associée (Shukla et al. 2012), qui à son tour peut faciliter la mycorhization des arbustes récemment plantés (Enkhtuya, Pöschl, and Vosátka 2005). La densité de spores et leur diversité dans les horizons profonds augmentent dans les parcelles agroforestières, contrairement aux monocultures dans le cas du caféier (Cardoso et al. 2003). Associés aux racines profondes des arbres, les champignons mycorhiziens augmentent l'acquisition de ressources hydriques et minérales, et donc la nutrition des espèces en co-culture (de Carvalho et al. 2010). Les arbres jouent aussi un rôle de « filet à papillon » : la canopée pouvant intercepter des propagules de CMA fixées à des particules de sol transportées par le vent (Chifflot et al. 2009).

Certaines activités anthropiques courantes en SAI peuvent affecter le potentiel endomycorhizogène des sols. Les racines pérennes des arbres permettent aux CMA de persister dans le sol d'une année à l'autre. La ligne d'arbre constitue un réservoir mycorhizien à partir duquel le réseau d'hyphes peut se propager vers l'allée cultivée, et induire la colonisation des premiers rangs de culture (Ingleby and Huddleston 2007). Les herbicides utilisés peuvent éliminer un hôte potentiel pour la survie des champignons (Chifflot et al. 2009), et peuvent avoir un effet délétère sur la germination des spores et leur propagation (Meena et al. 2020). Le cernage racinaire et l'utilisation de barrières en polyéthylène à l'interface arbre-culture sont préconisées pour atténuer la compétition entre les arbres et les cultures (Jose et al. 2000; Allen et al. 2004), mais ces pratiques empêchent les connexions de racines à racines par le réseau d'hyphes et son effet favorable sur le potentiel mycorhizogène du sol (Chifflot et al. 2009). Il en est de même pour le travail du sol par labour, qui consiste à retourner les horizons supérieurs du sol jusqu'à 30 cm de profondeur. Cette pratique permet la répression des adventices, l'aération du sol et l'incorporation des engrais, mais ce retournement a aussi pour conséquence la destruction mécanique des réseaux mycéliens mis en place (Peyret - Guzzon 2014).

4.3.2 SAI et dépendance mycorhizienne

La dépendance mycorhizienne indique dans quelle mesure la symbiose permet de satisfaire les besoins en Pi d'une plante lorsque le sol est incapable de répondre à ses exigences nutritionnelles. Dans la mesure où la plupart des essences feuillues utilisées en agroforesterie est hautement mycotrophe (Smith and Read 2009), la dépendance mycorhizienne en SAI est principalement influencée par la nature de la culture intercalaire, les conditions environnementales et l'itinéraire technique mis en oeuvre (**Tableau I.I**; (Chifflot et al. 2009)).

Les racines mycorhizées et les fragments mycéliens laissés par une culture intercalaire sont importants pour la colonisation de la culture suivante (Tommerup and Abbott 1981). Si la culture précédente n'est pas mycotrophe, les spores germées auront dégénéré, et la colonisation par les champignons MA de la culture suivante peut être réduite au détriment de son statut nutritionnel (Miller and Sharitz 2000).

Les champignons MA dépendent du carbone de la plante et tout facteur agissant négativement sur la photosynthèse peut réduire l'endomycorhization de la culture (Gavito et al. 2019). Pour les cultures intercalaires se développant à l'ombre des arbres, la colonisation endomycorhizienne et la sporulation peuvent diminuer comparativement à la même culture se développant en pleine lumière (Chifflot et al. 2009). Dans une noyeraie agroforestière française, la densité de spores de la culture intercalaire est plus faible au printemps comparativement à un système en monoculture (CASDAR 2009). L'ombrage des arbres retarderait la formation des arbuscules et la sporulation (Shukla et al. 2012). Chifflot et al. (2009) souligne également que la compétition pour l'eau réduit aussi la dépendance mycorhizienne de la culture, si cette compétition affecte la photosynthèse et l'assimilation du carbone par la plante. L'arbre peut créer un stress hydrique intense sur la culture, qui se traduit par une réduction de la photosynthèse (Miller and Pallardy 2001) et une surface foliaire plus faible (Jose et al. 2000).

Dans les environnements riches en minéraux, les racines des plantes sont capables de les acquérir sans être assujetties à la présence du CMA, ce qui diminue non seulement la dépendance mycorhizienne de la plante, mais aussi la diversité des communautés de CMA (Oruru and Njeru 2016). La SMA est plus efficace lorsque les conditions édaphiques sont contraignantes: par exemple, lorsque les ressources sont limitantes ce qui génère une compétition arbre-culture intercalaire (**Tableau I.I**; Chifflot et al. 2009). L'apport de fertilisants minéraux solubles augmente instantanément la biodisponibilité de ces éléments, ce qui tend à nuire à la dépendance mycorhizienne des arbres et des cultures (Chifflot et al. 2009). De très nombreuses études ont montré que dans ces conditions, le fonctionnement de la symbiose mycorhizienne est fortement réduit, voire supprimé (Grant et al. 2005). Par contre, les engrais qui libèrent très lentement les nutriments, comme les engrais de ferme (fumier, compost, résidus de culture, bois raméaux fragmentés) ne semblent pas avoir d'effets négatifs sur les champignons MA, et peuvent même les stimuler (Gosling et al. 2006). Seuls les systèmes de culture à bas niveau d'intrants ou ceux qui n'ont recours qu'à très peu d'engrais minéraux solubles semblent à priori favoriser la symbiose endomycorhizienne (Wani, McGill, and Tewari 1991).

5. Connaissances spécifiques aux interactions CMA – *Juglans* sp

Les mécanismes impliqués dans la nutrition carbonée et phosphatée des espèces mycotrophes pérennes restent encore peu documentés et concernent pour l'eseentiel la vigne (Valat et al. 2018) et le peuplier (Loth-Pereda et al. 2011; Calabrese 2019). Toutefois, dès les années 1980, de nombreuses études se sont interessées au rôle de la symbiose mycorhizienne à arbuscule dans l'établissement *in vitro* et *ex vitro* du noyer et d'autres ligneux et fruitiers.

Le chapitre suivant est consacré à synthétiser spécifiquement les connaissances acquises sur les interactions CMA-*Juglans* spp sous forme d'un article accepté pour publication le 12 octobre 2020 dans Agronomy for Sustainable Development.

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Chapitre II

Forty years of study on interactions between walnut tree and arbuscular mycorrhizal fungi. A review

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Abstract

Walnut trees are among the most important hardwood species in the northern hemisphere, ecologically and economically. They are mainly cultivated for timber and nut production, but are also attractive ornamental trees in parks. Establishing walnut orchards is difficult because seedlings have a coarse root architecture and few of them survive to transplanting. Planting success is mainly determined by the root system morphology and the nutrient status of the seedlings, so that rhizosphere conditions are critical for plant performance. Walnut trees can associate with soil-borne arbuscular mycorrhizal fungi, which are obligate biotrophs. In this association, plant-produced carbon compounds are traded against fungus-acquired soil mineral nutrients. The beneficial effect of arbuscular mycorrhizal symbiosis on hardwood seedling quality and field performance has long been known, but an integrated view is lacking about the effects of arbuscular mycorrhizas on walnut cropping. Therefore, we surveyed the literature published over the last forty years to provide up-to-date knowledge on the relationships between arbuscular mycorrhizas and walnut trees. Our review outlines the major following points: (**1**) the arbuscular-mycorrhiza-mediated nutrient uptake capacity of walnut trees is associated with first- to third-order roots, and fibrous tip-ended roots are dependent on arbuscular mycorrhizal fungi whereas pioneer roots are not, (**2**) early inoculation with arbuscular mycorrhizal fungi improves the survival and seedling performance attributes of transplanted walnut trees: biotization enhances walnut transplant success by increasing the number of lateral roots and plant P uptake, but these benefits are fungus- and host-dependent, (**3**) in the context of walnut agroforestry, deeply rooted walnut trees play a role as reservoirs of arbuscular mycorrhizal fungal propagules for the surrounding vegetation, but tree shade and soluble phosphate availability decrease walnut mycorrhizal dependency, and (**4**) the arbuscular mycorrhizal mycelium mediates the transport of juglone and thus plays a role in walnut tree allelopathy.

Keywords: *Juglans*, symbiosis, rootstock, acclimatization, fertilization, agroforestry, juglone, common mycelial networks

1. Introduction

Walnut is the common name given to twenty-one to twenty-five species of deciduous trees belonging to the genus *Juglans* (order Fagales, family Juglandaceae), characterized by a monoecious and dichogamous habit (Manning 1978; Gleeson 1982; Germain 1992; Willis 2000; Shah et al. 2018; Bernard et al. 2018). *Juglans* trees have been classified into four sections according to leaf, flower and fruit morphology (**Table II.1**), namely *Trachycaryon* (one species: *J. cinerea*), *Rhysocaryon* (black walnuts), *Cardiocaryon* (heartnuts), and *Dioscaryon* (one species: *J. regia*) (Manning, 1978; Bernard et al. 2018). The genus is mostly distributed across the temperate and subtropical regions of the northern hemisphere, and several species are also found in Central America and along the Andes Mountains in western South America (Bailey and Bailey 1976; Bernard et al. 2018). By providing wood, ornamental, and nutrition value to human beings, and food and a habitat to wildlife, walnuts are among the most important trees in the northern hemisphere, ecologically and economically (Bernard et al. 2018). The fruit is renowned as a rich source of unsaturated fatty acids, proteins, vitamins E and B1, selenium and iron (Bender and Bender 2005). It also contains a wide variety of flavonoids, phenolic acids and related polyphenols, which have antioxidant and anti-inflammatory properties (Martinez et al. 2010; Delaviz et al. 2017; Jaiswal and Tailang 2017). Bark or leaf extracts are notably used worldwide in traditional medicine (Amaral et al. 2004). Besides nuts and leaves, walnut is grown for its high-quality wood that is marketable for many uses, including furniture, gunstocks, veneers and panelling (Payghamzadeh and Kazemitabar 2011). In addition to direct economic benefits, various ecosystem services among which complementarities in resource-capture strategies and enhanced soil fertility have been reported from hardwood-based agroforestry systems (Jose 2009; Bainard et al. 2011; Shukla et al. 2012).

As listed in **Table II.1**, among the major species grown for commercial use are the Persian or English walnut (*J. regia* L.), the eastern black walnut (*J. nigra* L.), the northern California black walnut (*J. hindsii* Jeps.) and white walnut (*J. cinerea* L.). All species produce nuts, but *J. regia* is the main species widely cultivated for nut production, with worldwide in-shell walnut production exceeding 3,400 kt *per* year (www.nutfruit.org 2014; Bernard et al. 2018). The eastern black walnut (*J. nigra* L.) is also grown for its edible nuts, but is valued economically for its high-quality wood. The nut of the black walnut is of high flavor, but due to its hard shell and poor hulling characteristics it is not grown for commercial production (Verma 2014). Other walnut species used for valuable timber production include *J. regia, J. cinerea, J. major, J. neotropica, J. olanchana and J. mandshurica* (**Table 1**). The primary commercial importance of the Northern California black walnut (*J. hindsii*) is as a rootstock for commercial Persian walnut (*J. regia*) orchards or as a parent of the widely used hybrid rootstock 'Paradox' (*J. hindsii* x *J. regia*) (Verma 2014). Walnuts are now distributed across 60 countries around the globe as both commercial and ornamental trees (Avanzato et al. 2014).

Walnut cropping mainly relies on the propagation of cultivars of biological and economic interest. To ensure top-quality orchard trees, walnuts are mainly managed and grown to a suitable size in nurseries after grafting onto seedling rootstocks selected to provide the best anchorage, vigor, and resistance or tolerance to soil-borne pests and diseases (Lopez 2004; Verma 2014). However, poor survival after planting and slow growth rates are common difficulties encountered when establishing *Juglans* orchards (Jaynes 1979; Peixe et al. 2015). Studies on hardwood species indicate that the root system morphology is one of the major determinants of mineral nutrients such as nitrogen (N), phosphorus (P), and potassium (K) by seedlings having a particularly positive impact on their survival and development after planting (Landis 1985; Landis et al. 1989). In this context, the rhizosphere, namely the region of the soil in intimate interaction with the roots, is critical for plant performance as it contains a complex array of plant-associated communities of organisms vital for soil and plant health (Bowen and Rovira 1999; Buée et al. 2009). As such, ecosystem functions can be improved by managing the rhizosphere microbiome (Bender et al. 2016).

Table II.1. Main walnut species grown for commercial use according to Manning (1978), Bernard et al. (2018), and Shah et al. (2018). Abbreviations : SE southeastern ; SW southwestern ; NE northeastern ; NW northwestern.

Section	Species (vernacular name)	Geographic distribution	Commercial use
Dioscaryon (Common walnut)	*J. regia* L. (Persian or English walnut)	SE Europe, Iran to the Himalayas, China	Nuts - High-quality timber
Rhysocarion (Black walnut)	*J. nigra* L. (Eastern black walnut)	Eastern United States	Timber - Ornamental tree - Rootstock – Cosmetics - Abrasive and filtering properties
	J. hindsii Jeps. (Northern California black walnut)	Northern California	Rootstock orchard of *J. regia* - Ornamental tree - Timber
	J. microcarpa Berl. (Texas or little black walnut)	SW United States NW Mexico	Interbreeding with *J. major* and *J. nigra*
	J. major Heller (Arizona black walnut)	SW United States NW Mexico	Carpentry wood
	J. neotropica Diels (Andean, Ecuadorian or Columbian walnut)	NW South America	Wood (floor and decoration)
	J. olanchana Standl. & Williams (Cedro Negro)	Guatemala	Wood (furniture and lute-making)
Trachycarion (White walnut)	*J. cinerea* L. (Butternut)	Eastern United States	Lumber industry (furniture) - Medicine (cathartic properties)
Cardiocaryon (Heartnut)	*J. mandshurica* Maxim. (Manchurian walnut)	Manchuria, NE China, Korea	Timber - Ornamental tree
	J. ailantifolia Carr. (Japanese walnut)	Japan	Dye

The roots of most tree species are notably colonized by specialized soil-borne fungi that form symbiotic associations called mycorrhizas (Brundrett, 1991; Brundrett, 2009; Kariman et al. 2018). Mycorrhizal fungi play a key role in assisting plants in the acquisition of mineral nutrients, especially N, P, and K (Smith and Read 2008; Koide et al. 2014; Garcia and Zimmermann, 2014). Mycorrhizal fungi also confer protection against pathogens and root herbivores (Arya et al. 2010) and mediate carbon (C) transfer among plants (Simard et al. 1997; Lerat et al. 2002; Teste et al. 2009). *Juglans* hardwood species associate almost exclusively with arbuscular mycorrhizal (AM) fungi (Brundrett 1991; Brundrett, 2002; Comas and Eissenstat 2009; Comas et al. 2014), which belong to an ancient lineage of obligate biotrophs in the sub-phylum Glomeromycotina (Spatafora et al. 2016). AM fungi colonize plant roots to obtain plant-derived carbon in the form of sugars and lipids to sustain their growth and reproduction (Keymer et al. 2017). In return, AM fungi provide soil mineral nutrients to the host, which are acquired through the fungal extra-radical mycelium (ERM) that reaches soil volumes inaccessible to plant roots (Friese and Allen, 1991; Gutjahr and Parniske, 2013; Rich et al. 2017; Roth and Paszkowski, 2017; Wang et al. 2017). Fine (1-5 µm) fungal hyphae (Bago et al. 1998) give plants access to soil inorganic phosphate (Pi) and inorganic N in the form of nitrates (NO_3^-) and ammonium (NH_4^+) (Harrison et al. 2002; Hodge and Fitter, 2010; Bücking and Kafle, 2015; Chen et al. 2018). During mycorrhizal nutrient uptake, soil Pi and N are acquired by high-affinity transporters located in the extra-radical hyphae and translocated to fungal arbuscules (**Fig. II.1**). These tree-shaped invaginations develop in root cortex cells and enable nutrient exchanges between the two partners (Gutjahr and Parniske, 2013; Bücking and Kafle, 2015).

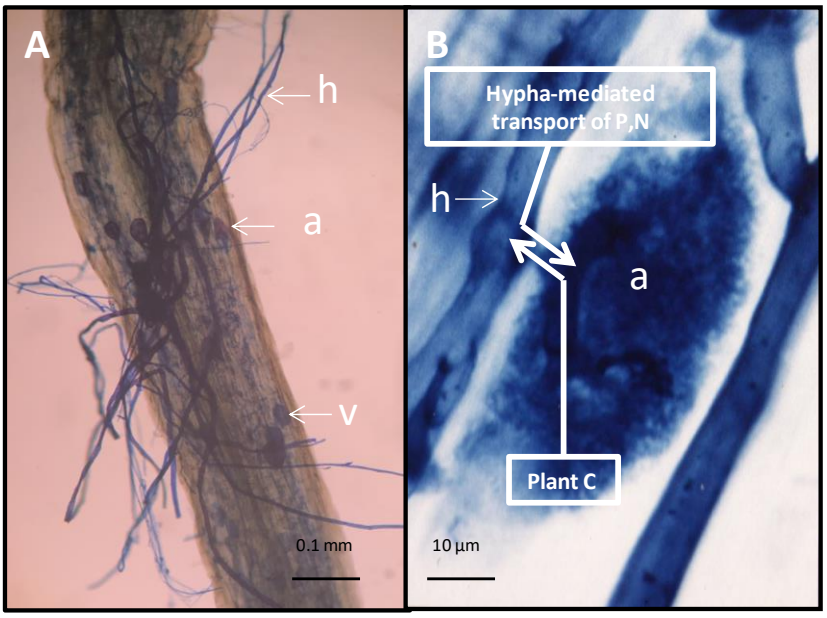

Figure. II.1. Illustrations after Trypan blue staining of **A**) AM fungal development in walnut roots with fungal hyphae (**h**), vesicles (**v**) and arbuscules (**a**), and **B**) an arbuscule consisting of highly-branched fungal hyphae that develop in root cortex cells where nutrient exchanges occur between the host and the fungus.

AM fungi act as a major biotic component of the rhizosphere because they improve plant development and nutritional status, reduce planting stress and increase the field survival rate of seedlings (Carpio et al. 2003; Davies, 2008). The role of AM symbiosis in improving hardwood seedling quality and field performance has long been known (Kormanik et al. 1982; Kormanik 1985; Cordell et al. 1987), but an integrated view of the agronomic role of arbuscular mycorrhizas in walnut cropping is still lacking. Based on a survey of the literature published over the last forty years, the present manuscript aims at providing up-to-date knowledge on arbuscular mycorrhiza - walnut tree relationships. As schematized in **Fig. II.2**, this review describes the AM colonization process of walnut as related to root morphology and anatomy. It further addresses benefits from AM biotization, which are fungus- and host-dependent. Finally, it highlights positive and negative feedbacks between walnut planting and AM fungi. The conclusion proposes future lines of research to bridge current knowledge gaps.

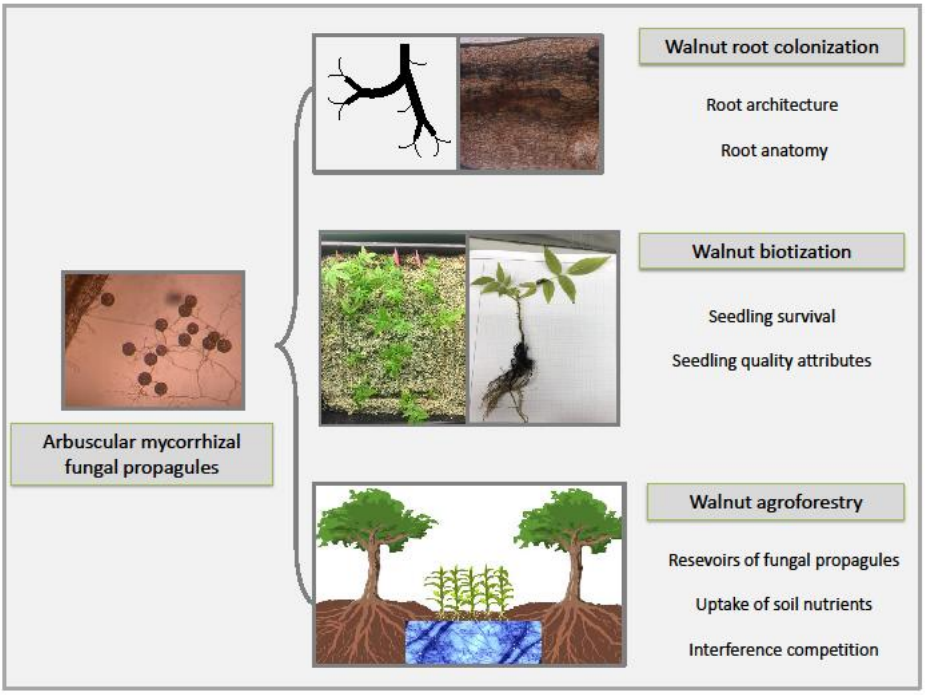

Figure. II.2. Walnut tree roots interacting with arbuscular mycorrhizal fungi.

2. Arbuscular mycorrhizal colonization of walnut is related to root architecture and anatomy

Trees usually form two main types of mycorrhizal associations: AM associations with fungi from the phylum Mucoromycota (Bonfante and Venice, 2020), and ectomycorrhizal (EM) associations with fungi mostly from the Ascomycota and Basidiomycota phyla (Wang and Qiu 2006; Brundrett 2009). Trees within a given genus usually have the same type of mycorrhiza, and these relationships are generally also consistent within families. Juglandaceae is an outlier family in which *Carya* spp. forms the EM type, whereas *Juglans* spp. predominantly displays the AM type (Brundrett, 1991). The mycorrhizal association type is not systematically related to the phylogenetic relatedness of the host tree: trees associated with different mycorrhizal types profoundly differ in root traits related to nutrient foraging (Liese et al. 2017; Kong et al. 2019). Knowledge about the root architectural and morphological features associated with AM colonization is therefore required to understand walnut acquisition of belowground resources.

2. 1 Root diameter and branching order of absorptive roots

Enhanced nutrient uptake is the major benefit for AM plants. Mycorrhizal dependency has thus been defined as the plant's inability to grow in the absence of mycorrhizas at a given soil fertility level (Gedermann 1975; Siqueira and Saggin-Júnior, 2001; Janos 2007). As such, mycorrhizal dependency is an intrinsic property of a plant species or genotype, and largely controlled by the root system architecture (Baylis 1975; Plenchette et al. 1983; Janos 2007). Traits of absorptive roots (roots less than 1 or 2 mm in diameter) are indicators of the nutrient and water uptake capacities (Pregtizer, 2002; Pregtizer et al. 2002; Guo et al. 2008). Plant species with thin absorptive roots are efficient in nutrient foraging and do not invest in AM fungi in P-limited soils (Bates and Lynch, 2001; Hodge, 2004; Liu et al. 2015). On the contrary, thick-root species have a limited intrinsic ability to absorb nutrients (Bates and Lynch, 2001). They are thus assumed to benefit from the presence of finely structured AM fungal hyphae that increase the surface area available for absorbing nutrients, especially P (Raven and Edwards, 2001; Comas et al. 2014;

Eissenstat et al. 2015; Liu et al 2019). Walnut absorptive roots have a coarse root architecture. Chen et al. (2016) reported contrasted root thicknesses for *Juglans nigra* (AM-type) and *Carya glabra* (EM-type), with mean root diameters of 0.36 mm and 0.19 mm, respectively. Thicker *Juglans* spp. absorptive roots are thus able to support more arbuscular mycorrhizas *per* unit root length or mass because AM fungi form associations within cortical cells along the root axis (Brundrett 2002; Brundrett, 2009; Guo et al. 2008; Zadworny and Eissenstat, 2011; Unger et al. 2017). In contrast, in EM associations, fungi predominantly form Hartig nets in the intercellular spaces of root tips, so that fine root systems are more adapted to EM fungal colonization (Brundrett 2002; Comas et al. 2014).

It has become increasingly clear in the last two decades that the terminal branched root system of perennial plants consist of individual units, with distinct traits (Guo et al. 2008; Salahuddin et al. 2018). Terminal root units consist of several orders that have been classified according to their branching position, with the thinnest, most distal ones termed 1st-order roots, as schematized in **Fig. II.3 A-B** (Pregitzer et al. 2002). The analysis of 23 temperate tree species, including *Juglans* spp., clearly showed that different branching orders display marked differences in anatomy (Guo et al. 2008). Based on (1) the stele-to-root-diameter ratio, (2) mycorrhizal colonization, and (3) the presence of secondary xylem (SX) and a continuous cork layer (CCL), five branching orders have been separated into two groups. In the Chinese key timber species *J. mandshurica* (**Table II.2**), the two or three distal orders have been linked to resource uptake, as inferred from the presence of mycorrhizal colonization, a low stele-to-root-diameter ratio, and no sign of secondary growth. Fourth- and higher-order roots have no mycorrhizal colonization and display a high stele-to-root-diameter ratio, indicating a limiting nutrient uptake capacity (Guo et al. 2008). As illustrated for *J. regia* in **Fig. II.3 C-D**, these results show that AM colonization occurs in first- to third-order roots, and is generally absent in the fourth and fifth orders. In summary, root anatomy and function change with position in a branching hierarchy and the AM nutrient uptake capacity of walnut trees is associated with first- to third-order roots.

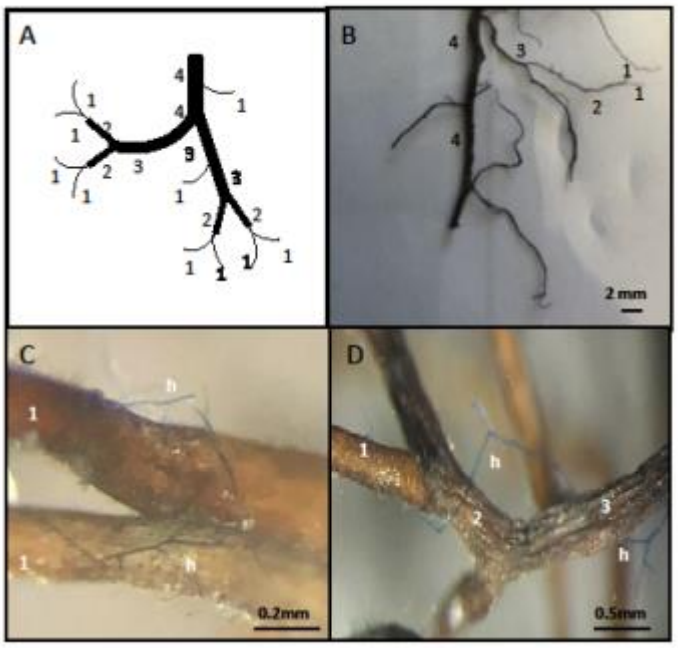

Figure II.3. Illustrations of **A**) root branch classification into orders numbered from 1 to 4, according to their position, with the thinnest, most distal roots identified as the first order; **B)** root branch order labelling of 2-month-old *J. regia* seedlings; Trypan blue-stained extra-radical hyphae

(**h**) of *Rhizophagus irregularis* DAOM 197198 in the vicinity of **C**) first-, or (**D**) second- and third-order roots of 2-month-old *J. regia* seedlings.

2.2 Fibrous vs. pioneer roots

Further work on AM tree species, including *J. nigra*, revealed that the roots emitted as first-order branches are not all the same (Zadorny and Eissenstat, 2011). All fine roots start their life as first-order roots, but only a subset grows into higher-order roots (Pagès, 2002). These pioneer roots explore the soil to expand the root system and have a relatively long mean lifetime because they undergo secondary growth (Wells and Eissenstat, 2001; Zadworny and Eissenstat, 2011). In contrast, fibrous roots are ephemeral, do not undergo secondary growth and are primarily associated with nutrient absorption (Wells and Eissenstat, 2001; Xia et al. 2010; Zadworny and Eissenstat 2011). In both young (< 14 day-old) and mixed-age *J. nigra* roots harvested after 2 years (**Table II.2**), the hypodermis of fibrous roots was composed of only one layer of cells, while the hypodermis of pioneer roots was multi-layered (**Table II.2**) (Zadworny and Eissenstat, 2011). In addition, the relative frequency of passage cells (cells with no evidence of secondary wall thickening) was lower in pioneer roots than in fibrous roots (**Table II.2**). Passage cells refer to hypodermal cells deprived of a suberin lamella; they are the only cells through which AM fungi gain access to the inner cortex (Smith and Read 1997; Brundrett and Kendrick, 1988). The abundance of passage cells *per* root explains almost all the variability in the number of AM fungi penetration points *per* root (Sharda and Koide, 2008). Importantly, Zadworny and Eissenstat (2011) revealed a complete lack of AM colonization in pioneer roots when compared to fibrous roots. Mycorrhizal colonization therefore strictly differs between pioneer and fibrous roots, and fibrous roots are dependent on AM fungi for nutrient acquisition.

3. Impact of arbuscular mycorrhizal biotization on walnut survival and seedling quality attributes

Walnut saplings are produced by seed propagation or plant tissue culture. Whatever the method, survival in field conditions is low (Jacobs, 2005a; Hacket et al. 2010). For most mycotrophic hardwood species, including *Juglans* spp., the successful establishment of seedlings largely depends on AM association and on the ability to acquire resources rapidly after planting (Smith and Read, 2008). This has led to considerable interest in evaluating the significance of inoculating walnut saplings (biotization) with selected AM fungi to enhance their survival. It is also important for nursery managers and reforestation silviculturists to identify seedling attributes quantitatively linked to planting success and to an improved field response (Burdett, 1983; Burdett, 1990; Grossnickle and Fork ,1993; Jacobs et al. 2005a; Haase ,2008; Grossnickle and MacDonald, 2018; Grossnickle et al. 2018). Seedling quality evaluation includes morphological quality based on physical attributes, and physiological quality based on metabolism. Both act as a proxy for interpreting the success of field establishment of the seedlings (Haase, 2008). Therefore above- and below-ground morphological and physiological parameters can help to grade the quality of mycorrhizal walnut saplings seedlings with regards to their planting success.

Table II.2. Arbuscular mycorrhizal (AM) colonization of *Juglans* spp. as related to root traits according to **[1]** Guo et al. (2008) and **[2]** Zadworny and Eissenstat (2011). Letters that differ within a line indicate significant ($P < 0.05$) differences; NA, not addressed.

***Juglans* species**	**Parameter**						**Ref.**
J. mandshurica (50 years old)	Root order	1	2	3	4	5	**[1]**
	AM colonization	Yes	Yes	Yes	No	No	**[1]**
	Presence rate of secondary xylem (%)	0	0	< 100	100	100	**[1]**
	Presence rate of continuous cork layer (%)	0	0	0	100	100	**[1]**
	Mean stele-to-root-diameter ratio	0.2[a]	0.3[a]	0.4[b]	0.7 [c]	0.8[c]	**[1]**
J. nigra (< 14 days old)	Root order	1 Fibrous roots	1 Pioneer roots	NA	NA	NA	**[2]**
	Mean number of hypodermal layers	1.0[a]	5.9[a]				**[2]**
	Passage cells (%)	15[a]	0[b]				**[2]**
	AM colonization (%)	21[a]	0[b]				**[2]**
J. nigra (> 2 years old)	Root order	1 Fibrous roots	1 Pioneer roots				**[2]**
	Mean number of hypodermal layers	1.0[a]	4.5[a]				**[2]**
	Passage cells (%)	10[a]	0[b]				**[2]**
	AM colonization (%)	34.5[a]	0[b]				**[2]**

3.1 Survival of walnut seedlings after *ex vitro* acclimatization and field transplanting

Due to the heterozygosity of walnut, the characteristics of agronomical interest of the chosen cultivar are not inherited *via* seed propagation (Sharma et al. 2003). Consequently, plant tissue culture plays a key role in mass propagation of high-quality walnut cultivars and rootstocks with desirable traits (Payghamzadeh and Kazemitabar, 2011). However, micropropagated walnut plantlets first undergo restricted Pi nutrition that limits biomass production (Barbas et al. 1993), and further display poor survival during acclimatization to the soil environment (Hacket et al. 2010). During this period, plantlets need to adapt to greenhouse conditions with decreased humidity and low sugar availability relatively to *in vitro* growth conditions (Fortuna et al. 1992; Schubert and Lubraco, 2000; Borkowska, 2002). The leaves of micropropagated plantlets display poor cuticular wax development, a low chlorophyll content and non-functional stomata (Rohr et al. 2003; Chandra et al. 2010). Those characteristics result in excessive transpiration rates leading to desiccation, reduced photosynthetic efficiency, and carbohydrate exhaustion before replenishment from photosynthesis. Earlier studies have highlighted a beneficial role of AM fungi during the *ex vitro* acclimatization (Azcón-Aguilar et al. 1992; Rai 2001), but to the best of our knowledge, it has been addressed only once for *Juglans* plantlets: Peixe et al. (2015) showed that inoculation with *Glomus* spp. did not improve *ex vitro* survival of *J. regia* x *J. hindsii* rootstocks. In this context, it is noteworthy that (1) AM colonization during *ex vitro* development only takes place in young secondary roots (Azcón-Aguilar and Barea, 1997), and (2) the rooting ability of micropropagated

walnut plantlets is genotype-dependent (Dolcet-Sanjuan et al. 1996). Further research is needed to assess the potential of AM fungi to improve the *ex vitro* acclimatization of micropropagated walnut trees as related to their rooting pattern and mycorrhizal dependency.

Field-transplanted nursery trees also present variable degrees of transplant stress, described as the disruption of physiological functions in seedlings. Transplant shock is mainly caused by low nutrient availability resulting from poor root-soil contact, low water porosity of suberized roots, and mechanical root damage (Reitveld, 1989; Haase and Rose, 1993; Grossnickle, 2005). Nurseries and reforestation programs can greatly benefit from AM biotization for the growth and establishment of seedlings used in forestry (Cordell et al. 1987; Perry et al. 1987; Pagano and Cabello 2011; Szabó et al. 2014). Early inoculation of walnut trees with AM fungi can help them survive once transplanted (**Table II.3**). This is especially mirrored by the calculation of the mycorrhizal response (MR) index (Plenchette et al. 1983) that represents the amount of plant gains from an AM fungal associate (Baon et al. 1993). A positive MR means that seedlings benefit from the AM symbiosis. A negative MR indicates that the costs of symbiosis for the plant exceed their benefits (Janos, 2007). **Table II.3** notably shows that the MR of walnut in terms of survival reaches up to 53 and 75% in *J. nigra* and *J. regia*, respectively. Consequently, even though indigenous AM fungi are present in the soil, early inoculation of walnut saplings with selected AM strains protects them against transplant stress.

3.2 Walnut seedling quality attributes

Seedling quality assessment aims to quantify the morphological attributes of seedlings associated with vigorous growth and development (Wilson and Jacobs, 2006). This involves a combination of several characteristics, including belowground and aboveground parameters (Jacobs et al. 2005a). As illustrated for *J. nigra* and *J. regia* (**Table II.3**), morphological grading of walnut saplings shows that AM inoculation of walnut significantly improves root biomass, total length and volume. The MR of walnut seedling lateral roots notably reaches 22% in *J. nigra* (**Table II.3**). Although this is not a general trait of mycorrhizal roots (Hetrick et al. 1988), AM colonization increases the number of lateral roots in most hardwood species and enhances the resource uptake activity (Guo et al. 2008; Zadworny and Eissenstat, 2011). Because rooting characteristics are not easily accessible, this has led to the identification of non-destructive measurements of aboveground parameters correlated to successful hardwood development.

Table II. 3. Examples of significant (P < 0.05) positive effects of arbuscular mycorrhizal (AM) fungal inoculation on the survival and quality attributes of *Juglans* saplings. AM, mean values measured in inoculated walnut seedlings. The mycorrhizal response (MR) was calculated according to Plenchette et al. (1983) as follows: MR = 100 x (mean value of AM plants–mean value of non-inoculated plants) / mean value of AM plants. ND, non-measured parameters. MPI, months post inoculation; n, number of replicates *per* treatment; Glomus is abbreviated G.

Parameter	AM	MR (%)	Inoculum	*Juglans* spp.	MPI	n	Substrate	Reference
Survival (%)	65	53.8	*G. fasciculatum*	*J. nigra*	5	5	Fumigated soil	Kormanik et al. 1982
	60	66.6	*G. mosseae* (BEG12)		9	30	Natural nursery field	Dolcet-Sanjuan et al. 1996
Root collar diameter (mm)	6.7	11.9	*G. mosseae* and *G. etunicatum*	*J. nigra*	5	4	Fumigated soil	Schultz et al. 1981
	7.9	27.8	*G. fasciculatum*		5	5	Fumigated soil	Kormanik et al. 1982
	6.5	12.3	*G. microcarpus* and *G. fasciculatum*		6	3	Steam sterilized soil	Melichar et al. 1986
	8.7	19.1	*G. deserticola*		4.5	6	Fumigated soil	Dixon, 1988
Root mass (g)	48.3	69.2	*G. mosseae* and *G. etunicatum*	*J. nigra*	5	4	Fumigated soil	Schultz et al. 1981
	75.4	80.2	*G. fasciculatum*		5	5	Fumigated soil	Kormanik et al. 1982
	102.0	52.9	*G.microcarpus* and *G. fasciculatum*		6	3	Steam sterilized soil	Melichar et al. 1986
Total root length (cm)	4897	20.8	*G. microcarpus* and *G. fasciculatum*	*J. nigra*	6	3	Steam sterilized soil	Melichar et al. 1986
	4500	33.3	*G. etunicatum*		4.5	6	Fumigated soil	Dixon, 1988
Root volume (ml)	55.6	14.4	*G. intraradices*	*J. nigra*	8	3	Fumigated soil	Brookshire et al. 2003
	59.1	19.5	*Gigaspora. margarita*					
Total number of lateral roots	132	22.7	*G. microcarpus* and *G. fasciculatum*	*J. nigra*	6	3	Steam sterilized soil	Melichar et al. 1986
Number of lateral roots > 2 mm	15.5	13.5	*Gigaspora. margarita*		4.5	6	Fumigated soil	Dixon, 1988
	17.2	22.1	*G. deserticola*					
Stem height (cm)	32.1	12.1	*G. deserticola*	*J. nigra*	4.5	6	Fumigated soil	Dixon, 1988
	49.6	10.1	*G. etunicatum*		8	3	Fumigated soil	Brookshire et al. 2003
	60.0	33.3	*Dentiscutata heterogama*	*J. venezuelensis*	3	10	Gamma irradiated soil	Fajardo et al. 2014

Stem mass (g)	3.3	21.2	*G. mosseae* and *G. etunicatum*	*J. nigra*	5	4	Fumigated soil	Schultz et al. 1981
	5.5	34.5	*G.microcarpus* and *G. fasciculatum*		6	3	Steam sterilized soil	Melichar et al. 1986
	5.6	30.3	*G. deserticola*		4.5	6	Fumigated soil	Dixon, 1988
	6.5	13.8	*G. intraradices*		8	3	Fumigated soil	Brookshire et al. 2003
Leaf area (cm^2)	1950	32.8	*G.microcarpus* and *G. fasciculatum*	*J. nigra*	6	3	Steam sterilized soil	Melichar et al. 1986
	726	20.9	*G. deserticola*		4.5	6	Fumigated soil	Dixon, 1988
	7098	45.3	*Dentiscutata heterogama*	*J. venezuelensis*	3	10	Gamma irradiated soil	Fajardo et al. 2014
Leaf count	ND	Positive	*G. mosseae* and *G. etunicatum*	*J. nigra*	5	4	Fumigated soil	Schultz et al. 1981
	17	29.4	*Dentiscutata. heterogama*	*J. venezuelensis*	3	10	Gamma irradiated soil	Fajardo et al. 2014
Leaf mass (g)	4.3	100	*G. fasciculatms*		5	5	Fumigated soil	Kormanik et al. 1982

The size of the root system and stem volume are an indicator of the seedling survival potential (Haase, 2008). In mycorrhizal *J. nigra* (Table 3), positive MRs have been recorded for the root collar diameter of *J. nigra* (**Table II.3**). Similar observations were done with stem biomass and height, which are usually used as a proxy of photosynthetic capacity (Haase, 2008; Fajardo et al. 2014). Leaf count, area and biomass were also significantly higher in inoculated seedlings than in the controls in *J. nigra* and in the timber species *J. venezuelensis* endemic to Venezuela (**Table II.3**). Positive mycorrhizal responses recorded for walnut aboveground parameters (**Table II.3**) have been ascribed to an increase in C assimilation by AM plants. More C is allocated to the leaves as a result of better nutrient availability to meet the growth demand (Fajardo et al. 2014). This was mirrored by increases in leaf P, N, and K concentrations of up to 35% following *J. nigra* and *J. venezuenlis* inoculation (**Table II.4**). As illustrated in **Fig. II4A** – a principal component analysis (PCA) of the data reported by Dixon (1988) – (see **Table II. 3**), the developmental and nutritional values of walnut saplings are positively correlated to each other and to the mycorrhizal status of seedlings. This result holds especially true for the walnut P content and the number of lateral roots. A PCA of the results reported by Kormanik et al. (1982) (**Table II.3**) also highlighted a positive correlation between the mycorrhizal status of walnut saplings, their survival and the total plant P content (**Fig. II.4B**). Taken together, these results largely support that biotization with AM fungi benefit walnut transplant success by increasing the number of lateral roots and plant P uptake. Multivariate analysis based on PCA also indicates that leaf area, stem height and root collar diameter are among the non-destructive aboveground morphological parameters correlated to AM-mediated walnut field survival.

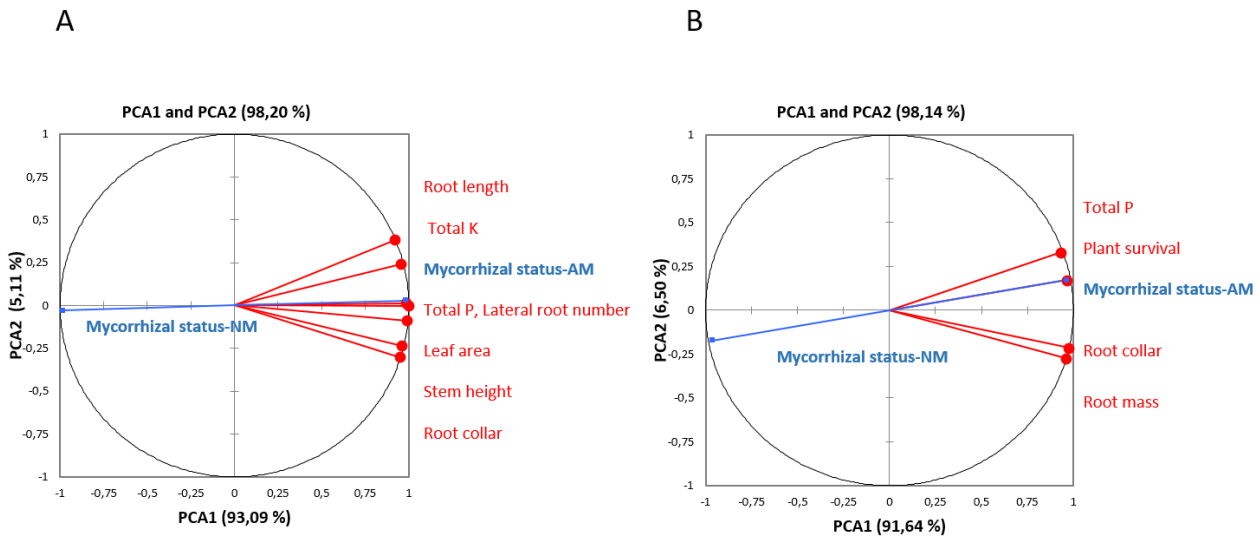

Figure II.4. Effect of mycorrhiza on walnut development and the mineral nutrient content. Growth and nutritional parameters of walnut seedlings under contrasted mycorrhizal statuses, as reported by **A)** Dixon (1988) and **B)** Kormanik et al. (1982), (both on *J.nigra*) were compared by principal component analysis, with PC1 explaining 93.09 and 91.64% of variance, respectively. AM and NM, mycorrhizal and non-mycorrhizal saplings, respectively.

Table II.4. Examples of significant (P< 0.05) positive effects of arbuscular mycorrhizal (AM) fungal inoculation on the mineral nutrition of *Juglans* saplings. AM, mean values measured in inoculated walnut. The mycorrhizal response (MR) was calculated according to Plenchette et al. (1983) as follows: MR = 100 x (mean value of AM plants–mean value of non-inoculated plants) / mean value of AM plants. ND, non-measured parameters. MPI, months post inoculation; n, number of replicates *per* treatment.

Sapling nutrient concentration (mg / g)	**AM**	**MR (%)**	**Inoculum**	***Juglans* spp.**	**MPI**	**n**	**Substrate**	**Reference**
Phosphorus								
Foliar	0.26	19.3	*Dentiscutata heterogama*	*J. venezuelensis*	3	10	Gamma irradiated soil	Fajardo et al. 2014
Foliar	0.27	22.2	*Rhizophagus manihotis*					
Total	0.69	21.7	*Glomus fasciculatum*	*J. nigra*	5	5	Fumigated soil	Schultz and Kormanik, 1982
Total	0.84	35.7	*Glomus* and *Gigaspora* species					
Total	1.20	33.3	*Gigaspora margarita*	*J. nigra*	4.5	6	Fumigated soil	Dixon, 1988
Total	1.30	38.5	*Glomus deserticola*					
Nitrogen								
Foliar	7.9	35.4	*Rhizophagus manihotis*	*J. venezuelensis*	3	10	Gamma irradiated soil	Fajardo et al. 2014
Total	2.1	23.8	*Gigaspora margarita*	*J. nigra*	4.5	6	Fumigated soil	Dixon, 1988
Total	2.1	23.5	*Glomus deserticola*					
Potassium								
Foliar	4.6	15.7	*Dentiscutata heterogama*	*J. venezuelensis*	3	10	Gamma irradiated soil	Fajardo et al. 2014
Foliar	4.2	7.4	*Rhizophagus manihotis*					
Total	5.7	28.1	*Gigaspora margarita*	*J. nigra*	4.5	6	Fumigated soil	Dixon, 1988
Total	6.4	35.9	*Glomus etunicatum*					

3.3 Benefits are fungus- and host-dependent

AM symbionts colonize a large number of host plant species (van der Heijden et al. 2015). Yet, the degree of root colonization by distinct AM inoculants does not correlate with the effects on the host plant. This result holds true for *Juglans* species, which do not respond in the same way to all AM fungal species (Schultz and Kormanik 1982; Dixon, 1988; Fajardo et al. 2014). Total P nutrition in *J. nigra* was greater in response to colonization with a mixture of *Glomus* and *Gigaspora* species than with *Glomus fasciculatum* alone (Schultz and Kormanik, 1982) (**Table II.5A**). In *J. venezuelensis*, *Dentiscutata heterogama* outperformed *Rhizophagus manihotis* with longer roots, and higher shoot-to-root mass ratio, leaf mass ratio, leaf area ratio, and chlorophyll *a* content. Although *J. nigra* roots were unequally colonized by distinct AM fungi (**Table II.5B**), similar symbiotic outcome in terms of leaf and stem weights (Kormanik et al. 1982), root length and the number of lateral roots (Dixon, 1988) were observed. The influence of the colonization of walnut roots by AM fungi of on gene expression patterns have not yet been done (Feddermann et al. 2010), but these results support the occurrence of functional diversity among AM symbionts not reflected by root colonization parameters.

Besides the AM fungal isolate, the walnut cultivar also impacts seedling quality attributes and the degree of mycorrhizal colonization of *J. nigra* seedlings (Dixon, 1988; Brookshire et al. 2003). Likewise, the seedling genotype/mycorrhiza interaction was significant for root collar diameter, leaf area, root weight and length, and colonization percentage, indicating a strong host-symbiont interaction effect (Dixon, 1988). Therefore, walnut responses to mycorrhization vary with the nature of the fungal and plant partners, and also with soil properties (Herrera-Peraza et al. 2011). As a result, one could recommend selecting an effective mycorrhizal inoculant to improve walnut seedling quality attributes based on bioassays integrating the morphological and physiological responses of each walnut cultivar.

4. Arbuscular mycorrhizal fungi in walnut agroforestry systems

Agroforestry is a land-use practice that involves planting woody perennials with other plants in a same area, simultaneously or sequentially (Nair, 1993; Garrity, 2012; Waldron et al. 2017). Relatively to tree monoculture, walnut agroforestry is based on the management of plant interactions to maximize tree growth and nut / timber productivity (Dupraz et al. 1999; Mary et al. 1998; Newman 2006; Malézieux et al. 2009; Rehnus et al. 2013). Mixed cropping has been documented for several walnut species and cultivars, including the common walnut (*J. regia*), various *J. nigra* x *J. regia* cultivars, the American black walnut (*J. nigra*), and *J. mandshurica*, the Manchurian walnut (Gordon and William 1991; Mosquera-Losada et al. 2009; Mohni et al. 2009; Yang et al. 2010; Salahuddin et al. 2018). Because fine roots are key for acquiring essential nutrients and water from the soil (McCormack et al. 2015), the sustainability of mixed-species plots largely depends on belowground complementary and competitive interactions (van Noordwijk et al. 1996; Jose et al. 2006). For mycotrophic hardwood species used in agroforestry, including *Juglans* spp., soil nutrient acquisition depends on rooting traits, and is also mediated by AM fungi (Janos, 2007; de Kroon et al. 2012). The role of these fungi in mixed plant communities is amplified by their low host specificity, so that extra-radical hyphae connect the roots of different plant species to form a common mycelial network (CMN). CMNs linking co-cultivated plant species can mediate facilitation *via* nutrient transfer (Martins and Cruz 1998; Fitter et al. 1998; He et al. 2003; Leake et al. 2004; Hauggaard-Nielsen and Jensen 2005; van der Heijden and Horton 2009; Walder et al. 2012; Felbaum et al. 2014; Gorzelak et al. 2015; Montesinos-Navarro et al. 2016), but also interference competition by releasing allelopathic chemicals that directly inhibit growth (Barto et al. 2012; Salahuddin et al. 2018). Understanding positive and negative feedbacks between mixed

walnut plantations and AM fungi is therefore of critical importance in the management of mixed cropping systems.

Table II.5. Examples illustrating that the degree of colonization of walnut tree roots by distinct arbuscular mycorrhizal (AM) inoculants does not reflect host plant responses. **A**) Significantly different *Juglans* spp. responses after inoculation with distinct AM fungi with equal compatibility in terms of root colonization. **B**) Similar *Juglans* spp. responses after inoculation with distinct AM fungi with significant different compatibility in terms of root colonization. For each parameter, different letters indicate significant ($P < 0.05$) differences. MPI, months post inoculation.

A) Equal compatibility		**Different plant responses**					
Degree of root colonization		**Plant responses**	**Inoculum**	***Juglans* spp.**	**MPI**	**Substrates**	**References**
Infection (%)	**Intensity** (%)	**P concentration (mg/g)**					
84.6[a]	3.0[a]	0.69[a]	*Glomus fasciculatum*	*J. nigra*	5	Fumigated soil	Schultz and Kormanik 1982
71.3[a]	2.7[a]	0.84[b]	*Glomus* and *Gigaspora* spp.				
Root colonization (%)	**Arbuscule/vesicle ratio**	**Total root length (m)**					
15.5[a]	1[a]	3.6[a]	*Gi. margarita*	*J. nigra*	4.5	Fumigated soil	Dixon 1988
15.8[a]	1[a]	4.5[b]	*G. etunicatum*				
Root colonization (%)	**Mycorrhizal root length**	**Total root length (m)**					
44.6[a]	6708[a]	118[a]	*Dentiscutata heterogama*	*J. venezuelensis*	3	Gamma irradiated soil	Fajardo et al. 2014
43.4[a]	9210[a]	234[b]	*Rhizophagus manihotis*				
		Leaf/plant mass ratio					
44.6[a]	6708[a]	31.7[a]	*D. heterogama*	*J. venezuelensis*	3	Gamma irradiated soil	Fajardo et al. 2014
43.4[a]	9210[a]	28.1[b]	*R. manihotis*				
		Leaf area ratio (cm^2/g)					
44.6[a]	6708[a]	80.6[a]	*D. heterogama*	*J. venezuelensis*	3	Gamma irradiated soil	Fajardo et al. 2014
43.4[a]	9210[a]	68.8[b]	*R. manihotis*		3		
		Shoot/root mass ratio					
44.6[a]	6708[a]	1.42[a]	*D. heterogama*	*J. venezuelensis*	3	Gamma irradiated soil	Fajardo et al. 2014
43.4[a]	9210[a]	1.20[b]	*R. manihotis*				
		Chlorophyll *a* ($\mu g/cm^2$)					
44.6[a]	6708[a]	5.9[a]	*D. heterogama*	*J. venezuelensis*	3	Gamma irradiated soil	Fajardo et al. 2014
43.4[a]	9210[a]	3.8[a]	*R. manihotis*				
B) Distinct compatibility		**Similar plant responses**					

Degree of root colonization		Plant responses	Inoculum	*Juglans* spp.	MPI	Substrate	Reference
Internal hyphae (%)	**Arbuscules (%)**	**Stem weight (g)**					
62[a]	29[a]	5.9[a]	*G. fasciculatum*	*J. nigra*	5	Fumigated soil	Kormanik et al, 1982
70[b]	26[ab]	3.3a	*G. mosseae* and *G. etunicatum*				
70[b]	24[b]	7.0a	*Glomus* and *Gigaspora* spp.				
Internal hyphae (%)	**Arbuscules (%)**	**Leaf weight (g)**					
62[a]	29[a]	4.3[a]	*G. fasciculatum*	*J. nigra*	5	Fumigated soil	Kormanik et al, 1982
70[b]	26[ab]	2.8[a]	*G. mosseae* and *G. etunicatus*				
70[b]	24[b]	4.8[a]	*Glomus* and *Gigaspora* spp.				
Root colonization (%)	**Arbuscule/vesicle ratio**	**Total root length (m)**					
40[a]	1[a]	4.5[a]	*G. etunicatum*	*J. nigra*	4.5	Fumigated soil	Dixon 1988
77[b]	-1[b]	4.5[a]	*G. deserticola*				
Root colonization (%)	**Arbuscule/vesicle ratio**	**Lateral root count >2 mm**					
40[a]	1[a]	15.8[a]	*G. etunicatum*	*J. nigra*	4.5	Fumigated soil	Dixon 1988
77[b]	-1[b]	17.2[a]	*G. deserticola*				

4.1 Arbuscular mycorrhizal inoculum density and diversity in walnut agroforestry systems

To form mycorrhizas, plant roots need to come into contact with AM fungal propagules – soil-borne spores, hyphae, or root fragments bearing fungal structures (internal hyphae or vesicles). These propagules represent the quantity of AM inoculum in the soil, i.e., the population of AM fungi (Plenchette et al. 2005). Compared to monoculture systems, the presence of trees in an agricultural system enhances AM fungal propagule abundance and diversity (Ingleby et al. 1997; Chifflot et al. 2009; de Carvalho et al. 2010; Bainard et al. 2011). Intercropping has been proposed to draw intermediate income before the plantation reaches economic maturity (van Sambeek and Garret, 2004; Monhi et al. 2009; van Sambeek et al. 2017; Wolz and DeLucia, 2019). In this case, the intercrop – winter cereals (*Triticum* spp), alfafa (*Medicago sativa*), soybean (*Glycine max*), or summer crops (e.g., maize) – is the only income during the first five to ten years; then, both trees and intercrops produce simultaneously (Mary et al. 1998; Huasen et al. 2014).

As woody perennial walnut roots allow for the year-in year-out persistence of AM propagules, intercropping with mycotrophic species increases mycorrhiza formation in the soil and thus the abundance of AM fungi populations. A higher AM spore density was observed in wheat roots intercropped with walnut than in crop monoculture (CASDAR, 2012). As previously indicated in other agroforestry systems, walnut trees act as reservoirs of AM fungi for crops or other annual vegetation (Ingleby et al. 2007; Kumar et al. 2007; Shulka et al. 2012). Walnut-wheat agroforestry plots displayed increased soil organic matter associated with a higher microbial biomass than monoculture plots (CASDAR, 2012). Both the number and diversity of AM spore were enhanced in the deep horizons of walnut-wheat agroforestry fields relative to conventional monoculture (PIRAT, 2012). When associated to deep walnut tree roots, AM fungi can increase the mycorrhiza-mediated uptake of soil nutrients, and thereby contribute to the nutrition of the co-cultivated species (Simard and Durall, 2004; de Carvalho et al. 2010). Because mycorrhizal types differ in their physiological traits, which facilitates dissimilar soil nutrient uptake processes, the presence and diversity of fungal associations should increase resource partitioning among the different plant species with which they associate (Ferlian et al. 2018). AM fungi associated with maize and walnut roots in a same field were recently found to differ in diversity (van Tuinen et al. 2020). Concomitantly, the analysis of ^{13}C from an AM mycelium taken from the surrounding environment of intercropped walnut and maize roots indicated that part of the carbon derived from walnut trees could be transferred to maize plants (van Tuinen et al. 2020). In summary, these results underline that walnut-tree-based intercropping enhances AM fungal richness compared to monoculture systems, and that AM fungi participate in the redistribution of nutrients between tree and crop roots.

4.2 Arbuscular mycorrhizal dependency in walnut agroforestry systems

As a genotypic property of plants, mycorrhizal dependency varies according to growing conditions and nutrient availability (Plenchette et al. 2005), with fine-root biomass generally lower in fertilized stands (Li et al. 2019). Compared to other deciduous angiosperms, walnut trees have great needs mainly in N, P, and K, and a relatively narrow range of soil conditions beneficial to their growth (Simorte et al. 2001; Bhattarai and Tomar 2009; Mohni et al. 2009; Gauthier and Jacobs, 2011). Based on analyses of nutrient levels in healthy leaf samples and comparisons with standards recognized to be non-limiting for plant growth, nutrient deficiency in the genus *Juglans* occurs for leaf N, P, and K contents below 22, 1, and 12 g *per* kg of dry matter, respectively (**Table II.6**). Fertilization enhances walnut value (Brockley, 1988; Jones et al. 1995; Jacobs et al. 2005b; Salifu et al. 2006). For example, annual applications of N and P at rates of 310 kg and 620 kg *per* ha for four years significantly increased *J. nigra* nut production and leaf nutrient levels (Ponder, 1998). Supplying plants with growth-limiting nutrients is one of the major factors in the control of fruit

yield and quality. However, plants have long been known to respond to long-term soil inorganic P or N levels in the range of 100 mg or more *per* kg of soil, with inhibition of AM symbiosis development (Baylis, 1967; Graham et al. 1981; Wipf et al. 2019 and references therein). As regards *Juglans* spp., a shift from 14 kg to 112 kg of both N and P *per* ha significantly reduced *J. nigra* root AM colonization intensity and the percentage of arbuscules (Schultz et al. 1981). In the same order of magnitude, mycorrhiza-induced growth benefits for black walnut were only reported for soil P levels below 170 kg *per* ha (Kormanik, 1985). Assuming that the total weight of one hectare of soil to a depth of 30 cm is approximately $3.9.10^6$ kg (Verheye, 2006), P fertility above 44 mg *per* kg of soil may inhibit AM colonization of walnut roots, decrease benefits of mycorrhiza, and enhance plant production costs for growers.

Besides chemical fertilization, the choice of the co-culture in walnut agroforestry has consequences on the mycorrhizal dependency of *Juglans* spp. The study of the acclimation of fine-root systems to long-term interspecific competition between larch (*Larix gmelinii*) and *J. mandshurica* trees showed that walnut displayed lower plasticity than larch as regards the branching patterns of the terminal root orders (Salahuddin et al. 2018). The arbuscular mycorrhizal colonization rates of the Manchurian walnut terminal root orders were also significantly lower (-28%) in the mixed plantation than in monoculture stands. The authors therefore proposed that the changes in mycorrhizal infection rates under interspecific competition are related to different nutrient availabilities, especially P. This hypothesis was indirectly supported by decreased specific root length and respiration rates of first-order walnut roots when grown in mixture with larch (Salahuddin et al. 2018). Consistently, Chen et al. (2001) showed that larch roots improved the mobilization of rhizospheric rock phosphate more efficiently. This resulted in an increased available P content in the rhizosphere of *J. mandshurica* in mixed plantation relatively to monoculture. In Manchurian walnut, higher P availability in mixed plantation thus leads to a decreased investment into mycorrhizal symbionts (Salahuddin et al. 2018).

As plant-produced C is exchanged for AM fungus-acquired soil mineral nutrients, factors that negatively affect photosynthesis can reduce AM plant root colonization, soil mineral nutrient uptake, and thus plant mycorrhizal dependency (Heinemeyer et al. 2003; Gavito et al. 2019). At the tree-crop interface, light is the main limiting factor for the growth of understory vegetation in agroforestry systems, where trees reduce the availability of light to intercrops (Reynolds et al. 2007). High light intensities and long day lengths improve AM colonization and spore production in many plants (Moses et al. 2013; Konvalinková and Jansa, 2016). On the contrary, tree shade, low light intensity, short day lengths, and defoliation reduce arbuscular development and spore formation because the photosynthate supply to the AM fungus decreases (Kumar et al. 2007; Shukla et al. 2009). In crop monoculture, tree shade has been evoked as the main factor responsible for the reduced AM spore density observed in agroforestry stands in spring when barley was intercropped with 30-year-old *J. nigra* and *J. regia* trees (CASDAR, 2012). Overall, these studies indicate that AM mycorrhizal dependency is responsive to seasonal variation and that tree canopy management is likely to increase the development of AM fungi in walnut intercropping systems.

Table II.6. Recommended leaf contents (% dry weight) of essential mineral macronutrients for adequate growth of *Juglans* spp, updated from Blinn and Bucker (1989) and Ponder (2004). ND corresponds to not determined.

	N	P	K	Ca	Mg	S	
Juglans spp	2.2-3.2	0.1-0.3	1.2	1.0	0.3	ND	Beutel et al. 1976
	2.2-3.2	0.14-0.3	1.2-1.7	> 1.0	> 0.3	ND	Beede et al. 2011
J. californica (California walnut)	> 2.5	> 0.11	> 1.00	ND	> 0.30	ND	Serr,1961
J. cinerea (Butternut)	1.79	0.44	0.82	1.11	0.72	0.25	Gerloff et al.19464
J. regia (Persian or English walnut)	2.6-3.5	0.19-0.24	1.3-2.3	ND	0.19-0.35	ND	Kopinga and van den Burg, 1995
	2.2-2.6	0.12-0.20	1.00-1.75	0.75-20.00	0.20-0.75	ND	Smith, 2003
	2.31-2.80	0.14-0.50	1.21-2.50	1.11-2.50	0.25-0.60	0.11-0.20	Olsen, 2006
J. nigra (Black walnut)	1.74	0.46	1.98	3.3	0.5	0.01	McHargue and Roy, 1932
	2.0-2.6	0.10-0.25	0.75-1.30	0.50-1.10	0.15-0.45	0.05-0.25	Phares and Finn, 1971
	2.1-2.6	0.15-0.21	1.05-2.00	ND	0.21-.30	ND	Kopinga and van den Burg, 1995
	1.92	0.54	1.48	0.95	1.01	0.14	Gerloff et al.1964
	2.47-2.98	0.16-0.24	1.32-1.47	1.9-2.0	0.51-0.64	0.15-0.16	Mills and Jones, 1996
	2.01-5.00	0.26-.0.39	2.10-4.00	0.51-1.00	0.26-2.50	0.22-0.55	Jacobs and Seifert, 2004
	2.2-3.5	0.20-0.33	0.9-2.0	1.2-2.5	0.3-0.6	ND	Reid et al. 2009

4.3. Arbuscular mycorrhizal fungal and interference competition in walnut agroforestry systems

Many trees of the Juglandaceae family, including *J. regia*, *J. nigra*, *J. cinerea*, *J. ailantifolia*, and *J. mandshurica*, produce juglone (5-hydroxy-1,4-naphthoquinone), an amber-colored phenolic compound poisonous to sensitive plants (Dana and Lerner, 1990; Jose and Gillespie 1998; Willis 2000; Yang et al. 2010). The presence of walnut trees has a natural inhibiting effect on several species, and acts as a growth-limiting factor in agroforestry systems (Strugstad and Despotovski, 2012). In some typical walnut intercrops, including *Zea mays* and *Glycine max*, juglone decreases and even inhibits shoot and root growth rates, leaf photosynthesis, transpiration, respiration and stomatal conductance. An inhibition threshold of 10^{-4} M (3 mg extractable juglone *per* kg of soil) was reported for maize and soybean (Hejl et al. 1993; Jose and Gillespie, 1998; von Kiparski et al. 2007 and references therein). The growth of the N_2-fixing walnut companion crop black alder and autumn-olive was depressed when hydroponically grown in solutions containing juglone at 10^{-5} M in chloroform (Rietveld, 1981).

Potential juglone abundance estimated in walnut leaves, hulls and roots ranges from less than 0.1% to 5% dry weight depending on when the samples were taken in the growing season and on the extraction techniques (Willis, 2000 and citations within). Juglone gets into the soil through rhizodeposition and leaching out of decomposing leaves, hulls, fruit or bark (Rietveld, 1983; Duroux et al. 1998; Appleton et al. 2014). Juglone release affects neighboring plants up to distances ranging from 4 m (Jose and Gillespie, 1998) to 27 m (Massey, 1925) from the trunk of walnut trees depending on their age. Toxicity persists after tree removal for up to one year because juglone is persistent in the soil (Strugstad and Despotovski 2012). However, juglone could easily be degraded by polyphenol oxidase, cellulose-decomposing microorganisms or the bacterium *Pseudomonas putida* (Yang et al. 2010 and references therein). As documented in root exudates of larch intercropped with *J. mandshurica*, the increased soil microbial populations and enzyme activities in a mixed-species plantation could lead to rapid degradation of juglone (Yang et al. 2010). Consistently, Manchurian walnut trees release a large quantity of juglone into the rhizosphere, but very little juglone reaches the bulk soil (Sun et al. 2013). These results point to soil microorganisms as an important determinant of the fate and activity of allelochemicals (Inderjit, 2005).

AM fungal connections transport purified or naturally released juglone (Achatz and Rillig, 2014; Achatz et al. 2014). The authors found an increase in juglone transport when a mycorrhizal hyphal network was present, resulting in reduced growth of the target tomato plants. The amount of extractable soil juglone in the tomato root compartment was 271% higher when the soil was connected to the walnut leaf litter by mycorrhizal hyphae (Achatz and Rillig, 2014). Because juglone is slightly hydrophobic, its movement through the AM mycelium most likely occurs *via* water flow along hyphae (Achatz and Rillig, 2014; Achatz et al. 2014). Overall, these studies underline that AM-hypha-mediated transport of juglone contribute to the allelopathic effect of walnut in agroforestry systems, but may be modulated by the presence of rhizosphere microbial communities able to degrade juglone.

5. Conclusion

Currently available knowledge indicates that walnut trees can benefit from the development of a functional AM symbiosis. Mycorrhizal inoculation notably helps *Juglans* species to establish, and improves planting performances in terms of plant survival and development. The effect of mycorrhization depends on the nature of the plant-fungus couple. As the coarse root architecture of walnut trees has a limited intrinsic ability to absorb soil nutrients, plant N and P concentrations are higher in AM-inoculated walnut seedlings than in non-mycorrhizal plants. Therefore, walnut trees benefit from symbiosis through the mycorrhizal network. However, despite the release of draft reference genomes for several *Juglans* spp. (https://harwoodgenomics.org; https://treegenesdb.org), no data is presently available on AM-specific walnut gene expression patterns. This also holds true as to the quantification of mycorrhizal symbiotic efficiency in walnut trees, i.e., C gained *via* the growth response to mycorrhizal colonization minus C spent to support the fungus. Fertilization was found to control the degree of AM colonization of walnut roots. This suggests that according to their nutrient status, walnut trees can stop allocating C to the symbiont when the cost of fungal maintenance exceeds the nutrient benefit. As an obvious implication in the context of walnut agricultural practices, fertilization with phosphate decreases AM colonization of walnut trees and reduces AM fungal community richness. However, in low-input agroforestry systems, deeply rooted mycorrhizal walnut trees act as reservoirs of AM fungal propagules for the surrounding vegetation. The existence of mycelial networks gathering trees and annual companion crops enhances complementarities in resource-capture strategies. While the transport of the allelochemical juglone by AM mycelial networks grown from walnut roots has been documented, no data is presently available on the role of CMNs in mediating N and P trophic plant-plant facilitation in walnut agroforestry. Finally, in the context of rootstock breeding and production, AM symbiosis may also find applications by potentially improving walnut plantlet acclimatization and adaptation to environmental conditions, including increased tolerance to various pests.

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Synthèse des axes de recherche développés dans le mémoire de thèse

En dépit de l'importance de la production de vitroplants de noyer fruitier en agroforesterie, la micropropagation des tigelles micropropagées est limitée par une nutrition en phosphate inorganique altérée qui inhibe la croissance des vitroplants (Introduction). De plus, si les pertes en porte-greffes micropropagés de noyer fruitier peuvent atteindre 100% après leur transfert *ex vitro*, très peu d'études ont été consacrées au rôle des CMA dans la survie de ces porte-greffes au cours de leur acclimatation et de leur *post*-acclimatation (Introduction, Chapitre II). Collectivement, les données bibliographiques soulignent que les bénéfices en termes de survie, de croissance et de nutrition phosphatée de la biotisation du noyer par les CMA dépendent du couple étudié (Chapitre II). La dépendance mycorhizienne des espèces mycorhizotrophes est en effet déterminée par le génotype de la plante hôte qui régit sa capacité à capter ce macroélément *via* des facteurs morphologiques incluant la géométrie du système racinaire et son efficacité métabolique (Chapitre I). De plus, le niveau de coopération des CMA estimée en coût carbone pour l'hôte par unité de Pi transféré, varie en fonction des espèces et des isolats. Ceci reflète en particulier des capacités différentes à développer un mycélium extra-radiculaire et à coloniser l'espace intra-radiculaire (Chapitre I).

Ces éléments indiquent que si la mycorhization est susceptible d'améliorer la croissance et la nutrition du noyer micropropagé lors de son acclimatation et *post*-acclimatation, évaluer les bénéfices de cette biotisation doit non seulement s'appuyer sur l'utilisation d'un CMA coopératif dont l'inoculum est disponible, mais aussi prendre en compte la diversité génétique des porte-greffes d'intérêt agronomique en termes de dépendance mycorhizienne (Introduction, Chapitres I et II). C'est pourquoi mes expérimentations se sont basées sur l'emploi du CMA coopératif *R. irregularis* DAOM 197198, champignon commercialisé dont le génome est séquencé (Agronutrition, France), et sur le screening de plusieurs génotypes (hybrides ou non) de *J. regia* (noyer fruitier) dont les porte-greffes d'intérêt économique sont micropropagés à grande échelle (Collaboration L. Jouve). Au regard des ressources minérales du sol, la dépendance mycorhizienne est essentiellement modulée par le phosphate inorganique. Afin d'appréhender la dépendance des différents porte-greffes micropropagés au cours de leur acclimatation et post- acclimatation (Chapitre III), deux concentrations en phosphate inorganique ont donc été utilisées.

La mise en évidence de bénéfices liés à la biotisation mycorhizienne lors d'une carence phosphatée chez certains porte-greffes implique de fait la contribution de la voie symbiotique à la nutrition minérale du noyer (Chapitre III). En l'absence d'études consacrées au programme symbiotique du noyer (Introduction, Chapitre II), le quatrième chapitre de ce mémoire a donc été dédié à une étude biomoléculaire basée sur l'orthologie pour identifier chez *J. regia* des transporteurs de Pi et des protéines de la biosynthèse et de transport des lipides impliqués dans la symbiose mycorhizienne à arbuscule

En systèmes agroforestiers, le noyer fruitier est très fréquemment associé à des cultures annuelles intercalaires comme le maïs, mais le rôle du réseau mycélien commun dans la nutrition carbonée et minérale des cultures associées est inconnu (Chapitre II). En utilisant un système agroforestier modèle, développé pendant ma thèse, connectant le porte-greffe et le maïs par le réseau mycélien commun, j'ai étudié dans le cinquième chapitre l'impact de la mycorhization du noyer sur la croissance et la nutrition de ces deux espèces végétales en conditions contrastées en phosphate disponible.

Partie 2 : Résultats

Chapitre III

Growth, nutrition, and quality of micropropagated walnut rootstocks after acclimatization and *post*-acclimatization, as dependent on phosphate fertilization and pre-inoculation with the commercial arbuscular mycorrhizal fungus *Rhizophagus irregularis*

1. Introduction

Walnut is the common name given to twenty-five species of deciduous trees belonging to the genus *Juglans* (order Fagales, family Juglandaceae) (Manning 1978; Gleeson 1982; Willis 2000; Shah et al. 2017). *Juglans* trees are mostly distributed across the temperate and subtropical regions of the northern hemisphere, and several species are also found in Central America and along the Andes Mountains in western South America (Bernard, Lheureux, and Dirlewanger 2018). The walnut tree nut is renowned in human nutrition as a rich source of proteins, polyphenols, fibers, sterols, essential beneficial unsaturated fatty and low content in saturated fats (Vinson and Cai 2012). For those significant health benefits, walnut is included in FAO list of priority plants (Gandev 2007). All species produce nuts, but *J. regia*, the English or Persian walnut, is the main species widely cultivated for nut production, with 3.8 million tons harvested worldwide in 2017 (http://www.fao.org/faostat/en/#data/QC). Production of walnut trees is commonly carried out by grafting selected varieties of English walnut scions onto seedling rootstocks selected to provide the best anchorage, vigour, and tolerance of pathogens such as *Phytophthora* spp., lesion nematodes, *Agrobacterium tumefaciens* and cherry leafroll virus (Lopez 2004; Verma 2014; Zhu et al. 2019). As such, the primary commercial importance of hybrid rootstocks 'Paradox' (*J. hindsii* x *J. regia*) is to provide tolerance of most unfavourable soil conditions as well as being relatively resistant to *Phytophthora* spp. and nematodes (McKenna and Sutter 1997; Hackett et al. 2010). Useful resistance to *Phytophthora* spp. and *A. tumefaciens* is also available among *J. microcarpa* x *J. regia* hybrids (Browne et al. 2015). The interest in using English walnut seedlings (*J. regia*) as rootstocks is related to their natural resistance to cherry leafroll virus (Liberato 2006). Due to walnut heterozygosis, the characteristics of agronomical interest of the chosen cultivar are not inherited *via* seed propagation (Sharma et al. 2003). Because walnut is difficult to propagate clonally from cuttings, *in vitro* plant tissue culture has increased in popularity over the past decades and plays a key role in mass propagation of high-quality walnut cultivars and rootstocks with desirable traits (Payghamzadeh and Kazemitabar 2011). The first *in vitro* propagation procedures for walnut trees were developed more than thirty years ago for the hybrid rootstock 'Paradox' (Driver et al. 1984), *J. regia* (McGranahan, Driver, and Tulecke 1987), and *J. regia x J. nigra* (Cornu and Jay-Allemand 1989). Advances in micropropagation and breeding technologies have further enabled the exploration of diverse *Juglans* species and hybrids for the improvement of walnut rootstocks (Leslie and McGranahan 1992; Yegizbayeva et al. 2021). Typically, walnut micropropagation essentially involves aseptic individual stem segments containing one bud that are placed into an agar medium containing a mixture of plant hormones and nutrients to promote bud growth. From

a single bud, many clonal microshoots can be produced and further used for rooting (Licea-Moreno et al. 2015).

However, a major hurdle in adopting clonal propagation for walnuts is that explants are much more difficult to root than rootstocks of other fruit and nut crops (Vahdati et al. 2004), and further undergo restricted inorganic phosphate (Pi) nutrition that limits biomass production (Barbas et al. 1993). In addition, saturated atmosphere and low light intensity on artificial culture media containing nutrients and growth regulators in concentrations exceeding levels recorded under natural environments cause developmental distortions (Novais et al. 2017; Vahdati et al. 2004; Licea-Moreno et al. 2015). These physiological, anatomical and morphological changes include a root system devoid of root hairs, which limits nutrient and water uptake, poor cuticular wax development, a low chlorophyll content, non-functional stomata and reduced photosynthetic efficiency (Rohr et al. 2003; Chandra et al. 2010; Isah 2015). Consequently, when transplanted *ex vitro*, micropropagated plantlets, including walnut, suffer from severe environmental stresses and substantial losses may occur during acclimatization to the *ex vitro* environment since both the nutrient capacity of the root system and the photosynthetic capability need to be improved (Cavallazzi et al. 2007; Hackett et al. 2010; Yegizbayeva et al. 2021). Walnut explants therefore need an acclimatization phase to repair the *in vitro* induced abnormalities and further require a *post*-acclimatization growth in greenhouse conditions when plantlets become photoautotrophic (Estrada-Luna, Davies Jr., and Egilla 2000; Chandra et al. 2010; Peixe et al. 2015). In this regard, pre-planting quality assessment is the keystone event in tree nursery production, because low nutrient availability resulting from poor root soil contact, low water porosity of suberized roots, and mechanical root damage is responsible for low survival of transplanted seedlings (Rietveld 1989; Haase and Rose 1993; Grossnickle 2012). Namely, a successful young tree stand closely depends on the quality of planted seedlings, which should be capable of resisting adverse field conditions (Binotto, Lúcio, and Lopes 2010). According to Fonseca *et al.* (2002), variables used for evaluating seedling quality should not be studied separately, that way avoiding the risk of selecting higher and yet weaker seedlings while discarding smaller, sturdier ones. Therefore, the Dickson quality index (Dickson, Leaf, and Hosner 1960), is considered as a reliable indicator of seedling quality because its calculation computes robustness and biomass distribution (Binotto, Lúcio, and Lopes 2010).

In their natural environment, plants are colonized by microorganisms both outside and inside their tissues. Some of them, particularly beneficial bacteria and fungi, can improve plant growth and health in particular under abiotic and biotic stresses (Kapoor, Sharma, and Bhatnagar 2008). The majority of woody and fruit trees, including walnut, often depend on symbiotic soil-borne arbuscular mycorrhizal (AM) fungi for normal growth and development due to the morphology of the root system, typically with coarse roots and relatively few roots per plant (Brundrett 1991; Comas and Eissenstat 2009; Comas, Callahan, and Midford 2014). AM fungi, which belong to an ancient lineage of obligate biotrophs in the sub-phylum Glomeromycotina (Spatafora et al. 2016), colonize plant roots to obtain plant-derived carbon in the form of sugars and lipids to sustain their growth and reproduction (Keymer et al. 2017). In return, AM fungi provide soil mineral nutrients, such as Pi, to the host, which are acquired through the fungal extra-radical mycelium that reaches soil volumes inaccessible to plant roots (Friese and Allen 1991; Gutjahr and Parniske 2013; Wang 2017). The stimulating effects of AM fungi on plant growth and physiology noticeably include a higher branching of the root system, an improved nutritional state due to an increased Pi supply, which in turn leads to increased photosynthetic rates and improved plant growth (Taylor and Harrier 2003; Kapoor, Sharma, and Bhatnagar 2008; Kaschuk et al. 2009; Gavito et al. 2019). Such characteristics have thus led to considerable interest in evaluating the significance of inoculating micropropagated plantlets (biotization *sensu* (Herman 1996)) with

selected AM fungi to contribute to their biohardening during the acclimatization and *post*-acclimatization stages (Samarina et al. 2017; Kanani, Modi, and Kumar 2020)**.** The beneficial effect of AM symbiosis on walnut quality seedling and field performance has long been known for the eastern black walnut *J. nigra*. For this specie that is valued economically for its high-quality wood (Verma 2014), mycorrhization enhances walnut transplant success by increasing the number of lateral roots and plant P uptake, and these benefits are fungus- and host-dependent (Schultz, Kormanik, and Bryan 1981; Kormanik, Schultz, and Bryan 1982; 1982, 0; Melichar, Garrett, and Cox 1986; Dixon 1988; Brookshire, Garette, and Robinson 2002). Fajardo et al. (2014) also showed that AM inoculation at time of transplanting improve the establishment of *J. venezuelensis*, a threatened walnut spp. endemic of Venezuela. More recently, application of AM fungi on 1-year-old *J. regia* seedlings (cv. Chandler) was found to improve growth and nutrition of walnut plants under drought stress (Behrooz et al. 2019). Likewise, Huang et *al*. (2020) demonstrated that mycorrhization of two-leaf-old germinated *J. regia* seedlings has positive effects on plant growth, root morphology, leaf gas exchange, and root nutrient acquisition after three months. However, the later studies were conducted on saplings grown from seedlings, which may be of limited relevance for micropropagated walnut plantlets because most of roots found during the first stage of acclimatization are *in vitro* formed (Huang et al. 2020; Yegizbayeva et al. 2021).

To the best of our knowledge, few studies have addressed the impact of AM fungi on the acclimatization and *post*-acclimatization of micropropagated *Juglans* spp. Peixe et al. (2015) showed that inoculation with *Glomus* spp. did not improve the *ex vitro* survival of micropropagated *J. regia* x *J. hindsii* rootstocks, while Dolcet-Sanjuan et al. (1996) reported that inoculation of micropropagated *J. regia* clones 'Serr' with the AM fungus *Glomus mosseae* or *Rhizophagus irregularis* (formerly *G. inraradices*) significantly improved plant survival when transferred to nursery. In addition, no data are available regarding the mycorrhizal behaviour of micropropagated walnut explants upon their pre-inoculation with an AM fungus on seedling quality, mineral nutrition, and growth responses, as referred to as mycorrhizal responsiveness and mycorrhizal dependency (David P. Janos 2007) (Waldemar Zangaro et al. 2005). Mycorrhizal responsiveness relates directly to host-growth rate and internal Pi demand, while mycorrhiza dependency is more related to the nutrient absorption ability of a non-colonized plant than to its nutrient requirement (Siqueira and Saggin-Júnior 2001). In this context, it is noteworthy that these mycorrhizal growth traits are determined by soil phosphate availability, but is also an intrinsic property of a plant species or genotype largely controlled by plant growth rate and the root system architecture (Baylis, n.d.; Plenchette, Fortin, and Furlan 1983; David P. Janos 2007). Mycorrhizal responses are for example negatively correlated with root morphological characteristics such as root length, root hair length, and density of root hairs (Tawaraya 2003). Because the rooting ability of micropropagated walnut plantlets is genotype-dependent (Dolcet-Sanjuan et al. 1996; McKenna and Sutter 1997; Yegizbayeva et al. 2021), further research is needed to assess the potential of the commercially available AM fungus *R. irregularis* to improve the acclimatization and *post*-acclimatization of micropropagated walnut explants as related to the impact of Pi supply and rootstock identity on their mycorrhizal seedling quality, nutrition, and growth responses. To address these questions at the acclimatization and *post*-acclimatization stages, we set up two experiments in which the mycorrhizal behaviours of seven rootstocks were recorded upon two contrasting Pi fertilization regimes.

Materials and Methods

2.1 Biological material

Seven micropropagated rootstocks were selected to evaluate the impact of the level of Pi fertilization and pre-inoculation with a commercially available AM fungus on walnut acclimatization and *post*-acclimatization, including three *J. regia* cultivars (RG2, RG6 and RG17) and four *Juglans* hybrids (RX1 (*J. microcarpa x J. regia*), Vlach (*J. hindsi x J. regia*), VX211 (*J. hindsii x J. regia*), and WIP3 ((*J. hindsii x J. regia*) *x J. regia*)). All micropropagated rootstocks were provided seven weeks after root induction by Dr. Laurent Jouve (L&J Biotech, Souillac, France) (**Fig III.1**). The model and commercially available AM fungus *R. irregularis* DAOM197198 (also known as DAOM181602) was purchased from Agronutrition (Labège, France) as an axenic suspension containing 200 spores per mL.

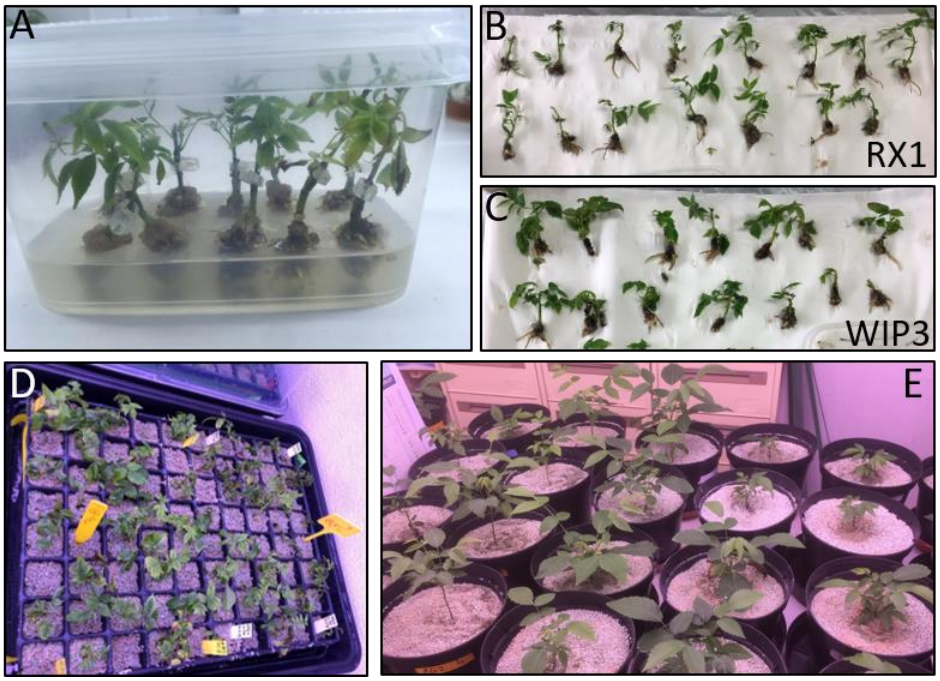

Figure III. *In vitro*-grown walnut rootstocks in their sterile container upon receipt (A) and before their transfer in mini-greenhouse (B, C). Experimental setups during the acclimatization stage in mini-greenhouse (exp. 1; D) and during the *post*-acclimatization stage (exp. 2; E).

2.2 Experiment 1. Nutrition, growth and quality of micropropagated walnut rootstocks after acclimatization as dependent on Pi fertilization and pre-inoculation with *R. irregularis*

Rootstocks were placed for two months in individual alveolar plates in closed mini-greenhouses (Rapid Grow, Nortene). Each alveolus was filled with an autoclaved (120°C, 2x 6h) silica sand (Biot, France): zeolite (Symbion, Czech Republic) substrate (1:1, v:v). Two days after their transfer in the sand-zeolite substrate, plants were either inoculated twice (mycorrhizal

treatment, AM) or not (non-mycorrhizal treatment, NM) with with 1 mL of the axenic spore suspension of *R. irregularis*. Plants were sprayed with water twice a week to maintain humidity and fertilized once a week with 15 mL per plant of either a low-Pi (P/10, 0.13 mM NaH_2PO4, $2H_2O$) or a full-strength Pi solution (PTOT, 1.3 mM NaH_2PO4, $2H_2O$) Hoagland solution (Bonneau et al. 2013). Before harvest, walnut cultures were maintained for two months in a growth chamber under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night, 100% relative humidity, 220 µE m^{-2} s^{-1}). The experiment was performed twice at a six-month interval with at least eight replicates per treatment.

2.2 Experiment 2. Nutrition, growth and quality of micropropagated walnut rootstocks after *post*-acclimatization as dependent on the Pi fertilization and pre-inoculation with *R. irregularis*

Rootstocks were placed for *ex vitro* acclimatization in closed mini-greenhouses (Rapid Grow, Nortene) filled with a sterilized (120 °C, 12h) quartz sand (Biot, France): zeolite (Symbion, Czech Republic) substrate (1:1, v:v). They were sprayed twice a week with tap water to maintain humidity and fertilized once a week with 15 ml per plant of a NPK 6-4-6 solution containing 2% of magnesium and oligoelements (Algoflash, France). Walnut cultures were maintained in a growth chamber under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night, 100% relative humidity, 220 µE m^{-2} s^{-1}). After two months of growth, plantlets were gradually exposed to reduced relative humidity by progressively removing the box covers during a further month, then rootstocks were transplanted in individual pots, each containing 3 L of the sterilized the sand-zeolite substrate. Two days after their transfer, plants were either inoculated twice (mycorrhizal treatment, AM) or not (non-mycorrhizal treatment, NM) with with 1 mL of the axenic spore suspension of *R. irregularis*. Plants were sprayed with water twice a week to maintain humidity and fertilized once a week with 15 mL per plant of either a low-Pi (P/10, 0.13 mM NaH_2PO4, $2H_2O$) or a full strength Pi solution (PTOT, 1.3 mM NaH_2PO4, $2H_2O$) Hoagland solution (Bonneau et al. 2013). Before harvest, walnut cultures were maintained for two months in a growth chamber under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night, 100% relative humidity, 220 µE m^{-2} s^{-1}). The experiment was performed twice with at least eight replicates per treatment.

2.2 Measurement of plant growth and nutritional parameters

Plant height (cm), branch number per plant, stem diameter at the soil line (mm), fresh shoot and root weights (g), and primary root length (cm) were measured at harvest. Roots were scanned and the resulting images were processed and analysed with ImageJ software (Schneider, Rasband, and Eliceiri 2012) to assess the root surface area. To determine dry matter weights (DW), shoot and root subsamples were separately weighed fresh, and subsequently dried for 2 days at 80 °C and weighed. For N and Pi measurements, dry root and shoot samples were finely ground using a Retsch Mixer ball mill (Haan, Germany). Soluble Pi was extracted from 5 mg dry matter samples as described by Grunwald et al. (2009). Free Pi was determined spectrophotometrically by measuring absorbance at 650 nm using the BIOMOL Green™ Reagent (Enzo Life Sciences, Villeurbanne, France) according to the instructions of the manufacturer. Dry matter aliquots (5mg) were analyzed for their nitrogen (N) contents at the Biogeosciences Laboratory of the University of Bourgogne Franche-Comté (Dijon, France) on a FlashSmart Elemental Analyzer (Thermo Scientific).

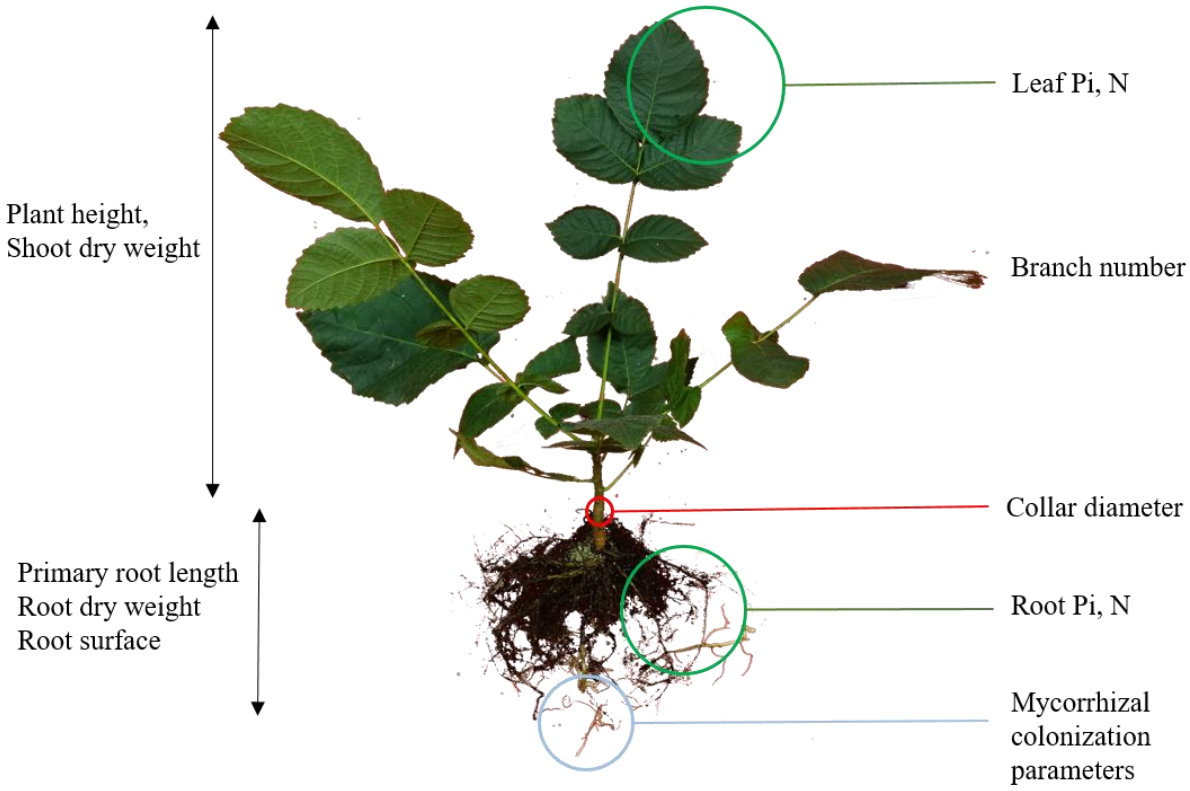

Figure III.2. Nutritional and growth parameters analysed in this study.

2.3 Staining of AM fungi in plant roots and quantification of root colonization

At harvest, root samples were randomly collected from each plant and dried for 2 days at 80 °C. They were cleared with at 90 °C for 30 min in 10% (w/v) KOH (Phillips et al. 1970). Walnut roots were additionally bleached with three drops of 30% H_2O_2 to remove any phenolic compounds left in cleared roots (Kormanik and McGraw 1982). Roots were rinsed with tap water before staining in a 0.05% (w/v) trypan blue solution in lactoglycerol (lactic acid-glycerol demineralised water, 1:1:1) for 30 min at 90 °C. Thirty fragments, ca. 10 mm long, were randomly collected from each sample, mounted in glycerol (15 fragments per slide) and observed at × 200 magnification as described in Trouvelot et al. (1986). Calculations of frequency of mycorrhization (F%), percentage of root cortex colonisation (M%) and percentage of arbuscules (A%) were carried out with the MycoCalc program (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

2.4 Metrics and statistical analyses

The Dickson Quality Index (DQI) was calculated individually for each plant according to the following formula (Dickson et al, 1960): DQI = (Shoot DW (g) + Root DW (g))/(Plant height (cm)/Stem diameter (mm) + Shoot DW/Root DW). The mycorrhizal dependency (MD) was calculated based on the average of total dry matter content of the plants (Shoot DW (g) + Root DW

(g)) according to Plenchette, Fortin, and Furlan 1983 : MD = 100 x (Dry matter of mycorrhizal plant - dry matter of non-mycorrhizal plant)/dry matter of mycorrhizal plant). The mycorrhizal growth response (MGR) was calculated based on the average total dry matter content of the plants according to Baon et al. (1993) : MD = 100 x (Dry matter of mycorrhizal plant - dry matter of non-mycorrhizal plant)/dry matter of non-mycorrhizal plant). The mycorrhizal quality response (MQR) was calculated in the same way, with values of DQI in the place of dry matter in the latter equation. Similarly, we determined the effect of pre-inoculation with *R. irregularis* on the shoot and root Pi content of the plant (mycorrhizal phosphate response; MPR) according to the following formula (Wang et al. 2016): MPR = 100 x (Pi content of mycorrhizal plant - Pi content of non-mycorrhizal plant)/Pi content of non-mycorrhizal plant). The results were further explored through a principal component analysis (PCA) performed with XLSTAT (Microsoft Excel®). As mycorrhizal colonization parameters represent percentages, all data were arcsine square root-transformed to obtain a distribution of values that could be checked for normality using the Kolmogorov-Smirnov test. Significant differences ($p < 0.05$) between transformed values recorded between the two contrasting Pi fertilization regimes were analysed using with XLSTAT by the Welch-test (degrees of freedom = n-1), which is compatible with unequal variances between groups (Staer et al. 2014). For each rootstock, the impact of the pre-inoculation treatment and the Pi fertilization regime on the growth and nutrition parameters was analysed using the Kruskal-Wallis test. When the Kruskal-Wallis test was significant ($p < 0.05$), a *post*-hoc analysis was performed to determine the extent to which variables differ from each other by using the Tukey's Honest Significant Difference (HSD) test. The chi-squared test was further used to assess whether the effect of the variability between the two experiments performed in this study was significantly higher than the effect of the inoculation or the Pi treatment. The three latter tests were performed under the Rstudio environment.

3. Results

3.1 Experiment 1. Nutrition, growth and quality of micropropagated walnut rootstocks after acclimatization as dependent on Pi fertilization and pre-inoculation with *R. irregularis*

Mycorrhizal root colonization parameters were recorded after two months of acclimatization in plants pre-inoculated (AM) or not (NM) with the AM fungus *R. irregularis* supplied with two contrasting levels of Pi fertilization. Fungal structures were not observed inside and outside the roots of NM walnut rootstocks. When considering plants inoculated with *R. irregularis*, the results displayed in **Figure III.3** show that mycorrhization developed to a similar extent in the two Pi fertilization regimes in the rootstocks RG2, RG6, RG17, RX1 VX211 and WIP3. Depending on the rootstock analysed, mean external frequency of mycorrhization of root fragments (F%), the intensity of root cortex colonization (M%) and mean arbuscule abundance (A%), ranged from 30 to 100%, 1.3 to 20%, and 0.9 to 15%, respectively. By contrast, in the rootstock VLACH, the three parameters F, M and A% were significantly ($p < 0.05$) lower in the highest Pi treatment. Noticeably, when compared to the M and A% that reached 21 and 18% in the P/10 condition, less than 2% of VLACH root length were colonized by intraradical hyphae, and only 1% root length formed arbuscules in the PTOT condition. These results indicate that rootstock genotype and Pi supply have an influence on the mycorrhizal colonization after the acclimatization stage.

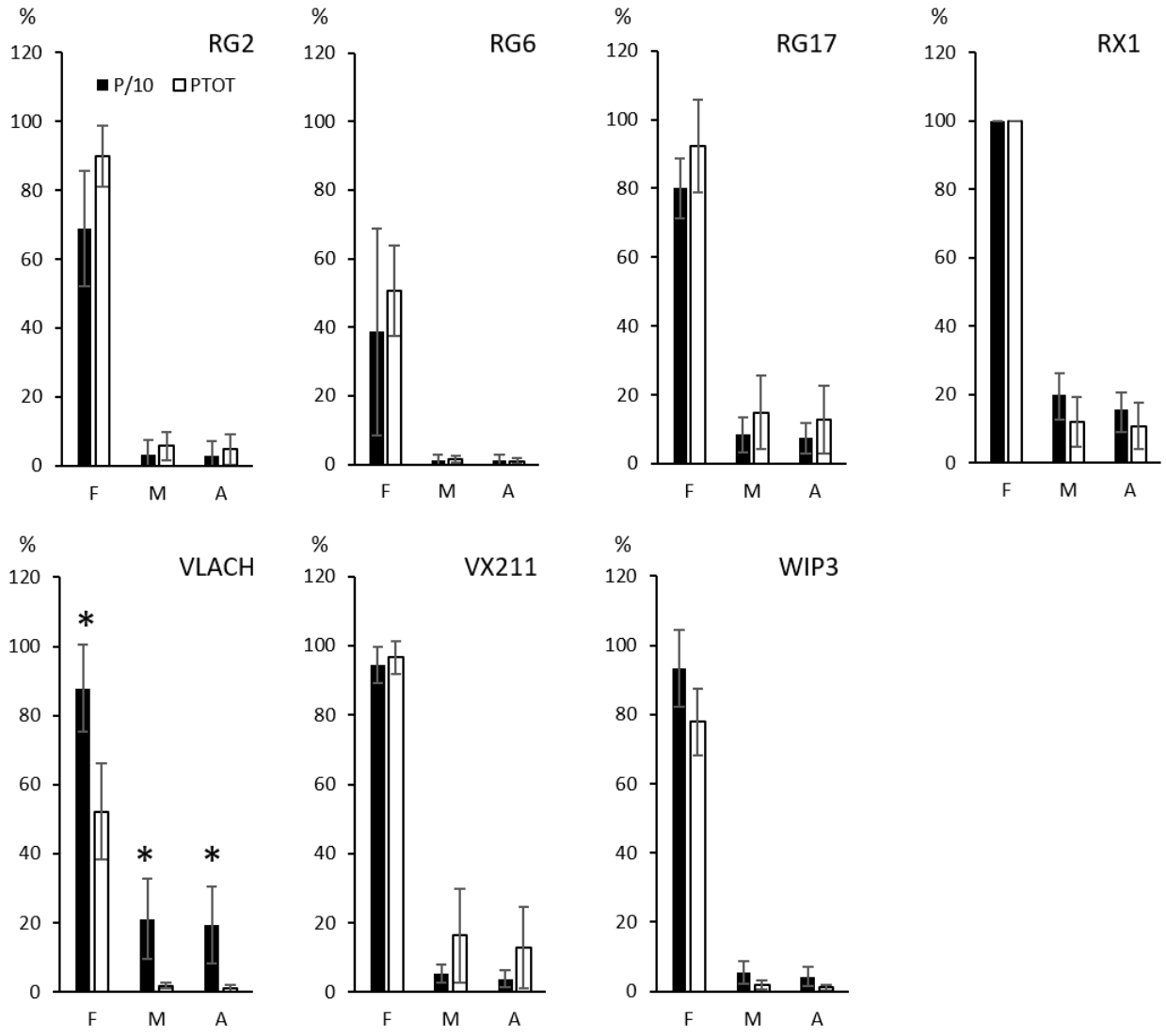

Figure III.3 Mycorrhizal root colonization after two months of acclimatization of walnut rootstocks pre-inoculated with the AM fungus *R. irregularis* under two contrasting Pi fertilization regimes. Mean data from a representative experiment out of two independent experiments are presented in the P/10 (dark bars) and PTOT (white bars) fertilization regimes. Error bars represent ± SE per treatment with $n \geq 8$. Asterisks indicate significant ($p < 0.05$) differences between the two Pi fertilization regimes.

To investigate the effects of pre-inoculation with *R. irregularis* on walnut rootstocks after the acclimatization stage, growth, quality and nutritional parameters were first compared between AM and NM plants under the two contrasting fertilization regimes. The data displayed in **Table SIII.1** show that for the rootstock RG2, leaf Pi concentrations were significantly ($p < 0.05$) lower in the P/10 treatment than in the PTOT condition in both NM and AM plants. Irrespective of the

Pi supply, mycorrhization did not affect the development and the nutrition of RG2, but the root DW was significantly higher in AM-PTOT plants than in AM-P/10 plants. Pre-inoculation of RG6 with *R. irregularis* had no impact in the P/10 condition, but led to a significant increase in root DW and rootstock quality, as measured by the Dixon quality index (DQI) in the PTOT treatment. In the two Pi fertilization regimes tested, root DW and DQI were higher in mycorrhizal than in non-mycorrhizal RG17 rootstocks. In parallel, AM RG17 walnuts showed a significantly lower S/R DW ratio than non-inoculated plants whatever the Pi supply. The latter result was also true for RX1. In this rootstock, mycorrhization led to a higher leaf N content in the P/10 condition. Under the PTOT condition, pre-inoculation of VX211 with *R. irregularis* resulted in a significant increase in leaf Pi concentration without affecting the rootstock morphological parameters. In the P/10 treatment, pre-inoculation of VX211 with *R. irregularis* resulted in a higher the Pi concentration and a lower S/R DW ratio relative to NM plants. Irrespective of mycorrhization, significantly lower primary root lengths and root surfaces, together with higher shoot DW and S/R DW ratios were recorded in the P/10 condition relative to the PTOT treatment. In the two fertilization regimes, mycorrhization of WIP3 led to a significant increase in leaf Pi concentrations without significantly affecting morphological parameters. In both AM and NM WIP3 plants, height, branch number and root surface were lower under the P/10 condition than under the PTOT fertilization. With regard to the rootstock VLACH, pre-inoculation with *R irregularis* resulted in higher leaf Pi concentrations (p = 0.09) relative to NM plants in the two fertilization regimes with different outcomes on morphological parameters **(Figure III.4A).**

In the PTOT treatment, the primary root length was lower in AM than in NM rootstocks **(Figure III.4H)**. In the P/10 condition, the collar, DQI, root, shoot and total DWs were significantly higher in AM than NM plants **(Figure III.4I, J, C, D, E)**. We also observed that relative to the NM-PTOT treatment, the NM-P/10 condition resulted in a significant ($p < 0.05$) decrease in leaf N content, primary root length, height, root DW, shoot DW, total DW and explant quality **(Figure III.4B, H, G, C, D, E, J)**, indicating that the lower Pi availability in the P/10 treatment limited the development, quality and nutrition of the rootstock VLACH. Noticeably, **Figure III.4** showed that AM symbiosis palliated the reduced Pi availability in the P/10 treatment for the rootstock VLACH, as inferred from the similar values recorded between the AM-P/10 and NM-PTOT conditions for parameters such as plant height, root DW, show DW, total DW, and quality index **(Panels G, C, D, E, J)**.

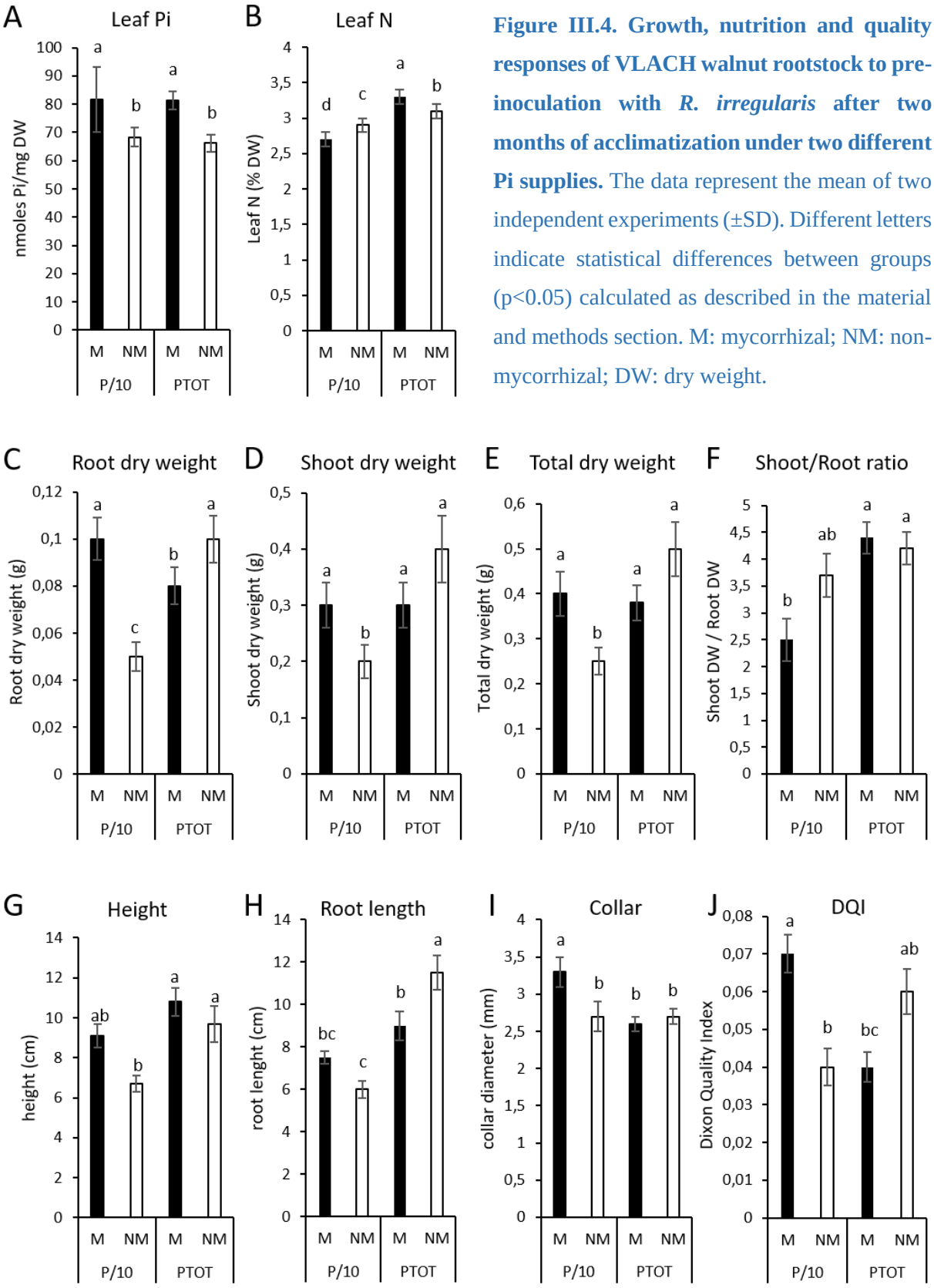

Figure III.4. Growth, nutrition and quality responses of VLACH walnut rootstock to pre-inoculation with *R. irregularis* after two months of acclimatization under two different Pi supplies. The data represent the mean of two independent experiments (±SD). Different letters indicate statistical differences between groups ($p<0.05$) calculated as described in the material and methods section. M: mycorrhizal; NM: non-mycorrhizal; DW: dry weight.

In a second time, to compare the impact of the differential Pi supply on the walnut overall behaviour in response to pre-inoculation with *R. irregularis* as dependent on the rootstock identity, we calculated in the two Pi fertilization regimes the mycorrhizal dependency (MD%), the mycorrhizal growth response (MGR%), the mycorrhizal shoot phosphate response (MPR_S%) and the mycorrhizal quality response (MQR%) (**Table III.1).** It showed that rootstock responses to pre-inoculation with *R. irregularis*, as dependent on the rootstock genotype and the Pi supply, ranged from negative, neutral, positive to highly positive, as indicated by bleu, grey, pink and dark pink-shaded values, respectively.

Table III. 1. Rootstock responses to pre-inoculation with *R. irregularis* after two months of acclimatization under two different Pi supplies. Values that correspond to mean data of two experiments, were categorized as follows: -5 %< neutral< 5% (grey values), negative :< -5% (blue values), 5 %< positive< 50% (pink values); highly positive≥ 50% (dark pink values). MD%, mycorrhizal dependency; MGR%, mycorrhizal growth response, MPRs%, mycorrhizal shoot phosphate response, MQR%, mycorrhizal quality response.

	RG2		RG6		RG17		RX1		VLACH		VX211		WIP3	
	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT
MGR %	17,6	14,2	-12,4	30,1	26,4	24,6	4,5	4,3	73,7	-14,9	26,9	-17,9	113,5	-20,2
MD %	15	12,5	-14,1	23,1	20,9	19,7	4,3	4,1	42,4	-17,5	21,2	-21,8	53,2	-25,2
MQR %	100	0	33,3	100	50	75	0	0	75	-33,3	66,7	-20	50	-20
MPRs %	34	-32,5	-9,6	-1,02	33,3	146	90	-9,3	80	-8	47,5	58,8	168,6	-35,9

Irrespective of the Pi supply, MGR, MD and MR% were neutral for RX1, while positive for RG6. On the opposite, all mycorrhizal responses were positive for VLACH and WIP3 in the lowest Pi condition, and negative in the highest Pi treatment (**Table III.1**). The results obtained were further explored through a principal component analysis (PCA) to assess the relationships between the different parameters. As displayed in **Figure III. 5A**, the first principal component (PC1), which explained 74.2% of the variation in the data, captured overall walnut mycorrhizal responses. Noticeably, MD, MGR, MPR_S, MQR% and P/10 fertilization were positively correlated to each other and negatively correlated to the highest Pi fertilization regime on this first axis. PC1 therefore represented the measurement of plant responses to mycorrhization: the more a rootstock lies on the positive side of this axis, the more its mycorrhizal behaviour in terms of growth, shoot Pi content and seedling quality is positive to the inoculation with *R. irregularis.* As shown in **Figure III. 5B**, the overall mycorrhizal responses of RX1 loaded negatively on PC1, irrespective of the Pi supply. In the lowest Pi supply (P/10: blue circles), the mycorrhizal behaviour of RG2, VLACH, VX211 and WIP3 clustered to the positive side of PC1, opposite to what observed upon the highest Pi fertilization regime (P: green circles). On the contrary, the mycorrhizal responses of RG6 and RG17 increased in the PTOT condition relative to the P/10 treatment. Taken together, these results show that after acclimatization, walnut shoot phosphate nutrition, growth and quality responses to pre-inoculation with *R. irregularis* depended on the rootstock genotype and the level of Pi supply.

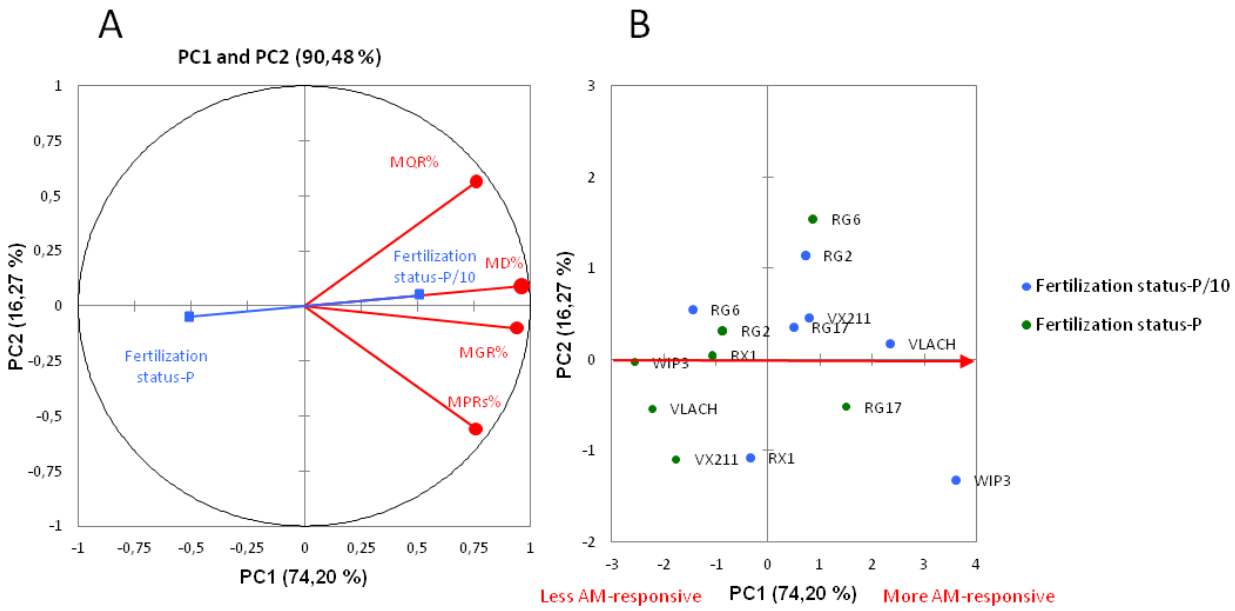

Figure III.5. Relationships between walnut rootstock responses to pre-inoculation with *R. irregularis* after two months of acclimatization under two different Pi supplies A) Circle of correlations showing that the positive side of axis PC1 is correlated to walnut responses to *R. irregularis* in the lowest Pi supply. Red and blue colours refer to active and supplementary variables, respectively. **B**) Responses to *R. irregularis* inoculation of walnut rootstocks under contrasting Pi fertilization regimes. Blue and green colours refer to P/10 and PTOT supply, respectively. Values correspond to mean data of two experiments. MD%, mycorrhizal dependency; MGR%, mycorrhizal growth response, MPRs%, mycorrhizal shoot phosphate response, MQR%, mycorrhizal quality response.

3.2 Experiment 2. Nutrition, growth and quality of micropropagated walnut rootstocks after *post*-acclimatization as dependent on Pi fertilization and pre-inoculation with *R. irregularis*

After acclimatization of the seven non-mycorrhizal rootstocks, walnut plants were either pre-inoculated or not with *R. irregularis* and further *post*-acclimatized in growth chamber conditions using two different Pi fertilization regimes. Mycorrhizal root colonization parameters were recorded after two months of *post*-acclimatization. Fungal structures were not observed inside and outside the roots of NM walnut rootstocks. When considering plants pre-inoculated with *R. irregularis*, the results displayed in **Figure III.6** show that mycorrhization developed to a similar extent in the two Pi fertilization regimes in the rootstocks RG2, RG6, RG17, RX1, VLACH and WIP3. Depending on the rootstock analysed, F, M, and A% ranged from 60 to 99%, 1.4 to 21%, and 0.7 to 13%, respectively. By contrast, in the rootstock VX211, the three parameters F, M and A% were significantly ($p < 0.05$) lower in the highest Pi treatment. When compared to the M and A% that reached 9 and 8% in the P/10 condition, less than 4% of VX211 root length were colonized by intraradical hyphae and only 1.6% root length formed arbuscules in the PTOT condition. These

results indicate that rootstock genotype and *post*-acclimatization stage influenced the mycorrhization parameters.

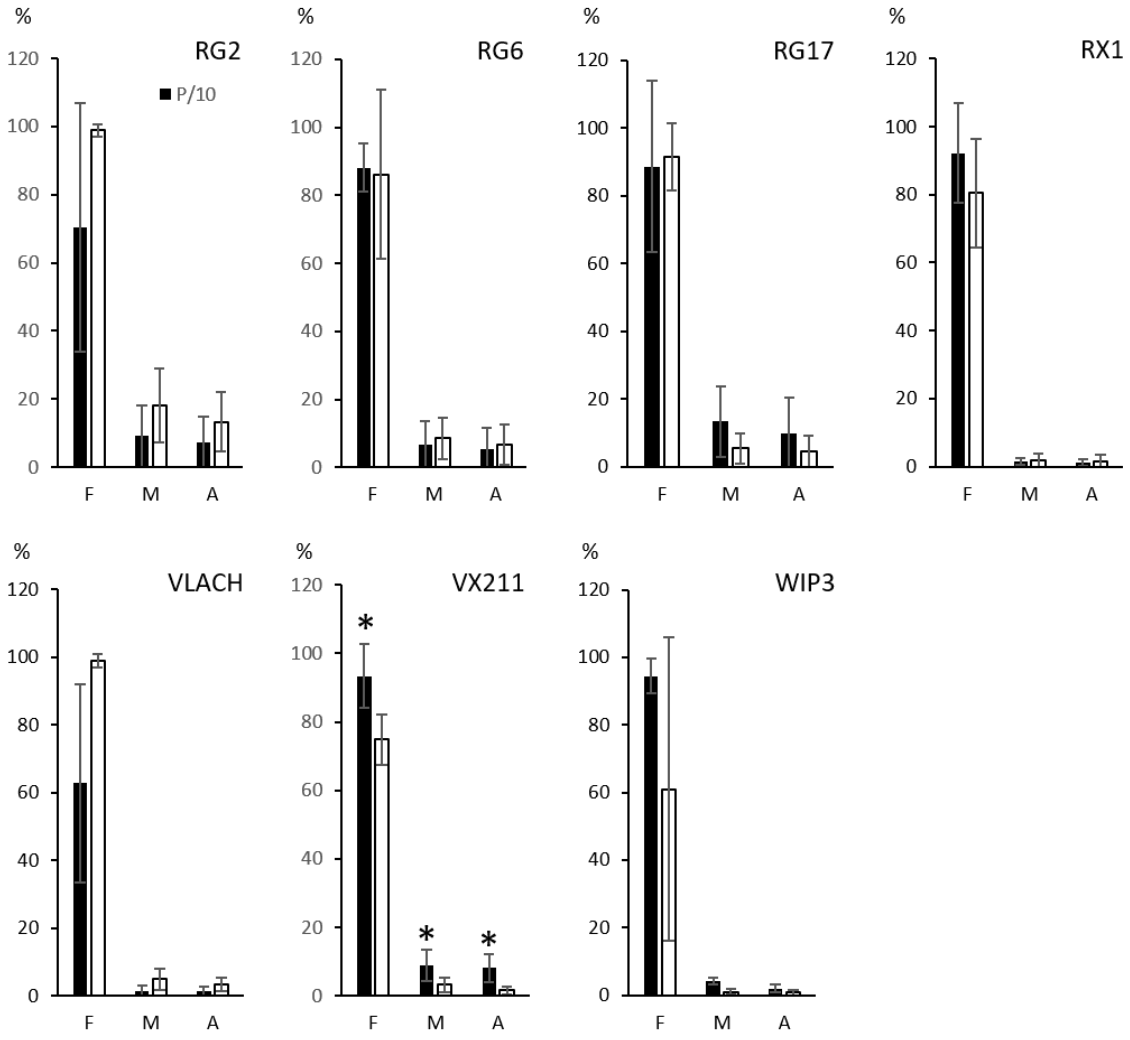

Figure III.6. Mycorrhizal root colonization after two months of *post*-acclimatization of walnut rootstocks pre-inoculated with the AM fungus *R. irregularis* under two contrasting Pi fertilization regimes. Mean data from a representative experiment out of two independent experiments are presented in the P/10 (dark bars) and PTOT (white bars) fertilization regimes. Error bars represent ± SE per treatment with $n \geq 8$. Asterisks indicate significant ($p < 0.05$) differences between the two fertilization regimes.

Growth, quality and nutritional parameters were further compared between AM and NM plants under the two contrasting Pi fertilization regimes in order to assess the effects of pre-inoculation with *R. irregularis* on walnut rootstocks after the *post*-acclimatization stage. The data presented in **Table III.S1** show that under the two fertilization regimes, AM RG2 rootstocks,

relative to NM controls, displayed higher leaf N content and Pi concentration, together with a lower primary root length. In both AM and NM RG2, root DW, total DW, DQI were significantly lower, and the S/R DW ratio significantly higher in the P/10 condition than in the PTOT condition. Under the two fertilization regimes, pre-inoculation of RG6 with *R. irregularis* did not significantly impact the development and nutrition of the rootstocks relative to NM saplings. In both AM and NM RG6, root and leaf N mean contents were lower, and S/R DW ratios significantly higher in the P/10 condition than in the PTOT treatment. Under the two fertilization regimes, AM RG17 rootstocks, relative to NM controls, displayed an increased height and root Pi concentration. In both AM and NM RG17 rootstocks, root N content, root and leaf Pi concentrations were inferior in the P/10 condition relative to the PTOT fertilization. With regard to RX1, whatever the fertilization regime, pre-inoculation with *R. irregularis* did not significantly impact the nutritional parameters relative to NM saplings, but significantly increased rootstock quality. AM RX1 displayed a higher total DW in the P/10 treatment and a higher shoot DW in the PTOT condition. Irrespective of the mycorrhizal status, we recorded a significantly lower root N content and higher root surface in the P/10 condition than under the PTOT condition. Under the two fertilization regimes, pre-inoculation of VLACH with *R. irregularis* resulted in a significant increase in the root Pi concentration without impacting morphological parameters. In both AM and NM VLACH saplings, root DW and DQI were higher in the lowest fertilization. Pre-inoculation of VX211 with *R. irregularis* had no significant impact on the nutrition and development of VX211 in the PTOT condition. Under the lower Pi supply (P/10) relative to NM controls, AM VX211 displayed a significantly increase in root and leaf Pi concentrations without any significant effect on morphological parameters. In this rootstock, irrespective of the mycorrhizal status, root surfaces, shoot and root PTOT concentrations and N contents were significantly lower under the P/10 fertilization than in the PTOT treatment, while S/R DW ratios were higher. Relative to NM rootstocks, pre-inoculation of WIP3 with *R. irregularis* under the P/10 condition resulted in increased height, branch number, root Pi concentration and N content, together with a lower root surface (**Figure III. 7, Panels G, H, A, C, E**). In the PTOT treatment, AM WIP3 displayed a higher height and branch number than NM rootstocks (**Figure III.7 G, H).** Irrespective of the mycorrhizal status, root surfaces were higher, while root Pi concentrations, leaf and root N contents were lower in the P/10 than on the PTOT condition (**Figure III.7 E, A, B, C)**. Noticeably, the total DW of AM WIP3 was higher in the P/10 than in the PTOT fertilization (**Figure III. 7 I**).

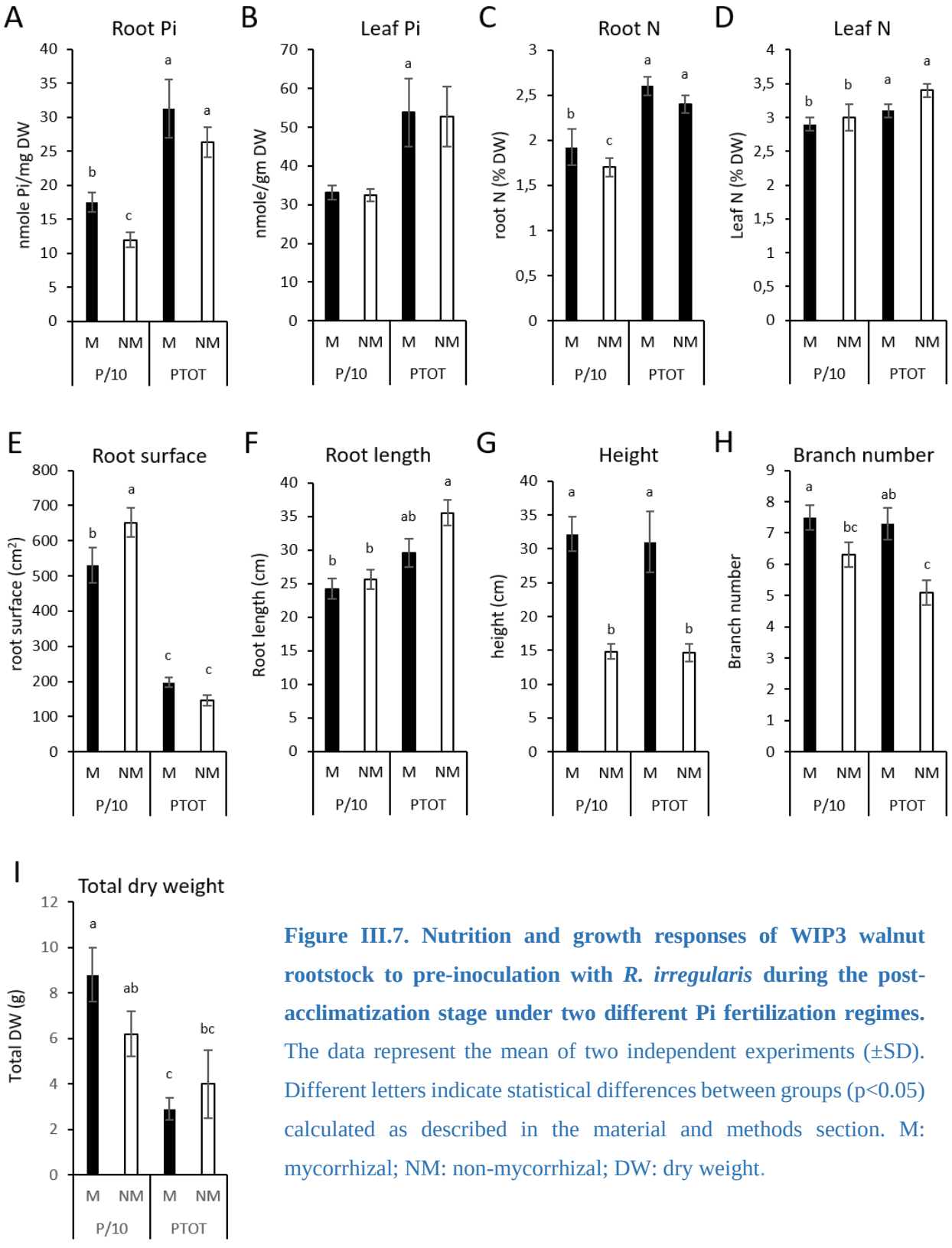

Figure III.7. Nutrition and growth responses of WIP3 walnut rootstock to pre-inoculation with *R. irregularis* during the post-acclimatization stage under two different Pi fertilization regimes. The data represent the mean of two independent experiments (±SD). Different letters indicate statistical differences between groups ($p<0.05$) calculated as described in the material and methods section. M: mycorrhizal; NM: non-mycorrhizal; DW: dry weight.

The comparison of growth, quality and nutritional parameters in the non-inoculated walnut plants between the two different Pi supplies, showed that the NM-P/10 condition relative to the NM-PTOT treatment, resulted in a significant ($p < 0.05$) decrease in plant primary root length (WIP3), collar diameter (RG2), root DW (RG2), total dry biomass (RG2, RX1), Dixon quality index (RG2), root surface (VX211), root Pi concentration (RG17, VX211,WIP3), leaf Pi concentration (RG17, VLACH, VX211), root N content (RG6, RG17, RX1, VX211, WIP3), and leaf N content (RG6,VX211, WIP3) (**Table III.S3**). These results indicate that the reduced Pi availability in the P/10 condition limited the development, quality, and/or the nutrition of the corresponding rootstocks. The analysis of these parameters between the NM-P, NM-P/10 and AM-treatments (**Table III.S3)**, underlined that pre-inoculation with *R. irregularis* after the acclimatization stage, either totally (RG17 root Pi concentration; RX1 total DW, Vlach leaf Pi concentration), or partially (VX211 root and leaf Pi concentrations, WIP3 root Pi concentration and root N content) relieved ($p < 0.05$) the above described features. For the other parameters, pre-inoculation with *R. irregularis* did not significantly rescue low Pi availability, especially in the rootstocks RG2 and RG6 (**Table III. S3)**. Taken together, these results demonstrate that AM symbiosis alleviated low Pi availability by significantly restoring or improving the nutritional status in VLACH, VX211 and WIP3 rootstocks (**Figure III.5**).

To further compare the impact of the Pi supply on the response of different micropropagated rootstock genotypes to pre-inoculation with *R. irregularis* at the *post*-acclimatization stage, we analysed in the two Pi fertilization regimes the relationship between MD, MGR MQR, the mycorrhizal shoot phosphate (MPR_S) and root phosphate (MPR_R) responses. Walnut responses to pre- inoculation with *R. irregularis* after the post-acclimatization stage, as dependent on the rootstock genotype and the Pi supply, ranged from negative, neutral, positive to highly positive, as indicated by blue, grey, pink and dark pink-shaded values, respectively (**Table III. 2**). Namely, irrespective of the Pi fertilization regime, MGR, MD and MR% were negative for RG2, RX1, while positive for RX1. On the opposite, all mycorrhizal responses were positive for WIP3 in the lowest Pi condition, and negative in the highest Pi treatment (**Table III.2**).

Table III. 2. Walnut responses to pre-inoculation with *R. irregularis* after two months of *post*-acclimatization under two different Pi supplies. Values that correspond to mean data of two experiments, were categorized as follows: -5 %< neutral< 5% (grey values), negative :< -5% (blue values), 5%< positive<50% (pink values); highly positive≥ 50% (dark pink values). MD%, mycorrhizal dependency; MGR%, mycorrhizal growth response, MPRr%, mycorrhizal root phosphate response; MPRs%, mycorrhizal shoot phosphate response, MQR%, mycorrhizal quality response

	RG2		RG6		RG17		RX1		VLACH		VX211		WIP3	
	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT	P/10	PTOT
MGR %	-6.4	-24	10.66	0	13.69	8.11	115	6.67	13.46	-2.85	20	-4.3	47.93	-27.5
MD %	-6.9	-32.8	9.63	0	12.05	21.82	53.49	6.25	11.86	-2.94	16.66	-4.5	29.54	-37.93
MQR %	-33.3	-60.6	0	-4.61	-26.6	-12.56	166	300	-16	-33	21.62	4.05	63.63	-37.62
MPRs %	77.45	88	9.89	-15.23	85.49	62.15	68.28	104	257.46	-15.32	160.51	37.24	53.24	-2.08
MPRr %	10,15	-27.2	46.17	-15.94	108.84	14.11	2.36	-123.4	49.89	38.36	120.88	41	64.06	-20.66

After a PCA to assess the relationships between the different parameters, **Figure III. 8A** shows that irrespective of mycorrhizal shoot phosphate responses, PC1, which explained 48.9% of the variation in the data, captured mycorrhizal responses with regard to MD, MGR, and MQR%. Noticeably, these parameters were positively correlated to each other and to the lowest Pi fertilization regime on this first axis. Mycorrhizal root phosphate response (MPR_R) loaded negatively on PC1, but positively on PC2. On this last axis that explained around 30% of the variation, MD, MGR MPR_R % and P/10 fertilization were positively correlated to each other and negatively correlated to the highest Pi fertilization regime and the MQR%. It therefore underlined the negative relationship between MPR_R and MQR%, as they were in the opposite directions on both PC1 and PC2 (**Figure III. 7A).** Overall, both PC1 and PC2 represented the measurement of plant responses to mycorrhization: the more a rootstock lies on the positive side of both axes, the more its mycorrhizal response, estimated thanks to growth, root phosphate content and seedling quality, is positive to the pre-inoculation with the AM fungus. As shown in **Figure III. 7B**, RG2, RG6 and RG17 belonged to the rootstocks for which the growth and quality responses loaded the weakest on PC1, irrespective of the Pi supply. In the lowest Pi supply (P/10: blue circles), the mycorrhizal behaviour of VLACH, VX211 and WIP3 clustered to the positive side of PC1 and PC2, opposite to what observed upon the highest Pi fertilization regime (P, green circles). In the lowest Pi supply, the overall mycorrhizal behaviour of RX1 lied positively both on PC1 and PC2, while its mycorrhizal root phosphate response decreased in the PTOT condition relative to the P/10 treatment. Overall, the mycorrhizal responses of RX1, VLACH and WIP3 to *R. irregularis* were superior in the P/10 treatment than in the PTOT condition. Taken together, these results show that after *post*-acclimatization, rootstock genotype and the level of Pi supply, influenced the response of walnut rootstock to *R. irregularis* biotisation.

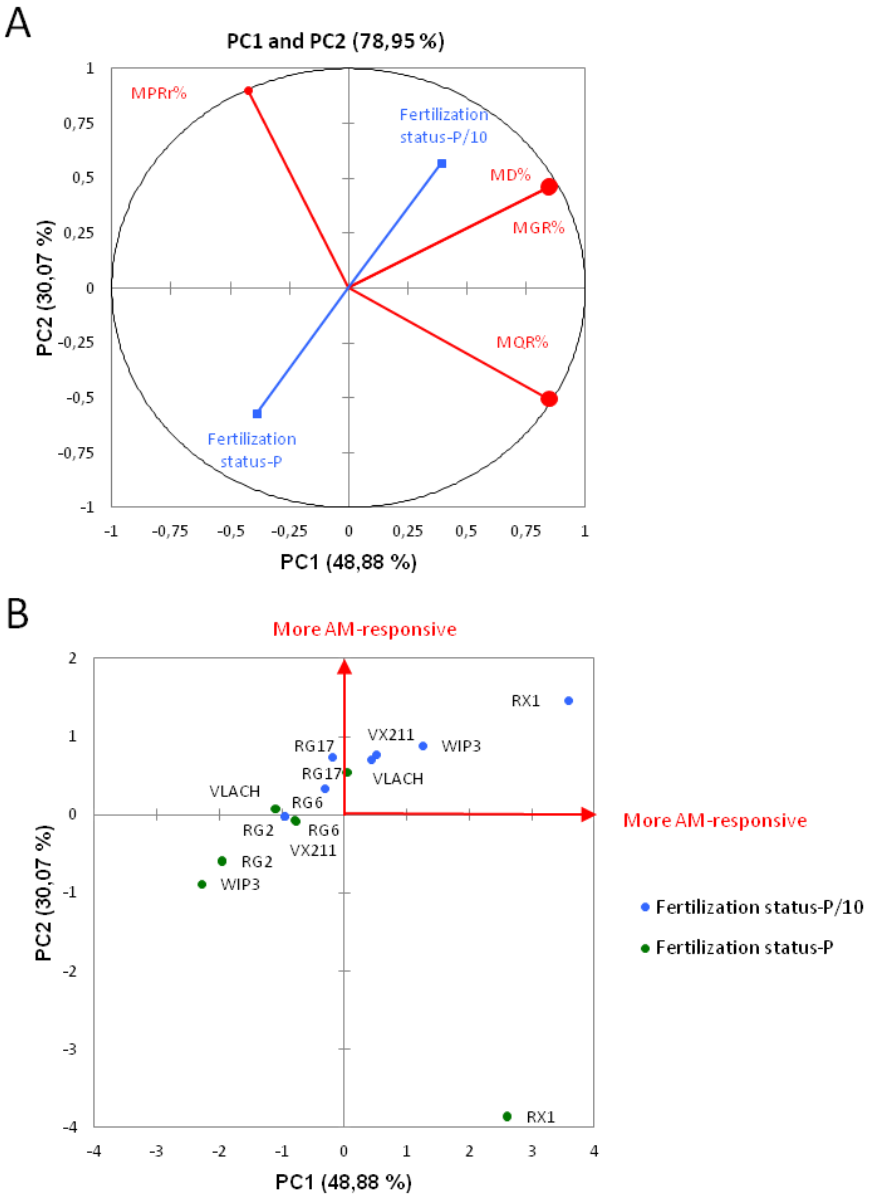

Figure III.8. Relationships between walnut rootstock responses to pre-inoculation with *R. irregularis* after two months of *post*-acclimatization under two different Pi supplies A) Circle of correlations showing that the positive side of axis PC1 is correlated to walnut responses to *R. irregularis* in the lowest Pi supply, with regard to MD, MGR and MQR values. On the contrary, root phosphate response (MPR_R) loaded negatively on PC1, but positively on PC2. Red and blue colours refer to active and supplementary variables, respectively. **B**) Responses to *R. irregularis* inoculation of walnut rootstocks under contrasting Pi fertilization regimes. Blue and green colours refer to P/10 and PTOT supply, respectively. Values correspond to mean data of two experiments. MD%, mycorrhizal dependency; MGR%, mycorrhizal growth response, MPRs%, mycorrhizal shoot phosphate response, MQR%, mycorrhizal quality response.

4. Discussion

Production of high quality, marketable walnut saplings is commonly carried out by grafting selected varieties of English walnut scions onto rootstocks obtained by micropropagation, a process that needs improvements owing to substantial rootstock losses after walnut *ex vitro* establishment (Hackett et al. 2010; Yegizbayeva et al. 2021). In spite of occasional reports on the impact of biotisation with AM fungus (Dolcet-Sanjuan et al. 1996; Peixe et al. 2015), little information is available regarding the growth, nutritional and sapling quality responses of walnut rootstocks pre-inoculated with *R. irregularis* after acclimatization and *post*-acclimatization. In addition, horticultural substrates for large scale production of walnut explants very often consist of peat supplemented with mineral nutrients able to depress mycorrhizal colonization (Javot, Pumplin, and Harrison 2007; Yegizbayeva et al. 2021). This study was therefore designed to assess the extent to which pre-inoculation with the commercially available AM fungus *R. irregularis* DAOM197198 may improve the acclimatization and *post*-acclimatization of micropropagated walnut explants as related to the impact of Pi supply and rootstock identity.

We first recorded root mycorrhizal parameters that reflect the degree of interaction between AM fungi and the host plant (Chen et al. 2020). Consistent with previous values obtained in *Juglans* spp. (Kormanik, Schultz, and Bryan 1982), external frequency of mycorrhization of root fragments (F%) and arbuscule abundance (A%), which represent root-intrinsic fungal structures involved in nutrient exchange, ranged from 30 to 100%, and 0.9 to 15%, respectively. Although less than 3% of arbuscule abundance were detected in the rootstock WIP3 after *post*-acclimatization under the P/10 fertilization regime, the benefit of mycorrhization relative to NM plants, could be demonstrated in terms of increased height, branch number, root Pi concentration, root N content and higher biomass production This is in agreement with the review of Chen et al. (2018) reporting improved biomass yield and/or mineral nutrition due to mycorrhization of micropropagated plantlets after *ex vitro* establishment as previously described in other fruit tress such as *Annona cherimola , Malus pumila, Prunus avium, Prunus cerasifera,* and *Vitis vinifera.* (Azcón-Aguilar et al. 1992; Cordier, Gianinazzi, and Gianinazzi-Pearson 1996; Schubert and Lubraco 2000; Feldmann 2008).

One can observe a negative impact of Pi supply on endomycorrhizal development in the rootstocks VLACH and VX211, as dependent on their development stage where biotisation was applied. Namely, the highest Pi fertilization regime significantly depressed the mycorrhizal colonization of VLACH after acclimatization but not after *post* acclimatization, opposite to what observed for VX211. According to Siqueira (1984) and Siqueira and Saggin-Júnior (2001), host nutrient requirements and their fulfilment in the absence of AM fungi are the major factors determining Pi effect on root colonization of woody plants. Consistently, we observed that after acclimatization, non-mycorrhizal VLACH contrary to NM VX211, underwent biomass and nutritional limitations under the P/10 fertilization regime (height, root DW, total DW, leaf N content), which were mostly relieved by mycorrhization. On the opposite, after *post*-acclimatization, non-mycorrhizal VX211 contrary to NM VLACH, displayed nutritional deficiencies under the P/10 fertilization regime (root and leaf Pi concentrations and N contents), which were partially palliated by mycorrhization**.** It has long been known that in response to high Pi levels in soil plants suppress the development of AM symbiosis (Baylis 1967; Mosse 1973; Sanders and Tinker 1973), a phenomenon referred to as Pi inhibition of mycorrhizal colonization (Graham et al. 1981). The adverse effect of high soil PTOT levels on AM formation has been ascribed to high PTOT concentrations in the roots (Sanders 1975), leading to a reduced carbon allocation to the AM fungus after several weeks of symbiotic functioning (Olsson et al. 2002;

Olsson et al. 2006, Javot et al. 2007). Although this hypothesis could not be tested after acclimatization due to tiny amounts of root materials, on could observe that inoculated VX211 walnuts after the *post*-acclimatization stage displayed the highest mean root Pi concentration (45.9 nmole Pi/mg DW) relative to the values recorded in the other rootstocks, i.e.: 27, 21, 21, 30, 21, and 33 nmole Pi/mg DW for RG2, RG6, RG17, RX1, VLACH and WIP3, respectively.

To investigate the extent to which rootstocks relied on the AM fungus for biomass production and Pi accumulation, the walnut overall behaviours in response to pre-inoculation with *R. irregularis* were compared as dependent on rootstock identity and Pi supply in terms of mycorrhizal dependency (MD%), mycorrhizal growth response (MGR%), and mycorrhizal phosphate response (MGP%). MD, MGR and shoot or root MGP% were positively correlated to each other under the lowest Pi fertilization regime tested (P/10), irrespective of the developmental stage. These results agree with the idea that in plant substrates with low available Pi, mycorrhizal growth responses (MGR%) and mycorrhizal dependency (MD%) of the plant are correlated, and may be a phenotypic response to the AM fungus facing with a greater carbon supply provided by the host (Zangaro, Bononi, and Trufen 2000). The extent to which walnut rootstocks depends on the AM fungus for dry matter production varied between rootstocks and the Pi fertilization regime: irrespective of root colonization parameters and developmental stages, mycorrhizal growth depression in the rootstock WIP3 after inoculation with *R. irregularis* was observed with increasing Pi availabilities in the substrate. These results also hold true for the rootstocks RG2, RG6, and RX1 after *post* acclimatization. As reviewed by Johri et al. (2015), decreasing or negative MGRs at high PTOT availabilities have often been attributed to the high carbon costs of the AM symbiosis that are not counterbalanced by a net gain in Pi. Consistently, the MPRr were always lower in these rootstock genotypes in the PTOT condition than in the P/10 treatment.

Plant dependence on mycorrhiza arises when uptake by roots alone of mineral nutrients at a given level of supply is not sufficient (Janos 1996), with root nutrient acquisition ability being largely dependent on the root surface (Eissenstat 1992). Within this line, RX1, which showed the lowest root surface (1 cm^2) after acclimatization in the NM-PTOT treatment relative to the other rootstocks, further displayed the highest mycorrhizal dependency (53.5%) after *post*-acclimatization under the P/10 condition. It is noteworthy that a large absorption area of the root system is an inherent property of the plant or can be induced by P deficiency (Marschener 1998). In this study, this was noticeably the case for the rootstocks RG2, RG6, RG17, RX1, VLACH and WIP3 in which the root surfaces were higher in the P/10 than in the PTOT fertilization regime in both NM and AM plants after *post*-acclimatization**.** A significantly reverse trend was noticed after acclimatization and *post*-acclimatization for the rootstock VX211, which displayed lower root surfaces in the P/10 than in the PTOT fertilization regime in both NM and AM plants. Owing to significantly increased leaf Pi levels in response to the AM fungus in the P/10 treatment whatever the developmental stage, these results suggest that *R. irregularis* provided greater effects in nutrient uptake than in the increase of root absorption area (Zangaro et al. 2005), a process which may be less costly for the plant than carbon allocation to root and mycorrhizal growth (Felderer and Homburg 2013).

To further assess the impact of pre-inoculation with *R. irregularis* on rootstock robustness and biomass distribution, we coined the mycorrhizal quality response (MQR%), which besides shoot and root dry weight matters, also integrated morphological traits such as plant height, stem diameter, and dry weight matter partitioning (Binotto, Lúcio, and Lopes 2010). MQR% ranged from negative to positive values depending on the rootstock identity and the Pi supply. MQR, MGR and MD% positively correlated to each other under the lowest Pi fertilization regime tested (P/10), irrespective of the developmental stage. These results underline that when grown under Pi

limitation, the rootstocks VLACH, VX211, and WIP3, showed a greater DQI than under full Pi fertilization regime. Higher DQIs in response to AM symbiosis have been previously reported in plants such as *Melia azedarach* and *Cannabis sativa*, relative to non-inoculated controls (Kakabouki et al. 2021). When investigating correlations between growth parameters and the Dixon quality index in forest seedlings, earlier studies have pointed to the shoot to root dry matter ratio and dry matter variables (Binotto, Lúcio, and Lopes 2010), as effective safe indexes to evaluate sapling quality. We found that the higher mycorrhizal quality response recorded in the P/10 than the in the PTOT fertilization regime was mainly driven by a higher root DW for WIP3, and a lower shoot to root dry matter ratio for RG2, VLACH, VX211 in AM plants than in non-inoculated controls after acclimatization, indicating a larger investment in root DW production. By contrast, after post-acclimatization, the increased shoot DW recorded for VLACH, VX211 and WIP3 in AM plants than in non-inoculated controls rather explained that the higher MQR% recorded in the P/10 than the in the PTOT fertilization regime**.** Taken together, these results support the idea that an increased ability to develop young roots is fundamental during the first acclimatization stage of walnut rootstocks (Yegizbayeva et al. 2021), which may be increased by mycorrhization. On the opposite, when micropropagated plantlets have an already developed root system after the acclimatization stage (Elsen et al. 2008; Liu and Yang 2008), they mainly rely on above ground expansion to develop their photosynthetic capacity in order to provide enough carbon to feed the AM fungi network which in return will bring back nutrients, instead of allocating carbon to root system development.

In conclusion, the main finding of this study relates to the ability of a commercially available AM fungus to decrease micropropagated walnut rootstock dependency on Pi fertilization both at the acclimatization and post-acclimatization stages, together with improving adequate sapling development and quality, especially in the hybrid rootstocks VLACH, VX211, and WIP3. Our results also revealed the substantial variation of the response to mycorrhization between the different rootstock genotypes. Such variability in the response to AM symbiosis can be explored to improve the production of biotisated micropropagated rootstock in nurseries and to test their interest for agroforestry systems.

Supplemental Table III.S2: Effects of *R. irregularis* and phosphate supply on walnut rootstocks growth and nutritional parameters during the acclimatization stage. The data represent the mean of two independent experiments (±SD). Different letters indicate statistical differences between groups ($p<0.05$) calculated as described in the material and methods section. Parameters showing statistical differences between groups are highlighted in bold. Batch effects are in blue. M: mycorrhizal; NM: non mycorrhizal; DW: dry weight; sw: specific water; DQI: Dixon Quality Index; MGR: Mycorrhizal Growth Response; MD: Mycorrhizal dependency.

	P/10		PTOT		
RG2	M	NM	M	NM	pvalue
Root length (cm)	8.1±0.7	8.0±0.7	8.0±0.9	10.7±1.7	0.37
Collar (mm)	2.5±0.2	2.2±0.2	2.9±0.2	2.7±0.2	0.15
Height (cm)	17.6±1.6	17.5±1.2	23.0±1.9	18.8±5.0	0.32
Branch number	18.0±1.2	16.6±2.0	20.6±1.2	13.3±3.8	0.23
Root dw (g)	0.07±0.01	0.05±7e^{-3}	0.08±0.02	0.07±0.02	0.63
Shoot dw (g)	**0.04±5.6e^{-3} b**	**0.05±6.6e^{-3} ab**	**0.07±7.e^{-3} a**	**0.06±8.3e^{-3} ab**	**0.03**
Total (g)	0.12±0.02	0.1±0.01	0.15±0.02	0.13±0.02	0.37
Shoot/root DW ratio	5.9±0.1	8.9±0.1	10.1±0.1	6.9±0.2	0.07
DQI	0.02±0.01	0.01±0.02	0.02±0.02	0.02±0.01	0.65
Root surface (cm²)	3.0±0.5	3.6±0.7	2.3±0.1	3.4±0.5	0.55
Leaf Pi nmoles Pi/mg DW	**27.2±7.0 bc**	**16.2±2.81 c**	**32.1±4.4 ab**	**55.5 ±4.4 a**	**0.02**
Leaf N (%DW)	3.1±0.1	3.0±0.1	3.0±0.1	3.3±0.1	0.27
Leaf C (%DW)	42.0±0.3	42.1±0.2	41.7±0.3	41.6±0.8	0.17
Leaf C(%DW)/ N(%DW)	14.0±0.3	14.4±0.2	14.7±0.3	13.3±0.5	0.1
Root SW mL/mg DW	5.8±0.5	5.6±0.7	4.8±0.4	6.3±0.7	0.32
Shoot SWmL/mg DW	5.4±0.1	4.7±0.1	4.8±0.2	5.3±0.5	0.05

	P/10		PTOT		
RG6	M	NM	M	NM	pvalue
Root length (cm)	7.56±1.4	8.38±1.2	5.04±1.4	7.56±1.0	0.36
Collar (mm)	2.33±0.1	2.34±0.1	2.17±0.3	2.33±0.1	1.0
Height (cm)	6.94±1.3	8.98±0.8	7.98±1.4	10.96±1.0	0.08
Branch number	12.2±1.7	13.2±1.2	9.6±2.2	10.43±1.7	0.4
Root dw (g)	**0.06±0.01** ^{ab}	**0.05±6.0e^{-3}** ^{ab}	**0.08±0.02** ^{a}	**0.03±6.4e^{-3}** ^{b}	**0.04**
Shoot dw (g)	0.2±0.04	0.2±0.07	0.2±0.07	0.2±0.03	0.97
Total (g)	0.2±0.05	0.3±0.07	0.2±0.08	0.2±0.03	0.95
Shoot/root DW ratio	2.9±0.6	4.3±1.0	2.6±0.7	5.6±0.7	0.07
DQI	**0.04±0.004** ^{a}	**0.03±0.004** ^{ab}	**0.04±0.001** ^{a}	**0.02±0.003** ^{b}	**0.03**
Root surface (cm^2)	3.1±0.5	1.9±0.4	1.5±0.6	2.1±0.4	0.4
Leaf Pi nmoles Pi/mg DW	36.9±7.2	40.8±9.4	48.6±5.1	49.1±6.7	0.47
Leaf N (%DW)	3.0±0.1	2.8±0.1	2.9±0.3	3.1±0.1	0.14
Leaf C (%DW)	42.3±0.3	42.14±0.3	41.70±1.3	41.6±0.3	0.37
Leaf $C_{(\%DW)}$/ $N_{(\%DW)}$	14.6±0.4	15.7±0.5	15.2±0.6	14.4±0.2	0.21
Root S.W mL/mg DW	7.5±0.4	6.7±0.5	5.0±1.6	7.7±0.7	0.3
Shoot S.WmL/mg DW	4.9±0.2	4.8±0.4	4.7±0.5	5.0±0.4	0.95

	P/10		PTOT		
RG17	M	NM	M	NM	pvalue
Root length (cm)	9.2±1.4	9.9±0.6	9.2±1.1	10.6±0.6	0.94
Collar (mm)	2.2±0.1	2.5±0.1	2.42±0.2	2.1±0.2	0.15
Height (cm)	11.9±1.2	10.7±1.1	14.4±2.2	13.3±1.6	0.61
Branch number	19.4±1.8	16.9±0.9	20.2±1.7	18.9±1.4	0.43
Root dw (g)	**0.12±0.03** ^{a}	**0.06±0.01** ^{b}	**0.15±0.03** ^{a}	**0.08±0.02** ^{b}	**0.02**
Shoot dw (g)	0.4±0.05	0.3±0.05	0.5±0.1	0.3±0.02	0.44
Total dw (g)	0.5±0.06	0.4±0.06	0.6±0.13	0.5±0.2	0.3
Shoot/root DW ratio	**3.8±0.5** ^{b}	**6.7±1.0** ^{a}	**3.7±0.7** ^{b}	**6.6±1.1** ^{a}	**0.02**
DQI	**0.06±0.001** ^{ab}	**0.04±0.007** ^{bc}	**0.07±0.01** ^{a}	**0.04±0.02** ^{c}	**0.01**
Root surface (cm^2)	4.4±0.6	3.8±0.8	1.8±1.0	3.39±1.2	0.42
Leaf Pi nmoles Pi/mg DW	36.5±5.6	36.5±6.1	56.5±8.9	38.35±4.6	0.23
Leaf N (%DW)	**2.9±0.1** ^{ab}	**2.8±0.1** ^{b}	**3.0±0.1** ^{ab}	**3.0±0.1** ^{a}	**0.02**
Leaf C (%DW)	**43.1±0.4** ^{b}	**44.4±0.3** ^{a}	**43.8±0.4** ^{ab}	**45.0±0.3** ^{a}	**<0.01**
Leaf $C_{(\%DW)}$/ $N_{(\%DW)}$	15.1±0.4	16.2±0.5	14.7±0.5	14.7±0.7	0.08
Root S.W mL/mg DW	**5.2±0.6** ^{b}	**4.4±0.4** ^{a}	**4.5±0.6** ^{b}	**5.25±0.5** ^{b}	**<0.01**
Shoot S.WmL/mg DW	4.8±0.1	4.4±0.2	4.5±0.2	4.5±0.2	0.18

	P/10		PTOT		
RX1	M	NM	M	NM	pvalue
Root length (cm)	**9.2±0.7 ^{ab}**	**7.9±0.6 ^{b}**	**9.6±0.5 ^{ab}**	**10.4±0.5 ^{a}**	**0.03**
Collar (mm)	*2.7±0.1*	*2.6±0.1*	*2.5±0.1*	*2.5±0.1*	*0.3*
Height (cm)	*12.44±0.4*	*12.5±0.7*	*13.7±0.8*	*11.7±1.0*	*0.3*
Branch number	*12.4±0.6*	*11.4±0.5*	*12.2±0.4*	*11.0±0.6*	*0.27*
Root dw (g)	0.08±7.4e^{-3}	0.1±0.03	0.05±3.6e^{-3}	0.05±5.5e^{-3}	0.5
Shoot dw (g)	*0.3±0.03*	*0.2±0.03*	*0.2±0.02*	*0.2±0.03*	*0.32*
Total dw	*0.3±0.03*	*0.3±0.05*	*0.3±0.02*	*0.3±0.03*	*0.7*
Shoot/root DW ratio	**3.8±0.4 ^{b}**	**6.6±0.3 ^{a}**	**3.7±0.3 ^{b}**	**6.6±0.3 ^{a}**	**0.01**
DQI	0.04±0.003	0.04±0.01	0.03±0.002	0.03±0.02	0.1
Root surface (cm²)	1.8±0.2	1.4±0.2	1.1±0.1	1.0±0.1	0.1
Leaf Pi nmoles Pi/mg DW	**20.5±1.6 ^{b}**	**16.2±2.8 ^{b}**	**32.1±3.0 ^{b}**	**35.4±2.8 ^{a}**	**<0.01**
Leaf N (%DW)	**3.0±0.1 ^{a}**	**2.8±0.1 ^{b}**	**3.0±0.04 ^{a}**	**3.0±0.1 ^{a}**	**<0.01**
Leaf C (%DW)	**43.1±0.3 ^{b}**	**44.4±0.4 ^{a}**	**43.8±0.2 ^{ab}**	**44.9±0.2 ^{a}**	**0.03**
Leaf C(%DW)/ **N**(%DW)	**15.1±0.3 ^{b}**	**16.1±0.3 ^{a}**	**14.8±0.2 ^{b}**	**15.0±0.3 ^{b}**	**<0.01**
Root S.W mL/mg DW	**8.5±0.7 ^{ab}**	**9.5±0.8 ^{a}**	**8.2±0.32 ^{a}**	**6.6±0.2 ^{b}**	**<0.01**
Shoots.W mL/mg DW	*4.4±0.2*	*4.0±0.3*	*4.3±0.2*	*3.6±0.2*	*0.8*

	P/10		PTOT		
VLACH	M	NM	M	NM	pvalue
Root length (cm)	7.5±0.3 ^{bc}	6.0±0.4 ^{c}	9.0 ±0.7 ^{b}	11.5±0.8 ^{a}	<0.01
Collar (mm)	3.3±0.2 ^{a}	2.7±0.2 ^{b}	2.6±0.1 ^{b}	2.7±0.1 ^{b}	0.02
Height (cm)	9.1±0.6 ^{ab}	6.7±0.4 ^{b}	10.8±0.7 ^{a}	9.7±0.9 ^{a}	<0.01
Branch number	10.7±0.5 ^{ab}	9.±30.5 ^{b}	12.1±0.5 ^{a}	11.5±0.9 ^{ab}	0.04
Root dw (g)	0.1±9.1e^{-3} ^{a}	0.05±6.3 e^{-3} ^{c}	0.08±7.9e^{-3} ^{b}	0.1±0.01 ^{a}	<0.01
Shoot dw (g)	0.3±0.04 ^{a}	0.2±0.03 ^{b}	0.3±0.04 ^{a}	0.4±0.06 ^{a}	<0.01
Total dw (g)	0.4±0.05 ^{a}	0.2±0.03 ^{b}	0.4±0.04 ^{a}	0.5±0.06 ^{a}	<0.01
Shoot/root DW ratio	2.5±0.4 ^{b}	3.7±0.4 ^{ab}	4.4±0.3 ^{a}	4.2±0.3 ^{a}	<0.01
DQI	0.07±0.005 ^{a}	0.04±0.005 ^{b}	0.04±0.004 ^{b}	0.06±0.006 ^{ab}	<0.01
Root surface (cm²)	1.7±0.1	1.2±0.2	1.4±0.1	1.4±0.1	0.15
Leaf Pi nmoles Pi/mg DW	81.7±11.4 ^{a}	68.3±3.3 ^{b}	81.3±4.7 ^{a}	66.2±3.3 ^{b}	<0.01
Leaf N (%DW)	2.7±0.1 ^{d}	2.9±0.1 ^{c}	3.3±0.1 ^{a}	3.1±0.1 ^{b}	<0.01
Leaf C(%DW)	42.0±0.3 ^{ab}	42.1±0.2 ^{ab}	41.7±0.2 ^{a}	41.6±0.3 ^{b}	0.03
Leaf $C_{(\%DW)}$/ $N_{(\%DW)}$	16.2±0.4 ^{a}	14.8±0.1 ^{b}	13.3±0.2 ^{d}	14.0±0.1 ^{c}	<0.01
Root S.W mL/mg DW	8.8±0.4 ^{ab}	10.6±1.0 ^{a}	7.5±0.4 ^{b}	9.0±0.7 ^{b}	<0.01
Shoots.WmL/mg DW	5.0±0.3 ^{b}	5.6±0.1 ^{a}	4.3±0.2 ^{c}	4.3±0.5 ^{c}	<0.01

	P/10		PTOT		
VX211	M	NM	M	NM	pvalue
Root length (cm)	**5.6±0.3 b**	**6.0±0.9 b**	**12.3±1.6 a**	**12.2±1.2 a**	**<0.01**
Collar (mm)	**2.1±0.1 ab**	**2.2±0.1 b**	**3.0±0.2 a**	**3.1±0.2 a**	**<0.01**
Height (cm)	*7.8±0.4*	*7.5±0.6*	*7.0±0.9*	*7.0±0.8*	*0.2*
Branch number	12.0±0.5	10.3±0.6	10.9±1.1	10.1±1.3	0.2
Root dw (g)	**0.09±8.8e^{-3} a**	**0.05±3. e^{-3} b**	**0.1±0.03 a**	**0.2±0.03 a**	**<0.01**
Shoot dw (g)	**0.1±0.01 a**	**0.1±0.02 a**	**0.05±9.5e^{-3} b**	**0.06±7.8e^{-3} b**	**<0.01**
Total dw (g)	0.2±0.02	0.2±0.02	0.2±0.04	0.2±0.04	0.3
Shoot/root DW ratio	**1.4±0.1 b**	**2.6±0.4 a**	**0.6±0.1 c**	**0.5±0.1 c**	**<0.01**
DQI	**0.05±0.004 b**	**0.03±0.002 b**	**0.08±0.02 ab**	**0.1±0.002 a**	**0.01**
Root surface (cm^2)	**1.1±0.1 b**	**0.9±0.1 b**	**2.8±0.3 a**	**6.5±0.9 a**	**<0.01**
Leaf Pi nmoles Pi/mg DW	**43.8±3.9 a**	**29.7±3.8 b**	**42.4±3.2 a**	**26.7±4.5 b**	**<0.01**
Leaf N (%DW)	2.3±0.1	2.4±0.1	2.6±0.1	2.6±0.1	0.08
Leaf C (%DW)	42.0±0.5	42.1±0.4	41.7±0.3	41.6±0.3	0.4
Leaf C$_{(\%DW)}$/ N$_{(\%DW)}$	19.2±0.5	18.6±0.4	17.7±0.7	17.3±0.8	0.09
Root s.w mL/mg DW	**9.9±0.7 a**	**9.0±1.3 a**	**4.8±0.8 b**	**4.6±0.4 b**	**<0.01**
Shoot s.w mL/mg DW	**3.6±0.1 a**	**3.6±0.2 ab**	**2.8±0.2 c**	**3.1±0.2 bc**	**0.02**

	P/10		PTOT		
WIP3	M	NM	M	NM	pvalue
Root length (cm)	7.0±0.4	7.7±0.6	9.0±0.8	9.8±0.9	0.07
Collar (mm)	*2.8±0.1*	*2.7±0.1*	*2.9±0.2*	*3.1±0.2*	*0.26*
Height (cm)	**8.34±0.5 ^{bc}**	**7.8±0.6 ^{c}**	**12.3±1.0 ^{a}**	**11.5±1.0 ^{ab}**	**<0.01**
Branch number	**10.6±0.4 ^{b}**	**9.7±0.6 ^{b}**	**13.1±0.7 ^{a}**	**14.0±1.0 ^{a}**	**<0.01**
Root dw (g)	*0.09±5.8e^{-3}*	*0.06±7.6e^{-3}*	*0.07±0.01*	*0.09±0.02*	*0.08*
Shoot dw (g)	*0.5±0.2*	*0.2±0.04*	*0.2±0.05*	*0.3±0.07*	*0.55*
Total dw (g)	*0.5±0.2*	*0.3±0.05*	*0.3±0.07*	*0.4±0.08*	*0.7*
Shoot/root $_{DW\ ratio}$	5.1±2.4	3.5±0.6	3.7±0.3	3.5±0.3	0.56
DQI	*0.06±0.004*	*0.04±0.006*	*0.04±0.009*	*0.05±0.01*	*0.65*
Root surface	**1.6±0.2 ^{b}**	**2.0±0.3 ^{b}**	**3.6±0.4 ^{a}**	**4.6±0.5 ^{a}**	**<0.01**
Leaf Pi $_{nmoles\ Pi/mg\ DW}$	**69.3±4.0 ^{a}**	**64±5.1 ^{b}**	**67.9±3.8 ^{a}**	**53.0±4.7 ^{b}**	**<0.01**
Leaf N $_{(\%DW)}$	**2.9±0.1 ^{a}**	**2.6±0.1 ^{b}**	**3.11±0.1 ^{a}**	**3.06±0.1 ^{a}**	**<0.01**
Leaf C $_{(\%DW)}$	**44.3±0.3 ^{a}**	**42.9±0.3 ^{b}**	**43.7±0.7 ^{a}**	**44.2±0.1 ^{a}**	**<0.01**
Leaf $C_{(\%DW)}$/ $N_{(\%DW)}$	**15.3±0.4 ^{b}**	**16.8±0.4 ^{a}**	**14.1±0.1 ^{c}**	**14.5±0.2 ^{bc}**	**<0.01**
Root $_{S.WmL/mg\ DW}$	**8.3±0.3 ^{ab}**	**9.8±0.5 ^{a}**	**7.96±1.1 ^{b}**	**7.5±0.6 ^{b}**	**<0.01**
Shoot $_{S.WmL/mg\ DW}$	**4.9±0.13 ^{a}**	**4.9±0.2 ^{ab}**	**4.0±0.3 ^{b}**	**4.9±0.5 ^{b}**	**0.01**

Supplemental Table III.S3: Effects of *R. irregularis* and phosphate supply on walnut rootstocks growth and nutritional parameters during the post-acclimatization stage. The data represent the mean of two independent experiments (±SD). Different letters indicate statistical differences between groups ($p<0.05$) calculated as described in the material and methods section. Parameters showing statistical differences between groups are highlighted in bold. Batch effects are in blue. M: mycorrhizal; NM: non mycorrhizal; DW: dry weight; sw: specific water; DQI: Dixon Quality Index; MGR: Mycorrhizal Growth Response; MD: Mycorrhizal dependency.

	P/10		PTOT		
RG2	M	NM	M	NM	pvalue
Root length (cm)	**20.2±1.1 ^{c}**	**31.4±2.3 ^{ab}**	**28.1±2.3 ^{b}**	**42.7±5.9 ^{a}**	**<0.01**
Collar (mm)	**6.9±0.2 ^{c}**	**7.1±0.4 ^{bc}**	**8.3±0.5 ^{ab}**	**9.2±0.8 ^{a}**	**0.02**
Height (cm)	26.3±1.3a	19.0±2.0	27.8±3.4	24.4±5.4	0.14
Branch number	9.9±0.3	9.9±0.2	9.6±0.4	9.3±0.4	0.92
Root dw (g)	**1.9±0.1 ^{b}**	**2.1±0.3 ^{b}**	**5.22±1.1 ^{a}**	**10.0±3.3 ^{a}**	**<0.01**
Shoot dw (g)	4.5±1.8	4.1±0.9	7.3±1.9	6.6±1.9	0.18
Total dw (g)	**5.8±1.7 ^{b}**	**6.2±1.0 ^{b}**	**12.5±2.7 ^{a}**	**16.6±4.8 ^{a}**	**<0.01**
Shoot/root DW ratio	**2.8±1.4 ^{ab}**	**2.2±0.4 ^{a}**	**1.4±0.3 ^{ab}**	**0.84±0.2 ^{b}**	**0.05**
DQI	**0.8±0.1 ^{b}**	**1.2±0.3 ^{b}**	**2.6±0.5 ^{a}**	**6.6±2.6 ^{a}**	**<0.01**
Root surface (cm²)	**692.2±70.2 ^{a}**	**593.8±35.5 ^{ab}**	**433.3±68.7 ^{bc}**	**314.0±47.1 ^{c}**	**0.01**

	P/10		PTOT		
RG2	M	NM	M	NM	
Root P nmoles Pi/mg DW	*17.7±1.2*	*14.54±1.5*	*27.5±5.76*	*19.72±4.09*	*0.09*
Leaf P nmoles Pi/mg DW	**36.7±2.0 ^{a}**	**22.7±2.7 ^{b}**	**37.8±7.2 ^{a}**	**22.2±3.2 ^{b}**	**0.02**
Root N (% DW)	*2.1±0.1*	*1.94±0.1*	*2.4±0.1*	*2.25±0.1*	*0.06*
Leaf N (% DW)	**3.1±0.3 ^{b}**	**2.8±0.1 ^{b}**	**4.1±0.3 ^{a}**	**3.4±0.2 ^{ab}**	**0.01**
Root C (% DW)	**41.6±0.4 ^{a}**	**42.1±0.5 ^{a}**	**38.5±1.3 ^{b}**	**38.2±0.9 ^{b}**	**<0.01**
Leaf C (% DW)	42.5±0.38	41.1±0.9	42.6±0.5	42.0±0.4	0.35
Root C/N (% DW)	**20.3±0.4 ^{ab}**	**21.9±0.3 ^{a}**	**16.5±0.3 ^{c}**	**17.1±0.4 ^{bc}**	**0.05**
Leaf C/N (% DW)	14.2±0.4 ^{a}	14.6±0.1 ^{a}	10.6±0.3 ^{b}	12.6±0.2 ^{ab}	<0.01
Root s w	**3.1±0.3 ^{a}**	**3.2±0.2 ^{a}**	**1.9±0.3 ^{b}**	**2.5±0.2 ^{ab}**	**<0.01**
Shoot SW mL/mg DW	**0.9±0.4 ^{b}**	**0.7±0.1 ^{b}**	**2.6±0.3 ^{a}**	**2.0±0.2 ^{a}**	**<0.01**

	P/10		PTOT		
RG6	M	NM	M	NM	pvalue
Root length (cm)	*27.1±1.5*	*29.6±1.6*	*33.9±3.7*	*31.6±2.0*	*0.64*
Collar (mm)	7.5±0.5	8.0±0.4	7.5±0.8	7.8±0.6	0.69
Height (cm)	*17.1±2.4*	*14.9±3.0*	*13.4±2.8*	*12.6±2.0*	*0.69*
Branch number	8.7±1.0	9.3±0.8	7.2±0.5	6.7±0.4	0.11
Root dw (g)	*3.0±0.6*	*2.35±0.3*	*6.4±1.4*	*7.4±1.8*	*0.09*
Shoot dw (g)	5.3±0.7	5.1±1.1	5.2±1.5	4.3±1.0	0.65
Total dw (g)	8.3±1.0	7.5±1.0	11.6±2.5	11.6±2.7	0.97
Shoot/root DW ratio	**2.5±0.5 ^{a}**	**2.7±0.7 ^{a}**	**1.0±0.3 ^{b}**	**0.7±0.1 ^{b}**	**<0.01**
DQI	*2.2±0.4*	*2.2±0.3*	*6.2±1.9*	*6.5±1.7*	*0.27*
Root surface (cm²)	510.6±77.8	629.5±67.2	364.7±51.8	454.5±46.5	0.65

	P/10		PTOT		
RG6	M	NM	M	NM	
Root P nmoles Pi/mg DW	*19.1±1.7*	*16.7±1.3*	*20.8±3.0*	*21.4±3.01*	*0.87*
Leaf P nmoles Pi/mg DW	27.6±2.9	26.1±3.0	27.9±5.0	40.0±7.0	0.31
Root N (% DW)	**2.1±0.1 ^{b}**	**2.0±0.1 ^{b}**	**2.4±0.1 ^{ab}**	**2.5±0.1 ^{a}**	**0.02**
Leaf N (% DW)	**2.9±0.12 ^{b}**	**2.9±0.1 ^{b}**	**3.4±0.1 ^{a}**	**3.5±0.10 ^{a}**	**<0.01**
Root C (% DW)	**40.7±0.3 ^{ab}**	**40.7±0.3 ^{ab}**	**41.0±0.3 ^{a}**	**38.5±1.0 ^{b}**	**0.03**
Leaf C (% DW)	*41.0±0.3*	*41.6±0.3*	*40.8±0.3*	*41.1±0.4*	*0.54*
Root C/N (% DW)	**20.9±0.3 ^{a}**	**21.1±0.4 ^{a}**	**17.2±0.2 ^{b}**	**16.4±0.2 ^{b}**	**<0.01**
Leaf C/N (% DW)	**14.1±0.2 ^{a}**	**14.5±0.2 ^{a}**	**12.4±0.1 ^{b}**	**11.6±0.1 ^{b}**	**<0.01**
Root s w	*3.0±0.21*	*5.3±1.78*	*2.2±0.2*	*2.6±0.12*	*0.09*
Shoot SWmL/mg DW	**1.2±0.3 ^{b}**	**0.98±0.2 ^{b}**	**2.6±0.3 ^{a}**	**3.0±0.5 ^{a}**	**<0.01**

	P/10		PTOT		
RG17	M	NM	M	NM	pvalue
Root length (cm)	31.6±2.4	42.3±2.3	39.7±4.3	47.3±6.0	0.15
Collar (mm)	*6.4±0.3*	*6.5±0.4*	*7.3±0.8*	*6.8±0.7*	*0.98*
Height (cm)	**13.5±1.9 a**	**8.0±1.3 b**	**15.6±1.4 a**	**11.8±1.4 ab**	**<0.01**
Branch number	**11.6±0.9 a**	**9.7±0.7 ab**	**8.1±0.7 b**	**8.4±1.0 b**	**0.02**
Root dw (g)	*3.0±0.4*	*3.6±0.6*	*5.7±1.4*	*5.3±1.2*	*0.62*
Shoot dw (g)	*5.3±0.7*	*3.7±0.9*	*5.6±2.11*	*3.6±1.1*	*0.24*
Total dw (g)	*8.3±0.8*	*7.3±1.4*	*11.4±3.2*	*9.0±2.0*	*0.7*
Shoot/root DW ratio	**2.03±0.3 a**	**1.0±0.1 ab**	**0.8±0.2 b**	**0.74±0.1 b**	**<0.01**
DQI	*2.35±0.3*	*3.2±0.4*	*3.9±1.1*	*4.46±1.3*	*0.6*
Root surface (cm²)	629.2±52.2	804.3±32.7	445.4±46.7	590.9±71.0	0.5

	P/10		PTOT		
RG17	M	NM	M	NM	
RootP nmoles Pi/mg DW	**18.6±1.5 b**	**13.2±0.9 c**	**30.2±1.0 a**	**27.0±4.4 b**	**<0.01**
Leaf P nmoles Pi/mg DW	**35.5±2.1 b**	**27.1±3.7 b**	**102.7±8.9 a**	**98.5±9.5 a**	**<0.01**
Root N (% DW)	**2.1±0.1 b**	**2.0±0.1 b**	**2.4±0.1 a**	**2.6±0.1 a**	**<0.01**
Root C (% DW)	38.9±1.3	41.1±0.8	39.2±1.0	40.7±0.4	0.27
Leaf C (% DW)	*42.3±0.3*	*42.3±0.3*	*41.0±0.5*	*41.1±0.5*	*0.09*
Root C/N (% DW)	**18.7±0.2 a**	**21.1±0.3 a**	**16.8±0.2 b**	**15.4±0.2 b**	**<0.01**
Leaf C/N (% DW)	14.0±0.1 a	13.3±0.1 ab	11.9±0.2 b	12.2±.1 b	0.01
Root s w	*3.3±0.3*	*3.1±0.3*	*3.3±0.2*	*3.6±0.6*	*0.75*
Shoot SWmL/mg DW	*2.3±0.2*	*1.5±0.2*	*2.3±0.2*	*2.6±0.4*	*0.06*

	P/10		PTOT		
RX1	**M**	**NM**	**M**	**NM**	**pvalue**
Root length (cm)	38.6±2.1	40.5±3.4	34.87±1.6	35.3±1.9	0.07
Collar (mm)	**5.5±0.4 a**	**4.7±0.4 ab**	**4.9±0.2 ab**	**3.9±0.2 b**	**0.02**
Height (cm)	**18.1±1.8 a**	**16.6±1.1 ab**	**13.2±1.1 b**	**14.9±1.2 ab**	**0.05**
Branch number	*6.1±0.6*	*6.8±0.7*	*5.6±0.2*	*5.8±0.4*	*0.53*
Root dw (g)	1.7±0.4	1.6±0.4	1.4±0.3	2.0±0.5	0.3
Shoot dw (g)	**2.7±0.6 a**	**1.8±0.5 a**	**1.8±0.4 a**	**1.0±0.2 b**	**<0.01**
Total dw(g)	**4.3±1.0 a**	**2.0±0.6 b**	**3.2±0.8 a**	**3.0±0.2 a**	**<0.01**
Shoot/root DW ratio	**2.5±0.6 a**	**1.4±0.4 a**	**1.9±0.5 a**	**0.6±0.1 b**	<0.01
DQI	**0.8±0.2 a**	**0.3±0.1 b**	**0.8±0.2 a**	**0.2±0.1 b**	**<0.01**
Root surface(cm²)	**501.4±67.8 a**	**532.2±66.3 a**	**149.2±40.3 b**	**98.3±9.5 b**	**<0.01**
	P/10		PTOT		
RX1	M	NM	M	NM	pvalue
Root P nmoles Pi/mg DW	17.6±2.1	18.3±3.1	21.2±1.4	16.93±2.1	0.24
Leaf P nmoles Pi/mg DW	26.7±2.3	23.8±2.0	36.5±4.03	32.2±6.0	0.14
Root N (% DW)	**2.0±0.1 b**	**2.1±0.1 b**	**2.5±0.1 a**	**2.6±0.1 a**	**<0.01**
Leaf N (% DW)	3.1±0.1	3.1±0.2	2.7±0.1	2.8±0.1	0.06
Root C (% DW)	*39.9±0.7*	*41.0±0.2*	*40.46±0.3*	*40.63±0.3*	*0.67*
Leaf C (% DW)	**45.9±0.3 a**	**44.9±0.4 ab**	**44.0±0.3 b**	**43.9±0.3 b**	**<0.01**
Root C/N (% DW)	**20.3±0.2 a**	**20.1±0.2 a**	**16.0±0.1 b**	**15.7±0.1 b**	**<0.01**
Leaf C/N (% DW)	14.8±0.1	14.7±0.2	16.4±0.1	16.0±0.1	0.07
Root SWmL/mg DW	**3.5±0.2 a**	**4.7±0.9 a**	**1.0±0.04 b**	**1.0±0.06 b**	**<0.01**
Shoot SWmL/mg DW	**1.0±0.2 b**	**0.5±0.1 b**	**6.01±0.3 a**	**5.6±0.46 a**	**<0.01**

	P/10		PTOT		
VLACH	M	NM	M	NM	pvalue
Root length (cm)	**29.4±2.2 ^{ab}**	**37.1±2.8 ^{a}**	**23.3±1.8 ^{b}**	**30.5±2.5 ^{ab}**	**<0.01**
Collar (mm)	7.2±0.7	6.4±0.4	5.7±0.5	5.6±0.5	0.13
Height (cm)	12.2±1.4	9.5±0.7	11.1±2.0	10.8±1.8	0.53
Branch number	6.1±0.6	6.1±0.5	6.4±1.1	5.6±0.8	0.9
Root dw (g)	**2.5±0.5 ^{ab}**	**3.0±0.6 ^{a}**	**1.0±0.2 ^{c}**	**1.5±0.4 ^{bc}**	**<0.01**
Shoot dw (g)	3.4±0.7	2.23±0.5	2.4±0.7	1.99±0.6	0.35
Total dw (g)	5.9±1.0	5.2±1.0	3.4±0.8	3.5±0.9	0.1
Shoot/root DW ratio	1.8±0.2 ^{a}	0.7±0.1 ^{b}	1.1±0.5 ^{ab}	1.2±0.4 ^{ab}	<0.01
DQI	**2.1±0.5 ^{a}**	**2.5±0.6 ^{a}**	**0.8±0.1 ^{b}**	**1.2±0.3 ^{ab}**	**0.02**
Root surface(cm²)	122.8±18.2	108.5±14.6	63.9±12.7	82.2±10.1	0.09

	P/10		PTOT		
VLACH	M	NM	M	NM	
Root P nmoles Pi/mg DW	**28.6±4.4 ^{a}**	**15.9±1.8 ^{b}**	**33.0±5.6 ^{a}**	**15.9±2.1 ^{b}**	**0.02**
Leaf P nmoles Pi/mg DW	**36.5±5.4 ^{a}**	**14.9±1.9 ^{b}**	**33.0±6.1 ^{a}**	**47.0±5.8 ^{a}**	**<0.01**
Root N (% DW)	2.6±0.1	2.5±0.1	2.4±0.1	2.50±0.1	0.3
Leaf N (% DW)	3.4±0.2	3.1±0.04	3.4±0.12	3.2±0.06	0.31
Root C (% DW)	35.2±4.5	40.1±0.9	40.9±0.4	40.2±0.3	0.18
Leaf C (% DW)	37.2±4.5	41.1±0.2	41.7±1.0	40.5±0.6	0.26
Root C/N (% DW)	16.5±0.7	15.8±0.3	17.2±0.1	16.4±0.2	0.41
Leaf C/N (% DW)	13.3±0.5	12.6±0.1	13.0±0.2	12.45±0.1	0.32
Root s w	2.5±0.3	2.1±0.7	6.1±2.4	3.7±1.1	0.36
Shoot SW mL/mg DW	**1.2±0.3 ^{b}**	**1.6±0.3 ^{ab}**	**3.3±1.0 ^{ab}**	**3.5±0.7 ^{a}**	**0.02**

	P/10		PTOT		
VX211	M	NM	M	NM	pvalue
Root length (cm)	*45.9±2.3*	*48.7±2.1*	*45.5±3.1*	*44.5±4.6*	*0.32*
Collar (mm)	*6.8±0.7*	*6.5±0.6*	*6.0±0.4*	*6.2±0.5*	*0.98*
Height (cm)	*10.6±1.8*	*10.6±1.6*	*9.4±0.8*	*9.6±0.8*	*0.92*
Branch number	*6.6±0.6*	*5.4±0.7*	*5.4±0.4*	*5.4±0.5*	*0.64*
Root dw (g)	*6.7±1.3*	*5.2±1.2*	*8.9±1.7*	*9.1±2.2*	*0.33*
Shoot dw (g)	*3.7±0.8*	*3.1±0.7*	*2.2±0.7*	*2.5±0.6*	*1.0*
Total dw (g)	*9.6±2.0*	*8.0±1.7*	*11.1±2.0*	*11.6±2.8*	*0.6*
Shoot/root DW ratio	**0.5±0.06 [a]**	**0.5±0.05 [a]**	**0.3±0.03 [b]**	**0.3±0.1 [b]**	**<0.01**
DQI	*4.5±0.8*	*3.7±0.6*	*7.7±1.8*	*7.4±1.8*	*0.44*
Root surface(cm²)	**169.5±19.9 [b]**	**197.93±20.5[b]**	**262.1±14.9[a]**	**255.7±12.9[a]**	**<0.01**

	P/10		PTOT		
VX211	M	NM	M	NM	pvalue
Root P nmoles Pi/mg DW	**18.0±1.0 [b]**	**10.5±1.1 [c]**	**45.9±6.4 [a]**	**36.2±4.4 [a]**	**<0.01**
Leaf P nmoles Pi/mg DW	**22.7±3.1 [b]**	**10.4±1.4 [c]**	**87.8±10.0 [a]**	**56.3±9.4 [a]**	**<0.01**
Root N (% DW)	**2.3±0.1 [bc]**	**2.2±0.1 [c]**	**2.5±0.1 [ab]**	**2.6±0.1 [a]**	**<0.01**
Leaf N (% DW)	**2.9±0.1 [b]**	**2.8±0.1 [b]**	**3.2±0.1 [a]**	**3.3±0.1 [a]**	**<0.01**
Root C (% DW)	*39.2±0.5*	*39.7±0.3*	*40.4±0.3*	*40.8±0.6*	*0.17*
Leaf C (% DW)	*41.1±0.4*	*41.1±0.5*	*41.4±0.5*	*41.5±0.4*	*0.87*
Root C/N (% DW)	**17.7±0.1 [a]**	**17.58±0.1 [a]**	**16.8±0.1 [a]**	**15.4±0.2 [b]**	**<0.01**
Leaf C/N (% DW)	*14.3±0.1 [a]*	*15.0±0.2 [a]*	*13.3±0.1 [ab]*	*12.5±0.1 [b]*	*<0.01*
Root s w	*1.9±0.3*	*1.7±0.4*	*2.2±0.2*	*2.6±0.13*	*0.13*
Shoot s.wmL/mg mL/mg DW	1.6±0.2	1.9±0.2	1.3±0.2	1.4±0.2	0.19

	P/10		PTOT		
WIP3	M	NM	M	NM	pvalue
Root length (cm)	**24.2±1.5 ^{b}**	**25.6±1.4 ^{b}**	**29.5±2.1 ^{ab}**	**35.5±1.9 ^{a}**	**<0.01**
Collar (mm)	6.6±0.2	6.1±0.2	6.2±0.5	5.6±0.2	0.11
Height (cm)	**32.2±2.6 ^{a}**	**14.8±1.1 ^{b}**	**31.0±4.5 ^{a}**	**14.7±1.3 ^{b}**	**<0.01**
Branch number	**7.5±0.4 ^{a}**	**6.3±0.4 ^{bc}**	**7.3±0.5 ^{ab}**	**5.1±0.4 ^{c}**	**<0.01**
Root dw (g)	3.6±0.7	3.2±0.4	1.6±0.3	2.4±0.9	0.06
Shoot dw (g)	4.8±0.5	3.2±0.6	5.0±0.8	5.0±0.9	<0.01
Total dw (g)	**8.8±1.2 ^{a}**	**6.2±1.0 ^{ab}**	**2.9±0.5 ^{c}**	**4.0±1.5 ^{bc}**	**<0.01**
Shoot/root DW ratio	1.8±0.3	1.1±0.2	1.2±0.2	1.5±0.2	0.16
DQI	1.8±0.4	1.1±0.19	1.31±0.27	2.1±0.7	0.4
Root surface(cm^2)	**529.5±50.0 ^{b}**	**651.4±41.2 ^{a}**	**197.3±14.1 ^{c}**	**146.1±15.2 ^{c}**	**<0.01**

	P/10		PTOT		
WIP3	M	NM	M	NM	
Root P nmoles Pi/mg DW	**17.5±1.4 ^{b}**	**12.0±1.1 ^{c}**	**31.3±4.3 ^{a}**	**26.3±2.2 ^{a}**	**<0.01**
Leaf P nmoles Pi/mg DW	**33.1±1.9 ^{a}**	**32.4±1.6 ^{a}**	**53.8±8.72 ^{b}**	**52.7±7.8 ^{b}**	0.06
Root N (% DW)	**1.92±0.2 ^{b}**	**1.7±0.1 ^{c}**	**2.6±0.1 ^{a}**	**2.4±0.1 ^{a}**	**<0.01**
Leaf N(% DW)	**2.9±0.1 ^{b}**	**3.0±0.2 ^{b}**	**3.1±0.1 ^{a}**	**3.4±0.1 ^{a}**	**<0.01**
Root C (% DW)	39.7±0.8	40.1±0.6	39.8±0.	40.2±0.5	0.95
Leaf C (% DW)	**42.6±1.3 ^{a}**	**42.7±0.3 ^{a}**	**39.6±0.7 ^{b}**	**40.0±0.8 ^{b}**	**<0.01**
Root C/N (% DW)	**20.9±0.2 ^{a}**	**23.0±0.2 ^{a}**	**16.1±0.2 ^{b}**	**16.7±0.3 ^{b}**	**<0.01**
Leaf C/N (% DW)	**14.7±0.2 ^{a}**	**14.9±0.2 ^{a}**	**11.5±0.1 ^{b}**	**11.7±0.1 ^{b}**	**<0.01**
Root s WmL/mg DW	4.3±1.1	3.13±0.3	2.8±0.8	1.7±0.3	0.65
Shoot SWmL/mg DW	**2.5±0.2 ^{ab}**	**2.2±0.3 ^{b}**	**3.8±0.9 ^{ab}**	**5.1±0.8 ^{a}**	**0.02**

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Chapitre IV

Mining for arbuscular mycorrhiza-conserved proteins from whole genome sequences: the phosphate and lipid exchange module under contrasting fertilization regimes as a case study in the walnut tree *Juglans regia*

1. Introduction

Phosphorus (P) is an essential macronutrient that plays a central role in all major metabolic processes in plants, particularly photosynthesis and respiration (Plaxton and Tran 2011; Plassard, Becquer, and Garcia 2019). A large fraction of soil P (50% to 80%) occurs as organic P of which phytic acid (inositol hexaphosphate) is a major component (Richardson, 1994). Only inorganic P (Pi) in the form of soluble orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) constitutes the plant available P (Marschner 1996; Poirier and Bucher 2002). P is abundant in the environment but the negative charge of the ionic forms are easily sequestered by cations (Adesemoye and Kloepper 2009). This results in tiny amounts of free Pi in the soil solution, where concentrations range from 1-10 μM, whereas plant cells require Pi in the millimolar range (Javot, Pumplin, and Harrison 2007). Moreover, the rate of absorption of Pi by growing roots is much higher than the rate of soil Pi diffusion, which leads to zone depleted in Pi around the roots thereby limiting the supply of phosphorus to the plant (Javot, Pumplin, and Harrison 2007; Balemi and Negisho 2012). Thus, application of large amounts of P fertilizers as soluble orthophossphate ions is the current agronomic practice to ensure plant productivity in most agricultural systems (Schachtman, Reid, and Ayling 1998; Smith et al. 2011). Generalized phosphorus fertilization has increased the crop production but it has tripled the rate of consumption of Pi and has contributed to soil degradation and water eutrophication (Conley et al. 2009). Pi is derived from mined non-renewable phosphate rock deposits and these are predicted to become a limiting factor for food production within the next century (Van Vuuren, Bouwman, and Beusen 2010). Consequently, appropriate agronomic management aiming to increase P use efficiency and minimize damage to the environment is of strategic importance for the development of sustainable agriculture (Liu et al. 2016).

An alternative to the input of Pi fertilizers to agricultural soils is to exploit the mechanisms developed by Pi-limited plants to improve Pi use efficiency in agro-ecosystems. Plant develop strategies which include the use of soil microorganisms that play key roles in plant P nutrition (Richardson and Simpson 2011; X. Liu et al. 2016). Among them, soil-borne arbuscular mycorrhizal (AM) fungi of the sub-phylum Glomeromycotina (Spatafora et al. 2016) engage in a mutualistic relationship with the roots of most of terrestrial plant species that encompass the majority of agricultural crops (Smith et al. 2011). AM fungi have been shown to benefit crop productivity and to support several ecosystem services including nutrient cycling (Gianinazzi et al. 2010). One prominent function of AM fungi is to assist their host plants with the acquisition of Pi that is absorbed from the soil solution through hyphal scavenging of soil volumes inaccessible to plant roots (Smith et al. 2011). It has been estimated that inoculation with AM fungi might result in a reduction of approximately 80% of the recommended fertilizer P rates (Jakobsen 1995).

When roots are colonized by AM fungi, soil Pi is acquired at the soil-fungus interface through high-affinity fungal phosphate transporters (PHTs) located in the extra-radical mycelium that develops outside of the host root (Harrison and Buuren 1995; Maldonado-Mendoza, Dewbre, and Harrison 2001; Benedetto et al. 2005; Fiorilli, Lanfranco, and Bonfante 2013; Xie et al. 2016). Pi accumulated in the vacuoles of extra-radical hyphae as polyphosphate (polyPi) chains (Ezawa et al. 2004) are further transferred by a tubular vacuolar network into the intra-radical hyphae and arbuscules (Uetake et al. 2002). Pi is hydrolysed from polyPi by fungal enzymes, after which it is released into the plant interfacial apoplast of arbuscules, where it is acquired by plant PHT located on the peri-arbuscular membrane (Harrison, Dewbre, and Liu 2002; Javot, Pumplin, and Harrison 2007). Two classes of plant PHT1 transporters which belong to the phosphate/H^+ symporter family are involved in Pi transport in the AM symbiosis (Pao, Paulsen, and Saier 1998). Mycorrhiza-inducible Pi transporters such as *StPT3* in potato (Rausch et al. 2001), *HvPt8* in barley (Glassop, Smith, and Smith 2005), and *OsPT13* in rice (Yang et al. 2012), are expressed in non-symbiotic roots, and over-expressed in symbiotic roots. Mycorrhiza-specific Pi transporters such as *OsPT11* in rice (Paszkowski et al. 2002), *MtPT4* in barrel medic (Harrison, Dewbre, and Liu 2002), *SlPT4* in tomato (Nagy et al. 2005), and *PtPT10* in poplar (Loth-Pereda et al. 2011) are specifically expressed in AM-colonized plants. The first mycorrhiza-specific Pi transporter genes *MtPT4* and *OsPT11* identified in dicot and monocot plants, respectively, have been localized at the arbuscule-branch domain of the peri-arbuscular membrane (Pumplin and Harrison 2009; Kobae et al. 2010), and are exclusively expressed in plant cells hosting arbuscules (Javot et al. 2007; Yang et al. 2012). Reduced fungal growth and altered arbuscule morphology in *Medicago pt4* and rice *pt11* loss-of-function mutants revealed that PT4 and PT11 are essentials for AM-mediated phosphate uptake and arbuscule maintenance (Javot et al. 2007; Yang et al. 2012). Likewise, *ZEAma:Pt1;6* is a mycorrhiza-specific Pi transporter gene of maize, and a knock-down *pht1;6* mutant showed reduced mycorrhiza formation (Willmann et al. 2013).

In exchange for soil Pi, AM fungi are supplied with plant organic carbon, which is essential to achieve their full life cycle (Gutjahr and Parniske 2013; Rich et al. 2017; Roth and Paszkowski 2017). Recent findings indicate that AM fungi are entirely dependent on lipid delivery by the plant for their growth, development and reproduction. The dependence on lipids may therefore be the prime reason for their obligate biotrophy (Wang 2017; Lanfranco, Bonfante, and Genre 2018). Genome analyses noticeably revealed that AM fungi do not possess genes coding the cytosolic fatty acid (FA) synthase subunits involved in *de novo* FA biosynthesis, indicating AM fungi are fatty acid auxotrophs (Wewer, Brands, and Dörmann 2014). Consistently, it was discovered that legume mutants with stunted arbuscules and reduced colonization are defective in AM-induced lipid biosynthesis genes, including *FATM* and *RAM2* that fine-tune lipid biosynthesis to promote AM development (Wang, Huang, and Gao 2012; Bravo et al. 2017; Q.-Y. Jiang et al. 2016, 201; Luginbuehl et al. 2017). Current knowledge indicates that the AM-essential plant thioesterase FATM releases 16:0 FAs (palmitic acid). When attached to CoA, 16:0 FAs are used as a substrate by the glycerol-3-phosphate acyl transferase RAM2 to produce 16:0 β-monoacylglycerol that can be exported across the peri-arbuscular membrane (PAM). In this regard, a pair of ABC half-transporters, named STR and STR2, necessary to support arbuscule formation, are localized in the PAM where they can export lipids into the apoplastic space between the plant and the AM fungus (Zhang, Blaylock, and Harrison 2010; Gutjahr et al. 2012). FATM, RAM2 and STR/STR2, which are essential to plants that engage in AM symbiosis, therefore form a functional module that enables the adjustment of lipid biosynthesis in colonized cells to synthesize sufficient lipids for AM fungi.

In response to high soil Pi levels in the range of 100 mg or more of Pi per kg of soil (Schubert and Hayman, 1978; Baon et al. 1992; Corkidiki et al. 2002), plants are known to suppress AM symbiosis (Baylis, 1967; Mosse, 1973; Sanders and Tinker, 1973). Pi fertilization interrupts the development of arbuscules at the coarse hyphal branch stage suggesting that the transcriptional regulation of genes involved in arbuscule branching may be suppressed during Pi fertilization (Park et al. 2015; Kobae et al. 2016). This indicates that plants allow continuation of arbuscular development and mycorrhizal fungal existence in roots only when soil P captured by the hyphae is transported into the roots (Javot et al. 2007). These finding support the model proposed by Fitter (2006) according to which a successful fungus must continue to provide the plant with Pi through the arbuscules in order to maintain a reciprocal carbon flux. Consistently, it has been shown that the host is able to reward the symbiont with sucrose for efficient symbiotic Pi delivery (Kiers et al. 2011). Recent studies also support a bidirectional exchange of phosphorus and lipid as a fundamental trait in AM symbiosis (Xue et al. 2018). These authors showed that in *Lotus japonicus*, the transcription factor CBX1 binds CTTC and AW-box CREs and co-regulates mycorrhizal phosphate transporter, H^+-ATPase, and RAM2 genes. Likewise, in *M. truncatula*, overexpression of the transcription factor WRI5a resulted in enhanced expression of *STR* and *MtPT4*, suggesting that WRI5a regulates bidirectional symbiotic nutrient exchange (Jiang et al. 2018).

Because fatty acids are thought to be the major form of carbon transferred from plant hosts to AM fungi (Wang 2017), regulatory inputs that influence *FATM, RAM2* and *STR2* expression might be expected as dependent of the nutritional status of the plant and the Pi delivered by the AM symbiosis. Little is known regarding the impact of Pi fertilization on the regulation of the lipid/transport module essential to AM symbiosis. Re-examining the free-market model on the basis of plant host-synthesized lipids (Wang 2017) is of noticeable relevance in agroforestry systems where tree and crop roots are connected by a common mycelium network. Unfortunately, AM-essential PHT1 and lipid synthesis/transport proteins are not yet available for many mycotrophic hardwood, fruit and nut bearing trees of agro-ecological interest. AM-related PHT1 have mainly been identified in legumes (e.g. *M. truncatula*; *L. japonicus*; *Glycine max*), cereals (e.g. *Oryza sativa*; *Zea mays*; *Hordeum vulgarum*; *Triticum aestivum*; *Setaria italica; Sorghum bicolor; Eleusine coracana; Brachypodium distachyon*) and *Solanaceae* (e.g. *Solanum lycopersicum, Solanum tuberosum; Nicotiana tabacum*; *Solanum melongena*) (Pudake et al. 2017; Wang 2017 and references therein). By contrast, little is known about the molecular basis of AM symbiosis-mediated Pi uptake in tree species other than poplar (*Populus trichocarpa)* (Loth-Pereda et al. 2011), grapevine *(Vitis vinifera)* (Valat et al. 2018), and upland cotton (*Gossypium hirsutum*) (Gao et al. 2020). Likewise, putative members of the plant lipid transfer module (Bravo et al. 2017), remain to be identified in trees. Overall, there is an urgent need to enlarge the understanding of the phosphate and lipid exchange module in hardwood species, as dependent of the Pi fertilization regime.

Genomic sequences coding target proteins in mycotrophic plants have been retrieved using BLAST searches (Loth-Pereda et al. 2011; F. Liu et al. 2018; Valat et al. 2018) or designing degenerate primers based on the conserved amino acid regions (Hu et al. 2017). These approaches are coupled to the fastidious transcriptional analysis of all identified sequences upon plant inoculation or not with AM fungi. The co-evolution of plants and AM fungi during the last several hundred-million years, the worldwide distribution of AM fungi and the apparent lack of host specificity in plant-AM symbiosis indicate that the cascade of events leading to the establishment of the functional AM symbiosis is conserved between evolutionary distant plant species (Karandashov et al. 2004). Several phylogenomics approaches to identify genes whose evolutionary history predicts conservation for AM symbiosis have been published in the last decade (Delaux et al. 2014; Favre et al. 2014; Bravo et al. 2016; Rich et al. 2021). As a result, 138 genes

from *M. truncatula* are predicted to function in AM symbiosis, including AM-essential PHT1 and lipid synthesis/transport proteins, and the accuracy of the predictions through reverse genetics analysis has been demonstrated (Bravo et al. 2016; 2017). Consequently, combining comparative genomics with differential gene expression analysis may allow the identification of widely conserved plant genes involved in AM symbiosis in trees, together with monitoring their regulation upon contrasting Pi fertilization regimes.

In this study, we therefore first used OrthoFinder to identify the proteins, and the corresponding genes, that are evolutionary conserved across plant species. To this aim, we compared the predicted proteomes of 25 mycotrophic species with substantial diversity and redundancy within dicots and monocots, including hardwood, fruit and nut-bearing trees such as *Julans regia, J. nigra, J. microcarpa, J. hindsii, Carica papaya, Citrus clementina, Eucalyptus grandis, Malus domestica, Olea europaea, Populus trichocarpa, Theobroma cacao, and V. vinifera.* After using OrthoFinder to define orthogroups between the 25 different species, based on an all-against-all comparison of the proteomes (Emms and Kelly 2019), we selected the proteome of the model legume *M. truncatula* to identify the orthogroups potentially essential to AM symbiosis. In a second time, to assess the extent to which Pi availability may regulate the phosphate and lipid exchange module in trees, we compared the expression pattern of genes coding orthologs to MtPHT1, MtFATM, MtRAM2 and MtSTR2 proteins in low-and high-Pi conditions upon mycorrhization or not, in the English walnut tree *J.regia*. This species widely cultivated for the production of edible nuts (Bernard, Lheureux, and Dirlewanger 2018), was selected as a case study of trees ideally suited for alley cropping owing to its short growing season, sparse canopy and deep rooting system (Wolz and DeLucia 2019).

2. Materials and Methods

2.1. Data collection, orthology and *in silico* analyses

We used Orthofinder (version 2.2.6; (Emms and Kelly 2015)) through standard mode parameters in the Diamond tool (v0.9.22.123) all-versus-all, to identify plant proteins orthologous to the AM-essential phosphate transporter MtPHT1;4 (MtPt4, (Harrison, Dewbre, and Liu 2002)), MtFATM, MtRAM2 and MtSTR2 (Bravo et al. 2016) in *M. truncatula*. To this aim, the complete predicted proteome sequences of 25 plant species were retrieved from the databases listed in **Table IV.S1**. Protein sequences were aligned using Muscle (Madeira et al. 2021)) and visualized using MView (Brown and Hu 1998). Protein domains were searched using PfamScan (https://www.ebi.ac.uk/Tools/pfa/pfamscan/) and InterPro (https://www.ebi.ac.uk/interpro/). TMHMM Server v. 2.0 (Krogh et al. 2001)) was used to predict transmembrane (TM) segments. BLASTP was used to search for close homologous sequences having an experimentally demonstrated localization in Uniprot (Uniprot.org). At least 60% pair-wise identity and a cut-off expectation value of e^{-40} were retained as thresholds for accurate sequence conservation of localization (Nair and Rost 2002). The 2-kb upstream regions of the identified genes were downloaded as putative promoter regions, and corresponding *cis*-regulatory elements were analysed using the PLACE database with manual adjustment (Higo et al. 1999). Phylogenetic trees were generated using bioinformatics tools available at www.phylogeny.fr, using the default settings: MAFFT was used for multiple alignment, BGME was used for alignment refinement, and FastME was used to generate the phylogenies (Letunic and Bork 2021).

2.2 Biological material, plant inoculation, and phosphate fertilization

The AM fungus *R. irregularis* DAOM197198 (also known as DAOM181602) was purchased from Agronutrition (Labège, France) as an axenic suspension containing 200 spores per mL. The micropropagated *J. regia* clone RG17 was provided by Dr. Laurent Jouve (L&J Biotech, Souillac, France). Rootstocks were received seven weeks after root induction and placed for *ex vitro* acclimatization in closed mini-greenhouses (Rapid Grow, Nortene) filled with a sterilized (120 °C, 12h) quartz sand (Biot, France): zeolite (Symbion, Czech Republic) substrate (1:1, v:v). They were sprayed twice a week with tap water to maintain humidity and fertilized once a week with 15 ml per plant of a NPK 6-4-6 solution containing 2% of magnesium and oligoelements (Algoflash, France). Walnut cultures were maintained in a growth chamber under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night, 100% relative humidity, 220 µE m^{-2} s^{-1}). After two months of growth, plantlets were gradually exposed to reduced relative humidity by progressively removing the box covers during a further month, then rootstocks were transplanted in individual pots, each containing 3 L of the sterilized the sand-zeolite substrate. Two days after their transfer, plants were either inoculated twice (mycorrhizal treatment, AM) or not (non-mycorrhizal treatment, NM) with 1 mL of the axenic spore suspension of *R.irregularis*. Plants were sprayed with water twice a week to maintain humidity and fertilized once a week with 15 mL per plant of either a low-Pi (P/10, 0.13 mM NaH_2PO4, $2H_2O$) or a full strength Pi solution (P, 1.3 mM NaH_2PO4, $2H_2O$) Hoagland solution (Bonneau et al. 2013). Walnut cultures were maintained for two months before harvest in a growth chamber under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night, 100% relative humidity, 220 µE m^{-2} s^{-1}). Plant harvest was performed two months after inoculation. The experiment was performed with at least eight independent replicates per treatment.

2.3 Measurement of plant growth and nutritional parameters

Plant height (cm), branch number per plant, stem diameter at the soil line (mm), fresh shoot and root weights (g), and primary root length (cm) were measured at harvest. Roots were scanned and the resulting images were processed and analysed with ImageJ software (Schneider, Rasband, and Eliceiri 2012) to assess the root surface area. To determine dry matter weights (DW), shoot and root subsamples were separately weighed fresh, and then dried for 2 days at 80 °C and weighed. For soluble Pi and %N measurements, dried root and leaf samples were finely ground using a Retsch Mixer ball mill (Haan, Germany). Soluble Pi was extracted from 5 mg dried samples as described by (Grunwald et al. 2009). Free Pi was determined spectrophotometrically by measuring absorbance at 650 nm using the BIOMOL Green™ Reagent (Enzo Life Sciences, Villeurbanne, France) according to the instructions of the manufacturer. Root and leaf dried aliquots (5 mg) were analysed for their %N contents at the Biogeosciences Laboratory of the University of Bourgogne Franche-Comté (Dijon, France) on a FlashSmart Elemental Analyzer (Thermo Scientific).

2.4 Staining of AM fungi in plant roots and quantification of root colonization

At harvest, root samples were randomly collected from each plant and dried for 2 days at 80 °C. They were cleared with at 90 °C for 30 min in 10% (w/v) KOH (Phillips et al. 1970). Walnut roots were additionally bleached with three drops of 30% H_2O_2 to remove any phenolic compounds left in cleared roots (Kormanik and McGraw 1982). Roots were rinsed with tap water before staining in a 0.05% (w/v) trypan blue solution in lactoglycerol (lactic acid-glycerol demineralised water, 1:1:1) for 30 min at 90 °C. Thirty fragments, ca. 10 mm long, were randomly collected from each sample, mounted 15 per slide in glycerol and observed at X 200 magnification as described

in Trouvelot (1986). Calculations of frequency of mycorrhization (F%), percentage of root cortex colonisation (M%) and percentage of arbuscules (A%) were carried out with the MycoCalc program (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

2.5 Primer design, RNA extraction, reverse transcription and qRT-PCR

At harvest, walnut root subsamples (1.5 g) were immediately frozen in liquid nitrogen and kept at -80 °C before use. They were ground to a fine powder with a pre-cooled mortar and pestle before RNA extraction. The SV Total RNA Isolation System (Promega, Madison, USA), which incorporates a DNase treatment step, was used to recover total root RNA from 50 mg ground tissues, according to the supplied instructions. For walnut, 50 mg of insoluble polyvinylpyrrolidone were added to root samples before RNA extraction in order to remove polyphenols (Hu et al. 2002). The purified RNA was quantified by measuring the absorbance at 260 nm with a Nanodrop (ND-1000 spectrophotometer, Isogen Life Sciences, Maarssen, Netherlands). Complementary DNAs (cDNAs) were obtained using the iScriptTMcDNA Synthesis Kit (BIO RAD Laboratories, Paolo Alto, CA, United States), using 300 ng of total RNA per reaction. On the basis of plant orthologs identified using Orthofinder, primers were designed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) on the corresponding gene sequences. All primers used are listed in **Table IV.S2**, including the reference genes used for expression normalization. Quantitative RT-PCRs were run in a 7500 real-time PCR system (Roche) using the following settings: 95 ◦C for 3 min and then 40 cycles of 95 ◦C for 30 s, 60 ◦C for 1 min and 72 ◦C for 30 s. The number of replicates comprised at least three biological and three technical replicates per treatment. Transcript levels were expressed as normalized expression to plant reference genes according to the following formula (with E referring to the PCR efficiency):

$$R = \frac{E_T^{\Delta Ct_T(Cm-I)}}{\sqrt{\left(E_{\text{ref1}}^{\Delta Ct_{\text{ref1}}(Cm-I)}\right)\left(E_{\text{ref2}}^{\Delta Ct_{\text{ref2}}(Cm-I)}\right)}}$$

2.6 Data analysis

As mycorrhizal colonization parameters represent percentages, all data were *arcsine square root*-transformed to obtain a distribution of values that could be checked for normality using the Kolmogorov-Smirnov test. Significant differences ($p < 0.05$) between Pi treatments were analysed using the Welch-test (degrees of freedom = n-1), which is compatible with unequal variances between groups (Staer et al. 2014). The impact of pre-inoculation with *R. irregularis* and Pi fertilization on the growth, nutrition, and gene expression parameters was analysed using the Kruskal-Wallis test. When the Kruskal-Wallis test was significant ($p < 0.05$), a *post*-hoc analysis was performed to determine the extent to which variables differ from each other by using the Tukey's Honest Significant Difference (HSD) test under the Rstudio environment.

3. Results

3.1 Identification of the orthogroups of MtPT4, MtFATM, MtRAM2, and MtSTR2 in *J. regia*

The comparative analysis of the 25 proteomes using Orthofinder led to the identification of 31,422 orthogroups (OG). Among them, the orthogroup OG0008521 of the AM-essential Pi transporter MtPT4 contained 43 proteins, including three proteins of *J. regia* (Jr13_30200_p1, Jr13_30210_p1, Jr16_00830_p1, further named JrPHT1;1; JrPHT1;2, JrPHT1;3, respectively). The orthogroup OG0009826 of the AM-essential ACP-thioesterase MtFatM consisted of 38 proteins, among which two proteins were retrieved for *J. regia*, namely Jr13_28660_p1 and Jr16_02040_p1 further referred to as JrFATM-1 and JrFATM-2, respectively. The orthogroup OG0006278 of the AM-essential glycerol-3-phosphate acyl transferase MtRAM2 also included two proteins of *J. regia* (Jr13_16580_p1 and Jr16_10950_p1, further named JrRAM2;1 and JrRAM2;2, respectively) within a total of 56 orthologs. Finally, the orthogroup of the AM-essential half-size ABC transporter MtSTR2 orthogroup OG0011040 contained 34 proteins, including two proteins of *J. regia* (Jr08_18980_p1 and Jr11_05690_p1, further named JrSTR2;1; and JrSTR2;2, respectively).

3.2 *In silico* characterization of the MtPT4 orthogroup in *J. regia*

The three proteins of *J. regia* (JrPHT1;1, JrPHT1;2 and JrPHT1;3) retrieved in the MtPT4 orthogroup displayed among others a typical Sugar transporter domain (PF00083) and the PHT1 signature consisting of the conserved sequence GGDYPLSATIxSE (**Figure IV. 1**) of 12 amino acid residues (Vladimir Karandashov and Bucher 2005). All of them were hydrophobic membrane proteins with 11 putative membrane-spanning regions with one intracellular central loop to transfer the phosphate molecule (**Figure IV. 1**). They also showed an accurate sequence conservation for localization to the plasma membrane (PM) with at least 60% pair-wise identity and a cut-off expectation value of e^{-40} (JrPHT1;1: 80%, e^{0}, JrPHT1;2: 77.4%, e^{0}; JrPHT1;3: 79,4%, e^{0}) to the peri-arbuscular membrane-resident protein MtPT4 (Harrison et al. 2002, Pumplin and Harrison 2009).

Figure IV.1. Sequence alignment of the *Medicago truncatula* PT4 (MtPT4; KEH40422.1) and the corresponding *Juglans regia* orthologs JrPHT1;1 (Jr13_30200_p1), JrPHT1;2 (Jr13_30210_p1) and JrPHT1;3 (Jr16_00830_p1). Protein sequences were aligned using Muscle (Madeira et al. 2019) and visualized using MView (Brown et al., 1998). Yellow bars indicate predicted transmembrane segments with TMHMM Server v. 2.0 (Krogh *et al.*, 2001). The sequence signature of PHT1 proteins (Vladimir Karandashov and Bucher 2005) was underlined by a red bar. The "Sugar (and other) transporter" domain PF00083 was identified from positions 23 to 499.

Analysis of the three corresponding promoter regions (**Figure IV. 2**) indicated the presence of several root motifs previously detected in plant *PHT* genes (Walder et al. 2015), but not the MYCS motif that has been detected in several AM-responsive *PHT1* (Favre et al. 2014, 20; Xue et al. 2018). The three promoters contained the Pi-responsive *cis*-regulatory elements P1BS, a known regulatory motif associated with Pi starvation signalling (Rubio 2001) and the AW-box recognized by the transcription factor CBX1 (Xue et al. 2018). The Pi-responsive *cis*-regulatory element PHO-binding sequence (Hu et al. 2017) was detected in the promoters of *JrPHT1;1* and *JrPHT1;2*. The promoters of *JrPHT1;1* and *JrPHT1;3* displayed a TTGACY W-box, a WRKY transcription factor binding element, which regulates expression of Pi-starvation responsive genes (Wang et al. 2014). OSE (organ-specific elements) previously revealed in several AM-inducible *PHT1* genes (Walder et al. 2015; Zhang et al. 2019), were retrieved in the promoters of *JrPHT1;2* (NODCON2GM) and *JrPHT1;3* (NODCON2GM, OSEROOTNODULE). The *cis*-element called 'AM-CYC box' recognized by CYCLOPS and computationally identified in several AM-induced genes (Favre et al. 2014), was only present in the promoter region of *JrPHT1;1*.

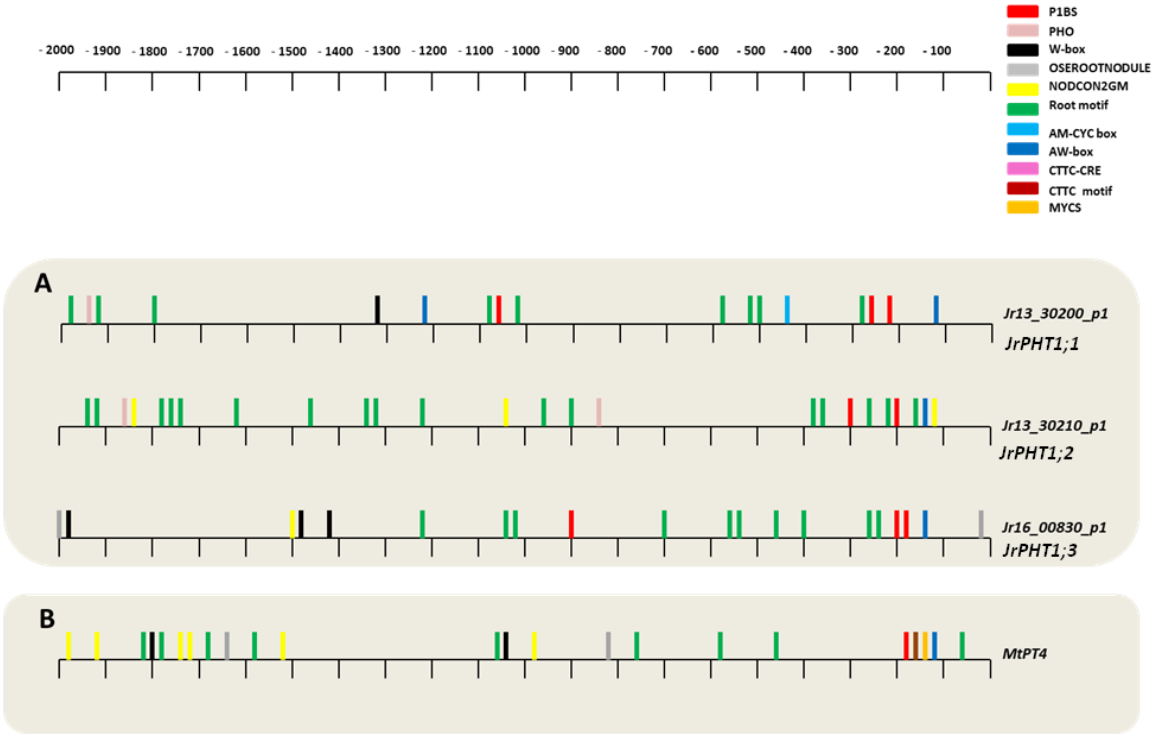

Figure IV. 2. Putative *cis*-regulatory elements involved in Pi and AM responses in the promoters (2-kb upstream regions) of **A**) the *J. regia* inorganic phosphate transporter (*PT*) genes orthologous to **B**) the mycorrhiza-essential gene *MtPT4* in *Medicago truncatula.* PIBS, GNATATNC; PHO, CACGTG; W-box, TTGACY; OSEROOTNODULE, AAAGAT; NODCON2GM, CTCTT; root motif, ATATT or AATAT; AM-CYC box, CGGCCG; AW-box, CNTNG(N)$_7$CG; CTTC CRE, CTTCTTGTTC; CTTC motif, TCT[T/C]GTT; mycorrhiza transcription factor binding sequence (MYCS), [T/C][T/G][T/A]CTTGTT[/C/T/G][T/C].

The phylogenetic tree of the PHT1 protein family clearly showed that the 26 newly identified proteins in *J. regia*, *J. hindsii*, *J. microcarpa*, *C. papaya*, *T. cacao*, *C. clementina*, *E. grandis*, *C. sativus*, *M. domestica*, *D. carota*, *H. annuus*, *O. europaea*, and *M. acuminata* belong to subfamily I, the members of which are induced in AM roots (**Figure IV.3**).

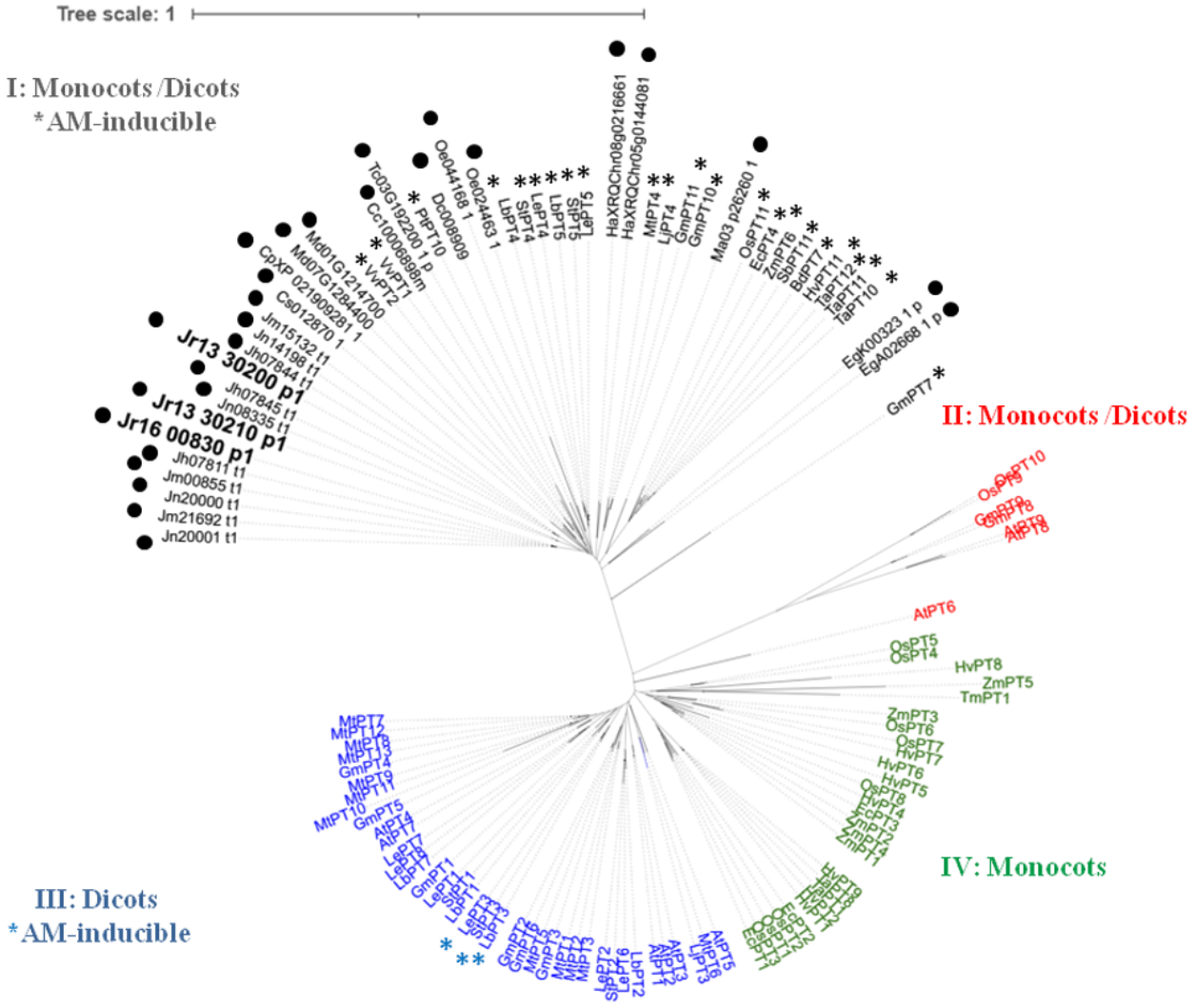

Figure IV. 3. Unrooted phylogenic tree for the amino acid sequences of plant PHT1 transporters. Accessions numbers and protein sequences are detailed in Table IV. S3. Roman numerical indicate the four PHT1 subfamilies thought to differ in evolutionary age (Nagy et al. 2005). Asterisks refer to AM-inducible *PHT1* genes (Table IV.S3). Circles indicate the 26 protein sequences orthologous to the AM-inducible transporter MtPT4 identified in this work. The three putative PHT1 transporters of *J. regia* orthologous to MtPT4 are indicated in dark bold characters. Abbreviations for plant species: At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon;* Cc: *Citrus clementina*; Cp, *Carica papaya*; Cs, *Cucumis sativus*; Dc, *Daucus carota*; Ec, *Eleusine coracana;* Eg, *Eucalyptus grandis*; Gm: *Glycine max*; Ha, *Helianthus annuus;* Hv, *Hordeum vulgare*; Jh, *Junglas hindsii*; Jm, *J. microcarpa;* Jn, *J. nigra*; Jr, *J.regia*; Lb, *Lycium barbarum*; Le, *Lycopersicon esculentum*; Lj, *Lotus japonicus*; Ma, *Musa acuminata* Md, *Malus domestica*; Mt, *Medicago truncatula*; Oe, *Olea europaea*; Os, *Oryza sativa*; Pt, *Populus trichocarpa;* Sb, *Sorgum bicolor*; St, *Solanum tuberosum*; Ta, *Triticum aestivum*; Tc, *Theobroam cacao;* Tm, *Triticum monococcum;* Vv, *Vitis vinifera*; and Zm, *Zea mays*.

3.3 *In silico* characterization of the MtFATM orthogroup in *J. regia*

The MtFATM orthogroup contained two proteins of *J. regia* namely Jr13_28660_p1 (JrFATM-1) and Jr16_02040_p1 (JrFATM-2), which displayed (**Figure IV. 4**) the PF01643 domain typical for acyl-acyl carrier protein thioesterases involved in fatty acyl group extension *via* hydrolyzing an acyl group on a fatty acid (Yuan et al. 1995). The two proteins also showed an accurate sequence conservation for localization to plastids with at least 60% pair-wise identity and a cut-off expectation value of e^{-40} (Jr13_28660_p1: 64%, 1.6^{e-173} ; Jr16_02040_p1: 67%, e^{0}) to the plastid-resident protein MtFATM (Bravo et al. 2017).

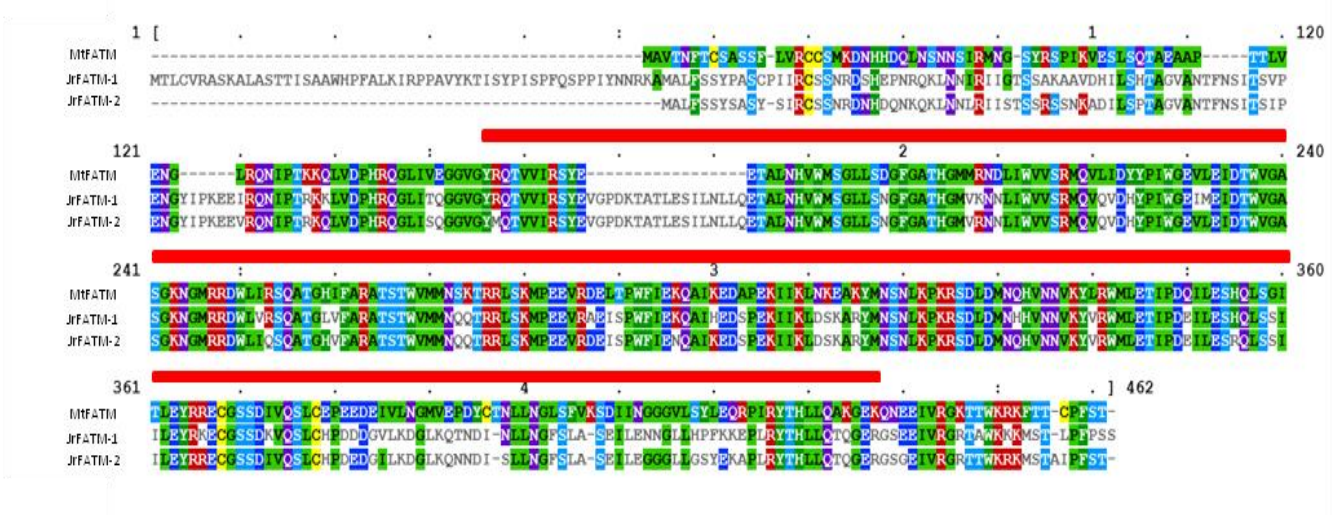

Figure IV.4 Sequence alignment of *Medicago truncatula* FATM (MtFATM; KEH44149.1) and the corresponding *Juglans regia* orthologs JrFATM-1 (Jr13_28660_p1), JrFATM-2 (Jr16_02040_p1). Protein sequences were aligned using Muscle (Madeira et al., 2019) and visualized using MView (Brown et al., 1998). The PF01643 domain typical for acyl-acyl carrier protein thioesterases is indicated by a red bar.

The further analysis of the promoter regions of *JrFATM-1* and *JrFATM-2* (**Figure IV.5**) indicated the presence of root motifs without the MYCS element, but with one or two AW-boxes. The Pi-responsive *cis*-regulatory element PHO-binding sequence, together with two CTTC motifs detected in AM-induced *PHT1* genes (Chen et al. 2011; Favre et al. 2014; Lota et al. 2013), were only observed in the promoter of *JrFATM1*. OSE motifs were also present upstream of *JrFATM-1* (one NODCON2GM) and *JrFATM-2* (two OSEROOTNODULE and one ODCON2GM).

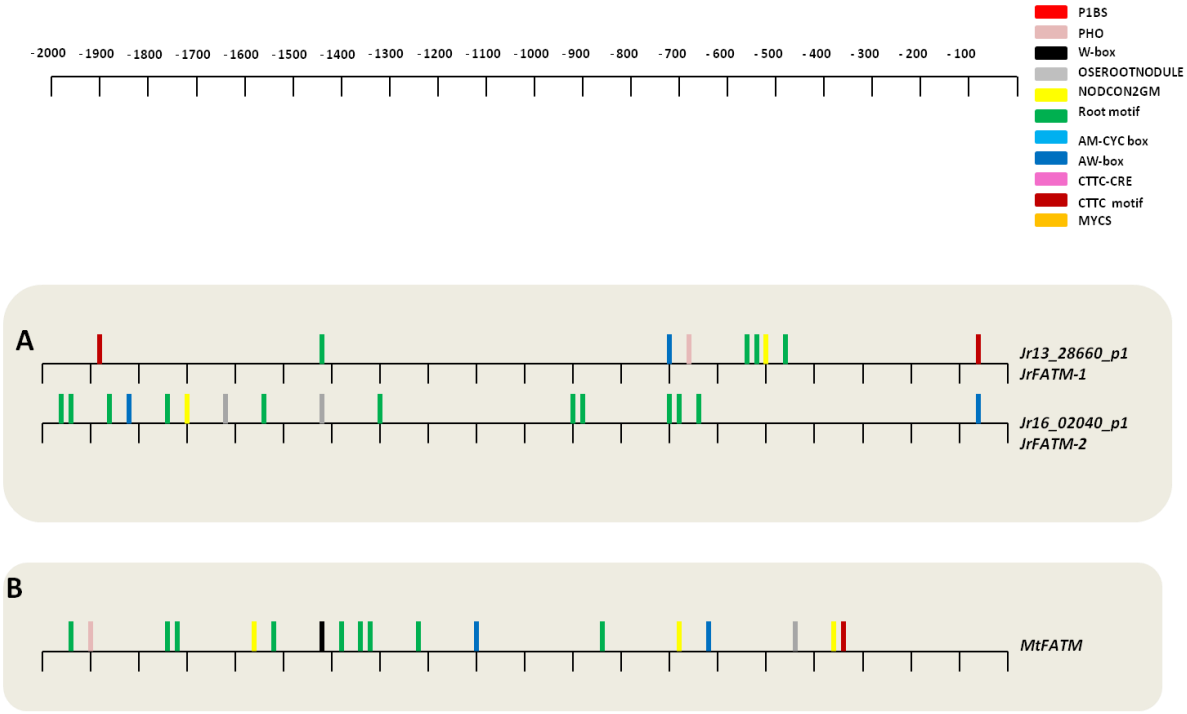

Figure IV. 5. Putative *cis*-regulatory elements involved in Pi and AM responses in the promoters (2-kb upstream regions) of **A**) the *J. regia* acyl-acyl carrier protein thioesterase-coding genes orthologous to **B**) the mycorrhiza-essential *MtFATM* (for FAT required for Arbuscular Mycorrhizal Symbiosis) in *Medicago truncatula*. PIBS, GNATATNC; PHO, CACGTG; W-box, TTGACY; OSEROOTNODULE, AAAGAT; NODCON2GM, CTCTT; root motif, ATATT or AATAT; AM-CYC box, CGGCCG; AW-box, CNTNG(N)$_7$CG; CTTC CRE, CTTCTTGTTC; CTTC motif, TCT[T/C]GTT; mycorrhiza transcription factor binding sequence (MYCS), [T/C][T/G][T/A]CTTGTT[/C/T/G][T/C].

The phylogenetic tree of FATM proteins (**Figure IV.6**) noticeably underlined that the 37 newly identified proteins in *B. distachyon; C. clementina*; *C. papaya*; *C. sativus*; *D. carota*; *E. grandis; G. max*; *H. annuus; J. hindsii*; *J. microcarpa; J. nigra*; *J. regia*; *M. acuminata; M. domestica*; *O. europaea*; *O. sativa*; *P. trichocarpa; S. bicolor*; *S. esculentum; S. tuberosum*; *T. cacao; V. vinifera*; and *Z. mays*, clustered with the AM-inducible FATM proteins of *L. japonicus* and *M. truncatula* (Keymer et al. 2017; Bravo et al. 2017).

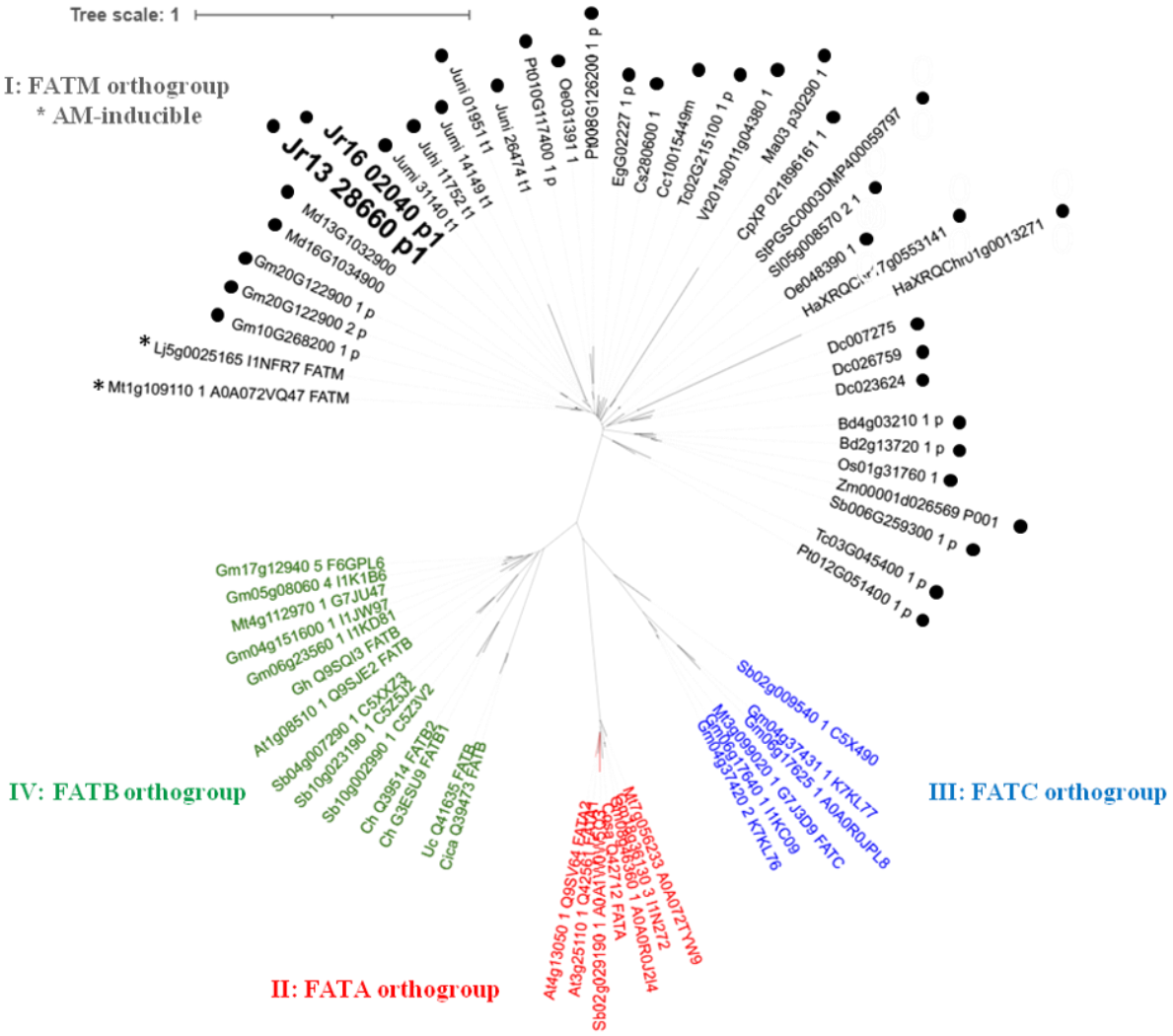

Figure IV.6. Unrooted phylogenic tree for the amino acid sequences of plant acyl-ACP thioesterases (FAT) that terminate chain elongation reactions of *de novo* fatty acid synthesis. Roman numerical indicate the four FAT subfamilies: **I**) FATM that shows preference for palmitoyl (16:0), is essential for AM colonization**,** while **II**) FATA, **III**) FATC, and **IV**) FATB supply fatty acids (predominantly 18:1Δ9 and 16:0) for regular AM-independent growth. Accessions numbers (Uniprot.org) refer to the protein sequences described by Brands et al. (2018) or reviewed in Uniprot (Cica; Cosa; Gh), with the exception of the 37 protein sequences orthologous to the AM-inducible MtFATM identified in this work indicated by circles. AM-inducible FATM proteins in *L. japonicus* (Keymer et al. 2017) and *M. truncatula* (Bravo et al. 2017) are indicated by asterisks.

The two putative proteins of *J. regia* orthologous to MtFATM are indicated in dark bold characters Abbreviations for plant species: At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon;* Cc: *Citrus clementina*; Cica, *Cinnamomum camphor;* Ch, *Cuphea hookeriana*; Cosa, *Coriandrum sativum;* Cp, *Carica papaya*; Cs, *Cucumis sativus*; Dc, *Daucus carota*; Eg, *Eucalyptus grandis;*Gh, *Gossypium hirsutum***;** Gm: *Glycine max*; Ha, *Helianthus annuus;* Jh, *Junglas hindsii*; Jm, *J. microcarpa;* Jn, *J. nigra*; Jr, *J.regia*; Lj, *Lotus japonicus*; Ma, *Musa acuminata* Md, *Malus domestica*; Mt, *Medicago truncatula*; Oe, *Olea europaea*; Os, *Oryza sativa*; Pt, *Populus trichocarpa*; Sb, *Sorgum bicolor*; Sl, *Solanum esculentum;* St, *Solanum tuberosum*; Tc, *Theobroam cacao;* Vv, *Vitis vinifera*; and Zm, *Zea mays*.

3 4. *In silico* characterization of the MtRAM2 orthogroup in *J. regia*

The MtRAM2 orthogroup contained two transmembrane proteins of *J. regia* namely Jr13_16580_p1 (JrRAM2;1) and Jr16_10950_p1 (JrRAM2;2) harbouring the PF01553 domain of acyltransferases involved in phospholipid biosynthesis (**Figure IV. 7**). These proteins also displayed the superfamily entry SSF69593 (https://www.ebi.ac.uk/interpro/entry/ssf/SSF69593/) typical of glycerol-3-phosphate acyl transferases from amino acid 215 to 488 and from 217 to 490 for JrRAM2;1 and JrRAM2;2, respectively.

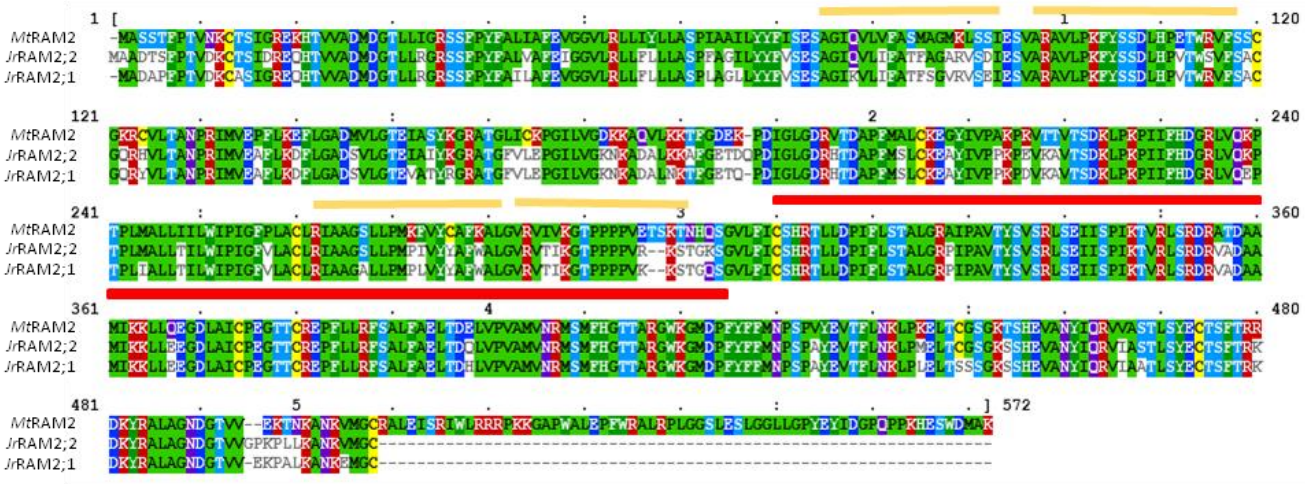

Figure IV.7. Sequence alignment of *Medicago truncatula* RAM2 (MtRAM2; AES60108.1) and the corresponding *Juglans regia* orthologs JrRAM2-1 (Jr13_16580_p1), JrRAM2-2 (Jr16_10950_p1). Protein sequences were aligned using Muscle (Madeira et al., 2019) and visualized using MView (Brown and Hu 1998). Yellow bars indicate predicted transmembrane segments with TMHMM Server v. 2.0 (Krogh *et al.*, 2001). The PF01553 domain typical of acyltransferases involved in phospholipid biosynthesis is indicated by a red bar.

Figure IV. 8 indicated the presence of root, NODCOM2GM, OSEROOTNODULE and P1BS motifs in the promoter of both *JrRAM2;1* and *Jr*RAM2;2. W- and AW-boxes were exclusive to the promoter of *JrRAM2;1*, while the CTTC motif was only detected upstream of *JrRAM2;2*.

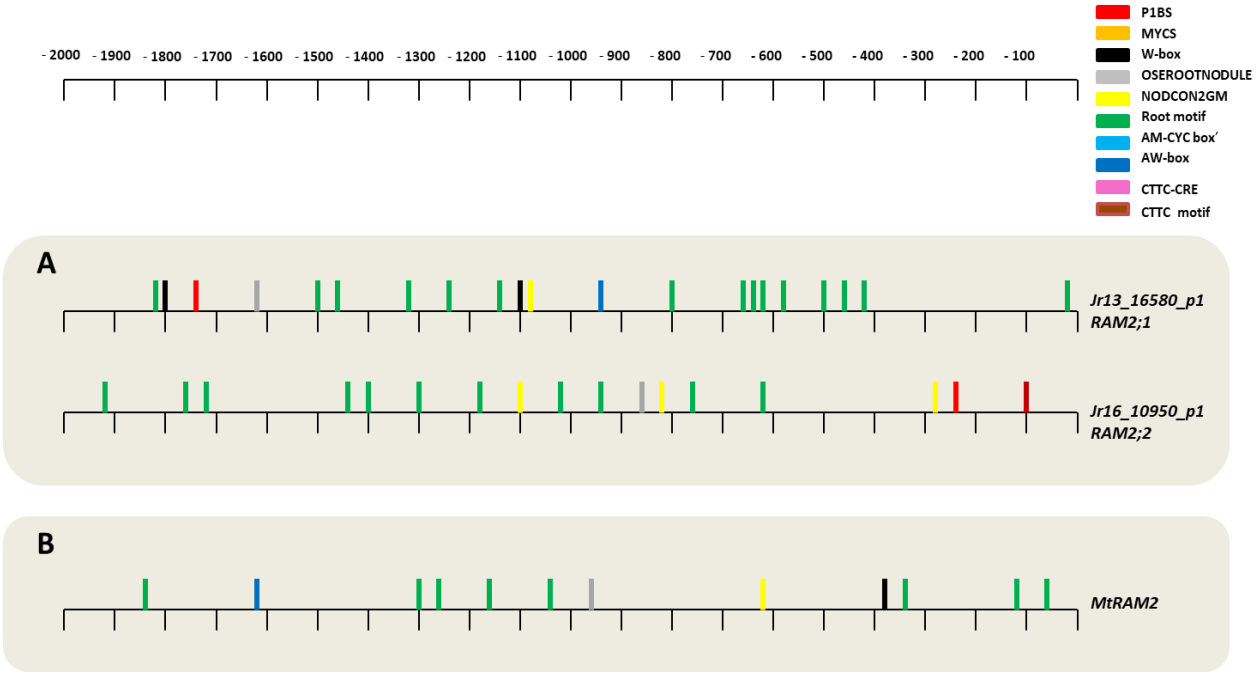

Figure IV. 8. Putative *cis*-regulatory elements involved in Pi and AM responses in the promoters (2-kb upstream regions) of **A**) the *J. regia* glycerol-3-phosphate acyl transferase (*GPAT*) genes orthologous to **B**) the mycorrhiza-specific GPAT-coding gene *MtRAM2* (for REDUCED ARBUSCULAR MYCORRHIZA 2) in *Medicago truncatula*. PIBS, GNATATNC; PHO, CACGTG; W-box, TTGACY; OSEROOTNODULE, AAAGAT; NODCON2GM, CTCTT; root motif, ATATT or AATAT; AM-CYC box, CGGCCG; AW-box, CNTNG(N)7CG; CTTC CRE, CTTCTTGTTC; CTTC motif, TCT[T/C]GTT; mycorrhiza transcription factor binding sequence (MYCS), [T/C][T/G][T/A]CTTGTT[/C/T/G][T/C].

The phylogenetic tree of plant glycerol-3-phosphate acyl transferases proteins (**Figure IV.9**) underlined that the 51 newly identified proteins in *B. distachyon*; *C. clementina*; *C. papaya*; *C. sativus*; *D. carota*; *E. grandis*; *G. max*; *H. annuus*; *J. hindsii*; *J. microcarpa*; *J. nigra*; *J. regia*; *M. acuminata*; *M. domestica*; *O. europaea*; *P. trichocarpa*; *S. bicolor*; *S. esculentum*; *S. tuberosum*; *T. cacao*; *V. vinifera*; and *Z. mays*, clustered with the AM-inducible RAM2 proteins of *L. japonicus* (Keymer et al. 2017), *M. truncatula* (Bravo et al. 2017), and *O. sativa* (Liu et al. 2021).

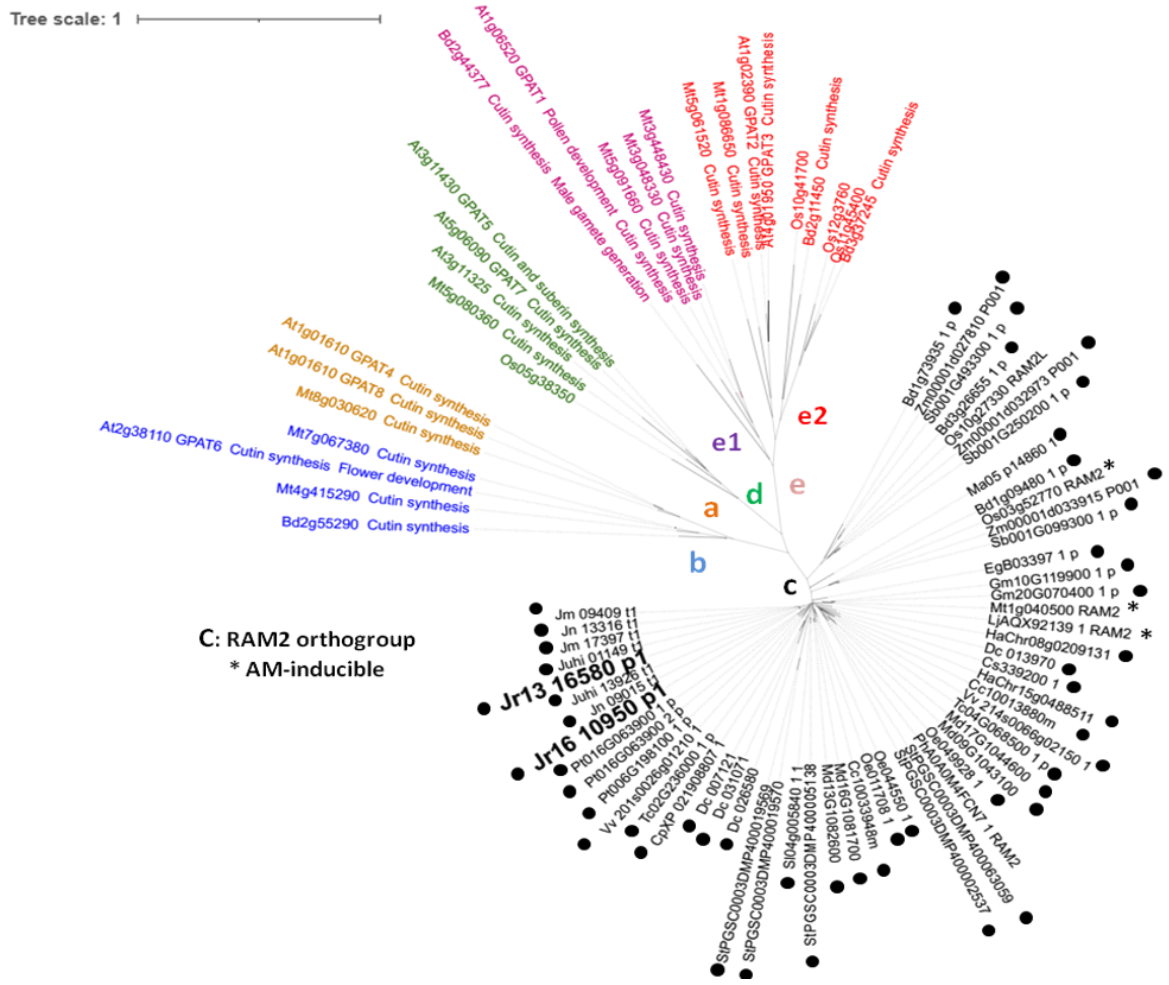

Figure IV.9. Unrooted phylogenic tree for the amino acid sequences of plant glycerol-3-phosphate acyl transferases (GPAT). Monophyletic subclades were named a, b, c, d, e1, and e2 according to Liu (2021). Accessions numbers refer to the protein sequences described by Wang (2012), Rich et al. (2015), Bravo (2017), Keymer (2017), Liu (2021), with the exception of the 51 protein sequences orthologous to the AM-inducible MtGPAT RAM2 identified in this work indicated by circles. Functional annotations were inferred from Uniprot.org. AM-inducible GPAT RAM2 proteins in *L. japonicus* (Keymer et al. 2017); *M. truncatula* (Bravo et al. 2017) and *O. sativa* (Liu et al. 2021) are indicated by asterisks. The two putative proteins of *J. regia* orthologous to the mycorrhiza-essential MRAM2 are indicated in bold dark characters. Abbreviations for plant species: At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon;* Cc: *Citrus clementina*; Cp, *Carica papaya*; Cs, *Cucumis sativus*; Dc, *Daucus carota*; Eg, *Eucalyptus grandis;* Gm: *Glycine max*; Ha, *Helianthus annuus;* Jh, *Junglas hindsii*; Jm, *J. microcarpa;* Jn, *J. nigra*; Jr, *J.regia*; Le, *Lycopersicon esculentum*; Lj, *Lotus japonicus*; Ma, *Musa acuminata* Md, *Malus domestica*; Mt, *Medicago truncatula*; Oe, *Olea europaea*; Os, *Oryza sativa*; Ph, *Petunia hybrida*; Pt, *Populus trichocarpa*; Sb, *Sorgum bicolor*; Sl, *Solanum esculentum;* St, *Solanum tuberosum*; Tc, *Theobroam cacao;* Vv, *Vitis vinifera*; and Zm, *Zea mays*.

3 5. *In silico* characterization of the MtSTR2 orthogroup in *J. regia*

As displayed in **Figure IV. 10**, the two proteins JrSTR2-1 (Jr08_18980_p1) and JrSTR2-2 (Jr11_05690_p1) of *J. regia* found in the MtSTR2 orthogroup, both harboured an ATP-binding cassette (ABC) domain (IPR03439) at the N-terminus and a six possible transmembrane region (IPR01325) at the C-terminus, indicating that they belong to family G of ABC transporters, which is implicated in the transport of lipids (Kerr, Haider, and Gelissen 2011). These two proteins also showed an accurate sequence conservation for localization to the PM with least 60% pair-wise identity and a cut-off expectation value of e^{-40} (Jr08_18980_p1: 71.4%, $e^{0;}$ Jr11_05690_p1: 72.1%, e^{0}) to the peri-arbuscular membrane-resident protein MtSTR2 (Zhang et al.2010).

Figure IV.10. Sequence alignement of *Medicago truncatula* STR2 (MtSTR2; AES95907.1) and the corresponding *Juglans regia* homologues JrSTR2-1 (Jr08_18980_p1), JrSTR2-2 (Jr11_05690_p1). Protein sequences were aligned using Muscle (Madeira et al., 2019) and visualized using MView (Brown et al., 1998). Yellow bars indicate predicted transmembrane segments with TMHMM Server v. 2.0 (Krogh *et al.*, 2001). Typical of family G of ABC transporters, red and pink bars underline the ATP-binding cassette (ABC) domain (IPR03439) at the N-terminus and the six possible transmembrane region (IPR01325) at the C-terminus, respectively.

Figure IV. 11 indicated the presence of root, NODCOM2GM, OSEROOTNODULE, P1BS, W- and AW-boxes motifs in the promoter of both *JrSTR2-1* and *JrSTR2-2*. The presence of MYCS elements and CTTC motifs was exclusive to the promoter of *JrSTR2-1* (*Jr08_18980_p1*).

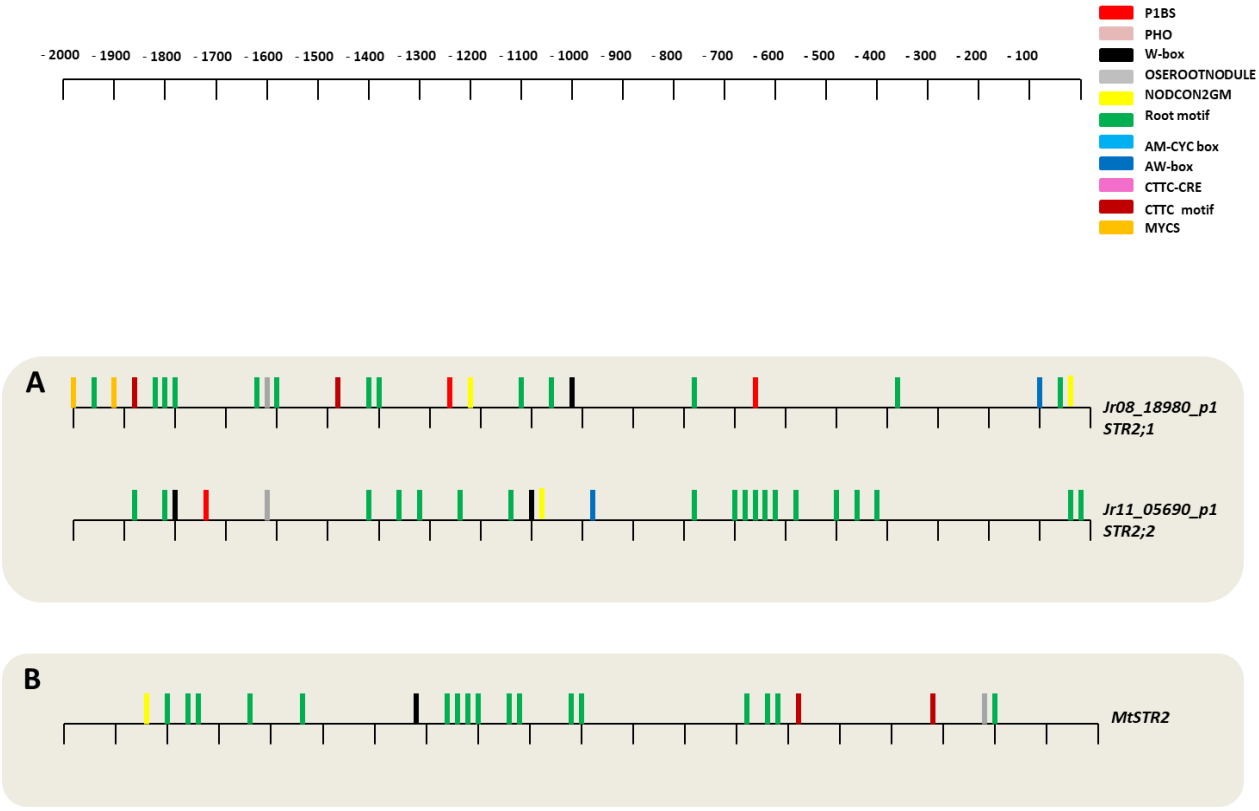

Figure IV. 11. Putative *cis*-regulatory elements involved in Pi and AM responses in the promoters (2-kb upstream regions) of **A**) the *J. regia* half-size ABC transporter *STR2* (for stunted arbuscule 2) genes orthologous to **B**) the mycorrhiza-specific *MtSTR2* in *Medicago truncatula*. PIBS, GNATATNC; PHO, CACGTG; W-box, TTGACY; OSEROOTNODULE, AAAGAT; NODCON2GM, CTCTT; root motif, ATATT or AATAT; AM-CYC box, CGGCCG; AW-box, CNTNG(N)$_7$CG; CTTC CRE, CTTCTTGTTC; CTTC motif, TCT[T/C]GTT;_mycorrhiza transcription factor binding sequence (MYCS), [T/C][T/G][T/A]CTTGTT[/C/T/G][T/C].

Finally, the phylogenetic tree of plant half-ABC transporter-G subfamily (**Figure IV.12**) further underlined that both the JrSTR2-1 and JrSTR2-2 sequences retrieved in this study, together with twelve newly identified proteins in *J. hindsii*; *J. microcarpa*; *J. nigra*; *O. europaea*; *S. esculentum*; *S. tuberosum*; and *V. vinifera* clustered with the AM-inducible STR2 proteins of *O. sativa* (Gutjahr et al. 2012) and *M. truncatula* (Zhang 2010).

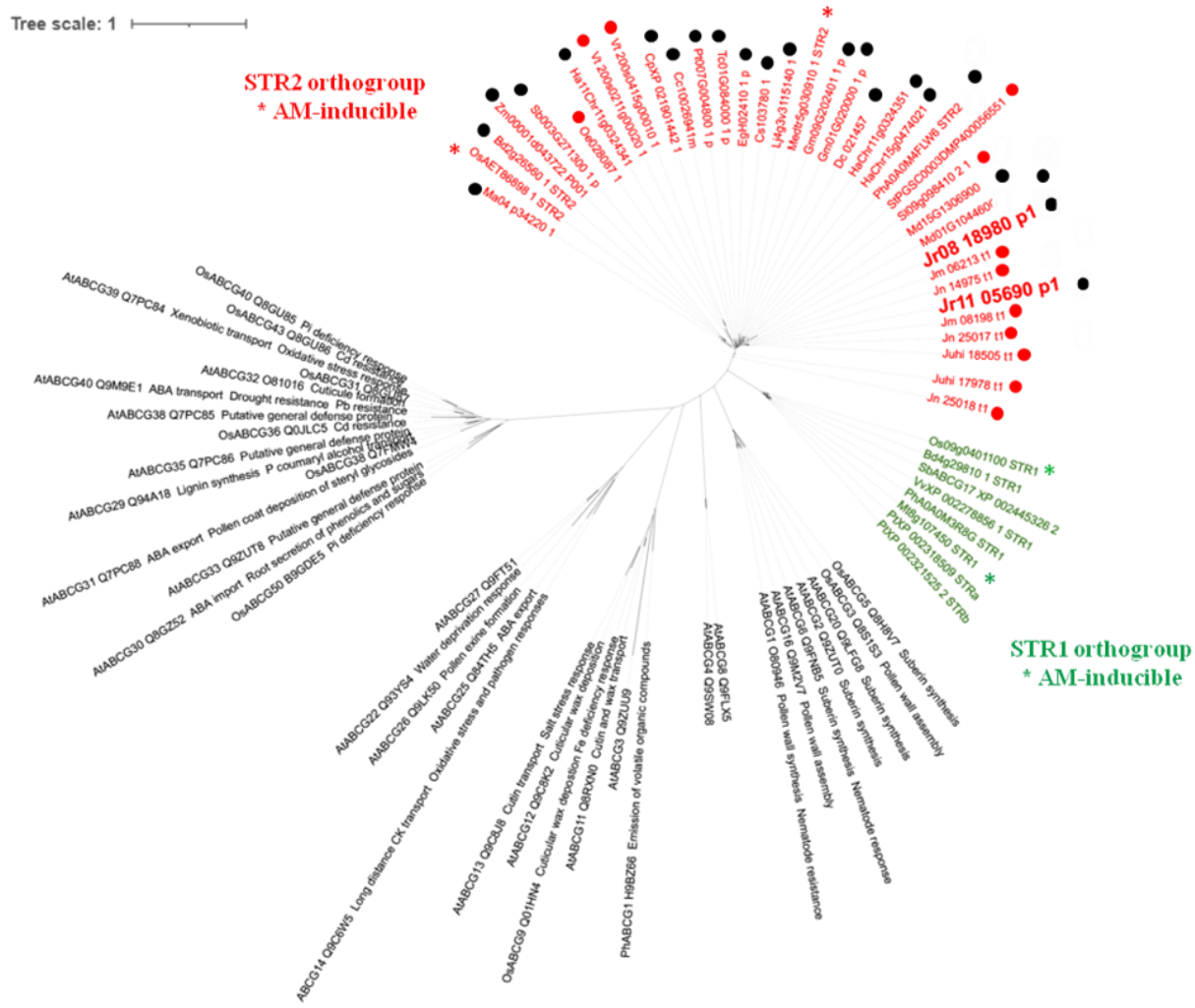

Figure IV.12. Unrooted phylogenic tree generated for the amino acid sequences of plant half-ABC (ATP Binding Cassette) transporter-G subfamily**.** Accessions numbers and annotations refer to the protein sequences described by Zhang (2010), Gutjahr et al. (2012); Nguyen (2015), Rich et al. (2017); Xin and Herburger (2021), with the exception of the 34 protein sequences orthologous to the AM-inducible MtSTR2 identified in this work indicated by circles. Dark circles refer to protein sequences recently described as AM-conserved (Radhakrishnan et al. 2020). AM-inducible STR proteins in *O. sativa* (Gutjahr et al. 2012) and *M. truncatula* (Zhang, Blaylock, and Harrison 2010) are indicated by asterisks. The two putative proteins of *J. regia* orthologous to MtSTR2 are indicated in red bold characters Abbreviations for plant species: At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon;* Cc: *Citrus clementina*; Cp, *Carica papaya*; Cs, *Cucumis sativus*; Dc, *Daucus carota*; Eg, *Eucalyptus grandis;* Gh**,** *Gossypium hirsutu*, Gm: *Glycine max*; Ha, *Helianthus annuus;* Jh, *Junglas hindsii*; Jm, *J. microcarpa;* Jn, *J. nigra*; Jr, *J.regia*; Lj, *Lotus japonicus*; Ma, *Musa acuminata* Md, *Malus domestica*; Mt, *Medicago truncatula*; Oe, *Olea europaea*; Os, *Oryza sativa*; Pt, *Populus trichocarpa;* Sb, *Sorgum bicolor*; Sl, *Solanum esculentum;* St, *Solanum tuberosum*; Tc, *Theobroam cacao;* Vv, *Vitis vinifera*; and Zm, *Zea mays*.

3.6 Nutrition and growth parameters of the walnut rootstock RG17 after *post*-acclimatization as dependent on Pi fertilization and pre-inoculation with *R. irregularis*

After acclimatization, RG17 walnut rootstocks were either pre-inoculated or not with *R. irregularis* and further *post*-acclimatized in growth chamber conditions using two different Pi fertilization regimes. Mycorrhizal root colonization parameters were recorded two months after pre-inoculation (AM) with *R. irregularis* or not inoculated (NM). No trace of fungal structure was observed in the roots of NM plants whatever the Pi supply. In walnut rootstocks inoculated with *R. irregularis*, the results of root fragment staining indicated that the level of Pi fertilization did not significant impact ($p > 0.05$) mycorrhizal colonization parameters. Mean external frequency of mycorrhization of root fragments (F%), the intensity of root cortex colonization (M%) and mean arbuscule abundance (A%), ranged from 88 to 91%, 5.4 to 13.4%, and 4.6 to 9.9%, respectively.

Table IV. 1. Nutrition and growth parameters of the walnut rootstock RG17 after *post*-acclimatization as dependent on Pi fertilization and pre-inoculation with *R. irregularis*. Data represent the mean of at least eight replicates for each treatment (±SD). Different letters indicate statistical differences between groups (p <0.05) calculated as described in the material and methods section. M: mycorrhizal; NM: non-mycorrhizal; DW: dry weight.

	P/10		PTOT		
RG17	M	NM	M	NM	pvalue
Primary root length (cm)	30.1±2.5	39.76±2.2	34.06±1.7	32.12±2.7	0.08
Collar (mm)	5.9±0.3	6.2±0.3	5.1±0.3	5.2±0.2	0.3
Height (cm)	**12.66±2.0 ^{a}**	**6.58±1.2 ^{b}**	**12.1±0.5 ^{a}**	**11.03±1.3 ^{ab}**	**0.03**
Branch number	**11.75±1.3 ^{a}**	**8.71±0.6 ^{ab}**	**7.5±0.8 ^{b}**	**7.33±0.6 ^{b}**	**0.04**
Shoot DW (g)	1.47±0.1	1.1±0.04	1.16±0.3	1.1±0.2	0.26
Root DW (g)	2.83±0.3	2.58±0.3	2.8±0.5	2.29±0.2	0.7
Shoot/root DW ratio	**2.10±0.4 ^{a}**	**0.95±0.2 ^{ab}**	**0.49±0.02 ^{b}**	**0.63±0.02 ^{ab}**	**0.02**
	P/10		PTOT		
RG17	M	NM	M	NM	pvalue
Root Pi (nmole/mg DW)	**18.55±2.1 ^{b}**	**12.85±1.1 ^{c}**	**30.21±1.5 ^{a}**	**26.96±6.5 ^{ab}**	**<0.01**
Leaf Pi (nmole/mg DW)	**40.15±0.8 ^{b}**	**36.55±0.7 ^{b}**	**102.70±13.2 ^{a}**	**98.45±14.2 ^{a}**	**<0.01**
Root N (%DW)	**1.98±0.1 ^{b}**	**1.73±0.1 ^{b}**	**2.47±0.2 ^{a}**	**2.76±0.1 ^{a}**	**<0.01**
Leaf N (%DW)	**2.96±0.1 ^{c}**	**3.068±0.1 ^{bc}**	**3.37±0.1 ^{ab}**	**3.63±0.2c ^{a}**	**0.01**

Upon the comparison of nutritional parameters in the non-inoculated walnut plants between the two different Pi supplies, the data presented in **Table IV.1** underlined a significant ($p < 0.05$) decreased root Pi, leaf Pi, shoot and root N contents in the NM-P/10 condition relative to the NM-PTOT treatment. These results indicate that the reduced Pi availability in the P/10 condition limited the nutrition of the rootstocks RG17. Noticeably, **Table IV.1** showed that AM symbiosis palliated the reduced Pi availability in the P/10 treatment, as inferred from the similar values recorded between the AM-P/10 and NM-PTOT conditions for the root Pi parameter. The morphological traits recorded in **Table IV.1** further highlighted a significantly higher height, branch number, and shoot to root biomass ratio in AM than in NM plants only under the P/10 fertilization regime. In both AM and NM RG17 rootstocks, root N content, root and leaf Pi concentrations were inferior in the P/10 condition relative to the PTOT fertilization regime.

3.7 Changes in RG17 of expression patterns of *JrPHT1, JrFATM, JrRAM2* and *JrSTR2* genes in response to *R. irregularis* inoculation and Pi fertilization

Transcript levels of *JrPHT1, JrFATM, JrRAM2* and *JrSTR2* genes were recorded in RG17 walnut roots either inoculated or not with *R. irregularis* under low and high Pi conditions**. Figure IV.13** showed that expression of *JrPHT1;1* and *JrPHT1;3* was slightly but significantly induced only in response to mycorrhization in the P/10 condition. Expression of *JrPHT1;2* was observed in both AM and NM plants only under the low Pi supply with a basal level in non-inoculated controls (NM-P/10) and a significantly higher transcript abundance in response to mycorrhization (M-P/10). These results indicated that the three *JrPHT1*genes are induced by *R. irregularis* under Pi starvation, with a higher mycorrhizal response recorded for *JrPHT1;2.*

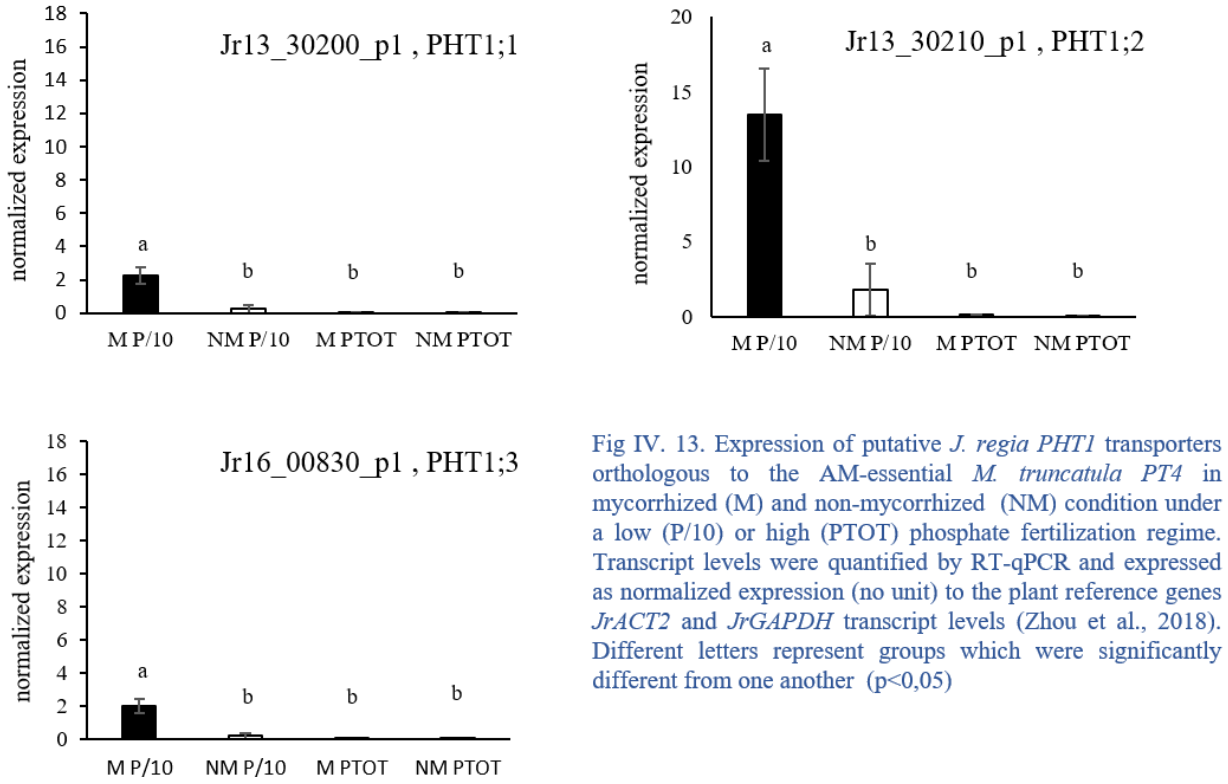

Fig IV. 13. Expression of putative *J. regia PHT1* transporters orthologous to the AM-essential *M. truncatula PT4* in mycorrhized (M) and non-mycorrhized (NM) condition under a low (P/10) or high (PTOT) phosphate fertilization regime. Transcript levels were quantified by RT-qPCR and expressed as normalized expression (no unit) to the plant reference genes *JrACT2* and *JrGAPDH* transcript levels (Zhou et al., 2018). Different letters represent groups which were significantly different from one another ($p<0,05$)

Analysis of transcript levels of *JrFATM* genes in walnut roots either inoculated or not with *R. irregularis* under low and high Pi fertilizations showed no expression of *JrFATM-2* whatever the treatment considered **(Figure IV.14)**. By contrast, *JrFATM-1* was expressed in all conditions to a similarly very slight extent in NMP/10, M-PTOT, and NM-PTOT treatments, but with a significantly higher transcript abundance in response to mycorrhization (M-P/10) relative to controls (NM-P/10). These results indicated that *JrFATM-1* is induced by *R. irregularis* under Pi starvation.

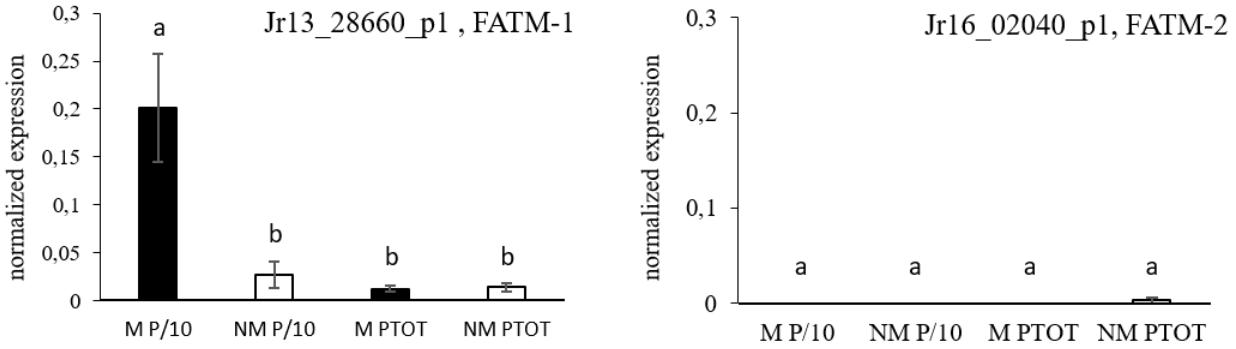

Fig IV. 14. Expression of putative *J. regia* RAM2 orthologous to the AM-essential *M. truncatula* FATM in mycorrhized (M) and non-mycorrhized (NM) condition under a low (P/10) or high (PTOT) phosphate fertilization regime. Transcript levels were quantified by RT-qPCR and expressed as normalized expression (no unit) to the plant reference genes *JrACT2* and *JrGAPDH* transcript levels (Zhou et al., 2018). Different letters represent groups which were significantly different from one another.

Figure IV.15 showed that *JrRAM2;1* is slightly expressed in RG17 walnut roots to similar levels whatever the treatments considered. Expression of *JrRAM2;2* was observed in both M and NM plants only under the low Pi supply, with a basal level in non-inoculated controls (NM-P/10) and a significantly higher transcript abundance in response to mycorrhization (M-P/10). These results indicated that *JrRAM2;2* is induced by *R. irregularis* under Pi starvation.

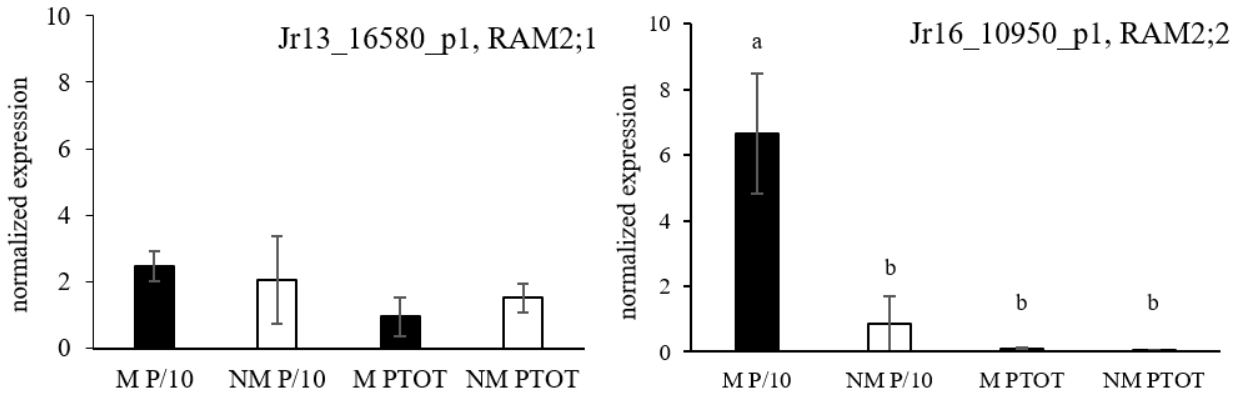

Fig IV. 15. Expression of putative *J. regia* RAM2 orthologous to the AM-essential *M. truncatula* RAM2 in mycorrhized (M) and non-mycorrhized (NM) condition under a low (P/10) or high (PTOT) phosphate fertilization regime. Transcript levels were quantified by RT-qPCR and expressed as normalized expression (no unit) to the plant reference genes *JrACT2* and *JrGAPDH* transcript levels (Zhou et al., 2018). Different letters represent groups which were significantly different from one another

Analysis of transcript abundance of the two *JrSTR2* genes in walnut roots either inoculated or not with *R. irregularis* under low and high Pi conditions showed no expression of both genes under the highest Pi level irrespective of mycorrhization (**Figure IV.16**). By contrast, expression of *JrSTR2;1* and *JrSTR2;2* was observed in both M and NM plants under the low Pi supply, with a basal level in non-inoculated controls (NM-P/10) and a significantly higher transcript abundance in response to mycorrhization (M-P/10). These results indicated that *JrSTR2;1* and *JrSTR2;2* are induced by *R. irregularis* under Pi starvation, with a higher mycorrhizal response recorded for *JrSTR2;1*.

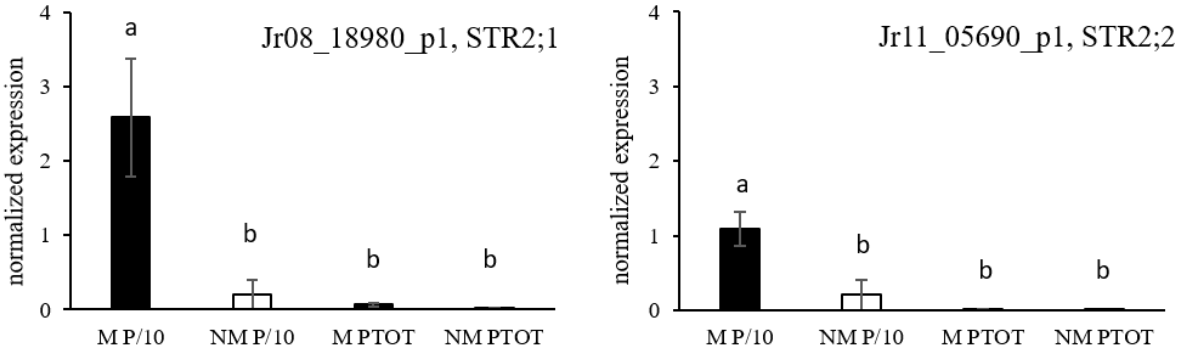

Fig IV. 16. Expression of putative *J. regia* STR2 orthologous to the AM-essential *M. truncatula* STR2 in mycorrhized (M) and non-mycorrhized (NM) condition under a low (P/10) or high (PTOT) phosphate fertilization regime. Transcript levels were quantified by RT-qPCR and expressed as normalized expression (no unit) to the plant reference genes *JrACT2* and *JrGAPDH* transcript levels (Zhou et al., 2018). Different letters represent groups which were significantly different from one another

4. Discussion

Little is known regarding the identity and regulation of AM-essential PHT1 and lipid synthesis/transport proteins in mycotrophic hardwood, fruit and nut bearing trees of agronomic interest. With the aim to fill this gap of knowledge, we first used a comparative approach using Orthofinder to identify evolutionary conserved plant proteins involved in AM symbiosis using the proteome of 25 mycotrophic species including hardwood, fruit and nut-bearing trees such as *Julans regia, J. nigra, J. microcarpa, J. hindsii, Carica papaya, Citrus clementina, Eucalyptus grandis, Malus domestica, Olea europaea, Populus trichocarpa, Theobroma cacao, and V. vinifera* (**Table IV. S1**). This led to the identification of 31 422 orthogroups (OG), which therefore provide a comprehensive resource to mine *M. truncatula* orthologs of biological interest in non-model plant species, including putative AM-essential proteins. Among them were members of the AM-essential phosphate and lipid exchange module, as represented by the Pi transporter MtPT4 OG0008521, the ACP-thioesterase MtFatM OG0009826, the glycerol-3-phosphate acyl transferase MtRAM2 OG0006278, and the half-size ABC transporter MtSTR2 OG0011040. Noticeably, the corresponding phylogenic trees allowed the identification of 26, 37, 51 and 12 previously unknown protein orthologs in different species that all clustered with the AM-required MtPT4, MtFatM, MtRAM2 and MtSTR2, respectively. Taken together, these results suggested that these newly identified proteins might be involved in AM-essential pathways.

To assess this possibility in the English walnut tree *J. regia* selected as a case study of mycotrophic trees ideally suited for alley cropping, we characterized, in a second time, the expression pattern of the retrieved PT4-, FatM-, RAM2- and STR2-like candidates in the walnut rootstock RG17 pre-inoculated with *R. irregularis* under different Pi fertilization regimes. In agreement with the data recorded in the previous study, the level of Pi fertilization did not significant impact mycorrhizal colonization parameters for RG17. In addition, for both AM and NM RG17 rootstocks, inferior root N content, root and leaf Pi concentrations in the P/10 condition relative to the P fertilization were also observed. However, significantly higher shoot to root biomass ratio and root Pi concentration were recorded in AM than in NM plants but only under the P/10 fertilization regime. Increased root Pi concentration along with a lower allocation to root biomass in RG17 AM-P/10 plants relative to the controls (NM-PTOT) therefore suggested that the mycorrhization, for soil Pi absorption (Smith et al. 2011), was only recruited in the lowest Pi supply in order to contribute to the mineral nutrition of the walnut rootstock RG17.

To test for this hypothesis, we further monitored in mycorrhizal and non-mycorrhizal walnut roots gwon under two contrasting Pi fertilization regimes, the expression of the three genes coding protein orthologous to the AM essential PHT1 MtPT4 identified in *J. regia via* Orthofinder. One could observe that *JrPHT1;1, JrPHT1;2 and JrPHT1;3* genes are induced by *R. irregularis* colonization only under Pi starvation. The higher expression of *JrPHT1;2*. than those of *JrPHT1;1* and *JrPHT1;3* suggests that JrPHT1;2 may be an AM-essential Pi transporter in *J. regia* RG17. The expression of *JrPHT1;2* in non-mycorrhizal walnut roots upon the lowest Pi fertilization regime, comforts the idea that *PT4*-like genes also belong to a Pi-sensing machinery at the root tip level, independently of AM fungi, as observed in *L. japonicus* and *M. truncatula* (Volpe et al. 2016). Building on promoter sequence features, the absence in the three *JrPHT1* upstream regions of the mycorrhiza transcription factor binding sequence (MYCS) indicated that the MYCS element may not be essential for the transcriptional induction of *JrPTH1* genes in walnut mycorrhizal roots as previously underlined in maize plants (Liu et al. 2016). This observation also hold true for the *cis*-regulatory elements CTTC (TCT(T/C)GTT) and CTTC CRE (CTTCTTGTTC), along with the W-box that was not detected in the promoter of *JrPHT1;2*. By contrast, the AW-box

(CNTNG(N)$_7$CG) was present in the promoters of *JrPHT1;1, JrPHT1;2 JrPHT1;3, and MtPT4*. This observation suggests the involvement of the transcription factor CBX1 that binds the AW-box, in the transcriptional activation of *PT4*-like genes (Xue et al. 2018; Limpens and Geurts 2018).

Concomitantly to the higher root Pi concentration recorded in AM than in NM plants only under the P/10 fertilization regime, mean biomass values were either similar (root and shoot DW) or higher (plant height) in AM-P/10 walnuts relative to the corresponding NM-P/10 controls. Taken together, these results indicated that plants could acquire sufficient Pi *via* the AM fungus to satisfy their demand for growth. Delegation of Pi acquisition to the AM fungi implies the expansion of extraradical hyphae in Pi-limiting conditions at the expense of plant carbon delivery (Felderer and Homburg 2013; Recorbet et al. 2021).

Because fatty acids are thought to be the major form of carbon transferred from plant hosts to AM fungi (Wang 2017), regulatory inputs that influence *FATM, RAM2* and *STR2* expression might be expected as dependent of the nutritional status of the plant and the Pi delivered by the AM symbiosis. To address this point, the expression of the genes coding protein orthologous to the AM essential MtFATM, MtRAM2 and MtSTR2 were recorded in mycorrhizal and non-mycorrhizal walnut roots under the two contrasting Pi fertilization regimes. In this respect, mycorrhization of RG17 with *R. irregularis* led to substantial changes in expression levels of several genes coding MtFATM, MtRAM2 and MtSTR2 orthologs in *J. regia*, and was dependent on the Pi fertilization regime. Most notably, the expression of *JrFATM-1, JrRAM2;2* and *JrSTR2;1* genes was strongly induced by *R. irregularis* under Pi starvation. All three remained not expressed under the highest Pi supply, irrespective of the mycorrhizal status. To our knowledge, this is the first report concerning the negative impact of Pi fertilization on the expression of genes coding MtFATM, MtRAM2 and MtSTR2 orthologs in mycorrhizal roots. Although the lost of lipid delivery to *R.irregularis* in the form of C16:0 β-monoacylglycerol (Luginbuehl et al. 2017) was not monitored, this finding suggested that in low Pi condition, the increased uptake of Pi by *R.irregularis* is rewarded by an increased lipid delivery from the host to the symbiont, as previously observed for sucrose (Kiers et al. 2011). Consistent with this finding, Konvalinková and Jansa (2016) showed that the carbon flow to AM fungi-signature fatty acid 16:1ὼ5 is reduced by P fertilization in leek and ryegrass. The further analysis of upstream coding regions indicated that compared to *JrFATM-2*, the promoter of the mycorrhiza-inducible *JrFATM-1* under Pi starvation was enriched in two CTTC and one PHO *cis-regulatory* elements (CRE). Likewise, relative to *JrRAM2;1*, the concomitant presence of one CTTC and one PIBS CREs were exclusive to the mycorrhiza-inducible *JrRAM2;2* under Pi starvation. The promoter of the mycorrhiza-inducible *JrSTR2;1* under Pi limitation displayed two MYCS, two CTTC and two P1BS CREs, which were absent in the upstream region of *JrSTR2;2*. Taken together, these results support the possible involvement of the transcription factor CBX1 that also binds the CTTC motif in the transcriptional activation of fatty acid biosynthesis and transport genes (Xue et al. 2018). They also revealed in the promoters of *JrFATM-1, JrRAM2;2* and *JrSTR2;1* recorded as mycorrhiza-inducible genes under Pi starvation, the unsuspected presence of motifs associated with Pi starvation signalling (P1BS, PHO). These findings support the transcriptional activation of *JrFATM-1, JrRAM2;2* and *JrSTR2;1* in response to Pi limitation.

In summary, we performed a comparative proteomic study that allowed to identify previously unknown protein orthologs as essential candidates to the AM symbiosis establishment. Differential gene expression analyses carried out in mycorrhizal and non-mycorrhizal walnut RG17 rootstocks grown under two contrasting Pi fertilization regimes led to the identification of *JrPHT1-, JrFATM-, JrRAM2, and JrSTR2*-like genes expressed in RG17 in response to *R. irregularis* colonization only under Pi starvation. *In silico* promoter analysis supports the possible involvement

of CBX1 in the transcriptional activation of mycorrhizal-essential Pi transport and fatty acid biosynthesis genes. In addition motifs associated with Pi starvation signalling were identified, which agree with the transcriptional activation of *JrFATM-1, JrRAM2;2* and *JrSTR2;1* in response to Pi limitation. Overall, we demonstrated that *FATM, RAM2* and *STR2* expression in mycorrizal-walnut plants is dependent on the Pi fertilization regime. Further lipidomic and transcriptomic studies will help to assess the extent to which Pi starvation is linked to host lipid delivery to the AM fungus.

Table IV.S1. List of the 25 proteomes analysed in this study and phylogenetic relationships between studied plant species

Clade	Class	Sub-class	**Order**	***Genus species*** (Common name)	**Assembly location**
Angiosperms	Eudicots	Rosids	Brassicales	*Carica papaya* (Papaya tree)	https://www.hardwoodgenomics.org/sites/default/files/sequences/pawpaw/carica_papaya_protein.clean.fasta
			Malvales	*Theobroma cacao* (Cacao tree)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Tcacao/Tcacao_523_v2.1.protein.fa.gz
			Sapindales	*Citrus clementina* (Clementine tree)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Cclementina/Cclementina_182_v1.0.protein.fa.gz
			Myrtales	*Eucalyptus grandis* (Fooded gum)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Egrandis/Egrandis_297_v2.0.protein.fa.gz
			Malpighiales	*Populus trichocarpa* (California poplar)	https://www.hardwoodgenomics.org/Genome-assembly/1919890/All gene models proteins
			Fabales	*Glycine max* (Soybean)	http://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Gmax/Gmax_508_Wm82.a4.v1.protein.fa.gz
				Medicago truncatula (Barrel medic)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Mtruncatula/Mtruncatula_198_protein.fa.gz
				Lotus japonicus (Birdsfoot trefoil)	https://lotus.au.dk/data/download/Lotusjaponicus_Gifu_v1.1_proteins
			Cucurbitales	*Cucumis sativus* (Cucumber)	https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Csativus/Csativus_122_v1.0.protein.fa.gz
			Fagales	*Juglans nigra* (Eastern black walnut),	https://treegenesdb.org/FTP/Genomes/Juni/v1.0/annotation/juni.1_0.peptides.fa
				Juglans regia (English walnut)	https://treegenesdb.org/FTP/Genomes/Jure/v2.0/annotation/Jure.2_0.pep.fa.gz
				Juglans hindsii (Northern California walnut)	https://www.hardwoodgenomics.org/sites/default/files/sequences/juglans_hindsii/Juhi.1_0.peptides.fa
				Juglans microcarpa (Texas walnut)	https://treegenesdb.org/FTP/Genomes/Jumi/v1.0/annotation/jumi.1_0.peptides.fa
			Rosales	*Malus domestica* (Apple tree)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Mdomestica/Mdomestica_491_v1.1.protein.fa.gz

			Vitales	*Vitis vinifera* (Grapevine)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Vvinifera/Vinifera_457_v2.1.protein.fa.gz
		Asterids	Apiales	*Daucus carota* (Wildcarrot)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Dcarota/Dcarota_388_v2.0.protein.fa.gz
			Asterales	*Helianthus annuus* (Common sunflower)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Hannuus/Hannuus_494_r1.2.protein.fa.gz
			Lamiales	*Olea europaea* (Olive tree)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Oeuropaea/Oeuropaea_451_v1.0.protein.fa.gz
			Solanales	*Solanum lycopersicum* (Tomato)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Slycopersicum/Slycopercum_514_ITAG3.2.protein.fa.gz
				Solanum tuberosum (Irish potato)	https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Stuberosum/Stuberosum_44_v4.03.protein.fa.gz
	Monocots	Commelinids	Poales	*Zea mays* (Corn)	ftp://ftp.ensemblgenomes.org/pub/release-43/plants/fasta/zea_mays/pep/Zea_mays.B73_RefGen_v4.pep.all.fa.gz
				Sorghum bicolor (Sorghum)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Sbicolor/Sbi.4pep.fa.gz
				Brachypodium distachyon (Purple false brome)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Bdistachyon/B distachyon_314_v3.1.protein.fa.gz
				Oryza sativa (Asian rice)	https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Osativa/Osativa_204.v7.0. protein.fa.gz
			Zingiberales	*Musa acuminata* (Edible banana)	https://banana-genome-hub.southgreen.fr/sites/banana-genome-hub.southgreen.fr/files/data/fasta/version2/musa_acuminata_v2_prot.faa

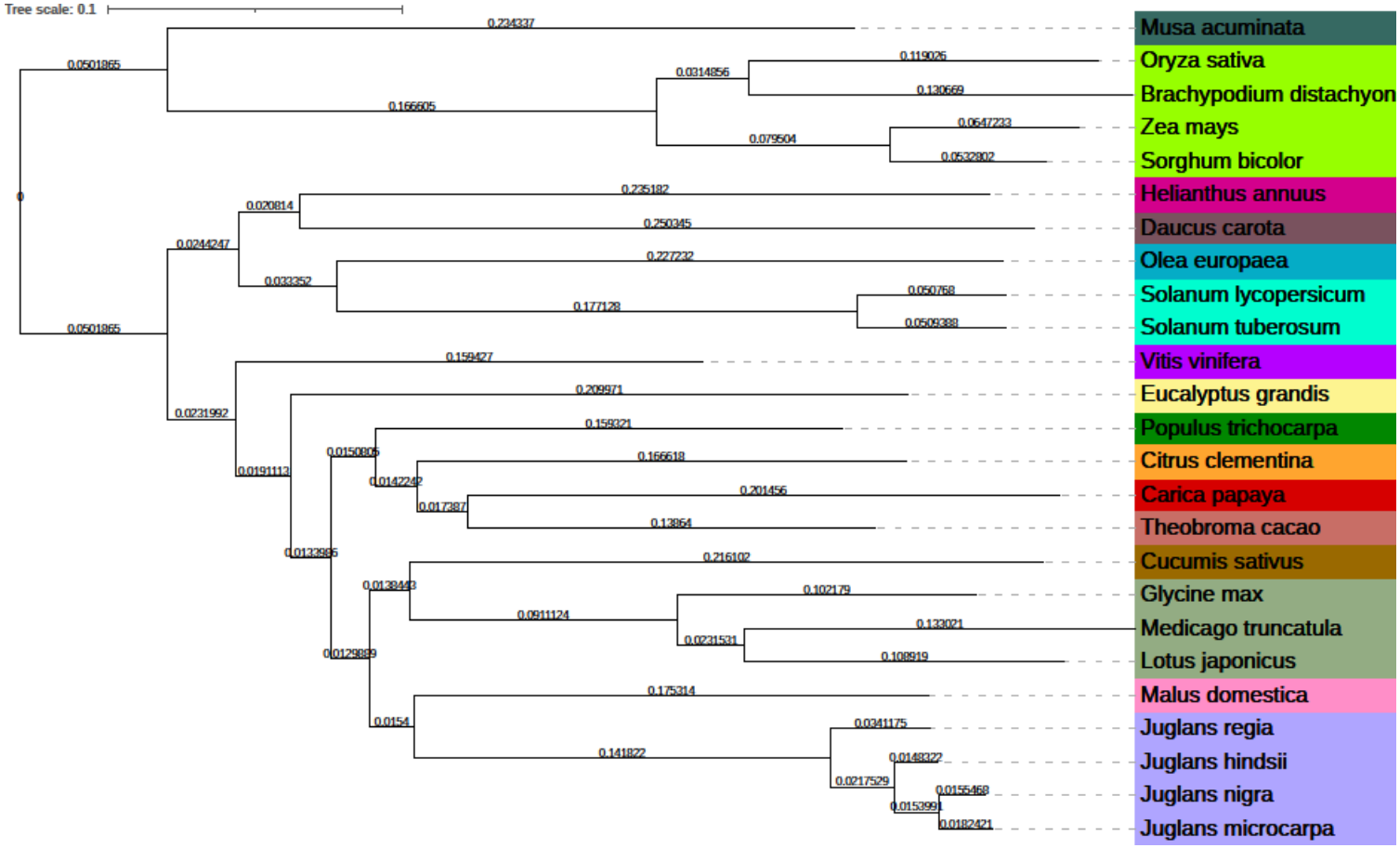
Tree scale: 0.1
Musa acuminata
Oryza sativa
Brachypodium distachyon
Zea mays
Sorghum bicolor
Helianthus annuus
Daucus carota
Olea europaea
Solanum lycopersicum
Solanum tuberosum
Vitis vinifera
Eucalyptus grandis
Populus trichocarpa
Citrus clementina
Carica papaya
Theobroma cacao
Cucumis sativus
Glycine max
Medicago truncatula
Lotus japonicus
Malus domestica
Juglans regia
Juglans hindsii
Juglans nigra
Juglans microcarpa

Table IV.S2. Primers used in this study

Oligoname	Code	Primers	Sequence	Transcript ID/Reference
J. regia				
JregGAPDHrefF	*1F*	Forward	ATCATGGGTAAAGACCCCGC	XM_018984056.1/Zhou et al. 2018 https://doi.org/10.1371/journal.pone.0209424
JregGAPDHrefR	*1R*	Reverse	TCAGTGACCGCATCCTTAGC	
JregACT2refF	*2F*	Forward	ATGGTCCCAAACATGACCCA	XM_018959949.1/Zhou et al. 2018 https://doi.org/10.1371/journal.pone.0209424
JregACT2refR	*2R*	Reverse	TGTTGGAGGAGCTTGTGCAG	
JregPT4-28771F	*3F*	Forward	TCGAGAAGCTGGGTCGTTTC	Jure_28771.t1=Jr16_00830_p1/This study
JregPT4-28771R	*3R*	Reverse	TTCAGGAGTGGTCCGAGTCA	
JregPT4-23100F	*5F*	Forward	GTTGCCCTTGTTGGTACCCT	Jure_23100.t1=Jr13_30200_p1/This study
JregPT4-23100R	*5R*	Reverse	AGCATATGGCGCAAAACACC	
JregPT4-23099F	*6F*	Forward	CCTGGCAGTAAGCCTGGAAA	Jure_23099.t1= Jr13_30210_p1 /This study
JregPT4-23099R	*6R*	Reverse	AGTTTATCACCCAGCCAGCC	
JregRAM2-09746F	*8F*	Forward	TTGCCGAACTAACCGACCAA	Jure_09746.t1=Jr16_10950_p1/This study
JregRAM2-09746R	*8R*	Reverse	AGGAGAGGACAGTGAAGGCTG	
JregRAM2-09371F	*9F*	Forward	GTGATCGCCGCAACTCTTTC	Jure_09371.t1=Jr13_16580_p1/This study
JregRAM2-09371R	*9R*	Reverse	GCTGGCTTCTCAACCACAGT	
JregSTR2-25696F	*10F*	Forward	CACAATCCACCAGCCCTCAT	Jure_25696.t1=Jr11_05690_p1/This study
JregSTR2-25696R	*10R*	Reverse	TACTGACACCTCCTCCCCTG	
JregSTR2-06098F	*11F*	Forward	CACAATCCACCAGCCCTCAT	Jure_06098.t1=Jr08_18980_p1/This study
JregSTR2-06098R	*11R*	Reverse	TCAGACCCGTAAGCGCAAAT	
JregFatM-25029F	*12F*	Forward	CAAGGCGCCTCTCCAAAATG	Jure_25029.t1=Jr16_02040_p1/This study
JregFatM-25029R	*12R*	Reverse	CAAGGCGCCTCTCCAAAATG	
JregFatM-13680F	*13F*	Forward	GGTGAGCGTGGAAGTGAAGA	Jure_13680.t1=Jr13_28660_p1/This study
JregFatM-13680R	*13R*	Reverse	AAATTTCTGGGACGGGGGAC	

Table IV.S3. FASTA sequences used for the phylogenetic analysis of plant PHT1 proteins.

>AtPT1 (NP_199149.1, Tabata et al. 2000; PUBMED 11130714)
MAEQQLGVLKALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCVSLVTKLLGRIYYFNPESAKPGSLPPHV
AAAVNGVALCGTLSGQLFFGWLGDKLGRKKVYGLTLVMMILCSVASGLSFGHEAKGVMTTLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGVGILAGGFVALAVSSIFDKKFPAPTYAVNRAL
STPPQVDYIWRIIVMFGALPAALTYYWRMKMPETARYTALVAKNIKQATADMSKVLQTDIELEERVED
DVKDPKQNYGLFSKEFLRRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPKAATMNATHEVFRI
ARAQTLIALCSTVPGYWFTVAFIDTIGRFKIQLNGFFMMTVFMFAIAFPYNHWIKPENRIGFVVMYSLTF
FFANFGPNATTFIVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSQDKAKVDAGYPPGIGVKN
SLIMLGVLNFIGMLFTFLVPEPKGKSLEELSGEAEVSHDEK

>AtPT2 (NP_001190462.1, Tabata et al. 2000; PUBMED 11130714)
MAEQQLGVLKALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCVSLVTKLLGRIYYFNPESAKPGSLPPHV
AAAVNGVALCGTLSGQLFFGWLGDKLGRKKVYGLTLIMMILCSVASGLSFGNEAKGVMTTLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGVGILAGGFVALAVSSIFDKKFPAPTYAVNRAL
STPPQVDYIWRIIVMFGALPAALTYYWRMKMPETARYTALVAKNIKQATADMSKVLQTDIELEERVED
DVKDPRQNYGLFSKEFLRRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPKAATMNATHEVFRI
ARAQTLIALCSTVPGYWFTVAFIDTIGRFKIQLNGFFMMTVFMFAIAFPYNHWIKPENRIGFVVMYSLTF
FFANFGPNATTFIVPAEIFPARLRSTCHGISAAAGKAGAIIGAFGFLYAAQNQDKAKVDAGYPPGIGVKN
SLIVLGVLNFIGMLFTFLVPEPKGKSLEELSGEAEVSHDEK

>AtPT3 (NP_199150.1, Tabata et al. 2000; PUBMED 11130714)
MADQQLGVLKALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCVSLVTKLLGRLYYFNPTSAKPGSLPPH
VAAAVNGVALCGTLAGQLFFGWLGDKLGRKKVYGITLIMMILCSVASGLSLGNSAKGVMTTLCFFRF
WLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGVGILAGGFVALAVSSIFDKKFPSPTYEQDRF
LSTPPQADYIWRIIVMFGALPAALTYYWRMKMPETARYTALVAKNIKQATADMSKVLQTDLELEERVE
DDVKDPKKNYGLFSKEFLRRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPKAATMNAIHEVF
KIARAQTLIALCSTVPGYWFTVAFIDIIGRFAIQLMGFFMMTVFMFAIAFPYNHWILPDNRIGFVVMYSL
TFFFANFGPNATTFIVPAEIFPARLRSTCHGISAATGKAGAIVGAFGFLYAAQPQDKTKTDAGYPPGIGV
KNSLIMLGVINFVGMLFTFLVPEPKGKSLEELSGEAEVDK

>AtPT4 (NP_181428.1, Lin et al. 1999, PUBMED 10617197
MAREQLQVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVEGAQKPGTLPPNV
AAAVNGVAFCGTLAGQLFFGWLGDKLGRKKVYGMTLMVMVLCSIASGLSFGHEPKAVMATLCFFRF
WLGFGIGGDYPLSATIMSEYANKKTRGAFVSAVFAMQGFGIMAGGIFAIIISSAFEAKFPSPAYADDALG
STIPQADLVWRIILMAGAIPAAMTYYSRSKMPETARYTALVAKDAKQAASDMSKVLQVEIEPEQQKLE
EISKEKSKAFGLFSKEFMSRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQSMNAIQEVFKI
ARAQTLIALCSTVPGYWFTVAFIDVIGRFAIQMMGFFFMTVFMFALAIPYNHWTHKENRIGFVIMYSLTF
FFANFGPNATTFVVPAEIFPARFRSTCHGISAASGKLGAMVGAFGFLYLAQNPDKDKTDAGYPPGIGVR
NSLIVLGVVNFLGILFTFLVPESKGKSLEEMSGENEDNENSNNDSRTVPIV

>AtPT5 (NP_180842.1, Lin et al. 1999, PUBMED 10617197)
MAKKGKEVLNALDAAKTQMYHFTAIVIAGMGFFTDAYDLFSISLVTKLLGRIYYHVDSSKKPGTLPPN
VAAAVNGVAFCGTLAGQLFFGWLGDKLGRKKVYGITLMLMVLCSLGSGLSFGHSANGVMATLCFFRF
WLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIVSLIVSSTFDHAFKAPTYEVDPVG
STVPQADYVWRIVLMFGAIPALLTYYWRMKMPETARYTALVARNTKQAASDMSKVLQVDLIAEEEEAQ
SNSNSSNPNFTFGLFTREFARRHGLHLLGTTTTWFLLDIAYYSSNLFQKDIYTAIGWIPAAETMNAIHEVF
TVSKAQTLIALCGTVPGYWFTVAFIDILGRFFIQLMGFIFMTIFMFALAIPYDHWRHRENRIGFLIMYSLT
MFFANFGPNATTFVVPAEIFPARLRSTCHGISAASGKAGAIVGAFGFLYAAQSSDSEKTDAGYPPGIGVR
NSLLMLACVNFLGIVFTLLVPESKGKSLEEISREDEEQSGGDTVVEMTVANSGRKVPV

>AtPT6 (NP_199148.1, Tabata et al. 2000; PUBMED 11130714)
MANEEQGSILKALDVAKTQWYHVTAVVVSGMGFFTDSYDLFVISLITKLLGRIYYQVPGSSSPGSLPDGI
SAAVSGVAFAGTFIGQIFFGCLGDKLGRKRVYGLTLLIMTICSICSGLSLGRDPKTVMVTLCFFRFWLGF
GIGGDYPLSATIMSEYSNKRTRGAFIAAVFGMQGIGILAAGAVSLLVSAVFESKFPSRAYILDGAASTVP
QADYVWRIILMVGALPALLTYYWRMKMPETARYTALVSKNAEQAALDMTKVLNVDIEASAAKNDQA
RVSSDEFGLFSMKFLRRHGLHLLGTASTWFLLDIAFYSQNLFQKDIFTTIGWLPSAKTMNAIQELYMIAK
AQTIIACCSTVPGYFFTVGFIDYMGRKKIQIMGFAMMTIFMLSLAIPYHHWTLPANRIGFVVLYSFTFFFS
NFGPNATTFIVPAEIFPARIRSTCHGISAASGKAGAMVGSFGFSALVKALGMSNTLYIMAGINLLGLLLTF
TIPETNGKSLEELSGETEPEKIKEKIVV

>AtPT7 (NP_001319749.1, Salanoubat et al, 2000; PUBMED 11130713)
MAGDQLNVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGSEKPGTLPPNV
SAAVNGVAFCGTLAGQLFFGWLGDKLGRKKVYGMTLMVMVLCSIASGLSFGSNPKTVMTTLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILTGGIFAIIVSAAFEAKFPAPTYQIDALAST
VPQADYVWRIILMVGALPAAMTYYSRSKMPETARYTALVAKDAKLAASNMSKVLQVEIEAEQQGTED
KSNSFGLFSKEFMKRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQTMNAIQEVFKIARAQ
TLIALCSTVPGYWFTVAFIDVIGRFAIQMMGFFFMTVFMFALAIPYDHWTHKENRIGFVAMYSLTFFFA
NFGPNATTFVVPAEIFPARFRSTCHGISAASGKLGAMVGAFGFLYLAQSPDKTKTEHGYPPGIGVKNSLI
VLGVVNLLGMVFTLLVPESKGKSLEEMSGENEQNDESSSSSNNNSNNAVSTA

>AtPT8 (NP_001323235.1, Theologis et al. 2000, PUBMED 11130712)
MPIKVLSSLDVARTQWYHFKAIIVAGMGLFTDAYDLFCIAPVMKMISHVYYNGDSINTAVLSTSYAIAL
LGTATGQLVFGYLGDRVGRRRVYGLCLIIMILSSFGCGFSVCTTRRSCVMVSLGFFRFFLGLGIGGDYPL
SATIMSEFANKRTRGAFIAAVFSMQGLGILVSSAVTMAVCVAFKRSGGGLEVDAAAPTEADLAWRLIL
MIGALPAALTFYWRMLMPETARYTALVENNIVQAAKDMQRVMSRSHISDEATTDPPPPPPPSYKLFSR
CFFRLHGRDLFAASFNWFLVDIVFYTSNLLLSHIFSHYSKKPSTAENVYDAAFEVAELGAIIAACSTIPGY
WFTVYFIDKIGRVKIQIMGFFFMAVIYLVAGIPYSWYWSKHEHNNKGFMVLYGLVFFFCNFGPNTTTFII
PAEHFPARFRSTCHGISGAAGKLGAIVGTVGFLWATKKMESDDKNQIYPEVNRMRIAFLILGGVCIAGIL
VTYFFTKETMGRSLEENEHDQDNNAESEDEPQIVDGQSSVSTLLQTR

>AtPT9 (NP_177769.1, Theologis et al. 2000, PUBMED 11130712)
MPELSLLSALDAARIQWYHFKAIIVAGMGLFTDAYDLFCIAPIMKMISQIYYHKDSIGTALLSTSYAIALL
GTALGQLIFGYLGDRVGRRKVYGLSLLIMVFSSFGCGFSVCTTRRSCVMVSLGFFRFVLGLGIGGDYPLS
ATIMSEFANKRTRGAFIAAVFSMQGLGILMSSAVTMVVCLAFKNAGEGSSEKTNVAGLETLAPPESDIA
WRLILMIGALPAALTFYWRMLMPETARYTALVENNVVQAAKDMQRVMSVSMISQITEDSSSELEQPPS
SSSYKLFSRRFLSLHGRDLFAASANWFLVDVVFYTSNLLLSQIFNFSNKPLNSTNVYDSAFEVAKLAAIV
AACSTIPGYWFTVYFIDKIGRVKIQMMGFFLMAVVYLVAGIPYSWYWSKHEKTNKGFMVLYGLIFFFS
NFGPNTTTFIIPAELFPARFRSTCHGISGAAGKFGAIVGTVGFLWATRHHEEDGFPDVKRVRIAFLILGGV
CIAGMIVTYLFTRETMGRSLEENEDEIVSTSAGSSPANELLRRQY

>BdPT7 (XP_003569484.1, Hong et al. 2012, PUBMED 22711284)
MAENESGGQNLAVLEALDSARTQMYHMKAIVIAGMGFFTDAYDLFCISTVSKLLGRIYYPLQNIDQGK
PGTLPVNVNNMVIGVALVGTLMGQLVFGYYGDKLGRKRVYGITLVLMAACAIGSGLSFGYTHRAVIG
TLCFFRFLLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGVGIIFAGLVSMIVSGLFLHYNPAPT
WSKVDPTLSNQTPAADYVWRIVLMIGAFPALATFYWRMKMPETARYTAIIEGNAKQASNDMQKVLEI
QIDDEQDKLAKFRAANEYPLLSKEFARRHGMHLIGTTTTWFLLDIAFYSQNLTQKDIFPAINLTDPPETM
NALKEMFVISRAMFLVALLGTFPGYWVTVAVIDKMGRYLIQLLGFFMMSVFMLVMGVKYEYLKDHN
HLLFAVLYALTFFFANFGPNSTTFVLPAELFPTRVRSTCHAISAASGKAGAIVAAFGVQRLTLKGDVKNI
TRALIILSVTNMLGFFFTFLVPETMGRSLEEISGEDGNTGNGAGAGAVSMSAADVSKDGKFPASSTEWQ
QPSMQA

>EcPT1 (AJD86034, Pudake et al. 2017, PUBMED 28391483)
MAGGQLKVLTTLDQAKTQWYHFMAIVIAGMGFFTDAYDLFCISLVSKLLGRLYYSEPNSPNPGSLPPN
VSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVVCSVASGLSFGHTAKGVIATLCFFRFW
LGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGILFGAIVALVVSSGFRNSYPAPSYEQNATAS
LVPEADFVWRIILMFGTIPAALTYYWRMKMPETARYTALIARNAQHAAANMSQVLNTEIVEDKDQAEL
ASASAGNNEWGLFSPQFLRRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSKVGWIPPPKTMNAIEEVFR
IARAQALIALCGTIPGYWFTVFFIDIVGRFAIQLMGFFMMTVFMLGLAVPYHHWTTAGHHTGFVVMYG
FTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAAGKAGAIIGAFGFLYAAQDPHKPEAGYSPGIGIRNA
LFVLAGTNFLGMIMTLLVPESKGLSLEEISKETVDDEEAA

>EcPT2 (AJD86035.1, Pudake et al. 2017, PUBMED 28391483)
MAGGQLNVLSTLDQAKTQWYHFLAIVIAGMGFFTDAYDLFCIALVTRLLGRIYYTDPTKPDPGSLPPNV
SAAVTGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVLCSMASGLSFGHTPKSVIGTLCFFRFWL
GFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILFGTIVALVVSGAFRNAYPAPAYYIDAKAS
LVPQADFMWRVILMFGTVPAALTYYWRMKMPETARYTALIARNTKQAAADMSKVLHKDIQEDEQVE
REVVATGDTWGLFSKQFLRRHGLHLLATTTTWFLLDIAFYSQNLFQKDIFTKVGWIPAGRTMNAIEEVF
RISRAQALIALRGTVPGYWFTVAFIDIIGRFWIQLMGFLMMTVFMIALAVPYEHWT

>EcPT3 (AJD86036.1, Pudake et al. 2017, PUBMED 28391483)
MARGGGDNLQVLSALDAAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGRIYYTDTSKPDPGSL
PPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLMLMVICSIASGLSFGHTPTGVIATLCFF
RFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIVTLIISAAFRTAYPAPAYHVDA

AASIVPQADFVWRIILMLGALPALLTYYWRMKMPNCGYTALVAKNAKQAAADMSKVLQPRSWTSRR
KLDDLVTRSATAAGLFSQEFARRPGCTYGTASTWFLLDIAFYSQNLFQKDIFTAINWIPKARTMSALEEV
FRISRAQTLIALCGTVPGYWFTVALIDIVGRFAIQLMGFFMMTVFMLGLAVPYHHWTTAGNHIGFVVM
YGFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAAGKAGAIIGSFGFLYAAQDPDH

>EcPT4 (AJD86037.1, Pudake et al. 2017, PUBMED 28391483)
MAESGGGANLAVLEALDSARTQMYHMKAIVIAGMGFFTXAYDLFCITTVSXLLGRLYYSEEGASKPGT
LPQRVNNMVIGVALVGTLIGQLVFGYFGDKLGRKRVYGITLVLMAACAIGSGLSFGKSPRAVMGTLCF
FRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGVGIIFAGLVSMIVSGIFLHYNPAKSFKED
PAGSTQQAADYMWRIVLMIGAVPALATFYWRMKMPETARYTALIEGNAKQAATDMTNVMEIQIDAE
QEKLAMFKPPNDYPLLSWEFARRHGMHLIGTATTWFLLDIAFYSQNLTQKDIFPAIHLTDKAENVNALQ
EVFQISKAMFLVALFGTFPGYWVTVALIDKMGRYLIQLIGFFMMSVFMMVMGVMYESLRTKQAVLFA
LLYXLTFFFANGPNSTTFVLPAELFPTRVRSTCHAISAASGKAGAIVAAYGVQSLTLNGQVKDIKKALIIL
SITNMLGFFFTFLVPETMGRSLEEISGEDGNAGIGGGASPGPAAGPGMDMSRDDKIPVSGTEWQSSMHA

>GmPT1 (NP_001239971.1, Fan et al. 2013, PUBMED 23510338)
MARDQLQVLNALDVAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYFEGHDKPGSLPSNV
SAAINGVAFCGTLAGQLFFGWLGDKMGRKRVYGMTLMLMVICSIASGLSFGKDPKAVMATLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGTVAIVVSSAFKALYPAPAFQVNPVL
STVPQADYVWRIILMFGALPALLTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVEIEAEQEK
VEQLDTRKGNEFGLFTKQFLRRHGLHLLGTAVTWFLLDIAYYSQNLFQKDIFSTIGWIPEAKTMNAIEEV
FKIARAQTLIALCSTVPGYWFTVALIDKMGRFTIQLMGFFFMTVFMFALAIPYHHWTMKGNQIGFVVLY
SLTFFFANFGPNATTFVPAEIFPARLRSTCHGISAAAGKAGAMVGAFGYLYTQNAIGLRNTLIVLGVVNF
LGLLFTFLVP

>GmPT2 (NP_001240239.1, Fan et al. 2013, PUBMED 23510338)
MAGGQLGVLNALDVAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRLYYTDITNPKPGVLPPN
VQAAVTGVALCGTLAGQLFFGWLGDKLGRKRVYGLTLMLMVVCSVASGLSFGDTPKGVMATLCFFR
FWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIVALIVSSAYDHKYDLPSYKDNP
AGSKVDSLDYVWRIILMFGAVPAGLTYYWRMKMPETARYTALVAKNAKQAASDMSKVLQVEVEAEE
DKLQHMVESEHQKYGLFSKEFAKRHGLHLVGTTVTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQDMNAI
HEVYRIARAQTLIALCSTVPGYWFTVAFIDIMGRFAIQLMGFFFMTVFMFALAIPYNHWKNHNNIGFVV
MYSFTFFFSNFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSTNPDKVDHGYPT
GIGVKNSLIVLGVINFFGMVFTLLVPESKGKSLEELSGENEDDGAEAIEMAGSARTVPV

>GmPT3 (NP_001241164.1, Fan et al. 2013, PUBMED 23510338)
MAGELGVLNALDVAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRLYYTEPGAPKPGSLPPRV
QASVTGVALCGTLAGQLFFGWLGDKMGRKRVYGLTLILMVVCSLASGLSFGSTPEGVMASLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGVVALIVSYAYDQKYKLPSYEQNPEA
SLDPAFDYVWRIVLMFGAVPAALTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVELEAEEE
KVKKLTENESNKYGLFTKEFVKRHGLHLLGTTTTWFLLDIAFYSQNLFQKDIFSAIGWIPPAKEMNAIHE
VYKIARAQTLIALCSTVPGYWFTVALIDYMGRFAIQLLGFFFMTVFMFALAIPYDHWSEKENRIGFVVM
YSFTFFFANFGPNSTTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSKDPSKTDAGYPTGI
GIKNSLIMLGVINFIGMLFTLLVPESKGKSLEELSGENGENDAEHAVSARTVPV

>GmPT4 (NP_001304639.2, Fan et al. 2013, PUBMED 23510338)
MAREQIQVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGAAKPGTLPPNV
SAAVNGVAFCGTLSGQLFFGWLGDKMGRKKVYGMTLMLMVICSIGSGLSFGHSAKSVIATLCFFRFWL
GFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILAGGIFAIIISVAFKERFDAPPYELDPAGSTV
AQADYIWRIIVMVGALPAALTYYWRMKMPETARYTALVAKNTKQAAADMSKVLQVEIQAEPQKEEQ
KANSYGLFSKEFLRRHGLHLLGTASTWFLLDIAFYSQNLFQKDIFSAIGWIPPAKTMNAIEEVYRIARAQ
TLIALCSTVPGYWFTVALIDKIGRFAIQLMGFFFMTVFMFALAIPYDHWTHKDNRIGFVVIYSLTFFFANF
GPNATTFVVPAEIFPARFRSTCHGISSASGKLGAIVGAFGFLYLAQNKDKSKADAGYPAGIGVKNALIVL
GVVNILGFFFTFLVPEANGKSLEEMSGENDEDVGTQEESEQSHSHNNNRTVPYV

>GmPT5 (NP_001304588.2, Wang et al. 2019, PUBMED 31412775)
MGKEQVQVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGAAKPGSLPPN
VSAAVNGVAFVGTLSGQLFFGWLGDKMGRKKVYGMTLALMVIASIASGLSFGHDAKTVMTTLCFFRF
WLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILAGGVFAIIIASVFKSKFDSPPYEVDPLGS
TVPQADYVWRIILMFGAIPAAMTYYSRSKMPETARYTALVAKNMEKAAADMSKVMNMEIQAEPKKE
EEAQAKSYGLFSKEFMSRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAKTMNALEEVFFIA
RAQTLIALCSTVPGYWFTVAFIDRIGRFAIQLMGFFFMTIFMFALAIPYDHWTLRENRIGFVVIYSLTFFF
ANFGPNATTFVVPAEIFPARFRSTCHGISSASGKLGAMVGAFGFLYLAQNQDPSKADAGYPAGIGVRNS

LLVLGVINILGFMFTFLVPEAKGRSLEEISGEQEEETKV

>GmPT6 (NP_001240032.1, Fan et al. 2013, PUBMED 23510338)
MAGGQLGVLNALDVAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRLYYTDIRNPKPGVLPPN
VQAAVTGVALCGTLAGQLFFGWLGDKLGRKRVYGLTLMLMVLCSIASGLSFGDTPKGVMATLCFFRF
WLGFGIGGDYPLSATIMSEYANKKTRGSFIAAVFAMQGFGIMAGGIVALIVSSAYDHKYDLPSYKDNPA
GSKVDSLDVWRIILMFGAVPAALTYYWRMKMPETARYTALVAKNAKQAASDMSKVLQVEVEAEEDK
LQHMVESENQKYGLFSKEFAKRHGLHLVGTTVTWFLLDIAFYSQNLFQKDIFTAIGWIPPAQDMNAIHE
VYRIARAQTLIALCSTVPGYWFTVAFIDIIGRFAIQLMGFFFMTVFMFALAIPYNHWKNHNNIGFVVMYS
FTFFFSNFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSTNPNKVDHGYPTGIG
VKNSLIVLGVINFFGMVFTLLVPESKGKSLEELSGENEDDGAEAIEMAGSARTVPV

>GmPT7 (NP_001239765.1, Chen et al. 2019, PUBMED 30317659)
MGFFTDAYDLFCITAVIKLIGRLYYYDPNSHSPGKLPKNVNNAITGVALCGTLAGQLFFGWLGDKLGRK
RVYGITLVTMVGCALASGLSFGSTAKSVVASLCFFRFWLGFGIGGDYPLSAVIMSEYANQKTRGAFVA
AVFAMQGVGILVAGGVAMLVSKLFLFAYPARIFAEDAVQSTQPEADFVWRIVLMFGAFPAALTYYWR
MKMPETARYTALVEGDHKKAVEDMAKVLDNDIPLEESNARVAATPGPSYGFFSSKFLEKHGLHLLGTT
STWFLLDIAFYSLQLTQKDFYPASGLVPKDSRMNAIEEVFLLSKAMFTVALFATVPGYWCTVYFIDKIG
RYKIQLVGFFVMSVCMWILGRKYGEYRGVDCSSDDRLEYCDGNLPMFIILFGLTLFFANFGPNSTTFIVP
AELFPARFRSTCHGISAAAGKAGAIIGAFVVQSYTDNAEDKIKGMKKALMTLSVVNFLGFFCTFLVPET
RGRSLEEISGEDKELPGNITQDNANETTTDKQKGTKNSNVEKLPETLMV

>GmPT8 (NP_001345390.1, Fan et al. 2013, PUBMED 23510338)
MARLKVLSALDTARTQYYHFKAIIIAGMGLFTDAYDLFSITLIIKIIGRVYYGHREGENRYETPPEVVSAL
VAVALLGTAVGQLVFGRLGDLKGRRRVYGFALLLMVFSSLASGFSICIRKTCVLLTLGFFRFFLGLGIGG
DYPLSSTIMSEFANKKTRGSFIAAVFSMQGFGILASSTVTMAVCSIFGAASKNSEADVAWRLILMLGSVP
AAMTYYWRMMMPETARYTALVEQNVMQAAKDMEKVLDVTLSQIAEEDPLPPTPHPYPLLSWEFLRR
HGPDLFACSSTWFLVDIVFYSQVLFQSEIYKRYLDKKEDVDVYQETFHAAWIQAVIAVCSTIPGYFFSM
YFIDKWGRVKIQMMGFFFMALAFFSIGIPYYSYWTTKEHHKNKVFMVLYGLAFFFANFGPNTTTFIVPA
ELFPARFRSSCHGISGAVGKVGAIIGSVGFLWASHRKKEDGYPKGIGMKVSLIILGGVCLLGMVITYFFT
RETMGRSLEENEVEIEEQDNL

>GmPT9 (NP_001241574.1, Fan et al. 2013, PUBMED 23510338)
MLWFKMARLKVLSTLDTSRTQYYHFKAIIIAGMGLFTDAYDLFSITLIIKIIGRVYYDHREGEHRYETPPE
VVSALVAVALLGTAVGQLVFGRLGDLKGRRRVYGFSLLLMVFSSLASGFSICRRKTCVLLTLGFFRFFL
GLGIGGDYPLSSTIMSEFANKKTRGSFIAAVFSMQGFGILASSTVTMAVCSIFRAASKNSEADLAWRLIL
MLGSVPAAMTYYWRMMMPETARYTALVEQNVMQAAKDMEKVLDVTLSQIAEEHPLPPTPHPYPLLS
REFLRRHGRDLFACSSTWFLVDIVFYSQVLFQSEIYKRYLDKKEEVDVYQETFHVAWIQAVIAVCSTIPG
YFFSVYFIDKWGRVKIQMMGFFFMALAFFAIGIPYYSFWTTEDHNMNKGFMVLYGLAFFFANFGPNTT
TFIVPAELFPARFRSTCHGISGAVGKVGAIIGSVGFLWASHKKKENGYPKGIGMEVTLIILGVVCLLGML
VTYLFTRETMGRSLEENEVESVNHEVEEHDNL

>GmPT10 (NP_001241400.1, Fan et al. 2013, PUBMED 23510338)
MALEVLEALDSARTQWYHVTAIVIAGMGFFTDAYDLFCISTVSKLLGRLYYFDPSTPTVPGKLPLNVNN
MVTGVALVGTLSGQLVFGWLGDKLGRKKVYGITLILMVFCAICSGLSFGATAKSVMGTLCFFRFWLGF
GIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGVGIIFAGLVSMALSGIFKYYYPAPAYIDNPVLSTQP
EGDLLWRLVLMIGSVPAMLTYYWRMKMPETGRYTAIIEGNVKQAAADMAKVLDIEIQAEQDKLAEFN
ANNNYPLWSNEFFKRHGRHLIGTMSSWFLLDIAFYSQNLTQKDIFPAVGLIHKDFEMDAIREVFETSRA
MFVIALLGTFPGYWFTVFFIEKIGRYKIQLIGFFMMSFFMFIIGVKYDYLKNEGKGYFALLYGLTFFFANF
GPNSTTFVLPAELFPTRVRSTCHALSAAAGKAGALVGTFGIQSLTVGGQSYKIKKVMIILAVTNLLGFFS
SFLVTETKGRSLEEISGEDGRESELTPTPNNRIQD

>GmPT11 (NP_001241127.1, Fan et al. 2013, PUBMED 23510338)
MALEVLEALDSARTQWYHVTAIVIAGMGFFTDAYDLFCISTVSKLLGRLYYFDPSTPNLPGKLPLSVNN
MVTGVAIVGTLTGQLIFGWLGDKLGRKKVYGITLILMVICAICSGLSFGATPKSVMGTLCFFRFWLGFGI
GGDYPLSATIMSEYANKRTRGAFIAAVFAMQGVGIIFAGLVSMTLSAIFKHYYPAPAYINDPVLSTQPEG
DLLWRLVLMIGAVPAMMTYYWRMKMPETGRYTAIIEGNAKQAAADMAKVLDIEIQAEQDKLAEFNA
SNNYPLWSNEFFQRHGRHLIGTMSSWFLLDIAFYSQNLTQKDIFPAIGLIDKDYQMDAIKEVFETSRAMF
VIALLGTFPGYWFTVFFIEKIGRYKIQLIGFFMMSFFMFVIGVKYDYLKNEGKGYFALLYGLTFFFANFG
PNSTTFVLPAELFPTRVRSTCHALSAAAGKAGALVGVFGIQCLTVGGESYKIKKVMIILAVTNLLGFFSS
FLVTETKGRSLEEISGEDGRESELTPTPNNRVQGSRTETM

>HvPT1 (AAN37900.1, Rae et al. 2003, PUBMED 14756304)
MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGRIYYTDPALNEPGHLPAN
VSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVLCSIASGLSFGHEAKGVMGTLCFFRFW
LGFGVGGDYPLSATIMSEYANKKTRGTFIAAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAASIG
PEADYVWRIIVMFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEEAGQGERAT
GDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFTKIGWIPPAKTMNALEELYRIARA
QALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTMMTIFMLAIAIPYDYLVKPGNHTGFVVLYGLTFFFA
NFGPNSTTFIVPAEIFPARLRSTCHGISAATGKAGAIIGAFGFLYASQDQKKPETGYSRGIGMRNALFVLA
GTNFLGLLFSLLVPESKGKSLEELSKENVGDDGIDA

>HvPT2 (AAO72434.1, Smith, F.W., Cybinski, D.H. and Rae, A.L. (1999) Regulation of expression of genes encoding phosphate transporters in barley roots. In: *Plant Nutrition – Molecular Biology and Genetics. Proceedings of the Sixth International Symposium on Genetics and Molecular Biology of Plant Nutrition, Elsinore, Denmark.* (G. Gissel-Neilsen and A. Jensen eds) Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 145– 150)
MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGRIYYTDPALNEPGHLPAN
VSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVLCSIASGLSFGHEAKGVMGTLCFFRFW
LGFGVGGDYPLSATIMSEYANKKTRGTFIAAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAASIG
PEADYVWRIIVMFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEEAGQGERAT
GDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFTKIGWIPPAKTMNALEELYRIARA
QALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTMMTIFMLAIAIPYDYLVKPGNHTGFVVLYGLTFFFA
NFGPNSTTFIVPAEIFPARLRSTCHGISAATGKAGAIIGAFGFLYASQDQKKPETGYSRGIGMRNALFVLA
GTNFLGLLFSLLVPESKGKSLEELSKENVGDDDAIAPTGV

>HvPT4 (AAO72437.1, Smith, F.W., Cybinski, D.H. and Rae, A.L. (1999) Regulation of expression of genes encoding phosphate transporters in barley roots. In: *Plant Nutrition – Molecular Biology and Genetics. Proceedings of the Sixth International Symposium on Genetics and Molecular Biology of Plant Nutrition, Elsinore, Denmark.* (G. Gissel-Neilsen and A. Jensen eds) Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 145– 150)
MARSEQQGLQVLSALDAAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGRIYYTDLSKPDPGSL
PPSVAAAVNGVAFCGTLAGQLFFGWLGDKMGRKSVYGMTLLLMVICSIGSGLSFAHTPKSVMATLCFF
RFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIVTLIISSAFRAGFHEPAYQDDR
VASTGTEADFVWRIILMLGALPALLTYYWRMKMPETARYTALVAKNAKLAAADMSKVLQVELEDET
EKMDEMVSRGANDFGLFSPQFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFTSINWIPKARTMSAL
DEVFRISRAQTLIALCGTVPGYWFTVFLIDVVGRFAIQLMGFFMMTVFMLGLAVPYHHWTTPGNQIGFV
VMYGFTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAMIGAFGFLYAAQDPHKPDAGYRP
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>HvPT5 (AAO72435.1, Smith, F.W., Cybinski, D.H. and Rae, A.L. (1999) Regulation of expression of genes encoding phosphate transporters in barley roots. In: *Plant Nutrition – Molecular Biology and Genetics. Proceedings of the Sixth International Symposium on Genetics and Molecular Biology of Plant Nutrition, Elsinore, Denmark.* (G. Gissel-Neilsen and A. Jensen eds) Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 145– 150)
MASRQQQQLQVLSALDGAKTQLYHFRAVVVAGMGFFTDAYDLFCISLVTKLLGRIYYADPSSPNPGSL
PPNVAAAVNGVALCGTLAGQLFFGWLGDRLGRKSVYGMTLILMVVCSVASGLSFGHTPASVMATLCF
FRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGVVTLVLSTVFRSAFPAPAYQT
DAAASTVPQADYVWRIILMLGALPAALTYYWRTKMPETARYTALVAKNAKKASLDMSKVLQSEVEA
EPEKLDEIMARGEDYGLLTSRFAKRHGLHLLGTATAWFLVDVAYYSQNLFQKDIFGSIGWIPKARTMD
ALEEVFRISRAQTLIALCGTVPGYWFTVFLIDVIGRFWIQLVGFAMMAVFMLGLAVPYHHWTTPGNHV
GFVVMYGLTFFFANFGPNATTFIVPAEIFPARLRSTCHGISAAAGKAGAIIGAFGFLYAAQSPDLAHVDA
GYKPGIGVQKALYVLAACNLLGFLVTFLVPESKGKSLEEMSGEADAEEGNGANNVRPSGEQLV

>HvPT6 (AAN37901.1, Smith, F.W., Cybinski, D.H. and Rae, A.L. (1999) Regulation of expression of genes encoding phosphate transporters in barley roots. In: *Plant Nutrition – Molecular Biology and Genetics. Proceedings of the Sixth International Symposium on Genetics and Molecular Biology of Plant Nutrition, Elsinore, Denmark.* (G. Gissel-Neilsen and A. Jensen eds) Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 145– 150)
MAREQLEVLSALDTAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYYREGADAPGSLPPNV
AAAVNGVAFCGTLSGQLFFGWLGDRMGRKRVYGMTLMCMVLCSIASGLSFGSTPGSVMATLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILTGGVVTLIVSAAFRAAFHAPAYEKGAVA
STPPQADFVWRFILMFGAVPALLTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVEIAAEDET
KDNDGAGEDRNSFGLFSGEFLRRHGLHLLGTATCWFLLDIAFYSQNLFQKDIFTAINWIPKAKTMSALE
EVHRIARAQTLIALCGTVPGYWFTVALIDRIGRFWIQLGGFFFMAVFMLGLAFPYHHWTTPGNHIGFVV

LYALTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAAGKLGAIVGSFGFLYLAQNQDPSKVDHGYKA
GIGVKNSLFILAACNFLGMAFTFCAPESNGISLEELSGENDDEAPAPATHARTVPV

>HvPT7 (AAO72436.1, Smith, F.W., Cybinski, D.H. and Rae, A.L. (1999) Regulation of expression of genes encoding phosphate transporters in barley roots. In: *Plant Nutrition – Molecular Biology and Genetics. Proceedings of the Sixth International Symposium on Genetics and Molecular Biology of Plant Nutrition, Elsinore, Denmark.* (G. Gissel-Neilsen and A. Jensen eds) Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 145– 150)
MAGDQVHVLAALDGAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLIGRIYYTVPGSPSPGSLPPTV
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GFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGILAGGGVAIGITALFRALFPAPPYAADPVAST
PDQADYVWRIVLMLGALPAALTFYWRMKMPETPRYTALIAKNAERAAADMSKVLNVEITKEQAGDLE
TAISIKSHTSPSFGLFSREFMRRHGLHLLGTASTWLLLDIAYYSQNLFQKDIFSAIGWIPPAPTMSALDEL
YHIARAQILIALCGTVPGYWFTVAFIDSVGRFKIQLMGFFMMTAFMLGLAGPYDYWTGQGHQVGFVV
MYALTFFFANFGPNATTFIVPAEIYPARFRATCHGISAASGKVGAIIGSFGFLYLAQSPDPAKTAHGYHPG
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>HvPT8 (AAO72440.1, , Smith, F.W., Cybinski, D.H. and Rae, A.L. (1999) Regulation of expression of genes encoding phosphate transporters in barley roots. In: *Plant Nutrition – Molecular Biology and Genetics. Proceedings of the Sixth International Symposium on Genetics and Molecular Biology of Plant Nutrition, Elsinore, Denmark.* (G. Gissel-Neilsen and A. Jensen eds) Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 145– 150)
MARQQLQVLHALDVARTQRYHAWAVVIAGMGFFADAYDIFCITLVTKLLGRIYYHVPGQPDPGMLPR
RIEAAINGVTFCGMIVGQLLFGWLGDKVGRKMFYGKTIMLMIMGSFLSGLSFGNTADGVMATLCFFRF
WLGVGIGGDYPLSATIISEYSNKRSRGSLIAAVFAMEGFGILAGCIVTLVVSATFQARFNPPAYEEDPMA
SVPPQADYVWRIILMVGAIPAVFTYRWRVMMPETARYTALVARDAEKAARDMSKVLKVEFTGEQDKI
ESFTRDRDYGVFSRRFARRHGWHLVGAVASWFVLDIVFYSQIILQEEIFRDVKWIPEARTMSALEEAYR
VARGQAIIALCGTLPGYWFTVAFVDVVGRKAIQFLGFTMMKGLMLVVAAFYHHLTQPGRRIWLVVMY
AFTFFFANFGPNSTTFIIPAEIFPAHVRTTCHGISSAAGKVGAIVGTFGFLYASQRADGSNEVKSGYPSGIG
VRASLFVLAACNVLGIIFTCLLPEPNGRSLEEVSGEPINREDADLGDSRVLPL

>HvPT9 (AM904733.1, Huang et al. 2011, PUBMED 21606317)
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VSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVLCSIASGLSFGHEAKGVMGTLCFFRFW
LGFGVGGDYPLSATIMSEYANKKTRGTFIAAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAASIG
PEADYVWRIIVMFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEEAGQGERAT
GDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFTKIGWIPPAKTMNALEELYRIARA
QALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTMMTIFMLAIAIPYDYLVKPGNHTGFVVLYGLTFFFA
NFGPNSTTFIVPAEIFPARLRSTCHGISAATGKAGAIIGAFGFLYASQDQKKPETGYSRGIGMRNA

>Jr13_30210_p1 This study, KAF5451116.1, Marrano et al. 2020, https://doi.org/10.1093/gigascience/giaa050)
MALAVLDALDSARTQWYHITAIIIAGMGFFTDAYDLFCISTVSKLLGRLYYAPGSKPGKLPHAVNNAVV
GVALVGTLTGQLFFGWLGDKLGRKKVYGLTLVIMVVCAICSGLSFGTTTAGSVITTLCFFRFWLGFGIG
GDYPLSATIMSEYANKTTRGAFIAAVFAMQGVGIIFAGLVSMILSKIFLSYYETVPFWDDHFLSVEREGD
YLWRIVLMIGAVPALLTYYWRMKMPETGRYTALIEGNAKQAAADMGRVLDLEIQAEQEKLSQFKAAN
SYPLLSMEFYNRHGLHLIGTTSTWFLLDIAFYSQNLTQKDIFPAMNLIKKDIDLSALREVFETSRAMFVL
ALLGTFPGYWFTVFFIEKLGRFKIQLMGFFMMSAFMLIIGVKYDYLKDHGTLFAALYGLTFFFANFGPN
STTFVLPAELFPTRLRSTCHAISAAAGKAGAMVGAFGVANYTLDGQAKDIKKAMMFLAVTNMLGFFC
TFLVTETKGRSLEEISGEDAGGNETQSSARQLPGANRTAGTHGEAM

> Jr13_30200_p1. This study, XP_018847378.1 Marrano et al. 2020, https://doi.org/10.1093/gigascience/giaa050)
MALDVLDALDSARTQWYHITTIVIAGMGFFTDAYDLFCISTVSKLLGRLYYYDPSTGKPGKLPHTVNNA
VVGVALVGTLTGQLVFGWLGDKLGRKKVYGVTLIIMVFCAICSGLSFGSSTQSVIGTLCFFRFWLGFGI
GGDYPLSATIMSEYANKKTRGTFIAAVFAMQGVGIVFAGLVSLILSKIFLDSCEKVGTFQEEHLFSTQPQ
GDYLWRIVLMIGAVPALLTYYWRMKMPETGRYTAIIEGNAQQAAADMGRVLDLEIQAQHDKLSQFKA
ANSYPLLSKEFLRRHGVHLIGTTTTWFLLDIAFYSQNLTQKDIFPAMGLIKKDVEMNALREVFETSRAM
FVIALLGTFPGYWFTVFFIEKMGRFMIQLIGFFMMSAFMLIIGVKYDYLKLHGSLFAVLYGLTFFFANFG
PNSTTFVLPAELFPTRVRSTCHALSAAAGKAGAIVGAFGVQNYTLDGNQDKIRKAMIFMAFTNILGFFFT
FCVVETKGRSLEEISGEDGGGNNTQGSTGVVGMTQITTGTHREYSM

>Jr16_00830_p1. This study, XP_035542482.1, 1 Marrano et al. 2020, https://doi.org/10.1093/gigascience/giaa050)
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VALFGTFPGYWFTVYFIEKLGRFTIQLVGFFMMSASMLIMGLQYQYLKNHGTTFAALYGLTFFFANFGP
NSTTFVLPAELFPTRLRSTCHALSAAAGKAGAMVGAFGVQNYTLDGNVNDIKKAMIFLAFTNMLGFFF
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>LbPT1 (AIU41746.1, Hu et al. 2017, DOI: 10.1093/treephys/tpw125)
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STVPQADFVWRIILMFGAIPAGMTYYWRMKMPETARYTALVAKNLKQAANDMSKVLQVEIEAEPEKV
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RIARAQTIIALCSTVPGYWFTVVFIDRIGRFAIQLMGFFFMTVFMFALAIPYHHWTLKDNRIGFVVMYSL
TFFFANFGPNATTFVVPAEIFPARLKSTCHGISAAAGKARAMVGAFGFLYAAQPQDPKKTDAGYPAGIG
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>LbPT2 (AIU41747.1, Hu et al. 2017, DOI: 10.1093/treephys/tpw125)
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YSLTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGVYGFLYAAQNKDPTKTDAGYPA
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>LbPT3 (AIU41748.1, Hu et al. 2017, DOI: 10.1093/treephys/tpw125)
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GFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILTGGIVALVVAGAFKNAYPSPIYSVNAKDS
TPPEADLVWRIILMFGAIPALLTYYWRMKMPETARYTALVAKNASKAAADMSKVLNVEIEAEKDKIEK
IVEEKGNNFGLFTKQFLRRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSKIGWIPHPETMNALDEVFKIA
RAQTLIALCGTVPGYWFTVAFIDKIGRFAIQLMGFFFMTVFMFALAIPYNHWTKKENRIGFVIMYSLTFF
FANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSTNPLKVDVGYPTGIGVKN
TLLVLGCVNLLGMLFTLLVPESKGKSLEEMSKENEGEEQNYGTETKGENAQTVPV

>LbPT4 (AIU41749.1, Hu et al. 2017, DOI: 10.1093/treephys/tpw125)
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AMFVIALFGTFPGYWFTVFFIEKIGRFRIQLMGFFMMSVFMAIIGIKYDYLRTEDHKWTLATLYGLTFFF
ANFGPNSTTFVLPAELFPTRVRSTCHALSAASGEAGAMISAFGIQQYTQDGSVKKIKKAMILLAVTNMV
GFCCTFLVTETKGRSLEEISGEDGRQNETQMKTSKPVSGHHDDGWE

>LbPT5 (AIU41750.1, Hu et al. 2017, DOI: 10.1093/treephys/tpw125)
MNSDNLTVLNALDTARTQWYHVTAVIIAGMGFFTDAYDLFCITTVSKLLGRLYYYDPTTHAPGKLPHN
VNNWITGIALVGTLTGQLVFGWLGDKLGRKKVYGLTLILMVICAISSGLSFGYSRKIVIGTLCFFRFWLG
FGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGVGIIFAGLVSMIVSKLFLMNYAGGAFKTDEVFST
EPEADYVWRIVLMLGALPALLTYYWRMKMPETGRYTAIIEGNAKQAAIDMGKVLEIEIQEDGEKLAKF
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MFVIALLGTFPGYWFTVFFIEKIGRFKIQLMGFFMMSVFMAIIGIKYDYLRNKEHKWTFAALYGLTFFFA
NFGPNSTTFVLPAELFPTRVRSTCHALSAASGKAGAMVSAFGVQQYTQDGNVHKIKQAMMFLAFTNMI
GFCCTFLVTETKGRSLEEISGEDEKQDEMQTKSTRPDSGRQDDGWD

>LbPT7 (AIU41751.1, Hu et al. 2017, DOI: 10.1093/treephys/tpw125)
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TVPQADYVWRIILMVGALPALLTYYWRLKMPETARYTALVAKNIQQATADMEKVVHVDIGTEKKEPA
ISSTTVIKSSNEFGLFTKQFVSRHGLHLLGTTSTWFLLDIAYYSQNLFQKDIFSAVGWIPAAKTMNAIEEV
YKIARAQTFIALCSTVPGYWFTVFLIDRIGRFTIQLLGFSMMTIFMFALAIPYDHWTHPGNHIGFVVLYSL
TFFFANFGPNATTFVVPAEIFPARLRPTCHGISAASGKLGAMVGAFGFLYLAQPQDKTRADAGYPAGIG
VRNSLIVLGVVNLLGTFFTFLVPESNGKSLEEMSRENDDSTEAEAEEENHSSDNRTVPV

>LePT1 (CAA74607.1, Daram et al. 1998, PUBMED 9737001)
MANDLQVLNALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCISMVTKLLGRLYYHHDGALKPGSLPPNV
SAAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLMIMVICSIASGLSFGHTPKGVMTTLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGMVAIIVSAAFKGAFPAPAYEVDAIGS
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KIARAQTLIALCSTVPGYWFTVAFIDKIGRFAIQLMGFFFMTVFMFALAIPYHHWTLKDHRIGFVVMYSF
TFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAMVGAFGFLYAAQPTDPTKTDAGYPPGHW
CEELVDRPWLCNFLGMLFTFLVPESNGKSLEDLSRENEGEEETVAEIRATSGRTVPV

>LePT2 (NP_001234043.1, Liu et al. 1998, PUBMED 9449838
MAVGDNDNNNLQVLNALDLAKTQLYHFTAIVIAGMGFFTDAYDLFSISLVTKLLGRLYYTKPDLLKPG
TLPPAVSASVTGVALVGTLAGQLFFGWLGDKMGRKKVYGMTLVLMVVCSVASGLSFGSTPKGVMTT
LCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILFSGIVALITAAGFDHAYRSPTF
EENAALSTVPQSDYIWRIILMFGSLPAALTYYWRMKMPETARYTALVAKDAKRAAQDMGKVLQVEIE
SEEAKIEQISRNETNQFGLFSWEFVRRHGLHLFGTCSTWFLLDIAFYSQNLFQKDVFSAVGWIPKAPTMN
AVQEVYKIARAQTLIALCSTVPGYWFTVAFIDIIGRFAIQLMGFFFMTVFMFAIAIPYHHWTLEANRIGFI
VMYSLTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAYGFLYAAQSKDPNKTDAGY
PAGIGIKNSLIVLGCINALGMLCTFCVPEPKGKSLEEASQETITGEA

>LePT3 (NP_001318089.1, Aoki et al. 2010, PUBMED 20350329
MAKDQLQVLNALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYHHEGDNKPGILPPGI
SAAVNGVAFVGTLSGQLFFGWLGDKMGRKRVYGMTLMIMVICSIASGLSFKTSSGVIATLCFFRFWLG
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PEADFVWRIILMFGAIPALLTYYWRMKMPETARYTALVAKNATKAASDMSKVLNVEIEAEKEKIVETQ
GNSFGLFTKEFLHRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSKIGWIPHPETMNALDEVFKIAKAQT
LIALCSTVPGYWFTVAFIDRMGRFAIQLMGFFFMTVFMFALAIPYNHWTKKENRIGFVIMYSLTFFFANF
GPNTTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSTDPSKVDTGYPTGIGVKNALIVLG
CVNFLGMLFTLLVPESKGRSLEEMSKENEGEEEITKGENAQTIPV

>LePT4 (NP_001234674.2, Xu et al. 2007, PUBMED 17545228; Nagy et al. 2005, PUBMED 15807785)
MIIHQPISSNTRKSNNMASDNLVVLNALDTARTQWYHVTAVIIAGMGFFTDAYDLFCITTISKLLGRLYY
YDPTTHAPGKLPHVANNWVIGVALVGTLSGQLVFGWLGDKLGRKKVYGLTLILMVLCALCSGLSLGY
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MRYEGEPFNVDEILSTEPQADYVWRIVLMLGALPALLTYYWRMKMPETGRYTAIIEGNAKQAAINMG
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NANTISALREMFETSRAMFVIALFGTFPGYWFTVFFIEKIGRFRIQLMGFFMMSVFMAIIGVKYDYLKSK
EHKWTFATLYGLTFFFANFGPNSTTFVLPAELFPTRVRSTCHALSAASGKAGAMISAFGIQQYTQDGNV
HKIKTAMILLAVTNMAGFCCTFLVTETKGRSLEEITGEDGGQNETQMKTSKPVSGHQDDGWE

>LePT6 (AJF19139.1, Chen et al. 2014, https://doi.org/10.1186/1471-2229-14-61)
MAVGDNDNNNLQVLNALDLAKTQLYHFTAIVIAGMGFFTDAYDLFSISLVTKLLGRLYYTKPDLLKPG
TLPPAVSASVTGVALVGTLAGQLFFGWLGDKMGRKKVYGMTLVLMVVCSVASGLSFGSTPKGVMTT
LCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILFSGIVALITAAGFDHAYRSPTF
EENAALSTVPQSDYIWRIILFGSLPAALTYYWRMKMPETARYTALVAKDAKRAAQDMGKVLQVEIESE
EAKIEQSRNETNQFGLFSWEFVRRHGLHLFGTCSTWFLLDIAFYSQNLFQKDVFSAVGWIPKAPTMNAV
QEVYKIRAQTLIALCSTVPGYWFTVAFIDIIGRFAIQLMGFFFMTVFMFAIAIPYHHWTLEANRIGFIVMY
SLTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAYGFLYAAQSKDPNKTDAGYPAGI
GIKNSLIVLGCINALGMLCTFCVPEPKGKSLEEASQETITGEA

>LePT7 (AJF19140.1, Chen et al. 2014, https://doi.org/10.1186/1471-2229-14-61)
MGGDMKVLNALDSAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGSSKPGSLPPNVS
TAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLMLMCICSIASGLSFSRDPKTVMATLCFFRFWL

GFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIFAIIISAAFEASFKAPPYQVDPLGSTV
PQADYVWRIILMVGSLPALLTYYWRMKMPETARYTALVAKNVKQATADMEKVMQVDIGTEQKEPAA
VSTVKSGNEFGLFTKKFLTRHGLHLLGTTSTWFLLDIAYYSQNLFQKDIFSAIGWIPAAKTMNAIEEVQK
IARAQTLIALCSTVPGYWFTVFLIDRIGRFTIQLIGFTMMTVFMFALAIPYHHWTLPGNHIGFVVLYSLTF
FFANFGPNATTFVVSAEIFPARLRSTCHGISAACGKVGAMVGAFGFLYLAQPQDKTKADAGYPAGIGVR
NSLIVLGVVNLLGLFFTFLVPESKGKSLEEMSRENEDSTEEGADNKTVPV
>LePT8 (AJF19141.1, Chen et al. 2014, https://doi.org/10.1186/1471-2229-14-61)
MAGDMKVLNALDSAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGSSRPGSLPPNVS
AAINGVAFCGTIAGQLFFGWIGDKIGRKKVYGVTLMIMSICSIASGLSFGKEPKTVMATLCFFRFWLGFG
IGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILGGGIFAIIISAVFQACFKAPAYQVDPLGSTVPQA
DYVWRIILMAGSLPALVTYYWRMKMPETARYTALVAKDVKQATADMEKVMQVDIETEQKELAVSSV
AVKSGNEFGLFTKQFLTRHGLHLLGTASTWFLLDIAYYSQNLFQKDIFSAIGWIPAAKTMNAIEEVYKIG
RAQTLIALCSIVPGYWFTVFLIDRIGRFTIQVIGFTMMTVFMFALAIPYHHWTLPGNHIGFVVFYSLTFFF
ANFGPNATTFVVPAEIFPARLRSTCHGISAACGKLGAMVGAFGFLYLAQPQDKTKADAGYPAGIGVKN
SLIVLGIVNLLGLFFTFLVPESKGKSLEEMSRENEDSAQPENHNRTVPV

>LjPT3 (BAE93353.1, Maeda et al. 2006, PUBMED 16774930)
MARGELGVLTALDTAKTQWYHFTAIIITGMGFFTDAYDLFSIPNVTKLLGRIYYTKEGSPKPGTLPANVS
VAVNGVALCGTLAGQLFFGWLGDKMGRKKVYGLTLAIMVVSSLASGLSFGHTPKGVISTLCFFRFWLG
FGIGGDYPLSATIMSEYANKKTRGAFIASVFAMQGFGILAGGIVSLIVSAAFDHAFKAPPYKDDPAASLV
PEADYVWRIILMFGAAPAALTYYWRMKMPETARYTALVAKNAKLAAQDMSKVLQMEVEAEQEKTD
KIAEPETQQNNKNSFGLFSKEFARTHGVHLLGTTSTWFLLDIAYYSSNLFQKDIYSSIGWLPHAEEMNAI
QEVYKVARASTLIALCGTVPGYWFTVAFIDHLGRFIIQLMGFFFMTVFMFALAIPYNHWTQKEHRIGFL
VMYAFTFFFANFGPNSTTFVVPAEIFPARLRTTCHGISSAAGKAGAIVGAFGFLYASQDKDEAKRDAGY
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>LjPT4 (BAG71408.1, Takeda et al. 2009, PUBMED 19220794)
MALEVLEALDSARTQWYHVTAIVIAGMGFFTDAYDLFCITTVSKLLGRLYYFDPSTGKPGKLPNNVNN
LVTGVALVGTLSGQLFFGYLGDKLGRKKVYGVTLILMVACAICSGLSFGASAKSVMGTLCFFRFWLGF
GIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGVGIIFAGLVSMCLSAGFKASYHAPSFHDDPIMSTQP
QGDLMWRLVLMIGAVPAAMTYYWRMKMPETGRYTAIIEGNAKQAAADMARVLDIEIQAEQDKLAEF
KAANDYPLWSNEFFTRHGRHLIGTMTSWFLLDIAFYSQNLTQKDIFPAMGLIDKDFEMNAIQEVFETSR
AMFVIALFGTFPGYWFTVFFIEKLGRYKIQLIGFFMMSVFMFIIGVKYDYLRNENSHMFALLYGLTFFFA
NFGPNSTTFVLPAELFPTRVRSTCHALSAAAGKAGAMVGAFGIQNYTQKGEQKQIKHAMMILAVTNLI
GFFCSFLVTETKGRSLEEISGEDGRESELTPTPPNNRVPTRQEPRSETM

>MtPT1 (AAB81346.1, MtrunA17Chr1g0167741, Medtr1g043220, Liu et al. 1998, PUBMED 9425684)
MSGELGVLNALDVAKTQLYHFTTIVIAGMGFFTDAYDLFCISLVTKLLGRIYYTEPNPTRPGTLPPSAQS
AVTGVALVGTLAGQLFFGWLGDKLGRKKVYGLTLILMVVCSVGSGLSFGSSPKSVMATLCFFRFWLGF
GIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILGGGIVALIVASIFDHKYKVPTFEENPATSLLVP
QFDYVWRLILMFGALPAALTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVELEVEEEKVQK
MTSDKRNSYGLFSKQFAARHGLALFGTCSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAKEMNAIHEVYKI
ARAQTLIALCSTVPGYWFTVAFIDHMGRFAIQMMGFFFMTVFMFGLAIPYDHWSKEENRIGFVVMYSL
TFFFSNFGPNAATFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSKDPTKTDKGYPTGIGIK
NSLIMLGVINFVGMLCTLLVPESKGKSLEELSGENEGEGAEATEQEGPRFENVA

>MtPT2 (AAB81347.1, MtrunA17Chr1g0167761, Medtr1g043290, Liu et al. 1998, PUBMED 9425684]
MSGELGVLNALDVAKTQLYHFTTIVIAGMGFFTDAYDLFCISLVTKLLGRIYYTEPNPTRPGTLPPSAQS
AVTGVALVGTLAGQLFFGWLGDKLGRKKVYGLTLILMVVCSVASGLSFGSSPKSVMATLCFFRFWLGF
GIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILGGGIVALTVASIFDHKYKVPTFEENPAASLLV
PQFDYVWRLILMFGALPAALTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVELEVEEEKVEK
MTSDKRNSYGLFSKQFAARHGLALFGTCSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAKEMNAIHEVYKI
ARAQTLIALCSTVPGYWFTVAFIDHMGRFAIQMMGFFFMTVFMFALAIPYDHWSKEENRIGFVVIYSLT
FFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSKDPTKTDKGYPTGIGIK
NSLIMLGVINFVGMLCTLLVPESKGKSLEELSGENEGEGAEATEQEGSRV

>MtPT3 (ABM69110.1, MtrunA17Chr1g0167731, Medtr1g043200, Liu et al. 2008, PUBMED 18596039)
MSGELGVLNALDVAKTQLYHFTTIVIAGMGFFTDAYDLFCISLVTKLLGRIYYTEPNPTRPGTLPPSVQS
AVTGVALVGTLAGQLFFGWLGDKMGRKKVYGLTLILMVVCSVASGLSFGSSPKSVMATLCFFRFWLG
FGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILGGGIVALIVVSIFDHKYKVPTFEENPAASLL
VPQFDYVWRLILMFGALPAALTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVELEVEEEKV
QKMTSDKRNSYGLFSKQFAKRHGWALFGTCSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAKEMNAIHEV
YKIARAQTLIALCSTVPGYWFTVAFIDHMGRFAIQMMGFFFMTVFMFALAIPYDHWSKEENRIGFVVM

YSLTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSKDPTKTDKGYPTG
IGIKNSLIMLGVINFVGMLCTLLVPESKGKSLEELSGENEGESVEDTRQEGSGV

>MtPT4 (Q8GSG4.1, MtrunA17Chr1g0158991, Medtr1g028600, Harrison et al. 2002, PUBMED 12368495)
MGLEVLEALDSARTQWYHVTAIVIAGMGFFTDAYDLFCISTVSKLLGRLYYFDPSTNKPGKLPPSVNNV
VTGVALVGTLSGQLVFGWLGDKLGRKKVYGVTLIIMVACAICSGLSFGSSAKSVMITLCFFRFWLGFGI
GGDYPLSATIMSEYANKRTRGAFIAAVFAMQGVGIIFAGLVSMVFSGIFKAYYQAPRFNEDPILSTQPEG
DLLWRLILMIGAVPAAMTYYWRMKMPETGRYTAIVEGNAKQAAADMARVLDIEIIAEQDKLAEFKAA
NDYPLWSSEFFNRHGRHLIGTMSCWFLLDIAFYSQNLTQKDIYPAMGLIRQDKEMNAIDEVFQTSRAMF
VVALFGTFPGYWFTVFFIEKLGRFKIQLVGFFMMSFFMFVIGVKYEYLKDENKNLFALLYGLTFFFANF
GPNSTTFVLPAELFPTRVRSTCHAFSAASGKAGAMVGAFGIQYYTLDGTPRKIRRAMMILAFTNLIGFFC
TFLVTETKGRSLEEISGEDGRESELTATPNDRAPGIRQDSRTEKM

>MtPT5 (ABM69111.1, MtrunA17Chr1g0186821, Medtr1g074930, Liu et al. 2008, PUBMED 18596039)
MAGQLGVLNALDVAKTQMYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYTDPTQPKPGVLPPGV
QAAVTGVALVGTLSGQLFFGWLGDKLGRKKVYGITLMLMVGCSLASGLSFGNSPKGVMATLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGIMAGGVFALIVAAAFDHKYQVPTYEENAK
ASLVLPAFDYVWRIILMFGAVPAALTYYWRMKMPETARYTALVAKNGKQAASDMSKVLQVEIEAEEE
KVQNLAENQNQKFGLFTRQFAKRHGLHLLGTTTTWFLLDIAFYSQNLFQKDIFTAIGWIPPAKEMNAIN
ELYRIARAQTLIALCSTVPGYWFTVAFIDYMGRFAIQLMGFFFMTVFMFALAIPYDHWTKKENRIGFVV
MYSLTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSKDPTKTDHGYPT
GIGIKNSLIVLGVVNFFGMVFTFLVPEPNGKSLEEMSGENEDDDAEAIEMAAGSARTVPV

>MtPT6 (XP_003601529.1, MtrunA17Chr3g0120761, Medtr3g082700, Breuillin-Sessoms et al. 2015, PMID: 25841038)
MAREQLGVLTALDAAKTQWYHFTAIIIAGMGFFTDAYDLFCIPNVTKLLGRIYYTHPGALKPGTLPPNV
SAAVNGVALCGTLAGQLFFGWLGDKMGRKKVYGLTLALMVFSSLASGLSFGHSAKGTIGTLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIASVFAMQGFGILAGGIVSLVVSTAFDHAFKAPPYKVDAAA
SLVPEADYVWRIILMFGAVPAGLTYYWRMKMPETARYTALVAKNAKQAAQDMSSVLQVEIEAEQEK
VDKIGVQDKNSFGLFSKEFLRRHGLHLLGTTSTWFLLDIAYYSSNLFQKDIYSSIGWLPPAQDMNAIHEV
FKVARATTLIALCGTVPGYWFTVAFIDVIGRFAIQLMGFFFMTVFMFALAIPYDHWTKRENRIGFLVMY
AMTFFFANFGPNSTTFVVPAEIFPARLRSTCHGISSAAGKAGAIIGAFGFLYASQSKDPKKRDAGYPAGIG
MKNTLILLAVVNCLGIFFTFLVPEANGKSLEEMSGENEEEEETEAAVVDPQASSNRTVPV

>MtPT7 (A0A072UDN5, MtrunA17Chr7g0261231, Medtr7g096870, Breuillin-Sessoms et al. 2015, PMID: 25841038)
MAKDQIQVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCVSLVTKLLGRIYYHVDGAAKPGTLPPN
VSAAVNGVAFCGTLSGQLFFGWLGDKMGRKKVYGMTLMMMVICSIGSGLSFGHTPKSVMTTLCFFRF
WLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILGAGIFAIIISSAFKARFDSPSYEVDPVGS
TVPQADYIWRIIVMVGALPAAMTYYSRTKMPETARYTALVAKNTAQAAADMSKVLQVEIEAEPQKAE
PEKAKAYGLFSKEFMSRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPPPQTMNALEEVYKIAR
AQTLIALCSTVPGYWFTVAFIDRIGRFTIQLMGFFFMTAFMFALAIPYEHWTHKENRIGFVVIYSLTFFFA
NFGPNATTFVVPAEIFPARFRSTCHGISSASGKLGAMVGAFGFLYLAQNKDKSKADAGYPAGIGMKNSL
ILLGVCNILGFLCTFLVPEANGKSLEEMSGENDEEVGSKDDVEKSHAQNNNTVR

>MtPT8 (A0A072U3J3, MtrunA17Chr7g0261241, Medtr7g096880, Breuillin-Sessoms et al. 2015, PMID: 25841038)
MAKEQIQVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGAGKPGTLPPNV
SAAVNGVAFCGTLTGQLFFGWLGDKLGRKKVYGMTLMIMVICSIGSGLSFGHNPKTVMATLCFFRFWL
GFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILGGGIFAIIVSAAFKAMFDSPSYEVDPVRSTV
PQADYIWRIIVMVGALPAAMTYYWRMKMPETARYTALVAKDTKQAAADMSKVLQVEIQAEPQQAEK
AKSYGLLSKEFMSRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQTMNALEEVYKIARAQT
LIALCSTVPGYWFTVALIDRIGRFKIQLMGFFFMTVFMFALAIPYDHWTHKDHRIGFVVLYSLTFFFANF
GPNATTFVVPAEIFPARFRSTCHGISSASGKLGAMVGAFGFLYLAQNKDKSKADAGYPAGIGVKNSLLL
LGVVNILGFLFTFLVPEANGKSLEEMSGENEEEAGTNGELEQSHSHNRTVSHV

>MtPT9 (A0A072VWQ9, MtrunA17Chr1g0183921, Medtr1g069935, Breuillin-Sessoms et al. 2015, PMID: 25841038)
MAKENLQVLNALDVAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGAEKPGSLPPN
VAAAVNGVAFVGTLSGQLFFGWLGDKLGRKKVYGMTLAVMVVCSIGSGLSFGHEPKTVLGTLCFFRF
WLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILAGGVFAIIVSIAFKSKFDAPAYMIDPLG
STVPQADYVWRIILMFGAIPAAMTYYSRSKMPETARYTALVAKNMEQAAADMSKVMNMDIQAETKN
VAAEEKKQFGLFSKEFMSRHGLHLLGTASTWFLLDIAFYSQNLFQKDIFSAIGWIPSAKTMNALEEVYKI

ARAQTLIALCSTVPGYWFTVAFIDRIGRFTIQLMGFFFMTVFMFALAIPYDHWTHKENRIGFVVLYSLTF
FFANFGPNATTFVVPAEIFPARFRSTCHGISSATGKLGAMVGAFGFLYLAQNQDPKKADAGYPAGIGVR
NSLILLGVVNILGFCFTFLVPETKGKSLEEMSGEDIDEIVVVTE

>MtPT10 (A0A072VKI6, MtrunA17Chr1g0183911, Medtr1g069930, Breuillin-Sessoms et al. 2015, PMID: 25841038)
MARENLRVLSALDVARTQCYHFTAIAITGLGFFTNAYDLFCISLVTRLLGRIYYYVDGAEEPGTLPPNVA
ATVNGLALVGTFTGQLFFGWLGDKLGRRTVYGITLNIMIICSLGSGLSFGHEPKMVLGTLCFFRFWLGF
GIGGDYPLSATIMSEYSNKKTRGAFVAAVFAMQGFGIVAGGVFAIIVSVAFKSKFDAPPYVVDPTGSTV
PEADYVWRIILMFGAIPAALTYYSRSTMPETARYTALVAKDMDKAVADMSKVLKMDIKAEPLKDDDV
EENKQFGLFSKEFMSRHGVHLLGTMSTWFFLDVVFHSMNLFQKDVFCTVGWIRYPKTMNAMEEVYIIA
KAQTLIALCSTVPGYWFTVALIDRMGRFTIQLMGFFFMTVFMFAIAIPFDHWTITENKIGFAVLYALTFFF
ANFGPNTTTFVVPAEIFPARFRSTCHGMSSAAGKLGAMVGAFGFLFLVQNQDPKKTDAGYPAGIGVRN
SLFCLGAVTILGFCFTFLVPEAKGRSLEEMSGEDIDDIVDDTISHSSSSNRMMSYP

>MtPT11 (A0A072VWQ9, MtrunA17Chr1g0183921, Medtr1g069935, Breuillin-Sessoms et al. 2015,PMID: 25841038)
MAKENLQVLNALDVAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGAEKPGSLPPN
VAAAVNGVAFVGTLSGQLFFGWLGDKLGRKKVYGMTLAVMVVCSIGSGLSFGHEPKTVLGTLCFFRF
WLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILAGGVFAIIVSIAFKSKFDAPAYMIDPLG
STVPQADYVWRIILMFGAIPAAMTYYSRSKMPETARYTALVAKNMEQAAADMSKVMNMDIQAETKN
VAAEEKKQFGLFSKEFMSRHGLHLLGTASTWFLLDIAFYSQNLFQKDIFSAIGWIPSAKTMNALEEVYKI
ARAQTLIALCSTVPGYWFTVAFIDRIGRFTIQLMGFFFMTVFMFALAIPYDHWTHKENRIGFVVLYSLTF
FFANFGPNATTFVVPAEIFPARFRSTCHGISSATGKLGAMVGAFGFLYLAQNQDPKKADAGYPAGIGVR
NSLILLGVVNILGFCFTFLVPETKGKSLEEMSGEDIDEIVVVTE

>MtPT12 (A0A072UDN5, MtrunA17Chr7g0261231, Medtr7g096870, Breuillin-Sessoms et al. 2015, PMID: 25841038)
MAKDQIQVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCVSLVTKLLGRIYYHVDGAAKPGTLPPN
VSAAVNGVAFCGTLSGQLFFGWLGDKMGRKKVYGMTLMMMVICSIGSGLSFGHTPKSVMTTLCFFRF
WLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILGAGIFAIIISSAFKARFDSPSYEVDPVGS
TVPQADYIWRIIVMVGALPAAMTYYSRTKMPETARYTALVAKNTAQAAADMSKVLQVEIEAEPQKAE
PEKAKAYGLFSKEFMSRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPPPQTMNALEEVYKIAR
AQTLIALCSTVPGYWFTVAFIDRIGRFTIQLMGFFFMTAFMFALAIPYEHWTHKENRIGFVVIYSLTFFFA
NFGPNATTFVVPAEIFPARFRSTCHGISSASGKLGAMVGAFGFLYLAQNKDKSKADAGYPAGIGMKNSL
ILLGVCNILGFLCTFLVPEANGKSLEEMSGENDEEVGSKDDVEKSHAQNNNTVR

>MtPT13 (A0A072U3J3, MtrunA17_Chr7g0261241, Medtr7g096880, Breuillin-Sessoms et al. 2015, PMID: 25841038)
MAKEQIQVLNALDVAKTQWYHFTAIIIAGMGFFTDAYDLFCISLVTKLLGRIYYHVDGAGKPGTLPPNV
SAAVNGVAFCGTLTGQLFFGWLGDKLGRKKVYGMTLMIMVICSIGSGLSFGHNPKTVMATLCFFRFWL
GFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGFGILGGGIFAIIVSAAFKAMFDSPSYEVDPVRSTV
PQADYIWRIIVMVGALPAAMTYYWRMKMPETARYTALVAKDTKQAAADMSKVLQVEIQAEPQQAEK
AKSYGLLSKEFMSRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQTMNALEEVYKIARAQT
LIALCSTVPGYWFTVALIDRIGRFKIQLMGFFFMTVFMFALAIPYDHWTHKDHRIGFVVLYSLTFFFANF
GPNATTFVVPAEIFPARFRSTCHGISSASGKLGAMVGAFGFLYLAQNKDKSKADAGYPAGIGVKNSLLL
LGVVNILGFLFTFLVPEANGKSLEEMSGENEEEAGTNGELEQSHSHNRTVSHV

>OsPT1 (Q8H6H4.1, Paszkowski et al. 2002, PUBMED 12271140)
MAGGQLNVLSTLDQAKTQWYHFMAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYTDDSKDTPGALPPN
VSAAVTGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVVCSVASGLSFGSSAKGVVSTLCFFRFW
LGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGILFGAIVALAVSAGFRHAYPAPSYSDNHAA
SLVPQADYVWRIILMFGTVPAALTYYWRMKMPETARYTALIARNAKQAAADMSKVLHTQIEESADRA
ETVAVGGESWGLFSRQFLRRHGLHLLATTSTWFLLDIAFYSQNLFQKDIFSKVGWIPPAKTMNALEELY
RIARAQALIALCGTIPGYWFTVAFIEIMGRFWIQIMGFAMMTAFMLGLAIPYHHWTTPGHHTGFIVMYG
FTFFFANFGPNSTTFIVPAEIYPARLRSTCHGISAAAGKAGAIIGAFGFLYAAQDQHKPEPGYPRGIGIKNA
LFVLAGTNFLGTIMTLLVPESKGMSLEVISQEVADGDDEEAAYPK

>OsPT2 (Q8GSD9.1, Paszkowski et al. 2002, PUBMED 12271140)
MAGSQLNVLVKLDQAKTQWYHFMAIVIAGMGFFTDAYDLFCIALVTKLLGRLYYTDITKPNPGTLPPN
VSSAVTGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVVCSIASGLSFGHTPKSVIATLCFFRFWL
GFGIGGDYPLSATIMSEYASKKTRGAFIAAVFAMQGFGILFGAIVALVVSAGFRHAYPAPSYAQNPAAS
LAPQADYTWRLILMFGTIPAGLTYYWRMKMPETARYTALVARNAKQAAADMSKVLHAEIEERPEVVE

SQVVAGETWGLFSRQFMKRHGMHLLATTSTWFLLDIAFYSQNLFQKDIFSKVGWIPPAKTMNALEELY
RISRAQALIALCGTIPGYWFTVAFIDIVGRFWIQIMGFFMMTVFMLALGVPYDHWTHPAHHTGFVVLYA
LTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAASGKAGAIIGAFGFLYAAQDQHNPDAGYSRGIGIRNA
LFVLAGTNFLGMLMTLLVPESKGLSLEEMSKDNVVDETAQEAIAQA

>OsPT3 (Q7XDZ7.1, Paszkowski et al. 2002, PUBMED 12271140)
MADGQLKVLTTLDHARTQWYHFMAIVIAGMGFFTDAYDLFCISLVSKLLGRIYYTDLAGDNPGSLPPN
VSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLVLMVVCSVASGLSFGRTAKGVVAXLCFFRF
WLGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGILFGAIVALVVSAGFRNAYPAPSYADGRA
ASLVPEADYVWRIILMFGTVPAALTYYWRMKMPETARYTALIARNAKQAAADMSKVLXTEIQEDXER
AEAVAAGGAGNEWGLFSRQFVRRHGVHLVATTSTWFLLDIAFYSQNLFQKDIFSKVGWIPPARTMNAV
EEVFRIARAQALIALCGTIPGYWFTVAFIDVAGRFAIQLMGFAMMTVFMLGLAAPYHHWTTPGNHTGF
VVMYGFTFFFANFGPNATTFIVPAEIYPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQDPHKPEAGYK
PGIGIRNALFVLAGTXFLGMLMTLLVPESKGMSLEEVSKENVADDEEATA

>OsPT4 (Q8H6H2.1, Paszkowski et al. 2002, PUBMED 12271140)
MAGELKVLNALDSAKTQWYHFTAIVIAGMGFFTDAYDLFSISLVTKLLGRIYYFNPASKSPGSLPPNVS
AAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLMLMVICCLASGLSFGSSAKGVMATLCFFRFW
LGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGNLTGGIVAIIVSAAFKSRFDAPAYRDDRTGS
TVPQADYAWRIVLMFGAIPALLTYYWRMKMPETARYTALVAKNAKQAAADMTQVLNVEIVEEQEKA
DEVARREQFGLFSRQFLRRHGRHLLGTTVCWFVLDIAFYSSNLFQKDIYTAVQWLPKADTMSALEEMF
KISRAQTLVALCGTIPGYWFTVFFIDIIGRFVIQLGGFFFMTAFMLGLAVPYHHWTTPGNHIGFVVMYAF
TFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAAGKAGAIVGSFGFLYAAQSTDASKTDAGYPPGIGVR
NSLFFLAGCNVIGFFFTFLVPESKGKSLEELSGENEDDDDVPEAPATADHRTAPAPPA

>OsPT5 (Q7X7V2.2, Paszkowski et al. 2002, PUBMED 12271140)
MVQDRKVLDALDTAKTQWYHFTAVVIAGMGFFTDAYDLFSISLVTKLLGRIYYFNPASKSPGSLPPNVS
AAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLMLMVICCLASGLSFGSSAKGVMATLCFFRFW
LGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGNLTGGIVAIIVSAAFKLRFDAPAYRDDRAG
XXADYAWRIVLMFGAIPALLTYYWRMKMPETARYTALVAKNDKKAAADMARVLNVELVDEQEKAA
AATAAAAEEEAARREQYGLFSREFARRHGHHLLGTTVCWFVLDIAYYSQNLFQKDIYTAVQWLPKAD
TMSALEEMFKISRAQTLVALCGTIPGYWFTVLFIDIVGRFAIQLGGFFLMTAFMLGLAVPYHHWTTPGN
HVGFVVMYAFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISSAAGKMGAIVGSFGFLYAAQSTDPSKT
DAGYPRGIGVRNSLFLLAGCNVVGFLFTFLVPESKGKSLEELSGENEMEAEPAAATNSYRQTVPDSGQS
E

>OsPT6 (Q8H6H0.1, Paszkowski et al. 2002, PUBMED 12271140)
MGGGGGEQQQLEVLHALDVAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGRIYYRVDGSPSP
GTLPPHVSASVNGVAFVGTLSGQLFFGWLGDKLGRKRVYGITLMLMVLCSLASALSFGHTPTSVMATL
CFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGIITGGLVAILVSASFRAAFPAPPYG
EDPVASTPPQADFVWRIILMLGALPAALTYYWRTKMPETARYTALVANNAKQAAADMSKVLQVVEM
RNIGNNGGSRRPFGLFSGEFVRRHGLHLVGTSATWLLLDIAFYSQNLFQKDIFSAVGWIPKAATMSALE
ELFRIARAQTLIALCGTVPGYWFTVALIDVVGRFKIQAVGFFMMTLFMLTLALPYHHWTAPGKNHVGF
LLLYGLTFFFANFGPNSTTFIVPAEIFPARLRATCHGISAASGKLGAIVGSFGFLYLAQSPDRSKTEHGYPP
GIGVRNSLFLLAACNLLGLLFTFLVPESKGKSLEEMSGDAEAQEEAPPPLQTVL

>OsPT7 (Q8H6G9.1, Paszkowski et al. 2002, PUBMED 12271140)
MAGDQMHVLSALDSAKTQWYHFTAIVIAGMGFFTDAYDLFCISLVTKLIGRVYYTADGASKPGSLPPN
VSAAVNGVAFVGTLTGQLFFGWLGDRVGRKSVYGMTLLLMIICSVASGLSFGDTPTSVMATLCFFRFW
LGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGILAGGAVAIGITAIFRSRFPAPPFAADPAAST
PPQADYVWRLILMFGALPAALTFYWRMRMPETARYTAIVAKNAERAAADMSKVLQVKITAEQAEMA
SPVDKPFTSKPFGLFSGEFARRHGFHLLGTTSTWLLLDIAYYSQNLFQKDIFSAIGWIPEAKTMSALDELY
HIARAQTLIALCGTVPGYWFTVALIDVVGRFKIQAAGFFVMTAFMLALAVPYDHWTAAGNQIGFVVLY
ALTFFFANFGPNATTFIVPAEIYPARLRATCHGISAASGKVGAIVGSFGFLYLAQSPVPAKAAAHGYPPGI
GVRNSLFALAGCSLLGFLLTFLVPEPKGKSLEEMSRENEVGQP

>OsPT8 (Q8H6G8.1, Paszkowski et al. 2002, PUBMED 12271140)
MARQEQQQHLQVLSALDAAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGRIYYTDLAKENPGS
LPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLLMMVICSIASGLSFSHTPTSVMATLCF
FRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIVTLIISSAFRAGFPAPAYQDD
RAGSTVRQADYVWRIILMLGAMPALLTYYWRMKMPETARYTALVAKNAKQAAADMSKVLQVEIQEE
QDKLEQMVTRNSSSFGLFSRQFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFTSINWIPKAKTMSA
LEEVFRIARAQTLIALCGTVPGYWFTVFLIDIVGRFAIQLLGFFMMTVFMLGLAVPYHHWTTKGNHIGF

VVMYAFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAAGKAGAIIGSFGFLYAAQDPHKPDAGYKP
GIGVRNSLFVLAGCNLLGFICTFLVPESKGKSLEEMSGEAEDDDDEVAAAGGGAAVRPQTA

>OsPT9 (Q8H6G7.2, Paszkowski et al. 2002, PUBMED 12271140)
MAPRIRVLAALDQARTQYYHFKAIVIAGMGLFTDSYDLFCISPVMKIFGRVYYAPSGSVDGSGSGPGVT
PPAVVSATVGVALLGAVAGNVVFGALGDRVGRRRVYGACLLLMVCSSVGSGLSVCRXRRCALASLCF
FRFLLGVGVGGDYPLSATIMSEFANRGTRGAFIAAVFSMQGFGILVSSAVTMAVAAAFDHYTGYPAPL
DTPECADLAWRIILMAGAVPAALTYYWRMSMPETARYTALVERDVVKATNDIGRVLADLDLAAVAEE
EVAAAALSPPPVTTPPPPRPSYGLFSRRFVRQHGRDLFACAAAWFLLDIPYYSSTLFQSQIYRPWFPPAA
KVNAFQEAFNVAKFQAVIAVASTIPGYFAAMLLIERAGRRRLQMAGFLLMAVFLFALAGPYDGYWRD
HAKTAGYIVLYSLTFFSANLGPNTTTFILPAELFPARFRSTCHGLSGAAGKLGALVGSIGFLWASQQKDG
AAAGHLPGIGMMYALFVLGGICLLGLALTYAFTPETMTRSLEENESSVQAQSQVGDGGSDAGNGSDGL
RFHELNVLMEAATKSPVSMASSHLSMSPILPHRMSL

>OsPT10 (Q69T94.1, Paszkowski et al. 2002, PUBMED 12271140)
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ATVGVALLGAVIGNVVFGALGDRVGRRRVYGACLLLMLRRERLLRVPDAPVRARQPLLLPFLLGVGV
GGDYPLSATIMSEFANRRTRGAFIAAVFSMQGFGILASSAVTMAVAAAFDHYTGYPAPLDTPECADLA
WRIILMAGAVPAALTYYWRMSMPETARYTALVERDVVKATNDIGRVLADLDLGAVAEEEVAAALSRP
PPPPRPSYGLLSRRFVRQHGRDLFACAAAWFLLDIPYYSSTLFQSQIYRPLFPAPGLINAFQEAFNVAKFQ
AVIAVASTIPGYFVAVLLIDRVGRRCLQMAGFLLMAVFLFALAGPYDGYWRDHGAHAGYIVLYSLTFF
SANLGPNTTTFILPAELFPARFRSTCHGLSGAAGKLGALVGSIGFLWASQQKDGAAAGHLPGIGMMYAL
FVLGGICLLGLALTYVFTPETMMRSLEENESDRAQTQVGDGGSDTEAAKSPASMASSHLSMSPILPARV
SV

>OsPT11 (Q94DB8.1, Paszkowski et al. 2002, PUBMED 12271140)
MADADGGSNLAVLDALDSARTQMYHMKAIVIAGMGFFTDAYDLFCISTVSKLLGRLYYQPDGSTDSK
PGALSKTANNMVIGVALVGTLMGQLVFGYFGDKLGRKRVYGVTLILMAACAIGSGLSFGSSRKAVIGT
LCFFRFWLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGVGIIFAGLVSMIVSSIFLTYNKAPSYK
GNHDLSRQMPAADYVWRIVLMIGAFPALATFYWRMKMPETARYTAIIDGNAKQAANDMQKVLSIEIE
AEQEKLAKFNAANNYPLLSMEFARRHGLHLIGTTTTWFLLDIAFYSQNLTQKDIFPAMGLISGAAEVNA
LTEMFQISKASFLVALLGTFPGYWVTVALIDKMGRYMIQLIGFFMMSMFMLAMGILYDYLKTHHFLFG
LLYALTFFFANFGPNSTTFVLPAELFPTRVRSTCHAISAAAGKAGAIVAAFGIQKLTYNSQVKSIKKALIIL
SITNMLGFFFTFLVPETMGRSLEEISGEDGNTGAGGGGAPAAANAGVGVSASDVSRDEKFPASSTEWQT
SMHA

>PtPT10 (XP_006374329, Potri.015G022800.1.p,POPTR_00015s 06110, PMID: 21705655, Loth-Pererda et al. 2011
MSSSNLAVLNALDNARIQLYHVTAIIIAGMGFFTDAYDLFCITTVSKLLGRLYYYDPITGNPGKLPTNVN
NVVTGVALVGTLSGQLVFGWAGDKLGRKKVYGVTLIIMVICAIGSGISFGSSTKSVITLCFFRFWLGFGI
GGDYPLSATIMSEYANKKTRGKFIAAVFAMQGVGIIFAGLVSMILSKIFLSRYHAVPFSKDPILSTQPQAD
FLWRIVLMLGALPAMLTFYWRMKMPETGRYTALIEGNAKKAAVDMGRVLDIDIQEESDKLSEIRASNN
YKLLSWEFFDRHGYHLIGTMSTWFLLDIAFYSQNLTQKDIFPAMGLTKQAVDVSALEEVYETSCAMFIV
ALLGTFPGYWFTVLFIESLGRFFIQVMGFIMMSSFMLLMGVFYDGLKEHKWLFALLYGLTFFFANFGPN
STTFVLPAELFPTRLRSTCHALSAAAGKAGAMIGAFVVQTYTLDGDVTKIKRALLALSFTNILGACFTFF
LSETKGKSLEEISGEDGGQNRTRTAARRSASWHDQLREVI

>SbPT11 (XP_002458253.1, Walder et al. 2015, DOI: 10.1111/nph.13292)
MAAEGGSNLAVLDALDSARTQMYHMKAIVIAGMGFFTDAYDLFCITTVSKLLGRIYYPNDNLYLDKPK
PGTLPVSTNNLVTGVALVGTLMGQLVFGYFGDKLGRKRVYGITLVLMAACAIGSGLSFGSTRHAVIGT
LCFFRFWLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGVGIIFAGLVSMVISGILLRYNPAPSW
TEDHDSSLGDQLPAADYMWRIVLMLGAFPALATFYWRMKMPETARYTALIEGNAKQAANDMQKVM
DVEIQSEQDKLARYKAANDYPLLSREFAQRHGLHLIGTATTWFLLDIAFYSQNLTQKDIFPAIKLTSPAG
DINPLKEVFEISKAMFLVALLGTFPGYWVTVALIDKMGRYLIQLIGFFMMSVFMLLMGIMYDDLKNKY
TTLFALFYALTFFFANFGPNSTTFVLPAELFPTRVRSTCHISAASGKAGAIVAAFGVQSLTLKGDIASIKK
ALIILAVTNMLGFFFTFLVPETMGRSLEEISGEDGNAGNGPGVPAGAAMGAADVSKDDKIPVSSTEWQS
SMQA

>StPT1 (NP_001275200.1, Leggewie et al. 1997, PUBMED 9090882)
MANDLQVLNALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCISMVTKLLGRIYYHHDNALKPGSLPPNV
SAAVNGVAFCGTLAGQLFFGWLGDKMGRKKVYGMTLMIMVICSIASGLSFGHTPKSVMTTLCFFRFW
LGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGMVAIIVSSAFKGAFPAPAYEVDALA
STVSQADFVWRIILMFGAIPAGLTYYWRMKMPETARYTALVAKNLKQAANDMSKVLQVEIEAEPEKV

GAISEAKGANEFGLFSKEFLRRHGLHLLGTASTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQTMNALEEV
YKIARAQTLIALCSTVPGYWFTVAFIDRIGRFAIQLMGFFFMTVFMFALALPYHHWTLKDNRIGFVVMY
SLTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAMVGAFGFLYAAQPTDPKKTDAGYPPGI
GVRNSLIVLGCVNFLGMLFTFLVPESNGKSLEEMSRENEGEEETVAEMRATSGRTVLFKF

>StPT2 (CAA67396.1, Leggewie et al. 1997, PUBMED 9090882)
MAVEDNNLQVLNALDLAKTQLYHFTAIIIAGMGFFTDAYDLFSISLVTKLLGRLYYTKPDLLKPGTLPP
ARVGLRHGCALVGTLAGQLFFGCARLAKMGRKKVYGMTLVLMVVCSVASGLSLGNTPKVVMTTLCF
FRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILFSGIVALITAAGFDHAYKAPTFAEN
AAVSTVPQADYIWRIILMFGSLPAALTYYWRMKMPETARYTALVAKDAKRAAQDMGRVLQVEIESEE
AKIEQISRDETNQFGLFSWEFVRRHGLHLFGTCSTWFLLDIAFYSQNLFQKDVFSAVGWIPKAPTMNAV
QELYKIARAQTLIALCSTVPGYWFTVAFIDIIGRFAIQLMGFFFMTVFMFAIAIPYHHWTLEANRIGFIVM
YSLTFFFANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAYGFLYAAQSKDPMKTDAGYPA
GIGIKNSLIVLGFINALGMVCTFCVPESKGKSLEEASQETISTGEA

>StPT3 (CAC87043.1, Rausch et al. 2033, PUBMED 11719809
MAKNQLQVLNALDVAKTQLYHFTAIVIAGMGFFTDAYDLFCISLVTKLLGRIYYHHEGAPKPGILPPGIS
AAVNGVAFVGTLSGQLFFGWLGDKMGRKRVYGMTLMIMVICSIASGLSFGKTPSGVIATLCFFRFWLG
FGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIVALVVSGAFKNAYPAPIYSVNNEGSTP
QEADIVWRIILMFGAIPALLTYYWRMKMPETARYTALVAKNATKAASDMSKVLNVEIEAEDDKIEKIV
AIQGNNFGLFSKEFLHRHGLHLLGTTSTWFLLDIAFYSQNLFQKDIFSKIGWIPHPETMNALDEVFKIAK
AQTLIALCSTVPGYWFTVAFIDRMGRFAIQLMGFFFMTVFMFALAIPYNHWTKKENRFGFVVMYSLTFF
FANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAIVGAFGFLYAAQSTDPSKVDAGYPTGIGVKN
ALIVLGCVNFLGMLFTLLVPESKGKSLEEMSKENEGEEEMTKVENAQTIPV

>StPT4 (AAW51149.1, Karandashov and Bucher, 2005, PUBMED 15642520)
MASDNLVVLNALDTARTQWYHVTAVIIAGMGFFTDAYDLFCITTISKLLGRLYYYDPTTHAPGKLPHV
VNNWVIGVALVGTLSGQLVFGWLGDKLGRKKVYGLTLILMVLCAICSGLSFGYSAKGVIGTLCFFRFW
LGFGIGGDYPLSATIMSEYANKATRGAFIAAVFAMQGVGIIFAGLVSMIISKLFLMKYGGKPFDVDEILS
TEPEADYVWRIVLMLGALPALLTYYWRMKMPETGRYTAIIEGNAKQAAINMGKVLDIEIQAEGDKLAQ
FKAANEYSLLSNEFFQRHGLHLIGTMSTWFLLDIAFYSQNLTQKDIFPVMGLTRKANTISALREMFETSR
AMFVIALFGTFPGYWFTVFFIEKIGRFRIQLMGFFMMSVFMAIIGLKYDYLKTKEHKWTFAALYGLTFFF
ANFGPNSTTFVLPAELFPTRVRSTCHALSAASGKAGAMISAFGIQQYTQDGNVHKIKTAMILMAVTNM
VGFCCTFLVTETKGRSLEEISGEDGGKNETQMKTSKPVSGHQDDGWE

>StPT5 (AAX85195.1, Nagy et al. 2005; PUBMED 15807785)
FTDAYDLFCITTVSKLLGRLYYYDPATHAPGKLPHSVNNWVTGVALVGTLTGQLVFGWLGDKLGRKK
VYGLTLILMVICALCSGLSFGYSRKVVIGTLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFIAAVF
AMQGVGIIFAGLVSMIVSKVFLMNFGGKAFTTDEVFSTEPEADYVWRIVLMLGALPALLTYYWRMKM
PETGRYTAIIEGNAKQAAIDMGKVLDIEIQAEGEKLAKFKAANEYSLLSNEFFMRHGHHLLGTMTTWFL
LDIAFYSQNLTQKDIFPTMGLVSNAKNISALREMFETSRAMSVIALLGTFPGYWFTVFFIEKIGRFKIQLM
GFFMMSIFMAIIGVKYDYLKTKEHKWTFAALYGLTFFFANFGPN

>TaPT1 (CAC69856.1, unpublished)
MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGRIYYTDPALNEPGHLPAN
VSAAVNGVALCGTLAGQLFFGWLGDKLGRKSVYGFTLILMVLCSIASGLSFGHEAKGVMGTLCFFRFW
LGFGVGGDYPLSATIMSEYANKKTRGTFIAAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAASIG
PEADYVWRIIVMFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEENVQGERAT
GDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFTKIGWIPPAKTMNALEELYRIARA
QALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTMMTIFMLAIAIPYDYLVKPGHHTGFVVLYGLTFFFA
NFGPNSTTFIVPAEIFPARLRSTCHGISAATGKAGAIIGAFGFLYASQDQKKPETGYSRGIGMRNALFVLA
GTNFLGLLFSLLVPESKGKSLEELSKENVGDDDTIVPTGV

>TaPT8 (AAP49822.1, unpublished)
MATEQLNVLKALDVAKTQLYHFKAVVIAGMGFFTDAYDLFCIALVTKLLGRIYYTDPALNEPGHLPAN
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LGFGVGGDYPLSATIMSEYANKKTRGTFIAAVFAMQGFGILFGTIVTIIVSSAFRHAFPAPPFYIDAAASIG
PEADYVWRIIVMFGTIPAALTYYWRMKMPETARYTALIAGNTKQATSDMSKVLNKEISEENVQGERAT
GDTWGLFSRQFMKRHGVHLLATTSTWFLLDVAFYSQNLFQKDIFTKIGWIPPAKTMNALEELYRIARA
QALIALCGTVPGYWFTVAFIDIIGRFWIQLMGFTMMTIFMLAIAIPYDYLVEPGHHTGFVVLYGLTFFFA
NFGPNSTTFIVPAEIFPARLRSTCHGISAATGKAGAIIGAFGFLYASQDQKKPETGYSRGIGMRNALFVLA
GTNFLGLLFSLLVPESKGKSLEELSKENVGDDDSIAPTGV

>TmPT1 (AAQ06280.1, unpublished)
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IAGMGFFTDAYDLFSASLIADLLGHIYYHSADGKLPGHVAGAVSGVALCGTVLGQLFFGWLGDRMGR
KRIYGVTLKLMVVCSLASGLSFHNKPKCVVATLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFIA
AVFAMQGLGNLAAGAVVLVLSASFKNTAAYDTDHLGQADYVWRIVLMLGAVPALLTYYWRMKMPE
TARYTALIAKNLKLAASDMAAVLDIDFVSDMDAEAVVKQDEFGLFSMEFLHKHGRQLLGTTVCWFVL
DVVFYSLNLFMKDIFNGIGWFGDAAEMSPLEQTYKIARTQAIIVVGGSLPGYFLTVLFVDRIGRIKIQLM
GFTMMTIFMIGLAAPYKFWSKPSMHAGFAIMYALILFFANFGPNSTTFILPTEIFPTRLRSTCNGISAAGG
KCGAIIGVLWFQYSHTSIRSSLLLLAGCNLVGVMFTLALPESKGMSLEDITGEMEEESEPSQESTVAEVE
FIHSVEILMFTTWPARRHACSSLFCHLHGAGSAMLYRVLDAVTSVKCETRRARKQIKVLEALDVAGTQ
LYHFTTIVIAGMGFFTDAYDLFSASLIADLLGHIYYHSADGKLPGHVAGAVSGVALCGTVLGQLFFGWL
GDRMGRKRIYGVTLKLMVVCSLASGLSFHNKPKCVVATLCFFRFWLGFGIGGDYPLSATIMSEYANKR
TRGAFIAAVFAMQGLGNLAAGAVVLVLSASFKNTAAYDTDHLGQADYVWRIVLMLGAVPALLTYYW
RMKMPETARYTALIAKNLKLAASDMAAVLDIDFVSDMDAEAVVKQDEFGLFSMEFLHKHGRQLLGTT
VCWFVLDVVFYSLNLFMKDIFNGIGWFGDAAEMSPLEQTYKIARTQAIIVVGGSLPGYFLTVLFVDRIG
RIKIQLMGFTMMTIFMIGLAAPYKFWSKPSMHAGFAIMYALILFFANFGPNSTTFILPTEIFPTRLRSTCNG
ISAAGGKCGAIIGVLWFQYSHTSIRSSLLLLAGCNLVGVMFTLALPESKGMSLEDITGEMEEESEPSQEST
VAEVEFIHSVEIL

>VvPT1 (XP_002267369.1, Valat et al. 2018)
MSDGLAVLHALDSARTQWYHITAIVIAGMGFFTDAYDLFCISTVSKLLGRLYYYDPTKDKPGKLPHGV
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ADYLWRIVLMLGALPALLTYYWRMKMPETGRYTALIEGNAKQAAADMGRVLEIEIQAEADKVAEFKA
ANEYSLWSREFFDRHGRHLIGTMSTWFLLDIAFYSQNLTQKDIFPAMNLVKKDYEVSALREMFETSRA
MFVVALLGTFPGYWFTVFFIDRIGRFIIQLVGFFMMSLFMLIIGIKYEYLRDDNKWLFAVLYGLTFFFAN
FGPNSTTFVLPAELFPTRVRSTCHAMSAAAGKAGAMIGAFVVATYTLDGKANEIRIAMITMACTNMLG
FFCTFLVTETKGRSLEEISGEDGGINETEMASRPS

>VvPT2 (XP_002267327.1, Valat et al. 2018)
MSNSLAVLHALDSARTQWYHITAIIIAGMGFFTDAYDLFCISTISKLLGRLYYYDPVGSPEKPGKLPHNV
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READFLWRIVLMLGALPALLTYYWRMKMPETGRYTALIEGNAKQAAADMGRVLEIEIQEEADKVAEF
KAANEYSLWSREFFDRHGLHLIGTMSTWFLLDIAFYSQNLTQKDIFPAMNLVNKDYQVSALREMFETS
RAMFVVALLGTFPGYWFTVFFIERIGRYIIQLVGFFMMSLFMLIIGIKYEYLRDDNKWLFAVLYGLTFFF
ANFGPNSTTFVLPAELFPTRVRSTCHAMSAAAGKAGAMIGAFVVATYTLDGKANEIRVAMITMACTN
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>ZmPT1 (NP_001105269.2, Wright et al. 005; PUBMED 16101924)
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NVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLMVMVICSVASGLSFGHTPTGVMATLCFF
RFWLGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGILAGGIVTLVISAAFRAAYPSPAYRDD
HFTSTVPQADIVWRVIVMLGAAPALLTYYWRMKMPETARYTALVAKNAKQAAADMSKVLHTEIVDE
QEKLDAAEGANSFGLFSREFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFTSINWIPKANTMSALEE
VYRISRAQTLIALCGTVPGYWFTVALIDVVGRFAIQLLGFFMMTVFMLGLAIPYHHWTTPGNHIGFVVM
YAFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAASGKAGAIIGAFGFLYAAQNQDRSKTDAGYPAGI
GVRNSLFVLAASNMLGFVLTFLVPESKGKSLEEMSGEAEDSEEEPVGARAVRPSETQMV

>ZmPT2 (NP_001105816.1, Schnable et al. 2009, PUBMED 27304955)
MARGGDGLQVLSALDAAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLLGRIYYTDTSKDNPGSLPP
NVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLMLMVICSVASGLSFGHTPTGVMATLCFFR
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FNSTVPQADYVWRIILILGAAPAMLTYYWRMKMPETARYTALVAKNAKQAAADMSRVLQTEIVDEQE
KLDEMVTAESNTFGLFSREFARRHGLHLVGTSTTWFLLDIAFYSQNLFQKDIFTSINWIPKANTMSALEE
VFRISRAQTLIALCGTVPGYWFTVALIDVVGRFAIQLLGFFMMTVFMLGLAIPYHHWTTPGNHIGFVVM
YAFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAASGKAGAIIGAFGFLYAAQNQDKSKADAGYPAGI
GVRNSLFVLAASNLLGFILTFLVPESKGKSLEEMSGEADDAEDDAVGTRAVRPSGTQMV

>ZmPT3 (AAY42387.1, unpublished)
MAAGDLEVLTALDTAKTQWYHFTAIVVAGMGFFTDAYDLFCISLVTKLLGRIYYTVEGSATPGTLPPH
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AASTPPQADFVWRIILMLGAMPAALTYYWRTKMPETARYTALVAKNAKQAAADMSKVLQVEISAGA
AEEGAAAATATATEPAPASASASFGLFSGEFLRRHGLHLLGTSLTWFLLDIAFYSQNLFQKDIFSAVGWI
PKAATMNALEELFSIARAQSLIALCGTVPGYWFTVALIDVLGRFAIQVTGFLMMTVFMLGLAVPYEHW
TTPGHHIGFIVMYGLTFFFANFGPNATTFIVPAEIFPARLRSTCHGISAASGKLGAIIGSFGFLYLAQNRDP
AKTDHGYPAGIGVRNSLFLLAGCNLLGLAFTFLVPESKGKSLEEMSGENDEAAAAAATPNYNNRTVPV

>ZmPT4 (AAY42388, unpublished)
2005MARGGDGLQVLSALDAAKTQWYHFTAIIVAGMGFFTDAYDLFCISLVTKLLGRIYYTDTSKDSPG
SLPPNVAAAVNGVAFCGTLAGQLFFGWLGDKLGRKSVYGMTLMVMVICSVASGLSFGHTPTGVMATL
CFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGFGILAGGIVTLVISAAFRAAYPSPAYR
DDHFTSTVPQADIVWRVIVMLGAAPALLTYYWLMKMPETARYTALVAKNAKQAAADMSKVLHTEIV
DEQEKLDAAEGANSFGLFSREFARRHGLHLVGTATTWFLLDIAFYSQNLFQKDIFTSINWIPKANTMSA
LEEVYRISRAQTLIALCGTVPGYWFTVALIDVVGRFAIQLLGFFMMTVFMLGLAIPYHHWTTPGNHIGF
VVMYAFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAASGKAGAIIGAFGFLYAAQNQDRSKTDAGY
PAGIGVRNSLFVLAASNMLGFVLTFLVPESKGKSLEEMSGEAEDSEEEPVGARAVRPSETQMV

>ZmPT5 (AAY42388.1, unpublished)
MALGQQLRVLHALDVARTQLYHFMATAIAGMGFFTDAYDFFAISLVMDLISYLYNEEQIDRGVKATIN
GIALCGAVPGQLVFGWLGDKMGRKRIYGVTLLLMVVTSLASGLYFGTNEASNVVAVLCFFRFWLGFGI
GGDYPLSATIMSEYANKRRRGAFIAAVFAMQGFGNLAAGIVAVVVSASFLRTNPRRNANFVWRIVLML
GAVPAILTYYWRMKMPETARYTALVAKDARKAASDMSSVLHVEIIPEDEAVRQDKYGLFSAQFLRYH
GTHLLATSACWLAVDITFYSLNLYMKDIFADVGLIDPPGNNDLFTRMTVTTLLHTGIALCGTLPGYFFT
VAFVDRIGRVRIQLLGFTMMSVLTAILAATYAYWKRQETIQRKMGFAVLYGLTNFFANFGPNTTTFIVP
AEIFPARMRATCHGIAGAFGKIGAIIGVFGFMSNMEEHGVVPRKLWALFASNLVGLVFTFLLPDSKGKS
LEEMAGETEEQQQQQDAAVVAAADHINLVPI

>ZmPT6 (NP_001105776.1, Liu et al. 2016, PUBMED 27304955)
MAAPGGSNLAVLDALDSARTQMYHMKAIVIAGMGFFTDAYDLFCISTVSKLLGRIYYPDDNLYIDKPK
PGTLPVSVNNMVTGVALVGTLMGQLVFGYFGDKLGRKRVYGITLVLMAACAIGSGLSFGSSAHAVIGT
LCFFRFWLGFGIGGDYPLSATIMSEYSNKKTRGAFIAAVFAMQGVGIIFAGLVSMIVSGILLHYHPAPAW
KENHDRSWQDQMPAADYMWRIVLMIGAFPALATFYWRMKMPETARYTALIEGNAKQAANDMQKV
MDVEIQAEQDKLARYKAANDYPLLSREFARRHGLHLIGTATTWFLLDIAFYSQNLTQKDIFPAIKLTSPV
DDINALKEVFEISKAMFLVALLGTFPGYWVTVALIDKMGRYLIQLIGFFMMSVFMLLMGVMYNDLKN
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HIKKALIILSVTNILGFFFTFLVPETMGRSLEEISGEDGNVENGPGAPAGVAMGVADVSKDDKMPVSSTE
WQSSMHA

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Chapitre V

Evidence that common arbuscular mycorrhizal network can overcome phosphate shortage in walnut and maize plants

1. Introduction

Inorganic phosphate (Pi) availability, as the orthophosphate anion, PO_4^-, is a major factor constraining plant growth and metabolism in many soils worldwide (Bates and Lynch 2001). Thus, application of large amounts of Pi fertilizers are used to ensure plant productivity in most conventional agricultural systems (S. E. Smith et al. 2011). From 34.3 Tg/year in 2000, Tilman (2001) predicted that the global consumption of P fertilizer will reach 83.7 Tg/year in 2050 because of worldwide intensification of agriculture to feed humankind. However, only about 10–30% of the P fertilizer applied in growing season is taken up by the roots, with a substantial part accumulated in the soil as residual P not readily available for plants (Syers, Johnston, and Curtin 2008). In addition, injudicious and untimely application of chemical fertilizer in agricultural field has generated environmental concerns (Tilman 2001; Foley 2005), including soil degradation and water eutrophication (Conley et al. 2009). To mitigate environmental degradation and because P fertilizer supplies are threatened due to finite phosphate rock resources (Cordell et al. 2009), it is therefore important to enhance P-use efficiency in crop production through optimized application of P fertilizers and utilization of residual P and other P pools from soils (Xue et al. 2016; Schneider-Maunoury et al. 2019). As a substitute to conventional cropping systems, which rely on large inputs of chemical fertilizers and other external inputs to sustain production, agroforestry is a low-input system which combines trees with annual crops in various combinations or sequences (Nair 1993). Agroforestry systems are designed with the aim of reducing the need for inputs through minimising losses and maximising internal cycling of nutrients (Smith, 2010). One of the major arguments in favour of agroforestry is the fact that a mixed system of trees and annual crops make a better use of natural resources such as light, water and nutrient more efficient than a monoculture. As resources are used more efficiently, less agrochemicals may be required to the benefit of the overall environment (Postma, 2005).

A fundamental aspect of agroforestry is to favour inter-specific facilitation between tree rows and cultivated alleys, where plants increase the growth and survival of their neighbours (Callaway 1998), particularly regarding limited resources such as nutrients (Schoeneberger et al. 2012; Battie-Laclau et al. 2020). In the maintenance of soil P fertility under alley cropping systems, the role of roots is at least as important as that of above-ground biomass (Young 1991; Young and Young 1997; van Noordwijk et al. 2015). Belowground facilitation occurs when one species makes previously unavailable P available to the other, due to the exudation of organic acids, phosphatases, or rhizosphere acidification resulting in increased P availability (Hinsinger et al. 2011). Since a decade ago, numerous studies have also pointed to the importance of soil-borne arbuscular mycorrhizal (AM) fungi of the phylum Glomeromycota (Tedersoo, Bahram, and Zobel 2020) in mediating plant-plant facilitation processes (van der Heijden, Bardgett, and van Straalen 2008; Montesinos-Navarro et al. 2016). In soils with strong Pi-fixing capacity, or where Pi is not adequately supplied, plant demand for this nutrient exceeds the rate at which it diffuses into the root zone, resulting in zones of Pi depletion surrounding the roots (Javot, Pumplin, and Harrison 2007; S. E. Smith and Read 2009). AM fungi help to overcome this limitation by extending their extra-radical hyphae from root surfaces to areas beyond the P depletion zone (Hayman 1983; Javot, Pumplin, and Harrison 2007). Consequently, AM fungi have the potential to maintain crop yields at low soil P levels

through a more effective exploitation of available P sources. It has been estimated that inoculation with AM fungi might result in a reduction of approximately 80% of the recommended fertilizer P rates under certain conditions (Jakobsen, Abbott, and Robson 1992). In return, AM fungi are supplied with sugar and lipid from the host plant (Gutjahr and Parniske 2013; Roth and Paszkowski 2017). Conversely, the importance of the role of mycorrhizal symbiosis in providing Pi to the plant is declining when soil phosphate concentration is elevated (Kiers et al. 2011).

As many other tree species used in tropical and temperate alley cropping systems such as ash, cocoa, coffee, eucalyptus, olive tree, paulownia, poplar, and rubber (Cardoso 2002; Janos 2007; de Kroon et al. 2012), walnut exhibits a high dependency on symbiotic AM fungi for their development due to coarse roots and relatively few roots per plant, which limit soil Pi uptake (Brundrett 1991; Comas and Eissenstat 2009; Comas, Callahan, and Midford 2014). Walnut is the common name given to twenty-one to twenty-five species of deciduous trees belonging to the genus *Juglans* (order Fagales, family Juglandaceae). By providing wood, ornamental, and nutrition value to human beings, and food and a habitat to wildlife, walnuts belong to the most important trees in the northern hemisphere, ecologically and economically (Bernard, Lheureux, and Dirlewanger 2018). Among them, the English walnut *J. regia* L. is the principal species cultivated worldwide for the production of edible nuts (Bernard, Lheureux, and Dirlewanger 2018b) with 3.8 million tons harvested worldwide in 2017 (fhttp://www.fao.org/faostat/en/#data/QC) on over an estimated six million hectares (Kostenko, Pechko, and Ivanova 2018). Because of its short growing season, sparse canopy and deep rooting system, walnut is an ideal species for agroforestry and belongs to the dominant trees used in temperate alley cropping (Wolz and DeLucia, 2018). Intercropping of *Juglans* spp. is aimed at increasing growth and quality of walnut trees together with providing an early financial return to help offsets the costs associated with establishing walnut orchards (Van Sambeek and Garrett 2004; Mohni, Pelleri, and Hemery 2009; Wolz and DeLucia 2019). Before the walnut plantation reaches economic maturity, the intercrop—winter cereals (*Triticum* spp), alfafa (*Medicago sativa*), soybean (*Glycine max*), or summer crops (e.g., maize) - is the only income during the first five to ten years; then, thereafter both trees and intercrops produce simultaneously (Mary 1998). Among these AM-dependent intercrops, maize (*Zea mays* L.), is one of the most cultivated crops for both staple food and industrial usage, in tropical and temperate soils worldwide, with phosphorus being a major growth limiting factor for commercial maize production (Brady & Weil, 2008).

The role of AM fungi in mixed plant communities is noticeably amplified by their low host specificity: extra-radical hyphae can connect the roots of different plant species to form a common mycelial network (CMN) and therefore enhance both tree and crop growth in alley agroforestry systems (Ingleby et al. 2007). This inter-plant mycorrhizal connection is able to promote facilitation through differential access to the nutrient pool from the common hyphal network (van der Heijden and Horton 2009), and to plant-to-plant nutrient transfers (Martins and Cruz 1998; Fitter et al. 1998; Leake et al. 2004; Hauggaard-Nielsen and Jensen 2005; van der Heijden and Horton 2009; Walder et al. 2012; Fellbaum et al. 2014; Gorzelak et al. 2015; Montesinos-Navarro et al. 2016). Noticeably, when associated to deep perennial tree roots, AM fungi can increase the mycorrhiza-mediated uptake of soil nutrients, and thereby contribute to the nutrition of the co-cultivated species (Simard and Durall 2004; de Carvalho et al. 2010; Bainard, Klironomos, and Gordon 2011; Bainard et al. 2012; Ingleby et al. 2007). In parallel, when focussing on the opposite, namely the effect of intercrops on tree growth, it has also been shown that the presence of trees and crops within agroforestry systems results in larger tree growth than that observed in a simple forestry systems, due to a better mineral nutrition of the trees even in low input systems (Jose et al. 2000). To date, improved tree growth has been ascribed to the likely ability of trees roots to intercept and uptake a significant proportion of nutrient fertilizer residues from the crop rooting zone (Smith 2010), but the contribution of

inter-plant mycorrhizal connections to this process yet has not been investigated. Because improving tree mineral nutrition through agroforestry systems is a low-cost means to increase tree growth and to shorten the development duration before tree harvesting (Chifflot 2009), this information is of importance to assess the overall costs or benefits of growing trees and annuals.

In this study, we investigated the extent to which intercropping with maize contributes to the mineral nutrition and biomass production of young hybrid walnut plants through the formation of a common AM fungi mycelial network. To this aim, we experimentally simulated a walnut agroforestry system in an home-made experimental device in which young walnut hybrid roostocks were connected or not by a common mycelial network to maize plants grown under contrasting Pi supply levels (0.13 *vs* 1.3 mM). This experimental-device was made of two separated compartments in which the walnut and the maize were planted and connected by a third compartment in which the extra-radical mycelium responsible for mineral nutrient supply for the plants, was separated by fine nylon nets from the associated host roots. We hypothesized that (1) the inoculum reservoir formed by walnut roots would allow the mycorrhization of maize roots through the formation of a CMN, thereby giving access to the maize nutrient pool; (2) hyphal connections under P deficient condition would result in a benefit comparable to P sufficient condition without a CMN; and (3) due to a slower growth rate than maize, walnut would invest more carbon in the development of extra-radical AM fungi hyphae than the maize.

2. Materials and Methods

2.1 Biological material

Maize (*Zea mays* L. var. Pompeo) seeds were surface-disinfected for 15 min in a commercial solution of sodium hypochlorite (1% available chlorine), and rinsed thoroughly. After imbibition in sterile water for 12 h in the dark at 4 °C, seeds were germinated at 20 °C with 16 h of fluorescent light (Osram Fluora L36W/77) on filter papers moist with sterile water. Maize plantlets were sown in microcosms at the third leaf stage. Micropropagated plantlets of the walnut hydrid rootstock RX1 (*J. regia x J. microcarpa*) (Browne et al. 2015) were provided by Dr. Laurent Jouve (LJ Biotech, Souillac, France). Rootstocks were received seven weeks after root induction and placed for *ex vitro* acclimatization in closed mini-greenhouses (Rapid Grow, Nortene) filled with a sterilized (120 °C, 12h) quartz sand (Biot, France): zeolite (Symbion, Czech Republic) substrate (1:1, v:v). They were sprayed twice a week with tap water to maintain humidity and fertilized once a week with 15 ml per plant of a NPK 6-4-6 solution containing 2% of magnesium and oligoelements (Algoflash, France). Walnut cultures were maintained in a growth chamber under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night, 100% relative humidity, 220 µE m-2 s-1). After two months of growth, plantlets were gradually exposed to reduced relative humidity by progressively removing the box covers during a further month, then RX1 rootstocks were sown in microcosms. The isolate DAOM197198 (also known as DAOM181602) of the model AM fungus *Rhizophagus irregularis* originally isolated from Pont Rouge, Canada (Stockinger et al. 2009) and commercialized by Agronutrition (Labège, France) was purchased from MYCAGRO (http://www.mycagrolab.com/31.html, Bretenière, France), as an axenic spore suspension (200 spores/ml).

2.2 Microcosm set-up, plant inoculation, and phosphate fertilization

Microcosms were set-up in an home-made device comprising tripartite compartments consisting of two root hyphal compartments, each connected to one central hyphal compartment (**Figure V.1**). Root hyphal compartments were made out of PVC tubes, each having a hole cut to allow the insertion of a horizontal central PVC tube (7.5 cm length, 5 cm inner diameter) containing the hyphal compartment. The walnut mycorrhizal donor plant compartment was designed wider (25 cm length, 12 cm inner diameter) than the maize plant receiver compartment (25 cm length 7.5 cm, inner diameter) to allow maize plants to explore beyond their own compartment (Teste et al. 2015). To create an air gap, a PVC ring (3.5 cm length, 5 cm external diameter) was vertically inserted in the central PVC tube and distributed at equal distance from each root hyphal compartment. In order to prevent a substrate-mediated diffusion of solutes between both sides of the PVC ring, the air gap was sandwiched between two 1-mm wire meshes using two PVC seals. Both sides of the air gap had a 40-µm nylon membrane (Anachem, Bedfordshire, UK) through which hyphae, but not plant roots, can grow (Focatelli and Smith, 2010). Two additional boxes were attached to the base of each of the root hyphal compartments to collect drainage solutions. Root hyphal and central compartments were filled with the corresponding volume of a sterilized (120 °C, 12h) quartz sand (Biot, France): zeolite (Symbion, Czech Republic) substrate (1:1, v:v). Walnut rootstocks were planted into the wider root hyphal compartment and maize seedlings into the other one. To assess whether mycorrhizal walnuts can benefit maize plant growth through a CMN, four treatments were compared. For the mycorrhizal modality, to check whether inoculated tree crop in the nursery should serve as infection sources for the interrow crop via a CMN, only donor walnut plants were inoculated (AM_Jug) or not (NM_Jug) with 1 ml of the spore suspension of *R. irregularis*. For the Pi modality, only recipient maize plants were fertilized in order to simulate nutrient fertilizer residues in the crop rooting zone. To this aim, maize plant received once a week 10 ml of either a low-Pi (Zea_P/10: 0.13 mM NaH_2PO4, $2H_2O$) or a full strength Pi solution (Zea_P: 1.3 mM NaH_2PO4, $2H_2O$) Hoagland solution (Bonneau et al. 2013), while walnut donor saplings received 10 ml tap water. For each treatment, the experimental setup was replicated at least six times. Plants were maintained in a growth chamber under controlled conditions with 16 h of light [220 µE m^{-2} s^{-1}] at 23 °C and 8 h of dark at 19 °C for two months before harvest. The experiment was performed with at least six independent replicates per treatment.

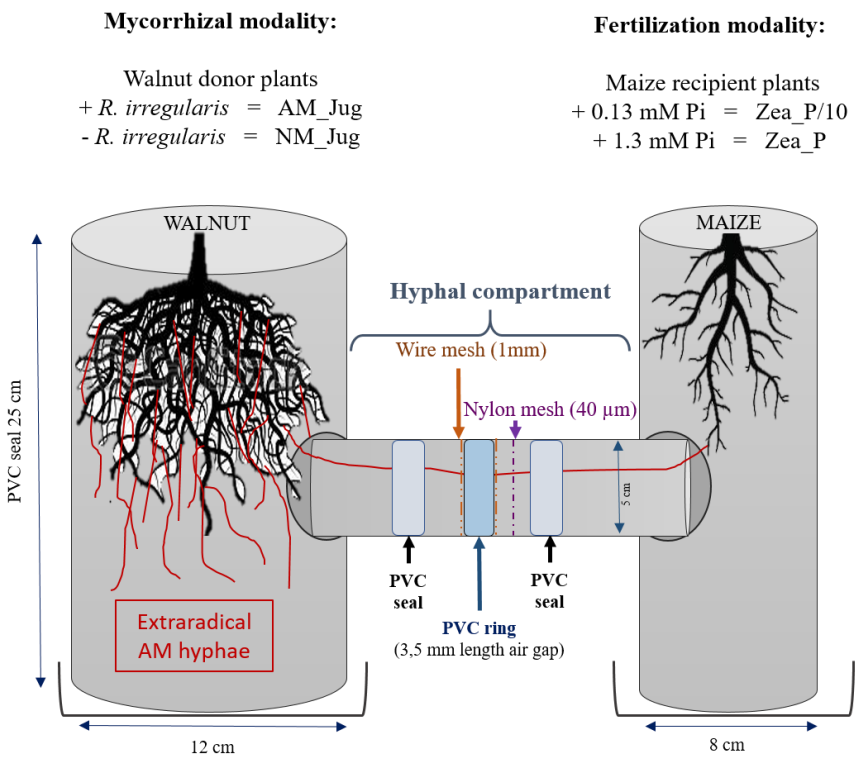

Figure V.1. Schematic representation of the home-made device comprising two planted microcosms connected by a third one specific for CMN of AM fungal hyphae and the four treatments tested in this study.

2.3 Measurement of plant growth and nutritional parameters

Before harvest, the leaf chlorophyll *a* fluorescence was quantified on attached donor and recipient leaves using a Pulse-Amplitude-Modulation (PAM) Chlorophyll Fluorimeter (Junior PAM, Walz, Effeltrich, Germany). Plants were dark-adapted for at least one hour and measurements were then performed on leaf adaxial side. From these measurements, the maximum photochemical quantum yield of PS_{II} (Fv/Fm) and the effective photochemical quantum yield of PS_{II} (Y_{II}) were calculated according to Kitajima 1975 and Genty 1989 (Kitajima and Butler 1975; Genty, Briantais, and Baker 1989), as described in the fluorimeter operator's guide. For plant growth parameters, plant height (cm), branch or leaf number per plant, stem diameter at the soil line (mm), fresh shoot and root weights (g), and primary root length (cm) were measured after two months of growth. To determine dry matter weights (DW), shoot and root subsamples were separately weighed fresh, and subsequently dried for 2 days at 80°C and weighed. For soluble Pi, $\delta^{13}C$, %C and %N measurements, dried root and leaf samples were finely ground using a Retsch Mixer ball mill (Haan, Germany). Soluble Pi was extracted from 5 mg dried samples as described by Grunwald et al. (2009). Free Pi was determined spectrophotometrically by measuring absorbance at 650 nm using the BIOMOL Green™ Reagent (Enzo Life Sciences, Villeurbanne, France) according to the instructions of the manufacturer. Root and leaf dried aliquots (5 mg) were analysed for their ^{13}C abundance, %C and %N contents at the Biogeosciences Laboratory of the University of Bourgogne Franche-Comté (Dijon, France) on a FlashSmart Elemental Analyzer (Thermo Scientific) coupled in continuous flow mode to a Delta V stable isotope ratio mass spectrometer (Thermo Scientific). USGS40 L-Glutamic acid (C = 40.8 wt%; δ13CVPDB=−26.39 ± 0.04‰) and IAEA-600 Caffeine (δ13CVPDB=−27.77 ± 0.04‰), with certified reference materials used for calibration. The carbon isotopic composition was expressed in delta notation and reported in per mile (‰)

relative to the Vienna Pee Dee Belemnite (V-PDB) standard; external reproducibility based on duplicate analyses of samples was better than ± 0.2‰ (1σ).

2.4 Staining of AM fungi in plant roots and quantification of root colonization

At harvest, root samples were randomly collected from each plant and dried for 2 days at 80 °C. They were cleared with at 90 °C for 30 min in 10% (w/v) KOH (Phillips et al. 1970). Walnut roots were additionally bleached with three drops of 30% H_2O_2 to remove any phenolic compounds left in cleared roots (Kormanik and McGraw 1982). Roots were rinsed with tap water before staining in a 0.05% (w/v) trypan blue solution in lactoglycerol (lactic acid-glycerol demineralised water, 1:1:1) for 30 min at 90 °C. Thirty fragments, ca. 10 mm long, were randomly collected from each sample, mounted 15 per slide in glycerol and observed at X 200 magnification as described in Trouvelot et al. (1986). Calculations of frequency of mycorrhization (F%), percentage of root cortex colonisation (M%) and percentage of arbuscules (A%) were carried out with the MycoCalc program (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

2.5 Mycelium harvest from the fungal compartment and quantification of root colonization mycorrhiza colonization

At harvest, the extra-radical mycelium developped in the hyphal compartment was collected by dispersing the substrate with tap water and fishing it from the surface using a 32 µM mesh. These steps were repeated several times. Afterwards, the hypha samples were dried for 2 days at 80°C and weighed. Root samples were randomly collected from each plant and dried for 2 days at 80 °C. They were cleared with at 90 °C for 30 min in 10% (w/v) KOH (Phillips et al. 1970). Walnut roots were additionally bleached with three drops of 30% H_2O_2 to remove any phenolic compounds left in cleared roots (Kormanik and McGraw 1982). Roots were rinsed with tap water before staining in a 0.05% (w/v) trypan blue solution in lactoglycerol (lactic acid-glycerol demineralised water, 1:1:1) for 30 min at 90 °C. Thirty fragments, ca. 10 mm long, were randomly collected from each sample, mounted 15 per slide.

2.6. Identification of proteins orthologous to the AM-essential phosphate transporter MtPT4 in *J. regia*, *J. microcarpa* and *Z. mays*

To identify walnut RX1 and maize proteins orthologous to the AM-essential phosphate transporter MtPT4 in *Medicago truncatula* (Harrison et al. 2002), we used Orthofinder (version 2.2.6; Emms and Kelly 2015) through standard mode parameters in the Diamond tool (v0.9.22.123) all-versus-all. To this aim, the complete predicted proteome sequences of 25 plant species were retrieved as described previously (Chapter IV, **Table IV.S1**). The proteome of *J. regia*, *J. microcarpa*, *M. truncatula* and *Z. mays* were download at https://treegenesdb.org/FTP/Genomes/Jure/v2.0/annotation/Jure.2_0.pep.fa.gz,https://treegenesdb.org/FTP/Genomes/Jumi/v1.0/annotation/jumi.1_0.peptides.fa,https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/Genomic sequence 5.0 and FASTA formatted 5.1.7 annotated features/MtrunA17r5.0-ANR-EGN-r1.7.prot.fasta, and ftp://ftp.ensemblgenomes.org/pub/release43/plants/fasta/zea_mays/pep/Zea_mays.B73_RefGen_v4.pep.all.fa.gz, respectively.

2.7 Primer design

On the basis of the plant orthologous proteins identified using Orthofinder, corresponding nucleotide sequences were retrieved to design couple of oligonucleotide primers with Primer-BLAST (https://www. ncbi.nlm.nih.gov/tools/primer-blast). To monitor Pi acquisition through the fungal side, expression pattern of the gene coding for the high affinity

transporter *RiPT1* previously found to be regulated by external Pi concentrations in *R. irregularis* (Calabrese 2019) was monitored in walnut and maize roots extracts of mRNA. All primers used are listed in **Table V.S1**, including the reference genes used for expression normalization.

2.8 mRNA extraction, reverse transcription and qRT-PCR

At harvest, maize and walnut root subsamples (1.5 g) were immediately frozen in liquid nitrogen and kept at -80 °C before use. They were ground to a fine powder with a pre-cooled mortar and pestle before mRNA extraction. The SV Total RNA Isolation System (Promega, Madison, USA), which incorporates a DNase treatment step, was used to recover total root RNA from 50 mg ground tissues, according to the supplied instructions. For walnut, 50 mg of insoluble polyvinylpyrrolidone were added to root samples before mRNA extraction in order to remove polyphenols (Hu et al. 2002). The purified mRNA was quantified by measuring the absorbance at 260 nm with a Nanodrop (ND-1000 spectrophotometer, Isogen Life Sciences, Maarssen, Netherlands). Complementary DNAs (cDNAs) were obtained using the iScriptTMcDNA Synthesis Kit (BIO RAD Laboratories, Paolo Alto, CA, United States), using 300 ng of total mRNA per reaction. Quantitative RT-PCRs were run in a 7500 real-time PCR system (Roche) using the following settings: 95°C for 3 min and then 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s. These analyses comprised at least five biologicals and three technical replicates per treatment. Transcript levels were expressed as normalized expression to reference genes according to the following formula (with E referring to the PCR efficiency):

$$R = \frac{E_T^{\Delta Ct_T(Cm-I)}}{\sqrt{\left(E_{\text{ref1}}^{\Delta Ct_{\text{ref1}}(Cm-I)}\right)\left(E_{\text{ref2}}^{\Delta Ct_{\text{ref2}}(Cm-I)}\right)}}$$

2.9 Statistical analyses

The plant mycorrhizal colonization parameters of each plant were compared between the two fertilization regimes with the Welch's t-test (unequal variances t-test), after *arcsin square root* transformation of percentages. For comparison of growth, nutritional and gene expression parameters between treatments, the Kruskal-Wallis H-test with post-hoc Tukey HSD (Honestly Significant Difference) were used under the Rstudio environment (version 4.03) due to non-Gaussian distribution of data and/or inequality of variances, as checked by the the Kolmogrov–Smirnov's and and Levene's tests, respectively. *P*-values less than 0.05 were used for statistical significance.

3. Results

3.1 Mycorrhizal colonization and extra-radical hypha development

In walnut roots inoculated with *R. irregularis*, the frequency of colonization (F%), the intensity of root cortex colonization (M%) and arbuscule abundance (A%) in the Zea_P/10 treatment reached 100, 20 and 10%, respectively (**Figure V.2A**). The intensity of root cortex colonization and arbuscule abundance in AM-Jug roots were significantly ($p < 0.05$) higher in the Zea_P/10 treatment when compared to the Pi-replete condition (Zea_P) (**Figure V.2A**). In non-inoculated maize roots connected to AM walnut plants, F, M, and A% values reached 100, 30 and 25%, respectively, in both Pi fertilization regimes (**Figure V.2B).** Whatever the fertilization regime applied to maize plants, extra-radical hyphae were visualized after trypan blue-staining of the substrate contained in the hyphal pipe close to the maize root compartment

(**Figure V.2C**). In both Pi fertilization regimes, walnut and maize plants were not colonized when the walnut donor plant was not inoculated.

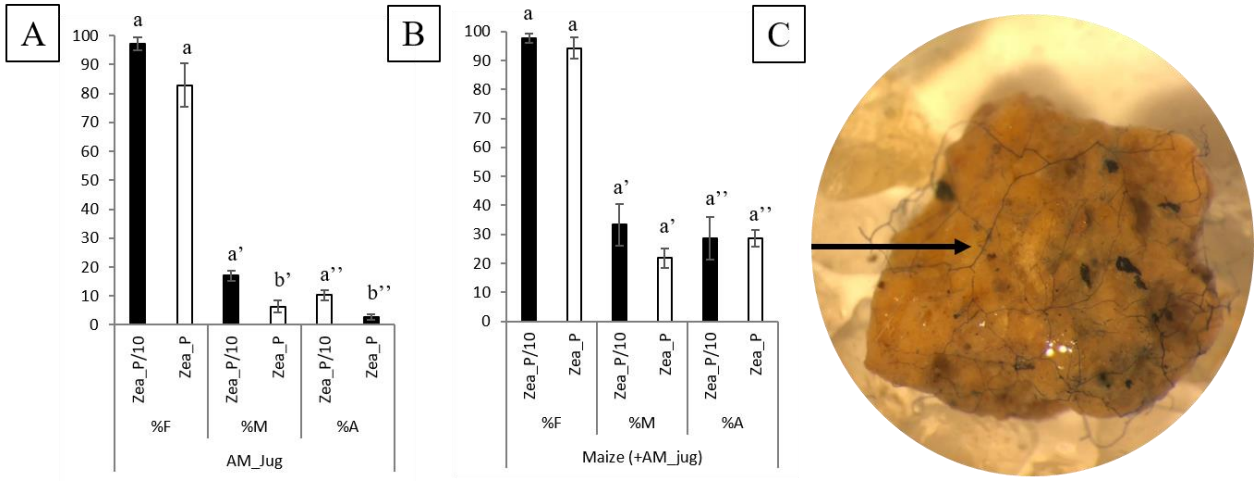

Figure V.2. Mycorrhizal colonization parameters measured two months after inoculation of walnut RX1 plants with *R. irregularis* under contrasting Pi fertilization regimes of maize (Zea_P/10, Zea_P) in (**A**) walnut donor and, (**B**) non-inoculated maize recipient roots. Values are means of six biological replicates. Error bars represent the SE. Lower case letters indicate significant difference (Welch's t-test after arcsin square root transformation; $p < 0.05$). Arrow show the development of the extra-radical mycelium in the hyphal compartment close to maize roots after trypan blue-staining, irrespective of the Pi fertilization regime of maize (**B**).

3.2. Effect of the mycorrhization of walnut and of the maize Pi availability on walnut growth and nutritional parameters

In walnut roots, in both Pi-limited (P/10) and Pi-replete (P) fertilization regimes of maize**,** mycorrhization of walnut (AM_Jug) led to a significant increase ($p < 0.05$) in root collar, root and shoot dry weights, relative to non-mycorrhizal (NM_Jug) plants (**Figure V.3A**). Under the maize Pi-limited condition, but not in the Pi-replete condition, AM walnut plants displayed a higher ($p < 0.05$) stem height, leaf Pi concentration and effective photochemical quantum yield of PSII (Y_{II}), together with a decreased root carbon content and shoot to root biomass ratio when compared to NM saplings (**Figure V.3B**). All the other parameters measured in walnut plants (the maximum photochemical quantum yield of PS II (Fv/Fm), primary root length, the number of branches, root soluble Pi, leaf %C, plant %N, $\delta^{13}C$, and leaf C/N ratio) were not significantly modified by either walnut mycorrhization or Pi fertilization regime applied to maize plants (**Table V.S2**).

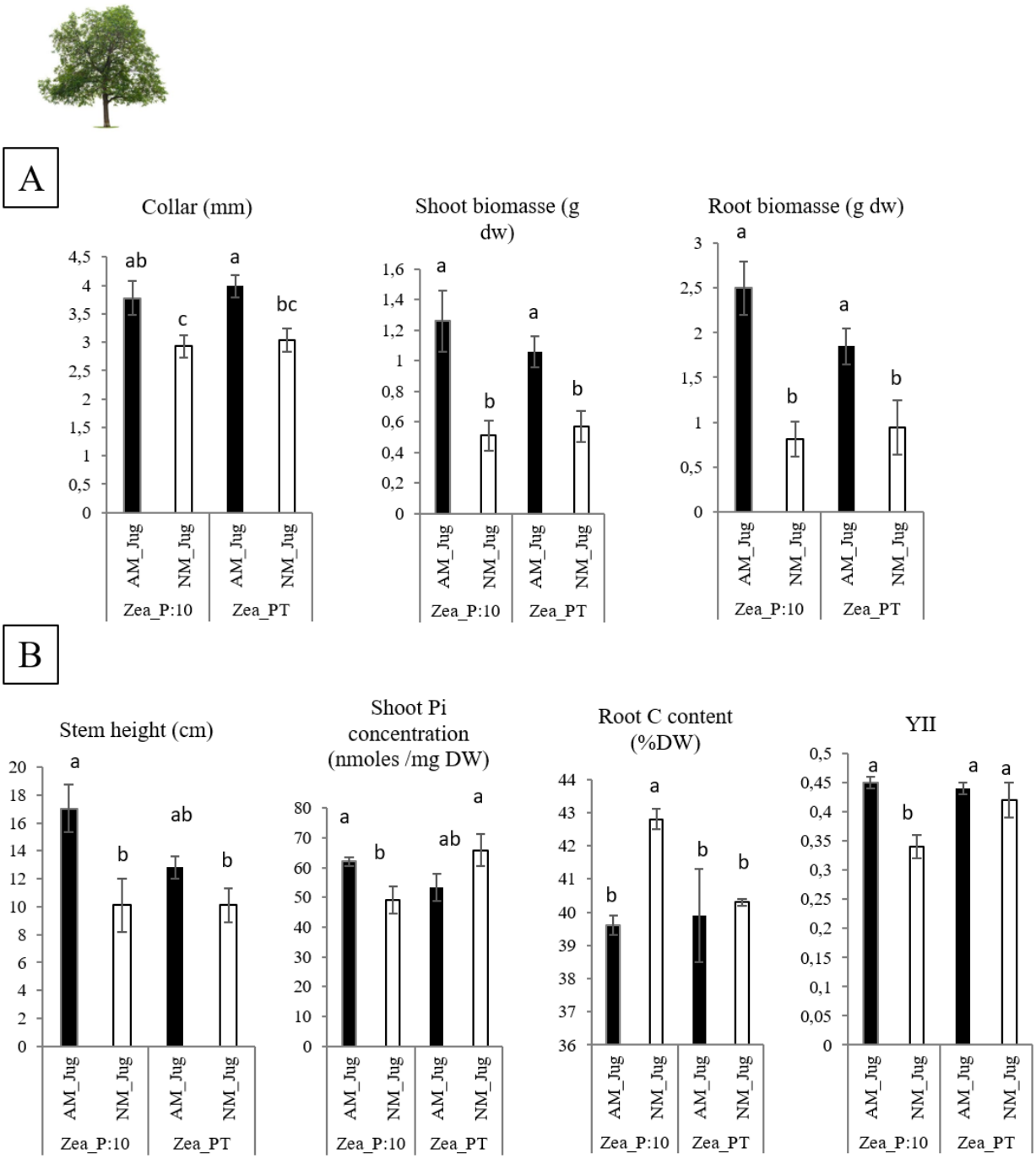

Figure V.3. Effect of the mycorrhization of the walnut (AM) as compared to non-mycorhizal controls (NM), growth and nutritional parameters of walnut irrespective of Pi fertilization regimes of *Z. maïs* (**A),** or only recorded in the P/10 condition (**B**). Values are means of six biological replicates. Error bars represent the SE. Lower case letters indicate significant difference (Kruskal-Wallis H-test with post-hoc Tukey HSD; $p < 0.05$).

3.4. Effect of the walnut mycorrhization and of the maize Pi fertilization on *Z. mays* growth and nutritional parameters

In maize plants, walnut mycorrhization with *R. irregularis* (AM_Jug) as compared to NM plants, led to a significant increase in root collar diameter, height, leaf number, root and shoot DW, leaf N content and Pi concentration, and to a decrease in leaf C/N ratio under the maize Pi-limited condition, but not in the Pi-replete condition (**Figure. V.4**). The other parameters measured in maize plants namely Y_{II}, Fv/Fm, primary root length, shoot to root biomass ratio, root Pi, root and leaf %C, %N, and $\delta^{13}C$, were not significantly modified by the mycorrhization of walnut with *R. irregularis* (**Table V.S2**). As regards Pi availability, the maximum photochemical quantum yield of PS_{II} (Fv/Fm) was significantly but slightly decreased only in NM plants under the low-Pi condition, when compared to the Pi-replete fertilization regime (**Table V.S2)**.

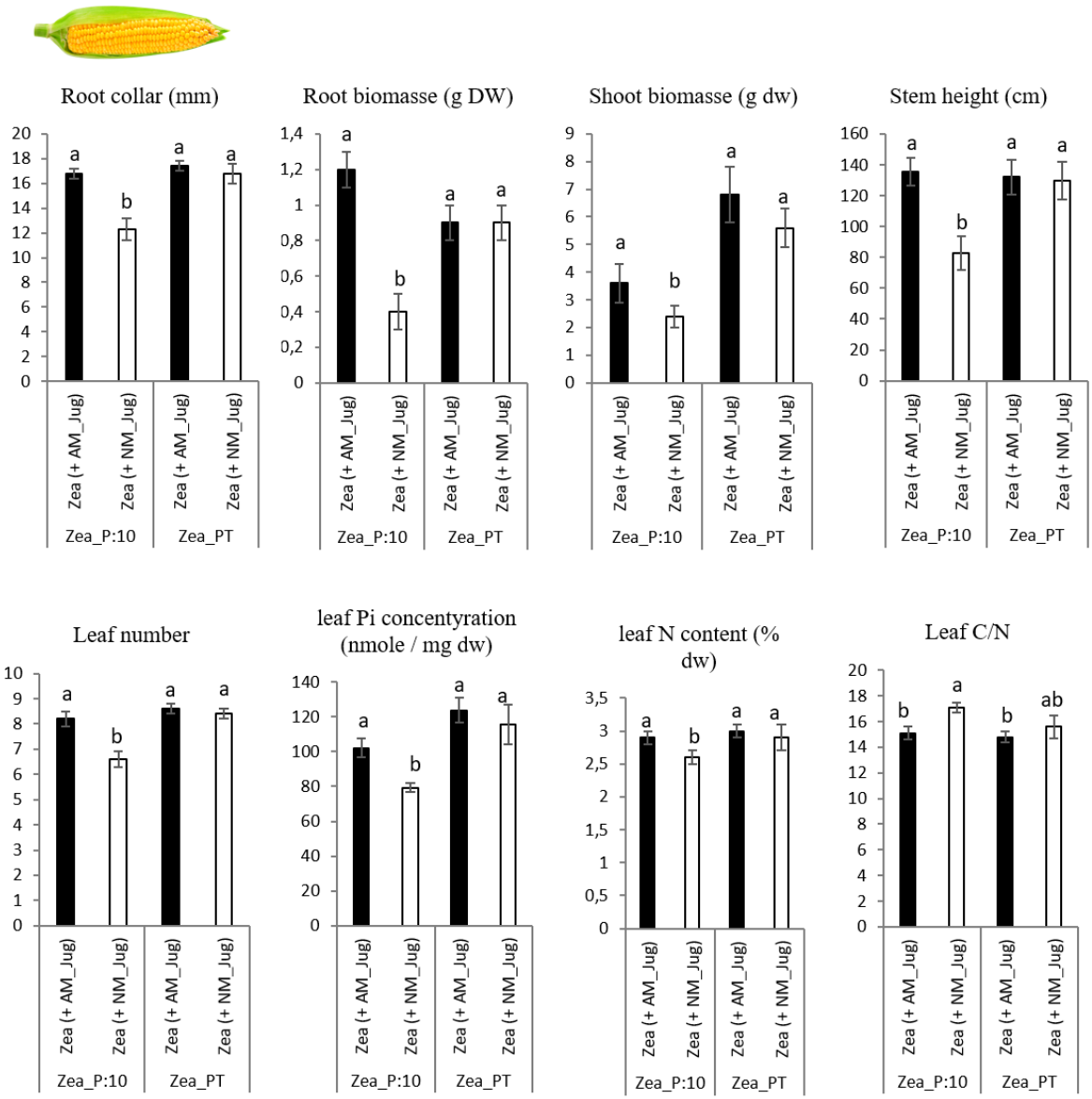

Figure V.4. Effect of the mycorrhization of walnut (AM) as compared to non-mycorhizal controls (NM), on growth and nutritional parameters of maize plants occurring under the Pi/10 condition. Values are means of five biological replicates. Error bars represent the SE. Lower case letters indicate significant difference (Kruskal-Wallis H-test with post-hoc Tukey HSD; $p < 0.05$).

3.5. Identification of walnut and maize proteins orthologous to phosphate transporter MtPT4 specific for AM symbiosis

To identify walnut and maize proteins orthologous phosphate transporter MtPT4 essential for AM symbiosis, we performed an OrthoFinder analysis that included the predicted proteome of *J.microcarpa*, *J. regia*, *Z. mays*, and the reference proteome of *M. truncatula*. OrthoFinder outputs (**Chapter IV**, **Table V.S1**) indicated that the AM-essential Pi transporter MtPT4 orthogroup contained three proteins of *J. microcarpa* (Jumi_00855.t1, Jumi_15132.t1, Jumi_21692.t1, further named JmPHT1;1, JmPHT1;2, JmPHT1;3, respectively), three proteins of *J. regia* (Jr13_30200_p1, Jr13_30210_p, Jr16_00830_p1, further named JrPHT1;1; JrPHT1;2, JrPHT1;3, respectively) and one protein of *Z. mays* (Zm00001d011498_P001), which turned out to be the AM-essential Pi transporter ZmPHT1;6 previously described in maize (Glassop, Smith, and Smith 2005; Tian et al. 2013).

3.6. Phosphate transporter gene expression

To monitor soil Pi acquisition through the AM fungal netwework, we monitored in walnut RX1 and maize roots the expression pattern of the gene coding the high affinity phosphate transporter *RiPT1* (called RiPT302-_Jug and RiPT302-_Zea) of *R. irregularis*. As expected, analysis of transcript levels in non-inoculated (NM_Jug) walnut and maize roots showed no expression of *RiPT1* **(Figure V. 5A, B**). By contrast, inoculation of RX1 (AM_Jug) with *R. irregularis* resulted in a significant ($p < 0.05$) induction of the expression of the gene *RiPT1* not only in walnut roots **(Figure V. 5A**), but also in maize roots **(Figure V.5B**), whatever the Pi fertilization provided to maize. These results indicated that the expression of the gene *RiPT1* is induced in the mycorrhizated donor RX1 and but also in the recipient maize roots. Similar expression levels of *R. irregularis* reference genes in walnut and maize roots. Further pair-wise comparison between AM walnut and AM maize roots, indicated a higher expression of the *RiPT1* gene in maize roots than in walnut roots both under the lowest (Zea_P/10, $p < 0.08$) and the highest Pi supply (Zea_P, $p < 0.03$). This showed that the contribution of the extra-radical mycelium of AM maize roots to soil Pi acquisition is higher than that of AM walnut roots.

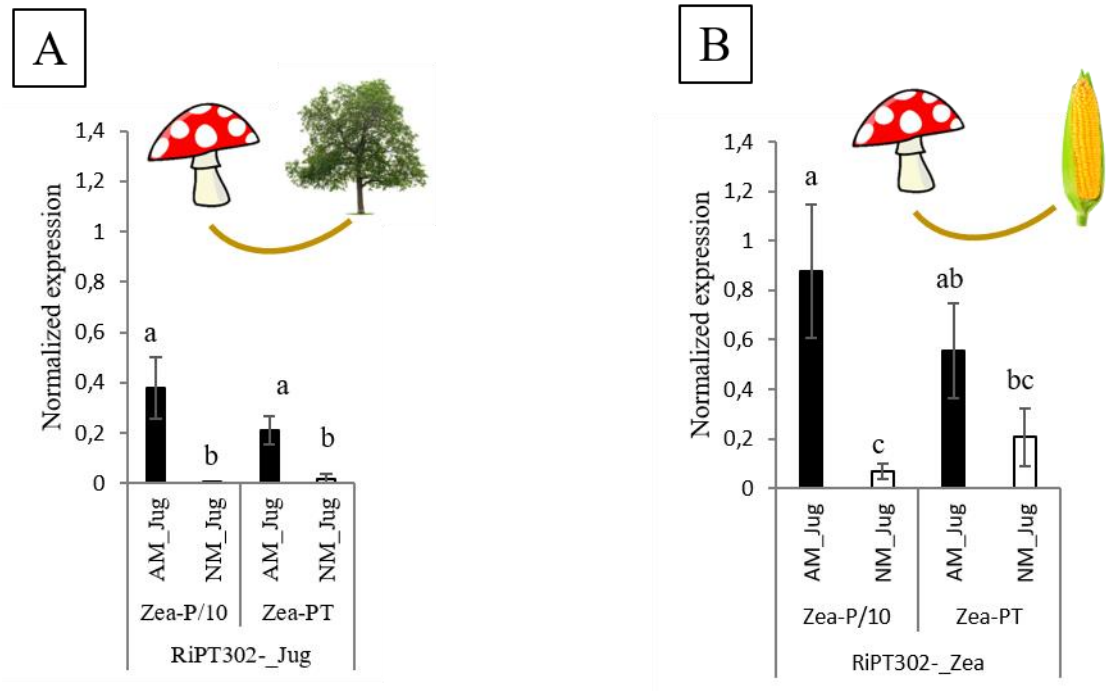

Figure V.5. Expression pattern of the gene *RiPT1* coding for the phosphate transporter of *R. irregularis* two months after its inoculation (AM_Jug) as compared to not inoculated (NM_Jug)

of walnut RX1 rootstocks under contrasting Pi fertilization regimes of maize (Zea_P/10, Zea_P) in walnut donor (**A**), and non-inoculated maize recipient roots (**B**). Expression patterns were quantified by RT-qPCR and expressed as normalized expression to the two fungal reference genes described in **Table V.S2** Values are means of at least five biological replicates. Error bars represent the SE. Lower case letters indicate significant difference ($p < 0.05$) according to the Kruskal-Wallis H-test with post-hoc Tukey HSD.

With respect to plant Pi acquisition through the mycorrhizal pathway, we analysed the expression of the maize AM-essential phosphate transporter *ZmPHT1;6* owing to its unique identification through Orthofinder Chapter IV, **Table V.S2**). As expected, analysis of transcript levels in non-mycorrhizal (NM_Jug) maize roots showed no expression of *ZmPHT1;6* **(Figure V. 6**). By contrast, inoculation of the donor walnut RX1 (AM_Jug) with *R. irregularis* resulted in a significant ($p < 0.05$) induction of *ZmPHT1;6* in recipient maize roots whatever the Pi fertilization provided to maize. These results comforted that in our experimental conditions, the expression of the gene *ZmPHT1;6* is induced by the colonization of maize roots with CMN *R. irregularis* network irrespective of the Pi supply.

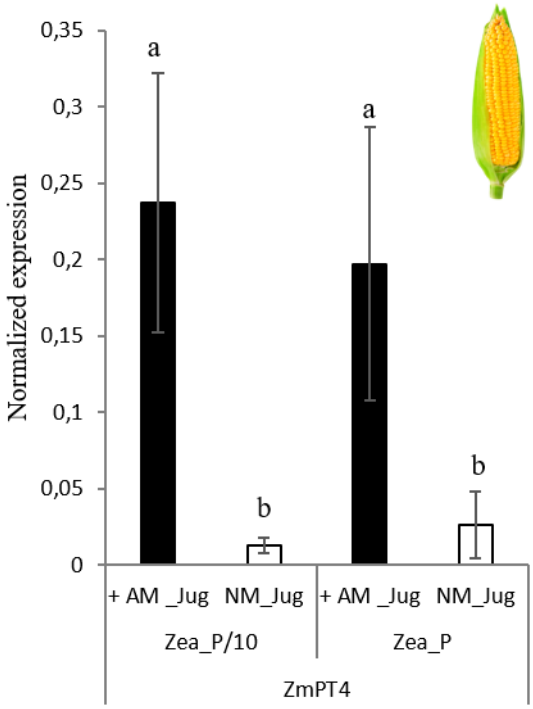

Figure V.6. Expression pattern of the gene *ZmPHT1;6* coding for a phosphate transporter in recipient maize roots two months after the inoculation (AM_Jug) of donor walnut RX1 with *R. irregularis* rootstocks or not (NM_Jug) under contrasting Pi fertilization regimes of maize (Zea_P/10, Zea_P). Expression patterns were quantified by RT-qPCR and expressed as normalized expression to the two maize reference genes described in **Table V.S2** Values are means of at least five biological replicates. Error bars represent the SE. Lower case letters indicate significant difference ($p < 0.05$) according to the Kruskal-Wallis H-test with post-hoc Tukey HSD.

Whatever the treatment of donor walnut plants, no expression could be detected in the roots of the hybrid rootstock RX1 for each of the three *J. microcarpa* putative AM-essential Pi transporters retrieved in the MtPT4 orthogroup (Jumi_00855.t1, Jumi_15132.t1, Jumi_21692.t1, data not shown). The analysis of the transcript abundance of the phosphate transporters *JrPHT1;1*; *JrPHT1;2*, and *JrPHT1;3* of *J. regia* in the roots of the hybrid walnut

RX1 showed only basal expression levels of *JrPHT1;3* in the four conditions tested (**Figure V. 7)**. On the opposite, inoculation of RX1 (AM_Jug) with *R. irregularis* resulted in a significant ($p < 0.05$) induction of *JrPHT1;1* and *JrPHT1;2* in AM-walnut roots, relative to NM_Jug controls whatever the Pi fertilization provided to maize plants (**Figure V. 7**). Expression levels of both *JrPHT1;1* and *JrPHT1;2* tend to be higher, although not significantly, under the P/10 than under the P supply to maize roots. Together these results indicated that the expression of the *JrPHT1;1* and *JrPHT1;2* genes are induced in the roots of the hybrid walnut rootstock RX1 by *R. irregularis* irrespective of the Pi level supplied to maize recipient plants.

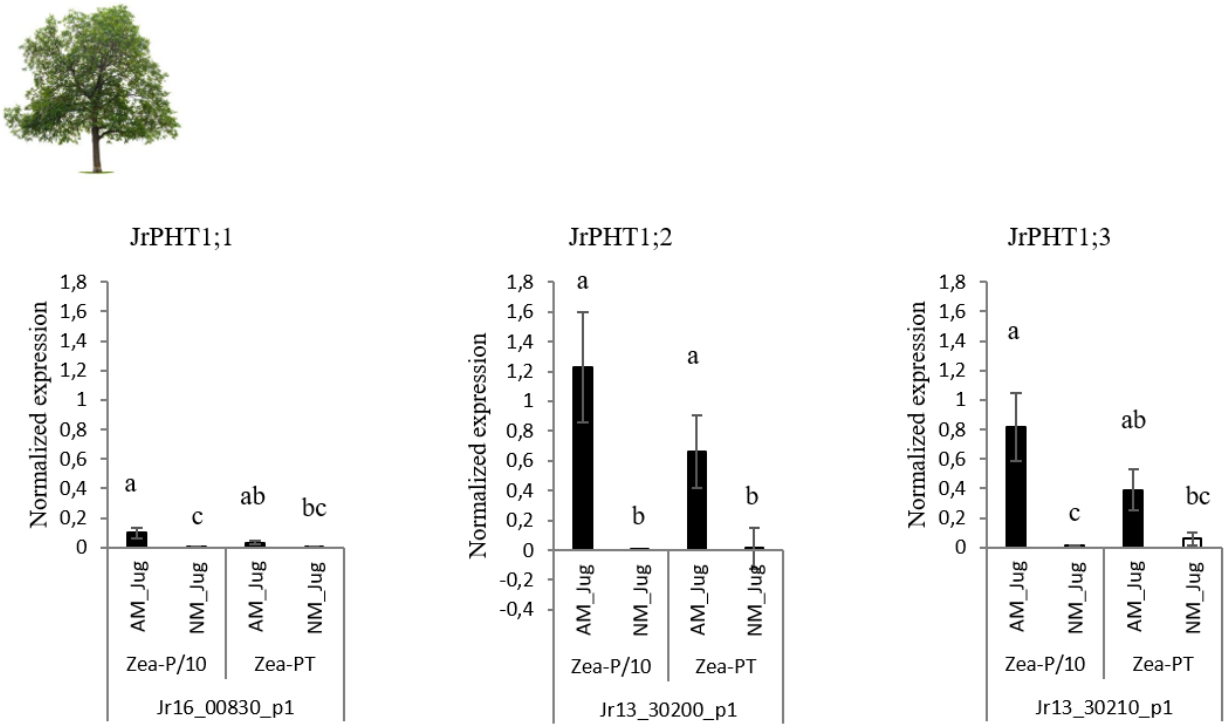

Figure V.7. Expression patterns of the genes *JrPHT1;1*; *JrPHT1;2*, *JrPHT1*;3 coding for orthologous to the phosphate transporter MtPT4*ZmPHT1;6* in donor walnut RX1 roots two months after their inoculation with *R. irregularis* (AM_Jug) or not (NM_Jug) under contrasting Pi fertilization regimes of maize (Zea_P/10, Zea_P) . Expression patterns were quantified by RT-qPCR and expressed as normalized expression to the two *J. regia* reference genes described in **Table V.S2** Values are means of at least five biological replicates. Error bars represent the SE. Lower case letters indicate significant difference ($p < 0.05$) according to the Kruskal-Wallis H-test with post-hoc Tukey HSD.

4. Discussion

4.1 The inoculum reservoir formed by walnut roots allows the mycorrhization of maize through the formation of a CMN that gives access to maize fertilization residues

Irrespective of the Pi supplied to *Z. mays* recipient plants, non-inoculated maize roots connected to mycorrhizal walnut rootstocks displayed F, M, and A% values reaching 100, 30 and 25%, respectively. These results demonstrated that whatever the Pi fertilization regime applied to maize, the inoculum reservoir formed by AM walnut roots enables the mycorrhization of maize recipient roots through the development of a common mycelial network (CMN). Consistently, extra-radical hyphae emanating from walnut root were visualized in the two Pi treatments after trypan blue-staining of the substrate contained in the fungal pipe close to *Z. mays* roots. This observation showed that the extra-radical mycelium that developed outside AM walnut roots have crossed the 40-µm nylon meshes to reach the recipient compartment and further colonize maize roots. Consistently, the high affinity transporter *RiPT1* of *R. irregularis* and the maize phosphate transporter *ZmPHT1;6* were only expressed in maize connected to mycorrhizal RX1 donor plants.

There was also evidence that AM walnut plants connected to recipient roots have access to maize Pi fertilization residues *via* the CMN. This conclusion was drawn from significantly higher stem diameter, shoot and root dry biomasses recorded in mycorrhizal RX1 saplings as compared to the non-inoculated walnuts in both Pi treatments (**Figure V.3A**). This finding demonstrated that walnut developing AM symbiosis gains growth benefit from the Pi supplied to maize through hyphal connections. In addition, we observed that AM walnuts connected to recipient plants are responsive to the extent of maize fertilization, as inferred from significantly lower M and A% in the P condition relative to the P/10 treatment (**Figure V.2A**). This result showed that Pi shortage in the maize compartment sustains walnut mycorrhization, while a ten-fold higher Pi fertilization restricts the intra-radical development of *R. irregularis* in RX1. This result is in accordance with many studies reporting a significant suppression of AM symbiosis upon a high Pi supply (Rausch et al. 2001; Breuillin et al. 2010; Bonneau et al. 2013; Kobae et al. 2016) because plants need less Pi through mycorrhizal hyphae that consume the plant's photosynthetically fixed carbon (Jakobsen and Rosendahl 1990; Smith and Read, 1997; Gavito et al. 2019). Consequently, the host plant may limit soil Pi uptake through the mycorrhizal pathway by reducing carbon allocation to the symbiont to save energy (Treseder & Allen 2002; Blanke et al. 2005). In agreement with this line, a decreased root carbon content, along with an increased leaf soluble Pi concentration and effective photochemical quantum yield of PS_{II} in AM walnuts compared to NM plants, was only significant in the Pi-limiting condition. Likewise, the mean expression of *JrPHT1;1* and *JrPHT1;2* genes coding for phosphate transporters tend to be higher in the roots of RX1, under the P/10 than under the P supply to maize roots. We concluded that in the walnut hybrid RX1, the AM fungus *R. irregularis* acts as a stronger carbon sink under Pi starvation (P/10) than under the Pi sufficient condition.

4.2 Common arbuscular mycorrhizal network can overcome phosphate shortage in walnut and maize plants

Relative to a 1.3 mM Pi supply, a 0.13 mM Pi fertilization of non-mycorrhizal maize plants led to a significant reduction in stem height and diameter, leaf number, Pi and N content, shoot and root dry mass, indicating that maize growth is limited by phosphate deficiency, a result previously underlined in many other studies (Usada and Kosuke, 1991; Kaeppler et al. 2000; Calderón-Vásquez et al. 2011; Almagrabi and Abdelmoneim 2012; Ma et al. 2021). In NM maize exposed to P sufficient treatments relative to Pi limiting conditions, higher leaf count, dry mass, plant height and stem thickness have also been reported (Coetzee, Ceronio,

and du Preez 2016). Notably, as an indicator of crop growth and development, when N is amply supplied, maize plant height is known to increase as P availability increases, similarly to stem stickiness (Aikins & Afukwa, 2010; Coetzee 2013). It has been proposed that both the phloem and vascular bundles, important for P nutrient transport, are higher due to increased cell division and elongation (Vasellati et al., 2001; Coetzee, 2013). In contrast to what observed in the NM controls, in AM maize plants cultivated under the P/10 supply, all the growth and nutritional parameters rose to a level similar to that recorded for NM plants under the P sufficient treatment, indicating that mycorrhization of maize plants alleviated Pi deficiency under the P/10 supply. These responses to mycorrhization in low P soil are in agreement with previous studies demonstrating that maize crop benefits from mycorrhizal associations (Calderón-Vásquez et al. 2011; Ma et al. 2021), and with the optimal trade principle predicting that plant growth will most strongly benefit from symbiosis with AM fungi under conditions of low soil phosphorus supply (Johnson, 2010; Johnson et al; 2015).Within this line, mean expression of the gene *RiPT1* in maize roots tended to be higher under the P/10 than under the P supply. We concluded that under maize Pi shortage, the mycorrhizal connection between walnut and maize roots alleviates Pi deficiency in the recipient plant through a better mineral nutrition. This resulted in maize growth and nutritional benefits comparable to the P sufficient condition without a common arbuscular mycorrhizal network.

As expected from the absence of mycorrhiza connections between plants when walnut donor roots were not inoculated with *R. irregularis,* the Pi fertilization regime of maize had no impact on the parameters measured in NM walnut plants. This result supported the absence of solutes diffusion through the air gap. Consequently, in our experimental conditions, NM walnut plants were not fertilized contrary to AM trees. When assessing the walnut growth benefits gained from the Pi supplied to maize through hyphal connections relative to non-fertilized NM walnut plants**,** one can observe that similarly to what observed in maize, a 0.13 mM Pi fertilization led to a significant increase in root collar, stem height, leaf Pi content, shoot and root dry DW, indicating that under maize low Pi fertilization, the mycorrhizal connection between walnut and maize roots alleviates Pi deficiency in the donor plant through a better mineral nutrition. Contrary to maize, mycorrhizal growth benefits of walnut plants in terms of root collar and dry mass are conserved under high Pi fertilization of maize, even though intra-radical colonization is reduced. Altogether, these results support the idea that walnut is more dependent on mycorrhization than maize to sustain its development owing to a coarse root architecture that has a limited intrinsic ability to absorb soil nutrients (Bates and Lynch 2001). We concluded that common arbuscular mycorrhizal network can overcome phosphate shortage in walnut and maize plants.

4.3 *R. irregularis* acts as a stronger carbon sink in walnut than in maize

When comparing mycorrhizal walnut and maize plants grown under the P/10 supply relative to NM plants, a significantly decreased root carbon content along with an increased effective photochemical quantum yield of $PS_{II,}$ was only observed in RX1, while both plants displayed a higher leaf Pi concentration. Consistently, when assessing the S/R biomass ratio as a measure of the carbon cost for AM establishment (Chen et al. 2010), mycorrhizal colonization decreased the shoot to root biomass partitioning only in RX1 grown under the P/10 supply. These findings suggested that AM fungal colonization could enhance walnut investment in root biomass development. Taken together, these results indicated that mycorrhization-induced Pi uptake generates a higher carbon cost for donor walnut plants than for maize plants by increasing walnut plant photosynthesis to provide the AM fungus with carbohydrate and lipid. In support for this rationale, when using walnut trees (*Juglans nigra* L., a C3-plant) and maize (a C4-plant), which display distinctly different ratios of $^{13}C/^{12}C$ ($\partial\ ^{13}C$), analysis of $\partial\ ^{13}C$ from an AM mycelium taken from the surrounding environment of intercropped walnut and maize roots indicated the transfer of walnut photosynthetates to the AM mycelium (van Tuinen et al.

2020). Unfortunately, in the current work, the fungal biomass collected in hyphal compartments was too small to meet $\partial^{13}C$ analysis needs and one cannot therefore not get this data (data not shown).

With regard to the potential mechanisms underlying a larger carbon investment in AM symbiosis of walnut than maize, Graham and Esseinstat (1994) proposed that a high mycorrhizal dependency, as displayed by walnut (Brundrett 1991; Comas and Eissenstat 2009; Comas, Callahan, and Midford 2014), may be a phenotypic response of the AM fungus when faced to a greater availability of the carbon supplied by the host. On the opposite, because plants with rapid growth rate need more carbon and absorb more soil nutrients to achieve fast growth (Lovelock 2008), less photosynthetates may thus be available to feed the CMN. According to Elser et al. (2000), plant growth rate is usually negatively related to C/N content ratio. In the current study, mycorrhization of maize with *R. irregularis* through the CMN led when compared to NM plants, to a significant decrease in leaf C/N ratio under the maize Pi-limited condition, but not in the Pi-replete condition. As this result was not observed in walnut rootstocks, our data supported the idea that at low soil fertility, walnut plant growth is limited more by nutrients than by carbon supply, and carbohydrates accumulate in plant organs (Poorter and De Jong 1999; Korner 2003). Under these conditions, it is more advantageous for walnut saplings than for maize plant to allocate assimilates to the AM fungus (van der Heijden and Horton 2009).

In summary, this study showed that that (1) the inoculum reservoir formed by walnut roots allows the mycorrhization of maize roots through the formation of a CMN, thereby giving access to the maize nutrient pool; (2) hyphal connections under shortage of P result in a benefit comparable to P sufficient condition without a CMN; and (3) due to a slower root growth rate than maize, walnut invests more carbon in the development of extra-radical hyphae than maize does. These findings support the agroforestry concept that the presence of a perennial root system support the maintenance of an active AM fungal network, which constitutes an AM reservoir enhancing root colonization of the annual crop. CMN formation is a facilitative mechanisms between trees and crops favourable to their growth and development (Bainard, Klironomos, and Gordon 2011; Bainard et al. 2012; Ingleby et al. 2007). These results also agree with the stress-gradient hypothesis that predicts that high-fertilised agricultural lands would benefit less from intercropping than low-fertilised lands (Brooker and Callaghan 1998; Rivest et al. 2010). Therefore, biotisation of tree saplings with a suitable AM fungus able to develop CMN favourable to alley annual crop may result in a benefit comparable to P fertilization and would therefore be a recommendable practice to achieve agroecological transition.

Table V.S1. Primer used for qRT-PCR in *J. regia*, *J. microcarpa*, *Z. mais*, and *R. irregularis*

Oligoname	Sequence	Transcript ID/Reference
J. regia		
JregGAPDHrefF	ATCATGGGTAAAGACCCCGC	XM_018984056.1/ Zhou et al. 2018
JregGAPDHrefR	TCAGTGACCGCATCCTTAGC	
JregACT2refF	ATGGTCCCAAACATGACCCA	XM_018959949.1/Zhou et al. 2018
JregACT2refR	TGTTGGAGGAGCTTGTGCAG	
JregPT4-28771F	TCGAGAAGCTGGGTCGTTTC	Jure_28771.t1=Jr16_00830_p1/This study
JregPT4-28771R	TTCAGGAGTGGTCCGAGTCA	
JregPT4-1F	GTTGCCCTTGTTGGTACCCT	Jure_23100.t1=Jr13_30200_p1/This study
JregPT4-23100R	AGCATATGGCGCAAAACACC	
JregPT4-23099F	CCTGGCAGTAAGCCTGGAAA	Jure_23099.t1= Jr13_30210_p1 /This study
JregPT4-23099R	AGTTTATCACCCAGCCAGCC	
J. microcarpa		
JmicGAPDHrefF	CCCTCACAGCGAAACGACC	Jumi_01067.t1/This study
JmicGAPDHrefR	TTTTTGAAGAATGGTGATGCAGGC	
JmicACT2refF	ACGGGTGGGGAAGATTTGAC	Jumi_26547.t1/This study
JmicACT2refR	GGAGGGAGCACCATGTATCC	
JmicPT4-2192F	ATGTCCCCGAACTCCTCTATCTT	Jumi_21692.t1/This study
JmicPT4-2192R	CGATTGTGTTGTCGCAGGG	
JmicPT4-15132F	GCCCAAGACTCCTTAACCCC	Jumi_15132.t1/This study
JmicPT4-15132R	CATCGACGAGGCTGATACGA	
JmicPT4-855F	AGAACCAGAGAACAGTGGCG	Jumi_00855.t1/This study
JmicPT4-855R	CATGGACCCTCACCTTCGAC	
Z. mays		
Zm B-tubulinrefF	CTACCTCACGGCATCTGCTATGT	Lin et al. 2014
Zm B-tubulinrefR	GTCACACACACTCGACTTCACG	
Zm-EF1αrefF	TGGGCCTACTGGTCTTACTACTGA	Lin et al. 2014
Zm-EF1αrefF	ACATACCCACGCTTCAGATCCT	
ZmPT1;6 F	TTCTGCATCTCCACCGTGTC	Zm00001d011498_P001= ZmPht1-6 / Glassop, Smith, and Smith (2005)
ZmPT1;6 R	CGCCTGTCACCATGTTGTTG	
R. irregularis		
Riα-tubulin a1 F	TGTCCAACCGGTTTTAAAGT	TC105406/Gomez et al. 2009
Riα-tubulin a1 R	AAAGCACGTTTGGCGTACAT	
RiTEFrefF	TGACAGGCGATCTGGTAAGG	Calabrese et al. 2019
RiTEFrefR	TCAGCGAAGGTCTCAACCAC	
RiPT1F	TGCTCGGTTGTGGTGCTATT	TC345640/Calabrese et al. 2019
RiPT1R	CGACGTCCGAAGTTGCTTTG	

Table V.S2. Effect of AM colonization and Pi availability on plant growth and nutritional parameters. Values are means of at least six biological replicates. Error bars represent the SE. Lower case letters indicate significant difference (Kruskal-Wallis H-test with post-hoc Tukey HSD; $p < 0.05$).

	Zea_P/10		Zea_P		
Walnut	M	NM	M	NM	pvalue
Root length (cm)	**33.13±5.1** ^{a}	**46.42±4.1** ^{a}	**38.10±4.3** ^{a}	**38.73±5.2**	**0.47**
Collar (mm)	**3.78±0.3** ^{ab}	**2.93±0.2** ^{c}	**3.99±0.2** ^{a}	**3.04±0.2** ^{bc}	**<0.01**
Height (cm)	**17.02±1.7** ^{a}	**10.10±1.9** ^{b}	**12.84±0.8** ^{ab}	**10.10±1.2** ^{b}	**0.02**
Leaf number	5.17±0.7	4.50±0.5	4.29±0.4	5.14±0.5	0.56
Shoot dw (g)	**1.26±0.2** ^{a}	**0.51±0.1** ^{b}	**1.06±0.1** ^{a}	**0.57±0.1** ^{b}	**<0.01**
Root dw (g)	**2.50±0.3** ^{a}	**0.81±0.2** ^{b}	**1.85±0.2** ^{a}	**0.94±0.3** ^{b}	**<0.01**
Shoot/root	**0.49±0.03** ^{b}	**0.86±0.2** ^{a}	**0.58±0.03** ^{ab}	**0.68±0.1** ^{a}	**0.05**
Fv_fm	0.72±0.02	0.71±0.02	0.70±0.01	0.71±0.01	0.90
Y_{II}	**0.45±0.01** ^{a}	**0.34±0.02** ^{b}	**0.44±0.01** ^{a}	**0.42±0.03** ^{a}	**<0.01**
Root $\delta^{13}C$ (‰)	-31.7±0.3	-32.5±0.3	-32.1±0.2	-32.7±0.2	0.08
Root N (%DW)	1.5±0.1	1.6±0.1	1.7±0.1	1.7±0.1	0.33
Root C (%DW)	**39.6±0.3** ^{b}	**42.8±0.3** ^{a}	**39.9±1.4** ^{b}	**40.3±0.1** ^{b}	**0.02**
Root P (nmole/mg)	40.18±4.2	45.97±6.3	50.56±2.6 ^{a}	41.82±3.1 ^{a}	0.13
Leaf $\delta^{13}C$ (‰)	-34.1±0.3	-34.5±0.3	-34.7±0.3 ^{a}	-34.9±0.3^{a}	0.37
Leaf N (% DW)	2.8±0.1	2.7±0.1 ^{a}	2.6±0.04 ^{a}	2.8±0.1 ^{a}	0.18
Leaf C (% DW)	**45.4±0.3** ^{a}	**44.3±0.4** ^{ab}	**44.5±0.1** ^{ab}	**43.8±0.4** ^{b}	**0.04**
Leaf P (nmole/ mg)	**62.0±1.6** ^{a}	**49.1±4.7** ^{b}	**53.3±4.5** ^{ab}	**65.9±5.4** ^{a}	**0.03**
Leaf C/N	16.2±0.5	16.6±0.6	17.3±0.3	15.6±0.9	0.3
	Zea_P/10		Zea_P		
Maize	M	NM	M	NM	pvalue
Root length (cm)	59.0±4.2	45.3±2.5	53.7±2.4	60.7±6.7	0.05
Collar (mm)	**16.8±0.4** ^{a}	**12.3±0.9** ^{b}	**17.4±0.4** ^{a}	**16.8±0.8** ^{a}	**<0.01**
Height (cm)	**135.5±8.8**^{a}	**82.6±10.8** ^{b}	**132.1±11.1** ^{a}	**129.9±12.3** ^{a}	**0.02**
Leaf number	**8.2±0.3** ^{a}	**6.6±0.3** ^{b}	**8.6±0.2**^{a}	**8.4±0.2**^{a}	**<0.01**
Shoot DW (g)	**3.6±0.7** ^{a}	**2.4±0.4** ^{b}	**6.8±1.0** ^{a}	**5.6±0.7** ^{a}	**<0.01**
Root DW (g)	**1.2±0.1** ^{a}	**0.4±0.1** ^{b}	**0.9±0.1**^{a}	**0.9±0.1** ^{a}	**<0.01**
Shoot/root	**5.6±0.5** ^{b}	**5.5±0.5** ^{b}	**8.1±0.6** ^{a}	**6.7±0.7** ^{ab}	**0.04**
Fv_fm	**0.8±0.01** ^{ab}	**0.8±0.01** ^{a}	**0.8±0.01** ^{a}	**0.8±0.01**^{a}	**<0.01**
Y_{II}	0.46±0.01	0.45±0.01	0.43±0.01	0.46±0.01	0.3
Root $\delta^{13}C$ (‰)	-14.8±0.1	-15.3±0.2	-15.2±0.1	-17.0±1.7	0.2
Root N (% DW)	1.7±0.2	1.7±0.1	1.5±0.2	1.7±0.1	1.0
Root C (% DW)	31.4±2.5	34.4±1.6	32.9±2.7	33.7±1.8	0.8
Root Pi (nmole Pi/mg)	66.3±7.7	42.9±1.9	52.4±3.3	47.6±8.1	0.1
Leaf $\delta^{13}C$ (‰)	-15.1±0.1	-15.1±0.1	-15.3±0.1	-15.5±0.2	0.1
Leaf N (% DW)	**2.9±0.1** ^{a}	**2.6±0.1** ^{b}	**3.0±0.1** ^{a}	**2.9±0.2** ^{a}	**0.02**
Leaf C (% DW)	44.1±0.3	44.1±0.1	44.3±0.2	44.7±0.2	0.1
Leaf Pi (nmole Pi/mg)	**102.19±5.6** ^{a}	**79.3±2.6** ^{b}	**123.8±7.2** ^{a}	**115.7±11.4** ^{a}	**<0.01**
Leaf C/N	**15.1±0.5** ^{b}	**17.1±0.4** ^{a}	**14.8±0.4**^{b}	**15.6±0.9** ^{ab}	**0.02**

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Partie 3 : Conclusion générale et perspectives

Conclusion générale et perspectives

Ce travail de thèse vise à contribuer au développement de systèmes agroforestiers comme un des leviers mobilisables dans la transition agroécologique des exploitations agricoles. Les systèmes agroécologiques présentent des performances tant sur le plan socio-économique qu'environnemental qui sont perçues comme des solutions durables et efficaces pour pallier les difficultés rencontrées par les systèmes de culture en raison de changement climatique et de la limitation des ressources naturelles mobilisables. C'est pourquoi des politiques publiques fortes telles que la loi d'avenir pour l'agriculture, l'alimentation et la forêt du 13 octobre 2014 permet la mise en œuvre concrète de l'agroécologie dans l'objectif d'une triple performance à la fois économique, environnementale et sociale de nos exploitations agricoles. L'objectif de cette loi et de plusieurs autres initiatives politiques est d'engager la transition agroécologique de la ferme France pour qu'elle puisse faire face aux enjeux du changement climatique et de la raréfaction des ressources naturelles tout en préservant l'outil de production et en limitant les impacts de l'agriculture sur la santé des écosystèmes et de l'Homme. Cette transition agroécologique induit des changements profonds des modes de production qui seront moins dépendants des intrants chimiques et qui reposeront davantage sur les bénéfices résultants des interactions biotiques entre les cultures de rente et les organismes vivants. Dans la transition agroécologique, la gestion durable des sols et des organismes vivants dans les sols, dont les microorganismes qui supportent de nombreuses fonctions écologiques, est primordiale pour maintenir la fonction de production primaire des sols mais également pour valoriser les autres fonctions écosystémiques remplies par ceux-ci, tel que le cycle des nutriments. La transition agroécologique va nécessiter la mobilisation de nouvelles connaissances, l'acquisition de nouvelles données scientifiques et le développement de nouvelles techniques dans des domaines différents pour accompagner les agriculteurs, les filières, les acteurs de l'agrofourniture et du conseil agricole.

Ce travail de thèse s'inscrit donc dans cette dynamique et il vise plus particulièrement à explorer de nouveaux modes de production dans la filière nucicole, et en particulier l'intérêt des interactions biotiques pour un système agroforestier à base de noyers fruitiers. La première étape de mon travail a consisté à faire un état de l'art sur la production nucicole afin d'en évaluer le potentiel pour l'agroforesterie en analysant notamment les interactions biotiques développées avec les champignons mycorhiziens à arbuscules. Sur la base cette étude bibliographique j'ai pu définir les hypothèses de mon travail de thèse qui a été décliné en trois axes principaux : (i) l'acclimatation et la biotisation avec des champignons mycorhiziens à arbuscule de porte-greffes de noyer propagés *in vitro*, (ii) l'identification chez le noyer fruitier des orthologues de protéines impliquées dans la symbiose, (iii) l'inclusion d'une plante de rente compagne dans un système agroforestier expérimental développé au cours de ma thèse.

La production *in vitro* des porte-greffes de noyers repose sur la multiplication végétative de tissus dédifférenciés qui permet la régénération à l'identique, avec la conservation des caractères génétiques, de la plante multipliée. L'une des principales difficultés de la production de plants micropropagés est la période cruciale de l'acclimatation *post-vitro* durant laquelle les plantules sont transplantés d'un milieu gélosé clos et stérile à un substrat dans lequel la plantule développe son appareil racinaire et devient autotrophe. A cette étape de nombreux vitro-plants sont perdus car ils ne résistent pas au stress engendré par la transplantation. J'ai étudié si la biotisation des vitro-plants avec un inoculum de champignon mycorhizien à arbuscule (CMA) pouvait améliorer leur survie lors de la transplantation et dans quelle mesure elle influençait les

paramètres biotiques de la plante. Ces études ont été menées dans des conditions contrôlées de température, d'humidité et d'éclairage en utilisant un substrat de culture inerte et une solution nutritive dont la composition est connue et reste inchangée tout au long de l'expérimentation afin de limiter autant que possible les variations expérimentales. Ces conditions expérimentales permettent d'étudier au mieux l'effet de la biotisation des vitro-plants par le CMA qui développe une symbiose jouant un rôle d'interface biotique et abiotique dans les relations que la plante entretient avec son milieu. La compréhension des mécanismes qui régissent les échanges entre la plante hôte et le champignon mycorhizien qui la colonise dans le cadre de cette symbiose doit permettre d'identifier des leviers biotechnologiques et agronomiques pour l'intégrer au mieux dans un système de culture agroforestier. En effet, le statut symbiotique de l'arbre permet d'améliorer sa nutrition minérale, de résister à différents stress abiotiques (dont la carence hydrique) et biotiques, et donc de diminuer le recours à l'agrochimie (engrais et produits de protection des plantes). Toutefois les multiples effets de la symbiose MA sont variables en fonction du pédoclimat et du contexte biotique mais aussi et surtout, en fonction du couple plante hôte / symbionte. J'ai donc dans un premier temps étudier la variabilité de la réponse de 7 cultivars de porte-greffe micropropagés biotisés au cours de leur acclimatation avec une souche de CMA.

Cette étude, conduite sur plusieurs lots de vitro-plants pour chaque variété de porte-greffe, montre que tous porte-greffes développent la symbiose avec le champignon mais que les effets de la symbiose sur les paramètres biotiques de la plante ne sont pas identiques et dépendent de plusieurs paramètres.

Ainsi la première expérience, consistant à biotiser les plants avec le champignon MA dès la phase d'acclimatation, révèle que le développement de la symbiose du cultivar VLACH avec le champignon MA conduit à pallier les impacts de la carence phosphatée observés sur le développement morphologique de la plante et sur sa nutrition. L'effet de la biotisation avec la même souche de champignon MA lors de la phase de transplantation des vitro-plants acclimatés a également été évalué sur les sept mêmes cultivars. Les résultats de cette étude mettent très clairement en évidence l'importance de l'impact du stade de développement de la plante pour l'établissement de la symbiose avec le champignon MA. En effet, le cultivar WIP3 répond le mieux à la biotisation de vitro-plants acclimatés avec notamment une amélioration significative de son développement physiologique et de sa nutrition en condition de carence phosphatée. Le principal apport de cette étude réside dans la mise en évidence de la capacité du champignon à diminuer la dépendance en fertilisation phosphatée des jeunes plants lors des phases d'acclimatation et de transplantation, le tout en impactant positivement le développement et la qualité des plants, plus particulièrement des hybrides VLACH, VX211 et WIP3. Cette étude permet d'envisager la mise en œuvre d'un nouveau process de production de porte-greffes de noyer micropropagés biotisés avec un CMA adapté au cultivar, dans le but de diminuer la dépendance des jeunes plants à la fertilisation phosphatée tout en améliorant le développement morphologique et la qualité du plant de porte-greffe. Cette première étude aux résultats prometteurs devra toutefois être complétée par de nouveaux essais visant à évaluer la performance des plants de porte-greffe biotisés suite à leur transplantation en pleine terre et à leur greffage avec différents types de greffon. Il serait notamment intéressant de suivre la persistance du CMA sur le système racinaire des plants biotisés au cours du développement des plants greffés et d'évaluer l'impact de la biotisation à plus long terme sur le développement des noyers et sur la qualité de la production de noix. De plus, même si les CMA ne présentent pas de spécificité d'hôte, il serait intéressant d'envisager un programme d'optimisation de la biotisation de porte-greffes en testant un plus large éventail de couples plante-CMA pour identifier les meilleures combinaisons deux à deux dans différents contextes agronomiques. La biotisation de plants de porte-greffe est une approche biotechnologique qui tire bénéfice de la symbiose développée par la plupart des plantes supérieures avec les CMA. Elle permet de promouvoir le développement, la résistance et la résilience de plantes d'intérêts agronomiques

et elle s'inscrit donc pleinement dans la transition agroécologique qui vise à diminuer la dépendance des systèmes de culture aux intrants chimiques.

Le deuxième objectif de ce travail de thèse était d'étudier à l'échelle biomoléculaire les mécanismes qui régissent l'interaction symbiotique entre une des variétés de porte-greffe (*J. regia*) et le CMA *R. irregularis*. Cette souche de champignon MA ubiquiste, communément étudiée, a été choisie non seulement parce que son potentiel symbiotique est élevé mais aussi parce que son génome est entièrement séquencé et annoté, permettant des études transcriptomiques. Afin d'étudier l'interaction symbiotique j'ai ciblé les gènes du noyer codant les transporteurs de phosphate et des protéines impliquées dans la biosynthèse des lipides connus pour être induits par la symbiose mycorhizienne chez d'autres plantes supérieures. La difficulté à laquelle j'ai été confrontée est que ces gènes n'étaient pas décrits chez le noyer. Nous avons donc conduit un travail d'analyse bioinformatique pour identifier les séquences orthologues au gène codant le transporteur de phosphate symbiotique de référence MtPT4 (*Medicago truncatula*) chez 25 espèces végétales dont *J. regia*. De la même manière, les gènes codants pour les protéines induites dans les voies de biosynthèse des lipides impliqués dans la symbiose MA ont été regroupées selon des orthogroupes comprenant les orthologues et paralogues de séquences de références, déjà connues et caractérisées. Ce jeu de données complet est maintenant accessible à l'ensemble de la communauté scientifique et a pour but de contribuer à la caractérisation de gènes codant les protéines essentielles aux interactions symbiotiques dans différentes espèces végétales. A partir de cette base de données, nous avons défini des couples d'amorces spécifiques de ces gènes afin d'étudier leur expression dans les plants biotisés. Nous avons ainsi montré que les gènes codant des transporteurs de phosphate et des protéines de la voie de biosynthèse des lipides étaient induits dans les plants de *J. regia* (RG17) biotisés avec *R. irregularis* (**Chapitre IV**). En revanche, nous avons pu observer que les gènes codant ces transporteurs n'étaient pas ou très peu induits lorsque les plants étaient non carencés en phosphate. Ces observations confirment donc que la symbiose MA produit des bénéfices sur les plants cultivés en condition de carence phosphatée pour lesquels le statut symbiotique représente un avantage biotique favorable à leur croissance. L'observation de l'induction des gènes codant des transporteurs de phosphate indiquent que la symbiose développée entre *J. regia* (RG17) et *R. irregularis* est fonctionnelle, et permet l'échange d'éléments minéraux dont le phosphate entre les deux partenaires, illustrant la propriété biofertilisatrice du champignon MA. Dans cette étude, j'ai étudié un seul couple plante/champignon MA et une approche ciblée sur des gènes codant des protéines connues comme des marqueurs fonctionnels de la symbiose mycorhizienne. Une approche holistique des interactions plante-microorganismes considérant le concept d'holobionte pourrait permettre de tirer profit de la biodiversité de microorganismes telluriques bénéfiques non seulement pour la culture des plantes de rente mais aussi pour les fonctions écologiques et les services écosystémiques des agrosystèmes.

Dans le contexte du changement climatique, les enjeux sanitaires et environnementaux auxquels doit faire face l'agriculture conduisent à promouvoir et développer de nouveaux modes de production tels que les systèmes agroforestiers. Ces systèmes intégrés, dynamiques et extensifs utilisent les interactions biotiques entre ses différentes composantes. Les systèmes agroforestiers associent la culture d'arbres et de plantes (de rente ou non) ; ils permettent non seulement de limiter l'érosion et de conserver le potentiel des terres agricoles *via* une couverture végétale annuelle, mais aussi de diversifier la production agricole, de manière simultanée. La plupart des essences d'arbres utilisées en agroforesterie forment des symbioses avec des champignons MA. La faible spécificité des champignons mycorhiziens à arbuscules vis-à-vis de leurs hôtes permet la formation de réseaux mycéliens communs (RMC) à l'arbre et à la culture associée. Afin de mettre en évidence le RMC formé entre *J.regia* x *J.microcarpa* (RX1), *R. irregularis* et *Zea mays*, nous avons développé un dispositif expérimental séparant les systèmes racinaires des plantes et les connectant via le CMA associé à RX1. Les résultats

présentés dans le **chapitre V** de la thèse, démontrent que le noyer constitue un réservoir mycorhizien à partir duquel un réseau d'hyphes se propage vers le compartiment du maïs, et permet sa colonisation. Une nouvelle fois j'ai pu observer que la colonisation des racines des plantes était fortement impactée par la quantité de phosphate apportée dans le dispositif. Ainsi la fertilisation phosphatée trop importante de la culture de maïs diminue de manière significative la colonisation racinaire de l'arbre et du maïs. De ce fait, l'amélioration de la croissance du maïs induite par la symbiose avec le champignon MA, provenant de l'arbre, n'est observée qu'en condition de phosphate carencée. L'étude biomoléculaire a permis de caractériser l'expression des gènes de RX1 codants les transporteurs de phosphate spécifiquement induits par la symbiose MA. Cette étude a pu être réalisée avec les données obtenues par l'analyse bioinformatique des différents orthogroupes des séquences génétiques d'intérêt chez *J.regia* et *J.microcarpa* qui par hybridation constitue le génome de RX1. L'étude de l'expression des gènes codants les transporteurs confirment sans ambiguïté l'importance de la teneur en phosphate pour l'établissement et le fonctionnement de la symbiose MA. En effet, les plantes cultivées en présence de phosphate ne présentaient pas de surexpression des gènes symbiotiques codants les transporteurs de phosphate contrairement aux plantes cultivées en carence de phosphate. Cette étude a permis de mettre en évidence le RMC établi entre le porte greffe de noyer biotisé et le maïs. Pour aller plus loin, il serait intéressant d'utiliser notre dispositif expérimental pour cribler différentes interactions tripartites (porte-greffe/champignon MA/plante cultivée) pour identifier les meilleures combinaisons pouvant être proposées aux agriculteurs dans différents contextes pédoclimatiques. Ces premiers résultats indiquent que l'arbre constitue un réservoir de réseau mycélien pouvant coloniser le système racinaire de la culture associée et ainsi optimiser le prélèvement de phosphate dans les sols et limiter le recours à la fertilisation phosphatée. Cependant la mise en place de ces systèmes est rendue délicate par la complexité des interactions à tous les niveaux et la maitrise temporelle et spatiale des cultures associées. Les systèmes agroforestiers ne sont pas des solutions uniques dans la quête d'une agriculture respectueuse de l'environnement, mais contribuent grandement à la diversité des espèces végétales, des microorganismes qui composent le sol et à la durabilité des écosystèmes par la stimulation des interactions naturelles entre la plante et son environnement. Ils doivent être appréhendés de manière intégrative pour évaluer plus globalement la performance sur le plan écologique, agronomique et économique. L'interdépendance des échelles mise en évidence dans ce travail de recherche permet de confirmer l'impact des pratiques culturales sur les interactions à l'échelle cellulaire entre une plante et un CMA. L'enjeu réside donc dans la difficulté à porter une réflexion à différents niveaux pour définir le meilleur assortiment de plantes (annuelles et pérennes) en adéquation avec les pratiques culturales, pour tirer tous les bénéfices des systèmes agroforestiers, tout en limitant l'apports des engrais phosphatés de synthèse. Dans le cadre des systèmes agroforestiers développés à partir des noyeraies en monoculture, il est important de considérer la production de juglone (5-hydroxy-1,4 naphthoquinone) des plants adultes du genre *Juglans* principalement. Ce composé aromatique inhibiteur de la respiration chez certaines plantes se trouve dans les feuilles, les fruits, l'écorce et les racines. Dans les systèmes agroforestiers, la juglone peut être transportée du noyer jusqu'aux racines de la plante intercalaire via les hyphes des CMA et avoir des effets délétères sur les plantes alentour. La gravité des symptômes toxiques que la juglone peut avoir sur des plants voisins du noyer varie selon l'espèce végétale. Dans le cadre de nos recherches, les jeunes plants de noyer produisent de la juglone en quantité trop faible pour nuire au maïs, mais il serait intéressant de mener des études complémentaires pour déterminer plus précisément les symptômes de toxicité attribuables au noyer dans le cadre d'un système agroforestier.

Pour conclure, ces travaux de recherche ont permis de déterminer les cultivars de porte-greffe de noyer fruitier les plus réceptifs à la symbiose mycorhizienne à arbuscule, en fonction de leurs stades de développement et de la fertilisation. Il est maintenant possible d'envisager la production de plants de noyers issus de micropropagation en symbiose avec une souche adaptée

de CMA dès les premiers stades de développement de la plante, et ce à grande échelle. Cette biotisation permet à la plante de s'adapter à des sols pauvres en phosphate et d'envisager à terme une diminution des apports en fertilisants de synthèse. Ce travail de thèse a mis en évidence qu'en situation de carence phosphatée, les transporteurs de phosphate induits par la symbiose mycorhizienne et les gènes codants les protéines impliquées dans les voies de biosynthèse des lipides impliqués dans la symbiose sont surexprimés chez *Juglans regia*. La dernière partie de ce travail de recherche a permis de définir l'ensemble des bénéfices tirés par un noyer et un maïs dans le cadre d'une simulation d'un système agroforestier. Ces bénéfices ont été observés à différentes échelles et contribuent à valoriser le développement de systèmes alternatifs qui réintroduisent la diversité des écosystèmes agricoles pour rétablir des équilibres biologiques naturelles. Ces bénéfices paraissent suffisamment clairs pour envisager la mise en place d'un système de ce type à plus large échelle. Ce travail conforte les données déjà disponibles relatives aux bénéfices observés et contribue à valoriser l'intérêt du développement de systèmes alternatifs qui réintroduisent la diversité des écosystèmes agricoles pour rétablir des équilibres biologiques naturelles.