Université de Montréal

Étude comparative des propagules extraracinaires et intraracinaires du champignon mycorhizien *Glomus irregulare*

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Ce mémoire intitulé :

Étude comparative des propagules extraracinaires et intraracinaires du champignon mycorhizien *Glomus irregulare*

Présenté par :
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a été évalué par un jury composé des personnes suivantes :

Dr Mohamed Hijri, président-rapporteur
Dr Anja Geitmann, directeur de recherche
Dr Marc St-Arnaud, membre du jury

Résumé

La germination des spores est une étape essentielle dans le cycle de vie de la majorité des champignons filamenteux. Les champignons mycorhiziens à arbuscules (CMA) forment un certain nombre de propagules infectieuses différentes qui augmentent leur potentiel à coloniser les racines. Parmi elles se trouvent les spores extraracinaires et intraracinaires. La paroi cellulaire des spores joue un rôle majeur dans la survie de ces propagules en étant une barrière physique et osmotique. Puisque une cellule peut faire des ajustements considérables dans la composition et la structure de sa paroi, en réponse aux conditions environnementales, il est possible que les parois des spores intraracinaires et extraracinaires montrent des propriétés mécaniques et osmotiques différentes affectant leur germination et leur survie. Pourtant, contrairement à la connaissance de la génétique moléculaire et de la formation de la paroi cellulaire des CMA, peu d'information est disponible au sujet de ces propriétés mécaniques. Les informations sur la germination des CMA dans des conditions hypertoniques sont aussi rares, et les modèles expérimentaux ne séparent généralement pas les effets directs de la forte pression osmotique externe sur la germination des champignons et les effets attribuables aux plantes. Cette étude avait pour but de répondre à deux importantes séries de questions concernant le comportement des spores mycorhiziennes. Nous avons d'abord déterminé la relation entre la composition de la paroi cellulaire, la structure et les propriétés mécaniques du champignon modèle *Glomus irregulare* (isolat DAOM 197198). La micro-indentation a été utilisée pour mesurer quantitativement les propriétés mécaniques de la paroi cellulaire. La composition (contenu de chitine et de glomaline) de la paroi cellulaire a été quantifiée par immunofluorescence tandis que la microscopie optique a été utilisée pour mesurer l'épaisseur de la paroi cellulaire. La densité locale en glomaline et l'épaisseur de la paroi étaient significativement plus élevées pour les parois des spores extraracinaires alors que la densité locale en chitine et la rigidité n'ont pas montré de variations entre les spores extraracinaires et intraracinaires. La grande variabilité dans les paramètres étudiés nous a empêchés de cibler un facteur principal responsable de la force totale de la paroi lors de la compression. La

diminution des concentrations de chitine et de glomaline a été corrélée à l'évolution de la paroi du champignon au cours de son cycle de vie. On a aussi observé une composition différentielle des couches de la paroi: les polymères de chitine et de glomaline furent localisés principalement dans les couches externes et internes de la paroi, respectivement. Dans la deuxième partie de notre travail, nous avons exploré les effets directs d'engrais, par rapport à leur activité de l'eau (a_w), sur la germination des spores et la pression de turgescence cellulaire. Les spores ont été soumises à trois engrais avec des valeurs de a_w différentes et la germination ainsi que la cytorrhyse (effondrement de la paroi cellulaire) des spores ont été évaluées après différents temps d'incubation. Les valeurs de a_w des engrais ont été utilisées comme indicateurs de leurs pressions osmotiques. L'exposition des spores de *Glomus irregulare* au choc osmotique causé par les engrais dont les valeurs de a_w se situent entre 0,982 et 0,882 a provoqué des changements graduels au niveau de leur cytorrhyse et de leur germination. Avec l'augmentation de la pression de turgescence externe, la cytorrhyse a augmenté, tandis que le taux de germination a diminué. Ces effets ont été plus prononcés à des concentrations élevées en éléments nutritifs. La présente étude, bien qu'elle constitue une étape importante dans la compréhension des propriétés mécaniques et osmotiques des spores de CMA, confirme également que ces propriétés dépendent probablement de plusieurs facteurs, dont certains qui ne sont pas encore identifiés.

Mots-clés : *Glomus irregulare*, pression osmotique, propriétés mécaniques, paroi cellulaire, spore, micro-indentation, germination, cytorrhyse, activité de l'eau, engrais, rigidité, chitine, glomaline, champignon mycorhizien arbusculaire (CMA), immunodétection

Summary

Spore germination is an essential developmental stage in the life cycle of many filamentous fungi. Arbuscular mycorrhizal fungi (AMF) form a number of different infectious propagules that increase their potential to colonize roots. Among them are extraradical and intraradical spores. The spore cell wall plays a major role in the survival of these propagules by being a physical and osmotic barrier. Because a cell can make considerable adjustments to the composition and structure of its wall in response to environmental conditions, it is possible that intraradical and extraradical spore walls show different mechanical and osmotic properties affecting their survival and germination. However, in contrast to the knowledge on the genetics and molecular composition of AMF cell wall, little is known about its mechanical properties. Information on the germination of AMF under hypertonic conditions is scarce, and experimental designs and methodologies have generally not allowed the direct effects of high external osmotic pressure on fungal germination to be separated from plant-mediated effects. This study had the goal to address two important sets of questions regarding the behavior of mycorrhizal spores. We first determined the relationship between cell wall composition, structure and mechanical properties of the model fungus *Glomus irregulare*. Micro-indentation was used to quantitatively measure the cell wall mechanical properties. Cell wall composition (chitin and glomalin content) was studied by immunofluorescence whereas optical microscopy was used to measure the cell wall thickness. Glomalin local density and wall thickness were both significantly higher for extraradical spore walls while chitin local density and rigidity were unaffected by origin of spores. High variability in results prevented us from identifying a primary factor responsible for overall wall strength during compression. Decreases of chitin and glomalin concentrations were correlated to the development of the fungal wall throughout its life-cycle. There was also differential association within the wall layers: The chitin and glomalin polymers were localized mostly in the outer and inner walls, respectively. In the second part of our work, we explored the direct effects of fertilizers, in relation to their water activity (a_w), on spore germination and cellular turgor

pressure. Spores were exposed to three fertilizers with different a_w and spore germination and cytorrhysis of spores were assessed after different times of incubation. Water activities of the fertilizers were used as indicators of their osmotic pressures. Osmotic shock exposure of the *Glomus irregulare* spores to fertilizers at a_w values between 0.982 and 0.882 caused gradual changes in cytorrhysis and germination. With the increase of external turgor pressure, cytorrhysis increased while the rate of germination decreased. These effects were most pronounced at high nutrient concentrations. The present investigation, while likely representing a significant step forward in understanding the mechanical and osmotic properties of AMF spores, also confirms that they might depend on many, as yet unidentified factors. Future research should examine differences in the physiology to discern reasons for such differences in spore properties.

Keywords : *Glomus irregulare*, osmotic pressure, mechanical properties, cell wall, spore, micro-indentation, germination, cytorrhysis, water activity, fertilizer, stiffness, chitin, glomalin, arbuscular mycorrhizal fungi (AMF), immunodetection

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Je dédie ce travail à ma mère et mon père
pour l'éducation qu'ils m'ont prodiguée; avec
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1 Introduction

1.1 Intérêts généraux de l'étude

Tout comme la disponibilité mondiale du pétrole a atteint son maximum au milieu des années 1970, le maximum de la disponibilité mondiale des phosphates a été atteint en 1988 (Fortin et al., 2008). Puisque cette ressource vitale ne connaît aucun substitut, elle limitera tôt ou tard la population mondiale. Avec cette baisse de disponibilité du phosphate prédite dans les années à venir, les pratiques agricoles devront se tourner vers d'autres alternatives que les applications d'engrais. L'utilisation de champignons mycorhiziens est parmi les plus prometteuses pour une meilleure gestion de la fertilisation, en particulier pour améliorer la nutrition phosphatée des plantes (Fortin et al., 2002 ; Declerck et al., 2005 ; Fortin et al., 2008). Les mycorhizes sont donc appelées à jouer un rôle clé pour contrer l'épuisement de cette ressource non renouvelable. Les champignons mycorhiziens vivent en symbiose avec les racines de la majeure partie des espèces végétales. La croissance des plantes est généralement augmentée en présence de ces champignons grâce à leur capacité à exploiter un plus grand volume de sol que les racines des plantes (Pfleger et Linderman, 1994; Hamel et Plenchette, 2007; Fortin et al., 2008). Par conséquent, il y a un grand intérêt dans l'utilisation de ces organismes dans les produits horticoles et agricoles.

Les objectifs de ce projet ainsi que les approches expérimentales sont basées sur les besoins de l'industrie des inoculants mycorhiziens pour une meilleure compréhension des caractéristiques des spores, forme sous laquelle ils sont vendus et appliqués dans le sol. Une spore est en fait une cellule de reproduction asexuée qui, une fois germée va permettre le développement des hyphes et la colonisation des racines et du sol. Les spores de champignons présentent des caractéristiques de résistance remarquables. En effet ces structures peuvent survivre pendant de longues périodes dans des conditions défavorables et permettre la dispersion de l'espèce.

1.2 Problématique

Les procédés de production, de formulation et d'emballage des inoculants mycorhiziens doivent assurer le maintien de la viabilité des propagules fongiques pendant de longues périodes de temps et la préservation de leur capacité à coloniser les plantes dans des conditions de terrain variées. Bien que les spores soient connues pour être des structures résistantes, lors des différents procédés industriels de formulation, elles sont soumises à certains stress de nature osmotiques et mécaniques pouvant les endommager voire même les rendre non viables.

1.3 Objectifs du projet

La reconnaissance de l'importance des propriétés biomécaniques au niveau cellulaire a donné lieu à une forte augmentation des applications cytomécaniques tant pour la biologie animale que végétale (Geitmann, 2006). Une combinaison de méthodes biomécaniques (micro-indentation), microscopiques (immunodétection, représentations 3D) et physiologiques (mesures de pression de turgescence) ont été employées pour contribuer à accroître les connaissances de base de la structure des spores de *Glomus irregulare* ainsi que leur comportement face aux chocs osmotiques. Les résultats de ce projet aideront donc à concevoir des produits commerciaux avec une viabilité et un potentiel infectieux améliorés en plus d'accroître les connaissances fondamentales sur la mécanique cellulaire des spores de champignons mycorhiziens.

2 Revue de littérature

2.1 La symbiose mycorhizienne

Le terme «mycorhize» (du grec «mycos » qui signifie champignon et « rhiza », qui signifie racine) décrit l'association symbiotique entre les racines d'une plante et certains champignons du sol. Selon les fossiles et les analyses moléculaires, l'origine de la symbiose mycorhizienne remonte au moins à l'Ordovicien, il y a 450 à 500 millions d'années (Remy et al., 1994;. Redecker et al., 2000). Il a été démontré que la colonisation des racines par des champignons mycorhiziens améliore la croissance et la productivité de plusieurs plantes en augmentant généralement l'absorption d'éléments nutritifs (Smith et Read, 2008).

Lorsque les spores de champignons mycorhiziens germent, des hyphes à paroi épaisse vont pénétrer la racine de l'hôte permettant une colonisation interne. La germination des spores peut être affectée par de nombreux facteurs, tels que: la nécessité d'une période de dormance (Juge et al., 2002), la présence d'exsudats racinaires et/ou volatiles; l'humidité du sol, la température, le pH, la lumière, le niveau de CO_2 (Bécard et al., 1992; Graham, 1982; Siqueira et al., 1982); la présence de flavonoïdes (Gianinazzi-Pearson et al., 1989; St-Arnaud et al., 1996), ainsi que la présence de certaines bactéries (Azcon, 1989). Les hyphes extraracinaires fonctionnent en tant que structures d'absorption des éléments minéraux et de l'eau. Comme ils peuvent s'étendre sur plusieurs centimètres de la racine, ils peuvent absorber les éléments immobiles qui se situent à l'extérieur de la rhizosphère. Ces champignons jouent également un rôle important dans la phytoremédiation, entre autres, des sols contaminés par des métaux lourds (Göhre et Paszkowski, 2006) et plusieurs études ont porté sur le rôle de la symbiose dans les échanges des éléments nutritifs, soit l'échange de carbohydrates en retour d'éléments nutritifs du sol, en particulier le phosphore (Smith et Read, 1997).

Il existe deux types abondants de mycorhizes, les ectomycorhizes et les endomycorhizes, définis par la combinaison plante / champignon et par la structure de la symbiose. Dans une ectomycorhize, qui existe chez la majorité des arbres dans les forêts tempérées, le partenaire fongique demeure à l'extérieur des cellules végétales du cortex racinaire, tandis que dans une endomycorhize, notamment chez les orchidées, les éricoïdes et les mycorhizes à arbuscules (MA), une partie des hyphes pénètre à l'intérieur des cellules corticales. Les champignons symbiotiques qui prédominent sont les champignons mycorhiziens à arbuscules (CMA).

2.1.1 Champignon mycorhizien à arbuscules

La MA, bien qu'on la retrouve chez certaines plantes aquatiques, est la symbiose terrestre la plus répandue. On la rencontre chez 80% des espèces de plantes terrestres. Des travaux récents sur la phylogénie des CMA ont fourni une base pour une nouvelle taxonomie (Schüßler et al., 2001) supprimant ces champignons du phylum polyphylétique des Zygomycota et en les plaçant dans un nouveau phylum monophylétique, les Glomeromycota (Fig.2.1).

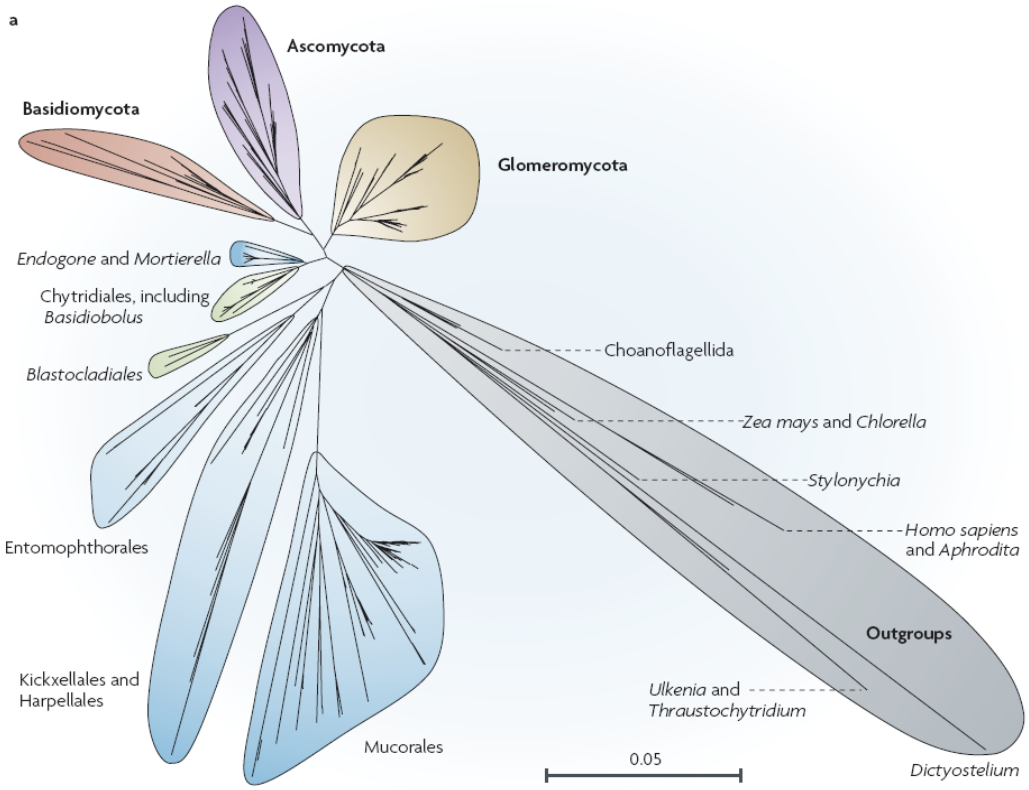

Figure 2.1 : Arbre phylogénétique montrant les Glomeromycota par rapport aux autres principaux phylum fongiques: les Ascomycota, les Basidiomycota, les Chytridiomycota et les Zygomycota. L'échelle représente le nombre de substitutions par site. Image tirée de Parniske (2008).

Le développement symbiotique des MA résulte en la formation de structures subcellulaires à l'intérieur des cellules corticales. Ces structures, connues sous le nom d'arbuscules (du latin « arbusculum », qui signifie arbuste), sont les principaux sites d'échanges de nutriments entre les partenaires symbiotiques (Fig. 2.2).

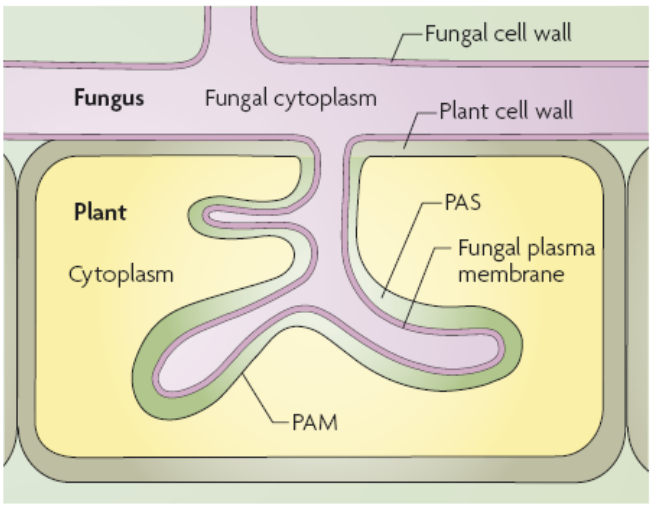

Figure 2.2 : Schéma du site d'échanges (arbuscule) dans une MA tiré de Parniske (2008).

La colonisation des cellules du cortex racinaire par les CMA induit des changements dramatiques dans l'organisation cytoplasmique et dans la morphologie des cellules colonisées. L'arbuscule fongique occupe une partie importante du volume des cellules végétales, mais est encore toujours séparé du protoplaste cellulaire par la membrane plasmique de l'hôte. L'espace résultant entre le protoplaste de la plante et le champignon se développe en un compartiment apoplastique qui représente l'interface symbiotique où se déroule la majeure partie des échanges (Bonfante et Perotto, 1995). Par la suite, certains groupes de CMA peuvent former des vésicules dans les cellules corticales qui fonctionnent comme des organes de stockage d'éléments nutritifs ou comme des propagules appelées « spores intraracinaires ».

En retour de l'approvisionnement en nutriments et en eau à la plante, le CMA obtient des glucides à partir des produits photosynthétiques de la plante. On estime à jusqu'à 40% la consommation des produits de la photosynthèse des plantes terrestres par les CMA (Bago et al., 2000). Par conséquent, la MA contribue de manière significative au

cycle global du phosphate et du carbone et influence la production primaire des écosystèmes terrestres. Les effets bénéfiques de la MA sont plus apparents dans des sols pauvres en nutriments et la colonisation des racines diminue généralement lorsque les nutriments sont disponibles. En conséquence, ce type de symbiose est considéré comme un élément clé de l'agriculture de type biologique (Bethlenfalvay, 1992) mais également en agriculture conventionnelle où les nutriments comme le phosphore sont fréquemment peu disponibles. Autre fait intéressant, la colonisation des racines par les CMA entraîne une inhibition de certains pathogènes s'attaquant aux plantes (Pozo et Azcon-Aguilar, 2007).

Les CMA sont des organismes particuliers en raison de leur mode de vie et de leur génétique. En effet, le réseau d'hyphes des CMA est généralement coenocytique, comprenant des centaines de noyaux qui partagent le même cytoplasme. De même, les spores individuelles contiennent des centaines de noyaux (Hijri et Sanders, 2005). Il est à noter que les spores de CMA peuvent germer en l'absence de plantes hôtes mais dépendent d'un partenaire végétal pour compléter leur cycle de vie et produire la prochaine génération de spores (Parniske, 2008).

2.1.2 Glomus irregulare

Dans la recherche sur les CMA, un champignon initialement appelé *Glomus intraradices* (DAOM197198), est le membre le plus fréquemment utilisé des Glomeromycota. En 2009, Stockinger et al., par le biais d'analyses moléculaires, a suggéré que le champignon modèle DAOM197198 ne serait pas un *G. intraradices* mais plutôt un *Glomus irregulare* et la communauté scientifique s'est officiellement statuée sur l'identité de la souche DAOM197198 comme étant *Glomus irregulare* en 2010 (Sokolski et al., 2010). À ce jour, plus de 1200 publications réfèrent cette espèce, et plus de 130 ont son nom dans le titre (Stockinger et al, 2009). Cette utilisation à grande échelle résulte du fait

que *G. irregulare* est le premier CMA à avoir été cultivé *in vitro* avec des racines transformées par *Agrobacterium tumefaciens* (Chabot et al., 1992). Les descendants de cette culture, sont largement utilisés dans la recherche fondamentale, par exemple pour des projets de séquençage génomiques (Martin et al., 2008) et pour démontrer des transformations génétiques transitoires (Helber et Requena, 2008). Il est également produit industriellement et constitue la composante principale de plusieurs inoculants commerciaux dont ceux offerts par Premier Tech Biotechnologies.

2.2 La paroi cellulaire fongique

Les cellules de *G. irregulare* sont entourées d'une paroi, cette structure est caractéristique des cellules fongiques, végétales et bactériennes tandis que les animaux et les protozoaires en sont dépourvues. Les matériaux constitutifs de la paroi cellulaire varient d'une espèce à l'autre mais son rôle demeure le même. Le rôle principal de la paroi cellulaire est de protéger le protoplaste des changements de pression osmotique et d'autres contraintes environnementales, sans interférer dans les interactions avec son environnement (Bowman et Free, 2006). Elle fournit également une protection mécanique pour la cellule car elle agit comme une barrière initiale qui est en contact avec des éléments rugueux rencontrés dans l'environnement. Par ailleurs, sa structure rigide sert à résister à une contrainte de compression lors de la pénétration de l'hyphe à l'intérieur de l'hôte lors de la colonisation. Dans chaque cas, la forme et l'intégrité du champignon dépendent de la résistance mécanique de la paroi cellulaire (Gooday, 1995). En dépit de son importance reconnue, la paroi cellulaire de la plupart des champignons reste insuffisamment étudiée et ses propriétés biomécaniques sont à nos jours mal comprises. Notre compréhension de cette structure se complique par le fait que ce "mur" n'est pas une structure inerte, mais bien un exosquelette dynamique qui change de composition biochimique au fil du temps et qui montre une variabilité spatiale.

En 1994, Maia et al. ont démontré à l'aide du microscope optique que la paroi des spores de *Glomus irregulare* est formée de deux zones distinctes: une zone interne composée de plusieurs couches et une mince zone externe qui se désintègre avec le temps (Fig.2.3). Ces deux zones se retrouvent aussi chez la paroi des hyphes de cette même espèce car la paroi des spores est en continuité avec celle des hyphes. La composition de la paroi sporique et hyphale est donc sensiblement la même mais diffère par leur épaisseur respective. Basé sur des analyses provenant de diverses espèces de champignons, la paroi fongique est principalement composée de chitine, de glucanes, de mannanes et de glycoprotéines, bien que sa composition varie souvent fortement entre les espèces (Adams, 2004). Certaines études démontrent aussi que la chitine, les glucanes et les glycoprotéines sont liés ensemble par des liaisons croisées de type covalentes (Bowman, 2006).

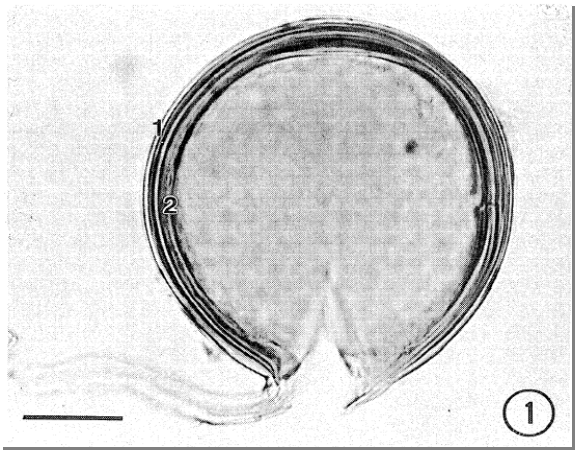

Figure 2.3: Spore de *G. irregulare* vue au microscope optique montrant une paroi composée de deux zones distinctes : une zone externe (1) et une zone interne (2) Échelle = 20 μm. Tiré de Maia (1994).

2.2.1 La chitine

Une des composantes principales de la paroi cellulaire des champignons est la chitine. La chitine est un polymère linéaire formé de monomères de β-1,4-N-acétylglucosamine (Fig.2.4) qui sert d'échafaudage pour la plupart des parois cellulaires chez les champignons, les arthropodes, les insectes et les crustacés (Bowen, 1992).

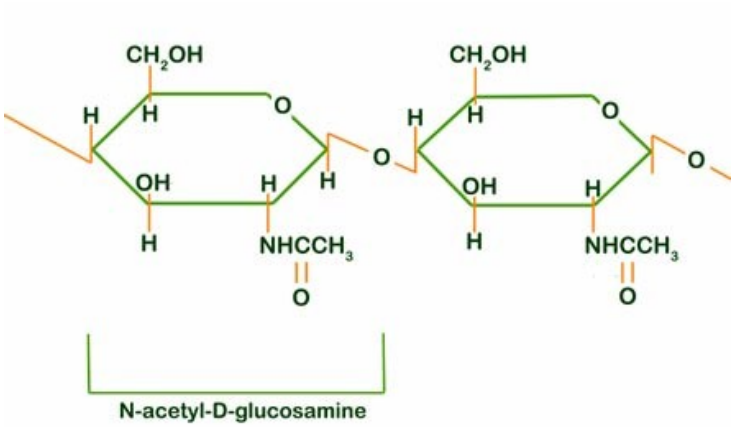

Figure 2.4 : Structure chimique de la chitine. Tiré du site web de University of Delaware (2007)

Elle forme des microfibrilles stabilisées par des liaisons hydrogène. Sous leur forme cristalline, les microfibrilles peuvent s'orienter de manière parallèle, antiparallèle ou en alternance entre parallèle et antiparallèle (Kramer et Muthukrishnan, 1998). Cette structure cristalline confère à la paroi cellulaire une plus grande résistance aux contraintes de tension. La structure de la chitine s'apparente beaucoup à la cellulose (polymère de glucoses liés en β-1 ,4) avec la substitution d'un groupe hydroxyle sur chaque monomère par un groupe amine acétyle chez la chitine. Cette substitution augmente le potentiel de liaison hydrogène entre les polymères adjacents, augmentant ainsi la force d'une matrice composée majoritairement de chitine. C'est pourquoi la chitine confère une grande résistance à la

paroi fongique, permettant aux hyphes de se développer dans des environnements hostiles (Garcia-Rodriguez, 2000).

2.2.2 La glomaline

La glomaline est une glycoprotéine produite en abondance par les hyphes et les spores des champignons mycorhiziens. On la retrouve en grande quantité dans le sol et dans les racines (Rillig et al., 2002) ce qui suggère que les champignons en produisent et en excrètent en excès. La découverte de la glomaline a d'abord été signalée en 1996 par Wright et Upadhyaya. On l'a d'abord remarquée pour deux caractéristiques apparentes : son abondante production par le champignon mycorhizien et la "ténacité" de la molécule. En effet, la glomaline s'avère très résistante à la décomposition microbienne (qui peut s'étendre sur 10 à 50 ans) et se dissout uniquement en conditions extrêmes : 121°C pendant au moins une heure dans une solution de citrate concentrée (Wright et Upadhyaya 1998). Ces propriétés en font un très bon agent protecteur. La composition chimique de la glomaline est encore peu connue, malgré cela Driver et al. (2005) ont démontré que cette substance est étroitement liée à l'intérieur de la paroi des hyphes et des spores, plutôt que d'être seulement relâchée dans l'environnement. Plusieurs hypothèses tentent d'expliquer la découverte de Driver et al. (2005). Parmi celles-ci, certains croient que la glomaline aurait un rôle similaire aux hydrophobines (Wösten, 2001). Les hydrophobines synthétisées par certains champignons filamenteux agissent comme une substance hydrofuge en abaissant la tension superficielle de l'eau et sont importantes dans l'attachement des hyphes à certaines matrices. La glomaline pourrait également jouer un rôle dans la prévention des brouteurs fongiques en diminuant la palatabilité des hyphes. Il se pourrait aussi que cette glycoprotéine augmente la résistance mécanique de la paroi cellulaire en renforçant les liaisons entre les molécules formant la paroi et ainsi offrir une plus grande intégrité à celle-ci.

2.3 Biomécanique de la paroi

Comme mentionné précédemment, la fonction primaire de la paroi est la protection mécanique. Pour mieux comprendre et évaluer cette fonction, il faut pouvoir analyser de manière quantitative les différents paramètres qui constituent la biomécanique. La biomécanique est l'application des principes mécaniques aux systèmes biologiques, comme les humains, les animaux, les plantes et les cellules telles que les spores. En 1974, Herbert Hatze l'a définie ainsi: « la biomécanique est l'étude de la structure et la fonction des systèmes biologiques au moyen de méthodes de la mécanique». La biomécanique est étroitement liée à l'ingénierie, certaines applications simples de la mécanique newtonienne et des sciences des matériaux peuvent fournir des approximations correctes à la mécanique de nombreux systèmes biologiques.

Pour mieux comprendre la biomécanique d'un système biologique comme les cellules végétales ou fongiques, il faut savoir que la paroi des cellules végétales et fongiques est considérée comme une matrice gélatineuse renforcée par différentes fibres (Geitmann, 2010). Dans les cellules végétales, la fibre se compose de microfibrilles de cellulose alors que dans les cellules fongiques, les microfibrilles se composent habituellement de chitine. Les parois cellulaires sont ainsi résistantes à la fois aux contraintes de traction et de compression. Le niveau de résistance à ces contraintes est influencé par certains facteurs tels que l'orientation, le degré de réticulation, la cristallinité ainsi que la longueur des microfibrilles (Geitmann, 2010).

Les deux paramètres quantifiables et qui ont une importance cruciale dans la résistance mécanique des cellules ayant une paroi sont la déformabilité de la paroi cellulaire ainsi que la pression hydrostatique. Leur quantification expérimentale permet la

détermination ou l'estimation du module d'élasticité volumétrique (ε) alors que la mesure de la pression hydrostatique cellulaire permet la détermination ou l'estimation de la pression de turgescence (P) (Geitmann et Ortega, 2009). De nombreuses méthodes micromécaniques ont été développées pour évaluer ces paramètres au niveau tissulaire, cellulaire et moléculaire (Geitmann, 2006).

2.3.1 Le micro-indenteur

Pour comprendre les propriétés physiques et les interactions mécaniques entre les différentes composantes structurelles de la cellule, diverses techniques ont été utilisées dans le passé. Toutes ces techniques avaient pour but de mesurer les propriétés physiques des composantes cellulaires ou des entités structurelles au niveau subcellulaire. Ces techniques incluent, entre autres, la micro-manipulation (Thomas et al. 2000; Wang et al. 2004), la microscopie à force atomique (Hassan et al., 1998) et la sonde de pression de turgescence (Tomos 2000). Une autre technique, conçue spécifiquement pour mesurer la déformabilité des cellules, a fait son apparition en 1982, il s'agit du micro-indenteur (Petersen et al., 1982). Le micro-indenteur (Fig. 2.5) est un instrument qui détermine les paramètres physiques liés au module de compression d'un matériau ou d'une structure. Normalement, la micro-indentation est utilisée pour des matériaux inorganiques, mais dans le laboratoire du Dr. Geitmann, les techniques de micro-indentation ont été modifiées pour mesurer la rigidité des tissus biologiques et de cellules végétales. Les mesures de déformabilité ont jusqu'ici donné une bonne compréhension des propriétés viscoélastiques des érythrocytes (Elson, 1988) et de la contribution de la dystrophine à la rigidité corticale des cellules musculaires (Pasternak et al., 1995). La micro-indentation s'est aussi avérée utile pour évaluer le rôle des pectines et de la callose dans la mécanique du tube pollinique (Parre et Geitmann, 2005a-b). L'utilisation de cette technique pour mesurer les propriétés mécaniques des champignons n'a jamais été faite à ma connaissance. La plupart des études mécaniques sur les champignons portent sur la force mécanique des hyphes pour la

pénétration des cellules hôtes et cette force est mesurée à l'aide d'une jauge à tension (« strain gauge ») ultra sensible (Money, 2001) ou par microscopie de type « waveguide » (Bechinger et al., 1999).

Il faut avouer que l'analyse des données obtenues par micro-indentation n'est pas toujours aisée. Lorsque les cellules ont des structures cytosquelettiques complexes, l'analyse mécanique tridimensionnelle s'avère plus difficile. L'anisotropie, c'est-à-dire un objet qui ne présente pas les mêmes caractéristiques dans les toutes directions, et l'asymétrie des cellules rendent la tâche encore plus complexe. L'analyse mécanique cellulaire est encore à un stade précoce mais des approches computationnelles telles que la modélisation par éléments finis s'avère grandement utile à l'analyse mécanique de cellules complexes tels que le tube pollinique (Bolduc et al., 2006; Fayant et al., 2010).

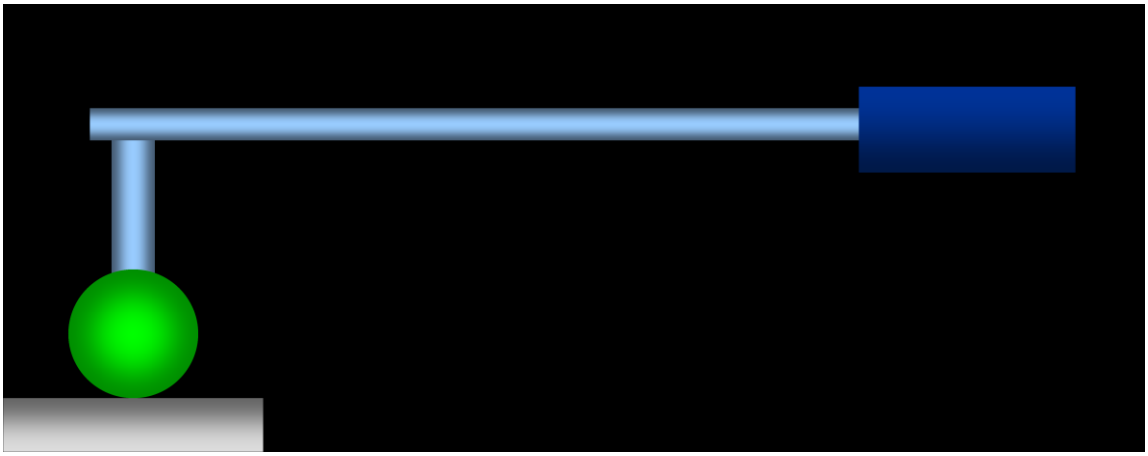

Figure 2.5 : Schéma du micro-indenteur, constitué du moteur piézoélectrique, de la tige, de l'aiguille et de la spore (le sujet de mesure).

Le principe de la micro-indentation est relativement simple: une aiguille microscopique attachée à une tige préalablement calibrée afin de connaître sa constante de flexion s'enfonce dans un objet dont la résistance mécanique provoque une déformation de la tige. Il est essentiel que l'extrémité de l'aiguille (d'un diamètre d'environ 8 μm) soit la plus plate possible pour éviter les risques de transpercement de la spore. La micro-indentation se fait à l'aide d'un microscope photonique inversé, permettant ainsi une visualisation directe et précise de l'indentation. Lors d'une micro-indentation (Fig. 2.6), le moteur effectue un déplacement vertical sur une distance contrôlée (Dm). Si l'aiguille n'entre pas en contact avec un objet, le déplacement du moteur et le déplacement de l'aiguille (Da) seront égaux : Dm = Da. Par contre, dans le cas où l'aiguille entre en contact avec un objet, son déplacement sera influencé par la résistance de l'objet et son déplacement sera invariablement inférieur à celui du moteur : Dm > Da. Ainsi la différence entre les distances de déplacement du moteur et de l'aiguille en fonction de la constante de flexibilité de la tige (k) permet le calcul de la force exercée : $F = k*(Dm - Da)$. Le principe de cette technique est donc similaire à celui utilisé dans la microscopie à force atomique laquelle est basée sur la présence d'une tige dont la déformation jauge la rigidité cellulaire locale. La différence réside dans le montage expérimental et l'ampleur des déformations qui est de magnitude supérieure à l'ampleur communément utilisée pour la microscopie à force atomique.

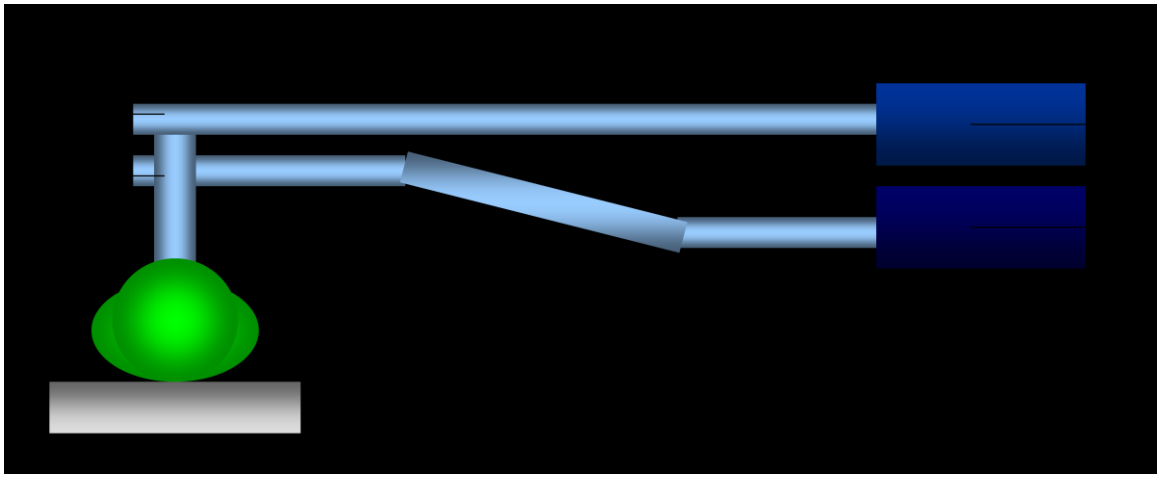

Figure 2.6 : Représentation du déplacement du moteur piézoélectrique et de l'aiguille lors d'une indentation. Dm représente le déplacement du moteur et Da représente le déplacement de l'aiguille. La force est calculée à l'aide de la formule F = k(Dm-Da)

On obtient ainsi le graphique de la force exercée par l'aiguille en fonction de sa profondeur d'indentation pour déformer l'objet ainsi que la force nécessaire pour que l'objet retrouve sa morphologie initiale après la déformation, comme illustré ci-dessous (Fig. 2.7).

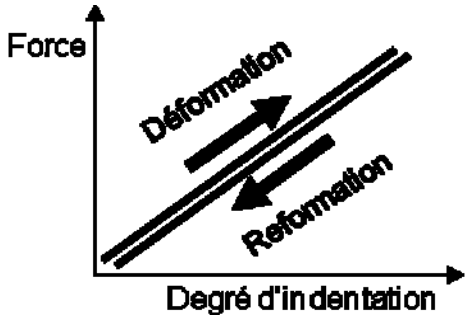

Figure 2.7 : Graphique de la force en fonction du degré d'indentation

2.3.2 Paramètres biomécaniques : rigidité et viscoélasticité

Le degré de déformation permet le calcul de la rigidité (plus l'objet sera résistant et moins grande sera la profondeur d'indentation pour une force donnée) alors que le temps de retour (hystérésis) à la position initiale indique le degré de viscoélasticité de l'objet. En d'autres termes, l'indenteur déforme l'objet en fonction de la charge choisie et la profondeur de l'indentation (de l'ordre de quelques micromètres) résulte de la déformation plastique et élastique. Au retour, lorsque la charge est enlevée, la déformation plastique reste alors que la composante élastique de la déformation retrouve sa forme originale, avec un retard en cas d'une déformation visco-élastique. Ainsi, le graphique de la force en fonction du degré d'indentation se compose de deux parties : la force nécessaire pour déformer l'objet et celle exercée pour que l'objet se reforme. La force nécessaire pour déformer l'objet est représentée par une droite dont la pente représente la rigidité (Fig. 2.8).

Figure 2.8 : Graphique de la force en fonction de la profondeur d'indentation pour deux objets de rigidités différentes.

Comme mentionné précédemment, la rigidité est représentée par la pente de la courbe, dans ce cas-ci l'objet A est plus rigide que l'objet B car la pente de l'objet A est plus élevée que la pente de l'objet B. Selon qu'un objet tend vers un modèle élastique,

viscoélastique ou plastique, l'allure du graphique de la force en fonction de la profondeur d'indentation différera. La figure 2.9 est une représentation typique d'un cas où l'objet déformé tend vers un modèle élastique. Dans ce modèle, l'objet reprend sa forme initiale immédiatement après l'indentation, les forces de déformation et de « reformation » sont égales et par conséquent le graphique représente deux courbes superposées.

Figure 2.9 : Graphique de la force en fonction de la profondeur d'indentation pour un objet élastique.

Dans le cas d'un objet qui est dit viscoélastique (Fig. 2.10), la « reformation » se fait plus lentement que la déformation, ce qui crée une aire entre la courbe de déformation et la courbe de « reformation ». Cette aire représente l'hystérésis, ou l'énergie dissipée à cause de la composante visqueuse dans le comportement du matériau. Plus l'objet est visqueux, plus cette aire est grande. Lorsque le retour à la forme initiale ne se fait pas complètement, cela est attribuable à la composante plastique de l'objet qui rend la déformation irréversible (Fig. 2.11).

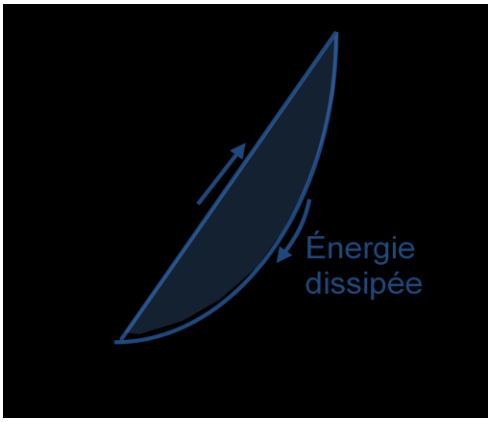

Figure 2.10 : Graphique de la force en fonction du degré d'indentation pour un objet viscoélastique. L'aire géométrique comprise entre les 2 courbes représente l'hystérésis ou l'énergie dissipée lors de la reformation de l'objet. Cette hystérésis est causée par la viscosité de l'objet.

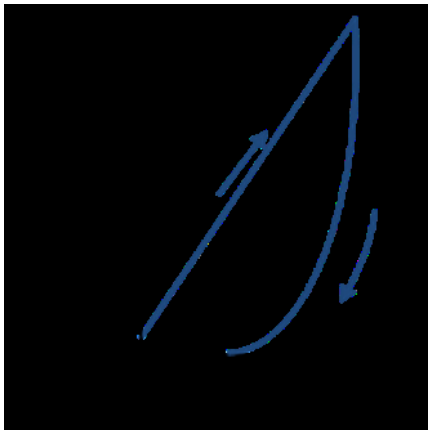

Figure 2.11 : Graphique de la force en fonction du degré d'indentation pour un objet plastique.

Pour une spore, les valeurs mesurées par micro-indentation sont influencées par deux facteurs, soit les propriétés mécaniques de la paroi ainsi que la pression hydrostatique interne.

2.4 Pression de turgescence

La pression de turgescence est la pression principale exercée par le contenu des cellules contre la paroi cellulaire et/ou la membrane cellulaire. La pression de turgescence est déterminée par la teneur en eau de la vacuole résultant de la pression osmotique, c'est-à-dire la pression hydrostatique provoquée par une solution dans un espace divisé par une membrane semi-perméable due à un différentiel de la concentration des solutés.

La pression de turgescence se développe à travers la paroi cellulaire rigide afin d'équilibrer le gradient de pression osmotique et est indispensable chez les CMA. En effet, la principale force pour trois des principaux mécanismes fongiques (croissance des hyphes, pénétration de substrat et décharge des spores) est communément attribuée à la pression de turgescence, dont les valeurs hyphales se situent entre 0.58 et 0.69 MPa selon les espèces fongiques (Money, 2004). La plupart des mycologues ont convenu que l'expansion de la paroi cellulaire à l'apex des hyphes en croissance est grandement affectée par la pression de turgescence (Wessels, 1993; Koch, 1994). Cette même pression permet aussi aux cellules spécialisées (appressorium et haustoria) de champignons de pénétrer une variété de structures solides telles que les racines d'une plante (Money, 2004) et enfin elle procure la force nécessaire afin de décharger les spores le plus loin possible (Trail, 2007). Certains chercheurs croient aussi que l'augmentation de la pression de turgescence dans une spore causée par une augmentation de la concentration de solutés (en particulier les tréhaloses) entraîne la germination de celle-ci (Undeen et Vander Meer, 1994). À l'inverse, une diminution de la pression de turgescence, causée par un environnement hyper-osmotique, entraîne une réduction de la germination chez certaines espèces de champignons (Jhoot et McKeen, 1965).

2.5 Méthodes indirectes pour mesurer la pression de turgescence

Puisque il a été démontré que la turgescence joue un rôle important pour différentes fonctions des champignons, il s'avère primordial de pouvoir la quantifier. Les méthodes indirectes utilisées pour déterminer la pression de turgescence des différentes espèces de champignons comprennent la plasmolyse naissante (lorsque 50% des cellules sont plasmolysées), la cytorrhyse naissante, l'osmométrie du point de fusion et l'osmométrie du déficit de pression de vapeur (Johns et al., 1999; Money and Hill, 1997; Money, 2001). Parmi celles-ci, nous aborderons uniquement la plasmolyse naissante ainsi que la cytorrhyse naissante.

2.5.1 Plasmolyse naissante et cytorrhyse naissante

Lorsqu'une cellule fongique est exposée à des concentrations élevées d'osmolytes, l'eau diffuse hors de la cellule. La perte de turgescence engendrée cause différents changements morphologiques selon la rigidité de la paroi cellulaire et la taille des osmolytes (Fig.2.12).

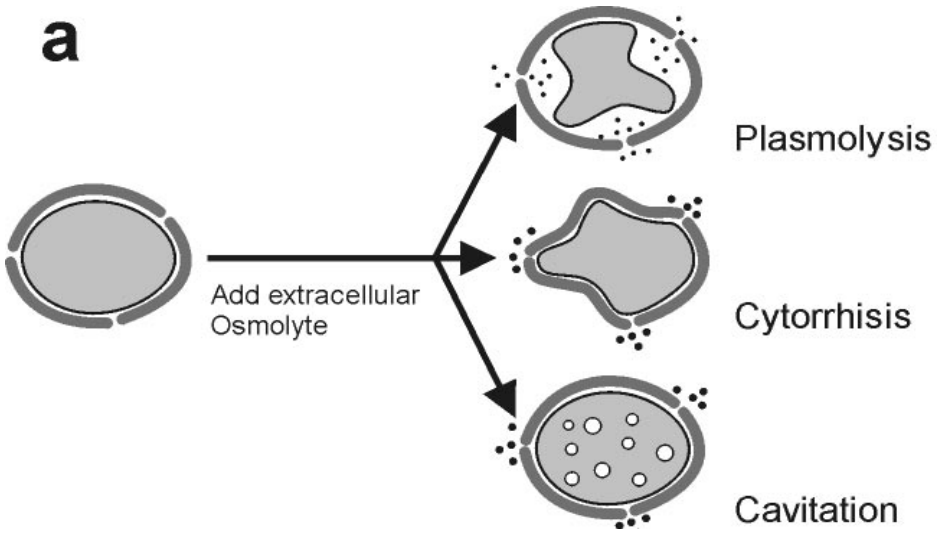

Figure 2.12 : Différents effets (plasmolyse, cytorrhyse et cavitation) d'une cellule soumise à un milieu hypertonique. Tiré de Bastmeyer et al. (2002) publié dans Annual Review of Biophysics and Biomolecular Structure.

La plasmolyse, c'est-à-dire la séparation de la membrane plasmique de la paroi cellulaire, se produit lorsque les osmolytes extracellulaires diffusent à travers la paroi cellulaire, mais sont exclus par la membrane plasmique. La plasmolyse naissante est atteinte lorsque le potentiel hydrique des solutions intracellulaires et extracellulaires est identique. Si la taille des osmolytes extracellulaires est plus grande que le diamètre des pores de la paroi cellulaire de la spore, l'eau de la spore suit le gradient de potentiel et diffuse vers l'extérieur de la spore. En conséquence, la spore fongique s'effondre, un phénomène appelé cytorrhyse (Money et Webster, 1988). Cependant, si la paroi cellulaire est très rigide de sorte que la cytorrhyse ne puisse se produire, le stress osmotique peut induire la formation de bulles de gaz dans le cytoplasme, un phénomène connu sous le nom de cavitation (Milburn, 1970). L'induction de plasmolyse naissante ou cytorrhyse naissante a été utilisée pour déterminer la pression de turgescence et la taille des pores de paroi cellulaire. Basé sur la valeur de la pression osmotique de la solution extracellulaire, une estimation de la pression de turgescence cellulaire peut être faite.

3 Mechanical properties and composition of spore cell wall of *Glomus irregulare*

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This manuscript will be submitted for publication in the near future.

3.1 Abstract

To survive and reproduce, microorganisms have to be able to adapt to changing environments. One of the features that render microorganisms resistant against environmental influences is the mechanical protection provided by the cell wall. To understand the biomechanical functioning of cellular features with structural function it is useful to analyze these from an engineering point of view. To do so, quantitative geometrical and mechanical information has to be gathered. Using model organism *Glomus irregulare*, a well-known arbuscular mycorrhizal fungus (AMF), a strategy will be described that allows for identification of biomarkers for robustness. Although fungal spores are known to be resistant structures, they are subjected to stress-inducing or mechanically damaging conditions during their life cycle which might compromise the viability of the spores, thus the efficiency of the symbiosis. In order to investigate the relationship between spore robustness and the preservation of their ability to colonize plants under various field conditions we used a wide array of techniques: 1) micro-indentation to determine spore stiffness and visco-elasticity, 2) confocal laser scanning microscopy in combination with histochemistry and immunodetection to determine biochemical spore wall composition. Chitin and glomalin are known to be major constituents of the fungal cell wall. We therefore assessed their distribution and relative abundance via a semi-quantitative fluorescence labeling method using Alexa-fluorophore 488 bound either with monoclonal antibodies for glomalin or with wheat germ agglutinin (WGA), a lectin that binds specifically to N-acetylglucosamine residues (chitin). Since *G. irregulare* produces intraradical and extraradical propagules, we compared the data obtained for each class depending on their origin, their size and their culture time. The results presented here show that cellular stiffness seems to vary depending on a combination of parameters such as wall chitin and glomalin, wall thickness, spore size and time of culture in growth media (age). Our data revealed a higher stiffness of extraradical spores compared to intraradical spores and also the fact that extraradical spore stiffness increases with age while the intraradical spore stiffness does not vary significantly over time. This last observation suggests that

spores are able to modulate their mechanical properties depending on the absence or presence of mechanical protection (the surrounding root tissue). We also found lower total content of chitin and glomalin in the cell wall of intraradical spores. This study demonstrates the power of using an array of complementary techniques for probing the organization of specific macromolecules and their role in determining the mechanical resistance of the spore cell wall.

Keywords

Glomus irregulare · Cell wall · Chitin · Glomalin · Arbuscular mycorrhizal fungus · Stiffness · Cell mechanics · Micro-indentation · Immunodetection · Spore

3.2 Introduction

Arbuscular mycorrhizal fungi (AMF) form symbiosis with the roots of many vascular plants, an association in which both partners exchange nutrients with each other (Bonfante, 1995). AMF play important roles related not only to the plant host but also to the structure of the soil such as by facilitating nutrient uptake by crops in low-input farming systems, where the concentration of soil phosphorus is generally low (Harley and Smith 1983). With the decrease of phosphate availability predicted for the coming years, agricultural practices will have to turn to alternative means to enhance plant growth. The use of mycorrhizal fungi is among the most promising avenues for a better fertilization management and more sustainable practices (Declerck et al., 2005). Therefore, there is a great interest in using these organisms in horticultural and agricultural products.

Spores of the arbuscular mycorrhizal fungus *Glomus irregular* isolate DAOM197198 are now produced on a large-scale to supply this increased demand in agriculture. Crucially, during this production process the viability of these spores must be maintained for extended periods of time and their ability to colonize plants under various

field conditions must be preserved. A better understanding of the biomechanical properties of the spores in relation to their physiological status will help to improve production processes and enhance spore fitness in the commercial products. Moreover, unraveling the relationship between biochemical and biomechanical properties will increase the understanding of fungal biology in general.

The recognition of the importance of biomechanical properties at cellular level has given rise to a surge in cytomechanical applications both in animal and plant biology (Geitmann 2006). Contrary to the burgeoning field of animal and plant cell mechanics, experimental studies on fungal spore biomechanics are extremely scarce. Spore ontogeny, morphology and factors affecting germination were investigated by Siqueira et al. (1982). Subsequent reports have reviewed spore germination in different species of Glomales (Safir et al. 1990; Meier and Charvat 1992). However, while we can rely on earlier studies of the anatomy of fungal spores (Maia and Kimbrough, 1994; Peterson et al., 2004; Massicotte et al., 2004), no biomechanical data on spore mechanical behavior and on the correlation with viability and the ability to germinate is available. Therefore, the present study will provide novel information and allow new insights into the field of mycorrhiza research.

Since the mechanical protection of fungal cells is attributed to their cell wall, we analysed this structure in detail. The spore wall in particular has been characterized biochemically in several studies (Grandmaison et al., 1988; Maia et al., 1993; Gianinazzi-Pearson et al., 1994). These studies have determined that it is composed mainly of a polysaccharide-based three-dimensional network. The cell wall used to be perceived as an inert exoskeleton, but nowadays it is seen as a live structure subject to changing if submitted to modification of culture conditions and environmental stresses (Latgé, 2007). **The objective of this study is the detailed and quantitative analysis of the biochemical composition and the biomechanical behavior of the spore cell wall of the fungus *Glomus irregulare*.** This fungus forms intraradical spores, also referred as vesicles in

litterature, inside the root and extraradical spores aggregated in the soil. Intraradical spores are usually ovoid to irregular whether extraradical spores are usually globose (Fig.3.1).

In order to investigate spore robustness, a combination of biomechanical and microscopic methods has been employed. Micro-indentation was used to determine spore stiffness and visco-elasticity whereas confocal laser scanning microscopy in combination with histochemistry and immunodetection has allowed to relate the biochemical composition to the other parameters.

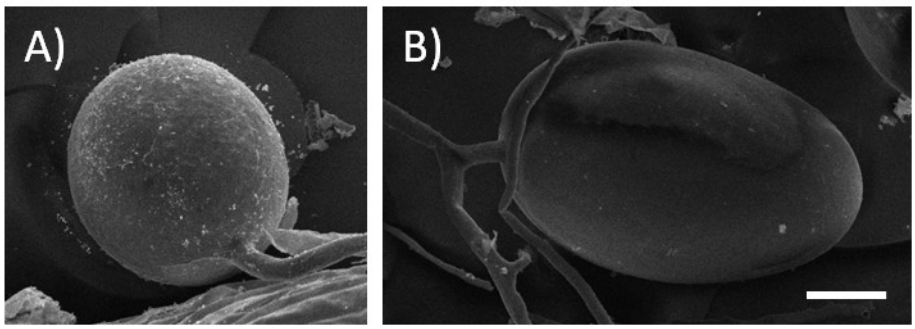

Figure 3.1 : Scanning electron microscopy images of *Glomus irregulare* A) extraradical spores which usually have a rough and round shape and B) intraradical spores which usually have a smooth and oval shape. Bar = 20 µm

3.2 Materials and methods

3.2.1 Isolation of spores by wet sieving

In order to study the different types of spores found in the commercially formulated product provided by Premier Tech Biotechnologies, spores were separated by wet sieving according to their size (sieves used : 150 µm, 75 µm and 38 µm), culture time and origin into the classes found in Table 3.1 and stored at 4°C in 0.1% peptone solution. The spores were grown with *Daucus carota* roots cultures in a proprietary large-scale production

process based on the two-compartment *in vitro* system described by St-Arnaud et al. (1996). This system allows the growth of the fungus and formation of extraradical spores on one compartment and restricts the growth of mycorrhizal roots and formation of intraradical spores to the other compartment. Time of culture started when a portion of colonized roots was placed in a new reactor and spores were harvested after 10, 20 and 40 weeks.

Table 3.1: Classification of spores by size fraction, culture time and origin

Class	Size fraction (µm)	Culture time(weeks) expressed as age	Origin
1	38-75	10	Extraradical
2	38-75	10	Intraradical
3	75-150	10	Extraradical
4	75-150	10	Intraradical
5	75-150	20	Extraradical
6	75-150	20	Intraradical
7	75-150	40	Extraradical
8	75-150	40	Intraradical

3.2.2 Preparation of sieves for immunohistochemistry

Preparation of small sieves to hold samples during incubations with antibodies/lectin and in washing steps were made from 6 cm blue pipette tips. Tips were cut into 2 cm sections. A fine nylon mesh (40 µm openings) was fused to one end of each section by heating, then the mesh was cut to fit the outer diameter. Sieves fit into wells of a 20-well tissue culture plates. To transfer samples, tubes were placed on a paper towel to blot off medium before submerging it into the following solution.

3.2.3 Spore fixation

Spores were fixed in 2% glutaraldehyde, 6% formaldehyde (freshly prepared from paraformaldehyde) in phosphate buffered saline (PBS) (0.2M, pH 7.4) as described by Declerck et al. (2000), with modified incubation times due to acceleration of fixation through microwaves (30 min fixation in microwave instead of 3 hours at room temperature). The microwave oven (Pelco Biowave 34700, Ted Pella Inc.) was used at maximum power, with a maximum temperature set at 26 ± 2°C and samples were washed 3 x 5 minutes with PBS 0.1M afterwards.

3.2.4 Freeze-shatter procedure

After fixation and washing, the samples were transferred to Eppendorf tubes. Care was taken to avoid crushing unfrozen spores. Grasping the Eppendorf tubes with tongs, the samples were lowered quickly into liquid nitrogen. After 1 minute, the tubes were removed from liquid nitrogen and placed on a cold block of aluminium. Using a miniature pestle shaped to fit in the Eppendorf tube, moderate pressure was applied to the samples to crush them. After 5 minutes, 0.1M PBS was added and the samples were transferred to staining sieves. Spores were then blocked to prevent non-specific adsorption of lectin/antibodies using 2% bovine serum albumin (BSA) in 0.1M PBS and incubated for 15 minutes and washed one time in PBS 0.1M.

3.2.5 WGA-Alexa Fluor 488 labeling of chitin

For chitin detection, spores were labeled with wheat germ agglutinin (WGA), conjugated to green fluorescent dye Alexa Fluor 488 (WGA-Alexa Fluor 488; Invitrogen Corp., Carlsbad, CA, USA). WGA is a carbohydrate-binding protein that selectively recognizes N-acetylglucosamine residues, such as chitin. After the freeze shatter procedure,

spores were incubated at a concentration of 10 µg/ml WGA in PBS 0.1M overnight at 4°C. After 3 x 5 min washes in 0.1M PBS, samples were placed on a glass slide and mounted in citifluor, a medium formulated to prevent rapid photobleaching and covered by a coverslip. 15 spores (chosen randomly) per class were analyzed by confocal laser scanning microscopy.

3.2.6 Immunofluorescent labelling of glomalin

The anti-glomalin monoclonal antibody (MAb32B11) was produced by overgrowth of the hybridoma cell line 32B11 (American Type Culture Collection) in Roswell Park Memorial Institute (RPMI) culture medium as described by Wright (2000). Antibodies in cell-free supernatant were stored at 4 °C as a sterile solution. Spore samples prepared by the freeze shatter procedure were incubated with the monoclonal antibody 32B11 (diluted 1:3 in PBS 0.1M) overnight at 4 °C. This antibody dilution was chosen because in preliminary experiments it has been shown to result in optimal labeling without background. After washes (3 x 5 min in PBS 0.1M), samples were exposed to a commercially available (Invitrogen) goat antimouse IgM antibody tagged with AlexaFluor 488 (diluted 1:100 in 0.1M PBS containing 0.1% BSA) for 2 h, washed three times and mounted as described above. 15 spores (chosen randomly) per class were analyzed.

3.2.7 Confocal laser scanning microscopy (CLSM)

Spectral and spatial distribution of Alexa Fluor 488 stained samples were visualized with a Zeiss LSM-510 Meta confocal laser scanning head fitted on an Axiovert 200 microscope (Zeiss, Jena, Germany). Because *G. irregulare* spores show strong autofluorescence, especially emitted from the cell wall, that interferes with the specific immunofluorescence (IF), linear unmixing was used. This algorithm removes autofluorescence by separating its spectrum from that of Alexa Fluor 488. Images were

collected with a Zeiss Plan neofluar 40X/1.3 oil objective, recorded using the 488 nm argon laser and emission channels in the 490 nm – 600 nm spectral range were used for fluorescence detection. In all cases, images were taken with the same parameter settings. To minimize the effect of photobleaching resulting from consecutive scans, data presented here were always recorded from the first scan of each cell.

3.2.8 Quantification of fluorescence intensity

Imaging data were analysed using ImageJ software to quantify fluorescence of the cell wall by measuring fluorescence intensity at several points (using a continuous line placed in the center of the wall drawn to follow its outline) and then averaged. The intensity of Alexa Fluor 488 fluorescence was quantified to semi-quantitatively compare the abundance of the respective cell wall components between different samples. All measurements were made under identical conditions. Signal deriving from cell wall autofluorescence was subtracted from the total signal through linear unmixing prior to determination of the specific fluorescence intensity. Averaging the values as described above provides an indication for the local density of chitin and glomalin in a unit volume of wall material. To obtain the total content of these substances produced by a single spore, the average fluorescence intensity representing the local density was multiplied by the total cell wall volume of each individual spore (calculated from spore diameter and wall thickness).

3.2.9 Micro-indentation

Spores were placed on a coverslip and incubated at 37°C for 3h. Samples were submerged in 0.1% peptone solution in the experimental chamber of the micro-indenter. The microindentation assembly consists of a vertical glass stylus whose circular end has a diameter of approximately 5 μm. This stylus is mounted on a horizontal glass cantilever 3

cm in length which in turn is mounted on a linear piezoelectric motor. Optical sensors monitor the vertical positions of the motor and the stylus. When the motor is activated the cantilever undergoes a corresponding vertical displacement. The positions of the poker tip and of the motor signal are identical when the stylus tip is not in contact with a surface. When it is in contact, the force exerted on the stylus by the object reduces its displacement and bends the cantilever. By using the formula F = k * X, where k is the bending force constant of the cantilever and X the displacement difference between the tip and the motor, it is possible to calculate the resistive force (F). In the experiments reported in this paper, the bending constant (k) = 2249 millidynes/μm, and the motor was programmed to execute a 20 μm vertical movement with a velocity of 10 μm s^{-1}. The cells were monitored using a Nikon TE2000 inverted microscope equipped with a Roper fx cooled CCD camera. Image acquisition was done with the software ImagePro (Media Cybernetics) and force was calculated using Matlab software. 10 spores (chosen randomly) per class were analyzed.

3.2.10 Cell wall thickness measurement

Spores were placed on a glass slide, mounted in 0.1% peptone solution and covered with a coverslip. Samples were visualized with the Axiovert 200 under DIC (differential interference contrast) illumination using a 100X oil objective (NA 1.4). Cell wall and spore diameter was measured using the ImageJ software. 20 spores (chosen randomly) per class were analyzed. Given that intraradical spores are usually oval in shape, we measured their short and long diameters to obtain the mean diameter.

3.3 Results

3.3.1 Older spores are protected by a thicker cell wall

The spore cell wall of *G. irregulare* (Fig.3.2) is known to be composed of two zones: the outer wall formed by two tightly linked layers (L1 and L2) that are somewhat translucent,

and an inner wall formed by a laminated layer (L3). To determine if there was a difference between wall thickness of extraradical and intraradical spores, we quantified the fungal spore wall thickness by DIC microscopy according to origin, age and size of spores. The thickness of the spore wall (including all three layers) varied with the origin (intra or extraradical) of the spores and the time of culture (age) of 75-150 µm spores (Fig.3.3B). Analysis of variance (ANOVA) showed that only age ($F = 20.39$, $p = 0.000$) exhibited significant differences in wall thickness with older spores having thicker walls. Extraradical spores had a tendency to display a thicker wall than intraradical propagules although this difference was not significant. Effect of spore size within the 10 week-old spores on cell wall thickness was also not significant between size classes 38-75 and 75-150 µm (Fig.3.3A) but when we subdivided these 2 classes into 7 classes, comparison of wall thickness with spore diameter yielded a weak but significant positive correlation of $r = 0.49$ (Fig.3.3C).

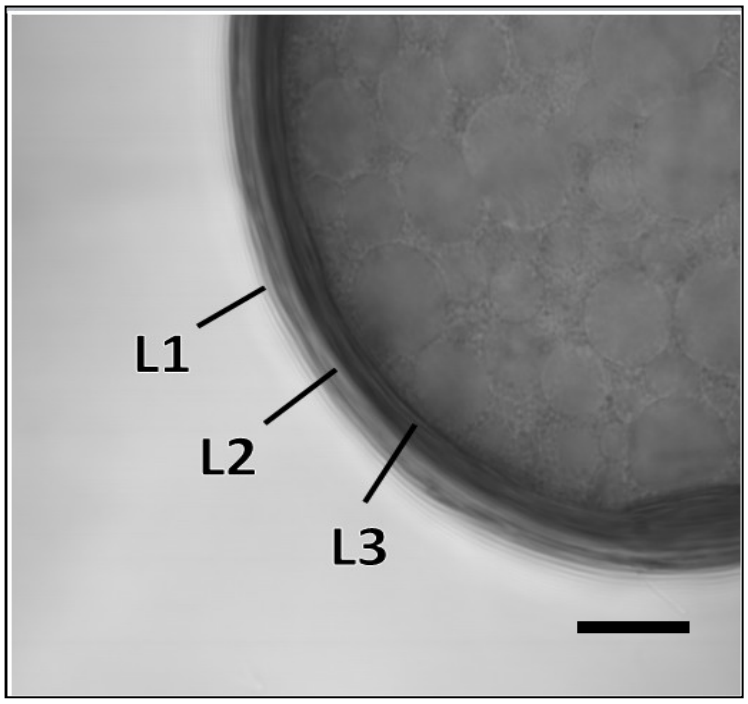

Figure 3.2: Brightfield micrograph of a spore of *Glomus irregulare*. The spore wall (arrow) comprises several layers (L1, L2, L3) according to Sturmer and Morton (1997). Bar = 10 μm

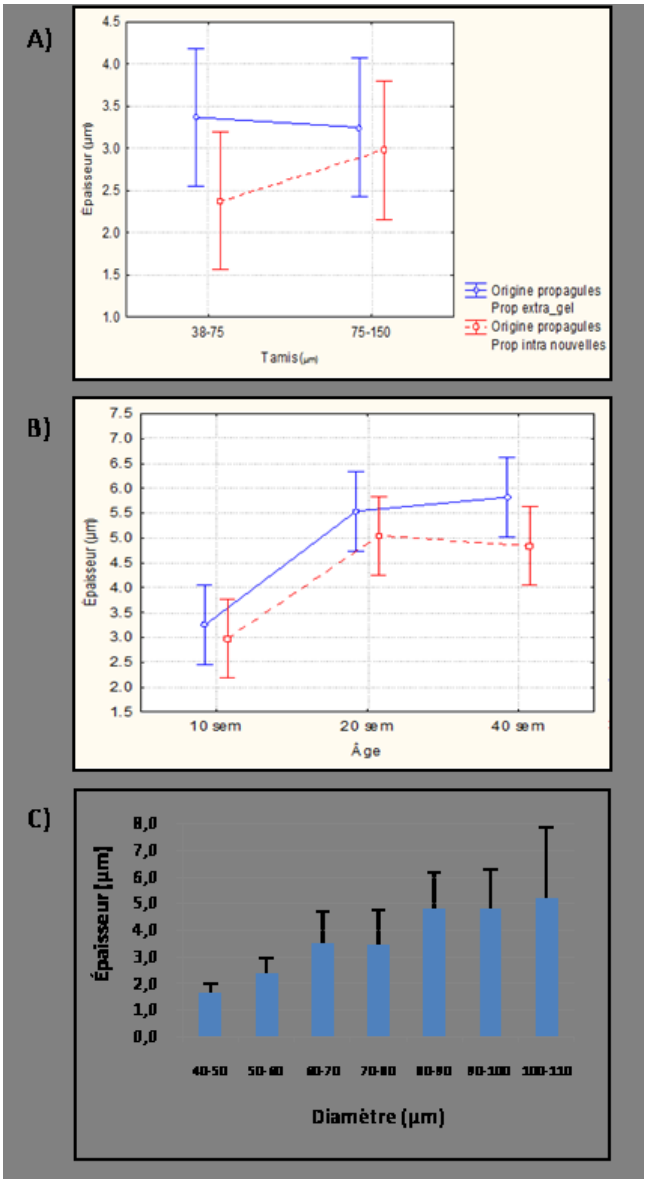

Figure 3.3: Spore wall thickness of *G. irregulare* measured by brightfield imaging as a function of A) origin and size fraction of 10 weeks-old spores, B) of origin and culture time of 75-150 µm spores and C) of spore diameter. The blue trace represents the extraradical spores and the red trace represents the intraradical spores.

3.3.2 Chitin is localized mainly in the outer wall

Chitin is one of the major cell wall components in many fungi. However, the biochemical composition of fungal cell walls changes during ontogeny, and no information

is available on the distribution of chitin and its developmental changes in *G. irregulare* spores. Cell wall chitin was localized in growing *G. irregulare* propagules using labeled wheat germ agglutinin (WGA), which binds to chitin, a homopolymer of N-acetylglucosamine. WGA has been used in several studies on chitin distribution in fungal walls (Ramonell et al., 2005; Vierheilig et al., 2005). Confocal laser scanning microscopy visualized WGA-488 Alexa conjugates in the outer layers (L1 and L2) of the cell wall (Fig.3.4).

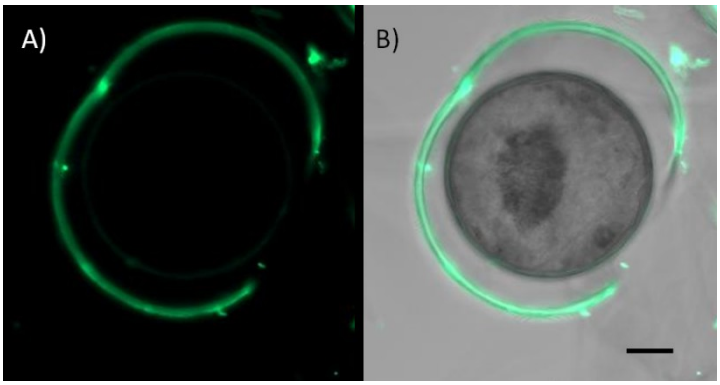

Figure 3.4: Confocal micrograph of chitin localization in spore wall of *G. irregulare*. A) Chitin specific fluorescence after subtraction of autofluorescence signal. B) Overlay of fluorescence micrograph on corresponding brightfield image showing that chitin localized mainly in the outer wall layers (L1 and L2 completely detached from the spore) and faintly in the inner wall layers (L3 still attached to the spore); Bar = 20 µm

Linear unmixing was used to separate the signal emitted by Alexa Fluor 488 from that of autofluorescence produced by the fungal wall. Chitin specific label was continuous in all layers but strong only in the outer layers (L1 and L2) of the spore wall, whereas the inner layer (L3) was only weakly fluorescent. This was particularly evident in spores in which the L2 layer had separated from the L3 layer. The center of the spore did not fluoresce in the Alexa Fluor 488 emission spectrum confirming the specificity of the label. The label pattern suggests that the outermost cell wall contains a high abundance of chitin.

3.3.3 Semi-quantitative comparison of chitin abundance

Quantification of the differences in labeling intensities between spore classes (Fig.3.5B) showed that chitin local density in the walls of spores from size class 75-150 μm varied significantly with culture time ($F = 5.28$, $p = 0.007$). Younger spores (10 weeks) in this size class showed more intense label than older spores (20 weeks and above). The origin of the spore did not affect chitin local density significantly, even if the extraradical spores seemed to have a tendency for displaying a higher chitin local density than intraradical spores. Within the 10 week-old spore class, spore size was not associated with significant differences in the local density of chitin (Fig.3.5B). Fig. 3.5C represents the total content of chitin in the wall of a single spore calculated by multiplying the local chitin density with the cell wall volume. This figure shows that extraradical spores have more total chitin content in their cell wall than intraradical spores.

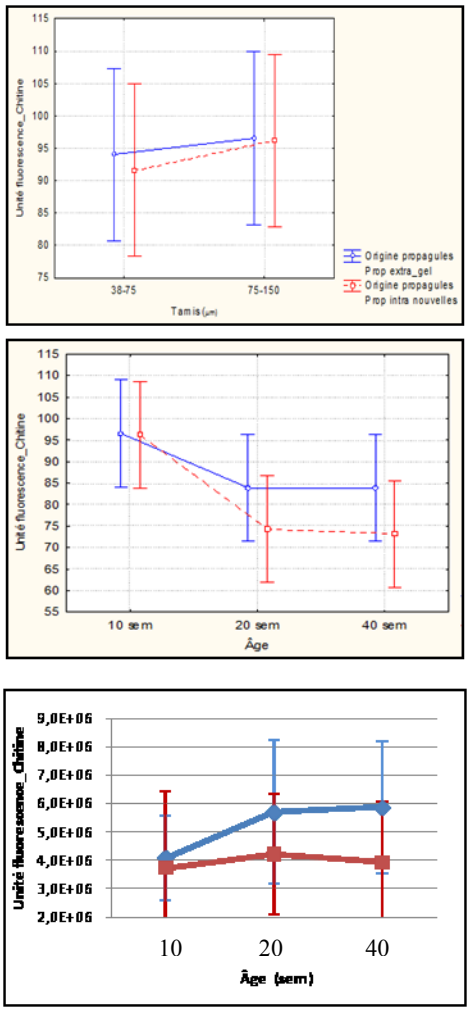

Figure 3.5: Local density of chitin expressed as relative fluorescence intensity after WGA–Alexa Fluor 488 label in the spore wall of *G. irregulare* as a function of A) origin and size fraction and B) origin and time of culture in the 75-150 µm fraction. C) Total chitin content in the spore wall as a function of origin and time of culture in the 75-150 µm fraction obtained from the multiplication of spore wall volume and local chitin density. The blue trace represents the extraradical spores and the red trace represents the intraradical spores.

3.3.4 Glomalin is localized mainly in the inner spore wall

The presence and relative abundance of glomalin in spores of *G. irregulare* was measured after immunolocalization with monoclonal antibody Mab32B11. Omitting MAb32B11 from the protocol resulted in the absence of specific label demonstrating that the AlexaFluor 488-labeled secondary antibody did not bind unspecifically to the spores (Fig. 3.6A). The immunolabel revealed differences between the wall layers (Fig 3.6C). Highest label intensity was present in the inner wall zone, corresponding to the laminated layer (L3). In the outer wall zone, composed of L1 and L2 layers, only L2 was labeled, but less intensely so when compared to L3. The intensity and spatial distribution of glomalin label varied within individual samples with some spores not showing any label at all in the cell wall (Fig.3.6B). Despite the variation, differences between spores of different ages could be detected. Generally, a continuous to semi-continuous layer surrounded young spores (10 weeks) (Fig.3.6D), while older spores typically displayed smaller patches of glomalin label (Fig.3.6E-F). This patchy labelling of glomalin could be explained by the evanescent layer (L1) on the spore surface as described by Morton and Benny (1990), usually thought to be due to the chitin degradation through microorganisms.

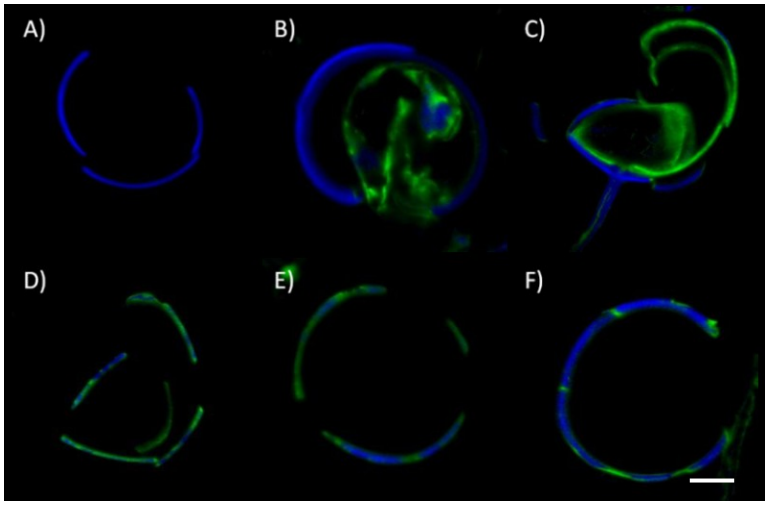

Figure 3.6: Fluorescence micrographs of glomalin labeled, freeze fractured extraradical spores of *Glomus irregulare*. (A) Control sample without primary antibody. The blue label corresponds to the autofluorescence emitted by the cell wall. (B-F) Spore walls labeled with a monoclonal antibody against glomalin showing: (B) no glomalin fluorescence in the cell walls, C) intense and uniform fluorescence emitted from the inner cell wall (L3) compared to the weaker and non-uniform fluorescence from the outer wall (L1 + L2), (D) young spore walls (10 weeks culture), (E) mature spore walls (20 weeks culture), F) old spore walls (40 weeks culture); Bar = 20 μm.

3.3.5 Assessment of relative glomalin abundance

The highest local density of glomalin in 10 week old spores was found in size class 38-75 μm of extraradical spores with a local density of almost twice that found in bigger and/or intraradical spores (Fig.3.7A). Extraradical spores had a higher glomalin local density than intraradical spores ($F = 5.39$, $p = 0.023$). Age did not significantly affect the glomalin local density within the 75-150 μm size fraction ($F=2.40$, $p=0.10$) (Fig.3.7B). However when these spores were analysed for their total glomalin content (fluorescence

intensity times cell wall volume), age seemed to increase the total content found in the extraradical spores whereas the intraradical spores seemed less affected (Fig.3.7C). It should be noted that estimates based on fluorescence intensity are semi-quantitative and cannot be equated with a fully quantitative biochemical analysis.

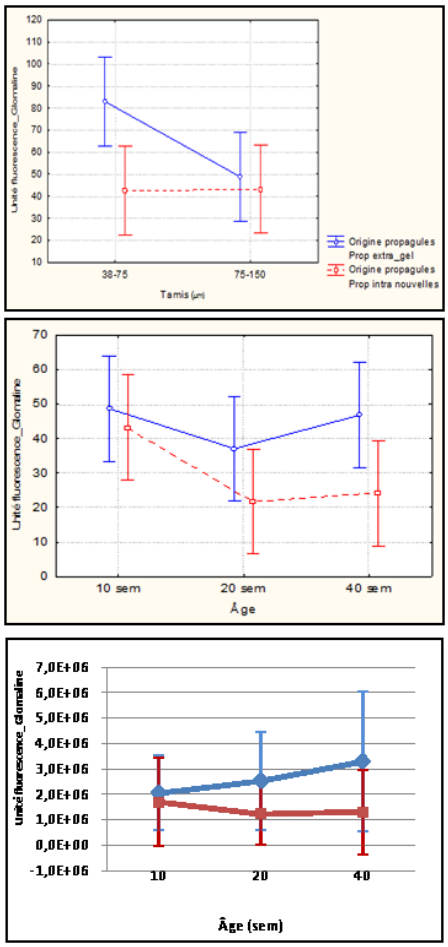

Figure 3.7: Glomalin local density measured as relative, local fluorescence intensity after label with monoclonal antibody (Mab32B11) in the spore wall of *G. irregulare* as a function of A) origin and size fraction for the 10 weeks old spores and B) spore origin and culture time in the 75-150 µm fraction. C) Total content of glomalin per spore as a function of origin and culture time in the 75-150 µm fraction obtained by multiplying spore wall volume with local glomalin local density.

3.3.6 Spores show a completely elastic behavior upon compression

To assess potential differences in the mechanical properties between spores of different ages and origins, we used micro-indentation to monitor their cellular stiffness. The method is based on a local, mechanical compression of the spore by a microscopical cylindrical probe or stylus mounted on an inverted microscope that allows the manipulator to see the point of contact between the stylus and the spore (see Fig.3.8).

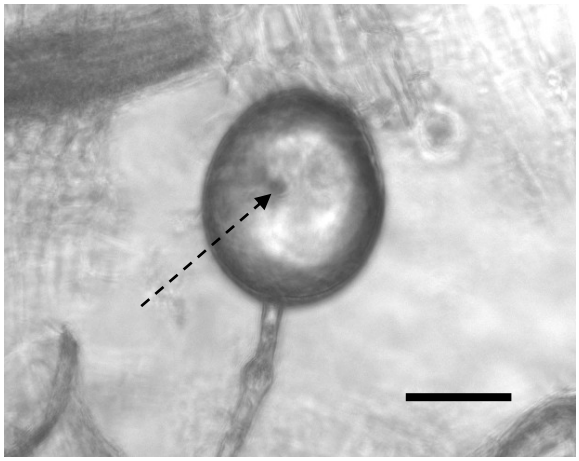

Figure 3.8 : Micrograph of a spore contacted by the indenter, the green arrow points at the area of contact which can be visualized by the shadow of the stylus above the spore. Bar = 50 µm

Fig. 3.9A shows a typical displacement curve of the micro-indenter probe indenting the spore. The total imposed vertical position change of the cantilever is 20 µm with a velocity of 10 µm s^{-1}. The tip position (green curve) shows the displacement of the stylus that is reduced compared to the motor when the stylus is in contact with an object such as a spore. From this curve, the force-displacement curve is calculated using the bending stiffness of the cantilever (Fig. 3.9B). This curve provides the force experienced by the stylus as a function of tip position (zero at the horizontal line and positive values above). The slope of the force-displacement curve represents the stiffness of the object. The blue

trace represents the loading/indenting process and the green trace is the unloading/release of the indenter from the spore.

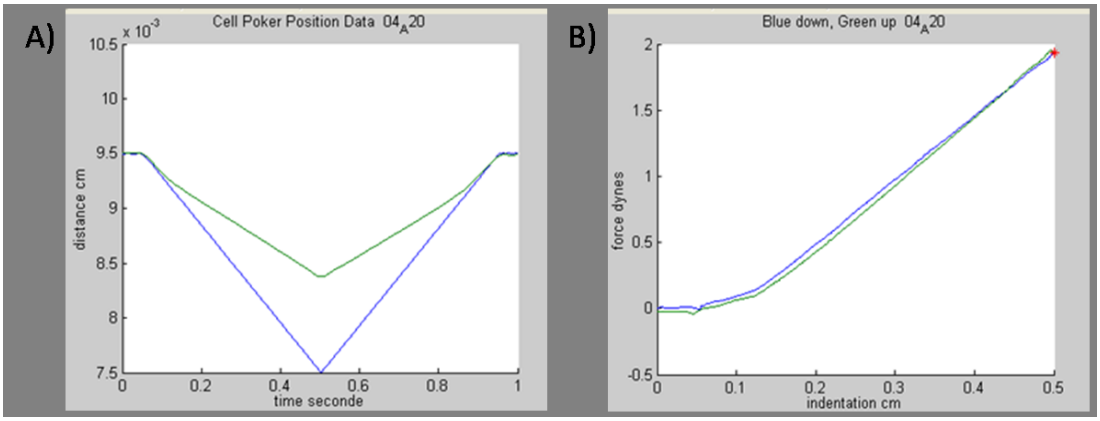

Figure 3.9: Deformation plot of a single micro-indentation experiment (A) Vertical position of the stylus (green) and the motor over time. (B) Force deformation plot representing the force experienced by the stylus at any given vertical position. Deformation (blue) and unloading (green) are identical indicating a purely elastic response

Prior to contact with the surface (at the extreme left of Fig. 3.9B), the cell force is zero but, as the probe deforms the surface, the force increases linearly. The linear nature of the curve indicates that at the indentation depths applied here the slope of the curve (rigidity) was not influenced by the depth of indentation. In the present example, an intraradical spore at 40 weeks of culture, the lower trace is almost exactly superposed to the upper trace, which means that there is no loss of energy in the return process, thus no hysteresis. The absence of hysteresis indicates that the spore surface was completely elastic. Elasticity and stiffness were fairly uniform across the surface, independently of the position of the stylus on the spore (not shown). Extraction of absolute mechanical properties would require the analysis of the precise geometrical situation of the indentation (Bolduc et al., 2006), since cellular stiffness is influenced by the mechanical properties of the cell wall, by the turgor pressure of the cell, and by the contact area between micro-indenter and spore surface. Extracting absolute values for the mechanical properties of the cell wall material

would require establishing a mechanical model of the spore, an endeavour that necessitates the collaboration with an engineer. Similar models have been established in the Geitmann lab in the past, but at present there is no modeler in the lab. However, semi-quantitative comparisons already provide valuable information in this case. They clearly show that the spores are much stiffer than growing pollen tubes, another walled cell type commonly measured in the Geitmann lab (Geitmann and Parre 2004, Zerzour et al., 2009). The linearity and elasticity of the spore behavior shown in Fig. 3.9 are typical for all the age-groups and origins measured in the present series of experiments. The reversability of the response and simultaneous microscopic observation indicate that the probe tip did not penetrate through the spore wall.

3.2.7 Extraradical spores are stiffer than intraradical spores

Analysis of variance (ANOVA) showed that spore origin had a significant effect on spore stiffness ($F=5.34$, $p=0.022$) (Fig. 3.10B) with the extraradical spores being stiffer when comparison is done within the 75-150 μm class. Extraradical spores seemed to show an increase in stiffness with longer culture times although the effect was not significant ($F=0.945$, $p=0.4$). The ANOVA performed on the 10 week old spores indicates that stiffness did not significantly change with spore origin and size, although bigger spores tended to be stiffer ($p=0.09$) (Fig. 3.10A). This would be consistent with the increase in wall thickness that is associated with spore size (Fig. 3.10B). These results indicate a high variability in spore stiffness between individual spores. Fig. 3.10C shows a weak but significant positive correlation ($p= 0.29$) between spore rigidity and spore diameter (all spores types, ages and sizes included). This relatively low correlation indicates that the rigidity of the cell is not influenced solely by the actual diameter of the cell.

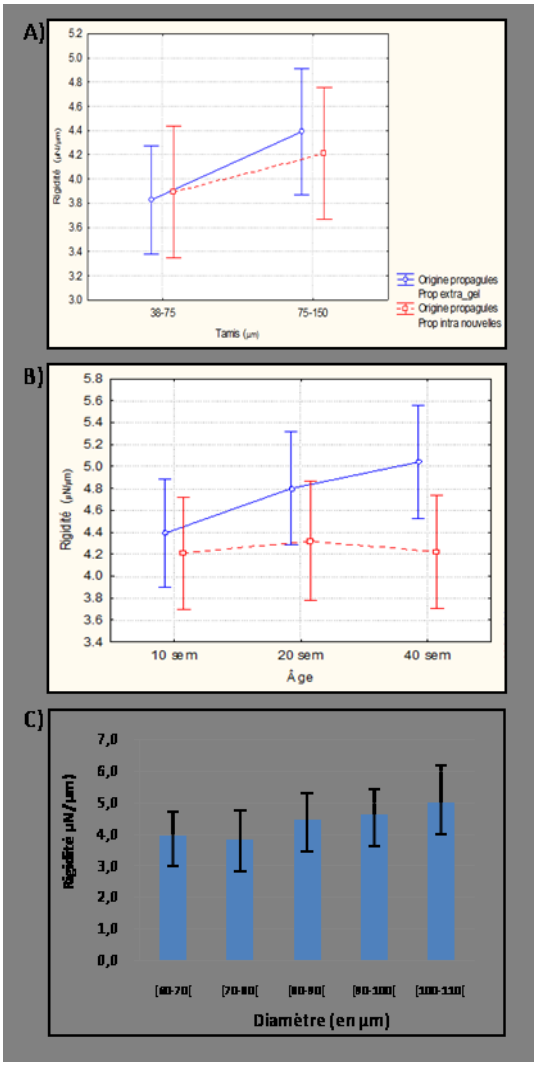

Figure 3.10: Stiffness of spores measured by micro-indentation as a function of A) origin and size fraction of 10 weeks old spores, B) origin and culture time of 75-150 µm spores and C) of spore diameter. The blue trace represents the extraradical spores and the red trace represents the intraradical spores.

3.4 Discussion

In industrial production and formulation processes, AM fungal spores are susceptible to the forces created by agitation, pump pressure and also to hydrodynamic stresses. The resistance of the spores against these mechanical forces depends mainly on the mechanical properties of their cell wall. The mechanical properties of the fungal cell wall and their dependence on environmental conditions remain poorly understood. In this study, the stiffness of spores of *G. irregulare* has been determined with the use of micro-indentation under physiological conditions. To be able to correlate the biomechanical behavior to structural features, the biochemical composition and the cell wall thickness were determined and the degree of correlation analyzed. This will equip us with an analytical tool set that allows us to predict cell mechanical properties in a given situation.

St-Arnaud et al. (1996) observed, after 40 h staining in 3-(4,5-dimethylthiazol-y1)-2,5-diphenyl-2H-tetrazolium bromide, that the percentage of non-viable *G. irregulare* spores under the culture conditions used was 18%. Since our method of culture was similar, we assume that viability in our samples was similarly around 80%. The percentage of non-viable spores therefore represents a non-negligible portion of the extraradical spore population. Cellular stiffness as determined by micro-indentation is potentially influenced by both mechanical cell wall properties and turgor pressure. Given that non-viable cells have no turgor pressure whereas viable cells are expected to have significant intracellular pressure (Reiser et al., 2003), approximately 20% of the measured spores should show significantly lower cellular stiffness if turgor had significant influence on cellular stiffness. However, despite a total number of 80 spores measured, no outliers with significantly lowered values were observed. This suggests that the spore wall is the dominating feature determining spore resistance to compression force. This finding is consistent with the significant thickness of the spore cell wall compared to the primary cell wall of the plant cells measured by micro-indentation (Geitmann et al. 2004; Lintilhac et al. 2000).

Our results suggest that the extraradical spores are stiffer than the intraradical spores although both respond in elastic manner. Furthermore, the stiffness of extraradical spores seemed to increase with the number of weeks of culture, whereas the stiffness of intraradical spores did not change over time. This finding is consistent with the marked differences in the total content of chitin and glomalin, which are both higher in the extraradical spore wall.

Although glomalin is known to be massively released into the environment by Glomales fungi, our study has shown that a significant amount is retained within the spore walls. This suggests that glomalin is an important structural component of the spore wall, consistent with quantitative measurements revealing that the majority (80%) of glomalin produced by the fungus is firmly incorporated into the hyphae and spore wall (Driver et al., 2005).

Our results (Fig. 3.7C) show that extraradical spores synthesize or retain more glomalin in their cell wall than the spores grown inside the roots. Furthermore, contrary to intraradical spores, the total content of glomalin in the spore wall of extraradical spores increased over time. This may be a response to the direct contact of these spores with the soil environment thus exposing them to mechanical stresses. Glomalin could be involved in the protection of the spores. This would be consistent with the finding that glomalin is produced by fungal hyphae in response to the interaction with changing environments comprising other soil organisms (defense, palatability, surface colonization) and soil surfaces (hyphal attachment, interaction with nutrients, etc.) (Driver et al., 2005). Compared to other soil fungi, AMF seem to be protected against grazers (Klironomos and Kendrick, 1996). It is therefore possible that glomalin may also reduce palatability of

spores (Purin and Rillig, 2007). These results open up exciting new avenues of research into the roles of glomalin in the ecophysiology of AMF.

As indicated during this time-course study, local glomalin density (not to be confounded with "total content" shown in Fig. 3.7C) decreases or becomes less immunoreactive as spore age increases (Fig.3.7B). The following hypotheses may explain this phenomenon: a) Glomalin is used by the spores to resist stresses primarily during the first stages of the spore life, when they is still fragile. This would be consistent with the finding that glomalin synthesis increases sharply under stress conditions (Lovelock et al., 2004). b) Glomalin in young spore walls may be more immunoreactive. This is supported by the fact that early tests with the Mab32B11 antibody yielded inconsistent results with spores and hyphae of AMF when tested as mature cultures or samples that had been dried for varying periods of time after being collected. MAb32B11 detected glomalin only in fresh active cultures of AMF (Wright et al. 1996). c) Microorganisms may degrade glomalin over time, since although we used an in vitro system (e.g., absence of soil) to produce the spores, harvesting was done in non-aseptic conditions. These hypotheses are not mutually exclusive.

Another aspect to be considered is that strong immunofluorescence was observed in the inner wall (L3) and weak immunofluorescence was observed in the outside zone which corresponds to L1 and L2 (Fig. 3.6C). As the spores have to be washed before fixation to remove residual culture medium, it is possible that some of the external antigen was removed, too, especially given the fragile nature of the L1 layer in most AMF.

The other cell wall component analysed, chitin, is a polysaccharide with monosaccharide units of N-acetylglucosamine joined by β-1,4 links which make chitin chains very straight and ribbon-like. These chains are arranged anti-parallel (α-chitin) and combined into a structure within which the sugar residues are heavily H-bonded making the chains very stiff and stable (Ruiz-Herrera et al., 2002). Cid et al. (1995) showed that when a

yeast daughter cell buds from a mother cell, a ring of chitin is found in the septum region to provide mechanical stiffness at this site. It is thus likely that chitin influenced the mechanical behaviour of the spore cell wall in our case.

In contrast to the glomalin distribution, specific fluorescence of chitin was mainly distributed throughout the outer wall layers (L1 + L2) (Fig. 3.4). The laminated layer (L3) only showed weak labeling, mainly restricted to its peripheral area. It has to be noted that the fluorescence intensities of glomalin and chitin label cannot be compared directly, since the ratio antibody/lectin: AlexaFluor 488 is different for each probe. Also, the number of antigens binding to each probe is not defined.

A correlation between the developmental stage of the spore and the chitin local density in the spore cell wall was evident. Our results (Fig. 3.5B) show that the local density of chitin decreased sharply (18%) after 10 weeks of culture and stabilized after 20 weeks. In the first place, the evidence strongly supports the notion that chitin is vital to protect the fragile young spores from mechanical damage. This high content of chitin could also have the function to protect the spore directly exposed to plant chitinases, as has been suggested for other fungal structures such as appressoria and haustoria (Ramonell et al., 2005). Therefore, it is likely that chitin is continuously produced during the course of spore growth and development. We also found lower total content of chitin in the cell wall of intraradical spores (Fig. 3.5C), suggesting that this type of spores does not require as much mechanical stiffness since they grow inside a protective matrix. Remarkably, the pattern in the variation observed in spore stiffness (Fig. 3.10B) is very similar to that of the differences in chitin content (Fig. 3.5C), corroborating that there might indeed be a correlation.

Not surprisingly, the rigidity of extraradical spores increased with age (Fig. 3.10B) but this effect was not dramatic. The rather large standard deviation could reflect variability of the measurements associated with independent spore cultures varying in time. A weak correlation was detected between spore diameter and rigidity. This is illustrated by Fig. 3.10C where smaller spores were found to be slightly more deformable than larger spores. However, wall thickness which is higher in bigger spores is more likely to be correlated with spore rigidity. Mechanical modeling on tubular cells has shown that if only wall mechanical properties are considered (not turgor), stiffness decreases with increasing tube diameter, whereas it increases with increasing wall thickness (Bolduc et al. 2006). In a spherical cell this effect is likely to be even more pronounced. The fact that the correlation between spore diameter and stiffness was weak (r=0.29), suggests that other factors might contribute to the rigidity. Wall thickness is likely to be important, but the correlation was relatively low as well (0.49) thus pointing at the biochemical composition to be crucial. Only few studies exist that relate the structure of walled cells to their stiffness. In pollen tubes, enzymatic modification of the cell wall changes cellular stiffness (Parre and Geitmann 2005a, 2005b) and the stiffness in baker's yeast was shown to only have weak dependence on cell size (Smith et al., 2000).

Wall thickness of spores harvested after 10 weeks was similar regardless of the origin of the material. Also, these spores had a significantly thinner wall (~40%) than those harvested at 20 or 40 weeks. Although the effect of origin was not significant ($p = 0.08$), extraradical propagules appeared to have a thicker wall (~12%) than intraradical spores. After 20 weeks of culture, the intraradical spore wall seemed to have attained its final dimension and did not thicken anymore even after a long period of incubation. This could be due to the added protection from the root cells. The overall thickness of the spore cell wall of *G. irregulare* seemed to be highly uniform over the surface of a single spore; this characteristic is known to increase the mechanical resistance to deformation. Li et al. (2005) found that this latter feature is reduced by about 53% as wall thickness changes

from uniform to completely non-uniform. It should be noted that in vitro culture is known to alterate morphological aspects such as wall thickness and diameter of fungal spores. Pawlowska et al. (1999) found that *Glomus etunicatum* spores formed in monoxenic culture were significantly smaller in diameter and had thicker laminated inner wall than soil-borne spores. Thus, it would be interesting to verify if our *in vitro* system also modify these characteristics in such way before extrapolating our results to the spores found in the environment.

While it would be a valuable exercise to compare our results with those from other studies, there are no previous estimates of AMF cell wall material properties to compare with. None of the available literature studies includes the exact experimental data needed on the mechanical properties of fungal cell walls. The only related study, to our knowledge, is the measurement of the stiffness of pollen tube determined by the exact same experimental technique. The cellular stiffness of these structures was estimated to be approximately 0.4 μN/μm (Zerzour et al., 2009), while our micro-indentation based quantification of *G. irregulare* spores were around 4.8 μN/μm, being about 12 times stiffer. Perhaps the comparison is not ideal, as the cell walls of both organisms possess different chemistry and structure as well as geometry, but the difference in one order of magnitude still suggests that the estimates obtained for *Glomus* spore cell walls are certainly reasonable. The differences in stiffness and strength are most likely due to the differences in their respective wall thickness. The cell wall of pollen tube is much thinner than *G. irregulare* spore wall ranging between 0.01 and 0.4 μm depending on the species (Picton and Steer, 1981; Derksen et al., 1995). Also, the load-bearing component is different; cellulose is the load-bearing component in plant cell walls whereas chitin is believed to be the load-bearing component of the AMF cell wall. It is also interesting to compare our data to those reported for other eukaryotic cell types. We note that our stiffness values are significantly larger (100x) than those obtained on animal cells (mice fibroblasts) using similar indentation measurements (Petersen, 1982). This is consistent with differences in

cell surface architecture; i.e., cell walls are not present in animal cells which are surrounded only by the pliable plasma membrane and occasionally by a proteic extracellular matrix. As a consequence, their stiffness derives largely from the intracellular cytoskeletal arrays. On the other hand, a crystal of tungsten (metal), known to have unusual strength and being very different from the complex, multilayered architecture of the fungal walls, has a stiffness approximately 40000x higher than a spore of *G. irregulare* (Oliver et Pharr, 1992). Clearly, further quantitative comparison of global and local measurements is needed to get a complete picture of the fungal cell wall stiffness.

3.5 Conclusion

This work represents the first estimate of AMF spore cell wall material properties and the first fundamental analysis of cell compression data. These results will be of considerable use in predicting spore stiffness and behaviour in different bioprocesses. Our long-term goal is to explain the structural basis of cell wall properties and their relationships to cellular mechanical properties. The present communication is meant to be an introduction to our approach and a preliminary survey of results. Although we cannot yet interpret our results in terms of detailed structural models, we can draw some general conclusions about the roles of the cell wall in determining cellular deformability.

In this paper we described a micro-indentation approach to measure cell wall mechanical properties of spores of the model fungus *Glomus irregulare*. The results presented in this paper show that cellular stiffness seems to respond to a combination of parameters such as wall chitin and glomalin, wall thickness, spore size and time of culture in growth media (age). It is difficult to extract quantitative material properties from these findings since the forces applied and deformations are measurements of the mechanical properties of the whole cell. Therefore, they are functions of each of the components which contribute to the overall mechanical behaviour of the cell. Nevertheless, our measurements have revealed several noteworthy characteristics of the spores of *G. irregulare* in culture.

These include the higher stiffness of extraradical spores over intraradical spores and also the fact that extraradical spore stiffness increases with age while the intraradical spore stiffness is not influenced by this condition. This suggests that the intraradical spores are protected by the root cells and they change their mechanical properties as a consequence of this protective layer. To a first approximation, biochemical composition seems to be mainly responsible for the stiffness of the spores. Both chitin and glomalin increase with age for extraradical spores (slope especially abrupt between 10 and 20 weeks) and do not vary much for intraradical spores and both are higher for extraradical spores. This pattern is similar to that of spore stiffness. But it is impossible, at this stage, to affirm which component influences the most the rigidity of the spore wall. For this we would have to degrade each component separately with enzymes such as chitinases and compare the stiffness of spores after each degradation. Still, the apparent influence of chitin and/or glomalin on stiffness provides further insight into the primary cause of the biological variability observed in micro-indentation measurements. These measurements are of considerable interest as they provide insight into the mechanical changes that accompany cell growth.

A quantitative interpretation of our measurements in terms of cellular material properties requires models for the observed mechanical behaviour to establish direct effects for each parameter on the shape of the force-deformation response and what impact this might have on determining cell-wall constitutive properties. Without a mechanical model of the cell it is extremely difficult to separate the contributions of each of the components on the overall stiffness of the cell. For a better understanding, further studies need to be carried out on how mechanical properties of the cell wall respond to various environmental factors.

4 Effect of fertilizers on osmotic pressure and germination of spores of *Glomus irregulare*

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This manuscript will be submitted for publication in the near future.

4.1 Abstract

Cellular turgor pressure and germination of *Glomus irregulare* extraradical and intraradical spores has been evaluated in relation to water activity (a_w) and the presence of nutritive solutes in fertilizers. We hypothesized that reducing sporal turgor by osmotic stress would inhibit germination of these cells because some experiments have suggested that germination requires an elevated sporal turgor pressure. Spores were incubated in three different fertilizers with aw values of 0.982, 0.964 and 0.882. At different intervals, spores were removed from the fertilizer and both the percentage of propagules which had germinated and the cytorrhysis of those propagules were determined. There was clear evidence that increasing concentration of nutrients inhibited germination of spores and subsequent hyphal growth and increased the number of collapsed spores i.e., spores that were cytorrhyzed. A positive correlation was obtained between percentage of normal-appearing spores and percentage of germination (r = 0.852). Both spore germination and normal-appearing spores were reduced as water activity decreased from 0.981 to 0.882. Fertilizer showing the lowest a_w tested inhibited germination of 65-70% of spores and cytorrhyzed 40% after 2 hours of contact, whereas fertilizers with levels of water activity of 0.964 and over had little or no effect. Decytorrhysis assay at the highest solute concentrations indicated that 75% of spores withstood the osmotic shock over the test period and regained their original shape. Because water activity (aw) is related to the osmotic pressure (p) of a solution with the following equation: $p = RT/Vw \ln(a_w)$, it is considered that the effects of fertilizers on *G. irregulare* spores should not be considered exclusively in terms of a_w, but also in conjunction with osmotic pressure. Thus, we highly suspect that the predominant inhibitory effects on germination of high nutrient solutions are due to the creation of an osmotic pressure across the spore cell wall. These results are consistent with the hypothesis that high external osmotic pressure reduces spore germination by an osmotic effect by inhibiting the pressure increase during germination that is thought to be caused by an endogenous increase in solute concentration.

Key words : *Glomus irregulare* – spore – germination – cytorrhysis – fertilizer – water activity (a_w) – osmotic pressure

4.2 Introduction

Arbuscular mycorrhizae (AM) are widespread fungi that form mutualistic relationships with the majority of terrestrial plants growing under natural conditions (Smith and Read, 1997). It is now very well established that colonization by AM, which act as extensions of the root system, contributes to increasing availability and uptake of water, diffusion-limited nutrients (N, Cu and Zn) and phosphorus (P). This latter effect is more pronounced in P-deficient soils (Smith and Read 1997). Because of this enhancement of nutrient absorption, AM fungi are of great value in promoting growth and yield of plants (Siddiqui and Mahmood 1998). Other beneficial effects are the biological control of root pathogens and hormone production. Moreover, plants colonized by mycorrhizae are usually more tolerant to drought. AM fungi also contribute to a better soil aggregate formation, which is important for soil structure and stability against erosion (Smith and Read 1997). All these advantages are key factors for successful low-input farming. Hence, mycorrhizal fungi are ubiquitous in agricultural systems and are expected to play an important role in sustainable agriculture (Jeffries et al., 2003). In agriculture, the use of AMF as biofertilizer to enhance plant growth and yield of many crops has gained popularity in recent years because of the higher cost and hazardous effects of heavy doses of chemical fertilizers. Biofertilizers are products containing living cells of different types of microorganisms, which have an ability to convert nutritionally important elements from unavailable to available form through biological processes (Vessey, 2003). They have emerged as an important component of the integrated nutrient supply system and hold a great promise to improve crop yields through environmentally better nutrient supplies. AMF are preferred over other microorganisms as biofertilizers inhabiting the soil as they are ubiquitous and form the largest group that is predominantly associated with agricultural crops (Smith and Read, 1997).

Glomus irregulare is an arbuscular mycorrhizal fungus in the phylum *Glomeromycota* (Sokolski et al., 2010). This fungus forms intraradical spores aggregated inside roots and extraradical spores in the soil. Intraradical spores are usually ovoid to irregularly shaped, whereas extraradical spores are usually globose (Chapter 2). The spore wall consists of two semi-permanent, hyaline outer layers and a laminate inner layer (Maia and Kimbrough, 1994). We found that the laminate innermost spore wall layer contains glomalin while the outer spore wall is rich in chitin (Chapter 2).

In agriculture, AM symbiosis is influenced by management practices, such as the amount and type of the supplied fertilizer (Vanlauwe et al., 2000). It is reported that high input of both nitrogenous and phosphatic fertilizers in soil is associated with low endomycorrhizal colonization and that the amount of soluble P, among other macronutrients, was the main cause for this effect (Hayman, 1980; Limonard and Ruissen, 1989; Ryan et al., 1994), although a recent study reported that long-term phosphorus fertilization does not affect AMF biomass in the soil (Beauregard et al., 2010). Nonetheless, colonization of roots by AMF and the fungal contribution to the nutrient uptake of the plant are often reduced by high levels of soluble phosphorus (Fabig et al. 1989). Thus, the limited AM colonization found in many studies seems to be related to the intensity of fertilizer input and the soluble nutrient contents, especially of P, in the soils. The mechanisms causing reduced mycorrhizal colonization upon P fertilization are not fully understood. Understanding these effects is important because, whether mediated by changes in nutrient availability or host plants, changes in mycorrhizal communities and the function of this symbiosis resulting from different agricultural management practices may have large effects on the structure and dynamics of host plant communities.

Regarding the positive effect of increasing AM colonization on plant responses, the question arises of how fertilizer application can be managed using the appropriate doses and sources for reaching optimum AM colonization, i.e. by ensuring abundant sporulation and successful germination of spores. However, sporulation of AMF is not always related

to the degree of colonization of the host root system (Coltman et al., 1988). Nor is it consistently related to P fertilization since applications of P to pot cultures of AMF can decrease (Gruhn et al., 1987) or stimulate (Sylvia & Schenck, 1983) sporulation. Because of these seemingly contradictory data, we will focus on the effect of fertilizers on fungal spore germination.

Germination of spores is affected by many factors: temperature, pH, concentration of heavy metals, soil moisture, osmotic pressure and other microorganisms in the soil (Xavier and Germida, 2003). The influence of other environmental factors, such as fertilization, has never been tested on spore germination of AMF. The exact biochemical connection between nutrient concentration in soil and its resulting effect on spore germination is not well understood. In cases where high phosphorus and nitrogen levels in the soil were found to be associated with low fungal colonization, it was unclear whether low spore germination and/or the inability of the germinated spores to colonize roots are responsible. One study has found that addition of even small amounts of phosphorus to the soil was associated with low colonization by AMF, despite a seemingly normal germination of the spores (Trinick, 1977), but more studies need to be done to confirm this observation to a wide range of AMF species.

One of the reasons why fertilizers may affect spore germination or fungal colonization could be their effect on cell turgor. Turgor pressure in fungal cells develops across the rigid cell wall in order to balance the osmotic pressure gradient. The maintenance of turgor pressure is indispensable in pathogenic and mycorrhizal fungal cells. Advances have been made in establishing the role of turgor in plant infection, using the rice blast fungus *Magnaporthe grisea* (Howard et al., 1991) and in the mechanism of hyphal extension growth (Harold et al., 1995). Preliminary results have shown that fertilizers influence the water potential of the environment and spore collapses were observed. However, little is known about the influence of various water potentials on physiology and germination of AMF spores. But a mechanism maintaining turgor pressure to a defined

value (turgor regulation) seems to be advantageous to walled cells, which are exposed to fluctuations in external osmotic pressure. For example, hyphae overcome the mechanical resistance of plant and animal tissues, and other substances through the secretion of digestive enzymes and the exertion of force. Unless the fungus can completely liquify its surroundings, its hyphae must use mechanical force to thrust forward. This force is derived from the osmotically generated turgor pressure within the hypha and is governed by wall loosening at the growing apex (Money, 2001).

In yeast cells, varying turgor pressure has been associated with an effect on the size of the protoplast or the entire cell. An elevated level in solute concentration in the surrounding medium causes either plasmolysis (detachment of the plasma membrane from the wall) or cytorrhysis (wall collapse or cell shrinking). The term "cytorrhysis" describes the phenomenon of osmotically related collapse of cells due to the impermeability of the cell wall for the applied osmolyte. Thus, the occurrence of cytorrhysis in the presence of certain solutes provides a criterion for cell wall permeability towards a given molecule.

To better understand the physiological behaviour of fungal spores, we have conducted a study to determine relationships between occurrence of cell collapse of *Glomus irregulare* spores in relation to their germination capacity after treatments in different solutions of inorganic fertilizers at known values of N-P-K and activity of water (a_w). The water activity is the degree of water availability for chemical activity and growth and is equivalent to the ratio of the vapor pressure of the medium to the vapor pressure of pure water. Thus, the purpose of the present article is to evaluate the effects of osmotic shock on viability of *G. irregulare* spores and the mechanisms used by fungi to regulate turgor pressure.

4.3 Materials and Methods

Unless stated, all experiments were performed at room temperature (21 ± 2°C with mature *Glomus irregulare* spores)

4.3.1 Organism and Growth Conditions

These studies were conducted with spores of strain DAOM 197198 of *G. irregulare* provided by Premier Tech Biotechnologies produced. The spores were grown in a large-scale industrial *in vitro* mycoreactor as described by St-Arnaud et al. (1996). Briefly, the spores were grown on *Agrobacterium rhizogenes* transformed roots of *Daucus carota* in a two compartment *in vitro* system. The growth of *D. carota* roots was restricted to one compartment while *G. irregulare* hyphae were permitted to grow on to the second compartment where they produce extraradical spores. Intraradical spores, which are often referred as "vesicles", were collected from the roots compartment by crushing the roots using a blender.

4.3.2 Measurements of Wall Pore Size and Spore Turgor

The pore size of the spore wall was estimated based on a solute exclusion technique (Money, 1990). Briefly, spores were incubated for 1 hour in concentrated solutions of polyethylene glycols (PEGs) of different average molecular weights and were screened for plasmolysis and cytorrhysis (collapse). Plasmolysis occurs only when the solute molecule can diffuse through the pores in the cell wall, whereas cytorrhysis is evidence for exclusion. This phenomenon is attributable to the fact that impermeability of the wall to external solutes results in little or no separation of the protoplast from the wall (Oparka, 1994). This closeness may lead to the collapse of the wall along with the protoplast if the external osmotic pressure is higher than the cellular osmotic pressure. Therefore, the diameter of the pores in the wall is estimated by the size of the smallest excluded molecules. These measurements were conducted on extraradical and intraradical spores of the same age.

4.3.3Morphological Effect of Fertilizers on Spores

Three fertilizers were used at concentrations recommended either for horticultural or agricultural applications. Fertilizers A and B, used in horticulture were produced by diluting fertilizer 20-8-20 in distilled water, whereas fertilizer C used in agriculture was applied without dilution. The recommended level of NPK in agricultural soil is : 85-115 ppm N, 25-30 ppm P and 25-35 ppm K. The fertilizers (Plant-Prod, Québec) tested were as follow :

- Fertilizer **A** : 350-140-350 ppm (N-P-K) ; a_w = 0,982
- Fertilizer **B** : 1500-600-1500 ppm (N-P-K) ; a_w = 0,964
- Fertilizer **C** : 60 000-240 000-60 000 ppm (N-P-K) ; a_w = 0,882

The levels of water activity (a_w) were obtained using the Hygro-Palm-AW1 with the Aw-Dio probe from Rotronic Instrument Corporation.

1 ml of 0.1% peptone water, a buffer solution suitable for storage of spores, containing ~2650 spores/ml was centrifuged at 1000 x g for 5 min and the pellet was resuspended in the fertilizer solution. Following a 2 hr, 24 hr, or 1 week incubation in fertilizer solutions, the proportion of normal and cytorrhysed spores was determined by confocal laser scanning microscopy based on n=25 spores per sample. The experiment was repeated once with similar results. To determine if cytorrhysis was reversible (decytorrhysis), spores that had been incubated in 1 ml fertilizer C were centrifuged at 1000 x g for 5 min. The fertilizer containing supernatant was removed and the pellet resuspended in 50 mM phosphate buffer pH 6.6. These washes were repeated 3 times to remove most of the fertilizer. Subsequently, spores were incubated for 24 hours to allow for recovery. To assess the degree of cytorrhysis spores were mounted directly in a drop (20 µl) of their last incubating solution (fertilizer for cytorrhysis test or phosphate buffer for decytorrhysis test) on a microscope slide and covered with a cover slip. Observations were made with a Zeiss LSM-510 Meta confocal laser scanning head fitted on an Axiovert 200 microscope (Zeiss,

Jena, Germany). Images were collected with a Zeiss Plan neofluar 40X/1.3 oil objective, recorded using the 488 nm argon laser for excitation. Emission at 490 nm – 600 nm was used exploiting the fact that the spore wall autofluoresces. To clearly detect the level of cytorrhysis, we used z-stacks to create 3D images of spores. Each 3D image of spores was examined and categorized into 1) morphologically normal spore, 2) weakly cytorrhyzed spore, 3) strongly cytorrhyzed spore and 4) burst spore.

4.3.4 Spore germination

Unless otherwise indicated, all germination studies were conducted in 10-cm-diameter plastic Petri dishes containing 20 ml of non-sterile vermiculite covered with a cellulosic membrane filter (Pall Corporation) (diameter 47 mm; pore size 0.45 μm). To determine whether the fertilizer affected spore germination, we tested fertilizers A, B and C after incubation times of 2 hours and 1 week.

The method was the same as the induction of cytorrhysis described above, except that the fertilizer was removed after the treatment by centrifuging the spore suspension at 1000 x *g* for 5 min. The supernatant was discarded and the pelleted spores were resuspended in fresh 0.1% peptone water. A second centrifugation was performed and the pelleted spores were again resuspended with fresh peptone water at a concentration of 100 spores/ml. 20 ml of vermiculite was uniformly distributed to cover the bottom of a 10 cm Petri dish. With small pliers, a Whatman #1 filter was deposited in the middle of the Petri dish and 17 ml of distilled water was deposited on the vermiculite and filter in order for the filter to adhere completely to the vermiculite. A cellulosic membrane filter (0,45 μm) was added on top of the humid Whatman #1 filter and 1 ml of spore solution was placed on the cellulosic membrane filter. Three replicate Petri plates were used for each treatment. Treatments were conducted only once. The Petri dishes were sealed with parafilm and incubated in the dark at 28°C. After a 2-week incubation, filters were removed, stained with 0.05% trypan blue, and assessed under a compound microscope (25X magnification) for

percent spore germination. Each spore was examined and categorized into 1) absence of germination, 2) germinated spore with a germ tube shorter than 500 μm, 3) germinated spore with a germ tube longer than 500 μm or 4) germinated spore with a germ tube that exceeds the field of view.

4.4 Results

4.4.1 Measurement of Wall Pore Size and Spore Turgor

To evaluate the pore diameter of the spore cell walls of *G. irregulare*, we incubated spores in PEGs solutions with different average molecular weights (PEG 200, 400, 600, 1000, 2000 and 6000 were tested) and verified which PEG was able to diffuse through the pores and induce plasmolysis. To better define the size of the molecules penetrating the cell wall, and to exclude any differential permeability due to the maturity of the spores, we used spores with similar age and size. Brightfield inspection revealed that none of the PEGs tested induced plasmolysis. Even PEG-200 induced cytorrhysis, indicating that while the walls were freely permeable to water, the pore size was smaller than the diameter of these molecules (i.e., <1 nm) (Scherrer and Gerhard, 1971). Following the reasoning of Carpita et al. (1979), the limiting pore size of spore cell walls appears to be under 1 nm since the osmolyte with the smallest molecule size tested (PEG-200) was not able to penetrate the cell wall, thus inducing cytorrhysis. The solute exclusion test experiment was performed twice with each molecular weight of PEG.

4.4.2 Effect of Fertilizers on Spore Morphology

To determine the effect of fertilizers on spore turgor pressure, spores were incubated in 3 different fertilizers (**A:** a_w = 0,982; **B** : a_w = 0,964 and **C** : a_w = 0,882) and the

morphological response of spores assessed in terms of the degree of cytorrhysis after 2h, 24h and 1 week of incubation (Fig. 4.1).

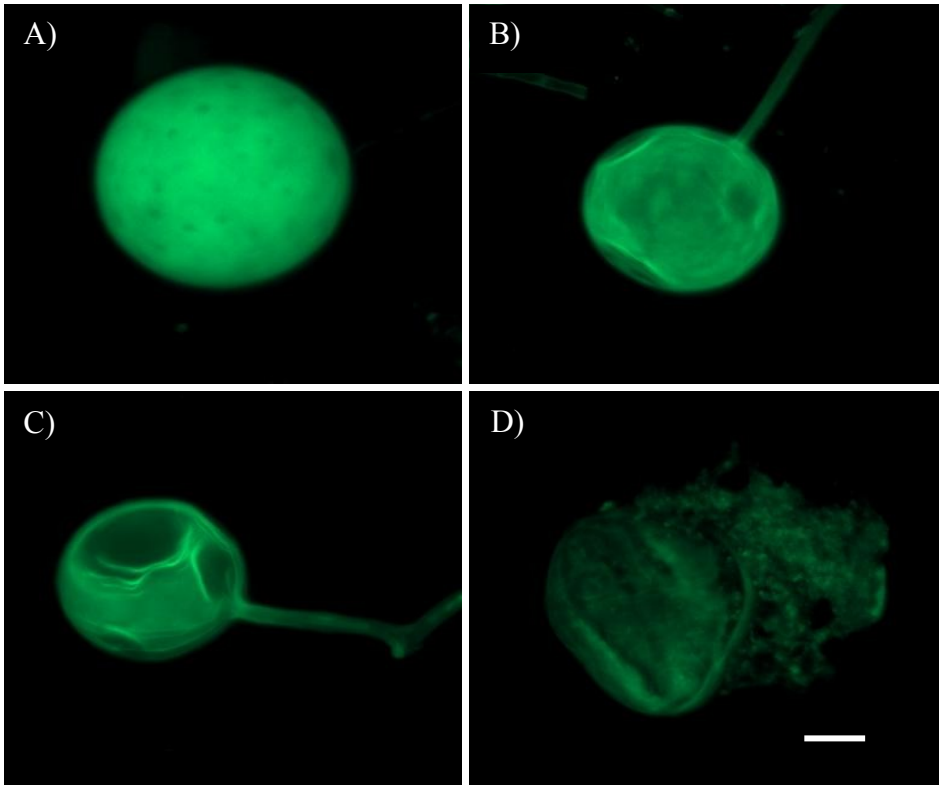

Figure 4.1 : Confocal laser scanning micrographs of *G. irregulare* spores. A) Normal spore, B) weakly cytorrhyzed spore, C) strongly cytorrhyzed spore and D) burst spore. All images are maximum projection of z-stacks. Bar = 25 µm

Collapse of the spore wall through cytorrhysis was demonstrated in every fertilizer tested for both intraradical and extraradical spores. The main results are summarized in Fig. 4.2 and Table 4.1. Factorial analysis of variance on percent of normal spores after exposure to the treatments showed that fertilizer type and concentration and exposure time affected the proportion of normal vs cytorrhyzed spores independently of the type of spores. Extraradical and intraradical spores generally had the same proportion of weakly cytorrhyzed spores after each treatment. When cytorrhysis occurred, the spores became indented and bowl-shaped. Of all the fertilizers used, solution C had the greatest impact on the integrity of the spores. After 2 h of contact, 30% of the spores had cytorrhyzed weakly and 10-15% had cytorrhyzed strongly. The effect increased with duration of exposure. After 24 h of incubation, 52% of the spores still had a normal appearance but after one week of incubation, this proportion fell to 26%. The effects of fertilizers A and B seemed to depend on the concentrations and exposure times. After 24 h of incubation in fertilizer A, 80-85% of the spores appeared normal. After 1 week of exposure, 75% of spores were still showing no sign of cytorrhysis. Fertilizer B had no effect after 2 h of incubation. After 24 h of contact, a significant proportion (20%) of spores was weakly cytorrhyzed. But this proportion did not increase even after one week exposure. The fertilizers A and B caused spores to collapse to a much lesser degree than the fertilizer C. Data presented in Fig.4.2 show that cytorrhytic effect of the solutions A and B after one week was less than the cytorrhytic effect of the solution C after two hours. We also noticed that spores floated in solution C only, suggesting a higher density of this solution compared to the density of the spores.

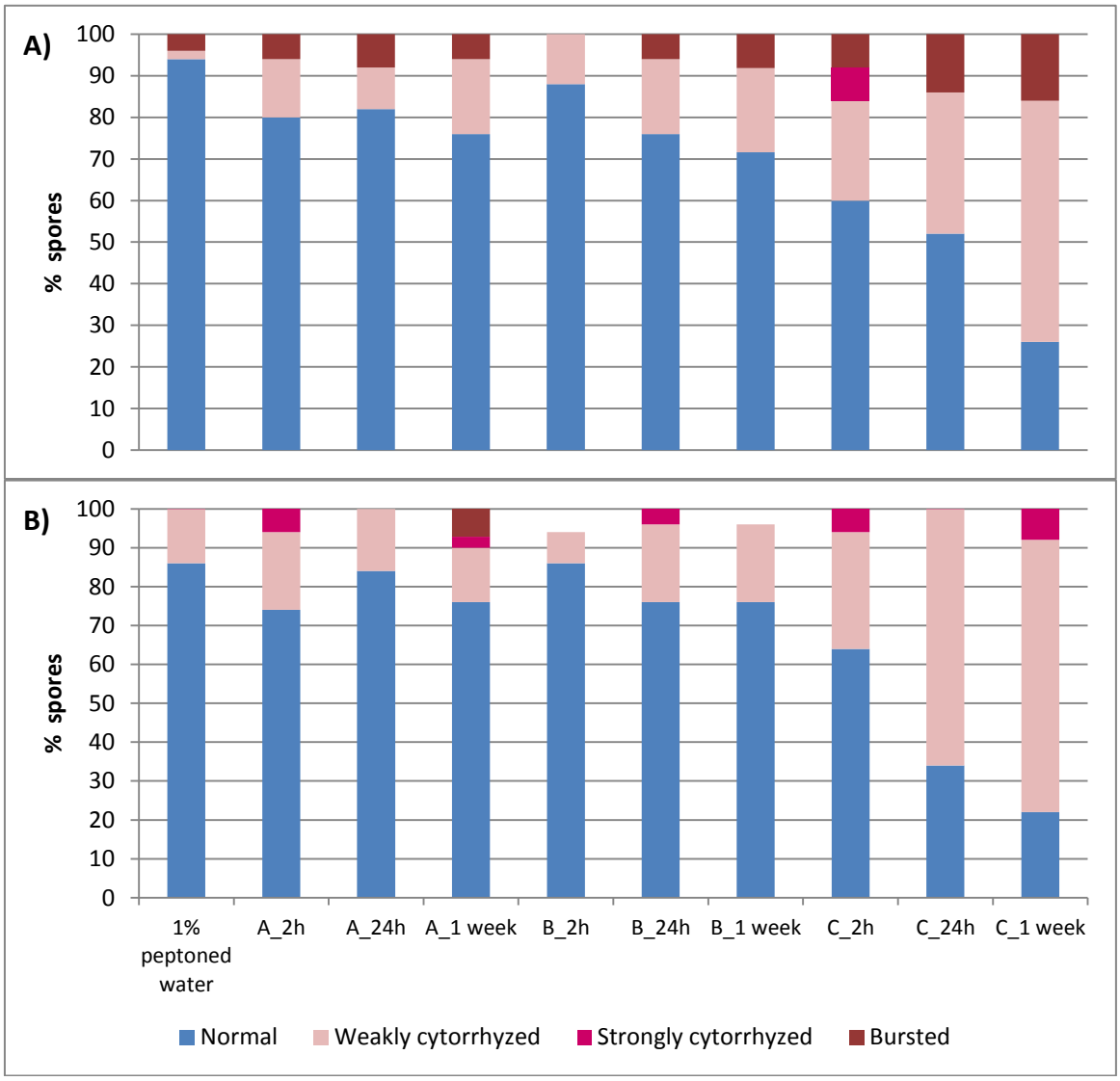

Figure 4.2 : Distribution of spore morphology (degree of cytorrhysis) in the presence of different fertilizers (A, B, C) on A) extraradical spores and B) intraradical spores after an incubation of 2 hours, 24 hours and one week. Fertilizers : (A: aw = 0,982; B : aw = 0,964 and C : aw = 0,882)

Table 4.1: Duncan analysis on percentage of normal spores after exposure to the different treatments. Treatments marked with **** within the same column are not significantly different (α=0.05)

Spore origin	Treatments	% normal spores	1	2	3	4	5	6
Extraradical	C_1w	26	****					
Extraradical	C_24h	52		****				
Extraradical	C_2h	60		****	****			
Extraradical	B_1w	72			****	****	****	
Extraradical	A_1w	76			****	****	****	
Extraradical	B_24h	76			****	****	****	
Extraradical	A_2h	80				****	****	****
Extraradical	A_24h	82					****	****
Extraradical	B_2h	88					****	****
Extraradical	1% Peptoned water	94						****
Intraradical	C_1w	22	****					
Intraradical	C_24h	34	****					
Intraradical	C_2h	64		****	****	****		
Intraradical	A_2h	74			****	****	****	
Intraradical	A_1w	76			****	****	****	****
Intraradical	B_24h	76			****	****	****	****
Intraradical	B_1w	76			****	****	****	
Intraradical	A_24h	84					****	****
Intraradical	1% Peptoned water	86					****	****
Intraradical	B_2h	86					****	****

4.4.3 Reversibility

To determine if cytorrhysis resulting from fertilizer contact was reversible, we incubated extraradical spores in fertilizer C and, after 6 h of contact, we transferred these spores to a phosphate (PO_4) buffer solution (50 mM, pH 6.6) for 24 h which is known to be adequate for spore survival. Reversibility of cytorrhysis (decytorrhysis) occurred in extraradical spores which were previously cytorrhyzed in solution C (Fig.4.3). If we exclude the percentage of spores (6%) that were already cytorrhyzed prior to incubation with the fertilizer, thus presumably dead, from the 1% peptoned water control test, a

significant proportion (75%) of spores that had been cytorrhyzed by treatment with fertilizer C recovered their original shape completely within 24 h after immersion in PO_4 buffer solution. Considering only the population of seemingly viable spores, cytorrhysis appeared to be permanent for only 12% of extraradical spores after 6 hours of incubation in fertilizer C.

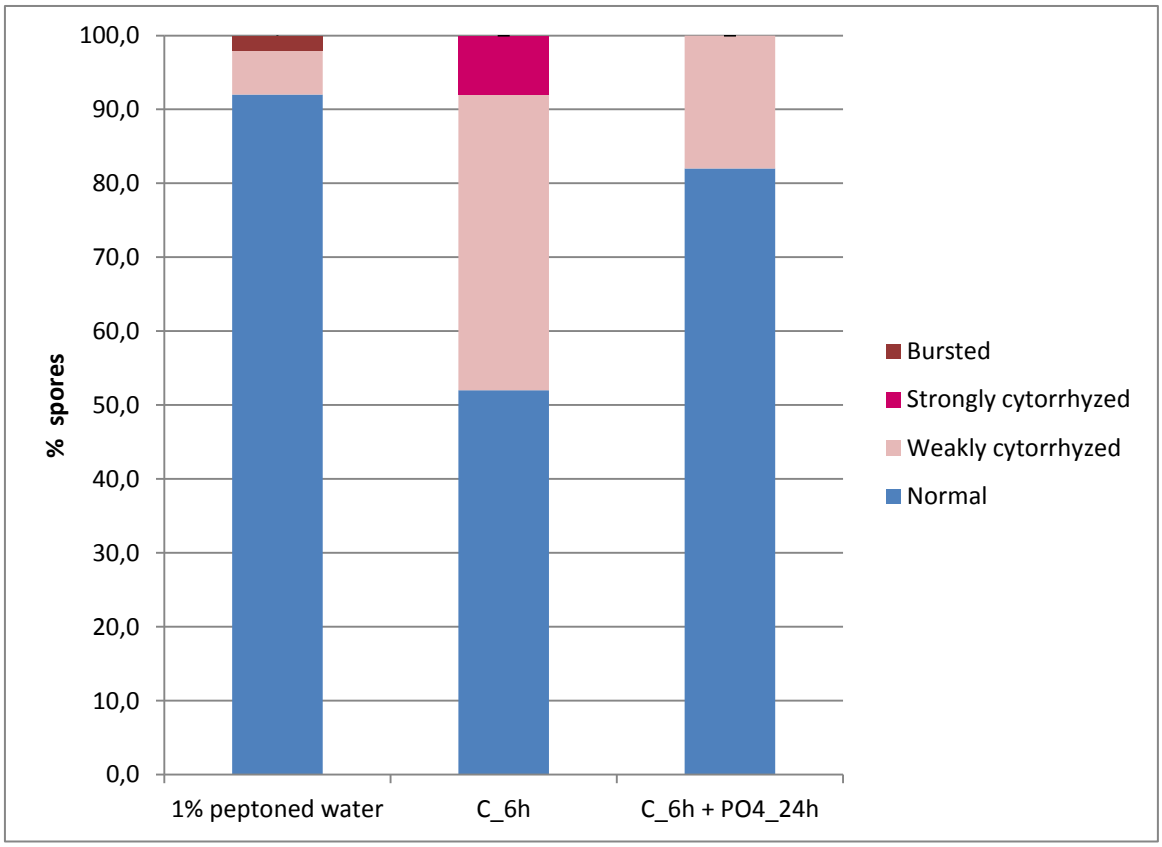

Figure 4.3: Cytorrhysis of spores of *G. irregulare* after a 6 hour incubation in fertilizer C and decytorrhysis of the same spores after a 24 hour incubation in PO_4 solution (50 mM, pH 6.6)

4.4.4 Spore germination

To determine whether the use of fertilizers affected spore germination of *G. irregulare*, we incubated spores in the same fertilizers mentioned previously (A, B and C) for 2h and 1 week and compared their effect on germination rate and vigor (expressed by the length of the germ tube) in Petri dishes by microscopic observations (Fig. 4.4).

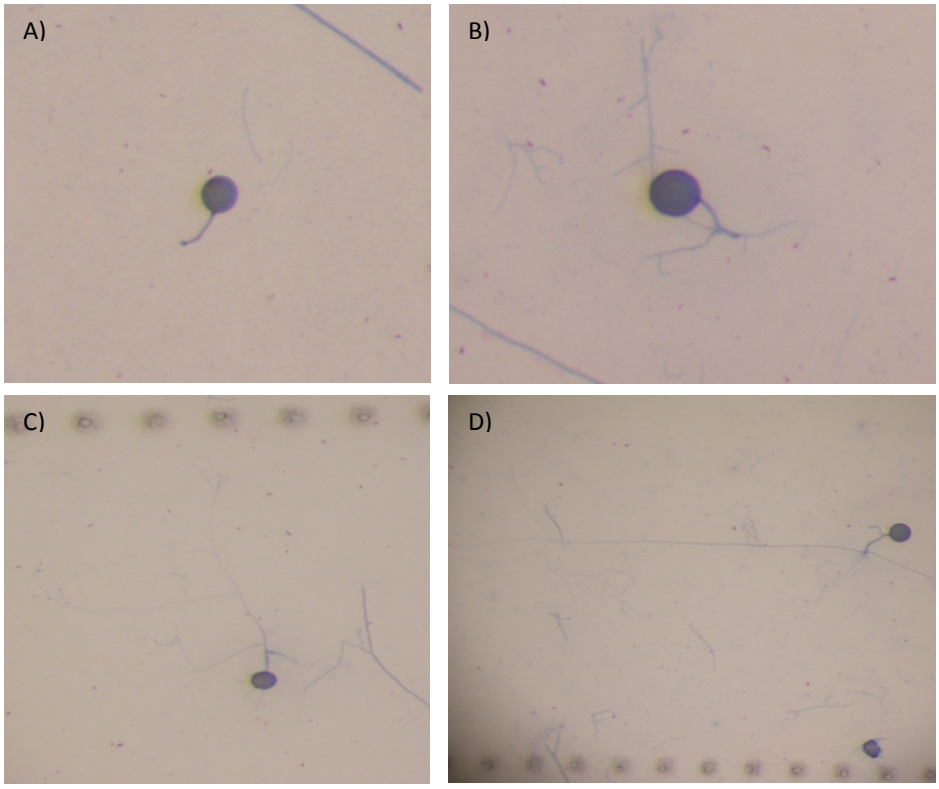

Figure 4.4 : Brightfield micrographs of *G. irregulare* spores stained with trypan blue showing varying degree of germination. A) No germination, B) weak germination, C) medium germination and D) strong germination.

Spores of *G. irregulare* germinated in non sterile soil without host plants through a new germ tube protruded through the spore wall. Germination of *G. irregulare* spores with and without treatments is shown in Table 4.2 and Fig. 4.5. Although 70 to 80% of untreated spores germinated, most of them showed weak germination (germ tubes < 0.5 cm after 2

weeks of incubation). This is not unusual on vermiculite which can sometimes reach a pH of 9.5. Since the germination assay has been conducted only once and on a vermiculite lot with high pH, results should be interpreted with care. The assay should be repeated either on another lot of vermiculite with a lower pH or on a different substrate in order to confirm the observations reported here. Exposure of the spores of *G. irregulare* to different concentrations of fertilizers for a period of 2 h and 1 week caused varying degrees of inhibition of spore germination. Factorial analysis of variance on the percent of germinated spores after exposure to the different treatments showed that the percentage of germination varied significantly with the type of spore and the type of treatment. There was a significant inhibition of fungal spore germination after treatments with the fertilizer C. The germination inhibiting effect of fertilizer C was rapid since an exposure time of 2 h was sufficient to reduce germination by half. Longer incubation times did not exacerbate this effect, suggesting that spores sensitive to the treatment suffer immediately, whereas those that are resistant, are not affected even by prolonged incubation.

Consistent with their low cytorrhytic effect and higher water activities, fertilizers A and B exhibited low degrees or even no germination inhibition compared with fertilizer C. Germination percentages were similar (60-73%) after incubation in both fertilizers but seemed to differ depending on the spore type with the extraradical spores showing a slightly higher percent of germination. Fertilizers A and B very weakly affected germination of both intraradical and extraradical spores even after one week of incubation. The percentage of germination of extraradical spores was 10% points higher than the percentage of germination of intraradical spores at the control test (78% compared to 68%).

Table 4.2: Duncan analysis on percentage of germinated spores after exposure to the different treatments. Treatments marked with **** within the same column are not significantly different (α=0.05)

Spore origin	Treatments	% spore germination	1	2	3	4	5
Extraradical	C_1w	26	****				
Extraradical	C_2h	35		****			
Extraradical	B_2h	68				****	
Extraradical	B_1w	71				****	
Extraradical	A_1w	71				****	
Extraradical	A_2h	73				****	
Extraradical	1% Peptoned water	79					****
Intraradical	C_1w	26	****				
Intraradical	C_2h	29	****				
Intraradical	B_1w	60			****		
Intraradical	A_1w	62			****		
Intraradical	1% Peptoned water	69				****	
Intraradical	B_2h	70				****	
Intraradical	A_2h	70				****	

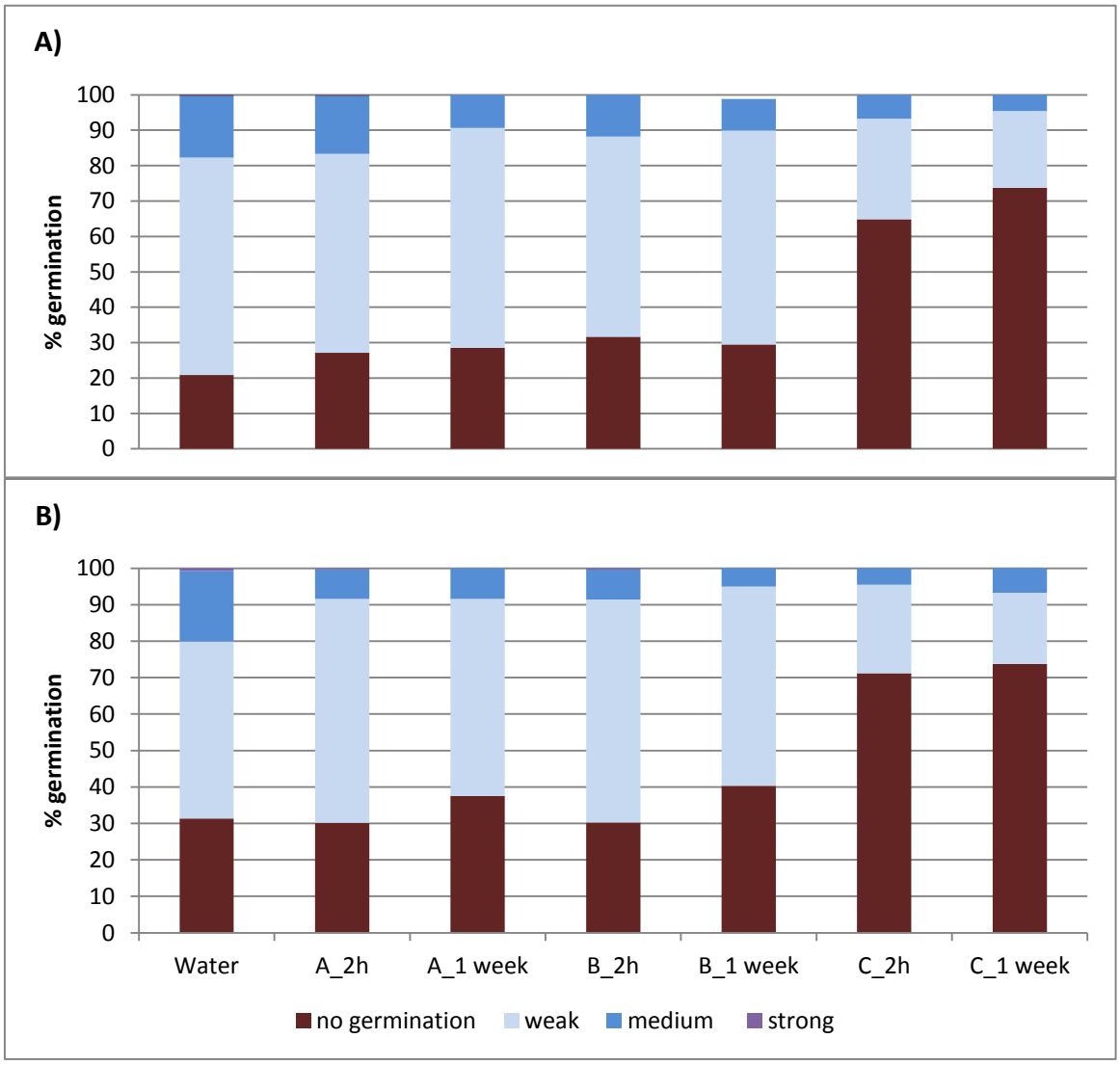

Figure 4.5: Effect of the concentration of different fertilizers on the germination of A) extraradical spores and B) intraradical spores after an incubation of 2 hours and one week. Fertilizers : (A: a_w = 0,982; B : a_w = 0,964 and C : a_w = 0,882)

Scatterplot of normal (absence of cytorrhysis) spores versus germinated spores (Fig.4.6) shows a positive correlation of 0.852. This figure suggests that high osmotic pressure which causes cytorrhysis of spores directly inhibits spore germination. Relating nutrient concentration, spore cytorrhysis and inhibition of spore germination indicates the

complex nature of the inhibitory action of the fertilizer. Fertilizers A and B apparently had no effect on the germination of spores of the fungus tested compared to the effect of fertilizer C. The absolute levels of NPK in the fertilizers used in these tests seem to influence germination. It has also been observed that those spores which germinated in presence of any fertilizer produced shorter germ tubes as compared to spores which were not subjected to fertilizer treatments. Hence, even if germination was successful, hyphal growth was still affected by the fertilizer treatment.

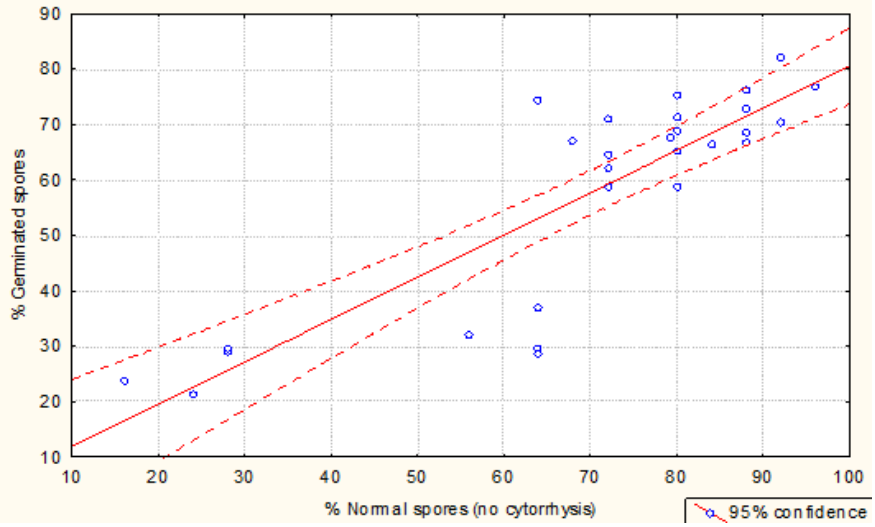

Figure 4.6: Scatterplot of normal (non-cytorrhyzed) spores and germinated spores (r=0.852).

4.5 Discussion

Using osmolytes with known molecular radii, we were able to investigate the permeability properties and the pore size of the *G. irregulare* spore walls. With the solute exclusion method we found that the cell wall is impermeable for molecules with a radius of more than 1 nm. This pore size is in the same range as the values found by Schübler et al. (1995) for the bladders of *Geosiphon pyriforme*, a *Glomus*-related fungus that has a

limiting pore radius of 0.5 nm. The results of the present study indicate that intraradical and extraradical spore cell walls seem to be similar in porosity, both showing a porosity between 0.278 nm (molecular size of water) and 1 nm (molecular size of PEG-200). The pore radii of *G. irregulare* cell walls are extremely small compared to that of yeast *Saccharomyces cerevisae* (~ 2.3 nm) or plant cell walls (~ 2 nm for pollen tubes and 1.7-2.6 nm for non-suberized walls) (Hoggart and Clarke, 1984; Carpita and Gibeaut, 1993, Gerhard and Judge, 1964).

The chemical nature of the substances limiting the permeability of the *G. irregulare* spore cell wall is not yet known. On the other side the electron microscopic studies on the cell wall of *G. irregulare* show a thin electron-dense band that separates the outer and inner walls of the spores and that seems to be made of sporopollenin (Maia, 1998). This substance is the most resistant polymer found in nature and it is believed to protect the spore from desiccation when exposed to air. An electron dense and mannoprotein containing outer layer has been shown to be the permeation barrier of *Saccharomyces cerevisiae* cell walls (De Nobel et al., 1990). The spore wall of the fungus *Entorrhiza* also has an electron dense layer of the cell wall that seems to be responsible for the impermeability (Fineran, 1993).

According to our results from Chapter 2, the specific binding of wheat germ agglutinin suggests that the outer layer of *G. irregulare* spore wall is chitinous and the inner layer seems to contain glomalin. Both these substances could be responsible for the low permeability of spores of *G. irregulare*. It should be noted that a low permeability of AM-fungal cell walls for fixatives and embedding media was reported previously (Maia et al., 1993). These studies demonstrate a satisfactory fixation and embedding for electron microscopy of AM fungal hyphae, arbuscules, and vesicles situated within the root, but not of the extraradical spores. Consistent with this, we experienced significant problems with the preparation of *G. irregulare* spores for transmission electron microscopy. In preliminary studies, the low viscosity resin LR White did not infiltrate the spores even after

8 days of incubation. Clearly, the walls of these spores are poorly permeable. The fact that the wall of *G. irregulare* spores represents a significant barrier to cellular exchanges has not only consequences for the osmotic behaviour but possibly also for the uptake of nutrients by the fungus. With the use of nuclear magnetic resonance spectroscopy, Pfeffer et al. (1999) found that extraradical hyphae of *G. irregulare* are unable to take up carbohydrates. Because molecules with diameters larger than the pore radii of *G. irregulare* walls are restricted in their ability to penetrate such a wall, it is logical to hypothesize that extraradical mycelium of *G. irregulare* is not able to acquire such essential molecules saprophytically from the soil, assuming a similar permeability between hyphae and spores. This finding supports the idea that uptake of carbohydrates by AMF have to be taken from the plant host via H^+-ATPases pumps predominantly located on arbuscular walls and intercellular hyphae (Gianinazzi-Pearson et al. 1991). Thus, our results reported here could be relevant for a better understanding of how AM fungi exchange matter with their environment and their symbiotic partners. It is important to keep in mind that our observations are based on walls of turgescent cells. They thus do not consider possible differences in permeability between stretched (turgescent) and non-stretched (cytorrhyzed) cell walls.

Our report appears to be the first use of cytorrhysis as a parameter to determine the effect of fertilizers on the spores of *Glomus irregulare*. Our observations on cytorrhysis of *G. irregulare* spores confirm that their cell walls are osmotic barriers. The rapidity and intensity of cytorrhysis of spores (extraradical and intraradical) was most drastic following their incubation in fertilizer C. After 2 h of contact, 45% of the spores had cytorrhyzed, 30% lightly and 15% strongly. Why cytorrhysis took place so slowly and weakly in the other fertilizer solutions is not clear but is probably related to the assumed lower osmotic potential of these solutions (assumption supported by measured water activities).

Ability of a cell to cytorrhyze and decytorrhyze usually indicates viability. Thus, the positive correlation between the ability of spores to cytorrhyze in solution C and to

decytorrhyze in 50 mM PO_4 solution indicates that the fertilizer solution was not lethal. Use of deplasmolysis to determine viability of oospores has been used (Jiang and Erwin, 1990) and thus decytorrhysis could also prove useful as a viability test. Although cytorrhyzed spores are readily identified (they are highly deformed), morphological criteria to distinguish non-cytorrhyzed but dead spores from viable spores have not been available. Data presented here might be useful to solve this problem. Other studies also showed that when spores are removed from low water potential treatments (NaCl, mannitol) and placed in water, they germinate normally (Hirrel, 1981).

This report also describes the complex effect of fertilizers on fungal spore germination. The results reported in this study indicate possible fungistatic activity of fertilizers depending on fertilizer type, concentration and exposure time, and provide some insights on the antifungal activity against germination of fungal spores. It must be stated that the germination assay has been performed as a complement to our observations of cytorrhysis. The assay was done only once in order to see to which extent the changes observed to spore structure under osmotic pressure can be correlated to a physiological response. Unfortunately, comparative statements regarding the fungitoxicity of fertilizers against various fungi by other workers could not be made due to the differences in the testing methods, most of them being field/greenhouse studies looking at long-term effect of fertilizers on colonization/sporulation (Wu et al., 2005; Joner, 2000; Douds and Schenck, 1990; Eom et al., 1999).

Our study indicated that spores of *G. irregulare* germinated readily *in vitro*, provided that they were viable, mature and were incubated at 25ºC for 15 days on a proper substrate. Because *G. irregulare* spores germinate without host regulation, the behavior of this species may exemplify evolutionary adaptation of this fungal symbiont with a wide range of hosts.

Sutherland and Cohen (1983) showed that germinability of oospores of *Phytophthora megasperma* was reduced drastically by storage in water (at 23ºC), but in our study mature spores of *G. irregulare* survived storage in water at 4ºC for at least 4-6 months. However, we found that intraradical spores have a germination percentage of 10% points lower than extraradical spores. The lower ability to germinate of intraradical spores after isolation and storage in distilled water at 4ºC than of extraradical spores could be due to damage in the extraction process or it is possible that intraradical spores take longer to germinate than extraradical spores and thus a longer incubation time would be needed to confirm this last hypothesis. L. Roy-Arcand observed this longer germination time for intraradical spores in certain conditions (personal communication). Juniper and Abbott (2006) also observed that intraradical spores of *Glomus sp.* germinated in the presence of 300 mM NaCl, but extraradical spores of the same isolate did not. This may indicate a difference in the amount of water and energy required to initiate germination.

It appeared that exposure time with fertilizers A and B had only a slight effect (about 10% reduction) on the germination of both extraradical and intraradical spores. The detrimental effects of fertilizer C on spore germination was very rapid compared to the others, consistent with its more dramatic cytorrhytic effect. But despite the considerable collapse of the cell wall, spores recovered after rinsing (decytorrhysis) since they were able to germinate.

It is known that soil temperature, moisture, and pH influence germination of fungal spores, but little is known about the influence of osmotic pressure, especially of fertilizers, on spore germination. Since the presence of solutes in an aqueous solution increases osmotic pressure and decreases the activity of water of the solution, a_w is inversely proportional to osmotic pressure. Thus, fertilizer C can be assumed to have the highest osmotic pressure among fertilizers tested because it is the one that had the lowest a_w (0.882). By comparison, a saturated solution of NaCl has a water activity of 0.755. It is well known that each microorganism has a critical a_w below which growth cannot occur. For

instance, bacteria *Clostridium botulinum* cannot grow at a_w <0.89 (Baird-Parker and Freame, 1967); while no growth occurs for the filamentous fungi *Trichoderma viride* and *Penicillium roqueforti* at a_w <0.92 and <0.86, respectively (Gervais et al., 1988). Thus, given the high permeability to water of biological membranes (Fettiplace and Haydon, 1980), the predominant mechanism of the inhibition of germination by solutes added to fertilizers might lie either in the inhibitory properties of a low a_w to fungal growth and metabolism *per se* or to the creation, by the extracellular solutes, of an osmotic pressure across the spore cell wall. Walter et al. (1987), after discovering that cells were much more strongly inhibited by "non-permeant" than by "permeant solutes", concluded that the predominant inhibitory effects on *Clostridium pasteurianum* of media of high solute content were due not to the low water activity but to the generation of an osmotic pressure, which seemingly acts to inhibit the glucose phosphotransferase system by which the organism effects glucose uptake. In this regard, it should be mentioned that Roth et al. (1985) have also shown that high osmotic pressures can indeed inhibit several sugar transport systems in *Escherichia coli*. In our case, a slight variation around the optimal water activity value led to a drastic decrease in germination. This could be explained by the fact that a variation of 10% in the a_w level corresponds to a variation of approximately 1000% in the osmotic pressure (Marechal and Gervais, 1991). It is thus possible that the reduced germination of spores after being incubated in fertilizer C could be due to the osmotic pressure solely. Curran (1931) found that germination of spores of *Bacillus mycoides*, a Gram positive bacterium, is most rapid at relatively low osmotic pressure and is inhibited at a limiting osmotic pressure of roughly 36-45 atmospheres (atm). Spore germination of *Nosema algerae*, a parasitic protozoa, was inhibited by sucrose and PEG at concentrations that produced between 40 and 70 atm pressure (Undeen and Frixione, 1990). Critical tolerance of sugar beets (*Beta vulgaris*) to osmotic pressure appear to be somewhere between 4 and 6 atm (Dubetz et al., 1959) and germination of alfalfa (*Medicago sativa*) seeds are inhibited at 12 to 15 atm by NaCl solutions (Uhvits, 1946).

Data on fungi show that osmotic pressure also has an impact on spore germination and growth of hyphae. Increasing osmotic pressure produced a linear reduction in germination of *Gigaspora gigantea* spores (Koske 1981) and *Erysiphe graminis* (Kettlewell et al., 2000). A similar effect was observed between 0.2 and 30.6 atm on germination of *Glomus epigaeus* spores (Daniels and Trappe 1980). Sylvia and Schenck (1983) found that germination at 10 atm was reduced by 95%, 92% and 67% from the maximum germination for *Glomus macrocarpum, G. clarum* and *G. etunicatum* respectively. The studies cited above all indicate a considerable inhibitory effect of high osmotic pressure on spore germination and this effect appears to be related to the ability of spores to take up and retain sufficient water from the substrate to become and remain hydrated. Since this inhibitory effect appears to be through osmosis, any fertilizer salt should have similar effects.

During fungal spore germination three steps can be distinguished: 1) activation of the resting spore in response to appropriate environmental conditions, 2) isotropic growth or swelling that involves water uptake and wall growth, and 3) polarized growth which results in the formation of a germ tube from which the new mycelium originates. In the present context, the swelling step is the one we are most interested in, but unfortunately, there is a remarkable lack of information in the literature on the precise mechanisms that control this step. Spores of *Nosema algerae* are thought to germinate after an increased osmotic potential and pressure within the spore derived from catabolism of a storage product, probably trehalose (Undeen, 1990). In fungal spores, trehalose may account for up to 15% of the dry mass and has been proposed to serve as a stress protectant (Sussman and Lingappa, 1959; Hottiger et al., 1994). Mobilization of the trehalose pool during spore germination suggests that it may also act as a reserve carbohydrate needed at the onset of this particular developmental stage.

Obviously, a substrate with high osmotic pressure could inhibit germination by counteracting the water uptake from these propagules. Hyperosmotic conditions seem to

prevent germination by eliminating the pressure differential and thus preventing spores from imbibing water in *N. algerae* (Undeen and Frixione, 1990). Thus moderate osmotic pressure (or reduced water activity) may serve as a signal to the spore that the environment is not conducive to future optimal growth. This response to hyperosmotic solutions that allows spores to stay dormant at this time of stress would insure that a portion of the population would survive to germinate and complete their life cycle later when soil water potentials would be less extreme. It seems that some barrier to the uptake of water exists in the resting spore, which may be suspended in water for prolonged periods of time without becoming hydrated, or swelling, germinating, or losing its viability. Heydecker and Wainwright (1976) reported that pre-treatment of crop seeds with osmotic agents such as PEG 6000 speeds up the rate of germination after transfer to distilled water. It would be interesting to verify if this treatment also enhances the rate of germination of *G. irregulare* spores particularly on annual crops where AM is most valuable when established as quickly as possible.

Conditions of high soil fertility are also known to inhibit mycorrhizal fungi (Hayman 1980). Application of fertilizers at the recommended level (85-115 ppm N, 25-30 ppm P, and 25-35 ppm K) reduced the number of infective propagules of mycorrhizal fungi and fertilizer application at 50 or 75% of the recommended level together with farmyard manure had no adverse effect on mycorrhizal sporulation. In our experiments, we found that immersion of spores into solutions of fertilizers A and B resulted in only slight reduction of spore germination and reduction of length of germ tube. The relatively high germination that occurred with these fertilizers and the fact that the germinated hyphae were shorter suggest that reduced infection observed in highly fertilized soils could probably result from failure of germinated spores to infect rather than from inhibition of germination. Kruckelmann (1975) suggested that the reduced infection may be due to decreased plant dependence on mycorrhizae when the soil is rich in nutrients but our study suggests that the fertilizers could also have a direct influence on this reduced colonization. However, our results have been obtained in the absence of soil contact. This can induce a

different spore germination response. Azcon-Aguilar et al. (1986) considered both the presence of other micro-organisms and direct soil contact to be important for AM spore germination and hyphal growth. In field experiments, water content in the soil would likely have a diluting effect on fertilizers which would result in a reduced inhibition of spore germination.

Fertilizer C had the highest concentration of P (240 000 ppm) and reduced the percentage of germination drastically for both type of spores. Phosphorus is typically the most limiting element for crop production in nearly all ecosystems, but the application of heavy doses of P fertilizer is known to reduce the symbiotic activity of AM fungi (Krishna and Bagyaraj, 1982; Guillemin et al., 1995). In general, the percentage of AM root colonization decreases as the P status of the host plant increases (Smith and Read, 1997). Results by Douds and Schenck (1990) indicate that production of spores by AM fungi was consistently increased when P was lacking in the soil nutrient solution. In fact, sporulation of AM fungi can be enhanced by manipulation of nutrient regimes in the pot culture, especially N:P ratios (Douds and Schenck, 1990). Solutions in which P concentrations were high produced less colonization than when N and P were in balance or solution without P, but their nutrient concentrations were lower (0-130 ppm) than concentrations tested in this study. Thus, a direct negative correlation between spore germination and available P fertilizer seems to exist but more detailed studies are required to understand these effects under natural conditions.

Glomus irregulare was shown to be 'P-tolerant' based on their increased sporulation after being drenched with super-phosphate (Sylvia and Schenck, 1983). The increased sporulation of *G. irregulare* under conditions of stress such as high P may be an adaptation to compensate for the reduced ability of individual spores to germinate. This would thus optimize colonization under the nutrient-rich conditions commonly found in crop fields.

We do not know by what mechanism exactly nutrient solutions affect spore germination but it could also be related to the bacteria found in the spore walls. Colonization of spore walls of AMF by bacteria has been shown in many *Glomus* species such as *G. clarum, G. constrictum, G. macrocarpum* (Bonfante-Fasolo and Schubert, 1987), *G. irregulare* (Maia and Kimbrough, 1994) and *G. caledonium* (MacDonald and Chandler, 1981). It is thought that bacteria located in the walls probably play an important role in wall degradation and spore germination by releasing nutrients or degrading toxic compounds that inhibit germination. Stimulation of spore germination of AMF by saprophytic bacteria has been reported (Azcon, 1989). Spore germination and hyphal growth of *Glomus versiforme* were significantly greater when spore-associated bacteria were present (Mayo et al., 1986). Thus, the process of maturation and eventual germination of AMF spores seems to benefit from the activity of the surface microorganisms degrading the outer hyaline layer. It is possible that fertilizer amendment affects the viability of these bacteria. Wani (1990) noted that the population size of N-fixing bacteria in soil deceased significantly after fertilizer was used. Additionally, a decrease in the overall diversity and heterogeneity of the bacterial community was observed after fertilizer amendments (Girvan et al., 2004) and, in a short-term study, Crecchio et al. (2001) found no changes in bacterial community structure after addition of municipal solid waste compost. However, to our knowledge, no published reports are available on the effect of fertilizers on the bacteria in the spore walls of AMF.

4.6 Conclusion

This paper used spore germination and cytorrhysis to examine the impact of fertilizers which can exhibit high osmotic potential. In this work, we showed that spore germination of *G. irregulare* can still occur after a one-week immersion in a fertilizer solution. Even after a strong collapse of the cell wall, about 30% of spores can still germinate. We believe that germination of the resilient spores is possible because decytorrhysis has occurred. High nutrient concentrations solutions such as fertilizer C that

have a low water activity and thus probably a high osmotic pressure have more impact on spores. We observed that collapsed cell wall (cytorrhysis) of *G. irregulare* was somewhat correlated with inhibition of spore germination shown by the length reduction of the germ tube. However, this should be confirmed by more analysis since the untreated spores had a weaker than expected germination and only one assay was performed. For the germination as well as for the integrity of spores of *G. irregulare*, water activity appeared to be an important and acute factor. These observations should justify regulating the water activity value of the substrate in cultures.

We also recommend that for enhancing AM symbiosis practices, factors favoring optimal soil properties, such as management of nutrient content for the development and activity of AM symbiosis should be chosen. AM propagules and their germination rate should be considered important to understand the influence of soil management strategies such as fertilization sources and doses. To increase P plant nutrition, nutrients have to be either supplied as fertilizer, or conditions for mycorrhizal formation have to be favored. A compromise between both components (fertilizer and AM inoculum) acting in a compatible way should be optimal. One way could be related to low-P or slow-release fertilizers since some studies showed that slow-release fertilizers had beneficial effects on the colonization of AM symbiosis (Douds and Schenck 1990; Rubio et al., 2003). The advantage of using soil amendments with less available P thus lies in a continuous slow release of P that prevents P levels from rising above the P level that is critical for a given fungus.

Also, Harinikumar and Bagyaraj (1989) found that fertilizers in organic form are less inhibitory to AM root colonization than synthetic mineral fertilizers. In any case, additional aspects of nutrient effects on germination warrant further study over wider ranges of concentrations and in various combinations. At this moment, we do not know if the N:P:K ratio or the osmotic pressure of the fertilizers (or both) is the chief cause of the reduced germination that was observed. It would be important to discriminate between these factors in future experiments by testing fertilizers with the same osmotic pressure and

varying N:P:K ratios, and fertilizers with the same N:P:K ratios but different osmotic pressures.

5. Conclusion générale

Ce projet de maîtrise a été conduit afin de répondre à deux questions à savoir s'il y avait des différences au niveau de la résistance biomécanique entre les spores extraracinaires et intraracinaires du champignon mycorhizien *Glomus irregulare,* et quels paramètres influencent ces propriétés mécaniques, et dans un deuxième temps d'évaluer si les fertilisants avaient une influence, via un effet osmotique, sur la germination des spores.

Pour la première expérience, bien qu'il soit difficile de séparer les contributions de chaque paramètre étudié sur la rigidité de la cellule entière, nos mesures ont révélé plusieurs caractéristiques remarquables des spores de *G. irregulare*. Les résultats ont démontré que la rigidité cellulaire semble répondre à une combinaison de paramètres tels que la quantité de chitine et de glomaline dans la paroi cellulaire, l'épaisseur de cette paroi et la taille des spores. Nos données ont révélé une plus grande rigidité chez les spores extraracinaires par rapport aux spores intraracinaires, ainsi que le fait que la rigidité, la quantité de chitine et de glomaline chez les spores extraracinaires augmentent avec l'âge alors que ces paramètres ne varient pas de façon significative au fil du temps pour les spores intraracinaires. Ces observations suggèrent que les spores sont capables de moduler leurs propriétés mécaniques en fonction de la présence ou l'absence de protection mécanique (le tissu racinaire environnant) et représentent donc un intérêt considérable car ils donnent un aperçu des changements mécaniques qui accompagnent le mode de vie des cellules. Cette expérience représente un premier pas dans la compréhension des propriétés mécaniques des spores de CMA et les résultats amassés serviront à modéliser les propriétés mécaniques qui ensuite aideront à prédire la rigidité des spores et leur comportement dans les différents bioprocédés.

Pour la deuxième expérience, nous avons émis l'hypothèse que la réduction de la turgescence interne de la spore par un stress osmotique inhiberait la germination de ces cellules. Nos résultats ont démontré que la germination des spores et le nombre de spores non-cytorrhyzées sont en lien avec l'activité de l'eau des engrais. Ainsi, nous soupçonnons

que les effets inhibiteurs des solutions nutritives sur la germination des spores sont dus à la diminution du potentiel osmotique dans l'environnement des spores (plus le potentiel osmotique d'un milieu est élevé, plus l'eau a tendance à s'en échapper). Un milieu hyperosmotique viendrait contrecarrer l'imbibition d'eau par les spores, une des étapes-clés de la germination. . Ces résultats sont cohérents avec l'hypothèse selon laquelle le champignon puise dans sa réserve de tréhalose au moment de la germination. Le catabolisme du tréhalose résulterait en une diminution du potentiel osmotique dans la cellule, laquelle favoriserait l'entrée d'eau et ainsi créerait une augmentation de la pression osmotique qui causerait le gonflement de la spore observé lors de la germination. Ces observations devraient justifier une régulation de la valeur de l'activité de l'eau dans les substrats utilisés lors de l'application des spores de CMA dans les champs agricoles.

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