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From characterization of aquaponic microbiota toward identification of potential biocontrol microorganisms against diseases caused by *Pythium aphanidermatum* on lettuce

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Abstract

Coupled aquaponics is defined as a sustainable and integrated system that combines aquaculture and hydroponic plant production in the same water loop. In such system, the use of phytosanitary treatments to control soil-borne plant pathogens is not recommended. Indeed, synthetic pesticides or water chemical treatments can be toxic to the fish and beneficial microorganisms (e.g., nitrifying bacteria) present in the same water loop. Furthermore, no biopesticides have been especially developed for aquaponic use. Among plant pathogens occurring in soilless plant culture, oomycetes pathogens responsible for root rot diseases, such as *Pythium aphanidermatum* (Edson) Fitzp., require special attention. Indeed, the disease epidemics can evolve very quickly because oomycetes produce numerous zoospores that can move freely and actively in liquid water. However, it was assumed that aquaponic systems could be naturally plant pathogen suppressive (i.e., antagonist).

The main aim of this study was to determine the potential suppressive activity of aquaponic water against *P. aphanidermatum* diseases of lettuce (*Lactuca sativa* L.) and to identify its origin. It was shown that microorganisms of aquaponic water can decrease *P. aphanidermatum* mycelial growth in *in vitro* bioassays. The suppressive effect of aquaponic water was then confirmed *in vivo*. Root rot disease of lettuce caused by *P. aphanidermatum* was suppressed using aquaponic water as nutrient solution for soilless lettuce growth, while lettuce grew in hydroponic water or in complemented aquaponic water (i.e., supplemented in mineral nutrients) failed to control the disease. Root microbial communities (i.e., microbiota) were analysed by high-throughput sequencing (HTS) to determine the origin of this suppressive action. The suppressive effect observed in the aquaponic treatment was correlated to rhizoplane microbiota composition and to a higher microbial species diversity in lettuce rhizoplane. A list of microbial taxa related to disease suppressiveness was also established and included taxa belonging to the genera *Methyloversatilis*, *Sphingobium*, *Hydrogenophaga* and *Catenaria*, and the family Burkholderiaceae. The subsequent aim was then to isolate these potential suppressive microorganisms to study them in *P. aphanidermatum* diseases biocontrol.

Based on the most promising genera identified by HTS, an innovative strategy to develop biocontrol of *P. aphanidermatum* was applied by isolating and testing biocontrol agents from aquaponic water. Eighty-two bacterial strains and 18 fungal strains were isolated, identified by sequencing of their rDNA 16s (bacteria) or ITS (fungi), and screened *in vivo* to control damping-off of lettuce seeds caused by *P. aphanidermatum*. Out of these 100 microbial isolates, eight controlled properly lettuce damping-off caused by *P. aphanidermatum*. Strains SHb30 (*Sphingobium xenophagum*), G2 (*Aspergillus flavus*) and Chito13 (*Mycolicibacterium fortuitum*) decreased seed damping-off at a better rate than a propamocarb fungicide or a

biocontrol agent registered for soil. At the seedling stage, lettuce mortality caused by the pathogen was prevented by the application of strains G2 and Chito13. Lettuce mortality and disease symptoms were eradicated by strain SHb30 in the first bioassay, but not in the second one. Foliar and root disease symptoms were each time reduced after the application of strain G2, which was at least as efficacious as the fungicide or the registered biocontrol agent controls.

SHb30, C13 and G2 were tested in combination to evaluate the efficacy of a consortium application to control *P. aphanidermatum* root rot disease on lettuce seedlings. Whatever the combination, all tested consortia containing SHb30, C13 and G2 were able to avoid lettuce mortality and to decrease disease symptoms at a similar level or even better than the fungicide and the registered biocontrol agent controls did. SHb30 and C13 combination increased biocontrol in comparison with their separated applications. The inclusion of G2 in a consortium with SHb30 and/or C13 did not significantly increase the biocontrol effect. Because of its multitask potential (i.e., soil bioremediation, disease biocontrol and plant biostimulation) and its safer use, SHb30 can be considered as the most promising strain for biocontrol in aquaponics. However, its biocontrol activity must be stabilized in consortium application or in an appropriate biopesticide formulation.

Résumé

L'aquaponie dite couplée est définie comme un système de production durable qui combine de l'aquaculture et de l'hydroponie dans un même circuit d'eau. Dans ce type de système, l'utilisation de traitements phytosanitaires pour contrôler les phytopathogènes du sol n'est pas recommandée. En effet, l'usage de pesticides de synthèse ou d'agent chimiques de traitement de l'eau n'est pas envisageable à cause la présence de poissons et de microorganismes bénéfiques (e.g., les bactéries nitrifiantes) dans le même circuit d'eau. En outre, jusqu'à maintenant, aucun biopesticide n'a été spécifiquement développé et conçu pour une application en aquaponie. Parmi les pathogènes racinaires pouvant infecter les systèmes hors sols, les oomycètes, tels que le phytopathogène *Pythium aphanidermatum* (Edson) Fitzp., sont particulièrement préoccupants. En effet, ces organismes ont une forte capacité de propagation dans les milieux aqueux grâce à leur forme mobile de dispersion que sont les zoospores.

L'objectif principale de ce travail a été de déterminer le potentiel suppressif de l'eau aquaponique contre *P. aphanidermatum* - un agent pathogène de la laitue (*Lactuca sativa* L.) - et d'en identifier l'origine. Il a été démontré que les microorganismes de l'eau aquaponique avaient la capacité de limiter la croissance mycélienne de *P. aphanidermatum* en culture *in vitro*. Il a aussi été montré que des laitues cultivées dans de l'eau aquaponique ne développaient pas les symptômes caractéristiques de la maladie de la pourriture racinaire causé par *P. aphanidermatum*, contrairement à des laitues cultivées dans de l'eau hydroponique ou de l'eau aquaponique complémentée (i.e., complémentée en sels minéraux). Les communautés microbiennes (i.e., le microbiote) racinaires de ses laitues ont été analysées par séquençage haut débit (i.e., high-throughput sequencing : HTS) afin de déterminer et de mieux comprendre l'effet suppressif aquaponique. L'effet suppressif observé a été corrélé à la composition microbienne de la rhizoplane en aquaponie et à une diversité spécifique plus élevée. Une liste de taxa microbiens impliqués dans l'effet suppressif a également été dressée. Les genres *Methyloversatilis*, *Sphingobium*, *Hydrogenophaga* et *Catenaria*, ainsi que la famille des Burkholderiaceae ont été considérés comme les principaux taxa impliqués dans l'effet suppressif. L'objectif subséquent a dès lors été d'isoler les microorganismes identifiés comme potentiellement suppressifs pour ensuite les étudier pour un effet de biocontrôle contre les maladies de la laitues causés par *P. aphanidermatum*.

Sur base des genres de microorganismes identifiés comme prometteur dans l'analyse HTS, une stratégie innovante d'isolement et de sélection d'agents de biocontrôle d'origine aquaponique a été développée pour contrôler *P. aphanidermatum* sur la laitue. Quatre-vingt-deux souches de bactéries et 18 souches de champignons ont été isolées, identifiées par séquençage Sanger, testées *in vivo* et

sélectionnées pour contrôler la maladie de la fonte de semis causé par *P. aphanidermatum* sur la laitue. Parmi ces 100 souches, huit se sont montrées particulièrement efficaces pour le biocontrôle de la fonte de semis. Les souches SHb30 (*Sphingobium xenophagum*), G2 (*Aspergillus flavus*) et C13 (*Mycolicibacterium fortuitum*) ont diminué la fonte de semis de manière plus efficace qu'un fongicide chimique à base de propamocrabe et qu'un agent de biocontrôle microbien homologué sur sol. Au stade plantule, l'application des souches G2 et C13 a permis de prévenir la mortalité de la laitue causée par le pathogène. La mortalité et les symptômes de la maladie ont été éradiqués avec l'application de SHb30 lors du premier bio-essai, mais pas lors du deuxième. Pour chaque bio-essai, l'application de la souche G2 a permis de réduire les symptômes racinaires et foliaires causés par la maladie à un niveau similaire, voire meilleur, comparé à l'application du fongicide ou de l'agent de biocontrôle homologué.

SHb30, C13 et G2 ont été testés dans différentes combinaisons pour évaluer la capacité d'un consortium à contrôler la maladie racinaire causée par *P. aphanidermatum* sur la laitue. Quelle que soit la combinaison réalisée, tous les consortia utilisés contant SHb30, C13 et G2 ont permis d'éviter la mort des plantules de laitue et de réduire les symptômes de la maladie avec un niveau similaire ou meilleur par rapport au fongicide et à l'agent de biocontrôle homologué. Combiner SHb30 et C13 permet d'améliorer l'effet de biocontrôle par rapport à leur application séparée. L'inclusion de la souche G2 dans un consortium comprenant SHb30 et/ou C13 n'améliore pas significativement l'effet de biocontrôle comparé à l'utilisation de G2 seul. Par son aspect multifonction (i.e., bioremédiation des sols, contrôle des maladies, et biostimulation des plantes) et son usage plus sûr que les autres souches, SHb30 est probablement la souche isolée la plus intéressante pour une utilisation de biocontrôle en aquaponie. Cependant, l'activité de biocontrôle de la souche devra être stabilisée, soit par une application en consortium, soit par une formulation adéquate.

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List of acronyms

ANOVA	Analysis of variance
AP	Aquaponic
AP-Pa	Aquaponic plus *P. aphanidermatum*
ATCC	American type culture collection
BCA	Biocontrol agent
BEA	Beef extract agar
BM	Biofilter media
BOD5	Biological oxygen demand in 5 days
C-	Negative healthy control
C+	Positive control
CAP	Complemented aquaponic
Cap	Aquaponic water control
CAP-Pa	Complemented aquaponic plus *P. aphanidermatum*
CBS-KNAW	Westerdijk fungal biodiversity institute
CDA	Chitosanase detection medium
Cf	Proplant® fungicide control
CFU	Colony forming unit
Chito.	Method of isolation for Burkholderiaceae family
CLPP	Community-level physiological profiles
CMA	Corn meal agar
CMC	Carboxymethylcellulose
Cpc	*Pseudomonas chlororaphis* tx-1 bio-fungicide control
CRRR	Corrected root rot rating
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
DS-FDR	Discrete false - discovery rate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E.g.	Exempli gratia
EC	Electroconductivity
EPA	Environmental protection agency
EpC	Epidemiological cycle
F-AP	Filtrated aquaponic water

F_dM	Foliar dry mass
F_dMD	Foliar dry mass decrease
F_fM	Foliar fresh mass
F_fMD	Foliar fresh mass decrease
FISH	Fluorescence in situ hybridization
FMI	Foliar mass improvement
ForE.	Method of isolation for *Methyloversatilis* genus using enrichment
ForG.	Method of isolation for *Methyloversatilis* genus without enrichment
F-RAS	Filtrated recirculated aquaculture water
FTD	Relative foliar turgidity decrease
FWC	Foliar water content
GAP	Good agricultural practices
HC	Healthy control
HP	Hydroponic
HP-Pa	Hydroponic plus *P. aphanidermatum*
HTS	High-throughput sequencing
HTSg	HTS-guided
I.e.	Id est
Ibid.	Ibidem
IL	Inoculated lettuce
IPM	Integrated plant pest management
ITS	Internal transcript spacer
KPBT	Kalium phosphate buffer and tween
Lact.	Method of isolation for *Lactobacillus* genus
LB	Luria-Bertani
Lit.	Literature-guided
LM	Lettuce mortality
MGR	Mean germination rate
MIN E	Mineral elements
MRS	De Man, Rogosa and Sharpe
MycBait.	Method of isolation for *Catenaria* and *Rhizophydium* genera using mycelium as bait

NA	Not applicable
NCBI	National center for biotechnology information
OD	Optical density
OosBait.	Method of isolation for *Catenaria* and *Rhizophydium* genera using oospores as bait
OTU	Operational taxonomic unit
PAFF Box	Plant and fish farming box
PAR	Photosynthetically active radiation
PBS	Phosphate buffered saline
PC1/2/3	Principal coordinates 1/2/3
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PDA	Potatoes dextrose agar
PERMANOVA	Permutational multivariate analysis of variance
PGPM	Plant growth promoting microorganisms
PGPR	Plant growth-promoting rhizobacterium
PYG	Peptone yeast extract
q2	Qiime 2
R2A	Reasoner's 2A agar
RAS	Recirculated aquaculture system
RB-S-F	Rose bengal, streptomycin sulphate and formalin
rDNA	Ribosomal DNA
RRR	Root rot rating
RSR	Root symptom reduction
S./H.	Method of isolation for *Sphingobium* and *Hydrogenophaga* genera
SE	Standard errors of the mean
Sh	Shaking
So	Sonication
Trich.	Method of isolation for *Trichoderma* genus
UV	Ultraviolet
WSR	Wilt symptoms reduction
YPSS	Yeast plus soluble starch

Preamble

Coupled aquaponics is defined as a sustainable and integrated system that combines aquaculture and hydroponic plant production in a same water loop. In aquaponics, research on microorganisms was mainly focused on nitrifying bacteria but little attention has been paid on microbial plant pathogens and methods to control them. The Chapter A introduces the state of the art of plant pathogens in aquaponics and the methods to control them with a special focus on *Pythium aphanidermatum*. In that chapter, it was highlighted that fungus-like pathogens able to produce a motile form of dispersion, such as oomycetes, are particularly problematic in aquaponic or hydroponic systems. Furthermore, available tools to control root pathogens in coupled aquaponic systems are still limited because of the toxicity risk of chemical control agents on fish and beneficial microorganisms. The model used in this study was the pathosystem *P. aphanidermatum – Lactuca sativa*. Then, the challenges related to *Pythium* diseases biocontrol in aquaponics were exposed. In addition, it was observed that aquaponics could naturally control plant pathogens. This natural antagonistic effect against plant pathogens was defined as **suppressiveness**. Methods to study suppressiveness and its origin were developed. Finally, a discussion on how the understanding of the mechanisms involved in disease biocontrol and suppressiveness could further drive the development of microbial consortia application to control soil-borne diseases was developed.

To answer efficiently the main aim of the thesis that is aquaponic water suppressiveness evaluation (Chapter B), an initial step of methodology development was necessary and is described in Chapter C. Firstly, a reliable method for root microbiota harvest was developed on lettuce. Composition and diversity of the microbiota harvested by diverse protocols were compared qualitatively and quantitatively by high-throughput sequencing (HTS) and by a culture-based method. The interest of several successive root washings to harvest root microbiota was also evaluated. The second step consisted in developing a protocol of lettuce infection by *P. aphanidermatum.* Inoculum production, inoculation timing, and definition of environmental conditions to allow disease symptoms on lettuce were studied.

After these methodological steps, aquaponic suppressiveness was assessed in Chapter D. Aquaponic water was used in bioassays to control *P. aphanidermatum* pathogen in the presence or not of its lettuce host. Microbial origin of the aquaponic suppressiveness was also analysed by HTS. Then, in Chapter E, a HTS-guided isolation of aquaponic microorganism was carried out and coupled with an *in vivo* screening to select novel *P. aphanidermatum* biocontrol agents. Most efficacious biocontrol agents identified were then combined in a consortium and studied to control the same pathogen (Chapter F).

Finally, methodology and experimental results are discussed in Chapter G. Future perspectives and considerations for biocontrol in aquaponics were also addressed.

Chapter A

Bibliographical Introduction

1. Plant pathogens and control strategies in aquaponics

The material presented in Section 1 is adapted from:
Stouvenakers, G., Dapprich, P., Massart, S., Jijakli, M.H., 2019. Chapter 14: Plant pathogens and control strategies in aquaponics, in: Simon Goddek, Joyce, A., Kotzen, B., Burnell, G.M. (Eds.), Aquaponics Food Production Systems. Springer, Cham, pp. 353–378. https://doi.org/doi.org/10.1007/978-3-030-15943-6_14

Abstract: Among the diversity of plant diseases occurring in aquaponics, soil-borne pathogens, such as *Fusarium* spp., *Phytophthora* spp. and *Pythium* spp., are the most problematic due to their preference for humid/aquatic environment conditions. *Phytophthora* spp. and *Pythium* spp. which belong to the Oomycetes pseudo-fungi require special attention because of their mobile form of dispersion, the so-called zoospores that can move free and actively in liquid water.
In coupled aquaponics, curative methods are still limited because of the possible toxicity of pesticides and chemical agents for fish and beneficial bacteria (e.g., nitrifying bacteria of the biofilter). Furthermore, the development of biocontrol agents for aquaponic use is still at its beginning. Consequently, ways to control the initial infection and the progression of a disease are mainly based on preventive actions and water physical treatments.
However, suppressive action (suppressiveness) could happen in aquaponic environment considering recent papers and the suppressive activity already highlighted in hydroponics. In addition, aquaponic water contains organic matter that could promote establishment and growth of heterotrophic bacteria in the system or even improve plant growth and viability directly. With regards to organic hydroponics (i.e., use of organic fertilisation and organic plant media), these bacteria could act as antagonist agents or as plant defence elicitors to protect plants from diseases. In the future, research on the disease suppressive ability of the aquaponic biotope must be increased, as well as isolation, characterization, and formulation of microbial plant pathogen antagonists. Finally, a good knowledge in the rapid identification of pathogens, combined with control methods and diseases monitoring, as recommended in integrated plant pest management, is the key to an efficient control of plant diseases in aquaponics.

Keywords: Aquaponics; plant pathogens; plant diseases; control strategies; suppressiveness

1.1. Introduction

Nowadays, aquaponic systems are the core of numerous research efforts which aim at better understanding these systems and at responding to new challenges of food production sustainability (Goddek et al., 2015; Villarroel et al., 2016). The cumulated number of publications mentioning "aquaponics" or derived terms in the title went from 12 in early 2008 to 215 in 2018 (January 2018 Scopus database research results). In spite of this increasing number of papers and the large area of study topics they are covering, one critical point is still missing, namely plant pest management (Stouvenakers et al., 2017). According to a survey on EU Aquaponic Hub members, only 40% of practitioners have some notions about pests and plant pest control (Villarroel et al., 2016).

In aquaponics, the diseases might be similar to those found in hydroponic systems under greenhouse structures. Among the most problematic pathogens, in term of spread, are hydrophilic fungi or fungus-like protists which are responsible for root or collar diseases. To consider plant pathogen control in aquaponics, firstly, it is important to differentiate between coupled and decoupled systems. Decoupled systems allow disconnection between water from the fish and crop compartment (Goddek et al., 2019). This separation allows the optimisation and a better control of different parameters (e.g. temperature, mineral or organic composition and pH) in each compartments (Goddek et al., 2016; Monsees et al., 2017). Furthermore, if the water from the crop unit doesn't come back to the fish part, the application of phytosanitary treatments (e.g., pesticides, biopesticides and chemical disinfection agents) could be allowed here. Coupled systems are built in one loop where water recirculates in all parts of the system (Goddek et al., 2019). However, in coupled systems, plant pest control is more difficult due to both presence of fish and beneficial microorganisms which transform fish sludge into plant nutrients. Their existence limits or excludes the application of already available disinfecting agents and chemical treatments. Furthermore no pesticides or biopesticides have been specifically developed for aquaponics (Rakocy et al., 2006; Rakocy 2012; Somerville et al., 2014; Bittsanszky et al., 2015; Nemethy et al., 2016; Sirakov et al., 2016). Control measures are consequently mainly based on non-curative physical practices (see Section 1.3.) (Nemethy et al., 2016; Stouvenakers et al., 2017).

On the other hand, recent studies highlighted that aquaponic plant production offers similar yields when compared to hydroponics although concentrations of mineral plant nutrients are lower in aquaponic water. Furthermore, when aquaponic water is complemented with some minerals to reach hydroponic concentrations of mineral nutritive elements, even better yields can be observed (Pantanella et al. 2010; Pantanella et al., 2015 Delaide et al. 2016; Saha et al. 2016; Anderson et al., 2017; Wielgosz et al., 2017; Goddek and Vermeulen, 2018). Moreover, some informal observations from practitioners in aquaponics and two recent scientific studies (Gravel et al., 2015; Sirakov et al., 2016) report the possible presence of beneficial

compounds and/or microorganisms in the water that could play a role in biostimulation and/or have antagonistic (i.e., inhibitory) activity against plant pathogens. Biostimulation is defined as any microorganisms or substances able to enhance plant quality traits and plant tolerance against abiotic stress.

With regard to these aspects, this chapter has two main objectives. The first is to give a review of microorganisms involved in aquaponic systems with a special focus on plant pathogenic and plant beneficial microorganisms. Factors influencing these microorganisms will be also considered (e.g., organic matter). The second is to review available methods and future possibilities in plant diseases control.

1.2. Microorganisms in aquaponics

Microorganisms are present in the entire aquaponics system and play a key role in the system. They are consequently found in the fish, the filtration (mechanical and biological) and the crop parts. Commonly, the characterisation of microbiota (i.e., microorganisms of a particular environment) is carried out on circulating water, periphyton, plants (rhizosphere, phyllosphere and fruit surface), biofilter, fish feed, fish gut and fish faeces. Up until now, in aquaponics, most of microbial research has focused on nitrifying bacteria (Schmautz et al., 2017). Thus, the trend at present is to characterise microorganisms in all compartments of the system using modern sequencing technologies. Schmautz et al. (2017) identified the microbial composition in different parts of the system whereas Munguia-Fragozo et al. (2015) give perspectives on how to characterize aquaponics microbiota from a taxonomical and functional point of view by using cutting edge technologies. In the following sub-sections, focus will be only brought on microorganisms interacting with plants in aquaponic systems organised into plant beneficial and plant pathogenic microorganisms.

1.2.1. Plant pathogens

Plant pathogens occurring in aquaponic systems are theoretically those commonly found in soilless systems. A specificity of aquaponic and hydroponic plant culture is the continuous presence of water in the system. This humid/aquatic environment suits almost every plant pathogenic fungus or bacteria. For root pathogens some are particularly well adapted to these conditions like pseudo-fungi belonging to the taxa of oomycetes (e.g., root rot diseases caused by *Pythium* spp. and *Phytophthora* spp.) which can produce a motile form of dissemination called zoospores. These zoospores can move actively in liquid water and thus are able to spread over the entire system extremely quickly. Once a plant is infected, the disease can rapidly spread out the system, especially because of the water´s recirculation (Jarvis 1992; Hong and Moorman 2005; Sutton et al., 2006; Postma et al., 2008; Vallance et al., 2010; Rakocy 2012; Rosberg 2014; Somerville et al., 2014). Though oomycetes are among the most prevalent pathogens detected during root diseases, they often form a complex with

other pathogens. Some *Fusarium* species (with existence of species well adapted to aquatic environment) or species from the genera *Colletotrichum*, *Rhizoctonia* and *Thielaviopsis* can be found as part of these complexes and can also cause significant damage on their own (Paulitz and Bélanger 2001; Hong and Moorman 2005; Postma et al., 2008; Vallance et al., 2010). Other fungal genera like *Verticillium* and *Didymella*, but also bacteria, such as *Ralstonia*, *Xhantomonas*, *Clavibacter*, *Erwinia*, *Pseudomonas*, as well as viruses (e.g. tomato mosaic, cucumber mosaic, melon necrotic spot virus, lettuce infectious virus and tobacco necrosis) can be detected in hydroponics or irrigation water and cause vessel-, stem-, leaf- or fruit damage (Jarvis 1992; Hong and Moorman, 2005). However, note that not all microorganisms detected are damaging or lead to symptoms in the crop. Even species of the same genus can be either harmful or beneficial (e.g., *Fusarium*, *Phoma*, *Pseudomonas*). Disease agents discussed above are mainly pathogens linked to water recirculation but can be identified in greenhouses also. Section 1.2.2. shows the results of the first international survey on plant diseases occurring specifically in aquaponics while Jarvis, (1992) and Albajes et al. (2002) give a broader view of occurring pathogens in greenhouse structures.

In hydroponics or in aquaponic systems, plants generally grow under greenhouse conditions optimized for plant production, especially for large scale production where all the environmental parameters are computer managed (Albajes et al., 2002; Vallance et al., 2010; Somerville et al., 2014; Parvatha Reddy, 2016). However, optimal conditions for plant production can also be exploited by plant pathogens. In fact, these structures generate warm, humid, windless, and rain-free conditions that can encourage plant diseases if they are not correctly managed (*ibid.*). To counteract this, compromises must be made between optimal plant conditions and disease prevention (*ibid.*). In the microclimate of the greenhouse, an inappropriate management of the vapour-pressure deficit can lead to the formation of a film or a drop of water on the plants surface. This often promotes plant pathogens development. Moreover, to maximise the yield in commercial hydroponics, some other parameters (e.g., high plant density, high fertilisation, to extend the period production) can enhance the susceptibility of plants to develop diseases (*ibid.*).

The question now, is to know by which route the initial inoculum (i.e., the first step in an epidemiological cycle) is brought into the system. The different steps in plant disease epidemiological cycle (EpC) are represented in Figure A-1. In aquaponics as in greenhouse hydroponic culture it can be considered that entry of pathogens could be linked to water supply, introduction of infected plants or seeds, the growth material (e.g., reuse of the media), air exchange (dust and particles carriage), insects (vectors of diseases and particles carriage) and staff (tools and clothing) (Paulitz and Bélanger 2001; Albajes et al., 2002; Hong and Moorman 2005; Sutton et al., 2006; Parvatha Reddy 2016).

Once the inoculum is in contact with the plant (step 2 in the EpC), several cases of infection (step 3 in the EpC), are possible (Lepoivre, 2003):

- The relationship pathogen-plant is incompatible (non-host relation), and disease does not develop.
- There is a host-relation, but the plant does not show symptoms (the plant is tolerant).
- The pathogen and the plant are compatible, but defence response is strong enough to inhibit the progression of the disease (the plant is resistant: interaction between host resistance gene and pathogen avirulence gene).
- The plant is sensitive (host-relation without gene for gene recognition) and the pathogen infects the plant, but symptoms are not highly severe (step 4 in the EpC).
- And lastly, the plant is sensitive and disease symptoms are visible and severe (step 4 in the EpC).

Regardless of the degree of resistance, some environmental conditions or factors can influence the susceptibility of a plant to be infected, either by a weakening of the plant or by promoting the growth of the plant pathogen (Colhoun 1973; Jarvis 1992; Cherif et al. 1997; Alhussaen 2006; Somerville et al., 2014). The main environmental factors influencing plant pathogens and disease development are temperature, relative humidity (RH) and light (*ibid.*). In hydroponics, temperature and oxygen concentrations of the nutrient solution can constitute additional factors (Cherif et al. 1997; Alhussaen 2006; Somerville et al., 2014) Each pathogen has its own preference of environmental conditions which can vary during its epidemiologic cycle. But in a general way, high humidity and temperature are favourable to the accomplishment of key steps in the pathogen's epidemic cycle such as spore production or spore germination (Figure A-1, step 5 in the EpC) (Colhoun 1973; Jarvis 1992; Cherif et al. 1997; Alhussaen 2006; Somerville et al., 2014). Colhoun (1973) sums up the effects of the various factors promoting plant diseases in soil whereas Table A-1 shows the more specific or adding factors that may encourage plant pathogen development linked to aquaponic greenhouse conditions.

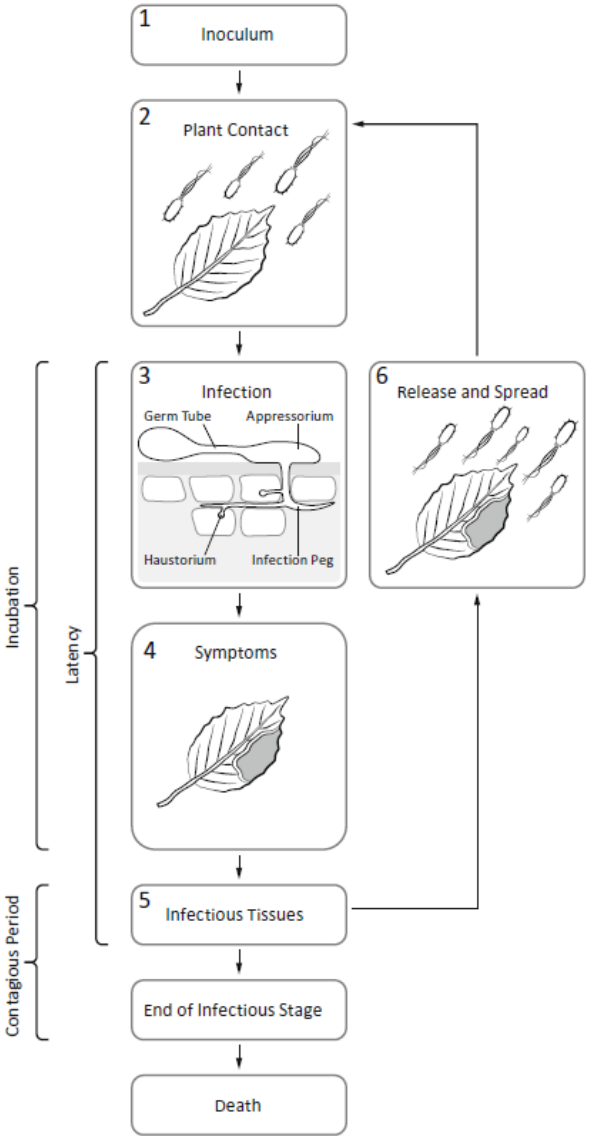


Figure A-1: Basic steps (*1* to *6*) in plant disease epidemiological cycle (EpC) according to Lepoivre (2003). (*1*) Arrival of the pathogen inoculum, (*2*) contact with the host plant, (*3*) tissues penetration and infection process by the pathogen, (*4*) symptoms development, (*5*) plant tissues become infectious, (*6*) release and spread of infectious form of dispersion.

In the epidemiological cycle, once the infective stage is reached (Figure A-1, step 5 in the EpC), the pathogens can spread in several ways (Figure A-1, step 6 in the EpC) and infect other plants. As explained before, root pathogens belonging to Oomycetes taxa can actively spread in the recirculating water by zoospores release (Alhussaen 2006; Sutton et al., 2006). For other fungi, bacteria and viruses responsible for root or aerial diseases, the dispersion of the causal agent can occur by propagation of infected material, mechanical wounds, infected tools, vectors (e.g., insects) and particles (e.g., spores and propagules) ejection or carriage allowed by drought, draughts or water splashes (Albajes et al. 2002; Lepoivre, 2003).

Table A-1: Adding factors encouraging plant pathogen development under aquaponic greenhouse structure compared to classical greenhouse culture.

Promoting factor	**Profiting to**	**Causes**	**References**
Nutrient film technique, deep flow technique	*Pythium* spp., *Fusarium* spp.	Easy spread by water recirculation; possibility of post contamination after a disinfection step; poor content in oxygen in the nutrient solution	Koohakan et al., 2004; Vallance et al., 2010
Inorganic media (e.g., rockwool)	Higher content in bacteria (no information about their possible pathogenicity)	Unavailable organic compounds in the media	Khalil and Alsanius 2001; Koohakan et al., 2004; Vallance et al., 2010
Organic media (e.g., coconut fibre and peat)	Higher content in fungi; higher content in *Fusarium* spp. for coconut-fibre	Available organic compounds in the media	Koohakan et al., 2004; Khalil et al., 2009; Vallance et al., 2010
Media with high water content and low content in oxygen (e.g., rockwool)	*Pythium* spp.	Zoospores mobility; plant stress	Van Der Gaag and Wever 2005; Vallance et al., 2010; Khalil and Alsanius 2011
Media allowing little water movement (e.g., rockwool)	*Pythium* spp.	Better condition for zoospores dispersal and chemotaxis movement; no loss of zoospore flagella	Sutton et al., 2006

High temperature and low concentration of DO in the nutrient solution	*Pythium* spp.	Plant stress and optimal conditions for *Pythium* growth	Cherif et al., 1997; Sutton et al., 2006; Vallance et al., 2010; Rosberg 2014
High host plant density and resulting microclimate	Pathogens growth, diseases spread	Warm and humid environment	Albajes et al., 2002; Somerville et al., 2014
Deficiencies, excess or imbalance of macro/micronutrients	Fungi, viruses, and bacteria	Plant physiological modifications (e.g., action on defence response, transpiration, integrity of cell walls); plant morphological modifications (e.g., higher susceptibility to pathogens, attraction of pests); nutrient resources in host tissues for pathogens; direct action on the pathogen development cycle	Colhoun 1973; Snoeijers and Alejandro 2000; Mitchell et al., 2003; Dordas 2008; Veresoglou et al., 2013; Somerville et al., 2014; Geary et al., 2015

1.2.2. Survey on aquaponic plant diseases

During January 2018, the first international survey on plant diseases was made among aquaponics practitioner members of the COST Action FA1305, the American Aquaponics Association and the EU Aquaponics HUB. Twenty-eight answers were received describing 32 aquaponic systems from around the world (EU: 21, North America: 5, South America: 1, Africa: 4, Asia: 1). The first finding was the small response rate. Among the possible explanations for the reluctance to reply to the questionnaire was that practitioners did not feel able to communicate about plant pathogens because of a lack of knowledge on this topic. This had already been observed in the surveys of Love et al. (2015) and Villarroel et al. (2016).

Key information obtained from the survey is:

- 84.4% of practitioners observe disease in their system
- 78.1% cannot identify the causal agent of a disease
- 34.4% do not apply disease control measures
- 34.4% use physical or chemical water treatment
- 6.2% use pesticides or biopesticides in coupled aquaponic system against plant pathogens.

These results support the previous arguments saying that aquaponic plants do get diseases. Yet, practitioners suffer from a lack of knowledge about plant pathogens and disease control measures actually used are essentially based on non-curative actions (90.5% of cases).

In the survey, a listing of plant pathogens occurring in their aquaponic system was provided. The Table A-2 shows the results of this identification. To remedy the lack of practitioner's expertise about plant disease diagnostics, a second survey version was sent with the aim to identify symptoms without disease name linkage (Table A-3). The Table A-2 mainly identifies diseases with specific symptoms, i.e., symptoms that can be directly linked to a plant pathogen. It is the case of *Botrytis cinerea* and its typical grey mould, powdery mildew (*Erysiphe* and *Podosphaera* genera in the table) and its white powdery mycelium/conidia, and lastly *Sclerotinia* spp. and its sclerotia production. The presence of 3 plant pathologists in the survey respondents expands the list, with the identification of some root pathogens (e.g., *Pythium* spp.). General symptoms that are not specific enough to be directly related to a pathogen without further verification are consequently found in the Table A-3. But it is important to highlight that most of the symptoms observed in this table could also be the consequence of abiotic stresses. Foliar chlorosis is one of the most explicit examples because it can be related to a large number of pathogens (e.g., for lettuce: *Pythium* spp., *Bremia lactucae*, *Sclerotinia* spp., beet western yellows virus), to environmental conditions (e.g., temperature excess) and to mineral deficiencies (nitrogen, magnesium, potassium, calcium, sulfur, iron, copper, boron, zinc, molybdenum) (Lepoivre, 2003; Resh, 2013).

Table A-2: Results of the first identifications of plant pathogens in aquaponics from the 2018 international survey analysis and from existing literature. Plant pathogens identified by symptoms in the aerial plant part are annotated by (a) and in root part by (b) in exponent.

Plant host	**Plant pathogen**	**References / survey results**
Allium schoenoprasu	*Pythium* sp. [(b)]	Survey
Beta vulgaris (swiss chard)	*Erysiphe betae* [(a)]	Survey
Cucumis sativus	*Podosphaera xanthii* [(a)]	Survey
Fragaria spp.	*Botrytis cinerea* [(a)]	Survey
Lactuca sativa	*Botrytis cinerea* [(a)]	Survey
	Bremia lactucae [(a)]	Survey
	Pythium dissotocum [(b)]	Rakocy, 2012
	Pythium myriotylum [(b)]	Rakocy, 2012
	Sclerotinia sp. [(a)]	Survey
Mentha spp.	*Pythium* sp. [(b)]	Survey

Nasturtium officinale	*Aspergillus sp.* [(a)]	Survey
Ocimum basilicum	*Alternaria* sp. [(a)]	Survey
	Botrytis cinerea [(a)]	Survey
	Pythium sp. [(b)]	Survey
	Sclerotinia sp. [(a)]	Survey
Pisum sativum	*Erysiphe pisi* [(a)]	Survey
Solanum lycopersicum	*Pseudomononas solanacearum* [(a)]	McMurty et al., 1990
	Phytophthora infestans [(a)]	Survey

Table A-3: Review of occurring symptoms in aquaponics from the 2018 international survey analysis.

Symptoms	**Plants species**
Foliar chlorosis	*Allium schoenoprasum* [1], *Amaranthus viridis* [1], *Coriandrum sativum* [1], *Cucumis sativu* [1], Ocimum *basilicum* [6], *Lactuca sativa* [4], *Mentha* spp. [2], *Petroselinum crispum* [1], *Spinacia oleracea* [2], *Solanum lycopersicum* [1], *Fragaria* spp. [1]
Foliar necrosis	*Mentha* spp. [2], *Ocimum basilicum* [1],
Stem necrosis	*Solanum lycopersicum* [1],
Collar necrosis	*Ocimum basilicum* [1]
Foliar Mosaic	*Cucumis sativus* [1], *Mentha* spp. [1], *Ocimum basilicum* [1],
Foliar wilting	*Brassica oleracea* Acephala group [1], *Lactuca sativa* [1], *Mentha* spp. [1], *Cucumis sativus* [1], *Ocimum basilicum* [1], *Solanum lycopersicum* [1]
Foliar, stem and collar mold	*Allium schoenoprasum* [1], *Capsicum annuum* [1], *Cucumis sativus* [1,] *Lactuca sativa* [2], *Mentha* spp. [2], *Ocimum basilicum* [4], *Solanum lycopersicum* [1]
Foliar spots	*Capsicum annuum* [1], *Cucumis sativus* [1,] *Lactuca sativa* [2], *Mentha* spp. [1], *Ocimum basilicum* [5]
Damping off	*Spinacia oleracea* [1], *Ocimum basilicum* [1], *Solanum lycopersicum* [1], seedlings in general [5]
Crinkle	*Beta vulgaris* (swiss chard) [1], *Capsicum annuum* [1], *Lactuca sativa* [1], *Ocimum basilicum* [1]
Browning or decaying root	*Allium schoenoprasum* [1], *Amaranthus viridis* [1], *Beta vulgaris* (swiss chard) [1], *Coriandrum sativum* [1], *Lactuca sativa* [1], *Mentha* spp. [2], *Ocimum basilicum* [2], *Petroselinum crispum* [2], *Solanum lycopersicum* [1], *Spinacia oleracea* [1]

Numbers in exponent represent the occurrence of the symptom for a specific plant on a total of 32 aquaponic systems reviewed.

1.2.3. Beneficial microorganisms in aquaponics – the opportunities

As explained in the introduction, several publications focused on bacteria involved in the nitrogen cycle while others already emphasise the potential presence of beneficial microorganisms interacting with plant pathogens and/or plants (Rakocy 2012; Gravel et al., 2015; Sirakov et al., 2016). This section reviews the potential of plant beneficial microorganisms involved in aquaponics and their modes of action.

Sirakov et al. (2016) screened antagonistic bacteria against *Pythium ultimum* isolated from an aquaponic system. Among the 964 tested isolates, 86 showed a strong inhibitory effect on *P. ultimum in vitro*. Further research must be achieved to taxonomically identify these bacteria and evaluate their potential in *in vivo* conditions. The authors assume that many of these isolates belong to the genus *Pseudomonas*. Schmautz et al. (2017) came to the same conclusion by identifying *Pseudomonas* spp. in the rhizosphere of lettuce. Antagonistic species of the genus *Pseudomonas* were able to control plant pathogens in natural environments (e.g., in suppressive soils) while this action also depended on environmental conditions. They can protect plants against pathogens either in an active or a passive way by eliciting a plant defence response, playing a role in plant growth promotion, compete with pathogens for space and nutrients (i.e., iron competition by release of iron-chelating siderophores), and/or finally by production of antibiotics or anti-fungal metabolites such as biosurfactants (Arras and Arru 1997;Ganeshan and Kumar 2005; Haas and Défago 2005; Beneduzi et al., 2012; Narayanasamy 2013). Although no identification of microorganisms was done by Gravel et al. (2015), they report that fish effluents have the capacity to stimulate plant growth, decrease the mycelial growth of *P. ultimum* and *Fusarium oxysporum in vitro* and reduce the colonization of tomato root by these fungi.

Information about the possible natural plant protection capacity of aquaponic microbiota is scarce, but the potential of this protective action can be envisaged with regard to different elements already known in hydroponics or in recirculated aquaculture. A first study was conducted in 1995 on suppressive action or suppressiveness promoted by microorganisms in soilless culture (McPherson et al., 1995). Suppressiveness in hydroponics, here defined by Postma et al. (2008), has "referred to the cases where (i) the pathogen does not establish or persist; or (ii) establishes but causes little or no damage". The suppressive action of a milieu can be related to the abiotic environment (e.g., pH and organic matter). However, in most situations it is considered to be related directly or indirectly to microorganisms activity or their metabolites (Borneman and Becker, 2007). In soilless culture, the suppressive capacity shown by water solution or the soilless media is reviewed by Postma et al. (2008) and Vallance et al. (2010). In these reviews, microorganisms responsible for this suppressive action are not clearly identified. In contrast, plant pathogens like *Phytophthora cryptogea*, *Pythium* spp., *Pythium aphanidermatum*, and *F. oxysporum f.* sp. *radicis lycopersici* controlled or suppressed by the natural microbiota are exhaustively described. In the various articles reviewed by Postma et al. (2008) and

Vallance et al. (2010), microbial involvement in the suppressive effect are generally verified via a destruction of the microbiota of the soilless substrate by sterilisation first and eventually followed by a re-inoculation. When compared with an open system without recirculation, suppressive activity in soilless systems could be explained by the water recirculation (McPherson et al., 1995; Tu et al., 1999, cited by Postma et al., 2008) which could allow a better development and spread of beneficial microorganisms (Vallance et al., 2010).

Since 2010, suppressiveness of hydroponic systems has been generally accepted and research topics have been more driven on isolation and characterization of antagonistic strains in soilless culture with *Pseudomonas* species as main organisms studied. If it was demonstrated that soilless culture systems can offer suppressive capacity, there is no similar demonstration of such activity in aquaponics systems. However, there is no empiric indication that it should not be the case. This optimism arises from the discoveries of Gravel et al. (2015) and Sirakov et al. (2016) described in the second paragraph of this section. Moreover, it has been shown in hydroponics (Haarhoff and Cleasby, 1991 cited by Calvo-bado et al., 2003; Van Os et al., 1999) but also in water treatment for human consumption (Verma et al., 2017) that slow filtration (described in Section 1.3.1) and more precisely slow sand filtration can also act against plant pathogens by a microbial suppressive action in addition to other physical factors. In hydroponics, slow filtration has been demonstrated to be effective against the plant pathogens reviewed in the Table A-4. It is assumed that the microbial suppressive activity in the filters is most probably due to species of *Bacillus* and/or *Pseudomonas* (Brand 2001; Déniel et al., 2004; Renault et al., 2007; Renault et al., 2012). The results of Déniel et al. (2004), suggest that in hydroponics, the mode of action of *Pseudomonas* and *Bacillus* relies on competition for nutrients, and antibiosis, respectively. However, additional modes of action could be present for these two genera as already explained for *Pseudomonas* spp.. *Bacillus* species can, depending on the environment, act either indirectly by plant biostimulation or elicitation of plant defences or directly by antagonism via production of antifungal and/or antibacterial substances. Cell wall degrading enzymes, bacteriocins and antibiotics, lipopeptides (i.e., biosurfactants) are identified as key molecules for the latter action (Pérez-García et al., 2011; Beneduzi et al., 2012; Narayanasamy 2013). All things considered, the functioning of a slow filter is not so different from the functioning of some biofilters used in aquaponics. Furthermore, some heterotrophic bacteria like *Pseudomonas* spp. were already identified in aquaponics biofilters (Schmautz et al., 2017). This is in accordance with the results of other researchers who frequently detected *Bacillus* and/or *Pseudomonas* in RAS (recirculated aquaculture system) biofilters (Tal et al., 2003; Sugita et al., 2005; Schreier et al., 2010; Munguia-Fragozo et al., 2015; Rurangwa and Verdegem 2015). Nevertheless, up until now, no study about the possible suppressiveness in aquaponic biofilters has been carried out.

Table A-4: Review of plant pathogens effectively removed by slow filtration in hydroponics.

Plant pathogens	References
Xanthomonas campestris pv. *Pelargonii*	Brand, 2001
Fusarium oxysporum	Wohanka 1995; Ehret et al., 1999 cited by Ehret et al., 2001; van Os et al., 2001; Déniel et al., 2004; Furtner et al., 2007
Pythium spp.	Déniel et al., 2004
Pythium aphanidermatum	Ehret et al., 1999 cited by Ehret et al., 2001; Furtner et al., 2007
Phytophthora cinnamomi	Van Os et al., 1999; 4 references cited by Ehret et al., 2001
Phytophthora cryptogea	Calvo-bado et al., 2003
Phytophthora cactorum	Evenhuis et al., 2014

1.3. Protecting plants from pathogens in aquaponics

At the moment aquaponic practitioners operating a coupled system are relatively helpless against plant diseases when they occur, especially in the case of root pathogens. No pesticide nor biopesticide is specifically developed for aquaponic use (Rakocy 2007; Rakocy 2012; Somerville et al., 2014; Bittsanszky et al., 2015; Sirakov et al., 2016). In brief, curative methods are still lacking. Only Somerville et al. (2014) lists the inorganic compounds that may be used against fungi in aquaponics. In any case, an appropriate diagnostic of the pathogen(s) causing the disease is mandatory in order to identify the target(s) for curative measures. This diagnosis requires good expertise in terms of observation capacity, plant pathogen cycle understanding and analysis of the situation. However, in case of generalist (not specific) symptoms and depending on the degree of accuracy needed, it is often necessary to use laboratory techniques to validate the hypothesis with respect to the causal agent (Lepoivre, 2003). Postma et al. (2008) reviewed the different methods to detect plant pathogens in hydroponics and four groups were identified:

(i) Direct macroscopic and microscopic observation of the pathogen,
(ii) Isolation of the pathogen,
(iii) Use of serological methods,
(iv) Use of molecular methods.

1.3.1. Non-biological methods of protection

Good agricultural practices (GAP) for plant pathogens control are the various actions aiming to limit crop diseases for both yield and quality of produce (FAO,

2008). GAP transposable to aquaponics are essentially non-curative physical or cultivation practices that can be divided in preventive measures and water treatment.

Preventive measures

Preventive measures have two distinct purposes. The first is to avoid the entry of the pathogen inoculum into the system and the second is to limit (i) plant infection, (ii) development and (iii) spread of the pathogen during the growing period. Preventive measures aiming to avoid the entry of the initial inoculum in the greenhouse are for example: a fallow period, a specific room for sanitation, room sanitation (e.g., plant debris removal and surface disinfection), specific clothes, certified seeds, a specific room for plant germination and physical barriers (against insect vectors) (Stanghellini and Rasmussen 1994; Jarvis 1992; Albajes et al., 2002; Somerville et al., 2014; Parvatha Reddy 2016). Among the most important practices used for the second type of preventive measures, are: the use of resistant plant varieties, tools disinfection, avoid plant abiotic stresses, good plant spacing, avoid algae development and environmental conditions management. The last measure, i.e., environmental conditions management, means to control all greenhouse parameters in order to avoid or limit diseases by intervening in their biological cycle (*ibid*.). Generally, in big scale greenhouse structures, computer software and algorithms are used to calculate the optimal parameters allowing both plant production and disease control. The parameters measured, among others, are temperature (of the air and the nutrient solution), humidity, vapour pressure deficit, wind speed, dew probability, leaf wetness and ventilation (*ibid.*). The practitioner acts on these parameters by manipulating the heating, the ventilation, the shading, the supplement of lights, the cooling and the fogging (*ibid.*).

Water treatment

Physical water treatments can be employed to control potential water pathogens. Filtration (pores size less than 10µm), heat, and UV treatments are among the most effective to eliminate pathogens without harmful effects on fish and plant health (Ehret et al., 2001; Hong and Moorman 2005; Postma et al., 2008; Van Os 2009; Timmons and Ebeling 2010). These techniques allow the control of disease outbreaks by decreasing the inoculum, the quantity of pathogens and their proliferation stages in the irrigation system (*ibid.*). Physical disinfection decreases water pathogens to a certain level depending on the aggressiveness of the treatment. Generally, the target of heat and UV disinfection is the reduction of the initial microorganisms population by 90 to 99.9% (*ibid.*). The filtration technique most used is slow filtration because of its reliability and its low cost. The substrates of filtration generally used are sand, rockwool or pozzolana (*ibid.*). Filtration efficiency is essentially dependent on pore size and flow. To be effective as disinfection treatment, the filtration needs to be achieved with a pore size less than 10µm and a flow rate of 100 $l/m^2/h$, even if less binding parameters show satisfactory performances (*ibid.*). Slow filtration does not

eliminate all of the pathogens; more than 90% of the total aerobic bacteria remain in the effluent (*ibid.*). Nevertheless, it allows a suppression of plant debris, algae, small particles, and some soil-borne diseases such as *Pythium* and *Phytophthora* (the efficiency is genus dependent). Slow filters do not act only by physical action but also show a microbial suppressive activity thanks to antagonistic microorganisms, as discussed in Section 1.2.3. (Hong and Moorman 2005; Postma et al., 2008; Van Os 2009; Vallance et al., 2010). Heat treatment is very effective against plant pathogens. However, it requires temperatures reaching 95° C during at least 10 seconds to suppress all kind of pathogens, viruses included. This practice consumes a lot of energy and imposes water cooling (heat exchanger and transitional tank) before reinjection of the treated water back into the irrigation loop. In addition, it has the disadvantage of killing all microorganisms including the beneficial ones (Hong and Moorman 2005; Postma et al., 2008; Van Os 2009). The last technique and probably the most applied is UV disinfection. 20.8% of EU Aquaponics Hub practitioners use it (Villarroel et al., 2016). UV radiation has a wavelength of 200 to 280 nm. It has a detrimental effect on microorganisms by direct damage of the DNA. Depending on the pathogen and the water turbulence, the energy dose varies between 100 and 250 mJ/cm^2 to be effective (Postma et al., 2008; Van Os 2009).

Physical water treatments eliminate most of the pathogens from the incoming water, but they cannot eradicate the disease when it is already present in the system. Physical water treatment does not cover all the water (especially the standing water zone near the roots), nor the infected plant tissue. For example UV treatments often fail to suppress *Pythium* root rot (Sutton et al., 2006). However, if physical water treatment allows a reduction of plant pathogens, theoretically, they also influence non-pathogenic microorganisms potentially acting on diseases suppression. In reality, heat and UV treatments create a microbiological vacuum, whereas slow filtration produces a shift in effluent microbiota composition resulting in a higher disease suppression capacity (Postma et al., 2008; Vallance et al., 2010). Even though UV and heat treatment in hydroponics eliminate more than 90% of microorganisms in the recirculating water, no diminution of the disease suppressiveness was observed. This was probably due to a too low quantity of water treated and a re-contamination of the water after contact with the irrigation system, roots, and plant media (*ibid.*).

Aquaponic water treatment by means of chemicals is limited in continuous application. Ozonation is a technique used in recirculated aquaculture and in hydroponics. Ozone treatment has the advantage to eliminate all pathogens including viruses in certain conditions and to be rapidly decomposed to oxygen (Hong and Moorman 2005; Van Os 2009; Timmons and Ebeling 2010; Gonçalves and Gagnon, 2011). However, it has several disadvantages. Introducing ozone in raw water can produce by-products oxidants and significant amount of residual oxidants (e.g., brominated compound and haloxy anions that are toxic for fish) that need to be removed, by UV radiation for example, prior to return to the fish part (reviewed by

Gonçalves and Gagnon, 2011). Furthermore, ozone treatment is expensive, irritant for mucous membranes in case of human exposure, it needs contact periods of 1 to 30 minutes at a concentration range of 0.1 to 2.0 mg/L, needs a temporal sump to reduce completely from O_3 to O_2, and can oxidize elements present in the nutrient solution, such as iron chelates and thus makes it unavailable for plants (Hong and Moorman 2005; Van Os 2009; Timmons and Ebeling 2010; Gonçalves and Gagnon, 2011).

1.3.2. Biological methods of protection

In hydroponics, numerous scientific papers review the use of antagonistic microorganisms (i.e., able to inhibit other organisms) to control plant pathogens but until now no research has been carried out for their use in aquaponics. The mode of action of these antagonistic microorganisms is according to Campbell (1989), Whipps (2001) and Narayanasamy (2013) grouped in:

i) Competition for nutrients and niches,
ii) Parasitism,
iii) Antibiosis,
iv) Induction of diseases resistance in plants.

The experiments introducing microorganisms in aquaponic systems have been focused on the increase of nitrification by addition of nitrifying bacteria (Zou et al., 2016) or the use of plant growth promoters (PGPR) such as *Azospirillum brasilense* and *Bacillus* ssp. to increase plant performance (Mangmang et al., 2015a, 2015c, 2015b, 2014b; Cerozi and Fitzsimmons, 2016; Bartelme et al., 2018). There is now an urgent need to work on biocontrol agents (BCA) against plant pathogens in aquaponics with regard to the restricted use of synthetic curative treatments, the high value of the culture and the increase of aquaponic systems in the world. BCA are defined, in this context, as viruses, bacteria and fungi exerting antagonistic effects on plant pathogens (Campbell 1989; Narayanasamy 2013).

Generally, the introduction of a BCA is considered as easier in soilless systems. In fact, the hydroponic root environment is more accessible than in soil and the microbiota of the substrate is also unbalanced due to a biological vacuum. Furthermore, environmental conditions of the greenhouse can be manipulated to achieve BCA growth needs. Theoretically all these characteristics allow a better introduction, establishment and interaction of the BCA with plants in hydroponics than in soil (Paulitz and Bélanger 2001; Postma et al., 2009; Vallance et al., 2010). However, in practice, the effectiveness of BCA inoculation to control root pathogens can be highly variable in soilless systems (Postma et al. 2008; Vallance et al. 2010; Montagne et al., 2017). One explanation for this is that BCA selection is based on *in vitro* tests which are not representing real conditions and subsequently a weak adaptation of these microorganisms to the aquatic environment used in hydroponics or aquaponics (Postma et al. 2008; Vallance et al. 2010). To control plant pathogens

and more especially those responsible for root rots, a selection and identification of microorganisms involved in aquatic systems which show suppressive activity against plant pathogens is needed. In soilless culture, several antagonistic microorganisms can be picked due to their biological cycle being similar to root pathogens or their ability to grow in aqueous conditions. Such is the case of non-pathogenic *Pythium* and *Fusarium* species and bacteria, where *Pseudomonas*, *Bacillus*, and *Lysobacter* are the genera the most represented in the literature (Paulitz and Bélanger 2001; Khan et al., 2003; Chatterton et al., 2004; Folman et al., 2004; Sutton et al., 2006; Liu et al., 2007; Postma et al. 2008; Postma et al., 2009; Vallance et al. 2010; Sopher and Sutton 2011; Hultberg et al., 2011; Lee and Lee 2015; Martin and Loper 2016 ; Moruzzi et al., 2017 ; Thongkamngam and Jaenaksorn 2017). The direct addition of some microbial metabolites such as biosurfactants has also been studied (Stanghellini and Miller 1997; Nielsen et al., 2006). Although some microorganisms are efficient at controlling root pathogens, there are other problems that need to be overcome in order to produce a bio-pesticide. The main challenges are to identify the means of inoculation, to determine inoculum density, the product formulation (Montagne et al., 2017), the production of sufficient quantity at low cost and the storage of the formulated product. Ecotoxicological studies on fish and living beneficial microorganisms in the system are also an important point. Another possibility that could be exploited is the use of a consortium of antagonistic agents, as observed in suppressive soil (Spadaro and Gullino 2005; Vallance et al., 2010). In fact, microorganisms can work in synergy or with complementary modes of action (*ibid.*). The addition of amendments could also enhance the BCA potential by acting as prebiotics (see Section 1.4.).

1.4. The role of organic matter in biocontrol activity in aquaponic systems

In Section 1.2., the suppressiveness of aquaponic systems was suggested. As stated before, the main hypothesis is related to the water recirculation as it is for hydroponic systems. However, a second hypothesis exists, and this is linked to the presence of organic matter in the system. Organic matter that could drive a more balanced microbial ecosystem including antagonistic agents which is less suitable for plant pathogens (Rakocy, 2012).

In aquaponics, organic matter comes from water supply, uneaten feeds, fish faeces, organic plant substrate, microbial activity, root exudates and plant residues (Naylor et al., 1999; Waechter-Kristensen et al., 1997, 1999). In such a system, heterotrophic bacteria are organisms able to use organic matter as a carbon and energy source, generally in the form of carbohydrates, amino acids, peptides or lipids (Sharrer et al., 2005; Willey et al., 2008; Whipps 2001). In recirculated aquaculture (RAS), they are mainly localised in the biofilter and consume organic particles trapped in it (Leonard et al., 2000; Leonard et al., 2002). However, another source of organic carbon for

heterotrophic bacteria is humic substances present as dissolved organic matter and responsible for the yellow–brownish coloration of the water (Takeda and Kiyono 1990 cited by Leonard et al., 2002; Hirayama et al., 1988). In the soil as well as in hydroponics, humic acids are known to stimulate plant growth and sustain the plant under abiotic stress conditions (Bohme 1999; du Jardin 2015). Proteins in the water can be used by plants as an alternative nitrogen source thus enhancing their growth and pathogen resistance (Adamczyk et al., 2010). In the recirculated water, the abundance of free-living heterotrophic bacteria is correlated with the amount of biologically available organic carbon and carbon-nitrogen ratio (C/N) (Leonard et al., 2002, 2000; Michaud et al., 2006; Attramadal et al., 2012). In the biofilter, an increase in the C/N ratio increases the abundance of heterotrophic bacteria at the expense of the number of autotrophic bacteria responsible for the nitrification process (Michaud et al., 2014, 2006). As implied, heterotrophic microorganisms can have a negative impact on the system because they compete with autotrophic bacteria (e.g., nitrifying bacteria) for space and oxygen. Some of them are plant or fish pathogens, or responsible for off-flavour in fish (Chang-Ho 1970; Funck-Jensen and Hockenhull 1983; Jones et al., 1991; Leonard et al., 2002, Nogueira et al., 2002; Michaud et al., 2006; Mukerji 2006; Whipps 2001; Rurangwa and Verdegem 2015). However, heterotrophic microorganisms involved in the system can also be positive (Whipps 2001; Mukerji 2006). Several studies using organic fertilizers or organic soilless media, in hydroponics, have shown interesting effects where the resident microbiota were able to control plant diseases (Montagne et al., 2015). All organic substrates have their own physico-chemical properties. Consequently, the characteristics of the media will influence microbial richness and functions. The choice of a specific media could therefore influence the microbial development so as to have a suppressive effect on pathogens (Grunert et al., 2016; Montagne et al., 2017, 2015). Another possibility of pathogen suppression related to organic carbon, is the use of organic amendments in hydroponics (Maher et al., 2008; Vallance et al., 2010). By adding composts in soilless media like it is common use in soil, suppressive effects are expected (Maher et al., 2008). Enhancing or maintaining a specific microorganism such as *Pseudomonas* population by adding some formulated carbon sources (e.g., nitrapyrin based product) as reported by Pagliaccia et al., 2007 and Pagliaccia et al., 2008 is another possibility. The emergence of organic soilless culture also highlights the involvement of beneficial microorganisms against plant pathogens supported by the use of organic fertilizers. Fujiwara et al., 2013, Chinta et al., 2014, and Chinta et al., 2015 reported that fertilization with corn steep liquor helps to control *F. oxysporum* f.sp. *lactucae* and *B. cinerea* on lettuce and *F. oxysporum* f.sp. *radicis-lycopersici* on tomato plants. And even if hardly advised for aquaponic use, 1 g/L of fish-based soluble fertilizer (Shinohara et al., 2011) suppresses bacterial wilt on tomato caused by *Ralstonia solanacearum* in hydroponics (Fujiwara et al., 2012).

Finally, though information about the impact of organic matter on plant protection in aquaponic is scarce, the various elements mentioned above show their potential capacity to promote a system-specific and plant pathogen suppressive microbiota.

1.5. Conclusions and future considerations

This chapter aimed to give a first report of plant pathogens occurring in aquaponics, reviewing actual methods and future possibilities to control them. Each strategy has advantages and disadvantages and must be thoroughly designed to fit each case. However, at this time, curative methods in coupled aquaponic systems are still limited and new perspectives of control must be found. Fortunately, suppressiveness in terms of aquaponic systems could be considered, as already observed in hydroponics (i.e., in plant media, water and slow filters). In addition, the presence of organic matter in the system is an encouraging factor compared to soilless culture systems making use of organic fertilisers, organic plant media or organic amendments.

For the future, it seems important to investigate this suppressive action followed by identification and characterization of the responsible microbes or microbe consortia. Based on the results, several strategies could be envisaged to enhance the capacity of plants to resist pathogens. The **first** is biological control by conservation, which means favour beneficial microorganisms by manipulating and managing water composition (e.g., C/N ratio, nutrients and gases) and parameters (e.g., pH and temperature). But identification of these influencing factors needs to be realized first. This management of autotrophic and heterotrophic bacteria is also of key importance to sustain good nitrification and keep healthy fish. The **second** strategy is augmentative biological control by additional release of beneficial microorganisms already present in the system in large numbers (inundative method) or in small numbers but repeated in time (inoculation method). But prior identification and multiplication of an aquaponic BCA should be achieved. The **third** strategy is importation, i.e., introduce a new microorganism normally not present into the system. In this case, selection of a microorganism adapted and safe for aquaponic environment is needed. For the two last strategies, the site of inoculation in the system must be considered depending on the aim desired. Sites where microbial activity could be enhanced are: the recirculated water, the rhizosphere (plant media included), the biofilter (such as in slow sand filters where BCA addition is already tested) and the phyllosphere (i.e., areal plant part). What the strategy is, the ultimate goal should be to lead the microbial communities to provide a stable, ecologically balanced microbial environment allowing good production of both plant and fish.

To conclude, following the requirements of integrated plant pest management (IPM) is a necessity to correctly manage the system and avoid development and spread of plant diseases (Bittsanszky et al., 2015; Nemethy et al., 2016). The principle of IPM is to apply chemical pesticides or other agents as a last resort when economic injury level is reached. Consequently, control of pathogens will need to be firstly based on

physical and biological methods (described above), their combination and an efficient detection and monitoring of the disease (European Parliament, 2009).

2. *Pythium* matter and suppressiveness study

2.1. Case of Pythium aphanidermatum – lettuce pathosystem

2.1.1. Working model

This study addresses the question of the natural suppressive action of aquaponic water against plant pathogens. To answer it, a relevant choice of the plant and its pathogens must be done. Lettuce *(Lactuca sativa* L.) is one of the most studied and produced vegetables in coupled aquaponics (personal observation from participiation in symposiums on aquaponics). Although it depends on the specific market studied, lettuce can be the most valuable vegetable in aquaponics (Bailey and Ferrarezi, 2017). Beside the price per head, lettuce presents the advantages to be planted at high density but, above all, to have a short growing period. These characteristics also make them an appropriate model for experimental studies. As mentioned in Section 1., research on plant diseases in aquaponics are scarce. However, we can consider that aquaponic lettuce receives similar culture conditions than in hydroponics. In these systems using recirculated irrigating water, plant pathogens adapted to aquatic environment, among which fungi or oomyctes able to produce mobile forms of dispersion are particularly problematic and well-studied. Among these plant pathogens, the well characterized *P. aphanidermatum* (Edson) Fitzp. oomycete was selected. On lettuce, but also on cucumber, *P. aphanidermatum* is one of the most common and damaging root pathogen in soilless conditions (Utkhede et al., 2000; Postma et al., 2008). The resulting model is therefore the interaction between aquaponic microorganisms, lettuce and the plant pathogen *P. aphanidermatum*.

2.1.2. *Pythium aphanidermatum*

Description

Pythium aphanidermatum is a fungus-like organism belonging to the Pythiaceae family inside the Oomycota phylum. Although *Pythium* spp. produce filamentous structures similar to fungi, they have been classified in the Chromista Kingdom. The Straminipila Kingdom or the term “stramenopile” are also used to differentiate them from pigmented algae also a member of the Chromista Kingdom. Oomycetes show coenocytic and cellulosic mycelia that differentiate (Figure A-2) either sporangia containing heterokont (i.e., stramenopile) zoospores during asexual reproduction, or either phylum characteristic globose oogonia during sexual reproduction (Hon, 2018).

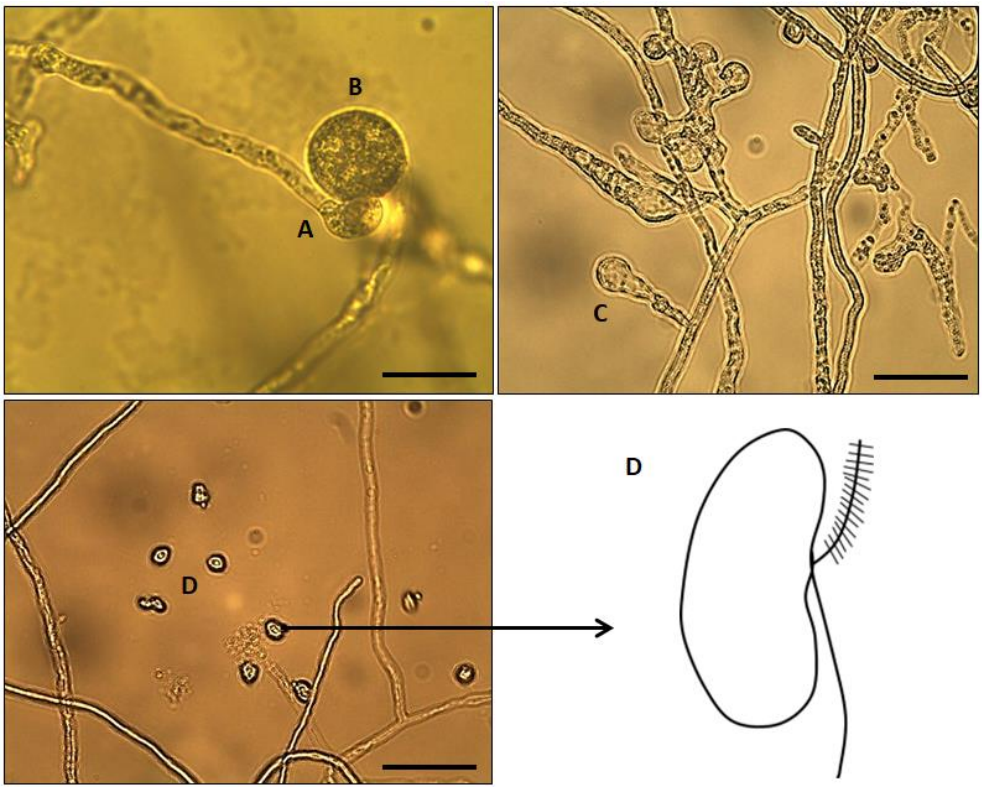


Figure A-2: *P. aphanidermatum* reproduction structures. Sexual mating of antheridium (A) and oogonium (B) structures. Asexual production of sporangia (C) containing heterokont zoospores (D) composed of tinsel and whiplash flagella. Bars of 20 µm. Personal pictures and representations.

Epidemiological cycle

Sutton et al. (2006) extensively reviewed the epidemiological cycle of *Pythium spp*. in hydroponics while Martin and Loper, (1999) reviewed it for soil. From these 2 references and personal observations, *P. aphanidermatum* disease development steps are summarized in this paragraph. *P. aphanidermatum* is one of the main species of *Pythium* able to cause root rot in the irrigation system. *Pythium* members also belong to the plant pathogen complex able to produce damping-off diseases. *P. aphanidermatum* can infect a wide range of host plants. In hydroponics, the most studied hosts for *P. aphanidermatum* are cucumber, pepper, tomato, and lettuce plants. *P. aphanidermatum* can release zoospores in high quantity from an outside vesicle on the tip of filamentous and lobed sporangia during asexual reproduction (Figure A-2). As others *Pythium* species, it can also produce oospores following the

sexual mating of an oogonium and an antheridium (Figure A-2). The initial infection by *P. aphanidermatum* occurs in the root zone through zoospores or mycelium. Zoospores could emerge from sporangia produced directly by mycelium differentiation or after oogonia/oospores germination (Figure A-3). The form of the initial inoculum is therefore quite large and can come from various introduction pathways (see Section 1.2.1. for disease primary inoculum sources in aquaponics). The secondary infection, i.e., from already infected plants in the system, is generally assumed by zoospores release in the recirculating water from the root zone. In this infection process, zoospores are chemotactically attracted by root exudates, they start adhering to roots and encyst themselves. Then spores germinate (9 min to 15 min after encystment for *P. aphanidermatum*) and produce germination tubes followed by penetration pegs or fine hyphae supported by enzymatic action to infect tissues. In some case *P. aphanidermatum* can produce appressoria or appressoria like structures. This *Pythium* specie can infect wounded but also non-wounded roots, with a preference for elongation zones and young root hairs.

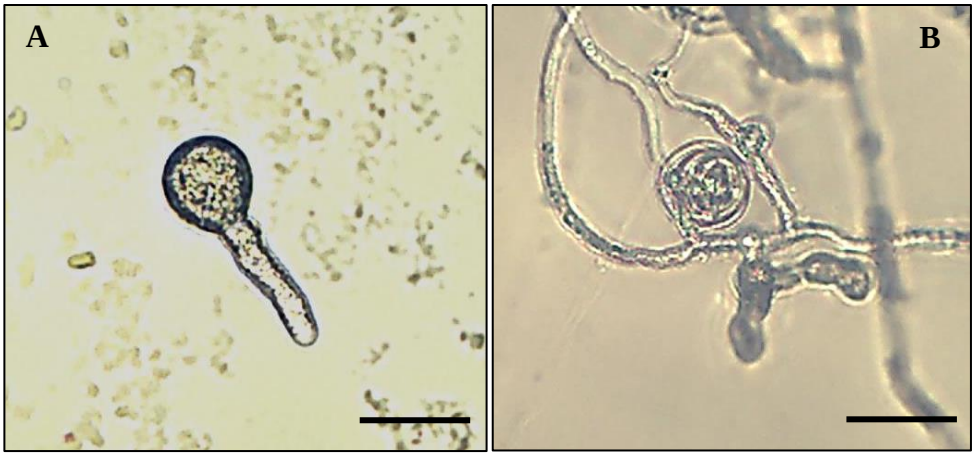


Figure A-3: *P. aphanidermatum* germinating oospore (A) and germinated oogonium with hyphae and sporangia production (B). Bars of 20 µm. Personal pictures.

P. aphanidermatum colonizes root tissue through both intercellular and intracellular ways. The first infection stages of this pathogen are often biotrophic and relatively asymptomatic. After this subclinical step, symptoms appear with the necrotrophic colonization of root tissue. The necrotrophic step is often induced by environmental conditions conducive to *P. aphanidermatum* (Alhussaen, 2006; Martin and Loper, 1999; Sutton et al., 2006). High temperature (from 23°C to 35°C) and moisture are the most determining factors (Sutton et al., 2006). The necrotrophic stage of *Pythium* could be generally observed or triggered by higher temperatures as already

highlighted on hydroponic lettuce (Funck-Jensen and Hockenhull, 1983; Alhussaen, 2006). In Stouvenakers et al. (2020), lettuce disease symptoms were initially the browning of root tips followed by a yellowing of roots sometimes accompanied by root architectural changes (Figure A-4). More severe symptoms were roots browning (browning caused by phenolic polymers accumulation according to Sutton et al. 2006) that degenerate into root necrosis and decay. At these last steps, lettuce was not able to provide its water demand and foliage rapidly turned into wilt and finally tissue necrosis (Figure A-4).

The epidemiological cycle of *P. aphanidermatum* in damping-off disease is relatively similar. The pathogen inoculum that infects seeds can also originate from different structures. Zoospores are attracted by seed exudates, encysts themselves, germinate and start seed colonization (Hendrix and Campbell, 1973; Jack and Nelson, 2010). *Pythium* spp. usually infect seeds or radicles that cause pre-emergence damping-off. Tissue of juvenile seedlings can also be infected by the pseudo-fungus and cause post-emergence damping-off. Symptoms are consequently non-sprouting seeds with radicle rot (if appeared) in pre-emergence (Figure A-4) or brown, water soaked and collapsed seedlings in post-emergence (Hendrix and Campbell, 1973).

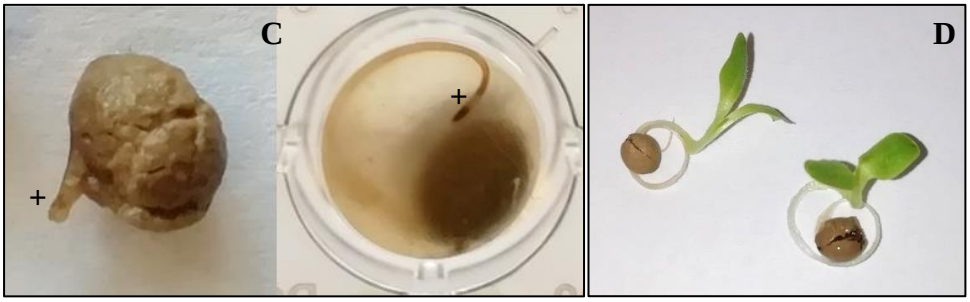


Figure A-4: Lettuce symptoms cause by *P. aphanidermatum* disease. Soft to medium symptoms of yellowing to browning roots with brown tips on hydroponic lettuce (A). Severe root rot symptoms with foliage wilt on hydroponic lettuce (B). Symptoms of pre-emergence damping-off with radicle rot (+) on pelleted lettuce seeds grown in 96-well plate (C) compared with healthy seedling of the same age (D). Personal pictures.

2.2. Control of Pythium diseases in aquaponics

2.2.1. Introduction

Control of zoosporic disease is complex in coupled aquaponic conditions and remains essentially based on preventive measures or physical water treatment such as UV water disinfection (see Section 1.3.1.). In soil, propamocarb is the sole active compound commercialized in Belgium to control *Pythium* disease on lettuce. However, chemical pesticides and disinfectants are not suitable for coupled aquaponics because of fish, nitrifying bacteria and plant presence in the same water loop (Stouvenakers et al., 2019; Folorunso et al., 2020; Rašković, Dvořák and Mráz, 2021). Moreover, most aquaponic practitioners publicize aquaponic sector as being sustainable and organic (Love et al., 2014; Goddek et al., 2015; Turnsek et al., 2020; Fruscella et al., 2021). Consequently, aquaponics should use of organic alternatives (e.g., biopesticides) although getting an organic label is currently not possible in EU.

2.2.2. Biocontrol by microorganisms application

In soil, many microorganisms were tested to suppress *Pythium* diseases, and some were approved as biocontrol agent by phytosanitary authorities. For example, *Trichoderma asperellum* T34 and *Clonostachys rosea* J1446 = (Gliocladium catenulatum J1446) are approved in Belgium against *Pythium* diseases on lettuce. However, antagonistic microorganisms used in soil were not developed for aquaponic or aquatic use. So far, no antagonistic microorganism was *in vivo* tested in aquaponic conditions while results in hydroponics often give variable *in vivo* efficacy against root pathogens due to the specific conditions of soilless cultures (Postma et al., 2008; Vallance et al., 2010; Montagne et al., 2017). Table A-5 presents the list of antagonistic microorganisms tested *in vivo* in hydroponic conditions against *Pythium*

disease. It could be noted that strains belonging to *Pseudomonas* species have been the most tested to control *Pythium* diseases in hydroponics. *Pseudomonas chlororaphis* strain Tx-1 was evaluated several times in hydroponics against *P. aphanidermatum* (Khan, Sutton and Grodzinski, 2003; Chatterton, Sutton and Boland, 2004; Liu et al., 2007). This strain seemed promising for hydroponic use because of its better adaptation to water environment (*ibid*.). The strain was notably EPA approved (BioJect®/Spot-less™) to treat golf courses against fungal diseases (including *Pythium* spp.) throuth application in the irrigation watering system (Sigler et al., 2001; Hardebeck et *al*., 2004). Efficacy results of the strain against *P. aphanidermatum* in hydroponics were valuable but variable (Khan, Sutton and Grodzinski, 2003; Chatterton, Sutton and Boland, 2004; Liu et al., 2007). Only 3 studies were found to use commercialized biofungicides in hydroponics against *P. aphanidermatum* (Utkhede, Lévesque and Dinh, 2000; Khalil and Alsanius, 2010; McGehee et al., 2019). In comparison with a *P. aphanidermatum* inoculated lettuce control, it was shown a slight decrease of disease severity while using *Bacillus subtilis* (Boost®), whereas the use of *T. harzianum* (Rootshield®) and *Gliocladium virens* (Soilguard®) were not beneficial (Utkhede, Lévesque and Dinh, 2000). However, disease severity of the non-treated control is complex to consider because of *P. aphanidermatum*-free control lack and was suspected to be low. On tomato plants, disease incidence, plant yields and pathogen presence were significantly improved by *Trichoderma polysporum* + *T. harzianum* (Binab-T®), *G. catenulatum* (Gliomix®) and *S. griseoviridis* K61 (Mycostop®) applications as well as the non-commercialized *Pseudomonas fluorescens* strain 5.014 (Khalil and Alsanius, 2010). Finally, on slightly symptomatic microgreens inoculated with *Pythium* spp., the use of *Bacillus subtilis* GB03 (Companion®), *T. harzianum* KRL-AG2 + *Trichoderma virens* G-41 (RootShield Plus®) and to a lesser extent, *Bacillus amyloliquefaciens* D747 (Triathlon BA®) were found to have a significant effect on disease incidence and severity but results were variable in time and dependent on the microgreen species (McGehee et al., 2019). To conclude, *Pythium* antagonistic microorganisms tested in aquatic environment are still limited in comparison to soil environment. First, these studies emphasised that *Pythium* virulence and symptoms are difficult to obtain in mock conditions because of the possible first saprophytic stage of *Pythium* spp.. Second, biocontrol level seems variable depending on hydroponic systems, environmental conditions, host plant and the formulation. More especially, time, place (seed, root, plant substrate or irrigation water), and biocontrol agent inoculum density also influence the biocontrol efficacy.

Table A-5: List of antagonistic bacteria and fungi tested in hydroponic system to control *Pythium* diseases.

Antagonistic bacteria	***Pythium* species**	**Host plants**	**References**
Bacillus cereus	*P. aphanidermatum*, *P. dissotocum*	Chrysanthemum	Liu et al., 2007
Bacillus subtilis (Boost®, Companion®)	*P. aphanidermatum*, *P. dissotocum*	Lettuce, microgreens (mustard, kale, arugula and radish)	Utkhede, Lévesque and Dinh, 2000; McGehee et al., 2019
Bacillus amyloliquefaciens (Triathlon BA®)	*P. aphanidermatum*, *P. dissotocum*	Microgreens (mustard, kale, arugula and radish)	McGehee et al., 2019
Burkholderia gladioli	*P. aphanidermatum*, *P. dissotocum*	Chrysanthemum	Liu et al., 2007
Comamonas acidovorans	*P. aphanidermatum*, *P. dissotocum*	Chrysanthemum	Liu et al., 2007
Enterobacter aerogenes	*P. aphanidermatum*	Lettuce	Utkhede, Lévesque and Dinh, 2000
Lysobacter enzymogenes	*P. aphanidermatum*	Cucumber	Folman et al., 2004; Postma et al., 2009
Pseudomonas aeruginosa	*P. splendens*	Tomato plant	Buyens, Höfte and Poppe, 1995
Pseudomonas chlororaphis	*P. aphanidermatum*, *P. dissotocum*	Pepper, chrysanthemum	Khan, Sutton and Grodzinski, 2003; Chatterton, Sutton and Boland, 2004; Liu et al., 2007; Sopher and Sutton, 2011
Pseudomonas corrugata	*P. aphanidermatum*	Cucumber	Paulitz, Zhou and Rankin, 1992; Rankin and Paulitz, 1994

Pseudomonas fluorescens (subgroup included)	*P. aphanidermatum*, *P. ultimum*	Cucumber, tomato plant	Paulitz, Zhou and Rankin, 1992; Rankin and Paulitz, 1994; Ongena et al., 1999; Gravel et al., 2006; Khalil and Alsanius, 2010
Pseudomonas koreensis	*P. ultimum*	Lettuce	Hultberg, Holmkvist and Alsanius, 2011
Pseudomonas marginalis	*P. ultimum*	Tomato plant	Gravel et al., 2006
Pseudomonas putida (subgroups included)	*P. aphanidermatum*, *P. ultimum*	Cucumber, tomato plant	Ongena et al., 1999 ; Gravel et al., 2006; Pagliaccia, Ferrin and Stanghellini, 2007
Pseudomonas syringae	*P. ultimum*	Tomato plant	Gravel et al., 2006
Streptomyces griseoviridis (Mycostop®)	*P. aphanidermatum*, *P. ultimum*	Tomato plant	Khalil and Alsanius, 2010
Antagonistic fungi	***Pythium* species**	**Host plants**	**References**
Gliocladium catenulatum (Gliomix®)	*P. aphanidermatum*, *P. ultimum*	Tomato plant	Khalil and Alsanius, 2010
Gliocladium virens (Soilguard®)	*P. aphanidermatum*	Lettuce	Utkhede, Lévesque and Dinh, 2000
Penicillium brevicompactum	*P. ultimum*	Tomato plant	Gravel et al., 2006
Penicillium solitum	*P. ultimum*	Tomato plant	Gravel et al., 2006
Trichoderma polysporum + *Trichoderma harzianum* (Binab-T®)	*P. aphanidermatum*, *P. ultimum*	Tomato plant	Khalil and Alsanius, 2010; Khalil and Alsanius, 2011
Trichoderma atroviride	*P. ultimum*	Tomato plant	Gravel et al., 2006
Trichoderma harzianum (Rootshield®)	*P. aphanidermatum*	Lettuce	Utkhede, Lévesque and Dinh, 2000

Trichoderma harzianum + *Trichoderma virens* (Rootshield Plus®)	*P. aphanidermatum*, *P. dissotocum*	Microgreens (mustard, kale, arugula and radish)	McGehee et al., 2019

In case of a wide number of isolates screened in a study, only microorganisms with a minimum of efficiency were listed.

2.2.3. Control by application of plant defense activators: silicon use matter

Microbial biocontrol agents can control plant diseases through plant defense elicitation as a mechanism of action (see Section 1.3.2). However, compounds or molecules can also be used directly as plant defense activator against plant pathogens. These compounds can be natural or synthetic (Walters et al., 2013; Wiesel et al., 2014). For example, natural compounds (i.e., biotic or abiotic) tested in hydroponics to control *Pythium* diseases are lipopeptides or biosurfactants produced by bacteria (Hultberg et al., 2010), glucosamine polymers such as chitosan (El Ghaouth et al., 1994; Postma et al., 2009), and mineral elements such as silicon (Chérif and Bélanger, 1992). Synthetic plant defense activator used in hydroponic to control *Pythium* diseases are mainly plant hormones, such as salicylic acid (Chen et al., 1999). Among these treatments, silicon addition is common in hydroponic crops (Belanger et al., 1995) and is also well studied to control *Pythium* diseases (Chérif and Bélanger, 1992; Chérif et al., 1992a, 1992b, 1994a, 1994b; Utkhede et al., 2000; Heine et al., 2007). Silicon is the second most abundant element on earth and plays a key role in aquatic ecosystems because of its involvement in diatoms formation, which is a first link in aquatic food chain (Linnik and Dikaya, 2014; Struyf et al., 2009). Silicon is not an essential element for terrestrial plants but many plants accumulate it (Epstein, 1994). Its availability also improves plant growth and tolerance to biotic and abiotic stresses (Epstein, 1994; Fauteux et al., 2005). Addition of silicon in recirculating hydroponic nutrient solution to control *Pythium* diseases showed interesting results (Chérif and Bélanger, 1992; Chérif et al., 1992a, 1992b, 1994a, 1994b; Utkhede et al., 2000; Heine et al., 2007). The addition of 100 mg/L of potassium silicate in recirculating nutrient solution increased foliar and root dry mass of lettuce inoculated with *P. aphanidermatum* by 25.7% and 30.8%, respectively (Utkhede et al., 2000). *P. aphanidermatum* and *P. ultimum* symptoms were also decreased in cucumber plants following the addition of silicon in nutrient solution (Chérif et al., 1994b; Chérif and Bélanger, 1992). Silicon mechanisms of action against plant pathogens were formerly attributed to a physical barrier linked to the cell wall reinforcement (Fauteux et al., 2005). However, this hypothesis is criticized at the expense of a plant defense induction (Fauteux et al., 2005; Gullino et al., 2015). In cucumber plants, it was stated that soluble silicon could induce a rapid plant defense response following *Pythium* infection. Indeed, activity of chitinases, peroxidases and polyphenoloxydases were enhanced following infection and an accumulation of phenolic compounds was observed in the plant (Chérif et al., 1992a, 1992b, 1994a). However, control effect of silicon against plant pathogens could be rapidly lost in cucumber if silicon feeding was stopped (Samuels et al., 1991). Because of its widely use in hydroponics and its safety for fish and plants, silicon could be considered in aquaponics to control *Pythium* diseases.

2.3. Suppressiveness study

2.3.1. *Pythium* suppressive biotopes

Soil-borne disease suppressive soils were recognized since the end of the 19th century (Expósito et al., 2017), while first studies on *Pythium* disease suppressive soil occurred in 20th century (Hancock, 1977; Kao and Ko, 1983; Martin and Hancock, 1986). Reviews and history of disease suppressiveness biotopes can be found in Expósito et al. (2017) and Bonanomi et al. (2018). Although closely related, it is generally accepted that suppressive soils are more driven (directly or indirectly) by the presence of antagonistic microorganisms than physical or chemical soil parameters (Bonanomi et al., 2007; Bongiorno et al., 2019). Presence and activity of the suppressive microbiota were found to be, *inter alia*, related to organic matter (Bonanomi et al., 2007). Organic amendment or crop management to increase carbon availability are factors helping suppressive soil establishment or conservation (Jambhulkar et al., 2015; Bongiorno et al., 2019). Moreover, a wide number of organic amendments such as compost were also described as disease suppressive (Bonanomi et al., 2007; Hadar and Papadopoulou, 2012). *Pythium* spp. were the second most studied pathogens after *Rhizoctonia solani* for suppression with organic amendments (Bonanomi et al., 2007). Two mechanisms of suppression can be differentiated: the general and the specific. In specific suppression, the disease antagonistic activity is related to a specific microorganism or a group of microorganisms. It was reviewed that *Pythium* disease suppression in soil or amendment was generally linked to general suppression because of the poor competitor ability of *Pythium* spp. and mainly during its saprophytic stage (Martin and Loper, 1999; Bonanomi et al., 2007; Jambhulkar et al., 2015). Indeed, microbiota richness, diversity and activity were often found positively correlated to *Pythium* suppression in soil (Garbeva, et al., 2004; Bonanomi et al., 2007). However, new molecular technologies such as high-throughput sequencing (HTS) have provided new ways to examined microbiota of suppressive soil (Schlatter et al., 2017). HTS analysis highlighted a possible specific *Pythium* suppression activity related to a specific group of microorganisms (Yu et al., 2015; Corato et al., 2019; Ros et al., 2019). From the discovery of soil-borne disease suppressive soils and suppressive organic amendments, a large isolation and screening campaign of antagonistic microorganisms was applied (Garbeva et al., 2004; Expósito et al., 2017; Bonanomi et al., 2018). Best known and described microorganisms studied to control *Pythium* diseases and derived from this campaign were *Pseudomonas*, *Bacillus*, *Streptomyces*, *Burkholderia*, *Paenibacillus*, *Enterobacter*, *Serratia* species for bacteria, and *Trichoderma*, *Gliocladium*/*Clonostachys* and non-pathogenic *Pythium* species for fungi (Martin and Loper, 1999; Kilany et al., 2015; Lamichhane et al., 2017).

Hydroponic suppressiveness and potentialities of aquaponics to suppress soil-borne diseases were already addressed in Section 1.2.3.. As explained, organic matter seems to play a key role in disease suppression in addition to the recirculated water

specificity of the system (Section 1.4.). However, in this type of soilless system, the existence of a *Pythium* specific suppression seems more pronounced than in soil. For example, pseudomonads were identified several times as antagonistic microorganisms linked to *Pythium* suppression in hydroponics (Déniel et al., 2004; Pagliaccia et al., 2007; Burgos-Garay, Hong and Moorman, 2014). Contradictory, the well-known soil antagonistic fungal genus *Trichoderma* and *Gliocladium*/*Clonostachys* were rarely related to hydroponic suppressiveness. When *Trichoderma* was found in the system, they were in organic plant substrates, i.e., substrates susceptible to be colonized by telluric fungi (Khalil and Alsanius, 2001; Gravel et al., 2006). Consequently, the strategy to isolate and screened *Pythium* antagonists adapted to hydroponic conditions and inert plant substrates targeted mainly specific bacteria such as pseudomonads. Results of this campaign could be illustrated by the list of non-commercialized antagonistic agents (i.e., without the "®" indication) cited in Table A-5. However, in aquaponic nutrient solution, the higher concentration of organic compounds than in hydroponics could then drive another group of specific suppressive microbiota. This specific aquaponic microbiota was studied in this thesis for isolation and screening of new antagonistic microorganisms adapted to such environment.

2.3.2. Microbial suppressiveness origin

Since their discovery, numerous studies have tried to identify the origin of suppressive biomes. Although dependent on each other, the main source of suppressiveness was generally linked to microorganisms action and to a lesser extent to abiotic parameters of the biotope, e.g., soil pH and EC (Bonanomi et al., 2007; Bongiorno et al., 2019; Hadar and Papadopoulou, 2012). Once suppressive activity linked to microorganisms is established, the following step is to determine which microorganisms could be responsible. Microbiota study in general suppression is hardly exploitable and remain fundamental because all the microbiome is considered responsible. Specific suppression is linked to specifics microorganisms and its study aims at transferring suppressiveness to other biotopes through specific microorganisms inoculation or promotion (Borneman and Becker, 2007; Weller et al., 2002). However, the question to answer in specific suppression is: how to discriminate responsible microorganisms?

Isolation-based methods for identification of suppressive microorganisms

Determination of specific suppressive microorganisms with culturation-based methods is often intrinsically linked to isolation. The former strategy was to use non-selective cultivation-based methods to isolate microorganisms without a priori and then screen them for specific antagonism versus a plant pathogen (Expósito et al., 2017). However, soils are very rich in microorganisms and only 0.1% to 1.0% of microorganisms in soil are culturable (Torsvik, et al., 1996). Moreover, because universal culture media for microorganisms does not exist, their use leads irremediably to the enrichment of certain microorganisms and lack of others (Davis,

et al., 2005). The method is also highly dependent of a following considerable screening step for biocontrol action. Nevertheless, this strategy resulted in the discovery of the later well-known biocontrol agents that belong, for example, to *Pseudomonas*, *Bacillus*, *Streptomyces*, or *Trichoderma* genera (Expósito et al., 2017). These large-scale isolation campaigns of biocontrol agents were of great value thereafter. Based on this knowledge, following studies tried to isolate specific microorganisms for their biocontrol action. For example, streptomyces are often targeted for their biocontrol activity against plant pathogens and selectively isolated (El-Tarabily, 2006; Evangelista-Martínez, 2014). It was the start of *a priori* isolation techniques. Another method derived from biocontrol agent study is the exploitation of antagonistic mode of action for functional isolation or functional isolates screening. For example, the ability of certain microorganisms to produce cell-wall-degrading enzymes involved in parasitism action could be used to isolate biocontrol agents of *P. aphanidermatum* (El-Tarabily, 2006). Specific reactive media or pre-enrichment procedure to isolate chitinase (Saima et al., 2013), chitosanase (Wangtueai et al., 2006), β-1,3-glucanase (Wu et al., 2018) and cellulase (Yin et al., 2010) producing microorganisms are examples of functional-based methods.

Community-based methods for for identification of suppressive microorganisms

The second approach to determine suppressive microorganisms in a biotope is based on community study. Numerous techniques are available to characterize microbiota composition and diversity. The Figure A-5 from Massart et al. (2015), compared these techniques depending on the quantity of data generated by the analyses and the study aim (from community description to functional understanding). Two approaches to study microbial community could be distinguished, culture-dependent and independent. One of the most known culturation-based method is community-level physiological profiles (CLPP). In CLPP, community are characterized depending on the carbon source utilization, for example, the BIOLOG® commercial system is widely used (Garland, 1997). However, this technique does not allow to identify microorganisms. The strategy applied by Folman et al. (2001) to find *P. aphanidermatum* biocontrol agents was first to compare carbon sources used by rhizospheric community with those used by the pathogen during its infection process. The second step was a screening of microorganisms able to exploit the same carbon sources and then potentially able to compete for nutrients and place with *P. aphanidermatum* (Folman et al., 2003). After this functional selection process, 127 bacteria were tested for disease suppression. However, only few bacteria were able to decrease disease symptoms and efficacy variability was detected between assays.

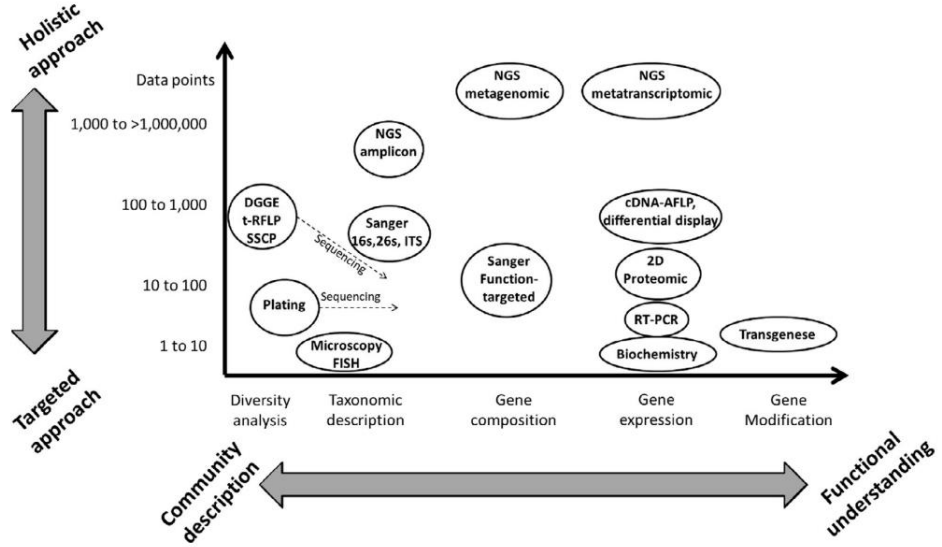


Figure A-5: Available technologies to study plant microbiota depending on the approach and the study goal. Ordinates are the generated information from data analyses expressed in data points. Graphic extracted without modification from Massart et al. (2015).

Culture-independent methods to study microbial community include phospholipid fatty acid analysis, fluorescence in situ hybridization microscopy (FISH microscopy) and nucleic acid-based techniques (Van Elsas et al., 1998). All these methods present advantages and drawbacks and could be compared depending on their output, i.e., from community description to functional understanding (Figure A-5). Nevertheless, a microbiota taxonomic composition is crucial to identify specific microorganisms involved in disease suppressiveness, and not all the methods are directly useful for that. Some methods must be coupled with another to allow identification. For example, denaturing gradient gel electrophoresis (DGGE) need a subsequent sequencing step for microorganisms identification (Muyzer Gerard, 1999). Most use culture-independent methods for identifying taxa associated with soil disease suppression are 16S/ITS amplicon sequencing, 16S/ITS microarray, 16S/ITS DGGE + sequencing (Expósito et al., 2017). The first thing is microbiota composition description but the second is to discriminate which specific microorganisms are responsible of disease suppression. To do so, the easiest way is probably to extrapolate a taxonomical composition to suppressive functions found in literature, such as in the aquaponic study of Eck et al. (2019). However, this strategy does not allow to identify novel biocontrol agents and could not be verified without deeper analysis. Specific microorganisms discrimination consequently need a comparison between two microbiota with contrasting level of suppressiveness (Borneman and Becker, 2007).

The most current comparison is between a pathogen suppressive and a pathogen conducive biotope. The microbiota could also be artificially modified by physical, biological or chemical additions to allow comparison between suppressiveness levels (Borneman and Becker, 2007). Time and sampling site location could also induce different levels of suppressiveness (Borneman and Becker, 2007). For example, the rhizosphere of a plant could be pathogen suppressive at a physiological stage but not to another. Microbial diversity in aquaponic rhizoplane could be linked to *P. aphanidermatum* suppressiveness but not in endosphere (Stouvenakers et al., 2020). The Figure A-6 illustrate in a simplified manner how microbial composition comparison can help to identify suppressive microorganisms. Because of their higher relative abundance in the pathogen suppressive biotope B, bacteria 4 and 5 seem related to disease suppressiveness (Figure A-6). However, high relative abundance of a taxa in a microbiota is not a guarantee of biocontrol action and *vice versa*. Identification should be strengthened by correlation or multivariate analysis integrating other parameters such as plant disease symptoms. Take into account physico-chemical, chemical or biological parameters of the biotope in the study is also important to distinguish if the suppressiveness is linked, partially linked or not to microbiota (Corato et al., 2019; Ros et al., 2019). EC, pH, chemicals, or organic substances can act directly or indirectly on the pathogen and on microbiota composition and functions (Bonanomi et al., 2007; Hadar and Papadopoulou, 2012).

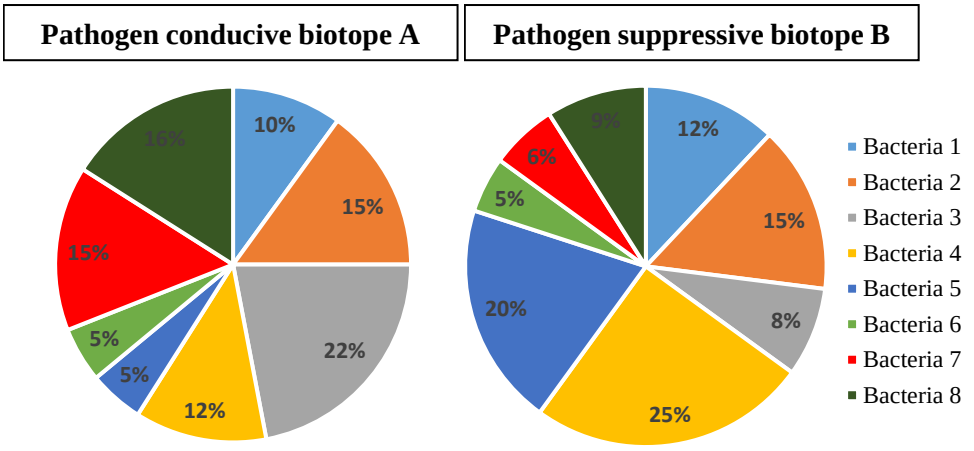


Figure A-6: Pie charts of bacterial relative abundance of two simplified theoretical biotopes composed each of the same eight bacteria. The biotope A is pathogen conducive and the B is pathogen suppressive. Personal representation.

Moreover, several approaches could be conducted in addition to metataxonomic (microorganisms identification with metagenomic techniques) to better understand

the microbial mechanisms involved in disease suppression and strengthen specific microorganism identification. In omic area, these additional approaches could be functional metagenomic, metatranscriptomic, metaproteomic, metabolomic and network analysis by bioinformatics (Expósito et al., 2017). For example, Chapelle et al. (2016) found with a metagenomic and metatranscriptomic approach that Oxalobacteraceae, Burkholderiaceae, Sphingobacteriaceae and Sphingomonadaceae families were linked to *Rizoctonia solani* suppression in sugar beet rhizosphere and that stress-related genes were upregulated after pathogen infection for these families. In Mehta et al. (2016), microbiota composition and macrocarpal, a secondary metabolite, were found associated to *R. solani* suppression in soil.

Whatever the culture-independent method used to identify microorganisms responsible of disease specific suppressiveness, the following logical step, according to the definition, is to transfer the suppressive activity to other biotopes. For that matter, two solutions are possible. Managing biotope parameters and/or content to promote specific microorganisms development, or inoculating specific microorganisms to the biotope to make it disease suppressive (Borneman and Becker, 2007; Weller et al., 2002). To apply the second solution, identified microorganisms must be isolated first. However, isolation of microorganisms identified by culture-independent methods is not ensured (Forbes et al., 2017; Garbeva et al., 2004; Stefani et al., 2015). First of all, metataxonomic methods and bioinformatics have their own bias that can introduce false identification (Forbes et al., 2017; Krakat et al., 2017) and then, false isolation. Identified microorganisms are not always viable in their initial environment (amplicon-based methods can detect dead microorganisms) or culturable in artificial media. Some microorganisms could be isolated easily and in quantity but the opposite too (Davis et al., 2005). Microbiota composition gave by metagenomic studies is not always deep enough to result in a species identification level. Moreover, once a target microorganism isolated, its ability to control plant pathogens must be verified. Indeed, a microbiota can contain distinct microorganisms for a same taxonomical level. Microbial isolates could have contrasting abilities for a same taxonomical level. For example, *Fusarium oxysporum* is often considered as a plant pathogen but some strains are pathogen antagonists, e.g., in lettuce (Thongkamngam and Jaenaksorn, 2017; Whipps, 2001). *Pythium* genus contains plant pathogen species but also beneficial one (Sankaranarayanan and Amaresan, 2020). For example, *P. oligandrum* can control *P. aphanidermatum* damping-off on soybean (Sankaranarayanan and Amaresan, 2020). Furthermore, biocontrol activity of a same microorganism can vary in time and with the growing environment, making the biocontrol validation difficult (Köhl et al., 2019).

2.4. Toward microbial consortia application for biocontrol

In Section 2., but also in literature, an important focus was made on the development of individual specific suppressive strains for controlling soil-borne diseases. The

initial idea was to transfer the suppressiveness of a biotope to another by a single biocontrol agent inoculation. However, soil suppressiveness is usually a complex process that cannot be summarized to the presence of one specific microorganism but rather needs to consider microbial communities interactions (Whipps, 2001; Massart et al., 2015; Expósito et al., 2017). Interactions are multiple and all components (biotic or abiotic) of the biotope might have a role (direct or indirect) in disease suppression (Ros et al., 2019; Whipps, 2001). Because of the diversity of soils and agricultural ecosystems, activity (e.g., antagonistic activity) of a single biocontrol agents in field could be variable and dependent on the environment (Niu et al., 2020; Pandey et al., 2012). The susceptibility to a biocontrol agent to get variable efficacy depending of its environment could be *inter alia* linked to the mode of action used by the microorganism (Köhl et al., 2019). For example, competition for nutrients and niches depend more on the environment than other mechanisms (Köhl et al., 2019). The application of a consortium of microorganisms was then proposed to solve the problem of variability in disease biocontrol. Biocontrol agents consortium is defined as the mixture of two or more microorganisms to improve stability and efficacy of a biocontrol action (Gopal, et al., 2013; Niu et al., 2020). Consortia can be composed by mixing biocontrol agents but also by the addition of helper strains to biocontrol agents. Helper strains do not have biocontrol action by their own but help biocontrol agents to improve their efficacy (Massart et al., 2015). Numerous studies have reported a better disease control with consortia application (Niu et al., 2020). Raison of consortia efficacity improvement on disease control can be explained by the addition of different features (Niu et al., 2020). In comparison to a single biocontrol agent application, a consortium to control soil-borne disease can (Niu et al., 2020):

- Improve antibiosis action. I.e., antimicrobial compounds production can be up regulated in a consortium and new compounds can emerge because of microbe – microbe interaction.
- Improve plant elicitation. I.e., elicitors are multiple and then the plant defense can be triggered by different pathways and express different ways of defense.
- Compete more efficiently, faster and for a broader range of nutrients and niches.
- Have a better stability but also a better adaptation to the environment.
- Promote rhizosphere colonization by a better:
    - Biofilm formation. I.e., multiple taxa and synergistic biofilm formation probably allowed by cooperation and signalling between microorganisms and extracellular matrix deposition increase.
    - Microbial growth. I.e., by combining metabolic abilities of microbial agents to catabolize different substrates that allows a syntrophic utilization of nutrients.

    - Migration. I.e., migration of certain microorganisms in the environment can be passively improved by other. Hyphal growth or motile microorganism can be used as vector for non or less mobile microorganism forms (e.g., asexual fungal spores, non-motile bacteria).
- Multiplied antagonistic modes of action (e.g., competition, parasitism, antibiosis and elicitation). Or combined biocontrol action with plant biostimulation.
- Have a higher influence on residing microbial by a network interaction.
- Protect plants against a broader range of plant pathogens.

To combine microbial agent in a biocontrol consortium, two approaches can be distinguished. The first is empirical and the second is based on synthetic communities (Niu et al., 2020). In empirical approach, microorganisms are often combined depending on their individual biocontrol activity. But criteria to select and combined microbial properties are still not standardized (Sarma et al., 2015). Before *in situ* application it is therefore advised to study compatibility of selected microorganisms, for example by *in vitro* co-cultivation (Niu et al., 2020). Indeed, microorganisms can interfere each other and produce detrimental effect or do not give additional biocontrol effect (Martin and Loper, 1999; Sarma et al., 2015; Whipps, 2001). Increasing taxonomic diversity is also a way to strengthen the establishment and the efficacy of the consortia in an specific environment (Niu et al., 2020). The second approach is to constitute a biocontrol consortium based on microorganisms and functions observed in a specific microbiome by a reductionist synthetic community designing (Liu et al., 2019; de Souza et al., 2020). The idea is then to mimic natural microorganism composition and interactions to transfer suppressiveness traits to the consortium.

Although biocontrol consortia have shown their utilities in soil-borne disease control, their commercial valorisation stay problematic. Mass production, formulation optimization and storage of a consortium ask more resources and research in comparison to a single biocontrol agent (Köhl et al., 2019). Furthermore, registration of a plant protection product is EU-regulated and individual approval must be carried out for each active substance, subsequently for each microorganism strain of the consortium (Köhl et al., 2019).

Chapter B

Thesis Aims

The **main aim** of the thesis was to evaluate the suppressive activity of aquaponic systems against plant pathogens (Chapter D).

Several key elements were first determined or developped:

- The pathosystem *P. aphanidermatum* – lettuce was selected for its pertinence in aquaponics (Chapter A-2).
- A reliable method of lettuce root microbiota harvest was developped as well as a protocol for lettuce infection by the pathogen (Chapter C).
- The Paff Box aquaponic system from Gembloux Agro-Bio tech at Uliege University in Belgium was selected as the source of aquaponic water and microbiota.
- Aquaponic suppressiveness was compared with hydroponic one.

Intrinsically, the **first sub-objective** was to determine the origin of aquaponic suppressiveness, i.e., beneficial compounds or beneficial microorganisms (Chapter D). Then the **second sub-objective** was to describe the composition and the diversity of the microbiota potentially linked to aquaponic suppressiveness (Chapter D). The **last sub-objective** was to isolate suppressive microorganisms from aquaponics and to evaluate their biocontrol activity alone or in a consortium against *P. aphanidermatum* diseases of lettuce (Chapter E and F).

Chapter C

Methodology Foundations

1. Standardization of plant microbiome studies: Which proportion of the microbiota is really harvested?

The material presented in Section 1 is adapted* from:

Sare, A.R.[+], **Stouvenakers, G.[+]**, Eck, M., Lampens, A., Goormachtig, S., Jijakli, M.H., Massart, S., 2020. Standardization of plant microbiome studies: Which proportion of the microbiota is really harvested? Microorganisms 8, 17. https://doi.org/10.3390/microorganisms8030342

*Only the matter of lettuce root microbiome was addressed in this section. Apple carposphere matter was removed but can be found in the original paper.

[+]These authors contributed equally to this work.

Abstract: Studies in plant-microbiome currently use diverse protocols, making their comparison difficult and biased. Research in human microbiome had similar challenges, but the scientific community proposed various recommendations which could also be applied to phytobiome studies. In the publication, we addressed the isolation of plant microbiota through apple carposphere and lettuce root microbiome while this section only focuses on the results obtained from lettuce roots. We demonstrated that the fraction of the culturable epiphytic microbiota harvested by a single wash might only represent half of the residing microbiota harvested after four successive washes. In addition, we observed important variability between the efficiency of washing protocols (i.e., 1.9-fold difference). QIIME2 analysis of 16S rRNA genes, showed a significant difference of the beta diversity between protocols. However, differences between protocols disappeared when sequences of the four washes were pooled. For a same protocol, a single wash was found enough to harvest a representative sample of the total root microbiota in terms of microbial richness and diversity. These results underline the interest of repeated washing to leverage abundance of microbial cells harvested from plant epiphytic microbiota whatever the washing protocols, thus minimizing bias.

Keywords: plant microbiome; bias; harvesting protocol; standardization; lettuce roots

1.1. Introduction

Plant tissues provide several niches for microbial growth and a rough distinction can be made between the aboveground plant organs, referred to as phyllosphere and the belowground microbial niches: the rhizosphere (the soil directly surrounding plant roots from which the physicochemical properties are influenced by the root); the rhizoplane (the root surface) and the root endosphere (the compartment formed by the apoplastic spaces between the root cells). Just as below-ground, the phyllosphere also comprises different compartments: the caulosphere, formed by the stems, the phylloplane, i.e., the leaf surface (with preferred habitats near nutrient rich specialized structures such as trichomes, stomata and veins), the anthosphere, i.e., the compartment formed by the flowers, the carposphere, i.e., the habitat created by the fruits and the spermosphere, shaped by the seeds.

Our current definitions of the different phyllosphere or root-associated communities are constrained by technical limitations (i.e., incomplete microbiome separation). The strength of the interaction of microbial cells with plants greatly varies within any single phytobiome. When considering the root system from the outside to the inside, the microbial diversity decreases while the degree of specialization and the strength of attachment and interaction increases (Reinhold-Hurek et al., 2015). Inhabitants of the rhizosphere exhibit several features enabling them to colonize the root system (Lugtenberg and Kamilova, 2009). It should be stressed that the diversity and the density of the bacterial community significantly varies between different regions along the root system (Compant et al. 2010). Moreover, differentiation between rhizosphere and rhizoplane is unclear regarding the continuum of microbial population variation from outside to inside the roots and is subject to variability between studies.

Plant surfaces harbour very diverse and abundant bacterial and fungal communities that provide specific functions and traits. Consequently, these communities are considered as a key factor for plant growth and health (Massart et al. 2015; Vorholt 2012). In recent years, and thanks to High Throughput Sequencing (HTS) technologies, increasing attention has been paid to the understanding of the relationship between the plants and their microbial communities.

Any microbiome study, also called phytobiome for plants, using HTS can include methodological biases at each step of the analysis, i.e., during: (i) microbiota harvesting, (ii) sample storage/preservation, (iii) sample preparation (DNA extraction and library preparation), (iv) sequencing, (v) bioinformatics analysis and (vi) data repository and experiment documentation in databases (Izard, 2014; Pollock et al., 2018; Boers et al., 2019). In order to efficiently use the increasing resources devoted to phytobiome studies, it is therefore of prime importance to pay careful attention to these methodological biases (Barillot et al. 2013; Knief 2014; Tian et al. 2017; Song et al., 2018). Nevertheless, one bias of plant microbiome study has not been studied

so far: the efficiency of the microbiota harvesting method and its effect on downstream molecular analyses. As shown in two papers on rhizosphere and rhizoplane microbiota sampling (Kloepper and Beauchamp 1992; Barillot et al. 2013), there is currently a large diversity of protocol to harvest epiphytic microorganisms. Some authors suggested the use of standardized protocols, even before the advent of HTS (Donegan et al. 1991; Barillot et al. 2013). International methods or standards of analysis (i.e., AOAC and ISO) exist but are related to specific microorganisms studies such as human pathogens detection for food safety. Only a few papers compare protocols efficiency such as made by Richter-heitmann et al. (2016) on rice root microbiota and Donegan et al. (1991) for *Enterobacter cloacae* recovering on bean leaves. A large diversity of protocols has been published so far to harvest epiphytic microbiota and Table C-1 illustrates the diversity of protocols for lettuce (4 protocols). Furthermore, a single washing step is commonly carried out without evaluating which fraction of the microbiota is really harvested and its representativeness of the whole community. The proportion of the microbiota which is harvested is barely ever mentioned whilst it is of utmost importance as it can generate quantitative or qualitative bias in the data interpretation during the downstream molecular analysis and can hamper comparison of results between studies. Therefore, in order to assess the impact of these parameters, we evaluated the effect of successive washes (with the same method and on the same plant sample) and of different washing protocols on the composition and quantity of microbiota harvested from lettuce roots.

Table C-1: Diversity of protocol for harvesting microbiomes from lettuce roots

Host	Buffer	Technic use	Purpose	Reference
Lettuce in soil	Sterile saline water	Shaking by rotary shaker at 307 rpm with glass bead for 1h	PCR-DGGE,	Grosch et al., 2012
	Sterile 0.9% NaCl + 0.02% Silwet L-77 solution	Vortexing twice for 15s	Plating	Bonaldi et al., 2015
Lettuce in aquaponics	Sterile ultrapure water	Vortexing for 2 min followed by 5 min ultrasonic bath	Illumina sequencing	Zala Schmautz et al., 2017
	Sterile peptone phosphate buffer (1g peptone + 1.21 g K_2HPO_4 + 0.34 g KH_2PO_4 + 1-liter deionized water	Crushing with a Tissue Lyser	T-RFLP	Mangmang et al., 2014a

1.2. Materials and methods

1.2.1. Microbiota recovery

Lettuce seedling of 11 days old (var. Grosse Blonde Paresseuse, Semailles, Belgium) were grown in an aquaponic system (described by Delaide et al., 2017) coupling Nile tilapia (*Oreochromis niloticus* L.) farming and hydroponic crop cultivation. Root samples were taken one month later at the morphological stage of 34 leaves. Root samples were washed following the four different protocols described in Table C-2. Two protocols were found in the literature; i.e., root shaking with isotonic water (NaCl) (Chave et al., 2008) or with $(NaPO_3)_6$+peptone (Rosberg et al., 2014). The two others were developed in our laboratory and consisted of root shaking (KPBT Sh) or sonication (KPBT So) in KPBT buffer. For each protocol, 2 g of roots coming from the same lettuce were collected and washed four times successively. Between each successive wash, roots were rinsed by vortexing in 10 mL of the corresponding buffer (5 mL for the NaCl protocol) and the rinsing solution was added to the previous washing to make sure that all the microorganisms were correctly gathered in the washing Falcon. Then, root washing waters were filtered through sterile cheesecloth to discard root debris.

Table C-2: Methods description of lettuce rhizoplane harvesting.

Treatment label	**(NaPO3)6 + peptone**	**NaCl**	**KPBT Sh**	**KPBT So**
Material	2 g of roots	2 g of roots	2 g of roots	2 g of roots
Solution	30 mL of a 2g/l (NaPO3)6 + 1g/L peptone sterile solution	5 mL of isotonic sterile water (0,85% NaCl)	30 mL of a sterile 0.05M potassium phosphate buffer + 0.05% tween80 pH 6.5	30 mL of a sterile 0.05M potassium phosphate buffer + 0.05% tween80 pH 6.5
Treatment 1	Shaking	Shaking	Shaking	Ultra-bath sonication
Duration 1	20 min	10 min	20 min	10 min
Intensity 1	200 rpm	150 rpm	150 rpm	-
Reference	Rosberg et al., 2014	Chave et al., 2008	Intern protocol	Intern protocol

1.2.2. Cultivable microbiota enumeration

Lettuce roots washes (collected twice independently from two different washing dates) were serial diluted (1:10 and 1:100) and plated in triplicate by addition of 100 µL of each successive wash suspension per petri dish. Plate media used were Luria-

Bertani agar medium (LB, 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar in 1 liter). Colony-forming unit (CFU) enumeration was achieved after Petri dishes incubation at 23±2 °C with 16/8 photoperiod for three days. CFU concentrations were calculated by gram of root.

1.2.3. Bacterial microbiota composition analysis by 16S rRNA gene

To gain a better understanding of the effect of the washing method and successive washes on the bacterial composition of the microbiota, the two protocols harvesting the maximum amount of CFU after plating were selected for HTS of 16S rRNA gene. Lettuce rhizoplane microbiota harvest was conducted in duplicate, i.e., in two different lettuce plant, and collected twice independently from two different washing dates with KPBT Sh and So protocols. The obtained microbiota was concentrated to a pellet by centrifugation at 2,350g for 20 minutes. DNA extractions for each of the four successive washes per protocol were performed by using the FastDNA spin kit (MP Biomedical) according to manufacturer's instructions. The Illumina MiSeq library preparation, sequencing and the quality filtering were performed at DNAVision (Gosselies, Belgium) in two different runs (2 x 300 nt for apple and 2 x250 nt for lettuce roots). The V1-V3 region was targeted using the Forward 27F and Reverse 534R Illumina primers used by Eck et al. (2019) in an aquaponic systems study.

Reads were demultiplexed and primers were trimmed at the sequencing center and obtained paired-end FASTQ sequences were analyzed with QIIME 2 (q2) version 2019-4 (Bolyen et al., 2019). Quality control and feature table construction was conducted with the q2 DADA2 method without trimming the sequences (Callahan et al., 2016). Features were classified with the q2 implemented VSEARCH method in the q2 feature-classifier plugin. SILVA_132 at 99% of sequence similarity was used as reference database for the taxonomy. Cytoplasmic contaminations (chloroplast and mitochondria sequences) were discarded with the q2 taxa filter-table script. The q2-diversity core-metrics-phylogenetic plug-in was then used to obtain ecological diversities (alpha and beta) information and for the comparison between the protocols and between the successive four washes. The diversity core-metrics were run on feature table rarefied at 19,986. PERMANOVA (9990 permutations) Kruskal-Wallis test was used to compare alpha diversity indexes (Observed OTUs, Faith Phylogenetic Diversity (Faith PD), Shannon and Pielou's Evenness), and beta diversity indexes (Weighted Unifrac distance metrics) were compared by the PERMANOVA (9990 permutations) pseudo-F test. Each PERMANOVA p-value was automatically corrected in QIIME 2 for multiple analysis of variance. Pseudo count of one were added to the feature tables and the q-2 ANCOM plug-in (Mandal et al., 2015) was used to compare differentially abundant features among washes and between the different protocols. Additionally, sequences of the four successive washes were pooled for each protocol and each repetition. Feature tables were then normalized by

rarefaction at 19,986. Then, diversity and ANCOM analysis as described above, were used to compare the protocols between them and also, the first single wash and the pool of the four washes.

Sequences are available on the National Center for Biotechnology Information (NCBI) under the sequence read archive accessions PRJNA592958.

1.3. Results

1.3.1. Lettuce rhizoplane microbiota

Cultivable microbiota enumeration

The results of the average cumulative number of CFU harvested by each protocol following successive washes are illustrated in Figure C-1. CFU enumeration showed that the first wash represented the major part of the microbiota recovered. Indeed, the first wash harvested respectively, 64.3%; 53.2%; 64.8% and 38.2% of the total microbiota (considered as the sum of the four successive washes) for KPBT So, KPBT Si, $(NaPO_3)_6$+peptone and NaCl method respectively. Regardless of the number of washes, KPBT So was the most efficient protocol. For example, KPBT So harvested at least twice as much bacteria compared to $(NaPO_3)_6$+peptone. After the fourth wash, the increase in harvested CFU ranged between 37 and 157 % compared to the first wash. An increase of 61 % was observed with KPBT So.

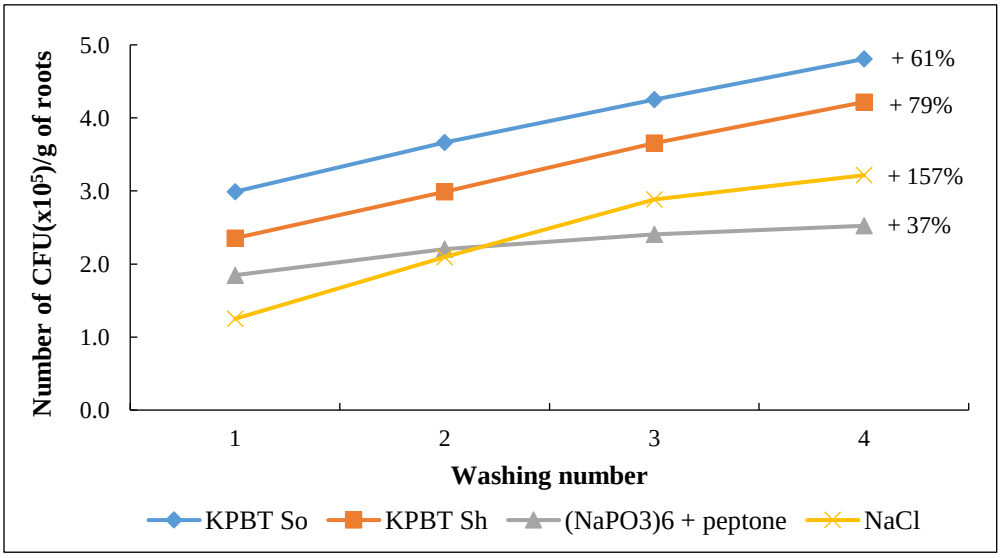


Figure C-1: Mean of the cumulative number of CFU per gram of roots for bacteria counted in the four successive washes for each protocol (KPBT So, KPBT Sh, $(NaPO_3)_6$ + Peptone and NaCl). The percentages on each graph represent the percentage increase of the sum of the four washes compared with the first.

Bacterial diversity analysis by 16s rRNA gene

The Illumina sequencing generated raw reads of an average Phred Q30 of 75.5 %. Due to an important loss of reads at the merging step of the analysis, only the forward reads were kept for analysis. The full quality control summary is available in interactive view (Figure C-S1). After the OTUs table cleaning, 1.9% of reads were unassigned in QIIME 2. Data were rarefied at 17,463 sequences per sample (individual and pooled samples) for the downstream analysis.

Table C-3: Alpha diversity comparison (pairwise Kruskal-Wallis q-value) between the protocols, between successive washes before pooling of washes and after the pooling of washes of lettuce rhizoplane; ns = not statistically significant; pd = phylogenetic diversity; Sh = shaking; So = sonication.

Comparison of non-pooled washes (rarefied at 17,463 sequences); all the successive washes were pairwise compared				
	Faith_pd	**Pielou_eveness**	**Observed_otus**	**Shannon**
KPBT Sh	106	0.73	764	6.99
KPBT So	85	0.73	622	6,75
Pairwise comparison between KPBT Sh and So	0.037	ns	0.028	ns
First wash	100	0.76	750	7.19
Second wash	98	0.73	780	6.96
Third wash	88	0.73	604	6.77
Fourth wash	100	0.71	670	6.65
Pairwise comparison (six) between the successive washes	ns for all	ns for all	ns for all	ns for all
Comparison of pooled washes (rarefied at 17,463 sequences)				
	Faith_pd	**Pielou_eveness**	**Observed_otus**	**Shannon**
KPBT Sh	120	0.73	925	7.2
KPBT So	80	0.72	609	6.95
Pairwise comparison between KPBT Sh and So	ns	ns	ns	ns
First wash	98	0.76	735	7.2

Pooled of the four washes	**116**	**0.72**	**885**	**7.2**
Comparisons between the first wash and the pooled of the four successive washes	ns	ns	ns	ns

Significant differences of alpha-diversity with Observed_Otus (q-value = 0.028) and faith-pd (q-value = 0.037) indexes were observed between the washing methods, i.e., KPBT with shaking or sonication (Table C-3). The same difference was highlighted with the β-diversity analysis (q-value = 0.018 for Weighted Unirac distance metrics) illustrated in Figure C-2. However, there was no significant difference of diversity when successive washes or pool of washes were compared between them or with the first wash respectively.

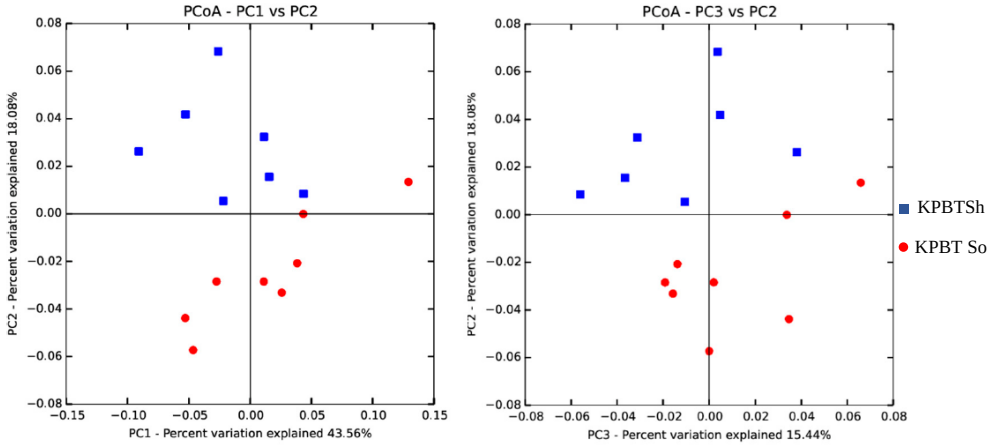


Figure C-2: Principal Coordinates Analysis (PCoA) plots of the weighted unifrac distance metrics by protocols (KPBT So and PBS So) of non-pooled washes. Each dot represents a successive wash. All samples were rarefied at 17,463 sequences.

Based on the QIIME 2 taxonomic assignation, the ANCOM analysis didn't reveal significant difference of relative abundance between methods, successive washes or between the first wash and 4 successive washes pooled. This is consistent with the relatively similar composition bar charts displayed on Figure C-3. All samples were dominated by the Burkholderiaceae family and the *Sphingobium* genus (Figure C-3).

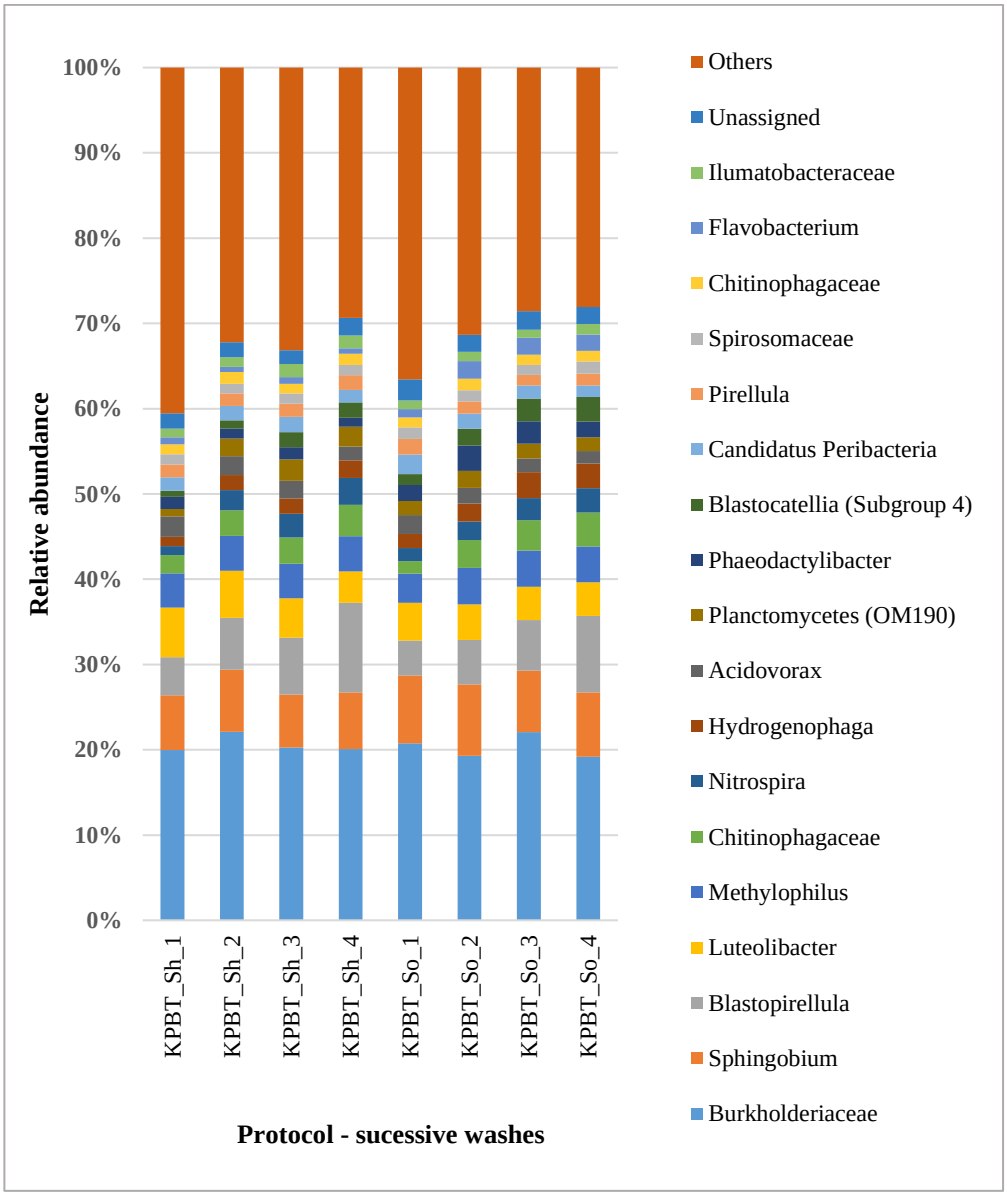


Figure C-3: Overview of the taxonomic profile at genera level of the metagenomic 16s rRNA gene analysis for lettuce rhizoplane obtained with QIIME 2. Each column represents all the detected genera of each of the four successive washes with KPBT So and KPBT Sh protocols. Each colour represents an OTU; Only the OTUs with high proportion (1% of the total reads) are presented and the rest are grouped as others. The numbers (from 1 to 4) associated to the protocols are successive washes.

1.4. Discussion

In the scientific history, any emerging concept relying on fast moving technologies has been prone to very important bias and errors at its infancy. The origin of bias was initially neglected in microbiome studies but gained more attention recently, including for phytobiome analysis. Over the years, the scientific community developed recommendations and best practices to improve the reliability of the HTS technologies to study plant microbial communities and to promote the comparativeness of the results. The most critical component when analysing the phytobiome is to ensure that the results are representative of the studied microbial community. In this context, phytobiome studies should directly benefit from standardization and recommendations which have been developed for the human microbiome. For instance, specific adaptations have been recommended such as the selection of primers (Hanshew et al., 2013), the concentration of DNA (Castle et al., 2018) or the sequencing technology (Song et al., 2018). Also bioinformatics "best practices" for microbiome HTS data analyses have been proposed (Knight et al., 2018) as well as recommendation for data storage and description.

In this paper, a potential bias never explored so far for phytobiome studies was studied: the impact of several successive washes on the harvested microbiota. Currently, there is a diversity of protocols available in the literature, even for a specific plant species and organ. The example presented in this paper focuses on lettuce rhizoplane, but the results warrant further investigation on the efficiency of harvesting strategies. To date, a systematic evaluation of microbiota harvesting efficiency and its potential effect on downstream molecular analyses is missing.

1.4.1. Impact of the protocol on the quantity and diversity of harvested microorganisms

Between studies found in the literature, protocols differ in washing buffers (water, phosphate, saline or phosphate saline), washing time, washing methodology [washing, grinding (therefore also including endophytes), shaking, sonication or a combination of them]. These variables might greatly impact the outcome of the experiments. For instance, with the grinding of tissues, the endophytes are also collected whilst this is not the case with a simple wash. Thus, it is difficult to compare the results from different publications. For root samples, rhizosphere microbiota is usually harvested by a simple wash to recover the microorganisms loosely attached to the root surface or which were contained in the soil surrounding the roots. While a more aggressive wash of "nude" root (without soil) as met in hydroponics through shaking, glass beads or ultra-sonication bath in a buffer is usually associated with the collection of the rhizoplane microbiota (Oh et al. 2012; Richter-heitmann et al. 2016). Furthermore, depending on the study, the distinction between rhizosphere and rhizoplane, their definitions or ways to harvest them are sometimes ambiguous (Kloepper and Beauchamp, 1992), especially in the case of plants grown in soilless systems.

The results of the plating of lettuce rhizoplane culturable microorganisms showed a huge variability of the number of cells recovered using the tested protocols. Furthermore, it was observed that sonication increases the microbial recovery for the tested protocols. These results are in accordance with the differences observed in other studies on culturable microorganisms (Kloepper et al., 1991; Richter-heitmann et al., 2016). Richter-heitmann et al. (2016) found the same trend for rice, clover and bean roots rhizoplane microbiota collection. Their results showed that only 45% of the rhizoplane microorganisms were harvested by vigorous washing, and that additional sonication process increased the detachment up to 78%. In our study, it was also noticed the efficiency of root sonication to harvest an increased quantity of microbial cells.

Results on culturable microorganisms were confirmed by the 16s rRNA genes HTS analysis. First, the beta diversity analysis showed that the harvesting methods significantly influenced the composition of the harvested bacterial community. This may then introduce bias between HTS studies comparison. Secondly, the measured alpha diversity indices (observed_otus and faith_pd) also confirmed a significant difference in the diversity of the harvested bacterial communities between washing protocols. With regard to Richter-heitmann et al. (2016), they did not observe differences with community fingerprinting of 16S rRNA genes by T-RFLP between washing methods on rice. However, they qualify these findings by proving that root morphological parameters strongly influence the efficiency of the washing method by testing other plant roots (bean and clover).

1.4.2. Impact of successive washes on the quantity and diversity of harvested microorganisms

The compositions of the successive washes were compared thanks to HTS tools. No qualitative differences were found amongst the successive washes for the same protocol. Indeed, the ANCOM test did not highlight any OTU which could be present in different abundances between the successive washes. This result indicates that for the same protocol, single washes can be fairly compared by 16S rRNA gene HTS analysis. However, concerning the quantity of microorganisms collected, the first wash of lettuce roots microbiota harvested less than half of the microorganisms collected after four successive washes, whatever the protocol. Taxa detected in this study were generally consistent with literature (Schmautz et al., 2017) until the class level but started to differ at a more precise taxonomic level. However, even with similar hypervariable regions (V1–V3), the alpha-diversity indices obtained in this study were much higher than the ones found in Schmautz et al. (2017).

This study has shown that pooling the successive washes allowed to increase the amount of culturable microorganisms with variable yields. Interestingly, the alpha- and beta-diversity between protocols were not significantly different anymore when the sequences of the four successive washes were pooled together. Therefore, pooling

several outwashes appears to allow fair comparison between studies using different protocols.

In addition, through the concentration effect of the quantity of microorganisms harvested with the successive washes pooled, it has the potential to limit bias linked to microbial DNA contaminations present in the extraction kits (Salter et al., 2014). Though only bacterial population were targeted in the 16S rRNA gene analysis, similar results need to be confirmed for fungal populations. Thus, there is also a need to pay attention to these parameters in further studies when harvesting microbial cells from plants.

1.5. Conclusion

This study cases indicate that the washing protocol significantly influences the quantity and the bacterial diversity of microorganisms harvested and that four successive washes can increase quantity of harvested microorganisms. In the tested conditions, the washing protocol significantly influenced bacterial beta diversity. There were no significant differences in bacterial alpha and beta diversity and OTU abundances from lettuce roots between washing protocols after pooling the four successive washes together, potentially indicating that each protocol repeated four times harvested nearly completely the rhizoplane microbiota. However, diversity of a single wash was not found different from pool of washes. Based on these results and the literature, we therefore recommend to carefully evaluate the opportunity to wash each sample several times in order to harvest plant epiphytic microbiota with limited bias. Such evaluation is an important, although currently neglected step toward a better comparability between phytobiome studies.

2. Lettuce infection by *P. aphanidermatum*

2.1. Lettuce infection

Pythium spp. are frequently detected in irrigation and hydroponic water (Utkhede, Lévesque and Dinh, 2000; Hong and Moorman, 2005; Alhussaen, 2006) and symptoms of *P. aphanidermatum* disease can be observed while less than 1 zoospore or 1 CFU per millilitre is detected in hydroponic systems producing lettuce (Utkhede, Lévesque and Dinh, 2000) or cucumber (Postma et al., 2001). However, the use of *Pythium* for experimentation could be more challenging than assumed. In literature, an important variability of *P. aphanidermatum* plant infection protocols is observed and the protocol should be adapted to each case (e.g., *Pythium* strain, plant host and plant growth system). In our understanding, the key points to reach measurable and reproducible symptoms are the pathogen inoculum and inoculation form, but also the environmental conditions. Temperature was the main factor affecting the development of measurable symptoms by *P. aphanidermatum* on soilless lettuce in artificial conditions (Sutton et al., 2006). For this study, a long time was spent to improve protocols of lettuce infection by *P. aphanidermatum* in soilless artificial conditions. Protocols optimisation and key advances gained to achieve lettuce disease with this pathogen are described in this section.

2.1.1. *P. aphanidermatum* inoculum production

For this thesis, the different forms of *P. aphanidermatum* inocula and how to produce them were studied. In literature, the two main forms of *P. aphanidermatum* inoculum used for plant inoculation are zoospores and propagules (mix of mycelium and reproduction structures). Zoospores are the natural mobile form of pathogen dispersion, consequently it was logical to focus on this strategy first. Zoospores have also the advantage to be easier to enumerate than propagules. In contrast, propagules of *P. aphanidermatum* are easier to produce but must be plated to enumerate viable structures. Another possibility of inoculum form, but less used in literature, is oogonia or oospores. Oogonia that become oospores after fecundation are the sexual form of conservation of the pathogen and could be at the origin of plant culture system infection. Oogonia/oospores production as inoculum source has different advantages. With their size of ± 10 µm, they are easier to count and allow a more reliable enumerating on haemocytometer than zoospores that are smaller and mobile. Oospores suspension is more homogenous that propagules suspension and is consequently more reliable when pipetting it. Their shelf live is longer (i.e., several months to one year) because they are thick-walled survival structure produced to preserve the pathogen to harsh environmental conditions (Martin and Loper, 1999). Importantly, the strain of *P. aphanidermatum* used in this study showed facilities to produce this sporulation form. In the literature, this form of *P. aphanidermatum* inoculum is often under-exploited and rather used to test different antagonistic

microorganisms, products or parameters for direct inhibition of oospores germination in laboratories (e.g., Johnson, 1988; El-Tarabily, 2006; Halo, Al-Yahyai and Al-Sadi, 2018; Elshahawy and El-Mohamedy, 2019; Halo et al., 2019). Table C-4 shows the protocols tested to produce inocula of *P.* aphanidermatum strain CBS 132490. Strain CBS 132490 was isolated from a diseased hydroponic cucumber system. The strain was deposited by A. Lévesque and used in several studies (Lévesque and De Cock, 2004; Adhikari et al., 2013) including biocontrol development in hydroponic systems growing lettuce (Utkhede, Lévesque and Dinh, 2000). For all tested protocols (Table C-4), the first step consisted in mycelium growth in solid or liquid culture at mesophilic temperatures. The second step aimed at initiating the production of sexual or asexual structures by applying stress conditions that often correspond to mycelium mat rinsing or incubation in distilled water. If the objective is to produce mycelium propagules and not reproductive structures, this second step is not carried out. To produce propagules and oospores, an additional step of blender is added to split produced structures. The final step is to harvest the suspension, filter it through cheesecloth (if needed) and enumerate inoculum structures on haemocytometer or in culture plates. In our experiment, the solution used to harvest inoculum structures was distilled water with sucrose and Tween 20. Tween was used to allow a better homogenization of the suspension and sucrose to allow a better germination of reproduction structures. If fact, it was determined that sucrose help taxis and germination of zoospores, and that sucrose is required for a better disease development (Johnson et al., 1981). Another adjuvant often used to increase oospores production and maturation in liquid culture of *Pythium* spp. is sterols (e.g., cholesterol) (Hendrix and Campbell, 1973) but it was not tested in our experiments. In clarified V8 juice + CaCO3 grow for 11 days, the addition of 30 mg/L of cholesterol had doubled the number of *P. aphanidermatum* oospores and increase their percentage of maturity by 31% (Johnson et al., 1981).

Result of tested protocols are shown in Table C-4. Propagules were the easiest form of inoculum to produce in comparison with zoospores and oospores. It was also observed that the strain CBS 132490 tended to initiate sexual reproduction rather than asexual reproduction. Consequently, oospores were easier to initiate and then to produce than zoospores. To produce zoospores inoculum, it was found that introduce a plant host in the process was necessary to increase sporangia production and zoospores release.

Table C-4: Tested protocols steps and results to produce *P. aphanidermatum* disease inoculum depending on the strategy use. CMA is corn meal agar medium (Merck Millipore), and PDA is potatoes dextrose agar medium (Merck Millipore).

Strategy of *P. aphanidermatum* inoculum production and references		**Mycelial growth**	**Propagules differentiation and harvest**	**Personal results and observations**
Zoospores release by flooding after solid growth	Rahimian and Banihashemi, 1982; Paulitz, Zhou and Rankin, 1992; Wulff et al., 1998; Chatterton, Sutton and Boland, 2004	Growth for 3 days on V8 juice agar with lighting at 25°C. Variants tried: Growth in CMA, PDA, or V8 juice agar with 3 g/L CaCO3	Successive flooding periods of culture plugs in 25 mL sterile distilled water. First flood at room conditions for 30 min. Second overnight flood at 35°C with lighting followed by a last incubation step for 4 to 48h at 20°C with lighting. Suspension harvest through cheesecloth.	No or few reproduction structures in mycelium when V8 juice agar, CMA or PDA is used. Oogonia/oospores production in mycelium with V8 juice agar + 3g/L CaCO3. Suspicion of zoospores in the suspension when V8 juice agar + 3g/L CaCO3 is used but suspension plating gives less than 1 CFU/mL.
	Royle and Hickman, 1964	Growth for 7 days on V8 juice agar with lighting at 25°C.	Successive flooding periods of culture plugs in 25 mL sterile distilled water. First flood at room conditions for 15h and then two successive floods for 1h at room conditions. Suspension harvest through cheesecloth.	No or few reproduction structures in mycelium. No zoospore in the suspension.

Zoospores release by flooding after liquid growth	Heungens and Parke, 2000; Folman, Postma and Van Veen, 2003	Growth on 20 mL of V8 juice + 3g/L $CaCO_3$ broth for 9 days. Variants tried: - Light or dark - 23°C or 25°C	Thrice washing of mycelial mat with sterile distilled water. Add 20 mL of sterile distilled water and incubate at 27°C. Suspension harvest through cheesecloth. Variants tried: - 6h, 24h or 48h of incubation. - Light, light + 1 to 4h of UV light.	No or few sporangia in the mycelium. Oogonia/oospores production in mycelium. Zoospores suspicion in the suspension for UV treatments but whatever the variants, suspensions plating gives a maximum of 30 CFU/mL.
Zoospores release by flooding and grass taxis after solid growth	Protocol adaptation after personal communication with Rintoul Tara and Lévesque André from the Agriculture and Agri-Food Canada organization.	Growth for 3 days on PDA with lighting at 25°C.	On a Petri dish, put 3 autoclaved pieces of wheat leave and add a culture plug on each wheat brand. Flood the Petri dish with 15 mL of sterile distilled water. Let stand at 25°C for 48h in the dark. Suspension harvest through cheesecloth.	Production of sporangia and zoospores in mycelium. Zoospores suspension enumeration on haemocytometer gives an average of $3x10^5$ zoospores/mL.

Propagules production after liquid growth	Personal protocol used in Chapter D based on Utkhede, Lévesque and Dinh, 2000; Postma et al., 2009	Growth in 25 mL of clarified V8 juice + 3g/L CaCO3 broth for 6 days at 23°C with 18h/6h day/night photoperiod.	Rinse the mycelial mat with a vortex and shaking in 15 mL of 0,85% NaCl water sterile solution. Repeat the action minimum twice more until no broth residue could be found. Drain the mycelial mat on sterile paper towel and weight it. At a proportion of 1 mL per 5 mg of mycelium, mix for 8 x 3s the mat with a hand blender in 10 mM sucrose + 0.05% Tween 20 water sterile solution.	Plating of the propagules suspension gives an average of $5.33x10^3$ cfu/mL.
Oospores production after liquid growth	Personal protocol used in Chapter E and F	Growth in 20 mL clarified V8 juice + 3g/L CaCO3 broth for 9 days at 23°C with 18h/6h day/night photoperiod.	Rinse the mycelial mat with vortex and shaking in 15 mL of sterile distilled water. Repeat the action minimum twice more until no broth residue could be found. Cut the mat in two and incubate each half mat in 20 mL of sterile distilled water for 24h at 28°C with lighting. Mix, for 8 x 3s with hand blender, 5 mycelial mats in 100 mL of 10mM sucrose + 0.05% Tween 20 water sterile solution. Suspension harvest through cheesecloth.	Production of oogonium and oospores in mycelium. Antheridium and oogonium mating can be observed on mycelium. Oospores suspension enumeration on haemocytometer gives a mean of $1.25x10^4$ oospores/mL.

2.1.2. *P. aphanidermatum* inoculum form for lettuce infection

Different forms of *P. aphanidermatum* inocula can be produced, but which one is the most favourable to infect lettuce and then produce disease symptoms? In a preliminary experiment to Chapter D, it was shown that hydroponic lettuce grew in hydroponic conditions had slightly more severe root rot symptoms when propagules suspensions were used instead of zoospores suspension (Table C-5). Concentration of both inoculum forms was $1x10^5$ cells/lettuce. Consequently, propagules were used as inoculum form for lettuce infection in Chapter D.

Table C-5: Evaluation of the impact of inoculum form (propagules or zoospores at a concentration of $1x10^5$ cells/lettuce) of *P. aphanidermatum* on disease symptoms in lettuce.

Lettuce inoculum form	**Disease symptoms**		
	Fresh leaf mass mean (g)	**Fresh root mass mean (g)**	**Root rot rating mean (0 to 6)***
No inoculation (i.e., healthy control)	42.78	2.32	1.25
Inoculation with propagules	27.27	1.97	2.00
Inoculation with zoospores	27.56	2.41	1.25

* See Chapter D for root rot rating. 0: no symptom; 6: brown-black decaying or dead roots.

In preliminary experiments to Chapter E, *P. aphanidermatum* inoculum form was studied to achieve lettuce seed damping-off disease. Only propagules and oospores inoculum forms were tested. It was first determined that a minimum concentration of $5x10^3$ propagules/mL or $1x10^4$ oospores/mL was needed to obtain seed damping-off with 100 µl of inoculum per seed. Results showed that propagules and oospores were even efficient to produce the disease. It was also found in that preliminary work that organic pelleted lettuce seed of the Millennia RZ and Lucretia RZ variety (Rijk Zwaan, Merksem, Belgium) were more susceptible to pre-emergence damping-off than post-emergence. Because of the better oospores enumeration reliability, this form was chosen for the following experiments (Chapter E and F)

2.1.3. Environmental conditions

Lettuce root rot disease

Even if the *P. aphanidermatum* is present in lettuce environment, the plant can remain asymptomatic. Indeed, after its inoculation, *Pythium* could be saprophytic. Moreover, once the tissue colonization process started, low symptom levels can be observed during the latency stage or then because of the possible biotrophic stage of

the pathogen (see Chapter A, Section 2.1.). However, more severe root rot disease symptoms are needed to make reliable scientific experiments. These more severe symptoms are produced during the necrotrophic stage of the infection.

To achieve measurable disease symptoms in Chapter D, several growth conditions parameters were preliminary tested to increase lettuce infection by *P. aphanidermatum*. For a given nutrient solution (i.e., with identical physicochemical water quality such as pH, DO, EC and mineral nutrients), a given timing of pathogen inoculation, and a given hydroponic lettuce growing system, it was found that the key point to obtain the necrotrophic stage of *P. aphanidermatum* strain CBS 132490 on lettuce was the temperature. Results of preliminary experiments showed that set the day temperature at 28°C for 31 days after the ten first days of germination did not induce lettuce symptoms development. Setting the day temperature at 35°C for the last five days (i.e., after 26 days at 28°C) of the lettuce growth cycle allowed to initiate the root necrotrophic stage of the disease but root rot symptoms level remained low. Finally, the day temperature was set at 35°C for the last ten days of the lettuce growth cycle. This setting allowed exacerbating root rot symptoms until decaying roots and led to the development of consequence symptoms on foliar part. It was then possible to observe and then measure the foliar wilt and necrosis. The Table C-6 showed the difference of symptoms levels observed for a temperature set at 35°C for the last five days or the last ten days of the lettuce growth cycle. With ten days at 35°C, the lettuce foliar fresh mass decrease was almost twice more. This foliar fresh mass decrease can be partially explained by foliar wilting (i.e., foliar turgidity was decreased by 12.2%) that was not present with the duration of 5 days. For Chapter D, the temperature was then set at 35°C for the last ten days.

Table C-6: Comparison of *P. aphanidermatum* disease symptoms on hydroponic lettuce grew with a final stage at 35°C for 5 days or 10 days.

	5 days at 35°C	**10 days at 35°C**
Average foliar fresh mass decrease	36,3%	66.6%
Average relative foliar turgidity decrease	-0.7%	12.2%
Root rot rating mean (0 to 6)	2	6

See Chapter D for disease symptoms indexes calculation.

Lettuce damping-off disease

In preliminary experiments to Chapter E, temperature was also found to impact the severity of seed damping-off disease of lettuce. It was determined that lettuce seeds (var. Millennia RZ and Lucretia RZ) infection by *P. aphanidermatum* was helped by temperature increase above 25°C. However, temperatures of 25°C or more were also found to directly inhibit seed germination in absence of the pathogen. Consequently, temperature was set at 23°C in Chapter E to avoid germination problems. Pathogen

timing of application on the seed was also preliminary tested. At 23°C, pre-emergence damping-off occurred when the pathogen is inoculated at the same time as seed hydration. The more seeds time germination increased before inoculation, the more disease incidence decreased at 23°C. For example, three days of germination before *P. aphanidermatum* inoculation decrease disease incidence by ± 20%. If seeds are inoculated after cotyledon emergence (11 days after sawing) and the temperature is set at 23°C, no seedlings damping-off occurred (i.e., disease incidence = 0%). Consequently, to obtain lettuce *P. aphanidermatum* disease at seedling stage, the temperature was set at 35°C for 21 days after pathogen inoculation (i.e., 10 days after sowing). In comparison, a temperature set at 28°C produced slight symptoms but mortality was null.

2.1.4. *P. aphanidermatum* conservation

Another difficulty met with *P. aphanidermatum* was to keep its pathogenicity and its virulence. Indeed, successive plating led to a lesser sporulation capacity and mycelial growth variability in culture media. It was then access different ways to store the first subculture of the initial ordered strain for further swabs. Freeze of mycelium PDA culture plugs in glycerol mix at 20, 25 and 30% in distilled or saline solution and store at -20°C or -80°C led to the death of the pseudo-fungi. Keep the strain at 4°C also kill the culture after one month. The solution found was to grow the pseudo-fungus in glass tubes poured with PDA and then flood the tubes with mineral oil (i.e., heavy liquid paraffin oil). Tubes are then stored in dark and at room temperature. By this way, *P. aphanidermatum* growth is stabilized, and the culture stay viable for several years. The followed culture swabs give revival with fast mycelial growth (i.e., 3 days to full a 9 cm Petri PDA dish at 23°C) and the sporulation capacity is kept.

Chapter D

Microbial Origin of Aquaponic Water Suppressiveness against *Pythium aphanidermatum* Lettuce Root Rot Disease

The material presented in this chapter is adapted from:
Stouvenakers, G., Massart, S., Depireux, P., Haïssam Jijakli, M., 2020. Microbial origin of aquaponic water suppressiveness against *Pythium aphanidermatum* lettuce root rot disease. Microorganisms 8, 1–25. https://doi.org/10.3390/microorganisms8111683

Abstract: Aquaponic systems are an integrated way to produce fish and plants together with mutual benefits. Fish provide nutrients to plants on the one side, and plant nutrients uptake allow water reuse for fish on the other side. In this kind of system, the use of phytosanitary treatments to control plant pathogens is sensitive because of the risk of toxicity for fish present in the same water loop, especially coupled aquaponics. Among plant pathogens, *Pythium aphanidermatum* is a most problematic microorganism due to the Oomycete's capacity to produce mobile form of dispersion (zoospores) in the recirculated water. Therefore, this study aimed at elucidating the potential antagonistic capacity of aquaponic water against *P. aphanidermatum* diseases. It was shown that aquaponic water presented an inhibitory effect on *P. aphanidermatum* mycelial growth in *in vitro* conditions. The same result was observed when lettuce plants growing in aquaponic water were inoculated by the same plant pathogen. Aquaponic lettuce was then compared to lettuce grown in hydroponic water or complemented aquaponic water (aquaponic water plus mineral nutrients). The disease was suppressed in the presence of aquaponic water, contrary to lettuce grown in hydroponic water or complemented aquaponic water. Root microbiota were analyzed by 16S rDNA and ITS Illumina sequencing to determine the cause of this aquaponic suppressive action. It was determined that the diversity and the composition of the root microbiota were significantly correlated with the suppressive effect of aquaponic water. Several taxa identified by metabarcoding were suspected to be involved in this effect. Moreover, few of these microorganisms, at the genus level, are known to have an antagonistic effect against *P. aphanidermatum*. These innovative results indicate that aquaponic water could be an interesting and novel source of antagonistic agents adapted to control *P. aphanidermatum* diseases in soilless culture.

Keywords: aquaponic; disease suppressive; *Pythium aphanidermatum*; lettuce; high-throughput sequencing; microorganism**;** bacteria; fungi

1. Introduction

In one loop (i.e., coupled) aquaponic systems, the control of plant pathogens is complex because of the simultaneous presence of fish and nitrifying bacteria in the same loop as plants. Indeed, the addition of chemical agents (e.g., disinfecting agents) and/or pesticides in the irrigation system could be toxic for both fish and nitrifying bacteria (Stouvenakers et al., 2019). In Europe, pesticides and antibiotics are forbidden in aquaculture and in crop agriculture, respectively. Presence and/or accumulation of pesticides in fish, greenhouse atmosphere, and in recirculated water (Hatzilazarou et al., 2004; Reinhardt et al., 2019) could also be problematic. Furthermore, in terms of biological alternative, no biopesticides have been especially developed and registered for aquaponic or hydroponic use. Evaluation and

development of microbial biopesticides in aquaponics are currently still at the early stages (Stouvenakers et al., 2019).

Oomycetes pseudo-fungi responsible for root rot diseases, such as *Pythium aphanidermatum* (Edson) Fitzp., are fungal protists able to produce mobile form of dispersion in recirculating water (reviewed by Sutton et al., 2006). This particularity makes them problematic because of their fast spread in the system and the scarcity of available methods to remove them in aquaponics (Stouvenakers et al., 2019). Etiology and epidemiology of *Pythium* species in hydroponics were extensively reviewed by Sutton et al., 2006. From this review, the following key elements could be exposed. *Pythium* diseases especially affect root zone and reduce plant yields and quality. First stages of the infection in root are normally biotrophic and asymptomatic. After these first stages of root colonization, *Pythium* spp. becomes necrotrophic and then induces symptoms. In general, symptoms translate into root discoloration turning in various shades of brown and finally degenerating in decaying and rotting roots. The foliage generally stays asymptomatic, with no perturbation of photosynthesis, for example, until severe root symptoms appear and produce leaf wilting as secondary symptoms. This lack of foliar symptoms makes the disease difficult to diagnose at early stages without inspecting the root zone. Furthermore, some *Pythium* spp. can remain asymptomatic until stressing conditions appear. High temperatures (from 23 °C to 35 °C, depending on the species) in the aerial zone or in the nutrient solution are one of the main factors encouraging fungal growth, while the plant is also directly affected by the stressing conditions of the high temperatures and the resulting decrease of dissolved oxygen in the nutrient solution.

Nevertheless, aquaponic systems could be more outfitted against plant pathogens than first expected. In fact, two recent studies (Gravel et al., 2015; Sirakov et al., 2016) and a recent review (Stouvenakers et al., 2019) reported the potentially suppressive (i.e., antagonistic) action of fish effluents or aquaponic water against plant pathogens by the natural presence of beneficial compounds and/or microorganisms. Suppressiveness in soilless culture has already been defined by Postma et al. (2008) as “referred to the cases where (i) the pathogen does not establish or persist; or (ii) establishes but causes little or no damage”. In aquaponic systems, in which water is recirculated, the presence of beneficial microorganisms and organic compounds are suspected to be the key sources of this suppressive action (Stouvenakers et al., 2019). Amongst beneficial microorganisms, antagonistic microorganisms are the ones suspected to act in suppressiveness against plant pathogens. In a more general way, modes of action of antagonistic microorganisms are commonly classified in: competition for nutrients and niches, parasitism, antibiosis, and/or plants defenses elicitation (Campbell, 1989; Narayanasamy, 2013a; Whipps, 2001). Concerning organic matter, its role in aquaponic suppressiveness could be related to the promotion of plant beneficial microorganisms and/or in plant biostimulation (Stouvenakers et al., 2019). In the review entitled “Microbial suppressiveness of *Pythium* damping-off

diseases", Kilany et al. (2015) also supported the importance of organic matter to control *Pythium* diseases. More generally, organic matter or amendments are known to be important factors for diseases suppressiveness (Bonanomi et al., 2018b; Garbeva et al., 2004).

Consequently, this study aimed at: (i) evaluating aquaponic water suppressiveness *in vitro* and for the first time *in vivo* on lettuce against *P. aphanidermatum*; (ii) differentiating the origin (microorganisms or dissolved compounds) of the *in vitro* suppressive action; (iii) analyzing and comparing aquaponic water microbiota with hydroponic and complemented (in nutrient salts) aquaponic water in the *in vivo* test through 16S rDNA and ITS Illumina sequencing; and (iv) identifying which specific microorganisms may be correlated with aquaponic water suppressiveness in the *in vivo* test

2. Materials and Methods

2.1. In vitro tests

In vitro tests aimed at evaluating the effect of aquaponic (AP) water recirculated aquaculture system (RAS) water and biofilter media (BM) microorganisms' suspension on the growth of *P. aphanidermatum* in a V8 CaCO3 broth (see Section 2.1.1). Origin and composition of these waters are detailed in Supplementary Material D. Briefly, waters were sampled in the RAS and AP system of Gembloux Agro-Bio Tech, University of Liege, in Belgium. BM microorganisms were recovered by washing biofilter media with 0.05 M Kalium Phosphate Buffer plus 0.05% Tween 80 (KPBT). These 3 types of water were also tested after a 0.2 µm filtration to remove microorganisms.

2.1.1. Methodology

Centrifuge tubes of 50 mL were inoculated with 5 mm plugs of 3-day-old culture of *P. aphanidermatum* (CBS 132490) grown in PDA (Potatoes Dextrose Agar) Petri dishes at 25 °C in the dark. These 50 mL tubes contained 20 mL of clarified V8 $CaCO_3$ broth with different compositions based on the modality (Table D-1). Two kinds of clarified and autoclaved V8 $CaCO_3$ broth were used. The first was a classical V8 $CaCO_3$ clarified broth (800 mL of distilled water, 200 mL of V8 juice, and 3 g of $CaCO_3$), and the second was a V8 $CaCO_3$ broth containing only 75% of its content in distilled water (V8-75% is composed of 550 mL of distilled water, 200 mL of V8 juice, and 3 g of CaCO3). RAS or AP test consisted in 10 centrifuge tubes with 15 mL of V8-75% plus 5 mL of RAS or AP water, respectively, 10 other tubes of 15 mL of V8-75%, plus 5 mL of 0.2 µm filtrated RAS (F-RAS) or AP (F-AP) water to remove microorganism, and 10 last tubes of 20 mL classical V8 $CaCO_3$ to serve as positive control for the growth of *P. aphanidermatum*. Lastly, the BM test was conducted by

using 10 tubes with 15 mL of V8-75% broth plus 5ml of BM water (obtained through washing of biofilter media in KPBT buffer; see Supplementary Material D for further details) and the positive control constituted in 10 tubes with 15 mL of V8-75%, plus 5 mL of KPBT. These 3 tests are summarized in Table D-1. After the broths' inoculation with the mycelial plugs, tubes were incubated at 25 °C in the dark for 5 days. The mycelium bulks thus produced were weighed after filtration and dried by centrifugation through a cheese cloth at 2350 g during 10 min. Three repetitions were carried out for each test (RAS, AP, and BM) over the course of 3 days, with aliquots of the same water sample kept at 4 °C. These repeated tests were also replicated twice with new water samples taken within one week of interval.

Table D-1: Broth composition depending on the test and the modality for *P. aphanidermatum* inoculation.

	Modalities		
Test name	**Broth composition with 25% of the water tested**	**Broth composition with 25% of the filtrated water tested**	**Positive control broth composition**
RAS*	15 mL of V8-75% + 5 mL of RAS water	15 mL of V8-75% + 5 mL of F-RAS water	V8
AP*	15 mL of V8-75% + 5 mL of AP water	15 mL of V8-75% + 5 mL of F-AP water	V8
BM*	15 mL of V8-75% + 5 mL of BM water		15 mL of V8-75% + 5 mL of KPBT

* The test was replicated thrice with different water samples taken within one week of interval and each replicated test was repeated thrice with the same water sample. RAS: recirculated aquaculture system, F-RAS: 0.2 µm filtrated recirculated aquaculture system, AP: aquaponic, F-AP: 0.2 µm filtrated aquaponic, BM: biofilter microbiota, V8: classical V8 CaCO3 broth, V8-75%: V8 CaCO3 broth containing only 75% of distilled water.

2.1.2. Statistical analysis

Statistical analyses were performed on Minitab v.19 software (Minitab Inc., State College, PA, USA). Assumptions of normality and homogeneity of variance were checked by Ryan-Joiner and Levene's tests. The significance of each modality (see Table D-1) on *P. aphanidermatum* mycelial growth for the 3 tests (RAS, AP, and BM) was determined by a partially hierarchized 3-way analysis of variance (ANOVA). The factors used are the modality, the repetition (3 repetitions for the same water sample) and the replication (3 replications with new water sample collected within one week of interval). The repetition factor was hierarchized to the replication factor. In case of interaction between factors, the 3-way ANOVA was decomposed in 2- or 1-way ANOVA. Dunnett's Multiple Comparison test was then used as a post hoc test to compare modalities means to the positive control.

2.2. In vivo tests

The *in vivo* test consisted of *P. aphanidermatum* inoculation on lettuce growing with specific environmental condition, in small raft boxes (description in Section 2.2.1.) containing aquaponic (AP) water, complemented aquaponic (CAP) water, and hydroponic (HP) water as treatment. Origin and composition of these waters are detailed in Supplementary Material D and in Table D-S1. This test was replicated once in time with water sampled in two different dates (trial 1 and 2 in Table D-S1). For each treatment (i.e., type of water), one raft box containing 4 lettuce plants was used as healthy control (HC modality, i.e., non-inoculated box), and the other one also containing 4 lettuce plants was inoculated by *P. aphanidermatum* (IL modality). Suppressiveness evaluation of AP water was made by suppressiveness indexes comparison with the other HP or CAP treatments. These indexes took into account the HC results-specific of each treatment (see 2.2.4 for suppressiveness indexes definition). Composition and diversity of lettuce root microbiota of the first replicate were also analyzed through 16S rDNA and ITS Illumina sequencing analysis (see Section 2.2.5).

2.2.1. Lettuce cultivation

Organic pelleted lettuce (Lactuca sativa) var. Millennia RZ (Rijk Zwaan, Merksem, Belgium) were sown in 36 × 36 × 40 mm rockwool cubes (Grodan B.V., Roermond, Holland) and placed in a phytotron (Fitotron® SGC 120 Plant Growth Chamber, Weiss Technik, Liedekerke, Belgium) with a day/night photoperiod of 16 h/8 h, a temperature of 22 °C/18 °C (16 h/8 h), and a relative humidity of 65%. Plugs were first placed in round plant trays with 2 cm of tap water over 11 days for the germination stage. The lighting system consisted in two 40-W LED panel of 120 × 30 cm, 6500 K (Novaled GmbH, Dresde, Germany) with specific wavelength spectrum designed for lettuce, and a photosynthetically active radiation (PAR) of 180 $\mu mol.m^{-2}.s^{-1}$. After this germination period, plugs were transplanted into homemade hydroponic boxes reproducing hydroponic raft system (deep water culture). These raft hydroponic boxes were composed of 30L Allibert Crownest boxes (Curver Benelux B.V., Rijen, Holland) of 36.3 × 42.5 × 26.3 cm (L × W × H) with a raft panel cut at box dimension in a rigid extruded polystyrene panel 3 cm thick. In this raft, 4 round holes were drilled in the 4 corners to welcome 5-cm rockwool baskets. The 6 hydroponic boxes were each filled with 20 L of the different water treatments and oxygenated 3 times a day for 15 min by 6 diffuser discs of 10 cm (Hi Oxygen disc, Aquatic Science, Herstal, Belgium) placed at bottom of each box and connected to a 40-W air pump (Hi-Blow 40, Aquatic Science, Herstal, Belgium) set at mid-air flow. These 6 boxes were placed in a shelf into the same Phytotron as for germination, 3 boxes on each floor. The same lighting panels were also used (one by floor) 19 cm over the top of the boxes for 16 h per day. After germination, lettuce plants were grown during 31 days in specific environmental conditions mimicking stressing

condition in greenhouse and suitable to *P. aphanidermatum* disease development. During the first 21 days, the phytotron was set at 28/25 °C (d/n; 16 h/8 h) for the temperature and at 65% for humidity. For the last 10 days, the temperature was set at 35/25 °C (d/n; 16 h/8 h) and the humidity at 92%.

2.2.2. Composition, formulation and management of AP, CAP and HP waters

Composition of AP, CAP, and HP waters are described in Table D-S1 in Supplementary Material D, for both trials. HP water is a nutrient solution composed of high purity mineral salts in demineralized water to reach the nutrients concentration recommended in Resh (2013) for hydroponic lettuce nutrient solution. The first week after transplantation of the seedlings in the boxes, only one half of salts quantity were added to the nutrient solution (½ HP) in order to avoid osmotic stress. The microorganisms' concentration at the beginning was determined by the inoculation and plating of 100 µl of ½ HP water on solid PDA (Potatoes Dextrose Agar) and LB (Luria-Bertani) Petri dishes. Number of Colony Forming Unit (CFU) was counted after 3 days of incubation at 23 °C with 18 h/6 h lighting. After this first week of lettuce adaptation, the rest of the salts were added to reach normal Resh nutrients concentration. AP water was characterized to determine its composition in macro and micronutrients, its Biological Oxygen Demand in 5 days (BOD5) and its concentration in cultivable microorganisms. After filtration at 0.45 µm, the concentration in NO^{3-}-N, NH^{+}-N, PO_4^{3-}-P, K^{+}, Ca^{2+}, Mg^{2+}, SO^{4-}-S, and Fe^{2+} was determined using a multiparameter spectrophotometer (HI 83200, HANNA instruments, Woonsocket, RI, USA) with the following reagents: HI 93,700 (TAN), HI 93,728 (NO^{3-}), HI 93,717 (PO_4^{3-}), HI 93,751 (SO_4^{2-}), HI 93,750 (K^{+}), HI 93,752 (Ca^{2+}), and HI 93,752 (Mg^{2+}). BOD5 was measured by OxiTop® (WTW Gmbh and Co, Welheim, Germany) manometer method following the standard method ISO 16072:2002. Microorganisms concentration was calculated as already described for HP water. Regarding CAP water, osmotic stress was also avoided the first week after transplantation by using the same classical AP water as described before but with a pH adjusted to 5.5–5.8. Thereafter, high purity nutrients salts were added to the solution to reach nutrients concentration levels of HP Resh nutrients solution. Salts used for HP and CAP were the following: $MgSO_4 \cdot 7H_2O$, $Mg(NO_3)_2.6H_2O$, NH_4NO_3, K_2HPO_4, $Ca(NO_3)_2 \cdot 4H_2O$, KNO_3, K_2SO_4, Fe-EDTA, $MnSO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, and H_3BO_3. Calculations of salts quantity needed for ½ HP, HP, and CAP waters were performed on HydroBuddy free software (http://scienceinhydroponics.com/category/hydrobuddy). For each type of water, pH and electroconductivity (Ec) were measured 3 times a week, and pH was adjusted to the right level with H_2SO_4 1M or NaOH 1M. For HP, AP, and CAP solutions, the pH was, respectively, kept between 5.5–5.8, 7.0–7.5, and 5.5–5.8. These parameters were measured with a multimeter (model HQ40d, HACH, Loveland, CO, USA) equipped with 2 probes (pH and Ec).

2.2.3. Lettuce inoculation by *P. aphanidermatum*

Pythium aphanidermatum (CBS 132490) was grown in PDA Petri dishes at 23 °C with 18 h/6 h lighting for 3 days. Sterile 150 mL Erlenmeyer flasks containing 25 mL of clarified V8 $CaCO_3$ broth (800 mL of distilled water, 200 mL of V8 juice, and 3 g of $CaCO_3$) were then inoculated by 5-mm plugs of the *P. aphanidermatum* culture. The Erlenmeyer was closed with a cotton ball and incubated during 6 days at 23 °C with 18 h/6 h lighting. The mycelial bulk thus produced was recovered and rinsed by vortexing in a 50 mL centrifuge tube filled with 15 mL of sterile isotonic water (0.85% NaCl). The operation was repeated minimum twice until V8 colour loss. Then the mycelium was drained on a sterile paper towel and mixed 8 times during 3 s with a hand blender (Braun Minipimer Control Plus, 300w) in a sterile solution containing 10 mM of sucrose and 0.05% of Tween 20 in distilled water. The proportion used was 5 mg of mycelium for 1ml of solution. The resulting propagules suspension corresponds to a mean concentration of 5.33×10^3 propagules/mL. Ten ml of this suspension were inoculated per rock-wool plug after 5 and 12 days (after seedlings transplantation) of lettuce growth. The 3 water treatments with the lettuce plants inoculated with *P. aphanidermatum* were then AP-Pa, HP-Pa, and CAP-Pa. For the healthy lettuce, 10 mL of sucrose plus tween solution was added per rock wool plug in the healthy controls (HC) boxes.

2.2.4. Suppressiveness measures

On the last day of the experiment, rating of root rot symptoms was recorded and lettuce plants were harvested to weigh fresh foliar mass. Leaves were then dried in a laboratory oven at 70 °C during 48 h and weighed. Root rot was recorded according to the following scale (adapted from Utkhede et al., 2000):

0 = 100% of healthy white roots, no discoloration;

1 = less than 50% of healthy light brown roots or white roots with brown apex;

2 = more than 50% of healthy light brown roots or white roots with brown apex;

3 = less than 50% of unhealthy medium brown roots with a possible decaying part;

4 = more than 50% of unhealthy medium brown roots with a possible decaying part;

5 = less than 50% of brown-black decaying or dead roots;

6 = more than 50% of brown-black decaying or dead roots.

To be able to compare water suppressiveness of each treatment on *P. aphanidermatum* independently of their respective performance without the disease, different indexes were calculated. Relative foliar turgidity decrease (FTD) represents the relative decrease in leaf water content of *P. aphanidermatum* inoculated lettuce (IL) compared to the water content mean of the corresponding healthy control (HC). FTD was calculated as follows:

$$\text{FTD} = 100 \times \left(FWC_{of\ HC\ mean} - FWC_{of\ IL} \right), \quad (1)$$

$$\text{FWC} = \frac{F_fM - F_dM}{F_fM}, \quad (2)$$

where FTD is the relative foliar turgidity decrease, FWC the foliar water content, F_fM the foliar fresh mass, F_dM the foliar dry mass, HC the healthy control, and IL the inoculated lettuce.

Foliar fresh mass decrease (F_fMD) was also calculated by comparison of IL foliar fresh mass with the mean of the corresponding HC foliar fresh mass. The equation is the following:

$$F_fMD = 100 \times \frac{F_fM_{\ of\ HC\ mean} - F_fM_{\ of\ IL}}{F_fM_{\ of\ HC\ mean}}, \quad (3)$$

where F_fMD is the foliar fresh mass decrease, and F_fM is the foliar fresh mass.

Foliar dry mass decrease (F_dMD) was calculated by comparison of IL foliar dry mass with the mean of the corresponding HC foliar dry mass. The equation is the following:

$$F_dMD = 100 \times \frac{F_dM_{\ of\ HC\ mean} - F_dM_{\ of\ IL}}{F_dM_{\ of\ HC\ mean}}, \quad (4)$$

where FdMD is the foliar dry mass decrease, and FdM is the foliar dry mass.

Lastly, a corrected root rot rating (CRRR) was calculated by taking into account the score of the corresponding HC.

$$CRRR = RRR_{of\ IL} - RRR_{of\ HC\ mean}, \quad (5)$$

where CRRR is the corrected root rot rating, and RRR is the root rot rating.

2.2.5. Statistical analysis of suppressiveness indexes

Statistical analyses were performed on Minitab v.19 software (Minitab Inc., State College, PA, USA). Assumptions of normality and homogeneity of variance were checked by Ryan-Joiner and Levene's tests. The significance of each kind of water (HP, AP, and CAP) on relative foliar turgidity decrease (FTD), foliar fresh mass decrease (F_fMD), foliar dry mass decrease (F_dMD), and corrected root rot rating (CRRR) was determined by a 2-ways analysis of variance (ANOVA). The factors used were the type of water and the replication. In case of interaction between factors, the 2-way ANOVA was decomposed in 1-way ANOVA. Tukey Multiple Comparison test was used as a post hoc test to pairwise compare types of water.

2.2.6. Microbiota analysis of the first test

Microbiota sampling

The microbial communities from 3 lettuce root compartments, the rhizosphere, rhizoplane, and endosphere, were sampled on the last day of the first in vivo experiment. In this experimental setup, the rhizosphere is the water area directly influenced by the roots. Consequently, in this experiment, the rhizosphere corresponds to the water in the boxes. Therefore, one water sample of 30 mL per box was taken, mixed with 10 mL of autoclaved glycerol and then immediately frozen in liquid nitrogen and stored at −20 °C. The rhizoplane is the roots surface including particles and microorganisms adhering on it. The rhizoplane microbiota was recovered as follows (Sare et al., 2020): 0.5 g of roots of each of the 4 lettuce plants were individually sampled and sonicated separately in 30 mL of KPBT (pH 6.5) during 10 min. Roots were removed from the 50 mL centrifuge tubes and 10 mL of autoclaved glycerol was added to the buffer containing the microbiota before flash freezing in liquid nitrogen and conservation at −20 °C. Roots used for the rhizoplane collection were then disinfected and washed for endospheric microbiota analysis. Disinfection was achieved by immerging the roots of each lettuce in alcohol (99%) for 1 min, then in sodium hypochlorite (3.78%) for 3 min, and then rinsed 3 times in sterile distilled water during 3 min. Disinfected roots were then flash frozen separately in liquid nitrogen and stored at −80 °C for further analysis.

Samples preparation and DNA extraction

All samples were processed under sterile conditions before DNA extraction. Rhizoplane and rhizosphere samples were defrosted and filtered through sterile cheesecloths to remove root residues. The filtrates were then vacuum filtered through sterile 0.2 µm filter (47 mm Supor® 200 PES Membrane Disc Filter, PALL Corporation, Portsmouth, UK). The filters were cut in small pieces and temporary stored at 4 °C before DNA extraction on the same day. Defrosted root samples (i.e., for the endosphere analysis) were grinded in mesh bags (12 × 12.5 cm, Agdia Biofords, Elkhart, IN, USA) containing KPBT buffer with the root:buffer ratio of 1:9. Root tissues inside the bags were grinded with a smooth disk tip mounted on a drill (model 850 W PowerPlus X0270, Varo, Lier, Belgium). Resulting root saps were recovered and filtered through sterile cheesecloths before being flash frozen and conserved at -20 °C with 25% autoclaved glycerol. After defrosting, root saps were concentrated through centrifugation at 2350 g for 20 min at 20 °C. Supernatant were removed and the concentrated part (1/4 of the volume) was used for DNA extraction.

FastDNA Spin Kit using Cell Lysis Solution TC (MP Biomedicals, Illkirch-Graffenstaden, France) was used according to manufacturer's instructions to extract DNA microbiota from filters for rhizosphere and rhizoplane samples and from concentrated sap for endosphere samples. DNA quality was checked with a Nanodrop

(Nanodrop ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, DE, USA) and then stored at −20 °C before amplification.

Amplification and sequencing

DNA amplification was performed with the 2X KAPA HiFi HotStart ReadyMix PCR kit (Kapa Biosystems) according to manufacturer's instructions. For bacterial community analyses, composite primers used for 16S rDNA amplification of V1–V3 hypervariable regions were the Forward 27F and Reverse 534R with Illumina sequencing adapters in 5′ 5' (Eck et al., 2019; Sare et al., 2020). For fungal community analyses, the ITS1 DNA region was targeted with the primers ITS1-F_ KYO2 and ITS2_KYO2 (Toju et al., 2012), with the same sequencing adapter in 5′. Amplifications were carried out on thermocycler with an initial denaturation step at 95 °C for 5 min followed by 25 (for all 16S rDNA samples), 30 (for ITS rhizosphere and ITS rhizoplane samples), or 35 cycles (for ITS endosphere samples) of denaturation at 95 °C for 20 sec, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. A final elongation step was performed at 72 °C for 5 min (Sare et al., 2020). The PCR products were further tagged and sequenced by paired-ends Illumina MiSeq at DNAVision (Gosselies, Belgium) with a run of 250 nucleotides.

Bioinformatics and statistical analyses

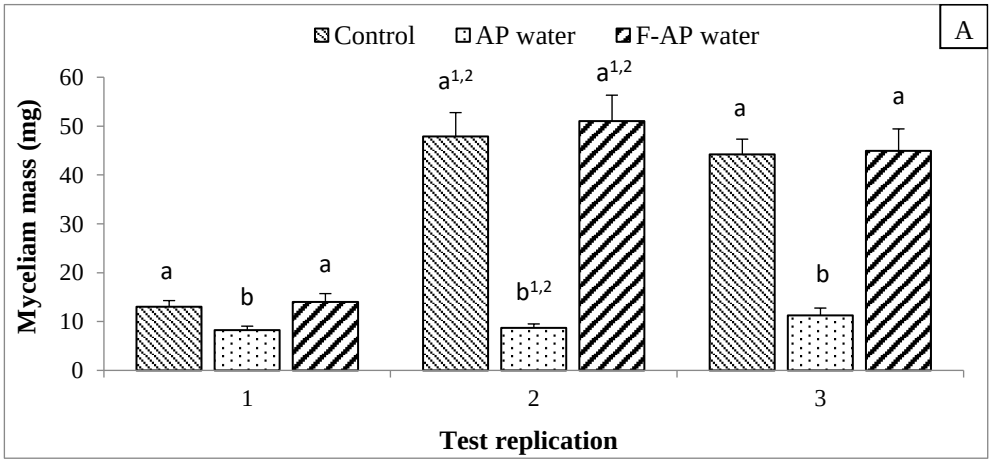
Demultiplexed data obtained from DNAVision were imported in the QIIME 2 software (q2) version 2019-4 (Bolyen et al., 2019) as single-end fastq files with forward reads only for 16S and paired-end fastq files for ITS. Sequences used are available on NCBI platform (https://www.ncbi.nlm.nih.gov/sra) with BioProject ID PRJNA662206. The workflow used was similar to Sare et al. (2020). Briefly, q2 VSEARCH feature-classifier plugin was used after quality control with DADA2 method. Reference database SILVA_132 release for 16S rDNA version 10.04.2018 and UNITE release for fungi version 18.11.2018 were used at 99% of sequence similarity. Q2 taxa filter-table script was run to discard cytoplasmic contaminations. Rhizosphere, rhizoplane and endosphere (i.e., type of microbiota) samples were separated with q2 feature-table script. Alpha and beta diversities were calculated using the q2-diversity core-metrics-phylogenetic plug-in with microbiota specific rarefaction. Rarefaction levels were chosen to keep the maximum of sequences by sample provided that a plateau is reached in the alpha rarefaction curves previously generated. Alpha diversity indexes (Observed OTUs number and Shannon index) were compared with the Kruskal-Wallis pairwise test. Beta diversity index (Weighted Unifrac distance metrics) were compared by the pairwise PERMANOVA (999 permutations) pseudo-F test. DS-FDR (Discrete False-Discovery Rate) tests with Kruskal–Wallis controlling procedure were carried out to compare OTUs relative abundance between treatments microbiota. Rhizosphere samples were composed of a unique liquid sample per treatment thus preventing statistical analysis of its microbiota. Linear correlations and ANOVA were done in R statistical software

version 3.6.0 between α-diversity indexes of rhizoplane HC of HP, AP, and CAP water, and suppressiveness indexes (FTD, FfMD, FdMD, and CRRR). Relationships between OTUs relative abundances at species level of rhizoplane HC of HP, AP, and CAP water, and suppressiveness indexes (FTD, FfMD, FdMD, and CRRR) were also tested in R. For these correlations, only the rhizoplane microbiota was selected in accordance with β-diversity results.

3. Results

3.1. In vitro test

For each water treatment (AP, RAS, and BM waters), statistics showed significant interactions (3-way ANOVA; $p \leq 0.05$) between factors (the modality, the replication, and the repetition), then inhibiting effect of the modality was tested for each replication independently (i.e., week replication 1, 2, or 3 done with different water samples) and represented in Figure D-1. An inhibiting effect of AP, RAS and BM waters on *P. aphanidermatum* mycelial growth was observed when used without previous 0.2µm filtration step (Figure D-1). Filtrated waters did not differ from the control. Although this observation was significant (2-way ANOVA; $p \leq 0.05$) for nearly all tests repetitions inside a week replication, some exceptions could be noticed after ANOVA separation, depending on the repetition factor (i.e., repetition 1, 2, or 3) and, thus, in case of interactions between the "modality" and "repetition" factors.



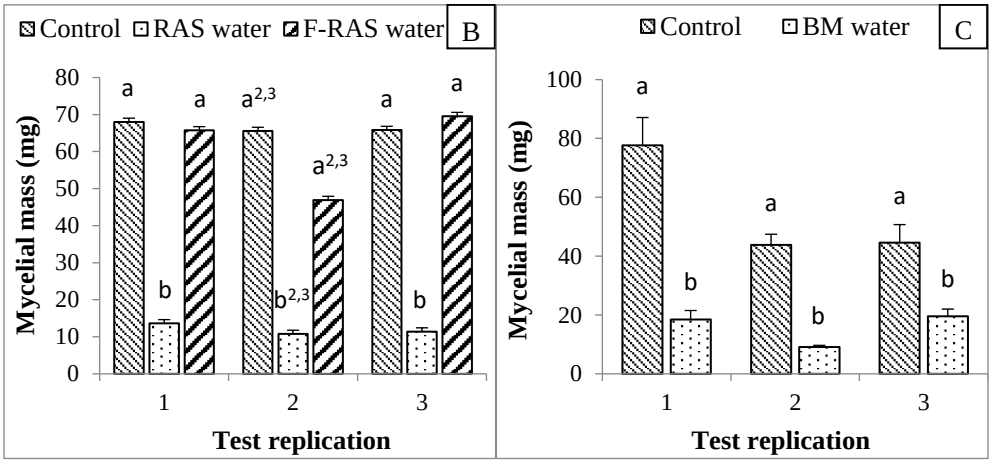


Figure D-1: *P. aphanidermatum* mycelial growth when testing (A): AP and F-AP water; (B) RAS and F-RAS water; and (C) BM water. Bars indicate the standard error of the mean. Different letters indicate significant differences by Dunnett's ANOVA post hoc test ($p \leq 0.05$) between modalities inside a same test replication, i.e., within the same date of water collection. Exponents in these letters indicate that the significance is valid only for the repetition (1, 2, or 3) mentioned by the numbers.

AP water had a significant inhibiting effect on mycelial growth during week replication 1 (-36.6%; $p = 0.005$) and 3 (−74.6% $p = 0.000$) by 2-way ANOVA, while F-RAS has no significant effect compared to the control. For the replication week 2, the significant effect of AP water was observed for the repetition 1 and 2 ($p = 0.000$ for both) but not for week 3 ($p = 0.098$) by 1-way ANOVA.

RAS water had a significant (2-way ANOVA; p = 0,000) inhibiting effect on mycelial growth during week replication 1 (−79.9%) and 3 (−82.7%), while F-RAS had no significant effect. In week 2, this significant effect of RAS water was also observed but only for the repetition 2 (1-way ANOVA; $p = 0.000$) and 3 (1-way ANOVA; $p = 0.000$).

BM water significantly (2-way ANOVA; $p = 0.000$) decreased mycelial growth of *P. aphanidermatum* for all 3-week replications compared to the control (−76.1%, −79.3%, and −56.2%, respectively). But it should be noted that the statistical repetition factor had a significant effect by 2-way ANOVA for the replication factor week 1 ($p = 0.023$) and 3 (p = 0,000).

3.2. In vivo test

3.2.1. Suppressiveness

Results indicated that AP lettuce stayed healthier than HP and CAP lettuce in the presence of the pathogen for the 4 suppressiveness indexes considered (Figure D-2).

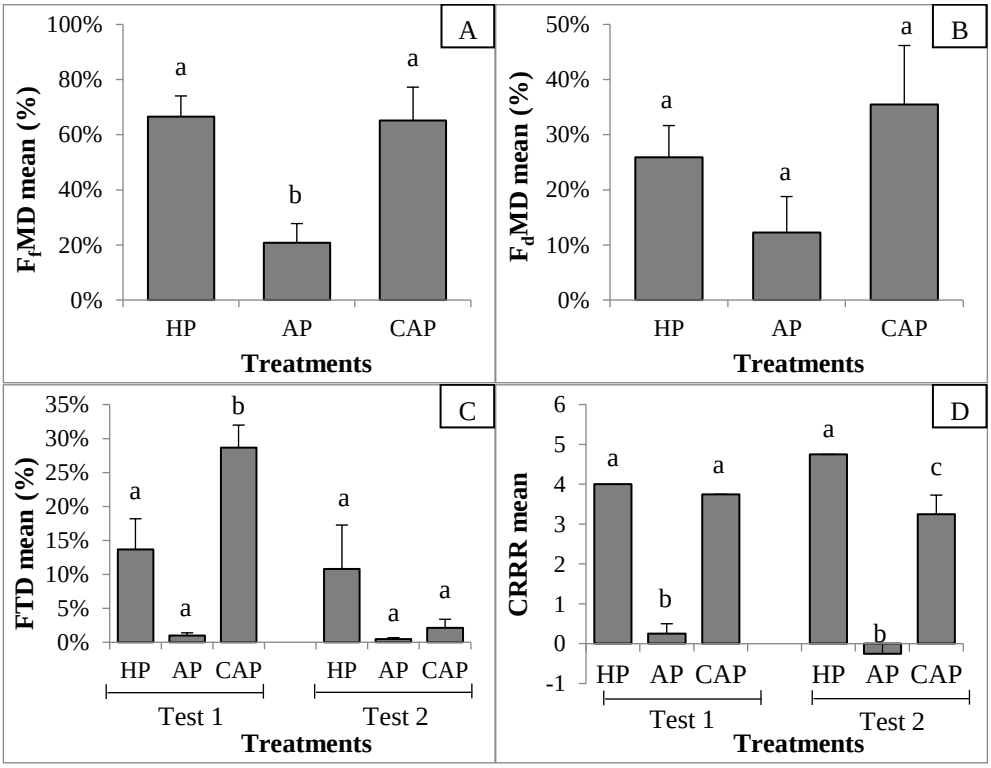


Figure D-2: Effect of hydroponic (HP), aquaponic (AP), and complemented aquaponic (CAP) treatment on (A) foliar fresh mass decrease (FfMD), (B) foliar dry mass decrease (FdMD), (C) relative foliar turgidity decrease (FTD), and (D) corrected root rot rating (CRRR) suppressiveness indexes. Bars indicate the standard error of the mean. Different letters indicate significant differences between treatments by Tukey's ANOVA post hoc test ($p \leq 0.05$).

Statistical analyses indicated significant effect of the replication on F_fMD and F_dMD (2-way ANOVA; $p = 0.001$ and $p = 0.006$, respectively) that were lower during the second trial. Concerning the effect of the treatment (HP, AP, or CAP water), F_fMD was significantly lower (2-way ANOVA; $p = 0.000$) when AP water was used compared to HP and CAP water (Figure D-2A). Means were 20.7% for AP, 66.6% for HP, and 65.1% for CAP. A similar trend (i.e., 12.3%, 25.9%, and 35.3% for AP,

HP, and CAP water, respectively) was also observed for F_dMD but was not significant (Figure D-2B). Interaction between the test replication (trial 1 or 2) and the treatment was recorded by 2-way ANOVA for FTD (p = 0.003) and CRRR (p = 0.028) suppressiveness indexes. Effects of the treatment were then analyzed separately depending on the replication, as illustrated in Figures D-2C and D-2D. Despite the fact that FTD of CAP was 28.7%, HP was 13.7%, and AP was 0.98% for the first trial, the Tukey's 1-way ANOVA post hoc test only highlights a significant FTD difference (p = 0.001) of CAP water treatment compared to the two other waters (which show no difference). During the second trial, no treatment difference was calculated by 1-way ANOVA for FTD but means tended to show a lower FTD for AP water. FTD were 2.1% for CAP water, 0.5% for AP water, and 10.8% for HP water. In regard to the CRRR index of the first trial, CRRR in AP (i.e., 0.25) was significantly lower (1-way ANOVA; p = 0.000) than in HP (i.e., 4.00) and CAP (i.e., 3.75) treatments. For the second trial, means of CRRR were -0.25 for AP, 4.75 for HP, and 3.25 for CAP. All treatments were different (1-way ANOVA; p = 0.000) between them.

3.2.2. Microbiota composition and diversity

Several samples were removed throughout the analysis. For the analysis of rhizoplane ITS community, 3 out of 4 CAP samples were not sequenced because no band was observed on the electrophoresis gel after PCR amplification. Another source of removal was the generation of a too low number of sequences. Consequently, 2 samples out of 4 were removed for AP, AP-Pa, and HP treatments in the 16S rDNA endosphere analysis. The last remaining CAP sample for ITS rhizoplane analysis was also removed for lack of sequences.

Microbiota composition

Bar charts with 16S rDNA and ITS relative composition, at family level, are presented in Supplementary Materials D (Figures D-S1 to D-S6).

The predominant family represented for bacteria was mainly the Bulkolderiaceae. This family was present in all treatments with a minimum relative abundance of 39.7%, 19.0%, and 12.7% in the endosphere, rhizoplane, and rhizosphere, respectively. However, in the rhizosphere, Methylophilaceae was present at higher abundance (17.6% at minimum), except in AP treatment (7.2%). In the endosphere, it is interesting to note that the Pseudomonadaceae family was important in all HC (8.7% at minimum) but that this ratio decreased when *P. aphanidermatum* was present. In other root microbiota, the Pseudomonadaceae family was lower than 1% of relative abundance. In the rhizoplane, Sphingomonadaceae and Lactobacilliaceae were also relatively abundant and more, especially, in AP, with a mean of 9.9% and 6.1%, respectively. The Xanthomonadaceae were also predominant in the rhizoplane but not in non-inoculated AP treatment.

Concerning ITS, most of the taxa were unassigned and/or only classified down to the *Fungi* kingdom. The part of unassigned sequences represented 11.59%, 52.9%,

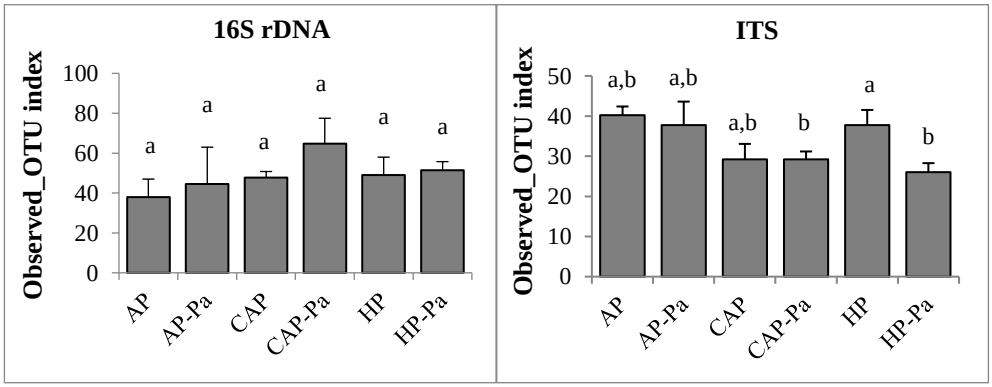
and 66.6% in average of the total OTUs number in endosphere, rhizoplane, and rhizosphere, respectively. However, a second analysis using a eukaryote UNITE database (results not showed) indicated that up to 5.5% of unassigned OTUs could be assigned to the Stramenipiles, Protista, and Viridiplantae kingdoms. Furthermore, manual blast on the NCBI platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) allowed the identification of some abundant OTUs to the Protista kingdom (see the ITS specific case in 3.2.3., where *Protista* represented an abundance of 11.74% on 52.44% of unassigned OTUs). Beyond that, Aspergillaceae were well represented (between 3.2% and 15.6%) in endosphere, as well as the Pleosporales, Ustilaginales, and Dothideales orders. In the rhizoplane, the Debaryomycetaceae was the most assigned family in all treatments at a minimum of 13.2%. The second most represented assigned family was Catenariaceae (4.1% at minimum) but not in HP and HP-Pa treatments, where it was lower than 1.7%. In the rhizosphere, AP water was dominated by Pleosporales, Ustilaginales, and Dothideales orders and, to a lesser extent, other treatments.

Microbiota α-diversity

Global views of α-diversity indexes (observed_OTU number and Shannon index) for the 3-root microbiota are shown in Figures D-S7 and D-S8 in Supplementary Material.

a. Endosphere

Species richness (observed_OTU number) and species diversity (Shannon index) of lettuce endosphere were shown in Figure D-3. Endosphere species richness (observed_OTU number) was relatively similar to all treatments. No statistical differences were found in bacterial analysis, while some pairwise differences ($p \leq 0.05$) were observed in ITS analysis by Kruskal-Wallis test.



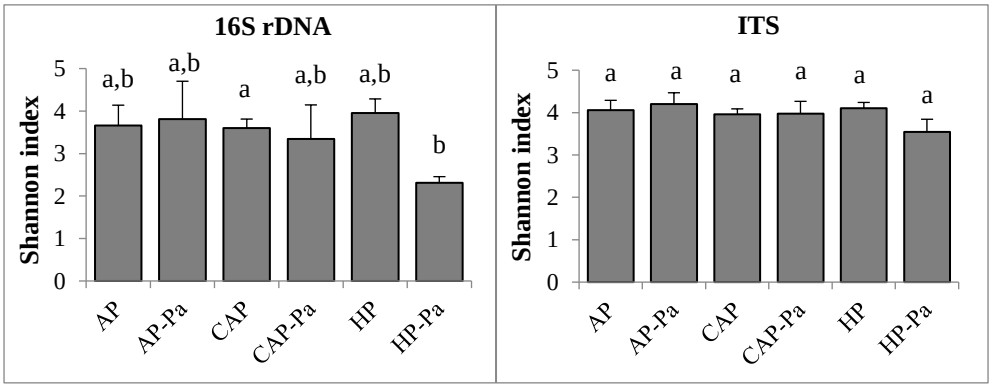
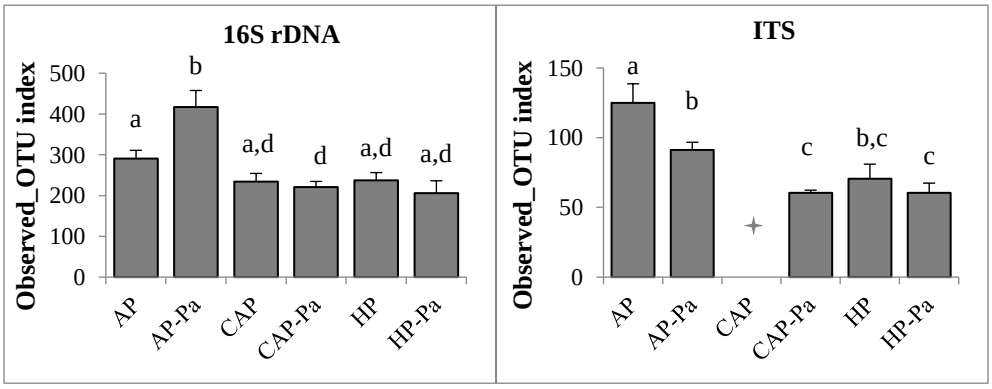


Figure D-3: Species richness (observed_OTU number) and species diversity (Shannon index) of lettuce endosphere of 16S rDNA and ITS analyses depending on the treatment (AP, AP-Pa, CAP, CAP-Pa, HP, and HP-Pa). Bars indicate the standard error of the mean. Treatments that do not share a same letter are significantly different by Kruskal-Wallis pairwise test ($p \leq 0.05$).

As for the richness, the species diversity (Shannon index) of the endosphere was relatively similar, whatever the treatment. Sole HP-Pa Shannon index was significantly different from CAP Shannon index (Kruskal-Wallis; $p = 0.02$) and only in the 16S analysis.

b. Rhizoplane

Concerning lettuce rhizoplane, species richness (observed OTU number) and species diversity (Shannon index) are shown in Figure D-4.



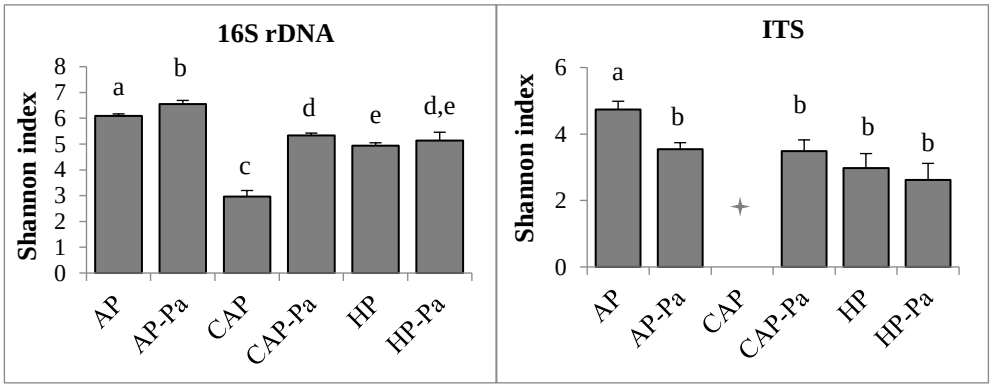


Figure D-4: Species richness (observed OTU number) and species diversity (Shannon index) of lettuce rhizoplane of 16S rDNA and ITS analyses depending on the treatment (AP, AP-Pa, CAP, CAP-Pa, HP, and HP-PA). Bars indicate the standard error of the mean. Treatments that do not share a same letter are significantly different by Kruskal-Wallis pairwise test ($p \leq 0.05$). ✦ CAP treatment in the ITS rhizoplane was removed by the rarefaction process during bioinformatic analysis.

Richness of AP-Pa was higher (Kruskal-Wallis; $p \leq 0.05$) than other treatments for bacteria. In ITS, both AP and AP-Pa were higher (Kruskal-Wallis; $p \leq 0.05$). For 16S species diversity, AP and AP-Pa Shannon indexes were significantly higher (Kruskal-Wallis; $p \leq 0.05$) than all other treatments. More especially, CAP species diversity was significantly reduced compared to AP (Kruskal-Wallis; $p = 0.021$), indicating that the modification of nutrient elements concentrations and pH of AP water decrease bacterial species diversity. In the ITS analysis, only AP Shannon index was statically different from the others (Kruskal-Wallis; $p \leq 0.05$) with the highest species diversity. It was also interesting to notice, in the rhizoplane study, that AP-Pa richness and diversity in the 16S analysis were significantly higher from all the others (Kruskal-Wallis; $p \leq 0.05$), while it was AP in ITS analysis (Kruskal-Wallis; $p \leq 0.05$).

c. Rhizosphere

Species richness (observed OTU number) and species diversity (Shannon index) of lettuce rhizosphere were shown in Figure D-5. Rhizosphere microbiota was not subject to statistics because of a unique sample per treatment. Consequently, non-statistical interpretations of the rhizosphere showed that AP-Pa had the higher richness number in bacteria followed by AP. In ITS analysis, AP had the lowest. Shannon indexes of AP and AP-Pa seemed higher in 16S rDNA analysis. In ITS, it was HP that showed the higher diversity but that decreased after *P. aphanidermatum* inoculation**.**

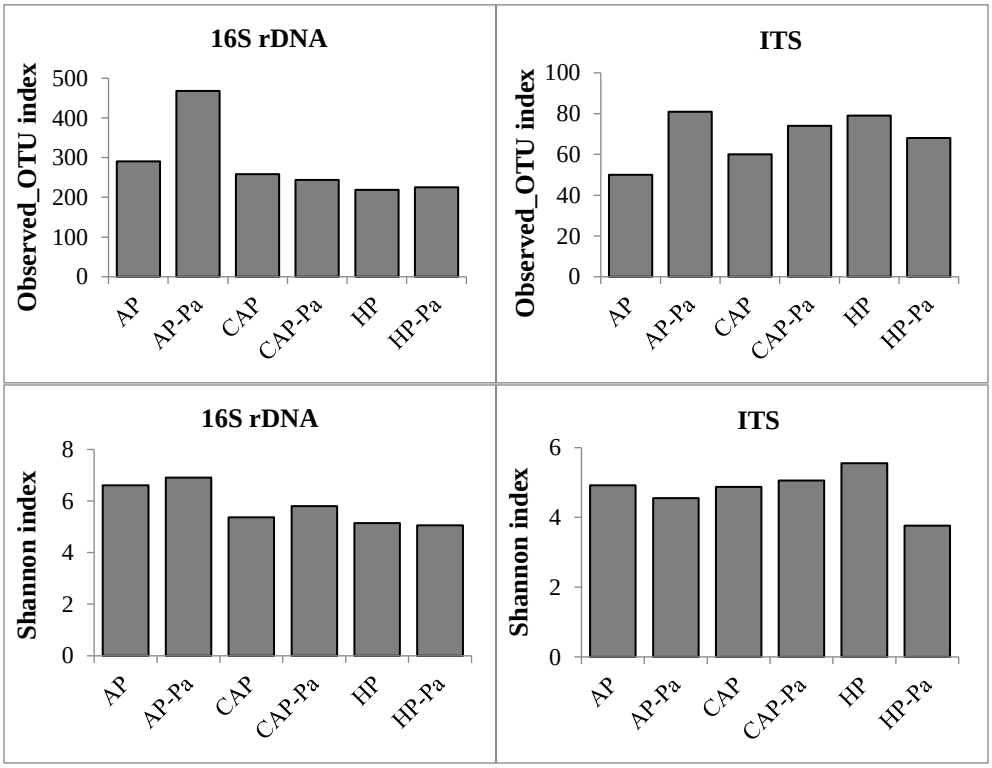
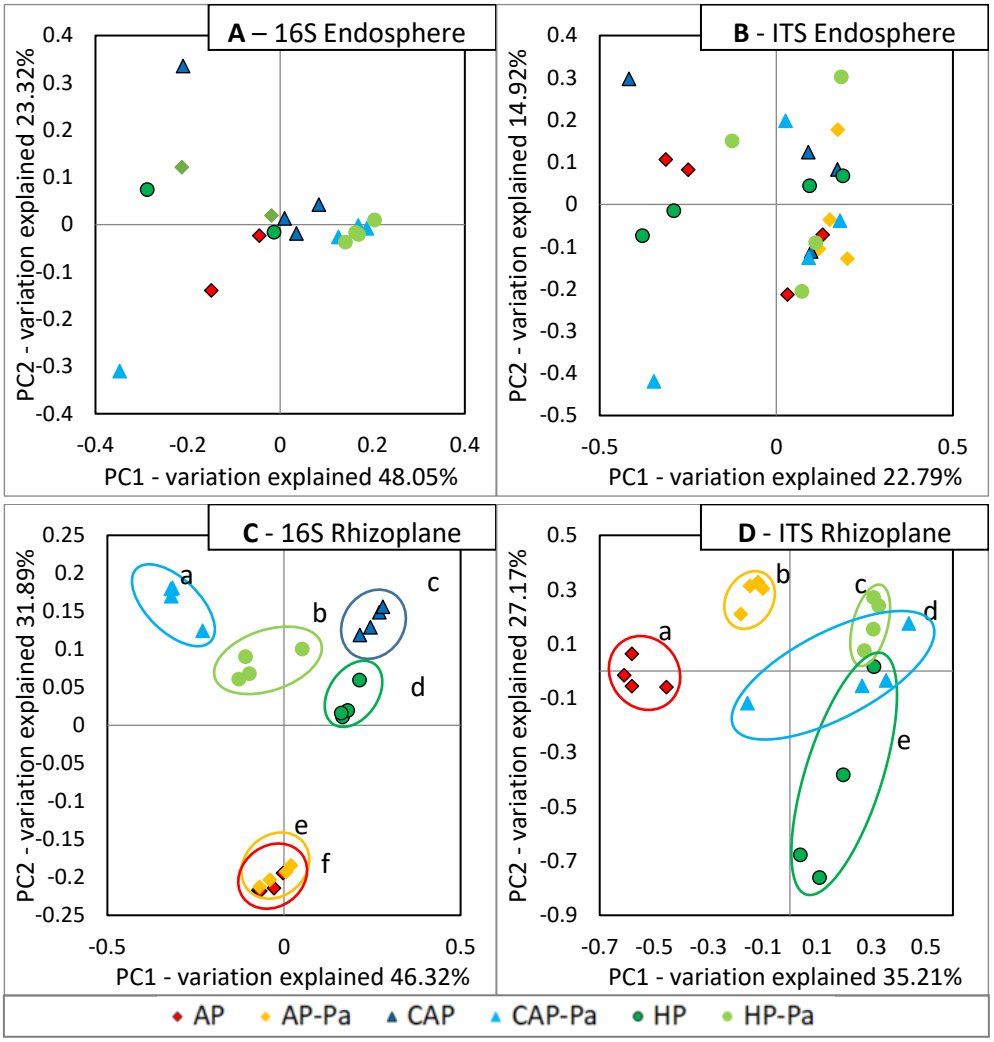


Figure D-5: Species richness (observed OTU number) and species diversity (Shannon index) of lettuce rhizosphere of 16S rDNA and ITS analyses depending on the treatment (AP, AP-Pa, CAP, CAP-Pa, HP, and HP-PA). Rhizosphere microbiota was constituted of a unique sample by treatment and was not subject to statistical analysis.

Microbiota β-diversity

β-diversity of endosphere, rhizoplane and rhizosphere samples are represented with Principal Coordinates Analysis (PCoA) plots in Figure D-6. Statistical differences of β-diversity with Adonis test were observed only in the rhizoplane for both 16S rDNA ($p = 0.001$) and ITS ($p = 0.001$) analyses. Each β-diversity treatment was significantly different ($p \leq 0.05$) from the other ones according to a PERMANOVA test. However, in 16S rhizoplane, AP and AP-Pa PCoA clusters stayed relatively close to each other (also in PC3 axis, with 0.04 of difference in mean) but still statistically different. AP and AP-Pa clusters were also well separated from the other treatments on the PC2 axis. Modifications of AP water to obtain CAP water (pH drop and salts addition) produced a shift of microorganisms visible in the β-diversity analysis of 16S rhizoplane. For CAP and HP treatments, the addition of *P. aphanidermatum* seemed to induce a translation toward negative value on PC1 in the 16S rhizoplane. In ITS

rhizoplane a positive translation on PC2 PCoA axis was visible in HP when *P. aphanidermatum* was present. In comparison with the 16S rhizoplane, *P. aphanidermatum* presence implied a higher β-diversity modification/translation between AP and AP-Pa samples in ITS rhizoplane PCoA. Concerning endosphere, no differences were observed, as well as real trends.



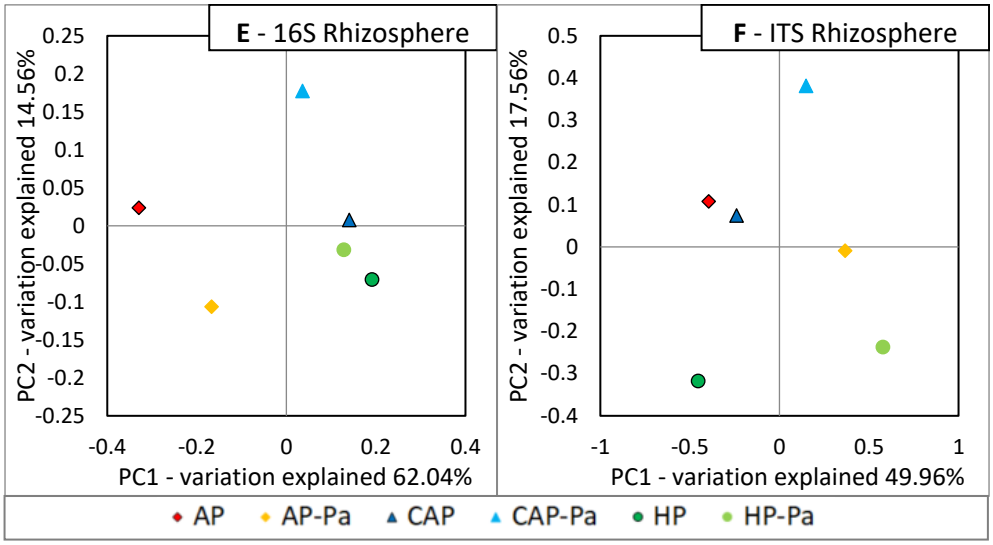


Figure D-6: PCoA plots (Principal Coordinates Analysis) of weighted Unifrac distance metrics of 16S rDNA lettuce endosphere (A), rhizoplane (C), and rhizosphere (E) and ITS lettuce endosphere (B), rhizoplane (D), and rhizosphere (F) analyses depending on the treatment (AP, AP-Pa, CAP, CAP-Pa, HP, and HP-PA). In case of differences ($p \leq 0.05$) with Adonis test, effect of the treatment was pairwise PERMANOVA analyzed, and differences ($p \leq 0.05$) were indicated by circles with different letters. CAP treatment in the ITS rhizoplane was removed by the rarefaction process during bioinformatic analysis. Rhizosphere microbiota is constituted of a unique sample by treatment and was not subject to statistical analysis.

3.2.3. Link between suppressiveness and microbiota

Alpha diversity correlation with suppressiveness indexes indicated that a higher richness (observed_OTU number) and diversity (Shannon index) correlated with disease suppression (i.e., lower suppressiveness indexes). This relation was represented by a negative correlation coefficient in Table D-2. This relation was significant (ANOVA; $p \leq 0.05$) for the species diversity essentially and less for the species richness.

Table D-2: Pearson correlation coefficients between species richness (observed_OTU number) or species diversity (Shannon index) of 16S rDNA or ITS analysis with suppressiveness indexes (F_fMD, F_dMD, FTD, and CRRR). Asterisks indicate statistically significant correlation by ANOVA ($p \leq 0.05$).

		Correlation coefficients with suppressiveness indexes			
	Alpha-diversity indexes	**F_fMD**	**F_dMD**	**FTD**	**CRRR**
16S	Species richness (Observed_OTU number)	-0.40	-0.24	-0.37	-0.55
	Species diversity (Shannon index)	-0.83*	-0.77*	-0.83*	-0.71*
ITS	Species richness (Observed_OTU number)	-0.82*	-0.57	-0.65	-0.78*
	Species diversity (Shannon index)	-0.86*	-0.75*	-0.84*	-0.79*

Correlations of relative 16S rDNA OTUs abundances with suppressiveness indexes indicated that 92 out of 1018 bacterial OTUs could be linked to suppressiveness. Out of these 92 OTUs, only 18 and 28 OTUs were, respectively, found in HP and CAP but with lower abundance than in AP. Correlations of the top 30 most relative abundant OTUs (representing an abundance of 72.8%) in AP were in most cases significantly correlated (ANOVA; $p \leq 0.05$) with suppressiveness indexes for 16 rDNA analysis (Table D-3). Furthermore, out of these 30 OTUs, only one (f_Methylophilaceae; *g_Methylophilus*; s_unculturedbact) was found not to be statistically different in abundance between treatments (AP, CAP, and HP) according to DS-FDR test. The 29 other OTUs were all significantly more abundant in AP compared to HP and CAP treatment (DS-FDR; $p \leq 0.05$). However, correlations in Table D-3 were less significant for F_dMD index and that could be explained by the fact that no significant differences were found between treatments for this index (see Section 3.2.1). All the values were negative, except for the last one. Negative correlations indicated that, the more abundant the microorganism, the more suppressed the disease was (i.e., lower suppressiveness indexes), and inversely. Moreover, OTUs significantly negatively correlated with suppressiveness indexes (ANOVA; $p \leq 0.05$) in Table D-3 were each time more abundant in AP than CAP or HP. *Methyloversatilis* was the most abundant genus link to suppressiveness followed by Burkholderiaceae family and *Sphingobium* genus. Moreover, these last two taxa were found several times in the top 30. Furthermore, inside Burkholderiaceae, the genus *Hydrogenophaga* was identified twice in the top 30.

Table D-3: Pearson correlation coefficients between taxa (OTU) relative abundances of 16S rDNA analysis and suppressiveness indexes (F_fMD, F_dMD, FTD, and CRRR). Only correlations with the 30 most abundant taxa in AP are reported (representing an abundance of 72.8%). Asterisks on OTU relative abundance indicate significant difference of abundance by Discrete False—Discovery Rate (DS-FDR) test ($p \leq 0.05$) between treatments (AP, CAP, and HP water). Asterisks on suppressiveness indexes indicate statistically significant correlation by ANOVA ($p \leq 0.05$).

Bacterial taxa of corresponding OTU and their mean abundance in AP	**Correlation coefficients with suppressiveness indexes**			
	F_fMD	**F_dMD**	**FTD**	**CRRR**
f_Rhodocyclaceae; g_Methyloversatilis: 8.02%*	-0.88*	-0.62*	-0.79*	-0.95*
f_Burkholderiaceae: 7.30%*	-0.89*	-0.61*	-0.75*	-0.98*
f_Sphingomonadaceae; g_Sphingobium: 5.61%*	-0.87*	-0.61*	-0.73*	-0.95*
f_Microscillaceae; g_uncultured: 5.01%*	-0.88*	-0.61*	-0.74*	-0.97*
f_Streptococcaceae; g_Streptococcus; s_uncultured: 3.97%*	-0.78*	-0.43	-0.58*	-0.89*
f_Lactobacillaceae; g_Lactobacillus; s_uncultured: 3.87%*	-0.77*	-0.45	-0.6*	-0.86*
f_Pedosphaeraceae; g_uncultured: 3.62%*	-0.88*	-0.60*	-0.73*	-0.98*
f_Burkholderiaceae: 3.51%*	-0.81*	-0.55	-0.63*	-0.84*
f_Sphingomonadaceae; g_Sphingobium: 3.46%*	-0.79*	-0.49	-0.65*	-0.92*
c_Blastocatellia (Subgroup4); o_11-24; f_uncultured:2.62%*	-0.90*	-0.60*	-0.75*	-1.00*
f_Burkholderiaceae: 2.62%*	-0.89*	-0.62*	-0.74*	-0.97*
f_Burkholderiaceae; g_Hydrogenophaga; s_uncult.: 2.46%*	-0.89*	-0.61*	-0.74*	-0.97*
f_Burkholderiaceae; g_Hydrogenophaga; s_uncult.: 2.17%*	-0.89*	-0.61*	-0.75*	-0.98*
c_Gammaproteobacteria; o_CCD24: 2.11%*	-0.88*	-0.61*	-0.74*	-0.97*
f_Burkholderiaceae: 1.77%*	-0.89*	-0.61*	-0.75*	-0.98*
f_Lactobacillaceae; g_Lactobacillus: 1.43%*	-0.87*	-0.55	-0.66*	-0.94*
f_Hyphomicrobiaceae; g_Hyphomicrobium:1.36%*	-0.82*	-0.53	-0.67*	-0.94*
f_Nitrosomonadaceae: 1.19%*	-0.87*	-0.59*	-0.75*	-0.97*
f_Nitrosomonadaceae; g_MND1; s_uncultured: 1.17%*	-0.85*	-0.58*	-0.73*	-0.94*
f_Saprospiraceae; g_uncultured; s_uncultured: 1.17%*	-0.82*	-0.56	-0.71*	-0.93*
f_Chromobacteriaceae; g_Vogesella; s_uncultured: 1.06%*	-0.81*	-0.58*	-0.65*	-0.87*
f_Fimbriimonadaceae: 0.99%*	-0.85*	-0.54	-0.73*	-0.96*
f_Propionibacteriaceae; g_Propionibacterium: 0.89%*	-0.75*	-0.48	-0.61*	-0.81*
f_Gemmataceae; g_uncultured; s_uncultured: 0.89%*	-0.81*	-0.53	-0.71*	-0.93*
f_Methylophilaceae; g_Methylophilus; s_uncultured: 0.82%	-0.56	-0.52	-0.51	-0.46

f_Reyranellaceae; g_Reyranella: 0.79%*	-0.84*	-0.57	-0.72*	-0.95*
f_Lactobacillaceae; g_Lactobacillus: 0.79%*	-0.82*	-0.52	-0.63*	-0.86*
f_Burkholderiaceae: 0.71%*	-0.90*	-0.62*	-0.75*	-0.98*
f_Sphingomonadaceae: 0.71%*	-0.86*	-0.57	-0.74*	-0.96*
f_Nocardiaceae; g_Rhodococcus; Ambiguous_taxa: 0.71%*	-0.28	-0.53	-0.48	0.08

Correlations of relative ITS OTUs abundances with suppressiveness indexes indicated that 35 out of 349 fungal OTUs could be linked to suppressiveness. Out of these 35 OTUs, only 11 OTUs were found in HP. Among these OTUs, only one OTU corresponding to *Meyerozyma* genus tended (DS-FDR; $p = 1$) to be more abundant in HP compared to AP (NB: CAP samples were removed from the analysis during rarefaction process). For ITS correlations, in comparison with 16S rDNA, less OTUs were significantly (ANOVA; $p \leq 0.05$) negatively correlated with suppressiveness indexes in the top 30 most abundant OTUs (representing an abundance of 83.0%) in AP (Table D-4). Out of these 30 OTUs, only 14 OTUs were found to be statistically different in abundance (DS-FDR test; $p \leq 0.05$) between treatments (AP and HP). All these 14 OTUs were found to be more abundant in AP treatment compared to HP treatment. Furthermore, most OTUs were unassigned in QIIME 2 and were thus manually blasted in NCBI platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for additional information. This manual blast highlighted that 11.74% of the 52.44% unassigned OTUs of Table D-4 were potentially members of the Protista Kingdom.

Table D-4: Pearson correlation coefficients between taxa (OTU) relative abundances of ITS analysis and suppressiveness indexes (F_fMD, F_dMD, FTD, and CRRR). Only correlations with the 30 most abundant taxa in AP are reported (representing an abundance of 83.0%). Unassigned OTUs and assignations limited to Fungi Kingdome were manually blasted and indicated for information in italic. Asterisks on OTU relative abundance indicate significant difference of abundance by DS-FDR test ($p \leq 0.05$) between treatments (AP and HP). Asterisks on suppressiveness indexes indicate statistically significant correlation by ANOVA ($p \leq 0.05$). CAP treatment is not part of the correlation analysis because it was removed by the rarefaction process during bioinformatics.

ITS taxa of corresponding OTU and their mean abundance in AP	**Correlation coefficients with suppressiveness indexes**			
	F_fMD	**F_dMD**	**FTD**	**CRRR**
k_Fungi; uncultured: 13.91%	-0.50	-0.11	-0.45	-0.64
k_Fungi; f_Debaryomycetaceae; g_Meyerozyma: 13.16%	0.54	0.78*	0.80*	0.27
k_Fungi: 9.30%	-0.50	-0.33	-0.42	-0.50
k_Fungi; f_Catenariaceae; g_Catenaria;s_unidentified:4.7%*	-0.77*	-0.36	-0.68	-0.90*
k_Fungi; uncultured: 4.30%*	-0.67	-0.32	-0.63	-0.78*

k_Fungi; uncultured: 4.09%*	-0.82*	-0.44	-0.70	-0.90*
k_Fungi; f_Catenariaceae;g_Catenaria;s_unidentified: 3.8%*	-0.79*	-0.33	-0.71*	-0.94*
k_Protista; c_Kinetoplastida: 3.70%*	-0.70	-0.26	-0.63	-0.85*
k_Protista; c_Kinetoplastida: 2.98%*	-0.68	-0.30	-0.60	-0.82*
k_Fungi o_Rhizophydiales: 2.76%*	-0.76*	-0.41	-0.69	-0.85*
k_Fungi; uncultured: 2.64%	-0.16	-0.11	-0.23	-0.22
k_Protista; g_Trypanosoma: 1.79%	-0.76*	-0.51	-0.75*	-0.73*
k_Protista; g_Trypanosoma: 1.52%	-0.73*	-0.40	-0.68	-0.75*
k_Fungi; uncultured: 1.47%*	-0.65	-0.20	-0.63	-0.83*
k_Fungi; o_Dothideales: 1.38%	-0.43	-0.29	-0.34	-0.36
k_Fungi; uncultured: 1.08%*	-0.81*	-0.37	-0.73*	-0.95*
k_Fungi; uncultured: 1.05%*	-0.81*	-0.40	-0.72*	-0.92*
k_Fungi; g_Cladosporium: 1.01%	-0.44	-0.31	-0.34	-0.37
k_Fungi; uncultured: 0.95%	-0.05	-0.45	-0.15	0.13
k_Protista; g_Trypanosoma: 0.93%*	-0.71	-0.40	-0.66	-0.77*
k_Viridiplantae; Embryophyta: 0.89%*	-0.80*	-0.41	-0.67	-0.86*
k_Protista; g_Trypanosoma: 0.82%*	-0.76*	-0.51	-0.68	-0.73*
k_Fungi; o_Ustilaginales: 0.77%	-0.35	-0.22	-0.27	-0.32
k_Fungi; f_Ustilaginaceae: 0.75%	-0.49	-0.32	-0.31	-0.42
k_Fungi; o_Pleosporales: 0.64%	-0.26	-0.15	-0.29	-0.26
k_Fungi; g_Cladosporium: 0.62%	-0.14	-0.11	-0.17	-0.07
f_Fungi; p_Ascomycota; Pezizomycotina: 0.52%	-0.40	-0.16	-0.45	-0.53
k_Fungus: 0.51%	-0.68	-0.34	-0.72*	-0.74*
k_Fungi; f_Aspergillaceae: 0.48%	-0.57	-0.05	-0.26	-0.72*
k_Fungi; f_Hypocreales_fam_Incertae_sedis: 0.46%*	-0.85*	-0.49	-0.74*	-0.92*

As for 16S rDNA, less significant correlations were found with F_dMD index. Among identified fungal taxa (for specific OTU) potentially linked to suppressiveness, *Catenaria* genus, Rhizophydiales, and Hypocreales (f_Hypocreales_fam_Incertae_sedis) order could be cited. *Meyerozyma* genus was significantly positively correlated for F_dMD (ANOVA; $p = 0.022$) and FTD (ANOVA; $p = 0.017$) indexes and tended to be more abundant in HP water, thus indicating that more it was abundant, more the symptoms tended to be high.

4. Discussion

Results of in vitro tests indicated that the pathogen was inhibited by microorganisms of AP, RAS and BM waters and not by the mineral or organic compounds found in the waters. However, significance was sometimes influenced by the replication (test replicated with different water samples) or the repetition (test repeated with a same water sample) factor. Interactions between these statistical factors and the mycelial growth were probably due to the growth variability of *P. aphanidermatum* (Martin and Loper, 1999). This assumption is strengthened by differences of growth observed for the controls between repetitions or replications. For example, there was a difference of 72.8% of mycelium mass growth for the control between the replication 1 and 2 in the AP test.

The *in vitro* effect of fish waters on fungal plant disease can be corroborated by other studies. Indeed, Gravel et al. (2015) reported that aquaculture effluents could promote plant growth, decreased *Pythium ultimum* and *Fusarium oxysporum* growth *in vitro* and also reduced tomato roots colonization by these fungi. *In vitro* growth inhibition of *Pythium ultimum* and *Fusarium oxysporum* were much higher when crude fish effluents were used compared to filtered sterilized or autoclave ones. In a paper by Sirakov et al. (2016), bacterial isolates from an aquaponic system showed an inhibitory effect on *P. ultimum* by agar diffusion method.

These *in vitro* trials informed on the direct effect of AP, RAS, and BM waters against *P. aphanidermatum* in absence of its plant host. However, plant defense elicitation by microorganisms is a possible antagonistic indirect path to control plant pathogens and cannot be discarded. These results also do not indicate if compounds in solution can help or not control the disease by an indirect way. For example, dissolved organic matter, such as humic acid, met in aquaponics (Hirayama et al., 1988, Takeda and Kiyono, 1990 cited by Leonard et al., 2002) can act on plant health by biostimulation or defense elicitation (Adani et al., 1998; Bohme, 1999; du Jardin, 2015). Moreover, plant defense elicitation by microorganisms or compounds is a possibility of indirect mode of antagonism that cannot be tested in this kind *in vitro* experiment.

In vivo experiments are better ways to testify the efficiency of antagonistic microorganisms against plant pathogens. In fact, complex interactions between microorganisms, plants, plant pathogens, and the environment are misrepresented in *in vitro* tests and often give incorrect results when compared to *in vivo* tests (Elsherif and Grossmann, 1994). However, in our case, *in vivo* tests results confirmed the suppressive action of AP water found *in vitro* against lettuce *P. aphanidermatum* disease.

Until now, articles studying microbial diversity in AP system are scare. No paper has described fungal composition, while 4 could be cited for bacteria in aquaponic system growing lettuce (Bartelme et al., 2019; du Jardin, 2015; Eck et al., 2019; Sare

et al., 2020). The two last references (Eck et al., 2019; Sare et al., 2020) originated from the same system than ours. When comparing the results of the 4 references with our study at the family level, *Burkholderiaceae* (including *Comamonadaceae* in UNITE taxonomy database) and Sphingomonadaceae are relatively common in aquaponic lettuce root microbiota. *Comamonadaceae* and *Sphingomonadaceae* were also major taxa in root microbiota of lettuce cultivated in soil (Cardinale et al., 2015). Moreover, according to the list of taxa associated with disease suppressive soils in Expósito et al. (2017) both families or their members are commonly identified and suspected to play a role in suppressive soil. In the lettuce bacterial endosphere of this study, *Pseudomonadaceae* was the second most abundant family after *Burkholderiaceae*. *Pseudomonadaceae*, especially *Pseudomonas* genus, is well known for its antagonistic activities against plant pathogens and was suspected to be an important player in aquaponic suppressiveness (Stouvenakers et al., 2019). Concerning fungal family found in the root zone in this study, particularly well abundant in the endosphere, the Aspergillaceae family (taxonomy of UNITE database) contains important genera known to be antagonistic fungi and component of suppressive soil. Two genera related to suppressive soil could be cited: *Aspergillus* and *Penicillium* (Expósito et al., 2017). Supplementary analyses using eukaryote UNITE database and manual blast on NCBI highlighted that a relatively important amount of ITS OTUs could belong to other taxa than fungi. In fact, plants and protists were identified. Among *Protista*, protozoa were especially abundant. Toju et al. (2012) had already expressed this risk of fungal ITS1 primers (ITS1-F_ KYO2 and ITS2_KYO2) matches with other eukaryotic sequences. ITS region is probably the most effective genetic marker and frequently used for fungi identification. However, its use can give limited results, namely because of its inter and intra-specific variability among fungi leading to weaker identification rates compared to bacteria (Begerow et al., 2010; Schoch et al., 2012).

In the PCoA plots, it could be noted that the inoculation of *P. aphanidermatum* produced a shift in microbiota β-diversity for most treatments in the rhizoplane. Although AP and AP-Pa are clustered differently, this shift was highly reduced for bacteria. This indicates a better resilience or resistance of the bacterial aquaponic microbiota to the entry of a perturbation, in this case, a plant pathogen. HP and CAP treatments induced a more disease conducive microbiota. The fact that bacterial diversity was quite similar after plant pathogen inoculation in suppressive environment is current in literature (Calvo-Bado et al., 2006; Kyselková et al., 2009; Vallance et al., 2012), while a disease conducive environment is more subject to bacterial modifications (Kusstatscher et al., 2019; Kyselková et al., 2009). However, the resulting microbiota on diseased plants (i.e., after *P. aphanidermatum* inoculation) cannot help identifying antagonistic microorganisms by comparison between treatments. Indeed, for the correlation analysis, only healthy controls of AP, CAP, and HP were chosen to link α-diversity indexes or relative OTU abundances with

suppressiveness indexes. The reason for this was that microbiota of diseased lettuce could result of post contamination of damaged/rotted tissues by opportunistic microorganisms masking the initial microbiota. Initial microbiota that was besides unable to control the pathogen entry in the case of CAP and HP treatments.

Initially, the presence of the CAP water treatment was added to counteract the potential bias in lettuce growth created by the lowest nutrients concentration in AP water compared to HP water. However, it was interesting to observe that CAP water (prepared from AP water) lost its potential disease suppressiveness by adding nutrient salts and lowered pH. Explanation could be linked to a microbiota modification as indicated in the α- and β-diversity and taxonomical composition of the rhizoplane. It was accorded that this type of parameters (e.g., pH and plant mineral nutrients) could impact microbiota diversity and also its suppressiveness (Garbeva et al., 2004). Moreover, modification of water quality parameters could also have an indirect impact by playing on lettuce health or a direct impact on *Pythium* spp. development. In soil, Martin and Loper (1999) reviewed the effect of pH on *Pythium* species. It was demonstrated in it that pH level impact disease development. Even if no general rules can be exposed, in some case, a pH under 7 can be linked to a better disease development on plant. This phenomena could be enlightened by a pH influence on zoospores production (in hydroponic system cropping lettuce in Funck-Jensen and Hockenhull, (1983)), on appressoria formation (Endo and Colt, 1974 cited by Sutton et al., 2006), on mycelial growth and on saprophytic activity of the pseudo-fungus (reviewed by Martin and Loper, 1999). Furthermore, mineral or organic components (where composition and availability are also influenced by pH) of the environment can also influence *Pythium* spp. development (Khalil and Alsanius, 2009; Mandelbaum and Hadar, 1990; Martin and Loper, 1999). In fact plant substrate or nutritive solution richer in nutrients can also sometimes enhance fungal diseases (Dordas, 2008; Geary et al., 2015; Veresoglou et al., 2013).

In the present study, higher α-diversity indexes were correlated with higher suppressiveness ability and more especially with species diversity where the relations were significant. In the literature about suppressive environments, this relation also appeared (Corato et al., 2019; Kusstatscher et al., 2019; Ros et al., 2019), but the reverse relation could be observed (Bonanomi et al., 2018a). However, some of these references compared the microbiota of healthy plants and that resulting of ill plants where secondary or opportunistic microorganisms appear after the initial infection.

Among microorganism significantly correlated with suppressiveness in rhizoplane AP water in this study, *Lactobacillus* was the most known genus to be a plant disease antagonist, with *Lactobacillus plantarum* as key species (Laitila et al., 2002; Wang et al., 2012). *Sphingobium* genus was also present and found to be antagonist of *P. aphanidermatum* in Burgos-Garay et al. (2014). *Catenaria* genus is mainly known to be a nematodes antagonist (Birchfield, 1960; Singh et al., 1996). However *Catenaria anguillulae* was also identified in Daft and Tsao (1984) as parasite of *Phytophthora*

cinnamomi and *parasitica;* two Oomycetes pathogens of citrus and avocado orchards. *Catenaria allomycis* was also described as fungus parasite on *Allomyces arbuscular* (Sykes and Porter, 1980). Rhizophydiales are, as *Catenaria* genus, member of Chytridomyces class. This class contains important parasite of invertebrates, protists and fungi. Notably, *Rhizophydium pythii*, who is a parasite of *Pythium* sp. (Sparrow, 1960 cited by Peter M. Letcher, 2012). An incertae sedis family belonging to *Hypocreales* was also correlated with disease suppression. Among this order, the well-known *Trichoderma* and *Gliocladium* genera could be cited for their antagonist activity against *Pythium* species (Martin and Loper, 1999). The most abundant genus in this study correlated with suppressiveness was *Methyloversatilis*. Today, it is never related to plant disease suppression. This genus describes as methylotroph has the particularity to degrade variety of C1 units and multicarbon compounds, such as aromatic compounds, organic acids, alcohols, and methanol or methylamine (Rosenberd, 2014; Smalley et al., 2015). After *Methyloversatilis*, the *Burkholderiaceae* family was the taxa most related to a suppressive action. Inside this family, the genus *Hydrogenophaga* was mentioned several times. *Burkholderiaceae* are linked to the bacterial composition of several suppressive soils (Benítez and McSpadden Gardener, 2009; Expósito et al., 2017). This family contains several species able to act against fungal plant pathogens such as *Burkholderia* or *Mitsuaria* species (Coenye, 2014), and notably against *P. aphanidermatum* (Benítez and McSpadden Gardener, 2009). The genus *Hydrogenophaga* is mainly known for its chemoorganotrophic or chemolithoautotrophic nutrition, using H_2 as energy source and CO_2 as a carbon source (Brenner et al., 2005). Moreover, it was cited and described as plant growth promotor essentially by acting in nitrogen cycle (denitrification and N_2 fixation) (Chanway and Holl, 1993).

HP water failed to support the inoculation of *P. aphanidermatum*. This difference with AP could be linked to a distinct microbiota composition and/or diversity in the rhizoplane, as well as difference of physicochemical water parameters (e.g., pH and mineral nutrients), these factors being closely related. This distinct and suppressive microbiota found in AP was probably driven by the presence of organic compounds in the nutritive solution. Stouvenakers et al. (2019) envisaged this possibility by making links with the suppressiveness met in some farming systems containing higher rates of organic carbons (e.g., organic hydroponics). However, it was supported in the literature that hydroponic systems without organic amendments could also express a suppressive activity against plant pathogens (reviewed by Postma et al., 2008). The explanation of why our HP water failed to suppress *P. aphanidermatum* disease development could be linked to an absence of system cycling before the experimentation. In fact the hypothesis behind the suppressive activity observed in hydroponics was the recirculated aspect of the nutrient water solution in the system (Calvo-Bado et al., 2006; McPherson et al., 1995; Postma et al., 2008; Tu et al., 1999).

This water recirculation created a stable and well-established microbiota after a certain period of cycling system.

In this in vivo study, growth of healthy lettuce was not compared between treatments, but raw data of fresh leaf mass (data not shown) indicated a lower foliar yield of AP lettuce compared to HP and similar yield between CAP and HP. This could be logical because AP water contained lower concentration of mineral nutrients. However, this is in contradiction with other papers where yields of AP were similar to HP, and CAP yields better than AP (Alcarraz et al., 2018; Delaide et al., 2016; Goddek and Vermeulen, 2018; Lennard and Ward, 2019; Monsees et al., 2019; Pantanella et al., 2012). In these papers, authors explained this contradiction (lower mineral nutrients concentrations but good yields) by the potential presence of microorganisms or compounds able to increase plant growth (Bartelme et al., 2018). This hypothesis was strengthened by Sanchez et al. (2019), who isolated several plant growth promoting bacteria in tilapia fish aquaculture. Explanations of the lower yield observed for AP lettuce in our experiment could be the bio-chemical variability of AP water or the stressing conditions applied to promote *P. aphanidermatum* infection that may impact development of plant growth promoting microorganisms.

5. Conclusion

Results of this study demonstrated that microorganisms of AP, RAS, and BM waters had a significant direct inhibitory effect on *P. aphanidermatum* growth in *in vitro* experiments. The suppressive effect of AP water was also shown on lettuce inoculated by *P. aphanidermatum* in *in vivo* conditions. Indeed, disease symptoms of AP lettuce were significantly reduced compared with CAP and HP lettuce. Root microbiota study suggested that AP water's suppressive effect was namely induced by differences in terms of microorganism composition and diversity. Moreover, it was shown that CAP water lost the natural suppressive capacity of AP water after addition of nutrient salts and pH modification of AP water to create CAP water. Several microorganisms were significantly correlated with the suppressive effect of AP water. Nevertheless, few of these microorganisms (at the genus level) were known to have an antagonistic effect against *P. aphanidermatum*. In conclusion, these results indicated that AP water could be an interesting and novel source of antagonistic agents able to control *P. aphanidermatum* diseases in soilless culture.

Chapter E

First Study Case of Microbial Biocontrol Agents Isolated from Aquaponics through the Mining of High-Throughput Sequencing Data to Control *Pythium aphanidermatum* on Lettuce

The material presented in this chapter is adapted from a manuscript accepted in Microbial Ecology:

Stouvenakers, G., Massart, S., Jijakli, M.H., 2022. First study case of microbial biocontrol agents isolated from aquaponics through the mining of high-throughput sequencing data to control *Pythium aphanidermatum* on lettuce. Microbial Ecology, p-p.

Abstract: Aquaponics is defined as a sustainable and integrated system that combines fish aquaculture and hydroponic plant production in the same recirculated water loop. A recent study using high-throughput sequencing (HTS) technologies highlighted that microbial communities from an aquaponic system could control one of the most problematic pathogens in soilless lettuce culture, namely *Pythium aphanidermatum.* Therefore, this study aims at isolating the microorganisms responsible for this biocontrol action. Based on the most promising genera identified by HTS, an innovative strategy for isolating and testing original biocontrol agents from aquaponic water was designed to control *P. aphanidermatum*. Eighty-two bacterial strains and 18 fungal strains were isolated, identified by Sanger sequencing, and screened *in vivo* to control damping-off of lettuce seeds caused by *P. aphanidermatum*. Out of these 100 isolates, the eight most efficacious ones were selected and further tested individually to control root rot disease caused by the same pathogen at a later stage of lettuce growth. Strains SHb30 (*Sphingobium xenophagum*), G2 (*Aspergillus flavus*) and Chito13 (*Mycolicibacterium fortuitum*) decreased seed damping-off at a better rate than a propamocarb fungicide and a *Pseudomonas chlororaphis* registered biocontrol agent did. In root rot bioassays, lettuce mortality was prevented by applying strains G2 and Chito13, which were at least as efficacious as the fungicide or biopesticide controls. Lettuce disease symptoms and mortality were eradicated by strain SHb30 in the first bioassay, but not in the second one. These results show that aquaponic systems are promising sources of original biocontrol agents, and that HTS-guided strategies could represent interesting approaches to identify new biocontrol agents.

Keywords: Soilless, root disease, biocontrol, *Sphingobium xenophagum*, *Aspergillus flavus*, *Mycolicibacterium fortuitum*

1. Introduction

Soil-borne plant diseases are in theory less common in soilless plant cultures than in soils (Vallance et al., 2010). However, some soil pathogens well adapted to aquatic environments can be highly virulent because they can produce mobile forms of dispersal that benefit from water recirculation (Sutton et al., 2006; Vallance et al., 2010). *Pythium aphanidermatum* (Edson) Fitzp. – an oomycete pathogen able to produce zoospores in water – can cause lettuce root rot and damping-off in soilless culture (Alhussaen, 2006; Stanghellini and Rasmussen, 1994). Soilless systems comprise hydroponic and aquaponic systems. Aquaponics is defined in this study as an integrated system combining fish and plant production in the same recirculated water loop. In coupled aquaponics, plant treatment with pesticides and chemical disinfection agents to control pathogens are inadvisable because they can be toxic to the fish and beneficial microorganisms (e.g., nitrifying bacteria) present in the same

water loop (Bittsanszky et al., 2015; Folorunso et al., 2020; Nemethy et al., 2016; Rakocy et al., 2006; Stouvenakers et al., 2019). Therefore, biocontrol in aquaponics is of prime importance but still understudied (Folorunso et al., 2020; Stouvenakers et al., 2019). In hydroponics, the use of biocontrol agents and related studies are not new (Paulitz, 1997). However, biocontrol research in hydroponics has often indicated poor adaptation and efficacy of common soil microbial biocontrol agents or microbial biopesticides to the specific aquatic conditions of soilless cultures. Until now, no biopesticide has been specifically marketed for aquaponic use (Folorunso et al., 2020; Stouvenakers et al., 2019) and only few products are available and useful to control root diseases in hydroponics (Postma et al., 2008; Vallance et al., 2010). Consequently, there is a need to develop new microbial biopesticides adapted to soilless conditions and more especially, to aquaponic conditions (Folorunso et al., 2020; Postma et al., 2008; Stouvenakers et al., 2019; Vallance et al., 2010). In regard to approved soil biocontrol agents whose isolation campaign started 50 years ago from soil-borne disease-suppressive soil (Deacon et al., 1988), this research has aimed to selectively isolate *P. aphanidermatum* biocontrol agents from the same aquaponic system where the suppressive *P. aphanidermatum* microbiota was characterized by Stouvenakers et al. (2020). The microorganisms to be isolated were selected based on the high-throughput sequencing (HTS) analysis achieved in Stouvenakers et al. (2020). Although numerous papers have characterized the suppressive activity of microbiomes by HTS (Expósito et al., 2017; Schlatter et al., 2017), few of them have used these results to selectively isolate new biocontrol agents (Liao et al., 2021; Niem et al., 2020). This strategy was used to discover, among others, new biocontrol agents as yet unknown for such activity. Once the biocontrol agents have been isolated, numerous *in vitro* bioassays are available to screen their antagonistic activity against a pathogen (Balouiri et al., 2016). Among the *in vitro* screening methods available, the best known and most used ones are the dual culture plate assays (Raymaekers et al., 2020). They are commonly used because they allow screening many isolates at the same time with minimum space needs. They are often used as a first screening step to decrease the number of strains before *in vivo* trials (Raymaekers et al., 2020). The *in vitro* first step was bypassed in the present study: the capacity of *P. aphanidermatum* to cause lettuce damping-off was used to develop a direct *in vivo* screening assay that combined the reliability of *in vivo* conditions with the advantages of *in vitro* bioassays. Once the best strains were screened, their efficacy was tested to control root rot of lettuce seedlings caused by the same pathogen. In summary, this study aims at isolating new biocontrol agents from aquaponics and testing them to control *P. aphanidermatum* lettuce diseases in soilless conditions.

2. Materials and Methods

2.1. Selection of antagonistic taxa

Microorganisms from aquaponic lettuce rhizoplane have been found correlated with *P. aphanidermatum* lettuce disease suppression (Stouvenakers et al., 2020). Using HTS analysis, a list of OTUs potentially linked to this suppressiveness was established (Stouvenakers et al., 2020). Then, bacteria and fungi (including Chytridiomycota phylum) to be isolated were selected based on this list and the literature. This strategy was named HTS-guided. The first criterion for taxon selection was a correlation of operational taxonomic units (OTUs) with suppressiveness in Stouvenakers et al. (2020). The second, optional criterion, was a documented pathogen suppressive action of the taxon in literature. Therefore, the OTUs considered as original, relatively abundant and *P. aphanidermatum*-suppressive in the study were selected. The selected OTUs and the isolation methods (further described) are summarized in Table E-1. If the taxonomic rank of a selected OTU was not deep enough, the key genera to be isolated inside the rank were selected according to their antagonistic potentialities in the literature and/or their potential identity after a nucleotide blast search on the NCBI platform (https://blast.ncbi.nlm.nih.gov). The targeted genera were *Burkholderia*, *Chitinimonas*, *Mitsuaria*, *Lactobacillus*, *Methyloversatilis*, *Sphingobium*, *Hydrogenophaga*, *Catenaria*, *Rhizophydium* and *Trichoderma*.

Table E-1: Target genera for selective isolation, and methods depending on OTU identification. CDA, MRS, R2A and RB-S-F are culture media. Chito., Lact., ForE., ForG., S./H., OosBait., MycBait., and Trich. are abbreviations of the isolation methods. Order, family, and genus taxonomic ranks were indicated by o_, f_, and g_ prefixes, respectively.

	OTU TAXA	TARGET GENERA	ISOLATION METHOD	ABBR.
Bacteria	f_Burkholderiaceae	*Burkholderia* *Chitinimonas* *Mitsuaria*	Chitosan degrading on CDA medium + colony morphological observation + Gram stain	Chito.
	g_*Lactobacillus*	*Lactobacillus*	MRS medium growth + colony morphological observation + Gram stain	Lacto.
	g_*Methyloversatilis*	*Methyloversatilis*	Enrichment or growth with formaldehyde and methanol + colony morphological observation + Gram stain	ForE. or ForG.

	g_*Sphingobium* g_*Hydrogenophaga*	*Sphingobium* *Hydrogenophaga*	R2A medium growth + colony morphological observation + Gram stain	S./H.
Fungi	g_*Catenaria* o_Rhizophydiales	*Catenaria* *Rhizophydium*	Oospores or mycelium baiting technique + morphological observation	OosBait. or MycBait.
	f_Hypocreales	*Trichoderma*	RB-S-F agar medium + morphological observation	Trich.

2.2. Strain isolation

The aquaponic samples used to isolate the targeted microorganisms were the -80°C frozen samples of lettuce rhizoplane washing water (plus 25% glycerol) taken during the first suppressive *in vivo* experiment by Stouvenakers et al. (2020**)**. When selective isolation was not conclusive, washing water of fresh lettuce rhizoplane was used instead. Lettuce plants for fresh rhizoplane isolation were grown in the PAFF Box aquaponic system of Gembloux Agro-Bio Tech, University of Liège (Belgium). The system is described in Stouvenakers et al. (2020**)**, and a block diagram is given in Eck et al. (2021). Rhizoplane water was collected by root sonication for 10 min in a 0.05 M kalium phosphate buffer plus 0.05% Tween 80 (KPBT), as described in Stouvenakers et al. (2020**)**. Growth rooms were set at a day/night photoperiod of 18/6h at 23°C or 28°C for all isolation protocols and incubating periods. After selective growth and selection processes, the selected strains were stored at -80°C in 0.85% NaCl sterile water plus 25% glycerol for further identification. During selective isolation, non-targeted microorganisms where voluntarily kept if they were described such as antagonistic microorganisms in the literature ('Lit' criterion, Table E-2) even though they were not initially targeted. Description of the methods used to isolate the targeted microorganisms is fully provided in Supplementary Materials S1 and summarized in Table E-1. Isolation and then the screening study was completed by testing specific ordered strains: *Methyloversatilis universalis* (DSM 25237) and *Hydrogenophaga pseudoflava* (DSM 1034) were received from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and *Catenaria anguillulae* (CBS 423.65) from CBS-KNAW (Westerdijk Fungal Biodiversity Institute). *Pseudomonas chlororaphis* Tx-1 (ATCC 55670) from the American Type Culture Collection (ATCC) was also ordered to be used as a bio-fungicide control (Cpc) in the *in vivo* screening. *P. chlororaphis* Tx-1 is an Environmental Protection Agency (EPA) registered biocontrol agent to control *Pythium* diseases in soil which was described as one of the most relevant microorganisms to control *P. aphanidermatum* in soilless culture (Chatterton et al., 2004; Khan et al., 2003; Liu et al., 2007).

2.3. Strain identification

DNA extractions were carried out from bacterial cells or fungal mycelial mats resuspended in sterile Milli-Q water. The FastDNA Spin Kit with TC cell lysis solution (MP Biomedicals, Illkirch-Graffenstaden, France) was used to start with, from 200 µl of suspension. The DNA extraction steps were adapted from the manufacturer's instructions, according to Eck et al. (2019). 16S rDNA and the ITS1-ITS4 regions were amplified for bacteria and fungi, respectively. Forward primer 16S A1 (5'- AGAGTTTGATCMTGGCTCAG-3') and reverse primer 16S B1 (5'-TACGGYTACCTTGTTACGACTT-3') were used for bacteria, while forward primer ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for fungi. PCR mixtures were prepared using the MangoTaq™ DNA Polymerase kit (Bioline, London, UK) manual. For bacteria, thermocyclers were run with an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 70°C for 2 min, and a final extension step at 72°C for 10 min. For fungi, the initial denaturation step was set at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were purified with QIAquick PCR Purification Kit (QIAGEN Benelux B.V., Antwerp, Belgium) before Sanger sequencing with the same primers at Macrogen Europe B.V. (Amsterdam, The Netherlands). Sequences were assembled using CAP3 program (Huang and Madan, 1999) and quality trimmed using Chromas software (http://technelysium.com.au/wp/chromas). The edited sequences were annotated by BLASTN analysis against the rRNA/ITS database using NCBI website (www.ncbi.nlm.nih.gov/blast) for closest identification with 97% identity minimum.

2.4. In vivo screening

Isolates and ordered strains were screened *in vivo* for their capacity to control lettuce damping off caused by *P. aphanidermatum*. Three different methods of seed treatment were tested in an experimental design set up using 96-well microplates.

2.4.1. Experimental and seed treatment setups

Organic pelleted seeds of lettuce (*Lactuca sativa*) var. Lucrecia RZ (Rijk Zwaan, Merksem, Belgium) were sowed without substrate in 96-well microplates (Greiner Bio-One B.V.B.A., Vilvoorde, Belgium) at a density of one seed by well. One microplate column (eight wells) corresponded to one replicate. Three methods were tested to treat seeds in relation to *P. aphanidermatum* inoculation: pre-inoculation, co-inoculation and biopriming (i.e., contrary to pre-inoculated seeds, germination of biopriming seeds was stabilized over time by air drying before sowing). Whatever the method, the seeds were treated with 10 µl of isolate suspension per seed on day 0 (see next section for the preparation of isolate suspensions). For pre-inoculation method,

microplates were directly sealed with self-adhesive film after seeds treatment. For biopriming, the seed pellets were left to dry under a laminar flow hood for 30 min before sealing. In the co-inoculation experiment, 100 µl of *P. aphanidermatum* oospores at a concentration of 10^4 oospores/ml were added *per* well before microplates sealing and just after the seed pellet had absorbed the treatment (see "Production of *P. aphanidermatum* inoculum" section for a description). For the pre-inoculation and biopriming treatments, oospores were added three days later after self-adhesive film removal. The microplates were then sealed again with a self-adhesive film that was punctured with a needle above each well to allow air exchange. The microplates were incubated at 23°C. Dark conditions were set before pathogen inoculation (i.e., only for the pre-inoculation and biopriming treatments), and a day/night photoperiod of 18/6h was set afterward. Seven days after *P. aphanidermatum* inoculation, seed damping off was binary scored. Dead seeds were scored 0, while healthy seeds with emerged cotyledons were scored 1. At the end of the screening assay, a strain was considered efficacious when a germination rate threshold of 37.5%, 12.5%, or 12.5% (i.e., three, one, or one seed out of eight, respectively) was reached in pre-inoculation, co-inoculation, or biopriming, respectively. Firstly, and because of the high number of isolates, only one strain *per* species accession was tested. Replications (i.e., columns of eight seeds) were carried out for the strains that protected at least 12.5% of the seeds. Other strains of a given species accession were screened (within a same treatment method) in three columns if the first tested strain in the accession allowed a seed germination rate of at least 12.5% in co-inoculation, 12.5% in biopriming, or 25.0% in pre-inoculation.
Four controls were used in each microplate at a configuration of one column (eight seeds) per control. Negative control (C-) seeds were treated with KPBT buffer and inoculated with the sucrose + Tween solution used for the oospore suspension. Positive control (C+) seeds were treated with KPBT buffer and inoculated with oospores. Fungicide control (Cf) seeds were treated with Proplant® (722 g/l propamocarb) at a concentration of 0.1% in KPBT buffer and inoculated with oospores. Proplant® is a propamocarb fungicide approved in Europe and was the sole chemical fungicide registered in Belgium to control *Pythium* diseases on lettuce. Bio-fungicide control (Cpc) seeds were treated with *P. chlororaphis* Tx-1 suspension (like the tested isolates were) and inoculated with oospores.

2.4.2. Isolate culture and suspension

Bacteria were grown on solid medium at 28°C for 3 days. R2A medium was used for all bacterial isolates except *Methyloversatilis* and *Hyphomicrobium* for which MIN E medium was used. All fungal isolates except *Catenaria anguillulae* were grown on PDA dishes at 23°C for 7 days. *C. anguillulae* grown on YPSS agar medium (20 g soluble starch, 1 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 15 g agar, 1000 mL distilled water) at 28°C was used to inoculate PYG agar plates (1.25 g peptone, 1.25 g

yeast extract, 3 g glucose, 1000 mL distilled water, pH 6.8). PYG plates were incubated at 28°C for 7 days to produce sporangia. Zoospores were washed off from the sporangia (based on Jansson and Thiman, 1992) by 1h flooding in KPBT solution. All bacterial cultures and fungal spores (i.e., fungal conidia and *C. anguillulae* zoospores) were harvested in KPBT buffer by surface scratching. Bacterial suspensions were diluted to reach 0.825 ±0.025 absorbance at 600 nm. An absorbance of 0.800 equalled to 5 x 10^7 CFU/ml for *P. chlororaphis* Tx-1. Fungal spore suspensions were cheesecloth-filtered and diluted to a concentration of 1x10^7 spores/mL after microscope count on a haemocytometer. When bacterial or fungal suspensions were not concentrated enough, they were centrifuged at 3000 g for 10 min and set to the right concentration after discarding the supernatant. After a first screening step at a concentration of 0.825 ±0.025 absorbance units, the most efficacious strains were tested at a 10-fold concentration (10x).

2.4.3. Production of *P. aphanidermatum* inoculum

Sterile 150-mL Erlenmeyer flasks containing 25 mL of clarified V8 $CaCO_3$ broth (800 mL distilled water, 200 mL V8 juice, 3 g $CaCO_3$) were inoculated with 5-mm PDA culture plugs of *P. aphanidermatum* (CBS 132490) grown at 23°C with 18 h/6 h lighting for 3 days. The flasks were closed with a cotton ball and incubated at 23°C with 18 h/6 h lighting for 9 days. Each mycelial bulk was recovered and rinsed by vortexing in a 50-mL centrifuge tube filled with 15 mL of sterile distilled water. The operation was repeated at least twice until V8 colour loss. Each mycelium bulk was cut in 2 pieces, and each half was incubated at 28°C with lighting for 24h in a 50-mL centrifuge tube filled with 30 mL of sterile distilled water. The mycelium pieces were recovered and mixed for 3 s 8 times with a hand blender (Braun Minipimer Control Plus, 300w) in a sterile solution containing 10 mM sucrose and 0.05% Tween 20 in distilled water. A proportion of at least one mycelium piece for 12.5 mL of solution was used with a minimum volume of 100 mL. The resulting propagule suspension was filtered through sterile cheesecloth to harvest the oospores, which were counted on a haemocytometer. The concentration was set at 1x10^4 oospores/mL.

2.5. Validation of biocontrol on lettuce seedlings

The eight most efficacious strains found in the *in vivo* screening assay were tested against root rot disease caused by *P. aphanidermatum* on lettuce seedlings. Lettuce seeds (see "*in vivo* screening") were sown in 25 × 25 × 40 mm rockwool plugs (Grodan B.V., Roermond, Holland) and placed in a phytotron, with a day/night photoperiod of 16 h/8 h, 22 °C/18 °C (day/night), and a relative humidity of 65% for the first 10 days of germination. See Stouvenakers et al. (2020) for lighting specificity. The seeds were inoculum-treated (1 mL *per* plug) on days 0 and 7. Each treatment occupied 2 plant trays (Ø 15 cm) containing nine rockwool plugs and one plant *per* plug. The eight treatments consisted of strains chito7, chito13, Mk, M25, M33,

SHb30, G2 and SHb18. The fungal strain G2 was produced in the same conditions as the screening conditions. Bacterial strains were grown in 250-mL Erlenmeyer flasks filled with 100 mL of the corresponding liquid medium (like the screening conditions, but without agar). After five days at 28°C and 100 rpm, the bacterial cultures were centrifuged at 4000 g. The pellets were rinsed with KPBT buffer, centrifuged again and finally resuspended in KPBT buffer. Bacterial and fungal cell suspensions were prepared at the concentration found most efficacious during the screening assay and with the same methodology as before. The controls were Proplant® fungicide (Cf) control), *P. chlororaphis* Tx-1 bio-fungicide (Cpc), aquaponic water control (Cap), negative healthy control (C-) and positive control (C+). KPBT was used to treat the rockwool plugs of C-, C+ and Cap. The fungicide was diluted at 0.1% in KPBT, and the *P. chlororaphis* Tx-1 suspension was set at $5x10^8$ CFUs/mL in KPBT. *P. chlororaphis* Tx-1 was produced in Erlenmeyer flasks, like the other bacterial strains. After 10 days of germination in tap water, a hydroponic solution (Hy-Pro A and B, Hy-Pro Fertilizers, Bladel, Holland) prepared according to the manufacturer's instructions was used to fill plastic trays (± 450 mL/tray). For Cap, aquaponic water from the PAFF Box aquaponic system (see Stouvenakers et al., 2020) was used throughout the experiment. On day 10, the rockwool plugs were inoculated with a suspension of *P. aphanidermatum* oospores prepared as before (1 mL *per* plug). The phytotron parameters were adjusted the same day to reach 35/25 °C (d/n; 16 h/8 h) and 92% relative humidity. Water evaporation/evapotranspiration from the trays was compensated for with nutrient solution and demineralized water three times a week. Twenty-one days after *P. aphanidermatum* inoculation (i.e., 31 days after sowing), foliar fresh mass (FFM), foliar dry mass (FDM), root rot rating (RRR) and lettuce mortality (LM) were measured according to Stouvenakers et al. (2020). Three indexes of disease symptom reduction were calculated from these raw data – root symptom reduction (RSR), foliar mass improvement (FMI), and wilt symptoms reduction (WSR) –, and expressed in percentages relative to C- and C+. WSR was based on foliar water content (FWC) calculated according to Stouvenakers et al. (2020). The formula used to calculate the disease symptom reduction indexes was as follows:

$$Index = \frac{\left(\frac{V_{mean\ of\ C-} - V_{mean\ of\ C+}}{V_{mean\ of\ C-}}\right) - \left(\frac{V_{mean\ of\ C-} - V_T}{V_{mean\ of\ C-}}\right)}{\left(\frac{V_{mean\ of\ C-} - V_{mean\ of\ C+}}{V_{mean\ of\ C-}}\right)}$$

Where V is the value of RRR, FFM or FWC depending on whether the calculated disease symptom reduction index is RSR, FMI or WSR, respectively. C- and C+ are the controls, and T is the treatment.

The experiment was performed twice, and RSR, FMI and WSR data were statically analyzed using Minitab v.19 software (Minitab Inc., State College, PA, USA). First the conditions of application were tested, and then 2-way analyses of variance

(ANOVAs) were performed with treatment and replicate factors. When a significant interaction between factors was observed, the 2-way ANOVA was decomposed in 1-way ANOVA. Tukey's multiple comparison post hoc test was used to compare treatments pairwise.

3. Results

3.1. Isolation

Eighty-two bacterial strains and 18 fungal strains were kept after selection; they are listed in Table E-2. Twenty-nine different bacterial species and eight different fungal species were identified among these 100 strains. They were used in the following *in vivo* screening assay. Among them, four were also potential suppressive microorganisms in the HTS study of Stouvenakers et al. (2020), and not known for this effect in the literature ('HTSg' criterion, Table E-2). Twenty-two species were identified as potential plant-beneficial microorganisms in the literature ('Lit' criterion, Table E-2). Seven species were identified as pathogen suppressive by the HTS study and as plant beneficial in the literature ('HTSg/Lit' criterion, Table E-2). In total, 43 % of the isolated strains had been identified as pathogen suppressive in the HTS study of Stouvenakers et al. (2020).

The targeted fungal genera *Catenaria, Rhizophydium* and *Trichoderma* were not isolated. However, non-targeted strains belonging to *Cladosporium*, *Aspergillus* and *Penicillium* were kept for screening because of their relatively high abundance (more than 0.5%), their aquaponic suppressiveness mentioned in Stouvenakers et al. (2020), and their potential antagonistic activities mentioned in the literature ('HTSg/Lit' criterion). Regarding bacteria, only *Sphingobium* was successfully isolated among the targeted microorganisms. Instead of *Methyloversatilis*, numerous non-targeted *Hyphomicrobium* spp. strains were isolated because of their formaldehyde resistance. However, they were kept because of their significant suppressiveness and higher abundance (1.36%) in Stouvenakers et al. (2020) study (HTSg criterion). Numerous non-targeted heterotrophic and/or methylotrophic bacteria were isolated. At the genus level, most of them were already listed in Stouvenakers et al. (2020), but in low abundant (<0.1%). Because most of these bacteria were found potentially beneficial to plants in the literature, they were all used in the screening study ('Lit' criterion). Among them, the genera *Microbacterium, Micromonospora, Mycolicibacterium, Nocardia* and *Streptomyces* were all actinomycetes, a group commonly described as plant beneficial (Reddy, 2014).

Table E-2: Isolate identification depending on the isolation method, the origin (1: frozen aquaponic rhizoplane water from Stouvenakers et al. (2020), 2: fresh aquaponic rhizoplane water, and 3: ordered strain), and selection criteria. Chito., Lact., ForE., ForG., S./H., OosBait., MycBait., and Trich. are isolation methods for Burkholderiaceae family, *Lactobacillus* genus, *Methyloversatilis* genus using enrichment, *Methyloversatilis* genus without enrichment, *Sphingobium*/*Hydrogenophaga* genera, *Catenaria*/*Rhizophydium* genera using oospores as bait, *Catenaria*/*Rhizophydium* genera using mycelium as bait, and *Trichoderma* genus, respectively. The species selection criteria for the screening study were as follows: 'HTSg', HTS-guided (i.e., potential suppressive pathogen identified by HTS in Stouvenakers et al. (2020) at the genus level); 'Lit', literature-guided (i.e., plant beneficial in the literature at the genus level, at least); 'HTSg/lit', both criteria-guided; NA, not applicable.

Species name	**Isolate abbr.**	**Method**	**Origin**	**Criteria**
Bacteria				
Bacillus flexus	SHb2	S./H.	2	Lit
Bacillus indicus	SHb31	S./H.	2	Lit
Bosea thiooxidans	SH6, SH9	S./H.	1	Lit
Enterobacter cloacae complex	L13	Lacto.	2	Lit
Hydrogenophaga pseudoflava	DSM 1034	/	3	HTSg
Hyphomicrobium sp.	M8	ForG.	2	HTSg
Hyphomicrobium vulgare	M18	ForG.	2	HTSg
Hyphomicrobium zavarzinii	M13	ForG.	2	HTSg
	M25, M27, M28, M31, M32, M34, M35, M36, M37, M38	ForG.	1	
	MetA, MetB, MetC, MetD, MetE, MetF, MetG, MetH	ForG.	1	
Methylorubrum podarium	Chito6	Chito.	1	Lit
Methylorubrum populi	Mc, Mk, Mq	ForE.	1	Lit
Methyloversatilis universalis	DSM 25237	/	3	HTSg
Microbacterium kitamiense	SHb4	S./H.	2	Lit
Microbacterium lacus	SHb23, SHb25	S./H.	2	Lit
Microbacterium paraoxydans	SHb18	S./H.	2	Lit
Microbacterium sp.	SH10, SH22, SH28	S./H.	1	Lit
Micromonospora maritima	SH32	S./H.	1	Lit

Mycolicibacterium aurum	M1, M2, M7, M15, M19, M23	ForG.	2	Lit
Mycolicibacterium fluoranthenivorans	M5, M6, M11, M16, M17	ForG.	2	Lit
Mycolicibacterium fortuitum	Chito1, Chito 5, Chito8, Chito11, Chito13, Chito16, Chito17, Chito18	Chito.	1	Lit
Mycolicibacterium sp.	Chito10	Chito.	1	Lit
Mycolicibacterium sp.	Chito2	Chito.	1	Lit
Mycolicibacterium wolinskyi	M33	ForG.	1	Lit
Nocardia fluminea	Chito7	Chito.	1	Lit
Novosphingobium aromaticivorans	SHb3, SHb10, SHb15, SHb16, SHb17, SHb21, SHb28	S./H.	2	Lit
Pedobacter solisilvae	SHb7, SHb26, SHb34	S./H.	2	Lit
Rhizobium sp.	SHb32	S./H.	2	Lit
Rummeliibacillus suwonensis	L2, L5, L9, L10, L11	Lacto.	2	NA
Sphingobium xenophagum	SHb9, SHb14, SHb27, SHb30	S./H.	2	HTSg
Streptomyces coelicoflavus	SHb13	S./H.	2	Lit
Fungi				
Aspergillus flavus	TS1 G2	Trich. MycBait	2 1	HTSg/Lit
Aspergillus fumigatus	G1	MycBait	1	HTSg/Lit
Catenaria anguillulae	CBS 42365	/	3	HTSg/Lit
Cladosporium halotolerans	TS6, TS10	Trich.	2	HTSg/Lit
Cladosporium ramotenellum	TS11	Trich.	2	HTSg/Lit
Cladosporium sp.	TS13	Trich.	2	HTSg/Lit
Cladosporium sphaerospermum	TS2, TS3, TS4, TS7, TS9, TS12, TS14	Trich.	2	HTSg/Lit
Penicillium citrinum	PC1, PC2, PC3, G3	MycBait	1	HTSg/Lit

3.2. In vivo screening

For this screening, a strain was considered efficacious to control *P. aphanidermatum* damping-off when a germination rate threshold of 37.5% or 12.5% was reached in pre-inoculation or in biopriming, respectively (see Section 2.4.). Efficacious strains represented by the most efficacious strain of each species are shown in Figure E-1A and E-1B for pre-inoculation and biopriming, respectively. No strain reduced seed damping-off following co-inoculation (data not showed). The full screening results are presented in Table E-S1. Firstly, seed germination was better following biopriming than following pre-inoculation. The mean germination rates of C- were 93.5% following biopriming and 60.0% following pre-inoculation. However, seed damping-off was more aggressive following biopriming than following pre-inoculation. The mean germination rates of C+ were 0.1% and 29.1% following biopriming and pre-inoculation, respectively. Cf, Cpc and Cpc.10x were not efficacious following pre-inoculation (≤ mean C+), while a minimal action was recorded for Cf (7.0%) and Cpc (6.4%) following biopriming (≥ mean C+). However, a better mean germination rate was observed following biopriming in Cpc.10x (28.1%).

Following pre-inoculation, seven bacteria and one fungus were found efficacious to control seed damping-off (Figure E-1A). They corresponded to *Mycolicibacterium fortuitum* (Chito13 type strain), *Nocardia fluminea* (strain Chito7), *Hyphomicrobium zavarzinii* (M25 type strain), *S. xenophagum* (SHb30 type strain), *Methylorubrum populi* (Mk type strain), *Mycolicibacterium wolinskyi* (strain M33), *Microbacterium paraoxydans* (strain SHb18) and *A. flavus* (G2 type strain). At a standard concentration (OD = 0.825 ± 0.025), Chito13, Shb30 and Mk were the most efficacious strains, and allowed for a mean seed germination rate of at least 50%. Moreover, Chito 13 allowed for a germination rate similar to that of C- (mean 58.4%). When the seeds were treated with 10x concentrated suspensions, the mean germination rates following Chito13, Chito7, and M25 treatments increased up to 79.2%, 75.0% and 66.7%, respectively. Moreover, these 10x treatments allowed for a better germination rate than the C- healthy control did.

Following biopriming, strain *Hyphomicrobium sp.* M8 and the 2 already known strains SHb30 and G2 highlighted by pre-inoculation also proved efficacious (Figure E-1B). At the standard concentration, the mean germination rates following SHb30, G2 and M8 treatment were 18.8%, 12.5% and 12.5%, respectively. When the 10x suspension was used, the mean germination rates increased up to 37.5% following the SHB30.10x and G2.10x treatments.

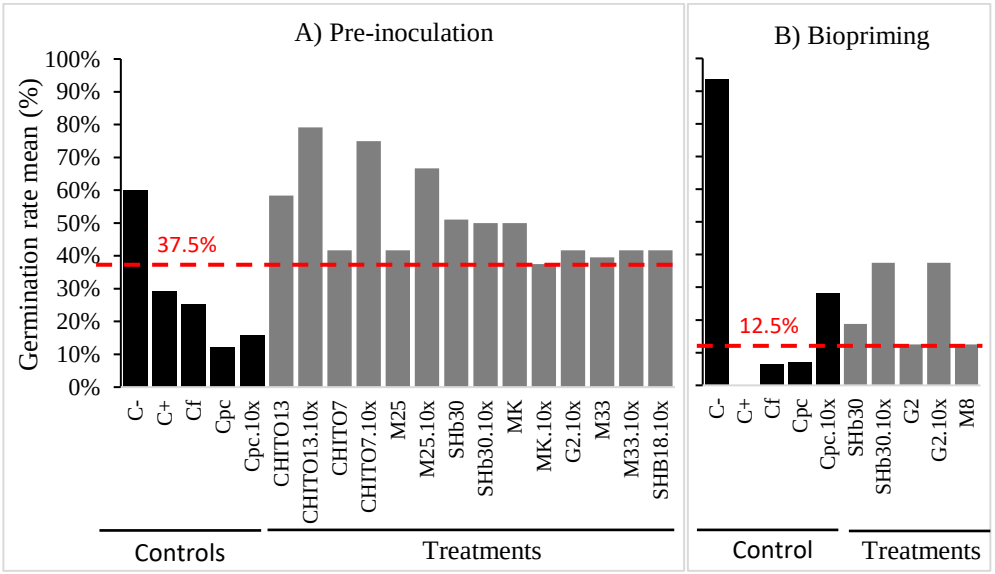


Figure E-1: Mean germination rates of lettuce seeds treated to control *P. aphanidermatum* damping-off depending on (A) pre-inoculation or (B) biopriming. Only the type strains that reached efficacy thresholds are showed in 'Treatments' (full results are in Supplementary Material E). Dotted lines, efficacy thresholds. ".10X", 10x concentrated treatments. C-, C+, Cf, and Cpc/Cpc.10x are the negative, positive, fungicide and biofungicide controls, respectively. Standard errors of the mean (SE) were not relevant and are not showed because of the binary scoring of the germination rate and the non-balanced data.

3.3. P. aphanidermatum control on lettuce seedlings

Two bioassays were carried out on lettuce seedlings to test the efficacy of the eight best strains found in the screening to control *P. aphanidermatum* root rot disease. Significant interactions (p=0.000) were found between the bioassay replicates and the treatment factors. The treatment effects were analyzed depending on the bioassay (i.e., 1 or 2).

In bioassay 1, SHb30, C13 and G2 were the only three treatments able to fully control lettuce mortality (i.e., LM = 0%), In comparison with controls, lettuce mortality of C+, Cf, Cap and Cpc were 33.3%, 0%, 0%, and 0%, respectively (Table E-3). Among treatments applied in the second bioassay, only G2 and Chito13 were able to inhibit lettuce mortality (i.e., LM = 0%), *versus* 38.9%, 0%, 0%, and 38.9% for C+, Cf, Cap and Cpc, respectively (Table E-3).

Considering the disease symptom reduction indexes (RSR, FMI and WSR) in bioassay 1, the best treatment was SHB30, followed by Chito13 and G2 (Figure E-2). The disease symptom indexes of SHb30-treated lettuce were all significantly lower

than those of C+ ($p \leq 0.05$), and no difference was found with C- ($p > 0.05$). The symptom reduction rates were 67.9%, 131.8%, and 83.2% for RSR, FMI and WSR, respectively. In particular, the high value of FMI (131.8%) following SHb30 treatment indicated that leaves tended to be more developed than in C- lettuce ($p > 0.05$), and that foliar symptoms were eradicated. Moreover, SHb30 tended to allow for a higher FMI than Cf did (62.4%; $p > 0.05$), and a higher FMI than Cpc did (54.7%; $p \leq 0.05$). Root protection following Chito13 and G2 treatments was intermediary (RSR = 44.9% and 47.1%, respectively) and at a similar level as in Cf (RSR = 29.4%) and Cpc (RSR = 42.2%) lettuce ($p > 0.05$) (Figure E-2A). FMI following Chito13 (93.0%) and G2 (66.8%) treatments was not significantly different than in C- and Cf lettuce (62.4%; $p > 0.05$) (Figure E-2B). Chito13 reduced leaf wilting (WSR = 72.1%) in a comparable way as in C- ($p > 0.05$; Figure E-2C). WSR following G2 treatment was more intermediary (52.2%). However, WSR following G2 and Chito13 treatments was not different than in Cf lettuce (67.7%; $p > 0.05$).

Table E-3: Lettuce mortality (LM) following treatment with the 8 microbial strains (Chito13, Mk, Chito7, M33, SHb30, M25, G2 and SHb18) against *P. aphanidermatum* disease on lettuce seedlings depending on the bioassay replicate (1 or 2). C+, C-, Cf, Cap and Cpc are the positive, negative, fungicide, aquaponic and biofungicide controls, respectively. Statistics not applicable.

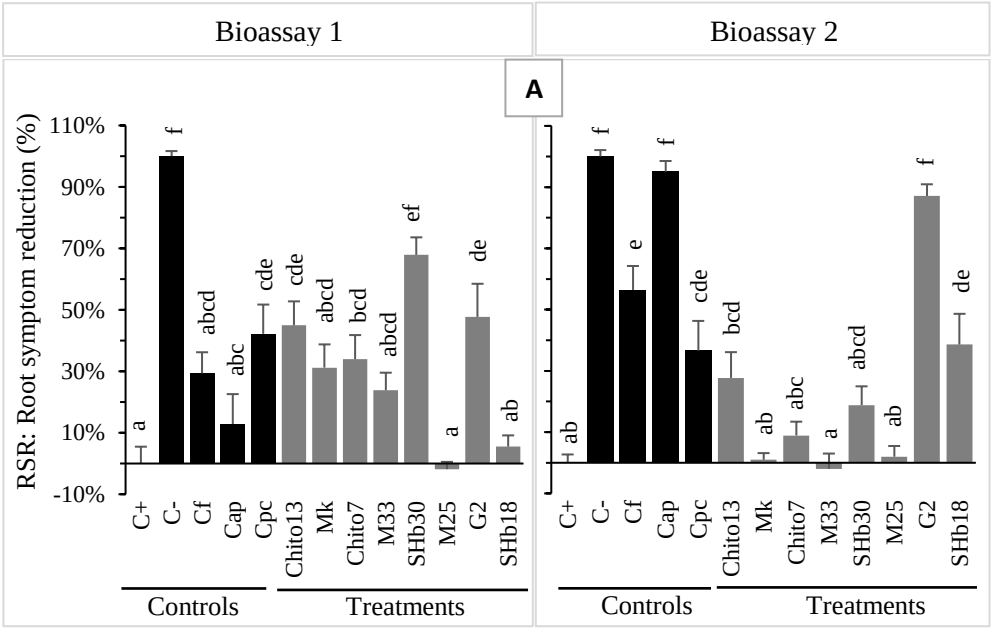
	LM: lettuce mortality (%)												
	Controls					Treatments							
Bioassay	C+	C-	Cf	Cap	Cpc	Chito13	Mk	Chito7	M33	SHb30	M25	G2	SHb18
1	33%	0%	0%	0%	0.%	0%	6%	23%	6%	0%	11%	0%	11%
2	39%	0%	0%	0%	39%	0%	39%	28%	28%	28%	28%	0%	33%

In the second bioassay, the best treatment was G2 in terms of symptom reduction (Figure E-2): root and foliar symptoms (RSR = 87.1%, FMI = 61.3% and WSR = 102.7%) were significantly reduced compared to C+, and no difference ($p > 0.05$) was found with C-. Depending on the measure, the protective action of G2 was consistently similar ($p > 0.05$) or better ($p \leq 0.05$) than on Cf and Cpc lettuce. Disease symptom reduction following Chito13 treatment was intermediate (RSR = 27.7%, FMI = 47.4% and WSR = 63.9%) and not significantly different than in Cf and Cpc lettuce (Figure E-2). SHb30 treatment was less efficacious in reducing disease symptoms in bioassay 2 than in bioassay 1. The symptom reduction rates following SHb30 treatment were quite low (RSR = 18.8%, FMI = 19.0% and WSR = 38.5%), and no difference with C+ was recorded ($p > 0.05$; Figure E-2).

M25 treatment was not efficacious in controlling the disease whatever the bioassay replicate. RSR, FMI and WSR were never different than in C+ lettuce ($p > 0.05$; Figure E-2). Although RSR, FMI and WSR of Mk, Chito7, M33 and SHb18

treatments were often not statistically different than in C+ lettuce, other comparisons were made (Figure E-2). The symptom reduction indexes of Mk, Chito7, M33 and SHb18 were not different ($p > 0.05$) than in Cf and Cpc lettuce in bioassay 1 (except for SHb18 WSR, lower than that of Cf). In bioassay 2, the symptom reduction levels following SHb18 treatment were similar to those of Cf and Cpc lettuce ($p \leq 0.05$), while Mk, Chito7, and M33 were less efficacious than Cf ($p \leq 0.05$).

Finally, no lettuce mortality was recorded in Cap control, but disease symptoms were reduced only during the second bioassay. This difference could be explained by the water sampling time that differed by 2 months.



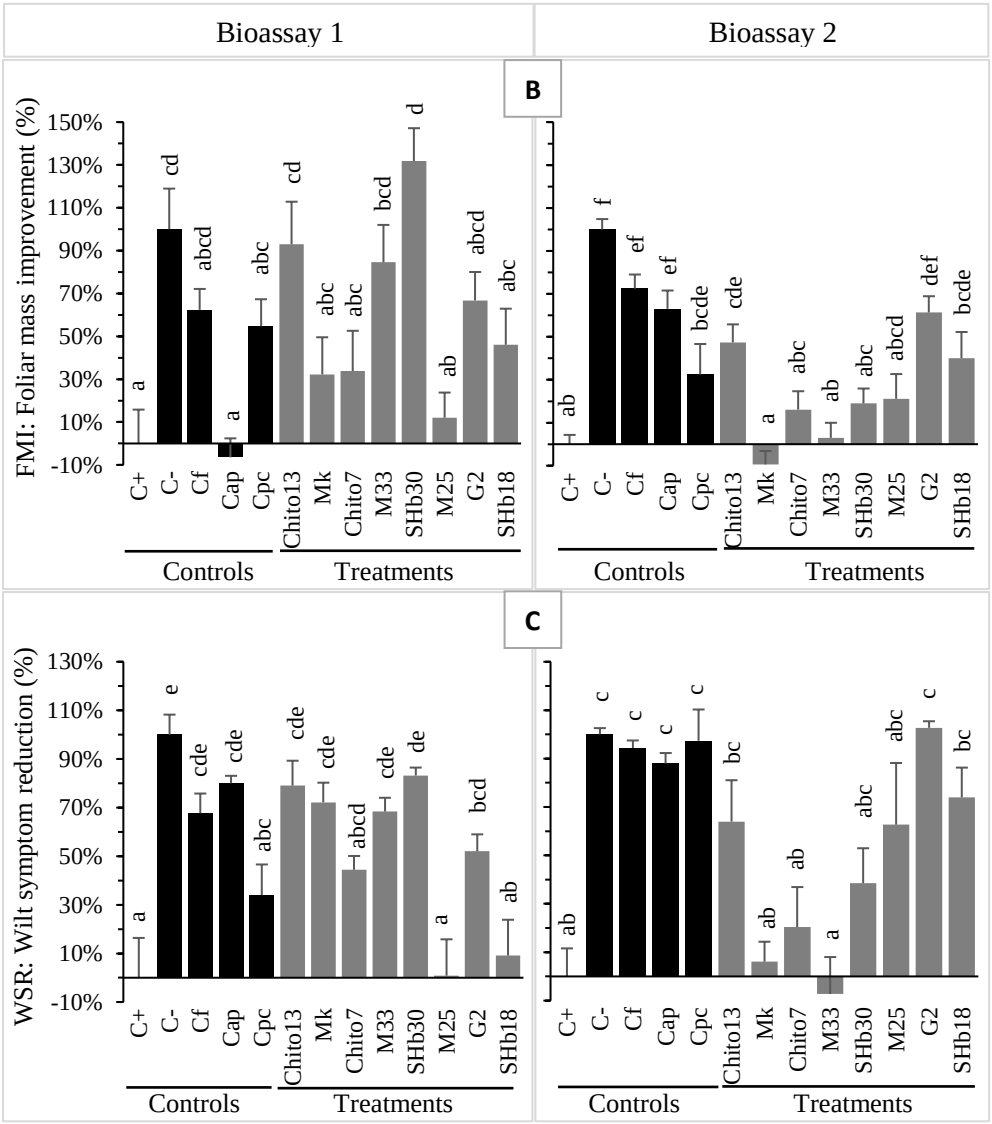


Figure E-2: Mean bar charts of (A) root symptom reduction (RSR), (B) foliar mass improvement (FMI), and (C) wilt symptom reduction (WSR) following treatment with the eight microbial strains (Chito13, Mk, Chito7, M33, SHb30, M25, G2 and SHb18) against *P. aphanidermatum* disease on lettuce seedlings depending on the bioassay replicate (1 or 2). C+, C-, Cf, Cap, and Cpc are the positive, negative, fungicide, aquaponic and biofungicide controls, respectively. Bars indicate the standard error of the mean (SE), and different letters indicate significant differences ($p \leq 0.05$) between treatments according to Tukey's ANOVA *post hoc* test.

4. Discussion

The strategy proposed in this study for isolating *P. aphanidermatum* biocontrol agents is original in several ways. The first one is the biotope used for isolation. The potential suppressiveness of aquaponic systems has been raised and discussed only recently. Before this study, only one had been devoted to isolating biocontrol agents from microbial populations of aquaponic systems (Sirakov et al., 2016). The second originality is the HTS-guided strategy used to select potential biocontrol agents to be isolated. The genera identified by HTS and bioinformatic analysis as interesting candidates for plant pathogen suppression in Stouvenakers et al. (2020) were targeted during the isolation step. The targeted lettuce rhizoplane microorganisms were selected for their high relative abundance and their correlation with suppressiveness in Stouvenakers et al. (2020). HTS-guided isolation of specific microorganisms is novel, and only 3 papers have been found using a similar strategy (Liao et al., 2021; Niem et al., 2020; Wu et al., 2018). Potential biocontrol agents have traditionally been mainly isolated in artificial broad-range media, using *a priori*-free approaches (Expósito et al., 2017). Due to its lack of selectivity, this isolation strategy is followed by a tedious screening step including many isolates (Raymaekers et al., 2020). Moreover, because universal media that suit all microorganisms are not available, their use unavoidably leads to enrichment in certain microorganisms that do not necessarily have biocontrol properties (Davis et al., 2005), while microorganisms of interest can be missed. The application of isolation methods that target specific microorganisms is an alternative solution for isolating biocontrol agents, but it requires *a priori* targeting relying on pre-existing data (El-Tarabily and Sivasithamparam, 2006; Williams and Wellington, 1982). For example, microorganisms can be selected based on previous biocontrol activity showed in similar pathosystems by other strains belonging to a certain species or genus or family (El-Tarabily, 2006; Evangelista-Martínez, 2014). This introduces a significant bias because different isolates from a single species can present contrasting properties (Nadeem et al., 2016). For example, the fungal species *Fusarium oxysporum* includes isolates or subspecies highly pathogenic or beneficial for lettuce (Thongkamngam and Jaenaksorn, 2017; Whipps, 2001). Relying on the composition of microbial communities in the studied pathosystem avoids this drawback and can identify biocontrol agents belonging to species so far unknown as biocontrol agents in the scientific literature. Niem et al. (2020), Liao et al. (2021) and the present study all show that isolating strains belonging to taxa identified as potential biocontrol agents by HTS is feasible. However, this novel strategy also suffers from the weaknesses of HTS. Bias may be introduced at each step, from microbiota sampling to bioinformatic analyses (Boers et al., 2019). These biases may have influenced the list of OTUs linked to suppressiveness in Stouvenakers et al. (2020), qualitatively and quantitatively. Therefore, the list of microorganisms selected for isolation in our study

is also potentially distorted by HTS bias. Only a few initially targeted microorganisms were successfully isolated (4.0% of the isolated strains), while microorganisms found at a low abundance in Stouvenakers et al., (2020) were unintentionally isolated (59% of the isolated strains). However, 43% of the strains (whether targeted or not) isolated at the genus level were identified as pathogen suppressive in Stouvenakers et al. (2020). In addition to HTS bias, these results are not surprising for several reasons. HTS is not a culture-dependent technique, and the targeted microorganisms are not always culturable (Forbes et al., 2017; Garbeva et al., 2004; Stefani et al., 2015; Torsvik et al., 1996). Furthermore, taxonomic abundance in metagenomics studies does not reflect the abundance found in culture-dependent techniques (Forbes et al., 2017; Garbeva et al., 2004; Stefani et al., 2015). Rare microorganisms could be abundantly and easily isolated in culture media, and vice versa (Davis et al., 2005). A high abundance does not entail biocontrol action (Köhl et al., 2019).

The isolates were screened for antagonistic activity *in vivo*. The method was designed to benefit from the logistic advantages of *in vitro* bioassays without their biases. Antagonistic modes cannot all be tested *in vitro*, and positive antagonism *in vitro* does not necessarily predict antagonistic activity in more complex assays including plant hosts (Köhl et al., 2019). However, contrary to *in vitro* methods, our *in vivo* screening was subjected to pathosystem variability. The seed itself (its microbiome or its germination rate for example), the variability of pathogen aggressiveness, the timing, and conditions of the treatment (T°, HR) can influence the disease and the results (Köhl et al., 2019; Martin and Loper, 1999). For example, *P. aphanidermatum* disease and oospore germination are promoted by temperature higher than 25°C (Adams, 1971; Sutton et al., 2006), while germination of our lettuce seeds drastically decreases above 25°C.

Retrospectively, the HTS-guided strategy coupled with the *in vivo* screening assay was a reliable approach for identifying new biocontrol agents. Two of the 3 most efficacious isolates for controlling *P. aphanidermatum* root rot disease on lettuce had been characterized as suppressive in Stouvenakers et al. (2020). The genus *Sphingobium* was present at a high relative abundance (2 OTUs at 5.6% and 3.5%, respectively) and correlated with aquaponic suppressiveness in Stouvenakers et al. (2020). The family Aspergillaceae to which *A. flavus* belongs was present in medium relative abundance (0.5%) and was correlated with root symptom reduction. However, 3 important taxa targeted by the HTS-guided strategy were not isolated, and type strains were ordered. They were not efficacious in controlling *P. aphanidermatum* disease, but this does not disprove the strategy because OTU identification in Stouvenakers et al. (2020) was at best at the genus level and because different strains for a same species can express different level of biocontrol action (Nadeem et al., 2016).

The 3 most efficacious isolates for controlling the disease *in vivo* were *S. xenophagum* strain SHb30, *A. flavus* strain G2 and *M. fortuitum* strain Chito13. *S.*

xenophagum and *M. fortuitum* had never been described as root disease biocontrol agents. The bacterium *S. xenophagum* can degrade xenobiotic aromatic compounds and is studied for bioremediation of contaminated environments (Song et al., 2019; Stolz et al., 2000). This species was already identified in the lettuce root zone and notably in aquaponics where the genus is among the most abundant ones (Cardinale et al., 2015; Sare et al., 2020; Schmautz et al., 2017). Sphingomonadaceae are generally also well represented in suppressive soils (Expósito et al., 2017). The range of action of *S. xenophagum* against plant diseases is still unknown, but volatile organic compounds produced by the bacterium decrease the growth of the plant pathogen *Botrytis cinerea in vitro* (Ortega et al., 2016). The species has also been described once as a plant growth-promoting rhizobacterium (PGPR), and can produce siderophores and indole-3-acetic acid (Wanees et al., 2018). *Mycolicibacteria* are common rhizosphere bacteria mainly studied for bioremediation of contaminated environments (Bisht et al., 2015; Miller et al., 2004). *M. fortuitum* could be the causal agent of fish tuberculosis in aquaculture (Fattah and Sayed, 2006) and could be an opportunistic human pathogen susceptible to cause nontuberculous mycobacterial infection (Faria et al., 2015). Human, animal and plant health is a prior concern in developing biocontrol agents, but pathogenicity is not necessarily dependent on the species (Alabouvette and Cordier, 2011). Indeed, for the same species, a strain could be pathogenic or not (Alabouvette and Cordier, 2011). For example, numerous strains of *Pseudomonas fluorescent* were commercialized as biocontrol agents while others were reported as pathogenic for human (Nadeem et al., 2016; Von Graevenitz and Weinstein, 1971). Contradictory to our results, *Mycolicibacterium* spp. have been found to enhance *P. aphanidermatum* growth *in vitro* and to be tobacco black rot disease conducive (Burgos-Garay et al., 2014; Kyselková et al., 2009). However, the genus has also been described several times as a PGPR (Reddy, 2014; Sanchez et al., 2019). As for *A. flavus* (G2 strain), the species is a saprophytic soil fungus mainly known to produce the secondary metabolite aflatoxin in infected crops. However, atoxigenic strains are also used and studied to control aflatoxin-producing ones (Amaike and Keller, 2011; Khan et al., 2021). Two *A. flavus* strains (AF36 and NNRL 21882) are already EPA registered as biopesticides in the USA (https://www.epa.gov/). Furthermore, *A. flavus* has been screened as an antagonist of *P. aphanidermatum* in dual culture (Shanmugan and Sakurana Varma, 1999).

5. Conclusion

The HTS-guided strategy for isolating aquaponic microorganisms coupled with *in vivo* screening led to the identification of original biocontrol agents of *P. aphanidermatum* lettuce disease. Out of 100 isolates, 8 were considered efficacious in controlling *P. aphanidermatum* lettuce damping-off and selected to be tested on lettuce seedlings. The 3 most efficacious isolates were *S. xenophagum* strain SHb30,

A. flavus strain G2 and *M. fortuitum* strain Chito13. Strains SHb30 and G2 were isolated and selected according to our HTS-guided strategy, while C13 was selected according to a literature-guided strategy. Seed treatment with each of these 3 strains decreased *P. aphanidermatum* damping-off and was more efficacious than the fungicide and biopesticide controls. Lettuce seedling mortality (LM) was 0.0% in bioassay 1 following treatment with strains SHb30, G2 and Chito13. In bioassay 2, LM was higher following SHb30 treatment, but remained at 0.0% following G2 and Chito13 treatments. The biocontrol action of SHb30 was high in bioassay 1, but more limited in bioassay 2. The biocontrol action of G2 and Chito13 was more constant. The foliar mass improvement (FMI) index showed that foliar symptom reduction following G2 and Chito13 treatments was at least as good as in lettuce treated with the fungicide control. In conclusion, these results indicate that aquaponics is an important source of novel biocontrol agents that could be more adapted to soilless conditions than common soil biocontrol agents are. However, safe use of G2 and Chito13 must be first checked. SHb30, G2 and Chito13 are promising but need to be further studied (e.g., mechanisms of action), and possibly developed in biopesticide formulation.

Chapter F

Application of Aquaponic Microorganisms Alone or in Consortium as Original Biocontrol Method of Lettuce Root Rots in Soilless Culture

The material presented in this chapter is adapted from a manuscript accepted in Acta Horticulturae:

Stouvenakers, G., Massart, S., Jijakli, M.H., 2022. Application of aquaponic microorganisms alone or in consortium as original biocontrol method of lettuce root rots in soilless culture. Acta Horticulturae, p-p.

Abstract: Root pathogens in hydroponic culture are often difficult to control without the use of synthetic pesticides. Moreover, most of the available biocontrol agents were isolated from soils. They were not developed for soilless application and often resulted in poor efficacy. It is therefore important to find novel sources of beneficial microorganisms that could grow and protect plant root in such aquatic environment. In this context, aquaponic systems that combines hydroponic plant culture and fish farming were described as a promising source of biocontrol agents. From a previous study, three aquaponic microorganisms were isolated and selected to evaluate their capacity to control *Pythium aphanidermatum* root rot disease on lettuce. *Sphingobium xenophagum* SHb30, *Mycolicibacterium fortuitum* C13 and *Aspergillus flavus* G2 were the three strains used alone or in consortium to protect soilless lettuce. Treatments were compared with a biocontrol agent registered against *Pythium* diseases, a propamocarb fungicide and the direct use of aquaponic water. G2 treatment alone protected lettuce as well as the fungicide and the foliar fresh mass of lettuce was similar to healthy lettuce. C13 had no effect on the disease, while SHb30 limited foliar yield loss. Consortium containing G2 gave similar results than G2 alone but the SHb30+C13 combination tended to increase the protective effect in comparison with separated application. This study highlighted that aquaponic water or some of its microorganisms applied alone or in consortium could control *P. aphanidermatum* root rot disease on lettuce in soilless conditions in a similar way than a fungicide and with a better action than a registered biocontrol agent. Further research will aim at developing appropriated formulation to stabilize and improve biocontrol efficacy of these strains.

Keywords: biocontrol, antagonist, consortium, hydroponics, aquaponics, *Pythium aphanidermatum*, lettuce

1. Introduction

Commercialized biocontrol agents used to protect plant against root diseases in soilless culture often lack high efficacy. In fact, most of biocontrol agents were isolated from soil, then studied and developed for soil uses (Postma et al., 2008; Vallance et al., 2010; Montagne et al., 2017). The consequence is therefore a poor adaptation of these microorganisms to aquatic conditions and the specific ecological niches in soilless systems often found under greenhouse structures (Postma et al., 2008; Vallance et al., 2010). Moreover, in these specific conditions, some root pathogens particularly adapted to water can rapidly spread the disease in the system. It is particularly true for Oomycetes pathogens that produce flagellated spores, such as *Pythium aphanidermatum* (Edson) Fitzp. This fungal pathogen causes root rot disease on lettuce (Sutton et al., 2006) in hydroponics and aquaponics. For aquaponics, chemical pesticides are inadvisable because of the presence of fish in the

same water loop as plants (Stouvenakers et al., 2019). To find novel biocontrol agents adapted to such environment, an isolation campaign of beneficial microorganisms found in hydroponics was led around 1995 and onward, but very few isolates led to commercialization (McPherson et al., 1995; Vallance et al., 2010). Nevertheless, it was recently highlighted that aquaponics could contain original microorganisms able to control *P. aphanidermatum* disease (Stouvenakers et al., 2020). From the list of potential antagonistic microorganisms set in it, a selective isolation was undertaken by Stouvenakers et al., 2022 and led to 100 isolates. The isolates were then screened to control *P. aphanidermatum* disease on lettuce. In the present study, the 3 most effective isolates detected in the past screening were applied alone or in consortium to control the same plant pathogen on lettuce seedlings.

2. Materials and methods

2.1. Treatments and controls

The three most effective isolates for controlling *P. aphanidermatum* on lettuce identified by Stouvenakers et al. (2022) were selected for this experiment. Two bacteria and one fungus were used alone or in consortium against the pathogen. They were *Sphingobium xenophagum* strain SHb30, *Mycolicibacterium fortuitum* strain C13 and *Aspergillus flavus* strain G2. The two bacteria were produced in liquid rich medium (R medium) that contained in 1 l of distilled water: 10 g peptone, 5 g yeast extract, 5 g malt extract, 5 g bacto-casamino acids, 2 g beef extract, 2 g glycerol and 1 g $MgSO_4$ (Hamana et al., 2015). Bacteria were incubated at 28°C with 100 rpm shaking for 3 days. Bacterial pellets were recovered by culture medium centrifugation at 4000G for 10 min. Pellets were rinsed with 0.05M Kalium Phosphate Buffer plus 0.05% Tween 80 (KPBT), centrifuged again and then resuspended in KPBT. Concentration of the suspensions were determined by spectrophotometer set at 600 nm and adjusted to $1x10^9$ cfu/mL in KPBT. G2 fungus was grown in potatoes dextrose agar (PDA, Merck Millipore) and incubated at 23°C for 7 days. Spores were scratched off in KPBT and filtered through cheesecloth. Spore concentration of the filtrate was measured in haemocytometer and fixed at $1x10^8$ spores/mL. Produced bacterial and fungal suspensions were used alone (SHb30, C13 or G2 treatments) or in consortium to treat lettuce in the experiment. Combinations made for consortium treatments were SHb30+C13, SHb30+G2 and SHB30+C13+G2. Mixtures were made with equal proportion of each constituent in described concentration. Controls used were a negative healthy control without the pathogen (C-), a positive control (C+), a biopesticide control (Cpc), an aquaponic control (Cap) and a fungicide control (Cf). C+, C- and Cap were treated with KPBT. Lettuce in Cap were grown in aquaponic water (instead of commercialized hydroponic solution) collected in the PAFFbox aquaponic system of Gembloux Agro-Bio Tech in Belgium (see Stouvenakers et al.,

2020 for the system description). For Cf, Proplant® (722 g/l propamocarb) fungicide was used at 0.1% in KPBT buffer. Finally, *P. chlororaphis* Tx-1 (ATCC 55670) suspension, produced as other bacteria and at the same concentration, was used for Cpc.

2.2. Pathogen inoculum preparation

According to Stouvenakers et al. (2022), stock mycelial culture of *Pythium aphanidermatum* (CBS 132490) was first reactivated on PDA for 3 days at 23°C with a day/night photoperiod of 18h/6h. Then, mycelial plugs of the active growing fungus were grown in Erlenmeyer flasks containing 25 mL of clarified V8 CaCO3 broth (800 mL of distilled water, 200 mL of V8 juice, and 3 g of CaCO3). After 9 days at the same conditions, mycelial bulk were recovered and rinsed several times in sterile distilled water. Mycelium bulks were then incubated for 24h at 28°C with lighting in sterile distilled water to initiate oospores formation and maturation. Mycelium bulks were then mixed with a hand blender (Braun Minipimer Control Plus, 300w) in a sterile solution containing 10 mM of sucrose and 0.05% of Tween 20 in distilled water. Oospores in suspension were then separated from other propagules by sterile cheesecloth filtration. Oospores found in the filtrate were then set at a concentration of $1x10^4$ oospores/mL after haemocytometer observation.

2.3. Experimental setup

Biocontrol experiment of *P. aphanidermatum* disease on lettuce seedlings was conducted as described in Stouvenakers et al., 2022. Organic pelleted lettuce seeds (*Lactuca sativa*) var. Lucrecia RZ (Rijk Zwaan, Merksem, Belgium) were sown in 25 × 25 × 40 mm rockwool plugs (Grodan B.V., Roermond, Holland). Plugs were put in square plant trays of 14 cm side and trays were then randomly placed in a phytotron set at 16h/8h (day/night) photoperiod, a temperature of 22°C/18°C (day/night), and a relative humidity of 65%. Excepted for Cap treatment, where aquaponic water was used all experiment long, tap water was used for the first week of germination and then hydroponic solution was used instead according to manufacturing instruction (Hy-Pro A and B, Hy-Pro Fertilizers, Bladel, Holland). Ten days after sowing, temperatures and humidity were increased to 35/25 °C (day/night) and 92%, respectively. Treatments were applied at a rate of 1 mL per plug on days 0 and 7. For each treatment, 2 plant trays were used containing each 9 rockwool plugs. On day 10 after sowing, plugs were inoculated by 1 mL of the pathogen suspension, excepted for C- where sucrose + tween solution was used instead. Lettuce mortality (LM), root rot symptoms (RRR: root rot rating) and foliar fresh mass (FFM) were recorded on day 31 as described in Stouvenakers et al. (2020). Statistics were achieved for FFM and RRR data on Minitab v.19 software (Minitab Inc., State College, PA, USA). Conditions of application were tested, and 1-way analysis of variance (ANOVA) were

performed with treatments as a factor. Tukey Multiple Comparison post hoc test was used to pairwise compare treatments.

3. Results

LM means were provided in Table F-1. RRR and FFM means were illustrated in Figure F-1. The highest lettuce mortality was observed in C13 treatment and Cpc control (33.3%, respectively), while lettuce mortality in C+ was 11.1%. Other controls or treatments used alone and in consortium had no LM.

Table F-1: Lettuce mortality (LM) of C13, SHb30, G2 treatments applied alone or in consortium to control *P. aphanidermatum* disease on lettuce seedlings. C+, C-, Cf and Cap were the positive, negative, fungicide and biofungicide controls, respectively.

LM: lettuce mortality (%)										
Controls					**Treatments**					
C-	**C+**	**Cf**	**Cap**	**Cpc**	**C13**	**SHb30**	**G2**	**SHb30 +C13**	**SHb30 +G2**	**SHb30 +C13 +G2**
0.0%	11.1%	0.0%	0.0%	33.3%	33.3%	0.0%	0.0%	0.0%	0.0%	0.0%

Although, low LM was observed in C+, disease was present with a RRR = 6.06 and a FFM = 1100.4mg for this positive control. In comparison, RRR and FFM of C- were 0.56 and 1844.9mg, respectively. Cf and Cap controls were effective ($p \leq 0.05$) to reduce RRR (2.17 and 2.14, respectively) and no FFM significant decrease ($p > 0.05$) was observed compared with C-. However, Cpc was not able to control the disease, with a RRR mean of 6.06 and a FFM mean of 788.2mg. C13 and SHb30 tended to decrease root symptoms (RRR= 5.2 and 4.7, respectively) but not significantly ($p > 0.05$). With the combination of the two bacteria (SHb30+C13), RRR dropped down ($p \leq 0.05$) to 2.81 compared with C+. Among treatments applied alone, G2 provided the best ($p \leq 0.05$) root disease protection with a RRR of 2.3, that was not different ($p > 0.05$) from Cf and Cap. All consortiums tested were effective to reduce RRR. In fact, their RRR levels were similar ($p > 0.05$) to RRR of Cf and Cap controls. Moreover, consortiums that contained G2 fungus (SHb30+G2 and SHb30+C13+G2) were not different from RRR of C- and G2 alone. In relation to FFM, C13 was not effective to control foliar loss (FFM = 902.79mg). In comparison with C+, SHb30 tended to improve FFM (FFM = 1341.0mg) but not significantly. FFM of SHb30 was, nevertheless, not different ($p > 0.05$) from Cf and Cap controls. Once combined, FFM of SHb30+C13 increased to 1694.5mg. This combination was different ($p \leq 0.05$) from C+ but not ($p > 0.05$) from C-. G2 and consortium containing G2 were all able to improve FFM to the same level as C- ($p \leq 0.05$) but no difference was found

between them. Excepted for C13 alone, all tested treatments applied alone or in consortium gave a FFM protection as good ($p > 0.05$) as Cf and Cap controls.

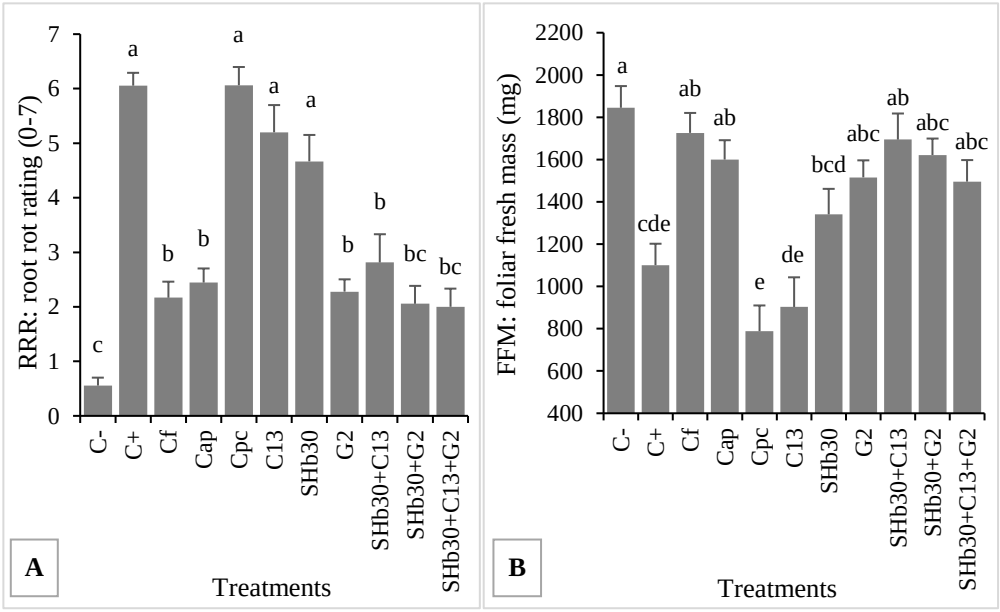


Figure F-1: Means bar charts of A: root rot rating (RRR), and B: foliar fresh mass (FFM) of C13, SHb30, G2 treatments applied alone or in consortium to control *P. aphanidermatum* disease on lettuce seedlings. C+, C-, Cf and Cap were the positive, negative, fungicide and biofungicide controls, respectively. Bars indicate the standard error of the mean and different letters indicate significant differences ($p \leq 0.05$) between treatments by Tukey's ANOVA post hoc test.

4. Conclusions and discussion

In this biocontrol study, it was shown that aquaponic water was able to control *P. aphanidermatum* lettuce disease. This suppressive action was already observed in Stouvenakers et al. (2020) and confirmed that aquaponics can be a source of antagonistic agents that should not be ignored for soilless use. In our study, the fungal *A. flavus* strain G2 was the best agent in sole application and was able to reduce lettuce root symptoms of *P. aphanidermatum* disease to a similar level than Cf and Cap controls. Moreover, no LM and no significant FFM loss were observed with this treatment. Although as effective as Cf and Cap, combination of G2 with C13 and/or SHb30 (both bacterial strains) did not bring significant additional effect. In relation to Stouvenakers et al., 2022, these results showed that antagonistic activity of G2 is reproducible over time. Studies on *A. flavus* use against *P. aphanidermatum* are scarce

and limited to few papers (Shanmugan et al., 1999; Stouvenakers et al., 2022), while atoxigenic strains were intensively studied to control aflatoxin in cereal crops (Khan et al., 2021). Activity of *S. xenophagum* strain SHb30 applied alone was less effective than G2 to control root rot but this treatment allowed to keep a FFM as good as Cf and Cap controls. Work must be undertaken to obtain stable action with SHb30 strain. *M. fortuitum* strain C13 was not able to control the disease in this test while Stouvenakers et al., 2022 reported a better efficacy. In our knowledge, this study and that of Stouvenakers et al., 2022 were the first reports of an antagonistic activity of *S. xenophagum and M. fortuitum* species against plant pathogens. Once SHb30 and C13 combined, an additional effect tended to be observed with efficacy similar to Cf and Cap controls. In literature, several strains of *P. chlororaphis*, including Tx-1 strain, were described as the most adapted biocontrol agents to control *Pythium* spp. diseases in hydroponics (Khan et al., 2003; Chatterton et al., 2004; Liu et al., 2007; Sopher and Sutton, 2011). However, its action remains variable as shown by its non-efficacy in this test and its medium efficacy reported in Stouvenakers et al., 2022. In conclusion, this study showed that aquaponics is an important source of antagonistic microorganisms that could control *P. aphanidermatum* disease on lettuce. G2 was effective to control the disease alone, while SHb30 and C13 were better in consortium.

Chapter G

General Discussion

1. Introduction

Considering all this thesis, several points can be discussed. Methodology used throughout the experiments were thought to efficiently reach research goals. Nevertheless, there is no perfect methodology (Section 2). Origin of aquaponic suppressiveness was assigned to rhizoplane microbiota. However, factors involved in disease suppressiveness are various and often interconnected (Section 3). Aquaponic water was described as plant pathogen suppressive, but this action was observed only in one system. Can we extrapolate this action to all systems, and what about decoupled aquaponic systems (Section 4 and 5)? Suppressive microorganisms in aquaponics were at the core of this thesis. However, they are not the only types of microorganisms that can play a role in plant health. Among plant beneficial microorganisms, plant biostimulant microorganisms will be tackled (Section 6). Finally, perspectives of plant pathogens biocontrol in aquaponics will be discussed according to the biocontrol agents discovered in the thesis (Section 7).

2. Methodology Importance

2.1. Variability in bioassays

Bias and limitations of *in vitro* bioassays to evaluate antagonistic activities of aquaponic water or biocontrol agents were considered throughout the experiments. The major concern was to maximize the chance of biocontrol effect in real environment. For that reason, *in vivo* bioassays were preferred to *in vitro* ones. Indeed, a positive antagonistic effect *in vitro* against a plant pathogen does not necessarily predict a biocontrol activity in more complex assays including plant hosts (Köhl et al., 2019). However, adding the plant host to the model increases its complexity. Furthermore, living biological material is susceptible to variability. First, the pathogen *P. aphanidermatum* can present variability in terms of aggressivity and pathogenicity (Martin and Loper, 1999; Stouvenakers et al., 2020) probably because of its first saprophytic stage before symptoms development (see Chapter A-2). The plant host also has its own variability in a specific environment, such as observed during lettuce experiments. With the same growth conditions and without pathogen application, variability in lettuce germination and growth were observed in bioassays. Finally, it is commonly accepted that biocontrol agent could give variable efficacy depending on their environment (Köhl et al., 2019). This *in vivo* variability was problematics in our experiments due to the small number of biological replicates imposed by the facilities. Indeed, biological variability coupled with few biological replicates can limit the observation of significant differences between treatments.

2.2. HTS bias

Microbial community analyses by HTS were an important component of the thesis. An important consideration is addressed on this technology to improve reliability, representativeness, and reproducibility between studies. In fact, bias can be introduced at any step of the protocol, i.e., from microbiota harvest to bioinformatic analyses (Boers et al., 2019; Izard, 2014; Pollock et al., 2018). Although important, the microbiota harvesting step was often underestimated in literature and lacks standardization (Barillot et al., 2013; Donegan et al., 1991). Microbiota harvest is the input of any microbial community studies and should receive much more consideration. For that reason, the first step of microbial analysis by HTS was to find the best practices to harvest rhizoplane microbiota from aquaponic lettuce (Chapter C-1). The best methodology workflow was then used throughout experiments. The selected method (root sonication in KPBT buffer) was quantitatively the best method to harvest culturable microorganisms. Furthermore, it was determined that a single washing step was sufficient to harvest a representative sample of the total rhizoplane microbiota in terms of microbial relative abundance, richness, and diversity.

Considering the Chapter D, several methodological points may have influenced HTS results. The selection of the genome region to sequence will directly influence HTS results. Indeed, the choice of primers and target regions is one of the most critical step in rDNA amplicons sequencing (Nikolaki and Tsiamis, 2013). A selected primer cannot equally anneal all microbiota members in a specific DNA region and will result in overestimation or underestimation of certain taxa (Nikolaki and Tsiamis, 2013; Pollock et al., 2018). For bacteria, the hypervariable region V1-V3 of the 16S rDNA was selected. This region was recommended for aquaponic microbiota studies by Munguia-Fragozo et al., 2015 and largely used in this specific domain (Eck et al., 2021, 2019; Schmautz et al., 2017). This choice could foster comparison between aquaponic systems although other steps and reagents can introduce bias. Primers importance is also particularly well illustrated in the fungal microbiota analysis of Chapter D. Indeed, the ITS1 primers used for fungi identification led to cross identification of non-targeted organisms. Numerous protozoa were identified in high relative abundance. This bias was already described by Toju et al. (2012) for the same set of primers. ITS is probably the most used and efficient region for fungi identification (Begerow et al., 2010; Schoch et al., 2012). However, a weaker assignation rate or level of taxonomic identification was observed for fungi in comparison with bacteria in Chapter D. Inter and intraspecific variability among fungi are more frequent and led to upper taxonomical rank identification (Begerow et al., 2010; Schoch et al., 2012). Moreover, fungal nucleotide databases are not yet enough trained in comparison with bacterial databases.

A mismatch between the microbial composition identified by HTS and culture plate was observed in Chapter E. It was for instance shown that most abundant taxa in HTS were not the most abundant in culture plates. Fifty-nine percent of isolated strains were found in low relative abundance in the HTS analysis. Certain isolated taxa were also not identified in the HTS analysis. In facts, HTS is not a culture-dependent technique, and identified microorganisms are not always culturable (Forbes et al., 2017; Garbeva et al., 2004; Stefani et al., 2015). Furthermore, it is commonly accepted that rare microorganisms can be abundantly and easily isolated in culture media, and *vice versa*. Universal media that suit to all microorganisms does not exist and culture media lead necessarily to a biased estimation of microorganisms abundance (Davis et al., 2005). It was, however, highlighted in Chapter E that a HTS-guided strategy to isolate potential biocontrol agents could be valuable. Indeed, 43% of isolated strains were identified as suppressive in the HTS analysis and among them 60% were found efficacious to control lettuce seed damping-off.

3. Origin of aquaponic suppressiveness

3.1. Suppressive microbial niches

To identify the origin of aquaponic suppressiveness against soil-borne diseases, root microbiota was analysed in Chapter D. I.e., microbiota of rhizosphere, rhizoplane and endosphere of aquaponic lettuce were considered for disease suppression. However, microbial niches influencing plant microbiota cannot be restricted to plant root. Microbiota of recirculating water, fish (e.g., , fish gut, scale and faeces), periphyton, biofilter and phyllosphere were the current locations studied in aquaponics (Eck et al., 2021, 2019; Schmautz et al., 2017; Sirakov et al., 2016; Sirsat and Neal, 2013). Schmautz et al. (2017) and Eck et al. (2021) reported that bacterial communities were niche dependent. However, Eck et al., 2021 identified that the Burkholderiaceae family was present in all compartments tested (i.e., sump water, biofilter and lettuce roots) of the Paff Box aquaponic system. Sphingomonadaceae family was also shared between the biofilter, and the root microbiota compartments. In Chapter D, these two families were correlated to aquaponic suppressiveness against *P. aphanidermatum*. This Chapter D also reported an *in vitro* microbial suppressive activity of sump water and biofilter water on *P. aphanidermatum* growth. This observation supported the idea that different compartments of the same system (e.g., the PAFF Box) could be plant pathogen suppressive. Furthermore, it was shown in Sirakov et al. (2016) that 14.6% of bacteria isolated from the root part of aquaponic tomato could inhibit *Pythium ultimum* in dual culture bioassays. Bacteria isolated from fish faeces, sump water and biofilter also showed a high proportion of *in vitro* suppressive bacteria, i.e., 13.3%, 10.4%, and 9.0%, respectively (Sirakov et al., 2016). In particular, the biofilter unit of aquaponic systems could be an important microbiota location for soil-borne disease

suppressiveness. It was assumed in Chapter A-1 that aquaponic biofilter could contain plant pathogen suppressive microorganisms. This assumption, later confirmed in Chapter D, was deducted from the microbial suppressive activity of some hydroponic slow filters or from taxa found in RAS biofilter that are often described as plant pathogen antagonist in literature (Tal et al., 2003; Sugita, et al., 2005; Furtner, et al., 2007; Schreier et al., 2010; Renault et al., 2012). Consequently, biofilters but also other components of aquaponic system should be further studied for their potential microbial suppressiveness.

3.2. A multifactorial suppressive origin

It was stated in Chapter D that the origin of aquaponic suppressiveness was linked to specific rhizoplane microorganisms. However, the relation is not so simple (see Chapter A, Section 2.2.3). In our *in vitro* suppressiveness bioassays, only the direct action of microorganisms was considered. Filtered aquaponic water (i.e., without microorganisms) was not able to suppress *P. aphanidermatum* mycelial growth. However, in the absence of the host plant, indirect mode of action such as plant elicitation or biostimulation by microbial or non-microbial compounds were missed. In *in vivo* bioassays, this indirect mode of action was nevertheless possible. In our *in vivo* bioassays, only the microbial component was taken in consideration for the suppressiveness study. Correlations were made between microbial factors and suppressiveness indexes but not with other physico-chemical, chemical, or biological factors. For example, some suppressiveness studies carried out multivariate analyses to have a better understanding of factors implied separately in disease suppression but also their possible interactions (Bonanomi et al., 2007; Bongiorno et al., 2019; Corato et al., 2019; Ros et al., 2019). Added factors in the analysis are often pH, EC and organic compounds presence. It was shown in Chapter D that aquaponic water loses its pathogen suppressive abilities after pH modification and mineral plant nutrient addition. However, only the microbial modification aspect was considered in the study to explain the loss of suppressiveness. For example, pH could have a direct effect on the *Pythium* pathogen by playing on zoospores production (Funck-Jensen and Hockenhull, 1983b), appressoria formation (Endo and Colt, 1974), mycelial growth and saprophytic activity of the pathogen (Martin and Loper, 1999). Moreover, richer plant substrate or nutrient solution can also directly influence plant diseases (Dordas, 2008; Geary et al., 2015; Veresoglou et al., 2013). Organic compounds can also have an indirect action on the pathogen by playing on plant health and growth (du Jardin, 2015). Organic compounds are considered as a main factor influencing suppressiveness by driving microbiota composition and diversity (Bonanomi et al., 2018b; Montagne et al., 2017). It is important to keep in mind that disease suppressive biotopes are complex environments that cannot be summarized in the "plant host – pathogen – biocontrol agents" trifocal approach (Expósito et al., 2017; Massart et al., 2015; Ros et al., 2019; Whipps, 2001). These three factors interact between them but

also with other microorganisms or more generally with the plethora of biotic and abiotic factors composing the environment (*ibid*.).

4. Is aquaponic suppressiveness universal?

4.1. Aquaponic suppressiveness in literature

The aquaponic water from the PAFF Box in Gembloux Agro-Bio Tech at ULiege University in Belgium was described in this thesis as plant pathogen suppressive. However, is this effect continuous and present in all aquaponic systems?

Besides our studies, only 2 papers evaluated the suppressive activity of aquaponic water. Firstly, Sirakov et al. (2016) isolated 86 bacteria in the aquaponic system of Zurich University in Switzerland that were able to control *P. ultimum* in dual culture bioassays. However, microbial composition and diversity of aquaponic samples were not studied and antagonistic bacteria were not identified. Furthermore, the general suppressive effect of aquaponic water was not tested *in vivo*. The second paper showed that strawberry plant grown in an aquaponic system at Seville University (Spain) showed greater suppressiveness levels to *Phytophthora cactorum* than in a hydroponic system (Suárez-Cáceres et al., 2021). However, this suppressive effect was variable depending on the growth stage of strawberry plants. Moreover, this same aquaponic system was not able to control *Fusarium oxysporum* f. sp. *lycopersici* on tomato plants. In comparison to our studies, the suppressive effect of aquaponic water was observed *in vivo* at lettuce seedling stage and later, but not at the seed stage against *P. aphanidermatum*. Furthermore, it was shown in Chapter E that aquaponic water sampled at different times could show different suppressive abilities. These reports emphasizing that aquaponic suppressiveness could be time dependent and not relevant for all pathosystems. The hypothesis that aquaponic suppressiveness can vary in time and depending on the system could be explained by the variation of aquaponic water composition and its parameters, as well as other environemnetal parameters. Water composition and parameters can directly influence the plant pathogen or indirectly by driving the suppressive microbiota (see Section 4.2.).

4.2. Factors influencing aquaponic microbiota

Numerous factors influence aquaponic microbiota composition and diversity. The modification of one factor will influence water composition and parameters and then aquaponic microbiota too. For example, it makes sense that two aquaponic systems growing different fish and plant species will drive different microbiota. Systems with different fish species have different needs in terms of water quality parameters and feed composition. Fish microbiota and excretion will be different too. At its scale, plant species, variety and growth stage will influence root microbiota composition and diversity.

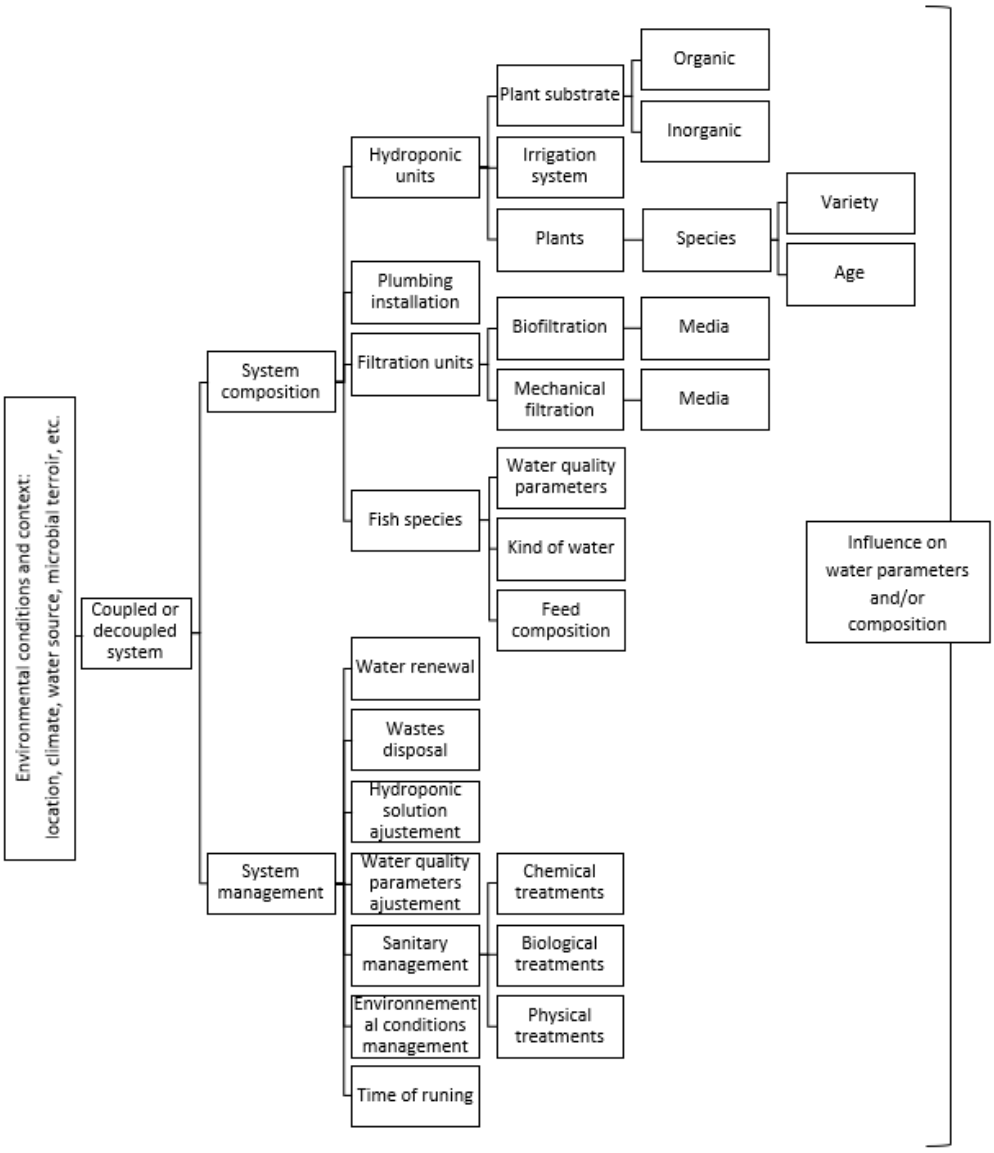


Figure G-1: Hierarchy of factors influencing aquaponic microbial composition and diversity by modifying water composition (mineral and organic compounds) and parameters (temperature, pH, gas content and hydraulic characteristic). Personal representation.

Based on aquaponics (Eck et al., 2021, 2019; Ichard et al., 2004; Munguia-Fragozo et al., 2015; Ru et al., 2017; Wang et al., 2016; Wongkiew et al., 2017) but more

especially on recirculated aquaculture (Blancheton et al., 2013; Itoi et al., 2006; Leonard et al., 2000; Michaud et al., 2014, 2006; Rurangwa and Verdegem, 2015; Schneider et al., 2007) and hydroponic articles or reviews (Chave et al., 2008; Postma et al., 2008; Strayer, 1994; Vallance et al., 2010), the Figure G-1 synthesizes the most important factors influencing aquaponic microbial composition and diversity. Each of these factors influence aquaponic microbiota by affecting water quality in terms of content (mineral, organic and gas content), hydraulic parameters (velocity, pressure, flow rate and turbulence), pH and temperature. The modification of a factor can affect the system at different levels. The impact of the factor can be high as the fish species that influences the kind of water (fresh, salt, or brackish), the feed, water quality parameters, etc., or low, like the plant age that influences the rhizosphere microbiota. Eck et al. (2019) compared bacterial communities of five aquaponic systems. Among them, 4 were decoupled system and one - the PAFF Box - was coupled. Systems displayed different fish species, plant species, feed (vegetarian or omnivorous), biofilter media, and kinds of water treatment (i.e., UV, ozone, or nothing). Bacterial taxa composition was different depending on the system. However, all aquaponic systems shared a core microbiota composed of 21 OTUs. Among these OTUs, Sphingomonadaceae and Comamonadaceae were identified. Moreover, these two families were also present in high abundance in our studies and found related to suppressiveness (Chapter D). In another article, Eck et al., 2021 studied bacterial community evolution over the course of a lettuce growth cycle in the PAFF Box aquaponic system. It was shown that bacterial diversity and composition in the root zone and in sump water was quite resilient throughout time and water parameters variation. Only the biofilter microbiota was more susceptible to water parameters modification (i.e., temperature, EC and nitrate concentration) caused by system management.

5. Matter of decoupled aquaponic system

Running coupled aquaponic systems needs to make compromises between fish and plant requirements. Indeed, fish and plant have their own optimum in terms of water quality and parameters (e.g., pH, and temperature) that are not fully met in coupled aquaponics (Delaide et al., 2016; Goddek et al., 2015). Furthermore, mineral nutrient concentration in aquaponic recirculating water is far less than recommended in hydroponic plant production (Delaide et al., 2016). To answer the problem, decoupled aquaponic systems were designed. This system separates the recirculated aquaculture unit to the hydroponic plant unit with only periodic water exchange (Goddek et al., 2016, 2015). In general, water exchange is a one-way direction, i.e., from fish to plants (Goddek et al., 2016; Monsees et al., 2017). This separation allows to monitor the two systems separately in order to reach the optimal plant and fish production. For the hydroponic part, it means that the nutrient pre-enriched water received from

aquaculture can be complemented by mineral nutrients and pH adjusted to suit optimal plant growth requirements (Delaide et al., 2016). Because fish are separated, decoupled aquaponic system also allows treatments to control plant pathogens in the hydroponic part. However, it was highlighted in Chapter D that complemented aquaponic water loses its plant pathogen suppressive ability after mineral salts supplementation and pH modification. This loss of suppressiveness was, *inter alia*, attributed to rhizoplane microbiota modification following the pH decrease and mineral salts supplementation. It is in contradiction with the resilient root microbiota observed in Eck et al. (2021). However, parameters variation measured in Eck et al. (2021) were less drastic than ours. Suggesting that rhizoplane aquaponic microbiota is resilient until a certain level. Furthermore, it is possible that the new water components and parameters applied were themselves more *P. aphanidermatum* conducive (Martin and Loper, 1999). It means that decoupled aquaponics could lose aquaponic suppressiveness because of the drastic changes imposed by the complementation and water parameters changes. To date, no other paper than ours studied the suppressive activity of complemented aquaponic water. Consequently, decouple aquaponic system can be a double-edge strategy. Plant growth conditions in decoupled aquaponics are better but the system could be more conducive to plant disease. If the system is more susceptible to plant disease outbreaks, the system will use more pesticides or chemical agents. These treatments influencing again microbial communities (Rosberg, 2014). On another side, certain hydroponic systems were already described as plant pathogen suppressive (McPherson et al., 1995; Postma, et al., 2008; Vallance et al., 2010), while hydroponic water was not found suppressive in Chapter D. This difference can be explained by the absence of a first step of system cycling before pathogen introduction in our experiments. Consequently, it is possible that complemented aquaponic water as well as hydroponic water were not disease suppressive in our study because of a weak establishment or too new establishment of microorganisms in the system. In conclusion, further research on the plant pathogen suppressive ability of decoupled aquaponic water should be led.

6. Plants biostimulation in aquaponics

Biocontrol agents are not the sole microorganisms playing a beneficial role in plant health management. Plant growth promoting microorganisms (PGPM) are an example of plant biostimulant involved in plant health. Biostimulants are microorganisms or compounds involved in growth promotion, quality traits improvement, nutrition efficiency and/or abiotic stress tolerance of plants (du Jardin, 2015). Microorganisms and compounds involved in plant biostimulation are also often found plant pathogen antagonist in literature. For example, pseudomonads are interesting candidates for both plant biostimulation and plant pathogen biocontrol (Nadeem et al., 2016), and chitosan is a compound that induces both biotic (e.g., plant pathogens) and abiotic

(e.g., drought) stress tolerances in plants (du Jardin, 2015). Although sometimes interconnecting, there is a consensus to separate plant biostimulation from plant pathogen biocontrol (du Jardin, 2015).

In aquaponic, it has been observed several times that plant yields can be similar to hydroponics, even though mineral nutrient concentration in the solution is lower (Pantanella et al., 2012, 2015; Delaide et al., 2016; Saha et al., 2016; Suhl et al., 2016; Anderson et al., 2017; Wielgosz et al., 2017). Yields could be even higher when aquaponic nutrient solution is complemented to reach hydroponic levels (Delaide et al., 2016; Suhl et al., 2016). To explain this unexpected plant growth enhancement, Gravel et al. (2015) and Delaide et al. (2016) suggested the presence of plant biostimulants in aquaponic water.

Until now, only Eck (2021) isolated biostimulant bacteria from aquaponic system. Sanchez et al. (2019) did the same but bacteria were isolated from recirculated aquaculture for aquaponic use. In both studies, isolated bacteria were *in vitro* screened for biostimulation traits. Thirty-one strains were screened by Eck 2021 and 61 by Sanchez et al. (2019). The percentage of positive bacterial strain for biostimulation traits is shown in Table G-1. Eck (2021) selected the three best strains and tested them to improve lettuce growth in aquaponic. Only *Serratia fonticola* strain T produced a slight biostimulant effect on roots. Strain T individually, or in a consortium with *Pseudomonas aeruginosa* strain A and *Chryseobacterium cucumeris* strain H improved plant growth in light stressing conditions but only in the first aquaponic assay. Lettuce seedling growth traits were also improved by the strain T and consortium application in nutrient stressing conditions (lettuce growth in demineralized or tap water). In the first *in vivo* bioassay to control *P. aphanidermatum* on lettuce in Chapter E, it was assumed that *S. xenophagum* strain SHb30 had a biostimulant effect. Indeed, foliar fresh mass of seedlings treated by SHb30 (and inoculated with the pathogen) tended to be improved in comparison with the healthy control.

Table G-1: Percentage of positive response of isolated bacteria depending on the biostimulation trait tested and the reference (i.e., Eck, 2021 and Sanchez et al., 2019).

	Percentage of positive strain (%)	
Biostimulation trait	**Eck, 2021**	**Sanchez et al., 2019**
Phosphorus solubilisation	29%	38%
Potassium solubilisation	42%	/
Ammonia production	61%	20%
Siderophores production	55%	46%
Indole-3-acetic acid production	26%	/

Results of Eck (2021) and ours indicated that PGPM application could be helpful to support plant growth in biotic and abiotic stressing conditions. Moreover, this section highlights that aquaponic could be an important bank of PGPM for further applications in soilless but also in soil conditions. Treatment with foreign PGPM (i.e., not from aquaponics) are also a possibility to increase plant growth and health in aquaponics (Mangmang, et al., 2014a, 2015b, 2015c; Cerozi and Fitzsimmons, 2016) (see chapter A-1).

7. Toward microbial biocontrol in aquaponics

7.1. Biocontrol agents isolated from aquaponics

In this work, three interesting biocontrol agents were isolated from aquaponic lettuce rhizoplane to control *P. aphanidermatum* diseases. They were strains SHb30 (*S. xenophagum*), G2 (*A. flavus*) and C13 (*M. fortuitum*) (Chapter E and F). C13 was the best strain to control lettuce damping-off, but its biocontrol effect was more variable for root rot disease bioassays. G2 and SHb30 were efficacious to control lettuce damping-off but at a lower rate than C13. On lettuce seedlings, SHb30 can show an extraordinary level of protection against root rot disease but its effect was variable depending on bioassays. G2 was the strain with the most constant efficacy to control lettuce root rot disease. In literature, *S. xenophagum* and *M. fortuitum* were never described for biocontrol activity against soil-borne disease. They are rather described as bacteria involved in bioremediation of contaminated environments (Bisht et al., 2015; Miller et al., 2004; Song et al., 2019; Stolz et al., 2000). Nevertheless, Sphingomonadaceae are often observed in suppressive soil or in aquaponics (Cardinale et al., 2015; Expósito et al., 2017; Sare et al., 2020; Schmautz et al., 2017). This suggesting that *Sphingobium* genus could be an important player in disease suppression. Furthermore, *S. xenophagum* was able to control *Botrytis cinerea in vitro* by volatile organic compounds production (Ortega et al., 2016). It has also been described once as a PGPM (Wanees et al., 2018). Antagonistic modes of action of SHb30, C13 and G2 were *in vitro* studied in our laboratory but results are not published. Competition and plant elicitation were not evaluated as modes of action. SHb30 shows biostimulant traits by acting on potassium solubilization, ammonia production and indole-3-acetic acid production. For biocontrol traits, SHb30 can degrade cellulose, a component of Oomycetes cell wall. G2 produces antimicrobial compounds that inhibit *P. aphanidermatum* growth in dual culture. G2 also produces lytic enzymes involved in plant pathogen suppression, i.e., protease, cellulase and amylase. C13 produces protease and amylase. *S. xenophagum* seems interesting for plant health because of its multitask potential, i.e., soil bioremediation, disease biocontrol and plant biostimulation. Moreover, *S. xenophagum* was never described

as detrimental for fish or plant. Unlike *M. fortuitum* that can be the causal agent of fish tuberculosis in aquaculture (Fattah and Sayed, 2006). *Mycolicibacterium* spp. have also been found to enhance *P. aphanidermatum* growth *in vitro* and to be tobacco black rot disease conducive (Burgos-Garay et al., 2014; Kyselková et al., 2009). *A. flavus* can be plant pathogen and the species are known to produce aflatoxin in infected crops. However, atoxigenic strains can be naturally found and are studied to control aflatoxin-producing ones (Amaike and Keller, 2011; Khan et al., 2021), such as *A. flavus* strains AF36 and NNRL 21882 that are EPA registered for biocontrol use. Consequently, G2 and C13 strains must be further studied to determine if C13 is a fish pathogen and if G2 can produce aflatoxin. SHb30 is the most promising strain but its biocontrol effect must be stabilized.

7.2. Formulation in biopesticides

Because of its multitask potential (i.e., soil bioremediation, disease biocontrol and plant biostimulation) and its safer use (i.e., for plant, fish and human health), SHb30 is probably the most valuable strain isolated by our for biocontrol in aquaponics. However, its biocontrol activity must be stabilized in an appropriate biopesticide formulation. Before application, the formulation is designed to stabilize the microorganism during production, distribution, and storage. The formulation will also enhance dispersion, attachment, and persistence of the biopesticide in the environment. And finally improve biocontrol agent activity (Jones and Burges, 1998). To improve efficacy, the selection of the good carrier substrate in formulation is probably the most important part (Keswani et al., 2016). The carrier will influence survival, antagonistic abilities and colonization of the microorganism on the target (Keswani et al., 2016). In our bioassays, SHb30 was inoculated in a mineral substrate (i.e., rockwool plug) that contained lettuce seed. Lettuce seedlings were grown in hydroponic conditions. It means that the sole source of organic nutrients was root exudates. It is then possible that SHb30 lacked organic nutrients for its establishment, its growth, or root colonization. The first solution could be to use a plant substrate composed of organic matter, such as peat, coconut-fiber or sawdust. The second solution to improve SHb30 efficacy and regularity could be to add organic matter in the formulation. Peat is often used alone or in mixture in the formulation but agricultural wastes (e.g., compost, manure, sawdust, cereal bran, crop filter cake) represent an important source of organic matter to explore (Hassan et al., 2015; Keswani et al., 2016; McLean et al., 2005; Yang et al., 2011). *Sphingobium* species are known to degrade a wide range of organic compounds and are often isolated in environments where organic substances tend to accumulate (Stolz, 2009). SHb30 was isolated from suppressive aquaponic microbiota. Consequently, organic matter found in aquaponic water should be identified and then studied in the formulation to improve SHb30 efficacy. Amino acids, organic acids or sugars identified in root exudates could also be studied to help the bacterium to colonized plant root (Neumann et al., 2014).

Moreover, in non-published experiments, a medium rich in amino acids and peptides were found to enhance SHb30 production in liquid broth. SHb30 was found to degrade carboxymethylcellulose (CMC), then this substance could be added to the formulation to enhance biocontrol action. Furthermore, CMC is a current carrier substrate that prevents desiccation of biocontrol agents (Keswani et al., 2016; Segarra et al., 2015). Lastly, the formulation of the biopesticide must be adapted to the application, i.e., seed, root, foliar or soil/nutrient water treatment (Narayanasamy, 2013b).

Another way to stabilize the biocontrol action of SHb30 could be to use it in a consortium. Indeed, it was shown in Chapter F that the combination of SHb30 with C13 or with G2 produced an additional biocontrol effect in comparison with SHb30 applications alone. This consortium biocontrol effect was found more stable between bioassays (non-published data). Interactions between microbial strains and the environment could be very complex (see Chapter A, Section 2.4.). Mixing biocontrol agents can combine mechanisms of action but they can also interact as helper strains. Root colonization by SHb30 was maybe enhanced by the capacity of C13 to form biofilm. Hyphal growth of G2 maybe helped the migration of SHb30 in roots. Or maybe nutrients were used syntrophicaly and then competitiveness or root colonization was improved. Unfortunately, biocontrol consortia are more difficult to formulate and registered (see Chapter A, Section 2.4).

Chapter H

Conclusions and Perspectives

It was stated that methods to control plant pathogens in coupled aquaponic systems are more limited than in hydroponics because of the presence of fish and nitrifying bacteria in the same water loop. However, it was assumed that some aquaponic systems could be plant pathogen suppressive. The aim of this thesis was then to test this hypothesis.

A method was developed to harvest lettuce root microbiota in aquaponics. The best methodology workflow was determined and used throughout the experiments. The selected method, i.e., root sonication in KPBT buffer, was quantitatively the best method to harvest lettuce rhizoplane microorganisms. It was also shown that a single washing step was enough to harvest a representative sample of the total rhizoplane microbiota in terms of microbial relative abundance, richness, and diversity. Microorganisms harvested by this method were then considered as a reliable input for further microbial community analysis.

The pathosystem *P. aphanidermatum* - lettuce was studied to test aquaponic suppressiveness. Results demonstrated that microorganisms of aquaponic water can decrease *P. aphanidermatum* mycelial growth in *in vitro* bioassays. The suppressive effect of aquaponic water was then confirmed *in vivo*. *P. aphanidermatum* root rot disease of lettuce was suppressed by using aquaponic water as nutrient solution for lettuce growth. On the other hand, lettuce grew in hydroponic water or in complemented aquaponic water failed to control the disease. The suppressive effect observed in the aquaponic treatment was correlated to a higher microbial species diversity in lettuce rhizoplane. A list of microbial taxa related to disease suppressiveness was also established. Burkholderiaceae, *Methyloversatilis*, *Sphingobium*, *Hydrogenophaga* and *Catenaria* were the main taxa highlighted.

A HTS-guided campaign of biocontrol agents isolation was led in aquaponic lettuce rhizoplane. Out of 100 microbial isolates, 8 were particularly efficacious to control lettuce damping-off caused by *P. aphanidermatum*. Strains SHb30 (*Sphingobium xenophagum*), G2 (*Aspergillus flavus*) and C13 (*Mycolicibacterium fortuitum*) were the most efficacious strains to decrease root rot disease severity on lettuce seedlings. No additional biocontrol effect was observed by using G2 in a consortium, but SHb30 and C13 combination tended to increase biocontrol in comparison with separated applications.

In brief, this study highlighted that aquaponic water of the PAFF Box system and their microorganisms can control *P. aphanidermatum* diseases of lettuce. However, this study was only a first insight into the broad field of aquaponic suppressiveness and a lot of work is still needed.

First, only one pathosystem was studied in this thesis. Suppression of *P. aphanidermatum* diseases in aquaponics should be studied for other hydroponic crops such as cucumber. Furthermore, *Pythium* spp. are not the sole pathogens able to cause damage in soilless crops. *Fusarium* spp. is an example of root pathogen that should be studied (e.g., *F. oxysporum* f. sp. *lactucae* on lettuce).
Second, only one aquaponic system was evaluated for disease suppression. It would thus be relevant to study further the suppressiveness in aquaponic systems from different localization and with different specificities. Influence of the fish species or management practices on disease suppressiveness and microbiota should be for example determined. Suppressiveness in decoupled aquaponic systems is also important to evaluate because they are developing for commercial applications. Is a running and an already cycled decoupled aquaponic system more able to suppress diseases than in our experiments in Chapter D?
Third, factors influencing aquaponic microbiota and interactions observed in the plant root zone are complex and not yet fully understood. Transcriptomic and proteomic can be used to have a better understanding of aquaponic microbiota – plant interaction. In particular, is aquaponic microbiota able to stimulate a plant defense reaction?
Fourth, the influence of organic matter in aquaponic disease suppressiveness should be investigated. Indeed, organic substances found in aquaponic water were never identified or quantified, while they were found involved in soil disease suppressiveness.
Fifth, from a more applied point of view, SHb30 biocontrol efficacy should be further studied. Because of its multitask potential (i.e., soil bioremediation, disease biocontrol and plant biostimulation) and its safer use, SHb30 is probably the most valuable strain isolated by our for biocontrol in aquaponics. However, so far, SHb30 has only been tested in hydroponic conditions. Moreover, its biocontrol activity in hydroponics was found variable over time. It is then proposed to study the addition of organic matter to the hydroponic system in order to improve SHb30 biocontrol efficacy (e.g., use organic plant substrate and/or organic fertilizer). The other possibility is to formulate SHb30 in a biopesticide containing adjuvants and carriers (e.g., organic) that will improve its antagonistic mechanisms of action or improve its establishment in the environment. SHb30 efficacy and safety should also be evaluated to be implemented in real aquaponic conditions. Mechanisms of action of SHb30 must be elucidated to gain a better comprehension of its biocontrol potential. Only antibiosis and parasitism enzymes production were studied as mechanisms. Plant defense elicitation and competition for nutrients and space should also be evaluated.
At last, from all this further research, a better understanding of aquaponic complexity can be gained and used to promote plant disease suppressiveness. For example, aquaponic disease suppressiveness could be conducted by an adequate system management (e.g., fish waste and environmental parameters management), the

addition of specific substances (e.g., specific organic compounds), or by the addition of specific biocontrol agents adapted to aquaponic conditions (e.g., SHb30).

To conclude, this thesis provided a first insight into aquaponic suppressiveness and paved the way for plant pathogen biocontrol in aquaponics. However, aquaponics is a complex and living system that only begins to yield its secrets.

Appendices

Supplementary material – chapter C

Lettuce rhizoplane enumeration - Bacterial diversity analysis by 16S rRNA gene

Figure C-S1: Bacterial diversity analysis by 16S rRNA gene can be found and visualized interactively at http://www.mdpi.com/2076-2607/8/3/342/s1

Supplementary material – chapter D

Water origin

For the *in vitro* tests, 3 different types of water were tested independently, i.e., recirculated aquaculture system (RAS) water, aquaponic (AP) water and washing water containing microorganisms of the AP biofilter media (BM for Biofilter Microbiota). For the *in vivo* test, AP water, complemented aquaponic (CAP) water and hydroponic (HP) water were tested. The RAS system is described in supplementary materials of Eck et al. (2019). Briefly it consists in a Nile Tilapia (Oreochromis niloticus) fish husbandry kept in 3 tanks of 380 litres at a density of 60kg/m3, a drum filter to remove sludge, a biofilter containing Biocerapond media to transform ammonia to nitrate, and a sump where water is pumped to go back in the fish tanks. The AP system named PAFF Box for Plant And Fish Farming Box is fully explained in Delaide et al. (2017). This system is composed of 2 tanks of 380 litres at a density of 60kg of Nile Tilapia per m3, a lamellar settler to remove solids (upgrade from Delaide et al. (2017), a sump where water is pumped through a microbeads biofilter (SHARK BEAD 45/25) and then goes upstairs in a greenhouse to supply a raft hydroponic system (deep water culture) composed of a majority of lettuces with other additional plants (basil, swiss chard, strawberries, parsley, coriander, cucumber, hot pepper, tomatoes and watercress). Fish in RAS and in AP system were fed with adapted level of tilapia feed (TI-4.5 Tilapia 4.9mm, Skretting, Fontaine Les Vervins, France). RAS and AP water samples were taken respectively in the RAS and in PAFF Box sump. The washing water with the BM was recovered as followed: centrifuge tubes of 50ml filled with 15g of microbead from the PAFF Box SHARK BEAD biofilter were sonicated in ultrasound bath during 10min with 30ml of 0.05M Kalium Phosphate Buffer plus 0.05% Tween 80 (KPBT) at pH 6,5. The washing solution containing microorganisms scraped off from the microbeads was filtrated in a cheese cloth to remove microbeads and biggest particles and then used for the experiment. CAP water is the water taken in the PAFF Box sump where mineral salts were added to reach hydroponic nutrients concentration. Finally, HP water is a hydroponic nutrient solution made by addition of mineral salts in demineralized water. For the *in vivo* test, composition of AP, CAP and HP water were summarized in Table D-S1.

Table D-S1: Bio-chemical parameters of aquaponic (AP), hydroponic with half nutrient salts (½ HP), hydroponic (HP) and complemented aquaponic (CAP) waters for the 2 tests replication (trial 1 and 2). The parameters measured were BOD5, microorganisms concentration, pH, Ec, and mineral nutrients concentration.

Water kind	BOD5 in mg/l	Microorganisms' concentration in CFU/ml		pH	Ec in µS/cm	Macronutrients in mg/l							Micronutrients in mg/l					
		PDA dishes	LB dishes			$NO3^-$ -N	NH_3^+ -N	PO_4^{3-} -P	K^+	Ca^{2+}	Mg^{2+}	SO^{4-} -S	Fe^{2+}	Zn	B	Mn	Cu	Mo
AP Trial 1	6.0	$0.9x10^3$	$9.0x10^3$	7.83	1380[y]	65.5	0.7	32.4	30.0	170.0	35.0	48.5	0.00	NM	NM	NM	NM	NM
AP Trial 2	4.6	$2.5x10^4$	$8.4x10^4$	7.38	1200[y]	43.3	0.8	25.0	90.0	100.0	30.0	66.7	0.12	NM	NM	NM	NM	NM
½ HP Trial 1	NM	$1.1x10^4$	$1.5x10^4$	5.72[z]	1161[x]	82.6	12.7	25.0	98.3	100.0	20.0	40.8	0.00	0.00	0.00	0.00	0.00	0.00
½ HP Trial 2	NM	$1.9x10^5$	$3.4x10^5$	5.57[z]	1083[x]	82.6	12.7	25.0	98.3	100.0	20.0	40.8	0.00	0.00	0.00	0.00	0.00	0.00
HP Trial 1	NM	NM	NM	5.67[z]	2210[x]	164.9	25.1	50.0	210.0	200.1	40.0	87.5	5.00	0.10	0.50	0.50	0.10	0.03
HP Trial 2	NM	NM	NM	5.64[z]	1971[x]	164.9	25.1	50.0	210.0	200.1	40.0	87.5	5.00	0.10	0.50	0.50	0.10	0.03
CAP[w] Trial 1	NM	NM	NM	5.77[z]	2515[y]	161.5	29.8	68.2	211.5	202.9	44.74	48.9	5.00	0.10	0.50	0.50	0.10	0.05
CAP[w] Trial 2	NM	NM	NM	5.72[z]	2245[y]	165.0	25.0	50.0	210.0	200.0	40.0	90.4	5.00	0.10	0.50	0.50	0.10	0.05

[w]The first week, CAP waters had the same composition than AP water with a pH adjusted to 5.5 - 5.8.
[x]Ec of ½ HP and HP waters differ between the trial 1 and 3 due to a different demineralized water quality (not measured).
[y]The organic matter and the non-linear NaCl concentration of AP water can disturb Ec measures.
[z]pH was measured after the first pH adjustment to the range 5.5 - 5.8
Sulfate was used as degree of freedom
NM = not measured

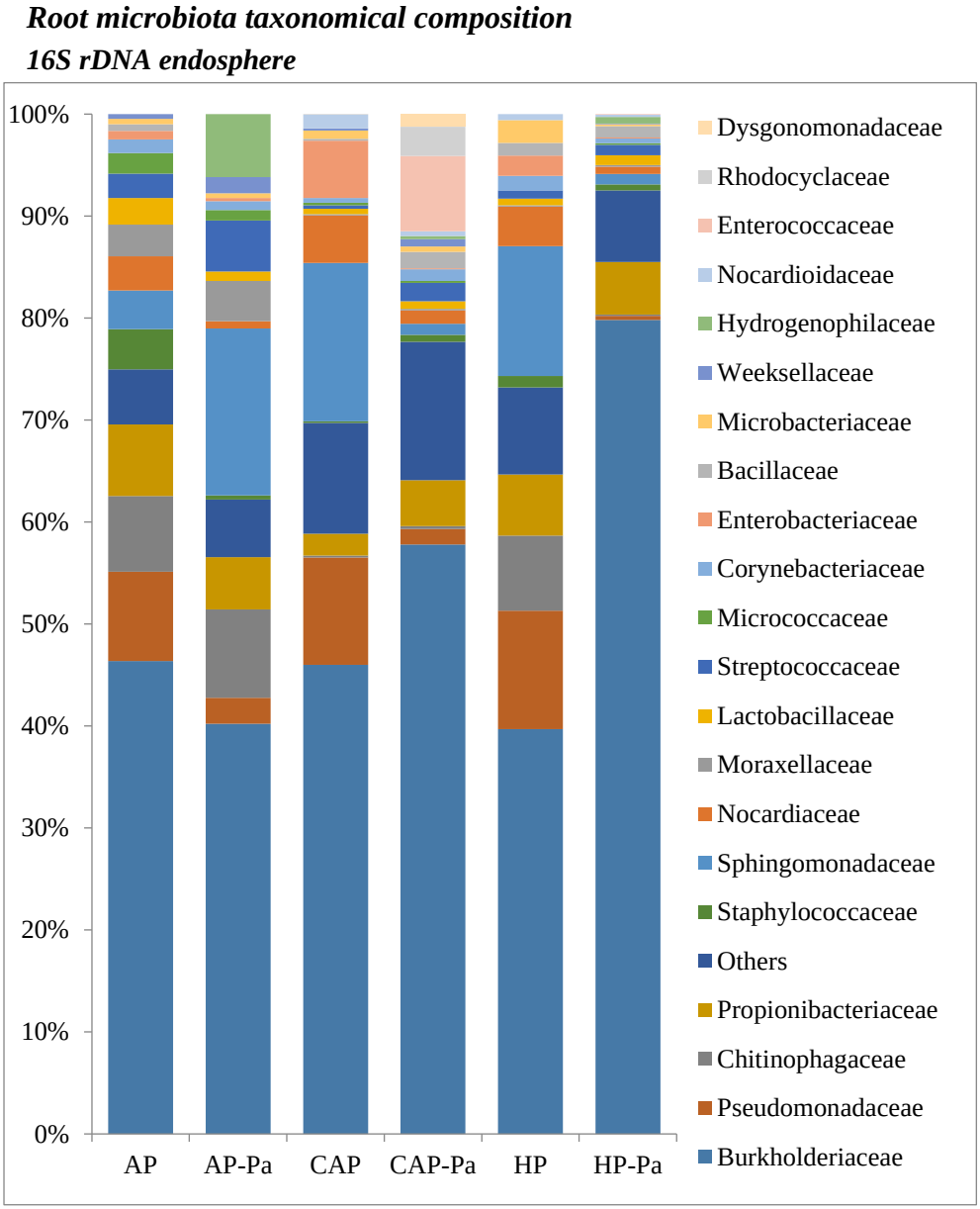


Figure D-S1: Means of bacterial relative composition, at family level, of lettuce roots endosphere depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum*. Only the OTUs with proportion higher than 1% were represented and the rest were clustered in "Others".

16S rDNA rhizoplane

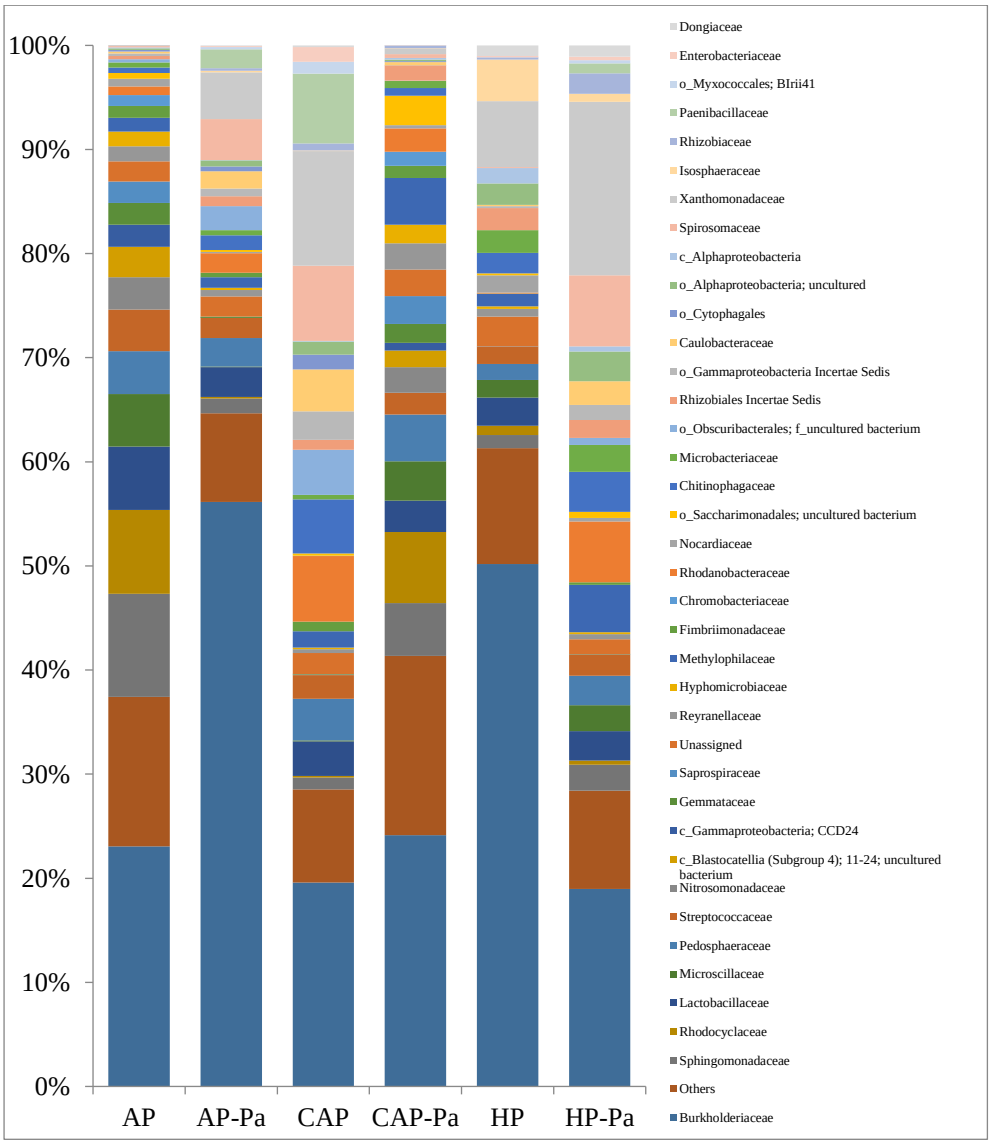


Figure D-S2: Means of bacterial relative composition, at family level, of lettuce roots rhizoplane depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum*. Only the OTUs with proportion higher than 1% were represented and the rest were clustered in "Others".

16S rDNA rhizosphere

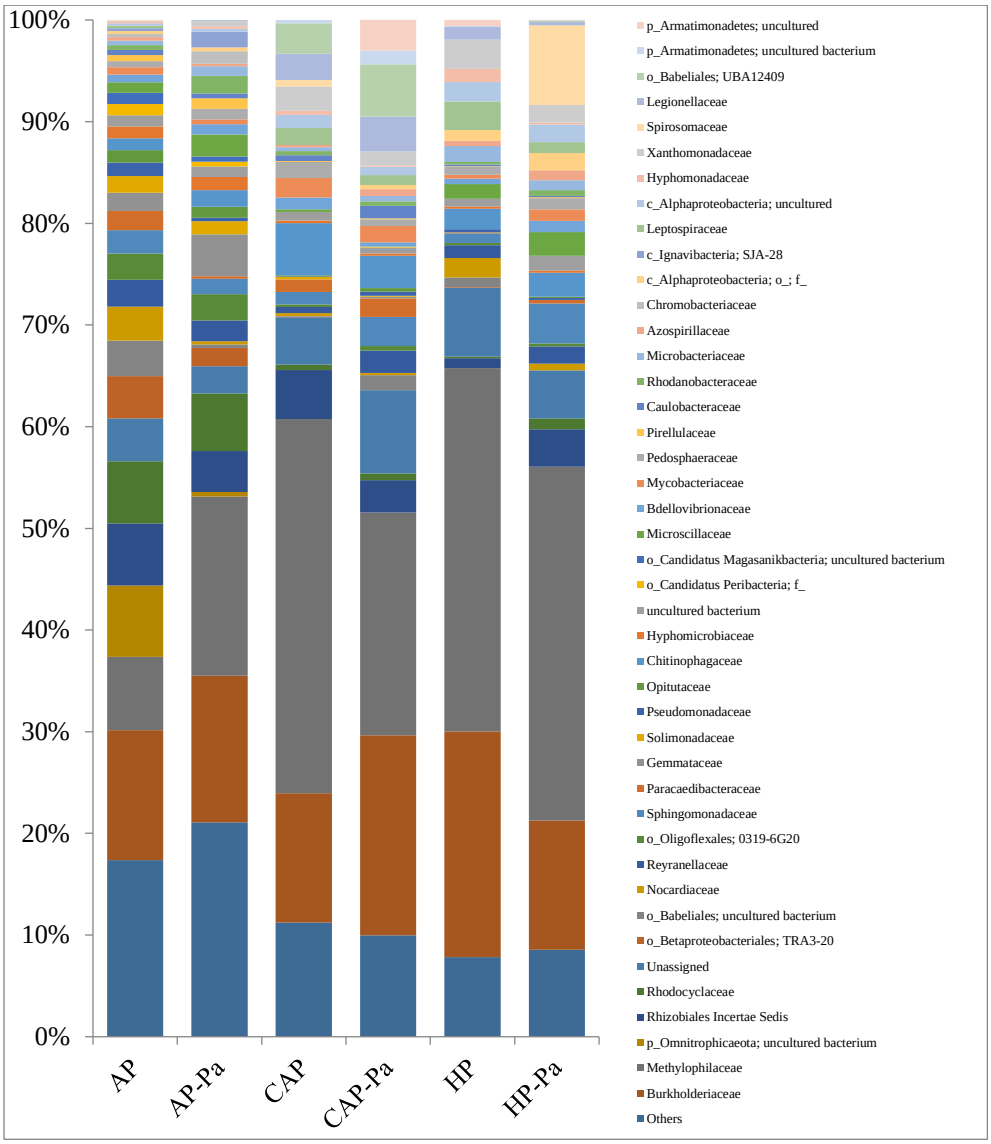


Figure D-S3: Bacterial relative composition, at family level, of lettuce roots rhizosphere depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum*. Only the OTUs with proportion higher than 1% were represented and the rest were clustered in "Others". Compositions were based on a unique microbiota sample.

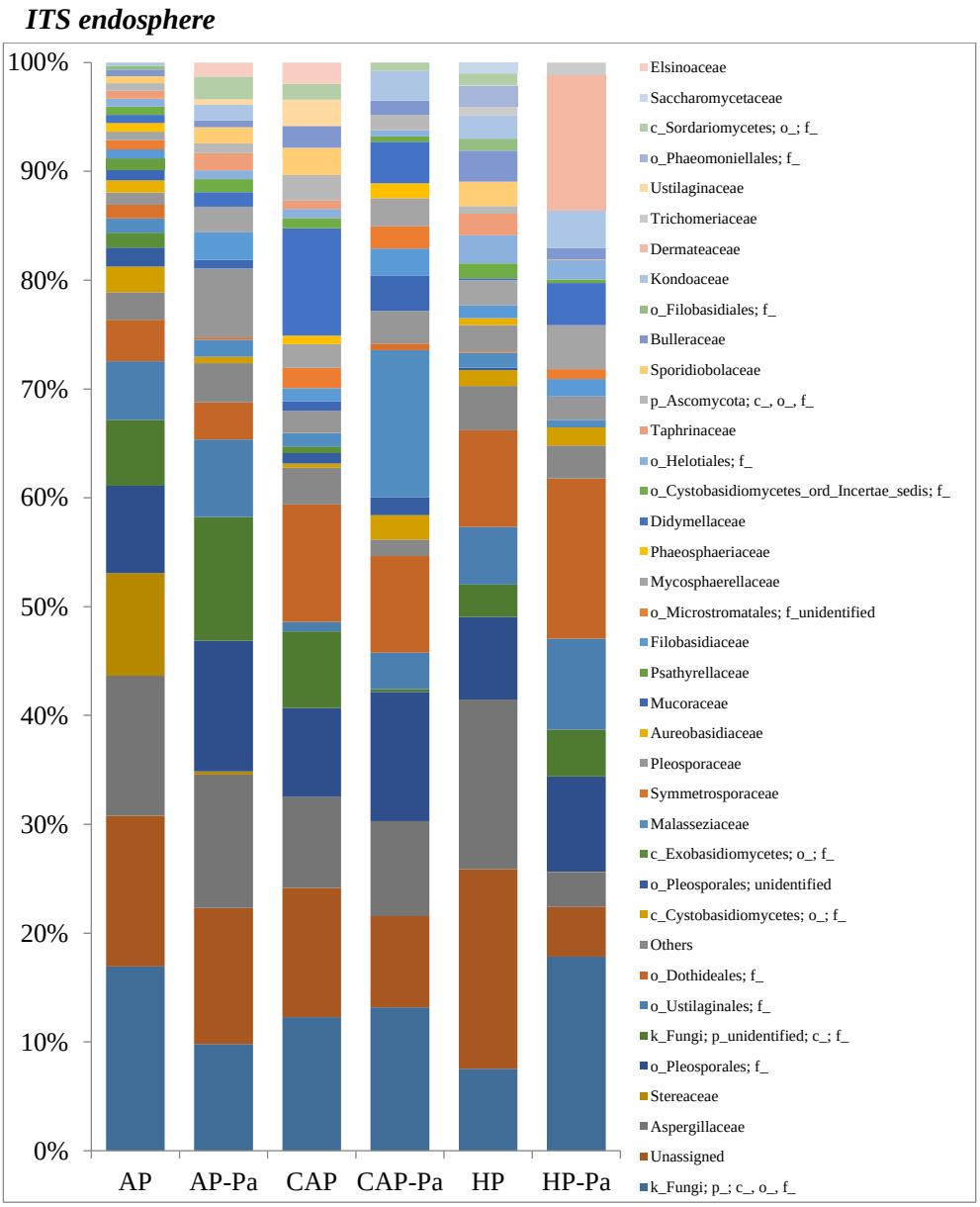


Figure D-S4: Means of fungal relative composition, at family level, of lettuce roots endosphere depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum*. Only the OTUs with proportion higher than 1% were represented and the rest were clustered in "Others".

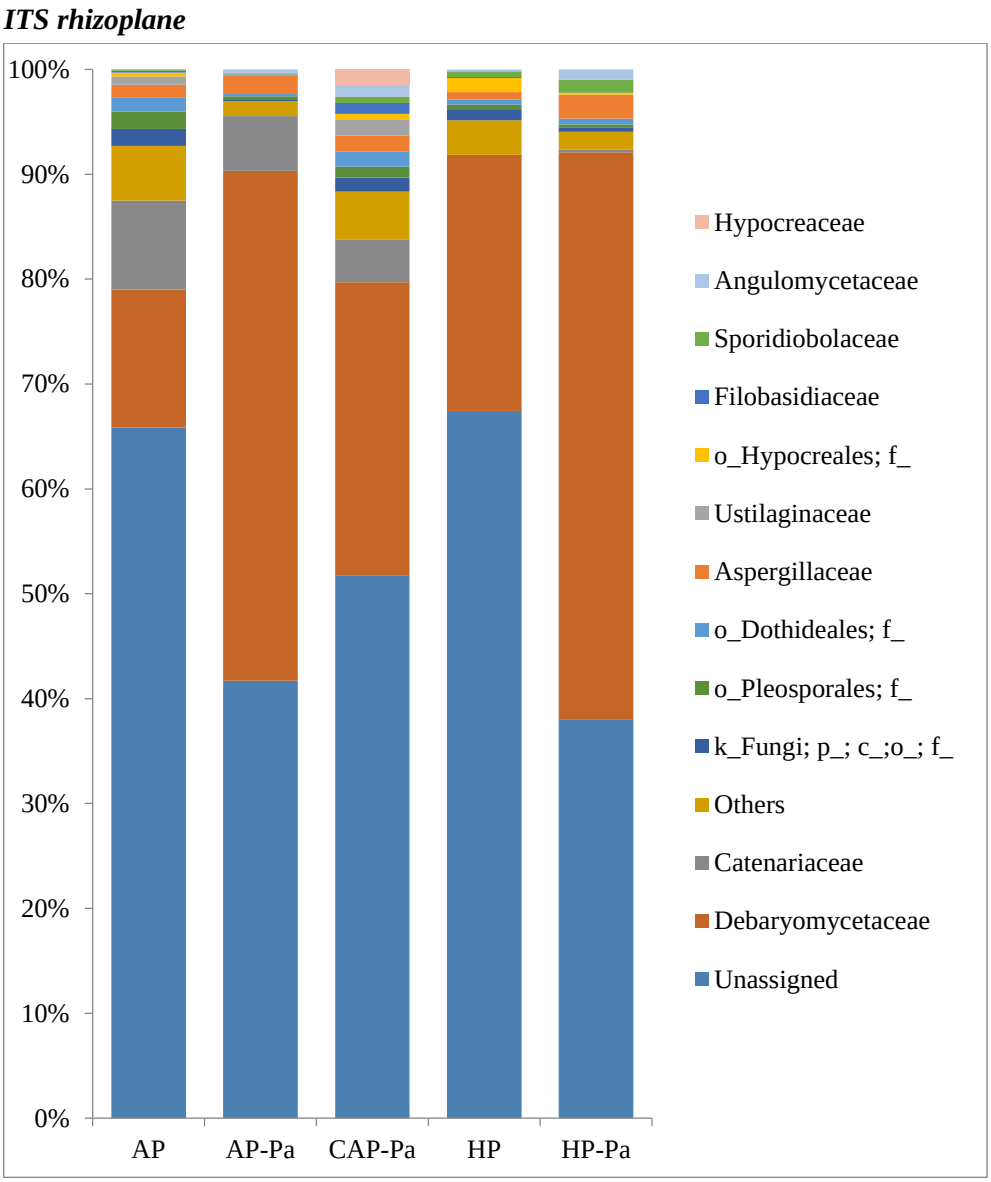


Figure D-S5: Means of fungal relative composition, at family level, of lettuce roots rhizoplane depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum*. Only the OTUs with proportion higher than 1% were represented and the rest were clustered in "Others". CAP water was removed from the analysis during de bioinformatics process.

ITS rhizosphere

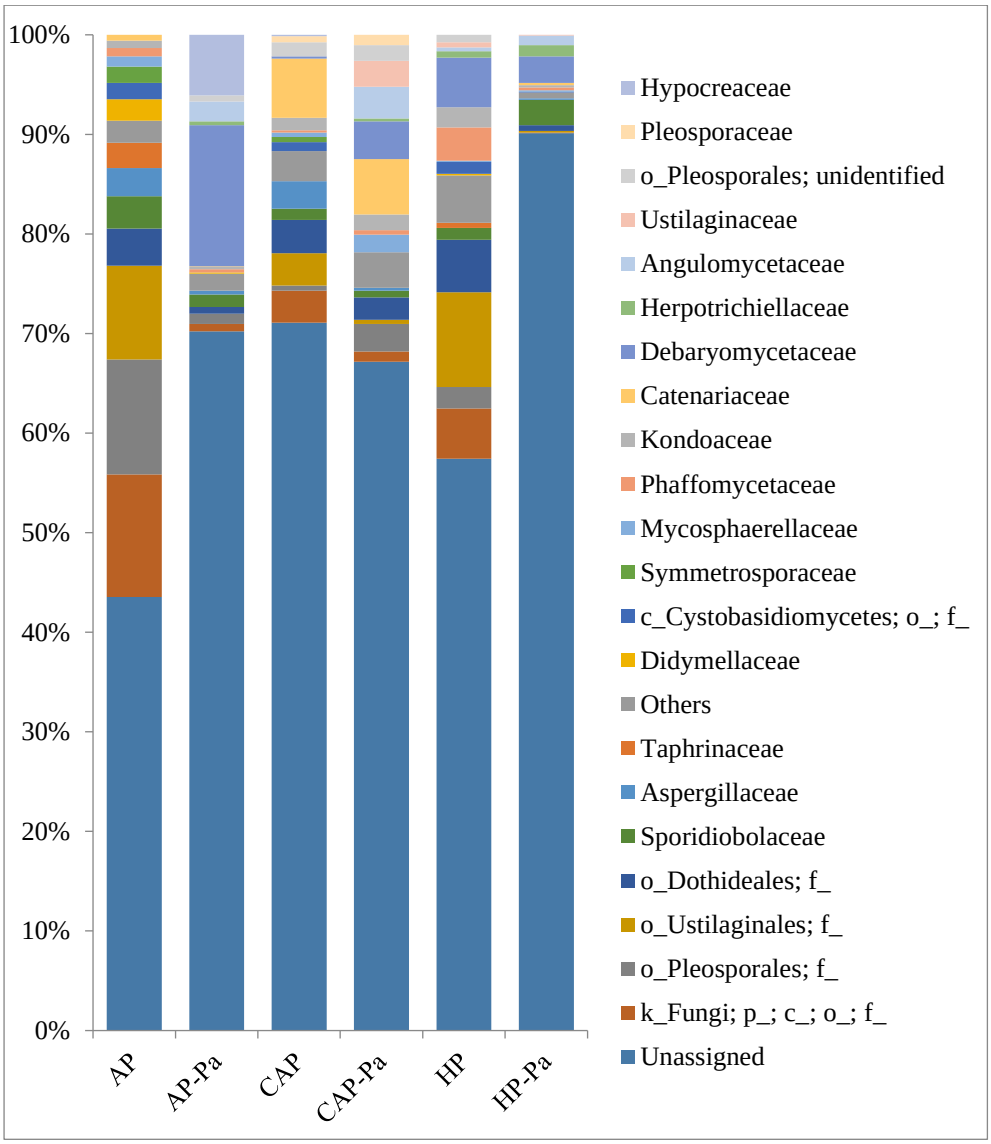


Figure D-S6: Fungal relative composition, at family level, of lettuce roots rhizosphere depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum*. Only the OTUs with proportion higher than 1% were represented and the rest were clustered in "Others". Compositions were based on a unique microbiota sample.

Microbiota α-diversity

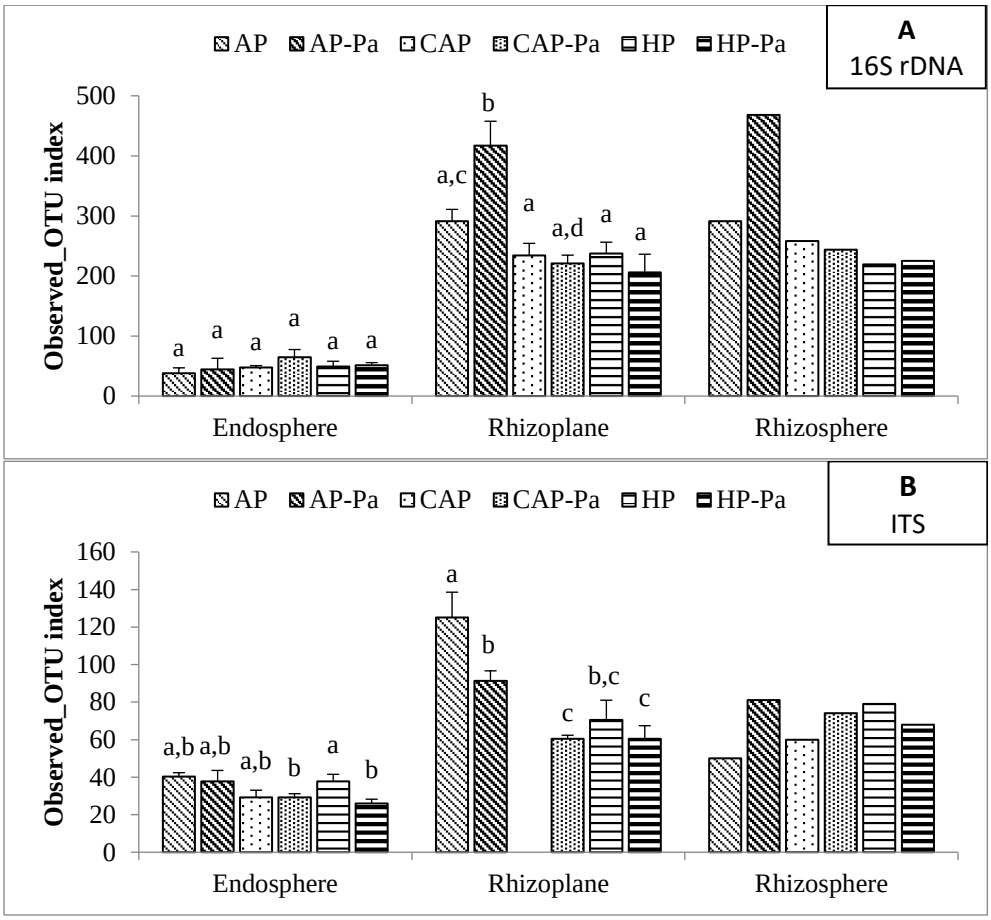


Figure D-S7: Species richness (observed_OTU number) of lettuce endosphere, rhizoplane and rhizosphere of (A) 16S rDNA and (B) ITS analyses depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum.* Bars indicate the standard error of the mean Treatments that do not share a same letter are significantly different by Kruskal-Wallis pairwise test ($p \leq 0.05$). CAP treatment in the ITS rhizoplane was removed by the rarefaction process during bioinformatic analysis. Rhizosphere microbiota was constituted of a unique sample by treatment and was not subject to statistical analysis.

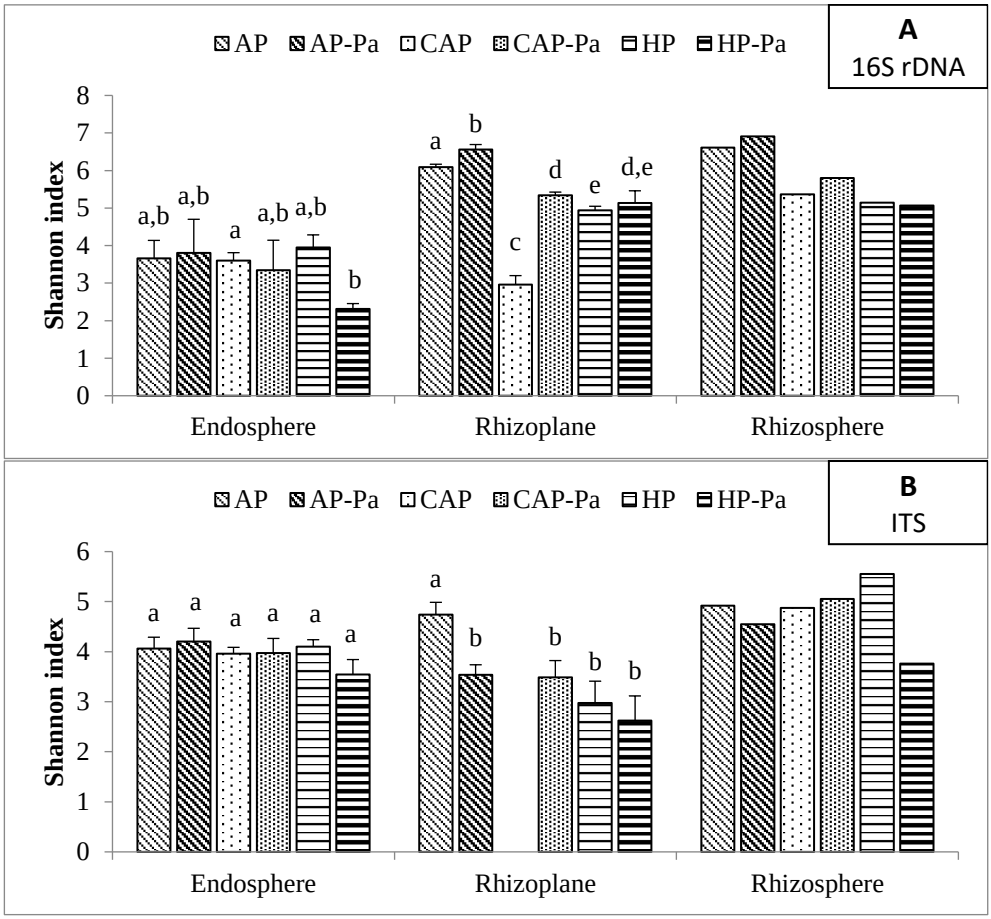


Figure D-S8: Species diversity (Shannon index) of lettuce endosphere, rhizoplane and rhizosphere of (A) 16S rDNA and (B) ITS analyses depending on the treatment. Treatments are aquaponic (AP), hydroponic (HP) and complemented aquaponic (CAP) waters and respectively AP-Pa, HP-Pa and CAP-Pa water after lettuces inoculation with *P. aphanidermatum*. Bars indicate the standard error of the mean. Treatments that do not share a same letter are significantly different by Kruskal-Wallis pairwise test ($p \leq 0.05$). CAP treatment in the ITS rhizoplane was removed by the rarefaction process during bioinformatic analysis. Rhizosphere microbiota was constituted of a unique sample by treatment and was not subject to statistical analysis.

Supplementary material – chapter E

Isolation of the targeted microorganisms

Burkholderiaceae isolation

Mitsuaria, *Burkholderia* and *Chitinimonas* were chosen as target genera among Burkholderiaceae. *Mitsuaria* was the closest blast hit of the OTUs identified as Burkholderiaceae in Stouvenakers et al. (2020), with possible cross identification with *Leptothrix* and *Roseateles*. *Mitsuaria, Burkholderia* and *Chitinimonas* are all chitosanase-producing bacteria (Amakata et al., 2005; Benítez and McSpadden Gardener, 2009; Coenye, 2014). A protocol for chitosanase detection on Petri plates was developed based on Cheng and Li, (2000), Öztopuz et al. (2018), and Yun et al. (2005). Chitosanase detection medium (CDA) with colloidal chitin as the sole carbon source was used. For 1 liter of distilled water, CDA was composed of 5.0 g colloidal chitosan, 1.3 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 0.24 g $MgSO_4$,$7H_2O$, 1.0g NH_4Cl, 0.01 g $CaCl_2$ and 20.0 g agar. The pH was adjusted at 6.5-7.0 with NaOH, and the medium was autoclaved at 121°C for 20 min. Colloidal chitosan used for CDA medium was prepared from deacetylated chitosan according to Yabuki et al. (1988). CDA plates were inoculated with serial-diluted rhizoplane water according to Stouvenakers et al. (2020) and incubated at 28°C for 15 days. The plates were checked for clear zones of chitosan hydrolysis around the CFUs. Then, the CFUs were purified and observed under a light microscope after Gram staining. Gram-negative rod bacteria were selected for further identification.

Lactobacillus isolation

Agar medium according to De Man, Rogosa and Sharpe (MRS agar, Merck Millipore) was used for selective isolation of *Lactobacillus* spp.. MRS agar plates were inoculated with serial-diluted melted rhizoplane water from Stouvenakers et al. (2020) or fresh aquaponic lettuce water, and incubated at 28°C for 3 to 5 days. CFUs were purified and observed under a light microscope after Gram staining. Gram-positive long rod bacteria potentially forming chains were selected for further identification.

Methyloversatilis isolation

Selective liquid medium enrichment and direct plating on selective solid medium were used. For enrichment, 150-mL Erlenmeyer flasks containing 20 mL of liquid mineral salt solution with 0.1% methanol (MIN E) were inoculated with 150 µl of melted rhizoplane water from Stouvenakers et al. (2020). MIN E was prepared according to the specifications of the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) for *Methyloversatilis* growth, with a methanol concentration

of 0.1%. Flasks were left to enrich at 28°C under shaking at 125 rpm. On day 5, formaldehyde (10-15% methanol) was added to obtain a concentration of 1 mM per flask. Nine days later, serial-diluted enriched samples were plated on solid MIN E and incubated at 28°C for 5 days. For direct plating, Petri plates with solid MIN E supplemented with 1 mM formaldehyde were inoculated with serial diluted melted rhizoplane water from Stouvenakers et al. (2020) or fresh aquaponic lettuce water. Then, the plates were incubated at 28°C for 11 days. Whatever the isolation technique, the plates were checked for white to brown CFUs with a diameter of 2 mm maximum. The selected CFUs were plated on 5 mM formaldehyde-supplemented MIN E and incubated at 28°C. Growing strains were purified and observed under a light microscope after Gram staining. Gram-negative rod bacteria were selected for further identification.

Sphingobium and Hydrogenophaga isolation

The capacity of *Sphingobium* and *Hydrogenophaga* spp. to produce yellow pigments in R2A agar (Merck Millipore, Merck Chemicals N.V./S.A, Overijse, Belgium) medium was used (Brenner et al., 2005). R2A plates supplemented with 50 mg/L nystatin were inoculated with serial-diluted melted rhizoplane water from Stouvenakers et al. (2020) or fresh aquaponic lettuce water and incubated at 28°C for 3 to 7 days. Plates were checked for light yellow to orange-brown CFUs 0.5 to 5.0 mm in diameter. The CFUs were purified and observed under a light microscope after Gram staining. Gram-negative rod bacteria were selected for further identification.

Trichoderma isolation

Rose bengal, streptomycin sulfate and formalin in peptone-dextrose agar (RB-S-F of McFadden and Sutton, (1975) was used as a solid medium for selective *Trichoderma* spp. isolation. For 1 liter of distilled water, RB-S-F agar medium was composed of 1 g KH_2PO_4, 0.5 g $MgSO_4$, 5 g peptone, 10 g glucose, 17 mg rose Bengal, 20 g agar, plus 0.2 mL formaldehyde and 30 mg streptomycin sulfate after autoclaving at 121°C for 20 min. Then, the RB-S-F plates were inoculated with serial-diluted melted rhizoplane water from Stouvenakers et al. (2020) or fresh aquaponic lettuce water, and incubated at 23°C for 21 days. The plates were checked daily for fast-growing molds with green to brown-green sporulation. The selected fungal spots were purified and observed under a light microscope. The strains of interest were kept for further identification.

Catenaria and Rhizophydiales isolation

The genera *Catenaria* and *Rhizophydium* (Rhizophydiales order) are both potential *Pythium* spp. parasites that can be isolated by baiting procedures. Baits were *P. aphanidermatum* oospores or mycelium. For oospores, the procedure was adapted from Sneh et al. (1977). Briefly, ± $1x10^5$ oospores (see *in vivo* screening Section for

oospore production) were fixed on a sterile 0.45-µm filter (47 mm Supor® 450 Membrane Disc Filter, PALL Corporation, Portsmouth, UK) by vacuum filtration. Instead of soil, one rockwool plug slice (36 x 40 x 8 mm) and 5 mL of fresh aquaponic lettuce rhizoplane water were deposited in each Petri dish. The plugs were either new ones or had been recently used for lettuce growth in the PAFF Box. The filters were placed oospore side up on the rockwool slices to allow water capillarity. Then, the dishes were incubated at 23°C. On day 7 or 14, the filters were recovered and the oospores were transferred to water agar 3% + streptomycin 10 mg/l according to Sneh (1977). Agar plugs were mounted for microscopy observation and observed daily for oospore parasite structures or zoospores.

For the mycelium baiting procedure, mycelium plugs of *P. aphanidermatum* were used. *P. aphanidermatum* was grown on potato dextrose agar (PDA, Merck Millipore) at 23°C for 3 days. The plugs were placed in 50-mL centrifuge tubes filled with 5 mL of 0.85% NaCl sterile water + 10 mg/l streptomycin. The tubes were inoculated with 200 µl of melted rhizoplane water from Stouvenakers et al. (2020) and incubated at 23°C. Water and mycelium were observed daily from day 7 for parasite oospore structures or zoospores. Observations were made visually, under a binocular microscope and under a light microscope after slide mounting. Whatever the baiting procedure, suspected parasite structures were isolated on corn meal agar (CMA, Merck Millipore) or beef extract agar (BEA, composed of 10 g peptone, 5 g NaCl, 3 g beef extract and 15 g agar per liter) supplemented with 10 mg/l streptomycin. Fungal spots were purified and observed under a light microscope for specific structures. The strains of interest were kept for further identification.

In vivo screening results

Table E-S1: Mean germination rate (MGR) of lettuce seeds following treatment against *P. aphanidermatum* with the selected microbial species depending on the inoculation method (pre-inoculation or biopriming). N: size of the population (i.e., number of treated seeds).

SPECIES OR CONTROL NAME	STRAIN ABBR.	PRE-INOCULATION		BIO-PRIMING	
		N	MGR	N	MGR
CONTROLS					
Negative control C-	/	288	60.0%	168	93.5%
Positive control C+	/	268	29.1%	148	0%
Fungicide control Cf	/	268	25.2%	148	6.3%
Biopesticide control Cpc	/	235	12.0%	148	7.0%
10x biopesticide control Cpc.10x	/	32	15.6%	32	28.1%

BACTERIA					
Bacillus flexus	SHb2	8	0%	8	0%
Bacillus indicus	SHb31	8	0%	8	0%
Bosea thiooxidans	SH9	24	20.8%	24	4,2%
Enterobacter cloacae complex	L13	48	27.1%	48	8.3%
Hydrogenophaga pseudoflava	DSM 1034	24	12.5%	24	4.2%
Hyphomicrobium sp.	M8	8	0%	8	12.5%
Hyphomicrobium vulgare	M18	24	20.8%	24	4,2%
Hyphomicrobium zavarzinii	M13	48	25.0%	24	8.3%
	M25	48	41.6%	NA	NA
	M25.10x	24	66.7%	NA	NA
	M28	24	33.3%	NA	NA
	M32	23	17.4%	NA	NA
	M35	24	37.5%	NA	NA
	M36	23	8.7%	NA	NA
	M37	24	29.2%	NA	NA
	M38	24	41.7%	NA	NA
Methylorubrum podarium	Chito6	56	23.6%	24	0%
Methylorubrum populi	Mc	16	62.5%	16	0%
	Mk	48	50%	24	8.3%
	Mk.10x	24	37.5%	NA	NA
	Mq	24	52.4%	16	6.2%
Methyloversatilis universalis	DSM 25237	56	33.3%	24	8.3%
Microbacterium kitamiense	SHb4	8	0%	8	0%
Microbacterium lacus	SHb23	32	15.6%	24	8.3%
Microbacterium paraoxydans	SHb18	56	25.5%	24	0%
	SHb18.10x	24	41.7%	NA	NA
Microbacterium sp.	SH22	8	0%	8	0%
	SH28	8	0%	8	0%
Micromonospora maritima	SH32	8	0%	8	0%
Mycolicibacterium aurum	M7	8	0%	8	0%
Mycolicibacterium fluoranthenivorans	M17	24	20.8%	24	0%
Mycolicibacterium fortuitum	Chito1	24	58.3%	24	4.2%
	Chito5	47	59.6%	24	4.2%

	Chito8	24	58.3%	NA	NA
	Chito11	24	62.5%	NA	NA
	Chito13	48	58.3%	NA	NA
	Chito13.10x	24	79.2%	NA	NA
	Chito16	23	56.5%	NA	NA
	Chito17	24	62.5%	NA	NA
	Chito18	24	29.2%	NA	NA
Mycolicibacterium sp.	Chito10	24	16.7%	24	0%
Mycolicibacterium sp.	Chito2	8	0%	8	0%
Mycolicibacterium wolinskyi	M33	48	39.6%	24	0%
	M33.10x	24	47.7	NA	NA
Nocardia fluminea	Chito7	48	41.7%	24	0%
	Chito7.10x	24	75.0%	NA	NA
Novosphingobium aromaticivorans	SHb28	8	0%	8	0%
Pedobacter solisilvae	SHb34	8	0%	8	0%
Rhizobium sp.	SHb32	8	0%	8	0%
Rummeliibacillus suwonensis	L2	8	0%	8	0%
Sphingobium xenophagum	SHb9	56	53.6%	47	27.7%
	SHb14	32	53.1%	24	25.0%
	SHb27	32	53.1%	24	16.7%
	SHb30	56	51.0%	48	18.7%
	SHb30.10x	24	50.0%	24	37.5%
Streptomyces coelicoflavus	SHb13	24	8.3%	22	9.1%
FUNGI					
Aspergillus flavus	TS1	8	0%	8	0%
	G2	48	16.7%	56	12.5%
	G2.10x	24	41.7%	24	37.5%
Aspergillus fumigatus	G1	8	0%	8	0%
Catenaria anguillulae	CBS 42365	8	0%	8	0%
Cladosporium halotolerans	TS6	24	20.8%	32	0%
Cladosporium ramotenellum	TS11	24	4.2%	32	3.1%
Cladosporium sp.	TS13	48	25.0%	32	0%
	TS13.10x	24	33.3%	NA	NA

Cladosporium sphaerospermum	TS4	24	25.0%	32	6.2%
Penicillium citrinum	PC3	24	12.5%	32	3.1%

N: size of the population (number of treated seeds).
".10x":10-times higher concentration compared to the standard concentration.
NA: not applicable.

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List of Publications

Sare, A.R.*, **Stouvenakers, G*.**, Eck, M., Lampens, A., Goormachtig, S., Jijakli, M.H., Massart, S., 2020. Standardization of plant microbiome studies: Which proportion of the microbiota is really harvested? Microorganisms 8, 17. https://doi.org/10.3390/microorganisms8030342

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