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Oxalotrophy as a biocontrol strategy

New insights in bacterial-fungal interactions

A dissertation submitted to the University of Neuchâtel, Switzerland

for the degree of *Docteur ès sciences*

by

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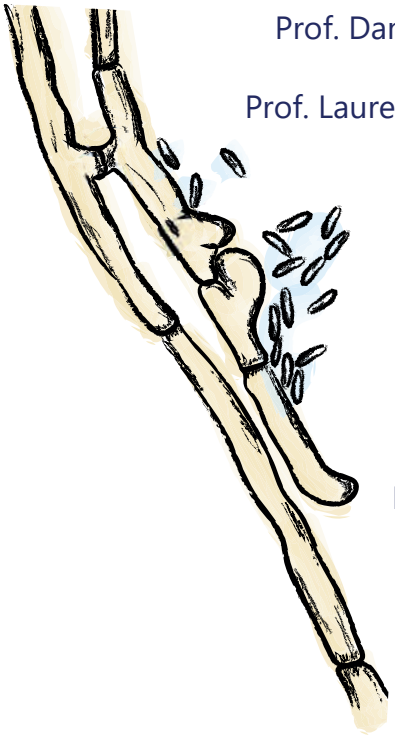
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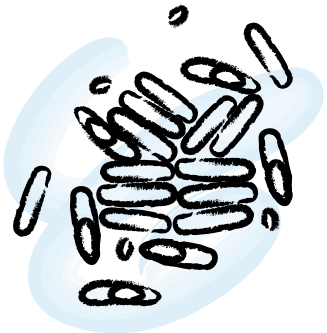
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"All these lights are colored in by you
All these times are precious due to you
Four seasons have passed with you
Four scents were left cause of you"

My you - Jungkook

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Summaries

Summary

Phytopathogenic fungi are a major threat to crops, and cause important economic losses every year. Furthermore, intensive and systematic fungicide application contributes to select resistant pathogenic strains and it is difficult to develop non-susceptible plant cultivars. In order to ensure the future of the agriculture, there is therefore a necessity to develop alternative pathogen control strategies, such as biocontrol. Many fungal phytopathogens use low molecular weight organic acids, and in particular oxalic acid, as a factor of pathogenicity. Oxalic acid is a highly versatile molecule mainly produced by plants and fungi, and ubiquitously found in nature. Numerous bacterial species can consume oxalate, the conjugated base of oxalic acid, as carbon and energy sources, and are thus called oxalotrophic bacteria. Bacterial oxalotrophy induces a strong alkalinisation locally, therefore modifying the physicochemical properties of the environment. Oxalotrophy as a biocontrol strategy has shown a great potential against oxalogenic phytopathogenic fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum*, two relevant pathogens for important crops. The aim of this thesis was therefore to provide new insights in bacterial-fungal interactions in the case of oxalotrophy as biocontrol strategy.

For this purpose, three methods to assess the interactions occurring between fungal resting structures, such as spores or sclerotia, and bacterial cells were developed. We investigated the control of germination of *B. cinerea* spores by two oxalotrophic soil bacteria, *Cupriavidus necator* and *Cupriavidus oxalaticus*, with two different media, diluted malt medium and Reasoner's 2 medium. The first medium favours fungal growth, while the second was originally developed for bacterial growth. Oxalic acid production by the fungus and growth control by the bacteria was assessed in both media. Mycelial growth control was only efficient in Reasoner's 2 agar or broth (R2A; R2B), the medium in which *B. cinerea* produced oxalic acid. Conversly, no oxalic acid was detected in diluted malt broth (MB1/10) and both bacteria were able to control the mycelial growth, but not the germination of *B. cinerea* spores. The control was attributed to the consumption of oxalic acid, however, as oxalotrophy impacts the physicochemical properties of the environment, the next step of this study was to disentangle oxalic acid consumption from the impact of bacterial development on pH.

To determine the effect of the pH change on the germination and the mycelial growth of *S. sclerotiorum*, a wild type (WT) strain of the pathogen and a mutant deficient in oxalic acid production (Δ*oah*) were confronted to *C. necator* and *C.oxalaticus* on the aforementioned media. Growth control was efficient only in R2B, the medium in which oxalic acid was produced by *S. sclerotiorum* WT. However, *S. sclerotiorum* Δ*oah* was also controlled on this medium, indicating that oxalic acid consumption was not the only mechanism controlling fungal growth. Further investigations showed that media alkalinisation driven by oxalic acid consumption or additional aspects of bacterial metabolism was responsible for the fungal growth control. Observations by microscopy of the interactions occurring during biocontrol

assays also showed that *C. necator* and *C. oxalaticus* had very different behaviours towards the fungal mycelium depending of the medium used. In R2B, bacteria were attracted to the fungus, while on MB1/10, they were clearly repulsed. Further experiments showed that the bacteria were not attracted by the spent medium of *S. sclerotiorum* cultures in R2B and that the attraction seemed to be dependant of a metabolite physically associated to the mycelium. For the repulsion, additional experiments showed that the repulsive metabolite had antibacterial properties and could be retrieved by selective binding to poly-l-lysine. Further studies are still required to determine the nature of the metabolite and its antibacterial potential against other Gram-positive and Gram-negative bacteria.

Finally, oxalotrophic endospore-forming bacteria (EFB) were isolated from a soil sample in order to assess their efficiency as biocontrol agents. Several oxalotrophic EFB were isolated. All the isolates were identical at more than 99% based on 16S rRNA gene sequencing and were closely related to *Ammoniphilus oxalaticus*, an obligate oxalotrophic bacterium. The isolated strain, *Ammoniphilus oxalaticus* strain HJ, was able to germinate and grow with calcium-oxalate (CaOx), potassium-oxalate (KOx) or oxalic acid as carbon source. However, the bacterium was only able to complete its life cycle (germination, growth, and sporulation) with CaOx. The germination and growth of *A. oxalaticus* strain HJ spores was also assessed in presence of actively growing mycelium of *B. cinerea* and *S. sclerotiorum*, in order to determine if the concentration of oxalic acid produced by these two oxalogenic fungi was sufficient to support the growth of *A. oxalaticus* strain HJ. Endospore germination and growth of *A. oxalaticus* strain HJ was observed only in the presence of *S. sclerotiorum*. Vegetative cells were found to be very active in proximity of the mycelium. Unfortunately, the potential of *A. oxalaticus* strain HJ as biocontrol agent could not be assessed during the course of this thesis. However, preliminary results showed that *S. sclerotiorum* growth was slowed down in presence of vegetative cells of *A. oxalaticus* strain HJ. Still, much remains to be investigated to assess the potential of *A. oxalaticus* strain HJ as biocontrol agent against oxalogenic phytopathogenic fungi.

Keywords

Oxalic acid, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Cupriavidus* spp., phytopathogenic fungi, bacterial biocontrol agent, *in-vitro* experiments, endospore-forming bacteria, *Ammoniphilus oxalaticus*, antimicrobial compound

Résumé

Les champignons phytopathogènes représentent une menace importante pour les cultures et causent chaque année d'importantes pertes économiques. De plus, l'application intensive et systématique de fongicides contribue à la sélection de souches pathogènes résistantes aux antibiotiques. Afin d'assurer le futur de l'agriculture, il est donc nécessaire de développer des stratégies alternatives de lutte contre les pathogènes, telle que le biocontrôle. De nombreux champignons phytopathogènes utilisent les acides organiques à faible poids moléculaire, et en particulier l'acide oxalique, comme facteur de pathogénicité. L'acide oxalique est une molécule aux capacités très polyvalentes, qui est produite principalement par les plantes et les champignons. L'acide oxalique est ubiquiste dans la nature et de nombreuses bactéries, appelées les bactéries oxalotrophes, utilisent l'oxalate (la base conjuguée de l'acide oxalique) comme source de carbone et d'énergie. L'oxalotrophie bactérienne induit une forte alcalinisation et modifie ainsi les capacités physico-chimiques de l'environnement. L'oxalotrophie en tant que stratégie de biocontrôle s'est révélé être très efficace contre les champignons pathogènes oxalogéniques tel que *Botrytis cinerea* ou *Sclerotinia sclerotiorum*, deux champignons attaquant des cultures économiquement importantes. L'objectif de cette thèse était donc fournir de nouvelles connaissances sur les interactions bactérie-champignon dans le cas de l'oxalotrophie en tant que stratégie de biocontrôle.

Pour ce faire, trois méthodes pour évaluer les interactions entre les spores ou les sclérotes fongiques et les cellules bactériennes ont été développées. Nous avons étudié le contrôle de la germination des spores de *B. cinerea* par deux bactéries oxalotrophes du sol, *Cupriavidus necator* et *Cupriavidus oxalaticus*, avec deux milieux différents, le milieu malt dilué et le milieu Reasoner's 2. Le premier milieu favorise la croissance fongique, tandis que le second a été initialement développé pour la croissance bactérienne. La production d'acide oxalique et le contrôle de la croissance ont été évalués avec les deux milieux. Le contrôle de la croissance a été efficace seulement avec le milieu Reasoner's 2 sur boîte (R2A) ou en liquide (R2B), le milieu dans lequel *B. cinerea* a produit de l'acide oxalique. L'acide oxalique n'a pas été détecté dans le milieu malt liquide (MB1/10). Les deux bactéries étaient capables de contrôler la croissance mycélienne, mais elles étaient incapables de contrôler la germination des spores de *B. cinerea*. Le biocontrôle a été attribué à la consommation d'acide oxalique, cependant, comme l'oxalotrophie impacte les propriétés physico-chimiques de l'environnement, la prochaine étape de cette étude a été de dissocier la consommation de l'acide oxalique et de l'impact du développement bactérien sur le pH.

Afin de déterminer quel effet le changement de pH avait sur la germination et la croissance de *S. sclerotiorum*, la souche originale du champignon (WT) et une souche mutant incapable de produire de l'acide oxalique (Δ*oah*) ont été confrontées à *C. necator* et *C. oxalaticus* sur les deux milieux mentionnés précédemment. Le contrôle de la croissance fongique n'était efficace qu'avec R2B, le milieu dans lequel *S. sclerotiorum* produisait de l'acide oxalique. *S.*

sclerotiorum Δ*oah* était aussi contrôlé par les bactéries oxalotrophes dans ce milieu, ce qui indiquait que la consommation de l'acide oxalique n'était pas le seul mécanisme derrière le contrôle du champignon. Des recherches supplémentaires ont montré que l'alcalinisation du milieu était responsable du contrôle de la croissance fongique. Durant les expériences de confrontation, des observations au microscope ont montré que *C. necator* et *C. oxalaticus* avec un comportement très différent envers le champignon en fonction du milieu de culture utilisé. Avec R2B, les bactéries étaient attirées par le champignon, tandis qu'avec MB1/10, elles étaient clairement repoussées par le mycélium. Des recherches plus poussées ont montré que les bactéries n'étaient pas attirées par le milieu de culture récupéré après la croissance de *S. sclerotiorum* dans R2B et que l'attraction semblait dépendre d'un métabolite associé au mycélium. Pour ce qui est du métabolite répulsif, nous avons montré qu'il se liait à la poly-l-lysine et qu'il avait des propriétés antibactériennes. Des expériences additionnelles restent à réaliser afin de déterminer la nature du métabolite et son potentiel contre d'autres bactéries à Gram-positif ou négatif.

Finalement, des bactérie oxalotrophes sporulantes ont été isolées à partir d'un échantillon de sol afin de déterminer leur efficacité en tant qu'agent de biocontrôle. Plusieurs bactéries ont été isolées. Les isolats étaient identiques à plus de 99% et apparentés à *Ammoniphilus oxalaticus*, une bactérie sporulante strictement oxalotrophe. La souche isolée, *A. oxalaticus* strain HJ, était capable de germer et de pousser avec de l'oxalate de calcium (CaOx), de l'oxalate de potassium (KOx) et de l'acide oxalique. Cependant, cette bactérie ne pouvait compléter son cycle de vie (germination, croissance et sporulation) qu'avec CaOx. Afin de déterminer si les concentrations d'acide oxalique produites par *B. cinerea* et *S. sclerotiorum* étaient suffisantes pour supporter la croissance de la bactérie, les endospores d'*A. oxalaticus* strain HJ ont été inoculées en présence de mycélium des deux champignons. Les endospores d'*A. oxalaticus* strain HJ n'ont germé et poussé qu'en présence de *S. sclerotiorum*, et les cellules végétatives étaient très actives à proximité du mycélium. Malheureusement, il n'a pas été possible de déterminer le potentiel d'agent de biocontrôle de cette souche durant cette thèse. Les résultats préliminaires montrent que la croissance de *S. sclerotiorum* était ralentie en présence des cellules végétatives d'*A. oxalaticus* strain HJ, cependant, il reste encore beaucoup à faire avant de pouvoir déterminer le potentiel d'*A. oxalaticus* strain HJ en tant qu'agent de biocontrôle contre les champignons pathogènes oxalogènes.

Mots-clés

Acide oxalique, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Cupriavidus* spp., champignons phytopathogènes, agent de biocontrol bactérien, expériences *in-vitro*, bactéries sporulantes, *Ammoniphilus oxalaticus*, composé antimicrobien

Introduction

Thesis outline

My PhD thesis is divided in four chapters, each one exploring a different aspect of *in-vitro* bacterial-fungal interactions (BFI) centred around oxalic acid, and their implications for the development of a biocontrol strategy against phytopathogenic fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum*. The focal point is bacterial oxalotrophy, a metabolism characterised by the consumption of oxalic acid as a carbon and energy source by so-called oxalotrophic bacteria. As an introduction, the reader will find a review about the numerous facets of oxalic acid in nature and its implications in BFI (DOI: 10.1016/bs.aambs.2018.10.001). The review was written with my fellow colleague, Dr Fabio Palmieri, and I contributed in the writing of all parts and in particular in sections about oxalic acid biosynthesis in plants and bacteria; bacterial oxalotrophy; roles in pathogenicity for phytopathogenic fungi; and oxalic acid cycling in soil as well as in plants. The rational and aims of the thesis follow the review, with a description of the model organisms used during the course of the thesis. Each chapter is then presented as a manuscript prepared for publication, supported by additional information. The thesis focuses on *in-vitro* experiments, in order to deepen our knowledge of BFI happening between oxalotrophic bacteria and oxalogenic fungi at specific time-points in their development such as during the germination of fungal resting structures.

The first chapter is about the development of novel methods to investigate BFI *in-vitro* and to assess the control of the germination of fungal spores by oxalotrophic bacteria. The aim was to develop easy to use innovative methods to perform confrontation assays with fungal spores, recover soluble metabolites and assess cell-to-cell interactions. The resulting work was published in FEMS Microbiology Letters (DOI: 10.1093/femsle/fnac034). The article is not presented with the original layout because of the journal copyrights, and is supported by supplementary information that were not included in the published article.

The second chapter is the core of the thesis and concern the biocontrol of oxalogenic phytopathogenic fungi by oxalotrophic bacteria. It is presented as a manuscript pending submission and is more specifically about the role of oxalic acid and pH in the control of oxalogenic phytopathogenic fungi. It was repeatedly shown that oxalotrophic bacteria can protect plants against infections by phytopathogenic fungi using oxalic acid as pathogenicity factor, but the mechanisms of biocontrol are still not entirely clear. Thus, the aim of this chapter was to use the methods developed in Chapter 1 to disentangle the effect of the consumption of oxalic acid from the subsequent alkalinisation of the medium in fungal growth control.

The third chapter is a direct consequence of the second is presented as a manuscript for future publication. In the Chapter 2, different BFI were observed during confrontation experiments, depending on the culture conditions, and bacteria were either attracted or repulsed by the growing fungal mycelium. The aim of this chapter was therefore to further investigate the

metabolites responsible for the observed BFI. Also, this chapter highlights the importance of novel experimental methods to investigate BFI.

The fourth chapter focuses on oxalotrophic endospore-forming bacteria (EFB) and touch the subject of their possible use as biocontrol agents. Oxalotrophic bacteria are found in all kind of environment, therefore, we evaluated the potential of oxalogenic fungi to sustain the germination and growth of oxalotrophic EFB isolated from a soil sample. The isolated strain also provided us with a new model for future studies about the potential of oxalotrophic EFB as biocontrol agents, and to simultaneously investigate oxalotrophy, germination and sporulation in EFB. As this chapter is also presented as a manuscript for future publication, further germination experiments under different culture conditions are presented in the supplementary information of the Chapter 4.

Finally, a summary and a discussion of the main findings and the future prospect of the study of BFI and the search of new biocontrol agents concludes this thesis.

Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions

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CHAPTER TWO

Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions

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Abstract

Oxalic acid is the most ubiquitous and common low molecular weight organic acid produced by living organisms. Oxalic acid is produced by fungi, bacteria, plants, and animals. The aim of this review is to give an overview of current knowledge about the microbial cycling of oxalic acid through ecosystems. Here we review the production

† These authors contributed equally to this work.

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and degradation of oxalic acid, as well as its implications in the metabolism for fungi, bacteria, plants, and animals. Indeed, fungi are well known producers of oxalic acid, while bacteria are considered oxalic acid consumers. However, this framework may need to be modified, because the ability of fungi to degrade oxalic acid and the ability of bacteria to produce it, have been poorly investigated. Finally, we will highlight the role of fungi and bacteria in oxalic acid cycling in soil, plant and animal ecosystems.

1. Low molecular weight organic acids (LMWOA) and oxalic acid (OA)

Low molecular weight organic acids (LMWOA) are compounds with a maximal, and arbitrary, molecular weight of approximately 300 g/mol, characterized by the presence of one or more carboxylic groups (Strobel, 2001). LMWOA can be found ubiquitously in all ecosystems. Examples of LMWOA include lactate, acetate, oxalate, glyoxylate, succinate, fumarate, malate, citrate, isocitrate or aconitate that are produced by most living organisms, including plants, animals, fungi, and bacteria; some LMWOA are also produced by archaea, although oxalic acid is not one of those (Yadav et al., 2015). These LMWOA are involved in many processes taking place in soil (Jones, 1998). Oxalic acid, one of the most prevalent LMWOA, seems to play a key role in the regulation of bacterial-fungal interactions (Deveau et al., 2018).

Either oxalic acid or oxalate, its conjugated base, can be found in various terrestrial, aquatic, and clinical environments (Chandra & Shethna, 1977; Dutton & Evans, 1996; Hervé, Junier, Bindschedler, Verrecchia, & Junier, 2016; Krieger et al., 2017; Sahin, 2003; Tamer & Aragno, 1980). Oxalic acid is a strong organic acid ($pK_{a_1} = 1.25$ and $pK_{a_2} = 4.27$) and therefore, in most environments, it is found as its conjugated base, oxalate (Strobel, 2001). Oxalate can form several salts depending on the metal availability and the corresponding salt solubility products (e.g., K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Cu^{2+}) (Gadd, 1999). As a result, metal-oxalate compounds are widespread in the environment, with the most common one in terrestrial ecosystems being calcium oxalate (CaOx). The two most common CaOx minerals occurring in soils are whewellite ($CaC_2O_4 \cdot H_2O$) and weddellite ($CaC_2O_4 \cdot 2H_2O$), the monohydrate and the dihydrate forms, respectively (Uren, 2018).

Because of its chemical versatility and widespread occurrence, it is increasingly evident that oxalic acid may play a major role in interactions and ecosystem functioning. Therefore, the aim of this review is to provide an overview of current knowledge about the roles of oxalic acid in different organisms, including the pathways of its production and degradation in both

fungi and bacteria. Finally, we will summarize what is currently known about the contributions of bacteria and fungi in the cycling of oxalate, as well as the role of oxalate as a modulator of bacterial-fungal interactions, using different model ecosystems, from humans to soils.

1.1 Roles of oxalic acid

Several roles have been proposed for oxalic acid in different types of organisms, and these are summarized in Table 1.

Table 1 Roles of oxalic acid in fungi, bacteria, plants and animals.

Organism	Role	References
Fungi	Pathogenicity	Dutton and Evans (1996), Moore, Robson, and Trinci (2011), and van Kan, Shaw, and Grant-Downton (2014)
	Mineral weathering and nutrients acquisition	Arvieu, Leprince, and Plassard (2003), Dutton and Evans (1996), and Schmalenberger et al. (2015)
	Wood degradation	Mäkelä, Galkin, Hatakka, and Lundell (2002), Shimada, Ma, Akamatsu, and Hattori (1994), and Tsao (1963)
	Metal tolerance (Cu, Zn, Pb, Cd)	Fomina et al. (2005)
Bacteria	Metal tolerance (Al)	Hamel, Levasseur, and Appanna (1999)
	Pathogenicity	Nakata (2011) and Nakata and He (2010)
	Mineral weathering	Becerra-Castro et al. (2013), Cheng, Wang, He, and Sheng (2017), Frey et al. (2010), and Hess, Coker, Loutsch, & Russ (2007)
Plants	Calcium regulation	Çalişkan (2000), Nakata (2003), and Smith (2002)
	Ion homeostasis	Çalişkan (2000) and Smith (2002)
	Heavy metal detoxification	Nakata (2003)
	Plant defenses	Nakata (2003)
Animals	Stimulation of Na^+, Cl^- and H_2O uptake	Markovich and Aronson (2007) and Wang, Egbert, Abbiati, Aronson, and Giebisch (1996)
	Production of H_2O_2 to increase phagocytosis	Albrecht, Brandl, and Schonfels (1994)

1.1.1 Oxalic acid in fungi

A wide range of fungi produce oxalic acid, and different environmental factors such as carbon or nitrogen availability, mineral type, and pH influence its production (Arvieu et al., 2003; Dutton & Evans, 1996; Schmalenberger et al., 2015). As a matter of fact, calcium oxalate is the most common oxalate mineral associated to fungal hyphae, but other metal oxalates are regularly observed in function of metal availability in the environment (Gadd et al., 2014). Given the large diversity of physiologies and ecologies found among fungi, several functions have been proposed for the role of oxalic acid in fungal metabolism. Secretion of oxalic acid by fungi can increase their pathogenicity (Dutton & Evans, 1996; Moore et al., 2011; van Kan, 2006). When this secreted oxalic acid accumulates in the host tissues, it lowers the pH, which stimulates the production and activity of fungal enzymes (Dutton & Evans, 1996). Furthermore, as oxalate is a strong metal chelator, it can extract and sequester calcium ions, which can have different physiological and structural roles in the host. For example, in the case of plants, the formation of calcium oxalate in the middle lamellae within the plant cell wall weakens the structure and facilitates infection and degradation by the fungal pathogen (Dutton & Evans, 1996; Yang, Tewari, & Verma, 1993). Moreover, oxalate can inhibit host defenses and induce programmed cell death, which is also beneficial for necrotrophic pathogens (de Oliveira Ceita et al., 2007; van Kan, 2006). However, oxalic acid can also have a protective role against pathogen attack. For example, oxalic acid produced by mycorrhizal fungi can act as an antimicrobial chemical and influence the growth of pathogenic fungi in the rhizosphere (Duchesne, Ellis, & Peterson, 1989).

Secretion of oxalic acid in soil does not only protect plants from undesirable fungi. Changes in soil pH that are induced by oxalic acid secretion play a major role in mineral weathering and thus in determining nutrient availability (Dutton & Evans, 1996). In particular, ectomycorrhizal fungi have been shown to produce large amounts of oxalic acid in response to the mineral composition of the medium (Landeweert, Hoffland, Finlay, Kuyper, & Van Breemen, 2001; Schmalenberger et al., 2015). In addition to this, oxalate can chelate aluminum and iron, thus increasing the availability of phosphate. Likewise, oxalic acid facilitates weathering of clays and therefore increases the availability of trace nutrients (Cromack et al., 1979; Graustein, Cromack, & Sollins, 1977; Landeweert et al., 2001). Oxalic acid also influences calcium cycling, as the formation of calcium oxalate efficiently traps calcium in the soil matrix and prevents its leaching (Cromack et al., 1979).

Oxalic acid also plays a major role in wood degradation (Mäkelä et al., 2002; Shimada et al., 1994; Tsao, 1963). Brown-rot fungi, which degrade only cellulose and hemicellulose, exploit the precipitation of calcium oxalate crystals in cell walls to aid the degradation of cell wall components (Dutton & Evans, 1996; Shimada et al., 1994). Moreover, cellulose degradation is more efficient at low pH and oxalic acid can act as a proton donor or provide reactive oxygen species, such as O_2^- and H_2O_2, required for hydrolytic and oxidative degradation. Oxalic acid also plays a key role in regulating the rate of enzymatic degradation of lignin in wood by manganese-dependent lignin peroxidases (Shimada et al., 1994) produced by white-rot fungi (Tsao, 1963). Oxalate chelates and stabilizes Mn(III), which otherwise converts to Mn(II). Mn(III) is a key co-factor of Mn-peroxidases, one class of enzyme acting on lignin degradation (Hakala et al., 2005; Watanabe, Hattori, Tengku, & Shimada, 2005). Finally, as wood has a low nitrogen content, wood-rotting fungi are thought to produce oxalic acid as a mean to dispose of the excess carbon in wood. This hypothesis is supported by the fact that oxalic acid production is stimulated by growth in media with a low nitrogen content (Kuan & Tien, 1993; Shimada et al., 1994). Interestingly, copper compounds are widely used in wood preservation and it appears that oxalic acid also plays a role in copper tolerance, by inducing the mineralization of copper oxalate from copper sulfate (Dutton & Evans, 1996). Other metals such as zinc, cadmium or lead are also detoxified by oxalic acid secretion and precipitation as metal-oxalate salts (Fomina et al., 2005).

1.1.2 Oxalic acid in bacteria

Up to now, only a few studies have reported oxalic acid production in bacteria. Hamel et al. (1999) showed that a strain of *Pseudomonas fluorescens* produces oxalic acid in response to aluminum stress. Other studies reported the use of oxalic acid as a pathogenicity factor in two bacterial species of the genus *Burkholderia* (Nakata, 2011; Nakata & He, 2010). Moreover, oxalic acid has been reported to participate in mineral weathering in several bacterial genera (Becerra-Castro et al., 2013; Cheng et al., 2017; Frey et al., 2010; Hess et al., 2007). However, given the limited number of studies on this topic, there is a clear gap of knowledge concerning the role of oxalic acid in bacterial metabolism.

1.1.3 Oxalic acid in plants

Most of plants are able to produce oxalic acid, and oxalate can be present in different forms in plant cells. Those forms include soluble oxalate salts

(potassium, sodium or magnesium oxalate) or insoluble oxalate salts (calcium oxalate). Oxalate generally accumulates within cells in vacuoles called crystal idioblasts, and it can represent between 3% and 80% of their dry weight (Franceschi & Horner, 1980; Franceschi & Nakata, 2005). Oxalic acid and oxalate are thought to play major roles in calcium regulation, ionic balance, heavy metal detoxification and in plant defense against herbivores (Çalişkan, 2000; Nakata, 2003; Smith, 2002). Details of each one of these roles are given below.

Calcium plays a major role in plant metabolism both through signal transduction and through biochemical regulation of cellular process (Smith, 2002). However, calcium is toxic to cells at a concentration of 10^{-6}–10^{-8} M and thus the formation of insoluble calcium oxalate complexes is a way to regulate its concentration in order to ensure the proper functioning of the cell. The size and quantity of calcium oxalate crystals varies as a function of the calcium concentration in the environment. In addition, calcium oxalate crystals can serve as a storage reservoir of calcium. When cellular calcium levels are too high more crystals will be formed, but if there is a deficiency in calcium, crystals will be dissolved to make calcium available for cellular function (Çalişkan, 2000; Nakata, 2003; Smith, 2002).

As plants do not have skeletal structure, ionic balance is essential to maintain the hydrostatic pressure required for a plant to stay upright. Therefore, oxalic acid could play a major role in ion homeostasis, as it can balance the charge of the positive ions and amino acids present in the cell (Çalişkan, 2000; Smith, 2002). Moreover, oxalic acid also plays a role in heavy metal detoxification (Nakata, 2003). Aluminum toxicity, for example, is a major problem for plants developing on acidic soils. Plants have evolved different mechanisms to prevent aluminum toxicity in these conditions. Both the exclusion of aluminum from plant roots and increased internal tolerance of aluminum are related to oxalate production. In either case, aluminum will be sequestered as non-toxic aluminum-oxalate salts, either in the rhizosphere or within the above-ground portion of the plant.

Oxalate may also provide herbivory protection in plants. Crystals may be produced pre-emptively as physical defenses or produced in leaves as a response to herbivory (Nakata, 2003).

1.1.4 Oxalic acid in humans

Although oxalate is a known metabolic by-product of cellular metabolism, only two potential functions in humans have been identified. First, oxalate has been shown to have a physiological function in the proximal tubule of

the nephron in human kidneys, where the transport of oxalate by the membrane transporter SLC26A6 stimulates the uptake of chloride, water and sodium (Markovich & Aronson, 2007; Wang et al., 1996). Second, another study has suggested that an oxalate oxidase produces H_2O_2 from oxalate, and by this mechanism, increases the burst of phagocytes (Albrecht et al., 1994). No other physiological roles in humans have been described to-date.

1.1.5 Oxalic acid in other organisms

Calcium oxalate crystals associated to sporophores of Myxomycetes are also mentioned in the literature (Clark & Haskins, 2014). However, to the best of our knowledge, the role of oxalic acid and/or oxalate in Myxomycetes has not been investigated further.

2. Oxalogenesis

2.1 Biosynthesis of oxalic acid in fungi

Oxalic acid can be synthesized through three different pathways in Fungi (Fig. 1): (i) the cytoplasmic pathway; (ii) the tricarboxylic acid (TCA) pathway; and (iii) the glyoxylate (GLOX) pathway. In the case of the cytoplasmic pathway, it first involves the fixation of carbon dioxide onto a molecule of pyruvate by the pyruvate carboxylase (PYC, EC 6.4.1.1) to produce oxaloacetate, which is then cleaved into oxalate and acetate by the Mn^{2+}-dependent oxaloacetate acetylhydrolase (OAH, EC 3.7.1.1) (Han et al., 2007). In the case of the TCA and GLOX pathways, in order to enter either cycle, pyruvate is first oxidized into acetyl-CoA by the pyruvate dehydrogenase multienzyme complex (PDC) (Mattevi et al., 1992). For the TCA pathway, which occurs in the mitochondria (Mäkelä, Hildén, & Lundell, 2010), oxalic acid is produced through the cleavage of oxaloacetate by the oxaloacetate hydrolase, as in the cytoplasmic pathway. In the case of the GLOX pathway, occurring in the glyoxysomes (Mäkelä et al., 2010), oxalic acid is synthesized through the hydrolysis of citrate into glyoxylate by the glyoxylate dehydrogenase (GLOXDH, EC 1.2.1.17) (Dutton & Evans, 1996; Plassard & Fransson, 2009). Once produced, oxalic acid is secreted in the environment through a dedicated transporter. Up to now, FpOAR, a membrane transporter found in *Fomitopsis palustris*, is the only one that has been characterized (Watanabe et al., 2010).

Oxalic acid production can be affected by numerous factors such as the source of carbon and nitrogen and environmental pH, as shown for

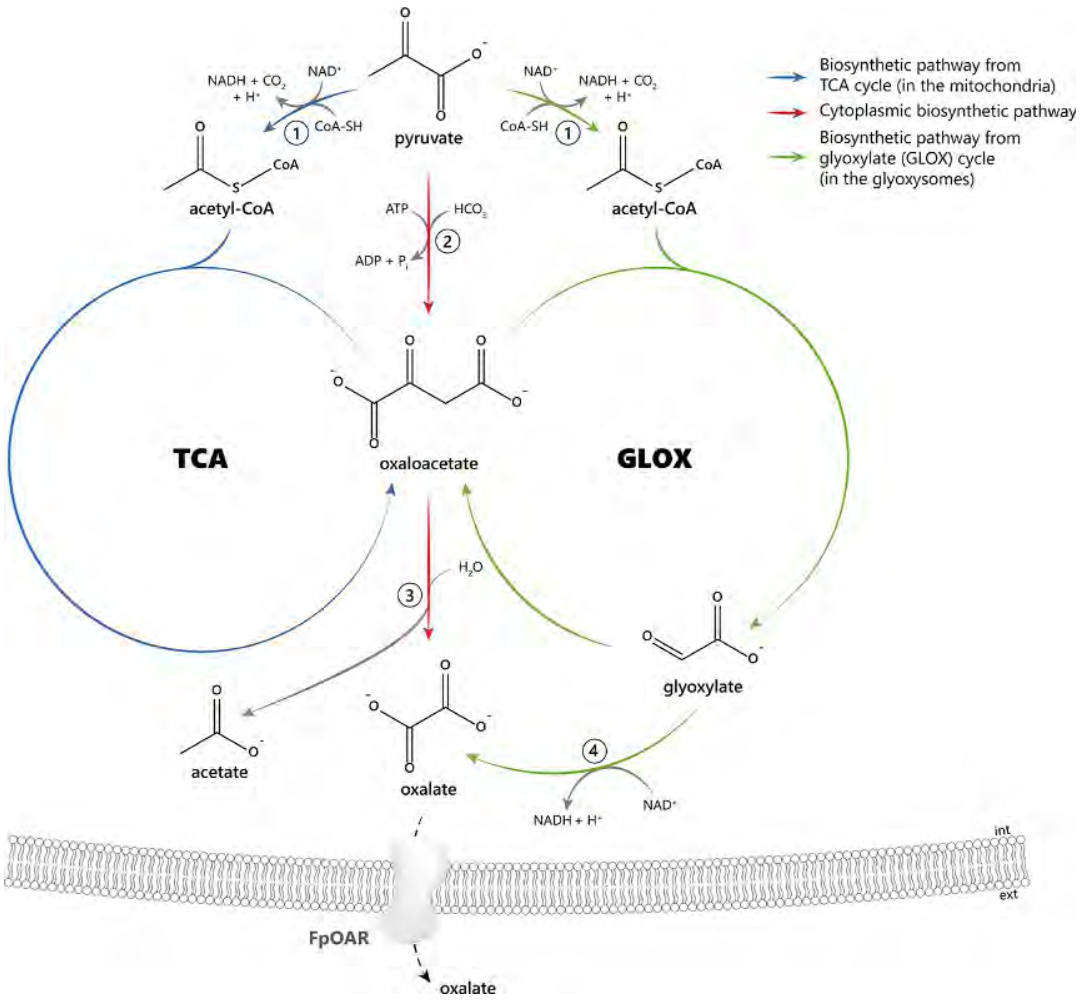


Fig. 1 Oxalogenesis pathways in fungi. Oxalic acid (OA) can be synthesized from a pyruvate precursor, which is either converted into acetyl-CoA by (1) the pyruvate dehydrogenase multienzyme complex that then enters the TCA or GLOX cycles, or is converted into oxaloacetate by (2) pyruvate carboxylase (PYC) (EC 6.4.1.1). The oxaloacetate (either produced from the TCA cycle or directly from pyruvate) can then be converted to oxalate by (3) oxaloacetate acetylhydrolase (OAH) (EC 3.7.1.1), whereas the glyoxylate from the GLOX cycle can be converted into oxalate by glyoxylate dehydrogenase (GLOXDH) (EC 1.2.1.17) (Dutton & Evans, 1996). Once produced, OA is secreted in the environment through an OA transporter. Up to now, FpOAR, an OA membrane transporter found in *Formitopsis palustris*, is the first one to be characterized (Watanabe et al., 2010).

Sclerotium rolfsii (Punja & Jenkins, 1984). Mineralogy has also been shown to influence oxalic acid production in the ectomycorrhizal fungus *Paxillus involutus* (Schmalenberger et al., 2015). Several studies have shown that the production of oxalic acid is stimulated by the presence of nitrate and inhibited by ammonium (Gharieb & Gadd, 1999; Kritzman, Chet, & Henis, 1977; Lapeyrie, Chilvers, & Bhem, 1987; Lapeyrie, Ranger, & Vairelles, 1991; Maxwell & Bateman, 1968). Optimal oxalic acid production occurs at pH 5–6 and is inhibited at a pH lower than 3, as shown in the case of the

well-known producer *Aspergillus niger* (Kubicek, Schreferl-Kunar, Wöhrer, & Röhr, 1988; Ruijter, van de Vondervoort, & Visser, 1999). Ruijter et al. (1999) also demonstrated that Mn^{2+} is co-factor altering the activity of the OAH. Carbonate and bicarbonate ions are also shown to stimulate oxalic acid production (Arvieu et al., 2003; Lapeyrie, 1988; Lapeyrie et al., 1987). The addition of divalent cations, such as calcium or metals (e.g., copper) favors the production of oxalic acid (Fomina et al., 2005; Guggiari et al., 2011).

2.1.1 Assessing the prevalence of oxalate producers among fungal species

In order to screen for the diversity of fungal species capable of synthesizing oxalic acid, we searched for putative OAH amino acid sequences in annotated sequences of 1091 fungal isolates from the Joint Genome Institute (JGI). The search was performed using a Hidden Markov model trained with fungal sequences having the systematic name "oxaloacetate acetylhydrolase" in NCBI's protein database. The *oah* gene was selected for this analysis because oxalic acid is synthesized mainly from oxaloacetate through the OAH pathway (Han et al., 2007; Kubicek et al., 1988; Rio, de Oliveira, de Tomazella, Silva, & Pereira, 2008). We created a rooted cladogram of the identified OAH sequences and assigned trophic groups to the fungi represented by these OAH sequences by matching species (when possible) or genus against the FUNGuild database (Nguyen et al., 2016). The identified OAH amino acid sequences were found in fungi from all seven trophic groups that are recognized in the FUNGuild database (Nguyen et al., 2016) (Fig. 2). When multiple OAH amino acid sequences were identified in the same fungal isolate, these sequences were generally most closely related, consistent with their sharing of a common evolutionary origin. However, OAH sequences identified from different fungi generally assorted into clades based on taxonomy instead of trophic group, suggesting that the evolutionary history of these amino acid sequences may not have been constrained by different nutrient acquisition strategies (Fig. 2).

2.2 Biosynthesis of oxalic acid in bacteria

There are only a few reports of oxalic acid production in bacteria. *P. fluorescens* ATCC 13525 produced oxalic acid in a mineral medium containing citric acid as sole carbon source; the oxalic acid was thought to be produced from a glyoxylate intermediate in response to aluminum stress (Hamel et al., 1999). Two genes, *obcA* and *obcB*, are required for oxalic acid biosynthesis and have been described in *Burkholderia mallei*. The *obcA* and

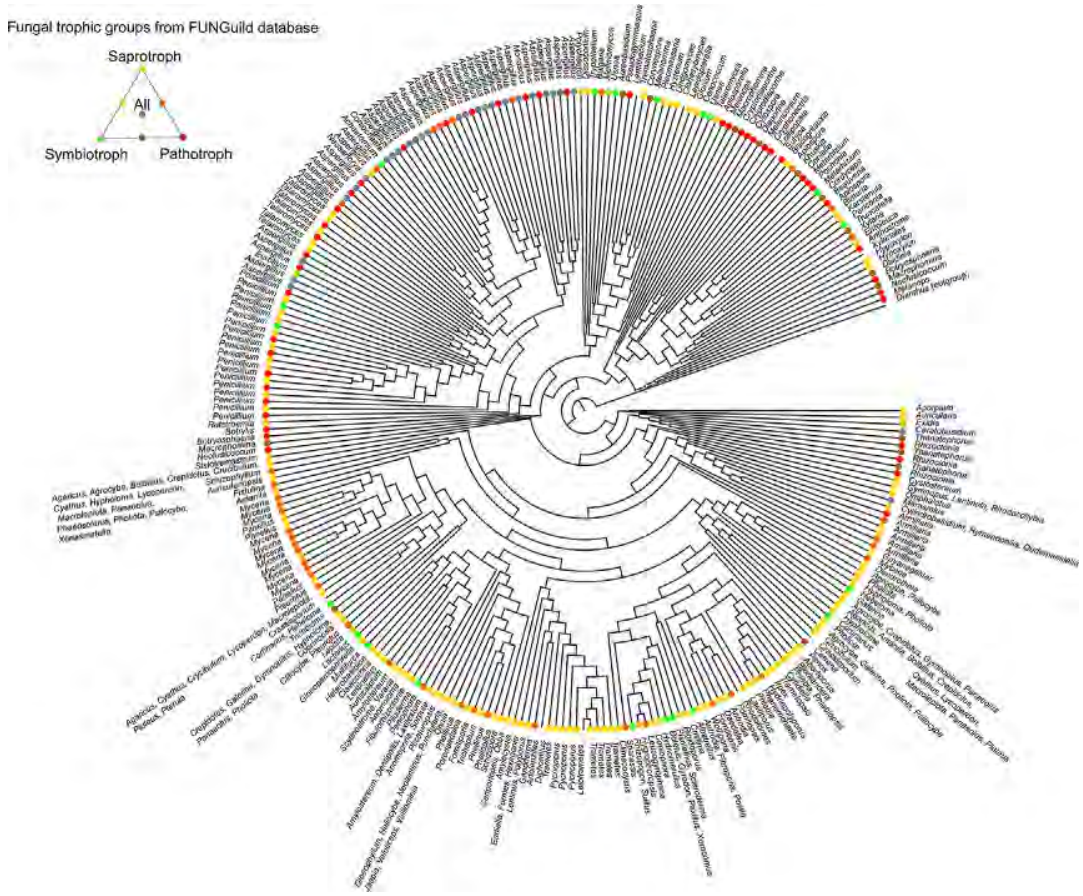


Fig. 2 Fungal *oah* gene screening. Cladogram of putative oxaloacetate hydrolase protein sequences (OAH) identified in fungal genera with diverse trophic strategies (different colors of points). The putative OAH amino acid sequences were identified using a Hidden Markov model using known fungal OAH sequences represented in the NCBI Protein database. This model was then used to screen all available fungal proteome datasets from JGI's Mycocosm portal (1091 fungal isolates) for putative OAH amino acid sequences. These putative OAH sequences typically group by genus instead of by trophic group, suggesting that they share common ancestry among closely related species instead of between fungi that share the same nutrient acquisition strategy.

obcB genes are located in the *obcAB* operon, which was also found in *Burkholderia glumae* (Nakata, 2011; Nakata & He, 2010). However, there is a significant gap of knowledge in the understanding of oxalogenesis in bacteria, as well as in the prevalence of this trait in bacteria.

3. Oxalotrophy

3.1 Oxalic acid degradation in bacteria

The first isolation of an oxalotrophic bacterium was reported more than a century ago (Bassalik, 1913). It has since become apparent that the taxonomic affiliation of oxalotrophic bacteria is heterogenous, with representatives

found among three different bacterial phyla: *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Hervé et al., 2016). Some oxalotrophic bacteria, such as *Oxalobacter formigenes* depend on the presence of oxalic acid for their survival (Anantharam, Allison, & Maloney, 1989; Smith, 2002). Others can use different carbon sources, including oxalic acid, for their metabolism (Müller, Schubert, Röst, Aebersold, & Vorholt, 2016; Sahin, 2003).

As oxalic acid is the most highly oxidized organic compound (Chandra & Shethna, 1977; Jakoby & Bhat, 1958), it must undergo an activation step before it can be used either as carbon or energy source (Jakoby & Bhat, 1958; Quayle, Keecht, & Taylor, 1961). Moreover, it must be chemically reduced before it can be used in condensation reactions to form the C_3 and C_4 units, essential for the synthesis of cell constituents (Harder, 1973; Kornberg, 1959; Quayle et al., 1961). As shown in Fig. 3, the first step for the assimilation of oxalic acid into cells is the transfer of coenzyme-A (CoA) from acetyl-CoA or succinyl-CoA to oxalic acid to form oxalyl-CoA (Jakoby & Bhat, 1958; Jakoby, Homura, & Hayaishi, 1956; Quayle et al., 1961). Oxalyl-CoA is the key molecule of the metabolism of oxalic acid. It can be (a) decarboxylated to formate in order to produce energy or (b) reduced to glyoxylate in order to be incorporated in the cell constituents (Chandra & Shethna, 1977; Jakoby et al., 1956; Quayle et al., 1961).

For energy production, oxalyl-CoA is decarboxylated to formyl-CoA via an oxalyl-CoA decarboxylase (Fig. 3, reaction 1) (EC 4.1.1.8) and thiamine pyrophosphate (ThPP) is required for this reaction (Blackmore & Quayle, 1970; Jakoby & Bhat, 1958; Jakoby et al., 1956; Kornberg, 1959; Turroni et al., 2007). Coenzyme-A is then recycled from formyl-CoA to a new oxalic acid molecule by a formyl-CoA transferase (Fig. 3, reaction 2) (EC 2.8.3.16) (Baetz & Allison, 1990; Sidhu et al., 1997; Turroni et al., 2007). The formate produced can be either transported outside the cell (Anantharam et al., 1989) or be further oxidized to CO_2 by a NAD-linked formate dehydrogenase (Fig. 3, reaction 3) (EC 1.6.1.5) to produce NADH (Blackmore & Quayle, 1970; Dijkhuizen, Wiersma, & Harder, 1977). Some anaerobic bacteria have an oxalate^{2-}:formate^{1-} antiporter (OxlT) in their membranes (Anantharam et al., 1989; Ruan et al., 1992). The presence of this heterologous antiporter that is associated with the consumption of one H^+ for the decarboxylation of oxalic acid leads to both a continued polarization of the membrane and also to an indirect proton pump, with a net stoichiometry of one H^+ per turnover (Anantharam et al., 1989; Ruan et al., 1992). Therefore, oxalic acid decarboxylation supports ATP synthesis by an ATP synthase, with a ratio of one ATP produced for every three

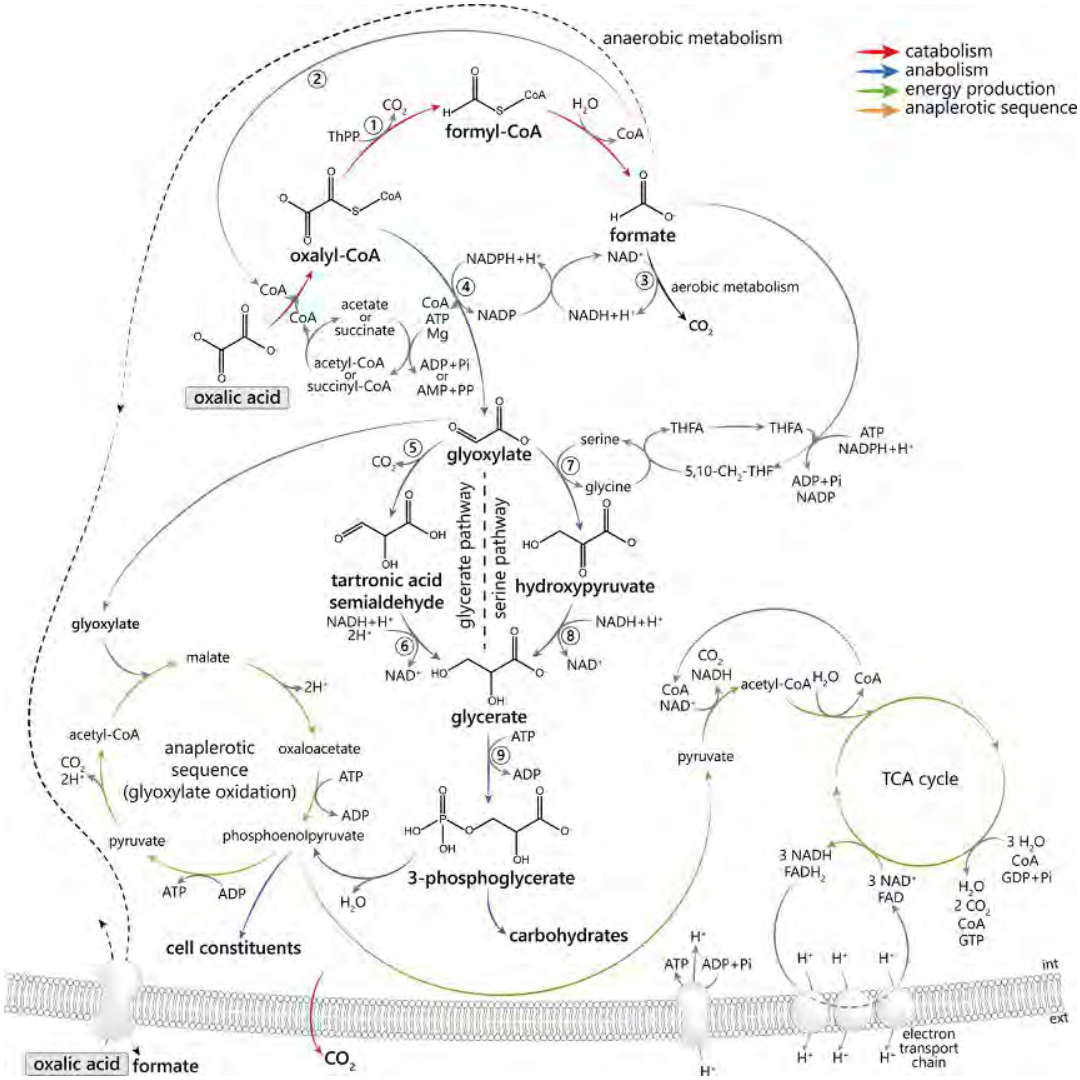


Fig. 3 Oxalotrophy in bacteria. In catabolic reactions (red arrows), oxalic acid (OA) is decarboxylated into formate (via an oxalyl-CoA intermediate) by (1) oxalyl-CoA decarboxylase (EC 4.1.1.8) and (2) formyl-CoA transferase (EC 2.8.3.16). In aerobic metabolism, the formate is then further oxidized in CO_2 by (3) NAD-linked formate dehydrogenase (EC 1.6.1.5); in anaerobic metabolism it is secreted via an oxalate:formate antiporter. For OA assimilation into cell constituents (blue arrows), oxalyl-CoA is converted to glyoxalate by (4) oxalyl-CoA reductase (EC 1.2.1.17) and is then converted into gylcerate either by the glycerate pathway: (5) glyoxylate carboligase (EC 4.1.1.47) followed by (6) tartronate semialdehyde reductase (EC 1.1.1.60), or by a variant of the serine pathway: (7) serine hydroxymethyl transferase (EC 2.1.2.1) followed by (8) hydroxypyruvate reductase (EC 1.1.1.29). Glycerate is finally phosphorylated into 3-phosphoglycerate by (9) glycerate 3-kinase (EC 2.7.1.31). The 3-phosphosglycerate can be used for carbohydrates synthesis or can act as a precursor either in the glyoxylate oxidation cycle (orange arrows) or in the tricarboxylic acid cycle (TCA) (green arrows) for the production of energy. For clarity, not all enzymatic reactions and co-factors required are depicted in this diagram of OA metabolism pathways in bacteria.

decarboxylated oxalic acid molecules (Anantharam et al., 1989). For aerobic bacteria, it is unclear how oxalic acid enters the cell (Dijkhuizen et al., 1977). However, it has been recently shown that OxlT is present in the aerobic bacterium *Methylobacterium extorquens* during the colonization of leaves, and that formate is then oxidized to CO_2 in the periplasm (Müller et al., 2016).

To be assimilated into cell components, oxalyl-CoA is first reduced in glyoxylate by an oxalyl-CoA reductase (Fig. 3, reaction 4) (EC 1.2.1.17) and then to glycerate by two different pathways (Blackmore & Quayle, 1970; Quayle et al., 1961). The first one is the glycerate pathway, where the glyoxylate is first transformed in tartronic acid semialdehyde by a glyoxylate carboligase (Fig. 3, reaction 5) (EC 4.1.1.47) and then reduced to glycerate by a tartronate semialdehyde reductase (Fig. 3, reaction 6) (EC 1.1.1.60) (Kornberg, 1959; Quayle et al., 1961). The second pathway is a variant of the serine pathway (Blackmore & Quayle, 1970; Chandra & Shethna, 1977) that uses conversion of glyoxylate to glycine by a serine hydroxymethyl transferase (Fig. 3, reaction 7) (EC 2.1.2.1) to produce hydroxypyruvate, that is in turn reduced to glycerate by a hydroxypyruvate reductase (Fig. 3, reaction 8) (EC 1.1.1.29). Glycerate is then phosphorylated into 3-phosphogylcerate by a glycerate 3-kinase (Fig. 3, reaction 9) (EC2.7.1.31) and can be used to synthesize carbohydrates or be further transformed in phosphoenolpyruvate (Chandra & Shethna, 1977). Phosphoenolpyruvate can be incorporated into cell constituents, act as precursor of pyruvate for the tricarboxylic acid (TCA) cycle, or as an intermediate for the glyoxylate oxidation, which is an anaplerotic sequence that renews intermediates for the TCA cycle (Kornberg, 1965).

Thus, oxalic acid metabolism has a low energy yield, because: (1) ATP and reducing power are both required to assimilate it in the cell constituents, with five ATP and two NADH required per mole of 3-phosphoglycerate produced and (2) because the complete oxidation of 1 mol of oxalic acid only gives 1 mol of NADH (Dijkhuizen et al., 1977; Harder, 1973). Oxalyl-CoA plays a major role in oxalic acid metabolism, and because it represents a required intermediate for both energy production and carbon assimilation, this metabolic trade-off contributes to the low growth yield of oxalotrophic bacteria (Chandra & Shethna, 1977). Furthermore, in pink-pigmented organisms that use a variant of the serine pathway to assimilate oxalic acid into the cell constituents, formate is used as a precursor for 5,10-Methylenetetrahydrofolate (5,10-CH_2-THF) (Fig. 3), which means that some of the formate is not oxidized in CO_2 to produce NADH, thereby

adding to the low energy yield of this metabolism (Blackmore & Quayle, 1970; Schneider, Skovran, & Vorholt, 2012). Because of the constraints in using oxalic acid as a sole energy and carbon source, oxalotrophic bacteria can use different input molecules for carbon assimilation and energy production. In *M. extorquens*, the two key enzymes for the conversion of oxalic acid into formate: oxalyl-CoA decarboxylase (encoded by the *oxc* gene) and formyl-CoA transferase (encoded by the *frc* gene), as well as the oxalate: formate antiporter—OxlT (encoded by the *oxlT* gene) are up-regulated during phyllosphere colonization (Baetz & Allison, 1990; Müller et al., 2016; Ruan et al., 1992; Sidhu et al., 1997). In contrast, the key enzyme for oxalic acid assimilation, oxalyl-CoA reductase is down-regulated. Overall, this indicates that oxalic acid is used only for energy production (Müller et al., 2016).

3.1.1 Assessing the prevalence of oxalate degraders among bacterial species

In order to better assess the diversity of bacterial species putatively capable of metabolizing oxalic acid, we screened bacterial proteomes for OXC and FRC sequences, which enable the use of oxalic acid as a carbon source under aerobic and anaerobic conditions, respectively (Fig. 3). All bacterial amino acid sequences annotated as either OXC or FRC were downloaded from the UniProt database. A subset of sequences from each represented species was randomly selected and used to train two independent Hidden Markov models (HMMs). These HMMs were used to identify OXC and FRC sequences within all (8648) UniProt reference bacterial proteomes. Nearly all bacterial strains identified as having these amino acid sequences contained either only OXC or both OXC and FRC; only 159 bacterial strains (2.5%) contained just FRC sequences (Fig. 4). The *oxc* and *frc* genes can co-occur in an operon (Azcarate-Peril, Bruno-Bárcena, Hassan, & Klaenhammer, 2006), and this may in part explain their co-occurrence as found here. Additional screening of proteome data could determine the location of these two genes to identify their potential to be encoded by an operon.

3.2 Oxidation of oxalic acid in fungi

Oxalic acid degradation activities have been documented in several fungi (Dutton & Evans, 1996; Mäkelä et al., 2010) and two enzymes involved in this degradation have been described: (1) oxalate decarboxylase (ODC, syn. Oxalate carboxy-lyase, EC 4.1.1.2) and (2) oxalate oxidase (OXO, oxalate: oxygen oxidoreductase, EC 1.2.3.4) (Mäkelä et al., 2010). ODC oxidizes

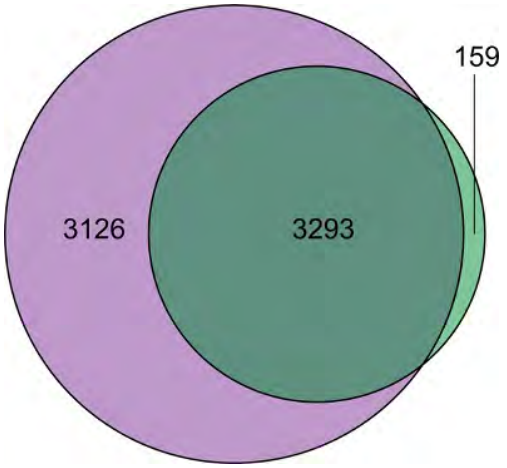


Fig. 4 Screening bacteria for OXC and FRC amino acid sequences. Counts of bacterial strains identified as only containing the oxalyl-CoA decarboxylase gene products (OXC) (purple), those only containing the formyl-CoA:oxalate CoA-transferase gene products (FRC) (light green), and those containing both gene products (dark green, overlap). Genes were identified using a Hidden Markov model using known bacterial OXC and FRC protein sequences from UniProt that had species-level assignment, and then querying all UniProt bacterial reference proteome accessions to independently identify those containing putative OXC sequences and those containing putative FRC sequences. The vast majority of proteomes contain only OXC sequences or both OXC and FRC sequences.

oxalic acid to formate and further to CO_2 and requires O_2 and Mn^{2+} as co-factors (Just et al., 2004). OXO, on the other hand, cleaves oxalic acid into two molecules of CO_2 and produces H_2O_2. OXO has been described in only two white-rot species so far, *Ceriporiopsis subvermispora* and *Abortiporus biennis* (Aguilar, Urzúa, Koenig, & Vicuña, 1999; Grąz, Jarosz-Wilkołazka, & Pawlikowska-Pawlęga, 2008). ODC seems to be the most prevalent oxalic acid degrading enzyme in the fungal world and has been shown to have a role in the maintenance of stable pH by controlling the levels of oxalic acid inside and outside fungal hyphae (Mäkelä et al., 2010; Micales, 1997). Formate dehydrogenase (FDH, EC 1.2.1.2 and 1.2.2.1) has been proposed to act sequentially with ODC to degrade formate—the end product of ODC—into CO_2 and NADH. This NADH produced through the combined activity of ODC and FDH could be used for ATP synthesis during fungal growth (Watanabe et al., 2005). Several fungal species have been shown to degrade oxalic acid in a calcium oxalate-amended mineral medium (Table 2, Guggiari et al., 2011; Simon, 2016). However, the mechanism of degradation is unknown and no enzymatic activity has been measured so far. Even if oxalic acid utilization as a carbon source has been described in many fungi, energy generation through oxalic acid consumption needs to be further investigated.

Table 2 Calcium oxalate (CaOx) mineral dissolution in CaOx-amended Schlegel mineral medium.

Species	CaOx dissolution on Schlegel-AB CaOx	References
Pleurotus tuber-regium	+	Guggiari et al. (2011)
Polyporus ciliatus	+	Guggiari et al. (2011)
Agaricus blazei	+	Guggiari et al. (2011)
Fusarium nygamai	+	Simon (2016)
Fusarium equiseti	+	Simon (2016)
Fusarium oxysporum	+	Simon (2016)
Fusarium chlamydosporium	+	Simon (2016)
Fusarium chlamydosporium	+	Simon (2016)

4. Cell-to-cell interactions

Oxalic acid has been shown to have a signaling role in the interaction between oxalogenic fungi and oxalotrophic bacteria. For instance, mycophagous bacteria of the genus *Collimonas* use oxalic acid to localize their fungal host and to move toward it by quorum sensing-dependent chemotaxis (Rudnick, Veen, & Boer, 2015). An earlier study by Mela et al. (2011) confronting *C. fungivorans* Ter331 with *A. niger* demonstrated that several genes encoding for oxalate:formate antiporter, formyl-CoA transferase and oxalyl-CoA decarboxylase were up-regulated in *C. fungivorans* Ter331, indicating a possible use of oxalic acid as carbon and/or energy source. However, *C. fungivorans* Ter331 does not seem to grow on oxalic acid (Mela et al., 2011; Rudnick et al., 2015). Four soil *Paraburkholderia terrae* strains have also been shown to be attracted by oxalic acid produced by two soil fungi, *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302. One of these strains, *P. terrae* BS001, is able to use oxalic acid as carbon source (Haq, Zwahlen, Yang, & van Elsas, 2018). These findings suggest that oxalic acid can be used as a signaling molecule for bacteria to locate fungi in soil in order for both partners to interact. A similar chemotactic behavior toward selected plant exudates such as oxalate has been demonstrated for rhizospheric bacteria (Alexandre, Greer, & Zhulin, 2000).

5. Bacterial-fungal interactions (BFI) in the framework of oxalic acid cycling: Parallels in soils, plants and animals

5.1 BFI and oxalic acid cycling in soils: The oxalate-carbonate pathway (OCP)

LMWOA are involved in many processes occurring in soil, particularly in the rhizosphere (Jones, 1998). By exuding LMWOA, plants and fungi create micro-niches for the development of bacteria around their roots or hyphae, respectively (Badri & Vivanco, 2009; Braissant, Verrecchia, & Aragno, 2002; De Boer, Folman, Summerbell, & Boddy, 2005). Indeed, LMWOA such as acetate, glyoxylate, malate or oxalate can be used as sole carbon and energy source by bacteria (Braissant et al., 2002). Despite the release of oxalic acid into the soil by both fungi and plants, either through exudates or through decomposition, oxalic acid appears not to accumulate in soils. While several soil properties, such as pH and sorption sites influence the presence of oxalic acid in soils, bacterial metabolism is the main factor influencing the persistence of this compound in soils (Martin et al., 2012; Uren, 2018).

Oxalotrophic bacteria readily metabolize oxalic acid and they play a major role in the oxalate-carbonate pathway (OCP) (Fig. 5), which is a biogeochemical process that provides two important ecosystem functions: (1) a potential sink for atmospheric CO_2 (Aragno & Verrecchia, 2012; Cailleau, Mota, Bindschedler, Junier, & Verrecchia, 2014; Verrecchia, Braissant, & Cailleau, 2006) and (2) an increase in soil nutrient content (Pons et al., 2018). Through photosynthesis, plants fix CO_2 and produce the precursors of oxalic acid (Franceschi & Horner, 1980). Decay of plant biomass and fungal excretion leads to the release of oxalate crystals in soils and to their availability as part of the soil carbon pool (Aragno & Verrecchia, 2012; Verrecchia et al., 2006). Oxalotrophic bacteria oxidize calcium oxalate to CO_2, which is then excreted in the local microenvironment and turns into one of the four carbonate species (CO_2*, H_2CO_3, HCO_3^-; CO_3^{2-}) in function of the alkalinity of the soil solution (Aragno & Verrecchia, 2012). The microbial metabolism of oxalic acid exchanges a strong acid—oxalic acid ($pK_{a_1} = 1.25$ and $pK_{a_2} = 4.27$)—for a weak one—carbonic acid ($pK_{a_1} = 6.35$ and $pK_{a_2} = 10.33$)—which may lead to a strong local increase of pH. Such an influence on the soil pH prevents cation leaching from soil as a result of low pH (Pons et al., 2018) and

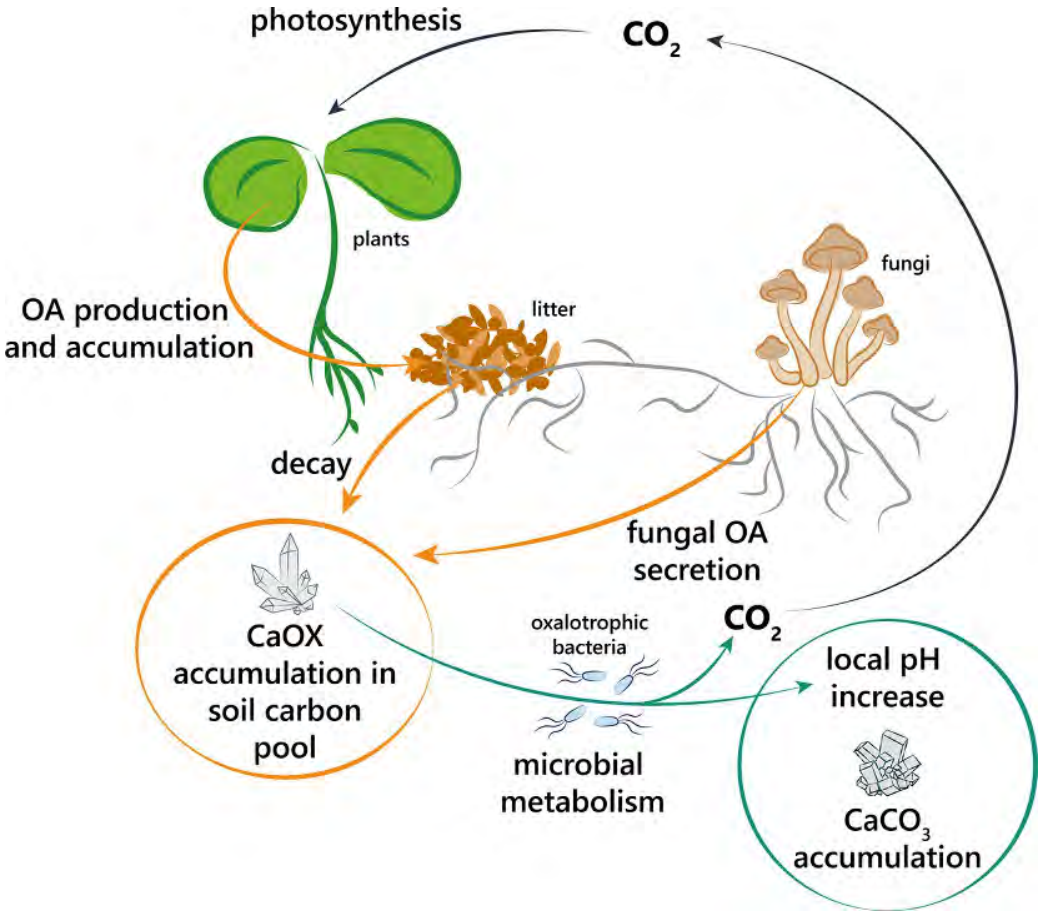


Fig. 5 Oxalate-Carbonate Pathway. Through photosynthesis, plants fix CO_2 and produce the precursors of oxalic acid (OA). Fungi also produce OA and upon decay of plant biomass, calcium oxalate (CaOx) accumulates in the soil. Oxalotrophic bacteria consume oxalic acid and the metabolic products lead to a local increase of pH, which is induced by the exchange of a strong acid (oxalic acid) for a weak one (carbonic acid). This may eventually lead to calcium carbonate precipitation if pH > 8. Excess CO_2 is released to the atmosphere and can enter the cycle again.

can eventually promote the precipitation of calcium carbonate ($CaCO_3$) if pH increases above 8 (Aragno & Verrecchia, 2012; Martin et al., 2012; Verrecchia et al., 2006). In this latter process, for each mole of calcium oxalate oxidized, 1 mol of CO_2 is released in the atmosphere, while the other one is sequestered in soils as biomineralized $CaCO_3$. The OCP may therefore act as an efficient carbon sink, as long as calcium originates from carbonate-free minerals (Cailleau et al., 2014; Martin et al., 2012).

One could think that fungi contribute to the OCP only by releasing oxalate into soils through exudates and by fostering the decomposition of plant biomass. However, it seems that fungi may play a more significant role in the oxidation of oxalic acid. Martin et al. (2012) showed that even if bacteria are the main organisms responsible for oxalate oxidation and consequent soil

alkalinization, their activity is dependent of the presence of fungi. We still do not have a clear understanding of the relationship between bacteria and fungi in the context of the OCP, but Martin et al. (2012) postulated that fungi could protect bacteria from the acidic conditions and help them to colonize the heterogeneous matrix of the soil to reach oxalate crystals. However, there is a lack of knowledge about the importance of oxalotrophic fungi and oxalogenic bacteria in oxalic acid cycling in soils in general and in the OCP in particular.

5.2 BFI and oxalic acid cycling in plants

Root exudates provide various carbon and energy sources for bacteria living in the rhizosphere and the different secreted compounds influence the composition of the microbial community (Jones, 1998). Oxalic acid has been shown to be a major component of the root exudates and to play an important role in the recruitment of bacteria (Dessureault-Rompré, Nowack, Schulin, & Luster, 2007). In *Burkholderia* species, for example, oxalotrophy has been shown to be associated strictly with strains that are beneficial to plant growth, and it is required for the successful colonization of the plant (Kost, Stopnisek, Agnoli, Eberl, & Weisskopf, 2013). As oxalotrophy decreases oxalic acid concentration in both leaves and rhizosphere, it has been postulated that oxalic acid degradation could be a function that protects plants (Kost et al., 2013; Müller et al., 2016). Oxalic acid is a pathogenicity factor of several phytopathogenic fungi and the degradation of this acid could provide protection to the plant by making the environment less favorable to fungi. Indeed, studies on *Arabidopsis thaliana* and *Phaseolus vulgaris* have shown that oxalotrophic bacteria offer protection against pathogenic fungi (Dickman & Chet, 1998; Schoonbeek, Jacquat-Bovet, Mascher, & Métraux, 2007). Dickman and Chet (1998) have found that *Pseudomonas* spp., which degrade oxalic acid, could offer protection for *A. thaliana* against infection from *Sclerotinia sclerotium*. To evaluate the potential of this approach for field applications, experiments with *S. rolfsii*, another oxalic acid-producing soil fungal pathogen, and *P. vulgaris* were conducted. With the addition of oxalotrophic bacteria, the disease incidence in beans was significantly reduced compared to the control by $60 \pm 11\%$ and $75 \pm 9\%$, respectively, for the two strains of bacteria used. Schoonbeek et al. (2007) also investigated the ability of four oxalotrophic bacterial strains that were isolated from agricultural soil samples to protect different crops and *A. thaliana* against the fungal pathogens *Botrytis cinerea* and *S. sclerotium*;

the bacteria increased protection against infection by 30–70%. Therefore, oxalic acid does not only play a major role in plant metabolism, but is also an important feature in microbial recruitment and protection against fungal pathogens.

5.3 BFI and oxalic acid cycling in the human and animal body

In mammals, oxalic acid in the blood can have an exogenous origin from the diet, or an endogenous origin from the metabolism of glycine, glyoxylate and ascorbic acid in the liver (Abratt & Reid, 2010) (Fig. 6). Typically, 20–40% of the oxalic acid originates from common food and beverages, where it is present in high concentrations, including coffee, tea, chocolate, rhubarb, spinach, and other fruits and vegetables (Abratt & Reid, 2010; Libert & Franceschi, 1987; Marengo & Romani, 2008; Stewart, Duncan, & Cave, 2006).

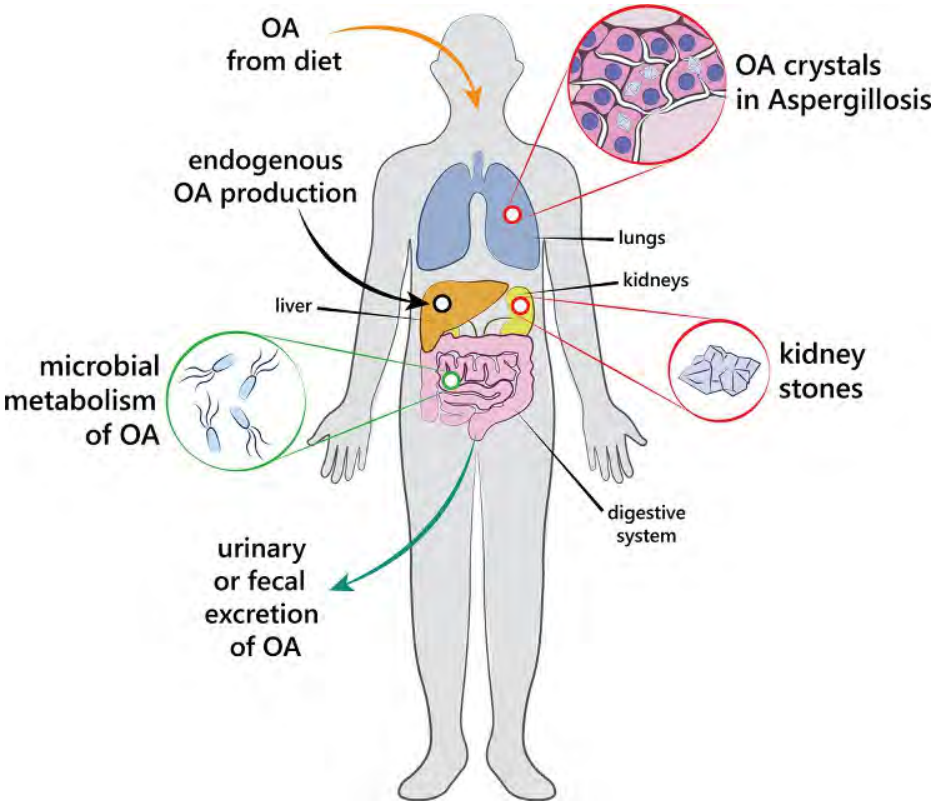


Fig. 6 Implication of BFI in the oxalic acid (OA) cycling in the human body. OA enters the body through the diet or is produced endogenously in the liver. It is then either excreted in the urine or sequestered in the form of calcium oxalate crystals and excreted with the feces. Gastrointestinal oxalate-degrading bacteria, such as *O. formigenes*, can degrade oxalic acid and thus participate to the maintenance of homeostasis. High levels of oxalic acid can lead to hyperoxaluria and the formation of kidney stones. In the case of pulmonary aspergillosis, the presence of calcium oxalate crystals has been reported, suggesting a potential link between oxalic acid and pathogenicity in *Aspergillus* fungi.

As humans lack the enzymes to degrade oxalic acid, it has to be either excreted or sequestered to maintain homeostasis. It can be absorbed in the kidneys and excreted in the urine or it can form insoluble calcium oxalate and be eliminated in the feces (Abratt & Reid, 2010) (Fig. 6). Moreover, oxalic acid can be degraded by gastrointestinal oxalic acid-degrading bacteria, such as *O. formigenes* (Allison, Cook, Milne, Gallagher, & Clayman, 1986; Stewart et al., 2006) (Fig. 6). A differential oxalic acid movement along the gastrointestinal tract has been demonstrated in humans (Hatch, Freel, & Vaziri, 1993): oxalic acid is absorbed in the distal colon while it is secreted in the ileum, jejunum and proximal colon.

High levels of oxalic acid can be toxic and cause pathologies like hyperoxaluria, a known risk factor for the formation of kidney stones, which are mainly composed of calcium oxalate (Fig. 6). The degradation of oxalic acid by gastrointestinal bacteria like *O. formigenes*, *Lactobacillus* spp. and *Bifidobacterium* spp. is supposed to decrease the rates of oxalic acid absorption and thus provides a potential target for probiotic treatment of hyperoxaluria (Abratt & Reid, 2010). In sheep, *O. formigenes* plays a major role in oxalic acid degradation (Smith, 2002). When they graze in spring pasture fields containing plants with high levels of oxalic acid, sheep die rapidly of renal failure. However, with a gradual exposure to oxalic acid, *O. formigenes*, which is lost from the gut during winter, is able to re-colonize the rumen and degrade oxalic acid, thus regulating the concentration of oxalic acid in kidneys, protecting sheep against disease (Smith, 2002).

Oxalic acid plays an important role in the pathogenicity of plant pathogens, and it might also play a role in human health by human fungal pathogens. However, a link between oxalic acid production and pathogenicity has not been yet reported for human pathogens. Fungi of the *Aspergillus* genus are widespread and some species have been associated with health issues ranging from allergies to life threatening systemic infections (Paulussen et al., 2017). Oxalic acid production by *A. niger* is well known (Payne, Dark, Conway, & Farina, 2017), and several studies reported the presence of calcium oxalate crystals in animal and human lungs in the pulmonary disease aspergillosis (Kauffman, Wilson, & Schwartz, 1984; Kurrein, Path, Green, & Rowles, 1975; Kuwabara & Shibayama, 2012; Maeno, Sasaki, Shibue, Mimura, & Oka, 2015; Muntz, 1999; Oda et al., 2013; Pabuççuoğlu, 2005; Payne et al., 2017). Therefore, oxalic acid could play a role in lung infection. The contribution of opportunistic fungal pathogens to the total oxalic acid pool of the human body and the link between pathogenicity and oxalic acid still need to be investigated.

6. Conclusions

In summary, this review shows that even if a large body of information has been generated in relationship to oxalic acid, there is still some significant gaps of knowledge. Furthermore, this review focuses on plants, bacteria, fungi and animals, but one can expect that oxalic acid is also a relevant metabolite in the case of protists and archaea. Oxalic acid production and its metabolic implications in plants and fungi are fairly well known, while only a few studies have focused on its metabolic implications in bacteria and animals, including humans. Conversely, oxalic acid consumption by bacteria has been extensively studied since the first isolation of an oxalotrophic bacterium. However, the potential ability of fungi to produce energy from oxalic acid remains largely under-investigated. These current knowledge gaps mean that the contribution of organisms such as oxalotrophic fungi or oxalogenic bacteria to oxalate cycling have not yet been investigated in detail. Moreover, as oxalic acid is a compound found ubiquitously in ecosystems, it could play a major role in microbial interactions but its use as a signaling molecule is still poorly understood. A similar knowledge gap exists in our understanding of the role that oxalic acid may play in animal health. Oxalic acid enters the body mainly through the diet and does not seem to have a major metabolic role. However, a balanced gut microbiota seems to be required to efficiently degrade oxalic acid in order to maintain an equilibrium between its uptake and excretion. Although oxalic acid can function as a pathogenicity factor in fungal pathogens of plants, it is still unclear whether this could also be the case for animal pathogens, despite the correlation between the occurrence of pulmonary aspergillosis and the presence of calcium oxalate crystals in the lungs. Therefore, the potential role of oxalic acid in human pathogenic fungi as well as the contribution of the host-associated microbiota in the defense of host still has to be addressed, especially because it may yield new ways to control pathogenic fungi amid the systematic emergence of antifungal resistance.

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Rationale, aims and objectives

The aim of this thesis was to provide new insights in bacterial-fungal interactions (BFI) in the case of oxalotrophy as a biocontrol strategy against phytopathogenic fungi, and to search for new bacterial biocontrol agents (BCA). Previous studies showed the potential of oxalotrophic bacteria to control fungal growth and protect different crops against phytopathogenic fungi producing oxalic acid as a pathogenicity factor (Dickman & Chet, 1998; Schoonbeek *et al.*, 2007; Marina *et al.*, 2019). However, most biocontrol studies target actively growing mycelium, whereas often the starting point of the infection process is the germination of spores or sclerotia (Agrios, 2004). Moreover, fungal growth control was always attributed to the consumption of oxalic acid. However, oxalotrophy not only removes oxalic acid from the system but also impacts the physicochemical properties of the environment. In order to address these knowledge gaps, the specific objectives of this thesis were first to assess the potential of oxalotrophic bacteria to control the germination of spores and sclerotia of phytopathogenic fungi and secondly, to disentangle oxalic acid consumption from pH changes in the control of fungal spores germination and mycelial growth. As *in-vitro* biocontrol confrontation assays are mostly designed to work with actively growing mycelium (Jin *et al.*, 2011; Droce *et al.*, 2013; Stanley *et al.*, 2014), the first step required to accomplish this objective was to develop methods to assess interactions between bacteria and fungal spores or sclerotia.

One important limitation in the use to bacteria in biocontrol is the development of a commercialised biocontrol agent. Indeed, there are only few biocontrol agents available in the market because of the inconsistent disease control under field conditions and the underestimated cost of production (Mathre *et al.*, 1999; Stewart, 2001; Fravel, 2005). Moreover, the formulation and delivery of the biocontrol agent need to be taken into account. Vegetative cells need to be encapsulated, but this can negatively affect the shelf-life of the agent, its performances or safety, and the capsule needs to be devised for long-term storage (Fravel *et al.*, 1985; Steddom *et al.*, 2002; Fravel, 2005; Weller, 2007; Bardin *et al.*, 2015). Therefore, endospore-forming bacteria (EFB), that are naturally encapsulated and can remain dormant and viable for a long time, have a strong potential as biocontrol agents (Moir & Smith, 1990; Pal & McSpadden, 2006; Madigan *et al.*, 2012; Wu *et al.*, 2015). This led us to the next specific objective of this thesis, which was to assess the potential of oxalotrophic EFB as biocontrol agents against phytopathogenic fungi. For this purpose, the first step was to isolate oxalotrophic EFB from a soil sample and to assess the efficiency of oxalic acid as trigger of germination.

Model organisms

Fungi

Botrytis cinerea is a saprotrophic and pathogenic fungus causing important economic losses world-wide (Schoonbeek *et al.*, 2007; van Kan *et al.*, 2014). It has a wide range of plant hosts and is the causative agent for the grey mould on harvested fruits and flowers (Williamson *et al.*, 2007; van Kan *et al.*, 2014). In the last decade, studies have shown that *B. cinerea* is not only a necrotrophic pathogen but also has a facultative endophytic lifestyle (van Kan *et al.*, 2014). Infection usually begins with the germination of *B. cinerea* spores and the formation of an appressorium to penetrate the host cells. Production of phytotoxic metabolites, enzymes and oxalic acid are required for the successful penetration of the cells (van Kan, 2006; Williamson *et al.*, 2007). Oxalic acid triggers the oxidative burst in the cells, a defence mechanism against pathogenic microorganisms, and causes the death of the cells (van Kan, 2006). Cell death is beneficial for necrotrophic fungi such as *B. cinerea*, as it facilitates the penetration of the cells and promotes disease incidence. Programmed cell death by oxalic acid is actually required for the successful colonisation of the host plant by *B. cinerea* (van Kan *et al.*, 2014). Diseases caused by *B. cinerea* can also remain asymptomatic for several weeks before the apparition of multiple infection sites with the flowers blossom (van Kan *et al.*, 2014). Root and seed infections, in lettuce for instance, also lead to asymptomatic and systemic infection of the plant host. Upon favourable conditions, the necrotrophic behaviour of the fungus takes over and *B. cinerea* produces abundant spores in order to spread and infect other crops (Williamson *et al.*, 2007; van Kan *et al.*, 2014). *B. cinerea* is particularly difficult to control due to its wide host range and dual lifestyle (Williamson *et al.*, 2007). As oxalic acid plays a major role for the successful establishment of *B. cinerea* in the host plant, the potential of oxalotrophic bacteria to control *B. cinerea* was assessed (Schoonbeek *et al.*, 2007; Marina *et al.*, 2019). Oxalotrophic bacteria isolated from soil offered up to 70% of protection against *B. cinerea* to *Arabidospis thaliania* and different crops (Schoonbeek *et al.*, 2007). Endophytic oxalotrophic bacteria were also able to successfully reduce cell penetration in *Arabidopsis* leaves by a combination of callose deposition and the triggering of the plant immune system (Marina *et al.*, 2019). Both *B. cinerea* and *Sclerotinia sclerotiorum* growth were successfully controlled by the *Stenotrophomonas* bacterial strains used (Marina *et al.*, 2019).

S. sclerotiorum is soil-born saprotrophic and necrotrophic plant pathogen that causes tremendous losses in economically important crops (Dickman & Chet, 1998; Cessna *et al.*, 2000; Jain *et al.*, 2015). It is responsible for the white rot on a large variety of plants and sclerotia is the state in which this fungus is usually found (Adams & Ayers, 1979; Hegedus & Rimmer, 2005). Sclerotia can germinate as mycelium or form an apothecia supporting ascospores, to spread over the crops (Hegedus & Rimmer, 2005). This necrotrophic fungus requires senescent cells for a successful colonisation of the plant host tissues and the role of oxalic acid in pathogenicity has been extensively investigated (Godoy *et al.*, 1990; Cessna

et al., 2000; Guimarães & Stotz, 2004; Williams *et al.*, 2011). Oxalic acid was first thought to be only responsible for the acidification of the tissues, which stimulate the production and activity of the fungal enzymes. However, additional studies have highlighted a much more complex role of oxalic acid in *S. sclerotiorum* pathogenicity (Williams *et al.*, 2011). *S. sclerotiorum* uses oxalic acid secretion to manipulate the redox equilibrium in the host's tissues in order to induce programmed cell death (Williams *et al.*, 2011). This regulation of the cellular death allows the fungus to control the plants defences and is essential for the successful establishment of the pathogen. Indeed, mutants deficient in oxalic acid production are unable to suppress the oxidative burst and to control the plant defences, and therefore to successfully infect the plant (Hegedus & Rimmer, 2005; Williams *et al.*, 2011; Jain *et al.*, 2015; Li *et al.*, 2018). Experiments with a mutant deficient in oxalic acid production of *S. sclerotiorum* engineered using CRISPR-Cas9, *S. sclerotiorum* Δ*oah* (Li *et al.*, 2018), have confirmed previous results obtained with UV generated S. sclerotiorum mutants, showing that oxalic acid is essential for the pathogenicity of the fungus. To engineer the mutant, a CRISPR-Cas9 system with a plasmid containing an hygromycin B resistance was used to remove the gene *Ssoah1*, required for oxalic acid production, from the genome of *S. sclerotiorum*. The double-strand break was repaired with the nonhomologous end joining pathway with a transformation vector. Successful deletion was determined on the basis of the hygromycin resistance and the inability of the strains to acidify the growth medium. The resulting *S. sclerotiorum* Δ*oah* strain was unable to produce oxalic acid and acidify the medium and were unable to successfully infect the plant host (Li *et al.*, 2018). Oxalotrophy as a biocontrol strategy was in fact first tested on *S. sclerotiorum* and showed great potential to protect *Arabidopsis* leaves against the pathogen (Dickman & Chet, 1998).

Bacteria

Cupriavidus necator and *Cupriavidus oxalaticus* are two Gram-negative flagellated soil bacteria and facultative oxalotrophs (Makkar & Casida, 1987; Şahin *et al.*, 2000; Sahin, 2003). *C. necator* was first described as a facultative predator of bacteria when environmental conditions were unfavourable (Makkar & Casida, 1987), while *C. oxalaticus* was initially named as *Pseudomonas oxalaticus* (Şahin *et al.*, 2000; Sahin *et al.*, 2002). Taxonomy was later revised and *P. oxalaticus* was renamed as *C. oxalaticus* (Vandamme & Coenye, 2004). Oxalotrophy was extensively investigated in *C. oxalaticus* and provided numerous information about oxalic acid assimilation by oxalotrophic bacteria (Quayle, 1963; Blackmore & Quayle, 1970; Chandra & Shethna, 1975, 1977; Dijkhuizen & Harder, 1979). The two bacterial strains used for this thesis were tagged with constitutively expressed fluorescent marker genes (*C. necator*-GFP (Sentchilo *et al.*, 2009) and *C. oxalaticus*-mCherry (Palmieri *et al.*, 2022)).

Lastly, an oxalotrophic endospore-forming bacterium (EFB) isolated from a soil sample during this thesis was also used. EFB produce endospores when the environment becomes unfavourable for their growth (Madigan *et al.*, 2012; Mandic-Mulec *et al.*, 2015). Endospores are resistance structures, able to withstand high and low temperatures, radiation or exposure

to chemical hazards (Moir & Smith, 1990; Madigan *et al.*, 2012). They disperse easily and can remain dormant but viable for years. In the other hand, germination can occur rapidly in presence of the right trigger (Foster & Johnstone, 1990; Moir & Smith, 1990). As long as environmental conditions are favourable for the EFB, it will divide and grow before sporulating when the conditions become unfavourable again. EFB are mainly Gram-positive bacteria from the Firmicute phylum and the most studied family of EFB is the *Bacillaceae*.

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Chapter 1

Improved methods to assess the effect of bacteria on germination of fungal spores

This chapter is based on the published article:

Estoppey, A., Weisskopf, L., Di Francesco, E., Vallat-Michel, A., Bindschedler, S., Chain, P. S. & Junier, P. (2022) 'Improved Methods to Assess the Effect of Bacteria on Germination', FEMS Microbiology Letters, 369, pp. 1–9. doi: DOI: 10.1093/femsle/fnac034.

Abstract

Bacterial-fungal interactions (BFI) play a major role on ecosystem functioning and might be particularly relevant at a specific development stage. For instance, in the case of biological control of fungal pathogens by bacteria, a highly relevant kind of BFI, *in-vitro* experiments often assess the impact of a bacterium on the inhibition of actively growing mycelia. However, this fails to consider other stages of plant infection such as the germination of a spore or a sclerotium. This study aims to present novel experimental platforms for *in-vitro* experiments with fungal spores, in order to assess the effect of bacteria on germination and fungal growth control, to recover the metabolites produced in the interaction, and to enhance direct visualisation of BFI. *Botrytis cinerea*, a phytopathogenic fungus producing oxalic acid (OA) as pathogenicity factor, was used as model. Given that oxalotrophic bacteria have been shown previously to control the growth of *B. cinerea*, the oxalotrophic bacteria *Cupriavidus necator* and *Cupriavidus oxalaticus* were used as models. The experiments performed demonstrated the suitability of the methods and confirmed that both bacteria were able to control the growth of *B. cinerea,* but only in media in which soluble OA was detected by the fungus. The methods presented here can be easily performed in any microbiology laboratory and are not only applicable to screen for potential biocontrol agents, but also to better understand BFI.

Introduction

Bacterial-fungal interactions (BFI) are involved in many processes, ranging from food production to nutrient cycling (Frey-Klett *et al.*, 2011). These physical or molecular interactions modulate the behaviour, reproduction and growth of microorganisms and result in the alteration of their environment. Thus, BFI are highly relevant in biogeochemical cycles or biocontrol of plant diseases (Frey-Klett *et al.*, 2011; Deveau *et al.*, 2018). The study of BFI in the laboratory involves the use of a wide array of experimental designs, including confrontation assays and metabolite characterisation. In traditional confrontation assays, a portion of mycelium is inoculated in the middle of an agar plate and bacteria are inoculated at a certain distance from the fungal inoculum (Levy *et al.*, 1988; Jin *et al.*, 2011; Swain *et al.*, 2017; Slama *et al.*, 2019). This straight-forward method assesses the effect of bacteria on mycelial growth, but provides little information about the effect of bacteria on other stages of the fungal life cycle. Furthermore, the use of agar hinders not only the recovery and characterization of extracellular metabolites, but also the quantification of fungal and bacterial biomass and of environmental parameters such as pH. Although it is possible to extract metabolites from agar, the procedure requires multiple steps (Li *et al.*, 2014). Growing fungi in liquid cultures is not an entirely suitable alternative either, as fungal growth is heterogeneous and locally produced metabolites would be homogenised and diluted (Madigan *et al.*, 2012; Droce *et al.*, 2013). One method previously proposed is the culture of fungi over glass beads immerged in liquid medium inoculated with bacteria (van Schöll *et al.*, 2006; Droce *et al.*, 2013; Essig *et al.*, 2014). However, the application of this method has been mainly restricted to the use of already growing mycelia.

Another problem of performing confrontations on agar plates is the difficulty to observe the interaction at a cellular level. The classical method for microscopy is to cut a thin agar slice colonized by mycelia, and to crush it in between a glass slide and a coverslip. Crushing the agar destroys the spatial structure, making this method inadequate for studying the spatial distribution in the BFI. Different microfluidic devices have been developed to offer a better visualisation of the interactions occurring at a cellular level (Stanley *et al.*, 2014, 2016; Stanley & van der Heijden, 2017). Although this approach offers a remarkable insight into BFI, it might not be affordable everywhere as it requires specialized equipment for building and imaging the devices.

Therefore, the aim of this study was to address the shortcomings enumerated above and to offer alternative methods for *in-vitro* experiments in the field of BFI. In particular, the goal was to develop methods to evaluate the bacterial inhibition of fungal spores' germination. A second, equally important objective of this work was for the methods to be easily performed in any laboratory, thus demanding minimal technical requirements. For this purpose, a confrontation assay where the bacteria are homogenously spread in the plate was compared to the traditional confrontation assay, to monitor fungal spores' germination in presence

of a potential bacterial biocontrol agent. Furthermore, a cell culture plate-based assay was developed to facilitate the recovery of soluble metabolites and the direct observation and monitoring of the interactions at the macro- and microscopic scale. Finally, live and direct imaging was made possible by using agarose pads in Gene frames.

To demonstrate the suitability of the methods, we used a model BFI consisting of a fungal plant pathogen and a potential biocontrol bacterium. *Botrytis cinerea* was used as a model pathogen. This necrotrophic fungus produces a wide array of enzymes and metabolites to penetrate and infect the host plant (van Kan, 2006; Sowley *et al.*, 2010). This fungus also produces oxalic acid (OA) as a co-factor of pathogenicity, to induce a local acidification and promote the production and activity of the secreted enzymes (van Kan, 2005, 2006; Williamson *et al.*, 2007). Oxalotrophic bacteria, which consume oxalate as carbon and energy source, have been shown to control the growth of oxalogenic fungal phytopathogens such as *B. cinerea* or *Sclerotinia sclerotiorum* (Dickman & Chet, 1998; Sahin, 2003; Schoonbeek *et al.*, 2007; Marina *et al.*, 2019). Thus, *Cupriavidus necator* and *Cupriavidus oxalaticus* were used as model oxalotrophic bacteria to control *B. cinerea* growth (Makkar & Casida, 1987; Şahin *et al.*, 2000). Using these microorganisms as a model of BFI with a potential in biocontrol, we were able to prove the efficacy and efficiency of the proposed methods to assess the impact of bacteria on the germination of spores and the interactions with growing mycelia.

Material & methods

Fungi and Bacteria

Botrytis cinerea BMM was obtained from the fungal collection of the Plant and Microbial Biology unit in the University of Fribourg, Switzerland. The bacteria *Cupriavidus necator*-GFP (Poehlein *et al.*, 2011) and *Cupriavidus oxalaticus*-mCherry (Şahin *et al.*, 2000) were obtained from the bacterial collection of the Laboratory of Microbiology, University of Neuchâtel, Switzerland.

Fungal and bacterial suspensions

Spores of *B. cinerea* BMM were conserved in 30 % glycerol at -80°C. Fresh spore suspensions were produced by cultivating 10 µl of thawed spores on potato dextrose agar (5 g/L potato infusion, 20 g/L dextrose, 17 g/L agar, pH 5.6 ± 0.2). After 14 days of growth in a climatic chamber (12 h day/night, 19°C, 70 % humidity), spores were harvested as described previously (2019) and diluted in MilliQ water to obtain a final concentration of $5*10^4$ spores/ml. Each confrontation assay was inoculated with 6 µl of fungal spore suspension.

Pure cultures of *C. necator*-GFP and *C. oxalaticus*-mCherry strains were conserved in 30% glycerol at -80°C. Ten µl of thawed bacterial suspension were spread on nutrient agar plates (8 g/L nutrient broth, 15 g/L agar), supplemented with 10 ppm Gentamycin for *C. oxalaticus*-

mCherry or 50 ppm Kanamycin for *C. necator*, and allowed to grow 24 h at 30°C. For bacterial suspensions, a single bacterial colony was re-cultured for 12-14 h in 5 ml of nutrient broth medium (8 g/L nutrient broth) at room temperature in a shaking incubator at 120 rpm. Bacteria were then centrifuged 10 min at 3000 g and the supernatant was discarded. The pellet was washed once with saline solution (9 g/L NaCl), resuspended in 5 ml of saline solution to be centrifuged at 3000 g for 10 min, and finally re-suspended in 3 ml of saline solution. After a measure of the optical density at 600 nm, bacteria were diluted in saline solution to obtain a final concentration of $1*10^8$ bacteria/ml.

Traditional confrontation experiments

Confrontation assays were conducted in 50 mm Petri dishes with two different media, diluted malt agar (MA1/10; 1,2 g/L malt extract, 15 g/L agar) or Reasoner'2 Agar (R2A; 0,5 g/L yeast extract; 0,5 g/L peptone; 0,5 g/L casamino acids; 0,5 g/L glucose; 0,5 g/L soluble starch, 0,3 g/L Na-pyruvate; 0,3 g/L K_2HPO_4; 0,05 g/L $MgSO_4 \cdot 7H_2O$; 15 g/L agar, in Milli-Q® water; Reasoner & Geldreich, 1985). MA1/10 contains oligosaccharides and amino acids and is mainly used to grow fungi, while R2A is a more complex medium with different carbon and nitrogen sources, specially developed for the cultivation of bacteria. These two media were chosen to compare the biocontrol activity on a medium favouring either fungal or bacterial growth.

Bacteria were inoculated as straight single lines on both sides of the fungal inoculum by pipetting in a line 10 µl of bacterial suspension. Each confrontation assay was done in triplicates and control plates were prepared by inoculating the fungus or the bacteria alone. Plates were incubated in the dark at 22°C. Pictures were taken after four and 30 days.

Confrontation experiments on soft agar plates

The protocol for the soft agar experimental design was adapted from Kropinski *et al.* (2009). Plates were prepared using two layers of medium with different agar concentrations, 15 g/L in the bottom layer and 9 g/L in the top layer (Fig. 1.1). The top layer (soft agar) was inoculated with bacteria prior to pouring. The plates (50 mm Petri dish) were done in two steps; the first layer was poured (8-10 ml of medium) and allowed to dry 20 min before being stored (Fig. 1.1; step 1). The second layer was made as follows: 2 ml of soft agar medium were autoclaved in pre-aliquoted glass test tubes (one tube per plate) and stored overnight in a 60°C incubator. Next day, the tubes were transferred to a 40°C water bath. Immediately before pouring the second layer, each tube was removed from the water bath, inoculated with 15 µl of bacterial suspension, quickly mixed and poured on top of the pre-solidified regular agar layer (Fig. 1.1; step 2). The second layer was allowed to dry 15 min before the plates were incubated at 22°C overnight. *B. cinerea* was then inoculated in the centre of the plates (Fig. 1.1; step 3), and the plates were incubated again at 22°C. Each confrontation assay and control plates were done in triplicates. Pictures were taken after four and 30 days.

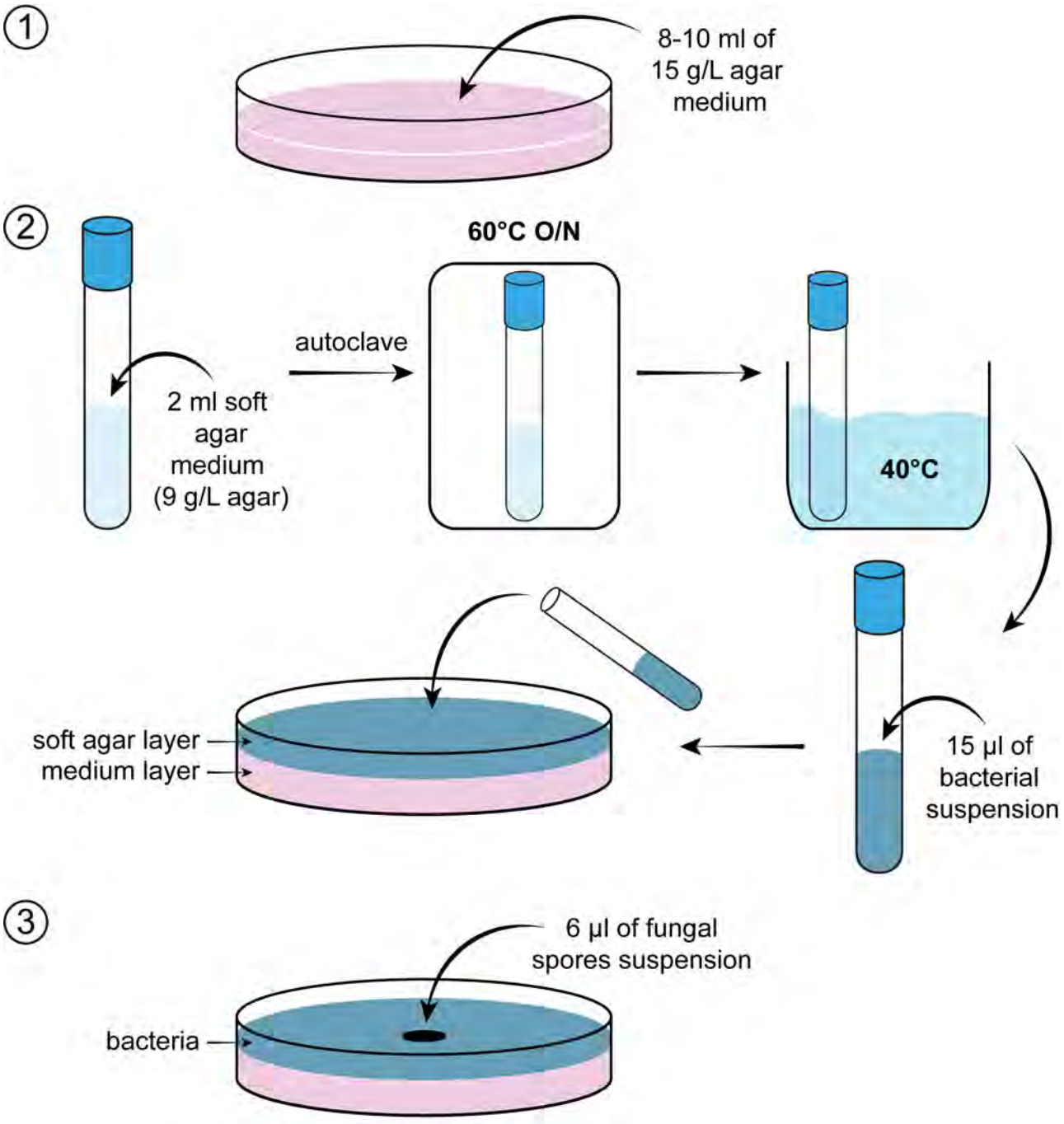


Figure 1.1. Preparation of the plates for the confrontation experiments on soft agar. *The plates are prepared by overlaying two layers of medium with different agar concentrations. The bottom layer (1) is prepared with 15 g/L agar medium, while (2) the upper layer contains only 9 g/L agar medium. The bacterial suspension is mixed with the soft agar before it is poured on the bottom layer. After an overnight incubation, the plates are inoculated with the fungal spore suspension (3) and incubated at 22°C.*

Area of fungal growth

In order to calculate the area covered by the fungal colony, the radius of fungal growth was measured from the diameter of the colony using ImageJ (2.0.0-rc65/1.51w software NIH, USA). Diameter was measured in duplicate on each plate (two perpendicular measurements) with the straight-line tool. The area formula ($A=\pi r^2$) was then applied. T-tests were used to compare the mean of the growth areas of the confrontation plates to the control plates.

Confrontations in cell culture Petri dish

The bottom of the cell culture plates is specially coated to promote cell adhesion and thus provides a stable surface for the fungus to attach and grow. For confrontation assays, 8 ml of liquid medium were added to a cell culture Petri dish (100 mm; Corning, Ref. 430167), with 15 µl of bacterial suspension and 6 µl of *B. cinerea* spore suspension. Each confrontation assay and control plates were done in triplicates and inoculated 10 days at 22°C. Growth control and bacterial viability was assessed after 10 days by inverted microscopy.

Oxalic acid concentrations

OA was measured using Ultra-High-Performance Liquid Chromatography (UHPLC) analysis. For this, 1,5 ml of medium were collected and centrifuged 10 min at 3000 g, transferring 1 ml of the supernatant to a new Eppendorf containing 500 µl of H_2SO_4 30 mM. After two hours of incubation at 60°C, samples were centrifuged again 10 min at 3000 g. One ml of the supernatant was then filtered at 0,22 µm (BGB, Ref. SF1309-1) and 200 µl of each sample were transferred to a HPLC vial (Interchim, Ref. 723594). Each sample was prepared in technical duplicates and kept at 4°C before UHPLC analysis. The UHPLC was coupled to a DAD detector set at 210 ± 2 nm (Ultimate 3000 RS-Dionex, Thermo Fisher Scientific). A 5 µL sample was injected onto a PrevailTM organic acid column (5 µm particle size, 150 x 4.6 mm, Grace Davison Discovery Sciences) with the temperature kept at 40°C. The mobile phase consisted of 50 mM phosphate buffer adjusted to pH 2.5 with phosphoric acid, with a flow rate of 1 ml/min. OA (Merck KGaA) was identified by retention time comparison with a certified standard and quantified by external calibration using a linear regression built from five calibration points (0,2 to 5 mg/mL). T-tests were used to compare the production of OA of the confrontation plates to the control plates.

Germination assays on agarose pads

To prepare the agarose pads (Fig. 1.2), a Gene frame (1 x 1 cm Gene frame; ThermoFisher Scientific, Ref. AB0576) was glued to a clean microscopy glass slide, keeping the upper protective plastic sheet covering the frame only. 100 µl of freshly prepared medium with 1% low melting point agarose (ThermoScientific, Ref. R0801) was pipetted in the Gene frame and a second glass slide placed on top of it, in order to spread the medium in the frame and level it out. The medium was set to dry for at least 20 min at 4°C in a glass Petri dish before use. To inoculate the pad, the top slide was removed by sliding it off, and the second protective plastic sheet was removed from the Gene frame. Two µl of fungal spore suspension were then inoculated in the middle of the pad and allowed to dry for 2-3 min before the Gene frame was carefully closed with a 24 x 60 mm coverslip by gently pressing it to seal it correctly. For confrontation assays, 15 µl of bacterial suspension and 6 µl of *B. cinerea* spores' suspension were mixed in a sterile Eppendorf and 2 µl of the mix were added to the pad. To follow *B. cinerea* germination and growth and its interaction with bacteria, time lapses were recorded on an inverted microscope at magnification 20x. Pictures were taken every minute for 20 h.

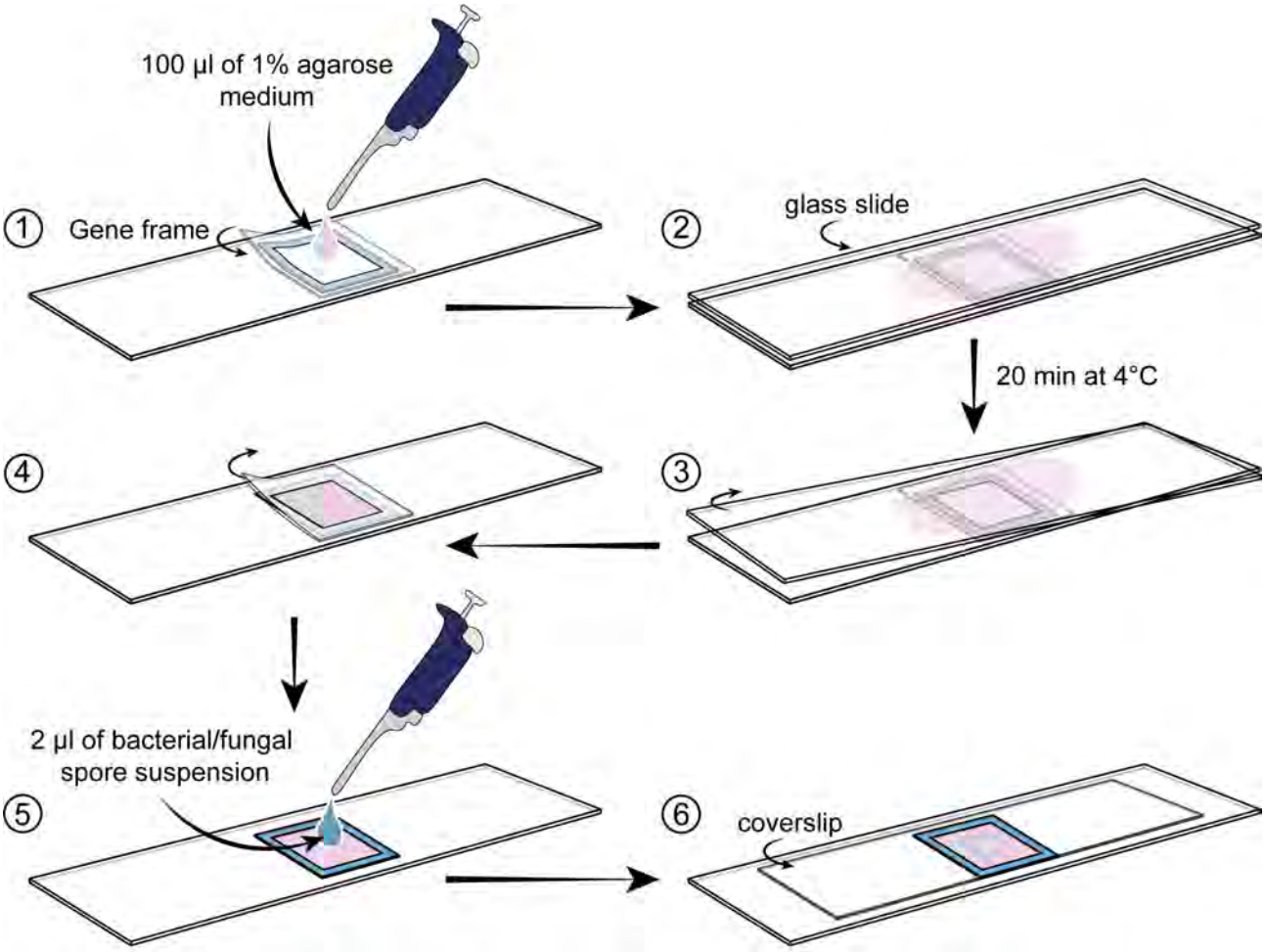


Figure 1.2. Preparation of slides for germination assays on agarose pads. *(1) A Gene frame with the protective sheet covering only the frame is glued to the glass slide and 100 µl of fresh 1% low melting point agarose medium is added to the chamber. (2) A second glass slide is deposited on top, in order to level the medium in the Gene frame. (3) After solidification of the medium, the second slide is removed, (4) as well as the protective sheet (only covering the frame) of the Gene frame. (5) Two µl of bacterial/fungal suspension are pipetted on the agarose pad and allowed to dry for a few minutes before (6) the Gene frame is sealed with a coverslip.*

Time lapse movies were produced using ImageJ, with 42 frames per second (fps).

Images and plots

All macroscopic pictures were taken with a Nikon 1 V2 and processed – adjustment of white balance and cropping – with Adobe Photoshop CC 2018. Inverted microscopy images were taken with the EVOS M5000 microscope. Fluorescent microscopy images were processed with ImageJ 2.0.0-rc65/1.51w to produce composite images of fluorescent and non-fluorescent pictures. Figures were made on Adobe Illustrator CC 2018 and Adobe Photoshop CC 2018. All graphical figures were done with GraphPad Prism 5.00.

Results

Traditional vs soft agar assays to monitor spore germination and early mycelium growth

B. cinerea spores germinated and grew regardless of the media or the confrontation assay used (Fig. 1.3). However, the extent of fungal growth control by the two oxalotrophic bacteria was different depending on the medium. On MA1/10, *B. cinerea* colonized the entire plate after 4 days when inoculated alone, but its growth was slowed down by the oxalotrophic bacteria in both confrontation assays (Fig. 1.3A-B). In the traditional confrontation assay, *B. cinerea* was able to grow past the bacterial inoculum, colonizing the entire plate after 30 days. In the soft agar method, fungal growth was delayed by the ubiquitous presence of oxalotrophic bacteria, especially by *C. necator* (Fig. 1.3A-B). The fungal growth was significantly reduced after four days in presence of the oxalotrophic bacteria compared to the control (*p*-values < 0,0001).

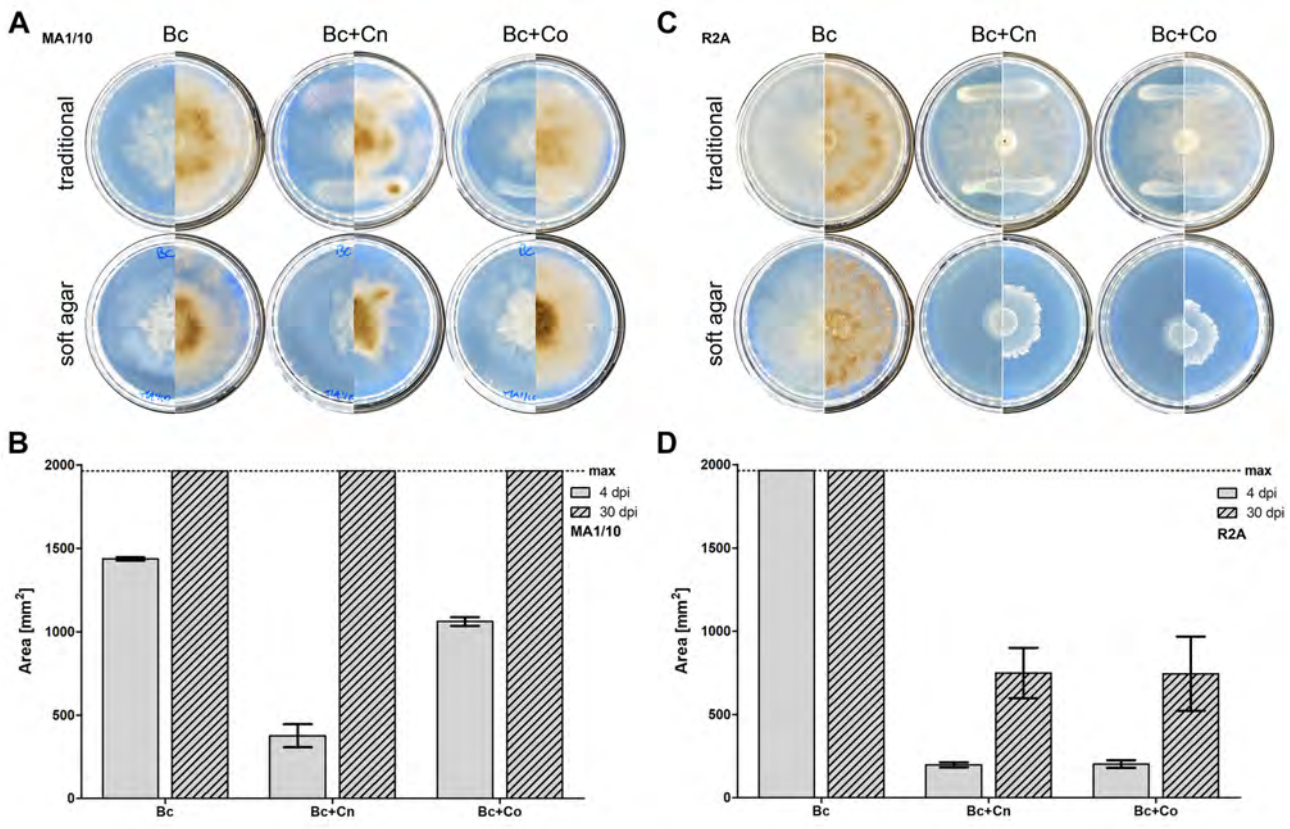


Figure 1.3. Traditional versus soft agar confrontation assays with Botrytis cinerea, Cupriavidus necator and Cupriavidus oxalaticus on MA1/10 (A-B) and R2A (C-D). *From left to right: B. cinerea (Bc), B. cinerea and C. necator (Bc +Cn); B. cinerea and C. oxalaticus (Bc+Co); left half picture was taken after four days and the right half after 30 days (50 mm Petri dishes). The area of mycelial growth was measured on the soft agar confrontations and plotted with the mean and the standard error of the mean in triplicates. After four days, the fungal growth area in presence of C. necator or C. oxalaticus was significantly different from the control (p-values < 0,0001) on both media, while after 30 days, it was only significantly different on R2A (p-values < 0,005). The dotted line indicates the maximum area of the plate.*

R2A media had a remarkable impact on the biocontrol of the fungus. In this medium, *B. cinerea* was not able to colonise the entire plate in presence of *C. necator* and *C. oxalaticus*, although it grew well when inoculated alone (Fig. 1.3C-D). With the traditional method, fungal growth stopped in contact with the bacterial inoculum and only a few hyphae managed grow past it after 30 days. In the soft agar method, *B. cinerea* growth was clearly hindered by the presence of both *C. necator* and *C. oxalaticus* (Fig. 1.3C-D). *B. cinerea* growth was significantly reduced in presence of the oxalotrophic bacteria after four (*p*-values < 0,0001) and 30 days (*p*-values < 0,005).

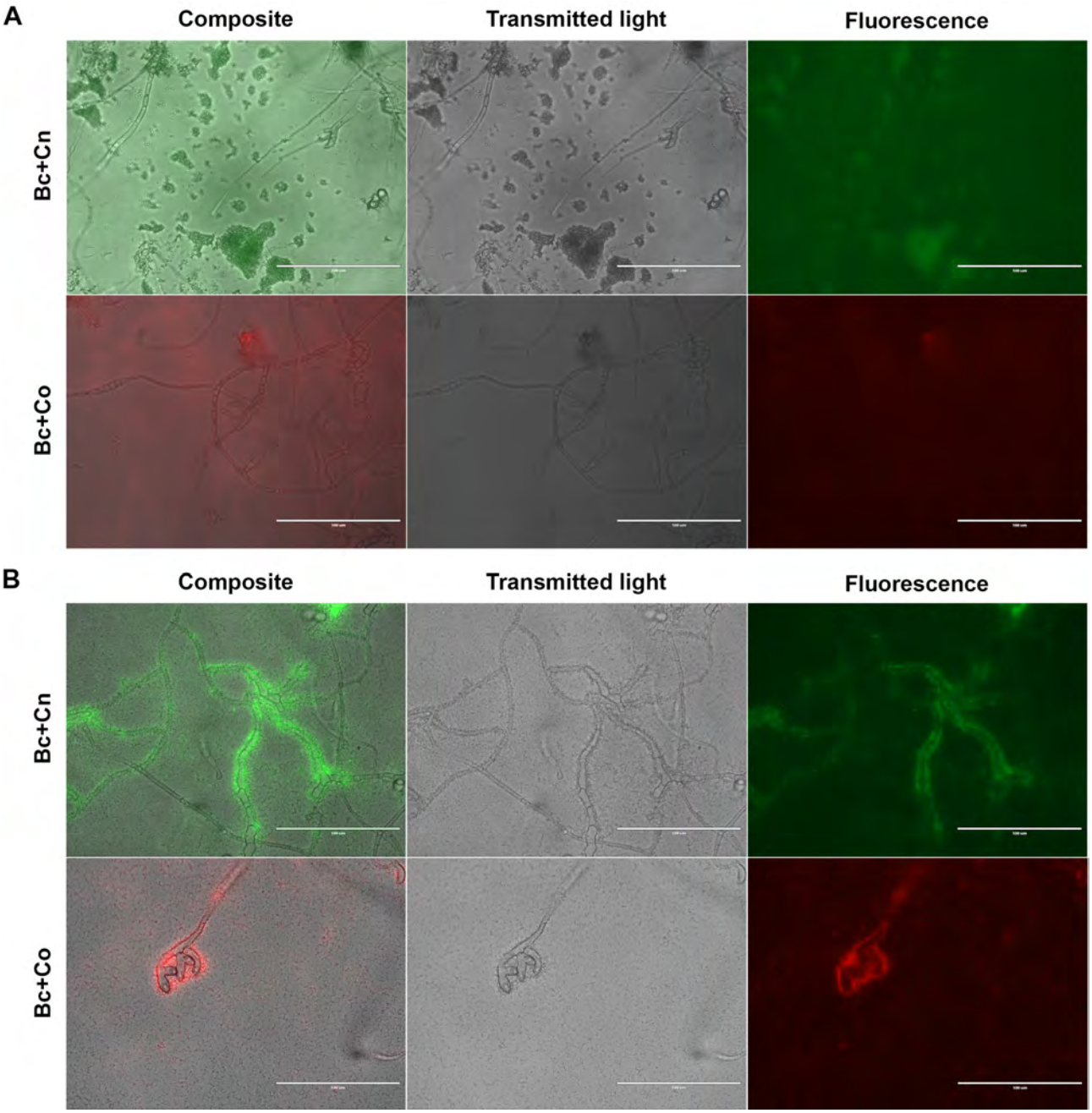


Figure 1.4. Bacterial-fungal interactions in cell culture Petri dishes in MA1/10 (A) and R2A (B) liquid media. *Images were acquired from the plates by inverted microscopy (100 µm scale bar) and epi-fluorescent images were combined with the transmitted light images to produce composite images. In MA1/10 (**A**), bacteria were found in heterogeneously distributed clumps in the plates while in R2A (**B**), both C. necator and C. oxalaticus were closely associated with B. cinerea.*

Cell culture confrontation assays to measure OA production by *B. cinerea*

B. cinerea spores germinated and grew as a mycelial colony in both media in the cell culture Petri dishes. In this experimental set-up BFI could be observed directly without any prior sampling step (Fig. 1.4). In MA1/10 (Fig. 1.4A), bacteria were found in clumps, with seemingly little to no contact with the fungus, while in R2A, bacteria were aggregated around the fungal mycelium (Fig. 1.4B). *C. necator* and *C. oxalaticus* did not prevent *B. cinerea* from germinating, but the mycelium was less dense in presence of the oxalotrophic bacteria, especially in R2A medium (Fig. 1.5A). After 10 days, samples of the spent medium were collected and analysed by UHPLC for OA (Fig. 1.5B). No OA was detected in samples from MA1/10 plates, even in pure *B. cinerea* cultures (data not shown). In contrast, 0,301 ± 0,012 mg/ml of OA were measured in *B. cinerea* cultures in R2A. The OA concentration dropped drastically in presence of *C. necator* and *C. oxalaticus* (0,001 and 0,002 ± 0,001 mg/ml, respectively).

Use of agarose pads to resolve BFI in time and space

B. cinerea spores germinated and grew in the Gene frames regardless of the medium used (Fig. 1.6, Sup. Data 1.2.1-2.2). On MA1/10, spores started to germinate after approximately 2h30 (Fig. 1.6A, Sup. Data 1.2.1) and mycelial growth continued well after 20h. On the other hand, germination happened later in presence of *C. necator* and *C. oxalaticus* (after 3 h and 3 h 30 min, respectively, Fig. 1.6A, Sup. Data 1.3-4). Moreover, the first ramifications occurred only 5 to 7 h later than spore germination, compared to already after 3 h in absence of oxalotrophic bacteria. On R2A, *B. cinerea* spores germinated after approximately 3 h 30 min (Fig. 1.6B, Sup. Data 1.2). Growth and exploration continued, with the apparition of more ramifications and secondary hyphae. In presence of *C. necator* and *C. oxalaticus*, spore germination was slightly delayed (Fig. 1.6B, Sup. Data 1.5-6) and all growth ceased after 8 h and 5 h 30 min, respectively.

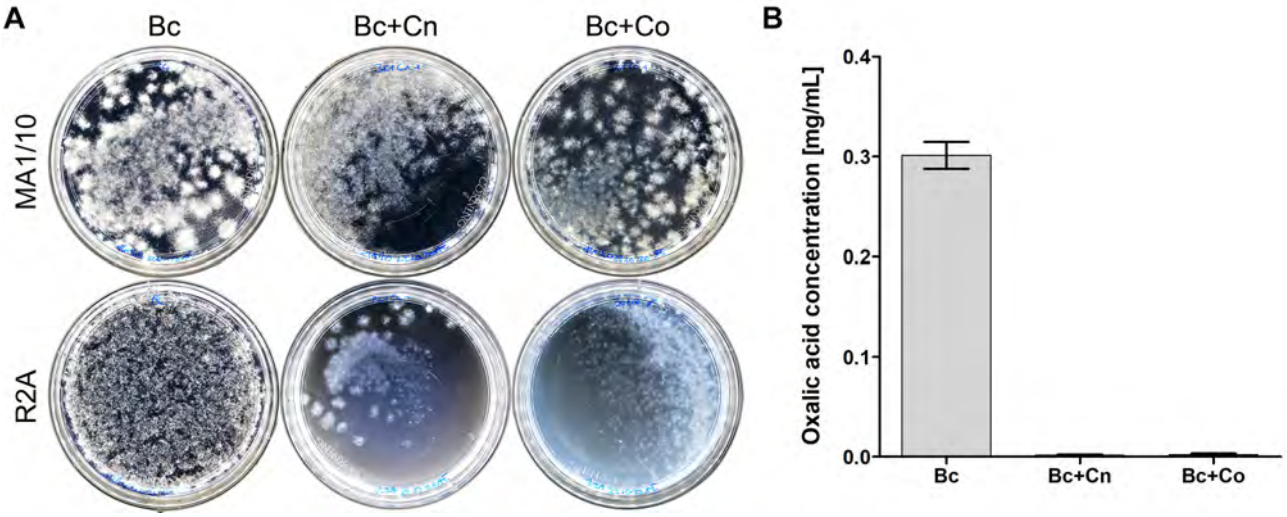


Figure 1.5. Confrontation assays in cell culture Petri dishes (A) and corresponding oxalic acid detection in R2A (B). *From left to right: B. cinerea (Bc), B. cinerea and C. necator (Bc+Cn); B. cinerea and C. oxalaticus (Bc+Co; 100 mm Petri dishes). For oxalic acid concentrations, the mean of three independent measures was plotted with the standard error of the mean. The oxalic acid concentration in presence of C. necator and C. oxalaticus was significantly different from the control (p-values < 0,005).*

BFI between *B. cinerea* and *C. necator* were also visualized using epi-fluorescence (Sup. Data 1.7-8). On R2A, *C. necator* cells were particularly active around *B. cinerea* spores before their germination (Sup. Data 1.7). Upon hyphal growth, some bacteria moved back and forth from the hyphal tip to the spore while others seemed to stay in contact with the hyphae. On MA1/10, *C. necator* cells were also active around the spores but they did not move along the hyphae (Sup. Data 1.8). As m-Cherry fluorescence is more difficult to visualise, we were not able to record any epi-fluorescent time-lapses in the case of *C. oxalaticus*.

Discussion

Our study presents alternative and easy-to-implement methods for *in-vitro* assays to evaluate the effect of bacteria on the germination of fungal spores. In many phytopathogenic fungi, the germination of the fungal spore is a crucial stage in the initiation of plant disease (Agrios, 2004). Biocontrol of *B. cinerea* by oxalotrophic bacteria has been previously described, but most studies focused on the inhibition of mycelial growth or the reduction of disease incidence (Dickman & Chet, 1998; Schoonbeek *et al.*, 2007; Marina *et al.*, 2019). Here, we showed that the inhibition of hyphal growth was consistent with the previous studies, but does not extend to the inhibition of spores' germination. The soft agar confrontation assay also offered new insights into the interactions occurring. Indeed, as bacteria are spread out in the entire plate, there is a constant contact between both organisms. Thus, the effect of oxalotrophic bacteria slowing down the growth of *B. cinerea* was more clearly observed than in the traditional confrontation assays.

The results were also strikingly different depending on the medium used. *B. cinerea* was able to colonise the entire plate on MA1/10, regardless of the presence of the bacteria, while its growth was clearly restricted on R2A. No OA was detected in MA1/10, while this organic acid was detected as a free fraction in R2A. It was previously shown that *in-vitro* production of OA depends of the carbon and nitrogen sources of the medium used (Punja & Jenkins, 1984; Dutton & Evans, 1996; Gadd, 1999). In the case of *B. cinerea*, it appears that OA is produced in higher amounts in R2A than MA1/10. The use of the cell culture Petri dishes allowed not only to show that *B. cinerea* produced OA in R2A, but also that its concentration decreased drastically in presence of *C. necator* and *C. oxalaticus*. However, as OA concentration was measured at only one time point and no correlated measures of fungal or bacterial biomass were performed, it is not possible to ascribe directly biocontrol of fungal growth to OA consumption. This disparity in the results shows that it is important to perform experiments on different media in *in-vitro* assays.

Another interesting feature of the cell culture Petri dishes is the possibility to perform inverted microscopy directly on the plates. As no prior sampling and preparation steps are required, this visualization method can be considered very close to an *in-situ* observation, offering a first overview of the cell-to-cell interactions occurring. Interestingly, *C. necator*

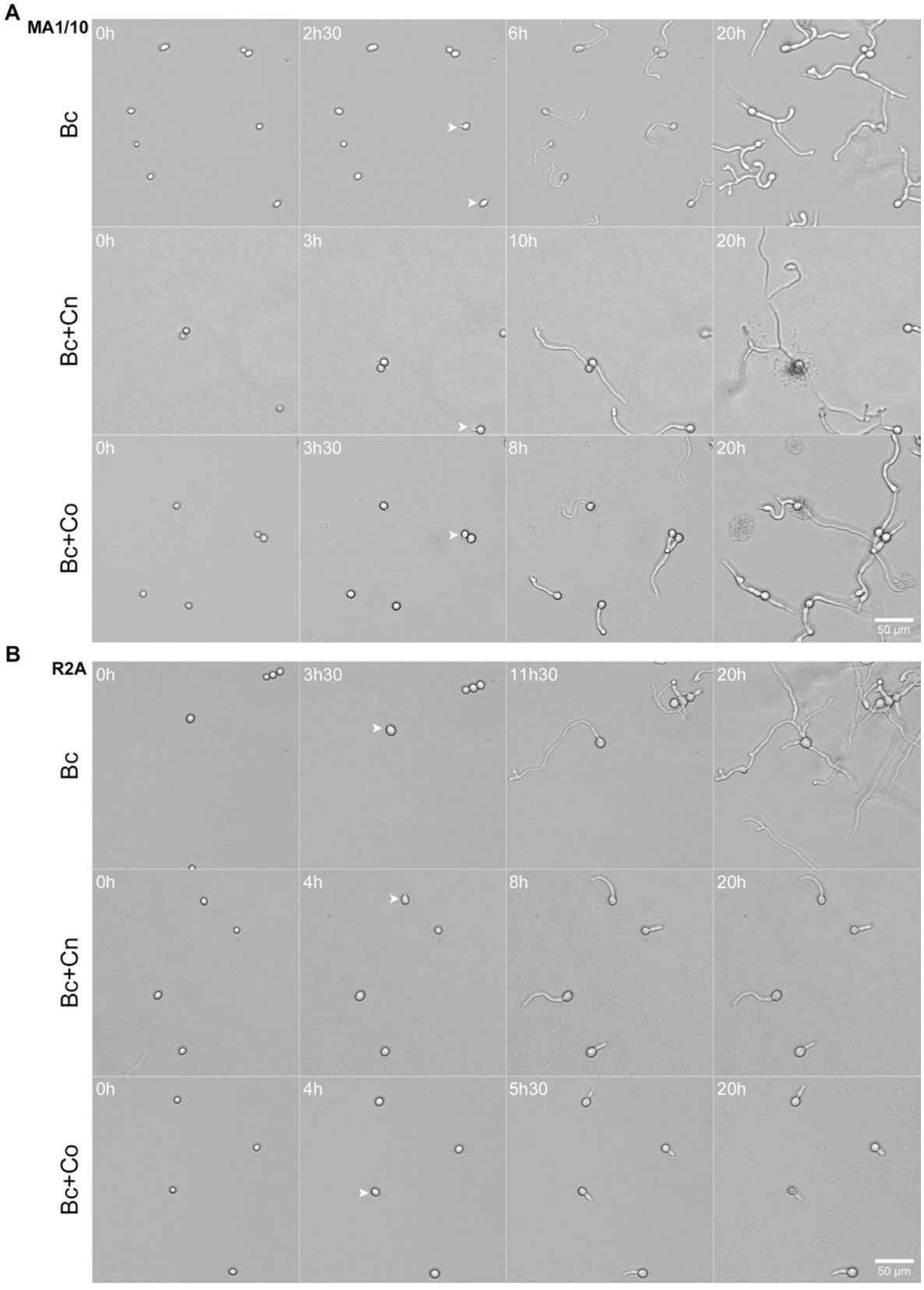


Figure 1.6. Snapshots of the germination of B. cinerea spores in presence of C. necator and C. oxalaticus on MA1/10 (A) and R2A (B) agarose pads. *All times indicated correspond to approximate times and the corresponding time lapses movies are available in Sup. Data 1-7. Germination occurred when spores were inoculated alone or with C. necator and C. oxalaticus. However, germination was delayed in presence of oxalotrophic bacteria. Ramifications happened later in presence of C. necator and C. oxalaticus on MA1/10 (**A**) but B. cinerea continued to grow after 20 h in all cases. On R2A (**B**), B. cinerea growth stopped after 8 h with C. necator and 5 h 30 min with C. oxalaticus. B. cinerea alone continued to grow well after 20 h.*

and *C. oxalaticus* did not seem to interact with *B. cinerea* in MA1/10. On the other hand, *C. necator* and *C. oxalaticus* aggregated around *B. cinerea* mycelium in R2A. This difference of behaviour on the two media could be linked to the production of OA. Indeed, OA is well-known to be involved in the chemotaxis of beneficial bacteria towards plants (Kost *et al.*, 2013; Liu *et al.*, 2019) or to act as signal molecule for mycophagous bacteria to detect their fungal prey (Rudnick *et al.*, 2015). Therefore, as no OA was detected in MA1/10, this could explain why *C. necator* and *C. oxalaticus* did not seem to interact with *B. cinerea*. Moreover, germination assays on agarose pads showed that *C. necator* behaved very differently in presence of *B. cinerea* on the two media. In R2A, *C. necator* was found to be very active around the fungal spores and the hyphae upon germination, while no such behaviour was observed on MA1/10.

Agarose pads in Gene frame offered a simple small chamber that fits on a glass slide and allowed visualisation at high magnification with any microscope. The gas-tight fit of the chamber prevents the dehydration of the medium, allowing monitoring for up to a week. Despite the fact that the medium and the oxygen supply are limited in the chamber, these agarose pads were powerful tools to perform direct imaging of cell-to-cell interactions with limited technical requirement. Ramification and secondary hyphae formation occurred later in presence of oxalotrophic bacteria on MA1/10 and the fungal growth appeared to stop entirely after a few hours on R2A.

Conclusions

The methods presented here are efficient and require minimal technical knowledge. Moreover, they can be applied to answer questions in multiple areas of BFI. The soft agar plates are easily replicable and, as the fungus is in contact with the bacterium from the beginning of the interaction, this method is suitable to work with spores sclerotia or active mycelium. Cell culture Petri dishes also allow to work with spores, sclerotia or active mycelium. The fungus grows as mycelium at the liquid-air interface, facilitating the measure of parameters such as pH or the recovery of soluble metabolites. Moreover, cell culture Petri dishes are made of optically-clear polystyrene, which allows to perform inverted microscopy directly on the plates. This gives the opportunity to obtain a direct insight of BFI or to visualise the mycelial architecture during fungal growth. Finally, agarose pads in Gene frames are a valuable alternative to more complex microfluidics devices. They are well suited to work with fungal spores or mycelium, or to follow the germination and growth of bacterial spores. In conclusion, our study offers useful and straightforward methods to expand our understanding of BFI or more broadly, our knowledge in fungal and bacterial growth.

Acknowledgements

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Supplementary data

Sup. Data 1.1. Time lapse movie of *B. cinerea* spores' germination on MA1/10. 20 h of time lapse, 1 picture/min, 42 fps.

Sup. Data 1.2. Time lapse movie of *B. cinerea* spores' germination on R2A. 20 h of time lapse, 1 picture/min, 42 fps.

Sup. Data 1.3. Time lapse movie of *B. cinerea* and *C. necator* on MA1/10. 20 h of time lapse, 1 picture/min, 42 fps.

Sup. Data 1.4. Time lapse movie of *B. cinerea* and *C. oxalaticus* on MA1/10. 20 h of time lapse, 1 picture/min, 42 fps.

Sup. Data 1.5. Time lapse movie of *B. cinerea* and *C. necator* on R2A. 20 h of time lapse, 1 picture/min, 42 fps.

Sup. Data 1.6. Time lapse movie of *B. cinerea* and *C. oxalaticus* on R2A. 20 h of time lapse, 1 picture/min, 42 fps.

Sup. Data 1.7. Close up of *B. cinerea* and *C. necator* interactions on R2A. 9 h 57 min of time lapse, 1 picture/min, 42 fps

Sup. Data 1.8. Close up of *B. cinerea* and *C. necator* interactions on MA1/10. 10 h of time lapse, 1 picture/min, 42 fps

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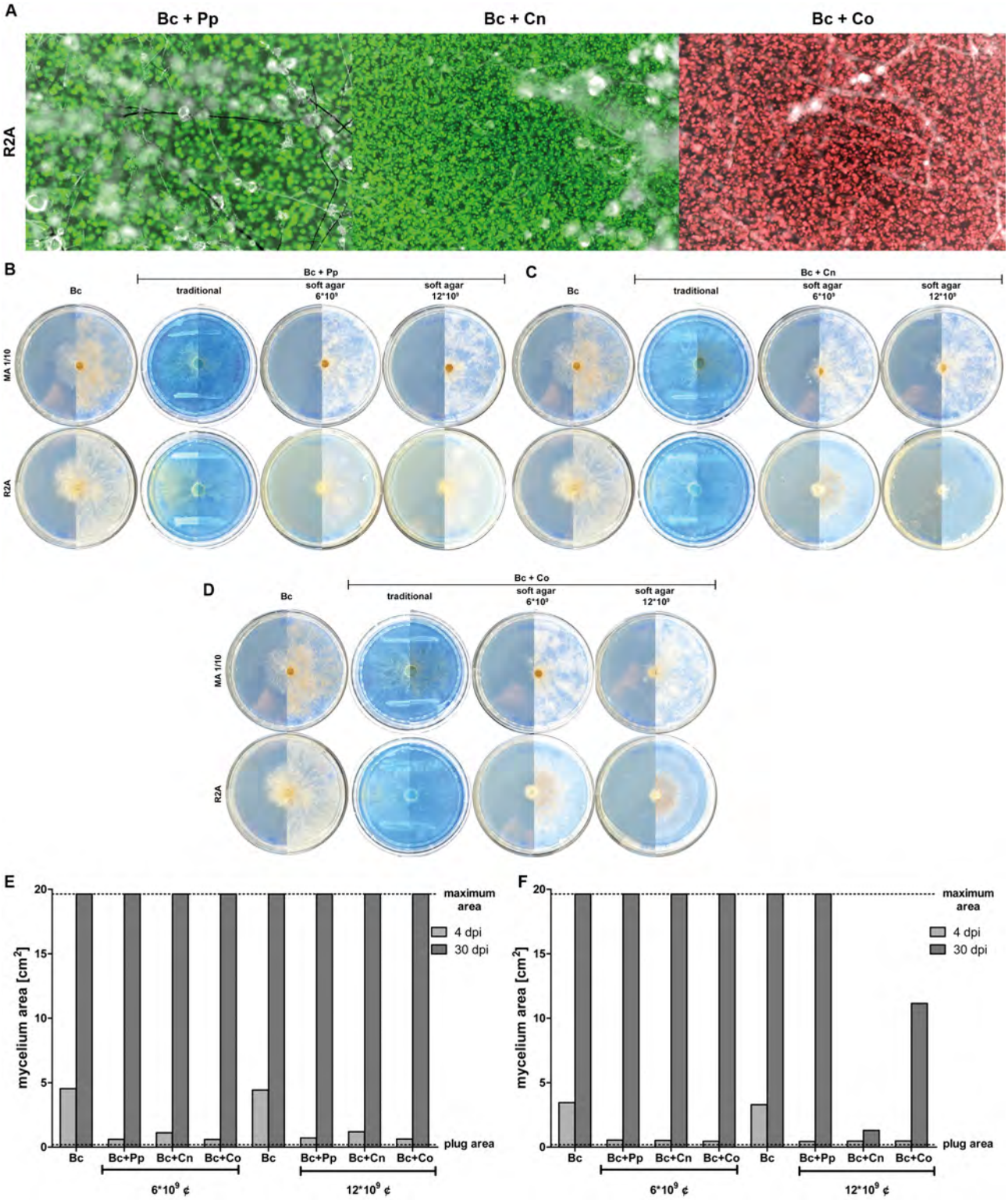
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Supplementary information

Soft agar experiments

The suitability of the soft agar design was verified with confrontation experiments with *B. cinerea* and three bacteria (Sup. Info. 1.1). *B. cinerea* was confronted to the two oxalotrophic bacteria *C. necator* and *C. oxalaticus*, and to the non-oxalotroph *P. putida* as control. Bacterial viability in the soft agar was assessed by fluorescent stereoscopy 48 h after inoculation (Sup. Info. 1.1A). Soft agar confrontations were performed with two concentrations of bacteria ($6{\cdot}10^9$ and $12{\cdot}10^9$ bacteria/ml) to determine the optimal concentration for further experiments, and compared to the traditional method (Sup. Info. 1.1B-D). Mycelium area was measured for the soft agar confrontation assays, in order to determine which concentration of bacteria was the most efficient to control the fungal growth (Sup. Info. 1.1E-F). As expected, no control occurred on MA1/10 and *P. putida* was unable to control the fungal growth (Sup. Info. 1.1B). On the other hand, both *C. necator* and *C. oxalaticus* were able to control *B. cinerea* over time on R2A (Sup. Info. 1.1C-D). The control was only efficient with $12{\cdot}10^9$ bacteria/ml (Sup. Info. 1.1F), therefore further experiments were performed with this concentration of bacteria.

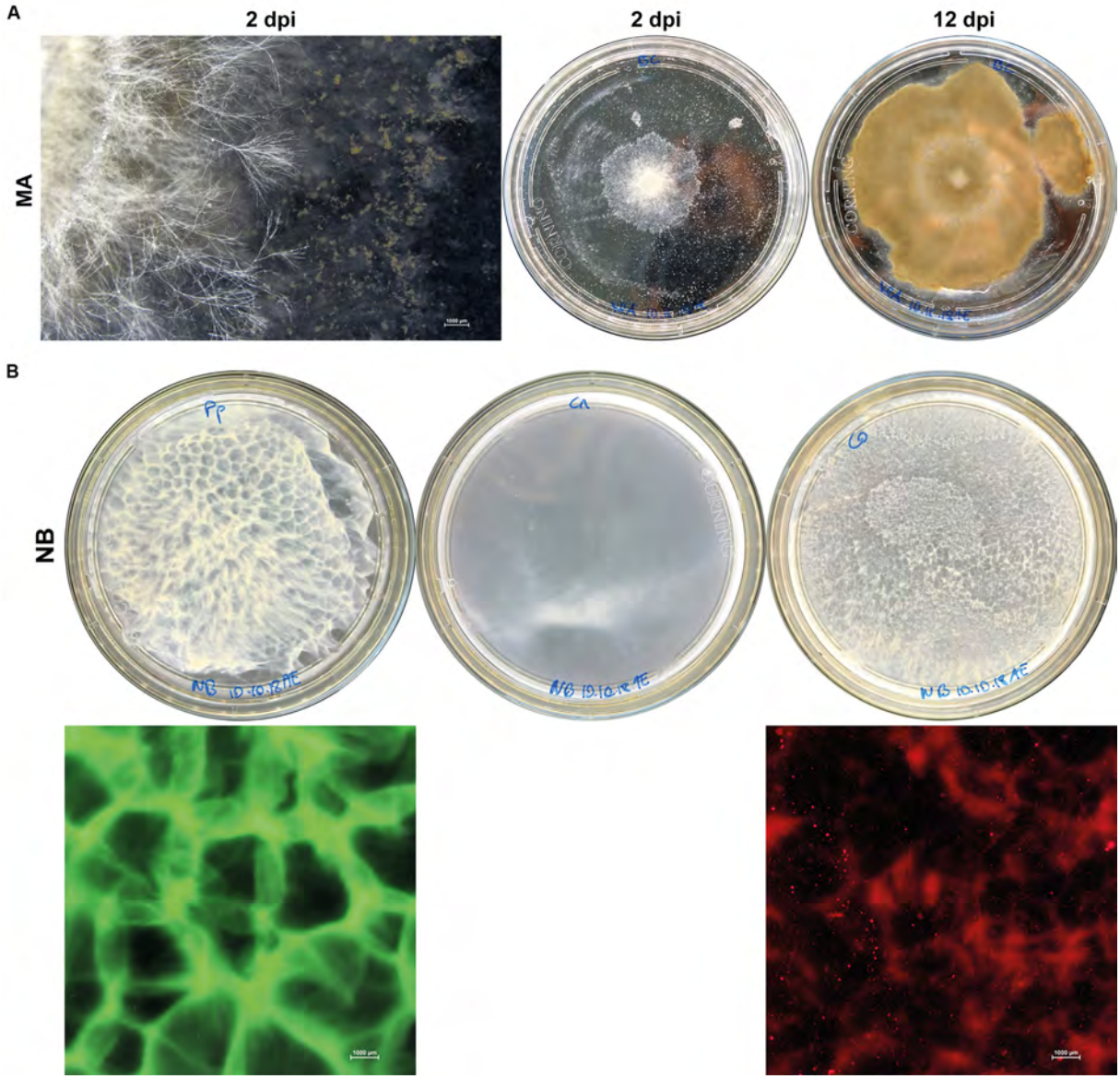


Sup. Info. 1.1. Soft agar experiments. ***A.*** *Bacterial viability was assessed by fluorescent microscopy 24 h after fungal inoculation. From left to right: B. cinerea + P. putida (Bc+Pp); B. cinerea + C. necator (Bc+Cn); B. cinerea +C. oxalaticus (Bc+Cn).* ***B.*** *Traditional and soft agar confrontation assays with B. cinerea and P. putida on MA1/10 and R2A, with two concentrations of bacteria (6·10⁹ bacteria/µl and 12·10⁹ bacteria/µl). Fungal growth was slow down on both media, but the control did not continue overtime.* ***C****. Traditional and soft agar confrontation assays with B. cinerea and C. necator different media. Fungal growth was slowed down on MA1/10 medium with both bacterial concentrations but not in the traditional design. On R2A medium, control was efficient after 4 days for each design, but only the plate with 12·10⁹ bacteria/µl showed a efficient control overtime. As the plate was contaminated, the result need to be taken with caution.* ***D****. Traditional and soft agar confrontation assays with*

B. cinerea and C. oxalaticus different media. Fungal growth was slowed down on MA1/10 medium with both bacterial concentrations but not in the traditional design. On R2A medium, control was efficient after 4 days for each design, but was more efficient in the traditional assay and the 12·10⁹ bacteria/µl plates. ***E – F.*** *Fungal growth measures on soft agar assays. Area covered by the mycelium was calculated for each plates on MA1/10 (**E**) and R2A (**F**) media, four and 30 days post inoculation (dpi). The plug area dotted line indicated the size of the initial inoculum, while the maximum area is the size of the plate. Control was efficient only for C. necator and C. oxalaticus when the initial bacterial inoculum was 12·10⁹ bacteria.*

Cell culture Petri dish

To verify the suitability of the cell cultures Petri plates for fungal and bacterial growth, *B. cinerea*, *P. putida*, *C. necator* and *C. oxalaticus* were inoculated in liquid malt broth or nutrient broth (Sup. Info. 1.2). *B. cinerea* was grown in liquid malt medium and colonised the plate in 12 days (Sup. Info. 1.2A). The three bacteria were inoculated in nutrient broth and viability was assessed after a few days of incubation by fluorescent stereoscopy (Sup. Info. 1.2B).



Sup. Info. 1.2. Fungal (A) and bacterial (B) growth in cell culture plates. A. *B. cinerea was inoculated in malt broth and colonised the plates after 12 days of inoculation.* ***B****. P. putida, C. necator and C. oxalaticus, were inoculated in nutrient broth and showed a good growth after a few days of incubation. Fluorescent stereoscopy was used to assessed the viability of the bacteria.*

Chapter 2

Impact of oxalic acid consumption and pH on the *in-vitro* biological control of the oxalogenic phytopathogen *Sclerotinia sclerotiorum*

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Abstract

The phytopathogenic fungus *Sclerotinia sclerotiorum* has a wide host range and causes important economic damage in crops worldwide. This pathogen uses oxalic acid as a virulence factor and, therefore, the degradation of this organic acid by oxalotrophic bacteria has been proposed as an approach of biological control. However, studies on the potential role of oxalotrophy in biocontrol did not investigate the respective role of biotic and abiotic factors on the outcome of the interactions between the pathogenic fungi and the oxalotrophic bacteria. In a similar fashion, the effect of oxalic acid consumption and the subsequent pH alkalinisation on the fungal growth has not been evaluated. In this study, confrontation experiments on different media between *S. sclerotiorum* WT and its oxalate-deficient mutant, strain Δ*oah*, with *Cupriavidus necator* and *Cupriavidus oxalaticus*, two soil oxalotrophic bacteria, showed a strong effect of abiotic factors on oxalic acid production, as well as on fungal growth control. Oxalotrophic bacteria were able to control *S. sclerotiorum* only in the medium in which oxalic acid was detected. However, the mutant deficient in oxalic acid production was also controlled in this medium. *S. sclerotiorum* WT acidified the medium when inoculated alone while for both fungi, the pH of the medium significantly changed from neutral to alkaline in presence of bacteria. Therefore, medium alkalinisation independent of oxalotrophy plays a major role in fungal growth control.

Introduction

Agriculture has developed through ages and we have reached the point where we are dependent of our crops for feeding an increasing global population. However, agriculture faces numerous problems to provide enough food. Phytopathogenic fungi are a major threat to crops and cause important economic losses each year (Tiwar *et al.*, 2004; Parikh & James, 2012; Mohd-Assaad *et al.*, 2016). Furthermore, the main control strategy, which is the intensive and systematic application of fungicides, exerts a strong selection pressure on resistant pathogenic strains, making them even more difficult to control (Dickman & Chet, 1998; Orton *et al.*, 2011; Mohd-Assaad *et al.*, 2016). Therefore, there is a necessity to develop and understand alternative pathogen control strategies to ensure our future food security.

One of these strategies is biocontrol, which can be defined as the deliberate use of a microorganism to control a phytopathogenic fungus responsible for a crop disease (Baker & Cook, 1974; Pal & McSpadden, 2006). Different kinds of biocontrol strategies exist, ranging from competition to antibiosis, and can target the pathogen, the host plant or the environment (Baker, 1968; Baker & Cook, 1974; Whipps, 2001; Pal & McSpadden, 2006). Biocontrol agents acting on the environment make it favourable for themselves, at the expense of the pathogenic fungus. In 1997, Dickman & Chet proposed a new approach of biological control, the degradation of oxalic acid, a metabolite required for fungal pathogenicity. Oxalic acid ($C_2H_2O_4$, pKa_1= 1,23, pKa_2= 4,19) is one of the low molecular weight organic acids (LMWOA) produced by fungi and the strongest polyprotic acid after sulfuric acid. This organic acid is excreted by several pathogenic fungi as a pathogenicity factor (Dutton & Evans, 1996; van Kan, 2005; Ren *et al.*, 2010; Moore *et al.*, 2011; Atkins & Jones, 2017). The versatility of oxalic acid provides many advantages for fungal virulence: the acidification of the host tissues stimulates the production and activity of fungal enzymes. In addition, thanks to its chelating-activity, oxalic acid can extract and sequester calcium ions from the wall pectin thus weakening the cell wall (Dutton & Evans, 1996; van Kan, 2006).

Oxalic acid is not only a factor of pathogenicity, but is one of the most commonly produced organic acids, being found in various environments, as well as in the atmosphere (Tamer & Aragno, 1980; Sahin, 2003; Rinaldi *et al.*, 2011; Hervé *et al.*, 2016). It plays a major role in the oxalate-carbonate pathway (OCP), a biogeochemical process acting as a potentially important sink for atmospheric CO_2 (Aragno & Verrecchia, 2012; Cailleau *et al.*, 2014; Pons *et al.*, 2018). Oxalic acid is mainly produced by plants, for regulation and detoxification of calcium or for protection against herbivory (Franceschi & Nakata, 2005). Fungal decay release oxalic acid in soils, provoking a strong local acidification and the precipitation of oxalate salts, mainly calcium oxalates (Verrecchia *et al.*, 2006; Plassard & Fransson, 2009; Aragno & Verrecchia, 2012). Yet, oxalates do not remain in soils and are used as carbon and energy sources by so-called oxalotrophic bacteria (Tamer & Aragno, 1980; Sahin, 2003; Verrecchia *et al.*, 2006; Hervé *et al.*, 2016). In aerobic metabolism, oxalotrophic bacteria oxidise oxalic acid in CO_2

and excrete it as the bicarbonate ion HCO_3^- (Quayle *et al.*, 1961; Quayle, 1963; Blackmore & Quayle, 1968; Dijkhuizen *et al.*, 1977). As carbonic acid is a weak acid (pKa_1= 6,37 and pKa_2= 10,25), its secretion will prompt local alkalinisation (Verrecchia *et al.*, 2006; Martin *et al.*, 2012; Atkins & Jones, 2017). When the pH reaches the equilibrium point of carbonic acid, calcium carbonate precipitation will happen. This important local alkalinisation is the reason why calcium carbonate precipitation and accumulation occur. Thus, the OCP exerts a strong influence on soil pH and soil physicochemical properties.

This propensity of the organisms involved in the OCP to modify the soil pH is the basis for the hypothesis of oxalotrophy as an effective biocontrol strategy (Fig. 2.1). Several studies already showed that oxalotrophic bacteria are indeed able to protect *Arabidopsis thaliana*, *Phaseolus vulgaris* and different crops against *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Botrytis cinerea*, three phytopathogenic fungi producing oxalic acid (Dickman & Chet, 1998; Schoonbeek *et al.*, 2007; Marina *et al.*, 2019). Both *in-vitro* and field experiments showed a significant reduction of disease incidence with the addition of oxalotrophic bacteria. However, none of these studies evaluated how the consumption of oxalic acid and the subsequent alkalinisation interfere with fungal growth and how biotic and abiotic factors influence the interaction between the oxalotrophic bacteria and the phytopathogenic fungus.

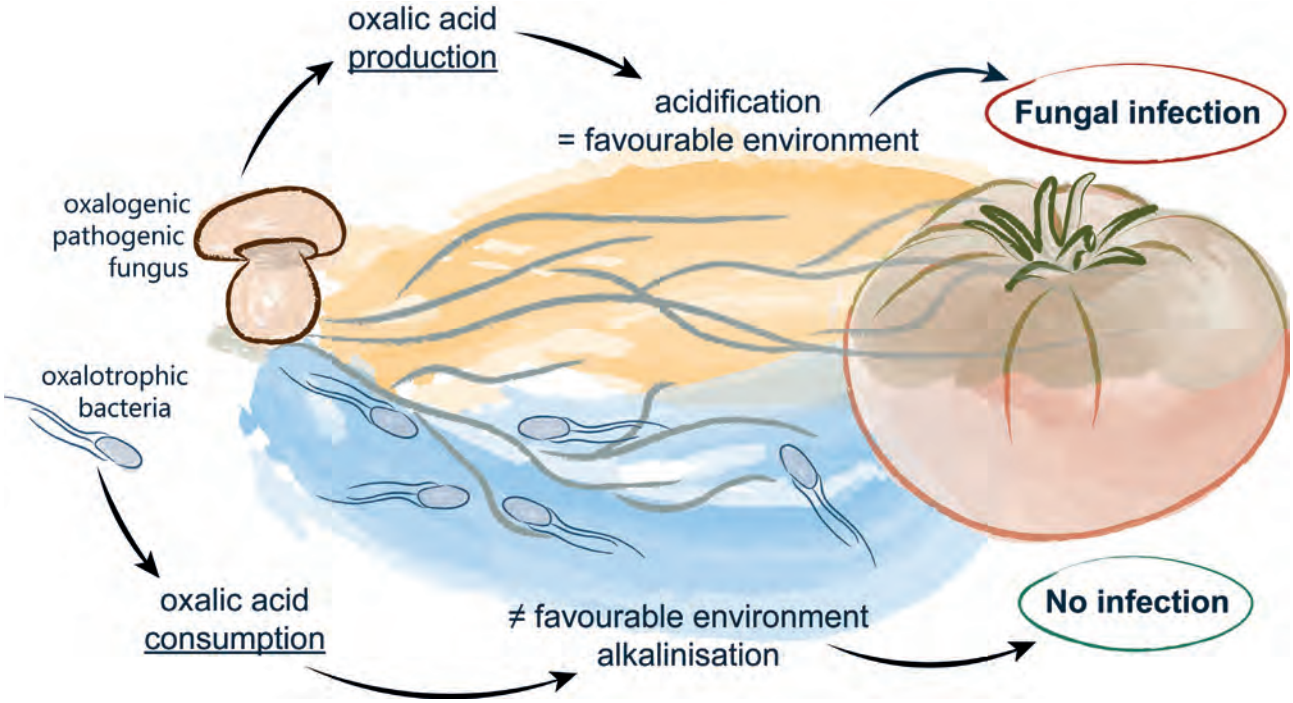


Figure 2.1. Schematic representation of the working hypothesis.

In order to disentangle the effect of oxalic acid consumption and pH change on fungal growth, we used a model interaction consisting of an oxalogenic phytopathogenic fungus and its oxalate-deficient mutant, as well as two oxalotrophic bacteria. *S. sclerotiorum* is a necrotrophic pathogen that causes important losses in economically relevant crops (Dickman & Chet, 1998; Cessna *et al.*, 2000; Hegedus & Rimmer, 2005). Oxalic acid production has been extensively investigated for this pathogen, demonstrating that oxalic acid is essential for

pathogenicity (Godoy *et al.*, 1990; Cessna *et al.*, 2000; Jain *et al.*, 2015). The oxalate-deficient mutant, *S. sclerotiorum* Δ*oah*, is unable to produce oxalic acid and is less virulent that the wild-type (Li *et al.*, 2018). *Cupriavidus necator* and *Cupriavidus oxalaticus*, two facultative-oxalotrophic bacteria from soil, were used as model bacteria for the interaction (Makkar & Casida, 1987; Şahin *et al.*, 2000). We monitored the biocontrol effect of *C. necator* and *C. oxalaticus* on different media, in order to investigate the influence of biotic and abiotic factors on the production of oxalic acid, changes in pH changes and the growth control of *S. sclerotiorum* WT and its mutant.

Material & methods

Fungi and bacteria

Two strains of *S. sclerotiorum,* WT and Δ*oah* (Li *et al.*, 2018), were kindly provided by Jeffrey Rollins from the Department of Plant Pathology, University of Florida. The bacteria *C. necator*-GFP (Poehlein *et al.*, 2011) and *C. oxalaticus*-mCherry (Şahin *et al.*, 2000), bearing a fluorescent tag (GFP and mCherry, respectively), were obtained from the bacterial collection of the Laboratory of Microbiology, University of Neuchâtel, Switzerland. In order to improve the readability of the text, the GFP and mCherry suffixes were omitted in the text.

Fungal sclerotia and bacterial suspensions

Sclerotia from *S. sclerotiorum* WT and Δ*oah* were harvested from 14 days old fungal cultures grown on potato dextrose agar (5 g/L potato infusion, 20 g/L dextrose, 17 g/L agar, pH 5,6 ± 0,2) and incubated at room temperature. Pure cultures of *C. necator* and *C. oxalaticus* were conserved in 30% glycerol at -80°C and suspensions were produced as described in Estoppey et al. (2022).

Soft agar confrontation assays and fungal growth area

Confrontation assays and control plates in soft agar were performed in triplicate in Reasoner'2 agar (R2A; 0,5 g/L yeast extract; 0,5 g/L peptone; 0,5 g/L casamino acids; 0,5 g/L glucose; 0,5 g/L soluble starch, 0,3 g/L Na-pyruvate; 0,3 g/L K_2HPO_4; 0,05 g/L $MgSO_4 \cdot 7H_2O$; 15 g/L agar, in Milli-Q® water; (Reasoner & Geldreich, 1985)) and diluted malt agar medium (MA1/10; 1,2 g/L malt extract, 15 g/L agar) as described in Estoppey et al. (2022). The fungal inoculum was a single sclerotium of *S. sclerotiorum* WT or Δ*oah* inoculated in the middle of the plate. Fungal growth area was calculated from the radius of the colony from pictures taken after 4- and 30-days post-inoculation (Estoppey *et al.*, 2022).

Liquid culture confrontation, oxalic acid concentration and pH

Confrontation assays and control plates in liquid media were performed in triplicate in 100 mm cell culture Petri dish, as described in Estoppey et al. (2022). The fungal inoculum was a single sclerotium of *S. sclerotiorum* WT or Δ*oah* placed in the middle of the plate. Oxalic acid

concentration in each assay was determined by HPLC, as described in Estoppey et al. (2022).

For the pH, measures were taken at the beginning and after 10 days of the experiment with the FiveEasy pH meter (Mettler Toledo). The initial pH of the media was pH 7,1 for R2B and 5,3 for MB1/10 (measured in triplicates). T-tests were used to compare the mean of the different samples.

Images, statistics and plots

Macroscopic images were taken with a camera Nikon 1 V2 and processed – adjustment of white balance and cropping – with Adobe Photoshop CC 2018. Inverted microscopy images were taken with the EVOS M5000 microscope and processed with ImageJ 2.0.0-rc65/1.51w to produce composite images of fluorescent and non-fluorescent pictures. Figures were made on Adobe Illustrator CC 2018 and Adobe Photoshop CC 2018. Graphical figures and statistics were done with GraphPad Prism 5.00.

Results

Confrontation assays were conducted with the oxalogenic phytopathogenic fungus *S. sclerotiorum* WT and its mutant, *S. sclerotiorum* Δ*oah*, which is deficient in oxalic acid production. Both fungi were confronted to two oxalotrophic bacteria, *C. necator* and *C. oxalaticus,* on two media, MA1/10 and R2A. Sclerotia of both fungal strains germinated and grew on the two media in absence of bacteria (Fig. 2.2). However, the growth pattern changed in presence of *C. necator* and *C. oxalaticus*. Mycelial growth was initially slowed down on MA1/10 in presence of the oxalotrophic bacteria, but the presence of bacteria did not control growth over time (Fig. 2.2A, C). On the other hand, *C. necator* and *C. oxalaticus* exerted a strong growth control over *S. sclerotiorum* WT and *S. sclerotiorum* Δ*oah* on R2A (Fig. 2.2B, D). In the case of the Δ*oah* mutant, growth was hindered on R2A in absence of bacteria, but fungal growth was still significantly controlled by *C. necator* and *C. oxalaticus* overtime.

In order to quantify oxalic acid production and its impact on the pH of the medium, the same confrontation assays were conducted in cell culture Petri dishes with a thin layer of liquid medium. Sclerotia of *S. sclerotiorum* WT germinated and grew in both liquid media in absence of bacteria (Fig. 2.3A). As in the confrontation assays on solid media, *C. necator* and *C. oxalaticus* were able to control *S. sclerotiorum* WT mycelial growth only in R2B (Fig. 2.3A). Measures of oxalic acid concentration showed that no oxalic acid was quantifiable in MB1/10 medium, while its concentration reached 0,65 g/L in R2B after 10 days of incubation (Fig. 2.3B). In R2B, oxalic acid production significantly decreased to 2 ± 3 mg/L in presence of oxalotrophic bacteria (Fig. 2.3B).

As expected, *S. sclerotiorum* WT significantly acidified the medium when inoculated alone.

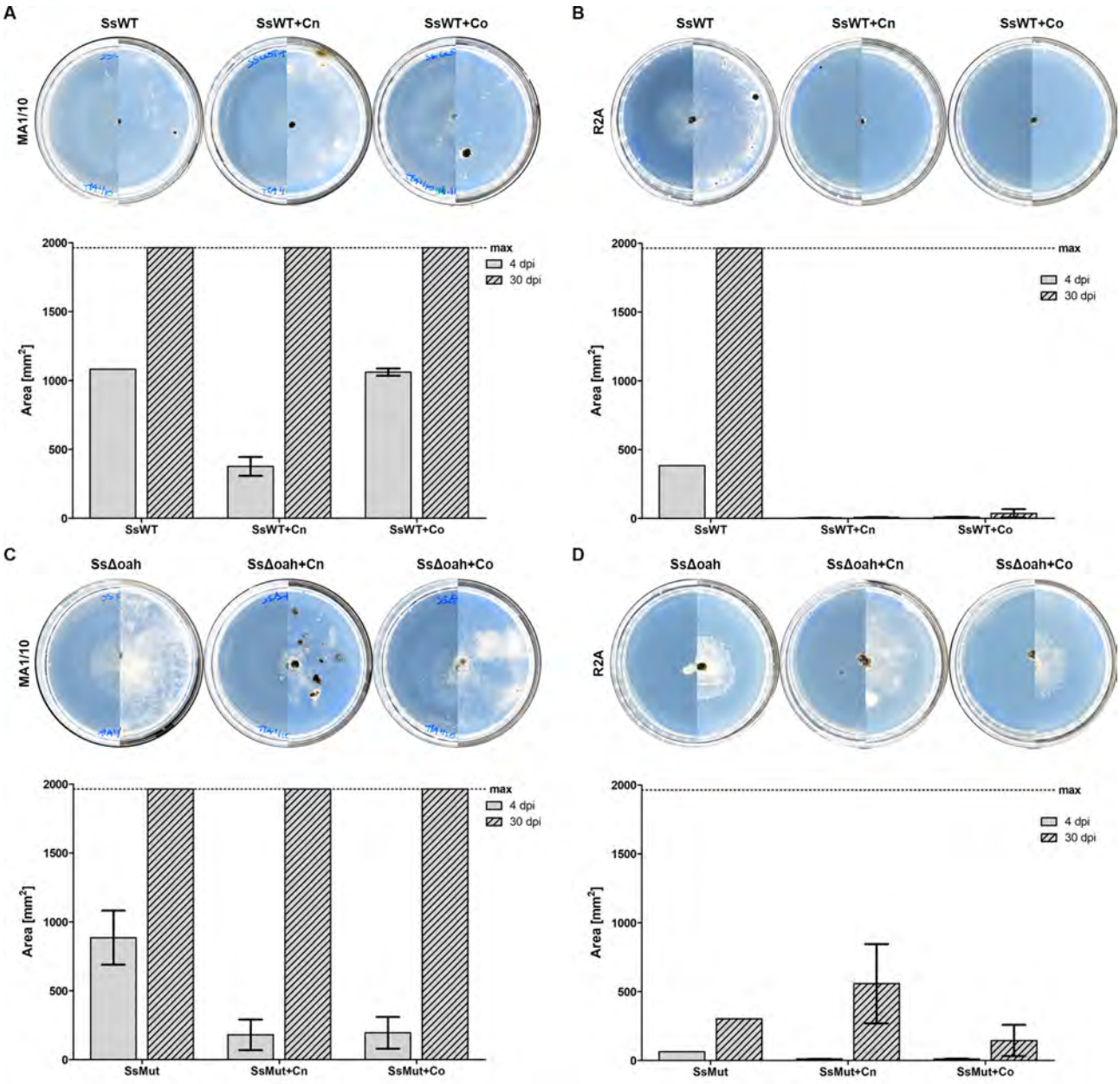


Figure 2.2. Soft agar confrontation assays between Sclerotinia sclerotiorum wild-type (WT), Cupriavidus necator and Cupriavidus oxalaticus on MA1/10 (A) and R2A (B); and with the non-oxalogenic S. sclerotiorum Δoah mutant, C. necator and C. oxalaticus on MA1/10 (C) and R2A (D). A-B*. From left to right: S. sclerotiorum WT (SsWT), S. sclerotiorum WT and C. necator (SsWT + Cn); S. sclerotiorum WT and C. oxalaticus (SsWT + Co).* ***C-D****. From left to right: S. sclerotiorum Δoah (SsΔoah), S. sclerotiorum Δoah and C. necator (SsΔoah + Cn); S. sclerotiorum Δoah and C. oxalaticus (SsΔoah + Co). The left half picture was taken after four days of incubation and the right half after 30 days of incubation. The area of mycelial growth was measured with ImageJ and plotted with the mean and the standard error of the mean in triplicates. After four days, the fungal growth area in presence of C. necator or C. oxalaticus was significantly different from the control (p-values < 0,05) for both fungi on both media, while after 30 days, fungal growth was only significantly different on R2A (p-values < 0,05). The dotted line indicates the maximum area of the plate.*

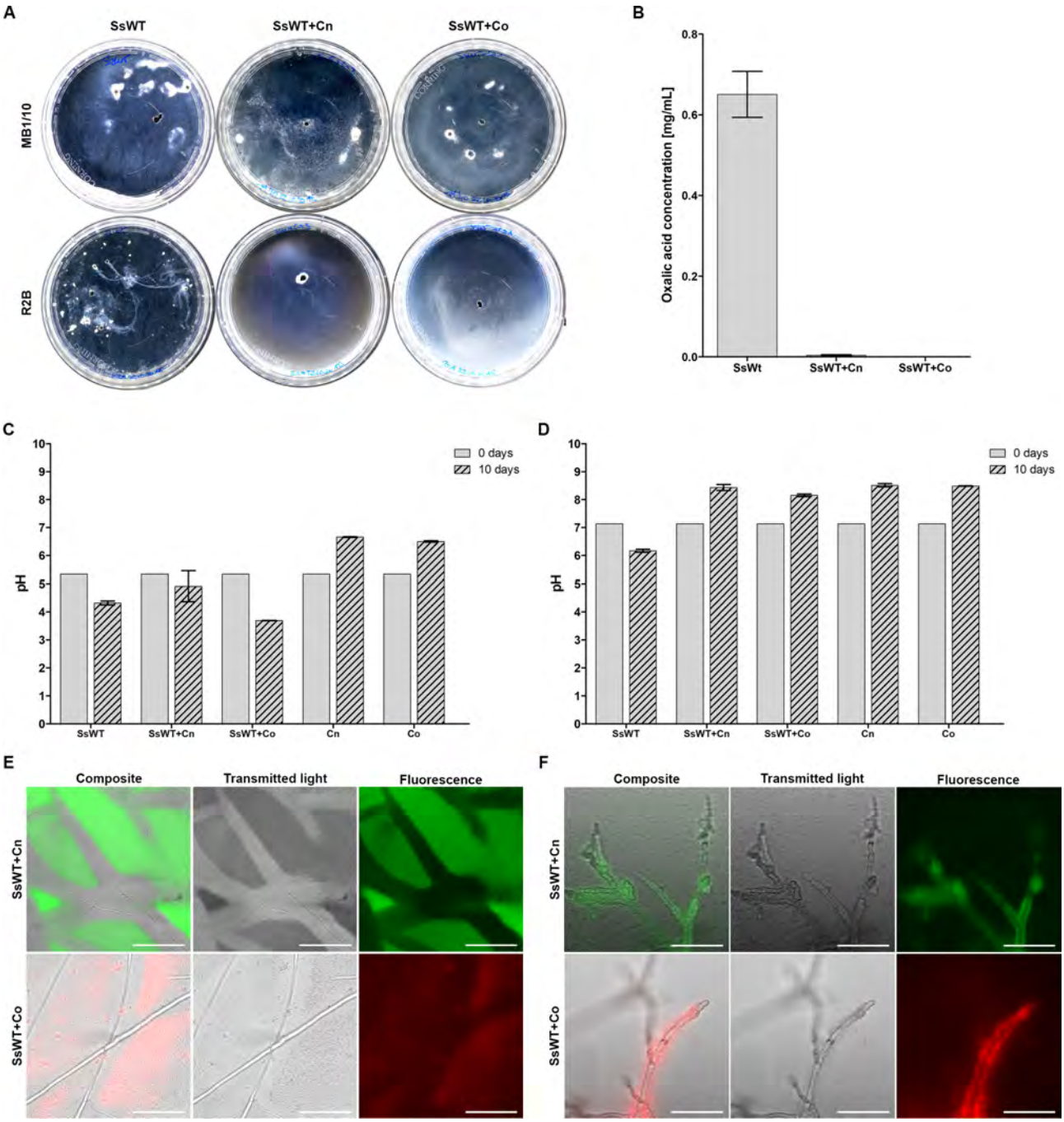


Figure 2.3. Confrontation assays in cell culture Petri dishes with S. sclerotiorum WT. A*. Images illustrating the growth on malt broth (MB1/10) on top and on Reasoner's 2 broth (R2B) on the bottom; from left to right: S. sclerotiorum WT (SsWT), S. sclerotiorum WT and C. necator (SsWT + Cn); S. sclerotiorum WT and C. oxalaticus (SsWT + Co).* ***B****. Corresponding oxalic acid detection in R2B.* ***C-D****. Initial and final pH measures in MB1/10 (****C****) and R2B (****D****).* ***E-F****. Inverted microscopy images in MB1/10 (****E****) and R2B (F; 100 µm scale bar). For oxalic acid concentrations (B), the mean of three independent measures was plotted with the standard error of the mean. The oxalic acid concentration in presence of C. necator and C. oxalaticus was significantly different from the control (p-values < 0,05). For pH measures (****C-D****), the mean of three independent measures taken at the beginning of the experiment and after 10 days was plotted with the mean and the standard error of the mean. After 10 days, the pH was significantly different from the initial pH in all cases except for S. sclerotiorum WT in presence of C. necator in MB1/10 (****C****). The images acquired from the plates showed that C. necator and C. oxalaticus are alive after 10 days in both MB1/10 and R2B media. The bacteria were found in large clumps away from the hyphae in MB1/10 (****E****), while they were closely associated to S. sclerotiorum WT mycelium in R2B (****F****).*

In MB1/10, the pH changed from 5,3 to 4,3 while in R2B, it changed from 7,1 to 6,2 (Fig. 2.3C-D). However, this effect on pH was drastically different in presence of the oxalotrophic bacteria in the two media. The final pH of the confrontation assays between *S. sclerotiorum* WT and the oxalotrophic bacteria in MB1/10 was significantly different from the initial pH, but not from the final pH of *S. sclerotiorum* WT single cultures (Fig. 2.3C). On the other hand, the final pH of the confrontation assays in R2B was significantly more alkaline than the initial pH or the pH of the control with fungi alone (Fig. 2.3D). Interestingly, *C. necator* and *C. oxalaticus* significantly alkalinised both media in absence of *S. sclerotiorum* WT (Fig. 2.3C-D).

Bacterial viability was assessed by inverted microscopy on the plates (Fig. 2.3E-F). *C. necator* and *C. oxalaticus* were viable in all cases but showed a different behaviour depending of the medium used. In MB1/10, both bacteria were found in clumps neatly organised away from *S. sclerotiorum* WT mycelium (Fig. 2.3E), while in R2B, the bacteria were closely associated to the hyphae (Fig. 2.3F). *S. sclerotiorum* WT mycelium also exhibited a different phenotype in R2B than in MB1/10 (Fig. 2.3E-F). Fungal cells were enlarged and distorted in R2B while on MB1/10, the hyphae were long and thin.

The same experiments were performed with the fungal mutant deficient in oxalic acid. Sclerotia of *S. sclerotiorum* Δ*oah* germinated and grew in both media in absence of bacteria (Fig. 2.4A). As expected, no oxalic acid was detected in *S. sclerotiorum* Δ*oah* cultures. However, *S. sclerotiorum* Δ*oah* still significantly acidified the medium when cultured in MB1/10, both in presence and in absence of oxalotrophic bacteria (Fig. 2.4B). On the other hand, *S. sclerotiorum* Δ*oah* did not acidify the R2B medium either alone or in presence of *C. necator* and *C. oxalaticus* (Fig. 2.4C). The pH of *S. sclerotiorum* Δ*oah* single cultures in R2B was slightly more alkaline than the initial pH, while it significantly changed from pH 7,1 to pH 8,4 in presence of the oxalotrophic bacteria. As with *S. sclerotiorum* WT, both bacteria were alive after 10 days and showed a similar behavioural pattern of interaction than the one observed with the wild-type fungus (Fig. 2.4D-E).

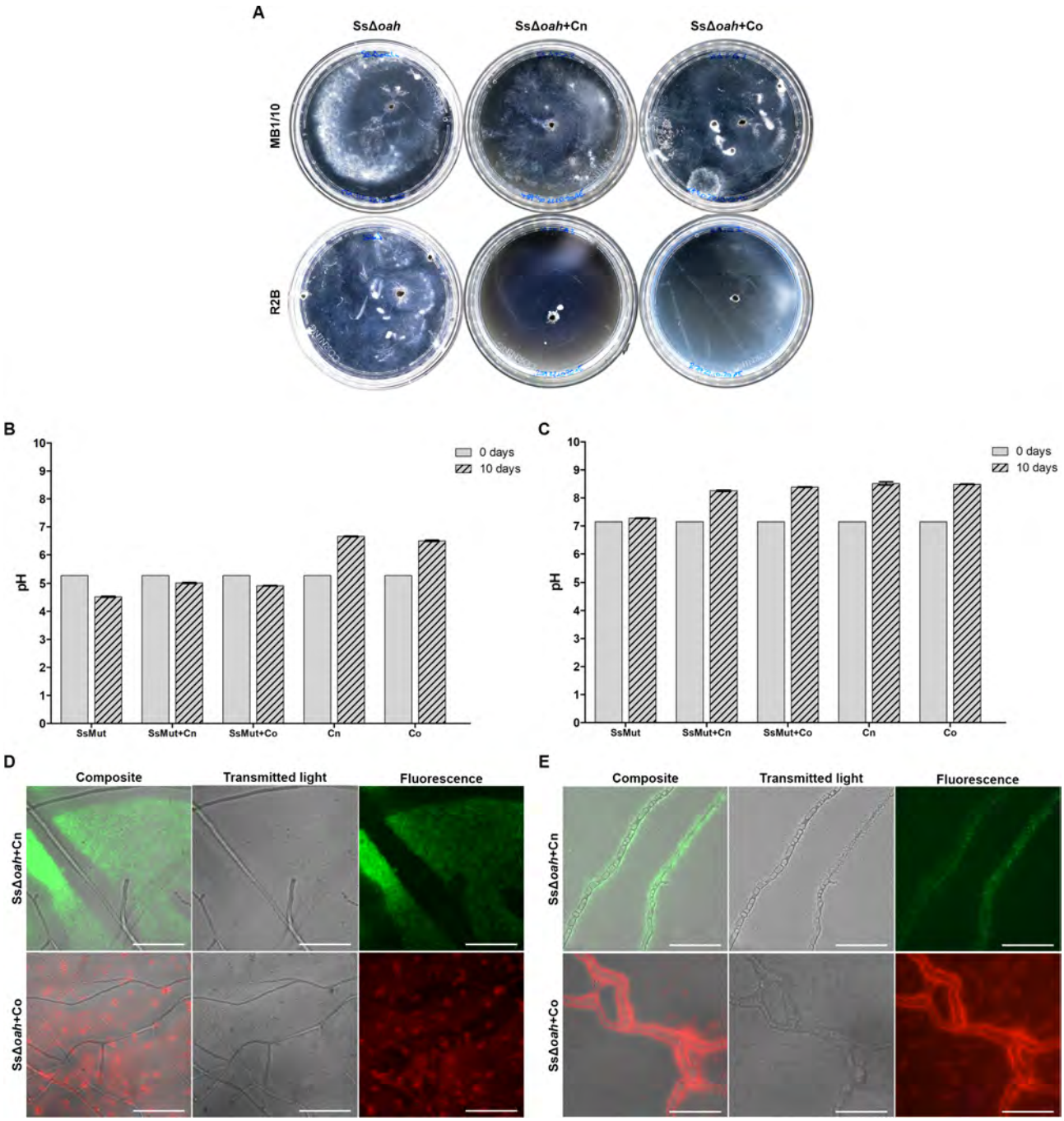


Figure 2.4. Confrontation assays in cell culture Petri dishes with S. sclerotiorum Δoah. A*. Images illustrating the growth on malt broth (MB1/10) on top and on Reasoner's 2 broth (R2B) on the bottom; from left to right: S. sclerotiorum Δoah (SsΔoah), S. sclerotiorum Δoah and C. necator (SsΔoah + Cn); S. sclerotiorum Δoah and C. oxalaticus (SsΔoah + Co).* ***B-C****. Initial and final pH measures in MB1/10 (****C****) and R2B (****D****).* ***D-E****. Inverted microscopy images in MB1/10 (****E****) and R2B (F; 100 µm scale bar). For pH measures (****B-C****), the mean of three independent measures taken at the beginning of the experiment and after 10 days was plotted with the mean and the standard error of the mean. After 10 days, the pH was significantly different from the initial pH in all cases. The images acquired from the plates show that C. necator and C. oxalaticus are alive after 10 days in both MB1/10 and R2B media. The bacteria were found in large clumps away from the hyphae in MB1/10 (****E****), while they were closely associated to S. sclerotiorum Δoah mycelium in R2B (****F****).*

Discussion

In order to assess the effect of oxalic acid consumption on fungal growth, the biocontrol effect of *C. necator* and *C. oxalaticus* on *S. sclerotiorum* WT and its oxalic-acid deficient mutant was monitored on two different media. In addition, as the production and consumption of oxalic acid is expected to have an impact on pH, this parameter was also monitored. Two different media were used to investigate the interaction: MA1/10, commonly used for fungal cultures, and R2A, developed to isolate bacteria from water samples. MA1/10 is a diluted version of malt agar medium, and is mainly composed of oligosaccharides and amino acids. On the other hand, R2A has various sources of carbon and amino acids, as well as potassium and magnesium sources (Reasoner & Geldreich, 1985). As both media have a very different composition, they supported oxalic acid and other LMWOA production and growth differently. The phytopathogen *S. sclerotiorum* grew faster on MA1/10 than on R2A, while the oxalotrophic bacteria showed a clear growth preference in R2A compared to MA1/10. *C. necator* and *C. oxalaticus* were able to control *S. sclerotiorum* WT only on R2A and oxalic acid was detected only in this medium.

Even though oxalic acid was detected only in R2B, both the wild-type and mutant fungi were able to acidify MB1/10. The fungus *S. sclerotiorum* is known to produce not only oxalic acid but also other LMWOA, such as succinic, malic or fumaric acids (Vega *et al.*, 1970; Corsini *et al.*, 1973). Most LMWOA are produced through the same three pathways, namely the glyoxylate pathway, the reductive and the oxidative tricarboxylic acids cycles (TCA) (Corsini *et al.*, 1973; Xu *et al.*, 2012; Martin-Dominguez *et al.*, 2022). However, most studies seem to agree that these acids are produced mainly by a branch of the reductive TCA, the oxaloacetate pathway (Kenealy *et al.*, 1986; Martin-Dominguez *et al.*, 2022). After glycolysis, pyruvate or phosphoenol-pyruvate is converted in oxaloacetate by the addition of CO_2 and the use of an ATP (Kenealy *et al.*, 1986; Martin-Dominguez *et al.*, 2022). Oxaloacetate can then be converted in oxalate and acetate, or in malate and then further processed in fumarate and succinate, or in citrate, to cite only a few possibilities (Kenealy *et al.*, 1986; Palmieri *et al.*, 2018; Martin-Dominguez *et al.*, 2022). Media composition plays a major role in the production of the different LMWOA as the medium as a direct impact on the efficacy of the enzymes involved in these pathways (Romano *et al.*, 1967; Corsini *et al.*, 1973; Gharieb & Gadd, 1999). It has been shown, for example, that oxalic acid production is stimulated in media with a high nitrogen content while fumaric acid production increase in nitrogen limiting conditions (Kenealy *et al.*, 1986; Gharieb & Gadd, 1999). This can explain the results obtained here, *S. sclerotiorum* WT produced oxalic acid only in the medium with a high nitrogen content (R2B), which indicates that MB1/10 is not a suitable medium to study oxalic acid production/consumption as part of a biocontrol strategy. However, as no other LWMOA were detected by HPLC, it is difficult to determine which LMWOA is responsible for the acidification in MB1/10 for both the wild-type and the mutant fungi, and to pin-point the exact reason why one LMWOA is produced preferentially compared to another. Interestingly, *S. sclerotiorum* Δ*oah* cannot produce oxalic

acid as it lacks one of the key genes required in the biosynthetic pathway, however this strain was not able to produce other LMWOA on R2B and therefore to acidify this particular medium. This demonstrates the importance of the medium composition on the metabolism of the fungus, the abiotic factors determine the LMWOA produced.

As the biocontrol strategy evaluated here was hypothesized to be mediated through oxalic acid consumption, the lack of oxalic acid production in MB1/10 medium explains the lack of biocontrol in this particular medium. As no oxalic acid was detected in MB1/10, the oxalotrophic bacteria would not able to raise the pH in confrontation assays, as the bacterial consumption of oxalic acid and the subsequent carbonic acid production are the key factors promoting alkalinisation. However, the alkalinisation of both media when *C. necator* and *C. oxalaticus* were cultured alone indicates that oxalotrophy is not the only mechanism of alkalinisation in place. In fact, amino acid catabolism, peptone degradation or different oxidative bacterial metabolisms result in the generation of molecules that can alkalinize the medium, and in particular, ammonia (Hugh & Leifson, 1953; Makkar & Casida, 1987; Farrell & Finkel, 2003; Madigan *et al.*, 2012). Ammonia production triggers an acute alkalinisation of the medium, which explain the change in pH by *C. necator* and *C. oxalaticus* cultures in both media. However, as ammonia is a weak base, it is not able to counteract the constant acidification of the medium by *S. sclerotiorum* when in confrontation in MB1/10. On the other hand, the combined action of oxalic acid consumption and ammonia production on R2B promote the strong alkalinisation of the medium. Interestingly, *S. sclerotiorum* Δ*oah* was also controlled on R2B. As no oxalic acid was detected in the mutant's spent medium, oxalic acid is clearly not the sole element involved in the biocontrol strategy investigated here.

The pH itself is an important element to be considered. Previous studies in oxalotrophy as a biocontrol strategy did not investigate the effect of pH on the fitness of the fungus, but showed that the control occurred from a combination of mechanisms (Dickman & Chet, 1998; Schoonbeek *et al.*, 2007; Marina *et al.*, 2019). *S. sclerotiorum* WT is known to acidify the environment to promote the production and activity of its enzymes and increase disease incidence (Magro *et al.*, 1984; Guimarães & Stotz, 2004; Hegedus & Rimmer, 2005). Other oxalogenic pathogenic fungi, such as *Aspergillus niger*, exhibit a wide array of mechanisms to maintain pH homeostasis in acidic environments (Hesse *et al.*, 2002). These fungal pathogens are heavily armed to survive in acidic conditions, while alkaline pH seems to exert an intense stress on them (Hesse *et al.*, 2002). *S. sclerotiorum* showed an acute response to alkaline pH, with a reduced growth and different hyphae morphology, possibly due to a disrupted membrane integrity. Indeed, alkaline pH is known to trigger changes in the membrane composition, as it plays a major role in pH homeostasis (Brown *et al.*, 2020). The production of glycosphingolipids and ergosterol in particular, ensure the formation of lipid rafts and the fluidity of the membrane (Brown *et al.*, 2020). These membrane changes are necessary to maintain on optimal cellular metabolism and defects in the sterol homeostasis pathway result in the inability of the fungi to grow in alkaline conditions (Brown *et al.*, 2020). Here, the

deformed hyphae of *S. sclerotiorum* in alkaline conditions could indicate a defective signalling in the membrane homeostasis, resulting in misshaped hyphae and reduced growth. In this case, the importance of the consumption of oxalic acid is lesser given the damages caused by the alkaline pH of the medium.

The initial pH of the medium also plays a role in the growth rate of the pathogenic fungus. As an acidic pH is required for the production and activity of the fungal enzymes, an initial acidic pH is beneficial for *S. sclerotiorum*, and its growth is impaired at an alkaline pH (Xu *et al.*, 2015, 2018). Both the mutant and the wild-type fungus grew faster on MA1/10 than on R2A. Since the initial pH of R2A is less favourable for *S. sclerotiorum*, its growth rate is reduced compared to MA1/10, where the initial pH is already in a range that is more favourable to its growth. On MA1/10 (pH 5,3), *S. sclerotiorum* does not need to produce oxalic acid but is able to simultaneously grow and acidify the medium, thus using the nutritional resources in a more optimal way. *C. necator* and *C. oxalaticus* metabolism and the subsequent alkalinisation of the medium are not sufficient to tip over the pH from an acidic to an alkaline one (Fig. 2.5A). In these conditions, *S. sclerotiorum* is therefore more successful than the oxalotrophic bacteria and is not controlled. On the other hand, on R2A (pH 7,1), *S. sclerotiorum* is in already in a less favourable environment and needs first to acidify the medium. Thus, its growth is significantly slowed down and the oxalic acid produced is rapidly consumed by the oxalotrophic bacteria. As *S. sclerotiorum* is unable to produce another LMWOA in this medium, the combination of oxalic acid consumption and the oxidative bacterial metabolism induce a constant alkalinisation of the medium. In these conditions, *C. necator* and *C. oxalaticus* are more successful than *S. sclerotiorum* and can therefore control its growth (Fig. 2.5B).

Conclusion

Although oxalic acid consumption was initially proposed as the basis of the biocontrol strategy of oxalogenic fungi tested here, we demonstrated that the subsequent alkalinisation of the medium is in fact responsible for fungal growth control. The use of different media, as well as a fungus deficient in oxalic acid production allowed us to disentangle the effect of the consumption of oxalic acid from that of the pH change in the control of fungal growth. Regardless of the driver of alkalinisation (i.e., oxalic acid consumption or bacterial metabolism), the pH change was ultimately responsible for the growth control of *S. sclerotiorum*.

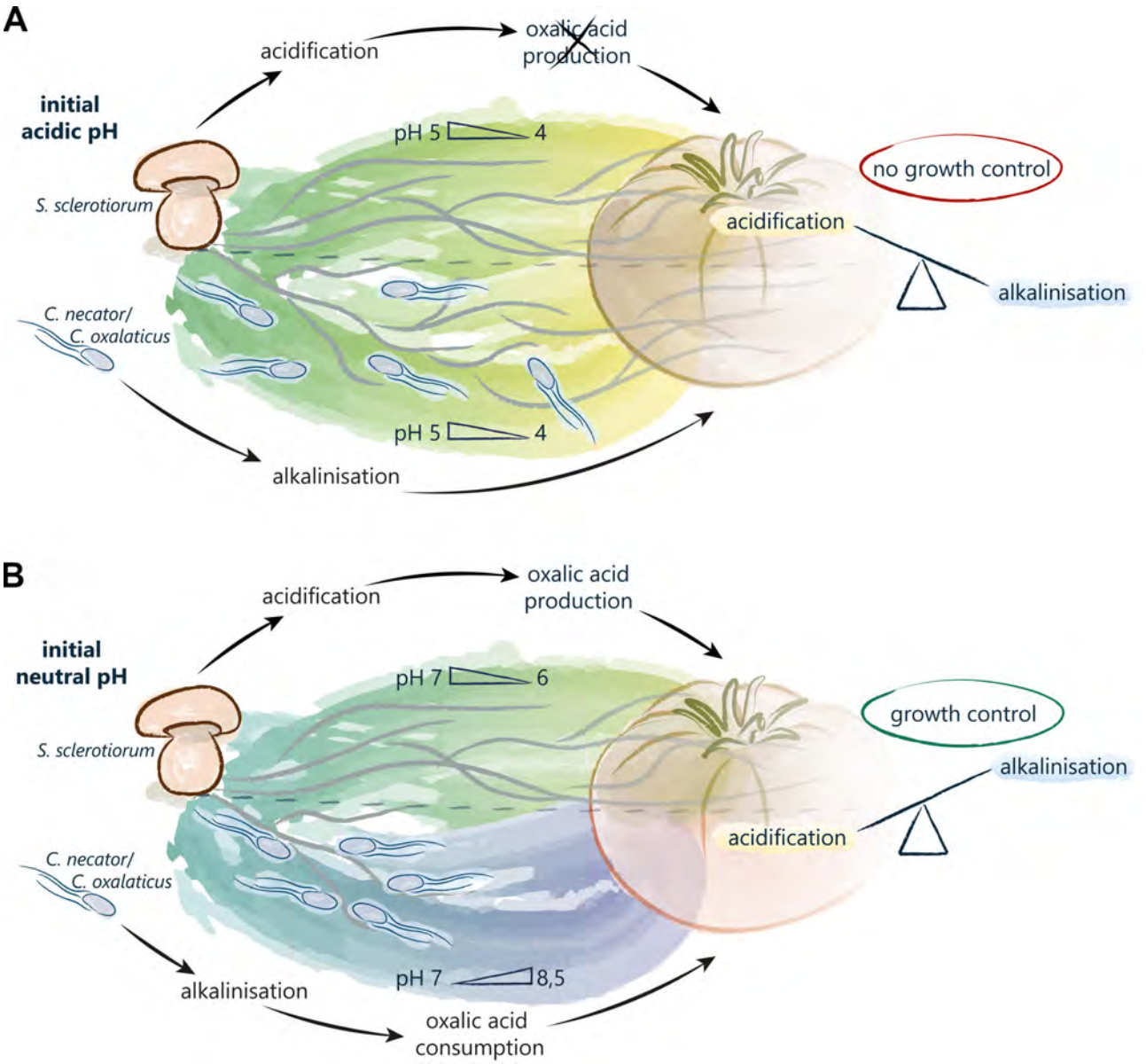


Figure 2.5. Schematic representation of the relation between the pH and the fungal growth control by the oxalotrophic bacteria when the initial pH is acidic (A) or neutral (B). *With an acidic initial pH (**A**), S. sclerotiorum acidify the medium but does not produce oxalic acid. Oxalotrophic bacteria metabolism is not sufficient to alkalinize the medium, therefore the fungal growth is not controlled. When the initial pH is neutral (**B**), S. sclerotiorum produces oxalic acid to acidify the medium. The combined action of oxalic acid consumption and bacterial metabolism promote the alkalinisation of the medium and control the fungal growth.*

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Chapter 3

Chemotactic signals regulate antagonistic interactions between *Sclerotinia sclerotiorum* and *Cupriavidus* strains

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Abstract

The sensing of chemical signals in their environment affects the behavioural response of microorganisms. Changes in the production of these chemical signals might not by the result of the metabolism of individual organism, but also of multiple interacting species. In this study, the interactions between *Sclerotinia sclerotiorum*, a soil-borne phytopathogenic fungus, and two soil oxalotrophic bacteria, *Cupriavidus necator* and *Cupriavidus oxalaticus* were investigated on two media. Different chemical stimuli were shown to influence the movement of these motile bacteria towards the fungus. In confrontation assays in Reasoner'2 broth, the bacteria were attracted to the fungus, while on diluted malt agar, *C. necator* and *C. oxalaticus* were repulsed by the fungus. Further experimentations showed that the repulsive metabolite produced by the fungus in diluted malt agar binds to poly-l-lysine and has antibacterial properties.

Introduction

Microorganisms exhibit different behaviours depending on their environment. One of the mechanisms by which bacteria sense the signalling molecule and react to it is chemotaxis. Chemical stimuli are known to affect the overall directionality of the movement of motile organisms to find a nutrient source or to avoid a potential danger. Chemotaxis relies on metabolites such as low molecular weight organic acids (LMWOA), amino acids or antibiotics, to modulate bacterial movement in response to environmental cues (Webre *et al.*, 2003; Madigan *et al.*, 2012; Deveau *et al.*, 2018). Oxalic acid, a LMWOA commonly found in nature, is widely used as an attractant chemotactic signal in bacteria (Frey-Klett *et al.*, 2011; Kost *et al.*, 2013; Rudnick *et al.*, 2015; Deveau *et al.*, 2018). Plant-associated *Burkholdeira* species use oxalic acid to localise their plant host, and the successful colonisation of lupin and maize plants is positively correlated with the oxalotrophic capacity of the beneficial *Burkholderia* strains (Kost *et al.*, 2013). Mutants deficient in oxalotrophy (Δ*oxc*) showed a reduced capacity to colonise the roots of their plant host, indicating that chemotaxis was effectively driven by oxalic acid (Kost *et al.*, 2013).

Oxalic acid is not only used to recruit beneficial bacteria and can also be used by mycophagous bacteria to localise their fungal prey (Rudnick *et al.*, 2015). For *Collimonas fungivorans* Ter331, a non-oxalotrophic mycophagous bacterium, fungal production of oxalic acid allows it to localise the hyphal tips (Rudnick *et al.*, 2015). Upon attachment to the hyphae, the bacteria consume cellular material from the fungal host and transform the fungal biomass into bacterial biomass. Fungal hyphae are also known to represent a hot-spot of nutrients for those bacteria able to colonise them (Leveau & Preston, 2008; Haq *et al.*, 2016). Upon attachment to the hyphae, bacteria thrive on fungal exudates (Frey-Klett *et al.*, 2007; Leveau & Preston, 2008; Haq *et al.*, 2016). For instance, *Burkholderia terrea* BS001 localises its fungal partner *Lyophyllum* sp. strain Karsten using chemotaxis driven by glycerol and oxalic acid produced by the fungus (Haq *et al.*, 2016). Upon adherence to the mycelium, *B. terrea* BS001 consumes the glycerol released by the fungus and converts it into bacterial biomass. Some chemical stimuli can also act either as attractant or repellent, depending on the bacteria sensing them. For instance, *Escherichia coli* uses amino acids as carbon and energy sources and is positively attracted by several different amino acids (Yang *et al.*, 2015). On the other hand, *Bacillus subtilis* only uses amino acids only as environmental cues to localise roots of a host plant. Certain amino acids such as valine or leucine, known for their antibacterial properties, act as chemorepellent and elicit a strong repulsive response in *E. coli*, while *B. subtilis* is positively attracted to them (Yang *et al.*, 2015).

In the Chapter 2, we observed that the interactions between the phytopathogenic fungi *Sclerotinia sclerotiorum* and two oxalotrophic bacteria, *Cupriavidus necator* and *Cupriavidus oxalaticus*, in cell culture Petri dishes coated with poly-l-lysine drastically changed depending on the medium used (Fig. 3.1). In diluted malt broth (MB1/10), bacteria were found in clumps

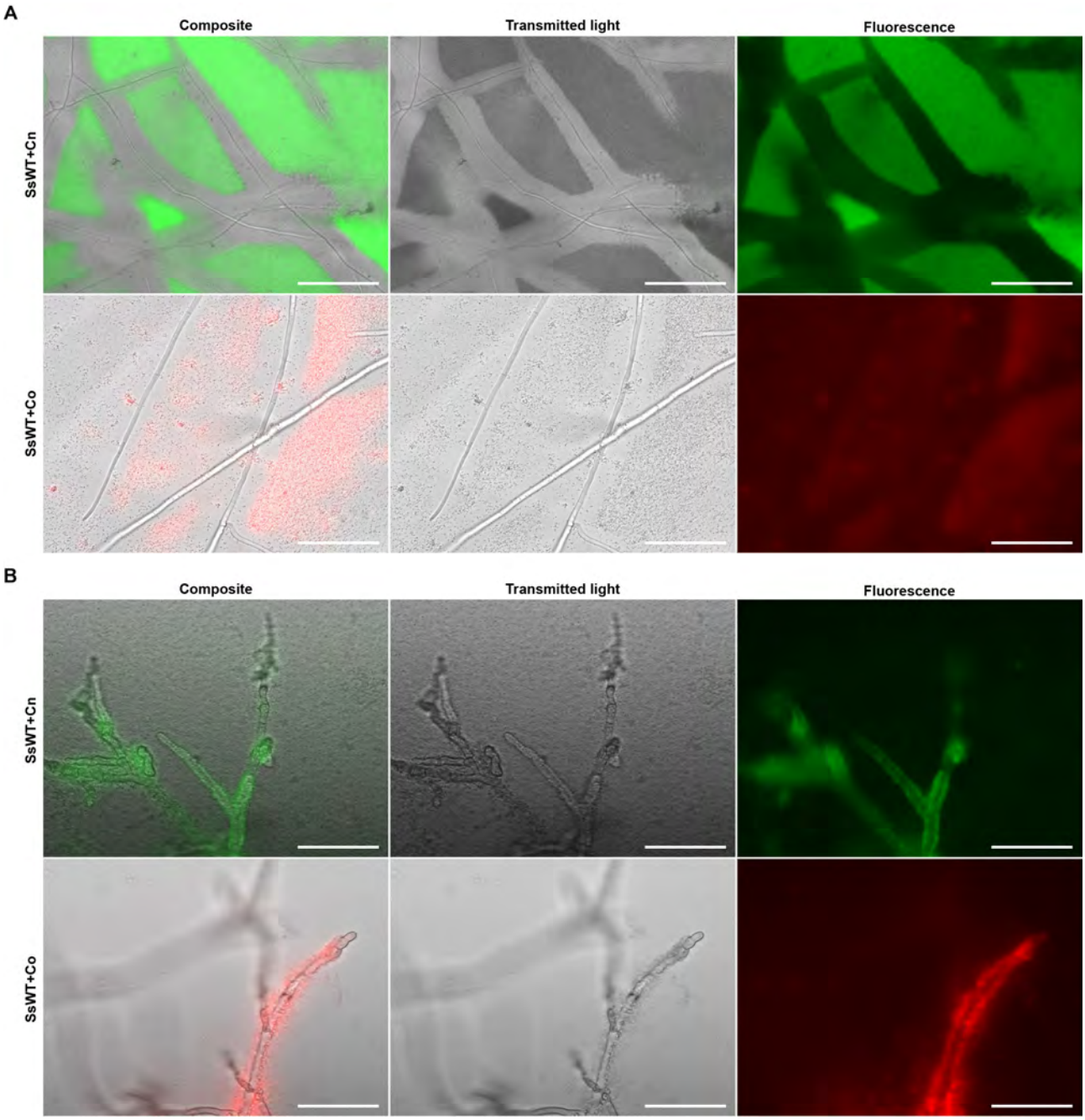


Figure 3.1. Confrontation assays. *Confrontation assays in MB1/10 (**A**) and R2B (**B**) assays of the oxalotrophic bacteria C. necator or C. oxalaticus and the oxalogenic fungus S. sclerotiorum. Inverted microscopy images in MB1/10 (**A**) and R2B (**B**; 100 µm scale bar) on the plates showed that C. necator and C. oxalaticus had a different behaviour towards the fungus depending on the medium. The bacteria were found in large clumps away from the hyphae in MB1/10 (**A**), while they were closely associated to S. sclerotiorum WT mycelium in R2B (**B**). Mycelium showed a different morphology in both media, the hyphae were long and thin in MB1/10 (**A**) while they were short and enlarged in R2B (**B**).*

at a regular distance from the hyphae and *S. sclerotiorum* growth was not controlled by the bacteria (Fig. 3.1A). On the other hand, *S. sclerotiorum* growth was controlled in Reasoner'2 broth (R2B) by these two oxalotrophic bacteria, and bacteria were closely associated to the hyphae (Fig. 3.1B). Therefore, as the bacteria seemed to be repulsed (in MB1/10) or attracted (in R2B) to *S. sclerotiorum* mycelium, we investigated those two chemotactic behaviours using spent media from single fungal cultures and confrontation cultures using 3D printed bridges

initially developed to evaluate bacterial dispersal along hydrated surfaces (Kuhn *et al.*, 2022). Here, we investigated their potential to study bacterial movement in presence of attractive or repulsive metabolites produced by fungi. The precise growth pattern of the cells in MB1/10 seemed to indicate a possible interaction between the putative repulsive metabolite and the poly-l-lysine coating the bottom of the plate. Therefore, we also investigated the potential of the putative repulsive metabolite to bind to poly-l-lysine using endotoxin removal columns containing beads coated with ε-poly-l-lysine (Thermofisher™) as molecules such as lipopolysaccharides (LPS: endotoxins). are known to have an high affinity for poly-L-lysine.

Material & methods

Fungal and bacterial cultures

S. sclerotiorum WT (Li *et al.*, 2018) was kindly provided by Jeffrey Rollins from the Department of Plant Pathology, University of Florida. The fungus was conserved as sclerotia in a sealed desiccator at room temperature. For fresh cultures, a single sclerotia was deposited in the middle of potato dextrose agar plates (PDA; 5 g/L potato infusion, 20 g/L dextrose, 17 g/L agar, pH 5,6 ± 0,2) and inoculated at room temperature for a week.

C. necator-GFP (Poehlein *et al.*, 2011) and *C. oxalaticus*-mCherry (Şahin *et al.*, 2000) were obtain from the bacterial collection of the Laboratory of Microbiology, University of Neuchâtel, Switzerland. Both strains carried a fluorescent tag (respectively GFP and mCherry), but the suffixes were omitted in the text in order to improve readability. Pure cultures were conserved in 30% glycerol at -80°C and fresh bacterial suspensions were produced as described in Estoppey et al. (2022).

Liquid cultures, confrontations and spent media

Confrontation assays and single fungal cultures in cell culture plates were performed as described previously (Estoppey *et al.*, 2022). For the fungal inoculum, agar plugs (5 mm Ø) were taken from fresh *S. sclerotiorum* cultures on PDA in the actively growing mycelium, and deposited in the middle on the cell culture plate. Cultures were done in diluted malt broth (MB1/10; 1,2 g/L malt extract) and in Reasoner'2 broth (R2B; 0,5 g/L yeast extract; 0,5 g/L peptone; 0,5 g/L casamino acids; 0,5 g/L glucose; 0,5 g/L soluble starch, 0,3 g/L Na-pyruvate; 0,3 g/L K_2HPO_4; 0,05 g/L $MgSO_4 \cdot 7H_2O$, in Milli-Q® water; (Reasoner & Geldreich, 1985)).

After 10 days of incubation at 25°C, the growth patterns were recorded by inverted microscopy performed directly on the plates. As the red fluorescence from the mCherry tag was more difficult to detect than the green fluorescence of the GFP, the following experiments were only performed with *C. necator*. Medium was then harvested from the plates and filtrated at 0,22 µm with nitrocellulose filters (MF-Millipore, ref. GSWP04700) to remove all microorganisms. Filtrations were performed in sterile filtration stations, in order to guaranty the sterility of the

spent media. Spent media were pooled by condition and conserved at 4°C until further use. Four different spent media were harvested, *S. sclerotiorum* in MB1/10, *S. sclerotiorum* with *C. necator* in MB1/10, *S. sclerotiorum* in R2B and *S. sclerotiorum* with *C. necator* in R2B.

Timelapse movies recording

To follow the interaction of *S. sclerotiorum* with *C. necator* in MB1/10 overtime, a timelapse in cell culture plate was recorded with an inverted microscope at magnification 20x. After two days of incubation, images were acquired every three minutes for 24 h. For the interaction in R2B, *S. sclerotiorum* and *C. necator* were inoculated in a BFI device filled with R2B (Stanley *et al.*, 2014). The BFI device was made of a polydimethylsiloxane (PDMS) part with microchannels and two inoculations sites (one for the fungal inoculum and one for the bacterial inoculum) sealed to a glass-bottom Petri dish. Sealing was performed using oxygen plasma treatment and the device was filled with R2B medium immediatly after sealing. The microchannels force the hyphea to grow in a constricted environement, which reduce the number of hyphea in each channel and allow the observation of cell-to-cell interactions with bacteria. *S. sclerotiorum* was inoculated first on the fungal side of the device and after 3 days of incubation, 10 µl of *C. necator* suspension was added to device trough the bacterial inoculation site. Images were acquired at magnification 20x every 30 seconds for 12 h. In both cases, images were acquired in transmitted light and in green fluorescence, to have a better visualisation of the bacterial cells. Inverted microscopy images were acquired with the EVOS M5000 microscope and processed with ImageJ 2.0.0-rc65/1.51w to produce composite images and timelapses.

Chemotaxis experiments using 3D printed bridges

Chemotaxis experiments were performed with the 3D printed bridges presented in Kuhn et al. (2022). Bridges were autoclaved in a water-filled container before use, and manipulated with sterile tweezers during the experiment. Each bridge was put across two wells in a 24-wells plate (Costar©, ref. 3526), defined then as "start" and "end" wells. Bacteria were always inoculated in the start well. Prior to placing the bridge, each well was filled with 2,75 ml of the respective medium containing 1% Ficoll (Roth, ref. CN90.3). Then, 5 µl of fresh *C. necator* suspension (optical density (OD) = 1 at 600 nm, corresponding to 10^5 bacteria/µl) was added to the start well, and finally the bridge was put across the start and end wells (Fig. 3.2).

For the experiments using MB1/10 spent media, the controls wells were filled with MB1/10 and the same apply for spent media from confrontations in R2B. For repulsive chemotaxis experiments, start wells were filled with spent medium from fungal cultures or confrontation assays, and the end wells were filled with fresh MB1/10. For attractive chemotaxis experiments, start wells were filled with fresh R2B and end wells were filled with spent media. For the controls, start and end wells were filled with fresh medium. Each condition was tested in triplicate. After inoculation, plates were incubated in the dark for 48 h at room temperature. After 48 h cells were counted by flow cytometry.

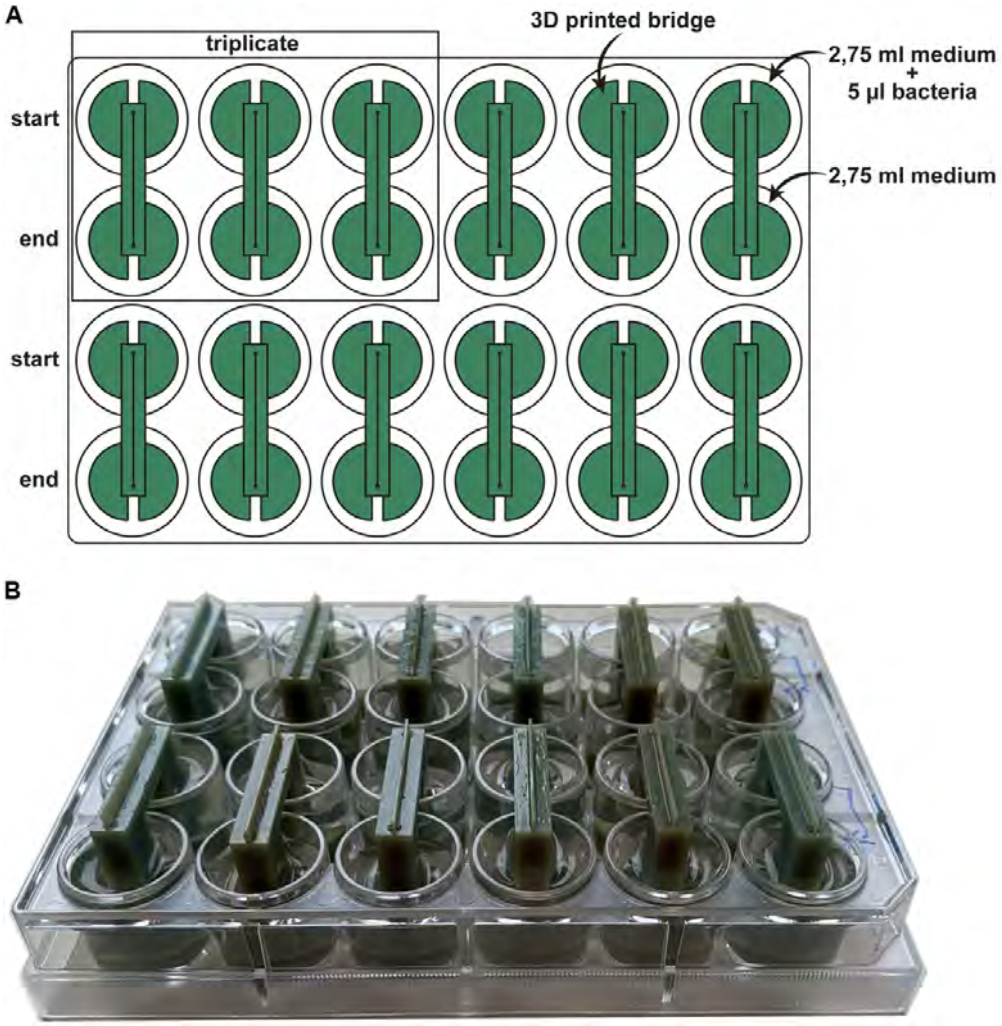


Figure 3.2. Schematic representation of the experimental design for the chemotaxis experiment using 3D printed bridges (A) and example of an inoculated plate (B). A. *Chemotaxis experiments were performed in 24-wells plates, which each well filled with 2,75 ml of spent or fresh medium. Bacteria were added to the start well before the bridge was put across the start and end wells. One plate allowed to tests up to four different conditions in triplicates.* ***B****. Example of an inoculated plate.*

Cells count by flow cytometry

After incubation, bridges were removed and cells were homogenized by repetitive up and down pipetting in the wells before cell counting by flow cytometry. In order to prevent false counts, samples were diluted with saline solution (0,9% NaCl in deionised water) and filtrated at 0,22 µm, to obtain a final rate of 500-1000 events/second at a flow rate of 1,4 µl/s. Cells counts were done with the BDAccuri™ C6 Plus flow cytometer. A 488 nm laser was used for excitation and GFP green fluorescence was collected on a 533/30 filter. In order to minimise the background noise, a threshold of 1000 was added to the side scatter (SSC) and of 3000 to the forward scatter (FSC) parameters. The bacterial population was gated on the SSC vs FSC plot, where the highest cell density was located. In order to analyse the alive and dead populations of *C. necator*, the FITC-A parameter (GFP) was plotted against FSC. The resulting plot was separated in four quadrants on the basis of GFP fluorescence and cell size. Cells in the upper right quadrant were positive for both fluorescence and size, while cells in the lower right quadrant were negative for fluorescence and positive for

the size. These two populations were considered as alive and dead populations. The same parameters were applied to all samples. The concentration of bacteria per µl for each pair of wells were plotted as floating bars with the median. A one-way ANOVA with a Tuckey post-test was performed to compare the statistical difference in the cell numbers for the different treatments. All graphs and statistics were done with GraphPad Prism 5.00.

Toxin-free spent media and concentrated toxin fraction

Pierce™ High-Capacity Endotoxin Removal Spin columns (Thermo Scientific™, ref: 88274) were used to separate the toxins from the spent media. Columns were regenerated as described in the providers' user guide with 0,2 N NaOH in 95% ethanol for 1 h at room temperature and the endotoxin-free buffer was 50 mM sodium phosphate buffer with 0,1 mM NaCl at pH 7. After regeneration, 4 ml of the spent media were incubated in the columns at room temperature for 1 h with gentle agitation (80 rpm). The toxin-free fraction was collected after centrifugation, transferred to a new sterile Falcon and conserved at 4°C. To collect the concentrated toxin fraction, 3,5 ml of 0,2 N NaOH in 95 % ethanol were added to the columns and incubated 1 h at room temperature. Concentrated toxin fractions were collected after centrifugation and transferred to new sterile Falcon. Columns were then regenerated for further use.

Growth curves

In order to assess the toxicity of the spent medium from MB1/10 cultures, growth curves were performed with *C. necator*. Growth was assessed in 96-wells plates (Costar©, ref. 3596), in eight replicates for each condition. The conditions were the following: fresh MB1/10; *S. sclerotiorum*; *S. sclerotiorum* + *C. necator*; *S. sclerotiorum* minus toxins; *S. sclerotiorum* + *C. necator* minus toxins. For each condition, 10 µl of *C. necator* suspension (OD 1) were added to 190 µl of medium. Growth was assessed with the OD measured at 600 nm every 30 min for 24 h with the Biotek Cytation 5 Imaging Reader. Shaking was applied for 5 sec before each measure. The mean of the OD was plotted against the time and a one-way ANOVA with a Tuckey post-test was performed to compare the statistical difference for the different treatments.

Results

BFI between *S. sclerotiorum* and *Cupriavidus* strains

The confrontation assay in cell culture Petri dishes in MB1/10 was observed overtime using a timelapse video (Fig. 3.3, Sup. Data 3.1), to better understand how the arrangement of the bacteria around the hyphae resulted in this very precise growth pattern of the bacterial cells. Microscopy images showed that bacteria seemed to move away when the tip of the hyphae approached and would then concentrate in areas in which hyphae were not observed. On the other hand, when the same bacteria were confronted with the fungus on R2B in a BFI

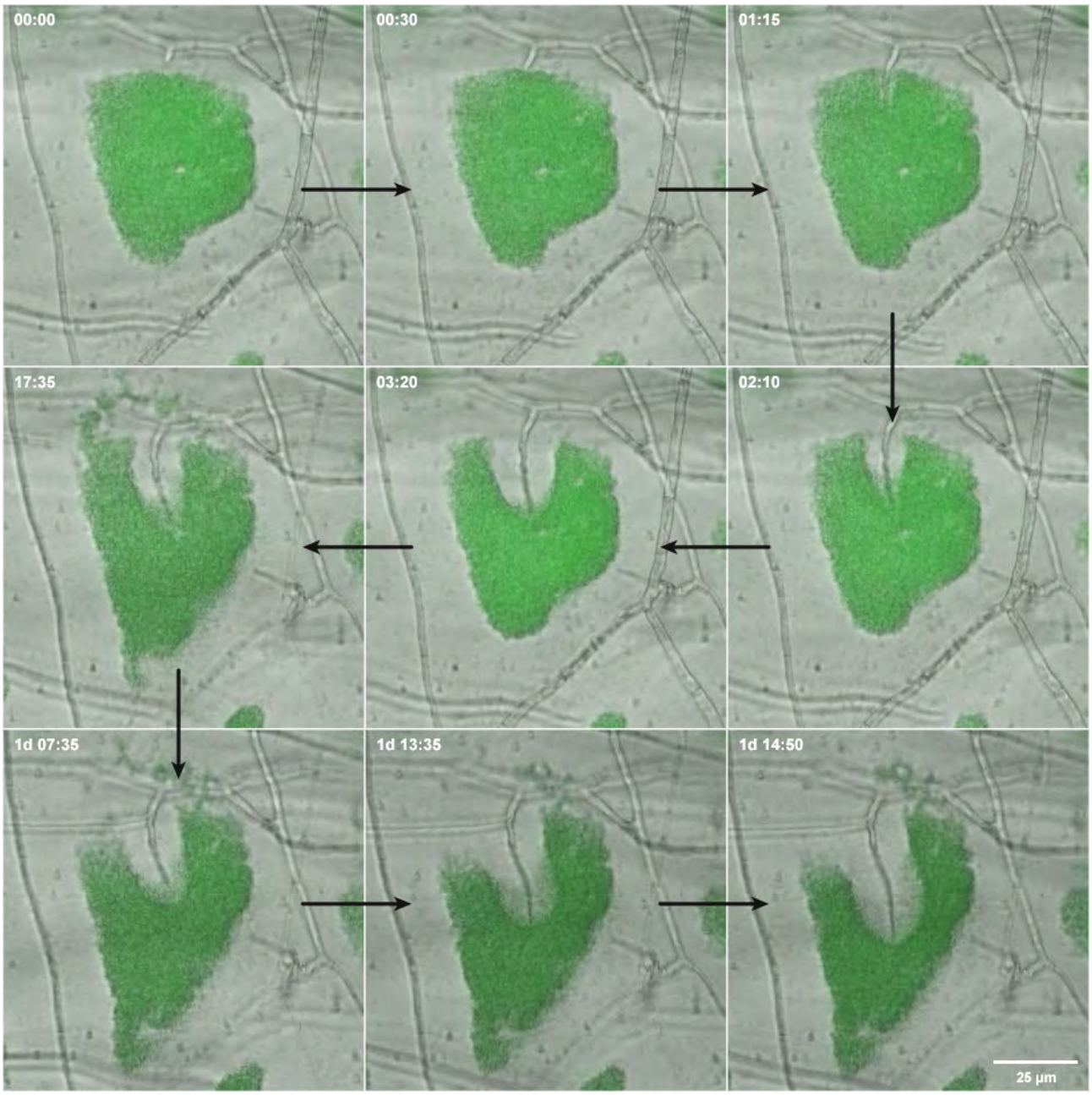


Figure 3.3. Interaction between C. necator and S. sclerotiorum in MB1/10. *Snapshots showing different stages of the interaction. Inverted microscopy images were taken every 3 minutes for 48h and the resulting timelapse movie is available in Sup. Data 1. The growing hyphae of S. sclerotiorum seemed to push the bacteria to the side, leaving a regular empty space on both sides of the mycelium.*

microfluidic device (Fig. 3.4, Sup. Data 3.2), bacteria first moved around the mycelium, but as soon as a cell attached to the hyphae, it was rapidly joined by additional cells. Unfortunately, it was not possible to discriminate if attached cells were dividing or not, and therefore, it is unclear if the groups of attached cells result from multiple cells being attracted together or from a single attached cell, which has divided.

Based on the recorded observations, we concluded that the two types of interactions resulted from the production of a soluble chemotactic metabolite by *S. sclerotiorum*. Therefore, spent media from the fungus was used to evaluate the nature of such metabolite as a repellent or attractant with chemotaxis assays.

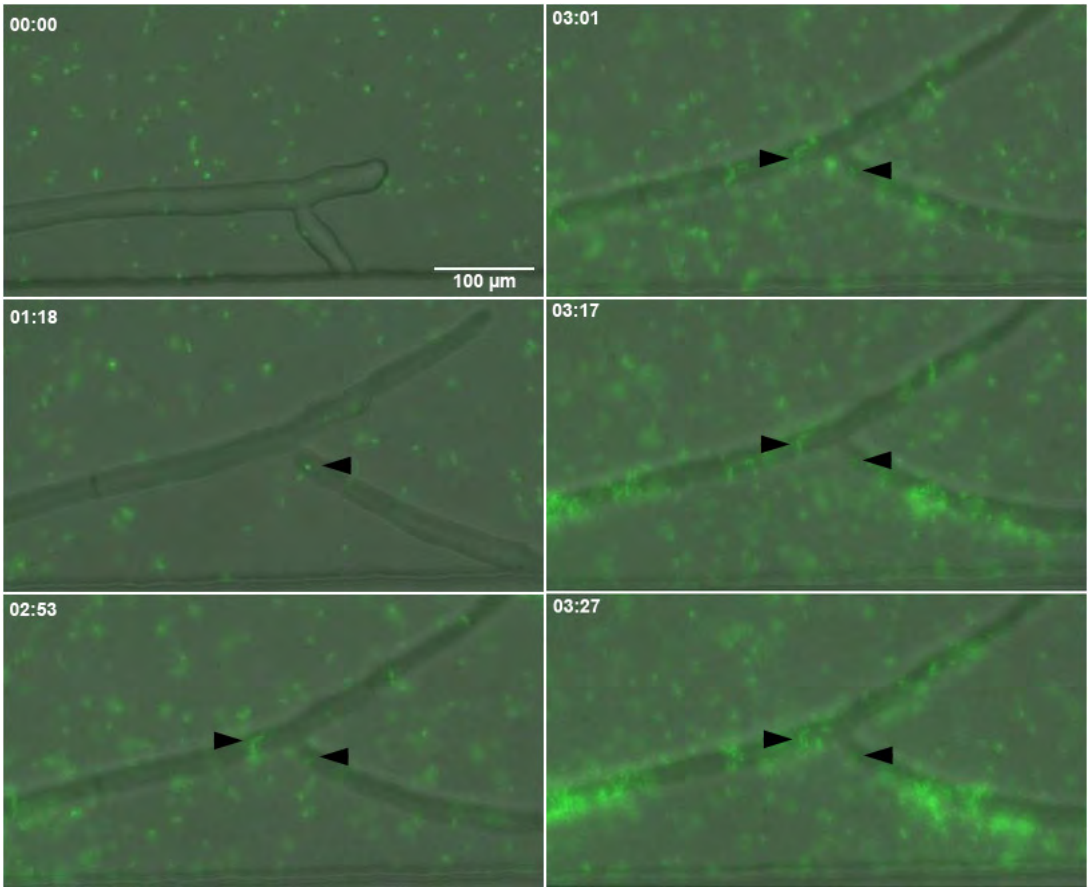


Figure 3.4. Interaction between C. necator and S. sclerotiorum in R2B. *Snapshots were taken every 30 seconds for 12h and the resulting timelapse movie is available in Sup. Data 3.2. C. necator was first swimming freely around the hyphae. A few cells attached themselves to the growing hyphae (black arrows) and were soon joined by additional cells.*

Attractive chemotaxis experiments with 3D printed bridges

The attractive effect produced by putative repellent chemotactic metabolites was assess with 3D printed bridges (Kuhn *et al.*, 2022). The attractive effect was assessed with filtrated spent R2B media from *S. sclerotiorum* cultures (SsWT) or confrontation experiments of the same fungus with *C. necator* (SsWTCn). In this case, fresh R2B was used as control medium (Fig. 3.5). The expected results with a chemo-attractant signal were for the bacteria to move from the fresh R2B medium towards the spent media, while in the control, a similar number of bacteria would be expected in both the start and end wells (Fig. 5A). After 48 h of incubation, cells were counted by flow cytometry in both the start and end wells. To discriminate the bacterial population from background noise, the whole population was gated based on size using the SSC vs FSC plot where the density of events was the highest (Sup. Data 3.3). This cell population was then divided using the green fluorescence to discriminate into alive and dead populations. Cells positive for fluorescence and size were considered as alive, while cells negative for fluorescence and positive for size were considered as dead.

In the control with R2B only, there was significantly less alive bacteria in the end well compared to the start well ($6{,}5 \cdot 10^2$ bacteria/µl and $4 \cdot 10^5$ bacteria/µl respectively; Fig. 3.5B). In the case of the dead cells, the concentration of bacteria was not significantly different between the start

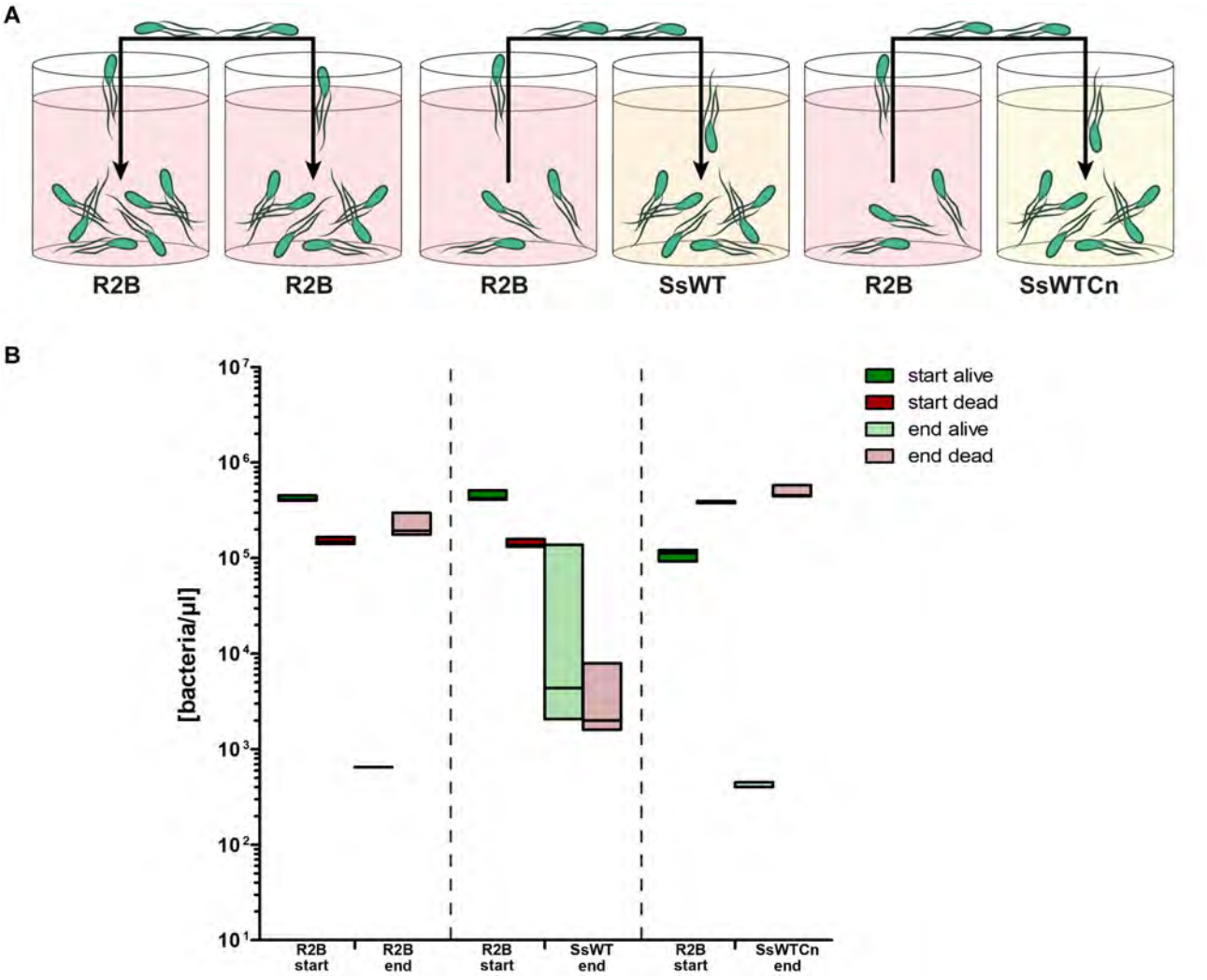


Figure 3.5. Attractant chemotaxis experiments. *Expected results for the attractive chemotaxis experiment using 3D printed bridges (**A**) and cells counts of C. necator after chemotaxis assays with R2B spent media (**B**) **A**. C. necator was expected to move from the fresh R2B to the spent media while similar concentration of bacteria would be found in both the start and end wells of the control. **B**. Alive and dead populations (respectively green and red) were counted by flow cytometry in the start and end wells after 48h of incubation. Cells counts were plotted with the maximum and minimum data and with the median, to indicate the variation between the replicates. Bacteria were inoculated in the start wells and wells filled with fresh R2B (R2B), or spent medium from S. sclerotiorum grown in R2B (SsWT), or spent medium from the interaction between C. necator and S. sclerotiorum grown in R2B (SsWTCn). Significantly less alive bacteria were counted in the end wells. The cells counts were not statistically different between the control and the treatments (N=3, Sup. Data 7).*

and end wells ($1{,}5 \cdot 10^5$ bacteria/µl and $1{,}9 \cdot 10^5$ bacteria/µl, respectively). In bridges with SsWT in the end well, there was significantly less alive and dead bacteria in the end well compared to the start well (respectively $4{,}2 \cdot 10^5$ and $1{,}3 \cdot 10^5$ bacteria/µl in the end well and $4{,}4 \cdot 10^3$ and $2 \cdot 10^3$ bacteria/µl in the start well). For SsWTCn, there was significantly less alive bacteria in the end well than in the start well ($4{,}5 \cdot 10^2$ and $1{,}1 \cdot 10^5$ bacteria/µl, respectively), however the number of dead cells in both wells was similar (respectively $3{,}8 \cdot 10^5$ and $4{,}5 \cdot 10^5$ bacteria/µl). The statistical differences in the concentration of alive cells between the control and the spent media, both in the start and end wells were not significant.

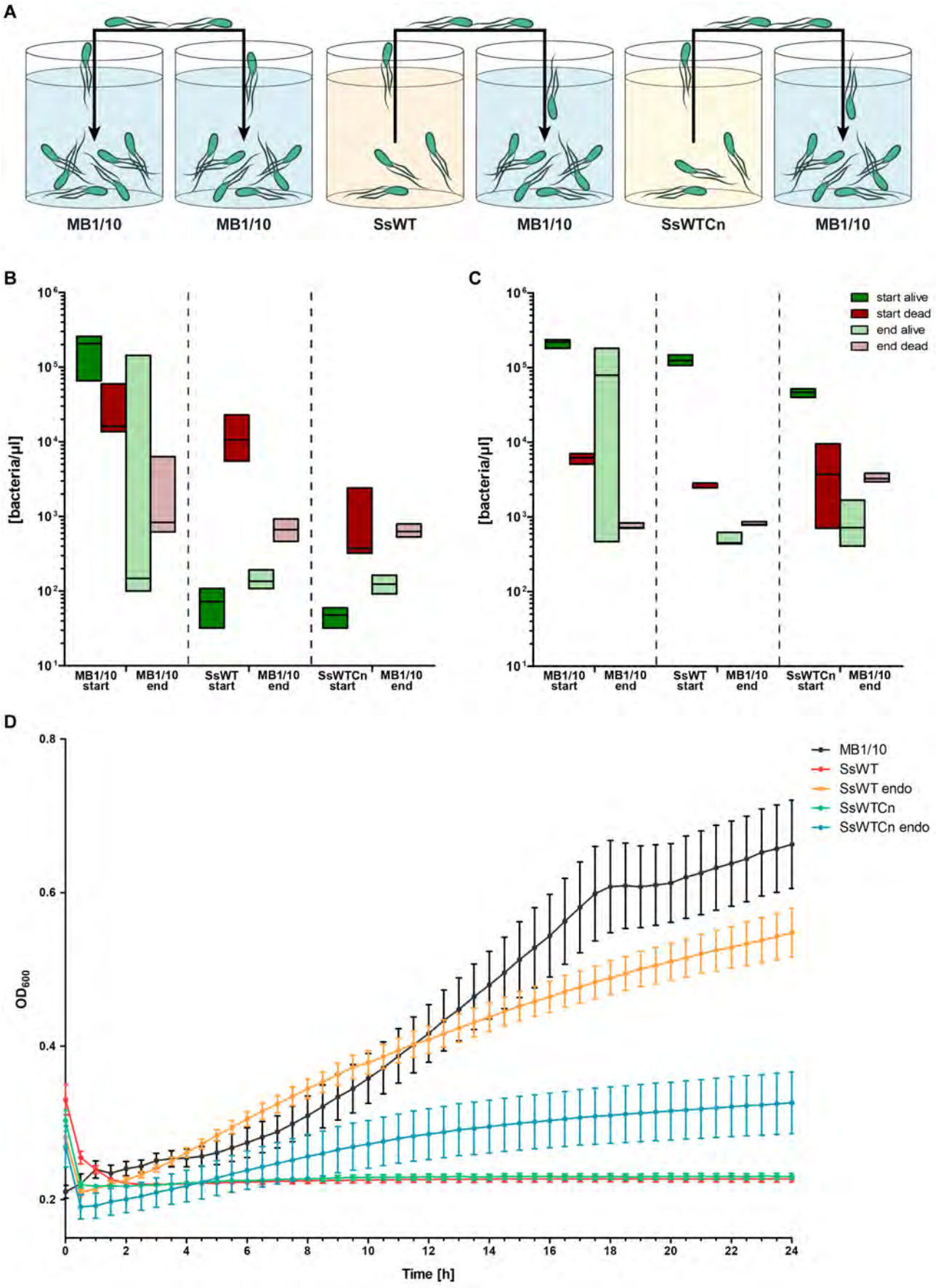


Figure 3.6. Repulsive chemotaxis experiments*. Expected results for the repulsive chemotaxis experiment using 3D printed bridges (**A**), cell counts of C. necator after chemotaxis assays with MB1/10 spent media (**B**) and toxin-free spent media (**C**) and growth curves for each condition (**D**). **A**. C. necator was expected to move from the spent*

media to the fresh MB1/10 while similar concentration of bacteria would be found in both the start and end wells of the control. ***B****. Alive and dead populations (respectively green and red) were counted by flow cytometry in the start and end wells after 48h of incubation. Cells counts were plotted with the maximum and minimum data and with the median, to indicate the variation between the replicates. Bacteria were inoculated in the start wells and wells filled with fresh MB1/10 (MB1/10), or spent medium from S. sclerotiorum grown in MB1/10 (SsWT), or spent medium from the interaction between C. necator and S. sclerotiorum grown in MB1/10 (SsWTCn). Cells showed a high mortality in the wells containing SsWT and SsWTCn media. Cells counts in start wells were significantly different from the control however, there was no statistical difference between the end wells (N=6, Sup. Data 3.4).* ***C****. After toxin removal, bacterial viability was recovered and there was no significant difference between the control and condition wells (N=3, Sup. Data 3.5).* ***D****. Bacterial growth was assessed in fresh MB1/10 as a control and in spent media. No growth occurred in SsWT and SsWTCn but bacterial growth was recovered and it was not statistically different from the control after toxin removal for SsWT spent medium (SsWT endo). While bacterial growth occurred after toxin removal for SsWTCn (SsWTCn endo), it was not statistically different from SsWTCn (N=8, Sup. Data 3.6).*

Repulsive chemotaxis experiments

Filtrated spent MB1/10 media from *S. sclerotiorum* cultures (SsWT) or from confrontation experiments of the same fungus with *C. necator* (SsWTCn) were used to test the presence of chemo-repellent metabolites. Fresh MB1/10 was used as control medium. The expected result was for bacteria to move away from the spent media (in the start well) to fresh MB1/10 (in the end well). In the control, a similar number of bacteria was expected to be measured in both the start and end wells (Fig. 3.6A). In the control bridges with MB1/10 in both wells (Fig. 3.6B, Sup. Data 3.4), there was significantly less alive bacteria in the end well ($1{,}5{\cdot}10^2$ bacteria/µl), as compared to the start well ($2{\cdot}10^5$ bacteria/µl). The same was true for the dead population ($1{,}6{\cdot}10^4$ bacteria/µl in the start well versus $8{,}3{\cdot}10^2$ bacteria/µl in the end well). In bridges with SsWT or SsWTCn in the start well (Fig. 6B), the alive population was not significantly different between the two conditions ($7{\cdot}10^1$ bacteria/µl and $4{,}8{\cdot}10^1$ bacteria/µl, respectively); however, this population was significantly different from the alive population in the control ($2{\cdot}10^5$ bacteria/µl). In both conditions, the number of dead cells in the start well was more than twice the number of alive cells ($1{\cdot}10^4$ bacteria/µl for SsWT and $3{,}7{\cdot}10^2$ bacteria/µl for SsWTCn). In the end wells, the concentration of both alive and dead cells was not significantly different from the control in both cases (respectively $1{,}4{\cdot}10^2$ and $6{,}7{\cdot}10^2$ bacteria/µl for SsWT; $1{,}2{\cdot}10^2$ and $6{,}3{\cdot}10^2$ bacteria/µl for SsWTCn; $1{,}5{\cdot}10^2$ and $8{,}3{\cdot}10^2$ bacteria/µl for the control).

To test whether the putative chemo-repellents bind to poly-l-lysine, the spent media were passed trough endotoxin removal columns containing beads coated with ε-poly-l-lysine. In the chemotaxis experiments performed with filtered spent medium there was no significant difference between the conditions (Fig. 3.6C). The pattern of alive and dead populations was similar for all conditions with a high concentration of alive cells in the start well ($2{,}2{\cdot}10^5$ bacteria/µl for the control; $1{,}2{\cdot}10^5$ bacteria/µl for SsWT; $4{,}7{\cdot}10^4$ for SsWTCn), and a thousand times less alive bacteria in the end wells ($7{,}9{\cdot}10^4$ bacteria/µl for the control; $4{,}5{\cdot}10^2$ bacteria/µl for SsWT; $7{,}2{\cdot}10^2$ for SsWTCn).

Considering the high mortality of the bacterial cells when inoculated in the spent media, we next measured bacterial growth on the different media used for the chemotaxis assays. Growth was assessed by OD measurements during 24 h using fresh MB1/10 as reference medium (Fig. 3.6D, Sup. Data 3.5). In the reference medium, the bacteria grew up to an OD of 0,66 with a lag phase of about 8 hours. When inoculated in SsWT spent medium free of toxin (SsWT endo), the bacterial growth was not significantly different from the control in MB1/10. Growth also occurred in SsWTCn spent medium free of the putative repellent metabolites (SsWTCn endo), however, it was not significantly different from SsWTCn spent medium. In contrast, no growth occurred in SsWT and SsWTCn spent media.

Discussion

In this study, we highlighted antagonistic BFI between *S. sclerotiorum* and *Cupriavidus* spp. with very different outcomes depending on the medium used. In R2B medium, bacteria exerted a control over fungal growth, while in MB1/10 medium, the fungus produced antimicrobial metabolites against the bacteria. In a previous chapter (cf. Chapter 2) it was shown that in R2B *S. sclerotiorum* produces oxalic acid and its growth is controlled through the combined consumption of oxalic acid and alkalinisation of the medium mediated by *C. necator* and *C. oxalaticus*. The detailed observations made here showed that bacteria were also attracted by the growing mycelium and were able to colonise the surface of the hyphae. We first thought that oxalic acid was the metabolite responsible for the attraction towards the mycelium as *S. sclerotiorum* WT produce oxalic acid in R2B medium. However, *Cupriavidus* spp. were also attracted to mutant fungus deficient in oxalic acid production (cf. Chapter 2), which indicated that another metabolite was responsible for the attraction. The experiments with 3D printed bridges performed in this study combined chemotaxis and dispersal. The bridges were first devised to study bacterial dispersal in absence of biotic interactions (Kuhn *et al.*, 2022). The difference between the interaction observed in plates and the results with the chemotaxis experiments using bridges highlighted the fact that the observed attraction seems to be inherently bound to the presence of the mycelium. Bacteria showed a clear attraction for *S. sclerotiorum* hyphae both in microfluidic devices and in cell culture plates but the dispersal towards the putative attractive spent media did not happen in absence of biotic interactions. While chemotaxis towards the mycelium can be driven by several secreted metabolites such as oxalic or fusaric acid (De Weert *et al.*, 2004; Rudnick *et al.*, 2015), it seems that other molecules are also involved in the colonisation of the fungus (De Weert *et al.*, 2004). For instance, spent media from *Fusarium oxysoporum* containing high concentrations of fusaric acid positively attracted *Pseudomonas fluorescens*, while spent media from the low fusaric acid producing strains did not attract the same bacterium. However, both fungal strains were colonised by bacteria, indicating that other non-soluble metabolites played a major role in the chemotactic signalling regulating the bacterial movement and the colonisation of the mycelium (De Weert *et al.*, 2004).

In MB1/10, the interaction between *S. sclerotiorum* and *Cupriavidus* spp. in MB1/10 had an opposite outcome. In this medium, *S. sclerotiorum* did not produce oxalic acid, was not controlled by the bacteria (cf. Chapter 2) and *Cupriavidus* spp. seemed repulsed by the fungal hyphae. This BFI outcome was first thought to revolve around a chemorepellent metabolite, but further investigations highlighted the antibacterial properties of the compound produced by the fungus. The recorded time-lapse of the interaction showed that bacteria were strongly repulsed by the growing hyphal tip and would then segregate on both sides of the hyphae. However, in the chemotaxis experiments, instead of using the bridges to escape the putative repellent metabolite, a high mortality in the spent media was observed. This was unexpected as repulsion was the only phenotype observed in the plates. The neat arrangement of the bacterial cells and the constant distance with the mycelium was highly interesting and appeared to indicate that the excreted metabolite could interact with the poly-l-lysine coating the plates. α-Poly-l-lysine is a synthetic polymer that improve the adhesion of the cells to solid surfaces and is commonly used to treat plates for tissue cultures (Mazia *et al.*, 1975). Cell adherence is promoted by the interaction between the positively charged polymer and the negatively charged cells. The biding of the deterrent metabolite to the poly-l-lysin could explain the very precise arrangement of the cells, as the metabolite would be trapped and form a repellent barrier for the bacteria on both sides of the mycelium. Upon sensing the metabolite, bacteria would escape death by staying away from this barrier. Toxic molecules such as LPS or endotoxins are known to have a high affinity for poly-l-lysine, therefore spent media were passed through endotoxin removal columns containing beads coated with ε-poly-l-lysine (Thermofisher™). Bacterial growth in spent media passed trough the columns was not significantly different from the growth in fresh medium, which indicated that the toxic metabolite was bound to the poly-l-lysine. Endotoxin production has not been reported for *S. sclerotiorum* and further experiments are required to determine the nature of the antibacterial metabolite. Amongst the metabolites produced by mycelium and sclerotia of *S. sclerotiorum*, fatty acids were found in large quantities both *in-vivo* and *in-vitro* (Sumner & Colotelo, 1970; Weete *et al.*, 1970; Colotelo *et al.*, 1971). Fatty acids could be candidates for the metabolite implicated in the BFI observed here. The antimicrobial properties of fatty acids have been extensively studied and have shown a great potential against both Gram-positive and Gram-negative bacteria (Desbois & Smith, 2010). Fatty acids from human sebum significantly reduced the growth of several Gram-positive human associated bacteria such as *Staphylococcus aureus* and *Streptococcus salivarius* (Wille & Kydonieus, 2003), while Gram-negative bacteria from the genus *Vibrio* were highly susceptible to unsaturated fatty acids from aquatic molluscs (Benkendorff *et al.*, 2005). Fatty acids also have a similar C-chain as the lipid-A of the LPS (Rietschel *et al.*, 1993, 1994), which is the part of the LPS molecule that interacts with the poly-l-lysine. This similarity between the molecules could explain the very effective removal of the putative toxin from the spent media after passage by the columns. The next steps will be to verify that the toxic metabolite was recovered from the columns, determine the chemical composition of the molecule and wether it is a bacteriostatic or bacteriocide.

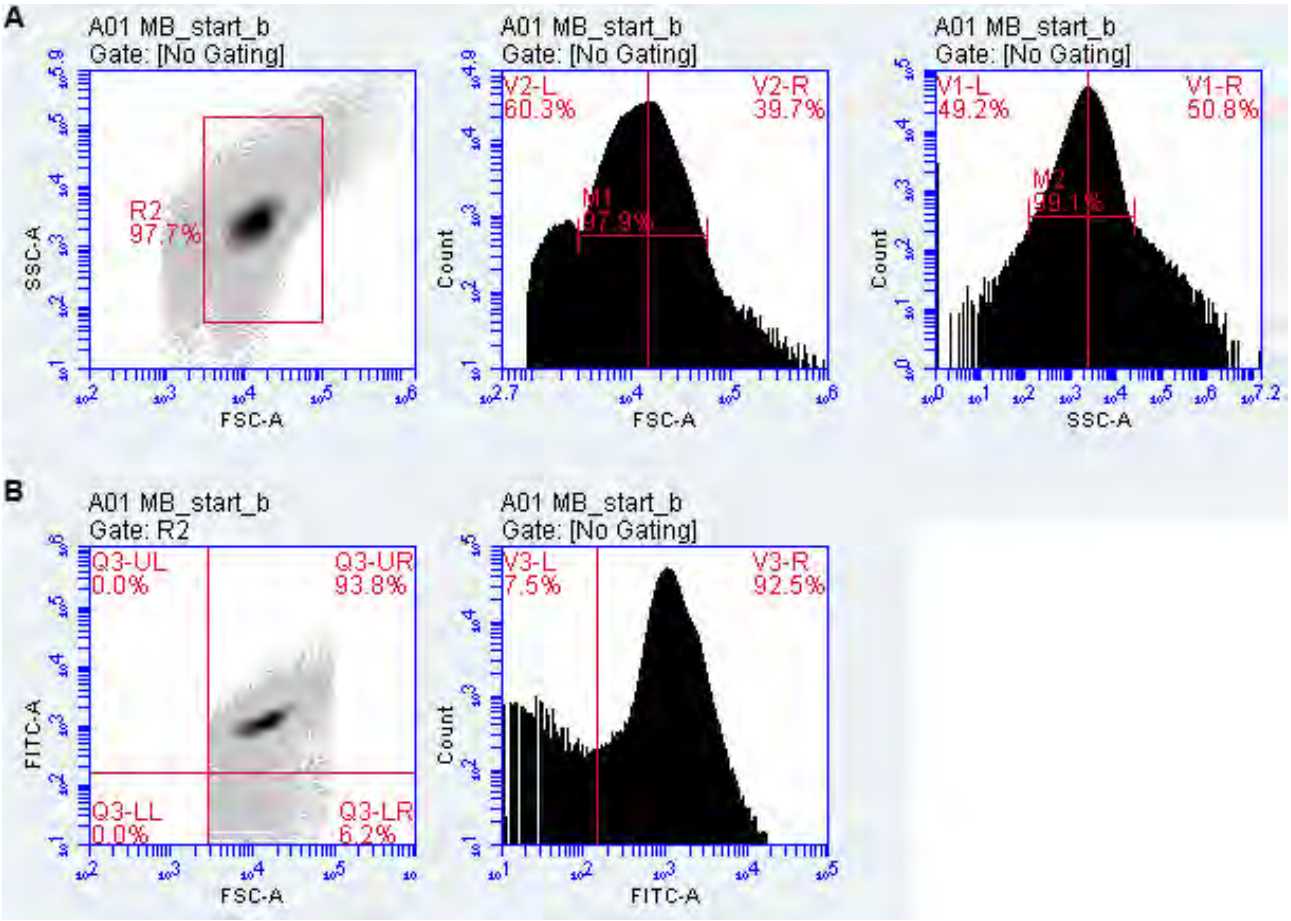
Conclusion

The use of non-traditional experimental designs to investigate BFI highlighted the importance of the medium on the interactions. In R2B, *Cupriavidus* spp. not only controlled fungal growth through medium alkalinisation, but also colonised the hyphae likely benefitting from fungal metabolites. On the other hand, when the bacteria were unable to control *S. sclerotiorum*, the fungus kept the bacteria at bay by producing metabolites whose antibacterial properties were subsequently demonstrated. This highlight the fact that much remains to be discovered in the chemical signals regulating bacterial chemotaxis and novel experimental designs are essential to further improve our understanding of BFI.

Supplementary Data

Sup. Data 3.1. Time lapse movie of the interaction between *C. necator* and *S. sclerotiorum* in MB1/10. 48 h of time lapse, 1 picture every 3 min, 42 fps.

Sup. Data 3.2. Time lapse movie of the interaction between *C. necator* and *S. sclerotiorum* in MB1/10. 48 h of time lapse, 1 picture every 3 min, 42 fps.



***Sup. Data 3.3. Gating example for the flow cytometry. A**. Gating for the initial population. **B**. Gating for the alive and dead populations based on the GFP fluorescence.*

***Sup. Data 3.4. Summary of the statistics for the chemotaxis experiment with MB1/10 spent media**. N=6.*

Alive				Dead			
start	end	Significant	summary	start	end	Significant	summary
MB1/10 vs	MB1/10	Yes	***	MB1/10 vs	MB1/10	Yes	***
SsWT vs	MB1/10	No	ns	SsWT vs	MB1/10	No	ns
SsWTCn vs	MB1/10	No	ns	SsWTCn vs	MB1/10	No	ns
MB1/10 vs	SsWT	Yes	***	MB1/10 vs	SsWT	Yes	*
MB1/10 vs	SsWTCn	Yes	***	MB1/10 vs	SsWTCn	No	ns
Start	*end*			Start	*end*		
MB1/10	*MB1/10*			MB1/10	*MB1/10*		
vs				**vs**			
SsWT	*MB1/10*	Yes	***	SsWT	*MB1/10*	No	ns
SsWTCn	*MB1/10*	Yes	***	SsWTCn	*MB1/10*	Yes	***
MB1/10	*SsWT*	No	ns	B1/10	*SsWT*	No	ns
MB1/10	*SsWTCn*	No	ns	MB1/10	*SsWTCn*	No	ns
Start	End			*Start*	End	significant	summary
MB1/10	MB1/10			*MB1/10*	MB1/10		
	vs				**vs**		
SsWT	MB1/10	No	ns	*SsWT*	MB1/10	No	ns
SsWTCn	MB1/10	No	ns	*SsWTCn*	MB1/10	No	ns
MB1/10	SsWT	No	ns	*MB1/10*	SsWT	No	ns
MB1/10	SsWTCn	No	ns	*MB1/10*	SsWTCn	No	ns
SsWT vs SsCn (start) alive		No	ns	SsWT vs SsCn (start) dead		No	ns

***Sup. Data 3.5. Summary of the statistics for the chemotaxis experiment with MB1/10 spent media after toxin removal.** N=3.*

Alive				Dead			
start	end	Significant	summary	start	end	Significant	summary
MB1/10 vs	MB1/10	Yes	***	MB1/10 vs	MB1/10	No	ns
SsWT vs	MB1/10	Yes	***	SsWT vs	MB1/10	No	ns
SsWTCn vs	MB1/10	Yes	**	SsWTCn vs	MB1/10	No	ns
Start	*end*			Start	*end*		
MB1/10	*MB1/10*			MB1/10	*MB1/10*		
vs				**vs**			
SsWT	*MB1/10*	No	ns	SsWT	*MB1/10*	No	ns
SsWTCn	*MB1/10*	Yes	*	SsWTCn	*MB1/10*	No	ns
Start	End			*Start*	End	significant	summary
MB1/10	MB1/10			*MB1/10*	MB1/10		
	vs				**vs**		
SsWT	MB1/10	No	ns	*SsWT*	MB1/10	No	ns
SsWTCn	MB1/10	No	ns	*SsWTCn*	MB1/10	No	ns
SsWT vs SsCn (start) alive		No	ns	SsWT vs SsCn (start) dead		No	ns

Sup. Data 3.6. Summary of the statistics for the chemotaxis experiment based on R2B spent media. *N=3.*

Alive				Dead			
start	end	Significant	summary	start	end	Significant	summary
R2B vs	R2B	Yes	**	R2B vs	R2B	No	ns
SsWT vs	R2B	Yes	***	SsWT vs	R2B	No	ns
SsWTCn vs	R2B	No	ns	SsWTCn vs	R2B	No	ns
R2B vs	SsWT	Yes	**	R2B vs	SsWT	No	ns
R2B vs	SsWTCn	Yes	**	R2B vs	SsWTCn	No	ns
Start	*end*			Start	*end*		
R2B	*R2B*			R2B	*R2B*		
vs				**vs**			
SsWT	*R2B*	Yes	**	SsWT	*R2B*	Yes	*
SsWTCn	*R2B*	Yes	*	SsWTCn	*R2B*	Yes	***
R2B	*SsWT*	No	ns	R2B	*SsWT*	No	Ns
R2B	*SsWTCn*	No	ns	R2B	*SsWTCn*	Yes	***
Start	End			*Start*	End		
R2B	R2B			*R2B*	R2B		
	vs				**vs**		
SsWT	R2B	No	ns	*SsWT*	R2B	No	ns
SsWTCn	R2B	No	ns	*SsWTCn*	R2B	No	ns
R2B	SsWT	No	ns	*R2B*	SsWT	No	ns
R2B	SsWTCn	No	ns	*R2B*	SsWTCn	Yes	*
SsWT vs SsCn (start) alive		No	ns	SsWT vs SsCn (start) dead		No	ns

Sup. Data 3.7. Summary of the statistics for the growth curves. *N=8*

	Significant? P < 0.05?	Summary
MB1/10 vs SsWT	Yes	***
MB1/10 vs SsWT endo	No	ns
MB1/10 vs SsWTCn	Yes	***
MB1/10 vs SsWTCn endo	Yes	***
SsWT vs SsWT endo	Yes	***
SsWT vs SsWTCn	No	ns
SsWT endo vs SsWTCn endo	Yes	***
SsWTCn vs SsWTCn endo	No	ns

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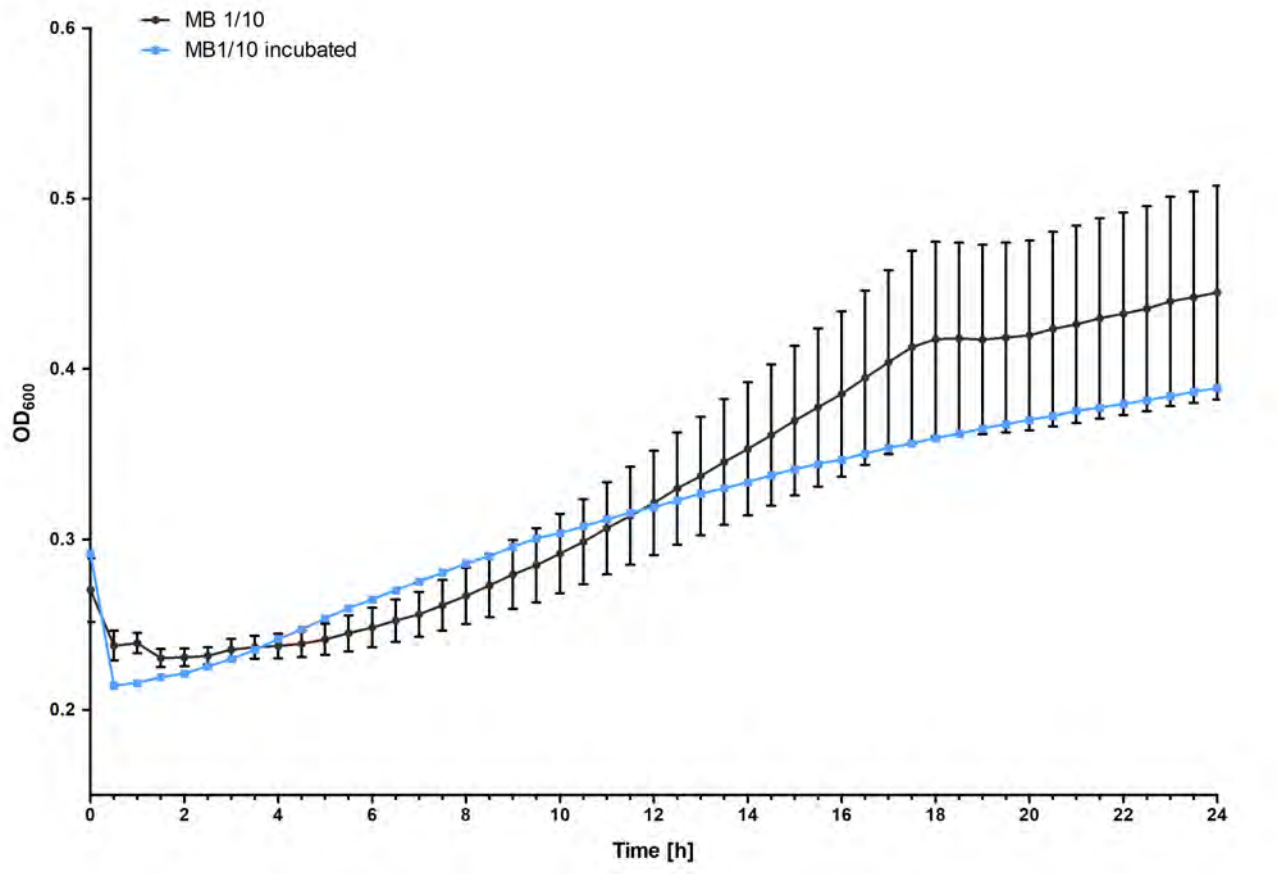
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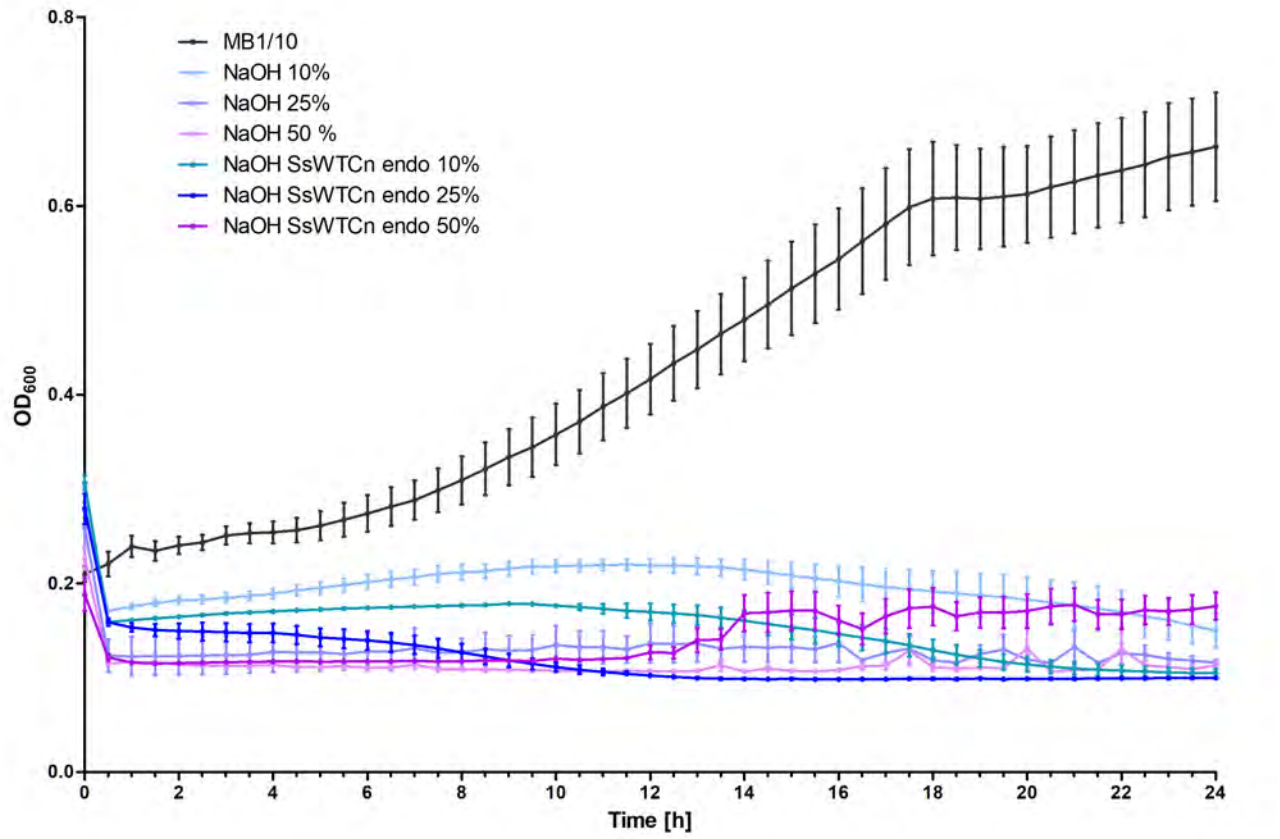
Supplementary Information

Growth curves

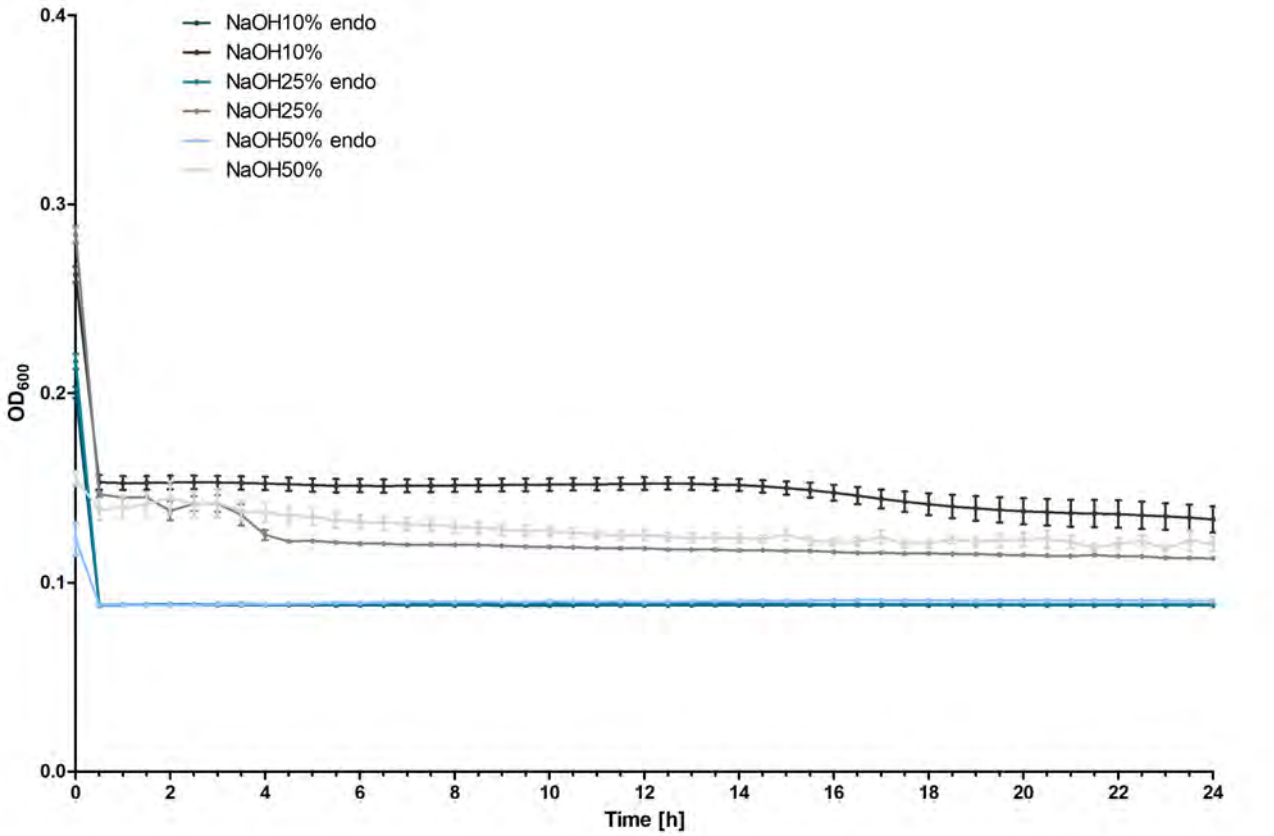
In order to verify that the bacterial growth was not impacted by the presence of poly-l-lysine in the plates, MB1/10 was incubated in a cell culture plate for 10 days before being filtered. *C. necator* growth was then assessed in both fresh and incubated MB1/10 (Sup. Info. 3.1). Bacterial growth happened in both media and no statistical difference was found between the treatments. The presence of the antimicrobial metabolite in the fraction eluted from the endotoxin removal columns was also assessed (Sup. Info. 3.2). The metabolite was eluted from the columns using NaOH, therefore growth curves were performed with several dilutions (10, 25, 50 %) of the eluant in fresh MB1/10 medium. Unfortunately, bacteria were not able to grow in the control wells with MB1/10 supplemented with NaOH. The experiment was repeated after neutralising the NaOH with HCl and bringing the alkaline pH to a neutral one (Sup. Info. 3.3). *C. necator* showed only minimal growth in the control media with neutralised NaOH, therefore further experiments need to be done to verify that the antimicrobial metabolite was effectively isolated.



Sup. Info. 3.1. Growth curves for C. necator inoculated in fresh MB1/10 and in MB1/10 incubated for 10 days in a cell culture plate. *OD (600 nm) was assessed every 30 min for 24 h. No statistical difference was found between both media.*



Sup. Info. 3.2 Growth curves of C. necator in MB1/10 supplemented with the eluant from the endotoxin removal columns. *Metabolites bound to the columns was recovered with a NaOH elution. Bacterial were not able to grow in control media supplemented with 10, 25, 50 % of NaOH, therefore it is not possible to draw any conclusion on the retrieval of the antimicrobial compound from the columns.*



Sup. Info 3.3. Growth curves of C. necator in MB1/10 supplemented with the neutralised eluant from the endotoxin removal columns. NaOH was neutralised with HCl before use. *Bacterial showed minimal growth in control media supplemented with 10, 25, 50 % of neutralised NaOH. However, growth curves were still significantly different from the neutralised eluant from the columns.*

Chapter 4

Chemogenic and biogenic oxalate sources as inductor of germination in oxalotrophic endospore-forming bacterium

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This chapter is a manuscript for future publication

Abstract

Endospores are one of the most resistant cell types produced by bacteria. They are resistant structures that can remain in dormancy for years; however, in presence of the right trigger, germination can happen within minutes. In soils, fungi promote the access to nutrients as well as the creation of new ecological niches for bacteria. One metabolite widely produce by fungi is oxalic acid, which is consumed as a carbon and energy source by so-called oxalotrophic bacteria. Oxalic acid from a fungal origin can theoretically constitute a germinant for oxalotrophic endospore-forming bacteria (EFB) co-existing with fungi in soils. The aim of this study was therefore to investigate the potential of oxalogenic fungi to create new ecological niches for oxalotrophic EFB. Several oxalotrophic EFB were isolated from a soil sample using as soil carbon source potassium and calcium oxalate (KOx and CaOx), two salts commonly associated with fungal mycelium. The isolated strains were identical and were closely related to *Ammoniphilus oxalaticus*, an obligate oxalotrophic EFB. The isolated strain, *Ammoniphilus oxalaticus* strain HJ, was able to germinate and grow with KOx, CaOx and oxalic acid as carbon source. However, it was only able to complete its life cycle (germination, growth and sporulation) with CaOx. Spent medium with oxalic acid produced by *Sclerotinia sclerotiorum* sustained the germination and growth of *A. oxalaticus* strain HJ on medium devoid of oxalate sources. *A. oxalaticus* strain HJ germinated and grew in presence of *S. sclerotiorum* mycelium and were observed to be highly active near the hyphae. This study opens up the possibility of selecting specific biocontrol EFB exploiting the ecological niche created by oxalogenic phytopathogenic fungi.

Introduction

Environmental stress has pushed microorganisms to develop survival strategies. Bacteria can produce dormant structures such as spores or spore-like structures to resist to unfavourable conditions over time (Lennon & Jones, 2011). One of the most successful strategies is the generation of endospores by endospore-forming bacteria (EFB). Endospores are sessile resistance structures able to withstand severe temperature changes, UV radiation or chemical hazards (Moir & Smith, 1990; Madigan *et al.*, 2012). These cells can remain dormant for years under unfavourable conditions. However, in presence of the right trigger, also called germinant, spores can germinate within minutes and return to an active vegetative state (Foster & Johnstone, 1990; Moir & Smith, 1990).

In soils, a large proportion of the bacteria are actually dormant and spend most of their time in an inactive metabolic state (Lennon & Jones, 2011). Soil is a heterogeneous environment in which resources are only locally available (Or *et al.*, 2007; Mandolini *et al.*, 2021). These resources are not easily accessible for bacteria, as hot spots of nutrients can be surrounded by air-filled pores (Or *et al.*, 2007). Fungal mycelium can connect these different micro-environments and provide a path for bacteria to cross the air-filled pores via fungal highways (Kohlmeier *et al.*, 2005; Simon, 2016; Deveau *et al.*, 2018). In addition to allowing bacteria to colonise new soil environments, fungi also promote the creation of new ecological niches, some of which can promote the germination of EFB (De Boer *et al.*, 2005; Frey-Klett *et al.*, 2011; Xiong *et al.*, 2022). As fungal hyphae can reach soil aggregates across air-filled gaps, they also have the ability to promote the access to nutrients and the formation of new ecological niches for sessile EFB (De Boer *et al.*, 2005). For instance, growing fungi produce metabolites and affect the physicochemical properties of the soil. This has been shown in the case of the fungus *Coprinopsis cinerea*, that promoted the germination of the strictly anaerobic EFB *Clostridium acetobutylicum* (Xiong *et al.*, 2022). In this case, oxygen consumption by the growing hyphae led to locally anoxic conditions and allowed the germination and the growth of *C. acetobutylicum* in an oxic environment. Upon growth, bacteria used the hypoxic hyphae of *C. cinerea* to reach additional anoxic micro-environments.

One interesting metabolite widely produced by fungi is oxalic acid. Oxalic acid (pKa= 1,2; 4,3), or oxalate (conjugated base), is a highly versatile molecule found in all natural environments, and is mainly produced by plants and oxalogenic fungi (Gadd, 1999; Nakata, 2003). Despite the fact that oxalate is the most oxidized compound after CO_2 (Gadd *et al.*, 2014), oxalotrophy is a widespread trait in soil bacteria (De Boer *et al.*, 2005; Hervé *et al.*, 2016). Oxalotrophic bacteria use oxalate as carbon and energy sources and are mostly found in the same environments as oxalogenic fungi. However, very little is known about the trophic interactions between oxalotrophic bacteria and oxalogenic fungi. Several oxalotrophic EFB have been isolated from environmental samples ranging from sediments to garden soil (Dehning & Sehink, 1989; Zaitsev *et al.*, 1998), in which oxalogenic fungi could provide a source of oxalate. Yet,

there is no data about the utilisation of fungal oxalic acid as a germinant and a nutrient for oxalotrophic EFB. Several examples of strict oxalotrophic EFB have been reported. Those include *Oxalophagus oxalicus*, an anaerobe isolated from freshwater sediments (Dehning & Sehink, 1989; Collins *et al.*, 1994). *O. oxalicus* grows in media supplemented with oxalate and acetate, but only when the initial inoculum was priorly pasteurized. Two aerobic oxalotrophic EFB closely related to *O. oxalicus*, *Ammoniphilus oxalaticus* and *Ammoniphilus oxalivorans*, were isolated from soil and wood samples (Zaitsev *et al.*, 1998). Both strains are ammonium-dependent and no prior pasteurisation was required for bacterial growth. In both studies, bacteria were cultured in media supplemented with ammonium oxalate (NH_4-Ox) in oxalic acid concentrations largely superior to those measured in natural environments (Gadd *et al.*, 2014).

Therefore, the aim of this study was first to isolate oxalotrophic EFB from a soil sample using potassium and calcium oxalate (KOx and CaOx), two oxalate salts commonly associated with fungal mycelium (Cromack *et al.*, 1977; Gadd *et al.*, 2014). Second, the germination and growth of the isolated strains was assessed using chemically-synthetized oxalate sources at different concentrations. Finally, the same experiments were performed in presence of actively growing mycelium of oxalogenic fungi. The two phytopathogenic fungi, *Sclerotinia sclerotiorum* and *Botrytis cinerea* were selected as models. The selection of these models is based on the fact that bacterial oxalotrophy has been shown to be an efficient biocontrol for oxalogenic fungal phytopathogens (Dickman & Chet, 1998; Schoonbeek *et al.*, 2007; Marina *et al.*, 2019), but their impact on germination of oxalotrophic soil EFB is entirely unknown. *S. sclerotiorum* is an oxalogenic necrotrophic pathogen that thrives in economically important crops (Dickman & Chet, 1998; Cessna *et al.*, 2000; Jain *et al.*, 2015). Oxalic acid production has been extensively investigated for this pathogen and it has been demonstrated that oxalic acid is essential for pathogenicity (Godoy *et al.*, 1990; Cessna *et al.*, 2000; Jain *et al.*, 2015). *B. cinerea*, that cause grey mould on harvested fruits and flowers, uses a wide array of phytotoxic metabolites and oxalic acid to penetrate and degrade the host cells (van Kan, 2006). Ten obligate oxalotrophic EFB were isolated from soil. The strains were able to germinate both in presence of chemically- synthesized oxalate sources and thrived in presence of *S. sclerotiorum* mycelium.

Material & methods

Enrichment and isolation of oxalotrophic EFB

A soil sample was taken under the oxalogenic shrub *Rheum* sp. (rhubarb) in Neuchâtel, Switzerland, at the coordinates 6°55'26,70" E, 46°59'41,52" N. In order to enrich only EFB, biomass was extracted from the soil sample and the spores were separated from the vegetative cells, using a method described previously for molecular biology studies in EFB (Wunderlin *et al.*, 2016). Filters obtained after the spore separation were cut in small pieces,

resuspended in 1,5 ml saline solution (NaCl 0,9% in deionised water) and then pooled together to obtain a spore suspension. The method of Tamer & Aragno (1980) was used for the enrichment of oxalotrophic bacteria. Briefly, 1 ml of spore suspension was added to 20 ml of liquid Schlegel AB (Tamer & Aragno, 1980) supplemented with 4 g/L of potassium oxalate (KOx) and incubated in a shaking incubator at 135 rpm at room temperature. In order to remove all possible source of carbon, the initial iron source, ammonium ferric citrate, was replaced with iron ammonium sulphate. After seven days, 1 ml of the enrichment culture was added to 20 ml of fresh medium and incubated again for seven days. Three successive enrichment transfers were performed. Isolation of oxalotrophic EFB from the last enrichment culture was carried out on Schlegel AB plates supplemented with 4 g/L of calcium oxalate (CaOx). From this, 100 µl of bacterial suspension were spread on plates and incubated at 24°C until the formation of dissolution halo around the oxalotrophic colonies. Subsequent pure cultures (strains A to J) were maintained on Schlegel AB-CaOx plates in order to retain the oxalotrophic capabilities of the individual strains.

Strains identification by sequencing

DNA extraction was performed using the *Quick*-DNA Fungal/Bacterial Miniprep Kit (D6005) from Zymo Research. The 16S rRNA gene was amplified by PCR using the forward primer EUB 9-27F (3'- GAG TTT GAT CCT GGC TCA G-5') and the reverse primer 907r (5'-CCG TCA ATT CCT TTG AGT TT-3'). The PCR program is available in Sup.Data 4. 1. Gene amplification was verified on an agarose gel before purification of the PCR products. Sanger sequencing was performed by Fasteris (Switzerland) and sequences were aligned and trimmed with BioEdit 7.2.5. Aligned sequences were compared to the NCBI database using BLAST to find matches.

A phylogenetic tree was constructed with the trimmed 16S rRNA gene sequences of the strains and available sequences on NCBI for *A. oxalaticus* strains RAOx-1 and RAOx-FF (Y14578.1; Y14579.1); *A. oxalivorans* (Y14580.1); *O. oxalivorans* (Y14581.1); *Aneurinibacillus humi* strain U33 (NR_169383.1); *Paenibacillus alvei* DSM 29 (NR_042091.1); *Cohnella thermotolerans* strain CCUG (NR_042400.1); *Cohnella hongkongensis* strain HKU3 (NR_041878.1); *Brevibacillus brevis* strain NBRC 15304 (NR_041524.1); *Brevibacillus borstelensis* strain Logan B4029 (NR_029131.1); *Saccharibacillus sacchari* strain GR21 (NR_044375.1); *Aneurinibacillus sediminis* strain 1-10M-8-7-50 (NR_157740.1); *Saccharibacillus endophyticus* strain JM-1350 (NR_153708.1). Strain F was removed because of the poor quality of the sequence. The maximum likelihood tree was generated with MEGA11 software, with 100 bootstraps replications and the Tamura-Nei model. The matrix of sequence identity of the aligned sequences was calculated with BioEdit 7.2.5.

Germination and sporulation experiments in one-condition microfluidic devices

Microfluidics experiments were carried out in Newcastle University, England, in collaboration with Prof. Anil Wipat, Dr. Lucy Eland and Dr. Valeria Verrone (ICOS Research, School of Computing Science, Newcastle University, Newcastle upon Tyne NE1 7RU). A microfluidic

chip with channels with 4% low melting point agarose (ThermoScientific, Ref. R0801) in Schlegel AB medium was freshly prepared and encased in a polydimethylsiloxane (PDMS) mould plasma bound (Harrick Plasma Cleaner, Ithaca, New York, Model PDC-002) to a glass coverslip. The mould had two holes to plug a pump and flow the system with medium at the rate of 10 µl/min. All pieces were plasma activated, 2 µl of bacterial spore suspension was added in the mould before the agarose chip was added and sealed in place with a piece of a glass slide. Sealing was tested by pushing medium in the system and non-sealed devices were discarded. Pictures were acquired automatically with a Nikon Eclipse, Ti-DH microscope coupled to a Camera QImaging RETIGA 2000R. For the different experiments, pictures were acquired at the rate of 1/3 min for 15 hours, 1/1h30 for 4 hours and 1/30 s for 30 min.

Germination on agarose pads

Agarose pads were prepared as described in Estoppey et al. (2022), with Schelgel AB medium or spent media. Spores were harvested from a two-weeks old culture on a Schlegel AB-4 g/L CaOx plate and resuspended in saline solution. For this, 5 ml of saline solution were added to the plate and cells were gently removed from the plate using a cell spreader. The spore suspension was then transferred to a sterile Eppendorf and conserved at 4°C for further experiments. Then 2 µl of this suspension was added to each agarose pad and allow to dry before the pad was closed with a coverslip. Microscopy images were acquired at the inoculation time and after 24h with the microscope Leica DM4 B coupled to the Leica DFC7000 T camera.

In order to verify that the strain was a strict oxalotroph, germination was assessed on media devoid of oxalic acid sources. Agarose pads were prepared with Reasoner's 2 medium (R2A; 0,5 g/L yeast extract; 0,5 g/L peptone; 0,5 g/L casamino acids; 0,5 g/L glucose; 0,5 g/L soluble starch, 0,3 g/L Na-pyruvate; 0,3 g/L K_2HPO_4; 0,05 g/L $MgSO_4 \cdot 7H_2O$; in Milli-Q® water (Reasoner & Geldreich, 1985)) and diluted malt medium (MA1/10; 1,2 g/L malt extract).

To assess the germination of the strain in presence of different oxalate sources, Schlegel AB was supplemented with different concentrations of calcium oxalate, potassium oxalate or oxalic acid (CaOx, KOx, OA). The concentrations selected corresponded to the concentrations of oxalic acid measured in *B. cinerea* (0,3 g/L) and *S. sclerotiorum* WT (0,65 g/L) cultures grown in R2B medium (cf. Chapters 1-2); the initial concentration of the medium (4 g/L), and the double amount of the medium concentration (8 g/L).

Fungal strains

The wild-type (WT) and the mutant deficient in oxalic acid production (Δ*oah*) of *S. sclerotiorum* were provided by Jeffrey Rollins from the Department of Plant Pathology, University of Florida, while *B. cinerea* was obtained from the fungal collection of the Plant and Microbial Biology unit in the University of Fribourg, Switzerland. Fungal strains were maintained on potato dextrose agar (PDA; 5 g/L potato infusion, 20 g/L dextrose, 17 g/L agar, pH 5,6 ± 0,2)

Germination test in the presence of fungi on agarose pads

To assess the interactions between the oxalotrophic EFB strain and a fungal partner, agarose pads were prepared with R2B medium. For *B. cinerea*, the fungal inoculum was 2 µl of a spore suspension prepared as described in Estoppey et al. (2022), while for *S. sclerotiorum* strains, a small amount of mycelium was spread on the agarose pad with a sterile platinum loop. In both cases, the bacterial inoculum was 2 µl of a spore suspension. Microscopy images were acquired at the inoculation time and after 24h with the microscope Leica DM4 B coupled to the Leica DFC7000 T camera.

Interaction with *S. sclerotiorum* in BFI devices

To inoculate the BFI devices (Stanley *et al.*, 2014), a small cube of agar was taken in the active growing mycelium (colony edge) from a fresh culture of *S. sclerotiorum* grown on a PDA. To prevent desiccation, 10 µl of R2B were added to the device trough the bacterial inoculation port before incubation. After three days of incubation, 10 µl of a spore suspension was added to the device. Images were acquired with the inverted microscope EVOS M5000 and processed with ImageJ 2.0.0-rc65/1.51w.

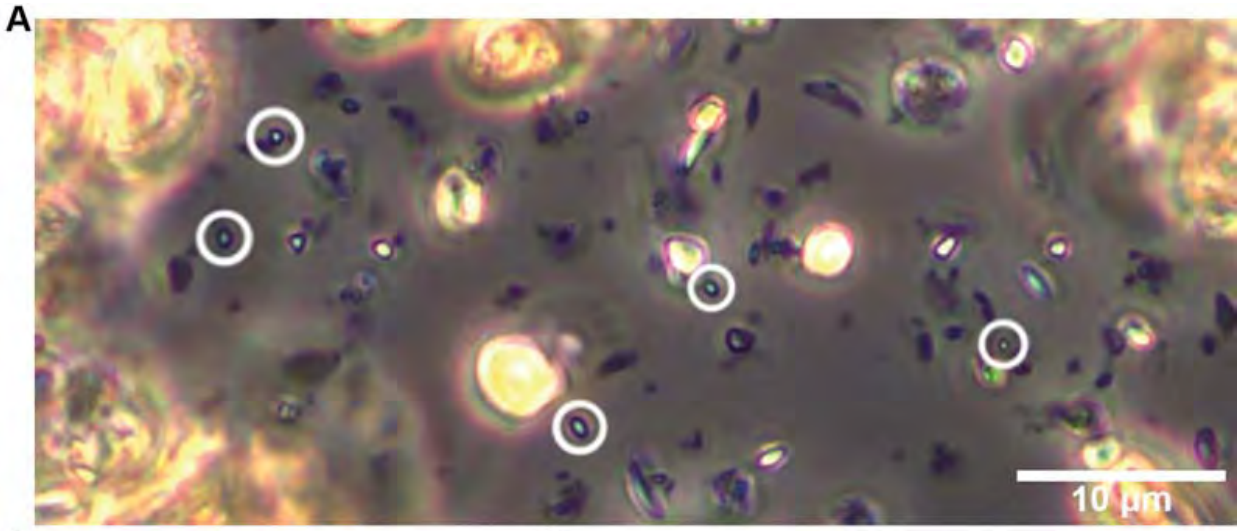
Results

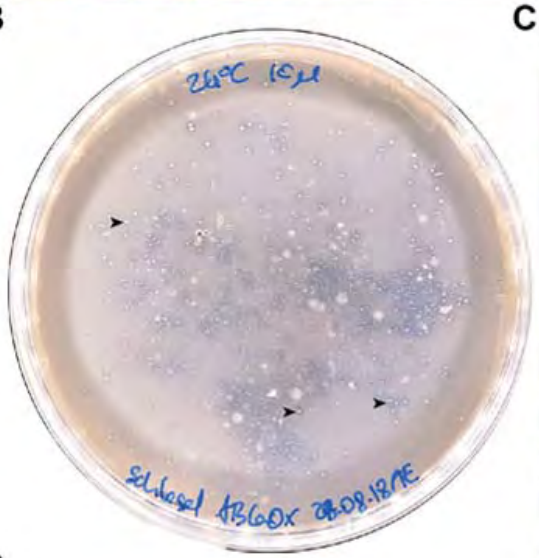
Isolation of an oxalotrophic EFB from a soil sample

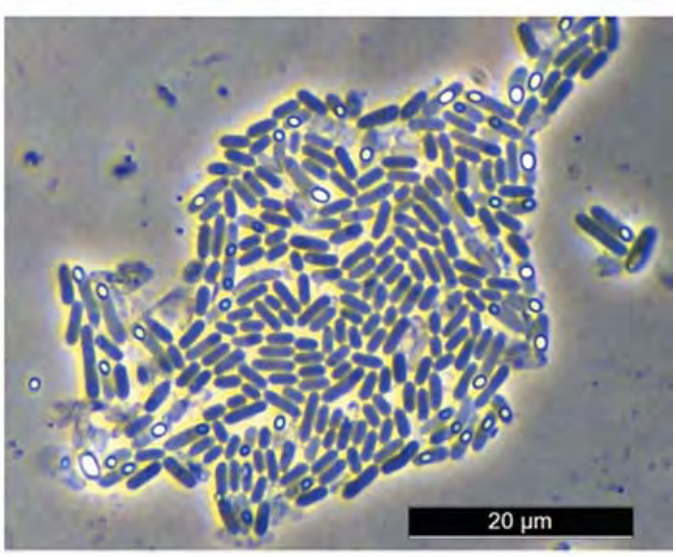
The procedure of extraction of biomass and separation from the vegetative cells using physical and chemical means resulted in the enrichment of EFB from the soil sample (Fig. 4.1A). Enrichment and isolation of oxalotrophic EFB was based on a combination of growth in liquid and on solid Schlegel AB media with KOx or CaOx as sole carbon source, respectively. In the solid medium, dissolution halos around the colonies indicated the degradation of CaOx and the presence of oxalotrophic bacteria (Fig. 4.1B). After several cycles of isolation, ten pure cultures (strains A to J) were obtained (Fig. 4.1C). The 16S rRNA gene sequences obtained for the strains were identical to each other at more than 99,4% and highly similar to several known oxalotrophic EFB (Table 1; Sup. Data 4.2). The most similar strains were *A. oxalaticus* and *A. oxalivorans* (99% of identity; Zaitsev et al., 1998) and *O. oxalaticus* (97% of identity; Dehning & Sehink, 1989), all of which are known strict oxalotrophic EFB. These three species belong to the *Aneurinibacillus* group in the Paenibacillaceae family. A phylogenetic tree with related species from the Paenibacillaceae family clearly show the placement of the isolated strains in close proximity to *A. oxalaticus* strain RAOx-FF (Fig. 4.1D).

Oxalotrophy

In order to verify that the strains isolated here were indeed obligate oxalotrophs, their growth was tested on Schelgel-AB medium devoid of oxalate, as well as on MA1/10 and R2A media (Fig. 4.2A, Sup. Data 4. 3). As the isolated strains grew slowly (first visible colonies


A
10 µm


B


C
20 µm

D

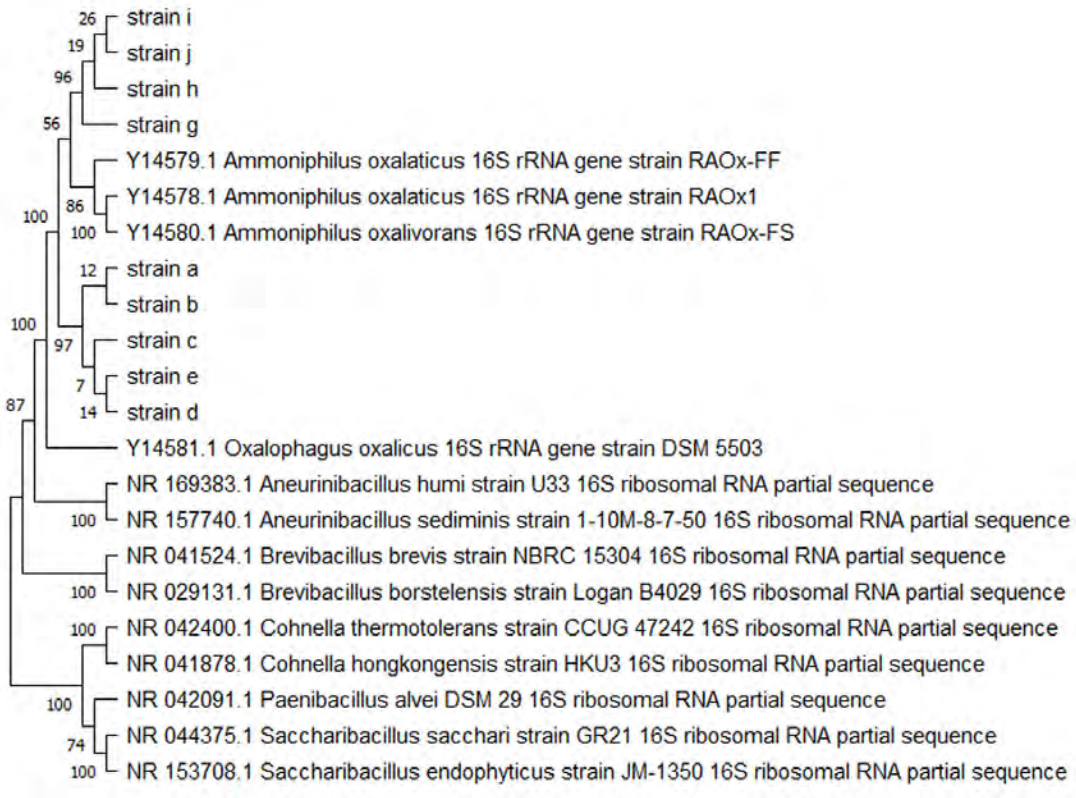

strain i
strain j
strain h
strain g
Y14579.1 Ammoniphilus oxalaticus 16S rRNA gene strain RAOx-FF
Y14578.1 Ammoniphilus oxalaticus 16S rRNA gene strain RAOx1
Y14580.1 Ammoniphilus oxalivorans 16S rRNA gene strain RAOx-FS
strain a
strain b
strain c
strain e
strain d
Y14581.1 Oxalophagus oxalicus 16S rRNA gene strain DSM 5503
NR 169383.1 Aneurinibacillus humi strain U33 16S ribosomal RNA partial sequence
NR 157740.1 Aneurinibacillus sediminis strain 1-10M-8-7-50 16S ribosomal RNA partial sequence
NR 041524.1 Brevibacillus brevis strain NBRC 15304 16S ribosomal RNA partial sequence
NR 029131.1 Brevibacillus borstelensis strain Logan B4029 16S ribosomal RNA partial sequence
NR 042400.1 Cohnella thermotolerans strain CCUG 47242 16S ribosomal RNA partial sequence
NR 041878.1 Cohnella hongkongensis strain HKU3 16S ribosomal RNA partial sequence
NR 042091.1 Paenibacillus alvei DSM 29 16S ribosomal RNA partial sequence
NR 044375.1 Saccharibacillus sacchari strain GR21 16S ribosomal RNA partial sequence
NR 153708.1 Saccharibacillus endophyticus strain JM-1350 16S ribosomal RNA partial sequence

Figure 4.1. Selective enrichment and isolation of an oxalotrophic endospore-forming bacterium from a soil sample, using an enrichment medium containing potassium oxalate (KOx) as sole carbon and energy sources. A*. Observation of the results of the biomass extraction procedure to identify the presence of spores (white circles).* ***B****. The ability of the bacteria growing on this selective medium to degrade CaOx is observed based on the dissolution of this mineral around the colonies (arrows).* ***C****. Mixed culture of vegetative cells, endospores and free spores of the isolated strain.* ***D.*** *Phylogenetic tree produced with the 16S rRNA gene sequences of the isolated strains and other related species.*

Table 4.1. Sequences identity matrix. *The percentage of identity was calculated for each sequences of the isolated strains (strains A-J) and the sequences of the most related species.*

Sequence	strain a	strain b	strain c	strain d	strain e	strain g	strain h	strain i	strain j	RAOx1	RAOx-FF	RAOx-FS	DSM 5503
strain a	ID	0,996	0,996	0,996	0,996	0,998	0,999	1	1	0,988	0,993	0,982	0,974
strain b	0,996	ID	1	1	1	0,994	0,995	0,996	0,996	0,989	0,995	0,985	0,975
strain c	0,996	1	ID	1	1	0,994	0,995	0,996	0,996	0,989	0,995	0,985	0,975
strain d	0,996	1	1	ID	1	0,994	0,995	0,996	0,996	0,989	0,995	0,985	0,975
strain e	0,996	1	1	1	ID	0,994	0,995	0,996	0,996	0,989	0,995	0,985	0,975
strain g	0,998	0,994	0,994	0,994	0,994	ID	0,997	0,998	0,998	0,986	0,991	0,981	0,972
strain h	0,999	0,995	0,995	0,995	0,995	0,997	ID	0,999	0,999	0,987	0,992	0,982	0,973
strain i	1	0,996	0,996	0,996	0,996	0,998	0,999	ID	1	0,988	0,993	0,982	0,974
strain j	1	0,996	0,996	0,996	0,996	0,998	0,999	1	ID	0,988	0,993	0,982	0,974
A. oxalaticus RAOx1	0,988	0,989	0,989	0,989	0,989	0,986	0,987	0,988	0,988	ID	0,992	0,991	0,968
A. oxalaticus RAOx-FF	0,993	0,995	0,995	0,995	0,995	0,991	0,992	0,993	0,993	0,992	ID	0,988	0,972
A. oxalivorans RAOx-FS	0,982	0,985	0,985	0,985	0,985	0,981	0,982	0,982	0,982	0,991	0,988	ID	0,965
O. oxalicus DSM 5503	0,974	0,975	0,975	0,975	0,975	0,972	0,973	0,974	0,974	0,968	0,972	0,965	ID

appeared after one week of incubation) experiments were conducted with the identical strains H and J (therefore called *A. oxalaticus* strain HJ), which were the two strains that showed the fastest growth rate among the ten isolated strains. MA1/10 and R2A do not contain oxalate, but contain other carbon and nitrogen sources instead. In MA1/10, the carbon and nitrogen are contained in the malt extract, while R2A contains various sources of carbon and nitrogen. No germination was observed on Schelgel-AB and MA1/10, while some spontaneous germination occurred on R2A (Fig. 4.2A, Sup. Data 4. 3). In contrast, when Schlegel AB medium was supplement with 4 g/L of KOx or CaOx (Fig. 4.2B-C, Sup. Data 4. 4-5), cells went from bright phase endospores to dark phase vegetative cells within minutes. The cells then divided rapidly after transition to dark phase cells. The germination to division process happened faster with KOx than with CaOx (Fig. 4.2B-C, Sup. Data 4-5). All spores went from bright phase to dark phase in less that 1 h 20 with KOx and the first cells emerged from the spore's coat after 1 h 50 (Sup. Data 4.4). The first complete cell division happened after 3 h and all cells had divided at least once after 5 h 20 (Fig. 4.2B, Sup. Data 4.4). On the other hand, it took 1 h 40 for all the spores to go from bright to dark phase with CaOx and exit from the spore's coat occurred after 2 h 30 (Fig. 4.2C, Sup. Data 4.5). With CaOx, the first division occurred after 4 h, and after 6 h, all cells had divided at least once (Sup. Data 4.5). The cycle was completed with the first sporulation events occurring between 10 to 18 h after inoculation (Fig. 4.2C. Sup. Data 4.5). With KOx, no sporulation was observed.

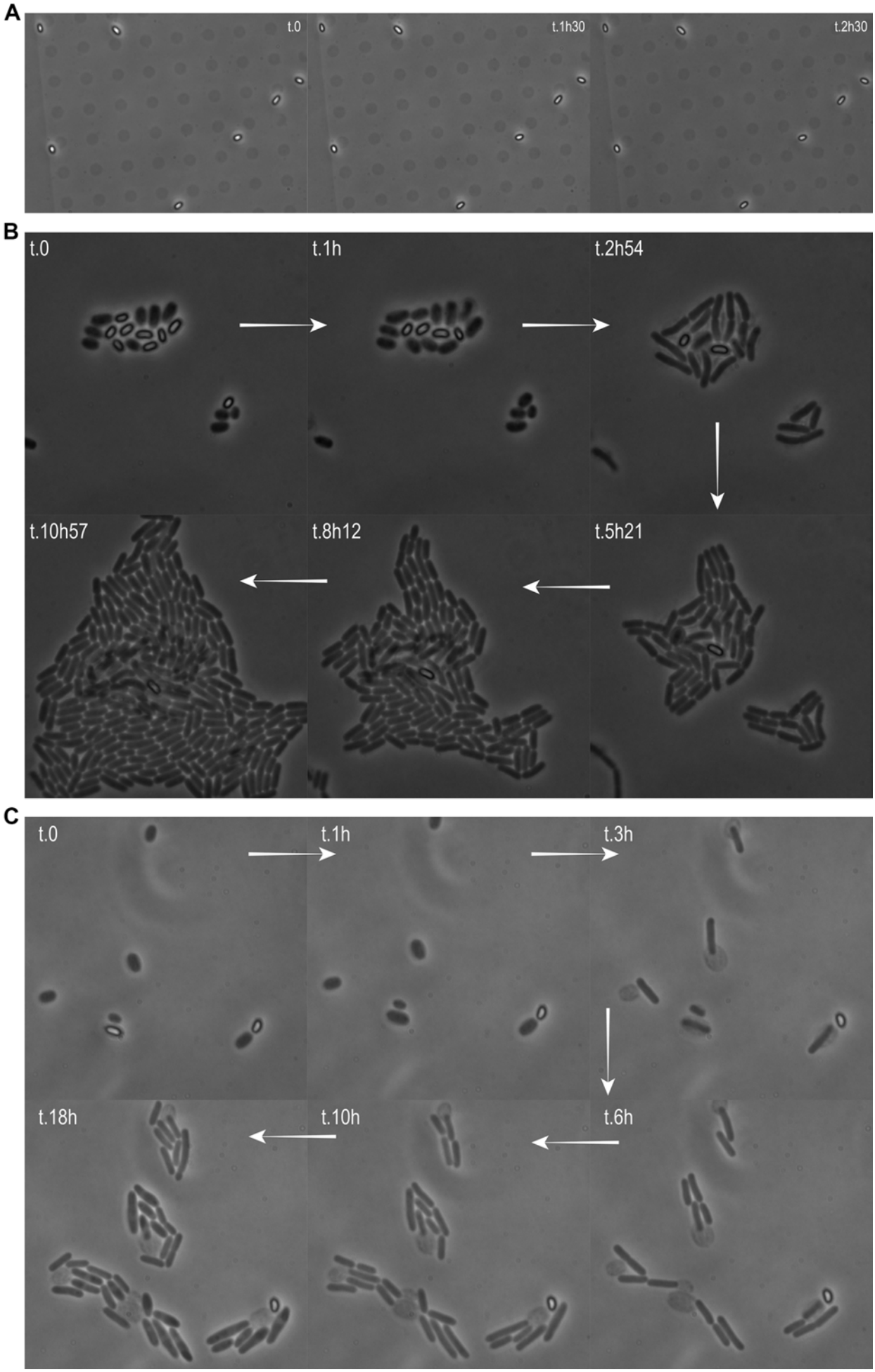

A
t.0
t.1h30
t.2h30
B
t.0
t.1h
t.2h54
t.10h57
t.8h12
t.5h21
C
t.0
t.1h
t.3h
t.18h
t.10h
t.6h

Figure 4.2. Germination in presence of KOx and CaOx. Snapshots of the germination and growth of Aox on Schlegel AB medium no supplemented in an oxalate source (A) or supplemented by 4 g/L of KOx (B) and CaOx (C). A*. Non-sealed microfluidic device was filled with Schlegel AB medium devoid of oxalate and no germination occurred upon time. Images at 100x were recorded at 3 time points.* ***B.*** *In a sealed microfluidic device filled with Schlegel AB-KOx, spores went from bright phase in less than one hour, as soon as the oxalate entered the system, and cells divided rapidly. No sporulation event was recorded. Images at 100x were recorded every 3 min for 15 hours and a full time-lapse movie of the germination and growth is available in Sup. Data 4.2.* ***C****. On a Schlegel AB-CaOx agarose pad, germination occurred in less than 3h and cells continued to divide well after 10h. Sporulation started between 10 to 18h but the complete cycle was not recorded. Images at 100x were recorded every minute for 10 hours and a second record was started after 18 hours. The entire time lapse movie is available in Sup. Data 4.3.*

Germination and growth with chemogenic oxalate sources

In order to characterise the response of *A. oxalaticus* strain HJ to different chemogenic oxalate sources, germination and growth was investigated on agarose pads at four concentrations ranging from physiological to extreme (0,3; 0,65; 4 and 8 g/L), and comparing three oxalate sources (CaOx, KOx and oxalic acid; Fig. 4.3). Images were taken at the inoculation time to verify the presence of spores and then every 24 h for several days to assess the germination and growth of the cells. On Schlegel AB-CaOx (Fig. 4.3A), *A. oxalaticus* strain HJ germinated and grew in all conditions except at 0,3 g/L. As pictures were not acquired at the same position for each time point, it was not possible to assess the growth rate at the different concentrations. *A. oxalaticus* strain HJ was able to germinate and grow in all conditions with KOx (Fig. 4.3B). On the other hand, too high concentrations of KOx had a deleterious effect on growth, which was highlighted by the deformed shape of the cells at 8 g/L. With oxalic acid as carbon source (Fig. 4.3C), *A. oxalaticus* strain HJ was only able to germinate at low concentrations, and 0,65 g/L was the sole concentration effectively supporting growth. However, no sporulation events were observed on this medium, even after several days of inoculation. Given the toxicity already observed at low concentrations, the highest concentration (8 g/L) was not investigated. Moreover, at this concentration, the high acidity prevented the jellification of the agar pads.

A. oxalaticus strain HJ interactions with oxalogenic fungi

A. oxalaticus strain HJ germination was then assessed in presence of growing mycelium of oxalogenic fungi. Bacterial spores were inoculated with *B. cinerea* spores or *S. sclerotiorum* WT mycelium on R2A agarose pads (Fig. 4.4). Microscopy images were taken at the inoculation time and after 24 h to assess bacterial growth. *B. cinerea* was able to sustain the germination of the bacterial spores (Fig. 4.4A). Clusters of ungerminated spores were found near the mycelium and germination appeared to happen near the hyphal tips. The presence of endospores already after 24 h seems to indicate that sporulation was triggered immediately after germination (Fig. 4.4A). In presence of *S. sclerotiorum* WT, spores germinated and vegetative cells were found to be actively moving around the mycelium (Fig. 4.4B, Sup. Data 4. 6). Germination happened not only in spores in direct contact with the hyphae, as shown

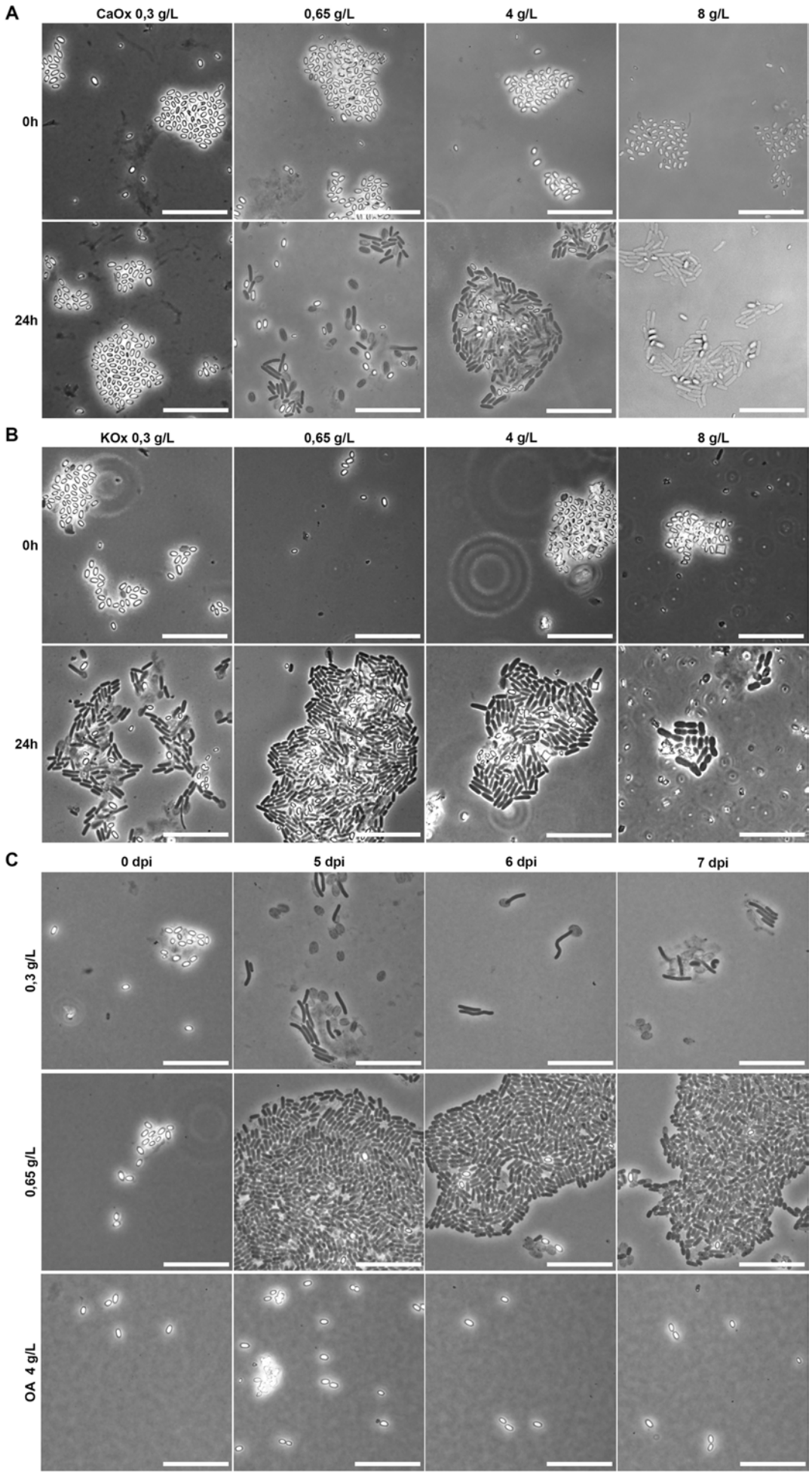

A
CaOx 0,3 g/L
0,65 g/L
4 g/L
8 g/L
0h
24h
B
KOx 0,3 g/L
0,65 g/L
4 g/L
8 g/L
0h
24h
C
0 dpi
5 dpi
6 dpi
7 dpi
0,3 g/L
0,65 g/L
OA 4 g/L

Figure 4.3. Germination and growth of in presence of different concentrations of CaOx (A), KOx (B) and oxalic acid (OA, C). *Snapshots of the growth of Aox on Schlegel AB agarose pads supplemented with 0,3; 0,6; 4 and 8 g/L of calcium oxalate (CaOx), potassium oxalate (KOx) and oxalic acid (OA). Microscopy images were taken at the inoculation time and after 24h for A-B, and after 5, 6, 7 days post inoculation for C (dpi; 10 µm scale bar).* ***A****. All CaOx concentrations except 0,3 g/L supported Aox germination and growth overnight.* ***B****. Aox was able to grow on all KOx concentration, however, higher concentrations of KOx had a deleterious effect on the cells.* ***C****. Aox was only able to grow with low oxalic acid concentrations.*

by the presence of vegetative cells in areas without mycelium. In BFI devices filled with R2B medium (Fig. 4.4C), spores also germinated in presence of *S. sclerotiorum* WT. Moreover, the presence of *A. oxalaticus* strain HJ vegetative cells appeared to slow down the growth of *S. sclerotiorum* WT. *A. oxalaticus* strain HJ germination was also investigated in presence of *S. sclerotiorum* Δ*oah*, the mutant strain deficient in oxalic acid production (Fig. 4.4D). Only some spores spontaneously germinated and the fungal growth was not impacted by the presence of the spores and the few vegetative cells observed.

Discussion

We demonstrated that it is possible to specifically isolate oxalotrophic EFB from a soil sample using oxalate sources commonly associated with fungal mycelium. *A. oxalaticus* strain HJ was able to germinate and grow in presence of different oxalates sources, as well as in presence of actively growing mycelium. Identification based on the 16S rRNA gene indicated that *A. oxalaticus* strain HJ was highly similar to *A. oxalaticus*, an ammonium-dependent obligate oxalotrophic bacteria (Zaitsev *et al.*, 1998). However, further investigations showed that the isolated *A. oxalaticus* strain HJ were different from the *A. oxalaticus* previously described. Colonies of *A. oxalaticus* strain RAOx-FF, the most similar strain, were described as "light brown, convex and circular with entire margin and smooth surface, ≥ 2 mm in diameter" (Zaitsev *et al.*, 1998) whereas *A. oxalaticus* strain HJ colonies were white and smaller than 2 mm. The other highly similar strains, *A. oxalaticus* strain RAOx1 and *A. oxalivorans* RAOx-FS, were also described as ammonium-dependent (Zaitsev *et al.*, 1998). *A. oxalaticus* strain HJ did not show specific requirements for ammonium and grew in aerobic conditions.

A. oxalaticus strain HJ was extremely sensible to the concentration and the source of oxalate in the medium. Growth was assessed in presence of three oxalate sources with different solubilities, ranging from extremely soluble (KOx; 392 g/L) to almost insoluble (CaOx; 6 mg/L). CaOx is the oxalate salt the most commonly found in association with fungal mycelium (Cromack *et al.*, 1979; Gadd, 1999; Verrecchia *et al.*, 2006). A concentration of up to 7 g/L has been detected in soils colonised by oxalogenic fungi (Cromack *et al.*, 1977). Qualitative observations of the germination and growth of *A. oxalaticus* strain HJ in presence of CaOx seemed to indicate that it was the preferential source of oxalate for *A. oxalaticus* strain HJ. Its growth was slow and steady with CaOx concentrations over 0,65 g/L, and it was the only oxalate source supporting the formation of endospores. On the other hand, a concentration

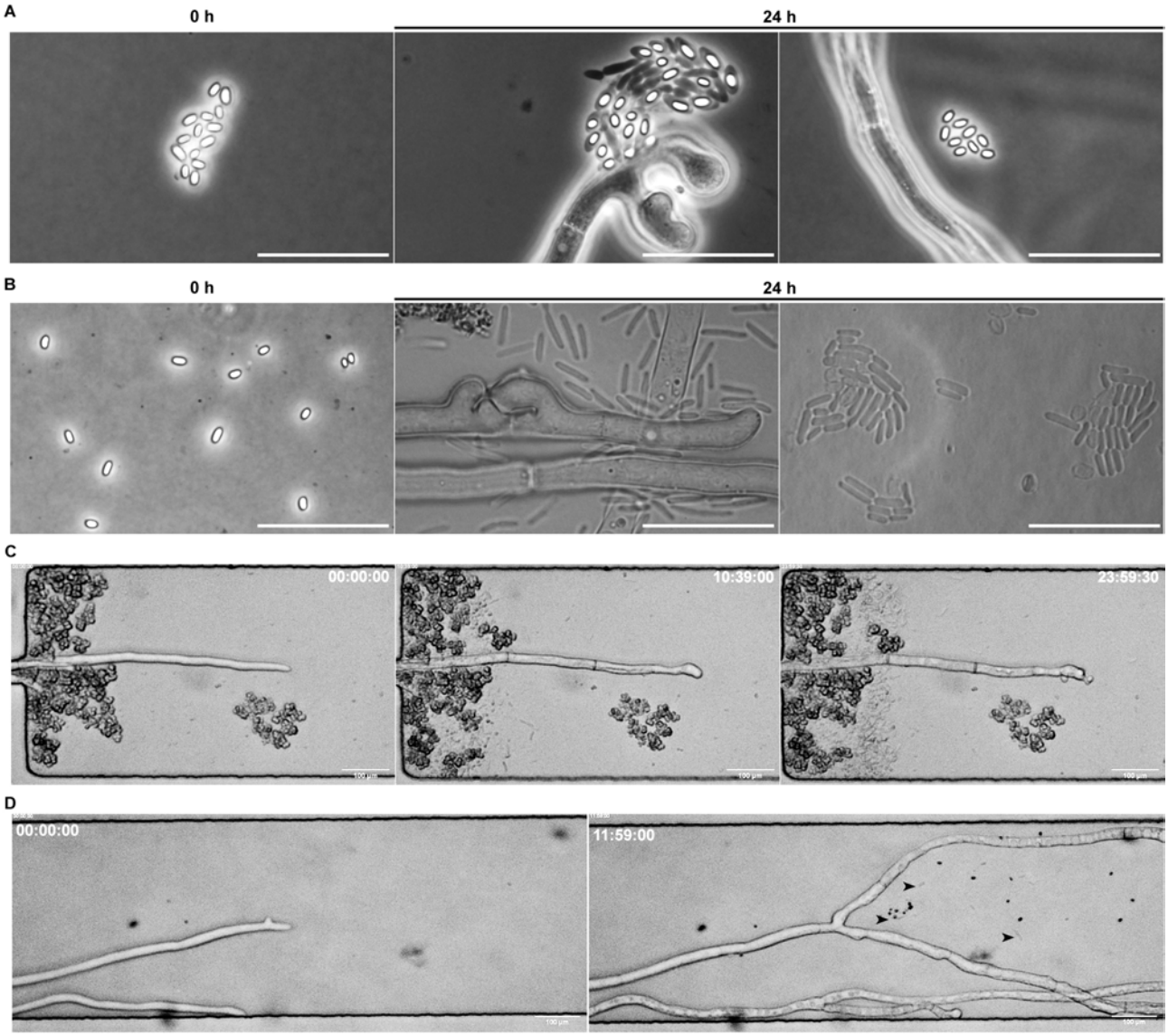


Figure 4.4. Germination and growth in presence of actively growing mycelium on agarose pads (A-B) and in BFI devices (C-D). A*. Bacterial spores inoculated with B. cinerea germinated when they were in presence with a hyphal tip. Scale bar 20 µm.* ***B****. In presence of S. sclerotiorum WT, cells were actively moving around the mycelium after germination. Germination also occurred when the spores were not in direct contact with the hyphae. Scale bar 20 µm.* ***C****. In BFI devices filled with R2B, spores also germinated with S. sclerotiorum WT. The presence of vegetative cells appeared to slow down the growth of S. sclerotiorum (Full timelapse movie in Sup. Data. 4.7)* ***D****. Some spontaneous germination occurred in presence of S. sclerotiorum Δoah mycelium in BFI devices, however, the growth of the fungus was not impacted by the presence of the bacteria (Full timelapse movie in Sup. Data. 4.8).*

of 8 g/L of Kox seemed to be deleterious for the bacterial growth. Indeed, germination and growth happened faster with KOx as carbon source and vegetative cells showed a deformed shape at this concentration. Only 0,3 and 0,65 g/L of oxalic acid, which is also soluble in water (13 g/L), supported growth. Germination was not observed at a concentration of 4 g/L. This indicates a potential toxicity of oxalic acid for the cells, which is consistent with the use of this chemical as a disinfectant (ref). Oxalic acid was shown to be effective against *Acidovorax citrulli*, responsible for the bacterial fruit blotch, at a concentration of 5 g/L (Gao *et al.*, 2017), but its overall toxicity for oxalotrophic bacteria is currently unknown. Soluble oxalate sources appeared to accelerate the growth of *A. oxalaticus* strain HJ without supporting

sporulation. No sporulation events were observed with KOx as carbon source, nor with oxalic acid. Divalent cations, in particular calcium ions, are required for the formation of the endospore and it was shown that insufficient concentrations of calcium in the medium reduces significantly the sporulation (Minh *et al.*, 2011; Sella *et al.*, 2014). It would therefore be interesting to investigate if the addition of a calcium source to the medium would enable *A. oxalaticus* strain HJ to complete its life cycle with KOx or oxalic acid as carbon sources. Germination and growth in presence of oxalogenic fungi was different depending on the fungus used. Both *B. cinerea* and *S. sclerotiorum* WT are known producers of oxalic acid (Hegedus & Rimmer, 2005; van Kan, 2005, 2006; Williamson *et al.*, 2007; Williams *et al.*, 2011). Measurements of the concentration of oxalic acid in liquid medium showed that *S. sclerotiorum* WT produces two-times more oxalic acid than *B. cinerea* in R2B (0,65 and 0,3 g/L, respectively; cf. Chapter 1-2). Observations with chemogenic sources of oxalate showed that a concentration of 0,3 g/L of oxalic acid was not sufficient to support the optimal growth of *A. oxalaticus* strain HJ and the same was observed in presence of the actively growing *B. cinerea* mycelium. Only a few spores germinated when in direct contact with the hyphal tip while ungerminated spores were found along the mycelium. This seems to indicate that the production of oxalic acid during the growth of *B. cinerea* is not sufficient to promote the formation of a niche for *A. oxalaticus* strain HJ. On the other hand, vegetative cells of the oxalotrophic EFB were found in abundance near *S. sclerotiorum* WT mycelium. Vegetative cells moved actively in the vicinity of the hyphae and no ungerminated spores were observed. When co-inoculated with the *S. sclerotiorum* mutant deficient in oxalic acid production, only a few spores germinated spontaneously and the vegetative cells were not seen to be actively moving. These observations indicate that oxalic acid production by *S. sclerotiorum* WT effectively supported the germination and the growth of *A. oxalaticus* strain HJ and promoted the formation of new ecological niches. Lastly, the presence of actively growing *A. oxalaticus* strain HJ cells seemed to slow down *S. sclerotiorum* WT fungal growth. Given that oxalotrophic bacteria have been shown previously to control the growth of *S. sclerotiorum* WT (Dickman & Chet, 1998; Schoonbeek *et al.*, 2007; Marina *et al.*, 2019), it would be interesting to further investigate the potential of oxalotrophic EFB as biocontrol agents against this oxalogenic pathogenic fungus *in vivo*.

Conclusion

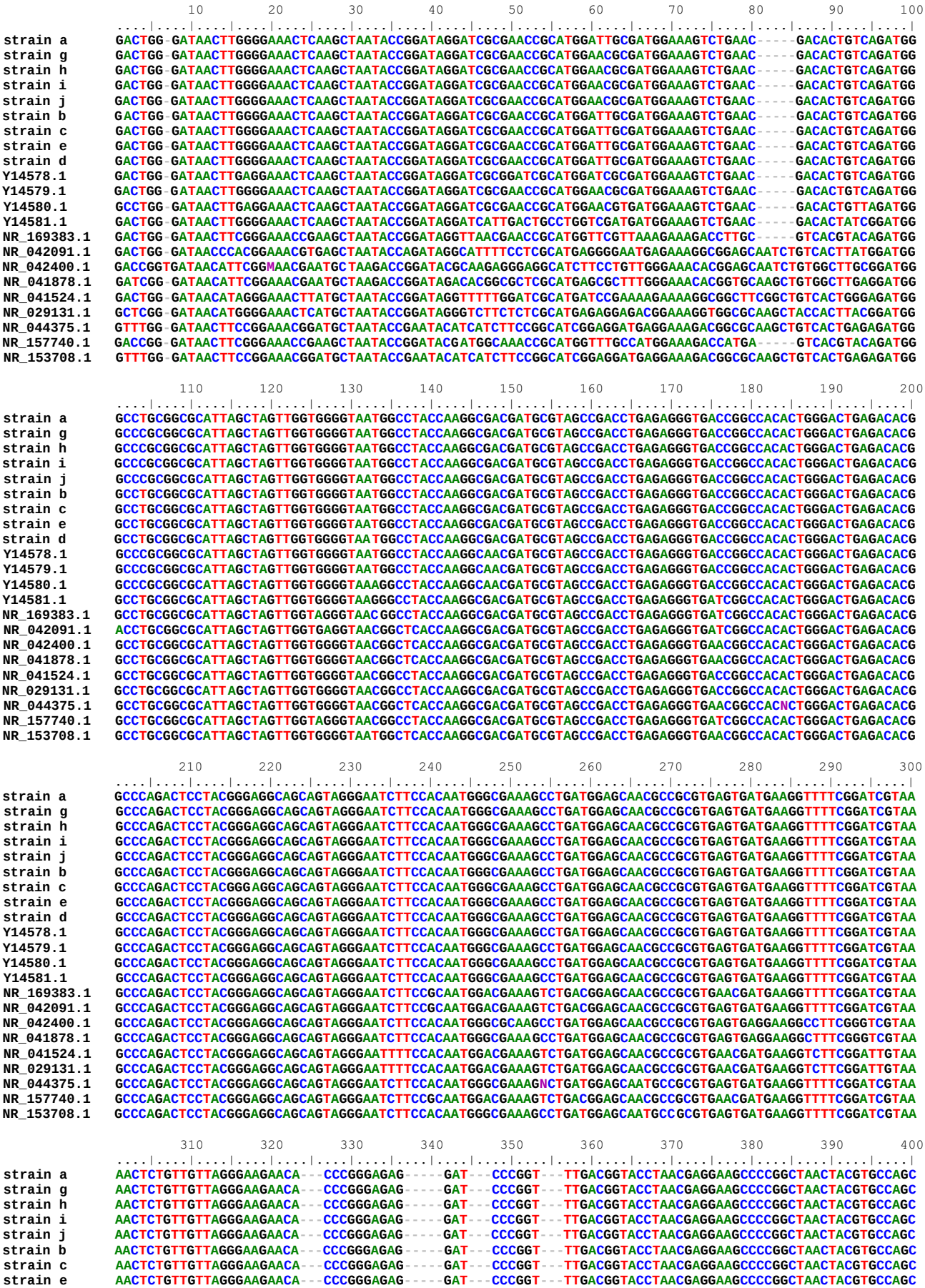
By combining a treatment to separate spores from vegetative cells and a traditional enrichment method, we were able to selectively enrich and isolate an oxalotrophic EFB using oxalate salts commonly found in association with oxalogenic fungi. The isolated strains showed different characteristics than strains previously known, indicating the potential for the isolation of new oxalotrophic EFB. *A. oxalaticus* strain HJ thrived in presence of *S. sclerotiorum* WT mycelium in medium otherwise unfavourable for its growth. This highlights the ability of oxalogenic fungi to promote the formation of new ecological niches for oxalotrophic bacteria in environments devoid of oxalic acid.

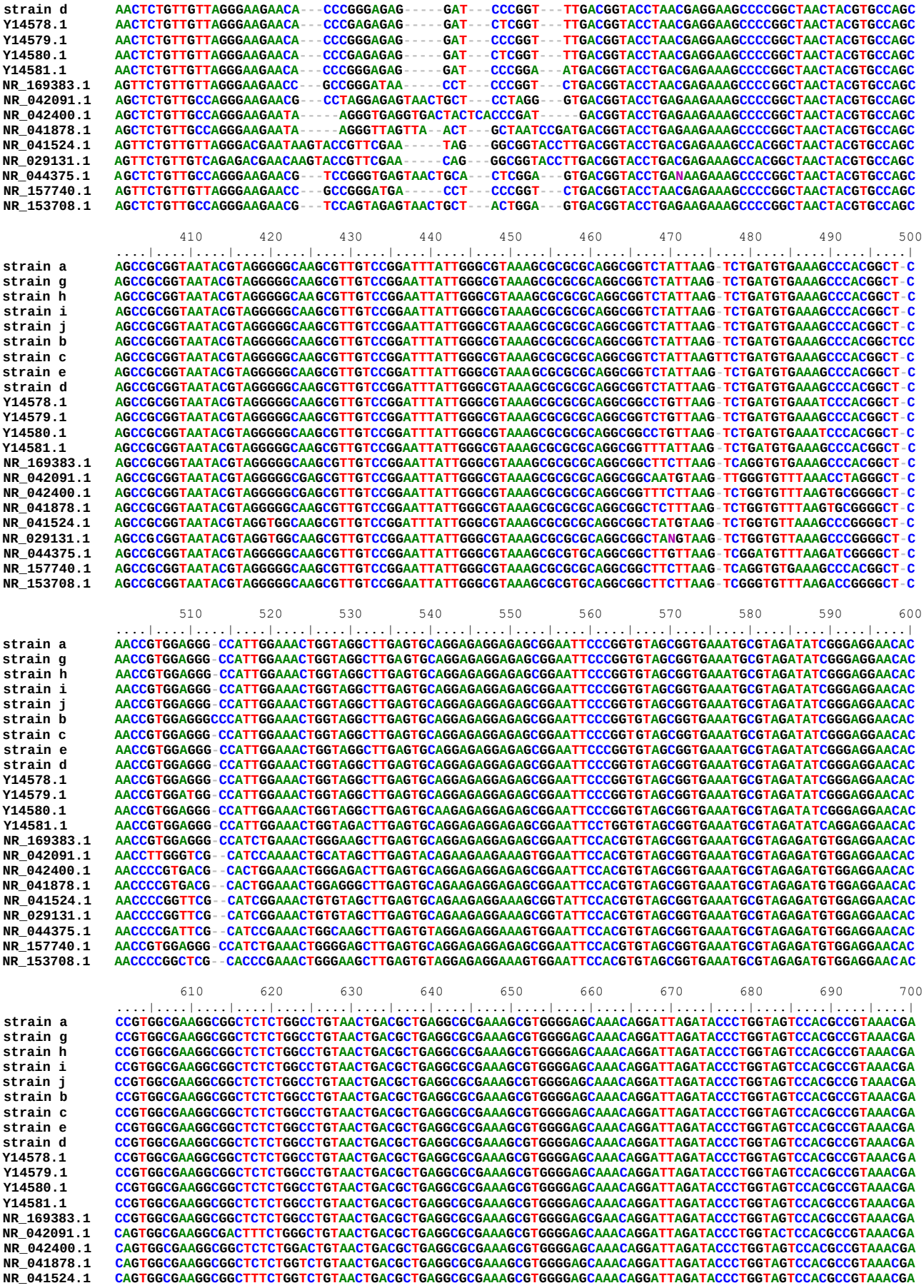
Supplementary data

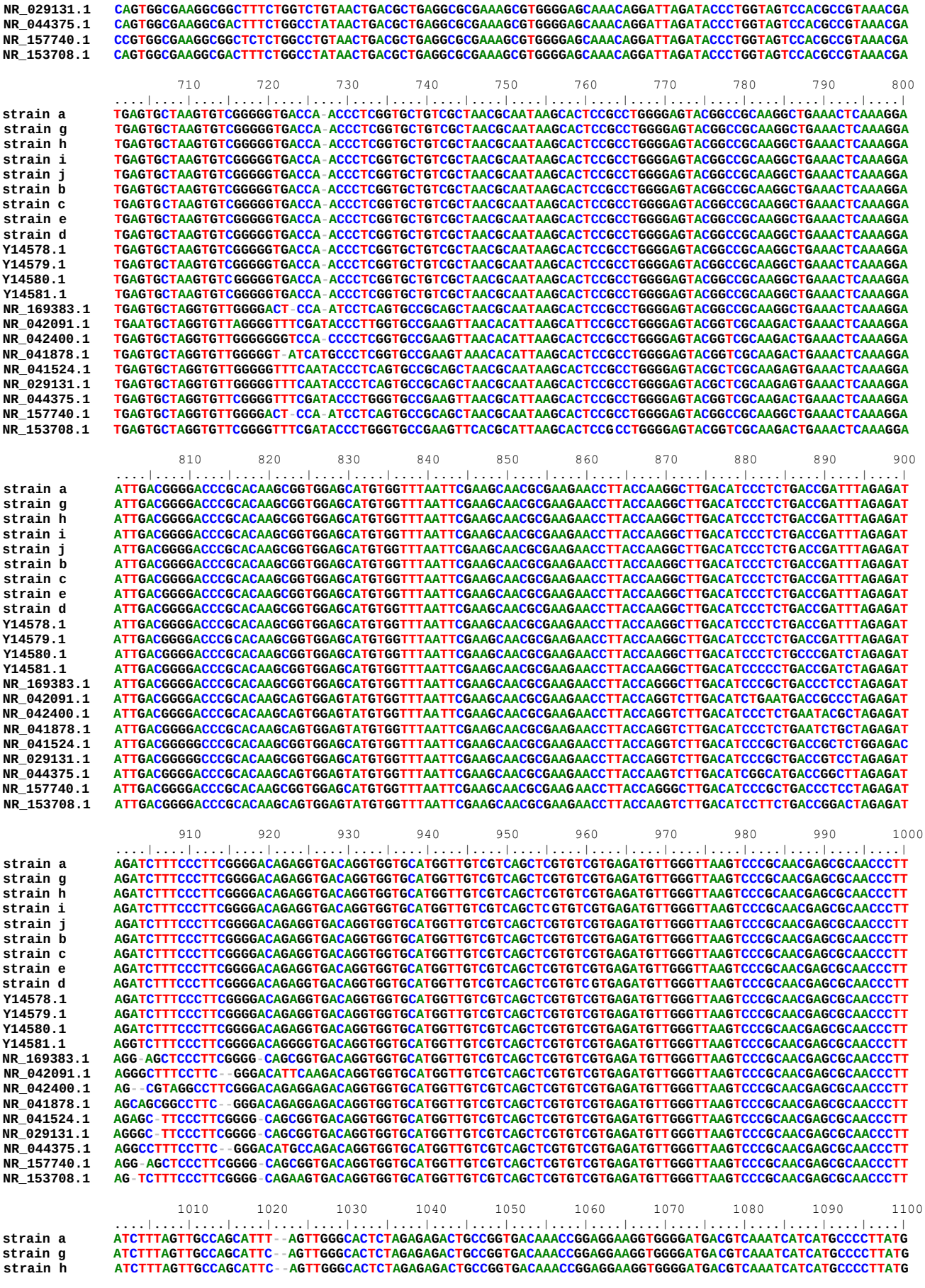
Program	T°C	Time
Initial phase		
Denaturation	95	1 min
Denaturation	95	15 sec
Annealing	59	15 sec
Elongation	72	30 sec
Cycles	40x	
Final		
Elongation	72	2 min
End	4	∞

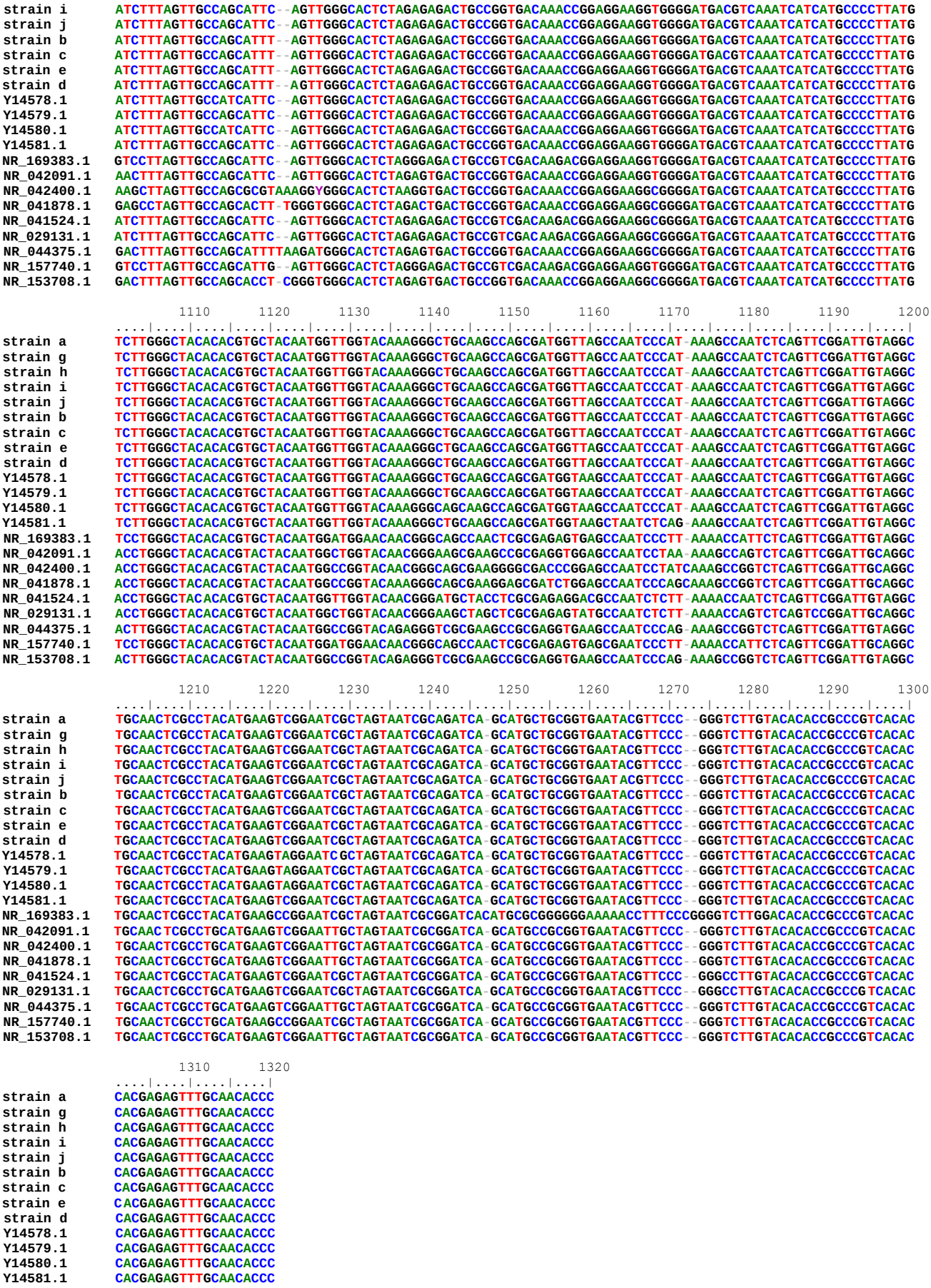
Sup. Data 4.1. PCR program for 16S rRNA gene amplification with the primers Eub 9-27F and 907r

Sup. Data 4.2. Aligned sequences used for the sequence identity matrix and the generation of the phylogenetic tree.

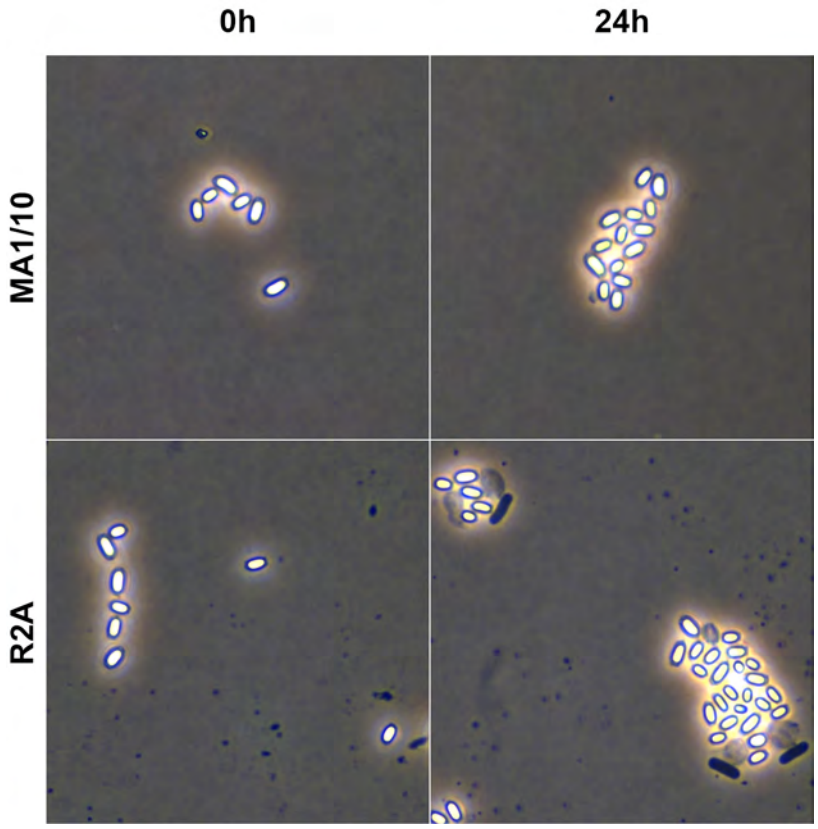



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NR_153708.1   CACGAGAGTTTACAACACCC
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Sup. Data 4.3. A. oxalaticus strain HJ spores on MA1/10 and R2A agarose pads. *No germination occurred on MA1/10, while some spores spontaneously germinated on R2A.*

Sup. Data 4.4. Timelapse of *A. oxalaticus* strain HJ spores germination and growth on Schlegel AB-KOx 4 g/L. Images were acquired every 3 min for 15 h.

Sup. Data 4.5. Timelapse of *A. oxalaticus* strain HJ spores germination and growth on Schlegel AB-CaOx 4 g/L. Images were acquired every minute for 15 h.

Sup. Data 4.6. Recording of *A. oxalaticus* strain HJ vegetative cells swimming around *S. sclerotiorum* WT mycelium on R2A agarose pads.

Sup. Data 4.7. Timelapse of *A. oxalaticus* strain HJ spores germination and growth in a BFI device filled with R2B and inoculated with *S. sclerotiorum* WT. Images were acquired every 30 sec for 24 h.

Sup. Data 4.8. Timelapse of *A. oxalaticus* strain HJ spores germination and growth in a BFI device filled with R2B and inoculated with *S. sclerotiorum* Δ*oah* . Images were acquired every 30 sec for 12 h.

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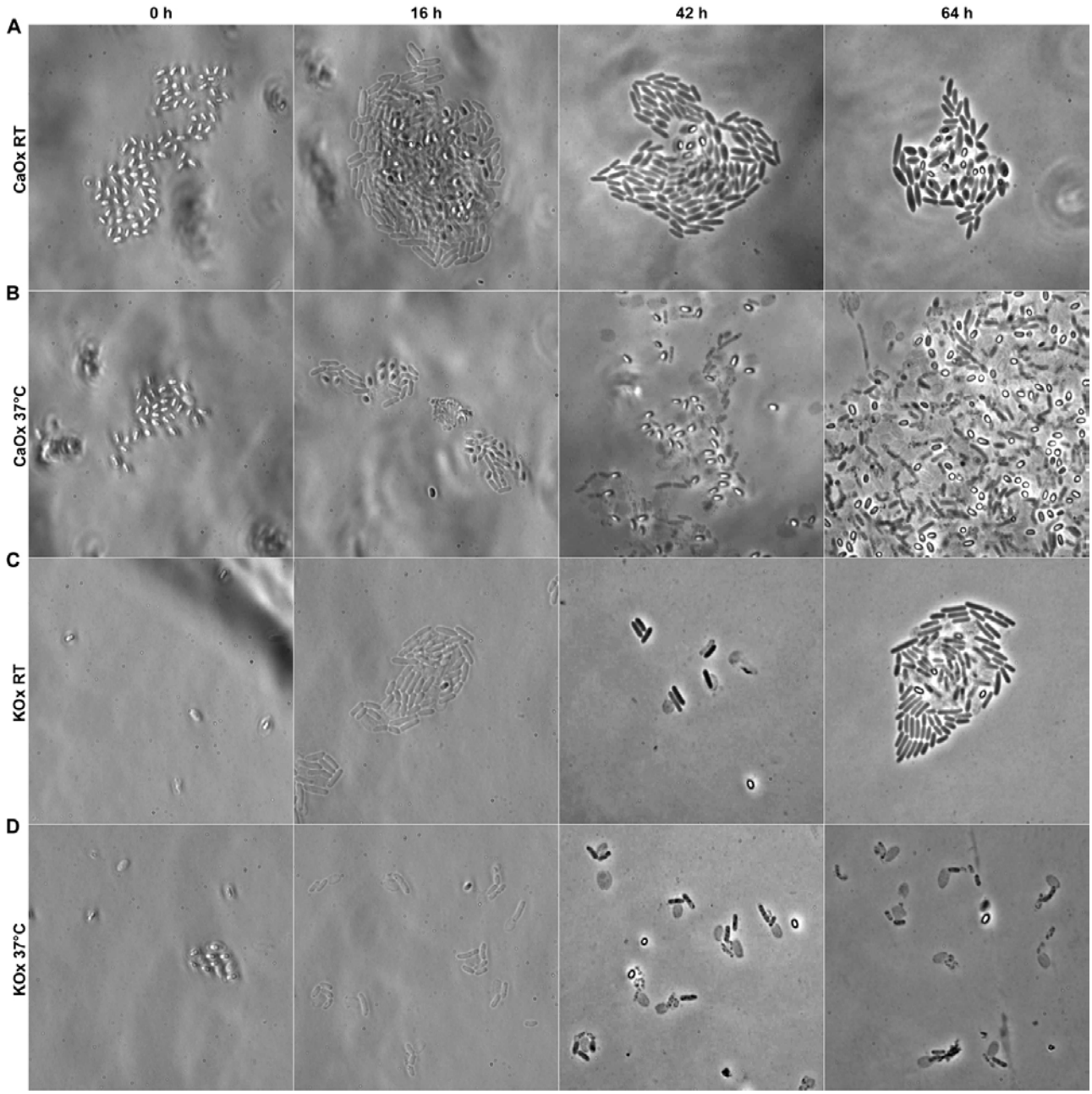
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Supplementary information

Germination with CaOx and KOx at two temperatures

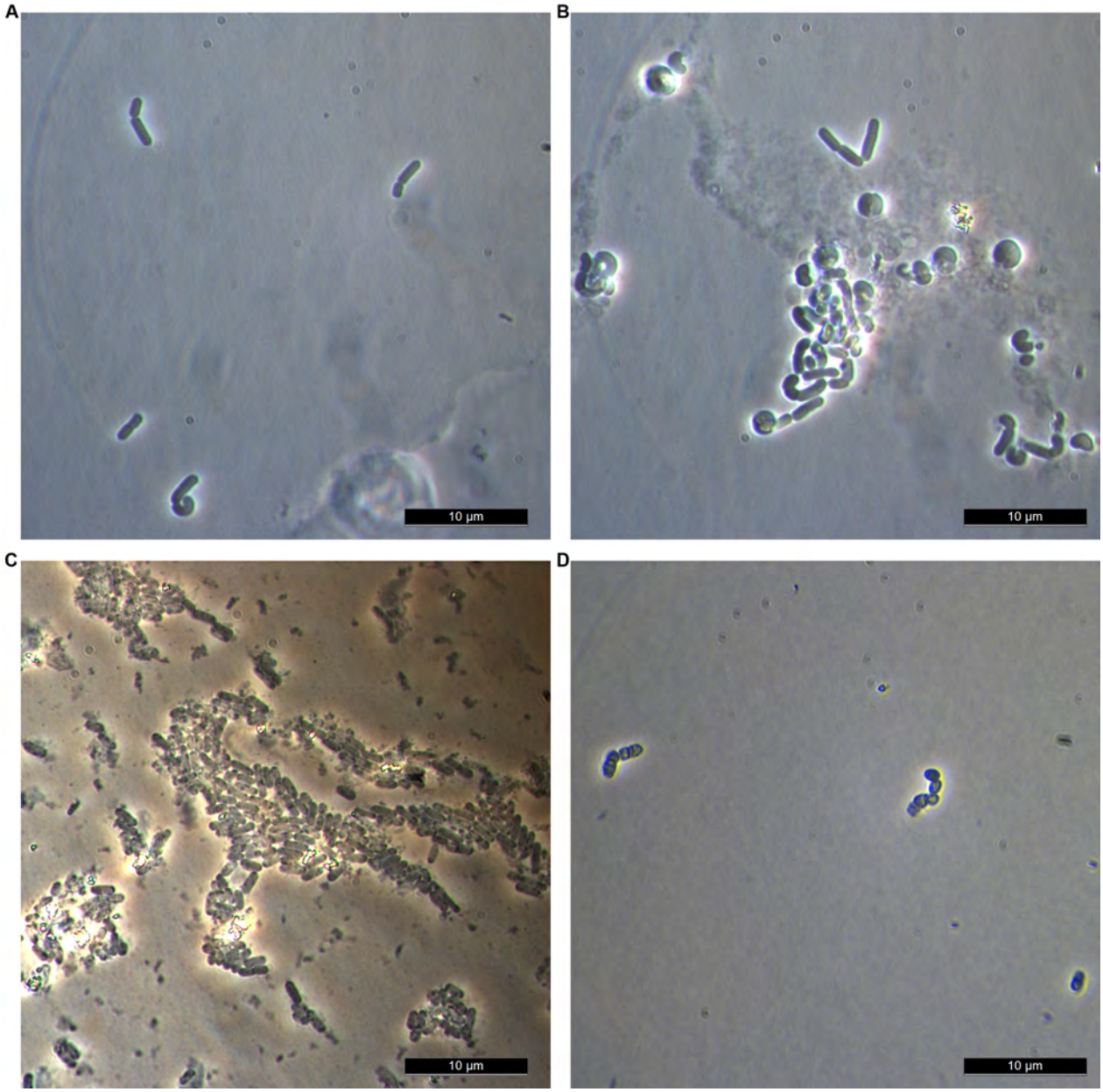
One aim of this chapter was to assess the potential of oxalotrophic EFB as biocontrol agents against plants and human fungal pathogens (Fabio Palmieri PhD thesis 2021). Therefore, germination and growth of the isolated strains were monitored overtime at room temperature and at 37°C, the human body temperature, on Schlegel AB agarose pads supplemented with 4 g/L of CaOx or KOx (Sup. Info. 4.1). The presence of spores was assessed at the inoculation time, then germination and growth were assessed quantitatively at 16, 42 and 64 h. At room temperature, germination and growth occurred in the first 16 h with CaOx as carbon and energy source (Sup. Info 4.1A-B). Sporulation seemed to happen after 42 h, several cells showed a terminal enlargement and endospores were visible in some cells after 64 h (Sup. Info. 4.1A). Sporulation happened faster at 37°C, and free spores were already present in the medium after 42 h (Sup. Info. 4.1B). Spores also germinated and grew in the first 16 h of incubation with KOx as carbon source (Sup. Info. 4.1C-D). Free spores were observed after 42 h of incubation, however, they are most likely ungerminated spores (Sup. Info. 4.1C-D). Germination also occurred at 37°C, however, only few vegetative cells were observed (Sup. Info. 4.1D). Moreover, cells appeared to lose their integrity after 42 h.



Sup. Info. 4.1. Germination and growth of A. oxalaticus strain HJ on Schlegel AB agarose pads using two sources of oxalate (at 4 g/L oxalate) and at two temperatures. A-B*. Germination and growth of A. oxalaticus on calcium oxalate (CaOx) at room temperature (**A**) and at 37°C (**B**). At room temperature sporulation occurred after 42 h, while at 37°C free spores were already present in the medium at the same time point.* ***C-D****. Germination and growth on potassium oxalate (KOx) at room temperature (**C**) and at 37°C (**D**). At room temperature A. oxalaticus germinated and grew, however no sporulation events occurred, while at 37°C, cells seemed to lose their integrity after 42 h. The free spores visible were likely ungerminated spores. Microscopy images taken with XX microscope at a 100x magnification.*

Liquid cultures in Schlegel AB medium supplemented with KOx, CaOx and NH_4-Ox

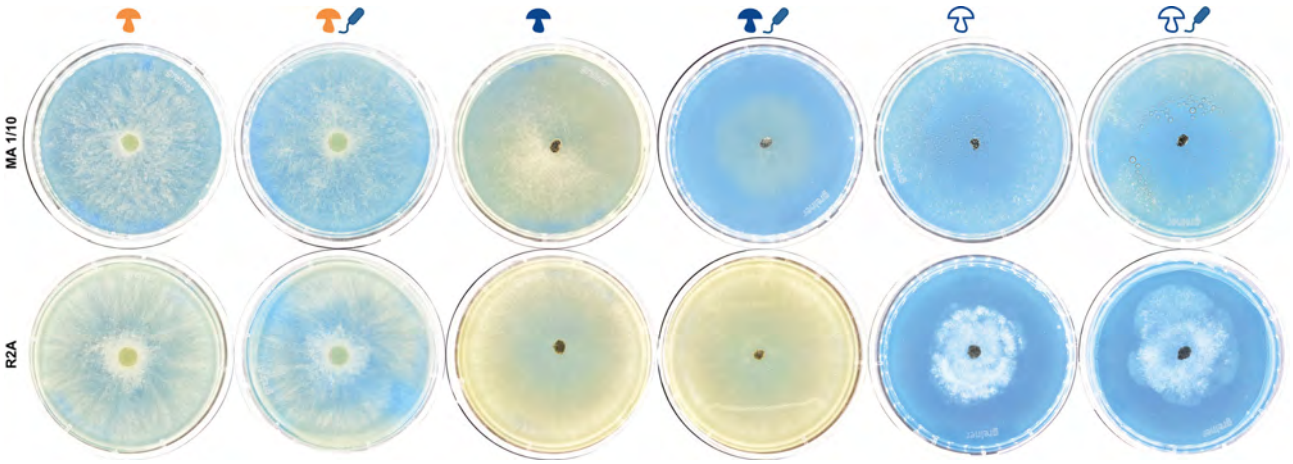
In order to produce large quantities of spores and extract genomic DNA, the isolated strains were inoculated in liquid Schlegel AB medium, supplement with 4 g/L of KOx, CaOx and ammonium-oxalate (NH_4-Ox). However, liquid cultures were quite unsuccessful (Sup. Info. 4.2). Spores germinated but the vegetative cells did not have the usual rod-shape. With KOx (Sup. Info. 4.2A-B), we either observe an asymmetrical division of the cells (Sup. Info. 4.2A) or curved little cells (Sup. Info. 4.2B). With CaOx (Sup. Info. 4.2C), cells were smaller that usual and their integrity seemed compromised. Finally, with NH_4-Ox, there was only very few vegetative cells and they were completely round (Sup. Info. 4.2D). No spores were observed after several days of incubation and we were unable to extract genomic DNA



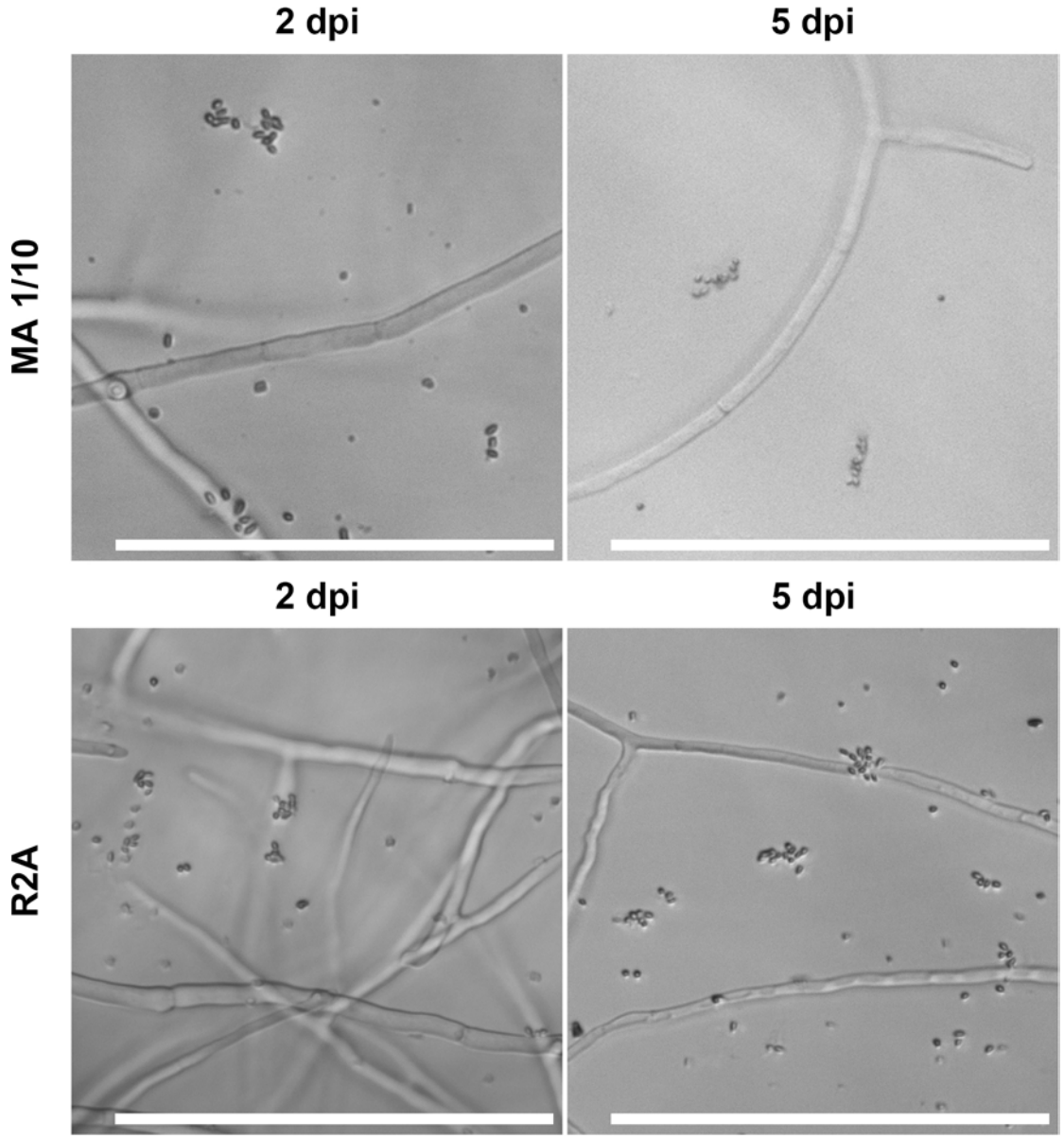
Sup. Info. 4.2. Microscopy images from liquid cultures in Schlegel AB medium supplemented with 4 g/L of KOx (A-B), CaOx (C) and NH_4-Ox (D). A-B. *With KOx,* ***o****nly a few vegetative cells were observed. The cells showed an asymmetric division (****A****) or a strong curvature which made them look like fortune cookies (****B****).* ***C****. Vegetative cells were found in large quantities with CaOx as carbon source, however, cells looked smaller and their integrity seemed compromised.* ***D****. With NH_4-Ox, vegetative cells were all round.*

Confrontation assays with *B. cinerea, S. sclerotiorum* WT and *S. sclerotiorum* Δ*oah*

To evaluate the potential of the isolated strains as biocontrol agents, they were confronted to *B. cinerea*, *S. sclerotiorum* WT and *S. sclerotiorum* Δ*oah* on MA1/10 and R2A (Sup. Info. 4.3). Fungal growth was not reduced or controlled in presence of the bacteria, and all fungi colonised the plates after 5 days of incubation. Confrontation experiments were also performed with *S. sclerotiorum* WT with the drops method (Matteo Buffi PhD Thesis), as inverted microscopy can be performed directly on the plates (Sup. Info. 4.4). No germination occurred when the spores were inoculated with the fungus.



Sup. Info. 4.3. Confrontation assays between A. oxalaticus strain HJ and the oxalogenic fungi B. cinerea and S. scletoriorum (wild type strain and oxalate deficient mutant) on MA1/10 and R2A plates. *From left to right: B. cinerea; B. cinerea + A. oxalaticus; S. scletoriorum WT; S. sclerotiorum WT + A. oxalaticus; S. sclerotiorum Δoah; S. sclerotiorum Δoah + A. oxalaticus. For each treatment, pictures were taken 5 dpi. Fungal growth was not controlled by A. oxalaticus.*



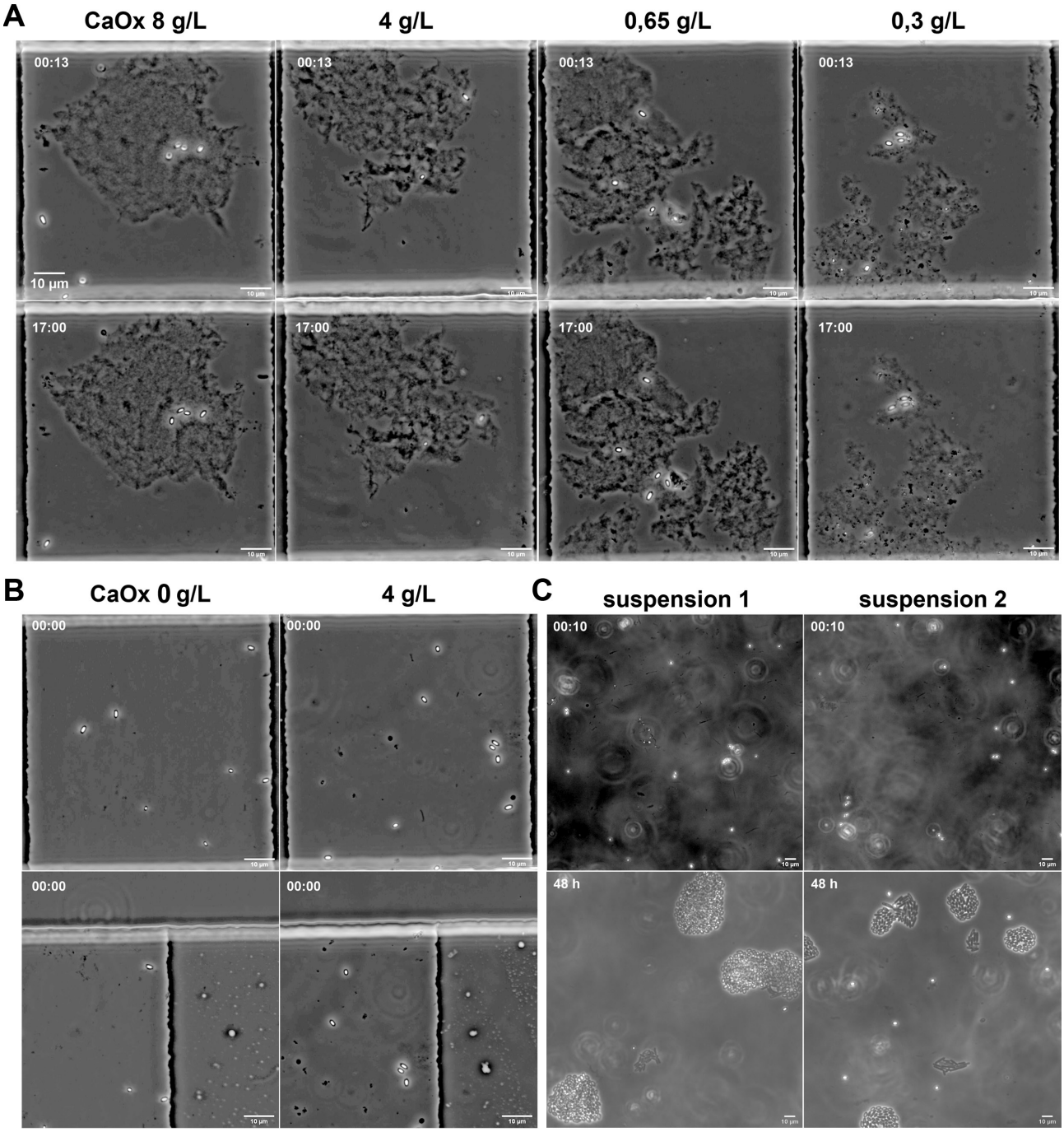
Sup. Info. 4.4. Confrontation assays between A. oxalaticus strain HJ and S. sclerotiorum WT in drops of MB1/10 (A) and R2B (B). *Inverted microscopy images were taken directly on the plates 2 and 5 days post inoculation (dpi; scale bar Xx µm). No germination occurred and only spores were found after incubation.*

Germination experiments in microfluidic devices

One of the limitations of the agarose pads is the impossibility to maintain the same concentration of oxalate over time. Therefore, germination experiments were conducted in microfluidic devices, in collaboration with Léa Bernier, a PhD student under the supervision of Dr Claire Stanley. We used the improved version of the two- and four-conditions devices from Täuber *et al.* (2020) that Léa Bernier is developing to follow the spores germination in presence of different sources of oxalate. A laminar flow of medium supplies chambers on both side of the supply canal by diffusion and there is normally no flow inside the chambers. In the two-conditions devices, changing the flow rate allow us to change the condition in the switch zone of the medium, thus controlling the concentration of oxalic acid in the medium.

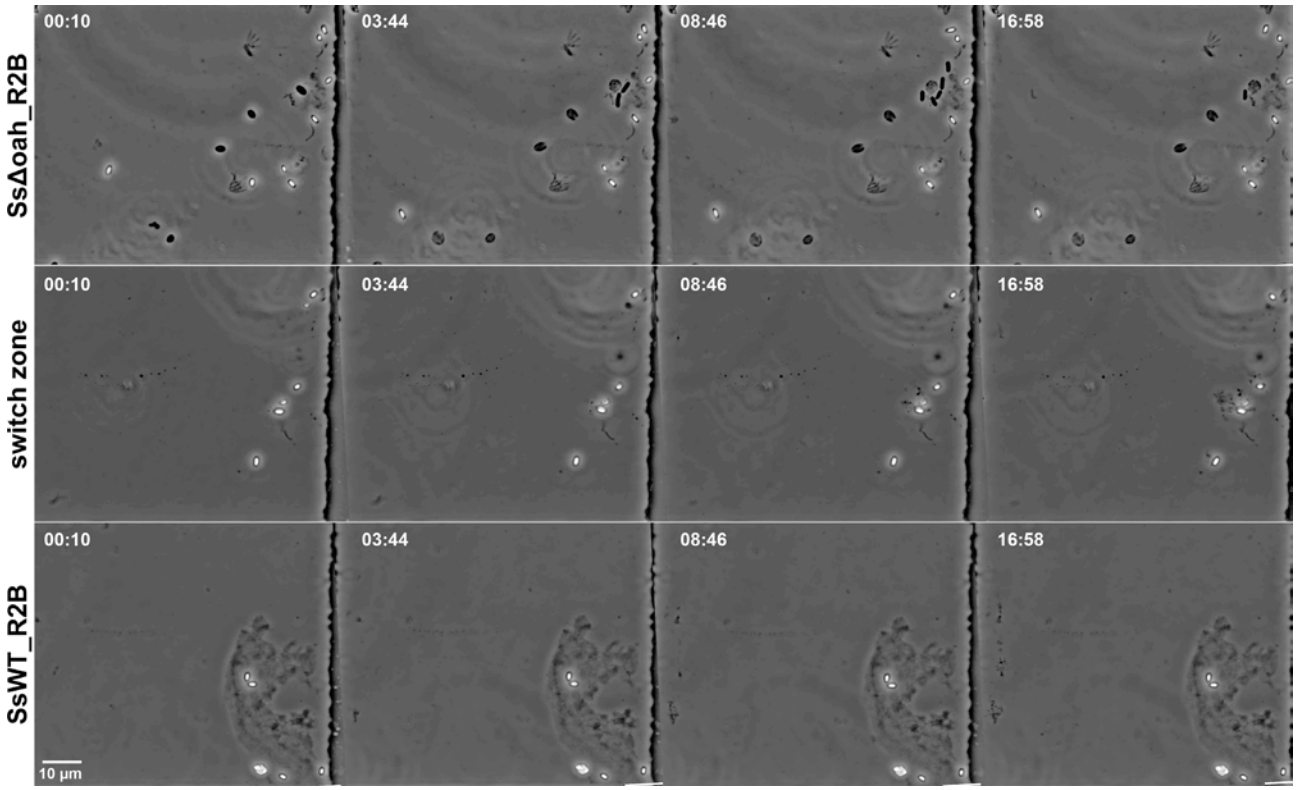
In order to test whether *A. oxalaticus* strain HJ spores were germinating in the device, spores were inoculated in a four-conditions device with liquid Schlegel AB medium with four concentrations of CaOx (0,3; 0,65; 4 and 8 g/L; Sup. Info 4.5A). However, no germination occurred after 17 h on incubation. As spores were found encased in agar wastes from the spore suspension, the experiment was repeated in a two-conditions device with 0 and 4 g/L of CaOx after washing the spores suspension to remove the agar wastes (Sup. Info 4.5B). A few spores were present in each chamber but no germination occurred after 3 h of incubation whereas spores usually germinate after 30 min. Spores viability from three different spores suspension was therefore assessed on Schlegel AB agarose pads supplemented with 4 g/L of CaOx (Sup. Info 4.5C). Spores germinated just fine on the pads, indicating that the spores and the medium were not the problem. Germination was then assessed in a two-conditions device with spent media from *S. sclerotiorum* WT (SsWT_R2B) and *S. sclerotiorum* Δ*oah* (SsΔ*oah*_R2B) cultures in R2B (Sup. Info 4.6). The spent medium from the mutant, devoid of oxalic acid was used as negative control. Interestingly, no germination occurred in the switch and positive side supplied by SsWT_R2B, the spent medium containing oxalic acid, while germination occurred in the negative side supplied with SsΔ*oah*_R2B, the medium devoid of oxalic acid. As the flow of medium in the device could have been a problem, we inoculated a device with spores and medium but did not apply any flow (Sup. info. 4.7A). Again, no germination occurred except for two spores highlighted by a red circle. All the results seemed to indicate that the problem was with the device itself, not the medium, the spores or the flow. We then decided to flush the devices with a saline solution before inoculation, in order to remove any uncured PDMS from the devices. After flushing, the device was inoculated with the spores and Schlegel AB-CaOx 4 g/L (Sup. info. 4.7B). Germination occurred as expected in less than 30 min in all acquired chambers (8/8 chambers). Therefore, an additional washing step was added in the preparation of the device, to guaranty that all uncured PDMS was removed from the microfluidic devices.

All problems seemed to be solved, therefore, a two-conditions device was inoculated with Schlegel AB-CaOx at 0 and 4 g/L (Sup. info. 4.8A). Again, no germination occurred after 16 h of incubation. A few spores germinated, but only when there was a high concentration of



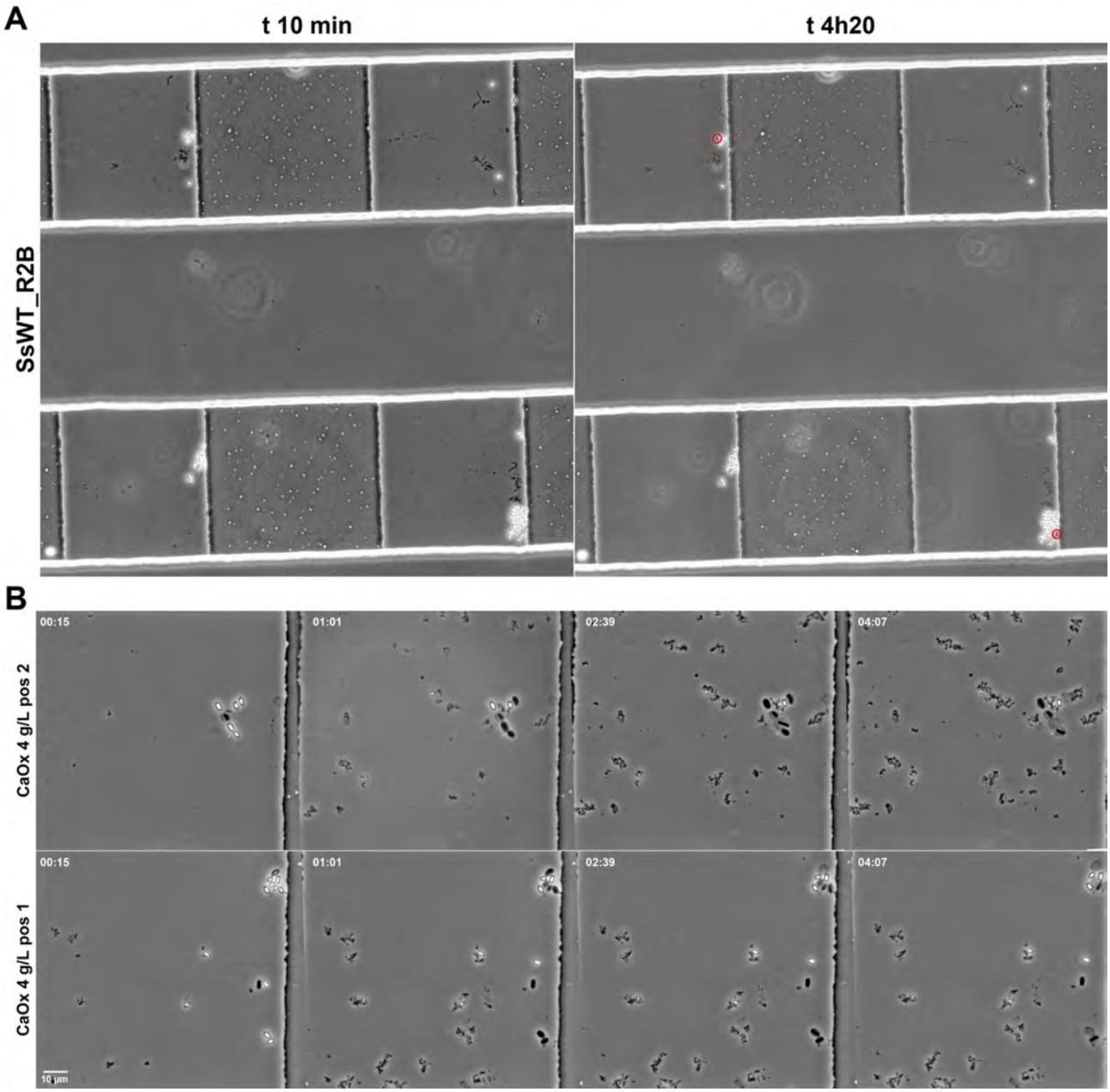
Sup. info. 4.5. A. oxalaticus strain HJ spores in a four-conditions device (A), a two-conditions device (B) and on agarose pads (C). A*. The device was filled with with Schelgel AB with 0,3; 0,65; 4 and 8 g/L of calcium oxalate (CaOx). No growth occurred after 17h of incubation and A. oxalaticus strain HJ spores were encased in agar wastes from the cell suspension.* ***B.*** *The two-conditions device was filled with Schelgel AB with 0 and 4 g/L of calcium oxalate (CaOx). Cell suspension was washed to remove agar waste but no growth occurred after 3h of incubation.* ***C.*** *In order to verify the viability of the cells, A. oxalaticus strain HJ spores were inoculated on agarose pads made of Schlegel AB medium supplemented by 4 g/L of CaOx. Microscopy images were taken after the inoculation and after 48h (10 µm scale bar).*

cells in the chambers. Also, as CaOx has a very low solubility in water, most of the oxalate precipitated in the supply syringe before entering the device. This result highlighted two problems, first, that *A. oxalaticus* spores germinated better when they were in a crowd and second, that Schlegel AB-CaOx was not a suitable medium to work with the devices

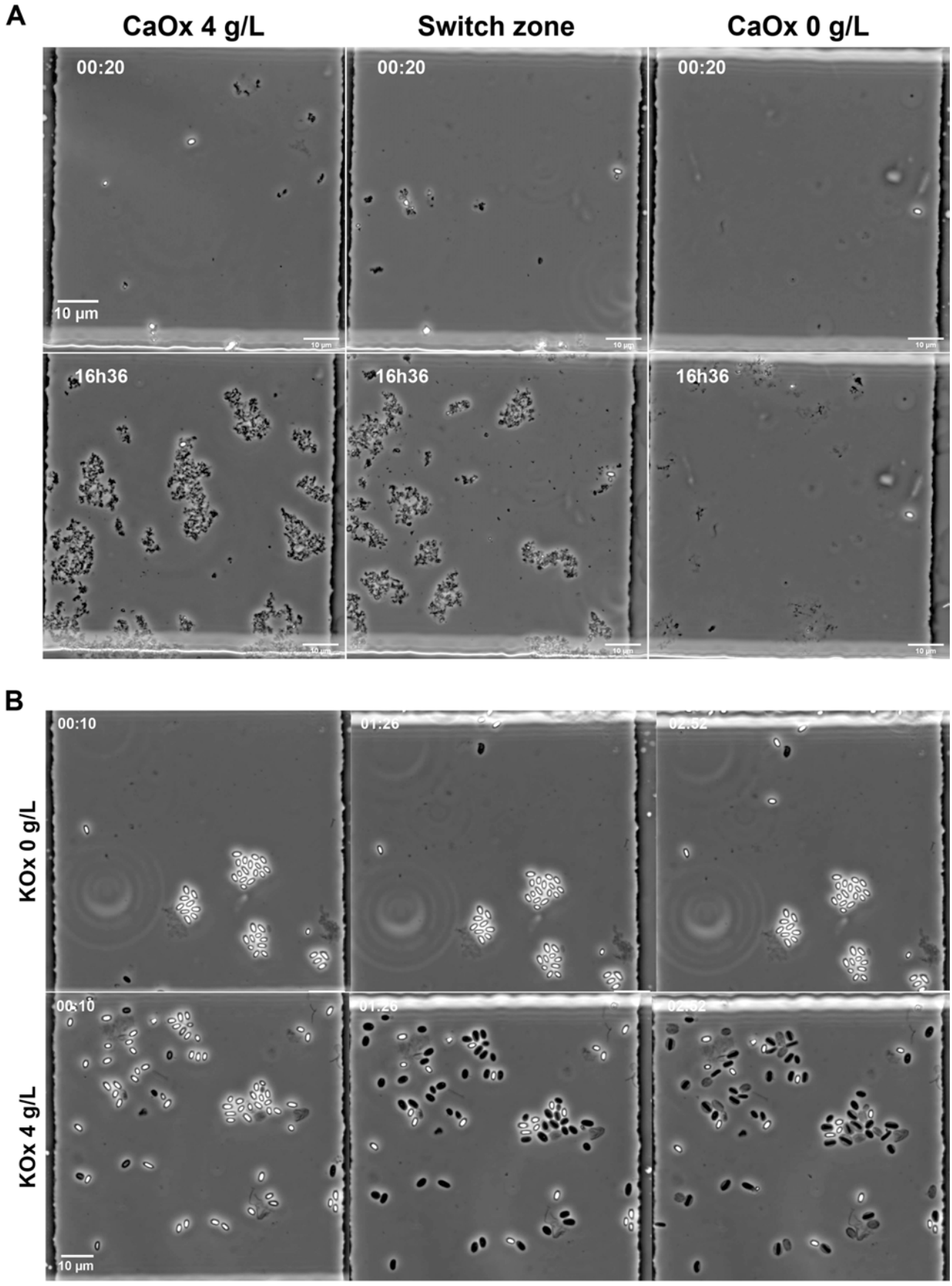


Sup. info. 4.6. Snapshots of A. oxalaticus strain HJ spores in a non-washed two-conditions device filled with spent medium from S. sclerotiorum WT (SsWT_R2B) or S. sclerotiorum Δoah (SsΔoah_R2B) cultures in R2B. *Germination occurred only in SsΔoah_R2B, the medium devoid of oxalic acid. No growth occurred in the both zones filled with SsWT_R2B (middle and last lines).*

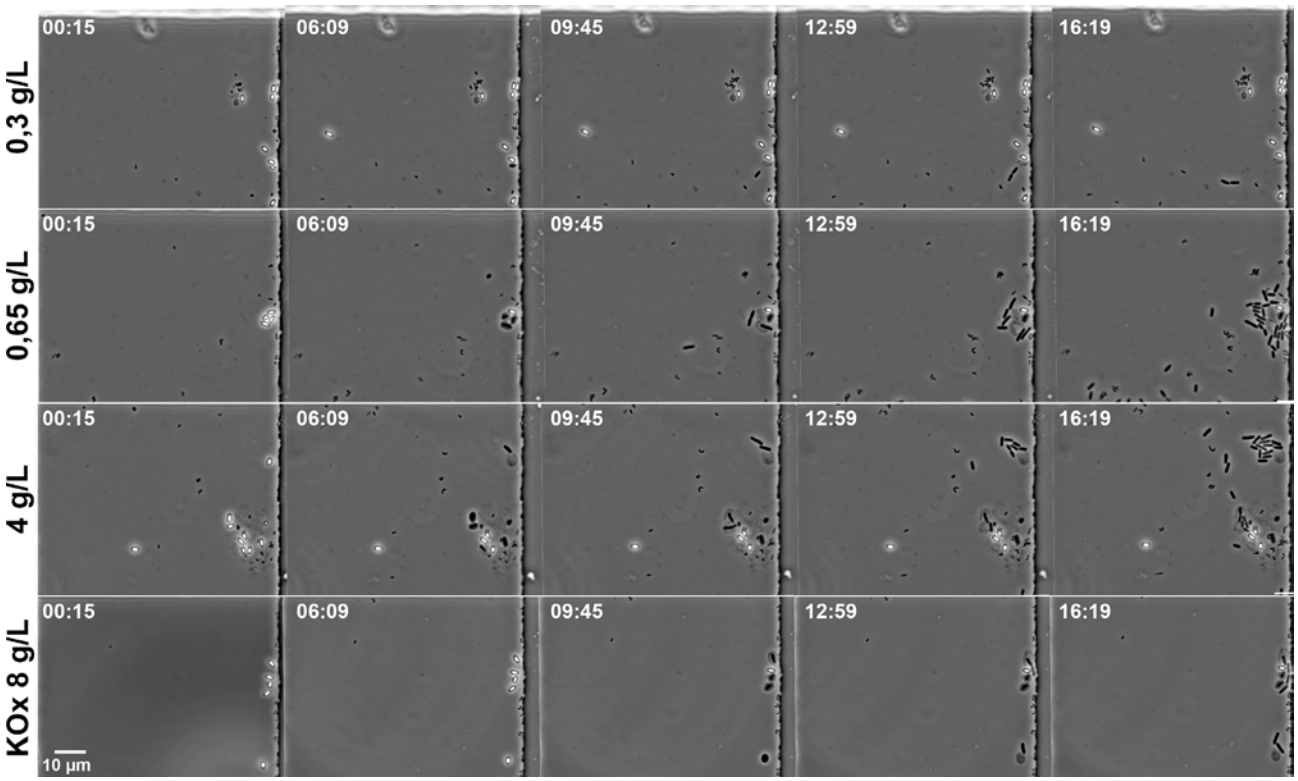
because of the low solubility of CaOx. We switched the medium to Schlegel AB-KOx, as KOx is more soluble in water, and repeated the experiment with 0 and 4 g/L of KOx and with a high concentration of cells in the chambers (Sup. info. 4.8B). Most spores germinated in all chambers recorded (8/10 chambers), however, another problem arose. *A. oxalaticus* strain HJ is a highly motile bacterium and upon germination, most cells would escape the chambers, making it difficult to follow their growth. Still, we decided to do a last experiment with the four-conditions devices, with Schlegel AB-KOx at four different concentrations (Sup. info. 4.9). The concentration of spores in each chamber was less than ideal, and there was a contamination, but we were still able to follow the growth of some individual bacteria over 16 h of incubation. Germination occurred before 6 h in 4 and 0,65 g/L (Sup. info. 4.9, second and third lines) in all four chambers recorded. With 0,3 g/L, germination occurred for one spore only (Sup. info. 4.9, first line). With 8 g/L of KOx, germination was delayed to 9 h after inoculation (Sup. info. 4.9, last line). As the device was unfortunately contaminated, it is not possible to draw any conclusions from this experiment. However, the results obtained with the microfluidic devices were not necessarily consistent with the results previously obtained. This suggests that this kind of device is maybe not the best experimental design to investigate the germination and growth of environmental bacteria with complex media and low solubility nutrients.



Sup. info. 4.7. A. oxalaticus strain HJ spores in a two-conditions devices with no flow (A) and in a flushed device (B). A. *The device was filled with with spent medium from S. sclerotiorum WT (SsWT_R2B) cultures in R2B and no flow was applied to the device, to determine if the flow was preventing the bacteria from germinating. Only two spores went form bright to dark phase in 4h.* ***B****. The device was flushed beforehand with a saline solution to remove any residues of PDMS before being filled with Schlegel AB with 4 g/L of CaOx.. As expected, A. oxalaticus spores went from bright to dark phase in less than 20 min and germination and growth occurred in 4h. Microscopy images were taken in different chambers in the device (pos 1, pos 2) every two minutes.*



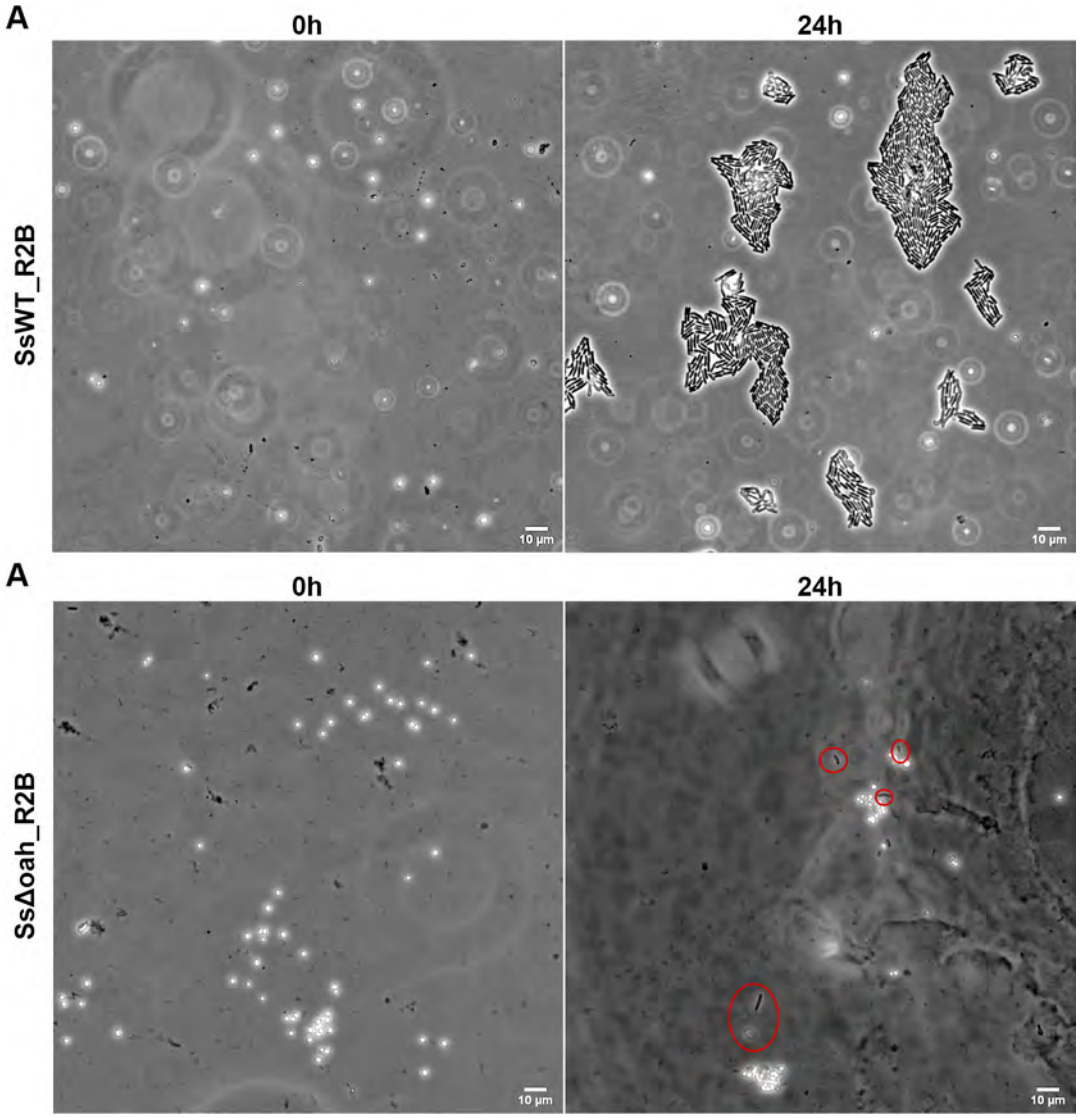
Sup. info. 4.8. A. oxalaticus strain HJ spores in a washed two-conditions device with Schlegel AB supplemented with CaOx (A) or KOx (B). A. *No germination occurred when the medium was supplemented with CaOx, as the CaOx was precipitating before entering the device.* ***B.*** *With KOx as carbon source, a more soluble source of oxalate, the spores germinated in all recorded chambers.*



Sup. info. 4.9. Snapshots of A. oxalaticus strain HJ spores in a washed 4-conditions device filled with Schlegel AB medium supplemented with 0,3; 0,65; 4 and 8 g/L of potassium oxalate (KOx). *Spore concentration per chamber was less than ideal, however, A. oxalaticus strain HJ germinated and grew in all conditions. Germination occurred first in 4 g/L, then in 0,65 and 0,3 g/L and lastly in 8 g/L.*

Germination and growth on agarose pads with *S. sclerotiorum* spent media

In order further characterise the full life cycle (germination, growth and sporulation) of *A. oxalaticus* strain HJ in presence of biogenic oxalate, germination was assessed on agarose pads prepared with spent media from *S. sclerotiorum* WT and Δ*oah* cultures in R2B (SsWT_R2B and SsΔoah_R2B respectively; Sup. Info. 4.10). With SsWT_R2B spent medium, *A. oxalaticus* strain HJ spores were able to germinate and grow in 24 h (Sup. Info. 4.10A). With spent medium devoid of oxalic acid, SsΔoah_R2B, only a few spores spontaneously germinated (Sup. Info. 4.10B).



Sup. Info. 4.10. Growth of A. oxalaticus strain HJ on agarose pads made of spent medium from S. sclerotiorum WT (SsWT_R2B; A) or S. sclerotiorum Δoah (SsΔoah_R2B; B) cultures in R2B*. Microscopy images were taken after the inoculation and after 24h (10 µm scale bar).* ***A****. SsWT_R2B supported A. oxalaticus strain HJ germination and growth.* ***B****. Aox spores showed spontaneous germination on SsΔoah_R2B (red circles), a spent medium devoid of oxalic acid.*

General discussion

Discussion

The aim of this thesis was to offer new insights in BFI in the case of oxalotrophy as a biocontrol strategy and in the search for new biocontrol agents against oxalogenic phytopathogenic fungi. From the initial screening to the commercialisation, the development of a successful biocontrol agent requires several steps (Fravel, 2005). Most of the potential BCA fail to enter into the market, due to the limitations of the agent to control the pathogenic microorganism in the field, its limited shelf-life or the cost of production (Stewart, 2001; Fravel, 2005). Moreover, when designing *in-vitro* biocontrol assays, it is important to target the right phase of the life cycle when screening for the most promising biocontrol agents, to understand the mechanism of action of the biocontrol agent, and to take in account that environmental factors play a major role in the efficacy of the agent to control a pathogenic fungus (Whipps, 1997; Fravel, 2005; Pal & McSpadden, 2006). Ideally, a successful BCA against phytopathogenic fungi would consist of a motile bacterium naturally encapsulated that would become active in presence of the specific fungal metabolite and return to dormancy once the pathogen has been controlled. Hence, an example of an ideal BCA would be that of an oxalotrophic endospore-forming bacteria (EFB) that germinate in presence of biogenic oxalic acid and that controls the growth of oxalogenic phytopathogenic fungi. However, the search of new BCA is not straightforward and several problems needed to be addressed before one can start to determine the potential of oxalotrophic EFB as biocontrol agents. Figure 5.1 presents the advances made during this thesis in the search of this ideal BCA, starting from the development of methods for the isolation of oxalotrophic EFB from a soil sample. The figure presents the main findings for each chapter in order (Chapter 1: Methods; Chapter 2: OA or pH with Chapter 3 about the chemotaxis signals; Chapter 4: EFB) and highlights the fact that much remains to be done to find the ideal BCA.

In order to assess the potential of oxalotrophic EFB as biocontrol agent, the first step was to develop methods tailored for *in-vitro* confrontation assays with the cellular structures that are more relevant during the early establishment of disease. Biocontrol assays are often conducted with actively growing mycelium whereas the starting point of a disease is often the germination of a spore or a sclerotia (Agrios, 2004). Most *in-vitro* biocontrol assays are performed on agar plates, with the bacteria inoculated as a droplet or streaked in the surface and with the fungus inoculated from a plug of actively growing mycelium in the centre of the plate (Li *et al.*, 2014; Gkarmiri *et al.*, 2015; Swain *et al.*, 2017). This is an easy and straight-forward method to assess the efficiency of a bacterium as biocontrol agent, as the non-contact zones observed around an efficient biocontrol bacterial strain are clearly visible. However, when the aim the biocontrol strategy is to test the impact of the bacterium on the germination of fungal spores or sclerotia, this inoculation mode is far from ideal as there is no initial contact between the two organisms. Moreover, the use of agar plates makes it difficult to recover soluble metabolites or to measure important environmental parameters such as pH. One method to recover soluble metabolites is to inoculate the fungus on a cellophane

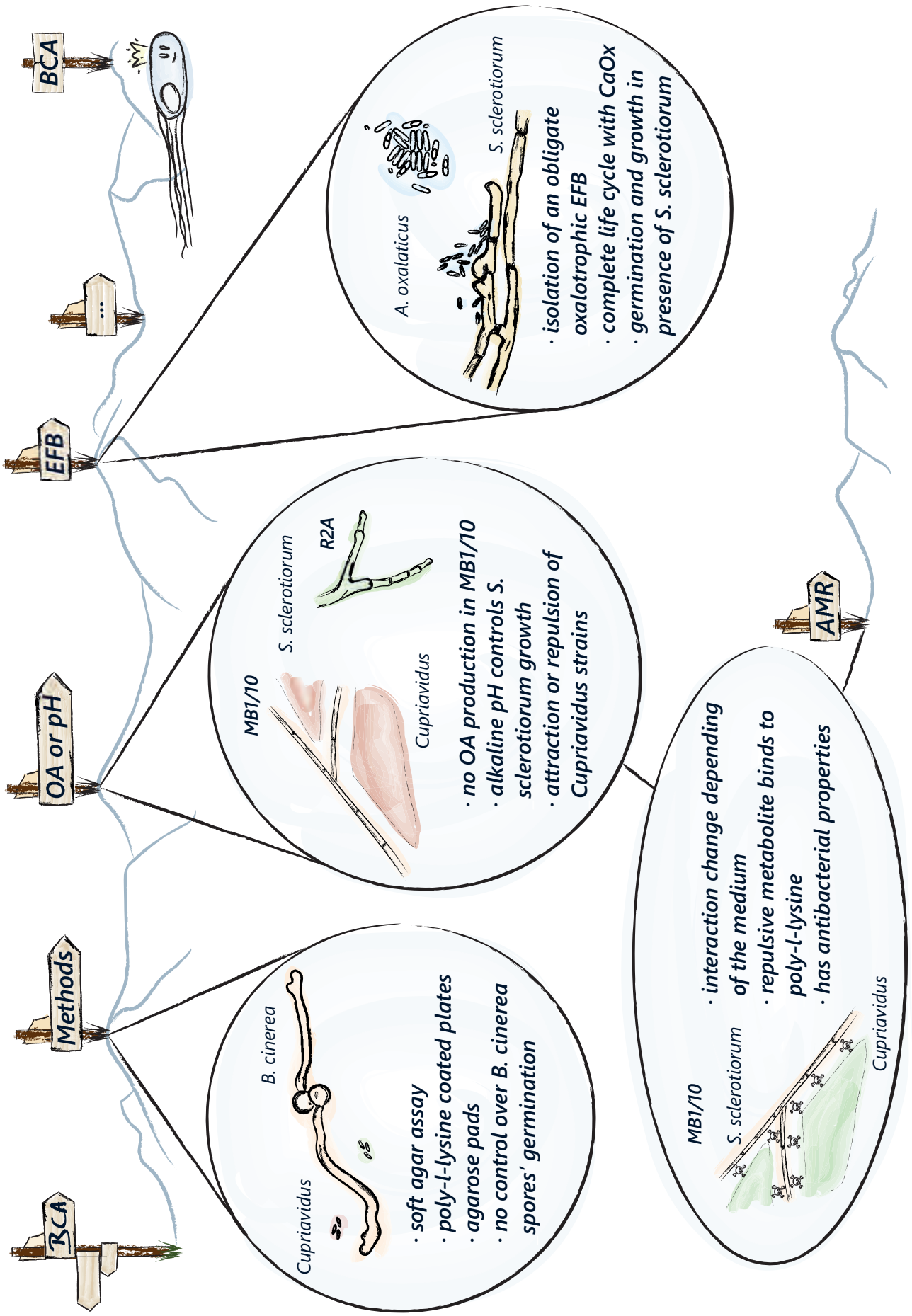


Figure 5.1. Summary of the mains findings of the thesis in the search of an ideal bacterial biocontrol agent (BCA). *From left to right: Chapter 1 (Methods); Chapter 2 (OA or pH) and Chapter 3; Chapter 4 (EFB).*

sheet deposited on top of glass beads, however, the whole process is long and tedious (Guennoc *et al.*, 2017). The three methods developed in Chapter 1 (soft agar confrontation assays, the cell culture (poly-l-lysine coated) plates and the agarose pads) allowed us to assess the control of the germination *Botrytis cinerea* spores by *Cupriavidus necator* and *Cupriavidus oxalaticus*. Each method allowed us to take a closer look at the interactions happening between this oxalogenic fungus and the oxalotrophic bacteria. As a result, we highlighted the fact that the medium composition played a major role in the production of oxalic acid, and that oxalotrophic bacteria were unable to control the germination of *B. cinerea* spores. Oxalic acid is required for the successful colonisation of the host plant by *B. cinerea*. However, it is difficult to pin-point the exact moment when oxalic acid is produced by the pathogen (van Kan, 2006; Williamson *et al.*, 2007). Our findings seem to indicate that the production of oxalic acid begins once the spore has successfully germinated, as the mycelial growth was rapidly hindered by the presence of *C. necator* on agarose pads. This is interesting and indicate that oxalotrophic bacteria might not be the ideal BCA choice to control the germination of *B. cinerea* spores.

One important thing to keep in mind with oxalotrophy as a biocontrol strategy is the fact that fungal growth control has always been attributed to oxalic acid consumption, whereas bacterial oxalotrophy also directly impacts the physicochemical properties of the environment (Aragno & Verrecchia, 2012). As a matter of fact, they are three factors important in the development of a disease: a susceptible host, a pathogen, and a favourable environment for the pathogen (Moore *et al.*, 2011). If one of the factors is removed from the equation, no disease occurs. Therefore, as acidic conditions stimulate the production and activity of fungal enzymes, our hypothesis was that the alkalinisation of the environment by the oxalotrophic bacteria would make the environment unfavourable for the fungal pathogen and therefore control its growth. Thus, the goal of Chapter 2 was to disentangle oxalic acid consumption from the impact of the BCA on pH. Confrontation experiments with *C. necator* and *C. oxalaticus* and *Sclerotinia sclerotiorum* showed that the alkalinisation of the medium was responsible for the control of fungal growth. Both bacteria were able to alkalinise the medium when inoculated alone, however, in confrontation assays, the bacteria were unable to successfully alkalinise MB1/10 medium and to control fungal growth. The fact that *S. sclerotiorum* Δ*oah*, the mutant deficient in oxalic acid production, was also controlled by the oxalotrophic bacteria raises up an interesting point. If the control of *S. sclerotiorum* is due to the alkalinisation of the medium and not directly to the consumption of oxalic acid, can we control the pathogen raising the pH by other means?

The importance of the medium composition on the production of oxalic acid by oxalogenic phytopathogenic fungi and on the outcome of the interaction with biocontrol bacteria was once again highlighted in this second chapter. Oxalic acid production occurred only in R2B but not in MB1/10. It is unfortunate that we were unable to determine what LMWOA was produced by *S. sclerotiorum* in MB1/10, as it would have given us more insights on the

metabolism of LMWOA production in oxalogenic fungi. Also, the opposite reactions of *Cupriavidus* strains towards *S. sclerotiorum* mycelium were puzzling. Further investigations on the metabolites regulating the chemotactic behaviour between *S. sclerotiorum* and the *Cupriavidus* strains (Chapter 3) showed that the spent medium with the putative attractant metabolite did not trigger an attraction while the repulsive metabolite was not only binding to the poly-l-lysine coating the plates, but also had antibacterial properties. Membrane proteins and polysaccharides were shown to play a major role in the successful attachment of bacteria to fungal hyphae (Raineyt, 1991; Gaddy *et al.*, 2009; Frey-Klett *et al.*, 2011). As the spent media from *S. sclerotiorum* cultures in R2B did not trigger chemotaxis in *C. necator*, it would therefore be interesting to further investigate the role of polysaccharides and other extracellular proteins in the attraction of *Cupriavidus* strains for *S. sclerotiorum* mycelium.

It would also be interesting to investigate the BFI occurring between *S. sclerotiorum* and the *Cupriavidus* strains in other media. The fact that *S. sclerotiorum* did not produce oxalic acid but an antibacterial metabolite in MB1/10 was unexpected and gave us the opportunity to study an interaction that we were not aware of. Most *in-vitro* confrontations experiments are performed only with one medium, usually PDA, a medium rich in carbon (Jin *et al.*, 2011; Droce *et al.*, 2013; Li *et al.*, 2014; Slama *et al.*, 2019; Ramírez-Cariño *et al.*, 2020; Kjeldgaard *et al.*, 2022). We showed here that the medium plays a major role on microbial metabolism and therefore using only one medium to test the biocontrol capacities of a given microorganism severely limits our understanding of the biocontrol mechanism. To expand our understanding of the multiple interactions occurring between the same set of microorganisms, we should systematically use different media, in order to assess the effect of trophic factors on the microorganisms and their interactions. We observed here two antagonistic interactions, but it would be interesting to know if we can observe other type of interaction by using a third medium. The use of the poly-l-lysine coated plates also played a major role in the discovery of the antibacterial properties of the metabolite produced. Without the observation of the neat arrangement of the bacterial cells at fixed distance from the hyphae, we would not have further investigated the interactions occurring and discovered that *S. sclerotiorum* was producing this antibacterial metabolite. The discovery of this metabolite is also particularly relevant in the light of the current antimicrobial resistance crisis occurring worldwide. Most antibiotics are used simultaneously in human health, crops, and animal husbandry, which has led to the emergence of multidrug-resistant pathogens (FOPH & FFSVO, 2022). The search for new antibiotics is also slow as the fraction of cultivable microorganisms is quite small. To overcome the problem of cultivability, different methods were developed to either cultivate microorganisms in their environment and to find genes responsible for the production of antibiotics in environmental DNA (Ling *et al.*, 2015; Hover *et al.*, 2018). With these methods, two new antibiotics were discovered, teixobactin and malacidin. Both showed a strong activity against multidrug-resistant bacteria and open the doors for the discovery of new antibiotics. However, we showed here that by using innovative methods and different media, we can also discover new antimicrobial metabolites in cultivable species. It would therefore

be interesting to further investigate if we can find other antimicrobial metabolites in the numerous microorganisms already used for regular work in laboratories around the world.

The last advance we made in search of an ideal BCA was the isolation of *Ammoniphilus oxalaticus* strain HJ, using oxalate salts commonly associated with fungal mycelium (Cromack *et al.*, 1979; Gadd, 1999). Several oxalotrophic EFB were isolated from soil and sediments samples; however, the oxalate source was NH_4-Ox, an oxalate source not reported to be associated with fungal mycelium (Dehning & Sehink, 1989; Zaitsev *et al.*, 1998). The fact that *A. oxalaticus* strain HJ was able to germinate and grow with oxalate sources both chemically and naturally produced, as well as in presence of *S. sclerotiorum* mycelium, is promising for its future use as BCA. Several endospore-forming *Bacillus* spp. are already used as BCA (Edwards & Seddon, 1992; Pal & McSpadden, 2006; Wu *et al.*, 2015; Srivastava *et al.*, 2016; Mejri *et al.*, 2017). Borriss, (2015) showed that *Bacillus* spp. germinated and colonised the roots of the plant host after inoculation; however, to the best of our knowledge, the germination of endospore in presence of a phytopathogenic fungi has never been assessed. The preliminary results showed that *S. sclerotiorum* fungal growth seemed reduced in presence of *A. oxalaticus* strain HJ vegetative cells, but numerous experiments still need to be performed in order to determine its potential as BCA.

It was unfortunate that experiments in microfluidics devices were not as successful as expected. However, all the experiments performed increased our knowledge of *A. oxalaticus* strain HJ metabolism. Indeed, the fact that *A. oxalaticus* strain HJ was only able to complete is life cycle with CaOx highlighted the importance of calcium in sporulation. Endosporulation has been extensively studied with *Bacillus* species and it has been shown that the sporulation process follows seven precise steps until the formation of a mature spore (Galperin *et al.*, 2012; Sella *et al.*, 2014; Tan & Ramamurthi, 2014). The core of the spore, that contains the genetic material, is surrounded by several membranes and the spore's coat (Sella *et al.*, 2014). To form the core, the cell undergoes several modifications, with the dehydration of the cytoplasm and the production of high concentrations of dipicolinic acid (DPA; Tan & Ramamurthi, 2014). DPA forms complexes principally with calcium ions, which increases to the resistance of the spores to adverse conditions (Sella *et al.*, 2014). DPA can also bind to other divalent ions; however, complexes with other ions decreases significantly the heat resistance of the spores, and removing calcium from the medium stops the sporulation process altogether (Minh *et al.*, 2011). As calcium is essential for the sporulation of *A. oxalaticus* strain HJ, we must first assess if the fungal exudates support the sporulation process. Indeed, one of the ideas behind the use of EFB as biocontrol agents is their capacity to sporulate once the fungal pathogen is controlled and their ability to germinate again in the event of a second fungal infection. Once this is assessed, numerous *in-vitro* and *in-vivo* experiments need to be done to assess the potential of *A. oxalaticus* strain HJ as biocontrol agent against oxalogenic pathogenic fungi.

In order to dwell deeper in the processes behind oxalotrophy as a biocontrol strategy, experiments were performed only *in-vitro* during this thesis. However, to validate the results obtained and further investigate the biocontrol strategy, *in-planta* experiments need to be performed. This is particularly important to assess the efficiency of *A. oxalaticus* strain HJ as a BCA against phytopathogenic fungi. Unfortunately, most successful BCA *in-vitro* fail to perform efficiently when confronted to pathogenic fungi in real conditions (Fravel, 2005). *In-planta* experiments pose several challenges, as the experimental conditions shift from strictly controlled conditions to complex ones (Besset-Manzoni et al., 2019). The first challenges will be to the survival of *A. oxalaticus* strain HJ and colonisation of the plant host, considering that this strictly oxalotrophic bacteria thrives only on media supplemented with oxalic acid. *A. oxalaticus* was isolated several times in soils samples, however it was never described as being part of the phyllosphere. Plants produce oxalic acid to ensure the proper functioning of the metabolism (Palmieri et al., 2018), therefore, the next step will be to assess the germination, growth and sporulation of the BCA in presence of oxalic acid produced by the host plant and then determine impact of the BCA on the plant and its microbial community. As the rational behind the use of oxalotrophic bacteria as biocontrol agents is that the spores would remain dormant in absence of the pathogenic fungus, the fact that plants naturally produce oxalic acid poses an additional challenge for future *in-planta* experiments. Indeed, it will be interesting to determine if *A. oxalaticus* strain HJ metabolisms changes the environmental pH and if this change alone is sufficient to prevent the germination and the growth of phytopathogenic fungi such as *B. cinerea* and *S. sclerotiorum*.

Conclusion

The research presented in this thesis offered new insights in BFI in the case of oxalotrophy as biocontrol strategy. Three novel methods to investigate BFI were successfully developed and published, and gave us the opportunity to deepen our understanding of oxalotrophy in biocontrol. This thesis also highlighted the importance of the trophic and abiotic factors on fungal metabolism and on the outcome of the interactions occurring between the model organisms investigated here. This showed the importance of innovative methods for the study of BFI, and that our understanding of said BFI is still quite limited. The discovery of the antibacterial metabolite produced by *S. sclerotiorum* also opened doors for further work on antibiotic development, which is currently a very important research topic. Finally, this thesis provides the basis for future studies with oxalotrophic EFB as biocontrol agents.

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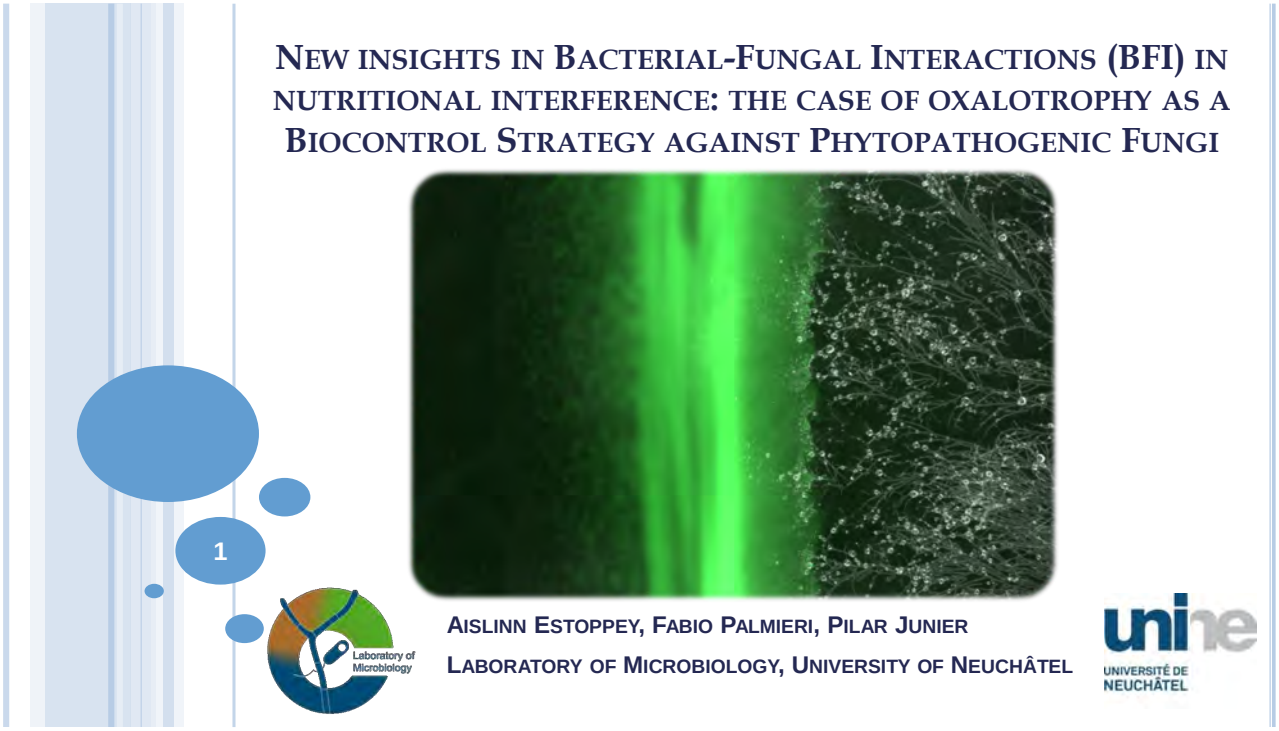
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Annexes

Presentations at national and international conferences

1 Oral presentation at the Swiss Society for Microbiology Annual congress 2018, CHUV – Lausanne, Switzerland, August 28-30, 2018



2 Poster presentation at the Annual PhD students meeting 2018 - Intellectual Property: from academia to industry, Neuchâtel, Switzerland, September 2018

Oxalotrophy as a biocontrol strategy

Aislinn Estoppey, Fabio Palmieri, Pilar Junier
Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Switzerland

Introduction

We need crops to feed the world !

BUT

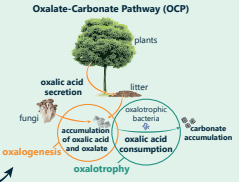
- important economical losses worldwide caused by photopathogenic fungi
- intensive use of fungicides → increased resistance to chemical weapons

► need of alternatives such as

BIOCONTROL

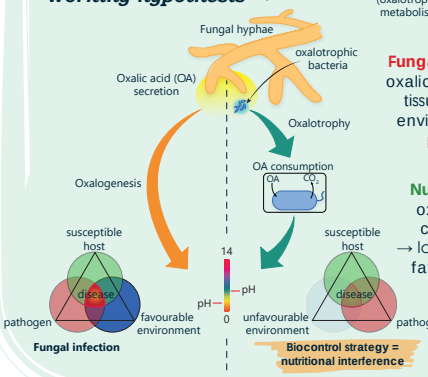
► understand fungal pathogenicity

- acidification of host tissues to stimulate production and activity of fungal enzymes
- toxic molecules, lytic enzymes, **oxalic acid**
- strong acid and metal chelator
- not only a factor of pathogenicity
- part of the **oxalate-carbonate pathway**



Plants and fungi produce oxalic acid (oxalogenesis)→ accumulation in soils as oxalic acid and oxalate. Oxalotrophic bacteria use oxalic acid as carbon and energy source (oxalotrophy) → precipitation of the products of metabolism → carbonate accumulation in soils

Working hypothesis



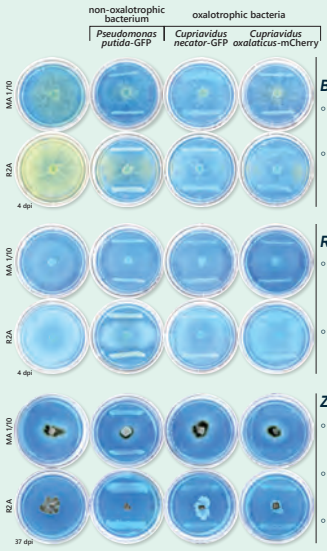
Fungal infection: secretion of oxalic acid to acidify the host tissues → more favorable environment for the fungus

► increase disease incidence

Nutritional interference: oxalotrophic bacteria consume oxalic acid → local pH increase → less favorable environment

► decrease disease incidence

Results



Botrytis cinerea
- fungal growth inhibition observed in all media
- contact between bacteria and fungus

Rhizoctonia solani
- no fungal growth inhibition by oxalotrophic bacteria
- inhibition by *P. putida* on R2A medium

Zymoseptoria tritici
- fungal growth inhibition observed in all media
- no contact between bacteria and fungus
- bacteria swarm towards the fungus

Confrontation assays on different media (MA1/10 and R2A) with three phytopathogenic fungi (*Botrytis cinerea*, *Rhizoctonia solani* and *Zymoseptoria tritici*) and two oxalotrophic bacteria (*Cupriavidus necator* and *Cupriavidus oxalaticus*). *Pseudomonas putida*, a non-oxalotrophic bacterium, was used as control. Pictures were taken after 4 days post inoculation (dpi) or 37 dpi.

Aims

To obtain new insights in bacterial-fungal interactions (BFI) and nutritional interference:
- influence of the fungal and bacterial partner
- influence of trophic factors

Conclusions

- BFI change depending on the fungal and bacterial partner and the trophic factors
- *B. cinerea* and *Z. tritici* are controlled by oxalotrophic bacteria on different media
- *P. putida* controls the fungal growth of *Z. tritici* and *R. solani*

Perspective 1

To investigate the effect of oxalotrophic bacteria on spore/sclerotia germination

WHY
- all studies so far focus on inhibition of mycelial growth
- allows to stop the pathogen before infection
- reduce spreading of disease

Perspective 2

To include the plant in the interaction

WHY
- investigate oxalotrophic bacteria-host plant interactions
- investigate BFI in a more complex system

HOW
- plant-bacterium-fungus interactions in a sterile environment



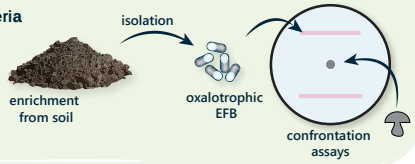
Lactuca sativa on R2A/MS medium

Perspective 3

Use of oxalotrophic endospore-forming bacteria (EFB) as biocontrol agents

WHY
- all studies used non-EFB
- can survive in harsh conditions

HOW
- isolation of oxalotrophic EFB by using oxalate as trigger of germination



Perspective 4

P. putida* as biocontrol agent against *Z. tritici

WHY
- very few biocontrol strategies against *Z. tritici*

HOW
- determine biocontrol mechanism
- design bioassays

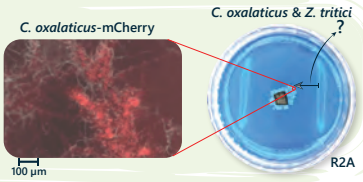


Perspective 5

To investigate the swarming behaviour of the bacteria in presence of *Z. tritici*

WHY
- novel behaviour

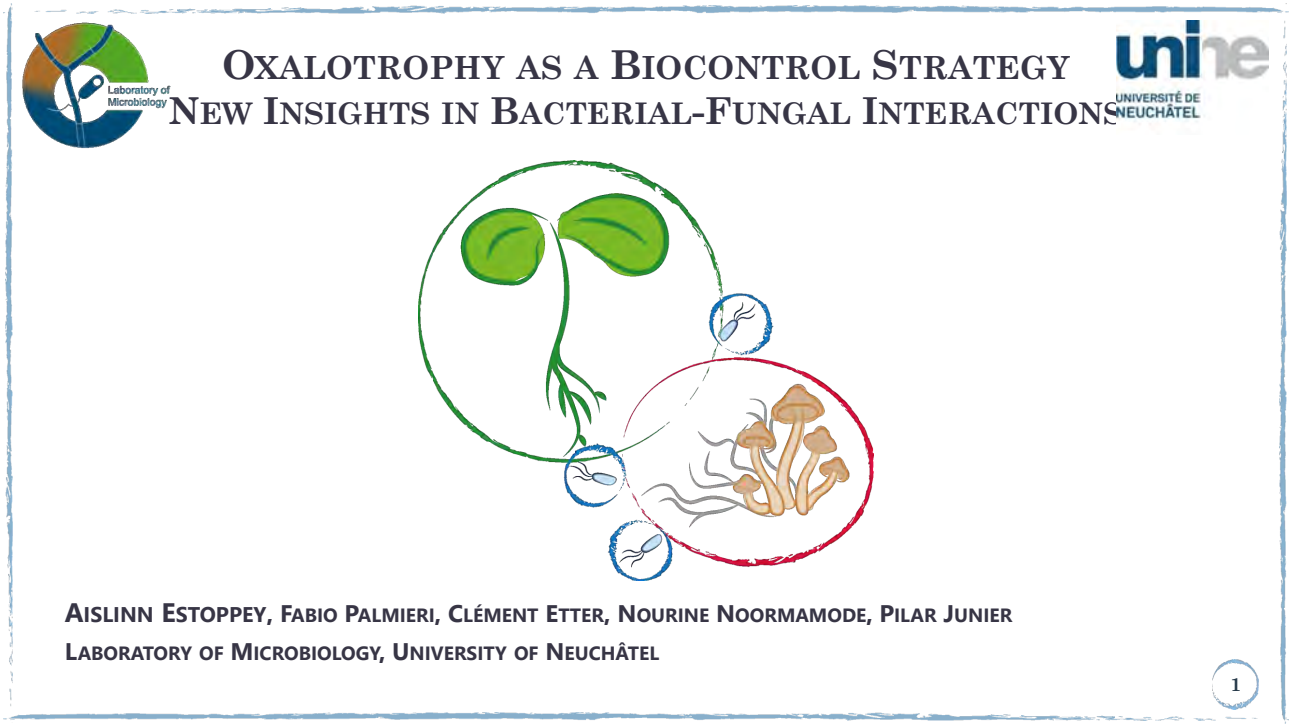
HOW
- transcriptomic analysis
- characterization of surfactants produced



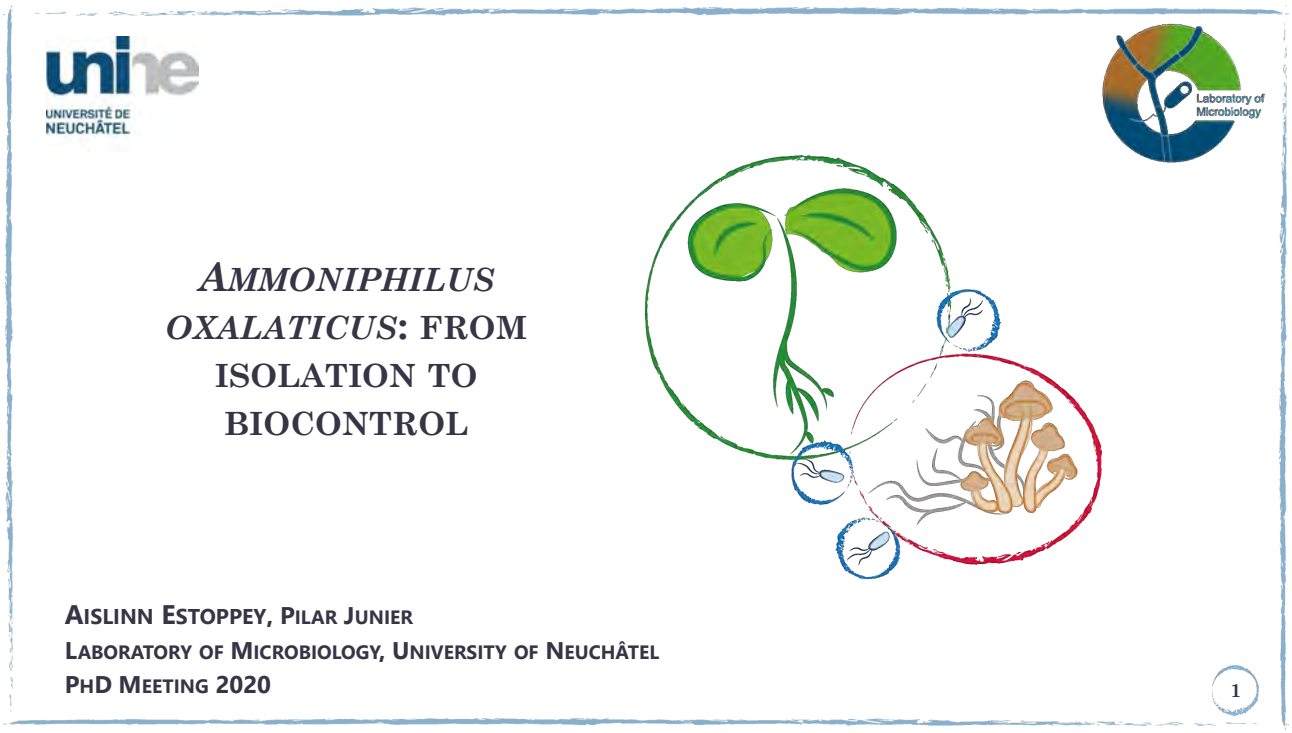
Acknowledgments
NOVARTIS FOUNDATION

Contact: aislinn.estoppey@unine.ch

3 Oral presentation at the 7th Swiss Microbial Ecology Meeting SME 2019, Aquatis Conference Center, Lausanne, Switzerland, January 30-February 1, 2019



4 Oral presentation at the Annual PhD Students Meeting 2020, Neuchâtel, Switzerland, September, 2019

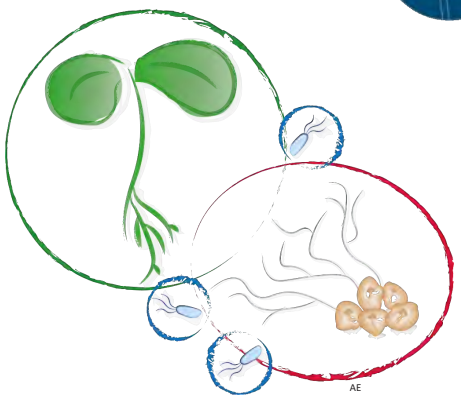


5 Oral presentation at the Zurich Mycology Symposium 2021, online, January 15, 2021

UNIVERSITÉ DE NEUCHÂTEL

Laboratory of Microbiology

Improved methods to assess bacterial biocontrol on germination of fungal spores or sclerotia



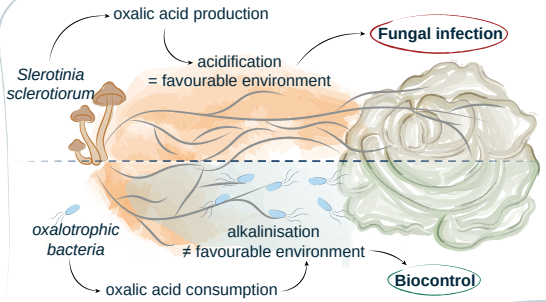
AISLINN ESTOPPEY[1], LAURE WEISSKOPF[2], SASKIA BINDSCHEDLER[1], PILAR JUNIER[1]
[1]LABORATORY OF MICROBIOLOGY, UNIVERSITY OF NEUCHÂTEL
[2]DEPARTMENT OF BIOLOGY, UNIVERSITY OF FRIBOURG
ZÜRICH MYCOLOGY MEETING 2021

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6 Poster presentation at ISME18 (18th International Symposium on Microbial Ecology), SwissTech Convention Center EPFL, Lausanne, Switzerland, August 14-19, 2022

Chemotactic signals regulating the interaction of *Sclerotinia sclerotiorum* and two soil oxalotrophic bacteria

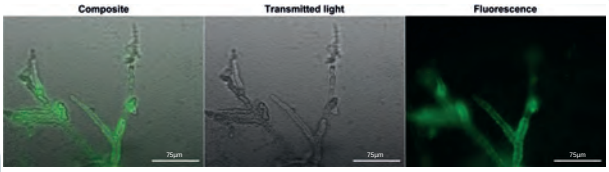
Aislinn Estoppey[1], Saskia Bindschedler[1], Thierry Kuhn[1 2], Armelle Vallat-Michel[3], Xiang-Yi Richter[2], Pilar Junier[1]
Laboratory of Microbiology[1], Laboratory of Eco-Ethology[2], Neuchâtel Platform of Analytical Chemistry[3], University of Neuchâtel, Switzerland



- *Sclerotinia sclerotiorum*, a phytopathogenic fungus, uses oxalic acid as a pathogenicity factor by regulating ambient pH to optimize the activity of fungal enzymes
- Oxalotrophic bacteria, such as *Cupriavidus necator* or *Cupriavidus oxalaticus*, consume oxalic acid, indirectly modifying the pH of the medium
- The change of pH from acidic to alkaline controls the growth of *S. sclerotiorum*
- Oxalotrophic bacteria exhibit contrasting chemotatic behaviours in presence of *S. sclerotiorum* in different media (attraction and repulsion)

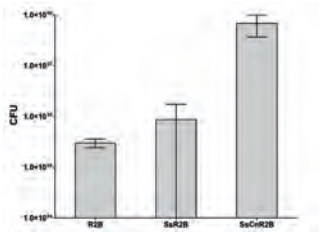
What compound or compound blend is responsible for these behaviours?
Is the chemotactic signal constitutively produced by the fungus?

Attraction



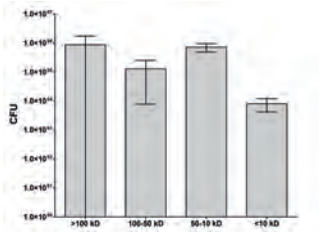
Images from inverted microscopy from a confrontation assay with *C. necator*-GFP and *S. sclerotiorum* in liquid medium (Reasonner'2 broth; R2B) in cell culture Petri plates[1].

- Fungal growth is limited and the bacteria are closely associated to the hyphea
- Similar results are obtained with *C. oxalaticus*-mCherry



- *C. necator* is significantly more attracted by the spent medium from the confrontation assay
- No significant difference of attraction between R2B or the spent medium of fungi grown in the absence of bacteria

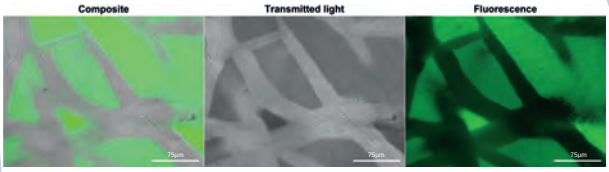
Colony Forming Units (CFU) from a chemotaxis experiment using bridges[2], with R2B, spent medium from *S. sclerotiorum* culture (SsR2B) and spent medium from a confrontation assay with *C. necator* (SsCnR2B).



- No siginificant difference of attraction between the different fractions of the spent medium from the confrontation assay with *C. necator*

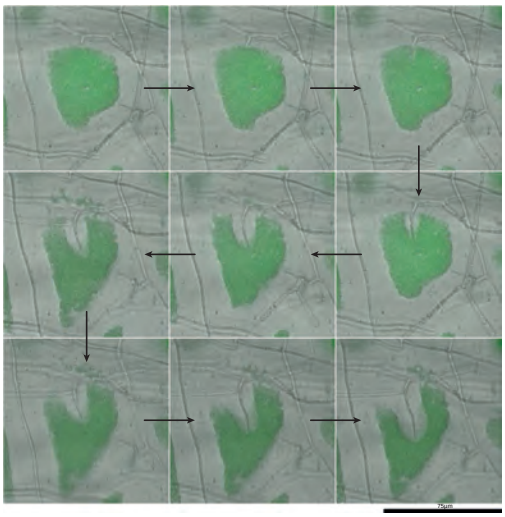
CFU from a chemotaxis experiment with fractions of different molecular weight from the spent medium of a confrontation assay with *C. necator*. Fractions were produced with the Amicon® Ultra centrifuge filters.

Repulsion



Images from inverted microscopy from a confrontation assay with *C. necator*-GFP and *S. sclerotiorum* in liquid medium (10x diluted matl broth; MB1/10) in cell culture Petri plates[1].

- The bacteria are clearly repulsed by the fungus and the hyphea form a net separation between the bacteria
- The same pattern is observed with *C. oxalaticus*-mCherry



Snapshots from a time-lapse video of *C. necator* and *S. sclerotiorum* interaction in MB1/10 in cell culture Petri plate. Images were taken every 5 min for 72h. Full video available via QRCode.

- The production of the attractant and the repellent by *S. sclerotiorum* is induced by the presence of the bacteria
- The chemotaxis experiment with the fractions was not conclusive to guide the search of the attractive compound
- Further investigations are needed to determine how the repulsive compound works

Methods
1. Estoppey A, Weisskopf L, Di Francesco E, Vallat-Michel A, Bindschedler S, Chain PSG, Junier Pilar. Improved methods to assess the effect of bacteria on germination of fungal spores. FEMS Microbiol Lett. 2022 Apr 21;369(1):fnac034. doi: 10.1093/femsle/fnac034. PMID: 35325127.
2. Kuhn T, Buffi M, Bindschedler S, Chain P, Gonzalez D, Stanley C, Wick L, Junier P, Li Richter XY. Design and construction of 3D-printed devices to investigate active and passive bacterial dispersal on hydrated surfaces. bioRxiv 2022.05.16.492069; doi: https://doi.org/10.1101/2022.05.16.492069

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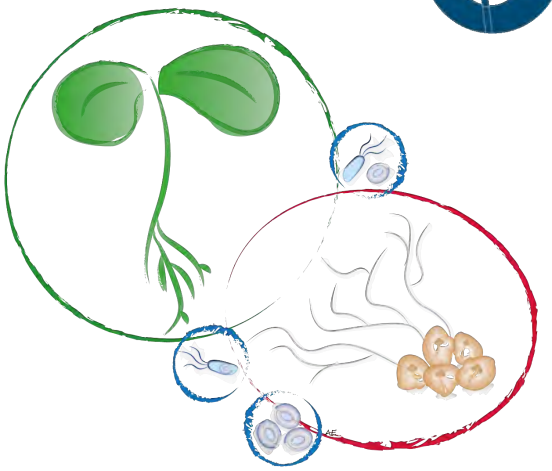


7 Oral presentation at the SGM·SSM annual meeting 2022, online, SwissTech Convention Center EPFL, Lausanne, Switzerland, August 30 - September 1, 2022

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Laboratory of Microbiology

AMMONIPHILUS OXALATICUS: OXALOTROPHY AND BIOCONTROL OF FUNGAL PATHOGENS



AISLINN ESTOPPEY, SASKIA BINDSCHEDLER, PILAR JUNIER
LABORATORY OF MICROBIOLOGY, UNIVERSITY OF NEUCHÂTEL
SSM22

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Liste of publications

Kuhn, T., Mamin, M., Bindschedler, S., Bshary, R., **Estoppey, A**., Gonzalez, D., Palmieri, F., Junier, P. & Richter, X. Y. L. (2022) '*Spatial Scales of Competition and a Growth-Motility Trade-off Interact to Determine Bacterial Coexistence*', Royal Society Open Science. Royal Society Publishing, 9(12). doi: 10.1098/rsos.211592.

Estoppey, A., Weisskopf, L., Di Francesco, E., Vallat-Michel, A., Bindschedler, S., Chain, P. S. & Junier, P. (2022) '*Improved Methods to Assess the Effect of Bacteria on Germination*', FEMS Microbiology Letters, 369, pp. 1–9. doi: DOI: 10.1093/femsle/fnac034.

Robinson, A. J., House, G. L., Morales, D. P., Kelliher, J. M., Gallegos-Graves, L. V., LeBrun, E. S., Davenport, K. W., Palmieri, F., Lohberger, A., Bregnard, D., **Estoppey, A**., Buffi, M., Paul, C., Junier, T., Hervé, V., Cailleau, G., Lupini, S., Nguyen, H. N., Zheng, A. O., Gimenes, L. J., Bindschedller, S., Rodrigues, D. F., Werner, J. H., Young, J. D., Junier, P. & Chain, P. S. G. (2021) '*Widespread Bacterial Diversity within the Bacteriome of Fungi*', Communications Biology, 4(1), pp. 1–13. doi: 10.1038/s42003-021-02693-y.

Junier, P., Cailleau, G., Palmieri, I., Vallotton, C., Trautschold, O. C., Junier, T., Paul, C., Bregnard, D., Palmieri, F., **Estoppey, A**., Buffi, M., Lohberger, A., Robinson, A., Kelliher, J. M., Davenport, K., House, G. L., Morales, D., Gallegos-Graves, L. V., Dichosa, A. E. K., Lupini, S., Nguyen, H. N., Young, J. D., Rodrigues, D. F., Parra-Vasquez, A. N. G., Bindschedler, S. & Chain, P. S. G. (2021) '*Democratization of Fungal Highway Columns as a Tool to Investigate Bacteria Associated with Soil Fungi*', FEMS Microbiology Ecology. Oxford University Press, 97(2), pp. 1–15. doi: 10.1093/femsec/fiab003.

Palmieri, F., Palmieri, I., Noormamode, N., **Estoppey, A**., Ishak, M. O., Kelliher, J. M., Vallat, A., Iyer, R., Bindschedler, S., Davenport, K., Chain, P. S. G., Harris, J. F., Junier, P., Division, B. & Alamos, L. (2020) '*Exploiting Oxalotrophic Bacteria to Control Aspergillus Niger Lung Infections*', BioRXiv, pp. 1–33. doi: 10.1101/2020.08.20.259929.

Palmieri, F*., **Estoppey, A***., House, G. L., Lohberger, A., Bindschedler, S., Chain, P. S. G. & Junier, P. (2018) '*Oxalic Acid, a Molecule at the Crossroads of Bacterial-Fungal Interactions*', in Advances in Applied Microbiology. Academic Press Inc., pp. 49–77. doi: 10.1016/bs.aambs.2018.10.001.

Paul, C., Filippidou, S., Jamil, I., Kooli, W., House, G. L., **Estoppey, A**., Hayoz, M., Junier, T., Palmieri, F., Wunderlin, T., Lehmann, A., Bindschedler, S., Vennemann, T., Chain, P. S. & Junier, P. (2018) '*Bacterial spores, from ecology to biotechnology*'. 1st edn, Advances in Applied Microbiology. 1st edn. Elsevier Inc. doi: 10.1016/bs.aambs.2018.10.002.

*equal contribution of the authors

Curriculum vitae

AISLINN ILOUKA MARIE ESTOPPEY

Education and Training

2018 – Present	PhD in Biology, University of Neuchâtel, Faculty of Sciences, Institute of Biology, Laboratory of Microbiology.
2016 – 2018	Master of Science in Biogeosciences, Universities of Lausanne and Neuchâtel, Faculty of Sciences, Institute of Biology, Laboratory of Microbiology.
2013 – 2015	Master of Science in Medical Biology, Specialization: Immunology & Cancer, University of Lausanne, Faculty of Biology and Medicine, School of Biology, Laboratory of Dr. Vladimir Katanaev, Professeur associé, Department of Pharmacology and Toxicology, University of Lausanne
2010 – 2013	Bachelor of Sciences in Biology, University of Neuchâtel

Research experience

2018 – Present	PhD Thesis, Laboratory of Microbiology, University of Neuchâtel. Thesis supervisor: Prof. Pilar Junier
2017 – 2018	Master thesis, Laboratory of Microbiology, University of Neuchâtel. "*Oxalotrophy as a biocontrol strategy*". Supervision: Prof. Pilar Junier, PhD Student Fabio Palmieri.
2014 – 2015	Master thesis, Laboratory of Dr. Vladimir Katanaev, Professeur associé, Department of Pharmacology and Toxicology, University of Lausanne. "*Regulation of EGFR endocytosis*". Supervision: Dr. Vladimir Katanaev, Dr. Gonzalo Solis
Oct. – Dec. 2013	Pre-master thesis, Laboratory of Dr. Denise Nardelli-Haefliger, PD, Urology Research Unit, CHUV. "*Expression of inhibitory molecules on dendritic cells*". Supervision: Dr. Laurent Derré, Dr. Mathieu F. Chevalier

Professional experience

2018 – 2023	Assistant for the practicals in Microbiology for Bachelor students, Laboratory of Microbiology, University of Neuchâtel
2018 – now	Expertises mycology (determination, cultures maintenance) in the Laboratory of Microbiology, University of Neuchâtel
Apr. – Oct. 2022	Mentor PROVOC for Clément Vanmerris, Travail de Maturité, Laboratory of Microbiology, "*Ammoniphilus oxalaticus, un potentiel agent de biocontrôle contr les champignons phytopathogènes?*"
2017 – 2018	Student Assistant for the practicals for Bachelor and Master Students, Laboratories of Microbiology and Functional Ecology

