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Ecological engineering: from concepts to applications

Biology and ecology of biofilms formed by a plant pathogen *Phytophthora parasitica*: from biochemical ecology to ecological engineering

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Abstract

In nature, the organisation of microbial species into biofilms has a great influence on local environments and in human or plant diseases. This important trait of prokaryotes and eukaryotes is poorly understood while the knowledge of the related biological processes could constitute a novel base for controlling diseases. A study is developed on the oomycete *Phytophthora parasitica* belonging to a major class of eukaryotic plant pathogens to understand molecular and ecological basis of biofilm formation. The identification of signalling molecules and the definition of their spectrum of activity within the biofilm community will improve our understanding of fundamental biological processes, our ability to forecast pathogen behaviour and to elaborate new tools dedicated to plant diseases management with low environmental impact.

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Keywords: biochemical ecology; biofilm; biomimetic materials; oomycete; plant disease management

1. Introduction

Biofilms are microbial communities living in co-operative groups attached to surfaces and embedded in a polymeric matrix they produce [1,2]. Their formation requires that planktonic (free-swimming or free-floating) cells first become attached to a solid surface, leading to the formation of microcolonies that then differentiate into exopolysaccharide-encased and fluid-filled channel-separated, mature sessile biofilms. For pathogens this trait contributes to the virulence as well as to the dynamics of interactions with their hosts [3-7]. Biofilms confer several

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advantages (i) favoring attachment on host surface, (ii) promoting virulence through aggregation, (iii) providing different protections (exopolysaccharidic matrix, slowed down metabolism, efflux pumps for toxic compounds) against host defences, biocide treatments or stressful environmental conditions, (iv) promoting dissemination through transition from the aggregated lifestyle to the planktonic one [8,9]. Biofilm generation has been first studied in details within the prokaryotic kingdom. There have also been several descriptions indicative of biofilm formation by yeasts, oomycete and filamentous fungi in different medical, environmental and industrial settings [5,10] comforting the idea that it is a trait of microbial life widely spread.

Among oomycetes, members of the genus *Phytophthora* represent a major class of eukaryotic and filamentous plant pathogens, causing devastating diseases in natural ecosystems and in numerous economically important crops. The adhesion on the host surface of a bi-flagellated and wall-less zoospore, followed by its encystment, cyst germination and germ tube penetration is believed to be the most important pathway in disease initiation [11]. In the species *P. parasitica* alternatively to this single cell behaviour, the early step of infection cycle may also involve cell population dynamics via the formation of biofilms in a cell density-dependent manner [12]. Initially the founder zoospores attracted by host signals adhere irreversibly to the plant surface and aggregate to form a microcolony (Fig. 1a). Then these cells emit their own signal (the autoinducer), drive the migration of a second wave of zoospores (several hundreds of cells) by setting up an external chemotactic gradient involving at least cAMP (adenosine 3′,5′-cyclic monophosphate) and leading to massive zoospore encystment and cyst-orientated germination (Fig. 1b). The structure is embedded within an extracellular matrix and is perforated by opening channels (Fig. 1c). Within 3-4 days zoosporogenesis and the subsequent zoospore release occur, each microcolony constituting by this way a new dissemination point (Fig. 1d). When microcolonies are incubated with rhizospheric samples the structure is rapidly colonized by different microorganisms forming a shared community in a mixed-species biofilm. Thus, the ability of oomycetes to form biofilm has an impact on the local environment and may contribute to disease outcome by causing a persistent chronic disease and by sheltering other potential pathogens.

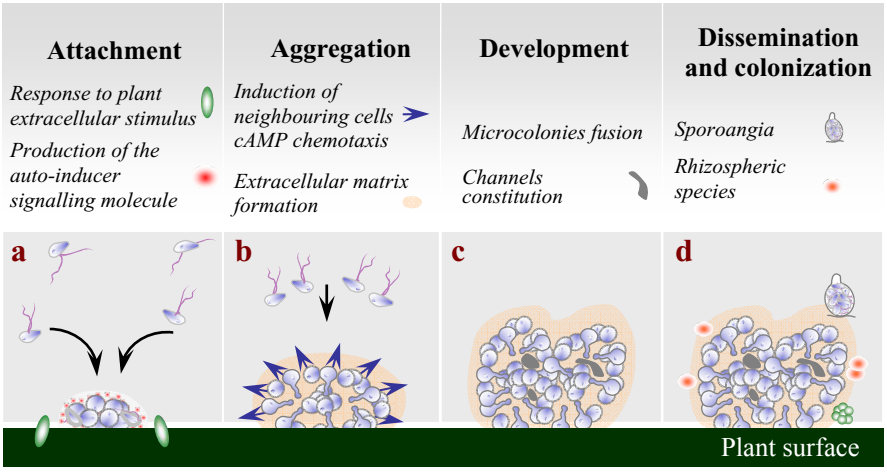

Fig. 1. Sequence of events leading to formation of *Phytophthora parasitica* biofilm

Using this model a biochemical ecology approach is developed. It consists first in the identification of molecules governing oomycete biofilm formation and then in the understanding of how they propagate or not their signal in the rhizospheric community associated with *P. parasitica* within the biofilm.

2. Characterization of host and oomycete molecules coordinating the behavior of zoospores

To identify these molecules a chromatographic purification (High-Performance Liquid Chromatographic, HPLC) and a structural elucidation (Mass Spectrometry) are combined with the analysis of microcolony transcriptome (comparison between standalone cysts and cysts structured in microcolonies).

P. parasitica zoospores (5 10^5-10^6 cells/ml) were incubated for 120 min in water in the presence of root or leaf pieces of its host *Nicotiana tabacum* in order to induce the formation of spheroid microcolonies (Figs. 2a and 2b). This simple experimental design allowed preparing an extract with strong chemoattraction and aggregation activities: an exogenously application of this extract to zoospores suspensions revealed that it contains molecules exhibiting properties of the host signal or of the *P. parasitica* autoinducer. A purification on reversed-phase HPLC led to three active fractions that controlled the movement of zoospores by causing strong chemoattraction at the application point. Each fraction corresponded to a single peak (Fig. 2c), each one showing different spectral signatures (obtained with the diode array detector, 200-400nm). First results obtained by mass spectrometry were indicative of low molecular weight (ranging from 200 to 500 Da) polar compounds. Theses analyses lead us to confirm the mass of the molecules and to obtain some structural features through fragments identification. In order to handle exact structures, we will carry out high resolution mass spectrometry and Nuclear Magnetic Resonance spectroscopy. To fully validate the proposed compounds, the organic synthesis of these molecules should be necessary, if they are not available in the chemolibraries. In addition, the possibility of having significant amount of products will allow the continuation of the biological tests (i) by testing the ability of each molecule to induce the regrouping of the zoospores and the formation of microcolonies on abiotic or biotic surfaces (ii) by improving their beneficial/toxic activities towards a set of other micro-organisms. Thus, the effective concentrations will be determined and compared to those measured *in vivo* during the biofilm formation, together with to their possible environmental effect.

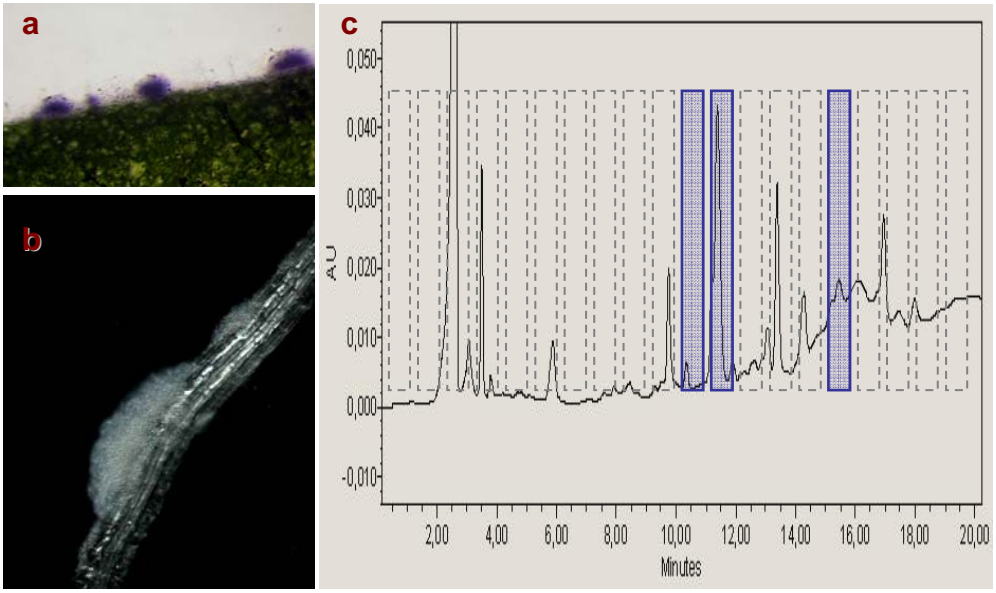

Fig. 2. Micrographs of microcolonies (a, b) and (c) HPLC chromatogram of a microcolony extract using a C18 column with a methanol/water gradient (detect 260nm). Blue bars indicate active fractions

In order to identify the cellular responses governed by these molecules, a second part of the study aimed to define the transcriptome of a microcolony. Using microarray slides containing probes for 4700 *P. parasitica* unigenes and targeted mRNA from microcolonies established on leaf surface (M situation), the mRNA abundance was determined compared to the isolated cysts germinated on Petri dishes (C situation). To establish correlations between the situations (M/C), a principal component factor analysis (PCA) was performed. Fig. 3 shows the results for each replicate from microcolonies (M) and isolated cysts (C). As expected it showed on the one hand a similar distribution of replicates from the M situation (in red) as well as of replicates from the C situation (in green). On the other hand it revealed that variables from M were poorly correlated with variables from C. Thus, analysis of transcriptional variations with this subset of genes allows distinguishing the microcolony and the cyst transcriptomes. Seventy three genes were identified with at least a 4-fold change (99% of confidence in student-t test) between the two situations and for both replicate sets. Using C as the control and M as the experiment, 26

genes were found up-regulated and 47 down-regulated. Validation of microarray data was performed by real time quantitative reverse transcription PCR for half of up-regulated genes. A general agreement was observed between the two sets of data. Finally the transcriptome of a *Phytophthora parasitica* microcolony was mainly characterized by the coordinated up-regulation of genes for extracellular matrix constituents and for export/ import of substrates. This set of genes is now functionally analyzed and used as molecular markers for characterization of molecules governing oomycete biofilm formation and characterized as described above.

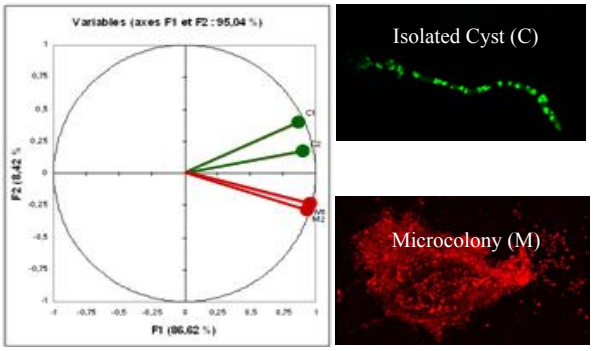

Fig. 3. PCA of microarray data. Variables (normalized intensity values) were plotted separately on graph by report of the first two principal components (F1 and F2). F1 and F2 represent 95,04% of the total variability

3. Characterization of biological activity within biofilm community and within the class of oomycetes

These molecules are not only evaluated for their biological activity on *P. parasitica* but also for their influence on the microbial community associated to the *P. parasitica* biofilm. This point is of particular interest since it should be of prime importance to delineate the impact of these molecules on the ecology of the rhizosphere. Thus the identification of microbial species that are able to colonize *P. parasitica* biofilm from the rhizosphere was undertaken. In this purpose three days-old mixed-species biofilms were dissociated and spread on agar plates. Colonies morphologically distinct from those formed *by P. parasitica* were isolated. Sequencing of bacterial 16S ribosomal RNA from some isolates indicated that bacteria were in particular *Pseudomonas, Pantoea, Shinella* each one of these genera including species able to form biofilms [9]. Interestingly the species identified as nearest to the isolates, based on the most similar BLAST hit [13], have been characterized for their tendency to form biofilms in plants. The identification of eukaryotic species is also in progress based on 18S ribosomal RNA sequencing.

4. Conclusions

In agriculture, one of the main current challenges is to reduce the environmental impact of crop treatments against pathogens while maintaining the economic profitability. The current development of studies on the biology and ecology of plant pathogen biofilms offers the opportunity to evaluate new environmental friendly agricultural practices. The emergence of cognitive bases on biofilm formation by major classes of plant pathogens such as Oomycetes will help to define the scientific/experimental framework to design new tools dedicated to plant disease management. For example, knowing the molecules that govern biofilm formation will help to elaborate biomimetic materials for the development of behavioural confusion techniques with low environmental impact. The design of molecular traps for pathogens associating specific attracting, aggregating and biocide molecules should also make it possible to diversify biologically-based alternatives to systemic treatments with synthetic fungicides. In addition, by determining which microorganisms among those neighbouring the pathogen influence the disease and how they do so, will allow both to develop and to vary biocontrol strategies.

The study of ecology of pathogens living with other species in highly organized biofilms also offers opportunities to extend our knowledge on causal relationships between biotic interactions and the diseases outcome. Until now the host-pathogen interaction attracts all the attention of studies aiming at developing the scientific bases for genetic engineering (breeding, GMO) of crop plants. To our knowledge, only few studies tackle the pathogen-pathogen interactions and the various interactions between the pathogen and the microbial community through cellular

dialogue. Studying biofilm formation by pathogens opens new ways (1) for the characterization of the nature of these two types of interactions, (2) for understanding their influence on diseases, and (3) for the development of new sustainable ecosystems that have both agricultural and ecological value.

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