# Ecosystem screening approach for pathogen-associated microorganisms affecting host disease

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# An ecosystem screening approach for pathogen-associated microorganisms affecting host disease

**Short title:** Microbe communities and disease

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## Abstract

The microbial community in which a pathogen evolves is fundamental to disease outcome. Species interacting with a pathogen on the host surface shape the distribution, density and genetic diversity of the inoculum, but the role of these species is rarely determined. The screening method developed here can be used to characterize pathogen-associated species affecting disease. This strategy involves three steps: (1) constitution of the microbial community, using the pathogen as a trap, (2) community selection, using extracts from the pathogen as the sole nutrient source, (3) molecular identification and the screening of isolates focusing on their effects on the growth of the pathogen *in vitro* and host disease. This approach was applied to a soilborne plant pathogen, *Phytophthora parasitica,* structured in a biofilm, for screening the microbial community from the rhizosphere of *Nicotiana tabacum* (the host). Two of the characterized eukaryotes interfered with the oomycete cycle and may affect the host disease. A *Vorticella* species acted through a mutualistic interaction with *P. parasitica*, disseminating pathogenic material by leaving the biofilm. A *Phoma* species established an amensal interaction with *P. parasitica,* strongly suppressing disease by inhibiting *P. parasitica* germination. This screening method is appropriate for all non obligate pathogens. It allows defining, for a given biotope, microbial species as promotors or suppressors of a disease. It should also help to identify important microbial relationships for ecology and evolution of pathogens.

## Introduction

Before infecting a host, a pathogen evolves within a microbial community that colonizes the host surface and may form mixed-species biofilms (9, 10). This community is capable of affecting disease and exerting selection pressure on the pathogen and the host (19, 30, 31). Investigations of the pathogenesis of several infections are currently moving away from a reductionist paradigm towards the view of the microbial community as a pathogenic unit (20, 29). Despite the growing recognition that this community is a driving force for natural selection and pathogenicity, the role of each microorganism associated with a pathogen is rarely identified. Current studies tend to focus on community structure, species richness and abundance (21). In the cases of plant diseases, examination of the microbial community compositions of pathogen-suppressive soils, soils in which the pathogen does not establish or persist, leads to found positive correlation between the population densities of some species and suppressiveness levels, suggesting that they may be involved in the disease suppressive process (6, 8). However in most cases microbial species promoting or controlling host diseases remains to be identify.

In this study the impact of rhizospheric microorganisms on the tobacco black shank disease caused by the soil-borne pathogen *Phytophthora parasitica* was investigated. *Phytophthora parasitica* is a filamentous eukaryotic plant pathogen (3), a member of the oomycetes group comprising several of the most devastating plant pathogens, causing diseases in natural ecosystems and in numerous economically important crops. This polyphagous species includes tobacco in its host range and causes the black shank disease. The infection cycle of *P. parasitica* may alternatively involve single cell behavior, via zoospore germination and germ tube penetration, or cell population dynamics of planktonic

zoospores, through the formation on the host surface of adherent microcolonies that develop into large biofilms (13).

*P. parasitica* biofilm was used to study the interations between the oomycete and the microorganisms from the rhizosphere of *Nicotiana tabacum*. This choice was based first on the principle that pathogens generally live in cooperative groups attached to surfaces. Biofilms contribute to the pathogen virulence as well as to the dynamics of interactions with host (5, 23, 26, 28, 32). They confer several advantages to pathogens favoring attachment on host surface, promoting virulence through aggregation and providing protections against host defences or biocide treatments (9). They also promote dissemination through transition from the aggregated lifestyle to the planktonic one (10, 15). This choice was also conditioned by the fact that in natural habitat biofilms constitute propitious niches for interactions between pathogens and other species (23). During *P. parasitica* biofim formation cAMP (adenosine 3′,5′-cyclic monophosphate) chemotaxis is suspected to be a key migration mechanism (13). cAMP-chemotactic migration is also known to regulate slug formation in the myxomycete *Dictyostelium* (12). The role of such molecule, so much ubiquist, in microbial aggregation processes makes possible a myriad of interactions between species. These interactions must be in addition tightly regulated for allowing optimal metabolic interactions and ensuring the ecological role of the community in the ecosystem.

To get insight into the ecological mechanisms of disease regulation, we developed a screening method for characterizing the repertoire of pathogen-associated species affecting a disease. The approach involves the trapping of species associated with a pathogen, the identification of those capable of growing in this environment and the assignment of ecosystemic functions in terms of pathological considerations.

## Materials and Methods

### *Plant material*

*Nicotiana tabacum* (cultivar Xanthi) plants were grown on compost (AGRI'OR) in a growth chamber at 24°C, with a 16 h photoperiod and at a light intensity of 100 $\mu Em^{-2}\ s^{-1}$. The same compost was used for all the experiments. Seeds were germinated in flowerpots (9 x 9 x 9.5 cm, SOPARCO). Two weeks after germination, 25 plants were transferred individually into flowerpots. NPK 15:12:30 fertilizer was applied once (100 ml per flowerpot), and the plants were then watered regularly and grown for three weeks. Leaves or roots were taken from 7- to 8-week-old.

### *Microbial strains*

*Phytophthora parasitica* strains 310, 329 and 408 were obtained from the INRA (Sophia-Antipolis) *Phytophthora* collection.

*Vorticella microstoma* strain 30897 was purchased from the ATCC (American Type Culture Collection) collection of protists (LGC standards). The cells of the ciliate were cultivated for 3-4 days in V8 liquid medium at 24°C with a 16 h photoperiod and at a light intensity of 100 $\mu Em^{-2}\ s^{-1}$.

The *Phoma* strain characterizing during this work is recorded in the national collection of Institut Pasteur (recording number CNCM I-4278**).**

### *Community constitution*

Microcolonies were prepared from strain 329, as previously described (13). Leaf pieces (5 cm x 0.5 cm) were inoculated in water for three hours at 25°C with a suspension of *P. parasitica* zoospores (400-600 cells ml/µL). Microcolonies formed on leaf surface were then isolated, washed with water before incubation with the rhizospheric samples.

For collection of soil samples from the rhizosphere, eight plants of similar size were chosen and the roots were collected, together with the soil clinging to them. The eight samples were pooled together, washed with sterile water (1/5, W/V) and filtered twice through a sieve with 100 µm pores. The resulting suspension was rapidly decanted (5 min). The supernatant (5 ml) was incubated in a 5.5-cm Petri dish at 25°C with 10-20 freshly formed and water-washed *P. parasitica* microcolonies.

The kinetics mixed-species biofilm constitution was defined on the basis of four independent experiments and similar observations obtained by microscopy under white light. The early formation of bacterial colonies was detected by DAPI staining (Axioplan fluorescence microscope, Carl Zeiss MicroImaging, Inc., Germany).

***Community selection***

After three days of incubation, the mixed-species biofilms obtained were rinsed three times in water and gently dissociated by mechanical trituration, consisting of 20 passes through the opening of a standard Pasteur pipette. The cell suspension obtained was spread on agar plates containing a *Phytophthora* extract as the sole nutrient source [*Phytophthora* crude extract 10 g/l; NaCl 10g/l; agar 1.5% (P/V)] and incubated at 25°C. The *Phytophthora* crude extract was prepared from a two-week-old mycelium of *P. parasitica* strain 329 (INRA, Sophia-Antipolis). The mycelium was rinsed in water, ground to a fine powder in liquid nitrogen and freeze-dried. Eukaryotes were selected on plates supplemented with 30 µg/ml chloramphenicol (in preliminary experiments chloramphenicol appeared as the most selective antibiotic --compared to ampicilin and kanamycin - for favoring growth of eukaryotes *versus* that of prokaryotes; data not shown). Colonies appeared within three to six days. The colonies, which were morphologically different from those formed by *P. parasitica,* were

isolated individually, transferred to 100 mm Petri dishes and expanded for mass cultures on V8 or malt agar.

***Molecular identification***

For each isolate, boiled cells were used as templates for PCR amplification. The template was prepared by suspending cells, spores or mycelium in boiling water for 3 min and then rapidly chilling on ice and centrifuging at 10,000×*g* for 3 min to remove debris. The supernatant (1 µl) was used for PCR amplification. The eukaryotic 18S rRNA gene was amplified with the forward primer EukA: 5'-CTGGTTGATCCTGCCAG-3' and the reverse primer EukB: 5'-TGATCCTTCYGCAGGTTC-3' (25). The PCR program included an initial denaturation at 94°C for 120 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 120 s.

***Fluorescence in situ hybridization (FISH)***

The probes Vortir339 (5'-Cy3- GACTGCCATGGTAGTCCAATACACT -3') targeting *Vorticella* ciliates and Eukr560 (5'Cy5-5'CGGCTGCTGGCACCAGACTTGCCCT-3') targeting all eukaryotes were used. Sample preparation and hybridization conditions were essentially as described in a previous study (16). Mixed-species biofilms were fixed by incubation in a 4% (wt/vol) paraformaldehyde solution for 4 h at 4°C, dehydrated by sequential washes in 50, 75, and 100% (vol/vol) ethanol (30 min each) and rehydrated sequentially in the same solutions in reverse order. Subsequently, 2 ml of hybridization solution [900 mM NaCl, 20 mM Tris-HCl [pH 7.4], 0. 1% [wt/vol] sodium dodecyl sulfate, 20% (wt/vol) formamide] containing 1 µM probe was added to the samples, which were incubated overnight at 45°C. Biofilms were washed twice, for 15 min each, in the hybridization solution at room temperature, placed on glass slides and

overlaid with an anti-fading reagent (VectaShield, "Vector") before observation under a Zeiss LSM 510 Meta confocal microscope. Merged images showing FISH staining and light micrographs (differential interference contrast) were generated.

***Screening of isolates for an effect on* Phytophthora *growth and plant disease***

Screening was performed *in vitro,* by co-incubating isolates and *P. parasitica* strains, and *in planta,* through co-infections. For *in vitro* confrontations, *P. parasitica* strain 329 was first grown separately with each isolate on malt agar. An agar disk (5 mm in diameter) bearing the oomycete mycelium (strain 329) was placed on the right-hand part of a fresh Petri dish containing malt agar; an agar disk carrying biological material for the isolate tested was placed on the left-hand part of the plate. The zone of growth inhibition seen around the disc corresponding to the isolate was used to evaluate anti-oomycete activity.

The influence of isolates on the germination of *P. parasitica* cysts was also assayed on 10-well slides (Dominique Dutscher). A 20 µl suspension containing zoospores (400 cells/µl of strains N°310, 329 or 408) was mixed with equal volumes of V8 medium and of isolate-conditioned water filtrate (Cells were previously and briefly vortexing to ensure synchronized germination). The filtrate was prepared from four mycelial discs incubated in water (1 ml) for 1 h at 25°C. After centrifugation at 2,000 × g for 2 min, the supernatant was passed through a filter with 0.2 µm pores. The percentage germination was determined for two replicates, after incubation for two hours at 25°C.

For *in planta* screening, parenchymatous leaf tissue was co-infected, to prevent interference with the resident flora in the rhizosphere. We infiltrated the right-hand parts of five leaves from three tobacco plants with a suspension (100 µl) containing 500 zoospores of *P. parasitica* strain 329 (INRA Sophia-Antipolis *Phytophthora* collection) and 500 spores of each isolate. For each isolate, spores suspension was prepared in water from two mycelial

discs (5 mm diameter) following incubation in water (1mL, 15 min, room temperature), counting using a Malassez chamber and calibration by dilution in water. The percentage of inoculated zones showing symptoms and the area damaged were determined two days after co-inoculation. We assessed the influence of isolates on the disease by comparing the results with those for the left-hand parts of the leaves, which were inoculated with a suspension (100 µl) containing 500 *P. parasitica* zoospores. None of the isolates induced the development of symptoms on the plant when used alone for inoculation (data not shown).

Data are expressed as the means ± SD of three independent experiments and statistical analysis was carried out by performing Student's t tests in Microsoft Excel 2003.

***Dissemination assay***

Eight leaf pieces (5 cm x 0.5 cm) harboring or not *P. parasitica* microcolonies formed on their section (2-3 microcolonies per cm) were incubated in water (10 mL) in the presence of cells of the *Vorticella microstoma* strain 30897 (1000 cells ml/L) for three hours at 25°C. The control without *V. microstoma* cells was adjusted with the appropriate volume of V8 medium. After incubation leaf pieces with anchored ciliates cells were rinsed two times with 20 mL of water to eliminate contamination of samples by circulating ciliate cells.

Dissemination assays were performed in a modified Boyden chamber (7). The apparatus consists of two well chambers separated by a filter containing pores of 200 µm (Buisine) to allow migration for propagules of large size. The lower chamber was created into a Petri dish (100 mm) pouring out 10 ml of a hot agar (2%) solution around the lower half of another Petri dish (60 mm) used as the chamber mould. The lower chamber was filled with water (15 mL) and then covered with the filter. Eight leaf pieces were added to the upper chamber. The assembled chambers were incubated for 72 hours at 25°C. Each 24 hours a sample of 500 µL was taken from the lower chamber to count both *Vorticella* cells, using a

malassez chamber, and migrated propagules using a black shank disease assay. The disease assay was performed by infiltration of tobacco parenchymatous leaf tissue with 100 µL of inoculum from a ten-fold serial (1, 1/10/, 1/100) dilution of cell suspension. Two days later the total number of inoculated zones showing symptoms was counted in order to determine the concentration of migrated propagules in the lower chamber. Each sample was tested in octoplicate. Statistical analysis was carried out on data from three independent experiments by performing Student's t tests in Microsoft Excel 2003.

***Germination inhibition assay***

The antigerminative properties of the *Phoma*-conditioned water filtrate was tested on 10-well slides *in vitro.* A 20 µl suspension containing zoospores from oomycete strains or spores from fungi (400-1000 spores/µl) was mixed with equal volumes of V8 medium and of a serial dilution of the *Phoma* filtrate. Zoospores were previously and briefly vortexing to ensure synchronized germination. The germination percentage was determined for two replicates, after incubation for 24h at 25°C. Two parameters were determined from data: the minimum inhibitory dilution (MID) corresponding to the lowest dilution that inhibits 99.9% of germination for the tested microorganism (expressed as a dilution factor); and the half inhibitory dilution corresponding to the dilution that inhibits 50% of germination for the tested organism (expressed as % v/v).

***Nucleotide sequence accession numbers***

Reported sequences are deposited in the GenBank databank. The accession numbers are indicated in Table 1.

## Results

### Screening of *P. parasitica*-associated species affecting tobacco black shank disease

A three-stage strategy was developed in order to identify *P. parasitica*-associated species affecting tobacco black shank disease. The first one was the constitution of the community, through the use of the pathogen as a trap for associated microorganisms in a natural habitat. The second one was the selection of microorganisms for their ability to grow in the vicinity of the pathogen. The third step was the identidication of those affecting the host disease (Fig. 1).

For the first step *P. parasitica* biofilms were used to trap oomycete-associated microorganisms. We first formed mixed-species biofilms from *P. parasitica* microcolonies and microbial samples representative of the natural ecosystem. Microcolonies of *P. parasitica* were incubated with samples from the rhizosphere of *Nicotiana tabacum*. Based on four independent experiments the kinetics of colonization appeared to consist in three main events. Invasion began with the formation of bacterial colonies, followed by the attachment of stalked ciliates (48-72 h) and the installation of yeast-like cells (96-144 h) (Fig. 2A). We then selected the microorganisms that survived and grew in the vicinity of the pathogen. Mixed-species biofilms were dissociated and spread on agar plates containing a *P. parasitica* extract as the sole nutrient source. This step was performed in order to avoid selection of microorganisms which interacted with the oomycete (due to cAMP chemotaxis for instance) but which were unable to grow in the immediate environment of *P. parasitica* because of any kind of metabolic incompatibility. The microorganisms forming colonies were isolated with chloramphenicol to focus on the selection of euraryotes. About 400 colonies grew in the presence of the antibiotic. From two independent experiments 50 clones were isolated

representative of the morphological diversity of the colonies. The entire strategy was applied to two independent sets of 11 and 20 isolates, corresponding to those that were able to grow easely in the *in vitro* conditions tested (Table1). The sequencing of 18S ribosomal RNA genes showed that the eukaryotes were mostly eumycetes, stramenopiles, red algae and ciliates. We mixed each eukaryotic isolate with *P. parasitica* to identify the isolates with effects on plant disease. We co-incubated hyphae and spores *in vitro*, and co-infected plants with spores of the two species. Only one of the 31 isolates (corresponding to at least 9 species) affected oomycete growth and disease (Fig. 2B, Table 1). No disease symptoms were observed in the presence of each isolate alone, in the absence of the pathogen, except for isolates with 18S rRNA gene sequences identical to that of *P. parasitica.* Furthermore, among ciliates colonizing *P. parasitica* biofilms (and only studied at the first step of this study, Fig. 2A), a *Vorticella* species was found to affect oomycete cycle (euk A in table 1).

**A *Vorticella* species facilitates the dissemination of *P. parasitica* propagules**

Based on the overall strategy, we have characterized two types of interactions between *P. parastica* and eukaryotes which might interfere with the disease cycle. We detected a mutualistic interaction involving a *Vorticella* species. This ciliate was initially identified on the basis of its morphological characteristics: about 120-150 µm in size, with a contractile stalk associated with a domed feeding zone (Fig. 3A). The identification was conforted by a specific staining with a *Vorticella* probe by Fluorescence in-situ hybridization (FISH). Double-labeling experiments were carried out with FISH probes specific for eukaryotes (Eukr560) and for the genus *Vorticella* (Vortir339). For all the mixed-species biofilms analyzed, cells with the typical characteristics of the ciliate were double-stained (Fig 3Bi, Biii and Biv). The other cells or structures present were either not stained or were stained with the

Eukr560 probe only, as shown for the sporangium of *P. parasitica* (Bi and Biii). The action of the ciliate on the biofilm was much like that of a pollinator on a flowering plant. Interaction with the oomycete began with the attachment of the ciliate to a *P. parasitica* microcolony (Fig. 3A, Movie S1, Sequence 1 ). Once temporarily rooted in the biofilm, the ciliate probably fed on bacteria, small protozoan or organic food (4, 27). *Vorticella* may then left the biofilm by swimming (Movie S1, Sequence 2). transporting with it material from the oomycete at the end of its stalk. This material could be important in size and include a *P. parasitica* sporangium (Movie S1, Sequence 3 and 4). In this way, each *Vorticella* cell swimming away from the biofilm facilitated dissemination of *P. parasitica* propagules. An analysis of the video sequences showed that the *Vorticella* cells transporting oomycete material were able to reach speeds of up to 100 µm/s. These observations indicated that *Vorticella* could ensure rapid dissemination the disease over large distances.

The dissemination by a *Vorticella* species of *P. parasitica* propagules was demonstrated *in vitro* using a Boyden chamber assay. Eight leaf pieces harboring both *P. parasitica* microcolonies and anchored cells from the *V. microstoma* strain 30897 were deposited in the upper part of the chamber. The ciliate cells were found to migrate gradually to the lower chamber reaching a cell density of 500 $\pm$ 84 cells/mL at 72 h (Fig. 3C). In these conditions migration properties of propagules causing tobacco black shank disease was also observed. In the lower chamber the migrated propagules increased with time and reached a concentration of 375 $\pm$ 83 propagules/mL at 72 h (VP in Fig. 3D). The detection of propagules in this chamber was dependent of *Vorticella* adhesion on *P. parasitica* microcolonies. The propagules concentration decreased drastically at each time point tested when pre-incubation with *Vorticella* cells was omitted, reaching 34 $\pm$ 27 at 72 h (P in Fig. 3D). Any propagules could be detected when leaf pieces harbored anchored cilates but not *P. parasitica* microcolonies in the upper chamber (V in Fig. 3D).

**A *Phoma* species suppresses black shank disease**

During the screening process, only one isolate (*Ieuk* 3) representing a *Phoma* species was found to affect oomycete growth and disease (Fig. 2B, Table 1). An amensalistic interaction between this *Phoma* species and *P. parasitica* was characterized. The presence of this fungus was detrimental to *P. parasitica* but its own growth was not affected by the presence of the oomycete (data not shown). The isolate was identified as most closely resembling *P. herbarum*, on the basis of the nucleotide sequence of the 18S ribosomal RNA gene for the closest match on BLAST analysis (1) (Table 1). The mycelium of the ascomycete sporulated laterally or by budding, forming aggregates of brown spores (Fig. 4A and 4B). It strongly suppressed the development of black shank disease (Fig. 2B). Following the inoculation of tobacco plants with a mixture of 500 spores from this *Phoma* species and 500 zoospores from *P. parasitica,* a mean of 95 $\pm$ 3% of the inoculated zones developed no symptoms, and no measurable area displaying disease symptoms could be identified. In these conditions, the inoculated parenchymatous tissue appeared healthy (Fig. 2B). By contrast, 100% of the zones inoculated with *P. parasitica* zoospores alone displayed disease symptoms within 48 hours, over a mean area of 1.8$\pm$0.3 $cm^2$. Further investigations indicated that the growth of the oomycete was inhibited by the presence of the ascomycete *in vitro*. A clear zone of growth inhibition was observed around the *P. parasitica* strain 329 mycelium when the two microorganisms were incubated together on the same medium (Fig. 4C). The fungus produced a metabolic compound (or a mixture) preventing *P. parasitica* germination, as demonstrated by the effects of a *Phoma*-conditioned water filtrate, which reduced cyst germination by up to 90% for strain 329 (Fig. 4D). Similar results were obtained for two additional *P. parasitica* strains, with the germination rates of strains 310 and 408 reduced by

98 and 96 %, respectively. These results suggest that this fungus may have broad-spectrum activity within the *P. parasitica* species.

The antigerminative properties of the *Phoma*-conditioned water filtrate was also investigated on three ascomycetes: *Penicillium griseofulvum* (*Ieuk2*), *Candida austromarina* (*Ieuk6*), *Botrytis cinerea*. Minimum inhibitory dilutions of 1:36 and 1:72 were found to completely inhibit the germination of *P. parasitica* strains for 24 h. Lower dilutions (ranging from 1:3 to 1:6) were required to observe the same effect on spores from the acomycetes (Fig. 4E). The values of HIDs confirmed the rather higher antigerminative properties of the *Phoma* species on *Phytophthora* strains. HID values were 1 and 1,5 % for strains 310 and 329 while they were 3, 5, 11 and for *U. isabellina, B. cinerea and P. griseofulvum,* respectively. Bacterial (*Escherichia coli*, DH5α) and yeast (*Saccharomyces cerevisiae*, JD53) growth was not impaired by exposure to *Phoma* filtrate at the lowest tested dilution (1:3) *in vitro* (data not shown).

## Discussion

We describe here a novel strategy for ecosystem screening of pathogen-associated microorganisms affecting host disease. The approach could be applied to any pathogen that can be cultured *in vitro,* and to any microbial sample easily recovered from the natural habitat of the pathogen. The different biological forms of a microorganism (cells, spores, zoospores, mycelium, biofilms,…) can be used in the first step to trap microorganisms interacting with a pathogen. The feasability of this method is demonstrated for the plant pathogen *Phytophthora parasitica,*

The use of this method allows characterizing the species interacting with a pathogen. A widely accepted system for classifying interactions between organisms has been developed by Odum (24). Interactions between two organisms are seen as having a negative effect (-), a positive effect (+), or a neutral effect (0) on each participant in the interaction. The extrapolation of this system may be proposed for the classification of biotic interactions involving a known pathogenic species, not in terms of the repercussion of the interaction on the two organisms, but in terms of the effects of the interaction on disease outcome. The species interacting with the pathogen would be considered to be promotors or suppressors of disease when they have positive or negative effects, respectively, on disease. For each of these species, and independently of the other species interacting with the pathogen studied, a disease index could be determined quantifying the intrinsic and individual influence of the species concerned on the disease. With the exhaustive characterization of most of the species affecting the disease, it would then become possible, for a given biotope, to calculate a community indicator of disease. This cumulative indicator would reflect the sum of individual indices weighted by the richness score for each species within the community. Its value would oscillate between two extremes: that for which all the biotic conditions are required for the occurrence of an epidemic and that for which these conditions would be most likely to

prevent an epidemic. Thus, by combining studies on community function, such as this one, with Metagenomics analyses providing a picture of a community structure, it should be possible to increase our ability to modify disease states, through the use of crucial data defining the status of a biotic environment with respect to a disease, and to forecast disease epidemics.

In the case presented here, the rhizospheric community screened was a mixed-species biofilm, the natural habitat of most microorganisms (9, 10). Two of the microorganisms trapped with the soilborne pathogen *P. parasitica* affected the biology of the pathogen and for one of them, an interference with *N. tabacum* disease was demonstrated. These results constitute the first characterization, for a plant disease, of the influence of biocenotic relationships within a microbial community considered as the pathogenic unit.

A mobile unicellular organism, *Vorticella*, was identified as a disseminator of *P. parasitica*. Further work is required to establish if the dessimination of oomycete propagules by *Vorticella* could contribute to disease propagation, if *Vorticella* may be a promotor of tobacco black shank disease. For *Phytophthora* species such as *P. parasitica*, which produce zoospores with swimming motility in the soil (17), this alternative route of dissemination may be seen as secondary. However, it should be noted that the ciliate may adopt a rectilinear trajectory when transporting large amounts of pathogenic material (Movie 1, sequence no. 2), and such trajectories are more efficient for long-distance exploration than the helical trajectory of zoospores (2). This mode of dissemination may predominate for non motile pathogens. In natural conditions, disseminator species such the *Vorticella* one describe here may increase the likelihood of the transported pathogen reaching a host of the appropriate genotype.

A *Phoma* species was identified as a suppressor of tobacco black shank disease. Experiments are needed to establish if the strain may be a suitable organism for biological control of *P. parasitica* in the field. Nevertheless, its characterization using the ecosystem screening approach (ESA) indicates that ESA applied to other pathogens could be fruitful to diversify material for biological control of some plant diseases.

Characterization of main suppressors should also help to study the evolution of pathogens. The presence of a suppressor species in the same habitat than *Phytophthora* limits the success of the oomycete, contributing to the overall control of the plant disease. Within the rhizosphere, interspecific competition may also cause the displacement of *Phytophthora* species towards another habitat, such as the plant roots, the nearest alternative habitat. During evolution, competitive displacement may have resulted in the selection, within ancestral populations, of new genetic traits contributing to development of virulence in plants within the *Phytophthora* lineage (22). Ecological studies could focus on the divergence of genetic characteristics between *Phytophthora* populations from biotopes with high levels of interspecific competition and those from biotopes with low levels of interspecific competition. Such studies could first mainly focused genome-wide catalogs of oomycete virulence effectors (18). This would provide an example of a localized blow to what Darwin referred to as the "yielding surface" of Nature, struck at the level of one of the ten thousand "wedges" packed closely together and representing different- species (11, 14). The shock resulting from the blow — in this case, biotic competition — creates ripples extending outwards over great distances, contributing here to the emergence of pathogenesis. In other words, some species may become pathogens to escape their competitors, with pathogenicity increasing the chances of survival for species subject to amenalism.

This study provides a general methodology for analyses of the biotic interactions of pathogens within natural microbial communities, identifying the major implications of each host-pathogen interaction from an agronomic or therapeutic viewpoint and identifying the microbial relationships worthy of study in evolutionary biology.

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## Figure Legends

**Figure 1**

**Scheme of microbial community screening for pathogen-associated microorganisms affecting host disease**

Example of analysis of the rhizosphere community associated, in biofilms, with the plant pathogen *Phytophthora parasitica*.

**Figure 2**

**Biofilm community and *in planta* screening**

**(A)** Illustration of a mixed-species biofilm after colonization of a *P. parasitica* microcolony.

**(B)** For *in planta* screening, *P. parasitica* zoospores were used alone (*Pp*) or with spores from isolates Ieuk1, Ieuk2, Ieuk3 ($I_1$, $I_2$, $I_3$) for inoculation. Only Ieuk3, corresponding to a *Phoma* species, suppressed the disease. The difference in the percentage area displaying symptoms between $I_3$ and Pp was highly significant in a Student's t test ($P < 0.0001$) in three independent experiments.

**Figure 3**

**_Vorticella-Phytophthora_ interaction**

**(A)** *Vorticella* species anchored in a biofilm. The inset illustrates a larger view of the attachment of a ciliate cell to a microcolony

**(B)** Confocal laser scanning microscopy images of a ciliate cell and a *P. parasitica* sporangium anchored in a mixed-species biofilm (Bii). Double FISH staining was performed for *Vorticella* (Bi) and eukaryotic (Biii) 18S rRNAs. The *Vorticella*-specific probe decorated only the ciliate cell, and did not stain the *P. parasitica* sporangium. The eukaryotic probe decorated both structures. Biv corresponds to the three merged images.

(**C**) Kinetics of *V. microstoma* dissemination in a boyden chamber. Leaf pieces harboring anchored *V. microstoma* cells and *P. parasitica* microcolonies were applied to the upper part of the chamber. At each time point *V. microstoma* cells concentration was determined in the lower part of the chamber. Data are expressed as mean ± standard deviation of three independent experiments.

(**D**) *P. parasitica* propagules dissemination in a boyden chamber at 24 (blue), 48 (red) and 72 (green) hours. Three types of source for inoculum are shown: Leaf pieces harboring anchored *V. microstoma* cells (V), leaf pieces harboring *P. parasitica* microcolonies (P), Leaf pieces harboring *V. microstoma* cells and *P. parasitica* microcolonies (VP). At each time point propagules concentration was determined in the lower part of the chamber using a black shank disease assay. Data are expressed as mean ± standard deviation of three independent experiments. Results were analysed statistically by means of a Student's t-test. Significant differences were noted between P and VP at 48 (P = 0,03; n=3, *) and 72 hours (P = 0,02; n=3, **).

**Figure 4**

***Phoma- Phytophthora* interaction**

**(A)** and **(B)** Micrographs of brown spores emerging laterally or apically from the *Phoma* mycelium .

**(C)** Agar plate showing a zone of inhibition of oomycete growth (right) by the mycelium of the *Phoma* isolate Ieuk3 (left).

**(D)** Effect of *Phoma*-conditioned water filtrate on *P. parasitica* growth, measured as the percentage of cysts germinating (black bar, micrograph on the right) and compared with that for water treatment (white bar, micrograph on the left). Statistical analyses were performed with Student's t test ($P < 0.001$). Error bars denote means ± SD. Bars: 10 µm.

(**E**) Comparaison of the effect of *Phoma*-conditioned water filtrate on germination of *P. parasitica* strains 310 (grey) and 329 (black) and of fungi: *Penicillium griseofulvum* (red), *Candida austromarina* (green), *Botrytis cinerea* (yellow). Data are means ± standard deviation (n=4) of a representative experiment from three.

**Supplemental Material**

**Movie SI 1 legend**

**Behaviour of *Vorticella***

Real-time video of the attachment of a *Vorticella* ciliate to a *P. parasitica* microcolony (sequence 1) and of dissemination of the oomycete material during the "free" swimming stage after release from the biofilm (sequence 2, 3 and 4).

Sequence 1: A *Vorticella* ciliate attached to a microcolony.

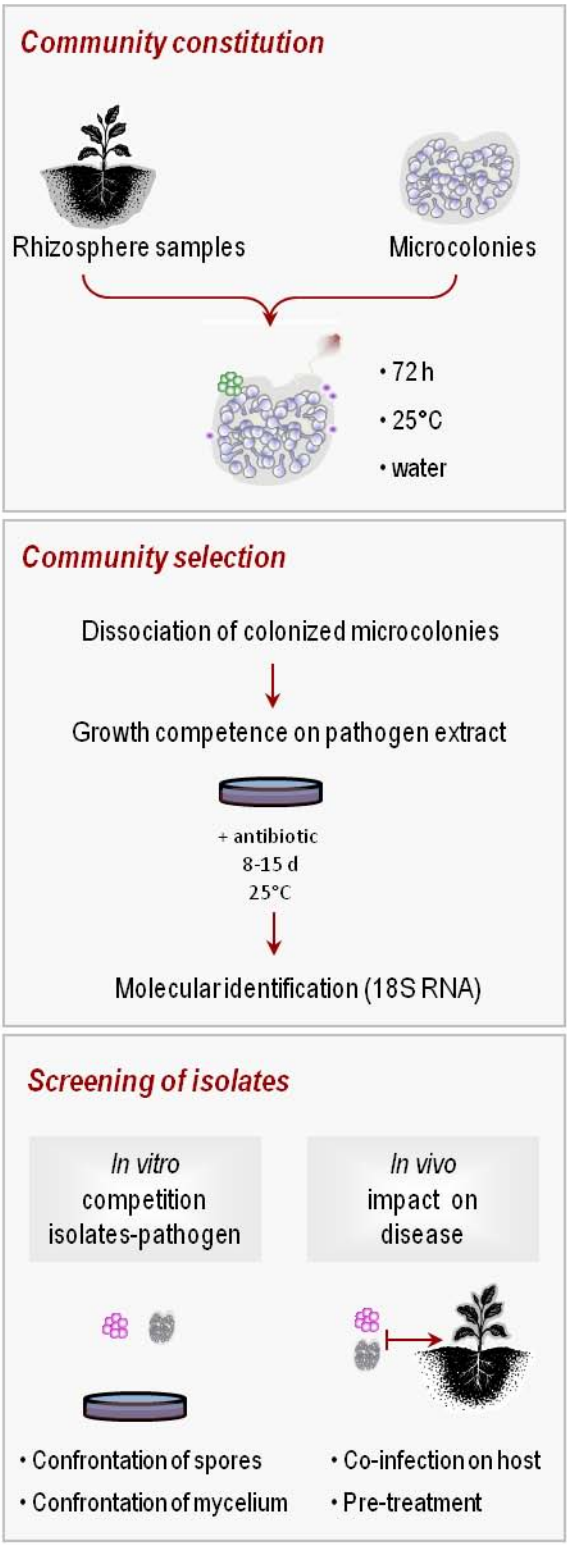
Sequence 2: A *Vorticella* cell released from a microcolony, swimming at a speed of 200 µm/s. The rectilinear trajectory (top-down along the vertical bar of 1000 µm in the sequence) ensures rapid propagation over long distances.

Sequence 3: A *Vorticella* cell released from a biofilm and transporting oomycete material at speeds of up to 100 µm/s.

**Table 1** ***Properties of eukaryotic isolates***

| *Isolate* | P. parasitica *growth* *(1)* | | *Black shank disease* *(2)* | | *Plant* *(3)* | *Molecular identification based on RNA 18S gene sequencing* *(4)* | | *(5)* | *Spore morphology* *(6)* |
|---|---|---|---|---|---|---|---|---|---|
| | Inhibition | Enhancement | Suppression | Promotion | Symptoms | Genus | Nearest species (Percent identity) | GenBank Accession n° | |
| *Ieuk1* | No | No | No | No | None | *ND* | *ND* | | ND |
| *Ieuk 2* | No | No | No | No | None | *Penicillium* | *P. griseofulvum (98%)* | HM161744 | round, fluorescent |
| *Ieuk 3* | Yes | No | Yes | No | None | *Phoma* | *P. herbarum (98%)* | HM161743 | round, smooth, brown |
| *Ieuk 4* | No | No | No | No | None | *Poterioochromonas* | *P. malhamensis (99%)* | HM161745 | elliptic, vacuolar |
| *Ieuk 5* | *ND* | *ND* | *ND* | *ND* | *ND* | *Phytophthora* | *P. nicotianae (99%)* | HM161752 | |
| *Ieuk 6* | No | No | No | No | None | *Candida* | *C. austromarina (99%)* | HM161746 | round, green |
| *Ieuk 7* | No | No | No | No | None | *Umbelopsis* | *U. isabellina (99%)* | HM161747 | round, smooth or spiny |
| *Ieuk 8* | No | No | No | No | None | *Poterioochromonas* | *P. malhamensis (99%)* | HM161748 | elliptic, vacuolar |
| *Ieuk 9* | *ND* | *ND* | *ND* | *ND* | *ND* | *Phytophthora* | *P. nicotianae (99%)* | HM161752 | |
| *Ieuk 10* | *ND* | *ND* | *ND* | *ND* | *ND* | *Phytophthora* | *P. nicotianae (99%)* | HM161752 | |
| *Ieuk 11* | No | No | No | No | None | *ND* | *ND* | | *ND* |
| *Ieuk 12* | No | No | No | No | None | *Penicillium* | *P. phialosporum (98%)* | HM161749 | *ND* |
| *Ieuk 13* | No | No | No | No | None | *Candida* | *C. lyxosophila (98%)* | HM161750 | *ND* |
| *Ieuk 14* | No | No | No | No | None | *Umbelopsis* | *U. isabellina (99%)* | HM161751 | round, smooth or spiny |
| *Ieuk 15* | No | No | No | No | None | *Candida* | *C. lyxosophila (98%)* | HM161753 | *ND* |
| *Ieuk 16* | No | No | No | No | None | *Cyanidioschyzon* | *C. merolae (94%)* | HM161754 | *ND* |
| *Ieuk 17-19* | No | No | No | No | *ND* | *ND* | *ND* | | *ND* |
| *Ieuk 20* | No | No | No | No | None | *Umbelopsis* | *U. isabellina (99%)* | HM161751 | *ND* |
| *Ieuk 21-31* | No | No | No | No | None | *ND* | *ND* | | *ND* |
| *euk A* | *ND* | Dissemination | *ND* | *ND* | *ND* | *Vorticella* | *ND* | | stalk , domed feeding zone |

Each isolate (Ieuk) is annotated for: ***(1)*** its effect on *P. parasitica* growth *in vitro*, ***(2)*** its impact on tobacco black shank disease when the plant was inoculated with both the isolate and the oomycete, ***(3)*** its ability to induce plant symptoms when used alone for inoculation, ***(4)*** the genus to which it belongs and the nearest species, based on closest match obtained with the BLAST algorithm (1), ***(5)*** the GeneBank accession number of the RNA 18S gene sequence, ***(6)*** the morphology of its spores. Two sets of 11 (Ieuk 1-11) and 20 (Ieuk 12-31) isolates were picked up from two independent experiments. ND for not determined.

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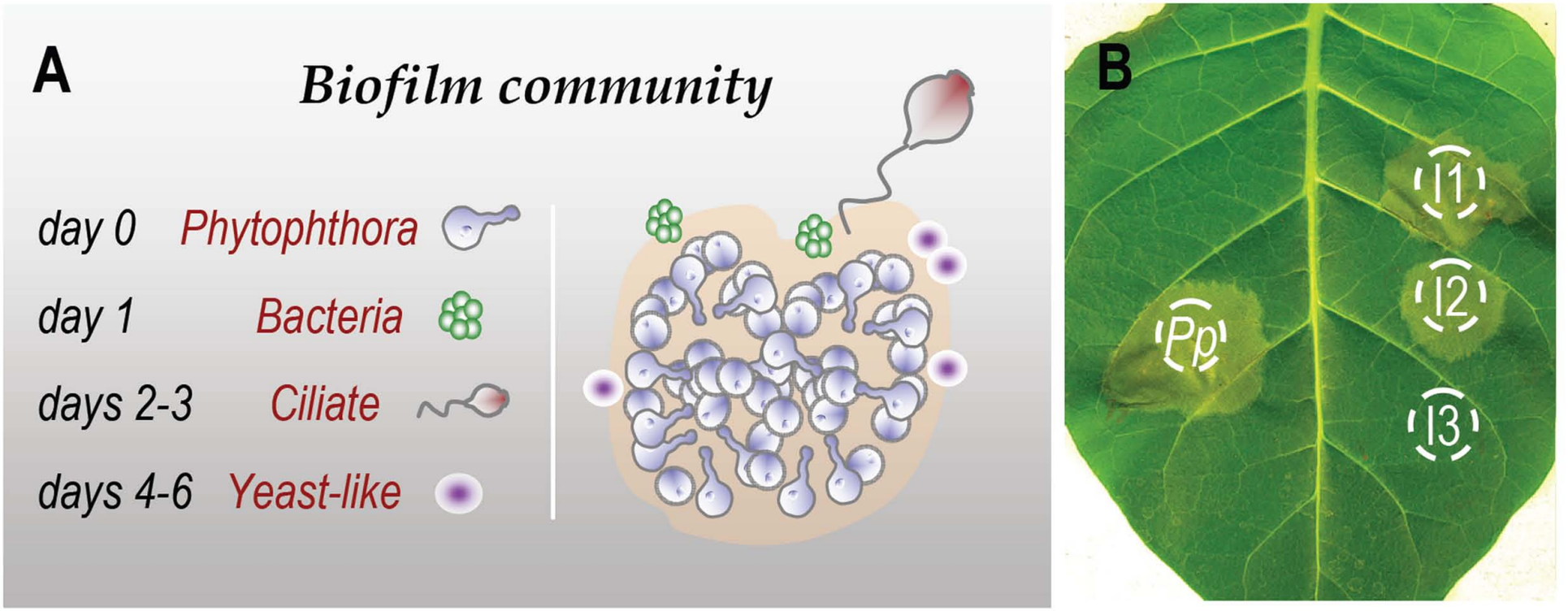


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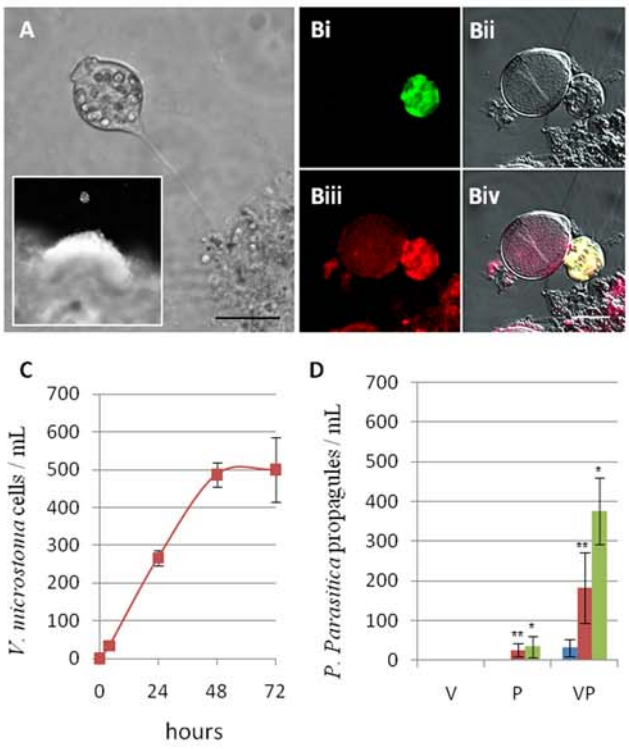


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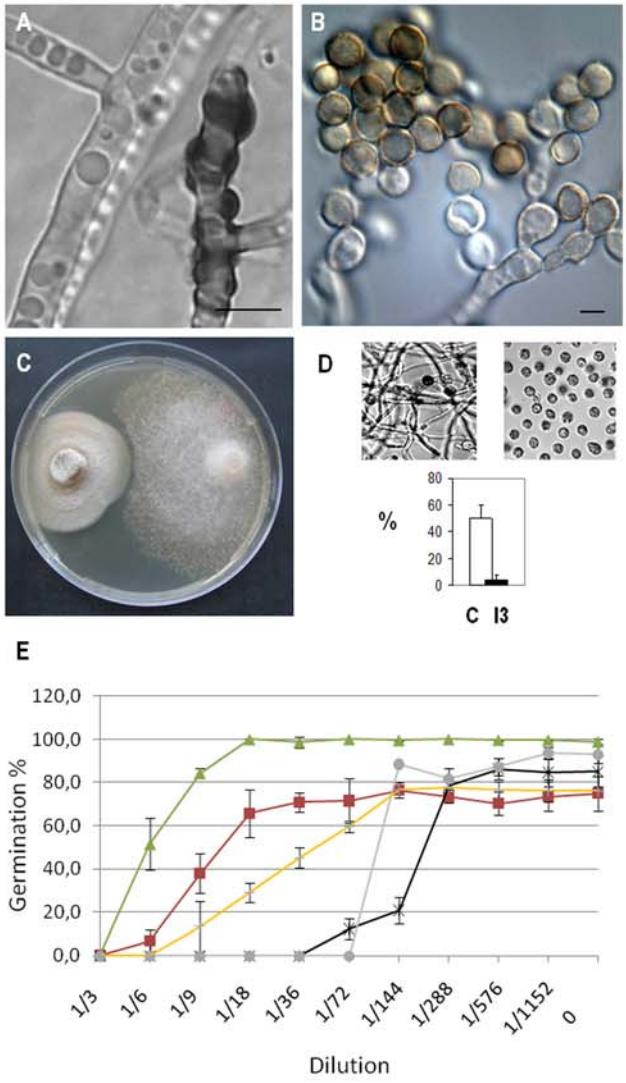


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