Applied and Environmental Microbiology, Sept. 2011, p. 6069–6075 Vol. 77, No. 17
0099-2240/11/$12.00 doi:10.1128/AEM.05371-11
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Ecosystem Screening Approach for Pathogen-Associated Microorganisms Affecting Host Disease▽†

Eric Galiana,[1,2,3]* Antoine Marais,[1,2,3] Catherine Mura,[1,2,3] Benoît Industri,[1,2,3] Gilles Arbiol,[1,2,3] and Michel Ponchet[1,2,3]

Institut National de la Recherche Agronomique, Unité Mixte de Recherche 1301 Interactions Biotiques et Santé Végétale, F-06903 Sophia Antipolis, France[1]; Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6243 Interactions Biotiques et Santé Végétale, F-06903 Sophia Antipolis, France[2]; and Université de Nice-Sophia Antipolis, Unité Mixte de Recherche Interactions Biotiques et Santé Végétale, F-06903 Sophia Antipolis, France[3]

Received 6 May 2011/Accepted 28 June 2011

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: (i) constitution of the microbial community, using the pathogen as a trap; (ii) community selection, using extracts from the pathogen as the sole nutrient source; and (iii) 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 nonobligate pathogens. It allows the definition of microbial species as promoters or suppressors of a disease for a given biotope. It should also help to identify important microbial relationships for ecology and evolution of pathogens.

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 (18, 29, 30). Investigations of the pathogenesis of several infections are currently moving away from a reductionist paradigm toward the view of the microbial community as a pathogenic unit (19, 28). 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 (20). In the case of plant diseases, the studies are mainly focused on microbial species controlling host diseases in pathogen-suppressive soils in which the pathogen does not establish or persist. The correlation between the high density of the populations of some species within a microbial community and the high suppressiveness level of the soil suggests that these species may be involved in the disease suppressive process (6, 8). In most cases microbial species promoting host diseases remains to be identified.

In the present study the impact of rhizospheric microorganisms on the tobacco black shank disease caused by the soilborne pathogen *Phytophthora parasitica* was investigated. *P. parasitica* is a filamentous eukaryotic plant pathogen (3), a member of the oomycete 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 of adherent microcolonies on the host surface that develop into large biofilms (13).

P. parasitica biofilm was used to study the interactions 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 pathogen virulence, as well as to the interaction dynamics with the host (5, 22, 25, 27, 31). They confer several advantages to pathogens favoring attachment on host surface, promoting virulence through aggregation, and providing protection against host defenses or biocide treatment (9). They also promote dissemination through transition from the aggregated to the planktonic lifestyle (10, 15). This choice was also conditioned by the

* Corresponding author. Mailing address: INRA, Unité Mixte de Recherche 1301 Interactions Biotiques et Santé Végétale, F-06903 Sophia Antipolis, France. Phone: 33(0)4 92 38 64 72. Fax: 33(0)4 92 38 65 87. E-mail: galiana@sophia.inra.fr.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 8 July 2011.

fact that in the natural habitat biofilms constitute propitious niches for interactions between pathogens and other species (23).

To get an 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 of the experiments. Seeds were germinated in flowerpots (9 by 9 by 9.5 cm; SOPARCO). At 2 weeks postgermination, 25 plants were transferred individually into flowerpots. Fertilizer containing nitrogen (N), phosphorus (P), and potassium (K), (15:12:30) was applied once (100 ml per flowerpot), and the plants were then watered regularly and grown for 3 weeks. Leaves or roots were taken from 7- to 8-week-old plants.

Microbial strains. *Phytophthora parasitica* strains 310, 329, and 408 were obtained from the Institut National de la Recherche Agronomique (INRA; Sophia-Antipolis, France) *Phytophthora* collection. *Vorticella microstoma* strain 30897 was purchased from the American Type Culture Collection (ATCC) collection of protists (LGC standards). The cells of the ciliate were cultivated for 3 to 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 characterized during the present study is recorded in the National Collection of the Institut Pasteur (recording number CNCM I-4278).

Community constitution. Microcolonies were prepared from strain 329, as previously described (13). Leaf pieces (5 by 0.5 cm) were inoculated in water for 3 h at 25°C with a suspension of *P. parasitica* zoospores (400 to 600 cells ml/μl). Microcolonies that formed on leaf surface were isolated and 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, mixed with sterile water (1/5 [wt/vol]), and filtered twice through a sieve with 100-μm pores. The resulting water suspension of microorganisms was rapidly decanted (5 min). The supernatant (5 ml) was placed in a 5.5-cm plastic petri dish, followed by incubation at 25°C in the presence of 10 to 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 (4′,6′-diamidino-2-phenylindole) staining using an Axioplan fluorescence microscope (Carl Zeiss Microimaging, Inc., Germany).

Community selection. After 3 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/liter; NaCl, 10 g/liter; agar 1.5% [wt/vol]) and incubated at 25°C. The *Phytophthora* crude extract was prepared from a 2-week-old mycelium of *P. parasitica* strain 329 (INRA). 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 of chloramphenicol/ml (in preliminary experiments, chloramphenicol appeared to be a more selective antibiotic than ampicillin and kanamycin for favoring growth of eukaryotes versus that of prokaryotes [data not shown]). Colonies appeared within 3 to 6 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 the cells, spores, or mycelium in boiling water for 3 min, which was then rapidly chilled on ice and centrifuged 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′) (24). 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.

FISH. The probes Vortir339 (5′-Cy3-GACTGCCATGGTAGTCCAATACA CT-3′) targeting *Vorticella* ciliates and Eukr560 (5′Cy5-5′-CGGCTGCTGGCA CCAGACTTGCCCT-3′) targeting all eukaryotes were used. The 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 a 1 μM concentration of probe was added to the samples, which were incubated overnight at 45°C. Biofilms were washed twice, for 15 min each time, in the hybridization solution at room temperature, placed on glass slides, and overlaid with an antifading reagent (VectaShield; Vector) before observation under a Zeiss LSM 510 Meta confocal microscope. Merged images showing fluorescence in situ hybridization (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 coincubating isolates and *P. parasitica* strains and in planta through coinfections. For the 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 the 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 from strain 310, 329, or 408/μl) was mixed with equal volumes of V8 medium and isolate-conditioned water filtrate (the cells were previously and briefly vortexed 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 2 h at 25°C.

For in planta screening, parenchymatous leaf tissue was coinfected 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 *Phytophthora* collection) and 500 spores of each isolate. For each isolate, spore suspensions were prepared in water from two mycelial discs (5 mm in diameter) after incubation in water (1 ml, 15 min, room temperature) and counted using a Malassez chamber and calibration by dilution in water. The percentage of inoculated zones showing symptoms and the area damaged were determined 2 days after coinoculation. We assessed the influence of isolates on the disease by comparing the results to 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 ± the standard deviations (SD) of three independent experiments, and statistical analysis was carried out by performing Student *t* tests in Microsoft Excel 2003.

Dissemination assay. Eight leaf pieces (5 by 0.5 cm) harboring or not *P. parasitica* microcolonies formed on their section (two to three microcolonies per centimeter) were incubated in water (10 ml) in the presence of cells of the *V. microstoma* strain 30897 (1,000 cells/ml) for 3 h at 25°C. The control without *V. microstoma* cells was adjusted with a volume of fresh and sterile V8 medium corresponding to the volume used for inoculation with the ciliate. After incubation leaf pieces with anchored ciliates, the cells were rinsed two times with 20 ml of water to eliminate contamination of the 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 of 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 mold. 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 h at 25°C. Every 24 h 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 10-fold serial dilution (1, 1/10, and 1/100) 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 octuplicate. Statistical analysis was carried out on data obtained from three independent experiments by performing Student *t* tests in Microsoft Excel 2003.

Germination inhibition assay. The antigerminative properties of the *Phoma*-conditioned water filtrate were tested on 10-well slides in vitro. A 20-μl suspension containing zoospores from oomycete strains or spores from fungi (400 to 1,000 spores/μl) was mixed with equal volumes of V8 medium and a serial dilution of the *Phoma* filtrate. Zoospores were previously and briefly vortexed to ensure synchronized germination. The germination percentage was determined for two replicates after incubation for 24 h 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 (HID), corresponding to the dilution that inhibits 50% of germination for the tested organism (expressed as a percentage [vol/vol]).

Nucleotide sequence accession numbers. Reported sequences are deposited in the GenBank databank. The accession numbers are given 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 step was the constitution of the community through the use of the pathogen as a trap for associated microorganisms in a natural habitat. Second, the microorganisms were selected on the basis of their ability to grow in the vicinity of the pathogen. The process was terminated by the identification of organisms 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 of three main events. Invasion began with the formation of bacterial colonies, followed by the attachment of stalked ciliates (48 to 72 h) and the installation of yeast-like cells (96 to 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 onto agar plates containing a *P. parasitica* extract as the main nutrient source. Since *P. parasitica* biofilm secretes cyclic AMP (cAMP) (13), a lot of microorganisms should be in the vicinity of biofilm in response to cAMP as a chemotactic signal (12). So this step was performed to focus on microorganisms interacting *with P. parasitica* and able to growth on biofilm matrix.

The microorganisms forming colonies were isolated with chloramphenicol to focus on the selection of eukaryotes. 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 underwent fast growth in the in vitro conditions tested (Table 1). The sequencing of 18S rRNA genes showed that the eukaryotes were mostly eumycetes, stramenopiles, red algae, and ciliates. We mixed each eukaryotic isolate with *P. parasitica* to identify the

TABLE 1. Properties of eukaryotic isolates[a]

Isolate(s)	*P. parasitica* growth		Black shank disease		Induction of plant symptoms	Molecular identification based on RNA 18S gene sequencing		GenBank accession no.	Spore morphology
	Inhibition	Enhancement	Suppression	Promotion		Genus	Nearest species (% identity)		
Ieuk1	No	No	No	No	None	ND	ND	ND	ND
Ieuk2	No	No	No	No	None	*Penicillium*	*P. griseofulvum* (98)	HM161744	Round, fluorescent
Ieuk3	Yes	No	Yes	No	None	*Phoma*	*P. herbarum* (98)	HM161743	Round, smooth, brown
Ieuk4	No	No	No	No	None	*Paterochromonas*	*P. malhamensis* (99)	HM161745	Elliptic, vacuolar
Ieuk5	ND	ND	ND	ND	ND	*Phytophthora*	*P. nicotianae* (99)	HM161752	
Ieuk6	No	No	No	No	None	*Candida*	*C. austromarina* (99)	HM161746	Round, green
Ieuk7	No	No	No	No	None	*Umbelopsis*	*U. isabellina* (99)	HM161747	Round, smooth, or spiny
Ieuk8	No	No	No	No	None	*Paterochromonas*	*P. malhamensis* (99)	HM161748	Elliptic, vacuolar
Ieuk9	ND	ND	ND	ND	None	*Phytophthora*	*P. nicotianae* (99)	HM161752	
Ieuk10	ND	ND	ND	ND	ND	*Phytophthora*	*P. nicotianae* (99)	HM161752	
Ieuk11	No	No	No	No	None	ND	ND		ND
Ieuk12	No	No	No	No	None	*Penicillium*	*P. phialosporum* (98)	HM161749	ND
Ieuk13	No	No	No	No	None	*Candida*	*C. fyxosophila* (98)	HM161750	ND
Ieuk14	No	No	No	No	None	*Umbelopsis*	*U. isabellina* (99)	HM161751	ND
Ieuk15	No	No	No	No	None	*Candida*	*C. fyxosophila* (98)	HM161753	Round, smooth, or spiny
Ieuk16	No	No	No	No	None	*Cyanidioschyzon*	*C. merolae* (94)	HM161754	ND
Ieuk17 to Ieuk19	No	No	No	No	ND	ND	ND		ND
Ieuk20	No	No	No	No	None	*Umbelopsis*	*U. isabellina* (99)	HM161751	ND
Ieuk21 to Ieuk31	No	No	No	No	None	ND	ND		ND
eukA	ND	Dissemination	ND	ND	ND	*Vorticella*	ND		Stalk, domed feeding zone

[a] Each Ieuk isolate is annotated for its effect on *P. parasitica* growth *in vitro*, its impact on tobacco black shank disease when the plant was inoculated with both the isolate and the oomycete, its ability to induce plant symptoms when used alone for inoculation, the genus to which it belongs and the nearest species, based on closest match obtained with the BLAST algorithm, the GenBank accession number of the RNA 18S gene sequence, and the morphology of its spores. Two sets of 11 (Ieuk1 to Ieuk11) and 20 (Ieuk12 to Ieuk31) isolates were picked up from two independent experiments. ND, not determined.

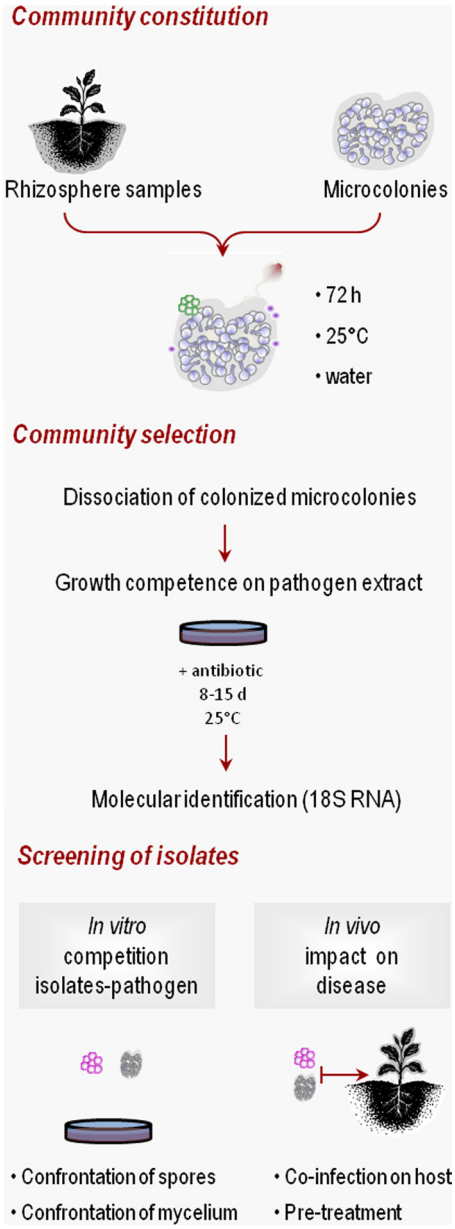

FIG. 1. Scheme of microbial community screening for pathogen-associated microorganisms affecting host disease. This represents an example approach to analysis of the rhizosphere community associated, in biofilms, with the plant pathogen *P. parasitica*.

isolates with effects on plant disease. We coincubated hyphae and spores in vitro and coinfected plants with spores of the two species. Of 31 isolates (corresponding to at least nine species), only one (*Phoma herbarum*) affected oomycete growth and disease (Fig. 2B and 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 the present study, Fig. 2A), a *Vorticella* species was found to affect the oomycete cycle (eukA in Table 1).

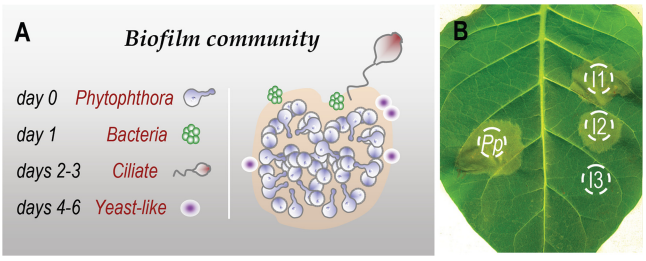

FIG. 2. Biofilm community and in planta screening. (A) Illustration of a mixed-species biofilm after colonization of a *P. parasitica* microcolony. (B) For in planta screening, *P. parasitica* zoospores were used alone (*Pp*) or with spores from isolates Ieuk1, Ieuk2, and Ieuk3 (I1, I2, and I3) for inoculation. 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 *t* test ($P < 0.0001$) in three independent experiments.

A *Vorticella* species facilitates the dissemination of *P. parasitica* propagules. Based on the overall strategy, we characterized two types of interaction between *P. parasitica* 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 to 150 μm in size, with a contractile stalk associated with a domed feeding zone (Fig. 3A). The identification was reinforced by a specific staining with a *Vorticella* probe by FISH. Double-labeling experiments were carried out with FISH probes specific for eukaryotes (Eukr560) and for the genus *Vorticella* (Vortir339). For all of 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* (Fig. 3Bi 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 and see Movie S1, sequence 1, in the supplemental material). Once temporarily rooted in the biofilm, the ciliate probably fed on bacteria, small protozoa, or organic food (4, 26). The *Vorticella* cell then left the biofilm by swimming (see Movie S1, sequence 2, in the supplemental material), transporting with it material from the oomycete at the end of its stalk. This material could be large and included a *P. parasitica* sporangium (see Movie S1, sequences 3 and 4, in the supplemental material). In this way, each *Vorticella* cell swimming away from the biofilm facilitated the 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 of the disease over large distances.

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,

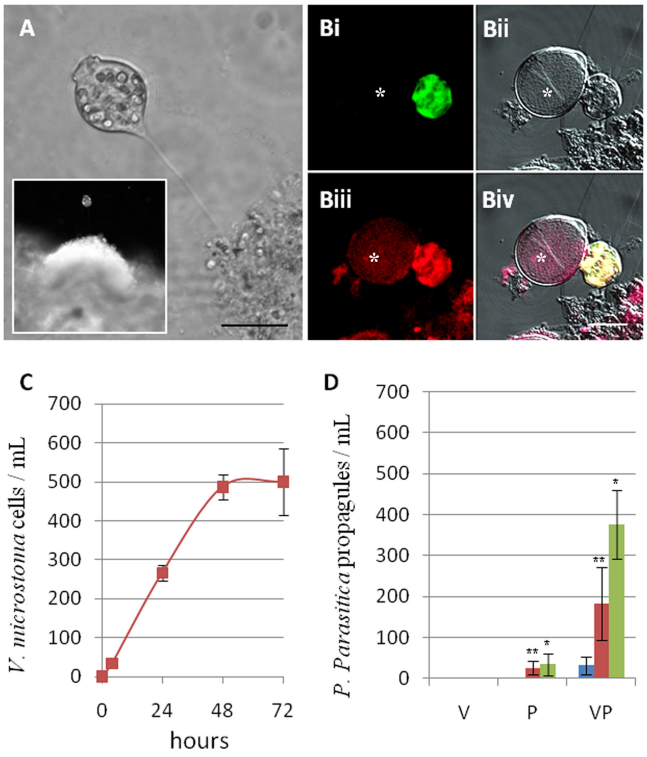

FIG. 3. *Vorticella-Phytophthora* interaction. (A) *Vorticella* species anchored in a biofilm. The inset illustrates a larger view of the attachment of a ciliate cell to a microcolony. (B) Confocal laser scanning microscopy images of a ciliate cell and a *P. parasitica* sporangium anchored in a mixed-species 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. Bars, 20 μm. (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. The data are expressed as means ± the SD of three independent experiments. (D) *P. parasitica* propagule dissemination in a Boyden chamber at 24 h (blue), 48 h (red), and 72 h (green). Three types of sources for inocula are shown: leaf pieces harboring anchored *V. microstoma* cells (V), leaf pieces harboring *P. parasitica* microcolonies (P), and leaf pieces harboring *V. microstoma* cells and *P. parasitica* microcolonies (VP). At each time point, the propagule concentration was determined in the lower part of the chamber by using a black shank disease assay. The data are expressed as means ± the SD of three independent experiments. The results were analyzed statistically by means of a Student *t* test. Significant differences were noted between P and VP at 48 (*, $P = 0.03$ [$n = 3$]) and 72 h (**, $P = 0.02$ [$n = 3$]).

reaching a cell density of 500 ± 84 cells/ml at 72 h (Fig. 3C). In these conditions migration properties of propagules causing tobacco black shank disease were also observed. In the lower chamber the migrated propagules increased with time and reached a concentration of 375 ± 83 propagules/ml at 72 h (VP in Fig. 3D). The detection of propagules in this chamber was dependent on *Vorticella* adhesion on *P. parasitica* microcolonies. The propagule concentration decreased drastically at each time point tested when preincubation with *Vorticella* cells was omitted, reaching 34 ± 27 at 72 h (P in Fig. 3D). No propagules could be detected when leaf pieces harbored anchored ciliates 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 (Ieuk3) representing a *Phoma* species was found to affect oomycete growth and disease (Fig. 2B and Table 1). An amensal 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 rRNA gene for the closest match by BLAST analysis (1) (Table 1). The mycelium of the ascomycete sporulated laterally or by budding, forming aggregates of brown spores (Fig. 4A and B), which strongly suppressed the development of black shank disease (Fig. 2B). After 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% ± 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 to be healthy (Fig. 2B). In contrast, 100% of the zones inoculated with *P. parasitica* zoospores alone displayed disease symptoms within 48 h, over a mean area of 1.8 ± 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), and *Botrytis cinerea*. MIDs 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 ascomycetes (Fig. 4E). The values of HIDs confirmed the rather higher antigerminative properties of the *Phoma* species on *Phytophthora* strains. The HID values were 1 and 1.5% for strains 310 and 329, while they were 3, 5, and 11% 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

The use of the ecosystem screening approach described here allows the characterization of species interacting with a pathogen and affecting host disease. A widely accepted system for

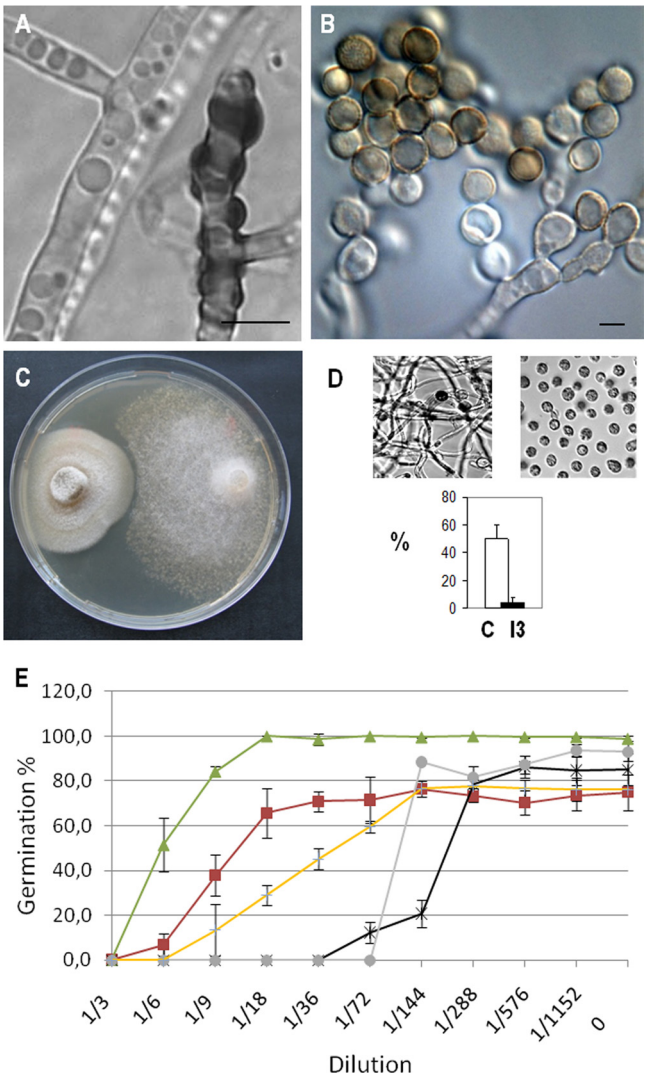

FIG. 4. *Phoma-Phytophthora* interaction. (A and B) Micrographs of brown spores emerging laterally or apically from the *Phoma* mycelium. (C) Agar plate showing a zone of inhibition of oomycete growth (right) by the mycelium of the *Phoma* isolate Ieuk3 (left). (D) Effect of *Phoma*-conditioned water filtrate on *P. parasitica* growth, measured as the percentage of cysts germinating (■, micrograph on the right) and compared to that for water treatment (□, micrograph on the left). Statistical analyses were performed with the Student *t* test ($P < 0.001$). Error bars denote means ± the SD. Bars, 10 μm. (E) Comparison of the effect of *Phoma*-conditioned water filtrate on germination of the *P. parasitica* strains 310 (gray) and 329 (black) and of the fungi *Penicillium griseofulvum* (red), *Candida austromarina* (green), and *Botrytis cinerea* (yellow). The data are means ± the SD ($n = 4$) of a representative experiment from three.

classifying interactions between organisms has been developed by Odum (23). 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 promoters 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 an eukaryotic microbial community considered as the pathogenic unit.

A mobile unicellular organism, *Vorticella*, was identified as a disseminator of *P. parasitica* in vitro. Further work is required to establish whether the dissemination of oomycete propagules by *Vorticella* could contribute to disease propagation, if *Vorticella* may be a promoter of tobacco black shank disease in the field. 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 (see Movie S1, sequence 2, in the supplemental material), and such trajectories are more efficient for long-distance exploration than the helical trajectory of zoospores (2). This mode of dissemination may predominate for nonmotile pathogens. In natural conditions, disseminator species such as *Vorticella* described 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 whether the strain may be a suitable organism for biological control of *P. parasitica* in the field. Nevertheless, its characterization using an ecosystem screening approach indicates that the application of this approach to other pathogens could be advantageous in diversifying 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. Within the rhizosphere, interspecific com-

petition may also cause the displacement of *Phytophthora* species toward 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 (21). 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 10,000 "wedges" packed closely together and representing different species (11, 14). The shock resulting from the blow—in this case, biotic competition—creates ripples extending outward 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 amensalism.

ACKNOWLEDGMENTS

We thank Julie Hopkins for revising the English. We also thank Véronique Saint-Ges for her constant encouragement and support.

REFERENCES

1. **Altschul, S. F., et al.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Appiah, A. A., P. van West, M. C. Osborne, and N. A. Gow.** 2005. Potassium homeostasis influences the locomotion and encystment of zoospores of plant pathogenic oomycetes. Fungal Genet. Biol. **42:**213–223.
3. **Attard, A., et al.** 2008. Strategies of attack and defense in plant-oomycete interactions, accentuated for *Phytophthora parasitica* Dastur (syn. *P. nicotianae* Breda de Haan). J. Plant Physiol. **165:**83–94.
4. **Bhamrah, H. S., and K. Juneja.** 2002. An introduction to protozoa. J. L. Kumar/Anmol Publication Pvt., Ltd., New Delhi, India.
5. **Blankenship, J. R., and A. P. Mitchell.** 2006. How to build a biofilm: a fungal perspective. Curr. Opin. Microbiol. **9:**588–594.
6. **Borneman, J., and J. O. Becker.** 2007. Identifying microorganisms involved in specific pathogen suppression in soil. Annu. Rev. Phytopathol. **45:**153–172.
7. **Boyden, S. V.** 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J. Exp. Med. **115:**453–457.
8. **Cook, R. J., and K. F. Baker.** 1983. The nature and practice of biological control of plant pathogens. American Phytopathological Society, St. Paul, MN.
9. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **21:**1318–1322.
10. **Danhorn, T., and C. Fuqua.** 2007. Biofilm formation by plant-associated bacteria. Annu. Rev. Microbiol. **61:**401–422.
11. **Darwin, C.** 1859. On the origin of species by means of natural selection, or the preservation of favored races in the struggle for life. John Murray, London, England.
12. **Devreotes, P.** 1982. Chemotaxis, p. 117–168. *In* W. F. Loomis (ed.), Development of *Dictyostelium discoideum*. Academic Press, Inc., New York, NY.
13. **Galiana, E., S. Fourré, and G. Engler.** 2008. *Phytophthora parasitica* biofilm formation: installation and organization of microcolonies on the surface of a host plant. Environ. Microbiol. **10:**2164–2171.
14. **Gould, S. J.** 2002. The structure of evolutionary theory. Belknap (Harvard University Press), Cambridge, MA.
15. **Hall-Stoodley, L., and P. Stoodley.** 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. **13:**7–10.
16. **Ishii, S., T. Shimoyama, Y. Hotta, and K. Watanabe.** 2008. Characterization of a filamentous biofilm community established in a cellulose-fed microbial fuel cell. BMC Microbiol. **8:**6.
17. **Judelson, H. S., and F. A. Blanco.** 2005. The spores of *Phytophthora*: weapons of the plant destroyer. Nat. Rev. Microbiol. **3:**47–58.
18. **Kent, A. D., and E. W. Triplett.** 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev. Microbiol. **56:**211–236.
19. **Kuramitsu, H. K., X. He, R. Lux, M. H. Anderson, and W. Shi.** 2007. Interspecies interactions within oral microbial communities. Microbiol. Mol. Biol. Rev. **71:**653–670.
20. **Mao-Jones, J., K. B. Ritchie, L. E. Jones, and S. P. Ellner.** 2010. How microbial community composition regulates coral disease development. PLoS Biol. **8:**e1000345.
21. **Morris, C. E., et al.** 2009. Expanding the paradigms of plant pathogen life history and evolution of parasitic fitness beyond agricultural boundaries. PLoS Pathog. **5:**e1000693.
22. **Morris, C. E., and J. M. Monier.** 2003. The ecological significance of biofilm formation by plant-associated bacteria. Annu. Rev. Phytopathol. **41:**429–453.
23. **Odum, E. P.** 1971. Fundamentals of ecology, 3rd ed. W. B. Saunders, Philadelphia, PA.
24. **Petroni, G., F. Dini, F. Verni, and G. Rosati.** 2002. A molecular approach to the tangled intrageneric relationships underlying phylogeny in *Euplotes* (Ciliophora, Spirotrichea). Mol. Phylogenet. Evol. **22:**118–130.
25. **Ramey, B. E., M. Koutsoudis, S. B. von Bodman, and C. Fuqua.** 2004. Biofilm formation in plant-microbe associations. Curr. Opin. Microbiol. **7:**602–609.
26. **Ravva, S. V., C. Z. Sarreal, and R. E. Mandrell.** 2010. Identification of protozoa in dairy lagoon wastewater that consume *Escherichia coli* O157:H7 preferentially. PLoS One **5:**e15671.
27. **Rigano, L. A., et al.** 2007. Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv. citri. Mol. Plant-Microbe Interact. **20:**1222–1230.
28. **Siqueira, J. F., Jr., and I. N. Rocas.** 2009. Community as the unit of pathogenicity: an emerging concept as to the microbial pathogenesis of apical periodontitis. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **107:**870–878.
29. **Stecher, B., and W. D. Hardt.** 2008. The role of microbiota in infectious disease. Trends Microbiol. **16:**107–114.
30. **Wolinska, J., and K. C. King.** 2009. Environment can alter selection in host-parasite interactions. Trends Parasitol. **25:**236–244.
31. **Yao, J., and C. Allen.** 2007. The plant pathogen *Ralstonia solanacearum* needs aerotaxis for normal biofilm formation and interactions with its tomato host. J. Bacteriol. **189:**6415–6424.