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

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

Keywords Soilless · Root disease · Biocontrol · *Sphingobium xenophagum* · *Aspergillus flavus* · *Mycolicibacterium fortuitum*

Introduction

Soil-borne plant diseases are in theory less common in soilless plant cultures than in soils [1]. However, some soil pathogens well adapted to aquatic environments can be highly virulent because they can produce mobile forms of dispersal that benefit from water recirculation [1, 2]. *Pythium aphanidermatum* (Edson) Fitzp.—an oomycete pathogen able to produce zoospores in water—can cause lettuce root rot and damping-off in soilless culture [3, 4]. Soilless systems comprise hydroponic and aquaponic systems. Aquaponics is defined in this study as an integrated system combining fish and plant production in the same recirculated water loop. In coupled aquaponics, plant treatment with pesticides and chemical disinfection agents to control pathogens are inadvisable because they can be toxic to the fish and beneficial microorganisms (e.g., nitrifying bacteria) present in the same water loop [5–9]. Therefore, biocontrol in aquaponics is of prime importance but still understudied [8, 9]. In hydroponics, the use of biocontrol agents and related studies are not new [10]. However, biocontrol research in hydroponics has often indicated poor adaptation and efficacy of common soil microbial biocontrol agents or microbial biopesticides to the specific aquatic conditions of soilless cultures [1, 11, 12]. Until now, no biopesticide has been specifically marketed for aquaponic use [8, 9] and only few products are available and

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useful to control root diseases in hydroponics [1, 11]. Consequently, there is a need to develop new microbial biopesticides adapted to soilless conditions and, more especially, to aquaponic conditions [1, 8, 9, 11]. In regard to approved soil biocontrol agents whose isolation campaign started 50 years ago from soil-borne disease-suppressive soil [13], this research has aimed to selectively isolate *P. aphanidermatum* biocontrol agents from the same aquaponic system where the suppressive *P. aphanidermatum* microbiota was characterized by Stouvenakers et al. [14]. The microorganisms to be isolated were selected based on the high-throughput sequencing (HTS) analysis achieved in Stouvenakers et al. [14]. Although numerous papers have characterized the suppressive activity of microbiota by HTS [15, 16], few of them have used these results to selectively isolate new biocontrol agents [17, 18]. This strategy was used to discover, among others, new biocontrol agents as yet unknown for such activity. Once the biocontrol agents have been isolated, numerous in vitro bioassays are available to screen their antagonistic activity against a pathogen [19]. Among the in vitro screening methods available, the best known and most used ones are the dual culture plate assays [20]. They are commonly used because they allow screening many isolates at the same time with minimum space needs. They are often used as a first screening step to decrease the number of strains before in vivo trials [20]. The in vitro first step was bypassed in the present study: the capacity of *P. aphanidermatum* to cause lettuce damping-off was used to develop a direct in vivo screening assay that combined the reliability of in vivo conditions with the advantages of in vitro bioassays. Once the best strains were screened, their efficacy was tested to control root rot of lettuce seedlings caused by the same pathogen. In summary, this study aims at isolating new biocontrol agents from aquaponics and testing them to control *P. aphanidermatum* lettuce diseases in soilless conditions.

Materials and Methods

Selection of Antagonistic Taxa

Microorganisms from aquaponic lettuce rhizoplane have been found correlated with *P. aphanidermatum* lettuce disease suppression [14]. Using HTS analysis, a list of OTUs potentially linked to this suppressiveness was established [14]. Then, bacteria and fungi (including Chytridiomycota phylum) to be isolated were selected based on this list and the literature. This strategy was named HTS-guided. The first criterion for taxon selection was a correlation of operational taxonomic units (OTUs) with suppressiveness in Stouvenakers et al. [14]. The second, optional criterion, was a documented pathogen suppressive action of the taxon in literature. Therefore, the OTUs considered as original, relatively abundant, and *P. aphanidermatum* suppressive in the study were selected. The selected OTUs and the isolation methods (further described) are summarized in Table 1. If the taxonomic rank of a selected OTU was not deep enough, the key genera to be isolated inside the rank were selected according to their antagonistic potentialities in the literature and/or their potential identity after a nucleotide blast search on the NCBI platform (https://blast.ncbi.nlm.nih.gov). The targeted genera were *Burkholderia*, *Chitinimonas*, *Mitsuaria*, *Lactobacillus*, *Methyloversatilis*, *Sphingobium*, *Hydrogenophaga*, *Catenaria*, *Rhizophydium*, and *Trichoderma.*

Strain Isolation

The aquaponic samples used to isolate the targeted microorganisms were the − 80 °C frozen samples of lettuce rhizoplane washing water (plus 25% glycerol) taken during the first suppressive in vivo experiment by Stouvenakers et al. [14]. When selective isolation was not conclusive, washing water of fresh lettuce rhizoplane was used instead. Lettuce

Table 1 Target genera for selective isolation, and methods depending on OTU identification

	OTU taxa	Target genera	Isolation method	Abbr.
Bacteria	f_Burkholderiaceae	*Burkholderia* *Chitinimonas* *Mitsuaria*	Chitosan degrading on CDA medium + colony morphological observation + Gram stain	Chito.
	g_*Lactobacillus*	*Lactobacillus*	MRS medium growth + colony morphological observation + Gram stain	Lacto.
	g_*Methyloversatilis*	*Methyloversatilis*	Enrichment or growth with formaldehyde and methanol + colony morphological observation + Gram stain	ForE. or ForG.
	g_*Sphingobium* g_*Hydrogenophaga*	*Sphingobium* *Hydrogenophaga*	R2A medium growth + colony morphological observation + Gram stain	S./H.
Fungi	g_*Catenaria* o_Rhizophydiales	*Catenaria* *Rhizophydium*	Oospores or mycelium baiting technique + morphological observation	OosBait. or MycBait.
	f_Hypocreales	*Trichoderma*	RB-S-F agar medium + morphological observation	Trich.

CDA, MRS, R2A, and RB-S-F are culture media. Chito., Lact., ForE., ForG., S./H., OosBait., MycBait., and Trich. are abbreviations of the isolation methods. Order, family, and genus taxonomic ranks are indicated by o_, f_, and g_ prefixes, respectively

plants for fresh rhizoplane isolation were grown in the PAFF Box aquaponic system of Gembloux Agro-Bio Tech, University of Liège (Belgium). The system is described in Stouvenakers et al. [14], and a block diagram is given in Eck et al. [21]. Rhizoplane water was collected by root sonication for 10 min in a 0.05-M kalium phosphate buffer plus 0.05% Tween 80 (KPBT), as described in Stouvenakers et al. [14]. Growth rooms were set at a day/night photoperiod of 18/6 h at 23 or 28 °C for all isolation protocols and incubating periods. After selective growth and selection processes, the selected strains were stored at − 80 °C in 0.85% NaCl sterile water plus 25% glycerol for further identification. During selective isolation, non-targeted microorganisms were voluntarily kept if they were described such as antagonistic microorganisms in the literature ("Lit" criterion, Table 2) even though they were not initially targeted. Description of the methods used to isolate the targeted microorganisms is fully provided in Supplementary Materials S1 and summarized in Table 1. Isolation and then the screening study were completed by testing specific ordered strains: *Methyloversatilis universalis* (DSM 25,237) and *Hydrogenophaga pseudoflava* (DSM 1034) were received from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and *Catenaria anguillulae* (CBS 423.65) from CBS-KNAW (Westerdijk Fungal Biodiversity Institute). *Pseudomonas chlororaphis* Tx-1 (ATCC 55,670) from the American Type Culture Collection (ATCC) was also ordered to be used as a bio-fungicide control (Cpc) in the in vivo screening. *P. chlororaphis* Tx-1 is an Environmental Protection Agency (EPA)–registered biocontrol agent to control *Pythium* diseases in soil which was described as one of the most relevant microorganisms to control *P. aphanidermatum* in soilless culture [22–24].

Strain Identification

DNA extractions were carried out from bacterial cells or fungal mycelial mats resuspended in sterile Milli-Q water. The FastDNA Spin Kit with TC cell lysis solution (MP Biomedicals, Illkirch-Graffenstaden, France) was used to start with, from 200 µl of suspension. The DNA extraction steps were adapted from the manufacturer's instructions, according to Eck et al. [25]. 16S rDNA and the ITS1-ITS4 regions were amplified for bacteria and fungi, respectively. Forward primer 16S A1 (5′-AGAGTTTGATCMTGGCTC AG-3′) and reverse primer 16S B1 (5′-TACGGYTACCTT GTTACGACTT-3′) were used for bacteria, while forward primer ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′) were used for fungi. PCR mixtures were prepared using the MangoTaq DNA Polymerase kit (Bioline, London, UK) manual. For bacteria, thermocyclers were run with an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 70 °C for 2 min, and a final extension step at 72 °C for 10 min. For fungi, the initial denaturation step was set at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products were purified with QIAquick PCR Purification Kit (QIAGEN Benelux B.V., Antwerp, Belgium) before Sanger sequencing with the same primers at Macrogen Europe B.V. (Amsterdam, The Netherlands). Sequences were assembled using CAP3 program [26] and quality trimmed using Chromas software (http://technelysium.com.au/wp/chromas). The edited sequences were annotated by BLASTN analysis against the rRNA/ITS database using NCBI website (www.ncbi.nlm.nih.gov/blast) for closest identification with 97% identity minimum.

In Vivo Screening

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

Experimental and Seed Treatment Setups

Organic pelleted seeds of lettuce (*Lactuca sativa*) var. Lucrecia RZ (Rijk Zwaan, Merksem, Belgium) were sowed without substrate in 96-well microplates (Greiner Bio-One B.V.B.A., Vilvoorde, Belgium) at a density of one seed by well. One microplate column (eight wells) corresponded to one replicate. Three methods were tested to treat seeds in relation to *P. aphanidermatum* inoculation: pre-inoculation, co-inoculation, and biopriming (i.e., contrary to pre-inoculated seeds, germination of biopriming seeds was stabilized over time by air drying before sowing). Whatever the method, the seeds were treated with 10 µl of isolate suspension per seed on day 0 (see next section for the preparation of isolate suspensions). For pre-inoculation method, microplates were directly sealed with self-adhesive film after seed treatment. For biopriming, the seed pellets were left to dry under a laminar flow hood for 30 min before sealing. In the co-inoculation experiment, 100 µl of *P. aphanidermatum* oospores at a concentration of 10^4 oospores/ml was added per well before microplates sealing and just after the seed pellet had absorbed the treatment (see "Production of *P. aphanidermatum* Inoculum" section for a description). For the pre-inoculation and biopriming treatments, oospores were added 3 days later after self-adhesive film removal. The microplates were then sealed again with a self-adhesive film that was punctured with a needle above each well to allow air exchange. The microplates were incubated at 23 °C. Dark conditions were set before pathogen inoculation (i.e., only

for the pre-inoculation and biopriming treatments), and a day/night photoperiod of 18/6 h was set afterward. Seven days after *P. aphanidermatum* inoculation, seed damping-off was binary scored. Dead seeds were scored 0, while healthy seeds with emerged cotyledons were scored 1. At the end of the screening assay, a strain was considered efficacious when a germination rate threshold of 37.5%, 12.5%, or 12.5% (i.e., three, one, or one seed out of eight, respectively) was reached in pre-inoculation, co-inoculation, or biopriming, respectively. First, and because of the high number of isolates, only one strain per species accession was tested. Replications (i.e., columns of eight seeds) were carried out for the strains that protected at least 12.5% of the seeds. Other strains of a given species accession were screened (within a same treatment method) in three columns if the first tested strain in the accession allowed a seed germination rate of at least 12.5% in co-inoculation, 12.5% in biopriming, or 25.0% in pre-inoculation.

Four controls were used in each microplate at a configuration of one column (eight seeds) per control. Negative control (C−) seeds were treated with KPBT buffer and inoculated with the sucrose + Tween solution used for the oospore suspension. Positive control (C+) seeds were treated with KPBT buffer and inoculated with oospores. Fungicide control (Cf) seeds were treated with Proplant (722 g/l propamocarb) at a concentration of 0.1% in KPBT buffer and inoculated with oospores. Proplant is a propamocarb fungicide approved in Europe and was the sole chemical fungicide registered in Belgium to control *Pythium* diseases on lettuce. Bio-fungicide control (Cpc) seeds were treated with *P. chlororaphis* Tx-1 suspension (like the tested isolates were) and inoculated with oospores.

Isolate Culture and Suspension

Bacteria were grown on solid medium at 28 °C for 3 days. R2A medium was used for all bacterial isolates except *Methyloversatilis* and *Hyphomicrobium* for which MIN E medium was used. All fungal isolates except *Catenaria anguillulae* were grown on PDA dishes at 23 °C for 7 days. *C. anguillulae* grown on YPSS agar medium (20 g soluble starch, 1 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 15 g agar, 1000 ml distilled water) at 28 °C was used to inoculate PYG agar plates (1.25 g peptone, 1.25 g yeast extract, 3 g glucose, 1000 ml distilled water, pH 6.8). PYG plates were incubated at 28 °C for 7 days to produce sporangia. Zoospores were washed off from the sporangia (based on Jansson and Thiman [27]) by 1-h flooding in KPBT solution. All bacterial cultures and fungal spores (i.e., fungal conidia and *C. anguillulae* zoospores) were harvested in KPBT buffer by surface scratching. Bacterial suspensions were diluted to reach 0.825 ± 0.025 absorbance at 600 nm. An absorbance of 0.800 equaled to 5×10^7 CFU/ml for *P. chlororaphis* Tx-1. Fungal spore suspensions were cheesecloth filtered and diluted to a concentration of 1×10^7 spores/ml after microscope count on a hemocytometer. When bacterial or fungal suspensions were not concentrated enough, they were centrifuged at 3000 × *g* for 10 min and set to the right concentration after discarding the supernatant. After a first screening step at a concentration of 0.825 ± 0.025 absorbance units, the most efficacious strains were tested at a tenfold concentration (10×).

Production of *P. aphanidermatum* Inoculum

Sterile 150-ml Erlenmeyer flasks containing 25 ml of clarified V8 $CaCO_3$ broth (800 ml distilled water, 200 ml V8 juice, 3 g $CaCO_3$) were inoculated with 5-mm PDA culture plugs of *P. aphanidermatum* (CBS 132,490) grown at 23 °C with 18 h/6 h lighting for 3 days. The flasks were closed with a cotton ball and incubated at 23 °C with 18 h/6 h lighting for 9 days. Each mycelial bulk was recovered and rinsed by vortexing in a 50-ml centrifuge tube filled with 15 ml of sterile distilled water. The operation was repeated at least twice until V8 color loss. Each mycelium bulk was cut in two pieces, and each half was incubated at 28 °C with lighting for 24 h in a 50-ml centrifuge tube filled with 30 ml of sterile distilled water. The mycelium pieces were recovered and mixed for 3 s eight times with a hand blender (Braun Minipimer Control Plus, 300 W) in a sterile solution containing 10 mM sucrose and 0.05% Tween 20 in distilled water. A proportion of at least one mycelium piece for 12.5 ml of solution was used with a minimum volume of 100 ml. The resulting propagule suspension was filtered through sterile cheesecloth to harvest the oospores, which were counted on a hemocytometer. The concentration was set at 1×10^4 oospores/ml.

Validation of Biocontrol on Lettuce Seedlings

The eight most efficacious strains found in the in vivo screening assay were tested against root rot disease caused by *P. aphanidermatum* on lettuce seedlings. Lettuce seeds (see "In Vivo Screening" section) were sown in 25 × 25 × 40 mm rockwool plugs (Grodan B.V., Roermond, Holland) and placed in a phytotron, with a day/night photoperiod of 16 h/8 h, 22 °C/18 °C (day/night), and a relative humidity of 65% for the first 10 days of germination. See Stouvenakers et al. [14] for lighting specificity. The seeds were inoculum treated (1 ml per plug) on days 0 and 7. Each treatment occupied two plant trays (Ø 15 cm) containing nine rockwool plugs and one plant per plug. The eight treatments consisted of strains chito7, chito13, Mk, M25, M33, SHb30, G2, and SHb18. The fungal strain G2 was produced in the same conditions as the screening conditions. Bacterial strains were grown in 250-ml Erlenmeyer flasks filled with 100 ml of the corresponding liquid medium

(like the screening conditions, but without agar). After 5 days at 28 °C and 100 rpm, the bacterial cultures were centrifuged at 4000 × g. The pellets were rinsed with KPBT buffer, centrifuged again, and finally resuspended in KPBT buffer. Bacterial and fungal cell suspensions were prepared at the concentration found most efficacious during the screening assay and with the same methodology as before. The controls were Proplant fungicide control (Cf), *P. chlororaphis* Tx-1 bio-fungicide control (Cpc), aquaponic water control (Cap), negative healthy control (C−), and positive control (C+). KPBT was used to treat the rockwool plugs of C−, C+, and Cap. The fungicide was diluted at 0.1% in KPBT, and the *P. chlororaphis* Tx-1 suspension was set at 5×10^8 CFU/ml in KPBT for Cf and Cpc. *P. chlororaphis* Tx-1 was produced in Erlenmeyer flasks, like the other bacterial strains. After 10 days of germination in tap water, a hydroponic solution (Hy-Pro A and B; Hy-Pro Fertilizers, Bladel, Holland) prepared according to the manufacturer's instructions was used to fill plastic trays (±450 ml/tray). For Cap, aquaponic water from the PAFF Box aquaponic system (see Stouvenakers et al. [14]) was used throughout the experiment. On day 10, the rockwool plugs were inoculated with a suspension of *P. aphanidermatum* oospores prepared as before (1 ml per plug). The phytotron parameters were adjusted the same day to reach 35/25 °C (d/n; 16 h/8 h) and 92% relative humidity. Water evaporation/evapotranspiration from the trays was compensated for with nutrient solution and demineralized water three times a week. Twenty-one days after *P. aphanidermatum* inoculation (i.e., 31 days after sowing), foliar fresh mass (FFM), foliar dry mass (FDM), root rot rating (RRR), and lettuce mortality (LM) were measured according to Stouvenakers et al. [14]. Three indexes of disease symptom reduction were calculated from these raw data—root symptom reduction (RSR), foliar mass improvement (FMI), and wilt symptoms reduction (WSR)—and expressed in percentages relative to C− and C+. WSR was based on foliar water content (FWC) calculated according to Stouvenakers et al. [14]. The formula used to calculate the disease symptom reduction indexes was as follows:

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

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

The experiment was performed twice, and RSR, FMI, and WSR data were statically analyzed using Minitab v.19 software (Minitab Inc., State College, PA, USA). First, the conditions of application were tested, and then two-way analyses of variance (ANOVAs) were performed with treatment and replicate factors. When a significant interaction between factors was observed, the two-way ANOVA was decomposed in one-way ANOVA. Tukey's multiple comparison post hoc test was used to compare treatments pairwise.

Results

Isolation

Eighty-two bacterial strains and 18 fungal strains were kept after selection; they are listed in Table 2. Twenty-nine different bacterial species and eight different fungal species were identified among these 100 strains. They were used in the following in vivo screening assay. Among them, four were also potential suppressive microorganisms in the HTS study of Stouvenakers et al. [14] and not known for this effect in the literature ("HTSg" criterion, Table 2). Twenty-two species were identified as potential plant-beneficial microorganisms in the literature ("Lit" criterion, Table 2). Seven species were identified as pathogen suppressive by the HTS study and as plant beneficial in the literature ("HTSg/Lit" criterion, Table 2). In total, 43% of the isolated strains had been identified as pathogen suppressive in the HTS study of Stouvenakers et al. [14].

The targeted fungal genera *Catenaria, Rhizophydium* and *Trichoderma* were not isolated. However, non-targeted strains belonging to *Cladosporium*, *Aspergillus*, and *Penicillium* were kept for screening because of their relatively high abundance (more than 0.5%), their aquaponic suppressiveness mentioned in Stouvenakers et al. [14], and their potential antagonistic activities mentioned in the literature ("HTSg/Lit" criterion). Regarding bacteria, only *Sphingobium* was successfully isolated among the targeted microorganisms. Instead of *Methyloversatilis*, numerous non-targeted *Hyphomicrobium* spp. strains were isolated because of their formaldehyde resistance. However, they were kept because of their significant suppressiveness and higher abundance (1.36%) in Stouvenakers et al. [14] study (HTSg criterion). Numerous non-targeted heterotrophic and/or methylotrophic bacteria were isolated. At the genus level, most of them were already listed in Stouvenakers et al. [14], but in low abundance (< 0.1%). Because most of these bacteria were found potentially beneficial to plants in the literature, they were all used in the screening study ("Lit" criterion). Among them, the genera *Microbacterium*, *Micromonospora*, *Mycolicibacterium*, *Nocardia*, and *Streptomyces* were all actinomycetes, a group commonly described as plant beneficial [28].

Table 2 Isolate identification depending on the isolation method, the origin (1: frozen aquaponic rhizoplane water from Stouvenakers et al. [10], 2: fresh aquaponic rhizoplane water, and 3: ordered strain), and selection criteria

Species name	Isolate abbreviation	Method	Origin	Criteria
Bacteria				
Bacillus flexus	SHb2	S./H.	2	Lit
Bacillus indicus	SHb31	S./H.	2	Lit
Bosea thiooxidans	SH6, SH9	S./H.	1	Lit
Enterobacter cloacae complex	L13	Lacto.	2	Lit
Hydrogenophaga pseudoflava	DSM 1034	/	3	HTSg
Hyphomicrobium sp.	M8	ForG.	2	HTSg
Hyphomicrobium vulgare	M18	ForG.	2	HTSg
Hyphomicrobium zavarzinii	M13	ForG.	2	HTSg
	M25, M27, M28, M31, M32, M34, M35, M36, M37, M38	ForG.	1	
	MetA, MetB, MetC, MetD, MetE, MetF, MetG, MetH	ForG.	1	
Methylorubrum podarium	Chito6	Chito.	1	Lit
Methylorubrum populi	Mc, Mk, Mq	ForE.	1	Lit
Methyloversatilis universalis	DSM 25,237	/	3	HTSg
Microbacterium kitamiense	SHb4	S./H.	2	Lit
Microbacterium lacus	SHb23, SHb25	S./H.	2	Lit
Microbacterium paraoxydans	SHb18	S./H.	2	Lit
Microbacterium sp.	SH10, SH22, SH28	S./H.	1	Lit
Micromonospora maritima	SH32	S./H.	1	Lit
Mycolicibacterium aurum	M1, M2, M7, M15, M19, M23	ForG.	2	Lit
Mycolicibacterium fluoranthenivorans	M5, M6, M11, M16, M17	ForG.	2	Lit
Mycolicibacterium fortuitum	Chito1, Chito 5, Chito8, Chito11, Chito13, Chito16, Chito17, Chito18	Chito.	1	Lit
Mycolicibacterium sp.	Chito10	Chito.	1	Lit
Mycolicibacterium sp.	Chito2	Chito.	1	Lit
Mycolicibacterium wolinskyi	M33	ForG.	1	Lit
Nocardia fluminea	Chito7	Chito.	1	Lit
Novosphingobium aromaticivorans	SHb3, SHb10, SHb15, SHb16, SHb17, SHb21, SHb28	S./H.	2	Lit
Pedobacter solisilvae	SHb7, SHb26, SHb34	S./H.	2	Lit
Rhizobium sp.	SHb32	S./H.	2	Lit
Rummeliibacillus suwonensis	L2, L5, L9, L10, L11	Lacto.	2	NA
Sphingobium xenophagum	SHb9, SHb14, SHb27, SHb30	S./H.	2	HTSg
Streptomyces coelicoflavus	SHb13	S./H.	2	Lit
Fungi				
Aspergillus flavus	TS1	Trich.	2	HTSg/Lit
	G2	MycBait.	1	
Aspergillus fumigatus	G1	MycBait.	1	HTSg/Lit
Catenaria anguillulae	CBS 42,365	/	3	HTSg/Lit
Cladosporium halotolerans	TS6, TS10	Trich.	2	HTSg/Lit
Cladosporium ramotenellum	TS11	Trich.	2	HTSg/Lit
Cladosporium sp.	TS13	Trich.	2	HTSg/Lit
Cladosporium sphaerospermum	TS2, TS3, TS4, TS7, TS9, TS12, TS14	Trich.	2	HTSg/Lit
Penicillium citrinum	PC1, PC2, PC3, G3	MycBait.	1	HTSg/Lit

Chito., Lact., ForE., ForG., S./H., OosBait., MycBait., and Trich. are isolation methods for Burkholderiaceae family, *Lactobacillus* genus, *Methyloversatilis* genus using enrichment, *Methyloversatilis* genus without enrichment, *Sphingobium*/*Hydrogenophaga* genera, *Catenaria*/*Rhizophydium* genera using oospores as bait, *Catenaria*/*Rhizophydium* genera using mycelium as bait, and *Trichoderma* genus, respectively. The species selection criteria for the screening study were as follows: HTSg, HTS-guided (i.e., potential suppressive pathogen identified by HTS in Stouvenakers et al. [10] at the genus level); Lit, literature-guided (i.e., plant beneficial in the literature at the genus level, at least); HTSg/lit, both criteria-guided; NA, not applicable

In Vivo Screening

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

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

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

P. aphanidermatum Control on Lettuce Seedlings

Two bioassays were carried out on lettuce seedlings to test the efficacy of the eight best strains found in the screening to control *P. aphanidermatum* root rot disease.

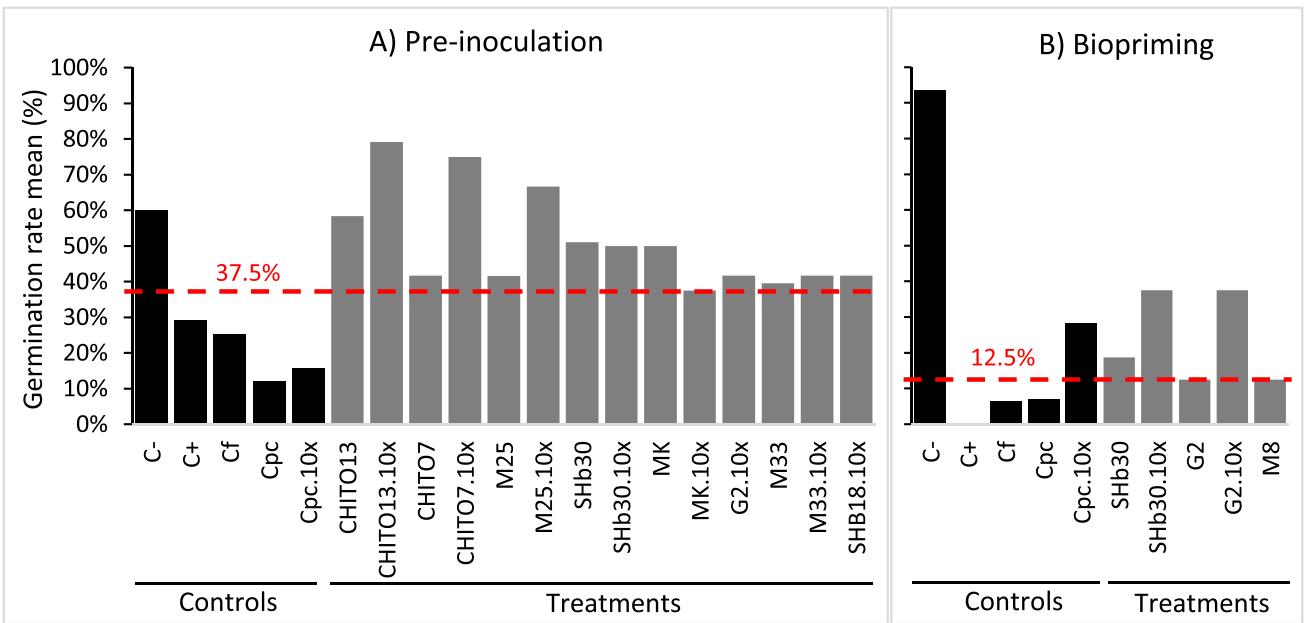


Fig. 1 Mean germination rates of lettuce seeds treated to control *P. aphanidermatum* damping-off depending on **A** pre-inoculation or **B** biopriming. Only the type strains that reached efficacy thresholds are showed in "Treatments" (full results are in Supplementary Materials). Dotted lines, efficacy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

Significant interactions ($p < 0.000$) were found between the bioassay replicates and the treatment factors. The treatment effects were then analyzed depending on the bioassay (i.e., 1 or 2).

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

Considering the disease symptom reduction indexes (RSR, FMI, and WSR) in bioassay 1, the best treatment was SHB30, followed by Chito13 and G2 (Fig. 2). The disease symptom indexes of SHb30-treated lettuce were all significantly lower than those of C+ ($p \leq 0.05$), and no difference was found with C− ($p > 0.05$). The symptom reduction rates were 67.9%, 131.8%, and 83.2% for RSR, FMI, and WSR, respectively. In particular, the high value of FMI (131.8%) following SHb30 treatment indicated that leaves tended to be more developed than in C− lettuce ($p > 0.05$), and that foliar symptoms were eradicated. Moreover, SHb30 tended to allow for a higher FMI than Cf did (62.4%; $p > 0.05$) and a higher FMI than Cpc did (54.7%; $p \leq 0.05$). Root protection following Chito13 and G2 treatments was intermediary (RSR = 44.9% and 47.1%, respectively) and at a similar level as in Cf (RSR = 29.4%) and Cpc (RSR = 42.2%) lettuce ($p > 0.05$) (Fig. 2A). FMI following Chito13 (93.0%) and G2 (66.8%) treatments was not significantly different than in C− and Cf lettuce (62.4%; $p > 0.05$) (Fig. 2B). Chito13 reduced leaf wilting (WSR = 72.1%) in a comparable way as in C− ($p > 0.05$; Fig. 2C). WSR following G2 treatment was more intermediary (52.2%). However, WSR following G2 and Chito13 treatments was not different than in Cf lettuce (67.7%; $p > 0.05$).

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

M25 treatment was not efficacious in controlling the disease whatever the bioassay replicate. RSR, FMI, and WSR were never different than in C+ lettuce ($p > 0.05$; Fig. 2). Although RSR, FMI, and WSR of Mk, Chito7, M33, and SHb18 treatments were often not statistically different than in C+ lettuce, other comparisons were made (Fig. 2). The symptom reduction indexes of Mk, Chito7, M33, and SHb18 were not different ($p > 0.05$) than in Cf and Cpc lettuce in bioassay 1 (except for SHb18 WSR, lower than that of Cf). In bioassay 2, the symptom reduction levels following SHb18 treatment were similar to those of Cf and Cpc lettuce ($p \leq 0.05$), while Mk, Chito7, and M33 were less efficacious than Cf ($p \leq 0.05$).

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

Discussion

The strategy proposed in this study for isolating *P. aphanidermatum* biocontrol agents is original in several ways. The first one is the biotope used for isolation. The potential suppressiveness of aquaponic systems has been raised and discussed only recently. Before this study, only one had been devoted to isolating biocontrol agents from microbial populations of aquaponic systems [29]. The second originality is the HTS-guided strategy used to select potential biocontrol agents to be isolated. The genera identified by HTS and bioinformatic analysis as interesting candidates for plant pathogen suppression in Stouvenakers et al. [14]

Table 3 Lettuce mortality (LM) following treatment with the 8 microbial strains (Chito13, Mk, Chito7, M33, SHb30, M25, G2, and SHb18) against *P. aphanidermatum* disease on lettuce seedlings depending on the bioassay replicate (1 or 2)

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

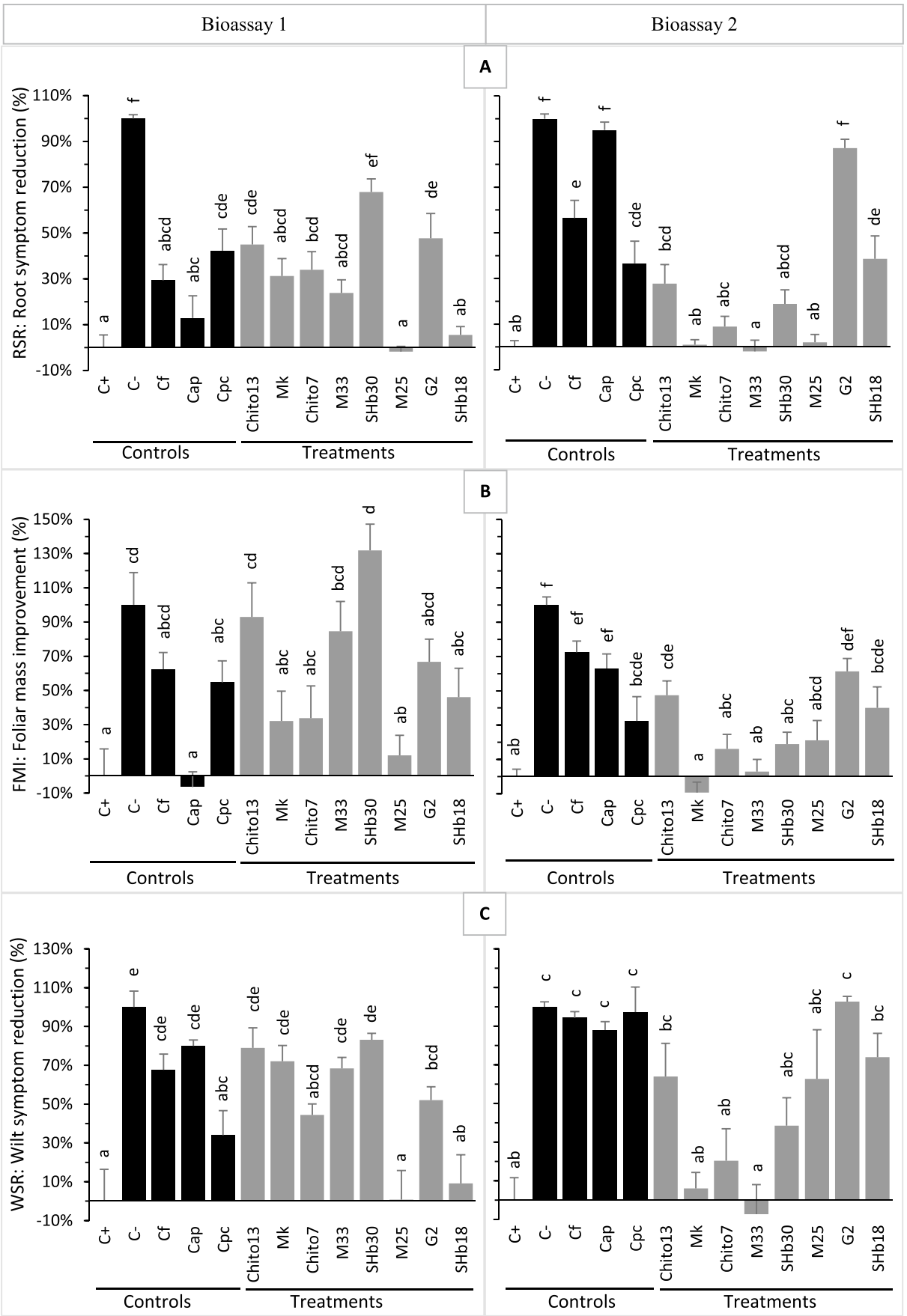
C+, C−, Cf, Cap, and Cpc are the positive, negative, fungicide, aquaponic, and biofungicide controls, respectively. Statistics not applicable

were targeted during the isolation step. The targeted lettuce rhizoplane microorganisms were selected for their high relative abundance and their correlation with suppressiveness in Stouvenakers et al. [14]. HTS-guided isolation of specific microorganisms is novel, and only three papers have been found using a similar strategy [17, 18, 30]. Potential biocontrol agents have traditionally been mainly isolated in artificial broad-range media, using a priori–free approaches [16]. Due to its lack of selectivity, this isolation strategy is followed by a tedious screening step including many isolates [20]. Moreover, because universal media that suit all microorganisms are not available, their use unavoidably leads to enrichment in certain microorganisms that do not necessarily have biocontrol properties [31], while microorganisms of interest can be missed. The application of isolation methods that target specific microorganisms is an alternative solution for isolating biocontrol agents, but it requires a priori targeting relying on pre-existing data [32, 33]. For example, microorganisms can be selected based on previous biocontrol activity showed in similar pathosystems by other strains belonging to a certain species or genus or family [34, 35]. This introduces a significant bias because different isolates from a single species can present contrasting properties [36]. For example, the fungal species *Fusarium oxysporum* includes isolates or subspecies highly pathogenic or beneficial for lettuce [37, 38]. Relying on the composition of microbial communities in the studied pathosystem avoids this drawback and can identify biocontrol agents belonging to species so far unknown as biocontrol agents in the scientific literature. Niem et al. [17], Liao et al. [18], and the present study all show that isolating strains belonging to taxa identified as potential biocontrol agents by HTS is feasible. However, this novel strategy also suffers from the weaknesses of HTS. Bias may be introduced at each step, from microbiota sampling to bioinformatic analyses [39]. These biases may have influenced the list of OTUs linked to suppressiveness in Stouvenakers et al. [14], qualitatively and quantitatively. Therefore, the list of microorganisms selected for isolation in our study is also potentially distorted by HTS bias. Only a few initially targeted microorganisms were successfully isolated (4.0% of the isolated strains), while microorganisms found at a low abundance in Stouvenakers et al. [14] were unintentionally isolated (59% of the isolated strains). However, 43% of the strains (whether targeted or not) isolated at the genus level were identified as pathogen suppressive in Stouvenakers et al. [14]. In addition to HTS bias, these results are not surprising for several reasons. HTS is not a culture-dependent technique, and the targeted microorganisms are not always culturable [40–43]. Furthermore, taxonomic abundance in metagenomics studies does not reflect the abundance found in culture-dependent techniques [40–42]. Rare microorganisms could be abundantly and easily isolated in culture media, and vice versa [31]. A high abundance does not entail biocontrol action [44].

The isolates were screened for antagonistic activity in vivo. The method was designed to benefit from the logistic advantages of in vitro bioassays without their biases. Antagonistic modes cannot all be tested in vitro, and positive antagonism in vitro does not necessarily predict antagonistic activity in more complex assays including plant hosts [44]. However, contrary to in vitro methods, our in vivo screening was subjected to pathosystem variability. The seed itself (its microbiome or its germination rate for example), the variability of pathogen aggressiveness, the timing, and conditions of the treatment (T°, HR) can influence the disease and the results [44, 45]. For example, *P. aphanidermatum* disease and oospore germination are promoted by temperature higher than 25 °C [2, 46], while germination of our lettuce seeds drastically decreases above 25 °C.

Retrospectively, the HTS-guided strategy coupled with the in vivo screening assay was a reliable approach for identifying new biocontrol agents. Two of the three most efficacious isolates for controlling *P. aphanidermatum* root rot disease on lettuce had been characterized as suppressive in Stouvenakers et al. [14]. The genus *Sphingobium* was present at a high relative abundance (2 OTUs at 5.6% and 3.5%, respectively) and correlated with aquaponic suppressiveness in Stouvenakers et al. [14]. The family Aspergillaceae to which *A. flavus* belongs was present in medium relative abundance (0.5%) and was correlated with root symptom reduction. However, three important taxa targeted by the HTS-guided strategy were not isolated, and type strains were ordered. They were not efficacious in controlling *P. aphanidermatum* disease, but this does not disprove the strategy because OTU identification in Stouvenakers et al. [14] was at best at the genus level and because different strains for a same species can express different level of biocontrol action [36].

The three most efficacious isolates for controlling the disease in vivo were *S. xenophagum* strain SHb30, *A. flavus* strain G2, and *M. fortuitum* strain Chito13. *S. xenophagum* and *M. fortuitum* had never been described as root disease biocontrol agents. The bacterium *S. xenophagum* can degrade xenobiotic aromatic compounds and is studied for bioremediation of contaminated environments [47, 48]. This species was already identified in the lettuce root zone and notably in aquaponics where the genus is among the most abundant ones [49–51]. Sphingomonadaceae are generally also well represented in suppressive soils [16]. The range of action of *S. xenophagum* against plant diseases is still unknown, but volatile organic compounds produced by the bacterium decrease the growth of the plant pathogen *Botrytis cinerea* in vitro [52]. The species has also been described once as a plant growth–promoting rhizobacterium (PGPR), and can produce siderophores and indole-3-acetic acid [53]. *Mycolicibacteria* are common rhizosphere bacteria mainly


Bioassay 1
Bioassay 2
A
RSR: Root symptom reduction (%)
110%
90%
70%
50%
30%
10%
-10%
a
f
abcd
abc
cde
cde
abcd
bcd
abcd
ef
a
de
ab
ab
f
e
f
cde
bcd
ab
abc
a
abcd
ab
f
de
C+
C-
Cf
Cap
Cpc
Chito13
Mk
Chito7
M33
SHb30
M25
G2
SHb18
Controls
Treatments
B
FMI: Foliar mass improvement (%)
150%
130%
110%
90%
70%
50%
30%
10%
-10%
a
cd
abcd
a
abc
cd
abc
bcd
d
ab
abcd
abc
ab
f
ef
ef
bcde
cde
a
abc
ab
abc
abcd
def
bcde
C
WSR: Wilt symptom reduction (%)
130%
110%
90%
70%
50%
30%
10%
-10%
a
e
cde
cde
abc
cde
cde
abcd
cde
de
a
bcd
ab
ab
c
c
c
c
bc
ab
ab
a
abc
abc
c
bc

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

studied for bioremediation of contaminated environments [54, 55]. Contradictory to our results, *Mycolicibacterium* spp. have been found to enhance *P. aphanidermatum* growth in vitro and to be tobacco black rot disease conducive [56, 57]. However, the genus has also been described several times as a PGPR [28, 58]. *M. fortuitum* could be the causal agent of fish tuberculosis in aquaculture [59] and could be an opportunistic human pathogen susceptible to cause non-tuberculous mycobacterial infection [60]. Human, animal, and plant health is a prior concern in developing biocontrol agents, but pathogenicity is not necessarily dependent on the species [61]. Indeed, for the same species, a strain could be pathogenic or not [61]. For example, numerous strains of *Pseudomonas fluorescens* were commercialized as biocontrol agents while others were reported as pathogenic for human [36, 62]. As for *A. flavus* (G2 strain), the species is a saprophytic soil fungus mainly known to produce the secondary metabolite aflatoxin in infected crops. However, atoxigenic strains are also used and studied to control aflatoxin-producing ones [63, 64]. Two *A. flavus* strains (AF36 and NNRL 21,882) are already EPA registered as biopesticides in the USA (https://www.epa.gov/). Furthermore, *A. flavus* has been screened as an antagonist of *P. aphanidermatum* in dual culture [65].

Conclusion

The HTS-guided strategy for isolating aquaponic microorganisms coupled with in vivo screening led to the identification of original biocontrol agents of *P. aphanidermatum* lettuce disease. Out of 100 isolates, eight were considered efficacious in controlling *P. aphanidermatum* lettuce damping-off and selected to be tested on lettuce seedlings. The three most efficacious isolates were *S. xenophagum* strain SHb30, *A. flavus* strain G2, and *M. fortuitum* strain Chito13. Strains SHb30 and G2 were isolated and selected according to our HTS-guided strategy, while C13 was selected according to a literature-guided strategy. Seed treatment with each of these three strains decreased *P. aphanidermatum* damping-off and was more efficacious than the fungicide and biopesticide controls. Lettuce seedling mortality (LM) was 0.0% in bioassay 1 following treatment with strains SHb30, G2, and Chito13. In bioassay 2, LM was higher following SHb30 treatment, but remained at 0.0% following G2 and Chito13 treatments. The biocontrol action of SHb30 was high in bioassay 1, but more limited in bioassay 2. The biocontrol action of G2 and Chito 13 was more constant. The foliar mass improvement (FMI) index showed that foliar symptom reduction following G2 and Chito13 treatments was at least as good as in lettuce treated with the fungicide control. In conclusion, these results indicate that aquaponics is an important source of novel biocontrol agents that could be more adapted to soilless conditions than common soil biocontrol agents are. However, safe use of G2 and Chito13 must be first checked. SHb30, G2, and Chito13 are promising but need to be further studied (e.g., mechanisms of action) and possibly developed in biopesticide formulation.

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Author Contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by GS. The first draft of the manuscript was written by GS, and all authors commented on the previous versions of the manuscript. All authors have read and approved the final manuscript.

Data Availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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