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Potential for Combined Biocontrol Activity against Fungal Fish and Plant Pathogens by Bacterial Isolates from a Model Aquaponic System

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Academic Editor: M. Haïssam Jijakli

Received: 16 September 2016; Accepted: 2 November 2016; Published: 7 November 2016

Abstract: One of the main challenges in aquaponics is disease control. One possible solution for this is biological control with organisms exerting inhibitory effects on fish and plant pathogens. The aim of this study was to examine the potential of isolating microorganisms that exert an inhibitory effect on both plant and fish pathogens from an established aquaponic system. We obtained 924 isolates on selective King's B agar and 101 isolates on MRS agar from different compartments of a model aquaponic system and tested them for antagonism against the plant pathogen *Pythium ultimum* and fish pathogen *Saprolegnia parasitica*. Overall, 42 isolates were able to inhibit both fungi. Although not yet tested in vivo, these findings open new options for the implementation of biological control of diseases in aquaponics, where plants and fish are cultivated in the same water recirculating system.

Keywords: aquaponics; beneficial bacteria; *Pythium ultimum*; *Saprolegnia parasitica*; screening tests

1. Introduction

Projections show that the human population will increase up to 9 billion in 2050. Therefore, food production must increase by 34% to 70% in comparison with today [1]. Future food production systems must be able to increase food safety, food security, and efficiency of production technology, while reducing environmental impacts.

One technology which has the potential to meet the aforementioned characteristics and could fairly be named a "technology of the future" is aquaponics (AP) [2]. It is an integrated technology combining aquaculture with plant cultivation in a single recirculating aquaponics system (SRAS) [3,4]. Despite the obvious advantages of this technology and the globally increasing interest, it also has inherent disadvantages that need to be addressed in the near future. One of the main challenges to successful aquaponics operation is connected with disease control. Pathogens can affect both main products: the fish in the aquaculture, and the plants in the hydroponic section. For plant treatment, conventional pesticides should not be used, as they are suspected to harm the fish [5]. On the other hand, medicines and chemicals for treating fish parasites and diseases cannot be applied, as plants may absorb and accumulate them [6]. This could cause harm to human health when consuming the crop products, as, for example, antibiotics entering the gastrointestinal tract of humans at concentrations below the minimum inhibitory concentration (MIC) may contribute to the enhancement of antibiotic resistance [7]. Furthermore, in some countries (e.g., Switzerland) pesticide application via the irrigation system is neither allowed nor registered. The Aquaponic Research Laboratory at the Zurich University of Applied Sciences in Wädenswil relies exclusively on integrated pest management for plant treatments,

using, for example, "Natural" from Andermatt Biocontrol (Grossdietwil, Switzerland) against aphids on salads, where fatty acids act as contact insecticide. On the other hand, the only possible treatment for fish is currently Wofasteril (Kesla Pharma Wolfen GMBH, Bitterfeld-Wolfen, Germany), a hydrogen peroxide-containing product that kills ectoparasites and leaves no residues in the system. From this point of view, the current options for disease management in AP are severely limited. Alternatives in comparable aquaponic systems are rarely reported, and lack sufficient information on their efficacy [5]. This led us to investigate the option of biological control using beneficial microorganisms, for which an extensive amount of information is available from soil-based agricultural systems [8].

The interactions between plants and microorganisms can be pathogenic, saprophytic, or beneficial [9]. The beneficial interaction between plants and micro-organisms can be either expressed in a plant growth-promoting effect in the absence of pathogens, or by protecting the plant against soil-borne diseases [10]. Beneficial bacteria for plants form stable biofilms on the roots. The most important group of these rhizobacteria—which have been well known for many years as plant biocontrol agents for different crops—are bacteria from the genera *Pseudomonas* or *Bacillus* [11–15]. Their plant growth-promoting activity has been attributed to a number of mechanisms, such as the production of antimicrobial compounds, competition for space and nutrients on the root system, induced resistance, and/or parasitism on plant-pathogenic organisms [8,16]. For another group of organisms, the so-called lactic acid bacteria, it was recently shown that they could also be used as biocontrol agents in crop production. Lactic acid bacteria possess antagonistic effects against phytopathogenic and spoilage microorganisms [17,18]. The mechanisms for biocontrol of this group have not yet been reported.

On the other hand, numerous studies have examined antagonistic effects of bacteria against fish pathogens and their potential use as biocontrol agents of fish diseases in aquaculture. The efficacy of bacterial strains from the genus *Pseudomonas* was shown to be high in in vitro and in vivo inhibition tests against *Aeromonas hydrophila* in tilapia (*Oreochromis niloticus*) [19], against *Vibrio* spp. [20], and against *Saprolegnia* spp. [21,22], showing the large potential of members of this genus.

To the best of our knowledge there are no studies dealing with the use of beneficial bacteria for simultaneous biocontrol of both plant and fish diseases. Therefore, the aim of this study was to examine the potential of isolating microorganisms that exert an inhibitory effect on both plant and fish pathogens. As model pathogens, we selected a *Saprolegnia parasitica* strain as fish pathogen, and an isolate of *Pythium ultimum* as plant pathogen, as these fungal pathogens can be problematic in aquaponics. Due to their common traits as members of the taxon Oomycota, we anticipated that the chance of obtaining a single biocontrol agent having an inhibitory effect against both might be higher than when using different pathogens. With the knowledge that aquaponics systems have a broad diversity of bacteria in the different compartments of the system [23], a sampling strategy throughout the system was applied to catch the broadest spectrum of biocontrol agents. Here we report the outcomes of the in vitro screening assays for antagonistic bacteria isolated from the aquaponics system.

2. Materials and Methods

2.1. Collection and Preparation of Samples

The aquaponic system used for collection of samples was situated in a foliar greenhouse (with 270 m^2 area) at Zurich University of Applied Sciences (ZHAW) in Wädenswil (Switzerland) [23]. The system consisted of a recirculating aquaculture unit growing tilapia (in total 3600 L) with a fish tank, drum filter for solids removal, moving bed biofilter, UV filter, and addition of pure oxygen, and a hydroponic section (in total 800 L) using three lines of tomato culture, comparing floating raft culture, nutrient film technique (NFT), and drip irrigation as alternative growing technologies. The fish and plant sections were connected by a collection sump with water level sensor. The system was operated for 6 months in a closed water loop (water withdrawal by fish sludge <0.4% per day) before the sampling took place. The samples for the isolation of bacterial strains were taken in October 2013 from

different compartments of the system (fish tank, biofilter, water from the hydroponics compartment), as well as from tomato roots and tilapia faeces and scales.

Water samples of 20 mL each were taken from within the fish tank, the biofilter, and from the collection sump of the hydroponic section. When sampling the biofilter, biochips were also included. Each sample was stirred for two minutes on a Vortex. Three tilapia (Til-Aqua, Someren, The Netherlands) of about 150 g were anesthetized by electrical stunning and killed by gill-cutting. Scales from fish were removed, and faeces taken from the tilapia's intestines. Samples from plant roots were taken from three tomato plants floating on Styropor® rafts (Dry Hydroponics, The Hague, The Netherlands) in the deep-water culture by cutting a widely ramified root string close to the main stalk in order to have both old and young roots. The sample volume from fish scales, fish gut, and plant roots were adjusted to 0.5 g and transferred to 50 mL centrifuge tubes. Saline (0.9% NaCl) was added up to 20 mL total volume, and the tubes were vortexed vigorously to release the attached bacteria.

For the enrichment of cultures, 500 µL of each sample were transferred to two 50 mL plastic tubes (Corning CentriStar, Corning, NY, USA). One tube contained 30 mL of King's B media broth (Fluka) amended with the antibiotics cycloheximide (100 $\mu g \cdot mL^{-1}$), chloramphenicol (13 $\mu g \cdot mL^{-1}$), and ampicillin (40 $\mu g \cdot mL^{-1}$) [24], described for a more selective enrichment of microorganisms from genus *Pseudomonas* and related organisms. The other tube contained 30 mL MRS broth according to De Man , Rogosa & Sharpe [25] (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), and is designated for specific isolation of lactic acid bacteria, but may also allow the growth of other organisms. The tubes were placed on a laboratory shaker and incubated for 4 days at a temperature of 24 °C. Isolation of bacteria from both enrichments was done by spreading 100 µL aliquots of serial dilutions (10^5 to 10^8) on petri dishes containing the corresponding medium. After 72 h incubation at 24 °C, colonies which had different morphological characteristics (colour, shape, and size) were picked up with sterile sticks and transferred to 2 mL of the corresponding medium in 24-well plates and incubated for 4 days at room temperature on a laboratory shaker. These cultures were used to test for antagonistic activity.

2.2. In Vitro Screening for Antagonistic Activity against P. ultimum and S. parasitica

The fungal isolates included in this study were *P. ultimum* strain 67-1 (obtained from Allelix Agriculture, Mississaugua, ON, Canada) and an *S. parasitica* strain obtained from the University of Bern, Switzerland. Both fungi were routinely maintained on 1.5% malt extract agar (MEA) plates (Sigma-Aldrich) and incubated at 24 °C.

Bacterial isolates were initially screened for inhibitory activity against *P. ultimum*, and the 50 strains presenting the strongest inhibition effect were subsequently tested against *S. parasitica*. A plug of an actively growing culture of *P. ultimum* or *S. parasitica* was placed in the centre of a MEA plate. On each plate, spots of 20 µL of three bacterial isolates were inoculated at a quartile on a circle of 3.2 cm from the centre of the plate. The fourth quartile was left open and used as control. The plates were closed with paraffin film and incubated for 3 days at 24 °C. After incubation, the fungal growth radius was measured from the centre up to its growing edges. The percentage of inhibition of mycelial growth was calculated by dividing the average radius of mycelial growth for a bacterial isolate by the average radius of the control quartile and multiplication with 100%. A strong inhibition effect was defined as the reduction of fungal growth >55% and used throughout.

3. Results

3.1. Bacterial Isolates

The aquaponics system at the Aquaponics laboratory of ZHAW was sampled in 2013 at the different compartments to obtain a representation of the entire bacterial community. Enrichment cultures for all sample points were used to enrich for fluorescent pseudomonads and lactic acid bacteria. The enrichments on King's B medium yielded a large number of colonies, whereas the

enrichments on MRS medium were less successful. In the end, a total of 1025 isolates was further tested, of which 924 isolates originated from King's B medium and 101 isolates from MRS medium.

3.2. In Vitro Screening Test against Plant Pathogen

As an initial test, the isolates were screened for their ability to inhibit the plant pathogen *P. ultimum* (Table 1). The experiment showed that the highest percentage of King's B isolates that exhibit an inhibition effect against *P. ultimum* were found on tomato roots (14.6%) and in the fish faeces (13.3%). The lowest percentage of *P. ultimum* growth-inhibiting isolates was found on the fish scales. In total, 86 of the 924 isolates from King's B medium (9.3%) were classified as strains presenting a strong inhibition effect. On the other hand, only one of the isolates from MRS medium was able to inhibit *P. ultimum* in the assay.

Table 1. Number of bacterial isolates obtained from the model aquaponic system, number of strains presenting a strong inhibition effect against the plant pathogen *Pythium ultimum* (>55% mycelial growth inhibition), and percentage of positive strains for both growth media.

Compartment	King's B Medium			MRS Medium		
	Total	Strong Inhibitive	Percentage	Total	Strong Inhibitive	Percentage
Fish tanks	144	7	4.9%	12	0	-
Tomato roots	144	21	14.6%	0	-	-
Biofilter	144	13	9.0%	24	0	-
Sump water	144	15	10.4%	17	0	-
Tilapia scales	168	6	3.6%	0	-	-
Tilapia faeces	180	24	13.3%	48	1	2.1%
Total	924	86	9.3%	101	1	1.0%

3.3. In Vitro Screening Test against Fish Pathogen

To investigate the dual ability of the bacterial isolates to control both plant and fish pathogens, we selected the 50 strains presenting the strongest inhibition effect against *P. ultimum* in a similar test against the fish pathogen *S. parasitica* (Table 2). All four isolates from the fish tank tested were able to reduce growth of *S. parasitica*, while the majority of strains from the other compartments were able to antagonize the fungus. The single MRS isolate that showed antagonistic effects against *P. ultimum* also showed suppressive effects (more than 55% inhibition effect) against *S. parasitica*. In total, over 80% of the tested isolates were antagonistic to both fungi.

Table 2. Number of selected King's B medium isolates with strongest inhibition effect from the model aquaponic system, number of effective isolates against the fish pathogen *Saprolegnia parasitica* (>55% mycelial growth inhibition), and percentage of positive isolates.

Compartment	Total	Strong Inhibitive	Percentage
Fish tank	4	4	100%
Tomato roots	11	9	81.8%
Biofilter	1	0	-
Sump water	5	4	80.0%
Tilapia scales	6	5	83.3%
Tilapia faeces	23	20	87.0%
Total	50	42	84.0%

4. Discussion

Although antagonistic effects of bacterial species against fungi have been known for a long time [8], their potential as biocontrol agents in aquaponics systems has up to now been neglected [5]. We have isolated a large number of potential biocontrol strains from enrichments with selective media.

Although the bacteria were not classified taxonomically, we assume that many of the isolates from King's B medium are members of the genus *Pseudomonas*, while the isolates of MRS belong to the group of lactic acid bacteria [24,25]. For the application as a biocontrol agent, a thorough identification of the candidate strain has to be performed, as several pseudomonads are known to have a clinical relevance, which subsequently cannot be used for food production purposes.

It is known that bacteria of the genus *Pseudomonas* are known to suppress different plant pathogens belonging to the fungal genera *Pythium* [26], *Fusarium* [27], and *Rhizoctonia* [28]. The inhibitory effect of lactic acid bacteria against *P. ultimum* was only discovered recently [18]. On the other hand, it was observed that *Pseudomonas aeruginosa* strains exhibit antagonistic activity against the fish pathogen *S. parasitica* [22]. Inhibitory effects of the lactic acid bacterium *Lactobacillus plantarum* FNCC 226 against the *S. parasitica* have also been described [29]. However, the combination of both antifungal activities has not been documented, and this study has now provided the first in vitro evidence that it is possible to have isolates that can effectively control both fish and plant pathogens.

We obtained an almost tenfold larger number of isolates from the enrichment with King's B medium than from the enrichments with MRS medium. A recent metagenomics study [23] that studied the same aquaponics unit showed that in the system, only a low amount of lactic acid bacteria was present, however a much larger number of pseudomonads. It also shows that the enrichment step on King's B medium is very effective, as in the fish faeces community sampled during the metagenomics study, only a small number of pseudomonads were present. On the other hand, bacteria closely related to or formerly named *Pseudomonas* were present in large amounts [23].

A closed recirculating AP is an especially susceptible system, because plants and fish are cultivated in the same recirculating water. Although hydroponic systems are easier to sterilize, reduced competition in these may make plant cultivation more susceptible to fungal infections [30], as the absence of soil as potential source of potential biocontrol strains reduces the chance that an effective population of biocontrol bacteria can establish. Additionally, the complexity of the system with several ecologically variable compartments, plant or fish pathogens could develop or sustain in the system without being observed. To reduce the risk of diseases and to improve fish and plant health, it would be possible to add biological control bacteria to protect both fish and plants simultaneously.

Whereas chemical antimicrobials and antifungals for the treatment of fish and plants in aquaponics are strongly undesirable [31], alternatives that could respond to disease control problems are needed. One possible solution is the use of antagonistic bacteria as a biocontrol additive in aquaculture [32], even though the possibility exists that the presence of these organisms in the ZHAW aquaponics system may have caused an inherent resistance already, implying that the addition of biocontrol is not necessary. The in vitro tests presented in this paper show on the other hand that it is possible to isolate biocontrol organisms on selective media that inhibit the growth of the plant pathogen *P. ultimum* and the fish pathogen *S. parasitica* with about the same effectiveness against both diseases. Nevertheless, this ability has not yet been tested in an operational aquaponics system. To obtain a sufficient level of biocontrol in practice, a lot of prerequisites have to be fulfilled, including the detailed identification of the biocontrol agent and knowledge of its effectiveness against further pathogens, its applicability as a biocontrol agent in vivo, and its compatibility with fish and plants.

5. Conclusions

The current study investigated bacteria that could have combined suppressive effects on fish and plant pathogens, which are both present in aquaponics and could play a central role for the successful and safe operation of this production technology. The initial results presented in this study suggest that biological control using bacterial biocontrol strains already present in the system could yield a promising addition for disease management in aquaponics. On the other hand, we need to consider that further research dealing with in vivo experiments with plants and fish as well as the mechanism of interaction between the bacteria and the pathogens is highly necessary. Up to then, we will have to deal with the insufficiency of the current disease control measures for aquaponics systems.

Acknowledgments: The research was carried out with the help of initial funding of the Technology Transfer Office of the ZHAW School of Life Sciences and Facility Management. Funding for Ivaylo Sirakov was provided by the Sciex-NMS^{ch}, Scientific Exchange Programme between Switzerland and the New Member States of the EU (Project code 12.352). Funding for Theo H. M. Smits was partially provided by the SNSF in the frame of the national research program "Sustainable use of Soil as a Resource" (NRP 68, Project 406840-143144).

Author Contributions: All authors collaborated for the completion of this work. Ivaylo Sirakov planned the study, conducted screening tests, performed data analyses and wrote the first draft of the manuscript. Ranka Junge, Theo H. M. Smits, Matthias Lutz and Andreas Graber planned the study, provided instructions on study design, supervised the data analyses, and reviewed the manuscript. Alex Mathis and Yordan Staykov supported the discussion on the experimental design.

Conflicts of Interest: The authors declare no conflict of interest.

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