Assessment the microbiological and molecular aspects of soil isolated bacteria that suppress *Pythium ultimum* in Abha/KSA

Mona Kilany[a,d], Essam H. Ibrahim[b,d], Saad A. Amry[d], Mohammed Hashem[c,d], Mohammad Hamada[d]

[a]Department of Microbiology, National Organization for Drug Control and Research (NODCAR), Cairo, Egypt, [b]Department of Blood Products, Quality Control and Research, National Organization for Research and Control of Biologicals, Cairo, Egypt, [c]Department of Microbiology, Faculty of Science, Asute University, Asute, Egypt, [d]Department of Biology, Faculty of Science, King Khalid University, Abha, Kingdom of Saudi Arabia

Correspondence to Mona Kilany, Department of Biology, Faculty of Science, King Khalid University, 61413, Abha, Kingdom of Saudi Arabia. Tel: +966 540 793 464; e-mail: mona.kilany@yahoo.com

Received 18 February 2016
Accepted 20 March 2016

Egyptian Pharmaceutical Journal
2016, 15:181–186

Background
Pythium ultimum is largely threatening many economically important plants by causing seedling damping-off disease. Microbial control approach is considered a new, effective, and safe trend in the eradication of phytopathogens.
Aims
The current work focused on the isolation and molecular identification of soil isolated bacteria that suppress the damping-off-causing pathogen (*P. ultimum*). Moreover, optimization of environmental factors and detection of the mode of action of *P. ultimum* suppression was taken into consideration.
Materials and methods
Soil bacteria were isolated and screened for their antagonistic potential toward *P. ultimum.* The most vigorous isolate was characterized and identified. Some environmental factors were optimized using a well-plate assay. The inhibitory effect of bacteria, whether fungistatic or fungicidal, was detected. Mode of action of fungal inhibition was studied as well.
Statistical analysis
Statistical analysis was carried out using one-way analysis of variance in Excel.
Results
The bacterial isolate was identified as *Enterococcus faecalis*. The extracellular filtrate presented higher fungal inhibition (68%) compared with the bacterial cells (53%). The environmental factors for fungal inhibition were optimized to be pH 8, 28°C, 100% inoculum size, and third day of incubation reaching maximal values of 75, 76, 81, and 83%, respectively.
Conclusion
E. faecalis is a promising fungicidal agent against *P ultimum* through the production of diffusible metabolites.

Keywords:
biocontrol, fungicidal, Pythium ultimum, soil

Egypt Pharmaceut J 15:181–186

1687-4315

Introduction

Damping-off is one of the most frequent plant diseases worldwide. Among the etiologic agents *Pythium spp*., which belong to fungal-like organisms called oomycetes [1]. It extensively induced the devastating root rot, causing seedling damping-off. Therefore, Pythium diseases are important limiting factors in the successful cultivation of crop plants and responsible for losses of multibillion dollars worldwide [2,3]. The control process would be difficult and is considered a very common problem in fields and greenhouses as *Pythium* spp. tends to be very generalistic and unspecific in its host range [4,5]. For many years, chemical pesticides have been extensively used to reduce crop diseases, despite the seriousness of pesticide residues in food and environment. Therefore, this necessitates toxicological safety and pathogen resistance, and thus increasing costs that are involved in pesticide development. In contrast, biological control is the widespread approach in agriculture as well as an environmental-friendly alternative to chemical use [6]. Actually, many microorganisms played a vital role in biocontrol of Pythium damping-off, such as *Trichoderma* spp (*T. virens* and *T. barzinum*), *Streptomyces griseoviridis* and *Gliocladium* spp [7–9]. *Pseudomonas* and *Bacillus* are the most common examples of biocontrol agents toward *Pythium ultimum*, leading to the high rate of seedling emergence of soybean crops [10,11]. Notably, indigenous agricultural soil bacteria were developed and formulated as biofungicides for diminishing the early crop loss caused by seedling damping-off and root rot [12]. Currently, Khabbaz *et al.* (2015) [13] explored novel strains of rhizobacteria, *Bacillus subtilis* Bs 8B-1,

 DOI: 10.4103/1687-4315.197587

Pseudomonas fluorescens Pf 9A-14, and *Pseudomonas* spp. P sp. 8D-45 having a broad spectrum antagonistic activity toward *Pythium* spp., resulting in the suppression of Pythium damping-off and root rot of cucumber. Many mechanisms were put forward to explain growth inhibition of one organism by another, such as competition, production of siderophores, antibiotics, enzymes, and volatile substances [14]. The objective of our study was to isolate and identify soil bacteria as a biocontrol agent toward *P. ultimum* and optimize the medium and growth conditions leading to the highest rate of *P. ultimum* inhibition. Moreover, the inhibitory effect of bacteria whether fungistatic or fungicidal was detected.

Materials and methods

Microorganisms and growth conditions

P. ultimum strain was provided by the Biology Department, College of Science, King Khalid University. *P. ultimum* was grown using both potato dextrose agar medium and potato dextrose broth medium and incubated at 28°C, pH 6 for 72 h. Antagonistic bacteria were isolated from rhizosphere soil in Abha (Kingdom of Saudi Arabia) on 12 November 2014 using nutrient agar medium and nutrient broth medium and incubated at 30°C, pH 7 for 24 h. All microorganisms were maintained at –4°C.

Isolation and screening of antagonistic bacteria

Isolation of soil bacteria was carried out using the serial dilution method. Colonies with different characteristics were selected. All isolates were screened for antifungal activity using the dual-culture plate assay. The inhibition percentage of the pathogen was calculated according to the following equation: Inhibition $\% = P–C/C$, where P is the diameter of the pathogen growth on the nearby site of the fungus disk that faces the bioagent isolate and C is diameter growth of the pathogen control.

Bacterial isolates that inhibited fungal proliferation were selected and preserved [15].

Characterization and identification of the bacterial isolate

The bacterial candidate exhibiting the highest antifungal activity toward *P. ultimum* was selected and characterized. It was identified using the 16S rRNA sequence method. Total genomic DNA was extracted from pure bacterial culture using DNeasy Blood and Tissue Kit (Qiagen, West Sussex, UK). PCR amplification of the 16S rRNA gene from bacterial isolates was performed using the universal primers 27F5′AGAGTTTGATCMTGGCTCAG3′and 1495′TACGGYTACCT GTTACGAC TT 3′. The amplified DNA fragments were gel-purified using QIA Quick Gel Extraction Kit (Qiagen, Valencia, USA) following the manufacturer's instructions and sequenced by Macrogen Inc. (Seoul, Korea) using an ABI3730 XL Automatic DNA Sequencer (Applied Biosystems, Renton, Washington, USA). Multiple alignments of sequences were carried out with Clustal X [16]. The evolutionary history was inferred using the maximum likelihood method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed and the phylogenetic analysis was conducted using Mega 6 (molecular evolutionary genetic analysis) software [17].

Detection of antifungal activity of extracellular filtrates

Two methods were adopted to estimate antifungal activity, well-plate assay and dual-culture plate assay, which were performed according to the method described by Petatán-Sagahón *et al.* (2011) [15].

Optimization of environmental conditions

Optimization of some environmental factors was carried out to achieve maximum antifungal activity using well-plate assay [15].

Detection of the best incubation period

The selected antagonist was cultured at the previous growth conditions for different incubation periods (1, 2, 3, 4, 5, 6, and 7 days). Afterwards, the antifungal activity was investigated.

Evaluation of inoculum size

Five dilutions (100, 50, 25, 12.5, 6, and 3%) from the bacterial isolate were prepared and used as initial inocula [18].

Evaluation of temperature

The bacterial isolate was incubated at different temperatures (5, 15, 20, 25, 28, 30, and 35°C), followed by measuring the antifungal activity.

Evaluation of initial pH

The bacterial isolate was incubated at different initial pH values (4, 5, 6, 7, 8, 9, and 10). Thereafter, the antifungal activity of each sample was determined.

Detecting whether the antagonist is fungistatic or fungicidal

The agar disc broth method was adopted [19]. After incubation, the agar disc was examined under light microscopy to determine the growth inhibition in comparison with a control disc. Later on, the fungal

disc was cultured to assess whether a particular antifungal isolate is fungistatic or fungicidal.

Biocontrol mechanism

Biocontrol mechanism of the bacterial candidate was predicted by checking the production of antifungal activity of the bacterial candidate was monitored through the production of diffusible metabolites.

Statistical analysis

All analyses were reported as the means of three replicates. The SD was determined for each mean. The obtained data were analyzed for significant variations ($P \leq 0.05$) of main effects using one-way analysis of variance in Excel.

Results

Isolation and screening of bacteria with antifungal activity

Thirty-seven bacterial isolates were isolated from two soil samples. Among them, 14 isolates could suppress *P. ultimum* with various potentials. The findings showed that the isolate number 3 was the most potent one that reduced the growth of *P. ultimum* by 53% and was chosen for subsequent work (Fig. 1).

Detection of antifungal activity of extracellular filtrates

The results showed that the extracellular filtrate exhibited higher antifungal activity compared with bacterial cells. The results confirmed that the isolate number 3 was the most potent one that significantly reduced the growth of *P. ultimum* by 68% (Fig. 2a and b). Furthermore, the extracellular filtrate completely inhibited fungal growth when it was mixed with the medium (Fig. 3a and b).

Characterization and molecular identification of bacterial isolate

The selected isolate was characterized as Gram-positive streptococci possessing yellow, round, entire, and opaque colony. According to 16S rRNA sequence, it is identified as *Enterococcus faecalis*. Fig. 4 shows the phylogenetic relationship between the isolated strains, other strains of *E. faecalis,* and other related bacterial strains found in the Gen Bank database.

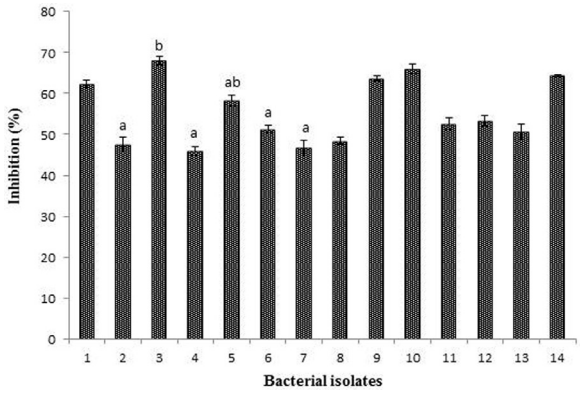
Evaluation of pH

The percentage of inhibition of *P. ultimum* significantly increased by elevating initial pH value, reaching the maximal value (75%) at pH 8 (Fig. 4).

Evaluation of temperature

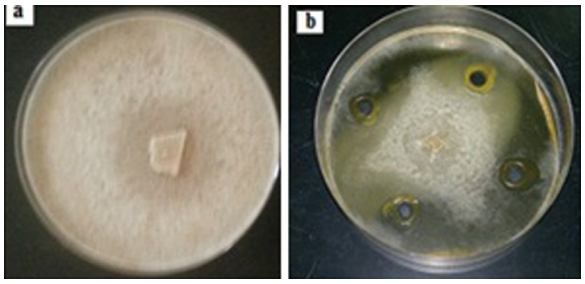
It was observed that the percentage of inhibition of *P. ultimum* increased gradually with the increase in

Figure 1



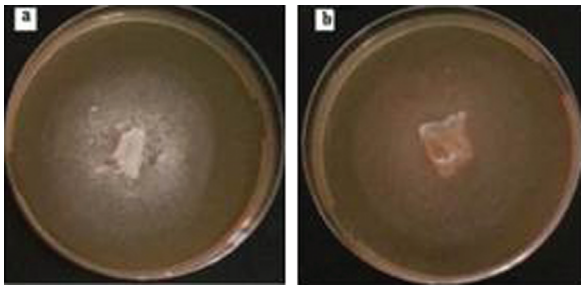
Antifungal activity of bacterial isolates against *Pythium ultimum.*

Figure 2



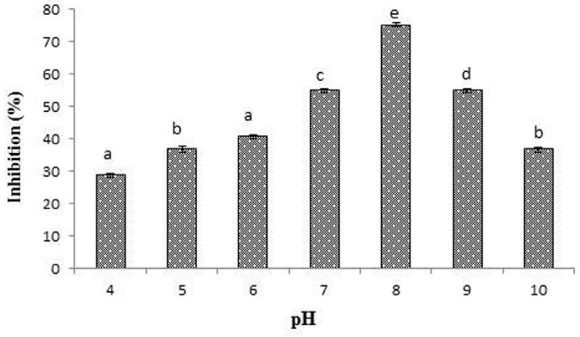
Antifungal activity of extracellular filtrate against *Pythium ultimum,* where (a) refers to the control and (b) refers to the sample.

Figure 3



Antifungal activity of extracellular filtrate of *Pythium ultimum* embedded in the medium, where (a) refers to the control and (b) refers to the sample.

Figure 4



Effect of pH on the stability of extracellular filtrates.

temperature, reaching the maximum value (78%) at 28°C, and then it began to decline (Fig. 5).

Evaluation of inoculum size

The results showed that the antifungal activity is directly proportional to the inoculum size, wherein 100% inoculum size exhibited the antifungal activity by 81%, and then it gradually decreased with the dilution of inoculum size (Fig. 6).

Effect of incubation period

The findings revealed that the antifungal activity of the bacterial candidate significantly increased with the increase in incubation period, reaching maximal value of 83% at the third day, and then it began to decline (Fig. 7).

Detecting whether the antagonist is fungistatic or fungicidal

Microscopic examination proved that there was no fungal growth. Concomitantly, a complete inhibition of fungal growth is the crucial evidence that the bacterial isolate is a fungicidal for *P. ultimum*.

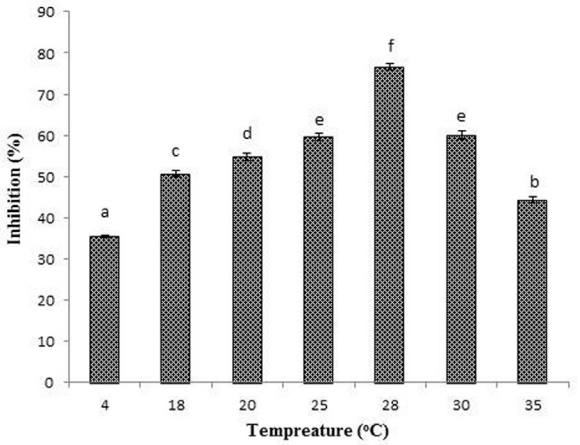
Biocontrol mechanism

Bacterial candidate produced diffusible metabolites, which inhibited the growth of *P. ultimum*.

Discussion

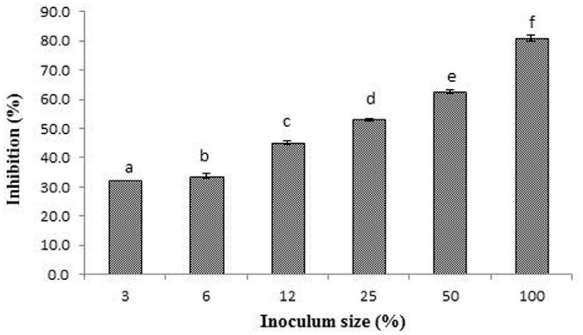
P. ultimum caused serious loss in a number of agricultural crops, which led to a considerable effort, devoted to the development of novel control agents. Microbial control of plant diseases offers a powerful and environmentally friendly alternative to dangerous chemical pesticides. Many promising approaches to explore microbial control of Pythium damping-off have been recorded [11,12]. In the current work, we focused on the same issue. *E. faecalis* is the most potent antagonist to *P. ultimum.* As such, the cell-free filtrates showed the large extent of inhibition of *P. ultimum* and it is more effective compared with the bacterial cells themselves. These results are in accordance with those of Chang *et al.* (2007) [20] and El Kahoui *et al.* (2011) [21] but not in accordance with the results of Petatán-Sagahón *et al.* (2011) [15] and Zamani *et al.* (2009) [22]. Essentially, the effects of bacterial filtrate may be due to the action of antibiotics that is associated with inhibition of spore germination or germ-tube elongation [23]. Apparently, the main reasons for the variability in biocontrol performance are varying environmental conditions, which affect survival, activity, and antibiotic production [24]. The highest antifungal production was observed at pH 8. This result is close to that reported by Ithnin (2007) [25]. Normally, pH is very important for bacterial metabolism and, consequently, for the biosynthesis of antimicrobial products. The elevation in pH is associated with permeability of the cell wall and membrane reflecting the peculiarities, either in the ion uptake by the cells or in a loss to the nutrient solution of soluble essential metabolites [26].

Figure 5



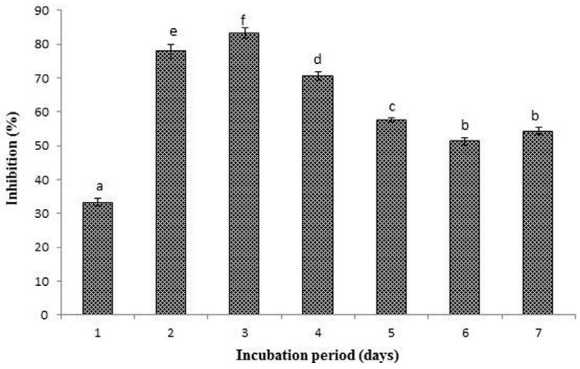
Effect of temperature on the stability of extracellular filtrates.

Figure 6



Evaluation of inoculum size of the bacterial isolate.

Figure 7



Effect of incubation period on the stability of extracellular filtrates.

Antifungal activity was also affected by temperature independently from growth. In the current study, the best fungal growth inhibition took place at lower temperatures. This may be due to denaturation of hydrolyzing enzymes at higher temperatures. This is in agreement with the findings of Schmidt *et al.* (2004a) [24] and Ithnin (2007) [25]. The inoculum size is one of the important factors affecting the antifungal activity. According to our findings, the antifungal activity is directly proportional to the inoculum size. Our results inferred that the increase of inoculum size lead to increase of antifungal activity of the bacterial isolate. This result is in agreement with that of Schmidt *et al.* (2004a,b) [24,27]. Incubation period is certainly an effective factor concerning antifungal activity. In this study, the maximum antifungal activity was achieved at the third day of incubation. These results are in accordance with other results, which reported that the synthesis of antimicrobial compounds generally starts at the end of the exponential phase and reaches the maximum level during the stationary phase [21,28,29]. In addition, the increase in incubation period and the decrease of nutrients in media, which lead to vulnerable growth of bacteria and in turn to deficiency in antifungal metabolite production. Generally, the mode of action of antagonistic bacteria involved the competition for nutrients and space or the production of extracellular lytic enzymes, siderophores, salicylic acid, antibiotics, or lipopeptides [30–32]. The bacterial isolate *E. faecalis* showed a fungicidal effect over the studied fungus through diffusible metabolites rather than through volatile metabolites. This result is in accordance with that of Abou-Zeid *et al.* (2008) [33]. Moreover, other substances exerted a fungistatic and fungicidal effect for *P. ultimum* such as fosetyl-Al and metalaxyl, respectively [34].

Conclusion

A bacterial candidate *E. faecalis* isolated from the rhizosphere would be a promising biocontrol agent, which acts against *P. ultimum* by excreting diffusible metabolites. Thus, this strain could be commercially exploited for the development of novel antifungal drugs. In the present study, an attempt has been made to isolate and identify antagonistic bacteria from soil (*E. faecalis*) against *P ultimum* and to optimize the environmental conditions leading to the highest antifungal activity through the production of diffusible metabolites. Thus, this strain could be commercially exploited for the development of novel antifungal drugs.

Acknowledgements

This work was supported by King Khalid University, Faculty of Science, Biology Department.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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