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Screening of Soil Isolates of Bacteria for Antagonistic Activity against Plant Pathogenic Fungi

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Abstract
The most common approach to biological control comprised of selecting antagonistic microorganisms, studying their modes of action and establishing a biological control product. The present study was an attempt to screening of soil isolates of bacteria for antagonistic activity against plant pathogenic fungi. Bacterial isolates from soil samples were identified by cultural, morphological and biochemical characterization. Bacterial isolates were then screened for their antagonistic activities against the tested plant pathogenic fungi. A total of 14 isolates belonging to 4 different species of bacteria with inhibitory activity against selected fungi were isolated from soil samples and identified biochemically. They were recognized in the genera *Bacillus* (*B. subtilis*, and *B. amyloliquefaciens*), *Klebsila* spp. *and Micrococcus* spp. The effect of the antagonistic activity of *Klebsila sp, Bacillus sp* and *Micrococcus sp* against plant pathogenic fungi was checked. The average diameter of inhibition zone (cm) of bacterial isolates against plant pathogenic fungi *Penicillium purpurogenum* was recorded as *Klebsila* spp. 2cm, *Bacillus subtilis* 2.6cm, *B. amyloliquefaciens* 0.866cm and *Micrococcus* spp. 1.6cm. With respect to the considerable tolerance of *B. subtilis* to environmental stresses and their facile production by current fermentation technology, bacterial isolates identified in this study with a diverse range of antifungal activities can be known as prospective resources of novel naturally occurring antifungal agents for controlling pathogenic fungi in medicine and agriculture. Biological control practices require an integrative approach, and more knowledge than chemical control.
Keywords: Biological control, antagonistic microorganisms, plant pathogenic fungi, inhibition zone.

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INTRODUCTION

The fungal kingdom consists of an estimated 1.5 million species on our planet. Among nearly 100,000 described species of fungi, approximately 400 species is now recognized as pathogens to humans, animals and plants. They include both molds and yeasts from different genera and species with the majority belonging to the genera *Fusarium* spp., *Alternaria chlamydospora* (Samson *et al*., 2000).

The fungi belonging to the genera *Fusarium* spp., *Alternaria chlamydospora* are important for not only causing life-threatening infections in humans and animals, but also producing toxic metabolites named "mycotoxins". *Fusarium* spp. is an important plant pathogenic fungus capable of producing different mycotoxins in food and agricultural commodities (Gallo *et al.,* 2015).

Effective control of plant pathogenic fungi involves different methods such as: cultural control, resistant cultivars and chemical control. Toxic compounds (potentially hazardous to humans and the environment) are accumulated by the rigorous usage of fungicides and also responsible for the resistance of the pathogens. In interpretation of these phenomenon and the presentation of biological control mediators (BCAs) appears to be one of the auspicious methods (Zivkovic *et al*., 2010). Synthetic chemicals including antifungal drugs and fungicides are widely used to handle injurious properties of fungi on human health and agriculture. Although fungicides are a key component of disease management programs, they grieve after large restrictions containing antagonistic responses on biological systems, resistance expansion of fungal pathogens and unwanted possessions proceeding non-target beneficial microorganisms. Thus, the optimization of environmentally- friendly fungicides is preferred (Ghisalberti, 2000).

The chief existing resources for antifungal identification are characterized by separating from the huge biodiversity ubiquitous in natural means (soil samples). The requirement for harmless and most functional agents

(antifungal agents) for the pathogenic fungus has generated significant attention for screening of new agents by natural means (Ranjbariyan *et al*., 2011). Bio control can be induced by nonpathogenic naturally existing microorganisms which can interact with the pathogenic microorganism for food, constrain pathogen reproduction by discharging chemical agents (antibiotics or toxins) (Zivkovic *et al*., 2010).

Similarly, pathogenic fungal resistivity to presently obtainable antifungal agents such as antibiotics has promote challenged the shortage of novel antibiotics (antifungal agent). Bacteria are gaining more importance regarding their ability to produce a wide range of bioactive metabolites with antimicrobial capabilities. At the present time, antifungal compounds have been obtained on the large scale from bacteria. Many of the antifungal agents are coming up by the researchers yet. Subsequently, antifungal metabolites construction in microorganisms such as bacteria is moderately reliant on the strain and species, continuing examination for identification of new bacteria to rise novel antifungals discovery is presently completed all over the world (Ranjbariyan *et al.,* 2011).

There is necessity to handle plant diseases, to sustain the worth and profusion of food, feed, and fiber formed by cultivators everywhere in the world. Various methods may be utilized to avoid, moderate or to control plant diseases. The present study was calculated to screen and separate potent bacteria from the rhizosphere soil samples for antimicrobial activity against plant pathogenic fungus.

MATERIALS AND METHODS

Collection of samples

A total of 10 soil samples were collected 4 to 5 cm deep from the surface using spatula from different sites in Yemen (Table 1). The collected samples were kept in sterile polythene bags and sealed properly. The samples were transferred to Microbiology Laboratory, University of Sana'a Yemen and kept at 4°C for further processing.

Tabie 1. Study sites in Yemen

Sr. No.	Region
1.	Alhodida
2.	Almahwite
3.	Amran
4.	Bni Al-harith
5.	Bni Matar
6.	Dhahban
7.	Ibb
8.	Kholan
9.	Taiz
10.	Wadi Dhahr

Tested fungal species

The fungal species were selected according to their Pathogenicity either to plant (Table 2) as recorded by (Lucas *et al.,* 1992).

Table 2. Pathogenicity of Tested Fungal Species to Plants

Pathogenic fungal species	Host	Disease
Fusarium spp.	Plant	Wilt
Phoma spp.	Plant	Rot and blight
Alternaria Chlamydospora	Plant	Spot diseases
Eurotium amstelodami	Plant	Hypersensitivity pneumonitis (p)
Penicillium purpurogenum	Plant	Fruit rots

Isolation of bacteria from soil samples

Samples were processed by dissolving 1 g of soil in 10 ml of sterile distilled water to make soil suspension in test tube. The test tube was vortexed for 2 to 3 minutes to remove soil, stones and debris. Supernatant was transferred to another test tube and ten-fold serial dilutions were prepared. Then spread 100µl of supernatant from each dilution on nutrient agar plates and kept at 37^0C overnight.

After 24hrs the colonies were picked and streaked onto Nutrient Agar plates for purification. Pure colonies were transferred from these plates to Nutrient Agar slants, incubated at 37^0C for 24 hr and stored at 4^0C until used.

Identification of bacterial isolates

After the purification, bacterial isolates were identified based on microscopic, morphological and biochemical charachers following Bergey,s Manual of systematic bacteriology (Bergey, 1984; Iqbal *et al.,* 2015; Yunus *et al.,* 2016).

Screening of bacteria isolates for anti-fungal activity

All bacterial isolates were streaked on Nutrient Agar at 37 ^{0}C for 24 hr. Target fungi were seeded in Sabouraud Dextrose Agar. Discs of NA with growth of bacteria isolates were cut by cork borer (1cm diameter) and transferred to the surface of seeded target microorganism plates under aseptic conditions. These plates were kept for 1hr in the refrigerator to facilitate diffusion then incubated at 28 ^{0}C for 3 days. Antifungal activity was recorded in term of inhibition zone of target fungal growth around the agar disc of bacterial isolates (Tepe *et al.,* 2004).

RESULTS AND DISCUSSION

The results showed that a total of 14 bacteria isolates were collected from 10 soil samples from different localities in Yemen (Table 3). These isolates belong to four different species *Klebsila* spp., *Bacillus subtilis*, *B. amyloliquefaciens* and *Micrococcus* spp.

Table 3. Distribution of bacterial isolates according to location

Bacteria	Location	No. of bacterial isolates
Micrococcus spp.	Wadi doher	1
Bacillus subtilis	Taiz	10
B. amyloliquefaciens	Kholan	2
Klebsiella spp.	Ibb	1
Total		14

The isolated bacterial genera were tested against fungal species for their antifungal activity. The highest inhibition was shown against fungus *Penicillium purpurogenum* (Table 4). The average diameter of inhibition zone (cm) of bacterial isolates against plant pathogenic fungi *Penicillium purpurogenum* was recorded as *Klebsila* spp. 2cm, *Bacillus subtilis* 2.6cm, *B. amyloliquefaciens* 0.866cm and *Micrococcus* spp. 1.6cm (Table 5).

Table 4. Antagonism between fungal pathogen and bacterial biocontrol agent

Fungal pathogen strain	Biocontrol agent	Location	Time cultivation (hours)		
			24	48	120
	Micrococcus spp.	Wadi doher	-	-	-
Fusarium spp.	*Bacillus subtilis*	Taiz	-	-	-
	B. amyloliquefaciens	Kholan	-	-	-
	Klebsiella spp.	Ibb	-	-	-
	Micrococcus spp.	Wadi doher	-	-	-
Phoma spp.	*Bacillus subtilis*	Taiz	-	-	-
	B. amyloliquefaciens	Kholan	-	-	-
	Klebsiella spp.	Ibb	-	-	+
	Micrococcus spp.	Wadi doher	-	-	-
Alternaria Chlamydospora	*Bacillus subtilis*	Taiz	-	-	-
	B. amyloliquefaciens	Kholan	-	-	-
	Klebsiella spp.	Ibb	-	-	-
	Micrococcus spp.	Wadi doher	-	-	-
Eurotium amstelodami	*Bacillus subtilis*	Taiz	-	-	-
	B. amyloliquefaciens	Kholan	-	-	-
	Klebsiella spp.	Ibb	-	-	-
	Micrococcus spp.	Wadi doher	-	+	++
Penicillium purpurogenum	*Bacillus subtilis*	Taiz	-	++	+++
	B. amyloliquefaciens	Kholan	-	+	+
	Klebsiella spp.	Ibb	-	++	+++

*Key: (+++ very good inhibition; + + good inhibition; + poor inhibition; - no inhibition)

Table 5. Effect of different bacteria on the antagonistic activity of *Klebsila* spp., *Bacillus* spp., *Micrococcus* spp. *against* plant pathogenic fungi *Penicillium purpurogenum*

Bacteria	Zone of inhibition (cm)
Micrococcus spp.	2
Bacillus subtilis	2.6
B. amyloliquefaciens	0.866
Klebsiella spp.	1.6

DISCUSSION

In the present study, to maximize the chance for isolating bacteria suitable as rich sources of antifungal bioactive metabolites as well as for possible expansion of naturally occurring antifungal compounds for pathogenic fungi, we isolated bacteria from Yemen (Alhodida, Almahwite, Amran, Bni Al-harith, Bni Matar, Dhahban, Ibb, Kholan, Taiz, Wadi Dhahr) soil samples. A total of 14 isolates belonging to 4 different species of bacteria with inhibitory activity against selected fungi were isolated from soil samples and identified biochemically. They were recognized in the genera *Bacillus* (*B. subtilis*, and *B. amyloliquefaciens*), *Klebsila sp, and Micrococcus sp* (an unidentified species). Inhibitory bacteria with antifungal activity against some selected fungi were evaluated. All these species exhibited considerable antifungal activity in various grades, indicate that diversity of population is an extremely important factor determining the potential for antagonistic activity of bacteria toward various microorganisms. The antagonism between microbial strains can be explained in a number of ways: the most common are synthesis of metabolites, competition and direct parasitism, but other mechanisms are involved, for example induced resistance sometimes linked with reduction of pathogen enzyme activity.

As reported by other researchers, the genera *Bacillus*, *Micrococcus* spp. and *Klebsila* spp. had

been well-thought-out as chief bacterial genera capable of showing antifungal activities (Ongena and Jacques, 2007). In the present study, the genus *Bacillus* was the most important antagonistic bacterial group as its isolates showed 0.866cm and 2.6cm zone on inhibition while the *Micrococcus* spp. showed 1.6 cm inhibition zone and the genus *Klebsila* spp. shown about 2 cm inhibition zone.

Within *Bacillus* species, *B. subtilis* as the most important species and in some extents other species such as *B. amyloliquefaciens* and *B. valismortis* are reported to produce a wide range of structurally related antimicrobial compounds and they are usually isolated from the soil as the main natural habitat (Stein, 2005; Arrebola *et al*., 2010). The strong inhibitory activity of *Bacillus* species isolated in the current research might aspect of manufacture of antifungal peptides by *Bacillus* strains (Reddy *et al*., 2009).

With respect to the considerable tolerance of *B. subtilis* to environmental stresses and their facile production by current fermentation technology, bacterial isolates identified in this study with a diverse range of antifungal activities can be known as prospective resources of novel naturally occurring antifungal agents for controlling pathogenic fungi in medicine and agriculture.

Clark (1996) stated that 2 or 3 antibiotics on average derived from microbes come in the marketplace every year. Similarly, bio-control products depending on bacteria have nowadays been economically established for the control of fungal diseases (Sharma *et al*., 2009). It has been shown that manufacture of tremendously widespread collection of antifungal composites by bacteria and their potential for use in bio control programs is fully dependent on limitations i.e; position of taxonomy and characteristics of physiology (i.e. species, varieties and growth cycle) conditions of geography, composition of soil, etc. Therefore, isolation of a huge amount of microbes commencing various geographical positions might raise the accidence of discovery of novel antifungal compounds.

CONCLUSION AND RECOMMENDATIONS

Despite a lot of progress is made in the knowledge of the modes of action of biological control agents (BCAs), practical application usually fails to control disease in the fields. One of the reasons illustrating this failure is that the bio-control product is used the same manner as a chemical product. Being biological these products have to be used according to their ecological requirements. Another approach consists of introduction of plant defense reactions. This can be achieved by application of natural substances obtained from microorganisms, plants, or algae. A third approach comprises of selecting cultural practices that might reduce the occurrence or severity of diseases. Future studies to identify antifungal metabolites of antagonistic bacteria isolated here, to determine their mechanisms of action on fungal cells and their evaluation as effective fungal bio control agents in the field are recommended.

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CONFLICT OF INTEREST

The authors declare that this article content has no conflict of interest.

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