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Antifungal Activity of *Bacillus* Species against Damping-off Fungi

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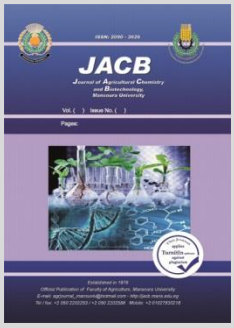

ABSTRACT

The current paper discuss the isolation of bacterial Bacilli from the rhizosphere soil of some seedlings plants to suppress growth plant pathogenic fungi. The 15 bacterial colonies were isolated and screened for their ability to antagonize damping-off fungi, *Rhizoctonia solani* and *Fusarium oxysporum. In vitro*, among them, four isolates were the highest effect in reducing growth of both mycelia for each fungi. These bacterial isolates exhibited multiple antifungal substances for example as: hydrogen cyanide (HCN), ammonia (NH_3), indole acetic acid (IAA), siderophore and hydrolytic enzymes (chitinase, cellulase, catalase). The most potent bacterial isolates based for morphological, biochemical and molecular characterizations. Four potent bacterial strains spore-forming were isolated on nutrient agar (NA) medium and identified as *Bacillus subtilis*, *Bacillus* sp., *Bacillus* sp. and *Bacillus amyloliquefaciens*, respectively. All strains were Gram-positive long rods, motile, spore forming bacteria. Strains *B. subtilis*-1 and *B. amyloliquefaciens*-14 were produced white pigment. Moreover, inhibition zone *in vitro* with strains *B. subtilis* -1 and *B. amyloliquefaciens*-14 higher than with strains *B.*sp.-4 and *B.*sp.-12. There were correlation between the *Bacillus* spp. strains against *R. solani* and *F.oxysporum* and its results of antifungal substances production. Finally, *Bacillus* spp. isolates have antagonistic activity for *R. solani* and *F.oxysporum* also, potenial to be used as a biocontrol agent. It is suggested that antifungal substances secreted by *Bacillus* spp. isolates should be involved in the suppression of the phytopathogenic fungal growth.

Keywords: *Bacillus* spp.*;* damping-off fungi; biocontrol

INTRODUCTION

Recantly, mentioned that bacteria especially genus *Bacillus* can be used as bio-control agents by antagonistic effects. *In vitro*, when samples of soil collected and isolated *Bacillus* was tested against the phytopathogenic fungi showed antagonism between various *Bacillus* species and pathogenic fungi (Sheikh *et al.* 2022).

Several species of *Bacillus* can be used as producers diverse classes of lytic enzymes and secondary metabolites (Lalitha and Nithyapriya 2020). Khan *et al.* (2018) repoted that microorganisms can be produce HCN, IAA, lytic enzymes and catalase to enhance plant health. The microorganisms can be found in the rhizosphere which decrease the deleteious effects of pathogens on crop yield through the production of hydrogen cyanide and siderophore (Larkin 2020; Mohammed *et al.* 2020).

A various species of bacteria including *Bacillus* spp. have the ability change iron formation of soluble Fe^{3+} by producton siderophores which are iron scavenging ligands produced from these bacterial species (Serrano, 2017). The outer layer of the the cell wall of fungi contain chitin, these compose with insoluble linear ß. 1, 4- linked polymer of N-acetylglucosamine (Gooday, 1990). Several microorganisms, including bacteria and many species of fungi (Bing-Lan liu, *et. al.*, 2003) can be using chitin as the sole source of carbon and energy (Kobayashi, *et. al.*, 1995). Most studies showed that inhibation of fungal pathogens as *Fusarium* as well as increase improvment plants growth by produced bacterial metabolites such as siderophores and indoles (Sheng *et al.* 2020).

A chitinase enzyme producing by bacterium isolate is very important product to reduce growth plant pathogenic fungi such as *Fusarium* sp. and *Rhizoctonia bataticola* (Vaidya, *et. al.*, 2001). Several studies have reported that *Bacillus* spp. reduced the growth of many plant pathogens by their antagonistic activity, with different modes of action such as production of enzymes (chitinase and 1,3-glucanase) (Sheikh *et al.* 2022).

Chitin is the main component of fungal cell wall, consists of glucans and other polymers (Agrios, 2005). Therefore, for industrial microbiology processes require suitable method of cultivation and optimizations for isolation of cultures from nature (Jeyanthi *et al.*, 2013). The chitinase producing by bacterial strains were isolated and identified as *Bacillus* spp. (Mathurot *et al.*, 2019).

The objectives of this paper was to know role of: (a) isolation *Bacillus* spp. from the rhizosphere seedlings plants (b) study antagonism between bacterial isolates and fungal phytopathogens (c) detection antifungal substances production by *Bacillus* isolates in suppression of damping-off fungi (d) idetentification the most strong bacilli isolates that reduced growth *Fusarium oxysporum* and *Rhizoctonia solani*.

MATERIALS AND METHODS

Pathogens

Fusarium oxysporum Schlech and *Rhizoctonia solani* Kühn were obtained from plant pathology Lab., A.R.C. Giza, Egypt. On potato dextrose agar (PDA) medium, fungal cultures slants were maintained for used (Murtado *et al.* 2020).

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Bacterial isolates

Bacillus as spore-forming bacteria were isolated from the soil by pasteurization soil suspension (15 min, 80ºC) (Ashour and Afify 1999) and the purified cultures were maintained on nutrient agar (NA) slants at 4-5ºC for used (Burcu and Bahri 2016).

***In vitro* screening of *Bacillus* spp.**

The antagonistic effect of *Bacillus* species against pathogenic fungi (*F. oxysporum* and *R. solani*) the test was described by Vidhyasekaran, *et. al.,* (1997) the zone of inhibition (mm) was recorded between the edges of the fungal growth and antagonistic bacteria. Mean from all treatments were carried out in triplicates.

Detection of antagonistic metabolites

Hydrogen Cyanide (HCN) production

Hydrogen cyanide production of the *Bacillus* strains was tested by measured the optical density (OD) values at 625 nm. This method as described by Castric (1975).

Ammonia (NH_3) production

The porduction of ammonia, the *Bacillus* strains were grown in peptone broth (10 mL) at 28-30ºC for 48h. Nessler's reagent was added (0.5 mL) to the bacterial broth and recorded change the color of brown to the yellow (Cappuccino and Sherman, 2002).

Siderophore production

For siderophore production, this method according to Sharma, *et al.* (2013).

Indole Acetic Acid (IAA) production

For this purpose, the IAA content was assayed according to Salkovski's method (Pandya *et al.* 2018).

Hydrolytic enzymes detection by *Bacillus* spp.

The activites of hydrolytic enzymes detected by streaking antagonistic *Bacillus* isolates individually on the medium containing enzyme substrate (Basha and Ulaganathan 2002). The estimation of enzyme was carried out according to Ngarajkumer *et al.* (2004). All treatments were carried out in triplicates.

Identification of potential *Bacillus* isolates

1. Morphological and biochemical characterizations

From *in vitro* tests, the highest four potent bacterial isolates studied for morphological and biochemical properties according to the Bergey's manual of determinative bacteriology (Krieg and Holt 1984).

2. Molecular characterization

16S rRNA gene sequencing of *Bacillus* spp. were amplified using forward primer 27 F 5' AGAGTTTGATCMTGGCTCAG3' and reverse primer 1792 R (5'- TACGGYTACCTTGTTACGACTT-3'). The sequences were subjected to homology search using BLAST programme (Altschul *et al.* 1990). The results were compared manually with the data in NCABI. The 16S rRNA gene sequences determined using the CLUSTAL W program version 2.1 (Chenna *et al.* 2003).

RESLTUS AND DISCUSSION

Inhibition of *F. oxysporum* and *R. solani* by *Bacillus* spp. Fifteen strains of *Bacillus* spp. were isolated from soil on nutrient agar (NA) medium. *In vitro* technique, the *Bacillus* species were tested for their activity to suppress the mycelial growth of *F. oxysporum* and *R. solani*. The results showed that only four bacterial isolates introduced most bacterial strains have different appearances of antagonistic activities against the pathogenic fungi. These *Bacillus* strains (No. 1, 4, 12 and 14) has shown are very important effects against all tested pathogenic fungi by remarkable antagonistic activity. From results found that, *B.* sp.-1 and *B.* sp.-14 were the higher effective in inhibiting growth of both fungi. These isolates *B.* sp.-1 and *B.* sp.-14 produced an inhibition zones of 12.8, 12.3 mm, respectively while, the isolates *B.* sp.-4 and *B.* sp.-12 recorded inhibition zones of 11.4, 10.6 mm, respectively (Table 1).

Table 1. Inhibition zones (mm) of mycelial growth of *F. oxysporum* and *R. solani* by *Bacillus* spp.

Isolates No.	Inhibition zone (mm)		Means
Bacillus spp.	*F. oxysporum*	*R. solani*	(mm)
*B.*sp. 1	11.8	13.8	12.8
2	2.0	2.2	2.1
3	5.1	6.1	5.6
4	10.4	12.4	11.4
5	2.0	2.0	2.0
6	5.1	4.1	4.6
7	1.1	1.3	1.2
8	7.9	8.7	8.8
9	8.2	7.0	7.6
10	2.0	3.6	2.8
11	4.2	4.0	4.2
12	11.6	9.6	10.6
13	1.5	1.3	1.4
14	13.0	11.6	12.3
15	2.2	2.0	2.1

This study indicates that the antagonistic activity of bacterial *Bacillus* spp. showed that the highest antagonistic activity against pathogenic fungi when tested by *in vitro* against the phytopathogenic fungi (Sheikh *et al.* 2022).

Detection of antagonistic substances by bacterial isolates

Results of bacterial activities as antagonistic substances are presented in Table (2). The four *Bacillus* isolates introduced HCN production by the change in the color of filter paper from yellow. The deep brown color of filter paper was observed as an indication of HCN production by bacterial strains *B.* sp.-1 and *B.* sp.-14. Also, bacterial isolates *B.* sp.-1 and *B.* sp.-14 produced copious ammounts of ammonia in pepton water. The production of IAA when the addition of Salkowski's reagent showed pink color by all isolates indicating IAA was production. In CAS assay *B.* sp.-1 showed a strong positive reaction (big orange color zone) for siderophore production. While, the other isolates produced weak reaction (small orange color zone) (Saha *et al.* 2016). Bacterial strains may protect plants from phytopathogens by production some materials such as ammonia, hydrogen cyanide and catalase (Kremer and Souissi 2001 & Khan *et al.* 2018). All of the products improved plant growth by the production growth regulators substances like IAA (Hameeda *et al.* 2008 & Afify and Ashour 2018). Among the four strains of *Bacillus* spp. (strains No.1,4,12,14) tested for production of hydrolytic enzymes as the antagonistic activities materials are presented in Table (2). Strain *B.* sp.-1 recorded the highest chitinase, followed moderate chitinase by *B.*sp. - 4., *B.* sp. - 14 and lowest chitinase by strain *B.* sp.-12. But, all strains produced catalase and not produced cellulase. Jaganmohan *et al.* (2010) demonstrated that some hydrolytic bacteria are very important agents for the protection many plants from phytopathogenic fungi. Thus, hydrolytic soil bacteria that are able to lyse hyphal fungi because fungi are an important

source of substrates for hydrolytic enzymes that produced by soil bacteria. Amrih and Elisa (2017) demonstrated that many reports introduced *Bacillus subtilis* with production of lytic enzymes, such as chitinase, protease, lipase, for inhibation various soil borne plant pathogens (Wietse De Bore *et al.* 1998). The study suggests that more than one mechanism may be involved in the reduction of *F. oxysporum* and *R. solani* by *Bacillus* spp. (Guetsky *et al.* 2002) and with antagonistic bacterial species. In the same Table a study by Kumar *et al.* (2022) showed that several genera of bacteria produce chitinase enzyme as the antifungal mechanism against plant pathogenic fungi. The biocontrol by chithnase enzyme can inhibit and control fungus growth by degraging chitin the fungal cell wall component ((Khan *et al.* 2018 & Khairah *et al.*, 2023). Additionally, some bacteria produce hydrolytic cellulases synergistic action by endoglucanase, exoglucanase or cellobiohydrolase and ß-glucosidase (Lynd, 2002). In addition, the bioagents materials showed that all bacterial strains produced ammonia and catalase enzyme (Afify and Ashour 2024).

Table 2. Detection of antagonistic substances by *Bacillus* spp.

Bacillus strains No.	production						
	HCN	NH_3	Siderophore	IAA	Chitinase	Cellulose	catalase
*B.*sp-1	+++	+++	+++	+	+++	-	+
*B.*sp-4	+	+	+	-	++	-	+
*B.*sp-12	+	+	+	-	+	-	+
*B.*sp-14	+++	+++	+	+	++	-	+

Indicator of production: +++ = high production
++ = moderate production + = few production

Identification of bioagents bacterial isolates

Fifteen *Bacillus* spp. strains were obtained from the rhizosphere seedlings plants plating on NA medium according to Sholberg *et al.* (2006) for classification as Genus: Bacillus as Domain: Bacteria, Phylum: Firmicutes, Class: Bacilli, Order: Bacillales, Family: Bacillaceae. The *Bacillus* strains were sclected from *in vitro* by antagonism test. The morphology of colony strains *Bacillus* shows white, with smooth texture (Felsensyein 1985). Microscopic examination and physiological characteristics showed endospore forming motile, long rod-shaped cells and Gram-positive. These characteristics as well as oxidase, catalase, methyl red test, V.P. and indole formation were consistent with the description of *Bacillus* group (Abirami *et al.* 2020). Based on the morphological, biochemical and physiological (as growth temperature ºC) characteristics in Table (3) (Schaad *et. al.* 2001).

Molecular characterisation

Morphological, biochemical and physiological tests followed by sequence results of 16S rRNA was exported to the database for the homlogous alignment (Saitou and Nel 1987). Based on the alignment results, the two strains were found as species of *Bacillus* sp. as: *Bacillus subtilis* (strain no.1) and *B. amyloliquefaciens* (strain no.14) which showed highest similarty between 97- 99%. the NCBI deposited isolates. The strains name were designated in Table (4).

Table 3. Morphological, biochemical and physiological characters of bioagents isolates on (NA) medium.

Character	No. of isolates			
	1	4	12	14
Cell shape	rod	rod	rod	rod
Spore formation	+	+	+	+
Gram stain	+	+	+	+
Motility	+	+	+	+
Pigment	-	-	-	-
Catalase reaction	+	+	+	+
Oxidase reaction	-	+	+	+
Casein hydrolysis	-	+	-	+
Voges-Proskauer (V.P.)	-	-	-	-
Indole production	+	-	-	+
Methyl Red	+	-	-	-
Growth temperature (30-50ºC)	+	+	+	+

+ = positive reactions , - = negative reactions

Table 4. Scientific name of *Bacillus* strains on (NA) medium.

No. of strain	Scientific name	Code name
1	*Bacillus subtilis*	*B.* sp.-1
4	*Bacillus* sp.	*B.* sp.-4
12	*Bacillus* sp.	*B.* sp.-12
14	*B. amyloliquefaciens*	*B.*sp.-14

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نشاط أنواع بكتريا الباسيلس المضاد لفطريات أمراض بادرات النباتات

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[2] قسم الميكروبيولوجيا الزراعية- كلية الزراعة- جامعة المنصوره- المنصوره- مصر

الملخص

أجريت هذه الدراسة لعزل البكتيريا العصوية (الباسيلس) من ريزوسفير بادرات بعض النباتات وتم الحصول على خمسة عشرة مستعمرة من هذه البكتيريا (الباسيلس) وبإجراء إختبار التضاد لهذه العزلات البكتيرية تجاه فطرين من الفطريات المسببة لأمراض بادرات النباتات فيوزاريوم أوكسيسبورم و ريزوكتونيا سولانى فقد سجلت نتائج إختبارات التضاد أن هناك أربعة عزلات من بكتيريا الباسيلس (أرقام 1 و 4و12 و 14) أعلى مناطق تثبيط للفطريات الممرضة وأن هذه البكتيريا لها القدرة على إنتاج بعض المواد المضادة لنمو الفطريات الممرضة مثل سيانيد الهيدروجين والأمونيا والسايدروفورز وكذلك مواد منشطة لنمو النبات مثل إندول حمض الخليك بالإضافة لإنتاج بعض الإنزيمات المحللة لجدر خلايا الفطريات مثل الكيتينيز والسليلوليز كذلك القدرة على إنتاج الكتاليز ولكن فى هذه الإختبارات إثنان من عزلات الباسيلس سجلت أعلى نتائج فى إنتاج المواد المضادة (أرقام 1 و 14) تم تعريف الأربعة عزلات البكتيرية وذلك بالإختبارات القياسية (المورفولوجية والبيوكيميائية ثم الجزيئية) وقد أكدت جميع العزلات أنها عصويات طويلة ومتجرثمة موجبة لجرام ومتحركة ومتحملة الحرارة العالية وبالتالى تتبع جنس الباسيلس ثم أظهرالتعريف الجزيئى لأنشط عزلتين أنهما باسيلس ستلس و باسيلس أميلوفاكشن. من هذه الدراسة نجد أن هناك علاقة بين نتيجة التضاد بين البكتيريا والفطريات الممرضة وبين مستوى إنتاج البكتيريا للمواد المضادة لنمو الفطريات الممرضة للنبات كما أن عزلات بكتيريا الباسيلس تمتلك القدرة لتثبيط نمو فطريات أمراض البادرات (فيوزاريوم أوكسيسبورم و ريزوكتونيا سولانى) وبناءا على ذلك يمكن إستخدامها كعوامل مقاومة حيوية لفطريات أمراض البادرات