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Antagonistic activity of Rhizospheric bacteria and Arbuscular Mycorrhiza isolated from Iraqi soil against fungal pathogen *Macrophomina phaseolina.*

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ABSTRACT

The antagonism of the rhizospheric bacteria toward pathogenic fungi *Macrophomina phaseolina* was investigated. Ten soil samples were collected from the rhizospheric zone around Cowpea root (*Vignaunguiculata* L.). These samples were used as the source of *Arbuscular Mycorrhizal* fungi (AMF) and rhizobacterial isolates. Twenty-five bacteria were isolated and evaluated as an antagonistic agent against pathogenic fungi. *M. phaseolina* was isolated from infected roots of Cowpea and used as a pathogen. Twenty-five bacteria were isolated and evaluated as an antagonistic agent against pathogenic fungi. *M. phaseolina* was isolated from infected roots of Cowpea and used as a pathogen. The synergistic effect between *A. siccitolerans* and (AMF) *Glomusmosseae,* was investigated on the Cowpea plant under greenhouse conditions. The results showed that either both bacteria and (AMF) or each of them was significantly increased most cowpea plant parameters (Vegetative weight, Dry weight, Lengths, Chlorophyll), compared with the plant alone or infected with a fungal pathogen.

Keywords: Antagonistic activity, Rhizobacteria, Arbuscular Mycorrhizal, biocontrol.

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INTRODUCTION

Cultivated cowpea (*Vignaunguiculata*) is an important legume crop in several countries, which belongs to the family of Fabaceae, cowpea are annual crops grown in tropical, subtropical and the semi-arid area around the world, especially Africa, Asia and America, Most of these plants have the ability to adapt to the dry and extreme environments such as savanna region of sub-Saharan Africa [1-3]. Cowpea plant can be infected with fungal diseases caused by phytopathogenic agent *M. phaseolina* (Tassi), this disease called Charcol rot; it is an economically important for a wide range of plant crops in Africa, Asia, North and South America such as legumes. This disease can infect all parts of the plant at any growth stage affecting seed, seedling and adult plant [4].

Many organisms colonize the zone of the soil adjacent to the roots of plants called rhizosphere microflora. They have the ability to interact with plant roots; and acquired the attention of many researchers in recent years [5]. One of the most predominant organisms found in the rhizosphere of plant roots is called *Arbuscular mycorrhizal* fungi (AMF), which can play an important role in providing the plant with numerous benefits. These benefits including, drought resistance, facilitate nutrient uptake or increase viability and resistance to pathogens and growth [6-9].

A Wide range of soil borne bacteria and Fungi especially rhizosphere bacteria including species of *Arthrobacter, Pseudomonas, Bacillus, Enterobacter, Azotobacter, Burkholderia* and *Klebsiella* and rhizosphere fungi belong to genus of *Trichoderma* and *Gliocladium* were detected as biocontrol agent for a wide range of plant disease caused by phytopathogenic microorganisms through many mechanisms that include induction of plant growth promoting substance and ,Antibiosis, Parasitism and compotation [10-14].

The genus of *Arthrobacter*was characterized as a high-GC-content Actenobacteria, and widely distributed in the soil and in the extreme environments [15]. New Species from these bacteria are described as a highly desiccation-tolerant such as *Arthrobacter siccitolerance* 4J27; in addition, its whole genome sequence was also determined [16,17].

Therefore, this study aimed to isolate the plant fungal pathogen *M. phaseolina* from infected Cowpea plant roots and determine the antagonistic activity of Rhizospheric bacteria and Arbuscular Mycorrhiza isolated from Iraqi rhizosphere soil towards fungal pathogen *M. phaseolina*.

MATERIALS AND METHODS

Soil samples

Ten rhizosphere soil samples were collected from three legumes farms in Kana'an village, Diyala Governorate, Iraq, during the period from October 2014 to January 2015. These samples were obtained from rhizosphere around Cowpea root plants (*Vignaunguiculata* L.), and used as a source of rhizobacteria and Arbuscular Mycorrhiza (AM) fungi.

Rhizosphere soil was selected for isolation of rhizobacteria by suspending ten grams of soil in 90 ml of sterilized peptone water and mixed well. Then, soil suspension was serially diluted10^1, 10^2 and 10^3.The dilutions were streaked out on nutrient agar plates as duplicates [18] these plates were incubated at 37°C for 24-48h.

Identification of Rhizobacterial isolates

Biochemical and physiological characterization of isolated bacteria were performed according to Berge's Manual of Systematic Bacteriology [19], bacterial isolates were characterized according to both their phenotypic traits: such as shape, size, margin, surface, elevation, color, pigmentation and staining by Gram stain, and also Biochemical test. The later was performed using standard biochemical and physiological testes that included, catalase test (3% H2O2), oxidase reaction (Kovacs method) and diffusible pigment production, In addition, carbohydrate fermentation was accomplished by using medium containing specific carbohydrate source (sucrose, glucose, lactose, mannitol, maltose, rhaminose), and starch hydrolysis was achieved by culture isolation on Starch nitrate agar medium, After incubation for 24 h, at 37 °C, the plate was covered with

iodine, and a clear zone around the growth of colonies was detected on the medium plate. Gelatin hydrolysis was achieved by stab tubes of nutrient agar containing gelatin, after inoculation with tested bacteria, liquefaction of gelatin was observed [20].

16S rDNA identification of most antagonistic bacteria

The molecular identification of the higher antagonistic activity *in vitro*(isolate No. 15) was done using the sequence analysis of 16S rDNA gene. The DNA was extracted from the bacteria by enzymatic lyses using lysozyme (20 mg/ml) and Proteinase K (1 mg/ml). Total genomic DNA was purified using phenol-cholrophorm extraction as described by [21]Polymerase chain reaction (PCR) amplification of the 16S rDNA genes was conducted using extracted DNA in the presence of the forward primer 16RW01 (5`-AGAGTTTGATCCTGGCTCAG-3`) and the reverse primer 16DG74 (5`-GGTTACCTTGTTACGACTT-3`). The final 50 µL reaction mixture contained 1× PCR buffer (NEB, England), 1 nmol of dNTPs, 1 pmol of 2 mM $MgSO_4$, 0.25 pmol of forward and reverse primers, 1 unit Taq DNA polymerase (NEB, England) and 10 µL template DNA.

The PCR amplification included initial denaturation of DNA at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, the mixture was kept for 10 min at 72°C for complete extension (X). The amplified PCR products were separated by electrophoresis on 1.5% agarose gel. The PCR product was purified by QIAquick Gel Extraction Kit (QIAGEN, USA) and electrophoresed on agarose gel to get the purified 16s DNA fragments for sequencing. Identification was achieved by comparing the contiguous 16S rDNA sequences obtained with the 16S rDNA sequence data from the reference and type strains available in public databases Gen-Bank using the BLAST (National Centre for Biotechnology Information).

Isolation of Plant Pathogen fungi

M. phaseolina is a fungus isolated from infected roots of *V. unguiculata* plants, after isolation the fungus was grown on potato Dextrose broth medium(PDB) in 250 ml Erlenmeyer flasks, The flasks were incubated at 25 °C in shaker incubator at 100 rpm, then the fungal pathogen is used in the antagonistic experiments.

Isolation and characterization of Arbuscular Mycorrhiza (AM) fungi

Arbuscular Mycorrhiza (AM) was isolated using wet sieving and decanting method [22]. The fungus hypha was observed and characterized using light microscope at 40 x magnification[23]. These processes were done in biotechnology lab, agriculture research Centre, Baghdad, Iraq. The pure cultures of Arbuscular Mycorrhiza were stored onto 1 soil: 3 beets sterilized twice in autoclave at 121 °C till to use as Cowpea inoculum.

Antagonistic activity by four equidistant point technique

The antagonistic activity of twenty five selected rhizobacterial isolates against the fungal pathogen *M. Phaseolina* was studied by the four equidistance method. Seven microliters of each rhizobacterial isolate suspension were inoculated in four equidistant points from the center of a petridish border of the Potato Dextroseagar medium, The Petri dish were incubated for 48 h. at 35-37 °C allow growth and production of diffusion of metabolites from rhizobacterial isolates into PDA medium. After this, 5 mm-diameter disc cut from pre growing culture of *M. phaseolina* was placed in the center of petri dish. The plates with only fungal disc without bacterial streaks served as control. All *in vitro* antagonism assays were done in triplicate. The diameter of pathogen colony and percent inhibition were determined after incubating for 5-6 days at 28°C by.

The percent of inhibition in growth of pathogen was calculated by the following equation:

$$\% \text{ inhibition} = R1 - R2 \times 100/R1$$

R1= radial growth of *M. phaseolina* in control treatment
R2= radial growth of *M. phaseolina* in four equidistant point inoculation treatment

Agriculture in pots under plastic houses

In this experiment, soil samples were collected from the banks of the Dijla (Tigris) River. These samples were sterilized by formalin and divided into plastic pots (1 kgm soil/ pot). The surface of each pot was sterilized by 5% sodium hypochlorite. The pots were inoculated by pathogenic fungi and Arbuscular Mycorrhiza (50 gm/ pot) as described by [24]. The bacterial strains were added separately. All microbial strains were added at depth of 3 cm and covered using thin layer of soil. Cowpea seeds were surface sterilized by 5% sodium hypochlorite for 10 min followed by washing three times with sterile distilled water to remove the remnants completely, sowed into plastic pots (5 seeds/ pot) and covered using suitable layer of soil. The field capacity of the pots was determined and maintained. The plants were followed-up and irrigated based on field capacity. This experiment was done in plastic house of Botanical Garden, Biology department, Faculty of science (for women), Baghdad University, Baghdad, Iraq.

Sampling and analysis

The Cowpea plants were harvested after 62 days from sowing. The shoots and roots of the plants were collected separately and stored in 4 °C using paper bags for further use, after being washed with tap water. The soil around Plants was collected separately in new nylon bags. All data of each treatment were recorded onto bags.

Determination of chlorophyll content (mg/L)

Leaf samples were collected from the plants growing under different treatments before being harvested. The samples were rinsed with tap water and dried. The weight of each sample was scaled precisely. After that, the leaves were grounded with 0.5 g of $CaCO_3$ in mortar, contained 80 % of acetone to break down leaves into pieces. The resulted extract was eluted with 80 % acetone and filter sterilized into 100 mL flasks. The collected samples were subjected to centrifugation for 10 min at 3000 rpm/minute. and the residual extract was measured. The raw chlorophyll extract was scanned at wavelengths of 645 and 663 nm in Hitachi U 2000 UV model 121-002 spectrophotometer. The absorption values were used in the following equation to calculate total chlorophyll.

$$\text{Total chlorophyll (CT)} = 20.2 \times A_{645} + 8.02 \times A_{663}$$

This equation gives the total chlorophyll contents existing in per liter of the extract. Then, this result was divided over leaves weight to determined chlorophyll content per gram of leaf.

Statistical Analysis

The effect of interaction between rhizobacteria and AMF on the growth of cowpea plants infected with *M. Phasoelina* was tested by using Analysis System [25]. Least significant difference (LSD) was used to compare the means in this study.

RESULTS AND DISCUSSION

Isolation and partially characterization of rhizosphere bacteria

Rhizosphere bacteria associated with cowpea plants roots were isolated after fresh soil samples were sampled. Twenty five bacterial isolates were isolated and the morphological and physiological characteristics were illustrated in Table (1). The results showed that the Bacilli shape was widespread in the tested samples. This result agrees with those obtained by [26] who found and isolate some Bacillus isolates, which were efficient against 32 isolates of *M. phaseolina*.

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Table 1. Morphological characteristics of bacterial isolates obtained from been rhizosphere soil.

Isolate No.	Oxidase	Catalase	Gram Stain	Shape of the bacteria
1	-	+	+	rod
2	-	+	+	rod
3	-	+	+	rod
4	-	+	+	Dram stick
5	-	+	+	rod
6	-	-	+	rod
7	-	-	+	rod
8	-	-	-	Cocci
9	-	+	+	Cocci
10	+	+	+	rod
11	-	+	-	rod
12	-	-	-	rod
13	-	+	+	rod
14	-	+	+	rod
15	-	+	-	Rod to cocci
16	-	+	+	rod
17	+	+	-	rod
18	-	-	-	Cocci
19	-	+	+	rod
20	-	-	+	rod
21	-	-	-	rod
22	-	+	+	rod
23	-	+	-	Cocci
24	+	+	+	Cocci
25	-	+	+	rod

Antagonistic effect between bacterial isolates and phytopathogenic fungi

In vitro efficacy of rhizobacterial isolates against *M. phaseolina* by using four equidistant point technique was achieved. The growth of *M. phaseolina* was inhibited at different levels, depending on the types of bacterial isolates. Rhizopshere bacteria isolates inhibited the *M. phaseolinain vitro* in the range from 12.5% to 87.5%. Six isolates namely (1, 11, 14, 15, 17 and 18) showed high antifungal activity more than 75 %. Interestingly, only one bacterial isolate (No15) showed the highest inhibitory activity toward the test plant pathogen; while the other isolates show varied inhibit growth of the fungus (Table 2 and Figure1). These results were in agreement with [27].

Table 2. Antagonistic activity of rhizobacterial isolates against *M. phasolina* by measuring of fungal colony diameter and percentage of inhibition

Isolates No.	Growth of fungal colony diameter in mm	% inhibition of pathogen in (mm)
1	20 mm	75%
2	30 mm	62.5%
3	50 mm	37.5%
4	30 mm	62.5%
5	50 mm	37.5%
6	30 mm	62.5%
7	40 mm	50%
8	50 mm	37.5%
9	40 mm	50%
10	60 mm	25%
11	20 mm	75%
12	50 mm	37.5%
13	30 mm	62.5%

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14	20mm	75%
15	10mm	87.5%
16	50 mm	37.5%
17	20 mm	75%
18	20mm	75%
19	40 mm	50%
20	60 mm	25%
21	70 mm	12.5%
22	70 mm	12.5%
23	50 mm	37.5%
24	50 mm	66.2%
25	70 mm	12.5%
Control	**80 mm**	**0%**

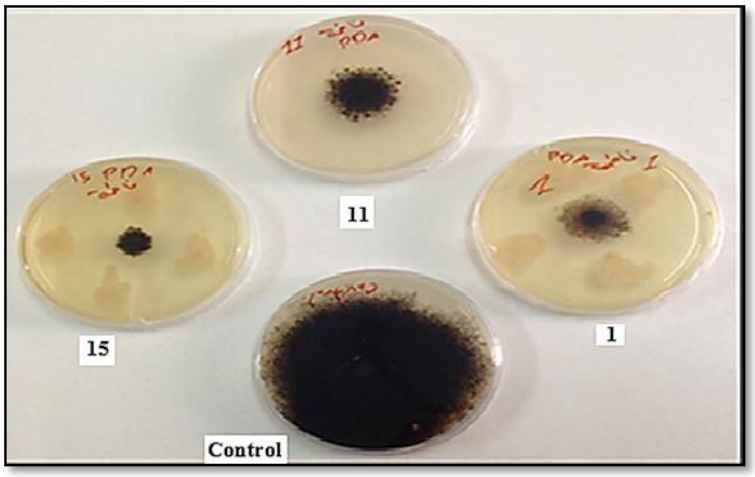

Fig. 1 The four-point technique for measure the antagonistic activity of the best three rhizobacterial isolates (1, 11 and 15) against fungal pathogen *M. phasolina*.

Identification of the most potent bacteria (isolate No. 15)

Isolate No 15 was the most potent bacterial isolate in inhibition of *M. phasolina* growth. This isolate was purified and subjected for colony characterization, Gram staining and motility tests. Then subjected for identification by using biochemical tests such as oxidation-fermentation reaction, oxidase, catalase and sugar fermentation tests. The results of morphological, cultural, biochemical and physiological characterization of this isolate were represented in Table (3). This isolate was Gram positive, motile, oxidase negative, catalase positive, positive for blood hemolysis and able to ferment the urea and some sugars. Based on these results, Phylogenetic, chemotaxonomic and phenotypic analyses indicated that isolate No 15 a new strain within the genus *Arthrobacter*.

Table 3. Morphological and biochemical characteristics of isolate No. 15.

No. of Test	Test	Result
1	Colony color	Creamy-white
2	Gram stain	Positive(+ve)
3	Motility	Positive(+ve)
4	Growth on MacConkey Agar medium	Negative(-ve)
5	Catalase	Positive(+ve)
6	Oxidase	Negative(-ve)
7	Blood Hemolysis	Positive(+ve)
8	Oxidation Test	A/K*
9	Urea Hydrolysis	Positive(+ve)

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10	Starch Hydrolysis	Negative(-ve)
11	Gelatin Hydrolysis	Negative(-ve)
12	Production of indol Acetic acid(IAA)	Positive(+ve)
13	Phosphate solubilzation	Positive(+ve)
Sugar Fermentation Test		
1	Sucrose	Positive(+ve)
2	glucose	Positive(+ve)
3	Lactose	Positive(+ve)
4	Mannitol	Positive(+ve)
5	Maltose	Positive(+ve)
6	Rhaminose	Positive(+ve)

The PCR product was 1301pb, and the resulted sequence of *Arthrobacter* sp. isolate was compared using the BLAST algorithm with the available 16S rDNA gene sequences from organisms in the GenBank databases. The analysis of 16S rDNA sequences indicated that Isolate No 15 deposited at GenBank under (accession number KX129776) and shared with a maximum 99% identity with *Arthrobacter siccitolerance* 4J27. Morever, Isolate No 15 clustered with an *A. siccitolerance* 4J27 type strain (GenBank accession number GU815139 and DDBJ/EMBL/GenBank databases under accession numbers CAQIo1000001 to CAQI01000064) [16,17]. The phylogenetic tree based on the 16S rDNA gene of *A. siccitolerans* compared with other strains found in gene bank was represented in Figure (3) and clearly demonstrating that the isolate was a member of *A. siccitolerans* of that level of 16S rDNA gene sequence homology.

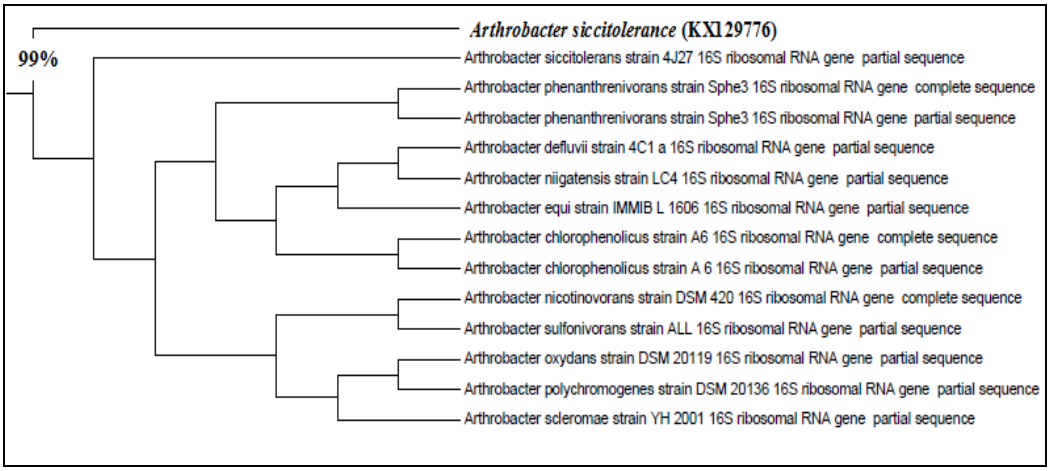

Fig. 3A neighbor-joining Phylogenetic tree based on the 16S rDNA genes of *A. siccitolerans*, showing the relationship between *A. siccitolerans* and other related *Arthrobacter species.*

The effect of the interaction between bio-agent *A. siccitolerans* and *G. mosseae* on the reduction of growth of plant disease caused by *M. phasolina* was accomplished by cultivation of Cowpea seed on the pots under plastic houses; the results of these experiments are shown in Table (4), which showed that all the tested biological microorganisms were significantly increase most of the vegetative, dry weight (Root and Steam), Lengths and chlorophyll, when they were individually or in combination.

Vegetative and dry weight of plant root and steam found to be enhanced to 0.60, 1.6 and 0.91, 0.6 g/ plant respectively in the treatment of interaction between (Plant + *M. phasolina +A. siccitolerans+G. mosseae*), compared to (0.34, 0.83 and 0.25, 0.34 g/plant vegetative root and 0.25 g/plant dry root), whereas, also the results revealed found significantly increased in the lengths of plant and the amount of chlorophyll (12.58 cm/plant and 9.83mg/g) respectively, compared with the treatment of control(10.21 cm/plant and 7.90 mg/g)respectively. While in the treatment of (Plant +*M. phasolina +A. siccitolerans*) results refer to moderate increasing in the vegetative and dry weight of root and steam, Lengths and Chlorophyll (0.58 g/plant, 1.1 g/plant, 0.82 g/plant, 0.58 g/plant, 12.60 cm/plant and 8.06 mg/g) respectively.

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Table 4. Efficiency of interaction between *A. siccitolerans* and *G. mosseae* against *M. phasolina*.

Treatment	Vegetative weight		Dry weight		Lengths/ cm	Chlorophyll /mg/g
	Root/g	Steam/g	Root/g	Steam/g		
Plant (control)	0.34	0.83	0.25	0.34	10.21	7.90
Plant + *M. phasolina*	0.12	0.20	0.24	0.12	5.90	6.21
Plant *M. phasolina* +*A. siccitolerans*	0.58	1.1	0.82	0.58	12.60	8.06
Plant + *M. phasolina* +*G. mosseae*	0.32	1	0.58	0.32	12.20	8.00
Plant + *M. phasolina* +*A. siccitolerans*+*G. mosseae*	0.60	1.6	0.91	0.60	12.58	9.83
LSD (P<0.05)	0.397	0.538	0.519	0.397	3.228	2.074

DISCUSSION

There are two broad groups of beneficial organisms that found in the rihzophere zone around the roots of plants, these organisms are classified according to their primary effect which include (i): microorganisms with direct effect on plant, thus occurs during facilitate the plant growth toacquire (N,P and essential nutrients) and induction of plant growth promoting which called (Plant growth promoting microorganisms PGPM) such as *Rhizobium* and *Glomus* sp. (ii) microorganisms play an important role in the controlling of plant disease, thus achieved indirectly, and called Biological control agent (BCA) such as *pseudomonas* and *Trichoderma* spp. [28,29]. From the previous studies, it could be concluded that the exploitation of interaction between rhizobia and plant growth promoting microorganisms is become an efficient strategy of enhancing the productivity of legumes, as well as to provide legume plants with natural bio-protection agent under sustainable agricultural system [30-33].

Arbuscular mycorrhizal fungi (AMF) inhibit both roots and surrounding soil and form a link between plant and soil by affecting plant growth, nutrient mobilization and by their antagonistic effect on plant pathogens. The beneficial effect of AMF increasing through their interact synergistically with bacteria to stimulate plant growth through some mechanisms that include improving of nutrients acquisition and controlling of plant pathogenic microorganisms [12,34,35].

The interaction between rhizobacteria and AMF was beneficial because greater growth and development occurred in the plants of Cowpea; increased vegetative and dry weight of Cowpea plants was obtained compared with treatment of AMF or *A. siccitolerans* and control only. The beneficial interaction between rhizobacterial isolate and AMF was also appeared in the results of the amount of chlorophyll, it was increased compared with others treatments, Efficacy of the interaction between both organisms exhibit the highest percentage of protection and lowest level of disease severity in Cowpea plant inoculated with *M. phaseolina*. Enhanced capacity in preventing of disease caused by plant pathogens by interaction or co-inoculation could result from a combination of various mechanisms, such as competition, altered root exudation, anatomical and morphological changes in the root system, antibiosis and induction of plant defense mechanisms by both antagonists and the saprophyte fungus [36,37]. There are many studies concern the effect of interaction and co-inoculation of Arbuscular mycorrhizal fungi and rhizosphere bacteria to biological control of phytopathogenic fungi [38,39,8].

In the present study, all rhizospheric bacterial isolates exhibits various levels in the inhibition of plant pathogen *M. phaseolina* in the four equidistant point technique; however, the highest inhibition in the media was accomplished by using the isolate NO. 15, that identified according to Morphological, biochemical and molecular identification as *A. siccitolerans* with similarity of 99 %, the inhibition of plant pathogen *M. phaseolina* by suing rhizobacteria *A. siccitolerans* may be related to some mechanisms such as production of an extracellular lytic enzyme complex that included chitinase which attack the hyphal walls of the fungal pathogens and weakening them and causing them to blot inner pressure and eventually to collapse [40], A few studies has been focused on the using of *Arthrobacter* spp. as a bio-control agent against phytopathogenic fungi, in the study of [41],refers to using the soil *Arthrobacter* sp. in the inhibition of plant pathogen *Fusarium moniliforme* var. *subglutinans*, the causes of pine pitch canker.

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CONCLUSION

The results of our study refer to the interaction between arbuscular mycorrhizal fungi and rhizosphere bacteria which can be used in combination with the biological control of phytopathogenic fungi *M. phaseolina* isolated form roots of Cowpea farm in Iraq. This may be based on the following items:

a. These bio control agents have adverse effect on the charcoal root rot disease affecting cowpea crop.
b. The combination of Arbuscular mycorrhizal fungi and rhizosphere bacteria do not have harmful effect on vegetative and dry weight of steam and roots, but can increase the vegetative and dry weight.
c. They play an important role in the increasing of the length of plants and chlorophyll amount when interaction occurs between them.

The results of present study may be encouraging and provide the probability of using the interaction between fungi and bacteria as good bio control agents against *M. phaseolina* that resulted in charcoal root rot and growth enhancing.

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