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EVALUATION OF THE ACTIVITY OF *BACILLUS SUBTILIS* AND *ACTINOMYCES SP.* AGAINST *MACROPHOMINA PHASEOLINA* (TASSI) GOID. CAUSATIVE AGENT OF CHARCOAL ROT ON SESAME

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Abstract

The study was carried out in Plant protection department/college of agricultural engineer science/university of Baghdad-Al-Jadrya, to evaluate the activity of the bacteria *Bacillus subtilis* and *Actinomyces sp*. to restrict infection of sesame plants with charcoal rot caused by *Macrophomina phaseolina* under potted and field conditions and to detect activity of control agents through appreciation total Phenol, Chlorophyll measurement Peroxidase enzyme, Phanylalanine ammonia lyase (PAL), Plant content of nitrogen and Protein. Six isolates of *M. phaseolina* were isolated and identified from infected sesame plants (Mp-1, Mp-2, Mp-3, Mp-4, Mp-5 and Mp-6) the color of the isolates colony were ranged from slight to deep brown as a results of forming irregular black sclerotia. The pathogenicity tests indicated that all the isolates induced reduction in sesame seed germination on PDA Mp-4 isolate was found the more pathogenic that completely inhibited seed germination, while the germination percentages with the other isolates ranged between 0.0-72% compared with 100% in control. The infection with *M.phaseolina* isolate, caused high reduction in sesame seeding growth with disease incidence between 20-100% and disease severity 12.6-92.6%. The isolate Mp-4 was the more active with disease incidence and severity 100 and 92.6% respectively. High antagonistic activity of *B.subtilis* and *Actinomyces sp*. against *M.phaseolina* (Mp-4) isolate on PDA was manifested that totally inhibited the fungal growth.

Key words: *Macrophomina phaseolina, Bacillus subtilis, Actinomyces sp.*

Introduction

Sesame, *Sesamum indicum* L. is one of the most important oil Crops in pedeliaceae family (Thabit, 2009). Sesame seeds reported to contain high percentage of oil, 45-60% and Considerable percentage of protein, 15%, as well as rich in minerals including Calcium and phosphorus (Yingxian *et al*., 1988). Sesame is Cultivates in tropical, subtropical and southern areas in the word and represent 60% of cultivation, in china and India (Banerjee and Kole, 2009). The total production of Sesame in Iraq in 2018 ranged between 110-600 Kg/ donum in area of 889 donum (Statistical center system, 2018). Sesame is Subjected to infection with many Fungi, The most important were, *Macrophomina phasolina*, *Fusarium solani* and *phytophthora rastica* (EL-Bramawy and Abd AL-wahid, 2009) charcoal rot disease caused by *M. phaseolina* is considered among The more Limitant of Sesame Cultivation and cause Losses in quality and quality of yield That reach to 100% (khadum, 2005). The control of The disease was restricted on Fungicides, but the excessive use of the Fungicides created enormous problems to human health and ecosystem as well as new strains of the pathogen resistance to Fungicides, were appeared. There fore efforts were spent to ward biological control using benefit rhizosphere microorganisms refer to as plant growth promoting rizobacteria (PGPR) including *Bacilus* and *Actinomycey sp*. alternative to Fungicide for disease management (Saharan and Nehran, 2011; Bhatti *et al*., 2017). Several genus and species at PGPR have been reported to show activity against different types of soil pathogenic Fungi including. The caused agent of charcoal rot as well as promote plant growth (Wehner *et al*., 2009). There is need to develop new programed methods to control the pathogens based on activation of plant defense mechanisms. It has been reported that is possible to induce resistance in plant using different agents, biotic and non-biotics (Han *et al*., 2000). The systemic resistance associated with increase in peroxidase, β- 1,3 -glucanase in plants (Nakkeeran *et al*., 2006). The study was conducted to manage charcoal rot disease on Sesame Caused by *Macrophomina*

phaseolina using the bio agents *Bacilus Subtilis* and *Actinomyces sp.* To induce Systemic resistance in the plants.

Materials and Methods

Isolation and Identification of *Macrophomina phaseolina*

Infected sesame plants, showing yellowing, dryness, black leg, were collected from, Madaen, Dorah, Taji, Radwonya, college of agricultural engineer in Baghdad, Shirkat in salah El-din areas. Parts at infected roots and stems were rinsed with water flow for 30 min and cut to small pieces, 0.5-1 cm, surface sterilized with 1% sodium hypochlorite for 2min. the pieces were rinsed with sterile distilled water, let to dry on filter paper and cultivated on PDA amended with 200mg /L of tetracyclin in 9cm dim petri plates, 4 pieces/ plate. the plates were maintained at 25±2°C for 3 days and the growing fungal colonies were purified by transferring a small part of colony border into petri plates containing PDA. the plates were maintained at 25±2°C for 5 days and the fungi were identified based on colony Characters, mycelium and sclerotia formed as described by (Holliday and Punithalingam, 1970). The fungal isolates were conserved in small vials containing PDA with sterile soil.

Pathogenicity of Macrophomina phaseolina isolates on sesame seeds

The pathogenicity of 6 isolates of *M.phaseolina* was carried out as described by Bolkan and Butler, (1974). Petri-plates, 9cm dim, containing (15-20 ml water agar/L water) were prepared. the plates were inoculated in the center with 0.5 cm dim discs, taken from colonies border on PDA, 5 days old, one disc/plate. The plates were maintained at 25±2°C for 3 days and cultivated with surfaces sterilized sesame seed with 1% sodium hypochlorite at plate border around fungal inoculum, 25 seed/plate with three replicates for each isolates. sesame seeds were cultivated on un inoculated PDA as control. The plates were maintained at 25±2°C for 7days and the seed germination percentages were estimated by the equation:

$$\text{Seed germination \%} = \frac{\text{Number of germinated seeds}}{\text{Number of total seeds}} \times 100$$

Effect of *Macrophomina phaseolina* on sesame seeds germination and seedling growth in pots:

The inoculum of 6 isolates of *M.phaseolina*, Mp-1 - Mp-6, were grown on sesame seeds. Sesame seeds in flask, were autoclaved at 121°C and 1.5 kg/cm^2 for 20 min., twice in two successive days. The flasks were inoculated with 0.5 Mm discs taken from *M.phaseolina* colony border, 3 days old, on PDA. the flasks were maintained at 25±2°C for 15 days with agitation every three days for verification and uniform distribution of the inoculum on the seeds (Dewan, 1989). The isolates inoculum on sesame seeds were separately added into sterile mixed soil (autoclaved at 121°C and 1.5 Kg/cm^2 for on hour, twice in two successive days) at 1% (W/W) in pots of 3 kg, the pots were watering and covered with drilled polyethylene sacs for 3 days, the pots were seeded with surface sterilized sesame seeds (Al-Rafidain) with 1% sodium hypochlorite for 2 min, 10 seeds, pot with 3 replicates. sesame seeds seeded in non-inoculated soil as control, the infection percentage was estimated after 7 days of cultivation as following.

$$\text{\% Infection} = \frac{\text{Number of infected seeds}}{\text{Total plants}} \times 100$$

The disease severity was determine after 30 days of cultivation by a scale of 5 degrees, where:

0 = healthy plants

1 = 1-25% yellow leaves

2 = 25-50% yellow leaves with slight browning of stem base

3 = 50-75% yellow leaves with deep browning of stem base

4 = 75-100% dry leaves, plant death with peeled stem base associated with sclerotia formation

The disease severity percentage was estimated by McKinney, (1923) equation:

$$\text{Disease severity \%} = \frac{\text{Number of plants from 0 degree} \times 0 + \cdots + \text{Number of plants fron 5} \times 5}{\text{Total number of plants tested} \times 5} \times 100$$

The bacteria

On isolate of each of *Bacillus subtilis* and *Actinomyces sp*., obtained from mycotoxin lab/ plant protection dept., college of agricultural engineer, were used. the two bacteria were grown in nutrient broth (NB) in 250 ml flasks. The flasks were separately inoculated with colonies of each bacteria (5 colonies /flask) on nutrient agar (NA), 24 hour old and maintained at 27±2°C.

Antagonistic activity of *Bacillus subtilis* and *Actinomyces sp*. Against *Macrophomina phaseolina* on culture medium

The antagonistic activity if *B.subtilis* and *Actinomyces sp*. Against *M.phaseolina* isolate-4 (Mp-4), the more pathogenic, was carried out as described by Raspor *et al*., (2010). A growth line of the two bacteria, from colonies on NA, 48 hrs old, was separately done by inoculation loop on PDA at one side of the plate, 2 cm from the border. A disc from fungal colony border on

PDA, 5 days old, was placed at the opposite side of the plate, 3.5 cm from the border three plates for each treatment were used with three plates with out bacteria for control. The plates were maintained at 25±2°C for 5 days and the mean fungal growth was calculated. the inhibition percentage at each treatment was estimated by the equation :

$$\text{Growth inhibition \%} = \frac{A}{A + B} \times 100$$

Where;

A = the distance between the growth line of bacteria and the end of fungal growth

B = fungal growth distance

Determination the active concentration of *Bacillus subtilis* and *Actinomyces sp*. For inhibiting *Macrophomina phaseolina* growth

Serial dilutions, 10^{-1} -10^{-10} in water from the bacterial suspension of both bacteria in NB, 48 olds, were alone in test tubes. One ml of each dilution was placed in petri plates containing NA before solidification with slight agitation for uniform distribution. the plates were inoculated in the center with 0.5 cm disc from the border of *M.phaseolina* isolate-4 (Mp-4)colony. 5 days old. Plates inoculated with fungus only were used as control. the plates were maintained at 28±2°C for 3 days and the fungal growth inhibition was estimated by the equation:

$$\text{Fungal growth inhibitin \%} = \frac{\text{Fungal growth in control - Fungal growth in treatment}}{\text{Fungal growth in control}} \times 100$$

Determination of *Bacillus subtilis* and *Actinomyces sp*. Cell numbers

The number of colony forming unit (CFU) in the convenient dilution of *B.subtilis* and *Actinomyces sp*. That inhibit *M.phaseolina* growth (10^{-8}) was determined by total colony count on NA. One ml of both bacteria was separately added to NA before solidification in petri plates with slight round agitation. the plates were maintained at 28±2°C for 24 hrs and the colonies growth were counted. the number of CFU per ml of the bacterial suspension was calculated by the equation:

Number of CFU/ml= number of bacterial colonies × dilution in version

$= x \times 10^{-8}$

Table 1: Isolates *Macrophomina phaseolina* and Collection Sites.

Collection Sites	Isolates
Dorah	Mp-1
Radwanya	Mp-2
Shirkatin Salah EL-din	Mp-3
Taji	Mp-4
Colleg of agricultural engineer in Baghdad	Mp-5
Madaen	Mp-6

Table 2: Pathogenic Isolates of *Mcrophomina phaseolina.*

Isolate	Germination %
MP-1	46.6
MP-2	72.0
MP-3	54.6
MP-4	0.0
MP-5	35.3
MP-6	22.3
Control	100.0
LSD	3.64
*each value represent mean of 3 replicate	

Results and Discussion

Isolation and Identification

Results of isolation and Identification of *Macrophomina phaseolina* from sesame plants infected with charcoal rot disease revealed the presence of 6 different isolates, Mp-1, Mp-2, Mp-3, Mp-4, Mp-5 and Mp-6 (Table 1). The isolates colony on PDA were characterized by white color converted gradually to brown in concomitant with the formation of small irregular seclorotia, slight difference in mycelium density between the isolates was observed, the mycelium is formed of hyaline hyphae in the early stages of growth, converted to black associated with hairy growth and formation of aerial branches over the colony within 5 days. Fig. 1, these results are in accordance with Barnett and Hunder, (1972); Ndiaye, (2007) results. *Macrophomina phaseolina* was previously isolated from sesame plant in fields of Agricultural college / Abu-Graib (Al-Ani, 1977). It has been reported that, *M.phaseolina* is one of fungi infecting wide range at hosts including, sunflower, Bean, Phaselus, Melon, watermelon (Al-Juboury *et al*., 2016; Khalaf,2017 and Hashim, 2017). The fungus survived in soil for long Period as sclerotia and the symptoms manifest at florescence although the infection begin early (Baird *et al*., 2003).

Pathogenicity of *Macrophomina phaseolina* Isolates

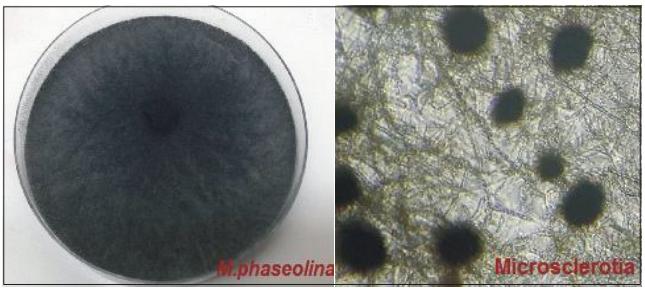

Fig. 1: *Macrophomina phaseolina* isolate-4 (Mp-4) on PDA and sclerotia under light Microscpe.

Table 3: Effect *M.phaseolina* on disease incidence and severity of Charcoal rot on sesame in pots.

Isolate	Disease incidence %	Disease severity %
MP-1	66.6*	52.6*
MP-2	20.0	12.6
MP-3	43.3	40.3
MP-4	100.0	92.6
MP-5	73.3	65.3
MP-6	83.3	77.0
Control	0.0	00.0
LSD	10.11	3.95
*each value represent mean of 3 replicate		

on sesame seeds

It was found that all the isolates of *M.phaseolina* caused reduction in sesame seeds germination that ranged between 0 to 72.0% Compared with 100% in control (Table 2). The isolate Mp-4 was the more effective that totally inhibited the germination, followed by Mp-6, Mp-5, Mp-1, Mp-3 with germination percentages 22.3, 35.3, 46.6, 54.6% respectively while Mp-2 isolation achieved the was highest seed germination attained 72.0%. The Variation between the isolates may be due to genetic variation and the ability of the isolates to produce lytic enzymes like polygalacturonase and cellulose (Mahtab *et al*., 2013). The Variation in isolates pathogenicity may came from their ability to produce secondary metabolites on the seeds and certains are toxic and affect seed germination - It was reported that *M.phaseolina* produce Isoasperlin, Phaseolinic acid, Phomenon, Phaseolinone and the isolate producing phaseolinone is more pathogenic. The virulence of the isolates depend one the quantity of toxin and the seed germination inhibition is correlated with the toxic produced (Kumar and Sharma, 2013).

Pathogenic activity of *Macrophomina phaseolina* on sesame plant in pots

Results, (Table 3) showed that all *M.phaseolina* isolates were pathogenic to sesame plants. The more effective isolate was found to be Mp-4 with disease incidence and severity, 100% and 92.6% respectively, followed by Mp-6 with disease incidence and severity 83.3% and 77.0% respectively. The disease incidence for the other isolates ranged between 20-73% and the disease severity between 12.6-65.3% respectively. The variation in pathogenicity between the isolates may be due to variation in isolates ability to produce toxic secondary metabolites and lytic enzymes degrading pectinase and cellulose in early stage of infection (Kumar and Sharma, 2013; Mahtab *et al*., 2013). It has been reported that *M.phaseolina* isolates varied in pathogenic ability on different hosts (Amusa, 2011; Al-Juboory *et al*., 2016). Hafed, (2001) found that *M.phaseolina* isolates differ in Pathogenic activity, where sesame isolate was the more effective than soybean and sunflower isolates. Some studies reported that *M.phaseolina* isolates possess different mechanisms to induce disease including close up the vessels by microsclerotia that interrupt transference water and nutritive minerals leading to wilt and plant death, as well as killing the tissue by the toxins and lytic enzymes produce which acts in combination with sclerotia to induce the disease (Khan, 2007).

Table 4: Effect Isolation of bacteria *Bacillus subtilis* and *Actinomyces sp*. In the growth of *Macrophomina phaseolina* on the agricultural medium PDA.

Treatment	% inhibiting the growth of fungi
*M.phaseolina(*Mp-4)	0.0
B.subtilis(Bs)+*M.phaseolina* (Mp-4)	100.0
Actinomyces sp(As) +*M.phaseolina* (Mp-4)	100.0
*each value represent mean of 3 replicate	

Antagonistic activity of *Bacillus subtilis* and *Actinomyces sp.* Against *Macrophomina phaseolina* on PDA

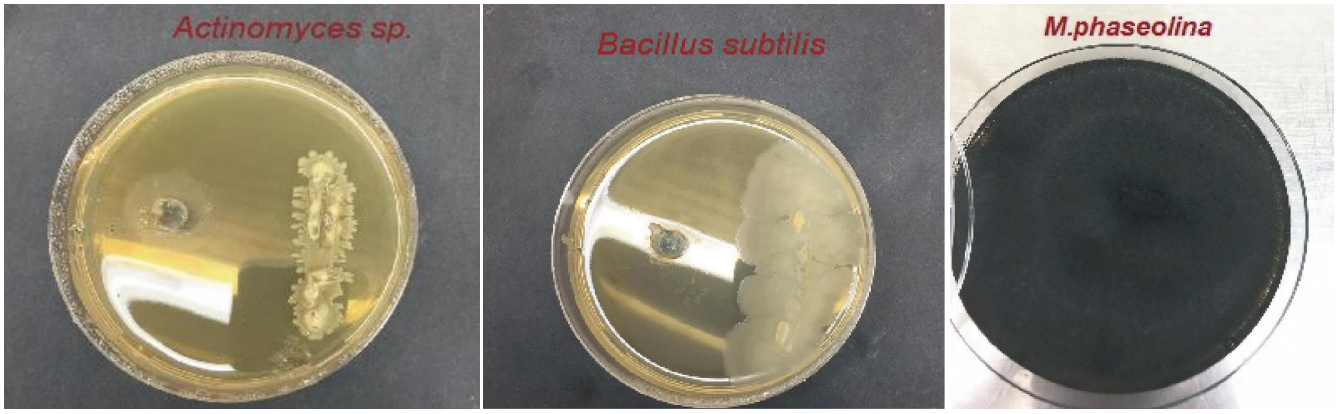

Fig. 2: Antagonistic activity of *Bacillus subtilis* and *Actinomyces sp*. Against *Macrophomina phaseolina* on PDA.

Results, (Table 4) and (Fig. 2) showed that *B.subtilis* and *Actinomyces sp*. Have totally inhibited *M.phaseolina* isolate-4 (Mp-4) On PDA. It was reported to the ability of *B.subtilis* to inhibit some pathogenic fungi including *Rhizoctonia solani* (Jaafer, 2011)**.** The ability of *B.subtilis* to inhibit fungal growth may be attributed to its rapid growth on the culture medium leading to stop fungal growth, as well as the ability at bacteria to produce many antibiotic like Bacillomycin, Bacitracin, Subtiline that acts as inhibitors to pathogenic fungi growth (Montealegre *et al*., 2003). It has been reported that *Bacillus* produced antibiotic and crystal protein as well as enzymes, Glutaminase, Protease, Amylase, Lipase that decompose polymeric compounds and restrict pathogens activity (EL-Hamshary and Khattab, 2008). *B.subtilis* has totally inhibited *M.phaseolina* growth on culture media through producing secondary metabolites including ring lipopeptide,Antifungal toxic and some lytic enzymes degrading fungal cell walls (Felip *et al*., 2017). Other studies reported that *Streptomyces* was active in restriction radial growth of *F. oxysporium sp. Lycopersici* causal agent of tomato wilt (Al-Dulaimy, 2019). The ability of *B.subtilis* to inhibit Mp-4 isolate can attributed to in ability to produce many antibiotics like Difficidin, Orydiffcidin (Stein, 2005) and produc Siderophore and lytic enzymes, Amylase, Proteases, Glutaminase (Gill *et al*., 1982). *Actinomyces sp*. Reported to express inhibition activity against many pathogenic fungi including *M.phaseolina* through producing small molecular weight compounds like HCN that inhibit pathogenic fungi at high concentration (Kumar and Kannabiran, 2010). The results of this study demonstrated that *Macrophomina phaseolina*, isolate-4 (Mp-4) was highly pathogenic to sesame plants and the addition of *B.subtilis* and *Streptomyces* into the culture medium caused total inhibition of Mp-4. This indicate that these bacteria may be promising in charcoal rot disease management.

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