MOLECULAR CHARACTERIZATION OF *M. PHASEOLINA* AND ITS MANAGEMENT USING AGROCHEMICALS AND *T. HARZIANUM*

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ABSTRACT

Systemic Acquired Resistance (SAR) induced by agrochemicals of chitosan (CH) and salicylic acid (SA) at (25, 50 and 100 ppm) , in addition to a biocontrol agent of *T. harzianum* (*Th*) at 4 ×10^6 were examined against *M. phaseolina* the causal agent of charcoal rot of sunflower. The results depended on estimation of diseases severity and microsclerotia density in the soil. Thus, the seeds immersion in CH 75 ppm for 6 h., gave the highest and considerable reduction (p=0.05) in disease severity by 48.25% and reduced microsclerotia survived in the soil up to 70%. Application of SA at 50 and 75 ppm proved an obvious reduction of charcoal rot severity by up to 39% and 37% for both concentrations, respectively and not varied with *Th*. The results also confirmed that CH at 75 ppm revealed significant reduction 40.63% in disease severity and similarized with SA at same concentration. However, the lowest dose of SA at 25 ppm realized the highest reduction of micro sclerotia density by 80.28 % compared to 74.91% when used CH at 75 ppm. For molecular identification of a pathogen Polymerase Chain Reaction (PCR) using ITS4 and ITS5 universal primers were applied to amplify and sequence of DNA for six isolates of *M. phaseolina* viz., OL901219, OL636051, OL901220, OL901204, OL636050 and OL636053 compared for identity of rDNA sequence according to NCBI GenBank databases by BLAST mode and the results showed the entire similarity ratio reached to 100%

Key words: chitosan, salicylic acid, *M. phaseolina*, *T. harzianum* , sunflower, PCR

مجلة العلوم الزراعية العراقية- 2024:55(1):569-578 سيدو وحسن

التشخيص الجزيئي للفطر *M. phaseolina* وإدارته باستخدام الكيماويات الزراعية و*T. harzianum*

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المستخلص

تم اختبار استحثاث المقاومة الجهازية المكتسبة (SAR) بواسطة كل من الكايتوسان (CH) وحامض الساليسيليك اسيد (SA) بالتراكيز (25 و 50 و 100ppm) ، اضافة إلى المقاوم الحيوي (*Th*) *T. harzianum* بتركيز 4× 10^6 كونيدة/ مل ضد التعفن الفحمي في زهرة الشمس المتسبب عن *M. phaseolina* . اعتمدت النتائج على تقدير شدة المرض وكثافة الاجسام الحجرية الدقيقة في التربة. وبذلك فان غمر البذور في CH بتركيز 75 ppm لفترة 6 ساعات اعطت أعلى اختزال لشدة المرض (p = 0.05) بنسبة 48.25٪ وكما واختزل كثافة الاجسام الحجرية الساكنة في التربة بنسبة اكثر من 70٪. اظهر استخدام SA بتركيز 50 و 75 ppm اختزالا واضحا في شدة التعفن الفحمي بنسبة اكثر من 39٪ و 37٪ وبكلا التركيزين على الترتيب دون اختلاف مع استخدام *Th*. أكدت النتائج ان استخدام CH بتركيز 75 ppm حققت اختزالا معنويا في شدة المرض بنسبة 40.63٪ ولم يختلف مع SA بنفس التركيز. عموما ، فإن أقل جرعة من SA عند 25 ppm اثبت أعلى انخفاض لكثافة الاجسام الحجرية وبنسبة 80.28٪ مقارنة بـ 74.91٪ عند استخدام CH بتركيز 75 ppm. تم تشخيص المسبب المرضي جزيئيا باستخدام تفاعل البوليميرات المتسلسلة (PCR) مع البادئات العالمية ITS4 و ITS5 لتوسيع وتحديد تسلسل DNA لستة عزلاتمن الفطر المدروس وهي OL901219, OL636051, OL901220, OL901204, OL636050 and OL636053 بالمقارنة مع تسلسل rDNA المشخص وفقا لقاعدة البيانات الدولية NCBI GenBank بواسطة BLAST mode ، أظهرت النتائج نسبة تشابه كاملة وصلت إلى 100٪ .

Received: 13/1/2022, Accepted:3/4/2022

INTRODUCTION

Charcoal root rot of sunflower caused by *Macrophomina phaseolina*, Ascomycete, Botryosphaeriaceae (20). This soil and seed borne fungus causes rot diseases on more than 500 plant species worldwide, particularly in the tropic reigns (63 , 27). The pathogen causes necrotic lesions on stems, branches, and peduncles (1), producing toxins such as phaseolinone and botryodiplodin that paving the infection (54, 14), and also affect plants by secreting a group of cell wall – degrading enzymes such as pectinase, cellulase , and proteinase (35). Furthermore, insufficient nutrients and water uptake by the host due to host tissue necrosis as well as fragility of root tissues (40). Foliar application or seed treatment of such agrochemicals as salicylic acid (SA) and chitosan (CH) increase resistance of plant against the diverse biotic and abiotic stresses (13). Furthermore, the effects of foliar spraying salicylic acid combined with chelated zinc on Halawani grapes were investigated in a study that revealed a rise in the leaf area and distance chlorophyll content in grape leaves (3). The foliar application of salicylic acid has been shown in several experiments to enhance vegetative characteristics (2, 5). El-Hai et al., (25) reported that SA and citric acid were used by seed soaking method and foliar spray to combat seedling against several *M. phaseolina* diseases on sunflower plant. Salicylic acid is a phenolic compound that works as a possible non-enzymatic antioxidant and contributes in the regulation of several physiological processes in plants, including stomatal closure, photosynthesis, and ion absorption, ethylene biosynthesis inhibition, transpiration, and stress tolerance (38, 6). The effect of spraying both salicylic acid and hydrogen peroxide on propagated via tissue culture date palm trees under salt stress conditions was challenged to estimate the gene expression of the superoxide dismutase enzyme (4). Shellfish byproducts are a common source of chitosan, a natural carbohydrate polymer comprised of randomly dispersed -(1 4) D-glucosamine and N-acetyl-D-glucosamine (10). It has sparked a lot of interest because of its potential for use in food and agriculture. Chitosan increases the activity of phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TYR), superoxide dismutase (SOD), catalase (CAT), and peroxidases (PODs), all of which are involved in defense mechanisms (16, 36). Chitosan also has the property of being an antibacterial agent (47) To comprehensively detect the pathogen, certain species-specific molecular diagnostic methods have recently been developed. Traditional polymerase chain reaction (PCR) markers based on the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region have been reported. by Babu et al. (8). Complex variation in fungus, soil colonization, and its survival of sclerotia makes its chemical control more complicate. Therefore, the maximum suitable technique to fight the pathogen is the usage of resistant varieties (37) and organic manage consisting of the usage of fungi consisting of *Trichoderma harzianum* (31). The resistance induction in plants plays a major role in suppression various pathogens. (42) , this depends greatly on such hormones as salicylic acid, the source responsible for activating the genes of systemic acquired resistance (SAR) and resistance genes (19, 43, 18). Furthermore, salicylic acid plays an important role in regulating most of biological activities such as growth, photosynthesis and regulation (52, 57, 56) and has a role in the cells permeability, ions translocation, and participates in stimulating certain changes in leaf anatomy and chloroplast structures. Therefore its signal transduction pathways stimulating defense against pathogens (32) , and it plays a role in stimulating systemic resistance by stimulating the production of pathogenesis related protein (PRP) (64, 22) and is involved in stimulating systemic acquired resistance (67). Treatment with medium doses of SA may be a promising way to increase the resistance - promoting flavonolignans in *Silybum marianum*, resulting in higher antioxidant and antimicrobial activity (51). The objective of recent study the role of such agrochemicals as salicylic acid, chitosan, and bicontrol agent of *T. harzianum* for induction SAR against charcoal root rot in the sunflower, and molecular characterization of a pathogen isolates.

MATERIALS AND METHODS

Isolation, preparation of *M. phaseolina* culture and plant inoculation: Diseased roots

washed thoroughly under tap water and small pieces of necrotic root surface disinfested in 2 % NaOCL for 2 min. under aseptic conditions and crown tissues were excised from roots and placed on potato dextrose agar (PDA) supplemented with chloramphenicol (250 mg/L) to avoid contamination according to (26, 59). Plates were incubated for 6–7 days at 28 ^{0}C ± 2^{0}C. Each isolate culture was purified and kept on PDA slants. A pure isolate (a3) of *M. phaseolina* was grown in flasks 250 ml contained substrate of millet seeds (*Pennisetum americanum*) as described by Edmunds (23). These seeds washed by distilled water, autoclaved and inoculated with five discs of the fungus before incubated at 28^{0}C ± 2^{0}C for 10 days, until production of extensive microsclerotial which observed over the surface of substrate. Four weeks after sowing seeds of sunflower, when plants reach to 20-25 cm height, the pots of each treatment were inoculated by mixing millet seeds with the surface of potting substrate at a rate of 5 gm per a pot. The results were recorded after 60 days including disease severity which estimated according to the scale consist of five scores and microsclerotia density in the soil. The later was assessed according to (50) for estimation the effects of agrochemicals and bioagent of *T. h.* on the microsclerotia density of a pathogen in the infested soil as follows: Ten gram was taken from infested soil surrounding roots at the end of the experiment after two months. This method is summarized by mixing infested soil in 100 ml of sterile distilled water, passing the mixture through two sieves of 175 and 38 mesh that placing them on top of each other and transferred to a beaker 250 ml containing 100 ml of 0.5% NaOCL for 5 minutes, then washed for one minute with sterile distilled water on a 38 mesh sieve to remove the remnants of sodium solution and then diluted to 1/10. One ml of diluted soil was transferred plates and sterile PDA was poured over it before incubated at 28 ^{o}C ± 2 °C for 48 hours. The sclerotia density for each treatment was estimated as colony forming unit (CFU).

Molecular identification

The growing mycelium was pelleted from liquid media and then frozen and stored at 20 ^{o}C. The protocol of Blood applied for DNA extraction, Tissue and Plant DNA kit AddPrep Genomic DNA Extraction Kit (Korea).The Nanodrop 2000-Spectrophotometer was used for measurement the purification and concentration of the extracted DNA. Depending on the optical density ratio at 260/280 nm DNA purity was assessed. Using universal primers, genomic DNA was employed as a template for PCR amplification of its stander for the ITS region of ITS5 and ITS4 (70). Amplified PCR products were checked by electrophoresis on an agarose gel (1%). After the agarose gel was polymerized and solidified, it had been transferred to the electrophoresis chamber. The later was done at 100V/ cm gel a voltage source (80V) for 45min. The sequencing was performed at Microgen Company (South Korea). The data for all trials analyzed using ANOVA and the difference between means was performed with DMRT at ≤0.05 using SPSS version 14.0 software.

RESULTS AND DISCUSSION

Effect of agrochemicals and *T. harzianum* on disease severity and microsclerotial density: Among agrochemicals of acetosalicylic acid (SA), chitosan (CH) and *T. harzianum* (*Th*) , the evident results exhibited that seeds immersion in CH 75 ppm for 6 hrs. gave the highest and remarkable reduction (p=0.05) in disease severity by 48.25% and reduced microsclerotia density survived in the soil up to 70%. The effect of CH may include its direct uptake, which could be utilized as nutrients for supporting plants health (49). Really, CH is a substantial source of N, Ca and other micro – nutrients such as Cu, Zn, and Fe (12, 55), in addition to the chelating properties of chitosan (39), it enhances the viability of rhizobacteria and fungi that promote plant growth (53, 29). Application of SA at 50 and 75 ppm and also resulted in obvious reduction of charcoal rot severity by up to 39 and 37% for both concentrations, respectively and no differs with *Th.* Literatures confirmed that high concentrations of SA, revealed a significant effect on reducing sclerotia density. The positive action of SA in stimulating (SAR) lies in the induction of genes encoding some pathogenesis related proteins (PRP) and the enzymes of chitinase and β – 1,3 glucanase (15, 21). In addition to the accumulation of

Hydrogen peroxide H_2O_2 and peroxidase that possess a great impact on analysis of fungal cell wall. No variance showed with CH 25 ppm and *Th* in disease severity when increasing seeds immersion period to 12hrs. though inequality results in survived inoculum of microsclerotia with 28.49%and 55.5% for both treatments, respectively. The lowest reduction in disease severity 20.53% observed when immersed seeds in SA at 25 ppm for 12 hours, whereas the lowest reduction in sclerotia density by 25.07 % shown when used CH 50 ppm for 12 hrs. Generally, *M. phaseolina* population density didn't coincide with the highest sunflower disease incidence, since the diversity of pathogen's inoculum of mycelium fructifications and pycnidia , in addition to microsclerotia. Colony-forming units (cfu) of *Macrophomina phaseolina* varied widely among soil samples collected during field investigations in different regions of India, while plant tissues carried more *M. phaseolina* cfu than soil samples (70). Therefore, the disease severity was affected by the infectious sclerosis population in the soil (37).

Table 1.% Reduction of micro sclerotia density and disease severity of charcoal rot on sunflower treated with agrochemicals and *T. harzianum*

Inducers	Duration (hrs)	Conc. (ppm)	% Reduction of disease severity *	% Reduction of microsclerotia density(cfu/g^{-1})× 10^{2}
Control	0	-	-	-
SA			21.15 ef **	81.24 ab
	6	25	(64.82) *	(3.67)
			21.28 ef	87.86 a
		50	(65.43)	(2.33)
			35.85 c	28.18 g
		75	(56.48)	(11.33)
			20.53 f	79.32 b
	12	25	(58.33)	(4.00)
			39.55 bc	59.28 d
		50	(48.52)	(7.33)
			37.65 bc	32.71 g
		75	(48.15)	(12.00)
CH			28.19 de	43.97 f
	6	25	(55.55)	(12.00)
			36.51 bc	49.71 ef
		50	(51.85)	(10.33)
			48.25 a	70.59 c
		75	(41.60)	(5.56)
			43.87 ab	28.49 g
	12	25	(47.42)	(20.00)
			32.32 cd	25.07 g
		50	(56.17)	(14.67)
			33.00 cd	79.23 b
		75	(50.93)	(3.67)
T. h.		4×10^{6}	34.79 cd	52.52 de
	6		(54.63)	(8.67)
			36.69 bc	55.52 de
	12		(61.11)	(9.33)

*Numbers between brackets constitute mathematical values computed using the equation of disease severity

** Means followed by the same letter (s) in each column are not significant different at ≤ 0.05

The results showed that seeds immersion in inducers solution of SA, CH and bio control agents of *Th*. revealed considerable reduction in disease severity (Fig. 1). The highest reduction 40.63% was resulted with CH at 75 ppm and no deference with SA application at the same concentration, in contrast, the lowest effect 20.84% observed in plants treated with SA at 25 ppm. This may be due to the impact of chitosan toxicity on pathogens fungi and bacteria similar as its antagonistic effect (24, 66) %, and this was more pronounced at high concentrations as well induction of resistance by one or more mechanisms (11).

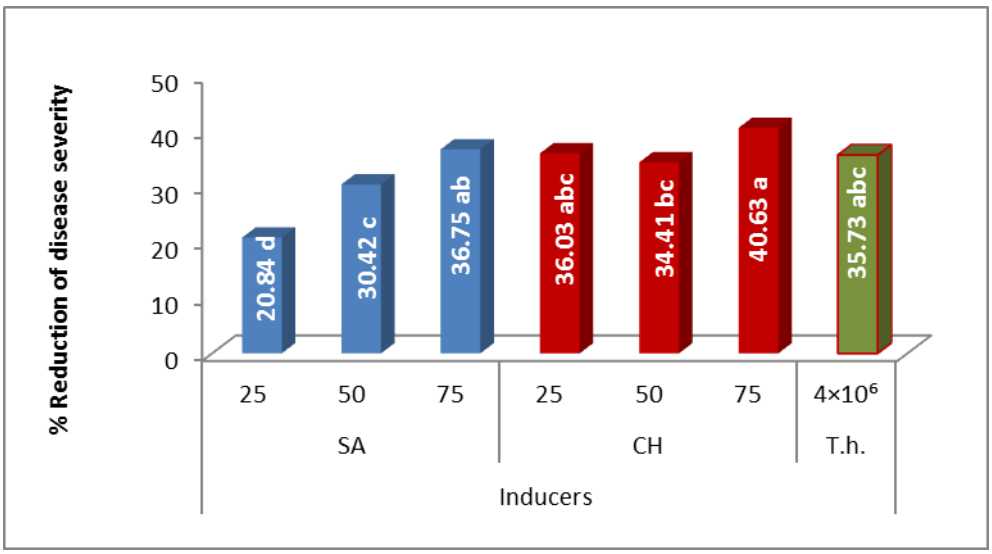

Figure 1. Effect of inducers concentration on the reduction of disease severity

Data in (Fig. 2) showed significant effects on a pathogen inoculum density when used inducers of SA at 25 ppm resulted in the highest reduction of micro sclerotia density by 80.28 %. In contrast, increasing CH dose to 75 ppm also lead to augmented reduction of survived inoculum by 74. 91%. The lowest reduction 30.44% was showed with SA at 75 ppm. In this aspect, toxicity of several soil fungi due to high dose of SA may limited their effectiveness against pathogens. Furthermore, SA may delayed or inhibited seed germination. However, the SA effects on plant growth depend on such factors as plant species, growth stage and SA concentration (60). Shakirova et al. (61) reported that the effects of SA on plant growth may be involved acceleration of photosynthesis, transpiration and stomata function that eventually reduces chlorophyll content (48) and distorted the leaf structure (68).

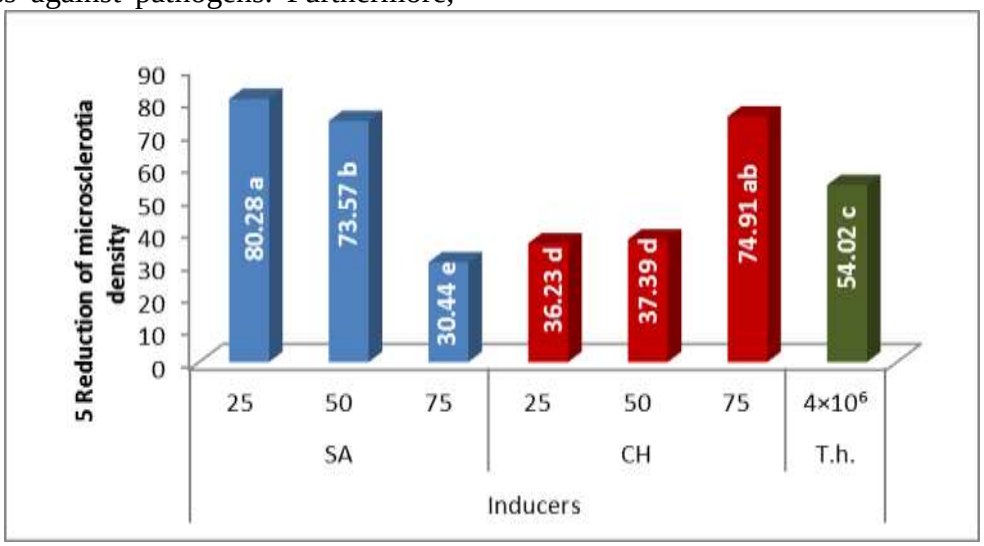

Figure 2. Effect of inducers concentration on the reduction of pathogenic microsclerotia density (cfu/ml) × 10^2

Sequencing and Phylogenetic Analysis

The length of the rDNA –ITS sequence of *M. phaseolina* isolates were 550-700 bp of ITS4-ITS5. A BLAST comparison showed the identify of rDNA sequence of the fungus *M. phaseolina* from NCBI (GenBank). Phylogenetic analysis, showed that the obtained sequences share 100% similarity to *M. phaseolina* strain of Mexico isolate (MT605403.1), India isolate (MT186840.1), Iran isolate (MZ31213.1), and South Korean isolate (OL455718.1) (Fig.3). The results showed that *M. phaseolina* was not restricted to a particular geographic area or host, except for some observations that the fungal strain showed host specificity, as suggested by (34, 9, 58, 61). Several literatures on the genetic, geodiversity, and variations of a pathogen

from Mexico and other countries have revealed distinct differences (69, 65, 44). Jana et al. (33) used a single RAPD primer to produce a taxonomic identifier for population research that identifies *M. phaseolina* isolates from soybean, sesame, ground nut, chickpea, cotton, common bean, and 13 additional hosts**.** *M. phaseolina* discovered from sunflower plants grown in different regions of Turkey's Adana province, a universal primers ITS4 and ITS5, DNA from *M. phaseolina* was amplified and sequenced, and the findings were compared to the identity of *M. phaseolina* rDNA sequences from NCBI GenBank databases using BLAST mode, with a similarity ratio of 100 percent (29).

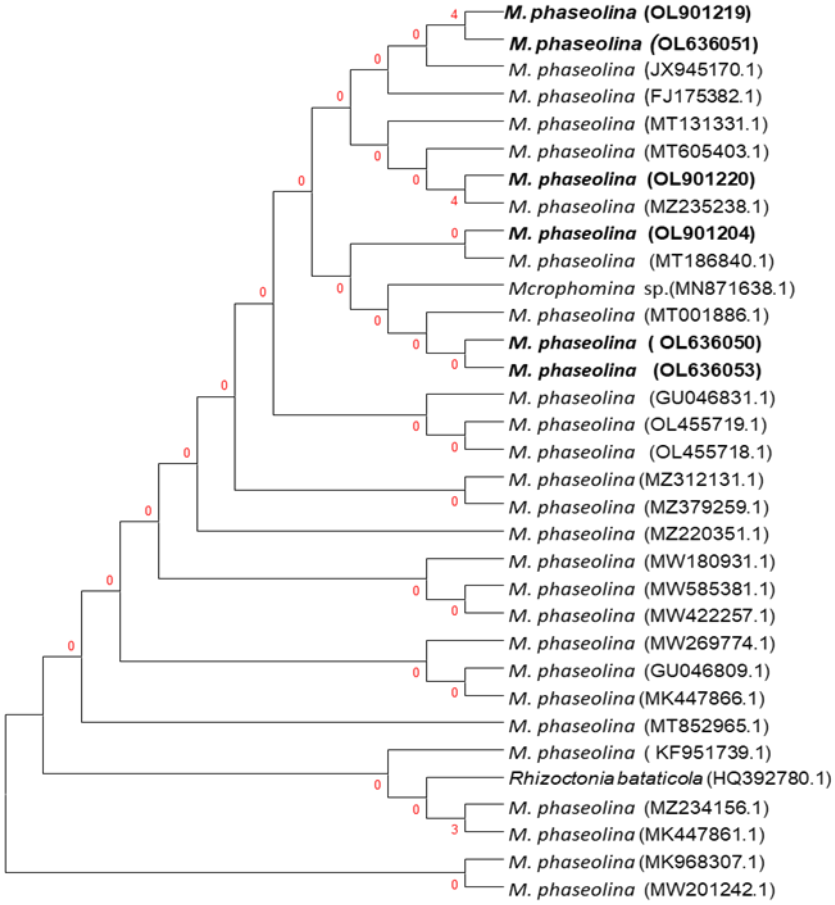

Figure 3. Phylogenetic tree of *M. phaeolina* based on neighbor-joining method – 1000 bootstrap replicas (bold) of ITS-rDNA sequence of Iraq strain and binding analysis with related Macrophomina species from GenBank. The accession number is displayed next to the species name

We conclude that *M. phaseolina* is a major pathogen of sunflower charcoal rot. Application of agrochemicals of chitosan(CH) and salicylic acid (SA) and *T. harzianum* exhibited obvious reduction in disease severity and inoculum density of microsclerotia (up to 70%) when applied CH at 75ppm followed by SA at 50 ppm and 75 ppm or bio agent of *Th* . The phylogenetic analysis of virulent isolates (OL901219, OL636051, OL901220, OL901204, OL636050 and OL636053) based on ITS4 and ITS5 showed high identify 100% with the sequence of *M. phaseolina* from the NCBI.

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