Methyl Jasmonate Improves Superoxide Dismutase Activity in Infected Sunflower Plants

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Abstract. Methyl jasmonate (Meja) is a volatile phythormone that contributes many plant critical processes, especially under biotic and abiotic stresses. Thus, Meja found to effectively regulating the biosynthesis of other plant hormones and/or enzymes, like Superoxide Dismutase (SOD). However, Meja specifically affects the plant antioxidant defense system, particularly SOD activity are still an area of ongoing research. The current results pointed to a clear effect of the applied Meja concentration in shaping the entire response to the biotic oxidative stress resulted from the three pathogenic fungi *Fusarium solani.* and *Macrophomina phaseolina.* The higher concentration of Meja had a greater effect on both gene expression and SOD enzymatic activity, which indicates the Meja importance in stimulating the defense system of sunflower varieties through which sunflower plants deal with the excessive production of reactive oxygen species (ROS) in response to the pathogenic infection. The different varieties showed different ability to cope with biotic stress represented by the three used pathogenic fungi, especially the antioxidants enzymatic activity. Sakha variety proved to have the most effective SOD enzymatic activity compared to the other two Ishaqi and Aqmar sunflower varieties. Meja concentrations in its maximum level (3 μM), was more effective in stimulating the antioxidant activity in term of enzymatic activity of SOD. Notably, *R. solani* had a greater effect on the antioxidant defense system, as it resulted in higher levels of SOD enzymatic activity in the three sunflower varieties. Further investigation maybe required to better understand the Meja ability to promote the plant antioxidant system, thus the possible protective mechanisms against plant pathogens.

1. Introduction

Sunflower (*Helianthus annuus* L.) is the most important oil crop belonging to Asteraceae family. The sunflower crop contains a variety of nutrients and is used in various industries such as the production of oils, livestock and poultry feed, and cosmetics. However, due to its wide range of environmental requirements, sunflower crop is exposed to many devastating fungal diseases [1].

The latest statistics showed that what was cultivated of this crop in Iraq, during the autumn season is amounted to 350 hectare, with a total seed yield up to 816 tons with an average of 2.3 ton h^{-1} [2].

Sunflower produces a large amount of oil per unit area, because its seeds contain a high percentage of oil that reaches 50%. However, the crop productivity has decreased significantly in recent years due to continue development of plant pathogens, which led to critical fluctuations in crop productivity [3]. Charcoal rot disease, caused by the *Macrophomina phaseolina*, is described as one of the most important determinants of sunflower crops and results in significant yield losses [4-5,16]. Also, root rot and seedling damping-off disease caused by *Rhizoctonia* is one of the most common soil- and seed-borne plant pathogenic fungi with a wide family range leading to high yield losses [1,17].

Fusarium wilt disease caused by *Fusarium spp*. is a soil-borne pathogen that tolerates unfavorable environmental conditions and infects plants at all growth stages. Fusarium wilt is one of the most widespread and important diseases that cause wilting, rotting and seedlings damping-off in several economically important plant species such as sunflower [6, 18-19].

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The emerging era of biotechnology and bioinformatics has significantly improved genetics approaches via which time and effort required for a certain result has been saved. The ITS technique (Internal transcribed spacer) is one of the most important DNA barcoding techniques that rely on analyzing a conservative domain in fungi genome to detect genetic variation at the level of single nucleotide polymorphism [7].
Methyl jasmonate (Meja) is a volatile phythormone that contributes many plant critical processes, acting as a signal in response to both biotic and abiotic stresses and modulating the biosynthesis of other plant hormones [8]. In normal conditions, Meja participates effectively in stimulating plant defense in response to both biotic and abiotic stress. The current study was designed to investigate the possible role of genetic background of sunflower varieties, Methyl jasmonate (Meja) concentrations and pathogenic fungi in stimulating SOD activity.

2. Materials and Methods

2.1. Field survey

Sunflower plants showing symptoms of wilting, yellowing, leaf drooping, and stem base coloration were separately collected from the regions of Baghdad and Anbar provinces during April, May and June of 2020. Plant samples were kept in polyethylene bags, marked with an area and date of collection and of collection for further use.

2.2. Isolation of fungi

Samples of sunflower plants that showed symptoms of wilting and yellowing were brought in polyethylene bags to the laboratory. Stems bases and roots were washed with tap water to remove stacked dirt and mud. The plant parts were cut into small pieces (0.5-1 cm) and sterilized with sodium hypochlorite NaOCl (5%) for three minutes. Sterile filtration to dry and grow on PDA medium (Potato Dextrose Agar) fortified with antibiotic (Amoxiline), autoclaved under a temperature of 121°C and a pressure of 1.5 bar. PDA plates of 9 cm containing five plant pieces were incubated at a temperature of 25 ± 2 °C for 5 days for diagnostic purposes.

2.3. Morphologic characterization of fungi

Based on their morphological characteristics, the isolated fungi were identified which were *M. phaseolina* according to Veverka [9], *R. solani* according to Parmeter and Whitney [10], *F. solani* according to McGovern [11] . The isolates were kept in test tubes containing sterile PDA supplied with antibiotic. Test tubes were placed until solidification, and the tubes were inoculated at a temperature of 25±2 for 5 days, then it was later kept in the refrigerator at a temperature of 4 °C until it is used in subsequent experiments.

2.3. Molecular characterization of fungi

2.3.1. Extraction of total genomic DNA

Molecular characterization work was conducted in Wahajd Al-Dana Center (Baghdad, Iraq). The DNA extraction was done from 50 mg fungal hypha of each isolates with the aid of ready kit Fungal/Bacterial/Yeast DNA MiniPrep™ (Irvine, CA 92614, USA). The manufacturer's instructions were followed literally. The DNA purity was investigated using the following formula: *DNA purity ratio= O.D.260/O.D.280*
DNA barcoding was done based on targeting the Internal Transcribed Spacer (ITS) with the aid of two specific primers, ITS1 F:5′- TCCGTAGGTGAACCTGCGG-3′ as forward and ITS4 R:5′-TCCTCCGCTTATTGATATGC-3′ as a reverse supplied by Integrated DNA Technologies company (Canada) Final volume of PCR reaction was of 25 µl containing 1.5 µl of DNA, 5 µl of Taq PCR PreMix, and 1 µl of each primer (10 picmol) then volume was completed with deionized distil water (DDW) to 25 µl. The following PCR programme was applied: Initial denaturation at 94 °C for 3 min., followed by 35 cycles of denaturation at 94 °C for 45s, annealing at 52°C for 1 min. and extension at 72 °C for 1 min. Final extension was at 72 °C for 7 min. using a thermal cycler (Geneamp, PCR system 9700; Applied Biosystem, Carlsbad, CA, USA). Agarose gel (1.5%) has been used to separate the amplified products, and ultraviolet light (302 nm) was used for visualization after electrophoresis and red staining.

2.3.2. Sequencing protocol

Vogelstein and Gillespie [12] procedure was adopted as sequencing protocol, where the gel extracted DNA was sequenced following Sanger sequencing procedure in the National Instrumentation Center for Environmental Management- NICEM (http://nicem.snu.ac.kr/main/?en_skin=index.html), Biotechnology lab using 3730XL Genetic Analyzer (Applied Biosystems, USA). Using Basic Local Alignment Search Tool (BLAST) software, homology test was conducted at the National Center Biotechnology Information (NCBI), (http:// www.ncbi.nlm.nih.gov) and BioEdit and MEGA6 softwares [13].

2.5. Pots experiment

The effect of three Meja concentrations (1, 2, and 3 µM) plus control in modulating SOD enzymatic activity in three sunflowers varieties namely Ishaqi, Aqmar, and Sakha was investigated in response to three pathogenic fungi. The Meja solution was prepared primary by dissolving 1 ml Meja in 2 ml ethanol then volume was completed to 500 ml by adding distilled water to prepare stock solutions. Dilutions were made to achieve 1, 2 and 3 µM plus control (ethanol+ distilled water). Seeds of the three sunflower varieties were soaked in Meja solution for 8 hours [14], and then seeds were washed in several change of sterile distilled water then left to complete dry on sterile filter paper for 2 hours. A total of 135 plastic pots were filled with aseptic soil-peat moss mixture (2:1) and treatments were distributed randomly. Previously sterilized pots were inoculated with the pathogenic fungi and three days later 5 seeds were sown in each treatment.

2.5. Analysis of Superoxide Dismutase activity (SOD)

Fresh leaves of 30 days Sunflower seedlings were collected in polyethylene bags and kept in icebox, then directly transferred to laboratory to spectrophotometrically assess (UV light with a wavelength of 240 nm.) Superoxide Dismutase (SOD) activity. SOD was extracted at 4°C using the relevant buffer and activity was analyzed at 30°C as described by Shannon et al. [15].

3. Results and Discussion

3.1. Phenotypic and molecular characterization of pathogenic fungi

According to the phenotypic characteristics the accompanying fungi are characterized to be *Rhizoctonia solani* (Figure 1, A and B), *Fusarium solani* (Figure 1, C and D) and *Macrophomina phaseolina* (Figure 1, E and F). Molecular size of the amplified ITS domain in the genomic fungi was about 550 bp for three characterized fungi, *R. solani F. solani* and *M. phaseolina* (Figure 1, G). The amplified sequences of ITS were registered in the National Center Biotechnology Information (NCBI), (http:// www.ncbi.nlm.nih.gov) with the accession numbers LC756195 for *R. solani* and LC756196 for *M. phaseolina*.

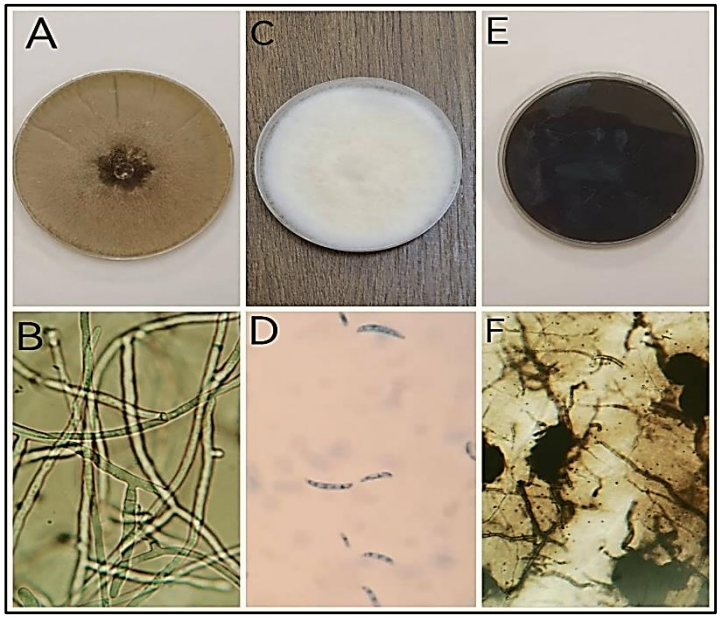

Fig. 1. Phenotypic characterization of the three pathogenic fungi *Rhizoctonia solani* (A and B) and *Fusarium solani* (C and D) and *Macrophomina phaseolina* (E and F).

3.1. Superoxide Dismutase activity, SOD (unit ml^{-1})

The results of Superoxide Dismutase activity SOD (Table 1, Figure 2) in sunflower varieties was affected by the different concentrations of methyl jasmonate (Meja) and the used three pathogenic fungi. Sakha variety (V3) showed the highest average activity of SOD up to 153.1 units ml^{-1}, followed by Aqmar variety (V2) that achieved an average activity of Superoxide Dismutase amounted to 129.8 units ml^{-1}. However, Ishaqi variety (V1) came last when it gave the lowest value for Superoxide Dismutase activity amounted to 103.9 units ml^{-1}. Notably, SOD activity varied according to the difference concentration of methyl jasmonate, as the maximum concentration of 3

µM revealed the highest activity of the investigated enzyme reached 169.5 units ml^{-1}, while the minimum concentration of 1 µM reflected a lower value for the enzymatic activity (112.6 units ml^{-1}) in contrast to the higher concentration of 2 µM which gave a value as low as 88.6 units ml^{-1}.

The role of pathogenic fungi was so clear in determining the efficiency of host plant's defense response, in which, *R. solani* was the most effective in stimulating the oxidative defense response when it had the highest average of Superoxide Dismutase activity of 177.5 units ml^{-1}, while the effect of *M. phasiolina* drooped to 131.1 units ml^{-1}, contrarily the effect of *F. solani*, which caused a decrease in the activity of the mentioned enzyme was greatly reduced by 94.0 units ml^{-1}.

The results in Table 1 and Figure 2 showed a different Superoxide Dismutase activity in response to the difference genetic background of sunflower varieties, and the used three concentrations of methyl jasmonate and plant infection with the pathogenic fungi. Aqmar variety (V2) was distinguished by the highest content of Superoxide Dismutase enzyme, up to 97.0 units ml^{-1}, meanwhile the enzymatic activity reached 74.5 units ml^{-1} for Ishaqi variety (V1).

Table 1. Superoxide Dismutase (SOD) activity (units ml^{-1}) in sunflower varieties treated with different methyle jasmonate (Meja) concentrations in response to *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani* .

No.	Treatments	SOD activity	No.	Treatments	SOD activity
1	V1	74.5	25	V2C2	85.8
2	V1M	241.7	26	V2C2M	55.2
3	V1Rh	228.9	27	V2C2Rh	29.4
4	V1F	68.1	28	V2C2F	204.8
5	V1C1	34.3	29	V2C3	346.2
6	V1C1M	108.3	30	V2C3M	318.9
7	V1C1Rh	44	31	V2C3Rh	23.1
8	V1C1F	79.4	32	V2C3F	60.1
9	V1C2	32.7	33	V3	27.9
10	V1C2M	13.4	34	V3M	376.8
11	V1C2Rh	26.3	35	V3Rh	270.7
12	V1C2F	146.9	36	V3F	16.7
13	V1C3	132.4	37	V3C1	235.3
14	V1C3M	18.3	38	V3C1M	19.9
15	V1C3Rh	359.1	39	V3C1Rh	243.3
16	V1C3F	53.6	40	V3C1F	68.1
17	V2	97	41	V3C2	196.7
18	V2M	42.4	42	V3C2M	10.2
19	V2Rh	224	43	V3C2Rh	220.8
20	V2F	71.3	44	V3C2F	40.8
21	V2C1	42.4	45	V3C3	52
22	V2C1M	97	46	V3C3M	270.7
23	V2C1Rh	365.5	47	V3C3Rh	95.4
24	V2C1F	13.4	48	V3C3F	304.4
		Means			
Varieties	**V1**	103.9	**V2**	129.8	**V3** 153.1
Methyl jasmonate concentrations	**C1**	112.6	**C2**	88.6	**C3** 169.5
Pathogenic fungi	**M**	131.1	**Rh**	177.5	**F** 94

Note: V1= Ishaq, V2= Aqmar, V3= Sakha. C1=1 µM, C2=2 µM, C3=3 µM. M= *M. phaseolina*, Rh= *R. solani*, F= *F. solani*

The different genotypic varieties of sunflower was clear in determining the response pattern to infection with the *M. phasiolina* pathogenic fungus through the variation in Superoxide Dismutase activity, which boosted in Sakha (V3M) and Ishaqi varieties (V1M) to reach 376.8 and 241.7 units ml^{-1}, respectively. Interestingly, *R. solani* effect was close to the enzymatic activity according to the different varieties used, as it reached 270.7, 228.9 and 224 units ml^{-1} for Sakha (V3), Ishaqi (V1) and Aqmar (V2) varieties, respectively. The enzymatic activity was generally low in different varieties scoring 71.3 and 68.1 units ml^{-1} for the two varieties Aqmar (V2) and Ishaqi (V1) respectively, whereas it was very low in the Sakha variety (V3) recording only 16.7 units ml^{-1}.

Methyl jasmonate had a clear effect in modulating oxidative enzymatic activity in sunflower varieties infected by soilborn pathogenic fungi (Table 1, Figure 1). Immersing sunflower seeds with the lowest concentration of methyl jasmonate (1 µM) increased the Superoxide Dismutase enzymatic activity in response to *R. solani* pathogenic fungus reaching 365.5 units ml^{-1} in Aqmar variety (V2C1Rh). On the other hand, *F. solani* induced an enzymatic activity of

13.4 units ml^{-1} for the same variety at a methyl jasmonate concentration of 1 µM. For Ishaqi variety (V1), the enzymatic activity was 108.3 units ml^{-1} in the presence of *M. phasiolina*, while the other two fungi *F. solani* and *R. solani* resulted in SOD activity of 79.4 and 44.0 units ml^{-1}, respectively. Considering Sakha variety (V3), its treatment with a concentration of 1 µM resulted in varied enzymatic activity in response to the different pathogenic fungi. The Superoxide Dismutase activity decreased in the same variety to be 243.3, 62.7 and 19.9 units ml^{-1} for fungi *R. solani*, *F. solani* and *M. phasiolina*, respectively.

Results presented in Table 1 and Figure 2 indicated a slight decrease in the oxidative stress in Ishaqi variety (V1), due to the increased concentration of methyl jasmonate up to 2 µM through a decreased Superoxide Dismutase activity that recorded 10.2 and 40.8 units ml^{-1} in the presence of the two fungi *M. phasiolina* and *F. solani*, respectively. However, the presence of *R. solani* boosted the targeted enzymatic activity up to 220.8 units ml^{-1}, corresponding to a similar performance in the Ishaqi variety (V1). The enzymatic activity was 13.4 and 26.3 in the presence of the fungi *M. phasiolina* and *R. solani*, respectively. The presence of *F. solani* pathogen boosted the enzymatic activity to 146.9 units ml^{-1} in the same variety. For Aqmar variety (V2), Superoxide Dismutase activity in the presence *of R. solani*, *M. phasiolina* and *F. solani* was 29.4, 55.2 and 204.8 units ml^{-1}, respectively.

The increased concentration of methyl jasmonate soaking solution to 3 µM, a clear effect was detected in varying Superoxide Dismutase activity, and thus increased efficiency of plant defense system against high levels of oxidative stress caused by pathogenic fungi. Sakha (V3) plants recorded high values of the enzyme activity of 304.4 and 265.3 units ml^{-1} after infection with *F. solani* and *M. phasiolina*, respectively. While it reached 90.0 units ml^{-1} in the presence of *R. solani*, however, Isaqi variety (V1) showed a high value of SOD activity in the presence of *R. solani* amounted to 359.1 units ml^{-1}. On the other hand, the other two fungi *F. solani* and *M. phasiolina*, the enzymatic activity reached 53.6 and 18.3 units ml^{-1}, but Aqmar variety (V2) showed enzymatic activity in the presence of *M. phasiolina* reached 318.9 units ml^{-1}, while it was 60.1 and 23.1 units ml^{-1} for *F. solani* and *R. solani*, respectively (Table 1, Figure 2).

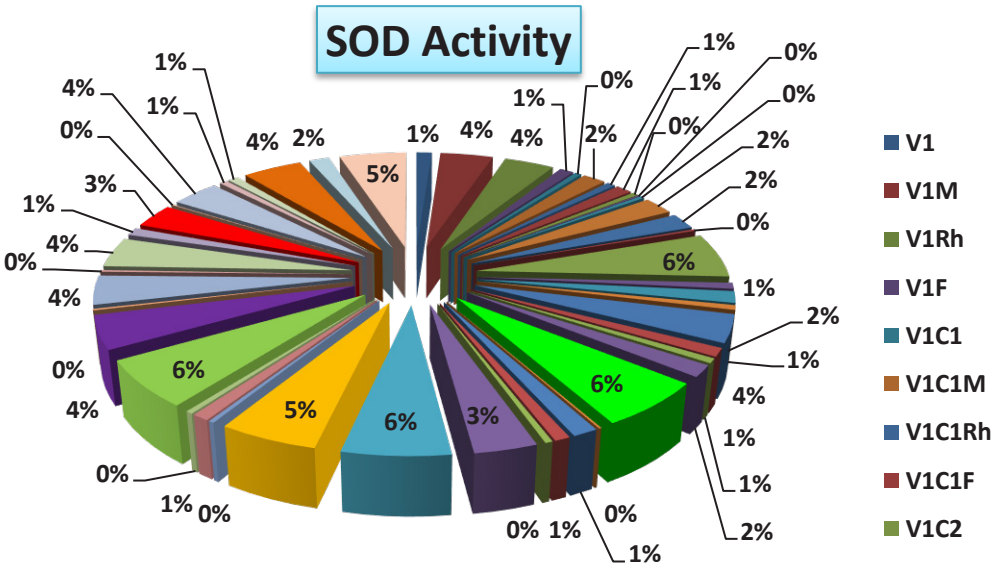

Figure 2. Superoxide Dismutase (SOD) activity in sunflower varieties treated with different methyle jasmonate (Meja) concentrations in response to *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium solani.*
Note: V1= Ishaq, V2= Aqmar, V3= Sakha. C1=1 µM, C2=2 µM, C3=3 µM. M= *M. phaseolina*, Rh= *R. solani*, F= *F. solani*

4. Conclusions

It seems clear that the three study factors, sunflower varieties, methyl jasmonate concentrations, as well as type of pathogenic fungi, had a very different effect of their applied levels on the Superoxide Dismutase activity. In general, Sakha variety (V3) showed the highest response in many treatments through high enzymatic activity values, followed by Aqmar (V2) and finally Ishaq (V1). The same applies to the concentrations of methyl jasmonates (Meja), of which the highest concentration (C3) showed the highest average induction of enzyme activity.

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