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Impact of plant growth promoting fungi on biochemical defense performance of Tomato under Fusarial infection

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Abstract

In the present study, ameliorative abilities of plant growth promoting fungi (PGPF) under fusarium infection have been examined through stimulation of biochemical defense and Physio-biochemical performance in tomato plants. A total of 25 fungal isolates were isolated from the *Beta vulgaris* Rhizosphere cultivated soil (Tamiya, Fayoum Governorate, Egypt). These fungal isolates have been characterized in terms of the production of certain plant growth promotion active metabolites that enhance plant growth and suppress diseases. Four fungal isolates were selected as the most for plant growth promotion. The four fungal isolates were identified morphologically as *Aspergillus niger, A. flavus, Mucor sp.,* and *Penicillium sp.* Under greenhouse conditions, tomato plant treated with these fungi separately showed significant reduction in wilt disease. Biochemical defense such as osmolytes, oxidative stress, and antioxidant enzymes activity were assessed at 60 days after planting. The results revealed that *Fusarium oxysporum* strain was highly destructive effect on tomato plants by PDI 87.5 %. Moreover, PGPF filtrates which applied to infected tomato improved osmolytes, total phenol and ascorbic acid. Interestingly, the deleterious impact of wilt disease on tomato plants were significantly reduced and it can be evident from reduced MDA and H_2O_2 levels. Therefore, these results highlighted that the soil harbors antagonistic fungi offering several plant growth-promoting fungi (PGPF), which can be exploited as a powerful biological control agent in tomato plant against *Fusarium* wilt.

Keywords**:** *Plant growth promoting fungi; Fusarium* oxysporum*; Biotic stress, Biochemical defense.*

Introduction

Under the threat of climate change and the spread of pathogens, improving crop productivity and avoiding the use of chemical pesticides is a major issue for the agricultural industry [1] . Fungal diseases are among the most dangerous biological stresses that cause severe damage to agricultural crops in many countries [2]. One of the most famous pathogens of fungal diseases, *Fusarium oxysporum*, causes a negative impact on crops, especially vegetable crops [3-5]. However, fungal wilt disease caused by *F. oxysporum* effecting severe injury through all phases of tomato growth [6,7]. Tomato is considered one of the most vital crops in Egypt for the local feeding and exportation[8]. Considering the importance of the tomato crop, the development of new management methods to improve resistance to biological stresses such as fungal may help in enhancing global food production that is safe and free of harmful chemical pesticides [9]. It is agreed that plant immunity against pathogen infection within plants can be activated by external spraying of biotic and abiotic stimuli or inducers. Biostimulants include non-pathogenic organisms or their produced fragments. Abiotic stimuli are chemicals that mimic a component of plant defense signaling pathways [10]. The use of the antimicrobial properties of microorganisms in the biological control of many plant diseases has been the main interest of several studies [11-13]. Bio fungicides are fungicides that can be produced by living organisms as microorganisms that are inhibit fungal pathogens but less toxic on human and ecofriendly agents that can be used efficiently in agricultural pest control [14-17]. The use of growth-stimulating microorganisms is a common strategy for researchers to enhance and improve the defense capacity and physiological immunity of plants as well as the bioavailability of minerals in the soil [18,19]. Growth-promoting microorganisms have been used as bio-nutrients to effectively increase the therapeutic nutrition content in crops [20,21]. Environmentally friendly biological agents can be used to improve the properties of the soil and secrete enzymes that facilitate the uptake of nutrients by the plant and at the same time do not lead to environmental pollution

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to achieve higher efficiency and produce more and higher value crops [22,19,23]. Stimulated resistance is a physiological state of protection potency produce by specific eco-friendly stimuli that act essential role against a broad range of plant pathogens including fungi [24]. Plant growing can be simply - stimulated by fungi through several mechanisms, such as systemic resistance's stimulation, plant nutrition enhancements, and via their toxicity to various pathogens [25,26]. Plant growth promoting Fungi produce chemical compounds with different benefits for the plant as HCN that recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens[27,28]. HCN is a broad-spectrum antifungal compound against fungal pathogens has been demonstrated in several studies [29,30,27,28]. HCN works direct on the cells of the pathogen by obstructive the cytochrome oxidase of the respiratory chain. Plant growth promoting Fungi produce were able to produce IAA [31]. IAA works a vital role in the improvement of plants by stimulating their growth when applied directly to the roots [83].Plant growth promoting Fungi were able to solubilize organic phosphates that play a role in enhancement plant health [32,33]. Herein, we are investigating the capabilities of these antagonists PGPF to produce plant growth promotion products that enhance plant growth and suppress diseases on the growing of tomato diseased with *F. oxysporum.* The novelty of this study is the use of non-pathogenic fungi isolated from Rhizosphere that have the ability to inhibit the harmful effect of Fusarium through improve plant resistance to biological stresses and supports immune responses. Our study opens the approach to an alternative and safety technique to plant growth promotion as well as increase immunity of tomato to resist the *Fusarium* wilt disease. Also, this study opens the way to an alternative and safety method to manage the wilt disease in tomato. We think this work is of great value and importance to many integrated management programs.

2.Materials and Methods

2.1. Isolation and identification of PGPF

Rhizosphere *Beta vulgaris* cultivated soil (29º 24' 06.5" N 30º 58' 10.4" E) (Tamiya, Fayoum Governorate, Egypt) was collected. Then, 10 g of soil was dissolved in 90 ml sterile distilled water. Serial dilution technique was performed from 10^{-2} to 10^{-6}. Aliquots of 0.1 ml were spread on sterile petri plates containing sterilized Potato Dextrose Agar (PDA) medium (Sigma Aldrich, Germany) amended with chloramphenicol (200 µg/L), then petri plates were incubated for 3-7 days at 30°C [34,35]. Fungal isolates were identified depending on their morphological characteristics according to [36-38]. Macroscopic morphological features including color, texture, diameter of colonies and microscopic characteristics including vegetative and reproductive structures of the fungi were noted.

2.2. Biochemical traits of fungal isolates

2.2.1. Production of hydrocyanic acid (HCN)

The ability of the tested isolates to produce hydrocyanic acid (HCN) was performed according to the protocol described by[39]. with slight modifications. Fungal isolates were inoculated on MEA medium supplemented with 4.4 g/l of glycine. Whatman paper discs were soaked in picrate carbonate solution (2.5% picric acid and 12.5% anhydrous sodium carbonate (Na_2CO_3). These discs were placed at the top of each Petri dish. Plates were sealed with parafilm, inverted, and incubated for 4 days at 27°C. A Petri dish inoculated only with SDW served as a negative control. HCN production is reflected in the color of 8 the Whatman paper (the development of yellow-to-yellow orange to brown or brown color).

2.2.2. Phosphate solubilization

The ability of the isolates to solubilize phosphate was tested on Pikovskaya (PVK) medium containing 0.5% tricalcium phosphate (Ca3 (PO4)2) as an insoluble phosphate source[40]. A volume of 10 µl of fungal suspension from a fresh culture of each fungal isolate was placed on the surface of the solid PVK medium. The dishes are incubated at 30°C for 7 days, the fungi having the capacity to solubilize the phosphates were surrounded by a clear halo.

2.2.3. Siderophores production

The isolated fungi were grown for 14 days on PDA medium at 28º C [41] . The culture supernatant of fungi was further subjected to Chrome Azurol Sulfonate liquid assay, for quantitative assessment of siderophore production. After CAS solution preparation, an equal volume was added to the culture supernatant; the positive result is the change of the blue color to orange or dark purplish red.

2.2.4. Production of Indole acetic acid

The ability of fungal isolates to produce Indole Acetic Acid (IAA) was determined by the colorimetric technique. Fifty milliliter of Potato dextrose broth (PDB) containing 0.1% tryptophan was inoculated with 500 µl of old fungal cultures and incubated in refrigerated incubator Shaker at 30±0.1 °C and 180 rpm for 3-5 days in dark. The fungal cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. [42]. Estimation of indole-3-acetic acid (IAA) in the supernatants was done using colorimetric assay. The development of a pinkish-red color indicates the production of IAA.

2.3. Source of pathogen

F. oxysporum f. sp. Lycopersici RCMB008001 was obtained from Regional Center for mycology at Al-Azhar University. then was confirmed by

pathogenicity test according to Hibar et al. [31], the pathogenicity test and inoculum were achieved according to [Aldinary et al. [11]]

2.4. Tomato Plant

Well recognized four weeks old -Tomato seedlings (*Solanum Lycopersicon* L. cv. Castlerock II PVP) were obtained from agricultural research center (ARC), ministry of agriculture, Giza, Egypt.

2.5. In vivo study

The ability of fungal isolates to stimulate biochemical defense performance of Tomato plants and to control wilt disease caused by *F. oxysporum* was assessed under greenhouse conditions using plastic pots (25 × 20 cm) Applied PGPF were applied one week before infection with *F. oxysporum*, then The pathogen was inoculated into cultivation soil .The pot experiments were achieved at the experimental farm station of Botany and Microbiology Department, Faculty of Science, Al-Azhar University. Seedlings were planted in 6 groups as following: (T1) plants without any treatment (healthy control), (T2) plants infected with *Fusarium oxysporum* (infected control), (T3) infected plants treated with *Mucor sp.*, (T4) infected plants treated with *Pencillium sp.*, (T5) infected plants treated with *A. niger*, (T6) infected plants treated with *A. flavus*. Disease development and severity were recorded 15 days post inoculation. The biochemical indicators for resistance assayed after 60 days of inoculation.

2.5.1. Disease symptoms and disease index

The disease symptoms were observed and disease severity and percentage the protection of PGPF were estimated by the equation: Protection % = A–B/A × 100% Where, A = PDI in infected control plants B = PDI in infected- treated plants according to Farrag et al. [7]

2.5.2 Biochemical defense indicators

Biochemical defense indicators were investigated as the following, the Contents of total soluble carbohydrates in dry leaves were determined using anthrone technique according to Irigoyen et al. [43]. Also, the content of total protein was measured in the dry leaves according to Vernon, Seely [44], and the free proline content in fresh leaves by method described by Bates et al. [45]. The phenol content of the dry leaves was also estimated according to Diaz, Martin [46]. The technique of Jagota, Dani [47] was used to estimate the ascorbic acid content of the fresh shoot. The content of Malondialdehyde (MDA) content in fresh tomato leaf was measured was measured using the thiobarbituric acid (TBA) method according to with slightly modification according to Hu et al. [48] . Hydrogen peroxide(H_2O_2) levels were determined as stated by Mukherjee, Choudhuri [49]. . The leaf was homogenized in 2 mL 0.1% trichloroacetic acid (TCA) solution. After centrifugation at 12,000×g for 15 min, 0.5 mL of the supernatant was added to the reaction mixture containing 0.5 mL 10mM K phosphate buffer (pH 7.0) and 1 ml of 1M KI. Absorbance was determined at 390 nm.

2.6. Statistical analyses

Valuation of pilot data was accomplished by using one-way analysis of variance (ANOVA). While, means differences by Duncan's multiple range test and the (L.S.D) at 5.0 % level of probability using Co State software [50,51].

3. Results and discussion

3.1. Isolation and screening of fungi according to HCN, IAA , Siderophores production and Phosphate solubilization

Twenty five fungal isolates (F1-F25) were isolated from rhizosphere *Beta vulgaris* cultivated soil; all fungal isolates were screened according to their potency to produce plant promoting substances. Plant growth promoting fungi produce chemical compounds with different benefits for the plant. Among them, HCN is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens [27,28]. This approach, seen as a biologically friendly alternative to chemicals, has established particular attention in recent years. Numerous fungi with a biological control effect are presently marketed in numerous countries[52]. Four fungal strains (*A. flavus* and *Pencillium sp.*, *Mucor sp.* and *A. niger*) were able to produce HCN (Table 1). *Mucor sp.* recorded the highest values, followed by *A. flavus*, *then Pencillium sp.* and *A. niger*. HCN is a broad-spectrum antifungal compound play a vital role in the biocontrol of fungal disease as has been demonstrated in several studies[29,30,27,28]. In this setting, [53] proposed that HCN compound works on the cells of the pathogen by obstructive the cytochrome oxidase of the respiratory chain.

IAA works a vital role in the improvement of plants by stimulating their growth when applied directly to the roots [83]. The results of the IAA test revealed all fungal strains (*A. flavus*, *A. niger*, *Pencillium sp.*, *Mucor sp.*) were able to synthesize IAA (Table 1). Also, data revealed that *A. flavus* the best isolates to produce IAA (+++) followed by *Mucor sp. (++)*, then *A. niger* and *Pencillium sp.* which gave the same result (+).

The results obtained in Table 1 showed that all fungal strains can reduce and secrete siderophores to scavenge iron from the extracellular environment. Siderophore–iron complexes are transported into the cell through receptors in the membrane [54,55]. According to this study, *A. flavus* and *Mucor sp.* were the best for siderophore production then *A. niger* and *Pencillium sp.* Phosphorus in its organic formulae, which will not be taken up by plants, seems to have substantial qualities. To be absorbed, organic phosphorus must first be changed into inorganic phosphorus by the action of microorganisms efficient of dissolving it. As a result, fungi that were able to

solubilize organic phosphates will play a role in enhancement plant health[32,33]. Our results showed that all fungal strains could solubilize tricalcium phosphate and make it bio-available (Table 1). According to this study *A. flavus* and *Mucor sp.* were the best strains able to solubilize phosphorus, followed by *A. niger* and *Pencillium sp.*. The repressive impact of our selected fungal isolates against *F. oxysporum* could be related to production of HCN, IAA and siderophores that limit the availability of iron. It is previously established that all tested isolates can induce Biochemical defense in plant [56,57].

3.2. Identification of most potent fungi

Results showed that the four fungal isolates F1, F9, F13 and F20 were the supreme producers plant growth promoting activity (HCN, IAA, Siderophore and phosphates solubilization).At morphological level, the fungal isolate F1 was identified as *Aspergillus flavus* where colonies grow rapidly reaching 3.0-5.0 cm diameter in 4 days at 28°C on PDA, dark yellowish green colonies, pale brown conidial head is Radiate and loosely columnar as shown in Figure 1 C&D. On the other side, F9 was identified as *Penicillium sp.* where colonies grow after 7 days at 28°C on PDA 25 to 40 mm in diameter as , usually plane, low and velutinous, occasionally floccose centrally or somewhat granular, mycelium inconspicuous conidial production moderate to heavy, greyish turquoise with whitish edges as shown in Figure 1 E&F.

Table 1: Screening of all fungal isolates according to HCN, IAA, Siderophore production and phosphates solubilization

Fungal isolates	HCN	IAA	Siderophore	Phosphates solubilization
FI	+	+	+	+
F2	-	-	-	-
F3	-	-	-	-
F4	-	-	-	-
F5	-	-	-	-
F6	-	-	-	-
F7	+	-	-	-
F8	-	+	-	-
F9	+	+	+	+
F10	-	-	+	-
F11	+	-	-	-
F12	-	-	-	+
F13	++	+++	+++	++
14 F	-	-	-	-
F 15	-	-	-	+
F 16	-	+	-	-
F 17	-	-	-	-
F 18	-	-	-	-
F 19	+	-	-	-
F 20	+++	++	+++	++
F 21	-	-	-	-
F 22	-	-	-	-
F 23	-	-	-	-
F 24	-	-	+	-
F 25	-	-	-	+

HCN: hydrocyanic acid ; IAA: Indole-3-acetic acid; (−): negative reaction. (+): positive reaction

The fungal isolate F13 was identified as *Mucor sp.*, where diameter of growth was 40-60 mm after 4 days at 28°C on PDA, where appeared pale grey to white in colour as shown in Figure 1 G&H. Furthermore, fungal isolate F20 was identified as *Aspergillus niger* where colonies grow rapidly reaching 30 to 50 mm diameter in 4 days at 28°C on PDA, showing rapid rate of growth with black or whitish black in color as shown in Figure 1 A&B.

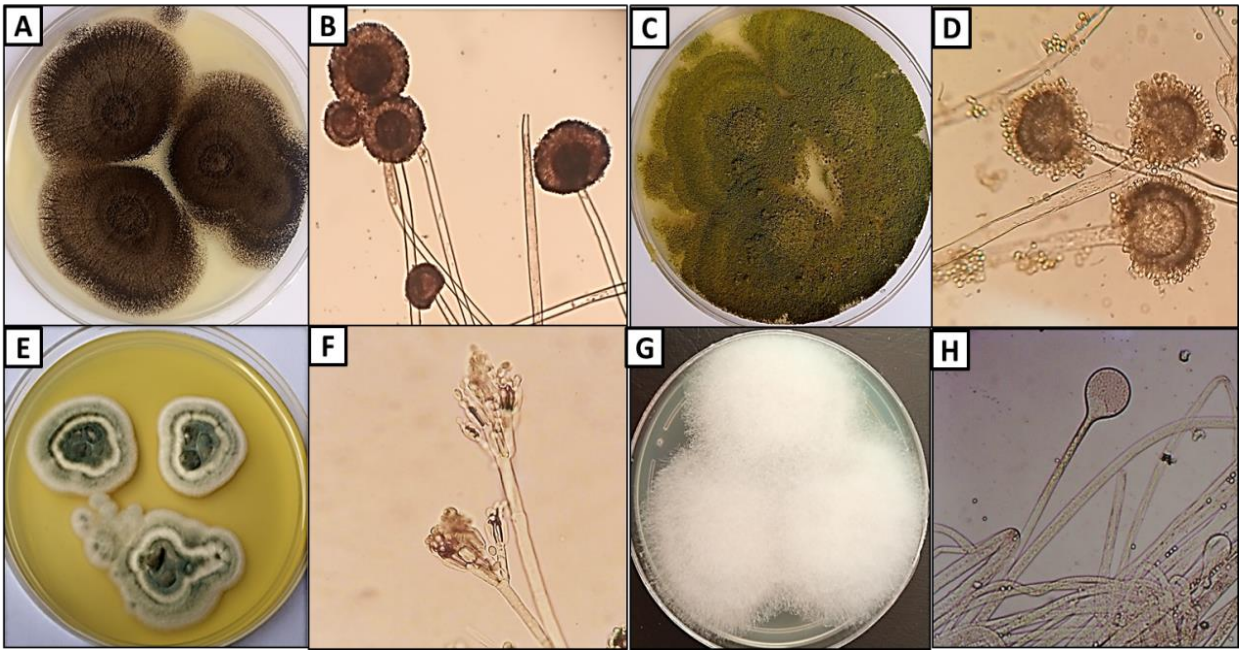

Figure 1: Surface colony of *A. niger* **(A)**, *A. flavus* **(C)**, *Pencillium sp.***(E),** *Mucor sp.***(G);** Conidiophore and conidia (400X) of *A. niger* **(B)**, *A. flavus* **(D)**, *Pencillium sp.* **(F);** Sporangiospores and sporangium of *Mucor sp* **(H);**

3.3. In vivo study

3.3.1. Disease severity (DS) and Protection%

Disease severity was the first guide to govern systemic resistance in treated plants by PGPF. Results in table 2 indicated that *F. oxysporum* highly destructive effect on tomato plants by PDI 90 % similar to heavily studies [11,7,13] .On the other hand, all applied PGPF reduced PDI (Table. 2) where *A. flavus*, *Mucor sp.*, *A. niger* and *Pencillium sp.* reduced the percentages of disease severity by 25, 30, 35, and 50 %, respectively. In addition, PGPF revealed high protection percentage of 72.2, 66.6, 61.1, and 44.4 % against Fusarium wilt. This protection effect of PGPF against *Fusarium* wilt reported by several studies which reported the ability of PGPF to suppress *Fusarium* pathogen [58,59]. This plant recovery may be due to PGPF were induced plant systemic resistance against *Fusarium* wilt [60].

3.3.2. Biochemical defense indicators

3.3.2.1. Osmolytes

The increase of osmolytes acts as a common phenomenon that plays an important role in ROS scavenging, provide plant cells with energy as well as modifying cell oxidoreduction [61,62]. As showing in Figure 2, results revealed that total soluble proteins and total carbohydrate of tomato declined significantly but free proline of tomato increased significantly in response to *F. oxysporum* infection that reported also by heavily studies [5,11,63]. It was noticed that, tested elicitors (*A. niger*, *Mucor sp.*, *A. flavus* and *Pencillium sp.*), respectively caused enhancement of total soluble proteins and carbohydrates compared to control plants. Total protein was determined as a response to the induction of systemic resistance [64]. The indirect effects of PGPF in the disease destruction include the activation of the plant defense mechanisms through the production of proteins [65,66]. In line with the conclusions of photochemistry and cell biology, we can say that physiological immunity results from many biological reactions, including changes in the cell wall and the synthesis of substances responsible for defense such as phytoalexins and proteins related to pathogenesis [67-69] . However, pre-treatment with *Mucor sp.* resulted in the most potent significant outcome in terms of the total soluble protein and total carbohydrate. These increasing of carbohydrates by PGPF play an important role in physiological

protection against several pathogens through stimulate hormones and various defense pathways which caused gene expression changes [70]. It is noticeable that, the greatest value recorded for the free proline was achieved by applied *Pencillium sp.* on the infected plants followed by *Mucor sp., A. flavus, and A. niger*. These results are in agreement with those reported by [28,71] they reported that infected plants treated with PGPR show a distinct increase in the levels of proline.

Table 2: Effect of PGPR on disease index of infected Tomato plants

Treatment	Disease symptoms Classes					PDI (disease index) (%)	Protection (%)
	0	1	2	3	4		
Control healthy	6	0	0	0	0	0	-
Control Infected	0	0	0	2	3	90	0
Infected + *A. niger*	1	2	1	1	0	35	61.1
Infected + *A. flavus*	1	1	2	0	0	25	72.2
Infected + *Mucor sp.*	1	2	2	0	0	30	66.6
Infected + *Pencillium sp.*	1	1	1	1	1	50	44.4

(0) no symptoms, (1) slight yellow of lower leaves, (2) moderate yellow plant, (3) wilted plant with browning of vascular bands, and (4) plants severely stunted and destroyed. PDI percent disease index.

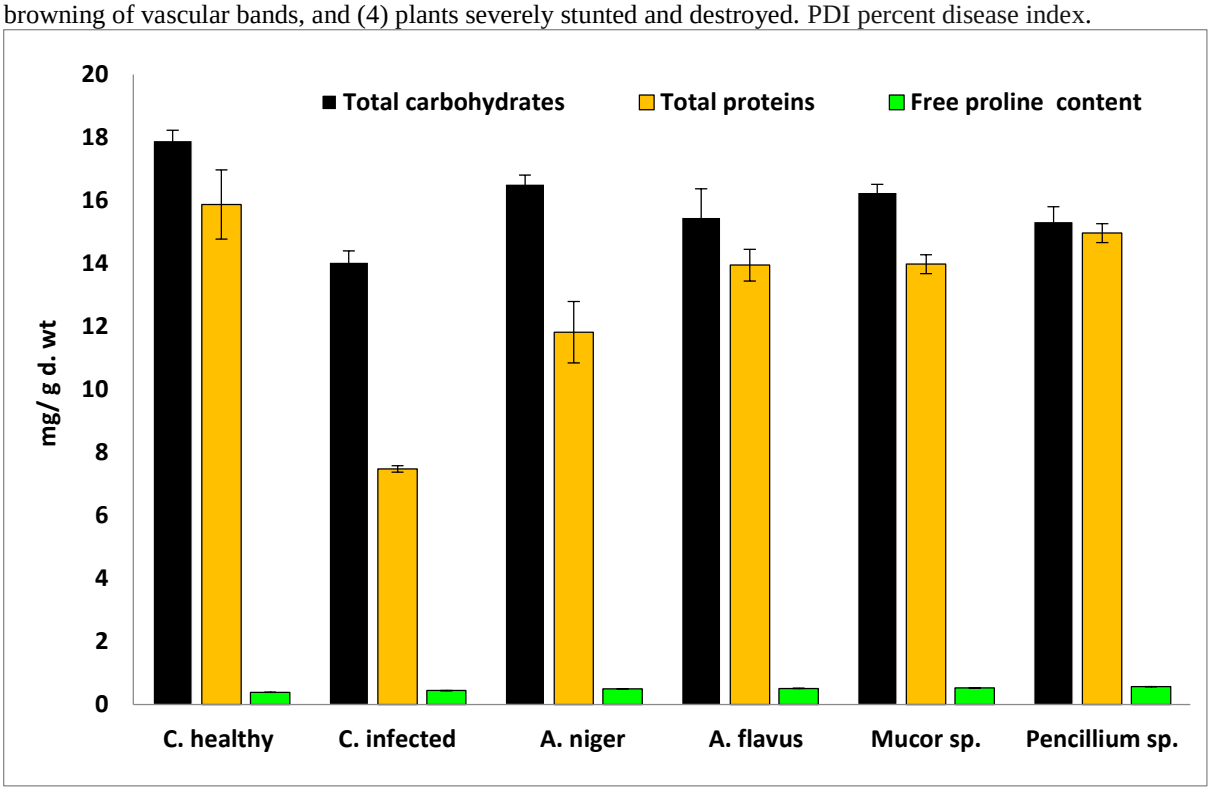

Figure 2: Effect of Fungal isolates (PGPF) on *Osmolyte*s contents

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3.3.2.2. Total phenols and Ascorbic acid

It is noticeable that, the greatest value recorded for the total phenols and ascorbic acid was achieved by applied *Mucor sp.* on the infected plants followed by *A. flavus, Pencillium sp. and A. niger*. Tomato plants grown under fusarial infection presented significant increases in contents of total phenols and ascorbic acid by 24.09 % and 35.4 % respectively, versus uninfected plants (Figure 3). Moreover, application of tested fungal strains resulted in a noticeable increase in the content of total phenols by 25.4 %, 16.3 %, 12.7 %, and 8 % at *Pencillium sp., Mucor sp., A. niger and A. flavus*, respectively, and in the content of ascorbic acid by 93.3 %, 58.3 %, 48.3% and 41.9 % at *Pencillium sp., A. flavus, Mucor sp.* and *A. niger*, respectively over infected plants only. The

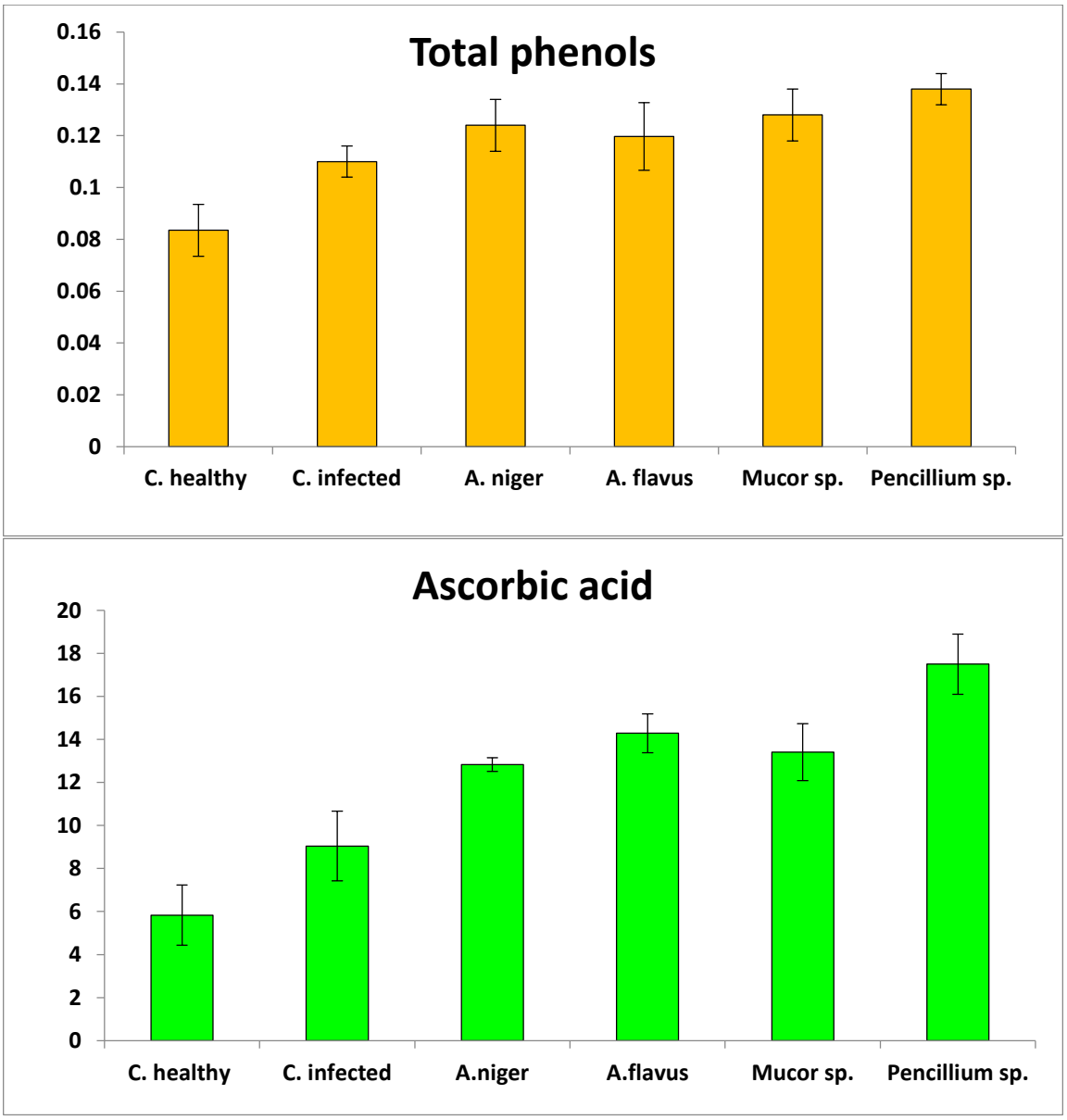

Figure 3: Effect PGPF on Total Phenols and Ascorbic Acid

increasing of total phenols play a vital role in metabolism regulation, vegetative growth and the lignin synthesis [72,73]. Our results are in harmony with other researchers [66–68]. Phenolic compounds and ascorbic acid assistance antioxidant functions by scavenging the free radicals, reduction their reactivity to the membrane components[74] [9,48].

3.3.2.3. Malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2).

Oxidative stress triggered by *Fusarium* infection led to dangerous disorder to plant cells and increased the contents of Malondialdehyde (MDA and Hydrogen peroxide (H2O2) in the leaves of tomato plants. These results agree with[75] [34,75,76]. The plants exposed to fusarium infection showed highly increase for MDA and H2O2 content in comparison to healthy control plants. Moreover, application of *PGPF* led to decrease in the content of MDA by 22.1 %, 16.5 %, 15.4 %, and 12.4 % at *Mucor sp., Pencillium sp.*, , *A. flavus and A. niger* , respectively, and in the content of H_2O_2 by 9.73 %, 6.5 %, 4.4 % and 1.9 % at *Pencillium sp.*, *Mucor sp.*, *A. flavus*, and *A. niger*, respectively compared to infected plants (Figure 4). These results similar to the study achieved by Badawy et al. [12] they reported that application of biological stimulators under stress conditions resulted to decreasing of MDA and H_2O_2

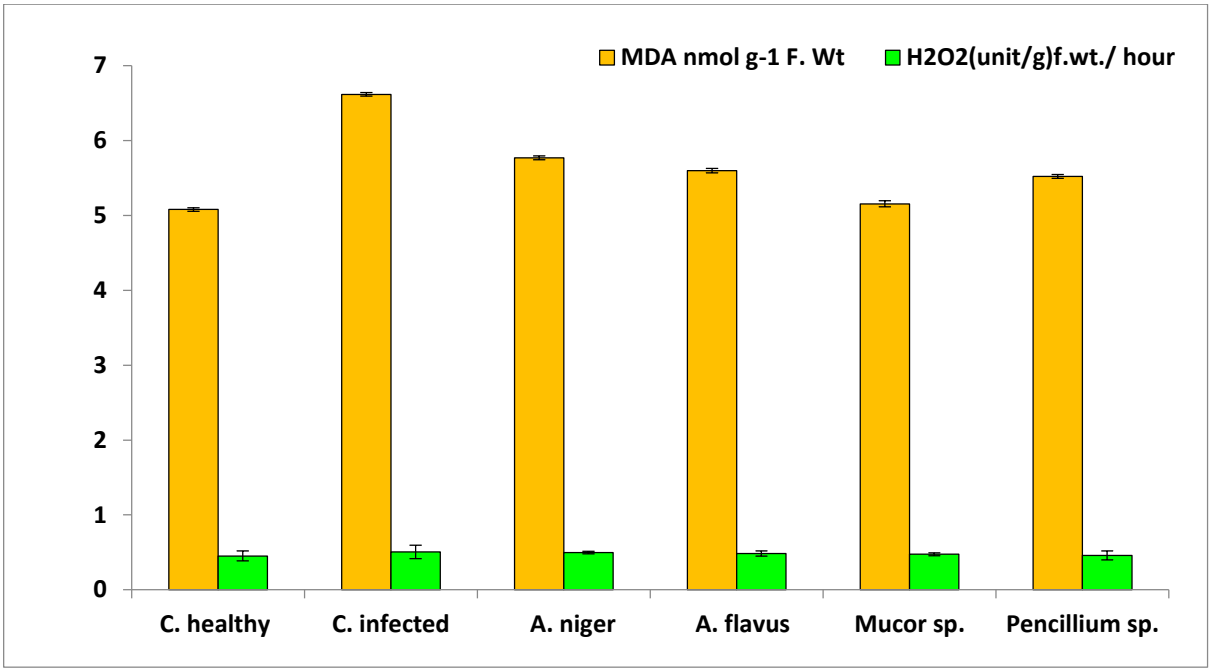

Figure 4: Effect of PGPF on Malondialdehyde (MDA and Hydrogen peroxide (H2O2)

4. Conclusion

In the current study, four soil fungi were isolated and identified morphologically as *Mucor sp., A. flavus, A. niger and Pencillium sp.* for tomato growth promotion and control of Fusarium wilt disease. In-vitro results revealed that all isolated PGPF have plant growth promotion compounds as HCN, IAA, Siderophores, also have ability to solubilize phosphate. Moreover, in-vivo results confirmed that all PGPF could stimulate biochemical defense and enhance the efficiency of tomato immunity against pathogens, especially *Fusarium* wilt. Finally, the use of these PGPF (*Mucor sp., A. flavus, A. niger and Pencillium sp.*) as biological agents and alternatives to chemical pesticides, and these results are promising in agricultural applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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