Mindanao Journal of Science and Technology Vol. 18 (2) (2020) 174-193

Bacteriological Therapeutic-Based Strategy for Management of Fusarium Wilt Disease in Tomato Plants

Abeer A. Ghoniem[1], Ehsan M. Rashad[2],
Ayman Y. El-Khateeb[3*] and WesamEldin I.A. Saber[1]
[1]Department of Microbiology, Soils, Water and Environment Research Institute
[2]Seed Pathology Research Department, Plant Pathology Research Institute
Agricultural Research Center
Giza, Egypt

[3]Department of Agricultural Chemistry
Mansoura University
Mansoura, Egypt
[*]*aymanco@mans.edu.eg*

Date received: March 12, 2020
Revision accepted: August 24, 2020

Abstract

Fusarium oxysporum f. sp. lycopersici (Sacc) causes a destructive wilt disease in tomato. With consideration to the environment and human health concerns, the bioagents as alternative procedures for defense against the plant pathogen were investigated. The potentiality of the antagonistic activity of Bacillus subtilis (ATCC® 11774™) reached 53% against the growth of F. oxysporum in a dual culture technique. The proteolytic; B. subtilis strain; was found to be a potent producer of amino acids under in vitro. Fractionation of the fermented hydrolysate showed the liberation of glutamic acid (760.15 mg/L), glycine (414.65 mg/L), aspartic acid (291.1 mg/L), cysteine (268.45 mg/L) and lysine (51.9 mg/L). In a greenhouse experiment, seed treatment with crude extract of amino acids and/or Bacillus subtilis cells demonstrated the greatest suppression of wilt symptoms on tomato seedlings. Moreover, pronounced plant growth promotion in root and shoot lengths, number of leaves, shoot fresh and dry weights of tomato plants was noticed. Biochemical parameters (photosynthetic pigments, total polyphenols, flavonoids, polyphenol oxidase, and peroxidase enzymes) were also upgraded. Antioxidant capacity of the plants – ABTS (%), DPPH (%) and reducing power – positively responded to the investigated treatments.

Keywords*:* antioxidant activity, amino acids, *Bacillus subtilis*, immunity, biological treatment

1. Introduction

The world production of tomato (*Solanum lycopersicum* L.), the most economically important vegetable plant, is approximately 130 million tons around the world (Rodriguez *et al*., 2018). It is one of the most favorable and consumed fruits with rich nutritional and high marketing values (Sun *et al*., 2018). However, tomato is perishable and susceptible to diseases caused by various fungi, which limit crop production leading to substantial economic damages (Zhang *et al*., 2011; Boukerma *et al*., 2017). *Fusarium oxysporum* f.sp. *lycopersici* (Sacc) is one of the highly destructive pathogens that causes the most epidemic disease to tomato plants either in the glasshouse or field inducing 50% yield loss (Sathiyabama and Charles, 2015; Mohammed *et al*., 2019).

Currently used synthetic fungicides for the management of tomato pathogens are highly effective; however, pathogens simultaneously developed resistance. Potential hazards also extended to human and environment wherein the emergence of alternative procedures with efficiency and low residue and/or less toxicity to humans and ecology had been investigated (Zhang *et al*., 2014). Induction of plant defiance to pathogens with biotics and/or abiotics has emerged as a potential procedure to alleviate the residual and toxicity of fungicides (Chen *et al*., 2017; Mohammed *et al*., 2019).

Various substances such as y-aminobutyric acid, arginine, and salicylic acid have been used as inducers for plant defense against pathogens (Zhang *et al*., 2011). A previous study investigated the role of the amino acids such as L-glutamate as an inhibitor for *Alternaria alternata* during inducing the resistance in tomato fruit (Yang *et al*., 2017). Moreover, endophytic bacteria have been found to play a potential role in plant growth promotion and phytopathogen control (Nejad and Johnson, 2000; Habtamu and Lamenew, 2020). Among endophytic bacteria, *Bacillus* species can colonize plants (Prabhukarthikeyan *et al*., 2014, Aloo *et al*., 2019), and suppress the phytopathogens. Another investigation stated that *Bacillus* sp. plays a role in the management of tomato pathogen during the production of antimicrobial lipopeptides and antifungal compounds like surfactin, iturin, bacillomycin and fengycin (Koumoutsi *et al*., 2004).

Furthermore, it has been found that *Bacillus* sp. induces systemic resistance in plants through the production of plant defense enzymes like polyphenol oxidase and peroxidase (Akila *et al*., 2011; Khan *et al*., 2020). *Bacillus*

amyloliquefaciens was found to manage the tomato fusarium wilt disease caused by *F. oxysporum* (Elanchezhiyan *et al*., 2018). Mohammed *et al.* (2019) stated that the application of *Pseudomonas fluorescens* and *Bacillus subtilis* may be a promising approach for biological control of the tomato bacterial wilt and may play an important role in sustainable agriculture.

The main aim of the current investigation was to manage Fusarium wilt disease using cells of *Bacillus subtilis* and/or crude amino acids mixture (the amino acids used herein were produced during the fermentation process by *B. subtilis*. The potential assets of immunity of tomato plants were determined against *F. oxysporum* f. sp. *lycopersici* in either sterilized or infested soil with the pathogen.

2. Methodology

2.1 Microorganisms and Dual Culture Assay

The *F. oxysporum* f. sp. *lycopersici* (ATCC® 201829™) was generously provided by the American Type Culture Collection (ATTC), Illinois, USA, and the *B. subtilis* (ATCC® 11774™) was acquired from the same source. Before fermentation trial, bacterial cells from culture aged at 24 h was scraped against the broth of the same fermentation medium to obtain an inoculum of 10^8 CFU/ml.

In vitro, an antagonistic assay, was performed between *F. oxysporum* and *B. subtilis* using the dual culture method adopted from Elkahoui *et al*. (2012).

2.2 Fermentation Medium

Besides being a bioagent against *F. oxysporum*, *B. subtilis* strain was also used for the production of amino acids. The proposed submerged liquid-state fermentation medium contained (gl^{-1}), peptone (8), KH_2PO_4 (2), olive cake (4) and corn steep liquor (4). The initial medium pH was adjusted to 7 and distributed in aliquots of 45 ml in 250-ml Erlenmeyer flasks, and then sterilized (121 °C for 15 min). The medium was inoculated with 5 ml of the bacterial inoculum and incubated at 35 °C under shaking (150 rpm). After 48 h, the fermented medium was centrifuged (5000 rpm for 15 min at 4 °C). The recovered bacterial cells were washed and re-suspended in sterile distilled water. The count of the cell suspension was adjusted to 108 cfu Ml^{-1}. The

resultant supernatant was then differentiated for the various amino acids content.

2.3 Separation of Amino Acid

Based on the official method of AOAC (2012), the Saykam S 443 amino acid analyzer (Sykam, Germany) was used for the separation of individual amino acids and estimation of the type and concentration of each amino acid. A non-inoculated medium was also injected into the amino acid analyzer to serve as a control and then subtracted from the fermented sample. The crude amino acids hydrolysate was kept freeze until use.

2.4 In vivo Evaluation of B. subtilis and/or its Amino Acids Mixture

Under greenhouse conditions, the application of *B. subtilis* and/or its supernatant was evaluated against tomato wilt disease. *F. oxysporum* was grown in a bottle containing sterilized sorghum, coarse sand, and water (2:1:2, v/v) medium and incubated at 25 ± 2 °C for 14 days. Pots (25-cm diameter) filled with sterilized soil was used. The upper layer of the soil was infested with the inoculum of the pathogen at 0.4% kg soil^{-1}.

Tomato seeds (Peto 86 variety) susceptible to Fusarium wilt infection were used in this study. Seeds were surface sterilized using sodium hypochlorite (1%), washed with sterile water, and then dried on filter paper. The seeds were soaked in the obtained bacterial cells, and bacterial supernatant or their combination for 1 h. Rhizolex-T (50% WP) at 3g/kg seed dressing as a chemical fungicide treatment (F) was used as control.

The treatments applied were; (1) negative control without any treatment, (2) *F. oxysporum* pathogen (P), (3) bacterial cells suspension (BC), (4) bacterial supernatant of the crude amino acids mixture (BS), (5) BC mixed BS (BCL), (6) P+BC, (7) P+BS, (8) P+BCL and (9) P + recommended fungicide (F). All pots were arranged in a randomized block-design and kept in the greenhouse.

2.5 Disease Assessment

The percentage of rotted seeds (un-germinated seeds), post infected seedlings (percentage of dead seedlings) and plant survival were recorded.

2.6 Growth and Physiological Parameters

Six-weeks after sowing, six plants with the entire root system were carefully pulled out, washed. The plant height, plant fresh and dry weights, and leaves number were then measured.

After 30 days from sowing, the spectrophotometric procedure for separation and capability for peroxidase (PO) and polyphenol oxidase (PPO) was employed (Seleim *et al*., 2014). The total phenolic content of fresh leaves was assessed using the Folin-Ciocalteu reagent method (Blainski *et al*., 2013). Total flavonoids content in fresh leaves was investigated according to the method of Li *et al*. (2015). After 45 days from sowing, total chlorophyll (Chl), Chl *a*, Chl *b* and carotene of leaves were also determined (Robinson and Britz, 2000).

2.7 Antioxidant Potentiality of Tomato Plants

The radical scavenging activity against 2,2'-azino-*bis* (3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) cation radical was performed, following the procedure of Christodouleas *et al*. (2015). The technique of Li *et al*. (2015) was used to assess the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The percentage of radical scavenging ability (RSA) by both ABTS and DPPH was estimated by the next Equation 1.

$$RSA\ (\%) = \frac{A_0 - A}{A_0} \times 100 \quad (1)$$

where:

A_0 = absorbance of the control
A = absorbance of the sample

The reducing power capacity of tomato plants was also evaluated, the procedure described by Li *et al*. (2015) was used to measure it. The greater reducing power ability was achieved by the higher reaction mixture absorbance.

2.8 Experimental Design and Statistical Analysis

All treatments were arranged in one-way randomized blocks. Data were subjected to analysis of variance using CoStat 6.4 software (CoHort Software, USA) followed by means separation using Duncan's multiple range test at probability (p) level ≤ 0.05.

3. Results and Discussion

3.1 Laboratory Experiment

3.1.1 Antagonism of *B. subtilis* towards *F. oxysporum*

A dual culture of *F. oxysporum* and *B. subtilis* has been conducted. The marked reduction of pathogen growth, with a ratio of 53% in growth diameter during an incubation period of 5 days has been obtained. The cytoplasm of the fungal mycelium showed a dramatic change as a result of bacterial antagonism (data not shown). Additionally, mycelium closest to bacterial colonies become yellow indicating some diffusers from bacteria that had reached this part of the mycelium.

3.1.2 Amino Acids Profile

B. subtilis was growing on an optimized batch fermentation medium and the associated amino acids had been differentiated. The profile of the amino acids associated with the fermented medium is presented in Figure 1.

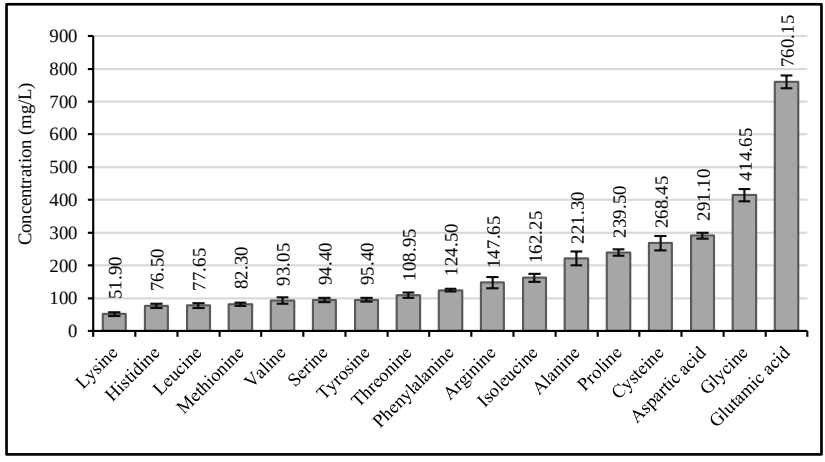

Figure 1. Profile of the differentiation of the amino acids produced by *B. subtilis* (ATCC® 11774™)

A total of 17 amino acids were detected, the kinds of recovered amino acids varied between essential ones (isoleucine, phenylalanine, threonine, valine, methionine, leucine, histidine and lysine) and non-essential ones (glutamic acid, glycine, aspartic acid, cysteine, proline, alanine, arginine, tyrosine, and

serine). The highest detected amino acids obtained were glutamic (760.15 mg/L) and glycine (414.65 mg/L), on the other side, lysine (51.90 mg/L) was the lowest. There are other amino acids in moderate amounts. Isoleucine (162.25 mg/L) was the highest essential amino acid detected.

3.2 Performance of Tomato Plants under Greenhouse Conditions

3.2.1 Response of *F. oxysporum* to the Bioagents

As shown in Table 1, data recorded significant variation in disease parameters of tomato plants as a response to the various *B. subtilis* treatments. In this respect, seed treatment with BS and BCL presented the highest protection levels of tomato seedlings from seed rot and wilt symptoms reaching 81.7 and 73.62% and 58.13 and 53.13%, respectively; and increased plant survivals, reaching 47.1 and 42.6%, respectively, compared with the untreated-infected control. BC and F treatments came in the second descending order among investigated parameters.

Table 1. Influence of *B. subtilis* and/or amino acids mixture on the development of seedlings mortality of tomato under greenhouse conditions

Treatment	Seed rot (%)	Infected seedlings (%)	Survivals (%)
P	23.50^{a}	16.00^{a}	60.50^{d}
C	1.700^{cd}	0.700^{e}	97.60^{a}
BCL	0.900^{d}	0.300^{e}	98.80^{a}
BS	0.600^{d}	0.200^{e}	99.20^{a}
BC	2.300^{cd}	0.900^{e}	96.80^{a}
BS+P	4.300^{cd}	6.700^{d}	89.00^{b}
BC+P	13.50^{b}	0.50^{bc}	76.00^{c}
BCL+P	6.200^{c}	7.500^{cd}	86.30^{b}
F+P	15.50^{b}	11.50^{b}	73.00^{c}

P – *F. oxysporum* pathogen, C – negative control, BS – bacterial supernatant, BC – bacterial cells, BCL – BS+BC and F – recommended fungicide. Means having the same letters in each column are statistically insignificant at P-value ≤ 0.0.

3.2.2 Bioagents and Plant Growth Features

The response of root and shoot lengths, number of leaves, plant fresh and dry weights of tomato plants to *B. subtilis* and /or a mixture of amino acids have been conducted (Table 2 and Figure 2). Generally, all the *B. subtilis* treatments and F showed no significant effect in increasing the root length parameter in comparison to the untreated-infected control. The highest significant increment was recorded in shoot length and leaves number due to BC and BCL seed treatments (63.38 and 55.56%; and 36.84 and 26.32%, respectively). The positive effect of the tested *B. subtilis* treatments extended to the fresh and dry

weight of the tomato plants, which reached one-fold or more in the case of BC treatment in comparison to infected control. The treatments of BS, BCL, and F came in the second descending order in this respect.

Table 2. Tomato growth characteristics as affected by application *B. subtilis* with and/or amino acids mixture treatments under greenhouse conditions

Treatment	Length (cm)		Leaves number per plant	Plant weight (g)	
	Root	Shoot		Fresh	Dry
P	6.750^{b}	11.25^{d}	4.75^{c}	1.97^{d}	0.046^{e}
C	10.00^{ab}	14.25^{bcd}	6.50^{a}	3.95^{ab}	0.093^{ab}
BCL	9.50^{ab}	15.25^{abc}	6.50^{a}	4.00^{ab}	0.096^{ab}
BS	9.250^{ab}	15.00^{bc}	6.75^{a}	3.93^{ab}	0.087^{bc}
BC	9.750^{ab}	13.50^{cd}	7.00^{a}	4.11^{a}	0.104^{a}
BS+P	8.250^{ab}	14.25^{bcd}	5.25^{bc}	3.53^{bc}	0.065^{d}
BC+P	11.38^{a}	18.38^{a}	6.50^{a}	4.04^{a}	0.081^{bc}
BCL+P	10.75^{a}	17.50^{ab}	6.00^{ab}	3.32^{c}	0.063^{d}
F+P	10.36^{a}	13.75^{cd}	4.75^{c}	3.37^{c}	0.074^{cd}

Means having the same letters in each column are statistically insignificant at P-value ≤ 0.05.

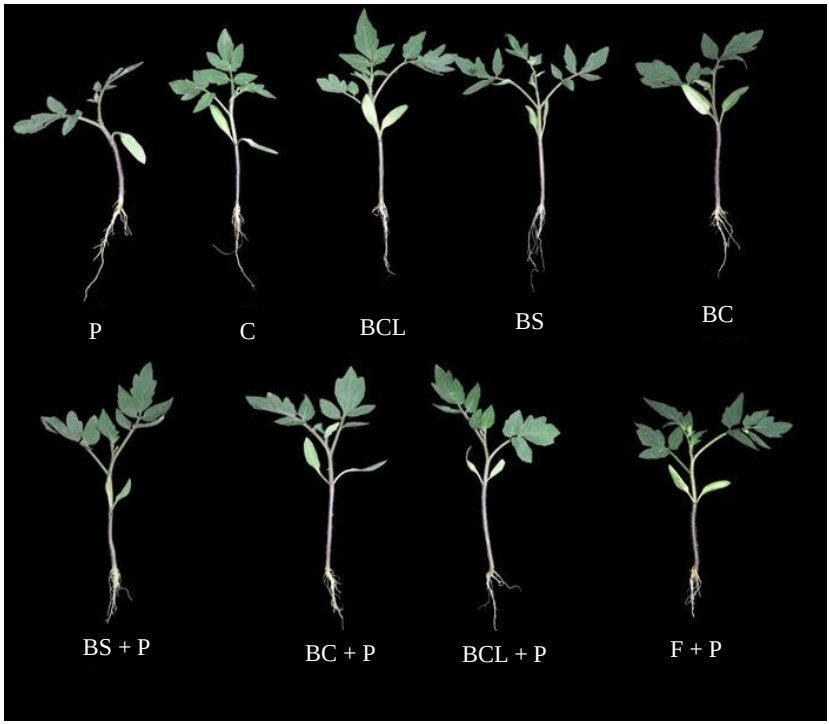

Figure 2. Tomato growth characteristics as affected by application with *B. subtilis* and/or amino acids mixture treatments under greenhouse conditions

3.3 Physiological Activities as Affected by the Bio-Agents

3.3.1 Photosynthetic Pigments

Concerning photosynthetic pigments (Table 3), generally, the presence of the pathogen markedly reduced the content of photosynthetic pigments of tomato leaves. Seeds treated with all *B. subtilis* treatments alleviated the harmful effect of the pathogen in which the highest increase of Chl *a* (62.28%), Chl *b* (114.49%) and total Chl (43.62%) were recorded by BCL application. Treatments of BC came next. As for the carotenoids, the BC presented the only threshold with significant increment among all *B. subtilis* and F treatments compared with infected control.

Table 3. Effect of treatments with *B. subtilis* and/or amino acids mixture on Chl and carotenoid contents of the tomato plant

Treatment	Chl contents (mg/g fresh weight)			
	Chl *a*	Chl *b*	Total Chl	Carotenoids
P	0.4411^{d}	0.2491^{cd}	0.7049^{e}	0.1530^{d}
C	0.8409^{a}	0.3608^{bcd}	1.2016^{a}	0.2614^{c}
BCL	0.9265^{a}	0.3620^{bcd}	1.2880^{a}	0.3426^{ab}
BS	0.9260^{a}	0.3054^{bcd}	1.2314^{a}	0.2866^{bc}
BC	0.8347^{a}	0.3856^{bc}	1.2203^{a}	0.2857^{bc}
BS+P	0.6856^{bc}	0.2491^{cd}	0.9347^{c}	0.1724^{d}
BC+P	0.5968^{c}	0.4430^{ab}	1.0400^{b}	0.3890^{a}
BCL+P	0.7158^{b}	0.5343^{a}	1.2502^{a}	0.1985^{d}
F+P	0.5774^{c}	0.2302^{d}	0.8076^{d}	0.1700^{d}

Means having the same letters in each column are statistically insignificant at P-value ≤ 0.05.

3.3.2 Physiological Performance

Table 4 displays the physiological response of tomato plants in terms of total polyphenols, flavonoids, polyphenol oxidase, and peroxidase enzymes contents to all investigated bioagents. The amino acids mixture of BS in sterilized soil was superior to the other ones, with values of 32.25 mg/g, 16.52 mg/g, 11.43 U/g, and 1.79 U/g, respectively, whereas the BCL came in the second-order. In general, total polyphenols, flavonoids and antioxidant enzymes (polyphenol oxidase and peroxidase) were significantly increased by BS, BCL and BC in infected soil compared with the recommended fungicide.

Table 4. Effect of treatments with *B. subtilis* and/or amino acids mixture on total polyphenols and flavonoids as well as defense-related enzymes contents of the tomato plant

Treatment	Total polyphenols (mg/g fresh weight)	Total flavonoids (mg/g fresh weight)	Polyphenoloxidase (U/g fresh weight)	Peroxidase (U/g fresh weight)
P	19.62^{h}	8.91^{i}	5.88^{i}	0.92^{i}
C	29.64^{c}	14.95^{c}	10.28^{c}	1.61^{c}
BCL	30.40^{b}	15.41^{b}	10.62^{b}	1.66^{b}
BS	32.25^{a}	16.52^{a}	11.43^{a}	1.79^{a}
BC	27.62^{d}	13.74^{d}	9.40^{d}	1.47^{d}
BS+P	24.74^{f}	12.00^{g}	8.13^{g}	1.28^{g}
BC+P	26.16^{e}	12.86^{e}	8.76^{e}	1.37^{e}
BCL+P	25.58^{e}	12.50^{f}	8.49^{f}	1.33^{f}
F+P	22.48^{g}	10.64^{h}	7.14^{h}	1.12^{h}

Means having the same letters in each column are statistically insignificant at P-value ≤ 0.05.

3.3.3 Antioxidant Capacity of Tomato

Generally, the stress effect of the infection was significantly reduced in terms of ABTS, DPPH and reducing power (Table 5) by the application of BS or BCL treatment compared with the untreated-infected treatment (P).

Table 5. Effect of treatments with *B. subtilis* and /or amino acids mixture on antioxidant capacity of the tomato plant

Treatment	ABTS inhibition (%)	DPPH inhibition (%)	Reducing power OD at 700 nm
P	15.42^{i}	26.50^{i}	1.34^{i}
C	25.87^{c}	42.98^{c}	2.35^{c}
BCL	26.65^{b}	44.22^{b}	2.42^{b}
BS	28.58^{a}	47.26^{a}	2.61^{a}
BC	23.76^{d}	39.65^{d}	2.14^{d}
BS+P	20.76^{g}	34.92^{g}	1.85^{g}
BC+P	22.24^{e}	37.26^{e}	2.00^{e}
BCL+P	21.63^{f}	36.29^{f}	1.94^{f}
F+P	18.41^{h}	31.20^{h}	1.63^{h}

Means having the same letters in each column are statistically insignificant at P-value ≤ 0.05.

Both BS and BCL enhanced the antioxidant capacity of tomato plants by 28.58 and 26.65% (ABTS), 47.26 and 44.22% (DPPH), and 2.61 and 44.78 (reducing power) compared with the other treatments. However, the BC came in next with antioxidant capacity of 26.65 and 44.22% of ABTS and DPPH, respectively. Commonly, the tested parameters of BS, BC, and BCL enhanced

significantly both the antioxidant capacity and reducing the power of tomato plants against *F. oxysporum* compared with recommended doses of fungicide.

Tomato is currently affected by Fusarium wilt disease that causes economic loss in productivity. Management of this disease through fungicides has been the prevailing control method for over 50 years. However, the persistence of fungicide in soil and adverse effect on beneficial soil microflora and human health is not acceptable. The adaptation of eco-friendly attitudes such as bio-fungicide and/or resistance inducing agents for controlling such diseases are always welcomed in this respect (Gowtham *et al*., 2016; Mohammed *et al*., 2019).

In this study, the results of the dual culturing technique showed that the radial growth of *F. oxysporum* was inhibited by *B. subtilis* strain at the ratio of 53%. These data are conceding with Elkahoui *et al*. (2012), who pointed out that *B. subtilis* might also affect pathogenic fungi by either producing antifungal metabolites or colonizing microsites faster than the surface of fungi. Furthermore, another investigation proved that *B. subtilis* produces lipopeptides belonging to the iturin and surfactin in the late phase of growth, which inhibits pathogen growth (Elkahoui *et al*., 2012).

The ability of *B. subtilis* to produce many hydrolytic enzymes (Khan *et al*. (2018), antimicrobial metabolites and siderophores was evidenced by the investigation of Mardanova *et al*. (2017). Additionally, phase contrast microscopy of the fungal mycelia in confrontation tests revealed alterations and irregular swelling, resulting in bulbous structures in the hyphal wall in comparison to mycelial control (Khan *et al*., 2018).

Moreover, 17 amino acids were found to be associated with the growth of the present *B. subtilis*, including essential and nonessential ones. The major amino acids detected being glutamic acid (760.15 mg/L) followed by glycine (414.65 mg/L). These data are in agreement with Ivanov *et al*. (2013) who reported the production of phenylalanine, threonine, and cysteine by *Escherichia coli*. Another investigation also reported the production of alanine and glycine by *Lactobacillus salivarius* (Lee *et al*., 2014).

In vivo results demonstrated that the crude amino acid mixture obtained by the current *B. subtilis* strain decreased root rot and infected seedling; and these results reflected on the survival percentage of plants. These may be due to the proteolysis process caused by *B. subtilis* during its growth on medium

containing olive cake and peptone, generating a measurable amount of amino acids, which were differentiated by an amino acid analyzer. These amino acids may be the key feature of the antagonistic effect of the bacterial strain. Their significant effect extended to vegetative growth and photosynthetic pigments. These results may be due to the important role of the secreted amino acids in boosting immunity and metabolic pathway of nutritional elements in tomato plants (Kadotani *et al*., 2016).

Another report illustrated that amino acids, namely proline, glutamine, and alanine showed to be effective inducers towards various types of bacteria, fungi, and oomycetes (Liu *et al*., 2010). Other studies pointed out that some of the biotic agents such as y-aminobutyric acid and arginine were found to induce plant defense against pathogens (Zhang *et al*., 2011). For example, L-glutamate inhibited the infection by *Alternaria alternata* in tomato fruit (Yang *et al*., 2017).

Amino acids and short peptides (10 to 50 amino acids), as boosting of plant immunity against phytopathogens, could sustain transduction of plant antimicrobial-defense proteins that synthesized constitutively or inducible by pathogen-associated molecular patterns (PAMP)-pattern recognition receptors (PRRs) or by the interaction of the Avr-R proteins, where these proteins present in an active-status in the response of phytopathogens infection (Silva *et al*., 2018).

Furthermore, the amino acids could be responsible for the constitution of some of the antimicrobial compounds such as glucosinolates, which are amino acid-derived phytoanticipins basically produced by *Brassica* plants, whereby accumulate methionine-derived aliphatic glucosinolates and tryptophane-derived indolic glucosinolates (Halkier and Gershenzon, 2006). These biomolecules decompose into various molecules with antimicrobial activity against *Pseudomonas syringae* (Fan *et al*., 2011). These previous findings may introduce explanations for the current data of in vitro antagonism between *F. oxysporum* and *B. subtilis*, which showed fungal growth reduction by 53%.

Interestingly, the current biological treatments played a marked role in decreasing significantly root rot, infected seedling and increasing survival of plants. These data are conceding with reported literature showing the efficiency of *Bacillus* species in the colonization of plants and suppress phytopathogens (Prabhukarthikeyan *et al*., 2014). Also, some of *Bacillus* species have a mechanism as plant-growth-promoting substances, in addition

to biocontrol agents (Khan *et al*., 2017). Generally, the kinetic of *Bacillus* spp. to protect the plants against phytopathogens could include two ways. The direct ways are phytohormones productivity, the acquisition of nutrients, (e.g. phosphorus and nitrogen) and control of pathogens (Khan *et al*., 2018). The indirect ways include protection from abiotic stress, (e.g. salinity and drought), induction of systemic defiance against pathogens and the suppression of free radicals (Martinez-Hidalgo *et al*., 2015).

Bacillus spp. have a kinetic of antagonism against phytopathogens through cross manufacture of various antimicrobial peptides (Falardeau *et al*., 2013, Aloo *et al*., 2019), secreted enzymes, proteins and volatile organic compounds (Baysal *et al*., 2013) and the secretion of cyclic lipopeptide; fengycin that plays a crucial role in the antagonistic process (Guo *et al*., 2014). This anti-biological activity against phytopathogens could be associated with the rhizosphere of plants, where bacilli and pseudomonads are the most public genera (Kumar *et al*., 2012). Hence, rhizobacteria is a promising agent for improving crop production and inducing immunity against pathogens, by which they support the genes coded for the production of secondary metabolites, pathogenesis-related proteins, as well as certain regulatory proteins involved in controlling defense mechanism (Dixon *et al*., 1994). Sun *et al*. (2018) suggested another mode of biological control action based on the presence of chitin that included in the cell-wall of the fungal pathogen, which induces plants and/or bioagents to enhances the actions of 6 defense-related enzymes (chitinase, superoxide dismutase, phenylalanine ammonia-lyase, peroxidase, β-1,3-glucanase, and catalase) by both plants and/or the bioagent.

The crucial role of BS and/or BC on boosting the immunity of tomato plants was confirmed in the present study, which significantly increased the total polyphenols, flavonoids, and antioxidant enzymes; they enhanced the antioxidant capacity of tomato plants, in which the inhibition percentage of ABTS and DPPH increased compared with control and recommended fungicide dose. These data concur with Ingle and Deshmukh (2010), who suggested that polyphenol oxidase and peroxidase are mainly responsible for the oxidation of phenols and polyphenols and lignification of the plant cell wall to prevent pathogen invasion.

Another parameter of the good performance of tomato plants was the improvement in photosynthetic pigments. Chls *a* and *b* were reported to play an important role in light absorption, as an intermediary in the transformation of the absorbed solar energy and synthesis of organic substances in plants during photosynthesis (Lobato *et al*., 2009). Chl *a* to *b* ratio was found to be

reduced in plants infected by *F. oxysporum*, which may occur mainly because of the decrease of Chl content. Besides, carotenoid levels presented significant reductions in infected plants (Tománková *et al*., 2006). On the other hand, *Bacillus* species were reported to induce positive physiological changes in plants by secretion of several metabolites that trigger plant growth and increase the synthesis of photosynthetic pigments. It also prevents pathogen infection and provides plant protection against adverse environmental conditions (Radhakrishnan *et al*., 2017). However, the increment in chlorophyll content reflects the health condition of the plant which enhances the efficacy of photosynthetic systems with a healthier potential for disease defense and alleviation of the photophosphorylation rate that takes place after the microbial invasion (Saber *et al*., 2009).

The induction immunity process of plants is usually associated with peroxidase and polyphenol oxidase enzymes, which are considered as biochemical markers for host resistance against pathogens. Also, phenylalanine ammonium lyase plays a role in the phenylpropanoid pathway with the conversion of phenylalanine into *trans*-cinnamic acid involved in phytoalexins and phenolics synthesis. Additionally, the expression of other pathogenesis proteins such as β-1,3 glucanases and chitinases causes lysis of fungal cell walls (Raju *et al*., 2008).

The antioxidant potentiality of tomato plants was determined in terms of ABTS, DPPH and reducing power capacity. All these antioxidation parameters were raised in tomato plants as a result of bacteriological therapeutic action. High antioxidant potentiality was reported to have several beneficial impacts. The antioxidant is a particle that hinders the oxidation of other molecules in which oxidation reaction transfers electrons or hydrogen from a molecule to an oxidizing agent. These radicals can start a chain of reactions causing damage or death to the cell. Antioxidants terminate this chain by removing free radicals' intermediates and inhibit other oxidation reaction by oxidizing themselves so antioxidants are often reducing agents. Therefore, insufficient levels of antioxidants cause oxidative stress that impairs cell function or damage its structure (El-Hersh *et al*., 2014; El-Awady *et al*., 2016). Amino acids may have antioxidant activity, representing an interesting additional chance to control fungal diseases within an eco-friendly integrated crop protection system through enhancing the defense of the plant to the pathogen (Abd El-Hai *et al*., 2019).

4. Conclusion and Recommendation

In conclusion, the successful biotechnological trend, using some of the bioagents for the challenge of the phytopathogens as inducers of the plant defense system, could be a part of defense strategy against plant pathogens without virulence to the ecosystem. Herein, amino acids and *B. subtilis* are recommended for management of Fusarium wilt disease in tomato, since both of them significantly decreased the root rot and infected seedling while sustaining high survival percentage of tomato plants against *F. oxysporum*. Improvement in growth, photosynthetic pigments, physiological features and antioxidant activity of plants was also observed. Generally, the sustainability of tomato plants under infection by *F. oxysporum* could be due to direct suppression action of *B. subtilis* and/or plant immunity-boosting by amino acids. More investigations are required to clarify the detailed biological control mechanism of *B. subtilis* and its individual amino acid.

5. Acknowledgement

The authors are grateful to Prof. Mohammed El-Hersh, Microbiology Department, Soils, Water and Environment Research Institute, Egypt, for revising the final manuscript.

6. References

Abd El-Hai, K.M.A., El-Khateeb, A.Y., Ghoniem, A.A., & Saber, W.I.A. (2019). Comparative response of cantaloupe features to amino acids, humic acid and plant oils towards downy mildew disease. Journal of Biological Sciences, 19(2), 122-130. http://dx.doi.org/10.3923/jbs.2019.122.130

Akila, R., Rajendran, L., Harish, S., Saveetha, K., Raguchander, T., & Samiyappan, R. (2011). Combined application of botanical formulations and biocontrol agents for the management of *Fusarium oxysporum* f. sp. *cubense* (Foc) causing Fusarium wilt in banana. Biological Control, 57(3), 175-183. https://doi.org/10.1016/j.biocontrol.2011.02.010

Aloo, B.N., Makumba, B.A., & Mbega, E.R. (2019). The potential of Bacilli rhizobacteria for sustainable crop production and environmental sustainability. Microbiological Research, 219, 26-39. https://doi.org/10.1016/j.micres.2018.10.011

AOAC. (2012). Official methods of analysis of AOAC International (19th Ed). Gaithersburg, Maryland, USA: AOAC International.

Baysal, Ö., Lai, D., Xu, H-H., Siragusa, M., Çalışkan, M., Carimi, F., de Silva, J.A.T., & Tör, M. (2013). A proteomic approach provides new insights into the control of soil-borne plant pathogens by *Bacillus* species. PloS One, 8(1), e53182. https://doi.org/10.1371/journal.pone.0053182

Blainski, A., Lopes, G.C., & de Mello, J.C.P. (2013). Application and analysis of the folin ciocalteu method for the determination of the total phenolic content from *Limonium brasiliense* L. Molecules, 18(6), 6852-6865. https://doi.org/10.3390%2Fmolecules18066852

Boukerma, L., Benchabane, M., Charif, A., & Khelifi, L. (2017). Activity of plant growth promoting Rhizobacteria (PGPRs) in the biocontrol of tomato Fusarium wilt. Plant Protection Science, 53(2), 78-84. https://doi.org/10.17221/178/2015-PPS

Chen, J., Cheng, H., Wu, D., Linhardt, R.J., Zhi, Z., Yan, L., Chen, S., & Ye, X. (2017). Green recovery of pectic polysaccharides from citrus canning processing water. Journal of Cleaner Production, 144, 459-469. https://doi.org/10.1016/j.jclepro.2016.12.140

Christodouleas, D.C., Fotakis, C., Nikokavoura, A., Papadopoulos, K., & Calokerinos, A.C. (2015). Modified DPPH and ABTS assays to assess the antioxidant profile of untreated oils. Food Analytical Methods, 8(5), 1294-1302. https://doi.org/10.1007/s12161-014-0005-6

Dixon, R.K., Solomon, A.M., Brown, S., Houghton, R.A., Trexier, M. C., Wisniewski, J. (1994). Carbon pools and flux of global forest ecosystems. Science, 263(5144), 185-190. https://doi.org/10.1126/science.263.5144.185

Elanchezhiyan, K., Keerthana, U., Nagendran, K., Prabhukarthikeyan, S.R., Prabakar, K., Raguchander, T., & Karthikeyan, G. (2018). Multifaceted benefits of *Bacillus amyloliquefaciens* strain FBZ24 in the management of wilt disease in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. Physiological and Molecular Plant Pathology, 103, 92-101. https://doi.org/10.1016/j.pmpp.2018.05.008

El-Awady, A.A., Saber, W.I.A., Hamid, N.M.A., & Hassan, H.A. (2016). Increasing antioxidant content of Broccoli sprouts using essential oils during cold storage. Agriculture (Polnohospodárstvo), 62(3), 111-126. https://doi.org/10.1515/agri-2016-0012

El-Hersh, M.S., Saber, W.I.A., & El-Fadaly, H.A. (2014). Amino acids associated with optimized alkaline protease production by *Bacillus subtilis* ATCC 11774 using statistical approach. Biotechnology, 13(6), 252-262. http://doi.org/10.3923/biotech.2014.252.262

Elkahoui, S., Djébali, N., Tabbene, O., Hadjbrahim, A., Mnasri, B., Mhamdi, R., Shaaban, M., & Limam, F. (2012). Evaluation of antifungal activity from *Bacillus strains* against *Rhizoctonia solani*. African Journal of Biotechnology, 11(18), 4196-4201. https://doi.org/10.5897/AJB11.3354

Falardeau, J., Wise, C., Novitsky, L., & Avis, T.J. (2013). Ecological and mechanistic insights into the direct and indirect antimicrobial properties of *Bacillus subtilis*

lipopeptides on plant pathogens. Journal of Chemical Ecology, 39, 869-878. https://doi.org/10.1007/s10886-013-0319-7

Fan, J., Crooks, C., Creissen, G., Hill, L., Fairhurst, S., Doerner, P., & Lamb, C. (2011). *Pseudomonas sax* genes overcome aliphatic isothiocyanate-mediated non-host resistance in *Arabidopsis*. Science, 331(6021), 1185-1188. https://doi.org 10.1126/science.1199707

Gowtham, H.G., Hariprasad, P., Nayak, S.C., & Niranjana, S.R. (2016). Application of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* for the management of Fusarium wilt in tomato. Rhizosphere, 2, 72-74. https://doi.org/10. 1016/j.rhisph.2016.07.008

Guo, Q., Dong, W., Li, S., Lu, X., Wang, P., Zhang, X., Wang, Y., & Ma, P. (2014). Fengycin produced by *Bacillus subtilis* NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. Microbiological Research, 169, 533-540. https://doi.org/10.1016/j.micres.2013.12.001

Habtamu, M., & Lamenew, F. (2020). Plant growth promoting rhizobacteria for plant growth promotion and biocontrol agent against tomato and pepper disease: A review. World News and Natural Sciences, 28, 13-23.

Halkier, B.A., & Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Annual Review of Plant Biology, 57(1), 303-333. https://doi.org/10.1146/annurev.arp lant.57.032905.105228

Ingle, R.W., & Deshmukh, V.V. (2010). Antifungal activity of PGPR and sensitivity to agrochemicals. Annals of Plant Protection Sciences, 18(2), 451-457.

Ivanov, K., Stoimenova, A., Obreshkova, D., & Saso, L. (2013). Biotechnology in the production of pharmaceutical industry ingredients: Amino acids. Biotechnology and Biotechnological Equipment, 27(2), 3620-3626. https://doi.org/10.5504/BBEQ.2012.0 134

Kadotani, N., Akagi, A., Takatsuji, H., Miwa, T., & Igarashi, D. (2016). Exogenous proteinogenic amino acids induce systemic resistance in rice. BMC Plant Biology, 16(1), 1-10. https://doi.org/10.1186/s12870-016-0748-x

Khan, N., Martínez-Hidalgo, P., Ice, T.A., Maymon, M., Humm, E.A., Nejat, N., Sanders, E.R., Kaplan, D., & Hirsch, A.M. (2018). Antifungal activity of *Bacillus* species against Fusarium and analysis of the potential mechanisms used in biocontrol. Frontiers in Microbiology, 9, 2363. https://dx.doi.org/10.3389%2Ffmicb.2018.02363

Khan, N., Maymon, M., & Hirsch, A.M. (2017). Combating Fusarium infection using Bacillus-based antimicrobials. Microorganisms, 5(4), 75. https://doi.org/10.3390/mic roorganisms5040075

Khan, A.R., El-komy, M.H., Ibrahim, Y.Y., Hamad, Y.K., Milan, Y.Y., & Saleh, A.A. (2020). Organic management of tomato Fusarium wilt using a native *Bacillus subtilis* and compost combination in Saudi Arabia. International Journal of Agriculture and Biology, 23(5), 1003-1012. https://doi.org/10.17957/IJAB/15.1379

Koumoutsi, A., Chen, X.H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., & Borriss, R. (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology, 186(4), 1084-1096. https://doi.org/10.1128%2FJB.186.4.1084-1096.2004

Kumar, P., Dubey, R.C., & Maheshwari, D.K. (2012). Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. Microbiological Research, 167(8), 493-499. https://doi.org/10.1016/j.micres.2012.05.002

Lee, K., Kim, H.J., & Park, S.K. (2014). Amino acids analysis during lactic acid fermentation by single strain cultures of lactobacilli and mixed culture starter made from them. African Journal of Biotechnology, 13(28), 2867-2873. https://doi.org/10.5897/AJB2013.13422

Li, J.E., Fan, S.T., Qiu., Z.H., Li, C., & Nie, S.P. (2015). Total flavonoids content, antioxidant and antimicrobial activities of extracts from *Mosla chinensis* Maxim.cv. Jiangxiangru. LWT-Food Science and Technology, 64(2), 1022-1027. https://doi.org/10.1016/j.lwt.2015.07.033

Liu, G., Ji, Y., Bhuiyan, N.H., Pilot, G., Selvaraj, G., Zou, J., & Wei, Y. (2010). Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in Arabidopsis. Plant Cell, 22, 3845-3863. https://doi.org/10.1105/tpc.110.079392

Lobato, A.K.S., Coimbra, G.K., Neto, M.A.M., Costa, R.C.L., Santos, F., Oliveira, N., Luz, L.M., Barreto, A.G.T., Pereira, B.W.F., Alves, G.A.R., & Monteiro, B.S. (2009). Protective action of silicon on water relations and photosynthetic pigments in pepper plants induced to water deficit. Research Journal of Biological Sciences, 4(5), 617-623.

Mardanova, A.M., Hadieva, G.F., Lutfullin, M.T., Khilyas, I.V., Minnullina, L.F., Gilyazeva, A.G., Bogomolnaya, L.M., & Sharipova, M.R. (2017). *Bacillus subtilis* strains with antifungal activity against the phytopathogenic fungi. Agricultural Sciences, 8(1), 1-20. http://doi.org/10.4236/as.2017.81001

Martinez-Hidalgo, P., Garcia, J.M., & Pozo, M.J. (2015). Induced systemic resistance against *Botrytis cinerea* by Micromonospora strains isolated from root nodules. Frontiers in Microbiology, 6, 922. https://doi.org/10.3389/fmicb.2015.00922

Mohammed, B.L., Hussein, R.A., & Toama, F.N. (2019). Biological control of Fusarium wilt in tomato by endophytic rhizobactria. Energy Procedia, 157, 171-179. https://doi.org/10.1016/j.egypro.2018.11.178

Nejad, P., & Johnson, P.A. (2000). Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. Biological Control, 18(3), 208-215. https://doi.org/10.1006/bcon.2000.0837

Prabhukarthikeyan, R., Saravanakumar, D., & Raguchander, T. (2014) Combination of endophytic Bacillus and Beauveria for the management of Fusarium wilt and fruit

borer in tomato. Pest Management Science, 70(11), 1742-1750. https://doi.org/10.1002/ps.3719

Radhakrishnan, R., Hashem, A., & Abd-Allah, E.F. (2017). Bacillus: A biological tool for crop improvement through bio-molecular changes in adverse environments. Frontiers in Physiology, 8, 667. https://doi.org/10.3389/fphys.2017.00667

Raju, S., Jayalakshmi, S.K., & Sreeramulu, K. (2008). Comparative study on the induction of defense related enzymes in two different cultivars of chickpea (*Cicer arietinum* L.) genotypes by salicylic acid, spermine and *Fusarium oxysporum* f. sp. ciceri. Australian Journal of Crop Science, 2(3), 121-140.

Robinson, J.M., & Britz, S.J. (2000). Tolerance of a field grown soybean cultivar to elevated ozone level is concurrent with higher leaflet ascorbic acid level, higher ascorbate-dehydroascorbate redox status, and long-term photosynthetic productivity. Photosynthesis Research, 64(1), 77-87. https://doi.org/10.1023/A:1026508227189

Rodriguez, M.H., Bandte, M., Gaskin, T., Fischer, G., & Büttner, C. (2018). Efficacy of electrolytically-derived disinfectant against dispersal of *Fusarium oxysporum* and *Rhizoctonia solani* in hydroponic tomatoes. Scientia Horticulturae, 234, 116-125. https://doi.org/10.1016/j.scienta.2018.02.027

Saber, W.I.A., Abd El-Hai, K.M., & Ghoneem, K.M. (2009). Synergistic effect of *Trichoderma* and *Rhizobium* on both biocontrol of chocolate spot disease and induction of nodulation, physiological activities and productivity of *Vicia faba*. Research Journal of Microbiology, 4(8), 286-300. http://doi.org/10.3923/jm.2009.286.300

Sathiyabama, M., & Charles, R.E. (2015). Fungal cell walls polymer-based nanoparticles in protection of tomato plants from wilt disease caused by *Fusarium oxysporum* f.sp. lycopersici. Carbohydrate Polymers, 133, 400-407. https://doi.org/10.1016/j.carbpol.2015.07.066

Seleim, M.A., Abo-Elyousr, K.A., Mohamed, A.A.A., & Al-Marzoky, H.A. (2014). Peroxidase and polyphenoloxidase activities as biochemical markers for biocontrol efficacy in the control of tomato bacterial wilt. Journal of Plant Physiology and Pathology, 2(1). https://doi.org/10.4172/2329-955X.1000117

Silva, M.S., Arraes, F.B.M., de Araújo Campos, M., Grossi-de-Sa, M., Fernandez, D., de Souza Cândido, E., Cardoso, M.H., Franco, O.L., & Grossi-de-Sa, M.F. (2018). Potential biotechnological assets related to plant immunity modulation applicable in engineering disease-resistant crops. Plant Science, 270, 72-84. https://doi.org/10.1016/j.plantsci.2018.02.013

Sun, C., Fu, D., Jin, L., Chen, M., Zheng, X., & Yu, T. (2018). Chitin isolated from yeast cell wall induces the resistance of tomato fruit to *Botrytis cinerea*. Carbohydrate Polymers, 199, 341-352. https://doi.org/10.1016/j.carbpol.2018.07.045

Tománková, K., Luhová, L., Petřivalský, M., Peč, P., & Lebeda, A. (2006). Biochemical aspects of reactive oxygen species formation in the interaction between *Lycopersicon* spp. and *Oidium neolycopersici*. Physiological and Molecular Plant Pathology, 68(1-3), 22-32. https://doi.org/10.1016/j.pmpp.2006.05.005

Yang, J., Sun, C., Fu, D., & Yu, T. (2017). Test for l-glutamate inhibition of growth of Alternaria alternata by inducing resistance in tomato fruit. Food Chemistry, 230, 145-153. https://doi.org/10.1016/j.foodchem.2017.03.033

Zhang, H., Ge, L., Chen, K., Zhao, L., & Zhang, X. (2014). Enhanced biocontrol activity of Rhodotorula mucilaginosa cultured in media containing chitosan against postharvest diseases in strawberries: Possible mechanisms underlying the effect. Journal of agricultural and food chemistry, 62(18), 4214-4224. https://doi.org/10.1021/jf500065n

Zhang, H., Li, R., & Liu, W. (2011). Effects of chitin and its derivative chitosan on postharvest decay of fruits: A review. International Journal of Molecular Sciences, 12(2), 917-934. https://doi.org/10.3390%2Fijms12020917