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Biocontrol of Tomato Mosaic Disease by Multiple Applications of Brown Alga (*Sargassum angustifolium*) Extract, *Pseudomonas fluorescens*, and *Bacillus subtilis*

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HIGHLIGHTS

- The combination of PGPR and brown alga promotes tomato growth and, consequently, improves the host tolerance to mosaic disease.
- ELISA extinction values (OD_{405}) were the lowest when *Pf+Bs+Sa* was applied to the rhizosphere of tomato plants.
- Chlorophyll content was the highest in *Pf+Bs+Sa* treated plants.
- The results of the current research open a new horizon of PGPR-algae interactions.

Abstract: Tomato mosaic disease caused by *Tomato mosaic virus* (ToMV) reduces tomato crop production globally. Biocontrol measures using various rhizobacteria and algae have been developed to reduce the adverse effects of plant diseases. To this end, two rhizobacteria (probiotic bacteria) including *Pseudomonas fluorescens*, *Bacillus subtilis*, and aqueous extract of brown alga (*Sargassum angustifolium*) were applied. A certain concentration of bacterial suspension was added to the tomato rhizosphere along with the aqueous extract of brown alga and the plants were subsequently inoculated with ToMV. Semi-quantitative indirect-ELISA was performed to estimate the virus titer within inoculated plants. Also, the disease severity index was determined by visual scoring of the plants at 14 and 28 days post-inoculation. Growth indices of plants were

evaluated and the data were statistically analyzed. The results showed that multiple treatments of the rhizobacteria and the aqueous extract of brown alga reduced the disease severity to 27.46%, and inhibit the ToMV accumulation up to 86.48% in tomato plants. Moreover, the growth indices of tomato plants pre-treated with a combination of the rhizobacteria and brown alga extract were significantly improved. Taken together, the results suggest that these biocontrol agents have a synergistic effect and their simultaneous application can, therefore, reduce the crop loss caused by ToMV.

Keywords: Growth index; Indirect-ELISA; Synergism; Virus accumulation.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most essential vegetables in Iran and many other countries. Viral diseases are responsible for qualitative and quantitative damage to this product. Tomato mosaic disease, mainly caused by *Tomato mosaic virus* (ToMV), is considered an emerging economically important disease worldwide [7]. ToMV was taxonomically placed in the genus *Tobamovirus* and the family *Virgaviridae* [2]. The yield of virus-susceptible cultivars may be reduced up to 25% by ToMV infection [12,28,35]. ToMV symptoms on tomato plants are varied; however, the most common symptoms include leaf mosaic, mottling, and deformation [41]. Viral disease management is commonly based on the use of resistant cultivars, insecticides, and agricultural practices to prevent the vector-mediated spread of the disease [5,16,17]. The methods mentioned earlier have limitations that restrict their use. For example, pesticide usage in the last century has resulted in removing beneficial microorganisms and caused environmental pollution [32, 34]. Given the limitations, applying environmental-friendly methods such as microbial agents to biocontrol plant pathogens seems necessary [19]. In recent years, the use of rhizobacteria has been introduced as an inducer of systemic resistance to control plant diseases. Among them, *P. fluorescens* and *Bacillus subtilis* are well-known probiotic bacteria with a high ability to biological control of plant pathogens [9,33,40]. It was shown that spraying a suspension of *B. amyloliquefaciens* on tobacco and pepper leaves can reduce *Cucumber mosaic virus* (CMV) infection in these plants [25]. Similarly, the treatment of tomato plants with rhizobacteria has resulted in significant enhancement of plant growth and protection against infection by CMV [30].

Algae are a large and diverse group of microorganisms that can carry out photosynthesis. They are also of great importance in agriculture due to their species diversity, wide distribution, and numerous unique physiological, and biological properties [31]. Today, natural compounds as a suitable alternative method to reduce the damage of plant pathogens that have been considered by many researchers. Various studies suggest that algae extract can improve crop tolerance to stress by increasing proline levels and activation of genes involved in resistance [21,23]. Many studies provide evidence that algae extract has antifungal, antibacterial, and antiviral properties [36,37]. Algae and bacteria have coexisted since the early stages of evolutions, and together, influence the ecosystem [37]. To date, scarce comprehensive studies have been performed on the simultaneous effect of rhizobacteria and brown alga on the biocontrol of viral pathogens. We hypothesize that these treatments will be useful for protection against ToMV damages in tomatoes. The objective of the above study was to achieve a combination of compatible rhizobacteria and brown algae to reduce the severity of ToMV-induced disease in tomatoes.

MATERIAL AND METHODS

Bacterial isolation and investigation of compatibility of isolates

Probiotic isolates of *P. fluorescens* and *B. subtilis* (Sc21 and Sc13, respectively) were provided from the microbial collection of the Plant Protection Department of Shahid Chamran University of Ahvaz (Ahvaz, Iran). Growth promoting traits and inhibitory effect of these isolates has been confirmed by Aeini and coauthors [4]. For testing antibiosis between PGPR strains, 100 µL of a 10^5 CFU/mL suspension of one PGPR strain was spread over the agar surface and dried. A sterile assay disk (6 mm in diameter; Schleicher & Schuell, Einbeck, Germany) was placed on the agar and a suspension of each challenged PGPR (10^{11} CFU/mL, 30 µL/disk) was dropped onto each disk. Plates were incubated at 28–30 °C for 24 h and examined for signs of clear zones indicating growth inhibition [18]. This test was repeated three times.

Preparation of bacterial suspension

The strains were removed from ultra-cold storage, streaked onto nutrient agar (NA) plates (Merck, Germany), and incubated at 30°C for 48 h to check for purity. Then 50-mL falcon tubes containing Luria

Bertani (LB) broth (Sigma-Aldrich, Germany) were inoculated with a singly colony of each isolate and incubated at 28 °C with shaking at 100 rpm. The optical density (OD) of each bacterial suspension was measured at 600 nm wavelength using a NanoDrop spectrophotometer (Thermo Scientific, USA). Based on optical densities, the bacterial suspensions were adjusted to 10^8 CFU/mL for each isolate. After the preparation of a single suspension for each isolate, two suspensions with the same volume were mixed to produce a combination of isolates.

Preparation of aqueous extract of alga

The brown alga (*S. angustifolium*) was collected from the shores of Chabahar, southeastern Iran (25.300278°N 60.612778°E), and transferred to the laboratory. The alga was identified according to Kokabi and Yousefzadi [24]. The alga was first washed with deionized distilled water (ddH_2O) to remove epiphytes and associated debris. After air-drying for ten days on Whatman no. 1 filter paper, the tissues were ground using a grinder. To prepare the aqueous solution, 50 g of alga powder and 500 mL of ddH_2O were shacked at room temperature. Then it was boiled for 60 min and filtrated. A stock solution of the extract with a concentration of 5 mg/ml was prepared and used for the assay [38, 42].

Virus inoculation

A previously characterized isolate of *Tomato mosaic virus* (ToMV-AWZHR-S95) was used. Since ToMV has a wide host range, is able to infect a large number of plant species such as tomato [14]. To infect tomato plants, ToMV-infected tomato tissue was homogenized into 0.05 M KH_2PO_4 buffer [pH 7] on ice. The resulting extract was mechanically inoculated to carborundum-dusted healthy tomato seedlings (2- to 3-leaf stage). The inoculated plants were kept in a growth chamber (25 °C, 40% relative humidity, 16L: 8D photoperiod) for symptom development.

Greenhouse assay

This experiment was performed in the growth chamber of the Faculty of Agriculture, Shahid Chamran University of Ahvaz in the winter of 2020, under controlled temperature, light, and humidity conditions. In this study, the tomato cultivar Ch-Falat (Falat Iran Co., Iran) was used as a commercially known cultivar to investigate the effect of probiotic bacteria and brown alga extract on tomato mosaic virus infection. The seeds were superficially disinfected by dipping them into 70% ethanol for one min and 1.5% NaOCl for ten min. The seeds were then washed 10 times with ddH_2O. The seeds were air-dried overnight. Four replications per treatment were used in a completely randomized design. Tomato seeds were then planted in sterile pots containing sterile peat moss and perlite (1: 3). The pots were irrigated with equal amounts of ddH_2O. The pots were kept in a growth chamber at a temperature of 25 °C with a relative humidity of 60-80%. Plants were mechanically inoculated with ToMV. Each pot was inoculated with 100 mL of bacterial suspension (alone or in a mixture of two isolates). Also, a certain volume (100 mL) of alga extract was added to the rhizosphere of plants. Bacteria and alga extract were added to the rhizosphere area seven days before inoculation of ToMV and repeated once every two weeks for a total of three times. The treatments used in this study included: *P. fluorescens* Sc21 (*Pf*), *B. subtilis* Sc13 (*Bs*), *S. angustifolium* (*Sa*), *P. fluorescens* Sc21 + *B. subtilis* Sc13 (*Pf*+*Bs*), *B. subtilis* Sc13 + *S. angustifolium* (*Bs*+*Sa*), *P. fluorescens* Sc21 + *S. angustifolium* (*Pf*+*Sa*) and *P. fluorescens* Sc21 + *B. subtilis* Sc13 + *S. angustifolium* (*Pf*+*Bs*+*Sa*). Controls included healthy plants (plant inoculated KH_2PO_4 buffer) and infected plants (plant inoculated with ToMV, not-treated with bacteria and alga extract). The pots were kept for 28 days. The experiment was repeated three times.

Measurement of plant growth indices

Wet and dry weight of roots and shoots and height of shoots and roots were measured at the end of the experiment. Plant height was measured from the crown to the end of the stem with a ruler. The plant samples were dried separately in paper bags for four days at 70 °C, and then using an auto-calibrated laboratory scale (AND, Japan) with an accuracy of 0.0001 g, the dry weight was calculated. The content of chlorophyll pigments in different treatments was read non-destructively using the SPAD chlorophyll meter (Minolta, Japan) [29]. Thus three-leaf areas including the tip, middle and terminal parts were subjected to chlorophyll assay and their average was recorded for each plant.

Evaluation of disease severity

Disease severity was determined at 14 and 28 days post-inoculation (dpi) using the following scale: 0 = asymptomatic plant, 2 = mild mosaic symptoms on leaves, 4 = severe mosaic on leaves, 6 = mosaic and leaf deformation, 8 = severe mosaic and leaf deformation, 10 = severe mosaic and leaf deformation with dwarfism. Determination of disease severity was calculated using the following equation [48]:

$$Disease\ Severity = \frac{\sum (disease\ index\ \times number\ of\ plant)}{(\text{total number of plants}\ \times \text{the highest disease index})} \times 100$$

Determination of virus titer

To estimate the relative concentrations of virus particles at 28 dpi in tomato plants, Indirect-ELISA was used according to Clark and Adams [8] in a semi-quantitative way. Sampling was done from all plants. Thus, one apical leaf was detached from each plant and the plant tissue was homogenized in extraction buffer in a ratio of 1:10, 1:20, 1:40, 1:50, and 1:60 (plant material: buffer). One hundred microliters of the obtained extract were added to ELISA plate wells and the plate was incubated overnight at 4 °C. ELISA plate was then washed and polyclonal antibodies (Bioreba, Switzerland) produced against ToMV were added to the ELISA plate and finally, enzyme-linked antibody (Sigma-Aldrich, Germany) containing alkaline phosphatase was added to the wells. Virus particles were detected by adding para-nitrophenyl phosphate (Sigma-Aldrich, Germany) as a substrate into the well. The OD of the samples was measured at 405 nm wavelength (OD_{405}) using an ELISA reader (Thermo Fisher Scientific, Germany). In this test, the leaf tissue of a healthy tomato plant was used as a negative control, and the leaf tissue of an infected tomato plant without biological treatments was used as a positive (infected) control. Also, three biological replications (plant leaves) and three technical replications (ELISA wells) were considered for each treatment. The data were statistically analyzed and the results of the 1:40 ratio, in which the OD_{405} was correlated with the extract concentration, were selected.

Molecular detection of ToMV

To detect ToMV within treated plants that showed positive results in ELISA, the total RNA of leaf samples was isolated using an RNA extraction kit (Denazist, Iran) according to the supplier's instructions. Complementary DNA (cDNA) was synthesized using QuantiTect Reverse Transcription Kit (QIAGEN, USA) and polymerase chain reaction (PCR) using specific Tob-Uni 1 and Tob-Uni 2 (5'-ATTTAAGT GGASGGAAAAVCACT-3' and 5'-GTYGTTGATGAGTTCRTGGA-3', respectively) primers were used to detect the virus [26]. The polymerase chain reaction was performed in a volume of 25 µL. The mixture was prepared containing 2.5 µL of cDNA synthesized as a template, 16.3 µL of ddH_2O, 2.5 µL of buffer (Ampliqon, Denmark), 1 µL of each primer, 1 µL of dNTPs, 1 µL of MgCl2 and 0.2 µL of *Taq* DNA polymerase Max. The temperature cycle used was to hold the resulting mixture for 4 min at 94 °C and then 35 cycles including 94 °C for 1 min, 50 °C for 45 secs, and 72 °C for 1 min, followed by 7 min of final extension at 72 °C. The PCR reaction product was loaded on 1% agarose gel containing DNA Green Viewer (Denazist, Iran) and analyzed under UV light. The PCR reactions were repeated twice.

Data analysis

All data collected during the experiment were edited using Excel software and prepared for statistical analysis. Data analysis was performed using SAS software (v. 9.4) [46]. The statistical design used in this study was a completely randomized design with four replications and the means were compared based on Duncan's multiple range test with an alpha error level of 5%.

RESULTS AND DISCUSSION

In this study, the effects of two probiotic rhizobacteria along with extract of brown alga on the severity of Tomato mosaic virus disease were investigated. At 14 to 19 dpi, leaf mosaic, leaf deformation, and stunting symptoms were developed on tomato plants (Figure 1). ELISA resulted in detecting ToMV within the tissues of virus-inoculated plants (Figure 2). It was found that the lowest ELISA extinction value (OD_{405}) belongs to the treatment of *Pf*+*Bs*+*Sa*. Although all the treatments significantly reduced the virus titer, the mean OD_{405} value of the *Sa*- and *Bs*-treated samples were significantly higher than that of other treatments. These results indicate that these agents can reduce ToMV titer during the infection. The OD_{405} values obtained from ELISA have been used to measure the concentration of viruses within plant tissues [22]. Although this is a relative method, it has been used as an effective tool for initial differentiation in pathogenicity studies [27].

Moreover, an 804 bp fragment of the ToMV genome was amplified through PCR assay in representatives of all treatments (Figure 3), which confirmed the presence of the virus within the plant tissues. The non-inoculated plants remained asymptomatic till the end of the experiment.

Figure 1. Dwarfism (a) and leaf mosaic and deformation (b, c, d) symptoms caused by ToMV-AWZHR-S95 on tomato (*S. lycopersicum* cv. CH-Falat) at 17 dpi. H: healthy, I: infected.

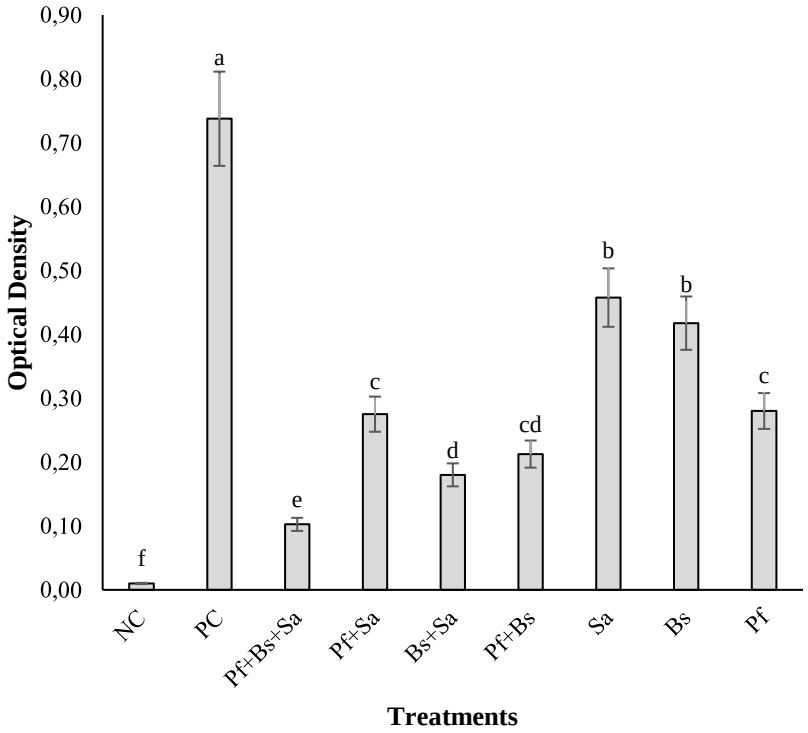

Figure 2. The results of semi-quantitative indirect-ELISA presented as the mean of optical density (measured at 405 nm wavelength [OD_{405}]) of samples from tomato plants treated with *S. angustifolium* extract (*Sa*) and probiotic rhizobacteria (*P. fluorescens* [*Pf*] and *B. subtilis* [*Bs*]) challenged with ToMV-AWZHR-S95. NC: negative control (healthy plant), PC: positive control (ToMV-infected plant). Means followed by different letters within a column represent a significantly different (P = 0.05) by Duncan's Multiple Range Test (DMRT).

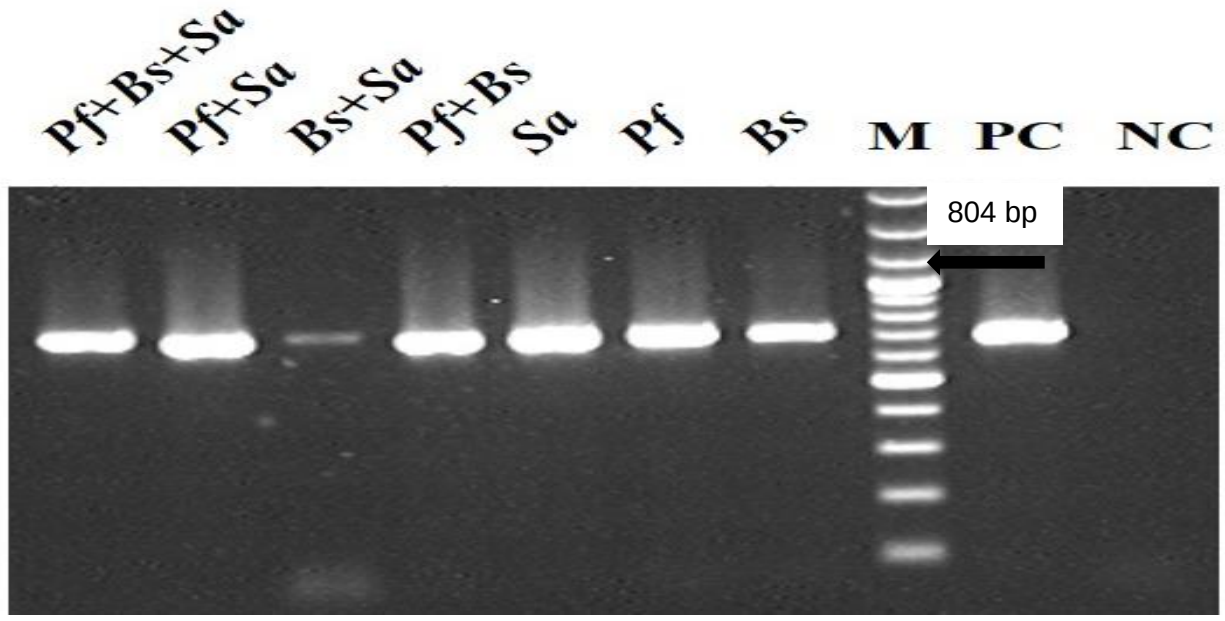

Figure 3. Agarose gel electrophoresis of PCR-products of ToMV and amplification products of 804 bp. M: 1 KB ladder (Thermo Fisher Scientific, German). PC**:** positive control (ToMV-infected plant), NC**:** negative control (healthy plant), M: Molecular marker 100 bp (Thermo Fisher Scientific, Germany), *Pf*: *Pseudomonas fluorescens*, *Bs*: *Bacillus subtilis*, *Sa*: *S. angustifolium* extract.

To determine the difference among the symptoms induced on plants treated with different agents, the disease severity of ToMV at intervals of 14 and 28 dpi was evaluated. The results of visual scoring of ToMV-infected plants showed that plants treated with *Pf*+ *Bs*+*Sa* had the lowest disease severity indices (Table 1). Thus, the severity of symptoms in plants treated with rhizobacteria showed a significant decrease compared to the positive control. The highest severity indices among the applied treatments were found on plants treated with *Pf* at 14 and 28 dpi (66.1 and 75.62, respectively). Comparatively, this treatment did not exhibit any suppressive effect on the symptoms induced by ToMV. Plants separately treated with *Bs* and seaweed extract did not show significantly different disease indexes at 14 and 28 dpi. There was no significant difference in the disease severity index of plants simultaneously treated with *Pf*-*Bs* and, or *Pf*+*Bs*+*Sa*. Finally, the lowest disease severity index belonged to plants simultaneously treated with *Pf*+*Bs*+*Sa* at 14 and 28 dpi (29 and 46.27, respectively). Healthy plants remained asymptomatic till the end of the experiment. Nevertheless, the highest inhibitory effect on the expression of symptoms was obtained when plants were treated with *Pf*+*Bs*+*Sa*, indicating the synergistic effect of these agents to induce resistance against ToMV in the tomato rhizosphere. In general, symptom ratings are qualitative, and one of the common methods in evaluating and comparing the effect of treatments on viral infection is the use of evaluation and scoring methods [27].

In this study, chlorophyll content was measured at 28 dpi. Comparison of the mean of the results obtained in this test showed that non-treated ToMV-infected plants had the lowest chlorophyll content (18.43) (Table 2). All bacterial and algal treatments, separately and in combination (*Pf*+*Sa* and *Bs*+*Sa*), resulted in a relatively lower chlorophyll content indicating the effect of viral infection on the photosynthetic capacity of plants. However, the chlorophyll content of plants in these treatments was significantly higher than that of ToMV-infected plants (Table 2), showing the inhibitory effect of these agents on ToMV-reduced chlorophyll content. However, the highest inhibitory effect was found when plants were pre-treated with *Pf*+*Bs*+*Sa* where the chlorophyll content was similar to that of healthy plants. Loss of chlorophyll structure and function leads to the appearance of symptoms associated with photosynthesis [47]. Based on the previous findings, infection with *Cucumber mosaic virus* causes the loss of photosynthetic pigments in tomatoes and cucumbers [43; 45]. Decreased chlorophyll content indicates a reduction in plant greening and consequently decreased plant chlorophyll which will eventually lead to reduced plant photosynthetic potential. Taken together, it can be concluded that simultaneous pre-treatment of tomato plants with the rhizobacteria and brown alga extract can increase in tolerance of tomato plants to tomato mosaic disease.

Table 1. Mean disease severity values for different treatments in tomatoes treated with probiotic bacteria and seaweed extract challenged with ToMV.

Treatment*	Disease Severity† (%)	
	14 dpi	28 dpi
Pf	66.1[b]	75.62[b]
Sa	55.66[c]	54[dc]
Bs	57.33[c]	53.3[c]
Pf +Sa	48.66[c]	47[d]
Bs+Pf	57.33[c]	54[dc]
Sa+Bs	56[c]	59.31[bc]
Pf +Sa+Bs	29[d]	27.46[e]
Positive control (ToMV-inoculated)	76.32[a]	79.65[a]
Negative control (healthy plant)	0[e]	0[f]
SEM	2.51	2.02
P-treat	*p*< 0.0001	*p*< 0.0001

* *Pf*: *P. fluorescens, Bs*: *B. subtilis*, *Sa*: seaweed extract, SEM: standard error of the mean.
† Same letters are not significantly different at the level of *P*=0.05 by DMRT.

Table 2. Means of chlorophyll index, and other plant growth parameters in ToMV-challenged tomato plants pre-treated with probiotic bacteria and seaweed extract.

Item	CI	H (cm)	RFW (gr)	SFW (gr)	RDW (gr)	SDW(gr)
Pf	30.03[b]	54.31[b]	6.39[cb]	29.63[b]	0.93[cb]	8.9[b]
Sa	28.16[b]	56.91[b]	6.61[cb]	32.63[b]	0.86[cb]	8[b]
Bs	25.43[b]	54.53[b]	6.93[cb]	28.57[b]	0.87[cb]	5.52[bc]
Pf+Sa	25[b]	57.5[b]	6.88[cb]	29.28[b]	0.99[ab]	6.03[bc]
Bs+Pf	27.5[b]	58.2[b]	6.77[cb]	31.86[b]	0.95[cab]	8.5[b]
Sa+Bs	28[b]	59.2[b]	6.70[cb]	31.81[b]	0.93[cb]	7.1[bc]
Pf+Sa+Bs	38.4[a]	66.33[a]	8[a]	48.1[a]	1.2[a]	12.3[a]
PC	18.43[c]	40.66[c]	5.3[c]	25.91[b]	0.70[c]	4.9[c]
NC	36.98[a]	54.09[b]	7.23[ab]	44.23[a]	1[ab]	13.4[a]
SEM	2.082	2.46	0.651	2.22	0.070	1.053
P- treat	*P*< 0.0002	*P*< 0.0013	*P*< 0.006	*P*< 0.0001	*P*< 0.0107	*P*< 0.0003

Pf: *P. fluorescens, Bs*: *B. subtilis*, *Sa*: seaweed extract, PC: positive control (ToMV-infected plant), NC: negative control (healthy plant), SEM: standard error of the mean, OC: optical concentration, CI: chlorophyll index, H: height, RFW: root fresh weight, SFW: shoot fresh weight, RDW: root dry weight, SDW: shoot dry weight. The same letters are not significantly different at the level of *P*=0.05 by DMRT.

Growth indices of the plants were investigated at 28 dpi and the results were presented in Table 2. All treatments could significantly increase the plants' height compared to non-treated ToMV-infected plants. The highest plant height (66.33 cm) was observed in the treatment of *Pf+Bs+Sa* compared to the infected control (40.66 cm). Also, the highest values of root and shoot fresh weights (8 g and 48.1 g, respectively) were recorded for plants treated with *Pf+Bs+Sa*. The highest root and shoot dry weights (1.2 g and 12.3 g, respectively) were observed for the plants treated with *Pf+Bs+Sa* (Table 2).

In this study, pre-treatment of plant growth-promoting bacteria (PGPR) and brown alga extract significantly increased chlorophyll content and growth indices (Table 2) which subsequently led to deteriorated symptoms in the ToMV-infected plants (Table 1). Improved growth indices due to pre-treatment of PGPR and alga extract are considered a tool in combating viral diseases in plants [10]. When plant growth indices are improved by probiotic agents, the severity of host infection by viral agents decreases [30], which is consistent with the results of this study. One possible reason is that increased growth rates in plants treated with probiotic rhizobacteria may reduce the time required for severe viral symptoms to develop [30].

Over the past few decades, algae were the only focus of aquaculture feed with few considerations to bacteria. However, the use of algae in agriculture was found to improve the growth indices by increasing the growth of shoots and chlorophyll content in the plant, and the use of these biological agents, therefore, has been considered a measure to manage diseases. Also, there are several reports on plant growth enhancement due to the use of algae [6,15]. For instance, using *P. stutzeri* in combination with the alga *Spirulina platensis* has increased vegetative growth in onions [13]. When a combination of PGPR is used, synergistic properties may appear which intensify their performance [1,3,18]. Probably one of the reasons for the multiple applications of PGPR is the use of different mechanisms by which they increase host growth and, indirectly, inhibit disease agents. Algae can be formulated with various agents; for example, they can

be used with PGPR [11]. Bacteria, along with algae, can produce plant growth stimulants or, conversely, have a negative effect on host growth and development. Also, these agents can induce disease resistance by induction of systemic resistance within plants [20]. According to the findings of this study, PGPR and brown alga extract can have a synergistic effect and be used as biocontrol agents in the ToMV-tomato pathosystem. Although these agents could reduce the adverse effect of ToMV infection on chlorophyll content and growth indices of tomato plants, they could not completely suppress the virus replication within the infected tissues. Although rhizobacteria have been widely used against plant pathogens, this is the first study on multiple applications of rhizobacteria and brown algae to decrease ToMV-induced disease in tomatoes. Therefore, more research on algal–bacterial interactions helps the commercialization stakeholders, and understand some of the basic and important questions. Since no research has been done on the synergistic effect of PGPR and brown alga, the results of this study will allow us to address such issues for future research of these biocontrol agents in various environmental and biotechnological applications.

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