Research Article

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Antiviral activity of titanium dioxide nanostructures as a control strategy for broad bean strain virus in faba bean

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ABSTRACT

BACKGROUND: This study fabricated titanium dioxide nanostructures (TDNS) to control broad bean stain virus (BBSV) in faba bean plants. Protection of faba bean against BBSV was evaluated biologically with respect to virus severity, reduction in BBSV accumulation and expression of a pathogenesis-related gene.

RESULTS: The results indicate that faba bean plants treated with TDNS show a significant reduction in disease severity relative to untreated plants. The regulatory and defense gene involved in the salicylic acid signaling pathway was highly expressed in faba bean plants treated with TDNS compared with untreated plants. The structural features of TDNS, such as the small particle size and suitable shape, contributed to its high efficacy against BBSV. Growth of faba bean plants treated with TDNS was significantly enhanced relative to untreated plants.

CONCLUSION: TDNS is an important, eco-friendly and safe strategy for controlling BBSV in faba bean and this study is the first report of this control strategy.

Keywords: plant virus; faba bean; nanoparticles; resistance induction; control

1 INTRODUCTION

Faba bean (*Vicia faba* L. Fabaceae) is one of the most important food crops in Egypt and the world. The beans are very sensitive to viral diseases, which cause a significant reduction in productivity worldwide.[1]

Large fluctuations in the productivity of the faba bean crop annually as a result of attack by plant viruses may be due to several factors, including location, cultured species and environmental factors.[2] Broad bean stain virus (BBSV) is one of the viruses that infect seeds in the soil and has been discovered in different regions of the world, for example, Africa, Asia, Europe and the Middle East. The virus is seed-borne in *Lens culinaris* (lentil), *Pisum sativum* (field pea), *Vicia faba* (faba bean, broad bean, tick bean) and *V. palaestina*.[3]

Control of plant pathogens can be achieved by direct antimicrobial activity or by inducing plant systemic resistance against the pathogen. Plant resistance to attack by pathogens can be triggered by treating plants with many chemicals or biological agents.[4] Stabilization of systemic acquired resistance (SAR) is related to the accumulation of pathogenesis-related (PR) proteins, salicylic acid (SA) and jasmonic acid (JA) throughout the plant.[5]

Nanomaterials always have good properties compared with the same materials at normal size.[6] Nanoscience has improved the efficiency and effect of materials by converting them to nanoparticles. Recently, there has been a new trend, the use of nanotechnology in the control of plant pathogens.[7] Titanium dioxide (TiO_2) has many applications due to its wide pH range, high chemical stability, high photoactivity, and the presence of many active adsorption sites on the surface of the molecule that help in absorbing pollutants. Because of its high photoactivity, TiO_2 is widely applied as a water treatment for the removal of different pollutants.[8] The antimicrobial effect of TiO_2 was first shown against *Escherichia coli*[9] and this was followed by several studies using TiO_2 against a wide range of microorganisms such as bacteria and viruses.[10,11] However, despite significant progress in understanding mechanized resistance by a plant against disease attack, there is little information on the mechanism of induced resistance in the plant using nanoparticles against viruses.

The efficiency of TiO_2 and its surface reactions are limited by several factors such as its surface morphology, crystalline structure and surface area to size ratio.[12] Scientists have focused on the synthesis of TiO_2 with new features and functionalities that make it suitable for green environmental applications such as the effective control of plant diseases.

The objectives of this study were to fabricate titanium dioxide nanostructures (TDNS) with unique characteristics and to evaluate the ability of TDNS to control BBSV in faba bean plants with respect to a reduction in disease severity, accumulation of BBSV in

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bean plants as measured by enzyme-linked immunosorbent assay (ELISA), and expression of regulatory and defense genes by reverse transcription polymerase chain reaction (RT-PCR). The effect of nanoparticles on some growth characteristics of faba bean was also examined.

2 MATERIALS AND METHODS

2.1 Chemicals

All chemicals were of analytical grade and were used without further purification. Titanium(IV) fluoride (TiF_4) was from Sigma-Aldrich Co., Ltd (St. Louis, MO, USA). Oleic acid was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Isopropanol was from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Nitrophenylphosphate, diethanolamine, ethylenediamine acetic acid and phosphate-buffered saline containing Tween (PBS-T) with a purity of 99.9% were from Sigma-Aldrich Co., Ltd (St. Louis, MO, USA).

2.2 Synthesis TDNS

TiO_2 nanostructures were from Nanotech Egypt Co. (Giza, Egypt). NanoTech Egypt is the only company in Egypt and the first in the Arab world to fabricate different nanomaterials. TiO_2 was synthesized through a self-assembly process as described by Goma *et al.*,[8] with some modification. Briefly, 2 g of TiF_4 was dissolved in 50 mL of isopropanol and Milli-Q H_2O (1:5:1 v/v/v) under mechanical stirring. When completely dissolved, 5 mL of oleic acid was added dropwise, and the solution stirred for 12 h, followed by autoclaving for 12 h in a Teflon-lined stainless-steel autoclave increasing the temperature at 5°C/min to 180 °C. The obtained white product was collected centrifugally after cooling and washed in Milli-Q H_2O and ethanol to remove soluble impurities. The powder was dried at overnight and finally calcined at 500 °C for 4 h under air to obtain the final hollow sphere nanostructures.

2.3 Characterization of fabricated TiO_2

Characterization of fabricated TiO_2 was carried out under supervision at the Nanotechnology Centre, Mansoura University. Shape surface topology and mapping data of TDNS were studied using a field emission scanning electron microscope (FE-SEM; JEOL JSM-7001F) at 20 kV and wide-angle powder X-ray diffraction (WA-XRD) was applied using an 18 kW diffractometer (Bruker D8 Advance) to investigate the phase and crystal structure of fabricated TDNS.

2.4 Treatment of faba bean plants

TDNS was applied to faba bean plants (30 plants in each treatment, each plant carrying four or five leaves and planted in a 30 cm diameter pot containing a sterilized planting mixture of clay/sand/peat moss; 1:1:1 w/w/w) by foliar spray and soil drench at a concentration of 150 μM. The foliar spray treatment was conducted by spraying faba bean seedlings with 200 mL of water containing TDNS. Faba bean plants were inoculated with BBSV after 24 h of TDNS spraying. The soil drench treatment was carried out by irrigating healthy faba bean seedlings with TDNS using 200 mL of water containing TDNS 24 h before virus inoculation. Inoculation of the plants with BBSV was performed mechanically.

2.5 BBSV inoculation

The BBSV inoculum used study consisted of infected faba bean leaf tissue ground in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.002 M EDTA. The inoculum was prepared by adding 1 g of tissue per 50 mL of sodium phosphate buffer. All materials used in the inoculation were incubated at 4 °C prior to inoculation and kept on ice during inoculation. Inoculation was carried out by rubbing the inoculum onto the oldest faba bean leaf 4 weeks after planting. Disease severity ratings for faba bean plants were: 0 = no symptoms; 2 = mosaic of the youngest two leaves; 4 = mosaic of the youngest two leaves with progression of symptoms into sequentially older leaves; 6 = mosaic progressed beyond the two youngest leaves, with six leaves expressing some form of BBSV-induced symptoms; 8 = mosaic progressed beyond the two youngest leaves, with all leaves expressing some form of BBSV-induced symptoms, with plants also having stunted growth (including both reduced internode extension and smaller leaves); and 10 = plants stunted with most of the leaves being small. The disease severity results were expressed as mean values of 10 samples in each treatment.

2.6 ELISA

BBSV concentration was determined by indirect ELISA, according to the method described by Elsharkawy and El-Sawy[13] with some modification. Leaf samples were collected 7 and 14 days after inoculation, ground in 50 mM carbonate buffer (pH 9.6) and added at a final dilution of 1:10 (g tissue per mL buffer) to microtiter plates. The plates were incubated overnight at 4 °C and washed three times with PBS-T. Anti-BBSV (primary antibody) was added to the plates at a concentration of 1 fg mL^{-1} in PBS-T. Plates were incubated at 37 °C for 1.5 h and washed three times with PBS-T. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase was diluted in PBS-T (1:7500 v/v) and then appliedto the plates. Plates were incubated at 37 °C for 1 h and washed three times with PBS-T. The reaction was allowed to develop at room temperature by addition of the substrate (*p*-nitrophenyl phosphate at 1 mg mL^{-1} in 10% diethanolamine, pH 9.8 (Sigma, St. Louis, MO, USA)). Absorbance at 405 nm was determined using a Bio-Rad model 550-microplate reader (Hayward, CA, USA). The immune analysis was repeated three times and each treatment consisted of four replicates of two leaves each.

2.7 RT-PCR analysis

To identify the possible mode action of TDNS against BBSV in faba bean, inoculated faba bean leaf tissue treated with TDNS and inoculated untreated plants were sampled 2 days after inoculation. A qRT-PCR was carried out as described by Wang *et al.*[14] using gene-specific primers (Table 1). The amount of target gene was normalized over the abundance of the constitutive *EF1a* gene (Table 1) to standardize the data. A 7500 RT-PCR system (Applied Biosystems) was used to perform qRT-PCR and the data were analyzed using the ABI PRISM 7500 Software Tool (Applied Biosystems, Foster City, CA, USA) that calculates the average expression ratio (standard curve method of relative gene quantification) and the *P*-values to assess the statistical relevance of any changes. To estimate the relative expression of the gene quantitatively the comparative 2-CT method was used and threshold values (CT) generated from the ABI PRISM 7500 Software Tool (Applied Biosystems) were employed.[15]

2.8 Direct effect of TDNS on the infection of BBSV under greenhouse conditions

To evaluate the direct antiviral activity of TDNS as a possible mode of action against BBSV, five faba bean seeds were sown

Table 1. Forward and reverse primers sequence for *PR1* and *EF1α* genes

Gene	Forward primer(5′–3′)	Reverse primer(5–3′)
PR1	CAGTGGTGACATAACAGGAGCAG	CATCCAACCCGAACCGAAT
EF1α	GTGAAGCCCGGTATGCTTGT	CTTGAGATCCTTGACT GCAACATT

in pots (25 cm in diameter) containing soil, sand, and compost at a ratio of 1:1:1. Six pots formed one replicate and there were four replicates for each treatment. Equal volumes of aqueous extracts from virus-infected faba bean leaves and TDNS (150 μM) were mixed and kept at room temperature (28 °C) for 10 min. The mixture was inoculated onto 3-week-old healthy faba bean plants. Disease severity and virus titer in treated plants relative to untreated plants were calculated on the basis of visible symptoms and ELISA, respectively.

2.9 Effect of TDNS on some growth characteristics of faba bean

To evaluate the effect of treatment by TDNS on some growth properties of faba bean plants, measurements were taken 6 weeks after planting (plant height, number of leaves and number of pods) in treated and untreated plants. The experiment was repeated in triplicate with 10 plants per replicate.

2.10 Data analysis

For analysis of variance (ANOVA) of the obtained data, XLSTAT PRO statistical analysis software (Addinsoft) was used. Fisher's least significant difference (LSD) test was used to separate the mean of each treatment. All analyses were performed at a significance of $P \leq 0.05$.

3 RESULTS

3.1 Characterization TiO_2 nanostructures

The crystal structure of the synthesized TDNS was characterized using WA-XRD analysis. The WA-XRD profile shows strong sharp and specific diffraction peaks for TDNS (4). The specific peaks of TDNS coincide with those of an anatase TiO_2 crystal shape (JCPDS: 96–900-9087 and 96–901-5930). The sharp peak at 2θ of 25.24° corresponds to the (011) plane, whereas the peaks at 37.8°, 48°, 53.9°, 55° and 62.66° could be assigned to the (004), (020), (015), (121) and (024) planes, respectively. The average crystal size of TDNS was calculated using the Scherrer equation, $t = 0.91\lambda/(B\cos\theta)$, where t is the crystal size, λ is the incident radiation wavelength (1.5406 Å), θ is the Bragg angle, and B is the full-width at half-maximum of the diffraction peak.

FE-SEM profiles (Fig. 1b) show the well-controlled synthesis of the TiO_2 structure with smooth inner and rough external surfaces, and low wall thickness. A top-view of TDNS shows a uniform sphere and a narrow size distribution of ~ 3–5 μm diameter, with open puncture having a width of ~ 700 × 900 nm. Moreover, the length and bottom width of the hollow sphere ranged between 3 and 4 μm, as shown in the plane and bottom-view profiles. The hollow-shape of TDNS may be formed by the gathering of many TiO_2 nanosheets. This may have occurred as a result of the formation of hydrogen bonds on the TiO_2 surface due to the presence of carboxylic groups of oleic acid.[16] These birdcage-like perforations contribute significantly to the increased exposed interaction area between the plant virus and TiO_2, and subsequently increase the antiviral activity of TDNS.

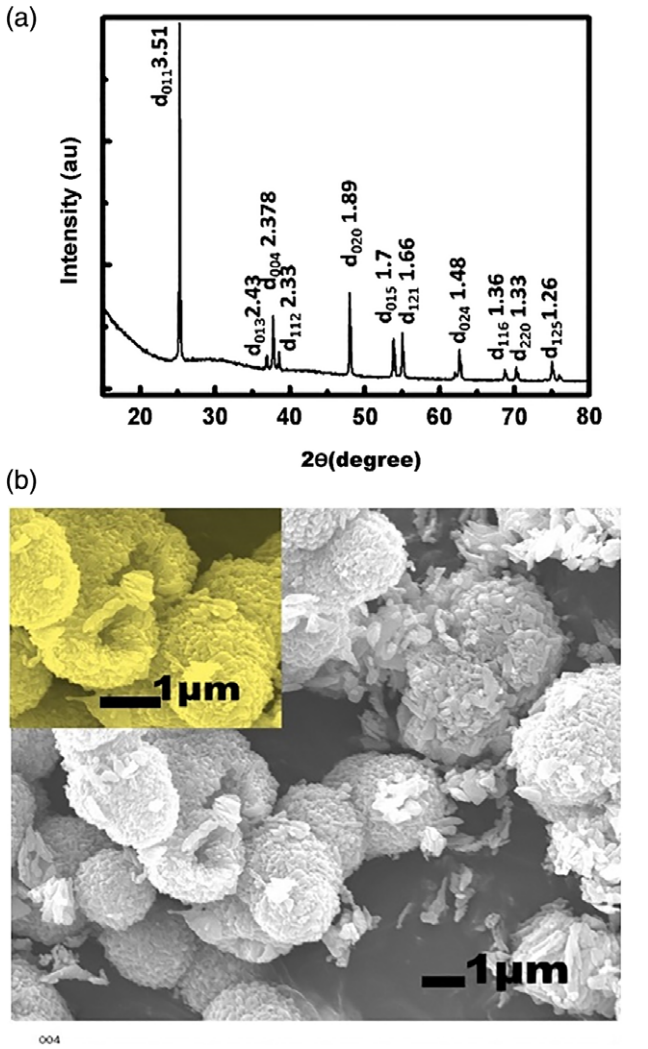

Figure 1. WA-XRD profile (a) and FE-SEM mapping (b) of TiO_2 nanostructures.

3.2 Systemic protection of TDNS against BBSV in faba bean

Symptoms of BBSV appeared 10 days after inoculation and ranged from mild mosaic in young non-inoculated leaves to severe mosaic with stunting. At 14 days after inoculation, faba bean plants treated with TDNS exhibited a dramatic reduction in BBSV symptoms compared with untreated control plants, which showed severe symptoms of mosaic with small deformed leaves (Fig. 2). Faba bean plants treated with titanium dioxide showed a significant decrease in disease severity 2 weeks after infection with BBSV compared with untreated plants (Fig. 3). Also, the decrease in disease severity in the faba bean plants treated with the TDNS by foliar spray was greater than in plants treated by soil drenching. Two weeks after inoculation, BBSV accumulation was significantly reduced in faba bean plants treated with TDNS compared with untreated plants (Fig. 3). BBSV accumulation was lower in faba bean plants treated with TDNS by foliar spray relative to plants treated by soil drench (Fig. 4).

3.3 Effect of TDNS on expression of an SA-inducible defense-related gene

Expression of the pathogenesis-related gene (*PR1*) in faba bean plants treated with TDNS either by foliar spray or soil drench was

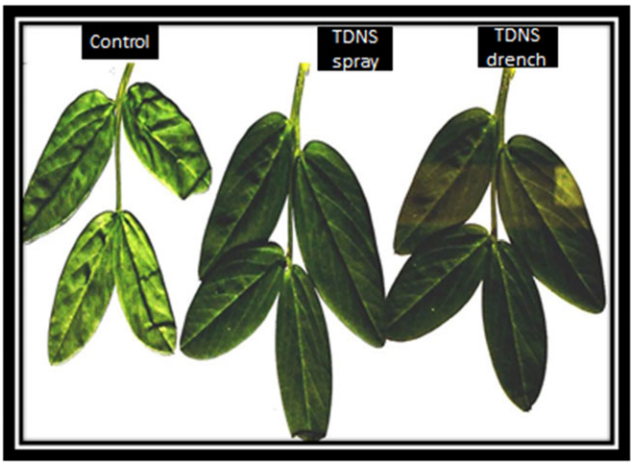

Figure 2. Disease symptoms caused by broad bean stain virus (BBSV) infection in untreated faba plants (control), and faba bean treated with titanium dioxide nanostructures (TDNS) by foliar spray and soil drench after 2 weeks of virus challenge inoculation.

initially detected 2 days after inoculation by BBSV. The *EF1a* gene was used as internal control. There was no significant difference in the expression of resistance genes in bean plants treated with spray or soil drench (Fig. 5). The target responsive gene showed weak stimulated expression after BBSV infection in untreated plants.

3.4 Direct antiviral activity of TDNS against BBSV

Direct antiviral activity of TDNS against BBSV was evaluated by mixing TDNS with BBSV and spraying the mixture onto faba bean plants. The results given in Table 2 show that the severity of BBSV was significantly reduced in sprayed faba bean plants relative to untreated plants (Table 4). Moreover, the ELISA-measured virus titer was significantly reduced in faba bean plants treated with TDNS relative to untreated plants.

3.5 Effect of TDNS on faba bean growth

Treatment of faba bean plants with TDNS significantly increased growth, as measured by plant height, and the number of leaves and pods in each plant compared with untreated plants (Table 3). The measured growth characteristics were greater in faba bean plants treated by foliar spray compared with soil drench (Table 3).

4 DISCUSSION

The increased costs of pesticide use to control pests, especially in poor areas of the world, and consumers' need for pesticide-free food led us to seek alternatives to pesticides. In addition, there are many viral plant diseases for which there are few or only ineffective chemical controls; sometimes there is no chemical control available.[17] Therefore, the use nanoparticles of suitable size and shape, as well as unique properties may provide a non-traditional solution to the problem of controlling viral plant diseases. The size and properties of these materials provide new uses in the control of plant diseases.[18] Plant protection against viruses by nanomaterials may occur by different mechanisms at the same time, including direct effects of nanoparticles on the viral cell including death, or by improving the capabilities of the plant defense by inducing resistance, taking into account the mechanics of occurrence and use in the field of plant protection.[19]

In this study, the use of metal oxides in nanotechnology is a new strategy in the fight against plant diseases, either by direct antiviral activity or by enhancing plant systemic resistance against the pathogen. In our study, control of the virus in bean plants (by direct mixing of TNDS with viral extract or application of TNDS to plants before inoculation with virus) was evaluated by determining the number of asymptomatic plants and the development of symptoms; the absence of virus or its accumulation were also estimated using ELISA. The results showed a significant decrease in disease severity in treated bean plants compared with untreated plants, and this was strongly correlated with the lower ELISA values in faba bean plants treated with TDNS compared with untreated plants. Also, our results showed the high efficacy of

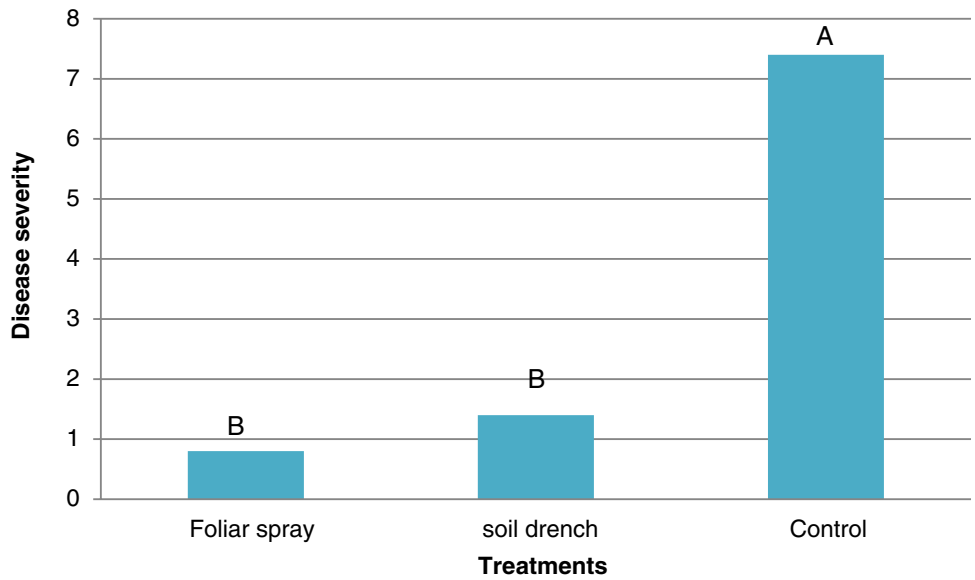

Figure 3. Disease severities in broad bean stain virus (BBSV)-inoculated faba bean plants treated with titanium dioxide nanostructures (TDNS) relative to untreated control plants after 14 days of inoculation. Columns represent mean values ($n = 10$, error d.f. = 27). Different letters above columns indicate significant differences by the Steel–Dwass test for faba bean ($P \leq 0.05$).

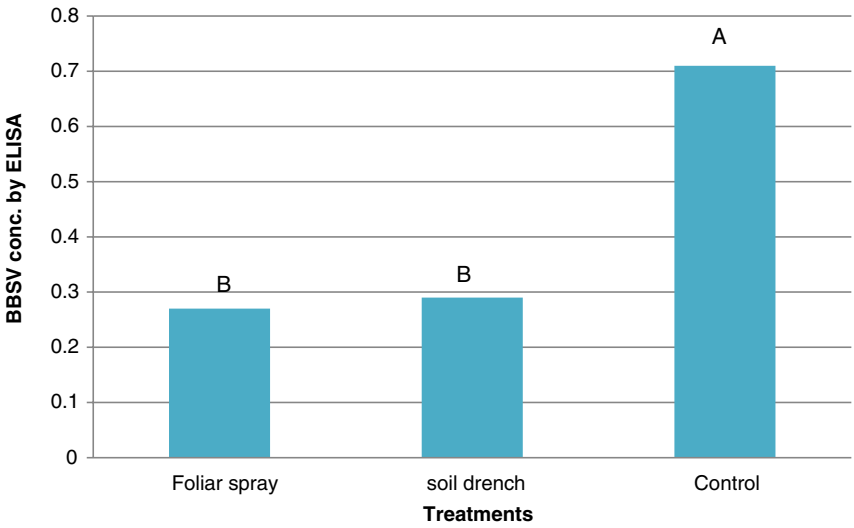

Figure 4. Broad bean stain virus (BBSV) accumulation in leaves of faba bean treated titanium dioxide nanostructures (TDNS) at after 14 days of inoculation. Columns represent mean values ($n = 4$, error d.f. = 9). Statistical comparisons are among treatments within primary inoculated or secondary non-inoculated leaves and the same line. Different letters indicate significant differences using Fisher's LSD ($P \leq 0.05$).

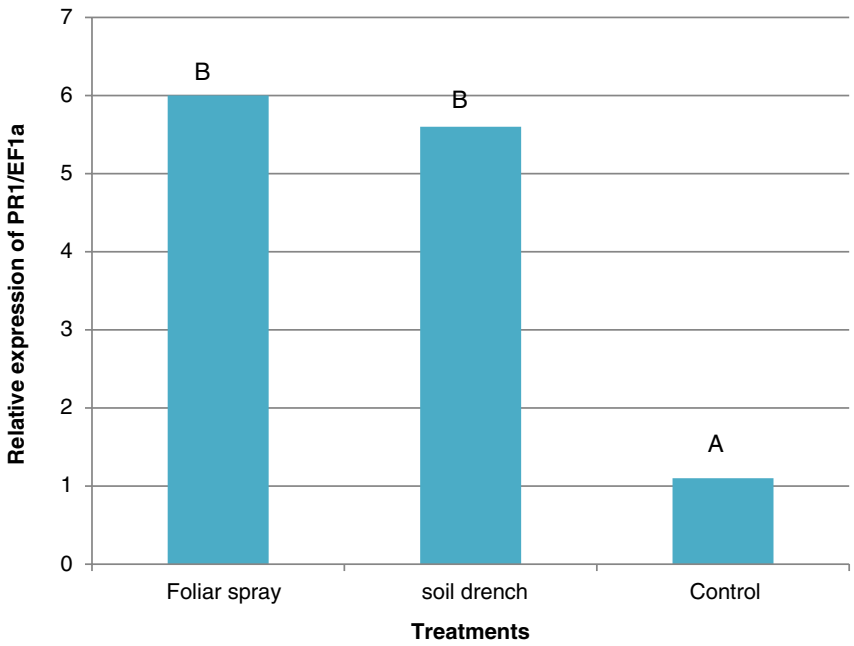

Figure 5. Expression of genes in leaves of faba bean plants treated with titanium dioxide nanostructures (TDNS) 1 day before challenge inoculation with broad bean stain virus (BBSV).

foliar spray treatment of TDNS against BBSV relative to soil drench treatment. This may be due to direct and rapid contact with the plant virus compared with soil drench or TDNS being adsorbed to soil particles, subsequently reducing the amount taken up by plants compared with foliar application.[20] Therefore, foliar spray was the most effective treatment for resistance induction against BBSV.

Molecular and genetic methods are used to explain the mechanism of plant protection against plant pathogens by resistance induction due to in evolution of defense characteristics between the plant and the pathogens that attack it.[21,22] Our data confirmed that application of TDNS upregulated expression of the SA-responsive PR gene (*PR1*), compared with the *EF1a* gene. Molecular and biochemical data in this study indicated that the mode of action of faba bean protection against BBSV may be because use of TDNS leads to the formation of mechanical barriers that prevent pathogenesis and improve system-induced resistance.[23–25] In our study, TDNS were capable of controlling viral infectivity of BBSV, most likely by blocking the interaction of the virus with the cell, which might depend on the size and zeta potential of the nanoparticles.[26] Application of TDNS places bean plants under stress and stimulates the plant to induce the resistance-related gene. The gene is expressed as a protein that blocks the plasmodesmata and prevents BBSV translocation and distribution inside bean plants. Alternatively, TDNS may activate expression of the defense-related gene and have an important role in the development of stress indicators in the plant such as SA, JA and ET.[27,28]

Table 2. Antiviral effect of titanium dioxide nanostructures (TDNS; 150 μM) against broad bean stain virus (BBSV) faba bean plants

Treatments	Disease severity	BBSV concentration
TDNS	1.30 ± 0.10^b	0.21 ± 0.01^b
Control	7.50 ± 0.27^a	1.12 ± 0.11^a

Different letters indicate significant differences by Fisher's LSD at $P \leq 0.05$.

Table 3. Effect of titanium dioxide nanostructures on some growth characters of faba bean plants

Treatment	Plant height (cm)	No. leaves	No. pods
Spray	72.40 ± 1.32^a	23.40 ± 0.51^a	7.00 ± 0.46^a
Soil drench	68.10 ± 2.11^a	22.70 ± 0.57^a	6.70 ± 0.33^a
Control	58.70 ± 1.35^b	19.80 ± 0.46^b	5.60 ± 0.42^b

Different letters indicate significant differences by Fisher's LSD at $P \leq 0.05$.

In this study, TDNS showed potential to protect faba bean against BBSV at very low concentrations. Therefore, to obtain effective control of this virus, faba bean plants should be treated with TDNS before emergence of viral disease symptoms to improve the self-defense ability of the plant towards the disease.

The impact of TDNS on the growth of faba bean plants and thus its productivity is an important factor that should be taken into account in addition to its ability to protect the plant against BBSV. The results of this study showed that all the growth parameters of the faba bean plants improved after treatment with TDNS. This is in agreement with Mahmoodzadeh and Aghili[29] who reported that TDNS improved germination and growth in treated plants. This increase may be because titanium stimulates the production of more carbohydrates and improves growth by increasing photosynthesis due to its ability to absorb light.[30–32] Titanium dioxide also regulates the activity of enzymes responsible for the nitrogen metabolism such as, glutamate dehydrogenase, nitrate reductase, glutamine synthase and glutamic–pyruvic transaminase which helps the plant to absorb nitrates. Moreover, these enzymes help in the conversion of inorganic nitrogen to organic in the form of protein and chlorophyll, which may be responsible for the observed increase in fresh and dry weight.[33,34] Finally, treatment of faba bean plants with TDNS reduces the severity of the pathogen and subsequently improves growth.

These results are important for future field applications where low amounts of TDNS can be used under controlled conditions on young plants, thereby reducing the risk of human and environmental exposure to nanostructures.[35] The study showed that TDNS can protect faba bean against BBSV by direct effects on the virus or by improving the plant's own defenses through induced resistance, or by both mechanisms together. Thus, this study provides an effective and environmentally friendly strategy to control BBSV in faba bean plants.

5 CONCLUSIONS

Based on estimations of disease severity, as well as genomic and molecular analysis, TDNS are promising for control BBSV infection in faba bean via inducing systemic resistance and direct antiviral activity that disease severity in treated faba bean. Moreover, the application of TDNS enhanced the growth of faba bean compared with untreated control plants. TDNS could be used as new a strategy to control BBSV in faba bean plants.

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