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Research Article

Seed priming enhances sugar beet resistance to beet curly tip Iran virus

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Abstract: This study assessed the effects of seed treatment with *Bacillus subtilis*, *Trichoderma zelobreve*, and β-aminobutyric acid (BABA) on sugar beet genotypes on growth, physiological characteristics, and responses to beet curly top Iran virus (BCTIV; *Becurtovirus betae*) infection. Two sugar beet genotypes, SB23 and 980043, were subjected to microbial and chemical seed priming, followed by agroinoculation with BCTIV under greenhouse conditions. Primed plants showed significantly lower symptom severity and higher BCTIV accumulation than non-primed controls ($P < 0.05$). Growth parameters, including shoot and root fresh and dry weights, chlorophyll and carotenoid contents, leaf area index, and plant height, were significantly enhanced in primed plants. Antioxidant enzyme activities, including polyphenol oxidase (PPO), peroxidase (POX), and catalase (CAT), were significantly elevated in primed plants, indicating activation of systemic defense responses. Treatments effectively reduced BCTIV impact by increasing resistance, which is associated with stronger biochemical defense responses and improved physiological status. These findings indicate that seed treatment with *B. subtilis*, *T. zelobreve*, and BABA promotes sugar beet growth, enhances antioxidant defense responses, and reduces BCTIV accumulation, representing a promising and sustainable strategy for disease management in affected areas.

Keywords: BCTIV accumulation, antioxidant enzyme activity, seed priming, quantitative PCR

Introduction

Sugar beet is susceptible to various pathogens, including fungal, bacterial, viral, and nematode pathogens. Among these, beet mosaic disease, beet rhizomania, and beet curly top disease are the most important. The primary causative agent of beet curly top disease, which affects sugar beet and several other dicotyledonous plants in Iran, is the beet curly top Iran virus (BCTIV; *Becurtovirus betae*). This disease severely reduces sugar beet yields, especially in arid and semi-arid regions (Yıldırım *et al*., 2022). BCTIV belongs to the family Geminiviridae, which is divided into 15 genera based on characteristics such as host range, genomic organization, sequence identity, number of genomic components, and insect vector (Roumagnac *et al*., 2022). The disease was first reported in Iran in 1967 (Gibson,

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1967) and affects more than 300 species across 44 plant families, including crops, ornamental plants, and weeds (Bennett, 1971).

Beet curly top disease in Iran is caused by beet curly top virus-Severe (BCTV-Svr; *Curtovirus betae*), beet curly top Iran virus (BCTIV; *Becurtovirus betae*), sesame curly top virus (STCV; *Turncurtovirus sesami*) (Hasanvand *et al.*, 2018), turnip curly top virus (TCuCV; *Turncurtovirus brassicae*) (Varsani *et al.*, 2014), and turnip leaf roll virus (TLRV; *Turncurtovirus rapae*) (Kamali *et al*., 2016; Heydarnejad *et al*., 2007; Yazdi *et al.*, 2008). Recently, BCTIV (*Becurtovirus betae*) has also been reported in Turkey, indicating its geographical expansion beyond Iran (Yıldırım *et al*., 2022). BCTIV is naturally transmitted by insects in a persistent non-propagative manner (Kamali *et al*., 2016; Varsani *et al*., 2014). The leafhopper *Circulifer haemataceps* is the vector of BCTIV in sugar beet fields in Iran (Taheri *et al*., 2012; Fatahi *et al*., 2012). Disease symptoms include stunted growth, interveinal chlorosis, leaf curling, and vein swelling (Heydarnejad *et al*., 2007; Strausbaugh *et al*., 2008Yıldırım *et al*., 2022).

Various strategies manage plant diseases. Among these, chemical priming and biopriming of seeds have gained attention as eco-friendly approaches that enhance plants' capacity to rapidly and robustly activate immune responses upon pathogen challenge. Biopriming with *Trichoderma harzianum* has reduced the incidence of BCTV-Svr in sugar beet by increasing peroxidase and polyphenol oxidase activities in primed plants compared with controls (Mafakheri *et al*., 2024). In addition, application of *Bacillus amyloliquefaciens* (Ba13) to tomato seedlings increased expression of systemic resistance-related genes (PR1, PR2, PR3) and activities of defense enzymes (phenylalanine ammonia lyase, polyphenol oxidase, peroxidase, β-1,3-glucanase, and chitinase), which led to the suppression of tomato yellow leaf curl virus (TYLCV) by decreasing the infection rate, disease severity, and virus accumulation (Guo *et al*., 2019).

Seed treatments with *Bacillus subtilis* followed by spraying the bacterial suspension onto tomato seedlings also reduced TYLCV severity by increasing peroxidase activity (Ghanem and Aqil, 2021). Similarly, lipopeptides from *Bacillus amyloliquefaciens*, including NPR1 and PR8 (chitinase), induce systemic resistance genes in sugar beet roots, which is associated with reduced infectivity of *Polymyxa betae*, the rhizome vector (Desoignies *et al*., 2013). Other examples of chemical defense inducers include the disease-suppressing effects of β-aminobutyric acid (BABA) seed treatment (Baccelli and Mauch-Mani, 2016). BABA seed treatment in sugar beet reduces susceptibility to BCTV-Svr, with lowered virus accumulation as measured by qPCR (Mafakheri *et al*., 2024). Similar results have been obtained in zucchini seeds soaked in 1% BABA (Rashid, 2014) and in tomato seeds treated with BABA to inhibit tomato mosaic virus spots on tomato (Khazir and Jarjis, 2012).

Seed treatments with beneficial fungi, antagonistic or symbiotic bacteria, and chemical inducers like BABA offer promising, sustainable disease management strategies by protecting vulnerable seedling stages and promoting germination and growth (Kazemi *et al*., 2026a).

This study aims to evaluate the impact of seed treatment with *B. subtilis*, *T. zelobreve*, and BABA on growth parameters, physiological traits, and defense-response activation in sugar beet plants challenged with BCTIV. Although these treatments have shown promise against other plant pathogens, their effectiveness against BCTIV in sugar beet has not yet been explored; this study addresses that gap.

Materials and Methods

Plant material and growth conditions

Two sugar beet (*Beta vulgaris* L.) genotypes, coded SB23 and 980043, were obtained from the Sugar Beet Seed Institute, Karaj, Iran. Seedlings were grown in an insect-proof greenhouse at 25 ± 2 °C with a 16-hour light/8-hour dark photoperiod during autumn and winter under natural light conditions. The experiments were conducted as a factorial experiment (2 genotypes × 4 priming treatments × 2 virus inoculation

levels) in a completely randomized layout with 31 replicates per treatment; each experiment was independently repeated twice to ensure reproducibility.

Experimental treatments

Plants were assigned to eight treatment groups: seeds treated with either *T. zelobreve* (10^9 CFU/ml), β-aminobutyric acid (BABA) (10 mM), or *B. subtilis* (10^8 CFU/ml), each with virus-inoculated and non-inoculated subgroups, along with two control groups: non-treated, non-inoculated (Control) and virus-inoculated only (V).

Seed treatment procedures

Preparation and application of *B. subtilis*

A fresh culture of *B. subtilis* was grown in Luria-Bertani (LB) broth for 24 hours at 28 °C with agitation at 160 rpm. Subsequently, 1 mL of this culture was inoculated into 100 mL of fresh LB medium and incubated for an additional 4 hours to reach approximately 10^8 CFU/ml. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 minutes at room temperature; the bacterial pellet was resuspended in sterile distilled water to obtain a homogeneous cell suspension, and finally resuspended in 1% (w/v) carboxymethyl cellulose (CMC) to prepare a bacterial suspension of 10^8 CFU/mL for seed coating. Sugar beet seeds were soaked in sterile water for 3 hours, surface-sterilized with 1% sodium hypochlorite for 1 minute, rinsed thoroughly with sterile distilled water, and soaked in the bacterial suspension with shaking at 150 rpm for 3 hours at room temperature. Seeds were then air-dried overnight in a laminar flow hood before sowing (Kazemi *et al*., 2026b).

Preparation and application of *T. zelobreve*

Wheat grains were boiled for 25-30 minutes to soften and sterilized by autoclaving twice at 121 °C for 20 minutes with a 24-hour interval. Each jar was filled with approximately 80 g of dry wheat (140 g after soaking), inoculated with ~0.5 cm agar plugs containing *T. zelobreve*, and incubated at 30 °C for 4-5 days with intermittent shaking to prevent clumping and ensure aeration. Spores were washed off with 120 mL sterile distilled water and centrifuged twice as above, resuspending the pellet in 1% CMC to obtain a 10^9 CFU/mL spore suspension. Surface-sterilized sugar beet seeds were soaked in this suspension with shaking (150 rpm) for 3 hours at room temperature and dried overnight under sterile conditions before planting (Kazemi *et al*., 2026b).

Preparation and application of β-Aminobutyric Acid (BABA)

A 10 mM BABA solution was freshly prepared in sterile distilled water. Seed treatment followed the protocol described above for bacterial and fungal treatments: seeds were soaked in BABA solution for 3 hours with gentle shaking at room temperature, then dried under sterile airflow before sowing.

Virus inoculation

Sugar beet plants were agroinoculated with an infectious clone of beet curly top Iran virus (BCTIV) (GenBank accession no. JQ707949) as described by Heydarnejad *et al.* (2013). Agroinoculation was conducted using *Agrobacterium tumefaciens* strain LBA4404 carrying infectious clones of BCTIV. The bacterial culture was grown in liquid LB medium supplemented with kanamycin (50 μg/ml) and rifampicin (50 μg/ml). As a control, a separate group of plants was agroinfiltrated with *A. tumefaciens* LBA4404 carrying the empty vector under the same conditions. Cultures were incubated at 180 rpm until the optical density at 600 nm (OD600) reached 0.2–0.6 (Grimsley *et al*., 1986). The cells were subsequently harvested by centrifugation at 6000 rpm for 10 minutes and resuspended in sterile distilled water containing 50 mM acetosyringone. Agroinoculation was carried out by leaf infiltration at the four- to six-leaf stage, approximately 40 days after sowing (Sedano *et al*., 2012). Symptom development was monitored (Montazeri *et al*., 2016; Kazemi *et al*., 2025a).

DNA extraction and Quantitative PCR (qPCR) analysis

Leaf tissue samples, all leaves except the virus-inoculated leaf, were collected at 40 days post-

inoculation (dpi). DNA was extracted using the CTAB method as described by Doyle and Doyle (1987).

Virus accumulation in plant tissues was quantified by qPCR using primers targeting a 127 bp segment of BCTIV (Table S1). qPCR was performed using RealQ SYBR Green PCR master mix (Amplicon, Denmark) and an ABI StepOne Real-Time PCR System (Applied Biosystems, USA). Reactions included four biological replicates (each a pooled sample of five plants) and two technical replicates per biological replicate. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method, normalized against the sugar beet small subunit (SSU) rDNA gene (Livak and Schmittgen 2001).

For each sample, ΔCt = Ct (virus) – Ct (SSU).

$\Delta\Delta Ct$ = ΔCt (sample) – mean ΔCt (virus-only control).

Fold change = $2^{-\Delta\Delta Ct}$

Growth parameter measurements

At 40 dpi, plants were harvested to measure the fresh weight of shoots and roots. Samples were then oven-dried at 70 °C for 48 hours to determine dry weight.

Enzyme activity assays

Activities of polyphenol oxidase (PPO), peroxidase (POX), and catalase (CAT) were determined at 2, 4, and 40 days post-inoculation (dpi) to evaluate both early and late biochemical responses induced by BCTIV infection and seed priming treatments. The PPO and POX activities were assayed according to the method described by Siguemoto and Gut (2017), based on absorbance measurements at 420 and 470 nm, respectively. CAT activity was determined following the method of Maehly and Chance (1954) by measuring absorbance at 240 nm. All enzyme assays were performed using a microplate reader (Epoch BioTek, USA). Leaf samples (all leaves except the virus-inoculated leaf) were collected, and 100 mg of tissue was homogenized in appropriate extraction buffers. Each enzymatic assay was performed using four biological replicates with two technical replicates.

Chlorophyll and carotenoid content

Chlorophyll a, chlorophyll b, and total carotenoid contents were quantified spectrophotometrically at 665, 652, and 470 nm, respectively, using the method of (Warren, 2008). Additionally, leaf greenness was assessed in vivo using a SPAD chlorophyll meter.

Assessment of rhizosphere microbial population dynamics

Rhizosphere soil samples were collected at 5 and 11 weeks after planting to evaluate the population dynamics of *B. subtilis* and *T. zelobreve*. Ten grams of soil per sample were suspended in 50 mL of sterile distilled water, serially diluted, and plated in duplicate on Potato Dextrose Agar (PDA) for fungal counts and Luria-Bertani (LB) agar for bacterial counts. Colony-forming units (CFUs) were counted after incubation (Fig. S1) (Mafakheri *et al*., 2024).

Root colonization assessment

Root colonization by *B. subtilis* and *T. zelobreve* was verified using modified root segment plating and staining methods (Phillips and Hayman, 1970). At 5 and 11 weeks post-planting, eight root segments per plant were surface sterilized (70% ethanol and 96% alcohol washes), dried, and plated on PDA amended with ampicillin (for *T. zelobreve*) or LB plates (for *B. subtilis*), and incubated at 28 °C. Colonization percentages were calculated based on the number of segments showing microbial growth relative to the total segments（Fig. S2).

Statistical analysis

All quantitative data were analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC). ANOVA was performed with the GLM procedure, and residual normality was checked using the UNIVARIATE procedure. Severity data, as ordinal data, were rank-transformed and analyzed using the nonparametric methods outlined by Shah and Madden (2004). Mean separations were conducted via LSD tests at $P \leq 0.05$. This analysis revealed no significant differences between the experiments, justifying

pooling the data for final evaluation. Composite analysis was performed with Minitab 18.1.

Results

Primed plants exhibited lower symptom severity of BCTIV

Symptom severity was assessed 40 days after inoculation. The virus-only (V) treatment and *B. subtilis* (BS)-treated plants of the SB23 genotypes, as well as virus-only plants of the 980043 genotypes, exhibited severe symptoms, including intense yellowing, extreme leaf twisting, reduced leaf area, and stunted growth. In contrast, *T. zelobreve* (TZ) and BABA treatments in SB23, and TZ treatment in 980043, showed the least symptom severity, characterized by bright veins with slight vein swelling and leaf crumpling. The incubation period from inoculation to symptom onset varied from 11 to 40 days, depending on genotype and treatment (Table S2, Fig. 1, Fig. S3).

Seed priming significantly increased chlorophyll a, chlorophyll b, and total chlorophyll contents compared to non-stressed (Ctrl) and virus-only (V) treatments.

The highest chlorophyll a content was recorded in the BABA-treated 980043genotypes, while the highest chlorophyll b and carotenoid contents were observed in the *B. subtilis*-treated SB23 genotypes. The lowest pigment contents were found in virus-only treatments of the same genotypes (Table S3, Table 1).

Growth parameters increased upon *T. zelobreve*, *B. subtilis*, and BABA treatments.

Fresh and dry weights of shoots and roots were measured at 40 dpi (Tables 2 and 3). *B. subtilis* treatment resulted in the greatest increase in fresh shoot weight in both genotypes compared to controls. BABA treatment yielded the highest fresh root weight in genotype 980043, whereas *B. subtilis* treatment resulted in the highest fresh root weight in SB23. Similarly, BABA-treated 980043 plants exhibited the highest shoot and root dry weights. In SB23, the highest root dry weight was observed in *B. subtilis*-treated plants, whereas the highest shoot dry weight was recorded in TZ-treated plants. Virus-infected plants showed the most pronounced reductions in fresh and dry weights across genotypes.

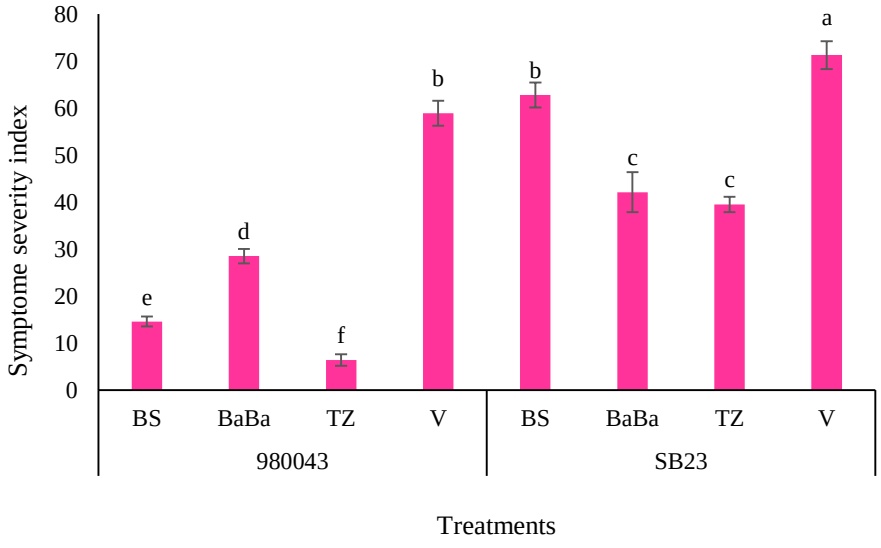


Figure 1 Effects of seed treatments with *Bacillus subtilis* (BS), *Trichoderma zelobreve* (TZ), and β-aminobutyric acid (BABA) on symptom severity index (SSI) in two sugar beet genotypes SB23 and 980043, infected with BCTIV, 40 dpi. SSI was scored on a 1–9 scale. Treatments were: inoculated with BCTIV only (V), other treatments, priming agent at the respective concentration + BCTIV. Values are means of 31 replicates ± SE. Bars marked with different letters indicate a significant difference (LSD test, $P < 0.05$).

Table 1 Effects of *Bacillus subtilis* (BS), *Trichoderma zelobreve* (TZ), and β-aminobutyric acid (BABA) on chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents (µg/mL) in sugar beet genotypes inoculated with BCTIV, 40 dpi. Treatments were: inoculated with BCTIV only (V), Control (no priming, non-inoculated), other treatments, priming agent at respective concentrations + BCTIV. For *B. subtilis* and *T. zelobreve*, the concentration is expressed in CFU/ml, and in the case of BABA, it is expressed in mM. Data are means of four biological replicates and two technical replicates.

Genotypes	Virus & Priming	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total chlorophyll (µg/ml)	Carotenoid (µg/ml)
980043	BS	5.75 ± 0.252 d	4.83 ± 0.044 c ††	10.58 ± 0.234 b	4.36 ± 0.047 b
	BABA 10 mM	8.91 ± 0.095 a	4.55 ± 0.042 d	13.46 ± 0.133 a	2.45 ± 0.157 e
	TZ	4.26 ± 0.125 g	3.62 ± 0.066 g	7.88 ± 0.176 e	3.38 ± 0.153 d
	Virus	3.28 ± 0.205 h	3.36 ± 0.036 h	6.64 ± 0.194 f	2.02 ± 0.176 f
	Control	7.44 ± 0.129 c	3.55 ± 0.063 g	10.99 ± 0.106 b	2.15 ± 0.08 ef
SB23	BS	7.65 ± 0.096 c	6.14 ± 0.075 a	13.79 ± 0.149 a	5.44 ± 0.068 a
	BABA 10 mM	8.21 ± 0.074 b	5.2 ± 0.055 b	13.42 ± 0.088 a	3.61 ± 0.193 cd
	TZ	5.33 ± 0.26 de	4.48 ± 0.076 de	9.81 ± 0.27 c	3.98 ± 0.141 c
	Virus	4.7 ± 0.169 fg	3.96 ± 0.085 f	8.66 ± 0.112 d	1.95 ± 0.042 f
	Control	5.17 ± 0.146 ef	4.33 ± 0.027 e	9.5 ± 0.16 c	3.54 ± 0.128 d

†† Means with common letters in each column indicate no significant difference based on the LSD test at the probability of 5%. Values are means ± SE.

Table 2 The effects of genotype, priming agents (*Trichoderma zelobreve* (TZ), *Bacillus subtilis* (BS), β-Aminobutyric acid (BABA) and virus on sugar beet growth parameters (shoot and root fresh weight, shoot and root dry weight). Data are means of 10 replicates ± SE. Different letters indicate significant differences (LSD test, $P \leq 0.05$). V0: control plants not inoculated with virus; V1: virus-inoculated plants.

Genotype	Priming	Virus	Shoot fresh weight	Root fresh weight	Shoot dry weight	Root dry weight
980043	BS	V0	5.71 ± 0.111 cd	0.85 ± 0.018 d ††	1.47 ± 0.021 c	0.31 ± 0.003 cd
		V1	5.36 ± 0.138 de	0.76 ± 0.016 e	1.39 ± 0.028 d	0.28 ± 0.009 de
	BABA 10 mM	V0	5.17 ± 0.13 ef	0.98 ± 0.014 b	2.32 ± 0.023 a	0.5 ± 0.003 a
		V1	4.43 ± 0.093 ij	0.76 ± 0.016 e	1.37 ± 0.03 d	0.2 ± 0.001 gh
	TZ	V0	4.78 ± 0.107 g-i	0.87 ± 0.021 cd	1.35 ± 0.026 d	0.27 ± 0.013 ef
		V1	4.58 ± 0.109 ij	0.66 ± 0.015 f	1.1 ± 0.026 fg	0.25 ± 0.017 f
	Control	V0	4.65 ± 0.114 hi	0.64 ± 0.021 fg	0.89 ± 0.023 i	0.18 ± 0.008 h
		V1	4.23 ± 0.062 jk	0.6 ± 0.015 gh	0.98 ± 0.025 h	0.13 ± 0.013 i
SB23	BS	V0	6.43 ± 0.209 a	1.14 ± 0.011 a	1.33 ± 0.032 d	0.34 ± 0.012 b
		V1	5.77 ± 0.15 bc	0.92 ± 0.021 c	1.08 ± 0.028 fg	0.2 ± 0.004 gh
	BABA 10 mM	V0	5.66 ± 0.128 cd	0.9 ± 0.026 cd	1.25 ± 0.016 e	0.31 ± 0.005 c
		V1	5.07 ± 0.173 e-g	0.65 ± 0.027 fg	1.04 ± 0.03 gh	0.2 ± 0.003 gh
	TZ	V0	6.1 ± 0.115 ab	0.78 ± 0.024 e	1.52 ± 0.02 bc	0.3 ± 0.003 cd
		V1	5.07 ± 0.131 e-g	0.57 ± 0.016 h	1.56 ± 0.016 b	0.25 ± 0.017 f
	Control	V0	4.96 ± 0.083 f-h	0.65 ± 0.017 fg	1.12 ± 0.027 f	0.2 ± 0.002 g
		V1	3.94 ± 0.105 k	0.61 ± 0.028 f-h	0.89 ± 0.018 i	0.2 ± 0 gh

Means with common letters in each column indicate no significant difference based on the LSD test at the probability of 5%.

Table 3 The effects of genotype, priming agents (*Trichoderma zelobreve* (TZ), *Bacillus subtilis* (BS), β-Aminobutyric Acid (BABA) and virus on sugar beet leaf area index.

Genotype	Prime	Virus	Leaf area (cm^2)
980043	BS	V0	35.67 ± 2.186 ab ††
		V1	31.33 ± 1.333 b
	BABA 10 mM	V0	37.67 ± 0.333 a
		V1	16 ± 1 de
	TZ	V0	35.67 ± 1.202 ab
		V1	25.33 ± 2.028 c
	Control	V0	14.67 ± 0.882 e
		V1	9 ± 0.577 f
SB23	BS	V0	35.67 ± 2.333 ab
		V1	19.33 ± 0.667 d
	BABA 10 mM	V0	26 ± 0.577 c
		V1	12 ± 1.155 ef
	TZ	V0	38.33 ± 1.764 a
		V1	25.33 ± 3.48 c
	Control	V0	12.33 ± 0.333 ef
		V1	4.33 ± 0.333 g

Means with common letters in each column indicate no significant difference based on the LSD test at the probability of 5%. Data are means of 10 replicates ± SE.

Seed priming increased leaf greenness and mitigated virus-induced reduction in plant height and leaf area

Genotype, priming, and virus had significant main effects ($P < 0.01$) on the greenness index, with primed plants showing greater leaf greenness than non-primed controls (Figs. 2, 3, Table S3, S4).

The interaction between genotype and priming was significant for plant height ($P < 0.01$), whereas the three-way interaction including virus was not significant. Priming treatments generally improved plant height, and some treatments helped maintain better plant growth under viral infection (Figs. 4, 5, Table 3). However, priming effects on leaf area varied by genotype and priming agent. Results of the variance analysis of leaf area are presented in Supplementary Table S5.

Antioxidant enzyme activities in primed plants

The activities of polyphenol oxidase (PPO), peroxidase (POX), and catalase (CAT) were evaluated at 48 hours, 96 hours, and 40 days post-inoculation (dpi) (F) with BCTIV. At all time points, plants treated with B. subtilis, T. zelobreve, and BABA showed significantly higher enzyme activities than untreated virus-infected plants, indicating enhanced activation of the plant antioxidant defense system.

In the 980043 genotype, PPO activity at 48 hours post-inoculation peaked for plants treated with *T. zelobreve* and *B. subtilis*, showing no significant differences between these two treatments (Fig. 6). Treated and virus-inoculated plants had significantly greater PPO activity than control plants. In the SB23 genotype, *T. zelobreve* treatment elicited the highest PPO activity at 48 hours; other priming treatments also showed elevated responses over controls. By 96 hours post-inoculation, PPO activities remained elevated for all primed groups in both genotypes. At 40 dpi (F), PPO activity was highest in treated plants, maintaining the trend of sustained enzyme induction after viral challenge.

POX activity mirrored PPO patterns, with *T. zelobreve* and *B. subtilis* treatments eliciting maximal activity in 980043 at 48 hours (Fig. 7). These treatments produced significantly higher POX levels compared to virus-only and control plants. In SB23, *T. zelobreve* treatment also induced the highest POX activity, with BABA and *B. subtilis* treatments showing intermediate increases. At 96 hours, primed plants maintained elevated POX activity, whereas virus-infected untreated plants showed a suppressed response, especially in SB23. At 40 dpi (F), POX activity remained significantly higher in primed plants compared to controls in both genotypes.

At 48 hours post-inoculation, *T. zelobreve* and *B. subtilis* treatments led to the highest CAT activity in 980043, significantly exceeding virus-only and control plants (Fig. 8). In SB23, BABA treatment induced the most notable CAT activity, while virus-inoculated plants showed a reduced enzymatic response compared to controls. CAT activity peaked at 96 hours in *T.*

zelobreve-treated 980043 plants and remained elevated compared to virus-only treatments. In SB23, *T. zelobreve* showed superior CAT induction, with virus-infected plants still showing decreased activity. By 40 dpi (F), CAT activity in primed plants continued to exceed that in virus-infected controls, indicating prolonged activation of antioxidant defenses. Variance analysis of enzyme activity is presented in Supplementary Table S6.

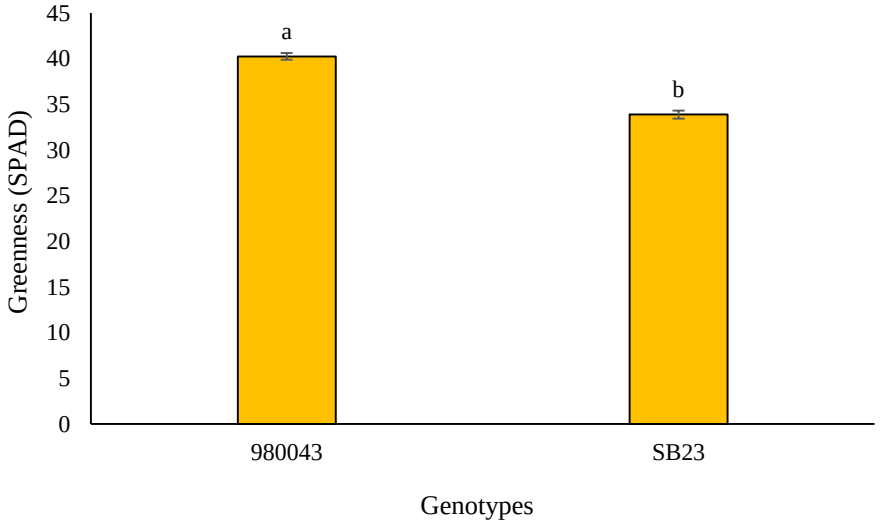


Figure 2 Effect of sugar beet genotype on greenness index under *Bacillus subtilis*, *Trichoderma zelobreve*, and β-aminobutyric acid (BABA): mean changes in greenness index measured under two sugar beet genotypes 980043 and SB23, regardless of priming and virus treatments. Data are means of 10 replicates ± SE. Different letters indicate significant differences (LSD test, $P \leq 0.05$).

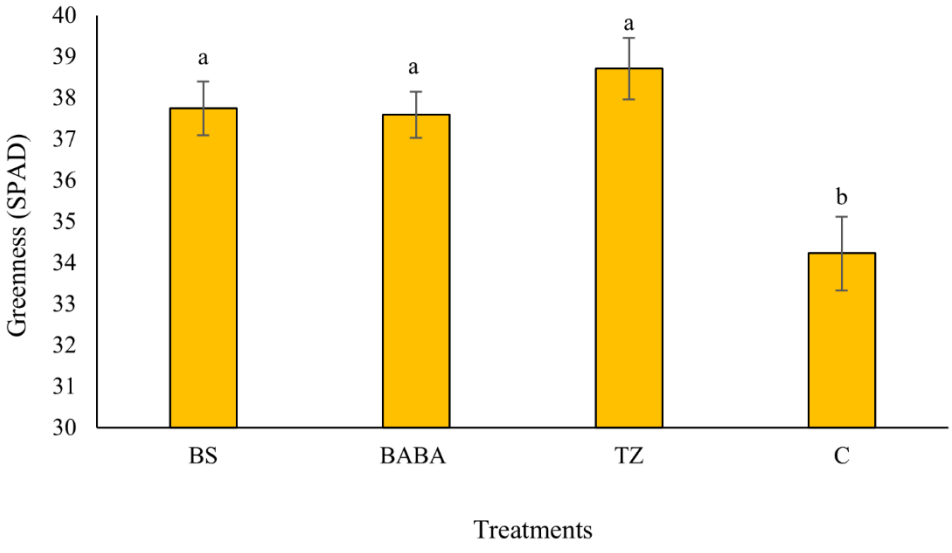


Figure 3 Effect of seed priming on greenness index across both sugar beet genotypes**.** Comparison of mean greenness index among different priming treatments (*Bacillus subtilis*, *Trichoderma zelobreve*, and BABA). All priming agents enhanced greenness compared to the non-primed control. Data are means of 10 replicates ± SE. Different letters indicate significant differences (LSD test, $P \leq 0.05$).

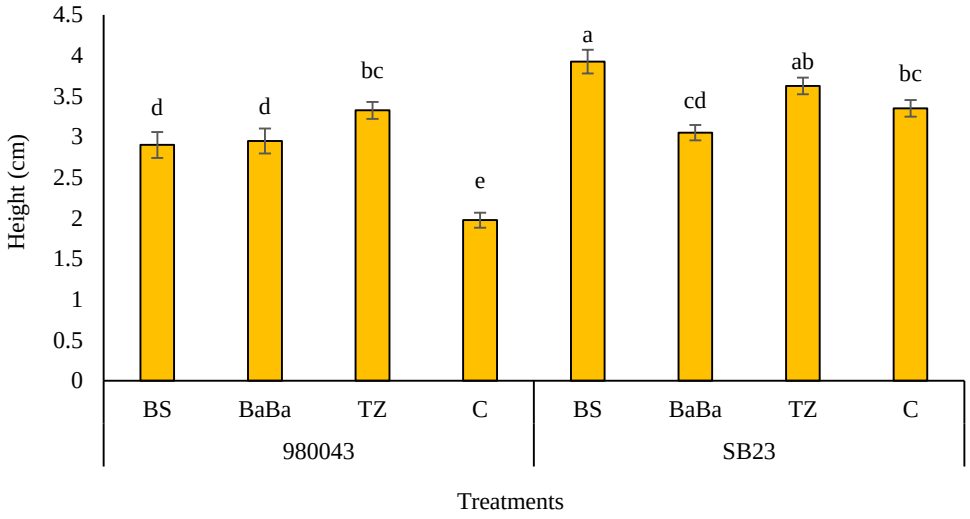


Figure 4 Effect of BCTIV inoculation on sugar beet plant height under different priming treatments. Comparison of mean plant height under BCTIV-inoculated conditions among various priming treatments and the control group. Primed plants exhibited less reduction in height compared to virus-infected non-primed plants. Data are means of 10 replicates ± SE. Different letters indicate significant differences (LSD test, P ≤ 0.05).

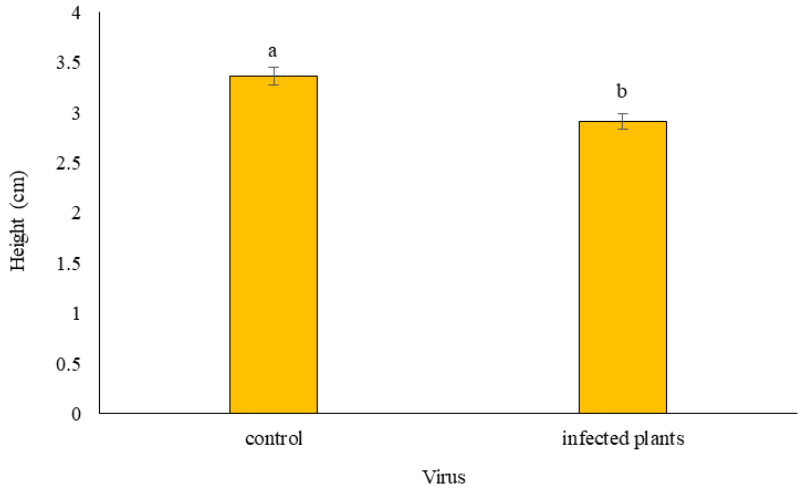


Figure 5 The main effect of virus and seed priming on plant height in sugar beet. Mean plant height measured across different combinations of sugar beet genotypes (980043 and SB23) and priming treatments (*Bacillus subtilis*, *Trichoderma zelobreve*, and BABA). Significant differences were observed among treatments. Data are means of ١٠ replicates ± SE. Different letters indicate significant differences (LSD test, P ≤ 0.05).

The consistent enhancement of PPO, POX, and CAT activities in primed plants across both sugar beet genotypes strongly suggests that *B. subtilis*, *T. zelobreve*, and BABA treatments prime the antioxidative defense system to suppress oxidative stress and contribute to resistance mechanisms against BCTIV infection.

Reduced virus accumulation in primed plants

BCTIV accumulation was quantified at 40 days post-inoculation using qPCR with specific primers targeting viral DNA. Relative BCTIV accumulation differed significantly among treatments (P < 0.01). Virus-inoculated control plants had the highest viral DNA levels in both

genotypes (Fig. 9). In cultivar 980043, *T. zelobreve* treatment resulted in the lowest relative BCTIV accumulation and was associated with reduced symptom severity. However, in genotype SB23, *B. subtilis* showed the lowest virus accumulation among the tested priming treatments, indicating a genotype-dependent response to priming. *B. subtilis* and BABA treatments also resulted in lower relative viral DNA levels compared with virus-inoculated controls. However, the magnitude of this reduction varied depending on the genotype and the treatment. Overall, priming treatments were associated with reduced BCTIV accumulation at 40 dpi and improved disease outcomes; however, further time-course analyses are required to determine whether these effects result from altered viral replication, delayed infection progression, or restricted systemic movement. A consistent trend was observed between relative BCTIV accumulation and symptom severity, with lower viral DNA levels generally associated with reduced disease symptoms (Table S7, Fig. 9).

Priming efficiency on virus control

The results show that priming sugar beet seeds with B. subtilis, T. zelobreve, and BABA significantly reduced BCTIV-induced symptom severity in both genotypes, 980043 and SB23, compared with non-primed plants (Fig. 1, Table 4). In genotype SB23, *T. zelobreve* and BABA treatments were associated with greater reductions in disease severity compared with the virus-inoculated control, showing only mild symptoms such as bright veins and slight vein swelling, while the virus-only and *B. subtilis*-treated plants showed more severe symptoms including intense yellowing, extreme leaf twisting, reduced leaf area, and stunted growth. In genotype 980043, *T. zelobreve* treatment also markedly reduced symptom severity compared to virus-infected controls.

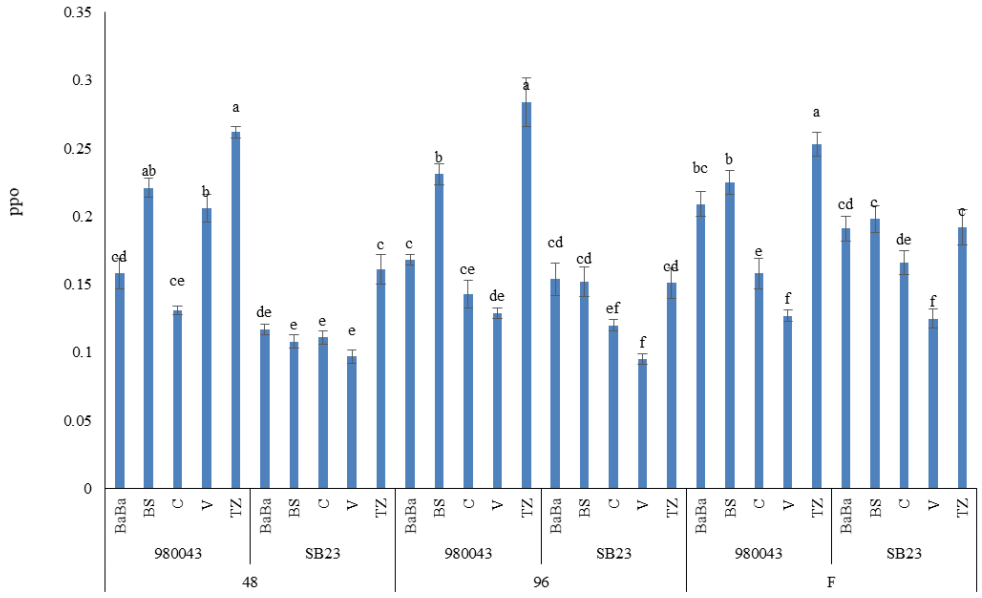


Figure 6 Effects of *Bacillus subtilis*, *Trichoderma zelobreve*, and β-aminobutyric acid (BABA) on polyphenol oxidase (PPO) (U mg^{-1} protein) of sugar beet genotypes inoculated with BCTIV 48 and 96 hours post-inoculation and 40 days post-inoculation (F), based on cutting at the Time point. Data are means of four biological replicates and two technical replicates. Different letters indicate significant differences (LSD test, $P \leq 0.05$). The data are presented as the mean, with error bars showing standard error (SE).

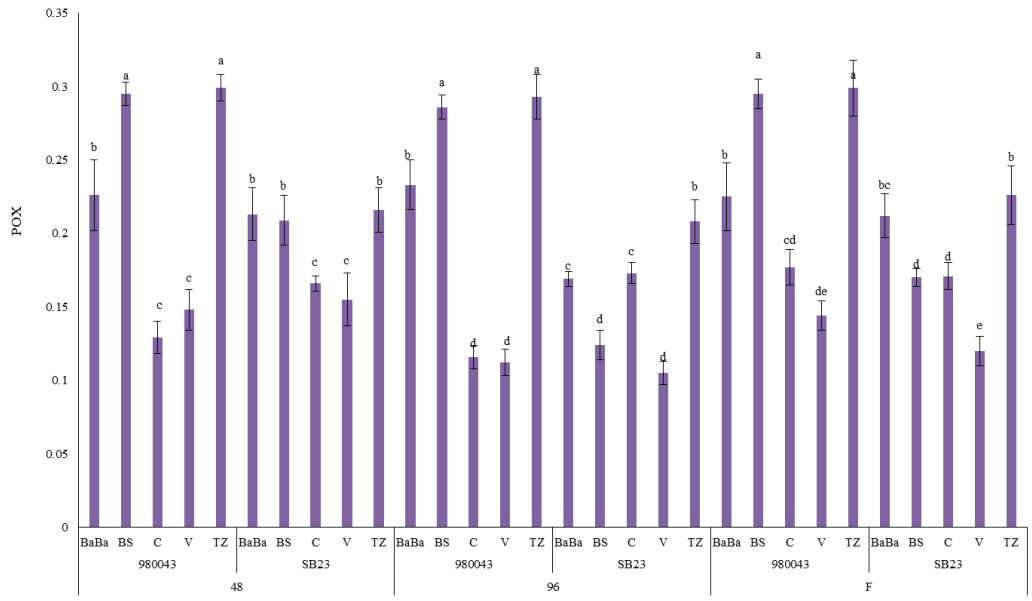


Figure 7 Effects of *Bacillus subtilis*, *Trichoderma zelobreve*, and β-aminobutyric acid (BABA) on guaiacol peroxidase (POX) (U mg^{-1} protein) of sugar beet genotypes inoculated with BCTIV 48 and 96 hours post-inoculation and 40 days post-inoculation (F), based on cutting at different time points. Data are means of four biological replicates and two technical replicates. Different letters indicate significant differences (LSD test, $P \leq 0.05$). The data are presented as the mean, with error bars showing standard error (SE).

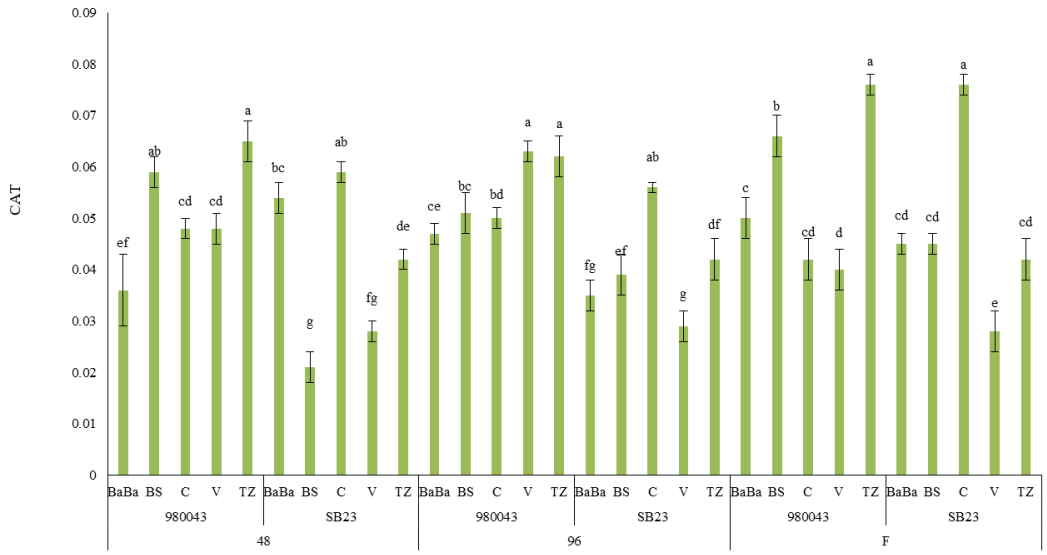


Figure 8 Effects of *Bacillus subtilis*, *Trichoderma zelobreve*, and β-aminobutyric acid (BABA) on catalase (CAT) (U mg^{-1} protein) of sugar beet genotypes inoculated with BCTIV 48 and 96hours post-inoculation and fourty days post-inoculation, based on cutting with Time point. Data are means of four biological replicates and two technical replicates. Different letters indicate significant differences (LSD test, $P \leq 0.05$). The data are presented as the mean, with error bars showing standard error (SE).

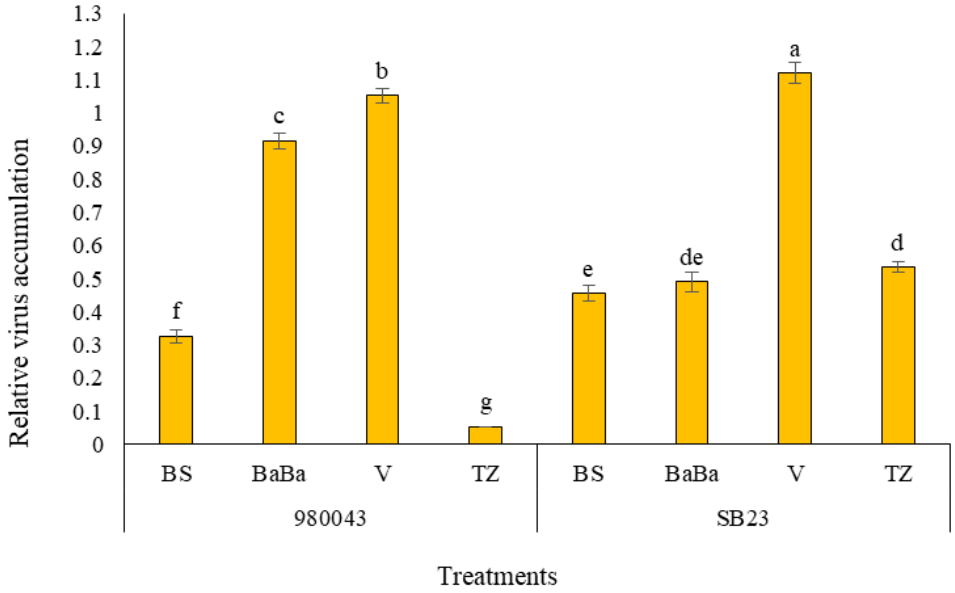


Figure 9 Effect of different treatments on relative BCTIV accumulation in sugar beet plants. Relative BCTIV accumulation was determined at 40 days post-inoculation using qPCR with specific primers targeting viral DNA. Treatments included *Bacillus subtilis*, *Trichoderma zelobreve*, BABA, and virus-inoculated controls. The effect of priming treatments on relative BCTIV accumulation varied between genotypes. In genotype 980043, *T. zelobreve*-treated plants showed the lowest relative BCTIV accumulation, whereas in genotype SB23, *B. subtilis*-treated plants exhibited the lowest numerical virus accumulation among priming treatments. Virus-inoculated control plants showed the highest relative BCTIV accumulation in both genotypes. Data represent means of four biological replicates with two technical replicates. Different letters indicate significant differences according to the LSD test ($P \leq 0.05$). Error bars represent standard error (SE).

Table 4 Effects of seed priming on disease severity, dry weight and virus accumulation in two sugar beet genotypes (980043 and SB23) 40 days post-inoculation with beet curly top Iran virus (BCTIV).

Genotype	Priming agent	Virus disease control (%)	Dry weight (% of control)	Virus accumulation (%)
980043	*Bacillus subtilis*	75.2	90.4	32.642
980043	*Trichoderma zelobreve*	88.9	79.8	5.361
980043	BABA	52.1	64.4	91.632
SB23	*Bacillus subtilis*	11.3	75.7	45.655
SB23	*Trichoderma zelobreve*	45.1	75.9	53.575
SB23	BABA	40.8	70.2	49.076

These observations indicate that seed priming with these biological and chemical agents enhances the plants' resistance to BCTIV infection, likely by stimulating early defense responses that delay symptom onset and reduce symptom intensity. The incubation period from inoculation to symptom appearance varied widely, from 11 to 40 days, reflecting the influence of both genotype and priming treatment on disease symptom progression.

Overall, the data confirm the efficacy of seed priming in controlling BCTIV severity, with *T. zelobreve* and BABA treatments showing the most pronounced improvements in virus resistance as evidenced by reduced symptom severity indices in both sugar beet genotypes.

Discussion

The study showed that seed priming with biocontrol agents, *B. subtilis* and *T. zelobreve*, and BABA increases sugar beet resistance to BCTIV by stimulating growth and activating natural defenses. These results confirm and add to the growing evidence that biopriming and chemical priming are sustainable, environmentally sound strategies for controlling viral diseases in crops (Kumar *et al*., 2023; Woo *et al*., 2023).

The reduction in symptom severity observed in certain priming treatments was accompanied by lower BCTIV accumulation, although the magnitude of this effect varied among treatments. Treatment with *T. zelobreve* resulted in particularly low virus accumulation, consistent with previous findings that Trichoderma species inhibit viral replication and systemic circulation across hosts by priming systemic resistance (Vitti *et al*., 2016). In certain seed-primed treatments, reduced viral accumulation was accompanied by lower symptom severity, suggesting that seed priming may help mitigate BCTIV infection.

Increased levels of antioxidant enzymes polyphenol oxidase (PPO), peroxidase (POX), and catalase (CAT) in primed plants confirm the role of oxidative stress-modulated antiviral enzymes in the homeostasis of reactive oxygen species (ROS) and in the fortification of the cell walls and signaling to activate the antiviral genes (Mittler, 2002). The long persistence of these enzymes, up to 40 days after inoculation, indicates a sustained priming effect that increases long-term BCTIV immunity. The induction of similar enzymes has been reported in cultures treated with *Bacillus* and *Trichoderma* spp. to overcome viruses such as tobacco mosaic virus (TMV; *Tobamovirus tabaci*), tomato yellow leaf curl virus (TYLCV; *Begomovirus coheni*), and cucumber mosaic virus (CMV; *Cucumovirus CMV*) (Beris *et al.*, 2018; Ghanem and Aqil, 2021; Abdelkhalek *et al*., 2022). Untreated plants showed the lowest enzyme activity and highest viral accumulation, whereas primed plants maintained significantly higher enzyme activity levels (Kazemi *et al*., 2025b).

The efficacy of BABA as a chemical priming agent is in line with its known role in the generation of systemic acquired resistance via salicylic acid-mediated pathways and modulation of the signaling pathways of reactive oxygen species (Baccelli and Mauch-Mani, 2016; Singh *et al*., 2023). Increased enzyme activity, reduced viral load, and improved growth parameters observed with BABA treatment reflect results documented in a variety of pathosystems, including tobacco, tomatoes, and cucumbers (Nazar *et al*., 2011; Rashid, 2014).

The difference in responses observed between two sugar beet genotypes highlights the influence of host genetic background on the efficacy and activation of antiviral defenses. Integrating priming agents with resistance-carrying strains can provide additive or synergistic benefits and advance durable treatment of curly top disease (Meesa *et al*., 2024). This genotype-dependent variability is consistent with previous studies in virus resistance breeding and biopriming (Fatahi *et al*., 2012; Mafakheri *et al*., 2024).

In addition to increasing resistance, the treatment improves physiological characteristics, including chlorophyll content, leaf area, and biomass accumulation, suggesting that the virus-induced growth retardation is alleviated and photosynthetic capacity is maintained. These findings align with reports on *B. subtilis* and *T.* spp. They promote nutrient uptake, stress tolerance, and growth vigor while priming the immune system (Mastouri *et al*., 2010; Tsotetsi *et al*., 2022; Etesami *et al*., 2023). This co-optimization of growth and defense addresses a classic compromise and is essential for sustainable crop productivity under pathogen pressure.

Successful colonization of *B. subtilis* and *T. zelobreve* in the sugar beet rhizosphere and root tissues (confirmed here) supports their ability to provide sustained protection by modulating the microbial community and directly antagonizing pathogens (Woo *et al*., 2023). This durable association is essential for the stable field efficacy of biocontrol agents.

In summary, this work confirms the potential of beneficial microbes and BABA as integral parts of

integrated BCTIV control strategies. Their multifaceted modes of action, including increased systemic resistance, activation of antioxidant enzymes, suppression of viral loads, and growth promotion, make them a promising alternative or complement to conventional chemical controls in sugar beet production systems.

Conclusion

This study provides strong evidence that treating sugar beet seed with B. subtilis, T. zelobreve, and BABA not only significantly reduces BCTIV severity and accumulation and sugar beet disease but also improves key plant growth and physiological parameters. By explaining the biochemical and physiological mechanisms behind this increased resistance, mainly through activation of key antioxidant enzymes and increased photosynthetic capacity, the study pioneers an integrated, sustainable approach to controlling a virulent disease in a major industrial crop. Responses in specific genotypes further highlight the potential for tailor-made disease management strategies that combine biopriming with host genetic traits to maximize efficacy.

This is a significant step in managing viral diseases, and the findings emphasize the need to promote chemical priming and biopriming as practical, scalable, and environmentally responsible alternatives that reduce chemical pesticide use and support sustainable sugar beet production. This research also addresses an important knowledge gap, as it is the first study to assess these priming methods against BCTIV in sugar beet, which could increase crop tolerance in areas where the disease is a threat, a timely and important finding that can serve as a foundation for translational research and commercial development of new types of biocontrol to protect food security and agricultural sustainability at a large scale.

Acknowledgments

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تأثیر آماده‌باش زیستی و شیمیایی بذر بر افزایش مقاومت چغندرقند به ویروس ایرانی پیچیدگی بوته چغندرقند

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چکیده: در این مطالعه تأثیر تیمار بذر ژنوتیپ‌های چغندرقند با *Bacillus subtilis*، *Trichoderma zelobreve* و ماده شیمیایی β-آمینوبوتیریک اسید (BABA) را بر رشد، ویژگی‌های فیزیولوژیک و پاسخ به آلودگی به ویروس پیچیدگی برگ چغندر ایران (BCTIV; *Becurtovirus betae*) بررسی شد. دو ژنوتیپ چغندرقند SB23 و ۹۸۰۰۴۳ تحت تیمار پرایمینگ میکروبی و شیمیایی با BABA قرار گرفت و سپس در شرایط گلخانه از طریق آگروواینوکولیشن با ویروس مایه‌زنی شدند. نتایج نشان داد که شدت علائم بیماری و میزان تجمع ویروس در گیاهان تیمار شده در مقایسه با گیاهان شاهد (آلوده بدون پرایمینگ)، به‌طور معنی‌داری کم‌تر بود. هم‌چنین شاخص‌های رشد شامل وزن تر و خشک اندام هوایی و ریشه، میزان کلروفیل و کاروتنوئید، شاخص سطح برگ و ارتفاع گیاه در گیاهان تیمار شده افزایش یافت. فعالیت آنزیم‌های آنتی‌اکسیدانی شامل پلی‌فنل‌اکسیداز، پراکسیداز و کاتالاز پس از تیمار افزایش پیدا کرد که نشان‌دهنده فعال شدن سیستم دفاعی گیاه است. این تیمارها با افزایش مقاومت، اثرات مخرب BCTIV را کاهش داده و این مقاومت با افزایش پاسخ‌های بیوشیمیایی دفاعی و بهبود وضعیت فیزیولوژیک گیاه همراه بود. این یافته‌ها نشان می‌دهد که تیمار بذر با *B. subtilis*، *T. zelobreve* و BABA باعث بهبود رشد چغندرقند و القای مقاومت سیستمیک به BCTIV می‌شود و می‌تواند راهکاری امیدبخش و پایدار برای مدیریت این بیماری در مناطق آلوده باشد.

واژگان کلیدی: تجمع ویروس، فعالیت آنزیم‌های دفاعی، آماده‌باش بذری، پی‌سی‌آر کمی

Supplementary tables and figures

Table S1 Primer pairs used in this study.

Primers name	Sequences (5'→3')	Product size (bp)	Function	Reference
DNA-BCTIV Forward	CGCATCCCTCCTAATCCGAT	127bp	Amplification of DNA of the virus	Kazemi *et al*., 2025
DNA- CTIV Reverse	TGGCTAGTGGTGCATTTTGG			
SSU DNA *B.Vulgaris* Forward	CGTTCTTAGTTGGTGGAGCG	245bp	Reference gene	Kazemi *et al*., 2025
SSU DNA *B.vulgaris* Reverse	AAGATTACCCGGACCTGTCG			

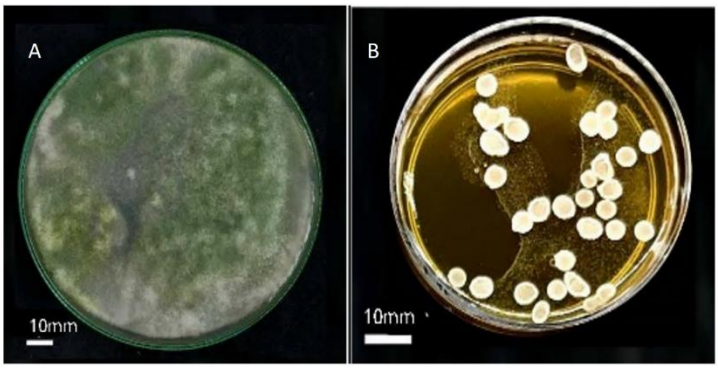


Figure S1 View of rhizospheric soil culture around the root of sugar beet treated with: A) *Bacillus subtilis* and B) *Trichoderma zelobreve.*

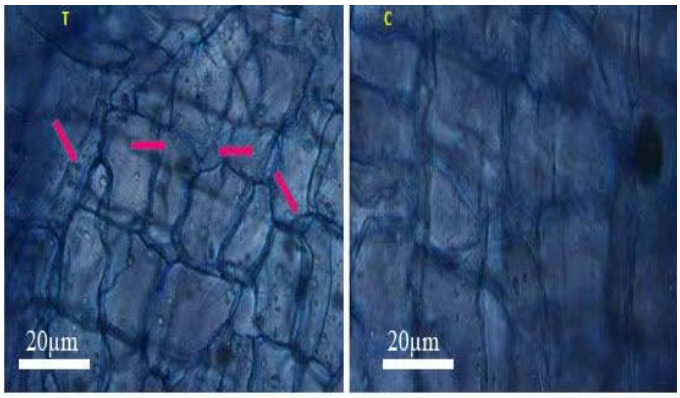


Figure S2 Pattern of sugar beet root colonization by *Trichoderma zelobreve*: Hyphae of *T. zelobreve* have penetrated the roots and grown in the intercellular spaces of the root epidermis.

Table S2 Mean Squares of the effect of cultivar and priming on Symptome severity index.

S. V.	df	Score
Cultivar (C)	1	117.18 **
Priming (P)	3	44.98 **
C × P	3	15.15 **
Error	72	0.382
CV (%)		10.18

** significant at 1 percent probability levels.

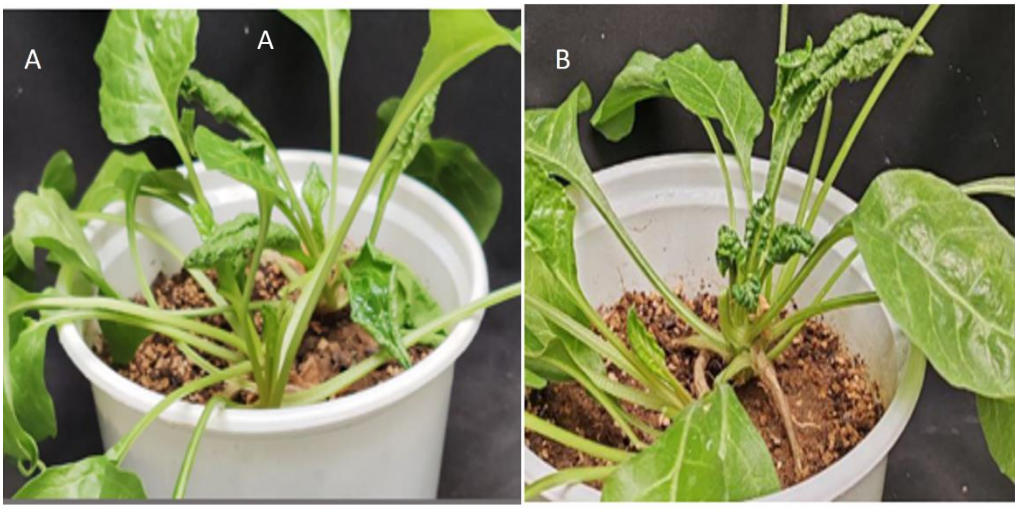


Figure S3 Sugar beet plants inoculated with beet curly top Iran virus (BCTIV), showing typical viral symptoms including reduced plant growth, leaf curling, and vein swelling. **A:** *Trichoderma zelobreve* treatment showing milder symptoms, including slight vein swelling and moderate leaf curling. **B:** Control treatment (virus-inoculated, untreated) exhibiting severe symptoms such as pronounced vein swelling and extensive leaf curling.

Table S3 Mean Squares of the effect of cultivar and prime on photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid) of sugar beet.

S. V.	df	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoid
Cultivar (C)	1	0.808 **	7.064 **	12.65 **	6.88 **
Prime (P)	4	25.23 **	4.57 **	44.80 **	9.44 **
C × P	4	6.008 $^{\times *}$	0.159 **	6.99 **	0.67 **
Error	30	0.111	0.014	0.117	0.067
CV (%)		5.50	2.71	3.27	7.88

$^{*, **}$ Significant at the 5 and 1 percent probability levels. Respectively.

Table S4 Mean Squares of the effect of cultivar, prime and virus on greenness index (SPAD), plant height and fresh and dry weight of shoots and roots.

S. V.	df	SPAD	Plant height	Fresh weight of aerial parts	Root fresh weight	Dry weight of aerial parts	Root dry weight	Total fresh weight	Total dry weight
Cultivar (C)	1	1621.80 **	19.60 **	10.44 **	0.007 ^{ns}	0.73 **	0.009 **	5.65 **	0.0002 ^{ns}
Prime (P)	3	153.47 **	5.78 **	12.57 **	0.645 **	2.03 **	0.122 **	20.70 **	1.22 **
Virus (V)	1	296.48 **	8.10 **	15.66 **	1.053 **	2.17 **	0.306 **	29.50 **	2.49 **
C × P	3	21.86 ^{ns}	3.60 **	1.36 **	0.226 **	1.89 **	0.041 **	3.52 **	0.29 **
C × V	1	1.93 ^{ns}	0.025 ^{ns}	1.57 **	0.016 *	0.184 **	0.006 **	0.68 *	0.002 ^{ns}
P × V	3	11.43 ^{ns}	0.212 ^{ns}	0.083 ^{ns}	0.073 **	0.557 **	0.070 **	0.70 **	0.234 **
C × P × V	3	3.23 ^{ns}	0.396 ^{ns}	0.45 *	0.011 *	0.579 **	0.042 **	1.58 **	0.077 **
Error	144	8.63	0.248	0.161	0.004	0.006	0.0008	0.168	0.004
CV (%)		7.92	15.88	7.84	8.09	6.07	10.92	6.40	6.57

$^{ns, *, **}$ non-significant and significant at the 5 and 1 percent probability levels, respectively.

Table S5 Mean Squares of the effect of cultivar, prime and virus on leaf area (LA) of sugar beet.

S. V.	df	LA
Cultivar (C)	1	192.00 **
Prime (P)	3	1152.05 **
Virus (V)	1	1633.33 **
C × P	3	47.40 **
C × V	1	16.33 ns
P × V	3	63.17 **
C × P × V	3	48.40 **
Error	32	7.00
CV (%)		11.18

ns ** non- significant and significant at 1 percent probability levels, respectively.

Table S6 Mean Squares of the effect of cultivar, prime, and virus on the activity of defense enzymes in sugar beet.

S. V.	df	PPO	PER	CAT
Time (T)	3	0.0015 **	0.060 **	0.0013 **
Timepoint (TM)	2	0.033 **	0.028 **	0.0002 **
Cultivar (C)	1	0.000012 ns	0.053 **	0.0013 **
Virus& Prime (VP)	4	0.010 **	0.052 **	0.00045 **
T × TM	6	0.0055 **	0.0021 **	0.0005 **
T × C	3	0.0026 **	0.006 **	0.0003 **
TM × C	2	0.142 **	0.084 **	0.005 **
T × TM × C	6	0.1 **	0.0053 **	0.00032 **
T × VP	12	0.1 **	0.008 **	0.00087 **
TM × VP	8	0.042 **	0.057 **	0.0043 **
C × VP	4	0.016 **	0.072 **	0.0007 **
T × TM × VP	24	0.012 **	0.015 **	0.0007 **
T × C × VP	12	0.011 **	0.016 **	0.0013 **
TM × C × VP	8	0.049	0.07 **	0.003 **
T × TM × C × VP	24	0.008 **	0.009 **	0.0008 **
Error	360	0.00032	0.0005	0.000011
CV (%)		10.62	11.15	6.97

ns. ** Non-significant and significant at 1 percent probability levels. Respectively.

Table S7 Mean Squares of Table of RT-PCR Data.

S. V.	df	Virus accumulation
Cultivar (C)	1	0.032 **
Priming + virus (PV)	3	1.02 **
C × PV	3	0.279 **
Error	24	0.002
CV (%)		7.49

** Significant at the 1 percent probability level.