

Xanthohumol and echinocystic acid induces PSTVd tolerance in tomato

Wenkun Tang^{1,2}  | Zhichao Tang^{1,2}  | Haiyi Liu^{1,2} | Jinbiao Lu^{1,2} | Qianyun Du³ | Huan Tian³ | Jingwei Li^{1,2} 

¹Vegetable Industry Research Institute, Guizhou University, Guiyang, China

²College of Agriculture, Guizhou University, Guiyang, China

³Guizhou Advanced Seed Industry Group, Guiyang, China

Correspondence

Jingwei Li, Vegetable Industry Research Institute, Guizhou University, Guiyang 550000, China.

Email: jwli3@gzu.edu.cn

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Abstract

Tomato is a popular vegetable worldwide; its production is highly threatened by infection with the potato spindle tuber viroid (PSTVd). We obtained the full-length genome sequence of previously conserved PSTVd and inoculated it on four genotypes of semi-cultivated tomatoes selected from a local tomato germplasm resource. SC-5, which is a PSTVd-resistant genotype, and SC-96, which is a PSTVd-sensitive genotype, were identified by detecting the fruit yield, plant growth, biomass accumulation, physiological indices, and PSTVd genome titer after PSTVd inoculation. A non-target metabolomics study was conducted on PSTVd-infected and control SC-5 to identify potential anti-PSTVd metabolites. The platform of liquid chromatography-mass spectrometry detected 158 or 123 differential regulated metabolites in modes of positive ion or negative ion. Principal component analysis revealed a clear separation of the global metabolite profile between PSTVd-infected leaves and control regardless of the detection mode. The potential anti-PSTVd compounds, xanthohumol, oxalic acid, indole-3-carbinol, and rosmarinic acid were significantly upregulated in positive ion mode, whereas echinocystic acid, chlorogenic acid, and 5-acetylsalicylic acid were upregulated in negative ion mode. Xanthohumol and echinocystic acid were detected as the most upregulated metabolites and were exogenously applied on PSTVd-diseased SC-96 seedlings. Both xanthohumol and echinocystic acid had instant and long-term inhibition effect on PSTVd titer. The highest reduction of disease symptom was induced by 2.6 mg/L of xanthohumol and 2.0 mg/L of echinocystic acid after 10 days of leaf spraying, respectively. A superior effect was seen on echinocystic acid than on xanthohumol. Our study provides a statistical basis for breeding anti-viroid tomato genotypes and creating plant-originating chemical preparations to prevent viroid disease.

KEYWORDS

anti-viroid metabolite, non-target metabolomics analysis, potato spindle tuber viroid, *Solanum lycopersicum*

Wenkun Tang and Zhichao Tang are the first authors.

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1 | INTRODUCTION

Cultivated tomatoes (*Solanum lycopersicum*) have narrow genetic variations (Baldo et al., 2007), making them insufficient to resist or tolerate the invasion of various pathogens including viroids. Various germplasms of semi-cultivated tomatoes of *Solanum lycopersicum* var. *Cerasiforme* with different phenotypes and levels of stress tolerance are important materials for breeding. Moreover, semi-cultivated tomatoes are widely used in Southwest China for their function of being processed as red sour soup hot pot (Xiong et al., 2021).

The sustainable production of tomato fruits is often threatened by abiotic stressors, including insects, fungi, bacteria, viruses, and newly emerged pathogens, such as viroids (Ling & Li, 2014). Viroids are the smallest pathogens in molecular weight comprised of circular single-stranded RNA without a protein coat; they are currently the smallest known pathogens. *Pospiviroidae* and *Avsunviroidae* have been classified as viroids (Adkar-Purushothama & Perreault, 2020; Ding, 2009; Flores et al., 2005). Potato spindle tuber viroid (PSTVd) is the first identified individual of the *Pospiviroidae* (Apostolova et al., 2020).

Solanaceae crops, for example, potatoes (*Solanum tuberosum*) and tomatoes, are easily infected with PSTVd (Matsushita & Tsuda, 2016; Verhoeven et al., 2004). Viroids cause dwarfing and necrosis of tomato plants a very short time after infection, and these plants accumulate a relatively high titer of this agent (Diermann et al., 2010; Hammond, 1994). Tomatoes symptomatically react to various PSTVd strains in about 2 weeks (Raymer & O'Brien, 1962). Unlike most viruses that are unable to infect the meristem and are transmitted by seeds, some viroids are infectious to the smallest cells in the shoot apical dome of the plant (Ebata et al., 2019), making this class of replicons a seed-borne disease (Dall et al., 2019). We have demonstrated through in situ hybridization technology that PSTVd can infect cells of our semi-cultivated tomato stem tip (data not shown). Viroids cause large yield losses and are a serious concern to the tomato industry, seed breeders, suppliers, and agricultural safety managers. The lack of efficient viroid control methods makes them a difficult problem for tomato cultivation.

Exploring highly resistant and highly sensitive genotypes for pathological study as well as anti-viroid breeding is of great importance considering the high risk of viroid disease on production, breeding, and ecological security. Meanwhile, searching for disease-resistant or -tolerant metabolites in plants and utilizing their own metabolites to develop agricultural chemicals can ensure the safety of crop disease and pest control. For instance, matrine extracted from *Sophora flavescens* (Chu et al., 2018) and berberine extracted from *Coptis chinensis* (Liu et al., 2022) have been widely used to protect crops from diseases and pests (Hwang et al., 2009; Li et al., 2018; Wang et al., 2019) for their efficiency and safety (Sun et al., 2022; Zhou et al., 2022).

The semi-cultivated tomatoes in this study are of great significance for the daily diet of local residents and the local fermented food production industry. The government of Guizhou Province requires

strict implementation of disease and pest control mainly based on biological agents in the vegetable cultivation industry. In this study, we assessed PSTVd tolerance in semi-cultivated tomatoes based on morphological traits, physiological changes, and the accumulation of replicons; the possible viroid-tolerance-inducing metabolites of the PSTVd-resistant tomato genotype were identified by non-target metabolomics analysis.

2 | MATERIALS AND METHODS

2.1 | Materials

Fresh red fruits from semi-cultivated tomatoes with the SC-5, SC-60, SC-96, and SC-128 genotypes were collected from dispersed villages in Chishui City, Guizhou Province, China. The fruits were fermented for 3 days, and the seeds were collected. The seeds were sowed in wet peat covered with transparent plastic wrap and cultivated in an artificial intelligence climate room at 25°C under a 16 h/8 h light/dark photoperiod until the two-euphylla stage. The seedlings were used as the plant material. The PSTVd-infected potato leaves were stored at less than -20°C at the Vegetable Industry Research Institute, Guizhou University.

2.2 | PSTVd detection and genome sequencing

Total RNA was extracted, and reverse transcription (RT) reaction and polymerase chain reaction (PCR) were conducted as described by Li et al. (2023). The primers, PSTVd-F: 5'-ATCGATGAGGAGCGC TTCAGGGATC-3' and PSTVd-R: 5'-GTCGACGGAGCTTCAGTTGTT TCC-3' were used to amplify 224 bp PSTVd bands. Annealing temperature for PCR was 56°C. The primers, PSTVdSq-F: 5'-ATCCC CGGGGAAACCTGGAGCGAAC-3' and PSTVdSq-R: 5'-CCCTGAAGC GCTCCTCCGAG-3', were designed to sequence the whole genome by next-generation sequencing (Leichtfried et al., 2019) and to analyze the secondary structure using Mfold (online tool). The RNA sequence was set to linear, the folding temperature was fixed at 37°C, the ion condition was 1 M NaCl, and there were no divalent ions.

2.3 | PSTVd infection and plant growing conditions

The conserved PSTVd-infected potato leaves were ground into a homogenate with sterilized quartz sand by friction inoculation. A .2-mL aliquot of the leaf-extract mixture was inoculated on the true leaves of the four genotypes of healthy semi-cultivated type tomato seedlings. An equal volume of healthy potato leaf extract-treated seedlings was taken as the control. Plants in each treatment were maintained under 100% humidity for 7 days in an artificial intelligence



climate room at 25°C under a 16 h light/8 h dark photoperiod. Twenty days after incubation, RT-PCR was conducted to check if healthy seedlings were successfully infected. The infected seedlings were cultivated in a phytotron with each plant covered with an insect screen net for 60 days.

2.4 | PSTVd titer detection by RT-qPCR

RT-qPCR was thereafter conducted as described by Li et al. (2023), and the value of PSTVd titer was calculated according to Livak and Schmittgen (2001). PSTVdq-F: 5'-ACAAGGCAGGGAGGAGACT-TACC-3' and PSTVdq-R: 5'-GAAGACGAACCGAGAGGTGATGC-3' were used for PSTVd titer detection by RT-qPCR. The *Actin* gene was taken as the internal control as described by Perveen et al. (2021).

2.5 | Growth measurements

Vegetative growth was assessed 60 days after inoculating and incubating PSTVd by measuring the average stem height, shoot diameter, leaf length, leaf width, root length, and weight of the roots. Reproductive growth was determined by average single fruit weight and total yield. These indices were compared between the control and infected plants of each genotype. Twenty randomly selected samples were measured for each replication.

2.6 | Detection of chlorophyll content, the sugar acid ratio, and soluble solids content

The content of chlorophyll was measured with a HED-YB portable chlorophyll meter (Horde, China). The ratio of sugar/acid was measured by dividing the percent total sugar by the percent titratable acidity according to Chauhan et al. (2020). The content of total soluble solids in the tomato fruits was measured with the Erma hand refractometer (0%–20%, Master-Agri, Japan). Each treatment group contained thrice replicates of 20 fruits or 20 plants.

2.7 | Non-targeted metabolomics assay

Tomato leaves were sampled equally from the healthy SC-5 (control) and PSTVd-infected SC-5 plants. They were washed, mixed, and ground in liquid nitrogen. Six replicates of each treatment were used. Sample preparation for the liquid chromatography-mass spectrometry (LC-MS) analysis was conducted as described by Li et al. (2022). LC-MS analysis, metabolomics data processing, and analysis were conducted according to Li et al. (2022) by LC-bio Co., Ltd (Huangzhou, China). Metabolites were both detected under a mode of positive ion and negative ion. Metabolites with a variable importance in projection (VIP) value of >1 were counted by Student's *t*-test. Significantly regulated metabolites were analyzed on a *p*-value of <.05.

2.8 | Measurement of anti-PSTVd effects of xanthohumol and echinocystic acid

2.8.1 | Instant effects measurement

The xanthohumol and echinocystic acid powder was first diluted with a small amount of 95% alcohol, then diluted with ddH₂O, and finally prepared as an aqueous solution. Shoot terminals of PSTVd infected SC-96 60-day seedlings carrying 1 youngest leaf and a shoot tip with 1 cm in length were harvested and incubated in PCR tubes loading 100 µL of xanthohumol (.0, 65.0, 13.0, or 2.6 mg/L) or echinocystic acid (.0, 2.0, 2.0, or .2 mg/L) aqueous solution; the cut of shoot terminals was immersed in different kinds of solution, each shoot terminal per tube. The lids of PCR tubes were tightly closed in case of the solution evaporation; shoot terminals were incubated under normal seedling culture condition. PSTVd titer detection of each treatment was performed 0, 24, 48, and 72 h post metabolite feeding by RT-qPCR as described above. Each treatment contained thrice of 10 shoot terminals.

2.8.2 | Long-term effects measurement

The PSTVd infection poses a heavier threat to the semi-cultivated tomato plants under high temperature and low rainfall conditions in the summer in the open field, causing much more serious symptoms. Considering that under more serious disease symptoms, the effect of metabolites is more pronounced. Therefore PSTVd-infected SC-96 seedlings were incubated under 33°C, 16 h/8 h light/dark photoperiod, and 30% air humidity for 20 days to induce more serious disease symptoms, which included plant wilting and leaf rolling. One hundred milliliters of xanthohumol (.0, 65.0, 13.0, or 2.6 mg/L) or echinocystic acid (.0, 2.0, 2.0, or .2) aqueous solution were evenly sprayed on the leaves for 0, 5, and 10 days under the same plant incubation condition. Disease incidence and index were thereafter measured according to Sofy et al. (2013). PSTVd titer of the treated leaves was detected by RT-qPCR as described above. Each treatment contained thrice of 10 plants.

2.9 | Statistical analysis

One-way analysis of variance or Student's *t*-test was used. Data are presented as mean value ± standard error. Significance was evaluated on a *p*-value of <.05.

3 | RESULTS

3.1 | PSTVd genomic sequence

The PSTVd genome isolated from PSTVd source potato leaves was composed of 360 nt bases and had the highest identity (99.29%) with

the PSTVd-JW1229 PSTVd sequence (accession number MW312744.1) as reported by Wu and Bisaro (2020). This novel PSTVd sequence was temporarily named “PSTVd-GZT” (tentative acronym). The primary and secondary structures are presented in Figure 1a. Two substitution mutations (A^{218} to C and A^{345} to G) were confirmed in PSTVd-GZT compared with the PSTVd-JW1229 sequence. In addition, a one-base deletion mutation ($^{221}\text{-GGA}^{223}$) and one base insertion mutation ($^{282}\text{-ACCT}^{286}$) resulted in an unchanged genome size (Figure 1b).

3.2 | Effects of PSTVd on vegetative growth and yield of four semi-cultivated tomatoes

Plant height was similar in the SC-5, SC-60, and SC-128 cultures of healthy and PSTVd-infected plants, whereas the plant height of healthy SC-96 (129.67 cm) is much higher than that of

PSTVd-diseased plants (99.68 cm) (Figures 2a and 3a–d). The PSTVd infection significantly limited shoot thickening in SC-96. The average diameters of the healthy and infected plants were 1.09 and .97 cm, respectively; however, no significant differences were found in the other genotypes (Figure 2b). PSTVd did not significantly induce changes in leaf area, and the healthy control and diseased plants produced similar leaf lengths and widths (Figures 2c,d and 3e–h). Unlike diseased SC-5, SC-60, and SC-128, diseased SC-96 germinated shriveled leaves (Figure 3g,g'). In the present study, the systematic infection of PSTVd did not inhibit the average single fruit weight of SC-5, SC-60, or SC-128 but did inhibit the weight of SC-96. The healthy control produced fruits weighing 7.21 g, whereas infected plants produced significantly smaller fruits, weighing 5.58 g (Figures 2e and 3i–l, i'–l'). A similar yield of tomato fruit was harvested in the control and PSTVd-infected SC-5 and SC-128 samples, but the viroid caused a significant loss of fruit yield in SC-60, SC-96, and SC-128. Healthy SC-60 plants produced fruits that weighed 489.44 g, but PSTVd

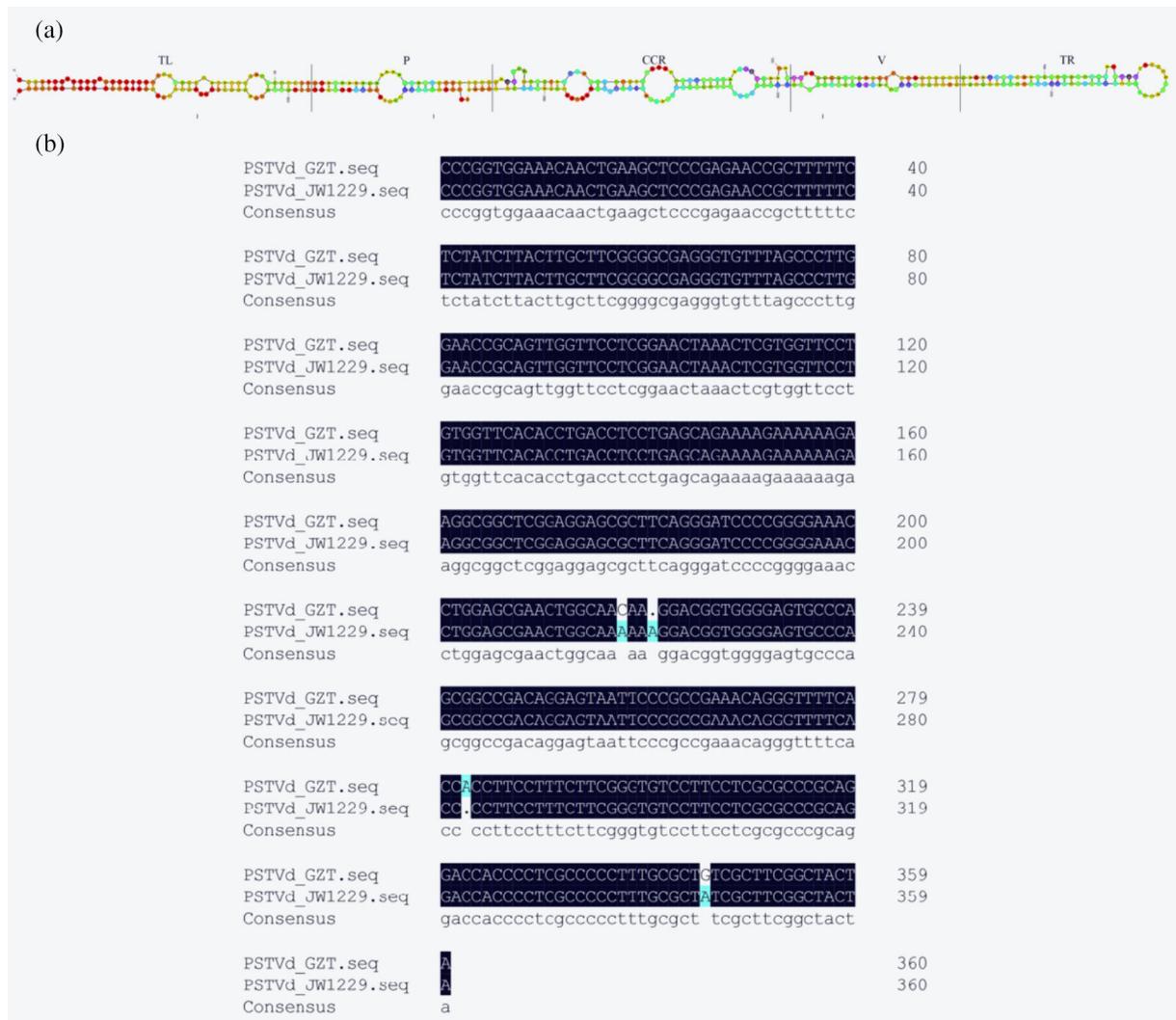


FIGURE 1 Structure and sequence of PSTVd (a) and the molecular characters compared with the PSTVd reference sequence (MW312744.1) (b). The secondary structure was analyzed using Mfold online software. TL refers to the terminal left region; P refers to the pathogenicity region; CCR is the central conserved region; V is the variable region; and TR is the terminal right region.

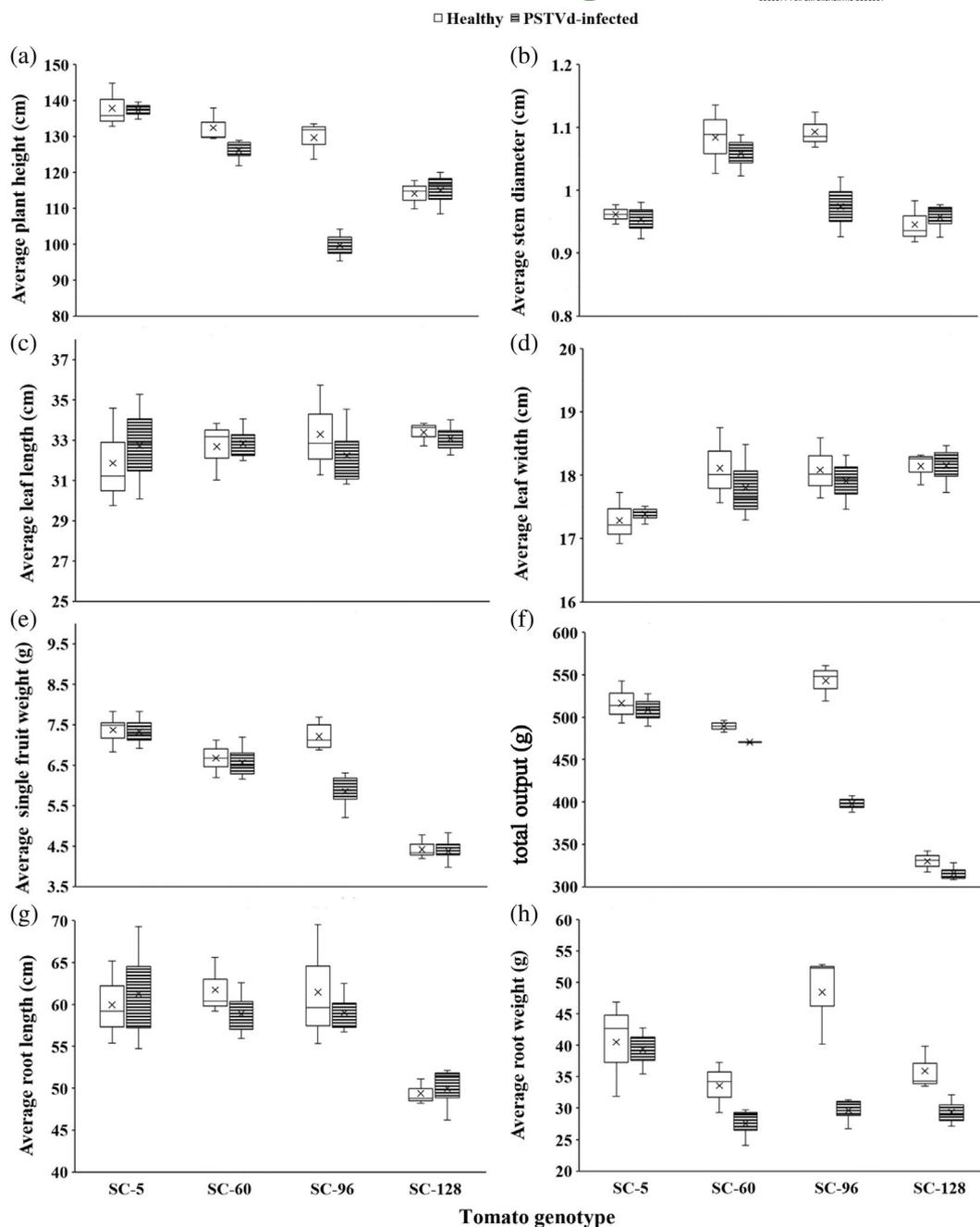


FIGURE 2 Plant height (a), stem diameter (b), leaf length (c), leaf width (d), single fruit weight (e), total output (f), root length (g), and root weight (h) of healthy and PSTVd-infected semi-cultivated tomatoes.

caused nearly 20 g of loss on a single plant. The SC-96 plants normally produced 542.85 g of fruits, but a 144.82 g loss was detected in PSTVd-diseased plants (Figure 2f). The longest roots produced by healthy samples were similar to those produced by PSTVd-infected one (Figures 2g and 3m-p), whereas fresh biomass was significantly greater in healthy SC-60, SC-96, and SC-128 plants than in viroid-diseased counterparts. The weights of the fresh root were 27.57, 29.63, and 29.37 g respectively in diseased samples. The fresh root weight of healthy control was similar to the PSTVd-infected cultures in SC-5 plants (Figures 2h and 3m-p). Seeds were isolated from

healthy and diseased tomato fruits, and no significant differences in the indices were detected, such as average seed number, weight, or germination rate (data not shown).

3.3 | RT-qPCR analysis of the PSTVd titer in four semi-cultivated tomato leaves

The PSTVd titer was significantly different among the four genotypes of semi-cultivated tomato leaves. Inoculating PSTVd on SC-5 leaves

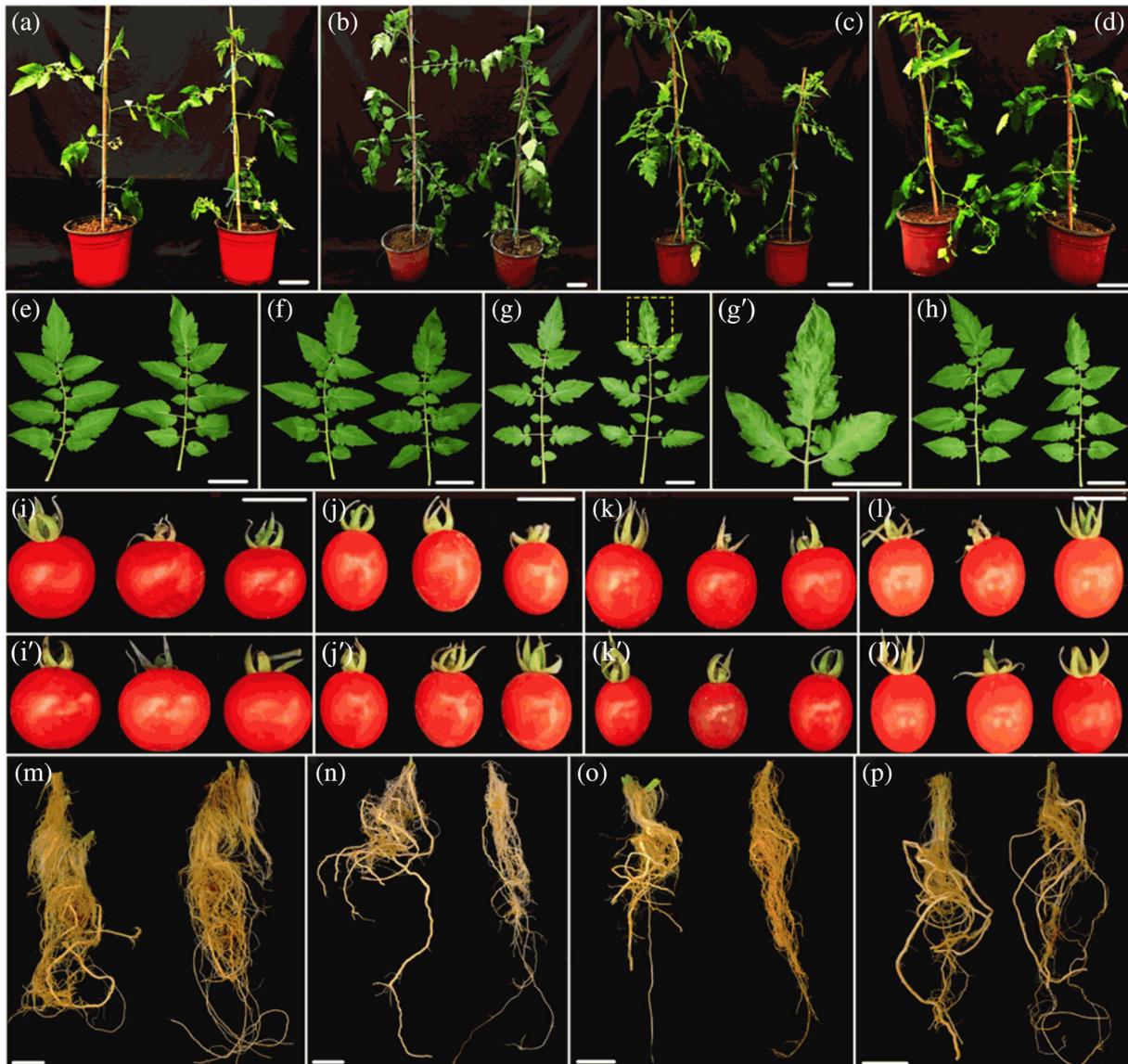


FIGURE 3 Plant vegetative growth (a–d), leaf (e–h, g'), tomato fruit (i–l, i'–l'), and root (m–p) morphology of healthy and PSTVd-diseased semi-cultivated tomatoes. The indices were determined 60 days post-inoculation. (i)–(l) indicate control fruits, and (i')–(l') indicate PSTVd-infected fruits. (a), (e), (i), and (m) represent SC-5; (b), (f), (j), and (n) represent SC-60; (c), (g), (k), and (o) represent SC-96, (g') is a close-up of a diseased SC-96 leaf; (d), (h), (l), and (p) represent SC-128. Bars indicate 1 cm.

resulted in the fewest PSTVd replicons, and the relative expression of PSTVd to actin was .204. The level of PSTVd replication was higher in SC-60 (.447) than in SC-5 but significantly lower than that in SC-128 (.744). PSTVd replicated the quickest in SC-96, and 1.133 PSTVd replicons were detected by RT-qPCR (Figure 4).

3.4 | Effects of PSTVd on chlorophyll biosynthesis and tomato fruit quality

Chlorophyll content was not significantly affected by infecting the SC-5, SC-60, and SC-128 leaves with PSTVd. The control values were 4.20, 43.30, and 47.72, and the infected sample values were 40.51,

42.44, and 47.42, respectively. PSTVd-infected SC-96 leaves had a significantly lower chlorophyll content (3.38) than the control (36.40).

To evaluate whether PSTVd infection decreases fruit quality and taste, changes in soluble solids content and the sugar/acid ratio of the tomato fruits were studied. Among the four phenotypes, a significant decrease in the content of soluble solids was only seen in diseased SC-96 (4.48%) compared with control (5.05%). The PSTVd infection did not stimulate the accumulation of soluble solids in SC-5, SC-60, or SC-128 (Figure 5b). Similarly, although PSTVd slightly inhibited the conversion from acid to sugar in all materials studied, a significant decrease in the sugar/acid ratio was only detected in SC-96. The SC-96 ddH₂O control value was 1.45, and that of its infected counterpart was 1.28 (Figure 5c).

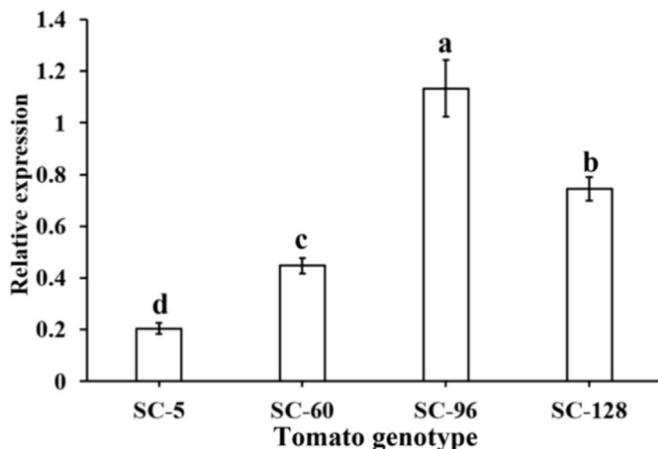


FIGURE 4 PSTVd titer of four infected semi-cultivated tomato leaves.

3.5 | Global metabolic characteristics of the SC-5 tomato response to PSTVd infection

According to the non-targeted metabolomics analysis in positive ion mode, the platform detected 158 differentially regulated metabolites (DRMs) for the partial least squares discriminant analysis (PLS-DA) model, of which 80 and 78 compounds were significantly upregulated and downregulated in the PSTVd-infected SC-5 plants, respectively. Sixty upregulated and 63 downregulated metabolites were detected in negative ion mode (Figure 6a). The significant DRMs are provided in Tables S1–S4. For pareto-scaled principal component analysis (PCA), in positive ion mode, the first principal component (PC1) explained 74.07% of the total variation, whereas PC2 explained 3.39% of the variation across the data set; in the negative ion mode, PC1 explained 7.07% of the variation and PC2 explained 7.32% of the variation. PC1 separated the PSTVd-infected leaves and those control samples under both positive mode and negative ion mode (Figure 6b).

The top 30 most PSTVd responsive metabolites in SC-5 tomato and their patterns are presented in Figure 6c. Only two-thirds of all compounds were identified with a definite name: topsentisterol A2, phenmetrazine, N-malonyltryptophan, fusarenone X, xanthohumol, dehydroeburicoic acid, and gamma-tocotrienol were upregulated by PSTVd infection in positive ion mode, whereas solasodine, mundulone acetate, nandrolone, L-pyroglutamic acid, 10'-apo-beta-caroten-10'-al, 3-hydroxy-7,12-diketocholanoic acid, 4-hydroxymandelonitrile, N6,N6,N6-trimethyl-L-lysine, protoporphyrin IX, 9s, 13r, 12-oxophytodienoic acid, tryptophol, and glutamine analog 1 were downregulated. In negative ion mode, guanine, 2-dehydro-D-gluconic acid, 3,4-dihydroxybenzoic acid, leucine, diethyl phosphate, 3-phosphoglycerate, dehydroepiandrosterone sulfate, inosine, protogracillin, guanosine, and kukoamine A were upregulated in response to PSTVd infection, whereas 3,4-dihydroxybenzaldehyde, quercetin 3-O-sophoroside, diethyl phthalate, 5-acetylsalicylic acid, 4-nitrophenol, daidzin, propylparaben, arctigenin, licochalcone A, and echinocystic acid-3-O-glucoside were downregulated (Figure 6c). The raw data of

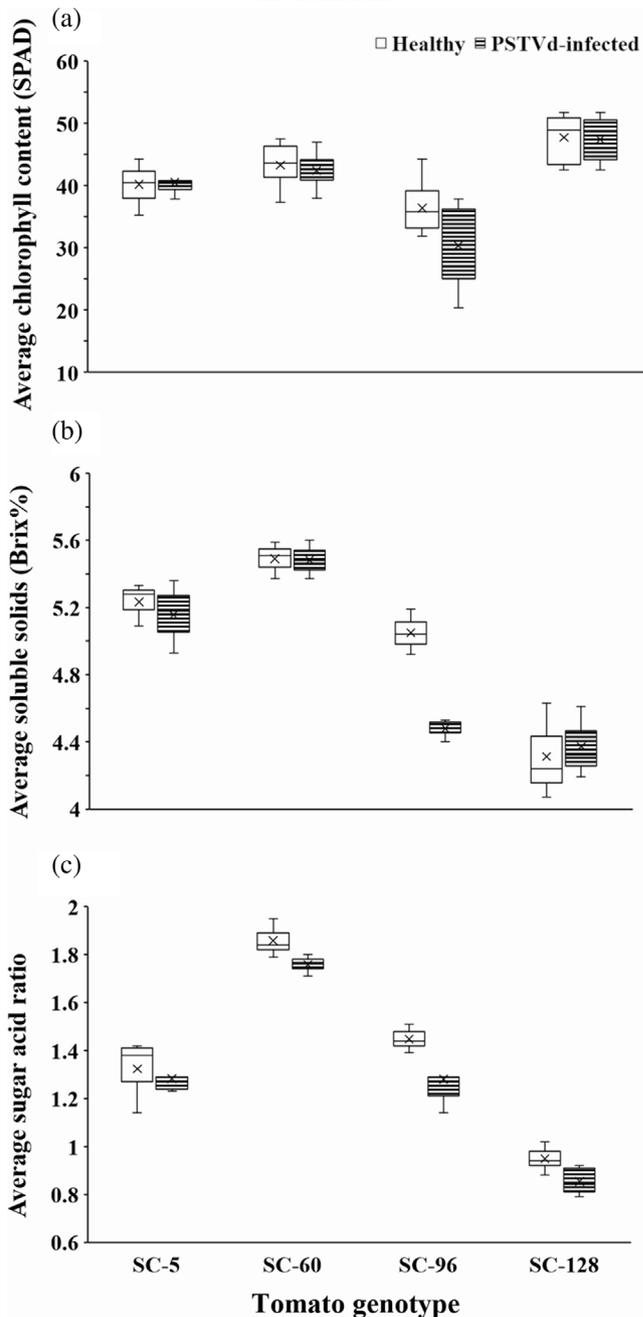


FIGURE 5 Leaf chlorophyll content (a), fruit soluble solids content (b), and the sugar/acid ratio (c) of control and PSTVd infected semi-cultivated tomatoes.

metabolomics analysis are deposited in the MetaboLights repository (MTBLS7976).

3.6 | Identification of potential anti-PSTVd metabolites

The potential anti-PSTVd compounds were isolated from the 20 top DRMs in modes of positive ion and negative ion, respectively, according to previous studies. Xanthohumol (fold change 112.3043),

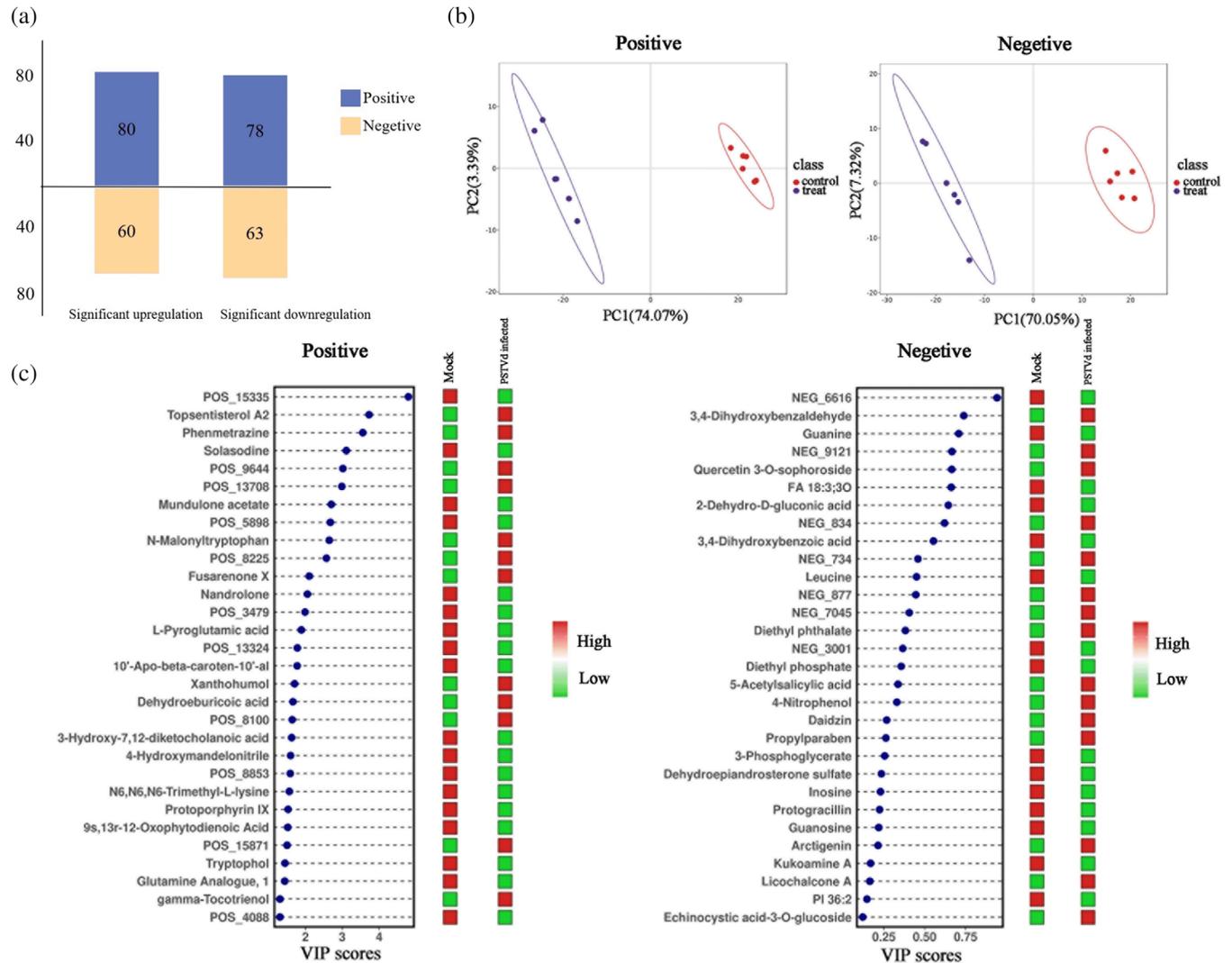


FIGURE 6 Analysis of DRMs between PSTVd-infected SC-5 tomatoes and control. (a) The number of up and downregulated DRMs in modes of positive ion and negative ion. (b) PCA of metabolic profiles of the control and susceptible groups. (c) Thirty top metabolites according to the VIP score for SC-5 tomato in response to PSTVd infection.

oxalic acid (fold change 7.7779), indole-3-carbinol (fold change 6.1781), and rosmarinic acid (fold change 5.9883) were selected among the upregulated metabolites in positive ion mode. Among those significantly downregulated DRMs in positive ion mode, cyclohexylamine (fold change .0271), protoporphyrin IX (fold change .0719), solasodine (fold change .0919), astragaloside (fold change .1199), and tryptophol (fold change .1690) were identified. Echinocystic acid (fold change 633.5949), chlorogenic acid (fold change 46.8765), and 5-acetylsalicylic acid (fold change 4.4690) were considered to be potential anti-PSTVd metabolites among the significantly upregulated DRMs in negative ion mode. Dronedarone (fold change .1414), 3,4-dihydroxybenzoic acid (fold change .1700), soyasapogenol B base + O-HexA-Pen-dHex (fold change .1717), and kaempferol-3-O-(6'''-trans-p-coumaroyl-2''-glucosyl) rhamnoside (fold change .1832) were selected among the downregulated counterparts. Among all of the potential anti-PSTVd compounds, PSTVd infection stimulated echinocystic acid the most (633.5949-fold), followed by xanthohumol

(112.3043-fold) (Table 1). All metabolites have been reported to be virus-resistant, anti-fungal, antioxidative, or anti-inflammatory according to previous studies.

These metabolites were selected from the top 20 significantly up and downregulated metabolites from data generated in positive and negative ion modes.

3.7 | Measurement of anti-PSTVd effects of important DRMs

Xanthohumol (fold change: 112.3) and echinocystic acid (fold change: 633.5) were the two most important upregulated metabolites after SC-5 tomato plants were infected by PSTVd. The effects of which on PSTVd-tolerance induction of seedlings of sensitive genotype SC-96 were conducted by exogenous application. Instant effects were measured by PSTVd titer detection. According to RT-qPCR value,

TABLE 1 Potential anti-PSTVd metabolites in PSTVd infected SC-5 tomato.

Name	VIP	Fold change	p-value	No. of KEGG	Subclass	PSTVd infected	Control
Upregulated metabolites in positive ion mode							
Xanthohumol	1.7067	112.3040	.0323	C16417	Chalcones and dihydrochalcones	627,829.2 ± 613,623.9	5590.4 ± 1686.9
Oxalicine B	.3602	7.7779	.0002		Terpene lactones	25,949.0 ± 9558.7	3336.2 ± 945.8
Indole-3-carbinol	.5011	6.1781	.0017		Indoles	59,024.4 ± 28,493.4	9553.8 ± 1948.8
Rosmarinic acid	.9302	5.9883	.0360		Hydroxycinnamic acids and derivatives	234,581.2 ± 197,512.5	39,173.1 ± 9217.7
Down-regulated metabolites in positive ion mode							
Cyclohexylamine	.3808	.0271	.0026	C00571	Cyclohexylamines	876.1 ± 276.2	32,279.6 ± 19,336.3
Protoporphyrin IX	1.5310	.0719	.0003	C05183	Porphyryns	35,053.1 ± 10,564.8	487,186.1 ± 207,549.2
Solasodine	3.1115	.0919	.0004			170,713.4 ± 19,771.7	1,856,511.3 ± 810,720.5
Astragalin	.5839	.1199	.0003	C12249	Flavonoid glycosides	8017.5 ± 2291.4	66,822.6 ± 27,282.6
Tryptophol	1.4451	.1690	.0097	C00955	Indoles	84,373.7 ± 18,713.5	499,175.8 ± 318,682.3
Upregulated metabolites in negative ion mode							
Echinocystic acid	.2887	633.5949	.0036			16,099.9 ± 10,438.2	25.4 ± 21.8
Chlorogenic acid	1.0429	46.8765	.0162	C00852	Alcohols and polyols	217,827.1 ± 180,984.6	4646.8 ± 2336.8
5-Acetylsalicylic acid	.3361	4.4690	.0001			24,477.0 ± 7725.9	5477.0 ± 981.6
Down-regulated metabolites in negative ion mode							
Dronedarone	1.7496	.1414	.0010		Carbonyl compounds	103,869.2 ± 20,754.4	734,344.1 ± 340,048.7
3,4-Dihydroxybenzoic acid	.5532	.1700	.0003	C00230	Benzoic acids and derivatives	12,842.2 ± 3680.3	75,522.2 ± 28,089.3
Soyasapogenol B base + O-HexA-Pen-dHex	.1457	.1717	.0050		Terpene glycosides	1027.9 ± 491.7	5984.4 ± 3354.6
Kaempferol-3-O-(6'''-trans-p-coumaroyl-2''-glucosyl)rhamnoside	.2374	.1832	.0265		Flavonoid glycosides	3141.6 ± 1431.6	17,147.8 ± 13,121.1

PSTVd titer of shoot terminals incubated with 13.0- and 2.6-mg/L xanthohumol for 24, 48, and 72 h were significantly lower than that of control and 65.0-mg/L treated samples. Further, 2.6 mg/L of xanthohumol generally resulted in the lowest titer of PSTVd regardless of incubation duration (Table 2). On testing with echinocystic acid, generally, a significantly lower PSTVd genome titer was identified on 2.0- and .2-mg/L treatments regardless of incubation duration. Except for 48 h of incubation, no significant difference was found between solvent control and 2.0-mg/L treatment. Among all treatments, 2.0 mg/L resulted in the highest efficiency on PSTVd titer inhibition (Table 2).

Middling seedling wilting and serious leaf rolling were observed when seedlings were cultured under environment of high temperature and low humidity; moreover, foliar solvent spraying (control) cannot alleviate those symptoms (Figure 7a,c,e,g,i,k). Solutions of xanthohumol and echinocystic acid were exogenously applied on SC-96 leaves for 0, 5, and 10 days for long-term effect measurement. The highest reduction of disease symptom was observed on 2.6 mg/L of xanthohumol or 2.0 mg/L of echinocystic acid 10 days treated seedlings. The

curled leaves on the diseased plant tend to flatten after treatment, and the plant changed from moderately wilted to upright (Figure 7b,d,f,h,j,l); the disease index decreased to 4.2 by 2.6 mg/L of xanthohumol and to 30.4 by 2.0 mg/L of echinocystic acid after 10 days of leaf spraying, whereas disease index was around 56.2–60.5 of control (Table 2). RT-qPCR value of PSTVd titer in each treatment may further confirm the above results. It was identified that the anti-PSTVd effect of 2.0 mg/L of echinocystic acid was higher and more stable than that of 2.6 mg/L of xanthohumol. Interestingly, 2.0 mg/L of echinocystic acid, which displayed a high instant effect on PSTVd titer inhibition, could not well inhibit the disease symptom on SC-96 seedlings (Table 2).

4 | DISCUSSION

Tomato is a sensitive crop to PSTVd and has long been used as an experimental plant in viroid studies (Diermann et al., 2010; Fujibayashi et al., 2021; Hammond, 1994). The expression of PSTVd symptoms in

TABLE 2 Effects of xanthohumol and echinocystic acid on induced PSTVd-tolerance of SC-96 tomato.

Concentration (mg/L)	Duration (in vitro)	PSTVd titer (relative accumulation)	Duration	Disease index	PSTVd titer (relative accumulation)
Xanthohumol					
.0	24 h	1.00 ± .16 a	0 days	59.4 ± 8.6 a	1.00 ± .36 a
65.0		1.05 ± .13 a		62.8 ± 4.6 a	.86 ± .12 a
13.0		.74 ± .32 b		65.3 ± 7.6 a	1.27 ± .27 a
2.6		.22 ± .03 c		60.8 ± 7.7 a	.76 ± .15 a
.0	48 h	1.00 ± .14 a	5 days	60.3 ± 5.4 a	1.00 ± .15 a
65.0		1.21 ± .18 a		62.8 ± 4.3 a	1.05 ± .14 a
13.0		.32 ± .04 b		53.6 ± 9.5 a	.57 ± .11 b
2.6		.34 ± .03 b		45.3 ± 2.2 b	.49 ± .02 b
0.0	72 h	1.00 ± .17 a	10 days	59.4 ± 9.2 a	1.00 ± .16 a
65.0		.84 ± .29 a		53.7 ± 4.3 a	1.21 ± .23 a
13.0		.23 ± .03 b		57.9 ± 2.3 a	.48 ± .02 b
2.6		.15 ± .03 c		4.2 ± 1.2 b	.33 ± .04 c
Echinocystic acid					
0.0	24 h	1.00 ± .17 ab	0 days	56.2 ± 7.4 a	1.00 ± .14 a
2.0		1.34 ± .19 a		58.8 ± 2.5 a	1.10 ± .10 a
2.0		.38 ± .03 c		53.6 ± 7.7 a	.76 ± .13 b
.2		.96 ± .05 b		6.2 ± 6.5 a	.69 ± .25 b
.0	48 h	1.00 ± .14 a	5 days	60.5 ± 8.7 a	1.00 ± .03 a
2.0		.46 ± .06 b		46.6 ± 3.4 ab	.45 ± .08 c
2.0		.18 ± .02 d		45.7 ± 2.7 b	.29 ± .11 d
0.2		.31 ± .04 c		54.7 ± 5.3 a	.76 ± .05 b
0.0	72 h	1.00 ± .27 a	10 days	57.3 ± 4.4 a	1.00 ± .27 a
20.0		.71 ± .14 a		45.6 ± 3.4 b	.20 ± .04 b
2.0		.26 ± .03 c		30.4 ± 4.6 c	.07 ± .01 c
.2		.41 ± .02 b		53.8 ± 9.7 a	.11 ± .05 b

tomatoes is affected by the strain and the environmental conditions as well as the plant host genotype (Mackie et al., 2019). A pair of PSTVd highly resistant (SC-5) and sensitive (SC-96) semi-cultivar tomato genotypes were identified in the present study (Figures 2 and 3). The PSTVd replicon titer was much higher in SC-96 than in SC-5. Dwarf and thin stems, reduced height, low fruit yield, and root biomass, as well as shrunken leaves, were seen on PSTVd-infected SC-96 plants, but not on the other genotypes. Similar results were reported for viroids inoculated on tomatoes by Mackie et al. (2019) and Kinoga et al. (2021). Infection usually causes malformed leaves, leaf chlorosis, reduced size, stunting, aborted flowers, necrosis of petioles, midribs, and stems, and decreases in fruit quality and yield. The irregular growth of diseased plants may be the result of reduced soluble sugar accumulation as identified in this study (Figure 5b,c). PSTVd replicates within the nucleus of host cells by the conserved sequence and/or the stem-loop structure it forms (Abraitiene et al., 2008), but it moves systemically throughout the plant and causes dysfunction of the chloroplast. Chlorophyll content significantly contributes to sugar accumulation and plant growth, whereas PSTVd infection decreased chlorophyll content (Figure 5a), thus restricting the growth of SC-96 tomatoes.

Viroids cause “viruela” symptoms as viruses on hosts, disordered plant growth (Rubio et al., 2013), and redox imbalance. Effective anti-viroid agents have not been developed or widely used. Thus, the potential anti-viroid metabolites were searched in PSTVd-resistant tomato cultivar SC-5 by non-target metabolomics analysis. As very few studies have isolated or verified anti-viroid compounds, metabolites that have been reported against virus disease, anti-inflammation, anti-oxidation, and/or induce tolerance to other pathogens were regarded as potential anti-PSTVd agents in PSTVd-resistant genotype in the present study.

Among the upregulated metabolites detected in positive ion mode, xanthohumol, a prenylated chalcone, was upregulated about 112-fold (Table 1). This compound exhibits antioxidant, anti-proliferative (Miranda et al., 1999), and viral-resistant activities (Buckwold et al., 2004). The effects on the resistance to bovine viral diarrhea virus, hepatitis B/C virus (HBV/HCV) of xanthohumol, were thereafter confirmed by in vitro experiments (Lou et al., 2014; Zhang et al., 2010). Oxalic acid, indole-3-carbinol, and rosmarinic acid reduce HIV-1 activities, human papillomavirus type 16 induces a malignant phenotype in cervical-vaginal cancer (Jin et al., 1999) and



FIGURE 7 Effects of xanthohumol and echinocystic acid on disease symptom inhibition of PSTVd-infected SC-96 tomato. (a) and (c): leaves (front and back) treated with solvent for 10 days; (b) and (d): leaves (front and back) treated with 2.6-mg/L xanthohumol for 10 days; (e) and (f): seedlings treated with solvent and 2.6-mg/L xanthohumol for 10 days; (g) and (i): leaves (front and back) treated with solvent for 10 days; (h) and (j): leaves (front and back) treated with 2.0 mg/L of echinocystic acid for 10 days; (k) and (l): seedlings treated with solvent and 2.0 mg/L of echinocystic acid for 10 days. Bar in (a), (b), (c), (d), (g), (h), (i), and (j) refers to 1 cm, and that in (e), (f), (k), and (l) refers to 2 cm. Leaves and seedlings were seldom selected from each treatment.

keratinocytes (Newfield et al., 1998), and replication of the Japanese encephalitis virus induces inflammation and Japanese encephalitis. Moreover, rosmarinic acid has strong antifungal properties, as it reduces the growth of *Candida albicans* (Gohari et al., 2009), *Alternaria alternate*, and *Penicillium digitatum* (Abdel-Rahman et al., 2020).

In the interaction between plants and pathogens, we hypothesized that there may be pathogens that downregulate resistant substances and immune systems through genetic or other biochemical regulation to achieve the goal of invading the host and replicating themselves. Thus, we selected the potential anti-PSTVd substances from the downregulated DRMs. Cyclohexylamine, protoporphyrin IX, solasodine, astragaloside, and tryptophol, which play important roles in resisting oxidation reactions, as well as anti-pathogen and anti-inflammation functions, were isolated from downregulated DRMs in positive ion mode (Table 1). Cyclohexylamine is toxic to the fungus *Pythium ultimum* (Gindrat, 1981), but its anti-viral effect has not been

reported. In vitro, protoporphyrin IX and derivatives show broad antiviral activities (Cruz-Oliveira et al., 2017; Figueira et al., 2020). A porphyrin derivative inhibits the virus entry on early stage (Lu et al., 2021) and its tin derivative is a prophylactic agent against HIV-1 (Figueira et al., 2020; Neurath et al., 1994). Solasodine, a derivative of tomatidine, has potent antiviral activity against Chikungunya virus in vitro. The effects of astragaloside are complex, including anti-oxidation, anti-viral, and anti-inflammatory effects (Choung et al., 2017). Luo and Li (2017) reported the inhibitory effect of astragaloside on HCV. Tryptophol reduces infection/replication of the GVE2 bacteriophage (Jin et al., 2015) and virus infection in shrimp (Zhu & Jin, 2015).

The potential anti-viroid agents echinocystic acid, chlorogenic acid, and 5-acetylsalicylic acid were isolated from the upregulated metabolites detected in negative ion mode (Table 1). Echinocystic acid is frequently used for inflammatory diseases in Asian (Lee et al., 2002; Ma et al., 2016). It exhibits potent anti-HCV entry activity (Wang

et al., 2013; Yu et al., 2016). Echinocystic acid was the most upregulated compound in the present study (Table 1), suggesting its important role in blocking PSTVd entry or reducing disease development. However, very limited information was found on the biosynthetic pathway of echinocystic acid and related enzymes or gene sequences. Chlorogenic acid reportedly inhibits inflammation (Zheng et al., 2015) and mammalian virus infection. Chlorogenic acid and crude extracts of coffee beans, which contain a high amount of chlorogenic acid, inhibit HBV activities. Moreover, chlorogenic acid sustains anti-enterovirus 71 activity (Li et al., 2013) and anti-human coronavirus NL63 activity (Weng et al., 2019) in vitro. Acetylsalicylic acid reduces the activities of plant and human viruses. Dhital et al. (2008) confirmed resistance of *Solanaceae* crop virus. Acetylsalicylic acid may induce an antioxidant environment to regulate HCV replication (Ibarra et al., 2013).

Dronedarone, 3,4-dihydroxybenzoic acid, soyasapogenol B, and kaempferol-3-O-rhamnoside were selected among the downregulated DRMs in negative ion mode. Gehring et al. (2014) reported the inhibitory function of dronedarone towards filovirus and New World arenavirus Guanarito activities, and the effects were dosage-dependent. 3,4-Dihydroxybenzoic acid is a well-known antioxidant (Bao et al., 2019). Ou et al. (2012) discovered its anti-infectious bursal disease virus and anti-HIV1 activities. Moreover, 3,4-dihydroxybenzoic acid is a TMV resistance agent (Tan et al., 2010). Soyasapogenol B has antiviral and anti-inflammatory activities (Amin & Mohamed, 2012). Hanna et al. (2012) successfully assessed its HCV, vesicular stomatitis virus, and hepatitis A virus inhibitory activities. These anti-viral effects were not detected for kaempferol-3-O-rhamnoside. Xi et al. (2016) confirmed that it inhibits inflammation and modulates antioxidant activity, and its biosynthesis was significantly affected by PSTVd-infection. We speculate that it may participate in plant immunity or damage replication.

Xanthohumol and echinocystic acid were substances in tomato SC-5 that regulated more than 100 folds in content after being infected with PSTVd. It was demonstrated that these two substances can inhibit the titer of PSTVd in both instant and long-term manner, reducing symptoms of wilting and leaf rolling caused by PSTVd infection. However, the inhibitory effect of Xanthohumol and echinocystic acid on PSTVd is significantly time- and concentration-dependent; specific concentrations and longer processing times were more effective (Table 2 and Figure 7). However, currently, we have only observed this phenomenon in terms of morphological characteristics and PSTVd titers. It is unclear how these two substances induce tomato SC-96 tolerance to PSTVd, and whether the regulatory mode is consistent with previous research on virus-induced diseases (Buckwold et al., 2004; Lou et al., 2014; Wang et al., 2013; Yu et al., 2016) deserves further exploration. In the previous study, extract of a xanthohumol enriched hop (*Humulus lupulus*) displayed a weak to mild BVDV (bovine viral diarrhea virus), HSV-1 (herpes simplex virus type 1), HSV-2 (herpes simplex virus type 1), and rhinovirus antiviral activity. Moreover, compared with purified isomer isoxanthohumol, xanthohumol was found to be a more potent antiviral agent against the above viruses (Buckwold et al., 2004). Lou et al. (2014) confirmed xanthohumol's anti-HCV (hepatitis C virus) activity

at concentrations ranging from 3.53 to 14.11 μ M. Echinocystic acid has a significant anti-HCV effect. Wang et al. (2013) established eight new metabolites of echinocystic acid with the chemical structure modified to assess the anti-HCV activity; however, most changes in its chemical structure cannot effectively improve its antiviral efficiency; instead, it had a certain degree of toxicity. Only two out of the eight types of echinocystic acid retain their antiviral properties, with a slight increase in antiviral efficiency. In the research of Yu et al. (2016), some echinocystic acid 28-COOH derivatives showed HCV moderate resistant activity, and one of them further removed hemolytic effect, which is commonly undesired. In addition, we observed that a high concentration (65.0 mg/L) of xanthohumol had no inhibitory effect on the titer of PSTVd at various treatment durations, but the effect of a lower concentration (2.6 mg/L) was more pronounced (Table 2). Whether there is a dose-dependent effect of xanthohumol on the inhibition of PSTVd titers, such as melatonin on *Alternaria brassicae* (Li et al., 2023), deserves further verification. Similar results were obtained by Lou et al. (2014), compared with 7.05 and 14.11 μ M, xanthohumol at 3.53 μ M had a stronger inhibitory effect on the HCV activity. This situation on echinocystic acid was not obviously seen.

There have been many successful cases of developing plant-originated metabolites as commercial biological stress resistance agents. For example, matrine developed from *Sophora flavescens* and berberine developed from *Coptis chinensis*. KNI3126 made by neem and matrine exhibited its promising inhibition efficiency against 6 pests, and it has shown the lowest biohazard (Hwang et al. (2009). Besides being used as an insecticide, .01% matrine reduced fungal pathogens by 44.5%–51.2% (Wang et al., 2019). Berberine is an environment-friendly fungicide with high efficiency in dealing with plant fungal diseases (Li et al., 2018). Knocking down the expression level of berberine bridge enzyme TaBBE64 compromised wheat's (*Triticum aestivum*) *Puccinia striiformis* resistance (Yu et al., 2023). Moreover, Guo et al. (2019) reported its promising function of anti-tobacco mosaic virus, further expanding its scope of application. The present study enriches the types of plant-derived disease-resistant metabolites; however, whether xanthohumol and echinocystic acid can be widely used as commercial agriculture chemicals needs further field experiments and environmental safety tests. In addition, based on the synthesis pathway of target plant-resistant metabolites, relevant metabolite synthesis genes can be excavated, and molecular breeding methods can be used to change gene expression levels and target metabolite synthesis levels, which can significantly alter target plant resistance traits. For instance, salicylic acid (SA) plays a crucial role in triggering system-acquired resistance of plants. Overexpress SA carboxyl methyltransferase or bacterial genes coding for enzymes that convert chorismate into SA would reduce or increase the SA-mediated plant resistance (Koo et al., 2007; Verberne et al., 2000). In addition, indirect regulation of SA signaling in plants through overexpression of VqWRKY31 enhances powdery mildew resistance in grapevine (*Vitis vinifera*) as well (Yin et al., 2022). Viroid-resistant/tolerant commercial tomato cultivars have not been developed, and key genes related to xanthohumol and echinocystic acid biosynthesis



in tomatoes have not been excavated yet; our results offered alternative molecular breeding targets.

With the continuous discovery of types of viroids, the danger of such diseases should be given attention in tomato breeding and cultivation. This study provides a statistical basis for breeding anti-viroid tomato cultivars and creating plant-originating chemical preparations for preventing viroid disease.

AUTHOR CONTRIBUTIONS

Wenkun Tang, Zhichao Tang, and Jinbiao Lu: conducting of experiments, data collection and analysis, and preparation of the manuscript; Haiyi Liu: assistance with the data collection; Qianyun Du and Huan Tian: assistance with the performance of experiments; Jingwei Li: experimental design and preparation of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The Authors did not report any conflict of interest.

DATA AVAILABILITY STATEMENT

No data were used for the research described in the article.

ORCID

Wenkun Tang  <https://orcid.org/0009-0005-2400-7762>

Zhichao Tang  <https://orcid.org/0009-0007-4644-6533>

Jingwei Li  <https://orcid.org/0009-0002-4342-5837>

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