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Investigating antiviral activities of iodine-conjugated organic agents against major viruses infecting pepper in Korea

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Abstract

Background Plant viruses cause economic losses by reducing the quantity and quality of major crops. This issue is a growing concern due to the expansion of global trade and climate change. In addition, the emergence of new pathogen strains increases the difficulty of controlling viral diseases. Effective management strategies are therefore needed. The control strategy for viral diseases relies primarily on non-chemical and cultural practices, as no commercial viricides are currently available. Some compounds have been identified as effective against certain viruses, but their use in the field is limited due to issues such as concentration, toxicity, and efficacy. Therefore, it is imperative to discover novel antiviral agents that address the existing challenges associated with the identified antiviral candidate compounds.

Results In this study, we evaluated iodine-conjugated organic compounds mixed with sialic acid, whey, and blood meal for virus disease management against seven viruses that cause significant yield losses and economic damage to plants. The candidate compounds reduced virus accumulation and symptom development. Treatment with candidate compounds, A4 and A5, reduced viral RNA accumulation to about half that of those in the control group and showed reduced symptoms along with healthier growth. In addition, we performed transcriptome analysis of treatment with two viruses, which suggested that the mechanism of viral RNA replication inhibition might relate to plant defense systems based on phytohormone pathways.

Conclusions This study demonstrated that treatments with naturally derived materials, such as iodine, nitrogen, and sialic acid-conjugated organic substances, may directly or indirectly impact the host plant's resistance to various virus infections. Moreover, our findings suggest that these natural candidate materials could be utilized for managing virus diseases in the field.

Keywords Antiviral agents, Inhibition of virus accumulation, Iodine, Metal–organic complex, Organic agent

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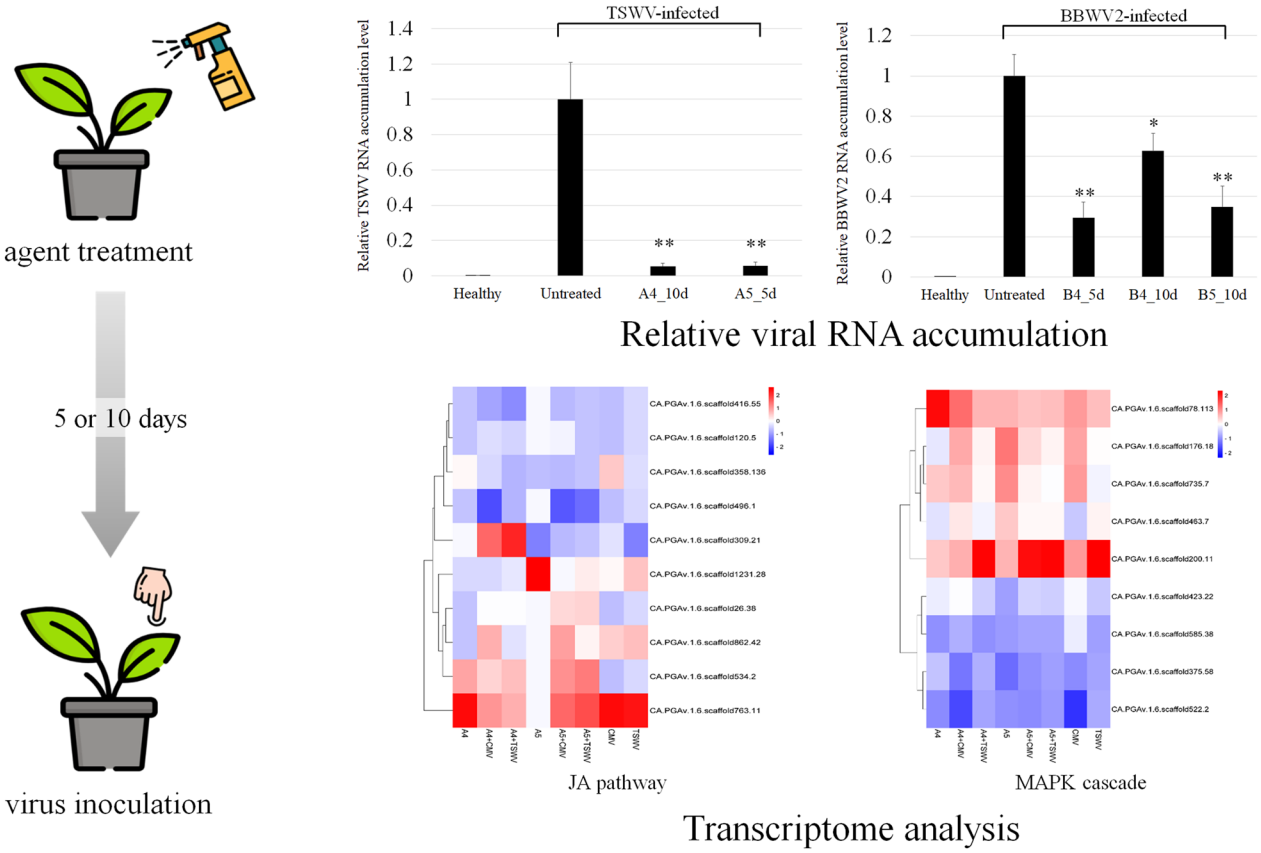
Graphical Abstract



Background

Plant virus infections profoundly impact plant growth, leading to various distortions in leaves, flowers, and fruits due to physiological stress in infected plants [1]. These virus infections often result in substantial economic losses due to reduced crop yields and compromised quality [2, 3]. In recent years, there has been a gradual increase in the incidence and pathogenicity of many plant viruses, potentially attributed to climate changes and the expansion of global trade. New pathogens and novel strains of established pathogens are also being identified annually [4]. These factors collectively contribute to the growing challenges posed by plant virus infections in agricultural systems.

As the prevalence of plant viruses continues to increase, so does the need for effective viral disease control. Current strategies include using virus-free plants/seeds, eliminating infected sources, controlling vector populations, and developing virus-resistant plants [5, 6]. However, these methods have limitations. Cultivating resistant varieties can be effective initially, but the development of new viral strains that can overcome resistance through mutations and recombination events can render resistant varieties ineffective over time [7, 8]. Insecticides, on the other hand, gradually lose their effectiveness as pests develop resistance to chemical substances over time [9]. Consequently, there is a persistent need for new and sustainable approaches to combat plant viral diseases.

Furthermore, curing virus-infected plants is challenging because there are currently no commercially available viricides specifically designed to target plant viruses. Unlike bacterial or fungal diseases that can be managed with antibacterial or antifungal agents, there is a lack of effective treatments for viral diseases [8, 10]. Previous studies have identified certain chemical compounds, such as ribavirin and 2-thiouracil, that have effectively inhibited virus replication and movement or induced host defense mechanisms [11, 12]. However, the use of these chemical compounds is associated with various

limitations. Firstly, the required concentrations of these substances to control the virus are often very high, which can be impractical and costly for large-scale applications. Secondly, some compounds may interfere with plant growth and exhibit toxicity toward the treated plants. Lastly, the amount of the compounds that reach the target organisms is often low, while the remaining bulk can contaminate the surrounding environment. Therefore, there is a pressing need to identify and develop new eco-friendly substances to manage virus diseases effectively. These substances should overcome the limitations of traditional chemical compounds and offer sustainable solutions for controlling viral infections in plants.

Plants require various nutrients for their growth and development. These nutrients can be classified into primary nutrients, secondary nutrients, and micronutrients based on their importance and the quantities in which they are needed. Interestingly, iodine is not traditionally included in the classifications of plant nutrients, and its importance in plant nutrition was not considered significant compared to other nutrients. Little is known about the role of iodine in plants. However, studies investigating the effect of iodine at various concentrations and forms on the growth of different crops have indicated that iodine may function as a plant micronutrient [13]. This emerging perspective suggests that iodine plays a role in plant nutrition, although further research is needed to fully understand its specific functions and requirements in plant metabolism.

As the understanding of iodine's potential impact on plants has grown, it has been suggested that iodine can act as a nutrient affecting plant growth and development. It has been observed that plants can absorb iodine through their roots or above-ground structures like stomata and cuticular waxes. When provided within a specific concentration range, iodine has been shown to influence plant growth, development, and even the plant's transcriptome [14, 15]. Iodine has been found to contribute to various nutritional and bioactive aspects in plants.

While there are limited published studies specifically focusing on the direct impact of iodine on plant pathogens, some research has indicated that iodine can enhance the production of antioxidants and improve resistance to specific abiotic stresses such as salinity and heavy metals [16, 17]. It is known that many stress factors increase the concentration of free radicals at the cellular level, and the induction of antioxidants is considered crucial for adaptive responses that confer stress resistance in plants [18]. In this regard, iodine, which functions as an antioxidant, may contribute to the induction of resistance against stressors, including potential pathogens [14]. Additionally, studies have shown that iodine-induced modifications in cuticular waxes can alter the interaction pattern between pathogens and plants [19].

Based on these findings related to plant responses to biotic and abiotic stresses, it is hypothesized that iodine may modulate defense mechanisms against viral infections. Therefore, iodine holds promise as a potential candidate to be explored as a new antiviral substance. This study aims to investigate and evaluate the antiviral activity of organic substances containing iodine against the major viruses that infect pepper and tobacco plants to discover new resources for antiviral agents.

Methods

Plant material and virus inoculation

Nicotiana benthamiana and *Capsicum annuum* cv. Chungyang used in this study were cultivated under controlled growth conditions. The plants were grown at 25 °C in a growth chamber with a photoperiod of 16 h of light and 8 h of darkness. To initiate viral infections, seven plant viruses were mechanically inoculated onto the plants. The viruses used in the study included broad bean wilt virus 2 (BBWV2; genus *Carmovirus*), cucumber green mottle mosaic virus (CGMMV; genus *Tobamovirus*), cucumber mosaic virus (CMV; genus *Cucumovirus*), pepper mottle virus (PepMoV; genus *Potyvirus*), and pepper mild mottle virus (PMMoV; genus *Tobamovirus*). Virus-infected leaf tissue was frozen and subsequently ground using 0.05 M potassium phosphate buffer (pH 7.4). For tomato spotted wilt virus (TSWV; genus *Tospovirus*), the infected leaf tissue was ground using 0.05 M potassium phosphate buffer (pH 7.0) containing 5% sodium sulfate. Prior to inoculation, carborundum was applied to the leaf surface, followed by gentle rubbing of the sap to ensure uniform distribution [20]. For the infection of *N. benthamiana* with tomato yellow leaf curl virus (TYLCV; genus *Begomovirus*), a leaf agroinfiltration method was employed. Experiments were performed with three biological replicates with at least three plants for each replicate. Plants were grown in growth chambers at 25 °C with 70% relative humidity and a 16/8 h photoperiod.

Agent treatment and validation of antiviral activity

In this study, various iodine-based formulations were evaluated as potential antiviral agents. The composition of these formulations varied, incorporating gradients of sulfur, sialic acid, whey, and blood meal. The specific candidate agents used in the study are listed in Table 1. To administer the treatments, the candidate compounds were diluted to their respective concentrations and applied to the test plants. The concentrations of the iodine and sulfur were determined by conducting

Table 1 Main ingredients and amount used in this study

Agent		Main ingredients and amount used per 100 L
I		Iodine-conjugated organic complex (1.806 g)
SI		Sulfur and iodine-conjugated organic complex (I: 1.806 g and S: 0.228 g)
M		Sialic acid, whey, and blood meal (230 g)
Type A	A4	Sialic acid, whey, blood meal, and iodine-conjugated organic complex (I + M)
	A5	Sialic acid, whey, blood meal, and iodine- and sulfur-conjugated organic complex (SI + M)
Type B	B4	Sialic acid, whey, blood meal, and iodine-conjugated organic complex (I + M)
	B5	Sialic acid, whey, blood meal, and iodine- and sulfur-conjugated organic complex (SI + M)

Type A: the non-separated form of sialic acid

Type B: the separated form of sialic acid

inductively coupled plasma analysis. To obtain the appropriate concentration of each compound, we prepared a series of dilutions for each compound without any contaminants and applied them to the plants by drenching and spraying. Drenching was performed using 40 ml of the diluted solution per plant, while spraying involved applying 5 ml of the solution per plant. The treatments were applied twice or three times before or after virus challenge inoculations.

Virus challenge inoculations were carried out using either sap inoculations or agroinfiltration inoculations, depending on the specific virus. The effects of each treatment were evaluated by observing symptom development and conducting a semi-quantitative polymerase chain reaction (PCR) analysis. The plants were monitored for up to 4 weeks following the challenge inoculations for visual observation of any changes or symptoms associated with virus infection.

Reverse transcription-PCR (RT-PCR) for virus detection

To extract total RNA from the systemic leaves, RNAiso Plus (TaKaRa, Japan) was used following the manufacturer's instructions. Approximately 2 µg of total RNA was then utilized for complementary DNA (cDNA) synthesis. The cDNA synthesis was carried out in a reaction volume of 10 µl, using random hexamers and GoScript™ Reverse Transcriptase (Promega, USA) according to the manufacturer's protocols.

Viral-specific primer sets were employed for the reverse transcription-polymerase chain reaction (RT-PCR). In the RT-PCR reaction, 20 ng of cDNA was added to the reaction mixture, which consisted of 1 µl of 10X PCR buffer, 0.8 µl of dNTPs, 0.1 µl of Ex-Taq DNA polymerase (TaKaRa, Japan), 0.5 µl each of forward and reverse primers (Additional file 1: Table S1), 2 µl of cDNA (20 ng/µl), and 5.1 µl of sterile distilled water. The PCR was performed using the following standard protocol: an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. A final extension step was conducted at 72 °C for 10 min. This RT-PCR protocol allowed for amplifying target viral sequences in the cDNA samples, providing information on the presence or abundance of the respective viruses in the systemic leaves.

Quantitative RT-PCR (RT-qPCR) analysis for determining virus accumulation level

After treating the agents with virus challenge inoculation, leaves from both the treated plants and the control group were collected at various time points. RT-qPCR was conducted using a CFX384 real-time PCR system (Bio-Rad, USA). In each 10 µl reaction mixture, 1 µl of cDNA (20 ng/µl) was combined with 5 µl of 2X iQ SYBR Green supermix (Bio-Rad, USA), 1 µl of each gene-specific primer set (Additional file 1: Table S2), and 2 µl of DEPC-treated water. The qRT-PCR was performed according to the following conditions: an initial pre-denaturation step at 95 °C for 3 min, followed by 40 cycles of programmed amplification, consisting of denaturation at 95 °C for 10 s, annealing and extension at 55 °C for 30 s, and a melt curve analysis ranging from 55 to 95 °C for 5 s.

The relative expression levels of genes were determined using the Bio-Rad CFX Manager software, version 1.6.541.1028 (Bio-Rad, USA), employing the $2^{-\Delta\Delta Ct}$ method [21]. The expression levels were normalized to the reference genes Eukaryotic initiation factor 5A2 (EIF5A2) and β-tubulin (β-TUB) [22]. Each treatment was evaluated in three independent experiments with at least three plants, including three technical replicates. The primer sets used for qPCR are shown in Additional file 1: Table S2. This allowed for the calculation of the relative expression levels of the target genes in comparison to the control group, providing insights into the gene expression changes during the experimental time course.

TSWV infection of *C. annuum* and leaf sampling to test the effect of the A5 compound on viral RNA replication

We conducted both chamber and field experiments to test the effect of the A5 compound on the inhibition of TSWV replication following TSWV infection. Briefly, *C. annuum* was sap-inoculated with TSWV at the 8-leaf stage. After confirmation of infection at 20 days post-inoculation (dpi) by conducting RT-PCR with a specific primer set using total RNA extracted from upper uninoculated leaves, plants were grown in a chamber at 25 °C with 70% relative humidity and a 16/8 h photoperiod and greenhouses located in Yongin, Gyeonggi-Do and Yeongyang, Gyeongsangbuk-Do, South Korea. There were 3 plots, and within each plot, there were 2 groups, i.e., A5-treated and an untreated control group. Each plot consisted of at least 30 TSWV-infected pepper plants; thus, we tested at least 90 plants per group in the chamber and in two greenhouses. A total of 45 ml of A5 or water was sprayed and drenched on each plant at 10-day intervals. Leaf tissue was collected from 10 plants and combined as a single sample at 20, 30, and 40 days post-A5 treatment (dpt) for analysis of both chamber and field samples. An additional sample was collected from the field at 60 dpt. Pepper fruits were harvested after 5 months of field growth. Nine pepper fruits from each group were randomly selected for quantifying relative TSWV RNA levels.

Statistical analysis

Statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Scheffe's test. A significance level of $P < 0.05$ was considered statistically significant, indicating a significant difference between the treatment and control groups.

Extraction of total RNA and preparation of RNA libraries for transcriptome analysis

To investigate the mechanism of action of A4 and A5 agents against TSWV and CMV, a total of 27 experimental conditions were created. The plant leaves were collected three days after treatment with A4 or A5 agents and virus inoculation. Total RNA was extracted from the collected samples using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of the extracted RNA samples was assessed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To prepare the libraries for sequencing, the TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) was used, and the libraries were sequenced using the NovaSeq 6000 system.

Mapping and identification of differentially expressed RNAs

The raw reads obtained from the sequencing were aligned to the cDNA databases for CM334, which were obtained from http://peppergenome.snu.ac.kr/, using the BBMap software (version 39.00). To identify the differentially expressed genes (DEGs), the read counts from all libraries were analyzed using DESeq2 in DEBrowser (version 1.24.1). DEGs were determined based on a log2-fold change (log_2FC) value greater than two and a p-value less than 0.05, indicating a significant increase or decrease in gene expression. A total of 27 datasets were generated, representing nine experimental samples with three replicates each for all comparisons. The transcriptome data used in this study were submitted to the NCBI as BioProjects (PRJNA975871).

Gene ontology (GO) enrichment and MapMan analysis

To gain insight into the functional implications of the differentially expressed genes (DEGs), a GO enrichment analysis was performed. To facilitate the analysis using *Arabidopsis* protein IDs, a BLASTP search was conducted to align the pepper protein sequences against the Arabidopsis proteins available in the TAIR database (version 10; https://www.arabidopsis.org/). The GO enrichment analysis was then carried out using the agriGO database (version 2.0), providing a comprehensive set of agricultural Gene Ontology analysis tools [23].

To further classify the gene expression patterns in response to treatment with iodine-conjugated compounds and virus infection, the MapMan software was utilized. The TAIR10 ID, which exhibited homology to the pepper protein sequences, was used in this analysis [24]. MapMan is a powerful tool that allows for the visualization and interpretation of gene expression data in the context of metabolic pathways and biological processes. These analyses aimed to provide a functional overview and categorize the gene expression changes induced by the treatment with iodine-conjugated compounds and virus infection.

Results

Antiviral effect of organic agents conjugated with sialic acid, iodine, and sulfur in *N. benthamiana* against virus challenge inoculations

To evaluate the inhibition activity of various organic agents conjugated with sialic acid, iodine, or sulfur (I and SI + M; Table 1), we applied these agents to the *N. benthamiana* by drenching method and inoculated five different viruses (CMV, BBWV2, TYLCV, CGMMV, PepMoV, and PMMoV). The treatment with these agents did

not show any side effects, such as stunting, yellowing, or plant death without virus inoculation.

The results show that against CMV and BBWV2 infection, both treated and untreated plants exhibited viral symptoms on the upper systemic leaves. However, the symptoms observed on I-treated plants were less severe compared to untreated plants (Fig. 1A). This suggests that agent I has some inhibitory effect on the severity of CMV and BBWV2 symptoms. RT-PCR analysis also confirmed lower RNA accumulation of CMV and BBWV2 in I-treated plants, further supporting their antiviral activity against these viruses (Fig. 1B).

In the case of TYLCV, treating plants with the agent I did not completely inhibit the virus movement to the upper leaves. However, mild symptoms were observed on the systemic leaves of agent-treated plants, indicating some level of suppression of TYLCV infection. RT-PCR analysis showed a lower accumulation level of TYLCV in treated plants compared to untreated plants, indicating the potential antiviral effect of agent I against TYLCV.

However, when it comes to CGMMV, treating plants with the agent I did not inhibit the virus accumulation in the upper systemic leaves. Additionally, no significant differences were observed between untreated and treated plants in terms of CGMMV accumulation, as determined by RT-PCR analysis using a virus-specific primer set (Fig. 1).

Overall, the results suggest that agent I has a mitigating effect on the severity of symptoms and viral RNA accumulations in CMV and BBWV2 infections, as well as a partial inhibitory effect on TYLCV infection. However, they may not inhibit CGMMV accumulation in *N. benthamiana* plants.

The results indicate that treating plants with SI and M agents led to mild symptoms of BBWV2 and TYLCV in systemic leaves (Fig. 2A). However, the RT-PCR analysis showed that virus accumulation of BBWV2 and TYLCV was not detected in SI and M agent-treated plants (Fig. 2B), suggesting a potential inhibitory effect on these viruses.

On the other hand, when it comes to PepMoV, CMV, and PMMoV, treating virus-infected plants with SI and M agents did not show a significant difference in symptom development or viral accumulation compared to control plants (Fig. 2A). This suggests that SI and M agents may not have strong inhibitory effects on PepMoV, CMV, and PMMoV infections in *N. benthamiana* plants.

Overall, the results indicate that SI and M agents have some inhibitory activity against BBWV2 and TYLCV but may not be as effective against PepMoV, CMV, and PMMoV in *N. benthamiana* plants. The response to treatment may vary depending on the specific virus species and the interactions between the agents and the viruses.

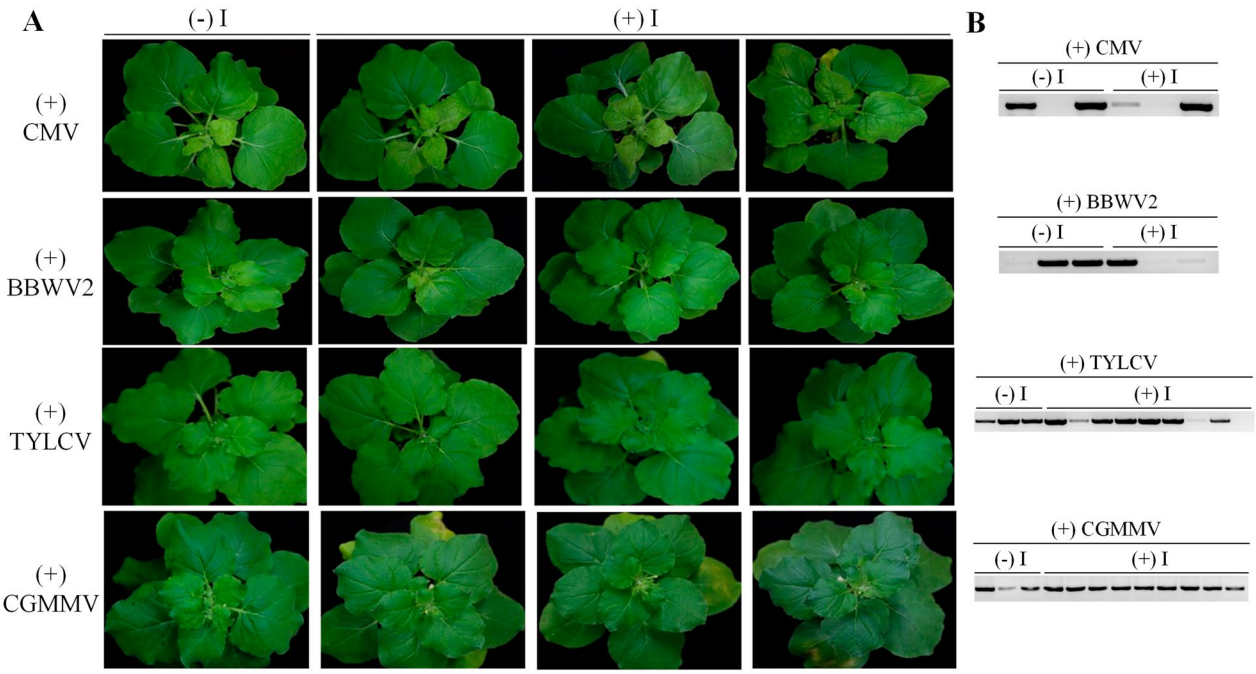


Fig. 1 The inhibitory effect of iodine agent (I) on CMV and BBWV2, TYLCV, and CGMMV infections in *Nicotiana benthamiana* plants. **A** CMV, BBWV2, and CGMMV were inoculated using virus-infected leaf tissue onto *N. benthamiana*. For the TYLCV inoculation, agro-infiltration using *Agrobacterium* was conducted. The plants inoculated by infected leaf tissue were observed under normal light. **B** The virus accumulation in systemic leaves was confirmed by performing RT-PCR

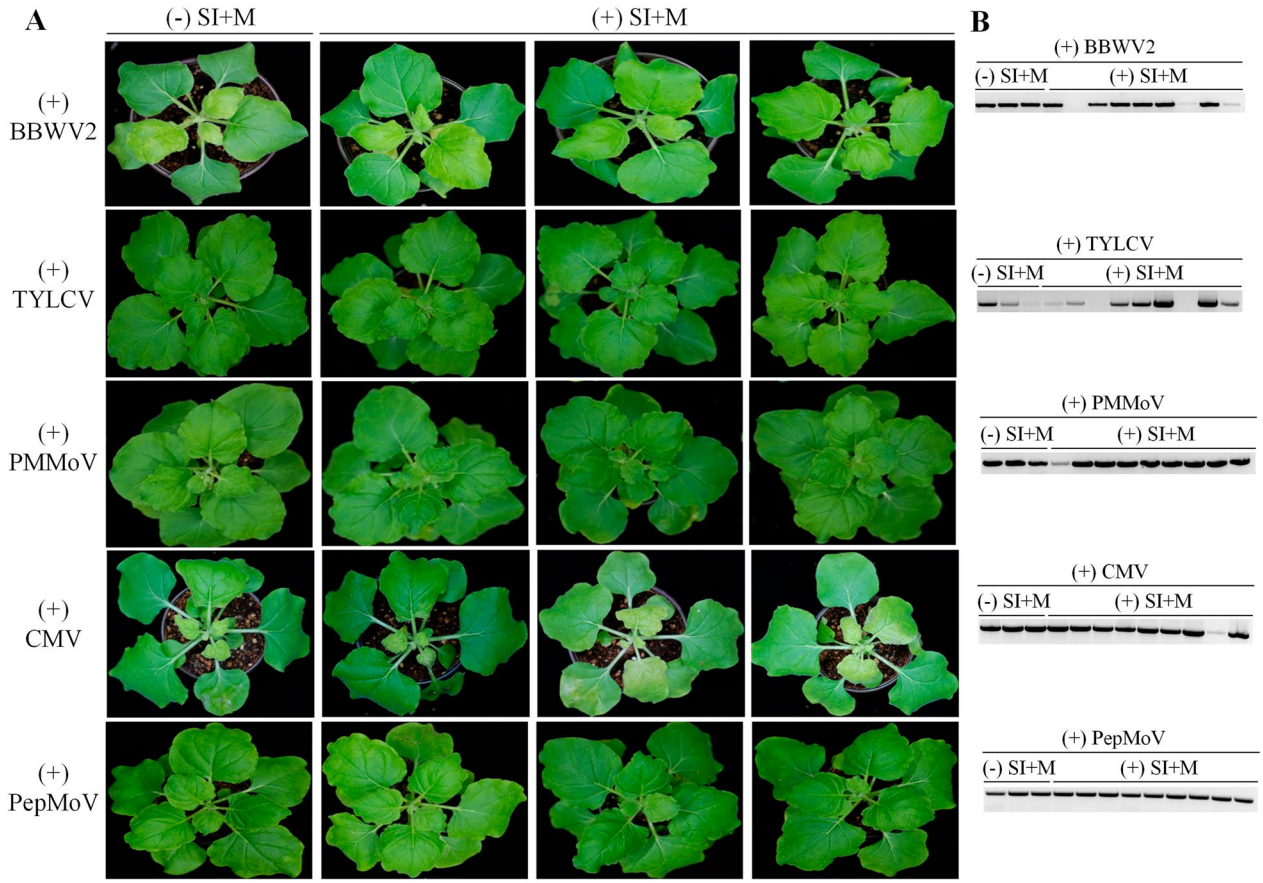


Fig. 2 The inhibitory effect of SI and M agents on five different virus infections on *N. benthamiana*. **A** Inoculation of BBWV2, PMMoV, CMV, and CGMMV were carried out using virus-infected leaf tissue onto *N. benthamiana*. For the TYLCV inoculation, agro-infiltration using *Agrobacterium* was conducted. The plants inoculated by infected leaf tissue were observed under normal light. **B** The viral accumulation in systemic leaves was detected by performing RT-PCR

Based on the results obtained with agents I, SI, and M in *N. benthamiana* plants, two types of organic compounds were formulated: type A and type B. Type A compounds were formulated using the non-separated form of sialic acid (A4 and A5). In contrast, type B compounds were formulated using the separated state of sialic acid obtained through enzyme digestion (B4 and B5). Compounds A4 and B4 were formulated with iodine-conjugated agents, while A5 and B5 compounds were formulated with both iodine and sulfur-conjugated agents.

These new organic compounds with different formulations were likely developed based on the observed antiviral activity and their potential to inhibit virus infection or reduce virus symptoms in previous experiments. The incorporation of iodine and sulfur in the formulation may have been aimed at enhancing the antiviral properties of the agents. We used these new organic compounds for further studies and experiments in *C. annuum* cv. Chungyang plants to evaluate the effectiveness of these newly formulated compounds and their potential application as antiviral agents in plant protection.

Antiviral effect of newly formulated agents based on mixtures of sialic acid, iodine, and sulfur in *C. annuum* against virus challenge inoculations

In the study evaluating the inhibition activity of type A and type B agents composed of a mixture of sialic acid, iodine, and sulfur, the agents were applied to pepper plants using a drenching method and inoculated three different viruses (TSWV, CMV, and BBWV2). The treatments were initiated 5 days after virus inoculation, and additional treatments were performed at intervals of either 5 days or 10 days. The treatment with these agents did not show any side effects in pepper plants. Leaf samples from the systemic leaves were collected for further analysis.

To assess the inhibition activity against TSWV infection, semi-quantitative PCR was performed. The results

indicated that the most significant inhibitory effects were observed with the following treatments: A4 treatment every 10 days and A5 treatment every 5 days (Fig. 3). These treatments showed reduced virus symptoms compared to the untreated control plants. The results also showed a significant reduction in the accumulation level of TSWV RNAs in plants treated with A4 every 10 days and A5 every 5 days compared to the untreated control plants (Fig. 3). These findings suggest that the A4 and A5 agents, formulated with a mixture of sialic acid, iodine, and sulfur have potential inhibitory effects against TSWV infection in pepper plants. The treatments at specific intervals demonstrated effective control of virus accumulation and symptoms.

In the study evaluating the inhibitory activity against CMV infection, the agent treatments demonstrated significant effects. Particularly, the treatments with B4 and B5 agents showed remarkable inhibition activity (Fig. 4). To further quantify the CMV accumulation, semi-quantitative PCR was performed. The results indicated that the 5-day interval treatments of B4 and B5 agents had significant effects on reducing CMV accumulation in pepper plants (Fig. 4). These findings suggest that the type B agents (B4 and B5), formulated with a mixture of sialic acid, iodine, and sulfur exhibited inhibitory activity against CMV infection in pepper plants. The treatments at a 5-day interval resulted in reduced CMV accumulation, indicating their potential for controlling CMV infection.

In the evaluation of the type A and type B agents' effectiveness against BBWV2, it was observed that treatment with type A and type B agents did not seem to alter symptom development and the accumulation of BBWV2 viral RNAs based upon semi-quantitative RT-PCR (Fig. 5). This might suggest that the treatment of newly formulated agents did not significantly inhibit

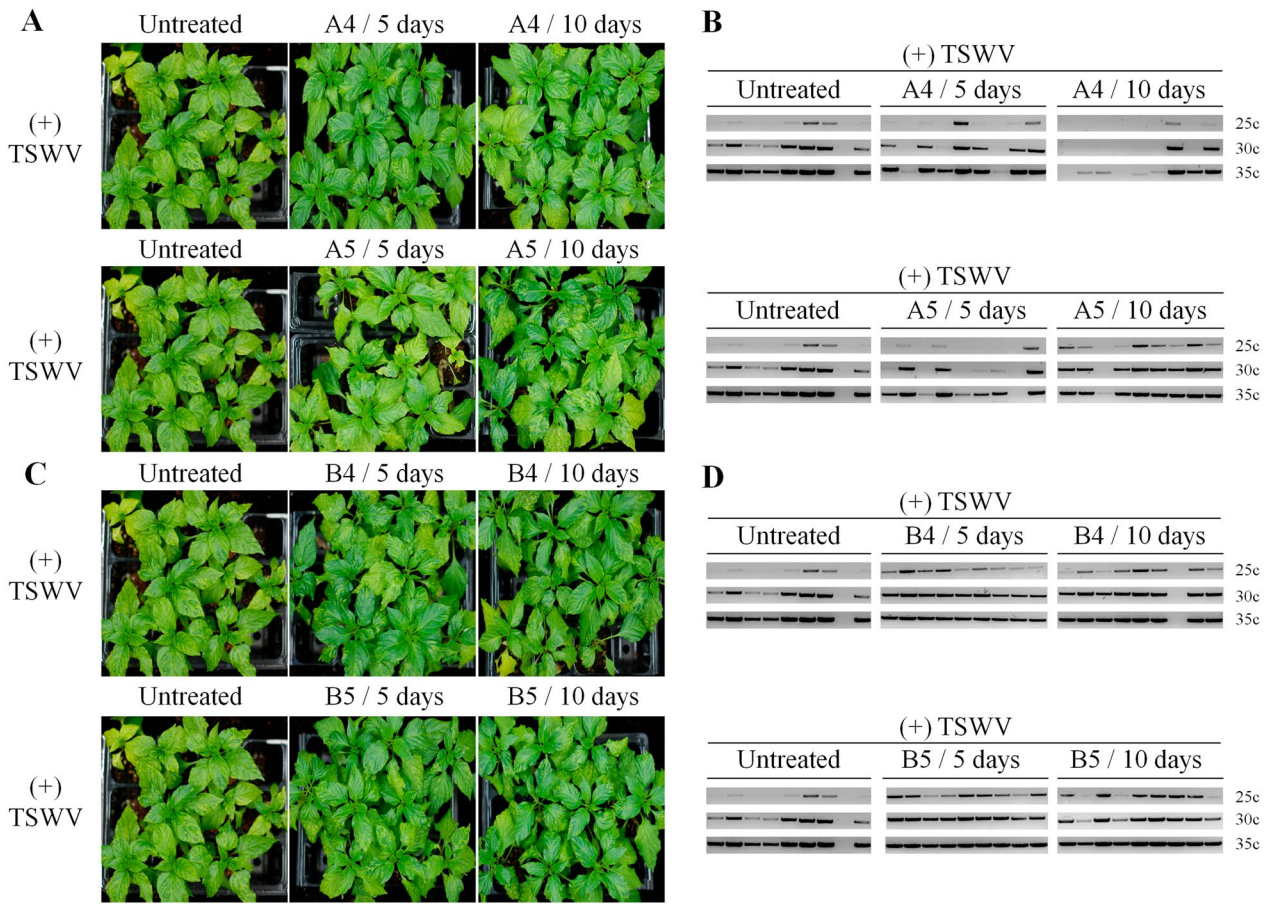


Fig. 3 Effect of type A or B agent treatment against TSWV infections in *Capsicum annuum*. **A**, **C** The plants inoculated by TSWV-infected leaf tissue were observed under normal light. The treatments were initiated 5 days after virus inoculation, and additional treatments were performed at intervals of either 5 days (5 days) or 10 days (10 days). The 'untreated' control image was re-used to provide a separate illustration with each treatment. **B, D** The viral RNA of TSWV in systemic leaves was detected by semi-quantitative RT-PCR using a TSWV-specific primer set. 25c, 30c, and 35c indicate PCR cycles used for amplification. The gel images representing amplified fragments from the 'untreated' control were re-used to provide a separate illustration with each treatment

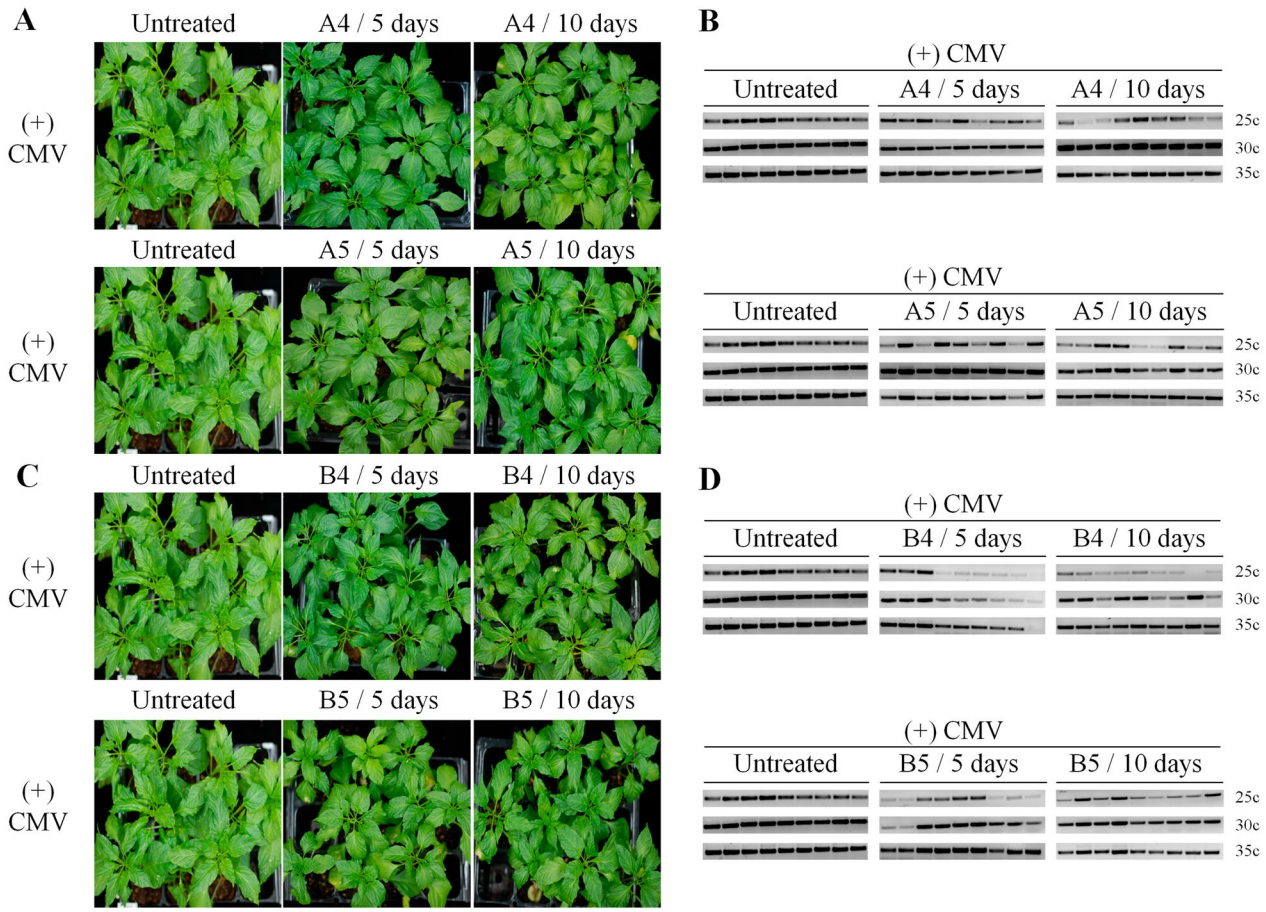


Fig. 4 Effect of type A or B agent treatment against CMV infections in *C. annuum*. **A, C** The plants inoculated by CMV-infected leaf tissue were observed under normal light. The treatments were initiated 5 days after virus inoculation, and additional treatments were performed at intervals of either 5 days (5 days) or 10 days (10 days). The 'untreated' control image was re-used to provide a separate illustration with each treatment. **B, D** The viral RNA of CMV in systemic leaves was detected by semi-quantitative RT-PCR using a CMV-specific primer set. 25c, 30c, and 35c indicate PCR cycles used for amplification. The gel images representing amplified fragments from the 'untreated' control were re-used to provide a separate illustration with each treatment

BBWV2 infection, as there were no noticeable changes in symptom severity or the accumulation of viral RNAs compared to the untreated control plants.

To further investigate the effectiveness of the type A and type B agents against three viruses infecting pepper plants (TSWV, CMV, and BBWV2), RT-qPCR analysis was conducted to quantify viral RNA accumulation. The results showed that the type A and type B agent treatments significantly reduced viral RNA accumulation for all three viruses. Specifically, A4 treatment with a 10-day interval and A5 treatment with a 5-day interval demonstrated over 90% inhibition activity against TSWV. CMV accumulation was effectively inhibited by A4 treatment with a 5-day interval, while B4 treatment with a 5-day interval showed the best inhibition effect against BBWV2 infection (Fig. 6).

It is interesting to note that although the reduction in BBWV2 RNA accumulation was not as significant as that observed for the other two viruses, there was still some degree of reduction observed in the B4- and B5-treated plants (Fig. 6). This suggests that the type B agents, particularly B4 treated with a 5-day interval, may have some inhibitory effect on BBWV2, although it may be less pronounced compared to the other viruses. Further investigation could confirm the potential antiviral activity of the agents against BBWV2.

Altogether, these findings suggest that the type A and type B agents, particularly A4 and A5 treatments, have the potential to effectively reduce viral RNA accumulation and inhibit the infection of TSWV and CMV in pepper plants.

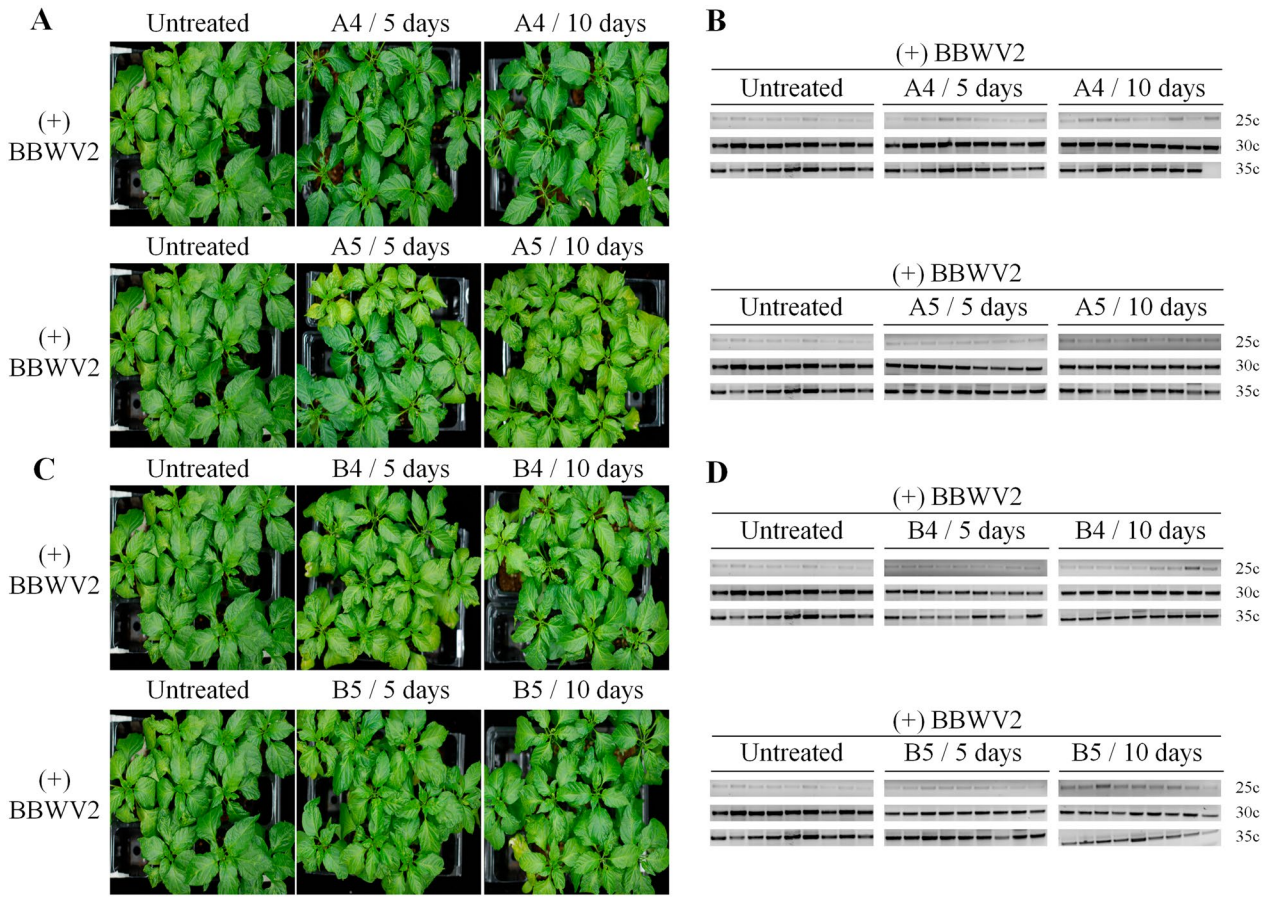


Fig. 5 Effect of type A or B agent treatment against BBWV2 infections in *C. annuum*. **A, C** The plants inoculated by BBWV2-infected *N. benthamiana* leaf tissue were observed under normal light. The treatments were initiated 5 days after virus inoculation, and additional treatments were performed at intervals of either 5 days (5 days) or 10 days (10 days). The 'untreated' control image was re-used to provide a separate illustration with each treatment. **B, D** The viral RNA of CMV in systemic leaves was detected by semi-quantitative RT-PCR using a BBWV2-specific primer set. 25c, 30c, and 35c indicate PCR cycles used for amplification. The gel images representing amplified fragments from the 'untreated' control were re-used to provide a separate illustration with each treatment

Effect of the A5 compound on the reduction of TSWV accumulation level in *C. annuum*

The effect(s) of the A5 compound on TSWV-infected pepper plants were further evaluated in the growth chamber and under field conditions. Each TSWV-infected plant was sprayed and drenched with 45 ml of 500X diluted A5 compound at 10-day intervals, and the leaf samples were collected from the 20 dpt at 10- or 20-day intervals up to 60 dpt. The field trial was conducted over 5 months until fruit harvest from each group of 90 pepper plants.

In the chamber experiment with controlled temperature and light interval, virus levels in the A5-treated group were reduced to approximately half of those in the control group and maintained decreased levels over time (Fig. 7A). The A5 compound-treated group generally showed reduced symptoms and healthier growth (data not shown).

In the field experiment with the same treatment in two different locations, we observed reduced levels of TSWV RNA replication in general. However, the relative levels of TSWV RNA accumulation at the same time point were not consistent in each location. For example, relative TSWV RNA accumulation levels in Yongin, Gyeonggi-Do were similar to untreated at 20 dpt and maintained reduced levels (45–58%) during 30–60 dpt (Fig. 7B). In contrast, reduced TSWV RNA accumulation levels were observed in Yeongyang, Gyeongsangbuk-Do at 20 and 30 dpt with 38 and 67% compared to the untreated samples, respectively (Fig. 7C). At 40 and 60 dpt, RNA accumulation levels increased over time.

We maintained the field in Yongin, Gyeonggi-Do, and harvested pepper fruits. The A5 group showed an outstanding yield of almost five times that of the control group, which was not treated after TSWV infection. Because the pepper plants were infected in the

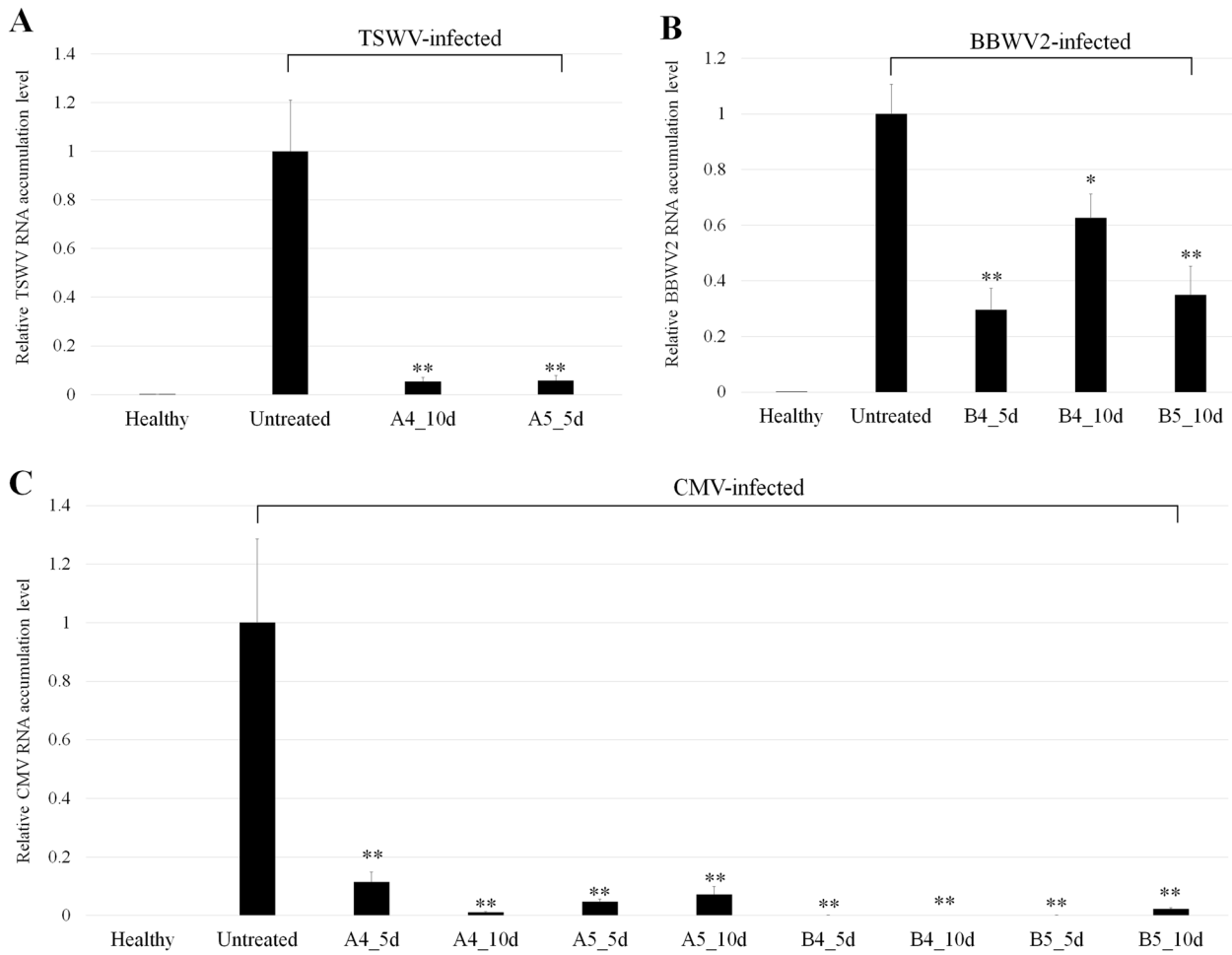


Fig. 6 The relative viral RNA accumulations of TSWV, CMV, and BBWV2 in *C. annuum* L. The y-axis indicated relative expression levels of TSWV (**A**), CMV (**B**), and BBWV2 (**C**) viral RNA accumulation. Relative viral RNA accumulation in the agent-treated plants was compared to positive controls for each virus infection plant without agent treatments. RT-qPCR results were normalized based on β-tubulin and EIF5A2 as reference genes. Each treatment was evaluated in three independent experiments with at least three plants, including three technical replicates

early stages of growth and transplanted to the fields, most of the untreated pepper plants were stunted. Some severely infected plants died. Symptoms on the leaves include stunting with necrotic spots or rings and eventually a wilted appearance. Developing green fruits showed diagnostic spots and concentric rings that were initially pale or yellow but became necrotic. Mature fruits were severely distorted and often showed necrotic rings. The diseased fruit rate was approximately 85–90% based on phenotypic observations. In contrast, most of the infected pepper plants survived and showed less severe symptoms on the leaves over time when we treated them with the A5 compound. Surprisingly, we observed recovery of symptoms in the fruit, so the rate of diseased fruit was approximately 3% based on phenotypic observations. Nine samples were then randomly selected from each of the control and A5 groups and the viral load in the flesh was quantified using RT-qPCR (Fig. 7D). All samples treated with A5 had qualification cycle (Cq) values higher than 33 or were undetectable, except for one sample with Cq = 30.19. This means that the virus was hardly transmitted to the pepper fruits. On the other hand, in the control group, all but one sample were detected at a relatively earlier time point (average Cq was 22.62).

We also tested whether A5 treatment prior to the TSWV infection would be helpful. A5 was applied 6 days and 1 day before inoculation. Pre-treatment with A5 did not reduce the infection rate. However, there was a similar reduction in the level of TSWV RNA accumulation compared to that of the post-treated group (data not shown).

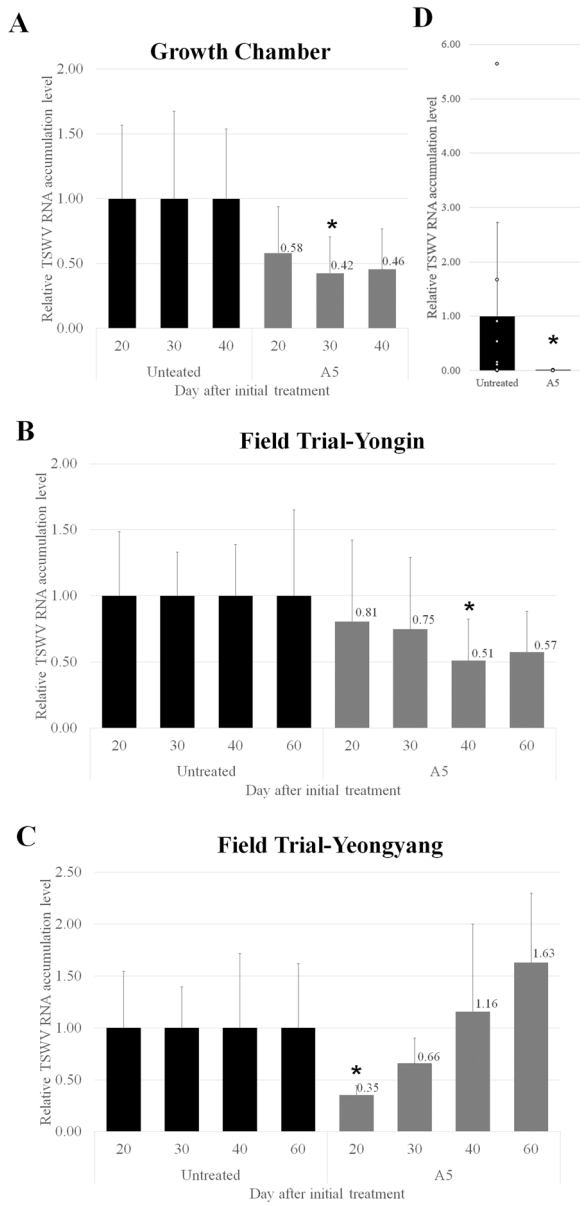


Fig. 7 The effect of prolonged A5 treatment on TSWV-infected *C. annuum*. A5 was treated with 10 days of interval after confirming the infection. Each group contained 30 plants with 3 replications, where it was grown on the plant growth chamber (**A**), the field in Yongin (**B**), or the field in Yeongyang (**C**). **D** The TSWV RNA level in the pepper fruits harvested from the field in Yongin. Nine individual fruits from each group were used for analysis. The asterisk indicates that the data were significantly different ($P < 0.05$) compared to that of the control based on the LSD test

DEG identification and GO enrichment analysis

The sequenced reads ranged from 56,632,978 (CMV-R1S2) to 89,413,592 (A4-R1S2), and the GC contents ranged from 42.59% (A5-CMV-R1S2) to 48% (A4-R3S2). Using the BBmap software, we mapped all the raw data files to the *C. annuum* cDNA database with default parameters. The mapping results were then used for further DEG analysis in the DeBrowser. In the A4 treatment alone without virus infection, a total of 1910 and 1551 genes were found to be up- and down-regulated, respectively, while 126 and 20 genes were up- and down-regulated with A5 treatment (Fig. 8A).

In the A4 treatment with CMV infection, a total of 136 genes were found to be upregulated, while 106 genes were downregulated (Fig. 8C and E). Among the DEGs, the top five upregulated genes were CA.PGAv.1.6.scaffold1312.7, CA.PGAv.1.6.scaffold134.58, CA.PGAv.1.6.scaffold773.10, CA.PGAv.1.6.scaffold501.4, and CA.PGAv.1.6.scaffold104.12. The top five downregulated genes were CA.PGAv.1.6.scaffold767.24, CA.PGAv.1.6.scaffold327.19, CA.PGAv.1.6.scaffold1185.2, CA.PGAv.1.6.scaffold511.7, and CA.PGAv.1.6.scaffold445.6 (Additional file 2: Table S3).

For the TSWV infection with A4 treatment, a total of 736 genes showed increased expression, while 522 genes showed decreased expression (Fig. 8D and F). The top upregulated genes in response to A4 treatment with TSWV infection were CA.PGAv.1.6.scaffold322.91, CA.PGAv.1.6.scaffold170.15, CA.PGAv.1.6.scaffold211.4, CA.PGAv.1.6.scaffold459.11, and CA.PGAv.1.6.scaffold553.18. On the other hand, the top downregulated genes were CA.PGAv.1.6.scaffold162.58, CA.PGAv.1.6.scaffold57.28, CA.PGAv.1.6.scaffold595.7, CA.PGAv.1.6.scaffold192.7, and CA.PGAv.1.6.scaffold584.37 (Additional file 2: Table S3).

In the A5 treatment with CMV infection, 276 genes in pepper cDNA were upregulated, while 92 genes were downregulated (Fig. 8C and E). Among the 368 genes differentially expressed in the A5 treatment and CMV infection condition, the top five upregulated genes were CA.PGAv.1.6.scaffold135.16, CA.PGAv.1.6.scaffold64.21, CA.PGAv.1.6.scaffold631.49, CA.PGAv.1.6.scaffold500.52, and CA.PGAv.1.6.scaffold553.18. The top five downregulated genes were CA.PGAv.1.6.scaffold1497.17, CA.PGAv.1.6.scaffold1473.1, CA.PGAv.1.6.scaffold595.7, CA.PGAv.1.6.scaffold79.34, and CA.PGAv.1.6.scaffold46.8 (Additional file 2: Table S3).

In the A5 treatment with TSWV infection, only 20 genes were upregulated, while four genes were downregulated (Fig. 8D and F). Among the 20 differentially expressed genes in the A5 and TSWV condition, the top five upregulated genes were CA.PGAv.1.6.scaffold358.11, CA.PGAv.1.6.scaffold338.10, CA.PGAv.1.6.scaffold608.7, CA.PGAv.1.6.scaffold1129.47, and CA.PGAv.1.6.scaffold134.124. The top four downregulated genes were CA.PGAv.1.6.scaffold160.11, CA.PGAv.1.6.scaffold631.22, CA.PGAv.1.6.scaffold874.74, and CA.PGAv.1.6.scaffold900.21 (Additional file 2: Table S3).

These DEG results provide valuable information about the gene expression changes in response to A4 or A5

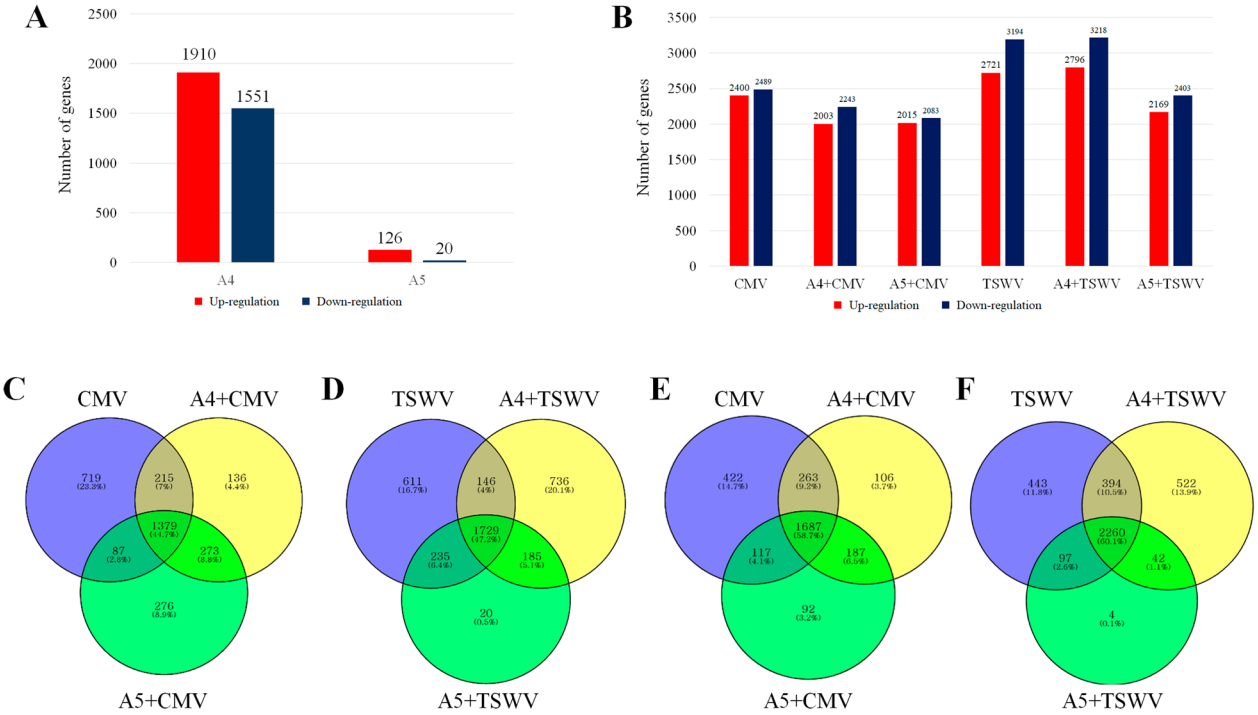


Fig. 8 The number of up- and down-regulated *C. annuum* genes in six different conditions. Differential gene expression was analyzed in response to iodine-conjugate compound treatment without virus inoculation (**A**) and with each virus inoculation (**B**). Venn diagram displayed the upregulated genes in the presence of A4 and A5 treatment with CMV (**C**) and TSWV infection (**D**), as well as the downregulated genes in the presence of A4 and A5 treatment with CMV (**E**) and TSWV infection (**F**)

treatments with CMV and TSWV infections. Further analysis and functional characterization of these DEGs can shed light on the molecular mechanisms underlying the antiviral activity of the A4 or A5 agent against these viruses.

In the GO enrichment analysis of the DEGs in response to agent treatments and virus infection, the DEGs were classified into the biological process (BP), cellular components (CC), and molecular function (MF) categories (Fig. 9A–C).

In the BP category of GO, it was found that 60 DEGs highly expressed in response to A4 treatment with CMV infection were enriched in the "response to single-organism process" (GO:0044699). Additionally, the 130 genes upregulated by A5 treatment with CMV infection were enriched in the "response to single-organism process" (GO:0044699) in the BP category of GO. This suggests these genes are involved in the cellular response to CMV infection.

Regarding the TSWV infection, 345 genes associated with the "cellular process" (GO:0009987) were identified in the A4 treatment with TSWV infection. This indicates that these genes play a role in various cellular processes related to TSWV infection. Based on the information provided, no DEGs were enriched in the biological process category in the presence of A5 treatment with TSWV infection.

In the cellular component category, 113 genes were identified for the "cell part" (GO:0044464) in A4 treatment with CMV infection, and 113 genes were associated with the "membrane" (GO:0016020) in A5 treatment with CMV infection.

In the cellular component category in response to TSWV infection with A4 treatment, most of the genes were enriched in the "cell part" (GO:0044464). Further classification of the cell part category revealed that 368 genes were associated with the "cytoplasm" (GO:0005737), and 150 genes were related to the "plasma membrane" (GO:0005886).

Regarding the molecular function category, 125 genes with "catalytic activity" (GO:0003824) were identified in A5 treatment with CMV infection. In A4 treatment with TSWV infection, 321 upregulated genes were involved in "binding" (GO:0005488). However, no DEGs were found in the molecular function category for A4 treatment with CMV infection and A5 treatment with TSWV infection.

The GO enrichment analysis provides valuable information about the functional categories and processes

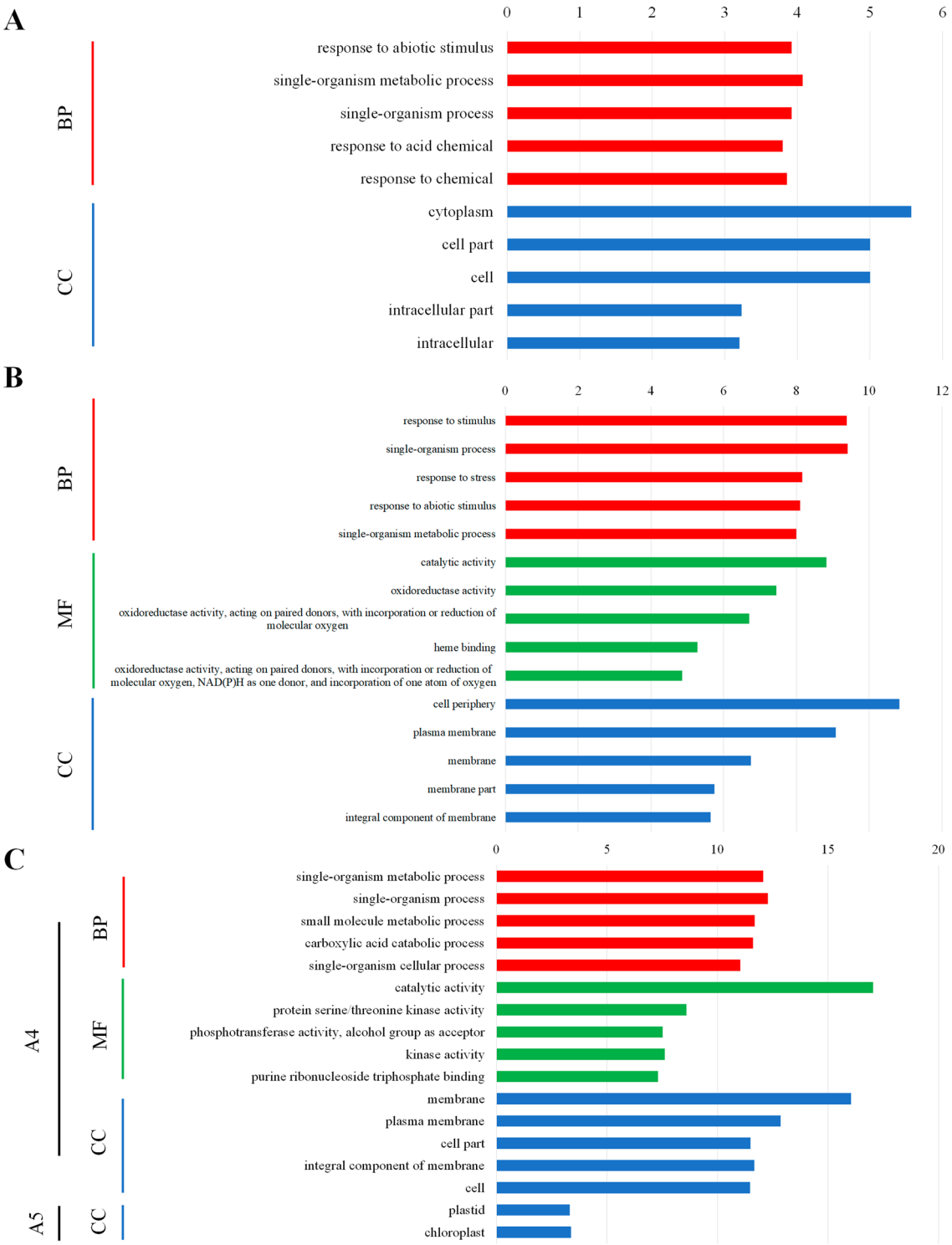


Fig. 9 Gene Ontology (GO) enrichment analysis of DEGs of *C. annuum* genes for six different conditions. GO term analysis showed enrichment of upregulated gene in A4 treatment with CMV infection (**A**), A5 treatment with CMV infection (**B**), and A4 or A5 treatment with TSWV infection (**C**)

that are affected by the agent treatments and viral infections. It helps to elucidate the biological significance of the differentially expressed genes and highlights the specific genes and processes that may play important roles in the defense mechanisms of pepper plants against viral infections.

Gene expression pattern of phytohormone pathways

Visualizing all DEGs using MapMan software provided insights into the antiviral mechanism of treatments A4 and A5 against CMV and TSWV infections. The analysis indicated that the inhibition of virus infections by these treatments was strongly associated with plant signal transduction and hormone signaling pathways (Fig. 10A and B).

Specifically, genes related to the ethylene and jasmonic acid (JA) pathways were upregulated in response to A4 and A5 treatments in the presence of both CMV and TSWV infections. This suggests these treatments may modulate the ethylene and JA signaling pathways to enhance the plant's defense against viral infections.

Furthermore, there was a significant upregulation of genes related to pathogenesis-related (PR) proteins, which are known to play a crucial role in plant defense against pathogens. The upregulation of PR proteins indicates an activation of defense responses in pepper plants treated with A4 and A5, further supporting their antiviral activity.

The heatmap analysis of gene expression patterns provided further insights into the relationship among ethylene, JA, and PR proteins in response to A4 and A5 treatments with CMV and TSWV infections (Fig. 10C). Among the ethylene-related genes, four genes (CA.PGAv.1.6.scaffold79.71, CA.PGAv.1.6.scaffold1856.9, CA.PGAv.1.6.scaffold78.126, CA.PGAv.1.6.scaffold624.33) showed notable increases in expression upon A4 and A5 treatments with both CMV and TSWV infections. This suggests that the treatments may stimulate ethylene signaling, potentially contributing to the antiviral response.

Regarding the JA pathway, two genes (CA.PGAv.1.6.scaffold34.2, CA.PGAv.1.6.scaffold763.11) were upregulated in response to A4 and A5 treatments with CMV and TSWV infections. This indicates that the treatments may activate JA signaling, which plays a role in defense responses against pathogens.

Furthermore, five genes (CA.PGAv.1.6.scaffold78.113, CA.PGAv.1.6.scaffold176.18, CA.PGAv.1.6.scaffold735.7, CA.PGAv.1.6.scaffold463.7, CA.PGAv.1.6.scaffold200.11) related to the MAPK cascade, which is involved in signaling pathways associated with plant defense responses, were upregulated in the presence of A4 and A5 treatments with virus inoculation. This suggests that the treatments may activate the MAPK cascade, contributing to the antiviral defense mechanism.

Moreover, four genes (CA.PGAv.1.6.scaffold143.31, CA.PGAv.1.6.scaffold58.59, CA.PGAv.1.6.scaffold257.39, CA.PGAv.1.6.scaffold504.17) related to the PR-protein pathway showed significant increases in expression upon A4 and A5 treatments with CMV and TSWV infections. This indicates that the treatments may induce the expression of PR proteins, which are known to play a role in plant defense against pathogens.

These findings suggest that treatments A4 and A5 activate signaling pathways and induce the expression of defense-related genes, including those involved in ethylene, JA, MAPK cascade, and PR protein pathways, to confer resistance against CMV and TSWV infections in pepper plants.

Discussion

With plant virus infections causing increasing damage worldwide, there is a pressing need for new materials to control these viruses. While previous chemical solutions have limitations, there is growing interest in organic substances with antiviral properties. We sought to identify organic substances that could provide plant nutritional benefits and enhance virus resistance. Our attention turned to iodine as a potential candidate, as recent studies have shown its potential to contribute to plant nutrition and resistance.

In our study, we developed an iodine-based agent to enhance plant virus resistance, focusing on the antiviral activity against different viruses, which are known to cause significant damage to peppers in Korea [25]. The iodine-based agent was developed using iodine and sialic acid as the main component, with the addition or subtraction of sulfur as necessary. Different treatment methods, including 5- to 10-day periods, were employed to enhance the effectiveness of the agent.

Importantly, the treated substances did not exhibit any side effects such as interference with plant growth or toxicity, which are common concerns when using chemical substances in crops. This finding is significant as it suggests that the ingredients used in the iodine-based agent can be effectively utilized as organic chemicals to protect crops against plant pathogens without harming the crops themselves.

The evaluation of antiviral activity showed that certain viruses' symptom severity was suppressed in plants treated with the iodine-conjugated agent. Notably, TSWV, CMV, and BBWV2 were significantly inhibited by the agent treatment. The accumulation level of TSWV RNAs was significantly reduced in plants treated with the A4 agent every 10 days and the A5 agent every 5 days compared to untreated control plants (Fig. 6A). For

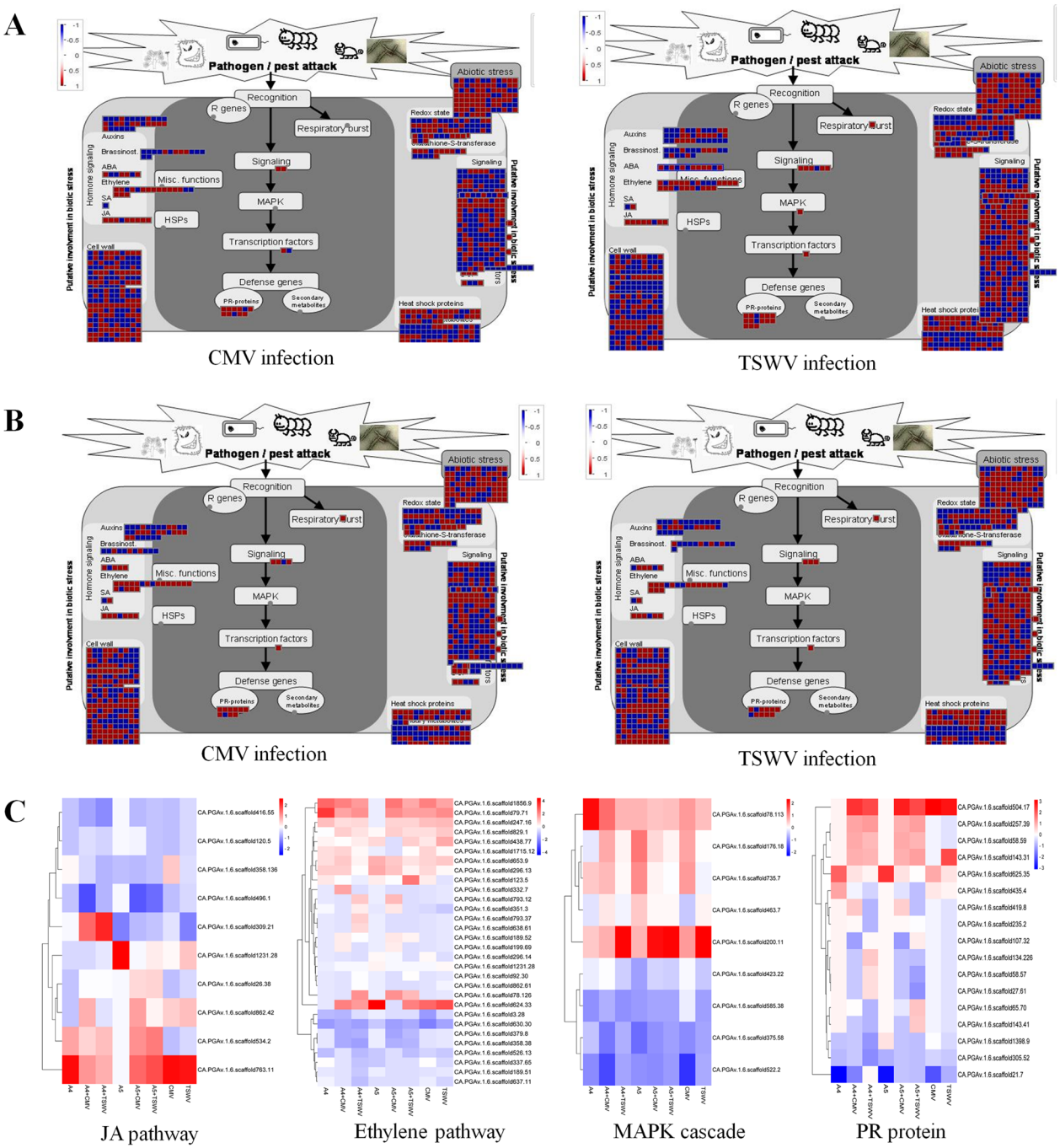


Fig. 10 Expression profile of phytohormone- and defense-related genes. MapMan analysis showed that overview of gene expression for biotic stress pathways in the presence of CMV infection or TSWV infection with A4 treatment (**A**) and A5 treatment (**B**). Heatmap analysis revealed the expression of phytohormone-related genes (**C**)

CMV, a remarkable inhibitory effect was observed with B4 and B5 agent treatments (Fig. 6B). However, the efficacy against BBWV2 infection was limited compared to TSWV and CMV (Fig. 6C).

To confirm the effect(s) of the iodine-based agent in preventing viral infection or in reducing viral RNA replication, we selected the A5 compound and tested the effect(s) of pre- and post-treatment in preventing TSWV

infection or in reducing TSWV RNA replication in a chamber condition and in the field. Although pre-treatment with A5 did not affect the TSWV infection rate, we observed reduced TSWV RNA accumulation in pre- and post-A5 treatments in the chamber condition. In the field conditions, we only tested the effect(s) of post-A5 treatment because we did not observe a difference in TSWV infection rate by pre-treatment of the A5. As described, there was inconsistency in the effect of A5 treatment in reducing TSWV RNA accumulation, but we observed reduced levels of TSWV RNA accumulation in general (Fig. 7).

Environmental conditions, such as temperature and soil conditions can significantly impact the levels of RNA virus replication. Plant RNA viruses typically exhibit optimal replication within a moderate temperature range, roughly 25–30 °C. At temperatures below or above this range, the replication rate might slow down, impeding the virus's ability to replicate and spread within the host plant [26, 27]. Some viruses replicate better in cooler conditions, while others prefer warmer environments [28]. While TSWV can replicate within a specific temperature range, differences in temperature or temperature fluctuations between two fields can affect its replication and overall impact on plant health. Soil conditions such as nutrient availability, soil pH, moisture content, and composition can also influence both the plant's health and the activity of viruses that infect the plant. These factors indirectly impact the plant's susceptibility to viral infections and, therefore, might result in differences in RNA replication with the same treatment play a significant role in its overall health and resilience against TSWV.

Our results suggest that the antiviral activity of the iodine-based agent depends on its composition and treatment interval. It also indicates that the conjugated organic substances in the agent may directly or indirectly affect the resistance response against virus infections in plants. Furthermore, the agents made of iodic acid and sulfur show potential as antiviral agents against several plant virus species. Overall, our study provides valuable insights into the development of organic substances, specifically the iodine-based agent, for enhancing plant virus resistance and protecting crops from viral infections without causing harmful side effects.

To elucidate the mechanism behind the antiviral activity of the iodine-conjugate agent against CMV and TSWV infections, we analyzed gene expression changes using RNA-Seq. The raw reads obtained were mapped to the pepper cDNA database, and DEGs were identified through GO term analysis and MapMan analysis. Interestingly, we observed a higher number of DEGs in the A4 treatment with both virus infections compared to the A5 treatment with both virus infections. This suggests that the A4 treatment induced significant changes in gene expression in pepper, resulting in the manifestation of resistance symptoms against virus infection. Further analysis provided insights into the involvement of phytohormone pathways in the antiviral mechanism of A4 and A5 treatments on pepper plants. Since we did not observe any deleterious effects of direct A4 or A5 treatment on virus-infected sap for 30 min at 25 °C on infection efficiency or viral RNA replication level, the upregulated genes in response to both iodine-conjugate compounds with or without sulfur treatments and virus infection might be related to the ethylene and JA pathways, PR proteins, and the MAP kinase cascade rather than the salicylic acid (SA) pathway.

Plants generally employ multiple defense pathways involving cross-talk among auxins, SA, JA, and ethylene to elicit various responses to infections [29]. JA and ethylene play crucial roles in systemic acquired resistance (SAR) triggered by bacterial, fungal, and viral infections [30, 31]. Sulfur is also involved in the activation of defense signaling pathways in plants [32, 33]. It plays a crucial role in the synthesis of signaling molecules such as SA and JA. These signaling molecules are involved in the regulation of defense responses, including the activation of defense genes and the induction of SAR. SAR is a defense mechanism that provides long-lasting protection against various pathogens, including viruses. Sulfur also influences the expression of defense-related genes, including those encoding PR proteins or sulfur-containing defense compounds, and thus provides pivotal roles in efficient defense responses to invading pathogens [33].

Additionally, ethylene has been shown to regulate the expression of PR genes encoding PR proteins through transcriptional activators [34]. Our study identified two genes related to JA signaling that exhibited high expression levels in response to iodine-conjugate compound treatment and virus infections. Among the ethylene-related genes, the expression of four genes was upregulated by an iodine-conjugate compound with or without sulfur treatment and virus infection. Furthermore, we identified five genes associated with the MAPK cascade and four genes involved in the PR-protein pathway. These findings provide evidence that the JA, ethylene, MAPK, and PR-protein signaling pathways are implicated in the virus inhibition mechanism of the iodine-conjugate compound against CMV and TSWV in pepper plants.

Previous studies conducted on various plant species, such as spinach, white clovers, tomatoes, perennial ryegrass, turnips, barley, flax, wheat, and mustard, have shown that iodine positively affects plant growth [13]. However, there was no significant difference in development in buckwheat, while oats and turnips exhibited

inhibitory effects on growth when treated with iodine at all tested concentrations [13]. These findings indicate that iodine can have a beneficial impact on plant development for several plant species. It can be inferred that when iodine is applied at specific concentrations, it may induce complex resistance responses by influencing the biological activity of host plant species. Furthermore, studies on tomato and *Arabidopsis* have suggested the potential involvement of iodine in plant defense mechanisms [14]. Considering that peppers and tomatoes belong to the same biological family, it is plausible to speculate that treating peppers with iodine may induce resistance through similar or shared mechanisms. This implies that iodine treatments have the potential to modulate the expression of multiple genes involved in plant defense and resistance responses, thereby providing protection to host plants against various stresses.

Based on the aforementioned studies and inferences, it is presumed that organo-iodine compounds with or without sulfur contribute to plant resistance mechanisms. This indicates that studying plant resistance reactions induced by iodine is of significant value. The present study has demonstrated a new potential for organo-iodine compounds with or without sulfur as agents for managing virus diseases and its contribution to plant resistance in real-world agricultural settings. As the iodine used in the study was conjugated with other elements, further experiments are necessary to investigate the interactions and potential effects of these elements on the management of virus diseases by iodine. Moreover, it is crucial to conduct in-depth research to elucidate the mode of action through which iodine, as the primary component of these agents, induces plant resistance. Such investigations will provide valuable insights into the underlying mechanisms of iodine-mediated plant resistance and enhance our understanding of its applications in agriculture.

Conclusions

In this study, we evaluated the efficacy of iodine-conjugated organic agents in enhancing resistance against seven virus diseases in tobacco and pepper plants. The accumulation of several viruses was reduced in plants pre-treated with agents before virus infection. The bioinformatics analysis revealed the involvement of phytohormone pathways in antiviral mechanisms derived by A4 and A5 treatment on pepper plants against CMV and TSWV. Our findings provide the possibility of iodine-mediated plant virus resistance in agriculture.

Abbreviations

BBWV2	Broad bean wilt virus 2
β-TUB	β-Tubulin
C. annuum	*Capsicum annuum*
CGMMV	Cucumber green mottle mosaic virus
CMV	Cucumber mosaic virus
DEG	Differentially expressed genes
EIF5A2	Eukaryotic initiation factor 5A2
JA	Jasmonic acid
N. benthamiana	*Nicotiana benthamiana*
PCR	Polymerase chain reaction
PepMoV	Pepper mottle virus
PMMoV	Pepper mild mottle virus
PR Protein	Pathogenesis-related protein
RT-PCR	Reverse transcription-PCR
RT-qPCR	Quantitative RT-PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
TSWV	Tomato spotted wilt virus
TYLCV	Tomato yellow leaf curl virus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00554-4.

Additional file 1: Table S1. List of primers used RT-PCR. **Table S2.** List of primers used for RT-qPCR.

Additional file 2: Table S3. Differentially expressed genes due to treatment and virus infection with gene description, DEG ID, annotation information, FPKM value, and P-value compared to the control.

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Not applicable.

Author contributions

HC, SJ, HKK, and K-HK conceptualized the manuscript; HC, SJ, S-JK, H-GK, and DL conducted the experiment; HC and SJ drafted the first manuscript; K-HK edited the manuscript. All authors have read this version of the manuscript.

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Availability of data and materials

The data presented in this study are available in the article. Further inquiries can be directed to the corresponding authors.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Hee K. Kim is employed by LFF Co. Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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