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Anti-TMV activity and functional mechanisms of two sesquiterpenoids isolated from *Tithonia diversifolia*

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ABSTRACT

Unlike chemical pesticides, antiviral plants are biodegradable, replenishable and safe. In this study, 14 sesquiterpene compounds from *Tithonia diversifolia* were tested for their activities against *Tobacco mosaic virus* (TMV) using the half-leaf method. Tagitinin C (Ses-2) and 1β-methoxydiversifolin-3-*O*-methyl ether (Ses-5) were found to have in vivo curative activities of 62.86% and 60.27% respectively, at concentrations of 100 μg/mL, respectively. In contrast, the in vivo curative inhibition rate of control agent ningnanmycin was 52.48%. Indirect enzyme-linked immunosorbent assay (ID-ELISA) also verified Ses-2 and Ses-5 had higher inhibition activities than the control agent ningnanmycin. Additionally, qRT-PCR showed that both Ses-2 and Ses-5 can partly inhibit the expression of CP and RdRp, two genes that play key roles in TMV infection. When TMV started to systemically spread, Ses-2 inhibited CP expression while Ses-5 inhibited RdRp expression. These results suggest that the two bio-agents have anti-TMV activities and may be used as bio-pesticides to control the plant virus.

1. Introduction

Tithonia diversifolia (Asteraceae) is widely distributed across tropical regions [1]. Its extracts have anti-inflammatory, analgesic, antimalarial, antimicrobial, antidiabetic, anti-cancer and anti-nematodosis effects [2,3,4]. Sesquiterpene lactones are a major constituent of *Tithonia diversifolia* secondary compound, and they have a wide spectrum of biological activities, including inhibition of *Leishmania* and bladder cancer [5,6]. Several findings have indicated that crude ethanol extracts from *Tithonia diversifolia* leaves have antiviral activity against TMV [7], but the underlying mechanism has remained unknown.

Tobacco mosaic virus (TMV) is one of the most damaging plant viruses [8], causing significant yield losses in crop production worldwide [9–11]. The TMV genome consists of a single strand of positive-sense RNA encoding at least four proteins [12]. RNA-dependent RNA polymerase (RdRp) mediates the genomic replication of TMV [13] and is also one of the key components necessary for cell-to-cell movement of the virus [14]. The coat protein (CP) plays a very important role throughout the life of the virus as it affects virion assembly [15] and is also required for systemic infection and viral replication [16–18]. Moreover, it greatly affects the development of symptoms in virus-infected plants [19]. Therefore, RdRp and CP are potential targets for the inhibition of viruses and should be examined in studies on the effects of anti-viral compounds on TMV replication.

Many methods have been recently described to counteract TMV infection or proliferation. Pesticides are used widely because they are inexpensive and easy to handle, but they are potentially toxic to many beneficial species and may leave residues that contaminate the environment. Thus, the development of biogenic pesticides is a current research priority. Several of these bio-agents with antiviral activity against TMV have been studied. Seco-pregnane steroids extracted from *Strobilanthes cusia* have exhibited against TMV and can suppress the expression of viral subgenomic RNA(s) without affecting the accumulation of viral genomic RNA [20]. Ningnanmycin, an antiviral agent isolated from *Streptomyces noursei* var. *x ichangensis*, has been shown to promote the systemic accumulation of pathogenesis-related proteins and the expression of resistance-related genes that are markers of systemic acquired resistance [21]. In addition, ningnanmycin also inhibited the assembly of the four-layer aggregate disk of CP [21,22]. A glycoprotein of BDP-30 from *Boerhaavia diffusa* L., suppressed TMV infection through inducing systemic resistance [23]. Other compounds, such as thiourea, schisanhenol derivatives, sulfated lentinan, and various polysaccharide peptides have exhibited activities and stimulated the contents of POD,

Abbreviations: TMV, Tobacco mosaic virus; ID-ELISA, Indirect enzyme-linked immunosorbent assay; CP, coat protein; RdRp, RNA-dependent RNA polymerase; qRT-PCR, Real-time fluorescence quantification.

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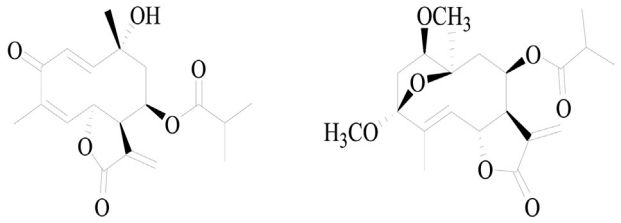


1: Tagitinin C (Ses-2); 2: 1β-methoxydiversifolin-3-*0*-methy1 ether (Ses-5)

Fig. 1. The molecular structure of Ses-2 and Ses-5. 1: Tagitinin C (Ses-2); 2: 1β-methoxydiversifolin-3-*0*-methyl ether (Ses-5).

SOD, and PAL proteins, which are related to plants pathogen resistance [24–27].

In the current study, we identified two sesquiterpenoid compounds from *Tithonia diversifolia*, Tagitinin C (Ses-2) and 1β-methoxydiversifolin-3-*0*-methyl ether (Ses-5), with anti-TMV activities and examined their potential anti-TMV mechanism. These two compounds could be developed as new biogenic pesticides with agricultural applications.

2. Materials and methods

2.1. Materials

TMV was isolated from diseased tobacco plants in our laboratory. Its CP nucleotide sequence shared the highest nucleotide identity (i.e., 99%) with that of the TMV-152 isolate. The virus was maintained and cultured in *Nicotiana tabacum* cv. K326. Purified virus particles were stored −20 °C. A rabbit antibody to TMV CP was prepared by our laboratory.

N. tabacum cv. K326 and *N. glutinosa* seeds were provided by the Yunnan Academy of Tobacco Agricultural Science.

The sesquiterpenoid compounds were provided by Professor Xiaojiang Hao's Laboratory. Ningnanmycin was purchased from Heilongjiang DeQiang Biology Co., Ltd. The molecular structures of the two compounds are shown in Fig. 1.

2.2. Purified TMV particles

TMV particles were purified using Gooding's method [28] and stored at −20 °C for later use. TMV particles were diluted to a concentration of 50 μg/mL with 0.01 M phosphate-buffered saline (PBS) before use.

2.3. Plant material preparation

N. glutinosa and *N. tabacum* cv. K326 plants were cultivated in an insect-free greenhouse. The experiments were conducted when the plants grew to the 6–8 leaf stage. *N. glutinosa* was used to assay inhibition activity, while *N. tabacum* cv. K326 was used to study the mechanism of the virus.

The whole plant of *Tithonia diversifolia* was collected in Xishuangbanna, Yunnan Province, China, in August 2013. The specimen was identified by Yu Chen of Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS). A voucher specimen (H20110805) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany.

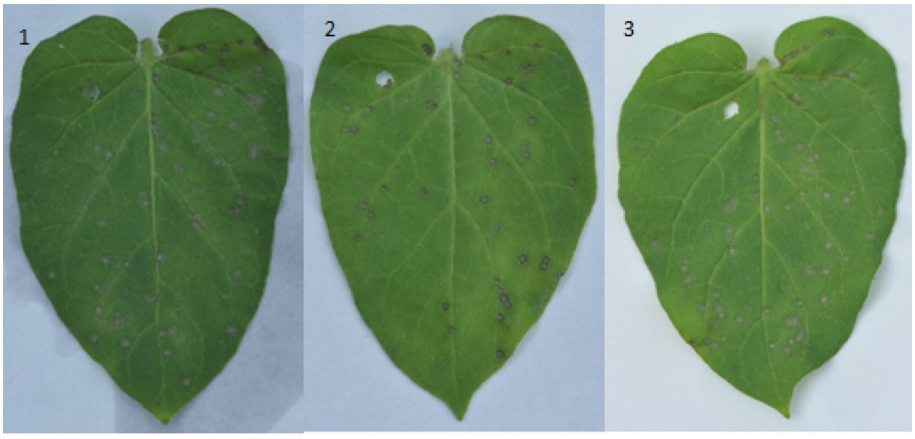
2.4. Compound separation and preparation

Dried powder of the whole plant of *Tithonia diversifolia* (18 kg) was extracted with MeOH (three times under reflux for 4, 4, and 3 h). The solvent was removed under reduced pressure to give a residue (2720 g, 15.1%), which was suspended with water and then extracted with petroleum ether and EtOAc successively. The extracts were evaporated under vacuum to afford the corresponding extracts of petroleum ether (315 g) and EtOAc (246 g). The EtOAc extract was separated to 10 fractions (A–I) by RP-18 silica gel column chromatography (20–45 μm, 49 × 460 mm, 440 g), eluted with MeOH–H_2O (v/v = 30:70, 40:60, 50:50, 60:40, 70:30, each 10 L).

Fraction E (25 g), a silica gel G column (100–200 mesh, 10 × 120 cm, 1.65 kg), eluted with petroleum ether/EtOAc (v/v = 9:1, 7:3, 6:4, 1:1, 0:1, each 30 L), to give five fractions (1–5). Fraction 3 (5.13 g) was extensively chromatographed over columns of silica gel and Sephadex LH-20 (CHCl3–MeOH, 1:1, 3.2 × 140 cm) to afford compounds (208.0 mg) and other compounds.

Fraction F (10.6 g). By silica gel G column (100–200 mesh, 20 × 150 cm, 1.0 kg) eluted with petroleum ether and EtOAc (v/v = 9:1, 7:3, 6:4, 1:1, 0:1, each 20 L), also gave five fractions (1–5). Fraction 2 (2.3 g) was subjected respectively to RP-18 silica gel column chromatography (20–45 μm, 30 × 460 mm, 150 g), eluted with MeOH–H_2O (v/v = 3:7, 5:5, 7:3, 8:2, 9:1, each 10 L), and Sephadex LH-20 (CHCl3–MeOH, 1:1, 1.8 × 120 cm), to give compounds (41 mg) and other compounds.

The compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with distilled H_2O to the required concentrations. A solution of equal DMSO concentration was used as a negative control agent (CK). Ningnanmycin was used as a positive control agent.



The whole leaf was inoculated with 50μg/mL TMV, 24 h later, left half leaf were smeared with agents. 1.100μg/mL Sec-5, 2. 100μg/mL Sec-2, 3. 80μg/mL Ningnanmycin

Fig. 2. Local lesions on the leaves of *Nicotiana glutinosa* in the curative effect treatments at 3 days postinoculation. The whole leaf was inoculated with 50 μg/mL TMV, 24 h later, left half leaf was smeared with agents. 1.100 μg/mL Sec-5, 2. 100 μg/mL Sec-2, 3. 80 μg/mL Ningnanmycin.

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Table 1
Inhibition effect of compound Ses-2, Ses-5 on TMV in *Nicotiana glutinosa* by half leaf method.

Compound name	Average inhibition rate (%)	
	Curative effect	Protective effect
Ses-2 (100 μg/mL)	62.86 ± 1.3a	33.6 ± 5.8b
Ses-5 (100 μg/mL)	60.27 ± 5.6ab	25.1 ± 5.3b
Ningnanmycin (NNM) (80 μg/mL)	52.48 ± 3.3b	52.1 ± 6.1a

CK+: positive control; CK-: negative control. All values are means ± SE. Means in a column followed by different letters are significantly different at $P \le 0.05$.

2.5. Protective effect in vivo

The compound solutions were smeared with a cotton swab onto the left side of *N. glutinosa* leaves along the main vein, whereas the DMSO solvent was smeared onto the right side of the same leaf as a negative control. After 6 h, TMV particles were inoculated onto the whole leaf. Each half of the leaf was smeared with 100 μL of TMV at concentration of 50 μg/mL, respectively. Each inoculated leaf was washed with water after 10 min. The numbers of local lesions were recorded 3–4 days after inoculation. Three replicates were conducted for each compound and control agent.

2.6. Curative effect in vivo

In contrast to the protective effect assays, the curative effect treatments began with inoculating TMV particles (50 μg/mL) onto whole leaves of *N. glutinosa* with cotton swabs. After 24 h, the compound solutions were smeared onto the left half of a previously TMV-inoculated leaf, while the DMSO solution was smeared onto the right side as a negative control. The local numbers of lesions were recorded 3–4 days after inoculation. Three replicates were conducted for each sample.

The TMV inhibition rates of the compounds were then calculated according to the formula. Inhibition rate (%) $= [(T - C) / T] \times 100\%$, where T is the average number of local lesions for the negative control and C is the average number of local lesions for the treatment.

2.7. ID-ELISA method

ID-ELISA was conducted according to a method previously described method with modifications [29] to determine the antivirus activities of each compound. Leaves (0.2 g) were ground with a mortar-grinder in a 1/3 dilution of PBS buffer (137 mM NaCl, 1 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 3 mM KCl). Crude extracts (100 μL) were added into one ELISA plate well for coating and incubated overnight at 4 °C or for 2 h at 4 °C. Then the plate was washed with PBST buffer (137 mM NaCl, 1 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$, and 3 mM KCl 0.05% Tween 20). The TMV CP rabbit antibody was diluted in conjugate buffer (PBST buffer containing 2% bovine serum albumin), and loaded into the plate wells (100 μL per well). Prior to incubation, 100 μL of AP-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) at a 1/5000 dilution was loaded into each well. The plates were incubated for 2 h at 37 °C, and washed with PBST buffer. A color-developing solution was prepared by dissolving *p*-nitrophenyl phosphate disodium hexahydrate (Sigma-Aldrich) in substrate buffer (9.7% diethanolamine and 3 mM NaN_3, pH 9.8) to a final concentration of 1 mg/mL, and 100 μL was loaded into each well. The absorbance at 405 nm was measured using a model *Elx* 808 microplate ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Crude extracts from healthy leaves was used as a negative control, PBS buffer were used as a blank control, and TMV-infected leaves were used as a positive control.

Table 2
The inhibitory effect of compound Sec-2 and Sec-5 on TMV in *Nicotiana tabacum* cv. K326 assayed by ID-ELISA.

Compound name	The concentration of virus (optical density OD450)	
	Treated leaves	Systematic leaves
Ses-2 (100 μg/mL)	0.181 ± 0.012b	0.175 ± 0.002b
Ses-5(100 μg/mL)	0.200 ± 0.016bc	0.364 ± 0.008c
Ningnanmycin (NNM) (80 μg/mL)	0.210 ± 0.013c	0.387 ± 0.029c
CK+	0.291 ± 0.033a	0.589 ± 0.047a
CK−	0.099 ± 0.001d	0.099 ± 0.001d

CK+: positive control; CK−: negative control. All values are means ± SE. Means in a column followed by different letters are significantly different at $P \le 0.05$.

Table 3
The inhibitory effect of compound Sec-2 and Sec-5 with different concentration on TMV in *Nicotiana tabacum* cv. K326 assayed by ID-ELISA.

Compound name Concentration	Ses-2	Ses-5
25 μg/mL	0.703 ± 0.02c	0.740 ± 0.05b
50 μg/mL	0.642 ± 0.01d	0.707 ± 0.007c
75 μg/mL	0.612 ± 0.04d	0.632 ± 0.02d
100 μg/mL	0.545 ± 0.03de	0.620 ± 0.02de
Ningnanmycin (80 μg/mL)	0.750 ± 0.07b	
CK+	1.312 ± 0.02a	
CK−	0.094 ± 0.001f	

CK+: positive control; CK−: negative control. All values are means ± SE. Means in a column followed by different letters are significantly different at $P \le 0.05$.

2.8. Real- time fluorescence quantification (qRT-PCR)

qRT-PCR was conducted to determine the absolute number of TMV CP and RdRp transcripts. Total RNA was extracted from tobacco leaves (0.2 g, fresh weight) using TriPure Isolation Reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's directions. The concentration of each RNA sample was measured with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Only the RNA samples with an A_{260}/A_{280} ratio (an indicator of protein contamination) of 1.9–2.1 and an A_{260}/A_{230} ratio (an indicator of reagent contamination) >2.0 were used for the analysis. The integrity of RNA samples was assessed by agarose gel electrophoresis. The primer pair TMV-CP-F (5′-ACGACTGCCGAAACGTTAGA-3′), TMV-CP-R (5′-CAAGTTGCAGGACCAGAGGT-3′) and TMV-RdRp-F (5′-TAGGCCAGCTCGCAAGATTTT-3′), TMV-RdRp-R (5′-ACTTTTGCTTGGGTTGTGCTTT-3′) were designed using Primer5 software that is a commercial software product based on the nucleotide sequences of the TMV CP and RdRp gene sequence deposited in GenBank (EMBL: AJ239099; NCBI: AY740529). First-strand cDNA was synthesized using One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen, Beijing, China). Then, qRT-PCR was conducted with TransStart Tip Green qPCR SuperMix (Transgen). Each 25 μL, reaction volume consisted of 0.26 μmol/L final concentration of primers, 12.5 μL of Tip Mix, 0.5 μL of Reference Dye II and 2 μL of cDNA, with the remaining volume filled by RNAse-free H_2O. Real-time thermo cycling process was performed on an Applied Biosystems Stepone Plus (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: denaturation for 30s at 94 °C, followed by 40 cycles of 5 s at 94

Table 4
TMV CP transcript levels in the inoculated leaves by qRT-PCR assays in the curative treatment.

Sample names	Concentration (ng/μL)		
	3 dpi ($\times 10^7$)	4 dpi ($\times 10^7$)	5 dpi ($\times 10^7$)
Positive control	8.70 ± 0.30a	17.80 ± 0.80a	31.26 ± 0.35a
Ningnanmycin	5.53 ± 0.50b	13.48 ± 0.26b	24.20 ± 0.53b
Ses-2	2.76 ± 0.23c	5.16 ± 0.21c	7.18 ± 0.06c
Ses-5	2.47 ± 0.27c	14.17 ± 0.45b	7.67 ± 0.06d

All values are means ± SE. Means in a column followed by different letters are significantly different at $P \le 0.05$.

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Table 5
TMV CP transcript levels in the systematic leaves by qRT-PCR assays in the curative treatment.

Sample names	Concentration (ng/μL)					
	1 dpi	2 dpi	3 dpi ($\times 10^5$)	4 dpi ($\times 10^5$)	5 dpi ($\times 10^5$)	6 dpi ($\times 10^5$)
Positive control	99.80 ± 4.38a	24,418.83 ± 3028.94a	9.89 ± 0.46a	16.33 ± 1.39a	483.4 ± 0.21a	578.90 ± 0.95a
Ningnanmycin	21.59 ± 0.26b	56.45 ± 7.40b	5.22 ± 0.54b	5.76 ± 0.46b	13.32 ± 0.17b	0.5 ± 0.05b
Ses-2	20.30 ± 1.24b	913.59 ± 11.37c	7.84 ± 0.16c	14.65 ± 0.61a	16.83 ± 1.21c	4.99 ± 0.31c
Ses-5	7.14 ± 0.84c	7565.99 ± 467.31d	1.03 ± 0.01d	11.84 ± 0.22c	19.65 ± 0.77d	4.38 ± 0.40c

All values are means ± SE. Means in a column followed by different letters are significantly different at $P \leq 0.05$.

°C for denaturation, 30 s at 55 °C for annealing, and 30 s at 72 °C for extension and fluorescent signal detection.

2.9. Standard curve

DNA fragments were amplified with the aforementioned primer pair, cloned into a pEASY-T1 vector (Transgen), and transformed into competent *Escherichia coli* strain DH5α cells. The insertion of PCR products was confirmed by colony PCR screening and sequencing. Recombinant plasmids were extracted with EasyPure Plasmid MiniPrep Kit (Transgen). The concentration of the purified plasmid DNA was measured with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies). Then, the plasmid DNA was diluted as the standard samples from a dilution series (of 10^{-2},10^{-3},10^{-4}, 10^{-5}, and 10^{-6} concentrations.

2.10. Statistical analysis

Data are expressed as mean ± SD. STATISTICA version 12.0 for Windows (StatSoft, Inc.) was used to perform the statistical analysis. To compare difference in means, SPSS statistics 17.0 was used to analysis of variance (ANOVA). The significance levels for tests were $P < 0.05$.

3. Results

3.1. Compound structural analysis

Tagitinin C (Ses-2), a colorless oil liquid, possessed the molecular formula $C_{19}H_{24}O_6$, as determined by the ESI-MS, ^{13}C NMR and DEPT data. Comparison of the 1D NMR spectroscopic data of 1 with those of Tagitinin C revealed that their structures were same each other. The structure was determined as Tagitinin C [30].

Tagitinin C (Ses-2): colorless oily; $C_{19}H_{24}O_6$; ESI-MS *m/z*: 371 [M + Na]+; ^1H NMR (CDCl3, 400 MHz) δ(H) (ppm): 6.92 (1H, d, J = 17.1 Hz, H-1), 6.31 (1H, d, J = 1.5 Hz, H-13a), 6.22 (1H, d, J = 17.1 Hz, H-2), 5.83 (1H, dd, J = 9.1, 1.2 Hz, H-6), 5.77 (1H, d, J = 1.5 Hz, H-13b), 5.40 (1H, d, J = 9.1 Hz, H-5), 5.30 (1H, ddd, J = 10.0, 6.3, 1.2 Hz, H-8), 3.53 (1H, d, J = 1.2 Hz, H-7), 2.44 (1H, dd, J = 14.0, 6.3 Hz, H-9), 2.38 (1H, m, H-2′), 1.96 (1H, dd, J = 14.0, 10.0 Hz, H-9), 1.91 (3H, s, H-15), 1.48 (3H, s, H-14), 1.02 (3H, d, J = 6.7 Hz, H-3′), 1.01 (3H, d, J = 6.7 Hz, H-4′); ^{13}C NMR (CDCl3, 100 MHz) δ_C (ppm): 197.0 (s, C-3), 176.3 (s, C-1′), 169.8 (s, C-12), 160.8 (d, C-1), 138.8 (s, C-4), 137.2 (d, C-5), 136.0 (s, C-11), 129.5 (d, C-2), 124.6 (t, C-13), 76.0 (d, C-6), 74.1 (d, C-8), 71.9 (s, C-10), 48.3 (t, C-9), 46.9 (d, C-7), 34.0 (d, C-2′), 28.8 (q, C-14), 19.6 (q, C-15), 18.8 (q, C-3′), 18.6 (q, C-4′).

1β-Methoxydiversifolin-3-*0*-methyl ether (Ses-5): a colorless oil liquid, possessed the molecular formula $C_{20}H_{28}O_7$, as determined by the ESI-MS, ^{13}C NMR and DEPT data. Comparison of the 1D NMR spectroscopic data of 2 with those of 1-methoxydiversifolin revealed that their structures were same each other. The structure was determined as 1-methoxydiversifolin [31].

1β-Methoxydiversifolin-3-*0*-methyl ether (Ses-5): colorless oily, $C_{20}H_{28}O_7$; positive ESI-MS *m/z*: 403 [M + Na]+; ^1H NMR (CDCl3, 400 MHz) δ_H: 6.23 (1H, d, J = 2.7 Hz, H-13a), 5.60 (1H, d, J = 1.8 Hz, H-5), 5.58 (1H, d, J = 2.7 Hz, H-13b), 5.39 (1H, ddd, J = 5.5, 3.9, 1.8 Hz, H-6), 4.08 (1H, ddd, J = 6.6, 4.8, 2.4 Hz, H-8), 4.02 (1H, dd, J = 5.5, 6.6 Hz, H-7), 3.38 (3H, s, H-OMe), 2.59 (1H, dd, J = 12.6, 6.5 Hz, H-1), 2.39 (1H, dd, J = 14.0, 6.5 Hz, H-2a), 2.13 (1H, dd, J = 14.0, 12.6 Hz, H-2b), 1.98 (1H, d, J = 13.9 Hz, H-9a), 1.81 (3H, s, H-15), 1.72 (1H, dd, J = 13.9, 4.8 Hz, H-9b), 1.51 (3H, s, H-14), 1.04 (3H, d, J = 7.0 Hz, H-3′), 1.03 (3H, d, J = 7.0 Hz, H-4′). ^{13}C NMR (CDCl3, 100 MHz) δ_C (ppm): 176.1 (s, C-1′), 170.0 (s, C-12), 140.4 (s, C-4), 136.2 (s, C-11), 128.7 (d, C-5), 122.7 (t, C-13), 103.5 (s, C-3), 86.3 (d, C-1), 81.9 (s, C-10), 75.4 (d, C-6), 70.5 (d, C-8), 58.6 (q, C-1-OCH3), 49.6 (d, C-7), 41.5 (t, C-2), 35.1 (t, C-9), 34.0 (d, C-2′), 27.2 (s, C-14), 22.5 (s, C-15), 19.1 (q, C-3′), 18.6 (q, C-4′).

3.2. Anti-TMV activities

The anti-TMV activity of 14 sesquiterpene compounds from *Tithonia diversifolia* was tested at concentration of 100 μg/mL in *Nicotiana glutinosa* using the half-leaf method. Based on the inhibition rates of local lesions on the leaves of *N. glutinosa* (Fig. 2), two of the 14 compounds, specifically Tagitinin C (Ses-2) and 1β-methoxydiversifolin-3-*0*-methyl ether (Ses-5) showed higher curative efficacy than ningnanmycin (Table 1). The curative inhibition rates of Ses-2 and Ses-5 against TMV were 60.27% and 62.86% respectively. However, these compounds exhibited no protective efficacies against TMV.

3.3. Verification of anti-TMV effects via ID-ELISA

To further confirm the anti-TMV activities of Ses-2 and Ses-5, the relative content of TMV was measured using Indirect enzyme-linked immunosorbent assay (ID-ELISA). The relative content of TMV in K326 tobacco leaves treated with Ses-2 and Ses-5 decreased significantly, indicating the accumulation of TMV in the treated leaves might be inhibited, especially in the systemic leaves of the plants treated with these compounds (Table 2).

Table 6
TMV RdRp transcript levels in the inoculated leaves by qRT-PCR assays in the curative treatment.

Sample names	Concentration (ng/μL)		
	1 d	2 d	3 d
Positive control	24.01 ± 5.93 × 10^7 a	251.41 ± 27.26 × 10^7 a	373.94 ± 3.48 × 10^7 a
Ningnanmycin	0.88 ± 0.04 × 10^7 b	103.28 ± 8.64 × 10^7 b	146.68 ± 12.59 × 10^7 b
Sec 2	9.72 ± 0.08 × 10^7 c	144.08 ± 9.05 × 10^7 c	234.22 ± 8.36 × 10^7 c
Sec 5	4.50 ± 0.05 × 10^7 d	2.54 ± 0.03 × 10^7 d	224.57 ± 11.55 × 10^7 c

All values are means ± SE. Means in a column followed by different letters are significantly different at $P \leq 0.05$.

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Table 7
TMV RdRp transcript levels in the systematic leaves by qRT-PCR assays in the curative treatment.

Sample names	Concentration (ng/μL)					
	1 d	2 d	3 d ($\times 10^5$)	4 d ($\times 10^5$)	5 d ($\times 10^5$)	6 d ($\times 10^5$)
Positive control	31.65 ± 0.04a	1688.48 ± 8.01a	11.19 ± 0.04a	28.24 ± 0.03a	39.57 ± 0.13a	42.11 ± 0.02a
Ningnanmycin	8.31 ± 0.34b	13.52 ± 1.82 b	0.32 ± 0.003b	2.07 ± 0.02b	11.55 ± 0.79b	2.30 ± 0.003b
Sec 2	24.59 ± 0.47c	460.66 ± 28.31c	5.27 ± 0.26c	12.25 ± 0.46c	6.94 ± 0.30c	2.29 ± 0.05c
Sec 5	4.42 ± 0.12d	417.11 ± 68.56c	3.58 ± 0.009d	4.77 ± 0.05d	11.27 ± 0.30b	2.74 ± 0.05d

All values are means ± SE. Means in a column followed by different letters are significantly different at $P \leq 0.05$.

3.4. Assaying anti-TMV activities across a concentration gradient

ID-ELISA showed the curative inhibition efficacies of Ses-2 and Ses-5 against TMV in K326 tobacco increased with compound concentration (Table 3). The overall anti-TMV activities of Ses-2 and Ses-5 increased with concentration.

3.5. Effect of Ses-2 and Ses-5 on TMV CP expression

Following the curative treatment, qRT-PCR assays revealed TMV CP transcript levels increased in the inoculated leaves with time, from 3 days post inoculation (dpi) to 6 dpi. However, transcript levels in the inoculated leaves treated with Ses-2 and Ses-5 were lower than those in the leaves of the inoculated leaves treated with ningnanmycin as well as in the positive control. TMV CP transcript levels in the systemic leaves treated with the two compounds and ningnanmycin were significantly less than those of the positive control (Tables 4, 5). Ses-2 and Ses-5 appeared to induce anti-TMV activities that could be transported to systemic leaves, as TMV CP expression was inhibited. TMV CP had been reported to be involved in long distance movement [16]. Ses-2 and Ses-5 did not disturb the long distance movement of TMV, but they did down regulate the expression and accumulation of TMV CP.

3.6. Effect of Ses-2 and Ses-5 on TMV RdRp expression

Following treatment to assess curative effects, qRT-PCR assays of TMV RdRp expression were conducted both in the inoculated and systemic leaves treated with ningnanmycin, Ses-2, Ses-5. Both Ses-2 and Ses-5 inhibited TMV expression (Tables 6, 7). However, the inhibition efficacy was only maintained for a relatively short time, i.e., for 1 day in the inoculated leaved treated with ningnanmycin and Ses-2 and 2 days under the Ses-5 treatment.

4. Discussion

Tithonia diversifolia can be grown under a range of different environmental conditions with respect to both climate and soil, it has high rates of dispersion and growth [32,33]. Some countries even used this plant as feed for dairy cows and goats [34,35]. Its extracts show a variety of biological activities [36–38]. The Ses-2 and Ses-5 compound each exhibited superior curative activity; hence, they may have applications as pesticides.

To further confirm the anti-TMV activities of Ses-2 and Ses-5, the relative content of TMV in inoculated and systematic leaves was measured using ID-ELISA. TMV infection of systemic leaves is the result of the long distance movement of virion from the inoculated leaves to systemic leaves. However, the significant decrease in TMV content in the systemic leaves indicates that Ses-2 and Ses-5 can induce systemic acquired resistance of the treated plants to inhibit TMV replication or coat protein synthesis.

The coat protein (CP) is required for systemic infection and viral replication [16–18]. RNA-dependent RNA polymerase (RdRp) mediates the genomic replication of TMV [13] and is also one of the key components necessary for cell-to-cell movement of the virus [14]. Anti-viral compounds inhibited virus infecting always through inhibiting viral replication. Li et al. first reported seco-pregnane steroid and its glycosides can inhibit replication of TMV and result in the failure of systemic virus infection [20]. Srivastava et al. had found BDP-30, a glycoprotein extract from Root of *Boerhaavia diffusa* L., inhibited TMV infecting through affecting TMV-CP replication [23]. RdRp and CP are potential targets for studying on the effects of anti-viral compounds on TMV replication. The qRT-PCR assays of CP and RdRp expression, showed that these two sesquiterpene compounds, Ses-2 and Ses-5, which are extracted from *Tithonia diversifolia*, can inhibit the infection or accumulation of TMV in treated tobacco plants by interfering with the expression of TMV CP for 24–48 h, but cannot halt the replication and movement of TMV as well as ningnanmycin. As a curative agent, these two compounds may inhibit the virus. Ningnanmycin is a TMV inhibitor that is used widely to control viral diseases throughout agriculture in China [39]. Ningnanmycin might promote the systemic accumulation of pathogenesis-related proteins (PRs), which are markers of SAR in inhibition of TMV infection [21]. Although a definite inhibition mechanism of Ses-2 and Ses-5 against TMV has remained unknown, our results suggested that these tested bio-agents have the ability to control viral infections, and their curative effects were stronger than those of ningnanmycin. Accordingly, these two sesquiterpene compounds exhibited potency as a bio-pesticide against plant viruses.

5. Conclusion

The anti-TMV activities of sesquiterpene compounds Ses-2 and Ses-5 were evaluated. The curative effect of these tested compounds was better than ningnanmycin, while the inhibition effect of compound Ses-2 was higher than the other tested compounds. qRT-PCR assay of TMV CP and RdRp in the inoculated and systemic leaves treated with Ses-2 and Ses-5 indicated that these two compounds could inhibit the infection of TMV by interfering with the expression of TMV CP. This is the first published report on the anti-TMV activities of these sesquiterpene compounds.

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