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Application of fatty acids as antiviral agents against tobacco mosaic virus

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Numerous studies reported fatty acids (FAs) affecting basal resistance to bacterial and fungal pathogens in plants, but limited reports focused on antiviral agents. In this study, oleic acid was separated from cottonseed oil sludge by antiviral bioassay-guided methods. Antiviral activity of FAs was compared with that of Ningnanmycin. Subsequently, effects of FAs on defense-related enzymes (PAL, POD) and defense-related genes (PR-1a, PR-5) were studied. FAs presented moderate antiviral activity, which is close to that of Ningnanmycin, and activities of PAL and POD were higher in oleic-acid-treated tobacco leaves than those inoculated with tobacco mosaic virus (TMV) and water-treated tobacco. In oleic-acid-treated tobacco, expression levels of PR-1a and PR-5 genes rapidly increased from days 1–3. All results showed that FAs can increase resistance against TMV, and related mechanism can be attributed to activated expressions of a number of defense-related genes, suggesting that FAs can potentially act as pesticides for integrated control of plant viruses in the future.

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1. Introduction

Fatty acids (FAs) are essential for all living organisms [1]. In plants, FAs modulate a variety of responses to abiotic and biotic stresses [1]; for instance, FAs regulate drought, heavy metals, and salt tolerance and provide defense against insect- and wound-induced responses and herbivore feeding in plants [2–5]. FAs also directly or indirectly participate in defense signaling in plants. Six unsaturated FAs (arachidonic acid, docosahexaenoic acid, linolenic acid, eicosapentaenoic acid, oleic acid and linoleic acid) were reported to induce systemic resistance against infection of potato by *Phytophthora infestans* [6]. Researchers also observed that increased levels of FAs possibly inhibit infection of soybean seed by the seed-borne pathogen *Diaporthe phaseolorum* [7]. In parsley, fungal infection rapidly induces transcription of linolenic-acid-synthesizing omega-3 FA desaturase; a sharp and rapid increase in 18:1 (18-carbon (C18) molecules with a single double bond are depicted as 18:1) levels was also observed in parsley cells treated with fungal elicitors [8].

Numerous research reported FAs and their derivatives and their effects on basal resistance to bacterial and fungal pathogens of plants, but studies regarding antiviral agents are limited. Plant viruses cause a variety of detrimental effects on agriculture and horticulture and are known as plant cancer [9,10]. Based on our previous study, which aimed at screening of plants for biologically active natural antiviral products, acetone extract of cottonseed oil sludge displayed moderate antiviral activity against plant viruses. In the present study, FAs were separated from cottonseed oil sludge as active compounds by antiviral bioassay-guided methods. Antiviral activity and antiviral mechanism of FAs were investigated.

2. Materials and methods

2.1. Reagents

All chemical reagents used in this study were of analytical grade. Reagents included acetone, petroleum ether (60–90 °C), ethyl acetate, and methanol and were obtained from Yangling Tiancheng Company (Xi'an, China). Oleic acid, stearic acid, linoleic acid, and palmitic acid were purchased from Sigma-Aldrich Co. LLC (Beijing, China). Soybean oil and rapeseed oil were purchased from Yangguang supermarket (Yangling, China). Ningnanmycin (8%) was purchased from Deqiang Biology Co., Ltd. (Harbin, China). Polyoxy ethylene nonyl phenyl ether was provided by Shaanxi Sunger Road Bio-science Co., Ltd. (Xi'an, China). Silica gel for thin–layer chromatography (TLC; HG/T 2354-92, GF254) and column

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chromatography (200–300 mesh) was purchased from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China).

2.2. Material

Cottonseed oil sludge was used as raw material and purchased from Jingyang Sanqu oil factory in August 2013 (Jingyang, China). A voucher specimen (No. 2013815) was deposited in State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University.

2.3. Instruments

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired with a Bruker DRX–500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) spectrometer (Bruker, Bremerhaven, Germany) in $CDCl_3$ with tetramethylsilane as internal standard at room temperature. Mass spectra were measured on a Waters High-performance liquid chromatography (HPLC)-Thermo Finnigan LCQ Advantage ion trap mass spectrometer (Milford, PA). UV spectra were obtained using a Shimadzu UV-2401 A spectrophotometer (Shimadzu, Tokyo, Japan); semi-preparative reversed-phase HPLC was performed on a Shimadzu LC20A (Shimadzu, Kyoto, Japan) apparatus equipped with a UV detector and a Hypersil BDS C_{18} (Thermo, 250 mm $*$ 10 mm) column at a flow rate of 2 mL/min. Polymerase chain reaction (PCR) was performed on a PCR Thermocycle Instrument made by Hangzhou Bioer Technology Co., Ltd. (Hangzhou, China).

2.3.1. Extraction and isolation

Air-dried and powdered sclerotia of cottonseed oil sludge (10 kg) were extracted thrice with ethyl acetate at room temperature (26 ± 1 °C). After filtration, the extracts were evaporated under reduced pressure. Ethyl acetate extract (362 g) was subjected to liquid–liquid fractionation with solvents, namely, petroleum ether (PE), AcOEt, and BuOH. EtOAc-soluble fraction (216 g), which showed the strongest anti-tobacco mosaic virus (TMV) activity, was applied to silica gel (200–300 mesh) column eluting gradient with $CHCl_3$–MeOH (10:0, 20:1, 9:1, 8:2, 7:3, 1:1, and 0:1), to obtain fractions A–G. Separation of fraction B (42.4 g) showed the strongest anti-TMV activity over silica gel column and was eluted with PE–acetone (10:1–1:2) to yield fractions B1–B6. Fraction B3 (11.3 g) was then subjected to semi-preparative reversed-phase HPLC (80% MeOH–H_2O for 20 min; detection at 210 nm; flow rate 2 mL/min) to produce **1** (1.23 g).

2.4. Virus purification

TMV isolates were provided by the State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University in the form of virus-infected plants. TMV was purified by Gooding's method [11], and *N. tabacum* cv.K326 was inoculated with TMV and used. Upper leaves were selected, ground in phosphate buffer, and filtered through a double-layer pledget. Filtrate was centrifuged at 10,000*g*, treated twice with polyethylene glycol, and centrifuged again. The whole experiment was performed at 4 °C. Absorbance values were estimated at 260 nm by ultraviolet spectrophotometer. Virus concentration was defined in the following formula.

$$\text{Virus concentration} = \frac{(A_{260} \times \text{dilution ratio})}{E_{1\ \text{cm}}^{0.1\%,260\ \text{nm}}}$$

2.5. Anti-TMV assays

N. glutinosa was used as a local lesion host and cultivated in insect-free greenhouse at 23–25 °C. Experiments were conducted when plants grew 5–6 leaves (approximately six weeks-old). Polyoxy ethylene nonyl phenyl ether was used to improve uniform distribution of FAs, because these compounds disperse hardly into water and influence antiviral effect. One gram of FAs were added to 0.05 of polyoxy ethylene nonyl phenyl ether, dissolved with acetone, and diluted with water at concentrations of 500 μg/mL to 1000 μg/mL. Ningnanmycin (8%) was diluted with water at concentration of 500 μg/mL as positive control. By using the half-leaf method, protective effect was tested.

2.5.1. Protective effect

Tested solutions and Ningnanmycin were smeared on the left side, and solvent used as control was smeared on the right side of same-age *N. glutinosa* leaves. After 24 h, leaves were inoculated with virus (TMV at 6×10^{-3} mg/mL) and washed with water. Local lesion numbers were recorded 3–4 days after inoculation. Each experiment was repeated thrice. In vivo inhibition rates of compounds were then calculated according to the following formula. Inhibition rate (%) = $[(C - T) / C] \times 100\%$. Where C is average local lesion No. of control, T is average local lesion No. of drug-treated tobacco leaves.

2.5.2. Leaf disk method

Growing leaves of *N. tabacum* cv.K326 were mechanically inoculated with equal volumes of TMV (30 μg/mL). After 72 h, smooth and thin leaf disks were cut from the leaf surface at an inner diameter of 1 cm. Leaf disks were floated on tested solutions and negative-control solvent. All leaf disks were stored in a culture chamber at 25 °C for 48 h. Then, coat protein of TMV was analyzed by Dot-enzyme-linked immunosorbent assay (ELISA) using a commercial kit per manufacturer's instructions (Neogen, Beijing, China).

2.6. Effect of FAs on PAL and POD activity in tobacco leaves

Enzyme extraction was previously described by Fan et al. [12] but with some minor modifications. A total of 0.5 g of tobacco leaves were weighed, and 1.0 mL of 0.2 M sodium borate buffer (containing 5 mM mercaptoethanol, 1 mM EDTA) with pH 8.8 was added; resulting mixture was ground into homogenate, and sodium borate with 1.0 mL wash buffer was added into the remaining part of the tube. Then, samples were centrifuged for 20 min at 4 °C with 20,000 g, under high-speed refrigerated system. Supernatants were stored at −40 °C until further use.

2.6.1. Determination of PAL activity

PAL activity was determined by using Zhu's method [13]. Reaction system consisted of supernatant (1 mL), sodium borate buffer (2 mL), and L-phenylalanine (1 mL) (in the control, L-phenylamine is replaced by the buffer). Reaction was conducted at 37 °C on a water bath for 30 min then on an ice bath to stop the reaction. Optical density (OD) value was read at 290 nm. One unit of enzyme activity (U) was defined as change in OD value by 0.01 per hour. Each experiment was repeated thrice.

2.6.2. Determination of POD activity

POD activity was determined by using Wang's method [14]. POD activity was assayed for 1 min in a reaction solution comprising crude extract (50 μL), guaiacol (20 mM), potassium phosphate buffer (100 mM, pH 7.0), and H_2O_2 (10 mM). Activity was measured by following changes in absorption at 470 nm. Each experiment was repeated thrice.

2.7. Gene expression analysis of PR-1a and PR-5 in FAs treated tobacco leaves

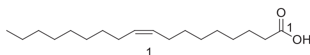
To assess relative expression levels of target genes in oleic-acid-treated tobacco, this study employed real-time PCR. Total RNA was extracted from leaf tissues using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The process for reverse transcription was as previously described by Zhao et al. [15], but with some minor modifications. In brief, 4 μL of total RNA was added

to a reaction mixture containing 2 U of ribonuclease inhibitor (Fermentas, USA), 4 pmol of oligod(dT)-18 primer (TaKaRa), and 5 mM dithiothreitol. After heating at 70 °C for 5 min, final reaction mixture was chilled on ice for 5 min. One microliter of 10 mM dNTP mixture, 2.5 U of reverse transcriptase (M-MLV, Fermentas, USA) and 4 μL of the 5× reverse transcription buffer (Fermentas, USA) were added to reaction mixture. Final reaction volume was adjusted to 20 μL with DEPC-treated water. Reverse transcription was performed at 42 °C for 1 h and followed by heating at 95 °C for 5 min. Primers for real-time PCR amplification were previously described by Chen et al. [16].

3. Results

3.1. NMR and mass spectrometry analysis

Finally, active compounds were obtained from ethyl acetate extract by bioassay-guided methods. Compound **1** was identified as oleic acid by comparison of spectroscopic data (NMR, and mass spectrometry) with data in literature. Oleic acid (**1**), Colorless oily liquid; ^{1}H NMR (500 MHz, $CDCl_3$,TMS): δ: 5.34 (2H, m, H-9, H-10), 2.34 (2H, t, J = 5.96, H-2), 2.01 (4H, d, J = 4.56 Hz, H-8, H-11), 1.63 (2H, m, H-3), 1.25–1.31 (20H, m, H-4-H-7, H-12-H-17), 0.80 (3H, t, J = 5.40, H-18); ^{13}C NMR (125 MHz, $CDCl_3$,TMS): δ: 180.3 (C-1), 34.0 (C-2), 24.6 (C-3), 28.9–29.1 (C-4-C-7), 27.2 (C-8), 130.2 (C-9), 129.7 (C-10), 27.1 (C-11), 29.2–29.9 (C-12-15), 31.8 (C-16), 22.5 (C-17), 14.0 (C-18); EIMS: m/z 281 $[M-H]^-$.



3.2. Anti-TMV assays

As shown in Table 1, four FAs showed similar anti-TMV activity levels regardless of used concentration (500 or 1000 μg/mL). Antiviral activities of the four FAs were close to that of Ningnanmycin when all of them were administered at the same concentration (500 μg/mL), with protection effect changing from 43.2% to 49.4% (Table 1). No phytotoxic activity was observed in treated plants throughout experiments.

According to results shown in Fig. 1, TMV content in oleic-acid-treated tobacco leaves decreased along with increasing concentration of oleic acid. This result indicates that oleic acid may inhibit replication of TMV in plant.

3.3. Effect of FAs on PAL and POD activity in tobacco leaves

As shown in Fig. 2(a), tobacco leaves were inoculated with TMV, followed by spraying with oleic acid. PAL activity in tobacco leaves at days 1–7 increased and reached the maximum on the seventh day and dropped at days 7–9. PAL activities in oleic-acid-treated tobacco leaves were higher than those inoculated with TMV and sprayed with water at days 1–9. Results showed that oleic acid may induce enhancement of PAL activity. These results can improve disease resistance of plants. As shown in Fig. 2(b), TMV inoculation of tobacco leaves followed by treatment with oleic acid significantly increased in POD activity in tobacco leaves at days 0–1 and 3–5 and reached the maximum on the 5th day. The activity during this period was higher than that after inoculation with TMV and water treatment.

Table 1
Anti-TMV activity of FAs and Ningnanmycin.

Compounds	Concentration (μg/mL)	Protection effect (%)
Oleic acid	500	47.5 ± 6.7
	1000	65.8 ± 5.4
Linoleic acid	500	43.2 ± 4.4
	1000	57.6 ± 3.5
Stearic acid	500	49.4 ± 4.2
	1000	59.6 ± 3.9
Palmitic acid	500	46.2 ± 5.6
	1000	62.1 ± 4.8
Soybean oil	500	40.2 ± 6.7
	1000	62.3 ± 1.6
Rapeseed oil	500	44.2 ± 3.9
	1000	59.5 ± 6.1
Ningnanmycin	500	49.7 ± 4.4

All results are expressed as mean ± SD; n = 3 for all groups.

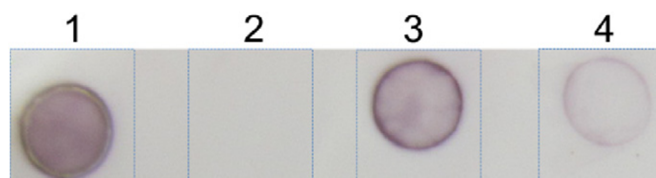


Fig. 1. Inhibitory effect of oleic acid on TMV in tobacco leaves, as tested by Dot-ELISA. Color depth shows highness and lowness of virus contents. 1: positive control, 2: negative control, 3: oleic acid at 500 μg/mL, 4: oleic acid at 1000 μg/mL.

3.4. Gene expression analysis of PR-1a and PR-5 in FAs treated tobacco leaves

Real-time PCR was conducted to determine gene expression analysis of PR-1a and PR-5 in oleic-acid-treated tobacco leaves. mRNA content of oleic acid-treated tobacco leaves for PR-1a gene increased after the first day, reached maximum on the third day, and decreased gradually

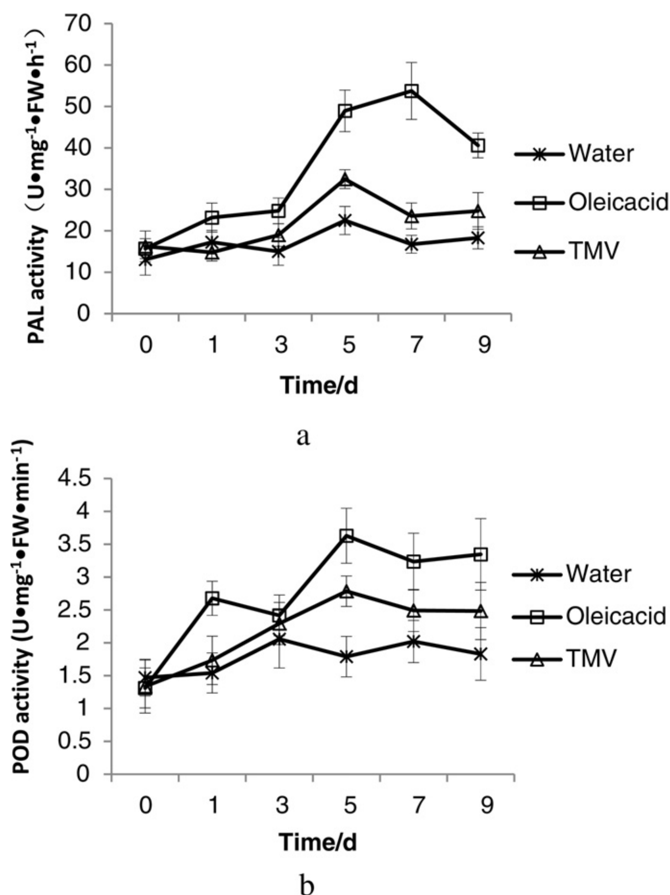


Fig. 2. Effect of oleic acid treatment on PAL and POD activities in *N. tabacum*. *N. tabacum* inoculated with TMV (30 μg/mL) was treated with oleic acid at 500 μg/mL for 0, 1, 3, 5, 7, and 9 days. Water: treated with water; Oleic acid: treated with TMV and oleic acid; TMV: treated with TMV. Data are expressed as means ± SD, n = 3 for all groups. One-way ANOVA revealed significant difference (P < 0.05).

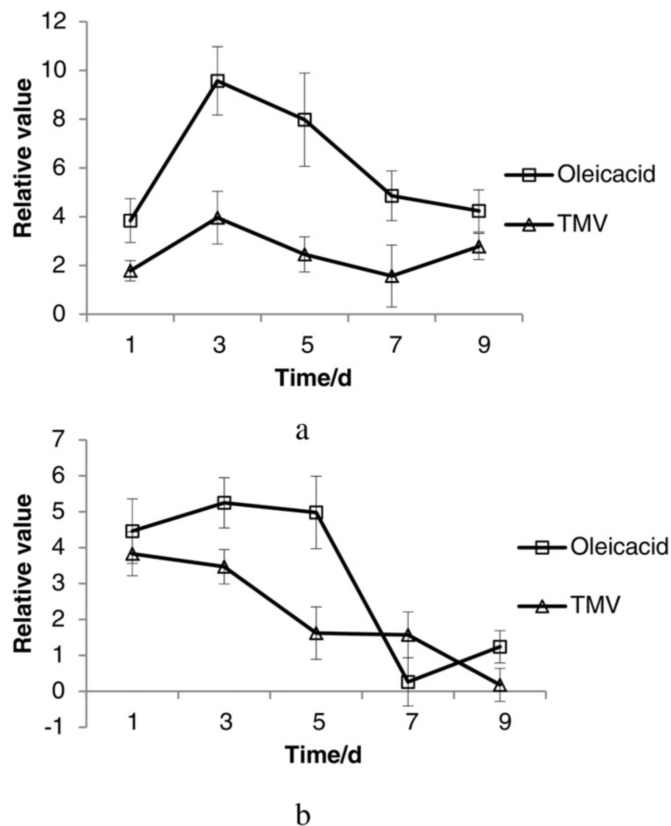


Fig. 3. Effect of oleic acid treatment on PR-1a and PR-5 gene expression in *N. tabacum*. *N. tabacum* inoculated with TMV (30 μg/mL) was treated with oleic acid at 500 μg/mL. TMV treatment and water treatment were used as positive and negative controls, respectively. β-actin gene served as internal reference gene. Semi-quantitative RT-PCR was used to analyze the RNA isolated from tobacco leaf for 1, 3, 5, 7, and 9 days. Fig. 3a: PR-1a gene; Fig. 3b: PR-5 gene. Data are expressed as means ± SD, $n = 3$ for all groups. One-way ANOVA revealed significant difference ($P < 0.05$).

afterward (Fig. 3a). However, in TMV-treated tobacco leaves, expression levels of PR-1a gene also increased from first to third day. Expression levels of PR-5 gene in oleic-acid-treated tobacco rapidly increased from day 1 and maintain high expression levels from third to fifth day before decreasing gradually (Fig. 3b), whereas in TMV-treated tobacco leaf, no significant increase was observed in PR-5 gene expression level.

4. Discussion

Plant viral diseases cause damage to agriculture. To combat plant viral diseases, researchers exerted considerable efforts to develop strategies for efficient control of plant viruses. Plant-derived antiviral drugs, are highly efficient, pollution-free, and can be bought at low costs; they demonstrated significant antiviral potential and attracted increasing attention in recent years. In this study, effective FAs were tested for their antiviral effect against TMV. Results demonstrated that FAs can induce resistance responses of tobacco to TMV, resulting in increased activity of PAL and POD and increased expression levels of PR-1a and PR-5 in tobacco leaves after treatment with oleic acid. Timely deployment of inducible defense responses is critical for effective resistance against pathogens [17]. Systemic acquired resistance (SAR) is one of the most common inducible defense mechanisms. Pathogenesis-related (PR) proteins are widely used as molecular markers for resistance response to SAR and pathogens [18]. Concurrent to these results, İlkay et al. [19] reported that some edible oils possess antiviral ability against *Parainfluenza.* Halldor et al. [20] reported that FAs and monoglycerides in human milk can inactivate enveloped virus. By contrast, Cao et al. [21] reported that antioxidant enzymes and FA composition is related to disease resistance in postharvest loquat fruit. The researchers observed higher levels of linolenic and linoleic acids and showed that higher activity of catalase and ascorbate peroxidase play roles in disease resistance of postharvest loquat fruit. All results suggest that FAs play important roles in plant resistance to pathogens.

To date, few FAs were developed as commercial disease protection agents. As shown by the present study, environment-friendly FAs hold potential for commercial application in the future. First, from economic considerations, starting material FAs are widely available in the world and can be purchased at low costs, as various FAs can be found in many plant materials, such as soybean, peanut, rapeseed, cottonseed, sunflower seed, palm, olive and corn. Second, FAs is very easy prepared despite equipment or technical conditions. Most importantly, antiviral assays demonstrated that FAs work effectively against anti-plant-viruses.

In summary, this study discovered that FAs possess superior activity against anti-plant-viruses, and their mechanism can be attributed to activation of expression of a number of defense-related genes. FAs may also play important roles in plant virus control and in reducing environmental pollution in agricultural ecosystems in the future. Antiviral spectrum and mechanism will be discussed in future course of investigation. Our results will hopefully provide some useful insights into future virus control strategies.

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