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Application of fatty acids as antiviral agents against tobacco mosaic virus

Lei Zhao [a,b,c,1], Yujia Chen [a,b,c,1], Kuan Wu [d], He Yan [e], Xingan Hao [a,b,c], Yunfeng Wu [a,b,c,*]

[a] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling 712100, China
[b] Key Laboratory of Crop Pest Integrated Pest Management on Crop in Northwestern Loess Plateau, Ministry of Agriculture, Northwest A&F University, Yangling 712100, China
[c] Key Laboratory of Plant Protection Resources and Pest Management, Ministry of Education, College of Plant Protection, Northwest A&F University, Yangling 712100, China
[d] Yangling Vocational & Technical College, Yangling 712100, China
[e] Research & Development Center of Biorational Pesticides, Northwest A&F University, Yangling 712100, China

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ABSTRACT

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.

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

* Corresponding author.
E-mail address: wuyf@nwsuaf.edu.cn (Y. Wu).
[1] These two authors contributed equally to the present study.

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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] × 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