Research Article

Study on the Absorption and Conduction Properties of Vanisulfane in Tobacco

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The purposes of this study were to explore the systemic properties of vanisulfane in tobacco plant and to provide a reference for the rational use of vanisulfane in the field. After the tobacco plants were treated by hydroponics and foliar spraying, the contents of vanisulfane in root and stem leaf were detected by high-performance liquid chromatography tandem high-resolution mass spectrometry (UPLC-HRMS), and the position of vanisulfane in root and stem leaf was real-time observed through fluorescence two-photon confocal microscope. UPLC-HRMS results showed that the contents of vanisulfane in root and stem leaf gradually increased with the extension of processing time, and after 12 h treatment, the contents of vanisulfane in root and stem leaf reached the maximum levels of 31.95 and 0.215 mg/kg, respectively. In addition, fluorescence two-photon confocal microscope results showed that vanisulfane could observe in the root and stem leaf. These results showed that vanisulfane had excellent upward and downward of systemic in tobacco plants, which is helpful to guide a reference for the rational use of vanisulfane in the field.

1. Introduction

Vanisulfane, 2,2′-(((4-((4-chlorobenzyl)oxy)-3-methoxyphenyl)methylene) bis-(2-hydroxyethyl) dithioacetal (Figure 1), is a novel antiviral agent that exhibits pronounced protection and curative activities against cucumber mosaic virus (CMV) with the half-maximal effective concentration (EC50) values of 186.2 and 206.3 μg/mL, respectively, which are superior to those of the commercial agents, such as ribavirin (766.5 and 858.2 μg/mL, respectively), dufulin (465.4 and 471.2 μg/mL, respectively), and ningnanmycin (405.3 and 426.1 μg/mL, respectively) [1]. Vanisulfane, whose CAS registry number and patent number are 2088490-79-1 and CN106467478A, was developed as a new dithioacetal class of antiviral agent. The patent of vanisulfane (CN106467478A) has been granted by the State Intellectual Property Office, the People’s Republic of China in March 2017.

In the last decades, many studies have reported the fluorescent label detection of other drugs [2–5]. Because farmers lack the means to carry out in situ assessment of leaf cover in real-time, they often overuse pesticides in order to prevent pests due to insufficient leaf coverage. In order to replace the excessive use of pesticides, a fluorescence method has been developed that can be quickly evaluated and used in the field to detect pesticides on plant leaves [6]. Ivy leaves were selected to study the fluorescence method for quickly assessing the coverage of pesticides and to overcome the matrix effects related to plant fluorescence emission and physiological spectral interferences. The results showed that fluorescent labeling agents in nano- and microcrystallites forms can be used to overcome matrix effects. By studying the characteristics of fluorescence quenching/enhancement, spectral shifts, and fluorescence lifetimes, it can be found that these spectral features are adequate for mapping the
pesticides on plant surfaces to assess their coverage [7]. Compared to other fluorophores, development of quantum dot (QD) based excellent and unique optical properties and have high fluorescence quantum yields has gained momentum in recent years. Their applications as fluorescent probes in the detection of pesticides in different media (including water, fruits, and vegetables) were studied. The low detection limits reported demonstrate the potential use of these methods as alternatives to expensive and time-consuming conventional techniques [8]. The fluorescence analysis method for detecting three organophosphorus pesticides using magnetic-assisted FAM-aptamer as probes was studied. The results showed that choosing 37°C and 150 min as the best combination temperature and time, respectively, the developed method exhibited a higher selectivity for organophosphorus pesticides (OPs) whose aptamers had the common sequence and trueness of the MA-FA method [9]. Many studies have shown that naringenin is a key signaling molecule in the control of root nodulation, a prerequisite for the plant nitrogen fixation. In this experiment, the appropriate positions of fluorescent labeling were found at the six chemically available positions of the flavonone core of naringenin. Fluorescence labeling was used to detect the bindings of naringenin to nod-D after its entry into Rhizobium cells. In the corresponding fluorescence imaging experiments, it was observed that the entry of naringenin into living Rhizobium cells was clear [10]. Methods with fluorescent labels have the potential to achieve the low limits of detection (LOD) imposed by legislation when interferences resulting from the sample matrix are reduced. In heterogeneous test formats, the background fluorescence inherent to real samples is reduced by means of a physical separation step shows that it is feasible to detect and accurately determine pesticides in low-limit real environmental samples [11]. Obviously, literature studies have shown that fluorescent labeling plays an important role in drug detection [12–14].

In addition, there is no literature report on the systemic conduction of vanisulfane in plants. Therefore, we investigated the systemic transmission of other drugs in plants as a reference [15–17]. The present investigation indicates that lignin is the most important sorbent responsible for the adsorption of pesticides in the apoplast. The adsorption of five labeled systemic fungicides and one herbicide, on lignins (stems of pepper plants and cotton plants, 120 and 90 days old, were used for lignin preparations) from three kinds of plant stems and on other components of plant tissue, was investigated. The dual character of car-bendazim-lignin binding is further corroborated adsorption of systemic fungicides by plant tissues by its decreased adsorption on lignin in the presence of mineral salts, on the one hand, and its increased binding to methylated cellulose on the other hand [18]. With the advent of industrialization, a variety of used chemicals and their derivatives will eventually enter the soil and pose a threat to plants. Some papers focus on the plant uptake capacity of various contaminants of emerging concern (CEC) in soil, such as pesticides [19–21] and pharmaceuticals [22, 23]. The results of some researchers indicated that the bioaccumulation of CEC in roots was higher than that in shoots. In addition, various plant species, pollutant types, and microbial interactions will affect the overall absorption [24]. The root pathway and seed pathway of maize seedlings during the growth and development process were studied. Experiments showed that the absorption and transportation of pesticides by these two pathways occurred at the same time. Compared with seeds, the root system has a stronger ability to absorb and transport pesticides. It has a greater contribution to the absorption and transport behavior of the coating agent [25]. The study using nanoparticle-immersed paper imprinting mass spectrometry imaging technology immersed in nanoparticles revealed the carrier-mediated systemic and transmission mechanism of modified chlorantraniliprole in plants. The results showed that the modified chlorantraniliprole was applied after foliar application. It has two-way conductivity in cabbage plants, and when the concentration of chlorantraniliprole in the phloem is too high, it can be transmitted to the xylem and then migrate to the leaves through the xylem [26].

To the best of our knowledge, there were only some researches on its synthesis, bioactivity, and the application methods of vanisulfane residues [27–29]. However, the fluorescent label detection and systemic conduction of vanisulfane in plants have not been reported yet. The fluorescent label detection and systemic conduction of vanisulfane was of great significance to the current situation in the field and scientific medication. Fluorescent labeling was a key technology, which was widely used in biological processes of cell and biochemistry. The two photon confocal technology undertakes the real-time observation and determination of the fluorescent molecular position. It can visually display the conduction of drugs in plants. In view of these considerations, this experiment took tobacco plants as the experimental object, using UPLC-HRMS [30] and fluorescent dual-light sub-confocal real-time imaging technology [10, 31, 32] to carry out the systemic conduction study of vanisulfane in the plant.

The purpose was to accurately and intuitively study the internal absorption and conduction performance of vanisulfane on tobacco plants, so as to provide a certain reference for the scientific and rational mode of administration of vanisulfane in the field. The objectives of the present study are as follows: (I) to synthesize fluorescent-labeled vanisulfane and compare the activity difference between vanisulfane and fluorescent-labeled vanisulfane, (II) to quantitative and

![Figure 1: The structure of vanisulfane.](image-url)
qualitative analysis vanisulfane in tobacco by UPLC-HRMS, and (III) to real-time imaging of vanisulfane in tobacco by fluorescent two-photon confocal.

2. Materials and Methods

2.1. Reagents and Instruments. Vanisulfane standard sample (99.6% purity) was provided by the Key Laboratory of Green Pesticide and Agricultural Bioengineering, Guizhou University (Guiyang, China). Methanol and acetonitrile (chromatographic grade) were purchased from Tedia High Purity Solvents (Darmstadt, Germany). Sodium chloride (analytical purity) was purchased from LookChem (Beijing, China), anhydrous sodium sulfate (analytical purity) was purchased from Jinshan Chemical Reagent Co. (Chengdu, China). Distilled water was obtained from Watsons Co. Ltd. (Dongguan, China).

Thermo Scientific UltiMate 3000 high-performance liquid chromatography and Thermo Scientific Q Exactive high-resolution mass spectrometer were purchased from Thermo Fisher Scientific, Inc. (Waltham, USA). BUCHI R-210 rotary evaporator was purchased from BUCHI Laborotechnik AG (Flawil, Switzerland). ALC-210.4 Electronic Balance was purchased from Sartorius (Darmstadt, Germany). MTV-100 Multitube Vortex Mixer was purchased from Hangzhou Allsheng Instruments Co., Ltd. (Hangzhou, China). TGL-20B centrifuge was purchased from Shanghai Anting Scientific Instrument Factory (Shanghai, China). KQ-100B Ultrasonic cleaner was purchased from Kunshan Ultrasonic Instruments Co., Ltd (Kunshan, China). Fluorescent Two-photon Confocal microscope Olympus FV 3000 was purchased from Olympus (Olympus, Japan).

2.2. The Absorption and Conduction of Vanisulfane in Tobacco

2.2.1. Internal Suction and Upward Conduction Test (Hydroponic Method). Vanisulfane aqueous solution (100 mL) with the concentration of 100 mg/L was added to 250 mL Erlenmeyer flask. The tobacco seedlings at the 3-4 leaf stage were dug out with roots and then washed away the soil with water. After that, the tobacco seedlings were inserted into a triangular flask for cultivation in a greenhouse (18-25°C) under natural light. The stems and leaves were cut off at 4, 8, 12, 24, 36, 48, 72, and 96 h, respectively, and then stored at -20°C for later test use.

2.2.2. Inward Suction Downward Conduction Test (Spray Method). Vanisulfane aqueous solution (100 mg/L) was uniformly sprayed on the leaf surface of the 3-4 leaf stage tobacco seedlings. Then, the leaf surface was sprayed with fresh-keeping film to prevent pollution. The plant samples were cultivated in a greenhouse (18-25°C) under natural light. The roots were cut and rinsed at 4, 8, 12, 24, 36, 48, 72, and 96 h, respectively, and then stored at -20°C for later test use.

2.3. Qualitative Analysis of Vanisulfane by UPLC-HRMS

2.3.1. Instrumental Method

(1) Liquid Condition. UPLC separations were obtained using a Thermo scientific Hypersil Gold C8 1.9 μm (2.1 × 100 mm) operating at 40°C in the isocratic elution mode. The mobile phase was component A (60% water with 0.1% formic acid): component D (40% acetonitrile). The flow rate was 0.3 mL/min, and the sample injection volume was 2 μL.

Table 1: Experiments of adding and recovering vanisulfane in tobacco root, stem leaf.

| Sample                      | Add level (mg/kg) | Recovery rate (%) | RSD_{A} (%) (n = 5) | RSD_{R} (%) (n = 15) |
|-----------------------------|-------------------|-------------------|---------------------|----------------------|
| Tobacco root and stem leaf  | 0.0005            | 101.12            | 99.98               | 101.03               |
|                             | 0.0010            | 77.93             | 80.21               | 81.11                |
|                             | 0.0100            | 86.69             | 85.55               | 84.32                |
|                             | 0.1000            | 82.10             | 83.29               | 80.23                |

Note: *intraday coefficient of variation; "intraday coefficient of variation.

Figure 2: Synthetic route of vanisulfane with fluorescent label.
Mass Spectrum Condition. ESI; negative ion mode; aux gas heater temperature at 300°C; capillary temperature at 300°C; and spray voltage at 3.0 kV, sheath gas, sweep gas, and auxiliary gas flow rates at 30, 3, and 10 a.u., respectively. Qualitative and quantitative ion: [M + HCOO]−, 459.07083 m/z.

2.3.2. Extraction Method of Vanisulfane from Tobacco Roots, Stems, and Leaves. In the experiment, 5 g of fresh tobacco (including roots, stems, and leaves) was accurately weighed into 50 mL centrifuge tubes, and vanisulfane standard samples were added to make the additive levels in tobacco are 0.0005, 0.01, 0.1, and 1 mg/kg, respectively. After the solvent evaporates, acetonitrile (20 mL), NaCl (2 g), and anhydrous Na2SO4 (3 g) were added to the centrifuge tubes. The homogenate was extracted for 3 minutes and centrifuged at 6000 r/min for 5 minutes. After that, 10 mL of the supernatant was sucked out and spin-dried. The dried sample was dissolved in 1 mL of methanol, and the content was determined by UPLC-HRMS. The additive recovery experiment was carried out for 3 consecutive days, and 6 parallels were performed for each additive level.

2.4. Synthesis of Fluorescent Labels of Vanisulfane. In order to further prove the law of internal absorption and conduction of vanisulfane in plants, the fluorescent-labeled of vanisulfane (Figure 2) was synthesized. Using rhodamine B as raw material, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 0.55 g), rhodamine B (0.92 g), 1-hydroxybenzotriazole (HOBt, 0.32 g), and tetrahydrofuran (20 mL) were added into a 50 mL three mouth flask and refluxed at 60 °C for 0.5 h. After that, vanisulfane (1 g) dissolved in 10 mL tetrahydrofuran was slowly added and stirred at 60 °C. After completion of reaction, the vanisulfane with fluorescent label was purified by column chromatography (Vethyl acetate : V dichloromethane : Vmethanol = 2 : 5 : 1) and characterized the structure by 1H NMR, 13C NMR, and HRMS. The 1H NMR, 13C NMR, and HRMS data are shown below. 1H NMR (500 MHz, CDCl3, ppm) δ 8.26 (d, J = 7.5 Hz, 1H), 7.72 (d, J = 11.9, 7.2 Hz, 2H), 7.3 (d, J = 13.0 Hz, 3H), 7.23 (d, J = 7.2 Hz, 1H), 7.07–6.91 (m, 4H), 6.88–6.63 (m, 6H), 5.09–4.87 (m, 3H), 4.13–4.03 (m, 2H), 3.86–3.16 (m, 14H), 2.72–2.44 (m, 4H), 1.25 (t, J = 6.7 Hz, 12H); 13C NMR (125 MHz, CDCl3, ppm) δ 165.00, 158.55, 157.86, 157.84, 155.63, 155.57, 149.79, 147.65, 135.70, 133.76, 133.64, 133.44, 133.06, 131.65, 131.36, 131.32, 130.54, 130.21, 130.07, 128.77, 128.74, 120.12, 118.17, 114.38, 114.27, 113.81, 113.61, 113.59, 111.49, 111.49,
96.56, 96.54, 70.44, 64.33, 61.58, 56.18, 53.26, 46.19, 46.17, 35.16, 31.03, 12.73; HRMS (ESI) theoretical value: m/z C_{47}H_{52}O_{6}N_{2}Cl_{2}S_{2} = 839.29498, measured value: m/z 839.29431, mass error, -0.79988 ppm (Shown in Supplementary Materials (available here).

2.5. Bioactivity Test of Vanisulfane with Fluorescent Label. Whether the CMV activity of vanisulfane after fluorescent labeling has changed remains to be determined. Therefore, the CMV activities of vanisulfane and vanisulfane with fluorescent label were determined and compared in this experiment. The curative and protection activity against CMV of the vanisulfane with fluorescent label at the concentration of 500 μg/mL was tested by the half-leaf dead spot method [1]. Methods: vanisulfane and vanisulfane with fluorescent label were accurately weighed 2 mg, respectively. The weighed samples were dissolved in 20 μL N,N-dimethylformamide (DMF), and 4 mL of 1% Tween water was added to prepare the compound to a concentration of 500 μg/mL. The anti-CMV activity of the compound was tested by the half-leaf dead spot method.

2.6. Real-Time Observation of Fluorescent Labels in Tobacco. The excitation and emission wavelength of the synthesized vanisulfane fluorescent label were measured. The detection wavelength of the two-photon confocal was set according to the measured excitation and emission wavelengths. According to the method of 2.2.1., the obtained tobacco plants were sliced, and the treated slices were observed under two-photon confocal microscope. The sampling time of fluorescence real-time imaging was the same as that of the hydroponic method.

3. Results and Discussion

3.1. Accuracy and Precision. According to the extraction method of vanisulfane in tobacco in 2.3.2, the experiments were carried out with different concentrations of vanisulfane, each with 5 mass concentrations in parallel, and the extraction was added for three consecutive days. As shown in Table 1, the recovery rate was 77.93-102.12%, and the coefficient of variation RSD was 1.34-5.05%. The experimental results showed that the accuracy and precision of the extraction method meet the requirements of pesticide residue analysis and detection [33].

3.2. The Quantitative Detection of Vanisulfane by UPLC-HRMS. The quantitative detection of the content of vanisulfane in tobacco at different times was carried out through design experiments using UPLC-HRMS. Figure 3(a) indicated that vanisulfane can be absorbed and transmitted downward to the root, and the conduction is slow. The content of vanisulfane in the sample reaches the maximum value of 0.215 mg/kg after 72 h of treatment. After that, the content gradually decreased with the extension of sampling time. At the end of sampling at 120 h, the content of vanisulfane in the sample was 0.022 mg/kg. It can be seen from Figure 3(b) that vanisulfane can be sucked inward and
uploaded to the stems and leaves. With the extension of treatment time, the content gradually increases. After 12 h of treatment, the content of vanisulfane in the sample reaches the maximum, which was 31.95 mg/kg. With the extension of sampling time, the content gradually decreased. At the end of 96 h, the content of vanisulfane in the sample was 3.85 mg/kg. According to the experimental data, vanisulfane can be conducted in tobacco, the process of downward conduction is slower than that of upward conduction, and the content is also lower. Many systemic pesticides can be transported up and down in plants [34–37]. It can be known that vanisulfane was a systemic agricultural antiplant virus agent.

3.3. Real-Time Fluorescence Two-Photon Confocal Imaging of Vanisulfane in Tobacco

3.3.1. Wavelength Determination of Vanisulfane Fluorescent Label. The excitation and emission wavelengths of vanisulfane with fluorescent label were determined by FLUOROMAX-4.
spectrofluorometer. The excitation wavelength was 568 nm, and the emission wavelength was 583 nm. The fluorescence intensity of vanisulfane fluorescent label compounds was different in different solvents. The determination results were shown in Figure 4. According to the measured excitation and emission wavelengths, the detection wavelength of the two-photon confocal was set 1165 nm.

3.3.2. Verification of the CMV Activity of Vanisulfane with Fluorescent Label. As shown in Table 2, the curative and protection activities against CMV of vanisulfane with fluorescent label were 51.23% and 53.12%, respectively, which were equal to those of vanisulfane (50.53% and 51.42%, respectively), demonstrating that there was no significant effect on the anti-CMV activity after connecting the fluorescent label.

3.3.3. Real-Time Imaging of Vanisulfane with Fluorescent Label in Tobacco. It can be seen from the results of two-photon confocal imaging Figure 5 that the fluorescence of fluorescent-labeled vanisulfane can be clearly observed in the root, stem leaf vein of tobacco in real-time. The excitation wavelength of two-photon was 1165 nm when the excitation wavelength of real-time imaging was 568 nm. Rhodamine B was selected as the fluorophore to avoid the interference of plant fluorescence, such as chlorophyll fluorescence (the excitation wavelength of chlorophyll was 439, and the excitation wavelength of two-photon was 915 nm). Figures 5(a) and 5(b) were comparison diagrams of tobacco stem cross-sections under bright field and fluorescent field positioning after partial magnification, and the fluorescent substance can be observed intuitively. The large number of fluorescent clusters was found in the xylem of tobacco root, which was transmitted along the xylem conduit (Figure 5(c)). The vessel of the plant can be clearly seen in the vertical section of the stem of tobacco, and the fluorescent substances were transmitted to all parts of the plant along the vessel (Figure 5(d)). In Figure 5(e), the fluorescence conduction can be clearly seen from the cross-section of the leaf vein. Figure 5(f) was shown that the presence of fluorescence can also be clearly seen on the leaf veins of tobacco.

Through two-photon confocal imaging, vanisulfane labeled with fluorescent can observe its internal absorption conduction in tobacco in real-time, which further verifies that vanisulfane has internal absorption conductivity in tobacco, which was consistent with the qualitative and quantitative results of high-resolution mass spectrometry. Both fluorescence two-photon confocal and UPLC-HRMS showed that vanisulfane had endothermic conductivity in tobacco plants.

3.3.4. Qualitative Determination of Vanisulfane with Fluorescent Label in Tobacco. Whether vanisulfane after being attached with a fluorescent tag was conducted in tobacco plants in addition to observing the conduction of fluorescence with two-photon confocal, whether vanisulfane after being fluorescently tagged was changed in plants remains to be determined. We therefore developed a high-resolution mass spectrometric qualitative assay of fluorescently tagged vanisulfane in tobacco (Figure 6).

Experimentally determined qualitatively by high-resolution mass spectrometry of the conducted fluorescently tagged vanisulfane in tobacco, mass range 839.29498 m/z peak pattern was able to be extracted in the total ion chromatogram (TIC), from which it was observed that the theoretical mass spectrum of fluorescently tagged vanisulfane had a peak at 839.29498 m/z, found 839.29364 m/z, which was -1.59983 ppm from the theoretical value, and was accurate in the 3 ppm range. The results of the experiment proved that the vanisulfane following the attached fluorescent tags was conducted in tobacco plants in vivo, but not the conduction of fluorescein itself in tobacco.

4. Conclusion

In this paper, the UPLC-HRMS technology was used to qualitatively and quantitatively analyze the internal absorption and conduction content of vanisulfane in tobacco plants after two treatments: hydroponics and foliar spraying. The properties of vanisulfane systemic upward and downward conduction in tobacco have been studied. The results showed that vanisulfane had good internal and upward conduction in tobacco plants. In addition, through fluorescent labeling of vanisulfane, using fluorescent two-photon confocal real-time imaging technology, we observed that vanisulfane can absorb and conduct in tobacco plants in real-time. The results can provide some reference for the scientific and rational drug use of vanisulfane in the field.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

None of the authors have any professional or financial conflicts of interest.

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Supplementary Materials

Vanisulfane with fluorescent label. The synthesized Vanisulfane fluorescent compound label has been characterized by $^1$H NMR, $^{13}$C NMR and high resolution mass spectrometry. $^1$H NMR, $^{13}$C NMR and high resolution mass spectrometry. HRMS (Supplementary Materials)

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