In situ sampling revealed that 2,4 DTBP contributes to continuous cropping obstacles in pepper

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Abstract

Continuous cropping (CC) obstacles resulting from the increase in planting frequency restrict the sustainable development of the vegetable industry. To understand the mechanisms causing continuous cropping obstacles in-depth, we analyzed pepper (Capsicum annuum L.) rhizosphere soils. Samples were collected using a new type of in situ sampler composed of microporous ceramic tubes. After elution, the samples were qualitatively and quantitatively analyzed via gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC). Then, the effects of the main possible allelochemicals on sensitive plants were investigated. Fourteen chemicals, including 2,4-di-tert-butyl-phenol (2,4-DTBP), diisobutyl adipate (DIBA), dibutyl phthalate (DBP) ,and limonene, were detected with GC-MS. The measured concentration of 2,4-DTBP increased with pepper growth. The results based on seed toxicity revealed that low-concentration (<50 mg·L-1) 2,4-DTBP improved lettuce seed germination rates and that high-concentration (>300 mg·L-1) 2,4-DTBP had significant ($p<0.05$) inhibitory effects. The results obtained from sensitive cucumber plants showed that the average plant height in the treatments containing 2,4-DTBP was lower ($p<0.05$). Significant increases in reactive oxygen species (ROS) levels and reductions in the photosynthetic rate (Pnet) were also detected ($p<0.05$). Leaf transpiration (Tr), stomatal conductance (Gs) and root activity also decreased significantly compared with those of the control. The comparison results between the 2,4-DTBP treatment and the pepper CC treatment showed that the cucumber was more significantly damaged in the latter. Similar trends also occurred in pepper. Our results indicate that the in situ sampler in this study can reflect the actual concentrations of possible allelochemicals in rhizosphere soil. 2,4-DTBP is one of the most important allelochemicals in pepper, and the accumulation of 2,4-DTBP may be an important factor inducing continuous cropping obstacles in pepper.

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Abstract

Continuous cropping (CC) obstacles resulting from the increase in planting frequency restrict the sustainable development of the vegetable industry. To understand the mechanisms causing continuous cropping obstacles in depth, we analyzed pepper (*Capsicum annuum* L.) rhizosphere soils. Samples were collected using a new type of in situ sampler composed of microporous ceramic tubes. After elution, the samples were qualitatively and quantitatively analyzed via gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC). Then, the effects of the main possible allelochemicals on sensitive plants

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Key words: in situ sampling; allelopathy; allelochemical; pepper, continuous cropping obstacle

Introduction

Pepper (*Capsicum annuum* L.) is an economically important vegetable crop species that is widely cultivated in temperate and tropical regions of the world. Its global annual yield is approximately 3.7 million tons (FAO, 2019). Pepper production in China accounts for 50.8% of global pepper production. Therefore, the stable development of the pepper industry has an important effect on vegetable industry development.

To ensure a sustainable supply, planting frequency in one year or the number of successive planting years has increased, which has resulted in continuous cropping (CC) obstacles that restrict the sustainable development of the vegetable industry (Li et al., 2019). This problem occurred in the pepper industry (Khan et al., 2015). CC obstacles slow crop growth and development, decrease plant vigor, increase the frequency of disease and directly decrease crop yield and quality (Li et al., 2015a, 2015b; Li et al., 2018; Li and Liu, 2018). This means that CC obstacles significantly influence the sustainable development of agriculture and have become one of the crucial problems to be solved in the field of agricultural science.

This negative effect of CC obstacles on crop growth is mainly caused by the accumulation of allelochemicals, causing self-allelopathy and allelopathy in the soil (Inderjit, 2006; Wang et al., 2019). Most allelochemicals in the soil are plant secondary metabolites. These natural compounds, such as sugar, amino acids, organic acids, phenolic compounds, flavonoids, and lipids, are mainly secreted by plant roots (Dong et al., 2018; Oburger et al., 2018).

Of these compounds, the effects of allelopathy from phenolic compounds on crop growth and development are particularly obvious (Batish et al., 2004; Khan et al., 2015; Verbon & Liberman, 2016). Chen et al. (2020) found that the accumulation of phenols in soil significantly inhibited strawberry growth and reduced the abundance of various beneficial bacterial groups; as a result, the quality and yield of strawberry fruit decreased. Wu et al. (2009) reported that the soil microbial ecological environment changed due to the accumulation of ferulic acid in cucumber soil, further inhibiting cucumber growth. Batish et al. (2007) found that when *Chenopodium murale* and wheat were intercropped, the phenolic substances secreted by *C. murale* roots hindered the growth and development of wheat. Thus, the study of allelochemicals and root system allelopathy contributes to understanding the mechanisms of and solving the problems caused by CC obstacles.

Determining how to sample allelochemicals in the soil is crucial for further analysis. Currently, root exudate sampling methods include the hydroponic method, tube method, agar culture method, air culture method, filter paper adsorption method, plant root box method, field investigation method, pot culture method, microsuction cup method, and others, the most popular of which is hydroponics (Wang et al., 2019). Many important advances in root exudate studies have been achieved with this method. However, because this method does not consider the decomposition and transformation of root exudates by soil microorganisms,

it is difficult to simulate the true concentrations of root exudates in the soil with hydroponics. Therefore, it is still challenging to compare the results obtained under hydroponic conditions with those from the real soil environment (Oburger & Jones, 2018). Instead, in situ soil collection has become the most promising technical means of solving the above problems because the exudates are undisturbed and remain closer to the actual secretion conditions. Proctor & He et al. (2017) transplanted *Rhododendron groenlandicum* from an ombrotrophic bog to a special root trap for cultivation and sampled root exudates without soil disturbance using a set of negative-pressure vacuum devices. Qualitative and quantitative analysis of the root exudates of different species revealed the differences in root exudates among different species and the variability in root exudates from roots with different morphologies. Oburger et al. (2013) invented an in situ culture box in which plant roots grew naturally into a hydroponic compartment containing an exchange membrane and root exudates were extracted under negative pressure. Then, the low-molecular-weight organic matter in the root exudates was quantitatively analyzed. In addition, an in situ root box was also used to culture individual roots to analyze the exudates with the aid of filter paper or resin (Haase et al., 2007; Shi et al., 2011).

In this study, to understand the allelopathy mechanism that results in pepper CC obstacles, we invented a new, noninvasive in situ sampler based on the principle of capillarity that extracts substances from the soil and identified the main potential pepper allelochemicals by qualitative and quantitative analyses. The mechanism of the main potential allelochemical was explored.

MATERIALS AND METHODS

Noninvasive in situ sampler

The in situ sampler, composed of microporous ceramic, was made by Yizhong Ceramic Technology Co., Ltd. (Yixing, China). The specifications of the in situ sampler were as follows: inner diameter, 10 mm; external diameter, 20 mm; length, 14 cm; average adsorption volume of tube wall, 33 cm^3; and intratubal hollow volume, 11 cm^3. The intratubal volume of the in situ sampler was filled with nonionic XAD-4 resin (Solarbio, Bei Jing, China) for sampling and extraction.

Collection of potential allelochemicals

Two wooden incubators with volumes of 1 m × 1 m× 0.3 m were placed in a greenhouse and then filled to a 20-cm depth with soil that had not been previously planted with pepper. Pepper seedlings of uniform height and good growth status were planted in one of the wooden incubators in a 5×5 pattern at a planting distance of 10-15 cm. The other wooden incubator was not planted with pepper seedlings to provide a control treatment. Nine in situ samplers were inserted into the soil in each incubator in a 3×3 arrangement. During each growth period, three of the in situ samplers were removed to analyze the components extracted from the rhizosphere soil. The XAD-4 resins removed from the sampler were eluted with 300 mL of methanol solution. The eluates were merged and concentrated to 5 mL by nitrogen purging. Then, the concentrates were filtered with a 0.45-μm filter membrane. The filtrates were stored at -20°C for gas chromatography–mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) analysis.

GC-MS analysis of potential allelochemicals in pepper root exudates

GC-MS analysis of the methanol extract was conducted with an Agilent 7890A GC system coupled to an Agilent 5975C mass selective detector (MSD) (Agilent, Technologies, Santa Clara, CA, USA). The GC was equipped with a two-way purged splitter installed after the analytical column (30 m × 0.25 mm, 0.25 μm, HP-5MS, J & W Scientific, USA). The inlet temperature was 250°C. The column temperature was initially held at 50°C and was gradually increased to 280°C at a rate of 10°C·min^{-1}. High-purity helium was used as the carrier gas at a flow rate of 1 mL·min^{-1}. The injection volume was 1 μL in splitless mode.

The mass spectrometry conditions were the following: electron bombardment source, electrospray ionization; bombardment voltage, 70 eV; scanning range m/z, 30-600 amu; scanning speed, 0.2 s whole-process sweep; ion source temperature, 200°C; and quadrupole temperature, 150°C.

Reconfirmation of in situ sampling effects

To evaluate in situ sampling, a given mass of 2,4-DTBP (400 mg·kg) was added to the soil. The traditional root soil extraction method (Yao et al., 2020) was used to quantitatively determine the content of 2,4-DTBP in pepper soil. Briefly, forty-five grams of soil was extracted with 100 ml of ethyl acetate for 24 h and centrifuged at 10 000 *g* (15 min). The supernatant was concentrated to 5 ml by rotary evaporation and filtered through a 0.45-μm filter. The filtrate was used for further comparison with in situ sampling. Five in situ samplers were put into one pot (3.571 L), and 5 pots were prepared. Water was added to the pot three times every day (total: 2 L). After 48 h, samplers were removed for quantitative analysis of 2,4-DTBP. The pepper CC soil was also treated with the same method.

Quantification analysis of 2,4-DTBP

The quantitative analysis of 2,4-DTBP in the concentrated solution was performed by an external standard method with an Agilent 1200s HPLC instrument (Agilent Co., Palo Alto, CA, USA) equipped with a C18 reversed-phase column. The mobile phase was methanol, and the flow rate was 1.0 mL min^{-1}. The column temperature was 38°C, and the injection volume was 20 μL.

Detection of 2,4-DTBP allelopathy

Lettuce seeds were placed on filter paper soaked with 2 mL of methanol solution containing 2,4-DTBP at concentrations of 200, 300, 500, 800, and 1,000 mg·L^{-1}. Fifty lettuce seeds were sown in each of three Petri dishes using filter paper as the substrate. Methanol solution without 2,4-DTBP was used as a blank control. All Petri dishes were placed in a dark room at 25°C for 6 days. The germination time was defined as the time until the radicle had protruded beyond the seed coat by at least 1 mm. The lengths of the radicle and hypocotyl of the lettuce seedlings were measured on the 17th day after germination.

Six treatments with 4 potential allelochemicals were established in the lettuce seed germination tests. In the treatments, the concentrations of 2,4-DTBP were 0, 10, 20, 30, 40, and 50 mg·L^{-1}. The concentrations of DIBA, DBP and limonene were 15 mg·L^{-1}. The lettuce seed germination rate was determined every 8 h from 24 to 48 h.

Greenhouse experiments

The phenolic compound 2,4-DTBP was obtained from Sigma Chemical Company (St. Louis, MO, USA). The 2,4-DTBP solution (1.18 g·L^{-1}) was prepared with methanol, and distilled water and methanol were used as controls. The experimental soil and pepper CC soil were obtained from the Horticultural Subacademy of the Heilongjiang Academy of Agricultural Sciences. The four treatments were designated CK (water), Me-CK (methanol), 2,4-DTBP, and pepper CC soil. Solutions of 2,4-DTBP, water and methanol were sprayed onto the soil. Cucumber (*Cucumis sativus* L. cv. holland No. 83-16) seeds were germinated in perlite. Ten-day seedlings with uniform growth vigor were selected for all treatments, and there were three replicates per treatment. The content of 2,4-DTBP in the soil was 23.6 mg·kg^{-1}. During the experiment, the air temperature was maintained at 25±2°C, and the relative humidity was between 50% and 70%. The plant height was measured at 3-day intervals. The experiment ended 25 days after inoculation with the 2,4-DTBP solution.

Four-week-old pepper seedlings were selected to test the effects of 2,4-DTBP on pepper. The same four treatments used for cucumber were established. After 3 weeks, the physiological indexes of pepper were measured.

Assay of antioxidant enzyme activity and malondialdehyde (MDA) conten *t*

Following the method of Li et al. (2011), 0.2 g leaves were selected and ground with 2 mL of cold PBS buffer (pH 7.8). After centrifugation at 4°C and 10,000 *g* for 20 min, the supernatant was extracted to determine the activity of superoxide dismutase (SOD). The reaction mixture was composed of 2.7 mL of 14.5 mM methionine, 90 μL of 50 mM phosphate buffer (pH 7.8), 10 μL of 20 μM riboflavin, 10 μL of 2.25 mM nitro blue tetrazolium chloride and 30 μL of enzyme supernatant, and the SOD activity was determined at 560 nm.

For the measurement of MDA, 0.2 g of leaf tissue was homogenized in 1.5 mL of 5% trichloroacetic acid (TCA) after being ground with liquid nitrogen. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. 1-mL aliquot of the supernatant was mixed with 2 mL of 0.6% thiobarbituric acid and heated at 95°C for 30 min. After being quickly cooled in an ice bath, the absorbance of the supernatant was recorded at 450, 532 and 600 nm (Dhindsa et al., 1981).

Assay of O_2^- and H_2O_2 formation rates

Following the method of Elstner and Heupel (1976), 0.2 g of leaves ground with liquid nitrogen were homogenized in 65 mM phosphate buffer (pH 7.8, 3 mL) and centrifuged at 4degC and 5,000 g for 10 min. The resultant supernatant (1 mL) was mixed with 0.1 mL of 10 mM hydroxylamine chlorhydrate and 65 mM phosphate buffer (0.9 mL) for 20 min at 25degC. Sulfanilamide (17 mM, 0.5 mL) and alpha-naphthylamine (7 mM, 0.5 mL) were added to the mixture (0.5 mL). After 20 min at 25degC, 3 mL of ethyl ether was added to the mixture for centrifugation at 4degC and 2,000 g for 15 min, and the absorbance was measured at 530 nm.

For the H_2O_2 content assay, 2 g of leaves was ground in liquid nitrogen together with 3 mL of acetone and centrifuged at 4degC for 20 min (15,000 x g). One milliliter of supernatant was mixed with 5% titanium sulfate (2.5 mL) and ammonium hydroxide (0.2 mL). After precipitation formation, the mixture was centrifuged at 5,000g for 20 min, and the supernatant was discarded. Five milliliters of 1 M sulfuric acid was added to stop the reaction. The absorbance was measured at 415 nm, and the formation rate of H_2O_2 was calculated from a standard curve of H_2O_2reagent.

Gas exchange measurements

The net photosynthetic rate (P_{net}), intercellular CO_2 concentration (C_i), leaf transpiration (T_r) and stomatal conductance (G_s) were measured after 15 days of treatment using a portable photosynthesis measurement system (CIRAS-1, PP System Company, UK). The CIRAS-1 homemade light source and a CO_2 cylinder were used to determine the gas exchange parameters. The light intensity was 800 μmol·m^{-2}·s^{-1}, and the concentration of CO_2 in the fixed system was μL·L^{-1}.

Root activity

For the root activity measurements, 0.2 g of root tip tissue was homogenized in a 10-mL mixture of 0.4% 2,3,5-triphenyltetrazolium chloride (TTC) and phosphate buffer (pH 7.8). The mixture was placed at 37°C in the dark for 3 h. Then, 2 mL of sulfuric acid was added to the mixture to terminate the reaction.

The grinding fluid was poured into a test tube. The residue was washed three times with a small amount of ethyl acetate. Finally, the residue was combined with 10 mL of ethyl acetate. The absorbance was measured three times at 485 nm. The TTC reduction amounts were calculated from a standard curve according to the formula:root activity = $\frac{TTC\ reduction\ quantity(\mu\gamma)}{}$

englishroot weigt$(g) \times time(H)$

The roots of the plants involved in the chlorophyll fluorescence assay were washed with distilled water to remove any impurities, and their morphology was characterized under a microscope (Nikon, TE2000).

Statistical analysis

Three different sets of lettuce seeds exposed to different concentrations of potential allelochemicals were used in the germination experiments. Statistical analyses were performed using the SPSS 20.0 statistical software program. Data were assayed in triplicate and expressed as the means ± standard errors. Differences were analyzed by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test. Differences were considered very significant at$p$ <0.05.

Results

Analysis of potential allelochemicals in pepper rhizosphere soil

In this study, we designed an in situ sampler, which is shown in Fig. S1. By the use of this in situ sampler, which relies on capillary action, rhizosphere chemicals were continuously collected without disturbing the plant roots. Twenty-eight organic compounds were detected in the pepper rhizosphere soils by GC-MS analysis, including 16 esters, 4 olefins, 2 phenols, 2 hydrocarbons, 1 aromatic, 1 alcohol, 1 ketone and 1 hydrazine (Table 1). Among them, their matching values to 14 standard chemical substances, including γ-sitosterol, DIBA, and tridecane, were higher than 90%. Most of these compounds were identified as the main allelochemicals in root exudates in previous reports (Shui et al., 2010; Sun et al., 2013). The results showed that it is feasible to use the in situ sampler developed in this study for the qualitative analysis of root exudates.

An in situ sampler in combination with GC-MS was used to quantitatively analyze the main chemical substances in pepper rhizosphere soil at different growth stages in one growing season. 2,4-DTBP, DIBA, DBP and limonene were the four most predominant substances throughout the whole growing season (Fig. 1). The accumulation of 2,4-DTBP with pepper growth was the most significant of all the compounds identified.

To test the effects of in situ sampling, we carried out reconfirmation experiments using a direct extraction method from rhizosphere soil. The results showed that the content of 2,4-DTBP in the soil of Y1 and the measured values (23.6 $mg \cdot kg^{-1}$; 22.4 $mg \cdot kg^{-1}$) from in situ sampling and direct extraction were not significantly different ($p > 0.05$). For the same pepper CC soil, the values (in situ sampling: 153.0 $mg \cdot kg^{-1}$; soil: 161.19 $mg \cdot kg^{-1}$) were also not significantly different ($p > 0.05$).

Effects of 2,4-DTBP on lettuce seed germination and growth

According to the GC-MS results, 2,4-DTBP, DIBA, DBP and limonene were the four main compounds in the rhizosphere. To test the allelopathy of 2,4-DTBP, we mixed these four substances and performed experiments with 0-50 $mg \cdot L^{-1}$ concentrations of 2,4-DTBP and fixed concentrations of 15 $mg \cdot L^{-1}$ DIBA, DBP and limonene. The germination of lettuce seeds was significantly promoted ($p < 0.05$) at low concentrations (<50 $mg \cdot L^{-1}$) (Fig. S3). To further clarify the allelopathic threshold, six concentrations of 0, 200, 300, 500, 800 and 1,000 $mg \cdot L^{-1}$ 2,4-DTBP were established. When the concentration of 2,4-DTBP was more than 300 $mg \cdot L^{-1}$, the germination time of lettuce seeds was significantly prolonged, and the growth of the radicle and hypocotyl was also significantly inhibited (Table 2) ($p < 0.05$). The germination rate of lettuce seeds decreased by 56.7%, 68.2% and 63.6% at 2,4-DTBP concentrations of 500, 800 and 1,000 $mg \cdot L^{-1}$, respectively, compared with that under no 2,4-DTBP exposure. The results showed that 2,4-DTBP at concentrations above 300 $mg \cdot L^{-1}$ had a significant inhibitory effect on seed germination.

Effects of pepper CC soil and 2,4-DTBP on the physiological and biochemical properties of cucumber and pepper leaves

To test the effect of 2,4-DTBP on plant growth, cucumber was first selected as the test object. Four treatments, namely, a no-addition control (CK), a methanol solvent-addition control (Me-Ck), a 2,4-DTBP treatment (2,4-DTBP) and a pepper CC soil treatment (pepper soil), were established. The 2,4-DTBP (23.6 $mg \cdot kg^{-1}$) and pepper CC soil (2,4-DTBP:153.0 $mg \cdot kg^{-1}$) treatments both significantly inhibited the growth of cucumber plants ($p < 0.05$). Compared with that in the CK treatment, the plant heights of cucumber in the 2,4-DTBP and pepper soil treatments were 34 cm and 45 cm lower, respectively. The growth rate and leaf area of cucumber treated with 2,4-DTBP and pepper soil were significantly lower than those of the corresponding control groups (Fig. 2 and Fig. 3) ($p < 0.05$). In particular, the growth rate of plants in the pepper soil was only 1.57 $cm \cdot d^{-1}$, which was 41.9% of that in the control group, and the leaf area of plants in the pepper soil was only 17.6% (17.5 cm^2) of the leaf area of the CK plants. The root activity in the pepper soil was significantly different from that in the other treatments. Compared with CK, the root activity of the cucumber in the pepper soil treatment was 55.2% lower. 2,4-DTBP decreased root activity by 19% compared with CK, but the difference was not significant. Based on the results for root activity, we further used fluorescence imaging to assess the root damage. The results showed that the fluorescence intensities for the CK, Me-CK, 2,4-DTBP and pepper soil treatments were 19.16, 21.61, 30.32, and 33.93 a.u., respectively.

The fluorescence intensities of 2,4-DTBP and pepper soil were significantly higher than those of the two control groups (Fig. 4) (p <0.05). The root activity of pepper decreased with continuous cropping years and showed significant differences between the treatments. Compared with CK, the root activity of Y1, Y2 and Y3 was 1.49, 0.93 and 0.28 mg TTF·g^{-1}·h^{-1}, respectively. The root fluorescence intensities resulting from the Y1, Y2 and Y3 treatments were 14.53, 19.94 and 24.00 a.u., respectively, which were significantly higher than those in CK (6.94 a.u.) (Fig. 5). In this study, there were no significant differences in root activity and root fluorescence intensity between CK and Me+CK.

Compared with those in CK and Me-CK, the SOD activity and MDA content in cucumber leaves grown in pepper soil significantly increased after 15 days of treatment, but the SOD activity and MDA content in cucumber leaves in the 2,4-DTBP treatment did not increase significantly (p <0.05). These results indicate that the damage to the cucumber leaf membrane was more serious in the pepper CC soil than in the 2,4-DTBP treatment. The 2,4-DTBP and pepper soil treatments significantly increased the level of reactive oxygen species (ROS) in the soil, i.e., the contents of H_2O_2 and O_2^-(p <0.05). The content of O_2^- was significantly different between the two treatments (Table 3). Compared with that in CK, the 2,4-DTBP and pepper soil had significant effects on leafP_{net} , decreasing it by 18.6% and 41%, respectively (p <0.05). 2,4-DTBP and pepper soil significantly reduced the C_i(p <0.05). The T_r andG_s in the 2,4-DTBP treatment were reduced by 36.4% and 33.3%, respectively. The T_r andG_s in the 2,4-DTBP treatment decreased by 50.8% and 53.3%, respectively (Table 4).

The results from pepper showed that the activity of SOD and the content of MDA and O_2^{*-} in pepper significantly increased when grown in CC soil for different years. The ROS level of pepper leaves was significantly increased (Table 3). TheP_{net} and WUE of pepper leaves were significantly affected by pepper CC years. The P_{net} values of Y1, Y2 and Y3 were 4.9, 0.91 and 0.82 μmol·m^{-2}·s^{-1}, and the WUE values of Y1, Y2 and Y3 were 3.12, 1.74 and 1.53, respectively. TheG_s of Y2 and Y3 were 22.39 and 22.10 mmol·m^{-2}·s^{-1}, and theT_r *of* Y2 and Y3 were 0.52 and 0.53 mmol·m^{-2}·s^{-1}, respectively. The CC years also had significant effects on the intracellular CO_2 concentration (C_i), which increased with the increase in CC years (Table 4). In general, with the increase in CC years, the photosynthesis of pepper was affected more significantly. The CC of pepper has a strong physiological toxic effect on itself. Compared with the allelopathic effect of CC of pepper on cucumber, the allelopathic effect of pepper was more significant.

Discussion

In situ sampling for the detection of possible allelochemicals in pepper rhizosphere soil

Considering the biological specificity of plant root structures and the spatial diversity of soil structures, the continuous, noninvasive extraction of plant root exudates under natural conditions is an urgent technical problem and a challenge for allelopathy research. Currently, most studies on root exudates are carried out by extracting the hydroponic solution from around the root or by indirectly converting the crude extract into an aqueous solution for analysis. Because these techniques separate plants from the soil environment, root growth, root cell activity and other physiological characteristics may change. When root metabolism changes, the natural compounds secreted by plant roots also change (Lucas Garcia et al., 2001; Canarini et al., 2016).

In this study, our in situ sampler was placed at the same depth as the roots of the plants. The tube wall came into close contact with the root system without causing physical damage to the root system in the collection process. Meanwhile, the capillary action of the tube wall naturally brought the root exudates from the soil into the tube without any external force. The in situ sampler used in this study collected the allelochemicals secreted by the soil roots without disturbance. After sampling with the in situ samplers, the samples were analyzed qualitatively and quantitatively. We also compared the concentrations of the exudates collected by the in situ sampler with the concentrations of exudates obtained through direct soil extraction analysis. The results showed that the differences in the concentrations of 2,4-DTBP between the samples from the in situ sampler and direct soil extraction were not significant. The results of the reconfirmation experiment with direct extraction and in situ sampling show that the in situ sampler could be suitable for sample collection.

This result illustrated that the in situ sampling technique could reliably and accurately reflect the actual concentration of allelochemicals from the plant in the soil (Holz et al., 2018; Oburger & Jones, 2018). The in situ sampler developed in our study is potentially suitable for noninvasive dynamic detection and analysis of root exudates.

Potential allelochemical analyses in pepper rhizosphere soil

By in situ sampling and GC-MS analysis of the pepper root exudates at different growth stages, twenty-eight potential allelochemicals were identified in the pepper root exudates. Most of them have been detected in a variety of plants. For example, dibutyl phthalate, isooctyl phthalate, 2,4-DTBP, benzyl benzoate, DBP, adipic acid, and diisobutyl ester were identified in the root soil of eggplant, garlic/eggplant CC or *Bidens pilosa* (Zhou et al., 2011, Wang et al., 2014, Shen et al. 2018). In this study, through the combination of in situ sampling and GC-MS analysis, 2,4-DTBP was identified in pepper soil (Fig. 1). The accumulation of 2,4-DTBP gradually increased as the pepper plants grew. The accumulation of allelochemicals is closely related to CC obstacles (Agegnehu et al., 2016; Chen et al., 2020). Through seed germination tests and plant growth tests with sensitive plants, we deduced that 2,4-DTBP is the main allelochemical substance in pepper rhizosphere soil. To the best of our knowledge, this is the first report that 2,4-DTBP is one of the main allelochemicals in pepper. According to previous reports, 2,4-DTBP has a wide range of biological functions. For example, 2,4-DTBP promotes senescence in human gastric adenocarcinoma cells by inhibiting the activity of the HDAC6 enzyme, induces mitotic mutations, produces polynuclear cells, increases the proportion of polymerized tubulin and has anticancer activity (Song et al., 2018). 2,4-DTBP is also an excellent antibacterial agent that is used as a biological fumigant to prevent and control diseases caused by*Botrytis cinerea* and *Rhizopus stolonifer* (Zhang et al., 2020). 2,4-DTBP can change the cell membrane properties of*Streptococcus pyogenes* and increase the sensitivity of *S. pyogenes* to antibiotics. In addition, 2,4-DTBP can be used in combination with antibiotics to achieve antibacterial efficacy and is considered a nonbactericidal and nontoxic drug candidate (Viszwapriya et al., 2016). In this study, we found that 2,4-DTBP is one of the main allelochemicals in pepper. The results of this study could aid in advancing functional research on 2,4-DTBP and solving the CC problem.

When the 2,4-DTBP concentration exceeded 300 $mg{\cdot}L^{-1}$, 2,4-DTBP significantly affected the rate and time of germination and the length of the radicle and hypocotyl of lettuce seeds. However, when the 2,4-DTBP concentration was below 50 $mg{\cdot}L^{-1}$, 2,4-DTBP significantly promoted the germination of lettuce seeds. This typical allelopathic phenomenon in 2,4-DTBP, i.e., high-concentration inhibition and low-concentration promotion, is similar to the results of previous studies on various allelochemicals. For example, Wang et al. (2007) found that low concentrations of cinnamic acid promoted the growth of cucumber seedlings under saline-alkali conditions, while high concentrations of cinnamic acid inhibited the growth of cucumber seedlings.

The effect of pepper CC soil and 2,4-DTBP on the physiological growth of cucumber and pepper

Phenolic compounds are very important and common allelochemicals in the ecosystem and generally exhibit obvious allelopathy. Ye et al. (2006) found that the oxidative stress caused by cinnamic acid had a significant inhibitory effect on cucumber growth and leaf area and increased the incidence of cucumber wilt. Huang et al. (2020) showed that the main phenolic substance (p-hydroxybenzoic acid (pHBA)) in the soil after three consecutive years of cucumber cropping reduced the activity of root meristems, thereby inhibiting the growth of cucumber roots. Most studies have suggested that allelochemicals have inhibitory effects on plant growth (Maberly, 2014; Asaduzzaman et al., 2016; Hussain and Reigosa, 2015). CC obstacles are an important ecological factor restricting vegetable production. The measures to solve or reduce the negative effects caused by CC are rotation or intercropping of different varieties of vegetables. As the main vegetable product, cucumber is usually intercropped with pepper (Dong et al, 1994; Zhao et al, 2016). The pepper CC soil and 2,4-DTBP had significant inhibitory effects on cucumber growth (Fig. 3). In particular, the cucumber plant height, leaf area and root vitality in the continuous pepper cropping soil treatment were significantly reduced compared with those in the CK treatment. The inhibitory effect of pepper CC soil was

the most significant in this study, indicating that this inhibitory effect was not caused only by the individual effect of 2,4-DTBP.

ROS, mainly H_2O_2 and O_2^-, are byproducts of many metabolic processes and play important signaling roles in all organisms (Asada, 2006). ROS are involved in many biological processes, such as plant system resistance, stomatal closure, programmed cell death, germination and root development. Allelochemicals can induce excessive ROS production (Xu et al., 2008). To reduce the damaging effects of ROS, plants have evolved enzymatic antioxidants, such as SOD. In many crops, such as tomato, kidney bean and green algae, environmental stress has been found to stimulate SOD activity (Zlatev et al., 2006; Hayat et al., 2018; Singh et al., 2018). The accumulation of membrane lipid peroxidation products (i.e., MDA) and ROS in plant tissues indicates that the permeability and integrity of plant cell membranes have been mostly destroyed (Sidhu et al., 2016). In plant cells, the oxidative effect of allelochemicals can increase the production of ROS, leading to the inhibition of photosynthesis (Garcia-Sanchez et al. 2012; Das & Roychoudhury, 2014). In this study, the pepper CC soil and 2,4-DTBP treatments both increased the ROS levels in the cucumber leaves. Similarly, these two treatments significantly reduced the net photosynthetic rate, transpiration rate, stomatal conductance, and intracellular CO_2 concentration in the plants and inhibited the photoconductive effect (Table 4). In particular, the decrease in stomatal conductance was more pronounced than the other effects, indicating that the closure of stomata may be an important factor leading to the decrease in photosynthesis and transpiration (Moles et al., 2016). The accumulation of ROS in cells under allelochemical stress can lead to pigment loss, reduced photosynthetic CO_2 assimilation, and decreases in protein and RNA levels (Rao et al., 2006). Therefore, the significant inhibitory effect of pepper CC soil and 2,4-DTBP on cucumber growth may be the result of diminished photosynthesis (Zhu et al., 2017). The SOD activity and MDA and ROS contents of the pepper CC soil treatment were significantly higher than those of the other treatments, indicating that the cell membrane structure of the cucumber leaves was more severely damaged, which in turn affected the photosynthesis and physiological growth of the cucumbers more significantly.

The mechanisms of CC obstacles are very complicated, but the accumulation of allelochemicals in the soil is certainly the main causative factor (Ahammeda et al., 2012; Huang et al., 2020). Many studies have found that CC leads to the continuous accumulation of phenols, which change the properties of the soil and have significant inhibitory effects on plant growth (Asao et al., 2008; Halvorson et al., 2009). In this study, the 2,4-DTBP content of the pepper CC soil was much higher than that of the unplanted soil and higher than that of other substances in the pepper CC soil. Experiments with pepper CC soil showed that the allelopathy of pepper CC was very obvious (Fig. S2). The self-toxicity of pepper CC soil is an inevitable phenomenon; therefore, we used cucumber, a common crop-rotation crop, for allelopathy tests involving CC obstacles. Then, we used pepper to test this effect. The results showed that the CC of pepper had a significant effect on the growth, photosynthesis and other physiological indicators of both cucumber and pepper. Thus, we deduce that the accumulation of 2,4-DTBP is an important obstacle to the CC of pepper.

Conclusion

Plant root exudates are closely related to CC obstacles, and there are many challenges in analyzing the components of root exudates accurately and in real time. In this study, we developed a new nondisturbing in situ sampler based on the capillary principle for the extraction and analysis of pepper root exudates. The sampler effectively collected possible allelochemicals without damaging the roots in the soil. The quantitative analysis of 2,4-DTBP showed that the content of 2,4-DTBP in the in situ sampler was close to that in the soil, indicating that the new sampling method could truly reflect the contents of the chemical substances secreted by roots into the soil. 2,4-DTBP had a typical allelopathic effect on seed germination. The growth, photosynthesis and root activity of cucumber were significantly affected by the 2,4-DTBP and pepper CC soil treatments. The 2,4-DTBP and pepper CC soil treatments significantly increased the activities of antioxidant enzymes and the contents of MDA and ROS in plant leaves. The results revealed that 2,4-DTBP is an important allelochemical in pepper CC soil. The accumulation of 2,4-DTBP may be a significant factor in CC obstacles.

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References

Agegnehu, G., Nelson, P.N., Bird, M.I. (2016) The effects of biochar, compost and their mixture and nitrogen fertilizer on yield and nitrogen use efficiency of barley grown on a Nitisol in the highlands of Ethiopia. *Science of the Total Environment* , 569-570, 869-879.

Asaduzzaman, M., Min, A., Pratley, J.E., Luckett, D.J., Lemerle, D., Coombes., N.(2016) The seedling root response of annual ryegrass (*Lolium rigidum*) to neighbouring seedlings of a highly-allelopathic canola (*Brassica napus*). *Flora* 219, 18-24.

Asada, K. (2006) Production and scavenging of ractive oxygen species in chloroplasts and their Functions. *Plant Physiology* ,141, 391-396.

Ahammeda, G.J., Wang, M.M., Zhou, Y.H., Xia, X.J.,Mao, W.H., Shi. K., Yu J.Q. (2012) The growth, photosynthesis and antioxidant defense responses of five vegetable crops to phenanthrene stress. *Ecotoxicology and Environmental Safety* , 80, 132-139.

Asao, T., Kitazawa, H., Ban, T., Pramanik, M.H.R., Tokumasa, K. (2008) Electrodegradation of root exudates to mitigate autotoxicity in hydroponically grown strawberry (*Fragaria* x *ananassa Duch.*) plants. *International Urology and Nephrology* , 43 (7), 2034-2038.

Batish, D.R., Setia, N., Singh, H.P., Kohli, R.K. (2004) Phytotoxicity of lemon-scented eucalypt oil and its potential use as a bioherbicide.*Crop Protection* , 23, 1209-1214.

Batish, D.R., Lavanya, K., Singh, H.P., Kohli, R.K. (2007) Root-mediated Allelopathic Interference of Nettle-leaved Goosefoot (*Chenopodiummurale*) on Wheat (*Triticum aestivum*). J. Agron. *Crop Science* , 193(1), 37-44.

Chen, P., Wang, Y.Z., Liu, Q.Z., Zhang, Y.T., Li., W.H. (2020) Phase changes of continuous cropping obstacles in strawberry (*fragaria*x *ananassa* Duch.) production. Appl Soil Ecology, 155, 103626.

Dong, L.L., Xu, J., Li Y., Fang H.L., Niu W.H., Li X.W., Zhang Y.J., Ding W.L., Chen S.L. (2018) Manipulation of microbial community in the rhizosphere alleviates the replanting issues in *Panax ginseng* . *Soil Biology and Biochemistry* , 125, 64-74.

Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A. (1981) Leaf senescence: correlated with increase leaves of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase.*Journal of Experimental Botany* , 32, 93-101.

Das, K., Roychoudhury, A. (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science* , 2, 53.

Elstner, E.F., Heupel, A. (1976) Inhibition of nitrite formation from hydroxyl ammoniumchloride: a simple assay for superoxide dismutase.*Analytical Biochemistry* , 70, 616-20.

FAO (Food and Agriculture Organization of the United Nations), (2019) FAOSTAT Database-Resources. Food and Agriculture Organization of the United Nations. FAO Statistical Yearbook 2019: World Food and Agriculture, Rome.

Garcia-Sanchez, M., Garrido, I., Casimiro, I.D.J., Casero, P.J., Espinosa, F.,Garcia-Romera, I., Aranda, E. (2012) Defence response of tomato seedlings to oxidative stress induced by phenolic compounds from dry olive mill residue. *Chemosphere* , 89, 708-716.

Haase, S., Neumann, G., Kania, A., Kuzyakov, Y., Roemheld, V., Kandeler, E. (2007) Elevation of atmospheric CO_2 and N-nutritional status modify nodulation, nodulecarbon supply, and root exudation of Phaseolus vulgaris L. *Soil Biology and Biochemistry* , 39, 2208-2221.

Huang, C.Z., Lei, X.U., Jin-Jing, S., Zhang, Z.H., Ke-Ke, Y.I. (2020) Allelochemical p-hydroxybenzoic acid inhibits root growth via regulating ROS accumulation in cucumber (*Cucumis sativus* L.). *Journal of Integrative Agriculture* , 19(2), 518-527.

Hussain, M.I., Reigosa, M.J. (2015) Characterization of xanthophyll pigments, photosynthetic performance, photon energy dissipation, reactive oxygen species generation and carbon isotope discrimination during artemisinin-induced stress in Arabidopsis thaliana. *PLoS One* , 10, e0114826.

Hayat, S., Ahmad, H., Ali, M., Ren, K., Cheng, Z. (2018)Aqueous garlic extract stimulates growth and antioxidant enzymes activity of tomato (*Solanum lycopersicum*). *Scientia Horticulturae* , 240, 139-146.

Halvorson, J.J., Gonzalez, J.M., Hagerman, A.E., Smith, J.L. (2009) Sorption of tannin and related phenolic compounds and effects on soluble-N in soil. *Soil Biology and Biochemistry* , 41(9), 2002–2010.

Inderjit. (2006) Experimental complexities in evaluating the allelopathic activities in laboratory bioassays: a case study.*Soil Biology and Biochemistry* , 38, 256-262.

Khan, M.A., Cheng, Z.H., Khan, A.R., Rana, S.J., Ghazanfar, B. (2015) Pepper-garlic intercropping system improves soil biology and nutrient status in plastic tunnel. *International Journal of Agricultural and Biological Engineering* , 17, 869-880.

Li, Q., Yu, B., Gao, Y., Dai, A.H., Bai, J.G. (2011) Cinnamic acid pretreatment mitigates chilling stress of cucumber leaves through altering antioxidant enzyme activity. *Journal of Plant Physiology* , 168, 927-934.

Li, W., Liu, Q. (2018) Changes in fungal community and diversity in strawberry rhizosphere soil after twelve years in the greenhouse.*Journal of Integrative Agriculture* , 18 (3), 677-687.

Li, X.Y., Liu, Q.Z., Wang, Y.Z., Sun, H.Y., Bai, C.Q., Lewis, E.E. (2015a) Different changes of soil nematode communities in replant and continuous-planting peach orchards and their indicative value for peach replant problem. *Helminthologia* , 52 (3), 261-269.

Li, H.Q., Zhang, L.L., Jiang, X.W., Liu, Q.Z. (2015b) Allelopathic effects of phenolic acids on the growth and physiological characteristics of strawberry plants. *Allelopath Journal* , 35 (1), 61-75.

Li, W., Liu, Q., Chen, P. (2018) Effect of long-term continuous cropping of strawberry on soil bacterial community structure and diversity.*Journal of Integrative Agriculture* , 17 (11), 2570-2582.

Maberly, S.C. (2014) The fitness of the environments of air and water for photosynthesis, growth, reproduction and dispersal of photoautotrophs: An evolutionary and biogeochemical perspective.*Aquatic Botany* , 118, 4-13.

Moles, T.M., Pompeiano, A., Huarancca, R.T., Scartazza, A., Guglielminetti, L. (2016) The efficient physiological strategy of a tomato landrace in response to short-term salinity stress. *Plant Physiology and Biochemistry* , 109, 262-272.

Oburger, E., Dell'mour, M., Hann, S., Wieshammer, G., Puschenreiter, M., Wenzel, W.W. (2013) Evaluation of a novel tool for sampling root exudates from soil-grown plants compared to conventional techniques.*Environmental Experimental Botany* , 87, 235-247.

Oburger, E., Jones, D.L. (2018) Sampling root exudates-Mission impossible? *Rhizosphere* , 6, 116-133.

Proctor, C., He Y.H. (2017) Quantifying root extracts and exudates of sedge and shrub in relation to root morphology. *Soil Biology and Biochemistry* , 114, 168-180.

Rao, K.V.M., Raghavendra, A.S., Reddy, K.J. (2006) Physiology and Molecular Biology of Stress Tolerance in Plants. Springer.

Sobolev, V.S., Potter, T.L., Horn, B.W. (2006) Prenylated stilbenes from peanut root mucilage. *Phytochemical Analysis* , 17, 312-322.

Shi, S., Condron, L., Larsen, S., Richardson, A.E., Jones, E., Jiao, J., O'Callaghan, M., Stewart, A. (2011) In situ sampling of low molecular weight organic anions from rhizosphere of radiata pine (*Pinus radiata*) grown in a rhizotron system. *Environmental Experimental Botany* , 70, 131-142.

Shui, J., An, Y., Ma, Y., Ichizen, N. (2010) Allelopathic potential of switchgrass (*panicum virgatum* L.) on perennial ryegrass (*lolium perenne* L.) and alfalfa (*medicago sativa* L.).*Environmental Management* , 46(4), 590-598.

Sun, H.Y.; Wang, Y. (2013) Hollow fiber liquid-phase microextraction with in situ derivatization combined with gas chromatography-mass spectrometry for the determination of root exudate phenyl-amine compounds in hot pepper (*Capsicum annuum* L.). *Journal of agricultural and food chemistry* , 61, 5494-5499.

Shen, Y., Sun, Z., Shi, P., Wang, G., Wu, Y., Li, S. (2018) Anticancer effect of petroleum ether extract from bidens pilosal and its constituent's analysis by GC-MS. *Journal of Ethnopharmacology* , 217, 126-133.

Song, Y.W., Lim, Y., Cho, S.K. (2018) 2, 4-Di-tert-butylphenol, a potential HDAC6 inhibitor, induces senescence and mitotic catastrophe in human gastric adenocarcinoma AGS cells. *Biochimica Et Biophysica Acta-molecular Cell Research* , 1865, 675-683.

Singh, R., Upadhyay, A.K., Chandra, P., Singh, D.P. (2018) Sodium chloride incites reactive oxygen species in green algae*chlorococcum humicola* and *chlorella vulgaris* : implication on lipid synthesis, mineral nutrients and antioxidant system.*Bioresource Technology* , 270, 489-497.

Sidhu, G.P., Singh, H.P., Batish, D.R., Kohli, R.K. (2016) Effect of lead on oxidative status, antioxidative response and metal accumulation in Coronopus didymus. *Plant Physiology and Biochemistry* , 105, 290-296.

Verbon, E.H., Liberman, L.M. (2016) Beneficial microbes affect endogenous mechanisms controlling root development. *Trends in Plant Science* , 21, 218-229.

Viszwapriya, D., Prithika, U., Deebika, S., Balamurugan, K., Pandian, S.K. (2016) *In vitro* and in *vivo* antibiofilm potential of 2,4-di-tert-butylphenol from seaweed surface associated bacterium*bacillus subtilis* against group a streptococcus.*Microbiological Research* ,191, 19-31.

Weiss, E.A., (2002) World Production and Trade. CABI Publishing, CAB International, Wallingford, UK.

Wang, M.Y., Wu, C.N., Cheng, Z.H., Meng, H.W., Zhang, M.R., Zhang, H.J. (2014) Soil Chemical Property Changes in Eggplant/Garlic Relay Intercropping Systems under Continuous Cropping.*P LoS ONE* , 2014, 9, e111040.

Wang, Y., Ren, W., Li, Y., Xu, Y., Teng, Y., Christie P., Luo Y. (2019) Nontargeted metabolomic analysis to unravel the impact of di (2-ethylhexyl) phthalate stress on root exudates of alfalfa (*Medicago sativa*). *Science of the Total Environment* , 646, 212-219.

Wang, X., Wang, H., Wu, F., Liu, B. (2007) Effects of cinnamic acid on the physiological characteristics of cucumber seedlings under salt stress. *Frontiers of Agriculture in China* , 1, 58-61.

Wu, F., Wang, X., Xue, C., 2009. Effect of cinnamic acid on soil microbial characteristics in the cucumber rhizosphere. European Journal of Soil Biology, 45 (4), 356-362.

Xu, C., Sullivan, J. H., Garrett, W. M., Caperna, T. J., and Natarajan, S. 2008. Impact of solar Ultraviolet-B on proteome in soybean lines differing in flavonoid contents. *Phytochemistry* , 69, 38-48.

Yao, C.Z., Jiang, Y.T., Yang Y.T., Ma Y.Y. , Pan, L.L. ,Li,Y.L. ,Sun Y.Q., Chen J.,Zhao, J. (2020) Allelopathic effect of extracts from Panax notoginseng mono-cropped soil on its root rot pathogens. *Chinese Journal of Applied Ecology* , 31(7) : 2227-2235.

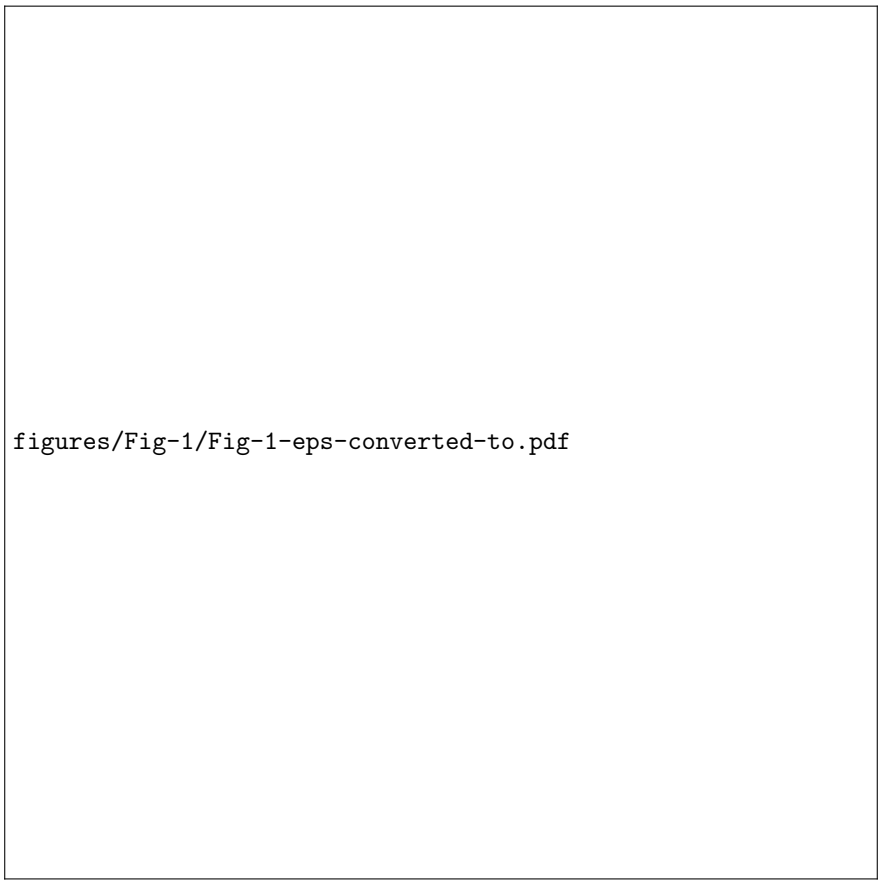
Ye, S.F., Zhou, Y.H., Sun, Y., Zou, L.Y., Yu, J.Q. (2006) Cinnamic acid causes oxidative stress in cucumber roots, and promotes incidence of fusarium wilt. *Environmental Experimental Botany* , 56(3), 255-262.

Zhou, B.L., Chen, Z.X., Du, L., Xie, Y.H., Ye, X.L. (2011) Allelopathy of the root exudates from different resistant eggplants to*verticillium* wilt (*Verticillium dahliae* Kleb). *Acta Ecologica Sinica* -China, 31, 3964-3972.

Zhang, X.Y., Gao, Z.F., Zhang, X.X., Bai W.B., Zhang L.X., Pei H.B., Zhang Y.J. (2020) Control effects of *Bacillus siamensis* G-3 volatile compounds on raspberry postharvest diseases caused by*Botrytis cinerea* and *Rhizopus stolonifer* . *Biological Control* , 141, 104135.

Zlatev, Z.S., Lidon, F.C., Ramalho, J.C., Yordanov, I.T. (2006) Comparison of resistance to drought of three bean cultivars. *Plant Biology* , 50, 389-394.

Zhu, W., Liu, J.W., Ye, J.L., Li, G.H. (2017) Effects of phytotoxic extracts from peach root bark and benzoic acid on peach seedlings growth, photosynthesis, antioxidance and ultrastructure properties.*Scientia Horticulturae* , 215, 49-58.



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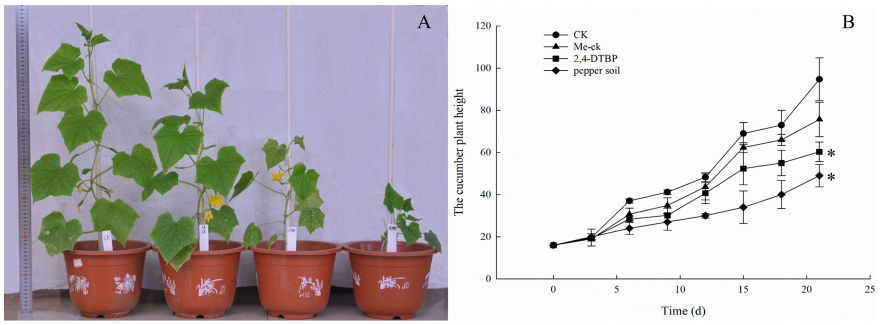

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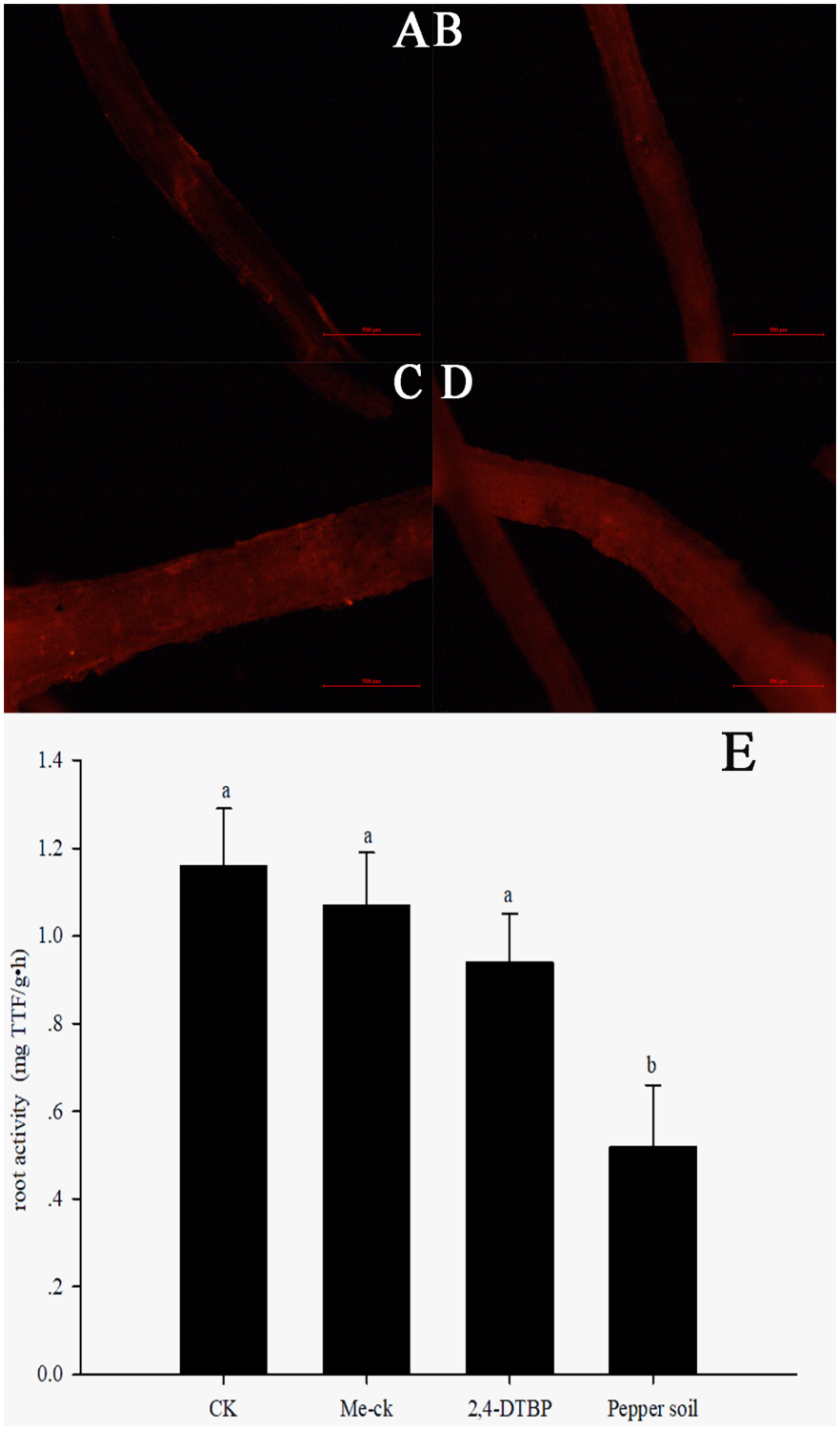

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