Types of Autotoxic Substances Secreted by Tobacco Seedling Roots and Effects of PAEs on Root Antioxidant Capacity (Postprint)

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Abstract

GC-MS was employed to identify root exudates of hydroponically grown tobacco varieties Burley and K326 at different growth stages during the seedling period; potted tobacco seedlings were also irrigated with solutions of dibutyl phthalate (DBP) and diisooctyl phthalate (DIOP) at various concentrations to investigate changes in root antioxidant capacity. The results were as follows: (1) Burley root exudates primarily contained three classes of compounds, among which the autotoxic substance phthalate esters (PAEs) exhibited relative contents of 7.6%, 0.3%, and not detected at the two-leaf, four-leaf, and six-leaf stages, respectively; whereas K326 root exudates primarily contained nine classes of compounds, with PAEs showing relative contents of 35.6%, 51.3%, and 2.2% at the two-leaf, four-leaf, and six-leaf stages, respectively. (2) PAEs at concentrations exceeding 0.1 mmol/L caused a significant ($P< 0.05$) increase in the production rate of superoxide anion radicals in roots; as DIOP and DBP concentrations increased, superoxide dismutase and catalase activities increased, reaching maxima at 0.5 mmol/L, then declining with further increases in treatment concentration. Malondialdehyde concentration increased with increasing treatment concentrations of these two PAEs. The results indicate that when autotoxic PAEs secreted by tobacco roots reach 0.5 mmol/L, they can reduce root antioxidant capacity, cause oxidative damage to the root tip cell membrane system, induce a series of physiological and biochemical changes such as root absorption function, and ultimately manifest autotoxic effects.

Full Text

Preamble

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Autotoxins Exuded from Roots and the Effects of PAEs on Antioxidant Capacity in Roots of Tobacco Seedlings

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Abstract

Root exudates from two tobacco (*Nicotiana tabacum*) varieties (Burley and K326) cultured in a hydroponic medium were collected and identified using gas chromatography-mass spectrometry (GC-MS). The effects of different concentrations of dibutyl phthalate (DBP) and diisooctyl phthalate (DIOP) on root antioxidant capacity were assessed using pot culture experiments. The results revealed three main compounds in Burley root exudates. The relative content of autotoxin phthalate esters (PAEs) at the two-, four-, and six-leaf stages were 7.6%, 0.3%, and not detected, respectively. However, nine main compounds were identified in K326 root exudates, with relative PAE contents of 35.6%, 51.3%, and 2.2% at the two-, four-, and six-leaf stages, respectively. PAEs such as DIOP and DBP were identified as the major autotoxins in root exudates of both tobacco varieties.

The rate of superoxide anion radical ($\cdot O_2^-$) generation in roots significantly increased at concentrations greater than 0.1 mmol/L DIOP and DBP ($P < 0.05$). Both superoxide dismutase (SOD) and catalase (CAT) activities increased with increasing DIOP concentrations, peaking at 0.5 mmol/L and subsequently decreasing at higher concentrations. Accumulated malondialdehyde (MDA) concentrations increased with increasing DIOP and DBP concentrations, and the magnitude of MDA content was DIOP > DBP, which indicated the order of their toxic effect. Finally, our findings also revealed that when PAEs reached 0.5 mmol/L, they decreased the antioxidant capacity of the root system, initiated oxidative damage of the root cell membrane system, and caused a further

decrease in root absorption and mineral metabolism, thereby leading to autotoxicity in tobacco plants.

Keywords: tobacco; root exudates; autotoxins; antioxidant enzyme activity

Introduction

Tobacco (*Nicotiana tabacum* L., Solanaceae) is an important economic crop in China. However, with decreasing arable land and the influence of economic factors on cropping systems, continuous tobacco cropping has become a serious problem. When certain substances secreted by tobacco roots accumulate in soil to a certain concentration, they become toxic to the crop itself, causing yield and quality decline—this is known as tobacco autotoxicity, the primary cause of continuous cropping obstacles. Autotoxicity directly harms normal plant growth and development, typically occurring at the seedling stage when plants are most vulnerable to autotoxic substances.

Previous research on plant autotoxicity has been limited. Jia et al. studied the effects of autotoxic substances from continuous tobacco cropping on seed vigor, root activity, seedling height, and biomass. Sun et al. investigated tomato autotoxins and their effects on growth. Liu et al. found that long-chain fatty acids in soil significantly inhibited peanut growth and soil enzyme activity when reaching certain concentrations. Guo et al. demonstrated that tobacco root exudates significantly reduced seedling root activity and nutrient uptake, with different components showing significantly different inhibitory effects, suggesting that tobacco root exudates contain multiple autotoxic substances. Yeasmin et al. cultured two asparagus varieties under different rotation systems and found that autotoxic substances such as succinic and tartaric acids differentially inhibited root and shoot growth and nutrient absorption.

Autotoxic substances are mainly released through volatilization, root exudation, and residue decomposition, with root exudates being a primary source. Cropping patterns and growth stages can affect the types and quantities of root exudates. However, the secretion characteristics of autotoxic substances at different seedling growth stages and the mechanisms of tobacco autotoxicity remain unclear. This study aimed to isolate and identify root exudates from two tobacco varieties at different seedling stages and investigate the effects of major autotoxic substances on superoxide anion generation rate, antioxidant enzyme activity, and lipid peroxidation in soil-cultured tobacco roots to elucidate the physiological and biochemical mechanisms of tobacco autotoxicity.

Materials and Methods

Plant Materials

Tobacco seeds of Burley and K326 varieties were provided by the Zhengzhou Tobacco Research Institute of China National Tobacco Corporation.

Collection of Root Exudates

Seeds were surface-sterilized and germinated on sterile filter paper. Uniform seedlings were pre-cultured in a glass culture dish containing agar-free MS medium (pH 5.70) with the following composition per liter: 1.65 g NH_4NO_3, 1.9 g KNO_3, 0.37 mg $MgSO_4 \cdot 7H_2O$, 0.17 g KH_2PO_4, 0.44 g $CaCl_2 \cdot 2H_2O$; 22.3 mg $MnSO_4 \cdot 4H_2O$, 0.83 mg KI, 0.025 mg $CuSO_4 \cdot 5H_2O$, 6.25 mg H_3BO_3, 0.025 mg $CoCl \cdot 6H_2O$, 8.65 mg $ZnSO_4 \cdot 7H_2O$, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$; 27.8 mg $FeSO_4 \cdot 7H_2O$, 37.3 mg Na_2EDTA. Each dish contained 20 seedlings and was placed in a growth chamber at 22–26 °C with 16 h light/8 h dark photoperiod.

When seedlings reached the two-, four-, and six-leaf stages, they were removed from the medium, rinsed, and placed in deionized water for 2 h to collect root exudates. The seedlings were then returned to fresh medium. This process was repeated three times per stage. The collected exudate solutions were combined and stored at 4 °C for analysis.

Sample Preparation for GC-MS Analysis

A 250 mL aliquot of root exudate solution was transferred to a separatory funnel and extracted three times with 50 mL dichloromethane for 30 min each. The organic phases were combined, passed through an anhydrous sodium sulfate column, and concentrated to dryness by rotary evaporation at 40 °C. The residue was reconstituted in 2 mL n-hexane and filtered through a 0.22 m membrane for GC-MS analysis.

GC-MS Analysis

GC-MS analysis was performed using a 450GC-320MS system (Bruker Daltonics Inc., USA) with a DB-5MS column (Agilent 19091S-433, HP-5MS, 5% Phenyl Methyl Siloxane, 30 m × 0.25 mm × 0.25 m). Operating conditions: injection temperature 250 °C; column flow 1.0 mL/min; splitless injection of 1 L; oven program: 130 °C for 4 min, ramped at 5 °C/min to 250 °C, held for 5 min, then ramped at 10 °C/min to 270 °C, held for 15 min; transfer line temperature 220 °C; ion source temperature 200 °C; interface temperature 250 °C; electron energy -70 eV; scan range m/z 50–500 amu; scan time 0.5 s. Compounds were identified by comparing mass spectra with Wiley 7n.L and NIST 05.L libraries, and relative contents were calculated using area normalization (JY/T003–1996).

PAE Treatment and Antioxidant Enzyme Assays

Tobacco seedlings were transplanted into pots (11 cm diameter, 1.5 L) containing soil from suburban Zhengzhou, Henan Province. Soil properties are shown in . Pots were placed in a greenhouse with temperature 25–35 °C, relative humidity 60–80%, and photosynthetically active radiation 320 $\mathrm{mol \cdot m^{-2} \cdot s^{-1}}$ during 16 h light period.

After establishment, seedlings were treated with 100 mL solutions of DBP or DIOP at concentrations of 0, 0.01, 0.1, 0.5, 1.0, and 5.0 mmol/L (based on concentrations detected in continuously cropped tobacco soils). Each treatment had three replicates. Roots were harvested 7 days after treatment for analysis.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the nitroblue tetrazolium method, expressed as U/g fresh weight. Catalase (CAT, EC 1.11.1.6) activity was determined by UV absorption at 240 nm. Malondialdehyde (MDA) content was measured by the thiobarbituric acid method. Superoxide anion ($\cdot O_2^-$) generation rate was determined by hydroxylamine oxidation.

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, USA). One-way ANOVA was used to analyze differences among treatments at $P < 0.05$ significance level.

Results

Identification of Root Exudates

GC-MS analysis identified different root exudate profiles between the two tobacco varieties at various growth stages. Burley root exudates contained three main compounds, while K326 contained nine main compounds. Phthalate esters (PAEs) were identified as major autotoxic substances in both varieties.

In Burley seedlings, the relative PAE content was 7.6% at the two-leaf stage, 0.3% at the four-leaf stage, and not detected at the six-leaf stage. The main PAEs included diisobutyl phthalate (DIBP), phthalic acid 3-hexyl isobutyl ester, and phthalic acid isobutyl nonyl ester .

In K326 seedlings, the relative PAE content was 35.6% at the two-leaf stage, 51.3% at the four-leaf stage, and 2.2% at the six-leaf stage. The main PAEs included diisooctyl phthalate (DIOP), dibutyl phthalate (DBP), butyl cyclohexyl phthalate (BCHP), and dioctyl phthalate (DOP) .

pH Changes in Culture Medium

The pH of the agar-free MS medium was 5.70. During tobacco seedling growth, the pH of the medium decreased in both varieties at all leaf stages compared to the initial pH, but gradually increased with seedling development. The pH values at different stages are shown in .

Effects of PAEs on Superoxide Anion Generation

Both DBP and DIOP significantly promoted $\cdot O_2^-$ generation rate in tobacco roots ($P < 0.05$). The generation rate increased with concentration, with DIOP showing a stronger effect than DBP. At concentrations above 0.1 mmol/L, the $\cdot O_2^-$ generation rate was significantly higher than the control [Figure 1: see original paper].

Effects of PAEs on SOD Activity

SOD activity increased with increasing DIOP concentration, reaching a maximum at 0.5 mmol/L, then decreasing at higher concentrations. DBP showed a similar trend but with less pronounced effects. The difference between the two treatments was significant ($P < 0.05$) [Figure 2: see original paper].

Effects of PAEs on CAT Activity

CAT activity increased with DIOP concentration up to 0.5 mmol/L, then decreased to levels similar to the control at higher concentrations. DBP treatment showed a similar pattern. The treatments had significant effects on CAT activity ($P < 0.05$) [Figure 3: see original paper].

Effects of PAEs on MDA Content

MDA content, indicating lipid peroxidation level, increased significantly with both DIOP and DBP treatments ($P < 0.05$). The increase was concentration-dependent, with DIOP causing greater MDA accumulation than DBP. At concentrations above 0.5 mmol/L, MDA content increased sharply, indicating severe membrane damage [Figure 4: see original paper].

Discussion

Autotoxicity represents a special form of intraspecific competition for limited resources, often manifesting as density-dependent effects or self-thinning. This regulates population density and favors natural selection by promoting healthier, larger individuals. Autotoxic substances are released through volatilization, root exudation, and residue decomposition, with root exudates being a primary source and important medium for root-soil exchange.

Plant species, cultivars, and growth stages influence root exudate composition. Schumacher et al. found that wild oat root exudates at specific leaf stages inhibited spring wheat growth, with two-leaf stage exudates showing the strongest toxicity. Similarly, our study revealed that both Burley and K326 tobacco varieties produced different types and amounts of root exudates at different leaf stages, with PAEs being the main autotoxic components.

Common autotoxic substances include water-soluble organic acids, aliphatic aldehydes and ketones, simple unsaturated lactones, alkaloids, cyanohydrins, benzoic acid derivatives, cinnamic acid derivatives, sulfides, glucosinolates, purines, and nucleosides. In tobacco seedlings, phthalate esters and dimethoxyphenol showed autotoxic effects, with PAEs being most abundant at early leaf stages (35.6% and 51.3% at two- and four-leaf stages in K326) and decreasing with plant development.

Phthalate esters are environmental endocrine disruptors with hormonal activity that can alter root cell physiological functions. Under stress, increased reactive oxygen species (ROS) production is a common response. Superoxide anion ($\cdot O_2^-$) is the first ROS generated and can be converted to more harmful radicals like hydroxyl ($\cdot OH$) and hydrogen peroxide (H_2O_2).

In our pot experiments, PAE application significantly increased $\cdot O_2^-$ generation rate in tobacco roots ($P < 0.05$), consistent with findings in tobacco and grapevine mesophyll protoplasts. Under normal conditions, ROS are effectively scavenged by antioxidant systems where SOD and CAT play key roles. SOD converts $\cdot O_2^-$ to H_2O_2, which CAT then decomposes.

Our results showed that SOD and CAT activities increased with PAE concentration up to 0.5 mmol/L, then decreased at higher concentrations. This biphasic response suggests that at moderate stress, the antioxidant system is activated, but under severe stress (>0.5 mmol/L), rapid $\cdot O_2^-$ generation overwhelms the scavenging capacity, causing enzyme inactivation and oxidative damage.

MDA content increased significantly with PAE concentration, indicating enhanced lipid peroxidation and membrane damage. The greater effect of DIOP compared to DBP reflects its higher toxicity. Membrane damage increases permeability, causing ion leakage and nutrient absorption imbalance, ultimately manifesting as autotoxicity.

Notably, the pH of culture media decreased during seedling growth, which may enhance toxicity by affecting nutrient uptake. The lipophilic nature of PAEs and pH changes likely act synergistically in tobacco autotoxicity.

Conclusion

Tobacco seedlings of both Burley and K326 varieties produce different types and relative amounts of root exudates at different leaf stages, with phthalate esters being the primary autotoxic substances. The relative content of PAEs decreases with seedling development. When PAE concentration exceeds 0.5 mmol/L, it significantly increases $\cdot O_2^-$ generation rate, initially enhances but then suppresses SOD and CAT activities, and causes MDA accumulation. When $\cdot O_2^-$ generation exceeds the antioxidant system' s scavenging capacity, oxidative damage to root tip cell membranes occurs, leading to increased membrane permeabil-

ity, ion leakage, nutrient absorption imbalance, and ultimately autotoxicity in tobacco plants.

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Note: Figure translations are in progress. See original paper for figures.