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The garlic allelochemical diallyl disulfide alleviates autotoxicity in the root exudates caused by long-term continuous cropping of tomato

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ABSTRACT: Continuous cropping obstacles seriously affect the sustainable production of tomatoes (*Solanum lycopersicum* L.). Researchers have found that intercropping with garlic (*Allium sativum* L.) could alleviate tomato continuous cropping obstacles. Diallyl disulfide (DADS) is the main allelochemical in garlic. However, the mechanism of DADS in alleviating tomato continuous cropping obstacles is still unknown. In this research, aqueous extracts of tomato continuous cropping soil were used to simulate the continuous cropping condition of tomato. Our results showed that DADS increased root activity and chlorophyll content, improved the activity of antioxidant enzymes (SOD, POD and PAL) and the metabolism of non-enzymatic antioxidants (GSH and GSSG) in tomato plants. DADS treatment reduced the content of fatty acid esters in tomato root exudates (e.g., palmitate methyl ester, palmitoleic acid methyl ester, oleic acid methyl ester) and increased the level of substances such as dibutyl phthalate and 2,2′-methylenebis(6-tert-butyl-4-methylphenol). The higher concentrations of palmitate methyl ester inhibited tomato hypocotyl growth, while oleic acid methyl ester inhibited tomato root growth. Moreover, the application of DADS significantly inhibited the secretion of these esters in the root exudates. Therefore, it suggests that DADS may increase tomato resistance and promote tomato plant growth by increasing root activity, photosynthetic capacity and development to reduce autotoxicity of tomato.

KEY WORDS: *tomato root exudates, garlic, diallyl disulfide, growth promotion, autotoxicity*

▪ Introduction

Tomato production in China, along with some other essential crops, is severely affected due to continuous cropping obstacles caused by long-term intensive cultivation under protective plastic tunnel systems. It is a repetitive agronomic practice can negatively affect soil physicochemical properties, secondary salinization and acidification, and soil-borne diseases, which severely affect crop productivity.[1] Besides, allelopathic autotoxicity is caused by secondary metabolites (e.g., organic acids, aldehydes, aromatic acids, coumarins, quinones, alkaloids, terpenoids and their decomposition products), has been proved to be one of the critical limiting factors for continuous plantation.[2,3]

The secretion of exudates from the roots is one of the main approaches of allelochemicals released into the rhizosphere. Continuous cropping obstacles are closely associated with the allelochemicals in root exudates.[4] Continuously cropping caused a decrease in root activity, chlorophyll content, superoxide dismutase (SOD) activity, net photosynthetic rate, stomatal conductance, transpiration rate, yield and biomass of tomato as the duration of successive planting.[5,6] Tomato root exudates inhibited seed germination and seedling growth of itself and the inhibition effects, mainly due to phenolic allelochemicals in root exudates [7]. Nowadays, resistant varieties, grafting technology, crop rotation and intercropping, straw mulching, plant disease biological control have been used in agriculture production to alleviate obstacles to continuous cropping.[2,8]

Garlic is a traditional vegetable and popular gardening crop in China. It is also an essential

allelopathic crop widely used in intercropping/rotation with other crop species.[9,10] Allelopathy contains beneficial and/or detrimental effects on target organisms. The beneficial allelopathic effects of garlic have been reported by many types of researches. Intercropping with garlic could improve the soil biology environment, thus alleviate the continuous cropping obstacle of cucumber.[11] Garlic straw and its decomposing products were also proved to have positive allelopathic effects on receiver plants and soil enzymes activities.[12,13] Low content of raw garlic straw (2%) could effectively suppress the incidence of *Meloidogyne incognita* and improve tomato yield in pot experiments.[14] Previously, aqueous garlic extracts application improved plant growth and physiology in a variety of treated plants such as cucumber,[15] tomato[16] and eggplant[17] under stress conditions.

Allelopathic effects of garlic are mainly caused by sulfur-containing compounds. The volatile diallyl disulfide (DADS) was found to be the leading allelochemical in garlic root exudates, garlic sprouts, garlic straw, and its decomposing products.[14,18] Recent studies reported that green garlic volatile organic compounds and DADS played vital roles in the reactive oxygen species (ROS) production and regulation of antioxidant enzymes in cucumber seedlings.[19] In our previous studies, we found that the DADS influenced the balance of phytohormones levels, gene expression and cellular mitotic division in the root tips of tomato[20] and cucumber.[21] However, it is still unclear whether or not the garlic allelochemical (DADS) bears the potential to alleviate autotoxicity under continuous cropping of tomato, which in turn may have an impact on root exudates and their autotoxicity activities.

In light of this serious threat of autotoxicity in continuous cropping of tomato, we decided to investigate the possible allelopathic potential of DADS and soil extracts (ATCS) from continuously cropped tomato fields to observe the growth and development responses in tomato seedlings. In the current study, changes in the root exudates of tomato treated with DADS and ATCS were determined by using Gas Chromatography-Mass Spectrometer (GC-MS), to explore the continuous cropping obstacles with autotoxic allelopathy. Therefore, our studies encompass physiological assessment of the plant's primary metabolism and root exudates identification and their effects on germination potential and seedlings growth of tomato. The findings of the current study will offer better insight into the allelopathic potential of DADS to alleviate autotoxicity or continuous cropping obstacles that significantly impair tomato production, particularly under the plastic tunnel production systems in China.

▪ Materials and methods

Soil aqueous extracts and DADS solution preparation. Soil samples were collected in the field that had been continuously cultivated with tomato for seven years in the Horticultural Station (34°17′N, 108°04′E) of Northwest A&F University. The dead leaves on the soil surface were removed and samples were randomly taken at 20 cm in the root rhizosphere. The collected samples were allowed to air dry at room temperature, crushed and then passed through a 1 mm sieve. The available nitrogen, phosphorus and potassium in the soil were 88.7, 87.5 and 251.8 $mg \cdot kg^{-1}$. To prepare the desired concentrations of ATCS, the air-dried soil was dissolved in 400 mL of distilled water and shaken for 48 h at room temperature. After siting, the supernatant was removed and subsequently diluted to 25, 50

and 100 g·L^{-1}. DADS stock solution was made by dissolving it in Tween-80 at a ratio of 1:2 (v:w) and the volume was raised to 100 mL with distilled water and stored at 4 °C for further use.[22] To prepare the desired concentrations, the DADS stock solution was subsequently diluted to 0.21, 0.41 and 0.69 mmol·L^{-1} respectively to perform the experiment.

Plant material and treatment. The whole experiment was conducted in growth chamber, at 25°C 16 h day/15°C 8 h night, 70% relative humidity, illumination at 30000 Lx. Tomato (*Solanum lycopersicum* L. var. Dongfen No.3) seedlings were grown in the sterilized perlite and irrigated with 1 × Hoagland's nutrient solution. DADS solutions (contain 1 × Hoagland's nutrient solution and different concentrations of ATCS) were applied at the 4-leaf stage of tomato seedlings. Tomato seedlings were treated with DADS every third day until 21 days. This experiment was performed with three replicates, and each replicate contained 15 tomato seedlings.

Plant morphological indices. Shoot length was noted using a measuring tape (in cm). Tomato seedlings were washed with distilled water to record the fresh weights using an electronic balance. Afterward, the plant materials were dried in an oven at 80°C for 2 days and their dry weights were recorded.

Chlorophyll content and root activity. The third leaf from the top was selected to measure chlorophyll content followed the method of Zhang.[23] Root activity was determined according to the method described by Khan et al..[24]

Defense enzymes activities, malonaldehyde (MDA), and soluble protein contents.

Activities of SOD, peroxidase (POD), phenylalanine ammonia-lyase (PAL), and MDA content were assayed according to Wang et al..[9] The content of soluble protein was determined using the method of Gao.[25]

H_2O_2 content. H_2O_2 content was assayed according to Sergiev et al..[26] The tomato root sample (0.5 g) was pestled with 5 mL trichloroacetic acid (0.1%, w/v) in an ice bath, then the homogenate was centrifuged for 20 min at 12000 r/min under 4°C. The supernatant was used for H_2O_2 determination. This reaction mixture contained 0.5 mL supernatant, 0.5 mL potassium phosphate buffer (0.1 mol·L^{-1}, pH 7.0) and 2 mL potassium iodide (1 mol·L^{-1}). After reacting in the dark for 1 h (25°C), the absorbance of the liquid was measured at 390 nm. H_2O_2 content was calculated according to the standard curve.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents. The contents of GSH and GSSG were assayed according to the method described by Anderson.[27] Sample (0.5 g) was pestled with 3 mL 6% metaphosphoric acid (including 1 mmol·L^{-1} ethylene diamine tetraacetic acid, pH 2.8) in an ice bath, then the homogenate was centrifuged for 15 min at 12000 r/min under 4°C. The supernatant was used to determine the contents of GSH and GSSG. For GSH, the reaction mixture included 0.5 mL supernatant, 0.5 mL ethylene diamine tetraacetic acid (10 mmol·L^{-1}), 1.7 mL phosphate buffer (pH 7.5), 0.2 mL NaOH (1 mol·L^{-1}) and 0.1 mL 5,5′-dithio-bis-2-nitrobenzoic acid (6 mmol·L^{-1}). After 5 min of water bath (25°C), the liquid was used to determine GSH content. The reaction mixture for total glutathione included 0.5 mL supernatant, 0.5 mL ethylene diamine tetraacetic acid (10

mmol·L^{-1}), 1.5 mL phosphate buffer (pH 7.5), 0.2 mL NaOH (1 mol·L^{-1}), 0.1 mL 5,5′-dithio-bis-2-nitrobenzoic acid (6 mmol·L^{-1}), 0.1 mL triphosphopyridine nucleotide (2.1 mmol·L^{-1}) and 0.5 units (U) of glutathione reductase (GR). The absorbance changes were recorded at 412 nm within 60 seconds.

Root exudates collection and identification. An additional forty more tomato plants were grown (having similar growth conditions as above) and treated with 0.21 mmol·L^{-1} DADS solution (containing 1× Hoagland's nutrient solution and 50 g·L^{-1} ATCS) at the 7-leaf stage. The root exudates were collected after 0, 1, 3, and 15 days since the treatment. The perlite was carefully washed with distilled water to avoid any root damage and then each plant was transferred to a 500 mL distilled water Erlenmeyer flask wrapped with black plastic for 24 h. Distilled water was added to replenish stomatal transpiration and tomato root exudates were concentrated through filtration and vacuum distillation on a rotary evaporator in a water bath at 40°C. Finally, the dry residue from evaporation was re-dissolved in 1-milliliter methyl alcohol and filtered through 0.22 μm membranes for GC-MS analysis.

GC-MS analysis was conducted on the GC-MS-QP2010Plus (Shimadzu, Japan) according to the previous method with some modifications.[28] The chromatographic conditions were Rtx-5 MS capillary column (30.0 m×0.32 mm, 0.25 μm film). The GC oven temperature was programmed at 50°C and held for 2 min, then increased up to 250°C at 6°C min^{-1} and held for 10 min. Helium was used as carrier gas at a constant flow rate of 1 mL·min^{-1}. The injector temperature was set at 230°C and 1 μL extract was injected. The MS was operated under the

following conditions: ion source voltage was at 70 eV, and the temperature was 200°C. The mass scan range was 30 to 600 amu. The compounds determination was carried out by matching their recorded mass spectra with those stored in the NIST08 mass spectral library of the GC–MS data system and the relative percentage was determined based on the peak area normalization method.

Biological test of tomato root exudates. Palmitate methyl ester and oleic acid methyl ester were selected for biological test according to the identified tomato root exudates. These chemicals were dissolved in ethanol and then diluted into 0.5, 5 and 20 $mmol \cdot L^{-1}$, respectively. The prepared solution (6 ml) was added to the petri dishes having double-layered filter paper and placed in an oven at 40 °C till the evaporation of alcohol. Ethanol was set as control. Forty tomato seeds were evenly placed on the filter paper, 6 mL of distilled water was added, and then 2 mL of distilled water was added on alternative days to maintain moisture for the germinating seeds. The petri dishes were maintained in a growth chamber under 25°C 16 h day/20°C 8 h night. Each treatment was repeated 4 times. Seeds germination (seeds with radicle emerged 3 mm or more in length) was recorded by counting the number of germinated seedlings for seven consecutive days. Root and hypocotyl length were measured using Vernier calipers (in mm). The fresh weight of the roots and aerial parts were recorded after 14 days of incubation.

Data analysis. Data were analyzed by SAS (SAS Institute, Cary, NC, USA). Fisher's Least Significant Difference (LSD) test was performed for the significance analysis with $P < 0.05$.

▪ Results

Effects of DADS on the morphology of tomato seedlings. To investigate the influence of

DADS on tomato growth under continuous cropping conditions, shoot length and dry weight of tomato plants were recorded (Table 1). Analysis of variance corroborated that shoot length were significantly inhibited by higher concentrations of ATCS but increased seedling dry weight at 50 $g \cdot L^{-1}$ ATCS in comparison with control seedlings (0 ATCS). Shoot length and dry weight were significantly promoted by DADS (0.21 $mmol \cdot L^{-1}$) compared to control (without DADS and ATCS). The DADS application at 0.21 $mmol \cdot L^{-1}$ improved shoot length in ATCS levels (25 and 50 $g \cdot L^{-1}$) with no apparent distinction but the increasing of dry weight showed significant differences under these conditions. As shown in Figure 1, the DADS treated seedlings were comparably healthier under various ATCS levels than ATCS treatments without DADS application.

Table 1. Effects of DADS on the morphology of tomato seedlings under ATCS levels.

Index	DADS	ATCS ($g \cdot L^{-1}$)			
	($mmol \cdot L^{-1}$)	0 (Control)	25	50	100
Shoot length (cm)	0	18.47 ± 0.55 b A	17.37 ± 0.44 b A	16.40 ± 0.75 b AB	14.57 ± 1.16 b B
	0.21	22.37 ± 0.47 a AB	22.63 ± 0.98 a A	23.13 ± 0.90 a A	19.63 ± 0.62 a B
	0.41	20.30 ± 1.04 ab A	20.83 ± 0.52 a A	21.07 ± 0.69 a A	18.87 ± 0.81 a A
	0.69	18.23 ± 1.07 b AB	20.63 ± 0.95 a AB	21.77 ± 0.66 a A	20.83 ± 1.30 a AB
Dry weight ($g \cdot plant^{-1}$)	0	1.52 ± 0.06 b B	1.41 ± 0.14 b B	1.90 ± 0.12 b A	1.58 ± 0.05 b B
	0.21	2.11 ± 0.05 a B	2.76 ± 0.23 a A	2.93 ± 0.18 a A	1.92 ± 0.10 a B
	0.41	1.62 ± 0.11 b A	1.72 ± 0.13 b A	1.74 ± 0.05 b A	1.60 ± 0.10 b A
	0.69	1.61 ± 0.11 b A	1.43 ± 0.09 b A	1.70 ± 0.21 b A	1.65 ± 0.16 ab A

Values are expressed as the mean ± SE (n = 5). Different lowercase letters within columns represent the significant difference at $p<0.05$ (LSD) among the DADS treatments under the

same conditions of ATCS. Different capital letters within rows represent the significant difference at $p<0.05$ (LSD) among ATCS treatments under the same DADS conditions.

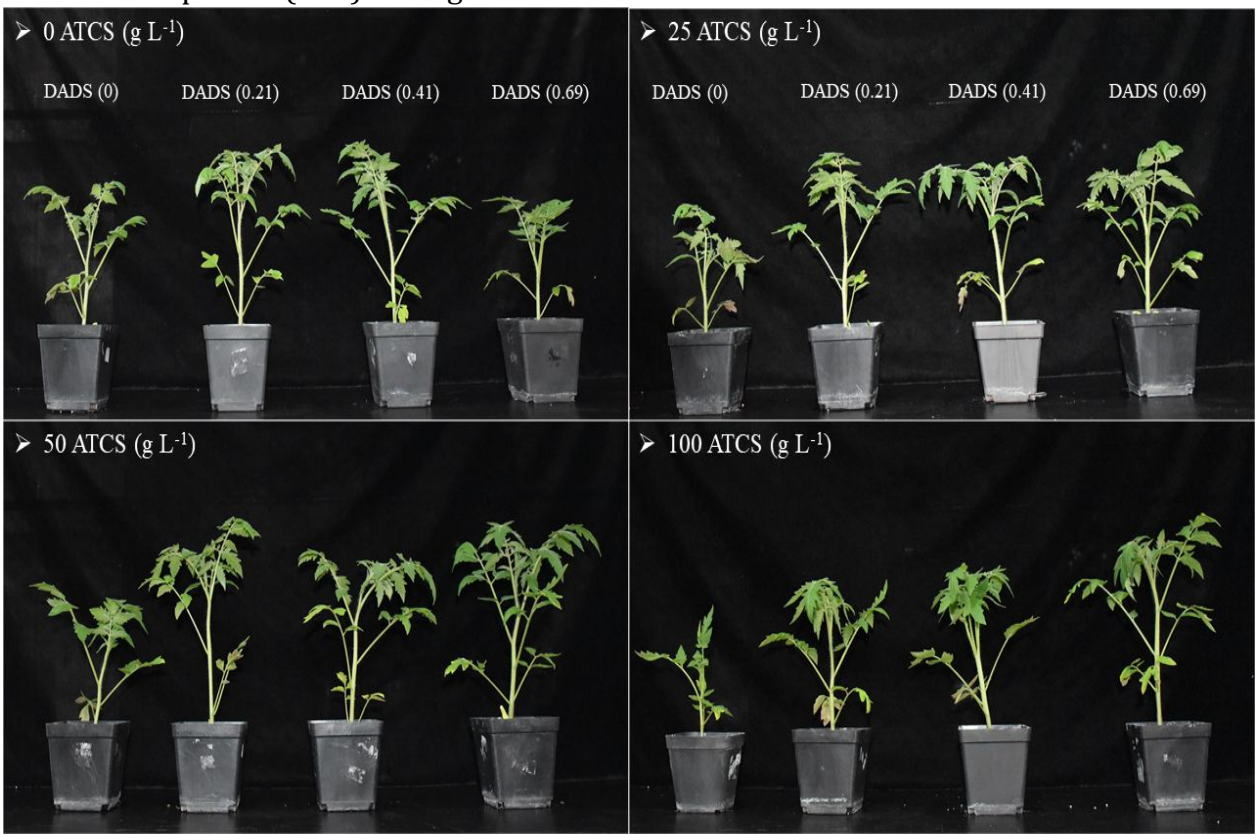


Figure 1. Effect of DADS on the growth of tomato seedlings under ATCS levels. Control seedlings (0 DADS + 0 ATCS + 1 × Hoagland's nutrient solution), DADS (Diallyl disulfide, 0.21, 0.41 and 0.69 mmol·L^{-1}) and ATCS (Aqueous extracts of tomato continuous cropping soil, 25, 50 and 100 g·L^{-1}).

DADS increased tomato chlorophyll content. DADS significantly increased the contents of chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll and carotenoid in tomato leaves (Table 2). The promoting effect was enhanced with the increase of DADS concentrations. However, there was no significant difference in chlorophyll contents and chl a/b ratio among ATCS treatments without the DADS application. In Chl a, the DADS at 0.21 mmol·L^{-1} showed higher result (2.45 ± 0.01 aA) in ATCS 25 g·L^{-1}, while the lowest was observed in ATCS 100 g·L^{-1} (2.03 ± 0.10 bcB). A similar pattern was observed in chl b and

carotenoid content. The promoting effect of DADS on chl b (17.2% - 40.9%) was greater than that of chl a (9.5% - 18.4%), which caused a decrease in chl a/b ratio.

Table 2. Effects of DADS on contents of chl a, chl b, total chl, carotenoid (mg·g^{-1} Fw) and chl a/b ratio of tomato seedlings under ATCS levels.

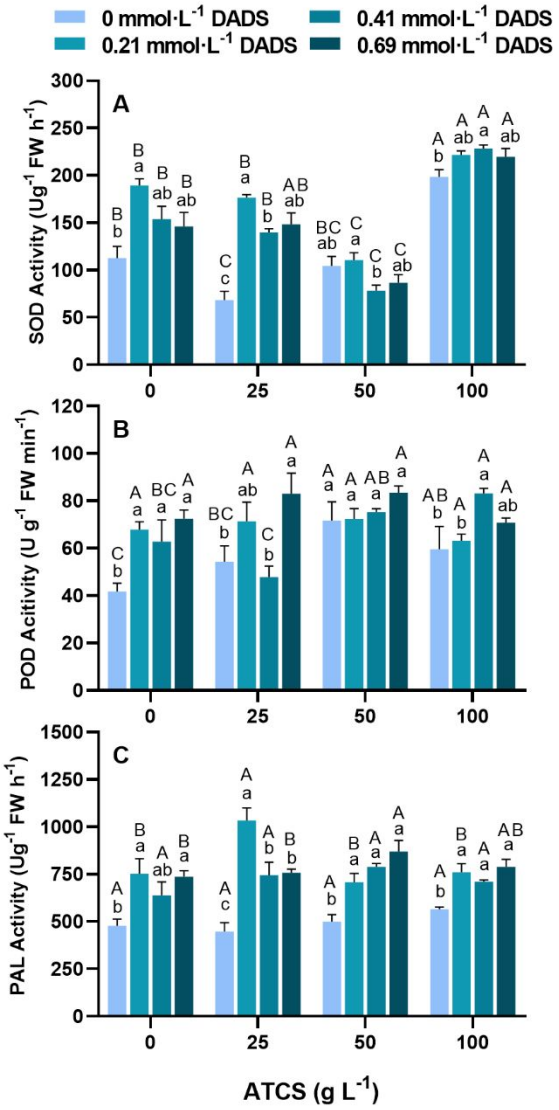
Index	DADS (mmol·L^{-1})	ATCS (g·L^{-1})			
		0 (Control)	25	50	100
Chl a	0	2.07 ± 0.03 c AB	1.98 ± 0.04 b AB	2.12 ± 0.04 b A	1.94 ± 0.08 c B
	0.21	2.27 ± 0.04 b A	2.45 ± 0.01 a A	2.31 ± 0.06 a A	2.03 ± 0.10 bc B
	0.41	2.30 ± 0.02 b BC	2.42 ± 0.04 a A	2.36 ± 0.04 a AB	2.21 ± 0.02 ab C
	0.69	2.45 ± 0.04 a A	2.36 ± 0.05 a A	2.38 ± 0.05 a A	2.41 ± 0.01 a A
Chl b	0	0.81 ± 0.02 c AB	0.77 ± 0.03 b AB	0.83 ± 0.03 b A	0.74 ± 0.04 c B
	0.21	0.95 ± 0.04 b BC	1.14 ± 0.01 a A	1.00 ± 0.06 a AB	0.79 ± 0.06 c C
	0.41	0.99 ± 0.02 b AB	1.11 ± 0.05 a A	1.03 ± 0.04 a AB	0.91 ± 0.02 b B
	0.69	1.14 ± 0.06 a A	1.06 ± 0.04 a A	1.05 ± 0.05 a A	1.08 ± 0.01 a A
Total Chl	0	2.88 ± 0.05 c AB	2.75 ± 0.07 b AB	2.95 ± 0.07 b A	2.67 ± 0.12 c B
	0.21	3.22 ± 0.08 b A	3.59 ± 0.02 a A	3.31 ± 0.11 a A	2.82 ± 0.15 bc B
	0.41	3.30 ± 0.04 b AB	3.53 ± 0.09 a A	3.39 ± 0.08 a A	3.12 ± 0.04 b B
	0.69	3.59 ± 0.10 a A	3.42 ± 0.09 a A	3.43 ± 0.11 a A	3.49 ± 0.02 a A
Carotenoid	0	0.69 ± 0.02 c AB	0.66 ± 0.01 b AB	0.71 ± 0.02 b A	0.62 ± 0.03 c B

	0.21	0.79 ± 0.03 b A	0.89 ± 0.01 a A	0.80 ± 0.03 a A	0.68 ± 0.04 bc B
	0.41	0.81 ± 0.01 b AB	0.87 ± 0.03 a A	0.84 ± 0.02 a A	0.76 ± 0.01 b B
	0.69	0.89 ± 0.03 a A	0.82 ± 0.03 a A	0.84 ± 0.04 a A	0.85 ± 0.00 a A
Chl a/b	0	2.57 ± 0.027 a AB	2.58 ± 0.036 a AB	2.54 ± 0.039 a B	2.64 ± 0.019 a A
	0.21	2.40 ± 0.045 b AB	2.16 ± 0.012 b C	2.33 ± 0.076 b BC	2.57 ± 0.055 a A
	0.41	2.32 ± 0.028 b AB	2.18 ± 0.063 b B	2.31 ± 0.053 b AB	2.43 ± 0.024 b A
	0.69	2.16 ± 0.072 c A	2.23 ± 0.052 b A	2.28 ± 0.074 b A	2.24 ± 0.024 c A

Values are expressed as the mean ± SE (n = 3). Different lowercase letters within columns represent the significant difference at $p<0.05$ (LSD) among the DADS treatments under the same conditions of ATCS. Different capital letters within rows represent the significant difference at $p<0.05$ (LSD) among ATCS treatments under the same DADS conditions.

DADS affected the activities of defense enzymes. ATCS notably increased SOD activity in tomato roots at 100 $g \cdot L^{-1}$ (Figure 2A); the activity of POD was also promoted by ATCS at 50 and 100 $g \cdot L^{-1}$ when DADS was not applied (Figure 2B). DADS treatments increased the activities of SOD and POD in tomato root without ATCS. DADS promoted SOD activity under a low concentration of ATCS (25 $g \cdot L^{-1}$). Both SOD and POD activities were not notably affected by DADS under the medium concentration of ATCS (50 $g \cdot L^{-1}$); however, DADS increased the activity of SOD and POD under high concentration ATCS (100 $g \cdot L^{-1}$) even though the significant difference was only observed at 0.41 $mmol \cdot L^{-1}$ DADS. The PAL activity was affected by both ATCS and DADS (Figure 2C). Compared with control, the activity of PAL

218 in tomato root was increased by DADS alone. Besides, PAL activity under different
219 concentrations of ATCS was significantly promoted by DADS.



220

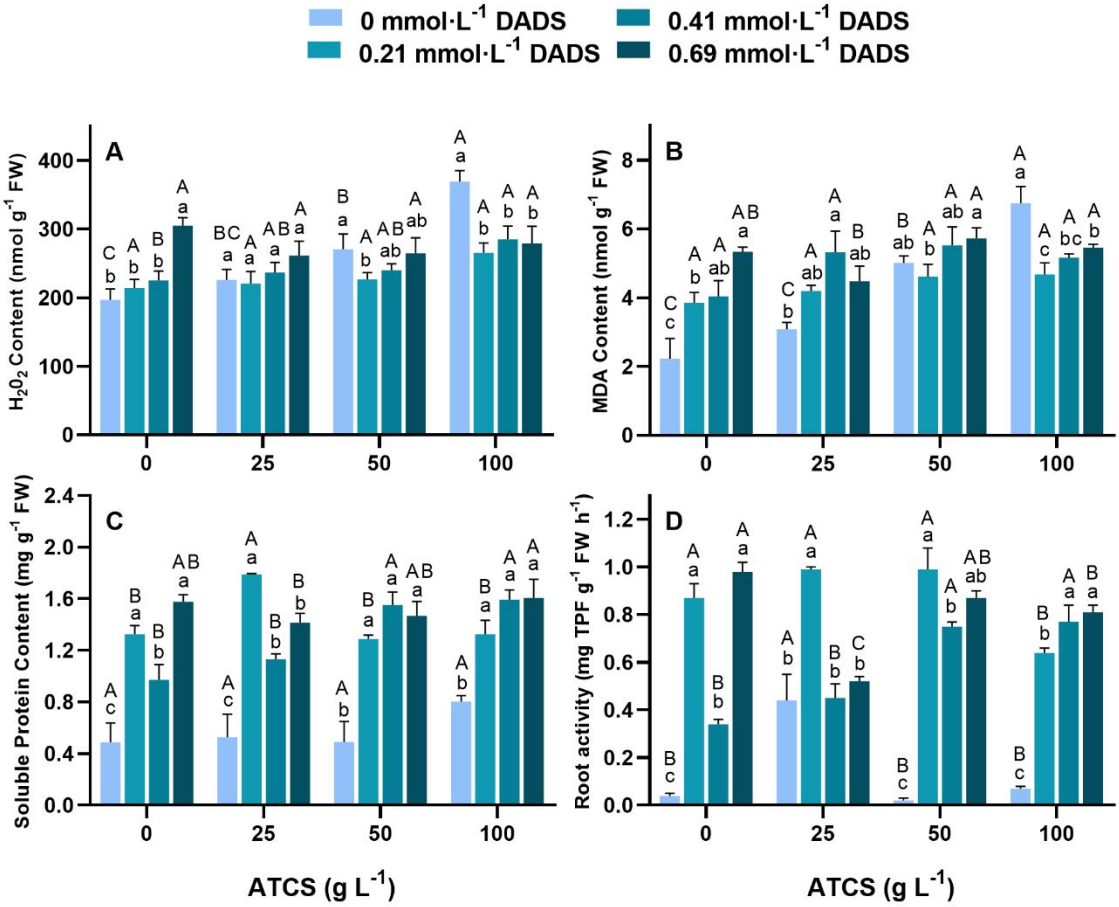
221 **Figure 2.** Effects of DADS on the activities of SOD, POD and PAL in tomato roots under ATCS
222 levels. Bars represent mean ± SE (n = 3). Different lowercase letters represent the significant
223 difference at $p<0.05$ (LSD) among the DADS treatments under the same conditions of ATCS.
224 Different capital letters represent the significant difference at $p<0.05$ (LSD) among ATCS
225 treatments under the same DADS conditions.

226 **H_2O_2, MDA and soluble protein contents.** The results of DADS on contents of H_2O_2, MDA,

soluble protein and root activity with/without ATCS are shown in Figure 3. The H_2O_2 content in tomato roots was significantly influenced by ATCS (Figure 3A). Compared to the control seedlings (0 ATCS), the increasing concentrations of ATCS increased H_2O_2 content in tomato seedlings. The DADS application slightly decreased the H_2O_2 content in ATCS levels. The lowest values were noted in DADS 0.21 mmol·L^{-1} at 25 g·L^{-1} ATCS (220.83) and 50 g·L^{-1} ATCS (227.22) followed by (265.56) at 100 g·L^{-1} ATCS. The results for MDA contents in tomato roots were significantly influenced by ATCS (Figure 3B). The increasing concentrations of ATCS exhibited higher levels of MDA content which was at a statistical difference when compared to the control seedlings (0 ATCS). The DADS application significantly decreased the MDA abundance; the lowest values were noted (4.61) in DADS 0.21 mmol·L^{-1} at 50 g·L^{-1} ATCS and (4.68) at 100 g·L^{-1} ATCS. Soluble protein contents in tomato roots were relatively low under ATCS conditions without DADS application and there was no significant difference among different treatments (Figure 3C). However, DADS treatments significantly increased soluble protein contents in tomato roots. Under different ATCS conditions, soluble protein contents were all dramatically increased by DADS from 0.21 - 0.69 mmol·L^{-1}. When the ATCS concentration was 25 g·L^{-1} and DADS was 0.21 mmol·L^{-1}, the soluble protein content was the highest which is about 3.4 times of that without DADS treatment. Root activity without DADS application under ATCS conditions showed a non-significant difference compared to control seedlings (0 ATCS), except for 25 g·L^{-1} ATCS which are significantly higher (Figure 3D). The DADS application significantly improved tomato root activity compared with control. Except for the 25 g·L^{-1} ATCS condition, the stimulatory effects of DADS on tomato root activity were

248 greater than the corresponding ATCS treatments. When ATCS concentrations were 25 - 50

249 g·L^{-1} and DADS was 0.21 mmol·L^{-1}, tomato root activity peaked.



250

251 **Figure 3.** Effects of DADS on H_2O_2, MDA, soluble protein contents and root activity in tomato
252 roots under ATCS levels. Bars represent mean ± SE (n = 3). Different lowercase letters
253 represent the significant difference at $p<0.05$ (LSD) among the DADS treatments under the
254 same conditions of ATCS. Different capital letters represent the significant difference at
255 $p<0.05$ (LSD) among ATCS treatments under the same DADS conditions.

256 **DADS increased GSH and GSSG contents.** ATCS at a higher level (100 g·L^{-1}) increased

257 GSH content of but did not affect the GSSG amount, and these caused great increase in the

258 ratio of GSH and GSSG (Figure 4). DADS treatments significantly increased GSH and GSSG

contents in tomato root system but reduced the ratio GSH and GSSG without ATCS conditions (Figure 4). Under the premise of ATCS, DADS treatments at different concentrations significantly increased GSH and GSSG contents. Except for the treatments 0.69 $mmol \cdot L^{-1}$ DADS under 50 $g \cdot L^{-1}$ ATCS and 0.41 $mmol \cdot L^{-1}$ DADS under 100 $g \cdot L^{-1}$ ATCS, the ratio of GSH and GSSG were notably decreased by DADS under corresponding ATCS conditions. Compared with DADS, ATCS has less effects on the content of GSH and GSSG. The increase of GSSG content was larger than that of GSH under DADS treatments.

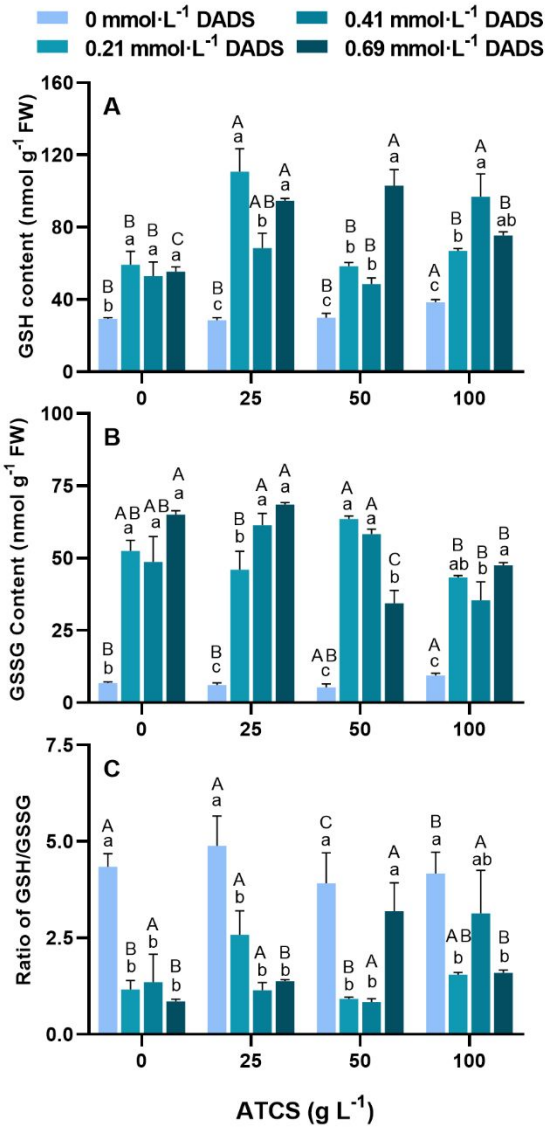


Figure 4. Effects of DADS on GSH and GSSG contents, and the ratio of GSH and GSSG in tomato roots under ATCS levels. Bars represent mean ± SE (n = 3). Different lowercase letters represent the significant difference at $p<0.05$ (LSD) among the DADS treatments under the same conditions of ATCS. Different capital letters represent the significant difference at $p<0.05$ (LSD) among ATCS treatments under the same DADS conditions.

Root exudates changes after DADS treatment. In the present research, hydrocarbons, esters, phenols, acids, aldehydes, alcohols, terpene and amines were identified in tomato root exudates, among which the types and relative amounts of ester substances were the largest (Table 3). For unsaturated fatty acid esters, the relative content of methyl

palmitoleate ranged from 2.26% to 19.82% and oleic acid methyl ester content is between 6.06% and 25.23%. The relative amount of saturated fatty acid ester, such as palmitate methyl ester ranged from 3.99% to 16.33%. After DADS application, the relative contents of fatty acid esters in tomato roots exudates were decreased on the first day and the third day, but increased on the 15th day while, the amounts of dibutyl phthalate and 2,2′-methylenebis(6-tert-butyl-4-methylphenol) increased post DADS treatment in the first day.

Table 3. The relative amounts (%) of different components in tomato root exudates different days after 0.21 mmol·L^{-1} DADS treatment under 50 g·L^{-1} ATCS level.

Name of components	**0 d**	**1 d**	**3 d**	**15 d**
Hydrocarbons				
Dodecane	0.17	0.16	0.20	0.12
Hexadecane	0.12	0.31	-	0.09
Heptadecane	0.20	0.32	0.24	0.21
Octadecane	0.30	0.34	0.19	0.51
2,6,10,14-tetramethyl pentadecane	0.68	0.86	0.47	0.77
Eicosane	0.38	0.29	0.24	-
2,6,10,14-tetramethyl hexadecane	0.66	0.79	0.50	0.95
Heneicosane	0.22	0.31	0.38	-
Tetracosane	-	-	-	0.47
Pentacosane	0.88	1.06	1.60	0.68
Dotriacontane	2.27	1.47	3.45	1.03
Tetratriacontane	1.93	1.59	4.41	1.20
Pentatriacontane	2.97	5.19	7.90	2.04
Hexatriacontane	1.08	0.87	3.66	0.62
Tetracontane	0.61	0.54	2.69	0.37
Phenols				
2,6-Bis(1,1-dimethylethyl) phenol	0.13	0.25	0.20	0.79
2,4-Di-tert-butylphenol	0.26	0.37	0.16	0.19
2,2′-Methylenebis(6-tert-butyl-4-methylphenol)	1.82	2.88	-	-
Esters				

Diethyl Phthalate	-	0.24	0.12	-
Methyl isomyristate	0.21	0.07	-	0.23
Methyl tetradecanoate	0.24	0.46	0.29	0.63
Dodecanoic acid, 4-methyl-, methyl ester	-	0.11	-	-
Pentadecanoic acid, methyl ester	0.47	0.20	0.19	2.94
Diisobutyl phthalate	0.11	0.17	-	0.47
Methyl 14-methylpentadecanoate	0.23	0.31	0.41	0.56
Methyl palmitoleate	6.80	3.42	2.26	19.82
Hexadecanoic acid, methyl ester	8.41	5.78	3.99	16.33
Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate	0.17	0.25	0.21	0.10
Dibutyl phthalate	0.24	0.45	-	-
Tridecanoic Methyl 4,8,12-trimethyltridecanoate	-	0.24	-	-
Heptadecanoic acid, methyl ester	0.35	0.45	0.27	0.23
Methyl 5-eicosenoate	-	0.20	0.29	-
cis-10-Heptadecenoic acid, methyl ester	1.82	0.28	0.20	3.19
Heptadecanoic acid, methyl ester	0.08	0.25	0.09	0.20
Linoleic acid, methyl ester	2.13	3.75	2.93	2.53
Oleic acid, methyl ester	12.17	7.59	6.06	25.23
Octadecanoic acid, methyl ester	3.44	3.64	2.66	2.46
cis-10-Nonadecenoic acid, methyl ester	-	-	-	0.31
2-Benzofurancarboxylic acid, 2,4,5,6,7,7a-hexahydro-4,4,7a-trimethyl-, methyl ester, cis-	1.80	1.50	2.65	0.76
Arachidonic acid methyl ester	-	0.54	0.68	-
Eicosatrienoic acid, methyl ester	-	0.17	0.59	-
Methyl 18-methylnonadecanoate	-	0.10	0.21	-
Methyl 4,7,10,13,16-docosapentaenoate	-	1.05	1.51	-
Methyl behenate	-	-	0.18	-
Phthalic acid, dioctyl ester	1.85	-	0.37	-
Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	2.43	-	0.83	-
Diisooctyl phthalate		0.55		0.19
Aldehyde				
Tetradecanal	0.06	-	-	0.23

Octadecanal	0.19	0.07	-	0.56
(Z)-9-octadecenal	-	-	-	0.42
Terpene				
Rishitin	-	-	-	0.19
Alcohols				
Behenic alcohol	-	-	-	0.22
Nonadecanol	-	0.35	0.39	0.16
Heptacosanol	-	-	-	1.83
Acid				
Tetradecanoic acid	0.42	0.78	-	0.73
cis-13-Eicosenoic acid	-	-	-	0.38
Icosapent	-	0.18	-	-
Amine				
Hexadecanamide	-	-	-	0.32
Erucylamide	-	0.60	0.71	0.73

- means not detected.

Tomato root exudates palmitate methyl ester and oleic acid methyl ester influenced tomato seed germination and seedling growth. According to the identified tomato root exudates, palmitate methyl ester and oleic acid methyl ester were selected for the biological test. As shown in Figure 5A, the germination rate of tomato seeds gradually decreased as the concentration of methyl palmitate increased, among which 5 $mmol \cdot L^{-1}$ of palmitate methyl ester significantly inhibited the germination rate. It did not affect the length and fresh weight of tomato root, but significantly inhibited the hypocotyl length and aerial part fresh weight at high concentrations. The effect of oleic acid methyl ester on germination rate and early seedling development of tomato are presented in Figure 5B. It reduced the germination rate of tomato seeds, but there was no significant difference as compared with the control. The high concentration of oleic acid methyl ester significantly inhibited tomato root length, but had no significant effect on hypocotyl length and aerial part fresh weight. The effect of oleic acid methyl ester on the fresh weight of tomato roots showed a trend of promoting and then inhibiting with the increase of its concentration.

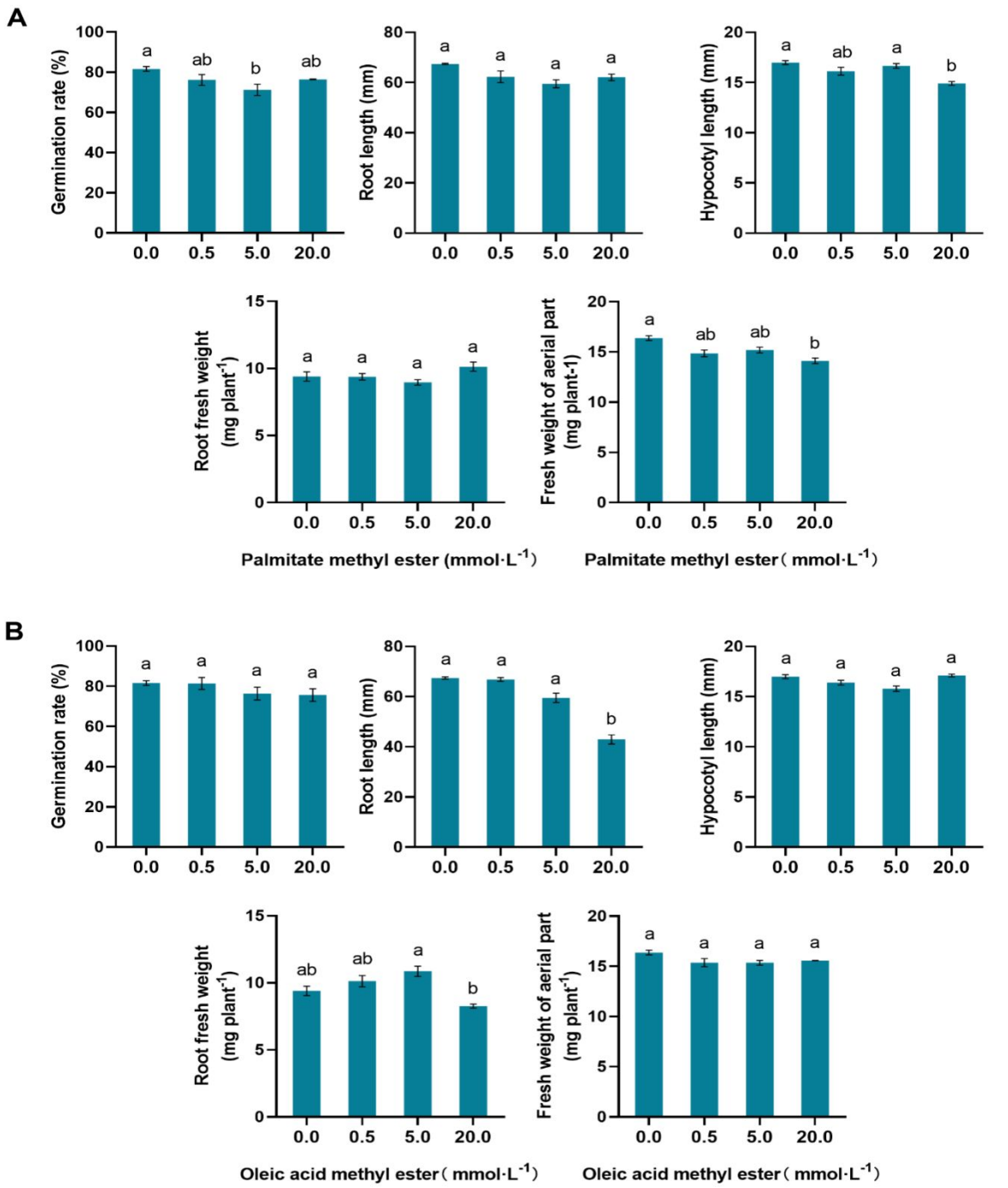


Figure 5. Effects of palmitate methyl ester (A) and oleic acid methyl ester (B) on seeds germination and early seedling growth of tomato. Germination rates (%) were measured at 7th day (40 seeds per replicate), hypocotyl length, fresh weight of aerial part, root length and root fresh weight were measured at 14th day of treated seeds with (0, 0.5, 5 and 20 mmol·L^{-1}) of palmitate methyl ester and oleic acid methyl ester. Bars represent mean ± SE for four replicates (10 seedlings per replicate). Bars caped with the same letters are not significantly different at $p < 0.05$ (LSD).

▪ Discussion

In our results, the long-term continuous cropping system negatively affected tomato plant biomass, root activity, defense systems and soil physicochemical properties. Similar results under long term intercropping were also reported by Fu et al..[6] Previous studies have found that intercropping/relay intercropping with garlic or onion could alleviate continuous cropping obstacles and promote the growth of receiver plants.[29,30] DADS is the organosulfur compound in Allium plants and the primary active substance in garlic allelopathy. DADS at 0.21 mmol·L^{-1} promoted seed germination and seedling growth of tomato seedlings.[20] Similar results were also observed under ATCS presence conditions in our work (Table 1). This growth promotion effect may be partly caused by the increased photosynthetic pigments (Table 2) which participate in photosynthesis, improve source-sink relationship and water and nutrition absorption. Moreover, previous studies also revealed that plant chlorophyll, carotenoid contents and net photosynthetic rate could be increased using aqueous garlic bulb extracts applied as a foliar spray,[31,32] adding decomposed garlic stalk to soil or interplanting with garlic.[13,33] These studies suggest that garlic allelochemicals (i.e. DADS) promote the synthesis of chlorophyll thus enhance plant photosynthesis and assimilation.

The tomato plants replanted continuously for more than three years were susceptible to plant disease in their reproductive stage, and the rate of disease infection increased significantly with the extension of continuous cropping year.[34] According to Ge et al.,[35] the continuous cropping obstacles in tomato grown in the field were observed after 4 - 5 years

328 of continuous cropping and gradually increased with prolonged duration. Tomato plants
329 grown in pots for two years showed inhibition in plant biomass.[5] In a meticulous study, it
330 was found that short-term continuous monoculture could improve soil quality.
331 Simultaneously, those greater than 4 years cropping had adverse effects on soil quality and
332 tomato yield.[6] The occurrence of continuous cropping obstacles is fluctuating which might
333 be due to the interaction between plants and soil microorganisms. Root growth and activity
334 were reduced by continuous cropping system in tomato plants. The application of garlic stalk
335 increased tomato root activity especially at the later growth stages and delayed the
336 senescence of roots.[8] A similar result was also observed in our work, suggesting that DADS
337 is the primary allelopathic substance in increasing root activity of receiver plants. However,
338 root activity was also increased by ATCS at 25 $g \cdot L^{-1}$, suggesting that certain chemicals in the
339 exudates might have a stimulating effect on the root activity.

340 The antioxidant enzymatic activity and MDA content were altered under a continuous
341 cropping system. According to our results, the contents of H_2O_2 and MDA were influenced by
342 ATCS (Figure 3). Moreover, the POD activity was increased by ATCS, and the activity of SOD
343 was decreased at a lower concentration of ATCS but increased at a high concentration of
344 ATCS. Similar results were also observed by Kang et al.,[34] who reported that POD activity in
345 tomato root increased within 5 years and decreased after 7 years during successive planting
346 years, the activity of SOD decreased and MDA content consistently increased throughout the
347 duration. Our results suggest that continuous cropping caused oxidative damage and a

disorder in the antioxidant system, which led to the inhibition of plant growth.

Plant defense enzymes such as SOD, POD and PAL play essential roles in plant response to biotic and abiotic stress. Several studies have observed that DADS exerted antioxidant protection via modulating antioxidant enzymes (e.g., glutathione-s-transferase, catalase, SOD) and non-enzymatic active substances such as increasing GSH content, and finally scavenged ROS and alleviated lipid peroxidation caused by abiotic stress.[36] According to Das and Chaudhri,[37] DADS could effectively attenuate the arsenite-induced cytotoxicity, ROS production, lipid peroxidation and control the activity of antioxidant enzymes (e.g., SOD and catalase) within the optimum range. DADS increased SOD and catalase activity and GSH content, reduced lipid peroxidation, and regulated the balance of oxidant and antioxidant during carcinogenesis.[38] In this study, the activities of SOD and POD were primarily increased by DADS with/without ATCS, which suggest that DADS induced tomato root resistance by modulating antioxidant enzymes.

PAL catalyzes the conversion of phenylalanine to trans-cinnamic acid, the first step in the phenylpropanoid pathway and the critical point between primary and secondary metabolism. PAL activity is affected by various biotic and abiotic stresses. According to Huang et al. [39] the *pal* mutant reduced salicylic acid (SA) content after pathogen infection. Plants with suppressed *PAL* expression could not develop systemic acquired resistance (SAR) in response to pathogen infection.[40] These results suggest that PAL is involved in the SAR mediated by SA. In this research, the activity of PAL was significantly increased by DADS

368 with/without ATCS treatment (Figure 2). This implies that DADS may induce tomato root
369 resistance by increasing salicylic acid content and stimulating the SAR process. Excessive
370 ROS causes redox imbalance and inhibits plant growth. However, the moderate amount of
371 ROS can act as signal molecules, which play essential roles in defense responses,
372 environmental adaptation, plant growth and development.[41] In the present results, the H_2O_2
373 content increased with increasing concentration of ATCS while DADS reduced the oxidative
374 stress caused by ATCS at a higher concentration compared to control (Figure 3). DADS
375 moderately increased H_2O_2 contents. Similar results were also found by Yang et al.,[19] where
376 DADS increased H_2O_2 contents in plants. The slight increase in H_2O_2 might regulate plant
377 response to biotic and abiotic stress such as continuous cropping obstacles.

378 GSH is a strong reductant for its thiol group and stable for the γ-peptide bond, which
379 protects it from being hydrolyzed by peptidases. It is the precursor of phytochelatins, which
380 participate in the detoxification of heavy metals, and also the substrate for GSH-s-
381 transferases (GST), which detoxify xenobiotic toxicity by catalyzing the conjugation of GSH
382 with dangerous xenobiotics.[42,43] In the present work, contents of GSH and GSSG were both
383 increased (Figure 4), which suggests that DADS treatment induced increase of total GSH and
384 GSSG content and promoted the circulation of GSH to increase the antioxidant capacity in
385 tomato root. In our previous research, the genes involved in GSH biosynthesis, such as *GSH1*
386 and *GR1*, and most *GST* genes were all up-regulated. However, the GSH degradation gene
387 *LapA2* was down-regulated by DADS treatment, which indicated that DADS treatment

induced increase of GSH content and GST activity.[44] It might imply that DADS could regulate activities of defense enzymes and contents of GSH and GSSG to maintain the redox state in tomato root to alleviate abiotic stresses.

Autotoxicity is one of the main factors that caused continuous cropping obstacles. Autotoxins influence the structure of soil microbial community, inhibit photosynthesis, defense enzymes activities, and nutrients uptake.[45,46] Yu and Matsui first found benzoic, phthalic, sinapic, palmitic acid, 4-hydroxybenzoic, phenylacetic, vanillic, ferulic, caffeic and 2-hydroxy-3-phenylpropanoic acids in tomato root extractions and determined that the first four substances are tomato autotoxins.[47] The phytotoxic substances i.e., benzoic, caffeic, chlorogenic, ferulic, p-hydroxybenzoic, salicylic and vanillic acid at the concentration of 400 $\mu mol \cdot L^{-1}$ significantly reduced fresh weight and dry weight of tomato plants, influenced the uptake of mineral nutrients and decreased bacterial counts in the culture solution.[48] The tomato root exudates in root stocks resistant to *M. incognita,* showed a significant increase in 2,2′-methylenebis (6-tert-butyl-4-methylphenol) and dibutyl phthalate.[49] This suggests that the above substances in tomato root exudates are responsible for tomato resistance to *M. incognita*. In recent research, Yang et al. found that tomato root exudate dibutyl phthalate suppressed egg hatching, increased second-stage juveniles mortality and reduced disease index of *M. incognita*.[50] It suggests that the root exudate may increase the nematode resistance of tomato plants. In this research, the contents of 2,2′-methylenebis (6-tert-butyl-4-methylphenol) and dibutyl phthalate in tomato root exudates increased within 24 hours

of DADS treatment but not been detected after 3 days. The decrease in 2,2′-methylenebis (6-tert-butyl-4-methylphenol) and dibutyl phthalate contents after 3 days of DADS treatment might be due to plant utilization of DADS and hence decreased the production of these compounds. In our study, the inhibition of germination, shoot, and root growth of tomato suggest that hexadecanoic acid methyl ester and oleic acid methyl ester may be the main autotoxins by hydroponics. The DADS is directly responsible for their inhibition and with the passage of time it might get completely utilized and results in increased production of these toxic compounds. Further work is necessary in order to explore various fat-soluble substances in tomato root exudates and their possible mechanism in tomato growth and development.

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Author Contributions

Fang Cheng: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing—Original draft preparation and Review, Muhammad Ali: Conceptualization, Data curation, Formal analysis, Methodology, Writing—Original draft preparation, Writing—Review and Editing, Ce Liu: Visualization, Investigation, Rui Deng: Software, Zhihui Cheng: Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

Conflicts of interests

The authors declare that they have no conflicts of interest.

ABBREVIATIONS

DADS, diallyl disulfide; SOD, superoxide dismutase; MDA, malonaldehyde; ATCS, aqueous extracts of tomato continuous cropping soil; POD, peroxidase; PAL, phenylalanine ammonia-lyase; GSH, glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; GC-MS, gas chromatography-mass spectrometer; chlorophyll a, chl a; chlorophyll b, chl b; SA, salicylic acid; SAR, systemic acquired resistance; ROS, reactive oxygen species; Fw, fresh weight

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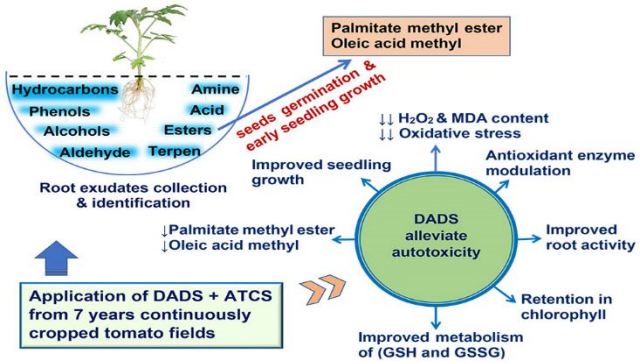

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