0971-4693/94 US $ 5.00
Allelopathy Journal 25 (1): 147-154 (2010) *International Allelopathy Foundation* 2010
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Alleviation of autotoxin-induced growth inhibition and respiration by sucrose in *Cucumis sativus* (L.)

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(Received in revised form: November 12, 2009)

ABSTRACT

The oil mobilization is a major source of sugar, which is substrate of carbon and energy metabolism during seed germination and seedling growth of cucumber. Here, we analyzed the effect of cinnamic acid (CA), one of the most important autotoxins in root exudates of cucumber on sugar content, respiration rate and expression of genes involved in oil mobilization in cucumber. Exposure of seeds to CA at 0.25mM significantly reduced the seedling growth followed by a decrease in total sugar, sucrose and hexose contents and this decrease was significantly attenuated by co-imbibitions with 0.5% sucrose. There was a significant decrease in the total respiration rate (V_T) and an increase in the KCN-resistant respiration rate (V_{KCN}) when treated with CA and an opposite trend was observed in seedlings exposed to sucrose. Transcript levels for genes involved in the oil mobilization were down-regulated in germinated seeds exposed to CA and up-regulated by sucrose. Notably, the presence of sucrose in the growth medium could attenuate the inhibitory effects caused by CA on respiration and gene transcripts.

Key words: Allelopathy, autotoxicity, cucumber, gene expression, glyoxylate cycle, respiration, sugar

INTRODUCTION

Allelopathy is the negative effect of chemicals released by one plant species on the growth or reproduction of another (5,12). Autotoxicity is a common phenomenon which occurs in many crops in both natural and manipulated ecosystems (15,22,27,28). Intriguingly, autotoxicity is implicated in the replant failure in monocropping systems (22,27). Benzoic and cinnamic acid derivatives have been identified from the cucumber root exudates (25), among them cinnamic acid (CA) is the principal autotoxin in root exudates of cucumber (25,26). Seed germination and subsequent seedling growth are the crucial stages in the life cycle of higher plants. The effects of allelochemicals on seed germination and seedling growth and they are more sensitive stages than other stages in mature plants (18,19,23). However, investigations on the effects of allelochemicals on metabolic processes during seed germination and seedling growth stage are limited. There are reports demonstrating that the plant growth inhibition mediated by sorgoleone or juglone was due to the disruption of mitochondrial function (10,20). It is indicated that the

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phytotoxicity of some allelochemicals to seedlings is largely attributed to their ability to disrupt normal metabolic processes in the plant.

Sugars are basic molecules in cell metabolism. Plant cells use sugars as respiration or metabolic intermediates and also as structural components in building their cell wall (7). In addition, sugars also play pivotal roles as signaling molecules (21). It has been demonstrated that local sugar concentration is positive to root elongation and branching (6). During seed germination and seedling growth of cucumber, oil is finally converted to sugars that can be transported around the seedling and used to support growth and respiration until the seedling becomes photoautrophic. The mobilization of storage oil involves the coordinated induction of a series of biochemical pathways, including β-oxidation, the glyoxylate cycle, and gluconeogenesis (8,11). Muscolo *et al.* (17) showed that the inhibition of *Pinus laricio* seed germination caused by phenolic compounds was linked to low utilization of storage lipids with consequent deficiency of glucose. It is necessary to investigate whether allelochemical-induced growth inhibition is related to metabolic dysfunction and sugar deficiency. By analyzing sugar content, respiration rate and expression of genes involved in glyoxylate cycle after treatment of CA, we determined the molecular mechanism by which CA inhibits cucumber seedling growth. Moreover, it is important to know whether supply of sugar could reverse the adverse effects of CA.

MATERIALS AND METHODS

Plant material

Cucumber seeds (*Cucumis sativus* L. cv. Jinyan No.4) were germinated at 25°C in darkness. Each twenty seeds were imbibed in a petri dish (9 cm dia) on two layers of Whatman filter paper saturated with 6 ml distilled water, 0.25% sucrose, 0.5% sucrose and 1% sucrose solutions, respectively. After 21 h, the seeds reached 100% germination and the radicle began to emerge from the seed coat, then the germinated seeds were transferred in petri dishes containing solutions of different combinations of cinnamic acid (0.25mM) and sucrose (0.25, 0.5 and 1%) for another 48 h. The distilled water served as control. The radicle length was measured 48 h after treatment. Each experiment consisted of three replications.

Measurement of sugar content

Germinated seeds were collected 48 h after treatment to determine sugars. Two hundred milligrams of freeze-dried germinated seeds was extracted in 50 ml 80% ethanol (v/v) overnight and the supernatant was analyzed for the contents of total soluble sugars, sucrose and hexose using a modified phenol-sulphuric acid method (4).

Measurement of respiration rate

Respiration of cucumber germinated seeds were measured as oxygen consumption in a 2 ml closed cuvette using a Clark type oxygen electrode (Oxygraph-lab, Hansatech, UK) at 48 h after treatment. Germinated seeds were kept in dark for 30 min before respiration measurements were taken. Samples (0.1 g fresh weight) were chopped and put into the reaction solution (20 mM phosphate buffer, pH 6.8) for measurement. The total respiration rate (V_T) was measured without any inhibitor. To measure the KCN-

resistant respiration rate (V_{KCN}), KCN was used as an inhibitor at a concentration of 10 mM (from a 100 mM stock solution in 20 mM phosphate buffer, pH 8.0). Temperature was maintained at 20 ^{o}C in a water bath set (2219 Multitemp II Thermostatic Circulator, Germany), which was connected to the measurement cuvette.

RNA extraction and gene expression analysis

Germinated seeds were collected at 24 h after treatment. Total RNA was extracted using TRIZOL reagent (Sangon, China) according to the supplier's instruction. A RevertAid^{TM} first strand cDNA Synthesis Kit (Fermentas) synthesized the first-strand of cDNA using 2 µg of purified RNA that was obtained using RNeasy Mini Kit (Qiagen). This initial strand of cDNA was used as the template for qRT-PCR. An iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA) performed the qRT-PCR. Each reaction (20 µl total volume) consisted of 10 µl iQ SYBR Green Supermix, 1 µl of diluted cDNA and 0.1 µM of forward and reverse primers. PCR cycling conditions were as follows: 95°C for 3 min, 40 cycles of 95°C for 10 sec, and 58°C for 45 sec. Fluorescence data were collected during the 58°C step. The cucumber *actin* gene was used as an internal control. The calculation of relative gene expression was conducted as described by Livak and Schmittgen (16). On the basis of EST sequences, the following gene-specific primers were designed and used for amplification: *Lox* (lipoxygenase), 5'-GGACCTCACTCCACCTTTGT-3' and 5'-GGATTGGGTCCTGCTAACAT-3'; *Icl* (isocitrate lyase), 5'-AAATCCAGCAACT- GTTTCCC-3' and 5'-AGCTTTGGGAGGC GTAAATA-3'; *Ms* (malate synthase), 5'-TGGCTGAAGTATGGAGTGGA-3' and 5'-CC CAAACAGTTCCTTGTTCA-3'; *Pck* (phosphoenolpyruvate carboxykinase), 5'-ACA TGTGCATTCGACCAACT-3' and 5'-CTGCCCAGCATGGTATATTG-3'; *actin*, 5'-TGG ACTCTGGTGATGGTGT- TA-3' and 5'-CAATGAGGGATGGCTGGAAAA-3'.

Statistical analysis

Seeds were arranged in three randomized blocks with three replicates per treatment. All results were subject to analysis of variance (SAS 8.0, SAS Institute, Cary, North Carolina) and the means were compared by the Tukey' HSD test at a significance level of $P \leq 0.05$.

RESULTS

Effects of cinnamic acid and sucrose on the radicle length in cucumber

Our previous studies demonstrated that cinnamic acid (CA) is an important autotoxin in cucumber root exudates (25,26). When the germinated seeds were exposed to 0.25 mM CA, the inhibition rate of radicle length reached 50.5% (Fig. 1). However, addition of different concentrations of sucrose (0.25, 0.5 and 1%) to the growth medium significantly alleviated the autotoxic inhibition by CA. For example, the radicle length recovered to the control level when the seeds were co-incubated with 0.5% sucrose and CA.

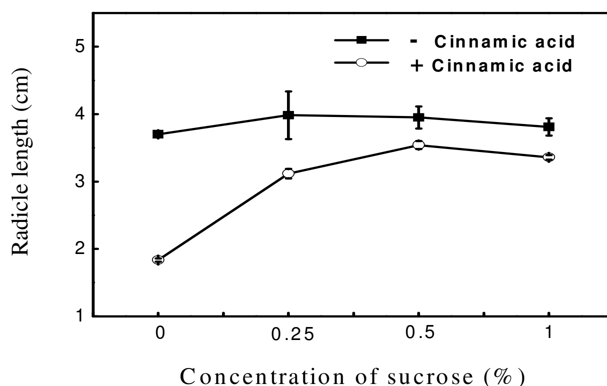

Figure 1. Effects of cinnamic acid with addition of different concentrations of sucrose on the radicle length in cucumber. The concentration of cinnamic acid (CA) was 0.25 mM. The concentrations of sucrose were 0.25, 0.5 and 1%. Distilled water (DW) was set as the control. Samples were taken 48 h after treatment. Each datum represents the average values from three replicates (30 individuals). Vertical bars represent standard errors.

Effects of cinnamic acid and sucrose on the soluble sugar content in cucumber

After exposure to CA, the total soluble sugar and sucrose content decreased by 35.97% and 59.08% compared to control. Meanwhile, there was a 30.03% reduction in hexose content (Table 1). Addition of 0.5% sucrose to the culture medium significantly increased total sugar and sucrose contents but had relatively little effect on hexose content as compared to control. When CA and sucrose were both present, total sugar and hexose contents were not different from those of control while sucrose content was lower than control. Total sugar, sucrose and hexose were increased by 34.74%, 70.40% and 78.08%, respectively, than CA treatment.

Table 1. Effects of cinnamic acid and sucrose on the soluble sugar content in cucumber germinated seeds

Treatment	Soluble sugar content ($mg \cdot g^{-1}$DW)		
	Total sugar	Sucrose	Hexose
Distilled water	9.62±1.23 b	6.11±0.74 b	3.11±0.11 a
0.25mM Cinnamic acid	6.16±0.47 c	2.50±0.13 d	2.19±0.38 b
0.5% Sucrose + 0.25mM Cinnamic acid	8.30±0.39 bc	4.26±0.43 c	3.90±0.14 a
0.5% Sucrose	16.17±1.93 a	11.58±0.92 a	3.84±0.64 a

Numbers with different letters in the same column refer to significant differences among the treatments ($P \leq 0.05$).

Effect of cinnamic acid and sucrose on the respiration rate in cucumber

To further investigate the effects of CA and sucrose on the energy metabolism, the respiration rates in cucumber germinated seeds after exposure to CA and sucrose were measured (Table 2). Compared to control, the total respiration rate (V_T) decreased by

Table 2. Effects of cinnamic acid and sucrose on the respiration rate in cucumber germinated seeds

Treatment	Respiration rate (μmol $O_2 \cdot g^{-1}$ $FW \cdot h^{-1}$)	
	V_T	V_{KCN}
Distilled Water	15.52±1.24a	5.10±0.43 b
0.25mM Cinnamic acid	9.02±0.23 b	6.45±0.31 a
0.5% Sucrose + 0.25mM Cinnamic acid	12.77±0.91a	5.05±0.31 b
0.5% Sucrose	15.53±0.65a	3.30±0.32 c

Numbers with different letters in the same column refer to significant differences among the treatments ($P \leq 0.05$).

41.88%, while the KCN-resistant respiration rate (V_{KCN}) was increased by 26.47% in CA-treated seedlings. In contrast, 0.5% sucrose treatment decreased V_{KCN} whilst V_T was almost independent on sucrose treatment. Importantly, CA-induced changes in respiration were attenuated when 0.5% sucrose were applied.

Effect of cinnamic acid and sucrose on the expression of genes involved in the oil mobilization

To investigate the molecular mechanism of CA-induced inhibition in cucumber post-germinative growth, we analyzed the transcript levels of *Lox*, *Ms*, *Icl* and *Pck* genes, which were involved in β-oxidation, the glyoxylate cycle, and gluconeogenesis, respectively. In comparison to control, the transcript levels of *Lox*, *Ms*, *Icl* and *Pck* declined to 24.24%, 45.67%, 55.84% and 60.29% of the control, when treated with 0.25 mM CA (Fig. 2). Exposure to sucrose resulted in 20.52%, 29.79% and 36.48% increase in the transcript levels in *Lox*, *Ms* and *Icl* but had little effect on the transcript level of *Pck*. Significantly, the transcript levels of *Lox*, *Ms*, *Icl* and *Pck* in the treatment of both sucrose and CA were higher than or not different from those of control.

DISCUSSION

Cinnamic acid is a principal autotoxin among different benzoic and cinnamic acid derivatives identified from cucumber root exudates (25,26). Previous studies showed that allelopathic agents of *Acacia nilotica* and *Eucalyptus globulus* decreased both the sugar content and seedling growth of pea and sorghum, respectively (1,2). In our experiment, exposure to CA resulted in significant decreases in both the radicle length and the sugar content in cucumber. It is interesting to note that addition of sucrose restored the inhibited growth of cucumber radicles induced by autotoxins, which is consistent with a recent report that sucrose could alleviate the inhibition of cress germination induced by 6-methoxy-benzoxazolin-2(3H)-one (MBOA) (14). Moreover, due to the lack of sugars, the substrates for respiration, the total respiration rate was also inhibited when treated with CA. However, the sugar content and the respiration rate were close to the level of the control when treated with the combination of CA and sucrose. These results suggest that shortages of sugars, which serve as life-sustaining compounds in plant growth, may be the major cause of the autotoxin-induced growth inhibitions.

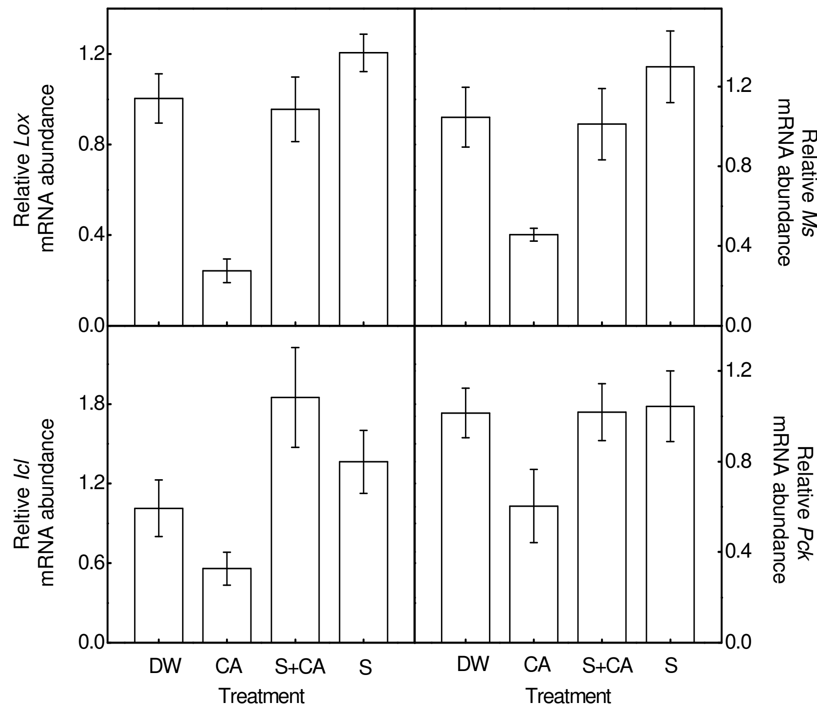

Figure 2. Effects of cinnamic acid and sucrose on the transcript levels of genes involved in the oil mobilization in cucumber. The concentrations of cinnamic acid (CA) and sucrose (S) were 0.25 mM and 0.5% respectively. Distilled water (DW) was set as the control. Samples were collected at 24 h after treatments. Data are the means of three replicates, with standard errors shown by vertical bars. Expression levels by qRT-PCR are expressed as a ratio of the control, which is set as 1.

In oilseed such as cucumber, sugar is synthesized from the stored oil in seed germination progression. The glyoxylate cycle operates during mobilization of seed triglycerides for seedling growth, where it has a key role in conversion of acetyl CoA from fatty acid β-oxidation into oxaloacetate and subsequently into sugar (4, 8). In this study, the expression of four key genes (*Lox*, *Ms*, *Icl* and *Pck*) involved in the conversion from oil to sugar during cucumber seed germination was analyzed. *Lox* (lipoxygenase) catalyzes triacylglycerol (TAG) to fatty acids, which was the first committed step of the turnover of the lipids. *Icl* (isocitrate lyase) and *Ms* (malate synthase) are two key enzymes unique to the glyoxylate cycle, which are used as marker genes in investigations of glyoxylate cycle. *Pck* (phosphoenolpyruvate carboxykinase) has been shown to play a critical role in gluconeogenesis (8). It was found that CA significantly repressed the expression of *Lox*, *Icl*, *Ms* and *Pck* genes, the process of oil mobilization was largely inhibited and thus the sugar synthesis was greatly impaired. In contrast, addition of sucrose, the final product of oil transformation, could restore the down-regulation of gene expression caused by CA.

Accordingly, addition of sucrose could effectively maintain the normal expression levels of genes involved in the oil mobilization, and finally make up the deficit in sugar content caused by CA.

It has been shown that 25 mM and 50 mM sucrose could repress the expression of *Ms* and *Icl* genes in cucumber roots due to a feedback inhibition mechanism (9,13). In our experiment, exogenous 0.5% sucrose (about 15 mM) did not inhibit the expression of those genes involved in the oil mobilization. This discrepancy could be attributed to the difference in the concentration of sucrose applied. On the contrary, CA inhibited seedling growth by inhibiting expression of these genes. Interestingly, the effects of CA and sucrose applied together were not additive in cucumber germinated seeds, suggesting that these two substances may function in interacting pathways to regulate gene expression and seedlings growth.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (2009CB11900), National Natural Science Foundation of China (40571083; 3050344; 30671428) and PhD Program of MOE.

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