INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17F–095/2017/19–6–1640–1644
DOI: 10.17957/IJAB/15.0508
http://www.fspublishers.org

Full Length Article

Responses of Root Border Cells of Cucumber (*Cucumis sativus*) and Pumpkin (*Cucurbita moschata* × *Cucurbita maxima*) Seedlings to Cinnamic Acid Stress

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Abstract

In the present study, effects of cinnamic acid (CA) concentrations on root border cells (RBCs) of the seedlings of cucumber and pumpkin were investigated. Two different concentrations of CA along with a control i.e., 0, 0.125 and 0.250 mmol L^{-1} were used. Their effects on numbers, survival rates, mucigel thicknesses, apoptosis rates of RBCs and the root activities were monitored. Survival rates of RBCs and root activities of both cucumber and pumpkin were decreased at 0.125 and 0.250 mmol L^{-1} CA. These inhibitory effects were more significant on cucumber. However, the mucigel thicknesses and apoptosis rates in RBCs of both cucumber and pumpkin were increased at 0.125 and 0.250 mmol L^{-1} concentrations of CA. In comparison with cucumber, greater mucigel thickness and lower apoptosis rates of RBCs were detected in pumpkin. At a concentration of 0.250 mmol L^{-1} CA, while RBCs attached to the root tips, root activities decreased by 18.8% in cucumber. This change was insignificant in pumpkin. After removing the RBCs, the root activities of both cucumber and pumpkin were sharply inhibited by 0.250 mmol L^{-1} CA. This inhibition was higher in cucumber than pumpkin. It has indicated that, RBCs could increase plant's resistance to CA stress, which was characterized by low apoptosis rate and thick mucigel. The resistance of RBCs in pumpkin to CA stress was stronger than RBCs of cucumber. © 2017 Friends Science Publishers

Keywords: Apoptosis; Cucumber; Pumpkin; Cinnamic acid; Stress; Root border cells

Introduction

In a large cultivated area with multiple cropping system, cucumber often suffer from succession cropping obstacle which was mainly caused by the autotoxin secreted by plant root (Qiao *et al*., 2014; Bu *et al*., 2016). Existence of autotoxin has commonly been observed in the extractives and exudates of taro, cucumber and watermelon (Yu *et al*., 2000; Asao *et al*., 2003). Growth of these plants is only inhibited by autotoxin formation, while it had no effects on some other plants (Ding *et al*., 2007).

Root border cells (RBCs) are living cells, which remain attached to root tips. These cells are able to protect the root tips from toxic substances and microbes (Iijima *et al*., 2003). Recent research results show that RBCs could protect root tips from toxins like Al^{3+}, Fe^{2+} and Cu^{2+} (Xing *et al*., 2008; Cai *et al*., 2013; Chen *et al*., 2017). Attack by parasitic nematodes, pathogenic bacteria and infectious fungi and toxicity produced by high-concentration of CO_2 could also be protected by RBCs (Zhao *et al*., 2000; Wuyts *et al*., 2006; Knox *et al*., 2007; Jaroszuk-Sciseł, *et al*., 2009). However, it is unclear how the RBCs protect root tips from autotoxins.

Cinnamic acid (CA), an important autotoxin from cucumber root exudate, was used as a typical allelochemical in many researches (Yu and Matsui, 1994; Ding *et al*., 2007). However, it has little effect on pumpkin, being common rootstock of cucumber. The aim of the present investigation was to find the possible reasons for the interspecific response differences due to autotoxin (CA) in both cucumber and pumpkin. Cucumber and pumpkin have been considered as CA-sensitive and CA-tolerated crops, respectively, used as experimental material in the present investigation. So, the final goal of the study was to find the effects of CA on the numbers, survival rates, mucigel thicknesses and apoptosis rates of cucumber and pumpkin RBCs as well as the root activities of seedlings.

Materials and Methods

Plant Materials and Treatments

Plant materials used in the present experiment were cucumber (*Cucumis sativus* L., cultivar: Jinyan No. 4) and pumpkin (*Cucurbita moschata* × *Cucurbita maxima*, cultivar: Japanese Cedar). Seeds of both the plants were disinfected with 70% ethyl alcohol for one minute followed by rinsing those 3 times with sterile distilled water. Thereafter the seeds were soaked in sterile distilled water for 4 h and

To cite this paper: Qiao, Y., Y. Zhang and H. Zhang, 2017. Responses of root border cells of cucumber (*Cucumis sativus*) and pumpkin (*Cucurbita moschata* × *Cucurbita maxima*) seedlings to cinnamic acid stress. *Int. J. Agric. Biol*., 19: 1640–1644

transferred to an incubator for germination at 28℃. The germinated seeds were kept in beakers and sealed by covering the top with plastic film and rubber band. The seeds were then sown onto the hole of filter paper (30 seeds for each beaker). The filter paper containing the seeds was placed onto an iron wire placed in a large beaker (1000 mL cap.) containing 150 mL distilled water. The whole setup was placed in an incubator at 28℃ in the dark for allowing the growth of root tips. When the root tip of seedlings reached about 5 mm in length, the beakers were divided into 2 groups with 24 in Group 1 and 12 in Group 2.

Group 1: Primary roots were sprayed with 0, 0.125 and 0.250 mmol L^{-1} CA (pH 6.0), respectively, 5 times at 2 h interval. The root activities and the numbers, survival rates, mucigel thickness, and apoptosis rate of RBCs were tested when the roots were grown to 20–25 mm.

Group 2: The primary roots were rinsed 5 times with small water flow (sterile distilled water) for about 30 s every 4 h and thereafter the RBCs were removed (Cai *et al*., 2013). The primary roots without RBCs were sprayed with 0, 0.125 and 0.250 mmol L^{-1} CA at 2 h intervals. The root activities were tested until the primary roots grown to 20–25 mm. In each batch 20 seedlings were used and all experiments were replicated three times.

Numbers and Survival Rates of RBCs

From the previously collected samples, 9 root tips were selected and placed in 200 µL tube and soaked in 50 µL distilled water for 1 min. The tube was gently stirred with Pasteur pipette to obtain considerable RBCs. From this suspension, 10 µL of RBCs was mixed with FDA-PI (fluorescein diacetate-propidium iodide) mixture (FDA 25 µg mL^{-1}, PI 10 µg mL^{-1}) for 10 min. A microscopic examination was conducted under the fluorescence microscope (Olympus BX 51-DP 70). Living cells were green (FDA fluorescence) while dead cells were red (PI fluorescence). Number of live and dead cells was measured by hemocytometer (Pan *et al*., 2004).

Mucigel Thickness of RBCs

A mixture, made up by mixing 10 µL RBCs suspension and 10 µL India ink was dripped onto a glass slide. The mucigel of RBCs was observed and measured with the help of an optical microscope. The mucigel thickness of RBCs was measured at 3 different positions and the mean values were taken (Cai *et al*., 2013; Qiao *et al*., 2013).

Apoptosis Rate of RBCs and Root Activity

The RBCs suspension was allowed to settle for 2 h, then the distilled water was removed and a little 95% ethyl alcohol was added and settled further for 30 min. The cell suspension was rinsed with phosphate buffer 2 times and then mixed with Hochest-33258 (10 µg mL^{-1}) staining liquor at a ratio of 1:1. Then the morphological characteristics and number of living and apoptotic cells were observed under a fluorescence microscope (Ma *et al*., 2011). Root activity was determined through triphenyl tetrazolium chloride method (Liu *et al*., 2014).

Statistical Analysis

A one-way analysis of variance was carried out through DPS7.05 software. Then a significance analysis was conducted using LSD test.

Results

Effects of CA on Number and Survival Rates of Cucumber and Pumpkin RBCs

Low concentration (0.125 mmol L^{-1}) of CA showed a slight inhibitory effect on the numbers of RBCs in cucumber and pumpkin. Whilst the high-concentration of CA (0.250 mmol L^{-1}) decreased higher number of RBCs in cucumber than RBCs of pumpkin. The rate of decrease of RBCs in cucumber was 29.2% while a slight change in pumpkin was observed (Fig. 1).

According to the results of FDA-PI dying test, the live RBCs were green and the dead RBCs were red (Fig. 2). High-concentration CA could inhibit the survival rate of cucumber RBCs more intensively compared with pumpkin RBCs (Fig. 1). The survival rates of cucumber RBCs treated with 0.25 mmol L^{-1} CA decreased by 14.6%, changing slightly for 0.125 mmol L^{-1} CA. The changes in the survival rate of pumpkin RBCs were not obvious for the above mentioned two concentrations of CA.

Effects of CA on Mucigel Thickness of Cucumber and Pumpkin RBCs

High-concentration of CA exhibited a promotive effect on the mucigel thickness of RBCs, which was higher in pumpkin (Fig. 3 and 4). After treatment with 0.125 mmol L^{-1} CA, the differences of the mucigel thickness of RBCs between cucumber and pumpkin were not significant. Compared with 0.125 mmol L^{-1} CA, the mucigel thickness of RBCs in cucumber treated with 0.250 mmol L^{-1} CA increased slightly, while 36.8% increase was found for the same treatment in pumpkin.

Effects of CA on Apoptosis Rates of Cucumber and Pumpkin RBCs

The cell nuclei of normal RBCs were round and small in shape and presented even texture and bright blue spots. However, the cell nuclei of apoptotic RBCs were diffused, irregular and larger. It presented bright and dark texture and blue fragments, which were dark (Fig. 5).

The apoptosis rate of pumpkin RBCs was lower than that of cucumber RBCs. CA had a promotive effect on the

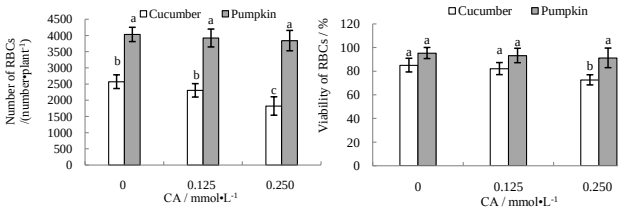

Fig. 1: Effect of CA on number and viability of RBCs in cucumber and pumpkin plants

Different letters in the figure mean significant at 0.05. The same as bellow

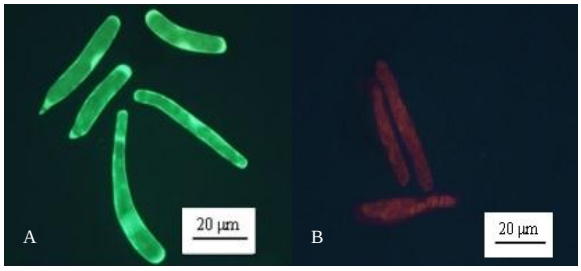

Fig. 2: RBCs of cucumber and pumpkin were dyed by FDA-PI

The live RBCs are green (A), the dead RBCs are red (B)

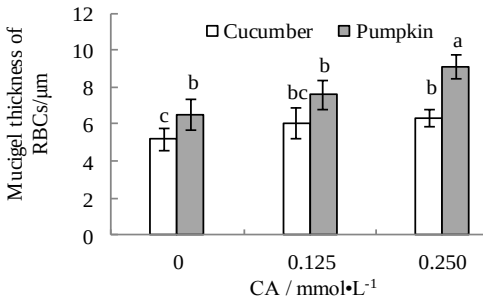

Fig. 3: Effect of CA on mucigel thickness of RBC of cucumber and pumpkin

apoptosis rate of RBCs in two crops, cucumber being obviously higher than pumpkin. After treated with 0.125 mmol L^{-1} CA, the apoptosis rate of cucumber RBCs increased by 2.2%, while no significant change was observed in pumpkin. However, after treatment with 0.250 mmol L^{-1} CA, the apoptosis rates of cucumber and pumpkin RBCs increased by 4.6 and 3.0%, respectively (Fig. 6).

Effects of CA and RBCs on Root Activities of Cucumber and Pumpkin

Root tips having RBCs attached to it and a treatment with 0.125 mmol L^{-1} CA showed no significant inhibitory effects on the root activities of both cucumber and pumpkin. The root activity decreased by 18.8% for cucumber, but slightly for pumpkin under a treatment of 0.250 mmol L^{-1} CA (Fig. 7).

After removing the RBCs, 0.125 mmol L^{-1} CA showed no significant inhibitory effects on the root activities of cucumber and pumpkin. But the root activities of both cucumber and pumpkin were significantly inhibited by 0.250 mmol L^{-1} CA. In this case, the root activities of cucumber and pumpkin decreased by 25.1 and 17.8%, respectively.

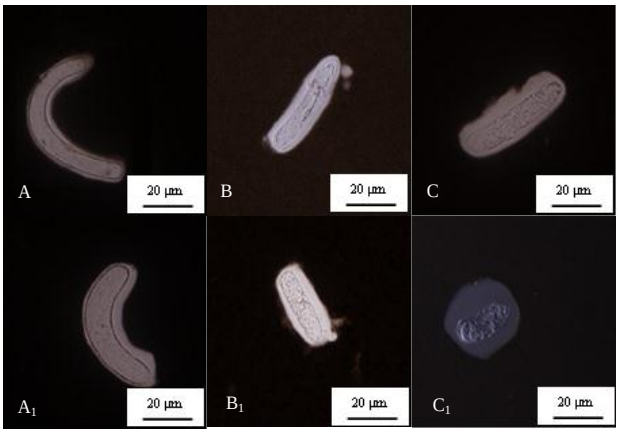

Fig. 4: Mucigel thickness around RBCs from cucumber and pumpkin subjected to treatment with CA

RBCs of cucumber (A-C) and pumpkin (A_1-C_1) exposure to 0 mmol L^{-1} CA (A and A_1), 0.125 mmol L^{-1} (B and B_1) and 0.250 mmol L^{-1} (C and C_1)

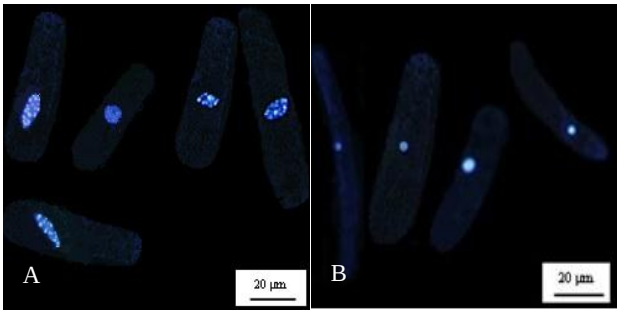

Fig. 5: The dispersed RBC from root-tips of cucumber and pumpkin were dyed by Hoechest-33258. Normal RBCs, (B) Apoptosis RBCs

Discussion

Cinnamic acid (CA), one of the major autotoxins, was an important inducer of succession cropping obstacle of cucumber (Yu *et al.*, 2009). According to the previous studies, pumpkin had a stronger stress resistance than cucumber (Ding *et al.*, 2007). There were some studies on the physiological differences, such as photosynthesis, nutrient uptake, the reactive oxygen species metabolism and gene expression between two crops under CA stress (Yu *et al.*, 2000; Ding *et al.*, 2007; Zhang *et al.*, 2009), but few researches were addressed to the response of root tips to CA.

The RBCs could resist stress from toxic metals like Al^{3+} and Cu^{2+} (Cheng *et al.*, 2008; Cai *et al.*, 2013; Peng *et al.*, 2016). However, it was unclear on the RBCs resistance to CA until now. We found that 0.250 mmol L^{-1} CA inhibited the

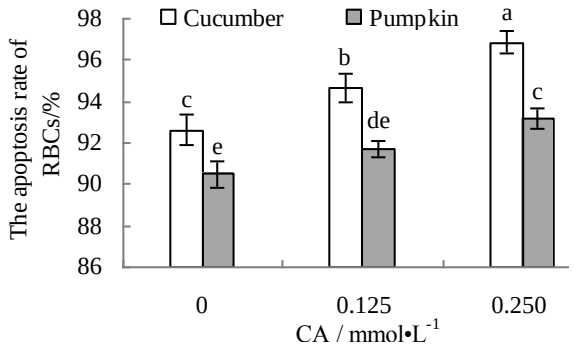

Fig. 6: Effect of different concentration CA on the apoptosis rates of root border cells of cucumber and pumpkin

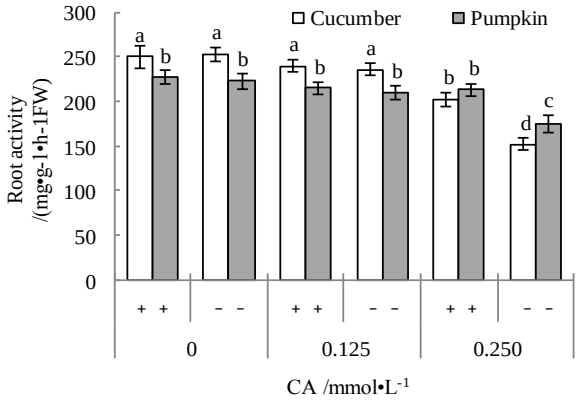

Fig. 7: Effect of CA and RBCs on root activity in cucumber and pumpkin

+: Root tips with attached RBC; -: Root tips with detached RBCs

root activity of cucumber significantly, and this inhibitory effect was stronger if the RBCs were removed from root tips. Under the protection of RBCs, 0.250 mmol L^{-1} CA had insignificant inhibitory effect on the root activity of pumpkin, the same concentration became significant when the root was not protected by RBCs (Fig. 7). CA inhibited the root activities of cucumber and pumpkin, and inhibitory effects could be alleviated by RBCs. Besides, the RBCs of different plants varied in the ability of CA resistance. In resisting CA stress of RBCs, cucumber was weaker than pumpkin (Fig. 7). In this respect, further studies are needed to find the differences of RBCs in resisting CA stress among other plants.

The RBCs were able to metabolize freely after they were separated from the root tips. As their separation from root tip proceeds, the cells synthesize and secrete hydrated mucilage that contains polysaccharides, secondary metabolites, antimicrobial proteins and extracellular DNA to protect the RBCs (Driouich *et al*., 2013). In this study, CA increased the mucigel thickness in two crops significantly (Fig. 3). The reason might be that when plant was subjected to allelochemicals toxicity, the RBCs would increase the mucigel layer by releasing chemicals outside to adsorb or chelate CA and block CA to permeate further into the cell. The process is related to extracellular DNA and proteins present in the mucigel layer (Driouich *et al*., 2013).

According to Ma *et al.* (2011), apoptosis occurs under physical or chemical stress. CA stress also caused dispersion of nuclei in RBCs of cucumber and pumpkin (Fig. 6) and raised apoptosis rate leading to final death of cells. These were accorded in the findings of present study, that CA decreased survival rate in CA treated RBCs (Fig. 1). The apoptosis rates and death rates of pumpkin RBCs were low than those of cucumber RBCs under CA stress. Further studies should be carried out to detect whether the present findings were suitable to other stress factors and species or not.

Conclusion

With low apoptosis rate, high survival rate and thick mucigel, pumpkin RBCs were much better able to resist CA stress than cucumber RBCs.

Acknowledgements

This work was financially supported by Natural Science Foundation of Hebei Province (C2015105091).

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(Received 17 August 2017; Accepted 11 October 2017)