Journal of Stress Physiology & Biochemistry, Vol. 9 No. 2 2013, pp. 307-317 ISSN 1997-0838

ORIGINAL ARTICLE

Phytotoxic Effects of Cinnamic Acid on Cabbage (*Brassica oleracea* var. capitata)

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Received January 15, 2013

The present study deals with the effects of exogenous application of cinnamic acid (CA) on growth and metabolism in growing seedlings of *Brassica oleracea* var. capitata (cabbage) in hydroponic culture. CA was added at 0.5, 1.0 and 1.5 mM concentrations. CA has shown inhibitory effects on shoot and root length, fresh and dry weight of seedlings. CA significantly decreased the photosynthetic pigments, nitrate reductase activity and protein content. Graded concentrations of CA increased lipid peroxidation and sugar content. The increasing concentrations of CA significantly increased the antioxidative enzyme activities viz. superoxide dismutase, catalase, peroxidase against the oxidative stress caused by CA.

Key words: Allelopathy, cabbage, cinnamic acid, lipid peroxidation

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Key words: Allelopathy, cabbage, cinnamic acid, lipid peroxidation

Abbreviations: CA, Cinnamic acid; CAT, Catalase; DW, Dry weight; EDTA, Ethylene diamine tetra acetic acid; FW, Fresh weight; LP, Lipid peroxidation; MDA, Malondialdehyde; NBT, Nitro blue tetrazolium; NEDD, N-1-napthyl-ethylene diamine dihydrochloride; NR, Nitrate reductase; POX, Peroxidase; ROS, Reactive oxygen species; SOD, Superoxide dismutase.

Allelopathy, an important component of plant interference has been accomplished in a variety of natural and managed ecosystems (Weston *et al*., 2003). Plants affect their neighbouring plants by releasing various secondary metabolites which are known as allelochemicals. Several allelochemicals have been identified and isolated in plants and they affect many ecological and physiological processes in plants, for examples, stomatal closure (Barkosky *et al*., 2000), plant water balance (Barkosky *et al*., 2003), cell division (Anaya *et al*., 1997), membrane permeability (Galindo *et al*., 1999), nutrient uptake (Baar *et al*., 1994), photosynthesis (Baziramakenga *et al*., 1994) respiration (Abrahim *et al*., 2000) and many other metabolic processes.

Cinnamic acid (CA) is a widespread phenolic acid released into soil by root exudates, leaf leachates

and decomposed plant tissues of different plants, for example, cucumber (Yu *et al*., 1997) and alfalfa (Chon *et al*., 2002), quack grass (Baziramakenga *et al*., 1994). Cinnamic acid is the principal autotoxin in root exudates of cucumber and the model allelochemical used in many studies (Ye *et al*., 2004). Benzoic acid and cinnamic acid at 0.02g/L inhibited seed germination and seedling growth of tomato are the main allelopathic substances (Yao, 2007). CA was identified as an allelochemical responsible for allelopathy in root exudates in cucumber (Politycka, 1996). It inhibits the germination and growth when applied exogenously (Chou *et al*., 1976). It was previously studied that different allelochemical including CA reduced seed germination and seedling growth of crops and grass species (Hussain *et al*., 2008).

It was well known that plants under the various stressful conditions such as sub-optimal temperature, high light and salinity and pathogen attacks may generate more reactive oxygen species (Yamamoto *et al*., 2003; Halliwell, 2006; Rhoads, 2006). Under the stress conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms (Foyer *et al*., 2005). ROS can affect the membrane permeability, cause damage to DNA and protein, induce lipid peroxidation and ultimately lead to the programmed cell death. Antioxidative enzyme activities viz. SOD, CAT, POX were found to be increased under allelochemical stress (Romero-Romero *et al*., 2005).

The aim of the present study was to investigate biochemical and biophysical changes in *B.oleracea* var. capitata during allelopathic stress caused by exogenous cinnamic acid in the hydroponic culture.

MATERIALS AND METHODS

The certified seeds of cabbage (*Brassica oleracea* var. capitata) were purchased from certified seed agency of Allahabad, Uttar Pradesh, India. The seeds were sown in February in nursery beds (1mx1m) for experimental plants in The Department of Botany, University of Allahabad (24°47' and 50° 47'N latitude; 81° 91' and 82° 21'E longitude; 78 m above sea level). The seed bed was irrigated as and when required. After 21 days the seedlings were uprooted and washed with distilled water to clean root. Seedlings were transferred in transparent plastic boxes (height 9 cm, width 17 cm, length 23 cm) each containing 2L of Hoagland solution (10 seedlings per box). Hoagland solution was prepared following the method of Hoagland and Arnon (1950). Cinnamic acid in concentrations of 0.5, 1.0 and 1.5 mM were prepared in distilled water and used for treatment. Seedlings in Hoagland nutrient solution without cinnamic acid were taken as control. The boxes were covered with black papers to avoid the algal growth. The experimental boxes were fitted with aerating tubes and mouth of each pore of plastic box was plugged with cotton to hold seedlings in vertical position. The Hoagland nutrient solution with and without CA was changed after 10 days. The experiment was done in the glass house. Boxes were continuously aerated. Sampling was done after 15 days of treatment for biochemical analyses. Morphological parameters were also recorded.

Determination of pigment and protein content

Chlorophyll of experimental plant was extracted with 80% acetone. The amount of photosynthetic pigments was determined as per Lichtenthaler (1987). Fresh leaf (10mg) was homogenized in 10 mL of 80% acetone and centrifuged. Supernatant was taken and optical density was measured at 663nm, 645nm and 470nm. Protein content was determined as per the method of Lowry *et al*.

(1951). The amount of protein was calculated with reference to standard curve obtained from bovine serum albumin.

Lipid peroxidation

Lipid peroxidation was measured in terms of malodialdehyde content as per the method of Heath and Packer (1968). Leaves (200 mg) of test plant were homogenized in 5 mL of trichloroacetic acid (0.1%w/v) and centrifuged at 10000 rpm for 10 min. Malondialdehyde level was used as index of lipid peroxidation and was expressed as nmol g^{-1} fresh weight. One mL supernatant was added to 4 mL 0.5 thiobarbituric acid prepared in 20% trichloroacetic acid. The mixture was incubated at 95^0 C for 30 min. followed by rapid cooling and centrifuged at 10000 rpm for 10 min. The absorbance of supernatant was recorded at 532 nm and corrected for non specific absorbance at 600 nm. Malondialdehyde content was determined using the extinction coefficient of 155 mM cm^{-1}.

Nitrate reductase

Nitrate reductase (EC 1.6.6.1) activity was assayed by modified procedure of Jaworski (1971) based on incubation of fresh tissue (0.25 g) in 4.5 mL medium containing 100 mM phosphate buffer (pH 7.5), 3% KNO_3 and 5% propanol. About 0.4 mL aliquot was treated with 0.3 mL 3% sulphanilamide in 3 N HCL and 0.3 mL 0.02% N-1-naphthyl ethylene diamine dihydrochloride (NEDD). The absorbance was measured at 540 nm. NR activity was calculated with a standard curve prepared from $NaNO_2$ and expressed as µ mol NO_2 g^{-1} FW h^{-1}.

Sugar content

Sugar content was estimated following Hedge and Hofreiter (1962). About 0.25 g of the sample was homogenized in 2.5 mL of 95% ethanol. After centrifugation, the sugar content was determined in the supernatant. The supernatant (1mL) was mixed with 4 mL of anthrone reagent and heated on boiling water bath for 8 min. Absorbance was taken at 620 nm after rapid cooling. Standard curve was prepared from glucose.

Antioxidant enzymes extraction and assay

Enzyme extract was prepared by homogenizing 500 mg leaves in 10 mL of 0.1 M sodium phosphate buffer (pH 7.0). The homogenate was filtered and centrifuged at 15000 g at 4^o C for 30 min. The supernant was collected and used for analyses of superoxide dismutase (EC 1.15.11), catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7).

Superoxide dismutase (SOD) activity was determined by the nitroblue tetrazolium (NBT) photochemical assay method following Beauchamp and Fridovich (1971). The reaction mixture (4mL) contained 63 µM NBT, 13 mM methionine, 0.1 mM ethylene diamintetra acetic acid (EDTA), 13 µM riboflavin, 0.5 M sodium carbonate and 0.5 mL clear supernatant. Test tubes were placed under fluorescent lamps for 30 min and absorbance was recorded at 560 nm. One unit of enzyme was defined as the amount of enzyme which caused 50% inhibition of NBT reduction.

Catalase (CAT) activity was assayed as per the method Cakmak and Marschner (1992). The reaction mixture (2mL) contained 25 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 0.2 mL enzyme extract. The activity was determined by measuring the rate of disappearance of H_2O_2 for 1 min at 240 nm and calculated using extinction coefficient of 39.4 mM^{-1} cm^{-1} and expressed as enzyme unit g^{-1} fresh weight. One unit of CAT was defined as the amount of enzyme required to oxidize 1 µM H_2O_2 $min^{-1.}$

Peroxidase (POX) (EC 1.11.1.7) activities were

assayed following Mc Cune and Galston (1959). Reaction mixture contained 2 mL enzyme extract, 2 mL potassium phosphate buffer, 1 mL 0.1 N pyrogallol and 0.2 ml 0.02% H_2O_2 and determined spectrophotometrically at 430 nm. One unit of enzyme activity was defined as the amount which produced an increase of 0.1 OD per minute.

Statistical analysis

Standard errors of means were calculated in triplicates. In addition, analysis of variance was carried out for all the data generated from this experiment, employing one way ANOVA test using GPIS software 3.0 (GRAPHPAD California USA).

RESULTS

The effects of cinnamic acid on shoot length, root length, fresh weigth and dry weigth of cabbage have been shown in Table 1. Shoot length and root length were decreased in dose dependent manner and maximum decrease of 19.59% and 36.43% was recorded in A_3, respectively. Adverse effect of CA on fresh weight (FW) and dry weight (DW) of cabbage was recorded. FW and DW were found to be reduced significantly in graded manner and maximum decrease of 57.15% and 38.39% respectively was observed at 1.5 mM concentration of CA.

Total chlorophyll and carotenoid contents in the cabbage seedlings significantly decreased in dose dependent manner. Total chlorophyll (51.64%) and carotenoid (78.64%) was maximally decreased in A_3 (Table 2).

The allelochemical decreased protein content. Control seedlings exhibited a maximum amount of protein in the leaves. Highest concentration of CA significantly decreased protein content as compared with control. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content. The MDA content increased significantly by the application of CA in dose dependent manner. Maximum increase of 97.84% was observed in A_3.The nitrate reductase (NR) activity in the leaves of CA treated cabbage seedlings was affected by allelochemical. Activity of NR was significantly decreased in dose dependent manner. The decrease was concentration dependent. Maximum inhibition of 83.56% was recorded in highest concentration. Sugar content was significantly increased with the increasing concentration of CA as compared with control. Maximum accumulation of sugar (2.37 times) was observed in A_3 (Table 3).

Important constituents of antioxidative enzyme system were also analyzed. When compared with control, the antioxdative enzymes viz. SOD, POX and CAT activities were enhanced by allelochemical in dose dependent manner in all treatments. Significant increase of 1.52, 1.77 and 1.56 times, respectively was recorded under A_3 treatment as compared with control (Table 4).

Table 1: Effects of cinnamic acid on shoot length, root length, fresh weight and dry weight of cabbage seedlings.

Treatments	Shoot length (cm)	Root length (cm)	Fresh weight (g/plant)	Dry weight (g/plant)
C	12.25 ± 0.72	24.7 ± 1.32	11.81 ± 0.34	1.39 ± 0.031
A_1	12.05 ± 0.37	22.4 ± 1.09	10.09 ± 0.24c	1.35 ± 0.004
A_2	11.85 ± 0.08	17.4 ± 1.18	7.02 ± 0.29al	0.85 ± 0.012al
A_3	9.85 ± 0.77	15.7 ± 1.96c	5.77 ± 0.32al	0.86 ± 0.005al

Data are mean of three replicates ± SEM. [a] $p<0.001$, [c]$p<0.05$ versus C, [l]$p<0.001$ versus A_1. C, control; A_1, 0.5mM A_2,1.0mM and A_3, 1.5mM concentrations of cinnamic acid.

Table 2: Effects of cinnamic acid on the pigment contents of cabbage seedlings.

Treatments	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total Chlorophyll (mg/g FW)	Carotenoids (mg/g FW)
C	0.849 ± 0.008	0.307 ± 0.019	1.156 ± 0.012	0.065 ± 0.016
A_1	0.751 ±0.002a	0.243 ± 0.006c	0.994 ± 0.008a	0.036 ±0.007
A_2	0.536 ± 0.003al	0.188 ± 0.007a	0.585 ± 0.003al	0.032 ± 0.001
A_3	0.397 ± 0.005alp	0.175 ± 0.008an	0.559 ± 0.004al	0.014 ± 0.001c

Data are mean of three replicates ± SEM. [a] p<0.001, [c] p<0.05 versus C, [l] p<0.001, [n]p<0.05 versus A_1, [P]p<0.001 versus A_2. C, control; A_1, 0.5mM: A_2, 1.0mM and A_3, 1.5mM concentrations of cinnamic acid.

Table 3: Effects of cinnamic acid on protein content, sugar, nitrate reductase activity and lipid peroxidation of cabbage seedlings.

Treatments	Protein (mg/g FW)	Sugar (mg/g FW)	NR (µmol NO_2 g^{-1} FW h^{-1})	LP (n mol g^{-1} FW)
C	27.85 ± 0.37	12.00 ± 1.44	91.25 ± 0.72	11.12 ±0.50
A_1	24.975 ± 1.16	14.00 ± 1.15	29.5 ± 2.59 a	16.87 ±0.64 a
A_2	24.775 ± 0.24	23.75 ± 2.16 bn	21. 25 ± 2.16 a	20.62 ± 0.36 am
A_3	18.1 ± 0.14 alp	28.50 ± 1.15 al	15.00 ± 1.44 am	22.00 ± 0.14 al

Data are mean of three replicates ± SEM. [a] p<0.001, [b]p<0.01 versus C, [l]p<0.001, [m]p<0.01, [n]p<0.05 versus A_1, [P]p<0.001 versus A_2. C, control; A_1, 0.5mM; A_2,1.0mM and A_3, 1.5mM concentrations of cinnamic acid.

Table 4: Effects of cinnamic acid on antioxidant enzyme activity of cabbage seedlings

Treatments	SOD (EU g^{-1} FW)	CAT (EU g^{-1} FW)	POX (EU g^{-1} FW)
C	63.52 ± 2.54	8.42 ± 0.28	570.27 ± 1.34
A_1	74.02 ± 8.05	10.08 ± 0.05c	581.72 ± 17.16
A_2	86.22 ± 0.84	11.25 ± 0.38a	631.47 ± 2.09c
A_3	97.13 ± 4.32b	13.22 ± 0.17alq	671.30 ± 8.34am

Data are mean of three replicates ± SEM. [a] p<0.001, [b]p<0.01, [c] p<0.05 versus C, [l]p<0.001, [m]p<0.01, versus A_1, [q]p<0.01 versus A_2. C, control; A_1, 0.5mM; A_2, 1.0 mM and A_3, 1.5mM concentrations of cinnamic acid.

DISCUSSION

A variety of physiological and biochemical processes were altered under the allelochemical stresses. Poor germination and seedling growth rate under allelochemical stress were observed in tomato (Sannigrahi *et al*., 2005). Under CA stress, the reduction in plant growth (root length, shoot length) and biomass was observed in *Lactuca sativa* (Hussain *et al*., 2010). Similar results were observed in literature (Ye *et al*., 2004). The CA (0.5-0.25 mM) has recently been shown to cause oxidative stress in cucumber roots (Ding *et al*., 2007). Altered root morphology in *Pisum sativum* was reported which was caused by the action of cinnamic acid derivatives (Vaughan and Ord, 1991). The effects of benzoic acid and cinnamic acids on the cell plasma membrane in intact soybean (*Glycine max* L. cv. Maple Bell) seedlings were reported by by Baziramakenga *et al*. (1995). Trans- cinnamic acid inhibited the root elongation of *L. sativa* L. (Fujita and Kabo, 2003). Likewise, Ding *et al*. (2007) reported that CA significantly inhibited the growth of cucumber. The three main processes of photosynthesis, stomatal control of CO_2 supply, thylakoid electron transport (light reaction), and carbon reduction cycle (dark reaction) were

significantly affected by allelochemicals (Zhou *et al*., 2006).

Protein content in all treatments was gradually reduced due to the effect of CA. Phenolic acids decreased the incorporation of certain amino acids into proteins and thus affected the rate of protein synthesis (Baziramakenga *et al*., 1997). Mersie and Singh (1993) demonstrated that ferulic acid, an allelochemical, also inhibited protein synthesis and reduced the incorporation of (^{14}C) leucine. They also reported that the maximum inhibition of protein synthesis by chlorogenic acid and vanillic acid was found in velvate leaf (*Abutilon theophrasti* Medik). In *Lactuca* protein synthesis was inhibited when treated with CA (Einhellig, 1996).

Under the allelochemical stress the reduction in the photosynthetic pigments was recorded. In previous studies, similar result was also obtained by Bagavathy and Xavier (2007) in sorghum plants. The reduction under allelochemical treatment may be due to the inhibition of chlorophyll biosynthesis and/or stimulation of chlorophyll degradation (Yang *et al*., 2004). Allelochemicals impeded the synthesis of porphyrin precursor of chlorophyll biosynthesis (Rice, 1984).

Declined carbon- skeleton, energy, electron donors which are prerequisite for NR activity may be due to the harsh reduction of photosynthethic machinery (Kaiser *et al*., 1993).The reduced synthesis or induction of enzymes may be another possible reason of reduced NR activity (Chen *et al*., 1983). Due to the effect of allelochemical the absorption of nitrate by roots decreased and the transport of nitrate from roots to leaves consequently reduced the foliar nitrate (Abd-El Baki *et al*., 2000).

CA treatment increased membrane damage and lipid peroxidation. Membrane damage and lipid peroxidation are common indicator of allelochemical stresses (Singh *et al*., 2006). Past studies of phenolic acid have indicated that they affect membrane permeability and plant growth (Doblinski *et al*., 2003).

The increased activity of SOD under allelopathic stress was observed by Bias *et al*. (2003) and Xiao *et al*. (2006). The enhanced activities of SOD and CAT were observed in various plants like cucumber (Romero-Romero *et al*., 2005), tomato (Macias *et al*., 2002), and mustard (Oracz *et al*., 2007) under the allelochamical stress. SOD scavenges the highly reactive free radicals ($O_2^{\cdot-}$) by converting them into H_2O_2. The toxic H_2O_2 was detoxifying by CAT and POX activities. The antioxidative enzyme system provides the better survival of plants under stressful condition (Mishra *et al*., 2006).

CONCLUSIONS

In the present study, cinnamic acid has shown inhibitory effects on *Brassica oleracea* var. capitata. CA decreased seedling growth by inhibiting root and shoots length, FW, DW, pigment and protein content. LP and sugar content were found to be increased under allelochemical stress. To cope with CA toxicity, cabbage induced several antioxidative enzyme activities viz. SOD, CAT, POX.

ACKNOWLEDGMENTS

The authors are thankful to University grants commission, New Delhi, India for the award of UGC-RGNF to Sunaina.

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