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Research article

A chlorophyll fluorescence analysis of photosynthetic efficiency, quantum yield and photon energy dissipation in PSII antennae of *Lactuca sativa* L. leaves exposed to cinnamic acid

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ABSTRACT

This study investigated the effects of cinnamic acid (CA) on growth, biochemical and physiological responses of *Lactuca sativa* L. CA (0.1, 0.5, 1.0 and 1.5 mM) treatments decreased plant height, root length, leaf and root fresh weight, but it did not affect the leaf water status. CA treatment (1.5 mM) significantly reduced F_v, F_m, photochemical efficiency of PSII (F_v/F_m) and quantum yield of PSII (ΦPSII) photochemistry in *L. sativa*. The photochemical fluorescence quenching (qP) and non-photochemical quenching (NPQ) were reduced after treatment with 1.5 mM CA. Fraction of photon energy absorbed by PS II antennae trapped by "open" PS II reaction centers (*P*) was reduced by CA (1.5 mM) while, portion of absorbed photon energy thermally dissipated (*D*) and photon energy absorbed by PSII antennae and trapped by "closed" PSII reaction centers (*E*) was increased. Carbon isotope composition ratios ($\delta^{13}C$) was less negative (−27.10) in CA (1.5 mM) treated plants as compared to control (−27.61). Carbon isotope discrimination ($\Delta^{13}C$) and ratio of intercellular CO_2 concentration (*ci*/ca) from leaf to air were also less in CA treated plants. CA (1.5 mM) also decreased the leaf protein contents of *L. sativa* as compared to control.

1. Introduction

Plants synthesize an array of chemicals that are involved in a variety of plant–plant, plant-microbe, and plant–herbivore interactions [34,61]. These chemicals are delivered into the rhizosphere mainly through volatilization, leaching, decomposition of residues, root exudates and mediate plant-to-plant interactions [28]. The chemical interactions have been proposed to have profound effects on the evolution of plant communities through the loss of susceptible species and by imposing selective pressure favoring individuals resistant to inhibition from a given allelochemical [3]. Investigation of the chemical composition of allelopathic plants reveals that a great number of secondary metabolites serve as allelochemicals. Among them, benzoic and cinnamic acid derivatives are frequently identified from soils or root exudates [10,34,64]. Root growth inhibition by benzoic and cinnamic acids has been widely observed [5,12,30,35,56]. Furthermore, exogenous addition of allelochemicals can influence physiological and biochemical reactions, such as photosynthesis, respiration, water and nutrient uptake, and generation of reactive oxygen species [17,19], as well as induce changes in gene expression [26,28]. All such physiological, biochemical, and transcriptional changes are implicated directly or indirectly in plant growth inhibition.

Cinnamic acid (CA) is a widespread phenolic acid released into soil by root exudates, leaf leachates, and decomposed plant tissues of different plants e.g. quack grass (*Elytrigia repens*) [9], cucumber (*Cucumis sativus* L.) [64], alfalfa (*Medicago sativa* L.) [12]. The CA, and *o*-,*m*-, and *p*-coumaric acids inhibited the growth of etiolated seedlings of lettuce at concentrations higher than 10^{-4} M and seed germination above 10^{-3} M [39]. Baziramakenga et al. [7–9], concluded that benzoic acid and *trans*-cinnamic acid were responsible for negative allelopathic effects of quack grass on soybean by inhibiting root growth, reducing the root and shoot dry mass, by altering ion uptake and transport, and by reducing chlorophyll content.

Abbreviations: Carbon%, carbon concentration; *ci*/ca, ratio of intercellular CO_2 concentration from leaf to air; *D*, portion of absorbed photon energy thermally dissipated; *E*, photon energy absorbed by PSII antennae and trapped by "closed" PSII reaction centers; F'_m, maximal fluorescence; F'_o, minimal fluorescence; F'_v, variable fluorescence level from light-adapted state; F_m, maximal fluorescence level from dark-adapted leaves; F_o, initial fluorescence level from dark-adapted leaves; F_v, variable fluorescence level from dark-adapted leaves; LOP, leaf osmotic potential; ΦPSII, maximum quantum yield of PSII electron transport; NPQ, non-photochemical fluorescence quenching; F_v/F_m, efficiency of photosystem II photochemistry in the dark-adapted state; *P*, Fraction of photon energy absorbed by PS II antennae trapped by "open" PS II reaction centers; Q_A, quinone type electron acceptor A; qP, photochemical quenching; RWC, relative water content; $\delta^{13}C$, composition of carbon isotope ratios; $\Delta^{13}C$, carbon isotope discrimination.

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Although the mechanisms by which physiological and metabolic processes are affected by allelochemical stress in higher plants are not fully understood [41], the photosynthetic process is often the first to be inhibited by allelochemical stress [29]. In the photosynthetic apparatus, light is absorbed by antenna pigments and excitation energy is transferred to the reaction centers of the two photosystems (PSI and PSII). There, the energy drives the primary photochemical reactions that initiate the photosynthetic energy conversion. Under optimal conditions, the primary photochemistry occurs with high efficiency and the dissipation of absorbed light energy by chlorophyll fluorescence is low and mainly emitted by PSII associated chlorophyll molecules [38,40]. This property of green plants is known to provide a simple and powerful non-intrusive means to determine the health state of plants [40]. In toxicity investigations, the saturation pulse method using pulse-amplitude-modulate (PAM) fluorometer is commonly used to study photosynthesis and is able to provide different fluorescence parameters giving reliable information of the effect of biotic and abiotic stress on plant physiology [37,58]. Among these parameters, the maximum quantum efficiency of PSII primary photochemistry (F_v/F_m) and, photochemical and non-photochemical quenching (qP and NPQ) are very useful for laboratory and field studies [14,52]. The two types of quenching occur in chloroplast: the photochemical quenching (qP) directly dependent to the electron transport, and the non-photochemical quenching (NPQ) representing all non-radiative mechanisms involved in dissipation of excess absorbed light energy. NPQ is composed of three different components according to their relaxation kinetics in darkness following a period of illumination, as well as their response to specific inhibitors [49]. Analysis of chlorophyll fluorescence emission can be used to gain information about the efficiency of photochemistry and thermal dissipation (D) in PSII complexes. Demmig-Adams et al. [15], present a method to assess the fraction of absorbed photon energy allocated to thermal dissipation on the basis of changes in F'_v/F'_m. The novel parameters they derive, D, is calculated as $1 - F'_v/F'_m$, and is useful, primarily because it allows for the direct comparison of allocation of photon energy to non-photochemical dissipation and to photochemistry, which is accessed via the parameter developed by Genty et al. [25].

Previously we have demonstrated that different allelochemicals including CA significantly reduced seed germination and seedling growth of crops and grass species [33], and it was subjected to further study. Since CA appeared to be a more potent inhibitor therefore the present study was undertaken to assess the impact of CA on growth and interference with chlorophyll fluorescence components, changes in excitation energy during photosynthetic induction and photon energy dissipation in lettuce (*Lactuca sativa*) and speculate on the mechanism of this inhibition. Acquisition of such knowledge may ultimately provide a rational and scientific basis for the design of safe and effective herbicides [18,19,42,44].

2. Results

2.1. Effect on plant growth

The inhibitory effect of CA on *L. sativa* growth is concentration-dependent. Exposure to 1.5, 1.0, 0.5 and 0.1 mM CA resulted in a significant reduction in leaf/root growth and leaf/root fresh biomass in *L. sativa*. Allelochemical, CA, at all concentration decreased fresh biomass of the leaves and roots. Both root and shoot length was decreased with increase in CA concentration. At 1.5 mM CA, there was nearly 39.60% reduction in shoot length compared to control, whereas in root length the reduction was 62.40%. The CA also reduced leaf/root dry weight of *L. sativa* however; the results were non-significant (Table 1).

2.2. Effect on leaf water status

There was non-significant differences were observed in leaf relative water (RWC) content as compared to control at all concentration of CA; however, RWC was relatively less at highest concentration of CA as compared to control (Fig. 1A). There was non-significant effect on leaf osmotic potential (LOP) in *L. sativa* among control and CA treated plants but there was a tendency of increase in LOP at lowest concentration (0.1 mM) of CA (Fig. 1B).

2.3. Chlorophyll fluorescence measurements

The inhibitory effect of CA (1.5 mM) in *L. sativa* was evaluated on the basis of six days treatment by analyzing several fluorescence parameters determined under dark-adapted and steady state conditions. After exposure to CA there was a significant reduction in maximum (F_m) and variable fluorescence (F_v) in *L. sativa* while a tendency of increase in initial fluorescence from dark-adapted leaves (F_o) in *L. sativa* was observed during days 2nd and 4th days (Fig. 2A, B, D). The efficiency of PSII reaction centers in the dark-adapted state (F_v/F_m) was decreased during different days after treatment with CA (Fig. 2C). The reduction in maximum fluorescence in light-adapted leaves (F'_m) was observed during all days (Fig. 3B). Meanwhile, CA reduced quantum yield of PSII photochemistry (ΦPSII) (Fig. 3C). The variable fluorescence (F'_v) in light adapted-state was significantly decreased after treatment with 1.5 mM CA (Fig. 3A). The efficiency of PSII photochemistry (F'_v/F'_m) by open reaction centers in the light state was reduced by CA during different days (Fig. 3D). There was decrease in photochemical fluorescence quenching (qP) during all days (Fig. 4A). CA also decreased the values of non-photochemical fluorescence quenching (NPQ) in *L. sativa* during day third, fourth and fifth while there was tendency of stimulation during initial two days (Fig. 4B). Furthermore, fraction of photon energy absorbed by PS II antennae trapped by "open" PS II reaction centers (P) values in *L. sativa* were decreased

Table 1
Effect of cinnamic acid at different concentrations on the leaf fresh weight, leaf dry weight, root fresh weight, plant height and root length of *L. sativa* measured a week after treatment.

Growth characteristics	Control	Cinnamic acid (mM)			
		0.1	0.5	1.0	1.5
Leaf fresh weight (g)	10.58 ± 0.60	7.29 ± 1.00*	6.87 ± 1.41*	6.03 ± 0.78*	6.18 ± 1.03*
Leaf dry weight (g)	1.63 ± 0.15	1.80 ± 0.20	1.39 ± 0.32	1.12 ± 0.38	1.04 ± 0.28
Root fresh weight (g)	6.31 ± 0.34	3.36 ± 0.49*	3.27 ± 0.69*	3.20 ± 0.96*	2.95 ± 0.40*
Root dry weight (g)	0.40 ± 0.04	0.30 ± 0.02	0.38 ± 0.08	0.30 ± 0.06	0.29 ± 0.05
Plant height (cm)	15.30 ± 0.23	8.63 ± 0.43*	8.60 ± 0.70*	7.17 ± 0.52*	6.06 ± 1.24*
Root length (cm)	27.77 ± 0.23	21.67 ± 2.20*	18.50 ± 1.25*	18.00 ± 0.57*	17.33 ± 0.60*

Each value represents the mean (±S.E.) of three replicates.
*Significant differences as compared to control for $p < 0.05$ according to Student's t-test.

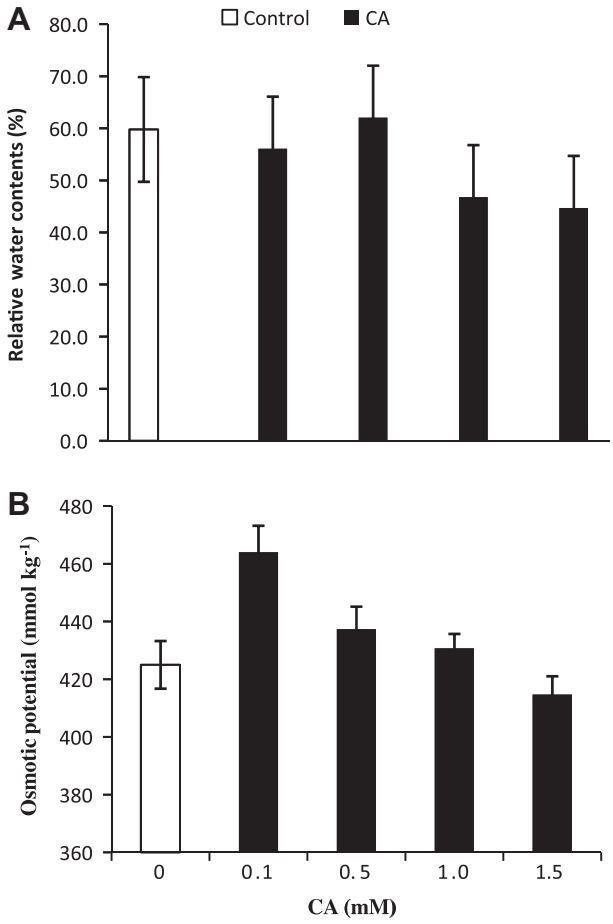

Fig. 1. Effect of CA at different concentrations on (a) relative water contents (%) and (b) osmotic potential (mmol kg^{-1}) in leaves of *L. sativa* measured a week after treatment. Every column in each bar represents the mean (±S.E.) of three replicates.

gradually starting from day first and highest reduction was observed on sixth day (Fig. 5A). The portion of absorbed photon energy that was thermally dissipated (*D*) and photon energy absorbed by PSII antennae and trapped by "closed" PSII reaction centers (*E*) was increased after CA exposure (Fig. 5B, C).

2.4. Carbon isotope composition analysis

The carbon isotope composition ratios ($\delta^{13}C$) were less negative (−27.10) in *L. sativa* leaves after treatment with 1.5 mM CA as compared to control (−27.61) (Fig. 6A). Carbon isotope discrimination ($\Delta^{13}C$) values in leaves of *L. sativa* were less (19.63) in CA treated plants at 1.5 mM concentration as compared to control (20.18) (Fig. 6B). The ratios of CO_2 concentration from leaf to air (*ci*/*ca*) were also significantly decreased after treatment with 1.5 mM CA (Fig. 7).

2.5. Protein contents

The CA (1.5 mM) significantly decreased leaf protein contents (1.035 mg g^{-1}) in *L. sativa* as compared to control (0.706 mg g^{-1}) (Fig. 8).

3. Discussion

Allelochemical appear to alter a variety of physiological processes in target plant species and it is difficult to separate the primary from secondary effects. Some have been explored as natural substitutes for commercial herbicides [18,19]. A number of studies have indicated that phenolic acids, including CA, affect membrane permeability and thus affect plant growth [16,36]. Here, the effect of putative allelochemical, CA on growth, photosynthetic capacity and stable isotopic characteristics of 30-days old *L. sativa* is reported. The plants growing in the presence of 1.5 mM CA were significantly smaller, with less fresh biomass and shorter root length. In general, the reduction was more in root length than shoot length (plant height) and this reduction was increased with increase in concentration from 0.1 to 1.5 mM CA. In response to 0.1 mM CA treatment, there was a reduction in root fresh weight that declined further due to increase of CA concentration. Plants exposed to highest concentration (1.5 mM) of CA were severely affected, with more than 50% reduction in root length as compared to control. The reduction in root growth has been considered one of the first effects of allelochemicals associated with reduction in plant growth and development. Similar results were reported in literature [63,64].

The greater phytotoxic effect on root is also reflected from higher levels of oxidative stress and induction of antioxidant enzyme system in the roots compared to leaves. In the present study, CA was applied at suitable concentrations (0.1–1.5 mM), since the concentration for allelochemicals was 0.1 mM in soils [59] after cucumber (*C. sativus* L.) cultivation and may reach to much higher levels in soils enriched in plant residues [1]. The CA (0.5–0.25 mM) has recently been shown to cause membrane peroxidation, increase H_2O_2 content and induction of oxidative stress in cucumber (*C. sativus* L.) roots [36]. Our observations are in agreement with earlier reports of the ability of phytotoxins to inhibit root growth [6,57]. Vaughan and Ord [60], observed that cinnamic acid derivatives altered root morphology in pea (*Pisum sativum* L.). The benzoic and cinnamic acid derivatives, depending on the extent and pattern of substitution, influence indole acetic acid (IAA) concentration in plant tissues by stimulating of inhibiting enzymes involved in IAA synthesis and destruction [60]. Baziramakenga et al. [8], reported the effects of benzoic and cinnamic acids on the cell plasma membrane in intact soybean (*Glycine max* L. cv. Maple Bell) seedlings. Treated intact root systems rapidly increased electrolyte leakage and ultraviolet absorption of materials into the surrounding solution. The two allelochemicals induced lipid peroxidation, which resulted from free radical formation in plasma membranes, inhibition of catalase and peroxidase activities, and sulfhydryl group depletion. Fujita and Kubo [24], reported that *trans*-cinnamic acid inhibited the root elongation of lettuce (*L. sativa* L.) by 50% at 8.1 μM. Likewise, Ju et al. [36], reported that CA significantly inhibited the growth of cucumber (*C. sativus*) but did not inhibit the growth of figleaf gourd.

The chlorophyll fluorescence is a useful measurement to study several aspects of photosynthesis because it reflects changes in thylakoid membrane organization and function and inhibition of photosynthesis and oxygen evolution through interactions with components of photosystem II [20,54]. In the present study, CA significantly reduced maximum (F_m) and variable fluorescence (F_v) in *L. sativa* while efficiency of "open" PSII reaction centers in the dark-adapted state (F_v/F_m) was also reduced. Damage to component of thylakoid membranes, especially those of PSII, and inhibition of energy transfers from antenna molecules to reaction centers can lead to lower F_v/F_m ratios [38], as well as photoinhibitory damage [13]. Meanwhile, Ye et al. [63], reported a decline in F_v/F_m, ΦPSII after CA exposure in *C. sativus*. Similarly, Zhou and Yu [65], concluded that allelochemicals can significantly affect the performance of the three main processes of photosynthesis: stomatal control of CO_2 supply, thylakoid electron transport (light reaction), and carbon reduction cycle (dark reaction).

Furthermore, our results clearly show that CA significantly inhibits the quantum yield of PSII electron transport (ΦPSII) in

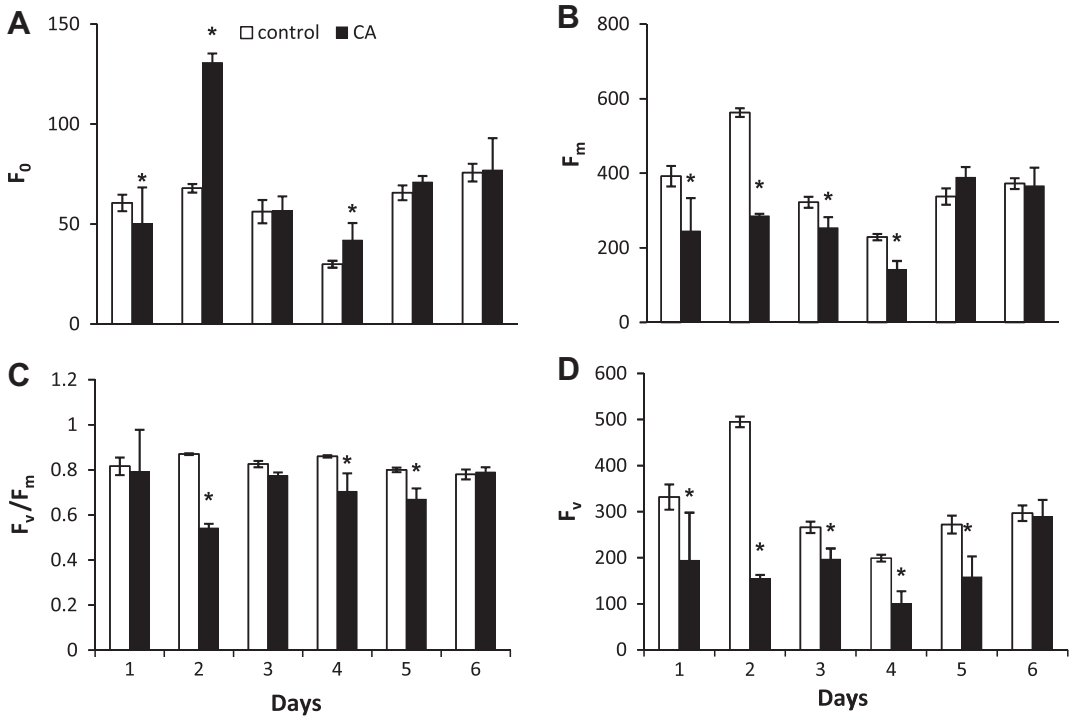

Fig. 2. Changes in chlorophyll fluorescence parameters (a) F_o, (b) F_m, (c) F_v/F_m and (d) F_v in *L. sativa* from days 1 to 6 following exposure to CA at 1.5 mM concentration. Every column in each bar represents the mean (±S.E.) of three replicates. *Asterisks indicate significant differences at level 0.05 with respect to control.

L. sativa seedlings. We also demonstrate that such a decrease in ΦPSII was associated to the alteration of qP. However, Huang and Bie [31], reported that 0.1 mM CA treatment decreased photosynthetic rate (Pn) and ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) activity, but it did not affect the maximal photochemical efficiency of PSII (F_v/F_m), photochemical efficiency of PSII (ΦPSII) and relative chlorophyll content. A decrease in qP induced by CA indicates a higher proportion of closed PSII reaction centers, which probably generates a decrease in the proportion of available excitation energy used for photochemistry. The significant

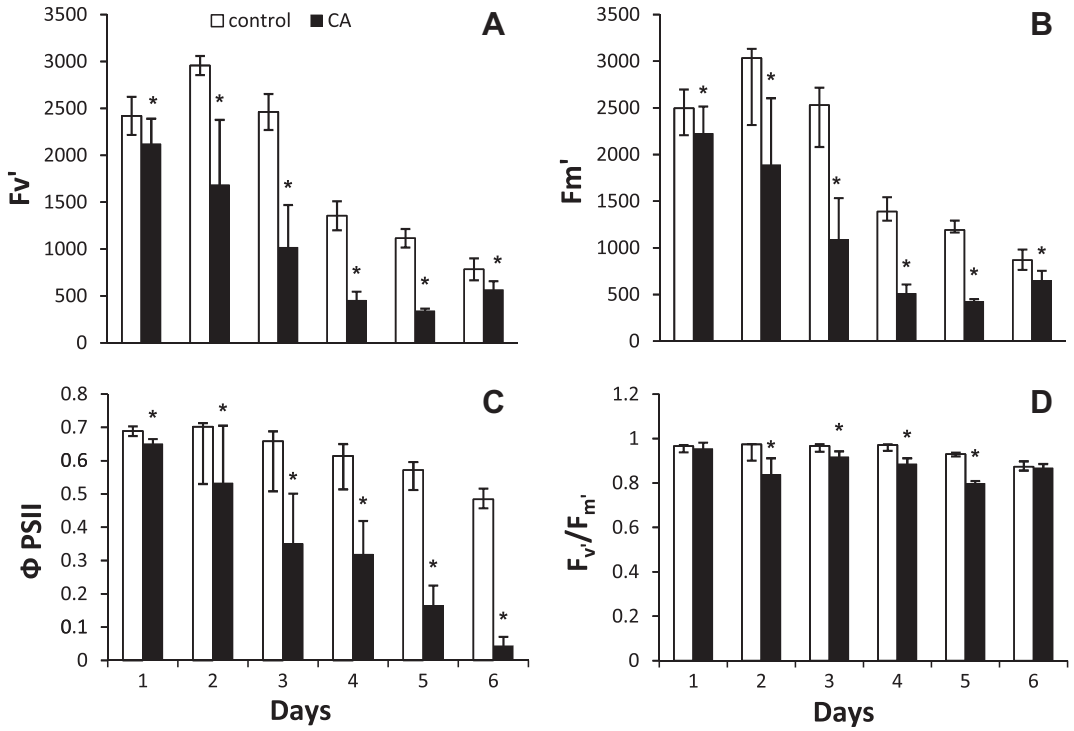

Fig. 3. Changes in chlorophyll fluorescence parameters (a) F_v', (b) F_m', (c) ΦPSII, and (d) F_v'/F_m' in *L. sativa* from days 1 to 6 following exposure to CA at 1.5 mM concentration. Every column in each bar represents the mean (±S.E.) of three replicates. *Asterisks indicate significant differences at level 0.05 with respect to control.

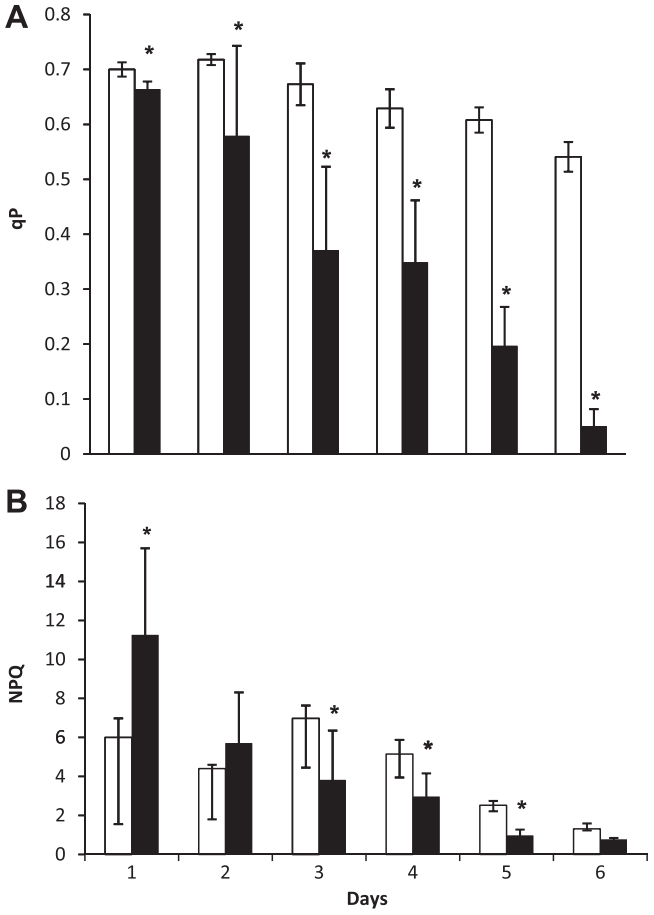

Fig. 4. Changes in fluorescence quenching coefficients (a) qP and (b) NPQ in *L. sativa* from days 1 to 6 following exposure to CA at 1.5 mM concentration. Every column in each bar represents the mean (±S.E.) of three replicates. *Asterisks indicate significant differences at level 0.05 with respect to control.

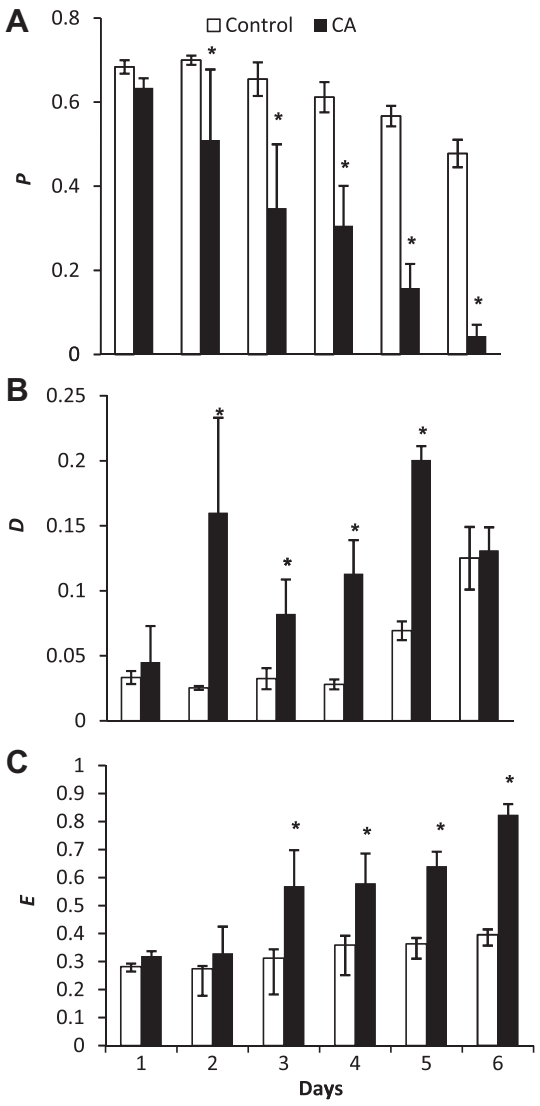

Fig. 5. Effect of 1.5 mM CA on the fraction of photon energy absorbed by PS II antennae trapped by "open" PS II reaction centers (*P*), portion of absorbed photon energy thermally dissipated (*D*) and photon energy absorbed by PSII antennae and trapped by "closed" PSII reaction centers (*E*) in *L. sativa* during 1–6 days. Every column in each bar represents the mean (±S.E.) of three replicates. *Asterisks indicate significant differences at level 0.05 with respect to control.

changes in PSII photochemistry in the light-adapted leaves, such as the decreased qP and F'_v/F'_m, as well as the increased of NPQ, can be seen as the regulatory response to down-regulate the quantum yield of PSII electron transport (ΦPSII) [25], that would match with the high decrease of CO_2 assimilation rate. It is suggested that the decay in ΦPSII of the CA stressed plants may be a mechanism to down-regulate the photosynthetic electron transport so that production of ATP and NADPH would be in equilibrium with the decreased CO_2 assimilation capacity in stressed plants. The decrease of PSII efficiency may be due to oxidative damage caused by possible stress factor [63,65].

Quantifying the energy dissipation as non-photochemical quenching does not allow comparing the flux of photon energy into thermal dissipation processes (*P*) [15]. Moreover, Maxwell and Johnson [46] pointed out that measurement of non-photochemical chlorophyll fluorescence quenching yield thermal dissipation relative to dark-acclimated state. Therefore, accurate interpretation of these measurements depends upon knowledge of the condition of photosynthetic apparatus in the dark-acclimated state. The ability to directly measure "*D*" with "*P*" even without knowledge of the previous status of the leaf is among the principal advantages of the method utilized in this study. Using this method of chlorophyll fluorescence analysis, we have been able to quantitatively compare the different processes of photon energy utilization during exposures of *L. sativa* leaves to CA stress. In the present study, CA gradually suppresses the portion of absorbed photon energy utilized in the PSII photochemistry (*P*). Presumably, the gradual decrease in "*P*" after treatment with CA may be the result of an inhibition of the activity of photosynthetic apparatus. The portion of absorbed photon energy that was thermally dissipated (*D*) and photon energy absorbed by PSII antennae and trapped by "closed" PSII reaction centers (*E*) was increased after CA treatment. It has been shown in many studies that an increase in the thermal dissipation in the PSII antennae competes with the excitation energy transfer from the PSII antennae to PSII reactions centers, thus resulting in a decrease in the efficiency of excitation energy captured by "open" PSII reaction centers F'_v/F'_m [15]. The "*E*" represents the absorbed photon energy that is neither allocated to "*P*" nor to "*D*" and it includes not only "excess" energy but also reflects non-light induced quenching processes. This portion of total absorbed photon energy has the greatest potential to cause PSII photoinactivation because it represents photon energy that is trapped by closed PSII reaction centers (i.e. those with quinine Q_A in the reduced state). The calculation of "*E*" includes the reduction state of Q_A (1 − qP) and the efficiency of

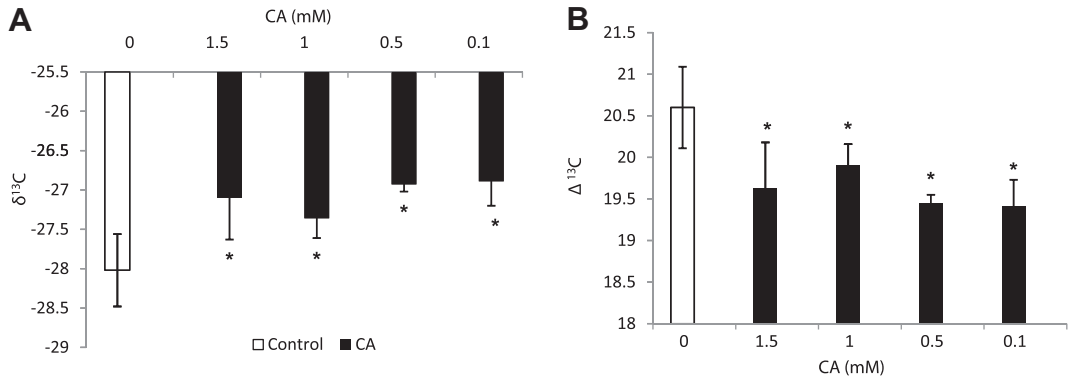

Fig. 6. Effect of different concentrations of CA on (a) carbon isotope composition ($\delta^{13}C$) and (b) carbon isotope discrimination ($\Delta^{13}C$) of *L. sativa*. Every column in each bar represents the mean value ± S.E. from three replicates. *Asterisks indicate significant differences at level 0.05 with respect to control.

the charge separation in PSII complexes in the light-acclimated state (F'_v/F'_m), which depends on the level of thermal dissipation [15]. The reduction state of Q_A and the extent of thermal dissipation are the factors affecting the probability of PSII photoinactivation [32]. Photon energy trapped by "closed" PSII reaction centers can result in the formation of triplet P680, double reduction of Q_A, and the generation of singlet oxygen, thereby creating conditions that favor PSII inactivation [48,51]. Accumulation of triplet P680 and singlet oxygen further enhances plant growth reduction and cause a severe oxidative stress resulting in disruption of metabolic activities in the plant cell. Baziramakenga et al. [8], demonstrated that benzoic and cinnamic acids induces generation of reactive oxygen species and results in increased peroxidase activity.

Plant photosynthesis discriminates against the stable ^{13}C isotope [21] when atmospheric CO_2 passes through stomata and during CO_2 carboxylation in RuBisCO. The ^{13}C discrimination decreases with a decrease in intercellular CO_2 concentration due to stomatal closure, and consequently with water use efficiency. Our results revealed that $\delta^{13}C$ was less negative after treatment with 1.5 mM CA. The $\Delta^{13}C$ were also less in CA treated plants as compared to control. The carbon isotope fractionation patterns indicate that limitations to the diffusion of CO_2 through the stomatal aperture can result in less negative $\delta^{13}C$ values [50]. Our data suggest that stomata's of *L. sativa* plants treated with 1.5 mM CA experienced some degree of closure, which ultimately leads to less discrimination against the heavier isotope. Previously, Barkosky and Einhellig [4], reported that $\delta^{13}C$ in leaf tissue of soybean (*G. max* L. Merr.) at the termination of the 28-day experiment showed significantly less discrimination (less negative $\delta^{13}C$) against ^{13}C in plants grown with 0.75 mM *p*-hydroxybenzoic acid. Meanwhile, there was non-significant effect on RWC as compared to control at any concentration of CA. But at the same time, there was a reduction in $\Delta^{13}C$ discrimination in *L. sativa* leaves during photosynthesis. These results strongly support to our data that reduction in $\Delta^{13}C$ discrimination in *L. sativa* leaves after treatment with CA, might leads to increased extent of stomatal closure which should result in decreased transpirational water loss (less reduction in RWC of leaf tissue). Different biotic and abiotic stress factors can cause degradation of proteins by senescence or by reduction in protein synthesis. In the present research, CA also decreased leaf protein contents in *L. sativa* at 1.5 mM concentration. Baziramakenga et al. [7], reported that phenolic acids reduced the incorporation of certain amino acid into proteins and thus reduce the rate of protein synthesis. The reduction in total soluble leaf proteins in Benzoxazolin-2(3H)-one (BOA) exposed lettuce (*L. sativa*) plants were also reported by Sánchez-Moreiras and Reigosa [57].

In conclusion, our results show that CA (1.5 mM) decreased the plant height and root length and significantly inhibited efficiency of photosystem II and coefficients of fluorescence quenching in leaves of *L. sativa*. Furthermore, photon energy absorbed by PS II antennae trapped by "open" PS II reaction centers, leaf proteins contents and $\Delta^{13}C$ were also decreased after treatment with CA in *L. sativa*. The CA has inhibitory effect on physiological and biochemical parameters as well as on the plant growth. These information's may provide useful clues and biochemical modes of action of CA that can be exploited by industry led natural herbicide discovery programmes [18,19]. Further field studies of the interactions of allelochemical with microorganisms, soil particles and movement in soil could provide useful clues to understand the allelopathic phenomenon under field conditions.

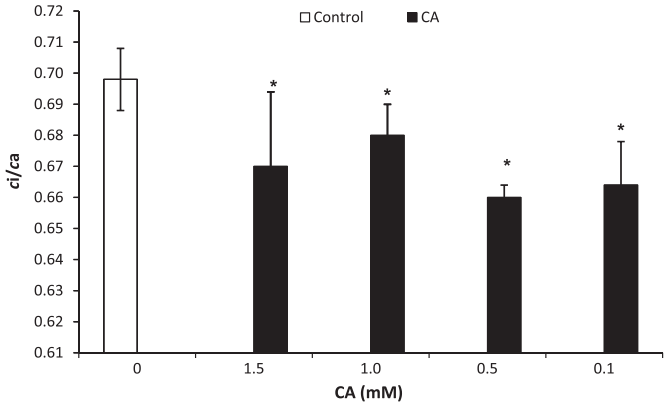

Fig. 7. Ratio of CO_2 concentration from leaf to air (ci/ca) in *L. sativa* following exposure to different concentrations of CA. Every column in each bar represents the mean (±S.E.) of three replicates. *Asterisks indicate significant differences at level 0.05 with respect to control.

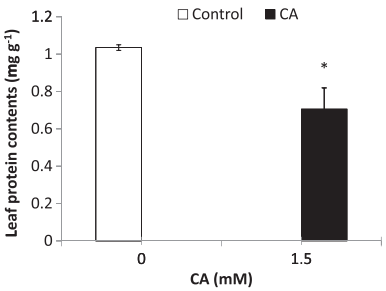

Fig. 8. Effect of CA (1.5 mM) on leaf protein contents (mg g^{-1}) in *L. sativa* measured a week after treatment. Every column in each bar represents the mean (±S.E.) of three replicates. *Asterisks indicate significant differences at level 0.05 with respect to control.

4. Materials and methods

4.1. Plant material and growth conditions

L. sativa L. cv. "Great Lakes" (lettuce) seeds were purchased commercially from Semillas Fito (Barcelona, Spain). Seeds were surface sterilized with sodium hypochlorite (0.1%, w/v) followed by washing with distilled water three times. Seeds were placed in plastic trays (32 × 20 × 6 cm) with a 5 cm deep layer (500 g/tray) of perlite in darkened at 20 °C temperatures in environmentally controlled growth chamber. Seeds were irrigated on alternate days with tap water until germination and thereafter with 500 ml 1:1 Hoagland solution/tray containing (KNO_3 (102 g/L), Ca $(NO_3)_2$ (50 g/L), $MgSO_4$ (49 g/L), $NH_4)_2$ HPO_4 (23 g/L), micronutrients: H_3BO_3 (2.86 mg/L), $MnCl_2$ (2.85 mg/L), $CuSO_4$ (0.08 mg/L), $ZnSO_4$ (0.4 mg/L), H_2Mo_4 (0.02 mg/L), $FeSO_4$ (2.8 g/L), Fe-ethylenediaminetetraacetic acid: Na_2EDTA (3.72 g/L), twice in a week. For seedling growth, the environmental conditions were as follows; temperature: 18/8 °C (day/night) and 12/12 h (light/darkness) photoperiod, 80% relative humidity and 200 μmol m^{-2} s^{-1} irradiance. One month old seedlings (three fully expanded leaf stage), were transferred to pots (10 cm) containing perlite (70 g) to stimulate the development of root system and shifted to the glass house with same growing condition and nutrient solution. The glass house was ventilated with outside air to ensure steady CO_2. In the present experiment, we have used perlite instead of soil because degradation of some allelochemical (BOA) has recently reported in great detail [42,43]. Since *trans*-cinnamic acid can be converted into *cis*-cinnamic acid by sunlight and by the presence of an electron-transfer facilitator, it is possible that the conversion of *trans*-cinnamic acid into *cis*-cinnamic acid is involved in the phytotoxic phenomenon [30].

4.2. Experimental treatments

Cinnamic acid (CA) (MW = 180.16) was purchased from Sigma Chemical Company, St. Louis, MO, USA. A stock solution (3 mM) of CA was prepared by dissolving requisite amount in methanol: water (20:80). The control was prepared with distilled water and methanol. The methanol was evaporated in a rotary vaporizer. The stock solution was further diluted with distilled water to get 0.1, 0.5, 1.0, 1.5 mM CA solution and adjusted to pH 6.0 with NaOH [45]. The final concentration of methanol in each solution including control was 0.1% (v/v), at which concentration methanol has a negligible effect on plants [64]. The concentrations used in present study were selected based on previous studies reported in literature for exploring mode of action of CA [9,33,63,64]. Test plants were selected randomly and assigned one plant per pot. Three replicates were maintained for each treatment in a randomized complete block manner. The treatments were applied three times (days 1, 3 and 5) with 100 ml solution of four concentration (0.1, 0.5, 1.0, 1.5 mM) of CA and control (distilled water) were checked against the target specie.

4.3. Plant growth measurements

Shoot/root length of *L. sativa* was obtained with a ruler and values were expressed in cm. The fresh and oven dry plant leaves and roots weight were obtained by first weighting independently fresh leaves and roots then drying these samples in a circulatory air oven at 70 °C for 72 h. The samples were weighed again to get dry weight of plant.

4.4. Determination of leaf water status

RWC (%) was calculated by measuring fresh (W_f), saturated (W_t) and dry (W_d) weights of *L. sativa* plant [53]. Leaf plant pieces from three replicates per treatment were weighed as fresh; saturates at 4 °C for 3 h, weighed again, oven dried at 70 °C for 72 h, and finally weighed once more for obtaining three necessary weights required for the following equation:

$$\mathrm{RWC} = W_f - W_d / W_t - W_d \times 100 \quad (1)$$

For osmolality measurements (mmol kg^{-1}) one leaf per replicate was transferred into 10 ml syringes and kept frozen at −80 °C until further analysis [27]. In analysis syringes were thawed until sample reached at room temperature and osmotic potential was measured on the second drop from collected sap by using a calibrated vapor pressure Osmometer (Automatic Cryoscopic Osmometer, Osmomat − 030, GmbH, Gonatec, Berlin, Germany) according to manufacturer's instructions.

4.5. Chlorophyll fluorescence measurement

Chlorophyll fluorescence was measured on fully expanded exposed leaves (one leaf per plant) using a pulse-modulated instrument fluorescence monitoring system (FMS) (Hansatech, Norfolk, England) by the method of Weiss and Reigosa [62]. At each measuring time, the plant leaves were kept in darkness for 20 min to allow all reaction centers to open and minimize fluorescence associated with the energization of the thylakoid membrane [38]. The parameters of chlorophyll fluorescence: initial fluorescence (F_o), maximum fluorescence (F_m), and variable fluorescence $F_v(F_m - F_o)$, were measured in the dark-adapted leaves using Walz leaf clips. The intensity of saturation pulses to determine the maximum fluorescence emission in the presence (F'_m) and absence (F_m) of quenching was 1800 μmol m^{-2} s^{-1} for 3 s. The steady state fluorescence (F_s), basic fluorescence after light induction (F'_o), maximum chlorophyll fluorescence (F'_m), variable fluorescence from light-adapted leaves (F'_v) were also evaluated by attaching the optic fiber to a leaf clip holder. The efficiency of photosystem II (PSII) photochemistry (F_v/F_m) in the dark-adapted state was considered a useful measurement of photosynthetic performance of plants and as stress indicators. Quantum yield of photosystem II (ΦPSII), photochemical quenching (qP) and non-photochemical quenching (NPQ) were also recorded [25]. The fraction of photon energy absorbed by PSII antennae that was trapped by "open" PSII reaction centers (centers with Q_A in the oxidized state) and utilized in PSII photochemistry was estimated as $P = F'_v/F'_m \times$ qP [25]. The portion of absorbed photon energy that was thermally dissipated was calculated as the difference between the maximal theoretical and actual levels of PSII efficiency ($D = 1 - F'_v/F'_m$). The fraction of absorbed radiation not attributed to P or D (denoted as "excess", E) and representing the photon energy absorbed by PSII antennae and trapped by "closed" PSII reaction centers (centers with Q_A in the reduced state) was estimated using $E = 1 - (D + P) = F'_v/F'_m \times (1 - \mathrm{qP})$ [15].

4.6. Carbon isotope discrimination analysis

The leaf samples were dried at 70 °C for 72 h in a forced-air oven (Gallenkamp oven, Loughborough, Leicestershire, UK) to constant weight and ground in Ball Mills (Retsch MM 2000, Haan, Germany). Dry ground plant material was weighed (1700–2100 μg) with weighing meter (Metler Toledo GmbH: Greifensee Switzerland), filled in tin capsules (5 × 3.5 mm, Elemental Microanalysis Limited, U.K.). Each tin capsule was entered automatically in combustion oven at 1600–1800 °C in the presence of oxygen and converted in to CO_2 and N_2. Subsequently isotope ratios were determined in an Isotopic Ratio Mass Spectrometer (Finnegan: Thermo Fisher Scientific, model MAT-253, Swerte Germany) coupled with an Elemental Analyzer (Flash EA-1112, Swerte Germany). The isotopic

ratio mass spectrometer has an analytical precision better than 0.3‰ for ^{13}C.

Carbon isotope compositions were calculated as;

$$\delta(‰) = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000 \quad (2)$$

where R_{sample} is the ratio of $^{13}C/^{12}C$ and $R_{standard}$ are standards used. Vienna PeeDee Belemnite (VPDB) is standard for carbon. Pure CO_2 ($\delta^{13}C = -28.2 \pm 0.1‰$) gas calibrated against standard CO_2 (−10.38‰) served as reference gas for $\delta^{13}C$. The accuracy and reproducibility of measurements of $\delta^{13}C$ was checked with an internal reference material (NBS 18 and IAEA-C6 for C), and Acetanilide for C %age ratios, respectively.

Carbon isotope discrimination is a measure of the carbon isotopic composition ratio in plant material relative to the value of the same ratio in the air on which plants feed:

$$\Delta(‰) = [(\delta a - \delta p)/1 + \delta p] \times 1000 \quad (3)$$

where Δ represents carbon isotope discrimination, δa represents C isotope composition in the source air, and δp represents C isotope composition in the plant tissue. Theory published by Farquhar et al. [21], and Farquhar and Richards [22], indicates that carbon isotope discrimination in leaves of plants can be expressed in relationship to CO_2 concentrations inside and outside as:

$$\Delta = a + (b - a)ci/ca \quad (4)$$

$$\Delta = 4.4 + (27 - 4.4)ci/ca$$

where a is discrimination that occurs during diffusion of CO_2 through the stomata (4.4‰), b is discrimination by RuBisCO (27‰), and ci/ca is the ratio of the leaf intercellular CO_2 concentration to that in the atmosphere. Equation (3) shows a direct and linear relationship between Δ and ci/ca. Therefore, measurement of Δ gives an estimation of the assimilation-rate−weighted value of ci/ca. Data of $\delta^{13}C_{air}$, ci/ca were obtained from McCarroll and Loader [47]and Francey et al. [23], whom used the high precision records of atmospheric $\Delta^{13}C$ from Antarctic ice cores and the atmospheric CO_2 concentration (ppm) from Robertson et al. [55]. Carbon isotope measurements were performed in CACTI (Centro de Apoio Científico Tecnológico a la Investigación, University of Vigo, Spain).

4.7. Determination of protein contents

Total leaf protein was quantified by using the Spectrophotometric Bradford methods as described by Bonjoch and Tamayo [11]. The *L. sativa* leaves (200 mg fresh weight) from three replicates per treatment were crushed in liquid nitrogen in cooled mortar with 0.05 g PVPP, and the powder was resuspended in 1 mL of Tris buffer containing 0.05 M Tris base, 0.1% w/v ascorbic acid, 0.1% w/v cysteine hydrochloride, 1% w/v PEG 4000, 0.15% w/v citric acid, and 0.008% v/v 2-mercaptoethanol [2]. The samples were centrifuged at 19,000 g_n and 4 °C for 20 min after resuspension. 0.1 mL supernatant was mixed for protein dye-binding reaction with 3 mL Bradford reactive containing 0.01% Coomassie® Brilliant Blue G-250, 4.7% v/v ethanol 95%, and 8.5% v/v phosphoric acid 85%. Absorbance was recorded at 595 nm after 5 min for quantification of the protein content. Commercial bovine seroalbumin (BSA) was used as standard. Values were expressed per gram of dry weight.

4.8. Statistical analysis

The experiment includes three replicates per treatment arranged in a randomized complete block manner. Data were analyzed with the statistical package SPSS® (version 15.00) for Windows® (SPSS Inc., Chicago, IL, USA). The data were analyzed with a one-way analysis of variance and differences between treatments were separated by the Student's t-test at the $P < 0.05$ level.

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