



Effect of elevated CO₂ and temperature on oxidative stress and antioxidant enzymes activity in coconut (*Cocosnucifera* L.) seedlings

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Abstract Increasing atmospheric CO₂ concentration is one of the factors driving climate change. We have investigated the individual effect of elevated CO₂ and temperature on oxidative stress and antioxidant enzymes activity in coconut (*Cocosnucifera* L.) seedlings. Three cultivars (WCT, LCT and COD) and two hybrids (WCT × COD and COD × WCT) were grown in six open top chambers (OTCs) and responses were analyzed after three years. In two OTCs the CO₂ was set at 550 and 700 μmol mol⁻¹ and control chamber had ambient level of 393 μmol mol⁻¹. In two separate OTCs, air temperature was elevated by 2 °C above the ambient control chamber. Remaining two chambers served as chamber control. Elevated CO₂ and temperature increased the activities of superoxide dismutase and catalase, whereas it reduced polyphenol oxidase activity. On the other hand, peroxidase

(POX) activity was decreased in elevated temperature, while it increased under elevated CO₂ conditions. The POX activity and membrane stability index (MSI) were positively correlated. By virtue of greater MSI and lower MDA content, WCT and hybrid COD × WCT were observed to be tolerant to oxidative stress among the five cultivars under elevated CO₂ and temperature conditions. These cultivars may adapt better to changing climates.

Keywords Climate change · CO₂ · Temperature · Membrane stability index · Antioxidant enzymes activity · *Cocos nucifera* L

Introduction

Global climate change is increasingly considered to be the most important external factor, which influences agricultural production including that of plantation crops. The atmospheric concentration of CO₂ and temperature are the two major climatic factors that can affect the growth, development and productivity of coconuts (NareshKumar et al. 2008). Among the greenhouse gases, the concentration of CO₂, which was at about 280 μmol mol⁻¹ in 1,850 (pre-industrial era) has rose to ~398 μmol mol⁻¹ in 2014 (NOAA/ESRL 2014) and multi model averages indicate maximum day temperature increase of about 1–3 °C by middle of 21st century and about 2–5 °C by the end of 21st century (IPCC 2012).

In plants, reactive oxygen species (ROS) are continuously produced. The removal of these ROS is done by efficient antioxidative systems consist of enzymatic and non-enzymatic pathways. The removal of ROS is disturbed by unfavorable environmental factors and that can cause serious injuries to plants (Parvaiz et al. 2008). Responses of

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antioxidative systems to elevated CO₂ and temperature varies in plants because of species and genotypic differences (Geetika 2003; Karnosky 2003). The failure of the defense metabolites and enzymes to detoxify ROS affects biological structures and decreases photosynthesis, and causes oxidation of amino acids, proteins, nucleic acids, pigments and lipids, leading to cell death and senescence (Xu et al. 2006; Naresh Kumar and Kasturi 2009; Kasturi et al. 2009). In coconut, drought stress is one of the major abiotic stress factors that increased the activities of enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and polyphenol oxidase (PPO) (Kasturi et al. 2009). In case of coconut seedlings no studies have been reported on the effect of high CO₂ and temperature on the activities of SOD, CAT, PPO and POX, and membrane damage as well as the production and removal of ROS. The coconut seedlings are very sensitive to external stresses with injury levels ranging from leaf senescence to seedling death, mainly due to oxidative stress and failure of antioxidative systems (Naresh Kumar and Kasturi 2009). As high CO₂ and temperature can increase the production of ROS in seedlings, it is important to study the antioxidative systems in coconut seedlings under elevated CO₂ and temperature conditions. The efficiency of the plant antioxidative systems makes them tolerant to oxidative stress originating from different abiotic stress factors. Thus, we investigated the activities of antioxidant enzymes as well as the membrane stability in coconut seedlings under elevated CO₂ and temperature conditions.

Materials and methods

The experiment was conducted in six open top chambers (OTCs) of 4 m³ size established at Central Plantation Crops Research Institute (CPCRI), Kasaragod, Kerala, India (12°18' N, 75° E, ~10.7 m elevation above mean sea level) during 2007–2010. One year old seedlings of three coconut cultivars popularly grown in southern India, viz., west coast tall (WCT), laccadive tall (LCT), chowghat orange dwarf (COD) and their two hybrids viz., WCT x COD (Kerasankara) and COD x WCT (Chandrasankara) were selected and grown in plastic containers filled with red sandy loam soil. The seedlings were maintained under uniform cultural practices recommended for coconut seedlings. The experimental site was humid (~88 % RH) with the mean maximum temperature of ~31.5 °C during summer and ~21.3 °C during winter and received ~3,400 mm rainfall annually.

In order to avoid the bright sunlight, green shade nets (with transmittance of 65 %) were spread inside the OTC walls. Among six OTCs, two had elevated CO₂ [550 μmol mol⁻¹; (CO₂)₅₅₀ and 700 μmol mol⁻¹;

(CO₂)₇₀₀] levels, two had elevated temperature (2 °C above ambient control chamber; [T] + 2 °C) levels and remaining two were used as ambient OTCs (served as chamber control; CC). All the OTCs were equipped with humidity and temperature sensors. The whole system of the OTC was controlled by the Supervisory Control and Data Acquisition (SCADA; Neogenesis, India) system. The required levels of CO₂ in the OTCs were attained by injecting air enriched with commercial-grade CO₂ with an air compressor (ELGI Equipment, India) and monitored with the help of SCADA linked infrared gas analyzer (IRGA; Neogenesis, India). In the OTCs with elevated temperature, reference temperature was obtained from the control OTC and air temperature was increased ~2 °C above control chamber by blowing of hot air, and controlled by the SCADA system.

The seedlings of both the cultivars and hybrids were exposed to elevated CO₂ and temperature for three years (2007–2010) and samples were collected during April 2010 between 0900 h and 1000 h. Leaf samples were collected in liquid nitrogen and stored in deep freezer at -40 °C. Enzyme extract was prepared by following the method of Chempakam et al. (1993) as standardized for coconut leaf tissue. Plant tissue sample (2.5 g) was homogenized in an ice jacketed pre-chilled motor and pestle with 20 ml sodium phosphate buffer (0.1 M; pH 7.6) containing β mercaptoethanol (0.2 ml) and insoluble polyvinyl pyrrolidone (PVPP) (1 g). This mixture was centrifuged using refrigerated centrifuge (Hareus, Germany) at 12,000 rpm at 4 °C for 15 min. Supernatant was collected and total protein was precipitated using ammonium sulphate (85 % saturation) and kept at 4 °C for 1 h. After 1 h, supernatant was centrifuged at 4 °C for 30 min at 12,000 rpm and the pellets were dissolved in 3 ml sodium phosphate buffer (0.1 M). The extract was dialyzed overnight with three changes of sodium phosphate buffer (0.1 M; pH 7.6). The dialyzed enzyme extract was transferred to micro tube and used for the assay of enzymes, viz., SOD, CAT, POX and PPO. Low temperature was maintained throughout the extraction procedure.

The specific activity of SOD (EC 1.15.1.1) was assayed by following the method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme required to bring about a 50 % inhibition of the rate of nitro-blue tetrazolium reduction measured at 560 nm and expressed as units min⁻¹ mg protein⁻¹. The specific activities of PPO (EC 1.14.18.1), CAT (EC 1.11.1.6) and POX (EC 1.11.1.7) were assayed by following the modified method of Kar and Mishra (1976), as standardized for coconut tissues (Kasturi Bai et al. 2005). The activity of CAT was determined as the decomposing rate of H₂O₂ monitored by change in absorbance at 240 nm and expressed in mM H₂O₂ consumed min⁻¹ mg protein⁻¹. Change in absorbance between

sample and blank was recorded using kinetic mode of spectrophotometer (Shimadzu UV160A, Japan). The assay mixture consisted of enzyme extract (0.5 ml), distilled water (1 ml), H₂O₂ (10 mM; 1 ml) and potassium phosphate buffer (50 mM; pH 6.8; 2 ml). The total reaction time (rate T) was 310 and 10 s lag time (lag T) was given to start the reaction. Absorbance was recorded at 30 s (interval T) intervals and the change in absorbance was calculated with molar extinction coefficient of H₂O₂ (43.6 M cm⁻¹). The activities of PPO and POX were determined by measuring change in absorbance by the formation of purpurogallin at 420 nm and expressed in absorbency at 420 nm (A₄₂₀ min⁻¹ mg protein⁻¹). The optical density readings were measured using UV- visible spectrophotometer (Shimadzu UV160A, Japan). The assay mixture for POX assay consisted of potassium phosphate buffer (50 mM; pH 6.8; 2 ml), pyrogallol (50 µM; 1 ml) and H₂O₂ (10 mM; 1 ml). To this, enzyme extract (0.1 ml) was added and kept at room temperature for 3 min for incubation. Reaction was stopped by adding sulfuric acid (2 N; 0.5 ml) and optical density was measured at 420 nm. The reagent blank was prepared by adding sulfuric acid to the reaction mixture before the incubation time. The assay mixture for PPO activity consisted of the same assay mixture as that of POX assay but without H₂O₂.

The malondialdehyde (MDA) content and leaf membrane stability index (MSI) were estimated by following the methods of Heath and Packer (1968) and Lu et al. (2003), respectively. Conductivity measurements (µS cm⁻¹) for the membrane stability index (MSI) were recorded using conductivity meter (Model PP20, Sartorius, Germany). The concentrations of total soluble protein in the leaflet were estimated by following the Bradford (1976) method. Statistical analysis was performed using AGRIS STAT software for calculating the critical difference (CD)

values. The Duncan's multiple-Range comparison test was performed using the SPSS package (SPSS Inc. version 16.0) for all set of data.

Results and discussion

The specific activities of enzymes in coconut seedlings were influenced by elevated CO₂ and temperature conditions. In general, activities of enzymes such as SOD, POX and CAT increased significantly under elevated CO₂ levels [(CO₂)₅₅₀ and (CO₂)₇₀₀]. At the same time elevated (T + 2 °C) increased the activities of SOD and CAT, but reduced the activity of POX. On the other hand, PPO activity decreased under both elevated temperature (T + 2 °C) and CO₂ conditions. The response of the coconut seedlings greatly varied depending on the cultivar and hybrid. Among them, LCT under elevated CO₂ [(CO₂)₅₅₀] and elevated temperature (T + 2 °C) and COD x WCT under elevated CO₂ [(CO₂)₇₀₀] had significantly higher SOD activity. The response in terms of activity of CAT differed in magnitude and direction. The hybrid of COD x WCT under elevated CO₂ [(CO₂)₅₅₀] and at T + 2 °C temperature, and cultivar LCT under elevated CO₂ [(CO₂)₇₀₀] had significantly higher CAT activity as compared to that in other hybrids and cultivars. The LCT under elevated CO₂ [(CO₂)₅₅₀] and COD under (CO₂)₇₀₀ had significantly higher POX activity. On the other hand, decrease in POX activity was highest in COD x WCT, while in case of WCT increase was observed only under elevated temperature (T + 2 °C) condition (Tables 1, 2).

Antioxidant enzymes are indispensable for the defense against ROS in C3 plants under different climatic stress conditions (Foyer 2002; Kasturi et al. 2009). Coconut is a source limited C3 plant (Naresh Kumar et al. 2002).

Table 1 Effect of elevated temperature and CO₂ conditions on superoxide dismutase and catalase specific activity in coconut cultivars and hybrids

Treatment/varieties	CC	T + 2 °C	(CO ₂) ₅₅₀	(CO ₂) ₇₀₀	CD (P = 0.01)
Superoxide dismutase activity (units min ⁻¹ mg ⁻¹ protein)					
WCT	2.6 ^b	4.2 ^b (+62 %)	1.6 ^c (-38 %)	1.6 ^d (-38 %)	Treatments (T) = 0.23 Varieties (V) = 0.27 T x V = 0.114 SEm ± 0.242
COD	3.0 ^a	4.5 ^a (+50 %)	2.1 ^c (-30 %)	2.3 ^c (-23 %)	
LCT	1.5 ^d	3.6 ^c (+140 %)	4.6 ^a (+207 %)	1.1 ^e (-27 %)	
WCT × COD	2.1 ^c	2.5 ^e (+19 %)	2.1 ^d (-2.0 %)	2.7 ^b (+29 %)	
COD × WCT	1.6 ^d	3.2 ^d (+100 %)	2.2 ^b (+38 %)	4.4 ^a (+175 %)	
Mean	2.2	3.6 (+67 %)	2.5 (+17 %)	2.4 (+12 %)	
Catalase activity (mM H ₂ O ₂ min ⁻¹ mg ⁻¹ protein)					
WCT	3.0 ^a	2.1 ^b (-30 %)	2.2 ^{bc} (-27 %)	5.2 ^a (+73 %)	Treatments (T) = 0.51 Varieties (V) = 0.42 T × V = 0.219 SEm ± 0.27
COD	3.9 ^a	3.3 ^a (-15 %)	4.6 ^a (+18 %)	2.4 ^c (-38 %)	
LCT	1.8 ^b	2.4 ^b (+33 %)	2.7 ^b (+50 %)	3.5 ^b (+94 %)	
WCT × COD	0.5 ^c	0.7 ^d (+43 %)	0.4 ^d (-10 %)	0.4 ^e (-18 %)	
COD × WCT	0.6 ^c	1.5 ^c (+173 %)	1.6 ^c (+191 %)	0.7 ^d (+24 %)	
Mean	1.9	2.0 (+3 %)	2.3 (+18 %)	2.4 (+25 %)	

High mean value is represented by letter a and low mean value by letter e

Table 2 Effect of elevated temperature and CO₂ conditions on polyphenol oxidase and peroxidase specific activity in coconut cultivars and hybrids

Treatment/varieties	CC	T + 2 °C	(CO ₂) ₅₅₀	(CO ₂) ₇₀₀	CD (P = 0.01)
Polyphenol oxidase activity (A ₄₂₀ min ⁻¹ mg ⁻¹ protein)					
WCT	0.21 ^c	0.12 ^b (-43 %)	0.06 ^d (-71 %)	0.06 ^c (-71 %)	
COD	0.28 ^b	0.20 ^a (-29 %)	0.08 ^b (-71 %)	0.11 ^b (-61 %)	Treatments (T) = 0.02
LCT	0.15 ^d	0.07 ^d (-53 %)	0.05 ^c (-67 %)	0.02 ^c (-87 %)	Varieties (V) = 0.07
WCT × COD	0.41 ^a	0.10 ^c (-76 %)	0.10 ^a (-76 %)	0.05 ^d (-88 %)	T × V = 0.04
COD × WCT	0.40 ^a	0.05 ^c (-83 %)	0.07 ^c (-83 %)	0.14 ^a (-65 %)	SEM ± 0.025
Mean	0.29	0.11 (-63 %)	0.07 (-75 %)	0.08 (-74 %)	
Peroxidase activity (A ₄₂₀ min ⁻¹ mg ⁻¹ protein)					
WCT	0.9 ^c	1.8 ^a (+100 %)	2.4 ^b (+167 %)	1.1 ^d (+22 %)	
COD	0.7 ^d	0.4 ^c (-43 %)	2.2 ^c (+214 %)	2.5 ^a (+257 %)	Treatments (T) = 0.04
LCT	0.5 ^e	0.4 ^b (-20 %)	2.0 ^d (+300 %)	1.3 ^c (+160 %)	Varieties (V) = 0.03
WCT × COD	1.4 ^a	0.2 ^d (-86 %)	0.5 ^e (-64 %)	0.5 ^e (-64 %)	T × V = 0.05
COD × WCT	1.1 ^b	0.1 ^e (-91 %)	2.6 ^a (+136 %)	1.5 ^d (+36 %)	SEM ± 0.181
Mean	0.9	0.6 (-40 %)	1.9 (+111 %)	1.4 (+50 %)	

Means followed by the same letter in a column do not differ significantly according to Duncan's multiple-range test. The values with in parenthesis are relative change due to treatment as compared to chamber control (CC)

Increase in photosynthesis and photorespiration can produce superoxide and H₂O₂ as intermediates (Foyer 2002). Oxidative stress has been reported to cause leaf scorching in coconut seedling and death in severe cases (Naresh Kumar and Kasturi 2009). The anatomical adaptations as one of the drought tolerant mechanisms has also been reported (Naresh Kumar et al. 2000). Hence, an increase in the activity of SOD, POX and CAT is essential for maintaining the integrity of the cell. Under elevated temperature condition activities of SOD and CAT increased significantly in spite of the decrease in the activity of POX. Increased activity of CAT might have compensated for decreased POX activity under elevated temperature condition as reported earlier for other species (Foyer 2002).

The highest decrease in PPO activity was observed in COD × WCT under elevated CO₂ [(CO₂)₅₅₀] and temperature (T + 2 °C) and the decrease was highest in WCT × COD under elevated CO₂ [(CO₂)₇₀₀] condition (Table 2). Interestingly, the MDA content in leaf significantly decreased under elevated CO₂, but under elevated temperature (T + 2 °C) it was similar to the values of chamber control. MSI slightly increased under elevated CO₂, whereas it decreased under elevated temperature (T + 2 °C) condition (Table 3). The ROS induced peroxidation of membrane lipid (in terms of MDA content) is considered as the manifestation of stress induced damage at the cellular level. The damage due to the lipid peroxidation of membrane can cause decreased membrane fluidity (Jain et al. 2001). The decrease in MDA content was highest in WCT under elevated CO₂ and temperature conditions. The MSI in coconut leaves indicated that WCT × COD and WCT had higher membrane stability under elevated CO₂. Similarly, under elevated temperature (T + 2 °C) conditions MSI was more in WCT followed by COD × WCT (Table 3). The reduced activity of PPO, decrease in MDA content and increased MSI in

seedlings of WCT and COD × WCT indicated that even under elevated temperature condition, elevated CO₂ reduced oxidative stress by the enzymatic protection mechanism in operation as compared to the other cultivars and hybrids. From these results it is evident that coconut seedlings were able to maintain the stability of membrane under elevated CO₂ and temperature conditions with higher activity of SOD, CAT and POX. The activity of PPO is reported to be high under different abiotic and biotic stress conditions as it is involved in the functions such as tissue browning, regulation of electron cycle in mahler reaction and protection from pathogens (Piyada et al. 2004, 2007; Sheela and Robina 2007). In the present study, activity of PPO is considered as an indicator of cell damage as reported earlier in coconut (Chempakam et al. 1993; Kasturi et al. 1996).

In coconut, drought tolerant varieties were characterized by high activities of SOD, POX and CAT and consequent lower level of MDA content and MSI. A negative correlation between leaf water potential and MDA content has been observed in coconut under drought conditions (Kasturi et al. 2009; Kasturi Bai et al. 2011). Coconut seedlings grown under elevated temperature condition had low water potential as compared to those under elevated CO₂ conditions (Sunoj et al. 2009). Results from earlier studies indicated that elevated CO₂ promoted growth in terms of carbon assimilation and dry matter production of coconut seedlings (NareshKumar et al. 2008). At the same time under elevated temperature condition increase in the activity of antioxidant enzymes supported seedlings in minimizing cell injury as is evident from the lower PPO activity, MDA content and MSI. This helped in maintaining growth at high temperature even at low water potential.

Elevated temperature (T + 2 °C) decreased the soluble protein concentration across the cultivars. But under elevated CO₂ conditions, variable response of cultivars was recorded. For instance, in case of WCT and LCT elevated

Table 3 Effect of elevated temperature and CO₂ conditions on lipid peroxidation and membrane stability index (MSI) of coconut cultivars and hybrids

Treatment/varieties	CC	T + 2 °C	(CO ₂) ₅₅₀	(CO ₂) ₇₀₀	CD (P = 0.01)
Lipid peroxidation (nmol malondialdehyde g ⁻¹ fr. wt.)					
WCT	44.97 ^c	39.35 ^c (-12 %)	26.71 ^d (-41 %)	21.81 ^d (-52 %)	
COD	57.03 ^b	58.06 ^b (+2.0 %)	56.00 ^a (-2.0 %)	50.00 ^b (-12 %)	Treatments (T) = 0.60
LCT	27.87 ^c	27.23 ^c (-2.0 %)	30.90 ^c (+11 %)	33.29 ^c (+19 %)	Varieties (V) = 0.64
WCT × COD	67.48 ^a	73.48 ^a (+9.0 %)	51.23 ^b (-24 %)	66.26 ^a (-2.0 %)	T × V = 1.0
COD × WCT	31.42 ^b	30.70 ^d (-2.0 %)	21.10 ^e (-33 %)	19.16 ^e (-39 %)	SEm ± 1.84
Mean	45.75	45.76 (+0.02 %)	37.19 (-19 %)	38.10 (-17 %)	
Membrane stability index (%)					
WCT	88.05 ^e	91.90 ^b (+4.0 %)	89.43 ^e (+2.0 %)	91.00 ^d (+3.0 %)	
COD	91.80 ^a	85.10 ^d (-7.0 %)	90.58 ^d (-1.0 %)	90.71 ^e (-1.0 %)	Treatments (T) = 0.57
LCT	91.69 ^b	91.26 ^c (-0.5 %)	92.95 ^a (+1.0 %)	91.68 ^b (-0.01 %)	Varieties (V) = 0.49
WCT × COD	89.77 ^d	84.90 ^e (-5.0 %)	92.22 ^c (+3.0 %)	91.25 ^c (+2.0 %)	T × V = 0.95
COD × WCT	91.37 ^c	92.42 ^a (+1.0 %)	92.59 ^b (+1.0 %)	93.51 ^a (+2.0 %)	SEm ± 0.26
Mean	90.54	89.17 (-1.6 %)	91.55 (+1.1 %)	91.63 (+1.2 %)	

Means followed by the same letter in a column do not differ significantly according to Duncan's multiple-range test. The values with in parenthesis are relative change due to treatment as compared to chamber control (CC)

Table 4 Effect of elevated temperature and CO₂ conditions on total soluble protein content in coconut cultivars and hybrids

Treatment/varieties	CC	T + 2 °C	(CO ₂) ₅₅₀	(CO ₂) ₇₀₀	CD (P = 0.01)
Total soluble protein (mg protein g ⁻¹ dry wt.)					
WCT	7.00 ^a	4.42 ^c (-37 %)	6.84 ^a (-2.0 %)	6.84 ^c (-2.0 %)	
COD	6.47 ^b	6.18 ^d (-4.0 %)	6.85 ^a (+6.0 %)	7.20 ^a (+11 %)	Treatments (T) = 0.24
LCT	7.00 ^a	5.09 ^b (-27 %)	5.90 ^b (-16 %)	6.29 ^d (-10 %)	Varieties (V) = 0.14
WCT × COD	6.97 ^a	5.30 ^a (-24 %)	6.87 ^a (-1.0 %)	6.99 ^b (+0.3 %)	T × V = 0.12
COD × WCT	6.95 ^a	4.18 ^d (-40 %)	6.86 ^a (-1.0 %)	7.27 ^a (+4.0 %)	SEm ± 0.11
Mean	6.89	5.03 (-27 %)	6.66 (-3.0 %)	6.98 (+0.6 %)	

Means followed by the same letter in a column do not differ significantly according to Duncan's multiple-range test. The values with in parenthesis are relative change due to treatment as compared to chamber control (CC)

CO₂ [(CO₂)₇₀₀] the protein concentration decreased as compared to that in the chamber control. The concentration of total soluble proteins decreased significantly in LCT under elevated CO₂ [(CO₂)₅₅₀] and under elevated temperature (T + 2 °C) in COD × WCT. On the other hand, increase in total soluble protein concentration was highest in COD under elevated CO₂ condition (Table 4). Studies on other species also reported decrease in total protein concentration under elevated CO₂ and temperature (Chaitanya et al. 2001; Taub et al. 2008). Such response of total protein concentration under elevated CO₂ condition can be attributed to the inadequate available soil nitrogen (Taub et al. 2008).

From the above results, it can be concluded that improved enzyme activities and membrane stability may benefit coconut seedlings under elevated CO₂ and temperature conditions. By virtue of greater membrane stability and lower MDA content, WCT and COD × WCT may be assumed as tolerant among the studied coconut

cultivars and hybrids for growing in changing climatic conditions. Since coconut is a perennial crop with a life span of 50–60 years, long term studies may provide better understanding on the efficiency of such cultivars.

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