Environ Sci Pollut Res (2015) 22:3936–3946
DOI 10.1007/s11356-014-3661-6

RESEARCH ARTICLE

Effects of ambient and elevated CO_2 on growth, chlorophyll fluorescence, photosynthetic pigments, antioxidants, and secondary metabolites of *Catharanthus roseus* (L.) G Don. grown under three different soil N levels

Aradhana Singh · Madhoolika Agrawal

Received: 11 April 2014 / Accepted: 25 September 2014 / Published online: 12 October 2014

Abstract *Catharanthus roseus* L. plants were grown under ambient (375±30 ppm) and elevated (560±25 ppm) concentrations of atmospheric CO_2 at different rates of N supply (without supplemental N, 0 kg N ha^{-1}; recommended N, 50 kg N ha^{-1}; and double recommended N, 100 kg N ha^{-1}) in open top chambers under field condition. Elevated CO_2 significantly increased photosynthetic pigments, photosynthetic efficiency, and organic carbon content in leaves at recommended (RN) and double recommended N (DRN), while significantly decreased total nitrogen content in without supplemental N (WSN). Activities of superoxide dismutase, catalase, and ascorbate peroxidase were declined, while glutathione reductase, peroxidase, and phenylalanine-ammonia lyase were stimulated under elevated CO_2. However, the responses of the above enzymes were modified with different rates of N supply. Elevated CO_2 significantly reduced superoxide production rate, hydrogen peroxide, and malondialdehyde contents in RN and DRN. Compared with ambient, total alkaloids content increased maximally at recommended level of N, while total phenolics in WSN under elevated CO_2. Elevated CO_2 stimulated growth of plants by increasing plant height and numbers of branches and leaves, and the magnitude of increment were maximum in DRN. The study suggests that elevated CO_2 has positively affected plants by increasing growth and alkaloids production and reducing the level of oxidative stress. However, the positive effects of elevated CO_2 were comparatively lesser in plants grown under limited N availability than in moderate and higher N availability. Furthermore, the excess N supply in DRN has stimulated the growth but not the alkaloids production under elevated CO_2.

Keywords Elevated carbon dioxide · Nitrogen · Oxidative stress · Growth · Alkaloids

Responsible editor: Philippe Garrigues

A. Singh · M. Agrawal (✉)
Laboratory of Air Pollution and Global Climate Change, Department of Botany, Banaras Hindu University, Varanasi 221005, India
e-mail: madhoo.agrawal@gmail.com

M. Agrawal
e-mail: madhoo58@yahoo.com

Introduction

As a result of anthropogenic activities, such as combustion of fossil fuels and biomass and deforestation, the atmospheric carbon dioxide (CO_2) concentration has increased from 280 to 398 ppm since the industrial revolution (NOAA 2013) and is expected to continue in the future by the end of this century (IPCC 2013). The level of CO_2 in the atmosphere has increased by 40 % compared with the level in 1750 (IPCC 2013). Elevated CO_2 stimulates net photosynthetic rate, growth, and development (Ainsworth and Long 2005; Ghannoum et al. 2010) and reduces photorespiration and oxidative stress (Vurro et al. 2009).

Nitrogen (N) is another important limiting resource and a major part of Rubisco and light reaction components involved in photosynthesis (Tissue et al. 1993). Previous studies with tree plants have shown that responses of plants to elevated CO_2 can be modified by N availability (Manderscheid et al. 2010; Zhang et al. 2011). Higher supply of N has been shown to increase plant growth, leaf N, net photosynthetic rate, and photosynthetic water use efficiency in *Eucalyptus* species grown under 760 ppm concentration of CO_2 in growth chambers (Novriyanti et al. 2012). However, Zhao et al. (2010)

reported a decrease in leaf chlorophyll and photosynthetic rate in sorghum plants at elevated CO_2 grown in N-deficient soil.

Elevated CO_2 reduced N content in plants due to dilution of N by increasing photosynthetic assimilation of carbon (Kumari et al. 2013). Nitrogen limitation stimulates formation of reactive oxygen species (ROS) such as H_2O_2, $^{\cdot}O_{2-}$ and OH^{-} in cellular compartments (Polle et al. 1997), which react with membrane lipids to generate lipid peroxides and other toxic reactive oxygen intermediates (Mittler 2002). Plant cells involve complex antioxidant defence mechanisms (enzymatic and nonenzymatic) against oxidative stress generated under challenging stress conditions (Matsuura and Fett-Neto 2013). Enzymatic antioxidant mechanisms include superoxide dismutase (SOD), catalase (CAT), and enzymes of ascorbate-glutathione cycle, such as ascorbate peroxidase (APX) and glutathione reductase (GR).

Elevated CO_2 increases the ratio of CO_2/O_2 at the site of photo-reduction because a greater amount of CO_2 diffuses inside the leaf (Robredo et al. 2007). Therefore, elevated CO_2 stimulates photosynthesis rate and inhibit the rate of photorespiration and ROS production, leading to downregulation of ROS-scavenging enzymes (Halliwell and Gutteridge 1989). Plentiful supply of N also reduces risk of damage caused by ROS, such as H_2O_2 and $^{\cdot}O_{2-}$ (Nakaji et al. 2001).

Secondary metabolites are a group of compounds synthesized in plants having antioxidant property against oxy radicals. Alkaloids, also a secondary metabolite exhibit biological activity, such as antitumoral, analgesic, antimicrobial, and insecticide. Other than improving the antioxidative capacity of plants, elevated CO_2 also enhances the production of alkaloids in medicinal plants (Ziska et al. 2008; Oliveira et al. 2010). It has been demonstrated that supply of N also enhances the synthesis of total alkaloids in *Catharanthus roseus* (Gholamhosseinpour et al. 2011) and in *Ilex vomitoria* (Palumbo et al. 2007). However, only limited studies are available on the combined effects of elevated CO_2 and nitrogen supply on oxidative stress, antioxidants status, growth, and secondary metabolism of medicinal plants. Polle et al. (1997) reported that elevated CO_2 with moderate nitrogen decreased the malondialdehyde content and SOD activity, leading to a reduction in oxidative stress. Matros et al. (2006) studied the accumulation of the secondary metabolites coumarins in tobacco plants at different CO_2 and N treatments in controlled environmental conditions. Higher concentrations of scopolin and scopoletin were observed in tobacco leaves grown at elevated level of CO_2 with higher N concentration. Plant growth as well as essential oil yield in lemon balm was increased under high CO_2 with additional supply of N (Shoor et al. 2012). Based on the above information, we hypothesized that with supply of N, elevated CO_2 would become more beneficial for plant growth, physiological performance, antioxidants, and alkaloids production. The present study was conducted to determine the responses of elevated CO_2 and N supply on photosynthetic pigments, chlorophyll fluorescence, oxidative stress, antioxidants, and secondary metabolites status in a medicinal plant, *C. roseus* (L.) G. Don, which is an important source of chemotherapeutic agents used in the treatment of several kinds of cancer.

Materials and methods

Experimental site and design

A field experiment was conducted in open top chambers (OTCs) during April to August 2011 in the Botanical garden of Banaras Hindu University, Varanasi, Uttar Pradesh (25° 18′ N latitude, 82° 01′ E longitude, and 76.19 m above sea level) situated in eastern Gangetic plains of India. During the experimental period, mean maximum temperature ranged from 32 to 40 °C, while minimum temperature varied from 20 to 25 °C. Mean relative humidity varied from 52.1 to 89.3 %, and total rainfall was recorded (763 mm) maximum during August. The experiment was designed as a split plot with CO_2 as main plot and nitrogen as subplot. There were two CO_2 treatments, i.e., ambient at 375± 30 ppm and elevated at 560±25 ppm. The elevated CO_2 concentration of 560 ppm was selected on the basis of IPCC (2007), which suggested that CO_2 will reach between 535 and 700 ppm by the end of this century. Three nitrogen concentrations were used for each CO_2 treatment viz. without supplemental nitrogen (WSN; no N was added above the residual N in the soil), recommended dose of nitrogen (RN), and double recommended dose of nitrogen (DRN); N was given as urea in the soil. Recommended and double recommended nitrogen doses were 50 and 100 kg ha^{-1}, respectively.

Open top chambers

OTCs of 1.9 m height and 2.05 m diameter were installed at the experimental site following the design reported by Kumari et al. (2013). Each chamber was attached with a high-speed blower for the continuous supply of air at the rate of three air changes per minute. Elevated CO_2 was supplied to OTCs via CO_2 cylinders connected with blowers and was maintained through a solenoid valve attached to a CO_2 analyzer. Measurements of microclimatic parameters (temperature, humidity, and light) were done within and outside OTCs during the experimental period. Mean temperature and relative humidity were 0.1–0.3 °C and 2–6 %, respectively, higher within the chambers than outside. Light intensity was 4–5 % less inside the chambers than outside.

Raising of test plants

Periwinkle (*C. roseus* (L.) G Don.), a perennial plant of the family Apocyanaceae was selected as test plant. The field was prepared by adding farmyard manure and ploughing up to 20 cm depth in the first week of April 2011. Eighteen OTCs were installed at the experimental site. There were three replicate chambers for each treatment. Soil from each experimental plot was collected for measurement of N content before transplantation of plantlets. Then, the deficit amount of N was added to the soil to attain the RN and DRN concentrations in two splits (Table 1). One third dose of nitrogen was given as basal dressing before transplantation, and two third dose was given as top dressing at 30 days after transplantation (DAT). Seeds of periwinkle were sown in a plot (2.5×2.5 m^2) without any N amendment in the last week of March 2011. Plantlets of 15 days were transplanted in rows in OTCs at 20 cm distance; there were a total of 15 plants in each chamber. Similar soil moisture at field capacity was maintained in each chamber through drip irrigation.

CO_2 monitoring

CO_2 monitoring was done inside and outside the OTCs using CO_2 analyzer (*LI-820*, *LI-COR* Biosciences, Lincoln, USA), and variations in diurnal concentration of CO_2 were recorded. Mean ambient concentration of CO_2 was recorded as 375± 30 ppm, and the elevated CO_2 concentration was 560± 25 ppm.

Plant sampling and analyses

Chlorophyll fluorescence

Chlorophyll fluorescence was determined between 10:30 and 11:30 hours using portable plant efficiency analyzer (Model, MK2, 9414, Hansatech Instrument Ltd., UK) on the third fully expanded mature leaf from the top of each plant. Leaf clips for dark adaptation were placed on the adaxial leaves for 30 min before measurement at an excitation irradiance of 3000 μmol m^{-2} s^{-1}. Minimum side of the fluorescence (F_0) and maximum fluorescence (F_m) were measured, from which variable fluorescence ($F_v=F_m-F_0$) and ratio of variable and maximum fluorescence (F_v/F_m) were calculated.

Table 1 Recommended, deficit, and actual amount of N added for attaining required amount of N in the soil

	WSN kg N ha^{-1}	RN kg N ha^{-1}	DRN kg N ha^{-1}
Recommended amount			
ACO_2	–	50	100
ECO_2	–	50	100
Deficit amount added			
ACO_2	–	26.7	75.8
ECO_2	–	25.3	75.4
Actual N concentration			
ACO_2	25.78	49.70	99.3
ECO_2	25.21	49.53	98.9

ACO_2 ambient CO_2, *ECO_2* elevated CO_2, *WSN* without supplemental N, *RN* recommended N, *DRN* double recommended N

Biochemical parameters

Biochemical parameters were analyzed in leaves at 60 DAT. Malondialdehyde (MDA) content was measured following the protocol given by Heath and Packer (1968). Superoxide radical ($O_2^{\cdot-}$) production rate was determined by monitoring the rate of nitrite formation from hydroxylamine in the presence of superoxide radicals (Elstner and Heupel 1976). Hydrogen peroxide content was measured after reacting with KI according to Alexieva et al. (2001). Total chlorophyll and carotenoids were estimated following Maclachlan and Zalik (1963) and Duxbury and Yentsch (1956), respectively. Estimation of ascorbic acid (AA) was done using the method of Keller and Schwager (1977). Total phenolics were extracted in 70 % acetone and then determined by adding folin ciocalteau reagent (Bray and Thorpe 1954).

For extraction of SOD enzyme (EC 1.15.1.1), 500 mg of fresh leaf sample was homogenized with 5 ml of extraction buffer (0.1 M phosphate buffer containing 0.5 mM ethylenediaminetetraacetic acid at pH 7.5) in a prechilled mortar and pestle. The homogenate was centrifuged at 15,000 rpm for 20 min. SOD activity was measured as percentage reduction of nitrobluetetrazolium (NBT) following the method of Fridovich (1974). APX (EC 1.11.1.11) activity was estimated by the method of Nakano and Asada (1987). Fresh leaf tissue (0.1 g) was homogenized with 5 ml of cold potassium phosphate buffer (0.05 M, pH 7.8) containing polyvinyl pyrolidone (1 %), ascorbic acid (1 mM), and phenylmethylsulfonyl fluoride (1 mM). The homogenate was centrifuged at 12,000 rpm for 15 min, and the extract was used for the determination of APX enzyme activity. For assay of GR (EC 1.6.4.2) enzyme, 200 mg fresh leaf sample was homogenized using chilled mortar and pestle in 5 ml of 50 mM Tris–HCl buffer (pH=7.6). The homogenate was centrifuged at 22,000×g for 30 min at 4 °C, and the supernatant was used for assay of GR activity by recording the decrease in absorbance of NADPH at 340 nm (Schaedle and Bassham 1977). Peroxidase (POX; EC 1.11.1.7) activity was determined by using the method of Britton and Mehley (1955). For extraction of enzyme, 100 mg fresh leaf sample was homogenized with 10 ml of 0.1 M cold phosphate buffer containing 5 mM cystein

at pH 6.8 in a prechilled mortar and pestle. The homogenate was centrifuged at 10,000×*g* for 10 min at 0 °C, and supernatant was used for estimating enzyme activity. Phenylalanine-ammonia lyase (PAL; EC 4.3.1.5), enzyme is extracted by homogenizing 1 g of leaf tissue in 0.2 M sodium borate buffer (pH 8.7) containing 2-mercaptoethanol. Estimation of enzyme activity as μmol *t*-cinnamic acid ml^{-1} was done by using phenyl alanine at 280 nm (Rao and Tower 1970).

Organic carbon and total nitrogen

Dried and sieved foliar samples were used for organic carbon content by wet digestion following the modified method of Walkley and Black (1934) and total foliar nitrogen was measured through Gerhardt automatic N analyzer (Model KB8S, Germany).

Extraction of alkaloids

Alkaloids were extracted from air dried and powdered leaf samples of *C. roseus* with three different organic solvents in separating funnel (Singh et al. 2000). Powdered sample (5 g) was extracted with 90 % (30× 3 ml, 12 h each) ethanol, and then extract was evaporated using an incubator till 10 ml of volume remains. Then, 10 ml of 3 % HCL and 10 ml of double distilled water were added. The solution was again extracted with ethyl acetate (30×3 ml, 12 h each). Then, pH of extract was made alkaline (8.5) by adding ammonia solution. Finally, it was extracted with chloroform (30×3 ml, 12 h each). Extract was collected and chloroform was evaporated to dryness and then weighed to obtain total alkaloids content.

Morphological parameters

Three plants were tagged randomly from each chamber for the measurement of morphological parameters. Stem height, number of branches, number of leaves, and number of flowers and fruits were measured in intact plants at 60 DAT.

Statistical analysis

Multivariate analysis of variance (ANOVA) tests were done for all the analyzed parameters with the factors CO_2, N, and their interactions. Significantly, different means between ambient and elevated CO_2 were calculated using "Student's *t* test." The entire statistical analyses were conducted by using SPSS software (SPSS Inc., version 16.0).

Results

Chlorophyll fluorescence and photosynthetic pigments

Elevated CO_2 significantly increased F_0 in WSN, while F_m, F_v, and F_v/F_m ratio in RN and DRN (Table 2). Total chlorophyll and carotenoid contents were significantly increased in RN and DRN under elevated CO_2 compared with ambient (Fig. 1). In WSN, photosynthetic pigments were reduced under elevated CO_2, but reduction was not significant for chlorophyll (Fig. 1). Results of two-way ANOVA test showed that F_v/F_m ratio, total chlorophyll, and carotenoids varied due to N, CO_2, and their interaction (Table 4).

Total nitrogen and organic carbon contents

Total N content was lowered under elevated CO_2 compared with ambient in WSN (Fig. 1). A significant increase in organic carbon under elevated CO_2 was observed in RN and DRN (Fig. 1). Total N varied significantly only due to N and CO_2 while organic carbon showed significant effects of N, CO_2, and N×CO_2 (Table 4).

Super oxide radical ($^{\cdot}O_2^-$) production rate, H_2O_2, and MDA contents

Elevated CO_2 significantly reduced the rate of $^{\cdot}O_2^-$ production in DRN by 43.2 % (Fig. 2). H_2O_2 content was also reduced significantly in RN (35.4 %) and DRN (49.3 %) under elevated CO_2 (Fig. 2). As compared with ambient CO_2, plants grown under elevated CO_2 showed reduced level of MDA content in WSN (25.1 %), RN (41.7 %), and DRN (32.2 %) (Fig. 2). Significant effects of N, CO_2, and N×CO_2 on $^{\cdot}O_2^-$ and H_2O_2, however, MDA content did not show a significant interaction effect (Table 4).

Antioxidants and metabolites

Elevated CO_2 caused significant reductions in the activities of SOD and CAT in RN and DRN; however, APX showed significant reduction only in DRN (Fig. 3). Elevated CO_2 significantly stimulated the activities of POX and PAL in WSN and RN, while GR activity was significantly increased in RN and DRN (Fig. 3). ANOVA results showed that SOD activity had significant effects of N and CO_2 individually. CAT and POX varied only due to CO_2, while APX varied due to N and N×CO_2 (Table 4). CO_2 and N×CO_2 had significant effects on GR, while PAL activity showed significant effects of all the individual factors and their interaction (Table 4).

Elevated CO_2 significantly increased total phenolics content in all N levels (Fig. 4). As compared with ambient, total alkaloids content was significantly higher in WSN and RN

Table 2 Initial fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence (F_v) and F_v/F_m ratio of *C. roseus* under different CO_2 and N treatments

Treatments	Parameters			
	F_0	F_m	F_v	F_v/F_m
WSN				
ACO_2	256±6.5	1151.7±48	895.7±16	0.777±0.005
ECO_2	265±5.7*	1185±8.6 NS	920±6.1 NS	0.776±0.004 NS
RN				
ACO_2	260.3±2.0	1129±14	868.3±7.6	0.769±0.004
ECO_2	243.6±6.4 NS	1293.6±51*	1056.0±53*	0.811±0.004*
DRN				
ACO_2	265.7±4.3	1147±9.2	881±11	0.768±0.004
ECO_2	249±2.3 NS	1311±11**	1061±16**	0.810±0.005*
Results of two-way ANOVA				
N				
F	1.52	2.16	2.58	5.43
P	0.25 NS	0.16 NS	0.117 NS	0.021*
CO_2				
F	4.07	24.28	28.01	57.53
P	0.07 NS	0.0*	0.0*	0.0*
N×CO_2				
F	4.53	3.14	4.55	15.08
P	0.034*	0.08 NS	0.034*	0.001***

Values are mean±SE

ACO_2 ambient CO_2, *ECO_2* elevated CO_2, *WSN* without supplemental N, *RN* recommended N, *DRN* double recommended N, *NS* not significant

* $p < 0.05$; ** $p < 0.01$; ***$p<0.001$—levels of significance between ambient and elevated CO_2 at a particular N treatment

under elevated CO_2; however, no significant change was observed in DRN (Fig. 4). Ascorbic acid content was significantly lower under elevated CO_2 in WSN (Fig. 4). Total phenolics and ascorbic acid showed significant effects of N and CO_2, while total alkaloids varied due to N, CO_2, and N×CO_2 (Table 4).

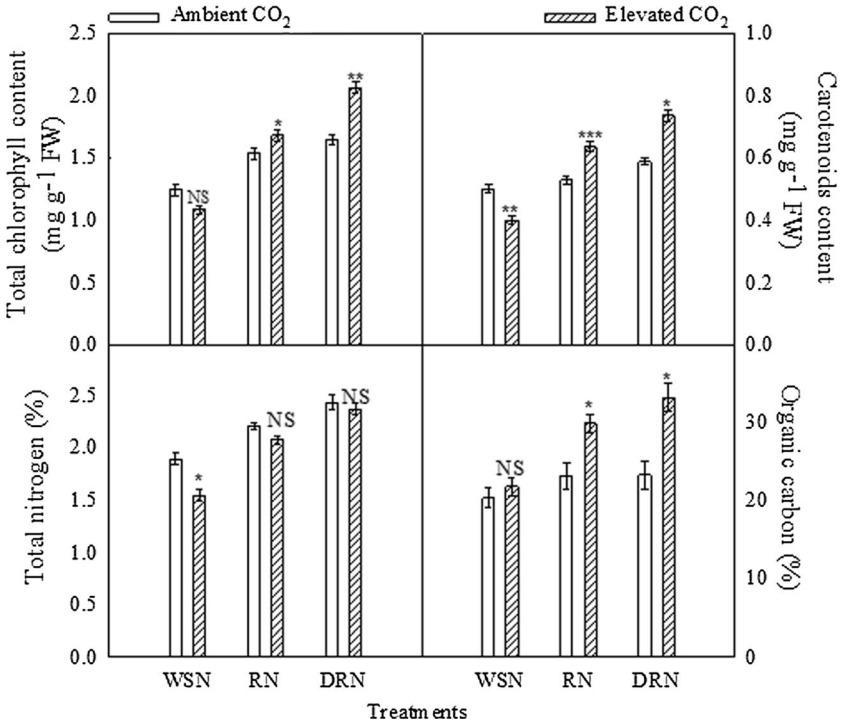

Fig. 1 Total chlorophyll, carotenoids, total nitrogen, and organic carbon contents in *C. roseus* at different CO_2 and N treatments. *Bars* represent mean± SE. Levels of significance between ambient and elevated CO_2 at a particular N; *NS* not significant, *$p<0.05$; **$p<0.01$; ***$p<0.001$, as determined by Student's *t* test. *WSN* without supplemental N, *RN* recommended N, *DRN* double recommended N

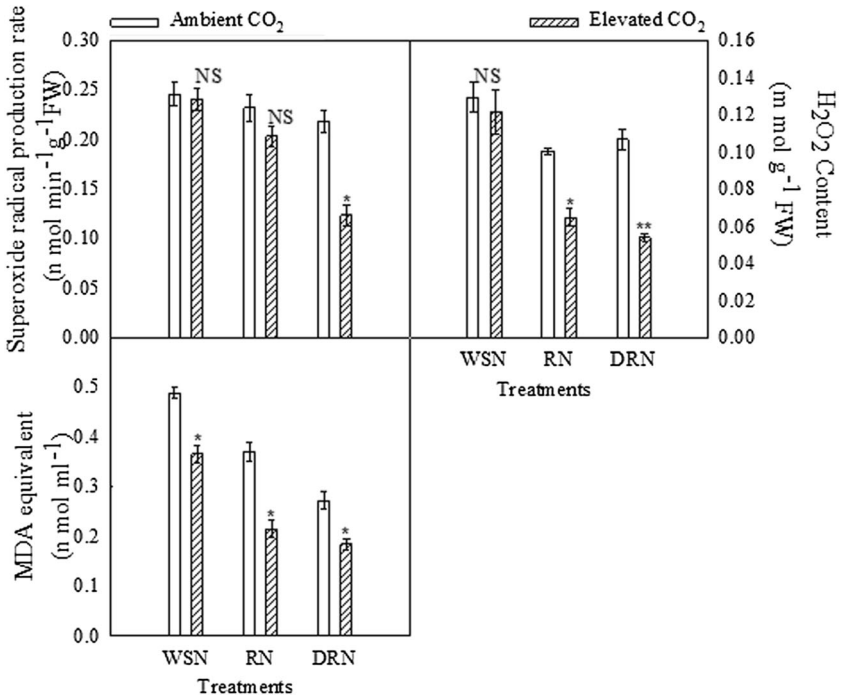

Fig. 2 Superoxide radical production rate, H_2O_2 and MDA contents in *C. roseus* at different CO_2 and N treatments. *Bars* represent mean±SE. Levels of significance between ambient and elevated CO_2 at a particular N; *$p<0.05$; **$p<0.01$; ***$p<0.001$, as determined by Student's *t* test. *WSN* without supplemental N, *RN* recommended N, *DRN* double recommended N

Growth

Plant height, number of leaves, and number of branches were significantly increased under elevated CO_2 in WSN, RN, and DRN (Table 3). Plants grown in RN and DRN produced significantly higher number of flowers under elevated CO_2 (Table 3), but the number of fruits was significantly higher in DRN (Table 3). ANOVA test showed significant effects of all the treatments and their combination on all the measured growth parameters except for the number of fruits (Table 4).

Discussion

The present study showed that elevated CO_2 had significant positive effects on growth and metabolism of *C. roseus* with increase in supply of nitrogen. Since carbon and nitrogen are the major components of photosynthetic pigments, elevated CO_2 increased chlorophyll and carotenoids contents in RN and DRN. Higher chlorophyll content is suggested to be an adaptation of the plants under elevated CO_2 to increase the photosynthetic activity (Bhatt et al. 2010). Li and Gupta (1993) reported an increase in chlorophyll content under elevated CO_2, with and without N supply. In the present study, photosynthetic pigments were reduced under limited supply of nitrogen (WSN), which may be ascribed to reduced uptake of N and increased photosynthetic activity due to elevated CO_2. Shangguan et al. (2000) also reported reduction in total chlorophyll content due to nitrogen deficiency.

Light energy absorbed by chlorophyll molecules present in photosystems and reaction centers can be converted into chemical energy via photosynthesis or dissipated as heat or re-emitted as chlorophyll fluorescence (Baker 2008). The efficiency and stability of PS II, a major component of the photosynthetic apparatus, have been widely monitored through the measurement of fluorescence of PS II (F_v/F_m) in dark-adapted leaves. In the present study, increase in F_0 indicates a lower re-absorption rate of the emitted fluorescence light, which might be due to a decrease in chlorophyll content (Barber 1998) under limited supply of N, though the reduction in chlorophyll content was insignificant. Furthermore, decrease in F_0 and significant increments in F_m and F_v under elevated CO_2 in RN and DRN suggests an increase in the rate constant of energy trapped by light harvesting complex (Havaux et al. 1991). In the present study, increase in F_v/F_m ratio under elevated CO_2 with additional N supply indicates increased efficiency of PS II and also reduced risk of damage caused to PSII by oxidative stress. This suggests that high soil-nitrogen supply enhanced not only the absorption rate of leaf due to increased chlorophyll content affecting F_0 but also the energy cycling between the reaction center and the chlorophyll pool, thus affecting F_m. Zhao et al. (2010) also reported an increase in F_v/F_m ratio in *Betula platyphylla* exposed to 700 ppm CO_2 at higher N.

Incomplete utilization of light energy absorbed by photosynthetic machinery leads to generation of ROS at the acceptor side of photosystem (Asada 1999). ROS such as $^{\cdot}O_2^-$ and H_2O_2 are highly sensitive and responsible for photo-inhibition through direct oxidative damage to photosystem. In the

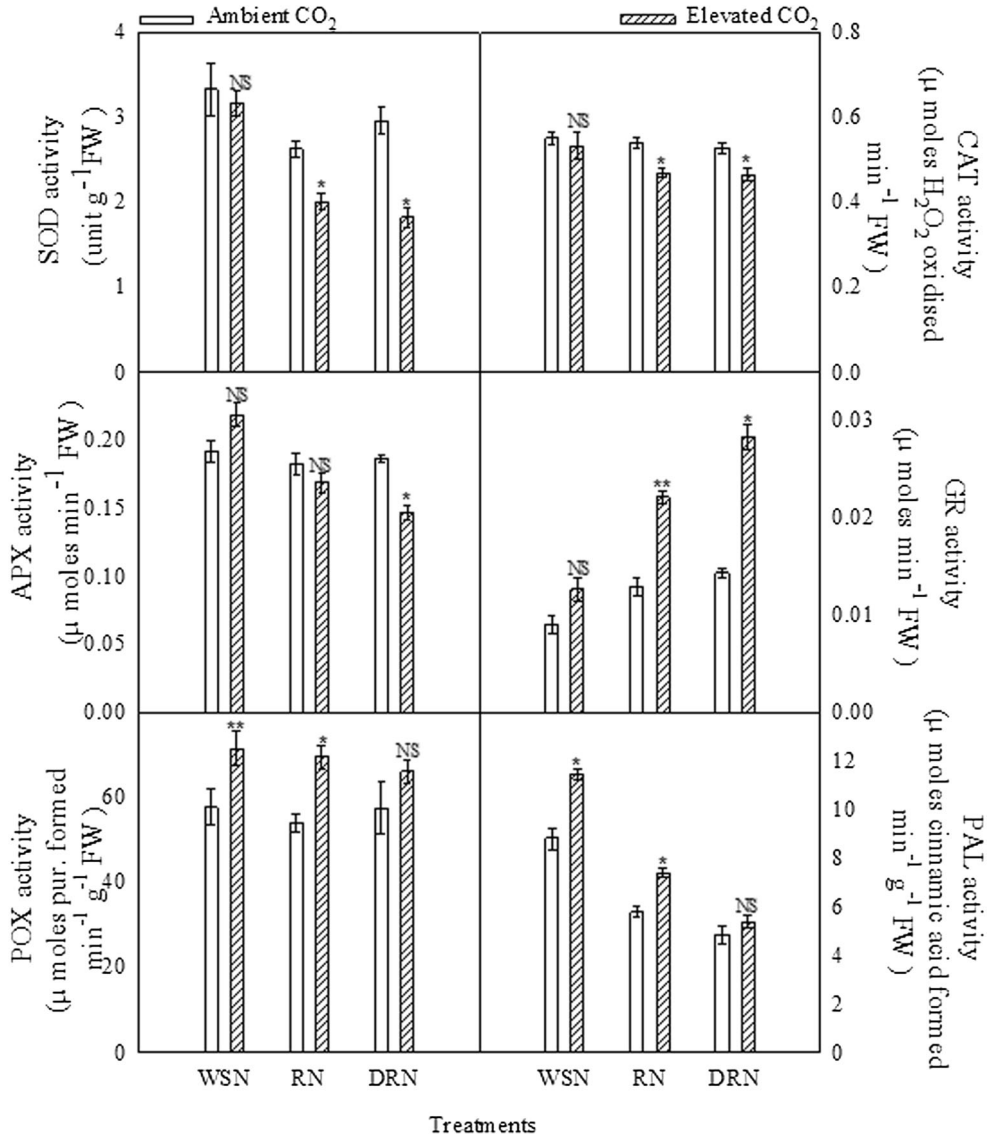

Fig. 3 Superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidises (*APX*) and glutathione reductase (*GR*), peroxidase (*POX*), and phenylalanine-ammonia lyase (*PAL*) activities in *C. roseus* at different CO_2 and N treatments. *Bars* represent mean±SE. Levels of significance between ambient and elevated CO_2 at a particular N; *$p<0.05$; **$p<0.01$; ***$p<0.001$, as determined by Student's *t* test. *WSN* without supplemental N, *RN* recommended N, *DRN* double recommended N

present study, significant reductions in $^{\cdot}O_2^-$ and H_2O_2 contents were observed under elevated CO_2 with higher N supply (RN and DRN). It indicates that elevated CO_2 and N supply reduces ROS generation by increasing pCO_2/pO_2 at the site of photo-reduction (Salazar-Parra et al. 2012) and increased the utilization of energy in photosystems leading to reduced availability of free electrons. Mishra et al. (2013) also reported that H_2O_2 content reduced by 41.6 % in wheat at 700 ppm CO_2. It seems that N limitation in WSN did not allow the positive effects of elevated CO_2 in reducing the oxidative stress and hence F_v/F_m ratio did not differ significantly in WSN between ambient and elevated CO_2 as mentioned earlier. Grossman and Takahashi (2001) reported accumulation of ROS in cells of photosynthetic organisms under nutrient limitation.

ROS interact with all biological molecules and cause cellular damage, such as lipid peroxidation, measured as MDA content during the present study. MDA and other peroxides formed during lipid peroxidation increase hydrophobicity of the inner side of the membrane (Frankel 1991), which hampers transport mechanism in cells leading to osmotic imbalance. Reduction in MDA content under elevated CO_2 may be due to reduced levels of H_2O_2 and $^{\cdot}O_2^-$ production rate during the present experiment. Higher reduction in MDA content under elevated CO_2 in RN and DRN further demonstrated that higher N availability protected the plants more against oxidative stress compared with WSN.

Plants possess a complex antioxidative defence system consisting of enzymatic and nonenzymatic components to scavenge ROS. SOD enzyme catalyzes dismutation of $^{\cdot}O_2^-$ into H_2O_2, which is further detoxified by CAT, APX, and POX. Reduced SOD and CAT activities under elevated CO_2 in RN and DRN may be directly correlated to a decrease in the production of $^{\cdot}O_2^-$ and H_2O_2 during the present study. A significant drop in SOD activity was reported under elevated

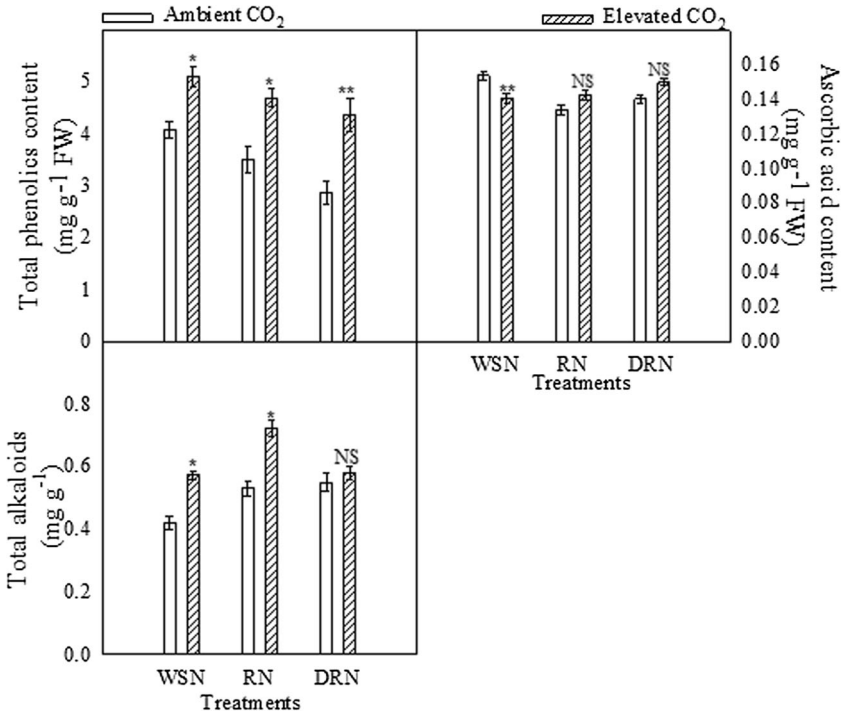

Fig. 4 Total phenolics, ascorbic acid, and total alkaloids contents in *C. roseus* at different CO_2 and N treatments. *Bars* represent mean±SE. Levels of significance between ambient and elevated CO_2 at a particular N; *NS* not significant, *$p<0.05$; **$p<0.01$; ***$p<0.001$, as determined by Student's *t* test. *WSN* without supplemental N, *RN* recommended N, *DRN* double recommended N

CO_2 (700 ppm) with an intermediate N supply (Schwanz and Polle 1998). Similarly, APX activity also reduced in DRN under elevated CO_2. Results of the present study suggest a downregulation of ROS-scavenging enzymes under elevated CO_2 at higher N supply. However, activities of GR, POX, and PAL enzymes were significantly upregulated under elevated CO_2. As GR is utilized in the regeneration of reduced glutathione for ascorbate-glutathione redox reactions, an increase

Table 3 Plant height, numbers of branches and leaves, and numbers of flowers and fruits in *C. roseus* under different CO_2 and N treatments

Treatments	Parameters				
	Plant height (cm)	Number of branches	Number of leaves	Number of flowers	Number of fruits
WSN					
ACO_2	22.0±1.5	2.0±0.32	26.4±1.33	2.0±0.32	0.4±0.24
ECO_2	51.5±2.3**	3.8±0.2*	46.6±5.40*	4.6±0.93 NS	0.6±0.60 NS
RN					
ACO_2	35.4±2.0	2.0±0.32	28.4±2.44	2.4±0.24	1.4±0.98
ECO_2	63.9±4.2**	4.6±0.81*	55.6±5.87*	7.4±1.03**	1.8±1.11 NS
DRN					
ACO_2	28.4±1.9	3.0±0.45	40.4±2.23	2.8±0.37	2.2±0.20
ECO_2	72.9±4.8**	7.8±0.66***	82.6±4.5***	10.2±0.86**	4.6±0.40**
Results of two-way ANOVA					
N					
F	12.71	14.05	21.47	10.38	9.12
P	0.00***	0.00***	0.00***	0.00***	0.00***
CO_2					
F	183.50	54.96	83.23	76.01	3.19
P	0.00***	0.00***	0.00***	0.00***	0.09 NS
N×CO_2					
F	4.11	4.70	3.93	5.84	1.57
P	0.03*	0.02*	0.03*	0.01**	0.23 NS

Values are mean±1 SE

ACO_2 ambient CO_2, *ECO_2* elevated CO_2, *WSN* without supplemental N, *RN* recommended N, *DRN* double recommended N, *NS* not significant

*$p<0.05$; **$p<0.01$; ***$p<0.001$—levels of significance between ambient and elevated CO_2 at a particular N treatment

Table 4 Results of two-way ANOVA test showing *F* and *P* values and levels of significance for biochemical parameters within and between N and CO_2 treatments

Parameters	N		CO_2		N×CO_2	
	F	*P*	*F*	*P*	*F*	*P*
Total chlorophyll	137.80	0.00***	15.04	0.00***	23.22	0.00***
Carotenoids	116.39	0.00***	21.74	0.00***	45.93	0.00***
Total phenolics	27.25	0.00***	133.78	0.00***	1.79	0.21 NS
Ascorbic acid	5.56	0.02*	47.68	0.00***	0.08	0.92 NS
Total alkaloids	16.08	0.00***	44.96	0.00***	6.89	0.01**
Total nitrogen	69.81	0.00***	12.54	0.00***	3.45	0.07 NS
Organic carbon	12.29	0.00***	23.52	0.00***	3.96	0.05*
PAL	148.59	0.00***	42.20	0.00***	6.19	0.01***
APX	17.65	0.00***	2.73	0.13 NS	11.79	0.00***
GR	2.78	0.10 NS	56.50	0.00***	4.48	0.04**
SOD	19.08	0.00***	20.32	0.00***	3.28	0.07 NS
CAT	3.67	0.06 NS	11.88	0.01**	1.24	0.33 NS
POX	0.34	0.72 NS	15.60	0.00***	0.42	0.67 NS
H_2O_2	28.08	0.00***	32.21	0.00***	5.54	0.02**
MDA	78.12	0.00***	85.25	0.00***	2.13	0.16 NS
$^{\cdot}O^{-}_{2}$	20.40	0.00***	21.30	0.00***	8.10	0.01**

NS not significant

*$p<0.05$; **$p<0.01$; ***$p<0.001$—levels of significance

in its activity under elevated CO_2 in higher N supply suggests an enhanced antioxidant regenerating potential during the present study. Increase in GR activity under elevated CO_2 might also be attributed to the increase in synthesis of photosynthetic nicotinamide adenine dinucleotide phosphate under increased pCO_2/pO_2 ratio (Vurro et al. 2009). POX enzyme plays an important role in detoxification of H_2O_2 (Jaleel et al. 2008) as well as synthesis of alkaloids in *C. roseus* (Sottomayor et al. 2004). As POX is also involved in secondary metabolism, increase in the availability of carbon assimilated may have acted as a signal for upregulation of this enzyme under elevated CO_2.

Organic carbon content (mainly structural and nonstructural carbohydrates) increased with supplemental N (RN and DRN) under elevated CO_2. This may be correlated with increase in photosynthetic carbon uptake due to CO_2 enrichment under adequate supply of N (Ainsworth and Long 2005). Kumari et al. (2013) also reported an increase in organic carbon content in leaves of *Beta vulgaris* grown under 570 ppm CO_2 concentration. In the present experiment, total foliar N was significantly declined under elevated CO_2 in WSN. The decrease in foliar N under elevated CO_2 is ascribed to the dilution by carbohydrates (Gifford et al. 2000) and reduced assimilation of nitrate into organic nitrogen compounds (Bloom et al. 2010).

It is widely recognized that elevated CO_2 with higher N availability increases photosynthetic rate leading to enhanced growth (Ainsworth and Long 2005). In the present experiment, more number of leaves produced by plants grown under elevated CO_2 led to enhanced conversion efficiency, which ultimately resulted in increased growth of other plant parts, with a greater extent in DRN. Enhanced production of photosynthates under elevated CO_2 directly affects cell division by stimulating cyclin-dependent protein kinase activity (Ranasinghe and Taylor 1996) and provokes the enhanced morphogenetic development (Morison and Lawlor 1999). During the present study, elevated CO_2 increased the numbers of leaves and branches and stem height in RN and DRN treatments. Hence, the present study proves the hypothesis that elevated CO_2 with higher N supply will lead to more growth enhancement compared with no additional supply of N. Significant positive effects of elevated CO_2 and higher N on growth of Eucalyptus plant were also reported by Novriyanti et al. (2012)

In the present study, phenolics content was significantly higher under elevated CO_2 in all N levels. This may be correlated with the fact that elevated CO_2 causes a stimulatory effect on the activity of the key enzyme PAL of phenylpropanoid pathway resulting in increased synthesis of phenolics (Mattson et al. 2005). The degree of increment in total phenolics was reduced with increasing N supply. The increase in N availability decreased the C/N ratio in plants resulting in decreased level of carbon-based secondary metabolites as resources allocated primarily toward growth due to more availability of N. In the present study, the lowest PAL enzyme

activity was observed in plants grown in DRN under ambient as well as elevated CO_2 treatments.

Furthermore, elevated CO_2 increased the total alkaloids content and enhancement was greater in RN than in WSN during the present study. Ziska et al. (2008) demonstrated that alkaloids production in wild poppy was significantly increased with increasing CO_2 (from 300 to 400 ppm). During the present study, total alkaloids content was lower in DRN than RN and WSN, but the difference between ambient and elevated CO_2 treatments was insignificant in DRN. However, growth enhancement was highest in plants grown in DRN under elevated CO_2. This result suggests that a greater proportion of photosynthate was diverted toward biomass production due to increase in sink strength under elevated CO_2 with excess N supply in DRN, hence alkaloids production was decreased. This observation did not approve the hypothesis that increasing N supply will increase alkaloid production under elevated CO_2. The decrease in POX activity in DRN compared with RN and WSN may be linked with decrease in alkaloids content as POX is a key enzyme in the formation of anticancer alkaloids in *C. roseus*.

Ascorbic acid, a secondary metabolite acts as a powerful antioxidant because of its capacity to donate electrons in a number of enzymatic and nonenzymatic reactions. In the present study, under elevated CO_2, ascorbic acid content was decreased in WSN, while no significant variations were observed in RN and DRN. By contrast, Wang et al. (2003) reported an increase in ascorbic acid content in strawberries grown at 600 ppm CO_2. This indicates a trade-off between secondary metabolite pool as other secondary metabolites (phenolics and alkaloids) increased under elevated CO_2 with no additional N supply.

Conclusions

Growth and metabolism of *C. roseus* plant were affected differently by elevated CO_2 and availability of N. Elevated CO_2 increased the photosynthetic efficiency of plant with both rates of supplemental N. Under elevated CO_2 and additional N supply, plants have developed different adaptive regulations in antioxidants pool to halt the propagation of oxidative reaction chain leading to a reduction in oxidative stress. Maximum growth at DRN suggests that the continuous stimulation of growth at elevated CO_2 requires additional N to maximize C assimilation in primary metabolites. However, N supply at recommended level was more favorable for alkaloids production than without supplemental N and double recommended N. A direct correlation between N availability, POX activity, and alkaloids production was found in the test medicinal plant. The study clearly suggests that most of the positive effects of elevated CO_2 may be diminished if plants grow under nitrogen insufficiency. Furthermore, projected level of CO_2 in the near future will be beneficial for overall performance of plant only with an adequate supply of N.

Acknowledgments The authors are grateful to the Head, Department of Botany, Banaras Hindu University for providing the necessary laboratory and field facilities. The authors are thankful to the Council of Scientific and Industrial Research, New Delhi for financial assistance in the form of a research project.

References

Ainsworth EA, Long SP (2005) What have we learned from15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytol 165:351–372

Alexieva V, Sergiev I, Mapellis S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344

Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50:601–639

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol 59:659–668

Barber J (1998) Photosystem two. Biochim Biophys Acta 1365:269–277

Bhatt RK, Baig MJ, Tiwari HS (2010) Elevated CO_2 influences photosynthetic characteristics of *Avenasativa* L. cultivars. J Environ Biol 31:813–818

Bloom AJ, Burger M, Rubio-Asensio JS, Cousins AB (2010) Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. Sci 328:899–903

Bray HG, Thorpe WY (1954) Analysis of phenolic compounds of interest in metabolism. Method Biochem Anal 1:27–52

Britton C, Mehley AC (1955) Assay of catalase and peroxidase. In: Colowick SP, Kalpan NO (eds) Method in enzymology. Academic, New York, p 764

Duxbury AC, Yentsch CS (1956) Plankton pigment monographs. J Mar Res 15:19–101

Elstner E, Heupel A (1976) Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. Anal Biochem 70:616–620

Frankel EN (1991) Review. Recent advances in lipid oxidation. J Sci Food Agric 54:495–511

Fridovich I (1974) Superoxide dismutase. Adv Enzymol 41:35–97

Ghannoum O, Phillips NG, Conroy JP, Smith RA, Attard RD, Woodfield R, Logan BA, Lewis JD, Tissue DT (2010) Exposure to preindustrial, current and future atmospheric CO_2 and temperature differentially affects growth and photosynthesis in eucalyptus. Glob Change Biol 16:303–319

Gholamhosseinpour Z, Hemati K, Dorodian H, Bashiri-Sadr GZ (2011) Effect of nitrogen fertilizer on yield and amount of alkaloids in periwinkle and determination of vinblastine and vincristine by HPLC and TLC. Plant Sci res 3:4–9

Gifford RM, Barrett DJ, Lutze JL (2000) The effects of elevated [CO_2] on the C:N and C:P mass ratios of plant tissues. Plant Soil 224:1–14

Grossman A, Takahashi H (2001) Macronutrient utilisation by photosynthetic eukaryotes and the fabric of interactions. Annu Rev Plant Physiol Plant Mol Biol 52:163–210

Halliwell BH, Gutteridge JMC (1989) Free radicals in biology and medicine, 2nd edn. Clarendon, Oxford

Havaux M, Strasser RJ, Greppin G (1991) A theoretical and experimental analysis of the qP and qN coefficients of chlorophyll fluorescence

quenching and their relation to photochemical and non-photochemical events. Photosynth Res 27:41–55
Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198
IPCC (2007) Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the IPCC. Cambridge University Press
IPCC (2013) The physical science basis. In: Stocker TF, Qin DH, Plattner GK, Stocker TF, Qin DH, Plattner GK (eds) Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, New York, p 1535
Jaleel CA, Gopi R, Manivannan P, Gomathinayagam M, Sridharan R, Panneerselvam R (2008) Antioxidant potential and indole alkaloid profile variations with water deficits along different parts of two varieties of *Catharanthus roseus*. Colloids Surf B Biointerfaces 62:312–318
Keller T, Schwager H (1977) Air pollution and ascorbic acid. Eur J Plant Pathol 7:338–350
Kumari S, Agrawal M, Tiwari S (2013) Impact of elevated CO_2 and ozone on *Beta vulgaris* L.: pigments, metabolites, antioxidants growth and yield. Environ Pollut 174:279–288
Li YC, Gupta G (1993) Photosynthetic changes in soybean with and without nitrogen and increased carbon dioxide. Plant Sci 89:1–4
Maclachlan S, Zalik S (1963) Plastid structure, chlorophyll concentration and free amino acid composition of a chlorophyll mutant of barley. Can J Bot 41:1053–1062
Manderscheid R, Pacholskib A, Weigel HJ (2010) Effect of free air carbon dioxide enrichment combined with two nitrogen levels on growth, yield and yield quality of sugar beet: evidence for a sink limitation of beet growth under elevated CO_2. Eur J Agron 32:228–239
Matros A, Amme S, Kettig B, Buck-Sorlin GH, Sonnewald U, Mock H-P (2006) Growth at elevated CO_2 concentrations leads to modified profiles of secondary metabolites in tobacco cv. SamsunNN and to increased resistance against infection with potato virus Y. Plant Cell Environ 29:126–137
Matsuura HN, Porto DD, Fett-Neto AG (2013) Bio-active alkaloids from South American Psychotria and related Rubiaceae. In: Ramawat KG, Mérillon JM (eds) Natural products, 1st edn. Springer, Berlin, pp 119–147
Mattson J, Julkunen-Tiitto RW, Herms DA (2005) CO_2 enrichment and carbon partitioning to phenolics: do plant responses accord better with the protein competition or the growth differentiation balance models? Oikos 111:337–347
Mishra AK, Rai R, Agrawal SB (2013) Individual and interactive effects of elevated carbon dioxide and ozone on tropical wheat (*Triticum aestivum* L.) cultivars with special emphasis on ROS generation and activation of antioxidant defence system. Indian J Biochem Biophys 50:39–149
Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410
Morison JIL, Lawlor DW (1999) Interactions between increasing CO_2 concentration and temperature on plant growth. Plant Cell Environ 22:659–682
Nakaji T, Fukami M, Dokiya Y, Izuta T (2001) Effects of high nitrogen load on growth, photosynthesis and nutrient status of *Cryptomeria japonica* and *Pinus desiflora* seedlings. Trees 15:453–461
Nakano Y, Asada K (1987) Purification of ascorbate peroxidase in spinach chloroplast, its inactivation in ascorbate depleted medium and reactivation by monodehydroascorbate radical. Plant Cell Physiol 28:131–140
NOAA/ESRL (2013) <http://www.esrl.noaa.gov/gmd/ccgg/trends/>
Novriyanti E, Watanabe M, Kitao M, Utsugi H, Uemura A, Koike T (2012) High nitrogen and elevated CO_2 effects on the growth, defence and photosynthetic performance of two eucalypt species. Environ Pollut 170:124–130
Oliveira VF, Zaidan LBP, Braga MR, Aidar MPM, Carvalho MAM (2010) Elevated CO_2 atmosphere promotes plant growth and inulin production in the cerrado species *Vernonia herbacea*. Funct Plant Biol 37:223–231
Palumbo MJ, Putz FE, Talcott ST (2007) Nitrogen fertilizer and gender effect on the secondary metabolism of yaupon holly, a caffeine-containing North American holly. Oecologia 151:1–9
Polle A, Eiblmeier M, Sheppard L, Murray M (1997) Responses of antioxidant enzymes to elevated CO_2 in leaves of beech (*Fagus sylvatica* L.) seedlings grown under a range of nutrient regimes. Plant Cell Environ 20:1317–1321
Ranasinghe S, Taylor G (1996) Mechanism for increased leaf growth in elevated CO_2. J Exp Bot 47:349–358
Rao PVS, Tower GHN (1970) L-phenylalanine ammonia-lyase (Ustilago hordei). In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic, New York and London, p 581
Robredo A, Pérez-Lépez U, de la Maza Sainz H, González-Moro B, Lacuesta M, Mena-Petite A, Muñoz-Rueda A (2007) Concurrent effects of CO_2 enrichment and water stress on barley plant water relations and gas exchange under controlled environment conditions. Environ Exp Bot 59:252–263
Salazar-Parra C, Aguirreolea J, Sánchez-Díaz M, Irigoyen JJ, Morales F (2012) Climate change (elevated CO_2, elevated temperature and moderate drought) triggers the antioxidant enzymes' response of grapevine cv. Tempranillo, avoiding oxidative damage. Physiol Plant 144:99–110
Schaedle M, Bassham JA (1977) Chloroplast glutathione reductase. Plant Physiol 59:1011–1012
Schwanz P, Polle A (1998) Antioxidative systems, pigment and protein contents in leaves of adult Mediterranean oak species (*Quercus pubescens* and *Q. ilex*) with lifetime exposure to elevated CO_2. New Phytol 140:411–423
Shangguan ZP, Shao MA, Dyckmans J (2000) Nitrogen nutrition and water stress effects on leaf photosynthetic gas exchange and water use efficiency in winter wheat. Environ Exp Bot 44:141–149
Shoor M, Mondani F, Aliverdi A, Golzardi F (2012) Interaction effect of CO_2 enrichment and nutritional condition on physiological characteristics, essential oil and yield of lemon balm (Melissa officinalis L.). Not Sci Biol 4:121–130
Singh DV, Prajapati S, Bajpai S, Verma RK, Gupta MM, Kumar S (2000) Simultaneous determination of important alkaloids in *Papaver somniferum* using reversed phase high performance liquid chromatography. J Liq Chrom & Rel Technol 23:1757–1764
Sottomayor M, Lopes Cardoso I, Pereira LG, Ros Barceló A (2004) Peroxidase and the biosynthesis of terpenoid indole alkaloids in the medicinal plant *Catharanthus roseus* (L.) G. Don. Phytochem Rev 3:159–171
Tissue DT, Thomas RB, Strain BR (1993) Long-term effects of elevated CO_2 and nutrients on photosynthesis and rubisco in loblolly pine seedlings. Plant Cell Environ 16:859–886
Vurro E, Bruni R, Bianchi A, di Toppi LS (2009) Elevated atmospheric CO_2 decreases oxidative stress and increases essential oil yield in leaves of *Thymus vulgaris* grown in a mini-FACE system. Environ Exp Bot 65:99–106
Walkley A, Black IA (1934) An examination of Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. Soil Sci 27:29–38
Wang SY, Bunce JA, Maas JL (2003) Elevated carbon dioxide increases contents of antioxidant compounds in field-grown strawberries. J Agric Food Chem 51:4315–4320
Zhang L, Wu D, Zhang C, Shi H, Zhan X, Zhou S (2011) Effects of elevated CO_2 and N addition on growth and N_2 fixation of a legume subshrub (*Caragana microphylla* Lam.) in temperate grassland in China. PLoS One 6(10):e62842
Zhao X, Mao Z, Xu J (2010) Gas exchange, chlorophyll and growth responses of *Betula Platyphylla* seedlings to elevated CO_2 and nitrogen. Int J Biol 2:143–149
Ziska LH, Panicker S, Wojno HL (2008) Recent and projected increases in atmospheric carbon dioxide and the potential impacts on growth and alkaloid production in wild poppy (*Papaver setigerum* DC.). Clim Chang 91:395–403