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Research article

Effect of elevated ozone and carbon dioxide interaction on growth, yield, nutrient content and wilt disease severity in chickpea grown in Northern India

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ABSTRACT

Wilt caused by *Fusarium oxysporum,* sp. Ciceris (FOC) is an important disease causing losses up to 10% in chickpea yield. Experiments were conducted growing chickpea in free air ozone and carbon dioxide enrichment rings under four treatments of elevated ozone (O_3) (EO:60 ± 10 ppb), elevated carbon dioxide (CO_2) (ECO_2:550 ± 25 ppm), combination of elevated CO_2 and O_3 (EO + ECO_2) and ambient control for quantifying the effect on growth, yield, biochemical and nutrient content of chickpea. For studying the impact on wilt disease, chickpea was grown additionally in pots with soil containing FOC in these rings. The incidence of Fusarium wilt reduced significantly ($p < 0.01$) under EO as compared to ambient and ECO_2. The activities of pathogenesis-related proteins chitinase and β-1,3- glucanase, involved in plant defense mechanism were enhanced under EO. The aboveground biomass and pod weight declined by 18.7 and 15.8% respectively in uninnoculated soils under EO, whereas, in FOC inoculated soil (diseased plants), the decline under EO was much less at 8.6 and 9.9% as compared to the ambient. Under EO, the activity of super oxide dismutase increased significantly ($p < 0.5$, 40%) as compared to catalase (12.5%) and peroxidase (17.5%) without any significant increase under EO + ECO_2. The proline accumulation was significantly ($p < 0.01$) higher in EO as compared to EO + ECO_2, and ECO_2. The seed yield declined under EO due to significant reduction ($p < 0.01$) in the number of unproductive pods and seed weight. No change in the protein, total soluble sugars, calcium and phosphorus content was observed in any of the treatments, however, a significant decrease in potassium (K) content was observed under EO + ECO_2. Elevated CO_2 (554ppm) countered the impacts of 21.1 and 14.4 ppm h (AOT 40) O_3 exposure on the seed yield and nutrient content (except K) in the EO + CO_2 treatment and reduced the severity of wilt disease in the two years' study.

Main finding

Elevated CO_2 countered the impacts of elevated O_3 exposure on the seed yield and nutrient content (except K) under the interaction EO + CO_2 treatment in the two years' study and significantly reduced wilt disease severity.

1. Introduction

Carbon dioxide (CO_2) and tropospheric ozone (O_3) are important components of global and regional climate change having strong impacts on growth and productivity of crop plants and likely to affect food production in the South Asian subcontinent (IPCC, 2014; Ghude et al., 2014). Climate trends over the past few decades have shown rapid increase in their concentrations in many agricultural regions around the world (Lobell and Gourdji, 2012). CO_2 typically stimulates plant productivity (Ainsworth and Long, 2005), whereas O_3 is phytotoxic to a range of plant species (Agathokleous et al., 2018). Increase in population, urbanization, higher irradiance, elevated temperatures, and the increasing levels of precursors emitted from urban areas are the best suited conditions for O_3 formation. Due to increase in the anthropogenic emissions, O_3 concentrations have risen from 10 ppb in the late 1800s to

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monthly average daytime levels of 40 ppb nowadays (Brauer et al., 2016) and the atmospheric CO_2 levels have increased from 320 ppm to 412 ppm from 1960 to 2019 (NOAA 2020). The IPCC Fifth assessment report projects an increase in background tropospheric O_3 on an average by about 8 ppb (25% of current levels) and CO_2 is expected to reach 700 ppm by 2100 (IPCC, 2014). South Asian region, will experience the highest increase in surface O_3 (average annual increase of 7.2 ppb) by 2030 (Lenka and Lenka, 2012).

Elevated levels of tropospheric O_3 may cause foliar injury in the susceptible plants, accelerate leaf senescence, alter photosynthetic activity and stomatal conductance in leaves, leading to reduced dry matter production and productivity of crops (Bhatia et al., 2012). As a strong oxidant, O_3 reduces important physiological functions, resulting in inferior crop quality (Avnery et al., 2011a). Under the IPCC SRES A2 Scenario, global yield losses due to O_3 are predicted to range from 5.4 - 26% for wheat, 15–19% for soybean, and 4.4–8.7% for maize, with total global agricultural losses in the range of $17 - $35 billion annually by 2030 (Avnery et al., 2011b). Another modelling estimate by Ainsworth (2017) predicts global yield losses due to current day O_3 levels ranging from 2 to 16% for wheat, rice, maize and soybean. There may be significant losses of crop yields in India due to rising tropospheric O_3 concentrations. In addition to direct effects on plants, O_3 may also influence plant response to other stresses such as pathogens and diseases. According to Paoletti et al. (2020) there is a need to quantify the O_3 impacts on crops along with other environmental stresses. Higher O_3 levels may increase the plant's susceptibility by seriously damaging the cuticle layer of the plants, leaving them exposed to pathogen and insect attack (Berner et al., 2015) and increase in plant diseases. Exposure to elevated O_3 doesn't have any direct effect on fungal pathogens, but may increase the tendency of necrotrophic pathogens to colonize plants weakened by O_3 (Manning and Tiedemann, 1995).

On the other hand, atmospheric CO_2 enrichment stimulates photosynthesis, increases leaf area index (LAI), enhances dry matter accumulation and delays senescence (Yadav et al., 2019). The ability of legumes to exchange carbon (C) for nitrogen (N) with their N_2-fixing symbionts has led to the hypothesis that legumes will have a competitive advantage over non-leguminous species when grown under elevated CO_2 in well managed systems (Rogers et al., 2009). A number of researchers have evaluated the effect of elevated CO_2 in leguminous crops such as chick pea (Saha et al., 2013; Chakrabarti et al., 2019), pigeon pea (Saha et al., 2012), red gram (Vanaja et al., 2010), bush bean (Elagöz and Manning, 2005), mungbean (Mishra and Agrawal, 2014), etc. Legumes have higher photosynthesis and reproduction efficiency than other plant groups under elevated CO_2 due to their ability to reduce carbon sink limitations through enhanced nitrogen uptake (Rogers et al., 2009).

Most studies on the effect of elevated O_3 and CO_2 have focused on wheat (Mishra et al., 2013; Piikki et al., 2008; Tomer et al., 2015) and few on other crops such as rice (Imai and Kobori, 2008; Bhatia et al., 2011), brassica (Singh et al., 2013; Berner et al., 2015), maize (Bhatia et al., 2013), potato (Kumari et al., 2015) and soybean (Mishra, 2008). However, how the legumes will perform under the interaction of elevated O_3 and elevated CO_2 has been studied mainly in Soybean (Dermody et al., 2006; Morgan et al., 2003), peanut (Burkey et al., 2007) and one season results on the growth and yield of chickpea have been reported by Singh et al. (2017).

Chickpea (*Cicer arietinum* L.) is the 3rd largest grain-legume crop consumed all over the world having a production of 13.12 million tons annually (FAO, 2018). Asia accounted for 84% of the chickpea production, India being the leading producer with 9.88 million tons of chickpea from 10.7 million hectares (IIPR, 2018). The average chickpea seed yield has been less than 1000 kg/ha due to the wilt caused by the fungal pathogen *Fusarium oxysporum (F. oxysporum),* a major soil-borne fungus affecting chickpea globally (Sankar et al., 2018). The pathogen penetrates the vascular bundles of the roots of infected plants and reduces water uptake to the foliage resulting in wilting and death (Kraft et al., 1994; Halila and Strange, 1996). It has been reported that besides crops, directly or indirectly their pests both insects and pathogens are either negatively or positively affected by the elevated levels of O_3 and CO_2 (Fuhrer, 2003). It has been reported that many abiotic stress conditions may weaken the defense mechanisms of plants and enhance their susceptibility to pathogenic infection (Atkinson and Urwin, 2012). Berner et al. (2015) observed that though the economic yield was not affected under CO_2 and O_3 interaction in brassica, however, the plants became more susceptible to pathogen and insect attack. Hemibiotrophic/necrotrophic pathogens favour stressed plants that are weakened or damaged (Manning and Tiedemann, 1995). It is known that O_3-induced metabolic changes can persist in plants over days or months (Sandermann, 2000), however, it is difficult to predict the effects of climatic variables on disease susceptibility (Eastburn et al., 2010). Under O_3 exposure both increase in disease susceptibility (Mina et al., 2016; Sandermann, 2000) and decrease in disease susceptibility (Coleman et al., 1988) have been reported.

F. oxysporum is considered a hemibiotrophic pathogen because it begins its infection cycle as a biotroph but later changes to a necrotroph. In a study by Sharma et al. (2014) at ICRISAT under elevated CO_2, the wilt caused by *F. oxysporum* increased and the pathogen became more aggressive and increased the infection. Altered plant physiological response under elevated CO_2 and O_3 could affect plant–pathogen interaction in relation to both availability and quality of nutrients for pathogen feeding. Changes in the content of stress compounds such as reactive oxygen species (ROS) may promote defense-like responses and affect the concentrations of antioxidant enzymes (Sandermann et al., 1998; Fiscus et al., 2005). Swarupa et al. (2014) reported the role of oxidative burst, ROS and antioxidant enzymes as an important defense response against *F. oxysporum* (). Plant pathogenesis-related (PR) proteins are also implicated in plant defense responses against pathogenic infection (Zuccarini, 2009). Production of PR proteins in the remote uninfected parts of plants can lead to the occurrence of systemic acquired resistance, protecting the affected plants from further infection (Ebrahim et al., 2011).

F. oxysporum is prevalent in the tropical and subtropical regions and its geographical range may extend due to climate change (Okubara and Paulitz, 2005). The classic disease triangle emphasizes the interactions between plant hosts, pathogens and the environment in causing disease (Grulke, 2011). Thus the present study was carried out to quantify the impact of elevated O_3 in combination with elevated CO_2 on the *Fusarium* wilt disease, growth, yield, biochemical and nutritional quality of kabuli chickpea.

2. Materials and methods

2.1. Experimental site, treatments and management

A field experiment was conducted growing chickpea (*C. arietinum L.*) cv. Pusa 5023-kabuli type) inside free air O_3 and CO_2 enrichment (FAOCE) rings at the experimental farm of ICAR-Indian Agricultural Research Institute, New Delhi (28°40′ N latitude, 77°12′ E longitude, altitude of 228.16 m above mean sea level). The mean maximum and minimum temperatures from November to April were 35.5 and 18.5 °C. Four treatments were taken in the FAOCE rings growing chickpea under ambient levels of O_3 and CO_2 (Amb O + CO_2) elevated O_3 (EO, 60 ± 10 ppb), elevated CO_2 (ECO_2, 550 ± 25 ppm) and a combination of elevated levels of both the gases (EO + ECO_2). The octagonal ring had a diameter of 6m and there were two replicate rings for each of the treatments. Each ring was further divided into four quadrants and each quadrant was taken as a replicate for all the measurements undertaken. The control plot was the ambient plot having ambient levels of CO_2 and O_3 concentrations. CO_2 and O_3 were released through horizontal perforated tubing's above the soil surface at the canopy level. The CO_2 sensor (NDIR based) was positioned at the center of each ring and regulated the rate of CO_2 gas released upwind for achieving the targeted CO_2 concentration. The CO_2 levels were elevated using highly pressurized CO_2 cylinders with the

help of dual stage regulators, gas flow meters and solenoid valves whereas the O_3 levels were elevated using an O_3 generator. The elevated levels were maintained post germination of chickpea seed to physiological maturity of the crop. Transparent poly carbonate circular sheets (1m in height) were placed at 2m distance around each octagonal ring to avoid cross contamination between the rings. The O_3 concentration was measured using an O_3 concentration analyzer (2B technologies). The O_3 fumigation began at 9:00 a.m. and continued until sunset but was discontinued on rainy days. In the ambient plots, plants were grown under ambient CO_2 and O_3 without the rings. Wind direction was measured with an anemoscope.

Chickpea variety Pusa 5023, an extra-large seeded kabuli variety with an average yield of 20q/ha, being moderately resistant to soil borne diseases was taken for the experiment. The chickpea seeds, were treated with fungicide Captan @ 2 g kg-^{1} seed and then with Rhizobium @12 gkg^{-1} seed and were sown on 16th Nov, 2016 and 16th Nov, 2017 in well prepared soil of the FAOCE rings with a row to row distance of 30cm and a plant to plant distance of 20 cm. In 2017 the seeds of chickpea were sown in only three quadrants of each of the rings. In the fourth quadrant, the studies on Fusarium wilt were carried out growing chickpea in pots.

Fertilizer NPK was incorporated prior to sowing @ 20:50:20 kg ha^{-1}. The alluvial soil of experimental site was silty clay loam (Typic Ustochrept) with bulk density of 1.38 g cm^{-3}, pH (1:2 soil:water) of 8.8, electrical conductivity of 0.43 dS m^{-1} and organic carbon, total N, Olsen P, and ammonium acetate extractable K contents of 3.5 g kg^{-1}, 0.32 g kg^{-1}, 0.009 g kg^{-1}, and 0.12 g kg^{-1}, respectively. O_3 exposure began on 30th November, 2016 and ended on 20 March, 2017 in the first year and on 1st December, 2017 and ended on 22nd March, 2018 in the second year. Crop was harvested at maturity on 11th April, 2017 and 14th April 2018.

2.2. Fusarium wilt studies and preparation of inoculum of FOC

For studying the impact of elevated CO_2 and O_3 on wilt disease incidence, chickpea was sown in earthen pots. Eight pots were kept in one quarter of each of FAOCE rings under the different treatments. Delhi isolate of *Fusarium oxysporum,* sp. Ciceris race 4 (FOC) used in this study was cultured on water soaked and autoclaved sorghum seed solid medium at room temperature. After 14 days of growth and at conidia forming stage, this FOC culture was mixed with pre sterilised soil (sterilised by spraying of 10% formalin/kg soil and covered with polyethene sheet for two weeks) in order to obtain final densities of 10^5 Conidia/gram of soil of *F oxysporum*.

Water soaked healthy seeds of chickpea were sown in pots (containing 16 kg soil having FOC inoculum). The pots were transferred to the FAOCE rings at the emergence of 4–5 leaves. The leaflets samples from fully matures leaves of chickpea plants grown in pots in the FAOCE rings were collected at the flowering stage. Collected leaflet samples were quickly frozen in liquid nitrogen to prepare the powdered sample. 1g of powdered sample, was extracted with 2 ml 0.1 M sodium citrate buffer (4 °C, pH 5.0) and 0.05 M sodium acetate buffer (pH 5.0) for analysis of Chitinase and β-1,3 glucanase enzyme activity respectively. The homogenate was centrifuged for 20 min at 12,000 g and the protein extracts obtained was used for estimation of activity of enzymes chitinase and β-1,3-glucanase. The changes in the activities of chitinase and β-1,3-glucanase was determined by colorimetric assays as described by Pan et al. (1991).

At the vegetative, flowering, pod filling and maturity stages, soil samples from the rhizosphere of chickpea plants under different treatments were collected and analysed for the changes in spore count/conidia per gram of soil.

Plants under each treatment were periodically monitored for the appearance of the symptoms of the wilt disease. The number of wilted plants was recorded and at maturity stage, the number of wilted and healthy plants in each treatment was recorded. The wilt incidence for each treatment was calculated by the following formula:

Wilt Incidence (%) = (Number of plants wilted /Total number of plants) X 100

At maturity *Fusarium* inoculated/wilted/diseased and uninoculated/healthy chickpea plants under each treatment were harvested and the shoot biomass and pod weight/plant was recorded.

2.3. Plant sampling for physiological and biochemical analysis

Each treatment had two replicate FAOCE rings. Plant samples from the three quadrants in each of the two replicate FAOCE rings were collected for studying the growth parameters at stem elongation and pod formation in 2016–17 and 2017–18 respectively. Shoot length, shoot dry weight, leaf area index, the number of side branches and the number of secondary branches were measured after each sampling. Measurements for the number of pods per plant, the number of seeds per pod, 100 seed weight and the seed yield was carried out after the final harvest. The seeds were separated from the pods, dried, and weighed.

Single-leaf net photosynthetic rates and stomatal conductance were measured with a portable photosynthesis system (LI-6400-40 Portable Photosynthesis System) at flowering. Total Chlorophyll content was estimated by the non-maceration method of Hiscox and Israelstam (1979). Activity of superoxide dismutase (SOD), peroxidase (POX), catalase (CAT) was also measured at flowering. The proline content was measured at stem elongation and flowering stages. The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Dhindsa et al., 1981). The assay of POX activity was carried out by measuring the decrease in absorbance at 420 nm due to the decomposition of H_2O_2 (Kar and Mishra., 1976). CAT activity was measured as the decline in absorbance at 240 nm due to the decomposition of H_2O_2 (Aebi 1983). Proline content was determined by the method of Bates et al. (1973). Total soluble sugars, starch, protein, P, K and Ca contents in the harvested seeds was estimated.

Leaf area Index (LAI) was measured at stem elongation, anthesis and pod formation using a plant canopy analyser (LICOR, LAI-2200 C, USA). The measurement of LAI was carried out at 15:30 h in each quadrant replicate from evenly spaced spots in two diagonal transects to maintain almost fixed incident solar angle with higher proportion of diffuse incident light at sunset.

2.4. AOT40

AOT40 is the sum of hourly average values of O_3 concentration beyond 40 ppb or accumulated exposure of O_3 over a threshold of 40 ppbv. The AOT 40 for the elevated O_3 treatments during the entire crop growth period was calculated from the differences between mean hourly concentrations (in ppb) and 40 ppb for each hour when the O_3 concentration exceeded 40 ppb, accumulated during the daylight hours.

2.5. Data analysis

All response variable data were analyzed by two-factor (O_3 and CO_2 level) analyses of variance (ANOVA). The treatment means were compared by Tukeys test when the anova was significant. Results were taken as significant at $p < 0.05$. Before the analysis, data were checked for normality (Kolmogorov–Smirnov test). All data analyses were carried using the SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Levels of O_3 and CO_2 in the different treatments

The ambient and elevated O_3 and CO_2 concentrations measured in the FAOCE rings during the crop growth period (November to April) in both the years of the experiment are shown in Table 1a. Critical levels for O_3 over 40 ppbv were calculated and the resulting index of AOT40 for the

Table 1a. Ambient levels of O_3 and CO_2 during crop growth period.

	2016			2017		
	Min	Max	Average	Min	Max	Average
Ambient O_3	7.5	58.6	30.3	10.7	60.8	24.2
Elevated O_3	54.2	73.1	68.6	51.0	68.4	65.3
Ambient CO_2	384	413	398	391	418	403
Elevated CO_2	530	570	554	527	580	558

entire crop growth duration was 21.112 and 14.152 ppm h in 2016–17 and 2017–18, respectively, in the elevated O_3 treatment.

3.2. Effect of elevated O_3 and CO_2 on crop phenology

No variation in the phenology was observed among the treatments initially. ECO_2 accelerated reproductive development and the onset of flowering was advanced by 3–4 days in the elevated CO_2 treatment during the two years of the study (Table 1b). No change was observed in the EO and EO + ECO_2 treatments as compared to the ambient during the two years. Under elevated CO_2 and EO + ECO_2 the days to pod initiation reduced by 2 and 3 days. The advance in pod initiation could be attributed to early translocation of photosynthates to the leaves. Under EO the pod maturity was earlier by 8 and 10 days as compared to the ambient during the two years which may be due to the source/sink imbalance under EO (Andersen, 2003). Elevated O_3 may inhibit sugar export from leaves (Grantz and Farrar, 2000), which could trigger early leaf senescence and early pod maturity.

3.3. Effect of elevated O_3 and CO_2 on the photosynthetic rate, stomatal conductance and total chlorophyll

A reduction in the stomatal conductance was observed under all the treatments as compared to the ambient. During the two years, 25 and 19% reduction in the stomatal conductance (gs) was observed in the EO treatment over the ambient treatment (Figure 1a). There was a significant reduction in the net photosynthetic rate (Pn) in EO (14% and 21%) over the ambient at 95 and 90 DAS during the two years. The decrease in gs in EO might have led to lower internal leaf CO_2 levels, thereby reducing the Pn. However, the increase in Pn by 17 and 11% was observed under ECO_2 over the ambient during the two years of the study (Figure 1b). An increase of 13 and 16% in the Pn was also observed under the EO + CO_2 interaction treatment over EO alone. This was due to the presence of higher CO_2 levels in this treatment.

The partial stomatal closure induced by elevated CO_2 decreased the impact of O_3 by restricting their uptake by stomata and also reducing transpiration losses and increasing water use efficiency (Booker et al., 2004). However, the exposure to O_3 alters the stomatal responses, thereby decreasing the ability of leaves to limit water loss and increasing transpiration in leaves (Hayes et al., 2012). The elevated CO_2 had a protective effect against O_3 injury and involved increased photosynthates availability to enable plants to maintain the growth that could be used for damage repair and detoxification processes (Booker et al., 2007). Morgan et al. (2003) had reported a 17.5% O_3induced reduction in gs in soybean when daily mean exposure levels of O_3 ranged from 30-79 ppb.

We observed significant increase in chickpea growth and dry matter allocation under elevated CO_2 due to the partitioning of photosynthates towards the different growing plant organs which led to an increased branching and leaf area index (Figure 2b, c). In our experiment, both elevated O_3 and elevated CO_2 reduced the stomatal conductance, which decreased even further when these two gases were combined. The reduced stomatal conductance could be due to changes in stomatal aperture under elevated CO_2 (Ainsworth and Long, 2005) as with increasing levels of CO_2 the stomata do not need to open as widely to allow sufficient CO_2 for photosynthesis to enter the leaf.

The total chlorophyll increased in ECO_2 (12 and 11%) as compared to the ambient at the flowering stage in both the years (Figure 1c). Being a legume, chickpea can fulfill its N requirement through symbiotic nitrogen fixation and plant N uptake may have accelerated under elevated atmospheric CO_2. This may have resulted in an increase in the foliar N concentration and leaf chlorophyll content (e.g. Cheng et al., 2010). The total chlorophyll increased significantly ($P < 0.5$) in our study under the interaction treatment EO + ECO_2 by 9 and 10 % over EO alone in both the years. The total chlorophyll under EO was lower than the ambient. In our study we found an increase in the activity of the anti-oxidant enzymes under EO treatment. The lowering in chlorophyll under EO could be due to O_3-mediated ROS accumulation which may lower the chlorophyll due to an insufficient leaf antioxidant capacity (Caregnato et al., 2013).

3.4. Effect of elevated O_3 and CO_2 on the growth parameters in chickpea

The shoot dry weight increased by 8 and 12% (significant at $p < 0.01$) under ECO_2 over Amb at stem elongation in the two years respectively. Shoot dry weight was lower by 12 and 14% under EO treatment at pod formation in both the years. A significant increase was observed at pod formation in all the growth parameters under the interaction treatment EO + ECO_2 as compared to the EO alone in both the years. The LAI decreased under EO and the decrease was more in the II^{nd} year (14%). LAI increased by 13 and 10 % in EO + CO_2 over EO and was statistically at par with the Amb (Figure 2c) treatment. Increased photosynthetic rates enabled the plants to utilize more amounts of phtosynthates for their growth with a simultaneous increase in LAI under ECO_2 and EO + CO_2. Chickpea being a leguminous crop may be able to fix increased nitrogen under higher CO_2, which is subsequently utilized by the plants to support the process of growth enhancement (Gamper et al., 2005). Higher leaf area index and a lower stomatal aperture in a CO_2-enriched

Table 1b. Effect of different treatments on days to key growth stages.

	Days to	Amb	EO	ECO_2	EO + ECO_2
2016	Flowering	82	82	79	82
	Pod initiation	112	115	109	110
	Pod maturity	147	139	141	139
2017	Flowering	85	85	81	85
	Pod initiation	115	120	112	113
	Pod maturity	150	140	144	141

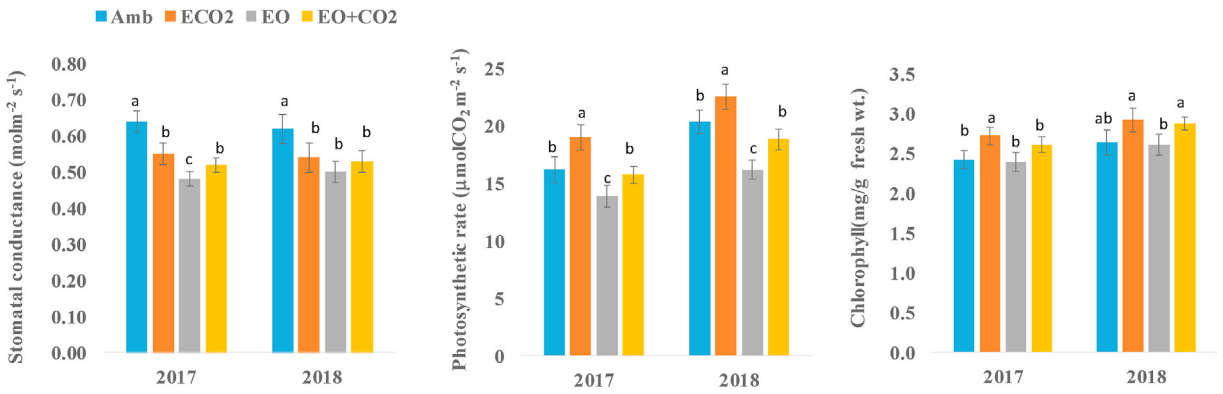

Figure 1. (a) Stomatal conductance (b) Photosynthetic rate (c)Total cholorophyll content at flowering stage in chickpea under different treatments (on the columns the same letter are not significantly different at P < 0:05); Amb: Ambient, ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

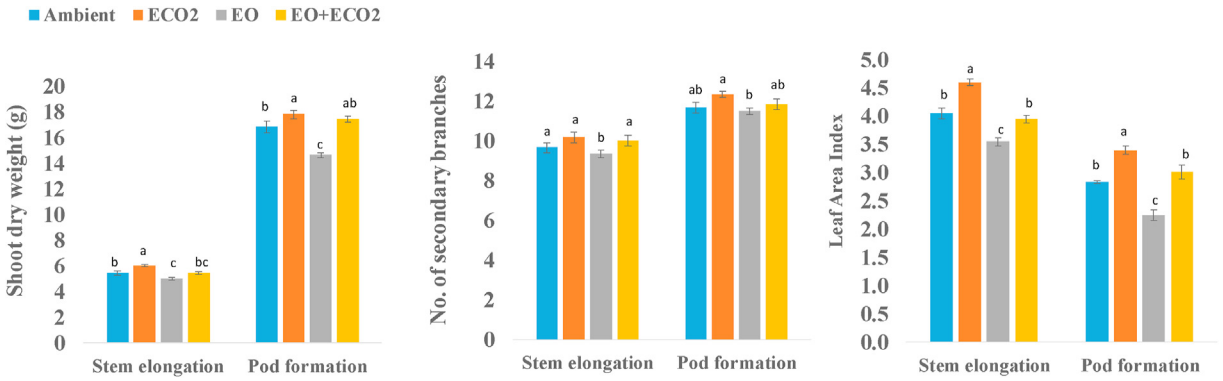

Figure 2. Effect of elevated CO_2 and O_3 on (a) shoot dry weight (b) No. of secondary branches/plant and (c) leaf area index of chickpea at stem elongation and pod formation stages (Average of two years). Means with at least one letter common are not statistically different ($p \leq 0.05$ Tukey's). Error bars indicate standard error; Amb: Ambient, ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

treatment may improve the water use efficiency at the leaf and canopy level (Mills and Harmens, 2011) resulting in better growth. Under EO, reduced photosynthetic rate, stomatal conductance and chlorophyll content resulted in a change in assimilate allocation and eventually in a decrease in the growth rate of the shoot. The number of secondary branches determines the total number of leaves and hence the total photosynthetic area. There was a significant decrease in the number of secondary branches under EO at pod formation (at $p < 0.05$) during the second year of the study. The number of secondary branches increased significantly (at $p < 0.05$) under ECO_2 at stem elongation and pod initiation during the first year.

3.5. Effect of elevated O_3 and CO_2 on the biochemical parameters

EO induced higher antioxidant enzyme activities in our experiment. Results showed that increase in Superoxide dismutase (SOD), Catalase (CAT), and Peroxidase (POX) activities under EO treatment may be related to the induction of antioxidant responses that protect the plant from oxidative damage. Superoxide dismutase (SOD) showed maximum increase in EO treated plants and less increase in the activities of CAT and POX. At flowering stage, under EO, SOD, CAT and POX activity increased significantly by 48, 16 and 25% in the first year and by 39, 9 and 20% in the second year respectively (Figure 3a). Thus it shows that SOD played a

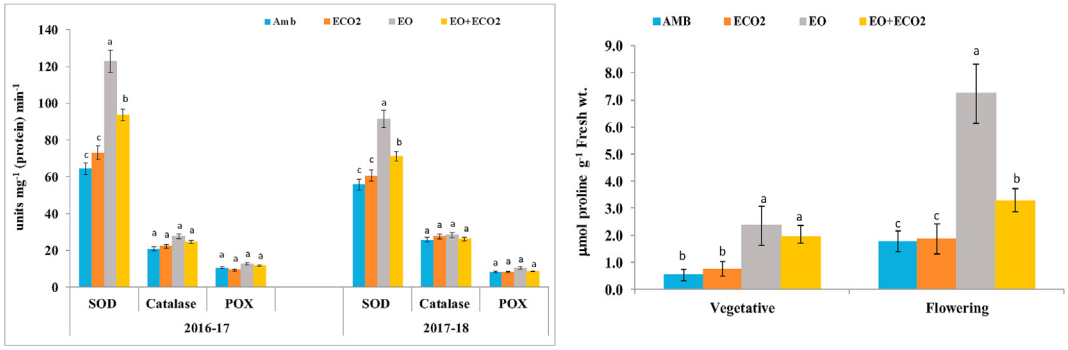

Figure 3. (a) Antioxidant enzymes at flowering stage (b) Proline content at vegetative and flowering stage in chickpea under different treatments in 2017–18 (on the columns the same letter is not significantly different at P < 0:05); Amb: Ambient, ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

greater role than CAT and POX in detoxifying the produced reactive oxygen species (ROS) since its activity increased more. Superoxide dismutase constitutes the first line of defence via detoxification of superoxide radicals (Sairam and Saxena, 2000), thereby maintaining the membranes of plant tissue, whereas, CAT consumes H_2O_2 by breaking it down directly to water and oxygen. The activity of SOD was significantly higher than Amb and ECO_2 under the interaction (EO + CO_2) treatment. Higher production of ROS due to O_3 stress in the plant may result in lower levels of anti-oxidants in the seed and lower nutritional quality (Daripa et al., 2016).

Proline accumulation is believed to play an adaptive role in plant stress tolerance (Verbruggen and Hermans, 2008). The proline content increased from stem elongation to flowering in all the treatments (Figure 3b). At flowering stage, the proline was significantly ($p < 0.05$) higher in EO as compared to EO + ECO_2, and ECO_2. Higher proline content was measured under EO + ECO_2 as compared to ECO_2 ($p < 0.05$) at both the stages. Plants can partly protect themselves against stress by accumulating osmolytes (Shinde and Thakur, 2015) and thus higher proline was observed under EO + CO_2. Accumulation of proline in the plant cell takes place in response to stress to protect the protein structure and to prevent the oxidative burst in plants by bringing concentrations of ROS within normal ranges (Hayat et al., 2012).

3.6. Effect of elevated O_3 elevated CO_2 on the seed yield

Elevated O_3 levels of AOT 40 of 21.112 and 14.152 ppm h led to an 18 and 15 % decrease in seed yield over Amb in the two years. Seed yield significantly increased by 31 and 26% in EO + ECO_2 treatment over EO and by 7 and 8.5% over Amb (not significant) in the two years (Figure 4b). The protective effect of CO_2 was due to increased photosynthetic rate, dry matter production, and more allocation of carbohydrate to the seed. The presence of elevated CO_2 along with EO thus countered the negative effect of O_3 and moderated the response, thereby increasing the yield (Burkey et al., 2007).

The yield contributing characters viz., number of pods/plant, number of unproductive pods, no. of seeds/pod, 100 seed wt., were negatively influenced by the EO levels (Figure 4a). There was no significant reduction in the total number of pods/plant under EO, however, there was a significant reduction in the total number of productive and unproductive pods under EO ($p < 0.01$). In ECO_2 treatment, no. of pods increased significantly ($p < 0.01$) by 10.8 and 6% over the Amb. The no. of productive pods/plant increased by 16 and 12 % in the EO + CO_2 treatment over EO alone in both the years respectively. It can thus be concluded that seed yield decreased due to higher number of unproductive pods under EO and reduced seed weight. The increase in the CO_2 concentration significantly increased all the major yield attributes and no significant difference was observed in the interaction treatment as compared to the Amb.

O_3 induced yield losses have often been attributed to reductions in photosynthetic activity and assimilate supply to support reproductive development and seed growth (Feng et al., 2010). Under EO the pod maturity was early by 8 and 10 days as compared to the ambient and this reduction in the length of reproductive period may have led to a lower seed weight and seed yield in our experiment. Declined photosynthetic activity under stress conditions may decrease assimilate translocation and carbon fixation affecting the reproductive organs, leading to fewer pods, lower seed set and declined sink activity in chickpea (Nadeem et al., 2019). Under stress, pollen tube growth rate may be reduced playing an important role in the pod and seed formation (Kaloki et al., 2019). It has been earlier reported that high temperature and heat stress in chickpea causes substantial loss in crop yield due to damage to reproductive organs, increased rate of plant development, and reduced length of the reproductive period (Gan et al., 2004), however there are no studies under O_3 stress.

3.7. Effect of elevated O_3 and CO_2 on the seed quality

A slight increase in the carbohydrate content of the seed was observed under the elevated CO_2 treatment (Figure 5a). The total soluble sugar and starch significantly increased ($p < 0.05$) by 7 and 4.5 % over the Amb in the ECO_2 treatment during the two years of the study (Figure 5a). In EO and EO + CO_2, no significant change was observed in the sugar or starch content in both the years of the study. O_3 is known to reduce photosynthesis, leading to lower translocation of carbon to the grain, resulting in reduced sugar and starch content in the grain (Bhatia et al., 2012, Daripa et al., 2016). However, in our study we did not observe any decrease in the carbohydrate content in chickpea.

No significant change in the seed protein content was observed in any of the treatments over the ambient control. Earlier researchers have reported a decline in the seed protein content under EO (Li et al., 2018; Chaudhary and Agrawal, 2015) in mungbean and pea due to lowered photosynthetic efficiency, and a decline in the seed protein under ECO_2 (Li et al., 2018; Singh et al., 2013) due to yield dilution effects. No significant decline in the protein content was observed under ECO_2 in our study. Being a legume, chickpea could probably fulfill its N requirement through symbiotic nitrogen fixation as plant N uptake was accelerated under ECO_2. This resulted in an increase in the leaf chlorophyll content which increased the foliar N content (Cheng et al., 2010) and the increased N fixation may increase the grain mass without actually decreasing its N concentration (Hampton et al., 2013).

The mineral nutrient content in the seed did not change under EO in both the years with the exception of K content reducing in year 1 of the

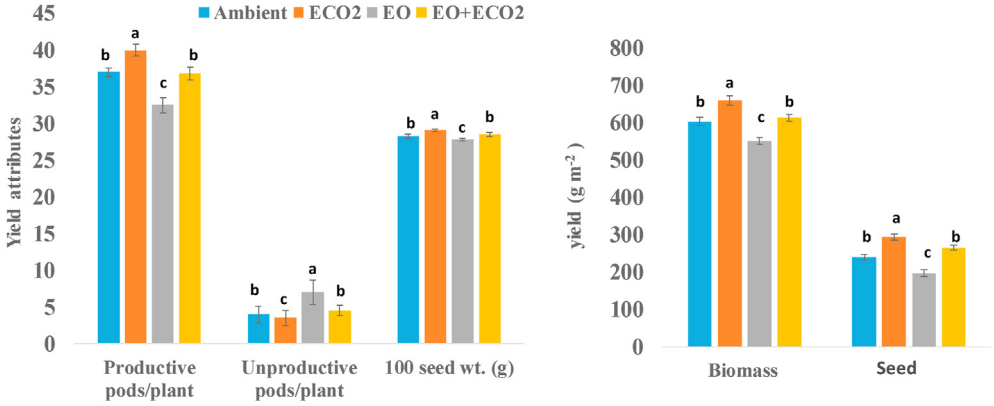

Figure 4. Effect of elevated CO_2 and O_3 on (a) yield attributes and (b) yield (Average of two years). Means with at least one letter common are not statistically different ($p \leq 0.05$ Tukey's). Error bars indicate standard error; ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

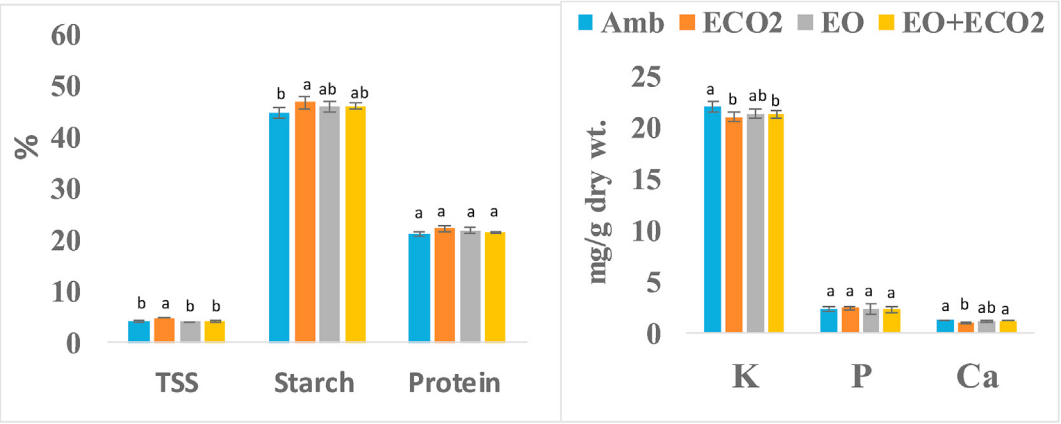

Figure 5. Effect of elevated CO_2 and O_3 on (a) Total soluble sugar, starch and protein and (b) mineral composition of seed. (Average of two years). Means with at least one letter common are not statistically different ($p \leq 0.05$ Tukey's). Error bars indicate standard error; Amb: Ambient, ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

study (Figure 5b). There was a significant decrease in the K content under the interaction treatment EO + ECO_2 as compared to the Amb. Potassium (K) and Calcium (Ca) content decreased significantly ($p < 0.05$) by 4.7 and 9.5% respectively under ECO_2 probably due to the yield dilution effect but no change was observed in Phosphorus (P) concentrations. No change or increase in the concentration of P was probably due to reduced transpiration under ECO_2 which may be beneficial for the diffusion of specific elements from the soil to the roots, thereby increasing their availability (Li et al., 2018). However, this mechanism fails to explain the decrease in the concentration of K and Ca in seeds under ECO_2.

3.8. FOC population dynamics in rhizosphere of chickpea

The number of FOC conidia per gram of rhizospheric soil sample analysed monthly at four growth stages of chickpea was the highest under Amb and lowest under EO in the range of 0.6×10^5–1.8×10^5 g^{-1} of soil (Figure 6a). The maximum FOC conidia load in soil was at the vegetative stage and the minimum was at the flowering stage. The flowering stage coincided with late December and early January months when average ambient temperature was below the optimum range for the growth of FOC pathogen. It has been reported that severe wilt develops at 20–30 °C and an inoculum density of FOC of at least 6 and 100 spores g^{-1} of soil, respectively (Navas-Cortés et al., 2007). The life cycle of the soil born FOC pathogen had a parasitic phase in the presence of host plant, chickpea in this study.

3.9. Effect of elevated O_3 and CO_2 on wilt disease incidence

In our study FOC acted as an obligate parasite with chickpea plants and caused the wilt incidence. Elevated O_3 levels significantly reduced (28.6%, $p < 0.05$) the wilt disease severity in EO and EO + CO_2 as compared to the ECO_2 and Amb (Figure 6b). The disease severity in plants under ambient conditions (56.8%) was significantly higher ($P < 0.05$) as compared to EO + CO_2 (42.9%) treatment, but at par with plants exposed to ECO_2 (50%). Ambient CO_2 may probably have little direct effect on soil inhabiting fungi pathogens, as they can tolerate more than 10- or 20-fold increases in CO_2, and might even be slightly stimulatory (Manning and Tiedemann 1995). Chakraborty et al. (2000) suggested that elevated CO_2 will directly alter the host physiology and morphology, bringing about a change in the light interception, modifying the micro-climate and leading to an increase in temperature which may increase or decrease the disease severity. In our study under EO and EO + ECO_2 the effect of O_3 was indirect by reducing the wilt disease severity by altering the host physiology (Manning et al., 1971) and directly by reducing the sporulation and growth of hyphae of obligate parasites (Violini, 1995). O_3 exposure may also activate plant defense and synthesis of pathogenesis-related (PR) proteins (Prasad et al., 2009). The increase in pathogenesis-related (PR) proteins as defense was probably the reason for least wilt incidence under the EO treatment in our study (discussed in next section).

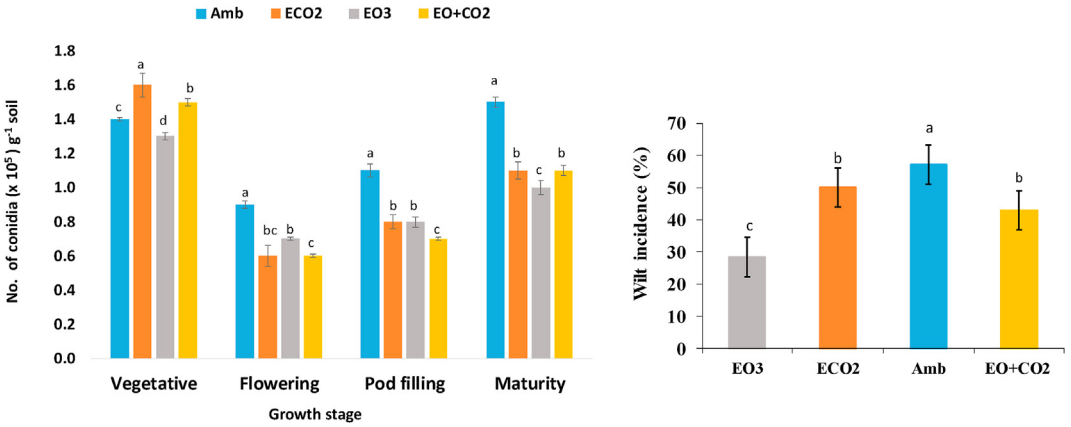

Figure 6. (a) Trend of *F. oxysporum,* sp. Ciceris race 4 population in rhizospheric soil of chickpea plants (b) Wilt incidence in chickpea plants under different treatment. Means with at least one letter common are not statistically different ($p \leq 0.05$ Tukey's). Error bars indicate standard error; Amb: Ambient, ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

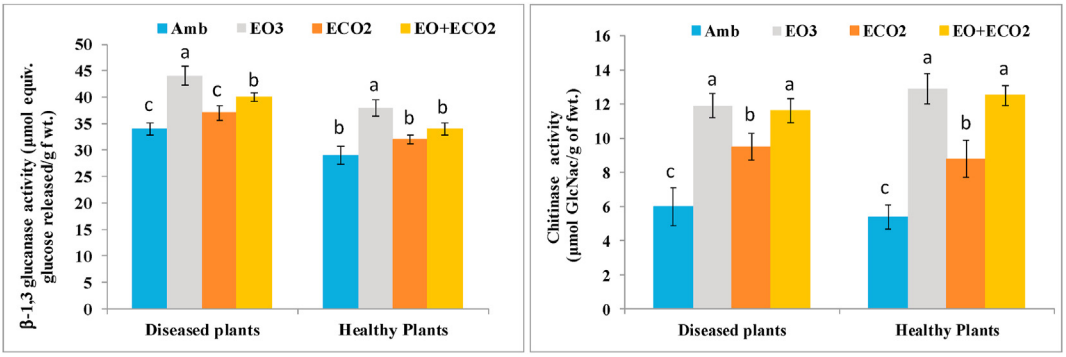

Figure 7. (a) Activity of β-1,3- glucanase PR protein and (b) activity of chitinase PR protein in chickpea plants at flowering stage under different treatment. Means with at least one letter common are not statistically different ($p \leq 0.05$ Tukey's). Error bars indicate standard error; Amb: Ambient, ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

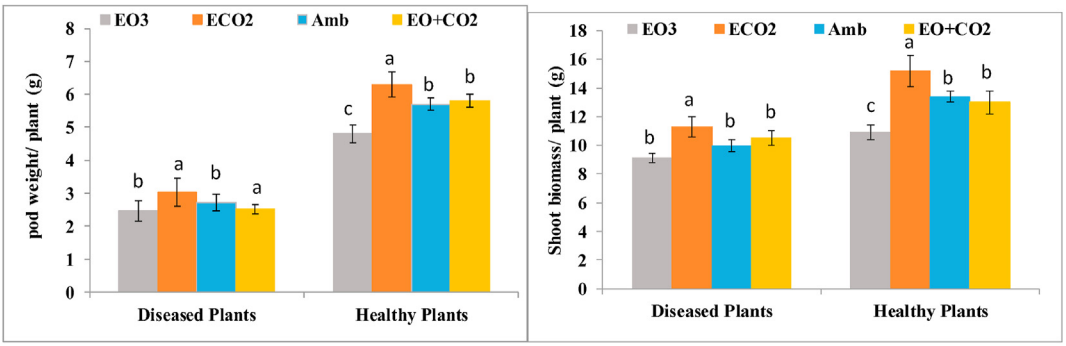

Figure 8. (a) Shoot biomass and (b) pod weight in diseased/inoculated and healthy/uninoculated plants under different treatments. Means with at least one letter common are not statistically different ($p \leq 0.05$ Tukey's). Error bars indicate standard error; Amb: Ambient, ECO_2: Elevated carbon dioxide, EO: Elevated ozone; EO + CO2: Elevated ozone and elevated carbon dioxide.

3.10. Effect of O_3, CO_2 and disease on pathogenesis-related (PR) proteins and yield attributes

Exposure to abiotic (O_3, CO_2) and biotic (FOC) stress induced defense response in both healthy and diseased plants and altered the levels of PR proteins- β-1,3- glucanase and Chitinase in our study. PR proteins chitinases and β-1,3-glucanases are two important hydrolytic enzymes that are abundant in many plant species after infection by different type of pathogens and exposure to abiotic stresses, thus they can be used as biochemical markers (Ebrahim et al., 2011). In plants exposed to only abiotic stresses of elevated O_3 and CO_2 (healthy plants), the activity of β-1,3- glucanase and chitinase was the highest under EO and the lowest under Amb. Plants that were diseased had higher activity of β-1,3-glucanase under ECO_2 treatments as compared to the healthy plants (Figure 7a). Elevated CO_2 may increase host resistance (Coakley et al., 1999) and lead to higher levels of PR proteins. The activity of chitinase was observed to be less in diseased plants as compared to the healthy plants under EO and EO + CO_2 treatments (Figure 7b).

Since the activity of β-1,3-glucanase was maximum under EO in both diseased and healthy plants, they developed resistance to wilt disease and thus the severity was found to be the lowest under EO treatment (Figure 6b). When a pathogen attacks, the PR proteins may accumulate in the vacuoles of the cell wall and intercellular spaces, thereby protecting the plants from further infection by not only accumulating locally in the infected and surrounding tissues but also in remote uninfected tissues (Datta and Muthukrishnan 1999). An increase in the activities of the PR proteins in plants susceptible to pathogens, under elevated O_3 and CO_2 concentrations may result in an improved resistance to the pathogens (Plessl et al., 2007).

The maximum reduction in the shoot biomass and pod weight was observed under EO treatment in both healthy and diseased chickpea plants. The shoot biomass and pod weight reduced by 18.7 and 15.8% respectively in healthy plants under EO as compared to the Amb (Figure 8 a,b). However, the decrease in shoot biomass and pod weight was lower at 8.6 and 9.9% respectively in the diseased plants under EO as compared to Amb (due to lower wilt incidence under EO). However, the presence of elevated CO_2 in the interaction treatment was able to counter the yield losses in the diseased plants. ECO_2 treatment positively influenced and nullified the adverse impact of EO on shoot biomass and pod weight in healthy plants, however, in diseased plants the biomass yield under the EO + CO_2 was at par with Amb but the pod weight significantly declined by 7.7% ($p < 0.5$) as compared to Amb.

4. Conclusion

Elevated ozone may directly affect the different growth and biochemical processes in chickpea plants; however, the increasing concentration of atmospheric CO_2 will likely ameliorate the deleterious O_3 effects on plants. The protein, starch and other mineral nutrients content in grain may not see a significant change under the elevated O_3 and CO_2 interaction, thus maintaining its nutritional quality. O_3 stress may induce a burst of reactive oxygen species, or induce enzymes, which triggers the plant defense system and the plant acquires systemic resistance to stress and disease. The incidence of wilt due to *Fusarium oxisporium* may be significantly lower under elevated O_3, but the interaction between O_3 and crop pathogens adds another dimension which may require more experimental studies for understanding at spatial and temporal scales.

More experiments are needed need to establish the productivity trade off in chickpea under elevated O_3 and biotic stress exposure.

Declarations

Author contribution statement

Arti Bhatia: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Usha Mina: Analyzed and interpreted the data; Wrote the paper.

Vinod Kumar, RN Singh: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Ritu Tomer: Performed the experiments; Analyzed and interpreted the data.

Amit Kumar, Bhupinder Singh: Contributed reagents, materials, analysis tools or data.

Bidisha Chakrabarti: Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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