Insight into Physiological and Biochemical Markers against Formaldehyde Stress in Spider Plant (Chlorophytum comosum L.)

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Abstract

Formaldehyde is a prominent volatile organic compound and also considered an indoor air pollutant. *Chlorophytum comosum*, an indoor plant, has been reported to metabolize indoor formaldehyde. But the phytotoxic effects of formaldehyde, being a pollutant, on *C. comosum* is not well explored. Furthermore, *C. comocum* responses that can be considered as markers at the physiological and biochemical level against formaldehyde stress are not yet investigated. Therefore, the current research study was aimed to evaluate such potential markers against formaldehyde in *C. comosum*. Briefly, *C. comosum* was exposed to 5, 10, and 20 ppm formaldehyde doses in an airtight glass chamber. Plant samples were then taken to analyze morpho-anatomical, physiological, and biochemical responses after short (2, 4, and 6 hours), medium (12 and 24 hours) and extended durations (48 and 96 hours) for each tested dose. Two-ways ANOVA followed by Tukey's test at $p \leq 0.05$ indicated that application of 10 and 20 ppm formaldehyde doses led to a significant incline in enzymatic antioxidants like CAT, GPX and SOD, and non-enzymatic parameters including TPC, TFC, T-AOC, carotenoids and intercellular CO_2. However, formaldehyde application negatively affected the physiological responses of *C. comosum* by reducing its photosynthetic rate, transpiration rate and stomatal conductance. Additionally, extended exposure of *C. comosum* to 10 and 20 ppm formaldehyde doses led to visible leaf damage. Principal Component Analysis indicated that enzymatic (SOD, CAT and GPX) and non-enzymatic (MDA, TPC, TFC, TAO, carotenoids, TSS and intercellular CO_2) parameters contributed the most to the total variance. Thus, these parameters have potential to serve as physiological and biochemical markers in *C. comosum* against formaldehyde stress.

Introduction

Indoor air pollution has become one of the major concerns for human health. Among the contributing pollutants, volatile organic compounds (VOCs) are major groups as their indoor release is continuous (Bacaloni et al. 2011). It's alarming that their indoor concentrations have become almost ten times higher than in the outdoor environment (Huang et al. 2011). Among VOCs, formaldehyde is one of the most abundant indoor air pollutants. It is continuously released from commonly used household items such as building materials, plywood, particleboard, furniture polish, carpets, glues, paints and deodorants (Salthammer et al. 2010). Indoor plants are commonly used for interiorscaping and also have the potential to remediate indoor levels of VOCs including formaldehyde. Various research studies have also documented the remediation of indoor formaldehyde via indoor plants including *Fatsia japonica* (Kim et al. 2008); *Epipremnum aureum* (Kim et al. 2009); *Chlorophytum bichetii* (Kim et al. 2013); *Sansevieria trifasciata* (Husti et al. 2016); *Scindapsus* (Li et al. 2020); *Dracaena sanderiana* (Wang et al. 2020) and *Chlorophytum comosum* (Qiao et al. 2023). *C. comosum* is well documented for the alleviation of toluene, n-hexane, benzene, ethylbenzene, xylene and formaldehyde (Yang et al. 2009).

However, indoor formaldehyde is also widely documented to affect indoor plants. These effects are majorly dependent on various factors such as the plant species being exposed, concentration, and exposure duration. Various research studies have reported formaldehyde to adversely affect plants by

causing destruction of spongy parenchyma and palisade cells, necrosis, stomatal damage, decreased peroxidase and catalase activity in *Rhapis excelsa*, *Epipremnum aureum* and *Ficus japonica* (Kim et al. 2013), enhanced plasma membrane permeability in *Tillandsia velutina* (Li et al. 2015), leaf injury in *Davallia bullata* and *Nephrolepis exaltata* (Wang et al. 2020), reduced chlorophyll content in *Peperomia tetraphylla* and *Dracaena sanderiana* and lower water soluble sugars in *C. comosum* (Wang et al. 2020). Plants also respond to the applied formaldehyde by activating its enzymatic machinery such as higher catalase, peroxidase and total antioxidant activity in *C. comosum* (Li et al. 2019); induction of redox and antioxidative enzymatic activities in *Taraxacum mongolicum* and *Plantago asiatica* (Zhao et al. 2019). It's well-known that under different stresses including formaldehyde, plants produce a relatively higher amount of reactive oxygen species (ROS). ROS mainly includes hydrogen peroxide, hydroxide ion, superoxide radicals, nitric oxide, singlet oxygen, peroxide ions and hydroxyl radicals. Additional ROS produced under stress conditions cause oxidative damage and oxidize organic molecules hence causing cellular malfunction in plants (Guo et al. 2023). To cope up with this situation, plants activate their defense mechanisms, consisting of enzymatic antioxidants including catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), and non-enzymatic antioxidants including total antioxidants (TAO), total flavonoid (TFC), total phenolics (TPC), carotenoid and proline (Gill et al. 2011). Among enzymatic antioxidants, SOD provides front line defense against superoxide radicals by dismutating them into O_2 and H_2O_2, hence reducing the chances of hydroxyl radical production (Mittler 2002). CAT transforms H_2O_2 into water H_2O and O_2 inside peroxisomes (Pan et al. 2022). APX has a higher potential for H_2O_2 compared to CAT and catalyzes the reduction of H_2O_2 to H_2O using ascorbate as an electron donor (Anjum et al. 2016). Peroxidases reduce H_2O_2 while oxidizing other electron donors including glutathione, ascorbate and phenolic compounds (Liebthal et al. 2018).

C. comosum is well documented to metabolize indoor formaldehyde (Yang et al. 2009; Gong et al. 2019; Li et al. 2019, Wang et al. 2020). Various research studies have primarily focused on evaluating the effects of indoor formaldehyde on *C. comosum*, however, the resultant responses which can be regarded as physiological and biochemical markers for formaldehyde stress have not been reported yet. Therefore, the present study was focused on exploring the periodic responses of *C. comosum* against different dose levels of formaldehyde that can be considered a marker of formaldehyde stress.

Methodology

Acclimatization of experimental plants

C. comosum plant with an average height of 25.33 ± 2.08 cm, a leaf width of 1.56 ± 0.20 cm, leaf length of 23.33 ± 4.04 cm and leaf area of 56.33 ± 8.78 cm^2 was selected for the current study. A single plant was transferred to pots (4 inches diameter) filled with peat moss, coco peat and perlite in a 2:2:1 proportion. Plants were acclimated in a specific growth room (temperature: 24°C; light period: 12 hours) 2 weeks prior to formaldehyde application (Khan et al. 2023).

Formaldehyde application

Three uniform sized glass chambers (0.128 m^3) were used for formaldehyde application. Each of the glass chambers was equipped with the top mounted small fan and an inlet for injecting formaldehyde into the chamber. Glass chambers were placed in a growth room with the same growth conditions set for the acclimatization of plants. Pots were covered with aluminum foil to avoid formaldehyde adsorption. Individual one potted plant was placed, and glass chambers were sealed with Teflon tape and cling film to ensure air-tight sealing during the experimentation (Fig. 1). Different doses of formaldehyde (5, 10 and 20 ppm) were injected into the chamber in liquid form via inlet port and the fan inside the chamber was kept operating for at least 30 minutes for its evaporation and homogenization in the air. The *C. comosum* plant was taken out of each glass chamber after short (2, 4 and 6 hours); medium (12 and 24 hours), and extended (48 and 96 hours) time durations and immediately investigated for their morpho-anatomical, physiological and biochemical analysis. Fully mature leaves (leaf area: 56 cm^2) of the plant were sampled. Plants were kept inside the glass chamber for the tested time durations without formaldehyde subjection to serve as respective controls. The experiment was carried out with six replicates.

Morpho-anatomical analysis

Leaf chlorosis and necrosis

To determine the visible phytotoxicity of formaldehyde, leaf chlorosis and necrosis (%) was calculated from the number of necrotic and chlorosis leaves out of the total number of leaves per plant (Wang et al. 2020).

Stomatal density

Microscopic slides were prepared from the abaxial side of mature leaves and examined under a light microscope (Optikam B6, Optika, Italy) at 40X times the original magnification (Hamill et al. 1992).

Physiological analysis

Photosynthetic and transpiration rates, intercellular CO_2 levels and stomatal conductance

IRGA instrument (Infrared gas analyzer, LI-COR, Lincoln, Nebraska) was used to determine the rate of photosynthesis, rate of transpiration, intercellular CO_2 levels and stomatal conductance on two mature leaves of each tested plant. Leaves were kept in the chamber for 5–15 minutes until the constant readings were shown (Arab et al. 2023).

Leaf pH

Fresh leaf material (0.2 g) was extracted in 1.5 mL deionized water, and supernatant was immediately used for determining the pH with a pH meter (Eutech PC 700, Singapore) (Husson et al. 2018).

Enzymatic antioxidants

Extraction for CAT, SOD and GPX enzymes

Freshly harvested leaf (0.25 g) was extracted in 2 mL of 0.1 M sodium phosphate buffer (pH 7.8) for CAT, SOD and GPX assay. The homogenates were centrifuged (HERMLE-Z 216 MK) for 10 minutes at 10,000 rpm (at 4°C). Supernatant was separated for further use.

Catalase

Sodium phosphate buffer (reaction buffer, pH 7.8), 1 mM H_2O_2 and 100 µL of extracted sample were taken and mixed in cuvette. Activity was immediately calculated by monitoring the reduction in absorbance at 240 nm via spectrophotometer (T80 + UV/VIS Spectrometer) for 75 seconds due to the decomposition of H_2O_2 (Aebi, 1984).

SOD

Reaction solutions containing sodium phosphate buffer (pH 7.8), methionine (130 mM), NBT (0.75 mM), EDTA (1mM), riboflavin (20 µM), and 100 µL of extracted sample were kept under a fluorescent lamp for 20 minutes. Absorbance was recorded at 560 nm (Baolong, 2016).

GPX

The elevation in absorbance at 420 nm based on the oxidation of guaiacol was monitored to calculate the activity. The reaction mixture was 0.1 M sodium phosphate buffer pH 7.0, 2% guaiacol, 1 mM H_2O_2 and extracted sample (Beyer and Fridovich, 1987).

Total antioxidants

Briefly, 80 mg of leaf material was ground fine and extracted in chilled methanol (80%). Afterwards, 2.7 mL of DPPH salt solution was taken in test tubes containing 300 µL of extracted sample and incubated for 50 minutes in the dark (Brand-Williams et al. 1995). Lastly, absorbance was recorded at 517 nm.

Non-enzymatic parameters

TPC and TFC

Fresh leaf material (80 mg) was first ground fine and extracted in chilled methanol (90%) for TPC and TFC quantification. The protocol of Ainsworth and Gillespie (2007) was followed using Folin-Ciocalteu reagent using for quantifying TPC. Lastly, absorbance was taken at 725 nm. Gallic acid was used as standard to quantify TPC in samples. TFC was measured using Aluminum trichloride method (Olajire and Azeez, 2011). Absorbance was taken immediately at 510 nm.

Proline content

The leaf material (0.25 g) was ground fine and extracted in sulfosalicylic acid (3%). Ninhydrin reagent method was employed to quantify proline content in leaves (Bates et al. 1973). The absorbance of the colored phase was recorded at 520 nm.

MDA content

A thiobarbituric acid reagent was used to quantify MDA content (Zeb & Ullah, 2016). Firstly, leaf sample was extracted in chilled glacial acetic acid and TBA (0.3%) was prepared in 20% trichloroacetic acid. TBA reagent (2 mL) was added to the extracted sample, mixed and kept in a water bath for 40 minutes at 95°C. Lastly, absorbance was measured at 532 and 600 nm.

Total protein

A fresh leaf sample (0.25 g) was fine ground and extracted in 0.1 M sodium phosphate buffer (pH 7.8) for 15 minutes at 10,000 rpm and the supernatant was taken. The extracted sample (200 µL) was added to Bradford reagent (1.4 mL) and incubated for 30 minutes in light (Bradford 1976). After incubation, absorbance was measured at 595 nm.

Total soluble sugars

Freshly harvested leaves (80 mg) were extracted in chilled ethanol (80%). Briefly, 100 µL of extracted sample was added to anthrone reagent (3 mL) and kept in a water bath at 95°C for 20 minutes. Absorbance was taken at 625 nm. Glucose was used as the standard to calculate standard curve (Razi et al. 1985).

Chlorophyll A, B, total chlorophyll and carotenoid content

In order to quantify the pigments, fresh leaf (100 mg) was ground fine and extracted in 80% acetone (chilled). The solution mixture was incubated overnight in the dark. Solution mixture was then centrifuged for 10 minutes at 5000 rpm and the supernatant was taken to measure absorbance at 664.2, 648.6 and 470 nm (Lichtenthaler and Wellburn, 1983).

Statistical analysis

The experiment was conducted in CRD (completely randomized design) with six replicates. The obtained data was then subjected to a two-way ANOVA, considering formaldehyde doses as one factor and tested durations as a second factor, followed by Tukey's test ($p \leq 0.05$) via IBM-SPSS (V-26) software. Pearson's correlation analysis was conducted to observe the relationship among the tested parameters. Principal component analysis was carried out to determine the contribution of tested parameters to the total variance.

Results

Morpho-anatomical responses

Application of formaldehyde to *C. comosum* led to an increase in stomatal density, leaf chlorosis and necrosis (Figs. 2 and 3). Table 1 shows that the control plant exhibited 36.83 mm^2 of stomatal density which increased significantly to 59.16 mm^2 in 20 ppm formaldehyde dose after 48 hours. Significant leaf

chlorosis (100%) and necrosis (93.14 and 100%) were observed in 10 and 20 ppm formaldehyde doses after 48 and 96 hours compared to control plants (table 1).

Physiological responses

Formaldehyde application resulted in a significantly reduced photosynthetic rate for each dose of formaldehyde. Compared to control plants (8.87 µmol $m^{-2}s^{-1}$), the photosynthetic rate declined significantly to 0.2 µmol $m^{-2}s^{-1}$ in 20 ppm dose after 96 hours as shown in table 1. Moreover, comparatively lower transpiration rate (1.64 mmol $m^{-2}s^{-1}$) and stomatal conductance (0.085 mmol $m^{-2}s^{-1}$) were exhibited by the control plants, which significantly inclined to 2.21 mmol $m^{-2}s^{-1}$ of transpiration rate and 0.23 mmol $m^{-2}s^{-1}$ of stomatal conductance in 20 ppm dose after 24 hours. No increase was observed in the 5 and 10 ppm formaldehyde doses for each tested duration compared to control plants (shown in table 1). Furthermore, application of formaldehyde also led to a higher intercellular CO_2 level (505.66 µmol mol^{-1}) in the 20 ppm dose after 6 hours duration compared to 187.83 µmol mol^{-1} in control plants. Whereas, the leaf pH of control plants (6.62) changed to 8.17 in the 20 ppm dose after 48 hours of duration (table 1).

Enzymatic antioxidants

Formaldehyde application significantly escalated the enzymatic activities of CAT, GPX and SOD in 10 ppm formaldehyde dose compared to their respective controls. Figure 4A displays a significant enhancement in SOD activity from 2.71 U/mg of protein (control plants) to 44.76 U/mg of protein after 12 hours of duration. CAT activity significantly rose from control plants (3.91 U/mg of protein) to 30.30 and 27.51 U/mg of protein after 4 and 12 hours, respectively (Fig. 4B). Likewise, a significant rise in GPX activity of 135.64 U/mg of protein was observed after 12 hours compared to control plants (9.13 U/mg of protein), shown in Fig. 4C. After subsequent inclination in GPX activity, it normalized back to control after 96 hours duration in each dose of formaldehyde. On the other hand, T-AOC significantly escalated in each tested dose of formaldehyde against tested durations. Activity rose to a maximum 85.05% in the 20 ppm dose after 2 hours of duration compared to 27.37% in control plants (Fig. 4D).

Non-enzymatic parameters

TPC and TFC significantly accumulated in leaves after formaldehyde application. A significant increase in TPC from 3.62 mg GAE/g (control plants) to 10.51 mg GAE/g was observed in the 20 ppm dose after 48 hours as shown in Fig. 5A. Similarly, TFC significantly rose to 0.955 mg/g in 10 ppm dose after 6 hours duration with respect to control plants (0.137 mg/g) (Fig. 5B). *C. comosum* exhibited higher proline, MDA and total protein content against formaldehyde application compared to their respective controls. Control plants exhibited 5.91 µg/g of proline content, whereas the content rose to 75.36 µg/g in the 20 ppm dose after 96 hours as shown in Fig. 5C. A significant rise in proline content was observed in each dose after 24 hours duration. Likewise, MDA content was significantly elevated (131.64 nmol/mg of protein) in 10 ppm dose after 12 hours of duration, compared to the control (12.77 nmol/ mg of protein). Whereas the

least incline was recorded in a 5ppm dose (Fig. 5D). Similarly, total protein content peaked from 0.35 mg/g (control plants) to 1.63 mg/g in a 5 ppm dose after 96 hours duration (Fig. 5E). Additionally, application of a 10 ppm dose for 12 hours led to a significant escalation in TSS content from 0.73 mg/g (control) to 4.72 mg/g. However, in all the treatments, the TSS content normalized to the control after 24 hours of duration as shown in Fig. 5F.

Pigments

Application of formaldehyde doses resulted in higher chlorophyll A, B, total chlorophyll and carotenoids compared to their respective controls. A significant rise in chlorophyll A content from 4.34 mg/g (control plants) to 8.43 mg/g (Fig. 6A), chlorophyll B content from 1.89 mg/g (control plants) to 5.30 mg/g (Fig. 6B) and total chlorophyll content from control plants (6.24 mg/g) to 13.73 mg/g was recorded in a 20 ppm dose after 48 hours of duration (Fig. 6C). In comparison to the control (0.07 mg/g), the highest total carotenoid content of 0.85 mg/g was recorded in a 10 ppm dose after 6 hours (Fig. 6D).

Pearson's correlation

Among the morpho-anatomical and physiological parameters, leaf necrosis significantly correlated with leaf chlorosis (0.923**) and pH (0.664**), whereas it has a significantly negative correlation of -0.449* with photosynthetic rate (Fig. 8). Additionally, a significantly positive correlation of stomatal conductance was observed with transpiration (0.852**) and photosynthetic rate (0.440*), respectively (Fig. 7). Whereas, among the enzymatic parameters, GPX significantly correlated with SOD (r^2 = 0.711**, $p < 0.01$) and other non-enzymatic studied parameters like MDA (0.878**), TPC (0.865**) and TFC (0.544**). SOD significantly correlated with CAT (0.556**) and MDA (0.914**). Furthermore, CAT also correlated with TFC (0.588**). However, among the non-enzymatic parameters, a significant correlation of MDA was observed with TPC (0.686**) and TFC (0.538**), respectively (Fig. 8).

Principal component analysis

According to PCA, three components collectively contributed 70.64% of the total variance (Fig. 9). Principal component 1 contributed 32.77% to the variance, compared to 22.62% in PC2 and 15.25% in PC 3. PC1 included GPX, MDA, TPC, SOD, TFC, TAO, CAT, carotenoids, TSS and intercellular CO_2 that contributed the most to the total variance.

Discussion

In the current study, higher stomatal density and leaf damage were recorded in formaldehyde exposed plants compared to the respective controls. The increase in stomatal density has also been reported in other indoor plants against formaldehyde application (Kim et al. 2013). Plants close their stomata to limit the entry of formaldehyde and tolerate its stress (Kvesitadze et al. 2006; Kim et al. 2013). Similarly, leaf damage has also been well documented in various plants under formaldehyde stress (Kim et al. 2013;

Nian et al. 2013; Li et al. 2021). VOCs are known to impart deleterious effects on the basic physiological processes of plants along with damage to cell ultrastructures (Yoo et al. 2006).

The physiological processes of *C. comosum* were significantly affected by formaldehyde application in the current study. The present study reports a decline in photosynthetic rate after formaldehyde application. Various studies have also reported this decline in different plants under VOCs and ozone stress (Phothi et al. 2016; Hörmann et al. 2018). VOCs are reported to cause damage to PS II quantum yield in light reactions of photosynthesis. Decreased absorption of photons in PS II reduces the formation of NADPH + H^+ and ATP which may reduce CO_2 fixation, hence leading to reduced photosynthesis (Yoo et al. 2006; Hörmann et al. 2018). Subjection to a higher dose of formaldehyde led to an increased transpiration rate and stomatal conductance in the present study. Gong et al. (2019) has also reported higher stomatal conductance in *Epipremnum aureum* against benzene stress. A higher transpiration rate reflects higher stomatal conductivity, which is essential for the removal of VOCs by plants (Jin et al. 2013). Whereas the transpiration rate and stomatal conductance significantly declined against low and moderate doses of formaldehyde in the current study. These findings are also supported by previous studies conducted on indoor plants (Yoo et al. 2006; Gong et al. 2019). VOCs including formaldehyde are reported to impart deleterious effects on the basic physiological processes of plants, and damage to cell ultrastructure (Yoo et al. 2006) and reducing the stomatal conductance due to the elasticity of damaged guard cells (Paoletti and Grulke, 2005). A significant rise in the intercellular CO_2 level was observed in the current study against formaldehyde doses. This finding is also supported by the research of Yoo et al. (2006) conducted on *Spathiphyllum wallisii* and *Hedera helix* under benzene and toluene stress. *C. comosum* metabolizes formaldehyde by hydrolyzing it into formate. Subsequently, the generated formate is then oxidized to CO_2 enzymatically by formate dehydrogenase (Wang et al. 2018). This oxidation could be a possible reason for elevated levels of intercellular CO_2 in plants under formaldehyde stress. Furthermore, acidic pH of the control plants shifted to basic after formaldehyde application in current study. Bharti et al. (2018) have also documented a pH shift to basicity in various plants against air pollution stress. Basic leaf pH is known to assist the tolerance capability of plants against air pollutants by enhancing the conversion rate of hexose sugar to vitamin C (Escobedo et al. 2008, Joshi and Bora, 2011). A change in pH is crucial for various enzymatic and physiological responses of plants under stress conditions (Escobedo et al. 2008).

In order to avoid the damage and maintain the balance of oxidation in the leaves, an enzymatic antioxidant system was activated in the current study. Significantly higher SOD, CAT, GPX activity and T-AOC were recorded in the current study. In support of our findings, various reports have documented higher SOD activity (Liang et al. 2019; Khan et al. 2023), CAT activity (Zhao et al. 2019; Li et al. 2021; Khan et al. 2023), GPX activity (Li et al. 2021; Khan et al. 2023)d AOC activity (Li et al. 2019; Khan et al. 2023) in various indoor plants against formaldehyde stress. SOD is an enzymatic antioxidant that catalyzes the elimination of the superoxide anion by dismutating it into O_2 and H_2O_2, eventually eliminating the chances of $OH^{\bullet}$ formation (Mittler, 2002). In the same way, catalase is reported to catalyzing the dismutation of H_2O_2 into H_2O and O_2 (Mittler 2002). H_2O_2 can cross membranes and cause oxidative damage by

resulting in 50% reduced activity of various enzymes and programmed cell death (Dat et al. 2000; Bienert et al. 2007). However, the CAT activity did not increase against the highest dose of formaldehyde in current study. The consistent trend of decreasing CAT activity under higher doses of formaldehyde is well documented in various studies (Xue et al. 2008; Linghu et al. 2011; Li et al. 2019). This decline is due to the interactions of formaldehyde with cell's thiol-disulphide status and the redundancy of reductive H_2O_2-metabolizing pathways (Mhamdi et al. 2010). Moreover, GPX is also a prominent antioxidant enzyme known to eliminate excessive H_2O_2 which thus aids the plant to withstand stressful environment (Asada 1999). Higher T-AOC is reported to aid plants in coping up various stresses (Li et al. 2019). TAOs are stable molecules known to donate an electron to a rampaging free radical and neutralize it. This reduces the potential for free radicals to cause cell damage (Lobo et al. 2010).

Current study reports a significant rise in TPC and TFC against formaldehyde application after tested durations. These findings are in alignment with the findings of Khan et al. (2023). Phenolic contents are a prominent non-enzymatic antioxidant (Samec et al. 2021) that aids plants by donating electrons to guaiacol-type peroxidases for the catabolism of H_2O_2 formed under harsh environmental conditions (Sakihama et al. 2002). Moreover, formaldehyde is shown to enhance the expression of enzymes that are involved in phenolic transformations, such as peroxidase (Peng et al. 2022). Whereas, flavonoids are reported to scavenge singlet oxygen and to reduce the damages to the outer envelope of the chloroplastic membrane (Agati et al. 2012).

Increased proline, total protein and MDA content was recorded in *C. comosum* plants against formaldehyde application in the current study. Various researchers have reported higher proline content (Wang et al. 2020; Markazi et al. 2022; Khan et al. 2023), total protein content (Li et al., 2019) and MDA content (Li et al. 2019; Wang et al. 2020; Qiao et al. 2023) in various indoor plants against formaldehyde stress. In stressful conditions, proline works as an osmo-protectant, maintains redox balance inside the plant cell and stabilizes cellular structures and enzymes (Meena et al. 2019). It quenches ROS including singlet oxygen, superoxide and hydroxyl radicals (Rehman et al. 2021). MDA is a byproduct of lipid peroxidation of polyunsaturated fatty acids caused by hydroxyl radicals, which is induced under formaldehyde stress in plants (Li et al. 2019). However, after subsequent increase, a reduction in MDA content at extended time durations was observed in the current study. This reduction is possibly associated with the decrease in formaldehyde level being metabolized by *C. comosum* (Li et al. 2021; Wu et al. 2022). Under abiotic stress conditions, ROS accumulation occurs in plants. This leads to the requirement of new protein synthesis to defend against ROS and replace existing damaged proteins (Qazi et al. 2019; Salih et al. 2020).

Formaldehyde application also significantly escalated the chlorophyll and carotenoid content in this study. This follows the pattern of previous studies conducted on *Pisum sativum* and *C. comosum* against formaldehyde stress (Erofeeva, 2018; Paresh et al. 2018; Khan et al. 2023). Under paradoxical effects, the toxic impact of higher formaldehyde level is reduced, leading to increased chlorophyll content in plants (Erofeeva, 2018). Additionally, a higher carotenoid content recorded in the current study could be a possible reason of an inclined chlorophyll content against formaldehyde. Carotenoids act as an

antioxidants to protect chlorophyll and photosynthetic apparatus by quenching of $^3Chl^*$ and $^1O_2$ by excitation transfer mechanism followed by thermal energy dissipation (Triantaphylidès & Havaux, 2009; Yuan et al. 2015).

Elevated TSS content in *C. comosum* against formaldehyde application is recorded in the current research, which is supported by the report of Skłodowska et al. (2023), conducted on *C. comosum* against formaldehyde stress. Formaldehyde is known to be oxidized and utilized in the Calvin cycle after being absorbed by plant leaves (Salonen et al. 2009; Kim et al. 2010). This could be a possible reason for inclined TSS content recorded in this study.

PCA indicated that SOD, CAT, GPX, MDA, TPC, TFC, TAO, carotenoids, TSS and intercellular CO_2 contributed the most to the total variance and can be considered biochemical markers for formaldehyde stress in *C. comosum*. Different researchers have documented these parameters individually to respond significantly against formaldehyde stress (Yoo et al. 2006; Liang et al. 2019; Li et al. 2019; Zhao et al. 2019; Wang et al. 2020; Li et al. 2021; Khan et al. 2023; Qiao et al. 2023; Skłodowska et al. 2023). However, according to our extensive literature review, no research study has documented these parameters as biomarkers in *C. comosum* against different levels of formaldehyde for a wide range durations.

Conclusion

The current research study shows that application of formaldehyde results in activation of anatomical, physiological and biochemical responses in *C. comosum*. Application of 20 ppm dose of formaldehyde for extended durations imparted morphological impairment compared to 5 and 10 ppm doses. However, activation of both enzymatic and non-enzymatic antioxidants was also observed. A Significant positive correlation was observed among enzymatic antioxidants such as SOD, CAT and CAT. However, among the non-enzymatic parameters, a significant positive correlation of MDA was observed with TPC and TFC, respectively. PCA indicates that among the eighteen studied parameters, SOD, CAT, GPX, MDA, TPC, TFC, TAO, carotenoids, TSS and intercellular CO_2 have a considerable contribution to variance, thus can be regarded as potential markers against formaldehyde stress in *C. comosum*. Current identification of biomarkers will enhance the understanding of plant's responses, early monitoring and detection of stress, against formaldehyde.

Declarations

Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Each author is willing to publish the manuscript.

Competing interests:

The authors declare no competing interests.

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Author contribution:

Hifza Imtiaz conducted the research and wrote the research article. Yasar Sajjad designed and supervised the study and performed data analysis, Sabaz Ali Khan and Amjad Hassan contributed to planning research and write up. Abdul Rehman Khan assists the data analysis and review. Ghazal Khurshid and Zahid Ahmad Khan contributed to the execution of the experiment.

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Data availability:

Available for reference and future use in research.

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Tables

Table 1 Periodic morpho-anatomical and physiological responses of *C. comosum* against different doses and duration of formaldehyde application

Dose (ppm)	Durations (hours)	SD (mm^2)	Leaf chlorosis (%)	Leaf necrosis (%)	PR (µmol m^{-2} s^{-1})	TR (mmol m^{-2} s^{-1})	SC (mmol m^{-2} s^{-1})	CO_2 (µmol m^{-1})	pH
5	0 (control)	36.67 ± 1.05 cde	00.00 ± 0.00 g	00.00 ± 0.00 h	8.98 ± 0.15 a	1.67 ± 0.05 b	0.078 ± 0.003 c	180.67 ± 8.80 fg	6.63 ± 0.01 n
	2	48.33 ± 3.07 abcd	11.43 ± 1.69 efg	15.87 ± 1.47 fgh	1.33 ± 0.01 hi	0.30 ± 0.02 fghij	0.020 ± 0.000 jkl	281.00 ± 7.87 d	6.89 ± 0.01 m
	4	42.50 ± 3.59 bcde	13.26 ± 1.87 ef	17.42 ± 3.26 fgh	0.75 ± 0.01 j	0.08 ± 0.00 j	0.010 ± 0.000 l	94.50 ± 10.28 h	6.82 ± 0.00 m
	6	55.00 ± 3.82 ab	15.45 ± 0.49 def	30.68 ± 2.85 efg	1.89 ± 0.03 fg	0.43 ± 0.02 defgh	0.022 ± 0.003 ijkl	224.17 ± 11.15 ef	7.21 ± 0.00 jk
	12	30.83 ± 1.54 e	26.19 ± 1.68 cd	33.33 ± 3.37 defg	2.28 ± 0.03 ef	0.52 ± 0.01 def	0.032 ± 0.003 ghijk	254.00 ± 4.59 de	7.52 ± 0.02 hi
	24	43.33 ± 3.07 bcde	20.95 ± 0.34 cde	37.30 ± 2.02 defg	2.65 ± 0.04 de	0.59 ± 0.03 d	0.017 ± 0.002 jkl	117.00 ± 3.97 h	7.94 ± 0.04 c
	48	41.67 ± 4.01 bcde	30.20 ± 0.58 c	67.35 ± 6.73 bc	3.10 ± 0.07 cd	0.46 ± 0.05 defg	0.042 ± 0.003 efghi	287.17 ± 7.99 d	7.94 ± 0.02 c
	96	35.00 ± 2.24 de	45.00 ± 3.54 b	87.08 ± 4.57 ab	0.92 ± 0.10 ij	0.19 ± 0.01 hij	0.012 ± 0.002 kl	272.33 ± 13.72 de	7.94 ± 0.02 c
10	0 (control)	36.67 ± 1.05 cde	00.00 ± 0.00 g	00.00 ± 0.00 h	8.63 ± 0.18 a	1.72 ± 0.06 b	0.078 ± 0.005 c	175.67 ± 7.74 fg	6.62 ± 0.01 n
	2	32.50 ± 2.14 e	20.33 ± 1.31 cde	39.00 ± 1.87 def	0.19 ± 0.01 k	0.22 ± 0.01 ghij	0.013 ±	274.50 ± 4.34 de	7.14 ±

Dose (ppm)	Durations (hours)	SD (mm^2)	Leaf chlorosis (%)	Leaf necrosis (%)	PR (µmol m^{-2} s^{-1})	TR (mmol m^{-2} s^{-1})	SC (mmol m^{-2} s^{-1})	CO_2 (µmol m^{-1})	pH
							0.002 kl		0.02 kl
	4	40.83 ± 1.54 bcde	13.93 ± 1.53 def	39.44 ± 1.79 def	2.84 ± 0.02 cde	0.22 ± 0.01 ghij	0.023 ± 0.004 ijkl	361.00 ± 3.57 c	7.07 ± 0.02 l
	6	38.33 ± 2.11 cde	9.39 ± 0.21 efg	31.21 ± 1.90 efg	0.50 ± 0.02 jk	1.16 ± 0.01 c	0.057 ± 0.002 de	430.00 ± 6.83 b	7.51 ± 0.01 i
	12	41.67 ± 3.57 bcde	13.10 ± 1.81 ef	29.90 ± 3.55 efg	0.79 ± 0.02 ij	0.12 ± 0.01 ij	0.023 ± 0.003 ijkl	247.17 ± 2.77 de	6.00 ± 0.00 o
	24	39.17 ± 1.54 cde	12.27 ± 1.83 efg	34.25 ± 2.08 defg	1.53 ± 0.02 gh	0.50 ± 0.02 def	0.048 ± 0.002 defgh	350.50 ± 2.88 c	7.77 ± 0.01 de
	48	30.83 ± 1.54 e	100.00 ± 0.00 a	100.00 ± 0.00 a	3.27 ± 0.03 c	0.58 ± 0.07 de	0.028 ± 0.003 hijkl	185.83 ± 16.60 fg	7.45 ± 0.01 i
	96	35.83 ± 2.01 de	100.00 ± 0.00 a	100.00 ± 0.00 a	4.66 ± 0.09 b	0.60 ± 0.09 d	0.035 ± 0.005 fghij	144.00 ± 35.35 gh	7.65 ± 0.02 fg
20	0 (control)	36.67 ± 1.05 cde	00.00 ± 0.00 g	0.00 ± 0.00 h	8.88 ± 0.32 a	1.64 ± 0.09 b	0.085 ± 0.004 c	187.83 ± 4.84 fg	6.62 ± 0.03 n
	2	45.00 ± 3.65 abcde	7.47 ± 1.54 fg	12.61 ± 0.59 gh	8.69 ± 0.27 a	1.63 ± 0.10 b	0.138 ± 0.004 b	254.83 ± 4.35 de	7.61 ± 0.01 gh
	4	43.33 ± 4.01 bcde	10.04 ± 1.20 efg	33.49 ± 1.14 defg	1.57 ± 0.03 gh	1.31 ± 0.06 c	0.053 ± 0.003 def	359.17 ± 6.17 c	7.71 ± 0.03 ef

Dose (ppm)	Durations (hours)	SD (mm^2)	Leaf chlorosis (%)	Leaf necrosis (%)	PR (µmol m^{-2} s^{-1})	TR (mmol m^{-2} s^{-1})	SC (mmol m^{-2} s^{-1})	CO_2 (µmol m^{-1})	pH
	6	43.33 ± 4.01 bcde	19.44 ± 3.00 cdef	27.31 ± 3.60 fg	4.66 ± 0.08 b	1.62 ± 0.03 b	0.068 ± 0.003 cd	505.67 ± 8.31 a	7.28 ± 0.01 j
	12	30.83 ± 2.01 e	28.68 ± 3.13 c	57.48 ± 5.90 cd	4.30 ± 0.05 b	1.87 ± 0.05 b	0.135 ± 0.006 b	358.50 ± 5.31 c	8.00 ± 0.00 bc
	24	50.00 ± 5.48 abcd	47.95 ± 2.92 b	53.70 ± 8.42 cde	2.45 ± 0.04 ef	2.21 ± 0.07 a	0.238 ± 0.011 a	361.17 ± 1.83 c	7.80 ± 0.02 d
	48	59.17 ± 3.00 a	100.00 ± 0.00 a	93.15 ± 1.30 a	0.91 ± 0.05 ij	0.33 ± 0.02 efghi	0.052 ± 0.004 defg	262.33 ± 6.58 de	8.04 ± 0.01 b
	96	51.67 ± 2.47 abc	100.00 ± 0.00 a	100.00 ± 0.00 a	0.20 ± 0.00 k	0.32 ± 0.02 fghij	0.015 ± 0.002 jkl	378.66 ± 1.23 bc	8.17 ± 0.01 a

Figures

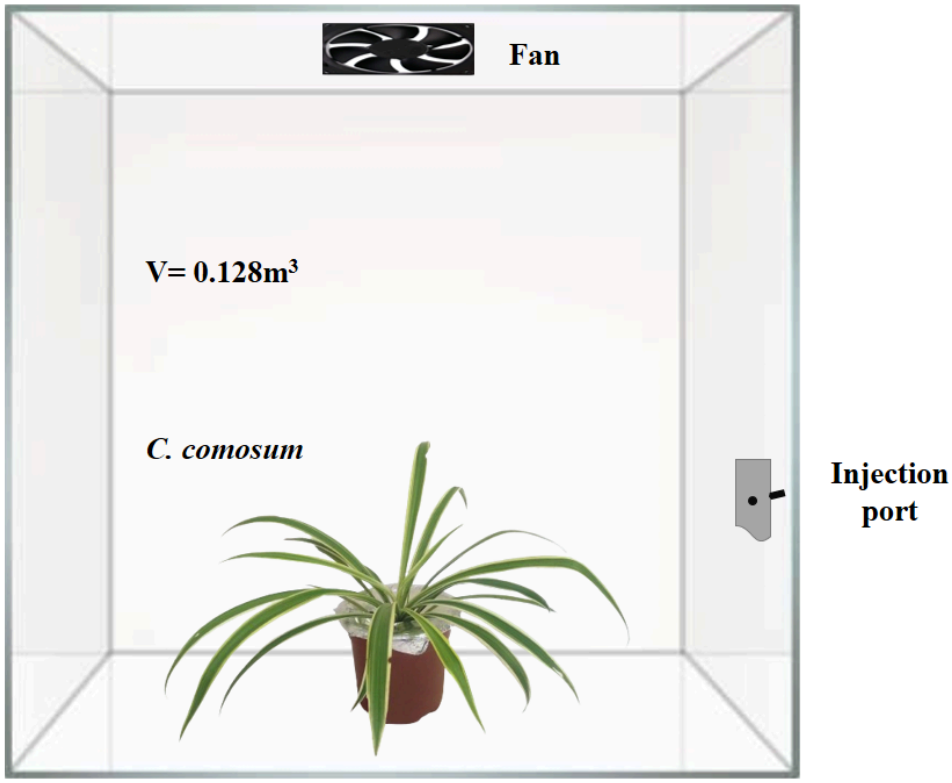

Figure 1

Air-tight glass chamber setup used for formaldehyde application

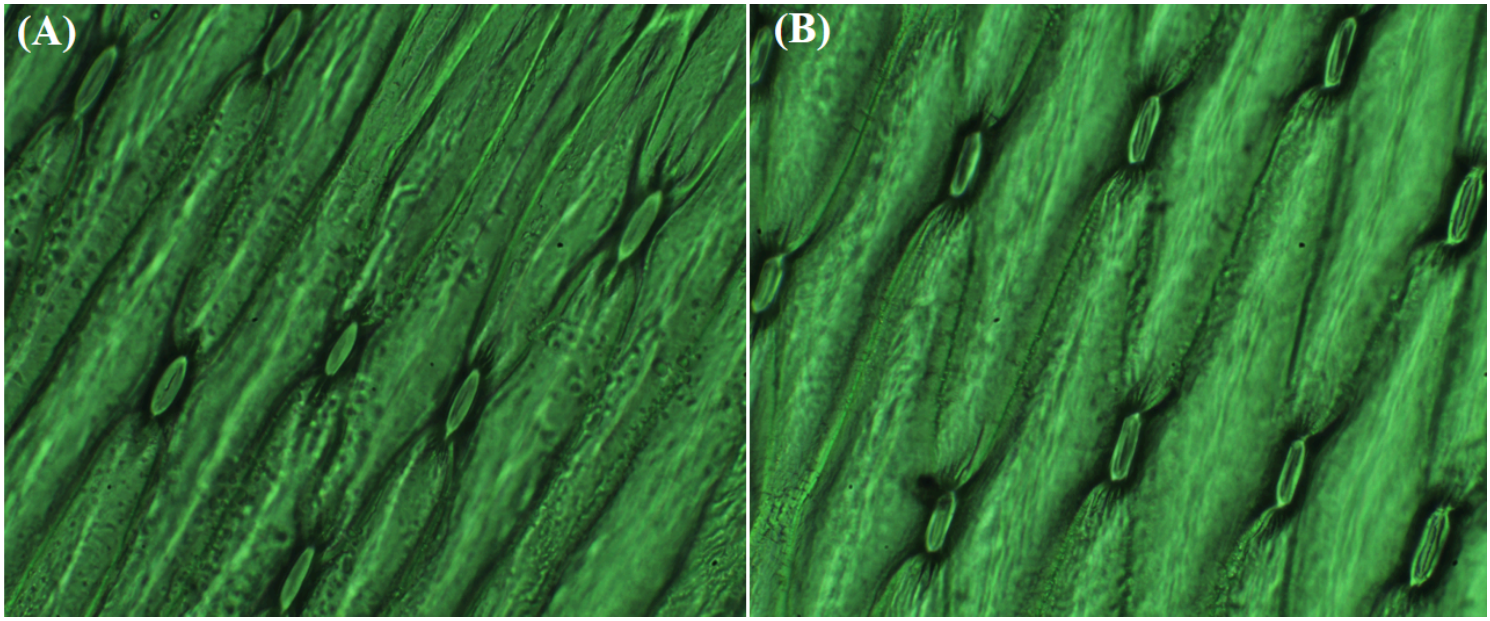

Figure 2

Stomatal density on leaf (abaxial side) of *C. comosum* at 400 magnifications. **(A)** Respective control and **(B)** after 20 ppm formaldehyde dose for 96 hours duration

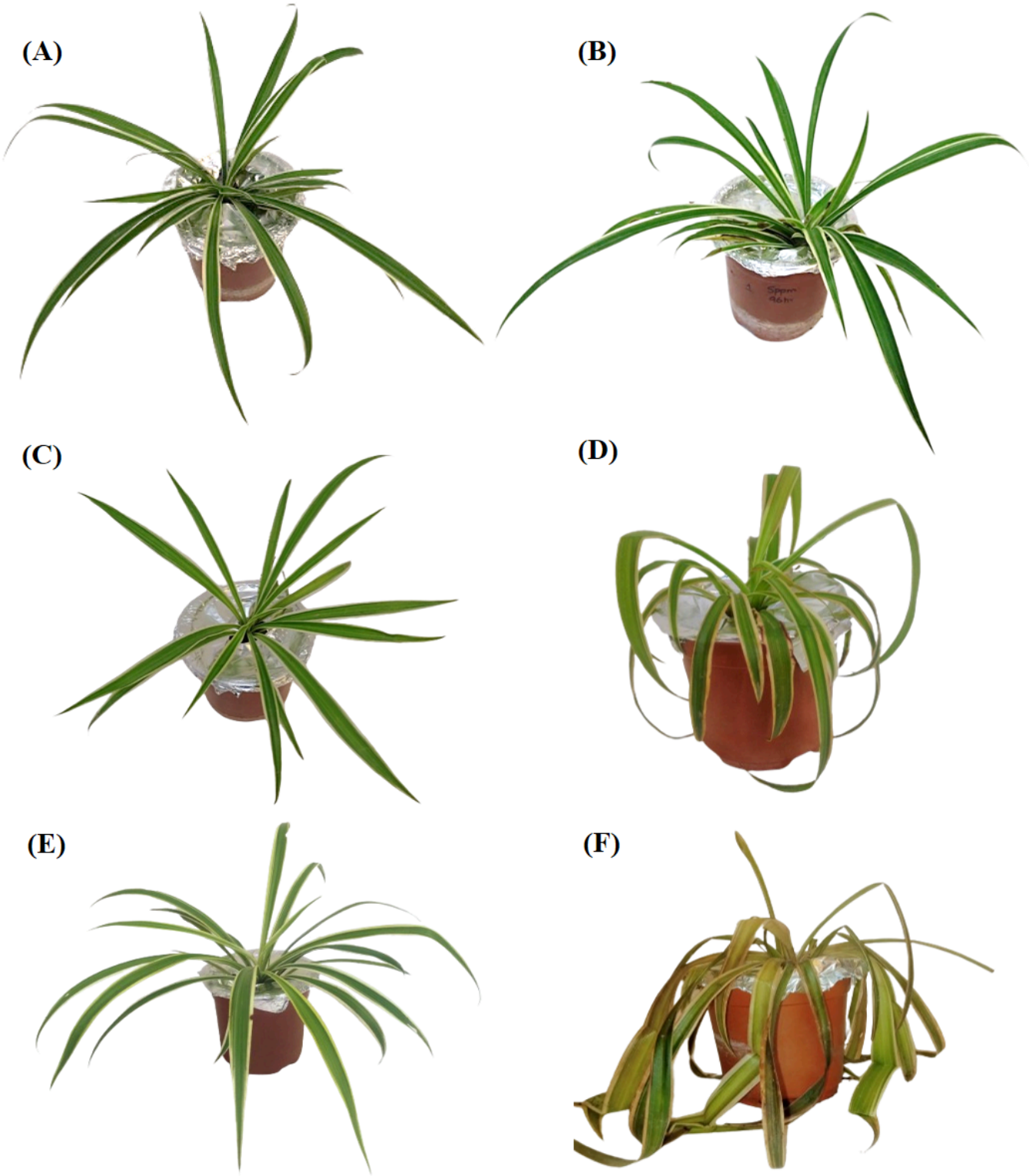

Figure 3

Leaf damage in *C. comosum* against formaldehyde application after 96 hours duration. **(A)** respective control and **(B)** 5 ppm formaldehyde; **(C)** respective control and **(D)** 10 ppm formaldehyde; **(E)** respective control and **(F)** 20 ppm formaldehyde

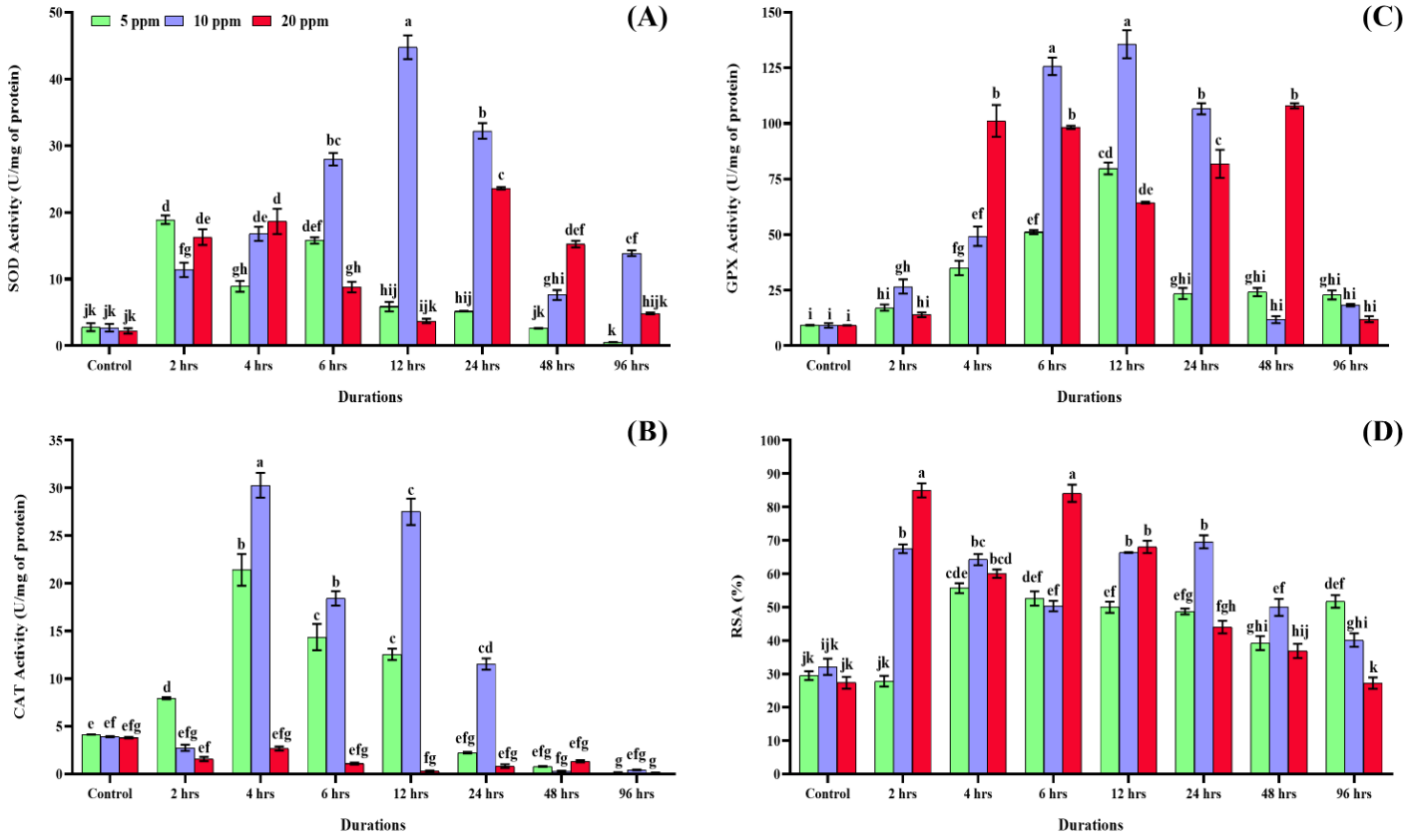

Figure 4

Periodic responses of *C. comosum* against applied doses of formaldehyde for different duration: **(A)** SOD; **(B)** CAT; **(C)** GPX and **(D)** total antioxidant activity. Means sharing different letters shows statistical difference by Tukey's test $p \leq 0.05$. Error bars represent standard errors, n=6

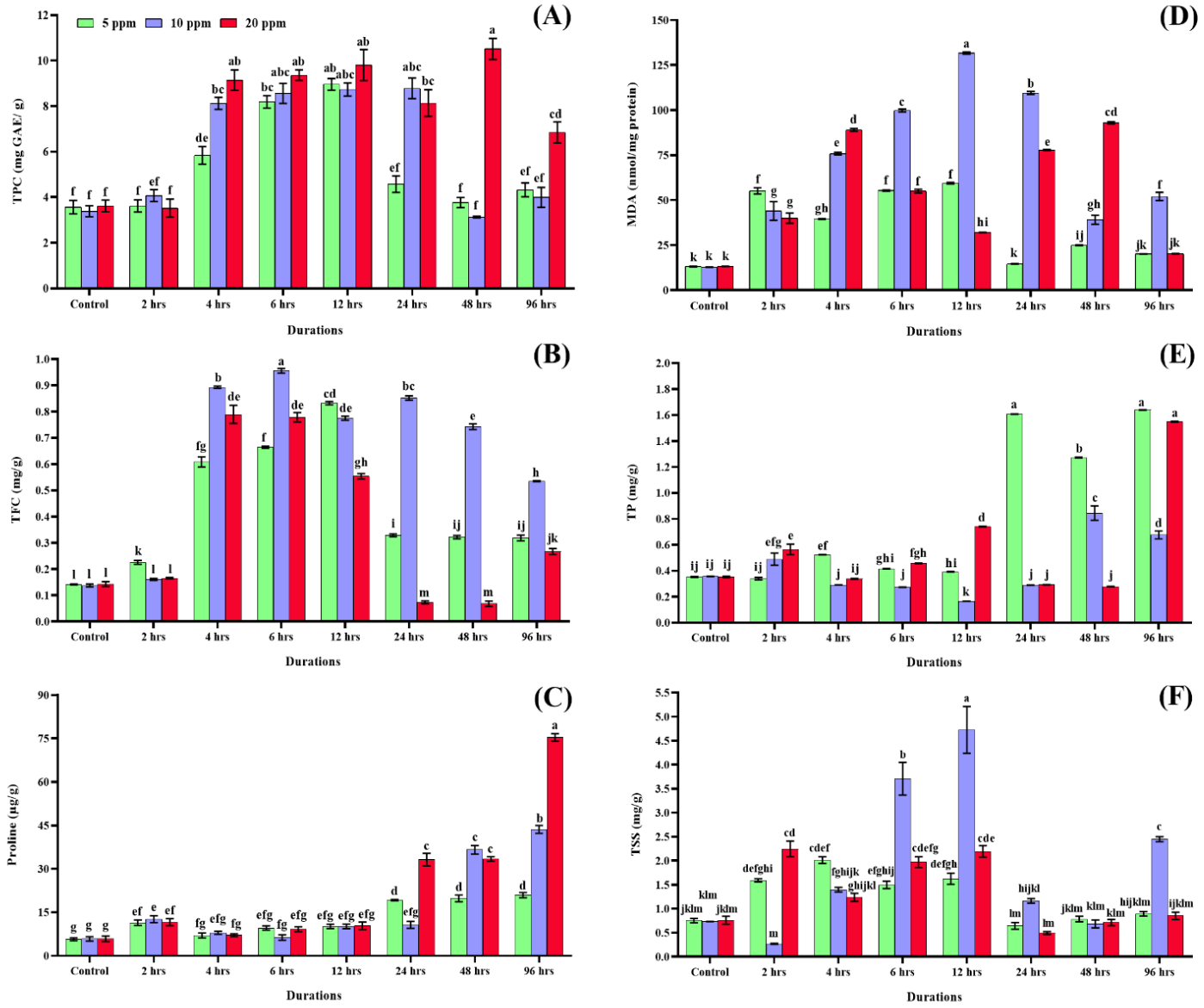

Figure 5

Periodic responses of *C. comosum* against applied doses of formaldehyde for different duration: **(A)** TPC; **(B)** TFC**; (C)** proline**; (D)** MDA**; (E)** TP and **(F)** TSS. Means sharing different letters shows statistical difference by Tukey's test at $p \leq 0.05$, Error bars represent standard errors, n=6

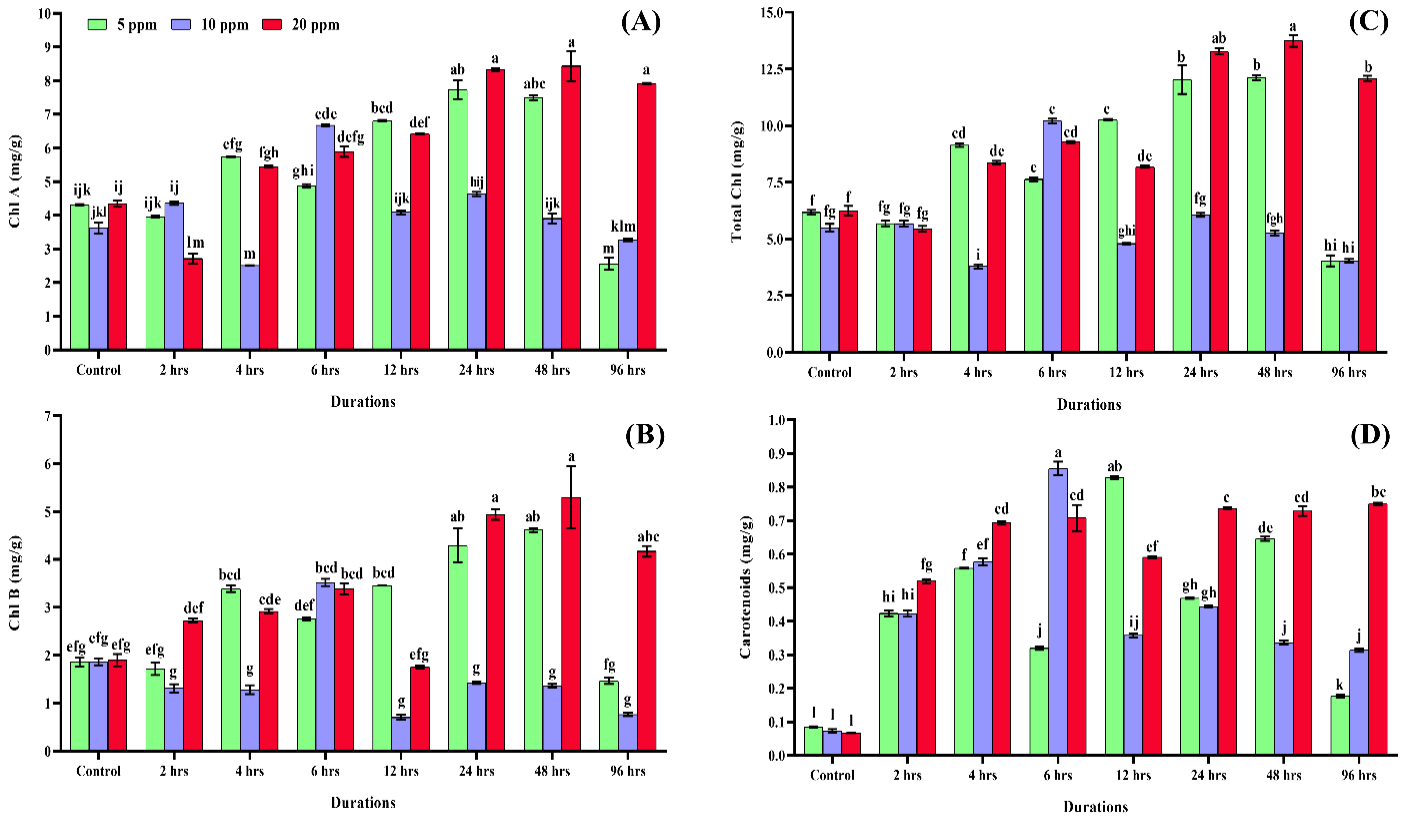

Figure 6

Periodic responses of C. comosum against applied doses of formaldehyde for different duration: (A) chlorophyll A; (B) chlorophyll B; (C) total chlorophyll and (D) carotenoid content. Means sharing different letters shows statistical difference by Tukey's test at $p \leq 0.05$, Error bars represent standard errors, n=6

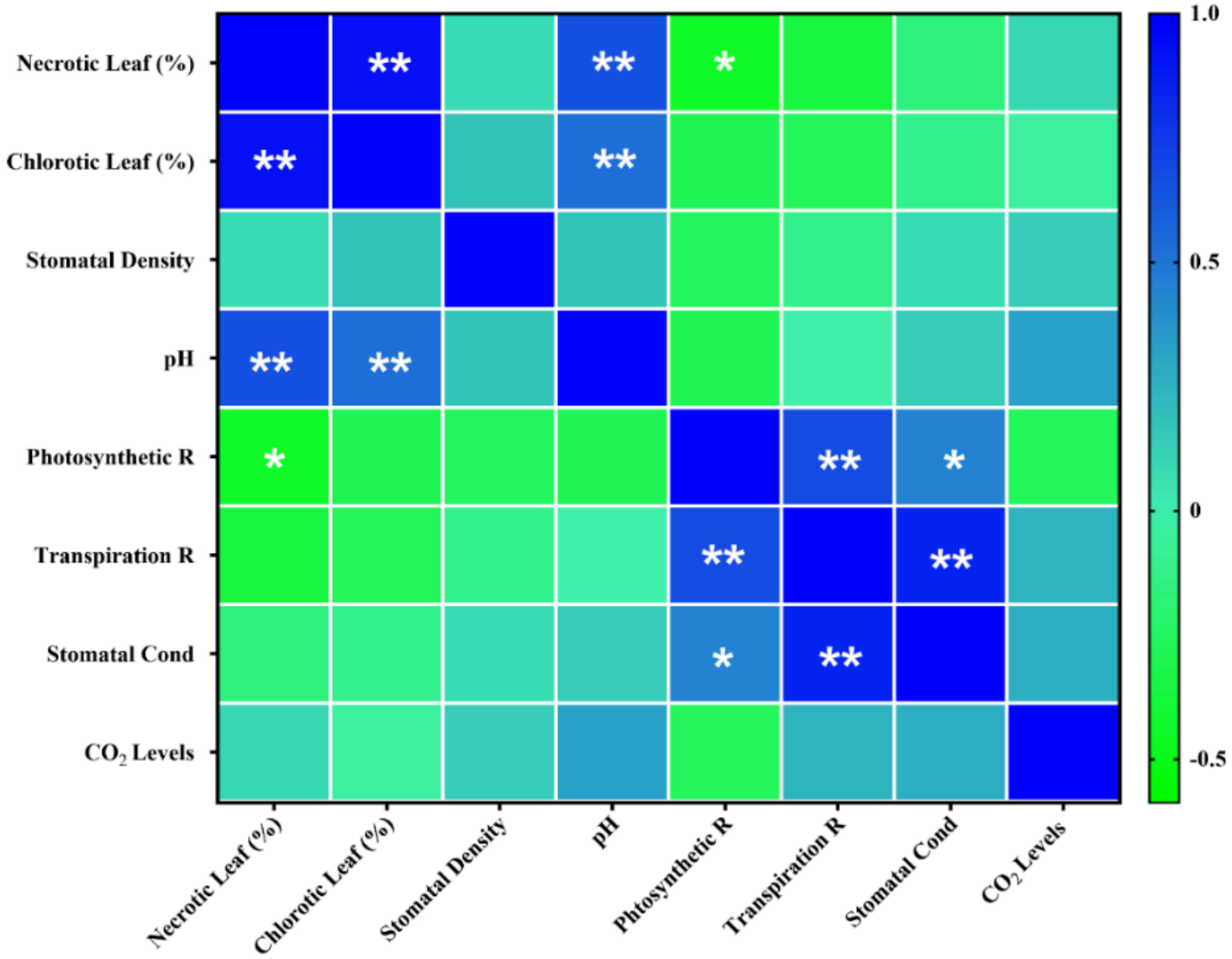

Figure 7

Pearson's correlation among leaf damage and physiological parameters. R (rate) and Stomatal cond (stomatal conductance)

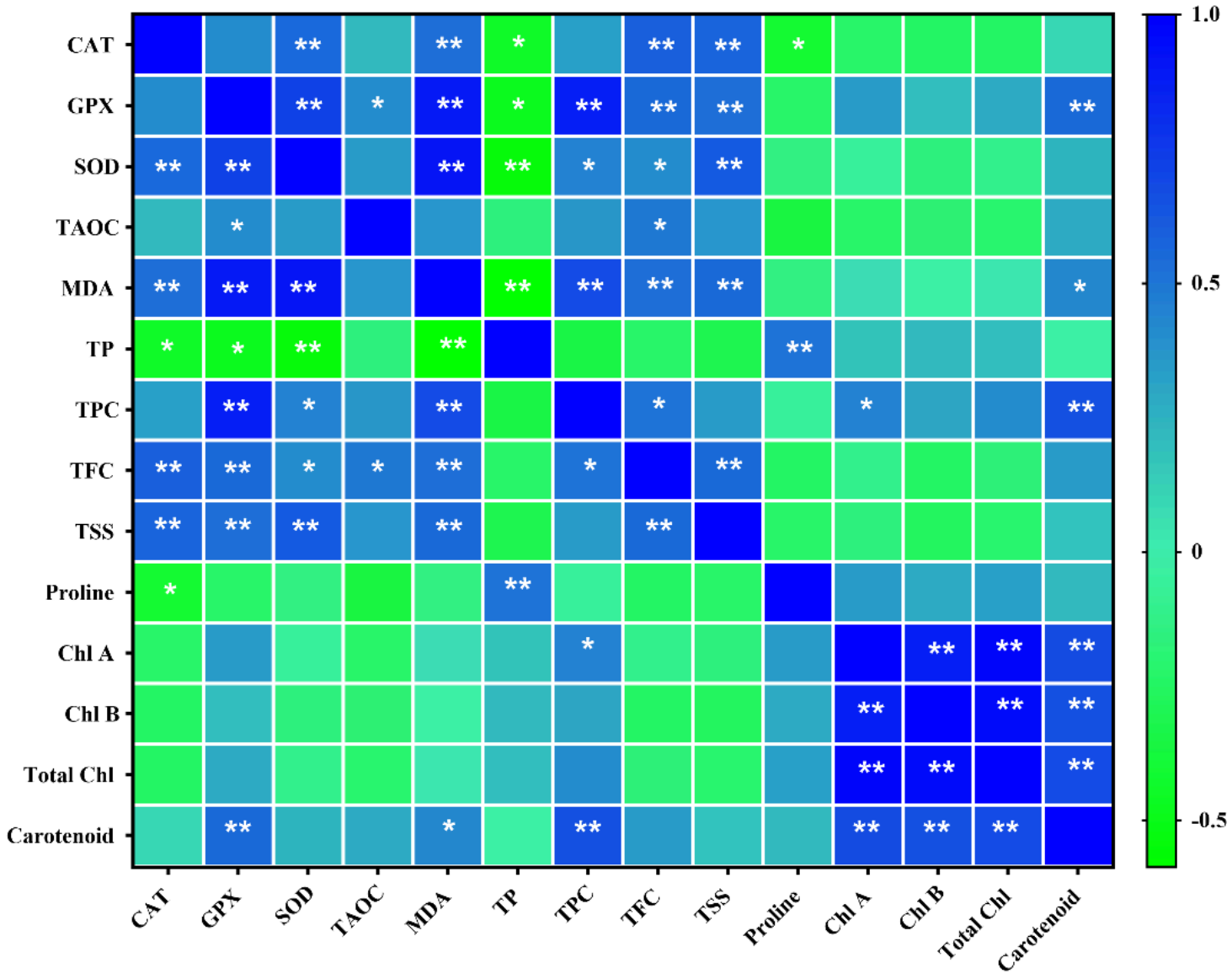

Figure 8

Pearson's correlation among tested enzymatic and non-enzymatic parameters. CAT (catalase); GPX (guaiacol peroxidase); SOD (superoxide dismutase); TAOC (total antioxidant capacity); MDA (malondialdehyde); TP (total protein); TPC (total phenolic content); TFC (total flavonoid content); TSS (total soluble sugars); Chl A (chlorophyll A); Chl B (chlorophyll B) and Total Chl (total chlorophyll). * Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level

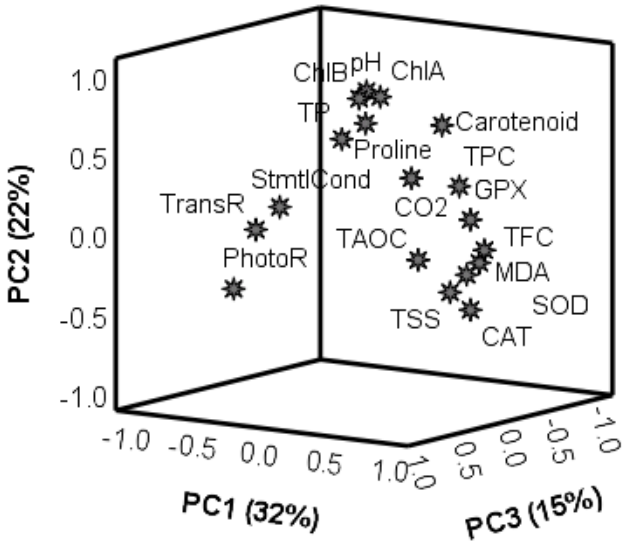

Figure 9

Three-dimensional principal component analysis of eighteen quantitative parameters. CAT (catalase); GPX (guaiacol peroxidase); SOD (superoxide dismutase); TAOC (total antioxidant capacity); MDA (malondialdehyde); TP (total protein); TPC (total phenolic content); TFC (total flavonoid content); TSS (total soluble sugars); ChlA (chlorophyll A), ChlB (chlorophyll B); stmtlCond (stomatal conductance); PhotoR (photosynthetic rate) and TransR (transpiration rate)