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PHYSIO-BIOCHEMICAL RESPONSES OF APPLE-OF-SODOM [*CALOTROPIS PROCERA* (AITON) W. T. AITON] TO VEHICULAR POLLUTION

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Abstract

Vehicular traffic has become a primary source of pollution along the roadsides which adversely affects plants and other biota. This study was conducted to assess the effects of vehicular pollutants on *Calotropis procera* growing at different sites along two major roads [Pindi Bhattian to Kala Shah Kaku (M-2) and Lahore to Gujranwala (G.T. road)] in the Punjab province of Pakistan. Control samples of *Calotropis procera* were collected at a distance of 50 m away from the roads. The photosynthetic rate (*A*), stomatal conductance (g_s), transpiration rate (*E*), water use efficiency (*WUE*), contents of photosynthetic pigments, total free amino acids and antioxidant activity of *C. procera* were determined. The results showed significantly lower contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids in *C. procera* along the roadside as compared to control plants. Similarly, stomatal conductance was also significantly lower, as was the case with photosynthetic and transpiration rates in *C. procera* growing along the roadside. The overall decrease in photosynthetic rate of *C. procera* was 34.90% and 44.79% along M-2 and G.T. road, respectively as compared to control. Reduced photosynthesis in roadside plants resulted in higher levels of water use efficiency. The higher contents of free amino acids and antioxidant activity were recorded and might be ascribed to the activation of the defense system of plants to cope with the detrimental effects of vehicle-related air pollutants. The correlation of different attributes of *C. procera* with traffic density was significant that suggested the direct impact of vehicular emissions on this species. Hence, *C. procera* can be used as a biomonitor of vehicular pollution.

Key words: Pollution, Roadside vegetation, *C. procera*, Gas exchange attributes, Biomonitor, Chlorophyll pigments.

Introduction

Rapid urbanization and industrialization accompanying with rise in vehicular traffic in the urban areas has become a great threat to quality of air, intimidating the very existence of the living organisms (Uka *et al*., 2017). The urban air pollution is one of the major ecological issues nowadays, and vehicular traffic is a significant contributor of this pollution (Patidar *et al*., 2016). Vehicular emissions in cities attribute more than 90% of air pollution (Anonymous, 2016). The major pollutants found in vehicular emissions include oxides of sulphur (SO_2), nitrogen (NO_x) and carbon (CO, CO_2), hydrocarbons (HCs), volatile organic compounds, suspended particulate matter and various heavy metals (Ibrahim, 2009; Bhandarkar, 2013). High concentrations of these pollutants in vehicular exhaust emissions having a detrimental affect on plants, humans and ecosystem (Hussain *et al*., 2013; Yaqub *et al*., 2015).

Plants along the roadsides are immediate and most important receivers of vehicular pollutants, as, they are immobile, so are constantly exposed to different pollutants from their environments (Khalid *et al*., 2018). The studies on the impact of vehicular pollutants on plants has attracted much interest. Several researches have reported a variety of plant responses to vehicular pollution, varying with the traffic. For instance, alterations in chlorophyll, relative moisture and protein contents in different plant species showed linear relation with traffic density in India (Shipra, 2017). Leaf senescence and photosynthetic activity in *Quercus ilex* were significantly altered with rise in density of traffic in Italy (Gratani *et al*., 2000). Likewise, variation in composition of plant community was observed near the roadsides (Bernhardt-Römermann *et al*., 2006). Generally, pollutants along the roadside substantially change the physiological behavior of plants (Ajoykumar & Sreya, 2018) and cause reduction in photosynthetic rate, stomatal conductance, transpiration rate and total proteins (Nawazish *et al*., 2012; Khalid *et al*., 2017). Pollution caused by vehicular emissions also influences human health accompanying with asthma, headaches, cardiovascular and lungs diseases (Kamal *et al*., 2015; Requia *et al*., 2016). Inhalation or ingestion of particulate matter present in vehicular emissions and consumption of contaminated roadside plants are the key factors which are responsible for exposure of humans to traffic pollution (Ogbonna and Ogbonna, 2011).

The effect of vehicular pollutants on vegetation was evaluated by biomonitoring, an important technique to examine the extent of pollution in the environment. Plants exhibit various types of responses to different air pollutants. They exhibit preliminary warning signs of pollution trends, hereafter, they can be employed to evaluate the quality of air. Various plants species e.g. *Nerium oleander*, *Robinia pseudoacacia* (Kaya *et al*., 2010) and *Celtis australis* (Ozturk *et al*., 2017) have been used previously for biomonitoring studies. In the current study, *C. procera* was used to study the impact of vehicular pollution because it is ubiquitously found at all the sites along G.T. road (Lahore-Gujranwala) and M-2 (Pindi Bhattian to Kala Shah Kaku), two highly busy highways in Pak-Punjab. Thus, the objective of the study was to assess the influence of vehicular pollution on physiology of *C. procera* along the roadsides.

Materials and Methods

Description of study area: Two highly busy roads [A section of Motorway (M-2) from Pindi Bhattian to Kala Shah Kaku and a section of Grand Trunk road (G.T. road) from Lahore to Gujranwala] in Punjab, Pakistan were selected to assess the effect of pollution caused by vehicular emissions (Fig. 1). Five sites were randomly selected on each of both roads. Roads varied in traffic density and type of vehicles running on them. On M-2, traffic comprises of vans, cars and buses, while, G.T. road remains busy round the clock with multi-wheeler loaders, trucks, rickshaws, buses, cars, motorcycles, and animal driven carts. Thus, vehicular emissions are likely to be much high nearby both the roads.

Calotropis procera: *C. procera* (Aiton) W.T. Aiton, a plant species in family Apocynaceae (previously placed in Asclepiadaceae), is a perennial shrub which quickly becomes established in open habitats with little competition, disturbed urban areas and along roadsides. It is native species of North Africa and Asia including Pakistan. It is a drought resistant and salt tolerant xerophytic species, which is capable to survive on a wide variety of soil types involving alkaline and saline soils however favors free-draining sandy soils. Its characteristics and widespread adaptability near the roadsides marks it an excellent tool for monitoring the impact of vehicular pollution.

Collection of leaf samples: Leaves were randomly collected from top, middle and base of *C. procera* from each selected site on both roads under study. Control plant samples were collected at a distance of 50 meter away from the roadside (Jian-Hua *et al*., 2009). All the sampled leaves were placed in labelled polythene zipper bags, stored in the cooling container and brought into the laboratory for chemical analysis.

Photosynthetic pigments: The procedure proposed by Arnon (1949) was followed to estimate the chlorophyll *a*, chlorophyll *b* and total chlorophyll while the method of Davis (1976) was used to calculate the carotenoids content. Readings were taken on spectrophotometer (IRMECO, UV-Vis, U2020).

Gas exchange parameters: Gas exchange attributes were measured for fully expanded leaves of *C. procera* by portable infra-red gas analyzer (IRGA), [Model LC pro + photosynthetic system; Analytical Development Company (ADC) Bioscientific, Hoddesdon, England]. The specifications/adjustments of IRGA were as follows: leaf surface area 11.34 cm^2, ambient CO_2 concentration (Cref) ranged from 467.52 µmol/mol, ambient temperature ranged from 22.4-27.9°C, temperature of leaf chamber (Tch) ranged from 31.5 to 37.8°C, water vapor pressure in the chamber ranged 7-11 mbar, ambient pressure 99.90 kPa, leaf chamber molar gas flow rate 313 µmol/sec, leaf chamber volume gas flow rate (v) 389 ml/min, molar air flow/unit area of leaf (Us) 404.3 $mol/m^2/sec$, PAR at leaf surface (Q leaf) was maximum up to 1694 $\mu mol/m^2$.

Free amino acids: Amino acids in plant samples were estimated through the procedure proposed by Hamilton & Van-Slyke (1943). Using leucine, a standard curve was prepared and the amino acids were estimated using the formula mentioned here under:

$$\text{Total free amino acids} = \frac{\text{Volume of sample} \times \text{Graph reading of sample} \times \text{Dilution factor}}{\text{Weight of fresh plant tissue} \times 1000}$$

Antioxidant activity: For the determination of total antioxidant activity, in a test tube, 1 g dried leaf sample was taken and 20 mL salt solution (0.45 %) was added. The mixture was heated for 20 minutes at 40°C in the water bath. After the centrifugation of the sample for 30 minutes at 3000 rpm, supernatant was separated. The collected supernatant was then stored (at -20°C) prior to start further analysis. Following Rahmat *et al*., (2004), ferric thiocyanate method was used for measuring the total antioxidant activity in plant samples.

Traffic density: The vehicular traffic data (average daily) were recorded at all the study sites along both M-2 and G.T. road for two hours at specific days (weekend, midweek). Data of traffic density were also taken from toll plazas near both selected roads.

Statistical analysis

Analysis of variance for all the attributes was calculated by COSTAT computer software package (Cohort Software, 2003, Monterey, California, USA). For all the parameters, mean values were compared by least significant difference test (LSD) at 0.05 level of significance (Liu *et al*., 2014). Correlation between traffic density and different plant attributes was computed using Pearson's correlation coefficient (two-tailed).

Results

The chlorophyll "*a*" content in *Calotropis procera* growing at different sites near both roads varied significantly ($p<0.001$). Along both roads, chlorophyll "*a*" content in *C. procera* growing near the roadsides was significantly lower as compared to control. However, along M-2, minimum chlorophyll "*a*" content (1.09 mg g^{-1}) was recorded in *C. procera* at Kala Shah Kaku site which differed non-significantly from that (1.16 mg g^{-1}) observed in *C. procera* at Sheikhupura site. Likewise, along G.T. road, *C. procera* exhibited minimum chlorophyll "*a*" content (0.98 mg g^{-1}) at Muridke site in comparison to control (Fig. 2a). Chlorophyll "*b*" concentrations were also lower significantly in *C. procera* growing along both roads as compared to control. *C. procera* at Kala Shah Kaku site along M-2 had lowest chlorophyll "*b*" content (0.54 mg g^{-1}) as compared to control, while, along G.T. road minimum chlorophyll "*b*" content (0.38 mg g^{-1}) was noted in *C. procera* at Muridke site (Fig. 2b). Similarly, total chlorophyll and carotenoids contents were also significantly lower in *C. procera* growing at all the sites along the roadsides in comparison to control plants (Fig. 2c & d).

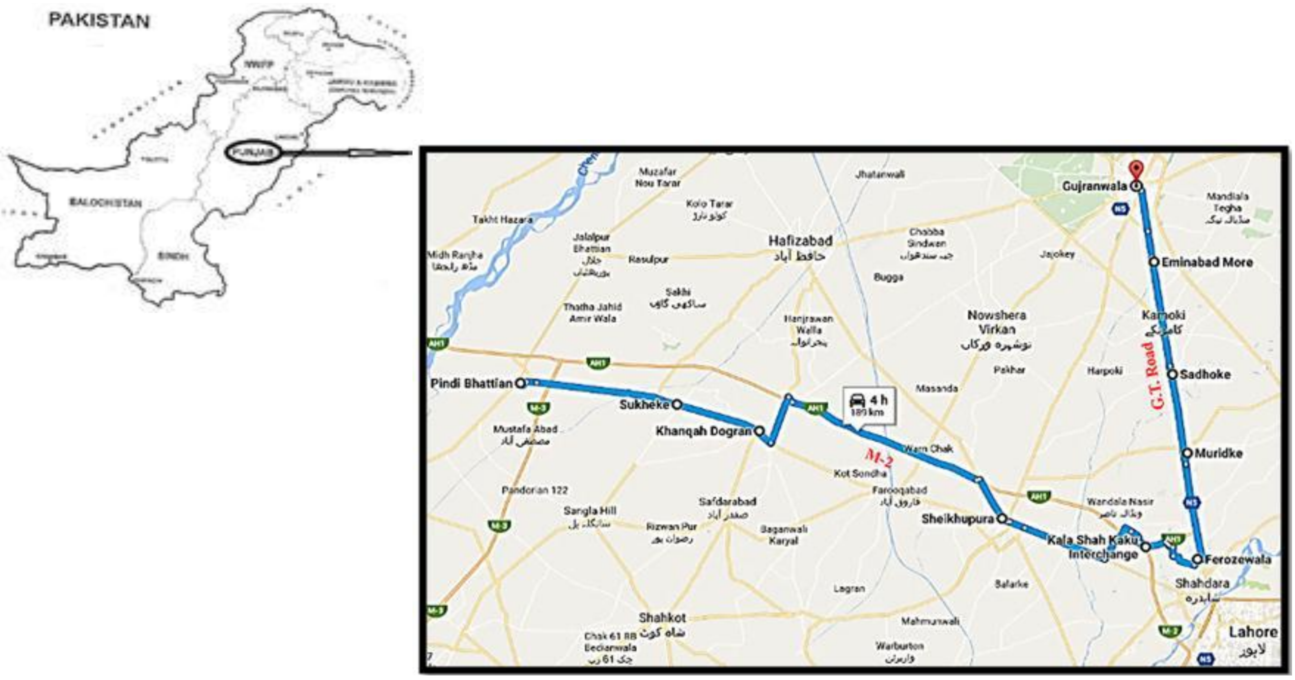

Fig. 1. Map showing study sites along G.T. road and M-2.

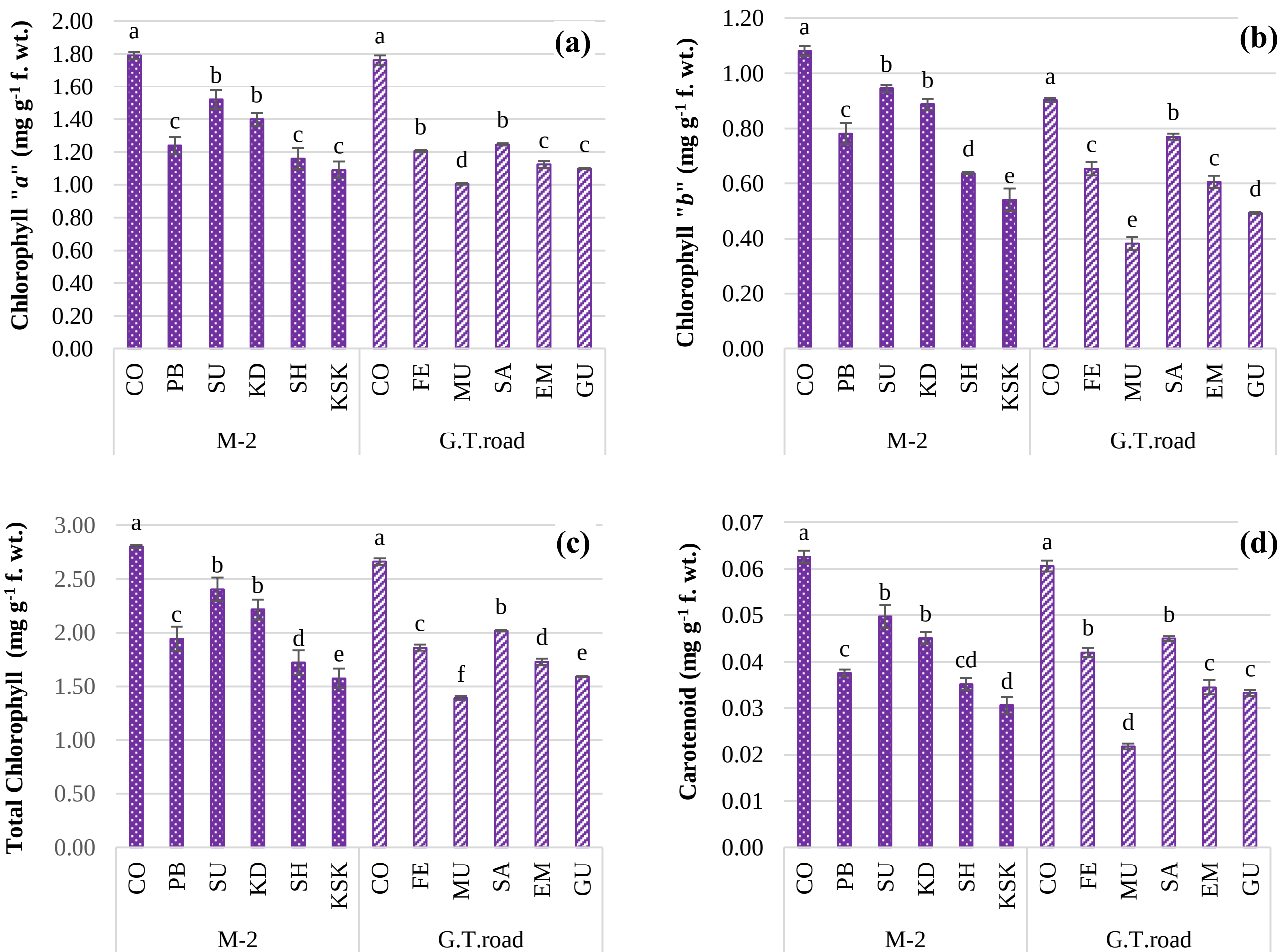

Fig. 2. The contents of photosynthetic pigments in *C. procera* growing at different sites along M-2 and G.T. road. Bars represent "mean of three values ± SE". Similar letters on bars denote non-significant differences. Different sites are abbreviated as, CO: Control, PB: Pindi Bhattian, SU: Sukheke, KD: Khanqah Dogran, SH: Sheikhupura, KSK: Kala Shah Kaku, FE: Ferozewala, MU: Muridke, SA: Sadhoke, EM: Eminabad More, GU: Gujranwala.

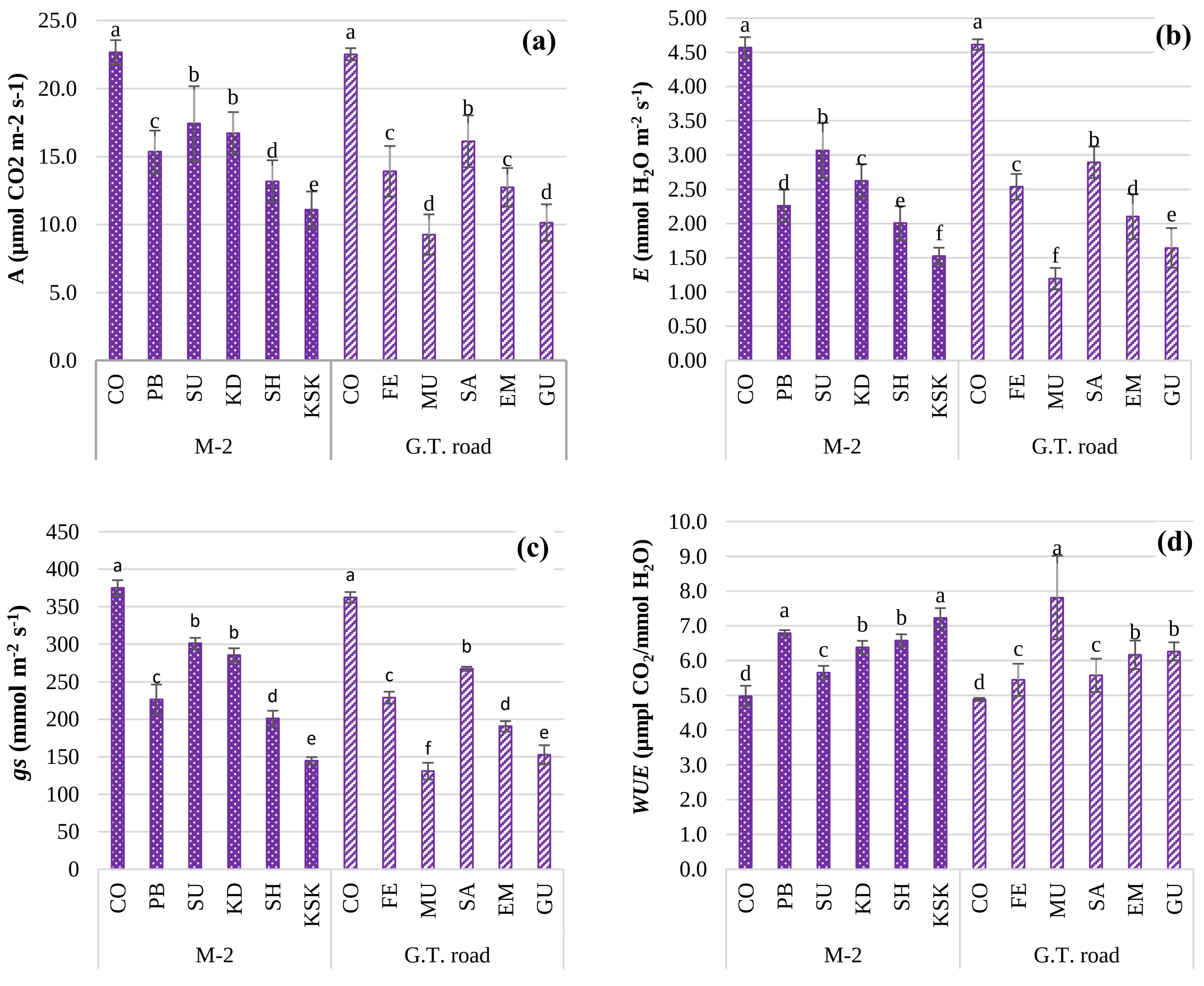

Fig. 3. The gas exchange parameters in *C. procera* growing at different sites along M-2 and G.T. road. Bars represent "mean of three values ± SE". Similar letters on bars denote non-significant differences. Different sites are abbreviated as, CO: Control, PB: Pindi Bhattian, SU: Sukheke, KD: Khanqah Dogran, SH: Sheikhupura, KSK: Kala Shah Kaku, FE: Ferozewala, MU: Muridke, SA: Sadhoke, EM: Eminabad More, GU: Gujranwala

Highly significant (p<0.001) variations were noted in photosynthetic rate of *C. procera* growing at different sites adjacent to both roads. *C. procera* growing at all the sites along the roadsides exhibited significantly lower photosynthetic rate as compared to control. However, along M-2, *C. procera* at Kala Shah Kaku site showed minimum photosynthetic rate (11.11 µmol CO_2 m^{-2} s^{-1}), whereas along G.T. road, least photosynthetic rate (9.25 µmol CO_2 m^{-2} s^{-1}) was noted in *C. procera* at Muridke site which differed non-significantly from that (10.12 µmol CO_2 m^{-2} s^{-1}) in *C. procera* at Gujranwala site (Fig. 3a.). Similarly, transpiration rate and stomatal conductance were also significantly lower in *C. procera* at all the sites close to both roads in comparison to control (Fig. 3b & c). Highly significant (p<0.001) differences in water use efficiency of *C. procera* growing at various sites were also noted along both roads. Water use efficiency was significantly higher in *C. procera* growing at all the sites along the roadsides as compared to control. However, *C. procera* exhibited maximum water use efficiency i.e. 7.8 µmol CO_2 $mmol^{-1}$ H_2O at Muridke site along G.T. road (Fig. 3d).

C. procera growing at different sites adjacent to both roads exhibited highly significant (p<0.001) variations in contents of free amino acids. Significantly higher contents of free amino acids were recorded in *C. procera* along the roadsides as compared to control. However, *C. procera at* different sites along G.T. road had higher concentrations of free amino acids in comparison to M-2 (Fig. 4a). Total antioxidant activity also varied highly significantly (p<0.001) in *C. procera* along both M-2 and G.T. road, and it was higher at all the sites along the roadsides as compared to control (Fig. 4b).

Strong negative correlation (r= 0.959) was observed between photosynthetic pigments in *C. procera* and traffic density along both M-2 and G.T. road. Likewise, among physiological parameters, photosynthetic rate, stomatal conductance and transpiration rate also showed negative correlation with traffic density along both roads. However, a significant positive correlation was found for water use efficiency, free amino acids and total antioxidant activity in *C. procera* with traffic density on both M-2 and G.T. road (Table 1).

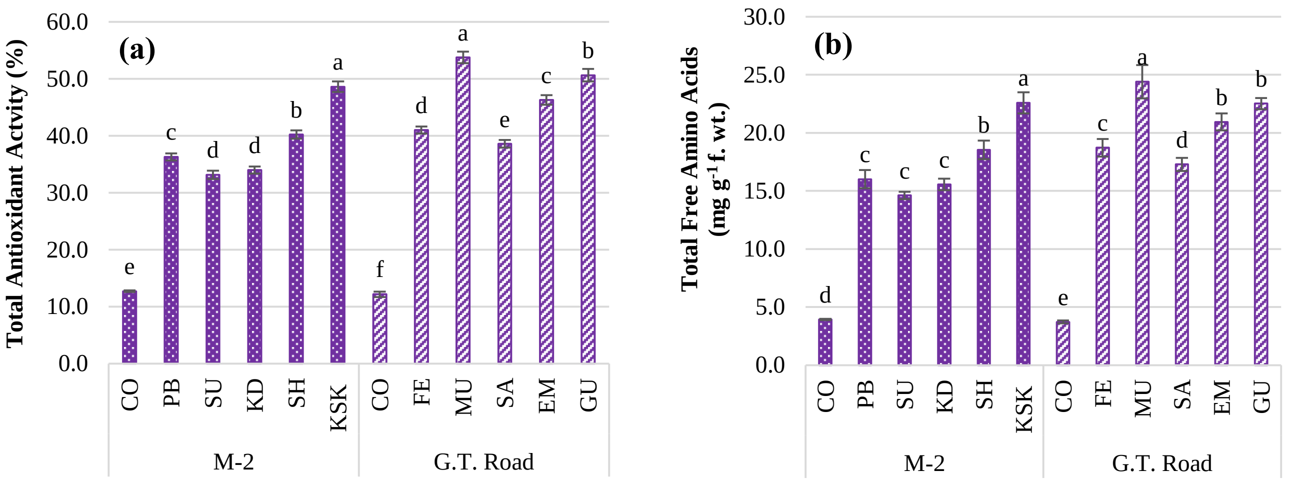

Fig. 4. Total antioxidant activity and free amino acids in *Calotropis procera* growing at different sites along M-2 and G.T. road. Bars represent "mean of three values ± SE". Similar letters on bars denote non-significant differences. Different sites are abbreviated as, CO: Control, PB: Pindi Bhattian, SU: Sukheke, KD: Khanqah Dogran, SH: Sheikhupura, KSK: Kala Shah Kaku, FE: Ferozewala, MU: Muridke, SA: Sadhoke, EM: Eminabad More, GU: Gujranwala.

Table 1. Pearson's correlation coefficient of traffic density with different plant attributes.

Traffic density	Photosynthetic pigments			
	Chlorophyll "*a*"	**Chlorophyll "*b*"**	**Total Chlorophyll**	**Carotenoids**
M-2	-0.991***	-0.986***	-0.995***	-0.996***
G.T. road	-0.948***	-0.957***	-0.959***	-0.943***
	Gas exchange parameters			
	Photosynthetic rate	**Transpiration rate**	**Stomatal conductance**	**Water use efficiency**
M-2	-0.973***	-0.988***	-0.994***	0.908**
G.T. road	-0.983***	-0.978***	-0.990***	0.814*
	Total free amino acids		Total antioxidant activity	
M-2	0.910**		0.921**	
G.T. road	0.981***		0.991***	

***, ** and * = Significant at 0.01, 0.05 and 0.1 levels, respectively

Discussion

Plants respond to environmental adversities, so can be used as indicators for assessing the environment quality. In this regard, the biochemical parameters such as contents of photosynthetic pigments are used as reliable indicators of the pollutants toxicity in plants (Gomes *et al*., 2014). Vehicular pollution caused deleterious effects on *C. procera* in this study. The concentrations of chlorophyll *a*, *b*, total chlorophyll and carotenoids were significantly lower in *C. procera* growing at all the sites close to both the roads as compared to control. Several workers have also reported a decrease in chlorophyll pigments of *Shorea robusta, Eucalyptus citrodora*, *Tectona grandis, Mangifera indica* (Joshi & Sawami, 2007), *Pongamia pinnata* (Bamniya *et al*., 2012) and *Artemisia maritima* (Laghari *et al*., 2015) under the effect of environmental pollutants. The chlorophyll and carotenoids are involved in mechanism of photosynthesis in plants, but toxic pollutants such as heavy metals present in the vehicular emissions significantly decrease their concentrations, thus disturbing photosynthesis (Chauhan & Joshi, 2008). Decline in chlorophyll pigments is observed as a common indication of toxicity of metals in various plants (Chauhan, 2010; Zouari *et al*., 2016). Among different sites along G.T. road, the highest reduction in photosynthetic pigments was found at Muridke site, recognized as the most polluted site. However, along M-2, the maximum decrease in chlorophyll pigments was observed at Kala Shah Kaku site, regarded as the most polluted site along this road. The metals and soot particles get deposit on the surface of leaves and block the stomata which causes decline in photosynthetic activity and chlorophyll content (Prajapati & Tripathi, 2008). The heavy metals in vehicular emissions could have disrupted the chlorophyll biosynthesis by the inhibition of enzymes i.e. δ-aminolevulinic acid dehydratase and protochlorophyllide reductase which are crucial for the synthesis of chlorophyll (Pourraut *et al.,* 2011; Rai, 2016). Decrease in carotenoid contents might have severe consequences because carotenoids function as non-enzymatic antioxidants for the free radicals and subsequent photochemical destruction (Strzalka *et al*., 2003; Sengar *et al*., 2008). Carotenoids quench the reactive oxygen species, triplet chlorophyll and several other excited molecules in pigment bed, which can disrupt metabolism by causing oxidative injury to cellular constituents

(Candan & Tarhan, 2003). However under stress, protective function of carotenoids transforms, leading to the degradation of pigments and cellular damage (Sharma & Tripathi, 2009). In a former study, Panda *et al*., (2015) reported that environmental pollution caused by soot could decrease the contents of photosynthetic pigments in plants. In another study, Iqbal *et al*., (2015) reported that pollutants released from vehicular activities reduced the amount of chlorophyll a, b and total chlorophyll in roadside plants in Karachi, Pakistan.

Regarding the physiological attributes of plants, gas exchange parameters are of great importance (Ashraf, 2009). In current study, stomatal conductance, photosynthetic rate and transpiration rate were lower, while, the water use efficiency was higher in *C. procera* growing at different sites close to the road as compared to control. Similar findings to our results have also been reported by Khalid *et al*., (2017). Bao *et al*., (2015) observed decrease in gas exchange attributes resulting a 16% decline in rate of photosynthesis in *Sophora japonica* under the effect of roadside pollutants. Likewise, Hassan *et al*., (2013) also noted alteration in gas exchange parameters of lettuce plants growing in some urban areas of Saudi Arabia, which was correlated significantly with metal pollutants. Decrease in photosynthetic rate. stomatal conductance, transpiration rate and photosynthetic pigments was also noted in *Alstonia scholaris* growing nearby some extremely busy roads in Lahore city of Pakistan (Muhammad *et al*., 2014). The particulate pollutants present in the vehicular emissions could have inhibited the conductance of stomata. Moreover, closure of stomata leads to decline in photosynthesis. Decrease in the conductance of stomata and photosynthetic rate in certain tree species growing along the roadsides was also reported by Chaturvedi *et al*., (2013). In another study, environmental pollutants decreased the stomatal conductance which lead to decline in the rate of photosynthesis and increase in intercellular CO_2 concentration in the leaves of *Azadirachta indica* (Qadir *et al*., 2016). The lower transpiration rate and higher water use efficiency of *C. procera* in current study could be due to the storage of water by plants when environmental contaminants and the resulting stresses restrict the ability of water uptake in plants via roots (Veselov *et al*., 2003). The rise in water use efficiency was found in plants under metal stress, because plants close their stomata to minimize the rate of transpiration (Greger & Johansson, 2006) under stressed conditions. The decrease in gas exchange attributes of plants growing near the roadsides occurs due to the deposition of Pb and Cd which could be involved in clogging stomata aperture leading to decreased rate of photosynthesis (Nawazish *et al*., 2012).

Amino acids are the precursors to and constituents of proteins, and serve a substantial role in stress metabolism of plants. Higher levels of amino acids and antioxidant activity were recorded in *C. procera* growing close to both M-2 and G.T. road in comparison to control. The results of this study are in agreement with several former researches, where rise in free amino acids and activity of antioxidants are reported under the impact of various environmental pollutants (Almohisen, 2014; Shackira *et al*., 2017). Plants under heavy metals stress accumulate various metabolites like betaine, proline, nicotianamine, antioxidants, polyamines and several other free amino acids, which help in reducing the stress by binding to the metallic particles. The accumulation of these metabolites occurs as a result of the activation of plant's defense system in response to metal stress (Sharma & Dietz, 2006). The increase in the level of free amino acids in current study might be attributed to the increase in level of heavy metal pollutants in the environment along the roadsides, because free amino acids in plants increase due to toxicity of metals and protect the plant (Clemens, 2001). Antioxidants are normally generated in plants, however under ecological abiotic stresses, their activities tend to rise, thus enable the plants to live in the stress conditions (Eraslan *et al*., 2016). Excessive accumulation of metals induces the generation of reactive oxygen species (ROS) that cause oxidative stress in plants (Kaur *et al*., 2015). To ease the detrimental effects of ROS under stress conditions, plants have developed antioxidant defense systems (Zouari *et al*., 2016; Akhtar *et al*., 2017) which restrains and reduces the oxidative damage, and enhances the stress resistance in plants (Suzuki *et al*., 2012). Moreover, sulphur dioxide (SO_2), a highly detrimental pollutant in the vehicular emissions also causes the generation of ROS and rise in the activity of antioxidants (Li & Yi, 2012). The robust and efficient response of antioxidants helps to evaluate the capability of plants to tolerate the environmental stress (Sidhu *et al*., 2016). The production and activity of antioxidants in plants under stressful conditions depends not only on the level of the stress, but also on the plant species, the duration of stress exposure, the extent of plant tolerance and the prevailing ecological conditions (Ahmed *et al*., 2010). Mohasseli *et al*., (2016) also noticed an elevated antioxidant activity of maple leaves due to heavy metal contamination along the heavily trafficked roads. Patidar *et al*., (2016) reported that traffic pollution caused an oxidative stress in plants growing along the roadside. Therefore, high antioxidant activity in roadside *C. procera* was a defensive response to stress injuries. This is in agreement with findings of Al-Hassan *et al*., (2017).

The significant negative correlation of chlorophyll pigments, stomatal conductance, photosynthetic rate and transpiration rate with traffic density along both M-2 and G.T. road showed that increase in damaging effects on *C. procera* was due to rise in vehicular traffic and environmental pollution. Likewise, the linear relation of level of amino acids and antioxidant activity with traffic density on both roads further supports the findings.

Conclusion

This study revealed a range of responses by *C. procera* to vehicular pollution at different sites along the two roads. The results showed that the alterations in physiology of *C. procera* was due to roadside pollution. The extent of effects caused by pollution along the two roads on *C. procera* though, varied according to road level. However, *C. procera* exhibited a decrease in all the studied photosynthetic pigments, stomatal conductance, rate of photosynthesis and transpiration with an elevated level of water use efficiency, amino acids as well as the activity of antioxidants at several different locations close to the selected roads. The plant's physiological parameters studied during the current investigation clearly revealed that they might be used as biomarkers for estimating and monitoring the preliminary effects of roadside pollution.

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