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Physiological and Morphological Responses to Ozone Exposure of Coleus (*Solenostemon scutellarioides* (L.) Codd)

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***Abstract*— The tropospheric ozone has been projected to increase, and it could harm humans, animals, and vegetation. This study investigates morphological changes in coleus leaves, to assess the change of pigment composition (chlorophyll, carotenoid, and anthocyanin), and to understand color dynamics due to ozone fumigation. Coleus Kong Green (fully green/FG), Coleus Kong Scarlet (green and purple/GP), Coleus Wizard Pastel (yellow and purple/YP), and Coleus Wizard Scarlet (reddish/RD) selected. The materials in this study tested for three different ozone concentrations which were CF (≤ 10 ppb ozone), CF+40 ppb ozone, and CF+150 ppb ozone for 8 hours/day for 30 days. Chlorosis in FG and curling leaf in RD were observed as the symptoms while other cultivars showed different appearances such as expanded purple area (GP and YP) and curled margin (RD). Chlorophyll and carotenoid content significantly decreased in all cultivars while anthocyanin was found increasing except in RD. The color change tended to redder and brighter in all cultivars except RD which was a stable and purple area in GP which was darker. Therefore, different coleus cultivars show different responses and it can be used as an ozone-plant model to investigate pigment composition under ozone exposure.**

***Keywords*— ozone exposure; physiology; morphology; coleus**

I. Introduction

Ozone (O3), an essential component of some areas of the stratosphere customarily recognized as the ozone layer is a troposphere part. The troposphere increased from the surface of Earth to between 12 and 20 kilometers over the level of sea consisting of numerous layers. Ozone is extra resolute more than the combined layer, or layer of earth. The ground level of ozone is more problematical since its health effects nonetheless with a reduction of powerful than ozone in the air. The reactions of photochemical and chemical concerning it make several of the chemical processes arise in the atmosphere either by day or by night. By peculiarly far above the ground concentrations by human activities principally deficient incineration of fossil fuels constitutes an impurity along with a part of haze. Ozone is a dominant oxidizing driving force enthusiastically responding through another chemical composite to construct various probably toxic oxides. Tropospheric ozone constitutes a greenhouse gas and commences the methane chemical elimination and other hydrocarbons on or after the atmosphere. Consequently, its concentration changes the length of the compounds stays in the air.

An increase of tropospheric ozone in ambient air reported in many regions in the tropical regions of the world. This increase may be caused by many different factors [1], [2] and will increase over the next hundred years [3]. Many researchers have produced studies to understand how plants respond to this environmental stress. These studies show a wide range of harmful effects from ozone [4]. To study about ozone in the individual level of the plant is very important due to the effect of ozone that caused reduction of total productivity of agriculture products [5]. Furthermore, it can affect food security by reducing food availability [6].

It has been found that antioxidants may have an essential role in the plant responses to ozone [7]. Pigments are thus among the more significant antioxidants [8] present in the plants' leaves [9]. Changes in the content and composition of pigments such as chlorophyll, carotenoid, and anthocyanin as also affect the visible color of the leaves [10]. Apart from the primary function of coloration in plants [11], photoprotective pigments [12], such as anthocyanin was found to be increasing because of H2O2, one of the oxidants in plant cells, in Pseudowintera colorata [13]. Anthocyanin is always found to increase in line with the appearance of oxidants [14]. The role of anthocyanin in leaf tissue is not fully known, though previous research suggests that it may be one of the antioxidants in some plants.

In contrast, carotenoid and chlorophyll have been examined more intensively regarding the ozone effect in physiological parameters in leaves. Several results indicated decreases of these pigments in leaves while some also found increases along with ozone exposure. A decrease in the state of both pigments found in Citrus Clementina [15], Oryza sativa L. [16], Trifolium repens [17], Tagetes erecta Linn. Moreover, Petunia hybrid Vilm. [18] whereas a stable state also found in Ailanthus altissima, Fraxinus Chinensis, Platanus orientalis and Robinia pseudoacacia [19] and an increased state of carotenoid in Fagus sylvatica [20].

Most of the studies have been conducted to investigate more about the ozone effect on plant crops and ozone-sensitive plants. Nonetheless, rich-anthocyanin content plants are not among the well-known plant in term of ozone stress response. It is interesting to understand how these highly pigmented plants respond to ozone regarding pigment content. While some plants produce more pigments such as anthocyanin and carotenoid, would this kind of plant respond to the same technique although the plants already have a high pigment content?

Coleus (Solenostemon scutellarioides (L.) Codd) as an ornamental plant that has been cultivated worldwide [21]. Some cultivars of this species have been tested to assess their response to environmental changes. The coleus is sensitive to environmental changes and may exhibit changes in leaf structure and the physiological condition of their leaves. In some studies, this plant was observed to respond to changes in the environment by changing the composition of chemicals in their leaves as an indication of metabolism change inside the tissue [22]. Some researchers have observed that environmental changes have resulted in changes in the color of the leaves [23], [24]. The changes in leaf color may be due to the plant producing more anthocyanin [25]. Notable increase and decrease regarding anthocyanin and carotenoid after environmental stress in which concentration levels of anthocyanins and carotenoids change in an opposite pattern, which points toward the response of this plant under environmental stress is improved to defend the plants against changes that induce damages [26].

Research into the changes in the physiological and biochemical state of ozone-affected plants is crucial and needs to be conducted on a broader range of plants than before [27]. Research needs to be done on the role of anthocyanin and how it responds to ozone [11]. There are fewer data about the relation between pigment composition and responses to ozone exposure, particularly regarding agricultural plants. This shortage of data is even more significant in ornamental plants. In this study, the research aims were to investigate and to distinguish the effect of ozone on visible appearance of Coleus, to assess the change of pigment composition (chlorophyll, carotenoid, and anthocyanin) in Coleus under ozone fumigation, and to understand the effect of ozone on leaf color.

II. Material and Method

A. Plant Material

The experimental plants consisted of four cultivars of Coleus (Solenostemon scutellarioides (L.) Codd) which are Coleus Kong Green (fully green/FG), Coleus Kong Scarlet (green and purple/GP), Coleus Wizard Pastel (yellow and purple/YP), and Coleus Wizard Scarlet (reddish/RD). Seeds were obtained from A.F.M. Flower Seed Co., Ltd. Company and had been germinated and grown for 4 months in 192 pots filled with compost soil called Terrahum sourced from Klassman-Deilmann Corp (pH 5.5-6.5, NPK 14:16:18 at 1.5 kg/m^3) in the greenhouse. Plants were well-watered regularly to avoid drought stress. Before fumigation, plants were transported to allow adaptation for a week in the chambers.

B. Ozone Exposure

The experiment conducted in six ozone-fumigation chambers that were ventilated with charcoal-filters (CF) to sustain the ozone background at less than 10 parts per billion (ppb). The chambers divided into three treatments. Two chambers had charcoal-filtered air (CF) only without additional ozone, two chambers contained charcoal-filtered air with new 40 ppb ozone concentration (CF+40 ppb), and two chambers with additional 150 ppb ozone concentration (CF+150 ppb). For CF+40 ppb ozone and CF+150 ppb ozone chambers, additional ozone generated by ozone generator Oz model 3020 from Ebase corp. Ltd. through Teflon pipes into the chambers. Before fumigation occurs, each chamber filled with air at the required ozone concentration, which monitored by ozone monitors Model 1008-PC from Dasibi Environmental Corp. and the concentration maintained through a 30-day period of fumigation (Fig. 1). The temperature inside the chambers maintained between 28-32˚C (day) and 25-28˚C (night), and it recorded by Testo 608-H1-Thermohygrometer (Testo Limited, UK). Two metal halide bulbs per chamber were used to provide light inside the chamber in which photosynthesis photon flux density (PPFD) was 700 µ mol s-1 m-2. Plants were fumigated for 4 weeks, 8 h/d, from 10.00 to 18.00, 7 days/week.

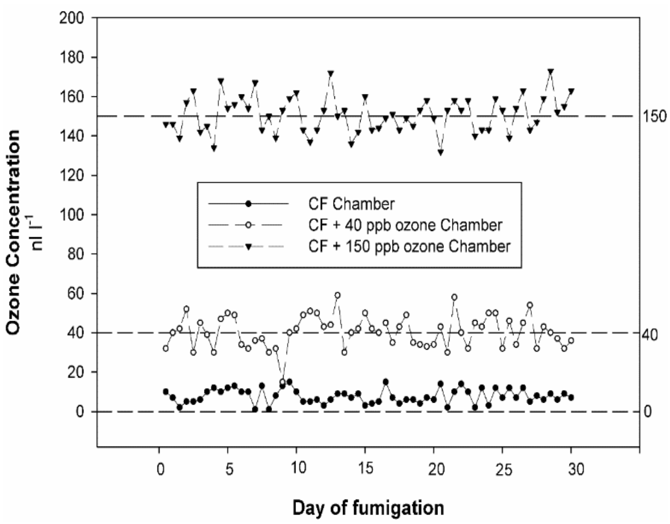

Fig. 1 The ozone concentration in six fumigation chambers over the 30-days of the experiment. Fumigation was done over an eight-hour period, starting at 8 o'clock in the morning. The monitoring was done twice at 8-hour intervals.

C. Morphological Assessment

The visible appearance of the leaves observed daily and symptoms recorded since the 10th day of fumigation when the first symptoms appeared. After the 30-day fumigation, the youngest expanded leaves in each plant per cultivar was selected to determine their color. Leaf color assessed with a colorimeter MiniScan XE Plus Model No. 45/0-S Hunter

Associates Laboratory, Inc. to examine the parameter (negatively refers to green, positively refers to red) as a and L main indicator apart from b.

D. Physiological Measurements

Chlorophyll and Carotenoid were extracted using a method described in the reference [28]. A sample of fresh leaves ground to a powder. The sample gradually dissolved in 250 ml Acetone/Tris buffer (80:20, vol: vol), centrifuged to remove the particulate and later add solvent to obtain the supernatant and it was read using Optizen 3220UV Spectrophotometer at 470, 537, 647 and 663 nm wavelength. The absorbance values thus calculated using equation from the same reference.

Total monomeric anthocyanin was calculated using a method from the reference [29]. One mg fresh leaf was ground with 5 ml pH 1.0 buffer and filtered into the volumetric flask 25 ml. Another 1 mg leaf was ground into 5 ml buffer pH 4.5 and put into another volumetric flask. Both were fulfilled with each similar until 25 ml volume reached. These solutions then were measured using a spectrophotometer at 510 and 700 nm. Absorbance then was calculated using an equation from the same source using cyanidin-3-glucoside molar extinction coefficient at 26900.

E. Data Analysis

Data were processed using SPSS program version 17.0. For the effect of ozone on each treatment, analyses of variance (ANOVA) performed on untransformed data for each variety with statistical significant $P < 0.05$. Correlation of each treatment and parameters was also measured using Duncan's Multiple Range Test (DMRT).

III. Results and Discussion

After 10-days fumigation, visible symptoms on leaves such as chlorosis observed in FG (Fig. 2), but there was no symptom occurring in other cultivars except several visible appearances such as a full purple area in GP and YP. In RD, the curl margin leaf symptom has been found and identified uniformly in CF + 150 ppb ozone chambers (Fig. 3). There was a trend of decreasing light (L) and increase redness (a) (Fig. 4). It is interesting to note that each cultivar that has green areas was paler in CF + 150 ppb ozone compared with CF and CF + 40 ppb ozone (Fig. 4). Overall, visible symptoms were found only in FG and RD, and there was no significant difference between CF and CF + 40 pp ozone in term of an obvious observation.

Pigment contents in four cultivars showed different trends after 30-days fumigation (Fig. 5). The anthocyanin content was found to be different in FG, GP, and YP, but tended to be stable in RD. FG showed significant differences in the three treatments, over the 30 days of the experiment. GP and YP showed only a slight increase towards the end of the experiment period. It is in line with the increase of units in colorimeter analysis where FG, GP, and YP were redder (displayed a more positive a unit) than CF or CF+150 ppb Ozone while in both a parameter and anthocyanin content RD showed no change. Furthermore, the light (L) of each cultivar and part of the leaves were differently changed (Fig. 4). The green area of YP and all of FG were getting darker, but GP showed a brighter leaf at CF+150 ppb ozone.

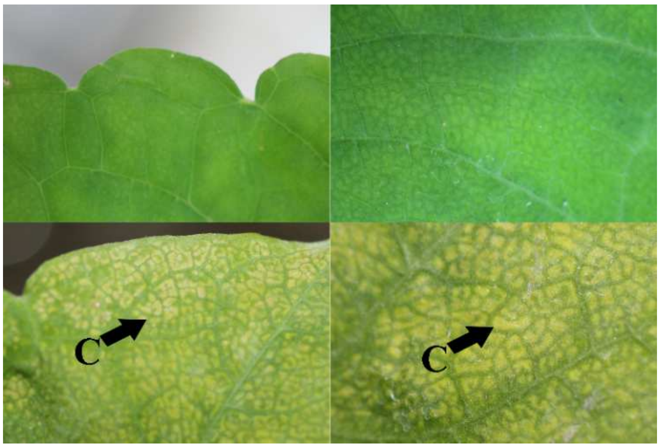

Fig. 2 Leaf surface of Coleus Kong Green cultivar (FG) in (left) margin and (right) middle of leaves under CF (upper) and CF+150 ppb ozone (lower) after 10-days fumigation. c: interveinal chlorosis.

In carotenoid analysis (Fig. 5), it found that there was a similar response in FG, RD, and GP in which they showed a notable decrease of carotenoid in CF+150 ppb ozone, and there was no significant difference between CF and CF+40 ppb ozone. YP did not demonstrate a significant difference between CF+40 ppb and CF+150 ppb ozone exposure, but there was a significant decrease from CF to CF+40 ppb ozone. Chlorophyll content in YP gradually decreased from CF, CF+40 ppb Ozone, and CF+150 ppb ozone. Nevertheless, other cultivars showed a significant decrease of chlorophyll only at CF+150 ppb ozone.

Generally, RD is stable in anthocyanin content after ozone exposure. It followed by GP and YP that only showed responses at CF+150 ppb ozone. FG showed an increase regarding anthocyanin content at CF+40 ppb ozone while YP showed a decrease regarding the carotenoid content at CF+40 ppb ozone. RD also shows a stable redness and brightness in all ozone concentration while other cultivars show a similar pattern of fading and redder colors.

After observation of all the responses under ozone exposure, and deliberating the analyzed data, it can point out that all of the cultivars responded to ozone exposure differently regarding morphological appearance and pigments contents. Some cultivars show an increase of environmental stress while the others slightly change in each parameter that tested. Morphologically, some cultivars showed a change in color, and regarding physiological changes and dynamic, some cultivars showed a variation of pigments compositions. Overall, FG is the most susceptible due to the change of physiological and morphological states while others reveal vary change in several morphological and physiological parameters.

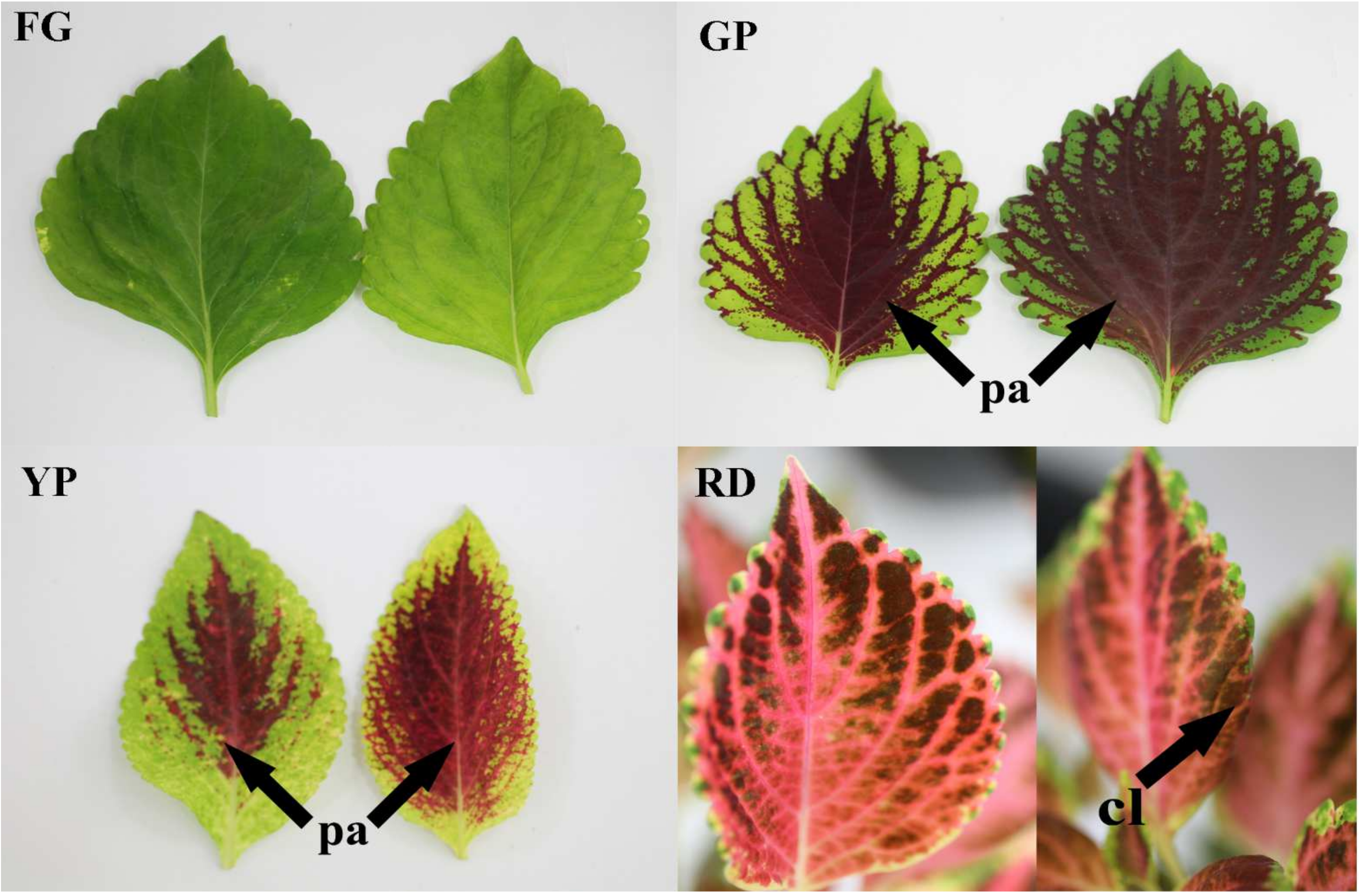

Fig. 3 Comparison between leaves from CF Chambers (left) and CF+150 ppb Ozone chambers (right) in Coleus Kong Green (FG), Coleus Kong Scarlet (GP), Coleus Wizard Pastel (YP), and Coleus Wizard Scarlet (RD) of Coleus after 15-days Fumigation. pa: purple area

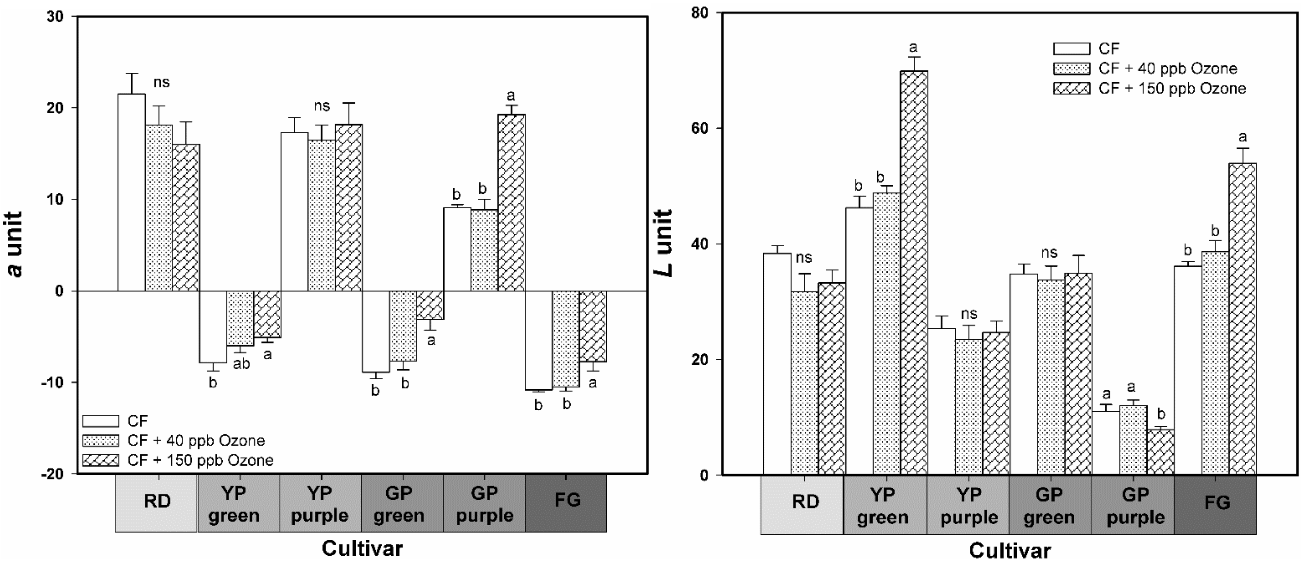

Fig. 4 Colorimeter analysis (*a* and *L* parameter) of Coleus Kong Green (FG), Coleus Kong Scarlet (GP), Coleus Wizard Pastel (YP), and Coleus Wizard Scarlet (RD). Green and purple address the green and purple area in the leaves. Different letters indicate significant differences among treatments (column) using Duncan's multiple range test (DMRT) with α=0.05 and n=10.

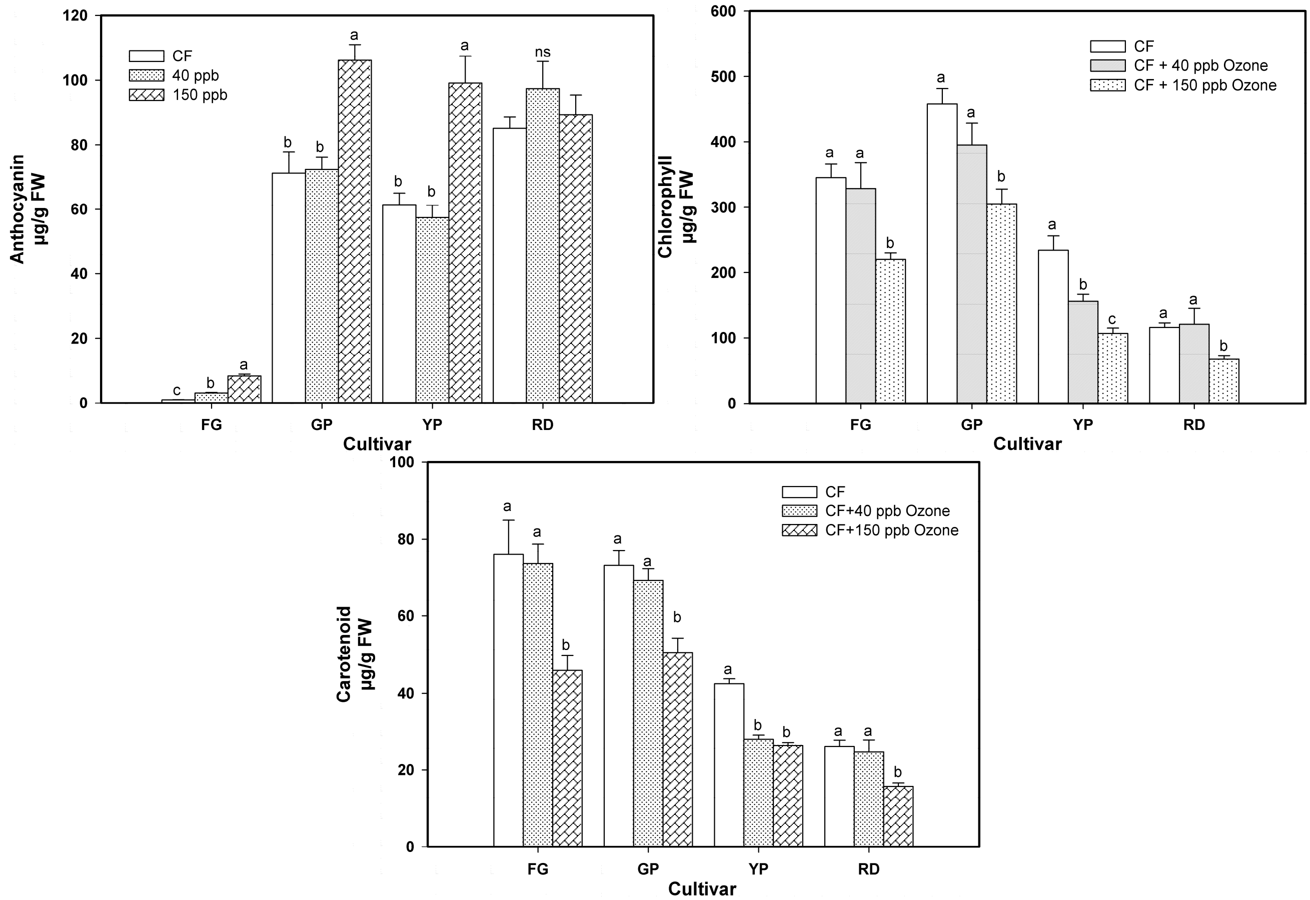

Fig. 5 Pigment content (mg/g FW) of Four different cultivars of *Coleus* (Coleus Kong Green (FG), Coleus Kong Scarlet (GP), Coleus Wizard Pastel (YP), and Coleus Wizard Scarlet (RD)) after 30-days ozone fumigation. Different letters indicate significant differences among treatments (column) using Duncan's multiple range test (DMRT) with α=0.05 and n=10.

Ozone studied for decades, and thus the effects of this air pollutant have been recorded in many species [30]. Among the results, there are many new changes due to ozone exposure, and it can be due to the specialization of each species. In many cropping and natural vegetation plants, to study varieties within the species is a standard way to generate susceptible and resistant varieties [31]. It is vital to obtain the resistant varieties to cultivate more yield, and therefore the agriculture sector can alter or stabilize production under ambient ozone that increases day by day [27]. Nonetheless, apart from cropping plants study and findings of different responses to ozone exposure among cultivars still in a low number.

Morphological and anatomical responses can be simple indicators to understand the response of plants. These two parameters examined for many years to assess plants resistance and susceptibility to ozone, In the same way, these parameters are also in effect on the ornamental plant under assessment of ozone exposure [32].

A full purple area in YP and FG caused by coloration of anthocyanin, carotenoid, and betalains [33], wherein previous research it found that that area addressed an increase in anthocyanin and decrease of chlorophyll [24]. Thus, the width and strength of the color are the units that determined by the pigments composition and therefore it is essential to a visual assessment of pigment content and proposed as a prior observation. The wider of this area is due to the increase of several pigments, and it can be included to be a response to ozone exposure.

Leaf curling is one of the effects of ozone [34, 35]. It purposed that ethylene produced because of an increase of arginine carboxylation and plant metabolism responds the increase by producing a high amount of ethylene [36]. Ethylene independently can be one of the indicators that plants have obtained stress from the environment. Moreover, in Arabidopsis thaliana, there is a significantly high amount of mRNA in the leaf tissues that contain code several ethylene-inducible genes after exposure to ozone [37]. An increase of ethylene as a cause of curling leaf in plants during ozone exposure is a sign of susceptibility of plants to ozone exposure [34].

Among the cultivars tested, FG and RD showed an apparent common symptom regarding their morphological response to ozone exposure. Chlorosis has been showed in FG to be a common symptom in many plants in response to ozone exposure [38], [39]. Chlorosis is among the initial symptoms of ozone exposure in many plant species [40]. Although chlorophyll that concentrated is essential to encounter free radicals as mentioned in [41], this situation of chlorophyll decreasing might be caused by either a pigmentation or a chlorophyll break down [42]. These parameters are physiological and produce visible symptoms that are related each other. Since the decrease of chlorophyll content is a physiological phenomenon as the color of FG gets paler because of the green color of the leaves is made up of chlorophyll.

A chlorophyll decrease due to ozone exposure is by the previous result of ozone fumigation in an acute state of exposure in same species [43]. An abundant state of reactive oxygen species and direct ozone reaction can cause the damage in biomacromolecules as shown in diminished chlorophyll concentration in leaf [44]. The reduction of chlorophyll after ozone exposure in several cultivars can also mean that there is an increase of antioxidant activity in which the system of antioxidant is expanding [45]. Furthermore, the different responses in several cultivars indicate that there are a variation biochemical traits among the cultivars [46]. Thus, in wheat (Triticum aestivum L.) has several cultivars that decrease chlorophyll content which called susceptible cultivars and several cultivars maintain the chlorophyll content in a stable state which known as resistant cultivars [47].

As another photosynthesis pigment, carotenoid has been decreasing as well. It is similar to the result of Populus deltoids that fumigated with ozone. The plant showed a decrease in both chlorophyll and carotenoid [48, 49]. This is because carotenoid is one of the antioxidants that has been used in plant tissue to scavenge the reactive oxygen species [50]. Nevertheless, carotenoid content in CF+40 ppb ozone and CF+150 ppb ozone is relatively similar in YP. Similar results found by Madkour and Laurence [51] who showed that ozone at low-level fumigation could cause a more significant loss of carotenoid. It indicates that this pigment is more responsible for neutralizing ozone. As a pigment, carotenoid becomes a chlorophyll protection agent [52].

The redness of the leaves tends to increase while the leaf color becomes lighter (fade) in several cultivars. Even though the pattern of fading is unclear yet similar fading results observed in other studies [53]. A similar result that related to ozone stress found in Rubus fruticosus which showed a reddening color that visibly appears as a part of ozone symptoms [54]. These trends can be related to the increase of anthocyanin content and a decrease in chlorophyll [24]. Higher a is related to high anthocyanin accumulation but lower chlorophyll content due to the excess light, e.g., sunlight in Coleus. In most of the case, a positive relation between leaf coloration and seasonal color of these plants has been found to be an impact of an increase of anthocyanin and a decrease of chlorophyll. The impact is the relation of color and content of anthocyanin and chlorophyll proven in several red-leaf kinds of grass (Imperata cylindrica, Panicum virgatum, Pennisetum advena, Pennisetum purpureum, and Schizachyrium scoparium) [55].

An increase of anthocyanin in FG has been measured in CF+40 ppb ozone and continued to increase in CF+150 ppb ozone. It addressed a sensitivity of FG cultivar to ozone exposure compared with other cultivars. Nevertheless, RD did not change at all after fumigation of CF+40 ppb ozone and CF+150 ppb ozone. It is related to the concentration of anthocyanin in this cultivar in which it was already high even in the normal condition (CF). Usually, an increase of anthocyanin is one of the responses of plants to counteract ozone exposure. Kovinich, Kayanja [56] found that Arabidopsis makes responses to abiotic stress by increasing total level of anthocyanin as well as anthocyanin profile accumulation. It is well-understood that ozone-free air makes less anthocyanin content in leaves compared with higher-ozone air [57]. Anthocyanin is an antioxidant that can scavenge free-radicals in leaves before they damage membranes in cells and the appearance of oxidative stress such as ozone, which associated with anthocyanin accumulation [9, 14]. Anthocyanin has been recognized as one of the stronger antioxidants by direct interaction as well as by being signaling chemical for balancing reactive oxygen species [58].

According to the change of pigment and visible symptoms, FG is the most sensitive cultivars to ozone exposure followed by RD, YP, and GP. The most resistant cultivar among these four experimental cultivars is GP. Ozone-resistant plant characteristics have been determined, based on plant stability under ozone exposure [59]. The characteristics are similar to what we have found in GP even though GP changed physiological measurements and RD was only showing a curling leaf without anthocyanin content change but the curling leaf is a sensitive plant characteristic to ozone exposure. Additionally, FG and RD can be the bio-indicators that show the symptoms under ozone stress in which this quest to find more bio-indicators regarding ozone was started over 60 years ago [60].

IV. Conclusion

Visible symptoms in coleus leaves are chlorosis in FG cultivar and curling leaf in RD cultivar. GP and YP cultivars show a full purple area in leaf appearance. Chlorophyll and carotenoid content tend to decrease as impacts of ozone exposure in all cultivars while anthocyanin content in FG, PG, and YP cultivars increases. FG, GP, and YP leaf colors are redder and darker in ozone fumigation compared with CF air. RD cultivar is stable in term of anthocyanin composition. The changes of leaf color tend to be redder and brighter in most of the cultivars, but the purple area in GP purple is darker. RD cultivars do not show any significant difference regarding the redness and brightness of leaves. As a result, different coleus cultivars show different responses, and it can be used as an ozone-plant model to investigate pigment composition under ozone exposure.

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