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DYNAMICS OF LEAD TOLERANCE IN TOBACCO (*NICOTIANA TABACUM* L.) GENOTYPES

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SUMMARY

Lead nitrate has reports of significantly inhibiting plant growth. Early exploration of the genotypic difference for lead nitrate stress in tobacco has started. The presented study had eight tobacco genotypes subjected to 200 µM lead nitrate (Pb $[NO_3]_2$) stress in a hydroponic culture. Lead stress treatment to plants for 14 days had data recording at three times intervals of stressed plants. Assessing photosynthetic and antioxidant enzymes' activities was in a time series order of one day, seven days, and 14 days. One-day, seven-day, and fourteen-day-old seedlings gained treatment of 200 µM lead nitrate stress and control. Soil Plant Analysis Development (SPAD) values for most genotypes decreased, while oxidant and anti-oxidant enzymes increased activity. Chlorophyll-a, chlorophyll-b, and total chlorophyll evaluated after lead nitrate toxicity showed reduced activity in studied tobacco genotypes compared with control as time passed. All chlorophyll contents, i.e., chlorophyll a, b, and total chlorophyll, declined with a longer span in lead nitrate solution. Genotype QVA-20 could benefit lead-salt tolerance and susceptible genotype 'long chang' cigarette based on chlorophyll content and SPAD values. Chlorophyll a capacity decreased as lead exposure to plants increased, but chlorophyll b increased in all genotypes on the 15th day. The MDA (malondialdehyde) content increased in all tobacco genotypes with increased lead nitrate exposure. Meanwhile, SOD (superoxide dismutase) contents decreased in genotypes RG-8, E1, and X6 with increased time, but POD (peroxidase) contents increased in all genotypes on the 14th day. Genotypes RG-8, E1, and X6 proved considerably tolerant of lead toxicity at 200 µM.

Key words: Lead phytotoxicity, tobacco genotype, chloroplast ultrastructure, *ceratophyllum demersum*, aminolevulinate dehydrogenase

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Key findings: The prevailing work reveals that genotype QVA260 exhibited tolerance to Pb stress as its SPAD value does not change significantly with time, followed by genotype E-1 and RG-11. Likewise, higher antioxidant enzyme activities disclosed the tolerance potential of QVA260, RG8, and E11..

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INTRODUCTION

Lead (Pb), a hefty metal, belongs to an anthropogenic origin (Mehla *et al*., 2017). Lead is a pollutant that persists in the environment as it accumulates in sediments, water, and soil (Alkhatib *et al*., 2019). The biological functions of Pb are unknown, and even at minimal concentrations, it is very poisonous to living organisms. Though it is an unnecessary element, some plant species grow in Pb-contaminated areas and store it in different parts. Roots are the first organ that various rhizosphere components come in contact (Alkhatib *et al*., 2019). Many studies indicated that greater concentrations of Pb give rise to harmful ramifications on plant growth (Mahmood *et al*., 2016). Clear indications of venomousness have smaller leaves specify these, with inhibited growth for both shoots and roots. Some chlorosis and necrosis symptoms arise in leaves, and roots become black and brown. Also, an increased concentration of Pb tends to decrease the dry weights of shoots and roots (Samreen *et al*., 2021).

Lead (Pb) gathers in various portions of the plant after being soaked up by the plant's root system. In contrast with other plant parts, Pb in the root system commonly becomes much more concentrated (Jung and Mun, 2018). Hence, as the distance from the root increases, Pb concentration in the aerial parts of the plant decreases. It happens due to the effect that the cell wall of roots accumulates a higher amount of Pb than other parts of the plant, and Pb can bind with the carboxyl groups of the carbohydrates galacturonic and glucuronic acids in the cell wall of the roots (Jung and Mun, 2018). Studies revealed that Pb probably intervenes and hinders several corporeal procedures. Those plants exposed to Pb ions show less rate of photosynthesis and transpiration. All these responses pertained to the effects, such as, lower chlorophyll content of the leaves, distortion of the chloroplast ultrastructure, CO_2 deficiency due to stomatal closure, and the hindered activities of the Calvin cycle enzymes (Zhou *et al*., 2017). *Ceratophyllum demersum,* commonly known as hornwort, when exposed to Pb $(NO_3)_2$, showed noticeable changes in chloroplast structure. Other plants exposed to hefty metals, including Pb, showed the absence or reduction of starch grains (Alkhatib *et al*., 2019).

Many crop plants exhibit genotypic variations in resistance to heavy metal toxicity (Metwally *et al.*, 2005). It is because of differences in strategies used by plants to cope with dense metal stress, including variations in antioxidant enzyme activities (Foroozesh *et al.,* 2012), heavy metal uptake, translocation, and accumulation capacity (Pourghasemian *et al.,* 2013), and the ability to sequester heavy metals to less vulnerable plant parts, such as vacuoles, through the action of metallothioneins, phytochelatins, and other metal-binding ligands (Wan *et al.*, 2003). Plants adopt many other mechanisms to evade the harmful effects of heavy metal toxicity, including the exudation of organic acids into the soil rhizosphere, which may lower the bioavailability of potentially toxic metals (Schwab *et al.,* 2005) and the action of heat shock proteins (Heckathorn *et al.,* 2004).

Tobacco is a crop of great economic importance, and because of widespread environmental pollution, some trees are planted in Pb-infested regions, resulting in yield reduction. The objectives of the presented study were to assess genotypic response in tolerance to Pb stress in different tobacco genotypes, to investigate the more tolerant Pb genotypes under high Pb stress,

and to evaluate the ultimate effect of Pb stress on the physiology of tobacco genotypes. The gathered findings can help to develop a comprehensive understanding of the principles influencing Pb stress resistance in tobacco. Notably, even a tiny amount of Pb reaches the chloroplasts and mitochondria of plants. Thus, investigating the photosynthetic physiological reaction of plants to heavy metal Pb requires immediate attention for identifying and preventing heavy metal contamination in agricultural production. Bear in mind this study came about to monitor the kinetic studies of lead tolerance in tobacco plants.

MATERIALS AND METHODS

Experimental site and materials

The research occurred at College of Agriculture and Biotechnology, Zhejiang University, China. Eight genotypes of *Nicotiana tabacum L. (N. tabacum L.),* applied with the tests, came from the said university.

Experimental method

Mature seeds of *N. tabacum L*. received diluted ethanol (70%) for about 3 to 5 min and were transmitted into mercury chloride ($HgCl_2$, 1%) for 10 and then washed with H_2O. Placing approximately 50 seeds of each variety proceeded in Petri dishes with moist filter paper and left overnight. After 24 h of germination, choosing randomly 20 seedlings for transfer to Petri dishes with two filter papers followed, with lead nitrate (Pb $[NO_3]_2$) solution (200 µM) added to each Petri dish. After 24 h, removing the overabundance solution, the seedlings received Hoagland's solution. Adding $PbNO_3$ to retain Pb concentrations and cures happened thrice. Seeds fertilization continued in a growth chamber with day/night temperatures of 25 °C/20 °C, a 16- h photoperiod, irradiance of 300 µmol m-2s^{-1}, and correlative dampness of 60%–70%.

After zero, seven, and 14 days, the plants' shoot and root separation transpired to calculate dry weights and other biological features. The SPAD (Soil Plant Analysis Development) meter, a hand-held chlorophyll device, took leaf chlorophyll, setting apart shoots for physiological measurements. Samples collected for enzymes and anti-enzymes analyses ensued. Stem and root lengths' calculation followed at the end of the experiment. Dry biomass (g per plant) evaluation used the method by Zhang *et al*. (2017).

Measurements: Determination of Pb contents in shoots and roots

Dehydrating the samples at 60 °C overnight occurred before converting them into ash in a Muffle furnace at 550 °C for 24 h to measure Pb contents in shoots and roots. Consequently, ash gestation followed using 31% HNO_3 and 17.5% H_2O_2 solution at about 70 °C for 2.5 h, then diffused in water. Fixing the Pb assimilation in the digest used an Atomic Absorption Spectrophotometer (PE-100, Perkin Elmer, USA).

Determination of chlorophyll contents

Mixing up pure acetone, pure ethanol, and distilled water (4.5:4.5:1) prepared the reaction solution or RS. Combining the RS and fresh leaf samples (small pieces) in small tubes (100:1), the tubes stored in the dark waited for the color of the leaf pieces to turn white. Undertaking chlorophyll readings were at 663 and 645 nm, for carotenoids at 470 nm (Croft *et al*., 2020).

Estimation of malondialdehyde (MDA) content

The cut plant tissue gained rinsing with distilled water. The weighed 0.5 g of sample continued its placement in a pre-cooled mortar on ice. Add 2–3 ml of pre-cooled Phosphate buffer solution with pH 7.8 ($Na_2HPO_4.12H_2O$ [16.385g]) + $NaH_2PO_4.2H_2O$ (0.663g) in distilled water to make the final volume of 1,000 ml. Sample homogenization consisted of it added on the ice to the buffer solution of 8 ml. Centrifuging the sample at 8,000–13,000 rpm for 15 min had a temperature of 4 °C.

Preserving the supernatant was at 4 °C in another small tube. The reaction solution comprised the following reagents:

(i) 5%Trichloro-acetic acid (TCA) = 25 g of TCA in 500 ml of water

(ii) Thiobarbitaric acid (TBA) = 2.5 g added in 500 ml solution of 5% TCA

Mixing 1.5 ml of the enzyme extract + 2.5 ml RS in a small tube followed with a hot water bath at 95 °C for 15 min, then immediately given an ice bath, and centrifuged at 4,800 rpm for 10 min. Using distilled water helped obtain a zero reading. Malondialdehyde (MDA) content measurement was according to a method described by Shu *et al*. (2012).

Determination of different enzyme activities

Acquiring enzyme and antioxidant activity consisted of 0.5 g leaves homogenized in 8 mL of 50 mM potassium phosphate buffer of pH 7.8. The homogenized solution centrifugation at 10,000 rpm for about 20 min had the pallet discarded, with only the supernatant utilized for the enzyme activities. Catalase (CAT, EC 1.11.1.6) activity calculation applied H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) for 1 min at A240 in 3 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA-Na_2, 10 mM H_2O_2, and 0.1 mL enzyme extract.

Attaining peroxidase (POD, EC 1.11.1.7) activity applied Zhou's *et al*. (2017) methodology.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity measurement used the technique of Zhang *et al*. (2017).

Statistical analysis

Statistical analysis employed a two-way ANOVA and the Duncan's multiple range tests.

RESULTS

Effects of Pb treatments on tobacco chlorophyll

Determining SPAD value, a representative of chlorophyll contents, transpired on days zero, seven, and 14. Varieties were significantly different in terms of SPAD value (Figure 1). On the first day, the SPAD value of genotype QVA260 (26.17) was maximum, followed by E-1 (24.97) and RG-8 (21.43), whereas a minimum SPAD value showed in X6 (17.26). It shows the typical genotypic response of tobacco genotypes against Pb. As days passed, the SPAD value of a control plant increased significantly, while lead-treated plants decreased. Although a SPAD value reduction appeared in all genotypes, a sudden decrease resulted in QVA260, but RG-8 remained constant on days seven and 14. Through time, most genotypes became stable for this level of Pb, possibly due to the activation of reactive oxygen species for protection against metal stress.

A decline in SPAD value of QUANDO-2 (19.93, 18.87, and 18.50), RG-8 (21.43, 20.97, and 20.20), and ZUNYAN-6 (17.93, 17.40, and 16.60) genotypes indicated that photosynthetic rate does not only depend on chlorophyll contents. Genotype QVA260 exhibited tolerance to Pb stress as its SPAD value did not change significantly through time, followed by genotypes E-1 and RG-11, which showed a slight decline in SPAD value as days passed. The decreased SPAD value could be due to reduced chlorophyll contents. In control plants, the trend of SPAD value through time revealed an increasing rate. The highest SPAD value occurred in genotype QVA260 on day zero (31.30), followed by E-1 (29.87). On day one, the maximum SPAD value emerged in genotypes QVA260 and E-1, with this trend continuing in the succeeding days. Hence, in terms of SPAD values, genotype QVA260 proved tolerant to 200 mM stress of lead (Figure 1).

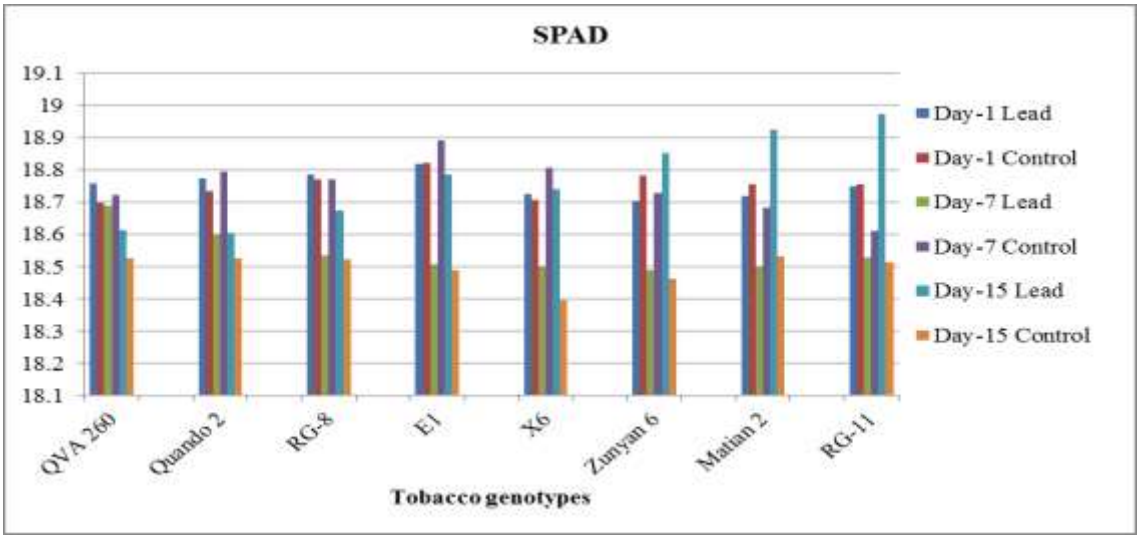

Figure 1. SPAD value for different tobacco genotypes under lead and control conditions.

The treatment sum of squares for some traits indicated the significance of chlorophyll-a, chlorophyll-b, and total chlorophyll contents on day seven, with the SPAD displaying influence on day one and day 15. Significant differences resulted in all genotypes' traits except the SPAD value on day seven. Variety versus treatment interaction revealed remarkable for chlorophyll a, b, and total chlorophyll contents on day seven and day 15, but for SPAD value, this interaction was notable on day seven. Gradual increase in SPAD contents could be seen in all genotypes under control conditions, whereas all genotypes showed decreasing behavior under lead treatment over time.

Effects of Pb treatments on tobacco chlorophyll contents

Chlorophyll-a contents emerged maximum on day one, then decreased over time. Although a decreasing trend occurred in all genotypes in the time series, chlorophyll-a amounts were more than usual in plants recorded in Quando2 at day 15 (Figure 2). Chlorophyll-b contents came out more in lead-treated plants rather than in normal ones. Decreasing trends are noticeable in all genotypes in the time series (Figure 3). Total chlorophyll contents showed minimum in genotype RG8 on day 15 and Zunyan6 on day seven, whereas genotype RG11 showed maximum entire chlorophyll contents on day seven (Figure 4).

Effects of Pb treatments on malondialdehyde (MDA) content

MDA activity increased over time in genotypes QVA260, RG-8, E-1, X6, and ZUNYAN-6. Maximum MDA contents appeared on day seven in genotype RG-8, and maximum MDA occurred in genotype E-1 on day 15. Genotype MATIAN-2 showed minimum contents on day one (Figure 5).

Effects of Pb treatments on different enzyme activities

SOD activity increased as time passed and then decreased on day 15. An increasing trend has continued in genotype QVA260 on day seven, while genotype E-1 showed maximum contents on day 15 (Figure 6). POD activity also increased over time, but it decreased on day 15. Utmost POD contents resulted in genotype RG-8 on day seven, followed by genotype QVA260 under lead treatment, whereas minimum POD activity occurred in genotype QVA260 under control conditions (Figure 7).

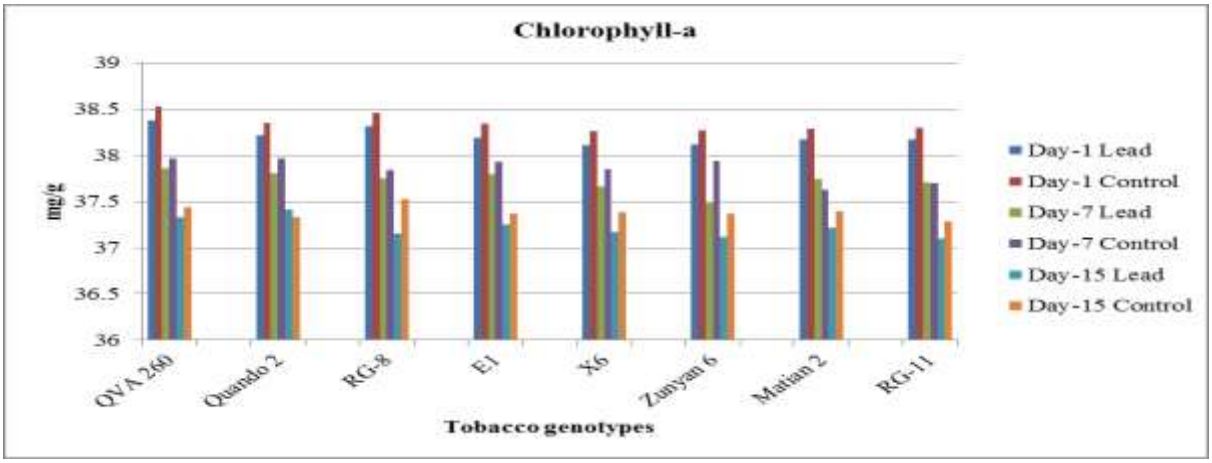

Figure 2. Chlorophyll-a contents in tobacco leaves under lead and control conditions.

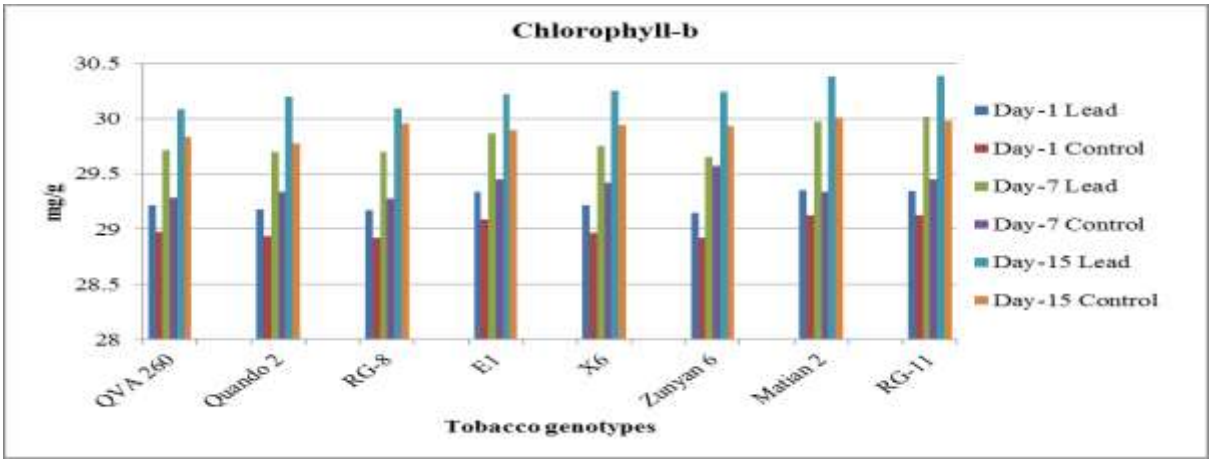

Figure 3. Chlorophyll-b contents in tobacco leaves under lead and control conditions.

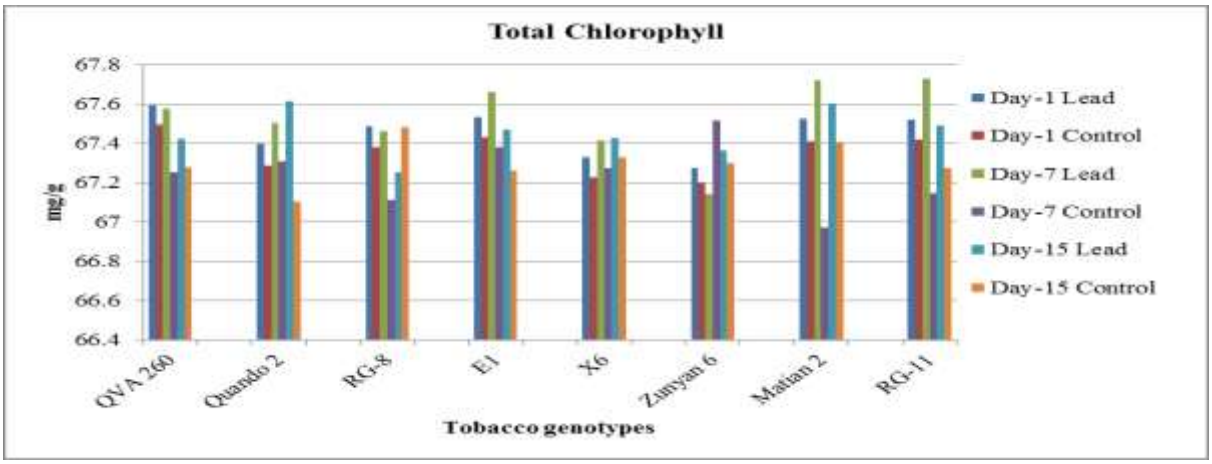

Figure 4. Total chlorophyll contents in tobacco leaves under lead and control conditions.

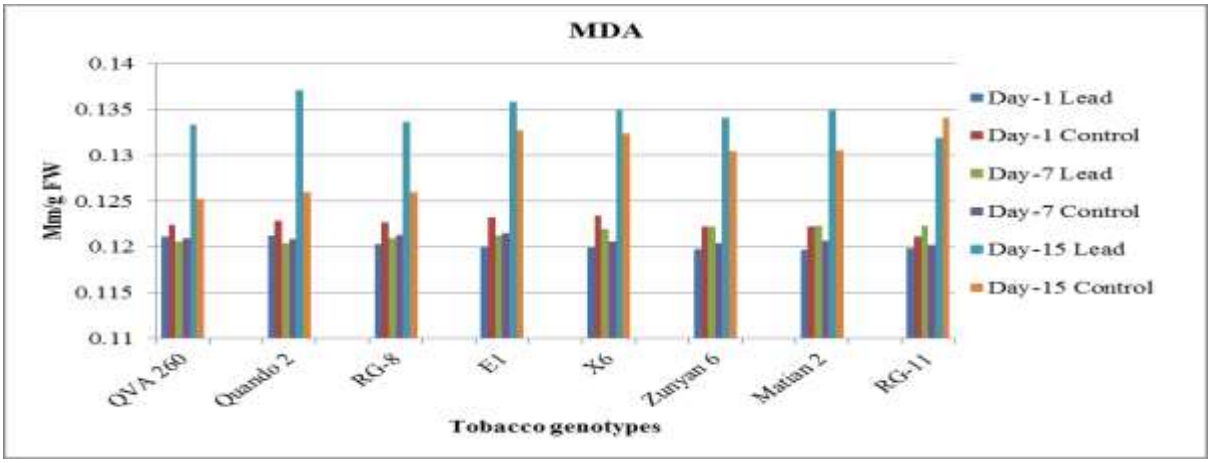

Figure 5. MDA contents in tobacco leaves under lead and control conditions.

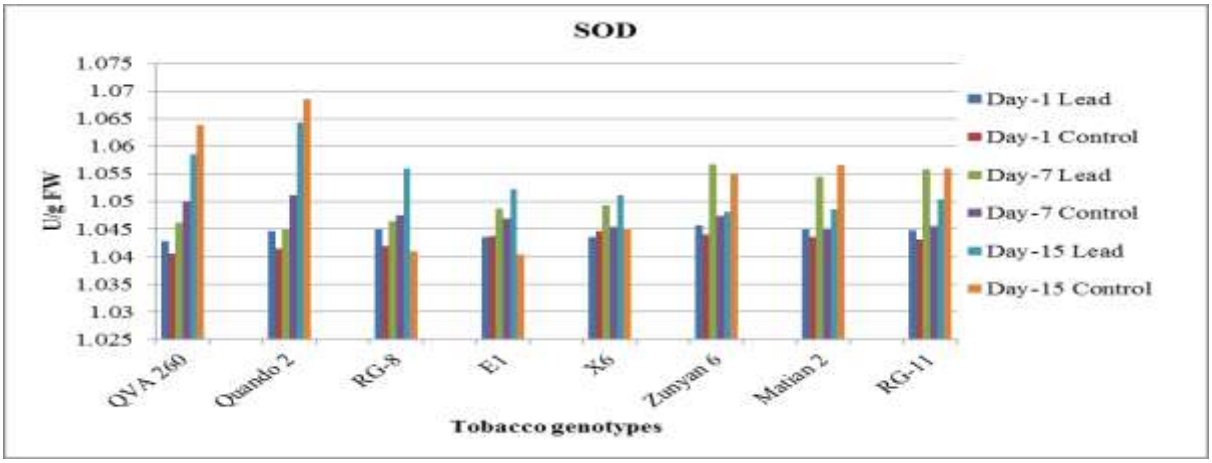

Figure 6. SOD contents in tobacco leaves under lead and control conditions.

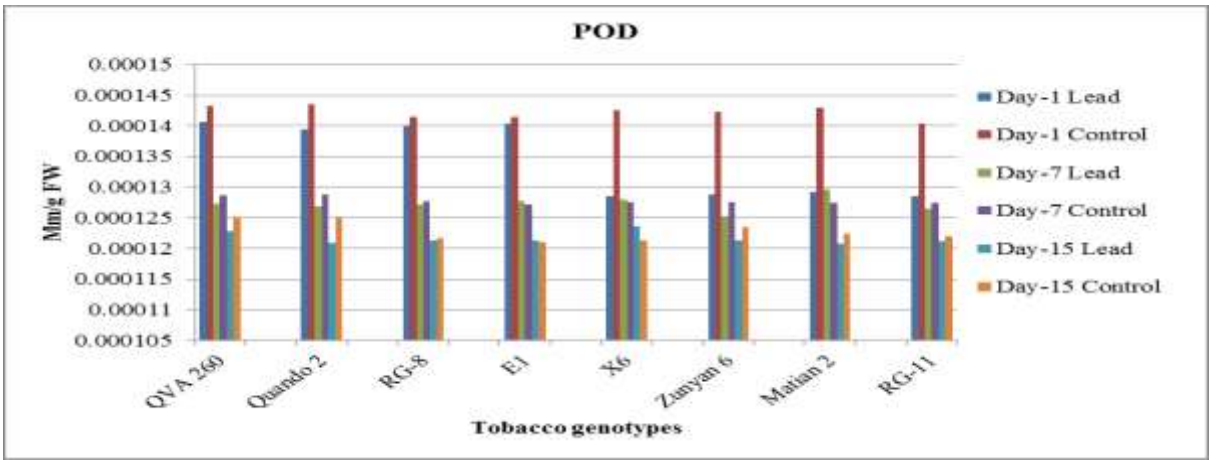

Figure 7. POD contents in tobacco leaves under lead and control conditions.

DISCUSSION

Excess Pb affects plants adversely by reducing photosynthetic activity, interacting with chlorophyll apparatus, inhibiting plastoquinone and carotenoids, Calvin cycle enzymes, and causing carbon dioxide depletion. When growing in Pb $(NO_3)_2$ solution, distinct changes have shown in the chloroplast's structure of *Ceratophyllum demersum* plants. Non-appearance of starch grains, a reduced stroma amount, and grana stacks related to the lamellar system have resulted in such plants. Several changes in the lipid composition of thylakoid membranes have also occurred in said plants (Ali and Nas, 2018).

SPAD value decline happened in all genotypes, but a sudden decrease resulted in QVA260, while RG-8 remained constant on days seven and 14. Significant differences appeared in all genotypes' traits except SPAD value on day-7. Chlorophyll-a contents came out maximum on day one and then decreased over time. Lead hinders the chlorophyll production by causing less uptake of essential elements, i.e., iron and magnesium, by the plants (Amin *et al*., 2018; Li *et al*., 2018). A report also stated that chlorophyll-a is less effective than chlorophyll-b with Pb treatment (Ali and Nas, 2018). It also inhibits the electron transport chain (Askerka *et al*., 2017).

The Pb-related effects have affected transmission to the electron donor and acceptor sites of photosystem-I (PSI), photosystem-II (PSII), and cytochrome b/f complex. Lead is generally recognized to more delicately hinder PSII electron transport than PSI electron transport (Lysenko *et al*., 2020; Zsiros *et al*., 2019). Lead also generates a hefty oxygen separation, developing external polypeptide of PSII and displacement of Ca, Cl, and Mn from the oxygen-growing multiplex (Iseki *et al*., 2020). Li *et al*. (2018) reported formal modifications have shown in light-reaping chlorophyll (LHC II) subunits after binding with Pb in vitro. They further suggested that formal modifications, prompted by Pb treatment, might accelerate insufficient collection from there on degradation (Li *et al*., 2018).

Kosobrukhovet's *et al*. (2004) findings revealed that photosynthesis in plants has several features directing it, together with number of stomata, stomatal cell size, conductance, and leaf area. Researchers observed increased chlorophyll content when examining the outcomes of Pb on the development of poplar plants and thylakoid of cucumber, which were either in the PSII core or LHC II at low concentrations of Pb treatment; however, a robust reduction in chlorophyll level of seedlings showed at the 50 mM Pb. The concentration of Pb inside the leaf may have been high enough to directly inhibit chlorophyll synthesis at a 50 mM Pb treatment level (Slima and Ahmed, 2020). MDA activity increased over time in genotypes QVA260, RG-8, E-1, X6, and ZUNYAN-6. With the increase in Pb concentrations, key variations ($p < 0.0001$) were notable in MDA content, bringing about considerable developments in MDA (Maodzeka *et al*., 2017).

The SOD activity increased through the days and then decreased on day 15. An increasing trend has resulted in genotype QVA260 on day seven, while genotype E-1 showed maximum contents on day 15. POD activity also increased over time, but it decreased on day 15. Utmost POD contents appeared in genotype RG-8 on day seven, followed by genotype QVA260 under lead treatment, while minimum POD activity surfaced in genotype QVA260 under control conditions. With the increase in Pb concentrations, significant differences ($p < 0.0001$) were remarkable for CAT and POD activities, causing dramatic increases in the recorded antioxidant enzymes (Maodzeka *et al*., 2017). The Pb treatment, just like other hefty metals, affects the performance behaviors of a large scale of enzymes involved in various metabolic tracks. Enzyme activity and behavior involved in various metabolic activities had Pb treatment influencing them. Recently, researchers have paid serious attention to studying the behavior of Pb on plants. The concentration of cadmium, which inhibits almost 50% of enzymes' activity, is known as the inactivation constant (Ki). The Pb interacts with the sulfhydryl (–SH) group of

enzymes as necessary for maintaining the tertiary structure of any enzyme. Blockage of carboxyl (COOH) groups also has reports of responding to Pb treatments.

Considerable attention has focused on comprehending the action of Pb on plant enzymes. At a concentration of about 10^{-5} to 2 × 10^{-4}M, Pb produces almost 50% inhibition of many enzymes. This value is said to be a deactivation constant (Ki). Many studies suggested that, due to the interplay of Pb with enzyme–SH groups, Pb exerted inhibition on enzyme activity (Karri *et al*., 2020). The –SH groups, which are available in the vigorous site of the enzyme, are vital for enzyme activity; Pb interacts with these free –SH groups that are important for stabilizing the enzyme tertiary structure. In addition to the reaction with –SH groups, Pb ions also block –COOH groups, which seem to play a key performance in inhibiting enzyme activity under Pb treatment. With the –SH group of cysteine, Pb forms a mercaptide, and with phosphate groups it forms complexes. A Pb treatment causes inhibition of metalloenzymes by displacing the basic metal by Pb.

Lead (Pb) ion firmly hinders the essential enzyme of chlorophyll biosynthesis and aminolevulinate dehydrogenase (Roychoudhury and Chakraborty, 2020; Samreen *et al*., 2021). A low $Pb(NO_3)_2$ concentration of 5 µM in leaf homogenates of spinach inhibited the activity of ribulose-bisphosphate carboxylase/oxygenase (Ali and Nas, 2018). A lesser decrease and similar activity have resulted from lactate dehydrogenase. However, $Pb(NO_3)_2$ has enhanced the activity of pyruvate kinase (Ali and Nas, 2018). A study has shown that Pb acetate dramatically lessened the performances of protease and α -amylase in rice endosperms after four days of germination; however, the activities of RNase and DNase are not much affected (Khan *et al*., 2018). In leaves of Pb-treated soybean plants, lesser action and low nitrate amount of glutamate dehydrogenase emerged, but insignificantly affecting malate dehydrogenase functions (Ribeiro *et al*., 2019). Inhibiting ATP synthetase/ATPase revealed Pb as highly effective (Zhang *et al*., 2017).

A Pb treatment expanded the activities of several enzymes. Extended enterprise of acid phosphatase, α-amylase, and peroxidase in leaves of soybean plants have occurred when grown in culture media holding 20–100 mg/L of Pb (Ribeiro *et al*., 2019). Parallels with the senescence of leaves and increased activity of hydrolytic enzymes along with peroxidase in soybean leaves under Pb treatment were prominent (Sandoval *et al*., 2019).

The enhancement in the performance of specific anti-oxidative enzymes has cropped out from Pb-treated plants, as Pb encourages the generation of reactive oxygen species in plants, giving rise to oxidative stress. Increased activities of the anti-oxidative enzymes superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and glutathione reductase in roots and leaves of rice plants arose when grown for 20 days in sand cultures holding 0.5 mM and 1 mM $Pb(NO_3)_2$ (Khan *et al*., 2018). But, when Pb displaces metals that are an essential part of the enzyme, activities of antioxidative metalloenzymes decline.

A Pb forbearance for plant types demonstrated different behavior of certain enzymes under Pb treatment. According to Alamri *et al*. (2018), the sequel of Pb on carbonic anhydrase activities in the forbearing and delicate types of melic grass (*Melica nutans*), it indicated that in forbearance melic-grass population, Pb activated carboanhydrase activity while in the fragile plants, the activity of this enzyme stood uninfluenced (Alamri *et al*., 2018).

CONCLUSIONS

The current work reveals that the genotypic distinction of tobacco plants in response to the Pb strain, no doubt, existed. Genotype QVA260 proved tolerant to Pb stress as its SPAD value does not change significantly over time, followed by genotype E-1 and RG-11. Higher antioxidant enzyme activities resulted in QVA260, RG8, and E11. Thus, the conclusion is that genotype QVA260 exhibited the highest Pb tolerance index, hence, the recommendation for lead-free tobacco production for smoking

purposes. Further research may lead to knowing extra Pb-resistant tobacco genotypes for cultivation in Pb-vulnerable zones, as it would lessen Pb aggregation and enhance the yield.

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