Amelioration of Lead Toxicity by Ascorbic Acid in Sugarcane under *in vitro* condition

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Abstract

Ameliorating role of ascorbic acid against Pb toxicity in three genotypes of sugarcane (YT-53, CP-77-400, NSG-59)under six different concentrations of $Pb(NO_3)_2$ (control, 0.1, 0.2, 0.3, 0.4, 0.5, 1mM) was studied under *in vitro* conditions. The morphological parameters like callus fresh weight and dry weight, shoot and root length, number of shoots and roots per cultured plant and biochemical parameters included antioxidants enzymes, total protein contents, ascorbic acid, Pb contents in Pb treated calli were compared with 0.5mM ascorbic acid pretreated calli at each level. It was observed that all the morphological parameters were negatively affected by Pb stress but ascorbic acid pretreatment recovered this damage. Biochemical parameters like antioxidants enzymes activity (POD, SOD, CAT) increased under elevated Pb concentration while ascorbic acid pretreatment further enhance the POD and SOD activity while CAT activity and total soluble protein contents did not change significantly. Different genotypic behaviors towards different treatments were also observed.

Introduction

Heavy metal pollution results in serious environmental threat on a global scale especially for agricultural lands around the world (Shifaw, 2018). Heavy metals are non-degradable, toxic and immobile. Their presence in soil has been a worldwide concern. Major concern associated with cultivated soils is sustainable global food demands for increasing population (Wu et al. 2019). Agriculture is the major source on the Earth for achieving future food security (Frona et al., 2019) but crops yield has been threatened by the subsequent rise in heavy metal stresses while very small net change in cultivated land in world over decades confound the problem (Raza et al., 2019). Agricultural sites across the world are polluted with various metals ions and lead (Pb) is the most perilous (Venkatachalam et al., 2017) among them. It is graded as number one heavy metal pollutant because of its wide occurrence while second among most hazardous substance by Agency for Toxic Substances and Disease Registry (ATSDR, 2019). Plants grown in contaminated soil is affected by Pb directly or indirectly. Even its small amount can induce adverse malfunctions in the series of biochemical, physiological and morphological changes in plants (Kushwaha *et al.*, 2018). Due to its toxicity for living organisms, widespread occurrence and persistence in the environment, the European Chemicals Agency (ECHA) considered it most concern metal for the environment (Pourrut et al., 2011). Pb has gained considerable attention and becomes a focus of study therefore, its toxicity must be addressed to mitigate its phytotoxic effects on agriculture crops (Nas and Ali, 2018).

Sugarcane is the oldest crop known to man that is grown in 120 countries (Ali et al., 2020). Besides providing 70% of worlds sugar it also provides raw material to other 25 industries through its byproducts including butanol, industrial enzymes, propanol, acetic acid, plywood and paper. Ethanol produced from sugarcane crop is commercially most efficient biofuel that reduce the 90% greenhouse gasses emission (Ashwath and Kabir, 2019; Karp et al., 2021).

Recent advances in biosciences like genetic engineering, plant stress physiology, transgenics and plant nutrition helped in identification and characterization the HM detoxification or tolerance in plants. However different strategies to develop heavy metal tolerant plant is a fervent issue that remain least discussed (Wani et al., 2018).

To combat abiotic stresses has been the major challenge to sugarcane breeders. Therefore sustainable and efficient methods are required to develop the stress tolerant cultivars so that the increased yield demand can be fulfilled for rapidly expanding global population (Godoy et al., 2021). Developing stress tolerant plants is a strategy to overcome this problem in plants. Therefore, it becomes the dire need to use new and innovative biotechnological methods for breeding and propagation of plants to obtain the resistant varieties against abiotic stress like heavy metasl (Ahmar et al., 2020). Conventional methods are more potent towards biotic and abiotic stresses but these are time consuming (Singhal et al., 2016). In this regard tissue culture provides easy tool as its application offers fast development and propagation of elite varieties for better yield in short time (Monthony et al., 2021) where abiotic tolerant plants could be acquired by applying the selection agent to the culture media. At this point, some chemical compounds are useful for enhancing the tolerance. Different chemicals like abscisic acid (AsA), citric acid (CC), salicylic acid (SA), polyamines (PA), ethylene (ET) have been used exogenously to improve the tolerance in plants and to minimize the damage caused by stress conditions (Yong et al., 2017). These chemicals regulate cell metabolism to activate the biological defense. Exogenous application of these chemicals under *in vitro* conditions can also be helpful to alleviate the adverse effects of heavy metals (Wiszniewska, 2021).

Taking into above consideration and recent literature the present paper reflects the impacts of Pb ions on physiological and cellular processes in sugarcane callus, mechanisms against detoxification of Pb ions, tolerance against Pb stress in sugarcane callus due to ascorbic acid pretreatment. It might helpful in developing HM tolerant plants and to deal with heavy metal induced threats to agricultural crops that ultimately leads in better economic yield of crops.

Materials And Methods

2.1 Media Preparation For callus induction plant growth regulators were added in Murashige and Skoog (MS) basal media (Murashige and Skoog, 1962) while MS media added with various level of Lead Nitrate ($Pb(NO_3)_2$) were used for lead (Pb) stress treatments. The pH was kept 5.65-5.75 with 1N HCl and 1N NaOH.

2.2 Plant Material The sugarcane genotypes (YT-53, CP-77-400 and NSG-59) were collected from the Shakerganj Sugarcane Research Institute Jhang Pakistan. The tops of 3-4 months old sugarcane plant were source of explants. Plant material was cleaned with 95% ethyl alcohol and inoculated on sterilized culture vessels and suitable conditions (temperature, light) for callus and regeneration were provide and recultured when requried.

2.3 Preliminary Test to Check the Effect of Lead (Pb) on Callus Thirty days old calli were shifted to MS media containing 6 different concentration of Lead Nitrate. Six different (0.1, 0.2, 0.3, 0.4, 0.5 and 1mM) concentrations were applied to callus cultures for each variety.

2.4 Morphological and Biochemical Studies Effect of various concentration of $Pb(NO_3)_2$ on callus was evaluated by morphological and biochemical studies. Superoxide dismutase (SOD), Peroxidase (POD), Catalase (CAT), protein, ascorbic acids, BCF, STI, RGR and Pb contents were assessed.

2.5 Ascorbic Acid Pretreatment Experiment Callus cultures were pretreated with 0.5mM Ascorbic acid (Ejaz et al., 2012) to find out the ascorbic acid impact on callus against Pb stress. Calli were kept for 24 or 48 hours on media supplemented with 0.5mM ascorbic acid and transferred to fresh MS medium with 0, 0.1, 0.2, 0.3,0.4,0.5 1mM. $(Pb(NO_3)_2$. All non-pretreated and pretreated cultures were then evaluated after 90 days,120 and 150 for callus necrosis, callus weight. Biochemical analysis of antioxidant enzymes, total soluble protein contents and BCF were also performed.

2.6 Estimation of Antioxidant Enzymes The, catalase, peroxidase and superoxide dismutase activities and soluble protein contents were estimated with the help of spectrophotometer (UV 4000) after day 90, 120 and 150. After 150 days Pb treated callus were transferred to regeneration media. For extraction, two-gram callus was crushed with 0.1g PVP and 4ml of 0.1 M phosphate buffer with pH 7.2 in ice-chilled pestle and mortar. The mixture was centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant attained after centrifugation was used for enzyme analysis.

2.7 Specific activity of Catalase estimated by Beers and Sizer (1952) method some changes.

2.8 Specific activity of Peroxidase estimated by Racusen and Foote (1965) method..

2.9 Superoxide dismutase(SOD) activity was assessed by Maral *et al*., (1977) method with modification that was based on principal that “Under specific condition one unit of SOD reduced the 50% of the maximum reduction of NBT”.

2.10 Total soluble Protein Contents estimated by Racusen and Johnstone, (1961) method.

2.11 Bio Concentration Factor (BCF)

BCF=Lead accumulation in callus / Pb concentration in medium mg/kg x 100 mg/kg(Kulkarni *et al*., 2014).

2. 12 Statistical Analysis Descriptive statistical analysis (mean and standard error of the means) was performed for two factors (varieties and treatments). Two-way full factorial design of experiment was applied. Then the data was subjected to univariate two-way analysis of variance (AONVA) using Statistix-8.1 software. As a post-hoc, least significant difference (LSD) test was applied using 95% level of confidence (p-value ≤ 0.05).

Results

To investigate the effect of pretreatment of ascorbic acid pretreatment on callus cultures, well proliferated calli were shifted to the MS medium supplemented with 0.5mM and 1mM ascorbic acid after 24 and 48 hours. It was observed that calli those were treated with 1mM ascorbic acid for 24 and 48 hour showed very slow growth while calli treated with 0.5mM AsA for 24hours showed normal growth. Therefore callus cultures treated with 0.5mM ascorbic acid for 24hrs were used for further analysis.

Calli those were not treated with ascorbic acid and $Pb(NO_3)_2$ were set as control (T_0) and shifted on already standardized media for callus (3.5mg/l 2,4-D for YT-53 and NSG-59 and 5mg/l CP-77-400) Non pretreated and ascorbic acid pretreated calli were compared at each concentration of $Pb(NO_3)_2$.

3.1 Effect of Ascorbic Acid Pretreatment on Lead Treated Callus Morphology

Calli treated with different concentration of $Pb(NO_3)_2$ show necrosis after certain time of inoculation in all three genotypes. NSG-59 start necroing earlier than other two genotypes and showed complete necrosis at 1mM after 90 days.

This necrosis was delayed in ascorbic acid pretreated calli at each level in all three genotypes.

3.2 Effect of Ascorbic Acid Pretreatment on Callus Fresh Weight and Dry weight in Sugarcane (Genotypes YT-53, CP-77-400, NSG-59)

Reduced fresh and dry weight at different concentration of $Pb(NO_3)_2$ was observed as compared to control in all three genotypes. Decrease in callus weight with respect to control at 0.1, 0.2, 0.3, 0.4, 0.5, 1mM concertation of $Pb(NO_3)_2$ was 16%, 28%, 37%, 53%, 84%, 81% in YT-53, 11%,25%, 48%, 71%, 89% in CP-77-400 and 16%, 27%, 46%, 59%, 78% in NSG-59 respectively. While ascorbic acid pretreated calli in YT-53 have 11%, 16%, 18%, 14%, 15%, 5% increase in fresh wight as compared to non pretreated call. This increases in fresh weight in CP-77-400 was 11%, 9%, 5%, 7%, 33% and 12%, 9%, 8%, 7%, 4% in NSG-59 .

Callus dry weight was also effected by different concentrations of $Pb(NO_3)_2$. Dry weight of YT-53 reduced to 12%,19%,33%,49%,85%,95%, Cp-77-400 7%, 28%,37%,57%,74%, NSG-59 17%,26%,34%,47%,58% as compared to their respective controls. Ascorbic acid pretreatment improved dry weight 17%, 15%, 13%, 12%, 6%, 3% in YT-53, 2%, 15%, 13%, 10%, 5% in CP-77-400 and 6%, 8%, 7%, 7%, 4% in NSG-59 as compared to non treated calli. Calli of CP-77-400 and YT-53 those did not survive at 1mM $Pb(NO_3)_2$ were survived after pretreatment of ascorbic acid.

3.1 Effect of Ascorbic Acid Pretreatment on Lead Treated Callus Morphology

To compare the effect of ascorbic acid pretreatment at different concentrations of $Pb(NO_3)_2$ regeneration potential in term of shoot morphology, shoot length, average number of shoots per plant and regeneration frequency for each genotype was compared to nonpretreated calli after 90 days under 16h photoperiod at 27 ± 2°C on their respective media 2mg/l kintine for YT-53 and NSG-59 and 1mg/l BAP for CP-77-400. The shoot regenerated from callus treated with different concentration of $Pb(NO_3)_2$ show necrosis after certain time. **C**alli pretreated with ascorbic acid show less necrosis as compared to non treated calli in all three genotypes.

Shoot length of YT-53 decrease 16%, 32%, 34%, 58%, 83%, CP-77-400 58%, 58%, 69% and NSG-59 16%, 33%, 46% while calli of CP-77-400 and NSg-59 didnot regenrate at 0.4, 0.5 and 1mM lead. Ascorbic acid pretreated calli improves shoot length 16%,27%,5%,12%,2% in YT-53, 28%,31%,56% in CP-77-400 and 20%,25%,26% in NSG-59 .

Shoot numbers at 0.1-1 mM $Pb(NO_3)_2$ decreased 24%, 41%, 41%, 62%, 86% in nonpretreated calli that was improved 21%, 20%, 15%, 23%, 7% in pretreated calli in YT-53. In CP-77-400 shoot numbers decreased 29%, 40%, 63% in nonpretreated calli and improve 12%, 13%, 11%, in pretreated calli while in NSG-59 21%, 36%, 57% decreased in nonpretrated calli was improved 1.4%,1.2%, 1% in pretreated calli. Non pretreated calli did not regenerated at 0.4, 0.5 and 1mM $Pb(NO_3)_2$ in CP-77-400 and NSG-59. However ascorbic acid pretreated calli regenerated at these concentration.

3.4 Root Induction in Ascorbic Acid Pretreated and Non Pretreated Callus Cultures in Sugarcane.

For root induction the well devloped plant were shifted into already standerized rooting plant regenerated from ascorbic acid pretreated and nonpretreated callus were compared.

In YT-53 the roots numbers and length didnot varry significantly in pretreated and non pretreated plants.While in CP-77-400 root numbers were increased but root length remain same in pretreated and nontreated plants.

Likewise CP-77-400 and NSG-59 showed more number of roots in plants developed from ascorbic acid pretreated callus than non pretreated while root length didnot show any significant difference among plants devloped from ascorbic acid pretreated and and

nonpretreated callus at all levels.

Root numbers that decreased 2%, 5%, 5%, 22%, 28% in YT-53, 2%,7%, 25% in CP-77-400 and 23% 38%, 49% was improved 3%, 0%, 2%, 5%. in YT-53, 6%,10%,22% in CP-77-400 and 14%, 27%, 26% NSG-59 in ascorbic acid pretreated calli.

3.5 Biochemical Analysis of ascorbic acid Pretreated and Nonpretreated Callus in Sugarcane (Genotype YT-53, CP-77-400, NSG-59)

3.5.1Total soluble Protein Contents Total soluble protein contents decreased gradually by increasing concentration of $Pb(NO_3)_2$ in callus culture media in all three genotypes as compared to control. Total soluble protein contents in ascorbic acid pretreated calli didnot show any significant difference as compared to non pretreated calli at all the levels of $Pb(NO_3)_2$.

3.5.2 Effect of $Pb(NO_3)_2$ and Ascorbic Acid Pretreatment on three Antioxidant Enzyme Activities in Genotypes of Sugarcane callus

3.5.2.1 Specific activity of Catalase (CAT) In all three genotypes specific activity of CAT increased significantly by increasing concentration of $Pb(NO_3)_2$ that started decline with time. Specific activity of CAT in ascorbic acid pretreated calli did not show any significant difference as compared to non pretreated calli at all the levels of $Pb(NO_3)_2$. Highest specific activity of catalase was recorded in YT-53 at 1mM $Pb(NO_3)_2$ at day 90.While this activity was lowest in NSG-59 among all three tested genotypes. Effect of pretreatment of ascorbic acid on specific CAT activity was non significant in all three genotypes in sugarcane under study.

3.5.2.2 Specific activity of Superoxide dismutase (SOD)

Specific activity of SOD in all three varieties increases by increases the concentration of $Pb(NO_3)_2$ callus culture media as compared to control. This activity start declining with time. Ascorbic acid pretreatment further enhanced specific activity of SOD significantly in calli maintained at different concentration of $Pb(NO_3)_2$ in all three genotypes.

3.5.2.3 Specific activity of Peroxidase (POD) Specific activity of POD increased by increasing the concentration of $Pb(NO_3)_2$ in callus culture media as compared to control in three genotypes. This activity start declining with time. Ascorbic acid pretreatment significantly effect specific activity of POD in all three genotypes. YT-53 showed highest POD activity among three genotypes.

3.6 Ascorbic acid Ascorbic acid concentration in callus increased with increasing concentration od $Pb(NO_3)_2$ in media in all three genotypes under observation while the ascorbic acid concentration in ascorbic acid pretreated calli were higher than non pretreated calli. Ascorbic acid concentration was highest at T6 in ascorbic acid pretreated calli in all three genotypes.

3.7 BCF (Bio Concentration Factor) BCF of callus decreased with increasing the concentration of $Pb(NO_3)_2$ in media while BCF of ascorbic acid pretreated calli didnot effect significantly in all three genotypes.

3.8 Pb (Lead) Contents In Callus Lead(Pb) contents in control calli were found zero. Pb (Lead) contents in callus of three sugarcane genotypes under 6 different concentrations of $Pb(NO_3)_2$ after 90 day is increases as concentration of $Pb(NO_3)_2$ increased in callus growing media. Maximum lead contents in YT-53were found in callus maintained on 1mM $Pb(NO_3)_2$ Lead contents in CP-77-400 and NSG-59 maximum lead contents were found in callus maintained on0.5mM $Pb(NO_3)_2$ Ascorbic acid treatment showed nonsignificant effect on lead(Pb) contents in calli of all three genotypes

Discussion

Brown appearance of callus was found to be the prime sign of stress. Browning reduced the growth and proliferation, lowered regeneration potential and even death of callus (Vijayalakshmi and Shourie, 2016). Ascorbic acid being non-enzymatic antioxidant plays vital role in plant growth by regulating various cellular processes like cell division, their differentiation and senescence. It enhance cell division and cell enlargement by improving membrane integrity effected by heavy metals that improve growth and biomass of stressed plant (Hassan et al., 2021; Akram et al., 2017). Improved growth and biomass of calli pretreated with ascorbic acid was reflecting that pretreatment of ascorbic acid enhances the callus tolerance against Pb toxicity.

Suppressed regeneration potential due to Pb toxicity was restored by ascorbic acid pretreatment. Exact mechanism of improvement of regeneration potential of calli pretreated with ascorbic acid is yet not well understood. It is believed that ascorbic acid improve the callus survival by reducing browning and improving antioxidant ability against metal stress through various physio biochemical process that ultimately improve the regeneration potential (Siddiqui et al., 2018). Significant role of ascorbic acid in improvement of

plant growth under abiotic stress in other plants like rice, (Alhasnawi et al., 2016) sugarcane (Rao and Jabeen 2013) and alfalfa (Arab and Ehsanpour, 2006) provide base to draw above conclusion.

In contrast to other studies growth parameters of root did not change significantly in plants regenerated from ascorbic acid pretreated and non-treated calli. In *Petunia*, ascorbic acid application stimulated the length of root and shoot, dry and fresh weight of petunia under saline condition but number of new shoots and roots did not change (Krupa-Malkiewicz and Smolik, 2019)..

Among non-enzymatic antioxidants, ascorbic acid being potential plant growth regulator enhances the plant tolerance against abiotic stress by up-regulating the abiotic stress-related genes(Rajput et al., 2021; Hameed et al., 2021).

Metal ions provoke the CAT activity in cells (Topal et al., 2019). This accelerated CAT activity under elevated concentration of Pb was also recorded in present study that started declining with time. Low concentration of stressor stimulate CAT activity but by gradual increase in its concentration, the accretion of reactive oxygen with time was farther than the adjustment ability of enzymes in plant body probably the reason of decline in CAT activity with time (Akberi *et al.*, 2020). According to some pervious studies CAT activity is accelerated by application of exogenous ascorbic acid (Noreen *et al.*, 2021; Elsiddig et al., 2021) while non significant impact of exogenous ascorbic acid on CAT has also been observed by other scientists (Agar et al., 2019) as observed during this study. This different behavior of sugarcane callus has also been reported by Munir and Aftab (2013) under salt stress conditions. Bhaduri and Flukar (2012) explained that ascorbic acid application triggers the APX activity that have greater affinity to scavenge H_2O_2 than CAT could be the reason of this peculiar behavior. Moreover at gene expression level, ascorbic acid remarkably down regulate the expression of CAT (Elkelish et al., 2020).

Data related to specific activity of Peroxidase (POD) indicated that POD activity also depend upon the concentration of Pb ions and its time of application. Results of present work are in conformation with findings for other plants under Pb stress as *Jatropha curcas* (Valadez-Villarreal, 2015) and *European Bluestar* (Acemi et al., 2020). Heavy metal stress in low concentrations for short time triggers POD activity but prolonged stress with high metal concentration results in enzyme inactivation (Lin et al., 2015) could be the reason of declining POD activity after certain period of time that was restored in ascorbic acid pretreated calli. This restoring ability of ascorbic acid can be justified by the fact that ascorbic acid is involved in transcription and antioxidative metabolism of anti oxidant enzymes under stress conditions (Alves et al., 2021; Elsiddig et al., 2021).

It is well known that SOD activity increases by increasing the Pb concentration (Rajput et al., 2021). Enhanced SOD activity under stress conditions is primary defense in plants as SOD harvest the ROS species by reacting with superoxide radicals to release H_2O_2. Specific activity of SOD depends upon the concentration of Pb and time of exposure as depicted in present study. In present investigation, pretreatment of ascorbic acid was found to be effective in amelioration of metal toxicity by escalating SOD activity. Thus, it becomes obvious that ascorbic acid is involved in the scavenging of ROS induced by Pb stress by activating the antioxidant enzymes like SOD (Lu et al., 2014).

Change in cell proteins correlated to change in the transcriptome profile of several enzymes (Pourrut et al., 2011). Although improved protein contents by external ascorbic acid application is well documented in other plant species (Xia et al., 2020; Alayafı, 2020) but such association was not observed during this study that reflect the limited role of ascorbic acid in protein improvement in sugarcane calli. Although finding of present investigation are not able to relate the ascorbic acid pretreatment to protein contents in sugarcane calli but are in line with the results of Ejaz et al., (2012) and Munir and Aftab (2011). The exact mechanism of this unusual behavior of sugarcane is not known and need more research.

Non significant effect of ascorbic acid on BFC value represent that it does not have any role in metal accumulation. Although the role of ascorbic acid in metal accumulation is rarely observed but from these results once can easily conclude that ascorbic acid provide additional defense against Pb toxicty but didnot effect the plant ability to accumulate the metal ions.

Therefore ascorbic acid is applied exogenously to enhance the internal ascorbic acid level to cope with stress and to improve its endogenous level to enhance stress tolerance in plants (Akram et al., 2017) Various evidence of enhanced endogenous level of ascorbic acid in plants under exogenously applied ascorbic acid have been reported in different plants (Khazaei & Estaji, 2020).

Conclusion

It was observed that Pb ions reduced the callus fresh weight, dry weight and shoot numbers, shoot length regenerated from Pb treated calli. While ascorbic acid pretreated calli showed less browning and improved weight than non pretreated calli. While antioxidant enzymes activity and protein contents did not change significantly by ascorbic acid pretreatment. From above results it was concluded that pretreatment of ascorbic acid to sugarcane callus improve the callus physically but not much as effective biochemically. Therefore further study for some stronger agent is required to enhance the tolerance again heavy metal stress like Pb.

Declarations

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Ethical Approval

Above research do not need any ethical approval

Consent to Participate

This statement is to certify that all Authors have seen and approved the manuscript “Amelioration of Lead Toxicity by Ascorbic Acid in Sugarcane under *in vitro* condition” being submitted. We assure that article is the Authors' original work. On behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission. We attest to the fact that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to the Environmental Science and Pollution Research**.**

Consent to Publish

All the authors have given their consent to publish this study.

Authors Contributions

All authors contributed to the study conception and design

Yasmmeen saleem Research work

Amir Ali Supervisor

Dr. Shagufta Naz Co-supervisor

Dr. Muhammad Jamil Stat analyst

Competing Interests

The authors declare no competing interests.

Availability of data and materials

All the data is takes by authors and not self manipulated

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Tables

Table Comparative fresh weight and dry weight in 0.5mM ascorbic acid pretreated and nontreated callus at six different concentration of $Pb(NO_3)_2$ in three varieties of sugarcane

Treatment detail		AsA	Fresh Weight (g)±SE			Dry weight (g)±SE		
			YT-53	CP-77-400	NSG-59	YT-53	CP-77-400	NSG-59
To	Control	NT	1.2±0.01b	0.9±0.004b	0.7±0.004a	0.07±0.00026b	0.06±0.0007b	0.05±0.002b
TA	0 mM Lead Nitrate + 0.5mM Ascorbic Acid	PT	1.3±0.011a	1±0.006a	0.8±0.008a	0.07±0.0003a	0.06±0.0004a	0.05±0.0004a
T1	0.1 mM Lead Nitrate	NT	1.±0.01c	0.8±0.004c	0.6±0.005b	0.06±0.0005d	0.05±0.0006c	0.04±0.0006d
T7	0.1 mM Lead Nitrate + 0.5mM Ascorbic Acid	PT	1.±0.01c	0.8±0.004c	0.6±0.004bc	0.06±0.0003c	0.05±0.001c	0.04±0.0003c
T2	0.2 mM Lead Nitrate	NT	0.9±0d	0.7±0.005d	0.5±0.009c	0.05±0.0006e	0.04±0.0005d	0.04±0.0009de
T8	0.2 mM Lead Nitrate + 0.5mM Ascorbic Acid	PT	0.9±0.01d	0.7±0.003d	0.4±0.067d	0.06±0.0005e	0.04±0.0005d	0.03±0.0004e
T3	0.3 mM Lead Nitrate	NT	0.8±0.01f	0.5±0.004e	0.4±0.01d	0.05±0.001g	0.04±0.0004e	0.03±0.0009f
T9	0.3 mM Lead Nitrate + 0.5mM Ascorbic Acid	PT	0.8±0.01e	0.4±0.005f	0.4±0.006d	0.05±0.0004f	0.03±0.0004f	0.03±0.0003f
T4	0.4 mM Lead Nitrate	NT	0.6±0.01g	0.3±0.005h	0.3±0.01e	0.04±0.0006h	0.02±0.0004g	0.02±0.001h
T10	0.4 mM Lead Nitrate + 0.5mM Ascorbic Acid	PT	0.5±0.012g	0.3±0.003g	0.3±0.004e	0.04±0.0004h	0.02±0.0004g	0.03±0.0003g
T5	0.5 mM Lead Nitrate	NT	0.2±0.011h	0.1±0.006j	0.2±0.006g	0.01±0.0002j	0.01±0.0009hi	0.02±0.0003i
T11	0.5 mM Lead Nitrate + 0.5mM Ascorbic Acid	PT	0.2±0.01h	0.14±0.009i	0.2±0.004f	0.02±0.0004i	0.01±0.0008h	0.03±0.0004gh
T6	1 mM Lead Nitrated	NT	0.1±0.011i	0.007±0.002k	0.004±0.003h	0.003±0.0003k	0.01±0.0003j	0.008±0.0008k
T12	1 mM Lead	PT	0.2±0.01h	0.02±0.0045k	0.01±0.003h	0.02±0.0002i	0.01±0.0003ij	0.01±0.0006j

Nitrate + 0.5mM Ascorbic Acid							
LSD 0.05		0.0192	0.0137	0.0532	0.0013	0.0018	0.0019
Mean square (df = 13)		1.548***	1.22***	0.58***	0.00498***	0.003***	0.0015***
Error (df = 126)		0.00047	0.00024	0.004	0.00000226	0.00000394	0.00000474
Source of variation (SOV)	df	MS	F	p-value	MS	F	p-value
Variety (V)	2	3.22	2238.8	<0.001	0.0057	1565.31	<0.001
Treatment (T)	13	3.17	2199.88	<0.001	0.0086	2352.40	<0.001
VxT	26	0.09	63.51	<0.001	3.84E-04	105.23	<0.001
Error	378	0.001			3.65E-06		

Table Comparative root number and root length in pretreated with 0.5mM ascorbic acid and nontreated callus at six different concentration of $Pb(NO_3)_2$ in three varieties of sugarcane.

Treatment details			YT-53		CP-77-400		NSG-59	
		AsA	Root length (cm)	Number of roots	Root length (cm)	Number of roots	Root length (cm)	Number of roots
To	Control	NT	1.1±0.06ab	14±0.37bc	1.±0.06b	9.4±0.2bc	1.3±0.058b	17±0.58b
TA	0 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1.1±0.06a	18±2.26a	1±0.04a	11±0.3a	1.4±0.041a	20±0.34a
T1	0.1 mM Lead Nitrate	NT	1.1±0.06ab	15±0.6b	1±0.04bc	9.2±0.4bc	1.2±0.051b	13±0.245d
T7	0.1 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1.1±0.04ab	14±0.51bc	1±0.03bc	9.8±0.4b	1.5±0.051a	15±0.245c
T2	0.2 mM Lead Nitrate	NT	1.1±0.07ab	14±0.4bc	0.9±0.04c	7.8±0.4d	1±0.02c	11±0.32e
T8	0.2 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1.±0.05cd	14±0.3bc	1±0.04bc	8.6±0.4cd	1.2±0.04b	14±0.316d
T3	0.3 mM Lead Nitrate	NT	1±0.037c	14±0.5bc	0.7±0.04d	5.8±0.6e	0.9±0.024d	9±0.316f
T9	0.3 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1±0.051bc	13±0.5c	0.9±0.02bc	8.6±0.245cd	1±0.037c	11±0.4e
T4	0.4 mM Lead Nitrate	NT	0.7±0.032d	11±0.4d	0±0g	0±0g	0±0g	0±0i
T10	0.4 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	0.9±0.04c	10±0.4d	0.5±0.051e	8±0.32d	0.64±0.04e	7±0.4g
T5	0.5 mM Lead Nitrate	NT	0±0f	0±0f	0±0g	0±0g	0±0g	0±0i
T11	0.5 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	0.5±0.12e	5±1.39e	0.3±0.011f	1.6±0.68f	0.44±0.02f	4±0.245h
T6	1 mM Lead Nitrated	NT	0±0f	0±0f	0±0g	0±0g	0±0g	0±0i
T12	1 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	0.44±0.024e	6±0.24e	0±0g	0±0g	0±0g	0±0i
$LSD_{0.05}$			0.156	1.53	0.124	1.043	0.097	0.876
Effect (Mean square) of Treatments (MS df = 13)			0.766***	162.6***	0.93***	80.91***	1.65***	248.02***
Error (df = 56)			0.0153	1.46	0.0095	0.68	0.006	0.48
Effect (Mean square) of Ascorbic acid pretreatment			0.315^{ns}	64.13*	0.28^{ns}	38.63*	1.57*	166.63*
			Two-way ANOVA of Root length			Two-way ANOVA of Number of roots		
Source of variation (SOV)		df	MS	F	p-value	MS	F	p-value
Varieties (V)		2	0.55	54.13	< 0.0001	476.41	552.75	< 0.0001
Treatments (T)		13	3.21	316.36	< 0.0001	449.46	521.47	< 0.0001
Interaction effect (VxT)		26	0.136	13.34	< 0.0001	27.53	31.94	< 0.0001
Error		168	0.01			0.86		

Table Comparative shoot number and shoot length in pretreated with 0.5mM ascorbic acid and nontreated callus at six different concentration of $Pb(NO_3)_2$ in three varieties of sugarcane.

Treatment details			YT-53		CP-77-400		NSG-59	
		AsA	Shoot length (cm)	Number of shoots	Shoot length (cm)	Number of shoots	Shoot length (cm)	Number of shoots
To	Control	NT	1.7±0.043b	15.8±0.98ab	1.96±0.051a	18.4±0.51a	1.56±0.051b	16.8±0.58b
TA	0 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	2.26±0.03a	21±0.83a	2.06±0.04a	19.4±0.4a	1.78±0.04a	19±0.44a
T1	0.1 mM Lead Nitrate	NT	1.78±0.02d	15.2±0.73c	0.92±0.037cd	12.8±0.49b	1.3±0.055c	13.2±0.37d
T1	0.1 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1.96±0.024c	18.4±0.93b	1.18±0.037b	12.4±0.245b	1.58±0.037b	15.4±0.51c
T2	0.2 mM Lead Nitrate	NT	1.44±0.05ef	11.8±0.37d	0.82±0.037d	11±0.32b	1.04±0.024d	10.6±0.51e
T2	0.2 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1.84±0.05d	14.2±0.66c	1.08±0.037bc	13.2±0.37b	1.1±0.045d	12.8±0.37d
T3	0.3 mM Lead Nitrate	NT	1.34±0.05f	11.8±0.37d	0.6±0.052e	6.8±0.71c	0.84±0.0245e	7.2±0.2f
T3	0.3 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1.48±0.037e	13.6±0.51c	0.94±0.04cd	12.2±0.37b	1.06±0.0245d	10.4±0.245e
T4	0.4 mM Lead Nitrate	NT	0.88±0.02h	7.6±0.245f	0±0g	0±0d	0±0g	0±0h
T4	0.4 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	1.2±0.055g	9.4±0.245e	0.54±0.024e	5.2±0.213c	0.78±0.049e	6.8±0.49f
T5	0.5 mM Lead Nitrate	NT	0±0j	0±0h	0±0g	0±0d	0±0g	0±0h
T5	0.5 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	0.92±0.037h	7.8±0.37ef	0.18±0.051f	2.2±0.36d	0.56±0.0245f	4.8±0.2g
T6	1 mM Lead Nitrated	NT	0±0j	0±0h	0±0g	0±0d	0±0g	0±0h
T6	1 mM Lead Nitrate + 0.5mM Ascorbic Acid	T	0.74±0.024i	3±0.265g	0±0g	0±0d	0±0g	0±0h
$LSD_{0.05}$			0.1	1.64	0.163	2.44	0.0933	0.9637
Mean square of Treatments (MS df = 13)			2.56***	230.18***	2.378***	243.44***	2.007***	223.97***
Error (df = 56)			0.006	1.67	0.0165	3.72	0.0054	0.579
Effect (Mean square) of Ascorbic acid pretreatment			2.84**	160.5*	100.8*	86.91*	1.6*	163.56*
			Two-way ANOVA of Shoot length			Two-way ANOVA of Number of shoots		

Source of variation (SOV)	df	MS	F	p-value	MS	F	p-value
Varieties (V)	2	4.496	1.96	0.145	175.66	88.25	< 0.0001
Treatments (T)	13	18.761	8.16	< 0.0001	678.61	340.93	< 0.0001
Interaction effect (VxT)	26	7.02	3.06	< 0.0001	9.49	4.77	< 0.0001
Error	168	2.298			1.99		

Figures

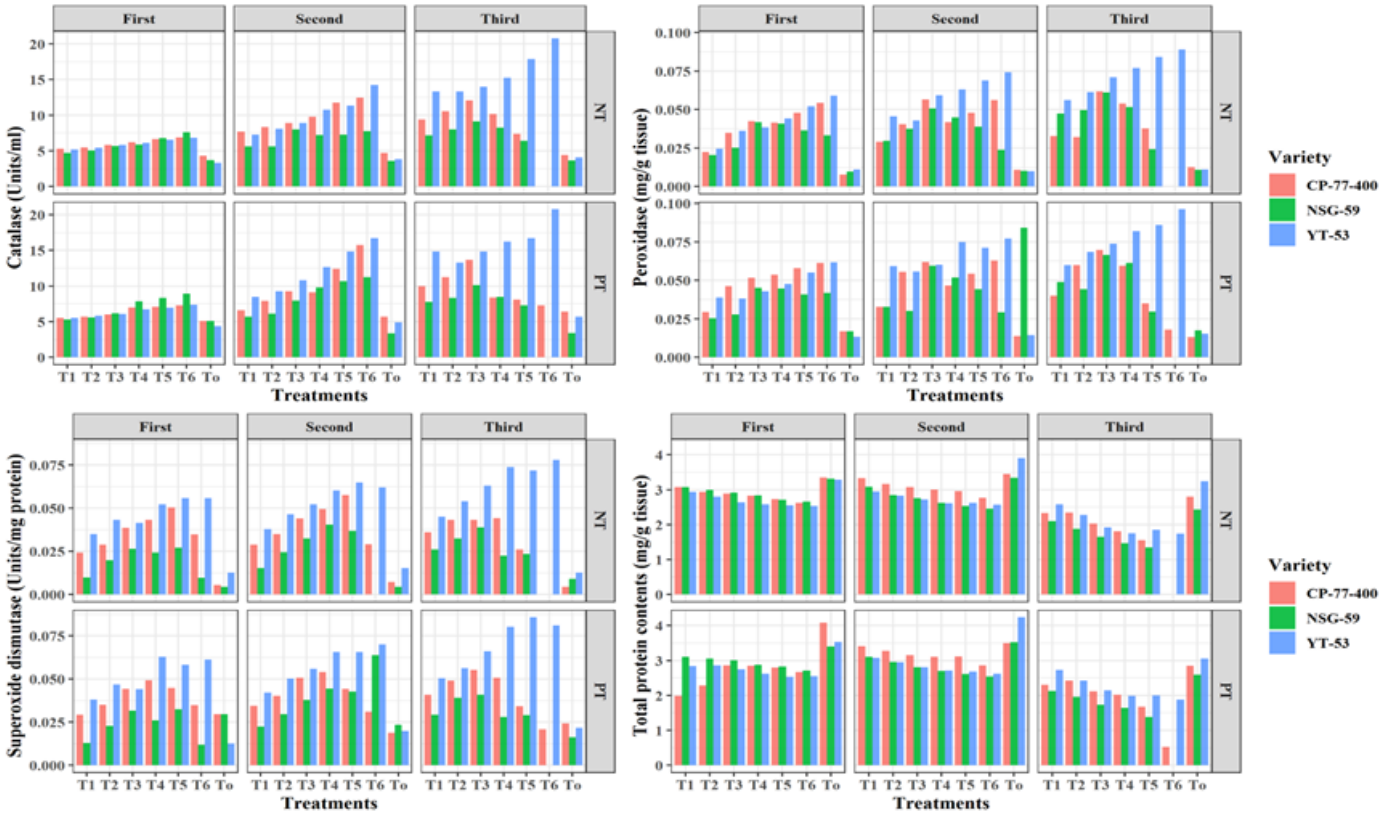

Figure 1

Specific activities of CAT, POD and SOD and total protein contents in 0.5mM ascorbic acid pretreated and nontreated callus maintained on their respective media with six different concentration of $Pb(NO_3)_2$ in three varieties of sugarcane after first second and third month

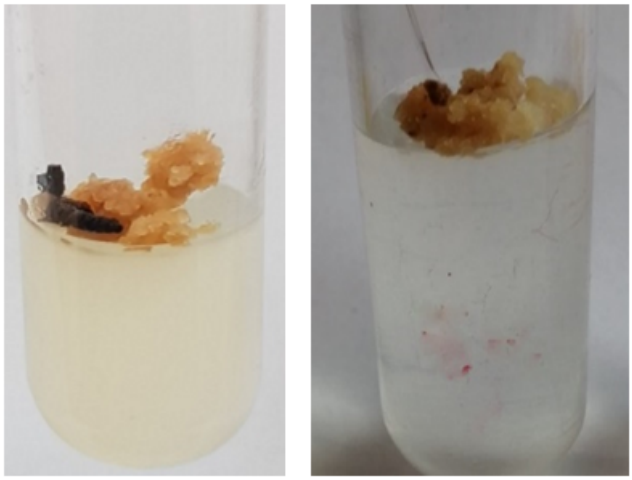

Figure 2

(a) Non pretreted callus culture of YT-53 maintained at 1mM $Pb(NO_3)_2$ + 3.5mg/l 2,4-D at day 150(2x)

(b)AsA pretreated callus culture of YT-53 maintained at 1mM $Pb(NO_3)_2$ + 3.5mg/l 2,4-D at day 150(2x)

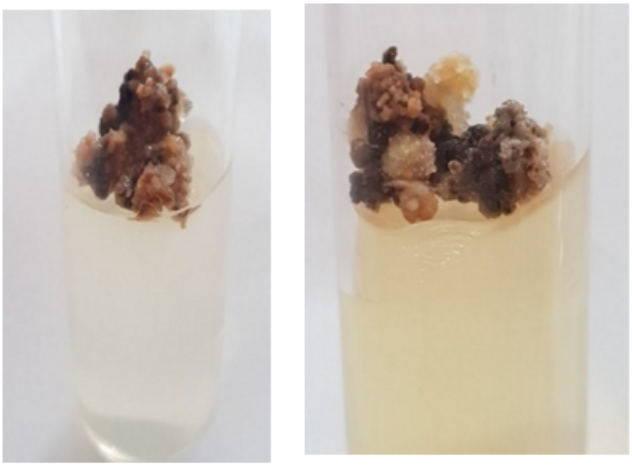

Figure 3

(a)Non pretreated callus culture of CP-77-400 maintained at 1mM $Pb(NO_3)_2$ + 5mg/l 2,4-D at day 150(2x)

(b)AsA pretreated callus culture of CP-77-400 maintained at 1mM $Pb(NO_3)_2$ + 5mg/l 2,4-D at day 150(2x)

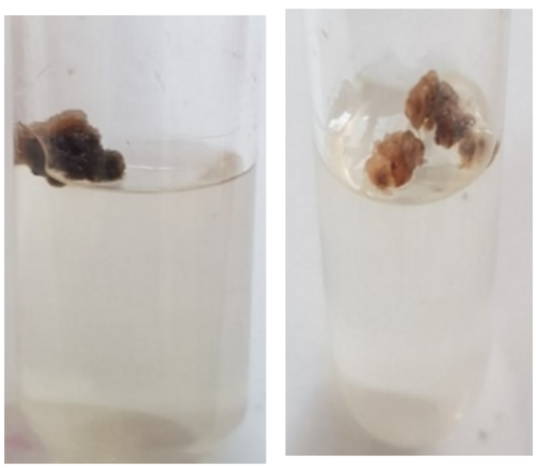

Figure 4

(a)Non pretreated Callus culture of NSG-59 maintained at 1mM $Pb(NO_3)_2$ + 3.5mg/l 2,4-D at day 150(2x)

(b)AsA pretreated Callus culture of NSG-59 maintained at 1mM $Pb(NO_3)_2$ + 3.5mg/l 2,4-D at day 150(2x)

Figure 5

(a) Regenerated plant of YT-53 developed from callus nonpretreated callus and maintained on 1mM $Pb(NO_3)_2$ +2mg/l kin at day 90(2x)

(b)Regenerated plant of YT-53 developed from 0.5mM AsA pretreated callus and maintained on 1mM $Pb(NO_3)_2$+2mg/l/l kin at day 90 (1x)

Figure 6

(a)Regenerated plant of CP-77-400 developed from non pretreated callus maintained on 0.5mM AsA maintained on $Pb(NO_3)_2$ +1mg/l BAP at day 90 (2x)

(b)Regenerated plant of CP-77-400 developed from 0.5mM AsA pretreated callus maintained on 0.5mM $Pb(NO_3)_2$ +1mg/l BAP at day 90 (1x)

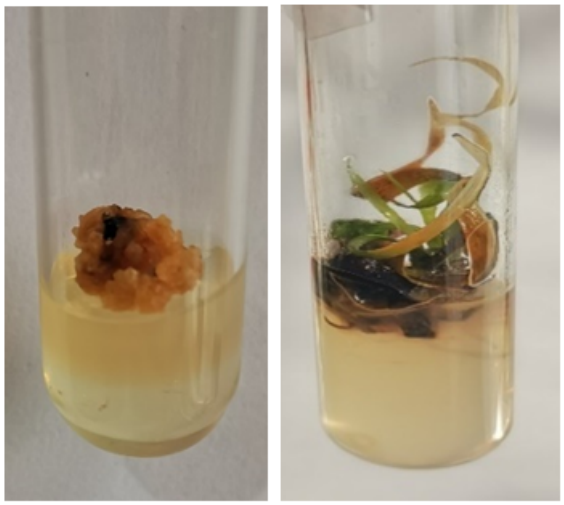

Figure 7

(a)Regenerated plant of NSG-59 developed from nonpretreated callus maintain at 0.5mM $Pb(NO_3)_2$ +2mg/l Kin. at day 90 (1x)

(b)Regenerated plant of NSG-59 developed from 0.5mM AsA pretreated callus maintain at 0.5mM $Pb(NO_3)_2$ +2mg/l Kin. at day 90 (1x)

Supplementary Files

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