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The Effect of Pro-oxidant, Anti-oxidant and Anti-Cancerous Drug on Colchicine Induced Polyploidy Cells of Grass Pea Seedling

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Abstract In an attempt to correlate polyploidy in grass pea and tumorigenicity, the effects of pro-oxidant, antioxidant and anti-cancerous drugs are evaluated. An increase in H_2O_2 content and lipid peroxidase (LPx) activity is associated with the stress along with the hike in the antioxidant enzyme levels in colchicine-induced polyploidy. However, ascorbic acid (AA) and methotrexate (MTX) cure oxidative damage owing to their free radical scavenging role and cell division arresting functionality and restoring the cells from polyploidy. This restoration is marked by a fall in the antioxidant enzyme activity namely catalase (CAT), LPx and superoxide dismutase (SOD). Although polyploidy has been controlled, normal growth is compromised as implied the change in radicle lengths of grass peas in various combinations of AA and MTX treatments. Soaking of seed with H_2O_2 aggravates stress and transforms polyploid cells into necrotic in the higher dose. This preliminary observation seems to be interesting to extrapolate the potentiality of antioxidants as well as anti-cancer drugs for retrieving cells from induced polyploidy.

Keywords Ascorbic Acid, Catalase, *Lathyrus Sativus* L., Methotrexate, Mitotic Index, Peroxidase, Polyploid Cell, Superoxide Dismutase

1. Introduction

In the plant kingdom, the manifestation of polyploidy is apparent in the chromosomal abnormalities and an increase in the chromosomal mass and number, leading to tumorigenicity [1]. Polyploidy bears an intricate relationship with tumorigenicity as indicated by the literature [2, 3]. Different sub populations of cancerous cells exhibit different levels of proliferation of the polyploid cells [4]. The above "Cause and Effect" relationship between polyploidy and tumorigenicity is evidenced in human cells like Pim 1 expressing human prostate and mammary epithelial cells, in genetically modified mice with Aurora A, Mad 2, Eg5, Apc oncogenes/tumor suppressors [5, 6]. Augmenting the expression of manganese superoxide dismutase, chromosomal instability is prevented in transgenic mouse models [7]. Similarly, an increase in polyploidy is visible in the liver subjected to oxidative stress [8].

Oxidative stress has originated from the generation of Reactive oxygen species (ROS) namely H_2O_2, superoxide, and singlet oxygen as a byproduct of cellular redox process [9]. ROS targets cellular macromolecules like carbohydrates, lipids, proteins and DNA and interferes with the regulation of transcription and translation [10]. Literature reveals an increase in the contents of reactive oxygen species in the cancerous cells compared to the

normal cells. It has myriad functional consequences and is considered to be an indicator of the onset of polyploidy and cancer [2, 11 – 13]. Oxidative stress induces apoptosis, necrosis, autophagy, mitotic catastrophe, and drug-resistant adaptation and very often causes oncogenic stimulation [6, 14].

Cells have several mechanisms to counteract oxidative stress. One of the pivotal players in this regard is the antioxidant enzymes that scavenge free radicals [15]. Most of the cancerous cells suffer from a fall in antioxidant enzymes compared to the normal cells [16]. Among the few anti-oxidant enzymes, Superoxide dismutase (SOD) converts superoxide radical to hydrogen peroxide [17]. On the other hand, Catalase (CAT) and peroxidase (PO) transform hydrogen peroxide into water [18]. Apart from antioxidant enzymes, a handful of low molecular weight antioxidants such as vitamin C, E and A along with glutathione combat oxidative stress as well [9, 19].

In the present study, an attempt has been made to assess the potential as well as the effect of a pro-oxidant (H_2O_2), antioxidant (ascorbic acid; AA) and an anticancerous drug (methotrexate; MTX) on induced polyploidy in grass pea seedling with respect to radicle length, polyploid cell frequency and different antioxidant enzymes activities. Although lowering in the antioxidant enzyme content is common in most human tumors as well as in salt-induced stressed plant cells, however, the role of antioxidants and pro-oxidants has not yet been studied in plant seedling that has undergone a status of polyploidy leading to tumorigenicity [16, 20, 21]. Subsequently, the role of an anticancerous drug (MTX) has also been studied to alleviate the state of polyploidy in plants in correlation to the antioxidant enzymes like CAT, SOD and LPx content and activity.

2. Materials and Methods

2.1. Germplasm and Treatments

Dry-filled seeds of grass pea (L. *sativus* L.; Family Fabaceae) were surface sterilized (0.1% $HgCl_2$) for 5 min and washed in distilled water (3 times, 10 min each). Washed seeds were soaked in distilled water for 6 h and then allowed to germinate in a cotton bed for 12 h. The seeds were then kept in an aqueous solution of colchicine (0.5%) for 8 h and then in different concentrations of ascorbic acid (0.5, 1.0, 1.5 and 2 mM), H_2O_2 (25, 50, 100 and 150 mM) and MTX (0.01, 0.1, 1 and 10 µM) for 3 days.

Seeds soaked in distilled water were marked as control A and in colchicine, as control B. All the dilution for preparing different concentrations was made with triple distilled water. Petri plates were kept at 25 ± 1 ℃ in an incubator.

2.2. Assessment of Radicle Length and Polyploidy Frequency

The radicle lengths (mm) were measured from germinating seedlings (randomly 25 seedlings) on the 3rd day of treatments. Seedling morphology was studied.

Polyploid frequency was measured from germinating root tips collected from control and treated materials (H_2O_2 and ascorbic acid treated). The root tips were fixed in 1:3 aceto alcohol overnight and preserved in 70 % alcohol. The root tips were stained in 2 % orcin-HCl (1 N) solution and squashed with 45 % acetic acid. The frequency of polyploidy cells was calculated using the formula: (polyploid cells/ total cell) × 100.

2.3. Antioxidant Enzymes Activity

The enzyme extract for SOD and CAT was prepared by grinding 0.5 g germinating seedlings in ice-cold extraction buffer (0.1 M potassium phosphate buffer of pH 7.6 containing 0.5 M EDTA) with a pre-chilled mortar and pestle. The homogenate was centrifuged at 4 ℃ in a refrigerated centrifuge (Remi C-24 plus) for 15 min at 10,000 rpm and the supernatant was used to determine enzyme activity.

SOD activity was estimated by recording the decrease in absorbance of nitro-blue tetrazolium dye [22, 23]. 3 ml of the reaction mixture was prepared which contained 100 mM potassium phosphate buffer (pH-7.6), 100 mM methionine, 1 mM nitro-blue tetrazolium (NBT), 0.5 M EDTA, 0.1 mL enzyme and distilled water for making up the mixture volume. The reaction was started by adding l00 µmol riboflavin and placing the tubes under three 5 W fluorescent lamps for 15 min. The reaction was stopped by the withdrawal of the light source. The reaction mixture without enzymes was used as a standard. A non-irradiated reaction mixture containing enzyme served as a blank. The tubes which were enzyme-less showed the maximum color and the blank showed no color development. Finally, absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbance reading of samples to 50% in comparison to reaction mixture lacking enzyme.

CAT activity was obtained by monitoring the decrease in absorbance due to hydrogen peroxide (H_2O_2) at 240 nm [23, 24]. The reaction mixture for measuring the CAT activity consisted of 3 mL of 50 mM potassium phosphate buffer (pH-7), 30% H_2O_2 and 50 µL enzyme extract, where only 50 mM potassium phosphate buffer (pH-7) was served as blank. The reaction began with the addition of H_2O_2 and the decrease in the absorbance value was recorded for a 30-sec interval of 5 min at 240 nm. Hence, by taking the standard curve of known concentrations of H_2O_2 as a reference the amount of H_2O_2decomposed was calculated and thus the amount of enzyme activity.

H_2O_2 activity was estimated by Velikova et al. [25]. The supernatant was collected in a separate test tube. 0.5 mL supernatant was added with 10 mM Phosphate buffer (pH-7.0) and 1 M of Potassium Iodide solution. The absorbance was recorded at 390 nm. Data were calculated by referring to a standard curve of known concentrations of H_2O_2.

The level of lipid peroxidation depends on the terms of thiobarbituric acid reactive substances (TBARS). Thus, the amount of TBARS was used to determine the lipid peroxidation level [26]. The protocol for estimation of LPx activity involved homogenization of the radicle sample (0.5 g) in 5 mL 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 15 min. The supernatant aliquot (2 mL) was added in 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was then heated at 95 ℃ for 30 min following which it was quickly cooled in an ice bath. The mixture was further centrifuged at 10,000 rpm for 10 min. The absorbance of supernatant was recorded at 532 nm and 600 nm. The TBARS content was calculated according to its extinction coefficient [155 mM^{-1} cm^{-1}]. The final result was obtained by subtracting the non-specific absorbance at 600 nm.

The protein sample was prepared by grinding 0.5 g radicle tissue in ice-cold extraction buffer (0.1 M potassium phosphate buffer of pH 7.6 containing 0.5 M EDTA) with a pre-chilled mortar and pestle. The homogenate was centrifuged at 4 ℃ in a refrigerated centrifuge (Remi C-24 plus) for 15 min at 10,000 rpm and the supernatant was used to determine total soluble protein using the Bradford method [27]. The absorbance of the blue color was read at 595 nm using a UV-VIS spectrophotometer. The amount of protein was quantified by using a standard curve of known concentration.

2.4. Statistical Analysis

Standard errors of the means were calculated and LSD ($P \leq 0.05$) was performed to check the level of significance of the difference with the help of ANOVA test using Microsoft Excel data analysis tools.

3. Results and Discussion

3.1. Effect of AA, H_2O_2 and MTX on Radicle Length and Polyploidy

Using Colchicine for the induction of polyploidy, various morphological features like radicle length and polyploid cell frequency of *L. sativus* have been studied in the presence of a different concentration of AA, H_2O_2 and MTX with respect to control A and control B. Distinct bulging of root tips and increase of nuclear volumes are associated with the induction of polyploidy i.e. control B (7.2%; Fig. 1:a-ii, b-ii, c-ii) compared to control A (Fig. 1:a-i, b-i, c-i)which is devoid of polyploid cells. However under the influence of various agents namely, AA, H_2O_2 and MTX in a range of concentrations (Fig. 1:a iii-iv, b iii-iv, c iii-iv) bulging of root tips as well as gradual changes in the radicle length is marked as presented in Fig. 1.

A decrease in the radicle length could be ascribed due to the remission of polyploidy under the influence of exogenous agents. Ascorbic acid is a ubiquitous biomolecule with a non-enzymatic antioxidant role that is crucial for both animal and plant cells. This plays a pivotal role in offering resistance to oxidative stresses namely heavy metal, saline, ultra-violet radiation, etc. [28]. Utilizing the Asada–Halliwell–Foyer pathway or by direct quenching of ROS it executes its protective role against oxidative stress [29, 30]. Keeping in mind that ascorbic acid is directly absorbed and transmitted within the plant following exogenous application, its role in colchicine-induced polyploidy has been judged in grass peas [31 – 35]. As indicated in Fig. 1, 2 although polyploidy has been controlled normal radicle length can't be retrieved in the presence of AA in grass pea. AA treatment decreases the bulging of root tips albeit in higher doses it stops the growth. H_2O_2 diminishes the tendency of polyploid cell occurrences (treatments 3.7 to 0.17%) and the radicle morphology becomes necrotic with increased concentration of H_2O_2. MTX reduces polyploidy by virtue of its inhibition of cell division in a dose-dependent manner [36]. Although the radicle length of grass peas in treated varieties is lesser than the control seeds with respect to its normal length.

The isolated cell fraction from the radicle was centrifuged (9000 rpm, 10 min) after 3 days of treatment and was utilized for assessment of antioxidant enzyme activity like catalase, SOD and lipid peroxidase. The total cellular content of H_2O_2 has also been estimated.

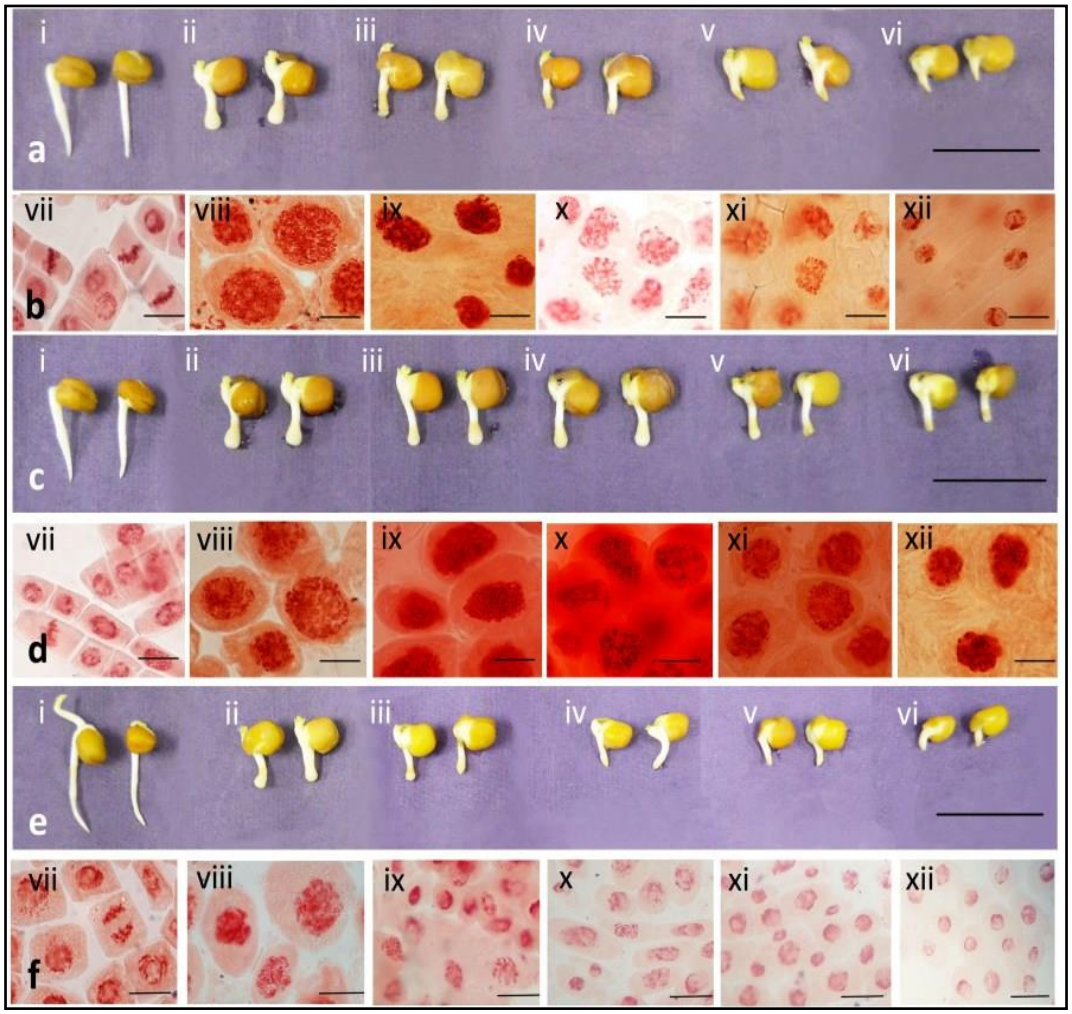

Figure 1. Effect of different concentrations of H_2O_2, ascorbic acid and MTX in *L. sativus.* Radicle morphology and divisional plates in the presence of **a,b** H_2O_2 **c,d** ascorbic acid **e,f** MTX (i. Control A, ii. Control B showing distinct bulging of root tips, iii-vii radicle morphology with the enhancement in concentration of treating agents). The divisional plates are represented as vii. Control A, viii. Control B, ix-xii respective divisional plates with different treatments). Scale bar=10 mm.

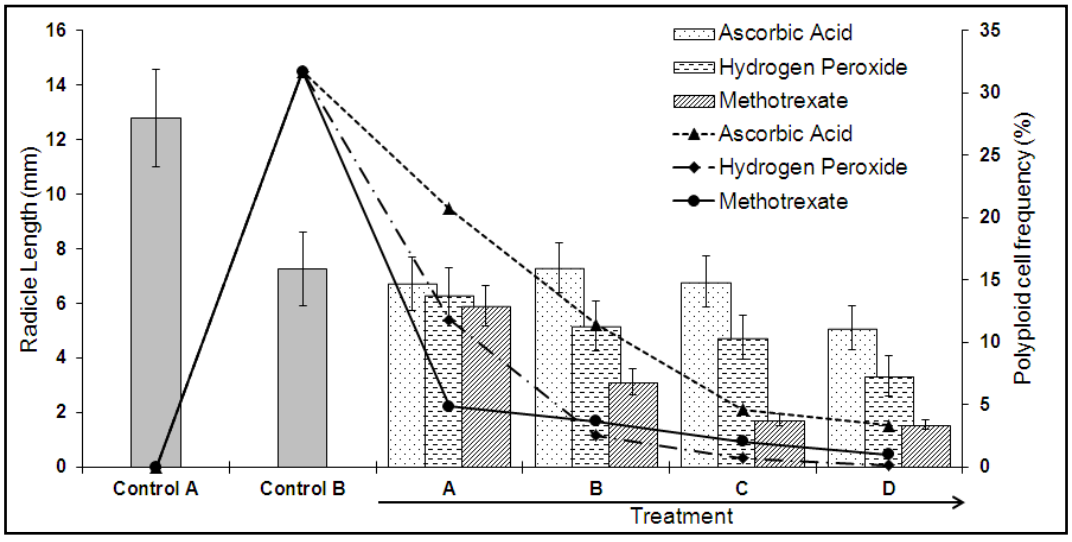

Figure 2. Assessment of radicle length and frequency of polyploid cells treated with colchicine followed by different concentrations of ascorbic acid, H_2O_2 and MTX (A, B, C, D - 0.5, 1.0, 1.5, 2.0 mM for ascorbic acid; 25, 50, 100, 150 mM for H_2O_2 and 0 01, 0.1, 1.0, 10 µmol for MTX)

3.2. Role of External H_2O_2 on Endogenous H_2O_2 Content and Lipid Peroxidation

When cells are subjected to stress due to the introduction of polyploidy, an inherent rise in the H_2O_2 content is manifested as an indication of oxidative stress. Compared to control A, in colchicine-treated cells (Control B) an obvious hike of 83.3% in the H_2O_2 level has been observed. However, in the AA-treated sets, there is a significant reduction of peroxide content (Fig. 3 A-D) which normalizes up to the H_2O_2 content of untreated cells (control A). AA quenches H_2O_2 owing to its natural free radical scavenging property. An enhancement in the concentration of H_2O_2, a common ROS has been observed in *L. stocksii* seedlings and in many other halophiles under higher salt circumstances [33, 37, 38]. Under the stressed conditions while cells are forced to divide in the presence of colchicine, a similar increase in H_2O_2 level has been noticed that has been augmented many folds (upto 45%) in the presence of externally added H_2O_2. However, MTX reduces H_2O_2 content by arresting polyploidy in A to D even below the control H_2O_2 content.

Compared to control A (1.46 µM TBARS content g^{-1}f.w.), the lipid peroxidation increases in control B (2.42 µM TBARS content g^{-1}f.w.) as more lipid synthesized by polyploidy cells. Lipid peroxidation can be considered cellular stress marker and has been inferred from malonaldehyde generated by the free radical oxidation reactions from cellular lipids. Increasing the concentration of MTX and AA, the LPx activity decreases gradually and at 10 µmol MTX, it is almost normalized (1.26 µM TBARS content g^{-1}f.w.). In the presence of H_2O_2, the peroxidation activity increases due to the rise in the total H_2O_2 content, however, at higher concentrations (C and D) it reduces due to cellular necrosis as apparent from Fig 4.AA due to its versatile, multifunctional character is offering an impasse inhibiting the increased lipid peroxidation and even it is more promising than MTX (D and C). In a report from the group of Ram ŕez et al. [39], it is known that AA recovers *Arabidopsis thaliana* from iron deficiency and prevents chlorosis. So AA-mediated remission of polyploidy as well as a decrease in lipid peroxidation is of significant biological importance to be studied in detail.

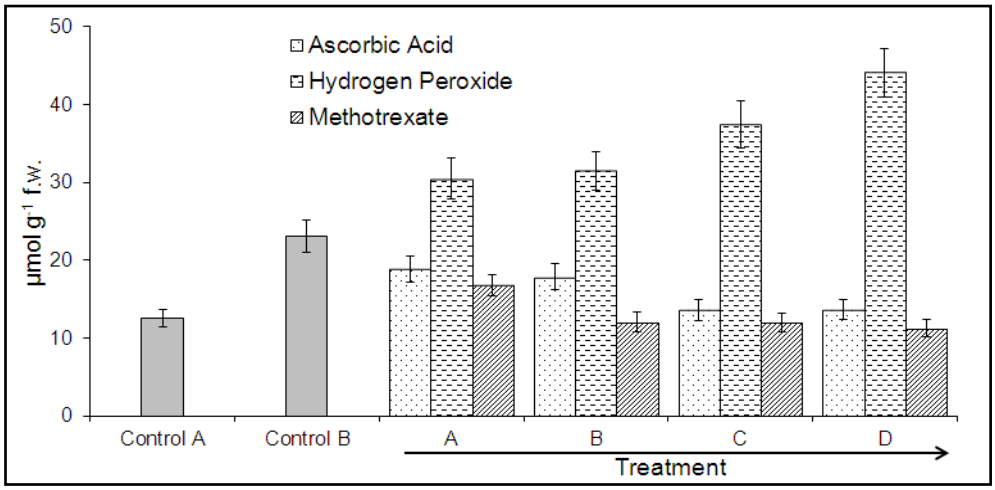

Figure 3. Effect of ascorbic acid, H_2O_2 and MTX on H_2O_2 content of *L. sativus* in the presence of different concentrations of ascorbic acid, H_2O_2 and MTX (A, B, C, D - 0.5, 1.0, 1.5, 2.0 mM for ascorbic acid, 25, 50, 100, 150 mM for H_2O_2 and 0.01, 0.1, 1.0 and 10 µM for MTX).

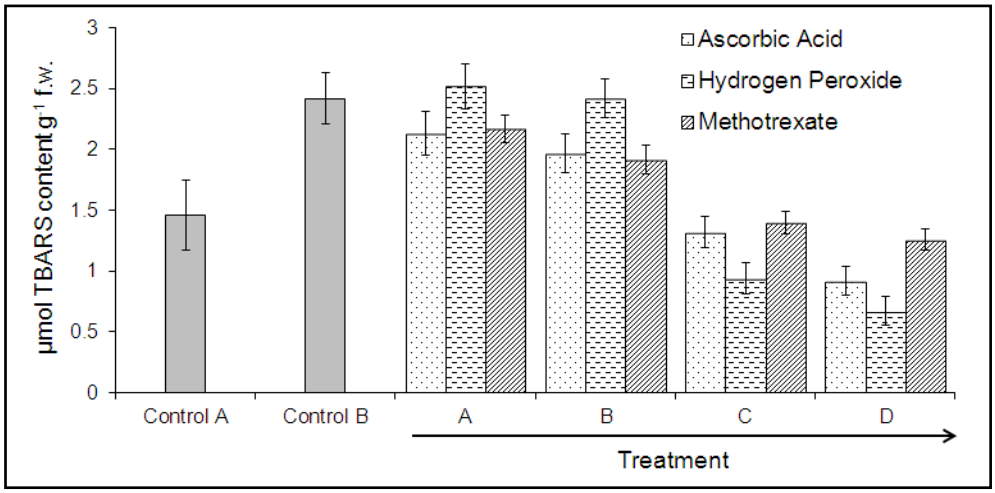

Figure 4. Effect of varying concentrations of ascorbic acid, H_2O_2 and MTX on LPx activity of *L. sativus*. Different concentrations of ascorbic acid, H_2O_2 and MTX were used (A, B, C, D - 0.5, 1.0, 1.5, 2.0 mM for ascorbic acid, 25, 50, 100, 150 mM for H_2O_2 and 0 01, 0.1, 1.0 and 10 µM MTX)

3.3. Effect of AA, H_2O_2 and MTX on Antioxidant Enzyme Activity in Correlation to Remission of Polyploidy

Antioxidant enzyme activity is regarded as the most effective defense mechanism against the threat of oxidative damage. Numerous reports exist in support of the protective effect of this antioxidant enzyme milieu against such an insult. To combat any stress generated following the onset of polyploidy, it is essential to check the profile of antioxidant enzyme activity. In the present study, SOD activity was calculated in control A and control B along with the sets where pre-incubation of seed has been performed with various concentrations of AA, H_2O_2 and MTX (as mentioned in legends). Compared to control A (3.6 Units min^{-1} mg^{-1} protein) more SOD activity wasobserved in control B (4.13 mg $protein^{-1}min^{-1}$) (Fig. 5). With increasing concentration of AA, MTX and H_2O_2, SOD activity decreases distinctly. All the results signify ($P \leq 0.05$) that both MTX and AA reduce the content of polyploidy cells (A) and also decrease SOD activity. However, in higher concentrations (B to D), all of them inclusive of H_2O_2 transform the polyploidy cells to a necrotic state due to enhanced stress with decreased SOD activity. Various reports concluded on the effect of salt stress on SOD activity shows increased salinity resulted in the rise of SOD function [20, 21]. The effect of various anticancer drugs on cell division and induced polyploidy of grass peas is also reported [40].

In addition to SOD, catalase activity has also been estimated. The Catalase activity is raised in control B where polyploidy has been induced (0.076 mM H_2O_2 min^{-1} mg^{-1} protein) as compared to control A (0.05 mM H_2O_2 min^{-1} mg^{-1} protein) to prevent oxidative damage induced by polyploidy (Fig. 6). In the presence of AA as well as MTX the enzyme activity decreases compared to control B level. In the presence of H_2O_2, the catalase action even reduces maintaining the same trend.

In comparison with the plant system, animal cancer cells produce less amount of antioxidant enzyme activity [16]. But in certain cases like adenocarcinoma, renal cell carcinoma and mesothelioma where an elevated level of SOD and CAT activity has been reported [16]. A 5-fold over expression of SODattenuates the malignant phenotype of human prostate carcinoma cells by inhibiting cell growth[41].

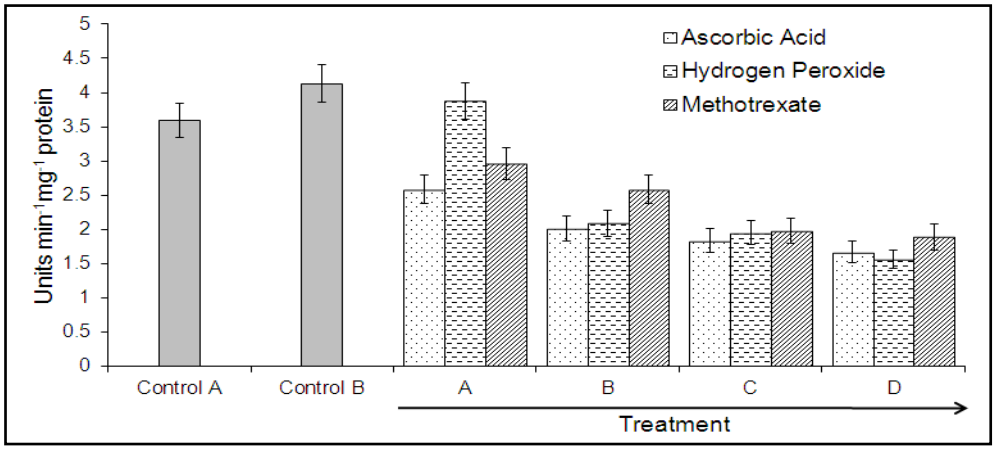

Figure 5. Effect of ascorbic acid, H_2O_2 and MTX on SOD activity of *L. sativus*. Different concentrations of ascorbic acid, H_2O_2 and MTX were used from A to D - 0.5, 1.0, 1.5, 2.0 mM for ascorbic acid, 25, 50, 100, 150 mM for H_2O_2 and 0 01, 0.1, 1.0, 10 µM for MTX

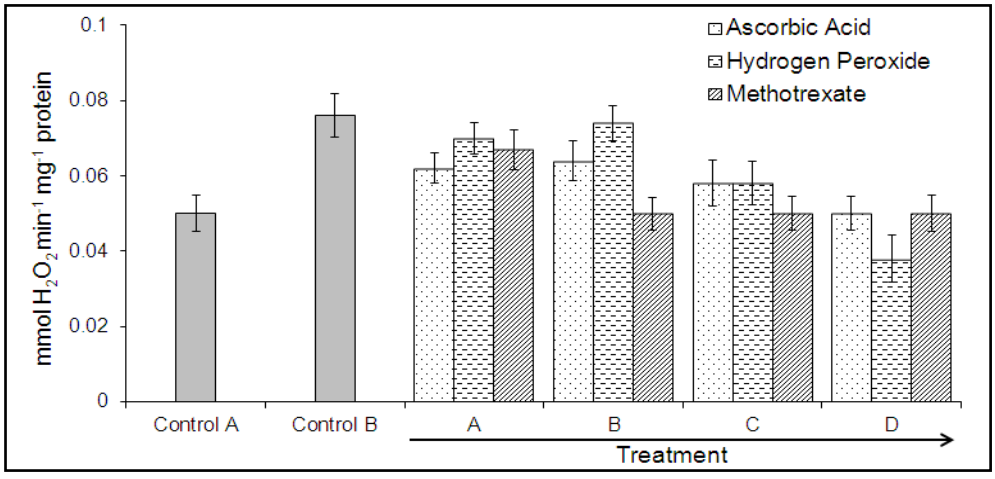

Figure 6. Effect of ascorbic acid, H_2O_2 and MTX on Catalase activity of *L. sativus* in the presence of different concentrations of ascorbic acid, H_2O_2 and MTX. (A, B, C, D - 0.5, 1.0, 1.5, 2.0 mM for ascorbic acid, 25, 50, 100, 150 mM for H_2O_2 and 0 01, 0.1, 1.0, 10 µM for MTX)

Plants are unique from animals w.r.t the presence ofplant-specific ROS-absorbing enzymes like Ascorbate peroxidase (APX), Monodehydroascorbate reductase (MDAR), and Dehydro-ascorbate reductase (DHR) [42]. Plants are better scavengers of ROS compared to animals as they can detoxify more ROS due to the presence of numerous oxidases and peroxidases. ROS at concentrations below the cytotoxic level and above cytostatic range drive biological redox reactions. It also promotes cell proliferation, stress management, defense against pathogens, transduces signals and triggers programmed cell death in plants.

4. Conclusions

In the present study, at higher doses of AA or MTX, the status of polyploidy has been controlled in grass pea seedlings; simultaneously the growth of grass pea seedlings is compromised. Although the production of ROS is detrimental to cell health and defense mechanisms are active for its prevention, still in a recent report it is described that "ROS is good". If the balance between ROS production and scavenging is disrupted, it results in the formation of smaller plants with limitations in growth and reproduction albeit they are alive. So, it can be appropriately concluded by Ron Mittler, in "*Trends in plant science*" that"if you are an aerobic organism, ROS are good, but too much or too little, of a good thing, can be bad for you". In the present context, however, it is manifested that pro-oxidant, antioxidant and MTX modulate the status of polyploidy in grass pea seedlings in a specific manner. However, detailed studies are needed to draw a definite conclusion.

Abbreviations

AA	Ascorbic acid
APX	Ascorbate peroxidase
CAT	Catalase
DHR	Dehydroascorbate reductase
EDTA	Ethylenediaminetetraacetic acid
LPx	Lipid peroxidase
LSD	Least significant difference
MDAR	Monodehydroascorbate reductase
MTX	Methotrexate
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

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Declarations

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable

Availability of Data and Material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interest.

Funding

Not applicable

Authors' Contributions

AS, TRM and SD performed the experiments, analyzed the data, and wrote the manuscript. AS, SG and SD designed the work and corrected the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest in the publication.

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