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Hydrogen peroxide promotes secondary cell wall biosynthesis and affects ultra-structural anatomy of Arabidopsis thaliana

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ABSTRACT

Reactive oxygen species (ROS) have been shown to act as messengers during plant growth and development. Among ROS molecules, hydrogen peroxide (H_2O_2) at an optimal level function as an important signaling molecule which promotes growth in plants. In the present study, we analyzed the role of exogenous H_2O_2 and demonstrated that H_2O_2 as an essential signaling molecules role in cell wall biosynthesis. In this study, interestingly, application of exogenous H_2O_2 (0, 2, 4, 6, 8, 10 mM) promotes secondary cell wall development as observed in microscopic studies. We also examined the germination of Arabidopsis seeds in presence of different concentrations of exogenous H_2O_2. H_2O_2 promoted different percentage of germination depending on the concentration of H_2O_2, showing that H_2O_2 acts as a signal molecule regulating germination in a concentration-dependent manner. The content of endogenous H_2O_2 in the leaves of Arabidopsis increased significantly following exogenous hydrogen peroxide treatment. The antioxidant activity of superoxide dismutase (SOD), ascorbate oxidase (APX) and catalase (CAT) activity were enhanced in Arabidopsis after treatment with exogenous hydrogen peroxide and remained at an elevated level after 5d of treatment in comparison with the non-treated plants. The increases in these antioxidant enzymes correlated with the increase in the H_2O_2 levels. The microscopic analysis showed that H_2O_2 induced structural changes related to plant secondary cell wall development. We further found that H_2O_2 affected yield, growth and biomass of Arabidopsis. These results suggest that H_2O_2 signaling may play an important role in plant development by modulating the secondary cell wall development pathway in *Arabidopsis*.

I. INTRODUCTION

Reactive oxygen species (ROS) control many different processes in plants (Miller et al. 2008). In the past many years, hydrogen peroxide (H_2O_2) is considered to be a toxic cellular metabolite in the environmental stress. Recently, many reports suggest that an H_2O_2 at low concentrations acts as a signaling molecule, while at higher concentrations it provokes the onset of cell death (Gechev and Hille 2005). H_2O_2 has been recognized as a signal molecule involved in gene expression regulation (Mittler et al., 2011). Plants have the effective enzymatic antioxidant defense system including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) enzymes. This system allows for scavenging of ROS leading to protection of plant cells from oxidative damage (Safar et al. 2009; Jaleel et al. 2008). Superoxide dismutase and catalase is responsible for the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. H_2O_2 also play a key role in the completion of germination and should be considered as messengers or transmitters of environmental cues during seed germination (Bailly, El-Maarouf-Bouteau & Corbineau 2008). Exogenous application of H_2O_2 improved seed germination in many plants, including camphor, barley, spinach, Zinnia elegans and almond (Chen and Lin 1994 Fontaine et al. 1994; Katzman, Taylor and Langhans 2001; Ogawa and Iwabuchi 2001; Zeinalabedini et al. 2009).

Plant cell walls are composed of cellulose, lignin and structural proteins in variable amounts. The cell wall is of critical importance for the cell shape and provides mechanical strength to withstand the turgor pressure. Secondary cell walls are deposited in specialized tissues such as xylem and sclerenchyma, providing strength, which allows plants to grow upright, and transport water efficiently (Wang et al., 2013; Zhong and Ye, 2015). Secondary cell walls are primarily made up of three polymers: cellulose, hemicellulose, and lignin. Phenylalanine ammonia lyase is one of the most frequently studied enzymes of the lignin biosynthesis pathway because it is the first enzyme in the pathway (Kováčik and Klejdus, 2012). Furthermore, there is evidence that this enzyme catalyzes the rate-limiting step in the phenylpropanoid pathway (Bate et al., 1994). H_2O_2 has also been involved in cell wall biosynthesis; it is now well proved that H_2O_2 is a regulator of a multitude of physiological processes like cell wall strengthening, senescence, photosynthesis, stomatal opening and the cell cycle (Bienert et al. 2006).

The objective of the present study is to investigate the role of H_2O_2 in secondary cell wall biosynthesis signaling and also on germination and yield in wild-type (Col-0) Arabidopsis plants. Our results suggest that at a low dosage of exogenously applied H_2O_2 functions as a developmental signal for the onset of secondary wall differentiation.

II. MATERIAL AND METHODS

Plant material, growth conditions, and hydrogen peroxide treatment

Seeds of WT Arabidopsis thaliana were grown in Murashige and Skoog (MS) medium for 10 days and transplanted thereafter to the soil mixture of vermiculite: peat moss: perlite (1:1:1) with MS medium and in the greenhouse under a 16 h light and 8 h dark cycle at 20±1º C and light intensity of 60-70 µmol PPFD m^{-2} s^{-1}. For stress treatment, 21d old seedlings of WT plants were sprayed with the desired concentration of H_2O_2 (0, 2, 4, 6,

8 and 10 mM). Three biological replicates were collected from each sample at respective time points after respective time points.

Gene-specific semi-quantitative PCR

Total RNA was isolated from H_2O_2 (0, 2, 4, 6, 8 and 10 mM) treated WT Arabidopsis plants using Total RNA extraction kit (Real Genomics). One microgram of total RNA was used for oligo (dT) primed first-strand cDNA synthesis in 20 ml reaction using of Superscript III Reverse transcriptase (Invitrogen). Constitutively expressed GAP-C (Glyceraldehyde-3-phosphate dehydrogenase C subunit) was amplified simultaneously in 27 cycles to ensure equal amounts of cDNA used. Details of the primers used in this study are given in Table 1.

Microscopic Analysis

Confocal microscopy analysis was done as described earlier Gill et al. (2010). The stem of WT was harvested and fixed in formalin, glacial acetic acid and 50% ethyl alcohol (FAA) (1:1:18) at room temperature. Samples were subsequently dehydrated in a tertiary butyl-alcohol series (Jensen, 1962); embedded in paraffin (melting point 58–60°C) and 8–10 mm sections were cut using a Finesse microtome. Sections were stained with 1% safranin in water and with 4% fast green in clove oil for 4 h and for 30 s, respectively. These were mounted in Canada balsam and examined using Confocal Laser Scanning Microscope (Zeiss LSM510 Meta Gmbh, Germany) equipped with a Zeiss Axiovert 100 M inverted microscope. Lignin auto fluorescence was collected by excitation/emission wave- lengths 488/505 nm.

Estimation of antioxidant enzymes

Leaf samples (100 mg) were homogenized in a pre-cooled mortar in homogenizing buffer containing 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5 % (v/v) Triton-X100 and 10 % (w/v) PVPP in 50 mM phosphate buffer pH 7.8. For APX activity, homogenizing buffer contained ascorbate in addition and the buffer pH was set to 7.0. The homogenate was transferred to 1.5 ml tubes and centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was collected and total SOD and APX activities were estimated. The total SOD activity was measured by adding 5 µl enzyme extract to the reaction mixture (200 µl) containing 1.5 µm riboflavin, 50 µm NBT, 10 mM Dl-methionine and 0.025 % (v/v) Triton-X100 in 50 mM phosphate buffer. One unit of enzyme activity was defined as the amount of enzyme required for 50 % inhibition of NBT reduction per min at 25 °C. Specific activity of SOD was calculated accordingly. APX activity was determined by following the oxidation rate of ascorbate at 290 nm as described by Nakano and Asada (1981). Catalase (CAT) activity was measured spectrophotometrically at room temperature by monitoring the decrease in absorbance at 240 nm resulting from the decomposition of H_2O_2. Catalase activity was measured according to the method of Aebi (1983). One unit (U) of catalase activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under assay conditions. The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2 and 100 µL of crude extract in a total volume of 3.0 mL. Protein content was estimated according to the dye binding method of Bradford using BSA as standard (Bradford 1976).

Measurement of hydrogen peroxide content

The level of H_2O_2 was measured following Sonja et al. (2002) method with some modifications. *Arabidopsis* leaves (100 mg) were ground to a fine powder in liquid nitrogen and the powder was extracted in 2 ml 1 M

$HClO_4$. Extraction was performed in the presence of insoluble PVP (5 %). Homogenates were centrifuged at 12,000×*g* for 10 min at 4 °C and the supernatant was neutralized with 2.5 M K_2CO_3 to pH 5.6 in the presence of 100 µl 0.1 M phosphate buffer (pH 5.6). The homogenate was centrifuged at 12,000×*g* for 1 min to remove $KClO_4$. The sample was incubated prior to assay for 10 min with 1 U ascorbate oxidase (Sigma Aldrich, India) to oxidize ascorbate. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5); 3.3 mM DMAB; 0.07 mM MBTH and 50 ng POX (Sigma Aldrich, India). The reaction was initiated by addition of an aliquot (50 or 100 µl) of the sample. The absorbance change at 590 nm was monitored at 25 °C. For each assay, H_2O_2 contents in the extract were quantified by reference to an internal standard (1.5 nmol H_2O_2, added to the reaction mixture on completion of the absorbance change due to the sample).

Statistical Analysis

All experiments were conducted with at least three independent repetitions in triplicates (biological triplicates). All values are shown as the mean ± the standard deviation. The statistical analysis was performed using Statistica software (*v.7*). The statistical significance of the mean values was assessed by analysis of variance (ANOVA) applying Duncan's Multiple Range Test (DMRT). A probability level of $P \leq 0.05$ was considered significant.

Germination

Arabidopsis plants were imbibed in dH_2O or H_2O_2 (0, 2, 4, 6, 8 and 10 mM) for 24 h. Then, seeds were washed twice with dH_2O and placed in Petri dishes. Seeds were incubated at 25 °C for 48 h, with a 12-h photoperiod (600-lux light intensity) or in darkness, at 4 °C. These experiments were repeated at least six times to determine the effect of H_2O_2 on germination.

III. RESULTS AND DISCUSSION

Effect of the H_2O_2 application on plant growth

Arabidopsis plants were subjected to different levels of exogenous H_2O_2 (0, 2, 4, 6, 8, 10 mM). After 1 week of H_2O_2 application, significant difference in terms of plant morphology were visible, indicating that H_2O_2 has some role to play in growth and development of Arabidopsis (Fig.1). Further, the growth rate of treated plants was faster as compared to untreated plants, which could be observed with the well-developed rosette formation in H_2O_2 treated plants (8 and 10 mM, Fig.1). ROS control many different processes in plants, including growth, development, and response to biotic and abiotic stimuli (Miller et al. 2008).

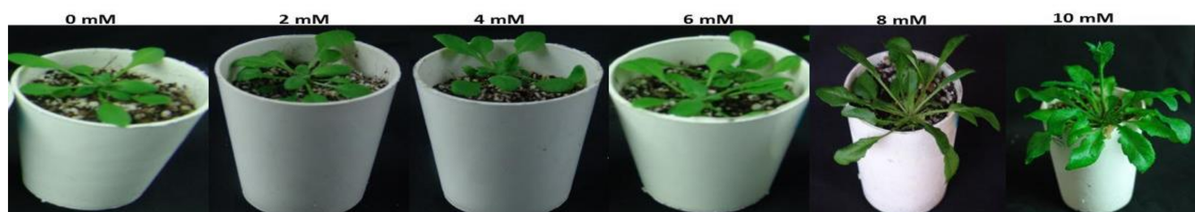

Fig.1 Growth of Arabidopsis under different concentration of hydrogen peroxide (0, 2, 4, 6, 8, 10 mM) after 3

H_2O_2 application induced structural changes in Arabidopsis

The ultrastructural study was performed on Arabidopsis supplemented with different concentration of H_2O_2 were done with bright field and confocal microscope (Fig.2A). Staining of histological sections revealed that the intensity of stain (Safranin) in vascular bundle of Arabidopsis stem sections increased with the increasing H_2O_2 levels (Fig.B). Similar results were observed from confocal micrographs, were the intensity of fluorescence increased with the higher concentration of H_2O_2 treatment (Fig.C). Exogenous H_2O_2 has been used to investigate its role in the mediation of cell wall stiffening (Schopfer, 1996). In cell suspension cultures of Picea abies, apoplastic H_2O_2 has been demonstrated to play a role in lignin formation (Karkonen and Koutaniemi, 2010). Exogenous H_2O_2 has also been demonstrated to mediate cell wall stiffening in maize coleoptiles (Schopfer, 1996). These results suggest that H_2O_2 may serve as a signal for the onset of secondary wall deposition.

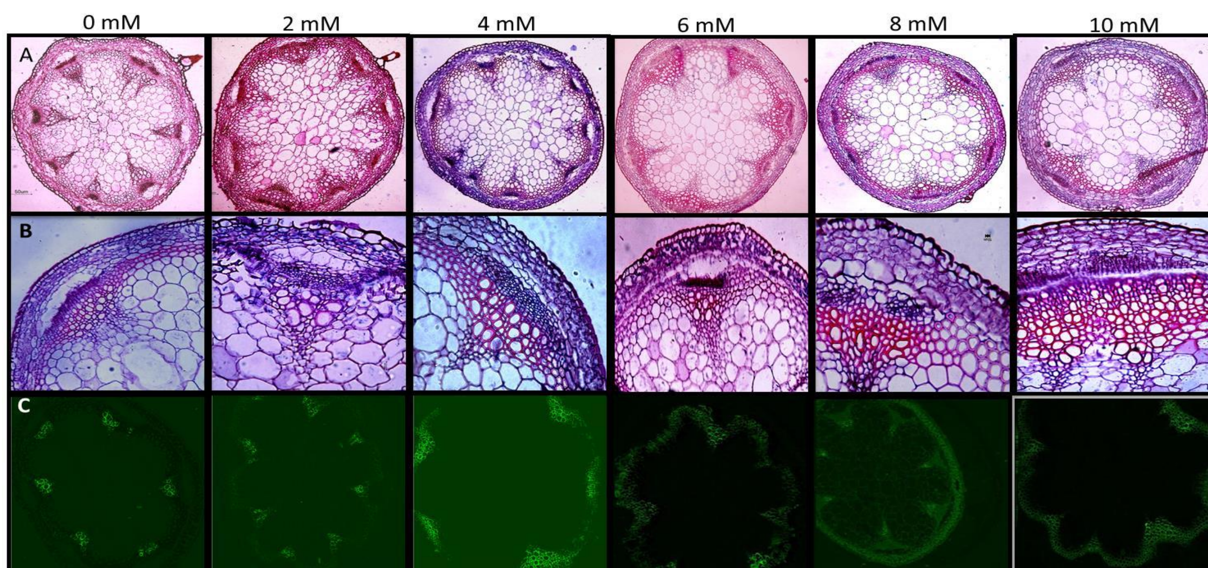

Fig.2 Anatomical studies of Arabidopsis stem sections under bright field microscope (A, B) and confocal microscope (C) at different concentrations of H_2O_2 (0 mM, 50 mM, 100 mM and 150 mM).

Secondary cell wall biosynthesis genes upregulation

To study the possible relationships between the induction of lignin accumulation, and differentiation of secondary walls, we examined the status of two important genes of lignin biosynthesis pathway PAL (Phenylalanine ammonia lyase), which catalyzes the first step of phenyl propanoid pathway and PXR (peroxidases) which helps in polymerisation of lignin monomers (Fig.3). It is recognized that ROS, especially H_2O_2, have a major role in cellular signaling pathways in plants (Neill et al. 2002; Hancock et al. 2006). This molecule plays a dual role in plants: at low concentrations it acts as a messenger molecule involved in signaling, triggering tolerance against various environmental stresses (Karpinski et al. 1999; Dat et al. 2000), whereas at high concentrations it orchestrates programmed cell death (Dat et al. 2003). It was observed that the expression of PAL and PXR genes increased after 8 and 10 mM H_2O_2 treatment, which indicates that the pathway has been influenced or induced by H_2O_2 signaling (Fig.3). Peroxidases need H_2O_2, a common reactive oxygen species (ROS) in plants, as a co-substrate. An excess of peroxidase activity, cross-linkable substrates and sufficient

amounts of H_2O_2 will favor the local stiffening of the wall, reduce cell wall expansion and thus strengthen the mechanical stability of the cells and organs (Tenhaken et al. 2014).

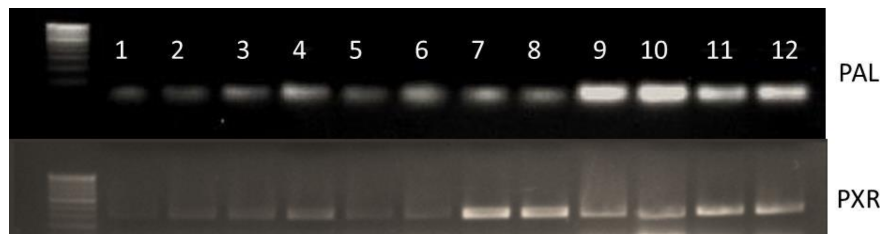

Fig. 3 Effect of H_2O_2 concentration on expression of Pal and PXR genes. Lane1-2 (0 mM), lane 3-4 (2 mM), lane 5-6 (4 mM), lane 7-8 (6 mM), lane 9-10 (8 mM) and lane 11-12 (10 mM).

Enhanced antioxidant gene activity at a different concentration of H_2O_2 treatment

The effect of H_2O_2 priming on the anti-oxidant metabolism was studied in Arabidopsis in order to correlate changes in these enzymes with changes in plant growth. The differences between the different light regimes were also analyzed. An array of antioxidants is constantly on the alert for rising H_2O_2 concentrations and provides effective scavenging for it (Apel and Hirt 2004; Miller *et al.* 2010). This antioxidant system consists of several enzymes, such as catalase (CAT), ascorbate (APX) etc (Noctor and Foyer 1998; Asada 1999; Miller *et al.* 2010). The activities of the enzymes SOD, APX and CAT were higher when plants were subjected to a 6mM H_2O_2. Thus H_2O_2 pre-treatment produced increased activities of these antioxidant enzymes (Fig.4). However, the increase was only statistically significant when 8 mM H_2O_2 was used. Under these conditions, a threefold increase in H_2O_2 was recorded after 5d of treatment (Fig.4D). H_2O_2 accumulation corresponded to the time of secondary wall biosynthesis (Fig.4D). It is probable that the decline was caused by elevated levels of ROS, which induced antioxidant enzymes.

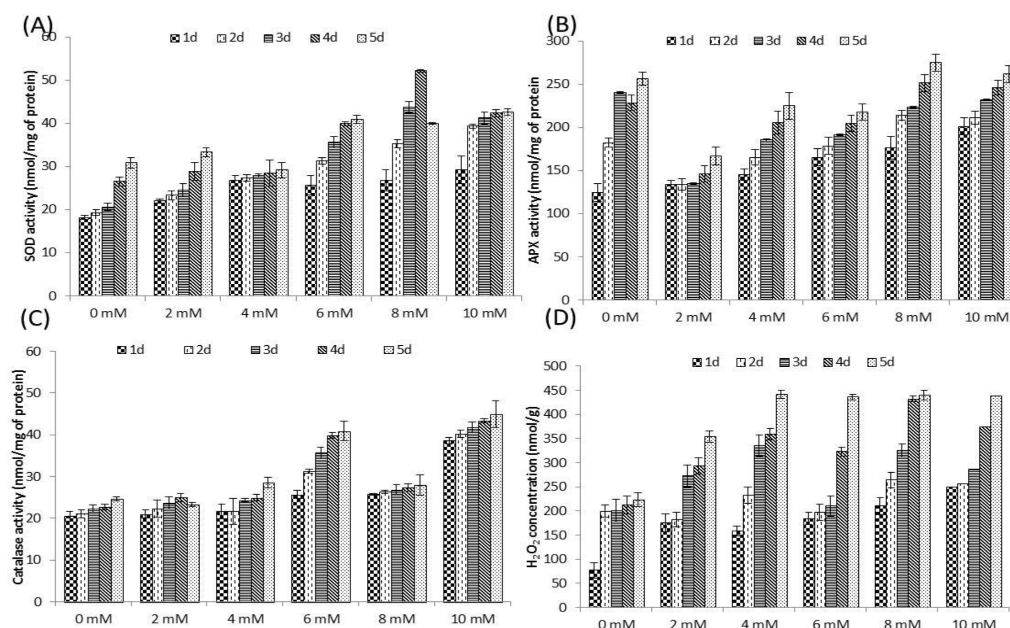

Fig. 4 Enzyme activity of Arabidopsis after 1d, 2d, 3d, 4d and 5d of 0, 2, 4, 6, 8 and 10 mM H_2O_2 treatment (A) SOD activity (B) APX activity (C) Catalase activity and (D) H_2O_2 concentration.

H_2O_2 application alters Arabidopsis yield, root and shoot biomass

The phenotypic changes were measured in terms of the shoot, root biomass, and seeds yield. There was an increase in the biomass and yield with the increase in the concentration of H_2O_2, but phenotypic differences were more pronounced after 4 mM H_2O_2 concentration (Fig.5). After 10 mM H_2O_2, the average weight of shoot, root biomass, and seed weight increased significantly and almost twice that of plants (0 mM).

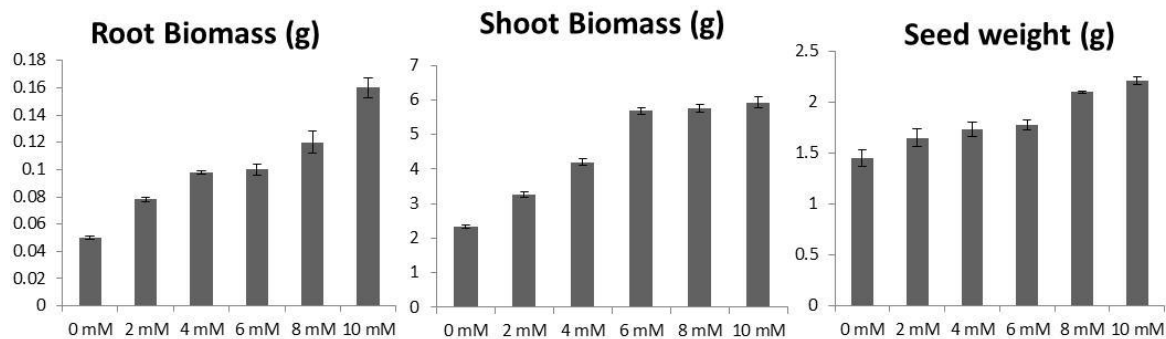

Fig. 5 Effect of H_2O_2 treatment on root, shoot biomass and seed weight

Germination percentage of Arabidopsis after exogenous H_2O_2 application

Germination of Arabidopsis seeds pretreated with different concentration H_2O_2 of was also calculated (Fig. 6). Nearly 70% of seeds had germinated at 24 h of incubation when imbibed with 10 mM H_2O_2. After 36 h of incubation, this percentage had reached nearly 84%, whereas the percentage of germination was around 60-70% with the 2, 4, 6, 8 mM H_2O_2 pre-treatment and 41% with dH_2O (0 mM). The percentage of germination reached 100% after 48 h for seeds pre-treated with H_2O_2, whereas the values for control seeds were around 60% after 48 h and 71% at 60 h (Fig. 6). This positive effect of H_2O_2 on seed germination was described previously in seeds from other plant species such as *Cinnamonun camphora* (Chen et al. 1993), *Hordeum vulgare* (Korytov and Nirimanov 1997), *Zinnia elegans* (Ogawa and Iwabuchi 2001), and almonds (Zeinalabedini et al. 2009). The positive effect of H_2O_2 on seed germination has been explained by the fact that the scavenging of H_2O_2 resulted in the production of O_2 for mitochondrial respiration and metabolic activities (Katzman et al. 2001). Another explanation is that H_2O_2 is helpful in cracking hard seeds, allowing them to interact with water (Chen et al. 1993).

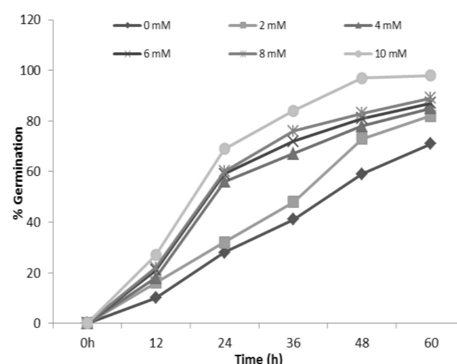

Fig. 6 Germination percentage of Arabdiopsis after exogenous application (0, 2, 4, 6, 8, 10 mM) of H_2O_2

IV. CONCLUSION

Overall, the data show that H_2O_2 stimulated germination and seedling growth and that these responses were correlated with the induction of genes related to plant cell growth, development, and signal transduction, as well as with the strong increase in the contents of SOD, APX, and CAT.

Table 1: Primer sequence, PCR conditions, and amplicon size for the PAL, PXR and GAP-C (reference gene) used for semiquantitative PCR

S.NO.	Genes	Primer Sequence 5′-3′	PCR conditions
1.	PAL (Phenylammonium lyase)	FW:GGAGATTAACGGGGCACACAAGAGC BW: AAGTTCCTTCTGAAGTGCGACACCG	94 °C - 3 min; 27cycles of 94 °C - 1 min, 62 °C- 1 min, 72 °C - 2 min; 72 °C - 7 min
2.	PXR (peroxidases)	FW: GCGAAGAGCTGTCCAAACGCAGAG BW:AGTCGTGAAATTACTCGTTGGAGGTGG	94 °C - 3 min; 27cycles of 94 °C - 1 min, 62 °C- 1 min, 72 °C - 2 min; 72 °C - 7 min
3.	GAP-C (Glyceraldehyde-3-phosphate dehydrogenase C subunit)	FW: CTTGAAGGGTGGTGCCAAGAAGG. BW: CCTGTTGTCGCCAACGAAGTCAG	94 °C - 3 min; 27cycles of 94 °C - 1 min, 55 °C- 1 min, 72 °C - 2 min; 72 °C - 7 min

REFERENCES

[1.] HE Aebi, Catalase. In: Methods of enzymatic analysis. Bergmeyer, H.U. (Ed.). Verlag Chemie Weinhem. 1983, 273-286.

[2.] K Apel, H. Hirt, Reactive oxygen species: metabolism, oxidative stress, and signal transduction, *Annual Review of Plant Biology, 55,* 2004, 373-399.

[3.] Asada K. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons, *Annual Reviews of Plant Physiology and Plant Molecular Biology, 50,* 1999, 601-639.

[4.] N. J. Bate, J. Orr, W. Ni, A. Meromi, T. Nadler-Hassar, P. W. Doerner, et al. Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis, *Proceedings of the National Academy of Sciences of the United States of America 91,* 1994, 7608–7612.

[5.] GP Bienert, J.K. Schjoerring, T.P. Jahn, Membrane transport of hydrogen peroxide, *Biochimica et Biophysica Acta , 1758*, 2006, 994-1003.

[6.] MM Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Annals Biochemistry, 72,* 1976, 248–254.

[7.] Z Chen, H. Silva, D.F. Klessing, Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid, *Science, 262,* 1993, 1883–1886.

[8.] J Dat, R. Pellinen, T. Beeckman, B. Van de Cotte, C. Langerbartels, J. Kangarjarvi, D. Inzé D. Van Breusegem F. Changes inhydrogen peroxide homeostasis trigger an active cell deathprocess in tobacco, *Plant Journal, 33,* 2003, 621–632.

[9.] J Dat, S. Vandenbeele, E. Vranova, M. Van Montagu, D. Inzé, F. Van Breusegem, Dual action of the active oxygen species during plant stress response. *Cellular and Molecular Life Sciences, 57,* 2000, 779–795.

[10.] TS Gechev, J. Hille, Hydrogen peroxide as a signal controlling plant programmed cell death, *Journal of Cell Biology, 168,* 2005, 17-20.

[11.] T Gill, Y. Sreenivasulu, S. Kumar, P.S. Ahuja, Over-expression of superoxide dismutase exhibits lignifications of vascular structures in Arabidopsis thaliana, *Journal of Plant Physiology, 167,* 2010, 757–760.

[12.] J Hancock, R. Desikan, J. Harrison, J. Bright, R. Hooley, S. Neill, Doing the unexpected: proteins involved in hydrogen peroxide perception, *Journal of Experimental Botany, 57,* 2006, 1711– 1718.

[13.] CA Jaleel, K. Jayakumar, Z. Chang-Xing, MM. Azooz, Effect of soil applied cobalt on activities of antioxidant enzymes in Arachis hypogaea, *Global Int J Mol Sci. 3,* 2008, 42-45.

[14.] WA Jensen, Botanical Histochemistry. 1962, p. 408. W. H. Freeman and Co., S. London.

[15.] A Karkonen, S. Koutaniemi, Lignin biosynthesis studies in plant tissue cultures, *Journal of Integrative Plant Biology, 52,* 2010, 176–185.

[16.] S Karpinski, H. Reynold, B. Karpinska, G. Wingsle, P.M. Mullineaux, Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis, *Science, 284,* 1999, 654–657.

[17.] LS Katzman, A.G. Taylor, R.W. Langhans, Seed enhancements to improve spinach germination, *Horticulture Science 36,* 2001, 979–981.

[18.] YN Korystov, A.A. Narimanov, Low doses of ionizing radiation and hydrogen peroxide stimulate plant growth. *Biologia, Bratislava, 52,* 1997, 121–124.

[19.] J. Kováčik, B. Klejdus, Tissue and method specificities of phenylalanine ammonia-lyase assay, *Journal of Plant Physiology, 169,* 2012, 1317–1320.

[20.] G Miller, N. Suzuki, S. Ciftci-Yilmaz, R. Mittler, Reactive oxygen species homeostasis and signalling during drought and salinity stresses, *Plant, Cell and Environment, 33,* 2010, 453-467.

[21.] G Miller, J. Coutu, V. Shulaev, R. Mittler, Reactive oxygen signalling in plants, In Intracellular Signalling in Plants (ed. Z. Yang) *Annual Plant Reviews, 33,* 2008, 189–201.

[22.] Y Nakano, K. Asada, Hydrogen peroxide is scavenged by ascorbate- specific peroxidase in spinach chloroplasts, *Plant Cell Physiology, 22,* 1981, 867–880.

[23.] SJ Neill, R. Desikan, A. Clarke, R.D. Hurst, J.T. Hancock, Hydrogen peroxide and nitric oxide as signalling molecules in plants, *Journal of Experimental Botany, 53*, 2002 1237–1247.

[24.] G Noctor, C.H. Foyer, Ascorbate and glutathione: keeping active oxygen under control, *Annual Reviews of Plant Physiology and Plant Molecular Biology, 49*, 1998, 249 -279.

[25.] K Ogawa, M.A. Iwabuchi, Mechanism for promoting the germination of Zinnia elegans seeds by Hydrogen peroxide, *Plant Cell Physiology, 42*, 2001, 286–291.

[26.] S. Rozen, H. Skaletsky, Primer3 on the WWW for general users and for biologist programmers, *Methods Mol. Biol. 132,* 2000, 365–386.

[27.] A Saffar, M.B.B. Najjar, M. Mianabadi, Activity of antioxidant enzymes in response to cadmium in Arabidopsis thaliana, *Pak J Biol Sci. 9,* 2009, 44-50.

[28.] P Schopfer, Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles, *Planta 199,* 1996, 43–49.

[29.] LZ Shi, R. Wang, G. Huang, P. Vogel, G. Neale, et al. HIF1a–dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells, *J. Exp. Med. 208,* 2011, 1367–1376.

[30.] R. Tenhaken, Cell wall remodeling under abiotic stress, *Frontiers in Plant Science, 5*, 2014, 77.

[31.] Y Wang, M. Chantreau, R. Sibout, S. Hawkins, Plant cell wall lignification and monolignol metabolism, *Frontiers in Plant Science, 4*, 2013, 220.

[32.] M Zeinalabedini, K. Majourhat, J. A. Hernández, F. Dicenta, P. Martínez-Gómez, Breaking seed dormancy in long-term stored seeds from Iranian wild almond species, *Seed Science and Technology, 37*, 2009, 267–275.

[33.] R Zhong, Z.H. Ye, Secondary cell walls: biosynthesis, patterned deposition and transcriptional regulation, *Plant Cell Physiology,* 56, 2015, 195–214.