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RESEARCH ARTICLE

The effect of perchloric acid as an oxidative stress trigger for protein threonine phosphatase in *Arachis hypogaea* L.

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ABSTRACT : The protein threonine phosphatases (PThPases) catalyze the reversible phosphorylation of threonine residues in proteins. They also regulate a large number of cellular processes including developmental and signaling pathways in eukaryotes.This study was conducted to determine the activity of PThPases as affected by different oxidizing agents in peanut (*Arachis hypogaea* L.) seedlings. In present work, the peanut seedlings were grown in dark on moistened whatman filter paper at 28±2°C and it was observed that the activity of PThPase increased several folds in 6-8 days growing seedlings (without stressed). The eight days growing seedlings were then further treated with 2 per cent solution of different oxidizing agents (Hydrogen peroxide, Nitric acid, Potassium dichromate, Perchloric acid, Potassium permagnate, Magnese dioxide, Lead dioxide and Ammonium persulphate).It was observed that the specific activity of PThPase got an appreciable reduction by perchloric acid ($HClO_4$).After treating the 8 days growing seedlings with different % $HClO_4$ (0.5-5%), it was observed that 3% $HClO_4$ shows a pronounced effect on PThPase activity and the specific activity was reduced upto 65 per cent. In addition,when seedlings were treated with 3% $HClO_4$ for different time intervals (1-6h),4h treatment causes more than 88 per cent reduction in specific activity.The different parts of seedling (stressed with 3% $HClO_4$ for 4h) show the maximum reduction in specific activity in hypocotyl followed by epicotyl, cotyledon and then root. The results suggest that the role of PThPase is stress-related cellular processes and likely to inhibit the activity of intracellular protein threonine phosphatases.

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Oxidative stress resulting from an imbalance in the accumulation and removal of reactive oxygen species such as H_2O_2, is a challenge faced by all aerobic organisms. In plants exposure to various abiotic and biotic stresses results in accumulation of H_2O_2 and oxidative stress. Increasing evidence indicates that H_2O_2 functions as a stress signal in plants, mediating adaptive responses to various stresses (Desikan *et al.,* 2001 and Chiarugi, 2005). The simultaneous addition of H_2O_2 and vanadate, the resulting pervanadate enhanced several folds the effects exerted separately by these two compounds (Veal *et al.,* 2007). Pervanadate has been shown to induce interferon and prolactin dependent transcription factors (Chiarugi, 2005). $HClO_4$ also act as the strong oxidative stress trigger for phosphatase enzyme activity. Protein phosphatases (PPases), in co-ordination with protein kinases (PKases), play crucial roles in regulation of signaling pathways. It can be presumed that the specific activities of

(PPases) *in vitro* are 10-1000 times higher than those of (PKases). Unlike most enzymes, the Serine/Threonine specific PPases show broad and overlapping substrate specificities *in vitro* and their classification requires the use of specific inhibitors and activators. By using these criteria, four major classes of PPase catalytic subunits have been identified in eukaryotic cells (Luan, 2003). The catalytic subunit of PP1 is highly conserved among all eukaryotes with approximately 70 per cent or greater protein sequence identity in any pair wise alignment. At least 100 putative PP1 binding R subunits have been identified with many more expected to be found (Cohen, 2004 and Torres, 2003). Type 2 phosphatase dephosphorylates the alpha subunit of phosphorylase kinase preferentially and are unaffected by the inhibitors. Type 2 phosphatases is comprise of three enzymes (PP2A, PP2B, PP2C) that can be distinguished by their requirement for cations (Cohen, 2004 and 1994). PP2A plays an important role in development, cell proliferation and death cell mobility cytoskeleton dynamics, the control of cell cycle, transcription of immediate early genes, cholesterol, protein biosynthesis and the regulation of numerous signaling pathways (Koch and Houf, 2010). It is also likely to be a tumor suppressor (Veal *et al.*, 2007 and Lowry *et al.*, 1951). PP2A is also a major protein phosphatase in all eukaryotic cells and has a wide range of biological functions. PP2B also termed calcineurin, is a calcium/calmodulin activated PSTPase consisting of a catalytic subunit 'A' and a regulatory or calcium binding 'B'subunit which makes this the only phosphatase directly regulated by second messesgers (Cohen, 1994). PP2C is Mg^{2+} dependent. It has been only isolated as monomeric protein. PP2C has broad and overlapping specificities *in vitro* and account for virtually all measurable activity in tissue extracts towards a variety of phosphoproteins that regulate metabolism, muscle contractility and other processes (Cohen, 1989).

The phosphorylation status of a protein or more specifically, the pattern of phosphorylation on a given protein can determine its activity. The presence or absence of a phosphate group can change the conformation of target protein. The balanced transfer of phosphoryl groups from one entity to another as catalysed by phosphatase and kinases is the basal mechanism by which cellular function is controlled (Cho and Xu, 2006). To identify PThPases and PSPases in the *Strongylocentrotus purpuratus* genome, 179 annotated sequences werestudied (122 PTPs, 57 ser–thr phosphatases). Sequence analysis identified 91, 1 Class III Cysteine-based PTP,1 Asp-based PTP and 25 ser–thr phosphatases (Byrum *et al.*, 2006). In the cytoplasm of eukaryotic cells most, if not all, phosphoseryl-phophothreonyl protein phosphatase activity can be accounted for by just four different catalytic subunit types PP1, PP2A, PP2B, PP2C.Various PP2A subunits mediate post translational regulation of metabolic enzymes and signaling components (Durian *et al.*, 2016). Many regulatory subunits also bind through the occupancy of an allosteric site on PP1 specific phosphoprotein substrate but depress activity with respect to others.

Phosphorylation of proteins is a prevalent post translation modification which affects intracellular signaling in many ways. About 2 per cent of eukaryotic genes code for PKases catalyzing phosphorylation events that have made it possible to identify thousands of phosphorylation sites simultaneously (Keyse, 1998 and Koch and Hauf, 2010). It is observed that phosphorylation process is directly affected when the seedlings are treated with different oxidizing agents.

Oxidative stress is characterized by high intracellular levels of ROS, which may function as physiological mediators of a number of cellular processes by acting a second messenger for specific signaling pathways (Moorhead *et al.*, 2009). ROS act as cellular messengers in cellular processes such as mitogenic signal transduction, gene expression, and regulation of cell proliferation, senescence and apoptosis. Redox regulated proteins include PThPase and PTKase, although with opposite regulation of enzymatic activity (Torres, 2003). Transient oxidation of thiols in PThPase leads to their inactivation by the formation of either an intramolecular S-S bridge or a sulfenyl-amide bond. Cellular responses to stimuli involve dynamic and localized changes in protein Kinases and phosphatases. During CVB3 infection, type I interferon signaling increases subcellular JNK I activity inhibiting nuclear JNK I activity that otherwise promotes viral protein synthesis in the infected host cell (Shah *et al.*, 2017). The tremendous efforts put forth by the academic research institutions and pharmaceutical industry to modulate phosphatase activity for the treatment of various disease states have also invigorated discussions regarding phosphatases as plausible drug targets. Several PThPases have been proved as important enzymes for the treatment of variety of diseases.

It is interesting to share that peanut plant is used in

the present study because the peanut seeds are easily available, easy to grow in every season and a sufficient amount of enzyme PPase is produced in the germinating seedlings.

EXPERIMENTAL METHODOLOGY

Seed germination and preparation of crude enzyme:

The peanut seeds were purchased from an authorized seed store and washed with double distilled water. The seeds surface was sterilized with 1% $HgCl_2$ and allowed to germinate under aseptic conditions for 10-14 days (Devi *et al.*, 2002). The seeds were germinated on autoclaved Whatmann filter paper. The whole plant and different parts of plant like root, hypocotyl, epicotyl and cotyledon (Fig. A) were crushed manually and the crude enzyme extract was prepared by homogenizing the plant tissue with extraction buffer in (1:3 ratio) at 0-4°C.The extraction buffer was made up of 100mM-Tris PH 7.5, 50mMNaCl, 10mMEDTA, 0.04 per cent beta mercaptoethanol. The homogenate was filtered through four layered cheese cloth. The filtrate was centrifuged at 10000 rpm for 30 min. The supernatant was used for enzyme assay.

Fig. A : Population of 0-14 days old germinating seedlings

Peanut (*Arachis hypogaea* L.) were germinated on Whatman (3 mm) filter paper moistened with sterile double distilled water in dark at 28±2°C under aseptic conditions in a seed germination chamber. Note the different anatomical parts which were clearly visible in the seedling only healthy seedlings were used.

Protein phosphatase and protein threonine phosphatase activity:

Protein phosphatase activity was assayed by using casein and protein threonine phosphatase activity was assayed by using O-Phospho L- threonine (Sigma).The reaction mixture is composed of 10µl substrate, 10µl enzyme 180µl Tris-HCl (100mM) (total volume 200µl). Now the reaction mixture is incubated for 30 minutes at 30°C. 10 per cent TCA solution is added to stop the reaction and the reaction is kept at ice for 5 minute and again it is centrifuged at 10000 rpm for 5 minutes in refrigerated centrifuge at 0-5°C, so that the protein is completely precipitated out. 200µl of supernatant of the reaction mixture was assayed for inorganic phosphate (Pi) release by the malachite green method (180 mg malachite green in 400 ml distilled water and 5.0 g Ammonium molybdate in 40 ml Conc. HCl, makeup volume 500 ml) (Lanzetta *et al.*, 1979).100µl supernatant is mixed with 1ml of malachite green solutionand the absorbance is measured by UV-visible spectrophotometer (Shimazdu). The similar method is also used for the protein threonine phosphatase (PTPase), protein tyrosine phosphatase (PTPase), protein serine phosphatase (PSPase), by using O-phospho-L-Threonine, O-phospho-L-tyrosine, O-phospho-L-Serine as substrate respectively. "One unit was defined as the amount of protein in mg that liberated one nano mole of inorganic phosphate (Pi) per minute under assay condition." The protein concentration is measured by the help of Lowry method (Lowry *et al*., 1951), in which bovine serum albumin is used as the standard substance.

Oxidative stress on peanut seedlings:

The oxidative stress is carried outby treating the germinated seedlings through the following steps:

- With 2 per cent solution of different oxidizing agents.
- With 0.5-5 per cent solution of $HClO_4$.
- With 3 per cent solution of $HClO_4$ for different time intervals.
- With 3 per cent solution of $HClO_4$ with different parts of germinated seedlings for 4hrs.

Following stress all other assays *viz.*, PPase, PThPase, protein were done as given above.

Molecular characterization of stressed and non-stressed protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis:

To deduce changes associated with oxidative stress in peanut seedlings on its protein content by SDS-PAGE was carried out according to the method of Laemmli

(1970) using 10 per cent polyacrylamide gel. Coomassie brilliant blue R-250 staining was carried out to visualize protein bands on the gels. The molecular weight of the protein was estimated by comparing the relative mobility of proteins of different molecular size using standard molecular weight marker (97.4-14.3kDa; Standard molecular mass markers: Lysozyme,14.3KDa; Trypsine inhibitor, 20.1 KDa; Carbonic anhydrase,29.5 KDa; Ovalalbumin, 45.0 KDa; Bovineserumal bumin, 66.0 KDa; Phosphorylase-b,97.4KDa). All the experiments were replicated at least thrice with three replicates each and the data was pooled to mean of the values obtained individually.

EXPERIMENTAL FINDINGS AND DISCUSSION

In recent years, various research groups have shown that oxidative agents such as H_2O_2 are able to activate the mitogen activated protein kinase cascade in both plants and animals, thus, provide the linkage between an upstream H_2O_2 signal and downstream gene expression *i.e*. leading to the modulation of gene expression (Janssens *et al*., 2001 and 2005; Luan, 2003 and Modesto *et al.*, 1999). The germinated seedlings are known to show high sensitivity to environmental and oxidative stresses. The present work shows the oxidative stress by different oxidizing agents such as Hydrogen peroxide (H_2O_2), Nitric acid (HNO_3), potassium dichromate ($K_2Cr_2O_7$), perchloric acid ($HClO_4$), potassium permagnate ($KMnO_4$), mangnese dioxide (MnO_2), lead dioxide (PbO_2) etc. however, a little work has been carried out by the use of $HClO_4$ as an oxidizing agent. The specific activity of PThPases when peanut seedlings are treated with 2 per cent aqueous solution of different oxidizing agents is shown in Table 1.

Specific activity of PThPases shows appreciable reduction by $HClO_4$ and itdecreases the activity of PThPase to a much lower extent as compared to all the other oxidizing agents. These results show that $HClO_4$ has a pronounced effect on the specific activity of PThPase suggesting thatoxidation cycle of PThPase can serve as a molecular switch of sorts that regulates catalytic activity. It is observed that there is almost 8.0 fold increase in specific activity (units /mg protein) of PThPase between 6-10 days growing seedlings (Fig. 1).

The 8 day-old growing seedlings show the maximum

Table 1 : Specific activity of PThPase with different oxidizing agents in 8 days growing peanut seedlings

Oxidising agents	PThPase
	Specific activity unit/mg-protein
*General	22.4
Hydrogen peroxide	13.9
Magnese dioxide	15.6
Nitric acid	18.5
Ammonium persulphate	27.7
Potasium dichromate	14.7
Tollens reagent	15.6
Perchloric acid	19
Potasiumpermagnate	16.5
Lead dioxide	16.8

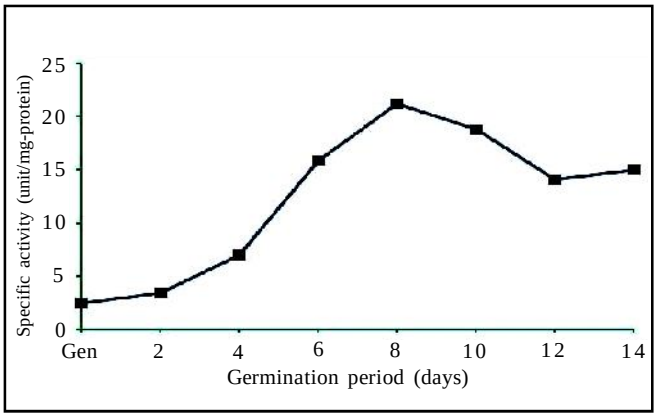

Fig. 1 : Distribution of PThPase specific activity in 0-14 days growing seedlings

Table 2 : Specific activity of PThPases in different per cent of $HClO_4$

% $HClO_4$	Protein (ug/ ml)	Specific activity (Activity of protein per mg protein) (um Pi/ml/min/mg-protein)	
		PPase	PThPase
0.5%	130	0.0269	0.0140
1%	52	0.0446	0.0309
2%	86	0.0253	0.0179
3%	100	0.0168	0.0105
4%	66	0.0255	0.0190
5%	70	0.0210	0.0140

specific activity. Initially the specific activity seems to increase upto 8th day but after that the specific activity of the enzyme PThPases is reduced. It indicates that the biosynthesis of the enzyme PThPases is enhanced during germination. These results demonstrate that the PThPase activity can be modulated by oxidizing agents and provide a framework to understand the effect of oxidative stress on PThPase signaling pathway (3). Physiological suppression of PThPase catalytic activity has been recognized as a key feature of their regulation within the cellular environment. The specific activity of PThPase when peanut seedlings were treated with different per cent of $HClO_4$ (0.5-5%) is shown in Fig. 2. It was observed that specific activity of PThPase get reduced to about 65 per cent, a much lower extent by use of a solution containing 3 per cent $HClO_4$ in immersed peanut seedlings.

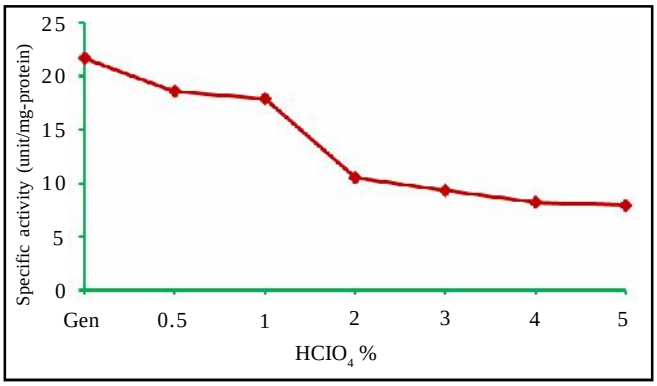

Fig. 2 : Specific activity of PThPase with different % of $HClO_4$ in 8 days growing peanut seedlings

Fig. 3 shows the trend of specific activity of PThPase when peanut seedlings are treated with

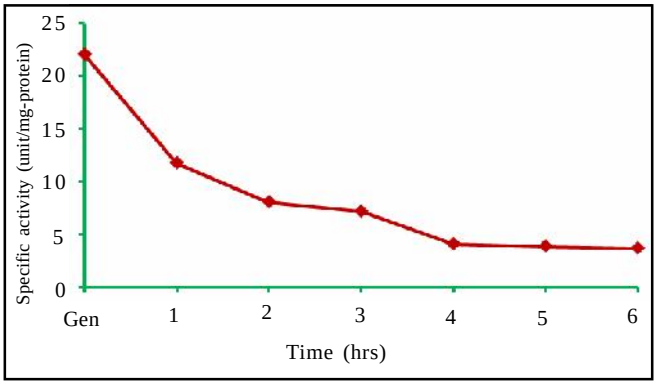

Fig. 3 : Specific activity of PThPase with 3% $HClO_4$ different time intervals in 8 days growing peanut seedlings

different 3 per cent of $HClO_4$ for different time intervals. It was observed that as the time increases the specific activity was decreases continuously. The maximum stress was observed at 4th hour treatment on PThPase. There is almost 5.0 fold decrement as compare to the 1hour treatment. The germinated seedlings are known to show high sensitivity to environmental and oxidative stresses. The present work shows the oxidative stress by $HClO_4$, however, a little work has been carried out by $HClO_4$.

Profile of protein bands by SDS-PAGE:

In the first hour treatment, the protein bands in the range of 29.0kDa to 20.1kDa were clearly appeared, while 2-6 h treatment shows the complete disappearance of same protein bands under the same condition.

SDS-PAGE profile of protein in 8 days old growing seedlings stressed by 3% $HClO_4$ for different time intervals (1-6 h). SDS-PAGE carried out under reduced and denaturating condition using 10 per cent polyacrylamide gel. Protein were stained by coomassie brilliant blue R-250. Lane1-Marker, Lane2-6 shows the protein band profile stressed with 3% $HClO_4$ for different time intervals (1-6hrs). Standard molecular mass markers: Lysozyme, 14.3KDa; Trypsine inhibitor, 20.1KDa; Carbonic anhydrase, 29.5KDa; Oval albumin, 45.0KDa; Bovine serum albumin, 66.0KDa; Phosphorylase-b,97.4KDa (Fig. 4).

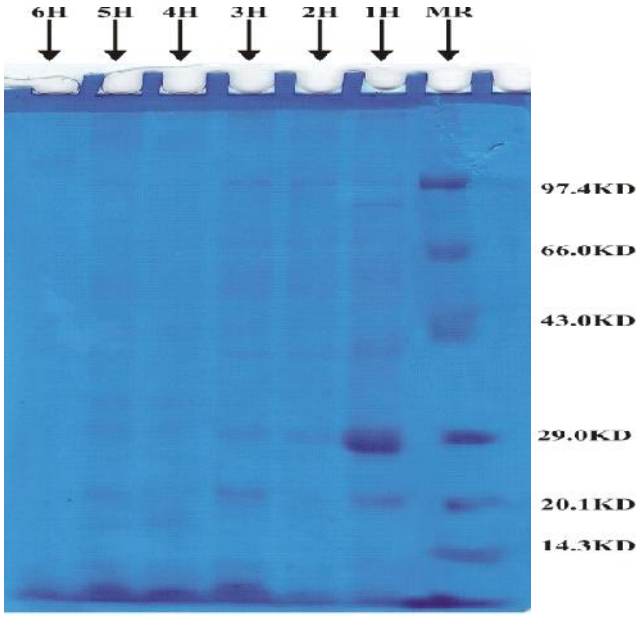

Fig. 4 : SDS-PAGE profile of protein 8 day old growing seedlings stressed by 3% $HClO_4$

Fig. 5 shows the trend of specific activity of protein threonine phosphstase (PThPase) when different parts of peanut seedlings were treated with 3 per cent of $HClO_4$ for 4 h. It is observed that the specific activity was reduced in case of stressed plant parts as compare to the general plant parts (without stressed). The minimum specific activity was observed in hypocotyls followed by epicotyl, cotyledon and then root. In addition, when different parts of germinated seedlings were treated with 3 per cent $HClO_4$ for 4 h, hypocotyl showed minimum activity.

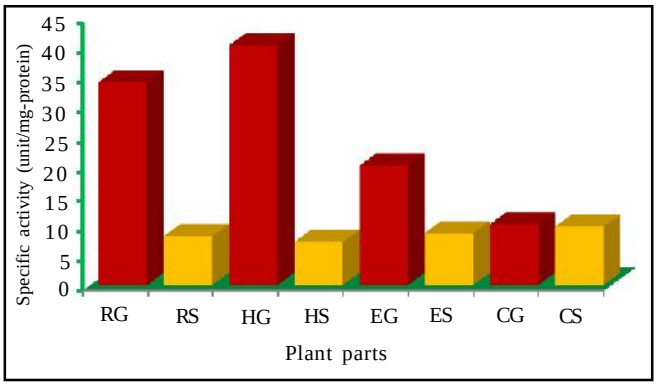

Fig. 5 : Specific activity of PTh Pase with 3% $HClO_4$ for 4h with different parts in 8 days growing peanut seedlings. (R: Root; H: hypocotyl; E: Epicotyl; C: Cotyledon; G: growth/control/unstressed; S: Stress)

Molecular characterization of stressed and non-stressed protein in different parts by SDS-PAGE:

The SDS-PAGE protein profiles of different parts of the peanut seedling before and after 4h treatment with 3% $HClO_4$ are shown in Fig. 6. In case of root, hypocotyls and epicotyl the 8-day-old germinating seedlings, the stressed tissue does not showany protein bands apparently. On the contrary, the protein profiles of untreated and treated cotyledon showed distinct differences as judged by the intensity of the protein bands separated by the SDS-PAGE. In cotyledon, the protein bands in the region of Mr from 97.4 to 120kDa suppressed. SDS-PAGE profile shows the disappearance of protein bands in stressed seedlings so it can be concluded that $HClO_4$ acts as the potent inhibitor of protein threonine phosphatase.

Fig. 6 SDS-PAGE (10%) profile of protein in different part of 8 day-old germinating peanut seedling with 3% $HClO_4$ for 4hrs (RT) revealed change in different protein band. Protein were stained by coomassie brilliant blue R-250. Lane1-Marker, 2,3,4-root general,

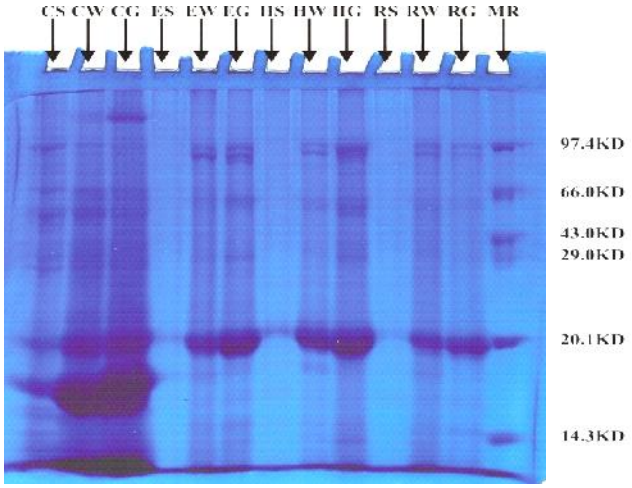

Fig. 6 : SDS-PAGE (10%) profile of protein in different part of 8 day-old germinating peanut seedling with 3% $HClO_4$

water and $HClO_4$ stressed whereas 5,6,7-hypocotyl general, water and $HClO_4$ stressed 8,9,10-are epicotyls, general, water and $HClO_4$ stressed,11,12,13-cotyledon general, water and $HClO_4$ stressed. Standard molecular mass markers: Lysozyme,14.3 KDa; Trypsine inhibitor, 20.1 KDa; Carbonicanhydrase, 29.5 KDa; Ovalalbumin, 45.0KDa; Bovine serum albumin, 66.0 KDa; Phosphorylase-b,97.4 KDa.

The chemistry by which oxidative species regulate PThPase is well characterized but the exact mechanism bywhich these species themselves are produced and regulated remain unclear (Kerk *et al*., 2002 and Rhee, 2006).

Reactive oxygen species (ROS) act as cellular messengers in cellular processes such as mitogenic signal transduction, gene expression, regulation of cell proliferation, senescence and apoptosis. Redox regulated proteins include PThPases and PTKases, although with opposite regulation of enzymatic activity (Vivancos *et al*., 2004 and 2006).

Conclusion:

3% $HClO_4$ reduces the maximum activity (almost 65%) of the 8 days growing seedlings. In addition, when different parts of germinated seedlings were treated with 3% $HClO_4$ for 4 h, hypocotyl showed minimum activity. SDS-PAGE profile shows the disappearance of protein bands in stressed seedlings so it can be concluded that $HClO_4$ acts as the potent inhibitor of protein threonine phosphatase. These results demonstrate that the PThPase

activity can be modulated by oxidizing agents and provide a framework to understand the effect of oxidative stress on PThPase signaling pathway. The studies show that the PThPase are deeply involved at many levels of biological cellular processes. Furthermore, the enzymes can be controlled and modulated in diverse and novel ways including oxidation, salt and surfactant tolerance. These results also suggest the role of PThPase in stress related cellular processes.

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