Chapter 2

Reactive Oxygen Species
2.1 Introduction

Being a redox active metalloid, As leads to over production of ROS in the cells, thereby creates oxidative stress condition inside (Srivastava et al. 2010; Namdjoyan et al. 2016). These ROS are a group of free radicals or ions, reactive to almost each of the cellular macro molecules (Abdrakhimova et al. 2015; Belikov et al. 2015). The most widely recognized ROS involve O$_2^\cdot$-, H$_2$O$_2$ and 'OH (Apel and Hirt 2004; Mattila et al. 2015). The reason of creating an oxidative burst inside the cells chiefly includes (i) an imbalance between ROS production and its detoxification due to disturbance in "normal" physiology of cells and (ii) ROS synthesis as a constituent part of stress signaling and immunity response needed for defense and adaptation. These mechanisms co-exist, because stress factors straight forward delivers ROS furthermore, that empower ROS generation by NADPH oxidase (Karuppanapandian et al. 2011; Inupakutika et al. 2016). About 1-2% of total O$_2$ consumed by any plant can be utilized for ROS formation (Bhattacharjee 2005).

Presence of As inside the cells, increases the amount of energy in the thylakoids than that of total energy driven away from the metabolic processes of the chloroplasts. As a consequence, in the thylakoid, process of electron transport gets hampered and leads to production of ROS (Garg and Singla 2011; Agnihotri and Seth 2016). In addition to this, the inter conversion of two forms of As i.e. As$^{III}$ and As$^{V}$, leads to release of electrons, that binds to O$_2$ and forms O$_2^\cdot$-, thereby increases ROS concentration (Meharg and Hartley-Whitaker 2002). These ROS has been shown to play dual role in the cells depending upon its concentration (Leymarie et al. 2012). Higher concentration of it results deleterious responses in cells, while at low level it act as a secondary messenger in intracellular signaling cascade that intervene a few responses inside plants (Bailey-Serres and Mittler 2006; Kumar et al. 2015c).

Mitochondria are major locations of ROS production (Noctor et al. 2007; Tripathy and Oelmuller 2012). Complex-I and complex-III of photosynthetic units are also known sites of ROS production (Gomes and Garcia 2013). Superoxide is the very first ROS to be generated here (Bovok et al. 2004). It is a moderately reactive ROS with a half-life of
approximately 2-4 µsec (Qin et al. 2016). It is the prime ROS produced in the cells and begins a cascade of reaction to generate "secondary" ROS, either directly or predominantly through enzyme or metal-catalyzed processes depending on the cellular compartments (Valko et al. 2005). These O$_2^-$ then possibly triggers generation of H$_2$O$_2$ which further produce 'OH after reacting with Fe$^{II}$ and Cu$^{II}$ (Sohal and Orr 2012). Dismutation of O$_2^-$ to H$_2$O$_2$ occurs either through non-enzymatic reactions or by SOD catalyzed responses (Gill et al. 2015).

Another moderately reactive species is the H$_2$O$_2$ and is relatively long-lived molecule with a half-life of 1 msec (Bienert et al. 2006). Unlike other ROS, it has no unpaired electrons and can readily diffuse through aquaporins of all the biological membranes, thereby cause oxidative damage to cellular molecules far from the site of its formation (Karuppananapandian et al. 2011). It has been well established that excessive existence of H$_2$O$_2$ in the plant cells leads to the oxidative stress (Ray et al. 2012; Sevilla et al. 2015). In plants, H$_2$O$_2$ plays a dual role: at low concentrations, it acts as a signaling molecule involved in the seed germination process and at high concentrations, it leads to death of the cells (Akter et al. 2015). Also, H$_2$O$_2$ may oxidize the thiol groups of enzymes, thus inactivates them and hinders various metabolic processes of the cell (Hung et al. 2005).

Among all the known ROS, most reactive is the 'OH, having a half-life of 1 nsec (Richards et al. 2015). Following Haber-Weiss reaction, formation of 'OH is dependent on both H$_2$O$_2$ and O$_2^-$ and is inhibited by both SOD and CAT (Sharma et al. 2012). At neutral pH and ambient temperature, O$_2^-$ and H$_2$O$_2$ produces 'OH via iron-catalysed, O$_2^-$-driven Fenton reaction. These 'OH produced inside the cells are thought to be largely responsible for mediating O$_2$ toxicity in vivo. It can potentially react with all the biological molecules like lipids, proteins and nucleic acids (del Rio 2015). It is considered to be highly reactive molecule because cells do not posses any enzymatic mechanism for the elimination of 'OH, hence excess accrual of it leads to cell death (Bhattacharjee 2005; Gechev et al. 2006).

To scavenge these ROS, plant cells have both enzymatic and non-enzymatic antioxidant defense systems (Jaspers and Kangasjarvi 2010). These components are involved in indirect scavenging of ROS (Dixit et al. 2016). Since, As has the ability to
directly bind with sulphydryl groups of both proteins and enzymes, therefore inhibits the activities of antioxidant enzymes (Nath et al. 2014; Chandrakar et al. 2016a, b). Arsenic has high affinity for non-enzymatic anti-oxidants also such as glutathione, phytochelatins, etc. (Sharma 2012). Due to As-induced inhibition/ lowering in the activities/ syntheses of antioxidant components, amount of ROS gets enhanced inside the cell (Siddiqui et al. 2015a; Begum et al. 2016).

Therefore, in the present Chapter, an attempt was made to monitor change in the ROS (O$_2^{•−}$, H$_2$O$_2$ and 'OH) production in response to As in Glycine max L. radicles. In addition, efforts were also invested to assess the impacts of DPI, EBL and Pro on ROS generation in Glycine max L. radicles on second and fifth days of As treatments. To authenticate the spectrophotometric data, growing radicles of Glycine max L. were taken into nitroblue tetrazolium chloride (NBT) and 3,3-diaminobenzidine (DAB) solutions separately, in view to scrutinize the exact locations of O$_2^{•−}$ and H$_2$O$_2$ synthesis, respectively.

2.2 Materials and Methods

2.2.1 Superoxide

_Localization_

To locate precise sites of O$_2^{•−}$ production in the Glycine max L. radicles, NBT staining protocol of Rughani et al. (2015, 2016) was followed. Excised radicles (5 Nos) of MW-grown control and different treatments of As, DPI, EBL and Pro were engrossed in 15 ml of 0.05 M NBT solution (prepared in 50 mM sodium phosphate buffer of pH 6.8), for 30 min, at laboratory condition. The stained radicles were washed three times in MW, to remove the extra amount of stain. Presence of deep blue/ purple coloration in the radicles was considered as sites of O$_2^{•−}$ production.

_Quantitative Estimation_

Liberation of O$_2^{•−}$ was measured following the procedure of Sangeetha et al. (1990). In this method, weighed amounts (0.2 g) of excised radicles were homogenized with 2 ml of cold sodium phosphate buffer (0.2 M, pH 7.2) consisting 0.001 M diethylthiocarbamate
(DDC, an inhibitor of SOD), in a pre-chilled pestle and mortar. The homogenate was then centrifuged at 11,000 rpm for 15 min at 4°C and the supernatant obtained was used as a source of \( \text{O}_2^{•−} \).

In this assay, \( \text{O}_2^{•−} \) was assessed by utilizing NBT as an indicator. The reduction of NBT by \( \text{O}_2^{•−} \) was detected by recording formation of a blue formazan (reduced NBT) at 540 nm. The calibration curve for pyrogallol auto-oxidation was prepared by determining the rate of NBT reduced by \( \text{O}_2^{•−} \), at 540 nm. The absorbance was zeroed by taking 2.7 ml of sodium phosphate buffer (0.2 M, pH 7.2) and 100 µl of NBT (2.5×10^{-4} M) in a quartz cuvette. The reaction was triggered by adding 500 µl of 0.2 mM pyrogallol (prepared in 10 mM HCl) and the change in absorbance was recorded at 540 nm after 6 min of incubation at laboratory condition.

The \( \text{O}_2^{•−} \) content was estimated by recording the kinetics of the reaction mixture containing 2.7 ml of sodium phosphates buffer (0.2 M, pH 7.2), 200 µl extract and 100 µl NBT solution, at 540 nm and values were expressed as \( \mu \text{mol min}^{-1} \text{ g}^{-1} \text{ FM} \).

2.2.2 Hydrogen Peroxide

**Localization**
Generation of \( \text{H}_2\text{O}_2 \) in treated as well as non-treated control *Glycine max* L. radicles was envisaged by DAB staining (Rughani et al. 2015, 2016). In this procedure, five radicles from control and each of the treatments were harvested and then immersed in sufficient volume of 50 mM sodium phosphate buffer (pH 6.8) comprising 1 mM DAB, for 30 min, at laboratory condition. Then, the stained radicles were washed thoroughly with MW. In this protocol, polymerization of DAB with \( \text{H}_2\text{O}_2 \) molecules results in the formation of a brown colored patching in the exposed radicles, which were identified as active sites of \( \text{H}_2\text{O}_2 \) generation.

**Quantitative Estimation**
Amount of \( \text{H}_2\text{O}_2 \) was determined after the protocol of Velikova et al. (2000). Weighed amounts (0.2 g) of radicles were extracted with 2 ml of 0.1% (w/v) trichloroacetic acid (TCA). This mixture was then centrifuged at 12,000 rpm for 15 min at laboratory
condition and supernatant was collected. In a quartz cuvette, 1 ml of supernatant was mixed with equal volumes of 10 mM potassium phosphate buffer (pH 7) and 1 M potassium iodide solution and absorbance of it was recorded at 390 nm. Content of H$_2$O$_2$ was calculated following an extinction coefficient 0.28 µmol$^{-1}$ cm$^{-1}$ and values were expressed as µmol g$^{-1}$ FM.

### 2.2.3 Hydroxyl Radical

**Quantitative Estimation**

Levels of •OH were measured following the method described by Kaur et al. (2012). For this, 0.2 g of treated and control *Glycine max* L. radicles were homogenized in 2 ml of sodium phosphate buffer (10 mM, pH 7.4) containing 15 mM 2-deoxyribose and then incubated at 37°C for 2 h. An aliquot (0.7 ml) of above solution was mixed with 3 ml thiobarbituric acid {TBA, 0.5% (w/v) prepared in 5 mM NaOH} and 1 ml glacial acetic acid. Now, this solution was heated at 100°C for 30 min and then cooled at 4°C for 10 min. Absorbance of it was recorded at 532 nm and corrected for non-specific absorbance at 600 nm. Content of •OH was calculated using an extinction coefficient of 0.155 mol$^{-1}$ cm$^{-1}$ and expressed as nmol g$^{-1}$ FM.

### 2.3 Results

#### 2.3.1 Superoxide

**Localization**

To make sure the precise locations of O$_2$•$^-$ production in the radicles of *Glycine max* L. underwent As-exposure for different time lengths, NBT staining technique was employed. Initially, appearance of few of the blue colored patches was identified at only tip portion of the protruded radicle, in response to minimum (10 µM) amount of As on both the days (second and fifth) of sampling (Figs. 2.1A and B). Further, progressive expansion in this blue coloration was investigated parallel to As supply as well as advancing exposure time. The 100 µM As-exposed radicles of fifth day incubation, appeared completely blue in color, exhibiting huge amount of O$_2$•$^-$ accumulation (Fig. 2.1B). However, exogenous application of DPI, EBL or Pro with 75 µM As resulted in
comparatively lesser colored patches/ $O_2^{-}$ production. Least coloration with NBT was observed in presence of Pro, thus suggesting it the most potent treatment in comparison to DPI or EBL.

**Fig. 2.1:** Localization of superoxide generation sites in *Glycine max* L. radicles exposed to various treatments of arsenic, diphenylene iodonium, 24-epibrassinolide and proline for two (A) and five (B) days.

**Quantitative Estimation**

During investigation of $O_2^{-}$ production, when *Glycine max* L. radicles were exposed to different concentrations of As (10-100 µM), significant upsurge in its level was observed, compared to control. In general, levels of $O_2^{-}$ estimated were quite more in the fifth day harvested radicles, than that of second day. On second day of analysis, the increments recorded in the $O_2^{-}$ generation was 200% and 2354%, under 10 and 100 µM As respectively, as compared to control (Fig. 2.2A). Further, on fifth day of investigation, significantly more amounts of $O_2^{-}$ accumulation was measured in the radicles maintained under all the tested concentrations of As (Fig. 2.2B). On the other hand, exogenous addition of DPI, EBL and Pro, separately, with 75 µM As considerably lowered the amounts of $O_2^{-}$ on both the days of radicle harvest. Gathered data suggested that Pro caused least accumulation of $O_2^{-}$, thus played prominent role than that of DPI and EBL, in counteracting deleterious impacts of As in *Glycine max* L. radicles.
**Chapter II Results**

2.3.2 Hydrogen Peroxide

**Localization**

To localize the accurate sites of H$_2$O$_2$ production in As-stressed *Glycine max* L. radicles, DAB staining protocol was adopted. On second day of harvest, in those radicles which were immersed in minimum (10 µM) concentration of As, a few small patches of brown color, probable sites of H$_2$O$_2$ production, were appeared on the basal and tip portions (Fig. 2.3A). Further, these patching increased considerably, parallel to As concentration and time of exposure.

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**Fig. 2.2:** Amounts of superoxide in *Glycine max* L. radicles grown in various experimental solutions for two (A) and five (B) days, separately. Each bar represents mean ± SE of five independent observations. Small letters represent significant difference at P < 0.05.
Fig. 2.3: Localization of hydrogen peroxide generation sites in *Glycine max* L. radicles exposed to various treatments of arsenic, diphenylene iodonium, 24-epibrassinolide and proline for two (A) and five (B) days.

Therefore, on fifth day of sampling, the radicle subjected to 100 µM of As, looks completely brown, witnessing huge amount of H$_2$O$_2$ production (Fig. 2.3B). Further, when DPI, EBL and Pro were applied separately with 75 µM As, comparatively lesser colored patches were observed. Least coloration with DAB was observed in presence of Pro, thus approving it the most effective treatment in comparison to DPI or EBL.

**Quantitative Estimation**

Increased supply of As caused simultaneous rise in the amounts of H$_2$O$_2$ generation, in *Glycine max* L. radicles. The level of H$_2$O$_2$ measured was quite high in the radicles harvested on fifth day of incubation, compared to those harvested on second day. In the control radicles, 0.23 and 0.36 µmol g$^{-1}$ FM of H$_2$O$_2$ was recorded on second and fifth days of their incubation, respectively. In case of 10 and 100 µM As-grown radicles, the level of H$_2$O$_2$ measured were 82 and 243% respectively, higher in comparison to control, on second day of As-exposure (Fig. 2.4A). Other side, on fifth day of radicle growth, under above discussed (10 and 100 µM) As treatments, amounts of H$_2$O$_2$ produced was recorded to be 50 and 244% respectively, than that of control (Fig. 2.4B). However, exogenous application of DPI, EBL or Pro with As, significantly reduced the amounts of H$_2$O$_2$ on both the days of investigation. On second day of analysis, when DPI, EBL and
Pro was separately applied along with 75 µM As, level of H$_2$O$_2$ was decreased by 22, 35 and 61% respectively as compared to As-treated samples, which becomes 42, 78 and 144% respectively, on fifth day of sampling. Data suggested that Pro played prominent role than DPI or EBL, to compensate the deleterious impacts of As in *Glycine max* L.

**Fig. 2.4:** Levels of hydrogen peroxide in *Glycine max* L. radicles harvested on second (A) and fifth (B) days of growth in respective experimental solutions. Each bar represents mean ± SE of five independent replications. Small letters represent significant difference at P < 0.05.

### 2.3.3 Hydroxyl Radicals

**Quantitative Estimation**

A remarkable and significant upsurge in the generation of *OH was documented in the growing radicles of *Glycine max* L., if subjected to rising concentrations (10-100 µM) of
As for two distinct periods, in general. In the control, MW grown radicles, negligible increase in 'OH production was noted in between second (5.47 nmol g\(^{-1}\) FM) and fifth (8.46 nmol g\(^{-1}\) FM) days of harvest. The levels of 'OH recorded on second day of growth in 10 and 100 µM As-exposed radicles were around 63 and 338% respectively, higher than that of control (Fig. 2.5A). On fifth day of sample harvesting, about 185% increase in 'OH was discernible in 75 µM As-subjected *Glycine max* L. radicles than that measured in control (Fig. 2.5B). Further, when DPI, EBL and Pro was separately applied with 75 µM As, 97, 113 and 130% decrease in 'OH content was measured.

![Graph A](image)

**Fig. 2.5**: Contents of hydroxyl radical in the radicles of *Glycine max* L. subjected to arsenic, diphenylene iodonium, 24-epibrassinolide and/ or proline for two (A) and five (B) days. Each bar represents mean ± SE of five independent replications. Small letters represent significant difference at P < 0.05.
Gathered data indicated least level of \textsuperscript{'}OH accumulation in the Pro treated samples (2.26 nmol g\textsuperscript{-1} FM) of second day, whereas, it was highest in the five days old, 100 µM As (28.71 nmol g\textsuperscript{-1} FM) exposed radicles.

2.4 Discussion

Arsenic is a toxic metalloid and ubiquitously present in the natural environment (Farnese \textit{et al.} 2013). Plants when exposed to phytotoxic amount of As, shows toxicity symptoms. The most common toxicity symptom appears in plant cell is the over production ROS in response to As. So, the Chapter undertaken evidently revealed the assessment of ROS in As-exposed \textit{Glycine max} L. radicles on second and fifth days of analyses. Exogenous addition of DPI, EBL and Pro, separately, into As solution (having LD\textsubscript{50}), reduced the amounts of ROS in \textit{Glycine max} L. radicles. However, among the applied treatments, Pro was found to be the most effective in reducing ROS accumulation than that of DPI and EBL.

Growing radicles of \textit{Glycine max} L. when exposed to series of As (10-100 µM) solutions, experienced excessive gathering of all the three ROS on both the days of investigation. In regard, Singh \textit{et al.} (2015a) demonstrated that interaction between As and intracellular components may results over production of ROS in stressed cells. A positive correlation exists between As level and ROS (O\textsubscript{2}\textsuperscript{−}: r = 0.83, H\textsubscript{2}O\textsubscript{2}: r = 0.81 and \textsuperscript{'}OH: r = 0.69) accumulation, on fifth day of radicle harvesting. Lovingness of As with sulphydryl groups, leads to inactivation of both proteins and enzymes involved in the defense system of the cell (Rosas-Castor \textit{et al.} 2014). Hence, the antioxidant enzymes become inefficient to counterbalance the over production of ROS, resulting in their excessive gathering (Nath \textit{et al.} 2014). Increase in the accumulations of ROS in response to As, clearly indicate that SOD is not available in sufficient amount to scavenge O\textsubscript{2}\textsuperscript{−} and CAT and APX to quench H\textsubscript{2}O\textsubscript{2} (Reddy \textit{et al.} 2015; Chandrakar \textit{et al.} 2016a). Hence, a negative correlation was found to exist between O\textsubscript{2}\textsuperscript{−} and SOD (r = -0.96) and H\textsubscript{2}O\textsubscript{2} and CAT (r = -0.98) and APX (r = -0.92). Similar change in free radical generation under As-stress has also been observed in \textit{Cucumis sativus} (Czech \textit{et al.} 2008), \textit{Brassica napus} L.
In the present study, As-stress-induced a significant accumulation of \( \text{O}_2^{-} \) and \( \text{H}_2\text{O}_2 \) in the radicles of \textit{Glycine max} L. and the extent of their productions were more pronounced in the samples treated with 100 \( \mu \text{M} \) As than that of other treatments on both the days of sample harvest. These results were also confirmed by histochemical studies with NBT and DAB staining (Figs. 2.1 and 2.3). The NBT reacts with dark blue \( \text{O}_2^{-} \) and forms insoluble blue formazan compound, hence can localize \( \text{O}_2^{-} \) production sites (Singh \textit{et al.} 2009a). While, in presence of peroxides, DAB is oxidized by \( \text{H}_2\text{O}_2 \) and turns the radicles brown in color (Kumar \textit{et al.} 2014b). Farooq \textit{et al.} (2016b) also confirmed the generation sites of both \( \text{O}_2^{-} \) and \( \text{H}_2\text{O}_2 \) with NBT and DAB staining respectively, in As-stressed roots of \textit{Brassica napus} L., keeping parity with our observations.

The results of the present study indicates that the As-induced oxidative damage in \textit{Glycine max} L. radicles was compensated remarkably by exogenous addition of DPI, EBL or Pro, by limiting ROS production. It is well known that DPI directly scavenges ROS or slow down ROS generation process via inhibiting \( \text{O}_2^{-} \) synthase enzyme (Ye \textit{et al.} 2012; Chandrakar \textit{et al.} 2017c). Similarly, EBL and Pro provides tolerance to oxidative stress either by accumulating endogenous osmolytes or activating antioxidant defense mechanism or both in the stressed tissues (Fariduddin \textit{et al.} 2014; Benitez \textit{et al.} 2016). Also, Pro protects the plants from ROS by donating an electron to photosystem-II, thus averts uncoupling of the oxygen thereby produces sufficient amount of NADPH (Oukarroum \textit{et al.} 2012). Moreover, Pro reduces ROS accumulation also by its ability to scavenge free radicals directly in the stressed cells (Jaarsma \textit{et al.} 2013). In this way, DPI, EBL and Pro lower downs the accumulation of ROS in stressed tissues, which was also in congruent with the observations of Fariduddin \textit{et al.} (2009), Sorkheh \textit{et al.} (2012) and Ye \textit{et al.} (2012) in \textit{Brassica juncea} L., \textit{Prunus dulcis} M. and \textit{Oryza sativa} L. respectively, under different abiotic stresses. These treatments stimulate (activate) the antioxidant components which are crucial for enhancing tolerance against varied abiotic stresses (Chandrakar \textit{et al.} 2017a).
Considering the results acquired herewith, it is now summarized that the radicles of *Glycine max* L. on exposure to As (10-100 µM) produces excessive amount of ROS like \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) and \( \text{\textquoteleft} \text{OH} \). Further, the levels of these ROS get lowered by the exogenous addition of DPI, EBL and Pro, separately, with As (75 µM) in *Glycine max* L. radicles, on both the days of harvesting. Due to the property of inhibiting NADPH oxidase, DPI successfully lowered the amount of ROS in stressed cells. Likewise, EBL helped in activating antioxidant system to combat against ROS. And Pro directly scavenged ROS and activated defensive mechanisms, that helped the stressed cells to fight against ROS.