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Arbuscular Mycorrhiza-Mediated Alterations in Redox Buffer Synchronize Antioxidant Network to Alleviate Salt Induced Oxidative Burden in Host Legumes and their Nodules : A Review

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Abstract

Legumes represent an important resource-conserving component of sustainable agricultural system as they fix atmospheric nitrogen and thus reduce the need for chemical fertilizers while enhancing overall crop productivity. However, most legumes are salt-sensitive, where adverse effects of salinity are referred through its detrimental effects on nitrogen fixing symbiosis in the nodules. High rates of respiration and the presence of O_2-sensitive metalloproteins and leghemoglobin in the nodules, particularly favor Reactive Oxygen Species (ROS) overproduction and thus makes nodule highly salt susceptible. Despite destructive activity of ROS, they are well-described second messengers in a variety of cellular processes, including nodule development and functioning. Under steady state conditions, antioxidative system comprising of the nonenzymic as well as enzymic antioxidants work in concert to control the cascades of uncontrolled oxidation and this redox balance orchestrates various metabolic activities, including nodulation process. However, salinity induced disturbance in cellular homeostasis eventually perturbs equilibrium between production and scavenging of ROS, thereby grounding progressive oxidative damage to proteins, DNA and lipids of both symbionts and thus induce nodular premature senescence and ultimately reduce legume productivity. Efficient antioxidative activity does not necessarily mean strong upregulation of full set of antioxidants, indeed redox balance as a result of synchronization between protective and regeneration antioxidant systems plays a crucial role in the regulation of nodule functioning and determines salt tolerance in most legumes. Apart from the intrinsic protective systems of legumes against stress, exploitation of arbuscular mycorrhizal fungi, another microorganism hosted by legumes, opens new alternatives for a pyramiding strategy against salinity induced oxidative damage. In the current review, we provide an inclusive update on the salt-induced oxidative burden in legumes, especially their nodules and recent advances on the emerging role of arbuscular mycorrhiza (AM) in imparting salt tolerance by altering redox buffers through fine regulation of ascorbate-glutatione (AsA-GSH) cycle.

1. Introduction

Leguminous plants play a critical role in natural ecosystems and agriculture owing to their capacity to fix atmospheric nitrogen by establishing intimate symbiosis with N_2-fixing soil bacteria collectively referred to as rhizobia. The process of biological nitrogen fixation (BNF) is of great agronomic interest, since symbiosis of rhizobia with more than one hundred agriculturally important legumes contributes at least half of the annual amount of nitrogen fixed in soil ecosystems (Peoples and Craswell, 1992). Moreover, BNF is required to replace tonnes of fertilizers that degrade lands (Burris, 1994) and pollute groundwater. For a successful symbiotic interaction to occur, the environmental requirements of both partners should be fulfilled (Araújo *et al*., 2015). However, salinity is one of the most widespread environmental constraints of arid and semiarid ecosystems that limit legume cultivation and productivity (Faghire *et al*., 2011). Low osmotic potential of soil solution under excessive salinization disturbs cellular osmotic balance resulting in "physiological drought" and prevention of water influx into the roots (Rewald *et al.,* 2013). The resulting drought conditions are additionally compounded by the presence of toxic ions, particularly Na^+ and Cl^- (Djanaguiraman and Prasad, 2013). Plasma membrane depolarisation together with the reduced Ca^{2+} concentrations disturbs K^+/Na^+ selectivity resulting in excessive influx of Na^+ and leakage of K^+ out of the cytosol (Tuna *et al*., 2007). Salinity caused disarray in transpiration, photosynthesis, respiration etc. further leads to decline in $NADP^+$ pool, which eventually grounds oxidative damage at the cellular level (Turan and Tripathy, 2013).

Legumes not only suffer directly from salt stress, their salt susceptibility is also potentially dependent on the salt tolerance of their micro-symbionts (Bruning and Rozema, 2013). This constraint restricts physiological and biochemical processes governing initiation, development and functioning of symbiotic nitrogen fixation more than either symbiotic partner by itself (Faghire *et al*., 2011). The complexity of the BNF process and the particular environment where it occurs, increase the number of possible factors altering this process, which include overloading of nodules with toxic ions, reduction in rhizobial survival and growth, hindrance in the infection process, suppression of nodule function and prevention of photoassimilates supply to bacteroids, oxygen limitation and reduction of nodule respiration, decrease of cytosolic proteins including Leghemoglobin (LHb) production and regulation of nitrogen metabolism (Bianco and Defez, 2009; Jebara *et al*., 2010; Larrainzar *et al*., 2014).

2. Differential Regulation of Reactive Oxygen Species (ROS) and Antioxidant Defense in Legumes

2.1. Under unstressed conditions

In plants, ROS [singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) species] are also continuously produced as by-products of various metabolic pathways that are localized in different cellular compartments. In the chloroplast, during photosynthesis, $O_2^{\cdot-}$ is produced mainly by electron leakage from Fe-S centers of PS I or reduced ferredoxin (Fd) to O_2 (Mehler reaction), is then converted to H_2O_2 by SOD. $O_2^{\cdot-}$ can also be produced by the leaking of electrons to O_2 from electron transport chains in PS I and II. Under excess light conditions PS II is able to generate $^1O_2$ by energy transfer from the triplet state chlorophyll (Asada, 2006). The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme, which catalyses the carboxylation of ribulose-1,5-bisphosphate (RuBP) during carbon assimilation, can also use O_2 to oxygenate ribulose-1,5-bisphosphate. Under unfavourable conditions, which impair CO_2 fixation in the chloroplast, the oxygenase activity of RuBisCO increases and the glycolate that is produced moves from the chloroplast to peroxisomes, where it is oxidized by glycolate oxidase (GO) forming H_2O_2 (Takahashi and Murata, 2008). In peroxisomes, H_2O_2 can also be formed directly from O_2 by enzyme systems such as xanthine oxidase (XO) coupled to SOD (Mhamdi *et al*., 2010). In mitochondria during respiration, $O_2^{\cdot-}$ are produced in two segments of the electron transport chain: in the flavoprotein NADH dehydrogenase (complex I) and in the ubiquinone zone. In glyoxysomes, acyl-CoA oxidase is the primary enzyme responsible for H_2O_2 generation. Plasma membrane-bound NADPH oxidases as well as cell-wall associated peroxidases are the main sources of $O_2^{\cdot-}$ and H_2O_2 producing apoplastic enzymes (Fotopoulos *et al*., 2010) (Fig. 1). ROS are key players in regulation of fundamental processes of plant metabolism, such as cellular growth (Foreman *et al*., 2003), stomatal closure (Pei *et al*., 2000). Moreover, ROS are known to orchestrate plant gene expression (Vanderauwera *et al*., 2005), as well as to modulate the activity of key signalling components, such as mitogen activated protein (MAP) kinases (Marino *et al*., 2011). Under optimal conditions, ROS production is controlled and deliberate, enabling plants to better adapt to the environment (Pitzschke *et al*., 2006).

During the earlier stages of legume-rhizobia interaction, the host plant reacts to the invasion of bacteria by over production of ROS to initiate the hypersensitive reaction (HR). The HR is characterized by a localized plant cell death at the site of infection that blocks further invasion of bacteria and by the induction of a systemic resistance to virulent pathogens (Santos *et al*., 2000). The ROS accumulation, detected few hours post treatment with Nod Factors (NFs), is linked to the NFs signaling transduction pathway as rhizobial mutants that produce altered NFs or a non-nodulating mutant are impaired in the ability to elicit ROS production (Ramu *et al*., 2002). Inhibition of this ROS production prevents root hair curling and formation of infection threads (Peleg-Grossman *et al*., 2007). This production of H_2O_2 is involved in the oxidative crosslinking needed for strengthening of the infection threads and cell wall formation. In contrast, H_2O_2 is not found in the nitrogen-fixing zone of the nodules (Cárdenas *et al*., 2008). In the plasma membrane, NADPH oxidases generate $O_2^{\cdot-}$ and H_2O_2 and perform important functions both in plant immunity and in the symbiotic interaction (Marino *et al*., 2012). Thus, the production of ROS is not a plant defense response to the microbe, but rather a process at multiple spatiotemporal steps that are needed for the development of a proper nodule formation (Chang *et al*., 2009).

On the other hand, there is an 'Oxygen paradox' in BNF which states that although elevated need of oxidative metabolic rates (higher respiration required to sustain BNF) favors an increased production of ROS, the activity of key enzyme i.e. nitrogenase is extremely sensitive to inhibition by oxygen and ROS (Alquéres *et al*., 2010). The most accepted hypothesis, called ''respiratory protection'', postulates that the increase in O_2 consumption during BNF would allow sufficient ATP

production and at the same time lower intracellular oxygen levels, preserving nitrogenase activity (Ureta and Nordlund, 2002). Legumes face oxidative risks beyond those associated with photosynthesis in other plants. Legume root nodules are especially at risk from oxidative damage by ROS because they contain all the ingredients required in Fenton chemistry, especially large amount of oxygen-labile proteins (nitrogenase, hydrogenase and ferredoxin) and potentially "catalytic Fe" available for free radical production (Marino *et al*., 2009). The main source of ROS in nodules is probably LHb, where only the ferrous form of LHb (LHb^{2+}) is able to bind O_2, forming oxyleghemoglobin ($LHb^{2+}O_2$). However, $Lb^{2+}O_2$ spontaneously autoxidizes to form ferric LHb (LHb^{3+}) and $O_2^{\cdot-}$, especially at acidic pH. The released $O_2^{\cdot-}$ can subsequently oxidize other molecules of $LHb^{2+}O_2$ to LHb^{3+}, thus enhancing the overall rate of LHb^{2+} and $LHb^{2+}O_2$ inactivation. Both $LHb^{2+}O_2$ and LHb^{3+} can also be oxidized by H_2O_2, which in turn may attack leghemoglobin, releasing catalytic Fe and producing the highly toxic $HO^{\cdot}$ radical through the Fe-catalyzed decomposition of H_2O_2 (Fenton reaction) (Halliwell and Gutteridge, 1989). The resulting Fe^{3+} can be reduced back to Fe^{2+} by the $O_2^{\cdot-}$ radical (Fe-catalyzed Haber-Weiss reaction) or by ascorbate, thus sustaining the Fenton reaction. At molar ratios of H_2O_2 to LHb^{3+} of 1:1 or 2:1, two LHb species are formed: ferryl LHb (LHb^{IV}), a relatively long lived species, and at least one globin radical. The MoFe (dinitrogenase) protein, and especially the Fe (dinitrogenase reductase) protein and the FeMo cofactor of nitrogenase, are irreversibly damaged by O_2. Ferredoxin, the proximal electron donor to nitrogenase, is also abundant in bacteroids. Based on the well-known ability of ferredoxins from bacteria and chloroplasts to reduce O_2 to $O_2^{\cdot-}$ (Misra and Fridovich, 1971), these powerful redox proteins are another likely source of ROS in nodules. ROS induce protein degradation, originating protein radicals and catalytic iron, which induce lipid peroxidation with generation of hydroxyl radicals and glutathione oxidation with generation of superoxide and oxygen peroxide (Chang *et al*., 2009; Marino *et al*., 2009). Because of the extreme reactivity and short life of the $OH^{\cdot}$ radical, it cannot be scavenged specifically in nodule cells and its formation has to be prevented by destroying H_2O_2 and $O_2^{\cdot-}$ radical and by sequestering Fe in forms inactive to catalyze free radical production, such as phytoferritin (Halliwell and Gutteridge, 1990).

Thus two, somewhat opposing, 'faces' of ROS, i.e. on the one hand, the damaging toxic molecule, and on the other hand, the beneficial signal transduction molecule, underscore the need to tightly control the steady-state concentrations of ROS in order to prevent oxidative damage in plant cells during normal metabolism (Miller *et al*., 2010). Plant cells are equipped with two intricate antioxidative systems to tightly regulate ROS homeostasis: one enabling the fine modulation of low levels of ROS [superoxide dismutase (SOD), guaiacol peroxidase (GPOX) and catalase (CAT)], and one regenerating the oxidized antioxidants [ascorbate peroxidase (APOX) and gluthatione reductase (GR)] (Araújo *et al*., 2015). Regeneration mechanism is fueled by ascorbate (AsA) and glutathione (GSH), which are major redox compounds also involved in other important processes such as photosynthesis, nitrogen fixation and organ development (Pignocchi *et al*., 2003). Under normal physiological conditions, AsA exists mostly in reduced form and regeneration of AsA is extremely essential because fully oxidized DHA (dehydroascorbate) has a short half-life and would be lost unless it is reduced back (Foyer and Noctor, 2011). The cellular redox state of AsA is regulated by its catalytic recycling, from either an unstable free radical-monodehydroascorbate (MDHA) or DHA, by monodehydroascorbate reductase (MDHAR) or dehydroascorbate reductase (DHAR), respectively (Vadassery *et al*., 2009). The electrons for MDHA and DHA reduction are provided by NADPH and GSH respectively and in doing so, GSH is oxidized to GSSG (Dunajska-Ordak *et al*., 2014). In the glutathione pool, NADPH-dependent GR reduces the disulfide bond of GSSG and sustains the reduced status of glutathione (Arora *et al*., 2002). Thus, maintaining balance between GSH and GSSG is the rate-limiting step and is critical for keeping a favorable redox status (Chellamma and Pillai, 2013). The key element for efficient protection against buildup of oxidant H_2O_2 is to maintain high levels of APOX and a high ratio of crucial redox buffers and sensors, i.e. GSH/GSSG and/or AsA/DHA (Fotopoulos *et al*., 2010; Foyer and Noctor, 2011) (Fig. 1).

Antioxidant defenses are indispensable to all aerobic life, but they are especially important for N_2-fixing organisms. In particular, nodules are very rich in antioxidants, probably as a result of the diverse reactions that generate ROS in nodule host cells and bacteroids (Puppo *et al*., 2005; Becana *et al*., 2010) (Fig. 2). Oxidative stress control in nodules is assured by changes in nodule cortex leading to the limitation of oxygen flux through the oxygen diffusion barrier and mainly the mobilization of antioxidant enzymes such as SOD, CAT and the enzymes of the AsA-GSH cycle (Mhadhbi *et al*., 2009). The abundance of AsA and thiols in the nodule-infected tissue strongly suggest that both types of redox metabolites cooperate in scavenging

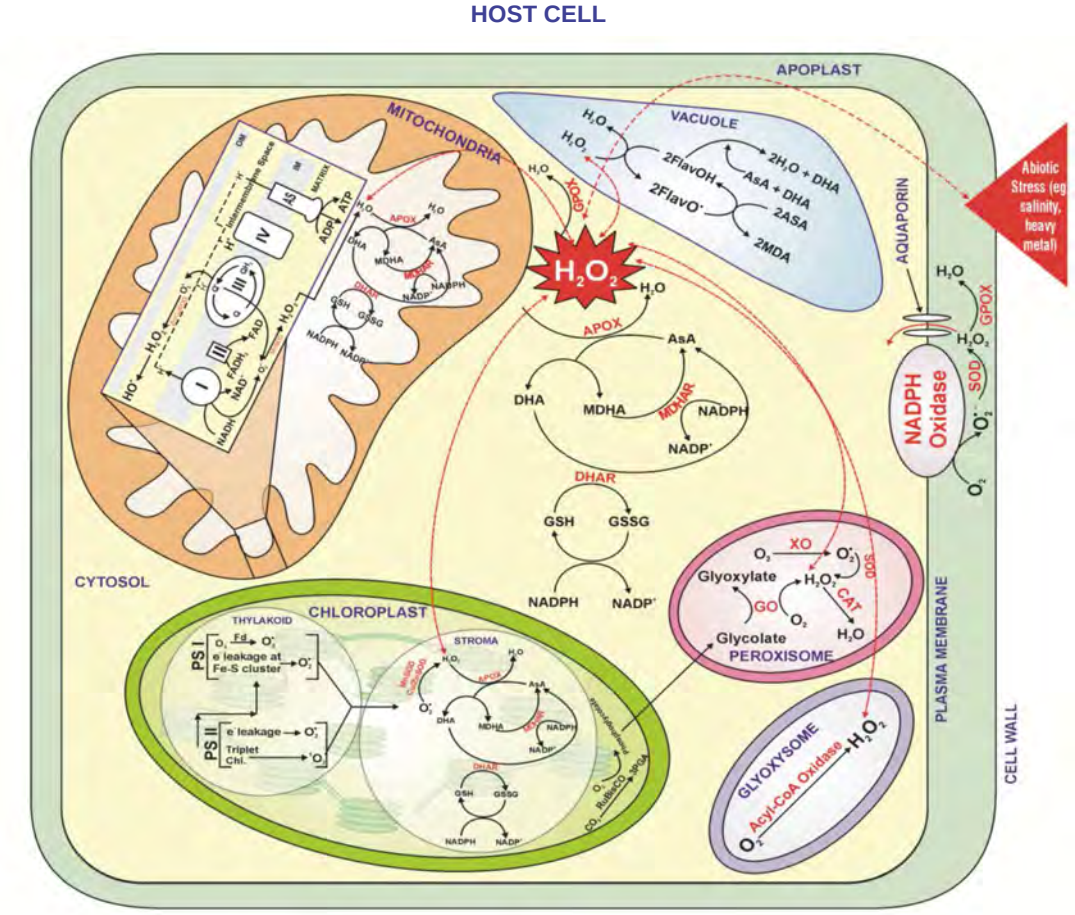

Fig. 1: Regulation of ROS generation and antioxidant machinery at various sites in host cell of legume

I: NADH-CoQ reductase, **II:** succinate-CoQ reductase, **III:** $CoQH_2$-cytochrome c reductase, **IV:** cytochrome c oxidase, **ADP:** Adenosine diphosphate, **APOX:** Ascorbate Peroxidase, **AS:** ATP Synthase, **AsA:** Reduced ascorbate, **ATP:** Adenosine triphosphate, **CAT:** Catalase, **CO_2:** Carbon dioxide, **Cu/ZnSOD:** Copper- and zinc-containing superoxide dismutase, **DHA:** dehydroascorbate, **DHAR:** Dehydroascorbate Reductase, **e- leakage:** electron leakage, **$FADH_2$:** hydroquinone form of Flavin adenine dinucleotide, **FAD:** quinone form of Flavin adenine dinucleotide, **Fd:** Ferredoxin, **FlavOH:** Flavonoid containing a free hydroxyl group, **FlavO$^{\cdot}$:** Flavonoid phenoxyl radical, **GO:** Glycolate oxidase, **GPOX:** Guaicol Peroxidase, **GR:** Glutathione Reductase, **GSH:** Reduced glutathione, **GSSG:** Oxidised glutathione, **H^+:** proton, **H_2O_2:** Hydrogen peroxide, **H_2O:** Water, **HO$^{\cdot}$:** Hydroxyl radical, **IM:** Inner membrane, **MDA:** Monodehydroascorbate, **MDHAR:** Monodehydroascorbate Reductase, **MnSOD:** Manganese Superoxide Dismutase, **NADPH:** Reduced Nicotinamide adenine dinucleotide phosphate, **NADP$^+$:** Oxidised Nicotinamide adenine dinucleotide phosphate, **OM:** Outer membrane, **$O_2^{\cdot-}$:** Superoxide radical, **O_2:** Oxygen, **3PGA:** 3-Phosphoglyceric acid, **PSII:** Photosystem II, **PSI:** Photosystem I, **Q:** Ubiquinone, **Q$^{\cdot}$:** transient semiquinone, **QH_2:** Ubiquinol, **RuBisCO:** Ribulose-1,5-bisphosphate carboxylase/oxygenase, **SOD:** Superoxide Dismutase, **Triplet Chl.:** triplet chlorophyll, **XO:** Xanthine Oxidase.

harmful concentrations of H_2O_2 in host cells by fueling the AsA-GSH cycle (Marino *et al*., 2009; Redondo *et al*., 2012). In some legume species and tissues, glutathione is partially or completely replaced by homoglutathione, another thiol tripeptide (where substitution of alanine for the terminal glycine takes place) which is synthesized in the cytosol and taken up by the mitochondria and bacteroids and has analogous functions (Loscos *et al*., 2008; Rubio *et al*., 2009). Dalton *et al*. (1998) proposed that the diffusion of O_2 into the nodule is regulated by adjustment of aerobic respiratory activity in parenchyma, with APOX functioning as a scavenger of H_2O_2 generated by respiration. Higher abundance of AsA and GSH in unstressed nodules, strongly suggests that their roles are not restricted to being substrates for APOX and DHAR. Both metabolites contribute to maintain the highly reducing conditions required for nitrogen

fixation and to protect nodule activity by direct scavenging of activated oxygen, especially of organic radicals (Halliwell and Gutteridge, 1989). Ascorbate and glutathione is involved in the regulation of quiescence, mitosis and cell growth and elongation (Potters *et al*., 2004). In nodules, the high mitotic activity in zone I (meristem) and the growth of infection threads in zone II (invasion) are therefore expected to require AsA and GSH. MDHAR is primarily localized in nodule cell walls, where it may regenerate the AsA consumed in the maintenance of redox status of cell wall proteins and lignifications (Matamoros *et al*., 2006). When soybean roots were supplied with exogenous ascorbate, striking increases in nodule nitrogenase activity and protection against oxidative damage were observed (Bashor and Dalton, 1999). Furthermore, the inclusion of ascorbate and ascorbate peroxidase in a model system containing *Bradyrhizobium japonicum* bacteroids and leghemoglobin resulted in large increases in nitrogenase activity and enhanced oxygenation of haem proteins (Puppo *et al*., 2005). In addition, GSH is required to maintain protein thiol groups in the reduced state and to activate transcription of defensive genes (Gogorcena *et al*., 1995). Moreover, glutathione has been shown to regulate auxin transport and root growth and higher GSH levels in the parenchyma are important for the efficient functioning of O_2 diffusion barrier. Thus antioxidants, especially GSH concentration regulates BNF efficiency in nodules and its deficiency impairs nodule growth (El Msehli *et al*., 2011). The concentrations of antioxidants in nodules are generally higher, which suggests an important connection between N_2 fixation and antioxidants (Becana *et al*., 2010). AsA concentrations were found to be about 10-fold higher in nodules than in roots of soybean (Bashor and Dalton, 1999). Concomitant with increases in ascorbate concentration during nodule development, enzymatic activities of APX, DHAR, and MDAR were at least twofold higher in nodules than in roots of soybean and common bean (Jebara *et al*., 2005). Greater differences in APX activity have been reported in alfalfa, pea, bean, and *Lotus japonicus*, for which activities were found to be between 12- and 38-fold higher in nodules compared with roots (Matamoros *et al*., 2006; Günther *et al*., 2007). Alquéres *et al*. (2010) did a cluster analyses based on common expression patterns and revealed the existence of a stable cluster made up of the genes encoding a-subunit of nitrogenase Mo-Fe protein (*nifD*), superoxide dismutase (*sodA*) and catalase type E (*katE*). They observed that controlled ROS production in nitrogen-fixing cells, due to the up-regulation of transcript levels of ROS-detoxifying genes, is an adaptive mechanism to allow nitrogen fixation. Moreover paraquat, a redox cycler that increases cellular ROS levels, was found to inhibit nitrogenase activity in a dose-dependent manner.

2.2. Under stressed conditions

However, when the plant is exposed to a rapid decrease in water potential, such as under salinity or drought stress (Mittler *et al.,* 2006), the reduction of photosynthesis is correlated with an increase in photorespiration. Linked with the photorespiration and/or as a consequence of imbalance between CO_2 and the electrons derived from light reactions, additional generation of ROS takes place. Thus, equilibrium between production and scavenging of reactive oxygen species (ROS) is overridden by the oxidative burst of ROS generation leading to disruption of cellular homeostasis (Ashraf, 2009). With an increase in the intensity of stress, the AsA/DHA redox state is directly affected by APOX activity and indirectly by impaired redox homeostasis of the glutathione pool, thereby leading to deterioration of the system (Dunajska-Ordak *et al*., 2014). Besides damaging plant tissues, excess ROS production can drastically damage bacteria. Salinity causes premature senescence of nodules and disturbs the delicate mechanisms of oxygen control that are essential for active nitrogen fixation (Sprent, 1981). This stress-induced senescence has been linked to the enhanced production of oxidants and the lowering of antioxidant defenses, enhancement of oxidized/ reduced thiol ratio and enhanced levels of malondialdehyde (MDA) (Hernández-Jiménez *et al*., 2002). In stressed *Medicago truncatula* root nodule proteome analysis, besides the dominating presence of leghemoglobin (LHb), Larrainzar *et al*. (2007) found the set of enzymes involved in ascorbate/ glutathione cycle, reflecting that the active antioxidant defense occurs within nodules. However, continued reduction in residual respiratory activity, possibly combined with a reduced efficiency of the O_2 diffusion barrier, leads to an increase in O_2 access in the infected area which consequently produces excess ROS. A marked decline in antioxidant protection results from interaction between catalytic Fe and the ROS (Groten *et al*., 2006). In this framework, diminution of GSH/hGSH and AsA pools was found to be correlated with a decrease in nitrogen-fixing efficiency during natural senescence in a number of legumes such as pea (Groten *et al*., 2006) or common bean (Loscos *et al*., 2008). Because of the high potential of nodules to generate activated oxygen and their large demand for antioxidant protection to preserve nodule functioning, the possibility exists that

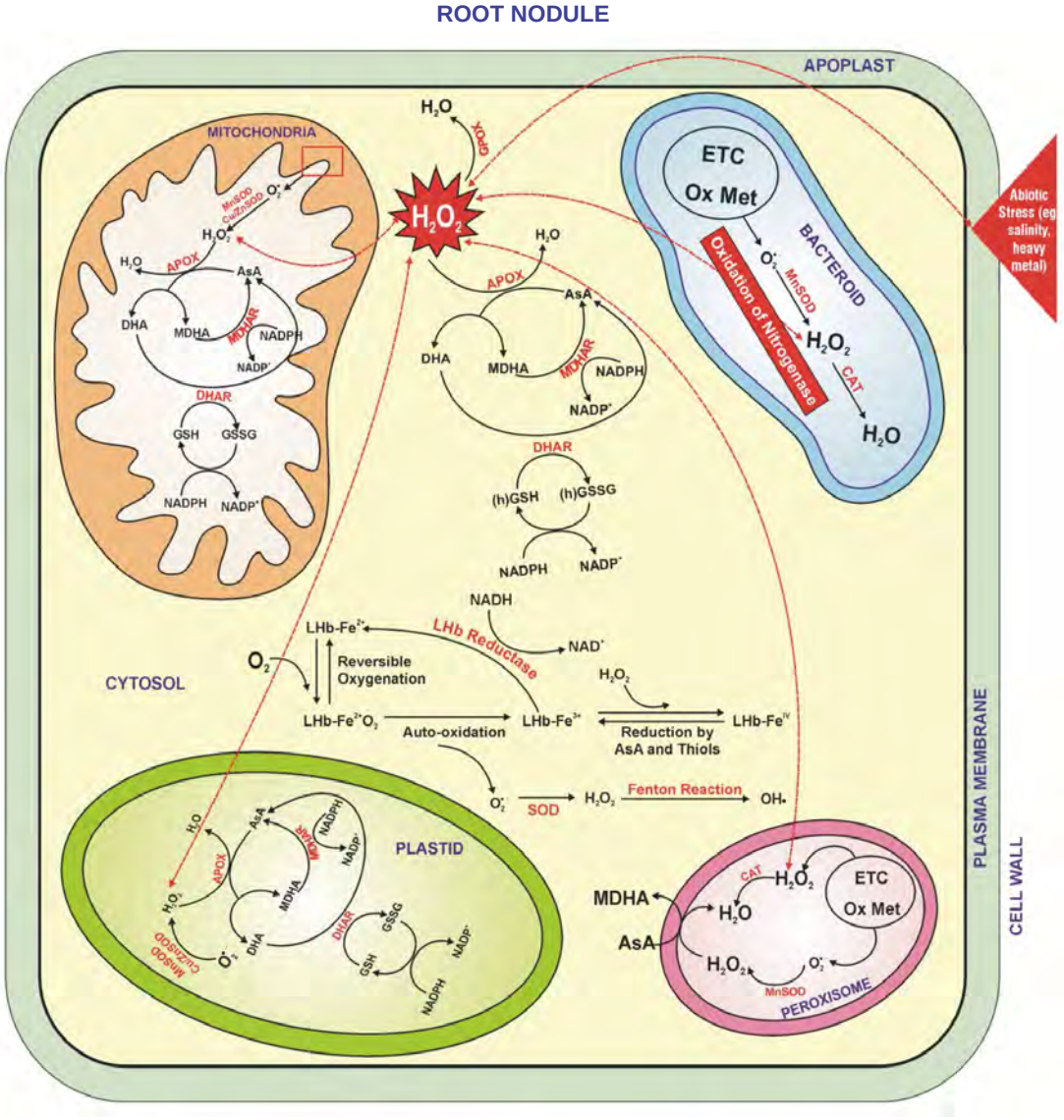

Fig. 2: Regulation of ROS generation and antioxidant machinery at various sites in root nodule of legumes

APOX: Ascorbate Peroxidase, **AsA:** Reduced ascorbate, **CAT:** Catalase, **Cu/ZnSOD:** Copper- and zinc-containing superoxide dismutase, **DHA:** dehydroascorbate, **DHAR:** Dehydroascorbate Reductase, **ETC:** Electron Transport Chain, **GPOX:** Guaicol Peroxidase, **GR:** Glutathione Reductase, **GSH:** Reduced glutathione, **GSSG:** Oxidised glutathione, **hGSH:** Reduced homoglutathione, **hGSSG:** Oxidised homoglutathione, **H_2O_2:** Hydrogen peroxide, **H_2O:** Water, **$HO^{\bullet}$:** Hydroxyl radical, **LHb-Fe^{2+}:** Ferrous form of Leghemoglobin, **LHb-$Fe^{2+}O_2$:** oxyleghemoglobin, **LHb-Fe^{3+}:** Ferric form of Leghemoglobin, **LHb-Fe^{IV}:** Ferryl form of Leghemoglobin, **LHb Reductase:** Leghemoglobin Reductase, **MDHA:** Monodehydroascorbate, **MDHAR:** Monodehydroascorbate Reductase, **MnSOD:** Manganese Superoxide Dismutase, **NADH:** Reduced Nicotinamide adenine dinucleotide, **NAD^+:** Oxidised Nicotinamide adenine dinucleotide, **NADPH:** Reduced Nicotinamide adenine dinucleotide phosphate, **$NADP^+$:** Oxidised Nicotinamide adenine dinucleotide phosphate, **$O_2^{\bullet-}$:** Superoxide radical, **O_2:** Oxygen, **Ox Met:** oxidative metabolism, **SOD:** Superoxide Dismutase.

stress shifts the balance between production and scavenging of activated oxygen, leading to oxidative stress (Gogorcena *et al.*, 1995). This excessive ROS production during nodule senescence and prolonged stress, can be lethal to cell integrity, owing to their capacity to damage proteins, lipids, and DNA (Rivero *et al.*, 2007).

3. Regulation of Antioxidants Machinery in Salt Stressed Legumes: Critical for Efficient *Rhizobium*-Legume Symbiosis

3.1. In non-mycorrhizal legumes

Various studies have emphasized a strong correlation between enhanced antioxidant enzyme activities and increased legume resistance to environmental stress (Mhadhbi *et al*., 2004; Tejera *et al*., 2004; Marino *et al*., 2008). Salt induced increase in the activity of antioxidative enzymes like SOD, CAT, POX, APOX and GR has been reported in leaves of mung bean (Dar *et al*., 2007) and *Glycine max*. L. (Doğan, 2011), roots of *Cicer arietinum* L. (Mishra *et al*., 2009) and many more. Higher salt resistance of tolerant genotypes of pea (Hernández *et al*., 2001), soybean (Malenčić *et al*., 2003), green pea (Yasar *et al*., 2008), pigeonpea (Garg and Manchanda, 2009) and chickpea (Garg and Singla, 2015) has been found to be associated with higher activity of antioxidant enzymes in their tolerant genotypes. Many studies on salt stressed legumes have also highlighted the differential regulation of different isoforms of antioxidant enzymes in a genotype and organ dependent manner. Corpas *et al*. (1991) established the subcellular location of three SOD isozymes (Mn-SOD, CuZn-SOD I and CuZn-SODII) in the leaves of salt-stressed *Vigna ungiculata* L. plants. Extensive studies conducted by Hernández *et al*. (1993) in salt stressed pea plants concluded that salt-induced higher enhancement in the rate of mitochondrial SOD activity (Mn-SOD) was concomitant with higher resistance of NaCl-tolerant plants as compared to the sensitive ones. Further, Hernández *et al*. (1999) reported that at 70 mol m^{-3} NaCl concentration, Fe-SOD and CuZn-SODII activity remained unchanged in pea leaves; while at 110-130 mol m^{-3} NaCl, activity of cytosolic CuZn-SODI, chloroplastic CuZn-SODII, mitochondrial and/or peroxisomal Mn-SOD increased, along with the increased activities of other antioxidant enzymes (APOX and MDHAR). Further, GR and DHAR activities were induced under 130-160 mol m^{-3} NaCl stress, thereby suggesting the differential induction of antioxidant defense depending upon the concentration of salt. When the effect of salt on antioxidant defenses was examined at the transcript levels of mitochondrial, chloroplast and cytosolic enzymes, an increase in all SOD isoenzymes was only found in tolerant pea plants suggesting that the induction of SOD activity is one component of the tolerance mechanisms of peas to long-term salt treatment (Hernández *et al*., 2000). In another study, Hernández *et al*. (1994) reported that differential regulation of SOD isoenzymes in different organelles of the leaves of salt-stressed cowpea plants was evidenced by significant reduction in mitochondrial Mn-SOD, slight decrease in cytosolic and mitochondrial Cu/Zn-SODI and no change in chloroplastic Cu/Zn-SODII. Bandeoglu *et al*. (2004) reported that root tissues of lentil plants were better protected from NaCl stress induced oxidative damage due to higher enhancement of total SOD activity (especially Cu/ZnSOD isoforms) together with higher levels of APOX activity in roots as compared to leaves. Similarly, Eyidogan and Öz (2007) found increased expression of isoenzymes of various antioxidants in salt-stressed chickpea seedlings and suggested that CAT and SOD activities played an essential protective role against oxidative burden. Relatively higher salt tolerance of Zhongmu 1 (Wang and Han, 2009) and Xinmu No. 1 (Wang *et al*., 2009) cultivar of alfalfa (*Medicago sativa* L.) was attributed to their higher capacity to up-regulate antioxidant enzymes in shoots and roots.

In legumes, besides antioxidant machinery in roots and aboveground plant parts, differential regulations of antioxidants have been reported to preserve rhizobial symbiosis from stress. In the study conducted by Molina *et al*. (2011) in salt stressed chickpea roots and nodules, diverse expression profiles were revealed by UniTags annotated to antioxidants of AsA-GSH cycle. Several of these transcripts (CATs, APXs, DHARs, GPOX, GRs) were very active in nodules even before the onset of the stress, probably due to the high metabolic activity of nodules. Interestingly, analysis of Jebara *et al*. (2005) on nodular antioxidant enzyme expression in salt stressed *Phaseolus vulgaris* genotype BAT477 inoculated with reference strain CIAT899 indicated that nodular isozymes have bacterial and root origins. Among CuZn, Fe, Mn SOD, CAT, APOX and GPOX isoforms expression in nodules; FeSOD, MnSOD and CAT isoforms were of rhizobial origin and thus suggested the bacterial contribution of nodule response to salt stress. In the study of Tejera *et al*. (2004), a more active symbiosis in salt stressed common bean plants inoculated with salt-tolerant *Rhizobium tropici* wild-type strain CIAT899 than its 'decreased salt-tolerance (DST) mutant' derivatives (HB8, HB10, HB12 and HB13) was attributed to higher antioxidant enzyme activities in wild-type nodules as compared to mutant nodules. Similarly in the investigations of Esfahani and Mostajeran (2011), relatively higher drought tolerance of *Cicer arietinum-Mesorhizobium ciceri* local C-15 strain symbiosis as compared to symbiosis with nonlocal CP-36 strain was associated with higher induction of POX and APOX activities in the former. The importance of redox homeostasis to symbiotic BNF process has also been indicated in studies of

Sinorhizobium meliloti strains, where mutants limiting in the antioxidant defense did not reach the differentiation stage of nitrogen-fixing bacteroids (Santos *et al*., 2000; Harrison *et al*., 2005). Similarly, a peroxiredoxin (prxS)/bifunctional catalase-peroxidase (katG) *Rhizobium etli* double mutant had significantly reduced symbiotic nitrogen fixation capacity (Alquéres *et al*., 2010).

The consequential depressive effect of oxidative damage, due to salt induced disturbance in the antioxidant defense mechanism, on LHb concentration, nitrogenase activity and nodule structure and functioning has been recorded in salt stressed chickpea (Mhadhbi *et al*., 2004), soybean (Borucki and Sujkowska, 2008), pigeonpea (Manchanda and Garg, 2011) and pea (Zilli *et al*., 2008), *Medicago ciliaris* (Ben Salah *et al*., 2010). Earlier studies of Comba and Benavides (1997) of salt-stressed soybean varieties reported that in contrast to tolerant variety (377), decrease in antioxidant enzymes (APX, CAT, GR and SOD) and glutathione in salt sensitive variety (411) had adverse effects on their nitrogen fixing ability and Comba *et al*. (1998) reported that at 50 mM NaCl, an overall increase in antioxidant enzymes maintained LHb content and nitrogenase activity in soybean root nodules; while at severe stress (200 mM NaCl), decline in LHb content and nitrogenase activity was accompanied by decline in APOX, CAT and GR activities (except SOD and GSH). Similarly Jebara *et al*. (2010) and Mhadhbi *et al.* (2011) reported that in salt-stressed *Phaseolus vulgaris* and *Medicago truncatula* genotypes, respectively, higher symbiotic performance of tolerant genotype as compared to sensitive ones was associated with higher induction and sustenance of highly regulated antioxidant mechanisms. Contrastingly in the study of Zilli *et al*. (2008), activity of most of the antioxidant enzymes (SOD, CAT and POX) except heme oxygenase (HO) decreased in nodules of salt stressed soybean (*Glycine max* L.) plants and the up-regulation of HO was suggested to be associated with protection of nitrogen fixation and assimilation under saline stress conditions. Recently, Ramírez *et al*. (2013) analyzed the global gene response of *Phaseolus vulgaris* nodules, subjected to oxidative stress, using the Bean Custom Array 90K [including probes from 30,000 expressed sequence tags (ESTs)] and found a total of 4280 ESTs differentially expressed in stressed bean nodules; of these, 2218 were repressed. (qRT)-PCR analysis of transcription factor (TF) gene expression showed that 67 TF genes were differentially expressed in nodules exposed to oxidative stress. The TF families that exhibited the strongest responses to oxidative stress were MYB, AP2/EREBP, C2H2(Zn), C2C2(Zn), GRAS, CCAAT, ARF and ZnF-CCHC, suggesting that they may constitute a core group of TFs relevant for the response of bean nodules to oxidative stress. Members of the GRAS TF family and MYB family were earlier reported to be involved in developmental processes like rhizobial Nod-factor induction and biotic and abiotic stress responses also (Ge *et al*., 2010).

Overexpression of antioxidant does not always result in the enhancement of antioxidative defence. Study of redox balance as a result of equilibrium between protective and regeneration antioxidant systems is a determinant of the competence of antioxidant system under environmental stresses (Rodrigues *et al*., 2013) and can be useful in understanding cultivation of legume plants in salt affected areas (Mhadhbi *et al*., 2011). Stress induced senescence of nodules and decrease in N_2-fixation has been found to be associated with simultaneous decrease in the levels of ascorbic acid (AsA) and APOX activity in salt stressed cluster bean (*Cyamopsis tetragonoloba* Taub.) (Bishnoi *et al*., 1997) and with the decrease of CAT, APOX, DHAR and GR activities in drought stressed pea (Gogorcena *et al*., 1995), thus shifting the redox levels of nodules towards the more oxidative side. Significance of redox homeostasis was also evidenced in the study conducted by Hernández *et al*. (2001), where an increase in DHAR and GR along with a decrease in APOX, MDHAR, AsA and GSH levels was associated with higher decline in AsA/DHA and GSH/GSSG ratios in the symplast from salt stressed *Pisum sativum* cv. Lincoln (salt-sensitive), while salt induced increase in DHAR, GR and MDHAR activity in cv. Puget (salt-tolerant) was associated with lower decrease in AsA/DHA and GSH/GSSG ratios. Similarly, in the study of Sumithra *et al*. (2006), higher activities of ROS scavenging enzymes and GSH concentration in the leaves of Pusa Bold imparted them superior salt tolerance than the leaves of CO 4 cv. of Pusa Bold holding higher GSSG concentration. In the study Rubio *et al*. (2009), relatively higher salt tolerance of *Lotus japonicus* was attributed to the capacity of plants to up-regulate gene encoding for cytosolic SOD, GPOX and DHAR, which possibly relates to its role in ascorbate recycling in the apoplast. On the other hand, in the study of drought stressed alfalfa nodules, Antolín *et al*. (2010) revealed that despite an increase in antioxidant enzyme activities (APX, GR and CAT), decrease in the concentrations of AsA compromised H_2O_2 detoxification through the ASC-GSH cycle. Large depletion of AsA and GSH in stressed nodules can be attributed to oxidation of both molecules by activated oxygen, which increases

because of the greater amounts of catalytic Fe present in stressed nodules. Moreover, as a result of decreased supply of NAD(P)H, senescent nodules have a lower capacity to regenerate AsA and GSH (Gogorcena *et al*., 1995). Recently, Hossain *et al*. (2011) reported that in salt stressed mung bean seedlings, stress induced sharp increase in GPOX and GR along with increase in GSH, GSSG content; while decrease in MDHAR, DHAR and CAT activities along with the reduced GSH/GSSG ratio and AsA content was associated with an increase in oxidative burden. Consequently the above scrutinization elucidates that the key element for efficient protection against salt stress-induced buildup of oxidants, is to maintain high levels of APOX and GR and a high ratio of crucial redox buffers and sensors, i.e. GSH/GSSG and/or AsA/DHA.

3.2. In mycorrhizal legumes

Fundamental paradigm for cost-effective and environmentally sound technology is to utilize beneficial micro-organisms in such a way as to improve crop productivity under stressed conditions (Hasanuzzaman *et al*., 2014). Arbuscular mycorrhiza (AM) fungi, belonging to the phylum Glomeromycota (Schüßler *et al*., 2001) are widespread in both natural and agricultural ecosystems, including salt-stressed areas (Wilde *et al*., 2009). Legumes are known to establish beneficial symbiotic relationships with both arbuscular mycorrhizal fungi and N_2-fixing bacteria; which develop the multifunctional legume mycorrhizosphere, a scenario of diverse activities relevant for legume productivity in sustainable agriculture (Azcón and Barea, 2010). Mycorrhizal symbiosis associated with legumes is an essential link for effective phosphorus (P) nutrition, leading to enhanced N_2 fixation that advocate a synergistic tripartite association (Mortimer *et al*., 2009; Azcón and Barea, 2010). It is well established that this three-way relationship benefits the host to a greater extent than singular inoculation with either symbiont, resulting in improved nutrition and growth of the host plant even under stress (Mortimer *et al*., 2008). Mycorrhization alters plant root plasticity, enhances photosynthetic activity and reduces toxic ion accumulation by immobilizing them in the fungal structures or by preferably allowing K^+ and Ca^{2+} uptake over Na^+ or up-regulating Na^+ exclusion and in turn can influence plant productivity (Ruiz-Lozano *et al*., 2012; Estrada *et al*., 2013; Hajiboland, 2013). Additional mechanisms have been proposed, such as enhanced osmotic adjustment (such as proline, glycine betaine, amino-acids etc.) and reduced oxidative damage by activating antioxidant defense (Cicatelli *et al*., 2012). Mycorrhization has been suggested to induce these antioxidants by improving assimilation of low mobility micronutrients utilized for proper activity of metallo-enzymes, for example, SOD, CAT, and APOX (Ruiz-Lozano *et al*., 2012).

Although mycorrhization have been reported to improve antioxidant defense mechanism in many plant species growing in stressed soils, a very little attention has been directed towards AM-mediated oxidative stress alleviation in salt-stressed legume plant system and their nodules. Ruiz-Lozano *et al*. (2001) reported that alleviation of oxidative damage to lipids and proteins in soybean nodules was involved in the protective effect of *Glomus mosseae* symbiosis against nodule senescence. They proposed two possibilities to explain the low oxidative damage found in nodules of mycorrhizal plants as compared to non-mycorrhizal ones: either mycorrhizal plants suffered less drought stress due to a direct water uptake by fungal hyphae from sources inaccessible to nonmycorrhizal plants and transfer to the host plant and thus kept plants protected against excessive ROS generation or mycorrhizal plants increased the activities of a set of ROS scavenging enzymes. However, as stated earlier, efficient destruction of H_2O_2 requires the action of several antioxidant enzymes acting in synchrony. Porcel *et al*. (2003) reported that out of the four ROS scavenging enzymes analyzed in root and nodule tissues of mycorrhizal soybean plants, only GR activity was higher in mycorrhizal roots and nodules, whereas SOD and CAT activities were similar and APOX activity was lower in the drought stressed mycorrhizal roots than in the corresponding nonmycorrhizal ones. Similarly SOD, CAT and APX activities were lower in the drought stressed mycorrhizal nodules than in the nonmycorrhizal ones. Porcel *et al*. (2003) suggested that consistently higher GR activity in roots and nodules of mycorrhizal plants might have generated reduced antioxidants (GSH), which helped to decrease the oxidative damage to biomolecules that is involved in premature nodule senescence. Similarly, association of improved antioxidant enzyme activities in mycorrhizal plants, as compared to non mycorrhizal ones, with improved longevity of the nodules structure and functioning has been reported in *Glomus fasciculatum* inoculated *Anthyllis cytisoides* L. subjected to low soil water content (Goicoechea *et al*., 2005) and *Glomus mosseae* inoculated pigeonpea plants (Manchanda and Garg, 2011). Similarly, Garg and Manchanda (2009) demonstrated that increase in the antioxidant enzyme activities in roots and leaves was also involved in the beneficial effects of mycorrhizal colonization in imparting salt tolerance to salt stressed pigeonpea

plants. Conversely, Kohler *et al*. (2009) suggested that suppression of antioxidant enzymes in salt stressed leaves of *Lactuca sativa* L. cv. Tafalla inoculated with *Glomus intraradices* (Schenk & Smith) or *Glomus mosseae* (Nicol & Gerd.) Gerd. & Trappe) as compared to that of non-mycorrhizal plants could be due to lower oxidative stress experienced by these plants. Sohrabi *et al*. (2012) reported that mycorrhizal colonization further improved drought induced antioxidant activities (POX, CAT and APOX) in chickpea leaves, where comparatively higher upregulation of POX and CAT in mycorrhizal plants of ILC-482 cultivar induced better resistance to water deficit than Pirouz cultivar.

Recently, Younesi and Moradi (2013) also reported that *G. mosseae* colonization notably increased the activities of SOD, CAT, POX and GR in the nodules of soybean (*Glycine max*) under salt stress and alleviated the salt-induced oxidative burden on symbiotic nitrogen fixation. Evelin and Kapoor (2014) reported that *G. intraradices* colonization-mediated attenuation of oxidative stress in in roots and leaves of fenugreek plants subjected to varying degrees of salinity was due to enhanced activity of antioxidant enzymes and higher concentrations of antioxidant molecules. Garg and Chandel (2015) reported that rapid generation of ROS in nodules of pigeonpea genotypes exposed to 4 and 6 dS m^{-1} NaCl stress was counteracted by *Funneliformis mosseae*-induced enzymatic activities (SOD, POD, CAT and GR) and nonenzymatic components (GSH-GSSG cycling, their ratio and total glutathione) and further stimulation of existing antioxidant enzymatic and non-enzymatic pool contributed in better nodule functioning in saline soils. Although, the role of AM symbiosis on the activities of some antioxidant enzymes have been reported, comprehensive study on AM-mediated equilibrium between protective ROS scavenging antioxidants and reparative enzymes of the AsA-GSH pathway in salt-stressed legume plants has not been carried out so far. Recently in our lab, Garg and Singla (2015) reported that, improved root and shoot growth of salt stressed chickpea plants inoculated with *Funneliformis mosseae* was strongly correlated with synchronized working of antioxidant machinery in mycorrhizal plants. Failure in H_2O_2 management and significant reduction in AsA/DHA and GSH/GSSG ratio by drastic oxidation of the ascorbate-glutathione pool made chickpea plants susceptible to salt-stress and thus, a significantly higher loss of redox equilibrium in DCP 92-3 genotype as compared to PBG 5 genotype was responsible for lower salt tolerance of the former, especially in roots. Therefore, in this investigation, rather than being an effective defense mechanism, higher activity of the antioxidant machinery in stressed plants was merely an indicator of ROS overproduction. The higher rate of MDHAR and DHAR activities compared to APOX activity in AM-inoculated plants provided more AsA for H_2O_2 reduction and was evidenced by better AsA/DHA redox status in these plants (as compared to their non-mycorrhizal counterparts). Higher DHAR activity in AM-inoculated plants corresponded with higher GSH availability and an improved GSH/GSSG ratio, which was due to a higher rate of increment in GR activity compared to DHAR activity. Thus the study suggested that, mycorrhization in chickpea plants improved the synchronization between the reparative enzymes of the AsA-GSH cycle and in so doing assisted ROS scavenging enzyme (APOX) activity with a higher magnitude of reduced AsA. Moreover, superior growth and functioning of nodules of salt stressed mycorrhizal chickpea genotypes (PBG 5 and DCP 92-3) could be attributed to relatively better synchronization between ROS scavenging enzymatic machinery and AsA-GSH pathways even in the nodules as compared to non-mycorrhizal ones (data unpublished). Moreover, superior upregulation of antioxidant defense mechanism in tolerant genotype as compared to the sensitive ones, could be attributed to genotypic variability in terms of per cent mycorrhizal colonization and responsiveness (Garg and Chandel, 2015; Garg and Singla, 2015). Similarly, our research group is carrying out extensive studies on AM mediated-alleviation of salt induced oxidative burden in other genotypes of salt stressed chickpea plants and other legumes species in order to authenticate the above observations. In the light of available literature, as cited above, a layout has been proposed illustrating the differential regulation of antioxidative machinery in non-mycorrhizal and mycorrhizal plants under salt stress (Fig. 3).

4. Conclusions

In conclusion, present review consolidates the evidence for adverse effects of salt-induced oxidative stress in legumes and potential role of arbuscular mycorrhiza in protecting host legumes by altering redox buffer and synchronizing antioxidant network. This review paves a new way to exploit legume tripartite association for sustainable agriculture in saline soils. While researches over many years have broadened our understanding of the multi-complex processes directing the use of plant-mycorrhiza symbiosis in ameliorating salt stress in plants, yet, there is only fragmentary understanding on the role of AM in alleviating oxidative stress. Future research should employ molecular and genomic techniques to decipher

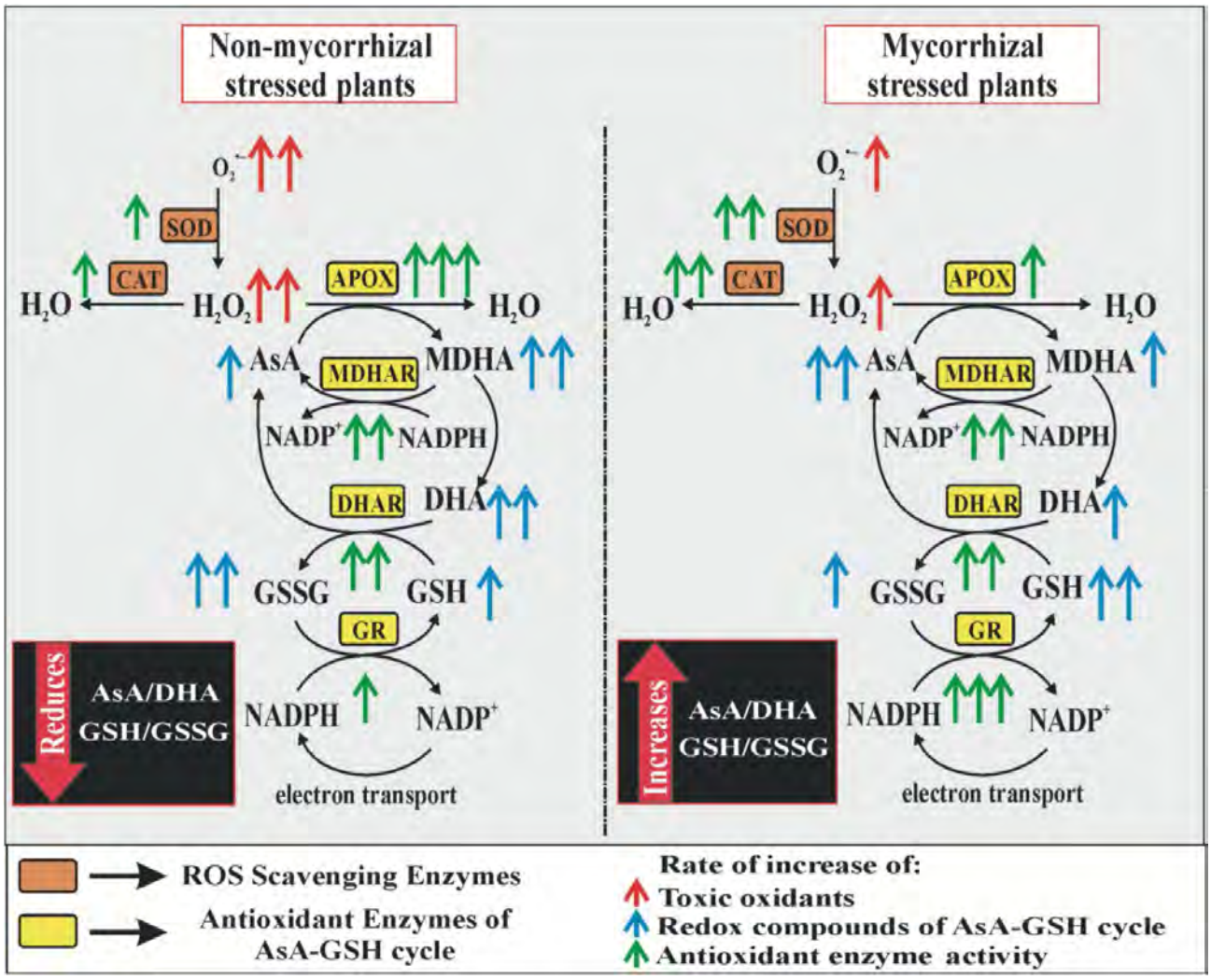

Fig. 3: Differential regulation of antioxidant machinery in non-mycorrhizal and mycorrhizal plants under salt stress

In salt stressed plants: Increase in SOD and CAT activities along with increasing AsA concentration in salt stressed plants cannot efficiently reduce H_2O_2, as indicated by high H_2O_2 concentration. Compared to AsA, DHA concentration increases at a higher rate with increasing salinity, thereby lowering the AsA/DHA ratio. Slower regeneration of AsA from MDHA and DHA under salt stress is due to slower rate of acceleration in MDHAR and DHAR activities compared to APOX. The rate of DHAR activity surpasses GR activity under salinity, suggesting that a predominant GSH oxidation takes place under salinity and lesser availability of adequate GSH (reducing power) turns DHAR activity incapable of providing sufficient AsA for APOX activity. Failure in H_2O_2 management and significant reduction in AsA/DHA and GSH/GSSG ratio by drastic oxidation of the ascorbate-glutathione pool make plants susceptible to stress.

In mycorrhizal stressed plants: Further increase in SOD activities under mycorrhization can efficiently scavenge $O_2^{\cdot-}$. Despite higher SOD activity, lower H_2O_2 concentration in AM-inoculated plants points toward rapid H_2O_2 scavenging by increased activities of CAT and peroxidases in AM plants. The higher rate of MDHAR and DHAR activities compared to APOX activity in AM-inoculated plants provide more AsA for H_2O_2 reduction. Higher rate of increment in GR activity compared to DHAR activity, makes GSH more available for DHAR activity in these plants. Mycorrhization can improve synchronization between reparative enzymes of the AsA-GSH cycle, increase AsA/DHA and GSH/GSSG ratio and in doing so can assist ROS scavenging enzyme activity with a higher magnitude of reduced AsA.

APOX: Ascorbate Peroxidase, **AsA:** Reduced ascorbate, **CAT:** Catalase, **DHA:** dehydroascorbate, **DHAR:** Dehydroascorbate Reductase, **GR:** Glutathione Reductase, **GSH:** Reduced glutathione, **GSSG:** Oxidised glutathione, **H_2O_2:** Hydrogen peroxide, **H_2O:** Water, **MDHA:** Monodehydroascorbate, **MDHAR:** Monodehydroascorbate Reductase, **NADPH:** Reduced Nicotinamide adenine dinucleotide phosphate, **$NADP^+$:** Oxidised Nicotinamide adenine dinucleotide phosphate, **SOD:** Superoxide Dismutase.

and understand the underlying AM-mediated mechanisms involved in altering redox buffer and synchronizing antioxidant network in salt-stressed host legumes.

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