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Antioxidant Responses of Pomegranate Due to Oxidative Stress Caused By ROS

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Abstract:- Pomegranate plant has wide usage because of its biologically active compounds with different biological activities, being present in different parts of plants. In this review, the point of focus is on antioxidant responses of this plant under conditions of oxidative stress due to reactive oxygen species. In pomegranate, ROS cause oxidative stress due to different biotic and abiotic stresses, some of them causing elevation of antioxidant level and some stresses cause decline in antioxidant level. But plant has ROS scavenging mechanism that is antioxidant defense system which can be enzymatic and non-enzymatic preventing damage caused by reactive oxygen species. By evaluating level of antioxidants in different parts of plant, we can decide about its therapeutic and commercial usage; this evaluation also acknowledges about its agricultural plantation at specific areas under specific stress conditions. Owing to more antioxidant activities in peel part, it can be used in prevention of food spoilage by enhancing the shelf life.

***Keywords*:-** *Biologically Active Constituents, Defense mechanism, Oxidative, Stress Pomegranate, ROS.*

I. INTRODUCTION

A. Introduction to Pomegranate

Pomegranate plant belongs to botanical family Punicaceae [1]. The worldwide production of pomegranate is approximately 1,500,000 tons [2]. Pomegranate (Punica granatum L.) is introduced from South East Asia. It is planted in Turkey, Iran, the USA, the Middle East, and Mediterranean and Arabic countries, as one of the significant fruits [3]. Pomegranate peel is taken into account as an agro-waste but it is a powerful source of compounds giving human health aids. Peel accounts for about 60 % of weight of pomegranate fruit [4]. Total Phenolic contents and antioxidant activity of pomegranate peel is greater than pulp [5]. Antioxidant activity of peel is also higher than flower, leaf and seed [6].

B. Biological Activities of Pomegranate Peel

Peel contains substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid [7]. Recently, addition of polyphenols to foods and biological systems remained point of focus. Polyphenols have ability to scavenge free radicals, i. e. antioxidant power. The generation of free radicals plays an important role in the progression of numerous pathological disturbances, such as atherosclerosis [8], brain dysfunctions [9] and cancer [10]. It was found that polyphenols from peel of pomegranate inhibit the proliferation and induced apoptosis of human prostate cancer cells [11]. Anti-inflammatory activity of pomegranate peel is due to gallagyldilacton, gallic acid, granatin B [12], [13]. Antioxidant activities of peel are due to Tannins like punicalin, punicalagin, pedunculagin, gallic acid and casuarinin [13]-[15]. It was found that the peel extract has tannins that made it effective virucidal agent [123] in opposition to genital herpes virus [16]. Numerous flavonoids in peel show antibacterial, antiviral, antioxidant, anti-inflammatory and antineoplastic bioactivities. These flavonoids are catechin, epicatechin, epigallocatechin-3-gallate, flavan-3-ol, kaempferol, kaempferolerol-3-O-glucoside, kaempferol-3-O-rhamnoglycoside, luteolin, luteolin 7-O-glucoside, Naringin, pelargonidin, prodelphindin, quercetin and rutin [4], [17]-[19].

Hydroxyl, carbonyl, and aromatic groups are also present in peel among significant amount of punicalagin [29], punicalin [14], granatin A [20], maleic acid [21], gallic acid [122], ursolic acid [26], and antioxidant constituents [5] and efficient constitutive chemical groups in the structure of these constituents could exhibit corrosion inhibition functioning. Phytochemicals are frequently mentioned to non-nutritive compounds assumed to be formed by plants, providing defense against harmful ultraviolet radiation, pathogens and herbivorous predators. Phytochemicals are of utmost significance for having prospective human health benefits and are valued for their biological and free radical scavenging actions. Peel of pomegranate has phenolic compounds, including flavonoids, anthocyanins and tannins that are the chief group of antioxidant phytochemicals [6]. Pomegranate fruit peel can be used as natural dye source for unmordanted fabrics. So, use of pomegranate peel in dyeing fabrics removes use of metallic mordants that have effect on color efficiency in process of mordanting. Unmordanted fabrics when dyed with pomegranate peel, show considerable antimicrobial activity [22]. One of the studies conducted in rats whose liver was made damaged due to CCl_4 showed that pretreatment with PPE boosts or preserves the action of hepatic enzymes superoxide dismutase, peroxidase and catalase. These enzymes have ability to scavenge free radicals, preventing their deleterious effects. PPE decreases lipid peroxidation to 54%. PPE cause considerable hypoglycemic effect by increasing insulin levels and renewal of pancreatic beta cells [23]. Blood

glucose level decreases due to components of pomegranate with known anti-diabetic activity. These components are ellagic, gallic, ursolic acids, uallic and oleanolic acids. Ellagic, gallic and ursolic acids are present in PPE [24].

Pomegranate has many flavonoids but only punicalagin has been identified to show inhibitory effect towards influenza virus. So, pomegranate extract may inhibit the viruses transmitted in our body. So, it can be concluded that PPE can be used for this purpose as having punicalagin [25]. One of the studies demonstrated PPE as to promote wound healing in skin by boosting up collagen synthesis, and also having antibacterial activity for wound bacteria comprising strains of Pseudomonas aeruginosa, E. coli, S. aureus, Klebsiella pneumoniae (K. pneumoniae), Streptococcus pneumoniae, Salmonella Anatum, S. Typhimurium [25]. Detailed description of bioactivity of active complements in peel of pomegranate is given in (Table-I) as follows:

Table-I: Biological activity of major tannins owing to its particular biologically active compounds (Sushil et al., 2013)

Compound	Bioactivity
Casuarinin	Antiviral, antioxidant
Corilagin	Antihypertensive, antineoplastic
Ellagic acid [EA]	Antineoplastic, skin whitening
Gallic acid	Antimutagenic, anti-inflammatory, antiviral, antioxidant
Methyl gallate	Antioxidant
Granatin A	Antioxidant, anti-inflammatory
Granatin B	Antioxidant, anti-inflammatory
Pedunculagin	Antineoplastic, antioxidant
Punicalagin	Antioxidant, antihypertensive, anti-hyperglycemic
Punicalin	Antioxidant, anti-HIV, anti-hyperglycemic

Table-II: Biological activities of major flavonoids and alkaloids owing to particular active constituents (Sushil et al., 2013)

Compound	Bioactivity
Catechin	Antineoplastic, antioxidant
Cyanidin	Antioxidant
Epicatechin	Antineoplastic
Epigallocatechin 3-gallate	Antineoplastic
Flavan-3-ol	Antineoplastic
Kaempferol	Antioxidant, anti-inflammatory
Kaempferol-3-0-glucoside	Antioxidant
Luteolin	Antioxidant, antioxidant
Luteolin-7-0-glucoside	Antioxidant
Quercetin	Antiviral, antioxidant, antineoplastic
Naringin	Antiviral, antibacterial
Major alkaloids of pomegranate peel	
Pelletierine	Antioxidant
Valoneic acid dilactone	Antidiabetic

C. Biological Activities of Pomegranate Fruit, Seeds, Leaves and Juice

Seeds, leaves and juices of pomegranate show antioxidant activities. Its fruit rind, flower and leaves exhibit anti-inflammatory and analgesic activities. Fruit of pomegranate also show anti-inflammatory activity. Leaves show antidiabetic, hypolipidemic and anti-obesity activities [121]. Principle constituents in juice of pomegranate are as: simple sugars aliphatic organic acids, gallic acid, ellagic acid, quinic acid, flavonols, amino acids, minerals, ascorbic acid [4]. Carbohydrates, reducing sugars, sterols, saponins, flavanoids, tannins, piperidine alkaloids, flavone, glycoside, and Ellagitannins, all of these are constituents of pomegranate leaves [14], [15].

II. REACTIVE OXYGEN SPECIES IN POMEGRANATE

A. ROS

ROS (reactive oxygen species) are free radicals such as hydroxyl radical (•OH), superoxide anion (O_2• −). Hydrogen peroxide (H_2O_2) is also ROS but non-radical molecule which is plentiful and more reactive in higher plants [27]. Each of ROS has distinct properties like half-life and mobility, mode of action, cellular sources. Scavenging system for each ROS is also distinct [28]. Following figure 1 shows the mechanism of ROS generation by energy transfer.

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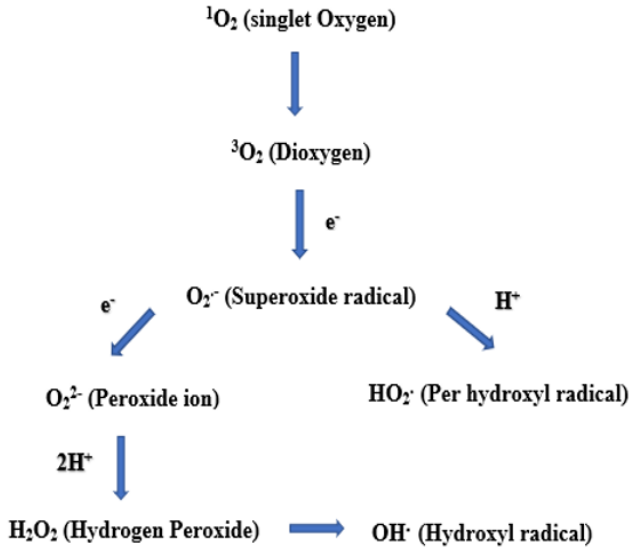

Fig.1. ROS generation by energy transfer

During energy transfer and electron transfer reactions ROS are produced from O_2. These reactions at initial require input of energy whereas next steps occur spontaneously as are exothermic [30], [31]. Under normal conditions in plant cells, there is equilibrium between ROS creation and elimination, but in case of any stress condition, this balance is upset in plants [28]. Molecular O_2 accumulate in atmosphere of earth due to which O_2 is used as terminal electron acceptor in aerobic organisms during process of cellular respiration [32]. Molecular O_2 is relatively unreactive, when reduced giving H_2O and ROS during normal metabolic activity of cell [30], [31], [33]. Extremely responsive ROS are produced due to O_2 reduction, when electron transport system not functions properly in stress condition [34]. ROS are overproduced by environmental factors like high light intensity, salinity, heat, chilling, wounding, ozone, heavy metals [28]. ROS have damaging effects to DNA, proteins, lipids and dysfunction metabolic activity in such a way that is irreversible causing cell death [35]. Figure 2 concludes the environmental factors which play role in ROS generation.

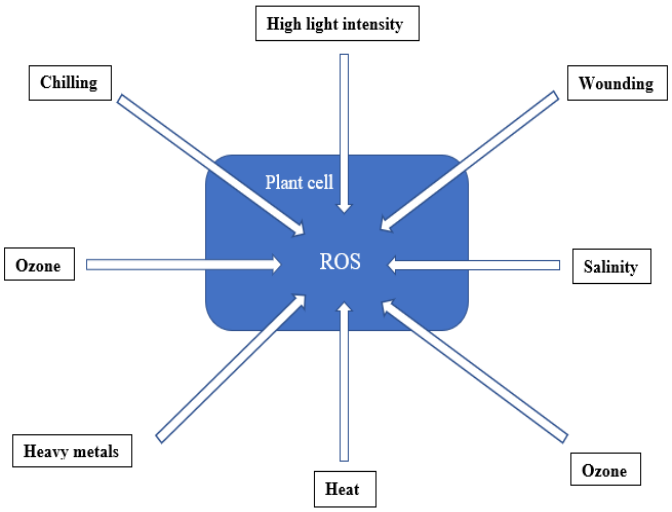

Fig. 2. Environmental factors causing ROS production

B. Dual Role of ROS

ROS play dual role, beneficial and deleterious depending on their concentration in plants. Beneficial effects are as, at lower concentration ROS act as signaling molecule and at higher concentration causes deleterious effects like ultimate death of cell [36], [37]. In plants ROS have dual behavior due to balance between ROS production and elimination, so act as signaling molecules at lower concentrations, regulating many physiological and developmental processes and perform damaging activity at higher concentration [38].

C. ROS Generation

These are produced in mitochondria, chloroplast and peroxisomes of plant cells [30]. ROS are also produced in other sites such as cytosol, cell wall and cytoplasm.

a.ROS generation in peroxisomes

ROS are generated in membrane and matrix of peroxisomes [39]. Peroxisome is membrane-bound subcellular organelle, present in nearly all eukaryotic cells and is major site of H_2O_2 production. Peroxisome has catalase and H_2O_2-producing flavin oxidase. Similar to glyoxysome, it has high concentration of catalase. Usually, peroxisomal respiratory pathway produce H_2O_2. Different flavin oxidases are involved in pathway. Following figure 3 shows the mechanism by which ROS are produced n chloroplast.

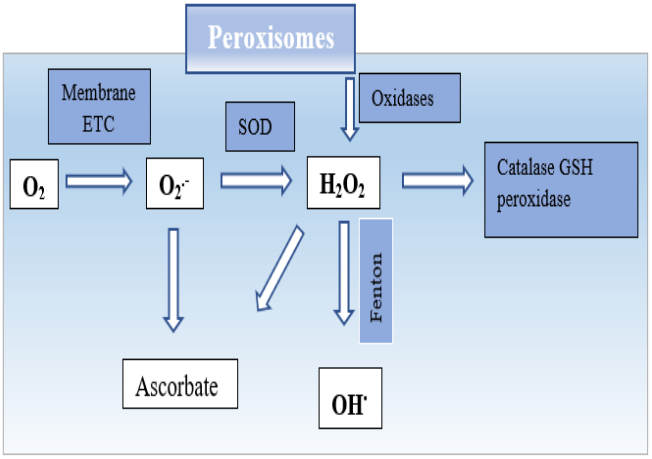

Fig.3. ROS generation and prevention in peroxisomes

In matrix, XOD catalyze oxidation of xanthin into uric acid. Super oxide radicals $O_2^{\cdot-}$ are converted to H_2O_2. Glycolate oxidase by oxidation of glycolate in process of photorespiration in peroxisome produce majority of H_2O_2. By beta oxidation of fatty acid, H_2O_2 also produced [28]. Electrons slip from PSI to PSII of peroxisome reacts with O2, giving Super oxide radicals $O_2^{\cdot-}$ is converted to Hydroperoxyl radical which ultimately converted to H_2O_2 [39].

b. ROS generation in Mitochondria

Mitochondria is known as major site where ROS like H_2O_2 are generated and is also considered as main site for ROS target [40]. In mitochondria, ROS are generated when

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O_2 is reduced by electrons that are present in ETC of mitochondria having sufficient free energy and this is known as primary source of ROS production in mitochondria [41]. When respiratory conditions are normal ROS are being generated, but their concentration reaches to high during biotic and abiotic stress conditions. In ETC of mitochondria, $O_2^{\cdot -}$ are generated at sites of complex I and complex III, this $O_2^{\cdot -}$ is dismutated by SOD into H_2O_2 [42]-[45]. About 1-5 % O_2 of mitochondria on consumption generate H_2O_2 [44]. Highly toxic $OH^{\cdot}$ is generated when H_2O_2 make reaction with Fe^{2+} and Cu^{+} (both are reduced forms), $OH^{\cdot}$ generated has no charge and is capable of penetrating membranes leaving mitochondria [41],[45]-[46]. ROS production may be controlled through energy dissipating systems in mitochondria [47]. Figure 4 shows how ROS are produced and scavenged in mitochondria.

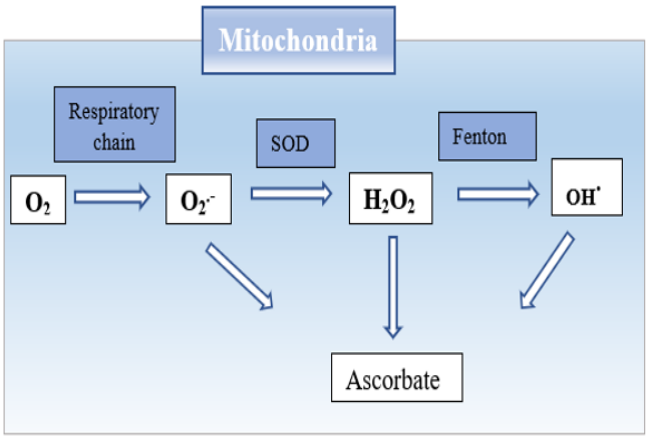

Fig.4. Production and prevention of reactive oxygen species in mitochondria

d. ROS generation in chloroplast

In chloroplast, $O_2^{\cdot -}$ produced when electrons are accepted by O_2 that is produced during photosynthesis. Sites of ROS production in chloroplast are chl. and ETC of PS I and PS II. Due to these sites, chloroplast is believed to be a major site where ROS are generated such as $O_2^{\cdot -}$, O_2 and H_2O_2. When electrons from photosystem centers are directed towards $NADP^{+}$, then reduces it into NADPH which reduces CO_2 (final electron acceptor) on entering the Calvin cycle and this happens under normal conditions. Through Mehler reaction, $O_2^{\cdot -}$ are generated when electrons reduce O_2 as flow of electrons gets diverted from ferredoxin to O_2, this happens when ETC is overloaded [48], [49]. $O_2^{\cdot -}$ are also produced when electrons get leakage at sides like QA, QB (acceptor sides of ETC in PS II) reaching the O_2 (M. Takahashi, 1988). Natural byproduct of photosynthesis is $^1O_2$ that is being formed during situation of low-light [50]. On dismutation of $O_2^{\cdot -}$ by Cu-Zn SOD, H_2O_2 generated at external membrane surface of stroma [51]. Mechanism of ROS generation and prevention in chloroplast is shown in Figure 5 as follows:

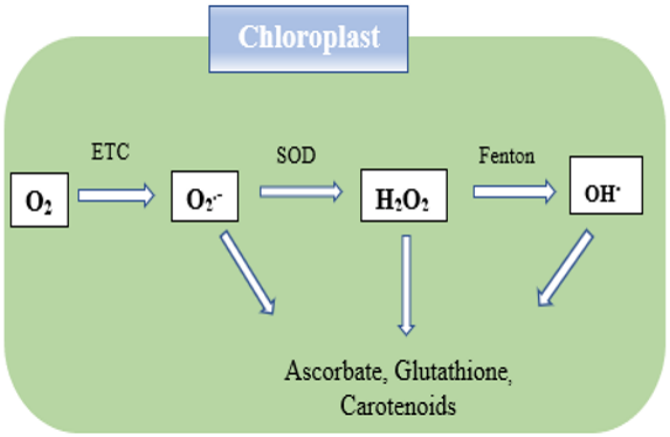

Fig.5 Generation and scavenge of reactive oxygen species in chloroplast

D. ROS Scavenging Mechanism in Pomegranate

Plants have natural defense mechanism due to which they show tolerance to stress. These involve antioxidant defense system that may be enzymatic and non-enzymatic. When ROS are produced under steady state, they are prevented from performing their damaging effects by antioxidant defense system [52], [53]. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX); all comprise enzymatic antioxidant defense system. Non-enzymatic antioxidant system comprises ascorbate (ASA), glutathione (GSH), α-tocopherol, carotenoids and phenolic compound that are non-enzymatic low molecular metabolites [54], [38] and [55]. Antioxidants have ability to scavenge free radicals.

III. ENZYMATIC DEFENSE MECHANISM

Types of antioxidants that are enzymatic in nature with their subcellular location are given in concise form in Table-III as follows:

Table-III Enzymatic antioxidants with subcellular localization (Kaushik et al., 2014)

Enzymatic antioxidants	Subcellular location
Superoxide dismutase (SOD)	Peroxisome, Mitochondria, Cytosol and Chloroplast
Catalase (CAT)	Peroxisome and Mitochondria
Ascorbate peroxidase (APX)	Peroxisome, Mitochondria, Cytosol and Chloroplast
Monodehydrascorbate reductase (MDHAR)	Mitochondria, Cytoplasm and Chloroplast
Dehydroascorbate reductase (DHAR)	Mitochondria, Cytoplasm and Chloroplast
Glutathione reductase (GR)	Mitochondria, Cytoplasm and Chloroplast
Guaiacol peroxidase (GPX)	Mitochondria, Cytoplasm, Chloroplast and ER

A. CAT

This is also named as H_2O_2 oxidoreductase [28]. This enzymatic antioxidant was firstly found and characterized. This enzyme has heme-group and is ubiquitous tetrameric. For H_2O_2, catalase is highly specific but against organic peroxides this enzyme displays weak activity. Reducing equivalents of cell are not required for catalase; therefore enzymes are unique in degrading H_2O_2 than other several types of H_2O_2-degrading enzymes. Rate of turnover is very quick in CAT, but it's affinity for H_2O_2 is much lower than APX. Peroxisome has high concentration of CAT. This enzyme is present in all aerobic eukaryotes, dismutating H_2O_2 into H_2O and O_2. Basically, it acts as an antioxidative enzyme that scavenges toxic level of H_2O_2 that is produced in peroxisome by oxidase of different metabolic oxidation reactions and also produced during oxidative stress. During stresses, peroxisome proliferates helping in removal of H_2O_2 [56].

H_2O_2 has ability to cross plant membranes. During photo respiratory oxidation, beta-oxidation of fatty acids and other enzymatic systems for example an enzyme system in which XOD coupled to SOD, H_2O_2 are generated and CAT scavenges these H_2O_2 [57], [58]. Cytosol, chloroplast and mitochondria all contain CAT but substantial CAT activity is no more pronounced in these organelles [59]. Subcellular localization of CAT is peroxisome, glyoxisome and mitochondria [28]. CAT is classified on basis of expression profile of genes of tobacco [60]. Light regulates class I CAT and expression of these CAT happens in photosynthetic tissues. Expression of class II CAT happens on vascular tissues with this expression occurring at high levels. Whereas in seedy and young seedlings, there is abundance of class III CAT. It is shown in figure6 how ROS generation is prevented. Equation (1) shows the function of CAT as follows:

$$H_2O_2 \longrightarrow H_2O + (1/2)\, O_2 \quad (1)$$

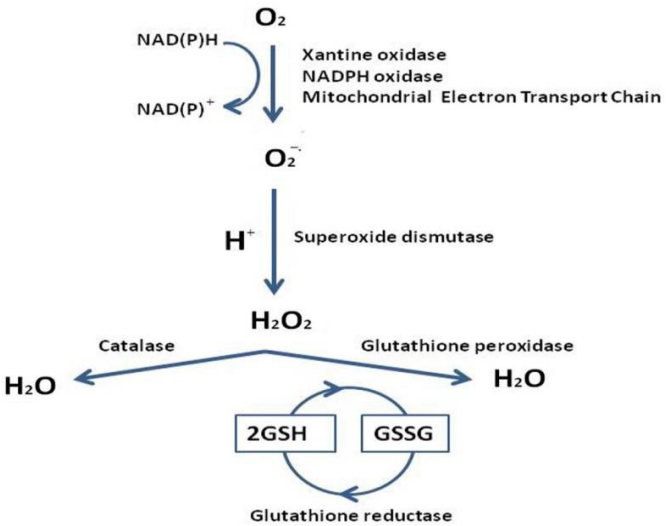

Fig. 6. Scavenging of ROS by catalase

It was concluded from one of study that there was decrease in CAT activity in pomegranate, in case of high salinity and decline in CAT activity indicated that CAT provided defense against salt stress with limited protection. The trend, about SOD activity in leaves of pomegranate, was as firstly increased then decreased [61]. One of study concluded that CAT activity increased under water stress in different species of pomegranate, except in one of them [62].

B. SOD

A metalloenzyme existing in all aerobic organisms, and subcellular components that are prone to oxidative stress, also contain it [63]-[65]. Numerous mechanisms can trigger O_2. When electronic configuration of O_2 is altered, then highly reactive $^1O_2$ (singlet oxygen) are created [28]. In all aerobic organisms, SOD mainly function in providing defense against oxidative stress [66]. SOD catalyzes dismutation $O_2^{\cdot -}$ to H_2O and O_2 as refer (2) as follows:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow 2H_2O + O_2 \quad (2)$$

As a result of classification of SOD on basis of metal cofactor, there are three types: (1) Fe-SOD that is contained in chloroplast, (2) Mn-SOD being located in mitochondria, (3) Third type is Cu-SOD contained in chloroplast, peroxisome and cytosol [67], [68]. Its structure differs from others two on basis of structure. It means that SOD has three isozymes, each having its own sensitivity level to H_2O. Nucleus encodes all forms of SOD and then through an amino terminal targeting sequence, these isoforms target specific subcellular compartments [212]. Cu/Zn-SOD exists as a dimer and is sensitive to cyanide in eukaryotes, but other two isoforms (Mn-SOD and Fe-SOD) are not sensitive to cyanide and existing as dimer or tetramer [66], [69]. Mechanism by which ROS are generated and prevented by SOS is shown in figure 7 as follows:

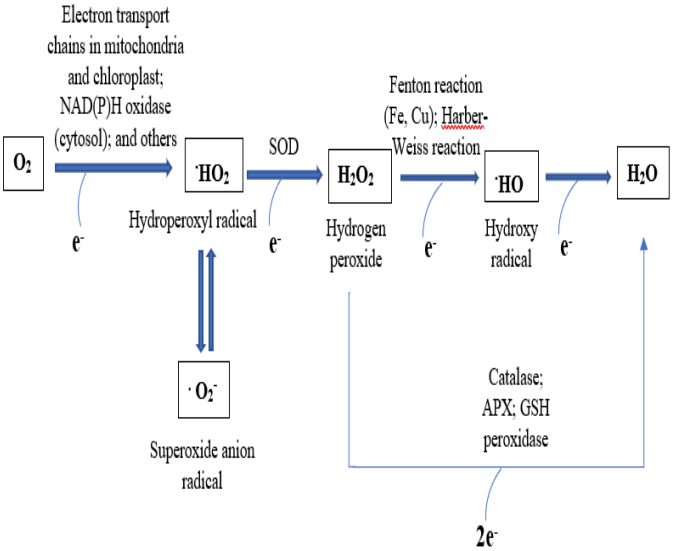

Fig. 7. Prevention of ROS by SOD

It has been reported that when plants are exposed to various environmental stresses including drought and metal toxicity, activity of SOD increases [70], [71]. It has been reported that SOD overproduction enhances tolerance of plants against oxidative stress [72]. When activity of SOD is increased, then plants more tolerate environmental stresses

[73]. It was concluded from one of study that there was decrease in SOD activity in pomegranate, in case of high salinity and decline in SOD activity indicated that SOD provided defense against salt stress with limited protection. The trend, about SOD activity in leaves of pomegranate, was as firstly increased then decreased [74]. One of study concluded that SOD activity increased significantly under water stress in different species of pomegranate, except in one of them [62].

C. Guaiacol peroxidase (GPX)

GPX is considered as one of the important enzymes. GPX is a protein having heme-group. When excess peroxides are produced during normal metabolism and also in stress situation, then GPX prevent cells from this excess of peroxide by oxidizing substrates, it usually oxidizes ascorbate. At the expenditure of H_2O_2, oxidation of substrate happens. Guaiacol is used as electron donor by GPX. It scavenges H_2O_2 generated in cytosol, vacuole and cell wall as well in extracellular space [28]. Equation (3) shows the Functioning of GPX as follows:

$$H_2O_2 + DHA \longrightarrow 2H_2O_2 + GSSG \quad (3)$$

D. Enzymes of Ascorbate-Glutathione Cycle

a. Ascorbate peroxidase

In AsA-GSH cycle, APX is a central component. To control levels of intracellular ROS, APX performs an important function. H_2O_2 is reduced by APX with usage of two molecules of AsA, along with two molecules of MDHA are generated. Class I superfamily of heme peroxidases has APX as its family member [75]. H_2O_2 and redox signal regulate APX [76]. APX has five isoenzymes, basing on amino acid sequence; these are distinct chemically and enzymatically. Higher plants contain these isoenzymes of APX at different subcellular localization. These isoforms of APX are present in cytosol, stroma, thylakoids, mitochondria and peroxisome [77]-[78]. It's one another form is bounded to membrane with role in scavenging of H_2O_2 and in controlling transport of electron in ascorbate-glutathione cycle. Equation (4) shows the function of APX as follows:

$$H_2O_2 + AA \longrightarrow 2H_2O_2 + DHA \quad (4)$$

APX is present in soluble form and is bound to thalakoids. H_2O_2 produced in organelles is scavenged by APX in these organelles, H_2O_2 that is produced in cytosol, apoplast or that diffused from organelles are eliminated by cytosolic APX [79]. APX isoforms that are produced in cytoplasm and cytosol act as electron donor [80]. Under stressful conditions, APXs are efficient scavengers of H_2O_2 and APX isoforms having greater affinity for H_2O_2 than CAT. APX is an antioxidant enzyme having wide distribution in plant cells [81]. It means that APX breakdown H_2O_2 into H_2O and MDHA (monodehydroascorbate), when uses ascorbate as H-donor and 2 cytosolic forms of APX perform function in defensive mechanism. At surface of membrane, $O_2^{\cdot -}$ is produced and trapped for conversion into H_2O_2. Then membrane-bound APX scavenge H_2O_2 [28].

b. Monodehydroascorbate reductase (MDHAR)

In reaction catalyzed by APX, MDHA radical is generated having decreased lifespan. AsA and DHA are generated on disproportionation of MDHA radical, this occurs when MDHA radical is not reduced rapidly [82]. AsA is regenerated from MDHA radical and this AsA regeneration is catalyzed by MDHAR, along with the usage of NAD(P)H as an electron donor. With H_2O_2, Horseredish peroxidase generates phenoxyl radicals, MDHAR has ability to reduce these radicals; and MDHAR uses MDA that is organic radical, as a substrate [83].

$$2MDHA + NADH \longrightarrow 2AA + NAD \quad (5)$$

Equation (5) shows the function of MDHAR as shown above. Several cellular compartments contain isozymes of MDHAR such as chloroplast, cytosol, mitochondria and peroxisomes all containing them [84]. Two physiological functions are performed by MDHAR in chloroplast: firstly, it regenerate AsA from MDHA, and secondly, when MDHA substrate is not present, then photo reduction of dioxygen to $O_2^{\cdot -}$ is mediated by MDHAR [85].

c. Dehydroascorbate reductase (DHAR)

DHAR is a thiol with monomeric form and dry seeds, roots and etiolated as well as green shoots contain abundance of this enzyme. DHAR reduces DHA to AsA, and GSH is used as reducing substrate [86]. So, maintaining reduced form of AsA is important function of DHAR. When AsA oxidation occurs in leaves and other tissues, some DHA is generated. DHA is a chemical having short lifetime, it can be hydrolyzed to 2, 3-diketogulonic acid in irreversible way or can be recycled by DHAR to AsA. It has been reported that DHAR has important role in determining AsA pool size [87]. Equation (6) shows functioning of DHAR as follows:

$$DHA + 2GSH \longrightarrow AA + GSSG \quad (6)$$

d. Glutathione reductase (GR)

Almost all cells contain GR as low molecular weight thiol compound and it is a ubiquitous tripeptide GSH that reduces disulphide thus protecting the enzymatic thiol groups. GR present in chloroplast, cytosol, mitochondria and peroxisomes. In the photosynthetic tissues, isoforms in chloroplast accounts for 80 % of GR activity [88]. H_2O_2 produced by Mehler reaction is detoxified by GSH and GR in chloroplast. GR reacts with $^1O_2$ and H_2O_2, also causes the regeneration of ascorbate. In GSSG, disulphide bond is formed and this depends upon NADPH with enzyme catalyzing the reaction, is GR. In this way, reduced pool of GSH is maintained vitally by GR. GR and GSH have role in scavenging H_2O_2 [89]. Equation (7) shows the functioning of GR is as follows:

$$GSSG + NADPH \longrightarrow 2GSH + NADP^+ \quad (7)$$

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In plants, GSH gets accumulate with increased activity of GR and finally plants are conferred stress tolerance [28]. In enzymic as well as non-enzymic oxidation-reduction cycles, GSSG by oxidation of GSH is generated and in such cycles, GR participate being as an antioxidant. In AsA-GSH cycle, DHAR catalyzes the reaction in which oxidation of GSH happens. GSSG is reduced to GSH by GR that is NAD(P)H – dependent enzyme, thus ratio of GSH / GSSG is maintained at high levels in cell. An essential disulfide group is contained in GR, and GR belongs to a group of flavoenzymes [90]. The mechanism of catalysis involves two steps: firstly, NADPH reduces flavin moiety, then oxidation of flavin occurs, and then a thiolate anion and a cysteine are generated on reduction of a redox active disulfide. Through disulfide interchange reactions, GSSG is reduced in second step [90]. Reduced enzyme can be reversibly inactivated if GSSG reoxidation not happens.

IV. NON-ENZYMATIC COMPONENTS OF ANTIOXIDANT DEFENSE SYSTEM

Type of non-enzymatic antioxidants with their particular function and subcellular localization are given in concise form in Table-IV as follows:

Table-IV: Non-enzymatic antioxidants with their subcellular localization (Kaushik et al., 2014)

Non-enzymatic antioxidants	Function	Subcellular location
Ascorbic acid	Detoxifies H_2O_2 via APX	Cytosol, Chloroplast, Mitochondria, Peroxisomes, Vacuole and apoplast
Glutathione	Act as detoxifying co-substrate for enzyme like peroxidases and GR	Cytosol, Chloroplast, Peroxisome, Vacuole and Apoplast
Alpha tocopherols	Guards against and detoxifies the products of membrane LPO	Mostly in membrane
Flavonoids	Direct scavenge of H_2O_2, $^1O_2$ and $OH^{\cdot}$	Vacuole

A. Ascorbate (AsA)

AsA exist in plenty with having low molecular weight and playing important role in case of enhanced level of ROS that cause oxidative stress. Number of enzymatic and non-enzymatic reactions receives electrons by AsA due to which AsA is taken into consider as a potent antioxidant. In addition to many physiological processes, growth, differentiation and metabolism are also such processes in which AsA display key function. Smirnoff-Wheeler pathway proceeds through GDP-D-mannose, L-galactose, GDP-galactose and L-galactone-1,4-lactone. This pathway also called as D-mannose / L-galactose, which provide bulk of AsA in plant. D-galacturonic acid is one of the intermediate of uronic acid which also synthesizes AsA [91]. Galacturonic acid reductase reduces D-galacturonic acid into L-galacturonic acid. L-galacturonic acid futher converts into L-galctono-1,4-lactone [92]. By oxidation of L-galactono-1,4-lactone, AsA is formed, enzyme catalyzing this oxidation reaction is L-galactono-1,4-lactone dehydrogenase (GALDH). So, GALDH synthesize AsA with this synthesis taking place in mitochondria. After its synthesis, transportation of AsA happens by the proton electrochemical gradient or facilitated diffusion to other components of cell.

It has been detected that majority of cell types of plants; organelles and apoplast in plants [93] have AsA and also found that photosynthetic tissues are plenty in AsA. Cytoplasm has almost more than 90% of AsA. To the apoplast, significant portion of AsA is transported with millimolar concentration existing there. This is a discrete feature of AsA from other soluble antioxidants. AsA provides actual defense mechanism against external oxidants with damaging capability. Oxidative damage to critical macromolecules is prevented due to AsA [94]. When physiological conditions are normal, in chloroplast most of AsA exist in reduced form and violaxanthin de-epoxidase is an enzyme there, for which AsA function as a cofactor, therefore dissipation of extra excitation energy is maintained by AsA [95]. AsA directly reacts with $O_2^{\bullet-}$ and H_2O_2 due to which membranes are protected and also from tocopheroxyl radical, alpha tocopherol is regenerated and enzymes that have ions of transition metal ions as prosthetic groups their activities are continued [89]. H_2O_2 are removed through AsA-GSH cycle in which AsA play important function for its removal [96]. AsA oxidation is accomplished in two steps, firstly (MDHA) is produced, DHA is generated next to it. In AsA-GSH cycle, H_2O_2 is reduced to H_2O through utilization of two molecules of AsA by APX along with MDHA production. Life time of MDHA is brief, as it is a radical. So, DHA and AsA are resulted due to impulsive (or spontaneous) dismutation of MDHA or NADP(H) dependent enzyme MDHAR reduces MDHA into AsA [97]. To prevent cleavage of DHA into tartarate and oxalate at more than 6.0 pH values [89], enzyme DHAR reduces DHA into AsA. It has been reported that level of AsA modifies during several stress conditions [71], [80] and [98]-[100]. Enzyme that synthesizes AsA, when overexpressed confers tolerance in plants to abiotic stress conditions. When two members of GME gene family are overexpressed, then buildup of ascorbate increases causing improved tolerance to biotic stress conditions in tomato plants [101]. When D-galacturonic acid reductase, which reduces D-galacturonic acid to L-galactonic acid and taking part in biosynthetic pathway of AsA, is overexpressed then AsA buildup giving enhanced tolerance to abiotic stresses in potato plants [102]. In Arabidopsis, when AsA level is increased, then it provides tolerance to oxidative stress [103].

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B. Glutathione (GSH)

GSH is one of the vital non-enzymatic antioxidants having low molecular weight and is non-protein thiol. It performs key function in by providing intracellular defense mechanism against oxidative damage triggered by ROS. Compartments of nearly all cells like cytosol, chloroplast, mitochondria, endoplasmic reticulum, and vacuoles contain this antioxidant. Biosynthesis of GSH happens in cytosol and chloroplast of plant cells. Isoforms of gema-glutamyl-cysteinyl synthetase that is specific to compartment of cell in plants and glutathione synthetase (GS) are actually involved in biosynthesis of GSH. To maintain redox state cell, it is important that there should be balance between GSH and glutathione disulfide (GSSG). GSH has key function in biological processes because of having reducing potential and these biological processes include cell growth, or cell division, sulfate transport regulation, protein and nucleic acid synthesis, enzyme regulation, metabolites conjugation, signal transduction, phytochelatin synthesis for metal chelation, expression of genes that response to stress; and xenobiotics detoxification [104]. By various ways, GSH can function as an antioxidant. It can directly scavenge free radicals by chemically reacting radicals for instance $O_2^{\bullet -}$, $^{\bullet}OH$ and H_2O_2. In glutathiolation process GSH react with electrophile and directly form adducts or GSH yield GSSG by acting as proton donor for ROS or organic free radicals. Due to adduct formation and proton donor capabilities of GSH, macromolecules are protected [105]. Though AsA-GSH cycle, antioxidant AsA is regenerated and GSH is takes part in this regeneration. From oxidized form to reduced form AsA is recycled by GSH, and enzyme involved in is DHAR [106]. At pH more than 7, DHA is reduced non-enzymatically through GSH with more than 1Mm concentration of GSH. When light is present, in chloroplast this may be an important pathway in which there is high GSH concentration as 5 mM, with pH approximately 8. Enzyme glutathione synthetase synthesizes GSH and GSH level is not affected by overexpression of this enzyme and also was not enough for enhancement in ozone tolerance [105].

C. Tocopherols

TOCs are lipophilic antioxidants that are effective in trapping free radical as in lipid autoxidation there is a step-in which chain propagation happens, tocopherols prevent this step. All plants synthesize these antioxidants and also biological membranes contain these antioxidants as their vital constituents [120]. Oxidizing radicals are repaired directly by tocopherol, and prevent chain propagation steps, so are known as chain-breaking antioxidant. In their polar head structure, phenolic rings present to which methyl groups are attached, due to pattern of attached methyl groups. Only green parts of plants have tocopherols and also only photosynthetic organisms synthesize these antioxidants. Two compounds homogenetistic acid (HGA) and phytyl diphosphate (PDP) act as precursors in pathway synthesizing tocopherols. Five enzymes synthesizing tocopherols are as 4-hydroxyphenyl-pyruvate dioxygenase (HPPD), homogentisate phytyl transferase (VTE2), tocopherol cyclase (VTE1), gamma-tocopherol methyltransferase (VTE4), and 2-methyl-6-phytylbenzoquinol methyltransferase (VTE3). But bypass pathway that involve synthesis and utilization of phytyl-tail is not included in tocopherol synthesis [107]. Due to chemical reactions of tocopherols with O_2 in chloroplast, lipids and other membrane constituents are sheltered and also structure and function of PSII are protected [108]. AsA, GSH [109] or Coenzyme Q [110] is capable of regenerating oxidized form of tocopherol back to reduced form of tocopherol.

It has been shown that in different species of plants, tolerance to salinity, chilling and water deficit is induced; when alpha-tocopherol get accumulated [111],[112]. During the oxidation of PUFA; $RO^{\cdot}$, $ROO^{\cdot}$ and ROO* are generated, alpha-tocopherol reacts with these lipid radicals. At interface of membrane-water, alpha-tocopherols and lipid radical both react with each other and during their reaction hydrogen atom is donated to lipid radicals by alpha-tocopherol resulting the formation of $TOH^{\cdot}$. Recycling of $TOH^{\cdot}$ back to alpha-tocopherol occurs, when $TOH^{\cdot}$ reacts with AA or another antioxidant [113]. Reduced form of alpha-tocopherol is regenerated from $TOH^{\cdot}$ Through GSH and AA. Alpha-tocopherol scavenge ROS especially $^1O_2$ and also through change transfer mechanism alpha-tocopherol deactivate $^1O_2$ physically. There is finding that membrane fluidity is modified by alpha-tocopherol and for glucose and proton, the permeability of digalactosyldiacyl glycerol (DGDG) vesicles is changed. Membrane structures are prevented from damaging effects of TOC's as TOC form complexes with FFA and lysophopholopids. In higher plants, genes that are responsible for synthesis of alpha-TOC, their expression via oxidative stress is triggered [28]. Lipid peroxy radicals, oxygen free radicals, and $^1O_2$ are scavenged by tocopherols [114]. During redox interaction with $^1O_2$, benzoquinone ring of tocopherol which is fully substituted and phytyl chain of tocopherol that is fully reduced, both these acts as antioxidants [115], [116]. In vitro, up to 220 molecules of $^1O_2$ are neutralized by a single molecule of alpha-tocopherol, so tocopherol more capably quench $^1O_2$ oxygen molecule [87].

D. Phenolics

Are secondary metabolites that are abundant in tissues of plants with also having diversity and include flavonoids, tannins, lignin and hydroxycinnamate esters. All of these have properties of antioxidants [117]. Biological activity such as antioxidant activity of polyphenols is due to -OH or OCH_3 substituents that are present in aromatic rings of polyphenols. It has been shown in in vitro antioxidant assays that polyphenols are highly capable of donating electrons or hydrogen atoms, therefore are more effective antioxidants than AsA and alpha-tocopherol. Phenolics contain properties of antioxidants due to following factors such as phenolics are highly reactive as donating electrons. Unpaired electrons are stabilized and delocalized due to ability of polyphenol-derived radical; it means that they have role in chain-breaking. Also transition metal ions are chelated by polyphenols. For scavenging of free radicals, polyphenols have perfect chemistry of structure and active oxygen species directly scavenged by polyphenols; these trap alkoxy radical so inhibiting lipid peroxidation. Kinetics of

peroxidation is modified because packing order of lipids altered by polyphenols thus reduced membrane fluidity [118]. Due to these alterations, free radical diffusion is sterically hindered, thus peroxidation reactions are restricted. Flavonoids show this ability [208]. Peroxidase oxidizes flavonoids and phenylpropanoids, and function in scavenging of H_2O_2, phenolic/AsA/POD system. There is evidence that during multiple stresses, metabolism of phenolic is induced [119].

V. ABBREVIATIONS

ROS: Reactive oxygen specie
XOD: Xanthin oxidase
ETC: Electron transport chain
SOD: Superoxide dismutase
PSI: Photosystem I
PSII: Photosystem II
NADPH: Nicotine amide adenine dinucleotide phoshphate
POD: Peroxidase
CAT: Catalase
APX: Ascorbate peroxidase
MDHAR: Monodehydrascorbate reductase
DHAR: Dehydroascorbate reductase
GR: Glutathione reductase
GPX: Guaiacol peroxidase
MDHA: Monodehydroascorbate
MDHAR: Monodehydroascorbate reductase
GR : Glutathione reductase
AsA: Ascorbate
GSH: Glutathione
GS: Glutathione synthetase
HGA: Homogenetistic acid
PDP: Phytyl diphosphate
DGDG: Digalactosyldiacyl glycerol
HPPD: 4-hydroxyphenyl-pyruvate dioxygenase
VTE2: Homogentisate phytyl transferase
VTE1: Tocopherol cyclase
VTE4: Gamma-tocopherol methyltransferase
VTE3: 2-methyl-6-phytylbenzoquinol methyltransferase

VI. CONCLUSION

It was described in one of study that antioxidant activities of pomegranate peel are more than in other parts, therefore it might be beneficial for human health benefits in therapeutic applications (because of medicinal properties of its compounds) and commercial applications such as for food storage for longer period of time. Although antioxidants in different parts of same plant may responds differentially to various stresses. None of research carried out for evaluating the non-enzymatic antioxidant level under various stressful conditions. A few of studies cover the antioxidant's responses of pomegranate under few of the stressful conditions. Under these stresses, antioxidant level might increase or decrease. Scientific research should be conducted to see the level of antioxidants in each part especially in peel of pomegranate under different stress conditions.

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