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**ROLE OF REACTIVE OXYGEN SPECIES IN PLANT REPRODUCTION**

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**ABSTRACT**

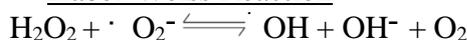
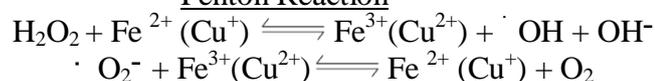
Reactive oxygen species (ROS) are generated by plants during photosynthesis and respiration. Initially ROS were considered to be by-products of metabolism and aerobic respiration. Studies in this direction, however, have now shown that ROS is an essential molecule and may have a role in growth and development of plant and may also act as signaling molecule. Role of ROS has been ascertained in cell proliferation, cell differentiation, programmed cell death, seed germination, gravitropism and growth of pollen tube. In reproductive biology, recent studies have indicated that ROS accumulate in the receptive stigma of plants. An accumulation of ROS in stigma signifies their role in recognition of pollen and further signalling in the pollen stigma interaction.

**Keywords:** Pollen, Peroxidase, Stigma, Superoxide dismutase, reactive oxygen species

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Higher plants require oxygen for the production of energy. During reduction of O<sub>2</sub> to H<sub>2</sub>O, different forms of reactive oxygen species (ROS) may be formed. ROS is a collective term for the activated forms of oxygen, such as superoxide anion (O<sub>2</sub><sup>•-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (•OH) and singlet oxygen (<sup>1</sup>O<sub>2</sub>). Under stress conditions, the balance between the production and removal of ROS is disturbed. ROS are capable of destroying cellular proteins, membrane lipids and DNA. They play important roles in regulating plant processes, such as growth and development, programmed cell death (PCD) and responses to biotic and abiotic stresses (Rogers, 2012). Superoxide is the first reduction product of ground state oxygen (O<sub>2</sub>), which is capable of oxidation and reduction. Superoxide is a byproduct of respiration and is produced by NADPH oxidases. Superoxide can also be produced from other forms of ROS and may undergo spontaneous or enzymatic dismutation to H<sub>2</sub>O<sub>2</sub>. Due to its negative charge, superoxides are unstable and are not able to diffuse through the membranes. Therefore, they are poor signalling molecules (Mittler et al., 2011).

Hydrogen peroxide is not a free radical but participates as an oxidant or reductant in many cellular reactions. It is a relatively stable molecule, selective in activity, and diffusible through membranes across aquaporins. H<sub>2</sub>O<sub>2</sub> has a role in various physiological activities in plants (Quan et al., 2008). It is synthesized during photosynthesis and respiration in plants. It is produced by NADPH oxidases, cell wall peroxidases, amine oxidases and other flavin-containing enzymes. H<sub>2</sub>O<sub>2</sub> mediates responses against biotic and abiotic stresses, such as pathogen attack (McInnis et al., 2005), wounding (Orozco-Cardenas et al., 2001), water deficit (Jubany-Mari et al., 2010), salinity (Miller et al., 2010), alteration in phytohormone levels (such as ABA) by systemic acquired resistance (SAR) (Zhang et al., 2001), hypersensitive resistance (HR) and programmed cell death (Gadjev et al., 2008). Recently, H<sub>2</sub>O<sub>2</sub> has also been reported to accumulate in the papillae of stigma where it is believed to play an active role in pollen-stigma interaction (McInnis et al., 2006b; Allen et al., 2011). H<sub>2</sub>O<sub>2</sub> can communicate with other signalling molecules, forming a part of complex signalling network. It can interact with Ca<sup>2+</sup> and calmodulin, which act as secondary messengers regulating different cellular processes in plants. H<sub>2</sub>O<sub>2</sub> also interacts with salicylic acid, nitric oxide, abscisic acid, ethylene and jasmonates (Quan et al., 2008). Superoxides and H<sub>2</sub>O<sub>2</sub> may either result in the slow formation of hydroxyl radical (•OH) by Haber-Weiss reaction or by Fenton reaction, which uses transition metals, especially iron (Fe) (Scandalios, 1993).

Haber-Weiss ReactionFenton Reaction

Hydroxyl radical ( $\cdot\text{OH}$ ) is the most reactive of all the ROS forms. It has the ability to damage cell structure and initiate radical chain reactions responsible for the irreversible modification of cellular macromolecules and damage to cell organelles. Plant cells are unable to scavenge this form of ROS and its production leads to programmed cell death (Karuppanapandian et al., 2011).

### SITES OF ROS GENERATION AND THEIR ROLES IN PLANTS

In green plants, chloroplasts and peroxisomes are the main ROS producers, whereas in non-green plants, mitochondria appear to be the main ROS producer (Moller, 2001; Karuppanapandian et al., 2011). In chloroplasts, photoactivated chlorophyll molecules transfer their excitation energy to photosynthetic reaction centres. Under conditions that prevent light energy from being utilized in the electron transport system, energy can excite oxygen from singlet to triplet form. PSI can reduce oxygen by Mehler's reaction and cause the formation of superoxides. In PSII, the oxidizing side facilitates the production of superoxide from  $\text{H}_2\text{O}$ , releasing triplet or ground state oxygen. During the electron transport chain in mitochondria, cytochrome oxidase consumes oxygen molecules. In situations when the chain is blocked, electrons are used for the reduction of oxygen, leading to the formation of superoxide and  $\text{H}_2\text{O}_2$ . Production of superoxide radical is linked with the rate of mitochondrial respiration and increase in oxygen, and respiratory activity further increases the amount of superoxide radicals (Zhu and Scandalios, 1993). Peroxisomes are involved in  $\text{C}_2$  photorespiratory cycle where they act in association with chloroplasts, resulting in the formation of ROS. Glycolate is translocated from chloroplasts to peroxisomes where its oxidation by glycolate oxidase causes the formation of  $\text{H}_2\text{O}_2$  (Apel and Hirt, 2004). NADPH-oxidase located on the plasma membrane, is also an important source of ROS. It causes the formation of  $\text{O}_2^{\cdot-}$ , which gets converted into  $\text{H}_2\text{O}_2$  and then enters the cytoplasm or exits to the outer environment. Peroxidases (including oxalate oxidases and amine oxidases) associated with the cell wall also participate in the production of  $\text{H}_2\text{O}_2$  (Smirnova et al., 2009).

In the presence of metal ions,  $\text{H}_2\text{O}_2$  may be reduced to much deleterious  $\cdot\text{OH}$  by superoxides. As there are no scavengers for  $\cdot\text{OH}$ , organisms have evolved enzymatic and non-enzymatic methods to maintain the concentrations of superoxides, hydrogen peroxide and transition metals (Cu and Fe) under control (Apel and Hirt, 2004). Non-enzymatic antioxidants include ascorbate, tocopherols, glutathione, flavanoids, alkaloids and carotenoids. Enzymatic ROS scavenging enzymes include superoxide dismutase, glutathione peroxidase, ascorbate peroxidase and catalase. (Apel and Hirt, 2004) Ascorbic acid and  $\alpha$ -tocopherol are effective antioxidants as they are poor electron donors under physiological conditions and act primarily by transferring single hydrogen atom. (Njus and Kelley, 1991) Ascorbic acid is a powerful antioxidant in plant cells and it can scavenge hydroxyl radicals, singlet oxygen, superoxide radical and hydrogen peroxide in all cell compartments, including the apoplast (Barceló, 1998; Smirnova et al., 2009).

Localized and temporal production of ROS is critical for the transduction of ROS signals. ROS-mediated signalling is controlled by a balance between the production and scavenging of ROS. However, much is yet to be known about ROS signalling, sensing and response mechanisms, and how a balance between their production and scavenging is controlled. ROS are important components of plant defence during stress conditions and affect the plant system in two ways:

1. They may react with biomolecules causing an irreversible damage leading to cell death
2. They may be involved in the expression of genes related to metabolic and signalling pathways. ROS may interact with certain target molecules that direct the plant responses to stress (Karuppanapandian et al., 2011). They can also play role/s in the regulation of cell wall stiffness by participating in the modification of polymer matrix of the cell wall (Smirnova et al., 2009).

### ROS ACCUMULATION

ROS are known to play signalling roles during pollen-stigma interaction. Recent reports have suggested that during stigma receptivity, angiosperms exhibit high levels of ROS, principally  $\text{H}_2\text{O}_2$  (McInnis et al., 2006a,b; Allen et al., 2011; Losada and Herrero, 2012). In *Seneciosqualidus* and *Arabidopsis thaliana*, ROS have been detected in the papillae, which play a crucial role in pollen-stigma recognition and subsequent events leading to the development of pollen tubes (McInnis et al., 2006a). McInnis et al. (2006a) proposed that the presence of high ROS/ $\text{H}_2\text{O}_2$  and peroxidase activity in stigma tissue leads to cross-linking of

pectins and extensins in the cell wall, thereby resulting in pollen adhesion and pollen tube growth in the stigma. Changes in the cell wall components are achieved by peroxidative or hydroxylic cycles of peroxidase. Elongation of cells results from a balance between cell wall loosening and stiffening. Cell wall rigidity is due to peroxidase-mediated cross-linking of several compounds, including polysaccharides, extensins and lignin monomers (Peroxidative cycle). Production of  $\cdot\text{OH}$  in the cell wall and apoplastic regions (hydroxylic cycle) leads to non-enzymatic loosening of the cell wall (Passardi et al., 2004).

ROS/H<sub>2</sub>O<sub>2</sub> play role/s in defence against pathogens. ROS may directly be toxic against the pathogenic microorganisms or they may trigger hypersensitive reactions and PCD at the site of pathogen attack (De Rafael et al., 2001). High levels of ROS have been detected in the floral nectar, which never experience microbial attack (Carter and Thornburg, 2004). Stigmas are also a source of nutrients for pollen grains and are prone to microbial attack. High amounts of ROS/H<sub>2</sub>O<sub>2</sub> in the stigma tissue may also act in the defense of stigma against microbial attack. Pollen has to, however, respond differently as compared to pathogen. In this context, a crosstalk between NO in pollen and ROS in stigma has been proposed (McInnis et al., 2006a). ROS/H<sub>2</sub>O<sub>2</sub> may further be involved in the signalling network, promoting pollen germination. Pollen grains attached to the stigma surface show ROS accumulation prior to germination (Speranza et al., 2012). The level of ROS detected, however, is lesser than that in the stigmatic papillae. ROS, thus, seems to have some function in the early development of pollen. ROS is known to be involved in the tip growth of pollen tubes and root hair. Localization of ROS in the tips of pollen tubes has been correlated with the activation of Ca<sup>2+</sup> permeable channels (Speranza et al., 2012). In olive, investigations on the localization of ROS have revealed maintenance of high levels of H<sub>2</sub>O<sub>2</sub> /ROS during early stages (unexposed) of stigma development. This is believed to be due to high metabolic activity of stigmatic papillae, which accumulate carbohydrates, lipids and pectins for the stigmatic exudates. A decrease in ROS accumulation has been observed on stigma receptivity coinciding with the adherence of pollen grains (Zafra et al., 2010). ROS has been localized at the germination apertures in kiwi fruits, suggesting a close association of ROS with pollen tube emergence (Speranza et al., 2012). Pollen tubes grow at a high rate and accumulate ROS at the tips. ROS accumulation is correlated with the activation of Ca<sup>2+</sup> permeable channels. It has been reported that ROS and generators of superoxide radicals cause pollen germination in *Hippeastrum hybridum* and spore germination in *Equisetum* sp. (Speranza et al., 2012).

### CHANGES IN SUPEROXIDE DISMUTASE ACTIVITY

Superoxide dismutase (SOD; EC 1.15.1.1) is a metalloenzyme that converts superoxides to H<sub>2</sub>O<sub>2</sub> and dioxygen. SOD was first isolated from bovine blood as a green copper protein (Scandalios, 1993). Based on the metal on their active site, three distinct types of SODs have been reported. These are copper and zinc (Cu/Zn SOD), manganese (MnSOD) or iron (FeSOD) containing SODs. Among higher plants, Cu/Zn SODs are commonly located in the cytoplasm and chloroplasts. MnSODs are present in the mitochondria and peroxisomes, whereas FeSODs are found in chloroplasts (Alscher et al., 2002; Wang et al., 2009). SODs form the first line of defence against ROS in the vegetative and reproductive plant tissues and cause the breakdown of superoxide anion (O<sub>2</sub><sup>-</sup>).

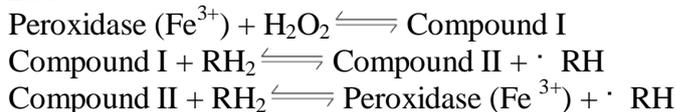


SODs have been linked with drought stress tolerance in maize, cotton and *Hordeum vulgare* (Acar et al., 2001). They are also involved in other stress conditions, such as salt stress, light stress, stress due to herbicide treatment and exposure to heavy metals (Alscher et al., 2002). SODs generate H<sub>2</sub>O<sub>2</sub> which contributes to the antimicrobial activity in the nectar (Carter and Thornburg, 2004). Nectar is a metabolically rich biological fluid containing sugars and metal ions, and it functions as a reward for the pollinator. Due to the presence of carbohydrates, it is also vulnerable to microbial attack. In *Nicotianatabacum*, five proteins accumulate in the nectar, which function in the nectar redox cycle, leading to the production of high levels of H<sub>2</sub>O<sub>2</sub>. Expression of NEC I for Nectarin I is associated with Mn(SODs) (Carter and Thornburg, 2004; Carter et al., 2007). In *Petunia*, SOD activity has been reported on the extracellular surface of stigma. This activity is dependent on the developmental stage of flower. The young bud stage exhibits the minimal activity whereas highest activity has been reported in the dehiscent flowers. SODs may have a role in plant defence mechanisms and other physiological aspects, as the stigma matures (Wang, 2006).

Distribution of superoxide anion has also been reported in the developmental stages of stigma of olive. Superoxide anion is the only detected ROS which increases as stigma matures. The increase has been proposed to be due to the presence of pollen grains and pollen tube on the surface of stigma having a high NADPH oxidase activity (Zafra et al., 2010). During self-incompatible reactions in *Lilium*, an induction of stress reaction has been reported to coincide with the accumulation of free radicals and an increase in the activity of SODs and PODs. The stress response could be induced after the recognition of incompatible pollen, and rejection occurs by inhibiting the pollen tube (Tezuka et al., 1997). It is known that SODs are present in the pollen grains of olive, *Camellia* sp. and *Nicotianatabacum*. Multiple forms SODs in pollen suggest their important roles in pollen tube growth and fertilization (Wang et al., 2009).

### MODULATION OF PEROXIDASE ACTIVITY

Peroxidases (EC 1.11.7) are heme-containing glycoproteins and can be divided into three different classes, namely Class I, II and III. Class I peroxidases include ascorbate peroxidase, cytochrome C peroxidase and catalase peroxidase. Class II peroxidases are encoded by fungi. Class III peroxidases are present in all land plants and are secreted into the cell wall or the surrounding medium and vacuole (Cosio and Dunand, 2009). Class III peroxidases can be further divided into acidic, basic or neutral, according to their isoelectric properties. These peroxidases can act in two different ways and have the ability to either produce or scavenge  $H_2O_2$  (Passardi et al., 2004). In the first case, they cause the reduction of  $H_2O_2$  by transferring electrons to various donor molecules, such as phenolic compounds, lignin precursors, auxin or secondary metabolites.



In a separate hydroxylic cycle, native peroxidases can be converted to oxyferro-peroxidases, leading to the formation of various forms of ROS. Peroxidases play a role in oxidative stress response, lignification, suberization, cross-linking of cell wall components, auxin metabolism and salt tolerance (McInnis et al., 2006a). In *Gossypiumhirsutum*, a class III peroxidase (GhPrx 37) has been reported to be specific for pollen. Three peroxidases are specific to stigma in *Arabidopsis* (AtPrx 28, AtPrx 39 and AtPrx 58). One peroxidase (SspPrx01) has been identified as stigma specific in *Seneciosqualidus*, and one putative stigma specific peroxidase (CavPrx) has been identified in hazelnut (Beltramo et al., 2012). Expression of stigma-specific peroxidases is developmentally regulated, their activity being maximally observed to accompany stigma receptivity (McInnis et al., 2005). Peroxidase activity has been reported in wet and dry types of stigma (McInnis et al., 2005). In *Arabidopsis thaliana*, *Petunia hybrida* and *Seneciosqualidus*, peroxidase activity increases as stigma matures and it is maximum at receptivity. This suggests their importance in the functioning of stigma (Dafni and Maués, 1998; McInnis et al., 2006b). Peroxidases are associated with a pellicle which overlays the cuticle in dry and semi-dry stigmas (McInnis et al., 2006b). They also play a role in defence against pathogens. Pistil expresses a number of pathogenesis-related proteins, some of which are peroxidases. A peroxidase (CaPrx02) in *Capsicum annuum* L. shows increased expression as a response to pathogen (Beltramo et al., 2012). Stigma-specific peroxidases are induced/upregulated in association with hypersensitive response or stress (McInnis et al., 2005).

Stigma-specific peroxidases point to their specific role/s during pollen-stigma interaction. Peroxidases form a component of signalling network, mediating species-specific pollen recognition (McInnis et al., 2005). In the stigmas of *Seneciosqualidus* (Asteraceae), five peroxidase isoforms have been localized, out which one is a stigma-specific (SSP). Peroxidases are present in the epidermal cells of the receptive surface of stigma, upon which pollen grains are captured. The papillae-specific peroxidase activity represents its importance for the proper functioning of stigma. Peroxidases cause a cross-linking of pectins and extensins in the cell wall. This could be important for pollen adhesion and growth of pollen tube (McInnis et al., 2006b). In olive, high levels of peroxides have been detected during early stages of stigma development which decrease at maturity, indicating the role of peroxidases (Zafra et al., 2010). High levels of peroxides may be dangerous for the growth of pollen tube and, therefore, peroxidases act in the destruction of excessive peroxides, so as to allow pollen germination and pollen tube growth (Beltramo et al., 2012). It has been reported that peroxidases have a role in self-incompatibility in *Petunia hybrida* (Carraro et al., 1986) and

*Nicotiana* sp.(Bredemeijer, 1984). Higher peroxidase activity has been observed after incompatible pollination than compatible pollination. The role of peroxidase in self-incompatibility is, however, debatable and requires further clarifications. Peroxidase activity may also increase during the development and senescence of plant tissue. In *Nicotianasp.*, an increased activity as well as induction of new isozymes has been observed upon ageing of the style (Bredemeijer, 1984).

Presence of Reactive oxygen species has been found in stigma of diverse species of angiosperms. The stigmas are rich in lipids, carbohydrates and protein, in comparison to other floral organ, where reactive oxygen species may function as a mechanism to provide defense to the plant stigmas. Scavenging of reactive oxygen species may be correlated with receptivity of stigma and the onset of pollen-stigma interaction (Zafra et al., 2016). Recent studies indicate that the level of H<sub>2</sub>O<sub>2</sub> decreases at the onset of pollen, triggering signaling events. These events are required for the navigation of the pollen tube, across the pistil tissue, towards the ovary. Additionally an increase of reactive oxygen species may also act as signaling mechanism for senescence at the later stages of stigma development.

In conclusion, it can be stated that ROS act as signaling molecule. The signaling capability of ROS depends on the fine balance in the production and scavenging of ROS through enzymes and antioxidants. The major ROS scavenging enzymes present in stigma and pollen include superoxide dismutase and peroxidases. The site of production of ROS, developmental stage of the plant, interaction of the plant with environment, interaction with other signaling molecules-lipid derivatives, hormones and Nitric oxide determine the chemical consequences and response of ROS in plants.

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