DOCTORAL THESIS

INVOLVEMENT OF REACTIVE OXYGEN SPECIES IN BIOPHOTON EMISSION FROM LIVING SYSTEM

BY

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OLOMOUC 2012

I would like to dedicate this thesis to my grandparents whose love will always remain in my heart

CONTENTS

IV

Acknowledgements

First of all, I would like to thank my supervisor, Doc. RNDr. Pavel Pospíšil, Ph.D. for supervising this research work and for warm and intensive discussions during my research activity.

I am also grateful to RNDr. Pavel Krchňák, Ph.D., who is unfortunately not among us anymore but even when the language barrier exist between us, he was always there to help me whenever I needed him during the measurement.

Next I would like to thank Dr. Arjun Tiwari, for his encouragement and help during my first years of the PhD study.

I am greatful to Dr. Jan Hrbáč, from the Department of Physical chemistry for the possibility to use the EPR spectrometer.

I am also thankful to Prof. RNDr. Petr Ilík, Ph.D., Prof. RNDr. Jan Nauš, Mgr. Iva Ilíková, Ph.D., and other faculty member of the Department of Biophysics, who was always there to help me during my stay in the laboratory.

I am also grateful to Prof. Claudio Rossi, Dr. Stefania Lamponi and Dr. Agnese Magnani with whom I worked at University of Siena during three months research stay. Apart of scientific discussion, their friendship and kindness made the time very pleasant in the warm weather of Italy. I thank Silvia, Alessandro, Claudia, Maso, Gemma, and Steven for the friendship and love during my stay in Siena.

I thank my friends Ankush for helping me during my experiments. Deepak, Zhara, Aditi, Manjul for helping me with my English. Shashikant and Sumit for arranging me the research articles. Without their help it might be much hard for me to reach this point.

I also thank all my colleagues in Department of Biophysics, namely Rakesh, Marika, Jan, Helena, Marek, and Mirek who made the environment much calm during the intensive research activity in the laboratory.

I thank my Friends, namely Vica, Jan and Ola who was always there to help me outside the laboratory and are filling my life with happiness. I would also thank Jan's family as they never let me feel I am abroad.

I thank my family, my parent for all their love and support which they provided me during the whole study period. Without the lessons from them, my study would never be

completed. I also thank my brother Pawan who bought me my first laptop and provided me his support which made it possible for me to come for PhD here in this university. I will also thank my sister, jiju and my nephew for their love and support from the distance. Last but not the least I thank Ismena for her love, interest and support during all this period.

Declaration

Hereby I declare that the Ph.D. thesis is my original work and that I have written it by myself using the literature listed in the section "References".

In Olomouc, ………   -----------------------

Anshu Rastogi

List of papers

The thesis is based on the following papers. These papers are referred in the text by the corresponding roman numerals and are enclosed at the end of the thesis.

I. **A. Rastogi** and P. Pospíšil (2010): Effect of exogenous hydrogen peroxide on biophoton emission from radish root cells. Plant Physiology and Biochemistry. 48, 117-123.

II. **A. Rastogi** and P. Pospíšil (2010): Ultra-weak photon emission as a non-invasive tool for monitoring of oxidative processes in the epidermal cells of human skin: comparative study on the dorsal and the palm side of the hand. Skin Research and Technology.16 (3), 365-370.

III. **A. Rastogi** and P. Pospíšil (2011): Spontaneous ultraweak photon emission imaging of oxidative metabolic processes in human skin - effect of molecular oxygen and antioxidant defense system. Journal of Biomedical Optics. 16: 096005.

IV. **A. Rastogi** and P. Pospíšil (Manuscript submitted): Production of hydrogen peroxide and hydroxyl radical in potato tuber during the late phase of oomycete *Phytophthora infestans* infection.

V. **A. Rastogi** and P. Pospíšil (Manuscript submitted): Ultra-weak photon emission as the non-invasive tool for the measurement of oxidative stress induced by UV-radiation in *Arabidopsis thaliana*.

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Attended 9th International Frohlich's Symposium on electrodynamic activity of living cells, held in Prague, Czech Republic (1st-3rd July 2011).

List of Publications

A. Rastogi and P. Pospíšil (2010): Effect of exogenous hydrogen peroxide on biophoton emission from radish root cells. Plant physiology and biochemistry 48, 117-123.

A. Rastogi and P. Pospíšil (2010): Ultra-weak photon emission as a non-invasive tool for monitoring of oxidative processes in the epidermal cells of human skin: comparative study on the dorsal and the palm side of the hand. Skin Research and Technology.16 (3), 365-370.

A. Rastogi and P. Pospíšil (2011): Spontaneous ultraweak photon emission imaging of oxidative metabolic processes in human skin - effect of molecular oxygen and antioxidant defense system. Journal of biomedical optics. 16: 096005.

A. Rastogi and P. Pospíšil (Manuscript submitted): Production of hydrogen peroxide and hydroxyl radical in potato tuber during the late phase of oomycete *Phytophthora infestans* infection.

A. Rastogi and P. Pospíšil (Manuscript submitted): Ultra-weak photon emission as the non-invasive tool for the measurement of oxidative stress induced by UV-radiation in *Arabidopsis thaliana*.

P. Pospíšil , **A. Rastogi** (Manuscript under preparation) Involvement of reactive oxygen species in ultra-weak photon emission from biological systems

Conference presentations

A. Rastogi and P. Pospíšil 2009 (Oral) Biophoton emission from radish root suspension, 7th International Conference of Ph.D. Students on Experimental Plant Biology, Brno, Czech Republic.

A. Rastogi and P. Pospíšil 2010 (Oral) Involvement of reactive oxygen species in biophoton emission, Young Biophoton Scientist Seminary (YBSS), Prague, Czech Republic.

A. Rastogi and P. Pospíšil 2010 (Oral) Ultra-weak photon emission as a non-invasive tool for monitoring of oxidative processes in the epidermal cells of human skin: comparative study on the dorsal and the palm side of the hand, 35th Meeting of the American Society for Photobiology, Providence, United States of America.

A. Rastogi and P. Pospíšil 2010 (Poster) Singlet Oxygen Imaging in Green Algae Chlamydomonas reinhardtii, 35th Meeting of the American Society for Photobiology, Providence, United States of America.

A. Rastogi and P. Pospíšil 2011 (Poster) Hydroxy radical production in potato tuber during the hypersensative response to fungus Phytophthora infestans. First International symposium on "Electromagnetic fields and Quantum phenomena in the Biological Systems" Poznań, Poland.

A. Rastogi and P. Pospíšil 2011 (Oral) Involvement of reactive oxygen species in biophoton emission from living system, 5th Young Biophoton Scientist Seminary (YBSS), Olomouc, Czech Republic.

Abbreviation

ROS	Reactive oxygen species
PMT	Photomultiplier tube
CCD	Charged coupled device
EPR	Electron paramagnetic resonance
SOSG	Singlet oxygen green sensor
NBT	Nitroblue tetrazolium
DAB	Diaminobenzidine
UVA	Ultra-violet A
H_2O_2	Hydrogen peroxide
$^1O_2$	Singlet oxygen species
$HO^\bullet$	Hydroxyl radical
OCl^-	Hypochlorite ion
$O_2^{\bullet-}$	Superoxide anion radical
$ROO^\bullet$	Peroxyl radical
$RO^\bullet$	Alkoxyl radical
ROOR	Dioxetane
ROOOOR	Tetroxide
$^3(C=O)^*$	Triplet excited carbonyls
$^3O_2$	Molecular oxygen
$HO_2^\bullet$	Perhydroxyl radical
EEG	Electroencephalography
Phe	Phenylalanine
Trp	Tryptophan
His	Hstidine
Cys	Cysteine
Fe^{3+}	Ferric iron
MDA	Malondialdehyde
HPLC	High performance liquid chromatography
MGDG	Monogalactosyldiacylglycerol

BSA	Bovine serum albumin
TEMP	2,2,6,6-tetramethylpiperidine
4-POBN	4-pyridyl 1-oxide N-tert-butylnitrone
OH$^-$	Hydroxy ion

ABSTRACT

All living organisms emit ultra-weak photon emission as the result of normal metabolic processes to its surrounding. This type of photon emission was termed as biophoton (from the Greek βιο; meaning "life" and φωτο; meaning "light"). Until recently, reactive oxygen species have been considered as the by-product of normal biochemical processes. In the current scenario, the belief that oxygen radicals are the hazardous by-product of normal metabolism is in progress to change. Plenty of evidence has been accumulated which indicates its important role in all aspects of regulation of biological processes. However, the role of reactive oxygen species in the regulation of biophoton emission is not obvious. The present research work shows the experimental evidences in favor of involvement of reactive oxygen species in biophoton emission from living system.

The spontaneous biophoton emission from radish root cells was observed to be 4 counts s^{-1}. Whereas when the different concentration of hydrogen peroxide was exogenously applied on the radish root cells the increase in biophoton was observed. The application of reactive oxygen species scavenger such as ascorbic acid and cysteine was found to prevent increase in biophoton emission from radish root cells. Spectral analysis indicates that the hydrogen peroxide-induced biophoton emission from radish root was mainly from red region of the spectrum. Our study of the human skin also shows the increase in biophoton emission with exogenous application of hydrogen peroxide, whereas the decrease in spontaneous biophoton emission was observed after the application of different scavengers of reactive oxygen species such as glutathione, ascorbic acid, coenzyme Q10 and α-tocopherol. The biotic stress such as the infection of *Phytopthora infestans* infecting the potato tuber was found to form hydrogen peroxide and hydroxyl radical with an increase in biophoton emission. The study with *Arabidopsis thaliana* shows an increase in biophoton emission due to oxidative stress caused by UV-radiation. Spectral analysis was performed to identify the molecular species responsible for UV-radiation induced biophoton emission and it has been found that the singlet excited chlorophylls and triplet excited carbonyls are responsible for biophoton emission.

Our study confirms the involvement of different reactive oxygen species in biophoton emission from different living system and introduces the technique as a non-invasive diagnostic tool for the measurement of oxidative stress in the living system.

Chapter 1

Overview

(Introduction, History, Hypothesis and Objective)

1.1 Introduction

Light is electromagnetic radiation which gives animals the sense of sight. Natural light sources such as the sun and stars have fascinated the human being and captured the attention of everyone irrespective of their background, such as: poets, writers, philosophers or scientists. The quest to understand and solve the questions regarding the generation, emission and characterization of light even now is the hot topic among the different branches of science. Apart of sun and starts, even some living system such as fireflies and jellyfishes have attracted humans because they emit light in the visible region. The phenomenon of light emission from such living systems is well characterized and is referred as bioluminescence [Harvey 1922]. These bioluminescences were recognized to have certain purposes, such as it works like mating signals or to attract the prey [Seliger 1975].

The human eye was found to be very much sensitive to the light and as minimum as 90 photons were required to elicit visual effect [Hecht et al. 1942]. Nevertheless, some light sources can emit even less than this value and so human eyes get insensitive to such radiation. With the recent development of technology, it got possible to design such instruments which have higher sensitivity than the human eyes. As a result it was discovered that every living system spontaneously emits light in the visible range but due to its low intensity the human eyes are not able to detect them. Such ultra-weak electromagnetic radiations are called by different names, such as biophotons, ultra-weak photon emission, autoluminescence, low-level luminescence, ultra-weak chemiluminescence etc [Popp et al. 1984, Campa and Cilento 1984, Yoda et al. 1985, Gumińska et al. 1997, Cohen and Popp 2003, Havaux et al. 2006, Cifra et al. 2007, Hossu et al. 2010]. Among them biophoton and ultra-weak photon emission are most common terms used for such type of photon emission. We have used biophoton emission and ultra-weak photon emission both interchangeably in our publications and as the topic of this thesis includes biophoton emission, the term biophoton emission is used in the rest of this thesis to explain such photon emission.

The spontaneous biophoton emission is very week which makes it hard to study the property of the biophoton emission. That's why the researchers often used an external stress factor to enhance the biophoton emission and study the property of such photon emission. The external stress factor is used in the form of physical (such as light and heat) [Jain et al. 2010, Hagens et al. 2008, Havaux 2003], chemical (such as hydrogen peroxide) [Cadenas et al. 1980, Cheun et al. 2007] or biological (fungi, bacteria) [Kobayashi et al. 2007, Floryszak-Wieczorek et al. 2011, Bennett 2005] stresses. The biophoton emission obtained after the application of external stress factors was termed as induced biophoton emission [Winkler et al. 2009].

1.2 History

The study of biophoton emission can be traced back to 1922, to a Russian scientist Alexander Gawrilowich Gurwitsch [Gurwitsch 1922]. He had performed his experiments on onion root. He relates that the cell-proliferation of an onion root (as a detector) was accelerated by directing the second onion root (as an inductor) towards it when separated by quartz, whereas when it was separated by glass no difference in cell-proliferation was observed [Beloussov and Voeikov 2006, Cifra 2006].
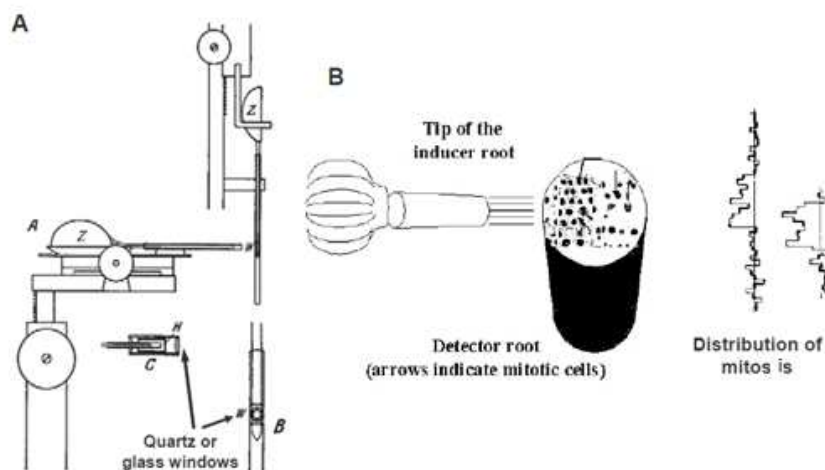
Fig. 1.1 Schematic presentation of "onion" experiment of Gurwitsch. **A.** Installation of an inducer root (horizontal) and a detector root (vertical) on moving tables of microscopes. Z-onion bulbs, C-tip of the inducer root fixed in an air-tight chamber, H- and W- quarts or glass windows. **B.** Sketch of experimental results evaluation. A detector root was sliced below and above the "irradiated" zone and an excess of mitotic cells on the left (irradiated) or right (non-irradiated) sides of the root on each slice was calculated. Two indented lines at the left illustrate the results of two representative experiments. Significant excess of mitotic cells on the left side over the average distribution was observed in the irradiated region. [Fig. adopted from Cifra 2006].

If the tip of the inducer was aimed at a metal mirror in such a way that its reflection fall onto the surface of the detector the cell-prolifrrating effect was again observed. According to observations, he proposed "Mitogenetic radiation theory" which state that the dividing cells emit ultra-weak far-ultraviolet light that is capable of inducing cell

division when incident on non-dividing cells [Gurwitsch 1988, Beloussov and Voeikov 2006, Cifra 2006 for original work in German refer Gurwitsch 1922, 1923,1924 and Gurwitsch and Gurwitsch 1924]. Due to the effect of radiation on cell proliferation, he termed it as mitogenetic radiation. But many organized experiments failed to prove mitogenetic radiation theory which made the theory controversial [Quickenden and Que Hee 1974]. As a result, the scientific activity has developed very slowly in this field. Regardless 0f this, in the 1970s with the development of sensitive devices such as photomultiplier tubes it became possible to detect such radiation and again the existence of biophoton came in light. Fritz-Albert Popp (Germany), Inaba (Japan), Boveris (USA), and Quickenden (Australia) started to work on biophoton approximately at the same time [Quickenden and Que Hee 1974,1976, Rattemeyer et al. 1981, Boveris et al. 1980, 1984, Inaba et al. 1982, Quickenden and Tilbury 1983, Popp et al. 1984,1988, Inaba 1988]. Even so, the puzzle around the biophoton remains in the form of questions such as: what are the sources and use of such photon emission in living system? Different scientific groups had proposed different hypothesis on the origin of biophoton emission, among them the coherence theory of biophoton emission and the biochemical theory of biophoton emission got much attention.

1.3 Hypothesis

1.3.1 Coherence theory of biophoton emission

The coherent theory of biophoton emission was proposed by Fritz-Albert Popp and was based on the understanding of biological systems as an open, non-linear and cooperative system [Gu 1998]. The coherent theory of biophoton emission is based on quantum physics formulas which are based on certain assumptions and is supported by a limited number of scientists. The researchers involved in the coherent theory have mainly focused the decaying property of the light-induced biophoton emission [Bajpai 2005, Bajpai and Drexel 2008, Popp and Yan 2002]. The coherence theory of biophoton emission proposes the DNA as the source of photon emission [Rattemeyer et al. 1981, Popp et al. 1984]. Coherence has been studied in many biological phenomenas and its existence cannot be ignored but there is a need to identify measurable holistic properties manifesting coherence at bio-molecular level [Saroval et al. 2010]. Lack of solid experimental evidence also made the coherence theory of biophoton emission less acceptable to the current research.

1.3.2 Biochemical theory of biophoton emission

The biochemical theory of biophoton emission relates biophoton's origin to the oxidative metabolic processes. It states that reactive oxygen species (ROS) produced inside the cells due to normal metabolic processes are responsible for the formation of excited molecules which finally results into biophoton emission while relaxing to its ground state [Boveris et al. 1980, Havaux et al. 2006, Hideg et al. 1990, Hideg and Inaba 1991]. Until recently, ROS have been considered as the by-product of normal biochemical processes. But recent study proved the involvement of ROS in signaling processes [Hoidal 2001, Thannickal and Fanburg 2000, Zhang and Gutterman 2007]. Many scientists proposed the involvement of ROS in biophoton emission, whereas countable experimental evidences are there which correlates biophoton emission to ROS. The lack of knowledge about the mechanism of the biophoton emission makes this theory also less acceptable to the modern research.

1.4 Objectives

The main goal of thesis is to fulfill the gap in the understanding of the biochemical theory of biophoton emission. To realize the goal following research subproject needed to be solved.

1. Perform the experiments on different living organisms to determine the involvement of ROS in biobhoton emission.

2. Correlation of the present study by other studies to identify the role of different ROS in biophoton emission such as superoxide anion radical, hydrogen peroxide and hydroxyl radical.

3. Discussion about the possible application of the detection of biophoton emission from living system.

Chapter 2

Concept

(Reactive oxygen species and photon emission)

2.1 Reactive oxygen species

Reactive oxygen species are the oxygen containing reactive compounds. It can be molecule such as hydrogen peroxide (H_2O_2), radicals such as hydroxyl radical ($HO^\bullet$), ion such as hypochlorite ion (OCl^-) or ion and radical both such as superoxide anion radical ($O_2^{\bullet-}$).
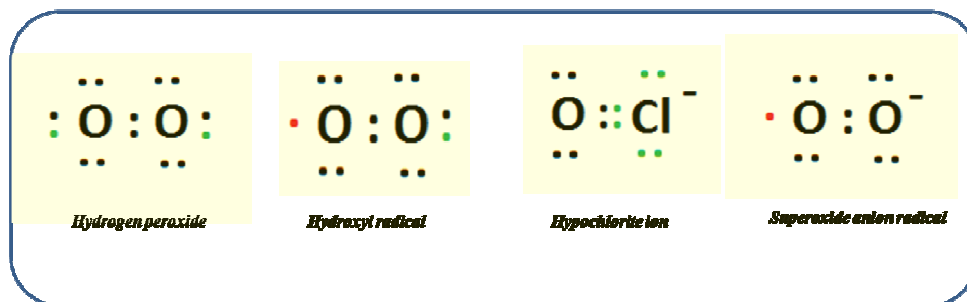
Fig. 2.1 Some of the ROS are shown with their electronic configuration. The unpaired electrons are shown in red dots whereas the green dots represent the electron from the atom other then the oxygen.

Reactive oxygen species are formed naturally in each living cell. Different metabolic activities within the cells are known to produce different ROS [Han et al. 2001, Devasagayam et al. 2004]. ROS are now proved to be crucial for normal physiology of the living cells, and are vital for the living systems. But when ROS are formed in excess, it is considered to be the source of many abnormalities in living systems which are responsible for diseases [Vincent and Crozatier 2010].

2.2 Formation of reactive oxygen species:

Reactive oxygen species can be formed inside the cell by following two mechanisms:

2.2.1 Energy transfer

Energy transfer from triplet state of photosensitizers such as chlorophylls and melanin to molecular oxygen can result into the formation of singlet oxygen species ($^1O_2$) [Pospíšil 2012].
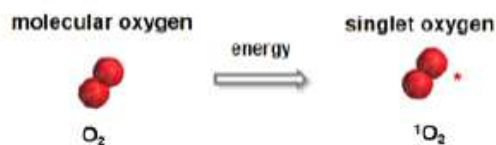
2.2.2 Electron transport

Electron transfer from one molecule to another, results into the formation of different ROS, such as $O_2^{\bullet-}$ is formed by one-electron reduction of molecular oxygen, H_2O_2 is formed either by one-electron reduction of $O_2^{\bullet-}$ or two electron reduction of molecular oxygen and $HO^{\bullet}$ is formed by one electron reduction of H_2O_2 [Nathan and Singer 1999, Turrens 2003].
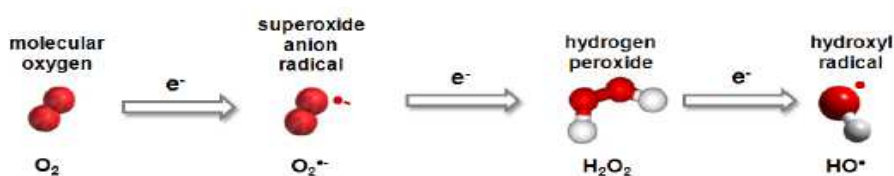
2.3 Organic radical and intermediates

Reactive oxygen species are known to react with organic molecule such as lipids, proteins and nucleic acids present in cells and form organic radicals [Liu et al. 2002].
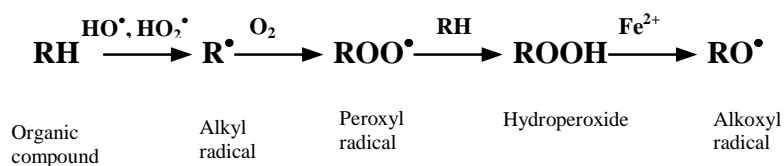
Organic radicals such as peroxyl radical ($ROO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$) can recombine or cyclize to form intermediates such as dioxetane (ROOR) and tetroxide (ROOOOR) [Cadenas 1989, Tímmins et al. 1997].

Recombination

$$ROO^\bullet + ROO^\bullet \longrightarrow ROOOOR$$

$$RO^\bullet + RO^\bullet \longrightarrow ROOR$$

Cyclization

$$ROO^\bullet \longrightarrow ROOR$$
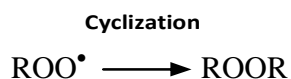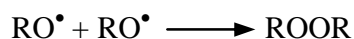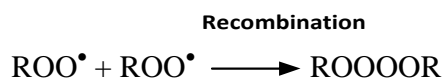
2.4 Electronically excited species

Dioxetane and tetroxide are very unstable molecules. Decomposition of tetroxide or dioxetane results in the formation of electronically excited species such as triplet excited carbonyls $^3(C=O)^*$ [Havaux et al. 2006, Velosa et al. 2007]. The $^3(C=O)^*$ can transfer its energy either to molecular oxygen to form $^1O_2$ or to different chromophores to form its excited state by triplet-singlet energy transfer method [Davies 2003]. The excitation energy transfer from $^3(C=O)^*$ to chlorophylls proceeds via the direct and induced reaction pathways [Marder et al. 1998]. In the direct reaction pathway, the excitation of chlorophylls directly results into the excited singlet state by the triplet-singlet energy transfer from $^3(C=O)^*$ to chlorophylls. On the other hand, in the induced reaction pathway, the chlorophylls are first excited to the triplet state by the triplet-triplet energy transfer by $^3(C=O)^*$ to chlorophyll and then overcome to the singlet excited state by the reverse inter-system crossing [Marder et al. 1998].

Formation of triplet excited carbonyls

$$ROOOOR \longrightarrow {}^3(R=O)^* + O_2 + ROH$$

$$ROOR \longrightarrow {}^3(R=O)^* + ROH$$

Triplet-singlet energy transfer

$$^3(R=O)^* + O_2 \longrightarrow {}^1O_2 + R=O$$

13
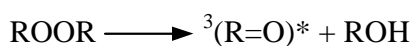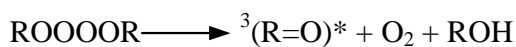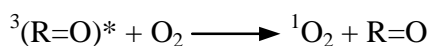
2.5 Photon emission

The molecules responsible for photon emissions from biological systems are

i) Excited triplet carbonyls

ii) Singlet oxygen

iii) Chromophores

2.5.1 Excited triplet carbonyls: Every excited molecules have tendency to go back to its ground state. Triplet excited carbonyls are known to emit light into the blue region of the spectra while overcoming from its excited state to the ground state. The spectral range of the photon emission was found to be in between 450-550 nm of the spectrum [Federova et al. 2007]

$$^3(R=O)^* \longrightarrow R=O + h\nu \text{ (450-550 nm)}$$
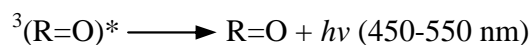
2.5.2. Singlet oxygen: Singlet oxygen is known to emit the visible light as the result of dimol reaction which results into the formation of one molecule of triplet oxygen. The spectral range of the photon emission due to dimol reaction of $^1O_2$ is in the range of 600 to 720 nm with the peak value at 634 and and 703 nm [Kazakov et al. 2007].

$$^1O_2 + {}^1O_2 \longrightarrow {}^3O_2 + h\nu \text{ (634 nm, 703 nm)}$$
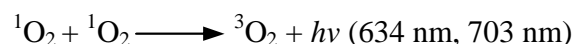
2.5.3. Chromophores: Chromophores such as chlorophylls when overcome from its excited state to ground state are known to emit light. The photon emission from chlorophylls was found in the spectral range from 660 nm to 740 nm [Rebeiz et al. 1973]. Whereas the photon emission of free chlorophylls is in the short wavelength range from 660 nm to 680 nm, the bound chlorophylls provide photon emission in the long wavelength range from 680 nm to 740 nm [Bassi and Simpson 1987].

Chapter 3

Literature review

(Involvement of Reactive oxygen species in biophoton emission)

3 Involvement of ROS in biophoton emission

As the concept of biophoton emission from living system came to light, its biochemical nature started to be discussed. But only in the 1970s some evidences were provided to correlate the biophoton emission to the molecular species such as ROS [Kakinuma et al, 1979]. Since than the direct or indirect experiments were performed to indicate the involvement of different ROS in biophoton emission [Cadenas et al. 1980, Mathew et al. 1992, Hagens et al. 2008, Van Wijk R et al. 2008, Prasad and Pospíšil 2011]. The experiments indicate the involvement of ROS such as $O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, and $^1O_2$ in biophoton emission.

3.1 Involvement of superoxide anion radical in biophoton emission

The chemical generation of $O_2^{\bullet-}$ by using xanthine/xanthine oxidase system made it possible to study the effect of $O_2^{\bullet-}$ on biophoton emission from different living system. One of the first reports on the involvement of ROS in biophoton emission came through the experiment on the human leukocytes. The exogenous application of xanthine/xanthine oxidase to human leukocytes brings a significant enhancement in biophoton emission, which indicates that $O_2^{\bullet-}$ is involeved in the biophoton emission [Rosen and Klebanoff 1976]. It has also been shown that the addition of myristic acid to polymorphonuclear leukocytes caused pronounced enhancement in biophoton emission [Kakinuma et al. 1979]. Based on the correlation between the enhancement in biophoton emission and oxygen uptakes, the authors proposed that $O_2^{\bullet-}$ is involved in biophoton emission. More recently, biophoton emission from rice cell culture has been demonstrated to rise after the addition of xanthine/xanthine oxidase system [Kageyama et al. 2006]. The biophoton emission from rice cells also get elicited by N-acetylchitooligosaccharide compared to control rice cells. N-acetylchitooligosaccharide is thought to enhance the production of $O_2^{\bullet-}$ in cells. On the basis of the results obtained, the author proposed the involvement of $O_2^{\bullet-}$ in the biophoton emission from the rice cells. The topical application of xanthine/xanthine oxidase on the human skin caused enhancement in biophoton emission upto three times, when compared with non-treated human skin [Prasad and Pospíšil 2011]. The authors proposed that $O_2^{\bullet-}$ is formed by one-electron reduction of molecular oxygen catalyzed by the complex I and complex III during the respiration in the mitochondria,

17

by NADPH oxidase during the respiratory burst in the phagocytic cells and xanthine oxidase in the cytoplasm of the cell.

Further evidences have been provided on the involvement of $O_2^{\bullet-}$ in biophoton emission by the application of non-enzymatic or enzymatic antioxidants. Kakinuma et al. (1979) showed that the myristic acid-induced biophoton emission was partially suppressed by the exogenous application of SOD. These observations indicate the involvement of $O_2^{\bullet-}$ in biophoton emission. The addition of exogenous SOD to spinach chloroplasts was found to suppress the biophoton emission significantly [Hideg and Inaba 1991]. On the basis of these observations, the authors proposed that $O_2^{\bullet-}$ is associated with biophoton emission. Mathew et al. (1992) demonstrated that the addition of exogenous SOD to the cotyledons of etiolated seedlings of *Cicer arietinum* L. previously subjected to the sudden freezing and thawing suppressed biophoton emission. The biophoton emission measured in SOD-deficient mutant of yeast *Saccharomyces cerevisiae* was found to be significantly high when compared to wild strain [Slawinski et al. 1992]. The two-dimensional imaging of biophoton emission from the tumor in nude mice transplanted with carcinoma cell lines AH109A, TE4 and TE9 demonstrated that the photon emission from the tumor is enhanced as compared to photon emission observed before transplantations [Takeda et al. 2004]. The authors proposed that decreased level of antioxidant defence system such as SOD is responsible for enhanced biophoton emission in tumor tissue.

It has been previously reported that $O_2^{\bullet-}$ is unable to oxidize polyunsaturated fatty acid or amino acid [Gebicki and Bielski, 1981, Aikens and Dix 1991]. Based on these observations, it is considered that the reactivity of $O_2^{\bullet-}$ toward the polyunsaturated fatty acids or amino acids are rather limited. Very few reports shows that the $O_2^{\bullet-}$ has a capability to oxidize iron-sulfur cluster or interact with radicals such as tyrosine phenoxyl radical formed by the hydrogen abstraction from the hydroxyl group of tyrosine [Ramsay et al. 1981, Hunter et al. 1989]. Although $O_2^{\bullet-}$ is relatively less reactive compared to another ROS, the potential deleterious effect of $O_2^{\bullet-}$ is due to ability to mediate reduction of H_2O_2 into highly oxidizing radicals such as $HO^{\bullet}$ or $RO^{\bullet}$. The protonated form of superoxide radical known as perhydroxyl radical ($HO_2^{\bullet}$) is considered to be more reactive than the anionic form of superoxide radical $O_2^{\bullet-}$. Many experimental evidences are

provided to indicate that $HO_2^\bullet$ is an oxidizing agent able to directly abstract hydrogen form polyunsaturated fatty acid or amino acid [Gebicki and Bielski, 1981, Aikens and Dix 1991]. The stronger oxidizing nature of $HO_2^\bullet$ is due to its higher oxidizing power (Em of $O_2^{\bullet-}/H_2O_2$ and $HO_2^\bullet/H_2O_2$ redox couple is 0.89 V and 1.06 V, respectively) and the lack of negative charge on the molecule. These considerations indicate that $O_2^{\bullet-}$ has a capability to induce biophoton emission.

3.2 Involvement of hydrogen peroxide in biophoton emission

Hydrogen peroxide is easily available and is the most stable among the ROS, it also lacks any net charge which makes the molecule easily diffusible across the biological membrane. There are numerous studies on the direct and indirect involvement of H_2O_2 in biophoton emission. The biophoton emission from rat perfused lung was found to increase by the addition of exogenous H_2O_2 and organic peroxide such t-butyl hydroperoxide [Cadenas et al. 1980]. Agatsuma et al. (1992) demonstrated that the addition of H_2O_2 to the healthy and hemodialysis plasma cells results in the biophoton emission predominantly in the red region of the spectrum with a peak at 680 nm. The biophoton emission from high-molecular-mass fraction obtained by HPLC-gel chromatography showed that the photon emission origins from proteins such as immunoglobin, albumin, cytochrome and microglobulin. The addition of H_2O_2 (several hundreds mM) in intact soybean seedlings caused an enhancement in the biophoton emission [Suziki et al. 2002]. When H_2O_2 was added in injured soybean seedlings, the biophoton emission was found to be three time higher as compared to the intact soybean seedlings. It has been observed that the biophoton emission after the exogenous application of H_2O_2 to the injured soybean seedling occurs in two phases i.e. fast and slow phase. The authors proposed that photon emission in the fast phase is due to the direct effect of H_2O_2, whereas in the slow phase molecular oxygen produced during the decomposition of H_2O_2 is involved in the photon emission. Kobayashi et al. (1999) demonstrated that spontaneous biophoton emission from a rat´s brain correlates with the metabolic activity of brain cells measured by electroencephalographic (EEG) activity. The photon emission from brain cells was enhanced by about 30 % in hyperoxia condition compared to normal conditions. The author proposed that enhancement of

biophoton emission is due to the higher generation of H_2O_2 in hyperoxia condition. It has been previously demonstrated that biophoton emission from rice cell culture increased after the addition of H_2O_2 (several hundreds mM) in a concentration-dependent manner [Kageyama et al. 2006]. Based on the correlation between H_2O_2-induced and elicitor-induced N-acetylchitooligosaccharide enhancement in biophoton emission the authors proposed that ROS formed during the phospholipid signaling pathway are involved in photon emission. The addition of H_2O_2 (several units mM) to Madin-Darby Canine Kidney (MDCK) cells results in the increase of biophoton emission [Cheun et al. 2007]. The oxidation of bovine serum albumin by H_2O_2 (several tens to several hundreds mM) was shown to enhance biophoton emission [Khabiri et al. 2008]. The enhancement in biophoton emission was shown to correlate with formation of protein carbonyls in a concentration dependence manner. The topical application of H_2O_2 and organic peroxide (benzoylperoxide) in the porcine ex vivo skin model caused pronounced increment in biophoton emission [Khabiri et al. 2008]. Spectral analysis shows that the photon emission is predominantly in the blue region of the spectra. The topical application of H_2O_2 (several tens mM) on the dorsal and the palmar side of the hand resulted in the increase of biophoton emission [Prasad and Pospíšil 2011]. The authors demonstrated that photon emission from the palmar side of the hand is significantly higher compared to biophoton emission from the dorsal side.

The addition of exogenous catalase to polymorphonuclear leukocytes treated with myristic acid partially suppressed biophoton emission [Kakinuma et al. 1979]. The simulatenous addition of exogenous SOD and catalase almost completely diminished the myristic acid-induced biophoton emission. These observations indicate the involvement of H_2O_2 in biophoton emission. Biophoton emission from the cotyledons of etiolated seedlings of *Cicer arietinum* L. subjected to the sudden freezing and thawing was found to be suppressed pronouncedly by the addition of exogenous catalase [Mathew et al. 1992].

It is well stabilized that H_2O_2 itself is a poorly reactive compound with almost no capability to oxidize the polyunsaturated fatty acid or having limited capability to oxidize amino acid even when used in high concentration [Mehdy 1994, McKersie 1996]. It has been reported that among all the amino acid, only few amino acids including cysteine,

20

tryptophan, tyrosine, histidine and methionine are oxidized by H_2O_2 [Subbaraman et al. 1971]. Even if H_2O_2 is poorly reactive compound when compared with another ROS, the potential deleterious effect of H_2O_2 in biological system is due to its ability to serve as a substrate for the formation of $HO^{\bullet}$ which is a strong oxidizing agent.

3.3 Involvement of hydroxyl radical in biophoton emission

Hydroxyl radical is most reactive among all the ROS and so is considered to be reacting with many biological molecules in its surrounding. The biophoton emission from hemodialysis plasma cells after the addition of H_2O_2 and iron sulfate was found to be predominantly in the blue region of the spectrum with a peak at 430 nm, attributed to the action of $HO^{\bullet}$ formed by the iron-catalyzed reduction of H_2O_2 [Agatsuma et al. 1992]. HPLC-gel chromatography of hemodialysis plasma cells was performed and the biophoton emission from low-molecular-mass fraction obtained showed that the photon emission originated from molecules such as vitamin B_{12} and uric acid. The addition of Fenton reagent (H_2O_2 and Fe^{2+}) in isolated amino acid such as Phe, Trp, His, and Cys or protein such as bovine serum albumin showed pronounced enhancement in biophoton emission [Khabiri et al. 2008]. The authors had linked the biophoton emission to the formation of $^3(C=O)^*$ and $^1O_2$ formed by recombination of peroxyl and alkoxyl radicals. Alternatively, biophoton emission is related to the decay of protein peroxides leading to the formation of excited amino acid. Interestingly, when Fenton reagent was added in combination of His to Trp more pronounced enhancement in photon emission was observed, when compared to pure amino acids. Spectral analysis of biophoton emission from pure amino acids showed that Trp and Cys exhibits comparable biophoton emission in the whole spectral ranges 420-550 nm, whereas His provide pronounced biophoton emission in the spectral range 420-455 nm. When biophoton emission was measured simultaneously from His to Trp, the photon emission in the spectral range 420-455 nm was found to be suppressed. Based on this observation the authors proposed that His transfers an excitation energy to Trp. The topical application of Fenton reagent (H_2O_2 and Fe^{2+}) on the dorsal side of the human skin results in an enhancement in biophoton emission [Prasad and Pospíšil 2011]. Based on the observation that the Fenton reagent provided the highest increase in biophoton emission compared to the topical application

of xanthine/xanthine oxidase and H_2O_2 the author confirmed that $HO^\bullet$ is the strongest oxidant in the human body.

The observation that the addition of mannitol and *t*-butyl alcohol suppressed significantly biophoton emission from the cotyledons of etiolated seedlings of *Cicer arietinum* L. subjected previously to the sudden freezing and thawing reveals that $HO^\bullet$ is involved in biophoton emission [Mathew et al. 1992]. The addition of iron chaletor such as desferrioxamine was found to supress biophoton emission, whereas the addition of exogenous ferric iron (Fe^{3+}) and iron-containing pigment (haematin) enhances biophoton emission [Mathew et al. 1992]. These observations indicates that $HO^\bullet$ involved in biophoton emission is formed by one-electron reduction of H_2O_2 by transition metals. The topical application of polyphenol antioxidant such as oligomeric proanthocyanidins on the human skin exposed to UVA radiation was found to suppress the biophoton emission [Van Wijk et al. 2010]. Considering that oligomeric proanthocyanidins has been shown to act as a scavenger of $HO^\bullet$ [Jordão et al. 2010], it is indicated here that $HO^\bullet$ formed in the human skin under UVA exposure was involved in the biophoton emission. Scavenging of $HO^\bullet$ by mannitol considerably suppressed biophoton emission measured after the addition of linoleic acid in the unicellular green alga *Chlamydomonas reinhardtii* [Prasad and Pospíšil 2011]. The authors proposed that the oxidation of linoleic acid by $HO^\bullet$ initiate lipid peroxidation resulting in the formation of $^3(C=O)^*$ and $^1O_2$ are responsible for the biophoton emission.

Due to the highly positive redox potential of $HO^\bullet/H_2O$ redox couple ($E_0{'}(HO^\bullet/H_2O) = 2.3$ V, pH 7), $HO^\bullet$ is highly reactive with macromolecules such as polyunsaturated fatty acids and amino acids. The hydrogen abstraction from polyunsaturated fatty acids and amino acids by $HO^\bullet$ forming lipid and protein alkyl radicals initiates a cascade of reactions leading to the lipid peroxidation and protein carbonylation. It is generally accepted that $HO^\bullet$ reacts with polyunsaturated fatty acids and amino acids in the proximity of the production site with limit diffusion to other target far away from the production site.

3.4 Involvement of singlet oxygen in biophoton emission

The addition of lipid vesicles (with different degree of unsaturated fatty acids) to cytochrome c/hydroperoxide system results into the enhancement in biophoton emission, the increase in biophoton emission was observed to be dependent on the degree of fatty acid unsaturation level [Cadenas et al. 1980]. The author proposed that the addition of $^1O_2$ formed by the decomposition of tetraoxide via Russel-type mechanism to double bonds of unsaturated fatty acid leads to the formation of dioxetane further the decomposition of dioxetane results into the formation of $^3(C=O)^*$. Biophoton emission from the two photosensitive mutants of Arabidopsis thaliana, the vtc2 single mutant deficient in ascorbate synthesis pathway and the vtc2 npq1 double mutant deficient in both ascorbate and zeaxanthin synthesis pathways was considerably higher as compared to WT [Havaux et al. 2006]. Further, the authors demonstrated that the level of lipid peroxidation monitored by MDA level correlates with the level of biophoton emission. These results indicate that the absence of zeaxanthin in the vtc2 npq1 double mutant results in the pronounced production of $^1O_2$ during the high light stress. Based on these considerations, it seems to be likely that $^1O_2$ formed during the high light stress initiates lipid peroxidation known to results in the formation of $^3(C=O)^*$ and $^1O_2$. The proposal that biophoton emission is caused by lipid peroxidation induced by $^1O_2$ was confirmed using isolated lipids such as monogalactosyldiacylglycerol (MGDG), linolenic acid (C18:3) and β-carotene [Birtic et al. 2011]. The authors demonstrated that the oxidation of isolated lipids by $^1O_2$ induced by methylene blue resulted in the biophoton emission. Interestingly, the oxidation of isolated proteins such as bovine serum albumin (BSA) and lysozyme resulted in the biophoton emission, whereas the oxidation of the isolated amino acids such as tryptophan (Trp), histidine (His) provided no photon emission. Similarly, when isolated DNA was oxidized by $^1O_2$ induced by methylene blue, no photon emission was observed. Based on these observation the authors proposed $^1O_2$ as a molecule responsible for biophoton emission.

23

Chapter 4

Methods

(Detection of biophoton emission and free radicals)

4 Detection of biophoton emission and free radicals

Instruments such as highly sensitive photomultiplier tube (PMT) and charged coupled device (CCD) camera were needed for the detection of biophoton emission. For the detection of ROS, spin trapping electron paramagnetic resonance (EPR) spectroscopy and imaging techniques by using probes such as singlet oxygen sensor green (SOSG) or diaminobenzidine (DAB) were performed.

4.1 Detection of biophoton emission

As the biophoton emission is a form of electromagnetic radiation different types of highly sensitive PMT and CCD camera can be used to detect it from the living system.

4.1.1 Photomultiplier tube (PMT): PMT is a device which works on the principle of photoelectric effect and converts photons into electron which further get multiplied and converted into voltage to give an output reading which is a digital number.

Photomultiplier tube consists of photocathode, dynode and anode. The photons are converted to electron at photocathode then get multiplied on several dynodes and finally converted to voltage at anode. The current is converted to digital signal by photon counting unit [Hamamatsu 2006]. There are two types of PMT:

4.1.1.1 Head-on PMT: Two major features of the head-on PMT are that the light enters

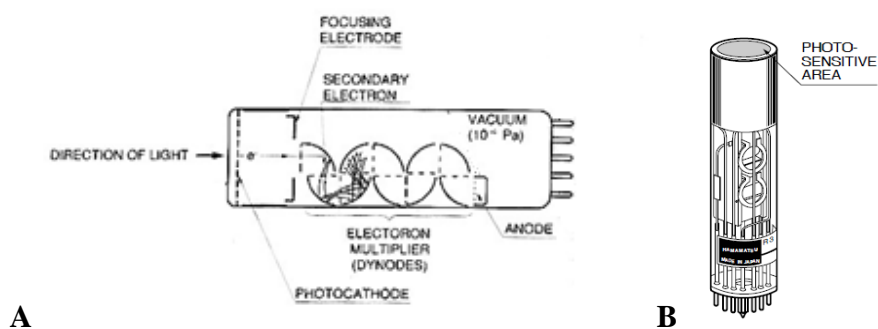A   B

Fig. 4.1 Head-on PMT: Figure [A] represents the assembly of a head on PMT. Whereas [B] shows a head on PMT unit with the indication of photosensitive area. [Fig. adopted from Hamamatsu photonics]

through the top of the device and the photoelectrons are transmitted to the dynodes [Fig. 3.1]

4.1.1.2 Side-on PMT: Two major features of the side-on PMT are that the light inters through the side of the device and the photoelectrons are reflected to the dynodes [Fig. 3.2]
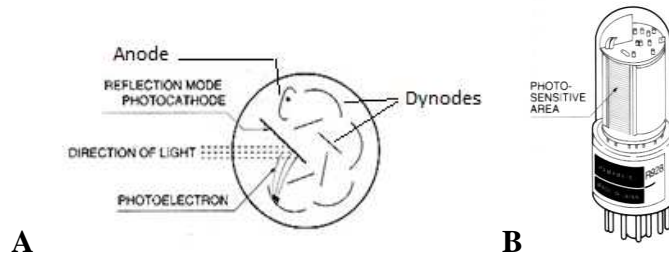

Fig. 4.2 Side-on PMT: Figure [**A**] represents the assembly of a side on PMT. Whereas [**B**] shows a side on PMT unit with the indication of photosensitive area. [Fig. adopted from Hamamatsu photonics]

Generally the PMT is cooled by either thermoelectric cooling device or liquid nitrogen to minimize the electrical noise.

The experiments in present work were performed by using PMT R7518P (Hamamatsu Photonics K.K., Iwata City, Japan) which is a side-on PMT. The PMT was cooled to -30°C using a thermoelectric cooler C9143 (Hamamatsu Photonics K.K., Iwata City, Japan). The photon counting unit C9744 (Hamamatsu Photonics K.K., Iwata City, Japan) was attached to the PMT for the output signal. The sensitivity of the instrument was within the range of 185 to 730 nm.
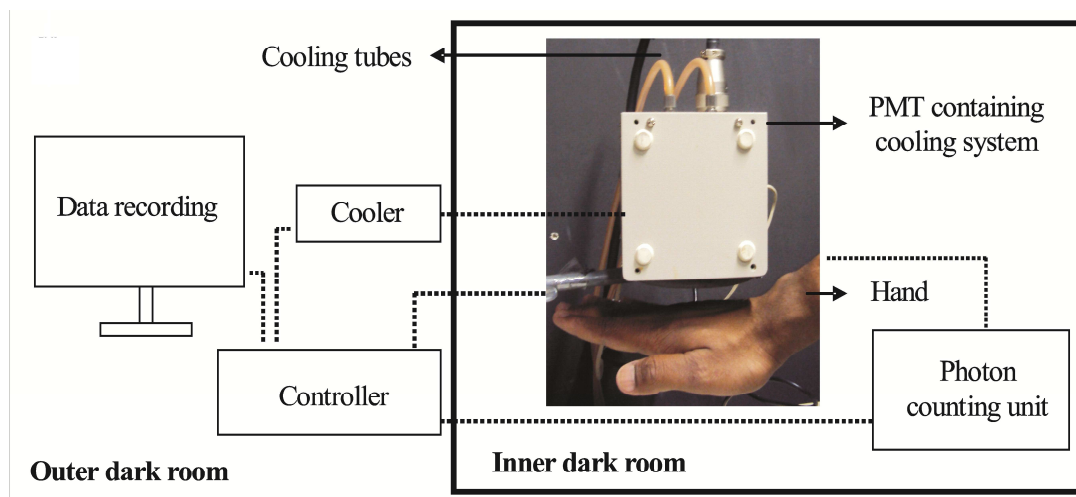

Fig.4.3 Represents the PMT setup used for the human skin experiment [Fig. adopted from Prasad and Pospíšil 2011].

28

4.1.2 Charged coupled device (CCD) camera: CCD camera is most appropriate device for the analysis of two dimensional imaging of the biophoton emission. CCD camera is a type of device which works on the principle of photoelectric effect and converts photons into electrons. The CCD camera consists of metal-oxide semiconductor capacitors (MOS) which can accumulate and store the charge generated due to photoelectric effect (Fig. 4.4) [Hamamatsu 2006].

Photons knock out electrons which are stored in a potential well resulting by applied voltage. These charges can be shifted from one pixel to another pixel by digital pulses applied to the top wells. In this way the charges are transferred though the pixels to the readout register. According to the orientation of the photosensitive area CCD camera can be divided into two types i) frontside illuminated CCD camera, and ii) backside illuminated CCD camera [Kraft et al. 1996].
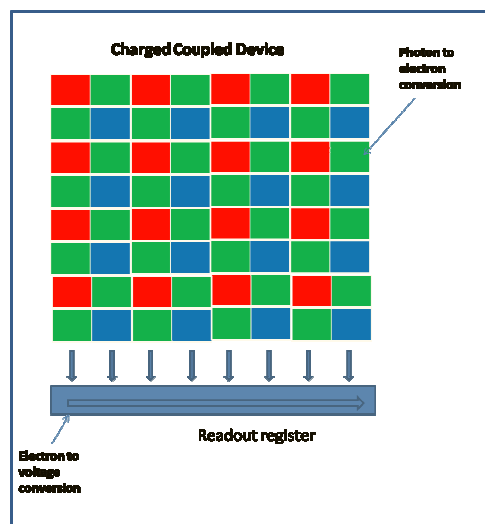
Fig. 4.4 Represents a CCD chip. The arrow indicates the direction of electron flow from pixels to the readout register where the electrons get converted to the voltage.

4.1.2.1 Frontside illuminated CCD camera: The most commonly used CCD camera is frontside illuminated CCD camera, where the light penetrate through photodiode array to reach photosensitive area (Fig. 4.5), in this process some of the light got reflected and lost.

4.1.2.2 Backside illuminated CCD camera: Due to the thin transparent silicon used the backlight illuminated CCD camera are more sophisticated in the production. The light

comes directly to the thinner transparent silicon plate, so are more sensitive and more efficient for the biophoton measurement.
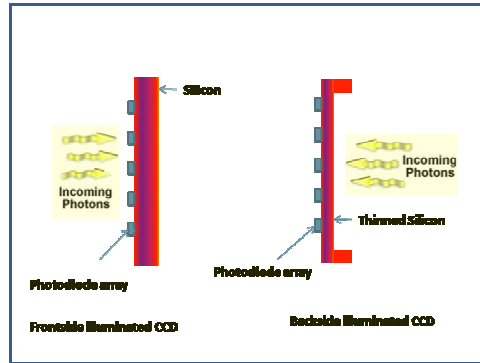
Fig. 4.5 Represents the systematic diagram of frontside illuminated CCD sensor and backside illuminated CCD sensor.

The two-dimensional imaging measurement during the research period was performed by using a backside illuminated CCD camera VersArray 1300B (Princeton instruments, Trenton, NJ, USA). The CCD camera was equipped with a 50-mm focal distance lens with an f-number of 1.2 (F mount Nikkor 50-mm, f:1.2, Nikon) to enhance the light collecting efficiency. The spectral sensitivity of the CCD camera was within the range of 200 nm to 1000 nm.

Fig.4.6 Represents the CCD camera setup used for the human skin experiment [Paper III]

Due to the lenses used, the spectral sensitivity of CCD camera was restricted to the visible range of the spectra. To reduce thermal electrons, the CCD camera was cooled to -

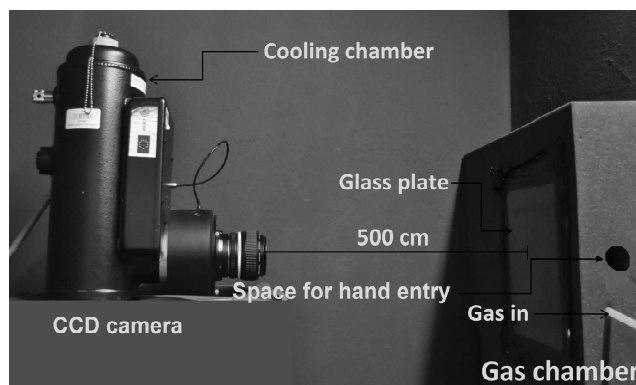110°C using liquid nitrogen. The camera was placed in the experimental dark room and controlled by the computer in the operational dark room. The data correction was made by subtracting the background noise before every measurement. The measurement was done in the image format of 1340 × 1300 pixels. CCD camera parameters were as follows: scan rate, 100 kHz; gain, 3; accumulation time, varies with different living system used for different studies.

4.2 Detection of ROS

There are many direct and indirect methods to detect the ROS from biological systems. The direct method of ROS detection include electron paramagnetic resonance (EPR) spectroscopy whereas a number of probes (such as singlet oxygen sensor green, nitroblue tetrazolium, di-amino benzidine etc) are commercially available that have a high affinity for specific ROS and that are effectively taken up by cells and tissues so works as another method for the detection of ROS. In the present work, both the methods have been used for the detection of ROS and its correlation with the biophoton emission.

4.2.1 Electron paramagnetic resonance (EPR) spectroscopy

It is a branch of absorption spectroscopy in which radiation having frequency in microwave region is absorbed by paramagnetic substance to induce transition between magnetic energy level of electron with unpaired spin. Magnetic energy splitting is done by applying a static magnetic field.
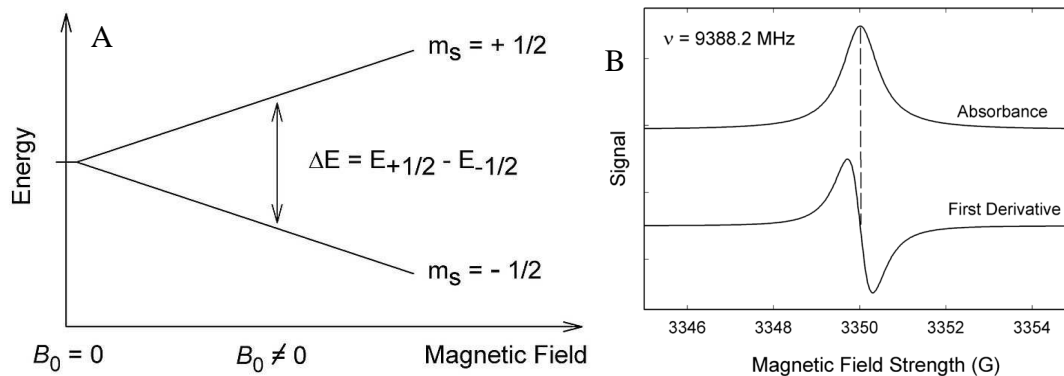
Fig 4.7 Represent the principle of EPR spectroscopy. As the magnetic field increases from $B_0 = 0$ the splitting of energy level takes place and at resonance condition ΔE the unpaired electron moves between two energy level by absorbing the electromagnetic radiation [A]. The absorbance is further converted into first derivative which is observed by most of the EPR [B] [Fig. adopted from Wikipedia http://en.wikipedia.org/wiki/Electron_paramagnetic_resonance].

Electron paramagnetic resonance spectroscopy is the most important technique for the study of radicals (having unpaired electron). On the basis of central atom with unpaired electron, oxygen, carbon, and nitrogen centered radicals have been differentiated into sub groups. Besides the direct detection of radicals in biological system, spin trap has been used for the study of free radicals generated from biological and chemical systems [Pincemail et al. 1990]. Electron paramagnetic resonance spin trapping in biological system is more suitable because the free radcals are less stable and high reactive. The EPR spin trapping is based on the diamagnetic property of the compounds, spin trap reacts with unstable radicals to form more stable paramagnetic compound which is known as spin adduct. Spin traps are generally specific to ROS and are having high reactivity with spin trap to form the spin adduct. 2,2,6,6-tetramethylpiperidine (TEMP) is the specific to $^1O_2$ whereas 4-pyridyl 1-oxide N-tert-butylnitrone (4-POBN) when added with ethanol are able to make the spin adduct with $HO^\bullet$.

For present work, spin-trapping EPR spectra were recorded by using X-band continuous EPR spectrometer MiniScope MS200 (Magnettech GmbH, Germany). Different conditions, and spin trap used for identification of different ROS from living system are maintained in the section material and methods of the related work attached at the end of this thesis.

4.2.2 *In-vivo* imaging of ROS

The in-vivo imaging of ROS can be used to indicate the presence and sites of production of ROS in biological system. There are many chemical probes with different sensitivity available which can react with different ROS to give a visible effect which further can be detected either through visible eye or though fluorescence/confocal microscopy. Some of the common probes used for ROS imaging are Singlet Oxygen Sensor Green (SOSG), nitroblue tetrazolium (NBT) and diaminobenzidine (DAB) [Fryer et al. 2002, Driever 2009].

4.2.2.1 Singlet Oxygen Sensor Green (SOSG): It is a stable fluorescein derivative compound highly specific for $^1O_2$. The probe exhibits weak fluorescence with excitation peaks at 372 and 393 nm and emission peaks at 395 and 416 nm. When SOSG reacts with $^1O_2$ it exhibit intensified fluorescence with excitation peak at 504 nm and emission peak at 525 nm [Flors et al. 2006, Driever 2009].
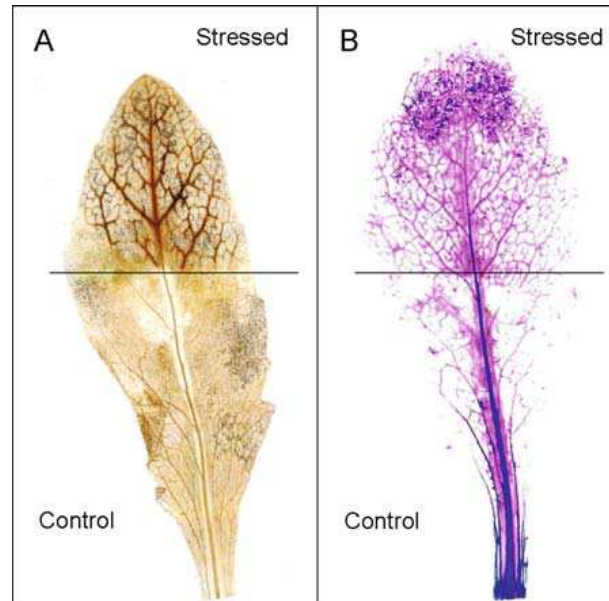

Fig 4.8 Imaging of hydrogen peroxide (A) and superoxide (B) production in leaves using DAB and NBT, respectively. Colour images are shown of *Arabidopsis* leaves infiltrated with DAB (A) and NBT (B). The lower half of each leaf was kept darkened as a control (Control). The upper halves of the leaves were exposed to a PPFD of 350 m mol m^{-2} s^{-1} for 60 min. Chlorophyll was extracted from the leaves before imaging. Marked superoxide production is observed in response to wounding in the control area of (B). [fig. adopted from Driever 2009].

4.2.2.2 Nitroblue tetrazolium (NBT): It is a yellow compound that is reduced by $O_2^{\bullet-}$ to form an insoluble blue formazan deposite. This blue colour can be seen after the extraction of chlorophyll from the plant leaves (Fig. 4.8) [Fryer et al. 2002, Driever 2009].

4.2.2.3 Diaminobenzidine (DAB): It is a photosensitive probe which reacts with H_2O_2 to form brown polymerization products, which can be directly imaged or imaged after extracting the chlorophyll when detected in leaves. Some of these probes were used for the detection of related ROS in living system (Fig. 4.8) [Fryer et al. 2002, Driever 2009].

The concentration and condition of applying the probes are maintained in the section material and methods of the related work attached.

Chapter 5

Result and Discussion

5 Result and Discussion

During the course of research, the involvement of ROS in biophoton emission was measured in different living systems, such as radish Root (Paper I), human hand skin (Paper II and III), potato tuber (Paper IV) and *Arabidopsis thaliana* plant and leaves (Paper V).

5.1 Biophoton emission from radish root cells (Paper I)

5.1.1 Effect of H_2O_2 and ROS scavengers on biophoton emission from radish root

Spontaneous biophoton emission was measured from freshly cut radish root and cell suspension. After subtraction of the noise value the spontaneous biophoton emission from radish root slide and cell suspension was observed to be 4 counts s^{-1} (Fig.1, Paper I). Whereas when H_2O_2 in various concentrations was exogenously added to the radish root cell suspension, a significant increase in biophoton emission was observed (Fig. 5.1).
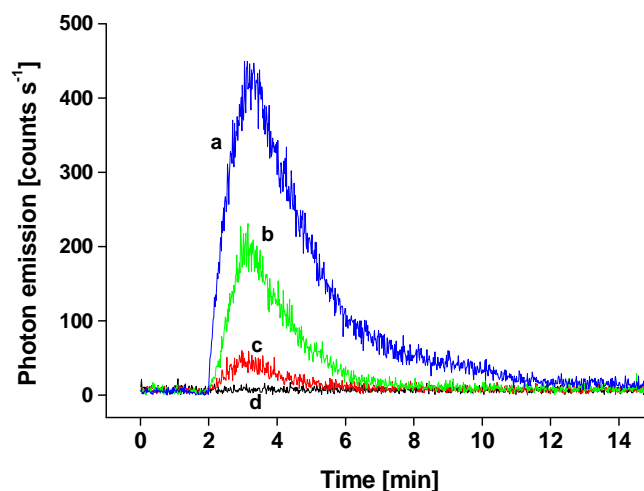
Fig 5.1 Effect of H_2O_2 on biophoton emission from radish root cells. H_2O_2 was added to radish root cell at concentartion of 10 mM (a), 2 mM trace (b) 0.5 mM (c) and 0 mM (d).

To test the involvement of ROS in biophoton emission, the effect of several ROS scavengers on biophoton emission was studied. It was observed that addition of 1 mM sodium ascorbate significantly lowered biophoton emission, whereas complete suppression of biophoton emission was observed with 5 mM sodium ascorbate (Fig.

37

5.2A). Similarly, biophoton emission was notably suppressed by 1 mM cysteine, whereas 5 mM cysteine caused complete inhibition of biophoton emission (Fig. 5.2B). These observations confirm that ROS are involved in biophoton emission.

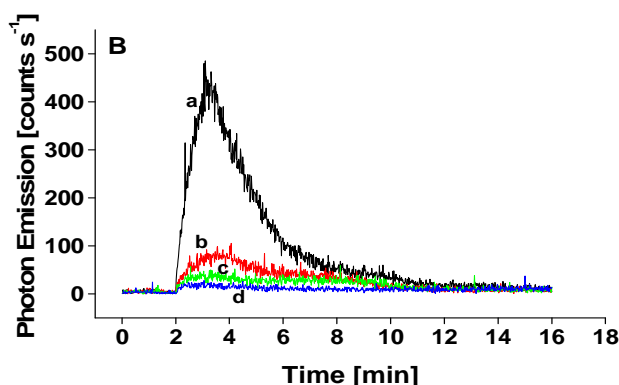**Fig. 5.2** Effect of sodium ascorbate (A) and cysteine (B) on biophoton emission from radish root cells measured in the presence of H_2O_2. Trace (a) is for the sample treated with 10 mM H_2O_2, traces (b), (c) and (d) are for the samples treated with 1 mM, 2 mM and 5 mM sodium ascorbate (A) or cysteine (B) with 10 mM H_2O_2 respectively.

5.1.2 Spectral analysis of the H2O2-induced biophoton emission

Different band pass and edge filters were used to study spectral properties of H_2O_2-induced biophoton emission. It has been observed that the H_2O_2-induced biophoton emission from radish root cells is emitted mainly in green-red region of the spectra, whereas the contribution of UV-blue and IR regions are negligible (Fig. 5.3). The biophoton emission in the red region indicates the photon emission might be due to $^1O_2$ as it is known that the dimole emission of $^1O_2$ is in the red region. To confirm the hypothesis the spin trapping EPR spectroscopy was done to detect the $^1O_2$ production and it has been found that the concentration of $^1O_2$ increases with the enhancement of H_2O_2-induced biophoton emission (Fig. 8 Paper I).
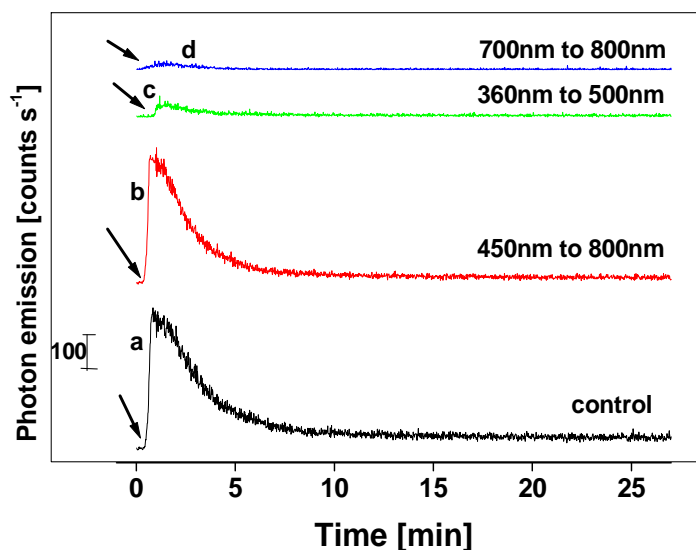
38

Fig. 5.3 Spectral properties of biophoton emission from radish root cells measured in the presence of H_2O_2. Biophoton emission was measured in the absence of filter (a) and in the presence of filters passing emission in the 450-800 nm (b), 360-500 nm (c) and 700-800 nm (d). Arrow indicates the point where 10 mM H_2O_2 was added.

These results confirm that ROS-initiated oxidation of biomolecules (proteins, lipids or nucleic acids) is a trigger reaction involved in biophoton emission (Scheme I, Paper I).

5.2 Biophoton emission from human hand skin (Paper II, III)

5.2.1 Spontaneous biophoton emission from the human hand skin (Paper II)

The biophoton emission from the dorsal side of human hand was observed to be 7 counts s^{-1} (Fig. 5.4 A). After subtraction of the dark count, the biophoton emission from the dorsal side of human hand was calculated to be 4 counts s^{-1}. Interestingly, when spontaneous biophoton emission was measured on the palm side of the human hand, net photon emission of 8 counts s^{-1} was detected (Fig. 5.4B). The observation indicates that spontaneous biophoton emission on the palm side of human hand is double compared to the biophoton emission on the dorsal side of human hand.

39
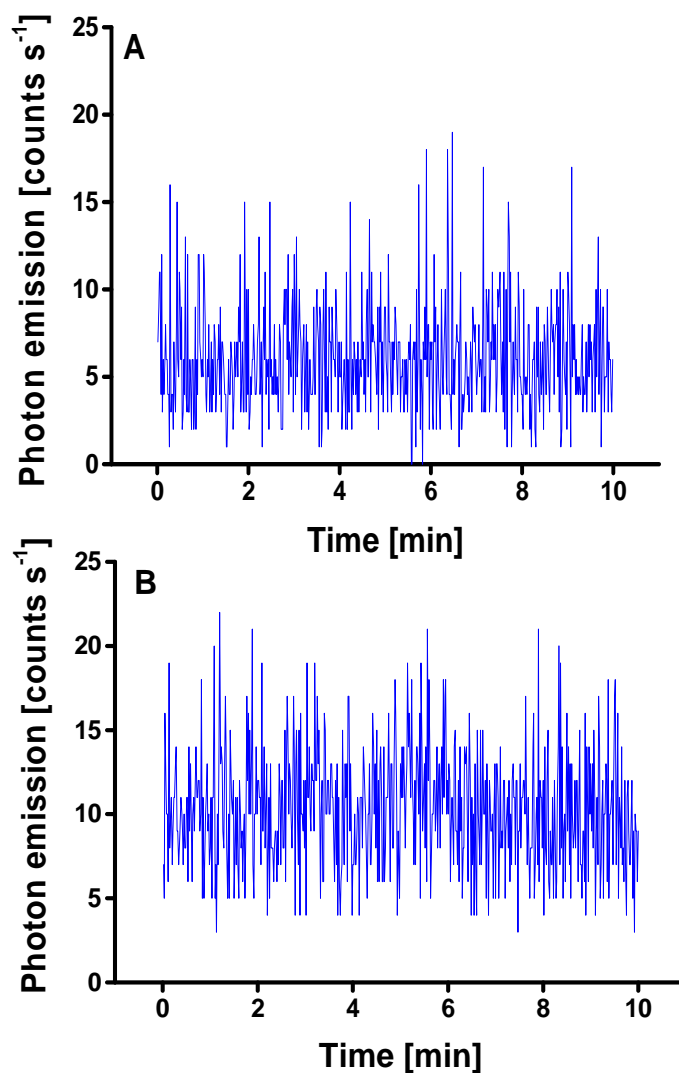
Fig. 5.4 Spontaneous biophoton emission measured from epidermal cell of skin on the dorsal (A) and palm (B) side of the human hand. The graph includes the noise level of the instrument which is 2 to 3 counts^{-1}, the value of which is comparable to dark count

5.2.2 Effect of exogenous application of H_2O_2 on biophoton emission from human hand skin (Paper II)

To study the effect of oxidative processes in the epidermal cell of the human skin on biophoton emission, H_2O_2 was applied exogenously to the fixed area of the skin surface. Topical application of H_2O_2 to the skin surface on the dorsal side of the human hand resulted in the sudden enhancement of biophoton emission. An increase in the

40

concentration of H_2O_2 exogenously applied to the skin surface caused gradual enhancement in the biophoton emission (Figure 2A, Paper II).
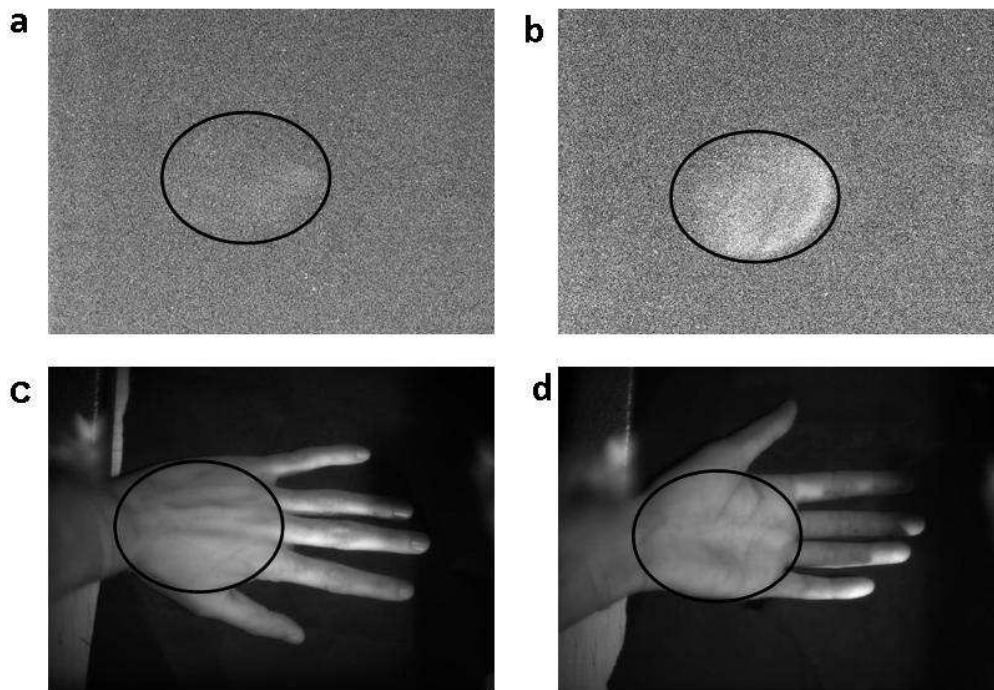
Fig. 5.5 Two-dimensional image of peroxide-induced biophoton emission from human hand measured after topical application of H_2O_2 to the dorsal (a) and palm (b) side of human hand and corresponding photographs of the dorsal (c) and palm (d) side of human hand.

Similarly, topical application of exogenous H_2O_2 to the skin surface on the palm side of the human hand brought about an enhancement in biophoton emission (Fig. 2B, Paper II). Interestingly, biophoton emission from the palm side of human hand was found more than the photon emission from dorsal side of the human hand. The two dimensional imaging was performed to check the effect of topical application of H_2O_2 on human skin. The imaging of spontaneous biophoton emission was not possible, whereas the portion of the skin appeared where the H_2O_2 was applied on the skin (Fig. 5.5). The higher biophoton emission from the palmer side of the human skin was correlated with higher turnover rate of the cells.

41

5.2.3 Effect of molecular oxygen on biophoton emission from human hand skin (Paper III)

To study the involvement of molecular oxygen in spontaneous biophoton emission from human skin, the photon emission was measured under aerobic, anaerobic and hyperaerobic conditions. To control gas environment around the human hand, the human hand was placed in a gas chamber (experimental set up is shown in Fig. 1 Paper III). Under aerobic conditions, the spontaneous biophoton emission from the dorsal side of the human hand was 2 counts s^{-1} (Fig. 3, Paper III). When the air in the gas chamber was replaced with nitrogen gas, spontaneous biophoton emission was suppressed to 1.25 counts per second (by 40 % compared to aerobic conditions). The supply of molecular oxygen in the gas chamber results in the enhancement in spontaneous biophoton emission to 3 counts s^{-1} (by 50 % compared to aerobic conditions). The two dimensional study of the effect of molecular oxygen on biophoton emission from human hand skin was measured and the image was found to be intensified in the presence of molecular oxygen whereas in the presence of nitrogen gas, the image intensity was found to be less (Fig. 5.6). These results reveal that the molecular oxygen is involved in spontaneous biophoton emission from the human skin.
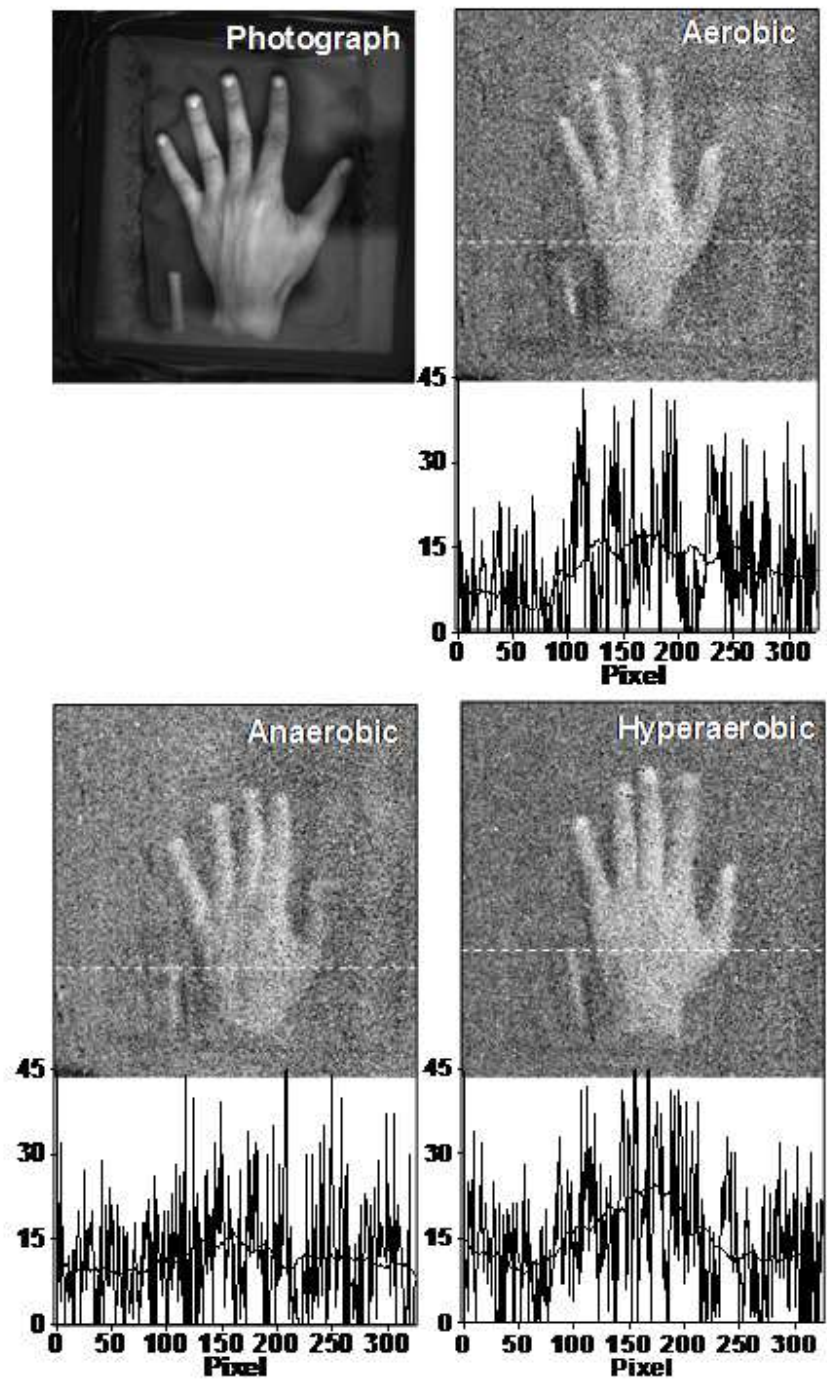
Fig. 5.6 Effect of molecular oxygen on two-dimensional spontaneous biophoton emission from the dorsal side of the hand. The bottom panel shows the spatial profile of photon emission in the middle strip of the image. Y axis denotes the number of counts accumulated after 30 minutes, where as X axis denotes the pixel of the image.

5.2.3 Effect of ROS scavengers on spontaneous biophoton emission from human hand skin (Paper III)

To study the involvement of ROS in biophoton emission, the effect of various ROS scavengers on spontaneous biophoton emission from the human hand was measured. When hydrophilic (sodium ascorbate and glutathione) and lipophilic (α-tocopherol and coenzyme Q10) ROS scavengers were topically applied on the human skin, the decrease in spontaneous biophoton emission to 1.6 counts per seconds (by 30 % compared to control) was observed (Fig. 5.7).
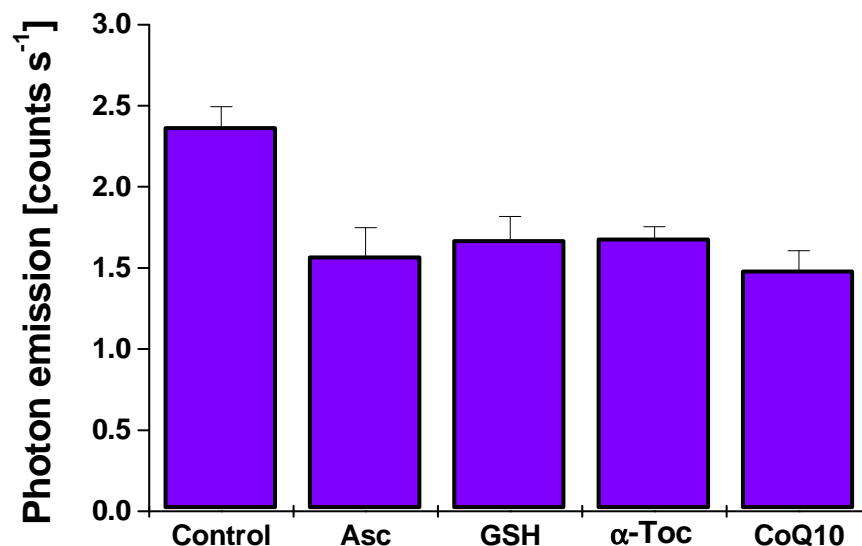
Fig. 5.7 Effect of ROS scavengers on one-dimensional spontaneous biophoton emission from the dorsal side of the hand. Prior to the measurement the hydrophilic (ascorbate and gluthatione) and the lipophilic (α-tocopherol and coenzyme Q10) ROS scavengers at the concentration of 5 mM were topically applied on the the dorsal side of the hand. Spontaneous biophoton emission from human hand was measured for 15 minutes. Each bar represents the mean value of at least three independent measurements ± SD made at the same diurnal time of three different days.

The effect of ROS scavengers on two-dimensional spontaneous biophoton emission from human hand skin was measured using CCD camera (Fig. 6, Paper III). Significant decreased in spontaneous biophoton emission was observed with the application of ROS scavengers. The observation indicates the involvement of ROS in spontaneous biophoton emission from the human skin.

44

5.3 Biophoton emission from potato tuber (Paper IV)

5.3.1 Detection of ROS from *Phytophthora infestans*-infected potato tuber

The *Phytophthora infestans* was used on potato tuber as the biotic stress factor to enhance the ROS production. To study the formation of ROS in the late phase of the oomycete *Phytophthora infestans* infection, the formation of H_2O_2 in *Phytophthora infestans*-infected potato tuber was detected by DAB imaging technique (Fig. 5.8).
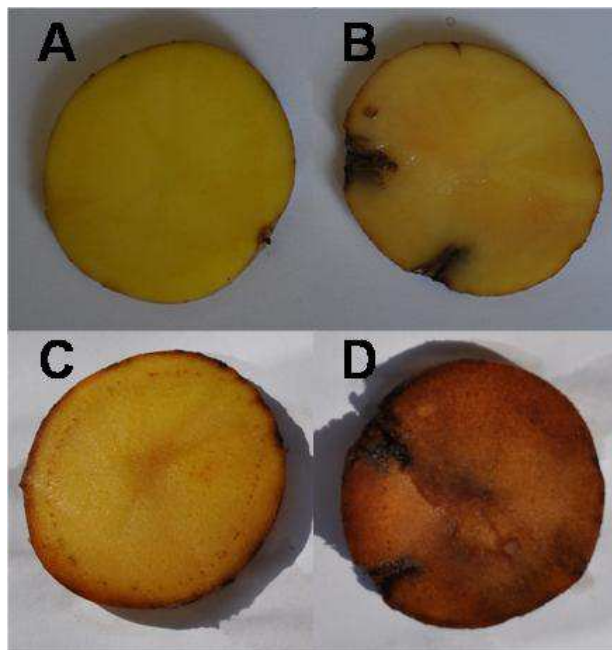
Fig. 5.8 Hydrogen peroxide formation detected by DAB imaging technique from *Phytophthora infestans*-infected potato tuber slice. The image of the slice from non-infected potato tuber (A), *Phytophthora infestans*-infected potato tuber (B), non-infected potato tuber infiltrated in DAB (C) and *Phytophthora infestans*-infected potato tuber infiltrated in DAB (D) were made 6 hrs after the cutting the potato slice. In (B and D), the potato tuber was used after three weeks of the inoculation with oomycete *Phytophthora infestans*. In (C-D), the potato tuber slices were incubated with DAB for 6 hrs.

To monitor whether the formation of H_2O_2 is linked to the formation of $HO^\bullet$ during the oxidative burst in the late phase of the *Phytophthora infestans* infection, the production of

HO$^\bullet$ was measured by EPR spin-trapping spectroscopy using 4-POBN/ethanol spin-trapping system (Fig. 5.9).

Fig. 5.9 Detection of HO$^\bullet$. 4-POBN-CH(CH$_3$)OH adduct EPR spectra were detected from non-infected potato tuber (trace a) and *Phytophthora infestans*-infected potato tuber for three (trace b) and five (trace c) weeks. Trace (d) represents 4-POBN-CH(CH$_3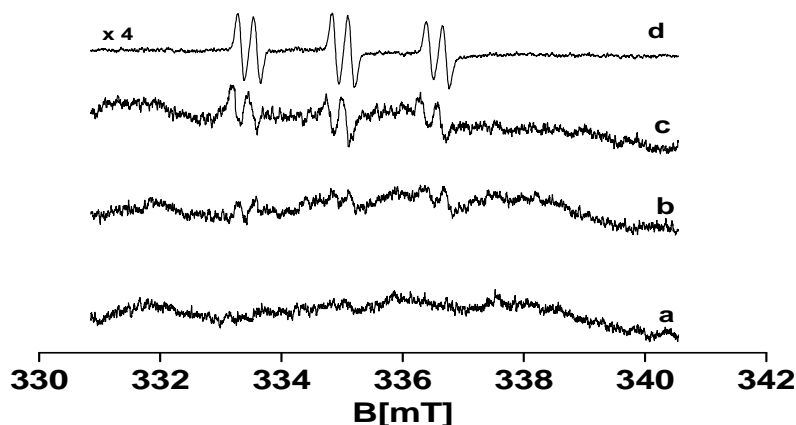$)OH adduct EPR spectrum observed from Fenton reagent system. In (a-c), the potato tuber slices were infiltrated with 50 mM 4-POBN with 170 mM ethanol for 6 hrs. In (d), 4-POBN-CH(CH$_3$)OH adduct EPR spectra was detected after the addition of 500 μM FeSO$_4$ in 20 μM H$_2$O$_2$ in the presence of 10 mM POBN and 170 mM ethanol (the spectrum 'd' was divided by 4).

5.3.2 *Phytophthora infestans*-induced biophoton emission from potato tuber

The biophoton emission from the non-infected and *Phytophthora infestans*-infected potato tuber was measured using highly sensitive PMT (Fig. 3, Paper IV). The dark count of PMT was measured to be 2 counts s^{-1}. The count rate from the non-infected potato tuber was observed to be 4 counts s^{-1} (Fig. 5.10A), whereas the count rate from *Phytophthora infestans*-infected potato tuber was 9 counts s^{-1} (Fig. 3B, Paper IV). After subtracting the dark count, the spontaneous biophoton emissions from non-infected and *Phytophthora infestans*-infected potato tubers were determined to be 2 counts s^{-1} and 7 counts s^{-1}, respectively (Fig. 3C, Paper IV). The presented data show that the biophoton emission from *Phytophthora infestans*-infected potato tuber was enhanced 2.5 times

when compared to the spontaneous biophoton emission from the non-infected potato tuber.

The observation indicates the enhancement in biophoton emission with the increased concentration of H_2O_2 and $HO^\bullet$ with the infection of *Phytophthora infestans* on potato tuber.

5.4 Biophoton emission from *Arabidopsis thaliana* (Paper V)

5.4.1 Effect of UVA on the biophoton emission from *Arabidopsis thaliana*

Biophoton emission was measured from the non-exposed and UVA radiation-exposed *Arabidopsis* plants using CCD camera. Two dimensional imaging of UVA radiation-exposed *Arabidopsis* plants shows significant increase in biophoton emission in comparison to UVA radiation-nonexposed plants (Fig. 1, Paper V). To study in more detail the spatial distribution of photon emission on the leaf surface, the two-dimensional biophoton emission was measured from the non-exposed and UVA radiation-exposed *Arabidopsis* leaves (Fig. 5.10).
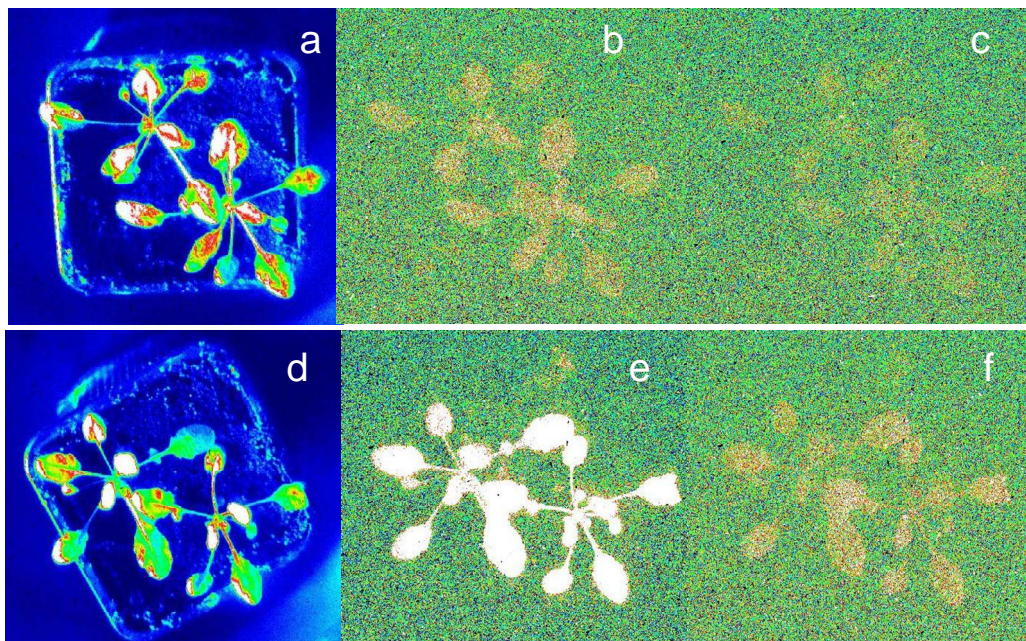
Fig. 5.10 Effect of UVA radiation on the two-dimensional biophoton emission from the *Arabidopsis* plant. The photographs of *Arabidopsis* plant were presented to show the dimension of the detached leaves in front of CCD camera (Figures a and d). Figures b and c represent the two-dimensional images of the biophoton emission from the non-exposed *Arabidopsis* leaves after the dark adaptation for 5 min and 50 min, respectively. Figures e and f represent the two-dimensional images of the biophoton emission from the

UVA radiation-exposed *Arabidopsis* plant observed after the dark adaptation for 5 min and 50 min, respectively. Biophoton emission imaging was measured with an integration time of 45 min.

5.4.2 Kinetic analysis of UVA radiation-induced biophoton emission

One-dimensional biophoton emission measured by low-noise photomultiplier tube (PMT) was used to study the kinetic property of biophoton emission from the non-exposed and UVA radiation-exposed *Arabidopsis* leaves (Fig. 5.11). When the biophoton emission was measured from the UVA radiation-exposed *Arabidopsis* leaves after 5 min of dark adaptation, a pronounced decay in biophoton emission was observed as compared to the non-exposed *Arabidopsis* leaves (Fig. 5.11). The biophoton emission from the UVA radiation-exposed *Arabidopsis* leaves shows two kinetically distinguished phases, the fast decay phase (0-6 min) and the slow decay phase (> 6 min). These observations indicate the possibility of two different phenomenons involved in the UVA radiation-induced biophoton emission.
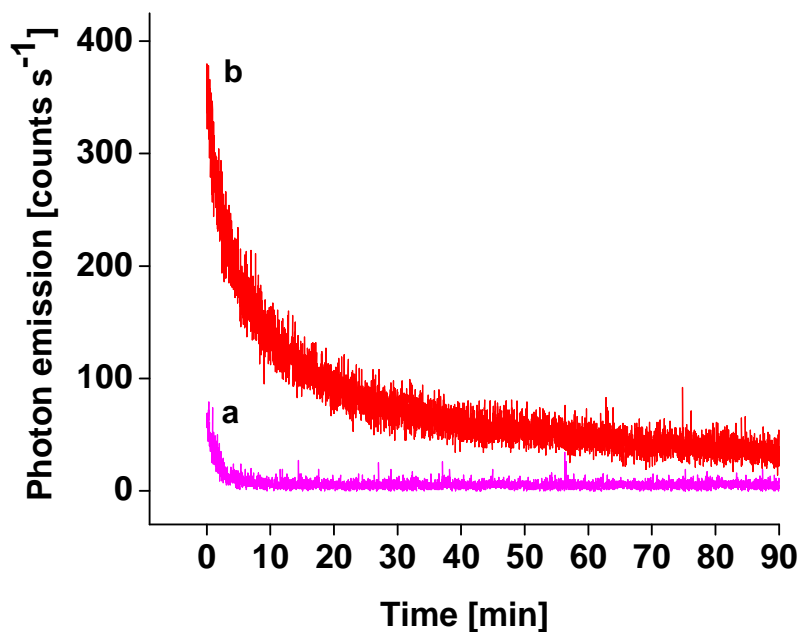

Fig. 5.11 Effect of UVA radiation on the one-dimensional biophoton emission from the *Arabidopsis* leaves: One-dimensional biophoton emission from the non-exposed (trace a) and UVA radiation-exposed (trace b) *Arabidopsis* leaves was measured using a PMT after the dark adaptation for 5 min.

5.4.3 Spectral analysis of UVA radiation-induced biophoton emission

To explore the origin of two kinetically distinguished phases of the biophoton emission, the spectral analysis of photon emission from UVA radiation-exposed *Arabidopsis* leaves was determined using band pass and interference filters. Without the filter, the biophoton emission was found to have the fast decay phase and the slow decay phase (Fig. 5.12A). When the red band pass filter (RG8) passing wavelengths longer than 680 nm was used, the biophoton emission during the slow decay phase was fully diminished (Fig. 5.12B). Similarly, when biophoton emission was measured with the interference filter with a maximum transmittance at 680 nm, the complete suppression of the biophoton emission during the slow decay phase was observed (Fig. 5.12C). To explore in more detail the biophoton emission in the red region of the spectrum, the UVA radiation-induced biophoton emission was further analyzed by using a set of different interference filter (Fig 5.12D). The UVA radiation-induced biophoton emission was found from 660 nm to 700 nm of the red spectrum with the peak value at 680 nm (Fig 5.12D). These observations reveal that the photon emission during the fast phase of biophoton emission is predominantly in the red region of the spectrum, whereas the biophoton emission during the slow decay phase is absent in the red region of the spectrum.

The observation indicates that the initial UVA radiation-induced biophoton emission was due to the chlorophylls, whereas at the late stage the photon emission was dominated by ^{3}(C=O)*.
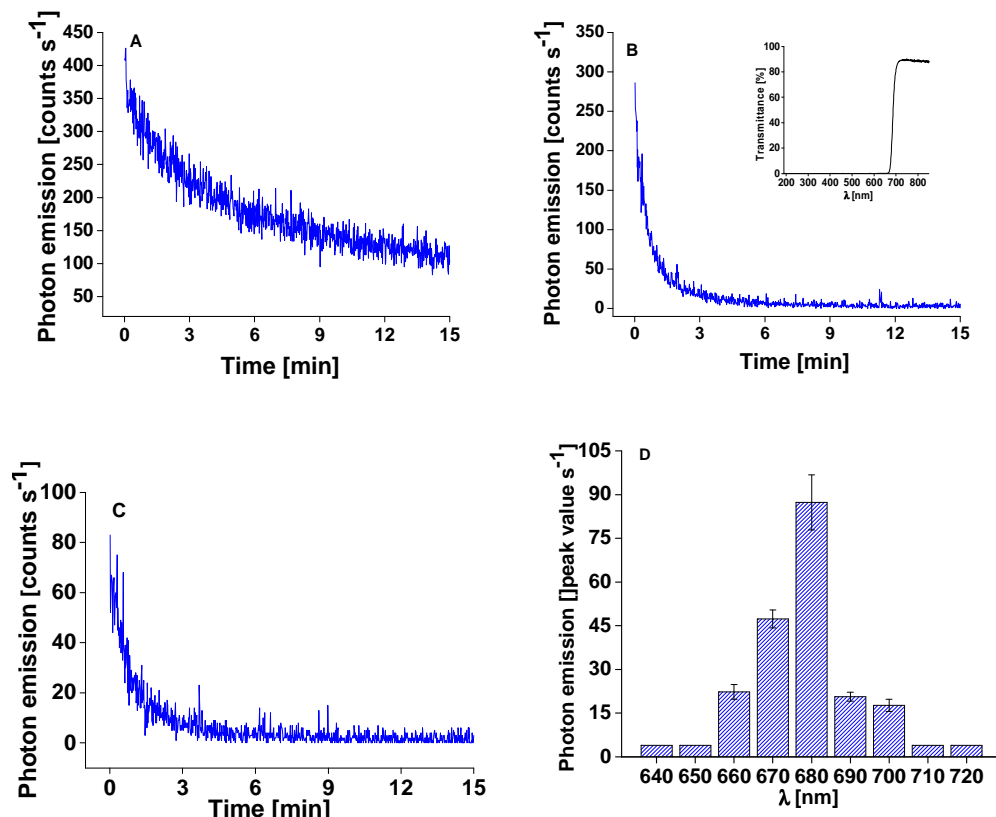
Fig. 5.12 Spectral analysis of the UVA radiation-induced biophoton emission from the *Arabidopsis* leaves: The biophoton emission from the UVA radiation-exposed *Arabidopsis* leaves was measured in the absence of filter (panel A), in the presence of the red band pass filter (RG8) (panel B) and the 680 nm interference filter. Panel D represents spectral property of UVA radiation-induced biophoton emission from *Arabidopsis* leaves in the red region of the spectra from 640 nm to 720 nm measured using a set of interference filters with a bandwidth of 10 nm. The different bars on the bar graph represent the biophoton emission observed with the interference filters centered at 641 nm, 651 nm, 662 nm, 671 nm, 683 nm, 691 nm, 702 nm and 711 nm. The presented data are expressed as the mean value and the standard deviation of at least three measurements (mean ± SD, n=3).

Chapter 6

Conclusions

On the basic of observations, the involvement of ROS in biophoton emission was confirmed and a scheme was proposed (Fig. 6.1) which indicates the mechanism of biophoton emission through ROS-induced lipid peroxidation and protein oxidation. One-electron reduction of molecular oxygen by highly reducing species forms $O_2^{\bullet-}$ (Fig. 6.1, reaction 1). The non-enzymatic and enzymatic dismutation of $O_2^{\bullet-}$ generates H_2O_2 (Fig. 6.1, reaction 2), the subsequent one-electron reduction of which leads to the formation of $HO^{\bullet}$ (Fig. 6.1, reaction 3).The triplet-triplet energy transfer from triplet chromophore to molecular oxygen results in the formation of $^1O_2$ (Fig. 6.1, reaction 4).The hydrogen abstraction from polyunsaturated fatty acid and amino acid (RH) by $HO^{\bullet}$ generates lipid and protein alkyl radicals $(R^{\bullet})$ (Fig. 6.1, reaction 5). The subsequent one-electron oxidation of $R^{\bullet}$ in the presence of molecular oxygen brings about the formation of lipid and protein peroxyl radical $(ROO^{\bullet})$ (Fig. 6.1, reaction 6). The subsequent hydrogen abstraction from another polyunsaturated fatty acid and amino acid by $ROO^{\bullet}$ forms protein and lipid hydroperoxide (ROOH) (Fig. 6.1, reaction 7). The one-electron reduction of ROOH results in the formation of alkoxyl radical $(RO^{\bullet})$ and hydroxy ion (OH^-) (Fig. 6.1, reaction 8). Dioxetane (ROOR) is formed by either the cycloaddition of $^1O_2$ to polyunsaturated fatty acid and amino acid (Fig. 6.1, reaction 9) or by the cyclization of $ROO^{\bullet}$ (Fig. 6.1, reaction 10). Tetroxide (ROOOOR) is formed by the recombination of either two $ROO^{\bullet}$ (Fig. 6.1, reaction 11) or two $RO^{\bullet}$ (Fig. 6.1, reaction 12).The decomposition of ROOR (Fig. 6.1, reaction 13) or ROOOOR (Fig. 6.1, reaction 14) results in the formation of triplet excited carbonyl $^3(C=O)^*$. The electronic transition from the triplet excited state to the ground state of the carbonyl is accompanied by the photon emission in the blue-green region of the spectrum (380-550 nm) (Fig. 6.1, reaction 15). The energy transfer from $^3(C=O)^*$ to chromophore (C) results in the formation of singlet excited state of chromophore (C^*) (Fig. 6.1, reaction 16).The electronic transition from the singlet excited state to the ground state of chromophore is accompanied by the photon emission in the green-red region of the spectrum (550-750 nm) (Fig. 6.1, reaction 17). The triplet-triplet energy transfer from $^3(C=O)^*$ to molecular oxygen forms $^1O_2$ (Fig. 6.1, reaction 18). Alternatively, $^1O_2$ is formed directly by the

decomposition of ROOOOR via Russell-type mechanism, while ground state carbonyls (C=O) and organic hydroxide are formed (Fig. 6.1, reaction 19). The dimol photon emission of $^1O_2$ is in the red region of the spectrum (634 nm, 703 nm) (Fig. 6.1, reaction 20) and the monomol photon emission of $^1O_2$ is in the infra-red region of the spectrum (1274 nm) (Fig. 6.1, reaction 21).
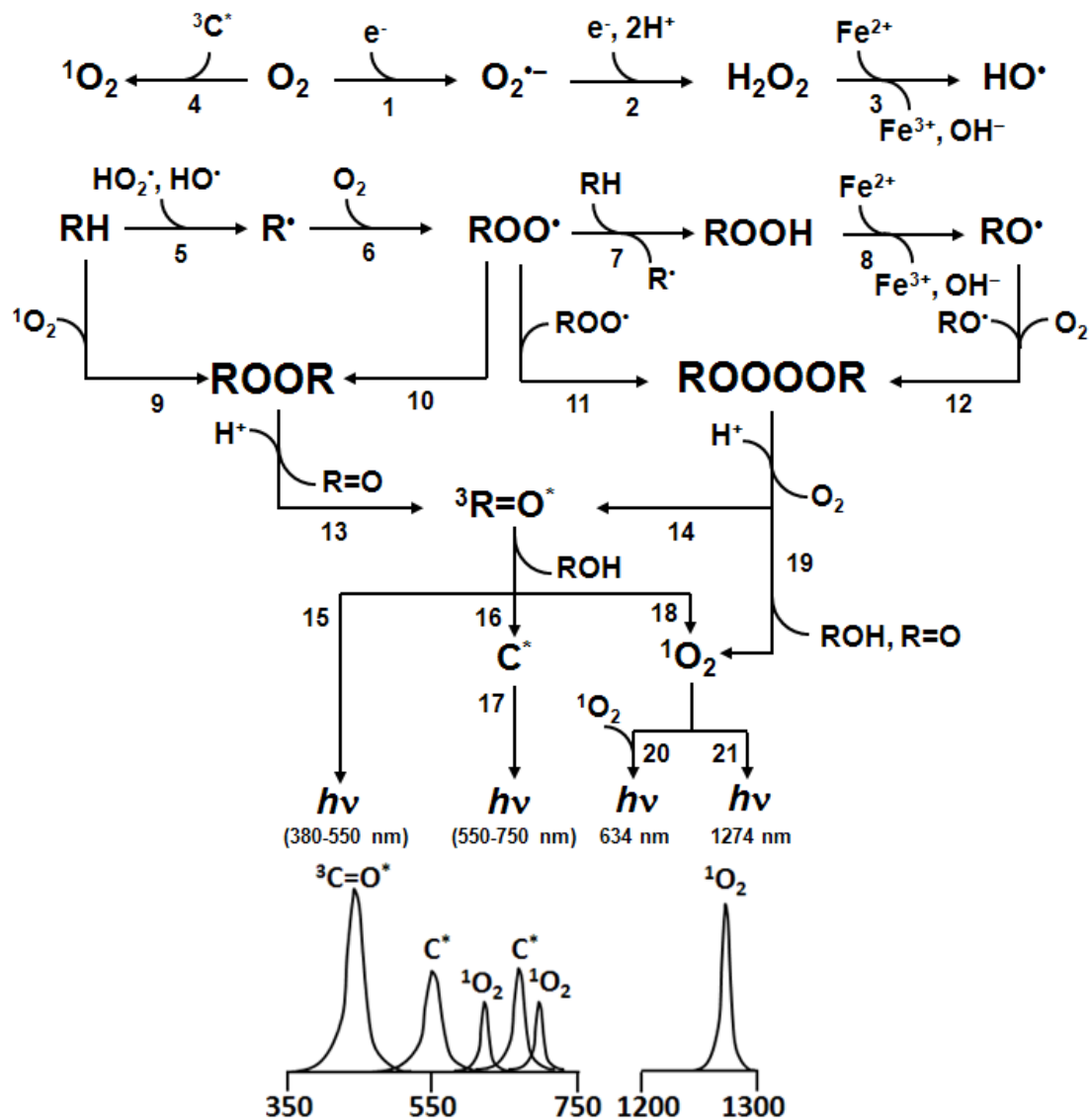

Fig. 6.1 Involvement of reactive oxygen species in biophoton emission [Fig. is adopted from review Pospíšil and Rastogi (manuscript under preparation)].

54

Chapter 7

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Chapter 8

Publications