Posudek oponenta habilitační práce

Uchazeč	**M.Sc. Ankush Prasad, Ph.D.**
Habilitační práce	Reactive oxygen species in the biological system: challenges and methodological advances
Oponent	doc. Ing. Petr Neugebauer, Ph.D.
Pracoviště oponenta, instituce	CEITEC VUT, Vysoké Učení Technické Brno Purkyňova 656/123, 612 00 Brno

Habilitační práce M.Sc. Ankushe Prasada, Ph.D. se věnuje problematice oxidačních procesů během metabolických reakcí, jako je dýchání, fotosyntéza nebo oxidační vzplanutí. U těchto procesů je známo, že jsou spojené s tvorbou reaktivních forem kyslíku a dusíku, radikálů. Autor se zejména zabývá výzkumem a identifikací cílových biomolekul nebo specifických zbytků, které odpovídají za tvorbu těchto radikálů a jejich umístění ve vnitrobuněčných oddílech. Získané poznatky jsou důležité pro pochopení jejich role ve zdraví a nemoci. Cílem předložené habilitační práce je studium oxidačních procesů. Práce poskytuje přehled o nejnovějších tendrech výzkumu volných radikálů v biologii a medicíně. V neposlední řadě práce shrnuje přínos autora k pochopení tvorby radikálů a její lokalizaci v živých systémech zdůrazňující nedávný vývoj v oblasti in-vivo detekce a vizualizace oxidačních procesů. Zvolené téma práce je velmi aktuální a výsledky důležité pro další vývoj v oboru.

The habilitation thesis of M.Sc. Ankush Prasada, Ph.D., deals with the issue of oxidative processes during metabolic reactions, such as respiration, photosynthesis or oxidative flares. These processes are known to be associated with the formation of reactive oxygen species and nitrogen radicals. The author mainly deals with research and identification of target biomolecules or specific residues that are responsible for the formation of these radicals and their location in the intracellular compartments. The knowledge gained is important for understanding their role in health and disease. The aim of the presented habilitation thesis is to study oxidation processes. The thesis provides an overview of the latest developments in free radical research in biology and medicine. Last but not least, the work summarizes the author's contribution to the understanding of radical formation and its localization in living systems, emphasizing recent developments in the field of in-vivo detection and visualization of oxidation processes. The chosen topic of the thesis is very current and the results are important for further development in the field.

Způsob zpracování tématu

Předložená habilitační práce je psaná v anglickém jazyce a je koncipovaná jako soubor komentovaných publikací. Tvoří komplexní celek, který shrnuje vše, co bylo autorem dosaženo ve vědecké oblasti studia oxidačních procesů a tvorbě reaktivních forem kyslíku a dusíku v biologických systémech. Téma práce je zpracováno přibližně na 180 stranách, včetně kopií 13 publikací, u kterých je habilitant převážně prvním nebo druhým autorem. Vědecké články byly publikovány v impaktovaných mezinárodních časopisech. V práci se dále odkazuje na 109 položek použité a citované literatury a seznam použitých zkratek. Po obecném úvodu se autor v první kapitole zabývá reaktivním kyslíkem v organismech (kapitola 1), následuje přehled detekčních technik (kapitola 2), popis oxidačních reakčních mechanismů (kapitola 3), v poslední kapitole popisuje výzvy v oblasti detekce a vývoji biosenzorů (kapitola 4). Poslední část je shrnutím předchozích kapitol a dosavadních výsledků výzkumné práce autora.

The submitted habilitation thesis is written in English and is designed as a set of annotated publications. It forms a complex unit that summarizes everything that the author has achieved in the scientific field of the study of oxidation processes and the formation of reactive forms of oxygen and nitrogen in biological systems. The topic of the thesis is processed on approximately 180 pages, including copies of

13 publications, in which the author is mostly the first or second author. Scientific articles have been published in impact international journals. The work also refers to 109 items of used and cited literature and a list of used abbreviations. After a general introduction, the author deals in the first chapter with reactive oxygen species (Chapter 1), followed by an overview of detection techniques (Chapter 2), description of oxidation reaction mechanisms (Chapter 3), in the last chapter describes challenges in detection and development of biosensors (Chapter 4). The last part is a summary of previous chapters and previous results of the author's research work.

Přínos habilitační práce

Hlavní přínos habilitační práce je popsán ve stěžejních čtyřech kapitolách s výsledky a s diskuzí. Je v nich dokumentován vlastní příspěvek habilitanta ve výzkumu a vědeckém bádání v oblasti reaktivních forem kyslíku v biologických systémech a jejich detekcí. Komentované výstupy byly publikovány ve velmi kvalitních recenzovaných impaktovaných vědeckých časopisech, jako například Scientific Reports, Frontiers in Physiology, Frontiers in Plant Science, PNAS atd. Jedná se o články převážně prvoautorské, spadající do kategorie Q1. To svědčí o vysoké kvalitě i původnosti dosažených výsledků a nemám k nim vážné připomínky nebo námitky. Prezentovaná práce je sestavena promyšleně a má potenciál stát se základem budoucího studijního materiálu zaměřeného na studium reaktivního kyslíku v biologických systémech.

The main contribution of the habilitation thesis is described in the main four chapters with results and discussion. They document the author's own contribution in research and scientific research in the field of reactive oxygen species in biological systems and their detection. The annotated outputs were published in high-quality peer-reviewed impact scientific journals, such as Scientific Reports, Frontiers in Physiology, Frontiers in Plant Science, PNAS, etc. These are mostly first-author articles, belonging to the Q1 category. This indicates the high quality and originality of the results achieved and I have no serious comments or objections to them. The presented work is compiled thoughtfully and has the potential to become the basis of future study material focused on the study of reactive oxygen in biological systems.

Závěrečné stanovisko

Habilitační práce i jednotlivé publikace jednoznačně představují přínos k problematice oxidačních procesů a potvrzují, že M.Sc. Ankush Prasad, Ph.D. tuto oblast zvládá po stránce teoretické i praktické. M.Sc. Prasad, Ph.D. (ORCID: 0000-0002-2009-8987) s aktuálním h-indexem 12 ve své dosavadní kariéře prokázal tvůrčí a vědeckou činnost, shrnutou ve 32 publikacích, v impaktovaných vědeckých časopisech se 495 citacemi dle Web of Science. Působil na zahraničních universitách, M.Sc and B.Sc tituly obdržel v Indii na Bundelkhanad Universitě, Jhansi, a Manipur Universitě, respektive. Dále strávil v souhrnu 6 měsíců v Italii na Universitě v Sieně a dva roky působil v Japonsku na Technologickém institutu v Tohoku. Dále je aktivní v pedagogické činnosti a výchově nových vědeckých pracovníků. Byl vedoucím několika magisterských a bakalářských prací, a to jak na své současné domovské univerzitě, tak i v zahraničí (Tohoku Institute of Technology, Sendai, Japonsko). Vede kurzy a přednáší v oblasti volných radikálů a biomedicíny na Palackého universitě v Olomouci a na Ostravské universitě. Z výčtu autorských publikací je průkazné, že habilitant v dané odborné oblasti během poslední dekády intenzivně bádá a publikuje. Je tak zřetelné, že jeho práce je aktuální a jádro habilitační práce bylo dostatečně publikováno ve velmi kvalitních recenzovaných mezinárodních časopisech a prezentováno na mezinárodních vědeckých konferencí. Předložená práce s přiloženými publikacemi a také seznam publikací z hodnocení jeho vědecké a tvůrčí činnosti dokazují přínos habilitanta k rozvoji vědeckého poznání v oboru biofyziky. Z posuzované práce a jeho osobních aktivit vyplývá, že MSc. Ankush Prasad, Ph.D. je pracovníkem s dostatečnou vědeckou erudicí. Předložená habilitační práce demonstruje potřebný nadhled habilitanta a schopnost analýzy a následné vědecké syntézy poznatků, a tak svědčí nejen o jeho odborných, ale i o pedagogických kvalitách a didaktických schopnostech. V práci jsem nenalezl žádné podstatné chyby, jen bych doporučil zvýšit aktivitu v oblasti získávání vlastních vědeckých grantů.

The habilitation work and individual publications clearly represent a contribution to the issue of oxidation processes and confirm that M.Sc. Ankush Prasad, Ph.D., manages this area from a theoretical and practical point of view and. M.Sc. Prasad, Ph.D., (ORCID: 0000-0002-2009-8987) has a current h-index of 12. He has demonstrated creative and scientific activity, summarized in 32 publications, in impact scientific journals with 495 citations according to the Web of Science. He has worked at foreign universities, with M.Sc and B.Sc degrees received in India at Bundelkhanad University, Jhansi, and Manipur University, respectively. He also spent a total of 6 months in Italy at the University of Siena and worked for two years in Japan at the Tohoku Institute of Technology. He is also active in teaching and educating new researchers. He has supervised several master's and bachelor's theses, both at his current home university and abroad (Tohoku Institute of Technology, Sendai, Japan). He leads courses and lectures in the field of free radicals and biomedicine at Palacký University in Olomouc and at the University of Ostrava. From the list of author's publications, it is evident that the author in the given professional field has been intensively researching and publishing in the last decade. It is so clear that his work is current and the core of the habilitation thesis has been sufficiently published in high-quality peer-reviewed international journals and presented at international scientific conferences. The presented work with the attached publications as well as the list of publications from the evaluation of his scientific and creative activity prove the contribution of the author to the development of scientific knowledge in the field of biophysics. The assessed work and its personal activities show that the MSc. Ankush Prasad, Ph.D., is a worker with sufficient scientific erudition. The presented habilitation thesis demonstrates the necessary overview of the author and the ability of analysis and subsequent scientific synthesis of knowledge, and thus testifies not only to his professional but also to his pedagogical qualities and didactic abilities. I did not find any significant mistakes in the work, I would just recommend increasing the activity in obtaining my own scientific grants.

Dotazy oponenta k obhajobě habilitační práce

1. Prostorové rozlišení používaných metod?
   Spatial resolution of used methods?

2. EPR zobrazování a „rapid scan" EPR?
   EPR imaging and rapid scan EPR?

Závěr

Habilitační práce M.Sc. Ankushe Prasada, Ph.D. „Reactive oxygen species in the biological system: challenges and methodological advances" **splňuje** požadavky standardně kladené na habilitační práce v oboru Biofyzika.

*The habilitation thesis of M.Sc. Ankush Prasad, Ph.D., "Reactive oxygen species in the biological system: challenges and methodological advances" **meets** the standard requirements for a habilitation thesis in the field of biophysics.*

V Brně dne 19.3.2022.

[redacted]

doc. Ing. Petr Neugebauer, Ph.D.

FACULTY
OF MATHEMATICS
AND PHYSICS
Charles University

prof. RNDr. Tomáš Polívka, Ph.D.
Katedra fyziky
Přírodovědecká fakulta JU
Branišovská 1760
370 05 České Budějovice

Prague, 24th February 2022

Subject: Review of Habilitation Thesis of MSc. Ankush Prasad, Ph.D.

The habilitation thesis of MCs. Ankush Prasad, Ph.D. entitled "Reactive oxygen species in biological system: challenges and methodological advances" summarizes the candidate's research in the field of detection of reactive oxygen species (ROS) in biological systems mainly during processes of photosynthesis and oxidative burst. The studied systems vary from living cells to plant and animal tissues. The main strength of the work is in very wide range of experimental techniques including luminescence, EPR, and electrochemical ones that were used to examine production of the ROS (both radical- and non-radical- ones) which makes comparison of the particular methods possible. The understanding of the function of ROS is very crucial in biological and medical research and thus the topics of the candidate's thesis is very important for a wide range of audiences.

The thesis itself consists from an introduction which covers in four chapters ROS in organisms, overview of the detection techniques, mechanisms of oxidative radical reactions and challenges in detection and development of biosensors. Unfortunately, this part of the thesis seem rather brief to me. It missed the opportunity to identify and highlight the common features of ROS in different environments and under varying conditions. It would be perfect to find and stress out unifying elements across the individual papers – in other words a kind of thread running through the candidate's scientific work. This part of the work would also deserve better proofreading. Especially, the frequent improper use of singular and plural forms of subjects and verbs is quite distracting.

I have the following questions and remarks to the first part of the thesis:

1. page 8, paragraph 1: Are non-radical ROS always less reactive than the radical ones, even in the case of $^1O_2$?

Department of Chemical Physics and Optics
Ke Karlovu 3, 121 16 Praha 2, Czech Republic
phone: +420 951 551 234
fax: +420 951 551 249
e-mail: Roman.Dedic@mff.cuni.cz

2. page 11, the last paragraph: "...this might be an indication that $^1O_2$ might be diffusing to some distance." However, $^1O_2$ is known to have a very limited diffusion length (generally only tens of nanometers) in cellular environment (e. g. K. Plaetzer et al., Lasers Med. Sci. 24 (2009) 259 or L. Ozog and D. Aebisher Eur. J. Clin. Exp. Med. 16 (2018) 123). Couldn't it be due to diffusion of the oxidized probe? Please discuss.

3. page 12, paragraph 3 – References to Fig. 2 and Fig. 3 should probably refer to Figs 3 and 4, respectively. And again later, Fig. 3 should be referred instead of Fig. 2 (the line above the last line on page 12 and the first line on page 13).

4. I wasn't able to find which Fig. is referred on the 4th line on page 13 (250 uM PMA for 72 hours).

5. page 17, table 1 should also mention direct luminescence detection of $^1O_2$ and singlet oxygen-sensitized delayed luminescence.

6. page 20: NOX (NADPH oxidase is not in abbreviations list)

7. reference 94 has its title in uppercase

The second part of the thesis consists of a set of 13 reprints of publications in respected scientific journals. The candidate is the first author of 10 of them. It's a pity that the supporting materials which are often referred in the papers are not included in the thesis too. That makes it more complicated to the reader who has to find the materials online.

I have also questions to some of the reprinted papers:

8. Article 1, Fig. 2B: What are the additional EPR bands in the spectrum of DCMU treated sample?

9. Article 1, Fig. 4: Why is the current decreasing during the dark period in the beginning of the time scale?

10. Article 2, page 5 and Fig. 5: "one-dimensional ultra-weak" In my opinion, one-dimensional means measurement along a line or a curve. What you probably mean is zero-dimensional or point-detection. How large is the measured are with photomultiplier detection?

11. Article 3: Would be possible to compare the rate of oxidation measurements via NADPH FLIM or via phosphorescence probes for oxygen concentration? Please discuss.

12. Article 5: What is the proposed mechanism of energy transfer from $^3L=O^*$ to ^1Chl – it is a spin-forbidden process?

13. Articles 5, page 3: You present the QE of CCD VersArray 1300B as 90 % here, but only 30 % in the Articles 2, but the camera seems to be the same. What is the right value?

14. Articles 5: The microscopic images lack scales.

15. Articles 6, Fig. 3: Why are the SOD data not presented?
16. Articles 6, Fig. 4A: Why the signal grows in later times?
17. Articles 7 Figs. 4B and 5B. The direct comparison shows that the signal is approximately 10 times lower with the filter – majority of photons originates from other sources – what they come from?
18. Articles 7, Fig. 6: Why was the strip selected in this way? It seems not very representative to me. Wouldn't averaging over a (part of) y axis provide more representative results?
19. Article 7: How selective is the sodium ascorbate as a quencher of $^1O_2$?
20. Article 7: Could be the dimol emission of $^1O_2$ proven using measurements with a band-pass filter?
21. Article 8, Fib. 8 caption: "...was measured in spectral range 505–508 nm (B) and 506–640 nm (B)" – one of them should be (A), which one?
22. Article 10: Fig 1, I: The description of A, B, C (given in the caption) is lacking in the figure.
23. Articles 10: Is the 543 nm wavelength red enough not to excite the fluorescence probes (mainly DHE Ox)?
24. Article 10, Fig 1, II, G: The fluorescence seems to be localized at cell boundaries of the lower levels of cells – please discuss.

The candidate demonstrated a deep and wide knowledge of the researched issues. His contribution to knowledge in the area is significant. Despite my comments and objections, the presented habilitation thesis gives evidence of highly valuable scientific work of the candidate and meets the usual requirements for habilitation theses. Therefore I recommend it for defense.

[redacted]

doc. RNDr. Roman Dědic, Ph.D.

Review, habilitation thesis by Dr. Ankush Prasad, PhD

Upon reviewing the habilitation thesis by Dr. Ankush Prasad, in the following is my overall conclusion: ACCEPT

The central theme of the 13 publications included for the habilitation of Dr. Ankush Prasad is constituted by the investigation of ROS formation and localization within living systems, detection of ROS-mediated reactive intermediates and their use as oxidative stress marker in both plant and animal models.

The research of the candidate is strictly focused on the development of innovative techniques to precisely measure reactive compounds and defining tools with higher sensitivity and specificity respect to previous published work. Major results of the applicant include the development of *in vivo* detection system for monitoring oxidative stress in real-time, also by applying innovative approaches such as electrochemical biosensors. Overall, these publications represent a novel and original contribution to the field of ROS detection.

The development of novel system for quantifying ROS-mediated biological responses represents a significant contribution of a dedicated scientist, I therefore recommend that the habilitation thesis by Dr. Ankush Prasad is ACCEPTED.

Yours sincerely,

Prof. Roberto Bassi

Verona, March 12th 2022

Review of the habilitation thesis of Dr. Ankush Prasad, PhD, entitled

"Reactive oxygen species in the biological system: challenges and methodological advances"

by Prof. Roberto Bassi, University of Verona, Italy.

The thesis consists of a brief introduction (14 pages; 9 Figures) to the formation of ROS during metabolic reactions, their effect on biomolecules, the major ROS detection techniques, and the mechanism of oxidative radical reactions. This is followed by 13 publications authored (11/13) or co-authored by Dr. Prasad. The publications are focused on *in vivo* detection systems for monitoring oxidative stress, including technical solutions aimed at following ROS release in real-time.
The selected papers are published in international, high rank, journals: PNAS, Scientific Reports, Frontiers in Plant Science, Frontiers in Physiology, Free Radical Biology.
Each publication represents an original contribution to the field of ROS biology, which led to a better understanding of the chemistry of lipid-peroxyl and protein-peroxyl radicals formation and in particular the development of novel sensor-based ROS detection platforms.

Original contributions to the field

In paper nr. 1, the experimental work is focused on hydrogen peroxide, a ROS generated in the chloroplast by both enzymatic and non-enzymatic mechanisms. Authors developed an electrochemical biosensor, based on catalytic amperometric device, for monitoring the production of H_2O_2 in sub-cellular organelles. In particular the biosensor device was obtained by an osmium-horseradish peroxidase-coating electrode, which worked as H_2O_2 detection system. The results revealed this system succeeded in following the production of H_2O_2 in PSII membranes in continuum, under light treatment. Being an effective system for qualitative determination of H_2O_2, it can find application in several field of biological research, including photosynthesis research.
In this paper, Dr Prasad contributed to the conception and design of the work, performed the measurements, analyzed and interpreted the data and drafted the manuscript.

In paper nr. 2, the work has been focused on singlet oxygen, measured in the green alga *Chlamydomonas reinhardtii* upon applying a heat stress. By-products of lipid peroxidation were detected by either fluorescence probes and confocal laser scanning microscopy, HPLC or ultra-weak photon emission, the latter originated by transfer of excitation energy from triplet excited carbonyls to either singlet excited state of chlorophyll or singlet oxygen. Interestingly, authors demonstrated that singlet oxygen is mainly formed enzymatically by lipoxygenases, indeed suppression of their activity by either cathecol or caffeic acid limits $^1O_2$ release.
In this paper, Dr Prasad contributed to the conception and design of the work, performed measurements, interpretation of data, analysis, and drafted the manuscript.

Paper nr. 3 refers to the production of superoxide anion radical in human phagocytic cells, which is coupled to an oxygen consumption when cells are treated with a differentiation inducer. Such a respiratory burst has been monitored by scanning electrochemical microscopy imaging (SECM), applied to a human monocytic cell line. Results confirmed SECM imaging as a reliable and highly

sensitive approach for measuring oxidative stress, than more traditional techniques used for the analysis of human cells, thus might find favorable applications in biomedical research and trials.
Paper nr. 4 was also dedicated to ROS produced in the human cells which mediate phagocytosis. In particular the study introduced a chip-type biosensor device with high specificity and sensitivity, based on microelectrodes working as O_2 and H_2O_2 detection sensors. The approach succeeded in simultaneous measurement of O_2 consumption and H_2O_2 release in animal cells upon exogenous addition of chemical inducer, indicating such a chip-type biosensor is a useful tool for real-time monitoring of the respiratory activity *in vivo*.

In paper nr. 5, Dr Prasad investigated the release of ROS associated to the local and systemic defense reactions induced by wounding of leaves. In particular, by using fluorescence probe (SOSG) and detection of ultra-weak photon emission *in vivo*, authors detected the formation of singlet oxygen after wounding of *Arabidopsis* leaves. Interestingly, treatment with the inhibitor catechol and analysis of *lox2* mutant lines showed that chloroplastic lipoxygenase are responsible for $^1O_2$ formation upon wounding. These evidences provide interesting and novel insights into signals involved in the local defense reaction.

In a similar research (paper nr. 6), Dr Prasad monitored by catalytic amperometry the superoxide anion release in leaves upon wounding. Results revealed continuous generation of the ROS, for minutes after wounding, followed by a decline. Treatments with exogenous SOD verified the electric signal was specific for the superoxide anion. The results confirmed polymeric iron-porphyrin-based carbon electrode is a sensitive system for superoxide detection, suitable for application in plant research.

In paper nr. 7, authors used the porcine skin as an *ex vivo* model system to investigate the role of $HO^{\bullet}$ in the initiation of lipid peroxidation, induced by topical application of Fenton's reagent and quantified by ultra-weak photon emission. Detection at different wavelengths (visible *vs.* near-infrared region of the spectrum) allowed to distinguish contributions by either singlet oxygen or triplet carbonyls. Authors claimed both species contributed to the ultra-weak emission in skin biopsies. The method may become a valuable tool for non-invasive clinical studies e.g. on pathological state of human skin.
In this paper, Dr Prasad contributed to the conception and design of the work, analyzed and interpreted the data, and drafted the manuscript.

Paper nr. 8 optimized the use of the fluorescence probe SOSG for singlet oxygen detection *in vivo*, in a number of species (*Synechocystis* sp. PCC 6803, *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*). This analysis confirmed SOSG is an appropriate stain for imaging $^1O_2$ release, although it can suffer for different permeability of the cell wall between different species. The work supported previous results which had shown that photosensitization of SOSG in white light can be prevented by illuminating the sample with red light.
In this work, Dr Prasad designed the research plan, performed experiments, and drafted the manuscript.

Paper nr. 9 involves the U937 monocytes cell line (human histiocytic lymphoma), employed to investigate the role of hydroxyl radical in the cellular differentiation. By applying a combined approach of EPR spectroscopy and confocal laser scanning microscopy, authors detected formation of $HO^{\cdot}$ within the cells undergoing differentiation, thus proposing a contribution of ROS metabolism in the development of pathological conditions.

In this work, Dr Prasad contributed to the conceptualization of the study, the data collection, the formal analysis, validation and writing of the original draft.

Similarly to paper nr.6, paper nr. 10 focused on the production of ROS in *Arabidopsis* leaves upon wounding, and confirmed previous results, suggesting that superoxide anion radical and singlet oxygen are locally produced upon wounding.
In all these papers, Dr Prasad contributed to the conception and design of the work, analyzed and interpreted the data, and drafted the manuscript

Paper nr. 11 addressed the functional significance of the lipid-soluble antioxidants of the chloroplast, focusing on tocochromanols, isoprenoid quinones and carotenoids. In particular, the interplay between these components was studied in *Arabidopsis* wild type and mutants *vte1* (tocopherol cyclase mutant), *vte2* (homogentisate phytyl transferase mutant) and a tocopherol cyclase overexpressor VTE1oex. Analysis of single oxygen release in the different lines showed this ROS was mainly quenched by α-tocopherol which, when consumed, got functionally replaced by zeaxanthin and plastoquinone. In particular, Zea catalyzes physical quenching of $^1O_2$, plastoquinone chemical quenching. Moreover, lipid peroxyl radicals were mainly scavenged by tocopherol. The research highlighted a functional hierarchy within the antioxidant network of the chloroplast, and the existence of an interplay between the different lipid-soluble antioxidants.
In this work, Dr Prasad performed confocal microscopy and ultra-weak photon emission analysis.

Research reported in paper nr. 12 is focused on Photosystem II, where electron transport resulted in formation of ROS including superoxide anion, and oxidative damages. Authors applied high-resolution tandem mass spectrometry to PSII sample from *Arabidopsis* WT and *vte1* (a tocopherol cyclase mutant), and pinpointed oxidation of D1 at the residue E130 to hydroxyglutamic acid, by O_2-. These evidences led authors to the conclusion that tocopherol should be localized near Pheo and the non-heme iron of subunit D1. Tocopherol is likely involved in preventing excess-light-induced oxidative modification of the complex, thus protecting photosynthetic electron transport. Overall, this work is pioneering in the sense that molecular details on PSII photoprotection by scavenging ligands are provided, and opens novel research questions. In this work, Dr Prasad performed research (*in vivo* ROS imaging).

In paper nr. 13, authors investigated the ROS-dependent activation of inflammatory macrophages, and found that such event is coupled to the formation hydroxyl radical, as confirmed by EPR spectroscopy combined with confocal laser scanning microscopy.
In all these papers, the applicant contributed to the conception and design of the work, analyzed and interpreted the data, and drafted the manuscript.

Methods and interdisciplinary approaches
The list of biological samples and organisms of choice, in the papers submitted for this habilitation, is wide and includes cyanobacteria, green microalgae, land plants, human cell cultures and animal tissues. These organisms served as models to set up and test novel approaches for detecting and monitoring ROS release *in vivo*. The scientific career of the proponent shows a research interest strictly focused on optimization of methods for detecting ROS, which led to the development of original methodologies and biosensors, optimizing and expanding current knowledge. This is the major strong point of the works presented by Dr. Prasad. Weak spot: the research activity resulted

mostly in technical advancements, while did not provide groundbreaking knowledge about a specific biological mechanism/problem.

International collaborations and visibility

I did not receive information different from the thesis and therefore it is difficult for me to reconstruct the international activity of dr. Pradad, Indeed it seems that most of the work was performed in the local university within the same research group(s), which by no means reduce the importance of the work.

Conclusion

De Prasad performed a thoroughly complete analysis of Ros detection methods, used them for confirming hypothesis on ROS production both temporally and topologically. Also, he devised new methods for continuous detection of ROS. Based on his production and large range of work in the field, he is probably one on the best expert in the field worldwide. I confirm my opinion that he deserve his habilitation.

Prof. Roberto Bassi [redacted]

Verona, March 12th 2022