REVIEW

on a dissertation submitted for the degree of **Doctor of Science** in the professional speciality **4.3. Biological sciences**, scientific speciality Plant Physiology

Author of the dissertation: Prof. Dr Violeta Borisova Velikova, Institute of Plant Physiology and Genetics (IPPG), Bulgarian Academy of Sciences (BAN)

Topic of the dissertation: Physiological role of biogenic isoprene in plants

Reviewer: Prof. Dr Andon Vasilev Andonov, Agricultural University of Plovdiv, professional speciality 4.3. Biological sciences, scientific speciality Plant Physiology, defined for a member of the scientific jury with Order № 971 / 17.12.2019 of the Director of IPPG-BAS

1. Short biographical and scientific-metric data of the candidate

Violeta Velikova holds a Master's Degree in Biology from the Faculty of Biology at Sofia University “St. Cl. Ohridski” in 1986 and educational and scientific degree “Doctor” in Plant Physiology at IPPG-BAS in 1998. She has been associated with the IFFG for more than 30 years of work, during which her career development has gone through various professional positions - from specialist biologist (1988-1998), through research associate (II-I degree) (1999-2006), associate professor (2006-2012) to professor (2012).

At present Prof. Velikova is a Chairman of the Scientific Board of the IFFG and a Head of the Laboratory "Photosynthesis - Activity and Regulation". She specialized in leading research centers in Italy, Germany, the United Kingdom, Portugal and Greece with financial support from prestigious foundations and programs such as Alexander von Humboldt, Maria Curie, NATO and others. She is a member of the Union of Scientists in Bulgaria, the Federation of European Societies of Plant Biology (FESPB) and the Society for Experimental Biology (SEB).

A prerequisite for obtaining the Doctor of Science degree is that the candidate meets the minimum national requirements, the minimum requirements of BAS and, in the specific case, the specific requirements of the IFFG for the professional field 4.3. Biological Sciences. The minimum scientific-metric data for this scientific degree are 100 points of scientific papers - monographs or publications referenced in the Web of Science and Scopus databases, and 100 points of citations of the papers in them. The presented scientific-metric data of Prof. Dr. Violeta Velikova shows that she meets the minimum requirements and significantly exceeds them. In connection with her dissertation, Prof. Velikova has presented 20 scientific publications (19 in Q1 and 1 in Q2), which form 495 points. The noticed citations of these publications in the mentioned databases up to 1.11.2019 are 1159, which form 2318 points.

2. Actuality of the scientific problem discussed in the dissertation

The problem of biogenic isoprene released by plants into the atmosphere is of growing relevance in at least two aspects.

From an *environmental* point of view, its relevance is related to the role and importance of isoprene emissions for the tropospheric chemistry and the climate change. Plants release isoprene into the atmosphere, which in the presence of light, nitrogen oxides and hydroxyl radicals initiates reactions leading to the formation of a number of toxic photochemical products and increased concentration of greenhouse gases, as well as to the accumulation of secondary aerosol particles. These negative effects alter the physical and the chemical properties of the atmosphere, including air quality. This motivates the need of studies on the dependence of biogenic isoprene emissions on major environmental factors in order to more accurately prediction of the atmospheric chemistry processes related to emerging climate change.

The importance of the second, *biological* aspect of the problem, is based on the presumed physiological role of isoprene in protecting plants from various abiotic stressors. Under optimal development conditions, plants "lose" 1-2% of the carbon assimilated during photosynthesis for isoprene synthesis, but in stressful situations this rate increases many times over. As the energy cost for the synthesis of isoprene is high (20 ATP molecules and 14 NADP-H molecules are consumed for the synthesis of 1 isoprene molecule), the question about the benefits of plants from this secondary metabolite is logical, as well as the mechanisms by which it is realized. Prof. Velikova's dissertation provides to large degree answers to these questions by providing a considerable amount of original scientific data and their in-depth discussion.

3. Analytical characteristics of the dissertation thesis

1. Volume and structure of the dissertation

The dissertation has a total volume of 422 pages and includes the following sections: Introduction (2 pages), Literature review (18 pages), Aim, hypotheses and tasks (2 pages), Approaches and research methods (8 pages), Results and discussion (94 pages), Conclusion (2 pages), Achievements (4 pages), References (20 pages) and Applications (272 pages). An exception to the traditional structure of the dissertation is the Applications section only. It presents the 20 scientific publications of the candidate on the topic of dissertation thesis. The dissertation is written in high scientific style. The text is tight, clear and facilitated by an appropriate list of abbreviations. A total of 379 references are cited.

2. Literature review

The literature review provides information about the environmental and the biological aspects of the problem under consideration. It is analytical, critical and sufficiently informative. The main questions are focused on: (●) Isoprene emissions from plants; (●) The role of isoprene in the atmospheric chemistry; (●) Effect of abiotic environmental factors on

the isoprene emissions and (●) Antioxidant and thermoprotective effects of the isoprene in plants. An interesting detail is the inclusion in the literature review of 8 scientific publications of the candidate, which are related to the dissertation. I think that the literature review correctly reflects the state of the art of the problem of biogenic isoprene.

The candidate concludes that the physiological role of isoprene in plants is related to its possible protective functions under abiotic stress conditions. At the same time, the known facts that not all plants release isoprene, and that its effect is seasonal, age- and temperature-dependent, show that the problem is not fully understood. The candidate considers that the putative mechanisms of the isoprene action in plants are only partially experimentally confirmed. This motivates carrying out next research with the following purpose and tasks.

3. Aim, tasks and hypotheses

The main purpose of the study is "to clarify the physiological role of biogenic isoprene as a plant protection means against abiotic stress".

The purpose is based on the assumptions and facts stated in the literature review about the protective action of the isoprene in plants, but during the design of the studies, they are summarized, further developed and formulated in the form of the following working hypotheses:

(●) Isoprene is essential to the tolerance of plants to a wide range of different abiotic stressors, which is manifested at different functional levels in the plant organism.

(●) Endogenous isoprene affects the accumulation of reactive nitrogen and oxygen products in cells and thus determines plant response to stress.

(●) Biogenic isoprene as part of the plant's antioxidant system works in synchron with other protective metabolites, providing better protection for plants under stress.

To achieve this aim, 4 logically related tasks are formulated.

(●) The first task aims to provide direct experimental evidence of the possible protective effects of the isoprene in plants in response to various abiotic factors (ozone, singlet oxygen, high temperature, high concentration of CO_2 in ambient air, drought, and anthropogenic pollution).

(●) The second task is aimed to elucidate the interactions between the endogenous isoprene and the signaling molecule nitric oxide (NO) in the implementation of plant response to oxidative stress.

(●) The third task seeks indirect evidence of the protective role of isoprene in plants by examining the effects of the isoprene emission inhibition on the chloroplast proteome, lipid and fatty acid composition of the photosynthetic membranes, and chloroplast ultrastructure.

(●) The fourth task clarifies the relationship between isoprenoids and phenylpropanoids in the plant protection system under optimal and stressful conditions.

4. Approach and research methods

In his research on the problem the candidate has used a wide range of *in vivo* and *in vitro* experimental designs and research methods, which are selected according to the

specifics of the tasks. I consider that the chosen methodological approach to combine different experimental designs as correct, because it allows, on the one hand, to make a complex characteristic of the studied problem and, on the other hand, to avoid the limitations that each experimental formulation brings.

Studies include experiments with plant species with different ability to release isoprene [e.g., strong emitter cane (*Phragmites australis*) and non-releasable isoprene Japanese grass Hakonechloa macra], as well as experiments with species with modified isoprene synthesis capacity as a result of gene manipulation - Arabidopsis thaliana, tobacco (*Nicotiana tabacum*, *cv.* Samsun) and gray poplar (*Populus x canescens*). In some experiments, leaves with manipulated isoprene emission by treatment with fosmidomycin were used, as well as leaves developed in an atmosphere with increased concentration of CO_2, leaves at different stages of their ontogenetic development, as well as leaves from plants with different age.

The researches have been carried out with the help of modern physiological, biochemical, proteomic and other research methods and highly sensitive scientific equipment, which undoubtedly increase the objectivity of the obtained results. Among these I would note photosynthetic gas exchange, chlorophyll fluorescence, biophysical indicators of thermostability of the thylakoid membranes (circular dichroism, electrochromic absorbance changes at 515 nm, thermoluminescence), antioxidant enzymes activity and antiradical activity, immunoenzymatic determination of abscisic acid, chromatographic determination of volatile isoprenoids emissions, individual carotenoids and phenylpropanoids, lipid profile of photosynthetic membranes, chemiluminescent determination NO emission into the leaves, the use of new techniques for the quantitative and qualitative analysis of plant proteins, the examination of chloroplast ultrastructure by transmission electron microscopy, and others.

The results obtained were processed by validated methods for statistical analysis. The reliability of the differences in the studied parameters between the different variants was proved mainly by ANOVA followed by Tukey or Student test. In some studies, the results were subjected to multivariate analysis with statistical methods known as Principal Component Analysis and Orthogonal Partial Least Squares.

5. Results and discussion

The section is differentiated into 7 sub-sections that finish with conclusions. Data are presented through 67 individual and composite figures and 1 table. Selected basic data are presented and discussed in the section, and other results and related information are given in the attached scientific publications. I consider the used approach to present the data as more appropriate for such type of dissertation. At the same time, it should be noted that it complies with the requirements set out in Article 3.2. of the Rules on the Specific Terms and Conditions for Acquisition of Academic Degrees and Occupation of Academic Positions at the IFFG.

As a result of the studies conducted, a considerable amount of new scientific information on the physiological role of isoprene in plants has been obtained. It has been established at various functional and structural levels in model plant objects and, in an integrated form, it provides conclusive evidence for the protective function of the isoprene in plants exposed to abiotic stress. The main results that support the hypotheses are the following:

(●) The endogenous isoprene has a proven ability to increase the plant tolerance to oxidative stress caused by ozone, singlet oxygen, high temperature, drought and anthropogenic heavy metal Ni contamination.

(●) The protective role of the isoprene is largely due to the ability of this molecule to influence the organization of thylakoid membranes and to reduce the formation of reactive oxygen and nitrogen forms.

(●) The isoprene improves the thermal stability of thylakoid membranes.

(●) The isoprene plays an additional "antioxidant" role, along with other volatile and non-volatile isoprenoids and phenylpropanoids, providing better protection to plants under stressful conditions, etc.

The obtaining these enviable volume and quality results would not be possible without comfortable research funding. Prof. Velikova has funded her research through a number of competitively won research projects funded by the Bulgarian Science Research Fund (TCB-1604 / 2007-2010; B02-8 / 2014-2017), Ministry of Education and Science (D01-168 / 2014-2016), bilateral projects with Italy (2004-2006; 2007-2009; 2010-2012) and Hungary (2010-2012).

4. Achievements of the dissertation thesis

As a result of the complex research, results have been obtained, most of which are innovative and can be considered as scientific achievements. It is accepted that the achievements may be differentiated into several groups, namely as new scientific facts, methodical and applied achievements. In this dissertation, 15 achievements have been formulated, which I fully accept. The main part of them belongs to the group "new scientific facts", in particular, to the subgroups: (●) Formulation and justification of new hypotheses and (●) Proving with new means significant new sides of already existing hypotheses. The most important achievements I would like to emphasize are the following.

- ✓ For the first time, it is demonstrated that endogenous isoprene has an important protective role in *Phragmites australis* (cane), exposed to ozone stress, quenching the accumulation of hydrogen peroxide in the leaves and reduces lipid peroxidation of cellular membranes.
- ✓ It is established that the isoprene has protective role against singlet oxygen. The quenching of singlet oxygen by the isoprene is explained by the availability of conjugated double bond structure in its molecule which facilitate energy transfer and heat dissipation

- ✓ For the first time it is presented experimental data directly supporting the hypothesis for the membrane stabilizing role of the isoprene. By the using different biophysical techniques it is demonstrated, that the isoprene improves the intactness of thylakoid membranes and their functionality at high temperature stress.

- ✓ For the first time, by the use of new approach for quantitative and qualitative proteomics based on stable isotope-coded protein labeling it is demonstrated that the suppression of isoprene synthesis and emission remodels the chloroplast proteome affecting the structural organization of photosynthetical membranes and decreases plant tolerance to oxidative stress.
- ✓ For the first time, isoprene has been shown to limit the accumulation of nitric oxide (NO) in the leaves of *Phragmites australis* that experience ozone stress. NO diffusely colonize the mesophyll cells of ozonized isoprene inhibited leaves. NO was not detected in ozonized isoprene emitting leaves. It is suggested that isoprene may be an effective mechanism to control active oxygen and nitrogen forms under stress conditions.

5. Critical remarks and questions

I have no critical notes on my dissertation.

6. Evaluation of the dissertation summary

The summary presented objectively reflects the structure and content of the dissertation.

7. Evaluation of the scientific publications summarized the dissertation

The candidate has presented 20 scientific publications related to the dissertation. He is the first author in 13 publications and in 7 of them is a corresponding author. He is the corresponding author also of the publication №20, in which he is not the first author. These facts clearly show her leading role in the carrying out of the scientific experiments and the writing of publications as well, which has been declared in some of them (№17). As already noted, all publications are in high impact factor journals (19 in Q1 and 1 in Q2). The total impact factor of the publications is 86.85.

CONCLUSION

The dissertation of Prof. Dr. Violeta Velikova shows that she possesses in-depth knowledge and professional skills in the field of Plant Physiology, which allow her to conduct research on important for the society and the science problems, such as the problem of the physiological role of biogenic isoprene in plants. It contains significant scientific results, representing an original contribution to the science and meeting all the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the

Rules for the application of the LDASRB and the Rules for the specific conditions and the procedures for acquiring scientific degrees and for employment of academic positions at the IFFG. The results of the dissertation have received international recognition and high response among the professional community, as they have been published in highly rated scientific journals and have been repeatedly cited.

The aforementioned gives me an argument as a reviewer to evaluate positively the dissertation of Prof. Dr. Violeta Velikova as well as to recommend to the distinguished members of the Scientific Jury to award her the degree of Doctor of Science in the professional direction 4.3. Biological sciences and the scientific specialty Plant Physiology.

10.03.2020 Reviewer:

/Prof. Dr Andon Vasilev Andonov/