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**Doctoral study – 1702 V 005 Biophysics - PhD. Thesis opinion request**

**Report on the thesis presented by Mgr. Anamika Mishra on the subject "Species Discrimination and Monitoring of Abiotic Stress Tolerance by Chlorophyll Fluorescence Transients"**

Already since a number of years, the technique of chlorophyll fluorescence has become ubiquitous in plant physiology and ecophysiology studies. This method became an important, standard method to investigate the photosynthetic performance of plants that is widely used not only in studies that just focus photosynthesis but also in many other studies by classic and molecular approaches in plant sciences. This trend has been fuelled to a large degree by the introduction of a number of highly user-friendly and portable chlorophyll fluorometers. The drawback in the past to using chlorophyll fluorescence with small sampling areas has been overcome by the development of chlorophyll fluorescence imaging systems that allowed the application of the technique for the screening of many plants simultaneously. The PhD work by Mrs. Mishra addresses three applications of the technique of chlorophyll fluorescence imaging, the species discrimination and the monitoring of two types of abiotic stress tolerance. Thus, Mrs. Mishra addresses timely topics that are not only relevant for basic research but also for practical applications in agriculture.

The cumulative manuscript is presented with two introductory chapters on plant photosynthesis and chlorophyll fluorescence and on advanced statistical tools, followed by a short material and methods chapter. The results are presented in the form of three articles either published or in press.

The introduction on photosynthesis and chlorophyll fluorescence is sufficiently comprehensive although it would have been preferable that the figures, and apparently also parts of the information in the text, would have been taken from reliable, resources such as established peer reviewed reviews or original publications and established text books rather than not filtered and reviewed internet resources such as Wikipedia or classroom materials. The introduction to the advanced statistical tools is very detailed and elaborate although the referencing to the recent and current literature is very limited.

The method section is very short and limited and essentially refers to the condenses method sections of the included publication. A more specific description that would allow direct repetition would have been desirable.

The first part of the combined results and discussion section addresses a very interesting application of chlorophyll fluorescence imaging, the discrimination of closely related species of the family Lamiaceae. In particular the analyses of mixed populations demonstrate the power and practical feasibility of the method for this application.

Chlorophyll fluorescence emission could be verified as appropriate reporter tool to screen for freezing tolerance. The analyses of various ecotype accessions of the model plant species Arabidopsis showed that the florescence data correlated nicely with classical measurements of electrolyte leakage.

Finally, transgenic plants engineered for increased drought tolerance were included in a simulated high-throughput screening procedure. Specific chlorophyll fluorescence parameters were shown to yield a good contrast between wild type and transgenic plants and thus demonstrate that they are suitable to identify elevated drought tolerance in large screening populations.

Since the experimental data are presented as original publication with several co-authors it would have been preferable to know the exact type and extend of the contribution of Mrs. Mishina to the individual studies. In the compilation on page iv in Czech language percentage numbers are given behind the references. I do conclude that they represent the contribution of Mrs. Mishina. If this assumption is correct they demonstrate that she contributed very substantial parts to papers 4.1 and 4.2 and a decent part to paper 4.3.

For a cumulative work I would have found it useful that a general result and discussion chapter would have been included to link the three individual and separate case studies. In such a chapter a general rational for the PhD work could have been elaborated and a generalized discussion under consideration of all results included to give the interpretations and the whole PhD work a broader, general perspective. This would have provided the opportunity to update the references of the published papers.

In conclusion, Mrs. Mishina mastered the concepts of chlorophyll fluorescence imaging and advanced statistical analyses with three case studies. The work has introduced new knowledge both on the technique and on the addressed biological questions.

Last but not least I would like to pose following questions to the candidate for further explanations:

1. What is the specific advancement with respect to image and statistical analyses compared to previously published similar studies and analyses within in the application of chlorophyll fluorescence imaging in the field of biotic plant stress response (e.g. in direct comparison with following papers that have been cited in the thesis: Matous et al. (2006) and Berger et al. (2007))?
2. For possible large scale screening purposes and practical field application in the field the advantages and disadvantages of chlorophyll fluorescence imaging versus classical spot measurements with fibre optics should be discussed.
3. Since the cited literature does not seem to be up to date and fully comprehensive, the findings should be discussed also with respect to the very recent literature within the field of study.

In summary the presented thesis demonstrates the qualification of Mgr. Anamika Mishra for independent creative work. I do recommend to accept her thesis as PhD work.

Graz, den 10.02.2012

Thomas Roitsch

Prof. Dr. Thomas Roitsch

Referee's opinion on PhD thesis of Anamika Mishra

entitled 'Species Discrimination and Monitoring of Abiotic Stress Tolerance by Chlorophyll Fluorescence Transients'

The thesis is non-conventional from viewpoint of classical plant physiology. Most of the thesis in plant physiology I had possibility to read repeat/refine the experiments already done by someone else; they do so usually by conventional techniques and under modified experimental conditions or plant species. This thesis is different, more innovative and ambitious in its approach. It is trying to find a new species specific signal emitted by a plant (plant's fluorescence image) or a unique image emitted at impact of environmental stress. From plethora of chlorophyll fluorescence transient parameters and their combinations, the author aimed to select those yielding maximum difference at the impact of stress or when compared to closely related species. Of course the selection process was not random but employed mathematical statistics, methods based on pattern recognition theory. This part is certainly innovative and, in my opinion, most valuable (unfortunately also far from my comprehensive understanding since I am not an expert in it). On the other hand, I think that the concept has similar weakness as, for example, the numerical- compared to analytical solution in engineering or mathematics: it has to be tailored for each single case (each kind of stress, plant species, a phase of ontogeny) due to missing physiological interpretation of the selected combination of fluorescence parameters or phase of fluorescence transition. This is also my first question to the Candidate Anamika Mishra. Is the technique really case-specific? Do you have to find the most contrasting image for each case without any knowledge when (in which phase of the transition) and why it occurs?

The thesis is suitably sub-sectioned into Introductions to Photosynthesis and to Statistical tools, Material and methods and Results and Discussions, Conclusions and Bibliography. The chapter 'Results and Discussion' consists from three published papers where the Candidate is the first author or co-author.

Notwithstanding my questions and minor comments below, I evaluate the text part of the thesis as very good. The science presented in the three studies is solid and proves ability of the Candidate to produce independent and high quality science. If the Candidate will be able to deal with the points below to the exam panel's satisfaction during the Defence of the Thesis, I recommend it be accepted for the award of a doctoral degree.

České Budějovice, 14.1.2012

Jiří Šantrůček

*Questions:*

You have mentioned in Chapter's overview and in the paper presented in section 4.3 that the transgenic tomato plants showed reduced stomatal density and pore size. As far as I know, this is rather unusual response in plants which use water more effectively than others (see the hyperbolic relationship between stomatal pore size and density among wide range of species e.g. in Fraks and Beerlinger, PNAS 106, 10343-10347, 2009). You have found 'good contrast' in several fluorescence parameters between wild and the drought tolerant transgenic tomato. Taking in mind the non-typical stomatal response, I wonder whether the fluorescence drought-response you found is typical also for another plant species or cultivars? Is your opinion based on published literature or your own experimental data?

A comment and question to the first (4.1) paper: It is amazing for me that you were able, by using fluorescence technique, to discriminate among the three related *Lamiaceae* species. My understanding of the process is very limited and thus my questions can be naive.

First: I would like to know whether the fluorescence parameter you found and used to distinguish among the species can be expressed in more explicit way (e.g. by arithmetic combination of commonly used parameters no matter how complicated the combination would have been).

Second: Can you speculate what is behind of the species-specific chl. fluorescence signal (which genetically-controlled process or structure affecting chl. fluorescence)?

Third: Is it necessary to go through the process of classifiers testing and feature selection for each combination of new species before using the fluorescence-based species recognition? Is it possible to automate the process by software packages similar to Pattern Recognition Toolbox which you mention you used?

It seems to me that the major sources of information were the www internet pages, for example majority from the introduction figures were taken from internet. In my opinion, internet pages are good source for general overview. However, mainly due to their evanescence and since they are not reviewed, they are not good enough for citations in scientific works.

*Minor comments and corrections:*

Missing "Pheo" and "$Y_Z$" in list of abbreviations.

P8: First group of fluorophores is mentioned with excitation and emission maxima but no chemical substance is specified. Can you give an example?P15: $F_0' > F_0$ (see Fig. 7)

P16 bottom: Should not be "*I is the total number ....*"?; similarly P17 top: ... $f_i(t_2)$ ....

P20, Eq.7: what the *T* in exponent stands for?

P33, Fig.16: the figure is properly located but not addressed in text

Typing errors: P3: carotenoids;

REVIEWERS' COMMENTS TO THE THESIS

**Title**: Species Discrimination and Monitoring of Abiotic Stress Tolerance by Chlorophyll Fluorescence Transients

**Author**: Anamika Mishra

The PhD Thesis „Species Discrimination and Monitoring of Abiotic Stress Tolerance by Chlorophyll Fluorescence Transients" written by Anamika Mishra is dedicated to the application of conventional chlorophyll fluorescence parameters as well as advanced statistical techniques of classifiers and feature selection methods (1) in discriminating three species of the same family *Lamiaceae*, (2) measuring cold tolerance in *Arabidopsis thaliana* plants, and (3) measuring drought tolerance in tomato plants. I found the topic of this thesis as actual and important with a high application potential in plant breeding, precise agriculture etc.

In general, the thesis consists of two major parts: literature overview and the presentation of three papers that have been previously published in the impacted journals (Journal of Fluorescence – IF=1.966; Plant Science – IF=2.481) and in the peer-reviewed journal (Plant Signalling and Behaviour). A. Mishra is the first author in two of them. Unfortunately, the hypotheses of the thesis or the hypotheses (and their presumptions) of the individual experiments are not explicitly presented in the thesis.

The literature overview is represented by two chapters, i.e. "Introduction to photosynthesis" (Chapter 1) and "Introduction to statistical analysis" (Chapter 2). These two chapters are written in a very general style typical rather for the school textbook than for the scientific literature overview. In my opinion it would be much more valuable to present, for example, the overview of the application of chlorophyll fluorescence techniques (traditional integrating imaging techniques) in the investigation of species discrimination and cold/ drought effects in plants. The introductions to papers presented in the chapter 4, according to journals requirements, are quite limited.

In addition, I have several critical comments / questions to the chapter 1:

- separation of the photosynthetic machinery to "light reactions" and "dark reactions" is not relevant. The "dark reactions" of carbon assimilation are also light-dependent since the change in pH in the chloroplast stroma, induced by electron transport, leads to the activation of several enzymes, e.g. Rubisco activase enzyme, involved in the carboxylation process. Therefore, the nomenclature "primary and secondary processes of photosynthesis" seems to be more relevant. What is your opinion?

- Chapter 1.4 (page 9): Is it possible to specify the fluorophores absorbing in the UV spectral range? The molecule of Chl *a* is mentioned as the second main fluorophore in plants. Why the Chl *b* is not contributing to the plant fluorescence emission? Determination of the fluorescence in intact leaves/plants is usually influenced by the phenomenon call as "re-absorption". Please, can you have a comment to this phenomenon?

The experimental part of the PhD thesis is based on three articles that have been already published in the reputable journals (see above) and thus reviewed by the relevant specialists. Therefore, I would like to ask only the following questions that should lead to the clarification of some parts of the text.

1. The quenching protocol has been applied on a "dark-adapted plant". How long the plants have been exactly dark-adapted? For example, it is known that there is a significant decrease in Rubisco activity after ca. 45 minutes of darkness, (e.g. earlier papers by RW Pearcy). May Rubisco deactivation influence the fluorescence quenching? Or may the time of dark-adaptation influence the chlorophyll fluorescence quenching?
2. In addition, may stomata influence the quenching of the fluorescence emission? For example, it is known that shade leaves have very low stomatal conductance during night periods that results in high intercellular CO2 concentrations compared to sun leaves. Therefore, shade leaves maintain higher portion of Rubisco in carbamylated active form enabling thus a faster photosynthetic induction after transient from low to high light conditions. What do you think about this mechanism? Are there any studies investigating the effect of stomatal conductance on fluorescence emission?
3. Under the quenching protocol the actinic lights of two different intensities has been used (50 and 200 umol m-2 s-1). Is the use of these actinic light intensities universal? Or should they be changed or modified according to the average growth light conditions?
4. Under the conditions of the non-ventilated laboratory, the CO2 concentration may significantly increase as compared to open-air conditions. Do you have an idea how the CO2 concentration can influence the fluorescence emissions and the fluorescence quenching?

5. What was the role of a leaf anatomy in the experiment number 1 (discriminating three different species of the same family *Lamiaceae*)?

The following questions should lead to the general discussion of the application of the fluorescence technique:

6. Under nature conditions, there is usually the multiple-complex of stressors that influence plant photosynthesis (fluoresce emission) and growth (e.g. cold x low nutrition uptake by roots, drought x high temperature stress etc.). Is it possible to distinguish the role of a single stressors by the fluoresce technique?
7. Based on Table 1, page 48, what is your opinion about the application potential of fluorescence in the discrimination of plant species in precise agriculture (discrimination between crops and weeds, discrimination between healthy leaves and leaves attacked by pathogen etc.)?

Finally, I would like to emphasize that the quality of the presented thesis shows that MSc. Anamika Mishra is able to provide independent scientific work including publication in the scientific journals.

In Brno, 12-1-2012

Mgr. Otmar Urban, Ph.D.