Evaluation of the doctoral thesis of MSc. Lena Hunt 'Physiological, structural, and biochemical leaf traits of selected Poaceae species involved in oxidative stress protection and acclimation to different light conditions'

Reviewer: Jiří Šantrůček, University of South Bohemia, Faculty of Science

Author of the dissertation, Lena Hunt, aimed to expand knowledge on the role of phenolic compounds in oxidative stress protection mainly in barley. Other three species of family Poaceae, other leaf traits and two environmental factors were also included. The dissertation consists of 22 pages of Introduction, Aims specification and Scientific Background, 11 pages summarizing substantial information on four published papers, three of which with Lena as the first author and one as co-author, 15 pages of Discussion, list of references on 16 pages and all four original papers in Supplement.

The feeling of heterogeneity in topics and rather opportunistic gluing of various subjects, which I had during reading of Introduction, disappeared when going through the chapter Scientific Background. This section deals with oxidative stress and ROS, phenolic compounds and their role specifically in Poaceae, irradiance effects on plant leaves, leaf functional traits, and evolution and ecological significance of Poaceae. The topics, thought apparently heterogeneous, are interlinked here and presented in a friendly-to-read and inspiring way indicating broad range of Lena's interest and knowledge. In my opinion, Lena Hunt submitted high quality thesis. In have only minor suggestions or comments, which are listed in the end of this review together with several questions, which could stimulate discussion.

The first presented paper, published in the journal Antioxidants (2021) by Lena Hunt as first author, is methodological in its significant part, and deals with histochemical detection of deposition of phenolic substances and determination of their chemical identity in leaf tissues of barley. Fluorescence imaging and MS detection were applied. The effect of different irradiance and atmospheric CO_2 concentration was investigated in two cultivars of barley with different sensitivity to oxidative stress.

The second paper published in Plants (2021) and concerning responsiveness of stomata to CO_2 and light, brings the main novelty in fast counting of stomata on micrographs using artificial intelligence. This is an important step forward in my opinion since counting of stomata, tedious and time consuming if doing manually, is a bottleneck of many studies. The developed method was used to count stomatal density on leaves of both barley cultivars grown under varying growth light and ambient CO_2 concentrations. Leaf gas exchange, ABA and saccharides level were also determined.

The third included paper was published in International Journal of Molecular Sciences (2022) by Radomír Pech as the first author. The study focus on the effect of growth-light wavelength on abundance and chemical composition of phenolic compounds in barley. Blue light was found to be the most effective in synthesis of antioxidant phenols and expression of genes coding antioxidant enzymes.

The fourth paper published in Plants (2023) is an ecophysiology and population dynamic study attempting to find leaf or plant traits responsible for the shifts in abundance of four grass species in a relict arctic tundra grassland in Krkonoše mountains. Changes in vegetation composition, retreat of *Nardus stricta*, were detected by several-year-long remote-sensing observation. Various leaf traits and phenology of species were studied. Presumably, the absence of grazing/mowing played important role together with species-specific phenolics profile.

Lena Hunt opens in her dissertation so far rather overlooked field of phenolic compounds in grass leaves. The ability of plants to synthetize phenolic compounds is regarded as one of the key features in plant terrestrialisation. The sessile character of dry-land plants, fluctuation of light environment and chemical nature of phenolics predetermined their antioxidative role and coupled their synthesis with ABA and other stress hormone signalling. Rich spectrum of phenolics, their species-specificity, organ specificity, dynamics during plant development and environmental modifications make this field ripe with questions and new discoveries prone. Specification of the role of different phenolic compounds in oxidative stress in barley is, in my opinion, the most important scientific outcome of Lena's thesis. I hope she will continue with this topic.

The role of phenolics is the most important but, certainly, not the only outcome. Another important contribution to current knowledge concerns the methodological progress in computer-aided stomata counting. There were several attempts to automatize stomata counting in last decades (including my own unsuccessful attempt) but only those from the last years using artificial intelligence seem to solve the difficult task. Lena with coworkers presented one of the attempt and I really appreciate their effort.

In summary, Lena Hunt has shown in her thesis that she has extensive knowledge of plant physiology ecology and biochemistry of secondary compounds, that she is able to combine them creatively and that she has the practical skills necessary for scientific work. Although I do not know the exact criteria of Charles University for awarding a doctoral thesis with the Cum Laude designation, in the case of Lena Hunt's thesis I would vote for it. **I therefore recommend her thesis for defence. After a successful defence, I recommend that Lena Hunt be awarded the degree of Doctor of Philosophy, Ph.D.**

Comments, Suggestions, Questions

I really enjoyed and highly evaluate the graphic arrangement of the thesis; Lena's own pen-drawings of investigated grass species and high quality of graphical abstracts. Nevertheless, In Figs. 3 and 5 Lena may have forgotten to cite the source unless she is the author.

When reading the sections 3.1 and 3.2 about "the double edged sword" of ROS, the following logical mismatch appears. Various kinds of stress result in ROS production. ROS facilitate crucial plant processes via cell signalling. Based on these two statements, we could say that stress facilitates the plant well-being. Or is the stress-induced ROS different from the signalling ROS? Or does plant need to keep a particular level of ROS (ROS homeostasis) to be happy? These questions are partly answered in the Discussion but still, can you comment?

In section 3.6 the author specifies very nicely what is meant by the term "leaf functional traits". Is it possible to define this term also by its negation? In other words, could you specify leaf traits that are non-functional? In my opinion, there are no such traits. And if there are, it is because we do not know yet their function.

What is behind the increase in phenolic compound under elevated ambient CO_2 concentrations? Is it just a 'drain' for excess carbon? Isn't it simply connected with higher rates of net photosynthesis (and, therefore, higher O_2 evolution and oxidative damage risk)? The same might be true with increasing light. Can you assess what fraction of leaf carbon is deposited in phenolics, the turnover of phenolics and compare it with carbon assimilation rate? In other way, can phenolic compounds in the leaf serve as significant sink for carbon?

Comment to Fig. 2, bottom part of the page 12 and Fig.3 of your paper Hunt et al. 2021: I always thought that phenolic compounds have blue autofluorescence when excited by UV light (360 nm) and it turns to green in alkaline media. It seems from Fig. 2 and its caption that I am wrong. What is responsible for the blue autofluorescence of cell walls mentioned in this caption? Is't it emitted by phenolic compounds embedded in cuticle and cuticular wax rather than by cell walls? Did you try to wash out the wax with chloroform and image autofluorescence after such treatment? I expect that the blue fluorescence of epidermal cells would mostly disappear.

I was rather confused by information concerning the temperature effect on accumulation of phenolic compounds (P. 39). It seems from the text that the phenolic accumulation and ROS production is higher under higher temperature. Is it true? I would expect, that the danger of oxidative damage of green leaves is highest under high light and low temperature, when the primary photosynthetic processes are running but CO_2 fixation is slow, so the electron transport chain can be overreduced and the production of antioxidants should be highest. If so, why is then sinapic acid with high ROS scavenging potential more beneficial at high temperature?

Why did you choose barley?

Minor comments:

P.10: "Upon excitation, the electron in the reaction center of chlorophyll moves to…". Where is the reaction center of chlorophyll? Better: the electron of chlophyll in the reaction center of photosystem moves to…

P.17, 4th line: hydroxybenzoic acid should be here instead of hydroxycinnamic acids

P.22, line: 12 Perhaps author could be interested in the proposed link between the origin of agriculture and the rise in atmospheric CO_2 concentration from 180 to 270 ppm between 15-12 thousand years ago (Sage, Global Change Biology, 1995).

P.33, line 13: 'Barke, the oxidative stress tolerant genotype,…' should be changed to 'Barke, the oxidative stress sensitive genotype,…'

In České Budějovice, August 21, 2023 Jiří Šantrůček