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Re: Review of the doctoral dissertation submitted by PREETI SHAKYA

Proteomic approach to understand the mechanisms underlying interactions between Hypericum perforatum L. and Agrobacterium tumefaciens

The topic of the dissertation is devoted to the study of the causes of recalcitrance of the medicinal plant *Hypericum perforatum* L. to *Agrobacterium tumefaciens*, whose Ti plasmid-based constructs are often used in the plant kingdom for genetic transformation. Clarifying the causes of recalcitrance and their eventual overcoming is therefore of great importance.

The doctoral thesis is composed of three published papers, two of them are review articles published in 2016 and 2019 and one is original research paper published in 2025. The submitted set of papers is supplemented by a commentary, which essentially only concerns the original paper (publication 3). The review (publication 1) represents literature review of the topic and may be considered as a theoretical background for experimental work. However, the review (publication 2) summarizes different elicitation approaches as tools for enhancement of secondary metabolism in *H. perforatum*. *Agrobacterium tumefaciens* is mentioned only by stating that an inactivated culture of *A. tumefaciens* affected xanthone metabolism in *H. perforatum*. My statement is also supported by the fact that the hypothesis formulated in the commentary as "Involvement of plant proteins during *Agrobacterium*-mediated transformation could give clue of recalcitrant mechanism" and also the specific objectives – “investigation of the behavior of *A. tumefaciens* during co-culture with *H. perforatum* cells, investigation of proteome response and assessment of physiological changes” relate exclusively to publication 3. It seems to be in contrast with the statement of the doctoral candidate that she “*presented the summary, introduction,*

materials and methods, results, discussion and conclusion from the conducted research for all the articles".

Based on the supervisors´ statement that the conditions for submission in the procedure for awarding the degree of Doctor of Philosophy are met and given the doctoral candidate statement that *"the form of the doctoral dissertation was prepared in accordance with the guidelines contained in the regulations of extramural mode procedure for performing activities in doctoral proceedings, habilitations, and awarding the title of professor at the Institute of Plant Genetics- Polish Academy of Sciences"* I assume this is a standard, acceptable procedure.

The submitted publications were subjected to a standard peer review before publishing, so I will focus on evaluating the results and raise a few questions for discussion.

The dissertation presents the results of a study of the physiological responses of *in vitro* cultured *H. perforatum* cells with *A. tumefaciens* suspension and of seedlings cultured in greenhouse conditions sprayed with *A. tumefaciens* suspension. The methods used in the experiments were appropriate. Physiological changes were studied in the short term, up to 24 hours after application. These changes are expressed by differentially expressed proteins (DEPs) in the case of cell cultures and changes in some physiological parameters in the case of seedlings.
From a methodological point of view, the interval of 24 hours after the treatment is too short and the reliability of the results would be supported by repeated analyses over a longer time period. For this reason, data-supported conclusions are limited.
The high mortality of bacterial cells during co-cultivation with *H. perforatum* cells suggests for the use of alternative methods of genetic transformation.

I kindly ask doctoral candidate to comment on the following questions:

1. It is natural for plant cells to respond to the presence of microorganisms by triggering defense reactions. In your opinion, what makes the responses found against *A. tumefaciens* specific compared to other microorganisms?

2. The number of up-regulated and down-regulated DEPs after 12 hr co-culture was 7 to 10 times higher than after 24 hr co-culture. How do you explain this fact? Do you expect this trend to continue after longer co-culture?

3. In the *in vitro* and *in vivo* experiments, you only observed the consequences of the "surface" interaction between *H. perforatum* and *A. tumefaciens* cells, i.e. you did not study the changes that occur after infection of injured tissues or cells with a disrupted cell wall, since this is precisely the condition for the entry and integration of T-DNA during genetic transformation. How can your results be extrapolated to the process of genetic transformation?

4. After applying *A. tumefaciens* suspension to young seedlings, you found changes in some physiological parameters. Why did you not analyze these parameters even after the plants reached maturity? Are the changes you recorded simply due to oscillations in physiological parameters caused by different genotypes in standard cultivation conditions?

Conclusion: The dissertation brings several original results that will require verification and deeper study. Based on internal rules, it meets the criteria for the award of the degree of Doctor of Philosophy (PhD.). **I recommend the thesis for awarding the academic title "Doctor of Philosophy".**

prof. RNDr. Eva Čellárová, DrSc.