



REVIEW

on dissertation for obtaining the educational and scientific degree "Doctor" in:
field of higher education: 6. Agricultural sciences and veterinary medicine,
professional field: 6.1. Crop production,
scientific specialty: Selection and seed production of cultivated plants

Author of the dissertation: Pervin Shengyun Halkoglu-Hristova - full-time doctoral student at the Department of Genetics and Selection at the Agricultural University, Plovdiv.

Topic of the dissertation: "*In vitro* cultures of *Fabiana imbricata* Ruiz. et Pav. as technological matrices for obtaining biologically active substances".

Supervisors: Associate Professor Dr. Svetla Yancheva. Corresponding Member Prof. D.T.S. Atanas Pavlov

Reviewer: Prof. Dr. Nasya Borisova Tomlekova.

Breeding Department, at the Maritsa Vegetable Crops Research Institute, Plovdiv.
field of higher education - 6. Agricultural sciences and veterinary medicine, professional field 6.1. Plant growing,
scientific specialty - Breeding and seed production of cultivated plants,
appointed a member of the scientific jury by order № RD-16-744./29.06.2021 by the Rector of the Agricultural University.

1. Brief introduction of the candidate.

Pervin Shengyun Halkoglu graduated from 2010 to 2015 with a bachelor degree in Agronomy - Plant Biotechnologist and a Master degree in Agronomy - Plant Protection at the Agricultural University in Plovdiv. In 2016, immediately after graduating from the university, she was enrolled in a full-time doctoral program in Plant Breeding (Plant Biotechnology) at the Agricultural University.

As a doctoral student, she specialized in Germany (Dresden Technical University). She took part in a DAAD-project and in a bilateral contract with the UFT program ENTER Expert Workshop and received a certificate for her training. There is no information in the documents for proficiency in a foreign language, but the specialization shows proficiency in such. She is a chief expert at the Laboratory of Plant Biotechnology and has taught plant biotechnology classes for bachelors at the Agricultural University of Plovdiv.

The set of paper materials presented by Pervin Halkoglu on the dissertation is in accordance with the requirements of the Law for development of the academic staff in the Republic of Bulgaria and the Regulations for its application.

2. Relevance of the problem.

Various ways of obtaining biologically active substances from plant species are known. The subject of the presented study, *Fabiana imbricata* Ruiz et Pav. is little known in Europe.

There are limiting factors that affect the efficiency of their production from this plant species. The work within the present dissertation contributes to overcoming the shortcomings that arose during its conventional reproduction and cultivation. Unlike classical breeding, *in vitro* cultivation methods ensure rapid reproduction and through them the production of biologically active substances is achieved under fully controlled conditions. Optimization of the critical factors for *in vitro* cultivation of the target species is crucial for their production.

The topicality of the topic must be considered in theoretical and practical aspect. Apart from solving the production of biologically active substances, which is of economic importance, their introduction into tissue cultivation is important for the research work of biotechnologists and for genetics and a number of other scientific fields.

3. Purpose, tasks, hypotheses and research methods.

The aim is to introduce the insufficiently studied plant species *Fabiana imbricata* Ruiz. et Pav. in *in vitro* cultures, which clearly focuses on the development of technological matrices for the production of biologically active substances.

The set tasks correspond to the stages of research of the developed problem and are carried out in a correctly chosen sequence.

The choice of plant species studied in terms of its value and characterized in terms of its susceptibility to *in vitro* cultivation is correct. *Fabiana imbricata* Ruiz. et Pav. has the potential to expand the spectrum for the production of bioactive substances with useful qualities and would also serve to solve a number of biotechnological problems. The starting plant material for *in vitro* experiments was supplied by Multicoflore - semences de plantes ornamentales and nursery Paddock Plants from England.

The idea of conducting scientific research is methodically constructed. The analyzes are performed with appropriately selected modern methods, described correctly, mastered and successfully applied by the doctoral student, as some of the work protocols are optimized. The following steps are described and used: Introduction to *in vitro* culture; Micropropagation of *Fabiana imbricata* Ruiz et Pav., optimizing the cultivation medium for multiplication, Adaptation of regenerants. The influence of the light source, the immersion type RITA® in the stage of multiplication has been studied. Morphological parameters of plants grown on different nutrient media were compared. The repetitions (24) made in the glass vessels, the number of explants and the reported parameters for evaluating the effectiveness of the newly introduced steps in the protocols were correctly selected. Additionally, the methods for obtaining callus cultures, plant cell and suspension cultures together with digital holographic microscopy have been selected, which makes an impressive number of applied methods for introducing the target plant species in *in vitro* culture. To these are added methods for extraction and analysis of polyphenols, spectrophotometric determination of their concentrations, determination of antioxidant activity by capturing the radical with DPPH method and ABTS method (modified in the dissertation), FRAP method (also modified), CUPRAC method (with modifications). Biochemical HPLC analysis for the determination of polyphenolic compounds and flavonoids (applied with a gradient in the elution and with 11 witnesses for phenols and 6 for flavonoids) is generally a third type of analysis and requires very specific knowledge. The number of applied methods is impressive, and it provides

completeness and depth of research and achievement of the set goal.

SPSS and Breeze TM, Excel are applied for processing the results, and they are formatted with Word. The reliability of the studies is ensured by a sufficient number of replicates corresponding to the requirements for the methods.

4. Visualization and presentation of the obtained results.

The dissertation is written on 118 pages. The title covers the content. The introduction hypothetically focuses on the target substances for research and approaches, the plant species, the identified problems, and the importance in solving them. After an extremely detailed and well-structured review of the existing scientific information, gaps in knowledge were found in specific areas with practical application. The reference consists of 191 literary sources, mainly in Latin and 3 in Cyrillic. They cover a 40-year review of the published literature on the subject. The conclusions made at the end of the section determine the need for this study.

The dissertation is well balanced by sections and includes 9 main sections, and in them subsections. The rich, well-illustrated, appropriately and purposefully systematized information in this section gives me reason to believe that the PhD student is very well informed about the main achievements related to the problems she is working on in her dissertation.

The goal is formulated precisely, and the tasks are chosen so as to ensure its implementation.

The material and methods are presented in detail in 6 chapters: seed germination test, biotechnological (introduction into cultures with different nutrient media, callus and suspension cultures, taking into account physical parameters, study of bioreactor system with temporary immersion), biochemical different types of analyzes of biologically active substances) and statistical.

The results (on 46 pages) are described in 4 chapters in the order of the experiments and the selected methods and are presented in detail, illustrated with 16 tables and 31 figures and good quality photos. The discussion follows the results. Attached are 2 tables with formulas and literature data for primary and secondary metabolites.

The conclusions are presented on 3 pages. A conclusion with contributions follows, and the latter are not formulated separately.

5. Discussion of the results and used literature.

The dissertation presents valuable scientific information obtained as a result of various experiments, described in four chapters. The presentation of the analyzed data in tables, photos and graphs makes an extremely good impression.

The results of the experiments are presented in detail. The initial explants were successfully sterilized and introduced into *in vitro* culture with initially hormonal medium and the effect of growth regulators BAP, IBA in different concentrations was established. The most suitable medium for shoot proliferation has been determined. Against the described vitrification, at certain concentrations of auxins in the medium, activated carbon was added, and the proliferation was doubled without hyperhydration. The use of activated carbon as an approach to overcoming a negative physiological phenomenon, such as vitrification, should be

noted as a success.

In all variants of the conducted experiments, rooting of the regenerants was ensured. The characteristics of the plants grown on different nutrient media are made in connection with obtaining successful rooting and adaptation. In this sense, the application of a hormonal environment in the latter subculture proves to be an extremely appropriate approach. The different spectrum of light, LED light source and fluorescent lamps affect the characteristics of plants differently, and fluorescent light is best suited for animation.

The tested automated temporary immersion system (RITA® type) demonstrated efficiency and higher values of growth characteristics were reported compared to the conventional *in vitro* system. An indisputable advantage of the bioreactor system is that it provides process automation, minimal space and production of large quantities of biomass and the value of the investment in equipment is reduced.

An original point in the dissertation is the use of digital holographic microscopy (DHM) to measure the size of cell clusters in suspension cultures of *F. imbricata*. DHM is used for the first time, proving a successful application for cell counting, measurement of cell size and cell clusters, analysis of cell culture viability and more.

The second part of the dissertation is focused on the analysis of biologically active substances in plants and *in vitro* cultures with different degrees of differentiation of the species *Fabiana imbricata* Ruiz et Pav.

The polyphenol profile was analyzed by HPLC of plants *in vitro*, *in vivo*, calluses, derived from them plant cell suspensions. Additionally, an analysis of key metabolites - gallic, protocatechuic, chlorogenic, vanilla, syringic, caffeic, salicylic and rosemary acid. The results are original in nature and allow the identification of target metabolites in *in vitro* cultures with varying degrees of differentiation under certain culture conditions. The high antioxidant capacity found is close to the values of a plant sample *in vivo*.

The successful combination of the competencies of the two supervisors is reflected in the overall development and interpretation of the results of the dissertation. The experiments were conducted in various laboratories of the University of Plovdiv, the University of Food Technology - Plovdiv, the branch of BAS - Plovdiv.

The design of the experimental work includes testing a large number of replicates and adequate statistical processing of the results. In the course of laboratory experiments, full-time doctoral student Pervin Chalkoglu has mastered the necessary methods for *in vitro* cultivation and chemical analysis of BAV.

The dissertation ends with the formulation of 15 conclusions, which reflect in detail the results obtained.

6. Contributions to the dissertation.

The dissertation is definitely an innovative type of research that brings both scientific and applied contributions. As the main ones I dare to point out:

Scientific contributions

- It has been shown that *in vitro* cultures of *Fabiana imbricata* Ruiz. et Pav. are suitable for

use in the production of broad-spectrum biologically active substances.

- The nutrient medium for micropropagation of *F. imbricata* is optimized, in which the balance of growth regulators provides a high percentage of multiplication.
 - Induced callus cultures differ in morphology and growth characteristics and on their basis 3 cultures are selected, suitable for cell suspensions.
 - For the first time, the number and size of cells and cell clusters in suspension cultures of *F. imbricata* were determined by digital holographic microscopy (DHM).
- By Using HPLC analysis, the polyphenolic profile of plants *in vivo* and *in vitro*, calluses and resulting cell suspensions of *F. imbricata* were established. The diversity of the synthesized polyphenolic compounds in the studied *in vitro* systems with different degree of differentiation has been proven.
- For the first time, spectrophotometric analysis of *in vivo* and *in vitro* cultures of *F. imbricata* was performed, which demonstrated high antioxidant activity of the analyzed extracts.

Scientific and applied contributions

- For the first time in Bulgaria, *in vitro* material from *F. imbricata* was obtained, suitable for use as a source of biologically active substances.
- The addition of activated carbon (AC) in the nutrient medium has a stimulating effect on the proliferation of shoots and leads to overcoming negative physiological conditions.
- The use of nutrient medium without growth regulators is a suitable approach for hardening of regenerants and ensures high survival of plants in *ex vitro* adaptation.
- For the growth and development of *F. imbricata* plants, white fluorescent light has been found to be most suitable for the multiplication stage compared to LED sources.
- Suspended cultures of *F. imbricata* have been shown to be suitable for the production of target metabolites because they reach a maximum increase in biomass in a short cultivation period (8-10 days) compared to callus cultures and *in vitro* plants (28 days).
- The potential of *in vitro* cultures of *F. imbricata* as technological matrices for obtaining target metabolites has been determined.

7. Critical remarks and questions.

I appreciate the merits of the presented topic and the combination of the methods used to achieve the main goal. The dissertation is a logically completed scientific development, which is planned, structured and derived using modern methodology.

The analysis of the results is in-depth and compared with other similar developments.

I would prefer the term "Determination of the content" of phenols, flavonoids and others. to be replaced by 'Determination of concentration', as they determine not the whole plant but the weight of the plant mass from which they are extracted. Witnesses for flavonoids are not listed in the "Material and methods" section. Not all photos and diagrams indicate authorship.

I would like to ask the following questions:

1. How was the species *F. Imbricata* chosen as a scientific object?
2. How would you explain the effect of the light spectrum on the change of the metabolic profile of different *in vitro* cultures.

8. Published articles and citations.

The presented reference on scientometric indicators contains sufficient publications that cover the minimum national requirements for awarding the educational and scientific degree "Doctor" and the requirements of the Regulations for the development of the academic staff at the Agricultural University of Plovdiv. The PhD student Pervin Halkoglu-Hristova has published her results in peer-reviewed and peer-reviewed journals in Scopus / Web of Science.

The prepared reference does not contain citations of the published results, but interest in the published works has been shown by various scientific journals.

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

CONCLUSION:

Based on various research methods learned and applied by the doctoral student, correctly performed experiments, commitments and conclusions made, considered to present a dissertation meeting the requirements of the Law on Agricultural Research and the Regulations of the Agricultural University for its application, which gives grounds to rate it POSITIVE.

I allow myself to suggest to the esteemed Scientific Jury that it also vote in favor and award

PERVIN SHENGYUN HALKOGLU-HRISTOVA

educational and scientific degree "Doctor" in the scientific specialty "Breeding and seed production of cultivated plants" (Plant biotechnology)

Date: 30.08.2021
Plovdiv

REVIEWER:
(Prof. Dr. Nasya Tomlekova)