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REVIEW

of the PhD thesis of landscape architect Vladimir Metodiev Ilinkin entitled

"Biotechnological approach for cultivation of култивиране на *Tanacetum cinerariifolium* (Trevir.) Sch.Bip. (Asteraceae)"

for obtaining the educational and scientific PhD degree

Higher education field: 4. "Natural sciences, mathematics and informatics Scientific field: 4.3. Biological sciences, Scientific topic: 01. 06. 03 Botany Department "Plant and fungal biodiversity and resources" Institute of Biodiversity and Ecosystem Research Bulgarian Academy of Sciences (IBER-BAS)

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> > Prepared by

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The present opinion has been prepared based on the Order № 35/11.04.2024 г. of the Director of IBER (BAS) - Sofia, Assoc. prof. Vladimir Vladimirov, PhD.

The author of the PhD thesis is a PhD student at the Department "Plant and fungal biodiversity and resources", IBER-BAS.

(1) General characteristics of the dissertation - volume and structure

The dissertation consists of 103 pages and includes 30 figures, 19 tables, 197 cited bibliographical references. The candidate's work is presented in the form of bound four thematically related scientific publications. A good impression is also made by the additional display and systematization of common connecting parts, which make the perception of the material logical and orderly. The Thesis has been structured as follows:

- 1. Introduction
- 2. Aims and Tasks
- 3. Bibliographical survey
- 4. Materials and Methods, Results and Discussion
- 5. Conclusion
- 5.1. Main outcomes

5.2. Declaration of originality and authenticity

6. Contributions

- 7. References
- 8. List of scientific publications with full bibliographic description

9. Supplementary 1. Protocol for *in vitro* micropropagation of *Tanacetum cinerariifolium* (Trevir.) Sch.Bip.

(2) Bibliographic knowledge and theoretical preparation of the candidate

An extremely extensive bibliographic survey has been made regarding the botanical characteristics and distribution, ecological requirements and cultivation of Dalmatian pyrethrum. Information regarding the content of the main biologically active substances has also been summarized, paying special attention to the content of pyrethrins both in natural populations and in introduced agricultural crops, including those available in Bulgaria. The available information in literature regarding various pyrethrin products, as well as their use not only as insecticides but also in medicine, has been reviewed. A number of synthetic analogues of natural pyrethrins have also been examined. Information has been presented regarding the possibilities of seed propagation of the species, as well as its vegetative and *in* vitro propagation. A separate references list of the literature review has been presented, systematized separately to the Introduction chapters of the bound publications. An excellent impression has been made by the extensive bibliography of studies by other authors on the factors related to seed germination and the further influence of the in vitro content of pyrethrins in different types of culture media and treatments carried out specifically on the studied species. This has enabled the candidate to address his research in an argument based and targeted way and to achieve in the current dissertation an upgrade over what is already available in literature.

(3) Methodological approaches

The methods adopted and applied in this dissertation are systematized as follows in chapter 4, in which the attached publications themselves are included:

4.1. Seed viability and germination

- 4.2. In vitro propagation and acclimation
- 4.3. Phytochemical analyses

In each of these sub-chapters, a summary of the methods is presented and reference is made to the relevant publication. The publications cover the application of the following methods:

First publication - "Germination and viability of seeds of *Tanacetum cinerariifolium* (Asteraceae)" – Vladimir Ilinkin, Elina Yankova-Tsvetkova, Marina Stanilova, Comptes rendus de l'Academie bulgare des Sciences, Tome 73, No 7, 2020, 965-970 - a comparative analysis of germination of fresh seeds in *in vitro* and *in vivo* (in petri dishes) conditions was performed, as well as *in vivo* of seeds stored for 1, 2 and 3 years. In the *in vivo* experiment, the vitality of the seeds was also monitored. The experiment was conducted with seeds collected in the area of Bogdan village, Plovdiv region. Methods for statistical processing of the results have been adopted and applied.

Second publication - "Germination of *Tanacetum cinerariifolium* seeds on different soil types" – Vladimir Ilinkin, Ecological Engineering and Environment Protection, No 2, 2019, p. 67-74 - an experiment was conducted to track the germination of seeds of the species in different types of soils (Skeletic Fluvisols, Rendzic Leptosol, Luvic Chernozem, Chromic Luvisols, Dystric-Eutric Cambisol, Pellic Vertisols) in controlled conditions. A set of physico-chemical soil indicators (total density, sand fraction, available phosphorus, total porosity, silt and clay fraction, acidity, carbonate content, total nitrogen content, total organic content) were studied and the statistical relationships between them and the germination of the seeds were analyzed.

Third publication - "In vitro clonal propagation of Tanacetum cinerariifolium and establishment of an ex situ collection of selected clones" - Vladimir Ilinkin, Boryanka Traykova, Marina Stanilova, BioRisk, 20, 2023, 97–114 - the work was performed with seeds collected from the area of the village of Bogdan, and for part of the experiments with seeds from natural deposits in Croatia. A protocol has been developed for introduction into in vitro culture, rapid multiplication, selection of promising clones and establishment of an ex situ collection. Experiments on the composition of the basic Murashige & Skoog culture medium were performed, and optimizations were carried out in terms of adding calcium carbonate to the media. The cephalosporin antibiotic ceftriaxone (Medaxone) has been used to influence the losses due to the occurrence of endophytic infections. For the study of micropropagation, experiments were also carried out in a liquid culture media under the conditions of a bioreactor system with controlled immersion (RITA temporary immersion system). The relevant morphometric characteristics were investigated and statistical processing of the results was performed. Successful ex vitro adaptation of four clones originating from the Croatian population was performed, and the ex vitro observed flowering characteristics were monitored and reported within two years, with a tendency to increase the number of flower heads formed in the second year.

Fourth publication - "Pyrethrins profiles and contents in seedlings, *in vitro* cultures, and acclimatized plants of *Tanacetum cinerariifolium* clones (Asteraceae)" – Vladimir Ilinkin, Milena Nikolova, Marina Stanilova, Strahil Berkov, Comptes rendus de l'Academie bulgare des Sciences, v. 76, No 7, 2023 - the composition of pyrethrins in hexane extracts obtained from several in vitro clones as well as from first and second flowering flower heads of *ex vitro* adapted plants was investigated by gas chromatography. In addition, phytochemical analysis was also performed on plant material obtained from seeds germinated in different soil types. Statistical analysis of the obtained results was performed.

(4) Significance and cogency of the obtained results, interpretations and conclusions

The summary of the experimental results is visible and traceable both in the chapters Results and Discussion and Conclusions in each of the presented bound publications, and is additionally systematized by the candidate in the general parts of the dissertation. The conclusions and discussions of the obtained results are strongly supported by the conducted statistical processing of the data, and their interpretation is discussed relevantly in the context of the developments and of other authors on the subject.

The dissertation presents a completed protocol for *in vitro* propagation of *Tanacetum cinerariifolium* (Trevir.) Sch.Bip. with starting material seeds. The influence of soil

characteristics on seed germination was also investigated. Comparative analyzes of the main biologically active compounds were made in *ex vitro* adapted and acclimatized plants from *in vitro* propagated selected clones. The clones differ among themselves in the total content of pyrethrins and in the ratio between the seven components, which makes the biotechnological breeding approach a suitable tool for the selection of highly productive clones.

The scientific results obtained in the dissertation are systematized as follows:

In vitro propagation Tanacetum cinerariifolium

• *Tanacetum cinerariifolium* seeds are characterized by low viability and germination (about 20%), which decrease progressively with storage time and no viable seeds are observed after a 3-year period. Freshly harvested mature seeds are most suitable for *in vitro* culture induction, as their *in vitro* germination rate is lower. Delayed onset and extended germination period are observed. The highest germination rate of pyrethrum seeds was observed in Rendzic type soils.

• Within 3 to 8 subcultures, plants die due to necrosis and development of endophytic microorganisms. Supplementation of Medaxone to the medium (at an optimal concentration of 200 mg/L) inhibited microbial growth and provided the highest productivity during 8 subcultures.

• On MS medium without growth regulators, well-formed and rooted *in vitro* plants were obtained, and the addition of the antibiotic did not affect the morphological development of the plants.

• Modification of the basic MS medium, with an increased concentration of calcium added as CaCO₃, was found to successfully reduce necrosis.

• Dozens of *in vitro* clones (induced from seeds and obtained by multiple subculturing) were found to differ in their reproductive ability, allowing for selection at the *in vitro* cultivation stage. Of the 19 branches that survived after 8 months of cultivation, 4 branches with the highest productivity were selected.

• The reproduction coefficient remained relatively constant for 5 subcultivations. Over time, a tendency to decrease its variation was established.

• The developed *in vitro* system for the cultivation of Dalmatian pyrethrum (Temporary Immersion System), in RITA® culture vessels and with added Medaxone at an optimal concentration, did not increase the reproduction coefficient of the tested clones. Agar-solidified medium of the same composition was shown to be more suitable for culturing the species.

• Under *in vitro* conditions, plants rooted spontaneously on all tested culture media, favoring *ex vitro* adaptation and resulting in high survival (88% survivors out of 360). Six weeks in a climate cabinet were shown to be enough to strengthen and grow the plants up to 10 cm in height. Almost all plants survived the next steps of adaptation, in a phytotron room and in an unheated greenhouse.

• The acclimatization of the plants in the ex situ collection of IBER was successful. All individuals of the species bloom already in the first growing season in June, secondary flowering was also observed in September-October. Differences were found in the branching of the stems and the number of flower heads. In the second year, flowering was more abundant, on average 328±138 flower heads per individual were formed in the most productive branch.

Phytochemical analyses:

• The presence of all pyrethrins, including the isomer of pyrethrin-I, was demonstrated in the examined samples. These metabolites were present in small amounts in both *in vitro* plants and *in vivo* germinants. Concentrations of the pyrethrin-I isomer were up to several times lower than those of pyrethrin-I.

• At primary flowering in June, in all four branches in the ex situ collection, the pyrethrin I/II ratio was found to be greater than 1, with pyrethrin-I being the major component. Statistically significant differences between clones (p < 0.05) were noted only for pyrethrin-II and the pyrethrin-I isomer. The content of pyrethrins in flower baskets was comparable to that reported for natural populations in Dalmatia.

• In the secondary flowering in September-October, the pyrethrin I/II ratio was less than 1, which was probably influenced by environmental factors (shorter sunshine as well as soil waterlogging in the ex situ collection) and the degree of ripening of the flower baskets during their collection.

• Variations in the content of pyrethrins were found in individual clones cultivated *ex situ*. For all 7 components, the variation is greatest in branch 2. The lowest variation of pyrethrins I was established in clone 1, and of pyrethrins II – in clone 3. Statistically significant differences in the ratio of pyrethrins I / II were found in clones 2 and 4.

(5) Critical Notes to the Dissertation

For future work with plant material with an increased risk of carrying endophytic infections, I would recommend experimenting with sterilization methods - optimizing the chlorine content in the sterilizing agent, experimenting with commercial products containing chlorine of different characteristics, using other sterilizing agents (e.g. mercury dichloride), varying ethanol pretreatment time, sterilizing agent treatment time, significantly reducing sucrose concentration, both in the initial germination media and in those for the initial passages of the resulting germinants. It would probably be helpful to increase the autoclaving time of the media, etc. In order to avoid the development of antibiotic resistance, as well as the presence of traces of the antibiotic in the obtained extraction material, I would only recommend a single passage through medium with an antibiotic.

(5.1.) Questions to the doctoral candidate

- The species was introduced into agro-culture in Bulgaria in the past. Is it currently cultivated in the field in our country?

- Does the species show sufficient frost resistance for our climatic conditions? Based on the results obtained in the dissertation, in which region of Bulgaria would you recommend its cultivation?

- On the basis of the results obtained in the dissertation, which of the approaches the candidate considers more feasible: to obtain pyrethrins biotechnologically in controlled conditions or to

apply the micropropagation method to obtain large quantities of planting material for the field cultivation of the species?

(6) Characterization of the scientific contributions.

An excellent impression is made by basing the experimental design in the thesis on what has already been achieved in the literature by other authors with *T. cinerarifolium*. This is reflected in the discussion and is a prerequisite for the original nature of the thesis contributions obtained by the candidate as a result of the experiments planned and carried out by him. Thus, for example, the inclusion of calcium carbonate in the culture medium is an authentic approach of the candidate, based on the ecological requirements of *T. cinerarifolium*. In the work of Vladimir Ilinkin, his ability to think independently and make decisions when performing the experiment is evident.

A Declaration of Originality and Credibility has also been submitted by the candidate. I support it and agree with its content. The contributions in the dissertation are structured as follows:

1. A new efficient protocol for *in vitro* micropropagation of *Tanacetum cinerariifolium* was established, providing several thousand in vitro plants in one year (Appendix 1) - original contribution.

2. It has been found that for the germination of the seeds of *T. cinerariifolium*, the most suitable soils of the Rendzic type - original contribution.

3. Four *in vitro* clones with high reproductive capacity were selected, characterized by different productivity in terms of the amount of flower heads in the conditions of the *ex situ* collection, which allows for additional selection - original contribution.

4. It was confirmed that *in vitro T. cinerariifolium* synthesizes all pyrethrins in a lower concentration compared to that in flower baskets of well-developed plants - within the specific lines obtained by the candidate within the experiment with the used from him seeds and culture media the contribution is original.

5. It was found that in *ex vitro* adapted and acclimatized plants in the *ex situ* collection, the ratio of pyrethrin I / II was different in some clones, which confirms the influence of the genotype in the biosynthesis of pyrethrin I and pyrethrin II and enables selection - within the specific lines obtained by the candidate within the experiment with the seeds and culture media used by him the contribution is original.

(7) Assessment of the quality of scientific works reflecting the dissertation research

The results of the candidate's work submitted for review for this dissertation have been published in four scientific publications. Two of them are in quartile Q2 and one in Q3. In one of them, the doctoral student is a single, and in the other three - the first and corresponding author. Three of the publications have an SJR index and two have an impact factor. The candidate's scientometric indicators, as well as his role in the publications presented for review, are an indicator of his ability to think independently and critically and to express and defend at an academic level the results obtained. The results of the dissertation were also presented in a total of nine participations at eight scientific forums, five of which were international.

(8) Motivated answer to the question to what extent the dissertation research is primarily the personal work of the doctoral student

The submitted dissertation shows that the candidate possesses the necessary qualities for generalization and critical analysis of bibliographic data, as well as diligence and persistence in learning and applying a wide range of experimental agro-ecological, biotechnological, phytochemical and statistical work methods. Interpretation, discussion and summary of the experimental results have been carried out, which shows the necessary scientific maturity achieved by Vladimir Ilinkin in the course of his training and work. Of the 250 required credits, the PhD candidate covers 630. All above is supportive to the conclusion that the research in the dissertation is primarily the personal work of the doctoral candidate.

(9) Motived conclusion

The dissertation submitted for review meets the requirements for structure, qualitative and quantitative criteria, and the candidate exceeds the minimum national requirements for obtaining the educational and scientific degree "doctor", defined in the Law on the Development of the Academic Staff in the Republic of Bulgaria, as well as the Regulations for the terms and conditions for acquiring scientific degrees and occupying academic positions at IBER-BAS. The dissertation, abstract and set of documents submitted by Vladimir Ilinkin are in accordance with the above requirements in order for him to be admitted to the defense of his dissertation.

All of the above gives me reason to confidently recommend to the members of the esteemed Scientific Jury to award the doctoral candidate Vladimir Ilinkin with the scientific and educational degree "doctor" in the field of Science: 4.3. "Biological Sciences", Scientific specialty: 01. 06. 03 "Botany".

Respectfully,Reviewer:София,/Assoc. prof. Kalina Danova, IOCCP-BAS/14.05.2024 г./Assoc. prof. Kalina Danova, IOCCP-BAS/