PhD THESIS

Research concerning passionflowers behaviour in controlled climate under the influence of conventional and unconventional technological factors

PhD student **Paula Boboc (căs. Oros)**

Scientific coordinator **Prof. univ. dr. Maria Cantor**

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INTRODUCTION

Flower production represents a huge potential for Romania, still unexploited, which could benefit both professionals and flower lovers.

The introduction of new species and varieties of flower crops in Romania is a necessity nowadays, especially since there is the possibility of monitoring ecological factors in a controlled climate. The current trends of the flower market in Romania involve the diversification of the assortment with exotic species. *Passiflora* is an extremely appreciated plant globally, both for its decorative value and for the multiple medicinal properties it has (BOBOC and CANTOR, 2017). Therefore, there is a need to produce vigorous plants for good adaptability and resistance to climatic conditions.

Production of *P. quadrangularis* plants by conventional methods has been shown to be problematic due to the low germination rate of the seeds. At the same time, the presence of disease-causing microorganisms significantly reduces the efficiency of propagation by cuttings (OŻAROWSKI and THIEM, 2013).

Regarding the possibilities of *in vitro* multiplication, the contamination of the vegetal material used for the initiation of cultures remains the biggest problem. The *in vitro* culture of Passifloraceae has also been associated with problems such as recalcitrance, sensitivity to ethylene accumulation, and browning of explants due to the presence of phenols in tissues and this doctoral thesis aims to provide solutions to these difficulties.

PURPOSE AND OBJECTIVES OF THE RESEARCH

The aim of the research undertaken in this doctoral thesis is to contribute to the optimization of propagation technologies in *Passiflora* and to determine the possibilities of vegetative propagation by conventional methods (cuttings) and unconventional (*in vitro*) as well as elements of culture technology (fertilization) in two species of *Passiflora*, *P. caerulea* respectively, *P. quadrangularis*. The experiments were organized in the form of three experimental series, based on the research and finding the following main and specific objectives:

I. Determining the degree of popularity of the genus *Passiflora* in Cluj-Napoca.

- Pre-orientation of information actions for local ornamental plant producers.

II. Establishing an optimal protocol for vegetative propagation by cuttings in *P. caerulea* and *P. quadrangularis.*

III. Establishing an optimal fertilization regime for controlled system culture in *P. caerulea* and *P. quadrangularis.*

IV. Testing and identification of the most suitable *in vitro* culture system for mass multiplication in *P. caerulea* and *P. quadrangularis*.

- Development of a regeneration protocol by direct organogenesis in *P. quadrangularis* that aims to solve two of the fundamental problems of the species: contamination and recalcitrance;
- Establishing the influence of phytohormones specific to each stage of micropropagation;
- Development of a regeneration protocol for *P. quadrangularis* plants *via* callus.
- Establishing the influence of the photoperiod and additional compounds in the case of different culture systems;

- Efficient acclimatization of plants and production of quality, disease-free planting material.

V. Determination of biochemical and bioactive compounds in *Passiflora* to confirm the quality of biological material obtained both by cuttings and *in vitro*.

DOCTORAL THESIS STRUCTURE

The doctoral thesis is organized into 10 chapters in two parts. The first part covers the current state of knowledge on conventional and unconventional culture in *Passiflora* and general aspects of systematic, morphology and ecology of species. The second part contains the material and working methods, the results obtained, the discussions about them and at the end, the conclusions and recommendations. At the end are found the bibliography, annexes and abstracts in Romanian and English. To achieve the expected objectives, 284 sources from the specialized literature were consulted. The scientific results obtained during the research period materialized through the publication of three *ISI* article (an *ISI* article (*IF*-2.331; *Q1*) and two *ISI Proceedings*), eight BDI articles and the participation in six International Conferences (see CV). The thesis contains several 27 tables and 41 figures and is structured in the form of three experimental series.

PERSONAL CONTRIBUTIONS

To achieve the proposed objectives and to obtain relevant scientific results, the studies were carried out using biological material from two species: *P. caerulea* and *P. quadrangularis*. Numerous chemicals, equipment and other materials were used to conduct the experiments. During these experiments, biometric parameters were analyzed, histological studies and growth or strucural observations were performed.

Study on the popularity of the genus *Passiflora*

The study on the degree of popularity of *Passiflora* plants in the population of Cluj-Napoca were conducted based on a short questionnaire consisting of nine questions that were applied to a sample of 273 randomly selected people.

Experimental series I - Studies on conventional culture at *Passiflora*

Studies on the possibilities of vegetative propagation by cuttings

The biological material used for the experiment was represented by cuttings of about 15 cm in length, respectively 2-3 knots from *P. caerulea* and *P. quadrangularis*. Three rooting biostimulators were used: Incit-8, Radistim-2 and Indolyl-butyric acid (AIB) 1000 ppm and four rooting substrates were tested: vermiculite (V), peat + vermiculite in a 1:1 mixture (T + V), peat + perlite in 1:1 mixture (T + P), peat + sand in 1:1 mixture (T + N). The combination of the three experimental factors resulted in a randomized experiment with 32 experimental variants. The rooting rate and biometric parameters were determined.

Studies on the influence of fertilization regime on morpho-decorative characters

The plant material that is the subject of the study of the fertilization regime for the culture in a controlled system consisted of one-year-old plants, obtained in the previous experiment. Two fertilizers were tested: Cropmax® and Nutricomplex® 20-

20-20 + microelements applied in three doses: 0.05%, 0.1% and 0.2%. Each species were analyzed individually, resulting in two completely randomized bifactorial experiments with nine experimental variants. Observations were made on morphological indices, relative growth rate (RGR) and qualitative parameters.

Experimental series II - Studies on the possibilities of multiplication by unconventional methods *in vitro* at *Passiflora*

The biological material comes from the species *P. caerulea* and *P. quadrangularis*, one-year-old plants, in the stage of vegetative growth. The plants destined for the experiments was grown in the Didactic and Experimental Greenhouse of the discipline of Ornamental Plants. The plant material used for the initiation of *in vitro* cultures by direct organogenesis were represented by nodal segments. Regarding callogenesis and somatic embryogenesis, the biological material used to initiate the callus cultures in *P. quadrangularis* were represented by four types of explant: internodal segments and leaf fragments, receptacle and sepals.

This experimental series is organized in the form of three studies performed on the stages of micropropagation:

- a study on multiplication by direct organogenesis in *P. caerulea* containing three secondary experiments;
- a study on multiplication by direct organogenesis in *P. quadrangularis* containing nine secondary experiments;
- a study on multiplication by indirect organogenesis and somatic embryogenesis in *P. quadrangularis* containing four secondary experiments.

Multiplication by direct organogenesis,

Asepticization of *P. caerulea* explants was performed using a 70% EtOH pretreatment for one minute, followed by a 15% NaClO treatment for 20 min.

For asepsis of *P. quadrangularis* explants from nodal segments, 15 treatments were tested. A total of 450 nodal explants were initiated in order to identify optimal disinfection treatments.

In the initiation stage the same types of culture media were used in both species, the MS culture medium was supplemented with 6-benzylaminopurine (BAP) alone (0.5-3 mg/l) or in combination with KIN (1 or 2 mg/l) resulting in a single-factor experiment with 13 experimental variants. In *P. quadrangularis* case, the experiment was followed by the study of the influence of the position of the explant and the culture medium on the axillary sprouting were realized by a bifactorial experiment of type 2 x 3 performed in three repetitions. The culture medium was then supplemented with additional compounds such as AgNO3 and Pluronic F-68 (PF-68) in order to reduce the effects of leavened phenols and the sudden browning of the explants resulting in a 3 x 6 bifactorial experiment performed in three repetitions, each rehearsal consisted of 10 trials. A number of 1020 explants were initiated in order to stabilize the *in vitro* culture for this species, compared to *P. caerulea* where 390 explants were initiated.

In both *P. caerulea* and *P. quadrangularis*, the subcultivation (multiplication) stage followed the same experimental pattern. *In vitro* regenerated shoots were multiplied by repeated subcultures on a fresh culture medium every four weeks. In the multiplication stage, a bifactorial study of type 2 x 4 was performed in which three

types of culture medium indicated in the literature were tested.

The miscroshoots obtained after the sixth subculture represented the biological material used to study the *in vitro* rooting capacity. Only *P. quadrangularis* microshoots passed through this stage because in *P. caerulea* the rhizogenesis took place during the successive subcultures. A one-factor study with three experimental variants was performed in three repetitions, each repetition consisted of 10 samples.

After *in vitro* rooting, the plants were transferred to pots containing a mixture of peat and perlite 1:1 or peat and vermiculite 1:1 for acclimatization were represented by the mixed substrate, with the two graduations. After four months of acclimatization, the survival rate of the plants were determined.

Multiplication by indirect organogenesis and somatic embryogenesis

The study of the influence of explant type and phytohormones on callogenesis was performed by an 11 x 4 bifactorial experiment. To facilitate the organization of the experiment, the combination of auxin + cytokinin in the culture medium was named as the treatment. Each treatment was applied for five explants and was performed in three repetitions so that a total of 660 explants were initiated.

The study of the influence of phytohormones and the photoperiod on the multiplication of the callus involves a bifactorial experiment of 3 x 2 type, from the interaction of the two factors resulting in six experimental variants.

The study of the influence of phytohormones and the culture system on the multiplication of the callus presupposes a bifactorial experiment of 3 x 2 type, from the interaction of the two factors resulting in six experimental variants. For each experimental variant, callus was inoculated into 10 culture flasks, the research being performed in three repetitions.

The study of the influence of the photoperiod and the additional compounds on the proliferation of embryogenic cultures involves a bifactorial experiment of 7 x 2 type, from the interaction of the two factors resulting in 14 experimental variants. The culture medium were supplemented with: PF-68 (0.2% and 0.4%), AgNo3 (1 mg/l and 2 mg/l) and coconut water (5% and 10%) as well as the variant without additional compound.

Experimental series III - Study on the determination of biochemical and bioactive compounds in *Passiflora*

Leaves and stems from mother and acclimatized plants, as well as friable callus of *P. caerulea* and *P. quadrangularis,* were analyzed to determine the content of total lipids and proteins, total polyphenols and flavonoids, respectively antioxidant capacity. A single-factor experiment was organized, where the biological material with the five graduations represented the experimental factor. The determinations of the 10 samples were performed in 3 repetitions, for each experimental variant.

RESULTS AND DISCUSSIONS

Results regarding the popularity of passionflowers

Regarding the degree of popularity of the genus *Passiflora*, there is a percentage of 63.5% of the total respondents state that the plant in the presented image (an image with the flower of *P. caerulea*) is known to them, and more than 98.4% of them have

knew how to call her.

Experimental series I - Results regarding the conventional culture at *Passiflora*

Results on the possibilities of vegetative propagation by cuttings

The cuttings of *P. caerulea* obtained an average rooting percentage of 79.78%, and those of *P. quadrangularis* of 74.57%. *P. caerulea* recorded the highest rooting percentage, of 96.7%, on the peat + vermiculite mixture substrate and *P. quadrangularis* 93.3% in vermiculite and in the mixture of peat and perlite. The length of the roots varies between 3.73 and 8.59 cm in *P. caerulea* and between 4.79 and 8.63 cm in *P. quadrangularis*. The results of the biometric parameters showed that the treatment with the biostimulator AIB 1000 ppm is the most suitable for rooting the cuttings in both studied species.

Results on the influence of the fertilization regime on morpho-decorative characters

The statistical interpretation of the results with Duncan MRT test highlighted the importance of fertilization using Cropmax. This organic fertilizer led to higher values of morpho-decorative parameters at both applied doses (0.2 and 0.1%, respectively), followed by Nutricomplex fertilizer (0.2%) which obtained statistically assured values for most of the analyzed characters. During the vegetation period, different growth rates were observed, thus establishing four-time intervals for which the evolution of the relative growth rate (RGR) was calculated. RGR obtained maximum values between June 4 and August 5, and Cropmax fertilizer led to the highest RGR in the analyzed time intervals for both species. The bifactorial analysis of the RGR for the experimental years 2019 and 2020 showed that in 2019 the RGR was higher in both species.

Experimental series II - Results on the possibilities of *in vitro* propagation by unconventional methods at *Passiflora*

Multiplication by direct organogenesis at *P. caerulea*

Axillary shooting in *P. caerulea* began in average after three days after initiation. The best results regarding the regeneration rate (88.22%) and biometric parameters (several 2.42 shoots per explant, with an average length of 2.10 cm) were recorded on the MS culture medium supplemented with 2 mg/l BAP + 1 mg/l KIN. In the sixth subculture, rhizogenesis were induced in over 80% of plantlets, which led to acclimatization without the rooting stage. On the peat + vermiculite substrate, 74.56% of the plants survived.

Multiplication by direct organogenesis at *P. quadrangularis*

This experiment aimed to perform the first complete *in vitro* regeneration protocol in *P. quadrangularis*, a recalcitrant species. Among a total of 15 treatments tested for explants asepsis, the most effective was the pretreatment of EtOH (70%, 1 min) + NaClO (50%, 10 min) followed by the treatment with the mixture Rifampicin (15 µg/ml) + Benomyl (2 g/l). By this method of disinfection, 61.67% survived. The best results regarding the regeneration rate and the number of shoots/ explant were obtained on the MS culture medium supplemented with 2 mg/l BAP (33.33%) and the

one with 2 mg/l BAP + 1 mg/l KIN (24.44%). To reduce the effects of leached phenols and sudden browning of explants, the culture media with the highest regeneration rates were supplemented with $AgNO_3$ and PF-68. Now, the regeneration rate has been increased to 84.44% for treatment with 0.2% PF-68 on MS medium supplemented with 2 mg/l BAP. The properties of $AgNO_3$ such as water solubility, easy availability, specificity and stability, find it effective for *in vitro* regeneration leading to a germination rate of 71.11%. From one shoot, a maximum of 7.17 shoots were obtained on the MS medium supplemented with 2 mg/l BAP and 1 mg/l TDZ in the sixth subculture. From the three culture medium variants tested in rooting stage, supplementation of ½ MS medium with 1 mg/l ANA led to rhizogenesis for 61.11% of shoots. At acclimatization two substrates were tested, on the peat + perlite 1:1 substrate, 73.33% of the plants survived.

Multiplication by indirect organogenesis and somatic embryogenesis at *P. quadrangularis*

The interaction between the type of explant and the phytohormones shows that the internode explants were 100% callused on the culture medium containing 2 mg/l 2,4-D and 0.5 mg/l BAP. On this culture medium, the leaf fragments also recorded the highest callogenesis rate (98.33%). For floral explants, culture media supplemented with PIC and KIN have proven to be the most prolific. The formation of the embryogenic callus is influenced both by the composition of the culture medium and by the period of illumination or the culture system. The absence of light associated with phytohormones in the culture medium had the effect of more than doubling the percentage of embryogenic callus, the values being statistically assured. Obtaining an average of 32.67 somatic embryos indicates the potential of multiplication by somatic embryogenesis compared to direct organogenesis. Callus proliferation was also tested depending on the growth system, the liquid medium proved to be superior both in terms of cell mass and dry matter content, which is explained by better access to nutrients and water. As the transfer time and the number of subcultures increase, a specific brown color is recorded as a result of the accumulation of phenols. By supplementing the culture media with additional compounds: $AgNO_3$, PF-68 and coconut water, it is shown that coconut water resulted in higher average values in terms of embryogenic callus (79.17% and 81.83%) and dry weight (0.31 g and 0.34 g) relative to photoperiod/ darkness. After the proliferation of the embryogenic callus, it was transferred to a semi-solid MS base medium supplemented with 0.5 mg/l GA_3 to mature the somatic embryos. Elongation of somatic embryos was observed after approximately 30 days of culture and after four subcultures, plantlets were transferred to MS culture medium with 2 mg/l BAP for development. Following this transfer, plantlets regeneration takes place within two months. Subsequently, they enter in the classic microshooting multiplication system.

Experimental series III - Results on the determination of biochemical and bioactive compounds in *Passiflora*

Biochemical compounds (lipids and proteins) as indicators of the biological quality of the propagated material are synthesized in both species, in both conventionally grown mother plants and acclimatized plants. In organized tissues,

total lipid content predominates in the leaf tissue. In the case of acclimatized plants, the total protein content is higher in the stems of both species and in the callus culture it is low due to the fact that the cells are not functionally specialized. The presence of polyphenols supports the method of unconventional multiplication as a potential source of mass production of phytotherapeutic compounds. The total content of flavonoids is predominantly in the leaves, whether it is the mother plant or the acclimatized plant, in the case of both species. The uniformity of callus cells led to the highest RSA percentage regarding the antioxidant activity of the species.

CONCLUSIONS

Conclusions on the popularity of the genus *Passiflora*

The results of the study highlight that the plants of the genus *Passiflora* stand out and arouse curiosity among people, due to the special flowers and the multiple possibilities of use and decoration that respondents largely know.

Experimental series I - Conclusions on conventional culture for *Passiflora*

Conclusions on the possibilities of vegetative propagation by cuttings

Cuttings treated with AIB 1000 ppm solution recorded the highest percentage of rooting in both species and the most effective rooting substrate proved to be vermiculite. The interaction of the biostimulator with the rooting substrate shows that the untreated cuttings recorded the lowest values of shoot length, which concludes the importance of using the biostimulators both to facilitate the emission of roots and for the good development of newly formed plants. The comparative analysis of the data shows that the rooting substrates do not have a significant influence on the number of shoots developed, but the cuttings rooted in vermiculite obtained more abundant shooting. In the case of *P. quadrangularis*, the data recorded indicates significantly higher values for the number of shoots issued, compared to *P. caerulea*, which indicates a high shooting capacity of the species.

Conclusions on the fertilization regime for controlled system culture

Regarding the average growth of plants in the first year after planting, in *P. caerulea*, fertilization with Cropmax led to the most vigorous growth, the plants reaching an average of 418.4 cm and in *P. quadrangularis* of 434.2 cm. Regarding RGR, significant influences were found especially in the first year. Also, the most intense period of plant growth takes place between June and August, both in *P. caerulea* (2.44) and in *P. quadrangularis* (2.61), following the application of Cropmax fertilizer.

Experimental series II - Conclusions on the possibilities of multiplication by unconventional methods (*in vitro*) for *Passiflora*

Conclusions on multiplication by direct organogenesis for *P. caerulea*

P. caerulea is a reference species for passionflowers micropropagation, the literature proves that it is intensively studied in all types of tissue cultures. The culture medium MS supplemented with 2 mg/l BAP and 1 mg/l KIN determined the regeneration of an average of 88.22% explants, generating several 2.42 shoots per explant, with an average length of 2.10 cm. Subculturing was performed with a rhizogenesis rate of over 80% in the sixth subculture on MS culture medium

supplemented with 2 mg/l BAP + 0.5mg/l ANA. In the acclimatization stage, on the peat + vermiculite mixture substrate, a plant survival rate of 74.56% was registered.

Conclusions on direct organogenesis multiplication for *P. quadrangularis*

The present study led to the development of the first complete regeneration protocol by direct organogenesis in *P. quadrangularis*. Starting from the nodal segments, the protocol proved to be efficient and reproducible. Following the study to determine the optimal disinfection treatment for nodal explants, the most effective formula proved to be 70% EtOH pretreatment, 1 min + 50% NaClO, 10 min followed by treatment with Rifampicin 15 (µg/ml) + Benomyl (2 g/l). In the preliminary stage of initiation on the culture medium MS supplemented with 2 mg/l BAP respectively, MS with 2 mg/l BAP + 1 mg/l KIN the best morphogenic responses were obtained. Supplementation of the culture medium with PF-68 0.2% led to a shooting capacity of 84.44% and PF-68 0.4% led to the maximization of biometric parameters. In the multiplication stage, the addition of phytoregulators BAP and KIN induced the proliferation of shoots which were significantly increased by the addition of PF-68 0.2% surfactant. For *in vitro* rooting, supplementation of ½ MS medium with 1 mg/l ANA proved to be the most prolific. In the acclimatization stage on the peat + vermiculite 1:1 mixture substrate, the highest plant survival rate was recorded.

Conclusions on multiplication by indirect organogenesis and somatic embryogenesis for *P. quadrangularis*

The absence of light associated with phytohormones in the culture medium had the effect of more than doubling the percentage of embryogenic callus. For *P. quadrangularis,* the liquid culture system proved to be superior both in terms of cell mass and dry matter content. Callus multiplication is stimulated by darkness and a rich nutrient substrate (coconut water). Somatic embryogenesis is an important way of *in vitro* clonal propagation for *P. quadrangularis* because recalcitrance is not manifested by this micropropagation technique and due to the high yield of somatic embryo formation it presents, on average 32.67.

Experimental series III - Conclusions on the determination of biochemical and bioactive compounds for *Passiflora*

Lipid and protein synthesis is stimulated in both *P. caerulea* and *P. quadrangularis*. *In vitro* culture is an effective approach to propagation methods, all the more, so as it offers the opportunity to develop the controlled production of important natural metabolites under monitored laboratory conditions, without depending the natural habitats of plants.

RECOMMENDATION

- Based on the study on the popularity level of the genus *Passiflora*, it is recommended to introduce Passifloraceae in the assortment of flowering plants grown in Romania and to promote them among the local producers of ornamental plants.
- Following the research concerning the possibilities of propagation by cuttings, the vermiculite, the mixture of peat + vermiculite 1:1 and the treatment of cuttings with AIB 1000 ppm at the rooting of *Passiflora* cuttings are recommended.

- Foliar fertilization of *Passiflora* is recommended to be done using Cropmax biostimulator due to the increase of biomass of ornamental plants but also in the case of an organic crop, for fruit cultivation, in alimentary purposes.
- *P. caerulea* is a species without micropropagation problems, therefore it is recommended to multiply it by direct organogenesis for industrial production of ornamental plants due to the ensured genetic stability and the quality of the propagating material.
- Although *P. quadrangularis* is recalcitrant to direct *in vitro* organogenesis propagation, this method of propagation is especially recommended for large-scale production of uniformly genetically plant material, but also for germplasm collections of *P. quadrangularis*.
- Somatic embryogenesis is recommended as a method of clonal propagation *in vitro*, on an industrial scale for *P. quadrangularis* due to the high yield of somatic embryo formation and lack of recalcitrance.
- The determination of biochemical and bioactive compounds is recommended in the case of plants important from a phytochemical point of view to confirm the biotechnological delivery of relevant primary and secondary metabolites structurally-cellular and therapeutically, especially since the mother plants are not grown in their native habitats.

ORIGINALITY AND INNOVATIVE CONTRIBUTIONS OF THE THESIS

- Establishing the possibilities of vegetative propagation by cuttings in *P. caerulea* and *P. quadrangularis* to obtain a high propagation efficiency.
- Establishing an optimal fertilization regime for the crop in a controlled system to improve the decorative morphological characteristics.
- Establishment of the first multiplication protocol by direct organogenesis in *P. quadrangularis* that solves the recalcitrance for the *in vitro* culture.
- Establishment of the first multiplication protocol by somatic embryogenesis, with a high yield of somatic embryo formation in *P. quadrangularis.*
- Use of coconut water in the proliferation of embryogenic cultures in *P. quadrangularis*.
- Comparative determination of biochemical and bioactive compounds in plants obtained from conventional and unconventional multiplication protocols.

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