Characterization of Cyclamen genotypes using morphological descriptors, bioactive compounds and DNA molecular markers

(SUMMARY OF THE DOCTORAL THESIS)

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SUMMARY

According to The World Health Organization [1] 60% of medicinal herbs may be used more frequently over the next twenty years, together with approximately 80% of individuals in underdeveloped nations (65% of the world's population) that currently use traditional medicine. The genus Cyclamen includes 24 species and is mainly distributed in the Mediterranean areas with temperate climate [2]. According to the International Union for the Conservation of Nature (IUCN) list *C. purpurascens* Mill. (purple cyclamen) is declining due to its collecting for medical uses in Croatia, whereas *C. libanoticum* Hildebr.(Lebanon cyclamen) is already endangered.

C. persicum is a popular decorative plant with significant commercial value, particularly in the Netherlands, Germany, and Italy. In addition to its ornamental and commercial value, cyclamen is very important in traditional and modern medicine [3-5]. Cyclamen's therapeutic benefits have been known to Romans, Greeks, and Egyptians since antiquity. Ground tubers are used as a traditional remedy to cover infected wounds, psoriases, dermatitis, ulcers, and other skin problems [6,7]. It is used to treat minor skin burns and diseases, as well as as an anti-helminthic, constipation, gainst arthritis, and rheumatism [8]. Cyclamen sp. exhibits significant antioxidant effects due to its phenolic content, and their anti-cancer properties have been demonstrated in HeLa, H1299 cells, HCT 116, HT-29, MDA-MB-231 and BJ cells [9-11].

Due to a scarcity of plant material, their usage in pharmaceutical industry has become a massive concern, contributing significantly to the extinction of plant populations and varieties, natural habitat destruction, and/or species extinction. Only a few Cyclamen species native to Turkey have had their morphological characteristics classified [12,13]. Gene bank materials, such as wild, cultivated, and chosen accessions, are valuable sources of variety for breeders. Thus, over the years, breeders used multiple techniques to examine and quantify the degree of diversity in plant populations [14]. In Cyclamen, genetic variation has been assessed using morphological characteristics and molecular markers such as, random amplified polymorphic DNAs (RAPD) [15], sequence-related amplified polymorphisms (SRAP) [16] and PCR-based start codon targeted (SCoT) markers [3].

The aim of this thesis was to determine germination capacity and morphological characterization of Cyclamen genotypes under GA3 application and different conditions, followed by anthocyanins, carotenoids identification and molecular characterization. The first direction was to evaluate the effects of GA_3 application on seed germination and plant development under different light exposure; germination capacity and plant development of heat-tolerant and heat-sensitive Cyclamen genotypes subjected to heat stress conditions. The second direction was to to determine the total phenolic, flavonoid, anthocyanins, and carotenoids content; identify and quantify the individual carotenoids and chlorophylls in the leaves, and anthocyanins in the flowers of Cyclamen; antioxidant, antimicrobial, and antitumoral activities; and determine the correlation between the above-mentioned activities and identified compounds using multivariate analysis. Additionally, comparison between clustering methods based on phenotypic and molecular markers (SRAP and SCoT markers) has been employed.

The experiments were performed at the Advanced Horticultural Research Institute of Transylvania, Laboratory of Cell Analysis and Spectrometry, Faculty of Horticulture in Business and Rural Development. Part of the experiments was carried out at Faculty of Animal Science and Biotechnologies and Faculty of Veterinary Medicine of of The University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca under the supervision of Acad. Prof. Doru Pamfil.

The main objectives of this PhD thesis were:

Cyclamen species (sowbread) are decorative and medicinal perenials mainly distributed in the Mediterranean area. It displays adaptability to ecological alterations and different environmental conditions, but its commercialization has had a deleterious impact on native populations. As a result of global warming, abiotic stress has emerged as a major source of worry in agricultural productivity. Significant research is being conducted to create techniques to dealing with abiotic challenges, as well as heat-, and drought-tolerant cultivars, and resource management approaches, among other things. Breeders are becoming interested in compounds that protect plants from a variety of severe climatic conditions. Exogenously applied growth regulators, such as gibberellic acids (GAs), proves to be advantageous in alleviating plants physiological status subjected to abiotic conditions.

In this regard, the aim of the thesis was to assess the effects of GA3 administration on seed germination and plant growth, as well as the ideal GA_3 dose applied to Cyclamen accessions as influenced by varying levels of sunlight. Additionally, water scarcity and other (a)biotic conditions may have an impact on crop quality and yield, which are both influenced by temperature and water accessibility. Considering these aspects, the present study aimed to non-destructively assess the germination capacity and plant development of heat-tolerant and heat-sensitive Cyclamen genotypes subjected to heat stress conditions. Furthermore, the present study aimed to analyze the genetic diversity of selected germplasm for abiotic stress tolerance and select genotypes with higher heat tolerance.

Apart from the ornamental quality, Cyclamen possesses strong antioxidant and anti-cancer activities. Considering this aspect, a second aim was to determine the total phenolic, flavonoid, anthocyanins, and carotenoids content from the leaves and flowers of Cyclamen. Furthermore, we aimed to determine the antioxidant, antimicrobial, and antitumoral activities; and finally determine the correlation between the above-mentioned activities and identified compounds using multivariate analysis.

The final part of the thesis presents the comparison between several correlation matrices and HCA, and evaluation of the genetic diversity and divergence in Cyclamen genotypes by the use of morphological, molecular, and combined data. Considering the importance of morphological and molecular characterization for the improvement of cultivated Cyclamen, this study evaluated 32 genotypes using 36 morphological traits, color indicators and molecular characterization (SRAP and SCoT markers) in a multivariate analysis.

In order to achieve the aim of the thesis, the following aspects were investigated and materialized into four objectives:

O.1. To assess the effect of different GA_3 concentrations on seed germination, growth, and ornamental quality of selected Cyclamen species under short- and long-day conditions.
O.2. Evaluate the germination and morphological parameters of heat-tolerant and heat-sensitive Cyclamen persicum accessions in the presence of different GA_3 solutions under ambient temperature and heat stress conditions;
O.3 Evaluate and compare in vitro antioxidant, antimicrobial, anticancer activities, and the individual carotenoids and anthocyanins content of methanol extracts of Cyclamen genotypes;
O.4. Investigation of the genetic diversity of Cyclamen genotypes using multivariate analysis for morphological traits and molecular characterization;

The results of this thesis were published in two review articles (one ISI indexed journal with IF 0.456 – in *Acta Poloniae Pharmaceutica* Journal; the second BDI in *BUASVM Cluj-Napoca. Horticulture*), and one book chapter to be published in Springer Nature. In addition, four original articles (one ISI indexed journal with IF 2.259 – in *Agronomy* Journal; the second ISI indexed journal with IF 6.313 – in *Antioxidants* Journal; the third ISI indexed journal with IF 4.658 – in *Plants* Journal; the fourth ISI indexed journal with IF 6.627 – in *Frontiers in Plant Science* Journal) were published.

The studies and experiments described in this thesis were performed within the Advanced Horticultural Research Institute of Transylvania from The University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca.

The PhD thesis is structured in two main parts: state-of-the art containing two literature review articles (**Chapter 1**) and original research containing the working hypothesis/objectives (Chapter 1 from the second part) and the general methodologies (**Chapter 2**), followed by the own research articles and one book chapter (**Chapters 3-7**), general conclusions and recommendations (**Chapter 8**), respectively the originality and innovative contributions of the thesis (**Chapter 9**).

Regarding the literature review articles, studies were identified by conducting PubMed, Web of Science Core Collection, Scopus, and Google Scholar electronic searches. After the literature screening presented within the state of the art, gibberelic acid proves to be an environmentally friendly bioregulator, which is widely used to enhance the productivity and phenotypic characteristics of multiple ornamental crops. Cyclamen genotypes prove to be useful as anticancer agents due to their highest phenolic content, but also demonstrate significant antioxidant and antimicrobial properties. The strategies investigated, like germination capacity, plant development, resistance to abiotic stresses, morphological and molecular characterization, support the importance of Cyclamen as ornamental and medicinal plants.

The experiments, which involved the submergence of Cyclamen seeds in different concentrations of GA_3 followed by screening of plants developmental stages, were conducted under controlled and stress conditions (**Figure 1**). Furthermore, morphological parameters of heat-tolerant and heat-sensitive *Cyclamen persicum* accessions in the presence of different GA_3 solutions were evaluated under control and heat stress conditions using multivariate analysis (**Figure 2**). The individual carotenoid and anthocyanin profiles of several Cyclamen genotypes were evaluated, along with the possible correlation between carotenoids, anthocyanins, and color characteristics as a starting point to establish a fast, cost-effective, and accurate pigment content estimation (**Figure 3**).

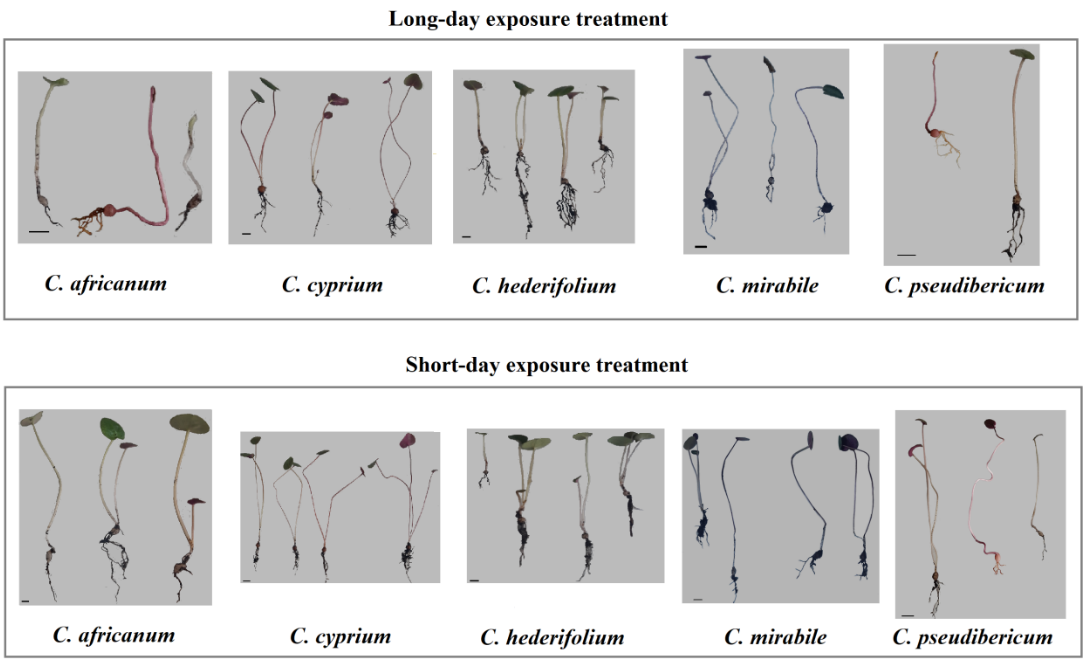

Figure 1. Variation in germination rate and seedling development under different GA_3 concentrations (control, 50 mg/L, 100 mg/L, 150 mg/L) in Cyclamen genotypes subjected to long-day or short-day conditions (https://doi.org/10.3390/agronomy10040516).

The last study presents the comparison of several correlation matrices and hierarchical clustering for investigating genetic diversity in Cyclamen, and evaluation of genetic diversity and divergence in Cyclamen genotypes by the use of morphological, molecular, and combined data (**Figure 4**). The analyses and determinations were performed in duplicates and triplicates for every individual study and analysis.

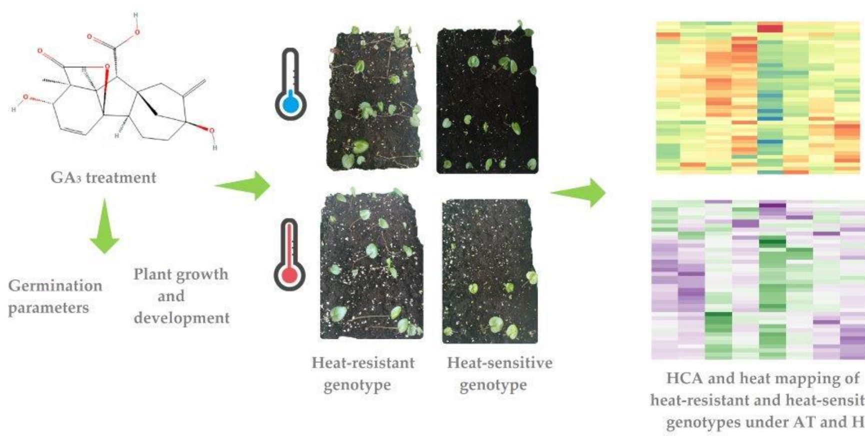

Figura 2. Variația în dezvoltarea plantelor la genotipurile de Cyclamen rezistente și sensibile la căldură în condiții de stres termic (https://doi.org/10.3390/plants11141868)

The statistical analyses were performed using GraphPad Prism 8.2.1.441 software (Graph Pad Software Inc., San Diego, CA, USA) and XLSTAT software (Addinsoft, New York, NY, USA). The results were expressed as the means ± standard deviation (SD). In the first study, the significance of the differences between GA3 treatments was tested by applying a one-way analysis of variance (ANOVA), at a confidence level of 95%. Tukey's Post hoc test was carried out to establish the statistically significant differences at p <

0.05. In the second study, data collected were analyzed with the analysis of variance technique (ANOVA) using HSD Tukey's test ($p < 0.05$) and presented as the interaction of ambient and high temperature stress and GA_3 treatment. Heatmap, dendrograms, correlation matrices and diversity matrices were generated using Paleontological Statistics (PAST) software (version 4.0, Oslo, Norway) and R software (version 4.2.2), respectively.

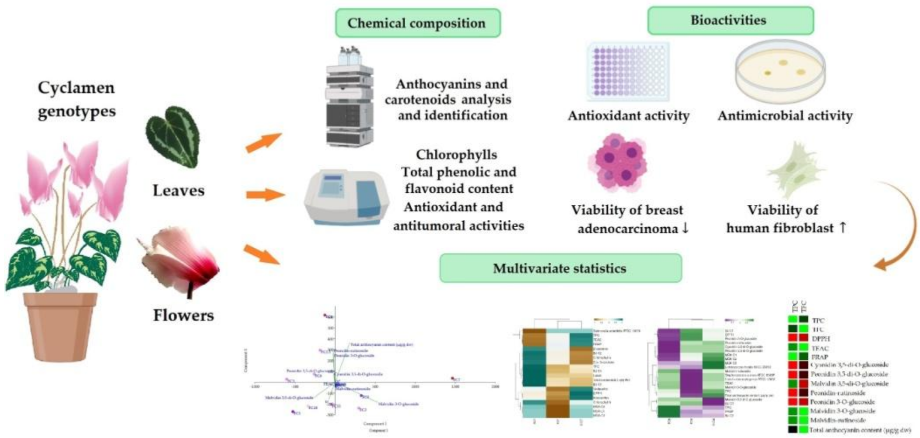

Figure 3. Carotenoids and Anthocyanins Identified in the Flowers and Leaves of Cyclamen Genotypes are associated with their antioxidant, antimicrobial and anticancer activities (https://doi.org/10.3390/antiox11061126)

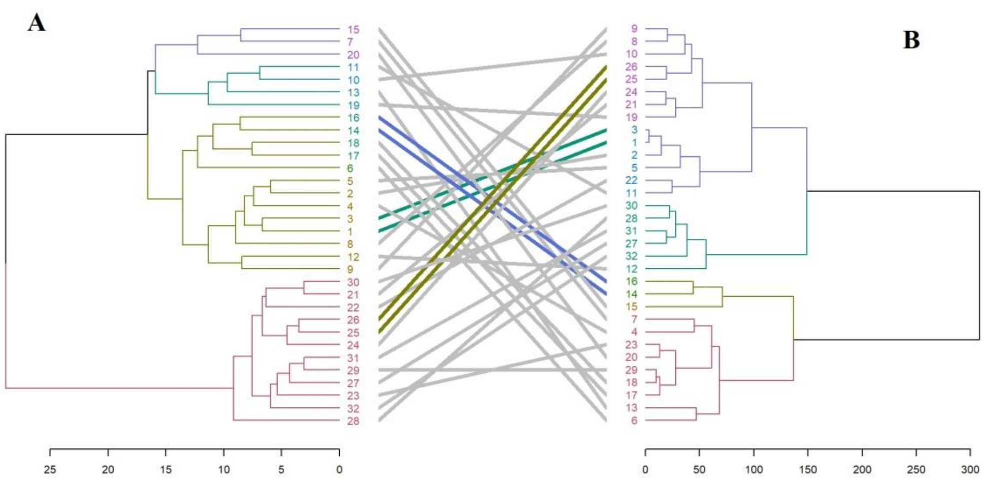

Figure 4. Comparison of dendrograms for the 32 Cyclamen genotypes from SRAP markers (A), and phenotypic data (B) with entanglement = 0.30. The grey lines in connecting the dendrograms correspond to mismatched genotypes whereas the colored lines are genotypes that maintained their position between clusters (https://doi.org/10.3389/fpls.2023.1100099).

The results obtained in the research articles mentioned above can be resumed as follows:

Chapter 3 presents the origin, distribution, taxonomy, economic significance, health advantages, genetic resources, cultivation practices, traditional breeding methods, and contemporary breeding methods utilized in the improvement initiatives of Cyclamen species.

Chapter 4 presents the response of long and short day exposed Cyclamen species under different GA3 concentrations. These findings reveal the response of these plants to high and low light intensity stress, as well as the germination capability induced by GA_3 treatment. All of the investigated species had unique morphological characteristics and germination parameters, allowing them to be easily differentiated based on germination properties.

Chapter 5 focuses on the priming effects of GA_3 on seed germination and plant growth, as well as the most beneficial GA_3 dose for Cyclamen accessions under ambient temperature and heat stress conditions This chapter demonstrates that the use of optimal GA3 concentrations is a promsisng step that leads to selection of resistant genotypes which might be used in future studies to assess their resistance under heat and/or drought stress, but also subjected to different growth hormone regulators.

Chapter 6 presents the antioxidant, antimicrobial, anticancer activities, and the individual carotenoids and anthocyanins content of methanol extracts of Cyclamen genotypes. This chapter the leaves of *C. persicum* accessions, *C. mirabile*, and *C. hederifoilium* are diverse in terms of their carotenoid profile and their antioxidant, antimicrobial, and antitumor activities. The Cyclamen flowers exhibited increased antibacterial and antitumor activities, emphasizing their importance as a natural source of antioxidants. The composition of anthocyanins and carotenoids contributes to the research of physiologically active substances discovered in the examined Cyclamen genotypes.

Chapter 7 presents the combination of phenotypic and genetic analyses, which increased the thorough assessment of true diversity in the Cyclamen genotypes. The current study's findings are valuable for assessing variety and genetic variability in Cyclamen breeding and genetic studies.

The general conclusions were:

1. In the long-day exposure treatment, the optimal GA3 concentration for plant growth was 150 mg/L in *C. cyprium* and *C. mirabile*. Increased plant elongation was seen in *C. cyprium* and *C. mirabile* in the short-day exposure treatment, followed by C. pseudibericum at concentrations of 50 and 100 mg/L, respectively.
2. Seedling vigor and ornamental quality were increased by GA_3 application, an economic strategy for enhancing germination and growth of selected Cyclamen species.
3. Heat tolerant genotypes, mainly C3 (6.42%), C15 (6.47%) and C16 (5.12%) had the highest germination rate with 90 mg/L GA3 treatment compared with control.
4. Under heat stress in heat-tolerant genotypes C9, C10 and C14 presented the highest plant development with 70 mg/L GA_3 treatment, with values between 12.79 and 15.43 cm.
5. Cyclamen leaves have significant contents of lutein and β-carotene with potential anticancer properties. The highest carotenoid content was noticed in *C. persicum* Merengue Magenta with 228.34 μg/g dw and the lowest in Origami with 79.62 μg/g dw.
6. The flowers of Cyclamen exhibited increased antimicrobial and antitumor activities, emphasizing their usage as natural antioxidant agents. The highest anthocyanins content was observed in *C. persicum* Halios falbala with 1673.6 μg/g dw and the lowest with 564.77 μg/g in *C. mirabile*.
7. *C. persicum* Halios falbala exhibited the highest antimicrobial activity that may be due to its high content in malvidin 3-O-glucoside and malvidin rutinoside.
8. The flowers of *C. hederifolium* demonstrated the highest activity against MDA-MB-231 cell lines regardless of the used concentration that might be due to its high phenolic and flavonoid content, but also due to its increased levels in malvidin 3,5-di-O-glucoside (357.53 μg/g dw), malvidin 3-O-glucoside (127.55 μg/g dw), and malvidin rutinoside (95.07 μg/g dw).
9. High cytotoxic activity against MDA-MB-231 and BJ cell lines was noticed in the leaves of *C. mirabile* that presented the highest levels in neoxanthin (31.13 μg/g dw) and violaxanthin (22.48 μg/g dw)

10. PCA usually supported the grouping of phenotypic features into species-specific clusters, as seen by C. persicum genotypes that clustered mostly in the second and third quadrants. PC1's significant variables were NS, PL, FA, LN, CA, and BCRD, as shown by the greatest eigenvalues, whereas PC2 accounted for flower color features.

11. Genetic diversity analysis generated with the SRAP (r = 0.95) and SCoT (r= 0.82) markers revealed a clear discrimination of the Cyclamen genotypes, as genetically-related genotypes clustered in the same group.

12. Combined molecular analysis revealed a more accurate genetic estimate between the genotypes according to their phylogeny, organizing the genotypes to their respective Gyrophoebe subgenera in the 2^{nd} cluster, whereas 3^{rd} cluster comprised genotypes corresponding to Cyclamen subgenus.

13. As seen by the high entanglement analysis (0.30) between the dendrograms, SRAP markers demonstrated to be effective tools for the separation of Cyclamen species and/or genotypes when combined with phenotypic data.

14. Colorimetric data for leaves (entanglement=0.47), flowers (entanglement=0.45) and SCoT markers revealed moderate similarities.

Future perspectives:

1. Treatment with GA_3 can improve seedling vigor and ornamental quality in a dose dependent manner that can be further used as a cost-effective technique for increasing germination capacity and development of Cyclamen genotypes.

2. The genotypes with the highest seed germination capacity and plant development may be chosen as heat-resistant genotypes to be deposited in germplasm banks and employed in subsequent improvement programs under biotic and/or abiotic conditions to obtain tolerant genotypes.

3. Chemical parameters and bioactivities evaluated using multivariate statistics (PCA, HCA, and dendrograms) could be a useful guidance for researchers and breeders in selecting genotypes based on their carotenoid and anthocyanin profile, but also on their potential therapeutic and ornamental quality.

4. Further in vitro pharmacological investigations of specific isolated compounds from Cyclamen are needed to better understand their use in several medicinal fields.

5. Multivariate analysis approaches can be further used to precisely classify various Cyclamen plants based on their agro-morphological, molecular, chemical composition, or bioactivities that are regularly compared with correlation coefficients. This may contribute to a more comprehensive genetic diversity analysis within and among species, phylogenetic investigations, and fingerprinting in Cyclamen.

ORIGINALITY AND PERSONAL CONTRIBUTIONS

The results presented in this thesis might be considered helpful for the scientific community from the biotechnology, breeding, and horticulture fields. This research can be considered a comprehensive study presenting the response of multiple Cyclamen species to different concentrations of GA3 under short, long day exposure and abiotic stress. These methods were studied as a potential way to shorten the germination and propagation period of these Cyclamen species and chosing heat-resistant genotypes. Furthermore, the present thesis aimed to non-destructively evaluate the germination capacity and plant development of heat-tolerant and heat-sensitive Cyclamen genotypes subjected to heat stress conditions. The heat resistant genotypes can be further used in breeding programs with different stress designs and hormone treatments to produce increased heat tolerance in crop generations that may lead to high production.

Individual carotenoids and anthocyanins in these selected Cyclamen species provide a foundation for future research to highlight the significance of their biological activity and therapeutic qualities. According to the most recent comprehensive review study on this topic, there are no scientific articles investigating these compounds in the Cyclamen genotypes used in the present thesis. The freeze-drying process was carried out to determine the water content and compare the experimental results with literature data often expressed as dry weight. The identification of carotenoid and anthocycanin profiles in selected Cyclamen genotypes may offer a better knowledge of the therapeutical value, since the separated chemicals may be employed as health-promoting agents in the treatment of various diseases. Also, the color parameters of Cyclamen leaves and flowers were measured, which may help in discriminating similar genotypes based on their color measurements. The overall level of polyphenolic and flavonoid components, which can act synergistically with the concentration of anthocyanins, can be associated with the potential antibacterial activity of *C. persicum*. Cyclamen species, particularly *C. persicum* and *C. hederifolium*, prove to be potential anticancer agents due to their significantly high content in lutein and β-carotene in leaves and malvidin 3-O-glucoside and cyanidin 3,5-di-O-glucoside in flowers.

A further objective was to better discriminate the samples using both PCA and HCA based on isolated compounds and biological activities. Both analyses showed the similarities and differences among flowers and leaves samples based on their antibacterial and citotoxicity activities.

Finally, our last objective was to compare several correlation matrices and HCA for investigating genetic diversity in Cyclamen, and evaluate the genetic diversity and divergence in Cyclamen genotypes using morphological, molecular, and combined data. The samples were grouped based on their similarities and differences of phenotypic, and color characterisitics. The analysis of genetic data revealed low variability between the genotypes used in the present thesis, with a few outliers indicated by the varied color spectrum. In contrast, higher variability was noticed between Cyclamen genotypes compared with *C. persicum* accessions. Entanglement analysis (0.30) among dendrograms obtained from the morphological and SRAP anlayses showed the highest association compared with the other analyses. Thus, colorimetric data for leaves and SCoT data presented lower similarities (entanglement=0.47) compared to colorimetric data for flowers and SCoT markers (entanglement=0.45). Conversely, the molecular markers employed (SRAP and SCoT) showed the lowest similarities with no entanglement, which is not surprising given that these primers are not specific for Cyclamen. To our knowledge, this is the first study that compares the phenotypic, molecular and combined data of Cyclamen genotypes using multivariate analysis techniques.

SELECTIVE BIBLIOGRAPHY

1. WHO. World Health Organization-Traditional Medicine; WHO: Geneva, Switzerland, 2008.
2. Grey-Wilson. Cyclamen (A Guide for Gardeners, Horticulturists and Botanists); Grey-Wilson: London, UK, 2015.
3. Cornea-Cipcigan M, Pamfil D, Sisea CR and Margaoan R (2023) Characterization of Cyclamen genotypes using morphological descriptors and DNA molecular markers in a multivariate analysis. Front. Plant Sci. 14:1100099. doi: 10.3389/fpls.2023.1100099
4. Sarikurkcu, C. (2011). Antioxidant activities of solvent extracts from endemic Cyclamen mirabile hildebr. tubers and leaves. Afr. J. Biotechnol. 10 (5), 831–839.
5. Turan, M., and Mammadov, R. (2018). Antioxidant, antimicrobial, cytotoxic, larvicidal and anthelmintic activities and phenolic contents of & cyclamen alpinum. J. Pharmacol. Pharm. 09 (04), 17. doi: 10.4236/pp.2018.94008
6. Ali-Shtayeh, M.S.; Yaniv, Z.; Mahajna, J. Ethnobotanical survey in the Palestinian area: A classification of the healing potential of medicinal plants. J. Ethnopharmacol. 2000, 73, 221–232.
7. Ali-Shtayeh, M.S.; Jamous, R.M.; Al-Shafie, J.H.; Elgharabah, W.A.; Kherfan, et al. Traditional knowledge of wild edible plants used in Palestine (NorthernWest Bank): A comparative study. J. Ethnobiol. Ethnomed. 2008, 4, 13
8. Blasdale,W.C. Cyclamen Persicum. Its Natural and Cultivated Forms; Stanford University Press: London, UK, 1953; p. 49.
9. Mihci-Gaidi, G., Pertuit, D., Miyamoto, T., Mirjolet, J.-F., Duchamp, O., Mitaine-Offer, A.-C., et al. (2010b). Triterpene saponins from cyclamen persicum. Natural Product Commun. 5, 1023–1025. doi: 10.1177/1934578X1000500707
10. Yildiz, M., Bozcu, H., Tokgun, O., Karagur, E. R., Akyurt, O., and Akca, H. (2013). Cyclamen exerts cytotoxicity in solid tumor cell lines: a step toward new anticancer agents? Asian Pacific J. Cancer Prev. 14 (10), 5911–5913. doi: 10.7314/ APJCP.2013.14.10.5911
11. Cornea-Cipcigan, M., Bunea, A., Bouari, C. M., Pamfil, D., Páll, E., Urcan, A. C., et al. (2022a). Anthocyanins and carotenoids characterization in flowers and leaves of cyclamen genotypes linked with bioactivities using multivariate analysis techniques. Antioxidants 11, 1126. doi: 10.3390/antiox11061126
12. Curuk, P., Sogut, Z., Bozdogan, E., Izgu, T., Sevindik, B., Mohammad Tagipur, E., et al. (2015). Morphological characterization of cyclamen sp. grown naturally in Turkey: Part I. South Afr. J. Bot. 100, 7–15. doi: 10.1016/j.sajb.2015.03.199
13. Curuk, P., Sogut, Z., Izgu, T., Sevindik, B., Tagipur, E. M., Da Silva, J., et al. (2016). Morphological characterization of cyclamen sp. grown naturally in Turkey: Part II. Acta Scientiarum Polonorum Hortorum Cultus 15, 205–224.
14. Bhandari, H. R., Bhanu, A. N., Srivastava, K., Singh, M. N., and Shreya, H. A. (2017). Assessment of genetic diversity in crop plants-an overview. Adv. Plants Agric. Res. 7 (3), 279–286.
15. Taşkin, B. G., Vardareli, N., Doğaç, E., Mammadov, R., and Taşkin, V. (2012). Genetic diversity of natural cyclamen alpinum populations. Turkish J. Biol. 36 (4), 413–422.
16. Simsek, O., Curuk, P., Aslan, F., Bayramoglu, M., Izgu, T., Da Silva, J., et al. (2017). Molecular characterization of cyclamen species collected from different parts of Turkey by RAPD and SRAP markers. Biochem. Genet. 55, 87–102.