

PYRETHRINS PROFILES AND CONTENTS IN SEEDLINGS,
IN VITRO CULTURES, AND ACCLIMATIZED PLANTS OF
TANACETUM CINERARIIFOLIUM CLONES
(ASTERACEAE)

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Abstract

Tanacetum cinerariifolium (Trevir.) Sch. Bip., Asteraceae, known as pyrethrum, is a perennial herb endemic to the eastern Adriatic coast. It is cultivated worldwide for its valuable secondary metabolites: pyrethrins, which are natural insecticides. Pyrethrins are monoterpene esters of chrysanthemum acid (Pyrethrin-I, Cinerin-I, Jasmolin-I) or pyrethric acid (Pyrethrin-II, Cinerin-II, Jasmolin-II) with lethal or knock-down insecticidal properties, respectively. The market demand of pyrethrin-based products is increasing due to the organic farming requirements. In the present study the content of pyrethrins was determined by GC/MS in n-hexane extracts of shoots of several in vitro clones and in flower heads of outdoor acclimatized plants from the same clones during primary and secondary blooming, as well as in seedlings grown on different soil types. Statistical analyses were done by One-way ANOVA Post Hoc LSD analysis. All pyrethrins were detected in all samples, including a Pyrethrin-I isomer but they differed in content and proportion. As expected, pyrethrins in in vitro shoots and in seedlings were in small amounts. However, in the flower heads of the acclimatized plants, pyrethrin content was comparable to

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that of the native Dalmatian populations. The main component in all samples was Pyrethrin-I, possessing the strongest insecticidal activity, with the highest content in the flower heads of the primary flowering: 4.87 mg/g with a CV of 24.9%. Statistical differences were noted between clones for Pyrethrin-II and the Pyrethrin-I isomer in June samples, and Pyrethrin-I in September samples ($p < 0.05$). A wide range of variation was also found in seedlings due to different soil types. Growing conditions should be optimized in terms of sunlight and watering, and further research on pyrethrin content should be carried out in several consecutive years.

Key words: Dalmatian pyrethrum, bio insecticides, *Chrysanthemum cinerariifolium*

Introduction. *Tanacetum cinerariifolium* (Trevir.) Sch. Bip., Asteraceae, commonly known as pyrethrum, is a perennial herb endemic to the eastern Adriatic coast. It is cultivated worldwide for its valuable secondary metabolites: pyrethrins, which are natural insecticides. They are contained in all parts of the plant, but mostly in the flowers, namely: 93.7% of pyrethrins accumulate in the seeds [1,2]. Dried parts of pyrethrum were used as an insecticide in folk medicine in Croatia [2]. Natural pyrethrins have some of the qualities of an ideal insect control agent [1,3], acting as a contact insecticide [2]. They are unstable when exposed to UV light, oxygen, water and at elevated temperatures, making them easily degraded [2,4]. The term “pyrethrins” refers to the six insecticidally active ingredients contained in *T. cinerariifolium* [5,6]. Pyrethrins-I (Pyrethrin-I, Cinerin-I and Jasmolin-I) are monoterpene esters of chrysanthemum acid with lethal insecticidal properties, while Pyrethrins-II (Pyrethrin-II, Cinerin-II and Jasmolin-II) are monoterpene esters of pyrethric acid, which have a knockdown effect on insects [6–10]. Pyrethrins are found in an approximate ratio of 1.08 Pyrethrins-I/Pyrethrins-II, although it showed uneven patterns over time [11]. According to some authors, Pyrethrin-II is most likely derived from Pyrethrin-I [9]. Accumulation of pyrethrins across flower development stages was studied in order to determine precisely the optimal harvest [11].

The market demand of pyrethrin-based products is increasing due to the organic farming requirements. In temperate latitudes pyrethrum plants bloom twice per year. Planting material of high quality is of crucial importance for establishment of pyrethrum plantation. In vitro clonal propagation is a powerful method for rapid multiplication of selected high-productive individuals. In 2020, as part of our previous research, in vitro obtained plants of four clones were ex vitro adapted and acclimated outdoor in the ex situ collection of IBER [12]. The present study aimed to check the content of pyrethrins in the shoots of several in vitro clones and to compare them with those accumulated in the flower heads of outdoor acclimatized plants from the same clones, collected during primary and secondary blooming. In addition, the content of pyrethrins was determined in seedlings grown on different types of soil.

Material and methods. Plant material. In vitro clones of pyrethrum were initiated, micropropagated, ex vitro adapted and outdoor acclimated as described previously [12]. Pyrethrins in the in vitro cultures were determined in 2 parallel samples per clone, each sample consisting of 32 dried in vitro shoots about 6–7 cm high. Four acclimated plants per clone were tested twice in full flowering in June and in September, 2 samples per plant per season, each comprising 10 dried flower heads. Seedlings were obtained from seeds germinated on six different soil types [13] and pyrethrins were determined in 2 parallel aggregated samples per soil type, consisting of 14 dried 2-month-old seedlings of about 10 cm height each.

Extraction. One hundred milligrams powdered plant material was macerated with 1 mL n-hexane in 2 mL Eppendorf tubes. Fifty μ L of nonadecanoic acid (1 mg/mL) were placed at the beginning of the extraction procedure as an internal standard. After 24 h of extraction at room temperature, an aliquot of 500 μ L of each sample was transferred to a glass vial.

Gas-chromatography-mass spectrometry analysis (GC-MS). GC/MS analysis of hexane extracts was recorded on a Thermo Scientific Focus GC coupled with a Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) was used. The conditions of the analysis were as described in [14]. The compounds were identified by comparing their mass spectra and retention indices (RI) with standard compounds.

Statistical analyses. Statistical analyses were done by One-way ANOVA Post Hoc LSD analysis.

Results and discussion. All pyrethrins were detected in all samples, including an isomer of Pyrethrin-I; however, their contents and proportions were different. As expected, pyrethrins in in vitro shoots and in seedlings were in small amounts (Fig. 1), because their normal localization is mainly in the flower heads [1,2]. In vitro clones differed both in total pyrethrin content and in proportions between the seven components (Fig. 1A). In the clones with the lowest average content of pyrethrins (clone 1 and clone 2) a higher content of Pyrethrin II was observed compared to Pyrethrin I, while in the other 3 clones Pyrethrin I prevailed over Pyrethrin II. In all clones examined, the content of Cinerin I was lower compared to Cinerin II, and the content of Jasmolin I and II were almost equal, with slightly higher levels of Jasmolin I. The isomer, Pyrethrin I, was in the lowest concentrations, compared to the other pyrethrins. In seedlings growing on different soil types, no clear regularity was observed in the ratio of Pyrethrin I and Pyrethrin II (Fig. 1B). Similar to the in vitro cultures, a predominance of Cinerin II content compared to Cinerin I was observed, and the content of Jasmolin I and II was almost equal. In both, in vitro shoots and seedlings, concentrations of the Pyrethrin I isomer were up to several times lower than those of Pyrethrin I.

In flower heads collected in June during primary flowering of plants acclimated outdoor, in all clones the average content of Pyrethrin I was higher than that of Pyrethrin II (Fig. 2). As in the in vitro clones, the content of Cinerin II was



Fig. 1. Content of pyrethrins [mg/g DW] and ratio between their seven components: **A)** in in vitro shoots; **B)** in seedlings growing on different soil types

higher than that of Cinerin I, except for clone 2. The amount of Jasmolin I slightly prevailed over that of Jasmolin II in clones 2 and 3, while in clones 1 and 4 the opposite trend was noticed. The greatest variation in both chrysanthemic acid esters (Pyrethrins I, including the Pyrethrin I isomer), and pyrethric acid esters (Pyrethrins II) was observed in clone 2. The variation of Pyrethrins I was least in clone 1 and that of Pyrethrins II was least in clone 3. A statistically significant difference ($p < 0.05$; LSD-Post Hoc) was observed in clones 2 and 4,

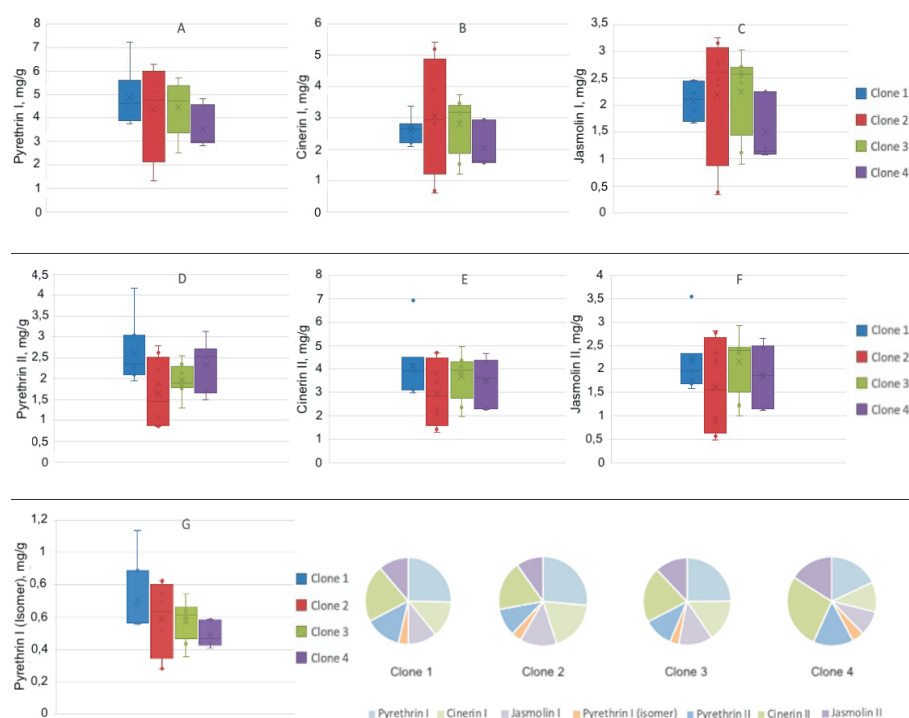


Fig. 2. Content of pyrethrins [mg/g DW] and ratio between the seven components in flower heads at primary flowering (June, 2020)

regarding the ratio of the corresponding chrysanthemum acid ester to the same pyrethric acid ester (Fig. 2).

During the secondary flowering, the levels of the studied secondary metabolites in the flower heads were many times lower (Fig. 3). The ratio between different pyrethrins also varied. Pyrethrin I content was lower compared to Pyrethrin II and Cinerin I content was higher compared to Cinerin II, both trends differing from both primary flowering and in vitro cultures. For six of the seven compounds tested, clone 4 showed the lowest variation, with only Jasmolin II having the lowest variation in clone 1. For most pyrethrins, the highest variation was observed in clone 3, and only for Pyrethrin I isomer and Jasmolin II the variation was greater in clone 2. Statistically significant differences ($p < 0.05$; LSD-Post Hoc) were found in the Pyrethrin I/II ratio between clones 2 and 3 and between clones 2 and 4. In the Jasmolin I/II ratio, statistically significant differences ($p < 0.05$; LSD-Post Hoc) were observed between clones 1 and 2; clones 1 and 3; clones 2 and 4. No statistically significant differences were observed between clones in terms of Cinerin I/II ratio. In all four clones, the Pyrethrin I/II ratio at primary flowering was greater than 1, while at secondary flowering in all clones, the Pyrethrin I/II ratio was less than 1. Similar trend was observed in several Dalmatian natural

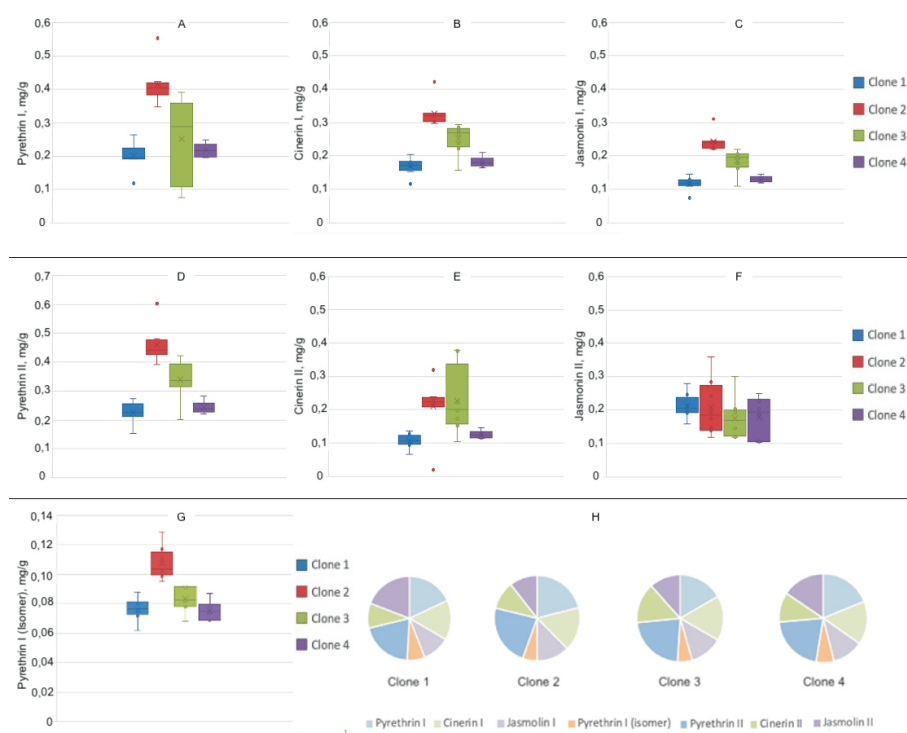


Fig. 3. Content of pyrethrins [mg/g DW] and ratio between the seven components in flower heads at secondary flowering (September, 2020)

populations [11]. The difference in pyrethrin content may be due to the uneven growth of the plants, due to insufficient spacing between them and the shading of the plants of clone 4 by a neighbouring tree, as noted in our previous studies [12]. According to recently reported results, flowering stage was found to be of crucial importance for total content of pyrethrins in Dalmatian populations studied, the maximal values detected at stage FS4 (2–5 rows disc florets open) [11]. As for our study, in primary flowering the flower heads were collected during this stage, but in secondary flowering they were collected later, during FS7 (ray florets dry; disc florets intact but with little colour [11]), which may be a significant reason for the lower pyrethrin content.

It is worth noting that the main component in all samples was Pyrethrin-I, whose highest content in the flower heads collected in June reached an average of 4.87 mg/g with a CV of 24.9%. This is important as this compound possesses the strongest insecticidal activity [2]. Statistical differences between clones were noticed only for Pyrethrin-II and the isomer of Pyrethrin-I in June samples, and Pyrethrin-I in September samples ($p < 0.05$). The ratio of Pyrethrins-I/Pyrethrins-II is considered to be an indicator of the quality of pyrethrin products, and this ratio varies widely in flower heads [6,15,16]. Depending on the pur-

pose of insect control, preparation with a predominance of Pyrethrin I or II may be preferred. High levels of variation were observed, especially for the summer flowering.

Data concerning aerial plant parts also showed a wide range of variation, not only in in vitro clones but also in seedlings growing on different soil types, thus confirming previous results regarding the influence of soil conditions on pyrethrin biosynthesis [5,17]. The complex impact of biotic and abiotic factors on the synthesis of the investigated pyrethrins has been discussed by various authors. Sunlight has been shown to be a factor crucial for the biosynthesis of pyrethrins [18]. The origin of the plant material, climatic and soil conditions are also essential [2,5,11,19]. In our case, the critical impact of sunlight as well as waterlogging of the soil may explain the significantly lower levels of pyrethrin content in the secondary bloom. The content of pyrethrins in flower heads collected in June was comparable to that reported for the native Dalmatian populations [11,17].

Conclusions. The use of plant biotechnology, namely in vitro clonal propagation, is promising for the rapid production of pyrethrum seedlings for agriculture. The established differences in the studied clones in terms of the content of pyrethrins and the ratio of the individual components allow the selection of suitable starting plant material, in accordance with the sought-after biological insecticide products on the market. More clones could be selected with reduced variation in pyrethrin content and different ratio of pyrethrins. This, in turn, would allow a better prediction of expected yields and a greater opportunity to create different commercial products differing in the ratio of pyrethrins. It is necessary to optimize the growing conditions (distance between plants, watering, and soil type) in relation to the climatic conditions in Bulgaria and to carry out additional research on the content of pyrethrins in several consecutive years during the optimal harvest time.

REFERENCES

- [1] JOVETIC S. (1994) Natural Pyrethrins and Biotechnological Alternatives, *Biotechnology and Development Monitor*, **21**, 12–13.
- [2] GRDIŠA M., K. CAROVIĆ-STANKO, I. KOLAK, Z. ŠATOVIĆ (2009) Morphological and biochemical diversity of Dalmatian pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Sch. Bip.), *Agr. Con. Scien. (ACS)*, **74**(2), 73–80.
- [3] PALMQUIST K., J. SALATAS, A. FAIRBROTHER (2012) Pyrethroid insecticides: Use, environmental fate, and ecotoxicology, *Insecticides – Advances in Integrated Pest Management*, 251–278.
- [4] GREENHILL M. (2007) Pyrethrum production: Tasmanian–success story, *Chr. Hor.*, **47**(3), 5–8.
- [5] McDONNELL C. C., W. ABBOTT, W. DAVIDSON, G. KEENAN, O. NELSON (1930) Relative insecticidal value of commercial grades of pyrethrum, *US Gov. Print. Off.*, **198**, 1–10.

- [6] HEAD S. W. (1973) Composition of pyrethrum extract and analysis of pyrethrins. In: *Pyrethrum: The Natural Insecticide*, 25–48.
- [7] ESSIG K., Z. ZHAO (2001) Preparation and characterization of a Pyrethrum extract standard, LC GC North Am., **19**(7), 722–730.
- [8] CASIDA J. (2012) *Pyrethrum: the natural insecticide*. Acad. Press, London, 323.
- [9] RAMIREZ A. (2013) *Pyrethrum Secondary Metabolism: Biosynthesis, localization and ecology of defense compounds*, Wageningen, Wageningen University, 190.
- [10] DAVIES T. G. E., L. M. FIELD, P. N. R. USHERWOOD, M. S. WILLIAMSON (2007) DDT, pyrethrins, pyrethroids and insect sodium channels, IUBMB Life, **59**(3), 151–162.
- [11] GRDIŠA M., N. JERAN, F. VARGA, T. KLEPO, T. NINČEVIĆ et al. (2022) Accumulation Patterns of Six Pyrethrin Compounds across the Flower Developmental Stages — Comparative Analysis in Six Natural Dalmatian Pyrethrum Populations, *Agron.*, **12**(2), 252.
- [12] ILINKIN V., B. TRAYKOVA, M. STANILOVA (2023) *In vitro* clonal propagation of *Tanacetum cinerariifolium* and establishment of an *ex situ* collection of selected clones, *BioRisk*, **20**, 97–114.
- [13] ILINKIN V. (2019) Germination of *Tanacetum cinerariifolium* seeds on different soil types, *Ecol. Eng. Environ. Prot.*, **2**, 67–74.
- [14] BERKOV S., D. PECHLIVANOVA R. DENEV, M. NIKOLOVA, L. GEORGIEVA et al. (2021) GC-MS analysis of Amaryllidaceae and Scylanthaceae-type alkaloids in bioactive fractions from *Narcissus* cv. Hawera, *Rapid Commun. Mass Spectrom.*, **35**(14), e9116.
- [15] MACIVER R. (1995) Constituents of pyrethrum extract. In: Casida J. E., Quistad G. B. (Eds) *Pyrethrum Flowers: Production, Chemistry, Toxicology, and Uses*, New York: Oxford University Press, 108–122.
- [16] MORRIS S., N. DAVIES, P. BROWN, T. GROOM (2006) Effect of drying conditions on pyrethrins content, *Ind. Crops Prod.*, **23**(1), 9–14.
- [17] GRDIŠA M., F. VARGA, N. JERAN, A. TURUDIĆ, Z. LIBER et al. (2022) Influence of bioclimatic conditions on the pyrethrin profiles of Dalmatian pyrethrum natural populations, *Int. Conf. on Biodiv. and Molec. Plant Breed.*, 8
- [18] GNADINGER C. B. (1936) *Pyrethrum flowers*, McLaughlin Gormley King Company, 380 pp.
- [19] GLYNNE-JONES A. (2001) Pyrethrum, *Pesticide Outlook*, **12**(5), 195–198.

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