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An Experimental Design for the Analysis of 5-Caffeoylquinic Acid (5-CQA) in Ethanolic Extracts of Hibiscus (*Hibiscus sabdariffa* L.) Flower

Kísérleti terv a hibiszkusz (Hibiscus sabdariffa L.) virág etanolos kivonatában található 5-koffeoil-kinsav (5-CQA) elemzésére

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Abstract: This study optimized the extraction and analysis of 5-caffeoylquinic acid (5-CQA), the predominant chlorogenic acid in hibiscus flowers, using a sustainable ethanolic extraction method combined with high-performance liquid chromatography (HPLC) analysis. To identify the optimal solvent extraction conditions for 5-CQA from hibiscus, Response Surface Methodology (RSM) was employed, incorporating three factors: pH levels (2, 4.5, 7), extraction times (20, 40, 60 minutes) and solvent compositions (25v/v%, 50v/v%, 75v/v% ethanol). The temperature was maintained at the boiling point throughout the extraction period. RSM identified 25% ethanol, pH 7, and 25-minute heat treatment as the optimal extraction conditions for maximizing 5-CQA yield. Two HPLC columns; InertSustain Phenyl (A) and Synergi Polar (B), differing in length, diameter, and particle size were evaluated for chromatographic performance monitoring HPLC-UV signals at a wavelength of 325 nm. The mobile phase for both columns consisted of acetonitrile and acidified water (with orthophosphoric acid). Both HPLC columns achieved high correlation coefficients (0.9908 and 0.9965, A and B) between extracted 5-CQA and standard peaks. Under optimized conditions, the yields of 5-CQA were 1.43 mg/g and 1.48 mg/g dry hibiscus flower for columns A and B, respectively. Compared to literature-reported methods, the developed protocol offers high efficiency, reduced solvent usage, and minimal heat treatment, establishing it as a convenient, and sustainable alternative for 5-CQA extraction.

Keywords: *5-caffeoylquinic acid; chlorogenic acid; ethanolic extract; hibiscus; HPLC-PDA*

Összefoglalás: Ez a tanulmány optimalizálta az 5-koffeoil-kinsav (5-CQA), a hibiszkuszvirágokban a domináns klorogénsav extrakcióját és elemzését, fenntartható etanolos extrakciós módszerrel kombinálva nagy teljesítményű folyadékkromatográfiás (HPLC) elemzéssel. A hibiszkuszból származó 5-CQA optimális oldószeres extrakciós körülményeinek meghatározásához Response Surface Methodology (RSM) módszert alkalmaztunk, amely három tényezőt tartalmazott: pH-szint (2, 4,5, 7), extrakciós idő (20, 40, 60 perc) és oldószer összetétele (25 v/v%, 50 v/v%, 75 v/v% etanol). A hőmérsékletet a forrásponton tartottuk az extrakciós periódus alatt. Az RSM a 25%-os etanolt (pH 7) és a 25 perces hőkezelést azonosította optimális extrakciós körülményként az 5-CQA hozam maximalizálásához. Két

HPLC oszlop; A hosszúságban, átmérőben és részecskeméretben eltérő InertSustain Phenyl (A) és Synergi Polar (B) kromatográfiás teljesítményét a HPLC-UV jelek 325 nm-es hullámhosszon történő monitorozására értékelték. Mindkét oszlop mozgófázisa acetonitrilből és (ortofoszforsavval) savanyított vízből állt. Mindkét HPLC oszlop magas korrelációs együtthatót ért el (0,9908 és 0,9965, A és B) az extrahált 5-CQA és a standard csúcsok között. Optimalizált körülmények között az 5-CQA hozama 1,43 mg/g és 1,48 mg/g száraz hibiszkuszvirág volt az A és B oszlopban. Az irodalomban közölt módszerekhez képest a kifejlesztett protokoll nagy hatékonyságot, csökkentett oldószerhasználatot és minimális hőkezelést kínál, így kényelmes és fenntartható alternatívát jelent az 5-CQA extrakcióhoz.

***Kulcsszavak**: 5-koffeoil-kinsav; klorogénsav; etanolos kivonat; hibiszkusz; HPLC-PDA*

1 Introduction

There is a whole family of chlorogenic acids (quinic acid esters with substituted cinnamic acids) generated from substitutions at the aromatic ring and the isomers and epimers in the cyclohexane part (Mok et al., 2022; Mukherjee et al., 2015; Sakai et al., 2022). The three major classes of chlorogenic acids are caffeoylquinic acid (CQA), dicaffeoylquinic acid (diCQA), and feruloyl quinic acid (FQA), and the three most available CQA isomers in plant source are; neochlorogenic acid (3-O-caffeoylquinic acid, 3-CQA), crypto chlorogenic acid (4-O-caffeoylquinic acid, 4-CQA), and chlorogenic acid (5-O-caffeoylquinic acid; 5-CQA). The most abundant among them is 5-CQA, a major polyphenol found in many plant sources such as green coffee beans, hibiscus, honeysuckle, and so on and is extensively studied because of its tremendous health benefits (Aree, 2023; Grujic et al., 2015). For instance, 5-CQA has received the attention of scientific communities mainly because of its antioxidant, anti-inflammatory, antibacterial, anti-cancer, and analgesic activities as well as beneficial regulatory effects caused on gut microflora. Hence, regular consumption of a diet rich in 5-CQA is known to reduce the occurrence of many non-communicable diseases (Bhandarkar et al., 2019; Plazas et al., 2013).

The calyces and leaves of *Hibiscus sabdariffa* L., also known as hibiscus and roselle, which belongs to the Malvaceae family are popular in tropical regions, especially in countries in Asia, South America, and Australia, for manufacturing herbal tea due to their therapeutic effects. Additionally, hibiscus calyces have been used as coloring, and flavoring agents in foods, medicines, and cosmetics (Chongwilaikasem et al., 2024). The aqueous and organic extracts of hibiscus calyces are rich in bioactive compounds including flavonoids, antocyanidins and chlorogenic acids. For instance, in a study by Chongwilaikasem et al (2024), the ethanolic extracts of hibiscus calyces have demonstrated moderated DPPH radical scavenging ability (EC_{50} = 289.61µg/mL) and strong antibacterial effects against several bacterial species including *Escherichia coli* and *Staphylococcus aureus*. Hence, hibiscus floral extracts have recently gained the attention of scientists in their uses in functional food applications (Paraı´so et al., 2019).

Although several studies are focusing on the overall bioactivity of hibiscus flower extracts and anthocyanin extraction from hibiscus flower, studies focusing on extraction of 5-CQA from hibiscus flower are minimal. The extraction process plays an important role in preserving the bioactivity and the food applications of the compounds of interest. For instance, the conventional extraction methods utilize large quantities of organic solvents which become toxic in food applications and increase the extraction procedure's cost. Additionally, in conventional methods, high-temperature long-time extractions are practiced which can accelerate the yields of hydrolysis products of 5-CQA (Pimentel-Moral et al., 2018). Therefore there is a requirement

to optimize a convenient method for the extraction of 5-CQA from hibiscus flowers using a minimum amount of solvent which is the least toxic in food applications, for example, ethanol. Additionally, the literature lacks evidence about the effect of the solvent mixture's pH on the extract's 5-CQA level. For instance, at higher pH values (5.0 to 9.0) CGA is easily hydrolyzed into products such as quinic acid, and caffeic acid, and with the effect of various conditions (e.g.; pH, and temperature), reversible isomerization can occur (Mok et al., 2022) which requires investigation in optimizing an efficient method.

Amongst various quantification methods of 5-CQA, high-performance liquid chromatography (HPLC) is the most dominating method even though it has several drawbacks. However, the extraction and analysis of 5-CQA in plant sources is challenging because of their diverse compositions and concentrations of active ingredients. For instance, in green coffee ethanolic extracts, interference of caffeine with 5-CQA at some wavelengths (274nm) can be observed in HPLC determinations. Additionally, the extraction solvents and method directly affect the determined 5-CQA levels in herbal infusions and there is a lack of information about HPLC methods optimized with each extraction method (Peng et al., 2005). Therefore, this study aimed to develop an efficient, convenient, and sustainable method for the extraction of 5-CQA from hibiscus flowers utilizing ethanol as the solvent and quantifying 5-CQA. Further, this study focused on evaluating the performance of two different analytical HPLC columns for the analysis of 5-CQA in ethanolic extracts of hibiscus flowers in terms of separation of 5-CQA, analysis time, and peak purity level. In this study, the optimization of the extraction method and 5-CQA quantification method was performed using Response Surface Methodology (RSM) utilizing a box-Behnken design which can investigate the effect of multiple independent variables on the dependent variable together.

2 Materials and Methods

2.1 Materials

Commercially dehydrated hibiscus (*Hibiscus sabdariffa* L.) flower samples used for the measurements were purchased from Natur Tea, Hungary. For extractions, Ultra-pure water was obtained from an ultra-pure water system (Milli Q-SQ 2 series, Germany), and absolute ethanol was purchased from VWR chemicals (France). The standard 5-CQA (purity ≥ 95%) was purchased from Merck Science Life Ltd. (Budapest, Hungary). For mobile phase preparation, acetonitrile of HPLC grade (Fisher Scientific, UK), and ortho-phosphoric acid of analytical grade (Lachner, Hungary) were used. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \times H_2O$), sodium acetate (CH_3COONa), acetic acid (CH_3COOH), and disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2H_2O$) were purchased from Merck, Germany.

2.2 Preparation of hibiscus extract

Hibiscus extracts were prepared in triplicates following a 3x3x3 factor-factorial design focusing on the effect of solvent composition, pH of the solvent mixture, and extraction time on the yield of 5-CQA extracted from the hibiscus flower. Treatment combinations described in Table 1 were selected after a preliminary study of existing literature. In all treatments, 1g of dehydrated hibiscus flowers was used to prepare 100 mL of extract. The pH adjustment was performed by using 10mL of respective buffer solution with 0.1M molarity; $NaH_2PO_4 \times H_2O$ with H_3PO_4 for the pH=2, CH_3COONa with CH_3COOH for the pH=4.5, and $NaH_2PO_4.H_2O$ with $Na_2HPO_4.2H_2O$ for the pH=7.0. All samples were maintained at the boiling temperature for the

respective extraction time mentioned in Table 1. After that, the extract was filtered into 1.5 mL vials using a syringe with a 0.22 µm hydrophilic filter for HPLC analysis.

Table 1 Factorial design (3^3) experimental values of tested independent variables for the extraction of 5-CQA from hibiscus flower

Treatment ID	Time (min)	Volume of ethanol (mL)	Volume of water (mL)	pH Value
T1	20	25	65	2.0
T2	20	25	65	4.5
T3	20	25	65	7.0
T4	20	50	40	2.0
T5	20	50	40	4.5
T6	20	50	40	7.0
T7	20	75	15	2.0
T8	20	75	15	4.5
T9	20	75	15	7.0
T10	40	25	65	2.0
T11	40	25	65	4.5
T12	40	25	65	7.0
T13	40	50	40	2.0
T14	40	50	40	4.5
T15	40	50	40	7.0
T16	40	75	15	2.0
T17	40	75	15	4.5
T18	40	75	15	7.0
T19	60	25	65	2.0
T20	60	25	65	4.5
T21	60	25	65	7.0
T22	60	50	40	2.0
T23	60	50	40	4.5
T24	60	50	40	7.0
T25	60	75	15	2.0
T26	60	75	15	4.5
T27	60	75	15	7.0

2.3 Preparation of 5-CQA standard solutions

A stock solution of 5-CQA at a concentration of 1mg/mL was prepared by dissolving 50.0mg of 5-CQA in 50 mL of ethanol. Working standard solutions of concentrations within the range of 5 to 50 µg/mL were prepared and injected into the HPLC system under determined conditions for developing a 5-CQA calibration curve by plotting concentration versus peak area. Separate 5-CQA calibration curves were developed for the two HPLC columns used in this study. Quantification of 5-CQA levels in each extract was performed using the calibration curves' regression equation of the best line of fit.

2.4 HPLC Analysis of 5-CQA in ethanolic hibiscus extracts (isocratic method)

The instrumental analysis of the 5-CQA was performed using an HPLC-PDA system (Jasco, Japan) equipped with a 4-line degasser (Jasco DG-2080-54), intelligent HPLC pump (Jasco PU-980), ternary gradient unit (Jasco LG-980-02), intelligent sampler (Jasco AS-2055), and a column thermostat (Phenomenex TS-130) maintained at 35˚C, and a column compartment. ChromeNAV software (Jasco, Japan) was used for data acquisition. Separation was achieved using two different columns (A: InterSustain Phenyl column with 5µm pore size, 3mm diameter, 150mm length, and B: Synergi Polar column with 4µm pore size, 4.6mm diameter, 250mm length) following an isocratic procedure. Columns were compared for the HPLC analysis of hibiscus extracts using a predetermined isocratic procedure based on preliminary studies. The mobile phase was composed of 7% acetonitrile, with 0.1% phosphoric acid, for column A, and 10% acetonitrile, with 0.1% phosphoric acid, for column B. Flow rate and injection volume in both columns were maintained as 1.2mL/min and 2µL respectively. The wavelength was set at 325 nm for monitoring the chromatographic profile. All measurements were done in triplicate.

2.5 Method Validation

Method validation was performed by calculating the calibration range, linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ) values for the calibration curves following the method explained by Chaowuttikul et al (2020). The specificity was analyzed by a peak purity test by comparing all the spectra within the chroatographic peak to the reference spectrum at the peak appex. The accuracy was tested by the recovery method. The percent recoveries based on each analytical column was calculated using a spiked test by injecting 100 µL of 1mg/mL 5-CQA standard solution into each sample (5 mL) in triplicates.

2.6 Experimental Design and Statistical Analysis

In this study, RSM occupying a box-Behnken design was used to assess the relationship between three independent variables (pH, ethanol concentration, and extraction time) which were coded into three levels (-1, 0, and +1), and the dependent variable (area of 5-CQA peak in chromatogram) and identify the optimal levels of the independent variables for the dependent variable. All analyses were conducted in triplicates. Minitab 19 software was used for the analysis of variance (ANOVA), response surfaces, regression equations, and designing the contour and surface plots. A confidence level of 95% was set as the basis for the determination of the significance of the difference. Validation of the RSM model was performed by analyzing the residual plots, model p-value, lack of fitness, determination coefficient (R^2), and $R^{2\ (adj)}$ values. Other data was analyzed using Microsoft Office Excel. All values were expressed as mean ± standard deviation (SD) values obtained in experiments performed in triplicate.

3 Results and Discussion

3.1 Optimization of HPLC conditions for the 5-CQA in hibiscus extracts

According to the literature, in HPLC, different chemicals such as formic acid, and phosphoric acid are used in the aqueous phase to improve the resolution, control ionization, and reduce peak tailing of compounds of interest (Chaowuttikul et al., 2020). In this study, after conducting preliminary trials with various mobile phases occupying numerous proportions of different aqueous phases and organic modifiers to separate 5-CQA in hibiscus, the most suitable mobile

phase that showed good resolution and symmetric peak shape was obtained using 7% acetonitrile and 0.1% phosphoric acid (column A) and 10% acetonitrile and 0.1% phosphoric acid (column B) with an isocratic program. The column temperature was held at 35˚C for the duration of the analysis to improve the retention time precision. In HPLC, the identification of compounds in herbal infusions depends on the retention time and UV-light spectral characteristics of the chromatographic peak of the standard solution. According to the literature, hydroxycinnamic acids have the maximum wavelength during 270 - 360 nm wavelengths (Yilmaz and Kolak, 2017). By comparing the UV spectra of standard 5-CQA at varying wavelengths, 325nm wavelength was identified as the optimum wavelength to detect 5-CQA.

3.2 Validation of the optimized method

To quantify the amount of 5-CQA in the herbal infusions of hibiscus, calibration curves for 5-CQA were prepared occupying each HPLC column used for the analysis in this study. The calibration curves for 5-CQA designed based on HPLC analysis with A and B columns are depicted in Figure 1. The regression equations for 5-CQA with A (y = 7364x - 3495) and B (y = 4859x - 3423) columns obtained in the form of y = ax + b demonstrated good linearity between concentration and peak area within the range of 5–50 µg/mL. The high correlation coefficient values (R_A = 0.99, R_B = 0.99) of 5-CQA denotes an excellent correlation between 5-CQA concentration and peak area. Similarly, the R^2 values were higher than 0.99 for both A and B columns, demonstrating the regression model fits the observed data satisfactorily. An analytical method is considered acceptable when the R^2 value is 0.99 or above (Chaowuttikul et al., 2020). Retention times for the standard 5-CQA in the HPLC analysis (at 325nm) using A, and B and columns were 5.69 and 12.1 min respectively. LOD and LOQ values for the 5-CQA with column A were 4.69µg/mL and 14.22µg/mL respectively. LOD and LOQ values for the 5-CQA with column B were 7.70µg/mL and 23.0µg/mL respectively. In quantification, it was confirmed that the spectral peaks for the hibiscus extracts were in line with the spectra of standard 5-CQA, which were further confirmed by spiked tests. Recovery percentages calculated with spiked samples were within the acceptable range of 80-120% (105.7% and 105.6% for columns A and B respectively). The percentage residual standard deviation (RSD %) values for the spiked samples were 2% and 3% for column A and B, respectively. RSD% values not more than 15% indicate the accuracy and repeatability of the tested RP-HPLC methods for the quantification of 5-CQA in hibiscus samples.

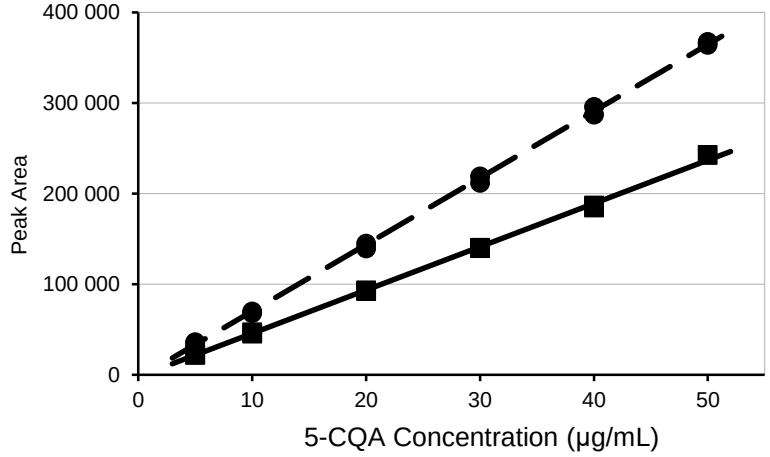

Figure 1 *Calibration curve for 5-CQA concentration developed for the two RP HPLC columns; InertSustain phenyl column depicted with a dashed line and round-shaped markers, Synergy Polar column depicted with a solid line with square-shaped markers*

According to the literature, when RP-HPLC is used for the separation of chlorogenic acids, the elution order of CGA isomers varies according to the provided conditions. For instance, the partition of an analyte between the column's packing material and eluent is affected by their polarities. In RP-HPLC, the polarity of the packing material is particularly lower than that of eluent. Hence, the analytes with higher polarity tend to distribute better in the eluent, eluting the analytes faster from the column. When referring to the structure, 5-CQA and 4-CQA have one axial and one equatorial hydroxyl residue making it less hydrophilic than 3-CQA which has two hydroxyl residues in an equatorial structure. Another main factor that determines the elution order and the degree of separation of the three CGA isomers under RP-HPLC is the functioning of the residual silanol group. Deineka et al (2019), revealed that when water–acetonitrile–formic acid or orthophosphoric acid is used as the mobile phase with an RP-HPLC column as the stationary phase with a low activity of silanol groups or polar functional, the order of CGA elution remains as 3CQA < 5QCA < 4CQA. Additionally, there is a substantial effect of the pH of the mobile phase on the retention of such acidic analytes (Jeon et al., 2017). However, the identification of 3-CQA and 4-CQA was not performed in this study due to the unavailability of standards, marking it as a limitation of this study. Figures 2 and 3 depict representative base-peak chromatograms of T2 hibiscus extract analyzed with the two HPLC columns. When the phenyl column was used for the analysis, at the occupied wavelength of 325nm, peak 2 was identified as 5-CQA by comparing with the standard 5-CQA spectra. However, when the polar column was used for the analysis, the elution of 5-CQA was recorded as the peak 3 (Figure 3). The extension of the retention times of 5-CQA in the polar column compared to the phenyl column can be due to the improved polar interactions offered by the polar column. Peak purity was calculated to determine the specificity of the method and confirmed that the purity levels of 5-CQA peaks were 81.28% and 79% in column A and B respectively. Peak purity test is important to find out whether the chromatographic peak of the analytes attributable with another compound. Chromatographic peaks which generate peak purity values of 100% confirm that no impurity was detected in the peak. However, the specificity reported in this study were comparatively low than the expected value demonstrating a possibility to contain some impurities. In contrast, the correlation coefficient values of the 5-CQA peak with 50µg/mL of 5-CQA standard were 0.9908 and 0.9965, in columns A and B, respectively, demonstrating both of these columns can be successfully used in the separation of 5-CQA in hibiscus extracts. Further, the comparison of 5-CQA yields from all studied hibiscus extracts as per the analysis with SynergiPolar and InertSustain Phenyl columns summarized that there is a determination coefficient of 0.9992 demonstrating the analytical values for the two studied RP-HPLC columns generate similar results (Figure 4).

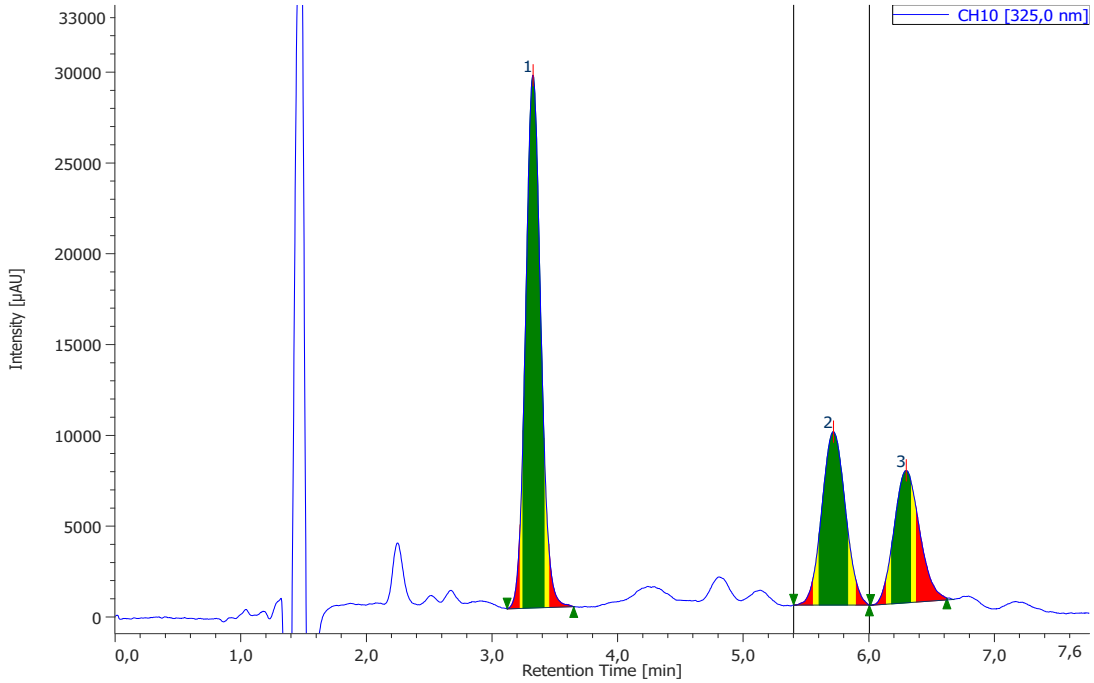

Figure 2 *Separation of chlorogenic acid with a mobile phase containing 7% acetonitrile and 0.1 % phosphoric acid with a flow rate of 1.2mL/min on InertSustain phenyl stationary phase at detector wavelength of 325 nm. Peak 2 was identified as 5-CQA. The purity of the 5-CQA peak was 81.28%. The green, yellow and red colours represent the high, medium and low purity at the peak's top position, respectively.*

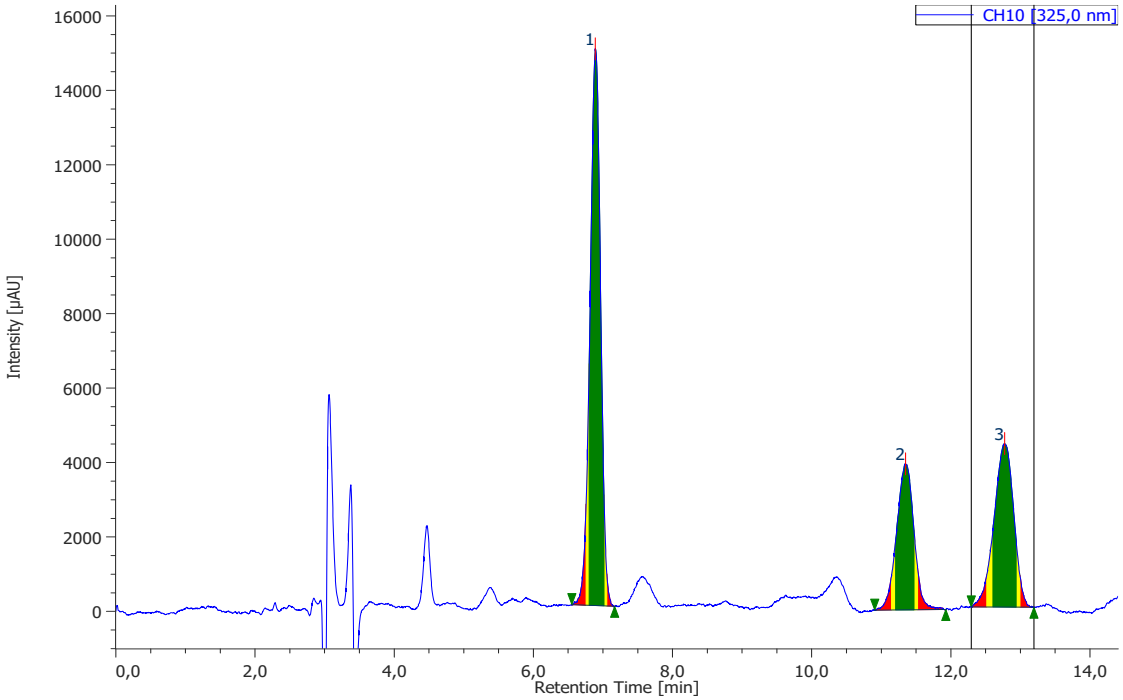

Figure 3 *Separation of chlorogenic acid with a mobile phase containing 10% acetonitrile and 0.1 % phosphoric acid with a flow rate of 1.2mL/min on SynergiPolar stationary phase at a detector wavelength of 325 nm. Peak 3 was identified as 5-CQA. The purity of the 5-CQA peak was 79%. The green, yellow and red colours represent the high, medium and low purity at the peak's top position, respectively.*

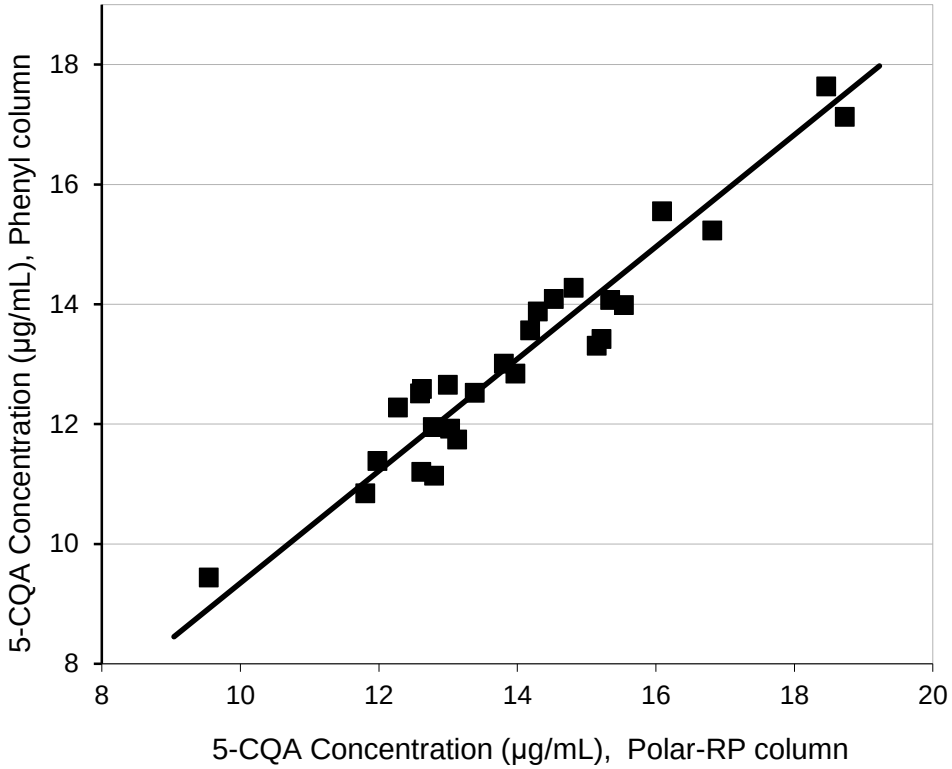

Figure 4 *Comparison of the 5-CQA concentration in hibiscus flower extracts analyzed with the two RP-HPLC columns; Phenylcolumn vs. Polar RP column*

3.3 Optimization of extraction conditions and quantification of extractable 5-CQA in hibiscus

The experimental results of 5-CQA detected in each hibiscus extract based on the HPLC analysis with two selected columns in this study are presented in Table 2. The 5-CQA levels were calculated using the equations generated according to the calibration curves. Accordingly, the 5-CQA concentration in 1mL of hibiscus extract ranged from 9.4 to 17.6 µg in HPLC analysis with the InertSustain column, while that was ranged from 9.5 to 18.5 µg in HPLC analysis with the SynergiPolar column. In both tested RP-HPLC columns, the highest 5-CQA yield (A=17.6µg/mL, B=18.5µg/mL) was reported in T2 extraction, which occupied 25% ethanol concentration, pH=4.5 buffer solution and 20min extraction time.

Table 2 The concentration of 5-CQA detected in Hibiscus flower extracts by HPLC analysis with InertSustain Phenyl Column and Synergi Polar Column

Treatment ID	InertSustain Phenyl Column	Synergi Polar Column
	Average 5-CQA concentration ± SD (µg/mL)	Average 5-CQA concentration ± SD (µg/mL)
T1	13.6±0.16	14.2±0.28
T2	17.6±0.24	18.5±0.18
T3	14.3±0.07	14.8±0.37
T4	14.1±0.23	14.5±0.44
T5	17.1±0.43	18.7±0.55
T6	13.3±0.63	15.1±0.30
T7	15.6±0.62	16.1±0.03
T8	11.2±0.23	12.6±0.20
T9	13.4±0.13	15.2±0.02
T10	11.4±0.25	12.0±0.20
T11	12.0±0.16	12.8±0.00
T12	13.9±0.09	14.3±0.18
T13	9.4±0.05	9.5±0.25
T14	11.7±0.24	13.1±0.15
T15	11.1±0.03	12.8±0.23
T16	14.1±0.18	15.3±0.12
T17	10.8±0.05	11.8±0.11
T18	14.0±0.08	15.5±0.48
T19	12.5±0.07	12.6±0.06
T20	12.5±0.13	13.4±0.45
T21	12.6±0.09	12.6±0.39
T22	12.8±0.07	14.0±0.15

T23	13.0±0.19	13.8±0.25
T24	15.2±0.30	16.8±0.02
T25	12.3±0.12	12.3±0.03
T26	11.9±0.09	13.0±0.26
T27	12.7±0.29	13.0±0.07

The effect of extraction time, solvent composition, and pH value on the peak area of the 5-CQA chromatogram was extensively investigated in this study. The residual plots (Figure 5) for the analysis of peak area based on the two analytical columns used in this study demonstrate that the data is randomly distributed being independent of each other following a normal distribution. Additionally, there is an equality of variance in the analyzed data for both columns. According to the multivariate regression analysis conducted occupying the RSM model, the R^2 was 62.34% while $R^{2(adj)}$ was 45% when the InertSustain column was used. The R^2 was 56.78% while $R^{2(adj)}$ was 37% when the SynergyPolar column was used. These R^2 and $R^{2(adj)}$ values indicate that the RSM model moderately fits with the analyzed data due to the significance of some factors considered in this study. The model p-values for columns A and B were 0.007 and 0.022 while the lack of fit values for both columns was 0.2 showing that this model can be used for further studies. However, it is suggested to improve the existing model further by considering cubic terms and including other random variables that affect the outcome as well.

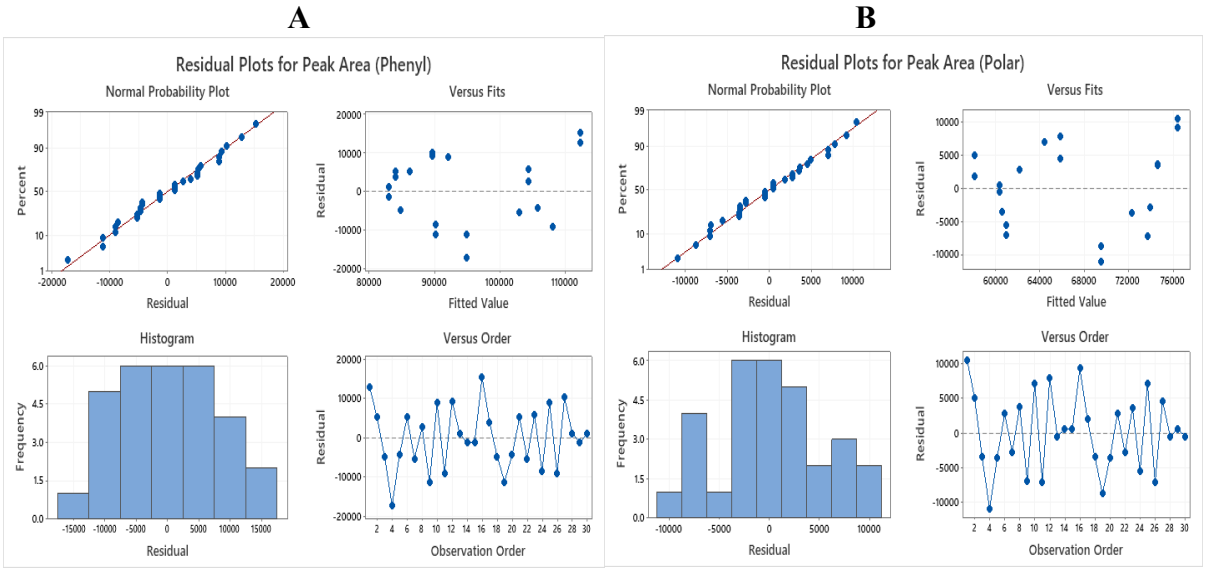

Figure 5 *Residual plots for the peak area of (A) InertSustain Phenyl column and (B) SynergiPolar column according to the response surface model*

For the area of the 5-CQA peak with column A, there was no significant effect from the linear variables ($p>0.05$), while time with ethanol concentration (C_{ETOH}) was the only significant two-way interaction ($p<0.05$) between the factors. However, in quadratic terms, there was a significant interaction with the square effect of time and pH ($p<0.05$). For the area of the 5-CQA peak with column B, pH was the only significant linear variable ($p<0.05$) while time with C_{ETOH} was the only significant two-way interaction ($p<0.05$) between the factors. Regarding the quadratic terms also, there was a significant interaction with the square effect of time and pH ($p<0.05$) (Table 3). Regression equations (Eq. 1, 2) designed based on the RSM models

depicted below describe the relationship between the independent and dependent variables. According to both equations, pH had a positive effect indicating that the increase in pH favored 5-CQA extraction while extraction time had a negative effect indicating that the increase in extraction time was not desired for the 5-CQA extraction. However, the effect of ethanol concentration on the dependent variable was negative according to column A and positive according to column B.

Table 3 ANOVA for polynomial surface response model of all variables depending on the two HPLC columns used for the analysis

Factors	P-value	P-value
	InertSustain Phenyl	SynergyPolar
Linear effect		
Time	0.073	0.175
C_{ETOH}	0.098	0.513
pH	0.121	0.047*
Quadratic effect		
Time*Time	0.035*	0.049*
C_{ETOH}*C_{ETOH}	0.384	0.796
pH*pH	0.019*	0.046*
Interaction effect		
Time*C_{ETOH}	0.010*	0.009*
Time*pH	0.137	0.261
C_{ETOH}*pH	0.146	0.244

**Significant at $p<0.05$*

$$Peak\ Area_{(InertSustain\ Phenyl)} = 153249 - 3153\ Time - 41\ C_{ETOH} + 1134\ pH + 29.15\ Time*Time + 0.01\ C_{ETOH}*C_{ETOH} - 3\ pH*pH + 5.13\ Time*C_{ETOH} + 57.6\ Time*pH - 53.8\ C_{ETOH}*pH \quad (1)$$

$$Peak\ Area_{(Synergy\ Polar)} = 90261 - 1945\ Time + 276\ C_{ETOH} + 2797\ pH + 18.12\ Time*Time - 2.98\ C_{ETOH}*C_{ETOH} - 205\ pH*pH + 2.62\ Time*C_{ETOH} + 26.1\ Time*pH - 18.9\ C_{ETOH}*pH \quad (2)$$

The contour plots and surface response plots of the peak area of 5-CQA with the interaction effect of C_{ETOH} with extraction time, pH with extraction time, and C_{ETOH} with pH, as per the HPLC analysis with columns A and B are depicted in Figure 6 and 7 respectively. It is a common fact that the chemical composition of hibiscus extracts obtained by different extraction methods can be different from each other depending on factors such as the type and concentration of solvent used, extraction temperature, time, and technology. Additionally, the HPLC conditions affect the separation of each bioactive compound. According to the extraction trends depicted in Figures 6 and 7, with extended extraction times, pH levels above 6 yield higher concentrations of 5-CQA than with lower pH levels, without being affected by the HPLC column used for the analysis. Similarly, according to the analysis with both columns, higher pH values generated higher yields of 5-CQA up to the 60% concentration of ethanol used for the extraction. This can be due to the reversible isomerization of hydrolyzed products of 5-CQA, which mainly involves acyl groups, at higher pH values with the effect of various conditions such as temperature, and solvent concentration (Mok et al., 2022). Moreover, with all tested ethanol concentrations and pH levels, lower extraction times yielded higher 5-CQA concentrations than extended heat treatments, when analyzed with both columns A and B.

It was determined from the RSM study that 25% ethanol, pH=7 buffer solution, and heat treatment for 25 minutes as the optimum parameters for maximizing the 5-CQA yield with a

composite desirability value of 0.9455 and 0.9498 for columns A and B, respectively. The experimental values for 5-CQA yield under the optimal conditions were 1.43mg and 1.48mg from 1g of dry hibiscus flower based on the analysis with columns A and B respectively. According to Mok et al (2019), a maximum of 0.12 mg of CGA was extracted from 1g of dry hibiscus flower using 50% methanol for 6 hrs. Accordingly, the present study reveals that the use of ethanol as the solvent for the extraction of 5-CQA is more effective than methanol. This can be further explained by the fact that the methods that can reduce the polarity of water by cleaving hydrogen ions in water can increase the solubility of 5-CQA in water because 5-CQA is a substance with low polarity. Ethanol is less polar than methanol due to the presence of a larger alkyl group in ethanol. Hence a solvent mixture with low polarity becomes ideal for the extraction of 5-CQA (Mok et al., 2022). Previous studies have also observed that the heat treatment significantly affected the extraction of 5-CQA. More particularly, 5-CQA has an acyl group bound to the hydroxyl group on carbon 5 of quinic acid which makes it more sensitive to heat treatments. Accordingly, with temperatures above 110˚C combined with longer extraction times, the yield of 5-CQA was decreased (Mok et al., 2022). However, the method presented in the current study becomes efficient and sustainable in terms of solvent usage and energy consumption for the heat treatment applied in 5-CQA extraction.

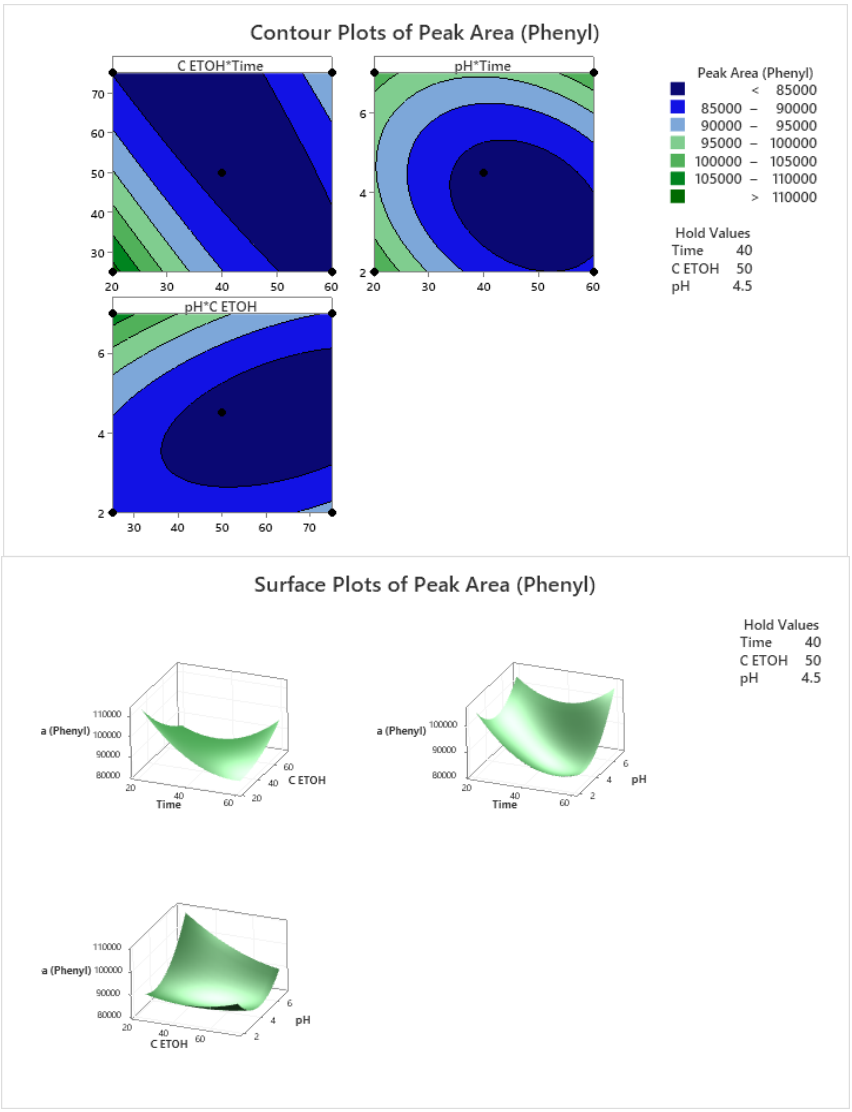

Figure 6. *Contour plots and surface plots of peak area of 5-CQA with the interaction effect of C_{ETOH} with extraction time, pH with extraction time, and C_{ETOH} with pH, as per the HPLC analysis with InertSustain phenyl column*

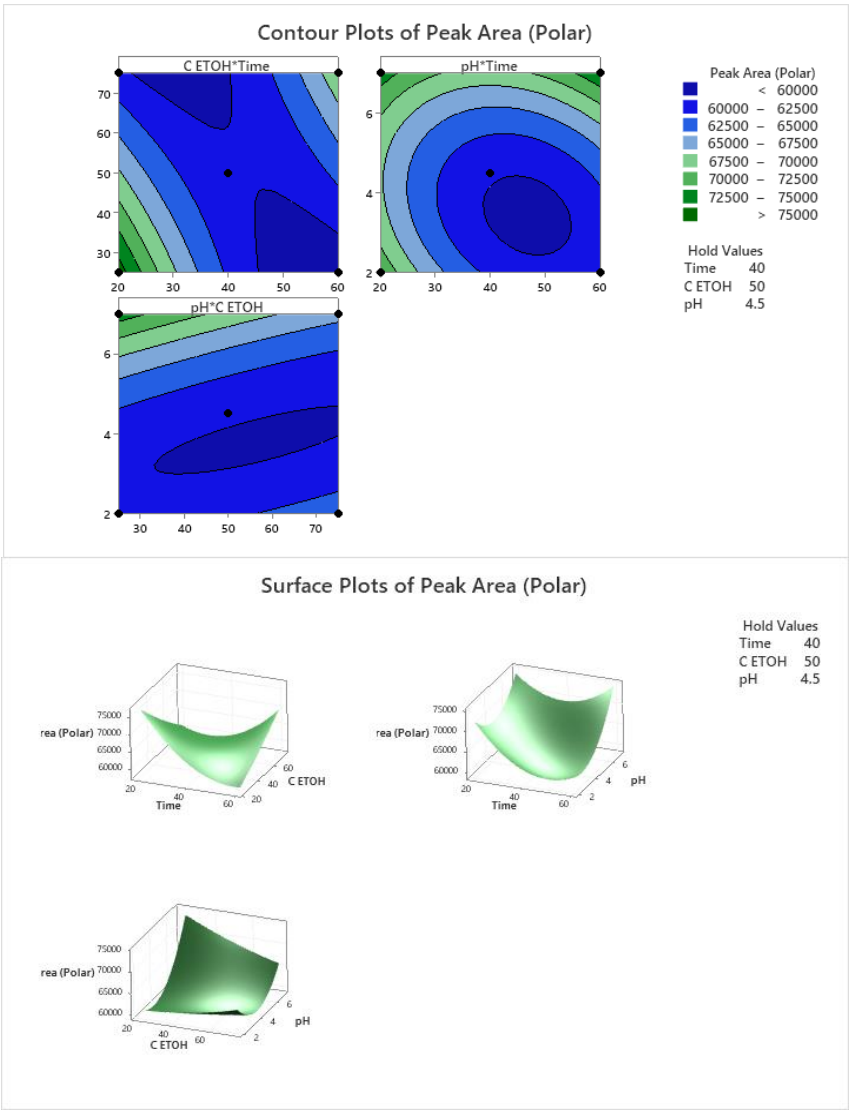

Figure 7. *Contour plots and surface plots of peak area of 5-CQA with the interaction effect of C_{ETOH} with extraction time, pH with extraction time, and C_{ETOH} with pH, as per the HPLC analysis with SynergyPolar column*

5. Conclusions

This study suggested that the optimum extraction conditions for maximum yield of 5-CQA from hibiscus flower are 25% ethanol, pH=7, and 20 minutes heat treatment concerning the HPLC analysis with both InertSustain phenyl and Synergi Polar columns. The experimental values for 5-CQA yield under the optimal conditions were 1.43mg and 1.48 mg from 1g of dry hibiscus flower, analyzed with InertSustain phenyl and Synergi Polar columns respectively. This extraction method can be justified from ecological and economic perspectives since it uses minimum amounts of ethanol, in addition to the short-period heat treatments, which can be effectively used in food manufacturing and pharmaceutical applications. The study revealed that the RSM model designed in this study can be used for the predictions in future studies, however, further improvements in the existing model by considering cubic terms and including other random variables that affect the outcome are suggested.

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Investigation on mulching weed control technologies of sweet potato (*Ipomoea batatas*)

Az édesburgonya (Ipomoea batatas) mulcsolásos gyomszabályozási technológiáinak vizsgálata

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Abstract: In our experiment, we investigated the weed control efficiency of organic mulches (wheat straw and grass cuttings) and agrotextile. The mulching materials reduced weed coverage and influenced the weed flora. However, their effectiveness significantly declined after approximately 4–6 weeks, necessitating supplementary weeding to prevent substantial yield losses. Agrotextile increased the yield by 43% (likely due to its effect on soil temperature and water management favorable for sweet potatoes), while organic mulches reduced the yield by 16–23%, even with supplementary weeding. In the weedy control, yield decreased by 97.2%. Agrotextile produced a marketable yield of 84.25 t/ha, whereas organic mulches resulted in 40.98–44.54 t/ha. Based on our results, agrotextile is the most recommended option for weed control in sweet potato, considering both the labor time required for weed management and the costs. Since agrotextile can be used for multiple years, its cost is not higher than straw mulch, and its environmental impact is lower compared to disposable plastic mulches.

Keywords*: sweet potato, weed control, mulching, agrotextile*

Összefoglalás: Kísérletünkben a szerves talajtakaró anyagok (szalma és fűnyesedék) továbbá az agroszövet mulcsolás gyomszabályozási hatékonyságát vizsgáltuk. A takaróanyagok csökkentették a gyomborítottságot és befolyásolták a gyomflórát. Hatékonyságuk kb. 4-6 hét elteltével jelentősen csökken, így kiegészítő gyomlálásokra is szükség van, hogy elkerüljük a jelentősebb terméscsökkenést. A termést az agroszövet 43%-kal növelte (ehhez hozzájárulhatott az agroszövet által kialakított, a batáta számára kedvezőbb talajhőmérséklet és vízgazdálkodás is), míg a szerves mulcsok 16-23%-kal csökkentették, még kiegészítő gyomlálásokkal is, a gyomos kontrollban pedig 97,2%-kal csökkent a termés. Az agroszövet 84,25 t/ha, míg a szerves talajtakarók 40,98-44,54 t/ha értékesíthető hozamot eredményeztek. Eredményeink alapján az édesburgonya gyomszabályozására leginkább az agroszövet ajánlható, figyelembe véve a gyomszabályozáshoz szükséges kézimunka idejét és a költségeket is. Mivel az agroszövet több évig használható, költsége nem nagyobb a szalma mulcsnál, továbbá a környezeti terhelése is kisebb, mint az egyszer használatos műanyag fóliáknak.

Kulcsszavak*: édesburgonya, gyomszabályozás, mulcsolás, talajtakarás, agroszövet*

1 Introduction

Sweet potato is the world's 7th most important food crop, cultivated on approximately 9-10 million hectares. It is a tuberous root plant propagated by seedlings. Despite its name, it is not part of the *Solanaceae* family like potato, but rather belongs to *Convolvulaceae*. Consequently, it faces significantly fewer plant protection challenges compared to potatoes. However, weeds also cause serious problems in its cultivation (Pepó, 2022).

The first half of the growing season is critical, as the plant exhibits extremely low weed suppression capacity from planting until the end of July. According to Seem et al. (2003), its critical competition period spans a four-week phase between the second and sixth weeks after planting. Currently, weed control is primarily managed through manual hoeing and ridging (Pepó, 2022). To replace these methods, mulching technologies already tested on potatoes and tomatoes may prove effective (Dezső and Pásztor, 2022; 2024).

Globally, plastic mulches (e.g., agrotextile and black PE film) are widely used in various crops. However, their use generates significant amounts of hard-to-recycle waste, prompting researchers and growers to experiment with organic and biodegradable mulches as alternatives to plastics (Cirujdea et al., 2012; Miles et al., 2012). It is important to note that mulches influence the weed flora as well as several important agrotechnical parameters, such as soil moisture, soil temperature, and organic matter content of the soil (Dezső and Pásztor, 2022; 2024; Schonbeck and Evanylo, 1988a-b).

In Hungary, sweet potato cultivation is gaining popularity in both large and small farms, as well as in home gardens, utilizing diverse production technologies, including black PE film mulching (INTERNET1; Kohut, 2023; Takácsné and Rubóczki, 2019).

2 Materials and Methods

The experiment was established in 2024 at the Dezső family farm in Nemespátró, Zala County, Hungary. Planting took place on May 22, with a row spacing of 70 cm and a plant spacing of 45 cm, using “Orange” variety and applying drip irrigation. Only biological pesticides (preparation of *Trichoderma asperellum* and *Beauveria bassiana* separately) were used.

For the experiment, 4 rows were designated, each 18 m long, divided into 8 treatments in a non-randomized arrangement (Figure 1). Each treatment was set up on a 9 m section, within which 4 plots were marked for data collection. The applied treatments were as follows: **C:** Weed-free control; **A:** Agrotextile mulch + 2 weeding; **S:** Straw mulch + 2 weeding; **G**: Grass cuttings mulch + 2 weeding; **WC:** Weedy control; **WA:** Agrotextile mulch without supplementary weeding; **WS**: Straw mulch without supplementary weeding; **WG**: Grass clippings mulch without supplementary weeding.

To assess the effectiveness of the treatments, weed surveys were conducted 4 times during the vegetation period (June 11, June 29, July 29, and October 1) using the Balázs-Ujvárosi method in 70 × 70 cm sampling areas. The impact of the mulching materials and weeds on soil temperature and soil moisture was measured using a specialized soil thermometer with a functionality to measure soil moisture on a 5-point scale. The temperature measurements had an accuracy of 1 °C. These measurements were conducted 3 times (June 14, June 19, and August 24). The growth of the plants was also monitored. At the beginning of the vegetation period, the length of the longest shoot was measured twice (June 13 and June 30). During the later surveys (July 29 and September 1), the ground cover by the plants was assessed. At the end of the vegetation period - during harvest (October 12) yield per plant was measured, and quality parameters (average tuber weight, proportion of damaged tubers, and marketable yield) were examined. The labor required for the application, maintenance, and weeding of the mulching materials was recorded, and the associated costs were quantified.

Data analysis was performed using MS Excel and IBM SPSS 27 software. The statistical methods applied included ANOVA, Welch test, Kruskal-Wallis test, and related post-hoc tests. Correlation and regression analyses were also conducted to examine the relationship between yield and weed coverage.

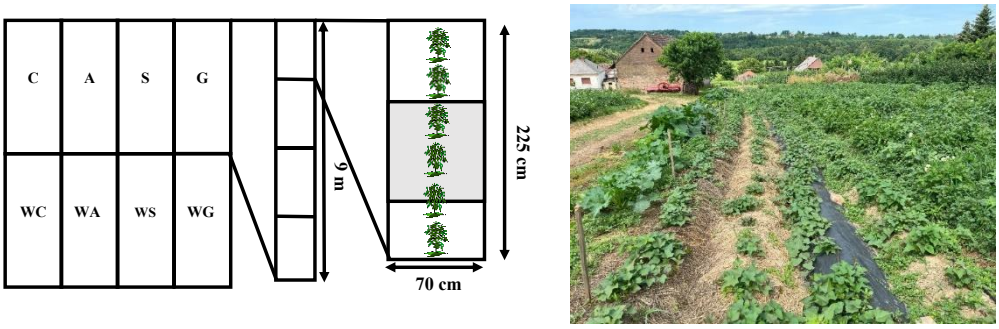

Figure 1. *Left: The design of the experiment Right: The experiment on June 20. 2024 (Dezső Dániel)*

3 Results/ Results and Discussion

3.1 Time requirement and cost of weed controll technologies

The most time-intensive treatment was the four weedings in Treatment C, requiring a total of 110 minutes (20–40 minutes per weeding). The use of mulch materials reduced the time needed for weed control by 65–82%. WA and WG required 28 minutes, WS alone required 20 minutes, straw (S) required 30 minutes, agrotextile (A) required 32 minutes, and grass clippings (G) required 39 minutes. In Treatment A, less time was needed for weeding compared to S and G (1–3 minutes versus 4–7 minutes per weeding) due to lower weed cover. The preparation and season-end removal of agrotextile required the most time in Treatment A. Significant advantage of organic mulches (S and G) is that they do not require removal, although collecting the mulch, especially in the case of G, demands substantial time (Figure 2).

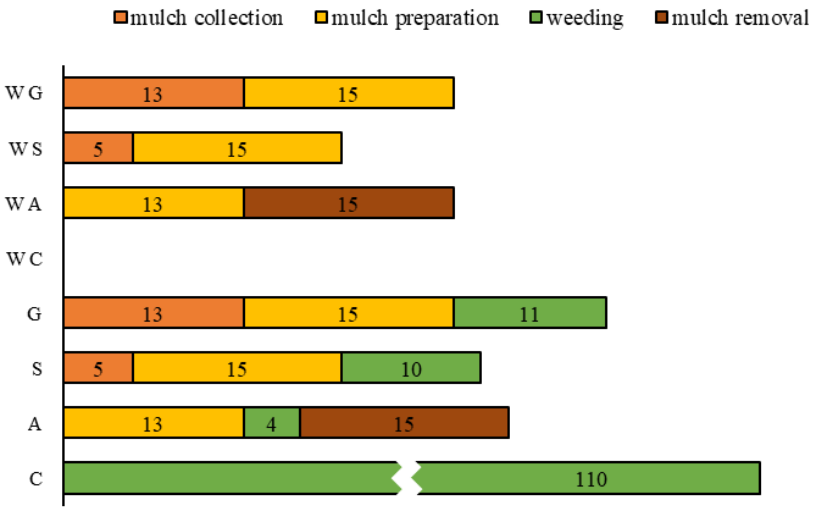

Figure 2 *Time requirement of weed controll in different treatments (min/10 m²) C-Weeded Control, A-Agrotextile, S-Stew, G-grass cuttings, WC-Weedy Control, WA-Weedy Agrotextile, WS-Weedy stew, WG-Weedy grass cuttings*

Grass clippings can be sourced for free from public areas or farmyards; however, mulching a 1.000 m² area requires a very large supply and/or tall grass. The cost of straw in 2024 was 65.000 HUF/1,000 m² (10 large round bales), while agrotextile costs ranged from 224.000 to

263.000 HUF/1,000 m². Agrotextile offers the advantage of being reusable for several years (at least 4 years which is the payback period compared to stew), based on our experience.

3.2 Evaluation of weed infestation and coverage

We found 21 weed species in the experiment across the vegetation period (Table 1). Most of the weeds in the experimental area were summer annuals (T4 species), such as *Amaranthus* spp., *Chenopodium album*, *Echinochloa crus-galli*, and *Ambrosia artemisiifolia*, the perennial *Convolvulus arvensis* was also prevalent.

Table 1 Weed species found in the experiment, ranking based on the average canopy across the vegetation period C-Weeded Control, A-Agrotextile, S-Stew, G-grass cuttings, WC-Weedy Control, WA-Weedy Agrotextile, WS-Weedy stew, WG-Weedy grass cuttings

Weed species	Life cycle[1]	Overall	Ranks in: C	A	S	G	WC	WA	WS	WG
Amaranthus blitoides	T4	1	2	3	2	2	6	4	1	2
A. retroflexus	T4	2	-	-	-	-	2	3	2	1
A. chlorostachys	T4	3	3	-	3	3	1	-	4	4
Convolvulus arvensis	G3	4	5	1	1	1	7	1	7	5
Echinochloa crus-galli	T4	5	4	-	8	6	-	-	3	3
Portulaca oleracea	T4	6	1	2	4	5	8	-	-	-
Chenopodium album	T4	7	9	-	5	-	-	2	5	8
Ambrosia artemisiifolia	T4	8	8	-	6	13	4	-	8	7
Solanum nigrum	T4	9	-	-	-	-	5	6	6	6
Galinsoga parviflora	T4	10	7	-	14	4	3	-	9	9
Digitaria sanguinalis	T4	11	6	-	9	10	10	-	-	-
Sonchus oleraceus	T4	12	12	-	-	9	9	5	-	-
Oxalis spp.	T4	13	-	-	7	-	-	-	12	-
Lolium multiflorum	T2	14	13	-	-	8	12	-	10	-
Taraxacum officinalis	H3	15	-	-	-	7	-	-	-	-
Urtica dioica	G1	16	-	-	-	-	-	-	11	-
Stellaria media	T1	17	10	-	11	12	-	-	-	-
Senecio vulgaris	T1	18	11	-	13	11	11	-	-	-
Setaria viridis	T4	19	-	-	10	-	-	-	-	-
Capsella bursa-pastoris	T1	20	-	-	-	12	-	-	-	-
Glechoma hederaceum	H1	21	-	-	12	-	-	-	-	-

[1] *T3-T4: summer annual weeds; T1-T2: winter annual weeds; G1, G3, H1, H3: perennial weeds*

In mulched and weeded areas (A, S, G), *C. arvensis* surpassed annuals in dominance. Table 1 shows that fewer weed species were present in mulched areas compared to WC (weed control). However, in three of the four survey periods, only Treatment A differed significantly from WC

($p<0.001$). On July 29 only, significantly fewer weed species were found in all mulched areas compared to WC. In Treatment G, the perennial *Taraxacum officinale* was introduced, likely via the grass clippings. Winter annuals (T1-T2 species) were more prominent in organically mulched and weeded areas, while the absence of weeding allowed summer annuals to dominate in WS and WG. Notably, no winter annual species were observed in these treatments.
Weed coverage differed significantly ($p<0.001$) across all four survey periods (Table 2). On June 11, all mulch materials significantly reduced weed cover. By June 29, A and WA significantly reduced weed cover compared to all other treatments. S and G showed significantly lower weed cover than C and WC. After June 29, no further weeding was performed in A, S, or G treatments, while C underwent two additional weedings. Consequently, A and C exhibited significantly lower weed cover in subsequent surveys compared to WC, WS, and WG. Organic mulches (S and G) performed similarly to WA and produced acceptable results due to the increased weed suppression from crop canopy development by the end of the vegetation.

Table 2 Weed coverage in the treatments C-Weeded Control, A-Agrotextile, S-Stew, G-grass cuttings, WC-Weedy Control, WA-Weedy Agrotextile, WS-Weedy stew, WG-Weedy grass cuttings

Time	C	A	S	G	WC	WA	WS	WG	p value[1]
June 11	78.13 b	1.12 a	13.28 a	14.06 a	90.63 b	3.90 a	14.06 a	17.19 a	<0.001
June 29	75 c	1.87 a	18.75 b	14.84 b	100 c	7.03 a	84.63 bc	81.25 bc	<0.001
July 29	4.69 a	2.89 a	21.87 ab	24.16 ab	90.68 c	29.69 b	84.38 c	87.5 c	<0.001
Oct. 1	7.81 a	9.38 a	23.44 ab	29.63 ab	83.13 c	37.5b	70.63 c	71.86 c	<0.001

[1]*Treatments labelled with the same letter are not statistically different, whereas those with different letters show significant differences; if a treatment receives multiple letters, it is not significantly different from any associated group.*

3.3 Plant growth and coverage

In our experiment, crop plants fully covered the soil by July 15, but their weed-suppressing ability was limited before then. During the first survey on June 13, Treatment A produced significantly ($p=0.014$) longer shoots (27.1 cm) than S and WS (19.4–19.6 cm), with no significant differences among other treatments. On June 30, A achieved significantly greater ($p<0.001$) shoot lengths (108.5 cm) than all other treatments (36.8–61.4 cm).
On July 29, all treatments except C exhibited significantly smaller plant cover than A (97.5%). While it was 87.5% in C and 72.5% in S and G. WA achieved 45% cover, significantly different than all other treatments, while WC, WS, and WG produced only 8.75–15% cover. By October 1, A, C, S, and G resulted in significantly greater ($p< 0.001$) plant cover (71.25–90%) compared to unweeded treatments (13.75–42.5%).

3.4 Soil Temperature and moisture

On June 14, Treatments A and C resulted in significantly higher ($p < 0.001$) soil temperatures (23.5 and 23.8 °C respectively) than other treatments (20.6–21.3 °C). Figure 3 illustrates daily soil temperature variations, showing more stable curves for organic mulches (S and G) compared to A and C, where afternoon temperatures rose to 30.5 °C. By contrast, temperatures in S and G treatments peaked at 24.5 °C. On June 29, C produced significantly higher soil temperatures than A and all other treatments. By August 24, no significant differences were

observed among treatments (p=70.245), as crop and weed cover had developed fully, overshadowing the effect of mulch type on soil heat dynamics.
No differences in soil moisture were observed in the first two measurements (June 14 and June 30). However, on August 24, C exhibited significantly lower soil moisture (p=0.011) than G and WC, while other treatments showed no significant differences.

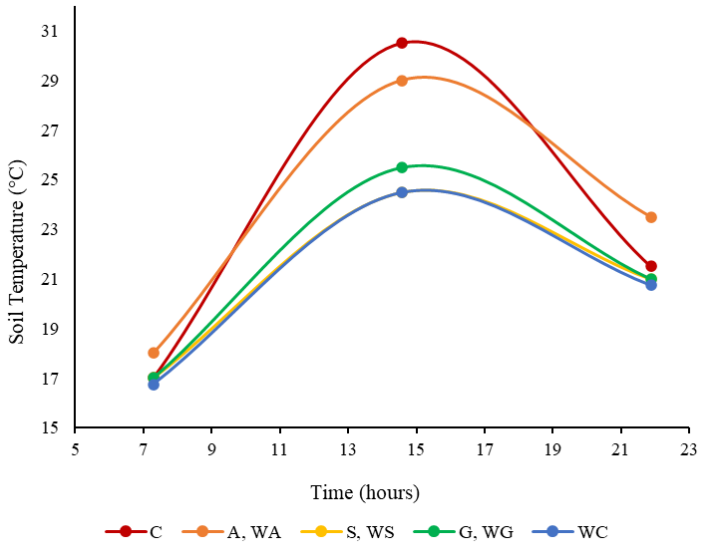

Figure 3 *The daily variation of soil temperature on June 14 in different treatments C-Weeded Control, A-Agrotextile, S-Stew, G-grass cuttings, WC-Weedy Control, WA-Weedy Agrotextile, WS-Weedy stew, WG-Weedy grass cuttings*

3.5 Assessment of yield and its connection with weed coverage

Treatment A produced the highest total yield, significantly exceeding all other treatments except C ($p<0.001$). S and G resulted in 16.4% and 23.3% lower yields than C, respectively, while agrotextile increased yield by 43.2%. Treatments without weeding exhibited yield reductions of 59.3–97.2%. The average tuber weight was highest in Treatment A, with acceptable values were recorded in C, S and G. Unweeded treatments (WC, WA, WS, WG) produced smaller tubers. The number of tuber per plant was significantly ($p<0.001$) highest in C, S and G smaller in WG, with no significant difference in A and WA and the smallest in WS and WC. Therefore, the use of agrotextile primarily increased the weight of the tubers rather than their number.
Pest damage (caused by wireworms and snails) affected 10–33% of the tubers, with no significant differences among treatments (p=0.159). Damaged tubers were still marketable as second-grade products. Treatment A resulted in the highest marketable yield, followed by significantly lower but acceptable values in C, S, and G. WA did not significantly differ from these, while WS and WG yielded much less, and WC produced no marketable yield (tubers were too small, averaging 23 g/tuber). The yield was also converted to a per-hectare basis; however, these data are for informational purposes only, allowing readers to compare the average yields of other crops. The gross production value was calculated per 1,000 m², with the highest value recorded in treatment A. All treatments without weeding produced significantly lower values, while treatments C, S, and G did not differ significantly from any treatment.
Yield showed a strong negative correlation with weed cover during the last three surveys, with correlation coefficients of -0.640, -0.890, and -0.899 ($p<0.001$). A linear regression model revealed that weed cover on October 1 most strongly predicted yield ($p<0.001$). The regression equation ($y=2356-28.44*x$) indicates that every 1% increase in weed cover reduces yield by 1.21% or 0.69 t/ha.

Table 3 Yield and qualitative parameters significant difference C-Weeded Control, A-Agrotextile, S-Stew, G-grass cuttings, WC-Weedy Control, WA-Weedy Agrotextile, WS-Weedy stew, WG-Weedy grass cuttings p<0.001 in every parameter (label letters as described in Table 2)

	C	A	S	G	WC	WA	WS	WG
total yield / plant [g]	1988 ab	2847 a	1662 b	1525 b	55 c	810 bc	261 bc	308 bc
avg. tuber waight [g]	243 b	425 a	193 b	192 bc	23 c	114bc	84 bc	82 bc
tuber/plant	10.13 a	7.5 ab	9.25 a	8.63 a	2.06 c	7.69 ab	3.25 bc	4 b
yield loss [%][1]	-	+43.2	16.4	23.3	97.2	59.3	86.9	84.5
marketable yield [g/plant] [2]	1789 b	2654 a	1403 b	1291 b	0 c	636 bc	159 c	203 c
marketable yield [t/ha] [2]	56.79 b	84.25 a	44.54 b	40.98 b	0 c	20.19 bc	5.05 c	6.44 c
gros income (HUF) [2]	3.041 ab	4.698 a	2.587 ab	2.339 ab	0 b	1.082 b	276 b	368 b

[1] Based on C (Controll)

[2] First grade (I.) + Second grade (II.)-damaged by wireworms and slugs (about 10-33%); net prices: II.: 450 HUF/kg, I.: 600 Ft/kg; gros income in thousend HUF/1000 m²

4 Discussion

The mulch materials significantly influenced weed coverage and weed flora, consistent with our previous research (Dezső and Pásztor, 2022; 2024). Additionally, they affected soil temperature, as seen in studies by Schonbeck and Evanylo (1988a) and Dezső and Pásztor (2024). However, their impact on soil moisture could not be demonstrated, probably due to continuous irrigation and the inaccuracy of the measuring instrument.

Strew and grass cuttings mulch resulted in acceptable yield loss compared to the weeded control but only with addition weeding, without weeding organic mulch does not have enough weed suppression ability as described in Dezső and Pásztor (2022). Agrotextile, however, resulted in significantly higher yields compared to the control treatment, likely due to improved water retention (besides the better weed suppression than only weeding) which warrants further investigation. The use of agrotextile is most recommended, as it produced significantly higher yields than organic mulches, required no substantially greater labor time, and, if used for at least four years, its costs are not higher than straw. However, it is made of plastic like other films that are highly polluting (Miles et al., 2012), it generates much less waste at the end of its usage. Nevertheless, microplastic pollution remains a concern. Although organic mulches can increase soil humus content according to Schonbeck and Evanylo (1988b), removing them from their source areas may decrease humus levels. Our current research did not address soil organic matter content or microplastic pollution, both of which merit further study. Another drawback of agrotextile is the time required for its removal. Moreover, the increased average tuber weight observed in our study may not always be advantageous as we harvested many tubers weighted 1 kg or larger witch are not always desirable in the market.

In the experiment, a relatively high proportion of tubers were damaged by terrestrial pests, however we used *Beauveria bassiana* preparations. Since currently this is the only other major pest issue in sweet potato cultivation, it would be worthwhile to conduct experiments on pest

control strategies, focusing on optimizing the application of biological methods, exploring chemical alternatives, and determining their effectiveness.

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Impact of crop site geographic altitude on drought indices of field crops in Hungary

A termőhely földrajzi paramétereinek meghatározó szerepe szántóföldi növényfajok aszályindexének alakulásában Magyarországon

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Abstract: Growth and development of field crops is highly influenced by the water availability of the crop site. In an assessment study at the MATE University, the magnitude of aridity in relation with the geographic location of the crop site has been evaluated. Six field crop species (Sugar beet *Beta vulgaris*, winter barley *Hordeum vulgare*, winter wheat *Triticum aestivum,* maize *Zea mays*, potato *Solanum tuberosum*, and alfalfa *Medicago sativa*) were involved in the research. Data bases of twelve meteorological stations (Békéscsaba, Budapest, Debrecen, Miskolc, Mosonmagyaróvár, Nagykanizsa, Nyíregyháza, Pécs, Siófok, Szeged, Szolnok, Szombathely) representing major geographic areas of Hungary were used in the evaluation. PAI indices of the stations involved were combined with vulnerability indices of the field crops studied. Upon the results of the study cereals proved to be the most tolerant, while potato and maize were highly influenced by aridity x vulnerability interactions. Considerable impact could be seen in the case of alfalfa and sugar beet. The geographic altitude of the crop site was in negative correlation with the magnitude of drought indices.

Keywords*: field crops, altitude, crop site, drought, vulnerability*

Összefoglalás: A szántóföldi növények növekedésében és fejlődésében döntő szerepe van a termőhely vízellátottságának. A MATE növénytermesztési kutatásaiban vizsgálták a termesztett növényfajok és a termőhely földrajzi paraméterei által meghatározott ariditási értékek összefüggését. Hat növényfaj (cukorrépa *Beta vulgaris*, őszi árpa *Hordeum vulgare*, őszi búza *Triticum aestivum,* kukorica *Zea mays*, burgonya *Solanum tuberosum*, és lucerna *Medicago sativa*) termesztési paramétereit elemezték tizenkét termőhely (Békéscsaba, Budapest, Debrecen, Miskolc, Mosonmagyaróvár, Nagykanizsa, Nyíregyháza, Pécs, Siófok, Szeged, Szolnok, Szombathely) meteorológiai állomásainak adatbázisán. A vizsgálat során az aszályindexek (PAI), illetve a sérülékenységi indexek (VI) kölcsönhatásait tanulmányozták. A kapott eredmények alapján igazolható volt, hogy a kalászos gabonák aszálytűrő képessége volt a legnagyobb, a kukoricáé és a burgonyáé pedig a legkisebb. A lucerna és a cukorrépa vízellátottsági kitettsége is jelentős volt. A termőhely földrajzi paraméterei közül a tengerszint feletti magasság az aszályindexekkel negatív korrelációt mutatott.

Kulcsszavak*: szántóföldi növények, földrajzi paraméterek, termőhely, aszály, érzékenység*

1 Introduction

All live systems depend on water availability. Water budget of crop sites is a determining factor regarding plant growth and development. Water deficiency of crops is labelled as aridity, however the physiological state my range from water scarcity to drought (Várallyay 2006). All physiological processes depend on the presence of moisture, like photosynthesis, osmosis, turgor, transpiration, respiration, as well as growth and development, and propagation. Water supply may have an influence in all of them. Aridity in general may obstruct growth and development, however drought is the most sever from among all types of water scarcity.

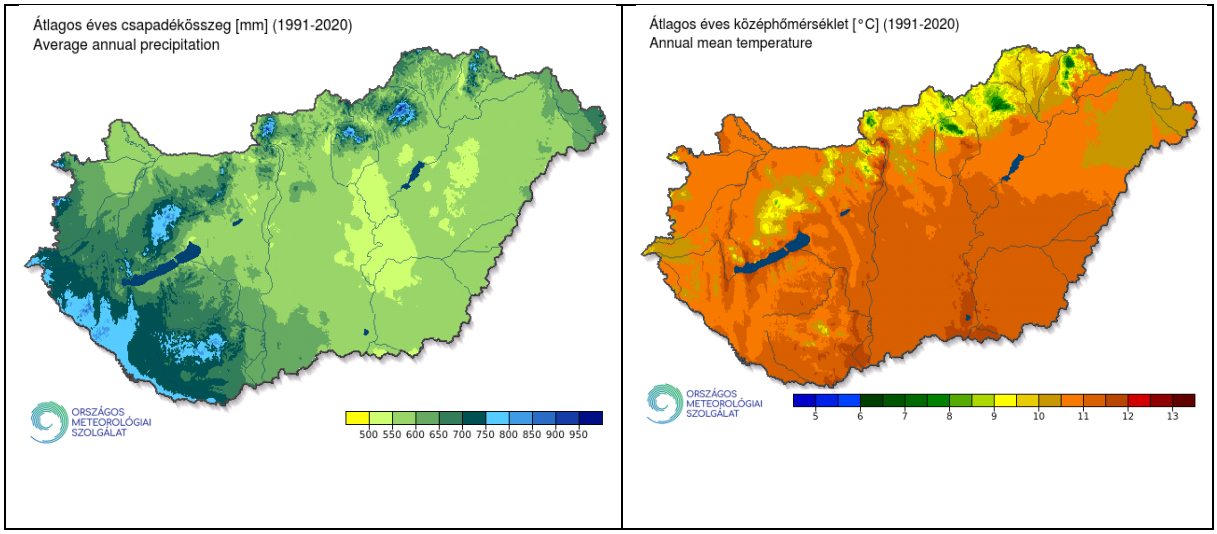

Figure 1 *Annual mean precipitation and temperature in Hungary on a 30 years' timescale (OMSZ 2022)*

Definition of droughts can be assessed in three main ways (Jolánkai et al 2012). Meteorological drought, hydrological drought and agricultural drought. The latter can be defined shortly, that drought is a phenomenon when a plant suffers irreversible physiological damages.

There are various assessments for quantification of water scarcity. Aridity indices are numerical indicators of the degree of dryness of the climate at a given location. A number of indices have been used in various parts of the world, like Köppen and Thornthwaite indices (UNEP 1992). These indicators serve to identify, locate or delimit regions that suffer from a deficit of available water, a condition that can severely affect the effective use of the land for such activities as agriculture or stock-farming. In Hungary the Pálfai Drought Index PAI is extensively used in agrometeorology (Pálfai 1990; Lakatos-Szalai 2010). In all aridity indices climatic components are expressed in mathematical formulas. Geographic locations in this context are very seldom examined upon the basis of crop site altitude, however the elevation may have a profound role in the utilization of natural water resources by the vegetation and so by the crop plants produced.

Agricultural crops have diverse reactions to water availability conditions. According to their taxonomy, life cycle, evapotranspiration patterns and the crop site characteristics, crop plants can be clustered to various vulnerability groups. The present study is dealing with the interaction between aridity and climatic vulnerability of some of the major field crop species of Hungary, as well as to evaluate changes in the aridity indices in relation with the geographic altitude of the crop site.

2 Materials and Methods

An assessment study has been done at the MATE University, Gödöllő to evaluate and identify the main factors of aridity. Almost all major field crop species were involved in the study, from which six species (Sugar beet *Beta vulgaris*, winter barley *Hordeum vulgare*, winter wheat *Triticum aestivum,* maize *Zea mays*, potato *Solanum tuberosum*, and alfalfa *Medicago sativa*) have been evaluated and presented. Crop vulnerability values (VI) were based on the mathematical model of Tarnawa et al (2010). In the survey databases of the Hungarian Meteorological Service (OMSZ) and the Ministry of Agriculture (AM) have been used (KSH 2022, OMSZ 2022). The use of Pálfai Drought Index (PAI) has been applied during the survey (Pálfai 1990, Bihari et al 2012). PAI values have been evaluated in a context of long-term databases. Regional evaluations were done respecting the databases of 12 meteorological stations chosen randomly to represent most of the regions of the territory of Hungary (Vermes 2011, Tarnawa et al 2012).

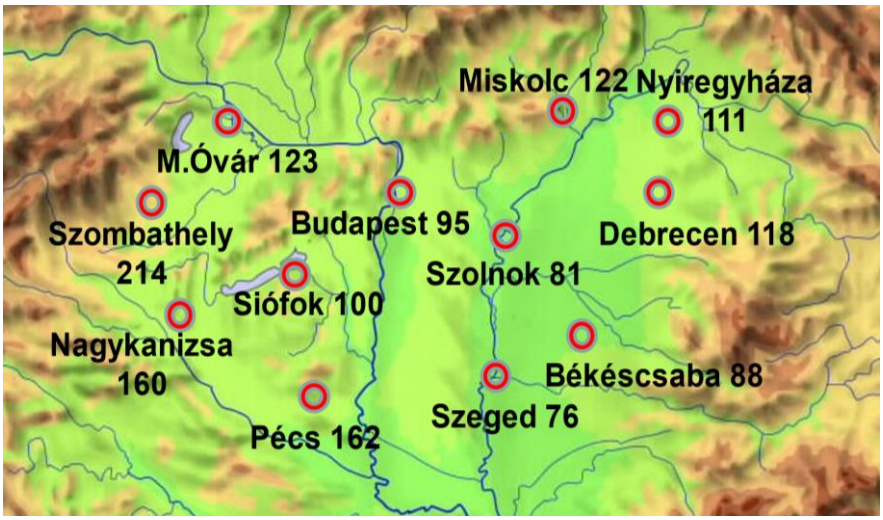

Figure 2 *Geographic altitude data of some meteorological stations in Hungary involved in the study, m*

Figure 2 presents geographic altitude data of twelve meteorological stations representing the territory of Hungary. Evaluating the long-term data bases the methodology of the state of the World's land and water resources for food and agriculture – Systems at breaking point (FAO 2021) was used. For statistical evaluations standard methods were applied; correlations, regression analysis, offered by Microsoft Office 2006.

3 Results and discussion

Pálfai Drought Indices of certain meteorological stations of the OMSZ Hungarian Meteorological Service (Békéscsaba, Budapest, Debrecen, Miskolc, Mosonmagyaróvár, Nagykanizsa, Nyíregyháza, Pécs, Siófok, Szeged, Szolnok and Szombathely) calculated on 50 years' averages were processed for each crop species studied. The results presented in Table 1 verify detectable differences between locations.

Twelve meteorological stations were randomly chosen to represent various levels of drought probability. The highest PAI indices were found in the case the central and the South-Eastern part of Hungary, while in the mountainous locations westward smaller figures were observed.

Table 1 Drought x crop vulnerability interactions regarding twelve meteorological stations and six field crop species based on 50 years' average

PAI (°C/100 mm)		wheat	winter barley	maize	potato	alfalfa	sugar beet	mean
VI indices		5,6	5,8	7,3	6,5	7,6	7,7	6,75
Békéscsaba	5.47	5,5	5,6	6,4	6,0	6,5	6,6	6,11
Budapest	5.85	5,7	5,8	6,6	6,2	6,7	6,8	6,30
Debrecen	4.91	5,3	5,4	6,1	5,7	6,3	6,3	5,83
Miskolc	4.18	4,9	5,0	5,7	5,3	5,9	5,9	5,47
Mosonmagyaróvár	4.69	5,1	5,2	6,0	5,6	6,1	6,2	5,72
Nagykanizsa	3.79	4,7	4,8	5,5	5,1	5,7	5,7	5,27
Nyíregyháza	5.23	5,4	5,5	6,3	5,9	6,4	6,5	5,99
Pécs	4.22	4,9	5,0	5,8	5,4	5,9	6,0	5,49
Siófok	5.07	5,3	5,4	6,2	5,8	6,3	6,4	5,91
Szeged	5.88	5,7	5,8	6,6	6,2	6,7	6,8	6,32
Szolnok	6.02	5,8	5,9	6,7	6,3	6,8	6,9	6,39
Szombathely	3.79	4,7	4,8	5,5	5,1	5,7	5,7	5,27
mean	4.92	5,2	5,4	6,1	5,7	6,3	6,3	5,83

Studying the interactions between drought and vulnerability the data suggest that crop species in accordance with their water consumption patterns and physiological characteristics may have a rather diverse performance in relation with the crop site PAI values.

Yield performance of the six crop species had a reducing trend due to the geographic elevation of the crop site. PAI x VI interactions have shown constant reduction of the crop yields. The trendline of this reduction was labelled by a strong linear regression. The lowest altitude was that of the Szeged crop site with 76 m above sea level, while the highest one of Szombathely had a geographic elevation of 214 m. The reason of this trend may be due to the annual mean temperature and the average precipitation of the location as this can be detected by the data of Fig 1. Central part of the lowland proved to have a higher temperature mean belonging to the annual 10-11 ºC isotherm range, while the same locations received an annual 500-550 mm precipitation over the long term.

Figure 3 presents data of the survey where the crop sites belonging to the certain meteorological stations were evaluated by their geographic altitude.

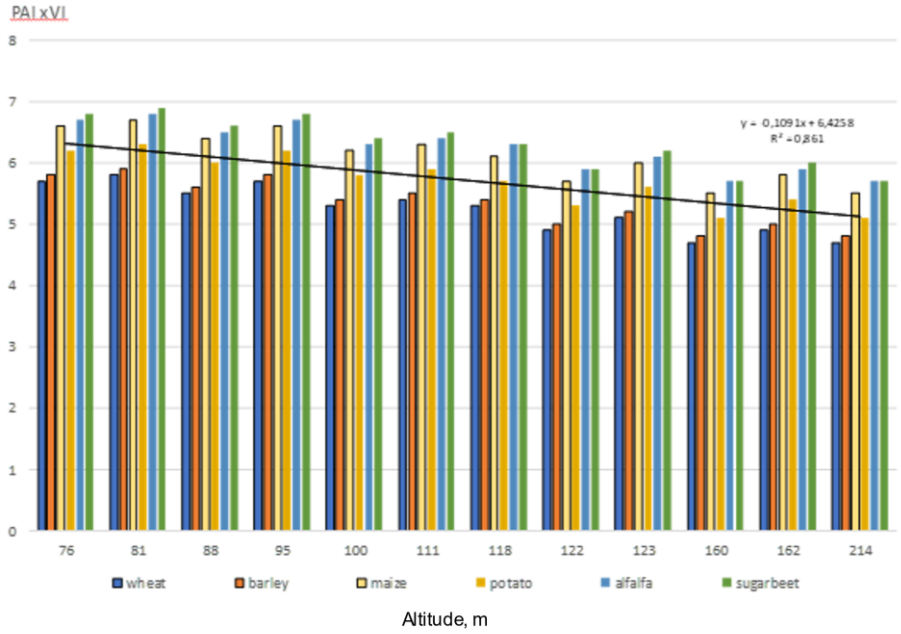

Figure 3 *Drought x crop vulnerability interactions of six field crop species by crop site altitude*

There were some alterations within the records since the most arid areas of the Great Plain belonging to the less than 500 mm range are located by the Szolnok station which is labelled by a higher PAI index than that of the Szeged crop site. Also, the highest altitude of this study at Szombathely with its 214 m had a medium level of PAI drought index. In the case of crop-species the trends were almost consequent but have shown detectable differences in accordance with their vulnerability. The results of the study support the postulate, that cereals were the least susceptible species, while potato and maize were proved to be highly influenced by drought x vulnerability interactions. Strong climatic impact could be detected in the case of alfalfa and sugar beet. Altitude of the crop site was in negative correlation with the magnitude of drought indices.

Figure 4 presents the regression data of the PAI drought indices related to the altitude of the meteorological stations of the crop sites. The equation can be expressed by a polynomial equation that is having a rather strong statistical value.

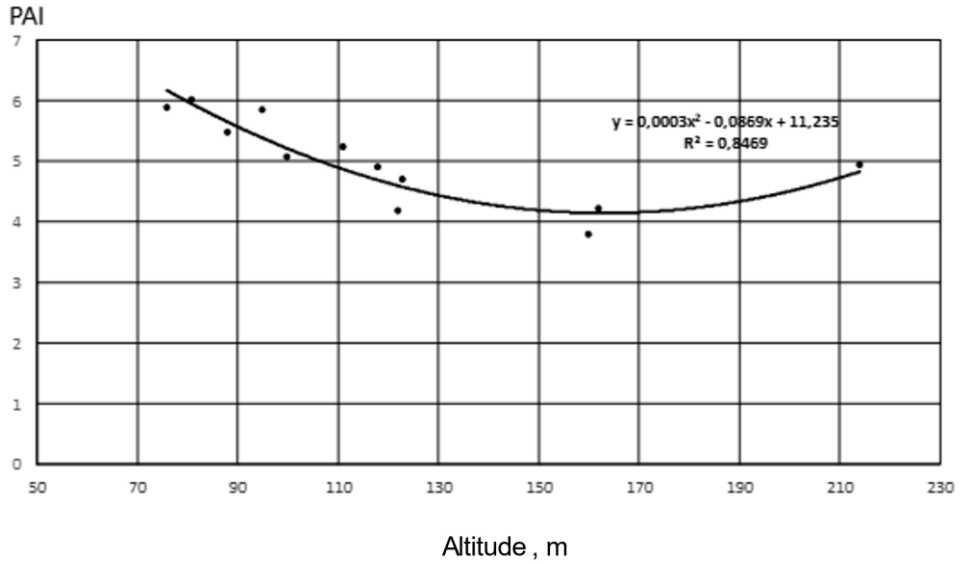

Figure 4 *Changes in the PAI indices due to the geographic elevation of crop sites*

4 Conclusions

As a conclusion of the study, it can be stated, that the geographic location may have a strong influence for the performance of various crop plant species. Certain crops like cereals are less susceptible to crop site conditions, while others, especially those with higher water demand like maize and potato are more exposed to that. Alfalfa and sugar beet were definitely proven to be the most vulnerable crops in this study. The interaction between PAI and VI indices were proved to be useful for characterising the crop site.

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Utilizing birds as a bioindicator species to monitor potentially toxic elements (PTEs) contamination in an ecosystem

A madarak bioindikátorként történő alkalmazása a potenciálisan toxikus elemek (PTE-k) szennyeződésének nyomon követésére az ökoszisztémákban

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Abstract: Potentially Toxic Elements (PTEs) is a versatile term which includes heavy metals, non-metals and even essential elements, that pose significant environmental and health risks to humans, animals, and plants. Bioindicator species, particularly birds, are valuable tools for monitoring PTEs contamination in ecosystems, offering insights into pollutant levels and their ecological impacts. Birds, as top predators with extensive mobility, absorb contaminants across large areas, making them effective bioindicators in freshwater ecosystems such as lakes, rivers, and wetlands. This study reviews recent research (2014-2024) on the use of birds for biomonitoring of PTEs, focusing on their behavior, feeding habits, and migration patterns, which influence contamination accumulation. Key findings indicate that bird species' diet, residency, and foraging behavior significantly affect PTEs bioaccumulation, with migratory species showing higher metal concentrations. Different sample types, including feathers, blood, and excrement, serve as non-destructive methods for assessing PTEs exposure in birds, with feathers possibly becomes a reliable indicator of metal accumulation in internal tissues. The review emphasizes the importance of selecting appropriate bird species and sample types to enhance the accuracy of environmental contamination assessments and underscores the utility of birds in understanding the broader ecological effects of PTEs pollution.

Keywords*: thematic review, potentially toxic elements (PTEs), bioindicator, birds, heavy metals*

Összefoglalás: A potenciálisan toxikus elemek (PTE-k) egy sokoldalú kifejezés, amely magában foglalja a nehézfémeket, a nem fémeket és akár az esszenciális vegyületeket is, amelyek jelentős környezeti és egészségügyi kockázatot jelentenek az emberekre, állatokra és növényekre. A bioindikátor fajok, különösen a madarak, értékes eszközök a PTE-k szennyeződésének monitorozására az ökoszisztémákban, mivel betekintést nyújtanak a szennyező anyagok szintjeibe és azok ökológiai hatásaiba. A madarak, mint csúcsragadozók, akik széles körű mobilitással rendelkeznek, képesek felhalmozni a szennyező anyagokat nagy területeket bejárva, így hatékony bioindikátorok az édesvízi ökoszisztémákban, mint tavak, folyók és mocsarak. Közleményünkben a madarak PTE-k biomonitoring célú felhasználásával

kapcsolatos legújabb kutatásokat (2014-2024) vizsgálja, kiemelve azokat a tényezőket, amelyek befolyásol(hat)ják a szennyeződés felhalmozódását, így a madarak viselkedését, táplálkozási szokásait és a migrációs mintáikat,. A legfontosabb megállapítások azt mutatják, hogy a madárfajok étrendje, tartózkodási helyük és táplálkozási magatartásuk jelentősen befolyásolják a PTE-k biokumulációját; a migráló fajok alapvetően magasabb fémkoncentrációkat mutatnak. A különböző típusú minták, például tollak, vér és ürülék, nem destruktív módszerekként szolgálnak a madarak PTE-k expozíciójának értékelésére, a tollak pedig megbízható indikátorrá válhatnak a fémek felhalmozódására a belső szövetekben. A tanulmány hangsúlyozza a megfelelő madárfajok és minta típusok kiválasztásának fontosságát a környezeti szennyeződés értékelésének pontosságának növelésében, és kiemeli a madarak hasznosságát a PTE-k szennyezésének szélesebb ökológiai hatásainak megértésében.

***Kulcsszavak**: tematikus áttekintés, potenciálisan toxikus elemek (PTE-k), bioindikátor, madarak, nehézfémek*

1 Introduction

Potentially toxic elements (PTEs) are a broad term used to describe contaminants which include heavy metals with density greater than 5 gcm^{-3} and non-metals. Heavy metals persist in the environment and can cause toxic effects to humans, animals and plants. Essential elements (micronutrients and trace elements) that are bioavailable at high concentration and become toxic are also consider as heavy metals. Whereas, non-metals also has its function in plants and humans at low concentration such as Arsenic (As) and selenium (Se) (Nieder & Benbi, 2024). These contaminants exist naturally in the environment with addition to anthropogenic sources (urbanization, manures, mining, fertilizers and pesticides, vehicle exhaust, coal burning). Once it is bioavailable, organisms at higher trophic level (i.e. birds) absorb these elements from macro-invertebrates and other abiotic elements which subsequently accumulated in their body (Sanchari, 2023).

Bioindicator is a measured sample that is taken from a biological species such as birds to monitor the burden of toxicants in the environment. Instead of studying a large sample from natural ecosystems, a fraction of sample from biological species could access the impact of contaminants of concern in an ecosystem (Egwumah et al., 2017). Typically, birds are an excellent species that could be used to observe metal burden, environmental health and their habitat habilitation (Siddig et al., 2016). Waterbirds are at the top of the food chain and have been shown to be appropriate for monitoring environmental contamination due to their long lifespan and great mobility, which allows them to absorb contaminants across a wide area (Kocagöz et al., 2014).

2 Methods

Relevant articles were scouted from scientific article search engine and database Google Scholar and PubMed. Discrete keywords such as ‘bioindicator species’, ‘birds biomonitoring’ and ‘wetlands contaminants’ were used. Only English articles from the year 2014 to 2024 were considered in this review. The articles that discussed contaminants in freshwater ecosystems which includes lakes, river and wetlands were considered in inclusion criteria while articles on biomonitoring of other organisms were omitted.

40 articles were considered and 21 were selected for this article. References management software (EndNote 20) was utilized to record and systemically review the articles based on

main keywords and assist for in-text citation. Thematic synthesis was conducted when reviewing the articles and main findings were extracted to integrate the theories to draw broader conclusions.

3 Results and Discussion

Bird species are one of the feasible vertebrates to be used as an indicator to measure the metals burden in the environment due to their morphology and physiology characteristics. Indirectly, when measuring the PTEs in birds, there are in-depth information regarding their health such as their dietary deficiency which makes them more prone to metal toxicity (Espín et al., 2024). This is crucial when selecting a specific avian species to study certain elements.

The source and distributions of contaminants could be traced down whether it is a point source or due to the trophic transfer, land use or the transport of contaminants based on the behavior, life history, migration and territory of the studied birds' species (Maznikova et al., 2024). Typically, bio-indicator species were chosen based on existing research, conservation status and their abundance in the study area to facilitate with the research trajectory and cross-referencing with the available information (Siddig et al., 2016).

3.1 Biomonitoring based on bird's ecology

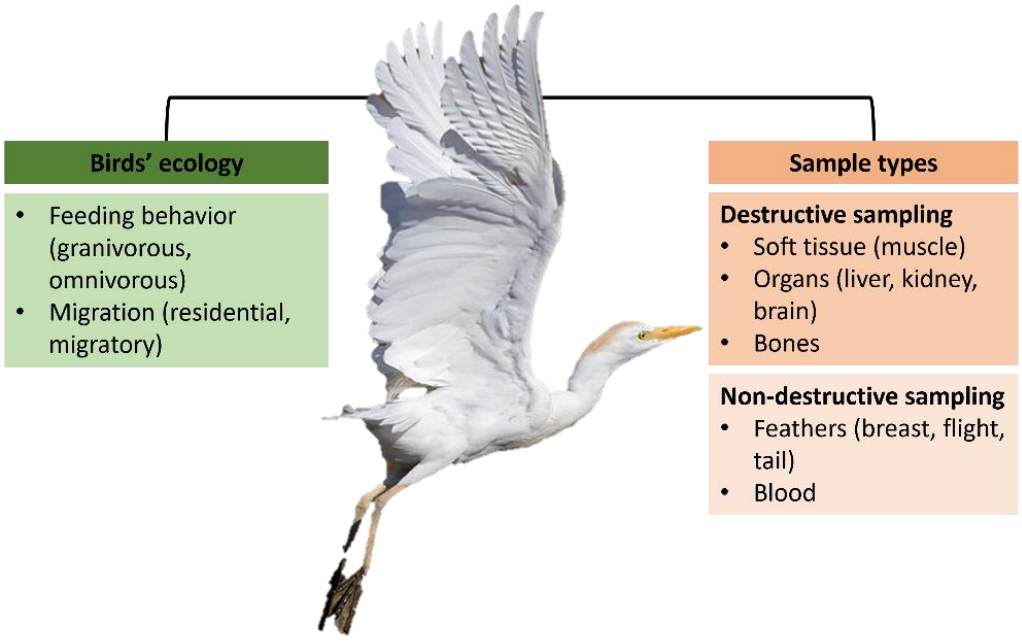

Figure 1 *Factors to consider when selecting bird's species as bioindicator to study PTEs contamination.*

The behavioral ecology and physiology characteristics of birds provide vast information on the fate of contaminations and how it manifested (Figure 1). There are variations between residential and migratory birds, feeding behaviors (granivorous and water birds), its diet and predator (Kocagöz et al., 2014; Samaraweera et al., 2022). Birds residing in the same geographical area potentially retain different concentration of PTEs depending on their residency pattern, diet and feeding behaviors, genetic and physiological characteristics (Khwankitrittikul et al., 2024).

Some birds who feed on invertebrate may retain higher lead (Pb), cadmium (Cd), nickel (Ni) and zinc in their liver and kidney compared to birds feeding on fish and other omnivorous species (Dahmardeh Behrooz & Burger, 2022). One of the many research projects on semi-aquatic birds revealed the residue of PTEs in different tissues. Copper (Cu), Pb, Cd and chromium (Cr) (in order of highest to lowest concentration) were presented in liver and blood of Cattle egret (*Bubulcus ibis*). The feeding behavior of this species on aquatic animals (i.e. fish) suggest that the PTEs except Cr can be transferred from lower to higher trophic level (Zaman et al., 2022). The ability of PTEs to bioaccumulate through trophic transfer could show potential threat in the ecosystems. For example, the concentration of Se increased when compared to prey and the eggs of passerine birds, Dippers (*Cinclus spp*). The passerines had consumed contaminated prey and then passed down during egg formation which proved a presence of threat to the health of local freshwater ecosystems (Maznikova et al., 2024).

In another research to study the effect of gender on PTEs accumulation in waterbirds by using their feathers shown different fate in different species. Gender of *Anas platyrhynchos* affect the accumulation of Pb while gender of *Anas crecca* affect the accumulation of Cu and Zn (Solgi et al., 2020). In contrast, a study on predatory bird in Hungary by using feathers sample shown no significant different of As, Cd, Pb and mercury (Hg) between genders (Grúz et al., 2019). This finding is also supported by a research in Iran where the level of Pb, Zn, nickel (Ni) and Cd are similar in both genders (Dahmardeh Behrooz & Burger, 2022). Although there is contrast in result between gender of birds, perhaps it is crucial to consider the birds species into consideration when drawing a conclusion.

The concentration of PTEs is high in migratory bird species compared to resident or local birds (Solgi et al., 2020). This could indicate that the accumulation in migratory birds happens over an extended period of time through different locations where they are overwintering. Interestingly, short distance migrant may accumulate higher PTEs than long distance migrant (Dahmardeh Behrooz & Burger, 2022). The reason of this disparity is likely due to localized exposure. Short distance migratory birds may spend longer time in high contaminated area while long distance migratory birds spend shorter time during stop-over and possibly avoiding the high contaminated areas. Hence, it is also crucial to consider using bioindicators that represent diverse habitats and foraging habits to determine the relationship between ecology of birds and its ability to accumulate PTEs.

3.2 Different sample types as bioindicator

There are two different sample categories when deciding to study the PTEs burden in birds which are destructive method and non-destructive or non-invasive methods. Commonly, muscles, bones and soft tissues such as liver were obtained for invasive sampling and rather less destructive catch and release methods to obtain feathers, blood and excrement sampling could be implemented.

Feathers can be obtained from the bird by catch and release method. Birds can be trapped using net, and species, gender identification and age estimation can be noted. 10 to 20 chest feathers as well as two secondary flight feathers can be plucked. The samples are then placed in a sealed bags and labelled with name, date and location. It is important to marked the birds before releasing them to avoid redundant sampling and aid in monitoring in the future (Yao et al., 2021). Other organic sample such as blood can be extracted from the bird's jugular vein by using heparinized syringe. Fresh excrement samples are usually taken during handling the bird for sample collection. Fresh sample need to be stored at -20^{o}C until assayed. Internal tissues and organs are retrieved during necropsy. Birds are euthanized by decapitation or shooting after the exercise is approved for ethical purpose by local regulatory body or national agency for research purpose (Berglund, 2018). The wet and dry samples are usually analyzed by

inductively coupled plasma mass spectrometry (ICP-MS) or inductively coupled plasma optical emission spectrometry (ICP-OES).

Feathers sample are a broad term for the sampling category. Researchers could select specifically different feather types such as from the body, wing, tail and breast (Rutkowska et al., 2018). Feathers are the most common non-destructive sampling because they are easy to handle which does not require deep freezing to store prior to the instrumental analysis. Besides, it is also possible to project the concentration of Pb and Cd in internal tissues once the concentration in feathers is determined (Varagiya et al., 2022). This sample type dictates the contamination source of entry in birds itself either through diets or exogenous factors. For example, Hg detected in feathers could come solely from food intake that transfer the metals through digestive tract into the bloodstream which then ended to the formation of keratin in feathers during detoxification. On the other hand, Cd and Pb detected in feathers can be originated from the metals present in the environment which then directly adhere to the keratin of the feathers (Rutkowska et al., 2018). A study conducted in a wildlife and bird sanctuary in India detected the contamination of Cu and Zn in all 11 studied bird species from the feathers sample. From the results, they could pin-point the sources of contamination whether it occurs naturally, manmade or through their diet by using compartments and multivariate analysis (Anbazhagan et al., 2021). Similar study in Pakistan on Cattle egrets (*Bubulcus ibis*) suggested that the bioaccumulation of contamination in their eggs, feathers, prey and sediment were due to their foraging habits (Abdullah et al., 2015).

Alternatively, using bird's feathers as bioindicator is deemed feasible to estimate the concentration of PTEs in other organs and soft tissues by using multiple regression and correlation analysis. For instance, feathers can be used to determine As, Cr and Pb in bone (sternum and femur) and internal organs (heart, liver, kidney) when the concentration is compared between different sample types to concentration in feathers (Khwankitrittikul et al., 2024; Mukhtar et al., 2020). On the other hand, specific tissue or organs sample could be selected to bio-monitor specific PTEs due to the sensitivity of these samples to certain elements. Based on previous studies, liver, feathers and kidney could be used to study Hg while liver, feather and kidney can be used to monitor Cd (Vizuete et al., 2019).

Blood and excrement were less discussed in published literature. However, they can be considered as a non-destructive sample to monitor the PTEs such as As, Cd, Zn, Pb and Cu. Berglund (2018) compare the heavy metal burden in blood and excrement then in then liver. Asymptomatic relationship was present for As, Cd and Pb when compared to liver and blood, excrement and liver and excrement and blood. However, at higher contamination area, using blood may underestimate the actual hazard. Meanwhile, excrement may overestimate the concentration at high exposure. Moreover, there is limited research on the PTEs level in the brain of bird. A study on birds' brain concluded that brain contain significant highest level of PTEs (Zn, Pb, Ni and Cd) than in liver, kidney and muscle (Dahmardeh Behrooz & Burger, 2022).

4 Conclusions

In conclusion, birds serve as an effective bioindicators for monitoring the presence and accumulation of potentially toxic elements (PTEs) in ecosystems. Their diverse ecological roles, behaviors, and mobility make them ideal for assessing environmental contamination over large areas and for extended periods. The study highlights the importance of considering factors such as residency patterns, diet, and migration in understanding how different bird species accumulate PTEs. Various sampling methods, including non-destructive techniques like feather analysis, offer valuable insights into the concentrations of toxic elements without compromising the health of the birds. The findings underscore the need for targeted research using specific bird species and appropriate sample types to improve the accuracy of environmental monitoring and better understand the impacts of PTEs pollution. By utilizing birds as bioindicators, we can gain crucial knowledge to inform conservation efforts and mitigate the environmental and health risks associated with PTEs contamination.

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An AI-driven image processing technique to simplify the pollen measurement in common ragweed (*Ambrosia artemisiifolia* L.)

Mesterséges intelligencia által vezérelt képfeldolgozó rendszer alkalmazása ürömlevelű parlagfű (Ambrosia artemisiifolia L.*) pollenmérésére*

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Abstract: *Ambrosia artemisiifolia* (common ragweed) is an invasive weed species that significantly impacts agriculture and public health. This study aimed to develop an automated AI-based object detection model using our annotated image recognition dataset for accurate pollen size measurement, focusing on repeatability and variability in pollen size among individuals with distinct morphological characteristics. The model can effectively streamline the traditionally labour-intensive process, achieving rapid, accurate data collection. Roboflow-based image analysis takes only milliseconds, which is significantly faster than traditional approaches, and a high repeatability index demonstrates a valid methodology for pollen analysis. The study suggests a relationship between pollen size variability and plant morphology, suggesting possible trade-offs between growth and reproduction or showing habitat-specific adaptations. Results may create valuable opportunities for plant biology or ecology, for instance, further investigation of plant-pathogen interactions and public health research. This innovative method represents a step forward in efficient pollen analysis and its integration into multidisciplinary studies.

Keywords*: automated measurement, image dataset, digital image processing, alien species, palynology, model training, artificial intelligence, machine learning*

Összefoglalás: Az ürömlevelű parlagfű (*Ambrosia artemisiifolia*) invazív gyomnövény, amelynek jól ismert a mezőgazdaságra és az egészségre gyakorolt negatív hatása. Munkánk során egy olyan mesterséges intelligencia alapú objektum felismerő modell kidolgozását tűztük ki célul, amely az általunk annotált képfelismerési adatbázist használja a pollenek méretének pontos meghatározására. Vizsgáltuk továbbá a pollenméret variabilitását eltérő morfológiai adottságokkal rendelkező egyedek között. A modell alkalmazása jelentősen leegyszerűsítette és felgyorsította a pollenmérés lassú és munkaigényes folyamatát. A hagyományos képelemző módszerekkel összehasonlítva, a Roboflow-alapú feldolgozás néhány milliszekundumra csökkenti a mérési időt. A repeatabilitás tesztelésekor, ugyanazt a pollent többször lemérve nagyon hasonló eredményt kaptunk, ami alapján kijelenthető, hogy a módszer alkalmas a pollenek méretének megbízható számszerűsítésre. Eredményeink egyedspecifikus összefüggést mutattak ki a pollenek méretében, ami mögött vélhetően trade-off kapcsolat állhat a növény növekedése és reprodukciója között. A jövőben érdemes lehet összefüggéseket keresni a morfológiai megjelenés és a pollen mennyisége és minősége között. A módszer és a belőlük származó kezdeti eredmények értékes kutatási potenciállal bír a növénybiológia vagy az ökológia területén, a növény-kórokozó kölcsönhatások további vizsgálatához, valamint a közegészségügyi vonatkozású kutatásokhoz. Ez az innovatív módszer fontos eszközként

szolgálhat a pollenek monitorozásához és más morfológiai kutatásokhoz, amely eredmények nagy érdeklődésre tarthatnak számot multidiszciplináris alkalmazhatóságuk révén.

***Kulcsszavak**: automatizált mérés, képi adatbázis, digitális képfeldolgozás, inváziós fajok, palinológia, modellképzés, mesterséges intelligencia, gépi tanulás*

1 Introduction

Ambrosia artemisiifolia (L.) is an annual invasive weed that has spread worldwide, including in many European countries (Leiblein-Wild et al., 2014). According to a survey by the Weed Science Society of America (2017), this species ranked among the top 10 of the most harmful weeds in North American crop production (Van Wychen, 2017).

Controlling common ragweed is critical for several reasons. In crop production, it negatively affects the yields of sunflower, corn, sugar beet, soybean, and cereal crops (Buttenschøn et al., 2010). Additionally, ragweed pollen is a significant public health hazard, being the main cause of allergic rhinitis and asthma (Buttenschøn et al., 2010). Studies have shown that even low pollen concentrations (5–10 grains per m^3) can provoke health issues in sensitive persons. Furthermore, in ragweed-infested areas, up to 12% of the human population suffers from respiratory diseases (Taramarcaz et al., 2005).

Pollen plays a crucial role in plant reproductive biology, so any methodical developments in quantifying their numbers, size, and other parameters represent an important methodological breakthrough. However, visual analysis of pollen images has traditionally been labour-intensive and time-consuming, requiring numerous manual steps for sample preparation and producing detailed microscopic images (Langford et al., 1990). In a study, an ImageJ software-based method proved to be a cheap, efficient, and reliable tool for pollen counting, adaptable to various pollen types and sizes (Costa & Yang, 2009). Efforts in development related to automated image processing and image-based recognition, an increasing number of image datasets are now available for analysing pollen grains (Rodrigues et al., 2015).

This study aimed to develop a rapid, automated, and user-friendly AI-based image processing method for accurately measuring pollen size. Therefore, our specific research aims were two-fold: 1) we tested the repeatability of the measuring procedures (i.e., how similar the size of two identical pollen grains after repeated measuring), and 2) we tested the variability of the pollen size among individuals. We hypothesised that if there is an individual-specific morphological trait, we will find differences in pollen sizes among individuals with different morphological appearances. Therefore, we selected common ragweed individuals exhibiting extremely different morphological characteristics to explore the influence of specific plant traits on pollen size.

2 Materials and Methods

2.1 Pollen collection

First, common ragweed individuals (N = 3) with various morphological appearances (1. Table) were selected for pollen collection. The samples were obtained from the same maize field population near Pilisvörösvár, Hungary (GPS: 47.627, 18.926; September 23, 2024). The collected plant material (i.e., inflorescence of male flowers) was transported in paper bags. Inflorescences were carefully separated and air-dried at room temperature (20-22 °C) to avoid tissue degradation. Other plant parts were dried in a drying oven (180 °C) for 2 hours. Subsequent laboratory measurements involved weighing the dry matter of flowers and plant parts.

Table 1 The main morphological characteristics of the studied common ragweed plants

Plant ID	longest shoot height (cm)	shoot radius (cm)	stem diameter (mm)	inflorescence number	plant biomass (g)	flower biomass (g)	pollen grains*
605	67	12	4.74	74	13.03	2.75	2.81
635	115	28	6.16	65	30.28	3.43	1.5
665	33	5	2.48	9	1.05	0.19	0.53

*1. Note *Number of pollen grains extracted from 0.15g dry male flower mass, expressed in millions*

Pollen was extracted following the method of Vaudo et al. (2020). Male reproductive parts of the flowers (0.15 g) were placed in a plastic sample collector and vortexed (20 s). Due to electrostatic forces, the charged pollen grains settle on the wall of the plastic centrifuge tube, allowing for easy separation from the remaining flower material. An aqueous pollen suspension was prepared by mixing ragweed pollen with sterilised distilled water (1 mL). The collected pollen samples were similar in size and visual appearance to the reference pollen grains images in the Palynological Database (Sam et al., 2020).

2.2 Image dataset, model training and pollen measuring

Pollen grains were placed into a Bürker chamber (standard environment for scaling the dimensions) and observed by a light microscope (Leitz LABORLUX S). The images were captured at 5× optical magnification with a Moticam 1080 HDMI & USB output microscope camera using MIDSDevices software.

Roboflow (Dwyer et al., 2024) was chosen as an end-user-friendly end-to-end computer vision platform for developing and running our Ambrart_pollends object recognition model. During the data annotation phase, images of pollen grains were labelled by annotating 49 images (pixel ratio: 1920×1080, size: 2.07 MB) with 2290 pollen grains using the smart polygon annotation feature. During the model training phase, the Roboflow 3.0 Object Detection (Fast) (ROD, hereafter) model was trained on the annotated dataset named Ambrart_pollends. The model was trained successfully three times, with increasing efficiency after each cycle (Table 2, Figure 1).

Table 2 The training process of Ambeart_pollends pollen recognition by Roboflow 3.0 Object Detection (Fast) model

ambrart_pollends versions	Total images	Train images	Valid images	Test images	mAP (%)	Precision (%)	Recall (%)
(1st)	7	3	2	2	53.80	59.70	46.60
(2nd)	27	21	4	2	77.40	85.30	52.80
(3rd)	34	27	5	2	98.20	93.00	97.10

2. Note mAP (Mean Average Precision); Precision (correct predictions); Recall (successfully identified relevant labels)

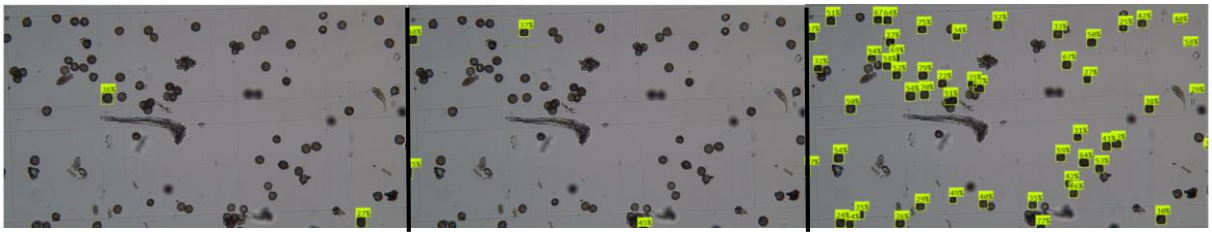

Figure 1 *The model development process demonstrates increasing effectiveness in recognition, as illustrated in the same image. (left: 1st version; middle: 2nd version, right:3rd version; see more details in Table 2.)*

After the training process, we measured the pollen regarding the focal plants by the trained model (2. Figure). The variables (position, width, height, recognition confidence, identification label) were extracted from all the recognised pollen grains and used for further statistical analyses. To determine the true size of pollen, the measurement was rescaled by the pixel µm ratio (median = 2.075003, SD = 0.0308, N = 10). For each pollen, we calculated the pollen size (ellipsoid area expressed in µm^2) using the following formula: $area = width \times height \times \pi$.

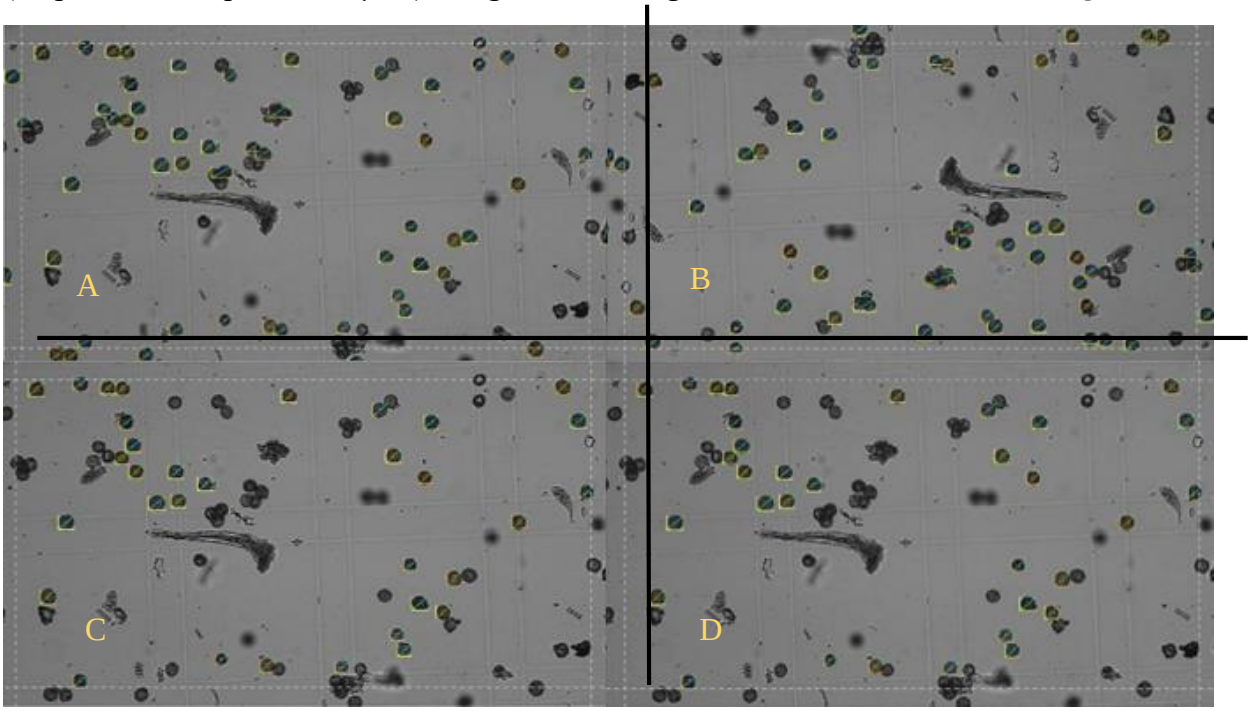

Figure 2 *Pollen recognition and measuring of the original (A, C) and rotated (B, D) versions before (A, B) and after (C, D) filtering out the false recognitions and measuring. Figure descriptions: red circle: identified pollen grains (correct recognitions and measures); blue circle: pollen grains with high asymmetry calculated from the width and height ratio (i.e., <0.9 or 1.1<; excluded from statistical analyses); yellow borders: measured width and height of given pollen grain with high recognition confidence (0.5<); orange borders: measured width and height of given pollen grain with low recognition confidence (<0.5); green line: diagonal of the fitted box; and grey dashed lines indicate the image centre (pollen grains were lying in the centre area (and without crossing these lines) were included into the statistical analyses. The recognition of the pollen grains was not completely identical comparing the original image and its rotated version.*

2.3 Statistical analyses

All statistical analyses were performed in the R statistical environment (version 4.3.1, 08-12-2024, R Development Core Team 2019).

We calculated the repeatability index by taking the ratio of within-group variance to total residual variance applied Markov Chain Monte Carlo sampler for multivariate Generalized Linear Mixed Models (MCMCglm) based on repeated measurements of the identical sample units (i.e., 'pollen_id'). The calculations were based on 1000 permutations using the '*MCMCglmm*' package (Hadfield 2010).

For testing the individual-specific variations of the pollen size, we fitted a linear mixed-effect model (LMM) structure using the '*lme4*' package (Bates et al. 2015), and the predictors were tested for significance using the Likelihood Ratio Test (LRT). In this model, the response variable was the pollen size, while the recognition confidence ('confidence', numeric, continuous), image type ('image set', factor, original or rotated, represented the repeated measuring), and plant identifier ('plant id', factor) testing the plant-specific differences. The pollen identifier ('pollen_id', factor) was included as a random variable, considering the data of the exact pollen grain from different images (i.e., original and rotated).

Table 3 Descriptive statistics of the pollen sizes and recognition confidence in each image set

Image set	Variable	Sample size	Range	Median	SD
1st	Area	101	281.23/502.89	369.24	31.21
1st	Confidence	101	0.17/0.84	0.53	0.15
2nd	Area	88	285.67/472.37	371.61	28.70
2nd	Confidence	88	0.22/0.91	0.56	0.16
Paired	Area	71 (142)	285.67/502.89	373.68	29.14
Paired	Confidence	71 (142)	0.2/0.91	0.53	0.16

3. Note The descriptive statistics suggested high similarities among the image sets. The numbers indicate unique pollen grains, while the repeated measurements of the same pollen grains are detailed in brackets.

3 Results

The three-step training process of the Ambrart_pollends pollen detection model has been applied successfully within the ROD framework. The comprehensive training for creating the training image dataset took approximately 40 work hours.

We obtained a high repeatability index (4. Table), indicating that the independent measurements of pollen grains and their rotated versions yielded highly similar, pollen-specific outputs by MCMCglm.

Table 4 Repeatability index and the corresponding Confidence Intervals (2.5 and 97.5 quartiles, CI_{95}) values by increasing the recognition confidence exclusion limits calculated by MCMCglm. These values suggested that the high overall repeatability was unaffected negatively by the data with low recognition confidence. Therefore, it was needless to exclude them from the statistical analyses

Confidence limit	R	$CI_{2.5}$	$CI_{97.5}$
0.0<	0.846	0.779	0.895
0.2<	0.847	0.778	0.897
0.3<	0.874	0.811	0.915
0.4<	0.862	0.780	0.912
0.5<	0.824	0.642	0.911
0.6<	0.809	0.106	0.958
0.7<	0.210	0.000003	0.911

The LMM revealed a significant relationship between recognition confidence and pollen size ('confidence': $F(1, 176.53) = 18.60$), $p < 0.001$), indicating that recognition confidence is positively increased with the pollen size. There is a detectable difference among plants ('plant id': $F(2, 297.40) = 3.87$, $p = 0.0219$), suggesting individual-specific, systematic variation in pollen size. In contrast, the effect of repeated measuring was unaffected ('image set': $F(1, 105.40) = 0.15$, $p = 0.7001$), suggesting that the image recognition and size measuring were unbiased by the repeated sampling sessions and produced consistent results.

4 Discussion

Our study demonstrated that the Ambrart_pollends detection model provides valid pollen size measurements, effectively streamlining a traditionally labour-intensive and time-consuming process. It can also be integrated easily with pollen counting. The developed platform reduces the need for manual measurements while allowing for the rapid collection of comprehensive pollen size data and providing accurate estimation for the concentration of a pollen suspension. Additionally, model confidence estimates can be adjusted to account for visual estimation errors. Roboflow-based image analysis is completed within milliseconds in each image, making it significantly faster than previous image-based analysis methods. Compared to Costa and Yang (2009), the analysis of individual images processed with ImageJ software ranged from 30 to 60 seconds. The high repeatability index clearly demonstrates that the AI-based measuring procedure can be used as a valid methodology for pollen measuring.

Every methodological approach has its pros and cons. One of the areas for improvement of Ambrart_pollends is the relatively long time required to train the model, as it relies on high-quality images and precise annotations of individual pollen grains. Pollen grains located at the edges of the image or overlapping with others are not always measured accurately and must be filtered out to obtain reliable pollen size data. Additionally, the costs of model training and financial maintenance could be a limiting factor.

Pollen size is related to the morphological properties of plants, though a large amount of data is needed to confirm the impact of these morphological differences. If pollen size can be used to infer trade-offs between growth and reproduction which traits vary across populations or habitats, it could provide valuable insights in plant biology and ecology. It is known that male reproductive investment in *A. artemisiifolia* increases with plant height, while female investment does not, indicating a male-biased sex allocation in taller plants (Nakahara et al., 2017). This suggests that height directly influences male reproductive success. The applications of our results extend beyond weed control and respiratory public health problems (Buttenschøn et al., 2010; Taramarcaz et al., 2005) or to the studies related to plant-pathogen interactions (Kocsis et al., 2022). Pollen grains significantly enhance the germination of plant-parasitic fungal spores, which effect is influenced primarily by the size of the pollen, as well as the specific plant taxon.

5 Conclusions

This study emphasized an efficient and accurate AI-based method for measuring ragweed pollen size, significantly reducing the human workload. With further development, this method has significant potential in plant biology, offering insights into growth-reproduction trade-offs and population- or habitat-specific variations.

Acknowledgements

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Results of weed surveys for different land uses

Gyomfelvételezések eredményei eltérő talajhasználatnál

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Abstract: Different land use patterns have a significant impact on the weed flora of agricultural land. Tillage, fertilisation and crop rotation have an important role to play in reducing weed infestation. The effects of conventional ploughing and reduced tillage (min-till) on weed control in maize fields were investigated in a maize-wheat biculture with different nitrogen application rates in a long-term Soil Tillage field Experiment set up in Keszthely, Hungary in 2023 and 2024. The results showed that either tillage or fertilizer use had an effect in minimizing weed infestation.

Keywords*: tillage, min-till, fertilizer, maize, weed cover*

Összefoglalás: Az eltérő talajhasználati módok jelentősen befolyásolják a mezőgazdasági területek gyomviszonyait. A talajművelésnek, a műtrágyázásnak és a vetésváltásnak fontos szerepe van a gyomosodás visszaszorításában. A keszthelyi Talajművelési Tartamkísérletben 2023-ban és 2024-ben vizsgáltuk a kukorica területeken a hagyományos szántásos talajművelés és redukált (min-till) talajművelés gyomosodásra kifejtett hatását, bikultúrás termesztésben, eltérő nitrogén adagok kijuttatása mellett. A kapott eredmények rámutattak, hogy nem elhanyogolható a talajművelés és az okszerű műtrágya használat a gyomosodás minimalizálásának érdekében.

Kulcsszavak*: talajművelés, min-till, műtrágya, kukorica, gyomosodás*

1 Introduction

Throughout the history of agriculture, the purpose of tillage has been to prevent, or at least minimise, the damage caused by weeds to the crop.Tillage can kill weeds by breaking them up, tearing them apart or pulling them out of the soil, causing them to dry out (desiccation), covering the soft tissues with soil, inhibiting photosynthesis and depleting stored nutrient reserves. It has been shown that proper soil tillage reduces the weed seedbank, as well as the number of vegetative reproductive formations in the soil. Weed seeds brought close to the soil surface by tillage can germinate and be killed by repeated tillage (Hunyadi et al., 2000).

The importance of tillage is its weed controlling effect on the area, such as ploughing or disking (Ujvárosi, 1973). The weed control effects of conventional, min-till and no-till systems have been investigated (Tuesca et al, 2001; Shrestha et al., 2002; Kismányoky, 2010). The results of several studies support the idea that conventional inversion tillage has a weed-controlling effect, whereas no tillage can be considered as weed-supportive technology (Kismányoky, 2010).

Autumn deep ploughing is of great importance for deep-rooted perennial weeds. In addition to deep ploughing in autumn, spring seedbed preparation is also important, because if deep ploughing is carried out early in autumn, several weeds, mainly of T_1 and even T_2 life forms, may emerge in autumn, but these can be controlled by spring seedbed preparation (Hunyadi et al., 2000).

It is important to note that the negative effects of weed competition for nutrients cannot be counteracted by increasing soil nutrient content alone, since weeds are very strong competitors and use available nutrients in extra quantities compared to cultivated plants (Vengris et al., 1955). The former statement is also supported by the finding that weeds often absorb nutrients faster and in larger quantities than cultivated plants. Thus, fertilization of weeds with high weed cover stimulates their development to such an extent that they outgrow and suppress the cultivated plants (Alkämper, 1976).

There are, however, cases where the optimal rate of fertilizer, or even the timing of application, helps to control weeds and reduce their harmful effects on the crop.

A study in maize showed that the increase in yield with N fertilizer was much more pronounced than the development of monocotyledonous weeds (Nieto-Staniforth, 1961). In studies in wheat, it was found that the number of weed species and their biomass was significantly reduced with increasing N fertilizer rates (Lehoczky, 1995).

Ultimately, it can be concluded that increasing nutrient supply does not control weeds, i.e. it is not a substitute for other control methods, since in most cases it only increases the high nutrient uptake by weeds and promotes their intensive growth. As described so far, the correct application of phosphorus and nitrogen can be incorporated into integrated weed management in some cases. Since most of our soils are moderately well supplied with phosphorus, its control is not really feasible, while nitrogen, which does not accumulate in the soil, is easier to control (Hunyadi et al., 2000).

2 Materials and Methods

The studies were carried out in the Keszthely Soil Tillage Experiment, managed by the Institute of Agronomy, Georgikon Campus, Keszthely, Hungarian University of Agricultural and Life Sciences, which was set up in 1972 to develop time and energy saving methods for different tillage methods and different nitrogen fertilizer rates. The experiment has a two-factor split-plot design with four replicates in which three different tillage variants and five different rates of nitrogen fertilizer are studied on winter wheat and maize indicator crops. The winter wheat and maize are rotated every two years (winter wheat- winter wheat- maize- maize) in a bicultural system. Three different tillage options are used in the trial: conventional deep inversion tillage in autumn, shallow disk tillage in autumn and minimum tillage system,(min-till), also disking just before drilling. The same crop protection treatment was applied to all plots in the area, and fertilizers with the active ingredients P_2O_5 and K_2O were applied uniformly at a rate of 100-100 kg/ha.

We continued our studies in the maize crop in 2023 and 2024, Previous crop of maize was winter wheat (2023) and then (2024). For the experiment evaluation, we compared the conventional tillage and minimum tillage systems.

N Fertilizer rates:

- B1: 0 kg/ha
- B2: 120 kg/ha
- B3: 180 kg/ha
- B4: 240 kg/ha
- B5: 300 kg/ha

Weed surveys were carried out using the Balázs - Ujvárosi method at different stages of maize development. Timing of weed surveys: early summer (8-10 leaf developement stage) and flowering. In presenting the results, we would like to present the results of the maize at flowering, since at this stage we have no control of weeds and the maize absorbs most of the nutrients until the end of flowering, so these data are also important for competition.

3 Results

In 2023, winter wheat was the preceeding crop before maize in the Keszthely Soil Tillage long-term field experiment. Weed surveys were carried out at the time of maize flowering (Figure 1.). The results showed that the average weed cover was lower in the conventional tillage fields than in the reduced tillage fields. For both tillage systems, weed cover was highest where no nitrogen fertiliser was applied. Among the ploughed plots, the lowest average weed cover was found in treatment B3 with 180 kg nitrogen/ha, whereas in the Min-Till treatment, the lowest average weed cover was found in treatment B5 (300 kg/ha).

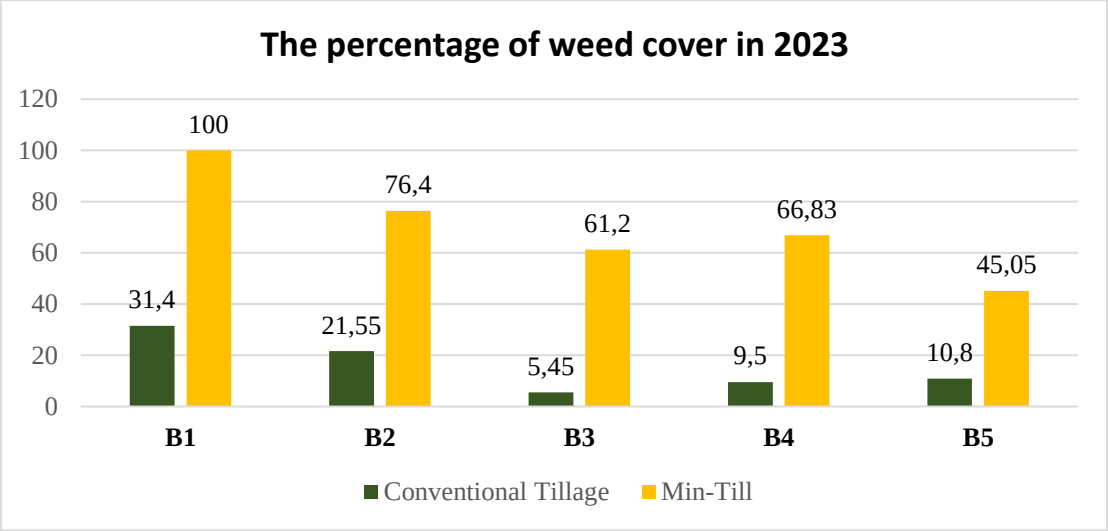

Figure 1 *The percentage (%) of weed cover in 2023*

In the year 2024, maize followed itself. The results of the weed surveys carried out at flowering are shown in Figure 2. The results were similar to 2023, as again the conventional inversion tillage resulted in lower weed infestation than the reduced tillage plots in all treatments. However, in contrast to 2023, the ploughed areas had the lowest weed cover at the highest applied nitrogen rates (300 kg/ha), while the highest was also where no nitrogen fertiliser was applied. In the min-till fields, similar results were obtained as in the conventional fields, as the highest weed infestation was also in the untreated fields and the lowest in the 300 kg/ha treatment.

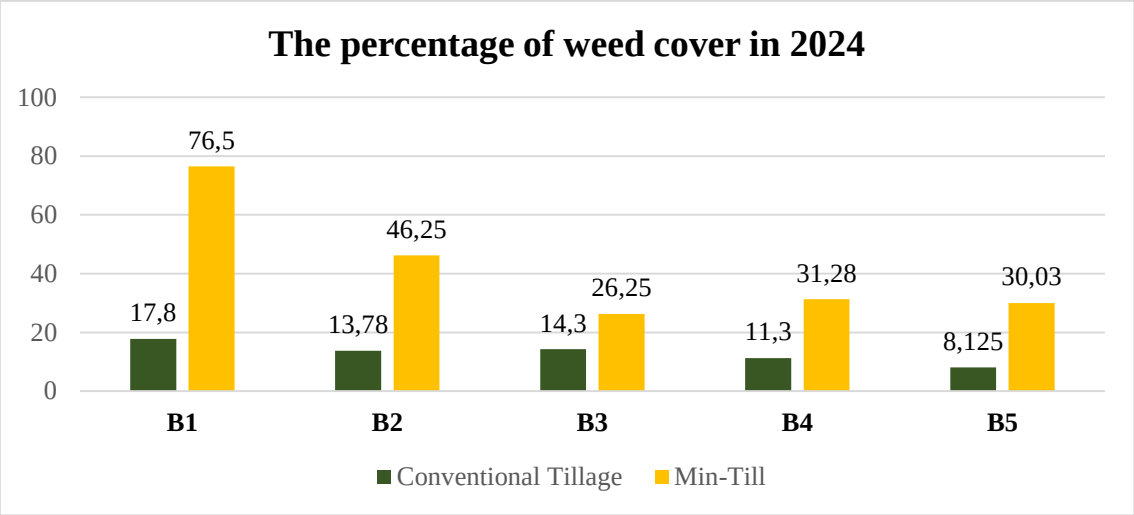

Figure 2 *The percentage of weed cover in 2024.*

Table 1 Average weed cover of the main weeds in the experiment by treatment and tillage type

	Average weed cover (%)										Year
	B1		B2		B3		B4		B5		
	Conventional tillage	Min-Till	Conventional tillage	Min-Till	Conventional tillage	Min-Till	Conventional tillage	Min-Till	Conventional tillage	Min-Till	
Digitaria sanguinalis	10	4	2	5	0,5	15	1,05	8,3	2,55	4	2023
	3,4	6,5	3,75	15	3,5	2	3,3	7,5	2,37	6,67	2024
Echinochloa crus-galli	-	2	0,1	10	0,1	-	5	-	0,1	2,5	2023
	5	-	0,1	2	1	3	1	1	2	-	2024
Setaria pumila	-	10	5	6,6	2,5	5	1,87	10	1,86	10	2023
	7	15,75	2	13	3	4	3,5	7,3	2,025	6,75	2024
Setaria viridis	6,7	6	3,5	5	0,1	1	-	5	0,1	5	2023
	2	5	2	4	2	5	-	-	1,05	-	2024
Panicum miliaceum	15	3,5	5	-	-	-	-	12,5	-	-	2023
	-	-	-	-	0,1	-	-	-	1	-	2024
Ambrosia artemisiifolia	6,7	13,3	2,5	8,3	0,1	0,1	-	6,6	-	8,3	2023
	-	27,3	-	2	10	-	2	-	2	-	2024
Polygonum aviculare	6,7	-	8,3	-	1,73	5	5	8,3	8,33	-	2023
	-	-	-	-	-	-	0,1	-	-	-	2024
Convolvulus arvensis	6,7	25	5	30	3,37	28,3	5	21,6	3,5	20	2023
	6,5	9,75	4,75	10	10	18	3,5	13,3	4	8,25	2024
Cirsium arvense	5	18,36	2,55	13,3	-	13	-	5	0,1	13,36	2023
	6	16	1	9	8	4	5	10	-	9,3	2024

The first table shows the main weeds that were prominent in the fields. The table shows the average percentage cover of weeds by year and by treatment. The presence of *Digitaria sanguinalis* is outstanding, being present in the fields in all treatments in both years. In 2023, it was present in all but the B1 treatment with lower cover in ploughed fields than in min-till fields. *Setaria pumila* is also an important monocot weed, as it was present in min-till fields in all years, in all treatments, and its percent cover exceeded the results in conventional tillage fields with the same nitrogen supply. *Panicum miliaceum*, which is emerging as an increasingly important species in the weed flora of domestic maize production, was more abundant in the 2023 year of the tank trial, while in the 2024 year it was only marginally present. It is worth pointing out that *Polygonom aviculare* was present in all treatments in the year 2023, when winter wheat was the pre-sown maize in the ploughed fields, but in the min-till fields it was only present in treatments B3 (5%) and B4 (8.3%). Conversely, in 2024, it appeared only in the conventional tillage B4 treatment with an average cover of 0,1%.

Examining the data on perennial weeds, we found that *Cirsium arvense* and *Convolvulus arvensis* were the most important, but *Lathyrus tuberosus*, *Cynodon dactylon* and *Plantago major* species were also present in the areas, but their presence was more significant in the min-till areas. *Convolvulus arvensis* was present in all plots in both years, irrespective of tillage and nitrogen treatment, but it is important to highlight that it was more dominant in the min-till areas than in the ploughed areas under the different treatments. *Cirsum arvense* never exceeded an average cover percentage of 10% in conventionally tilled areas and its presence was most significant in the year 2024, when maize was followed by maize. Based on the weed survey results for the min-till cultivated areas, it can be said that *Cirsium arvense* was present in all years, in all treatments and most often had a higher percent cover than in the ploughed areas.

4 Discussion

Long-term field experiments are of particular importance as they provide essential information and shed light on relationships that cannot be studied in short-term experiments, or only incompletely. It is possible to study the biological and physical parameters of the soil and the results obtained can be useful not only for farmers and researchers, but also as a basis for sustainable agricultural production (Kismányoky-Jolánkai, 2009). In the long-term experiments, we investigated the maize fields of the years 2023 and 2024 from the point of view of weed infestation. The weather in the growing season of the two years was extremely different, which may have had a major impact on the development of the weed flora, which is beneficial for us because it gives a more complex picture of the factors influencing weed infestation. Summarizing the results, we can say that, independent of the effect of the year, the average weed cover was significantly higher in the reduced tillage areas than in the conventional inversion tillage areas. However, it can also be concluded that the growing season 2024 was drier, but with lower weed cover in all treatments in the min-till fields than in the previous year, but not in the conventional fields. The results of the different nitrogen treatments also show that fertilisation can have a significant effect on the development of weed flora, because, as fertilisers help plants to grow intensively, they can easily compete with weeds and then suppress them. In all cases, weed cover was higher where nitrogen fertiliser was not applied, but ploughing was also found to be better in terms of weed control in untreated areas.

In the areas studied, T_4 weed species typical of the domestic maize areas were common, among which it is important to highlight the monocots, which are very difficult to control by maize growers. Both *Digitaria sanguinalis* and *Setaria pumila* have higher average weed infestations with Min-Till than with conventional tillage, so it is possible that rotational tillage can help to control these species. *Cirsium arvense* and *Convolvulus arvensis* were also present in the plots studied and are important perennial weed species in maize production, which can only be effectively controlled by chemical means. The experiment showed that the application of nitrogen fertilizers and inversion tillage can help to control these weed species.

In the future, we would like to continue these studies, complemented by soil weed stock studies, and to investigate the N, P and K content of maize plants and weeds to get a more complex picture of the effects of different soil uses and to apply the knowledge gained for sustainable crop production.

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Virological survey of walk-in plastic tunnel grown pepper seedlings and forced pepper varieties in Western Hungary in 2023-2024

Nyugat-Magyarországon fóliasátorban nevelt paprikapalánták és hajtatott paprikafajták virológiai vizsgálata 2023-2024-ben

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Abstract: Peppers can be infected by approximately 50 plant viruses of which the most common species causing significant economic losses in Hungary are *Potato virus Y* (PVY) and *Cucumber mosaic virus* (CMV) in outdoor cultivation and *Tomato spotted wilt virus* (TSWV) and tobamoviruses in indoor cultivation. In addition to vectors' activity, the infected propagating material also plays a very important role in the spread of pepper infecting viruses. Thus, besides the use of virus-free propagating material and effective protection against vectors, the cultivation of virus-resistant varieties and application of hygiene regulations are essential in the control of pepper viruses. Even with the most precise cultivation techniques, virus infections can occur during indoor cultivation, not only in plantations which are already producing but also in seedling nurseries, therefore monitoring these is crucial to achieve virus-free fruiting pepper stand. In 2023–2024, virological survey of pepper seedlings and fruiting plants collected from two horticultural farms in Zala county and grown in walk-in plastic tunnel was performed using RT-PCR technique to test leaf and fruit samples. During the analysis only the presence of TSWV was identified in 13 samples, while infection of CMV, PVY and tobamoviruses was not detected. It is interesting to highlight that TSWV was detected, despite the black necrosis that developed, in the fruits of the TSWV resistance gene carrying pepper cultivars Antal F_1 and Zalkod F_1. These results indicate that the use of TSWV resistant pepper varieties or hybrids does not provide complete protection against TSWV damage as symptoms may also appear on resistant individuals not only on susceptible ones due to the vectors' activity. Therefore, monitoring the vectors that can transmit TSWV and timely control them are essential for indoor pepper cultivation. Without these even TSWV resistant plants will not be able to produce marketable crop.

Keywords*: Capsicum annuum, pepper, virus, RT-PCR, resistance*

Összefoglalás: Megközelítőleg 50 növényi vírus képes fertőzni a paprikát, melyek közül hazánkban szabadföldön a burgonya Y vírus (*Potato virus Y*, PVY) és az uborka mozaik vírus (*Cucumber mosaic virus*, CMV), hajtatásban pedig a paradicsom foltos hervadás vírus (*Tomato spotted wilt virus*, TSWV) és a tobamovírusok okozzák a legnagyobb problémát. A paprikát fertőző vírusok terjesztésében a vektorokon felül kiemelkedő szerepe van a fertőzött szaporítóanyagnak is. Így a paprikavírusok elleni védekezésben is elengedhetetlen a vírusmentes szaporítóanyag használata és a vektorok elleni hatékony védelem, ezeken felül

pedig a vírusrezisztens fajták termesztése és a higiéniás rendszabályok betartása is fontos. Még a legprecízebb termesztéstechnológia ellenére is előfordulhatnak a zárt téri termesztés során is vírusfertőzések nem csupán a termő állományokban, de már a palántanevelőkben is, így ezek monitorozása kiemelten fontos a vírusmentes termő állományok kialakításához. 2023–2024-ben két Zala vármegyei kertészetből, fóliasátorból, paprika palántákról, illetve termő növényekről gyűjtött levél- és termésminták virológiai vizsgálatát végeztük el RT-PCR technikával. A vizsgálat során csak a TSWV jelenlétét tudtuk igazolni, 13 minta esetében kaptunk pozitív eredményt, míg a CMV, a PVY és tobamovírusok előfordulását egyetlen mintában sem tudtuk kimutatni. Érdekes megfigyelés volt, hogy a TSWV ellen rezisztenciagént hordozó Antal F_1 és Zalkod F_1 paprikafajták terméseiben a kialakult fekete nekrózisok ellenére is kimutattuk a TSWV-t. Eredményeink alapján megállapítható, hogy a TSWV rezisztens paprikafajták, illetve hibridek alkalmazása nem jelent teljes védelmet a TSWV kártételével szemben, ugyanis a vektorok kártételének következtében a TSWV rezisztens egyedeken is megjelenhetnek tünetek éppúgy, mint a rezisztencia gént nem tartalmazó, fogékony növényeken. Ezért a hajtatásban történő paprika termesztés alapvető feltétele a TSWV-t terjesztő vektorok monitorozása és a védekezés időben történő megkezdése. Ennek hiányában a TSWV rezisztens növények sem képesek értékesíthető termést hozni.

***Kulcsszavak**: Capsicum annuum, paprika, vírus, RT-PCR, rezisztencia*

1 Introduction

Sweet pepper (*Capsicum annuum* L.) is a herbaceous, thermophilic plant belonging to the *Solanaceae* family. It originates from the tropical areas of South America, where the indigenous Indians used it for various medicinal purposes (Barboza et al., 2005). It was probably introduced to Hungary by Turkish merchants in the 16th century. Hungarian pepper become world famous in 1937 with Albert Szent-Györgyi's research on vitamin C (Balázs, 1994).

Nowadays, approximately 24-25 million tons of pepper are harvested annually from an area of 2 million hectares worldwide. Hungary accounts for nearly 1% of the world's pepper production. In 2023, 90 thousand tons were harvested from 1281 hectares in Hungary. Outdoor pepper production area shows a decreasing trend, and currently pepper is typically grown in plant growing structures (Takácsné, 2017; KSH, 2024; FAO, 2024).

Peppers can be infected by approximately 50 plant viruses (Edwardson and Christie, 1997) of which the most common species causing significant economic losses in Hungary are *Potato virus Y* (PVY) and *Cucumber mosaic virus* (CMV) in outdoor cultivation and *Tomato spotted wilt virus* (TSWV) and tobamoviruses in indoor cultivation. PVY can be transmitted by more than 50, while CMV by more than 80 aphid species. The most important insect vector of TSWV is the western flower thrips (*Frankliniella occidentalis*), while in the case of tobamoviruses vector transmission is currently unknown. In addition to vectors' activity, the infected propagating material and disregarding of cultivation hygiene standards also play very important role in the spread of pepper viruses.

Thus, besides the use of virus-free propagating material and effective protection against vectors, the cultivation of virus-resistant varieties and application of hygiene standards are essential in the control of pepper viruses.

Even with the most precise cultivation techniques, virus infections can occur during indoor cultivation, not only in plantations which are already producing but also in seedling nurseries, therefore monitoring these is crucial to achieve virus-free fruiting pepper stand.

Between 2023 and 2024 a virological testing of 31 leaf and fruit samples from pepper seedlings and fruiting plants collected from two horticultural farms in Zala county and grown in walk-in plastic tunnel was carried out to determine the virus infection of the pepper samples.

2 Materials and Methods

In 2023–2024, pepper leaf and fruit samples from 27 pepper individuals - 17 seedlings and 10 fruiting plants - belonging to 11 different pepper varieties and hybrids (Table 1) grown in walk-in plastic tunnels in two horticultural farms (Kiskanizsa and Várfölde) in Western Hungary (Zala County) were collected. Overall 31 samples, 4 fruit and 27 leaf samples were tested. During sample collection, symptoms observed on the pepper plant parts and the presence of trips in the walk-in plastic tunnel were noted. In the case of seedlings only leaves (symptomatic or not) were sampled, while in the case of fruiting pepper, in addition to the symptomatic leaves, in some cases fruits were also sampled. 1-3 plant parts per plant were collected and stored at -70 ºC until use.

Total nucleic acid (TRNA) extraction was performed using a simplified CTAB method (Xu et al. 2004, Sáray et al., 2022). 0.2 g of plant tissue was used per sample for total nucleic acid extraction. The total nucleic acid extracts were visualized on a 1% TBE agarose gel containing a fluorescent dye and stored at -70 °C until further use.

RT-PCR was carried out for the molecular detection of viruses. Viral cDNAs were synthesised by reverse transcription (RT) of the TRNAs extracted from pepper leaves or fruits using Random primer (Thermo Fisher Scientific) and RevertAid Reverse Transcriptase according to the manufacturer's instructions (Thermo Fisher Scientific). The success of the cDNAs synthesis were checked for each sample by PCR using primers designed for pepper actin gene (Capsicum_actin_for and Capsicum_actin_rev; Li et al., 2016).

Species-specific primer pairs were used for CMV (CMV_rev and CMV_for; Nemes and Salánki, 2020), PVY (PVY_rev and PVY_for; Nemes and Salánki, 2020) and TSWV (TSWV_rev and TSWV_for; Nemes and Salánki, 2020) and a universal genus-specific primer pair (UniTobamo5-for and UniTobamo3-rev; Kálmán et al., 2001) was used in the PCR for tobamoviruses. All PCRs were performed in a final volume of 25 µl. In addition to the nucleic acid, the reaction mixture contained 12.5 µl DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 10 pmol each of antisense and sense primers, and 10 µl of nuclease-free water. Sterile distilled water was used as a negative control, and the following parameters were set for each PCR: 95 °C for 5 min, for 35 cycles 95 °C for 30 sec, 60 °C for 30 min, 72 °C for 1 min, and finally 72 °C for 10 min. The PCR amplicons were visualized on a 1% TBE agarose gel staining with fluorescent dye.

Table 1 Tested pepper varieties and hybrids, and their resistances

Varieties and hybrids	Resistances
Armand F1	*HR Tm 0*
Amy	-
Antal F1	*HR Tm 3, IR TSWV*
Cassovia F1	*HR Tm 2, IR TSWV*
Blumen	-
Hétvezér F1	*HR Tm 0*
Eszter F1	-
Promontor F1	*HR Tm 0*
Rédei fehér	-
Senator	-
Zalkod F1	*HR Tm 2, IR TSWV*

Legend: HR: High resistance; IR: Intermediate resistance; Tm 0: TMV resistance; Tm 2: TMV and ObPV resistance; Tm 3: TMV, ObPV and PMMoV resistance; TSWV: TSWV resistance.

3 Results

Among the 27 tested plant individuals, 26 showed some symptoms and one was asymptomatic. The symptoms observed on pepper samples were variable (Figure 1.). All the fruiting pepper plants were damaged by thrips, while the presence of thrips was not detected in the seedling nursery.

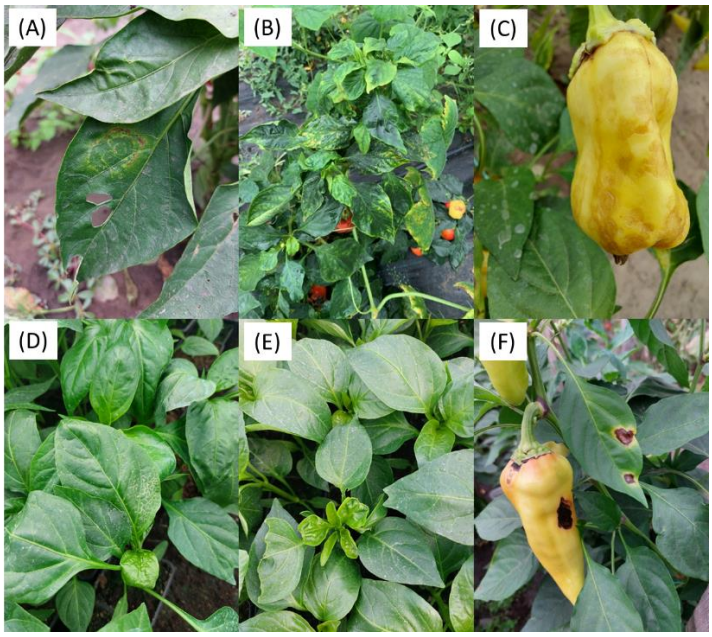

Figure 1 *Symptoms on pepper plants: (A) Chlorotic necrotic rings on Armand F1 hybrid leaf (Photo: Fehér), (B) Chlorosis on Eszter F1 hybrid leaf (Photo: Fehér), (C) Ringspots on Hétvezér F1 hybrid fruit (Photo: Szendrei), (D) Vein lightening on Amy seedling leaf (Photo: Szendrei), (E) Two-pointed leaf on Promontor(6) seedling (Photo: Szendrei), (F) Dark necrotic areas on Zalkod F1 hybrid fruit and leaf (Photo: Szendrei)*

Actin test gave positive results for all 31 pepper samples. In the cases of all samples the ~230 bp long DNA fragments were amplified during RT-PCR. In the case of control water samples negative results were obtained. In the RT-PCR assays for virus testing using the virus species-specific and genus-specific primer pairs positive results were obtained only when using the TSWV-specific primer pair. In the case of 13 samples the ~350 bp long PCR products were detected. While for the control water samples, and RT-PCR tests for CMV, PVY, and tobamoviruses gave negative results and no PCR products was detected. All the TSWV positive plants showed symptoms, while none of the monitored viruses was detected in the asymptomatic seedling. 43.33% of the symptomatic samples, 9 leaf and 4 fruit samples were proved to be infected with TSWV. The presence of TSWV was detected in at least one tested sample of 8 tested varieties out of the 11. In the case of Cassovia F_1, Amy and Senator varieties none of the tested samples were found to be infected with any of the tested viruses. There were no varieties for which all samples tested were infected with TSWV, although one sample [Promontor (2)] was asymptomatic. The highest infection rate (75%) was observed in the case of Armand F_1. Three out of four leaf samples collected from four different Armand F_1 hybrid seedlings were found to be infected with TSWV (Table 2).

Table 2 Results of RT-PCR tests

No.	Pepper sample	Plant part	Symptom	Presence of vector	CMV	PVY	TOBAMO	TSWV	*C. annuum* actin
1.	**Armand(1)**	leaf	+	+	-	-	-	+	+
2.	*Armand(2)*	leaf	+	-	-	-	-	-	+
3.	*Armand(3)*	leaf	+	-	-	-	-	+	+
4.	*Armand(4)*	leaf	+	-	-	-	-	+	+
5.	*Amy*	leaf	+	-	-	-	-	-	+
4.	**Antal(1)***	leaf	+	+	-	-	-	-	+
5.	**Antal(2L)***	leaf	+	+	-	-	-	-	+
6.	**Antal(2F)***	fruit	+	+	-	-	-	+	+
7.	**Antal(3)***	leaf	+	+	-	-	-	-	+
9.	*Blumen(1)*	leaf	+	-	-	-	-	-	+
10.	*Blumen(2)*	leaf	+	-	-	-	-	+	+
11.	**Cassovia***	leaf	+	+	-	-	-	-	+
12.	**Hétvezér(1)**	leaf	+	+	-	-	-	-	+
13.	**Hétvezér(2L)**	leaf	+	+	-	-	-	+	+
14.	**Hétvezér(2F)**	fruit	+	+	-	-	-	+	+
15.	**Eszter(1)**	leaf	+	+	-	-	-	-	+
16.	**Eszter(2L)**	leaf	+	+	-	-	-	-	+
17.	**Eszter(2F)**	fruit	+	+	-	-	-	+	+
18.	*Promontor(1)*	leaf	+	-	-	-	-	+	+
19.	*Promontor(2)*	leaf	-	-	-	-	-	-	+
20.	*Promontor(3)*	leaf	+	-	-	-	-	+	+
21.	*Promontor(4)*	leaf	+	-	-	-	-	+	+
22.	*Promontor(5)*	leaf	+	-	-	-	-	-	+
23.	*Promontor(6)*	leaf	+	-	-	-	-	-	+
24.	*Rédei(1)*	leaf	+	-	-	-	-	-	+
25.	*Rédei(2)*	leaf	+	-	-	-	-	-	+
26.	*Rédei(3)*	leaf	+	-	-	-	-	-	+
27.	*Rédei(4)*	leaf	+	-	-	-	-	+	+
29.	*Senator*	leaf	+	-	-	-	-	-	+
30.	**Zalkod(L)***	leaf	+	+	-	-	-	-	+
31.	**Zalkod(F)***	fruit	+	+	-	-	-	+	+

*Legend: Samples marked with * collected from TSWV resistant plants; samples from fruiting plants are marked in bold, samples from seedlings are marked in italics; +: positive result, - negative result.*

4 Discussion

Virus infection of more than 40% of the symptomatic samples were determined during virological testing of pepper samples, however, of the four monitored viruses that most commonly infect pepper in Hungary (CMV, PVY, TSWV, tobamoviruses), only the presence of TSWV was detected.

In a virological survey, Sáray et al. (2021) found that more than half of the tested greenhouse pepper samples (58%) were infected with viruses and most of the positive samples (67%) was infected with TSWV, which is in line with our results. However, in the contrary to our results

they also confirmed the presence of tobamoviruses (19%), CMV (12%), and PVY (2%) in the tested samples.

The pepper samples were collected from varieties and hybrids with or without virus resistance against plant viruses. The leaves and fruits of the TSWV-resistant Antal F_1, Cassovia F_1 and Zalkod F_1 hybrids showed symptoms. The necrotic spots observed on Antal F_1 and Zalkod F_1 plants clearly indicated the presence of the resistance gene against TSWV and that the normal strain of TSWV was present in the walk-in plastic tunnel (Tóbiás et al., 2014). However, in the fruits of Antal F_1 and Zalkod F_1 the presence of TSWV was determined despite the presence of TSWV resistance gene and black necroses that developed. It is interesting to note that in the case of these two varieties the presence of TSWV was only detected in the fruit but not in the leaf sample showing necrotic symptoms and originating from the same pepper individual. TSWV infection was also identified only in the fruit but not in the leaf sample of Eszter F_1 hybrid collected from the same symptomatic plant individual.

Although symptoms were observed on all plants except for one seedling we were unable to detect the monitored viruses in more than half of the samples, which confirms the well-known finding that macroscopic symptoms observed on plants can easily be confused with changes caused by certain abiotic factors, such as lack of micro- or macroelements or heat stress. However, it cannot be excluded that other viruses that were not examined might infect these symptomatic pepper plants.

Nearly half of the samples collected from a plant growing structure where thrips were noticed, while the other half collected from a walk-in plastic tunnel where the presence of thrips were not observed. Thrips, especially the western flower thrips, is the main vector of TSWV and is responsible for the main virus transmission (Whitfield et al., 2005). However, the virus can also be transmitted mechanically and by seed (Wang et al., 2022) which may have contributed to that TSWV was detected in leaf samples from locations where thrips were not detected.

5 Conlusion

Based on our results it can be concluded that the use of TSWV resistant pepper varieties or hybrids does not provide complete protection against TSWV damage as symptoms may also appear on resistant individuals due to vectors' activity. Therefore monitoring vectors transmitting TSWV and timely control them are essential for indoor pepper cultivation. Without these even TSWV resistant plants will not be able to produce marketable crop. Mechanical and seed transmission may also have a significant role in spread of TSWV. Thus continuous disinfection of the tools used during cultivation and growing seedlings from virus-free seeds are also essential conditions for successful cultivation.

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Efficacy of biological control against soil-borne pathogens in *Catharanthus roseus*

A biológiai védekezés hatékonysága a *rózsás metény* (Catharanthus roseus) *talajból fertőző kórokozói ellen*

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Abstract: The cultivation of Madagascar periwinkle (*Catharanthus roseus* L.) is affected by many pathogens, including soil-borne pathogenic fungi such as *Fusarium* spp., *Phytophthora* spp., and *Pythium* spp. The importance of biological control methods has been increasing due to the ongoing phase-out of chemical pesticides. Biocontrol agents like *Bacillus amyloliquefaciens*, *Trichoderma asperellum*, and *T. atroviride* are increasingly used due to their pathogen suppression capabilities and ability to enhance plant resilience to stress. This study evaluated the *in vitro* efficacy of *T. asperellum* against *F. oxysporum*. Significant inhibition of *F. oxysporum* growth was observed in dual culture confrontation test. Our results highlighted the potential of *T. asperellum* as a biocontrol agent for managing *F. oxysporum* in *C. roseus* cultivation. The application of these agents can reduce the dominance of chemical pesticides and promote sustainable agriculture practices.

Keywords: *Madagascar periwinkleis; Trichoderma asperellum; Fusarium wilt*

Összefoglalás: A rózsás metény (*Catharanthus roseus* L.) termesztését számos kórokozó befolyásolja, beleértve a talajból fertőző kórokozókat is, mint például a *Fusarium*, *Phytophthora* vagy *Pythium* fajokat. Egyre inkább előtérbe kerül a biológiai növényvédőszerek használata a kémiai növényvédőszerek hatóanyagainak folyamatos kivonása miatt. A kórokozók elleni védekezésben egyre gyakrabban használnak antagonista szervezeteket, mint a *Bacillus amyloliquefaciens*, a *Trichoderma asperellum* és a *T. atroviride.* Ebben a tanulmányban a *T. asperellum in vitro* hatékonyságát vizsgáltuk *F. oxysporum* ellen. A konfrontációs teszt során a *F. oxysporum* növekedését jelentősen gátolta az antagonista gomba. Ez előrevetíti *T. asperellum* használatának a lehetőségét *C. roseus* termesztésében *F. oxysporum* ellen. Alkalmazása csökkentheti a kémiai növényvédő szerektől való függőséget és elősegítheti a fenntartható mezőgazdasági gyakorlatot

Kulcsszavak*: Madagascar periwinkleis; Trichoderma asperellum; fuzáriumos hervadás*

1 Introduction

The cultivation of Madagascar periwinkleis (*Catharanthus rosaeus* L.) is highly affected by many pests and pathogens, particularly soil-borne fungi. These pathogens mainly infect seedlings causing root rot, wilting and eventually the death of seedlings. Mature, but less

vigorous plants can also be attacked, leading to foliage wilting. These symptoms are often caused by plant pathogenic fungi belonging to different genera, including *Fusarium* spp., *Pythium* spp., *Phytophthora* spp., *Alternaria* spp. (Farr et al., 2021). In the case of Madagascar periwinkleis, several plant pathogenic fungi such as *Rhizoctonia solani*, *Fusarium equiseti*, *F. oxysporum* and *F. solani* have been reported to cause wilt (Yasir and Almaliky, 2023). Infected plants showing these symptoms become unsellable, causing serious economic losses for the growers. Possibilities in chemical control against these pathogens is negatively affected by the continuous withdrawal of pesticides. However, biological control methods for soil-borne pathogens have proven to be a promising alternative. Several biocontrol products are currently approved in Hungary, including different strains of *Bacillus amyloliquefaciens*, which have a broad spectrum of activity and can be applied directly in the soil. In addition, *Trichoderma asperellum* (formerly known as *T. harzianum*) and *T. atroviride* are also approved. These mycoparasitic fungi not only suppress plant pathogens but also enhance plant tolerance to both biotic and abiotic stresses (Singh et al., 2018).

The aim of this study was to evaluate the effectiveness of the antagonist organism, *Trichoderma asperellum*, in inhibiting the growth of *Fusarium oxysporum,* and we assumed that the *Trichoderma* species inhibits and parasitizes the tested pathogen in laboratory conditions.

2 Materials and Methods

Experimental Setup

The effectiveness of *Trichoderma asperellum* as an antagonist against *Fusarium oxysporum* plant pathogen was tested using a dual-culture agar confrontation test in Petri dishes. PDA (potato dextrose agar) medium was prepared and poured into sterile Petri dishes (90 mm diameter) under a laminar flow cabinet. Seven to ten-day-old *Fusarium oxysporum* culture from the Department's culture collection (isolated from *Catharanthus roseus,* 2023) were used to cut mycelial discs (7 mm diameter) with sterile tools. These pathogen discs were inoculated on one side of the Petri dishes, 1.5 cm from the edge.

Mycelial discs of the mycoparasitic antagonist organism were prepared similarly and placed on the opposite side of the Petri dishes, 1.5 cm from the edge. in a total of 40 replicates. Control dishes contained only the mycelium of the pathogen, antagonistic, *Trichoderma* was replaced with a sterile PDA disc. All dishes were sealed with Parafilm, labelled, and incubated at room temperature (23±2 °C).

Growth Measurements

Pathogen growth was assessed by measuring the radial growth of colonies at 48-hour intervals, starting 48 hours post-inoculation. This measurement was repeated twice.

Statistical Analysis

The impact of the antagonist was analyzed using a Linear Mixed-Effect Model (LMM) in R (Version 4.1.0, 2019). Colony radius was square root-transformed to approximate a normal distribution. The model included transformed colony radius as the dependent variable, with the presence of antagonist ('Treatment'), measurement days ('Time'), and their interaction as fixed effects. The unique identifier of each Petri dish was included as a random factor.

3 Results

In all cultures, the colonies increased in size, but with different growth dynamics as shown in Table 1. The significant interaction ('Treatment×Time') indicates that the two fungi showed a different growth pattern. The antagonist organism significantly ('Treatment(Treated)') inhibited the growth of *Fusarium oysporum* in the dual cultures, successfully demonstrating the *in vitro* efficacy of *Trichoderma asperellum* as a biocontrol agent against the pathogen (Fig. 1).

Table 1 Effects of the *T. asperellum* treatments and the time on the growth of *F. oxysporum using LMM*.

Predictors	Estimate	Std. Error	df	t value	P value
Intercept	1.72	0.01	48	125.94	<0.001 ***
Treatment(Treated)	-0.39	0.02	48	-25.80	<0.001 ***
Time	0.37	0.01	98	34.53	<0.001 ***
Treatment(Treated)×Time	-0.31	0.01	98	-25.73	<0.001 ***

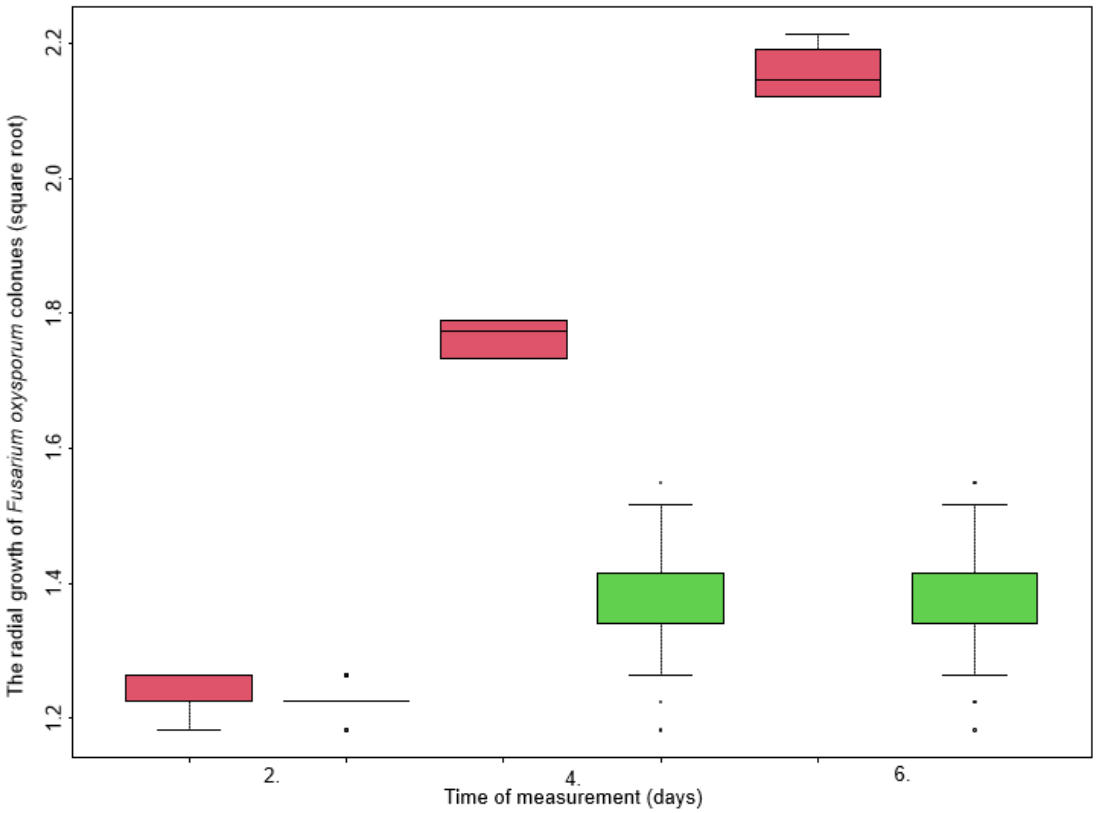

Figure 1 *Whisker's plot shows the effect of antagonistic fungi on the growth of* Fusarium oxysporum *colonies:*

Red indicates control Petri dishes, and green indicates colonies growing with the antagonist. The horizontal line in the diagram represents the median. The boxes indicate the interquartile range. Whiskers indicate 95% confidence intervals.

4 Discussion

The results demonstrate that *Trichoderma asperellum* acts as an effective biocontrol agent against *Fusarium oxysporum in vitro*. The different growth pattern may be the result of the pathogen development in the treated Petri dishes having stopped when the two species interacted. The significant inhibition of pathogen growth in the dual cultures highlights the antagonistic interaction between *Trichoderma asperellum* and *Fusarium oxysporum*.. This inhibition suggests that the antagonist's mechanisms, such as mycoparasitism or competition for space, may play a role in limiting pathogen growth. Similar interactions have been observed in previous studies involving *Fusarium oxysporum* and other *Fusarium* species (Ghanbarzadeh *et al*., 2014; Larran *et al*., 2020).

Trichoderma species are widely distributed in soils and could be effectively used in integrated pest management due to their ability to degrade the hyphal cell wall thereby reducing disease symptoms and limiting the spread of pathogens including *Fusarium* species (Monte, 2001; Woo *et al*., 2013).

These findings support the potential application of *T. asperellum* in managing *F. oxysporum* infection in *Catharanthus roseus* cultivation.

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