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Preliminary Study of Control and Biochemical Characteristics of Giant Hogweed (*Heracleum sosnowskyi* Manden.) Treated with Microwaves

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Abstract: There is an urgent need to develop new compensatory and human-safe methods for controlling invasive *Heracleum* species. This research aimed to determine the effectiveness of *H. sosnowskyi* control under microwave radiation and biochemical changes in tissues and sap after irradiation. In field experiments in southern Poland, the plants were cut and irradiated with a device generating electromagnetic waves (2.45 GHz, 32.8 kW/m^2). The control efficacy of plants in the rosette phase irradiated for 5, 10, or 15 min was 20%, 100%, and 100%, respectively. The control efficacy of plants in the flowering phase irradiated for 7.5 or 15 min was 66% and 100%. The metabolomic analysis of tissues and sap of irradiated *H. sosnowskyi* showed significant changes, mainly in the content and composition of proteins and sugars. In tissues, the treatments resulted in protein denaturation and significant changes in the metabolism of amino acids and the glyoxylate and galactose pathways. The sap was rich in sugars, glutamic acid, glutamine, homoserine, serine, and methionine. More changes in metabolite levels were observed in the tissues irradiated for 7.5 min. In conclusion, microwave radiation of *H. sosnowskyi* for longer times, e.g., 10 and 15 min, efficiently controls it.

Keywords: non-chemical control; physical control; metabolomics of stems and sap

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1. Introduction

There are over 20 species of plants of the genus *Heracleum* known in Europe. Three species of this genus, so-called giant hogweeds, i.e., *Heracleum sosnowskyi* (Manden.), *Heracleum mantegazzianum* (Sommier & Levier), and *Heracleum persicum* (Desf. ex Fischer), from the Central and Eastern Caucasus, Trans-Caucasus, and Turkey, are invasive plants in Europe [1,2]. These plants pose a serious threat to habitats under changing climatic conditions [3], being highly competitive with the native flora [4]. Invasive hogweeds are characterized by an intensive growth rate, huge size (up to 4 m high), and a high reproductive rate [2,5]. Studies have proven that their competitive abilities also result from allelopathic interactions [6]. The invasive *Heracleum* species cause health problems in humans and animals due to the content of chemical compounds, i.e., furanocoumarins, in their tissues. Direct contact with the plants or even being in their vicinity may often result in skin and eye irritation and skin burns [7,8] and special cases of poisoning accompanied by disorders of the nervous system and heart muscle [9].

Out of the three invasive Hogweeds in Poland, two are a serious problem, i.e., *Heracleum sosnowskyi* and *Heracleum mantegazzianum*; they occur in various habitats, along roads and railways, on the banks of rivers and irrigation canals, and in agricultural areas

and national parks [10,11]. Their dynamic spread in recent decades throughout northern Europe is partly explained by the increase in winter temperature [12,13]. In the European Union, the invasive Hogweeds are included in the Invasive Alien Species of Union list EU [14], which obliges the EU countries to limit their spread or eliminate them.

This research focused on *Heracleum sosnowskyi* due to the frequency of occurrence and the area occupied in southern Poland, which varies from a few square meters with a few specimens to several hectares of dense stands [15]. The biological and ecological characteristics of *Heracleum sosnowskyi* make it a species difficult to control. Mechanical mowing does not give the desired effects because plants grow back, and herbicides are a significant threat to the environment [16–20]. Therefore, there is an urgent need to develop new compensatory methods for controlling invasive *Heracleum* [21,22].

As a result, in this research, to destroy *Heracleum sosnowskyi* plants, the authors used a device emitting electromagnetic (microwave) radiation within the limits of radiofrequency radiation. Out of the wide range of microwave frequencies, only a few are more widely used, according to the regulations of the Federal Communications Commission. Metal reflects microwaves, which pass through electrically neutral materials such as glass, most plastics, ceramics, and paper, and when they are absorbed, they generate heat in the absorbing material. Two particularly important properties of microwaves are volumetric heating and inverted temperature gradients in the heated material [23–25]. Currently, the use of microwaves in many areas is becoming common, for example, in food processing and agriculture [26–31], sterilization of seed material [32] and soil [33], or in forestry to improve the quality of seed germination [34], for the disinfection of soil [35], and for the drying of wood and wood-based products [36–39]. It is known from the available literature that microwave radiation also affects the growth and development of plants. However, the effects of microwaves on plants depend on the plant family and growth stage involved [40,41], and the exposure duration, frequency, and power density [42]. The number of published studies is insufficient to conclude on the effects of microwaves on plants [43]. Thus far, studies that show the possibility of combating plants with microwaves and the biochemical changes in plants under the influence of exposure to microwave radiation are few [44–46]. For example, millimeter-wave irradiation of low intensity improves the growth of chickpea [47] and soybean seedlings, promoting glycolysis- and redox-related pathways under flooding conditions and activating sugar metabolism, especially trehalose synthesis [48]. Since the final effect of microwaves on plants depends on the intensity and duration of microwave treatment, we hypothesize that the stimulating effects will change under high microwave intensity to inhibiting ones. Hence, the study aimed to determine the effectiveness of Sosnowsky's hogweed (*Heracleum sosnowskyi* Manden.) control under microwave radiation and to determine the biochemical changes in tissues and sap after irradiation.

2. Materials and Methods

2.1. The Description of the Microwave Device for the Destruction of Heracleum sosnowskyi Plants

A device emitting microwave radiation with an open stream directed at the plant was built (Figure 1) based on the elements of a microwave oven. Before disassembling its elements, the output microwave power was determined following the Polish norm PN-EN 60705. The device used a magnetron to generate electromagnetic waves with a frequency of 2.45 GHz and a power density of 32.8 kW/m^2. A voltage-increasing transformer powered the magnetron through a voltage multiplier consisting of a capacitor and a diode. Both the magnetron and the transformer were cooled by the air stream forced by the fan. In addition to these elements, the system included fuses, bimetallic thermal switches, and a filter that eliminated disturbances in the power supply network.

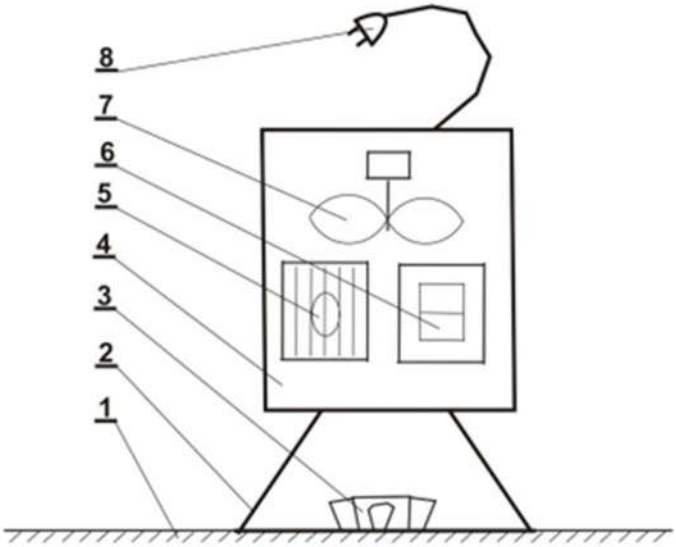

Figure 1. The scheme of action of the microwave destruction device. 1—ground; 2—horn microwave antenna; 3—cut stem of Sosnowsky's hogweed; 4—microwave emitter; 5—a magnetron that produces microwaves; 6—converter; 7—fan cooling magnetron and converter; 8—a plug with a microwave emitter power cord.

The wiring diagram is shown in Figure 2. The average surface power density for the dimensions of the constructed antenna for the magnetron was 30 kW/m^2. The antenna was positioned centrally to emit microwave radiation to the center of the plant, and a raise in temperature destroyed the plant.

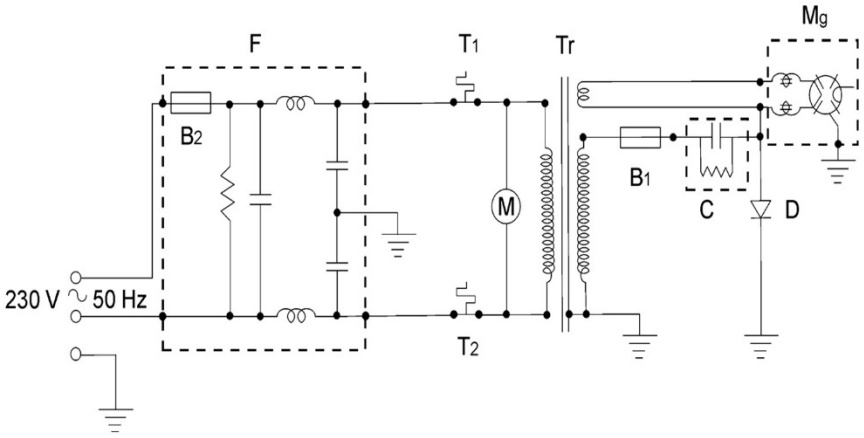

Figure 2. The electrical diagram of the device for microwave disinfection of soil. F—filter, T1, T2—temperature switches, Tr—high-voltage transformer, M—the motor of magnetron cooling fan, Mg—magnetron, D—high-voltage diode, C—high-voltage capacitor, B1, B2—fuses.

2.2. Microwave Treatment in the Rosette Phase of Heracleum sosnowskyi

The treatment was carried out on 15 June 2021, at the Experimental Station of the Faculty of Biotechnology and Horticulture of the University of Agriculture in Krakow, which is located in Garlica Murowana (50°8′26.65″ N, 19 ° 56′5.26″ E; 274.4 m a.s.l.), ca. 5 km north of Krakow (Figure 3 left). The experimental plot was a seminatural plant community of the Molinio-Arrhenatheretea class in secondary succession. In the western part of the plot, on 800 m^2, there was a dispersed population of Sosnowsky's hogweed (*Heracleum sosnowskyi* Manden.) composed of ca. 25 specimens (Figure 3 right). Tall grasses and *Solidago canadensis* surrounded the hogweed plants. The treatment began around 10 a.m., when the air temperature was ca. 23–24 °C, and there was no wind, and was completed in the afternoon, around 4 p.m., when the air temperature increased to 30 °C. Twenty plants

of Sosnowsky's hogweed in the rosette phase (height about 50–60 cm) were selected. The plants were cut with a sickle to 4 cm from the soil surface. Next, the microwave device was placed centrally over each cut stem and irradiated for 5, 10, or 15 min. Each irradiation was carried out in 5 replications, and the remaining 5 stems served as non-treated control.

Figure 3. Location of field studies in Poland (**left**) and Garlica Murowana (**right**).

2.3. Microwave Treatment in the Flowering Phase of Heracleum sosnowskyi

The treatment was conducted in natural conditions on a 1200 m^2 area covered by a dense population of Sosnowsky's hogweed (*Heracleum sosnowskyi* Manden.) on 5 July 2021. The area is located in the western part of the Wielkie Błoto PLH 120080 peatbog (50°1′11,326″ N 20°15′53,481″ E; 195.6 m a.s.l.), about 20 km southeast of Kraków (Figure 4). The hogweed population was located near the local road. On the eastern side, it was limited by the post-mining reservoir in the overgrowing phase with rush vegetation belonging to the *Phragmitetea* class.

The microwave treatment of hogweed was performed in the early morning at an air temperature of 18 °C and air humidity of 85%. Eighteen flowering plants, ca. 3 m tall and with similar physiognomy, were selected. Whole plants were cut with a sickle at ca. 4 cm above the ground, and their location was marked with a plastic plate. Next, the microwave device was placed over each of the cut stems. Six cuts were irradiated for 7.5 min (R1) and another six for 15 min (R2). The remaining six cuts were the control without microwave irradiation (R0). In treatments R1 and R2, the microwave antenna was positioned above the cut stem to emit the radiation centrally on the stem.

Figure 4. Location of field studies in Poland (**left**) and Wielkie Błoto peat bog (**right**).

2.4. Soil Analyses

The soil samples from both locations, Garlica Murowana and Wielkie Błoto, were analyzed in the accredited laboratory (AgroEkspert, Kościelec, Poland) according to the following methods: soil particle size: sand (2.0–0.05 mm), coarse silt (0.05–0.02 mm), fine silt (0.02–0.002 mm), clay (<0.002 mm) PN-R-04032: 1998 (for samples with organic matter content < 5%); total nitrogen content by the Kjeldahl method; soil pH-PN-ISO 10390: 1997; available P_2O_5-PN-R-04023: 1996, K_2O-PB-1 (Ed. of 20 February 2013), Mg-PN-R-04020: 1994 + Az1: 2004; microelements: Mn-PN93/R-04019 (1 M HCl), Zn-PN92/R-04016 (1 M HCl), Cu-PN92/R-04017 (1 M HCl), Fe-PN-R-04021: 1994 (1 M HCl), B-PN93/R-04018 (1 M HCl); heavy metals Cd, Ni-PN-ISO 11047: 2001. The soils composition from both sites is presented in Table 1.

Table 1. Mechanical and chemical characteristics of soils from sites in Garlica Murowana and Wielkie Błoto.

Analysis	Garlica Murowana	Wielkie Błoto
Soil particles	sand: 23% coarse silt: 36% fine silt: 33% clay: 8%	sand: 89% coarse silt: 6% fine silt: 5%
N total (g/kg d.m. soil)	2.1	10.1
pH KCl	7.3	7.1
Macronutrients (mg/100 g soil)	P_2O_5: 26.2 (very high) K_2O > 35.0 (very high) Mg: 7.6 (high)	P_2O_5: 6.7 mg (low) K_2O: 14.8 mg (high) Mg > 15.0 (very high)
Micronutrients (mg/100 g soil)	Mn: 247.3 (medium) Zn: 24.5 (high) Cu: 4.5 (medium) Fe: 2092.7 mg/kg soil (medium) B: < 0.3 mg/kg soil (low)	Mn: 535.7 (high) Zn > 50.0 (high) Cu: 6.3 (high) Fe: 3425.7 mg/kg soil (medium) B: 0.9 mg/kg soil (low)
Heavy metals (mg/kg soil)	Cd < 5.0 Ni: 15.2	Cd < 5.0 Ni: 15.0
Soil humidity	86%	89%

2.5. Assessment of the Heating Value of Microwaves and Plant Destruction of Heracleum sosnowskyi in Both Populations

The diameter of each cut stem of hogweed was measured using a roller with an accuracy of ±1 mm, at two cross-cutting positions, as the stems were elliptic. Next, the area of the stem was calculated using the equation:

$$P = \pi ab, \quad (1)$$

where a and b are the ellipse driveshafts.

Before and right after each irradiation process, the temperature of the cut stems of hogweeds was measured with a thermal imaging camera FLIR E60 equipped with Wi-Fi (producer: FLIR Systems, Inc., Wilsonville, OR, USA); the camera measures temperature in a range from −20 to +120 °C (±2 °C). The camera was placed at ca. 0.5–1 m above each stem, and photos of a resolution of 320 × 240 pixels were taken. Next, each photo was analyzed using the FLIR ResearchIR MAX (producer: Teledyne FLIR LLC, Wilsonville, OR, USA), based on the mathematical processing of the pixels' color scale. The average temperature values were calculated for each treated plant and analyzed further using regression analysis in the Excel worksheet.

The level of plant destruction following irradiation was monitored visually based on the regrowth of new leaves. The plant population in Garlica Murowana was monitored at

16 and 94 days after irradiation (DAI), and the population in Wielkie Błoto was monitored at 7 and 75 DAI.

2.6. A Sampling of Plant Material for the Metabolomic Analyses

Five hours after the R1 and R2 treatments, from the population in Wielkie Błoto, the transparent sap, excreted to the internal parts of the cut stems of hogweed, was collected separately from each plant using sterile syringes and containers of 20 mL volume. Additionally, the internal tissues of stems were cut with a sterile lancet, at 3 cm in length and about 0.3–1 cm in depth, and placed in sterile containers of 20 mL volume, separately from each hogweed plant. The collection of plant material was carried out at an air temperature of 34 °C and air humidity of 60%.

In total, 12 samples with sap and 18 samples with stem tissues were collected. The sap samples were filtered in the laboratory on the same day through Pureland PP (Stargard, Poland) syringe filters with a nominal pore size of 0.45 µm, and 0.2 mL of the filtrate was poured into the sterile Eppendorf vials. The collected plant tissues were gently scrubbed with sterile lancets to remove all the surface impurities and placed in sterile containers of 20 mL volume. The material was lyophilized at −40°C in Labconco FreeZone (Kansas City, MO, USA) freeze dry system for ca. 30 h, to quench the metabolism, and stored at room temperature.

2.6.1. Metabolite Extraction and Derivatization

Fifty milligrams of lyophilized plant material was used for each sample and replicated. Sample extraction was carried out following the protocol Lisec et al. [49] proposed with some modifications. In particular, 1400 µL of cold methanol (at −20 °C) was added to the sample. After vortexing, 60 µL of ribitol stock solution (internal standard), used at the concentration of 0.2 mg/mL in ddH_2O was added. Samples were heated (70 °C) and shaken (950 rpm) for 10 min to enhance metabolome extraction and then centrifuged for 10 min at 11,000× *g*. The polar and non-polar classes of metabolites were separated using 750 µL $CHCl_3$ (−20 °C), and 1500 µL ddH_2O (4 °C) was sequentially added to the methanolic extracts. All the samples were vortexed and centrifuged for 15 min at 2200× *g*. For each sample, replicate, and treatment, 150 µL of the upper polar phases were collected and dried in a vacuum concentrator at room temperature. Then the samples were methoximated, adding 40 µL methoxyamine hydrochloride (20 mg/mL in pyridine), followed by incubation at 37 °C for 2 h in a thermomixer (950 rpm). Successively, the methoximated samples were further silylated adding 70 µL of N-methyl-N-trimethylsilyltrifluoroacetamide. Samples were then incubated for 30 min at 37 °C in a thermomixer (950 rpm). For the GC-MS analysis, derivatized samples (110 µL per each sample and treatment) were then transferred into GC-MS glass inserts.

2.6.2. GC-MS Analysis

The derivatized extracts were injected into a MEGA 5-MS capillary column (30 m × 0.25 mm × 0.25 µm + 10 m of pre-column) using a gas chromatograph apparatus (GC Agilent series 7890A) equipped with a single quadrupole mass spectrometer (MSD 5975C inert XL Perf Turbo). A CombiPAL autosampler was used for sample handling and injection.

The injector, transfer line, and source were set at 250, 250, and 260 °C, respectively. One microliter of the sample was injected in splitless mode with a helium (Elio Bip 6.0, SAPIO, Monza, Italy) flow of 1 mL/min using the following programmed temperature: isothermal 5 min at 70 °C followed by a 5 °C/ min ramp to 350 °C and a final 5 min heating at 330 °C. Mass spectra were recorded in the electronic impact (EI) mode at 70 eV, scanning at the 40–600 m/z range, with a scan time of 0.2 s. The mass spectrometric solvent delay was set as 9 min. n-Alkane standards (C10–C40 all even) and blank solvents (pyridine) were also injected for instrumental performance, tentative identification, and monitoring of shifts in retention indices (RIs).

2.6.3. GC/MS Data Analysis and Statistical Analyses

The open-source software MS-DIAL, equipped with open-source publicly available EI spectral libraries, was used for data baseline filtering and calibration, peak alignment, deconvolution analysis, peak identification, and integration. The software was employed for the analysis as previously described by Misra et al. [50].

Compounds were identified based on the mass spectral pattern compared to EI spectral libraries, using commercial (NIST17/2017) and publicly available libraries. The public libraries were archived from the MSRI spectral libraries from Golm Metabolome Database, MassBank, and MoNA (Mass Bank of North America). Metabolite annotation and assignment of the EI-MS spectra were carried out following the metabolomics standards initiative (MSI) guidelines [51], using Level 2 (identification based on the spectral database (match factor > 80%) and Level 3 (putatively characterized compound class based on spectral similarity to known compounds of a chemical class as suggested).

A completely randomized design with five biological replications was adopted. Samples were divided into two groups (stem tissue and sap) and separately analyzed. The statistical analysis was performed using MetaboAnalyst version 5.0 [52]. Briefly, relative abundance values from the MS-DIAL outputs were normalized per the internal standard, Log2 transformed, and Pareto scaled. Successively, univariate analysis (ANOVA using LSD test as post hoc $p \leq 0.05$, evaluated through the false discovery rate), multivariate analysis (unsupervised principal component (PCA) analysis and supervised partial least squares discriminant analysis (PLS-DA) and machine learning analysis (random forest) were used to highlight the main metabolites involved in group separation as well as potential biomarkers.

The PLS-DA model was built using the two main latent variables (PC1 and PC2), which explained more than 70% of the total variability. The model affordability was then confirmed through cross-validation and the permutation test analysis. In particular, for cross-validation, the leave-one-out cross-validation (LOOCV) procedure and Q2 for performance evaluation were used. The separation distance statistical test, setting the permutation number to 20, was used for the permutation. The random forest analysis was built using 500 trees and 7 predictors with a randomness option. Enrichment and pathways analysis was carried out only on stem tissue samples using the MetaboAnalyst 5.0 tools and setting *Arabidopsis thaliana* as a metabolome reference database.

3. Results

3.1. The Effectiveness of Destroying Hogweed in the Leaf Rosette Phase

In the experiment in Garlica Murowana, a soil of medium agronomic category was found, with a clay structure and an average moisture content of 84% (Table 1). The average area of cut hogweed shoots in the rosette phase was 3.14–78.5 cm^2. The mean temperature of the plants untreated with microwaves was 21.5 °C, and immediately after microwave exposure, the temperature increased to 68, 81.8, and 94.6 °C after 5, 10, and 15 min, respectively (Figure 5).

All control plants that were cut only re-grew (Figure 6). After 16 days from treatment, the effectiveness of destroying Sosnowsky's hogweed with microwaves within 5 min was 20%. Sixteen days after the treatment, four plants irradiated for 5 min showed signs of regeneration out of the five irradiated (Figure 7). The regenerated plants developed 1–2 new leaves. Sixteen and 94 days after the irradiation for 10 and 15 min, the effectiveness of destroying hogweed was 100% (Figures 8 and 9).

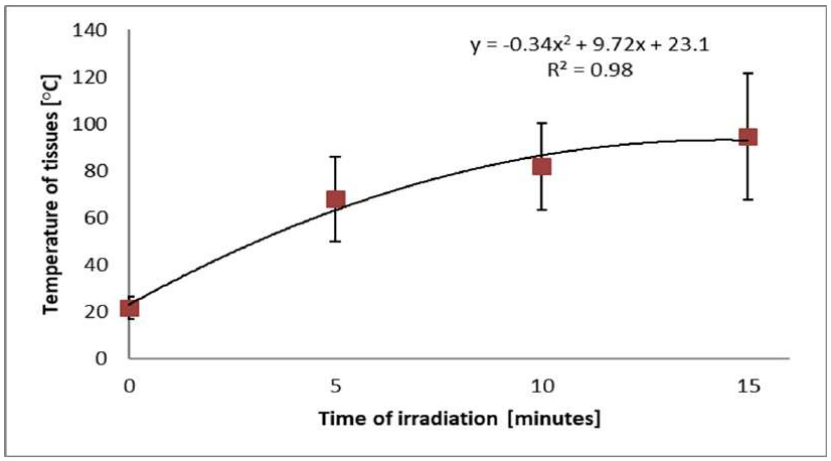

Figure 5. The temperature of hogweed stems in Garlica Murowana before (0) and immediately after microwave exposure for 5, 10, or 15 min; n = 5.

Figure 6. Re-growing control plant 16 days after cutting.

Figure 7. Re-growing plant irradiated for 5 min, 16 days (**left**), and 94 days (**right**) after the irradiation. Stick marks the place of the irradiated plant.

Figure 8. Plant irradiated for 10 min, 16 days (**left**), and 94 days (**right**) after the irradiation.

Figure 9. Plant irradiated for 15 min, 16 days (**left**), and 94 days (**right**) after the irradiation.

3.2. The Effectiveness of Destroying Hogweed in the Flowering Phase

In the experiment carried out in the Wielkie Błoto population, the soil found was loamy sand, a very light soil according to the agronomic category, with an average humidity of 89% (Table 1). The average area of cut hogweed shoots in the flowering phase was 33.7–45.4 cm^2. The average temperature of the plants untreated with microwaves was 15.4 °C. Immediately after microwave exposure, the temperature of the plants increased to 62.7 and 76.3 °C after 7.5 and 15 min, respectively (Figure 10).

Seven days after the treatment, the effectiveness of hogweed irradiation for 7.5 min was 66%, which means that two plants (out of six) re-grew a new leaf (Figure 11). Seventy-five days after treatment, three plants were found to regenerate following 5-min-long irradiation; those plants were growing about 4–6 new leaves each (Figure 12). Microwave irradiation for 15 min caused 100% of plant destruction, as found 7 and 75 days after treatments (Figures 13 and 14). Figure 15 shows a control plant that was cut only 7 and 75 days after cutting.

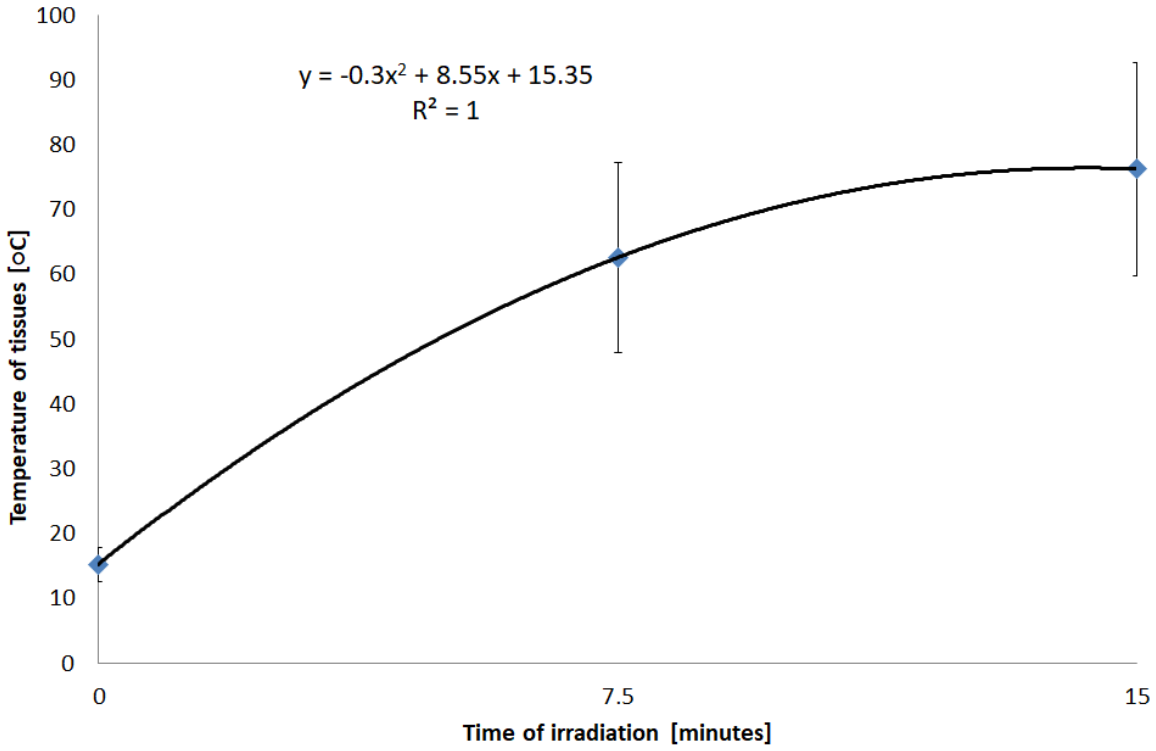

Figure 10. The temperature of hogweed stems in Wielkie Błoto before (0) and immediately after microwave exposure for 7.5 or 15 min; n = 6.

Figure 11. Plants irradiated for 7.5 min, 7 days after irradiation. Plant sample 9 (**left**) is destroyed and plant sample 11 (**right**) is re-growing.

Figure 12. Plants irradiated for 7.5 min, 75 days after irradiation. Plant sample 9 (**left**) is destroyed and plant sample 11 (**right**) is re-growing several new leaves.

Figure 13. Plants irradiated for 15 min (**left** and **right**), 7 days after irradiation. Both plants are destroyed.

Figure 14. Plants irradiated for 15 min (**left** and **right**), 75 days after irradiation. Both plants are destroyed.

Figure 15. Re-growing control plant 7 days (**left**) and 75 days (**right**) after cutting.

3.3. GC-MS-Driven Untargeted Metabolomic Analysis of Stem Tissues of Hogweed Irradiated in the Flowering Phase

The metabolic changes in *H. sosnowskyi* stem tissues were assessed following the microwave treatments using a GC/MS-driven untargeted-metabolomic analysis. The analysis allowed us to annotate and quantify 82 putatively annotated metabolites and extract 965 unknown EI-MS shared features. The data analyzed by univariate one-way ANOVA allowed us to identify 65 significantly altered features, for which the concentrations for each replicate have been reported in the text as a heat map (Figure 16).

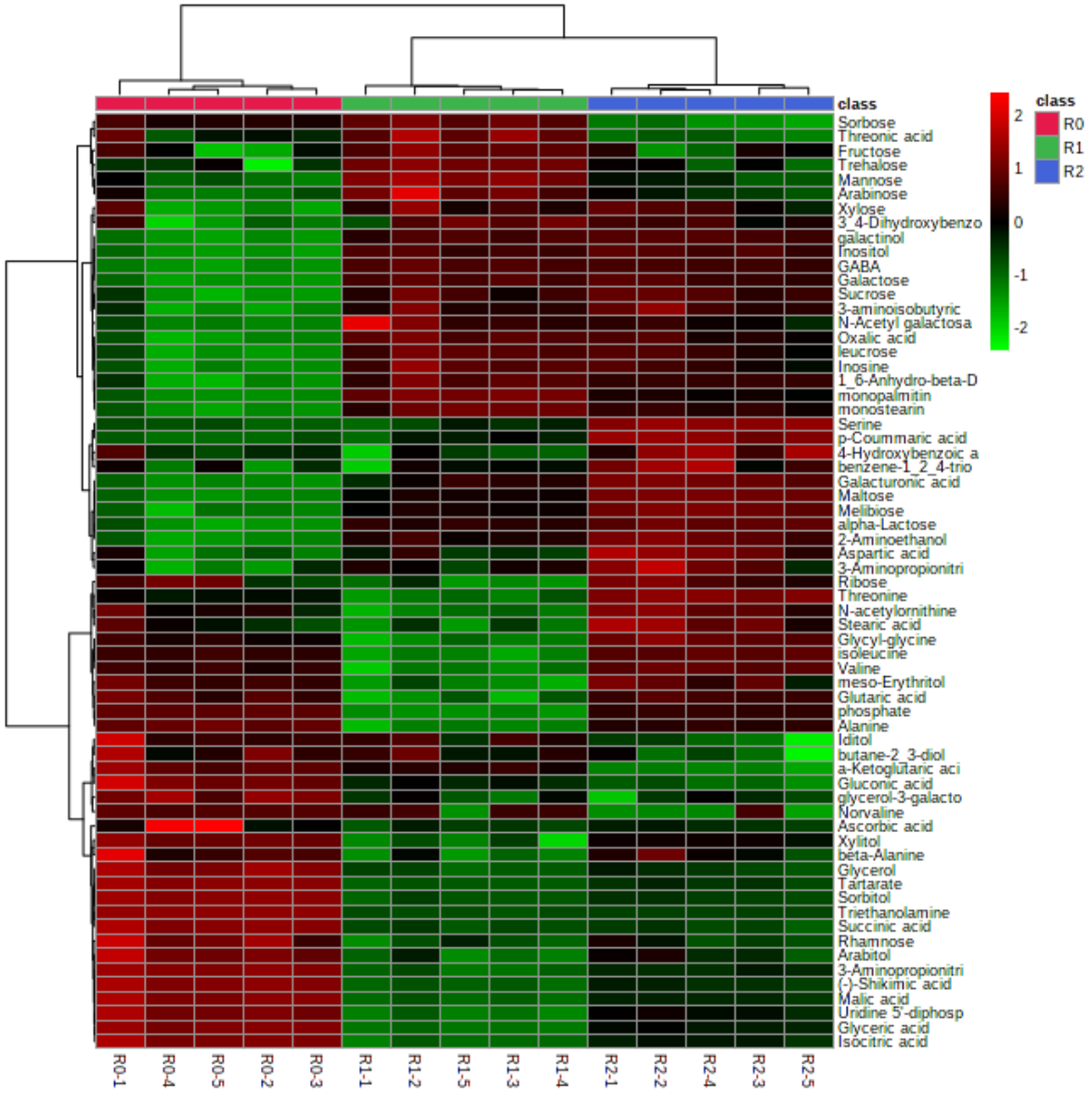

Figure 16. A heat map with 65 significantly altered features following the *H. sosnowskyi* (Manden.) stems' exposure to microwaves for 0 (R0), 7.5 min (R1), and 15 min (R2), as analyzed by univariate one-way ANOVA; n = 5.

Successively, to identify differences among the groups and the molecules involved in those differences, the data were analyzed through multivariate analysis using the unsupervised PCA (Figure 17a), revealing clear discrimination among the three sample groups with no outliers. In particular, PCA was conducted based on the first two components (PC1 and PC2), which accounted for 77.3% of the total variance. PC1 described 53.9%, whereas PC2 explained 23.4% of the total variance (Figure 17a). The PCA loading plots were analyzed to highlight the main contributors to sample separation. PC1 was mainly dominated by triethanolamine, phosphate, 3-aminopropionitrile fumarate, galactinol, and alpha-lactose, whereas for PC2 it was isoleucine, threonine, serine, phosphate, and norvaline. A partial least squares discriminant analysis (PLS-DA) was successfully applied (Figure 17b). The results confirmed the separation of groups. The PLS-DA-derived variable importance in projection (VIP) scores (built on the metabolites with a VIP score higher than 1) revealed triethanolamine, serine, alpha-lactose, maltose, melibiose, and galactinol, among others, as the main metabolites involved in group separation (Figure 17c). Finally, the machine learning random forest analysis revealed xylitol, melibiose, isocitric acid, valine, gluconic

acid, alpha-ketoglutaric acid, and shikimic acid, among others, as the metabolites with the highest mean decrease accuracy (features ranked by their contributions to classification accuracy) for the three sample groups (Figure 17d).

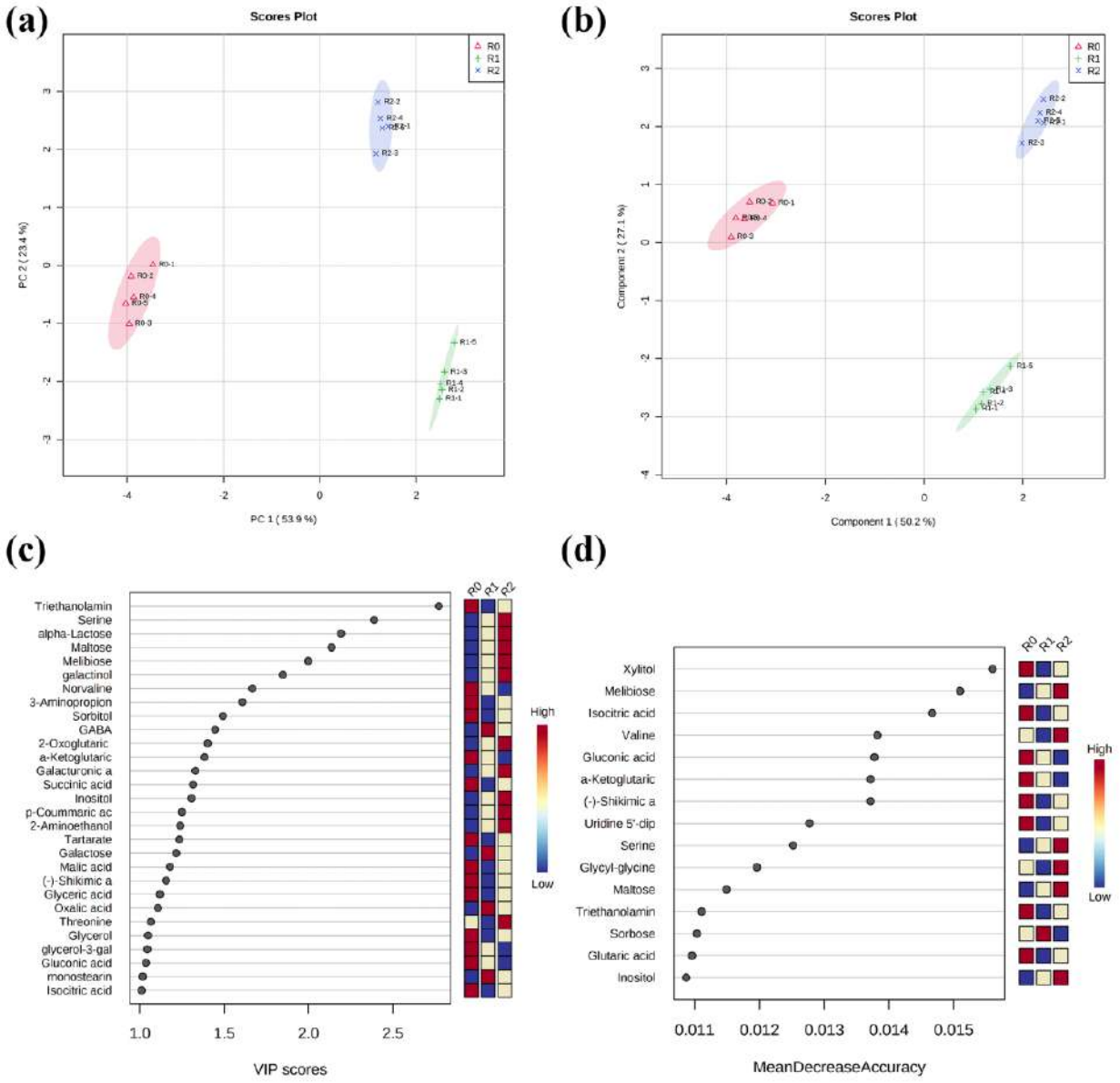

Figure 17. Discrimination through principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) of the metabolite patterns in *H. sosnowskyi* (Manden.) stems exposed for 0 (R0), 7.5 min (R1), and 15 min (R2) to microwave treatments. (**a**) PCA and (**b**) PLS-DA showed score plots that allowed group discrimination by the first two principal components (PCs). (**c**) Variable importance of projection (VIP) features for the groups from PLS-DA analysis. (**d**) Random forest analysis displaying the mean decrease accuracies; n = 5.

Finally, a KEGG-based pathway analysis, which combines enrichment and topology analysis, revealed that 22 pathways were significantly altered by the microwave treatment (Table 2). The treatments significantly impacted the amino acid metabolism and the glyoxylate and the galactose pathways.

Table 2. The results from "Pathway Analysis" carried out on the metabolite identified in *H. sosnowskyi* (Manden.) stem tissues exposed for 0 (R0), 7.5 min (R1), and 15 min (R2) to microwave treatments.

Metabolic Pathways	Total Cmpd	Hits	Raw p	FDR	Impact
Glycine serine and threonine metabolism	33	4	1.10×10^{-12}	5.15×10^{-11}	0.355
Alanine aspartate and glutamate metabolism	22	5	3.87×10^{-6}	1.81×10^{-5}	0.327
Glyoxylate and dicarboxylate metabolism	29	6	1.62×10^{-5}	3.62×10^{-5}	0.295
Galactose metabolism	27	7	3.00×10^{-6}	1.81×10^{-5}	0.249
Carbon fixation in photosynthetic organisms	21	3	1.02×10^{-7}	1.97×10^{-6}	0.149
Citrate cycle (TCA cycle)	20	3	0.00034	0.000533	0.146
Pentose phosphate pathway	19	3	1.25×10^{-7}	1.97×10^{-6}	0.144
Butanoate metabolism	17	3	0.000336	0.000533	0.136
Pantothenate and CoA biosynthesis	23	3	0.04018	0.047212	0.117
Glycolysis/Gluconeogenesis	26	3	0.004828	0.005971	0.103
Inositol phosphate metabolism	28	2	3.13×10^{-6}	1.81×10^{-5}	0.103
Starch and sucrose metabolism	22	2	0.00016	0.000279	0.099
Fructose and mannose metabolism	20	2	4.93×10^{-6}	1.81×10^{-5}	0.098
Phenylalanine tyrosine and tryptophan biosynthesis	22	1	7.26×10^{-6}	1.81×10^{-5}	0.080
Arginine and proline metabolism	34	2	0.000167	0.00028	0.075
Glutathione metabolism	26	2	0.040112	0.047212	0.071
Sulfur metabolism	15	1	6.04×10^{-6}	1.81×10^{-5}	0.033
Phosphatidylinositol signaling system	26	1	6.85×10^{-6}	1.81×10^{-5}	0.033
Glycerolipid metabolism	21	2	7.57×10^{-6}	1.81×10^{-5}	0.017
Fatty acid biosynthesis	56	2	0.00435	0.005526	0.011
Glycerophospholipid metabolism	37	1	1.90×10^{-5}	3.88×10^{-5}	0.009
Purine metabolism	63	1	6.98×10^{-5}	0.000126	0.001

Total Cmpd: the total number of compounds in the pathway; Hits: the matched number from the uploaded data; Raw p: the statistical *p*-value, FDR: the false discovery rate applied to the nominal *p*-values to control for false-positive findings; Impact: the pathway impact value calculated from pathway topology analysis.

3.4. GC-MS-Driven Untargeted Metabolomic Analysis of Hogweed Sap

It was observed that plants treated with microwaves exuded a transparent sap inside the cut stem as an effect of radiation. For this reason, the chemical composition of the sap was analyzed. Metabolomic data concerning the sap produced by microwave-treated plants at the end of the experiments were handled as previously described for the stem tissue metabolome. The metabolomic analysis identified 1498 unknown molecules and 142 putatively annotated metabolites belonging to the class of amino acids, sugars, sugar alcohols, and organic acids. Among the annotated compounds, the ANOVA analysis pointed out that 111 out of 142 were significantly altered by the time of exposition to the microwave treatment. In particular, the analysis pointed out that the exposition time to microwaves was increasing/decreasing specific classes of compounds. The sap composition differed significantly among the three treatments. A clear separation was observed on PCA and PLS-DA analysis (Figure 18a,b). In particular, the PCA using the first two components explained 80.4% of the total variability. The metabolites participating in the separation for the two components were mannose, galactose, glutamic acid, *N*-acetyl-L-tyrosine, palmitic acid, glucose, *N*-acetylmannosamine, glucarate, and ferulic acid for PC1 and hexitol, 4-methylbenzoic acid, L-5-oxoproline, citramalic acid, pyrogallol, 3,4-dihydroxybenzoate, galactose, *O*-acetyl-L-serine and methylsuccinic acid for PC2.

Moreover, after its validation, the PLS-DA model further confirmed a clear separation among the groups, pointing out more than 30 metabolites with a VIP score higher than 1 (Figure 18c). Several sugars, i.e., galactose, glucose, and mannose, and amino acids, i.e., glutamic acid, glutamine, homoserine, serine, methionine, etc., should be annotated. Finally, the random forest analysis revealed that citric acid, norvaline, and threonic acid, among others, were the potential biomarkers of the different treatments (Figure 18d).

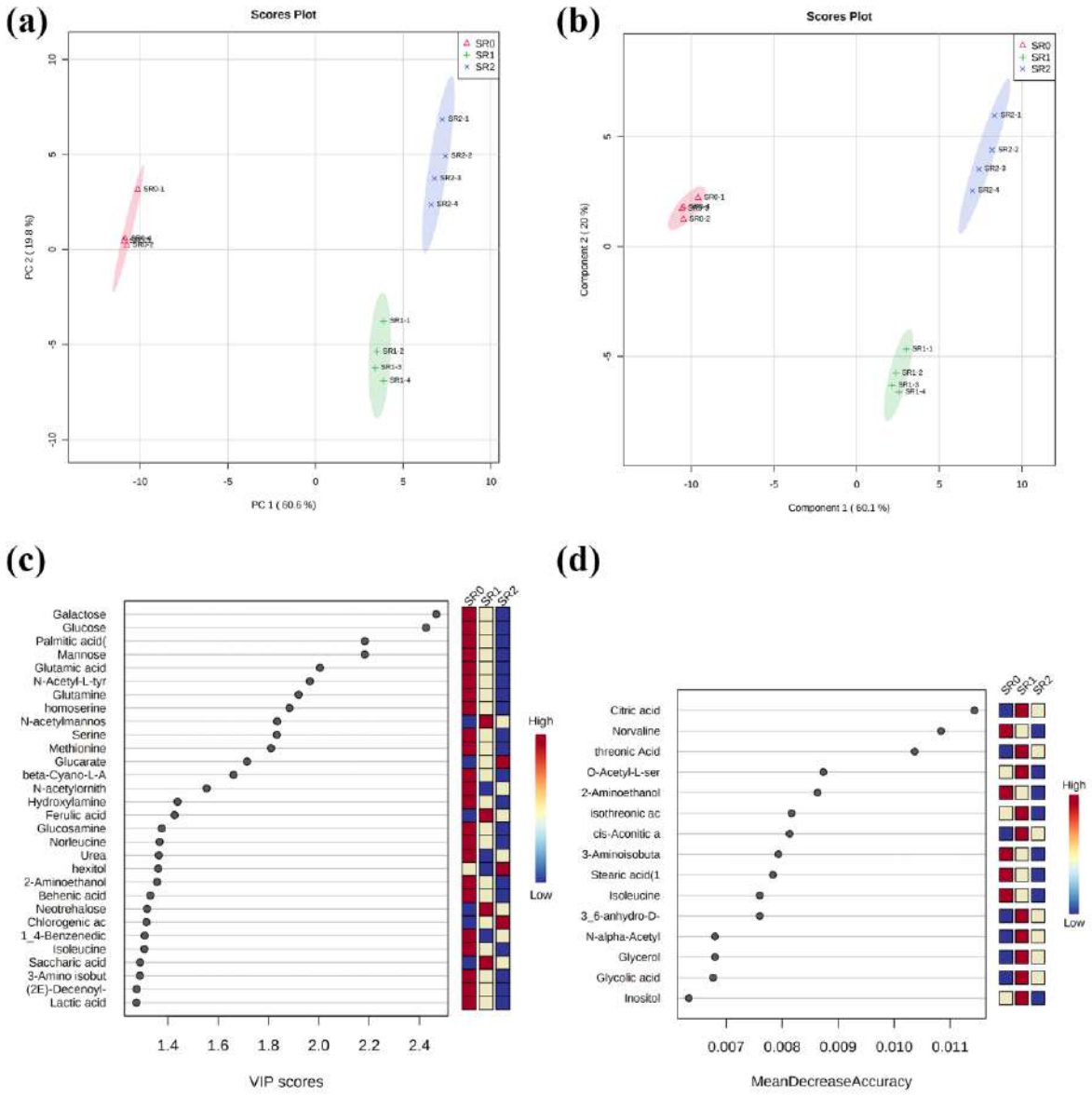

Figure 18. Discrimination through principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) of the metabolite patterns in *H. sosnowskyi* (Manden.) sap exuded by the roots exposed for 0 (R0), 7.5 min (R1), and 15 min (R2) to microwave treatments. (**a**) PCA and (**b**) PLS-DA showed score plots that allowed group discrimination by the first two principal components (PCs). (**c**) Variable importance of projection (VIP) features for the groups from PLS-DA analysis. (**d**) Random forest analysis displaying the mean decrease accuracies; n = 4.

4. Discussion

The results presented in this study on the control of Sosnowsky's hogweed with microwaves are pioneering. The effectiveness of combating hogweed plants in the leaf rosette and flowering phase was demonstrated using a device emitting microwaves at frequency 2.45 GHz and a power density 32.8 kW/m^2. It was found that, the longer the exposure of plants to microwaves, the lesser the subsequent regeneration. In the case of hogweed, 10 and 15 min of irradiation effectively destroyed 100% of plants in the leaf rosette and flowering phases, respectively. Previous studies on microwave exposure on plants have revealed variations in the sensitivity of plants to different microwave frequencies, exposure durations, and power intensities. For example, a stimulating effect was observed at low-power microwave irradiation on in vitro Sequoia plants [53]. Continuous microwave energy at 2.45 GHz with 0.5–1.2 mW/cm^2 caused no difference in growth and biomass accumulation

between control and the microwave treatment of alfalfa [42]. Furthermore, horizontally and vertically polarized 2 GHz continuous microwaves at 1.8 W m^{-2} power density did not alter the growth parameters, e.g., shoot length, stem diameter, and internodal length of *Myriophyllum aquaticum* plants [54]. Contrarily, a long-term signal at 915 MHz with a maximum power density of 10 mW/m^2 caused significant morphological modifications in *Phaseolus vulgaris* [55].

The other factors responsible for the irradiation effect include biological and environmental factors, particularly soil moisture. Research shows that the higher the soil moisture at irradiation, the higher its effectiveness [40]. For these reasons, in the present study, irradiation was carried out in the early morning when the humidity of both soils was high, 84% and 86%. The high humidity of the soils guaranteed a high degree of moisture in plant tissues. Therefore, their longer exposure time caused a significant increase in temperature, up to almost 95 °C, after 15 min of plant irradiation in the leaf rosette phase. As shown by Radzevičius et al. [56], slightly elevated temperatures (up to +30 °C) combined with 9.3 GHz frequency microwaves for 4 µs cause an incentive effect on plants, e.g., improved saccharide distribution in seeds or plants and significantly reduced content of non-structural carbohydrates, e.g., raffinose, glucose, fructose, and sucrose.

Contrarily, in our treatments, the high temperature of tissues during irradiation disintegrated the hogweed cells and tissues, resulting in the excretion and accumulation of sap in the irradiated stems. The metabolomic analysis of tissues and sap showed significant changes mainly in the content and composition of proteins and sugars. In the stem tissues, the treatments caused the denaturation of proteins and significant changes in the amino acid metabolism and the glyoxylate and the galactose pathways. The exuded sap was rich in sugars, mannose, galactose, and glucose and also in amino acids, i.e., glutamic acid, glutamine, homoserine, serine, and methionine. The random forest analysis revealed high citric acid, norvaline, and threonic acid contents in the sap. Following microwave irradiation, the observed changes in Sosnowsky's hogweed plants could be compared to heat changes caused by the flame method in soybean crops [57]. Under high-temperature conditions, over-fluidity of membranes appears, leading to an outflow of ions. Ion efflux leads to a reduction in the activity of many enzymes [58]. The most susceptible physiological process under microwave treatment is photosynthesis [54,55]. As found by Upadhyaya et al. [59], the exposure of plants to 900 MHz electromagnetic waves for up to 72 h indicated significant detriment in phenolic compounds by 32.12%, flavonoids by 14.89%, reduction in DPPH radical scavenging activity by 56.33%, and total antioxidant activity by 42.01%.

More changes in the levels of metabolites were observed in the stem tissue after 7.5 min of microwave treatment. Some plants re-grew after this treatment, suggesting that some of the observed changes could be of defensive character. Interestingly, in the sap of plants irradiated for 7.5 min, high levels of citric acid were found. Farid et al. [60] report that citric acid alleviates environmental stress in microwave-irradiated *Brassica napus* L. by initiating an antioxidant defense system.

5. Patents

Application number: PL43727921A, Title: Device for controlling weeds and their seeds, especially Sosnowsky's hogweed (*Heracleum sosnowskyi* Manden.). Publication date: 2022-01-17.

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