



Agitated and temporary immersion bioreactor cultures of *Reynoutria japonica* Houtt. as a rich source of phenolic compounds

Wojciech Makowski¹ · Aleksandra Królicka² · Julia Sroka¹ · Alicja Matyjewicz¹ · Marta Potrykus² ·
Paweł Kubica³ · Agnieszka Szopa³ · Barbara Tokarz¹ · Krzysztof M. Tokarz¹

Received: 10 April 2024 / Accepted: 4 August 2024 / Published online: 19 August 2024
© The Author(s) 2024

Abstract

Reynoutria japonica Houtt. (Japanese knotweed) is an invasive plant belonging to the Polygonaceae family. However, being native to East Asia, it has been used in natural medicine for ages because of its broad range of biological activity. Although *R. japonica* is known as a rich source of phenolic compounds, plant biomass collected from the field may be contaminated with toxic elements like heavy metals, and the level of metabolite accumulation depends on environmental conditions. Therefore, the aim of this study was to derive Japanese knotweed tissue cultures and investigate biomass production and phenolic compound synthesis in in vitro conditions. Plants were cultivated in a traditional agar-solidified medium, in a liquid medium with rotary shaking (agitated culture), and in a temporary immersion bioreactors Plantform™, as well as in soil (ex vitro conditions). Analyses of the growth index and dry weight accumulation were performed on the collected material. In the extracts obtained from examined plants, qualitative and quantitative analysis of phenolic derivatives using DAD-HPLC was conducted to determine the sum of phenolic compounds, as well as the quantity of selected phenolic acids, catechins, and other flavonoids. Results have shown that agitated cultures and temporary immersion bioreactors increased biomass accumulation compared to solid medium cultures. Tissue cultures of *R. japonica* had increased synthesis of phenolic compounds compared to plants from ex vitro conditions. Shoots and roots from agitated cultures were 2.8- and 3.3-fold richer in catechins, respectively, compared to plants cultivated in soil. Based on the obtained results it can be concluded that agitated and bioreactor cultures are the best source of Japanese knotweed biomass rich in valuable secondary metabolites.

Key message

Reynoutria japonica Houtt. plants in agitated culture and temporary immersion bioreactor are a rich source of flavonoids, including catechin derivatives.

Keywords In vitro · Liquid medium · Japanese knotweed · Phenolic compounds

Communicated by So-Young Park.

✉ Wojciech Makowski
wojciech.makowski@urk.edu.pl

¹ Department of Botany, Physiology and Plant Protection, Faculty of Biotechnology and Horticulture, University of Agriculture in Kraków, 29 Listopada 54, Kraków 31-425, Poland

² Intercollegiate Faculty of Biotechnology UG and MUG, Laboratory of Biologically Active Compounds, University of Gdańsk, Abrahama 58, Gdańsk 80-307, Poland

³ Chair and Department of Pharmaceutical Botany, Collegium Medicum, Jagiellonian University, Medyczna 9, Kraków 30-688, Poland

Abbreviations

PPFD	Photosynthetic photon flux density
SM	Solid medium
AC	Agitated culture
TIB	Temporary immersion bioreactors
GI	Growth index
DW	Dry weight
FW	Fresh weight
DAD-HPLC	High-pressure liquid chromatography with a diode array detector
SD	Standard deviation
SPC	Sum of phenolic compounds
PAL	Phenylalanine ammonia lyase

ROS Reactive oxygen species

Introduction

Phytotherapy is becoming increasingly popular around the world and is a major area of natural medicine. According to reports from the World Health Organization approximately 80% of people in the world use natural methods to complement conventional treatment (Lachowicz and Oszmiański 2019). Moreover, the demand for substances of natural origin that have a wide spectrum of biological activity is still growing (Krychowiak et al. 2014). Therefore, plants that can synthesize potent secondary metabolites are of interest to science and industry.

Reynoutria japonica Houtt. (syn. *Fallopia japonica* (Houtt.) Ronse Decr.; *Polygonum cuspidatum* Sieb. & Zucc.) called Japanese knotweed is a plant belonging to the family Polygonaceae. This herbaceous perennial geophyte is native to eastern Asia (Stachowicz et al. 2021). *R. japonica* is naturally rich in many secondary metabolites belonging to the group of phenolic compounds, like stilbenes, quinones, lignans, flavonoids, coumarins or phenolic acids, and it was used in natural Asian medicine for over 1800 years (Bozin et al. 2017). These metabolites are synthesized in plants as a part of their defense system in response to environmental factors (Labudda et al. 2020a; Muszyńska et al. 2021). On the other hand, because of their properties, they also belong to substances used in medicine (Makowski et al. 2020).

R. japonica is included in Chinese Pharmacopoeia, where it is believed to treat nervous system disorders, bronchitis, high blood pressure, and jaundice (Wang et al. 2022b). It has been proved, that Japanese knotweed has anticancer (Zhang et al. 2019; Kim et al. 2019) and antioxidant activity (Bozin et al. 2017), as well as may be applied in the prevention of the circulatory and digestive systems diseases (Liu et al. 2019), and in respiratory infections (Wang et al. 2022b). It is considered that the rhizome and roots of Japanese knotweed are the richest in phenolic compounds with a broad range of biological activity (Lachowicz and Oszmiański 2019). Therefore, the majority of available research is focused on phytochemistry and biological activity of underground parts of *R. japonica*—rhizome and roots (Bozin et al. 2017). Moreover, until now, the only research available on Japanese knotweed was performed with the use of plants grown in their natural environment (collected from uncontrolled conditions). Thus, the content of biologically active secondary metabolites in plants may be biased because of different collection locations. Therefore, the application of controlled conditions during the cultivation of Japanese knotweed seems to be a reasonable solution for obtaining plant material with a repeatable phenotype.

Japanese knotweed was introduced in 1825 to Europe and after, in the late nineteenth century, to North America as an ornamental plant, as well as a fodder and erosion control plant. Over time, it has become one of the most invasive plants on these continents (Zubek et al. 2022). Today, *R. japonica*, with two other representatives of this botanical genus, *Reynoutria sachalinensis* and *Reynoutria x bohemica*, is included in the top 100 world's most dangerous invasive alien species being a huge threat to native ecosystems (Vidican et al. 2023). This plant can rapidly colonize various environments and predominantly grows over secondary anthropogenic habitats and areas polluted by industry, but less frequently in natural niches, like forest edges or river valleys. The invasion strategy of Japanese knotweed in various habitats is related to its ability to synthesize a large number of biologically active compounds that influence the behavior and/or composition of soil microbial communities, as well as allelopathic compounds synthesis (Stefanowicz et al. 2017, 2021; Zubek et al. 2022; Soln et al. 2022).

As far as Japanese knotweed appears to be a promising source of secondary metabolites with potential application in the pharmaceutical industry its acquisition from natural sites is impossible. *R. japonica* is found in polluted habitats, often contaminated with heavy metals and other toxic elements (Vidican et al. 2023). Lerch et al. (2022) showed the ability of *R. japonica* to accumulate Cd, Cu, Zn, and Ni and to distribute those elements from the roots to the stem and leaves. Rahmonov et al. (2014) reported the presence of Pb and Cr in the tissue of Japanese knotweed collected from various sites. Valorization of plant biomass for industrial purposes harvested from the places of Japanese knotweed natural occurrence threatens its pollution with toxic elements (Lerch et al. 2022).

Considering that the content of secondary metabolites in the tissues of wild plants depends on variable environmental factors, and at the same time wild plants can accumulate substances harmful to health, plant material from natural sites is not suitable for industrial use. Thus, it seems necessary to make its acquisition independent of populations occurring in the natural environment. Derivation of Japanese knotweed tissue cultures will, therefore, allow for cultivation in strictly controlled conditions, where the obtained plants will accumulate valuable secondary compounds at a constant level. Moreover, the introduction of knotweed to in vitro cultivation will allow the future use of biotechnological tools, such as elicitation or genetic transformation, aimed at increasing the content of phenolic compounds in its tissues. Therefore, in this study, for the first time, controlled in vitro conditions were used for the cultivation of *R. japonica* plants.

Plant tissue culture technology gives the possibility to produce valuable and standardized plant material. Plants

grown in in vitro conditions are free from contaminants that occur in the natural environment and have an even phenotype (Grzegorzczak-Karolak et al. 2024). Many authors reported effective plant biomass production by micropropagation (Grzegorzczak-Karolak et al. 2019; Kunakhonnuruk et al. 2019; Makowski et al. 2023). Depending on the plant genus and the aim of cultivation various modifications of traditional agar-solidified medium for tissue cultures may be used. Cultivation of plants in liquid media with rotary shaking (agitated cultures) or in temporary immersion bioreactors may lead to increased biomass and/or secondary metabolite production (Kikowska et al. 2020; Makowski et al. 2023). Such platforms are efficient for scaling up the production of plant-derived compounds. Therefore, developing Japanese knotweed cultivation methods in in vitro conditions can provide pollution-free plants, rich in secondary metabolites of a medicinal nature. We hypothesized, that due to better distribution and availability of nutrients in agitated cultures and in bioreactors, plant biomass accumulation may increase as compared to culture in agar-solidified medium. Also, mechanical stimulation of agitated culture, as well as periodic flooding in bioreactors may induce stress reactions in plants, which in turn results in increased levels of phenolic compound production in *R. japonica* tissue.

The aim of this study was to: (1) introduce, for the first time, *R. japonica* into in vitro culture conditions, (2) evaluate its growth and secondary metabolites production in agar-solidified medium, agitated cultures and in temporary immersion bioreactor Plantform™, compared to plants cultivated in soil (ex vitro conditions). The research will enable the selection of the best conditions for growing knotweed in vitro to obtain secondary metabolites.

Materials and methods

Plant material for tissue culture initiation

The primary explants for the *R. japonica* tissue culture initiation were side buds located on knotweed rhizomes. Buds from the intact plants were collected in March 2021 in Kraków, Poland, occurred on the banks of the Rudawa River (50°03'35.5"N, 19°54'03.7"E). Buds were used as explants to establish and stabilize shoot cultures in in vitro conditions.

Shoot culture initiation

The explants were surface disinfected according to the method described by Makowski et al. (2023), with modifications. Briefly, buds were washed in tap water with Tween 20 (Sigma Aldrich, Germany). Then, explants were treated

with 70% (v/v) ethanol for 30 s. In the next step, buds were surface sterilized with 5.25% calcium hypochlorite for 20 min. Next, sterile explants were rinsed three times in sterile distilled water and placed in 250 mL flasks with 50 mL of ½ MS medium solidified with agar (0.8%) (Murashige and Skoog 1962). The medium had no growth regulators and contained 3% of sucrose. pH of medium was 5.8, adjusted prior autoclaving. Flasks with initial buds were placed in the growing room at 21 ± 2 °C, under white fluorescence light characterized by a photosynthetic photon flux density (PPFD) of $50 \mu\text{mol}\times\text{m}^{-2}\times\text{s}^{-1}$ and a photoperiod of 16 h/8 h light/dark cycle. After 3–4 weeks newly grown shoots of *R. japonica* started to appear on initial buds. Shoots were cut off and subcultured to fresh medium with the same composition as described above. Shoots rooted spontaneously without addition of plant growth regulators. Plants were subcultured every 4–5 weeks and served as an initial material for the further experiments.

In vitro and ex vitro cultivation of *R. japonica*

Plants in each applied system: in solid medium culture, agitated culture, temporary immersion bioreactors and in ex vitro conditions were cultivated at 21 ± 2 °C under white fluorescence light with a PPFD of $100 \mu\text{mol}\times\text{m}^{-2}\times\text{s}^{-1}$ and a photoperiod of 16 h/8 h light/dark cycle.

Cultures in solid medium (SM)

In this experiment two-leaf plants from previously obtained cultures were subcultured to 250 mL Erlenmeyer flasks (5 explants per flask) with 50 mL of ½ MS medium solidified with 0.8% agar, without growth regulators, containing 3% sucrose, with pH 5.8 (adjusted prior to autoclaving). Explants were weighted and measured. After 5 weeks of cultivation plants were collected for further analysis. The experiment consisted of 15 flasks ($n = 15$).

Cultures in liquid medium with rotary shaking– agitated cultures (AC)

For AC of *R. japonica* initiation, two-leaf plant explants placed in 250 mL Erlenmeyer flasks (5 plants per flask) with 50 mL of liquid ½ MS medium, without growth regulators, containing 3% sucrose, with pH 5.8 (adjusted prior to autoclaving). Explants were weighted and measured. Cultures were placed on a rotary shaker (Phoenix RS-LS 20, DanLab, Poland) with continuous mode at 140 rpm. After 5 weeks plants were collected for further analysis. The experiment consisted of 15 flasks ($n = 15$).

Cultures in temporary immersion bioreactors (TIB)

TIB cultures were grown in a Plantform™ temporary immersion system (Plant Form, Sweden). 15 explants from previously obtained starter cultures were transferred to each bioreactor. One bioreactor contained 500 mL of liquid ½ MS medium, without growth regulators, containing 3% sucrose, with pH 5.8 (adjusted prior to autoclaving). Explants were weighted and measured. The immersion and aeration period in the bioreactors was programmed in the following cycle: 30 min of immersion, 20 min of gravitational fall of the medium, and 10 min of aeration. After 5 weeks of cultivation plants were harvested for further analysis. The experiment consisted of 5 bioreactors ($n=5$).

Plants cultivated in ex vitro conditions (ex vitro)

For this experiment, 4 weeks-old, rooted and healthy plants obtained from in vitro cultures from solid medium were transferred to the pots with peat and perlite (3:1) for the acclimatization to ex vitro conditions (one plant in one pot). Plants were weighted and measured before transfer. Pots with *R. japonica* were placed in growing chamber with initial humidity 90%. The humidity was decreased 10% each week to final humidity 50%. After 4 weeks plants were fully acclimatized to ex vitro conditions. The experiment consisted of 20 pots with plants ($n=20$).

Preparation of plant material for biochemical analysis

After the experiment with different cultivation systems plants were harvested, and fresh biomass of shoots and roots (separately) was weighted separately from each flask/bioreactor/pot. Also, photo documentation and the observation of plants' health status and plants' morphology was performed. Plants were packed in paper envelopes and freeze-dried for 24 h. Dry shoots and roots from each experimental conditions were weighed and homogenized to the powder. Plant's material was stored in plastic falcons in $-20\text{ }^{\circ}\text{C}$.

Shoot length and multiplication

To estimate shoot length shoots of plants from each experimental conditions were measured with ruler. Also, the number of shoots grown on one explant in in vitro conditions and one acclimatized plant to ex vitro conditions were calculated.

Growth index (GI) and dry weight (DW) estimation

Estimation of GI was performed according to Tokarz et al. (2018). The GI was calculated according to the following formula: $\text{GI} [\%] = (\text{FW}_2 - \text{FW}_1)/\text{FW}_2 \times 100$, where FW_1 is the fresh weight of the plants at the beginning of the experiment and FW_2 is the final fresh weight. Determination of DW content in shoots and roots of *R. japonica* plants from each experimental condition was performed according to Tokarz et al. (2020). The DW content in the plant tissue was calculated according to the following formula: $\text{DW} [\%] = \text{DW}_2 \times 100/\text{FW}_2$, where DW_2 is the dry weight after freeze-drying.

Determination of phenolic compounds presence in *R. japonica* tissue

Extraction procedure

Plant extracts were prepared according to the procedure described by Makowski et al. (2021b), with modifications. For the preparation of methanolic extracts, 100 mg of homogenized lyophilized tissue powder were weighed separately for shoots and roots, from each tested cultivation system. The tissue samples were subjected twice to extraction with 6 mL of 80% methanol (HPLC-grade) by sonication in an ultrasonic bath (POLSONIC 2, Poland) for 30 min, $21 \pm 2\text{ }^{\circ}\text{C}$. Sample for each research object (shoots/roots from each applied cultivation systems) was prepared in 5 biological repetitions ($n=5$). The obtained extracts were centrifuged (10000 RPM), and then filtered through sterilizing syringe filters ($0.22\text{ }\mu\text{m}$, Millex® GP, Millipore) prior to HPLC analysis to the 1,5 mL vials.

Phytochemical analysis

To estimate the accumulation of phenolic compounds in plant tissue high-pressure liquid chromatography with a diode array detector (DAD-HPLC) was used. The quantitative analyses of selected secondary metabolites in the extracts were conducted with a validated method, using an apparatus of Merck-Hitachi (LaChrom Elite) with a DAD L-2455 detector and on a Purospher RP-18 ($250 \times 4\text{ mm}$; $5\text{ }\mu\text{m}$, Merck, Germany) column according to former established and validated method according to Sułkowska-Ziaja et al. 2017 and Szopa et al. 2020. The flow rate was $1\text{ mL} \times \text{min}^{-1}$, the temperature was set to $25\text{ }^{\circ}\text{C}$, and the injection volume was $10\text{ }\mu\text{L}$. The detection wavelength was set to 254 nm. The mobile phase consisted of A—methanol, 0.5% acetic acid in ratio 1:4, and B—methanol (v/v). The gradient program was as follows: 0–20 min, 0% B; 20–35 min, 0–20% B; 35–45 min, 20–30% B; 45–55 min,

30–40% B; 55–60 min, 40–50% B; 60–65 min, 50–75% B; and 65–70 min, 75–100% B, with a hold time of 15 min. Identification was performed by comparison to the retention times and UV spectra of phenolic compounds: caftaric acid ($y=1E+08x-13409$; $R^2=0.9903$; $RT=5.52$ min), protocatechic acid ($y=1E+078x+159880$; $R^2=0.9991$; $RT=6.98$ min), chlorogenic acid ($y=4E+07x-45223$; $R^2=0.9997$; $RT=11.67$ min), caffeic acid ($y=6E+07x-169512$; $R^2=0.9996$; $RT=16.96$ min), ferulic acid ($y=6E+07x+30835$; $R^2=0.9983$; $RT=36.91$ min), epigallocatechin ($y=3E+06x-30719$; $R^2=0.9999$; $RT=7.53$ min), catechin ($y=6E+06x+54243$; $R^2=0.9991$; $RT=8.51$ min), epicatechin gallate ($y=3E+07x-148672$; $R^2=1.0$; $RT=15.43$ min), epicatechin ($y=6E+06x-48481$; $R^2=0.9994$; $RT=19.03$ min), isoquercetin ($y=9E+07x+1113000$; $R^2=0.9999$; $RT=45.42$ min), trifolin ($y=8E+07x+583711$; $R^2=0.9994$; $RT=49.25$ min), avicularin ($y=1E+08x-1E+09$; $R^2=0.9995$; $RT=49.40$ min), quercitrin ($y=7E+07x-813339$; $R^2=0.9979$; $RT=49.90$ min), and apigenin ($y=6E+07x-1E+06$; $R^2=0.9999$; $RT=64.65$ min) (acquired from Sigma-Aldrich Co., Germany). The above-mentioned compounds were presented in the chromatogram of the extract from *R. japonica* shoots cultivated in vitro (Fig. 1). The quantification was performed based on the calibration curves method. Samples were prepared and analyzed in five

replications. The results were expressed in $\text{mg} \times 100 \text{ g}^{-1}$ DW.

Sum of phenolic compounds (SPC) calculation

SPC was calculated by summing up the content of all tested phenolic derivatives: protocatechic acid, chlorogenic acid, caffeic acid, ferulic acid, caftaric acid, catechin, epigallocatechin, epicatechin gallate, epicatechin, isoquercetin, quercetin, avicularin, trifolin and apigenin. Results were expressed as $\text{mg} \times 100 \text{ g}^{-1}$ DW.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to determine significant differences between means during statistic elaboration of growth index of plants. Two-way analysis of variance (ANOVA) was performed to determine significant differences between means during the statistical elaboration of the rest presented parameters: dry weight, the sum of phenolic compounds, accumulation of phenolic derivatives, as well as the sum of phenolic acids, the sum of catechin derivatives and the sum of flavonoids (Tukey post-hoc test at $p < 0.05$). STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA) was used to conduct statistical analyses. The principal component analysis (PCA) was performed

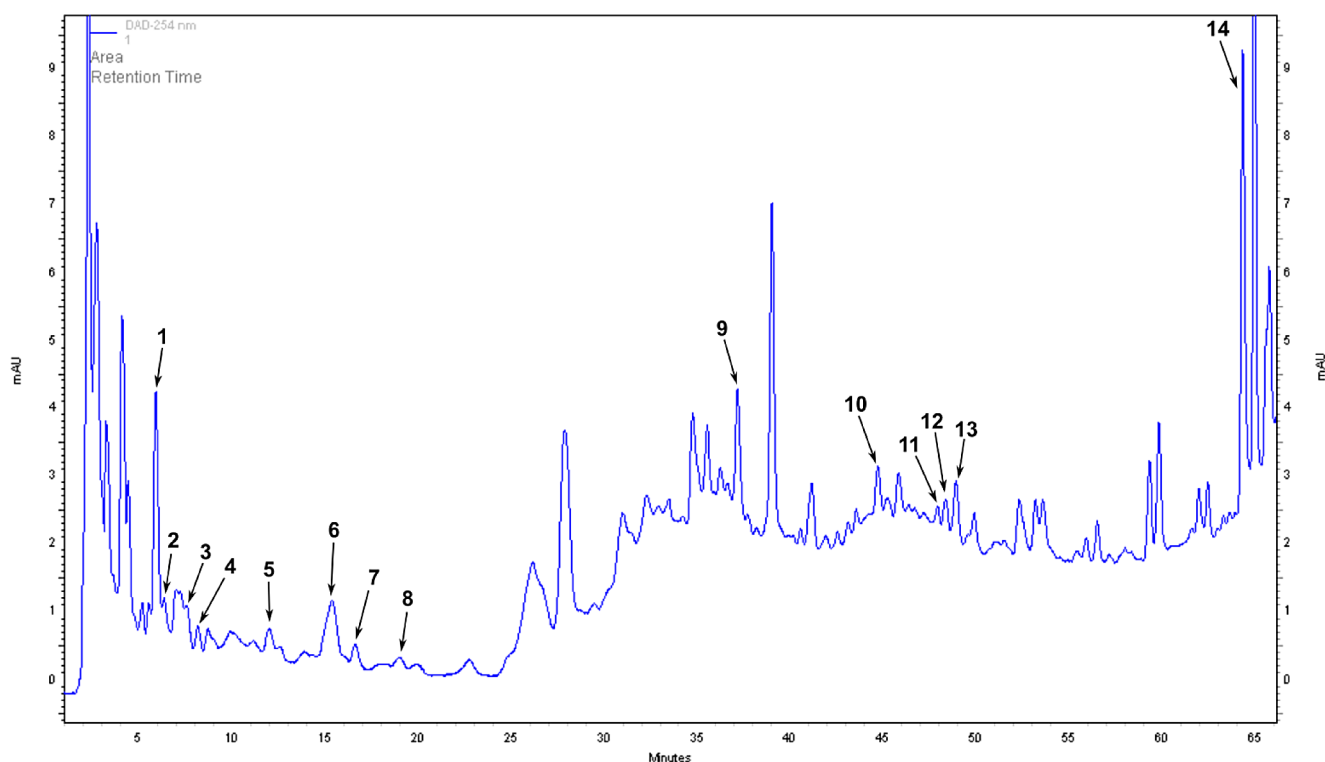


Fig. 1 Example DAD-HPLC ($\lambda=254$ nm) chromatogram of extract from *Reynoutria japonica* shoots cultivated in vitro conditions. Identified compounds: 1 - caftaric acid, 2 - protocatechic acid, 3 - epi-

gallocatechin, 4 - catechin, 5 - chlorogenic acid, 6 - epicatechin gallate, 7 - caffeic acid, 8 - epicatechin, 9 - ferulic acid, 10 - isoquercetin, 11 - trifolin, 12 - avicularin, 13 - quercitrin, 14 - apigenin

Fig. 2 *Reynoutria japonica* plants after 5 weeks of cultivation in solid (left) and agitated (right) culture (A, B). Scale bar = 1 cm



Fig. 3 *Reynoutria japonica* plants after 5 weeks of cultivation in temporary immersion bioreactor Plantform™ (A, B). Scale bar = 1 cm

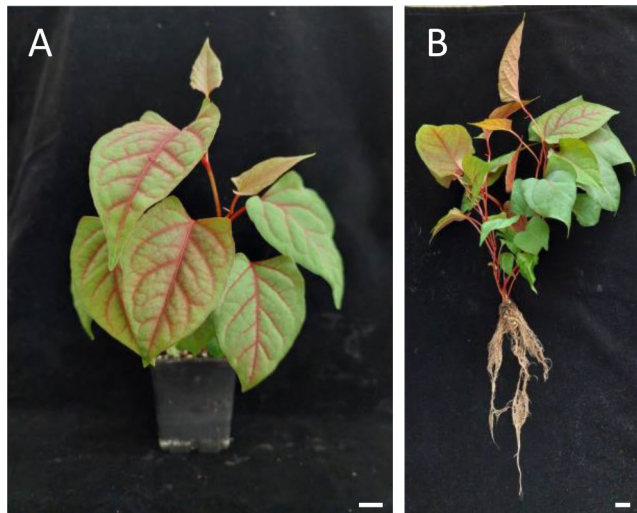
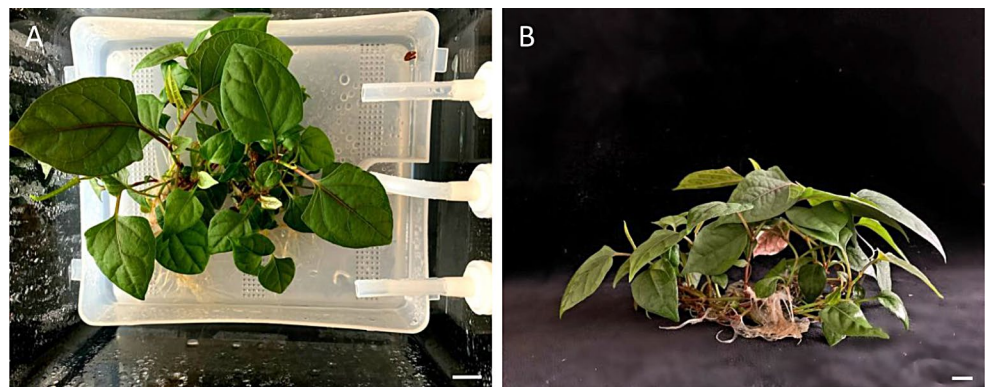


Fig. 4 *Reynoutria japonica* plants after 5 weeks of cultivation in soil (ex vitro) (A, B). Scale bar = 1 cm

with the use of Past software (Hammer and Harper 2001) to show global differences and/or similarities between culture types and between plant parts (shoots and roots) based on the sum of phenolic acids, catechin derivatives, and flavonoids. Before PCA analysis, the data were normalized by calculating their $\log_{10}$ values.

Results

Biometric parameters and plant morphology

An important parameter in the evaluation of plant viability cultivated in liquid media is the lack of abnormal morphology and hyperhydricity (Kikowska et al. 2020). In this study, after 5 weeks of *R. japonica* cultivation in all tested conditions (SM, AC, TIB, ex vitro), the plants showed no signs of stress or hyperhydricity (Figs. 2, 3 and 4). Furthermore, plants harvested from ex vitro conditions (Fig. 4) and temporary immersion bioreactors (Fig. 3) had bigger leaf area and shape than those from SM and AC (Fig. 2).

Moreover, in TIB and ex vitro conditions length of shoots was larger than in SM or AC (Table 1). TIB conditions led to the highest shoot multiplication, having the biggest number of shoots per one explant (Table 1).

The obtained results demonstrated that the growth index of plants cultivated in AC, TIB, and ex vitro conditions increased significantly 1.5-, 1.8- and 2.7-fold as compared to SM, respectively (Fig. 5).

In presented research DW was measured separately for shoots and roots of *R. japonica* from each experimental condition. The obtained results showed that shoots from AC,

Table 1 Shoot length [cm] and number of shoots per explant of *Reynoutria japonica* in: solid culture, agitated culture, temporary immersion bioreactor Plantform™ and soil (ex vitro). Different letters indicate statistical significance of means acc. Two-way ANOVA, post hoc Tuckey test at $p < 0.05$, SD—standard deviation. Statistical analysis performed for each of the tested parameters separately

Type of culture	Shoot length cm [$\pm$ SD]	Number of shoots per explant [$\pm$ SD]
Solid medium culture	7.3 $\pm$ 1.9 ^a	3.5 $\pm$ 1.4 ^{ab}
Agitated culture	9.2 $\pm$ 1.5 ^a	5.0 $\pm$ 1.2 ^b
Temporary immersion bioreactor	15.8 $\pm$ 1.5 ^b	13.4 $\pm$ 1.5 ^c
Ex vitro	18.7 $\pm$ 3.6 ^b	1.9 $\pm$ 0.9 ^a

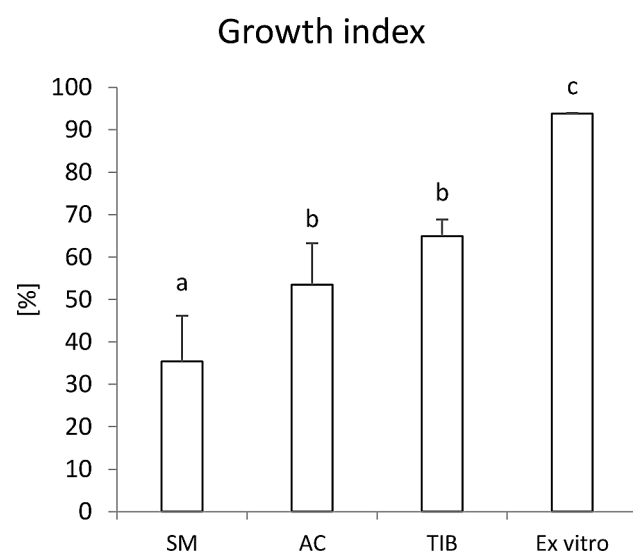
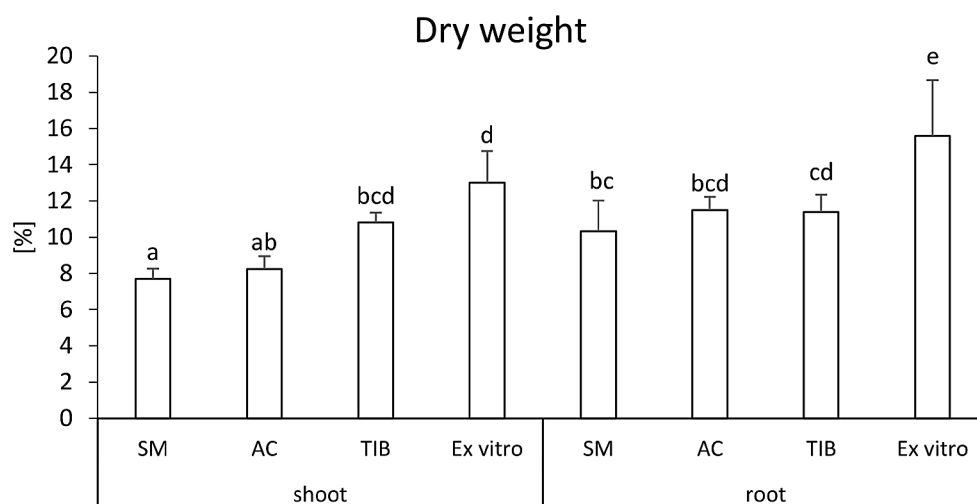


Fig. 5 Growth index [%] in *Reynoutria japonica* plants after 5 weeks of cultivation in: solid medium (SM), agitated culture (AC), temporary immersion bioreactor Plantform™ (TIB) and soil (Ex vitro). Upper case letters indicate statistical significance of means acc. one-way ANOVA, post hoc Tuckey test at $p < 0.05$; the bar represents the standard deviation

Fig. 6 Dry weight [%] content in *Reynoutria japonica* shoot and root after 5 weeks of cultivation in: solid medium (SM), agitated culture (AC), temporary immersion bioreactor Plantform™ (TIB) and soil (Ex vitro). Upper case letters indicate statistical significance of means acc. one-way ANOVA, post hoc Tuckey test at $p < 0.05$; the bar represents the standard deviation



as well as shoots and roots from ex vitro conditions had increased dry weight content by 1.4; 1.7; 1.5-fold, respectively, as compared to SM (Fig. 6).

Sum of phenolic compounds

In the current study the concentration of total phenolic compounds content in in vitro and ex vitro propagated *R. japonica* plants was evaluated and expressed as a sum of phenolic compounds (SPC) estimated with the use of DAD-HPLC method (Fig. 7). The highest SPC was obtained for shoots from AC (3188.1 mg $\times$ 100 g⁻¹ DW). Shoots from TIB had significantly lower SPC than shoots from AC, with a value of 2638.0 mg $\times$ 100 g⁻¹ DW. Interestingly, a similar amount of SPC was found in shoots of plants cultivated in SM and in ex vitro conditions as well as roots obtained in AC culture (2312.4, 2149.3, and 2123.4 mg $\times$ 100 g⁻¹ DW, respectively). The lowest SPC was reported for roots of *R. japonica* cultivated in ex vitro conditions (785.70 mg $\times$ 100 g⁻¹ DW). The level of phenolic compounds in the roots grown on SM (1830.5 mg $\times$ 100 g⁻¹ DW) was higher by 16% than in the roots of plants grown in TIB (1569.3 mg $\times$ 100 g⁻¹ DW) (Fig. 7).

The variable presence of phenolic acids, catechins and other flavonoids in *R. japonica* in vitro cultures

Using the DAD-HPLC method qualitative and quantitative analysis of extracts from Japanese knotweed plants was performed. In the analyzed samples from in vitro conditions, the same qualitative composition of phenolic derivatives was found, as in the plants cultivated in soil. The presence of 5 phenolic acids: ferulic, caftaric, chlorogenic, protocatechic and caffeic acid was confirmed (Table 2). Moreover, the presence of 9 flavonoids, including 4 catechins: catechin, epigallocatechin, epicatechin gallate and epicatechin

Fig. 7 Sum of phenolic compounds [$\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$] in *Reynoutria japonica* shoot and root after 5 weeks of cultivation in: solid medium (SM), agitated culture (AC), temporary immersion bioreactor Plantform™ (TIB) and soil (Ex vitro). Upper case letters indicate statistical significance of means acc. one-way ANOVA, post hoc Tukey test at $p < 0.05$; the bar represents the standard deviation; DW—dry weight

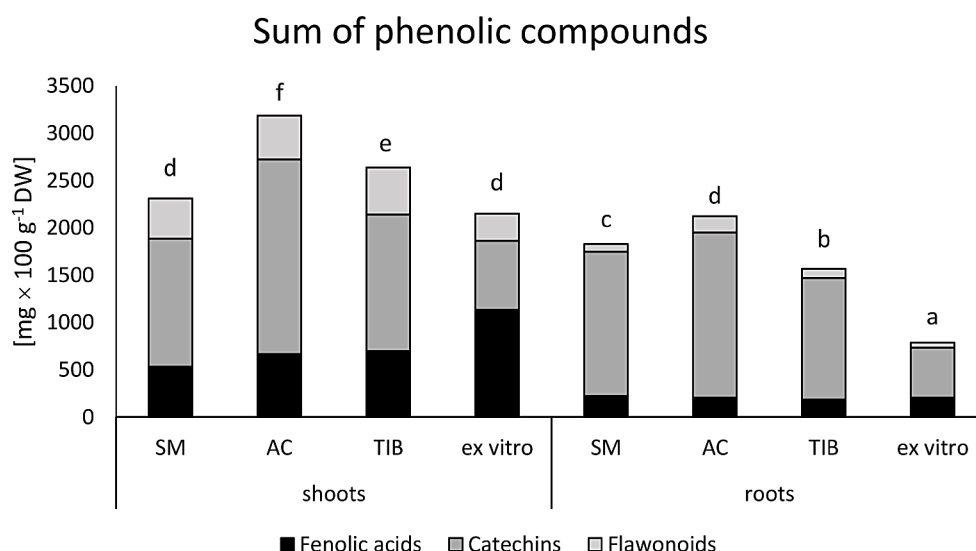


Table 2 Accumulation of phenolic acids [$\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$] in *Reynoutria japonica* shoot and root cultivated in: solid culture (SM), agitated culture (AC), temporary immersion bioreactor Plantform™ (TIB) and soil (ex vitro). Different letters indicate statistical significance of means acc. Two-way ANOVA, post hoc Tukey test at $p < 0.05$, SD—standard deviation, DW—dry weight. Statistical analysis performed for each of the tested parameters (the content of a given compound and the sum) separately

Phenolic acids $\text{mg} \times 100 \text{ g}^{-1} \text{ DW} [\pm \text{SD}]$	Shoot				Root			
	SM	AC	TIB	Ex vitro	SM	AC	TIB	Ex vitro
Ferulic acid	40.9 $\pm$ 0.9 ^b	66.8 $\pm$ 6.1 ^c	70.0 $\pm$ 3.5 ^c	38.7 $\pm$ 3.8 ^b	8.6 $\pm$ 0.4 ^a	40.5 $\pm$ 5.3 ^b	31.7 $\pm$ 3.6 ^b	146.4 $\pm$ 16.3 ^d
Caftaric acid	435.7 $\pm$ 21.2 ^c	531.9 $\pm$ 5.9 ^d	579.9 $\pm$ 14.4 ^e	981.2 $\pm$ 26.6 ^f	98.6 $\pm$ 4.0 ^b	99.1 $\pm$ 3.1 ^b	71.3 $\pm$ 2.8 ^b	12.1 $\pm$ 1.2 ^a
Chlorogenic acid	47.9 $\pm$ 2.5 ^{cd}	59.2 $\pm$ 6.1 ^e	36.4 $\pm$ 0.9 ^b	93.1 $\pm$ 4.6 ^f	92.7 $\pm$ 4.5 ^f	42.5 $\pm$ 3.8 ^{bc}	53.3 $\pm$ 2.9 ^{de}	4.8 $\pm$ 0.4 ^a
Protocatechic acid	3.5 $\pm$ 1.0 ^a	2.8 $\pm$ 0.8 ^a	4.7 $\pm$ 1.6 ^a	5.4 $\pm$ 0.6 ^{ab}	14.3 $\pm$ 1.1 ^d	11.4 $\pm$ 2.6 ^{cd}	9.0 $\pm$ 1.3 ^{bc}	6.2 $\pm$ 0.9 ^{ab}
Caffeic acid	3.5 $\pm$ 0.7 ^a	7.6 $\pm$ 2.0 ^{ab}	4.6 $\pm$ 0.3 ^a	17.5 $\pm$ 1.8 ^{cd}	7.1 $\pm$ 0.8 ^{ab}	13.2 $\pm$ 0.7 ^{bc}	20.8 $\pm$ 5.3 ^d	35.4 $\pm$ 3.9 ^e
Sum of phenolic acids	531.5 $\pm$ 21.0 ^b	668.2 $\pm$ 6.1 ^c	695.7 $\pm$ 15.4 ^c	1135.8 $\pm$ 29.1 ^d	221.3 $\pm$ 8.7 ^a	206.6 $\pm$ 10.2 ^a	186.0 $\pm$ 3.0 ^a	204.9 $\pm$ 17.7 ^a

Table 3 Accumulation of catechin derivatives [$\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$] in *Reynoutria japonica* shoot and root cultivated in: solid culture (SM), agitated culture (AC), temporary immersion bioreactor Plantform™ (TIB) and soil (ex vitro). Different letters indicate statistical significance of means acc. Two-way ANOVA, post hoc Tukey test at $p < 0.05$, SD—standard deviation, DW—dry weight. Statistical analysis performed for each of the tested parameters (the content of a given compound and the sum) separately

Catechin derivatives $\text{mg} \times 100 \text{ g}^{-1} \text{ DW} [\pm \text{SD}]$	Shoot				Root			
	SM	AC	TIB	Ex vitro	SM	AC	TIB	Ex vitro
Catechin	385.2 $\pm$ 30.6 ^b	647.6 $\pm$ 26.5 ^{cd}	550.9 $\pm$ 63.6 ^c	350.5 $\pm$ 17.0 ^b	607.1 $\pm$ 42.1 ^{cd}	670.0 $\pm$ 24.4 ^d	438.1 $\pm$ 37.8 ^b	204.5 $\pm$ 4.2 ^a
Epigallocatechin	610.9 $\pm$ 19.8 ^e	729.5 $\pm$ 23.6 ^f	419.7 $\pm$ 38.1 ^d	203.1 $\pm$ 10.7 ^b	387.9 $\pm$ 9.5 ^d	375.1 $\pm$ 10.1 ^d	267.2 $\pm$ 24.1 ^c	96.9 $\pm$ 6.1 ^a
Epicatechin gallate	76.5 $\pm$ 4.5 ^b	153.9 $\pm$ 12.6 ^c	89.8 $\pm$ 3.1 ^b	50.7 $\pm$ 3.4 ^{ab}	164.1 $\pm$ 29.4 ^c	216.2 $\pm$ 18.4 ^d	224.8 $\pm$ 28.8 ^d	16.8 $\pm$ 1.4 ^a
Epicatechin	283.4 $\pm$ 22.2 ^{bc}	523.2 $\pm$ 37.3 ^c	384.7 $\pm$ 27.7 ^d	121.4 $\pm$ 5.6 ^a	366.6 $\pm$ 22.2 ^d	483.3 $\pm$ 32.5 ^e	353.3 $\pm$ 39.2 ^{cd}	212.0 $\pm$ 6.3 ^b
Sum of catechin derivatives	1356.0 $\pm$ 50.3 ^{cd}	2054.3 $\pm$ 79.2 ^f	1445.1 $\pm$ 68.3 ^{cd}	725.8 $\pm$ 31.3 ^b	1525.6 $\pm$ 92.9 ^d	1744.7 $\pm$ 61.0 ^e	1283.3 $\pm$ 95.2 ^c	530.1 $\pm$ 16.8 ^a

(Table 3), as well as other flavonoids: isoquercetin, quercetin, avicularin, trifolin and apigenin was determined (Table 4).

Shoots produce more phenolic acids than roots, while the highest sum of phenolic acids was obtained in shoots from ex vitro plants (1135.8 $\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$) (Table 2). Among all compounds from this group the dominant in shoots and roots (excluding roots of plants in soil) was caftaric acid. AC

and TIB increased accumulation of ferulic acid in shoots, with the concentration 66.8 and 70.0 $\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$, respectively. Interestingly, the highest level of ferulic acid was obtained for roots from ex vitro conditions (146.4 $\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$). Production of chlorogenic acid reached the highest level in shoots from ex vitro conditions (93.1 $\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$) and roots from SM (92.7 $\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$). Protocatechic and caffeic acids were accumulated

Table 4 Accumulation of flavonoids [$\text{mg} \times 100 \text{ g}^{-1} \text{ DW}$] in shoot and root of *Reynoutria japonica* cultivated in: solid culture (SM), agitated culture (AC), temporary immersion bioreactor Plantform™ (TIB) and soil (ex vitro). Different letters indicate statistical significance of means acc. Two-way ANOVA, post hoc Tuckey test at $p < 0.05$, SD—standard deviation, DW—dry weight. Statistical analysis performed for each of the tested parameters (the content of a given compound and the sum) separately

Flavonoids $\text{mg} \times 100 \text{ g}^{-1} \text{ DW} [\pm \text{SD}]$	Shoot				Root			
	SM	AC	TIB	Ex vitro	SM	AC	TIB	Ex vitro
Isoquercetin	43.8 ± 9.8^f	36.9 ± 4.8^{def}	28.2 ± 2.6^{cde}	15.0 ± 3.1^{ab}	23.7 ± 1.4^{cd}	40.9 ± 6.1^{ef}	34.2 ± 3.0^{def}	4.1 ± 0.3^a
Quercetin	192.4 ± 13.7^b	229.7 ± 23.6^b	269.2 ± 25.7^c	212.9 ± 9.6^b	6.6 ± 2.2^a	29.5 ± 4.5^a	12.4 ± 2.2^a	16.7 ± 1.5^a
Avicularin	10.9 ± 1.0^c	16.5 ± 2.2^d	15.2 ± 0.5^d	9.8 ± 1.4^c	0.3 ± 0.0^a	2.1 ± 0.2^{ab}	1.9 ± 0.5^{ab}	3.6 ± 0.6^b
Trifolin	4.4 ± 0.8^{bc}	17.1 ± 1.6^c	8.5 ± 1.4^d	0.6 ± 0.2^a	0.6 ± 0.1^a	2.9 ± 0.4^{ab}	0.9 ± 0.2^a	6.0 ± 0.4^{cd}
Apigenin	173.5 ± 13.6^d	175.4 ± 10.6^d	176.0 ± 17.4^d	49.4 ± 3.5^b	52.3 ± 6.7^b	96.6 ± 1.8^c	50.7 ± 5.2^b	20.4 ± 2.2^a
Sum of flavonoids	425.0 ± 38.1^d	465.7 ± 29.3^{de}	497.2 ± 44.0^e	287.7 ± 16.2^c	83.5 ± 6.9^a	172.0 ± 8.9^b	100.0 ± 4.6^a	50.7 ± 4.3^a

more efficiently in roots, than in shoots. SM was the most effective platform for protocatechic acid accumulation in roots, with concentration of $14.3 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$, while $35.4 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$ of caffeic acid was found in roots from TIB (Table 2).

The highest sum of catechin derivatives was obtained in shoots from AC ($2054.3 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$), and then for the roots from the same type of culture ($1744.7 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$). Moreover, AC plants were characterized by high content of catechin, epigallocatechin and epicatechin in shoots: 647.6 , 729.5 and $523.2 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$, respectively. In roots, $670.0 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$ of catechin were accumulated in AC, while 387.9 and $375.1 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$ of epigallocatechin from SM and AC, respectively. Epicatechin gallate was found at the highest level in roots for AC ($216.2 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$) and TIB ($224.8 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$) and epicatechin in roots from AC ($483.3 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$) (Table 3).

In vitro conditions: SM, AC and TIB led to an increased sum of flavonoids in shoots (425.0 , 465.7 and $497.2 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$, respectively), compared to ex vitro plants. The highest concentration of flavonoids in roots was obtained from AC and TIB (172.0 and $100.0 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$). SM and AC conditions promoted synthesis of isoquercetin in shoots, while AC and TIB in roots. The highest quercetin level ($269.2 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$) was found in shoots from TIB. The highest avicularin yield was observed in shoots from AC and TIB (16.5 and $15.2 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$), trifolin in shoots from AC ($17.1 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$), and apigenin from SM, AC and TIB (173.5 , 175.4 , $176.0 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$) (Table 4).

Variable influence of cultivation systems on *R. japonica* secondary metabolites production

To verify the general patterns of phenolic compounds distribution in *R. japonica* roots and shoots cultivated in different conditions (SM, AC, TIB, ex vitro) a principal component analysis (PCA) was performed (Fig. 8). By combining the data of sums of phenolic acids, sums of catechin derivatives

and sums of other flavonoids (8 objects $\times$ 3 variables), as much as 80.3% (PC1) and 18.14% (PC2) of the data variability among plant parts (shoots vs. roots) and growing conditions could be explained. Moreover, the variances of the variables describing the content of other flavonoids and phenolic acids were mainly explored by PC1, while the variabilities of catechin derivatives content was mainly explained by the PC2. The analysis clearly discriminates shoots and roots of *R. japonica*. Moreover, the content of phenolic compounds is more similar between different in vitro cultivation methods (SM, AC, TIB) than in soil (ex vitro) (Fig. 8). Interestingly, the roots' phenolic compounds content from SM and TIB is almost identical, contrary to shoots from the same conditions. This may suggest that roots were exposed to similar stress conditions in SM and TIB, while for shoots it was different. It seems that the content of phenolic compounds was affected the most by AC growing conditions, which may be considered the most stressful for the plant (constant shaking, oxygen limitation). Indeed, in this condition, the production of phenolic compounds was the largest both among tested shoots and roots (Fig. 7). Since the growth index and dry weight of the plants from AC conditions were like TIB conditions (Figs. 5 and 6), it can be considered that growing *R. japonica* plants in liquid culture with rotary shaking is the most promising when looking at the number of secondary metabolites it produces.

Discussion

Invasive plant species are one of the most important problems in environment protection and preservation of the natural habitats. On the other hand, many of invasive plant species have unique adaptational strategies, often based on the ability to synthesize biologically active secondary metabolites in their tissue (Stefanowicz et al. 2021). Therefore, they can be a source of valuable compounds with medical potential. This is why plant biotechnology looks up for novel strategies to propagate invasive species in separation from the natural environment, in stable, controlled

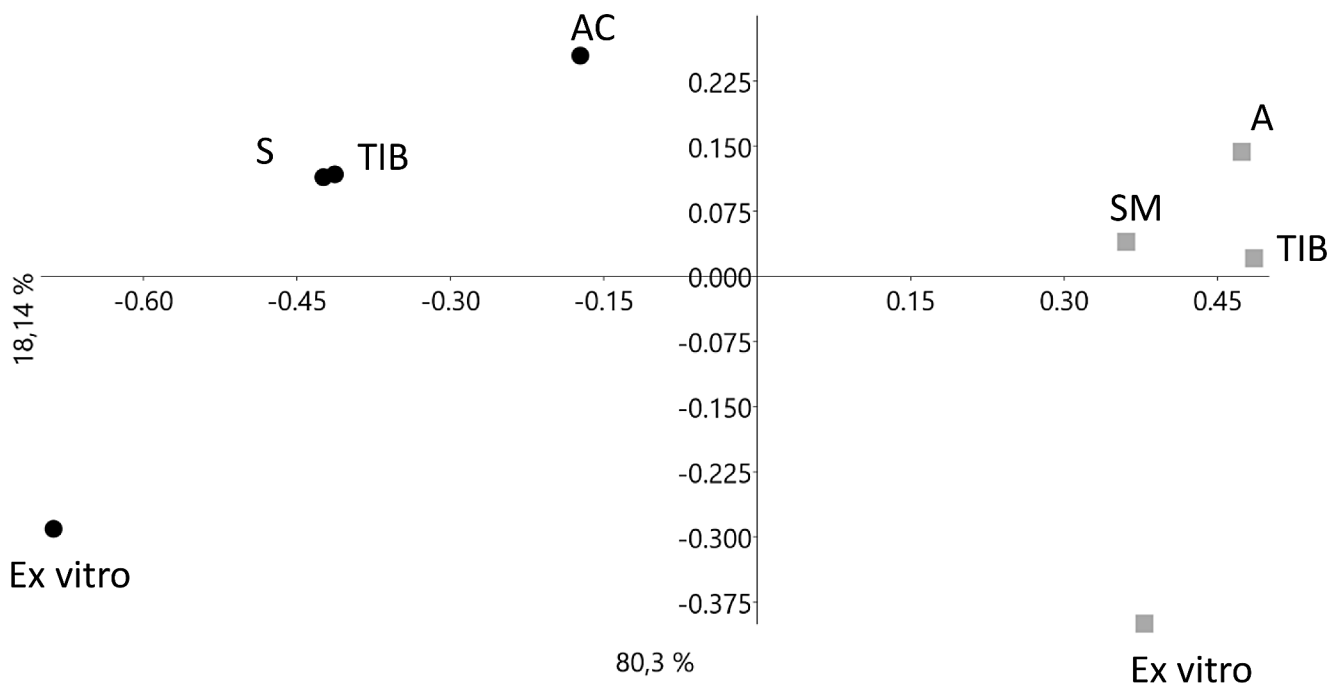


Fig. 8 Comparison of plant cultivation method effect on the content of phenolic acids, catechin derivatives and other flavonoids in roots (black dots) and shoots (grey squares) as measured with principal com-

ponent analysis. Solid medium (SM), agitated culture (AC), temporary immersion bioreactor Plantform™ (TIB) and soil (Ex vitro)

conditions to produce plant biomass rich in plant-derived chemicals. To the best of our knowledge this is the first report about application of micropropagation in *R. japonica* plants cultivation. The present work focuses on biomass accumulation and synthesis of secondary metabolites from the group of phenolic acids, catechins, and other flavonoids in Japanese knotweed tissue harvested from different cultivation modes, including cultures in solid medium (SM), agitated cultures (AC), temporary immersion bioreactor (TIB) and plants cultivated in soil (ex vitro conditions). Creation of *R. japonica* tissue culture is also the first necessary step to enable further research that will aim to increase the synthesis of the secondary metabolites in its tissues with the use of biotechnological tools.

Application of liquid medium and shaking technology, as well as bioreactors as the modifications of traditional agar-solidified, stationary plant cultures may be a chance for increasing the yield in medical plant cultivation (Makowski et al. 2020; De Carlo et al. 2021; Hwang et al. 2022). Many authors reported possible changes in growth effectivity and dry weight accumulation in plants depending on the method of cultivation in in vitro conditions (Moreira et al. 2013; Malik et al. 2016; Kunakhonnuruk et al. 2019; Szopa et al. 2019). Tissue cultures in liquid medium with rotary shaking were reported as an effective mode of cultivation for plant biomass production for *Dionaea muscipula*, *Hypericum perforatum* and *Knautia sarajevensis* (Makowski et al. 2020; Kwiecień et al. 2015; Karalija et al. 2017).

Nevertheless, some studies indicate liquid systems as the unfavorable conditions for plant tissues multiplication due to limited oxygen exchange and/or hydromechanical stress (Savio et al. 2012; Grzegorzczuk-Karolak et al. 2015). *Pontechium maculatum* plants grown in liquid medium with rotary shaking presented a lower growth index than plants grown on agar-solidified medium (Makowski et al. 2023). Furthermore, *Scutellaria alpina* cultivated in stationary and agitated liquid medium did not derive shoots in the research of Grzegorzczuk-Karolak et al. (2017). Effectiveness of plant propagation as an agitated culture relates to the plant's sensibility to constant immersion and possible oxygen limitation (Savio et al. 2012), as well as mechanical stimulation during shaking (Makowski et al. 2020). *R. japonica* naturally occurs in wet habitats, like river valleys or forest edges, which may explain its good adaptation to liquid culture conditions (Rahmonov et al. 2014). Both methods of cultivation connected with liquid medium (AC and TIB) stimulated biomass growth and shoot multiplication compared to SM. Moreover, the increased growth index of plants from AC compared to SM may relate to better availability of nutrients in liquid medium. What is more, in TIB, medium flow during the flooding and aeration phase may contribute to a better distribution of medium ingredients thus making them more available to plants (Kunakhonnuruk et al. 2019). Similarly, increased growth of *Schizandra chinensis* was obtained in Plantform™ bioreactors by Szopa et al. (2019). The best proliferation and fresh weight yield

for sundew were observed for its growth in the bioreactor system as reported by Kunakhonnuruk et al. (2019).

In our previous study conducted on *P. maculatum*, plants obtained from TIB, and ex vitro conditions had different morphology (bigger leaves and callus tissue) than plants cultivated in SM and AC (Makowski et al. 2023). Maliński et al. (2019) also observed a bigger leaf and shape of *Lychnis flos-cuculi* acclimatized to ex vitro conditions. In the presented study, *R. japonica* plants showed a bigger leaf shape in TIB compared to SM. It may be related to a bigger volume of the container, larger medium volume per one bioreactor (0.5 L) and ethylene elimination during the aeration phase (Bello-Bello et al. 2010). In turn, the highest growth of plants in soil (acclimatized to ex vitro conditions) is probably related to the effective photosynthesis process (Soni et al. 2021).

One of the important parameters of plant growth and development is the dry weight (DW) accumulation (Isah et al. 2018). This study showed increased DW accumulation for shoots in AC and whole plants (shoot and root) from ex vitro conditions. Interestingly, a study on *D. muscipula* plants cultivated in liquid medium with rotary shaking did not show differences in DW accumulation when compared to plants grown in SM (Makowski et al. 2020). Moreover, in another study, only shoots of *P. maculatum* plants cultivated ex vitro had increased DW content as compared to plants cultivated in SM, AC, and TIB (Makowski et al. 2023). Similarly, Kunakhonnuruk et al. (2019) reported an increase in DW accumulation in *Drosera communis* plants grown in a temporary immersion system compared to a semi-solid medium and permanent-immersion system. The higher DW of Japanese knotweed shoots obtained in AC is probably related to the acclimation strategy to constant mechanical stimulus (during shaking). However, increased DW of ex vitro plants may be related to cell wall rebuilding during the acclimation process and increased photosynthesis activity (Salgado Pirata et al. 2022).

For the first time, the content of phenolic compounds in shoots and roots of Japanese knotweed from in vitro conditions was evaluated and compared to plants cultivated in soil. According to Wang et al. (2022b) rhizomes and roots of *R. japonica* were reported in Chinese Pharmacopoeia as a potent source of secondary metabolites with a broad range of biological activity, used in traditional Chinese medicine. Interestingly, our research showed that shoots of *R. japonica* plants from in vitro conditions contain between 25 and 68% more phenolic compounds than roots, depending on the growing conditions (Fig. 7). Moreover, the type of cultivation method significantly affected the content of phenolic compounds in plant organs. The sum of phenolic compounds was highest in Japanese knotweed from AC. *P. maculatum* plants grown in liquid medium with rotary

shaking presented an increased total phenolic content in shoots when compared to SM, while the highest value of total phenolic accumulation was obtained in shoots from TIB, and ex vitro cultivated plants (Makowski et al. 2023). Similarly to *R. japonica* cultures, the lowest accumulation of total phenols was observed in *P. maculatum* roots in ex vitro plants. On the other hand, *Eryngium alpinum* intact plant roots had higher levels of phenolic compounds than those cultivated in tissue cultures (Kikowska et al. 2020). Similarly, Szopa et al. (2019) reported that leaves of *S. chinensis* intact plants accumulate more phenolics than plants obtained during in vitro cultivation. On the other hand, *Salvia viridis* shoot culture appeared as a more effective source of phenolic compounds than plants from ex vitro conditions (Grzegorzczak-Karolak et al. 2019). Phenolic compounds accumulate in plant tissue in response to stress factors (Caretto et al. 2015). An increase in the synthesis of secondary metabolites in *R. japonica* observed in AC and TIB may be induced by mechanical and/or flooding stress. Also, generally higher values of phenolic metabolites in above-ground parts of examined plants compared to underground parts may relate to the fact, that Japanese knotweed in tissue cultures, as well as acclimatized to ex vitro conditions (cultivated in pots with soil) did not produce the rhizomes. Moreover, our study presents data obtained from the experiment on plants cultivated in ex vitro conditions, but in the controlled identical environment as for the cultivation of plants in in vitro conditions. Other available literature data focused on the phytochemistry of Japanese knotweed were obtained from the plants grown in the field/natural environment (Lachowicz and Oszmiański 2019).

Cultivation of medical plant species in in vitro systems may lead to changes in secondary metabolite composition in comparison to plants from ex vitro conditions (Muraseva and Kostikova 2021). Nevertheless, the results from presented experiments (SM, AC, TIB, ex vitro) confirmed the presence of phenolic acids and flavonoids that were previously described in shoots and roots/rhizomes of *R. japonica* obtained from the field environment, which were reported by Bozin et al. (2017), Lachowicz et al. (2019), Lachowicz and Oszmiański (2019), Stefanowicz et al. (2021), Stefanowicz et al. (2022).

The level of phenolic acid concentration in *R. japonica* was dependent on the cultivation system, as well as the type of the plant organ. The highest concentration of phenolic acids was accumulated in shoots from ex vitro conditions, where the dominant derivative—caftaric acid obtained a concentration $981.2 \text{ mg} \times 100 \text{ g}^{-1} \text{ DW}$. Lachowicz and Oszmiański (2019) studied the content of phenolic compounds in leaves, stalks, and roots of *R. japonica* collected from field plants. They reported the following concentrations of caftaric acid: 440.0, 20.0 and $10.0 \text{ mg} \times 100 \text{ g}^{-1}$

DW in leaves, stalk and roots, respectively. Phenolic acids are synthesized in plants through the shikimic acid by phenylpropanoid pathway. The crucial reaction of phenylalanine conversion into trans-cinnamic acid is catalyzed by phenylalanine ammonia lyase (PAL) (Kumar and Goel 2019). PAL activity, and hence, phenolic acid synthesis relates to stress responses, as well as to primary and secondary metabolism (Labudda et al. 2020b). Plants cultivated in ex vitro conditions have increased activity of primary and secondary metabolism pathways due to being completely autotrophic, compared to the plants from in vitro conditions. Effective photosynthesis derives resources for secondary compound synthesis, but on the other hand, together with cellular respiration produce reactive oxygen species (ROS). Phenolic acids may reduce ROS and prevent oxidation of lipid membranes. AC and TIB conducted to 1.25- and 1.31-fold increase in the sum of phenolic acids in shoots, compared to SM. Similarly, TIB stimulated phenolic acid production in *P. maculatum* shoots (Makowski et al. 2023), while agitated culture of *E. alpinum* synthesized more phenolic acids than SM plants (Kikowska et al. 2020).

Phytochemical analysis allowed for quantification of nine flavonoids in examined extracts. Four of them: catechin, epigallocatechin, epicatechin gallate and epicatechin belong to the catechin derivatives and constitute an important group of secondary metabolites characteristic of Japanese knotweed (Lachowicz et al. 2019). Both shoots and roots of *R. japonica* obtained from each cultivation method were rich in catechins (Table 3). Three of them, catechin, epigallocatechin, and epicatechin, were dominant relative to epicatechin gallate. The most popular raw material, famous for its high content of catechin derivatives, is green tea. The health-promoting properties of green tea result from the catechins it contains (Musial et al. 2020). We can hypothesize that the presence of various catechins in *R. japonica* tissue causes several of its biological activities. Lachowicz et al. (2019) studied the concentration of catechins in the leaves and rhizomes of *R. japonica* plants collected from the garden. They reported 145.4 and 8.10 mg $\times$ 100 g⁻¹ DW of catechin in leaves and rhizome, respectively. In the same study epicatechin content in leaves was 329.58 and in rhizome 74.14 mg $\times$ 100 g⁻¹ DW. Our results indicate the advantage of growing *R. japonica* in in vitro conditions, because of higher catechin synthesis from 87 to 183% in shoots and from 142 to 229% in roots. The most favorable growing conditions AC and TIB increase catechin content probably through stress induction. Mechanical stimulation during agitating, as well as temporary flooding in bioreactors, may induce the defense response in plants, manifested by increased secondary metabolite synthesis (Makowski et al. 2023).

The content of the rest of the flavonoids identified and analyzed in Japanese knotweed is, above all, determined by the plant organ. The shoots of *R. japonica* accumulated more flavonoids than the roots (Table 4). Nevertheless, cultivation methods used for plant propagation in the experiment also affected flavonoid content. Stefanowicz et al. (2021) reported the following concentrations of quercetin in leaves, stem, and roots/rhizomes of *R. japonica* collected from the field: 63.0, 68.0, 220.0 mg $\times$ kg⁻¹ DW, while in this study quercetin level in TIB shoots obtained 269.2 mg $\times$ 100 g⁻¹ DW. The obtained results showed that controlled in vitro conditions stimulate flavonoid synthesis in Japanese knotweed plants.

Conclusions

We can conclude that the most suitable methods for the cultivation of Japanese knotweed rich in phenolic metabolites are agitated cultures and temporary immersion bioreactors Plantform™. These modes of cultivation accelerate the growth of plants as compared to traditional cultures on solid medium, probably because of the better availability of nutrients from liquid media. Moreover, agitated and bioreactor cultures accumulate more phenolic compounds, than plants from solid medium and ex vitro conditions. Increased synthesis of secondary metabolites is probably the response to stress induced by shaking of agitated cultures and flooding (hypoxia stress) in bioreactors. We can conclude that *R. japonica* plants obtained through in vitro propagation are a rich source of biologically active polyphenols and may be used as a safe material for the propagation of plants for pharmacological use.

Author contributions W.M. Conceptualization, Methodology, Investigation, Data curation, Writing— original draft, Review & editing. A.K. Data curation, Methodology, Review & editing J.S. Data curation. A.M. Data curation. M.P. Data curation, Methodology, Review & editing. A.Sz. Data curation, Investigation, Methodology, Review & editing. P.K. Data curation. B.T. Review & editing. K.M.T. Review & editing.

Funding The study was funded by the Ministry of Science and Higher Education of Poland as a part of a research subsidy to the University of Agriculture in Krakow (050012-D017).

Data availability All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interest The authors declare no conflict of interest exists. Agnieszka Szopa was an editorial board member of Plant Cell Tissue and Organ Culture at the time of submission. This had no impact on the peer review process and the final decision.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Bello-Bello JJ, Canto-Flick A, Balam-Uc E, Gomez-Uc E, Robert ML, Iglesias-Andreu LG, Santana Buzzy N (2010) Improvement of in vitro proliferation and elongation of habanero pepper shoots (*Capsicum chinense* Jacq.) By temporary immersion. HortScience 45:1093–1098. <https://doi.org/10.21273/HORTSCI.45.7.1093>
- Božin B, Gavrilović M, Kladar N, Rat M, Anačkov G, Gavarić N (2017) Highly invasive alien plant *Reynoutria japonica* Houtt. Represents a novel source for pharmaceutical industry—evidence from phenolic profile and biological activity. J Serb Chem Soc 82:803–813. <https://doi.org/10.2298/JSC170306054G>
- Caretto S, Linsalata V, Colella G, Mita G, Lattanzio V (2015) Carbon fluxes between primary metabolism and phenolic pathway in plant tissues under stress. Int J Mol Sci 16:26378–26394. <https://doi.org/10.3390/ijms161125967>
- De Carlo A, Tarraf W, Lambardi M, Benelli C (2021) Temporary Immersion System for production of Biomass and Bioactive compounds from Medicinal plants. Agronomy 11:414. <https://doi.org/10.3390/agronomy11122414>
- Grzegorzczak-Karolak I, Rytczak P, Bielecki S, Wysokińska H (2015) The influence of liquid systems for shoot multiplication, secondary metabolite production and plant regeneration of *Scutellaria alpina*. Plant Cell Tissue Organ Cult 122(3):699–708. <https://doi.org/10.1007/s11240-016-1126-y>
- Grzegorzczak-Karolak I, Rytczak P, Bielecki S, Wysokińska H (2017) The influence of liquid systems for shoot multiplication, secondary metabolite production and plant regeneration of *Scutellaria alpina*. Plant Cell Tiss Organ Cult 128(2):479–486. <https://doi.org/10.1007/s11240-016-1126-y>
- Grzegorzczak-Karolak I, Kuźma Ł, Lisiecki P, Kiss A (2019) Accumulation of phenolic compounds in different *in vitro* cultures of *Salvia viridis* L. and their antioxidant and antimicrobial potential. Phytochem Lett 30:324–332. <https://doi.org/10.1016/j.phytol.2019.02.016>
- Grzegorzczak-Karolak I, Tabaka P, Weremczuk-Jeżyna I (2024) Enhancing polyphenol yield in *Salvia viridis* L. shoot culture through liquid medium optimization and light spectrum manipulation. Plant Cell Tiss Organ Cult 156:88. <https://doi.org/10.1007/s11240-024-02713-9>
- Hammer Ø, Harper RP (2001) Past: paleontological statistics software package for education and data analysis. Palaeontol Electron 4:9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm
- Hwang HD, Kwon SH, Murthy HN, Yun SW, Pyo SS, Park SY (2022) Temporary Immersion Bioreactor System as an efficient method for Mass production of in vitro plants in horticulture and Medicinal plants. Agronomy 12:346. <https://doi.org/10.3390/agronomy12020346>
- Isah T, Umar S, Mujib A, Sharma MP, Rajasekharan PE, Zafar N, Frukh A (2018) Secondary metabolism of pharmaceuticals in the plant in vitro cultures: strategies, approaches, and limitations to achieving higher yield. Plant Cell Tiss Organ Cult 132:239–265. <https://doi.org/10.1007/s11240-017-1332-2>
- Karalija E, Zeljković SC, Tarkowski P, Muratović E, Parić A (2017) The effect of cytokinins on growth, phenolics, antioxidant and antimicrobial potential in liquid agitated shoot cultures of *Knautia sarajevensis*. Plant Cell Tissue Organ Cult 131:347–357. <https://doi.org/10.1007/s11240-017-1288-2>
- Khalil AAK, Park WS, Lee J, Kim H-J, Akter K-M, Goo Y-M, Bae J-Y, Chun M-S, Kim J-H, Ahn M-J (2019) A new anti-helicobacter pylori juglone from *Reynoutria japonica*. Arch Pharm Res 42:505–511. <https://doi.org/10.1007/s12272-019-01160-x>
- Kikowska M, Thiem B, Szopa A, Ekiert H (2020) Accumulation of valuable secondary metabolites: phenolic acids and flavonoids in different *in vitro* systems of shoot cultures of the endangered plant species — *Eryngium alpinum* L. Plant Cell Tiss Organ Cult 141:381–391. <https://doi.org/10.1007/s11240-020-01795-5>
- Kim BR, Ha J, Lee S, Park J, Cho S (2019) Anti-cancer effects of ethanol extract of *Reynoutria japonica* Houtt. radix in human hepatocellular carcinoma cells via inhibition of MAPK and PI3K/Akt signaling pathways. J Ethnopharmacol 11:2179. <https://doi.org/10.1016/j.jep.2019.112179>
- Krychowiak M, Grinholc M, Banasiuk R, Krauze-Baranowska M, Głód D, Kawiak A, Królicka A (2014) Combination of silver nanoparticles and *Drosera binata* extract as a possible alternative for antibiotic treatment of burn wound infections caused by resistant *Staphylococcus aureus*. PLoS ONE 9:e115727. <https://doi.org/10.1371/journal.pone.0115727>
- Kumar N, Goel N (2019) Phenolic acids: natural versatile molecules with promising therapeutic applications. Biotechnol Rep 24:e00370. <https://doi.org/10.1016/j.btre.2019.e00370>
- Kunakhonnuruk B, Kongbangkerd A, Inthima P (2019) Improving large-scale biomass and plumbagin production of *Drosera communis* A.St.-Hil. By temporary immersion system. Ind Crops Prod 137:197–202. <https://doi.org/10.1016/j.indcrop.2019.05.039>
- Kwiecien I, Szydlowska A, Kawka B, Beerhues L, Ekiert H (2015) Accumulation of biologically active phenolic acids in agitated shoot cultures of three *Hypericum perforatum* cultivars Elixir, Helos and Topas. Plant Cell Tissue Organ Cult 123:273–281. <https://doi.org/10.1007/s11240-015-0830-3>
- Labudda M, Tokarz K, Tokarz B, Muszyńska E, Gietler M, Górecka M, Różańska E, Rybarczyk-Płońska A, Fidler J, Prabucka B, Dababat AA, Lewandowski M (2020a) Reactive oxygen species metabolism and photosynthetic performance in leaves of *Hordeum vulgare* plants co-infested with *Heterodera filipjevi* and *aceria tosichella*. Plant Cell Rep 39:1719–1741. <https://doi.org/10.1007/s00299-020-02600-5>
- Labudda M, Muszyńska E, Gietler M, Różańska E, Rybarczyk-Płońska A, Fidler J, Prabucka B, Dababat AA (2020b) Efficient antioxidant defence systems of spring barley in response to stress induced jointly by the cyst nematode parasitism and cadmium exposure. Plant Soil 456:189–206. <https://doi.org/10.1007/s11104-020-04713-y>
- Lachowicz S, Oszmiański J (2019) Profile of Bioactive compounds in the Morphological Parts of Wild *Fallopia japonica* (Houtt) and *Fallopia sachalinensis* (F. Schmidt) and their antioxidative activity. Molecules 11(7):1436. <https://doi.org/10.3390/molecules24071436>
- Lachowicz S, Oszmiański J, Wojdyło A, Cebulak T, Hirnle L, Siewiński M (2019) UPLC- PDA-Q/TOF-MS identification of bioactive compounds and on-line UPLC-ABTS assay in *Fallopia japonica* Houtt and *Fallopia sachalinensis* (F. Schmidt) leaves and rhizomes grown in Poland. Eur Food Res Technol 245:691–706. <https://doi.org/10.1007/s00217-018-3191-4>
- Lerch S, Sirguy C, Michelot-Antalik A, Jurianz S (2022) Accumulation of metallic trace elements in *Reynoutria japonica*: a risk

- assessment for plant biomass valorization. *Environ Sci Pollut Res* 29:67390–67401. <https://doi.org/10.1007/s11356-022-20485-7>
- Liu S, Zhang R, Zhang X, Zhu S, Liu S, Yang J, Li Z, Gao T, Liu F, Hu H (2022) The Invasive species *Reynoutria japonica* Houtt. As a Promising Natural Agent for Cardiovascular and Digestive System Illness. *Front Pharmacol* 13(13):863707. <https://doi.org/10.3389/fphar.2022.863707>
- Makowski W, Tokarz KM, Tokarz B, Banasiuk R, Witek K, Królicka A (2020) Elicitation-based method for increasing the production of antioxidant and bactericidal phenolic compounds in *Dionaea muscipula*. *J Ellis Tissue Molecules* 25(8):1794. <https://doi.org/10.3390/molecules25081794>
- Makowski W, Królicka A, Nowicka A, Zwyrtková J, Pecinka A, Banasiuk R, Tokarz KM (2021a) Transformed tissue of *Dionaea muscipula* J. Ellis as a source of biologically active phenolic compounds with bactericidal properties. *Appl Microbiol Biotechnol* 105:1215–1226. <https://doi.org/10.1007/s00253-021-11101-8>
- Makowski W, Królicka A, Tokarz B, Miernicka K, Kołton A, Pięta Ł, Malek K, Ekiert H, Szopa A, Tokarz KM (2021b) Response of physiological parameters in *Dionaea muscipula* J. Ellis teratomas transformed with *rolB* oncogene. *BMC Plant Biol* 21:564. <https://doi.org/10.1186/s12870-021-03320-y>
- Makowski W, Królicka A, Tokarz B, Szopa A, Ekiert H, Tokarz KM (2023) Temporary immersion bioreactors as a useful tool for obtaining high productivity of phenolic compounds with strong antioxidant properties from *Pontechium maculatum*. *Plant Cell Tissue Organ Cult* 153:525–537. <https://doi.org/10.1007/s11240-023-02487-6>
- Malik S, Sharma M, Ahuja PS (2016) An efficient and economic method for *in vitro* propagation of *Arnebia euchroma* using liquid culture system. *Am J Biotechnol Med Res* 1(1):19–25. <https://doi.org/10.5455/ajbmr.20160501100040>
- Maliński MP, Kikowska M, Kruska D, Napierała M, Florek E, Sliwińska E, Thiem B (2019) Various *in vitro* systems of ragged robin (*Lychnis flos-cuculi* L.): a new potential source of phytoecdysteroids? *Plant Cell Tiss Organ Cult* 139:39–52. <https://doi.org/10.1007/s11240-019-01660-0>
- Moreira AL, Silva AB, Santos A, Reis CO, Landgraf PRC (2013) *Cattleya Walkeriana* growth in different micropropagation systems. *Cienc Rural* 43(10):1804–1810. <https://doi.org/10.1590/S0103-84782013001000012>
- Muraseva DS, Kostikova VA (2021) *In vitro* propagation of *Spiraea betulifolia* subsp. *aemiliana* (Rosaceae) and comparative analysis of phenolic compounds of microclones and intact plants. *Plant Cell Tiss Organ Cult* 144:493–504. <https://doi.org/10.1007/s11240-020-01971-7>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Musial C, Kuban-Jankowska A, Gorska-Ponikowska M (2020) Beneficial Properties of Green Tea Catechins. *Int J Mol Sci* 21:1744. <https://doi.org/10.3390/ijms21051744>
- Muszyńska E, Tokarz KM, Dziurka M, Labudda M, Dziurka K, Tokarz B (2021) Photosynthetic apparatus efficiency, phenolic acid profiling and pattern of Chosen Phytohormones in Pseudometallophyte *Alyssum Montanum*. *Sci Rep* 11:4135. <https://doi.org/10.1038/s41598-021-83695-y>
- Rahmonov O, Czylok A, Orczewska A, Majgier L, Parusel T (2014) Chemical composition of the leaves of *Reynoutria japonica* Houtt. And soil features in polluted areas. *Cent Eur J Biol* 9:320–330. <https://doi.org/10.2478/s11535-013-0267-9>
- Salgado Pirata M, Correia S, Canhoto J (2022) Ex Vitro Simultaneous Acclimatization and Rooting of *In Vitro* propagated Tamarillo plants (*Solanum betaceum* Cav.): effect of the substrate and Mineral Nutrition. *Agronomy* 12(5):1082. <https://doi.org/10.3390/agronomy12051082>
- Savio LEB, Astarita LV, Santarém ER (2012) Secondary metabolism in micropropagated *Hypericum perforatum* L. grown in non-aerated liquid medium. *Plant Cell Tissue Organ Cult* 108(3):465–472. <https://doi.org/10.1007/s11240-011-0058-9>
- Šoln K, Žnidaršič N, Dolenc Koče J (2022) Root growth inhibition and ultrastructural changes in radish root tips after treatment with aqueous extracts of *Fallopia japonica* and *F. xbohemica* rhizomes. *Protoplasma* 259:343–355. <https://doi.org/10.1007/s00709-021-01668-4>
- Soni V, Keswani K, Bhatt U, Kumar D, Singh H (2021) *In vitro* propagation and analysis of mixotrophic potential to improve survival rate of *Dolichandra unguiculata* under ex vitro conditions. *Heliyon* 7(2):e06101. <https://doi.org/10.1016/j.heliyon.2021.e06101>
- Stefanowicz AM, Stanek M, Nobis M, Zubek S (2017) Few effects of invasive plants *Reynoutria japonica*, *Rudbeckia laciniata* and *Solidago gigantea* on soil physical and chemical properties. *Sci Total Environ* 574:938–946. <https://doi.org/10.1016/j.scitotenv.2016.09.120>
- Stefanowicz AM, Kapusta P, Stanek M, Frąc M, Oszust K, Woch MW, Zubek S (2021) Invasive plant *Reynoutria japonica* produces large amounts of phenolic compounds and reduces the biomass but not activity of soil microbial communities. *Sci Total Environ* 767:145439. <https://doi.org/10.1016/j.scitotenv.2021.145439>
- Stefanowicz AM, Frac M, Oszust K, Stanek M (2022) Contrasting effects of extracts from invasive *Reynoutria japonica* on soil microbial biomass, activity, and community structure. *Biol Invasions* 24:3233–3247. <https://doi.org/10.1007/s10530-022-02842-2>
- Sułkowska-Ziaja K, Maślanka A, Szewczyk A, Muszyńska B (2017) Physiologically active compounds in four species of *Phellinus*. *Nat Prod Commun* 12:363–366. <https://doi.org/10.1177/1934578X1701200313>
- Szopa A, Klimek-Szczykutowicz M, Kokotkiewicz A, Dziurka M, Łuczkiwicz M, Ekiert H (2019) Phenolic acid and flavonoid production in agar, agitated and bioreactor-grown micro-shoot cultures of *Schisandra chinensis* cv. Sadova 1—a valuable medicinal plant. *J Biotechnol* 305:61–70. <https://doi.org/10.1016/j.jbiotec.2019.08.021>
- Szopa A, Dziurka M, Granica S, Klimek-Szczykutowicz M, Kubica P, Warzecha A, Jaferník K, Ekiert H (2020) *Schisandra Rubriflora* Plant Material and *In Vitro* Microshoot cultures as Rich sources of Natural Phenolic antioxidants. *Antioxidants* 9(6):488. <https://doi.org/10.3390/antiox9060488>
- Tokarz K, Makowski W, Banasiuk R, Królicka A, Piwowarczyk B (2018) Response of *Dionaea muscipula* J. Ellis to light stress in *in vitro*: physiological study. *Plant Cell Tissue Organ Cult* 134(1):65–77. <https://doi.org/10.1007/s11240-018-1400-2>
- Tokarz B, Wójtowicz T, Makowski W, Jędrzejczyk RJ, Tokarz KM (2020) What is the difference between the response of grass pea (*Lathyrus sativus* L.) to Salinity and Drought stress? — a physiological study. *Agronomy* 10(6):833. <https://doi.org/10.3390/agronomy10060833>
- Vidican R, Mihaiescu T, Pleșa A, Mălinaș A, Pop B-A (2023) Investigations Concerning Heavy Metals Dynamics in *Reynoutria japonica* Houtt. - Soil Interact Toxics 11:323. <https://doi.org/10.3390/toxics11040323>
- Wang Z-J, Trill J, Tan L-L, Chang W-J, Zhang Y, Willcox M, Xia R-Y, Jiang Y, Moore M, Liu J-P (2022a) *Reynoutria japonica* Houtt for Acute respiratory tract infections in adults and children: a systematic review. *Front Pharmacol* 13:787032. <https://doi.org/10.3389/fphar.2022.787032>
- Wang ZJ, Trill J, Tan LL, Chang WJ, Zhang Y, Willcox M, Xia RY, Jiang Y, Moore M, Liu JP, Hu XY (2022b) *Reynoutria japonica* Houtt for Acute respiratory tract infections in adults and children: a systematic review. *Front Pharmacol* 13:787032. <https://doi.org/10.3389/fphar.2022.787032>

- Zhang XG, Ma GY, Kou F, Liu WJ, Sun QY, Guo GJ, Ma X-D, Guo S-J, Jian-Ning Z (2019) *Reynoutria japonica* from traditional Chinese medicine: a source of competitive adenosine deaminase inhibitors for anticancer. Comb Chem 22(2):113–122. <https://doi.org/10.2174/1386207322666190415100618>
- Zubek S, Kapusta P, Stanek M, Woch MW, Błaszowski J, Stefanowicz AM (2022) *Reynoutria japonica* invasion negatively affects

arbuscular mycorrhizal fungi communities regardless of the season and soil conditions. Appl Soil Ecol 169:104152. <https://doi.org/10.1016/j.apsoil.2021.104152>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.