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ORIGINAL ARTICLE

Temporary immersion bioreactors as a useful tool for obtaining high productivity of phenolic compounds with strong antioxidant properties from *Pontechium maculatum*

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Abstract

Pontechium maculatum (Russian bugloss) is a medical plant belonging to the family Boraginaceae. Although this species is known as a medical plant rich in biologically active secondary metabolites, biotechnological studies about this valuable plant is still missing. The scientific objectives of this study were to investigate the biomass production, synthesis, and productivity of various phenolic acids, flavonoids, and shikonin in *P. maculatum* cultivated in various breeding systems. Additionally, the antioxidant activity of plant-derived extracts was evaluated. Plants were cultivated in a traditional agar-solidified medium, a liquid medium with rotary shaking, and a temporary immersion bioreactors Plantform™ (TIB), as well as cultivated 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, total phenolic content was estimated, and qualitative and quantitative analysis of phenolic derivatives using DAD-HPLC was conducted, simultaneously with an analysis of antioxidant capacity. TIB stimulated the highest synthesis of all examined phenolic acids and shikonin. In TIB-cultivated shoots level of rosmarinic acid obtained a concentration of 3160.76 mg × 100 g^{-1} dry weight (DW), and shikonin obtained a concentration of 77.26 mg × 100 g^{-1} DW. Furthermore, plants from TIB were characterized by the highest productivity of all studied phenolic derivatives, what makes it very effective platform for the synthesis of biologically active secondary metabolites in Russian bugloss. Moreover, this article shows that *P. maculatum* is a rich source of various phenolic derivatives with high antioxidant potential.

Key message

Temporary immersion bioreactors are suitable system for production of phenolic compounds in tissue cultures of *Pontechium maculatum* plants.

Keywords Agitated culture · Flavonoids · In vitro · Phenolic acids · Shikonin · Temporary immersion bioreactors

Introduction

The family Boraginaceae includes about 2000 species occurring worldwide, mainly in Europe and Asia. This group of species is important in pharmacology because of its various medical properties. The therapeutic effect of these plants comes from their numerous biologically active secondary metabolites, including phenolic compounds such as naphthoquinones, flavonoids, and phenolic acids (Dresler et al. 2017). Russian bugloss (*Pontechium maculatum* (L.) U.-R. Böhle and H.H. Hilger) was previously known as *Echium russicum* J.F.Gmel (Nowak et al. 2020). It was

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distinct as a monotypic genus in the family Boraginaceae in the year 2000 (Hilger and Böhle 2000). This rare plant naturally occurs in meadows, sand, and uncultivated slopes, as well as sunny forest steppes (Dresler et al. 2017). Moreover, as reported by Jakovljević et al. (2019), P. *maculatum* can occur in heavy-metal-contaminated areas, being a facultative metallophyte. The Russian bugloss can synthesize a broad range of phenolic compounds, including the red dye shikonin (1,4-naphtoquinone derivative) and phenolic acids, especially rosmarinic acid and flavonoids (Dresler et al. 2015). In the natural habitat, these secondary metabolites allow plants to acclimate to unfavorable environmental conditions (Cheynier et al. 2013). On the other hand, plants rich in secondary metabolites from the group of phenolic compounds have many applications in the pharmaceutical industry. A survey of the literature by Eruygur et al. (2016) indicates that various Boraginaceae plants have been used in folk medicine. The presence of phenolic compounds derivatives like phenolic acids or 1,4-naphtoquinones was reported in *Echium arenarium*, *Echium angustifoliumi, Echium gaditanum* or *Echium italicum* (Kefi et al. 2018; Eruygur 2018; Duran et al. 2017 and Dresler et al. 2017, respectively).

Although plants from the Boraginaceae family have beneficial features, their potential application in medicine remains unclear. Furthermore, phytochemical analysis of these species seems desirable (Dresler et al. 2017). Phytochemical studies of yet-unrecognized plant species may provide new knowledge about valuable sources of natural bioactive compounds. To the best of our knowledge, until now apart from the works of Dresler et al. (2015 and 2017) only Olennikov et al. (2017) gave phytochemical insight into Russian bugloss. They showed that in roots *P. maculatum* can accumulate 10 derivatives of shikonin and 5 derivatives of rosmarinic acid.

Given that *P. maculatum* is a rare and endangered species, obtaining this plant from its natural habitat is impossible. This is why Nowak et al. (2020) showed the possibility of Russian bugloss propagation in in vitro conditions for horticulture and plant preservation. Furthermore, Zare et al. (2010) stated that the production of shikonin from wild plants of the Boraginaceae would fail because a large amount of the plant material is needed for this purpose. However, plant tissue cultures are an excellent tool that surmounts this problem (Zare et al. 2010). The aspect of phenolic compounds synthesis in some medical plants tissue cultures was studied (Giri and Zaheer 2016; Chandran et al. 2020), while studies on tissue cultures of *P. maculatum* in the context of biomass and phenolic compounds production and biological activity are still missing. This article is the first report on the synthesis of phytochemicals in Russian bugloss tissue cultivated in various breeding systems as well as in plants cultivated in ex vitro conditions.

Modifying traditionally used plant tissue cultures cultivated in agar-solidified media is an effective platform for scaling up the production of plant-derived secondary metabolites. As shown by Kikowska et al. (2020) and Szopa et al. (2019a), the accumulation of phenolic compounds in medical plants could be positively influenced using agitated cultures (shoot cultures in liquid media with rotary shaking) or bioreactors with a temporary immersion system. Moreover, such modifications of breeding strategies can affect biomass accumulation and growth dynamics. Nevertheless, to verify possible strategies for scaling up the production of medicinal plants in tissue cultures, it is important to compare the results with those obtained for intact or ex vitro grown plants.

This study provides insight into the phytochemistry of Russian bugloss as the medical plant that cannot be obtained from natural environment. Moreover, presented article shows new data about possible application of various modifications of plant tissue cultures for synthesis of biologically active phenolic derivatives, especially phenolic acids (among others rosmarinic acid) and 1,4-naphtoquinone (shikonin). Therefore, obtained results contribute to the development of the broadly understood biotechnology of medicinal plants.

The main goals of the research presented here were to: (1) study the phytochemical composition of *P. maculatum* plants grown in ex vitro and in vitro conditions, (2) check if the cultivation of *P. maculatum* plants in in vitro conditions is a better strategy for biomass and phenolic compounds production than conventional in-soil cultivation, (3) check if shaking cultures in a liquid medium or temporary immersion bioreactors are effective platforms for the production of phenolic compounds in *P. maculatum*, and (4) compare the antioxidant properties of *P. maculatum* plants from various growing conditions. We hypothesized that a liquid medium with rotary shaking and temporary immersion bioreactors could increase plant biomass production because of a better distribution of nutrients contained in the liquid medium. On the other hand, we expected that the hydromechanical stress resulting from shaking in agitated cultures, as well as limited oxygen availability while flooding in temporary immersion bioreactors, would increase the production of phenolic compounds through the stress response induction.

Materials and methods

Initiation of in vitro culture

The seeds of *P. maculatum* (obtained from the collection of the Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Poland) were treated with 70% (v/v) ethanol for 30 s and surface-sterilized with 5.25% calcium hypochlorite for 15 min. Then, the seeds were rinsed three times in sterile distilled water and placed in 250 mL flasks with 50 mL of MS medium (Murashige and Skoog 1962) solidified with 0.8% agar, without growth regulators, containing 3% sucrose, with pH 5.8 (adjusted prior to autoclaving). Germination was performed in the dark and first seedlings were observed after 7–8 weeks. Plantlets were subcultured every 4 weeks to a fresh medium with the composition described above. Plants were cultivated at 21 ± 2 °C, under white fluorescence light characterized by a photosynthetic photon flux density (PPFD) of 120 µmol × m^{-2} × s^{-1} and a photoperiod of 16 h/8 h light/dark cycle. The grown plants were the material to initiate the experiment.

In vitro culture systems and ex vitro plants cultivation

Solid medium cultures (SM)

SM cultures were cultivated in 250 mL Erlenmeyer flasks. For the experiment, a 1 g of rooted plants per flask was used. Plants were placed in 50 mL of MS medium (Murashige and Skoog 1962) solidified with 0.8% agar, without growth regulators, containing 3% sucrose, with pH 5.8 (adjusted prior to autoclaving). Biomass was collected after 6 weeks of growth cycles. Tissue cultures of *P. maculatum* plants in SM were cultivated at 21 ± 2 °C under white fluorescence light with a photosynthetic photon flux density (PPFD) of 120 µmol × m^{-2} × s^{-1} and a photoperiod of 16 h/8 h light/dark cycle. The experiment consisted of 10 biological repetitions (n = 10).

Liquid medium cultures with rotary shaking (LM)

LM cultures were maintained in 250 mL Erlenmeyer flasks containing 50 ml of liquid MS medium (Murashige and Skoog 1962), without growth regulators, containing 3% sucrose, with pH 5.8 (adjusted prior to autoclaving). For the inoculum, 1.0 g of rooted plants was used. Cultures were placed on a rotary shaker (Phoenix RS-LS 20, DanLab, Poland) at 120 rpm. Tissue cultures of *P. maculatum* plants in LM were cultivated at 21 ± 2 °C under white fluorescence light with a photosynthetic photon flux density (PPFD) of 120 µmol × m^{-2} × s^{-1} and a photoperiod of 16 h/8 h light/dark cycle. Biomass samples from LM cultures were collected after 6 weeks of growth cycles (n = 10).

Cultures in temporary immersion bioreactors (TIB)

TIB cultures were grown in a Plantform temporary immersion system (Plant Form, Sweden). The immersion and aeration period in the bioreactors were programmed in the following cycle: 20 min of immersion, 30 min of gravitational fall of the medium, and 10 min of aeration. These parameters were optimized for *P. maculatum* plants in a preliminary experiment (data not shown). The bioreactor was inoculated at 10/500 (g/mL) rooted plants to medium ratio. Tissue cultures of *P. maculatum* plants in TIB were cultivated at 21 ± 2 °C under white fluorescence light with a photosynthetic photon flux density (PPFD) of 120 µmol × m^{-2} × s^{-1} and a photoperiod of 16 h/8 h light/dark cycle. Biomass samples from TIB cultures were collected after 6 weeks of growth cycles (n = 10).

Ex vitro plants cultivation (in soil)

One-month plants was transferred from in vitro conditions to pots with peat and sand (3:1) for acclimatization to ex vitro conditions (one plant per one pot). *P. maculatum* plants in soil were cultivated at 21 ± 2 °C under white fluorescence light with a photosynthetic photon flux density (PPFD) of 120 µmol × m^{-2} × s^{-1} and a photoperiod of 16 h/8 h light/dark cycle. The initial humidity was 90% and was lowered each week by 10% to final humidity at 50%. After 4 weeks, the plants were acclimatized to ex vitro conditions. Biomass samples from ex vitro plants were collected 6 weeks after acclimatization (n = 10).

Determination of biometric parameters

To estimate the growth of *P. maculatum* plants in the experimental conditions, the tissue cultures and plants from the ex vitro conditions were harvested and weighed immediately. The growth index (GI) was calculated according to the following formula: GI [%] = (FW_2 - FW_1)/FW_2 × 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. To determine dry weight (DW) accumulation, the plants were freeze-dried for 48 h, and the shoots and roots/callus were weighed separately. The DW content in the plant tissue was calculated according to the following formula: DW [%] = DW_2 × 100/FW_2, where DW_2 is the dry weight after freeze-drying. The freeze-dried plant tissue, with the shoots and roots/callus separated, was homogenized to a powder, and stored at -20 °C for further analysis.

Extraction procedure for spectrophotometric analysis

DW plant tissue with a mass of 0.1 g per sample was placed in a 15 mL flask and subjected to extraction in 10 mL of 80% methanol (HPLC grade purity) by sonication in an ultrasonic bath (2 × 30 min, 22 ± 3 °C). To prevent temperature rise, the water was exchanged before each extraction cycle. The samples were centrifuged for 15 min (15,000 g, 4 °C). The resultant extracts were filtered through sterilizing syringe filters (0.22 µm, Millex®GP, Millipore). Samples of each object (shoots or roots/callus from all experimental conditions) were prepared in five repetitions. The extracts were stored at -20 °C for up to one week and used for all spectrophotometric and DAD-HPLC analysis performed during the study.

Total phenolic content estimation

The total phenolic content (TPC) was estimated using the spectrophotometric method with Folin–Ciocalteu's reagent (Swain and Hillis 1959) after modifications (Tokarz et al. 2018). Previously obtained methanolic extract (procedure described above) was 25 times diluted with distilled water. 1.0 mL of diluted extract was mixed with 0.2 mL of Folin's reagent (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany) and 1.6 mL of 5% Na_2CO_3 and then incubated for 20 min at 40 °C. The absorbance of the mixture was measured at 740 nm. Chlorogenic acid (Sigma-Aldrich Chemie, GmBH, Steinheim, Germany) was used as a reference standard. The results were expressed as milligrams of chlorogenic acid equivalents per 1 g of DW. Analyses were performed in five biological replicates.

Phenolic compounds estimation using DAD-HPLC

The phenolic compounds were estimated in a methanolic extract prepared from 500 mg of DW tissue in 10 mL of 80% HPLC-grade methanol. Tissue was extracted in methanol using sonication (two times for 30 min at 25 ± 2 °C) (Polsonic). Samples were centrifuged (25 255×*g* for 15 min at 4 °C). The resultant supernatant was filtered through syringe filters (0.22 µm Millex®GP, Millipore, Merck, Darmstadt, Germany) for analysis with high-pressure liquid chromatography with a diode array detector (DAD-HPLC). The quantitative analyses of phenolic compounds 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 × 4 mm; 5 µm, Merck, Germany) column (Sułkowska-Ziaja et al. 2017; Szopa et al. 2020) The flow rate was 1 mL × min^{-1}, the temperature was set to 25 °C, and the injection volume was 10 µL. The detection wavelength was set to 254 nm. The mobile phase consisted of A—methanol, 0.5% acetic acid 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 standards (acquired from Sigma-Aldrich Co., Germany). The quantification was performed based on the calibration curves method. Samples were prepared and analyzed in five replications. The results were expressed in mg × 100 g^{-1} DW ± SD.

Productivity of phenolic compounds

The productivity (P) of each phenolic compound was calculated according to the formula (Makowski et al. 2021): P [mg of phenolic compound/6weeks/flask] = A × B, where A is the content of the phenolic compound in plant tissue per 1 g DW after 6 weeks of growth, and B is the content of DW in one flask/bioreactor/pot.

Ferric-reducing antioxidant power assay

The FRAP (ferric-reducing antioxidant power) assay was performed according to Benzie and Strain (1996), with modifications (Tokarz et al. 2020). The FRAP working solution was prepared afresh by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (Sigma Aldrich) in 96% ethanol, and 20 mM $FeCl_3$ (10:1:1, v:v:v). Next, 3 mL of the working solution was mixed with 0.1 mL of the diluted methanolic extract and 0.4 mL of water (extract obtained as described above for spectrophotometric analysis, diluted 8 times with 80% methanol). The absorbance was measured at 595 nm after 5 min. The results were expressed as mMol Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma–Aldrich) per 1 g of DW. Analyses were performed in five replicates.

Cupric-ion-reducing antioxidant capacity assay

The CUPRAC (cupric-ion-reducing antioxidant capacity) assay was performed according to Apak et al. (2007), with modifications (Kostecka-Gugała et al. 2020). A volume of 1 mL of 10 mM $CuCl_2$, 1 mL of 7.5 mM neocuproine (Sigma–Aldrich) in 96% ethanol, and 1 mL of 1 M NH_4Ac buffer (pH 7.0) were mixed with 0.3 mL of the diluted methanolic extract and 0.8 mL of water (extract obtained as described above for spectrophotometric analysis, diluted 20 times with 80% methanol). The absorbance was measured

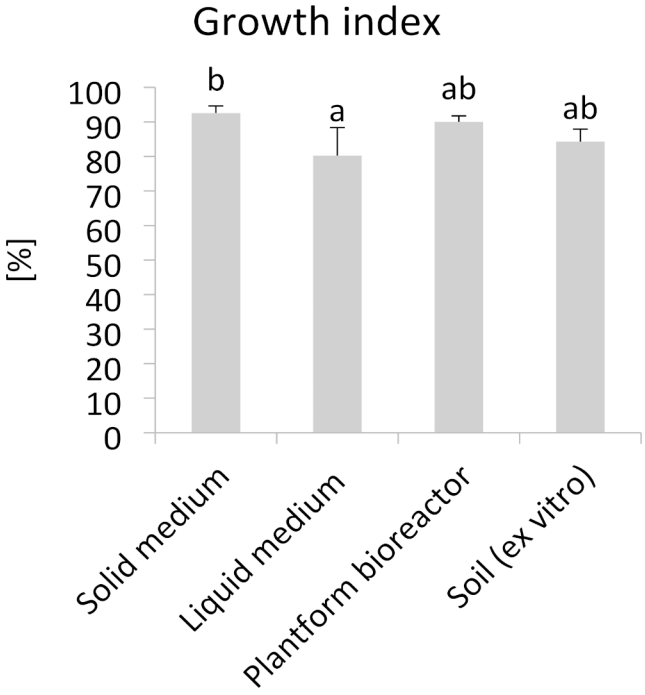

Fig. 1 Growth index (%) of *Pontechium maculatum* cultivated in: solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Upper case letters indicate statistical significance of means acc. one-way ANOVA, post hoc Tuckey test at $p < 0.05$; the bar represents the standard deviation

at 450 nm after 5 min. The results were expressed as mmol Trolox (Sigma–Aldrich) per 1 g of DW.

Statistical analyses

A one-way analysis of variance (ANOVA) was performed to determine significant differences between means during statistic elaboration of growth index of plants and productivity of phenolic derivatives. Moreover, two-way analysis of variance (ANOVA) was performed to determine significant differences between means during statistic elaboration of the rest presented parameters: dry weigh, total phenolic content, FRAP, CUPRAC and accumulation of phenolic derivatives (Tukey test at $p < 0.05$ level). STATISTICA 12.0 (StatSoft Inc., Tulsa, OK, USA) was used to conduct statistical analyses.

Results

Biometric parameters and plant morphology

After 6 weeks of cultivation, the *P. maculatum* plants were harvested, and their biometric parameters were evaluated. The liquid medium with a rotary shaking system (LM) led to a decrease in the plant growth index (by about 12%) compared to the plants cultivated in the solid medium (SM) (Fig. 1).

The highest accumulation of dry weight (11%) was found in the shoots cultivated in ex vitro conditions (Fig. 2). Observations after 6 weeks of the experiment showed that the plants cultivated in the temporary immersion bioreactors (TIB) had changed their morphology. In the bioreactors, the plants formed callus lumps instead of roots. Moreover, shoots were formed on the callus lumps. The leaves grown on the callus lumps were shorter and rounder than those obtained in the other cultivation conditions. The *P. maculatum* plants cultivated in SM, in LM or ex vitro conditions exhibited normal morphology—the plants developed shoots and roots (Figs. 3 and 4).

Total phenolic content

The modifications of the way of cultivation affected the sum of phenolic compounds accumulated in the shoots and roots/callus of *P. maculatum*. The highest concentration of phenolic compounds in shoots was found in the plants grown in TIB and soil (an over 1.5-fold increase compared

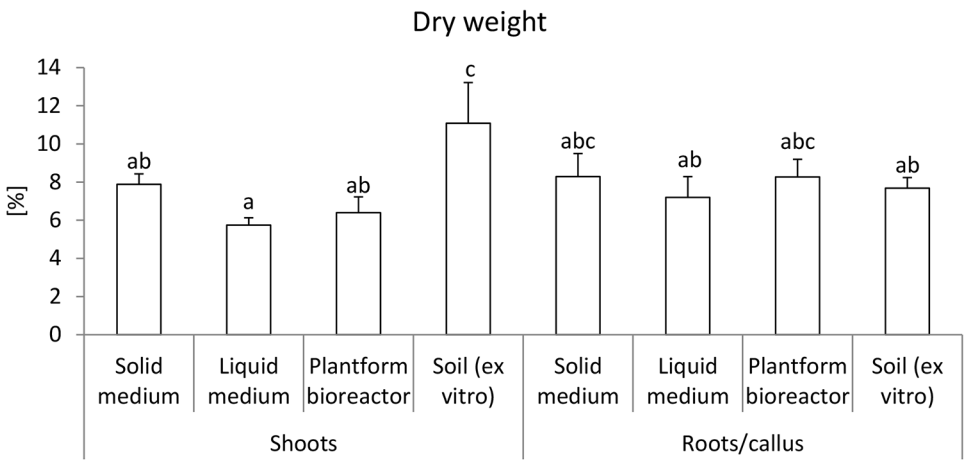

Fig. 2 Accumulation of dry weight (DW) (%) in shoots and roots of *Pontechium maculatum* cultivated in: solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Upper case letters indicate statistical significance of means acc. two-way ANOVA, post hoc Tuckey test at $p < 0.05$; the bar represents the standard deviation

Fig. 3 *Pontechium maculatum* after 6 weeks of cultivation in: solid medium (A), liquid medium with rotary shaking (B), temporary immersion bioreactor Plantform (C), and soil (ex vitro conditions) (D). Scale bar = 1 cm

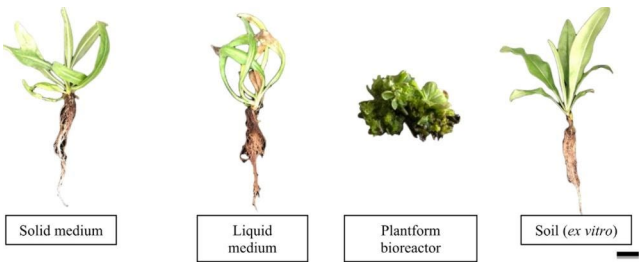

Fig. 4 Morphology of *Pontechium maculatum* after 6 weeks of cultivation in: solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Scale bar = 2 cm

to SM). The LM system boosted the synthesis of phenolic compounds in the shoots of *P. maculatum* over 1.2-fold in comparison to SM. In roots, the total phenolic content increased in LM up to 18% and decreased by about 64% in soil (ex vitro conditions) (Fig. 5).

Accumulation of phenolic derivatives

In extracts of the *P. maculatum* plants, numerous compounds of the polyphenolic structure were detected. The phenolic acids found were: chlorogenic, cryptochlorogenic, caffeic, ferulic, o-coumaric, isoferulic, isochlorogenic, and rosmarinic acid. The extracts also contained a few flavonoids: rutin and kaempferol and shikonin (belonging to the class of 1,4-naphtohinones). The amounts of these compounds depended on the type of organs (shoots or roots/callus) and the growing conditions, both ex vitro and in vitro. The highest content of phenolic acids (chlorogenic, cryptochlorogenic, caffeic, and ferulic) was found in the *P. maculatum* shoots cultivated in TIB (Table 1). Compared to SM, the in vitro model of shoot breeding had an influence on, e.g., the production of caffeic acid, which increased 11.7-fold, and the level of ferulic acid (a 12.8-fold increase was seen). Moreover, the plants cultivated in TIB exhibited the highest level of o-coumaric acid in their shoots and callus tissue. The accumulation of isoferulic and isochlorogenic acids increased in the shoots cultivated in TIB compared to the shoots cultivated in SM. The LM model of in vitro tissue cultivation was found to favor the highest synthesis of those compounds in roots. Chromatographic analyses revealed very high yields of rosmarinic acid in the in vitro cultures propagated in TIB, where the shoots and the callus tissue accumulated 3160.76 mg × 100 g^{-1} DW and 1641.76 mg × 100 g^{-1} DW of this compound, respectively. For flavonoids, the accumulation of rutin increased 7.8-fold in the *P. maculatum* shoots cultivated in TIB compared to the SM mode of breeding. The most effective system for kaempferol synthesis in roots was LM cultivation (Table 1). The only naphthoquinone compound detected -shikonin - a characteristic compound of *P. maculatum.* It increased in the shoots and the callus of the TIB-grown plants by 3.5-fold and 3.2-fold, respectively (Table 1).

Fig. 5 Total phenolic content in shoots and roots of *Pontechium maculatum* cultivated in: solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Upper case letters indicate statistical significance of means acc. two-way ANOVA, post hoc Tuckey test at $p < 0.05$; the bar represents the standard deviation

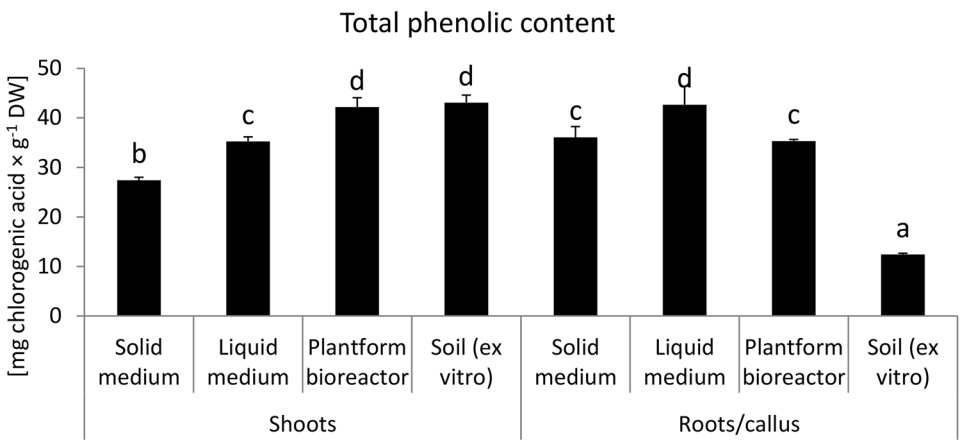

Table 1 Accumulation of phenolic derivatives in shoots and roots of *Pontechium maculatum* cultivated in: solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Different letters indicate statistical significance of means acc. two-way ANOVA, post hoc Tuckey test at $p < 0.05$, SD—standard deviation

Phenolic compound		Shoots				Roots/callus			
		Solid medium	Liquid medium	Plantform bioreactor	Soil (ex vitro)	Solid medium	Liquid medium	Plantform bioreactor	Soil (ex vitro)
Chlorogenic acid	mg × 100 g^{-1} DW [± SD]	5.74 ± 0.95^{ab}	7.06 ± 0.58^{b}	30.44 ± 0.34^{d}	14.23 ± 0.32^{e}	11.21 ± 2.48^{e}	12.73 ± 1.00^{e}	6.41 ± 2.07^{ab}	3.29 ± 0.58^{a}
Cryptochlorogenic acid		17.21 ± 2.62^{d}	18.03 ± 1.40^{d}	31.55 ± 0.38^{e}	7.78 ± 0.05^{b}	10.96 ± 3.63^{bc}	21.19 ± 2.64^{d}	15.47 ± 2.69^{cd}	6.21 ± 0.85^{a}
Caffeic acid		5.13 ± 1.78^{a}	7.00 ± 0.25^{b}	60.09 ± 0.63^{f}	14.15 ± 0.09^{e}	24.69 ± 4.40^{d}	40.75 ± 4.32^{e}	16.49 ± 1.66^{c}	11.11 ± 1.12^{bc}
Ferulic acid		7.17 ± 1.60^{ab}	8.78 ± 0.16^{ab}	91.78 ± 3.16^{e}	13.39 ± 3.71^{b}	14.37 ± 0.68^{b}	26.07 ± 6.18^{c}	48.47 ± 2.85^{d}	2.87 ± 1.88^{a}
O-coumaric acid		52.43 ± 4.12^{d}	50.44 ± 1.54^{cd}	218.02 ± 2.41^{e}	40.16 ± 5.44^{c}	14.47 ± 5.52^{b}	44.66 ± 3.43^{cd}	238.41 ± 3.62^{f}	11.9 ± 3.47^{a}
Isoferulic acid		30.95 ± 1.99^{b}	38.45 ± 1.01^{bc}	64.92 ± 1.61^{d}	40.16 ± 4.06^{bc}	50.24 ± 11.39^{c}	74.78 ± 2.55^{d}	34.21 ± 1.78^{b}	9.78 ± 2.92^{a}
Isochlorogenic acid		55.59 ± 5.62^{b}	55.87 ± 1.42^{b}	304.14 ± 6.99^{d}	54.31 ± 11.82^{b}	105.95 ± 24.23^{c}	304.20 ± 5.97^{d}	103.69 ± 2.17^{c}	11.54 ± 3.33^{a}
Rosmarinic acid		486.69 ± 15.54^{b}	745.58 ± 20.18^{c}	3160.76 ± 34.23^{g}	893.20 ± 23.96^{d}	784.55 ± 82.51^{c}	1433.8 ± 13.41^{e}	1641.76 ± 24.74^{f}	72.72 ± 9.78^{a}
Rutin		21.57 ± 1.76^{ab}	30.72 ± 6.15^{abc}	167.67 ± 2.04^{e}	48.69 ± 8.46^{c}	42.50 ± 13.00^{bc}	76.06 ± 6.83^{d}	94.80 ± 10.58^{d}	14.04 ± 4.30^{a}
Kaempferol		27.03 ± 0.92^{a}	47.12 ± 0.82^{cd}	33.24 ± 0.45^{ab}	57.92 ± 0.92^{d}	41.19 ± 11.89^{bc}	90.86 ± 0.58^{e}	23.93 ± 0.28^{a}	22.44 ± 1.19^{a}
Shikonin		22.19 ± 0.31^{ab}	20.74 ± 1.62^{ab}	77.26 ± 2.07^{c}	25.81 ± 0.24^{b}	17.70 ± 0.33^{a}	16.65 ± 0.46^{a}	70.41 ± 7.26^{c}	17.74 ± 0.28^{a}

Productivity of phenolic derivatives

Of all the examined phenolic compounds, the best productivity in the *P. maculatum* plants was obtained in TIB. For instance, compared to the plants cultivated on SM, the productivity of ferulic acid increased 66.8-fold, rosmarinic acids increased 38.9-fold, and shikonin increased 38.2-fold (Table 2).

Antioxidant properties

The antioxidant power of plant tissue was examined using two spectrophotometric methods: FRAP and CUPRAC. Both methods revealed a similar trend. The highest ferric-ions-reducing potential was found in the shoots of *P. maculatum* obtained from soil (ex vitro conditions) and the roots cultivated in LM (Fig. 6). However, the highest cupric-ions-reducing potential was observed in the case of the roots obtained in the LM system (Fig. 7). Both testing methods revealed the lowest antioxidant strength for the extract derived from the callus tissue cultivated in TIB.

Discussion

Phytochemical studies focusing on the synthesis, accumulation, and productivity of phenolic compounds in plant tissue have great value for basic knowledge as well as the industry (Albuquerque et al. 2021). *P. maculatum*, being a rich, natural source of phenolic acids, flavonoids, and 1,4-naphtoquinones (shikonin), is a good example of a species for such research (Dresler et al. 2015). To the best of our knowledge, this is the first report on the use of this unique plant in green biotechnology. The present work looks at the impact of various breeding systems on biomass accumulation and the synthesis of phenolic compounds in *P. maculatum* plants. The breeding systems included modifications of the traditional in vitro cultures on a solid medium (SM), a liquid medium (LM) with rotary shaking and temporary immersion bioreactors (TIB), as well as cultivation in soil (ex vitro conditions). Moreover, the antioxidant properties of the plant tissue cultivated in those various conditions were compared.

Advanced culture techniques, such as agitated cultures or cultures in bioreactors, can be used to increase the yield of plant tissue, which is hard to obtain with conventional cultures in solid media or traditional in-soil plant cultivation (Moreira et al. 2013; Kunakhonnuruk et al. 2019) demonstrated that cultivation of the medical plant *Drosera communis* in LM and a temporary immersion system increased the biomass production, dry weight accumulation, number of shoots, and diameter of the plant. An increased growth rate of plants cultivated in LM (agitated culture) compared

Table 2 The productivity of phenolic derivatives (mg × container^{-1} × 6 weeks $^{-1}$ [± SD]) in *Pontechium maculatum* cultivated in: solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Different letters indicate statistical significance of means acc. one-way ANOVA, post hoc Tuckey test at $p < 0.05$, SD—standard deviation

Phenolic compound		Solid medium	Liquid medium	Plantform bioreactor	Soil (ex vitro)
Chlorogenic acid	mg × container $^{-1}$ × 6 weeks $^{-1}$ [± SD]	0.86 ± 0.09^{a}	0.82 ± 0.04^{a}	19.14 ± 1.23^{b}	0.55 ± 0.01^{a}
Cryptochlorogenic acid		1.42 ± 0.31^{a}	1.62 ± 0.06^{a}	24.42 ± 1.47^{b}	0.44 ± 0.03^{a}
Caffeic acid		1.51 ± 0.31^{a}	1.97 ± 0.18^{a}	39.77 ± 1.13^{b}	0.79 ± 0.04^{a}
Ferulic acid		1.09 ± 0.09^{a}	1.44 ± 0.25^{a}	72.83 ± 0.79^{b}	0.51 ± 0.10^{a}
O-coumaric acid		3.38 ± 0.24^{a}	3.92 ± 0.13^{a}	237.03 ± 2.50^{b}	1.63 ± 0.17^{a}
Isoferulic acid		4.10 ± 0.52^{b}	4.67 ± 0.09^{b}	51.48 ± 0.46^{c}	1.56 ± 0.12^{a}
Isochlorogenic acid		8.16 ± 1.24^{b}	14.85 ± 0.21^{c}	211.79 ± 2.83^{d}	2.06 ± 0.35^{a}
Rosmarinic acid		64.17 ± 4.67^{ab}	89.91 ± 1.18^{b}	2493.95 ± 30.60^{c}	30.17 ± 0.69^{a}
Rutin		3.23 ± 0.66^{a}	4.40 ± 0.38^{a}	136.30 ± 6.52^{b}	1.96 ± 0.27^{a}
Kaempferol		3.44 ± 0.60^{b}	5.69 ± 0.03^{c}	29.69 ± 0.26^{d}	2.51 ± 0.06^{a}
Shikonin		2.01 ± 0.01^{a}	1.54 ± 0.05^{a}	76.69 ± 4.69^{b}	1.36 ± 0.01^{a}

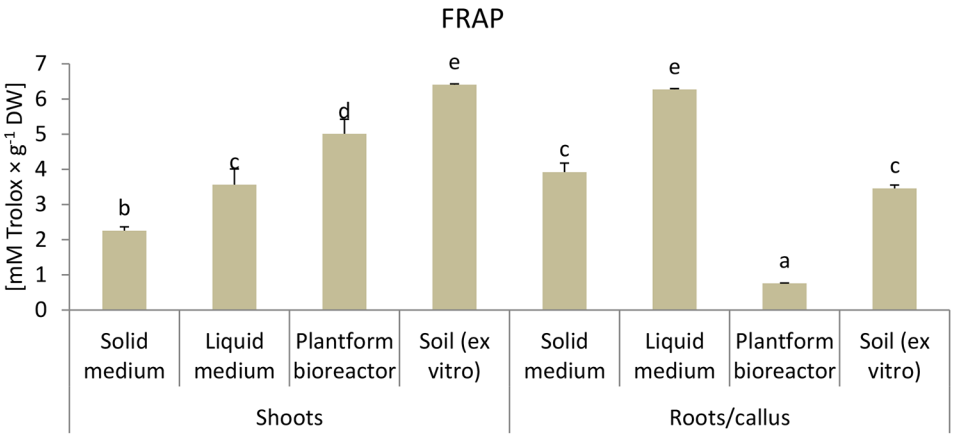

Fig. 6 Ferric reducing antioxidant power (FRAP) of extracts derived from shoots and roots of *Pontechium maculatum* cultivated in solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Upper case letters indicate statistical significance of means acc. two-way ANOVA, post hoc Tuckey test at $p < 0.05$; the bar represents the standard deviation

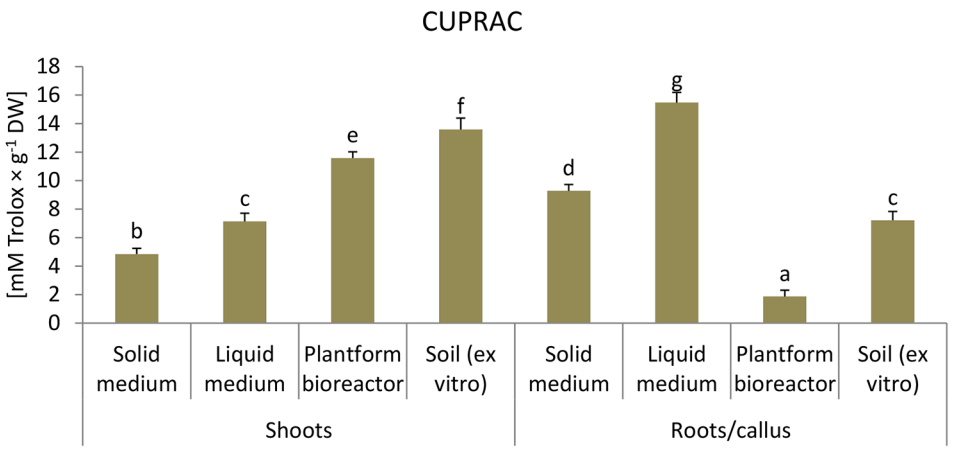

Fig. 7 Cupric-ion-reducing antioxidant capacity (CUPRAC) of extracts derived from shoots and roots of *Pontechium maculatum* cultivated in solid medium, liquid medium with rotary shaking, temporary immersion bioreactor Plantform, and soil (ex vitro conditions). Upper case letters indicate statistical significance of means acc. two-way ANOVA, post hoc Tuckey test at $p < 0.05$; the bar represents the standard deviation

to plants from SM was reported by Makowski et al. (2020) in the case of *Dionaea muscipula* plants. Similar breeding strategies resulted in a higher biomass growth for *Scutellaria alpine* and *Arnebia euchroma* Grzegorczyk-Karolak et al. 2017; Malik et al. 2016b, respectively). Optimized conditions for the cultivation of *Nasturtium officinale*, *Dracocephalum forrestii*, or *Rhododendron tomentosum* in temporary immersion bioreactors were previously demonstrated by Klimek-Szczykutowicz et al. (2021), Weremczuk-Jeżyna et al. (2020), and Jesionek et al. (2018), respectively. Both LM and TIB can be more effective for plant biomass production because of better nutrient distribution and oxygen availability in the growing medium (Klimek-Szczykutowicz et al. 2021). Moreover, the aeration phase

in the growing cycle in TIB can lead to ethylene elimination from the growing chamber (Bello-Bello et al. 2010). In the experiments described in this article, the cultivation of *P. maculatum* plants in LM was conducted to decrease the growth index. Lower biomass accumulation can be an effect of hydromechanical stress appearing in such conditions (Makowski et al. 2020). Mechanical stimulation can affect plant defense mechanisms, manifested by a decreased growth rate and up-regulation of pathways connected with secondary metabolite production (Lattanzio et al. 2018). Moreover, our findings show that the shoots of the *P. maculatum* plants cultivated in ex vitro conditions exhibited increased levels of dry weight. This may be related to the rebuilding of the cell wall during the process of acclimatization from in vitro to ex vitro conditions (Salgado Pirata et al. 2022). Furthermore, in ex vitro conditions, plants perform effective photosynthesis, directly affecting the dry matter accumulation in photosynthetic organs (Soni et al. 2021).

Manipulation in tissue culture conditions, including temporary immersion systems, can lead to spontaneous changes in plant morphology (Debnath 2016). In our study, the *P. maculatum* plants rising in TIB produced callus lumps with emerging shoots with a different morphology than those from the other in vitro or ex vitro conditions. It is likely that TIB induced hormonal changes in the plant tissue, leading to callus proliferation instead of root formation (Arigundam et al. 2020). It is a well-known fact that flooding can increase ethylene accumulation in plant tissue (Grichko and Glick 2001). We hypothesize that the ethylene accumulation promoted the callus formation in the upper parts of the plants rather than root development. Additionally, ethylene accumulation may have led to an increased synthesis of phenolic compounds in *P. maculatum* organs obtained from TIB (Bidelington 1992).

The concentration of plant secondary metabolites depends on the type of tissue, age of the organ, and cultivation conditions. Moreover, it has been demonstrated that the accumulation of phenolic compounds differs between plant tissue cultures and intact plants (Muraseva and Kostikova 2021). This is why one of the aims of the research presented here was to study the changes in phenolic compound production in *P. maculatum* plants cultivated in various conditions. As far as we know, there are no reports regarding the synthesis of phenolic compounds in in vitro cultures of *P. maculatum* plants. Phenolic compounds are plant secondary metabolites produced as defense chemicals protecting against various environmental stressors (Cheynier et al. 2013). In our study, the highest total phenolic content (TPC) was obtained in the shoots of the plants cultivated in TIB and ex vitro as well as in the roots from LM, reaching a concentration of 40–45 mg × g^{-1} DW. The lowest TPC value was seen in the *P. maculatum* roots harvested from soil (ex vitro conditions). This result contradicts the data presented by Dresler et al. (2015). In their work, the roots of *P. maculatum* harvested from intact plants were reported as the richest source of valuable phenolic metabolites. Additionally, Kikowska et al. (2020) reported a higher concentration of phenolic acids and flavonoids in the roots of *Eryngium alpinum* intact plants than in the roots from in vitro conditions. On the other hand, some research in the field of medical plants indicates that an increased accumulation of phenolic compounds is obtained in plants cultivated in in vitro conditions in comparison to plants cultivated in soil. Grzegorczyk-Karolak et al. (2019) obtained the highest accumulation of phenolic compounds in *Salvia viridis* shoot cultures. Similar observations were published by Costa et al. (2013), where an in vitro culture of *Lavandula viridis* had a higher concentration of phenolic compounds than intact plants. Nevertheless, our results obtained for TPC in *P. maculatum* plants indicated that modifications in culture conditions, such as LM or TIB, can affect the synthesis of valuable plant-derived chemicals in shoots and/or roots.

HPLC-DAD analyses of the *P. maculatum* shoots and roots confirmed the presence of eight phenolic acids: chlorogenic, cryptochlorogenic, caffeic, ferulic, o-coumaric, isoferulic, isochlorogenic, and rosmarinic. Moreover, a phytochemical analysis confirmed the accumulation of two flavonoids: rutin and kaempferol, as well as one 1,4-naphtohinone - shikonin. The qualitative composition of the phenolic compounds was the same for the plants cultivated ex vitro and the tissue cultures cultivated in various conditions (SM, LM, or TIB). The application of these various breeding strategies highly influenced the quantitative composition of the plant-derived extracts. Rosmarinic acid was a qualitatively dominant phenolic acid, the best source of which were the shoots and callus tissue of the *P. maculatum* harvested from TIB (the total amount of rosmarinic acid in the shoots and callus lumps from TIB was 4802.52 mg × 100 g^{-1} DW). Additionally, a very high concentration of rosmarinic acid was detected in the *P. maculatum* roots cultivated in LM with rotary shaking (1433.86 mg × 100 g^{-1} DW). An analysis of phenolic compound productivity showed that by using TIB, it is possible to obtain nearly 2.5 g of rosmarinic acid per one bioreactor in 6 weeks. Our results clearly indicate that *P. maculatum* plants cultivated in TIB are a very rich source of rosmarinic acid compared to well-known medical plants in which this phenolic acid is also a dominant phenolic derivative. For example, in an in vitro culture of *S. viridis* shoots, the level of rosmarinic acid was up to 1150.00 mg × 100 g^{-1} DW (Grzegorczyk-Karolak et al. 2019; Kikowska et al. 2020) found that rosmarinic acid was also a dominant secondary compound in *E. alpinum*, and its highest concentration of 358.05 mg × 100 g^{-1} DW was reached for intact plants.

The Plantform bioreactor was the most effective system for synthesizing a few other phenolic acids in *P. maculatum* shoots. The chlorogenic acid accumulation was 30.44 mg × 100 g^{-1} DW, with a productivity of 19.14 mg × container $^{-1}$ × 6 weeks $^{-1}$. Moreover, the *P. maculatum* callus tissue in TIB accumulated the highest concentration of o-coumaric acid (238.41 mg × 100 g^{-1} DW). On the other hand, isoferulic and isochlorogenic acids had the highest levels of synthesis in the shoots of the plants cultivated in TIB and the roots from LM with rotary shaking. The best source of rutin was the *P. maculatum* shoots in TIB, while the accumulation of the kaempferol increased the most in the roots from LM. Increased syntheses of phenolic acids or flavonoids in plants cultivated in temporary immersion systems have also been reported for *S. viridis*, *S. chinensis*, and *D. forrestii* (Grzegorczyk-Karolak et al. 2022; Szopa et al. 2019b; Weremczuk-Jeżyna et al. 2020). In our study, we demonstrate that TIB is the most effective system for the productivity of the selected phenolic acids and flavonoids. This parameter is important when a medical plant species is used as a source of some plant-derived chemicals for industry. Increased synthesis of valuable secondary metabolites, such as phenolic compounds in temporary immersion systems, may result from the flooding of plant material during the growth cycle (Klimek-Szczykutowicz et al. 2021). Such conditions may lead to limited oxygen availability and hypoxia stress. In response to stress conditions, a plant can produce more phenolic compounds as part of its defense against an increased synthesis of reactive oxygen species and protect lipid membranes from lipid peroxidation (Cheynier et al. 2013). Furthermore, changes in the accumulation of phenolic compounds in agitated cultures may be related to hydromechanical stress and attrition connected with shaking during the cultivation cycle (Makowski et al. 2020; Szopa et al. 2017).

P. maculatum plants can accumulate the 1,4-naphtoquinone derivative shikonin (Dresler et al. 2015). Shikonin is a commercially important plant secondary metabolite known for various biological activities, such as antimicrobial, insecticidal, antitumor, and antioxidant. These compounds are colored and therefore have applications in the food, textile, and cosmetic industries (Malik et al. 2016a). In the research presented here, we found that the TIB system is the most useful for the synthesis and productivity of shikonin in *P. maculatum.* The level of this compound reached 77.26 mg × 100 g DW and 70.41 mg × 100 g^{-1} DW in the shoots and the callus tissue from TIB, respectively. Moreover, the productivity of shikonin increased 38.2-fold in the *P. maculatum* culture growing in TIBP compared to the plants from SM. Our results concur with the findings of Gupta et al. (2014), where the highest productivity of shikonin in the cell lines of *Arnebia* sp. was obtained in air-lift bioreactors compared to LM with shaking. Furthermore, it has been shown that biotechnological tools such as plant transformation or modifications of breeding strategies in plant tissue cultures can be useful in shikonin production and its upscaling (Pal et al. 2019; Sykłowska-Baranek et al. 2012).

All the plant-derived extracts described here were characterized by significant antioxidant activity. However, various breeding strategies of *P. maculatum* influenced the antioxidant potential of the extracts obtained. Similar observations were made by Grzegorczyk-Karolak et al. (2019), where the extracts taken from *S. viridis* plants cultivated in various conditions had different biological activities. Additionally, the same authors concluded that the antioxidant activity measured by the DPPH and FRAP assay was strongly correlated with TPC in the plants used in their research (Grzegorczyk-Karolak et al. 2019; Costa et al. 2013) reported a higher antioxidant potential of wild *L. viridis* plants than the antioxidant potential of the plants from in vitro conditions, which concurs with our results. In the research described here, the highest antioxidant capacity was observed for the shoots from ex vitro conditions, independent of the method applied (FRAP or CUPRAC). Moreover, the results obtained for TPC followed a similar trend to that seen in the results for antioxidant activity. It was previously reported by Makowski et al. (2020) that the antioxidant capacity of plant-derived extracts is affected by the amount of phenolic acids. In our study, the highest concentrations of phenolic acids were found in the shoots/callus tissue of *P. maculatum* cultivated in TIB. Nevertheless, the antioxidant capacity of various phenolic derivatives is different, and in our study, the accumulation of only several phenolic compounds from the examined plants was analyzed by DAD-HPLC. The presence of other phenolics included in plant-derived extracts could be important for the antioxidant capacity. This issue is the basis for further research on the biologically active properties of individual secondary metabolites contained in tissues of *P. maculatum* plants.

Conclusions

This article describes the first research focused on the synthesis and productivity of the phenolic compounds in *Pontechium maculatum* plants in in vitro conditions. The results clearly indicate that Russian bugloss plants cultivated in temporary immersion bioreactors Plantform are the best source of phenolic acids, flavonoids, and the 1,4-naphtoquinone derivative shikonin compared to the other cultivation strategies we tested. Our study shows that *P. maculatum* tissue cultures may be a source of extracts with high antioxidant potential, while the highest antioxidant potency was

found in the roots of examined plants obtained from a liquid medium with rotary shaking.

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Author contributions W.M. designed the experiment. W.M., A.K. and K.M.T. interpreted, and discussed the data. W.M. performed the statistical analysis, prepared the graphical part of the manuscript, and wrote the manuscript. W.M. and A.K. contributed to data acquisition. A.S. and H.E. developed the analytical method for the determination of phenolic compounds using DAD-HPLC. W.M., A.K., B.T., K.M.T., A.S. and H.E. checked and corrected the manuscript. All authors proofread the manuscript, agreed on its contents, and consented to its submission.

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

Declarations

Conflict of interest The authors declare no conflict of interest exists.

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