ORIGINAL ARTICLE

Optimization of cultivation conditions of *Salvia viridis* L. shoots in the Plantform bioreactor to increase polyphenol production

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Abstract

Salvia viridis is a rich source of pharmacologically-important polyphenolic compounds with anti-inflammatory, antioxidant and anticancer activities. The aim of the present study was to optimise the large-scale cultivation of sage shoots in the Plantform bioreactor. It evaluated the effect of culture duration (two or three weeks), the use of a semi-continuous system and the immersion frequency in a liquid medium (every 80 or 170 min) on the growth and accumulation of secondary metabolites. The content of bioactive phenolic acids and phenylethanoids in the obtained material was determined using HPLC. The most efficient growth parameters, expressed as fresh (26.99 g) and dry weight (3.51 g) as well as proliferation ratio (18.6), were observed for shoots grown in a fed-batch system, immersed every 80 min; this approach yielded a 33-fold biomass increase within four weeks. However, the most efficient production of bioactive compounds was achieved for shoots grown for three weeks in a batch system, immersed every 80 min: total phenolic acid, total phenylethanoid and total phenol contents of 18.3, 11.4 and 29.7 mg/g DW. In these conditions, within three weeks, total phenolic acid level was almost 10 times higher than that found in the aerial parts of four-month-old soil-grown plants with a similar phenylethanoid level. Hence, the described *S. viridis* shoot culture cultivated in the Plantform bioreactor may be an alternative efficient source of plant material rich in valuable compounds.

Key message

Optimization of culture conditions (culture duration, the use of fed-batch mode, frequency of the medium application) for growth and productivity of bioactive compounds in *Salvia viridis* shoots cultivated in the Plantform bioreactor.

Abbreviations

MS	Murashige & Skoog medium
IAA	Indole-3-acetic acid
m-Top	Meta-topolin
DW	Dry weight
FW	Fresh weight
TIS	Temporary immersion system
HPLC	High-performance liquid chromatography
RA	Rosmarinic acid
VB	Verbascoside
MRA	Methyl rosmarinate
MART	Martynoside
LEU	Leucosceptoside A
ISOVB	Isoverbascoside
CA	Caffeic acid
CAH	Caffeic acid hexoside
SAF(I)	Salvianolic acid F isomer (I)
SAF(II)	Salvianolic acid F isomer (II)
SAB	Salvianolic acid B

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Introduction

Salvia viridis L. (syn. *Salvia horminum* L.), also commonly-known as red-topped sage, painted sage, annual clary and Joseph's sage, is an annual or biennial member of the *Lamiaceae* family (Bown 2002). Being native to the Mediterranean countries and Anatolia (Turkey), it prefers dry, rocky areas as well as fields, wastelands and dunes. It can also be found in Iran and in the Caucasus (Davis 1982) where it has long been known for its medicinal properties in traditional medicine. For centuries, it has been used as a remedy for infectious diseases and inflammatory disorders, especially affecting the eyes and oral cavity (Dweck 2000; Ghorbani 2005).

S. viridis is rich in biologically-active chemical compounds, most of which are polyphenols and terpenoids; however, mono- and sesquiterpene hydrocarbons are also present in the essential oil (Yayli et al. 2010). The aerial parts are mainly rich in polyphenolic derivatives such as phenolic acids, phenylethanoids and flavonoids, with rosmarinic acid, verbascoside and glycoside derivatives of apigenin and luteolin dominating (Grzegorczyk-Karolak and Kiss 2018). In the roots of the species, polyphenolic acids and diterpene derivatives predominate with small amounts of flavonoids and phenylethanoids also being present (Rungsimakan 2014; Zengin et al. 2019).

While *S. viridis* is mainly cultivated as an ornamental plant in Europe, increasing attention is being focused on the multidirectional pharmacological potential of its shoots and roots. Rosmarinic acid and verbascoside demonstrate rich and well-documented biological activity, and are present in significant amounts in extracts from *S. viridis*. The varied chemical composition of the plant makes it potentially useful in disorders such as diabetes (Naimi et al. 2017; Wu et al. 2020), Alzheimer's (Habtemariam 2018) and Parkinson's diseases (Lv et al. 2019) and depression (Liang et al. 2016; López-Rodríguez et al. 2019). It may also be used to treat inflammatory disorders (Lee et al. 2013; Alipieva et al. 2014; Luo et al. 2020), as well as these caused by bacteria such as *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli* or *Proteus vulgaris* (Ulubelen 2003; Grzegorczyk-Karolak et al. 2019) and fungi such as *Candida* spp. and *Aspergillus brasiliensis* (Grzegorczyk-Karolak et al. 2019). Compounds isolated from the plant also show antiviral activity; they have been found to inhibit *Herpes simplex* virus type 1 attachment to host cells and affect their penetration, inhibit HIV-1 integrase, an enzyme essential for viral replication, and reduce influenza-induced lethality and clinical syndromes in mice in vivo (Tewtrakul et al. 2003; Astani et al. 2014; Hu et al. 2016). They also show effective action against several species of protozoa, e.g.

Leishmania donovani, L. amazonensis, or *Trypanosoma brucei rhodesiense* (Wu et al. 2020). Thanks to its antioxidant activity, the species is a valued agent in the cosmetic and food industries (Grzegorczyk-Karolak and Kiss 2018). *S. viridis* extracts have also been found to have anticancer (Nadeem et al. 2019; Wu et al. 2020) and anti-angiogenic (Vieira et al. 2020) properties. Other biological activities of this plant include: photo- (Alipieva et al. 2014), gastro- (López-Rodríguez et al. 2019), cardio- (Ulubelen 2003; Quan et al. 2021), hepatoprotective (Elufioye and Habtemariam 2019) and even immunosuppressive (Yun et al. 2003) effects.

As the plant demonstrates such broad therapeutic potential, including civilization diseases, and considering that it is an annual plant, requiring annual sowing, there is a clear need to develop effective methods for its cultivation. Due to the almost unlimited demand for high-quality standardized sources of bioactive compounds, which regrettably results in the extinction of species, alternative methods of meeting them, such as in vitro plant cultures, are being investigated.

Plant biotechnology allows fast, efficient and easily reproducible production of plant raw materials and their isolated compounds. In vitro cultures offer many advantages over traditional plant cultivation methods, such as independence from climatic and geographical conditions, possibility of efficient propagation of species that are difficult to reproduce in vivo or obtaining large amounts of raw material in a short time from a small quantity of explants (Sharma et al. 2015; Espinosa-Leal et al. 2018). However, an important step in the optimization of cultivation, necessary for its profitability, is increasing its scale; one approach to this is through the use of special bioreactors for the propagation of plants and their organs.

Bioreactor cultivation allows manual operations to be streamlined and automated, saves time and reduces risk of infection, thus reducing costs of procedure. It also offers the possibility of monitoring and adjusting culture conditions at different stages. Additionally, the use of liquid media gives explants better access to nutrients evenly dispersed in the medium, which often increases yield. Moreover, unlike solid media, the nutrients are not absorbed by gelling agents, whose removal from the media results in additional reduction of culture costs (Tripathi and Tripathi 2005).

Recent reports indicate that temporary immersion systems (TIS) seem to be particularly beneficial for plant breeding. They allow to avoid complete and continuous immersion of plant organs in the medium, which often results in various types of anomalies and growth disorders, including hyperhydricity (Damiano et al. 2005). An example of such a system is the Plantform bioreactor (Plant Form, Sweden). So far, it has been successfully used for the propagation of a some of medical plants, including *Olea europaea* L. (Benelli and De Carlo 2018), *Phoenix dactylifera* L. (Almusawi et al.

2017), *Rubus idaeus* L., *Echinacea purpurea* (L.) Moench. (Welander et al. 2014), *Vaccinium corymbosum* L., *V. angustifolium* L. (Welander et al. 2017) or *Schisandra chinensis* (Turch.) Baill. (Szopa et al. 2017). However, no studies appear to have examined the in vitro cultivation of *Salvia spp.*, including *S. viridis*, in this type of bioreactor.

Therefore, the aim of this study was to establish a culture of *Salvia viridis* L. shoots in the Plantform bioreactor and evaluate the level of bioactive phenols in the obtained plant material. Previous biotechnological studies on *S. viridis* have focused on differentiated and undifferentiated cultures of this species (Grzegorczyk-Karolak et al. 2019), as well as on the optimization of the conditions for their growth and bioactive metabolite production (Grzegorczyk-Karolak et al. 2020). Other studies have examined the genetic transformation of *S. viridis* by *Agrobacterium rhizogenes* and obtaining hairy root cultures which, after their optimization, produced 10-times higher levels of polyphenolic acids compared to the roots of naturally-grown plants (Grzegorczyk-Karolak et al. 2018; Grzegorczyk-Karolak 2020).

Since the growth of cultures in a bioreactor system and their secondary metabolite accumulation could strongly depend on the cultivation conditions, the present study examines the effect of changes in culture duration (i.e. two or three weeks) and the frequency of immersing the culture in the medium (i.e. every 80 or 170 min). The effect of modifying the culture by adding a fresh portion of the medium after two weeks (fed-batch system) was also tested.

Materials and methods

Plant material

The explants used in the study were obtained from *S. viridis* shoot culture maintained as described earlier (Grzegorczyk-Karolak et al. 2019) and grown on MS (Murashige and Skoog 1962) solid (0.7% agar) medium with 0.1 mg/L IAA and 1 mg/L m-Top. This growth regulator combination has been experimentally established as optimal during previous research (Grzegorczyk-Karolak et al. 2020).

Shoot culture in the Plantform bioreactor

Shoots were cultivated in a bioreactor with the Plantform temporary immersion system (PlantForm, Hjärup, Sweden). The bioreactor consists of a transparent polycarbonate container, a frame with four legs and a polypropylene chamber. Air flow is controlled through ports equipped with 0.2 μm filters, two of which are connected through polypropylene sterilized filters to the system that pumps sterile air into the lower chamber of the bioreactor, which contains the medium. The chamber includes a basket with holes, on which the plant material is placed. The apparatus is closed from the top with a snap polypropylene lid, isolated with silicon ring from the inside. The aeration and immersion period of the culture was regulated using two Hailea air pumps (Guangdong Hailea Group Co., Ltd, Guangdong, China): ACO-9610 (power 10 W; capacity 10 L/min) and ACO-9602 (power 5 W; capacity 7.2 L/min) connected with bioreactor via silicon tubes. Immersion time and frequency were programmed with a timer (ESIC AX300) connected to the device. More detailed information about the bioreactor system is available on the manufacturer's website. Before use, the bioreactor and the medium were sterilized at a temperature of 121 °C, under a pressure of 1 atm., for 17 min.

For cultivation, about 0.84 g FW (0.11 g DW) inoculum, comprising 18 shoots containing the apical meristem approximately 1 cm in length, was placed in Plantform bioreactor (Fig. 1A). Initially, the bioreactor contained 500 mL of MS medium with 0.1 mg/L IAA and 1 mg/L m-Top. The explants was immersed with the medium every 80 min for 10 min. The cultures were grown in batch mode, and the shoot growth parameters were estimated after 2 and 3 weeks.

The next stage of the experiment tested the fed-batch culture system. Briefly, the bioreactor initially contained 450 mL of the growth medium; 2 weeks after inoculation, this amount was supplemented with 300 mL fresh medium, making a total of 750 mL. In such cases, the growth parameters were determined after a further week and two weeks: the total duration of the culture was 3 and 4 weeks.
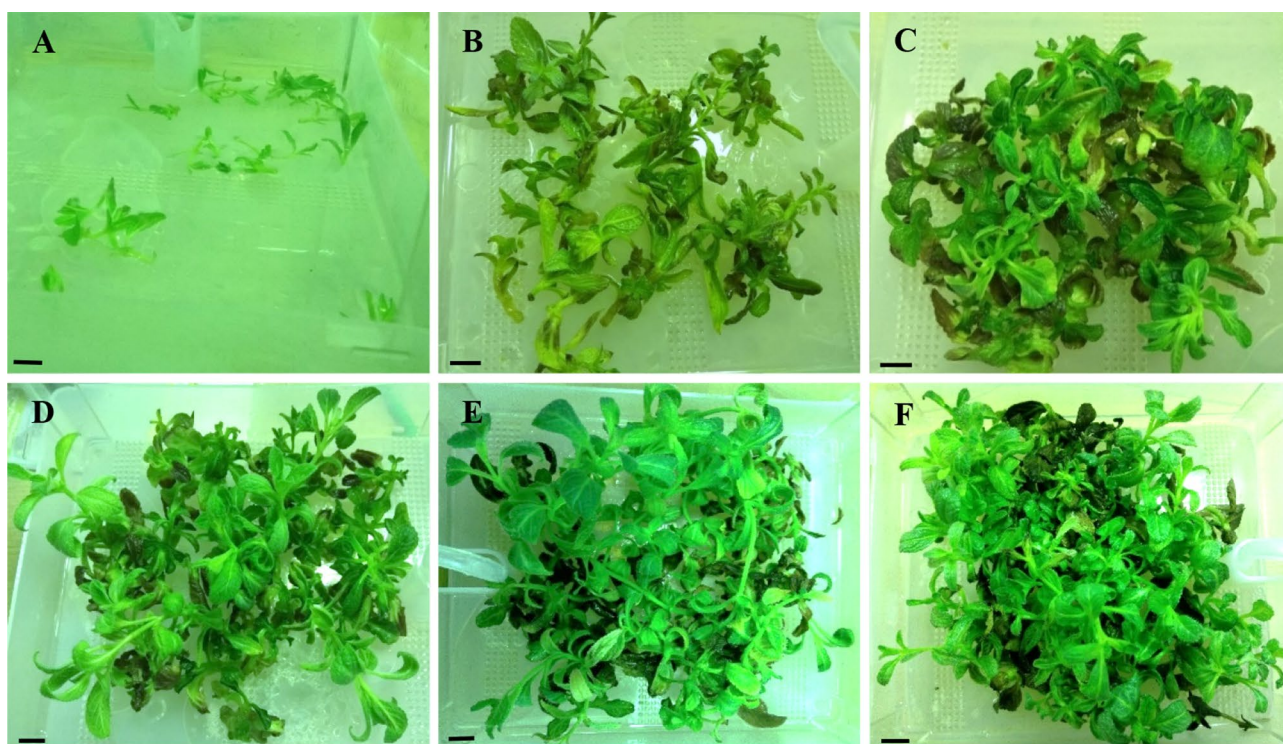
In the last stage of the experiment, the number of daily immersion periods was halved (the culture was immersed every 170 min for 10 min). In this experiment, the culture was kept under earlier established optimal growth conditions; after two weeks the medium was partially refilled, and the growth parameters were determined four weeks after inoculation.

All cultures were grown in a growth chamber at 26 ± 2 °C, 80–90% humidity, under a 16 h photoperiod in artificial light (fluorescent lamps; 40 μm m^{-2} s^{-1}). After a cultivation time appropriate to the treatment, the mean number of buds/shoots formed per explant (proliferation ratio), the mean shoot length and the ratio of obtained shoots to buds (structures less than 0.5 cm long) were determined. Shoot morphology and fresh (FW) and dry weight (DW) of culture [g/culture] were also established.

Phytochemical analysis

Following cultivation, shoots from all treatments were collected for analysis. The plant material (100 mg) was lyophilised, pulverized, extracted three times using methanol/water (8:2 v/v) as described earlier (Grzegorczyk-Karolak and Kiss 2018), and combined extracts were evaporated under reduced pressure.

with the one-way ANOVA test and multivariate analysis of variance, followed by the Tukey's test. Values marked with the same letter were not significantly different (at $p \leq 0.05$).

Results

Propagation and biomass growth

The effect of different cultivation variants on *S. viridis* culture growth and production of secondary metabolites was evaluated. In all protocols, the shoots cultivated in Platform bioreactor showed vigorous growth and normal morphology, regardless of treatment.

Initial observations concerned the optimal time for cultivating shoots in a bioreactor with a temporary immersion system. Most explants from the batch culture immersed in the medium 10 min every 80 min demonstrated shoot and/or bud formation after two weeks (Fig. 1B). After this cultivation time, the proliferation ratio was 8.2 (Fig. 2A), and the fresh weight of the culture reached 9.1 g (Fig. 2C), while the dry weight was 1.38 g (Fig. 2D). Extending the duration of the culture by another week resulted in intense growth of the culture (Fig. 1C). Although the proliferation index increased only slightly during third week of batch culture (8.9), and the mean length of the main and axillary shoots did not change significantly (Fig. 2E-F), the biomass of the culture doubled during this time. A significant change was also observed in the percentage ratio of obtained shoots to buds (from 23 to 37%) (Fig. 2B). However, at the end of the third week of culture, the shoots, especially their lower leaves, started to darken, which could indicate aging of the culture due to depletion of nutrients in the medium. Further extending the duration of culture in the batch system proved impossible.

The next stage of the experiment was based on a semi-continuous system with supplementation with extra medium two weeks after initial inoculation. The immersion frequency of *S. viridis* shoots in the medium was not changed. Evaluation of growth parameters three weeks after initiation of culture (Fig. 1D) revealed 30% higher fresh and 20% higher dry weight (Fig. 2C-D) than that obtained at the same time without medium supplementation. The changes in biomass were associated with a statistically significant increase in the proliferation ratio (13.4), a significant increase in the length of the main shoots and a significantly higher ratio of shoots to buds formed on the explants (Fig. 2B). After supplementation, the culture demonstrated active growth for three weeks and the shoots showed no changes in morphology or necrosis. This was decisive in extending the duration of the fed-batch culture by another week, up to four weeks. After another week, only a slight increase in biomass was observed; however, a further increase in the proliferation ratio and elongation of shoots, especially the main shoots,

was observed. These two parameters reached their optimal values in this cultivation treatment: proliferation ratio of 18.6 and elongation above 10 cm (Fig. 2). At the end of this period, however, aging and shoot darkening were observed (Fig. 1E).

The last stage of the research examined the effect of modifying the immersion frequency of the shoots in the fed-batch cultivation variant optimized for culture growth. In this variant, the shoots were again immersed in the medium for a period of 10 min, but at half the frequency, i.e. every 170 min (Fig. 1F). The *S. viridis* shoot culture immersed at this new frequency demonstrate slightly higher DW and FW, compared to those immersed every 80 min (Fig. 2C-D). They also demonstrated a higher ratio of axillary shoots to buds, i.e. from 38 to 51% (Fig. 2B), and greater axillary shoot length, i.e. about 1.2 times (Fig. 2F). On the other hand, fewer shoots/buds developed for the new variant (Fig. 2A), and the main shoots were significantly shorter (Fig. 2E). It was also observed that the leaves of shoots immersed every 170 min started to darken earlier, but the process was slower and less intensive in comparison with culture immersed twice as often.

Phenolic compound production

Six phenolic acids and four phenylethanoids were identified in the *S. viridis* shoot extract using UPLC-PDA-ESI–MS/MS, as described earlier (Grzegorczyk-Karolak et al. 2020). The same compounds were revealed in all extracts taken from all bioreactor cultivation variants; however, quantitative differences were revealed by HPLC analysis (Fig. 3). The content of individual compounds is presented in Table 1. In all samples, the predominant phenolic acid was rosmarinic acid (RA), and the predominant phenylethanoid was verbascoside (VB); the level of RA was higher than VB.

Longer cultivation time, regardless of whether the batch or fed-batch system was used, favored higher levels of most bioactive compounds. However, while the fed-batch system, i.e. where further medium was added during culture, was very beneficial for the growth of the culture, it turned out to be less effective for the production of polyphenolic compounds: the levels of most of the analyzed metabolites, including all phenylethanoids was lower. Also, lower biosynthesis of most bioactive compounds was observed when the frequency of immersion was reduced.

Ultimately, the highest levels of all phenylethanoids and most polyphenolic acids, including RA, were observed in the three-week batch culture (Table 1). In this protocol, the RA content (16.6 mg/g DW) was over 20% higher than in shoots grown for two weeks, and over 40% higher than in shoots from the four-week-old fed-batch culture. In the three-week batch system, the highest content, or close to it, was achieved by all polyphenolic acids, and thus the greatest
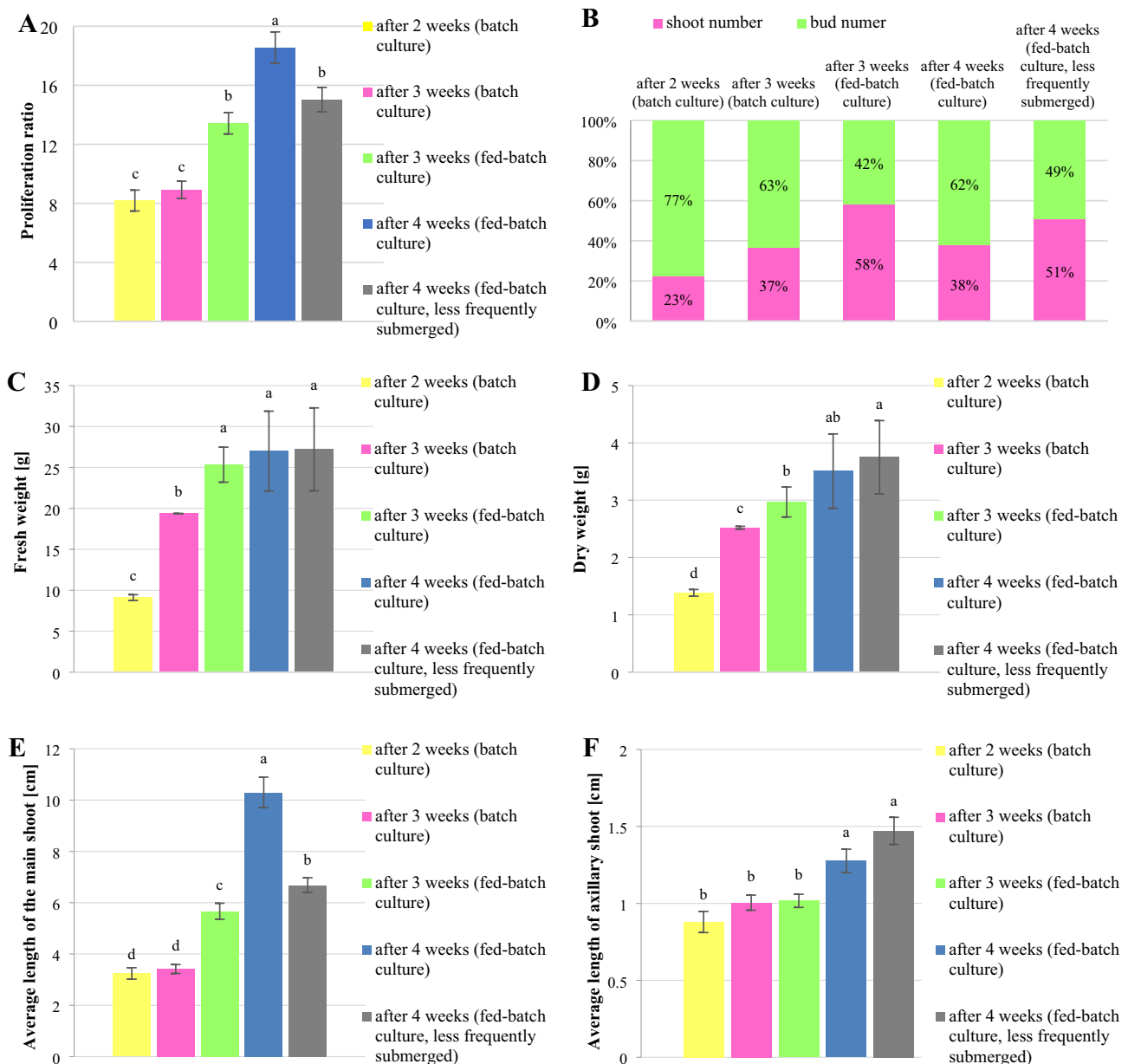
Fig. 2 Effect of different cultivation variants of *Salvia viridis* L. shoots in the Plantform bioreactor on: **A** proliferation ratio, **B** ratio of shoots to buds, **C** fresh weight, **D** dry weight, **E** length of the main shoot, and **F** length of axillary shoots. The results presented are mean values ± standard error (SE). Results were compared with the Tukey test at 5% statistical significance. Values marked with the same letter were not significantly different (at $p \leq 0.05$)

total polyphenolic acid content was reported (Table 1). However, the levels of other polyphenolic acid derivatives identified in the tested material were significantly lower than RA content; apart from methyl rosmarinate, they did not exceed 0.5 mg/g DW.

The three-week batch culture was also the most favourable for the biosynthesis of all phenylethanoids. The verbascoside (VB) level achieved was 9.80 mg/g DW. The conversion of the batch cultivation system to the fed-batch system reduced the VB content in the shoots by almost half, but

extending the duration of the fed-batch culture by another week increased production, and the final level was only 11% lower than observed for the three-week batch culture, which gave optimal phenylethanoid production (Table 1). The three-week cultivation variant also proved to be the most effective for producing the other tested phenylethanoids. The second most abundant compound in this group was LEU, the level of which exceeded 1 mg/g DW for the optimal cultivation variant. The three-week batch system thus ultimately proved to be the most efficient in terms of phenolic
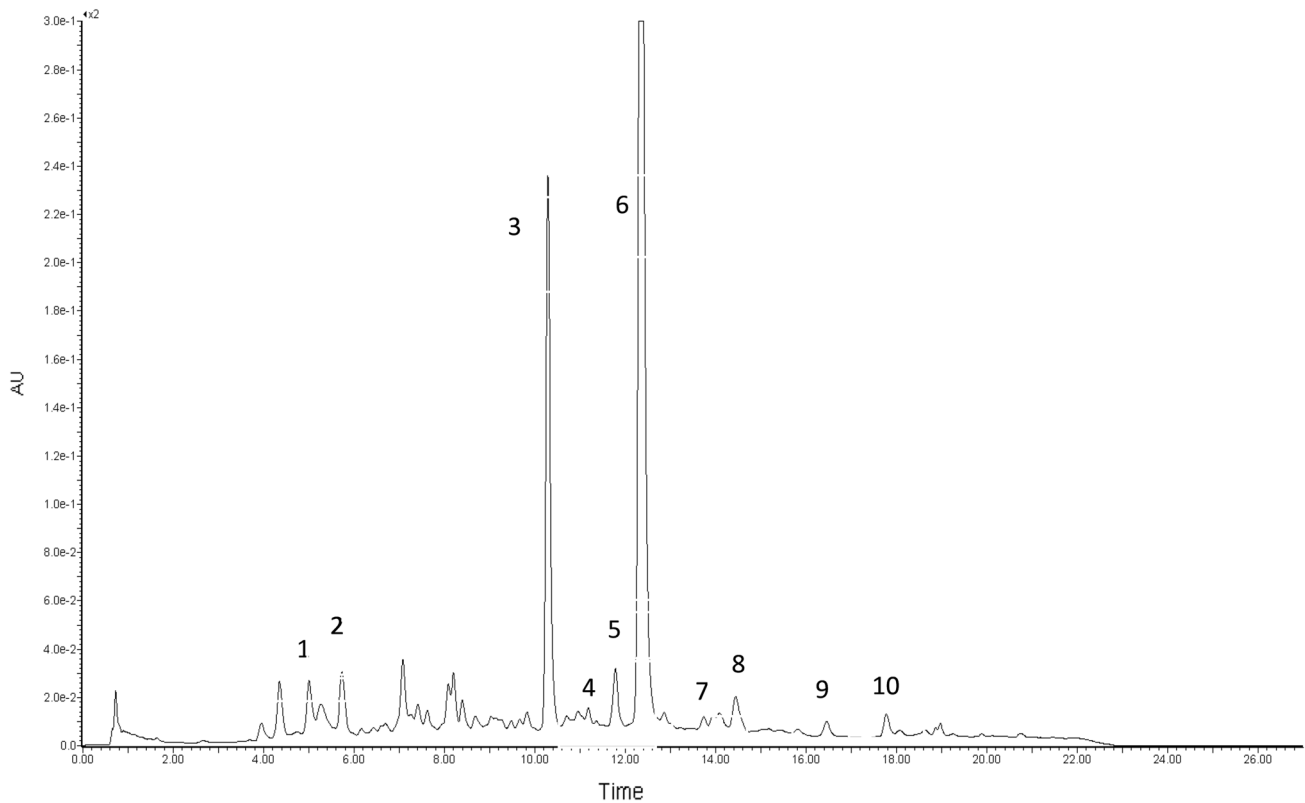
Fig. 3 Sample HPLC chromatogram of the hydromethanolic extract from Plantform bioreactor-cultivated shoot culture of *S. viridis*. 1-CAH, 2-CA, 3-VB, 4-ISOVB, 5-LEU, 6-RA, 7-MART, 8-MRA, 9-SAF(I), 10-SAF(II)

compound production, followed by the four-week fed-batch culture system (Table 1).

Since the most efficient for growth cultivation variant did not turn out to be optimal for the production of bioactive metabolites, it was therefore necessary to calculate the metabolite production in the biomass obtained for the different treatments during a single bioreactor cycle (Fig. 4). The highest total phenol production in *S. viridis* shoots during a single culture cycle was observed for the four-week fed-batch system (80.8 mg) followed by the three-week batch culture (74.8 mg); however, the differences were not statistically significant. Similar observations were made for total phenolic acid and phenylethanoid content; although in the case of the latter group, the difference between the two treatments was statistically significant, being approximately 20% higher than the three-week batch culture. On the other hand, some individual metabolites demonstrated optimal production in the three-week batch system. Meanwhile, similar production of RA, the predominant metabolite in the cultured in vitro sage shoots, was observed for the three-week batch system and four-week fed-batch cultures, at both the higher (every 80 min) and lower (every 170) immersion frequencies: 41.7 mg, 40.6 mg and 37.6 mg, respectively (no statistical differences) (Fig. 4).

Discussion

In recent years, numerous studies have been conducted to assess the potential of natural raw materials and the possibility of their use in the prevention and treatment of various diseases. One example of such a species is *Salvia viridis*, a plant used in traditional medicine in Turkey and Iran, and one confirmed to have pharmacological potential. To harness the potential of a locally-occurring species on a large scale, it is necessary to identify methods that will provide unlimited access to standardized material, rich in bioactive compounds, regardless of its natural occurrence. In addition, to be effective and economically profitable, the production of plant material must be carried out on a large scale; such approaches are used for fungal or bacterial metabolites, where production is often carried out on an industrial scale in bioreactors with volumes of several thousand litres. However, changing the cultivation of plant organs from solid to liquid media is much more difficult (Grzegorczyk-Karolak et al. 2021). It requires not only upscaling: the use of larger culture vessels with higher volumes of medium, it also entails other technical issues, such as the sensitivity of plant tissues to the shear forces occurring during mixing or the limited access to gases when the explants are completely

Table 1 Contents of phenolic compounds [mg/g DW] in *Salvia viridis* L. shoots cultured in the Plantform bioreactor, in MS liquid medium with 0.1 mg/L IAA and 1 mg/L m-Top under different cultivation conditions

Compound	Content of phenolic compounds [mg/g DW]				
	After 2 weeks (batch culture)	After 3 weeks (batch culture)	After 3 weeks (fed-batch culture)	After 4 weeks (fed-batch culture)	After 4 weeks (fed-batch culture, less frequently submerged)
Phenolic acids					
Rosmarinic acid	13.40 ± 0.21b	16.57 ± 0.68a	6.86 ± 0.15e	11.57 ± 0.21c	10.03 ± 0.16d
Methyl rosmarinate	0.56 ± 0.006a	0.49 ± 0.01b	0.50 ± 0.01b	0.42 ± 0.004c	0.41 ± 0.004c
Caffeic acid hexoside	0.13 ± 0.005d	0.25 ± 0.01b	0.17 ± 0.006c	0.49 ± 0.04a	0.18 ± 0.04c
Caffeic acid	0.19 ± 0.004b	0.24 ± 0.008a	0.18 ± 0.005b	0.10 ± 0.03c	0.09 ± 0.05c
Salvianolic acid F (I)	0.31 ± 0.01a	0.33 ± 0.009a	0.33 ± 0.04a	0.35 ± 0.01a	0.16 ± 0.01b
Salvianolic acid F (II)	0.24 ± 0.01c	0.39 ± 0.01b	0.45 ± 0.01a	0.48 ± 0.02a	0.16 ± 0.02d
Phenylethanoids					
Verbascoside	3.47 ± 0.08d	9.80 ± 0.43a	5.10 ± 0.12c	8.68 ± 0.22b	5.50 ± 0.08c
Leucosceptoside A	0.75 ± 0.02b	1.13 ± 0.05a	0.55 ± 0.022c	0.72 ± 0.017b	0.52 ± 0.009c
Isoverbascoside	0.17 ± 0.01c	0.33 ± 0.03a	0.15 ± 0.02c	0.13 ± 0.03c	0.26 ± 0.02b
Martynoside	0.07 ± 0.05b	0.16 ± 0.02a	0.05 ± 0.04 c	0.07 ± 0.01bc	0.05 ± 0.01c
Total compound content					
Phenolic acid	14.8 ± 0.25b	18.3 ± 0.73a	8.5 ± 0.19d	13.4 ± 0.25b	11.0 ± 0.20c
Phenylethanoid	4.5 ± 0.11d	11.4 ± 0.54a	5.8 ± 0.17c	9.6 ± 0.25b	6.3 ± 0.12c
Phenol	19.3 ± 0.36c	29.7 ± 1.27a	14.3 ± 0.36e	23.0 ± 0.5b	17.3 ± 0.32d

The results presented are mean values ± standard error (SE) of nine replicates for each plant material

Results were compared with Tukey's test at 5% statistical significance

Values marked with the same letter were not significantly different (at p ≤ 0.05)

Fig. 4 The effects of different cultivation variants for *Salvia viridis* L. shoots in the Plantform bioreactor on total phenolic acid (TPA), phenylethanoid (TPE) and polyphenol (TP) content. The results presented are mean values ± standard error (SE) of nine replicates for each plant material. Results were compared with Tukey's test at 5% statistical significance. Values marked with the same letter were not significantly different (at p ≤ 0.05)
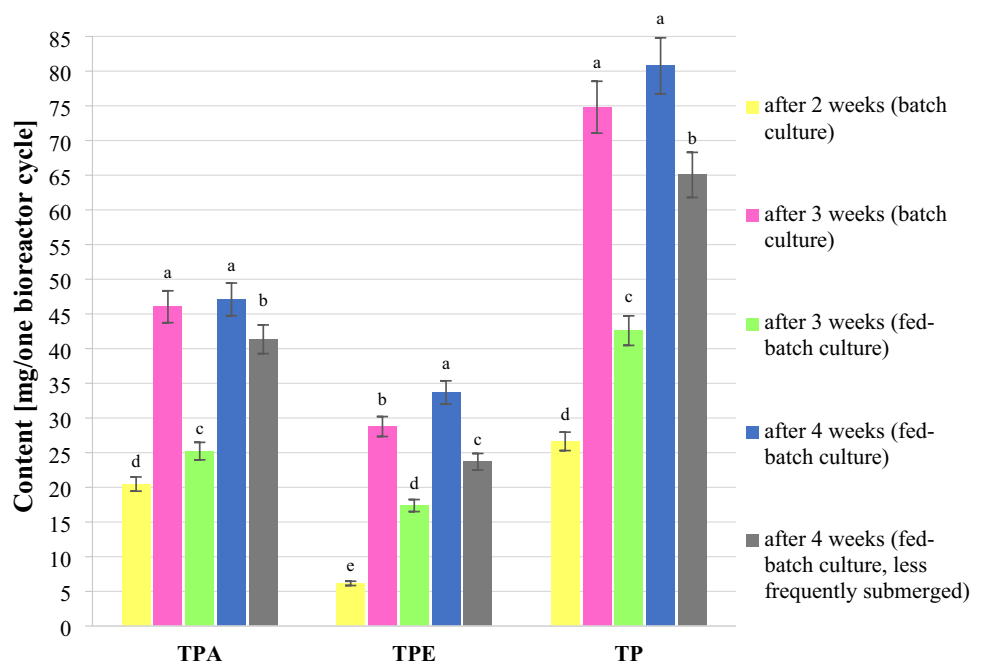
immersed in culture medium. Therefore, to address these issues and allow effective cultivation, the correct bioreactor type and cultivation conditions need to be identified.

The present study demonstrates the influence of various cultivation conditions on the growth and the production of bioactive metabolites in *S. viridis* shoots cultured in the

Plantform bioreactor. No data currently exists on the large-scale in vitro cultivation of this species, nor on the use of Plantform bioreactors for the cultivation of other species of *Salvia*. This bioreactor type has previously demonstrated promising results when cultivating plant tissues in temporary immersion systems and has been successfully used for other medicinal species, such as *Echinacea purpurea*, *Rubus idaeus* (Welander et al. 2014) or *Schisandra chinensis* (Szopa et al. 2017). Temporary immersion systems (TISs) such as the Plantform system are characterized by extremely good tolerance for plants, they are cheap and easy to use, and they can be upscaled to industrial levels thanks to the possibility of serial connection. Such a solution could be useful for the effective production of large biomasses, which can produce large amounts of bioactive secondary metabolites.

However, like others, this system requires optimization for a given species. Various factors can influence the multiplication efficiency and production of secondary metabolites in shoot cultures grown in TISs. Two key parameters are the immersion frequency of plant material in the medium and the length of contact with the medium during the cycle. These factors influence the amount of nutrients delivered and the humidity inside the culture vessel, and can trigger stress associated with complete immersion in the medium; this can affect the amount of biomass obtained and the proliferation ratio, as well as the quality of the plant material obtained, its hydration level and vitreousness (Teisson et al. 1996).

Pérez-Alonso et al. (2009) report that the frequency of immersion in the medium (two minutes of immersion, every 2, 4, 6 or 12 h) significantly influences the biomass of *Digitalis purpurea* L. shoot culture, its degree of hydration and proliferation ratio; however, immersion frequency did not appear to have any significant effect on shoot length. In the case of the *S. viridis* shoot cultures grown in the Plantform bioreactor, no significant differences in culture biomass were found between the two immersion frequencies (every 80 and 170 min). Despite this, it was found that *S. viridis* shoots immersed more frequently in the medium demonstrated a higher proliferation ratio (18.6) than those immersed less frequently, despite being cultivated for the same amount of time. The plant material which had less frequent contact with the medium was characterised by shorter main shoots (approximately 1.5-fold), but slightly longer axillary shoots (1.2-fold).

The differences observed in response to frequency of immersion between *Salvia viridis* and *Digitalis purpurea* L. cultures may be specific to the species, but could also be related to the duration of submergence in the growth medium: in the present study, *S. viridis* culture was submerged for five times longer (10 min) than described by Pérez-Alonso et al. for *Digitalis purpurea*. In other studies, out of two immersion frequencies, viz. 15 min/4 h and 15 min/8 h, the latter was found to be more favourable for growth of *Myrtus communis* L. (Aka Kaçar et al. 2020). Similarly, immersion every eight hours was found to be optimal for the multiplication of *Stevia rebaudiana* Bert. (Ramírez-Mosqueda et al. 2016). In contrast, however, changing the frequency of immersion (i.e. every 4, 8 and 12 h) did not result in any differences in biomass or multiplication for *Anthurium andreanum* Lind. (Martínez-Estrada et al. 2019).

Another factor influencing the amount of plant material obtained and the production level of secondary metabolites is the duration of culture. Prolonging the culture of *Schisandra chinensis* shoots in RITA® and Plantform bioreactors has been found to result in the biomass and proliferation ratio more than doubling; however, this resulted in a lower production of lignans, which may indicate a depletion of nutrients in the supplied medium during the experiment (Szopa et al. 2017). In the present study, prolonging the duration of culture also proved to be more beneficial for the growth of *S. viridis* shoots in the Plantform bioreactor. From two to three weeks of culture (at the same immersion frequency) a twofold increase in FW and DW was observed, together with elongation of the main and axillary shoots, although the proliferation ratio increased only slightly (from 8.2 to 8.9). However, the ratio of shoots to buds increased from 23 to 37%, suggesting that this procedure may make it possible to obtain more material capable of subsequent micropropagation without the need to further stimulate bud elongation. Longer culture durations were associated with adverse morphological changes, such as intense darkening of the lower parts of the shoots, and shoot necrosis. Similar effects were observed for *S. chinensis* (Szopa et al. 2017); the authors attribute this to the decrease in nutrient concentration in the medium or the accumulation of toxic metabolites inhibiting shoot growth. Therefore, the present study also examined the effect of adding a fresh portion of medium during the culture.

Some literature data indicate the possibility of using a semi-continuous system (fed-batch) to optimise plant culture growth and the biosynthesis of bioactive metabolites. The fed-batch system was compared with the batch system by Ritala et al. (2014), who cultivated the transformed tobacco (*Nicotiana tabacum* L.) roots in a bioreactor. Briefly, 20% of the initial volume of fresh medium was added to the culture after 8, 15 and 22 days. The procedure resulted in a 56% increase of DW in comparison to the batch culture and 1.6 times higher geraniol biosynthesis in the obtained biomass. Similarly, in the present study, the addition of fresh medium increased the proliferation of *S. viridis* shoots in the Plantform bioreactor. After three weeks, the fed-batch culture demonstrated a 31% increase in FW, 18% increase in DW, a 50% increase in proliferation ratio, and a 66% increase in main shoot length compared to the batch culture. No significant differences were observed in the case of axillary shoot

length. However, the shoot to bud ratio increased from 37 to 58%. In addition, the duration of culture could be extended up to four weeks.

The growth dynamics of the *S. viridis* shoot culture in the Plantform bioreactor were significantly different to a similar culture performed on the solid agar medium of identical composition (MS with the addition of 0.1 mg/l IAA and 1 mg/l m-Top) (Grzegorczyk-Karolak et al. 2020). In the latter, the shoot growth cycle lasted 5 weeks, during which time, a 20-fold increase in biomass was achieved; in contrast, a 33-fold increase in biomass was obtained in the semi-continuous bioreactor culture within four weeks. This more intensive increase of culture mass may be attributed to differences in shoot length: the main and axillary shoots grown in the bioreactor significantly longer. In addition, the proliferation ratio was four times higher. In conclusion, the *S. viridis* shoots cultured in liquid medium in the Plantform bioreactor demonstrate faster growth processes than those on solid medium, probably due to the better availability of nutrients contained in the medium. Immersion in the medium allowed the shoots to absorb substances necessary for growth with their whole surface, not only through the lower parts. Similar observations were made in an experiment in which *Salvia officinalis* L. shoots were grown in a liquid medium (Grzegorczyk and Wysokińska 2008); this culture demonstrated more intensive growth than that grown on agar medium of the same composition for two weeks longer. The shoots obtained in the conditions of a liquid medium produced more leaves, which reached larger sizes. While both cases demonstrated similar numbers of formed shoots, liquid culture demonstrated greater biomass and a considerably shorter cultivation time.

Another aim of the present study was to optimise the cultivation conditions of *S. viridis* shoots grown in the Plantform bioreactor to allow efficient biosynthesis of secondary metabolites. It was found that the production of bioactive compounds differed according to the choice of culture technique, its duration and immersion frequency, and that the conditions that were most favourable for culture growth were not optimal for secondary metabolite production. This may have been due to a significant change in the growth cycle and metabolism of the culture caused by the supplementation. The most efficient biosynthesis of phenolic acids and phenylethanoids, with total content of approximately 30 mg/g DW, was observed in the three-week batch culture, immersed every 80 min (Table 1). The two main secondary metabolites were rosmarinic acid, at a concentration of 16.6 mg/g DW, and verbascoside, at a concentration of 9.8 mg/g DW. The RA levels were twice those obtained in shoots cultivated on the solid medium, and 13 times more than in aerial parts of soil-grown plants (Grzegorczyk-Karolak and Kiss 2018; Grzegorczyk-Karolak et al. 2020). For VB, this value was similar to that of the soil-grown shoots and 1.8 times

higher than the in vitro cultures grown on solid medium. It should also be noted that the material could be obtained more quickly by liquid culture: 5 weeks were needed for agar culture and 16 weeks for soil cultivation. Plantform bioreactor culture thus appears to be an exceptionally advantageous method of obtaining biologically-active compounds from *Salvia viridis*; in addition, unlike traditional field cultivation, it offers the possibility of continuous, reproducible continuation of cultivation, regardless of climate or season.

The potential for bioreactor cultivation to deliver larger amounts of secondary metabolites in a shorter time has also been noted in previous studies. For example, nutrient sprinkle bioreactor culture of *Salvia officinalis* L. shoots yielded 26.2 mg/g DW of rosmarinic acid after three weeks; this value was more than twice that obtained for 10-week-old shoots grown in soil, i.e. 12.2 mg/g DW (Grzegorczyk and Wysokińska, 2010). Ruta et al. (2020) also note that the total phenolic content (calculated as gallic acid) in *Lycium barbarum* L. shoots cultivated in the Plantform bioreactor was more than twice (23.6 mg/g DW) that found in shoots grown in the natural environment (11.98 mg/g DW). Previous studies also suggest that shoot cultures grown in bioreactors with a temporary immersion system offer promising potential for efficient production of a variety of secondary metabolites with therapeutic properties: lignan biosynthesis in *Schisandra chinensis* (Szopa et al. 2017), cardenolide glycosides in *Digitalis purpurea* (Perez-Alonso et al. 2009) or volatile compounds in *Rhododendron tomentosum* Harmaja (Jesionek et al. 2017).

Finally, it should be remembered that several factors influence the effectiveness of the culture, not only the final content of compounds in the obtained plant material and the increase in biomass, but also the duration of the culture and the cost of preparation and supplementation of the medium. The three-week batch culture and four-week fed-batch system offered comparable efficiency in the production of bioactive compounds in *S. viridis* shoot culture (Fig. 4). In fact the former appears more efficient and cost effective: despite weaker culture growth and proliferation, the obtained plant material was richer in bioactive secondary metabolites; in addition, culture time was shorter and did not require further medium supplementation. There was also no chance of culture contamination associated with the addition of the medium. Ultimately, within three weeks, 46 mg of phenolic acids were obtained, including 41.7 mg of RA and 28.8 mg of phenylethanoids, including 24.7 mg of VB.

Conclusions

The presented findings indicate for the first time that effective cultivation of *Salvia viridis* shoots in the Plantform bioreactor with a temporary immersion system is possible.

The duration and technique of culture (batch or fed-batch) and the frequency of immersion in the liquid medium were shown to affect the biomass, proliferation ratio and the production of secondary metabolites. The obtained results are innovative and have practical applications, because culture obtained large amounts of plant material rich in the bioactive compounds. The most favourable growth parameters were achieved for plants grown for 4 weeks in a semi-continuous (fed-batch) system, in which 26.99 g FW and 3.51 g DW of shoots were obtained, with a proliferation ratio of 18.6. However, ultimately, the highest production of bioactive phenolic compounds was found in the 3-week batch culture: the obtained total phenolic acid content was about 10 times higher than in plants grown in soil for four months and more than two times higher than in shoot culture grown for 5 weeks on solid medium. In addition, after three-week batch culture in the Plantform bioreactor, the total phenylethanoid level in *S. viridis* shoots was 40% higher than that found in shoots from five-week solid culture and similar to that in the aerial parts of plants grown in the soil for 16 weeks. These figures demonstrate the potential of such culture methods, both in industry, for producing medically important compounds, and in research, for obtaining large amounts of valuable biomass for further research.

Data Availability The data that support the findings of this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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